Preface from the Editor

In order to disseminate to broader community, a proceeding consisted of scientific papers presented at the 2nd International Conference on Pharmacy and Advanced Pharmaceutical Sciences, held in Yogyakarta, Indonesia, 19 – 20 July 2011, is produced. The proceeding is divided into two books i.e. Clinical and Social Pharmacy and Pharmaceutical Science and Technology.

The conference was organized by the faculty of Pharmacy Universitas Gadjah Mada in collaboration with the Nara Institute of Science and Technology and Deutscher Akademischer Austausch Dienst-German Academic Exchange Service. This event was part of the faculty’s 65th Anniversary celebration as well as the 62th Anniversary of the Universitas Gadjah Mada. In this conference participants from 6 countries have participated of which 14 lectures within the field of Pharmaceutical care and sciences were presented by our invited speakers, followed by presentation of 160 researchers in form of oral and poster presentation. On behalf of the organizing committee, I would like to thank all invited speakers and presenters for participating the in International Conference on Pharmacy and Advanced Pharmaceutical Sciences and for giving valuable contribution to this proceeding.

Acknowledgements are addressed to the Rector of Universitas Gadjah Mada, the Nara Institute of Science and Technology, Japan, Deutscher Akademischer Austausch Dienst-German Academic Exchange Service in collaboration with Federal Foreign Office as well as all sponsors for the nice collaboration in bringing forth the conference. Furthermore, personally, I would like to express my deep appreciation to the members of the Organizing Committee for the good teamwork and the great effort to bring success to the conference and in producing the proceeding.

Finally, I hope this proceeding will give a remarkable contribution to broad scientific research, especially in the field of pharmacy and pharmaceutical sciences.

Yogyakarta, July 2011

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Yuliana Setiarini, S.Mn
Welcome Message from Organizing Committee

Distinguished Ladies and Gentlemen,
On behalf of the Scientific and Organizing Committee, it is such a great pleasure for me to welcome all participants to Yogyakarta, to the 2nd International Conference on Pharmacy and Advanced Pharmaceutical Sciences 2011.

The conference is organized by the faculty of Pharmacy Universitas Gadjah Mada in collaboration with the Nara Institute of Science and Technology and Deutscher Akademischer Austausch Dienst-German Academic Exchange Service. This event is as part of the faculty’s 65th Anniversary celebration as well as the 62th Anniversary of the Universitas Gadjah Mada. In this conference participants from 7 countries have participated of which 14 lectures within the field of Pharmaceutical care and sciences will be presented by our invited speakers, followed by presentation of 160 researchers in form of oral and poster presentation. Herewith we would like to express our gratitude to all speakers and presenters for joint us today to share advance knowledge and expertise in this scientific event.

The Organizing Committee gratefully acknowledges the Rector of Universitas Gadjah Mada, the Nara Institute of Science and Technology, Japan, Deutscher Akademischer Austausch Dienst-German Academic Exchange Service in co-operation with Federal Foreign Office well as all sponsors for the nice collaboration in bringing forth this conference. Furthermore, personally, I would like to express my deep appreciation to the members of the Organizing Committee, for the good teamwork and their great effort to bring success to the conference.

Finally, I wish all participants could benefiting from the Conference and have an enjoyable moments in Yogyakarta

Thank you very much
Remarks of the Dean of Pharmacy Faculty UGM
Prof. Dr. Marchaban, DESS., Apt

Assalamu’alaikumwr. wb.
Distinguished ladies & gentlemen.
First of all, on behalf of the Faculty of Pharmacy Universitas Gadjah Mada, I would like to welcome to all of you in Yogyakarta, thank you very much for your attention to come and to attend the 2nd International Conference on Pharmacy and Advanced Pharmaceutical Sciences. I hope we are all in health condition.

Ladies and gentlemen,
The conference is organized by the Faculty of Pharmacy UGM in collaboration with the Nara Institute of Science and Technology (NAIST) Japan and DAAD, Germany and held as part to celebrate the 65th anniversary of the Faculty of Pharmacy UGM and the 62nd anniversary of Univeristas Gadjah Mada. In the conference, I hope we can communicate our recently information concerning social / clinical pharmacy and pharmaceutical sciences. I hope the conference will be very fruitful, very useful for all of us.

I address special thanks to the plenary speakers both from domestic and abroad, the oral and poster presenters, as well as to those who come just to know the development of clinical or social pharmacy and pharmaceutical science. Your willingness to come, to communicate and to share your experiences is highly appreciated.

Therefore, during almost whole day discussing scientific matter related to human health and welfare, I hope we can make a wonderful opportunity to make a scientific closer relationship while we enjoy the cultural performances of Yogyakarta presented by our pharmacy student.

Finally, I hope that this meeting will give benefits to all of us, and we may see each other again in a similar event in the near future.

I look forward to thank you all for attending this event
Wassalamu’alaikum warahmatullahi wabarakatuh,
Opening Remarks by Rector of Universitas Gadjah Mada
Prof. Ir. Sudjarwadi, M. Eng., Ph. D
Bismillahirrahmanirraahim
Assalamu’alaikum wr wb
Chairman of Presidential Advisory Board, Prof. Dr. Emil Salim

Distinguished guests, ladies and gentlemen,
Good morning. It’s truly a privilege for me to be able to welcome all of you to The Second International Conference on Pharmacy and Advance Pharmaceutical sciences in Yogyakarta with theme “Bridging Science to Pharmacy Practice” that is organized in cooperation with DAAD and the Nara institute of Science and Technology Japan to celebrate the 65th Anniversary of Faculty of Pharmacy UGM. This scientific gathering is aimed to update recent findings in pharmacy and advanced pharmaceutical sciences. It is expected that researchers, academia, practitioners, policy makers as well as students and other participants can learn and share with each other about the latest developments in pharmacy and pharmaceutical science. This meeting is much required to develop the participant’s knowledge in the science and address the pharmacological issues that arise. Moreover, in the fast developing condition of pharmaceuticals due to the increasing demands and mobility of people around the world and identifications of diseases that emerge, it is highly important for scientists to better contribute their knowledge to the wider public.
Participants here all invited to seek for and introduce new ways on how to bring this knowledge to the pharmacy practices to be able to benefit the public. I hope that in these two days you will all be involved in insightful and inspiring discussions that can produce such new ways, new findings and alternatives as well as solutions to current problems in the area of pharmacy. It is my belief that all of us will benefit much from this special meeting. I wish you have a good and enjoyable gathering while in Jogja. Lastly, my sincere thanks and deep appreciation go to everyone who have made this seminar a success.

Thank you very much.
Wassalamu’alaikum wr wb.
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DISCUSSION
ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF ETHANOLIC EXTRACT OF HEDYOTIS CORYMBOSA (L.) LAM ON BREAST CANCER CELL LINE OF T-47D

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ABSTRACT

Herb of Hedyotis corymbosa (L) Lam (known as Rumput Mutiara) is member of Rubiaceae and traditionally have been used for fever treatment. This medicinal herb is also believed has potency as hepatoprotector and anticancer activities. In order to evaluate its antiproliferative activity in vitro, ethanolic extract of H. corymbosa (L) Lam was tested against breast cancer cell line of T-47D using enzymatic assay of 3-(4,5 dimethylthiazol-2-yl)- 2, dipheniteterazolium bromide (MTT). In addition, its antiradical scavenging activity was also determined using 1,1-Diphenyl-2-picryl hydrazyl (DPPH) method. The result showed that Hedyotis corymbosa herbs which were extracted with 70% ethanol, significantly inhibit T47-D cell proliferation where IC_{50} value was 119 µg/ml. Moreover, the extract was also exhibit a potential antioxidant activity. However, it needs further assay to determine in which mechanism the extract inhibit cancerous cell proliferation.

Key words : Hedyotis corymbosa, cytotoxicity, MTT, DPPH, and antioxidant activity.

INTRODUCTION

Rumput mutiara (Hedyotis corymbosa (L) Lamk is one of the important medicinal plants has been used as antitumor drugs which described the immunocompetent activity to prevent haematopoietic damage when used in combination with radiotherapy (Yang et al.1997). The ethanolic extract of this plant exhibit antihepatotoxic which showed decreasing of SGOT and SGPT levels and stimulated liver cell proliferation of acetaminophen-induced rabbit (Tatang et al.2006). Then the methanolic extract its produced significant hepatoprotective effects as evidenced by decreased serum enzyme activities, SGPT, SGOT, SAKP and serum bilirubin and an almost normal histological architecture of the liver in treated mice compared to the controls. And the extract also shortened hexobarbitone-induced sleeping time in mice, besides showing significant antilipid peroxidant effect in vitro (Sadasivan et al. 2006). Subsequently, the ethanolic extract also its has a potential activity to be developed as co-chemotherapeutic agent, and the combination of this extract with doxorubicin induced apoptosis and decreased Bcl-2 expression. (Haryanti et al. 2009). Combination of Hedyotis corymbosa, Andrographis paniculata extracts and curcumin indicated an effective of herbal anti-malarial drugs (Misra et al. 2009). Beside exhibit anti-tumor and hepatoprotective activity, the ethanolic extract of rumput mutiara also has an antioxidant activity similar to α-tocopherol using thioarbituric acid (TBA) method (Amelia, 2006). Currently, methanolic extract its exhibit high antiradical activity against DPPH, ABTS, nitric oxide and hydroxyl radicals, the antioxidant activity of the extract was comparable with that of standard butylated hydroxyl toluene (BHT), and the main antioxidant compound was polyphenol (Sasikumar et al. 2010).

The aim of the research was to evaluate its antiproliferative activity in vitro of ethanolic extract of H. corymbosa (L) Lam against breast cancer cell line of T-47D using enzymatic assay of 3-(4,5 dimethylthiazol-2-yl)-2, dipheniltevezolium bromide (MTT), and determined of its extract as antiradical scavenging activity using 1,1-Diphenyl-2-picryl hydrazyl (DPPH) method.
METHODOLOGY
Extraction of rumput mutiara herb (*Hedyotis corymbosa* L.)

The dried Rumput mutiara herbs were ground and macerated with ethanol 70% solvent. Then, filtrate were collected and evaporated by vacuum rotary evaporator to get the viscous extract.

Cytotoxic analysis

Human Breast cancer cell line of T47D was maintained as monolayer cultures in RPMI 1640 medium, supplemented with 1% antibiotics (50 IU/ml penicillin and 50 µg/ml streptomycin) and 10% heat inactivated of fetal bovine serum, in a humidified incubator containing 5% CO2 at 37°C. Subcultures were obtained by trypsin treatment of confluent cultured. The cells were seeded in 100 µl of medium in 96 microwell plate at a density of 5 x 10^3 cells/well, and the plates were placed in a 37°C, 5% CO2 incubator. One day later the cell culture was added with 100 µl/well medium containing the indicated concentrations (0, 10, 20, 50, 100, and 250 µg/ml) of extract in triplicate. After 24 hours of treatment, washed twice with phosphate buffer saline, then the medium was changed and 100 µL MTT[3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] 0.5 mg/mL was added and maintained at 37°C in 5% CO2 incubator for 4 hours to allow MTT to be converted to formazan crystals by reacting it with metabolically active cells. The viable cells were directly proportional to the production of formazan. The reaction was stopped by adding SDS (sodium dodecyl sulfate) 10%, and the cell viability was measured at 570 nm using a plate reader. IC50 value of extract was determined based on the equation of linear regression of log concentration vs % cell viability (Wang et al. 2000).

DPPH antiradical scavenging assay

Assay of the antioxidant activity of extracts carried out by spectrophotometric methods using 1,1-diphenyl-2-picrylhydrazyl (DPPH). As positive controls used vitamin C (ascorbic acid) and vitamin E (α-tocopherol). Methanolic extracts of Rumput mutiara herbs was prepared with concentrations of 1000 ppm, and 0.0079% DPPH solution (7.9 mg in 100ml methanol). Later, 1000 µl of each sample solution with a concentration of 5.10, 25, 50 and 100 ppm was mixed with 500 µl of DPPH solution in the cuvet. Positive control of vitamin E (α-tocopherol) was prepared at the same concentration (5.10, 25, 50 and 100 ppm) and vitamin C (ascorbic acid) with a concentration of 4, 6, 8, 10 and 12 ppm. Observations were made at minutes 1, 10, 20, and 30, using UV-Vis spectrophotometer Genesys 20 equipment at a wavelength of 517 nm. The antioxidant activity was calculated based on the decrease in DPPH absorption due to the addition of the test sample, as follows:

\[
\text{Radical scavenging activity} \% = \left( \frac{\text{Abs DPPH} - \text{Abs sample}}{\text{Abs DPPH}} \right) \times 100
\]

RESULTS AND DISCUSSIONS

Cytotoxic activity

Observations cytotoxicity testing was intended to observe the inhibition of cell proliferation and obtain IC50 values. Observations after 24 hours of extracts treatment showed cytotoxic effects of extracts on T47D cells with IC50 value of 119 ppm and in normal CHO cells around 375.74 ppm, whereas on the positive control of cisplatin IC50 about 17-18 ppm on both two cells tested (Table 1).

Tabel 1. The IC50 value of ethanolic extract of Rumput mutiara (*Hedyotis corymbosa*) against T47D cancer cells and normal CHO cells.

<table>
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<th>Extract / positive control</th>
<th>IC50 (ppm)</th>
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<tr>
<td></td>
<td>T47D cell</td>
</tr>
<tr>
<td>Rumput mutiara extract</td>
<td>119</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>17,19</td>
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<table>
<thead>
<tr>
<th>Extract concentrations (ug/ml)</th>
<th>Proliferative inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>T-47D cell</td>
</tr>
<tr>
<td>10</td>
<td>3.64</td>
</tr>
<tr>
<td>20</td>
<td>5.19</td>
</tr>
<tr>
<td>50</td>
<td>17.55</td>
</tr>
<tr>
<td>100</td>
<td>39.62</td>
</tr>
<tr>
<td>250</td>
<td>47.57</td>
</tr>
</tbody>
</table>

From the test results showed the selective properties of the extract where the cytotoxic effects of extracts of T-47D cancer cells is higher than the normal CHO cells, although lower than the positive control of cisplatin.

Cytotoxic activity of extracts also proven by inhibitory effect of cell proliferation in both cell tested, CHO cell and T47D cell (Table 2). Increasing the extract concentration from 10 ppm to 250 ppm on T-47D cell showed a significant increased in proliferation inhibitory effect from 3.64% to 47.57%. In contrast, at the lower concentration of extract was not exhibit any inhibitory effect on normal cell CHO proliferation, the inhibitory effect of normal occurred after addition of 100 and 250 ppm of extract, which inhibits proliferation of 18.49 - 28.55% compared to untreated cell.

In our research was not determine yet how the mechanism of the extract inhibit cell proliferation, but another research revealed that the extract exhibit apoptotic cell on different cancer cell. Ethanolic extract of Rumput mutiara induced apoptotic in breast cancer cell MCF-7, which stained with acridine orange-ethidium bromide and observed under fluorescence microscope. All cells in control cell gave green fluorescence showing there was no death cell. The extract treatment caused some cells gave orange and red fluorescence which signed the increasing of cell membrane permeability as the indicator of apoptosis. The nuclear of several cells were fragmented and formed apoptotic bodies (Haryanti et al. 2009).

**DPPH antiradical scavenging assay**

The DPPH antiradical scavenging activity of Rumput mutiara (*Hedyotis corimbosa* L.) extract observed in the first minute and 10 minutes incubation showed that the longer the incubation time increased radical scavenging activities, likewise increasing the concentration of extracts also increased radical scavenging activity specially in the concentration 25ppm – 100 ppm of extract (Figure 1). Furthermore observation in 20 minutes and 30 minutes incubation showed the same pattern where the increasing incubation time and extract concentration increased radical scavenging activities (Figure 2).

![Figure 1](image1.png)

Figure 1. DPPH antiradical scavenging activity of Rumput mutiara (*Hedyotis corimbosa* L.) extract compared with Vitamin E and vitamin C : a. 1st minutes observation, b. 10th minutes observation.
At the end of assay the activities of rumput mutiara extract still lower that both of positive control Vitamin E and Vitamin C even at 100 ppm of extract. May be increasing the extract concentration would be increase the radical scavenging activity. In this research the highest concentration of extract treated was 100 ppm, another researcher used the higher concentration until 500 ppm and 1000 ppm. The methanolic extract oh this plant at 1000 ppm caused significant elevation of scavenging activity on DPPH radical comparable to 1000 ppm of that positive control of butylated hydroxyl toluene (BHT) (Sasikumar et al., 2010).

Previous research comparing antioxidant activities of coronet and root of ethanolic extract with α-tocopherol using thiobarbituric acid (TBA) method which based on the specific reaction between TBA and malondialdehyde (MDA). The addition of coronet and root extract inhibit the rate of MDA formation, that mean improved the antioxidant activity. The result indicated that 500 ppm and 200 ppm coronet extract and 500 ppm extract showed similar inhibitory effect to 200 ppm α-tocopherol on MDA formation (Amelia, 2006).

CONCLUSION

1. The ethanolic extract of *Hedyotis corymbosa* herbs has selective properties which significantly inhibit T47-D cell proliferation, but less toxic on normal cell line CHO
2. The extract was also exhibit a potential antioxidant activity on DPPH antiradical scavenging, although at 100 ppm the activity was still lower than Vitamin E (α-tocopherol) and Vitamin C (ascorbic acid).
3. It needs further assay to determine in which mechanism the extract inhibit cell proliferation.

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MOLECULAR DYNAMIC SIMULATION OF SIRNA AND MODIFIED SIRNAS

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ABSTRACT

Short interfering RNA (siRNA), the active agent of RNA interference, shows promise of becoming a valuable tool in both basic and clinical research. RNAi has been used to prove that RNA molecules were behind some gene silencing. Application of siRNAs in vivo and their possible use as therapeutics still face several critical hurdles, among others, the stability. Many efforts have been comprehensively done to address this issue, for instance by chemical modifications and various mutations. In the present work, we compare the stability of siRNA (PDB entry:1SI3) with modified siRNA by replacing the non-canonical end base pair CxU with the canonical base pair CxG and dangling ends using molecular dynamic simulation. Our results showed that the modified siRNA exhibited significantly good stability compared with the wild type during 10 ns simulation time. This great improvement indicated the role of end base pair and dangling ends in the stability of siRNA duplex.

Key words: md simulations, siRNA, canonical base pair, RNA stability

INTRODUCTION

The substantial progress in biology in recent decades has been the knowledge that small RNAs can affect gene expression. It begins, when Andrew Fire, Craig Mello and colleagues announced their finding of RNA interference (RNAi), the silencing of gene expression by double-stranded RNA molecules, in nematode worms (Caenorhabditis elegans) (Fire, 1998). It was reported that double-stranded RNAs (dsRNAs) can trigger silencing of complementary messenger RNA sequences. Shortly afterwards, short dsRNAs or short interfering RNAs (siRNAs) were created artificially and used to show that this process also happens in mammalian cells, usually, but not always, without triggering the innate immune system, which normally recognizes RNAs as part of an antiviral defence mechanism (Cullen, 2009).

RNA-based therapeutics is one of the most promising new strategies for disease therapy. Only six years after the discovery of RNAi (in 2004), the first siRNA-based human therapeutics was developed as treatments for wet age-related macular degeneration and entered phase I clinical trials. (Cejká, 2006, Castanotto, 2009). In particular, the development of siRNA-based therapies has progressed rapidly. Given the ability to specifically silence any gene of interest, siRNA offers several advantages over conventional drugs as potential therapeutic agents for the treatment of human maladies including cancer, genetic disorders, and infectious diseases (Zhou, 2011).

Although much is known about the mechanisms of RNAi, there are a number of challenges that applications of this gene-silencing technology need to overcome. For instance, siRNA delivery, bio-stability, pharmaco-kinetics and specificity, including off-target effects, will be major topics of further investigation.

One critical advance was chemically modifying siRNA to create molecular structures without compromising gene-silencing efficiencies and applying more rigorous bioinformatics to siRNA design (Elmén, 2005, Pande, 2008). Here, we undertook the present computational study of duplexes based on crystal structure data from PDB 1SI3 (Ma, 2004). The duplexes of interest are shown in Figure 1. Duplex II-IV derived from duplex I were used to study effect of closing base pair and dangling residues on the duplex stability.
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... structure III were the most rigid compared to other structures and had the smallest mean value of RMSF 1.7 ± 1.1 Å.

METHODOLOGY

The MD simulations of four duplexes in Figure 1 were carried out in explicit solvent using GROMACS package 4.0.3 (van der Spoel, 2005, Berendsen, 1995) and Amber force field 2003 (Duan, 2003) for 10 ns. A starting structure was obtained from PDB 1SI3 (Ma, 2004) and the other modified structures were generated using Nucleic Acid Builder software package (Macke, 1998). The duplex was placed in a rhombic dodecahedron box (edge length approximately 6 nm), which subsequently filled with about 5000 TIP3P water molecules (Jorgensen, 1983). To neutralize the system, 16 sodium ions were placed randomly in simulation box. The simulations were performed in the NPT ensemble with periodic boundary conditions, a temperature of 300 K and at pressure 1 atm. The temperature and the pressure were kept constant by Berendsen’s weak coupling scheme (Berendsen, 1984) with coupling parameter of 0.1 ps for temperature, 1.0 ps for pressure, and the isothermal compressibility of 4.5-10-5 bar-1. Constraint were used for bond lengths using the LINCS algorithm for the duplex (Hess, 1997), and SETTLE for the water (Miyamoto, 1992). A twin-range cut off was used for van der Waals interactions. The electrostatic interactions were evaluated by using particle mesh Ewald (PME) method (Darden, 1993).

RESULTS AND DISCUSSIONS

The all MD simulations of four structures in Figure 1 were performed for 10 ns simulation time and the root mean standard deviation (Figure 2) were examined against all atoms after fitting on backbone atoms (C4’, C3’, O3, P, O5, C5’ atoms). The RMSD values were obtained 7.3 ± 1.2 Å, 5.5 ± 1.0 Å, 3.8 ± 0.6 Å, and 4.3 ± 0.8 Å for structure I – IV respectively. These values revealed the difference between the simulated and starting structure.

As can be seen in Figure 2, the RMSD values of the first structure was increasing which suggested that the simulation did not lead to a stable structure. By contract, after changing the closing base pairs and dangling ends, the RMSD values were decreased significantly. However, structure III and IV demonstrated smaller RMSD values compared to structure II. It is noteworthy, that the dangling ends do not interact directly by hydrogen bonding in the duplexes but contribute more to the stability of duplex.

The individual atomic motions within the siRNAs were also calculated. The fluctuation of an atom relative to its average position (root mean square fluctuation of RMSF) indicate the extent motion experienced by this atom. Figure 3 depicted RMSF of all atoms for four structures. Each residues had a peak due to the especially flexible phosphate group, while the ribose and base parts were more rigid. Upon closer inspection it turned out, however, that the atoms of structure III were the most rigid compared to other structures and had the smallest mean value of RMSF 1.7 ± 1.1 Å.

Figure 1. Sequences of the duplexes used in this study. The modified residues shown in blue color are derived from naturally occuring siRNA (structure I from PDB 1SI3) (Ma, 2004).
The fraction of hydrogen bonding between the base pairs during the 10 ns simulation time is displayed in Figure 4. There were 6 base pairs in the duplexes and the time evolution of the occurrence of base pairing in the duplexes are also illustrated. We adopted the H-bond criteria: H-bond donor or acceptor heavy atom distance D..A < 3.5 Å D-H..A angle > 120°. Here, most of the hydrogen bonds were maintained during the simulation for modified structure of duplexes. The hydrogen bonding in canonical Watson-Crick base pair C-G can understandably provide a significant effect on the stability of duplexes. Moreover, as already noted above, the dangling ends contribute even greater than the base-pairing effect on the stability of duplexes.

Figure 2. Time-course of the RMSD (in Å) of the siRNAs in Figure 1 against the starting structure after fitting on the backbone atoms

Figure 3. Atomic RMS fluctuation (RMSF) for all atoms relative to the atom average positions for nucleotide atom types. The red lines indicate the backbone atoms. Some residue codes are shown, and the codes in blue indicate the modified residues.
CONCLUSION

Using MD simulations with ffamber03, we have compared the global structural features of a siRNA and modified siRNAs during the time simulation of 10 ns. The changing of closing base-pair and dangling ends affected significantly the RMSD, RMSF and fraction of H-bonds of the duplexes by hydrogen bonding and base stacking. Therefore, these interaction might affect both backbone rigidity and stability of RNA structure.

ACKNOWLEDGEMENT

This research funded by a grant from DP2M DIKTI (grant no. 327/SP2H/PP/DP2M/2010).

REFERENCES


FAST DISSOLVING TABLET FORMULATION OF METOCLOPRAMIDE HYDROKLOKLORIDE BY ADDITION OF KOLLIDON CL-F AS SUPERDISINTEGRANT

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ABSTRACT

Metoclopramide hidrokloride is antiemetic and gastroprokinetic, mainly used to treat nausea and vomiting. Patients who have it is difficult to swallow anti vomiting conventional tablets. So that anti-vomiting drugs tablets better if it made with a fast dissolving tablet technology. Fast dissolve tablet (FDT) is a solid dosage forms that disintegrate in the oral cavity leaving an easy-to-swallow residue. The disintegration times are generally less than one minute. For fast dissolving tablets, taste-masking of bitter or objection-tasting drug substances is critical. The influence use of Kollidon CL-F as super desintegrant concentration of 10% (F1), 15% (F2) and 20% (F3) compared with the F0 are not using Kollidon CL-F, carried out to determine the variation of disintegration time, dissolution rate, water absorption ratio and wetting time of tablets in each formula. The assessment of the tablet so the four formulas also qualify include uniformity of size, weight, hardness and friability test. The results showed that the dissolution rate of metoclopramide HCl with the addition of Kollidon CL-F are better than tablets that not using Kollidon CL-F as desintegrant. Percentage of metoclopramide HCl dissolution was at minute 30 of F0, F1, F2 and F3 in the media aquadest row is 74.62%, 84.47%, 87.08%, 84.65%.

Keywords: Metoclopramide Hydrochloride, Fast dissolving tablet, Kollidon CL-F

INTRODUCTION

Oral drug delivery is a way of giving the most important to obtain systemic effects. Of drugs given via the oral, solid dosage form that is more favored, in this case the tablet dosage form because it is more practical and easy to use.

However, not all patients can perform oral therapy using a tablet, especially in children and elderly as well as some patients with symptoms of difficulty in swallowing. The situation also occurs in patients who are experiencing nausea and vomiting conditions (emesis).

So this needs to be handled by making rapid dissolve tablet. Tablets dissolve quickly (Fast dissolving tablets) is a unit of solid dosage forms which terdesintegrasi or dissolve quickly in the mouth without chewing and water, so it takes a strong desintegrant that can destroy the drug without water.

One substance that can be used in fast dissolve tablet formulations (Fast dissolving tablets) as superdisintegrant is Kollidon CL-F is disintegrator that have characteristics that are very hydrophilic, fast wetting and has the ability to develop excellent. With a surface area that is very specific polymer causes disintegrator has a high capillary activity and hydration capacity are great. As a result, water is more easily entered into the tablet. This increased volume creates pressure in excess of tablet strength and caused a rapid disintegration time of tablets. That is why Kollidon CL-F categorized as superdisintegrant.

Efficacious antiemetic metoclopramide hydrochloride and gastroprokinetik primarily used to treat nausea and vomiting, and to facilitate gastric emptying in patients with gastroparesis. Anti-emetic action of metoclopramide is due to antagonistic activity at D2 receptors in chemoreceptor trigger zone (CTZ) in the central nervous system (CNS), this action prevents nausea and vomiting triggered by most stimuli. At higher doses, 5-HT3 antagonist activity may also contribute to the anti-
emet effect. Metoclopramide prokinetic activity mediated by muscarinic activity, D2 receptor antagonist activity and 5-HT4 receptor agonist activity. Influence prokinetic itself can also contribute to the anti-emet effect.

In a previous study on the formulation and evaluation of tablets rapidly dissolve Clonazepam which uses three types namely desintegran Crosspovidone, Croscarmellose-Na and sodium starch glycolate resulted in some concentration in vitro dissolution time, wetting time and water absorption ratio is good. (3)

In this study metoclopramide hydrochloride tablet dosage form by direct compression. Preparation of tablets by direct compression is most likely used because it gives many advantages such as quick-making process, both to substances that are unstable to heat and humidity, the possibility of faster dissolution due to the direct tablet broken into powder. It is very important in the manufacture of tablet formulation by direct compression method is the selection of materials used, the excipients used must have properties that can support the process of making tablets. Tablets must contain a sweetener to mask the active substance, because the tablet will be destroyed while still in the mouth.

Based on the above, in this study about the development of metoclopramide hydrochloride tablets with various concentration of Kollidon CL-F as superdisintegrant, so that can know the effect of adding several concentrations of Kollidon CL-F on the dissolution rate of tablets and tablet quality that meets the requirements.

This study aimed to obtain the formula tablets metoclopramide hydrochloride with Kollidon CL-F superdesintegran which can result in the rate of disintegration time, the ratio of absorption and wetting time, dissolution rate and tablet quality is best.

**METHODOLOGY**

**Material**
The materials used in this study are as follows: metoclopramide hydrochloride (KF Research and Technology), Kollidon CL-F (BASF), Amprotab, Avicel, PH 102, Spray Dried Lactose, Hydroxy propyl cellulose (PT. Brataco), Stearic acid (PT. Brataco), Talc (PT. Brataco), Aerosil (PT. Brataco), Stevia, Mannitol (Ristek KF), distilled water, mercury (II) acetate, acetic anhydride, glacial acetic acid (PT. Brataco), perchloric acid 0.1 N, Potassium Hydrogen Pthalat, and Crystal Violet Indicator.

**Research Methods**
The study begins with the materials used for metoclopramide hydrochloride and Kollidon CL-F based on the Indonesian Pharmacopoeia Fourth Edition and includes organoleptic properties, solubility, and purity. Ibuprofen purity raw materials was done by UV spectrophotometry and titration of free water.

The next process is the sifting of raw materials, followed by mixing all the components and the manufacture of tablets and tablet with some formula Kollidon CL-F. Formula 0 without the addition of Kollidon CL-F (amprotab), formula 1 with the addition of Kollidon CL-F 10%, 15% of formula 2 and formula 3 with the addition of Kollidon CL-F 20%. Before compressing the previous tablet, tablet compressed evaluation period covers the homogeneity test by setting the levels of ultra violet spectrophotometry, the water content, flow properties, angle repose and the density of granules.

The tablets that have been compressed evaluated include weight uniformity test, size uniformity, tablet hardness, friability test, determination of the active substance in tablets, water content, disintegration time test, the wetting time, water absorption ratio, and dissolution test.

**Research Results**
Examination of Identity and Purity of Raw Materials Raw Material Inspection metoclopramide Inspection carried out to identify the raw materials which include metoclopramide-HCl organoleptic examination and solubility. Examination procedure in accordance with Indonesian Pharmacopoeia Fourth Edition 1995. Metoclopramide-HCl purity test. Inspection of Raw Material Purity of
metoclopramide HCl. Metoclopramide–HCl purity examination conducted by the free water titration method. Standardization performed volumetric solution 0.1 N perchloric acid. Carefully weighed 200 mg of potassium hydrogen phthalate, solution in 15 mL glacial acetic acid. Add 5 drops of acetic acid anhydride and 2 drops of crystal violet indicator. Then titrated with 0.1 N glacial acetic acid the determination of purity of raw materials metoclopramide. Carefully weighed raw materials metoclopramide–HCl 300 mg, inserted into the lid 125 mL erlenmeyer flask, then added 10 mL of mercury (II) acetate LP, 2 mL acetic anhydride and added to 30 mL of glacial acetic acid P. Allow a few moments and titration with perchloric acid 0.1 N, calculated levels of metoclopramide–HCl. Examination Kollidon CL-F Kollidon CL-F reviewed by Hand Book of Pharmaceuticals Excipients ed.IV Fourth Edition 2003. Examination includes organoleptic test and solubility test. Wavelength Determination and Calibration Curves Determination of Maximum Absorption Wavelength metoclopramide–HCl Carefully weighed as much as 50 mg of metoclopramide–HCl. Then put in 50.0 mL measuring flask and dissolved in distilled water until a limit (1000 μg / mL). The solution of 5.0 mL and diluted with distilled water to 50.0 mL (100 μg / mL). Then a solution of 2.0 mL added and diluted with distilled water to 25.0 mL (8 g / mL). Then the absorbance was measured at a wavelength of 200-400 nm. The results obtained by measuring the wavelength of 309.0 nm.

Preparation of Calibration Curve of metoclopramide–HCl Carefully weighed 50.0 mg of metoclopramide–HCl, then dissolved in distilled water to 50.0 mL (1000 g / mL). The solution of 5.0 mL added and diluted with distilled water to 50.0 mL (100 μg / mL). From the solution made metoclopramide–HCl solution with concentrations of 2, 4, 6, 8, 10, 12 μg / mL. Each concentration was measured absorbance at a wavelength of 309.0 nm, then made a standard curve by plotting the absorption against concentration. 5.3. Soluble Tablet Making Fast metoclopramide–HCl. The composition of the fast dissolving tablet with various concentrations of Kollidon CL-F can be seen in the table below:

<table>
<thead>
<tr>
<th>Formula of metoclopramide HCl Fast Dissolving Tablets</th>
<th>F0</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metoclopramide–HCl (mg)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Kollidon CL-M (%)</td>
<td>-</td>
<td>10</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Dry starch (%)</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Avicel pH 102 (%)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Spray Dried lactose (%)</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Hydroxy propyl Cellulose (%)</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Talc (%)</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Aerosil (%)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stearic acid (%)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Stevia (mg)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mannitol until (mg)</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

Weighed materials as needed, the material was not crushed because the expected shape of granular excipients. Then mix all ingredients (except magnesium stearate, talc and Aerosil). Ingredients are mixed for 15 minutes until homogeneous, then add magnesium stearate, talc and Aerosil mixed for 2 minutes. Conducted an evaluation of the tablets, including weight type, grade incompressible, flow rate, moisture content. Then compress the granules using 8 mm punch. Tablets produced and be evaluated.

**Evaluation the Tablet**

Homogeneity Test Homogeneity test carried out by specifying the content of the print sample tablets by UV spectrophotometry. Samples will be weighed the equivalent of 10 mg metoclopramide–HCl. Samples were dissolved in 100.0 mL of distilled water. Dipipet as much as 1.0 ml solution add 50.0
mL with distilled water in a flask peck. Then the absorbance was measured at a wavelength of 309.0 nm. Each sample was tested 3 times.

**Powder Density**

**True Density**

Determined by picnometer using liquid paraffin. Density measurement was performed in the 25 mL pycnometer.

**Real density**

Defined by using the measuring cup 250 mL. 100 g powder print the tablets inserted into the measuring cup and measure its volume. Apparent density was calculated using the formula:

\[
\text{Real BJ} = \frac{\text{Weight}}{\text{Volume}}
\]

**Density incompressible**

Determined on a tap. 100 g powder of the print is inserted slowly into 250 mL measuring cup, then the volume of compressed powder by tapping a measuring cup with a speed of one beat per second. Having obtained a constant volume of incompressible (v), density (ρt) can be calculated using the formula:

\[
\text{Density incompressible} = \frac{\text{Weight}}{\text{Volume}}
\]

The results can be seen in Appendix 4, Table V.10

**Flow rate**

Defined by the funnel method. 100 g powder of the print is inserted into the funnel contained in the instrument. Prepared at the bottom of the container receptacle. Close the funnel was opened for powder flow. Flow rate is determined by calculating the time required by a number of powder to fall through the funnel flow rate testers.

The flow of granules will be considered good if the print through a funnel as much as 4 grams per second.

**Determined with a moisture balance.** 1 g of powder is put into the aluminum plate, flatten and then insert it into the tool. Heat at a temperature of 70 °C, and then look at the numbers listed on the appliance.

**Tablet Evaluation**

**Organoleptic evaluation**

Visually observed, including appearance, odor and taste. Observed whether there unhomogeneity colors or not, the form of tablets, the surface defect or not, and should be free of stains or spots.

**Size Uniformity**

Measured diameter and thickness of tablet of 20 times using a shove. Tablet diameter not more than three times and not less than one third of the thickness of the tablet.

**Weight Uniformity**

20 tablets taken randomly and then carefully weighed each tablet. Calculated weighted average and deviation of average weight. No one deviates tabletpun weight greater than average weight set of columns A and B.

**Tablet Hardness**

Performed using a hardness tester with 20 tablets taken randomly. Then calculated the average.

**Friability test**

Performed using tools friabilator on 20 tablets taken randomly. Tablets taken at random and then cleaned one by one with a fine brush, and then weighed. Then entered all the tablets into the appliance, and then rotated by 20 turns per minute for 5 minutes. Then the tablets be cleaned again and weighed to determine the reduced weight.

\[
\text{Percentage of Friability} = \frac{(\text{initial weight-final weight tablets tablets})}{(\text{initial weight of the tablet})}
\]

**Content Uniformity**

Content uniformity test done because the weight of the active substance <50 mg, or weight of the active substance ≤ 50% weight of the tablet. 30 tablets taken randomly, and then taken 10 tablets and are determined one by one at a wavelength of 309.0 nm. If there is a tablet that is beyond the
limits 85-115%, prescribed 20 tablets remaining are considered eligible if only one tablet of 30 tablets that provide results beyond 85-115%.

**Disintegration time**

One tablet inserted in each tube of the basket, one disc is inserted in each tube and then the appliance. Used aquades at 37 ° ± 2 ° C as the media.

Water Absorption Ratio and wetting time. Conducted to determine the ability to absorb water tablets and find out the speed of a tablet to absorb water. Performed on 5 cm diameter Petri dish, lined with 2 layers of tissue that had formed a circle with a diameter of 5 cm. Next to it is added 6 mL distilled water. Let stand until water can be absorbed by the entire surface of the tissue. Weighed tablets initial weight (Wa), then place the tablet test at the center of a petri dish. Calculated time required for water to wet the entire surface of the tablet. Weigh the tablet that has been wetted (Wb). Then the calculated value of absorption ratio with the formula:

\[ \text{Absorption ratio} = \frac{W_b - W_a}{W_a} \]

**Dissolution test**

The tools used dissolution method stirrer wing, consisting of a glass vessel with a round base, which is placed in a water bath equipped with a thermostat to maintain the temperature to remain constant. In rowing vessel is placed in the middle of the rotation speed 50rpm. Used distilled water as much as 900 mL of dissolution media and the temperature maintained at 37 ° ± 2 ° C by the thermostat.

![Dissolution rate profiles of metoclopramide HCl fast dissolvinf tablet](image)

**DISCUSSION**

Metoclopramide hydrochloride is used to treat nausea and vomiting. Patients with both nausea and vomiting caused by pregnancy, chemotherapy drugs, gastroenteritis, intestinal tract disorders, or...
peritonitis, postoperative. Patients who experience nausea and vomiting is often difficult to swallow tablets usual anti-vomiting conventional. Thus, anti-vomiting drugs tablets better if made with the technology Fast dissolving or sometimes called Desintegration Orally Tablet.

The materials used for metoclopramide hydrochloride and Kollidon CL-F is important because both of these raw materials which play an important role in fast-dissolve tablet formulations such. Results from pemeksaan both meet the requirements of Indonesian Pharmacopoeia IV edition, and Handbook of Eksipient edition IV. Checking the purity of metoclopramide hydrochloride titration carried out free water, and obtained the results of 100.84%.

In this research, the Fast dissolving tablets made with variation 3 formulas and a formula as the formula used for comparison. Direct compression method was used because this method is more practical and easily understood than the method of making another Fast dissolving tablet. Although it should be used eksipient helpers with better quality the price is quite expensive when compared with conventional tablet excipients, such as spray dried lactose as filler. This formula should also use a fairly dry binder as to ensure the ability to be compressed even compress for Avicel PH 102 at a concentration of 20-90% can be used as a binder.

In the first formula (F0) is not used Kollidon CL-F, only used amprotab since F0 is used as a comparison. Used Kollidon CL-F 10% (F1), 15% (F2) and 20% (F3). This is done to determine the variation of disintegration time, dissolution rate, the ratio of water absorption and wetting time of tablets in each formula.

A tablet will be uniform content of each tablet when secured by the homogeneity of the print, the results of the evaluation showed good results for the homogeneity of the print that will be felt. Because this formula is made direct print, then the print must mempunyasi kompressibilitas flow rate and good, so you can easily print, has a weight uniformity and good uniformity of dosage. Flow rate improved with the use of excipients which granular form to reduce fines when printed, so use Lactose Spray Dried for filler in combination with Avicel PH 102. Evaluation of flow rate and kompressilitas show good results

Hygroscopic properties of Kollidon CL-F and mannitol hard to avoid, although storage with silica gel and the use of the dryer cabinet, but on the F3 with the composition of CL-F Kollidon highest (20%) still have a high moisture content is 4.85%. However, during the printing process, it does not cause capping, lamination or picking.

Evaluation carried out on tablets so from the four tablet formula includes the examination of the uniformity of size, weight uniformity, hardness and brittleness, water absorption ratio, wetting time. In organoleptic, shape and surface of the tablet F0, F1 and F2 both flawless, but the F3 surface brittle and bad. This is caused by poor tablet hardness, which is caused by the composition of Kollidon CL-F high which resulted in the more humid tablet printing so difficult to print well. Metoclopramide bitter taste is still difficult to offset with the use of mannitol and stevia, but if the concentration os stevia be increased the tablets will taste more bitter because stevia has distinguished bitter after taste. If the amount of mannitol who improved, print the tablets would be difficult to flow due to very poor flow properties of mannitol and tablets will stick to the punch because mannitol is very hygroscopic. Uniformity test metoclopramide hydrochloride tablet size is done by measuring the diameter and thickness of 20 randomly selected tablets of each formula with a shove. Diameter of tablets of each formula is the same as used the same punch that is 0.830 cm, while the thickness of the tablet from the formula ranges from 0.410 to 0.435 cm and acceptable and fulfill the requirements of the third edition of Pharmacopoeia Indonesia tablet diameter not more than 3 times and not less from 1 1 / 3 times the thickness of the tablet.

Test results metoclopramide hydrochloride tablet weight uniformity of F0, F1, F2 and F3 have a pretty good weight uniformity of 204.09 ± 3.14; 201.50 ± 7.25; 204.89 ± 5.52; 200.87 ± 5.58 mg. Deviations from the entire weight of the tablet formula ranged from 1.127 to 2.763%. The whole formula tablet weight uniformity requirements based on the Indonesian Pharmacopoeia third edition
of not more than 2 tablets each weight deviates from its average weight is greater than 7.5% and not a single tablet that weighs even deviate from its average weight is more than 15%.

Results The average hardness test for tablets F0, F1, F2 and F3 respectively was 4.7 ± 0.48; 4.6 ± 0.52, 3.6 ± 0.52, 2.5 ± 0.53 Kg/cm³. Tablet hardness tests carried out to observe the strength of tablets. In addition, tablet hardness may reflect its ability disintegrated in the dissolution media. Tablet F0, F1 and F2 showed good results as per the requirement, namely 4-6 Kg/cm³. However F3 showed poor tablet hardness, it caused the print kompressibilitas higroskopisitas and F3, so it is difficult at Felt. Test results for tablet keregasan F0, F1, F2 and F3 respectively 0.46%, 0.41%, 0.27% and 1.10%. The test results for F1, F2 and F3 meet the requirements of not more than 1%. The test results for F3 bad, because poor compressibility test keregasan tablet tablet aims to find the resistance against friction and mechanical shocks during the manufacturing process and delivery.

The result of content uniformity test of tablets F0 - F2 either of between 100.52 to 100.97% with relative standard deviation ranged from 2.27 to 3.61. The results show that the formulas F0, F1 and F2 meet the requirements of content uniformity based on the Indonesian Pharmacopoeia IV edition that is not less than 90.0% and should not be more than 110.0%, but in F3 there is a tablet that contains 89.77% so that transactions are carried out tests on 20 tablets remaining. Of the 30 tablets F3, not to exceed 1 tablet whose levels are less than 90% or exceed 110%. So F3 still qualify tablet content uniformity, which is worth 99.81%.

Testing the disintegration time is important to know the time terdispersinya drug, because the purpose of the making of this formula is expected velocity dispersion so that the tablet can be destroyed very quickly. Kollidon CL-F has the ability berdispersi with swelling mechanism or expand, forming large pores so that water can quickly get into the inside of the tablet. Tablets which will expand very easily destroyed. From the evaluation results, tablet disintegration time F0, F1, F2 and F3 respectively in the second, scoring 168 ± 15.31; 28 ± 3.61, 20.67 ± 1.53 and 10 ± 2.65. According to several journals, destroyed a good time for quick dissolve tablet is less than 30 seconds. F0 as the comparison does not have destroyed a good time.

Test the water absorption ratio and time of wetting aims to determine the capabilities and speed of a fast dissolving tablet to swell by absorbing saliva which is expected later broken tablet in the mouth. The ratio of water absorption and wetting time is an important criteria for understanding the capacity desintegran which showed the drug more easily swallowed with a little water. From the evaluation results, the ratio of water absorption of F0, F1, F2 and F3, respectively, 12.21% ± 1.29; 57.96% ± 11.39; 97.98% ± 4.42 and 103.14% ± 4.01; 71.4 ± 0.53, 68.17 ± 3.05 and 66.73 ± 1.55 seconds. The evaluation results of water absorption ratio and wetting time indicates F0 as a blank tablet has a poor ability to absorb water, when compared with F1, F2 and F3 as F0 not using Kollidon CL-F as desintegretor. F0 tablet ability to absorb water that is long enough for 103.33 seconds. F3 shows the water absorption ratio of the largest and most rapidly absorb water.

Metoclopramide tablet dissolution test was performed in about 900 mL of distilled water as a medium. Dissolution test results of metoclopramide hydrochloride in formulas F0, F1, F2 and F3 respectively has increased significantly. There are significant differences when viewed between F0 with F1, F2 and F3. F0 as an blank showed good dissolution results, which is just as much as 74.62% are substances that dissolve for 30 minutes. Meanwhile, according to the requirements of Indonesian Pharmacopoeia IV edition, metoclopramide hydrochloride tablets tolerance must be dissolved within 30 minutes of not less than 75% of the solute of concentration on the label.

CONCLUSION

From the research it can be concluded that the fast dissolving tablet formulation of metoclopramide hydrochloride using superdisintegran Kollidon CL-F with a concentration of 10%, 15% and 20% can increase the disintegration time, the ratio of water absorption and dissolution. Kollidon CL-M proved to be used as a good superdisintegant, shown from the results of the evaluation of
Fast Dissolving Tablet Formulation

This is because the mechanism of the disintegration of Kollidon CL-F that expands thereby increasing the porosity and water absorption into the tablet. From the three formulas are added Kollidon CL-F as a superdisintegrant, only 2 formulas that show the optimal results overall evaluations conducted. The most optimal formulation is F2, a solid tablet with good quality for the process been seen packing and distribution of the friability test while fulfilling the requirements of disintegration time, water absorption ratio and time of wetting and dissolution rate as the main requirement of a rapid dissolve tablet.

In addition, the dissolution of good support from the success of both the formula. F3 said is not good enough because the tablets are brittle so there are many defects due to the composition of Kollidon CL-F which resulted in too many tablets to be very hygroscopic and brittle.

REFERENCES

IN VITRO ANTIBACTERIAL ACTIVITY OF NIGELLA SATIVA SEEDS AGAINST STREPTOCOCCUS PYOGENES

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ABSTRACT

Antibacterial activity of Nigella sativa seeds against Streptococcus pyogenes was investigated. The seed extracts were prepared by soxhlet extracted method using n-hexane, ethyl acetate, and ethanol. Several concentrations of Nigella sativa extract were tested by the well diffusion method. The inhibition zones of the Muller Hinton agar in different extract concentrations such as 20%, 40%, and 80%, showed that n-hexane extract was found to be inactive against Streptococcus pyogenes, while ethanolic extract at 80% showed the biggest inhibition zones. Bioautography assay of ethyl acetate extract and ethanolic extract showed there were flavonoid and saponin compounds that inhibited Streptococcus pyogenes growth.

Key words: Nigella sativa, antibacterial activity, bioautography, Streptococcus pyogenes

INTRODUCTION

Nigella sativa is commonly known as black seed which belongs to botanical family of Ranunculaceae. The seeds have been claimed to have several traditional medicinal properties (Roshan, et al., 2010). The seed extracts and its essential oil have been reported to exhibit many pharmacological effects including antidiabetic (Khanam, et al., 2008), antiinflammatory (Elbandy, et al., 2009), antibacterial (Zuridah, et al., 2008), anti oxidant (Yoruk, et al., 2010), and anti stress (Roshan, et al., 2010).

Pharyngitis (strep throat) is common illness in Indonesia. It may happen because of bacteria, such as Streptococcus pyogenes. Beside the anti-inflammatory agent, it needs an antibacterial agent to cure the illness. Nigella sativa is becoming the alternative medicine for strep throat, because of it anti-inflammatory activity and antibacterial activity. The aim of this study is to determine the antibacterial activity of several seed extracts from Nigella sativa against Streptococcus pyogenes.

METHODOLOGY

Plant material and extraction procedure

The Nigella sativa seeds were purchased from a local herbal shop in Semarang, Indonesia. A modification of soxhlet extraction procedure by Ali, et al. (2007) was used. The seeds of Nigella sativa were powdered in a mixer. 30 g Nigella sativa seed powder placed in a soxhlet and extracted with 300 ml n-hexane at 70°C. The plant residue in soxhlet, was then re extracted with 300 ml ethyl acetate at 80°C. The lastly, the plant residue in soxhlet was extracted with 300 ml ethanol at 70°C. All the extract, n-hexane, ethyl acetate, and ethanolic extracts were each evaporated after extraction using a vacuum rotary evaporator.

Bioautography assay

Volatile oil, flavonoid and saponin compound of n-heksan extract, etil acetate extract, and ethanolic extract have been analyzed using thin layer chromatography (TLC) with silica gel GF 254 plates (Merck), according to the method of Wagner and Bladt (1996) and contact bioautography assay procedure by Gende, et al. (2008). N-hexane extract was used for volatile oil identification using toluene-etil asetat (93:7) as the mobile phase. Etil acetate extract and ethanolic extract were used for flavonoid and saponin identification. Flavonoid identification used butanol-asam asetat-air (4:1:5) as the mobile phase. Saponin identification used kloroform : metanol : air (64 : 50 : 10) as the mobile phase.
The stationary phase that used for identification, was placed on the inoculated plates within 30 minutes. The plates were incubated at 37°C for 24 hours. Microbial growth inhibition zone was determined by measuring the area of inhibition zones. The inhibition zone was calculated as average of three measurements per TLC plate.

**Determination of antibacterial activity**

*Nigella sativa* seeds extract were tested against *Streptococcus pyogenes*. This strains were obtained from Health Laboratory Office, Semarang.

Antibacterial activity was determined by the well diffusion method. Muller Hinton agar plates were cultured with *Streptococcus pyogenes* for 24 hours. The inoculum size was adjusted to 1 MacFarland I turbidity standard (3x10^8 cfu/ml). Muller Hinton agar plates were inoculated with *Streptococcus pyogenes* suspensions using spread method. The dried plant extracts were dissolved in polyethyleneglicol (PEG) to give concentrations of 20%, 40%, and 80% (w/v). Wells (1 cm diameter) were cut into the agar and filled with 50µl of the plant extract. The inoculated plates were incubated at 37°C. The antibacterial activity was evaluated by measuring the diameter of the inhibition zone. The experiment was repeated five times for each concentration and the mean of diameter of the inhibition zones was calculated.

**RESULTS AND DISCUSSIONS**

The ability of an antibacterial agent to inhibit bacterial growth in vitro may be estimated by the diffusion method. This study investigated the antibacterial effect of n-hexane, ethyl acetate, and ethanolic seed extract on *Streptococcus pyogenes*. The n-hexane extract did not show the antibacterial activity. TLC analysis of volatile oil in n-hexane extract showed there was one blue colour spot (Rf 0.28) using anisaldehyde-sulphonic acid, but did not show inhibitory effect against *Streptococcus pyogenes* on bioautography assay. TLC analysis of flavonoids in ethyl acetate extract and ethanolic extract showed one spot (Rf 0.67) in each extract. It is possible that flavonoid can be extracted with both ethyl acetate and ethanol. Bioautography assay of flavonoids in ethyl acetate extract and ethanolic extract showed an inhibitory zones with 1.363 cm diameter for ethyl acetate extract and 1.281 cm for ethanolic extract. TLC analysis of saponins in ethyl acetate extract and ethanolic extract showed different spots with different values of Rf, 0.88 cm (ethyl acetate extract) and 0.77 cm (ethanolic extract). The diameters of the inhibition zones of saponin for ethyl acetate extract and ethanolic extract were 1.296 cm and 1.371 cm (Figure 1).

The bioautography technique was employed to define the active constituents (Iskan, et al., 2002). There are two constituent in *Nigella sativa* seeds which are flavonoid and saponin can inhibited *Streptococcus pyogenes* growth. Saponin have shown activity against a broad range of microorganism including bacteria, filamentous fungi and yeast (Kredy, 2010).

![Bioautography assay of flavonoids (A) and saponin compound (B) in ethyl acetate extract and ethanolic extract](image-url)

Figure 1. Bioautography assay of flavonoids (A) and saponin compound (B) in ethyl acetate extract and ethanolic extract
In this study, we investigated the antibacterial effects of n-hexane, ethyl acetate, and ethanolic seed extracts on *Streptococcus pyogenes* (Table 1).

Table 1. Inhibition zones of ethyl acetate extract and ethanolic extract at various concentration against *Streptococcus pyogenes*.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Zone of inhibition (cm)</th>
<th>20%</th>
<th>40%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate extract</td>
<td></td>
<td>1.358</td>
<td>1.608</td>
<td>2.177</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td></td>
<td>1.963</td>
<td>2.163</td>
<td>2.319</td>
</tr>
<tr>
<td>Positive control</td>
<td></td>
<td>2.292</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

There are significantly different inhibition zones from various kind of solvent and concentration extract. The ethanolic extract gave higher value compared to ethyl acetate extract. It is possible because of the polarity of the flavonoid and saponin are similar to ethanol. So, it can good extracted by ethanolic solvent. The best inhibition was seen at 80% ethanolic extract. The inhibition zones showed higher value on the higher concentration, due to the concentration of constituents of the extract.

The end-point of inhibition is judged by the naked eye at the edge where the growth starts, but there are exceptions like the heaped-up growth characteristics (Zuridah, et al., 2008). Ciprofloxacin (50mg/L) and PEG were used on the MHA for positive control and negative control, respectively.

In this preliminary study, Minimum Inhibitory Concentration (MIC) of the extract was not carried out, however the diameters of *Streptococcus pyogenes* were reported here. This study showed that the ethanolic extract of *Nigella sativa* seed at 80% concentration had the best antibacterial activity.

**CONCLUSION**

In our study we have found that ethyl acetate and ethanolic extracts of *Nigella sativa* seed, which its compounds were flavonoid and saponin, could be used as antibacterial agent against *Streptococcus pyogenes*. More studies may be needed to make final conclusion regarding the use of *Nigella sativa* seed as a good medicinal plant for strep throat.

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EXTRACTION OF ANTIOXIDANT FROM SHIITAKE MUSHROOM (LENTINULA EDODES) ~AN INITIAL STUDY TO FIND NEW ANTIOXIDANT SOURCE~

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ABSTRACT

Shiitake mushroom (Lentinula edodes) is a very popular food in Asia, especially in Japan, China, and Korea. It is cultivated in oak log or sawdust block (mushroom bed). We obtained extract from shiitake mushroom and mushroom bed, and tested their antioxidant activity. The result indicated that the extracts contain antioxidant materials which was proven by DPPH radical scavenging activity. IC_{50} was 1.08mg/mL for the extract from mushroom cup, and 1.33mg/mL for that from mushroom stem. Instead of the mushroom, the mushroom bed also contained antioxidant as well as the mushroom. The extract of the mushroom can be applied for antioxidant source for medicine, while that of the mushroom bed may be applied as an additive substance for animal feed.

Key words: shiitake mushroom, antioxidant activity, DPPH.

INTRODUCTION

Shiitake mushroom (Lentinula edodes) is a popular food in Asia, especially China, Japan, and Korea. It is available in the market as fresh and dried vegetables. Shiitake attributes not only nutritional value, but It is also known as medical mushroom due to its potential for therapeutic applications, such as depressed immune function, cancer, environmental allergies, and so on (Bisen et al., 2010).

Shiitake mushrooms have a high nutritional value and contain several bioactive compounds, including polysaccharides, dietary fiber, ergosterol, vitamin B1, B2, and C, folates, niacin and minerals (Jiang et al., 2010). Shiitake mushroom also contains vitamin D, especially after exposed under sunlight. Moreover, mushrooms contain various polyphenolic compounds recognized as excellent antioxidants (Ishikawa et al., 1984).

In the past, shiitake was cultivated natural hardwood log, but recently it is also cultivated in sawdust block. The sawdust block is used only once for shiitake cultivation, and after mushroom harvesting, the block remains as by-product.

In the present study we extracted shiitake mushroom and the sawdust block after shiitake mushroom cultivation (by-product), and investigated their antioxidant activity. We expected that not only the mushroom having antioxidant activity, but the remaining sawdust block is also contains antioxidant. The objective of the study is to find new source of natural antioxidant.

METHODOLOGY

Materials

Shiitake mushroom was purchased from supermarket, distributed by JA (Japan Agriculture cooperation) central of Ehime Prefecture, Japan. By-product of Sawdust block (hereafter called as mushroom bed) was obtained from a shiitake farmer in Ozu city, Ehime Prefecture, Japan. DPPH (2,2-Diphenyl- 1-picryl-Hydrazil) was purchased from Sigma-Aldrich, Inc., Japan. 11.8mg of DPPH was dissolved in 100mL of 99.9% ethanol (Kyowa Hakko Bio, CO., LTD.). MES [2-(N-Morpholino) ethane sulfonic acid] buffer was prepared by dissolving 0.70g of MES (Nacalay Tesque, Inc., Japan) in 200mL of...
distilled water, then pH was adjusted to 7.4 by adding 0.1M NaOH aqueous solution. 100ppm (+)-catechin was made by dissolving 0.010g (+)-catechin hydrate (Tokyo Chemical Industry, Co. LTD., Japan). This cathecin was used as positive control for DPPH radical scavenging analysis. Linoleic acid (Nacalay tesque, Inc., Japan) was used to test the peroxide value.

**Extraction process**

Shiitake cup was cut into small pieces (about 3-5mm in size). Fifty grams of it was transferred into beaker glass, and was added with 150 mL of distilled water, then was heated at 80°C for 2hrs. Extract of the shiitake mushroom was separated by centrifugation at 3500 rpm for 10 minutes. Another 150 mL of distilled water was added again, then the heating and extract separation process was repeated. The extracts were collected, and the volume was adjusted to 300mL. Similar extraction procedure was also done for mushroom stem, with the ratio of stem and distilled water of 8g/100mL. Mushroom bed was cut into small pieces. Above extraction process was carried out for the mushroom bed with final solid/solution ratio of 50g/1000mL. We did different solid-solution ratio, because the mushroom bed has very low bulk density.

Water content of the mushroom and of the bed was measured by heating the materials at 70°C for 24h. The difference between fresh weight and dry weight is water content. Finally the extract was diluted so as to give same ratio of solid/solution (distilled water). Solid content of the extracts were determined, by evaporating 10 mL of each extract in evaporation dish.

**DPPH radical scavenging activity**

DPPH radical scavenging activity of the extracts was determined by the method of Hu et al. (2009). Various amounts (0.05mL to 1mL), of the extracts were put in test tube glass, then ethanol was added to each sample in order to make total volume of 1.0mL. One mL of MES buffer was added, followed by addition of 1.0mL of DPPH. The sample was mixed well, then was allowed for 30 minutes. After 30 minutes the absorbance of the samples were measured at 522nm. Relative scavenging activity was calculated as follow:

\[
\text{RSA} \% = \frac{(A_0 - A_1)}{A_0} \times 100 \%
\]

Where A0 is absorbance value of DPPH blank, A1 is absorbance value of sample.

**Inhibition of Linoleic oxidation**

Inhibition of linoleic oxidation was assessed from peroxide value after oxidation of linoleic acid. To each 0.2g of linoleic acid was added with various amounts of the mushroom extract from 1mL to 5mL, then was heated at 60°C in an oven for 12hours, to accelerate oxidation process. After the oxidation, peroxide value was measured according to European Pharmacopoeia 5.0 (2005), with little modification of solvent from chloroform and glacial acetic acid (2:3) to cyclohexane and glacial acetic acid (1:1). A blank run was done without adding shiitake mushroom extract. Same experiment was carried out for the extract of mushroom bed. Lower peroxide value means greater inhibition of linoleic oxidation (greater antioxidant activity).

**RESULTS AND DISCUSSIONS**

Water content of the samples was 90.20%, 40.14%, for Shiitake mushroom and mushroom bed, respectively. The mushroom bed extract was diluted so as to give same solid/solution ratio with that of shiitake mushroom (16.77g/L, based on dry weight). The solid content of the both extracts was not so different: 6.27mg/ml and 6.07mg/mL for mushroom extract and mushroom bed extract, respectively. Figure 1 shows DPPH radical scavenging activity of the samples.
Fig. 1 DPPH radical scavenging activity of the extract samples

The synthetic DPPH free radical is widely used to determine antioxidant activity. Absorption maximum at 517nm and its purple colour (Debnath et al., 2010). When it is mixed with antioxidant, the antioxidant compound will donate an electron to DPPH, so the purple colour will be changed to light purple or light yellow, depending upon the antioxidant content. Our measurement of DPPH absorbance showed that DPPH has absorption maximum at 522nm, so we measured the absorbance at 522nm.

Relative scavenging activity (RSA) increases with increasing amounts of the extract. The RSA is greatest for mushroom bed, followed by shiitake cup and shiitake stem. The high RSA for shiitake mushroom bed may be not only come from shiitake, but also the bed itself (sawdust) contains antioxidant compounds.

Fig. 2 Effect of mushroom and mushroom bed extract on peroxide value of linoleic acid after oxidation

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IC$_{50}$ is effective concentration of an antioxidant compound to inhibit half of free radical (RSA equal to 50%). IC$_{50}$ is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. The IC$_{50}$ of the extracts was 1.33mg/mL, 1.08mg/ml, and 0.30mg/mL for the shiitake cup, shiitake stem, and mushroom bed, respectively. The IC$_{50}$ of (+) cathecin was 4.16µg/mL. It is expected that the shiitake mushroom contains ergothioneine as antioxidant compound. It is necessary to analyze ergothioneine content.

Figure 2 shows effect of the mushroom and mushroom bed extract for inhibition of linoleic oxidation, assessed by peroxide value. Peroxide value is an empirical measurement of the amount of oxidation that has occurred to oils or fats that have occurred during storage.

We used peroxide value to assess the inhibition of lipid oxidation by the mushroom extract. Peroxide value is the number that expresses in milliequivalents of active oxygen contained in 1000g of substance. Linoleic acid was used as representative of lipid. Linoleic acid is unsaturated lipid possessing two double bonds, at atom C number six and nine, so it is called as omega-6 (Ω-6). The principle of the experiment is that when unsaturated lipid is oxidized, peroxide is formed, then the formed peroxide is reduced by I$_2$ (KI) to form I$_2$. Thereafter the I$_2$ is titrated with 0.01M NaS$_2$O$_3$, with soluble starch as indicator.

![Chemical structure of linoleic acid](image)

$2\text{I}_2 + \text{H}_2\text{O} + \text{R-O-O-H} \rightarrow \text{ROH} + 2\text{H}^+ + \text{I}_2$

$2\text{S}_2\text{O}_3^{2-} + \text{I}_2 \rightarrow \text{S}_4\text{O}_6^{2-} + 2\text{I}^-$

The results indicated that without adding of mushroom extracts peroxide value of linoleic acid considerably increased from 19 to 1529 meq/kg after oxidation (heating at 60°C for 12h). However, with mushroom extract treatment the peroxide value is lower as compare to the blank (without extract treatment). This indicates that shiitake mushroom extract inhibits oxidation of linoleic acid. Inhibition degree increased with increasing amounts of extract, and reached optimum at 18.8 mg/0.2g linoleic acid. It seems that ergothioneine contained in the mushrooms play important role in inhibitions effect of oxidation of linoleic acid. Our molecular orbital calculation proved that deprotonation occurs at S-H functional group in the structure of ergothioneine (calculation result is not shown here). The released proton inhibits oxidation of linoleic acid.

In case of mushroom bed extract, small amount of extract is very effective to prevent oxidation of linoleic acid. By adding 6.1mg of the extract, the peroxide value decreased from 1529 meq/kg (blank) to 148 meq/kg. However, the degree of inhibition of linoleic oxidation decreased with increasing amounts of the extracts, may be due to mushroom bed extract contains many substances that give positive and negative effects to the oxidation process.

Both shiitake mushroom and mushroom bed have antioxidant activities, so it is possible to apply this kind of extract for the human medicines and for additive substance for animal feed. Our previous project showed that mushroom bed extract gave inhibitory effect on oxidation of dark flesh of yellow tail fish.
CONCLUSION
Shiitake mushroom extract and mushroom bed extracts contain antioxidant substance. The presence of the antioxidants was proven by DPPH radical scavenging activity and inhibition of linoleic oxidation.

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SYNTHESIS AND ANTICANCER ACTIVITY OF ANTIMYCYIN A₃ ANALOGUE

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ABSTRACT

Novel polyhydroxylated 18-membered analogue of antimycin A₃ was synthesized. Our synthesis was commenced with Boc-L-Threonine and was achieved by way of one-pot homocoupling of ring-closing metathesis reaction and Sharpless asymmetric dihydroxylation. The analogue exhibited a greater anticancer activity against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells than that of the original antimycin A₃.

Key words: Synthesis, Anticancer, Antimycin A₃, Analogue

INTRODUCTION

Antimycin A₃, a nine-membered dilactone which was isolated from Streptomyces sp. in 1949, is one of the most active agents that inhibits the electron transfer activity of ubiquinol-cytochrome c oxidoreductase and prevents the growth of human cancer cells. Antimycin A₃ was also found to induce apoptosis of cancer cells by selectively killing the cancer cells that expressed high levels of anti-apoptotic Bcl-2 and Bcl-xL.¹⁻⁴ Studies on the structure-activity relationship of antimycin A₃ by Miyoshi et al. in 1995 revealed that the hydroxyl group and 3-formamido group in the salicyl moiety, as well as amide bond were necessary for anticancer activity. On the other hand, the nine-membered dilactone core in antimycin A₃ was found less effective for anticancer activity than 3-formamidosalicyl moiety.⁵ These facts indicated that, it is quite possible to carry out the synthesis of antimycin A₃ analogue by replacing the nine-membered dilactone core with another active core that contributes to the improvement of its anticancer activity. Therefore, in this work, we focused on the synthesis of antimycin A₃ analogue (I), in which the nine-membered dilactone core of antimycin A₃ was replaced by a polyhydroxylated 18-membered tetralactone core in our analogue (Figure 1). It has been reported that 18-membered ring of polylactone, such as respirantin which has the similar structure with antimycin A₃ showed a potent antitumor activity against human cancer cell lines with IC₅₀ range 0.00018 to 0.47 µg/mL. Furthermore, respirantin with IC₅₀: 0.0037 µg/mL showed a stronger cytotoxicity than antimycin A₃ (IC₅₀: 0.015 µg/mL) against mouse Leukemia P-388 cells.⁶ It is also known that the presence of hydroxyl groups in biologically active compound significantly increases its biological activity due to the enhancement of its solubility in water, which is the one of important factors influencing the efficacy of drugs.⁷ Thus, the polyhydroxylated 18-membered tetralactone core in our analogue is expected to be active as an anticancer agent. Moreover, introduction of the 3-formamidosalicyl group into this core, is expected to synergistically improve its anticancer activity compared to that of the original compound antimycin A₃.

Figure 1. Structure of antimycin A₃, respirantin, and desired antimycin A₃ analogue (I)
**METHODOLOGY**

The retrosynthetic analysis of antimycin A analogue is outlined in Scheme 1. As shown, analogue 1 can be built by amidation of tetraol 3 with 3-formamidobenzoic acid (2), followed by hydrogenolysis of the benzyl group. Tetraol 3 can be prepared from Boc deprotection of 4, whereby 4 can be derived from 18-membered tetractone 5 by way of Sharpless dihydroxylation. Tetraactone 5 can be constructed from one-pot homocoupling ring-closing olefin metathesis of diester 6, and 6 can be synthesized from two-step esterification of commercially available Boc-L-Threonine (7).

![Scheme 1. Retrosynthetic analysis of antimycin A₁ analogue (1)](image)

**RESULTS AND DISCUSSIONS**

Scheme 2 outlines the synthesis of 18-membered tetraol 3. Starting from esterification of Boc-L-Threonine (7) with allyl bromide under basic condition was conducted according to procedure provided by Wu et al.,12 afforded Boc-L-Threonine-allyl ester (8) in 96% yield. Condensation of ester 8 with acrylic acid employing the combination of DIC and DMAP occurred easily to afford 92% yield of diester 6. In the next step, Ru-catalyzed homocoupling ring-closing metathesis (RCM) was used to transform diester 6 into a 18-membered tetractone 5. Three kinds of Ru-catalyst, Grubbs 1st generation, Grubbs 2nd generation, and Zhan catalyst 1B,13 were tested in this metathesis reaction (Figure 2). Homocoupling RCM of diester 6 with the less reactive Grubbs 1st catalyst was failed to form tetractone 5, while the use of the more reactive Grubbs 2nd catalyst provided 5 in only 32% yield. The best result, 73% yield of 5 was achieved by performing the reaction with 4 mol% of Zhan catalyst 1B in concentration 0.1 M at 45°C. Subsequently, Sharpless asymmetric dihydroxylation of 5 with OsO₄, (DHQ)₂PHAL ligand and K₃Fe(CN)₆ as a co-oxidant gave 67% yield of a 12:1 diastereomeric mixture of tetraol products. This mixture can be separated by medium pressure liquid chromatography to afford 61% yield of pure major tetraol 4 which was then treated with with HCl/EtOAc to afford the polyhydroxylated 18-membered tetractone 3 in 81% yield as a white solid. The X-ray crystallographic analysis of 3 clarified its stereochemistry as depicted in Figure 3.
Scheme 2. Synthesis of 18-membered tetraol 3. Reagents and conditions: (a) Allyl bromide, Na$_2$CO$_3$, DMF, rt (96%); (b) Acrylic acid, DIC (diisopropyl carbodiimide), DMAP, CH$_2$Cl$_2$, 0°C to rt (92%); (c) Zhan catalyst 1B, CH$_2$Cl$_2$, 45°C (73%); (d) OsO$_4$, K$_2$Fe(CN)$_6$, K$_2$CO$_3$, NaHCO$_3$, (DHQ)$_2$PHAL, MeSO$_2$NH$_2$, t-BuOH-H$_2$O, 0°C (61%); (e) HCl, EtOAc, rt (81%); (f) 2, DIC/DMAP or EDCI/DMAP or EDCI/HOBt or EDCI/HOSu, NMM.

Figure 2. Structure of Grubbs 1$^{st}$, Grubbs 2$^{nd}$ and Zhan 1B

Figure 3. ORTEP drawing of 3·2H$_2$O

With key intermediate 3 in hand, our subsequent plan was to conduct the amidation of tetraol 3 with 3-formamidobenzoic acid 2 which was prepared according to known procedure as reported previously by Pettit $et$ $al.$ to give tetraol diamide 9. Unfortunately, after extensive attempts with a variety of amidation reagents, such as DIC/DMAP, EDCI/DMAP, EDCI/HOBt and EDCI/HOSu under many reaction conditions, the desired tetraol diamide 9 was not obtained (step f in Scheme 2). We postulated that not only the amine groups but also four hydroxyl groups of 3 can react with carboxylic acid 2, and it might cause byproducts instead of the desired tetraol diamide 9. To solve this problem, we abandoned this strategy, and proposed a new one. The revised synthetic strategy for the synthesis of antimycin A$_3$ analogue 1 is summarized in Scheme 3. We planned to carry out the amidation of non-hydroxylated tetrалactone 11 with acid 2 to give diamide 10 in the initial stage, and then introduce the hydroxyl groups into diamide 10 by Sharpless AD followed by deprotection of Bn group in the late stage, to generate desired analogue 1. By this method, we hoped the amidation of non-hydroxylated
tetralactone 11 with acid 2 will go well without the formation of complicated mixture of byproducts which might be obtained from the esterification of a polyhydroxylated tetralactone with acid 2. The non-hydroxylated tetralactone 11 derived from Boc deprotection of 18-membered tetralactone 5, which we had already prepared in the former strategy.

Scheme 3. The revised retrosynthetic analysis of antimycin A3 analogue (1)

As shown in Scheme 4, tetralactone 11 as bis ammonium chloride salts was formed from Boc deprotection of 5 with HCl. Amidation of 11 with acid 2 could be performed using the base NMM and the combination of EDCI/HOBt with THF as a solvent, afforded diamide 10 in 45% yield. In the next step, Sharples dihydroxylation of 10 in the presence of 6 equiv of NMO with 50 mol % of both OsO4 and (DHQ)2PHAL proceeded smoothly to give tetaol diamide products in 66% yield as a 4:1 inseparable diastereomeric mixture, with 9 as a major diastereomer. As the final step, hydrogenolysis of this diastereomeric mixture with 10% Pd/C resulted in simultaneous cleavage of both Bn group, and afforded a 4:1 diastereomeric mixture of the corresponding Bn deprotected tetaol diamide products, which was successfully separated in this step by medium pressure liquid chromatography to give a pure major analogue 1 in 51% yield. Subsequently, the analogue 1 could be recrystallized readily by methanol. The X-ray crystallographic analysis of 115 has assigned its stereochemistry as shown in Figure 4.

Scheme 4. Completion of the synthesis of antimycin A3 analogue (1). Reagents and conditions: (a) HCl, EtOAc, rt (90%); (b) 2, EDCI, HOBt, NMM, THF, rt (45%); (c) OsO4, NMO, (DHQ)2PHAL, t-BuOH-THF-H2O, rt (66%, 4:1 dr); (d) 10% Pd/C, H2, MeOH, rt (51%).

After completion of the synthesis, cytotoxicities of the analogue 1 and two intermediate products, 18-membered tetaol 3 and 18-membered tetralactone 11 were evaluated as inhibitors of cancer cell growth versus HeLa cells, breast cancer MDA-MB-231 cells, and prostate cancer PC-3 cells (Table 1). Tetaol 3 with IC50 over 4000 µM showed no cytotoxicity against all tested cancer cells. In contrast to tetralactone 11, tetaol 3 which has four hydroxyl groups showed the cytotoxicity in...
concentration 250 µM, 112 µM, and 205 µM against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells, respectively. The cytotoxicity of tetraol 3 is greatly improved by the presence of the hydroxyl groups compared to that of tetralactone 11. This fact suggested that the hydroxyl groups are very important for the anticancer activity. Compared to analogue 1, tetraol 3 which does not possess 3-formamidosalicylyl group, showed less cytotoxicity, suggesting that the introduction of 3-formamidosalicylyl group on 18-membered tetraol moiety in analogue 1 was potentially responsible for increase in its anticancer activity. Moreover, analogue 1 which contains a polyhydroxylated 18-membered core and two parts of 3-formamidosalicylyl groups exhibited a greater anticancer activity than that of the original antimycin A₃ on all cancer cells tested, with IC₅₀: 39, 40, and 43 µM against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells, respectively.

Table 1. Cytotoxicities (IC₅₀, µM) of tetralactone 11, tetraol 3, analogue 1, and antimycin A₃ against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>HeLa</th>
<th>Breast MDA-MB-231</th>
<th>Prostate PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetralactone 11</td>
<td>4514</td>
<td>4289</td>
<td>4002</td>
</tr>
<tr>
<td>Tetraol 3</td>
<td>250</td>
<td>112</td>
<td>205</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>39</td>
<td>40</td>
<td>43</td>
</tr>
<tr>
<td>Antimycin A₃</td>
<td>60</td>
<td>79</td>
<td>50</td>
</tr>
</tbody>
</table>

These results indicated that introducing two parts of formamidosalicylyl groups and replacing the nine-membered dilactone ring of antimycin A₃ with a polyhydroxylated 18-membered ring in analogue 1 was successfully improved its anticancer activity. Thus, analogue 1 which strongly inhibited the growth of HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells, should be considered as a promising candidate for the treatment of human cervical, breast, and prostate cancers.

CONCLUSION

We have synthesized a novel analogue of antimycin A₃ (1) from Boc-L-Threonine through a sequence of reactions. Analogue 1 showed a greater anticancer activity against HeLa cells, breast MDA-MB-231 cells, and prostate PC-3 cells compared to the original antimycin A₃.

ACKNOWLEDGEMENTS

This research was supported partially by grant-in-aid for scientific research from Nara Institute of Science and Technology (NAIST), which is gratefully appreciated. We also thank to Ms. Yoshiko
Nishikawa for High Resolution Mass Spectrometry Measurement, and Mr. Shouhei Katao for X-Ray Crystallographic analysis.

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Crystallographic data for analogue 1 is deposited with the Cambridge Crystallographic Data Centre (Supplementary Publication No: CCDC 813907) and can be obtained, free of charge, on application to CCDC, 12 Union Road Cambridge CB2 1EZ, UK. (email:deposit@ccdc.cam.ac.uk).

Crystallographic data for tetraol 3 is deposited with the Cambridge Crystallographic Data Centre (Supplementary Publication No: CCDC 813908) and can be obtained, free of charge, on application to CCDC, 12 Union Road Cambridge CB2 1EZ, UK.

Surya Dwira, et al.

INTRODUCTION

Malaria is a very dangerous disease and 1.2% of total human deaths caused by this disease. Outbreaks of malaria in Indonesia increased. On the other hand, the malaria parasite, Plasmodium falciparum was resistant to commonly used malaria drugs, so it needs develop new antimalarial drugs. WHO has recommended treatment of malaria with Artemisia annua L. in combination with another drug called Artemisinin Combination based Therapy (ACT), to overcome multiple drug resistance malaria. Artemisinin is very effective against P. falciparum. Artemisinin has a problem like a short plasma half-lives, limitation of bioavailability, less solubility in oil and water performance and the results of artemisinin from natural sources is low. This research is to detect the effectiveness of a drug insilico with docking approach and in-vitro antimalarial test against hemin bioavailibilitas. This is because the chemical structures, physical-chemical properties, chemical reactivity and the ability of drugs to interact with the receptor depends on the electronic structure, composition and interactions of all electrons with molecules. From the research, interaction between hemin with antimalarial in-silico by docking approach indicates that artemisinin derivatives group artesunat (ARTS) has the lowest binding energy compared with other derivatives, and also the level of hydrogen bonding. The interaction of hemin with antimalarial through UV-Vis test showed that at a wavelength of 400 nm, ARTS has a lower free energy of interaction compared with other ligands artemether (ARTE) and artemisinin (ARTM). The existence of a significant interaction between hemin with antimalarial artemisinin derivatives showed no different between insilico and UV-Vis identification.

Key words: artemisinin, insilico (docking), hemin, antimalarial, invitro

ABSTRACT

WHO has recommended the treatment of malaria with Artemisia annua L. in combination with another drug called Artemisinin Combination based Therapy (ACT), to overcome multiple drug resistance malaria. Artemisinin is very effective against P. falciparum. Artemisinin has a problem like a short plasma half-lives, limitation of bioavailability, less solubility in oil and water performance and the results of artemisinin from natural sources is low. This research is to detect the effectiveness of a drug insilico with docking approach and in-vitro antimalarial test against hemin bioavailibilitas. This is because the chemical structures, physical-chemical properties, chemical reactivity and the ability of drugs to interact with the receptor depends on the electronic structure, composition and interactions of all electrons with molecules. From the research, interaction between hemin with antimalarial in-silico by docking approach indicates that artemisinin derivatives group artesunat (ARTS) has the lowest binding energy compared with other derivatives, and also the level of hydrogen bonding. The interaction of hemin with antimalarial through UV-Vis test showed that at a wavelength of 400 nm, ARTS has a lower free energy of interaction compared with other ligands artemether (ARTE) and artemisinin (ARTM). The existence of a significant interaction between hemin with antimalarial artemisinin derivatives showed no different between insilico and UV-Vis identification.

Key words: artemisinin, insilico (docking), hemin, antimalarial, invitro

INTRODUCTION

Malaria is a very dangerous disease and 1.2% of total human deaths caused by this disease. Outbreaks of malaria in Indonesia increased. On the other hand, the malaria parasite, Plasmodium falciparum was resistant to commonly used malaria drugs, so it needs develop new antimalarial drugs. WHO has recommended treatment of malaria with Artemisia annua L. in combination with another drug called Artemisinin-based Combination Therapy (ACT), to overcome multiple drug resistance malaria. Artemisinin is a product of secondary metabolites from plant Artemisia and is highly effective against P. falciparum. Preparation of artemisinin by synthesis is difficult and not economical. Easy and cheap way is to extract from the plant Artemisia. The existence of plants as medicine indicate that it contains bioactive compounds in plants. This compound in Indonesia has been recommended by Department of Health as a drug combination malaria, but artemisinin has a problem, like a short plasma half-lives, limited bioavailability, less solubility in oil and water and low results of artemisinin from natural sources, has urged scientists to develop new synthesis of artemisinin derivatives. One way of research for antimalarial drug design and to investigate the effectiveness of new drugs of the artemisinin derivatives is to use interaction analysis of Hemin on the antimalarial compound artemisinin derivatives. Drug design and effectiveness of a drug can be examined in in-silico and in vitro, this is because the chemical structures, physical-chemical properties, chemical reactivity and the ability of drugs to interact with the receptor depends on the electronic structure, composition and interactions of all electrons with a molecule (Nogrady, 1992).

The development of computational chemistry and Bioinformatics allows for the calculation of quantum mechanics and the calculation of free energy of a compound that can be obtained from the compound which can be expressed with a solubility parameter, Acceptor donor, inhibition concentration and the degree of IC50 of a drug. In addition to insilico test, in vitro test is needed if the process of healing or drug treatment, can be viewed as a process of molecular interaction between
micromolecule compounds with biological molecules from the source or cause disease. This interaction is not static but constantly developing in accordance with the conditions and situational and solvents conditions and environment. Likewise, the interaction between hemin and artemisinin, in the presence of hemin in a large number of malaria parasites cause hemoglobin digested (Sharma, 1984) need prevention of parasitemia in hemoglobin. For optimal function of Antimalarial drug in bioavailability conditions, the solvent must be considered. In previous research, it has been conducted interaction between hemin and antimalarial in aqueous solution (water as the solvent) (Chou et al, 1980). In this medium, the solubility antimalarial is small, antimalarial drug soluble in alkaline solution because of hemin stable in neutral or slightly acidic solution can not be achieved due to a dimer (Bilia et al, 2002).

**METHODOLOGY**

**Preparation of in-silico Test**

**Hemin Preparation**

Three-dimensional structure of hemin can be downloaded from the chemspider. Optimization and minimization of three-dimensional structure of the enzyme were employed using the software of MOE 2009.10. with addition of hydrogen atoms. Protonation was employed with protonate 3D programs. Furthermore, partial charges and force field was employed with MMFF94x. Solvation of enzymes was performed in the form of a gas phase with a fixed charge, RMS gradient of 0.05 kcal / Åmol, and other parameters using the standard in MOE 2009.10 software.

**Ligand Preparation**

Artemisinin derivatives consisting of artesunat, arteether and artemisinin is modeled into three-dimensional structure. This modeling is performed using ACD Lab software. Three-dimensional shape was obtained by storing in the 3D viewer in ACDLabs. Furthermore, the output format was changed into Molfile MDL Mol format using software Vegazz to conform for the docking process. Ligand was in the wash with compute program, adjustments were made with the ligand partial charge and partial charge optimization using MMFF94x forcefield. The conformation structure energy of ligands was minimized using the RMS gradient energy with 0,001 Kcal / A mol. Other parameters were in accordance with existing default in the software.

**Docking simulation of Hemin Docking with Artemisinin and its derivatives:**

The docking process was begun with the docking preparation, that was employed using a docking program from MOE 2009.10 software. Docking simulations were performed by the Compute-Simulation dock program. Placement method was conducted using a triangle matcher with 1.000.000 repetition energy readings each position and other parameters were in accordance with existing default in the MOE software. Furthermore, scoring functions used london DG, refinement of the configuration repetition forcefield with 1.000 populations. The first repetition of 100 times and the second setting was shown only one of the best results.

**Preparation of invitro Test**

**Preparation of Hemin solution and Artemisinin**

Hemin stock solution of 40% aqueous DMSO, 306 µmol / L was prepared with 10 mg gram dissolved in 20 mL DMSO, then added 30 mL of tris buffer. Tris buffer was prepared by mixing different amounts of 0.2 mol / L tris (hidroksimetil)-methylamin and 0.2 mol / L HCl to give pH atmosphere. Then 0.1 mol / L Tris-HCl buffer used in all-aqueous solution of 40% DMSO

**Measurement of pH and Absorption**

pH measurement is done by using a pH meter and previously performed calibration with standard aqueous buffers. Then absorbance measurements were taken using UV-Vis spectrophotometer. Measurements with spektrofotometr to identify the chromophore group of artemisinin and its derivatives which consist of artesunat, artemether. Artemisinin group absorbed weakly at short wavelengths and more difficult for quantification. Then, with titration, characteristics of hemin will appear at a wavelength of 402 by mixing a volume of 0.1 mL solution of hemin with the variation of the volume of drug solution (artemisinin, artesunat and artemether) and then dissolved in
10 mL with the addition of Tris-DMSO mixture. Thus the constant of hemin concentration is 3\(\mu\)mol / L with a range of drug concentrations between 0-200 \(\mu\)mol / L. Before each absorbance is measured, the solution was incubated at 37\(^\circ\)C. UV-Vis spectrophotometer can read the spectrum after the incubation period of 10 to 24 hours. The spectrum reading is in the wavelength of 200-450 nm.

**RESULTS AND DISCUSSIONS**

**Interaction of Hemin with Artemisinin derivatives in the In-silico.**

The docking results showed that the ligand of artemisinin derivatives that can inhibit hemin better has a lower Gibbs energy. The result of docking of artemisinin derivatives as Table 1.

<table>
<thead>
<tr>
<th>Ligan</th>
<th>Compound 1 (ARTM)</th>
<th>Compound 2 (ARTE)</th>
<th>Compound 3 (ARTS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR</td>
<td>282.336</td>
<td>312.406</td>
<td>383.417</td>
</tr>
<tr>
<td>LogP</td>
<td>2.361</td>
<td>2.870</td>
<td>0.907</td>
</tr>
<tr>
<td>TPSA</td>
<td>152.990</td>
<td>145.150</td>
<td>202.350</td>
</tr>
<tr>
<td>HDon</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>HAcc</td>
<td>8</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Energi (kcal/mol)</td>
<td>-3.317</td>
<td>-3.7001</td>
<td>-8.922</td>
</tr>
</tbody>
</table>

Stability and interaction strength non covalent on hemin-ligand complexes can be understood from the formation large bond energy. Bond energy derived from free energy released during the interaction of hemin-ligand complex. From Table 1 there are three ligands; ligand 1, ligand 2 and ligand 3 which has a low \(S\) value. Ligand 3 (ARTS) has a lowest \(S\) value. From the results of binding energy level, then the compound ARTS has a higher stability compared with other artemisinin. It is one of the factors that cause ARTS ligand has a smaller bond energy (-4.8922 kcal / mol) compared with standard ARTM ligand (-3317 kcal / mol). This hydrogen bonding contributes ligand affinity to hemin because the electrostatic interaction between oxygen or nitrogen atom of ligand with a hydrogen or Oxygen atom of hemin or vice versa. Hydrogen bonding occurs between hemin with ligand as Figure 1.

**Ligand conformation Against Hemin**

Hemin has an active site of N, Fe and Cl that can be inhibited by the presence of active compounds. If the active compounds enter and interact with hemin then these compounds can bind hemin. Based on the spectrum of electrostatic potential, the ligand position or ligand conformation in a hole is observed by using the software MOE as in Figure 1.

In Figure 1, the ligand is shown by sticks colored gray and red, hemin is shown with ball and stick gray, red, green and hydrogen bonds formed is shown by dashed lines broken gray. ARTS ligands form hydrogen bonds with the active site hemin and its conformation towards hemin, so ARTS ligands can act as potential inhibitors that can bind to hemin more effective than the ARTM and ARTE ligands.

The docking results indicate that both ATRE and ARTS ligands have appropriate shape and conformation structure to bind N present in hemin. Conformation of the active site of hemin in the form of cavities that can be bound by the active compounds of artemesinin and its derivatives. Based on the spectrum of electrostatic potential, figures 1b and 1c indicated that ARTS ligand forms conformation in accordance with ARTE ligand which can bind N of hemin. The difference charge of
binding site with the ligand allows the interaction between the binding sites with groups N of hemin with -OH group or =O group of ligand that would increase the affinity and stability complex of hemin-ligand. Stability and affinity complex of the active side with ligand is influenced by the bond distance. This indicates that the ARTS ligand form conformation that can change the conformation or in other words can inhibit hemin.

Figure 1. Hydrogen bond between hemin and derivatives of artemisinin

![Heme-ARM](image1)

![Heme-ARTE](image2)

![Heme-ARTS](image3)

Figure 1. Hydrogen bond between hemin and derivatives of artemisinin
Interaction of Hemin with Artemisinin derivatives in the In-silico

Hemin binding reaction with artemisinin, artesunat, and artemether in DMSO-water mixture can be observed from changes in the typical spectrum as shown in Figure 2. The Change of peak absorbance value of absorption spectrum of hemin after addition artemisinin, artesunat, and artemether can be seen in Table 2.

Table 2. The Change of peak absorbance value of absorption spectrum of hemin after addition of artemisinin, artesunat, and artemether

<table>
<thead>
<tr>
<th>ADD (µmol/L)</th>
<th>% change absorbance after ARTM addition</th>
<th>% change absorbance after ARTS addition</th>
<th>% change absorbance after ARTE addition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>55</td>
<td>73</td>
<td>58</td>
</tr>
<tr>
<td>20</td>
<td>72</td>
<td>78</td>
<td>65</td>
</tr>
<tr>
<td>50</td>
<td>82</td>
<td>83</td>
<td>69</td>
</tr>
<tr>
<td>100</td>
<td>84</td>
<td>85</td>
<td>65</td>
</tr>
</tbody>
</table>

Hemin binding reaction with artemisinin, artesunat, and artemether in DMSO-water mixture can be observed from changes in the typical spectrum as shown in figure. And we can conclude that the strength and stability of the interaction between artemisinin molecules or its derivatives against hemin can be seen from the change in absorbance value of the hemin spectrum after addition of these compounds. The Change of peak absorbance value of hemin spectrum after addition of artemisinin, artesunat, and artemether can be seen in Table 2.

Results of interaction analysis of artemisinin compounds or their derivatives with hemin in vitro by spectrophotometric method are consistent with molecular mechanical calculations using molecular docking which indicates that the interaction artesunat has a lower free energy compared with other ligands (ARTE and ARTM). This means that artesunat will be more effectively used as anti-malarials.

CONCLUSION

Interaction analysis of artemisinin compounds or their derivatives with hemin in vitro by spectrophotometric method are consistent with molecular mechanical calculations using molecular docking which indicates that the interaction artesunat has a lower free energy compared with other ligands artemether and artemisinin (ARTE and ARTM).
ACKNOWLEDGEMENT
This research support by Early Grand Researsh of Indonesia University 2010.

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DETERMINATION OF ARTEMISININ IN ETHYL ACETATE FRACTION FROM THE STEMS, ROOTS, LEAVES AND FLOWERS OF MUTANT STRAINS OF Artemisia vulgaris L. BY HPLC

Faridah *, Aryanti**, Desi Nadya Aulena*
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** Laboratory of Plant Breeding, PATIR-BATAN – Jakarta

ABSTRACT

Artemisia vulgaris L. included in the Asteraceae family. This plant contains a bitter substance artemisinin, which is one of secondary metabolite. Artemisinin is an antimalarial agent was found in Artemisia sp medicinal plant, but it is in small quantity. Irradiated plant with better properties than its parent is called a mutant. Before getting the mutant plants, there will be the selection process. The plants that are in the selection process is called a mutant lines. This study aimed to compare the levels of artemisinin in ethyl acetate fraction from the stems, leaves and flowers and roots in mutant lines Artemisia vulgaris L and control plant by High Performance Liquid Chromatography (HPLC) with conditions : mobile phase acetonitrile-water (7:3), the stationary phase C18 flowrate 1 ml / min, UV detector at wavelength 252 nm. The results showed that artemisinin content in Artemisia vulgaris L. control plants is 0.07% (stems), 0.14% (roots), 0.95% (leaves) and 3.70% (flowers), whereas artemisinin content in mutant lines is 0.24% (stems), 0.59% (roots), 10.56% (leaves) and 12.46% (flowers). The largest artemisinin content found in the flower of the mutant lines. Kruskal-Wallis test showed that at 95% confidence level there are significant differences between the control plants with a mutant lines of Artemisia vulgaris L.

Key ords : Artemisia vulgaris L., artemisinin, HPLC.

INTRODUCTION

Indonesia is geographically located on the equator so it has a tropical climate characterized by high air temperatures and rainfall are influenced by the season. So many living things reproduce here, including plasmodium cause malaria. Malaria is a disease caused by single-celled parasites classified as obligate intracellular protozoan of the Plasmodium falciparum which is the most dangerous species to human because it can cause acute and severe infection and can cause death. During this mosquito eradication efforts carried out by spraying, but this has caused a new problem such as environmental pollution. While the treatment of malaria with chloroquine synthetic compounds also cause resistant plasmodium.\(^1\)

Treatment of malaria with artemisinin has been recommended by the World Health Organization (WHO) in the form of Artemisinin Combined Theraphy to treat malaria in areas of Africa and Indonesia.\(^2\) One of the medicinal plants that will be examined is the new China (Artemisia vulgaris L) belonging to families Asteraceae and widely grown in the mountains with a height of 1000 meters above sea level.\(^3\)

Baru Cina plant (Artemisia vulgaris L) containing artemisinin compounds. This compound is in a very small quantity in the plant.\(^4\) Treatment plants with gamma irradiation can enlarge the genetic diversity so that the opportunity to perform selection and obtain the desired properties can be achieved. Low-dose gamma irradiation is administered continuously in a certain period of time is expected to provide a direct physiological effect on cells that can be selected at the level of development of a somaclonal network, in order to obtain new varieties hat bring better properties than its parent.\(^5\)

Radiation plants with better properties than the parent is called a mutant, before getting the selection and mutant plants that are in the stages of selection referred to as the mutant strains.
Irradiation of the plant *Artemisia vulgaris* L. expected to increase the content of artemisinin compounds. Therefore, to determine levels of artemisinin in the plant needs to be done research on the stem, roots, leaves, and flowers *Artemisia vulgaris* mutant strains of L. so that can know which parts of the plant with highest levels artemisinin.

**MATERIALS**

Dry simplicia from mutant strains of *Artemisia vulgaris* L. and control plant (irradiation dose = 10 Gy, Age of plants = 4 months), the reference standard artemisinin (Sigma - Aldrich).

**INSTRUMENT**

High Performance Liquid Chromatography (Shimadzu SPD-20A/LC-20AD), analytical balance (Sartorius 1872), the separation column (Sep-Pak Catridges Milford, MA 01 757), Siring Lux Bland, filter paper and 0.4 0.2 Millipore Millipore, vacuum oven.

**METHODOLOGY**

Preparation of mutant strains of *Artemisia vulgaris* L and Extraction.

The material used in this study is simplicia dry (stems, roots, leaves and flowers) of mutant strains of *Artemisia vulgaris* L. which is the harvest of field trials in Bogor, dried then mashed in a blender until a powder. As much as 30 g of dry powder simplicia weighed, extracted by maceration with methanol until the solvent is extracted perfectly. Then the extract was evaporation to obtain viscous extract. Then separated using a column separator sep-pak C18 catridges with gradient solvent system of n:hexane, n:hexane:ethyl acetate (1:1), ethyl acetate. Ethyl acetate fraction was isolated further.

**Loss of drying**

Weighed as much as 1 g into container container that already has a constant weight, keep it in oven (105 °C for 1-2 hours) repeatedly until has a constant weight.

**System suitability test**

Solution containing a 10 mg / 10 ml of artemisinin standard, a concentration of 1000 ppm of 20µl injected and measured peak area. The execution is repeated up to five repetitions. From the peak areas obtained, the calculated relative standard deviation. HPLC conditions for the assay of artemisinin. The control system: Shimadzu SPD-20A/LC-20AD, Column: C18 (Okta decyl silane) Shim Pack. Detector: UV. λ max: 252 nm. Mobile phase: Acetonitrile-water (7:3). Flow rate: 1 ml/min Injection volume: 20µl

**Linearity test**

The experiment was conducted to determine the relationship between concentration and peak areas. To test the linearity made a series of solutions consisting of five different concentrations. Preparation: A total of 25 mg of raw artemisinin is weighed and then introduced into 25 ml volumetric flask and dissolved in methanol to the mark and shaken homogeneous. Solution A, B, C, D: A total of 0.5 ml, 1.0 ml, 2.5 ml and 5 ml pipetted respectively into 10 ml volumetric flask, then diluted with methanol to the mark and shaken homogeneous.

How to setting: Some 20 mL of each solution injected on the HPLC mobile phase has flowed elected in optimum condition. Curve is made then the relationship between the concentration of artemisinin as the x-axis and the peak areas as the y-axis and is made and the regression line equation calculated the regression coefficient values.

**The recovery test**

Recovery test used to assess the accuracy of the method. This test is done by adding a certain number of reference standard solution of artemisinin into the sample of known purity. Injection of
Determination of artemisinin content in *Artemisia vulgaris* L. and plant control

**Sample preparation.**
Total of 10 mg each of ethyl acetate fraction of the mutant lines and control plants were weighed, then diluted with 10 ml of solvent.

**Assay procedure.**

**Preparation of standard solution.**
A 10 mg of artemisinin standards were weighed approximately added to 10.0 ml methanol and mix until homogeneous.

**Preparation of test solution.**
A number of 30 mg of test substance ethyl acetate fraction was weighed. Then the test substance dissolved in 10.0 ml of ethyl acetate and shake until homogeneous, then filtered using a 0.2 Millipore filter paper. Some 20 mL test solution and standard solution injected into HPLC, with mobile phase acetonitrile-water (7:3).

**RESULTS AND DISCUSSION**

**Extraction results**
Extraction by maceration techniques and yield of the mutant strains of *Artemisia vulgaris* L. details are shown in table V.1, namely:

<table>
<thead>
<tr>
<th>Simplicia</th>
<th>Powder Weight (g)</th>
<th>Extract Methanol Weight (g)</th>
<th>Rendemen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stems</td>
<td>24,725</td>
<td>1,507</td>
<td>6,1</td>
</tr>
<tr>
<td>Roots</td>
<td>54,820</td>
<td>0,771</td>
<td>0,01</td>
</tr>
<tr>
<td>Leaves</td>
<td>11,769</td>
<td>3,249</td>
<td>27,6</td>
</tr>
<tr>
<td>Flowers</td>
<td>17,295</td>
<td>3,290</td>
<td>19,0</td>
</tr>
<tr>
<td>Stems</td>
<td>49,810</td>
<td>2,743</td>
<td>5,5</td>
</tr>
<tr>
<td>Roots</td>
<td>44,520</td>
<td>1,089</td>
<td>2,5</td>
</tr>
<tr>
<td>Leaves</td>
<td>63,370</td>
<td>2,208</td>
<td>3,5</td>
</tr>
<tr>
<td>Flowers</td>
<td>4,940</td>
<td>0,313</td>
<td>6,3</td>
</tr>
</tbody>
</table>

**Loss of Drying results**
Loss of drying is one of the parameters to determine the quality of the extract. The results obtained from *Artemisia vulgaris* L. simplicia powder 0.2% (stem), 0.2% (root), 0.4% (leaf), and 0.1% (flowers). General requirements usually is 10%

**System suitability test result**
System suitability test results based on the injection of a standard solution five times as much as 20 mL of 1000 ppm, namely:
Based on the results obtained by testing the relative value of standard deviation is 0.0143% artemisinin obtained values qualify the relative value of standard deviation of ≤ 2.0% based on the requirements of Indonesian Pharmacopoeia IV edition. HPLC methods can be used for the assay of ethyl acetate fraction of artemisinin in *Artemisia vulgaris* mutant strains of L.
Tabel V.2. System suitability test Result

<table>
<thead>
<tr>
<th>Retention time (minute)</th>
<th>Area</th>
<th>Average</th>
<th>Standard Deviation SD</th>
<th>Relative Standard Deviation SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.171</td>
<td>25209</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.161</td>
<td>25154</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.155</td>
<td>25953</td>
<td>25501</td>
<td>364,3398</td>
<td>0.0143 %</td>
</tr>
<tr>
<td>6.129</td>
<td>25367</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.167</td>
<td>25822</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Linearity test results**

Determination of calibration curve based on the chromatogram data describes the relationship between the concentration of standard solution with area at retention time (tR) 6.1 minutes.

Table V.3. The results of linearity tests

<table>
<thead>
<tr>
<th>No.</th>
<th>Concentration (ppm)</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>50</td>
<td>1083</td>
</tr>
<tr>
<td>2.</td>
<td>100</td>
<td>2439</td>
</tr>
<tr>
<td>3.</td>
<td>250</td>
<td>5891</td>
</tr>
<tr>
<td>4.</td>
<td>500</td>
<td>13189</td>
</tr>
<tr>
<td>5.</td>
<td>1000</td>
<td>25209</td>
</tr>
</tbody>
</table>

Based on the above data obtained by the following equation: $Y = -146.4660 + 25.5491x$ and $r = 0.9994$. Thus there is a good linear relationship between the concentration of standard solutions with an area. So HPLC conditions can be used in the analysis of the assay.

**The recovery test result**

Table V.4. Obtaining Test Results Back In The Trunk, The Roots, Leaves and Flowers

<table>
<thead>
<tr>
<th>Sample</th>
<th>Weight (g)</th>
<th>Sample area</th>
<th>Standard area</th>
<th>Total area</th>
<th>Sample concentration (µg/g)</th>
<th>Sample concentration average (µg/g)</th>
<th>Recovery (%)</th>
<th>Average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stems</td>
<td>1,0068</td>
<td>186614</td>
<td>25271</td>
<td>210185</td>
<td>7384,5</td>
<td>7395,3</td>
<td>87,16</td>
<td>87,46</td>
</tr>
<tr>
<td></td>
<td></td>
<td>187045</td>
<td>25256</td>
<td>210207</td>
<td>7406,0</td>
<td>7406,0</td>
<td>87,75</td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td>1,0037</td>
<td>450636</td>
<td>25452</td>
<td>474090</td>
<td>17705,3</td>
<td>17705,3</td>
<td>88,19</td>
<td>82,12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>450804</td>
<td>25536</td>
<td>474105</td>
<td>17653,7</td>
<td>17653,7</td>
<td>89,90</td>
<td>85,16</td>
</tr>
<tr>
<td>Leaves</td>
<td>1,0001</td>
<td>5441165</td>
<td>25283</td>
<td>5464981</td>
<td>215210,4</td>
<td>215210,4</td>
<td>94,20</td>
<td>92,05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5441200</td>
<td>25278</td>
<td>5464986</td>
<td>215254,4</td>
<td>215254,4</td>
<td>93,00</td>
<td></td>
</tr>
<tr>
<td>Flower</td>
<td>1,0000</td>
<td>3183252</td>
<td>25247</td>
<td>3206477</td>
<td>126084,4</td>
<td>126084,4</td>
<td>88,90</td>
<td>90,95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3183770</td>
<td>25255</td>
<td>3206472</td>
<td>126064,9</td>
<td>126064,9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Recovery test results was 87.46% (stem), 85.16% (root), 92.05% (leaves) and 90.95% (flowers) as shown in Table V.4. Thus the method used HPLC method was sufficiently accurate.
Artemisinin content in plant control and in ethyl acetate fraction in mutant strain Artemisia vulgaris L. by HPLC

HPLC analysis results indicated the increasing of artemisinin in mutant strains of Artemisia vulgaris L. in the ethyl acetate fraction compared with control plants, both on the stems, roots, leaves and flowers. Also can be seen that the content of artemisinin in ethyl acetate fraction has the highest in the flowers of 3.70% (control plants) and 12.46% (mutant strains). This is because the flowers is part of the plant is getting lots of sun exposure. Also flowers is also a place of reproduction of plants that would later become candidates for new fruits and plants. So most of the artemisinin compounds accumulate in this section with a better metabolism than other plant parts.

<table>
<thead>
<tr>
<th>Simplicia</th>
<th>Control Area</th>
<th>Content (%)</th>
<th>Control Mutan strain Area</th>
<th>Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stems</td>
<td>55091</td>
<td>0.07</td>
<td>186614</td>
<td>0.24</td>
</tr>
<tr>
<td>Roots</td>
<td>207369</td>
<td>0.14</td>
<td>450636</td>
<td>0.39</td>
</tr>
<tr>
<td>Leaves</td>
<td>4119671</td>
<td>0.95</td>
<td>5441165</td>
<td>10.65</td>
</tr>
<tr>
<td>Flowers</td>
<td>946266</td>
<td>3.70</td>
<td>3183252</td>
<td>12.46</td>
</tr>
</tbody>
</table>

CONCLUSION

Artemisinin content in ethyl acetate fraction from the plant Artemisia vulgaris L. significantly different from each other, the highest content of artemisinin obtained in the flowers at the rate of 12.46% (mutant strains) and 3.70% (control plants). Based on the results of Kruskal-Wallis test using SPSS 13.0 for windows on a 95% confidence level (α = 0.05) obtained a significant difference between the levels of artemisinin in plants of control and mutant strains of Artemisia vulgaris L.

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Rezeki S. Comparison of levels of artemisinin in methanol and ethyl acetate fraction of methanol extract of Artemisia vulgaris mutant strains of L by HPLC (Thesis). London: Faculty of Pharmacy, University of Pancasila; 2009. h. 5-11.

ABSTRACT

Traditionally, herb of Typhonium flagelliforme (Lodd.) Blume (known as Keladi Tikus), member of Araceae is believed as medicinal plants and being used as cancer treatment, including for breast cancer. In order to evaluate its antiproliferative activity in vitro, ethanolic extract Typhonium flagelliforme was tested against breast cancer cell line of MCF-7 using enzymatic assay of 3-(4,5-dimethylthiazol-2-yl)-2, dipheniltetrazolium bromide (MTT). The extract was also assayed against non-cancerous cell line of Chinese Hamster Ovary Cells (CHO-T120) to look for selectivity of the extracts in inhibiting cancer cell lines. The result of this preliminary study showed that ethanolic extract of T.flagelliforme inhibited breast cancer cell line of MCF-7 where the IC-50 was 51.1 ppm. Meanwhile, at the same concentration, the IC-50 value on CHO-T120 cell lines was 308.2 ppm. This result indicated that ethanolic extract of T.flagelliforme herb has potency as source of anti cancer compound, where it inhibit the breast cancer cell line of MCF-7 but less inhibit non-cancerous cell of CHO-T120.

Key words: ethanolic extract, Typhonium flagelliforme, MTT, MCF-7 and CHO-T120 cells line.

INTRODUCTION

Typhonium flagelliforme which is commonly known as ‘keladi tikus’ has been reported widely as a traditional herbal medicine. This plant has been used as an alternative traditional medicine for breast cancer treatment. In ancient medical tradition, the root of the plant was boiled with water at 100°C. Typhonium flagelliforme consisted of alkaloid, triterpenoid, and lignins (polyfenol) have demonstrated a variety of effects in the body, hence it is good enough to inhibit the growth of breast cancer cells. Typhonium flagelliforme is a member of plant family Araceae which growing up to 25-30 cm in height, with leaf blade 5-25 cm. It grows best on shallow water by streams, water fields, moist meadows; sea level to 400 m and spread in some regions such as Guangdong, Guangxi, SE Yunnan [Bangladesh, Bhutan, Cambodia, India, Indonesia, Laos, Malaysia, Myanmar, Philippines, Singapore, Sri Lanka, Thailand and Australia (Heng & Hetterscheid, 2010).

Researchs on Typhonium flagelliforme against various cancer cells had been studied previously. For example, the biological effect of the ethanolic extracts on lung cancer cells (NCL-123) where IC-50 was 7.5 ug/ml (Sheen, et al, 2008), and breast cancer cell (MCF-7) with IC-50 value of 89.15 ug/ml (Sheen, et al, 2008). Moreover, Typhonium flagelliforme also contains antioxidant and antibacterial activity. Phenol as an antioxidant which is one of the compound found in the tuber, was extracted using 5.21±0.82b dichlomethane. The extract has also showed has antibacterial activity against gram negative bacteria of Pseudomonas aeruginosa and Salmonella choleraesuis with inhibition zone area of 11±1.0 mm and 12±1.1 mm, respectively (Mohan. S, et al, 2008).

In this research, the tuber of Keladi tikus was extracted by ethanol before it was assayed in vitro toxicity against breast cancer cells (MCF-7) and normal cells (CHO). This preliminary research is to determined wether the ethanolic extract of Typhonium flagelliforme has an anti-cancer properties without inhibiting the growth of the normal cells surrounded.
**EXTRACTION METHOD**

Typhonium flagelliforme tubers were dried and grinded before it was extracted with ethanol. In every one gram of dried Typhonium flagelliforme, 10 ml of ethanol was added and macerated and mixed the solution with speed of 250 rpm. The solution was then filtered and evaporated at 40°C and pressure of 90-120 mbar.

**CELL CULTIVATION METHOD**

The MCF-7 (breast cancer cells) and CHO-T120 (Chinese Hamster Ovary, normal cells) were cultured in Tc-75 flasks with RPMI 1640 supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C, 5% CO2 and 95% humidity. When the cell confluence reached 70-80% density, the cells were harvested with 0.05% Trypsin EDTA. The cells were counted using by haemocytometer with Trypan blue exclusion assay. A number of 5x10^3 cells were grown in 100 ml culture media in each 96-well plate. The cells were incubated at temperature of 37oC, 5% CO2 for 24 hours.

**MTT ASSAY METHOD**

Twenty mg ethanolic extracts of Typhonium flagelliforme diluted in 400 µl of DMSO. Furthermore, culture media were diluted to achieve a serial concentration of 10, 20, 50, 100, 250 and 500 ppm. The extract samples were added on cells and incubated for 24 hours, at 37°C, 5% CO2 and 95% humidity. Culture medium was then aspirated and the cells were washed with 1X PBS. One hundred of MTT (3-(4,5 dimethyl-thiazol-2-yl)-2, dipheniltetrazolium bromide) solution (0.5 mg/ ml) was added into every well. After 4 hours incubation, 100 ul of 10 % SDS solution (in 0.1 N HCl) was added into the cells to stop the reaction. The cells were then incubated overnight in a dark room before the absorbance values were read using Elisa reader with a wavelength of 570 nm.

**Table 1. IC-50 values of ethanolic extract from Typhonium flagelliforme on MCF 7 and CHO-T120 cell lines.**

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC-50 (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF 7</td>
<td>51</td>
</tr>
<tr>
<td>CHO-T120</td>
<td>308</td>
</tr>
</tbody>
</table>

Figure 1: Typhonium flagelliforme (A), leaf (B), flower (C) and tuber (D)
RESULT AND DISCUSSION

The IC-50 values of Typhonium flagelliforme extracts on cancer cells of MCF-7 and normal cells of CHO-T120 were 51 and 308 ppm, respectively. This suggests that ethanolic extracts of Typhonium flagelliforme inhibited the growth of cancer cells MCF-7, but less toxic on normal cell growth of CHO-T120 (Table 1). This fact was further confirmed by Figure 2. There was not much different on cells of CHO treated with and without the extract (Figure 2A and 2B). However, as shown in Figure 2C and 2D, the extract exhibits differently on MCF-7 cell lines, where the growth of the cells were inhibited. Furthermore, as shown in Figure 3, the growth of MCF-7 cell lines were inhibited significantly compared to CHO cell lines.

CONCLUSION

The results of toxicity assay of ethanolic extract of Typhonium flagelliforme on normal cells of CHO-T120 and cancer cells of MCF-7 showed that the extract has toxic effect on cancer cell line of MCF7 (IC-50 of 51 ppm) but do not have toxic effect on normal cell line of CHO-T120 (IC-50 of 308 ppm). This suggests that ethanolic extract of Typhonium flagelliforme inhibit breast cancer cell lines of MCF-7 but do not inhibit the growth of normal cells of CHO.

REFERENCES


Fery Azis Wijay

Figure 2. Normal cells of CHO-T120 without treated (A) and treated with Typhonium flagelliforme ethanolic extract (B). Cancer cells of MCF 7 without treated (C) and treated with ethanolic extract of Typhonium flagelliforme (D).

Figure 3. The growth of normal cells of CHO-T120 and cancer cells of MCF-7 by adding ethanolic extract of Typhonium flagelliforme.


SCREENING OF BIOACTIVE COMPOUNDS FROM OLEA EUROPAEA AS GLUTAMINE SYNTHETASE A INHIBITOR OF BACTERIAL MENINGITIS HAEMOPHILUS INFLUENZAE TYPE B THROUGH MOLECULAR DOCKING SIMULATION

*Hadi Sunaryo and Rizky Arcinthya Rachmania
*Department of Pharmacy, Faculty of Mathematic and Science, University of Muhammadiyah Prof. Dr. Hamka, Jakarta, 13460

ABSTRACT

Haemophilus influenzae type b (Hib) is a leading cause of bacterial meningitis and pneumonia in children worldwide, resulting in at least 3 million severe illnesses and 386,000 deaths each year. The progress to against this disease in developing countries has been relatively slow due to drug resistance and financing for the vaccine. In this research, we have conducted molecular docking simulation to screen bioactive compounds from Olea europaea which has a role as antibacterial activity to against Haemophilus influenzae. Glutamine synthetase A (glnA) which has a role in peptidoglican synthesis of bacterial cell wall, were selected for drug target. The docking result identified that cornoside and demethyl-oleuropein compound has affinity and ability to inhibit glnA. There are four residues contact of cornoside compound and six residues contact of demethyl-compound to glnA enzyme that formed hydrogen bonds with domain catalytic. The docking result showed that cornoside and demethyl-oleuropein has binding energy and affinity than other bioactive compounds and the standard compound. Moreover, our study concludes that cornoside and demethyl-oleuropein can be used as the candidates of glnA inhibitor to against bacterial meningitis Haemophilus influenzae type b.

Key words: bacterial meningitis, Olea europaea, screening, antibacterial, molecular docking

INTRODUCTION

Haemophilus influenzae type b (Hib) is a leading cause of bacterial meningitis and pneumonia in children worldwide, resulting in at least 3 million severe illness and 386,000 deaths each year (Lewis, 2008). Hospital-based studies show that H. influenzae type b (Hib) is a major cause of bacterial meningitis in Philippines, India, Thailand, Malaysia, Indonesia and Vietnam (Lolekha et al, 2000). The universal routine immunization of children with conjugate vaccines has reduced the occurrence of invasive diseases H. influenzae. Although measures have been taken to immunize infants and children against Hib in developing countries, the progress has been relatively slow, partly because of financing for the vaccine, the sustainability of the immunization programs, and the need for data on the burden of invasive disease. Several factors may be relevant to increase the incidence, i.e. age at exposure to the bacterium, social/economic conditions, and genetic differences in the ability to mount an immune response. Major neurologic sequelae of Hib meningitis include behavior problems, language disorder, delayed language development, impaired vision, mental retardation, motor abnormalities, ataxia, seizures and hydrocephalus (Muliawan, 2008).

Haemophilus influenzae is a bacterium that exists in two forms: capsular and non-capsular. Capsular (typable) form have a polysaccharide covering that is responsible for the organism’s virulence and stimulation of immunity. Six distinct capsular serotypes have been described; they are designated types ‘a’ through ‘f’. Of these, type ‘b’ is almost always responsible for serious disease in children, such as meningitis, pneumonia and septicaemia (i.e. invasive Hib disease). Noncapsular (non-typable) forms of Haemophilus influenzae mostly colonise the upper respiratory tract without causing illness. Peptidoglycan is a polymer of sugars and amino acids that forms a homogeneous layer outside the plasma membrane. It serves a structural role in the bacterial cell wall, giving it shape and strength, as well as counteracting the osmotic pressure of the cytoplasm. The peptidoglycan layer is a crystal lattice formed by linear chains of N-acetylglucosamine and N-acetylmuramic acid that are connected by short [4-5 residues] amino acid chains. Glutamine synthetase [EC 6.3.1.2] is an enzyme that plays an essential
role in the metabolism of nitrogen by catalyzing the condensation of glutamate and ammonia to form glutamine. The peptidoglycan synthesis is an effective pathway with numerous enzymes which are potent drug targets. Glutamine synthetase A (glnA) is an enzyme that has a role of peptidoglycan synthesis of bacterial cell wall can be used for drug target (Kasturi et al, 2011).

The increasing resistance of Hib to antibacterial agents such as ampicillin, cotrimoxazole, chloramphenicol and cefalosporins has been reported from many parts of the world. The Hib immunization has to be a larger extent decreased the occurence of the disease in developed countries. Various studies of herbs, explained that Olea europea has antibacterial activity. Phenolic and glucoside compounds in Olea europea has the ability to inhibit respiratory tract pathogens, namely Haemophilus influenzae (Khan et al., 2007; Pereira et al., 2007).

In this research, we have studied the interactions between bioactive compounds with glutamine synthetase A (glnA) using computer software applications (in silico) (Elkins, 2007). The interaction with bioactive compounds was performed through molecular docking simulations. We generated the docking as screening process between the molecules of bioactive compounds that can bind to the domain catalytic of glnA enzyme. Analysis was based on binding energy values, inhibition constant as affinity, and hydrogen bonds as the interaction between glnA and ligands.

METHODOLOGY

Protein Preparation

The target protein of glnA was retrieved from NCBI database (http://www.ncbi.nlm.nih.gov) and composed of 472 amino acid residues. Multiple sequence alignment method was used BLASTp in NCBI. Homology modeling was performed using the Swiss Model (http://swissmodel.expasy.org/SWISS-MODEL.html). Optimization and minimization of three-dimensional structure of the glnA enzyme were employed using the MOE software with addition of hydrogen atoms. Protonation was employed with protonate 3D programs. Furthermore, partial charges and force field was employed with MMFF94x. Solvation of enzymes was performed in the form of a gas phase with a fixed charge with a RMS gradient of 0.05 kcal / A0 mol, and other parameters using the standard in MOE software.

Preparation of Bioactive Compounds as a Ligand

Bioactive compounds of Olea europea were modeled into three-dimensional structure using ACD Labs software. Three-dimensional shape was obtained by storing in the 3D viewer in ACDLabs. Furthermore, the output format was changed into Molfile MDL Mol format using software Vegazz to conform for the docking process. Ligand was in the wash with compute program, adjustments were made with the ligand partial charge and partial charge optimization using MMFF94X forcefield. The conformation structure energy of ligands was minimized using the RMS gradient energy with 0.001 Kcal / A mol. Other parameters were in accordance with existing default in the software.

Docking Simulation

The docking process was begun with the docking preparation, that was employed using a docking program from MOE software. Docking simulations were performed by the Compute-Simulation dock program. Placement method was conducted using a triangle matcher with 1,000,000 repetition energy readings each position and other parameters were in accordance with existing default in the MOE software. Furthermore, scoring functions used london DG, refinement of the configuration repetition forcefield with 1,000 populations. The first repetition of 100 times and the second setting was shown only one of the best results.

RESULTS AND DISCUSSIONS

Twelve bioactive compounds as ligands were obtained from Olea europaea. These bioactive compounds contained of glycoside groups, phenolic groups, terpen units (Khan et al., 2007). The docking results showed that from 12 ligands there are two bioactive compounds which have a lower binding energy than other bioactive compounds and standard ligand cefuroxime.
Table 1. Analysis Docking

<table>
<thead>
<tr>
<th>Ligands</th>
<th>Binding energy (kcal/mol)</th>
<th>pKi (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornoside</td>
<td>-13.1105</td>
<td>11.598</td>
</tr>
<tr>
<td>Demethyl oleuropein</td>
<td>-12.4742</td>
<td>12.020</td>
</tr>
<tr>
<td>Oleuropein isomer</td>
<td>-12.2390</td>
<td>6.643</td>
</tr>
<tr>
<td>Oleoside methylester</td>
<td>-11.7709</td>
<td>9.821</td>
</tr>
<tr>
<td>β-glucopyranosil (c)</td>
<td>-11.3987</td>
<td>7.977</td>
</tr>
<tr>
<td>β-glucopyranosil (b)</td>
<td>-11.3908</td>
<td>9.088</td>
</tr>
<tr>
<td>Secoiridoid glucoside</td>
<td>-11.3578</td>
<td>9.191</td>
</tr>
<tr>
<td>Hydroxytyrosol malic ester</td>
<td>-11.2086</td>
<td>4.560</td>
</tr>
<tr>
<td>Ligstroside</td>
<td>-10.1386</td>
<td>8.088</td>
</tr>
<tr>
<td>β-glucopyranosil (a)</td>
<td>-9.5346</td>
<td>9.155</td>
</tr>
<tr>
<td>Glucosite methylester</td>
<td>-9.3229</td>
<td>9.092</td>
</tr>
<tr>
<td>Cefuroxime (standard ligand)</td>
<td>-8.8694</td>
<td>7.921</td>
</tr>
<tr>
<td>Oleicester tyrosol</td>
<td>-7.9399</td>
<td>4.689</td>
</tr>
</tbody>
</table>

Table 2. Hydrogen bonds between ligands to glnA

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Residues of amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornoside</td>
<td>Lys32, Lys32, Glu33, Glu33, Lys244, Lys244, Lys244, Arg349, Arg344, Arg364</td>
</tr>
<tr>
<td>Demethyl-oleuropein</td>
<td>Glu217, Glu217, Glu225, Arg349, Arg344, Arg364</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>Lys32, Lys32, Lys32, Glu248</td>
</tr>
</tbody>
</table>

*Bold letter : amino acid residues in catalytic domain

There were only 2 ligands bioactive compounds, cornoside and demethyl-oleuropein that have a lower binding energy than ligand standard cefuroxime. Analysis of docking results was shown in Table 1. Cornoside and demethyl-oleuropein has a lower binding energy value, -13.1105 kcal/mol and -12.4742 kcal/mol than standard ligand cefuroxime -8.8694 kcal/mol. The docking result showed that cornoside and demethyl-oleuropein ligand have pKi value 11.598 µM and 12.020 µM, this value indicated that cornoside and demethyl-oleuropein ligands have stronger affinity and interaction to form complex compounds with glnA, if it compared with cefuroxime with the pKi value 7.921 µM (Table 1).

The identification of hydrogen bonds between glnA amino acid residues with cornoside, demethyl-oleuropein and standard ligands is shown in Table 2. Cornoside ligand, demethyl-oleuropein ligand and cefuroxime ligand have 8, 6 and 4 hydrogen bonds, respectively. Cornoside ligand binds to the catalytic domain into the site of Glu241, Lys244, Lys244, Phe260, which formed hydrogen bonds with the catalitic domain as many as 4 bonds, demethyl-oleuropein ligand binds to the catalytic domain into the site of Glu217, Glu217, Glu225, Arg349, Arg344, Arg364 which formed hydrogen bonds with the catalitic domain as many as 6 bonds and standard ligand cefuroxime has 1 hydrogen binding to catalytic domain of glnA (Table 2). Cornoside and demethyl-oleuropein ligand has more binding site to the domain catalytic than the standard. Based on hydrogen bonds, cornoside and demethyl-oleuropein have formed interaction with glnA.

Conformation of the cornoside ligand, demethyl-oleuropein ligand and cefuroxime to the glnA is shown in figure 1. The binding of cornoside and demethyl-oleuropein ligands allowed the interaction between domain catalytic residues with functional group such as glucoside and carboxyl group of the ligand. These interaction showed that these ligands are able to interact with glnA so that it will increase the affinity and stability of the enzyme-ligand complex. Molecule structures of cornoside and demethyl-oleuropein are shown in Figure 2a and 2b.
Figure 1. (a.1), (b.1), (c.1) are hydrogen bonds as the interaction between glnA with cornoside, demethyl-oleuropein and cefuroxime, respectively. (a.2), (b.2), (b.3) are conformations of konformasi cornoside, demethyl-oleuropein and cefuroxime in catalytic domain of glnA.
CONCLUSION

In this study, we have screened bioactive compounds from *Olea europaea* which are expected to be potential as inhibitor glnA to against bacterial meningitis *Haemophilus influenzae*. We have employed 12 compounds of *Olea europaea* to figure out some targeting antibacterial candidates for the treatment of bacterial meningitis. After the overall procedures presented, we indeed find a compound of potent antibacterial target candidates. Among them, cornoside and demethyl-oleuropein ligand which produced 4 and 6 hydrogen bonds to domain catalytic in glnA. The binding energy of cornoside and demethyl-oleuropein are -13.1105 kcal/mol and -12.4742 kcal/mol, than standard ligand cefuroxime -8.8694 kcal/mol. pKi as affinity of cornoside and demethyl-oleuropein are 11.598 µM and 12.020 µM, respectively. Those value are greater than cefuroxime as standard. Thus, cornoside and demethyl-oleuropein has the ability to be candidate of glnA inhibitor to against bacterial meningitis *Haemophilus influenzae* type b.

ACKNOWLEDGEMENT

The researchers are grateful to the Chairman of Department of Pharmacy, Faculty of Mathematics and Science, University of Muhammadiyah Prof. Dr. Hamka for the support of this research.

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**FLAVOURING AGENT OPTIMATION OF KALANCHOE PINNATA, PERS. CRUDE EXTRACT LOZENGES**

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**ABSTRACT**

Kalanchoe pinnata, Pers. leaves contain vitamin C which give sour taste, alkaloid which give bitter taste, tannin, and flavonoid. Flavouring agent was added to the formula of lozenges to cover the sour taste, cool, smell and astringent, so the consumers like the lozenges. In this study, the ethanol extract of Kalanchoe pinnata leaves was dried by freeze drying method using 10% maltodextrin concentration. Kalanchoe pinnata leaf extract powder was formulated into lozenges with a wet granulation method using 3 of flavourings agent with various concentrations. Results showed that the 9th formula with 0.3% essence lemon and 0.03% sunset yellow had good characteristics of lozenges. Lozenges are yellow, lemon smell, sweet with sour taste, uniformity of weight 2.0345 g; uniformity of size tablet with a diameter of 20.11 mm and thickness of 5.72 mm; friability of 0.41%; hardness of 11.55 kg/cm²; and disintegration time of 13.86 minutes. A Kruskal Wallis method of non parametric analysis was used to analyze lozenges hardness and hedonic test for taste and color of the lozenges. Based on the analysis for lozenges hardness, results shown that variation of flavorings agent was not affect the hardness (α (sign) > 0.05). Hedonic test was done for analyzed the taste and color of the lozenges using 50 panelists. The result shown that variation of flavorings agent affected the taste and color so analysis continued to the Bonferroni test (α (sign) < 0.05).

**Key words**: Kalanchoe pinnata Pers., freeze drying, lozenges, flavouring agent, hedonic test

**INTRODUCTION**

Kalanchoe pinnata, Pers. is one of the ornamental plant whose leaves can be efficacious as an antiseptic which have a acid taste, cold, odor less, and astringent. Compounds of Kalanchoe pinnata, Pers. is known to have antimicrobial activity as is saponin, bryophyllin, and phenolic compounds (Okwu, 2006). The ethanol extract of Kalanchoe pinnata, Pers. can prevent Streptococcus mutans growth caused odor in the oral cavity with Minimum Inhibitory Concentration (MIC) is 5% (Firdaus, 2010). That extract is acid at range pH of 4 (Yantih, 2010). The acid taste, cold, odor less and astringent can covered by flavouring agent (essence and colouring) (Siregar, 2008).

In this research leaves of Kalanchoe pinnata, Pers. will be made into crude extract which made into powder using freeze dryer with maltodextrin 10% and made into lozenges with wet granulation method (Kartiningsih, 2010). The variation of concentration and different types of essence and coloring were optimized to obtain the best formula of lozenges which is like by the consumers.

**METHODOLOGY**

**Materials**

Crude extract of Kalanchoe pinnata, Pers., mannitol, PEG 6000, PVP, essence strawberry, essence lemon, essence orange, sunset yellow, erythrosin, orange I.

**Tools**

Freeze dryer equipment (LABCONCO), Karl Fisher moist meter (Mitsubishi), Oven (Memmert), tablet compression machine, Tablet hardness tester, powder compressibility testing devices (Omron H3Cr), shieve shaker machine, disintegrator time of testing devices (Omron H3BA), analitical scales (AND GR-200 type), friabillator.
Table 1. The design of dosage formulation of lozenges

<table>
<thead>
<tr>
<th>Formulation Ingredient</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Extract powder of crude extract</td>
<td>0.52</td>
</tr>
<tr>
<td>PVP</td>
<td>0.1</td>
</tr>
<tr>
<td>PEG 6000</td>
<td>0.1</td>
</tr>
<tr>
<td>Essence strawberry</td>
<td>0.002</td>
</tr>
<tr>
<td>Erytroshin</td>
<td>0.0002</td>
</tr>
<tr>
<td>Essence orange</td>
<td>-</td>
</tr>
<tr>
<td>Orange I</td>
<td>-</td>
</tr>
<tr>
<td>Essence lemon</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol (to)</td>
<td>2</td>
</tr>
</tbody>
</table>

Tableting of lozenges with wet granulation method

The active ingredient is crude ethanol extract of *Kalanchoe pinnata*, Pers. and the excipients, e.g.: mannitol as filler once a sweetener, PVP as binder, PEG 6000 as glidan, Essence as enhancer taste, and coloring that are listed below (Table 1). Lozenges was formulated at 2 g.

Powder extract of *Kalanchoe pinnata*, Pers. was added mannitol. PVP was dissolved into ethanol 96% and added coloring, and than mixed well. PVP solution was added into powder mixture, and mixed until granul mass form. Granul was shive with shieving no.12 mesh, dried in oven with 40°C. Next, dry granul was shive with shieving no.16 mesh. Granul mass have been evaluated flow properties, particle size distribution, compressibility and water content. Finally, the bulk was added PEG 6000 and essence, and then compressed into lozenges (Larry, 2008; Kartiningsih 2010).

**Evaluation of lozenges**

**Organoleptic Test**

Organoleptic include: coloring, smell and taste.

**Uniformity of Size Test**

By random sampling, the diameter and thickness of 20 lozenges were measured using a shove.

**Weight Uniformity Test**

Lozenges (20 pieces) were weighed and then was calculated the average weight per lozenges.

**Friability Test**

Lozenges (20 pieces) were taken randomly suction, weighed and cleaned of dust. Then was put in friabilator which was run for 4 minutes with speed of 25 rpm. Lozenges that have been test was re-cleaned and then weighed (terms: < 1 %).

**Hardness Test**

Lozenges (20 pieces) were taken randomly and determined by its hardness tools tablets. (terms: 10 – 20 kg/cm²).

**Desintegration Time Test**

A total of 6 lozenges prepared. One tablet was inserted into each table to the basket, put on 1 disc on each tube and run the tool. Water temperature was set at 37±2°C as the media. The desintegrator was driven and observed the process of destruction of lozenges. Lozenges should be destroyed completely in less than 30 minutes. If 1 or 2 lozenges were not destroyed completely, the...
test have been repeated with 12 other lozenges. No less than 16 lozenges than 18 lozenges were destroyed completely.

**Hedonic test**
Hedonic test was done to the 50 panelis with kuesioner method. Panelis was asked for their argument about the colour and taste of the lozenges. Panelis was given one tablet per formula and they were give a skor from 1 (not like) until 5 (extremely like).

**RESULT AND DISCUSSION**

**Organoleptic Examination Results**
Results of organoleptic evaluation examination lozenges of crude extract of *Kalanchoe pinnata*, Pers. leaves shown formulas I—IX different results for each formulation. Because of different types of essence and coloring give different smell and color for each formula. Increasing concentration of essence and coloring, so color and smell of lozenges of *Kalanchoe pinnata*, Pers. leaves increase too (Table 2). The white with yellowish from powder of *Kalanchoe pinnata*, Pers. leaves which have a dislike colour can be covered by coloring agent, so lozenges were looked more better. Beside that, aromatic smell of powder of *Kalanchoe pinnata*, Pers. leaves can be covered by essence strawberry, essence orange and essence lemon so the lozenges has a better taste. Acid taste of *Kalanchoe pinnata*, Pers. powder which contained ascorbic acid, lemon acid and caffeoat acid can not be covered because of essence and mannitol used with low concentration for each formulas.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Color</th>
<th>Smell</th>
<th>Taste</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Soft pink</td>
<td>Strawberry</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>II</td>
<td>Pink</td>
<td>Strawberry</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>Strawberry</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>IV</td>
<td>Soft orange</td>
<td>Orange</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>V</td>
<td>Orange</td>
<td>Orange</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>Orange</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>VII</td>
<td>Soft yellow</td>
<td>Lemon</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>VIII</td>
<td>Yellow</td>
<td>Lemon</td>
<td>Sweet and acid</td>
</tr>
<tr>
<td>IX</td>
<td></td>
<td>Lemon</td>
<td>Sweet with acid</td>
</tr>
</tbody>
</table>

**Tablet Weight Uniformity Evaluation**
At tablet uniformity test, the weight of fifth qualifying formulas were no more than 5% and 10% of the average weight of each tablet formulation. It was also related to flow properties and particle size distribution of the mass granul which made the lozenges have a small variation weight. Different types and concentration of essence and coloring shown formula I—IX do not have a weight variation because it has a small SD.

**Size Uniformity Evaluation**
Result of evaluation of the uniformity of the size of formula I—IX shown the diameter of the tablets were from 20,10—20,11 mm and thickness were from 5,72—5,75 mm (Table 3). The results shown that different types and concentration of essence and colouring did not have a weight variation because it has a small SD. Beside the colouring and essence, the size uniformity of lozenges will effect the esthetics.

**Evaluation of Friability**
The required of tablet friability is < 1% (Larry, 2008). The results shown that formula I—IX were eligible (Table 3). Because of PVP 5% as a wet binder has a goodability to bind all particles and have a less fines so the lozenges have a small friability. Small friability was expected to the tablet is not
fragile and not much eroded during the printing process until the distribution to consumers. Different types and concentration of essence and colouring do not affected the friability with a small SD.

### Tablet Hardness Test

Terms of hardness lozenges were 10—20 kg/cm$^2$. The results shown that formula I—IX were eligible with value more than 10 kg/cm$^2$. Because of PVP 5% as a wet binder could bind all particle, with a low concentration but could made a tablet eligible.

Different types and concentration of essence and colouring do not affect the hardness with a small SD. Based on statistical tests of tablet hardness using Kruskal Wallis non parametric method, different types and concentration of essence and colouring do not provide a significant difference to the hardness ($\alpha$(sign) > 0,05).

### Disintegration time test

Terms of disintegration time for the tablet suction was eroded slowly in 30 minutes or less (Table 3). The evaluation shown that the disintegration time lozenges met the requirements of less than 30 minutes so time lozenges in the mouth was longer cause the inhibitory effect of microbial populations better.

Different types and concentration of essence and colouring agent do not affected disintegration time of lozenges with a small SD.

Table 3. Results of evaluation of lozenges

<table>
<thead>
<tr>
<th>Evaluation of tablet</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>Uniformity of size</td>
<td></td>
</tr>
<tr>
<td>- Diameter (mm)</td>
<td>20,11</td>
</tr>
<tr>
<td>- Thickness (mm)</td>
<td>5,69</td>
</tr>
<tr>
<td>Weight uniformity test (g)</td>
<td>2,035</td>
</tr>
<tr>
<td>Friability (%)</td>
<td>0,42</td>
</tr>
<tr>
<td>Hardness (kg/cm$^2$)</td>
<td>11,33</td>
</tr>
<tr>
<td>Disintegration test (menit)</td>
<td>13,72</td>
</tr>
</tbody>
</table>

### Hedonic test

Hedonic test was done to know acceptance of panelis for taste and colour of *Kalanchoe pinnata*, Pers. lozenges. Different types and concentration of essence and colouring agent were variated into 3 taste and colour with 3 different concentration. Next, 50 panelis were given by kuesioner. The results were done in numeric scale 1—5 (1=dislike, 2=neutral, 3=a little like, 4=like, 5=extremely like).

The results shown that panelis could be accepted the lozenges especially formula IX with lemon taste and yellow colour. Because of the powder extract of *Kalanchoe pinnata*, Pers. with acid taste and yellow with brown colour so essence and colouring agent were suitable with the characteristic of the powder extract of *Kalanchoe pinnata*, Pers. Based on statistical tests of hedonic test using Kruskal Wallis method, different types and concentration of essence and colouring agent provides a significant difference to the hedonic test ($\alpha$(sign) < 0,05).

### CONCLUSION

The powder extract of *Kalanchoe pinnata*, Pers. can be formulated into lozenges with a wet granulation method. The formula IX was a good formula with weight of 2,0345 g, diameter of 20,11
Kartiningsih

mm, thickness 5.72 mm, friability 0.41%, hardness of 11.55 kg/cm² and the disintegration time of 13.86 minute.

Flavouring agent in formulation of lozenges can be covered a colour and aromatic smell of the powder extract of *Kalanchoe pinnata*, Pers., The acid taste of the powder extract of *Kalanchoe pinnata*, Pers. can not be covered but the lozenges can be accepted by consumers (panelis).

**ACKNOWLEDGEMENT**

Special thanks are devote for Directorate General of Higher Education of Indonesia which has given grants in this research.

**REFERENCES**


EFFECTS OF FERTILIZERS AND DIFFERENT LIGHT INTENSITY ON GROWTH AND ANDROGRAPHOLIDE CONTENT OF SAMBILOTO (*Andrographis paniculata*. Ness)

**ABSTRACT**

This study aims to evaluate the effect of fertilization and different light intensity on growth and andrographolide contents of bitter plant (*Andrographis paniculata* Ness). The study was conducted using Completely Randomized Design (CRD) with factorial 3 x 3. The main factor is fertilization treatment which consist of three levels: NPK (Urea 1.2 grams / plant, TSP 2.4 g / plant and KCl 0.6 g / plant), animal manure (1.2 kg / plant) and without fertilization. The second factor is the different light intensity which consists of three levels: full light intensity, half-shade and full shade. For Each treatment combination 3 replicates were used. Vegetative growth was observed 1 and 2 months after application of fertilizer and different light intensity. Parameters observed were plant height, leaf number, number of tiller, plant fresh weight. Levels of secondary metabolite was determined by HPLC method. Data were analyzed using the Analysis Of Variance (ANOVA). The results obtained showed plant growth response of bitter plants were different under different type of fertilization and different light intensity. Application of NPK on plants grown under 40 % light intensity (P2N1) gave highest plant height. Application of manure on plants grown under full sunlight gave higher number of leaves and tillers. Fresh weight of *sambiloto* increased when the plants were applied with NPK fertilizer and grown under 60% light intensity or applied with manure and grown under full sunlight. Light intensity effects the synthesis of secondary metabolite of bitter plant, when the harvest was conducted at 1 month after planting, the highest levels of secondary metabolite was found on plants applied with NPK fertilizer or without fertilizer and grown under full sunlight. When the plants were harvested at 2 months after planting, there was no effect of light intensity and fertilizer on the content of secondary metabolite.

**Key words:** Growth, fertilizers, light intensity, andrographolide

**INTRODUCTION**

*Sambiloto* (*Andrographis paniculata*. Ness), is one kind of medicinal plants traditionally utilized by people in Indonesia. The folkloric actions of sambiloto crude extract were as a remedial for typhus, atshma, cough and fever (Fauzi, 2008). This plant contain secondary metabolites such as deoxy-andrographolide, neoandrographolide and homoandrographolide (Hardiana, 2006).The secondary metabolite profiles and its accumulation in plant tissues can be influenced by both internal and external factors such as light intensity, temperature, fertilizer, etc. Odabas et al. (2009) reported that increasing light intensity and temperature caused an increased in secondary metabolites (hypertorin, hypericin and pseudohypericin) of *Hypericum perforatum* L. Combination of nitrate supply and light intensity also influenced the metabolite levels in tomato (*Solanum lycopersicum*) plant. Urbanczyk-Wochniak and Fernie (2005) showed that under high-light regime and low supply of nitrate the content of dopamine was increased where as the content of nicotinate, gluconate and norvaline were decreased. In tomato plants grown under the hight-light regime and high nitrate supply, the content of nicotinate, noradrenaline, norvaline and spermidine were increased whereas dopamine content was reduced. Most of the metabolite were not detected when the tomato plants grown under low-light regime. In other plant species such as lyquorice (*Glycyrrhiza uralensis* Fisch), low light intensity
Effects of Fertilizers and Different Light.............

decreased leaf thickness, photosynthesis and biomass, but increased leaf area and chlorophyll content. In addition, the content of glycyrrhizic acid and liquiritin in the root were increased (Hou et al., 2010). The physiological age of plant could also determine the content of secondary metabolites. It has been reported that coumarine contents in the young leaves of *Mikania glomerata* grown under full sunlight increased two-fold compared to the adult plants grown under similar light intensity (Castro et al., 2006). These results showed that both internal and environmental factors differently affecting the content of secondary metabolic in a certain plant. The objectives of this present study were to evaluate the effect of light intensity and fertilizer application on the growth of *A. paniculata* Ness. and its andrographolide content on the leaves that were harvested at different growth stage.

**METHODOLOGY**

The present study was carried out at Kebun Pendidikan, Penelitian dan Pengembangan Pertanian (KP4) of Gadjah Mada University from June to November 2010. The *A. paniculata* seeds were obtained from Research Centre of Medicinal Plants at Tawangmangu, Central Java, Indonesia. The seeds were germinated and after one month the seedlings were transplanted into the polybag containing soil (10 kg soil per polybag).

**Experimental design**

This experiment used 3 x 3 factorial arranged in complete randomized design. The first factor was fertilizer application which consists of three levels namely: without fertilizer (control), organic fertilizer of 1.2 g/plant, and synthetic fertilizer (Urea 1.2 g/plant, TSP 2.4 g/plant, KCl 0.6 g/plant). The second factor was light intensity which consists of three levels namely: full sunlight, 60% shade, and under full canopy of “Durian” plants. For each treatment combination 3 replicates were used. Fertilizer application was conducted one week before seedlings were transplanted into the polybag. Watering was done regularly and the leaves were harvested one and two months after seedlings were grown in the polybag under different light intensity. Other parameters observed were plant height, number of leaves and tillers, as well as fresh and dry weight of plants.

**Secondary metabolite analysis**

The content of andrographolide was detected using HPTLC method. Dry leaves of 50 mg was macerated using 60% ethanol for 30 minutes and this procedure was repeated three times. Samples were heated at 80 °C for 90 minutes then eluted with ethanol : ethyl acetate (1 : 5 v/v), vortexed for 10 minutes and then applied on TLC Aluminium plate pre coated with Silica gel60 GF254 and developed using Toluene : Ethyl acetate : Formic acid (5:4.5:0.5) v/v as a mobile phase. The plate was sprayed (derivatized) with Anisaldehyde- Sulphuric Acid reagent followed by heating at 110 °C for 10 minutes and detection and quantification were carried out densitometrically using an UV detector at wavelength of 232 nm.

**Data analysis**

Data were evaluated using Analysis of Variance (ANOVA). The significant difference amongst treatments were determined by Duncan’s Multiple Range Test (DMRT) at P = 5%.

**RESULTS AND DISCUSSIONS**

The growth of *A. paniculata* after one or two months grown under different light intensity and fertilizer application is presented in Fig. 1. The average of plant height increased on plants applied with organic or anorganic fertilizer and grown under either full sunlight or 60% shade. However, those plants grown under ‘Durian’ canopy showed very poor growth and there was no effect of fertilizer applied on the average of plant height. After grown for two months, the average plant height of *A. paniculata* was doubled on plants grown under full sunlight and applied by anorganic fertilizer compared to those plants applied with organic fertilizer or no fertilizer. This results indicate that anorganic fertilizer provide available nutrients that can be absorbed by plants quicker compared to organic fertilizer.
Fig. 1. Average plant height of *A. paniculata* treated with different light intensity and fertilizer.

The average leaves number of *A. paniculata* at 1 or 2 month following treatment was presented in Table 1 and 2.

**Table 1.** Average leaves number of *A. paniculata* at 1 month after subjected to different light intensity and fertilizer.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>N₀</th>
<th>N₁</th>
<th>N₂</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₀</td>
<td>66,33&lt;sup&gt;d&lt;/sup&gt;</td>
<td>27,00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8,00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33,78&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>P₁</td>
<td>46,30&lt;sup&gt;c&lt;/sup&gt;</td>
<td>27,67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6,67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26,89&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td>P₂</td>
<td>42,33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>42,33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8,00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30,89&lt;sup&gt;pq&lt;/sup&gt;</td>
</tr>
<tr>
<td>Average</td>
<td>51,67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32,33&lt;sup&gt;y&lt;/sup&gt;</td>
<td>7,56&lt;sup&gt;x&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Average leaves number of *A. paniculata* at 2 months after subjected to different light intensity and fertilizer.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>N₀</th>
<th>N₁</th>
<th>N₂</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>P₀</td>
<td>170,00&lt;sup&gt;c&lt;/sup&gt;</td>
<td>101,00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9,00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>93,33&lt;sup&gt;p&lt;/sup&gt;</td>
</tr>
<tr>
<td>P₁</td>
<td>435,33&lt;sup&gt;d&lt;/sup&gt;</td>
<td>87,67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8,00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>177,00&lt;sup&gt;q&lt;/sup&gt;</td>
</tr>
<tr>
<td>P₂</td>
<td>164,67&lt;sup&gt;c&lt;/sup&gt;</td>
<td>110,67&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9,33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>94,89&lt;sup&gt;p&lt;/sup&gt;</td>
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<tr>
<td>Average</td>
<td>256,67&lt;sup&gt;z&lt;/sup&gt;</td>
<td>99,78&lt;sup&gt;y&lt;/sup&gt;</td>
<td>8,78&lt;sup&gt;x&lt;/sup&gt;</td>
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</tbody>
</table>

The results showed that at the early growth of *A. paniculata*, there was no effect of fertilizer on the average of leaves number, but light intensity influenced the average leaves number. The lower light intensity the smaller number of leaves were formed. At two months following treatment with different light intensity, the average leaves number of *A. paniculata* was highest in those plants grown under full sunlight and fertilized with organic fertilizer, however when plants were grown at lower light intensity, there was no effect of fertilizers on the average leaves number. This results indicate that reduced height of those plants fertilized by organic fertilizer and grown under full sunlight to make more leaves compared to those plants fertilized by anorganic fertilizer and having a larger height.
It was found that when the plants were harvested at 1 month, there was no significant difference in the average of fresh weight amongst those plants treated with different light intensity and fertilizers. However, when the harvest was conducted at 2 months following treatment, it was found that those plants applied with organic fertilizer and grown under full sunlight showed the highest fresh weight. It is due to the more bushier leaves of the plants compared to other treatments.

The effect of light intensity and fertilizer on the average of fresh weight was presented in Fig. 2.

![Fig. 2. The average fresh weight of A. paniculata at 1 or 2 months following grown under different light intensity and fertilizer.](image)

The andrographolide content was determined in the leaves of plants harvested after 1 or 2 months following treatment with different light intensity and fertilization (Fig. 3). It was found that the plants grown under full canopy of “Durian” plants grew very poor and its andrographolide content can not be detected.

![Fig. 3. The average content of andrographolide in A. paniculata at 1 or 2 months following grown under different light intensity and fertilizer.](image)

When the plants were harvested at 1 month following treatment, the andrographolide content was low in the leaves of plants treated with organic fertilizer, on the contrary, when harvest was conducted 2 month following treatments, the content of andrographolide in the leaves of plants treated with organic fertilizer was similar compared to those plants treated with anorganic fertilizer or control plants. The biosynthesis of secondary metabolite often influenced by different kind of stress conditions such as, low light intensity, salt stress, etc. For A. paniculata, it seems that 60% shade net
only reduced the fresh weight of plants and not affecting the synthesis of andrographolide. It will be interesting to observe whether there are fluctuation in the content of other metabolites in \textit{A. paniculata}.

**CONCLUSION**

Growth of sambiloto (\textit{A. paniculata}) was very poor when grown under full canopy of plant. Anorganic fertilizer increased plant height of \textit{A. paniculata} grown under full sun light or 60\% shade net. The highest leaves number and fresh weight were obtained in plants treated with organic fertilizer and grown under full sunlight. Andrographolide content of \textit{A. paniculata} treated with organic fertilizer and harvested at 1 month old was still low compared to those applied with no fertilizer or anorganic fertilizer. However, when plants were harvested at 2 months old, there was no different in the andrographolide contents

**ACKNOWLEDGEMENT**

The authors would like to thank Head of “Laboratorium Fitokimia Farmasi”, UGM; Staff of KP4, UGM and “Balai Besar Pengembangan Tanaman dan Tanaman Obat Tradisional, Tawangmangu” for providing the equipments and materials required in this research. This research is funded by I-MHERE (Indonesia Managing Higher Education for Relevance and Efficiency), of Fakultas Farmasi UGM.

**REFERENCES**


ACUTE TOXICITY TEST AND LOZENGES TABLET FORMULATION OF KALANCHOE PINNATA P. ETHANOL EXTRACT

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ABSTRACT

Leafs of Kalanchoe pinnata P can be used as antiseptics because it contains polar compounds such as kaffeoat acid, brofilin, bufadenolida, and phenol. In previous study, MIC of extract powder can inhibit growth of Streptococcus mutans which caused halitosis. The K. pinnata P leafs crude extract was freeze dried with maltodekstrin 10%. In this study, will be carried out the acute toxicity test of K. pinnata P powder crude extract and granule crude extract to find out the LD$_{50}$ value, to know the safety dose of granule crude extract and powder crude extract which will be tested to male and female mice. Base on the calculation of toxicity test by Weill CS methods, the LD$_{50}$ value was got as much as 16g/kg body weight of granule crude extract and equivalent to 16g/kg of powder crude extract. So, K. pinnata P granule crude extract and powder extract belong to the category of practically non toxic. Afterwards, K. pinnata P powder crude extract was formulated become a lozenges tablet to get an easy in use and can be hold in longer time in mouth. So, inhibition effect on growth of microbe can reached. The formulation of lozenges tablet with wet granulation methods by using PVP as a binder at a PVP concentration of 5%, 7.5%, and 10%. The tablets are creamy; aromatic smell; sweet sour; has a little bitter taste; uniformity thickness of 5.71 mm; friability value 0.2470%; hardness value 18,975 kg/cm$^2$, and disintegration time of 28.25 minutes. Based on statistical analysis using ANAVA I-direction methods and Tukey test, the friability value, hardness value, and disintegration time of tablets, was found that variation on PVP concentration gave significant differences of friability valu, hardness value and diintegration time (α(sign) < 0.05).

Key words: Acute toxicity test, Kalanchoe pinnata P. leaf ethanol extract, wet granulation, PVP-ethanol, lozenges tablet.

INTRODUCTION

Leafs of Kalanchoe pinnata P can be used as antiseptics because it contains polar compounds such as kaffeoat acid, brofilin, bufadenolida, and phenol. In previous study, MIC of extract powder can inhibit growth of Streptococcus mutans which caused halitosis. The K. pinnata P leafs crude extract was freeze dried with maltodekstrin 10%. In this study, will be carried out the acute toxicity test of K. pinnata P powder crude extract and granule crude extract to find out the LD$_{50}$ value, to know the safety dose of granule crude extract and powder crude extract which will be tested to male and female mice. Base on the calculation of toxicity test by Weill CS methods, the LD$_{50}$ value was got as much as 16g/kg body weight of granule crude extract and equivalent to 16g/kg of powder crude extract. So, K. pinnata P granule crude extract and powder extract belong to the category of practically non toxic. Afterwards, K. pinnata P powder crude extract was formulated become a lozenges tablet to get an easy in use and can be hold in longer time in mouth. So, inhibition effect on growth of microbe can reached. The formulation of lozenges tablet with wet granulation methods by using PVP as a binder at a PVP concentration of 5%, 7.5%, and 10%. The tablets are creamy; aromatic smell; sweet sour; has a little bitter taste; uniformity thickness of 5.71 mm; friability value 0.2470%; hardness value 18,975 kg/cm$^2$, and disintegration time of 28.25 minutes. Based on statistical analysis using ANAVA I-direction methods and Tukey test, the friability value, hardness value, and disintegration time of tablets, was found that variation on PVP concentration gave significant differences of friability valu, hardness value and diintegration time (α(sign) < 0.05).
METHODOLOGY

Material and tools

*K. pinnata* P. leafs crude extract; maltodekstrin; manitol; polivinyl pirolidon; PEG 6000; ethanol 96%; male and female mice DDY strain; distilled water; analytical scales (AND type GR-200); glass tools and volumetrical tools (Pyrex); freeze drying equipment (LABCONCO); filter paper Whatman No. 42; rotary evaporator; Karl Fischer moisturemeter (Mitsubishi); oven (Memmert); tablet compression machine; tablet hardness tester; granule compressibility testing devices (Omron H3Cr); sieving analitical devices; desintegration time testing devices (Omron H3BA), friability tester; flow rate tester; animal scales; stomach sonde; mice box.

Acute toxicity test of granule

*K. pinnata* P. leafs crude extract granule was dissolved in water and administered orally to male and female mice of DDY strain (20-25g body weight) using stomach sonde with the dose of 16g/kg body weight that is equivalent to 16g/kg crude extract powder. The toxic effect was observed during 24 hours after administration. The number of died mice during this period were noted. The 50% mortality of mice that is expressed as the LD$_{50}$ of the crude extract granule and crude extract powder were calculated by the Weill (1952) methods.

Tableting of lozenges tablet

The active ingredient is *K. pinnata* P. crude extract granule. Additional ingredients (excipients) i.e.: PVP as binder, mannitol as sweetener and filler, PEG 6000 as glidan. Tablet was designed in 3 formulae by wet granulation method as shown in Table 1.

Table 1. The weight (g) composition of ingredient in lozenges tablet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Formulae</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Powder extract (g)</td>
<td>I 0,52</td>
<td>II 0,52</td>
<td>III 0,52</td>
</tr>
<tr>
<td>PVP (g)</td>
<td>0,1</td>
<td>0,15</td>
<td>0,2</td>
</tr>
<tr>
<td>PEG 6000 (g)</td>
<td>0,1</td>
<td>0,1</td>
<td>0,1</td>
</tr>
<tr>
<td>Mannitol (g)</td>
<td>2,00</td>
<td>2,00</td>
<td>2,00</td>
</tr>
</tbody>
</table>

Note: Mannitol was used to fix the weight of tablet.

All of the ingredient were calculated in weight (g). PVP was dissolved in ethanol 96% . *Kalanchoe pinnata* P. leafs crude extract powder was added to mannitol and homogenized. The PVP solution was added to mixed of mannitol-crude extract, then it was homogenized become a granule. Granule was sieved with mesh 12, then dried in oven at 40$^\circ$C, and sieved again with mesh 16. Granule was then evaluated on flow property, particle size distribution, compresibilility property, and moisture with added PEG 6000. Finally the granule was then compressed into lozenges tablet with the weight of each tablet was 2 g [3].

Evaluation of lozenges

Organoleptic test

Organoleptic test i.e.: shape, colour, smell, and taste

Size uniformity test

At amount of 20 lozenges tablet were taken out randomly, and its diameter and thickness were measured.

Weight uniformity test

At amount of 20 lozenges tablet were taken out randomly, and each tablet weight was measured, and the average weight of tablet was calculated.
Friability test
At amount of 20 lozenges tablet were taken out randomly, put in the suction tool, weighted, and clean up of dust, it then put in the friability tool. After running for 4 minutes (speed 25 rpm) the tablets were cleaned and weighted (terms : < 1%) [4].

Hardness test
At amount of 20 lozenges tablet were taken out randomly and its hardness were measured (terms: 10 -20 kg/cm²).

Disintegration time test
At amount of 6 lozenges tablet were taken out randomly. One tablet was put in each tube in the disintegration test basket, then put on 1 disc of each tube and run the tool. Water temperature was kept at 37 ± 2°C as the media. The basket was up and down 30 times per minute, regularly. At the end of test, the basket was lift up and the tablets were observed. The tablets must be completely disintegrated or destroyed in less than 15 minutes. If there was 1 or 2 tablets not disintegrated or destroyed completely, the testing must be repeated with 6 other tablets.

RESULTS AND DISCUSSIONS
LD₅₀ value of *Kalanchoe pinnata* P. leafs crude extract granule.
The LD₅₀ value of *Kalanchoe pinnata* P. leafs crude extract granule to mice was 16g/kg body weight. It is equivalent to 16g/kg leafs crude extract powder. There was not mouse died. Based on the table of the substances toxicity classification (Gleason, 1969), the *Kalanchoe pinnata* P. leafs crude extract granule was belongs to practically non toxic substances (LD₅₀ >15g/kg body weight).

Organoleptic test
The lozenges tablet of *Kalanchoe pinnata* P. leafs crude extract with 3 formulae revealed that all have similar properties. The result of evaluation was shown in Table 2. The tablet has homogeneous colour; has a specific odor/aromatic smell, may be due to the active substance is a natural product which has a specific and strong odor; has a sweet and cool taste due to the addition of manitol that is able to cover a sour taste of caffeoic acid and ascorbic acid come from *Kalanchoe pinnata* P. leafs crude extract powder; whereas the formulae III had more bitter taste due to the amine compound of PVP.

Table 2. Organoleptic evaluation resultsof 3 formulaes

<table>
<thead>
<tr>
<th>Formulae</th>
<th>Colour</th>
<th>Odor/Smell</th>
<th>Taste</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Yellowish-white</td>
<td>Aromatic</td>
<td>Sweet</td>
</tr>
<tr>
<td>II</td>
<td>Yellowish-white</td>
<td>Aromatic</td>
<td>Sweer</td>
</tr>
<tr>
<td>III</td>
<td>Yellowish-white</td>
<td>Aromatic</td>
<td>Sweet, more bitter</td>
</tr>
</tbody>
</table>

Weight uniformity test
The weight uniformity of 3 lozenges tablet formulaes was evaluated and the result is presented in Table 3.

Table 3. Weight uniformity of 3 lozenges tablet formulaes

<table>
<thead>
<tr>
<th>Formulae</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>2.0103</td>
<td>2.0110</td>
<td>2.0185</td>
</tr>
<tr>
<td>Maksimum</td>
<td>2.0300</td>
<td>2.0311</td>
<td>2.0320</td>
</tr>
<tr>
<td>Average</td>
<td>2.0209</td>
<td>2.0212</td>
<td>2.0276</td>
</tr>
<tr>
<td>SD</td>
<td>0.0066</td>
<td>0.0058</td>
<td>0.0039</td>
</tr>
</tbody>
</table>
According to Pharmaceutical Techno-logy requirement, a tablet that has weight more than 300 mg must have weight deviation not more than 5% of the average weight (A column) and not more than 10% of the average weight (B column) of each tablet formulae. Result revealed that there was not tablet has weight deviation more than 5% of the average weight (A column) and more than 10% of the average weight (B column) of each tablet formulae. The weight variation of tablet was decreased with increasing the concentration of PVP. The weight variation of tablet is related to granule flow property and particle size distribution that was formed from more homogeneous spherical granule with increasing of PVP content.

Size uniformity test
The size uniformity of 3 lozenges tablet formulae was evaluated. At amount of 20 tablet of each formulae was taken out and the size of each tablet was measured. Result of measurement is presented in Table 4. Result revealed that the diameter of each tablet was ranged between 10-20,12 mm and the thickness ranged between 5,65-5,80 mm. The size variation was increased with increasing the PVP concentration in the formulae.

Table 4. Size uniformity of 3 lozenges tablet formulaes

<table>
<thead>
<tr>
<th>Size uniformity of lozenges tablet (mm)</th>
<th>Formulae</th>
<th>I</th>
<th>II</th>
<th>III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>D</td>
<td>T</td>
<td>D</td>
<td>T</td>
</tr>
<tr>
<td>Minimum</td>
<td>20.10</td>
<td>5.65</td>
<td>20.10</td>
<td>5.65</td>
</tr>
<tr>
<td>Maximum</td>
<td>20.12</td>
<td>5.80</td>
<td>20.12</td>
<td>5.78</td>
</tr>
<tr>
<td>Average</td>
<td>20.11</td>
<td>5.71</td>
<td>20.11</td>
<td>5.70</td>
</tr>
<tr>
<td>SD</td>
<td>0.0081</td>
<td>0.0578</td>
<td>0.0083</td>
<td>0.0484</td>
</tr>
</tbody>
</table>

Note: D = diameter; T = thickness

Friability test
According to Pharmaceutical Techno-logy requirement, a good tablet must have a friability value less than 1%. The friability value of 3 lozenges formulae of this research is presented in Table 5.

Table 5. Friability value of 3 lozenges tablet formulae

<table>
<thead>
<tr>
<th>Formulae</th>
<th>Initial weight (g)</th>
<th>Final weight(g)</th>
<th>Friability (%)</th>
<th>Average (%)</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>40.4180</td>
<td>40.2492</td>
<td>0.4176</td>
<td>0.4193</td>
<td>0.0176</td>
</tr>
<tr>
<td></td>
<td>40.4235</td>
<td>40.2466</td>
<td>0.4376</td>
<td>0.4193</td>
<td>0.0176</td>
</tr>
<tr>
<td></td>
<td>40.4168</td>
<td>40.2541</td>
<td>0.4026</td>
<td>0.4193</td>
<td>0.0176</td>
</tr>
<tr>
<td></td>
<td>40.4248</td>
<td>40.2965</td>
<td>0.3174</td>
<td>0.4193</td>
<td>0.0176</td>
</tr>
<tr>
<td>II</td>
<td>40.4431</td>
<td>40.3101</td>
<td>0.3289</td>
<td>0.3279</td>
<td>0.0100</td>
</tr>
<tr>
<td></td>
<td>40.4370</td>
<td>40.3006</td>
<td>0.3373</td>
<td>0.3279</td>
<td>0.0100</td>
</tr>
<tr>
<td></td>
<td>40.5361</td>
<td>40.4363</td>
<td>0.2462</td>
<td>0.3279</td>
<td>0.0100</td>
</tr>
<tr>
<td>III</td>
<td>40.5012</td>
<td>40.3988</td>
<td>0.2528</td>
<td>0.2470</td>
<td>0.0054</td>
</tr>
<tr>
<td></td>
<td>40.5270</td>
<td>40.4289</td>
<td>0.2421</td>
<td>0.2470</td>
<td>0.0054</td>
</tr>
</tbody>
</table>

According to Pharmaceutical Techno-logy requirement, a good tablet must have a hardness value ranged between 10-20 kg/cm². The hardness value of 3 lozenges formulae of this research is presented in Table 6. Result of hardness value of 3 formulae was eligible to have a hardness value less than 20 kg/cm². It is, probably, due to the amount of binder in the formulae was sufficient. The results indicated that increasing the PVP concentration as binder resulting a much more bond among the particles, to form a compact granule and contained less amount of fine particles. PVP also make a
tablet has a good compresibility thereby increasing resilience against shocks and friction inter tablet, and as expected, the tablet is not fragile and not much eroded during mold process and product distribution to consumers. Based on the result of statistical analysis of tablet hardness using Tukey test method, the concentration of PVP provides a significant difference to the tablet hardness ($\alpha_{\text{sign}} < 0.05$).

**Hardness Test**

According to Pharmaceutical Technology requirement, a good tablet must have a hardness value ranged between $4 - 8 \text{ kg/cm}^2$. The hardness value of 3 lozenges formulae of this research is presented in Table 6.

Table 6. Hardness value of 3 lozenges tablet formulae

<table>
<thead>
<tr>
<th>No.</th>
<th>Formulae I</th>
<th>Formulae II</th>
<th>Formulae III</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11</td>
<td>16.5</td>
<td>19</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>16</td>
<td>18.5</td>
</tr>
<tr>
<td>3</td>
<td>11.5</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>16</td>
<td>18.5</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>17</td>
<td>18.5</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>16.5</td>
<td>18.5</td>
</tr>
<tr>
<td>7</td>
<td>11.5</td>
<td>16</td>
<td>19.5</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>17</td>
<td>18.5</td>
</tr>
<tr>
<td>10</td>
<td>12</td>
<td>16</td>
<td>18.5</td>
</tr>
<tr>
<td>11</td>
<td>11</td>
<td>16.5</td>
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</tr>
<tr>
<td>12</td>
<td>11</td>
<td>17</td>
<td>19.5</td>
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<tr>
<td>13</td>
<td>11.5</td>
<td>17</td>
<td>18.5</td>
</tr>
<tr>
<td>14</td>
<td>12</td>
<td>16.5</td>
<td>18.5</td>
</tr>
<tr>
<td>15</td>
<td>12</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>16</td>
<td>11</td>
<td>17</td>
<td>18.5</td>
</tr>
<tr>
<td>17</td>
<td>11.5</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>18</td>
<td>12</td>
<td>16.5</td>
<td>19</td>
</tr>
<tr>
<td>19</td>
<td>11.5</td>
<td>17</td>
<td>19.5</td>
</tr>
<tr>
<td>20</td>
<td>12</td>
<td>16</td>
<td>18.5</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>11.525</strong></td>
<td><strong>16.575</strong></td>
<td><strong>18.975</strong></td>
</tr>
<tr>
<td><strong>SD</strong></td>
<td><strong>0.4435</strong></td>
<td><strong>0.4375</strong></td>
<td><strong>0.5730</strong></td>
</tr>
</tbody>
</table>

According to Pharmaceutical Technology requirement, a good tablet must have a hardness value ranged between $10 - 20 \text{ kg/cm}^2$. The hardness value of 3 lozenges formulae of this research is presented in Table 6. Result of hardness value of 3 formulae was eligible to have a hardness value less than $20 \text{ kg/cm}^2$. It is, probably, due to the amount of binder in the formulae was sufficient. The results indicated that increasing the PVP concentration as binder resulting a much more bond among the particles, to form a compact granule and contained less amount of fine particles. PVP also make a tablet has a good compresibility thereby increasing resilience against shocks and friction inter tablet, and as expected, the tablet is not fragile and not much eroded during mold process and product distribution to consumers. Based on the result of statistical analysis of tablet hardness using Tukey test method, the concentration of PVP provides a significant difference to the tablet hardness ($\alpha_{\text{sign}} < 0.05$).
Disintegration time test

According to Pharmaceutical Technology requirement, a good tablet must be completely disintegrated or destroyed in less than 30 minutes. The disintegration time test result of 3 lozenges formulae of this research is presented in Table 7.

Table 7. Disintegration time of 3 lozenges tablet formulae

<table>
<thead>
<tr>
<th>Formulae</th>
<th>Disintegration time (minute) of tablet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
</tr>
<tr>
<td>1</td>
<td>13.86</td>
</tr>
<tr>
<td>2</td>
<td>13.91</td>
</tr>
<tr>
<td>3</td>
<td>13.90</td>
</tr>
<tr>
<td>Average</td>
<td>13.89</td>
</tr>
<tr>
<td>SD</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Result of disintegration time of 3 formulae was eligible to have a disintegration time less than 30 minutes, but the best formulae was formulae III because it has a longest time for tablet to be completely destroyed. The longer time for lozenges tablet being in the mouth the longer or the better of inhibitory effect on microbial growth in the mouth. Based on the result of statistical analysis of tablet disintegration time using Tukey test method, the concentration of PVP provides a significant difference to the tablet disintegration time (\( \alpha \) sign) < 0.05).

CONCLUSION

The LD\(_{50}\) value of *K. pinnata* P. leaf crude extract granule and leaf crude extract powder using male and female mice was 16g/kg body weight, so that the *K. pinnata* P. crude extract granule and crude extract powder were belong to practically non toxic substances.

The *K. pinnata* P. leaf crude extract granule and leaf crude extract powder was formulate become a lozenges tablet with wet granulation methods using PVP as a binder at the concentration of 5%, 7.5%, and 10%. The result revealed that formulae III with PVP concentration of 10% is the best formulae. The tablet properties were, the average weight was 2.0276 g; diameter was 20.11 mm; thickness was 5.71 mm; friability value was 0.2470%; hardness value was 18.975 kg/cm\(^2\); and disintegration time was 28.25 minutes.

ACKNOWLEDGEMENT

The authors thank to the Directorate General of Higher Education of Republic of Indonesia for funding this research.

REFERENCES


SIMULTANEOUS DETERMINATION OF CAFFEINE AND NICOTINAMIDE IN ENERGY DRINKS BY FIRST-ORDER DERIVATIVE SPECTROPHOTOMETRY

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ABSTRACT

A simple analytical method is described for the simultaneous determination of caffeine and nicotinamide in powder form of energy drinks. The first-order derivative UV-spectrophotometry was employed using water as solvent, the zero-crossing wavelengths were found at 273 and 261 nm for caffeine and nicotinamide, respectively. The calibration graphs were rectilinear up to 25.4 µg/mL and the relative standard deviations values were lower to 1.7 % for both analytes. The limits of detection were 0.669 µg/mL caffeine and 0.550 µg/mL nicotinamide, respectively with recovery values of 93.44 to 104.65% for caffeine and 88.89 to 104.26% for nicotinamide.

Key words: energy drink, caffeine, nicotinamide, zero-crossing method

INTRODUCTION

Caffeine and nicotinamide are two of substituents that always could be found in energy drink. Numbers of reported method were already available for the individual determination of both compounds. Caffeine has been determined by titration in nonaqueous solvent (Depkes RI 1995); high performance liquid chromatography (HPLC) (BP Commission 2009; USP Convention 2009). Nicotinamide has been determined by titration in nonaqueous solvent (Depkes RI 1995; BP Commission 2009) and HPLC (USP Convention 2009). Caffeine in beverage has been estimated using derivatives spectrophotometry (Aldpogan et al 2002) and HPLC (Aditia 2005). No spectroscopy method has been developed for the simultaneous determination of them in mixture. Derivative spectroscopy provides a greater selectivity than common spectroscopy and offers a powerful approach for resolution of band overlapping quantitative analysis of multicomponent mixture (Hayun et al 2006). The aim of the present study is the development of a simple, accurate, precise and sensitive derivative spectroscopic method for the determination of caffeine and nicotinamide in energy drinks on the basis of zero-crossing measurement.

METHODOLOGY

Chemicals and Reagents

Caffeine and nicotinamide were obtained from BPOM. All the chemical and solvents used in the present study were a kind gift from Faculty of Pharmacy, Pancasila University. Commercial energy drinks was purchased from the local supermarket in South Jakarta.

Instrumentation

Spectroscopic analysis was carried out on Shimadzu UV 1700 double beam UV/Visible spectrophotometer. The zero order absorption spectra were recorded over the wavelength range of 200 to 400 nm, against solvent blank, in quartz cuvettes with 1 cm diameters. For all solutions, the derivative spectra were obtained over 210 – 400 nm range at 2.4 nm.

Standard and calibration solutions

Standard stock solutions of a concentration of 250 µg/mL of caffeine and nicotinamide were prepared separately using water as solvent. Appropriate volume of standard stock solution was diluted.
Simultaneous Determination of Caffeine

with water to get concentration of 10 µg/mL of both analytes. Further dilutions were made from stock solutions in the same solvent to get linearity concentrations 5-25 µg/mL for caffeine and nicotinamide.

**Linearity and Range**

Calibration curves were constructed by analysis of working standard solutions of caffeine and nicotinamide with at least 5 different concentrations in the range between 5 - 25 µg/mL. Each concentration was analysed in triplicate. First derivative values (D1) of caffeine and nicotinamide were measured at 261 and 273 nm respectively. Calibration curve was plotted by taking first derivative values (D1) on Y-axis and concentrations on X-axis. The relation between analyte concentration (x) and its corresponding D1 value (y) i.e., expressed by the equation y = mx + b, where m is slope and b is intercept.

**Limit of Detection and Quantitation**

The limit of detection (LOD) and limit of Quantitation (LOQ) was estimated from the standard calibration curve. The residual standard deviation of regression line or standard deviation of y intercepts of regression lines used to calculate LOD and LOQ. Here, LOD=3.3 D/S and LOQ=10 D/S. Where, D is the standard deviation of y intercept of regression line and S is the slope of calibration curves.

**Precision and Accuracy**

Precision of procedure was calculated from analysis of the powder of energy drinks that made six replications of synthetic mixture and two commercial brand name. Quantities of the energy drinks powder equivalent to 50 mg of caffeine and 20 mg of nicotinamide were accurately weighed and transferred in a 100 mL volumetric flask. Weighed powder was dissolved in 50 mL of water, shake well. Then the volume made up to 100 mL with water. Solution obtained was filtered and diluted with water to get the concentration within linearity and used for the measurement of caffeine and nicotinamide derivative spectra. The concentration of caffeine and nicotinamide in energy drinks were calculated using the corresponding calibrated curve.

Accuracy of method was measured as percentage of deviation between added and measured concentrations (recovery study). Quantities of the energy drinks powder equivalent to 37.5 mg of caffeine and 15 mg of nicotinamide plus 12.5 mg of caffeine and 5 mg of nicotinamide were accurately weighed, transferred in a 100 mL volumetric flask. Weighed powder was dissolved in 50 mL of water, shake well. Then the volume was made up to 100 mL with water. Solution obtained was filtered and diluted with water to get the concentration within linearity and used for the measurement of caffeine and nicotinamide derivative spectra. The concentration of caffeine and nicotinamide in energy drinks were calculated using the corresponding calibrated curve. The other one, quantities of the energy drinks powder equivalent to 25 mg of caffeine and 10 mg of nicotinamide plus 25 mg of caffeine and 10 mg of nicotinamide were accurately weighed, transferred in a 100 mL volumetric flask. The next step were done as above.

**RESULTS AND DISCUSSIONS**

The zero-order spectra of caffeine and nicotinamide were found in Fig. 1 and 2. As a result, the simultaneous determination of the two compounds cannot be possible by direct absorbance measurements. The first order spectra of them were shown in Fig 3 and Fig 4.

Derivative spectroscopy, based on mathematical transformation of spectra zero-order curves into derivative spectra, allows a fast sensitive and precise resolution of a multi-component mixture and overcomes the problem of overlapping of a multicomponent system. Derivative spectroscopy on the basis of zero-crossing measurements involves measurement of absolute value of total derivative spectrum at an abscissa value corresponding to the zero-crossing wavelength of the derivative spectra of individual components, which should be only the function a the concentration of the other component.
Zero-crossing points of caffeine and nicotinamide were found to be 273 and 261 nm respectively (Fig. 3 and Fig. 4). The measurements exhibited the best linear response and have given a near zero intercept on the coordinate of the calibration graph, and is less affected by the concentration of any other component. Caffeine was determined by measurement of its D1 amplitude at the zero-crossing point of nicotinamide (at 261 nm). Nicotinamide was determined by measurement of its D1 at the zero-crossing point of caffeine (at 273 nm).

Linearity, Range, LOD and LOQ

The developed UV derivative method has shown the linearity in range of 5 to 25.4 µg/mL for caffeine and nicotinamide. The calibration curves were constructed by plotting the D1 value against caffeine (at 261 nm) or nicotinamide (at 273 nm) concentration at zero-crossing wavelength of caffeine and nicotinamide, respectively. The results obtained are summarized in Table 1. The linearity of the calibration curves and the adherence of the method to Beer’s law are validated by the high value of correlation coefficient. The LOD and LOQ values are also summarized in Table 1.
Table 1. Regression analysis data for the determination of caffeine and nicotinamide using first order derivative spectra.

<table>
<thead>
<tr>
<th>Statistical Parameter</th>
<th>Caffeine</th>
<th>Nicotinamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (nm)</td>
<td>261</td>
<td>273</td>
</tr>
<tr>
<td>Regression Equation</td>
<td>$y = 0.00017 + 0.00191x$</td>
<td>$y = -0.00030 - 0.00175x$</td>
</tr>
<tr>
<td>Correlation coefficient ($r^2$)</td>
<td>0.9997</td>
<td>-0.9998</td>
</tr>
<tr>
<td>Limit of Detection (µg/mL)</td>
<td>0.669 µg/mL</td>
<td>0.550 µg/mL</td>
</tr>
<tr>
<td>Limit of Quantitation (µg/mL)</td>
<td>2.230 µg/mL</td>
<td>1.835 µg/mL</td>
</tr>
</tbody>
</table>

* $Y = mx + b$ where, $y$ is the D1 amplitude at specified wavelength, $x$ is the concentration of drug in µg/mL, $m$ is slope and $b$ is intercept.

Table 2. Accuracy and precision data for determination of caffeine and nicotinamide

<table>
<thead>
<tr>
<th>Name of sample</th>
<th>analyte</th>
<th>Amount (mg/sachet)</th>
<th>Precision</th>
<th>% recovery in addition of analyte (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic</td>
<td>Caffeine</td>
<td>50</td>
<td>100.23 ± 0.493</td>
<td>0.492</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Nicotinamide</td>
<td>20</td>
<td>103.22 ± 0.441</td>
<td>0.427</td>
</tr>
<tr>
<td>Brand A</td>
<td>Caffeine</td>
<td>50</td>
<td>101.50 ± 0.657</td>
<td>0.647</td>
</tr>
<tr>
<td>Brand A</td>
<td>Nicotinamide</td>
<td>20</td>
<td>103.33 ± 1.698</td>
<td>1.643</td>
</tr>
<tr>
<td>Brand B</td>
<td>Caffeine</td>
<td>50</td>
<td>96.65 ± 0.512</td>
<td>0.530</td>
</tr>
<tr>
<td>Brand B</td>
<td>Nicotinamide</td>
<td>20</td>
<td>109.59 ± 0.791</td>
<td>0.722</td>
</tr>
</tbody>
</table>

Accuracy and Precision

Data of these tables showed a good accuracy and precision over the entire concentration range. The relative standard deviation (RSD%) values were less than 1.7% for both analytes in all three kind sample. The percentage recovery in each case was calculated. The results obtained from the recoveries of both analytes showed good accuracy. (Table 2). No interference was observed from the presence of the other constituent in energy drink.

Applicability of the proposed methods for the simultaneous estimation of caffeine and nicotinamide was studied by assay of commercial powder of energy drinks (Brand A and Brand B). The results obtained are given in Table 2. The results obtained are in good agreement with the labeled content.

CONCLUSION

A rapid, simple and specific UV first derivative spectroscopic method has been developed for the simultaneous determination of caffeine and nicotinamide. Method is also successfully applied for determination of both compounds in energy drinks.

ACKNOWLEDGEMENT

The Authors are grateful to Kopertis Wilayah III for research funding and also Faculty of Pharmacy, Pancasila University for extending the facilities.

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Liliek Nurhidayati


MODULATION OF MACROPHAGE IMMUNE RESPONSES OF EXTRACT MIXTURE OF BETEL LEAF (PIPER BETLE, L), GAMBIER (UNCARIA GAMBIER, ROXB) AND CALCIUM HYDROXIDE ON PHAGOCYTIC CELLS OF MICE

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ABSTRACT

The mixture of betel leaf (Piper betle L), gambier (Uncaria gambir, Roxb) and slaked lime (calcium hydroxide) has been used as one of traditional medicine in Indonesia and other countries with multi purpose for along time. Generally, the mixture of extract is used for chewing in their natural raw condition along with many other ingredients like betel, areca nut, gambier, cardamom, slaked lime, etc. some people use it as an immunomodulatory agent. To ensure the effectiveness of the mixture of betel chewing as an immunomodulatory agents. Materials and A mixture that consists of water extract of betel leaves, gambir and slaked lime, the extract was given to 7 groups of mice for 14 days with doses 100, 200 and 400 mg/kg body weight. Two kinds of Immunomodulatory drug in syrup form commonly used in the treatment of infectious was used as positive controls. Then the peritonial fluid of mice containing macrophage cells was isolated by performing surgery. This study calculated the number of phagocytosis activity and capacity of macrophage cells of mice, after one hour intra peritoneal injection Staphylococcus epidermidis on each group of mice. The betel chewing component mixture group compared with negative controls showed an increase of phagocytosis activity of 86.6%, 94.2% and 88.3% for doses of 100, 200 and 400 mg/kg body weight, respectively. Meanwhile the phagocytosis capacity was found for an average of 50 macrophage cells was 626.1, 806.4 and 754.5 bacteria for dose of 100, 200 and 400 mg/kg body weight respectively. For the doses administered the 200 mg/kg dose showed the most efficacy where both the phagocytic activity and capacity was calculated statistically significant (p≤0.05)

Key words: Immunomodulatory, betel, gambier, slaked lime.

INTRODUCTION

Chewing of betel nut is an ancient custom in Indonesia and in some countries, such as, several parts of south-east Asia, south Asia, the south Pacific islands and Taiwan. This practice dates back several thousand years and is deeply entrenched in the culture of the population. It is estimated, there are about 600 juta people chewing betel nut in the world, the habit of chewing of betel nut is a habit the second largest after smoking in the world (Nelson and Heischober, 1999).

Chewing of betel nut is traditionally known to be useful for the treatment of various diseases such as, bad breath, boils and abscesses, conjunctivitis, constipation, headache, hysteria, itches, mastitis, mastoiditis, leucorrhoea, otorrhoea, ringworm, swelling of gum, rheumatism, abrasion, cuts and injuries etc, as folk medicine. (Nair et al, 2004).

In Indonesia generally, the people chewing betel nut is a mixture of betel leaf, gambier, slaked lime and areca nut. In this study, areca nut was not used, because according to the results of research conducted by several researchers, areca nut has been related mainly to oral, pharyngeal and oesophageal cancer. Areca-nut alkaloids such as, arecoline, arecaidine, guvacoline and guvacine through metabolism in the body will transformed into nitrosamine derivatives, in which these compounds are the causes of oral cancer (Canniff et al, 1981; Chang, et al, 2002; Nair et al, 2004).

On the other hand, betel leaves, gambier and slaked lime are materials of natural medicines, almost have no side effects, but have many benefits for body health, especially to combat infectious
diseases. (Nair et al, 2004) So far, most researchers have been conducted research for the components of chewing betel for the treatment of infectious diseases, while for immnomodulatory agent in the form of a mixture has not been found in existing publications. However, eugenol as the main content of betel leaf and catechin as the main content of Gambier have been known as immunomodulatory agents (Chikara, 2005; Biswas et al, 2002).

Therefore, this study aims to determine the effectiveness of a mixture of betel chewing component as immunomodulatory agents. Two kinds of Immunomodulatory drug in syrup form commonly used in the treatment of infectious was used as positive controls

**METHODOLOGY**

**Materials**

The materials of each plant were obtained from a single source, Betel (*Piper betle*, L). leaves from Bogor (Balitro), gambier (*Uncaria gambir*, Roxb) from Payakumbuh (West Sumatra), while, sliced lime was obtained from E-merck.

**Preparation of crude aqueous extract of a mixture chewing of betel**

The preparation of a mixture was conducted by blending betel leaf, gambier and sliced lime with a ratio 421: 70: 9, add water 500 mL. The mixture was filtered with Whatman paper No.IV. Then, dried with a freeze drier and calculated the yield was obtained.

**Preparation of experimental animals**

The animals were used for experiments acclimatization for 14 days, then, selected animals were eligible. The number of animals for each group were calculated with the formula Federer (Each group consisted of for mice).

Animals were divided into 7 groups randomly, ie; extract mixture of low-dose group (100 mg/kg body weight of mice), extract mixture of medium-dose group (200 mg/kg body weight of mice), extract mixture of large-dose group (400 mg/kg body weight of mice), normal control, negative control (CMC 0.5%), positive control 1 (Echinacea syrup 3,1 ml/kg body weight of mice), positive control 2 (Phylanthus niruri syrup 6,2 ml/kg body weight of mice).

**Preparation of bacterial suspension**

The *Staphylococcus epidermidis* was obtained from microbiology laboratory Indonesian Institute of Sciences, Cibinong, Bogor. The stock of these bacteria was kept in nutrient agar, then inoculated into the broth medium, incubation at shaker incubator with speed 120 rpm, temperature 30 °C, for 24 hours until reach the active phase. Adjust the amount of bacteria by using spectrophotometer UV-visible ±10^5 cfu / ml (T= 25%, λ = 580 nm)

**Treatment of experimental animals** (Kusmardi et al, 2006)

Each group of experimental animals were administered the test preparation once daily according to with groups and doses, as mentioned above, for 14 days. On the 15th day, experimental animals was injected intra peritonial with 0.5 ml of Staphylococcus epidermidis (10^9 cfu/ml). After one hour of injection, the experimental animals were carried out surgery. Into the peritonium cavity was injected 1 ml phosphate buffered saline solution and taken fluids peritoneum.

**Preparation of glass slides for analysis** (Dey et al, 1996)

Put 100 µl peritoneal fluid on an object glass, fixation with absolute methanol for 5 minutes, do the staining with Giemsa 4%, leave for 45 minutes, dip into 0,1 M acetic acid, washing with distilled water, dried and observed by light microscopy.

**Determination of macrophage phago-cytosis activity and capacity** (Dey et al, 1996)
The determination value phagocytosis activity of macrophages was conducted with calculate the amount of macrophages that carried out phagocytosis activity of 100 macrophages against *Staphylococcus epidermidis*, the selection of macrophages was conducted as randomly. Perform the calculate was conducted three times from different slide for one experimental animal.

The determination value phagocytosis capacity of macrophages was conducted with calculate the amount 50 of macrophages that still active carried out phagocytosis against *Staphylococcus epidermidis*, the selection of macrophages was conducted as randomly. Perform the calculate was conducted three times from different slide for one experimental animal.

**RESULTS AND DISCUSSIONS**

From the research results was found the yield of extract mixture of betle, gambier and slaked lime with ratio 421 : 70 : 9 about 9.79% dry extract. The determination of ratio number is based on preliminary experiments, where the number 421 gram is converted from the people chewing betel for small doses 4 pieces betel leaf once a day. The based on of this dose conversion, for medium doses was administered 8 pieces and for large doses was administered 16 pieces betel leaf once a day. The yield of dry extract was found just about 9.79 %, because, the fresh betel leaves contain about 85-90% water (Guha, 2006).

<table>
<thead>
<tr>
<th>Name of sample</th>
<th>Phagocytosis activity</th>
<th>Phagocytosis capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>63.58 ± 1.78</td>
<td>362.67 ± 8.48</td>
</tr>
<tr>
<td>Negative control</td>
<td>70.92 ± 0.90</td>
<td>395.17 ± 4.43</td>
</tr>
<tr>
<td>Positive control 1</td>
<td>92.00 ± 0.27</td>
<td>770.83 ± 14.89</td>
</tr>
<tr>
<td>Positive control 2</td>
<td>87.33 ± 0.72</td>
<td>756.08 ± 13.82</td>
</tr>
<tr>
<td>100 mg/kg of body weight</td>
<td>86.58 ± 0.65</td>
<td>626.08 ± 12.68</td>
</tr>
<tr>
<td>200 mg/kg of body weight</td>
<td>94.17 ± 0.88</td>
<td>806.43 ± 15.11</td>
</tr>
<tr>
<td>400 mg/kg of body weight</td>
<td>88.33 ± 1.15</td>
<td>754.50 ± 9.38</td>
</tr>
</tbody>
</table>

The statistical test (Table 1) showed that the treated groups for phagocytosis activity and capacity (doses 100, 200 and 400 mg/kg body weight) are significantly different from the normal control, and negative control, while for doses 200 mg/kg of body weight beside are significantly different for normal control and negative control also for both of positive control (p<0.05).

Phagocytosis activity increase from dose 100 mg to dose 200 mg/kg body weight, ie; from 86.8% to 94.17%, while, from dose 200 mg to dose 400 mg/kg body weight was occurred the opposite, ie; Phagocytosis activity decrease from 94.17% to 88.33%.

Likewise, for Phagocytosis capacity increase from dose 100 mg to dose 200 mg/kg body weight, ie; from 626.08 to 806.42, while, from dose 200 mg to dose 400 mg/kg body weight was occurred the opposite, ie; Phagocytosis capacity decrease from 806.43 to 754.50.

In research was carried out by Domingues et, al (2011) with topic; immunomodulatory effect of *Uncaria tamentosa* Aqueous-ethanol Extract Triggers an Immunomodulation toward a Th2 Cytokine Profile, in this study also was occurred at doses of 125 mg/kg body weight works as stimulant and at a dose of 500 mg work as immunosuppression, as shown in the Figure 2 and Figure 3.
Modulation of Macrophage Immune Response

Figure 1. Effect phagocytosis activity of mixture of betle, gambier and slaked lime to macrophage cell of mice

Nor con = Normal control, Neg con = Negative control,
Pos con 1 = Positive control 1, Pos con 2 = Positive control 2
S dos = 100mg/kg bw   M dos = 200 mg/kg bw   L dos = 400 mg/kg bw

Figure 2. Effect phagocytosis capacity of mixture of betle, gambier and slaked lime to macrophage cell of mice

According to Labro (2000) this occurs due to the large doses will cause metabolic disorders immune system or destroy of certain parts on the macrophage cell, because the mechanism action of macrophage cells not only work as phagocytosis of bacteria or foreign substances in the body, but macrophage cell also released some mediator chemical for the interaction between the immune system in the body, when metabolism of immune system is disturbed, so cause phagocytosis activity and capacity also will be disturbed. However, when the dose is administered appropriate, macrophage cells will work together with other parts of the immune system in against the bacteria, as occurs in a dose of 200 mg/kg body weight, the activity and capacity of macrophage cells already reached the optimum condition, as shown in the Figure 1 and Figure 2.

Each of the materials was used to make the mixture in this study, have activity as antibacterial. Betel leaf has been known as an antibacterial against several bacterial pathogens, such as; Proteus mirabilis, Proteus vulgaris, Salmonella typhymurium, Shigella flexneri Staphylococcus aureus, Streptococcus mutans, Staphylococcus faecalis, Candida albicans, Vibrio cholerae, Diplococcus pneumoniae.
Muhammad Yanis Musdja

and *Klebsiella aerogenes* (Musdja 1. et al, 2011; Kumar et al, 2010; Shitut et al, 1999). Gambier has been known as an antibacterial against several bacterial pathogens, such as; *Staphylococcus epidermidis, Staphylococcus aureus Bacillus subtilis, Shigella flexneri, Proteus aeruginosa, Escherichia coli, Proteus vulgaris and Proteus mirabilis.* (Musdja 2. et al, 2011). Slioked lime (CaOH₂) with concentration 0,005% already has activity as antibacterial (Asada et al, 2001).

According to Labro (2000), drug which have effect as immunomodulatory agent and antibacterial will be better to preventive and therapy for infectious diseases, due to the synergistic effect of the immunomodulatory agent with antibacterial, when compared to the drug that only have the effect as immunomodulatory agent, or is only have an effect as antibacterial.

**CONCLUSION**

The mixture of betel leaf (*Piper betle L*), gambier (*Uncaria gambir, Roxb*) and slaked lime (calcium hydroxide) has effect as immunomodulatory agent. Dose of 200 mg/kg body weight is better than dose of 100 mg / kg and dose of 400 mg/kg body weight.

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THE TLC PROFILE CHARACTERIZATION OF ETHANOL EXTRACT OF KALANCHOE PINNATA WITH OR WITHOUT DRYING BY FREEZE AND SPRAY DRYING

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ABSTRACT

Extract has been dried to reduce water content and improve its quality. This research aims to determine the effect of drying techniques on the profile of compounds in the ethanol extract of Kalanchoe pinnata, which were observed based on the results of TLC analysis. By using of Silica Gel GF 254 and methanol-water (9:1) as mobile phase, the TLC profile of ethanol extract of Kalanchoe pinnata with or without drying by freeze and spray drying were not different. The results showed that the freeze and spray drying processes did not affect the profile of compounds in ethanol extract of leaves of Kalanchoe pinnata on TLC plates.

Key words: Kalanchoe pinnata leaf, ethanol extract, freeze drying, spray drying, TLC

INTRODUCTION

Kalanchoe pinnata is used in ethnomedicine generally for the treatment of ear-ache, cough, diarrhoea, dysentery, abscesses, ulcers, insect bites, heart-troubles, epilepsy, arthritis, dysmenorrhea, inflammation, pain, and fever (Gill, 1992). The leaves of this plant have been reported to possess antimicrobial activity (Obaseiki, 1985; Ofokansi, 2005; Akinsulire, 2007). Other works have also shown that this plant possesses analgesic, anticonvulsion, antiinflammatory, antiarthritic and antispasmytic properties (Theophil, 2006). The ethanol extract of Kalanchoe pinnata showed greater antimicrobial activity (MIC 5 %) than the water extract (MIC 20 %) (Firdaus, 2010).

One of the factors that affect the quality of extract is water and residual solvent content. The recently research shown that The water content of ethanol extract of Kalanchoe pinnata was higher than the requirement of The National Agency of Drug and Food Control (NA-DFC) (Yantih, 2010), so is important to be dried by a method of extract dried, such as by freeze and spray drying (Kartiningsih, 2010). The process of drying extract also has a role in determining the quality of the resulting dry extract (Lieberman, 1989).

The objective for this study was to determine the effect of drying techniques on the profile of compounds in the ethanol extract of Kalanchoe pinnata, which were observed based on the results of TLC analysis.

METHODOLOGY

Plant Collection and Determination

Kalanchoe pinnata leaves was collected from Balitro Garden, Bogor, and determinated by Research Center for Biology, Indonesian Institute of Sciences.

Preparation and Extraction of Plant material

Extract preparation was by macerating according to method of Salahdeen, 2006. The plant extract was prepared by blending and macerating 500 g of the fresh leaves of Kalanchoe pinnata with 500 ml of 96% ethanol at 40 ºC for 24 hours. The resulting mixture was filtered. The extracts solution of 96% ethanol was evaporated through vacuum rotavapor.

Freeze and Spray Drying

Drying viscous extract process using freeze dryer and spray dryer. Firstly, maltodextrin dissolved in distilled water. The maltodextrin solution and the crude extract of Kalanchoe pinnata was mixed and
homogenized. At the freeze drying techniques, the mixed solutions were first frozen and then dried by freeze drying at 48 hours, 5µmHg pressure and temperature of -50°C (Kartiningsih, 2010). The temperature inlet was 180°C by the Spray drying. Out put from this process are powder extracts (Kartiningsih, 2010).

**Thin Layer Chromatography System**

The separation of thin layer chromatography (TLC) was performing by using of Silica Gel GF 254 and methanol-water (9:1) as mobile phase. The TLC profile of ethanol extract of Kalanchoe pinnata with or without drying by freeze and spray drying.

**RESULTS AND DISCUSSIONS**

The yield of ethanol extract from fresh leaves *Kalanchoe pinnata* was about 3%. Because of the water content of the ethanol extract did not met the requirement of The NA-DFC (Yantih, 2010), the freeze and spray drying was carried out to improve the quality of the extract.

The method of drying the extract may affect the content of compounds in the extract. In this study observed the effect of drying on the content of the compounds in ethanol extract of leaves of *Kalanchoe pinnata* based on the results of analysis by TLC. The observations carried out to compare the profile spots of condensed ethanol extract of the leaves before and after drying using the method of freeze and spray drying.

![Figure 1. Chromatogram of ethanol extract of *Kalanchoe pinnata* with (S) and without (E) freeze drying on ordinary light (a), UV 254 nm (b), and 366 nm (c).](image)

By using of Silica Gel GF 254 and methanol-water (9:1) as mobile phase, the TLC profile of ethanol extract of *Kalanchoe pinnata* with or without drying by freeze and spray drying were not different. The results shown that there is no influence of both drying process on the content of the compounds in ethanol extract of leaves of *Kalanchoe pinnata* (Figure 1 and 2).

The chromatogram before and after drying the extract were detected on UV 254 dan 366 nm, and also on the ordinary light. However, require further study the effect of drying on the extracts using other analytical methods.
Figure 2. Chromatogram of ethanol extract of *Kalanchoe pinnata* with (S) and without (E) spray drying on ordinary light (a), UV 254 nm (b), and 366 nm (c).

From the results obtained by freeze and spray drying powder, water content obtained from the three temperature is eligible under the legislation of traditional medicine that was ≤ 10% (Anonim, 2000).

**CONCLUSION**

The freeze and spray drying processes did not affect the profile of compounds in ethanol extract of leaves of Kalanchoe pinnata on TLC plates.

**ACKNOWLEDGEMENT**

Special thanks are devoted for Directorate General of Higher Education of Indonesia which has given grants in this research.

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FORMULATION DEVELOPMENT AND EVALUATION OF CLARITHROMYCIN GEL FOR INJECTION INTO PERIODONTAL POCKET

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ABSTRACT

Clarithromycin loaded sorbitan monostearate organogels (SMS organogels) and hydrophobic amphiphilic gel were formulated then mixed with various concentrations of mucoadhesive polymers. All gel formulations were tested for flow property, mucoadhesive property, spreadability and work of syringeability. Amphiphilic gels could provide desired mucoadhesive property with acceptable spreadability and work of syringeability. The formulations can adhere on the porcine buccal mucosa up to 30 hours. On the other hands, SMS organogel could not provide a good mucoadhesive property although they provide good syringeability and spreadability. Those amphiphilic gel formulations are the suitable formulations for sustaining clarithromycin from periodontal pocket for a long period of time. 

Key words: periodontal disease, organogels, amphiphilic gels, clarithromycin, sustained release

INTRODUCTION

Periodontal disease refers to the inflammations and infections including gingivitis and periodontitis. These conditions can affect to the supporting structure of the teeth [M.A. Listgarten., 1987]. The result is the formation of pockets between gingiva and tooth that causes gingival margin retraction and the development of an ideal environment for anaerobic bacteria growth responsible for the disease. Moreover, periodontal disease

Antimicrobial agents are orally administered to produce a systemic effect, but this application provide some disadvantages including systemic side effects [A. Mombelli.et al. , 1997, C.M. Bollen. Et al., 1996] and insufficient drug concentration at the treating site for the long period of time [B.N.A. Vandekerckhove. et al., 1997]. A solution to these problems could be the local administration of the drug formulated in a controlled release delivery system to be placed directly on the action site.

Clarithromycin, has similar antimicrobial spectrum as erythromycin but is more effective against certain gram-negative bacteria

Organogels are semisolid systems, in which an organic liquid phase is immobilized by a three-dimensional network composed of a self-assembled gelator [K. Almdal.,1993, T. Pénzes.et al., 2005]. There are several types of organogels have been investigated extensively, including amphiphilic gels and sorbitan monostearate organogels.

Sorbitan monostearate gels are opaque, thermoreversible semisolids, and they are stable at room temperature for weeks. Amphiphilic gels, with one surfactant causing the gelation of another liquid surfactant, are so named because of the amphiphilic nature of the lipid phase. The gelators of amphiphilic gels are also generally amphiphilic substances, such as sorbitan monostearate (Span 60) and sorbitan monopalmitate (Span 40) [N. Jibry.et al., 2004, S. Murdan. et al.,1999].

The purpose of this study was to formulate sorbitan monostearate (SMS) organogels in liquid paraffin and amphiphilic gels and evaluate in vitro sustained-release ability to produce of organogel matrices for injection at periodontal pocket.
METHODOLOGY

Formulation of Clarithromycin-containing sorbitan monostearate organogels

Sorbitan monostearate (20% w/v) and polysorbate 20 (2% w/v) and liquid paraffin was mixed and heated to 60 °C then cool to room temperature to obtain an opaque, thermoreversible semisolid gel. The gel was mixed with ethyl cellulose gel and various concentrations of mucoadhesive polymers (Sodium alginate and Carbopol 934 P) prior to add Clarithromycin.

Formulation of Clarithromycin-containing amphiphilogels

Sorbitan monostearate (20% w/v) and polysorbate 20 (2% w/v) were weighed into a vial, and sorbitan monooleate was mixed and heated to 60 °C then cool to room temperature to obtain an opaque, thermoreversible semisolid gel. The gel was mixed with ethyl cellulose gel and various concentrations of mucoadhesive polymers (Sodium alginate and Carbopol 934 P) prior to add Clarithromycin.

Rheology measurement

The rheological data of each formulation was performed by using HAAKE Roto Visco 1 using spindle number 35. Measuring at 25 °C. Then the relationship between shear rate and shear stress was plotted.

Evaluation of Spreadability

The spreadability was tested to determine the gel movement under the force or pressure. Using parallel - plate method. In brief, 1 g of gel between two 20 x 20 cm glass plates. Then the weight 125 g was placed onto the upper plate. The spreading diameter was measured after 1 minute. All formulations were measured at 25±1 °C.

Examination of the work of syringeability

The syringeability of each formulation was determined by Lloyd® universal testing machine equipped with 5 kg cell in compression mode. In brief, gel formulations were transferred to identical plastic syringe to the constant height. The content of each syringe was fully expressed through a 21-gauge needle of diameter 0.33 mm using texture analyzer with the speed 1 mm/min and the resistance to the expression was determined from the area under the resultant force-displacement plot.

Evaluation of the ex vivo mucoadhesion force

The ex vivo mucoadhesion force of each formulation was determined by Lloyd® universal testing machine in tensile mode. The porcine buccal mucosa (2 cm x 2 cm) was attached to both upper and lower metal support using cyanoacrylate adhesive. A 500 mg sample of the gel was placed on the lower mucosa. The upper mucosa was then lowered to the gel surface until contact was made between the surfaces and the thickness of the gel layer was kept constant (0.5 mm) for 5 minutes. After 2 min in contact, the upper mucosa was withdrawn upwards at a speed of 1 mm/minute until failure occurred between the surfaces. The whole experiment was performed at room temperature and a relative humidity of 50%.

Evaluation of the ex vivo mucoadhesion time

The ex vivo mucoadhesion time was performed triplicate after application of gel preparation on porcine buccal mucosa (size 2 x 2 cm). The tissue were fixed on microscopic slide (slanted to 45°) with plastic clamp. The mucosal side was flushed with 20 ml/min of simulated saliva solution until the gel was removed. During the experiment, the time was recorded.
RESULTS AND DISCUSSIONS

After preparation, syneresis cannot be observed in amphiphilic gel formulation but can be seen in the SMS organogel formulation added with 5 and 10 % carbopol 934 P thus two formulations have to be excluded from further study.

The rheological properties of SMS organogel formulations and amphiphilic gel formulations are illustrated in Figure 1 and 2 respectively. Both mucoadhesive materials added in gel formulations can change their rheological property. Blank SMS organogel and amphiphilic gel formulations provide pseudoplastic flow whereas formulations mixed with sodium alginate show different rheological data. For amphiphilic gel formulations, increasing percentage of carbopol 934 P increases the yield value.

The spread diameters of all formulations are presented in Figure 3. Most of gel formulations have spread diameter between 60 - 75 mm. Increasing concentration of carbopol 934 P in gel formulation significantly decreases the spread diameter of gel formulations. On the other hands, increasing concentration of sodium alginate cannot decrease the spread diameter of gel significantly. This may relate to the viscosity of the gel formulation.

The works of syringeability of each formulation are shown in Figure 4. The works of syringeability of SMS organogel formulations are less than those of amphiphilic gel formulations. Implying easier for injection. Moreover, increasing concentration of carbopol 934 P in gel formulation significantly increases the work of syringeability whereas concentration of sodium alginate has no significant effect for spread diameter.
The ex vivo mucoadhesion force of all formulations are presented in Figure 5. Mucoadhesion forces of amphiphilgel are more than those from SMS organogel. Indicating that amphiphilgel formulations provide more mucoadhesive property than SMS organogel formulations. Furthermore, Increasing concentrations of carbopol 934 P increase the force to detach gel from porcine buccal mucosa. However, this effect can not be seen when increasing the percentage of sodium alginate in gel formulations.

The ex vivo mucoadhesion time of all formulations are presented in Figure 6. Amphiphilgel formulations show more ex vivo mucoadhesion time than SMS organogel formulations. The amphiphilgel formulation containing 10 % carbopol 934 P can adhere on porcine buccal mucosa up to 30 hrs.

CONCLUSION

Amphiphilgel formulations can provide good mucoadhesive property with acceptable syringeability and spreadability. Those formulations have a potential to be the suitable vehicle for maintaining clarithromycin at periodontal pocket for the long period of time.
ACKNOWLEDGEMENT

The authors would like to thank IIAC, Chulalongkorn University Centenary Academic Development Project for research funding. Clarithromycin from the Siam Pharmaceutical Co., Ltd. is also appreciated.

REFERENCES


A-GLUCOSIDASE INHIBITORY AND ANTIOXIDANT ACTIVITIES OF ASPERGILLUS TERREUS MC751

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ABSTRACT

Hyperglycemia causes increased production of free radicals and reduced antioxidant defense, which further mediate the initiation and progression of diabetes-associated complication. The combination of α-glucosidase inhibitors and antioxidants will be more effective for preventing further development of type 2 diabetes. The objective of this study was to evaluate the antidiabetic and antioxidant properties of the ethyl acetate extract of mycelia (AtKM) and filtrate (AtKF) extracts from the Aspergillus terreus MC751 in Czapek-dox medium through in vitro models. The AtKM extract showed a stronger α-glucosidase inhibitory activity than AtKF with IC₅₀ value are 13.38µg/ml and 28.81µg/ml respectively. Kinetic studied revealed of AtKM showed uncompetitive inhibition whereas AtKF showed non-competitive inhibition on α-glucosidase. The antioxidant activities were measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay. The AtKF showed a higher radical scavenging activity with IC₅₀ value is 22.98 µg/ml than AtKM (IC₅₀ = 35.25µg/ml). The higher total phenolic content was observed in AtKF extract (ca 278.41 mg gallic acid equivalent/g of extract). Based on its strong α-glucosidase inhibitory and antioxidant activities, the ethyl acetate extracts of A. terreus appears to be a potential as a good resource for future development of antioxidant and antidiabetic drugs.

Key words: Aspergillus terreus, α-glucosidase inhibitory activity, antioxidant activity

INTRODUCTION

Hyperglycemia has been a classical risk factor in the development of diabetes and its complications associated with diabetes (Ramkumar 2009). This uptake of excess sugar causes an imbalance between oxidant and antioxidant in the human body, a condition called oxidative stress (Mayur, 2010). The increase in free-radical production and reduction of antioxidant defense may partially mediate the initiation and progression of diabetes-associated complication (Yao, 2010).

Several management strategies have been proposed for their early stage of dysglycemia with the aim of preventing further development. α-Glucosidase inhibitor is usually used to prevent or medically treat type 2 diabetes. These inhibitors combine with intestine α-glucosidase and block the uptake of postprandial blood glucose (Johnston, 1998). The combination of α-glucosidase inhibitors and antioxidants will be more effective for preventing further development of type 2 diabetes (Shibano, 2008).

A number of plant and mushrooms (fruiting body) are commonly known to produce therapeutic agent, but there are few report on lower fungi. The fungi are nature source of biotechnological interest in the fermentative processes that culminate in the production of secondary metabolites (Fox & Howlett, 2008). They are exploited in medicine and industry and considered to be potential sources of new therapeutic agents.

Aspergillus terreus is a common soil saprophyte that has been recovered from desert soil, grassland, and compost heaps, as well as contaminants of stored corn, barley, and peanuts A. terreus produce a variety of secondary metabolites that are economically significant, such as lovastatin, antihypercholesterolemic drug (Balajee, 2009). Compare to study as anticholesterol there have

The objective of this study is to evaluate the antidiabetic and antioxidant activities of ethyl acetate extracts of filtrate culture (extracellular) and mycelia (intracellular) were obtained from Aspergillus terreus MC751 in Czapek-dox medium through various in vitro models.

METHODOLOGY

Microorganism and Culture condition
Aspergillus terreus MC751 were obtained from Research Center for Chemistry-Indonesian Institute of Sciences. The stock culture of A. terreus was grown on PDA, incubated for seven days 25°C from which two discs (8mm) of fungal mycelia were used to inoculate 50 ml Czapek-dox's broth (sucrose 3%, sodium nitrate 0.2%, K₂HPO₄ 0.1%, magnesium sulphate 0.05%, potassium chloride 0.05%, ferrous sulphate 0.001%). After incubation for 10 days at static condition (25°C), the culture broth was filtered through Whatman filter paper no.2 due to separate filtrate culture and mycelia. The mycelia and filtrate culture were extracted with ethyl acetate by shaking for 20 min at 250 rpm in room temperature. The extracts were filtered and evaporated to dryness in vacuo. The dry extracts were used for various assays.

α-Glucosidase inhibitory assay
The enzyme inhibition activity for α-glucosidase was assessed according to the methods reported by Kim et al 2004, with minor modifications. The reaction mixture contained 250 µl of 3mM p-nitropheynl α-D-glucopyranoside (PNPG), 495 µl of 100mM phosphate buffer (pH 7.0) adding to flask contain 5 µl of sample dissolved in DMSO at various concentrations (5 to 50µg/ml). The reaction mixture was pre-incubated for 5 min at 37°C, the reaction was start by adding 250 µl α-Glucosidase (0.065unit/ml) (EC 3.2.1.20 from Wako Pure Chemical Industry) incubation was continued for 15 min. The reaction stopped by adding 1ml of 0.1 M Na₂CO₃. Activity of α-glucosidase was determined by measuring release of p-nitrophenol at 400 nm.

Kinetics of inhibition against α-Glucosidase
The inhibition type against α-glucosidase has evaluate through increasing concentration of PNPG was used as substrate in control (no inhibitor), quercetin (reference) and At-M and At-F (inhibitors). The inhibition type of the data was determined by Lineweaver-Burke plot analysis of the data

Scavenging effect on DPPH radical
The scavenging effect of the extracts on the DPPH radicals was estimated according to the method of Yen, 1995. Each sample (10-200 µg/ml) in 5 ml of methanol was mixed with 1.0 ml of a solution of 1 mM DPPH radical in methanol. The mixture was shaken vigorously and kept for 30 min, and the absorbance of the mixture at 517 nm. All the tests and analyses were performed in triplicate and averaged

Determination of total phenolic content (TPC)
The total polyphenolic content was determined colorimetrically using the Folin-Ciocalteau (FC) method according to Singleton, 1999. with some modifications. Test sample (0.5 ml) was mixed with 0.5 ml of 2N FC reagent and allowed to stand for 8 min to which 1.5 ml of 20% sodium carbonate was added and mixed completely. The reaction mixture was incubated at room temperature for 2 hours. Absorbance of the reaction mixture was measured at 765 nm. Gallic acid was taken as standard.

RESULTS AND DISCUSSION
In the present study, the hypoglycemic potential of ethyl acetate extracts of filtrate culture (At-F) and mycelia (At-M) of A. terreus was evaluated by the α-glucosidase inhibition assay. The At-M extract
showed a stronger α-glucosidase inhibitory activity than At-F with IC₅₀ value are 13.38µg/ml and 28.81µg/ml respectively. Quercetin was used as positive control with IC₅₀ value 3.10µg/ml. The assay presented dose dependent inhibition (Figure 1).

![Figure 1](image1.png)

Figure 1. α-Glucosidase inhibitory effect of At-M and At-F (5-100µg/ml) Quercetin was taken as reference standar.

The mode of inhibition of the At-M and At-F extracts against α-glucosidase was also investigated by Lineweaver-Burk plot analysis. This showed different type inhibition between At-M (un-competitive) and At-F (non-competitive) against α-glucosidase (Figure 2), which indicated that the active compound of At-M binds to complex substrate-enzyme (E-S), whereas At-F binds to a site other than the active site of the enzyme. These result indicated that the active compound(s) of those extract might have different structure. Therefore to isolate the active compound(s) from those extract will help identify the potent natural inhibitor(s) of α-glucosidase, in turn helping to prevent and/or to treat diabetes.

![Figure 2](image2.png)

Figure 2. Lineweaver-Burk plots of kinetic analysis of α-glucosidase treated with various concentrations of PNPG in the absence or presence of extract (At-M and At-F) and quercetin as reference.

In this study we used quercetin as standard due to several reports that quercetin a phenolic compound (flavonoid) have stronger inhibition of α-glucosidase from yeast *S. cereviceae* than
acarbose (Tadera, 2006; Li, 2009; Jo, 2009). As reported previously, α-glucosidase broadly consists of type I from yeast S. cerevisiae and type II from the mammalian species, and there are structural differences between these types (Kim, 2005). There are reports that voglibose and acarbose have high inhibitory effects on mammalian α-glucosidase, but no inhibitory activity for yeast S. cerevisiae (Kim, 2004). These different inhibitory activities may be caused by structural differences due to the origin of the enzyme.

The free radical scavenging activity of At-M and At-F extracts from A. terreus, measured by the DPPH method, was shown in Figure 3. At-F extract had stronger antioxidant activity (IC\textsubscript{50} = 22.98 µg/ml) than At-M extract (IC\textsubscript{50} = 35.25 µg/ml). However, quercetin which was used as positive control showed better radical scavenging effect (IC\textsubscript{50} = 10.06 µg/ml).

![Figure 3. Antioxidant activity of the At-M and At-F extracts measured by the DPPH method and compared with quercetin as reference.](image)

The radical scavenger activity of the ethyl acetate extracts of A. terreus may be because of the presence of phenolic compounds as antioxidant compound. The higher phenolic content in At-F extract (278.41 µg/g) might account for stronger antioxidant activity than At-M extract (194.61 µg/g). Total phenol content of extracts were determinant from regression equation of standard curve gallic acid (Y=0.1006x-0.0014, R\textsuperscript{2}=0.9986) and expressed in gallic acid equivalent (GE). Some reports have strongly suggested a relationship between polyphenolic content of the extract and activity as antioxidant and inhibition of α-glucosidase (Tadera, 2006, Ramkumar, 2009, Mayur 2010). The polyphenolic compounds are the secondary metabolites commonly found in plant, mushrooms, and fungi and have been reported to possess biological effects such as anti-inflammatory, antibacterial, antitumor, antiartherosclerotic anticarcinogenic, antimutagenic, and cardio protective actions including antioxidant activity such as hispidin, 3,14′-bihispidinyl, hypolomine B, and 1,1-distyrylpyrylethen from fungi Inonotus xeranticus and Phellinus linteus (Jung, 2008) and 3,3′-dihydroxyterphenyllin, 3-hydroxyterphenyllin, and candidusin B from A. candidus (Yen, 2001, and Yen, 2003). The bioactivity of phenolics may be related to their ability to chelate metals, inhibit lipoxygenase, and scavenge free radical (Decker, 1997).

**CONCLUSION**

Based on its strong α-glucosidase inhibitory and antioxidant activities, the ethyl acetate extracts of A. terreus (both of extra and intracellular metabolites) appears to be a potential as a good resource for future development of antioxidant and antidiabetic drugs. However, we need further study to identify the active fractions responsible for hypoglycemic activity and to clarify the mechanism of the effect.
ACKNOWLEDGEMENT
We thank Dr. M. Hanafi from the Research Centre of Chemistry, Indonesian Institute of Sciences, Serpong, Indonesia, for providing the culture of A. terreus MC751 for this study.

REFERENCES
FINGERPRINT STUDY OF FOeniculum Vulgare Mill. FOR STANDARDIZATION OF TRADITIONAL MEDICINE EXTRACT

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ABSTRACT

Foeniculum vulgare Mill. could improve the immunological functions (Hua-ze, D., et al 2009); antinociceptive and antiinflammatory activity in mice (Nassar, M.I., et al 2010). The fennel oil of F. vulgare Mill. could be a source of antimicrobial agent (Gulfraz, M., et al 2008). To ensure the quality of its extract, study of fingerprint or chromatogram profile is needed. This can be an alternative method when its marker is unavailable. In this research, the fingerprint study was carried out by TLC (Thin Layer Chromatography) scanner (254 and 366 nm). Fingerprint had been analysed from 4 extracts of F. vulgare Mill. i.e. extract of n-hexane, chloroform, ethyl acetate, and ethanol respectively. From the result, elution by n-hexane: ethyl acetate (7: 3 v/v) showed the best separation which had the specific retention factor (1) \( \lambda = 254 \text{ nm} : \) at 0.21, 0.32, 0.37, and 0.90 for n-hexane extract; at 0.20, 0.31, 0.37, and 0.91 for chloroform extract; at 0.22, 0.33, 0.39, and 0.87 for ethyl acetate extract; and 0.23; 0.34; and 0.41 for ethanol extract; (2) \( \lambda = 366 \text{ nm} : \) at 0.32, 0.68, 0.75, and 0.90 for n-hexane extract; at 0.31, 0.64, 0.72, and 0.90 for chloroform extract; at 0.33, 0.67, 0.72, and 0.91 for ethyl acetate extract; and 0.35; 0.70; and 0.48 for ethanol extract. Compare to others, ethyl acetate extract had the best TLC profile; therefore it can be used for standardization of F. vulgare Mill. extract.

Keywords: Foeniculum vulgare Mill., TLC scanner, fingerprint, and retention factor

INTRODUCTION

Along with the development of civilization, health has become a priority health needs after a major human food. Therefore, natural medicine as one of the alternative media to maintain the health has been interest to be developed. In order to improve the quality, safety, and efficacy of natural medicines, this research had been conducted.

There are some methods to control the quality of simplicia, extract, or natural medicine products, i.e.: 1. Fingerprint or chromatogram profile of medicinal plant extracts.
2. Identification and determination of specific chemical compound (marker) contained in a medicinal plant.

One of the medicinal plants that abundant in Indonesia is Foeniculum vulgare Mill. Foeniculum vulgare Mill. could improve the immunological functions (Hua-ze, D., et al 2009); antinociceptive and antiinflammatory activity in mice (Nassar, M.I., et al 2010). The fennel oil of F. vulgare Mill. could be a source of antimicrobial agent (Gulfraz, M., et al 2008). Because of its benefits, F. vulgare Mill had been selected in this study.

The characteristic volatile constituents of F. vulgare Mill. are trans-anethole, cis-anethole, estragole, fenchone, p-anisaldehyde, eucalyptol and camphor (Chun, X.J., et al 2005). GC-MS analysis of essential oil obtained from the seed of Foeniculum vulgare showed the presence of 31 components containing 95.2% of the total amount and the major component was trans-anethole (70.1%) (Gulfraz, M., et al 2008).

This study aims to obtain a fingerprint/chromatogram profile F. vulgare Mill. as a basic for standardization of F. vulgare Mill. extract.
 METHODOLOGY

Material

_ Foeniculum vulgare_ Mill. fruit were collected from Indonesian Medicinal and Aromatic Corps Research Institute and was determined in Herbarium Bogoriense, Research Centre for Biology, Indonesian Institute of Science, Bogor, Indonesia with determination letter number 439/IPH.1.02/If.8/VI/2011. Solvent used in this research were n-hexane, chloroform, ethyl acetate, ethanol and methanol (Merck Co.)

Equipment

Evaporator Buchi R-200 (Buchi Co.), 60 GF254 silica plates (Merck Co.) , Linomat 5 TLC Spotter (CAMAG), TLC Scanner 3 (CAMAG), TLC Documentation System Reprostar3 (CAMAG) and UFLC LCk20 series (Shimadzu, Kyoto, Japan)

Methods

Extraction

Dried powder material (30 g) of _Foeniculum vulgare_ Mill. leaves were macerated in 4 kinds of solvents (n-hexane, chloroform, ethyl acetate, and ethanol) each was 300 mL for 24 hours (three times repeated). Each macerate was evaporated (by rotary evaporator), then weight the extracts and determined the yields.

Analysis

TLC analysis of each extract was carried out on silica gel GF 254 as stationary phase, and there were 3 mobile phases which were applicable for _Foenicullum vulgare_ Mill extract: a) n-hexane : ethyl acetate (7 : 3 v/v); b) chloroform : ethyl acetate (9 : 1 v/v); c) toluene : chloroform : ethyl acetate (3 : 6 : 1 v/v/v). The TLC analysis results were detected by TLC Scanner and recorded TLC Documentation System.

RESULTS AND DISCUSSIONS

The yields of n-hexane, chloroform, ethyl acetate, and ethanol extracts were showed on Table 1. The chloroform extract gave the highest yield. It was caused by the polarity of chloroform. Chloroform has a low polarity index, and the main compound of _Foenicullum vulgare_ Mill. (anethol) also has a low polarity index. Therefore, chloroform could bind the main compound of _F. vulgare_ Mill.

The TLC analysis profiles of each extract on silica gel GF 254 using mobile phase: a) n-hexane : ethyl acetate (7 : 3 v/v); b) chloroform : ethyl acetate (9 : 1 v/v); c) toluene : chloroform : ethyl acetate (3 : 6 : 1 v/v/v) which were recorded by photo documentary system can be seen on Figure 1 and 2. All eluents above had a low polarity index or classified as a non polar – semi polar eluents. Those eluents could separate the compounds contained in the extract clearly therefore it can be concluded that _F. vulgare_ Mill extracts had the best separation result in a non polar to semi polar eluent/mobile phase.

The chromatograms profile (254 nm and 366 nm) of TLC scanner can be seen at Figure 3 and 4. The chromatograms profile showed that n-hexane: ethyl acetate (7: 3 v/v) had the best separation which had the specific retention factor (1) \( \lambda = 254 \text{ nm} \) : at 0.21, 0.32, 0.37, and 0.90 for n-hexane extract; at 0.20, 0.31, 0.37, and 0.91 for chloroform extract; at 0.22, 0.33, 0.39, and 0.87 for ethyl acetate extract; and 0.23; 0.34; and 0.41 for ethanol extract; (2) \( \lambda = 366 \text{ nm} \) at 0.32, 0.68, 0.75, and 0.90 for n-hexane extract; at 0.31, 0.64, 0.72, and 0.90 for chloroform extract; at 0.33, 0.67, 0.72, and 0.91 for ethyl acetate extract; and 0.35, 0.70, and 0.48 for ethanol extract.

Elution with chloroform : ethyl acetate (9 : 1 v/v) showed the specific retention factor (1) \( \lambda = 254 \text{ nm} \) : at 0.12, 0.43, and 0.48 for n-hexane extract; at 0.04, 0.12, 0.44, and 0.75 for chloroform extract; at 0.13, 0.44, 0.50, 0.56, and 0.87 for ethyl acetate extract; and 0.04; 0.12, 0.15, 0.46, and 0.79 for ethanol extract; (2) \( \lambda = 366 \text{ nm} \) at 0.16, 0.50, 0.54, 0.76, 0.84, and 0.93 for n-hexane extract; at 0.05, 0.16, 0.35, 0.53, 0.84, and 0.97 for chloroform extract; at 0.05, 0.35, 0.58, 0.80, 0.85, and 0.94 for ethyl acetate extract; and 0.05, 0.37, 0.49, 0.56, 0.80, 0.88, and 0.97 for ethanol extract.
Figure 1. Chromatogram profile ($\lambda = 254$ nm) on TLC silica plates of n-hexane, chloroform, ethyl acetate, and ethanol extracts after elution with A) n-hexane : ethyl acetate (7 : 3 v/v); B) chloroform : ethyl acetate (9 : 1 v/v); C) toluene : chloroform : ethyl acetate (3 : 6 : 1 v/v/v).

Figure 2. Chromatogram profile ($\lambda = 366$ nm) on TLC silica plates of n-hexane, chloroform, ethyl acetate, and ethanol extracts after elution with A) n-hexane : ethyl acetate (7 : 3 v/v); B) chloroform : ethyl acetate (9 : 1 v/v); C) toluene : chloroform : ethyl acetate (3 : 6 : 1 v/v/v).
Figure 3. The chromatograms profile (254 nm) of TLC scanner n-hexane, chloroform, ethyl acetate, and ethanol extracts eluted with A) n-hexane : ethyl acetate (7 : 3) v/v; B) chloroform : ethyl acetate (9 : 1 v/v); C) toluene : chloroform : ethyl acetate (3 : 6 : 1 v/v/v).

Figure 4. The chromatograms profile (366 nm) of TLC scanner n-hexane, chloroform, ethyl acetate, and ethanol extracts eluted with A) n-hexane : ethyl acetate (7 : 3) v/v; B) chloroform : ethyl acetate (9 : 1 v/v); C) toluene : chloroform : ethyl acetate (3 : 6 : 1 v/v/v).
And elution with toluene : chloroform : ethyl acetate (3 : 6 : 1 v/v/v) showed the specific retention factor (1) $\lambda = 254$ nm : at 0.10, 0.12, 0.38, 0.42, 0.60, 0.74 and 0.95 for n-hexane extract; at 0.10, 0.12, 0.38, 0.60, 0.75, and 0.94 for chloroform extract; at 0.08, 0.13, 0.39, 0.45, 0.61, 0.65, 0.90, and 0.96 for ethyl acetate extract; and 0.11, 0.13, 0.40, 0.62, 0.77, and 0.92 for ethanol extract; (2) $\lambda = 366$ nm at 0.42, 0.61, 0.65, 0.71, and 0.80 for n-hexane extract; at 0.24, 0.40, 0.61, 0.72, 0.81, and 0.94 for chloroform extract; at 0.24, 0.45, 0.61, 0.72, 0.80, and 0.96 for ethyl acetate extract; and 0.27, 0.40, 0.63, 0.73, and 0.97 for ethanol extract.

Every chromatogram of all extracts in the same eluent has the similar pattern. It means that they have some similar retention factors value. Compare to others, ethyl acetate extract had the best TLC profile; therefore it can be used for standardization of $F. vulgare$ Mill. extract.

CONCLUSION
According to the results above, it can be concluded that the fingerprint/ chromatogram profile of ethyl acetate extract of $F. vulgare$ Mill. can be used for standardization of its traditional medicine extract.

ACKNOWLEDGEMENT
This paper is acknowledgement to Research Centre for Drug and Food - The National Agency of Drug and Food Control for funding this research.

REFERENCES
**ABSTRACT**

One requirement of semisolid dosage forms that applied to open wound is sterile. Sterilization commonly used is dry and wet sterilization. The objective of this research was to search the influence of dry and wet heat in sterilization process to the change of physical properties of gel base obtained. In wet heat sterilization, gel base was sterilized with autoclave at 115 °C for 30 minutes. In dry heat sterilization, CMC Na was put on an oven at 160 °C while Ca Alginate at 150 °C, each of them for an hour. Then CMC Na and Ca Alginate were turned into gel base. In addition, gel base without sterilization is made as comparison. The next day, the physical properties i.e. viscosity and spreadability of the gel bases were measured. The influence of wet heat to viscosity and spreadability is compared to that if dry heat. The influence is shown by the change of viscosity and spreadability of the gel base to the gel base without sterilization process. The result showed that dry heat had bigger influence to viscosity and spreadability of gel base than wet heat. In wet heat, the gel viscosity decreased to 4.17 dPaS and 23.33 dPaS in dry heat at high level of the mixture of CMC Na and Ca Alginate. The same result emerges at intermediate and low level of the mixture. In wet heat, gel spreadability increased to 0.0123 g.cm/sec and 0.1846 g.cm/sec in dry heat at high level of the mixture of CMC Na and Ca Alginate. The same result emerges at intermediate and low level of the mixture.

**Keyword**: dry heat, wet heat, CMCxNa, Ca-Alginate, physical properties

**INTRODUCTION**

Hydrogels are three dimensional network that are formed by physically and chemically crosslink of polymer in water. Hydrogels have to be sterile before being applied to open wound (Moyhan and Crean, 2009; Adel, et al, 2010)

Sodium Carboxymethylcellulose (CMC Na) is an anionic polymer available at various grades that differ in degree of substitution and molecular weight (Zatz and Kushla, 1996). Figure 1 shows an ideal CMC Na structure that has 1 degree of substitution. Water molecule at any temperatures can not force the chain to hydrate them. It explains that CMC is water insoluble (Hoefler, 2011)

CMC Na can be sterilized in the dry state by maintaining it at temperature of 160 °C for 1 hour. This process results significant decrease in viscosity of the solution prepared from sterilized material. Aqueous solution of CMC Na may be sterilized by autoclaving. This process reduces the viscosity of the CMC Na solution for about 25%. However, this reduction of viscosity is less than CMC Na solution prepared from material sterilized in the dry state (Rowe et al, 2006)
Alginate is a polysaccharide that contains of several units (typically 100 -3000) monomer linked together in flexible chain. Alginate is linear co-polymer of α-L-guluronate dan α-β-mannuronate. Its gelling properties are derived from the binding of Ca ions localized between homopolymeric blocks of guluronate residues (Funduenu et al, 1999). Ca Alginate may be sterilized by autoclaving at 115 °C for 30 minutes or dry heat at 150 °C for 1 hour (Rowe et al, 2006).

**Figure 2. Alginate structure.**

**METHODOLOGY**

**Preparation of hydrogel base**

<table>
<thead>
<tr>
<th>R/</th>
<th>Carbopol 941</th>
<th>0.75</th>
</tr>
</thead>
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<tr>
<td>CMC Na</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Ca Alginate</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>Triethanolamine1</td>
<td>Glycerol</td>
<td>12.5</td>
</tr>
<tr>
<td>water ad</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Mixture of CMC-Na and Ca-Alginate at various level

<table>
<thead>
<tr>
<th>level</th>
<th>CMC-Na (g)</th>
<th>Ca-Alginate (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>Intermediate</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Low</td>
<td>0.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

On dry heat sterilization. CMC Na is put in an oven at 160 °C for an hour while Ca Alginate at 150° C, each of them for an hour. In the aseptic room, CMC Na is poured to aqua p.i and stirred with mixer at 400 rpm for 10 minutes. It is added with Ca Alginate, stirred for 10 minutes at 400 rpm also. Then, it is added with sterilized solution mixture of TEA, Glycerol, and Carbopol, stirred for 10 minutes at 400 rpm. Then, the physical properties of the gel base are determined.

On wet heat sterilization. CMC Na is poured to water and stirred with mixer at 400 rpm for 10 minutes. It is added with Ca Alginate and stirred for 10 minutes at 400 rpm. Then, it is added with Glycerol and Carbopol, stirred until it becomes homogeneous. Then, it is added with TEA. It is sterilized with autoclave 115 °C for 30 minutes. Then, the physical properties of the gel base are determined.

**Determination of physical properties**

Viscosity determination. Gel base is put in to a container. Then, the portable viscometer is put in the container. Viscosity is obtained by monitoring the moving of the viscosity pointer.

Spreadability determination. The gel base weights 2 g is put in the middle of the ground glass slide. The gel is sandwiched between two ground glass slides. A 1 kg weight is placed on the top of the two slides for 3 minutes. The top slide is subjected to pull of the 80 g. The time and the distant needed to separate the two slides are noted. Spreadability is then calculated using the following formula $S = \frac{M \times L}{T}$.
Where, S = is spreadability, M = is the weight in the pan (tied to the upper slide), L = is the length moved by the glass slide, and T = represent the time to separate the slide completely from each other.

RESULTS AND DISCUSSIONS

One requirement of semisolid dosage forms that applied to open wound is sterile. Sterilization process commonly used is wet sterilization. Wet sterilization using autoclave decreases viscosity of the gel base (Rowe et al, 2006). Decreasing of viscosity may be caused by depolimerization of polymer at wet sterilization process. During wet sterilization process hydrolysis may occur. It causes depolymerization occur. On the dry sterilization, depolymerization may be caused by oxidation process. It is interesting to study the phenomena happened during sterilization process that influences physical properties of gel base. The objective of this study is to search the influence of wet heat and dry heat during sterilization process to the physical properties of the gel base containing CMC Na and Ca Alginate. Physical properties studied are viscosity and spreadability. This study is applied to three levels of the mixtures of CMC Na and Ca Alginate, i.e. high, intermediate and low levels.

![Figure 3. Viscosity of hydrogel base](image1.png)

![Figure 4. Spreadability of hydrogel base](image2.png)

The influence of the sterilization heat to gel base physical properties is shown by the change of viscosity and spreadability of the gel base. The changes of gel base viscosity sterilized by wet heat are less than that sterilized by dry heat (figure 3). Similar result shown in the spreadability properties. The changes of gel base spreadability sterilized by wet heat are less than that sterilized by dry heat (figure 4). In other word, wet heat sterilization has smaller influence to the viscosity and spreadability of gel base than dry heat sterilization. The same result emerges at high, intermediate and low level of the mixture.
Table 2. Viscosity and spreadability of gel base

<table>
<thead>
<tr>
<th>Level</th>
<th>Heat</th>
<th>Viscosity</th>
<th>Change of viscosity</th>
<th>Spreadability</th>
<th>Change of spreadability</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>Wet heat</td>
<td>260.00±12.25</td>
<td>4.17</td>
<td>0.2046±0.00145</td>
<td>0.0123</td>
</tr>
<tr>
<td></td>
<td>Dry heat</td>
<td>232.50±8.22</td>
<td>23.33</td>
<td>0.3919±0.0600</td>
<td>0.1846</td>
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<tr>
<td></td>
<td>Without heat</td>
<td>255.83±10.21</td>
<td></td>
<td>0.2073±0.0104</td>
<td></td>
</tr>
<tr>
<td>Intermediate</td>
<td>Wet heat</td>
<td>125.83±3.76</td>
<td>30.83</td>
<td>0.9018±0.0512</td>
<td>0.4114</td>
</tr>
<tr>
<td></td>
<td>Dry heat</td>
<td>97.50±2.24</td>
<td>59.17</td>
<td>1.8435±0.2391</td>
<td>1.0102</td>
</tr>
<tr>
<td></td>
<td>Without heat</td>
<td>156.67±2.58</td>
<td></td>
<td>0.8333±0.0433</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>Wet heat</td>
<td>67.92±2.46</td>
<td>4.17</td>
<td>5.0672±0.2686</td>
<td>0.4492</td>
</tr>
<tr>
<td></td>
<td>Dry heat</td>
<td>40.83±3.04</td>
<td>31.25</td>
<td>8.5817±0.6212</td>
<td>3.6974</td>
</tr>
<tr>
<td></td>
<td>Without heat</td>
<td>72.08±2.46</td>
<td></td>
<td>4.8843±0.7019</td>
<td></td>
</tr>
</tbody>
</table>

Hoefler (2011) said that raising or lowering the CMC Na solution temperature has no permanent effect to the viscosity properties. Depolymerization of CMC Na occur when the solution is flattened extremely high temperature for long time heating. Depolymerization causes decreasing viscosity of the gel base. The temperature of the wet heat sterilization is not high enough to degrade the cellulose.

To form a gel, Alginates must contain a sufficient level of guluronate monomer in a block to react with Calcium. The gel properties of Alginate are derived from the interaction between Calcium ion and guluronate blocks. Serp (2002) said that temperature and duration of thermal treatment of Alginate gels influence the polysaccharide network.

On the dry heat sterilization, temperature (160 °C) and duration of thermal treatment (60 minutes) have higher value than wet heat sterilization process (115 °C, 30 minutes). Therefore, the possibility of depolimerization of CMC Na is bigger on dry heat than on wet heat sterilization. It is proven by the fact that the decreasing of viscosity and the increasing of spreadability of gel base sterilized by dry heat is bigger than those sterilized by wet heat. On the other hand, the raise of temperature and duration of thermal treatment will decrease the viscosity of the Ca Alginate solution due to rearrangement and leakage of homopolymer blocks of Ca Alginate.

Dry heat sterilization has bigger influence to viscosity and spreadability of gel base than wet heat sterilization. It is because of temperature and duration of thermal treatment on dry heat sterilization have higher value than wet heat sterilization, so that the possibility of depolimerization of CMC Na and Ca Alginate is bigger.

CONCLUSION

Dry heat sterilization has bigger influence to viscosity and spreadability of gel base than wet heat sterilization.

ACKNOWLEDGEMENT

Enade P. Istyastono for some references.

REFERENCES


SAFETY EVALUATION OF URIC ACID-LOWERING NATURAL PRODUCT IN RAT: SUBCHRONIC 16-WEEK TOXICITY WITH BLOOD BIOCHEMISTRY AS PARAMETER

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ABSTRACT

Arthritis gout is a degenerative disease characterized by high blood uric acid level due to inhibition of xanthin oxidase enzyme. Selederi (Apium graveolens L.) and salam (Syzygium polyanthum (Wight) Walp) are kind of plants containing some principles that inhibit this enzyme. Combination of both these plant-derived extracts, supplemented with jinten crude oil (Nigella sativa L.), known as JAMU ASAM URAT (JAU), had been claimed to inhibit xanthine oxidase enzyme in rats. This sub-chronic 16-week toxicity study was conducted in order to evaluate the adverse effects and raise the quality of JAU as well based on “Pedoman Uji Praklinik Obat Tradisional” protocol issued by Badan POM Ministry of Health R.I. The arranged doses of JAU, 40, 200, 1000 mg/200 g bw, were administered in Sprague dawley male and female rats for 16 consecutive weeks orally. Analyses of blood biochemistry content were performed before (0), at 8 weeks and 16 weeks after treatment using the available diagnostic kit. The result showed that no-dose related effects of blood biochemistry were observed from all doses at the each point of measurements and no mortality was examined during the period of experiment. In addition, the weight of all rats showed a significant increase 16 weeks after treatment. The recovery groups, groups of rats that were left 16 weeks after treatment, demonstrated no different results after for 2 weeks normal treatment. Finally, it could be concluded that no-observe effect level (NOEL) of JAU especially on blood biochemistry content was considered to be 40, 200 and 1000 mg/200 g bw both in male and female Sprague dawley rats. In order to assess the safety level of JAU, it was still required further toxicity studies comprehensively.

Key words: subchronic toxicity, blood biochemistry, rat, JAMU ASAM URAT.

INTRODUCTION

Arthritis gout is one kind of metabolism diseases characterized by high blood uric acid content. Uric acid, the end product of purine compounds metabolism, is often deposited both in and around of joints, kidney and causes formation stone in urine tracts (Edu S. Tehupeior, 2000). The accumulation of uric acid is triggered by some situation such as consumption of high purine compounds in foodstuff (beans, red meat etc.), taking alcohol, medication (niacin, salicylic compound, and levodopa) and obesity as well (Pittman JR. and Bross MH., 1999). Recently, there is a few number of chemical drugs for high uric acid treatment, one of which that is well-known is allopurinol. This drug acts by xathine oxidase inhibition, thus decreasing the formation of uric acid and lowering blood uric acid (N. Zollner, 1982).

It had been studied that many of medicinal plants containing chemical properties show xanthine oxidase inhibitory activity. This enzyme metabolizes purine compounds to both hipoxanthin and xanthin and then converts them into uric acid (Pittman JR. and Bross MH., 1999). Previous research showed that tannin compounds of salam leaves (Syzygium polyanthum (Wigh.) Walp), family of Myrtaceae, inhibited xanthine oxidase in vitro. Relevant research claimed that salam had anti-inflammation activity in which its activity gave benefit of it in use for arthritis gout treatment (Suprapto Maat, 2001). Besides salam, Seledri (Apium graveolens L.), belonging to apiaceae family, demonstrated
xanthine oxidase inhibitory activity as well. This activity was performed by epigenine-flavanoid compound with the IC50 value about 0.70 ± 0.23 µM (Cos P. et al., 1998).

JAU is one of traditional products consisting of plant-derived extracts both salam extract and seledri extract supplemented with jinten crude oil (*Nigella sativa* L.). Our previous study claimed that JAU was able to decrease high blood uric acid in potassium oxonic-induced hyper-uric acid rats (data was not published).

Even though JAU had provided preclinical efficacy study, it was still required to evaluate the toxicity properties. Both WHO and Badan POM Depkes R.I. proposed a protocol to conduct toxicity test for traditional medicines and they stated that there was a correlation between the period of toxicity study and expected period of clinical use (Anonym, 2000; anonym, 1991).

Sub-chronic toxicity test reveals series of toxic effects that is due to the administration of drug substances or chemicals products in various doses for a particular time. In addition, the test is also useful to look at the target organ, where the samples are going to give toxic reactions. This study aims to conduct sub-chronic 16-week toxicity test of JAU with blood biochemistry as observed parameters. This study was carried out in order to evaluate the adverse effect based on “Pedoman Uji Praklinik Obat Tradisional” protocol issued by Badan POM Ministry of Health R.I.

Finally, by supporting of comprehensively preclinical information (efficacy and toxicity studies), the quality and acceptance of JAU will raise widely and clinicians will be more convince in use JAU as alternative drug for treatment arthritis gout patient.

**METHODOLOGY**

**Materials**

**Plant-derived extracts.**

All plant-derived extract (salam, seledri and jinten) were prepared by maceration method at room temperature with ethanol as solvent. Each of macerates was collected and dried under vacuum at 40-50°C until semisolid and or oily mass was obtained.

**Animals.**

*Sprague dawley* male and female rats were purchased from Badan POM Depkes RI at 4-5 weeks old and 90-120 gram of body weight. All rats were housed in polycarbonate individual cage by sex with 2 rats /cage and free accessed for normal chow and tap water ad libitum. The laboratory environment was kept at temperature of 22°C±2°C with humidity 50-70% and dark-light 12 h cycles. Animals were acclimatized for 7 days prior to toxicity testing and then healthy rats were chosen for the experiment.

**Experiments**

Sub-chronic 16-week toxicity experiment was conducted based on “Pedoman Uji Praklinik Obat Tradisional” protocol issued by Badan POM Ministry of Health RI. Healthy rats were divided into 7 groups randomly by 10 rats of both sex each that were 3 treatment groups (D1, D2 and D3), 3 recovery groups (S1, S2 and S3) and 1 normal group. Each group received JAU that was suspended in CMC 0.5% at the respective dose of JAU perorally for 16 consecutive days by gavage. Three level of JAU doses were 40, 200 and 1000 mg/200 g bw. Normal group was treated with carrier solution, CMC 0.5%.

Blood samples were collected before treatment, at 8 weeks and 16 weeks after administration and a 2-week post-treatment for recovery groups. The 14-16 hour fasting blood of each rats was drained from sinus orbitalis vena and EDTA-plasma was obtained by centrifugation at 10000 rpm for 5 minutes at temperature of 4°C. The observed blood biochemistries were blood glucose, creatinine, urea, alanine amino transferase, and aspartate aminotransferase using available diagnostic kit Diasys®.

Body weights of all rats were recorded before treatment and every weeks during period of study.
Data analyses

Blood biochemistry data were calculated based on protocol of each diagnostic kit and presented as mean. Each tested parameter between groups was analyzed statistically using SPSS 15 program at the significant value < 0.05 (α < 0.05). Body weight of each group was curved against to time of measurement.

RESULTS AND DISCUSSIONS

Based on toxicity protocol, the sub-chronic toxicity is conducted on minimal three level doses. This 16-week sub-chronic toxicity of JAU experiment was carried out in three dose level viz. 40, 200 and 1000 mg/200 g bw. The low dose, 40 mg/200 g bw, p.o. was the effective dose as lowering blood uric acid in potassium oxonic-induced hyperuric acid rats in vivo (data not published). The high one, 1000 mg/200 g bw, was the highest dose that was purposed delivering toxic effect without due to significant mortality of rats, besides that, this dose was still be able to be intubated by gavage orally. And the medium one, 200 mg/200 g bw, was the middle dose between two doses mentioned before. This experiment was aimed to evaluate the sub-chronic effect of JAU at the arranged doses on the blood biochemistry especially. From these experiments were found that, basically, the 16-week exposure of SD rats to all level doses of JAU p.o. did not show a dose-related effect on blood biochemistry content. Even though there were fluctuating data between groups after 16 weeks treatment of JAU, statistic analysis showed no difference value of each group significantly.

The glucose level of male rats, 16 weeks after treatment, varied between groups rather than in female rats. Blood glucose content of high dose group, 1000 mg/200 g bw, was slightly lower than normal group, viz. 111 mg/dl and 122 mg/dl, respectively. Although a little decline, there was no significantly statistic difference. And also, this level turned into normal after the rats were maintained for 2 week under normal condition (Table a, the high dose of recovery group reached 120 mg/dl). The decrease of blood glucose level was likely to due to the composition of JAU. Previous research claimed that salam extract administration resulted lowering high glucose level both in vivo study and on clinical test (Siti Sapardiyah Santos dan Yulfrra Media, 2003; Kusnardar Anggadiredja, 2006). In addition to, jinten oil, the other component of JAU, was stated to have insulinotropic properties in streptozotocin-nicotinamide-induced diabetic hamster p.o. (Fararh KM, 2002) and to enhance glucose-induced insulin release in rat pancreatic cells (Rchid H et al., 2004). So that, the combination of both extract at high dose was very likely to decline blood glucose. However, at the effective dose of JAU, 40 mg/200 g bw, did not exhibit hypoglycemic effect.

The effect of JAU on kidney organ was observed from the blood biochemistry level of creatinine and urea. The creatinine level both in male and female rats 16 weeks after treatment was relatively not various between groups. The same result was also evaluated from blood urea level. Although the value was various between groups, statistic analyses showed no difference significantly.

Aspartatransferase (AST) and alaninetransferase (ALT) are two kinds of enzymes produced and stored in the liver cells. AST is also found in other parts of bodies such as the brain, pancreas, heart, skeletal muscles, kidneys and lungs. If the level of both enzymes rises from normal, it indicates the damage of hepatocyte cells happen (Anonym, http://hepatitis.about.com/od/diagnosis/a/LiverEnzymes.htm). After 16-week treatment of JAU, the level of AST and ALT indicated various by sex and dose. These results did not differ compared to normal group statistically.

Actually, the sub-chronic toxicity studies related to these three medicinal extracts had been under taken by previous researchers. It had been reported that either chemical properties of jinent, such as tymoquinone and its derivatives, or crude extract showed protective activities against nephrotoxicity and hepatotoxicity by either disease or chemical (B. H. Ali and Gerald Blunden, 2003). Related research was reported by Abdel-Razik H. Farrag and his team. They found that jinent crude extract protected in liver damage due to lead induction in rats. Combined treatment between lead-induced rats and jinent showed marked improvement in blood biochemical and liver histopathological (Abdel-Razik H. Farrag et al., 2007).
Tabel a: The average blood biochemistry level of male rats from each group

<table>
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<th>Observed parameters</th>
<th>Points of measurements</th>
<th>Group</th>
<th>Statistic analyses</th>
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<td></td>
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<td>N</td>
<td>D1</td>
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<td></td>
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Tabel b: The average blood biochemistry level of female rats from each group

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<td>Aspartat transferase/AST</td>
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<tr>
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<td>67</td>
<td>-</td>
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</table>
n.d. not different statistically compared to normal group at each point of measurement. 1, 2, 3 = points of blood biochemistry measurement before, at 8 weeks and 16 weeks after treatment, respectively. 4 = after two weeks in normal maintenance after 16-week treatment. N = normal group, D1, D2, D3 and S1, S2 and S3 = treatment and recovery groups received low, medium and high doses of JAU, viz. 40, 200 and 1000 mg/200 g bw, respectively.

Figure 1. Diagram of the body weight change from each group of male (A) and female (B) rats. D1, D2 and D3 were given JAU at dose of 40, 200 and 1000 mg/200 g bw, respectively, so do S1, S2 and S3 groups, respectively. Normal group was given carrier, CMC 0.5% solution. JAU suspended in CMC 0.5% was dosed by gavage orally for 16 weeks. Body weight was recorded each week during the period of experiment. W# = week after JAU treatment. D# = day on the normal maintenance after 16 weeks treatment. n = 10 rats/group each sex.

Sub-chronic toxicity reports of selederi seeds had also been performed by earlier researchers; one of which was by M. C. Powanda and K. D. Rainsford. They confirmed that dosing ethanolic extract of selederi seed in rats at dose of both 150 and 5000 mg/kg bw for 28 consecutive days did not cause appearing of sub-chronic toxic effect significantly compared to control group (M. C. Powanda and K. D. Rainsford, 2010). The records related to salam toxicity studies were still rare. However people especially in Asia countries have utilized this plant in culinary as seasoning since long time ago. And it was no toxic effect reported due to this plant.

The body weights of all rats from each group were recorded every week during the period of experiment and, especially, for recovery groups, recording was every day for 14 days on normal...
maintenance after 16 week treatments (Figure 1). JAU on three doses regiment did not markedly influence on the change of body weight. Rat's body weights tend increase on all of dose groups comparable with those of the normal group. Although the high dose of JAU caused a little fluctuation body weight in the female rats, basically, it still showed an increase tendency. In this sex, the decrease weight observed at the 9 weeks and 16 weeks after treatment was likely due to blood taking, so that the rat felt a little inconvenient condition.

Generally, in the toxicity studies, animals receiving high dose would be characterized by losing of body weight or wasting syndrome. The marked changing of body weight was a valuable indicator in evaluating the toxicity of compounds or extract preparations in which this parameter could be seen easily (Abiodun Humphrey Adebayo et al., 2010, Toshihiro Kojima et al., 1993).

CONCLUSION
From this study could be concluded that no-observe effect level (NOEL) of JAMU ASAM URAT especially on blood biochemistry content was considered to be 40, 200 and 1000 mg/200 g bw both in male and female Sprague Dawley rats. In order to assess the safety level of JAMU ASAM URAT, it was still required further toxicity studies comprehensively.

ACKNOWLEDMENTS
We would like to thank for research funding from join research between The Center for Pharmaceutical and Medical Technology BPPT and PT Jamu Jago Semarang in 2002-2003 so that the research could be taken place. Thanks are also extended to Julham Efendi and Nuralihi who had provided technical assistances.

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Sri Ningsih


FINGERPRINTS STUDY OF GUAZUMAULMIFOLIA LAMK LEAVES FOR STANDARDIZING TRADITIONAL MEDICINE EXTRACT

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ABSTRACT

Guazumaulmifolia Lamk. (Sterculiaceae) or jatibelanda leaves extract is generally contained in traditional body slimming to reduce body weight (Rahardjo et al, 2006). The aim of the research was to obtain fingerprint that can be used for identification and standardization qualitatively of its extracts when its marker is unavailable. In this research, fingerprint study was carried out by Thin Layer Chromatography (TLC) scanner. From the results, ethanol extract showed a better separation when eluted with toluene-chloroform-ethyl acetate (5:3:2, v/v/v) and had specific retention factors (Rf) 0.17, 0.36, 0.74, 0.87, 0.92 (λ 254 nm) and 0.04, 0.06, 0.09, 0.36, 0.61, 0.74, 0.87, and 0.92 (λ 366 nm). Ethyl acetate extract with toluene-diethyl ether-ethyl acetate (6:3:1, v/v/v) had specific Rf 0.06, 0.23, 0.36, 0.71, 0.79, 0.86, 0.91 (λ 254 nm) and 0.07, 0.23, 0.36, 0.71, 0.86, 0.91 (λ 366 nm) while chloroform extract with toluene-diethyl ether-ethyl acetate (6:3:1, v/v/v) had specific Rf 0.05, 0.36, 0.79, 0.86, 0.92 (λ 254 nm) and 0.05, 0.36, 0.65, 0.80, 0.86, 0.92 (λ 366 nm). Moreover clear separation of n-hexane extract was showed in toluene-chloroform-ethyl acetate (5:4:1, v/v/v) with specific Rf 0.19, 0.46, 0.57, 0.69, 0.75, 0.78, 0.97 (λ 254 nm) and 0.54, 0.58, 0.69, 0.75, 0.89, 0.96 (λ 366 nm). According to the TLC chromatogram, ethanol extract had the best chromatogram profile therefore it can be used as fingerprint for standardization of Guazumaulmifolia Lamk. leaves extract.

Key words: Guazumaulmifolia Lamk leaves, fingerprint, TLC scanner

INTRODUCTION

Fingerprint/chromatogram profile can be used in identification and standardization processes of traditional medicine extract. The aim of this research was to obtain fingerprints that can be used in identification and standardization of Guazumaulmifolia Lamk leaves extracts. Chromatogram profile of Guazumaulmifolia Lamk leaves was developed using TLC technique.

Guazumaulmifolia is widely used as traditional medicine in Indonesia especially for body slimming. It is also used traditionally in some countries such as Brazil, Guatemala, Haiti, Mexico, and Belize to threaten bronchitis, burn wounds, diarrhea, asthma, inflammation, and alopecia (Lopes et al, 2009). All plant parts can be used as medicine. Traditionally the leaves efficacious as a medicine slimming body and lower body fat levels. Seeds are used for upset stomach and abdominal bloating while the fruits can be used as a cough medicine (Kloppenburg, 1983). Dekok of the bark can be used to threaten malaria, diarrhea, and syphilis (Van Vankelburg and Bunyapraphatsara, 2002).

Some results of recent studies mentioned that the bark extracts have antibacterial activity (Camporesie et al, 2003 and Caceres et al, 1990). The ethanol extract of leaves could decrease rat serum lipase activity thus reducing fat and cholesterol absorption in the intestine (Rahardjo et al, 2006). Ethanol extract of leaves and flowers (aerial part) were capable of protecting gastric mucosa against injury from NSAIDs, particularly through the mechanism of anti-inflammatory and free radical capturing (Berenguer et al, 2007). Ethyl acetate extract of Guazumaulmifolia could inhibit the replication of polio virus type I (P-1) and bovine herpesvirus type I (BH-1) (Felipe et al, 2006).

Chemical constituents of Guazumaulmifolia such as sugar, starch, proteins, phenols, lipids (Shekhawat et al, 2010), tannins, alkaloids, and saponins (Rahardjo et al, 2006), flavan-3-ol,
procyanidin, menisdaurin, epicatechin, ent-gallocatechin, epigallocatechin, epiafzelechin-epicatechin, epicatechin-catechin, epicatechin, epigallocatechin, α-methy-epiafzelechin (Lopes et al., 2009), cyanogenic glycosides (taxiphyllin) (Seigler et al., 2005), proanthocyanidin, flavonoids (Hor et al., 1996), epigallocatechingallate, procyanidin C1 (Terra et al., 2011), caryophyllene, farnesol, friedelin, kaurenoic acid, precocene I, procyanidin B2 (epicatechin-epicatechin), procyanidin B5, caffeine, friedelin-3α-acetate, friedelin-2β-ol, and β-sitosterol (Anonymous, 1996).

METHODOLOGY

Materials

Guazumaulmifolia leaves was collected from Indonesian Medicinal and Aromatic Crops Research Institute and was determined in Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Science, Bogor, Indonesia.

Chemicals

Silica gel 60 F254 TLC glass plates (20x20 cm), ethanol p.a, ethyl acetate p.a, chloroform p.a, n-hexane p.a, toluene p.a, and diethyl ether p.a. were purchased from Merck.

Extraction

Dried powder material (30 g) of Guazumaulmifolia leaves was macerated in 300 mL various solvents as follows: ethanol, ethyl acetate, chloroform, and n-hexane for 24 hours (three times repeated). Each macerate was evaporated using Rotary evaporator Buchi R-200 (Buchi Co.). Extracts were weighed and the yields were determined.

TLC and TLC Scanner Study

TLC analysis was performed on silica gel 60 F254 TLC glass plate (20x20 cm). Concentration of samples (20 mg/mL) were reapplied as 8 mm bands using CamagLinomat 5 automated TLC applicator. After completion of sample application, the plate was eluted in Camag glass tank pre-saturated with mobile phase for 2-3 hours. The TLC run was performed under laboratory conditions. After elution, the plate was taken off and dried in room temperature. The dry plate was documented using Camag TLC Documentary System model 3 to get the picture of the plate. The spots were analyzed using Camag TLC Scanner model 3 equipped with CamagWincats software. The retention factor (Rf) was automatically calculated by the Wincats software on wavelength (λ_max) 254 nm and 366 nm. Rf is defined as the distance of the compound eluted divided by the distance of the eluent.

RESULTS AND DISCUSSIONS

Extraction

Extract yields can be seen in Table 1. Yield decreases with decreasing polarity means that the number of non-polar compounds in the Guazumaulmifolia leaves was less than semi-polar or polar compounds. That was supported by data from Shekhawat et al., that the leaves contain soluble sugar 129 mg/gdw (gram dry weight), protein 67 mg/gdw, starch 29 mg/gdw, phenol 20 mg/gdw, and lipid 10 mg/gdw. Otherwise, according to Arriaga et al., the major components in the leaves are precocene I (56.0%), β-Caryophyllene (13.7%), and (2Z,6E)-farnesol (6.6%). They are triterpenoids. Other components are caffeine (0.14%) and kaurenoic acid (Anonymous, 1996). It can be estimated that ethanol extract contain polar compounds such as phenolic compounds (tannins), glycosides, and chlorophyll. The semi-polar extracts (ethyl acetate and chloroform extract) contain semi-polar compounds such as flavonoids, alkaloids, and tannins. Tannins are polyphenol compounds that have broad solubility due to differences in the number and position hydroxyl group. The n-hexane extract can dissolve non-polar compounds such as terpenoids and steroids/lipids.
Table I. Extracts Weight and Yields

<table>
<thead>
<tr>
<th>Extract</th>
<th>Weight of dry extract (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol</td>
<td>7.125</td>
<td>23.522</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>4.787</td>
<td>15.890</td>
</tr>
<tr>
<td>Chloroform</td>
<td>1.3582</td>
<td>4.524</td>
</tr>
<tr>
<td>N-hexane</td>
<td>0.3777</td>
<td>1.254</td>
</tr>
</tbody>
</table>

Figure 1. TLC plates of all extracts on visible light, λ254 nm, and 366 nm (left to right) with three different volume of sample i.e 5, 10, and 15 µL on each plate (sample concentration 20 mg/mL).

Figure 2. Chromatogram profile of ethanol extract on λ 254 nm (a) and 366 nm (b) using mobile phase toluene-chloroform-ethyl acetate (5:3:2, v/v/v) with three different volume of sample i.e 5, 10, and 15 µL (front to back) on each plate (sample concentration 20 mg/mL).

Chromatogram Profile

The TLC elution carried out by trying various eluent/mobile phase for all extracts and the best eluent was chosen. Based on the trial, mobile phase for TLC of ethanol extract was toluene-chloroform-ethyl acetate (5:3:2, v/v/v), then toluene-diethyl ether-ethyl acetate (6:3:1, v/v/v) for ethyl acetate and chloroform extract. While toluene-chloroform-ethyl acetate was used as mobile phase for n-hexane extract. All extract spots using their mobile phase can be seen in Figure 1. The chromatograms which performed by TLC scanner can be seen in Figure 2 until 5.
Figure 3. Chromatogram profile of ethyl acetate extract on λ 254 nm (a) and 366 nm (b) using mobile phase toluene-diethyl ether-ethyl acetate (6:3:1, v/v/v) with three different volume of sample i.e 5, 10, and 15 µL (front to back) on each plate (sample concentration 20 mg/mL).

Figure 4. Chromatogram profile of chloroform extract on λ 254 nm (a) and 366 nm (b) using mobile phase toluene-diethyl ether-ethyl acetate (6:3:1, v/v/v) with three different volume of sample i.e 5, 10, and 15 µL (front to back) on each plate (sample concentration 20 mg/mL).

Figure 5. Chromatogram profile of n-hexane extract on λ 254 nm (a) and 366 nm (b) using mobile phase toluene-chloroform-ethyl acetate (5:4:1, v/v/v) with three different volume of sample i.e 5, 10, and 15 µL (front to back) on each plate (sample concentration 20 mg/mL).
Generally all extracts show almost similar spot pattern except n-hexane extract that has more different, because ethanol is universal solvent which dissolve polar until non polar compound. The spots analysis on wavelength 254 nm and 366 nm using TLC scanner showed specific retention factor. Ethanol extract showed pretty good separation when eluted using mobile phase toluene-chloroform-ethyl acetate (5:3:2, v/v/v) and had specific retention factors (Rf) 0.17, 0.36, 0.74, 0.87, 0.92 on λ 254 nm and 0.04, 0.06, 0.09, 0.36, 0.61, 0.74, 0.87, and 0.92 on λ 366 nm (Figure 2).

Ethyl acetate extract when eluted using mobile phase toluene-diethyl ether-ethyl acetate (6:3:1, v/v/v) had specific Rf 0.06, 0.23, 0.36, 0.71, 0.79, 0.86, 0.91 on λ 254 nm and 0.07, 0.23, 0.36, 0.71, 0.86, 0.91 on λ 366 nm. Furthermore, chloroform extract when eluted using same mobile phase produced specific Rf 0.05, 0.36, 0.79, 0.86, 0.92 on λ 254 nm and 0.05, 0.36, 0.65, 0.80, 0.86, 0.92 on λ 366 nm. While n-hexane extract should be developed with mobilephasecomposition that more nonpolar than the previous mobile phase to separate the compounds dissolved in the extract. Therefore the fraction of polar solvent in the mobile phase was reduced. The mobile phase composition selected to toluene-chloroform-ethyl acetate (5:4:1, v/v/v) had specific Rf 0.19, 0.46, 0.57, 0.69, 0.75, 0.78, 0.97 on λ 254 nm and 0.54, 0.58, 0.69, 0.75, 0.89, 0.96 on λ 366 nm. Some spot that appeared on λ 254 nm and λ 366 nm had same Rf, so that can be concluded that they were same compound.

CONCLUSION

According to the TLC chromatogram, ethanol extract had the best chromatogram profile therefore it can be used as fingerprint for standardization of *Guazumaulmifolia* Lamk. leaves extract.

ACKNOWLEDGEMENT

The authors express thanks to Research Center for Drug and Food, NADFC Republic of Indonesia for the financial support and laboratory facilities.

REFERENCES


PROTECTIVE EFFECT OF EGCG ON OXIDATIVE DAMAGE IN ENDOTHELIAL PROGENITOR CELLS

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ABSTRACT

A number of studies have examined that tea consumption decreases cardiovascular risk, but the mechanisms remain undefined. Endothelial dysfunction has been correlated with coronary artery disease and circulating endothelial progenitor cells (EPCs) is contributed of this repair process. Endothelial dysfunction is associated with increased oxidative stress and may be reverse by antioxidant. Epigallocatechin-3-gallate (EGCG), the major phenolic in green tea, is known as free radical scavenger which has a powerful antioxidant action. The aim of this study is to investigate whether EGCG can protect EPCs from oxidative stress through antioxidant protective mechanism. Total mononuclear cells (MNCs) were isolated from peripheral blood by Ficoll density gradient centrifugation. The cells were then plated on fibronectin-coated culture dishes. EPCs were characterized as adherent cells double positive for DiLDL-uptake and lectin binding under fluorescent microscope. Further characterizations were done by demonstrating the expression of CD34/45, CD133, and VEGFR-2 with flow cytometry. After being cultured for 7 d, EPCs were then induced for oxidative stress using various concentrations of H₂O₂ and incubated with or without EGCG. Cell viability was measured by quantitative colorimetric assay with MTS. The level of intracellular reactive oxygen species (ROS) was quantified by fluorescence with 2',7'- dichlorofluorescein diacetate (DCF-DA) using flow cytometry. The result showed that EGCG may ameliorate cell viability by decreasing accumulation of intracellular ROS H₂O₂-induced EPCs.

Key words: endothelial progenitor cell, oxidative stress, reactive oxygen species, EGCG, antioxidant, endothelium, coronary diseases

INTRODUCTION

Tea, a product made from Camellia sinensis, is the second most widely consumed beverage in the world after water and well ahead of coffee, beer, wine, and carbonated soft drinks (Costa, et al., 2002; Macfarlane & Macfarlane, 2004; Rietveld & Wiseman, 2003). A number of studies have examined the relation between tea consumption and cardiovascular risk (Hertog, et al., 1993; Geleijnse, et al., 1999; Sesso, et al., 1999). The reduction of cardiovascular risk by tea consumption is suggested due to its flavonoid compound (Knekt et al., 1996; Hertog et al., 1995). This suggestion is convincing by other studies that dietary intake of flavonoid from tea and other sources (onions, apples, red wine) is associated with reduced in cardiovascular risk (St Leger et al., 1979; Knekt et al., 1996; Yochum et al., 1999).

Tea flavonoid contain of catechins (30 to 36% of dry weight) including Epigallocatechin-3-gallate (EGCG), which constitutes up to 63% of total catechins (Mannning & Roberts, 2003). The antioxidant activity of EGCG has been shown to be 25 to 100 times more potent than vitamins C and E (Doss, et al., 2005). One benefit of the dietary flavonoids is their antioxidant properties. Flavonoids are well known for their free radical scavenger, such as reactive oxygen species (ROS) (Robak & Gryglewski, 1988). Other studies suggest that flavonoid may prevent LDL oxidation, a key early occurrence in atherosclerosis development (Diaz et al., 1997). Recent studies also suggest that flavonoid may favourably affect endothelial function (Fitzpatrick, et al., 1995; Andriambeloson, et al., 1997).
ROS are form of unpaired electron, such as superoxide anion (O$_2^-$) and hydroxyl radical (HO'), and also non-free radical species such as hydrogen peroxide (H$_2$O$_2$) (Halliwell, 1995). In the cardiovascular system, ROS are recognized as important signaling molecules. Long-term exposure to ROS can damage diverse macromolecules, including proteins, carbohydrates, lipids, and DNA. These damaging actions cause in vascular injury and result in endothelial dysfunction (Bauer & Bauer, 1999).

Endothelial dysfunction has been correlated with coronary artery disease and observed in patients with established coronary artery disease or coronary risk factors (Drexler, 1997). Endothelial progenitor cells (EPCs), a kind of stem cell that makes a vessel in peripheral blood, play important roles in maintaining the vessel tone and have role to repair the endothelial cell injury, an early stage of atherosclerosis caused by cardiovascular risk factor (Asahara, et al., 1997; Walter et al., 2002; Sata, 2003; Gill, et al., 2001). Recent a number of studies found that the number of circulating EPCs were reduced when there were more atherosclerosis risk factors causing the process of atherosclerosis (Vasa et al., 2001; Hill et al., 2003).

Endothelial dysfunction in atherosclerosis is close related with increased oxidative stress and might be reversed by antioxidant treatment (Diaz, et al., 1997). We hypothesized that EGCG protect EPC from oxidative stress through antioxidant protective mechanism, which contributes to protective effect on endothelial cells. To test this hypothesis, we assessed the protective effect and ROS-inhibiting effect of EGCG on H$_2$O$_2$-induced oxidative damage in human EPCs.

**METHODOLOGY**

**Isolation and Cultivation of EPCs**

EPCs were cultured according to the previously described method (Chen et al., 2004). Total mononuclear cells (MNCs) were isolated from peripheral blood of healthy young human volunteers by Ficoll-paque plus (GE Healthcare) using density gradient centrifugation method. MNCs were then plated on culture dishes coated with human fibronectin (Roche) and cultured using VascGrow™ (Stem Cell and Cancer Institute). After 4 days in culture, new media were applied and the culture was maintained through day 7. Informed consent was provided from all volunteers. All of the procedures were done in accordance with ethical clearance board.

**EPC Characterization**

EPCs were characterized as adherent cells after 7 days in culture. Direct fluorescent staining was used to detect dual binding of Ulex europaeus agglutinin I conjugated with fluorescein isothiocyanate (FITC-UEA-I; Sigma) and 1, 1-dioctadecyl- 3, 3, 3, 3-tetramethylindo carbocyanine-labeled acetylated low density lipoprotein (DiI-acLDL; Invitrogen). To detect the uptake Dil-acLDL, adherent cells were incubated with DiLDL (1mg/ml) at 37°C for four hours. Cells were then fixed with 3% paraformaldehyde for 10 minutes. After washing, cells were incubated with FITC-UEA-I (1 mg/ml) at 37°C for one hour. Cells also were nucleus-stained using DAPI (Invitrogen). After staining, cells were then observed using inverted fluorescent microscope (Axiovert 40 CFL, Zeiss). Cells that were double positive for DiLDL and lectin were defined as EPCs (Kalka et al., 2000; Vasa et al., 2001).

Further identified of EPCs were done using Fluorescence-activated cell sorting (FACS). Adherent cells were detached using 2 mM ethylene diamine tetra acetate (EDTA). 1 x 10$^5$ cells were pre-incubated for 15 minutes at room temperature with FcR Blocking (Miltenyi Biotech). Cells were then incubated at 4°C with FITC-conjugated anti-CD45/phycocerythrin -conjugated anti-CD34 (BD Biosciences) and phycoerythrin-conjugated anti-CD133 (Miltenyi Biotech) for 15 minutes, and PE-conjugated VEGF R2/KDR (R&D System) for another 40 minutes. Isotype-identical antibodies served as negative controls. Quantitative FACS was performed on a FACSCalibur Flow Cytometer (BD Biosciences).

**EPC Cytotoxicity Assay**

EPC cytotoxicity was done to determine the maximal tolerance concentration of EGCG on EPC culture and to determine the optimal oxide damage concentration of H$_2$O$_2$ for the following
experiments. Cytotoxicity of EPC was determined by CellTiter® (Promega) based on quantitative colorimetric assay. After 7 days in culture and identification of EPC, cells were then digested with Trypsin-EDTA and were cultured at a density of $5 \times 10^4$ cells/mL on 96-well tissue culture plates using serum-free medium and cultured 24 h before treatment (Bickford et al., 2006; Chen, et al., 2004; Gu, et al., 2006). Cells were then treated with different concentration of EGCG (ranging from 1.56 – 50 mg/L) and $H_2O_2$ (ranging from 12.5 – 400 µM) for 24 h. EPCs were supplemented with 20 µL of CellTiter® each well and incubated for another 4 hours. OD value was measured at 490 nm using microplate reader (Bio-Rad).

**Assessment Protective Effect of EGCG on Oxidative Damage in EPC**

After 7 days in culture, EPCs were then digested with Trypsin-EDTA and were cultured at a density of $5 \times 10^4$ cells/mL on 96-well plates using serum-free medium and cultured 24 h before treatment. Culture medium was replaced with fresh medium containing various concentration of $H_2O_2$ (50, 100, 200 µM). EGCG (6.25 mg/L) was added 1 h before treatment with $H_2O_2$ for a subsequent 24 h (Jie, et al., 2006). Cell viability was measured by CellTiter® assay (Promega). Control was done by treated cells without $H_2O_2$. The value of different absorbance was expressed as a percentage of control.

**Measurement of Intracellular Reactive Oxygen Species.**

Quantification of intracellular ROS level was done by fluorescence with 2',7'- dichlorofluorescein diacetate (DCF-DA; Invitrogen) using modification methods from Stolzin & Scutt (2006) and Jie et al. (2006). After 7 days in culture, EPCs were then digested with Trypsin-EDTA and 1 x $10^5$ cells were incubated with 10 µM DCF-DA for 30 min at 37 °C. After the incubation, the excess probes were washed out with PBS+KCl, then incubated with EGCG (6.25 mg/L) for 30 min. Cells were then incubated with $H_2O_2$ for final concentration 50, 100, 200 µM for another hour. The intracellular ROS levels were measured using FACSCalibur Flow Cytometer (BD Biosciences). Control was done by treated cells with $H_2O_2$ without EGCG pre-treatment. The measured fluorescence values were expressed as a percentage of control cells.

**RESULTS AND DISCUSSIONS**

**EPC Characterization**

When cultured in in vitro system, EPCs will attach to the fibronectin-coated dish and proliferate rapidly forming spindle-shaped cells within 4–7 days of culture (Hristov & Weber, 2004). Beside cell morphology, functional assay also be used to demonstrate that putative progenitors have endothelial cell potential including uptake of Dil-labeled acetylated-low density lipoprotein (Ac-LDL) (Voyta, et al., 1984) and binding of fluorescently labeled *Ulex europaeus agglutinin 1* (*UEA*-1) plant lectin (Suzuki, et al., 1990). Cell surface markers assay has been used for convincing the EPC identification.

In the present study, MNCs isolated and cultured for 7 days resulted in an attached cell with spindle-shaped morphology (Fig 1). EPCs were characterized as adherent cells double positive for DiLDL uptake and lectin binding (Fig 2). Further characterization were demonstrating the expression of CD34/45 (0.13 ± 0.041%), CD133 (0.14± 0.035%), and KDR (0.23±0.031%) (Fig 3).

**EPC Cytotoxicity Assay**

The cell viability was measured using colorimetric method for determining the cytotoxicity assay. CellTiter® solution (Promega) has been used in this study. The solution contains a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl) -5- (3-carboxy methoxyphenyl)- 2 - (4-sulfophenyl)- 2H-tetrazolium (MTS). The MTS tetrazolium compound is bioreduced by cells into a colored formazan product due to conversion by dehydrogenase enzymes in metabolically active cells (Berridge & Tan, 1993).
The protective effect of EGCG on oxidative damage was studied using EPCs. EPCs were cultured on VascGrow™ medium and exhibited a spindle-shaped morphology on day 4 and more defined patterns on day 7.

Figure 1. EPC morphology characterization. MNCs (A, 400X) were cultured on VascGrow™ medium and started to exhibit a spindle-shaped pattern on day 4 (B, 400X) and more defined patterns on day 7 (C, 400X; D, 800X).

Figure 2. EPC functional characterization. Adherent cells were stained with DAPI (A), binding lectin (B) and taking up Dil-acLDL (C). Panel (D) was obtained by merging (A), (B), and (C). A-D were assessed under inverted fluorescent microscope, magnification 800X.

The result of cytotoxicity effect from EGCG and H₂O₂ is shown in Figure 4 (A and B). After treatment for 24h, the cells that were treated with EGCG at concentration of 1.56-6.25 mg/L had relatively no effect on cell cytotoxicity. However, after treatment at concentrations 12.5, 25, and 50 mg/L, the viability of cells started to decrease about 15, 60, and 88%, respectively, relative to the negative control. Interestingly, when EPC were treated at concentration 6.25 mg/L of EGCG, the increased of cell viability was about 9.6% higher than that of negative control. From this data, EGCG may have activity to promote the cell division and the detailed mechanism of this promoting cell growth needs further study.
Figure 3. EPC marker characterization. Cells were demonstrating the expression of CD34/45 (A, isotype; B, marker CD34/45), CD133 (C), and KDR (D).

Figure 4. Effect of EGCG (A) and \( \text{H}_2\text{O}_2 \) (B) on cytotoxicity of EPC. EPC were cultured on 96-well tissue culture plates (5 X 10^4 cells/well) and treated with EGCG and \( \text{H}_2\text{O}_2 \) at a wide range of doses for 24 hours. After treatment, cells were prepared for CellTiter® analysis of cell cytotoxicity as described in Methods. Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 when compared with that of the negative control (untreated cells). NS denotes no statistical difference.

The cells that were treated with \( \text{H}_2\text{O}_2 \) gave no oxide damage effect to the cells at concentrations of 12.5-25 \( \mu \text{M} \) and start to decreased the viability of EPC for about 21, 55, 75, and 84% relative to the negative control for the concentration of 50, 100, 200, and 400 \( \mu \text{M} \), respectively. Concentration 12.5 mg/L of EGCG and 50, 100, and 200 \( \mu \text{M} \) of \( \text{H}_2\text{O}_2 \) have been choose for the following experiments for independent and dependent concentrations, respectively.

**Protective Effect of EGCG on Oxidative Damage in EPC**

ROS have important role on oxidative stress which can damage diverse macromolecules and result in decreasing the cell viability. Thus the oxidative stress occurrence might be pressured by antioxidant treatment (Diaz, et al., 1997). EGCG, the most abundant component in green tea, has a potent antioxidant property and shown to be 25 to 100 times more potent than vitamins C and E...
EGCG actions are very diverse and include direct free radical scavenging, antioxidant, anticancer, antibacterial, and antiviral activities (Cabrera, et al., 2006). Several studies also have demonstrated that EGCG can protect heart, kidney, and brain from oxidative injury (Fu & Ko, 2006; Hirai, et al., 2007; Itoh, et al., 2005). EGCG provide protective effect from oxidative stress through a variety mechanisms (Chung, et al., 2003; Guo, et al., 1999). Even though protective effect of EGCG has been reported in various subject, there are few studies about the protective effect of EGCG against ROS-induced stem cells, especially human progenitor cells.

Figure 5. Effect of EGCG on \( \text{H}_2\text{O}_2 \)-induced EPC. Viability of cell was estimated by CellTiter\textsuperscript{®} assay after treatment with 50, 100, 200 µM \( \text{H}_2\text{O}_2 \) with/without 6.25 mg/L EGCG for 24 hours. Data were expressed as mean (percentage over negative control) ± Standard Deviation (\( n = 3 \)). \( *p < 0.05 \) when compared with that of the negative control (treated only with \( \text{H}_2\text{O}_2 \)). NS denotes no statistical difference.

Figure 6. Intracellular ROS levels in EPC. The cells were incubated with 10 µM DCF-DA for 30 min and exposure to several doses of \( \text{H}_2\text{O}_2 \) with/without EGCG treatment. The basal level of ROS with no exposure to \( \text{H}_2\text{O}_2 \) (B) was obtained by gating the ROS level with control unstained-DCFDA cells (A). The cells treated with of 50 µM (C), 100 µM (D), 200 µM (E) \( \text{H}_2\text{O}_2 \) exhibited increasing in ROS level. Pararel samples were treated with 6.25 mg/L EGCG and the ROS level in cells were decreased to the control (\( \text{H}_2\text{O}_2 \)-induced cells) for the concentrations \( \text{H}_2\text{O}_2 \) of 50 µM (F), 100 µM (G), 200 µM (H).
Figure 7. Fluorescence intensity of ROS from H$_2$O$_2$-induced EPC. EGCG showed its antioxidant capacity to reduce the ROS level in the cells. The cells were incubated with 10 µM DCF-DA for 30 min and exposure to several doses of H$_2$O$_2$ with/without EGCG treatment. Data were expressed as mean (percentage over negative control) ± Standard Deviation (n = 3).

The protective effect of EGCG on H$_2$O$_2$-induced EPC is shown in Figure 5. Treatment with 50, 100, 200 µM H$_2$O$_2$ for 24 hours decreased the viability cells about 34, 56, and 66 %, respectively, relative to the negative control. After treatment with 6.25 mg/L of EGCG, the viability of cells was ameliorated in all the concentration of H$_2$O$_2$ (50, 100, and 200 µM). At the concentration of 50 and 100 µM H$_2$O$_2$, the viability of cells were about have the same percentage (21 and 20 %, respectively), higher than that of the negative control group (p < 0.05). Even though at the concentration of 200 µM H$_2$O$_2$ had no statically difference, the viability of cells was about 9% higher than that of the negative control group. These data showed that EGCG protect the cells from oxidative damage and were ameliorated the H$_2$O$_2$-induced loss of EPC cell viability.

**Measurement of Intracellular Reactive Oxygen Species**

In this study, intracellular ROS level measurement was done by fluorescence with 2',7'-dichlorofluorescein diacetate (DCF-DA; Invitrogen). More than decades, DCF-DA has been employed for several studies dealing with the effect of ROS in cell culture (Saez, et al., 1987; Scott, et al., 1988; Murphy, et al., 1989). DCF-DA is crosses membranes of viable cells and is enzymatically hydrolyzed by intracellular esterases to 2',7'-dichlorofluorescein (DCFH) without fluorescence. DCFH is rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS within the cells. DCF remains trapped within the cell and can be measured to represent the intracellular ROS level (Lebel et al., 1992; Jie, et al., 2006).

Representative dot blots of intracellular reactive oxygen species (ROS) levels in EPC is shown in Figure 6. The level of fluorescence intensity is an indicator of ROS production. The basal level of ROS with no exposure to H$_2$O$_2$ was found about 7-16 % (Fig. 6B) compare to that control cells (unstained-DCFDA cells; Fig. 6A). After treatment with different doses of H$_2$O$_2$ (50, 100, 200 µM) for 1 hour in EPC, the level of ROS in the cells increased for about 7-34 % (Fig. 6C,D,E) in comparison with that of negative control (untreated cells; Fig. 6B). When the cells were treated with 6.25 mg/L of EGCG, the ROS levels were decreased dose-dependently for about 80.7 ± 10.84 %, 90.73 ± 7.88 %, and 92.05±0.31% compare to the control on H$_2$O$_2$-induced EPC for the concentrations of 50, 100, 200 µM, respectively (Fig. 6F,G,H). These results indicated that EGCG treatment reduced the accumulation ROS level in H$_2$O$_2$-induced cells. The graphic for fluorescence intensity of ROS level on EPC (Figure 7).

**CONCLUSION**

In conclusion, EGCG may protect EPC from oxidative damage by ameliorating the H$_2$O$_2$-induced loss of EPC cell viability and decreasing its accumulation intracellular ROS.
ACKNOWLEDGEMENT
This work was funded by grant from Ministry of Research and Technology and supported by Stem Cell and Cancer Institute (SCI), Jakarta.

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COMPARISON ACTIVITIES OF LEAVES AND STEM BARK OF ETHYL ACETATE EXTRACT FROM BAWANG HUTAN PLANT (Scorodocarpus borneensis Becc. (OLACACEAE))

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ABSTRACT

Bawang hutan plant (Scorodocarpus borneensis Becc) is one of Olaceae family which contains polysulphide compound like as in Allium species plants. The aims of research is to know comparison activities (antioxidant, antibacteri, and toxicity) of ethyl acetate extract of bawang hutan plant. Extraction is carried out by reflux and maceration with methanol and then portioned to ethyl acetate, n-BuOH and water. All extracts are tested to antioxidant (free radical scavenging method); antimicrobes (diffusion agar method) and toxicity (Brine Shrimp Lethality test). The result showed that ethyl acetate extract of stem bark (reflux and maceration) gave activities as anti oxidant are IC₅₀ 39.66 g/mL and 87.08 g/mL, respectively. While, ethyl acetate extracts of leaves (reflux and maceration) had IC₅₀ 182.90 ug/mL and 1145 ug/mL. The anti microbe showed ethylacetate of stem bark (reflux) inhibited growth of Staphylococcus aureus, and ethyl acetate extract of stem bark and leaves (all racemarian) inhibited growth of E. coli, and the stem bark of ethyl acetate (reflux) has the highest toxicity than others edtracts (LC₅₀ 40.53 ppm).

Key words: : bawang hutan; Scorodocarpus borneensis Becc.; Olaceae; antioxidants; antimicrobial, toxicity (BSLT)

INTRODUCTION

Bawang hutan (Scorodocarpus borneensis Becc) is one of plant that can be used as drug. The study of literature, in native region of Sumatera and Kalimantan, these plants are widely used as a spice in cooking because the smell similar to garlic, so these plants contain compounds that are similar to polysulphide Allium species. Therefore, the plant is thought to have antioxidant activity and has a toxicity effect. Other parts of the plants that have been reported to be used as traditional medicine is the leaf that is as anti diarrheal, and contain flavonoids. While the results of the research before, it is known that ethanol extract of S. borneensis bark containing flavonoids and methylthiomethyl, methyl disulphide as having antimicrobial activity, and leaves of S. borneensis are suspected has the potential to produce compounds that have activities as an antioxidant, antimicrobial, and has the effect of toxicity. In this study conducted comparison of the activities of ethyl acetate extract (bark and leaves, extraction by reflux and maceration), includes testing of antioxidants with DPPH reagent, antimicrobial test with gelatin diffusion method against Gram-positive bacteria (Staphylococcus aureus) and Gram negative (Escherichia coli), and toxicity test using BSLT (Brine Shrimp Lethality Test). The cytotoxic properties shown in toxicity tests on larval shrimp Artemia salina Leach. with the observed death rate (mortality) of shrimp larvae.

METHODOLOGY

Material

Materials research stem bark powder, dry simplicia (chopped) bark and leaves of bawang hutan (Scorodocarpus borneensis Becc.) collected from East Kalimantan
Chemicals and reagents

95% ethanol, methanol, ethyl acetate, n-butanol, n-hexane, DPPH (1,1-diphenyl-2-pikrilhidrazil), suspension of the test microbes (Staphylococcus aureus, Escherichia coli), Nutrient Agar (NA), to powder, peptone broth, salt water solution (NaCl), eggs Artemia salina Leach., dimethyl sulfoxide (DMSO) 1%, 1% cerium sulfate, silica gel 60, celite 545.

Method

Methanol extracts of stem bark and leaves are obtained by reflux and maceration. Simplicia dry stem bark and leaves 1 kg and 500 g respectively, refluxed at a temperature of 60°-70 °C by using methanol solvent repeatedly (3 times), each for 3 hours and concentrated. The extracts partitioned to ethyl acetate, n-butanol, and water. The next phase of ethyl acetate was collected and evaporated to obtain viscous extract of ethyl acetate, and then extracts ethyl acetate tested antioxidant activity with DPPH free radical method of arrest, antimicrobials by agar diffusion method, and toxicity tests with the method BSLT. Then the ethyl acetate extract had the highest activity class of chemical compounds identified by Farnsworth method.

RESULTS AND DISCUSSIONS

Preparation of extracts

The weight of methanol extract of the bark is thick extract reflux is obtained 12.30 g (1.230%), and thick leaves extract 14.98 g (2.996%). The results of the manufacture of methanol extract of the bark maceration 5.60 g (1.867%), and the viscous extract 13.85 g (6.925%). Extraction results are presented in Table 1.

Table 1. The results of extraction of the bark and leaves of S.borneensis with the methanol

<table>
<thead>
<tr>
<th>Extraction process</th>
<th>Methanol extract</th>
<th>Weight, g (%)</th>
<th>color</th>
</tr>
</thead>
<tbody>
<tr>
<td>stem bark</td>
<td>Reflux</td>
<td>12.30 (1.23)</td>
<td>black brownish</td>
</tr>
<tr>
<td>leaves</td>
<td></td>
<td>14.98 (2.99)</td>
<td>green blackish</td>
</tr>
<tr>
<td>stem bark</td>
<td>Maceration</td>
<td>5.60 (1.867)</td>
<td>black brownish</td>
</tr>
<tr>
<td>leaves</td>
<td></td>
<td>13.85 (6.925)</td>
<td>green blackish</td>
</tr>
</tbody>
</table>

Partitioning results show that ethyl acetate extract of the stem bark from S. borneensis maceration and reflux are 3.05 g (0.305%) and 1.47 g (0.490%). While the ethyl acetate extract of leaves of 8.75 g (1.750%) and 7.93 g (3.965%). Extraction results are presented in Table 2

Table 2. The results of ethyl acetate extract partition stem bark and leaves of S.borneensis

<table>
<thead>
<tr>
<th>Extraction process</th>
<th>Ethyl acetate extract</th>
<th>Weight, g (%)</th>
<th>color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stem bark</td>
<td>Reflux</td>
<td>3.05 (0.305)</td>
<td>black</td>
</tr>
<tr>
<td>leaves</td>
<td></td>
<td>8.75 (1.750)</td>
<td>green blackish</td>
</tr>
<tr>
<td>Stem bark</td>
<td>Maceration</td>
<td>1.47 (0.490)</td>
<td>black brownish</td>
</tr>
<tr>
<td>leaves</td>
<td></td>
<td>7.93 (3.965)</td>
<td>green blackish</td>
</tr>
</tbody>
</table>

Ethyl Acetate Extract Activity Test

Antioxidant test

Testing the antioxidant activity of ethyl acetate extract (stem bark and leaves) by reflux and maceration, with concentrations (100, 50, 25 ug / mL) showed that a sample has antioxidant activity when giving IC50 values < 200 ug / mL. The results of the fourth test extracts and positive control of vitamin are presented in Table 3.
The results showed that the ethyl acetate extract of stem bark (reflux or maceration) and ethyl acetate extracts of leaves (reflux), has a reduction potential of free radicals with IC₅₀ < 200 µg/mL. There were significant differences between the percent inhibition of ethyl acetate extract reflux and maceration, the percent inhibition of the ethyl acetate extract reflux was much larger compared with maceration.

**Antimicrobial Test**

Table 4. The test results of antimicrobial activities ethyl acetate extracts & chloramphenicol against the bacterium *Escherichia coli*

<table>
<thead>
<tr>
<th>Microbe Test</th>
<th>Samples</th>
<th>Concentration (µg/mL)</th>
<th>DIG (mm)</th>
<th>Concentration (µg/mL)</th>
<th>DIG (mm)</th>
<th>Concentration (µg/mL)</th>
<th>DIG (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>The ethyl acetate extract of the bark (Reflex)</td>
<td>1500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1000</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1500</td>
<td>9</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>The ethyl acetate extract of the bark (Maceration)</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1500</td>
<td>9</td>
<td>9</td>
<td>9,5</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>The ethyl acetate extract of leaves (Reflex)</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1500</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>The ethyl acetate extract of leaves (Maceration)</td>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1500</td>
<td>7,5</td>
<td>7,8</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>Chloramphenicol (positive control)</td>
<td>1500</td>
<td>23</td>
<td>23</td>
<td>23,3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>20</td>
<td>20</td>
<td>20,5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>18</td>
<td>18,6</td>
<td>19</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Note**

DIG (-): Diameter of Inhibition Growth
(-): No inhibition
The test results of antimicrobial activity of ethyl acetate extract of the bacterium *Escherichia coli* are presented in Table 4. These results indicate that only the ethyl acetate extract of the stem bark and leaves of *S. borneensis* by maceration that have inhibition a concentration of 1500 µg/mL, while the stem bark and leaves extracts by reflux has no inhibition.

The results of antimicrobial activity of ethyl acetate extract against *Staphylococcus aureus* bacteria are presented in Table 5. The results showed that the ethyl acetate extract of the bark from *S. borneensis* (reflux) that have inhibition against bacteria *Staphylococcus aureus* at concentrations 1500 µg/mL, whereas the ethyl acetate extract of the leaves (reflux), stem bark and leaves by maceration has no inhibition against *S. aureus* bacteria.

<table>
<thead>
<tr>
<th>Microbe Test</th>
<th>Samples</th>
<th>Concentration (µg/mL)</th>
<th>IGD (mm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>The ethyl acetate extract of the bark</td>
<td>1500</td>
<td>8</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>(Reflux)</td>
<td>1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>The ethyl acetate extract of the bark</td>
<td>1500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(Maceration)</td>
<td>1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>The ethyl acetate extract of leaves</td>
<td>1500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(Reflex)</td>
<td>1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>The ethyl acetate extract of leaves</td>
<td>1500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>(Maceration)</td>
<td>1000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>chloramphenicol (positive control)</td>
<td>1500</td>
<td>27</td>
<td>27.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1000</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>500</td>
<td>22</td>
<td>22.6</td>
</tr>
</tbody>
</table>

**Toxicity test (BSLT)**

Table 6. LC$_{50}$ value of the results BSLT ethyl acetate extract of the bark and leaves from *S. borneensis* extraction by maceration and reflux.

<table>
<thead>
<tr>
<th>Ethyl acetate extract</th>
<th>Concentration (µg/mL)</th>
<th>mortality (%)</th>
<th>LC$_{50}$ (µg/mL)</th>
<th>information</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem bark</td>
<td>1000</td>
<td>100.00</td>
<td>40.53</td>
<td>most active</td>
</tr>
<tr>
<td>(reflux)</td>
<td>100</td>
<td>93.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>100.00</td>
<td>63.67</td>
<td>active</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>69.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stem bark</td>
<td>1000</td>
<td>100.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Maceration)</td>
<td>100</td>
<td>69.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf (reflux)</td>
<td>1000</td>
<td>100.00</td>
<td>15.37</td>
<td>active</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>18.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leaf (Maceration)</td>
<td>1000</td>
<td>100.00</td>
<td>100.25</td>
<td>active</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>40.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>11.54</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The results of the ethyl acetate extracts showed that the ethyl acetate extract of the stem bark from *S. borneensis* by (reflux) has the highest activity with LC$_{50}$ value 40.53 ug / mL, followed by ethyl acetate extract of the stem bark in maceration with LC$_{50}$ 63.67 ug / mL, ethyl acetate extract leaves in the maceration with LC$_{50}$ 100.25 ug / mL and ethyl acetate extracts of *S. borneensis* leaves by refluxed with LC$_{50}$ 155.37 ug / mL (Table 6).

The observations of ethyl acetate extract has the effect due to its toxicity LC$_{50}$ < 1000 ug / mL.

From the above data, all ethyl acetate extract are toxic, and the highest activity are the ethyl acetate extract of the bark from *S. borneensis* extracted by reflux (LC$_{50}$ 40.53 ppm). The identification of chemical compound group showed that in the powder and ethyl acetate extracts contain flavonoid, steroid, triterpenoid coumarin and sulphide compound.

Table 7. The identification of chemical compound group for stem bark of *S. borneensis*

<table>
<thead>
<tr>
<th>No.</th>
<th>Chemical compounds</th>
<th>Powder symplisia</th>
<th>Extract of ethyl acetate (reflux)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Flavonoid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Saponin</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Tannin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Steroid/triterpenoid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Quinone</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Minyak atsiri</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Coumarin</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Sulphide</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Information:

+: Give positive results

+: Give negative results

CONCLUSION

The result of the research comparison activities of leaves and bark of ethylacetat extract from bawang hutan plant (*Scorodocarpus borneensis* Becc) are:

1. The stem bark's extract (reflux and maceration) has the highest antioxidant activity.
2. The stem bark and leaf's extract (maceration) has inhibition against *E. coli*.
3. The stem bark's extract (reflux) has toxicity against Artemisia larvae.

ACKNOWLEDGEMENT

The Authors thanks to Ms. Rinawati for assistance in this research.

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Kartika R. Isolasi Senyawa Antibakteri Dalam Kulit Batang Kayu Bawang (*Scorodocarpus borneensis* Becc) (tesis). Bogor: Institut Pertanian Bogor; 1999. h.4; 22


DISSOLUTION PROFILE OF ACETAMINOPHEN TABLET AND IBUPROFEN TABLET WITH L–HPC 21, L–HPC 22, AND SODIUM STARCH GLYCOLATE AS DISINTEGRANT IN WET GRANULATION METHOD

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ABSTRACT

The dissolution of tablets is one of a drug absorption determinant. Disintegrant agent has play an important role on determining the dissolution of tablets. In this experiment, we studied the differentiation dissolution profiles of Acetaminophen Tablet and Ibuprofen Tablet with various disintegrant agent such as Low substituted – Hydroxypropyl Cellulose (L–HPC) 21, L–HPC 22 and Sodium Starch Glycolate (SSG) as comparator. All disintegrant agent used at three concentration (6%, 7% and 8%) for every tablets formula. Tablets made by wet granulation method and pressed using single punch 13 mm flat E. Korsch machine. Evaluation of each tablets was conducted for uniformity of weight and size (diameter and thickness), hardness, friability, disintegration time and dissolution. The dissolution profile on Acetaminophen Tablets showed that only tablet with 6 % L–HPC 21 was not agree with FI IV (Q = 80%, 30 minutes), but on Ibuprofen Tablets where was agree with FI IV (Q = 80%, 60 minutes) only tablet with 8% L–HPC 21, 7% and 8% SSG. From that results suggested that L–HPC have more disintegrant character at hydrophilic active ingredients.

Key words: Acetaminophen Tablet, Ibuprofen Tablet, SSG, L-HPC 21 and 22, Dissolution Profile

INTRODUCTION

Compressed tablets continue to enjoy the status of being the most widely used oral dosage form. The composition of all compressed tablets should, in fact, be designed to guarantee that they will readily undergo both disintegration and dissolution in the upper gastrointestinal (GI) tract.

Dissolution tests are used nowadays in a wide variety of applications: to help identify which formulations will produce the best results in the clinic, to release products to the market, to verify batch-to-batch reproducibility, and to help identify whether changes made to formulations or their manufacturing procedure after marketing approval are likely to affect the performance in the clinic. Further, dissolution tests can sometimes be implemented to help determine whether a generic version of the medicine can be approved or not (Dressman J., 2005).

Dissolution tests can be used to predict the in vivo performance of the dosage form when release of the drug is the limiting factor in the absorption process. The drug release profile influenced by complex factors, one of all is excipient selection in tablet formulation (Dressman, 2000).

Standard in vitro dissolution testing models include two processes: the release of drug substance from the solid dosage form and drug dissolution. Drug release is determined by formulation factors such as disintegration/dissolution of formulation excipients or drug diffusion through the formulation. Drug dissolution is affected by the physicochemical substance properties (e.g., solubility, diffusivity), solid-state properties of the substance (e.g., particle surface area, polymorphism), and formulation properties (e.g., wetting, solubilization). In vitro dissolution testing should thus provide predictions of both the drug release and the dissolution processes in vivo. To reach this goal, the choice of dissolution apparatus and test medium should be carefully considered (Gibson, 2009).

Disintegration/dissolution by formulation factors effect from excipient which adding as desintegrant such as Low-substituted hydroxypropyl cellulose (L–HPC) and Sodium starch glycolate (SSG). L–HPC is widely used in oral solid-dosage forms. It is primarily used as a disintegrant, and as a
binder for tablets and granules in wet or dry granulation. Whereas SSG commonly used in oral pharmaceuticals as a disintegrant in capsule and tablet formulations. Usually disintegration occurs by rapid uptake of water followed by rapid and enormous swelling (Rowe, et al, 2009).

In this experiment, we want to studied effect of L–HPC 21, L–HPC 22 and SSG as comparator on little hydrophilic substance such as Acetaminophen Tablet and hydrophobic substance such as Ibuprofen Tablet in the differentiation dissolution profiles. Result from this experiment can be use by formulator pharmacist as recommendation in design-ing solid dosage formulation.

**METHODOLOGY**

**Materials**

Acetaminophen Ex Hebei (from Pharmacy Distributor PT. Bratachem), Ibuprofen (kindly provided by PT. Indofarma Tbk.), Low-Substituted Hydroxy propyl Cellulose (L–HPC) 21 and 22 Ex Shin-Etsu Chemical Co., Ltd. (kindly provided by PT. Lawsim Zecha), Sodium Starch Glycolate as Primojel Ex Gujarat Microwax Ltd., Starch for tablet / Ampro-tab (generously donated from PT. Holi Farma), Potassium dihydrogen Phosphat, Silicon dioxide as Aerosil and Magnesium Stearate (from Pharmacy Distributor CV. Quadrant Lab.), Talcum (generously donated from PT. Kimia Farma), NaOH (from Pharmacy Distributor PT. Bratachem), Aquadest.

**Methods**

**Tablet Formulation**

Table 1. Composition of tablet excipients in Acetaminophen Tablets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>F_A (%)</th>
<th>F_B (%)</th>
<th>F_C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>77</td>
<td>77</td>
<td>77</td>
</tr>
<tr>
<td>Lactose</td>
<td>11.2</td>
<td>10.2</td>
<td>9.2</td>
</tr>
<tr>
<td>Amylum for Paste</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>L-HPC 21</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>L-HPC 22</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Primojel</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mg Stearate</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aerosil</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Talcum</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Composition of tablet excipients in Ibuprofen Tablets

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>P_A (%)</th>
<th>P_B (%)</th>
<th>P_C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>61.5</td>
<td>61.5</td>
<td>61.5</td>
</tr>
<tr>
<td>Lactose</td>
<td>18.75</td>
<td>17.75</td>
<td>16.75</td>
</tr>
<tr>
<td>Amylum for Paste</td>
<td>11.25</td>
<td>11.25</td>
<td>11.25</td>
</tr>
<tr>
<td>L-HPC 21</td>
<td>6</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>L-HPC 22</td>
<td>-</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td>Primojel</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mg Stearate</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Aerosil</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Talcum</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>
Granules Evaluation

Evaluation for granules include loss in drying, bulk density, tap density, compressibility, flow ability and angle of repose. If all of granules fulfill all of requirements that has a good criteria such as good compressibility and smooth fluently. Then all can pressed into tablet.

Tablet Evaluation

After all of granules passed from evaluation, granules will be press by 13 mm flat single punch E. Korsch Tablet Machine. Then quality of tablet will be check for hardnesses, friabilities, weight and thickness uniformities include desintegration time and dissolution.

RESULT AND DISCUSSION
Granules Evaluation

Table 3. Granule Evaluation for Compress mass (granules) Acetaminophen Tablets

<table>
<thead>
<tr>
<th>Formula</th>
<th>L-HPC 21</th>
<th>L-HPC 22</th>
<th>Primojel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F_A1</td>
<td>F_A2</td>
<td>F_B1</td>
</tr>
<tr>
<td>Loss on drying (%)</td>
<td>1.8</td>
<td>0.62</td>
<td>0.4</td>
</tr>
<tr>
<td>Bulk density (g/ml)</td>
<td>0.55±</td>
<td>0.541±</td>
<td>0.561±</td>
</tr>
<tr>
<td>Tap density (g/ml)</td>
<td>0.008</td>
<td>0.015</td>
<td>0.008</td>
</tr>
<tr>
<td>Compressibility (%)</td>
<td>0.606±</td>
<td>0.612±</td>
<td>0.646±</td>
</tr>
<tr>
<td>Flow ability (g/detik)</td>
<td>0.018</td>
<td>0.011</td>
<td>0.021</td>
</tr>
<tr>
<td>Angle of repose (°)</td>
<td>±1.64</td>
<td>±1.61</td>
<td>±1.94</td>
</tr>
</tbody>
</table>

Table 4. Granule Evaluation for Compress mass (granules) Ibuprofen Tablets

<table>
<thead>
<tr>
<th>Formula</th>
<th>L-HPC 21</th>
<th>L-HPC 22</th>
<th>Primojel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P_A1</td>
<td>P_A2</td>
<td>P_A3</td>
</tr>
<tr>
<td>Loss on drying (%)</td>
<td>1.64</td>
<td>1.61</td>
<td>1.94</td>
</tr>
<tr>
<td>Bulk density (g/ml)</td>
<td>0.449±</td>
<td>0.442±</td>
<td>0.444±</td>
</tr>
<tr>
<td>Tap density (g/ml)</td>
<td>0.005</td>
<td>0.003</td>
<td>0.005</td>
</tr>
<tr>
<td>Compressibility (%)</td>
<td>0.557±</td>
<td>0.557±</td>
<td>0.560±</td>
</tr>
<tr>
<td>Flow ability (g/detik)</td>
<td>0.004</td>
<td>0.012</td>
<td>0.012</td>
</tr>
<tr>
<td>Angle of repose (°)</td>
<td>±1.12</td>
<td>±1.76</td>
<td>±0.905</td>
</tr>
</tbody>
</table>

Tablet Evaluation

From granules evaluation we find all of granules meet requirements standard for tablet compress. So, all of granules suitable to pressed into tablet with 13 mm flat single punch E. Korsch Tablet Machine. After that, tablets was evaluated for checking tablet quality, such as below.
Physically all of tablets in good condition if we looked at weight and thickness uniformity or hardness and friability. But differently when we looked at desintegration time and solubility percentage (dissolution). There is something make some formula out of standard. If we looked at tablet evaluation result, we find that only Acetaminophen tablet in formula F_{A1} not meet the requirement for desintegration time (15 minutes) and dissolution (30 minutes, 80%). Differently at Ibuprofen tablet all of tablet were not passed from desintegration time but on the dissolution only formula P_{A3}, P_{C2} and P_{C3} were passed from the test (60 minutes, 80%).

From this result we find that L-HPC 21 has more desintegrant character than L-HPC 22 especially at hydrophobic API.

**CONCLUSION**

From the result showed that tablet with 6 % L–HPC 21 was not agree with FI IV (Q = 80%, 30 minutes), but on Ibuprofen Tablets where was agree with FI IV (Q = 80%, 60 minutes) only tablet with 8% L–HPC 21, 7% and 8% SSG.

Based on above result we conclude that L–HPC have more disintegrant cha-racter at hydrophilic active ingredients. Between those L–HPC, L–HPC 21 has more disintegrant than L–HPC 22.

**ACKNOWLEDGEMENT**

The authors would like to thanks to PT. Lawsim Zecha, PT. Holi Farma, PT. Kimia Farma and PT. Indofarma for their kindly materials provided, and to Irene Natalie and Randy Andrian Herawan for the data collection.

**REFERENCES**


PREPARATION AND CHARACTERIZATION OF PROTEIN-LOADED CHITOSAN NANOPARTICLES WITH ALUMINIUM HYDROXIDE GEL AS NASAL DELIVERY SYSTEM

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2 Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, 10330, Thailand.
3 Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, 10330, Thailand.

ABSTRACT

The aim of this study was to prepare novel nasal vaccine adjuvant as combination of chitosan nanoparticles (CSNPs), an effective mucosal adjuvant, with aluminium hydroxide gel, a currently licensed adjuvant and to investigate the potential to be a carrier system for the nasal delivery of model antigen. Combination adjuvant was prepared by mixing of chitosan nanoparticles, prepared by ionic cross-linking of chitosan solution with sodium tripolyphosphate (TPP), with aluminium hydroxide gel suspension at ambient temperature while stirring. Bovine serum albumin (BSA) used as model antigen. The effect of factors such as CS concentration, weight ratio of CS:TPP, solution pH and aluminium hydroxide gel suspension concentration on the size and zeta-potential were studied. The results showed that low concentration of CS solution gave smaller size of nanoparticles and suitable weight ratio of CS:TPP was 3:1. However, increasing of CS solution pH resulted bigger nanoparticles. The BSA loaded nanoparticles, with CS solution pH of 4.5, without and with 0.1 mg/ml aluminium hydroxide gel had average size of about 110 nm and 135 nm and zeta-potential of about 18 mV and 20 mV respectively. The particle size increased significantly when aluminium hydroxide gel suspension concentration was increased but the zeta potential was slightly increased. Morphology of CSNP was studied by Transmission electron microscopy (TEM). It indicated spherical nanoparticles covered with aluminium hydroxide. Circular dichroism (CD) spectra showed no significant change of encapsulated BSA conformation compared to native BSA. Additional studies such as % loading, protein stability would be discussed.

Key words : chitosan nanoparticles, aluminium hydroxide gel, combination adjuvant, bovine serum albumin, nasal delivery system

INTRODUCTION

Intranalsal administration has many advantages for the local and systemic delivery of many kinds of therapeutic compounds and also protein-based antigens. It is needleless, non-invasive, painless and can be self-administrated. Nasal mucosa has a relatively large absorptive surface and low proteolytic activity (Illum, et al., 2001). Essentially, nasally administratered vaccines can induce both local and systemic immune responses (Amidi, et al., 2006). However, short residence time and poor permeability of protein formulations are the big obstacles to be overcome.

CS is a natural material, nontoxic, biocompatible, biodegradable and cationic polysaccharide (Onishi and Machida, 1999). Moreover, CSNPs has mucoadhesive properties and facilitate opening of the epithelial tight junctions (Yamamoto et al, 2005). It also can be used to enable the controlled release of drugs and enhance membrane permeability.

Aluminium hydroxide gel is one of a few currently licensed adjuvants. Combination of adjuvants showed some evidences of improvement of adjuvant effects. In this study, combination of CSNPs, an effective mucosal adjuvant, with aluminium hydroxide gel has been investigated the potential to be a carrier system for the nasal delivery of model antigen.
METHODOLOGY

Materials
Chitosan (Low molecular weight), Sodium tripolyphosphate (TPP) and Bovine serum albumin (BSA) were purchased from the Sigma-Aldrich Co. (Singapore). Aluminium hydroxide gel (Rehydragel HPA), was purchased from the Reheis Inc. (USA) All other chemicals were of analytical grade.

Preparation of CSNPs
CSNPs were prepared based on the ionotropic gelation of CS with anion of TPP. Briefly, CS was dissolved at a concentration of 0.1 and 0.2% (w/v) in solution with 2% acetic acid overnight and adjusted to pH 4.5, 5.5 and 6.5. The particles were formed spontaneously upon slowly drop-wised addition of different volumes of an aqueous solution of TPP (1 mg/ml) to 20 ml of a CS solution at ambient temperature under magnetic stirring. 0.5 ml of 4% tween 80 and various volume of aluminium hydroxide gel suspension were added after particles were formed. The volume of the TPP solution were calculated to have CS:TPP ratios of 6:1 to 2:1.

BSA loaded nanoparticles were prepared, with an optimized condition, as described above by incorporation of BSA solution in TPP solution before adding in CS acidic solution to have final concentration of 30 and 60 µg/mL.

Characterization of nanoparticles

The morphological examination of nanoparticles was performed by transmission electron microscopy (TEM) (Jeol, JEM-2100, Japan). Particles size and zeta potential were evaluated by a Dynamic Light Scattering (DLS) technique with a Zetasizer ZS (Malvern Instruments, UK). Zeta potential determinations were based on electrophoretic mobility of the nanoparticles in aqueous suspensions. These measurements were performed in triplicate with independent particle batches.

Fourier-transform infrared measurement

Fourier-transform infrared (FTIR) analysed for CSNPs (with or without BSA) were performed using a FTIR spectrometer (Perkin Elmer, Spectrum 400) Freeze dried sample was placed on IR crystal window and subjected to light within the infrared region. The instrument was operated with resolution of 1 cm$^{-1}$ and scans with frequency range of 400-4000 cm$^{-1}$.

Loading efficacy of BSA from chitosan nanoparticles

BSA-loaded CSNPs were prepared as described above. The amount of protein entrapped in the nanoparticles was calculated from the difference between the total amount added to the loading solution and the amount of non-encapsulated protein remaining in the supernatant. BSA concentrations in the supernatants were measured by the QuantiPro BCA Assay (Sigma-Aldrich, USA). Since Tween 80 in the supernatant interfered with the protein assay, for this study the BSA-loaded CSNPs were prepared without Tween 80. Aliquots of the resulting nanoparticle suspension were centrifuged for 30 min at 60,000 rpm and 8°C and the supernatants were then separated from the nanoparticles. The amount of no-encapsulated protein remaining in the supernatant was measured by the QuantiPro BCA Assay (Sigma-Aldrich, USA). A non-loaded CSNP suspension without Tween 80 was used as a blank to correct for interference by CS. Loading efficiency (LE) was calculated as follows.

$$\text{LE} = \frac{\text{Total amount of BSA} - \text{Free BSA}}{\text{Total amount of BSA}} \times 100\%$$

Circular dichroism (CD) measurements

Circular dichroism spectroscopy (CD) (Jasco, J-715 spectropolarimeter, Japan) was used to measure the conformational change of the encapsulated BSA compared with the native one. Solution of the native BSA or the encapsulated BSA was prepared to 30 and 60 µg/ml, supernatant of CSNPs was used as solvent and blank, and scanned over the wavelength range 200-250 nm, using a 1-mm quartz cylindrical cell.
RESULTS AND DISCUSSIONS
Preparation and characterization of CSNPs

CSNPs were prepared based upon the ionotropic gelation of CS contact with the TPP anions. In order to study the appropriate conditions for encapsulation of BSA, it was first evaluated the influence of critical formulation parameters. The results of concentration of CS solution showed that lower concentration promoted formation of smaller particles and higher zeta potential. Particle size and zeta potential of 0.1% and 0.2% CS solution with TPP solution at CS:TPP weight ratio of 2:1, 2.5:1 and 3:1 were 104.5, 103.6, 109.3 nm / 21.0, 31.1, 35.1 mv and 679.1, 115.3, 121.6 nm / 17.5, 27.2 / 33.2 mv respectively. Thus, it was supposed that a relatively lower viscosity of CS with lower concentration promoted the formation of nanoparticles between CS and TPP (Tang et al., 2007).

With the above results, 0.1% CS concentration at pH 4.5 with 3:1 of CS:TPP weight ratio was selected for further evaluation. Then, aluminium hydroxide gel suspension in different volume was added to the formulation to have final concentration of 0.1, 0.2 and 0.4 mg/ml. The results showed that particle size and zeta potential were 135.48±7.9, 191.02±38.62, 444.69±94.09 nm / 20.36±1.39, 22.08±3.65, 24.53±1.91 mV and 135.48 ±12.79, 203.37±31.09, 363.90±39.12 nm / 21.88 ±1.48, 22.37 ±1.93, 26.29±1.62 mV for 30 and 60 µg/mL respectively.

The shape of nanoparticles were examined by TEM. Figure 2. shows the size and physical appearance of CSNPs which is around 110 nm and in spherical structure, while CSNPs with aluminium hydroxide gel has slightly bigger size (about 130 nm) and a compact core surrounded by a fluffy coat of aluminium hydroxide gel.

Figure 1:
Effect of pH of CS solution on size of nanoparticles.
1-5 are 2:1, 3:1, 4:1, 5:1 and 6:1 of CS:TPP weight ratio respectively.

Figure 2. TEM photograph of a. CSNPs and b., c. CSNPs with aluminium hydroxide gel
Fourier-transform infrared measurement

Figure 3 shows FTIR spectra of CS, CSNPs, BSA-loaded CSNPs and BSA. Three characteristics absorption bands observed in CS, at 3435, 1650 and 1321 cm\(^{-1}\), as in the given order are due to the N-H, amide I and amide III groups present in CS. According to Yuan et al. (Yuan et al., 2010), the peak of 3435 cm\(^{-1}\) corresponds to stretching vibration of N-H in CS but it has shifted to lower wavenumbers, 3412 cm\(^{-1}\) and widened in CSNPs indicating enhanced hydrogen bonding (Wu et al., 2005). Primary amines also show sharp peak between 3500 and 3400 cm\(^{-1}\) which could be attributed to the asymmetric and symmetric stretching of the N-H bonds (Yuan et al., 2010). The peak in CS and CSNPs appears broad in this region due to the contribution of O-H stretching peaks and hydrogen bonding (Zhang & Kosaraju, 2007). FTIR Spectra of BSA showed peaks around 1656, 1534 and 3314 cm\(^{-1}\), reflecting the acetylamino I, acetylamino II and (NH\(_2\)) groups, respectively (Zhang & Kosaraju, 2007).

Acetylamino I at 1656 cm\(^{-1}\) and acetylamino II at 1534 cm\(^{-1}\) in BSA overlapped 1650 cm\(^{-1}\) of amide I and 1602 cm\(^{-1}\) in CS, therefore higher intensive peaks appeared for the BSA-loaded CSNPs.

Figure 4 shows FTIR spectra of BSA-loaded CSNPs with aluminium hydroxide in different concentration. For aluminium hydroxide, absorption band at 3632 cm\(^{-1}\) \([\nu(O-H)]\) is assigned to the stretching vibration of Al–OH and the other are at 914 \([\delta (Al–Al–O)]\) (Wu et al., 2005). Additionally, there were three peaks at 626, 522 and 464 cm\(^{-1}\) in CSNPs with aluminium hydroxide. These peaks were more intensity in more amount added aluminium hydroxide and suggest the possibility of a strong interaction between CSNPs and aluminium hydroxide.

Figure 3 shows FTIR spectra of CS, CSNPs, BSA-loaded CSNPs and BSA.

Figure 4 shows FTIR spectra of BSA-loaded CSNPs without and with aluminium hydroxide in concentration of 0.1, 0.2 and 0.4 mg/mL.
Loading efficacy of BSA from chitosan nanoparticles

The CSNPs-BSA with aluminium hydroxide of 0.1, 0.2 and 0.4 mg/mL displayed loading efficiency (LE) of 52.47±2.99%, 55.65±0.56%, and 56.56±0.66% and 49.79±4.98%, 50.34±4.77%, and 50.67±2.22% for 30 and 60 µg/mL samples respectively, while showed 47.92±3.66% and 47.35±4.47% for samples without aluminium hydroxide. The results indicated that some of the BSA was absorbed onto the aluminium hydroxide layer on the surface of the particles.

Circular dichroism (CD) measurements

CD was used to measure the conformational change of the encapsulated BSA compared with the native one. The native BSA has two extreme valleys at 208 and 222 nm. Figure 5a shows CD spectra of native BSA and BSA encapsulated in CSNPs, the shape of spectra was not different in each concentration, but ellipticity (θ) of BSA encapsulated in CSNPs was slightly less than in native BSA. Figure 5b shows CD spectra of BSA encapsulated in CSNPs without and with aluminium hydroxide in concentration of 0.1, 0.2 and 0.4 mg/mL, the shape of spectra and ellipticities were not different. The results indicated that there was no significant conformational change in BSA encapsulated in CSNPs with aluminium hydroxide.

CONCLUSION

In this study, CSNPs were prepared under mild condition by crosslinking with TPP and loaded with BSA as model antigen. By optimized conditions small size and narrow size distribution were obtained. Moreover positive zeta potential of prepared particles indicated their potential to provide bioadhesion on nasal mucosa. Aluminium hydroxide was added after CSNPs were formed and coated as fluffy layer on the surface of the particles. Loading efficiency of CSNPs was around 47% and slightly increased with addition of aluminium hydroxide. CD spectra showed no significant conformational change in BSA encapsulated in CSNPs with aluminium hydroxide compared with native BSA. These results showed that with some enhanced properties, CSNPs with aluminium hydroxide gel has potential to be a potential carrier for nasal delivery system for protein antigens, and further investigations have to be conducted then.

ACKNOWLEDGEMENT

The authors would like to thanks research funding from NRU Project of CHE and Ratchadapiseksompot (HR1164A). Financial support from IIAC, Chulalongkorn University Centenary Academic Development Project is also appreciated.
REFERENCES
REDDUCTION OF BLOOD GLUCOSE LEVELS OF ETHANOLIC EXTRACT OF BUNGUR (LAGERSTROEMIA SPECIOSA [L.] PERS) LEAVES IN ALLOXAN INDUCED DIABETIC RATS

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ABSTRACT

An investigation on hypoglycemic effect of ethanolic extract Bungur leaves (Lagerstroemia speciosa [L.] Pers) in alloxan induced diabetic rats had been studied. This study was aimed at testing the effects of antidiabetic extract of Bungur leaves ethanolic extract in diabetic rats that had been induced with alloxan monohydrate. In this study, Bungur extract was prepared by cold extraction (percolation), using ethanol as solvent. Twenty five of male Wistar rats weighing of 100-150 g were used. Each rat was induced with 15 % Alloxan monohydrate intramuscularly (i.m). After the rats became diabetic (Blood Glucose Level > 200mg/dL), the rats were divided into five groups, each group consisted of 5 rats. Group I was the negative control group which was given CMC Na 0.5% orally, group II, III and IV group received Lagerstroemia speciosa [L.] Pers ethanolic extract of 250 mg/kgBW, 500 mg/kgBW and 1000 mg/ kgBW orally, and group V as comparison, which was given Metformin with the dose of 63 mg/kgBW orally. The effect of ethanolic extract of Lagerstroemia speciosa [L.] Pers on blood glucose levels of all group was determined at 0 and 8 days. One Way Anova calculation showed that $F_{\text{count}} > F_{\text{table}}$ (46.818 > 2.87), which meant there were significant differences between the five treatments. The percentage of reduction in blood glucose level by Bungur extract at a dose of 250 mg/kgBW, 500 mg/kgBW and 1000 mg/kgBW was 78.23%, 80.49% and 83.53% respectively. Based on the results obtained, it was concluded that the ethanolic extract of Bungur leaves (Lagerstroemia speciosa [L.] Pers) has hypoglycemic effect, and the highest hypoglycemic effect was shown by the dose of 1000 mg/kgBW by 83.53%, which is 1.17 times higher than positive control (Metformin) which reduced blood glucose level by 71.35%.

Keywords: hypoglycemic, ethanolic extract, Lagerstroemia speciosa [L.] Pers, rats, diabetic, alloxan

INTRODUCTION

The globalization of today’s rapidly evolving society have influenced the pattern of life. Unhealthy eating patterns, such as fast food less in fiber, drinks with sugar and blended synthetic dyes and preservatives can cause various diseases.

One of the degenerative diseases of concern to governments and communities is a Diabetes Mellitus (DM) or commonly called diabetes. DM is a disease characterized by chronic hyperglycemia, that is blood sugar levels higher than normal. This situation is related to the abnormal metabolism of carbohydrates, fats, and proteins, and presence of hormonal disorders such as insulin, glucagon, cortisol and growth hormone. DM can be the cause of various diseases such as hypertension, stroke, coronary heart disease, kidney failure, cataracts, glaucoma, retinal damage which can lead to blindness, impotence, impaired liver function, longer time taken to heal wounds, especially in the legs that leads to amputation. Diabetes can be caused also by impaired insulin production.

Statistical Report of the International Diabetes Federation (IDF) said that now there are about 230 million people with DM. This figure continues to grow by 3% or about 7 million people each year. Thus, the number of people with DM is predicted to reach 350 million by 2025 and half of that number are in Asia, especially India, China, Pakistan and Indonesia. DM patients in Southeast Asia were found in Singapore as much as 10.4%, Thailand 11.9%, Malaysia more than 8% and Indonesia 5.7%. In 1995, Indonesia was the 7th country with the highest DM patients in the world, and in 2025 is estimated to
rise to be 5th on the world. At this time, it was reported that in big cities like Jakarta and Surabaya, was nearly 10% of the population suffer from DM (Tandra, 2008).

Indonesia’s natural wealth of source material has been used by Indonesian people through generations. The culture to back to nature, or better known as Back to Nature is now a trend worldwide, including Indonesia. The result of research conducted by Andayani in 2000 showed that the crude extract of chickpea can lower blood glucose levels up to 30% in rabbits alloxan-induced diabetes. Bitter leaf extract at a dose of 0.5 g / kg bw, 1 g / kg bw, and 1.5 g / kg bw was able to inhibit the increase in blood glucose levels in normal rats (Suryadhana, 2000).

Several research reports indicated the potential of Bungur leaf extract in lowering blood glucose levels in DM patients (Liu et al., 2001, Hayashi et al., 2002, Saha et al., 2009). Based on the studies of water extract of leaves Bungur (EADB) it was shown that hypolipidemic activity was found at all doses of treatment, 0.1 g/200gBW, 0.2 g/200gBW and 0.5 g/200gBW. The dose of 0.5 g/200gBW Bungur leaf water extract treatment showed a higher hypoglycemic activity compared with glibenclamide treatment (Hernawan et al., 2004).

In this research the effect of decreasing blood glucose levels of the ethanol Bungur leaf extract at a dose 250mg/kgBW, 500mg/kgBW, and 1000mg/kgBW given orally to male Wistar albino rats that have been induced alloxan were determined. Alloxan inhibit insulin secretion from pancreatic β cells and stimulated a type 2 diabetes mellitus model, so that we could predicted the influence of ethanol extract of Bungur leaves to decrease blood glucose levels in hyperglycemic conditions, as well as the use of metformin at the dose of 63 mg / kgBW as a comparison.

**METHODS**

**Plants**

Bungur leaves were collected and determined from Purwodadi Botanical Garden, Pasuruan, East Java. Bungur leaves were dried and then extracted with 70% ethanol by maceration. The extract obtained was concentrated with an evaporator.

**Animals and diet**

25 male Wistar Rats (*Rattus norvegicus albinus*), 2 months old, weight between 100-150 gram were used for the present study. They were housed in room temperature at 26±2 °C with a 12-h light and 12-h dark cycle. The animals were kept in the experimental animal room for 7 days with free access to food and water. Blood samples were withdrawn from the vein tail for glucose determinations.

**Estimation of blood sugar**

Blood sugar estimation was estimated by using standard glucose kit essentially followed by glucose oxidase-peroxidase (GOD-POD) methods.

**Experimental protocol**

25 male albino rats were induced with alloxan monohidrate i.m. at the dose of 150 mg / KgBW to create conditions of type 2 diabetes mellitus (Kresnamurti and Sugiarso, 2003). Blood glucose levels were measured every day after induction. Seven days after the occurrence of hyperglycemia, all rats were randomly divided into five groups where each group consisted of five rats, the first group was given 0.5% CMC Na, the 2nd, 3rd, 4th groups were given the suspension of the ethanolic extract at the dose of 250, 500, and 1000 mg / kgBW and the 5th group was given metformin as comparison group at the dose of 65 mg / KgBW. The Blood glucose level were measured every day until 8 days administration of extracts.

**Data analysis**

All data are expressed as mean ± S.E.M. ANOVA and Tukey test were used for statistical analysis. Values were considered to be significantly different when the p value was less than 0.05.
RESULTS AND DISCUSSION.

Table 1. Average of Rat Blood Glucose Levels All Groups on the 8th day after 7th Days of Treatments

<table>
<thead>
<tr>
<th>No</th>
<th>Treatment Groups</th>
<th>Δ Blood Glucose Level (mg/dl) After 7th days of treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Na CMC 0.5%</td>
<td>184.40 ± 70.12</td>
</tr>
<tr>
<td>2</td>
<td>Bungur Ethanol extract 250 mg/KgBW</td>
<td>-340.60 ± 147.06</td>
</tr>
<tr>
<td>3</td>
<td>Bungur Ethanol extract 500 mg/KgBW</td>
<td>-340.80 ± 192.12</td>
</tr>
<tr>
<td>4</td>
<td>Bungur Ethanol extract 1000 mg/KgBW</td>
<td>-376.40 ± 151.47</td>
</tr>
<tr>
<td>5</td>
<td>Metformin 63 mg / KgBW</td>
<td>-297.40 ± 48.19</td>
</tr>
</tbody>
</table>

Fig 1. Percentage of blood glucose level vs time (days)

Table 2. Percentage of Reduction of Blood Glucose Level All Groups at the 8th day After 7th days of Treatments

<table>
<thead>
<tr>
<th>No</th>
<th>Treatment groups</th>
<th>Percentage of reduction of Blood Glucose Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ethanolic extract 250 mg/KgBW</td>
<td>78.23 %</td>
</tr>
<tr>
<td>2</td>
<td>Ethanolic extract 500 mg/KgBW</td>
<td>80.49 %</td>
</tr>
<tr>
<td>3</td>
<td>Ethanolic extract 1000 mg/KgBW</td>
<td>83.53 %</td>
</tr>
<tr>
<td>4</td>
<td>Metformin 63 mg / KgBW</td>
<td>71.35 %</td>
</tr>
</tbody>
</table>

Based on the results of the study all doses of ethanol extract of leaves Bungur given orally for 7 days gave high decreasing effect of the blood glucose level of diabetic rats which more than 75% higher than metformin which was 71.35%. Increasing doses of ethanol extract of leaves Bungur enhanced the effect of blood glucose levels, but not statistically significant by Tukey test at α > 0.05.

Blood glucose lowering effect of leaves Bungur was thought to originate from the content of the ellagitannin or ellagic acid from Bungur leaves extract that can be dissolved in 70% ethanol. Ellagitannin is hydrolyzed tannin compound which is exactly a heksaoxidiphenate acid esters. Ellagitanin consists of lagerstroemin, flosin B, and reginin A which has properties similar to the hormone insulin (insulin-like compound). In vitro, all three compounds are able to increase the activity
of glucose transport in adipose cells. The Ability of lagerstroemin and flosin B is almost half time the ability of insulin to increase glucose transport rate. In fact, reginin A has a capability similar to insulin (Hayashi et al., 2002). From the description it was known that the hypoglycemic activity of leaves Bungur occur through the increment of glucose transport rate. The involvement of transport speed occurs via the same pathway with the hormone insulin pathway. It is based on in vitro studies that show the work of ellagitannin in the Bungur leaves which can be inhibited by wortmannin compound, i.e a compound that is able to close the path furanosteroid the hormone insulin through PI3-kinase activation barriers (phosphatidylinositol 3-kinase) in insulin signaling pathway.

It is estimated that the mechanism of action of Bungur leaves active compounds i.e. ellagitannin are as follows the active compound of Bungur leaves binds to the insulin receptor (IR) protein, causing activation of tyrosine kinase autophosphorylation and phosphorylation of Tyr residues followed. These reactions cause the activation of insulin receptor substance that generate docking sites of SH2-containing protein molecule (a protein subunit of p85/p110 on PI 3-kinase). Then the molecule PI 3-kinase becomes active and produces PIP3 (phosphatidylinositol 3,4,5-phosphate) and then binds to the PH-domain of PDK-1 (PIP3-dependent kinase) and Akt (protein Ser / Thr kinase B). This reaction lead to Akt and PDK-1 becomes active. Active Akt molecule can cause the translocation of GLUT4 proteins that mediate the glucose transport mechanism.

From these findings, it is very likely that the hypoglycemic activity of bungur ethanolic extract is derived, at least in part, from the decrease in insulin resistance, presumably because of the increase in GLUT4 translocation in total muscle membrane. These results suggest that the clinical use of bungur ethanolic extract in the treatment of diabetes mellitus, especially type 2 diabetes, may be appropriate.

ACKNOWLEDGEMENT

Researchers would like to acknowledge the advice and input provided by Dr. Junaidi Khotib, Dr. Bambang Prayoga, and Dr. Ratna Megawati Widharna on this project.

REFERENCES


METAL CHELATING ACTIVITY OF RICE BRAN AND RICE HUSK

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ABSTRACT

Free radical-induced oxidative stress is the root cause for many human diseases. Naturally occurring antioxidant supplements from plants are vital to counter the oxidative damage in cells. The main objective of the present study was to explore and compare the antioxidant activity of rice bran and rice husk extracted from rice milling waste. N-hexane extracts of rice bran and rice husk were used to study their in vitro antioxidant activities using metal chelating activity (iron (II)-phenanthroline complex). Vitamin E was used as standard material. The ability of the sample to chelate metal ion (ferrous ion) was calculated relative to the control and expressed as % inhibition. % inhibition of two samples were analyzed with student test (P=0.05). The results have shown that at the same concentration (10 ppm), rice husk extract, rice bran extract and vitamin E have the different activity, i.e.: 0.51%, 2.27% and 5.55% in inhibition of chelat formation, respectively. In conclusion, metal chelating activity of rice husk extract is smaller than rice bran extract. Activity of rice bran extract is almost a half from vitamin E, so this extract is still potential to be developed as source of antioxidant compounds.

Key words: rice bran, rice husk, metal chelating activity, antioxidant

INTRODUCTION

Rice bran is a rich source of natural antioxidants which can be used as free radical scavengers. It is widely recognized that many of the today’s diseases are due to the oxidative stress that result from an imbalance between formation and neutralization of pro-oxidants (Hazra et al., 2008; Braca et al., 2002). Cells have developed antioxidant mechanisms to quench the free radicals but when the generation of free radicals exceeds the scavenging capacity of the cell, the excess free radicals seek stability through electron pairing with biological macromolecules such as proteins, lipids and DNA in healthy human cells resulting the induction of lipid peroxidation which leads to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases (Rao et al., 2010). The free radicals are known to be scavenged by synthetic antioxidants, but due to their adverse side effects leading to carcinogenicity, search for effective and natural antioxidants has become crucial (Choi et al., 2007; Adeolu et al., 2009).

Rice bran is a by-product of rice milling which contains a significant amount of natural phytochemicals including sterols, higher alcohols, gamma-oryzanol, tocopherols, tocotrienols and phenolic compounds (Nam et al., 2006; Isao et al., 2004; Devi & Arumughan, 2007). These bioactive molecules have known to reduce serum cholesterol, decrease the incidence of atherosclerosis and also have antitumor properties (Deepa et al., 2008; Simi & Abraham, 2008; Halliwell, 1992; Itani & Ogawa, 2004).

In addition to rice bran, a by-product of rice milling is rice husk. This process yields rice husk and rice bran between 15-20% and 8-12%, respectively. Husk is outer skin of rice, while bran is epidermis of rice. If the national dry paddy production is 49.8 million tons/year, therefore the products of husk and bran are 7.5-10 million tons/year and 4-6 million tons/year, respectively. Utilization of rice by-products are still limited, sometimes even pollute the environment. These materials actually have an economic value well if be handled correctly. They can increase the value added in agro-industry system of rice. Some alternatives include the use of rice husk as a growing medium for mushrooms and ornamental plants, fuel, ash scrub, a mixture of tiles and biodiesel makers (Rachmaniah et al., 2007).
Bran can be utilized in the manufacture of breakfast cereals and in the increasing of dietary fiber (Hermanianto et al., 1999; Widowati, 2001).

Antioxidant activity of rice bran has been studied previously using several methods, i.e.: reducing power, total antioxidant activity, nitric oxide scavenging capability and DPPH scavenging assay. The reducing power of the rice bran extracts increased with the increasing concentration and a significant change was observed at 0.1 to 0.5 mg/ml. Total antioxidant activity of the rice bran extracts also increased with the increasing concentration of the extracts and a significant change was observed at 0.02 to 0.1 mg/ml. On DPPH test, the extracts showed activity with an IC\textsubscript{50} 30.85 µg/ml (Rao et al., 2010).

Since rice husk is paddy skin, hence it is supposed contain antioxidant compounds. At the other hand, there is no antioxidant activity test on rice husk recently. Based on the background mentioned, it is important to study the antioxidant activity of rice husk and compared it to rice bran and synthetic antioxidant.

There are many mechanisms of action of antioxidant compounds in counteracting the effects of oxidants. Antioxidant activity test was used in this study is metal chelating activity. This method is useful to investigate the function of antioxidants that can bind metals (Limantara et al., 2009). Solvent was used to extract the rice husk and rice bran is n-hexane. It was subjected to attract non-polar compounds contained in both materials. Antioxidant activity of rice husk and rice bran were also compared to synthetic antioxidant vitamin E. The antioxidant capacity is determined from the IC\textsubscript{50} (Inhibition Concentration 50), the concentration of test material that can inhibit 50% the formation of chelate (Lim et al., 2007).

**METHODOLOGY**

**Materials and equipments**

The samples of rice bran and rice husk were obtained from the rice milling unit, UD Eka Jaya located at Surabaya, East Java in september 2010. Standard α-tocopherol was purchased from Sigma-Aldrich Chemical Co. n-hexane and methanol were purchased from Mallinckrodt, while FeSO\textsubscript{4}, 1,10-phenantrline and ethyl acetate were purchased from Merck. Demineralised water was used as reagent's solvent.

Equipments were used include rotary evaporator (IKA® WERKE RV06-ML), analitical balance (Sartorius), Spectrophotometer UV-Vis (Shimadzu), vortex (Branson 1200), Maxi-mix (Thermolyne tipe 1600), and glassware equipments.

**Methods**

**Preparation of rice husk and rice bran extract**

Rice husk was washed and then dried at ambient temperature. After that, 150 g of dried rice husk was extracted thrice with certain volume of n-hexane for 2 h in an electrical stirrer at room temperature. Next, the extracts were filtered and evaporated under vaccum using a rotary evaporator (50°C) until thick extracts were obtained. Rice bran extracts were prepared in same manner without washing on starting materials. Finally, rice bran and rice husk extracts were dissolved in methanol until certain concentration (called as mother extract). From this concentration, working extracts were prepared in several concentrations.

**Metal chelating activity**

The metal chelating activity of extracts were measured qualitatively by the decrease in the intensity color of the iron (II)-phenantrline complex. Rice husk and rice bran extracts were applied on TLC plate Si Gel 60F\textsubscript{254}, eluted with n-hexaneethyl acetate (17:3) and sprayed with mixture of 1.68% FeSO\textsubscript{4} and 0.32% 1,10-phenantrline. Metal chelating inhibition was shown by light spot color with orange background.

Metal chelating activity tests were performed quantitatively according to Lim et al. (2007) with modification, as follow: 2 ml of 0.056% FeSO\textsubscript{4} and 2.0 ml of 0.0108% phenantrline were mixed with 1.0 ml of sample (with different dilutions). The mixture was allowed to equilibrate for 10 min before
measuring the absorbance at 509 nm. Sample solutions with appropriate dilutions were used as blanks as the extracts may also absorb at this wavelength. The ability of the sample to chelate ferrous ion was calculated relative to the control (consisting of iron and phenantroline only) using the formula,

\[
\text{Chelating effect} (\%) = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

Where \( A_{\text{control}} \) is the absorbance of the control reaction (containing all reagents except the test compound) and \( A_{\text{sample}} \) is the absorbance of test sample.

**Statistical analysis**

The measurements of samples were replicated at four times and the standard at twice. The results were statistically analysed with Student Test. Statistical significance was accepted at a level of \( P < 0.05 \)

**RESULT AND DISCUSSION**

In this study, the using of n-hexane as menstrum was subjected to extract the non polar compounds such as sterols, higher alcohols, gamma-oryzanol, tocopherols and tocotrienols. These compounds have been supposed to have antioxidant activity. From 150.0 g rice husk and rice bran were obtained 74.6 mg (0.05%) and 1.2 g (0.8%) dry extracts, respectively. The metal chelating activity of rice husk and rice bran extracts are illustrated qualitatively in fig. 1.  

![TLC profile](image)

Fig. 1. TLC profile of rice husk extract (a), rice bran extract (b), vitamin E (c)* and catechin (d)* on Si gel 60 F254, eluted with n-hexane:ethyl acetate (17:3) under vis (A), uv 254 (B), and sprayed with mixture of 1.68% FeSO\(_4\) and 0.32% 1.10-phenantroline (C)

*not eluted

From the fig. 1 (C), there are three white spots (a, b, c) and a black spot (d) with orange background. This indicated that both rice bran and rice husk extracts contain some compounds which inhibit the metal chelating reaction.

The chelating ability of the extract measures how effective the compounds in it can compete with phenantroline for ferrous ion. The iron–phenantroline complex has maximum absorbance at 509 nm and a large decrease in absorbance indicates strong chelating power. By forming a stable iron(II) chelate, an extract with high chelating power reduces the free ferrous ion concentration thus decreasing the extent of Fenton reaction which is implicated in many diseases (Halliwell & Gut-teridge, 1990).
Data of ferrous chelating activity of rice bran and rice husk are shown in table 1. Although there have been performed activity test with several concentrations of sample (both rice bran and rice husk extracts at 7-21 ppm), but only one concentration showed inhibition activity, i.e: 10 ppm. Therefore IC$_{50}$ of rice bran extract can not be calculated and neither can rice husk extract. From T test, there is found a significant difference between rice bran and rice husk extracts (t stat: 314.3911 > t critical two-tail: 2.447)

Table 1. Metal chelating activity of rice husk and rice bran extracts

<table>
<thead>
<tr>
<th>Replication</th>
<th>Rice bran extract</th>
<th>Rice husk extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ppm)</td>
<td>Inhibition (%)</td>
<td>Concentration (ppm)</td>
</tr>
<tr>
<td>1</td>
<td>10.62</td>
<td>2.28</td>
</tr>
<tr>
<td>2</td>
<td>10.53</td>
<td>2.26</td>
</tr>
<tr>
<td>3</td>
<td>10.62</td>
<td>2.26</td>
</tr>
<tr>
<td>4</td>
<td>10.35</td>
<td>2.26</td>
</tr>
<tr>
<td>Mean</td>
<td>10.53</td>
<td>2.27</td>
</tr>
</tbody>
</table>

It is found that rice bran and rice husk extract have low chelating power. For comparison, at approximately same concentration (10 ppm) vitamin E has inhibition 5.52%. This value is derived from linear correlation equation between concentrations of vitamin E versus % inhibition (table 2).

Table 2. Metal chelating activity of vitamin E

<table>
<thead>
<tr>
<th>Replication</th>
<th>Concentration (ppm)</th>
<th>$A_{sample}$</th>
<th>$A_{control}$</th>
<th>Inhibition (%)</th>
<th>IC$_{50}$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.3</td>
<td>0.803</td>
<td>7.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.6</td>
<td>0.778</td>
<td>10.06</td>
<td>234.4 (y = 4.3098 + 0.1949x)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>49.2</td>
<td>0.77</td>
<td>16.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>61.5</td>
<td>0.719</td>
<td>18.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>73.8</td>
<td>0.703</td>
<td>21.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>86.1</td>
<td>0.675</td>
<td>6.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12.1</td>
<td>0.808</td>
<td>10.54</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24.2</td>
<td>0.772</td>
<td>11.24</td>
<td>244.4 (y = 3.9635 + 0.1884x)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>48.4</td>
<td>0.766</td>
<td>13.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>60.5</td>
<td>0.749</td>
<td>16.57</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>72.6</td>
<td>0.72</td>
<td>22.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>84.7</td>
<td>0.666</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Although the metal chelating activity of rice bran and rice husk extract are low, but it can not be concluded that both have low antioxidant activity. This can be caused by many mechanisms of action of antioxidant. For comparison, guava has very potent primary antioxidant property but its function as secondary or preventive antioxidant is poor. At the other hand, langsat though acts as a weak primary antioxidant, can act as a moderate secondary antioxidant. Primary antioxidants scavenge radicals to inhibit chain initiation and break chain propagation. Secondary antioxidants suppress the formation of radicals and protect against oxidative damage such as bind to metal ions.

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CONCLUSIONS

Rice bran extracts have shown metal chelating activity greater than rice husk extracts, respectively 2.26% and 0.51% at 10 ppm of extract. At 10 ppm, vitamin E has inhibition 5.52% and IC$_{50}$ equivalent to 239.4 ppm. Although inhibition activity of rice bran extracts are smaller than vitamin E, but there is a good possibility to use this waste material as antioxidant sources.

ACKNOWLEDGEMENTS

The authors greatly appreciate Drs. Soediatmoko Soediman, M.Si for assistance in optimization of reactions.

REFERENCES


LONG TERM EFFECT OF ETHANOLIC EXTRACT OF FENUGREEK SEEDS (TRIGONELLA FOENUM-GRAECUM L.) ON WHITE RAT KIDNEY FUNCTION

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ABSTRACT

Fenugreek seed (Trigonella foenum-graecum L.) was known having activity to handle some of degenerative diseases such as diabetes mellitus, hypercholesterolemia and also postmenopausal symptoms. Our last researches proved that ethanolic extract of fenugreek showed potency as phytoestrogen. This further study was conducted to investigate the long term used of ethanolic extract of fenugreek seeds on white rat, especially to evaluate its effect on kidney function. This in vivo assay referred to WHO protocols for toxicity assay of natural medicines. We used Spraque dawley white rats, female and male, 6 weeks age, which divided into three groups; namely normal group, treatment groups and recovery groups. Sample was orally treated for 16 weeks, divided into three doses, 80mg/200gBW, 400mg/200gBW dan 2000mg/200gBW, both in treatment and recovery groups. Blood and urine were sampled and analyzed in week 0, 8 and 16 (18 for recovery groups). At the last week of treatment, animals were autopsied to take the kidney organ. While the recovery group left for two weeks without treatment and autopsied at week 18. Parameters tested were urea and creatinine from blood plasma and urine, histological preparation of kidney was done using Hematoxylin-Eosin staining. Other physical urine parameters were also analyzed, such as pH, density, volume, and color. All the data were analyzed with two way ANAVA (α=0.05). Results showed that ethanolic extract of fenugreek seeds treatment on all groups did not cause a significant (α=0.05) difference on urea and creatinine from blood plasma and urine, also kidney histopathology of rat with treatment duration until 16 weeks.

Key words: Fenugreek seeds, Kidney Histopathology, Urea, Creatinine, Trigonella foenum-graecum L.

INTRODUCTION

Recent research in Indonesian medicinal plants growing so fast, especially for formally medical purposes. The potency of our biodiversity, especially medicinal plants and their empirical data, could become a good point to start proofing their scientific clarification. So that products will be able to use in medical world. Mostly, medicinal plants are used for handling and maintenance the degenerative diseases and disruption of metabolism function. Nowadays, it is become important to increase their quality by completing the data with activity and toxicity assay, as much as their growing. So in the future, the Indonesian natural products not only have empirical proven but also scientifically proven through preclinical assay. Badan Pengawasan Obat dan Makanan (BPOM) or the Indonesian Food and Drug Administration has determined our natural products into three classification, they are Jamu, Standardized Herbs and Phytopharmaca. Jamu is the original natural products having only empirical data. To achieve the Standardized Herbs level, we need to do the preclinical assay using animal test including activity and toxicity assay from acute until chronic assay. The highest natural products level is Phytopharmaca, which has been completed with preclinical and clinical data in humans by following the rules of Good Clinical Practice Test.

Toxicity assay is one of preclinical assay to investigate the effect of long term use of the products, which is divided into three categories. They are acute toxicity assay, sub chronic toxicity assay and chronic toxicity assay. Based on protocol issued by World Health Organization protocol.
(WHO, 2000) and BPOM (2000), several provisions of chronic toxicity assay was arranged, such as the use of animals, animal sex, treatment groups, duration of treated sample and parameters of analyses that must be observed.

Fenugreek seed or Foenigraeci semen is dried seed from Trigonella foenum-graecum L., Leguminosae, (MMI, 1979). Fenugreek was predicted having estrogenic like effect because of some sapogenin steroid ingredients, e.g. diosgenin, precursor for sexual hormone (Evans, 2002), its isomer Yamogenin (Dewick, 1997), gitogenin, tigogenin, and trigoneoside (saponine like estrogen) which have effect as phytoestrogen for menopause symptoms therapy (Hoffman, 2004). Fenugreek contains diosgenin in base free form 0.8 – 2.2 % (Wiryowidagdo, 2000). Besides that, fenugreek also contains fatty oil 20-30%, alkaloids (trigonelline, an alkaloid pyridine, gentianin and karpain), flavonoids e.g. vitexin in glycoside or ester form, isovitexin, orientin, vicenin, quercetin and luteolin (Hoffman, 2004), essential oil, saponine, nicotinamide, choline, bitter compound and mucilage (Evans, 2002). Fenugreek can induce uterine contraction, so it can’t consume during pregnancy (Hoffman, 2004). Phytoestrogen is used as alternative for Hormone Replacement Therapy (HRT) to help reducing menopause symptoms. It can be used for long term until the body can make adaptation on the new level hormone (Badziad, 2003).

Fenugreek seed also has medicinal properties for hemorrhoids, asthma, ulcers, and muscle pain and often used as a preventative hair loss and skin softener. In previous research data showed that the LD50 of acute toxicity is more than 2500mg/kgBW so we can say that ethanolic extract of fenugreek is practically non toxic. On the activity assay on white wistar strain rats, data showed that fenugreek seed has effect as phytoestrogen (Agustini, 2007), antidiabetic, as well as anticancer and for hypercholesterolemia handling(Mills, 2000).

This experiment was done to investigate the effect of Trigonella foenum-graecum L. extract for long term use, due to kidney function. Parameters has been analyzed to investigate the kidney function are blood urea nitrogen (BUN) and creatinine of blood plasma. While kidney histological analyzed by Haematoxylin-Eosin staining.

Level of urea (BUN) and creatinine blood plasma could predict kidney disruption. Urea was produced from protein metabolism and will be excreted in urine. Increase of BUN level showed disrupted of glomerolus. Urea was distributed into all intracellular and extracellular fluid. Majority of urea was excreted by kidney, and also excreted by sweat and gastrointestinal in small amount. Urea contains ammonia which is produced from α-amino nitrogen on amino acids. Toxic mechanism of ammonia still not clear, but body secrete ammonia by change it first into non-toxic metabolite, urea. Sometimes, BUN level also could be influenced by food and hepatotoxicity, which is one of common toxicity effect.

Creatinine is a metabolite which is excreted in urine by filtration process in glomerolus. The increase of blood creatinine level indicated kidney damage too. Blood creatinine level also can be use to predict glomerular filtration rate (GFR), one of kidney analyses parameter (Murray, 1992). Analysis is using colorimetric or spectrophotometric.

METHODS

Extraction of Fenugreek extract

Dried Fenugreek seed was obtained from Balai Penelitian Tanaman Obat (BPTO) Tawang mangu, Solo and deteriorate by Lembaga Herbarium Bogoriense LIPI, Bogor. Fenugreek was macerate five times with distilled ethanol and then dried through vacuum evaporator. The crude extract was stored in desiccators until the weight is constant. Sample were suspending with CMC Na 0.5%.

Animal preparation

Fifty five (55) female and fifty five (55) male Sprague-Dawley rats, 4 months old, 150-200 grams Body Weight (gBW), were purchased from Indonesian Food and Drug Administration (FDA), Health Ministry, Jakarta. Rats were caged and acclimatized for one week in Animal Laboratory.
Experimental protocol

Each group contains 10 male and 10 female rats, except the recovery groups, each 5 male and 5 female rats. Each group was fed one of diets describe in Table 1, per-oral by gavages into gastric, once a day for 16 weeks. Before treatment, at weeks 8 and the end of treatment, all of animals were took blood sample for creatinine and urea analysis.

Table 1. Groups of animal treatment

<table>
<thead>
<tr>
<th>Normal Groups</th>
<th>N</th>
<th>Normal group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose Groups</td>
<td>D1</td>
<td>Sample 80mg/200gBW</td>
</tr>
<tr>
<td></td>
<td>D2</td>
<td>Sample 400mg/200gBW</td>
</tr>
<tr>
<td></td>
<td>D3</td>
<td>Sample 2000mg/200gBW</td>
</tr>
<tr>
<td>Recovery Groups</td>
<td>RD1</td>
<td>Sample 80mg/200gBW</td>
</tr>
<tr>
<td></td>
<td>RD2</td>
<td>Sample 400mg/200gBW</td>
</tr>
<tr>
<td></td>
<td>RD3</td>
<td>Sample 2000mg/200gBW</td>
</tr>
</tbody>
</table>

At weeks 16 all animals in treatment groups were autopsied. While Recovery Groups were autopsied at weeks 18. Kidney organs were quickly removed, weighed and immersed in Bouin’s solution.

Creatinine and urea level were analyzed with Diasys® kit reagent and were measured by spectrophotometer. Histological preparation used paraffin method and stained with hematoxylin-eosin. Slides were examined under microscope for analyzed the diameter of centralist vein and endothelial cell of liver organs.

Statistical analysis

All quantitative data were analyzed using computer programme, Statistical Product and Service Solution (SPSS) 11.5 for windows. Normality test with Kolmogorov-Smirnov and homogeneity test with Levene variance. ANAVA test continuing with LSD test. A p value below 0.05 was chosen as the limit of statistical significance.

RESULT AND DISCUSSION

This assay is further research of toxicity assay after the efficacy assay was done. Three variance doses used were calculated until 25 times the effective dose. This high dose sample was fed to male and female rats for 16 weeks. Animal models used were normal rat male and female, to reduce sex variance, which was divided into Dose groups and Recovery groups. The aim of recovery group is to investigate the recovery effect after treated with high dose of ethanolic Fenugreek extract for 16 weeks.

Analysis of blood biochemistry of kidney function was done in three times evaluation, before treatment, in the middle of treatment (week 8) and at the end of treatment before autopsy (except recovery groups). Result of BUN and creatinine analysis in male and female rats were showed in figure 1-4. Furthermore, data were analyzed with statistical method, SPSS, to investigate whether there is difference significance or not. ANAVA analysis showed that there are differences between groups in different times. But overall, there are no differences between treated groups and normal group significantly (α=0.05) in the same time sampling. We can say that high dose sample treatment for 16 weeks, did not cause decreasing blood level of Urea and Creatinine.

Increasing of BUN and creatinine indicates nephotoxic (kidney damage phenomena). The main function of kidney is to filtrate the unusefull metabolite and toxic compound from body into urine. Mainly metabolites from protein metabolism process are urea and creatinine which are secreted from the body through filtration process in glomerulous kidney. With this concept, so increasing BUN and creatinine level will indicate something disruption of kidney function. At picture 3 and 4 show that at week 16, all BUN and creatinine blood level were decreased as well as normal control group,
both in male and female rats. We can say that treatment of high dose (till 25 times effective dose) everyday for 16 weeks, did not cause increasing level of BUN and creatinine blood. So we can predict that there is no disruption of filtration process in glomerulus or bowman’s capsule in kidney. Further investigation was done by analyzing histological preparation of kidney organ.

After autopsied, the kidney organs were weighed and macroscopic investigated. While histological preparations were investigated under microscope. In figure 5 shows there is no significance (α=0.05) difference between groups and time on male rats. On female rats, there are significance (α=0.05) differences between treatment groups comparing with normal group, but the differences still in the normal limit, meaning still in the range of two deviation standard. So we can say that this differences did not show significance changes for this parameter.
Figure 7. Weight of kidney organ in male and female rats after treated with ethanolic Fenugreek extract for 16 weeks

Figure 8. Body weight data of male rats before treatment, week 8, week 16 and for Recovery groups in week 18.

Figure 9. Body weight data of female rats before treatment, in week 8, week 16 and for Recovery groups in week 18.

**Histological preparation of Male kidney organs**

Figure 10. Histological preparation of kidney from each groups after treated 16 weeks and 18 weeks for recovery groups.

In figure 6 shows that there is no difference between groups in male and female groups. ANAVA analysis shows there is no difference of weight kidney organs significantly ($\alpha=0.05$).
Kidney as an excretion organ receive 25% of blood from cardiac system, which is transport, mainly, metabolite and toxic compounds. This flow will be filtrated in cortex contain glomerolus. If there is damage in the epithelial cell in varietal layer in glomerolus, because of their sensitivity to the toxic compounds, the filtration process will be disrupted too. Accumulation of non filtration toxic compound will make more damage of the cell in glomerolus. In the histological preparation, the damage can be showed by analyses of cells of bowman’s capsule in glomerolus, diameter and space between. The diameter will decrease and damage of cells and visceral layer cause space between bowman’s capsule become increase.

Another parameters is body weight. In figure 7 and 8 shows that long term treatment of high dose sample for 16 weeks, did not increase or decrease body weight of treated rats comparing with normal rats. ANAVA analyses showed that there are no significance (α=0.05) difference between groups in male and female rats.

CONCLUSION
High dose treatment (2000mg/200gBW) of Ethanolic Fenugreek extract for long term used (16 weeks) did not cause negative effect on rats kidney organ.

ACKNOWLEDGEMENT
This study was funded by DIPA 2007 of Center of Pharmaceutical and Medical Technology, BPPT, Jakarta. We would like to thank Julham Effendi (BPPT) and Vio Araneta (Pharmacy Faculty, University of Pancasila) for extract preparation and handling animals during toxicology study.

REFERENCES
MOLECULAR DOCKING OF SEVERAL COMPOUNDS OF BUNGUR (LAGERSTROEMIA SPECIOSA [L.] PERS) LEAVES TO (IGF-1R) RECEPTOR TYROSINE KINASE WITH MOLEGRO VIRTUAL DOCKER

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ABSTRACT

The binding possibility of several active compounds of Bungur (Lagerstroemia speciosa [L.] Pers) leaves to type 1 insulin-like growth receptor (IGF-1R) had been evaluated by molecular modeling and molecular docking using Molegro Virtual Docker. Based on several previous researches, there were five active compounds of Bungur extract having anti-diabetic activity used in this study, i.e. ellagitannin, Penta-O-galloyl-glucopyranose (PGG), corosolic acid, lageflorin A, and lageflorin B. The docking experiment gave several parameters used to determine the stability of the ligand-receptor complex formed, i.e. MolDock Score, Rerank Score, RMSD, and H Bond. Corosolic acid were found to be the most stable ligand for IGF-1R, followed by ellagitannin and PGG, because it showed the smallest values of MolDock Score, Rerank Score, and H Bond i.e. -198.918; -151.231; and -23.4225; respectively. The results of this study have been justified the previous researches or proposed theories concerning the mechanism of lowering blood glucose level of these three active compounds of Bungur leaves, i.e. by its binding to the insulin receptor that will initiate a signaling cascade in target tissues. On the contrary, lageflorin A and B have higher MolDock Score and Rerank Score and no hydrogen bond interaction. Thus, these two compounds were proposed to have different mechanism in its anti-diabetic activity.

Keywords: Lagerstroemia speciosa [L.] Pers, Molegro Virtual Docker, IGF-1R, ellagitannin, corosolic acid, PGG, lageflorin

INTRODUCTION

Type 2 diabetes mellitus or non-insulin-dependent diabetes mellitus is a widespread syndrome characterized by fasting and post-prandial hyperglycemia affecting increasing number of the world population (Richard, 2004). According to The Statistical Report of the International Diabetes Federation (IDF) there are now about 230 million people suffering this disease. This figure continue to grow by 3% or about 7 million people each year, and is predicted to reach 350 million by 2025, with half of this number are contributed by people in Asia, especially India, China, Pakistan, and Indonesia. In 1995, Indonesia was the 7th country with the most people with diabetes mellitus in the world, and in 2025 is estimated to rise to be the 5th at the world. At this time, it was reported that almost 10% of the population in big cities of Indonesia, like Jakarta and Surabaya, suffer diabetes mellitus (Tandra, 2008).

Despite considerable progress in the management of diabetes mellitus with synthetic drugs, the search for indigenous natural anti-diabetic agents is ongoing. Based on a large number of chemical and pharmacological research work, numerous bioactive compounds have been found in Indonesian medicinal plants for diabetes. One of the potential plants used to treat diabetes mellitus is Bungur (Lagerstroemia speciosa [L.] Pers). Several research reports showed that Bungur leaf extract lowered blood glucose levels in diabetes mellitus patients (Liu et al., 2001, Hayashi et al., 2002, Saha et al., 2009, Hoyosama et al., 2003, Klein et al., 2007, Okada et al., 2003, Hernawan et al., 2004). The potential of Bungur leaf extract as anti-diabetic agents definitely related to the biological activities of the bioactive compounds contained in it.
The main content of Lagerstroemia sp. leaf extract which has a blood glucose lowering effect is polyphenol (Hayashi et al., 2002). Ellagitannin (Klein et al., 2007) and Penta-O-galloyl-glucopyranose (PGG), a gallotannin compound, has been reported to have high activities in stimulating glucose transport (Li et al., 2005). Corosolic acid, a triterpenoid compound in Bungur leaf extract, has been reported also having hypoglycemic activity by stimulating glucose uptake through phosphorylation of insulin receptor (Klein et al., 2007). In L. parviflora, other species of Lagerstroemia, also found lagerflorin which is a pentacyclic form of triterpenoid (Barik and Kundu, 1988).

![Chemical structures](image)

Figure 1. Chemical structure of (a) ellagitannin; (b) Penta-O-galloyl-glucopyranose (PGG); (c) corosolic acid; (d) lageflorin A; (e) lageflorin B

There are several possible mechanisms in lowering blood glucose effect of bioactive compounds. One possible mechanism is binding of the bioactive compounds to the insulin receptor, that will initiate a signaling cascade in target tissues as the first step in the regulation of metabolic homeostasis. In this study, the binding possibility of L. speciosa active compounds to insulin receptor will be evaluated by molecular modeling and molecular docking technique. Molecular modeling is the evaluation of molecular properties and structures using computational chemistry and molecular graphics to provide three-dimensional visualization and representation of molecules. These techniques nowadays have been used widely in modern drug design or to better understand the drug-receptor interaction.

**METHODS**  
**Hardware and Software**

Several softwares used in this study were ChemBioDraw Ultra 11.0 (for 2D structure construction and determining of physicochemical properties), ChemBio3D Ultra 11.0 (for 3D structure construction), and Molegro Virtual Docker 3.0 (for Docking experiment). The Molegro Virtual Docker 2008 is a free molecular docking program that could be downloaded from [http://www.molegro.com](http://www.molegro.com). The computer used in this study is based on Windows with dual core processor.
Molecule Structures and Optimization

All of 2-dimension molecule structures of bioactive compounds of *Lagerstroemia speciosa* [L.] Pers used in this study (Figure 1) were constructed by ChemBio Draw Ultra 11.0 2008. These structures then converted into 3-dimension geometrical structures using ChemBio 3D Ultra 11.0 2008. The 3-D structures of all molecules then optimized further by minimizing its energy using MM2/MMFF 94 tools in ChemBio 3D Ultra 11.0 2008, and saved in SYBIL2 (mol2) files. These 3-D structures are presented in Figure 2.

The protein structure used in this study is type 1 insulin-like growth factor receptor (IGF-1R), a member of the receptor tyrosine kinase family, and was retrieved from Protein Data Bank ([http://www.rcsb.org](http://www.rcsb.org)) with PDB ID 3EKK. The existed ligand was extracted from the protein before the docking experiment.

Molecular Docking

The docking of five molecule structures in *Lagerstroemia speciosa* [L.] Pers extract to the binding site of IGF-1R was done by using Molegro Virtual Docker Program with its Sybil2 engine. This algorithm utilize an algorithm detected cavity to detect the potential binding site of protein-ligand. The screening of most stable ligand structure was done by MM2/MMFF 94 tools in ChemBio 3D Ultra 11.0 2008 and was combined with conformation seeking to produce the ligand pose that most consistent and precise with protein binding site by Docking Wizard tools in Molegro Virtual Docker Program. Docking experiment was done with standard arrangement, of in Molegro Virtual Docker using cavity 1 (volume 134.656) among 5 cavities detected in IGF-1R (PDB ID 3EKK) protein. The scoring function was MolDock Score (GRID) with grid resolution 0.30 Å. The search algorithm used was MolDock SE with number of runs 10 and constrain poses to cavity. The parameter settings included maximum iteration 1500 and maximum population size 50. The energy threshold of pose generation was set to 100.00, while the simplex evolution used in maximum step 300 and neighbor distance factor 1.00.

Figure 2. 3D structures with minimized energy of (a) ellagitannin; (b) Penta-O-galloyl-D-glucopyranose (PGG); (c) corosolic acid; (d) lagerflorin A; (e) lagerflorin B, save in Sybil2 (mol2) files.
Scoring Function

The conformation forms that have been docked into the protein were printed further using different scoring functions available in [Sybil2]. The Molegro Virtual Docker algorithm uses internal scoring function where DockScore is used to choose and distinguish the position of each compounds. DockScore is a simple force field based on scoring function that estimates the summing energy of interaction of ligand-protein and internal energy of ligand. The force field in Molegro Virtual Docker is used to solve electrostatic parameters for DockScore. The putative 3D positions yield scores that are saved in Sybil2 file. Every minimized docking was purely used DockScore, molecular mechanic of scoring function and unique poses, to be further presented with other scoring functions, including Rerank Score, RMSD (Root Mean Standard Deviation) and H bond.

DISCUSSION

The insulin receptor (IR) and the type-1 insulin-like growth factor receptor (IGF-1R) are homologous multidomain proteins that bind insulin and IGF with differing specificity (Lou et al., 2006). Insulin binds with high affinity to the IR and with low affinity to IGF-1R (Zhao et al., 2009). Both of these receptor proteins, are member of the receptor tyrosine kinase family, and are large, transmembrane, glycoprotein dimer consisting of several structural domains. The N-terminal half of the ectodomain contains two leucine-rich repeat domains (L1 and L2) separated by a cysteine-rich region (CR). The C-terminal half of the IR ectodomain consists of three fibronectin type III domains, the second of which contains an insert region of ≈ 120 residues (Lou et al., 2006).

Besides their structural homology, IR and IGF-1R have a similar binding mechanism to their cognate ligands (insulin and IGF-I, respectively). They have equivalent binding surfaces on the ligands and receptor, with an additional interaction of the IGF-I C domain with the cysteine rich (CR) domain of IGF-1R found to contribute its high affinity binding (Alvino et al., 2009). In this study, IGF-1R was chosen as receptor for several active compounds of Lagerstroemia speciosa [L.] Pers, in order to predict the mechanism of their lowering blood glucose level through their binding to insulin receptor or its analog. The IGF-1R protein structure in complex with 4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidines was retrieved from PDB ([http://www.rcsb.org](http://www.rcsb.org)) with PDB ID 3EKK, elucidated with X-Ray Diffraction method with 2.10 Å resolution (Figure 3). This 3EKK IGF-1R protein structure was only the kinase domain, chain A of the whole structure, with 307 amino acids. The ligand was then extracted to leave a cavity, used as binding site of the examined ligand compounds. The cavity placed by this ligand was further recognized as cavity 1 among the 5 cavities detected by the Molegro Virtual Docker Program. The volume of cavity 1 is 134.656, and was the largest one.

![Figure 3](image-url)

Figure 3. The structure of type 1 insulin-like growth factor receptor (IGF-1R), with PDB ID 3EKK, complexed with an inhibitor (4,6-bis-anilino-1H-pyrrolo[2,3-d]pyrimidines) as ligand in cavity 1, (a) in ball and stick mode, (b) in secondary structure mode
The docking experiment was started with the replacement of initial ligand in cavity 1 with the compound to be analyzed. The structure of the five compounds to be analyzed should be converted first into 3-D structure in the most stable conformation by minimizing its energy using MM2/MMFF 94 tools in ChemBio 3D Ultra 11.0 2008 program, and then save in the SYBIL2 (mol2) format. After the placement of the analyzed compound to the binding site of the protein, the docking experiment could be go on using the docking wizard tools of this program, with specific arrangement of the parameters as described in previous section. All of the docking experiment parameters were set the same for all of the analyzed compounds, to make an equal comparison condition in the further analysis step.

The result of docking experiment could be presented in several ways. Figure 4 showed the ligand-receptor interaction in the secondary structure view. All of the analyzed compounds were bound in the same place, i.e. the cavity 1, further called as the binding site of IGF-1R receptor. This binding site is located at the cavity formed by two adjacent anti-parallel beta sheets in the 3-D structure of the protein.

Figure 4. The secondary structure view of IGF-1R that bind five active compounds of Lagerstroemia speciosa [L.] Pers extract, (a) ellagitannin; (b) Penta-O-galloyl-D-glucopyranose (PGG); (c) corosolic acid; (d) lageflorin A; (e) lageflorin B

The binding affinity of ligand to its receptor can be evaluated also by their hydrogen bond interaction. Figure 5 indicated the hydrogen bond formation between five active compounds of L. speciosa [L.] Pers extract and IGF-1R. PGG was shown to have high affinity to IGF-1R, because it could form 14 hydrogen bonds to 12 amino acids of the protein. This is not surprisingly due to the number of hydroxyl groups possessed by this compound. Ellagitannin, having 4 hydroxyl groups, was able to form seven hydrogen bonds in ellagitannin-(IGF-1R) complex. These hydrogen bonds were formed between ellagitannin and four amino acids of IGF-1R. On the other case, corosolic acid was only able to form three hydrogen bonds with the IGF-1R. But, the hydrogen bond energy formed was the highest among other complexes, higher than the hydrogen bond energy form in PGG-(IGF-1R) complex. We proposed this higher energy was developed due to the closer distance formed between corosolic acid’s pharmacophores and IGF-1R amino acid. There was one conserved amino acid used in hydrogen bond formation in all of three ligand-receptor complexes, i.e. Asp1150, while Met1079 and Lys1030 were used in two ligand-receptor complexes.
Figure 5. The hydrogen bonding between amino acids of IGF-1R and five active compounds of Lagerstroemia speciosa [L.] Pers extract, (a) ellagitannin; (b) Penta-O-galloyl-D-glucopyranose (PGG); (c) corosolic acid; (d) lageflorin A; (e) lageflorin B

Table 1. Docking Parameters of L. speciosa [L.] Pers Active Compounds to IGF-1R

<table>
<thead>
<tr>
<th>Ligand Names</th>
<th>MolDock Score</th>
<th>Rerank Score</th>
<th>RMSD</th>
<th>H Bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagitannin</td>
<td>-99.7117</td>
<td>-74.3641</td>
<td>8.97307</td>
<td>-5.61265</td>
</tr>
<tr>
<td>Penta-O-galloyl-glucopyranose</td>
<td>-75.7993</td>
<td>-70.9837</td>
<td>4.82563</td>
<td>-10.5656</td>
</tr>
<tr>
<td>Corosolic acid</td>
<td>-198.918</td>
<td>-151.231</td>
<td>9.79921</td>
<td>-23.4225</td>
</tr>
<tr>
<td>Lageflorin A</td>
<td>-98.7657</td>
<td>-69.2041</td>
<td>10.1633</td>
<td>0</td>
</tr>
<tr>
<td>Lageflorin B</td>
<td>-93.3974</td>
<td>-56.9037</td>
<td>5.93393</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2. Physicochemical Parameters of L. speciosa [L.] Pers Active Compounds *)

<table>
<thead>
<tr>
<th>Ligand Names</th>
<th>Log P</th>
<th>Clog P</th>
<th>MR</th>
<th>CMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagitannin</td>
<td>1.05</td>
<td>0.376463</td>
<td>70.24</td>
<td>7.0416</td>
</tr>
<tr>
<td>Penta-O-galloyl-glucopyranose</td>
<td>2.42</td>
<td>2.62922</td>
<td>211.18</td>
<td>21.0514</td>
</tr>
<tr>
<td>Corosolic acid</td>
<td>7.22</td>
<td>6.2972</td>
<td>138.04</td>
<td>13.9847</td>
</tr>
<tr>
<td>Lageflorin A</td>
<td>7.34</td>
<td>7.772</td>
<td>131.22</td>
<td>13.3264</td>
</tr>
<tr>
<td>Lageflorin B</td>
<td>7.34</td>
<td>7.772</td>
<td>131.22</td>
<td>13.3264</td>
</tr>
</tbody>
</table>

*) computed by ChemBioDraw Ultra 11.0 Program
Molecular Docking of Several Compounds

On the contrary, 2 isomer forms of Lageflorin, couldn’t form hydrogen bonds with IGF-1R, because they don’t possess any hydroxyl group needed in hydrogen bond interaction with the protein. It seemed like these compounds use other interaction in its ligand-receptor binding mechanism, presumably hydrophobic interaction due to its high hydrophobicity (shown by its high LogP and ClogP values, table 2). Figure 6 showed the hydrophobic interaction between five ligands and IGF-1R. Different compounds will give different hydrophobic environment in the binding site of IGF-1R.

The total interaction energy between ligand and receptor was calculated in Molecular Virtual Docker Program, and reported further in several scoring function parameters, which were MolDock Score, Rerank Score, RMSD and H bond. The main docking parameters used in determining the most stable ligand-receptor complexes are MolDock Score and Rerank Score. The smaller number of MolDock Score and Rerank Score the more stable is the ligand-receptor complex formed. Thus, from table 1, we can predict that corosolic acid form the best or most stable conformation of ligand-receptor complex with IGF-1R protein, because it had the lowest number of MolDock Score, Rerank Score and H bond Score. Ellagitannin and PGG were predicted to be able to form a moderate stable conformation with IGF-1R. But, lageflorin A and B, were predicted to be weakly bound to IGF-1R.

These results then can be used to predict (because there are no comparable data to further analyze), to give justification, or understand better the mechanism of these five compounds in lowering blood glucose level. We proposed that corosolic acid, ellagitannin, and PGG, act as an insulin-like compound that will be bound to the insulin receptor, and thus will initiate a signaling cascade in target tissues as the first step in the regulation of metabolic homeostasis, especially related to uptake glucose to target tissues and lowering the blood glucose level. Lageflorin, in the other way, will use different mechanism in its activity as anti-diabetic agent. Further studies are needed to explain the exact mechanisms of these active compounds in its anti-diabetic activity.
ACKNOWLEDGMENTS
We thank Prof. Dr. Siswandono, Apt. for his invaluable advices and discussions.

REFERENCES
THE EFFECT OF GIVING TOMATO JUICE (*LYCOPERSICUM ESCULENTUM MILL.*) TO THE AMOUNT OF PLATELETS, BLEEDING TIME AND COAGULATION TIME OF WHITE MALE RATS OF WISTAR STRAIN

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ABSTRACT

Tomato has a compound which is able to prevent coagulation of blood. This research objective aimed to know the effect and effective dose of giving tomato juice to the amount of platelets, bleeding time and coagulation time of white male rats of wistar strain. The research used 5 white male rats from wistar strain of 3-4 months of age that weigh between 200-300 grams as subjects, all of which was divided into 5 treatment groups (TG). TG I was treated as negative control with the administration of aqua distillate. TG II, III, and IV were then treated with administration of tomato juice at the dose of 12.60 g/kg of BW, 15.75 g/kg of BW and 18.90 g/kg of BW. TG V was treated as positive control and treated with the administration of 12.60 mg/kg of BW aspirin suspension. Each TG was treated for 21 days and was tested for its bleeding time, coagulation time and the amount of platelets on the day 0, 7, 14 and 21. The measurement process of the data used one way anova, non-parametric likes Kruskal-Wallis test and Mann-Whitney test, and paired sample t-test analyse with 95% result accuracy using SPPSS version 15. The analysis showed that administration of tomato juice affects to bleeding time, coagulation time and the amount of platelets of white male rats of wistar strain. Three of the dose administered to the subjects were able to prevent coagulation of blood by enduring the bleeding time and coagulation time, decreasing the amount of platelets as well. The three different dose showed almost similar result but the most effective dose is 15,75 g/kg of BW at the 7th day.

Keywords: tomato juice, the amount of platelets, bleeding time, coagulation time

INTRODUCTION

Haemostatic is the way of body to discontinue bleeding at injured vessel (Ganiswara, 2007). Haemostatic process involves three main processes, namely vasoconstriction (shrinkage) of blood vessels, the activity of circulating platelets, and the activity of platelet clotting factors. When there is injury to the blood vessel, shrinkage occurs in the injured blood vessels and reflexes constriction of small arteries and arterioles in the vicinity led to the initial slowing of blood flow to the area of injury. Reduced blood flow allows the contact activation of platelets and coagulation factors in causing blood clots. If freezing is not controlled, then the small blood vessels in certain areas may stop. If there is a blockage of blood vessels in brain, it can cause a stroke. Blockage of blood vessels in the heart can cause heart attacks (Pettit and Hoffbrand, 2005).

Tomatoes (*Lycopersicum esculentum Mill*) contain a compound called lycopene. Lycopene, the powerful weapon in tomatoes is a class of carotenoids and antioxidant compounds found in most red tomatoes. The results of Rowett Research Institute in Aberdeen, Scotland, found that the yellow gel that covers tomato seeds can prevent clotting and blood clots that may cause strokes and heart disease. (Sutomo, B., 2008: http://budiboga.blogspot.com/2008/02/menguakmanfaat-tomat.html)

In this research, tomato juice will be tested against the anti-aggregation power of platelets to prevent clotting or blood clots compared with asetasol that is already known to inhibit platelet aggregation. The power of anti-platelet aggregation tomato juice in this research can be seen from the test performed such as the amount of platelets, bleeding time, and coagulation time.

The use of synthetic drugs nowadays is still giving considerable side-effects. As absiksimab drugs that are inhibitors to glycoprotein IIb/IIIa included in these anti-platelet drugs that has side-
effects such as bleeding and thrombocytopenia. Therefore, nowadays, people use many herbs, including the tomatoes, to treat diseases, including those associated with the circulation system.

RESEARCH METHODS

Materials
Tomato juice, distilled water, sodium carboxymethylcellulose, asetosal, and Ress-Ecker reagents.

Tools
Glassware, injection syringes with needles blunt-ended (sonde), filter paper, cotton, scissors, non-heparin hematocrit, stopwatch, a set of erythrocytes pipettes, watch glass, deck glass, and microscope.

Tested Animals
Test animal used were white male rats of wistar strain aged 3-4 months and weight 200-300 grams. Test animal obtained from Laboratorium Penelitian dan Pengujian Terpadu (LPPT-Gajah Mada University).

Tomato-Juice Making
Tomatoes are steamed for 10 minutes and then blended for 2, 5 minutes.

Platelet Andragegast-Effects Testing
In the test, rats are divided into 5 groups that each consists of five rats. Testing is done by giving the test material for 21 days. The dozes of tomato juice used were 12, 60 g/kg of BW, 15, 75 g/kg of BW, and 18, 90 g/kg of BW. Positive controls used were asetosal 12, 60 mg/kg of BW and the negative control was distilled water. It conducted three tests of bleeding time, coagulation time, and the amount of platelets on 0, 7th, 14th, and 21st days. Data testing results statistically tested using t-student.

Measurement of Bleeding Time
Blood obtained from the end tail of rat, the blood coming out and absorbed by filter paper. Bleeding time is recorded when the blood started coming out until the blood stopped flowing and no longer absorbed by filter paper (Gandasoebrata, 2008).

Measurement of Coagulation Time
Blood coming out from the end tail of rat housed in non-heparin hematocrit up to full. Hematocrit is broken every 30 seconds to form fibrin threads. Coagulation time is recorded when the blood out through the establishment fibrin threads at the breaking of hematocrit (Gandasoebrata, 2008).

Measurement of the Amount of Platelets
Blood coming out from the end tail of rat housed in a bottle containing Na EDTA. Numbers of platelets are recorded by using the method of RessEcker (Gandasoebrata, 2008).

RESULT AND DISCUSSION
This research aimed to observe the effect of tomato juice as an anti-blood clotting by preventing aggregation of platelets (anti-platelets) that is given to tested-animal of white male rats of wistar strain. There are three parameters observed, namely: bleeding time, coagulation time, and the amount of platelets.

Bleeding time is an examination to assess the function of primary haemostatic. Primary haemostatic played by platelets and blood vessels. The first primary haemostatic involved in the process of cessation of bleeding in case of injury or trauma. Primary haemostatic begins with vasoconstriction of blood vessels and the formation of platelet plaques cover the wound and stop bleeding. Vasoconstriction causes blood to flow more slowly on the wound or trauma. This situation would facilitate the adhesion of platelet receptor. Gplb stick to subendotel blood vessels (adhesion) with the mediation of van Willebrand factor. Activated platelets cause platelets receptor to be adjacent to each other and followed the occurrence of platelet aggregation enclosed with establishment platelet plaques that cover the wound or trauma. This blockage is temporary. The existence of anti-platelet aggregation effect is shown by a longer bleeding time after administration of the test material.
Table 1. Rats’ bleeding time at testing the effects of platelet anti-aggregation.

<table>
<thead>
<tr>
<th>Group</th>
<th>The 0 day</th>
<th>The 7th day</th>
<th>The 14th day</th>
<th>The 21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (-)</td>
<td>106,60 ±</td>
<td>128,80 ±</td>
<td>100,40 ±</td>
<td>129,00 ±</td>
</tr>
<tr>
<td></td>
<td>17,358</td>
<td>49,494</td>
<td>7,092</td>
<td>35,064</td>
</tr>
<tr>
<td>Dose of 12, 60 g/kg BW</td>
<td>128,00 ±</td>
<td>165,20 ±</td>
<td>275,80 ±</td>
<td>121,80 ±</td>
</tr>
<tr>
<td></td>
<td>6,633</td>
<td>14,307</td>
<td>22,654</td>
<td>13,103</td>
</tr>
<tr>
<td>Dose of 15, 75 g/kg BW</td>
<td>132,60 ±</td>
<td>194,00 ±</td>
<td>425,80 ±</td>
<td>123,60 ±</td>
</tr>
<tr>
<td></td>
<td>22,300</td>
<td>24,352</td>
<td>5,450</td>
<td>13,390</td>
</tr>
<tr>
<td>Dose of 18,90 g/kg BW</td>
<td>133,60 ±</td>
<td>177,00 ±</td>
<td>201,26 ±</td>
<td>120,00 ±</td>
</tr>
<tr>
<td></td>
<td>14,258</td>
<td>9,028</td>
<td>8,228</td>
<td>21,760</td>
</tr>
<tr>
<td>Control (+)</td>
<td>153,10 ±</td>
<td>256,10 ±</td>
<td>196,10 ±</td>
<td>276,10 ±</td>
</tr>
<tr>
<td></td>
<td>27,318</td>
<td>101,471</td>
<td>35,557</td>
<td>109,452</td>
</tr>
</tbody>
</table>

Description: * In contrast significantly with the 0 day at p < 0.05

Picture 1. Graph profiles of rats’ bleeding time after being given the test material.

Time coagulation is a process of secondary haemostatic that is the continuing process of primary haemostatic. Secondary haemostatic is determined by a clotting factor and anti-coagulating.

Secondary haemostatic begins with activation of coagulation through the extrinsic and intrinsic pathways. Extrinsic pathway that is independent network and are bound to factor VII, leading to active factor VII (FVIIa) also activate factor X into factor Xa and together with factor V and PF3 form a complex protombinase. Besides activating factor X, factor VII also activate factor IXa in the intrinsic pathway. Protrombinase complex activates prothrombin to thrombin and thrombin will break down fibrinogen into fibrin. Fibrin will replace platelet plugs until the formation of the wound happened. Anti-platelet effects shown by the coagulation time which is getting longer after giving the test material.

Test materials are given in the preparation of tomato juice that has been steamed for 10 minutes until the outer skin peeled off. By doing this way, it is easier to get lycopene from the cells of fruit and it is also more soluble in the body so that the lycopene is more easily absorbed by the body. Tomato product which is processed by heating like tomato sauce apparently produces six times more lycopene compared with intact and fresh tomatoes (Silalahi, J., 2006).
Table 2. Rats’ coagulation time at testing the effects of platelet anti-aggregation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Coagulation Time (second)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>The day</td>
</tr>
<tr>
<td>Control (-)</td>
<td>58.20 ±</td>
</tr>
<tr>
<td></td>
<td>14.446</td>
</tr>
<tr>
<td>Dose of 12.60 g/kg BW</td>
<td>66.80 ±</td>
</tr>
<tr>
<td></td>
<td>5.805</td>
</tr>
<tr>
<td>Dose of 15.75 g/kg BW</td>
<td>70.20 ±</td>
</tr>
<tr>
<td></td>
<td>8.167</td>
</tr>
<tr>
<td>Dose of 18.90 g/kg BW</td>
<td>66.40 ±</td>
</tr>
<tr>
<td></td>
<td>12.122</td>
</tr>
<tr>
<td>Control (+)</td>
<td>77.40 ±</td>
</tr>
<tr>
<td></td>
<td>7.021</td>
</tr>
</tbody>
</table>

Description: * In contrast significantly with the 0 day at p < 0.05

Results obtained from this research are:

Time bleeding

On the 7th and the 14th day, each treatment dose gets a higher increase of bleeding time compared to day 0, the day before treatment (Table 1). On the 7th day the dose of 15, 75 g/kg BW have the same effect with asetosal 100 mg. On the 14th day, it has the same effect with asetosal 100 mg that is the dose of 18, 00 g/kg BW (Picture 1). On the 21st day, each treatment dose gets decrease bleeding time and after being statistically tested, the results didn’t differ significantly with the 0 day (Table 1).

Coagulation Time

On the 7th and the 14th day each treatment dose gets increase of coagulation time compared to the 0 day, that is day before treatment (Table 1). On the 7th day, the dose of 15, 75 g/kg BW have the same effect with asetosal 100 mg.

On the 14th day, it has the same effect as asetosal 100 mg, that is the dose of 12, 60 g/kg BW and 18, 90 g/kg BW (picture 2). On the 21st day, each treatment dose gets decreased coagulation time and after statistically tested, the results didn’t differ significantly with the 0 day (Table 2).

The Amount of Platelets

Normal range of human platelet counts ranged from 150-400x109 per liter. Platelet function was balance when the numbers at normal levels, but worsened when the platelet count goes down.
Table 3. Amount of The Rats’ platelets at testing the effects of platelet anti-aggregation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Amount of Rats' platelets (mm$^2$)</th>
<th>The 0 day</th>
<th>The 7th day</th>
<th>p</th>
<th>The 14th day</th>
<th>p</th>
<th>The 21st day</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (-)</td>
<td></td>
<td>293,400 ±</td>
<td>308200 ±</td>
<td>0.234</td>
<td>295000 ±</td>
<td>0.861</td>
<td>291200 ±</td>
<td>0.854</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18649,397</td>
<td>10039,821</td>
<td></td>
<td>15811,388</td>
<td></td>
<td>14042,792</td>
<td></td>
</tr>
<tr>
<td>Dose of 12.60 g/kg BW</td>
<td></td>
<td>267200 ±</td>
<td>224400 ±</td>
<td>0.018*</td>
<td>197800 ±</td>
<td>0.002*</td>
<td>230800 ±</td>
<td>0.083</td>
</tr>
<tr>
<td></td>
<td></td>
<td>24162,875</td>
<td>12116,105</td>
<td></td>
<td>13423,859</td>
<td></td>
<td>15401,299</td>
<td></td>
</tr>
<tr>
<td>Dose of 15.75 g/kg BW</td>
<td></td>
<td>210800 ±</td>
<td>220000 ±</td>
<td>0.005*</td>
<td>168800 ±</td>
<td>0.012*</td>
<td>232800 ±</td>
<td>0.399</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30182,782</td>
<td>22360,680</td>
<td></td>
<td>27188,233</td>
<td></td>
<td>39864,771</td>
<td></td>
</tr>
<tr>
<td>Dose of 18.90 g/kg BW</td>
<td></td>
<td>248000 ±</td>
<td>223600 ±</td>
<td>0.041*</td>
<td>210600 ±</td>
<td>0.042*</td>
<td>264000±</td>
<td>0.344</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33166,248</td>
<td>31761,612</td>
<td></td>
<td>22167,755</td>
<td></td>
<td>38000,000</td>
<td>*</td>
</tr>
<tr>
<td>Control (+)</td>
<td></td>
<td>293100 ±</td>
<td>238100 ±</td>
<td>0.002*</td>
<td>223600 ±</td>
<td>0.003*</td>
<td>212000 ±</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16118,313</td>
<td>19256,168</td>
<td></td>
<td>27173,621</td>
<td></td>
<td>22934,690</td>
<td>*</td>
</tr>
</tbody>
</table>

Description: * In contrast significantly with the 0 day at p < 0.05

On the 7th and the 14th day each treatment dose decreased the amount of platelets compared to the 0 day that is the day before treatment (Table 3). On the 7th day the entire dose has the same effect with asetosal 100 mg.

On the 14th day, it has the same effect as 100 mg, that is the dose of 12, 60 g/kg BW and 18, 90 g/kg BW (picture 3). On the 21st day, each treatment dose gets the increasement of coagulation time and after statistically tested, the results didn’t differ significantly with the 0 day (Table 3).

CONCLUSION

Test results show that the effective dose of tomato juice as an anti-platelet aggregation is the dose of 15, 75 g/kg BW on the 7th day. This is evidenced by test results that showed increasement of bleeding time, coagulation time and decreased platelet count, that caused not so many platelets aggregate.

BIBLIOGRAPHY


DOCKING STUDIES RHINACANTHIN DERIVATIVES AS A POTENTIAL INHIBITOR OF POLO-LIKE KINASE 1 (Plk1)

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ABSTRACT

Rhinacanthins are naphthoquinone ester derivatives isolated from the methanolic extract of the roots of the medicinal plant *Rhinacanthus nasutus* (Acanthaceae). Some of these compounds have been reported to exhibit cytotoxicity against P388, A-549, HT-29, and HL-60 cancer cell lines. Polo-like kinase 1 (Plk1) is overexpressed in many types of human cancers, and has been implicated as an adverse prognostic marker for cancer patients. Plk1 localizes to its intracellular anchoring sites via its polo-box domain (PBD). The PBD of Plk1 has a crucial role in proper subcellular localization and mitotic functions of Plk1. In this work, we studied rhinacanthins-M, -N, -Q and 39 novel naphthoquinone esters to carry out PBD of Plk1 inhibitor binding interactions, free energy binding and estimated Ki values using docking method. The Docking study was performed using Molecular Operating Environment (MOE 2008.10) software. The results suggested that free binding energies and Ki value of rhinacanthin derivatives were lower than the parent compound’s. Low value of ∆Gbind indicated the strong favorable bond between enzyme and ligand and parallel with the Ki values. It could be estimated that the reaction equilibrium shifted to the complex formation. In general, all calculated Ki values were relatively low (within micromolar range), indicating the formation enzyme-ligand complexes is stable. Every ligands showed reasonably low internal energy. It indicates that the docked conformation of the ligands were in their most favourable conformations. The result was predicted docking energy and residu contact which reflected the increased binding affinities.

Key words: (Rhinacanthin derivatives, Plk1, inhibitor, docking)

INTRODUCTION

The serine/threonine kinase Polo-like kinase 1 (Plk1) is overexpressed in many type of human cancers, and has been implicated as an adverse prognostic marker for cancer patients. Plk1 has been studied most extensively because of its ability to override cellular checkpoints and induce genetic instability, leading to oncogenic transformation of human cells [1,2,3]. Plk1 localizes to its intracellular anchoring sites via its polo-box domain (PBD). The PBD of Plk1 has a crucial role in proper subcellular localization and mitotic functions of Plk1. Reind et al. (2008) had studied that Plk1 can be inhibited by small molecules which interfere with its intracellular localization by inhibiting the function of the PBD [4].

Naphthoquinone derivatives congeners were chosen to be interesting candidates for possible therapeutic agents primarily in the field of cancer chemotherapy due to their cytotoxic activities data against various cancer cells.

Rhinacanthins are naphthoquinone ester derivatives isolated from the methanolic extract of the roots of the medicinal plant *Rhinacanthus nasutus* (Acanthaceae). Some of these compounds have been reported to exhibit cytotoxicity against P388, A-549, HT-29, and HL-60 cell lines. In Thailand, the roots and leaves of *R. nasutus* are used for the treatment of cancer. Kongkathipet. Al (2004) have been synthesized rhinacanthin-M, -N, -Q and 39 related naphthoquinone esters, together with their cytotoxicities against human carcinoma cell lines, KB (oral human epidermoid carcinoma), HeLa (human cervical carcinoma), and HepG2 (human hepatocellular carcinoma). They also conducted preliminary studies on the mode of action of some of these naphthoquinone esters by computer modeling using Autodock version 3.05.
In this study, we carried out docking simulation of rhinachanthin derivatives to demonstrate inhibitory interaction activities toward Plk1 in silico (Figure 1). The binding interactions between these inhibitors and Plk1 were studied by molecular docking methods using MOE software [5].

The aims of this study is to get drug ligand that could inhibit interaction of Plk1, and to understand the interactions with in the inhibitor and the enzyme’s binding sites via computational docking methods. It is expected that result from this study will provide information about the design development of anticancer drugs.

Fig 1. Structure of Rhinacanthin M, N, Q and 39 related naphthoquinone esters

**METHODOLOGY**

Protein structure
Three dimensional (3D) structure of the polo-like kinase 1 complex was retrieved from RCSB (Research Collaboratory for Structural Bioinformatics)[6]. The 3D structure was feed to the MOE software with pdb file format.

**Building ligand structures**

In this study, 42 compounds were obtained from Kongkathip (2004). The ligands consist of Rhinanchanthin and naphthoquinone esters (Fig.1). The ligand structures were built and minimized optimization by using ChemBioDraw Ultra 12.0. Amino acid residues have been chosen from template box in database menu to draw the two dimensional structure of the ligands. The output is MDL Molfile. And then, the output format was converted to MDL Mol format with VEGA ZZ software [7].

**Docking**

Docking files were prepared by using MOE 2008.10 software. In the MOE software, the default non-stochastic Triangle Matcher placement method, followed by molecular mechanics refinement and London dG scoring, GBVI, and no return poses 1000, was operated for the docking runs [8,9]. The default of MOE parameters was used for the other parameters in docking simulation.

**RESULTS AND DISCUSSIONS**

Crystal structures of the Plk1 PBD in complex with artificial phosphopeptides optimized for PBD binding have revealed that the PB1 and PB2 motifs have identical βα folds and form a heterodimericphosphopeptide binding module. The phosphopeptide binds to a cleft formed between PB1 (residues 411-489), PB2 (residues 511-592) and interacts with key amino acid residues from both polo boxes, His 538 and Lys 540 from PB2 are pivotal for electrostatic interactions with the negatively charged phosphate group of phosphorylated serine or threonine residues, whereas Trp 414 from PB1 is crucial for the selection of serine. Molecular surface of the Plk1 crystal structure had the hydrophobic surface and hydrogen bonds (Fig. 2). Hydrophobic is represented in green, hydrogen bond in pink, and polar interaction is in blue.

![Figure 2](image-url) Crystal structures of the Plk1 PBD.

**Parameters Docking**

The protein structure was prepared for docking as described previously. This included the addition of missing hydrogens. During docking, series of poses (ligand-protein complexes) were generated for each molecule. The quality of each pose was further assessed by the London dG (LdG) scoring function (SF) which estimated the binding free energy of the ligand and a set of the highest score poses were chosen for each molecule (pose) docked [10,11]. In this study, MMFF94 and MMFF94x were used for forcefield minimization. MMFF94x was reported as the efficient forcefield for minimizing ligand-protein complexes [12,13], furthermore, the MMFF94 variant ensures planar geometry of the peptide bonds [14]. Triangle Matcher [15,16] was applied to orient the ligands in the active site based on charge groups and spatial fit. Triangle Matcher performs a random walk with the ligand in the active site to define the optimal binding orientations. Docking simulations carried out on

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The pocket, which is composed of Trp 414, Arg 516, His 538, and Lys 540 residues. This hydrophobic pocket give contribution to the interaction of Plk1 PBD [1]. Based on docking simulation result, naphthoquinone derivatives, could be proposed as a potential inhibitor to the interaction of Plk1 PBD 8-{[2-methoxy-5-(4-methylpiperazin-1-yl)phenyl]amino}-1-methyl-4,5-dihydro-1H-pyrazolo[4,3-h]quinazoline-3-carboxamide (071), which became as comparative ligands, have already designed earlier by other research group.

**Estimated docking energy**

The calculated free binding energy (ΔGbind) of flexible-ligand docking simulation from the best ligand is shown in Table 1. Free energy of binding for all the designed ligands were lower than the comparative ligand. The free energy of all the designed ligands was negative value. The negative and low value of ΔGbind indicated the strong favorable bond between enzyme and ligand.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Estimated ΔG (kcal/mol)</th>
<th>Estimated Ki (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>071</td>
<td>-9.1434</td>
<td>0.578</td>
</tr>
<tr>
<td>Rhinacanthin-M</td>
<td>-16.4709</td>
<td>6.53x10^-7</td>
</tr>
<tr>
<td>Rhinacanthin-N</td>
<td>-15.9464</td>
<td>9.84x10^-8</td>
</tr>
<tr>
<td>Rhinacanthin-Q</td>
<td>-15.1760</td>
<td>1.44x10^-5</td>
</tr>
<tr>
<td>(13)</td>
<td>-19.7035</td>
<td>1.63x10^-7</td>
</tr>
<tr>
<td>(4)</td>
<td>-18.4780</td>
<td>1.01x10^-6</td>
</tr>
<tr>
<td>(25)</td>
<td>-18.2865</td>
<td>1.39x10^-7</td>
</tr>
</tbody>
</table>

These ΔGbind values are parallel to the Ki values observed in Table 1. All the designed ligands showed the lowest Ki value, it could be estimated that the reaction equilibrium shifted to the complex formation. In general, all calculated Ki values were small (within micromolar range), indicating the formation of stable enzyme-ligand complexes. Every ligands showed reasonably low internal energy. It indicates that the docked conformation of the ligands were in their most favourable conformations. In this docking simulation, we utilized gas phase solvation.

Figure 3. Interaction plots illustrating between ligand and the respective amino acid residues, rhinacanthins-M (a), rhinacanthin–N (b), rhinacanthin–Q(c), 071(d).
Binding interaction between enzyme and ligand

We presented residue contact of naphthoquinone derivatives ligands. All ligands have residue contact with Trp 414, His 538, and Lys 540 residues.

If we compare all of the ligand, compound [13] are the best ligand. Hopefully, this ligand can block the interaction of Plk1 PBD. In Fig. 3 and Fig. 4, we presented the ligand-receptor interaction diagrams. This is a depiction of relatively strong connections among hydrogen bonds as well as electrostatic or charge-transfer interaction between a ligand and the protein residue.

CONCLUSION

Rhinacanthin M, N, Q, naphthoquinone esters (13,14,25) could be proposed as a potential inhibitor of Plk1 PBD.

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INTERACTION BETWEEN BETAMETHASONE-17-VALERATE AND NEOMYCIN SULFATE

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ABSTRACT

The investigation of interactions between betamethasone-17-valerate (BV) and neomycin sulfate (NS) was carried out by various methods. This study provides an attempt to observe the BV-NS physical interactions with and without heating. The temperature-composition phase diagram of the BV-NS binary system was achieved using differential scanning calorimetry (DSC). Contact method of solutions was used to detect the possibility of physical interaction of these drug combinations. The mixture containing 1:1 mol fraction mixture of the BV-NS was prepared by solution crystallization and solvent drop grinding method and characterized by DSC, powder x-ray diffractometry (PXRD), fourier transform infra red (FTIR), scanning electron microscopy (SEM), solubility- and dissolution-test methods. Binary phase of diagram shows no interaction between both substances. Observation under polarization microscope of contact method of both solutions, NS amorphous solids always has been closed to the BV crystals. SEM photomicrograph of BV-NS (1:1) that prepared by solvent drop grinding for 15 and 30 min showed changes crystals habit of BV from prismatic became irregular with smaller size. Solubility and dissolution rate of BV increased from BV-NS (1:1) that prepared by solvent drop grinding. The changes of solid state characteristics of BV and NS in the mixture that investigated by DSC, XRD. SEM indicate an improvement of physical properties of the BV.

Keywords: Interaction, betamethasone-17-valerate, neomycin sulfate, DSC, XRD, SEM

INTRODUCTION

Drugs combination in pharmaceutical dosage form could lead to a physical interaction or interaction intermolecular. Such interaction brings consequences not only limited to the difficulty during processing into the dosage form, but also in many cases can influence the efficacy of that combination of drugs. In many years, research on the drug-drug or drug-excipient interaction were carried out intensively in order to providing an effective and safe drug (Sundani, et al., 2008; Sundani, et al., 2007; Bettinetti, et al, 2000; Li and Mroso, 1984). Drug interactions can change the way our medications act in the body. Most drug interactions are taken place either through pharmacodynamic or pharmacokinetic in nature. The effect may be an increase or a decrease in the action of each substance, or it may be an adverse effect that is not normally associated with each drug.

![Fig. 1 The chemical structure of betamethasone-17-valerate (a) and neomycin sulfate (b)](a) (b)
In pharmaceutical sciences, the most common interaction is eutectic mixture. Eutectic is a mixture of chemical compounds or elements that has a lower melting point than any of its single pure component. Normally, the eutectic formation cause increasing of moisture in the powder mixture and very rare followed by changing of biological effect. Such condition can be easily overcome by addition some amounts of adsorben. Whilst, there are some interaction that almost similar with eutectic phenomenon be able to influence the efficacy, even toxicity of drugs combination. In general, there are two types of physical interactions or intermolecular interactions, namely: 1) conglomerate without mixed crystal formation and 2) conglomerate with mixed crystal formation. All of them showed almost similar phenomenon, that is melting point decreasing.

Corticosteroids are frequently used as topically drugs. Despite of their demonstrated effectiveness for the treatment of psoriasis and atopic dermatitis, topical corticosteroids are associated with various side effects that may limit their use (Del Rosso and Friedlander, 2000; Wiedersberg et al., 2008). Betamethasone-17-valerate (BV) (Fig. 1a) is the gold standard of these agents and serves as a reference in the clinical studies for the registration of new glucocorticosteroids (Sivaramakrishnan et al., 2004). It is a medium potency glucocorticoid, lacking mineralocorticoid properties, currently available in topical dosage forms: cream, ointment, lotion and foam all with a strength of 0.1% (w/w) (expressed as betamethasone base) (Franz et al., 1999). A number of antibacterial/steroid topical combinations are commercially available. Betamethasone-17-valerate is often combined with antibacterials, such as neomycin sulfate (NS) (Fig 1b). This combined drugs mainly used as topical anti infection, in intestinal inflammation and infected eczema with secondary infection.

The treatment failure of combination between BV and NS was predicted, caused by drug-drug physical interaction. The easiest method to observe drug-drug physical interaction is thermal contact method by hot stage microscope (HSM). This method requires drugs should be stable before, during and after its melting point. BV does not degraded, but NS degraded before its melting point. In this case, thermal contact method cannot be used. But, some methods are available for identifying of solid state drug, such as DSC, PXRD, FTIR, SEM, solubility- and dissolution-test. Those methods are also expected to be used to identify the changes the solid state drug due to drug-drug physical interaction.

The aim of this research was to determine the type of physical or intermolecular interaction between BV-NS and physical properties changed of either BV or NS.

**METHODOLOGY**

**Material**

Betamethasone-17-valerate (BV) and neomycin sulfate (NS) commercial material with purity of >99% were purchased from PT. Kimia Farma, tbk, Indonesia. Methanol, ethanol, and other reagents were obtained from E-Merck without any purification.

**Methods**

**Binary phase of diagram**

The samples containing physical mixture of BV and NS in different weight ratio (10:0, 9:1, 7:3, 1:1, 3:7, 1:9, and 0:10) were prepared and measured by Perkin-Elmer DSC-6 differential scanning calorimeter. Endothermic peak (melting events) of interaction was plotted against the weight ratio of the mixture.

**Contact method of solution**

A drop of BV solution in methanol was placed on object glass and covered, then allowed to recrystallize. One droplet of NS solution in methanol was placed at the other side of cover glass. NS solution left in contact with the crystal BV. Optical microphotographs of crystal were taken under polarizing microscope (Olympus BX-51). The microscopic images were recorded with a Olympus SC-30 digital color camera attached to the Olympus BX-51 microscope.
Preparation of BV-NS (1:1) by solution crystallization

A 1:1 mol fraction mixture of BV (95.31 mg, 0.2 mmol) and NS (142.54 mg, 0.2 mmol) was added to 20 ml of ethanol 70% in a 25 ml conical flask and gently heated. The solution was allowed to evaporate slowly at the ambient conditions. The predicted BV–NS crystals were then scaled up to 2 g for further analysis.

Preparation of BV-NS (1:1) by solvent drop grinding experiments

A 1:1 mol fraction mixture of BV (95.3 mg, 0.2 mmol) and NS (142.5 mg, 0.2 mmol) were placed in a mortar and five drop of methanol was added. The mixture was grinded for 15 and 30 min in Retsch RM 100 mortar grinder using a fresh batch for each grinding period.

Powder X-ray diffraction (PXRD)

PXRD data were collected with Shimadzu XRD-7000 X-ray powder diffractometer. The sample was scanned within the scan range of 2θ = 5° to 35° continuous scan, at a scan rate of 2°/min.

Thermal Analysis by DSC

Differential scanning calorimetry (DSC) was performed with a Perkin Elmer DSC-6. 2-5 mg of each crystal sample was placed in crimped sample pan. The sample was heated from 30 to 350°C at a heating rate of 10°C/min. The samples were purged with a stream of flowing nitrogen at 20 mL/min.

Fourier Transform Infra Red (FTIR)

The FTIR spectra of each sample were recorded on Perkin-Elmer FTIR.

Scanning Electron Microscopy

The electron microscopy measurements were performed at a Carl Zeiss scanning electron microscope (SEM).

Determination of BV solubility

The solubility of BV in water were determined at room temperature using an orbital shaker on each sample. Excess amounts of compound were added to 5 mL of the media, mix it continuously and then filtered after 48 h of equilibration. The bulk solutions were measured spectrophotometrically using Shimadzu 1601-PC spectrophotometer at 242 nm. The calibration curve for BV (y=0.0327+0.003 was linear from 1.31 to 20.00 µg/ml (r=0.9999). The experiments were carried out in triplicate.

In vitro dissolution of BV

Dissolution test of BV were carried out in water (900 ml, 37±0.5°C, 100 rpm) for 90 min using the USP XXIII paddle apparatus (ZRS-6G, Tianjin, China). At predetermined time intervals, 5 ml samples were withdrawn and spectrophotometrically assayed (Shimadzu 1601-PC spectrophotometer) for drug concentration at 242 nm.

RESULTS AND DISCUSSION

Thermoanalysis offers significant advantages in saving both time and the number of substance as well. In principle, makes it possible to detect compatibility/incompatibility directly on physical mixture, avoiding the time consuming step of the annealing of the mixtures under stress conditions (Brown et al, 1999; Jacobson and Reier, 1969). Based on the melting points of BV–NS physical mixture containing various weight ratios of the components, phase diagram have been constructed (Fig. 2). The melting point of pure BV was 192.6°C, while NS was 221.4°C followed by decomposition. DSC-curve showed two endothermic events for each composition. The melting poin of BV fixed in each weight ratio. Binary phase of diagram shows no interaction between both substances.

Fig. 3 present optical microscope photos of crystals with larger size and long needle shape of BV (3A) and amorphous state of NS (3B). Fig. 3C shows photo after contact between BV crystal and NS solution. NS is always in amorphous state, diffuse form and always been cover center of several BV crystals. There was no a new habit formed. This photo indicates an interaction-like between BV and NS.

The X-ray diffraction patterns of pure BV, NS, and the mixture of B:V (1:1) that prepared by solution crystallization and solvent drop grinding are offset in fig 4. The PXRD-spectra patterns exhibit hollow form of the pure NS (amorphous solid). All mixtures shows the two highest intensities of characteristic interferences of BV, i.e. at 14.1° and 17.1° 2θ. The mixture of B:V (1:1) that prepared by solvent drop grinding for 30 min has the lowest relative intensity for both characteristic interferences of BV.
Fig. 2. Binary phase diagram of physical mixture of BV and NS

Fig. 3. Polarized microscope photos of BV (A), NS (B), and result of contact method solution between BV and NS (C) recrystallized in methanol with magnification 200x.

Fig. 4. PXRD spectra intensity (arbitrary units) as a function of 2θ of BV (A), NS (B), (B:V=1:1) after solution crystallization (C), (B:V=1:1) after solvent drop grinding for 15 min (D), and 30 min (E)

DSC-curve of pure BV, NS, and the mixture of B:V (1:1) that prepared by solution crystallization and solvent drop grinding are shown in fig 5. A broad endotherm peak at around 100 °C after crystallization and grinding process, indicates of water losses. That condition due to the extremely hygroscopic properties of pure NS, after crystallized from solution and grinded. The longer time of grinding, the larger the endothermic peak area around 100°C. The melting point of BV at 192.6°C decreases in the mixture of B:V (1:1) that prepared by solution crystallization and solvent drop grinding. This is agreement with relative intensity obtained with XRD. Thus, preparation of mixture B:V (1:1) by solution crystallization and solvent drop grinding changed physical state of either components.
Fig. 5 DSC-thermogram of BV (A), NS (B), (B:V=1:1) after solution crystallization (C), (B:V=1:1) after solvent drop grinding for 15 min (D), and 30 min (E).

Fig. 6 Scanning electron micrographs of BV (A), NS (B), (B:V=1:1) after solution crystallization (C), (B:V=1:1) after solvent drop grinding for 15 min (D), and 30 min (E).
Fig. 7 FTIR spectra of BV (A), NS (B), (B:V=1:1) after solution crystallization (C), (B:V=1:1) after solvent drop grinding for 15 min (D), and 30 min (E)

Fig. 7 shows FTIR spectra of pure BV, NS, the mixture of B:V (1:1) that prepared by solution crystallization and solvent drop grinding. There was no new peak seen in spectra of the mixture of B:V (1:1) that prepared by solution crystallization and solvent drop grinding.

Solubility experiments showed that solubility of BV in water increased in the mixture of B:V (1:1) that prepared by solvent drop grinding. There was significant increasing of solubility of BV in the mixture of B:V (1:1) that prepared solvent drop grinding. The existence of particles of NS around the BV crystals causes BV easily wetted, so that its solubility increases. This is agreement with XRD results. This result indicate an interaction between BV and NS.

**Fig. 6** shows representative SEM pictures of some of the samples. The morphology of the particles showed prismatic shape of BV and irregular shape of NS. In the mixture of B:V (1:1) that prepared solvent drop grinding for 15 and 30 min, the habit of BV crystals become irregular and smaller.

**Fig. 8** shows profile dissolution of pure BV(-♦-), physical mixture of BV:NS (1:1) (-■-), (B:V=1:1) after solution crystallization (-▲-), (B:V=1:1) after solvent drop grinding for 15 min (-x-), and 30 min (-●-).
Tabel 1. Solubility of betamethasone-17-valerate

<table>
<thead>
<tr>
<th>Material</th>
<th>Solubility (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betamethasone-17-valerate</td>
<td>13.11±0.33</td>
</tr>
<tr>
<td>B:N (1:1) solution crystallization</td>
<td>10.78±0.47</td>
</tr>
<tr>
<td>B:N (1:1) solvent drop grinding for 15 minutes</td>
<td>29.94±0.97</td>
</tr>
<tr>
<td>B:N (1:1) solvent drop grinding for 30 minutes</td>
<td>56.77±1.11</td>
</tr>
</tbody>
</table>

The profile dissolution of pure BV and the mixture and the mixture of B:V (1:1) that prepared by solution crystallization and solvent drop grinding are showed in fig 9. The dissolution percentage of BV from the mixture of B:V (1:1) that prepared solvent drop grinding increased. There was no significant difference in dissolution profile between pure BV, physical mixture of B:V (1:1) and that prepared by solution crystallization. The dissolution percentage at 90 min ($DP_{90}$ min) of the mixture of B:V (1:1) that prepared by solvent drop grinding for 30 min was 43.50% higher than others.

**CONCLUSION**

The mixture of BV:NS (1:1) that prepared by solvent drop grinding showed an interaction between BV and NS. Solubility and dissolution rate of BV increased from BV-NS (1:1) that prepared by solvent drop grinding. The changes of solid state characteristics of BV and NS in the mixture that investigated by contact method of solutions, DSC, PXRD, SEM, solubility- and dissolution-test indicate an interaction between the BV-NS.

**REFERENCES**


HEPATOPROTECTIVE EFFECT OF WARU (HIBISCUS TILIACEUS) LEAVES INFUSION IN PARACETAMOL INDUCED HEPATOTOXIC RATS

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ABSTRACT

Paracetamol is one of hepatotoxic agent which cause centrilobular necrosis. The aim of this study was to identify the fitochemistry compounds and to investigate the hepatoprotective effect of infusion from Waru (Hibiscus tiliaceus) leaves in paracetamol induced hepatotoxic rats based on ALT-AST serum levels and liver histopathology examination. Twenty five male Wistar rats, age 2-3 months and weight 150-200 gram each, were randomly divided into 5 groups. Group I (control) was given toxic dose paracetamol (2000 mg/kg of body weight) orally, Group II (placebo) was given aquadest, whereas group III, IV, V were given H. tiliaceus leaves infuse daily 250, 500 and 1000 mg/kg of body weight orally, for six days. Paracetamol was given on the 6th day, half hour after administration of H. tiliaceus leaves infuse. Blood samples were collected for determining the ALT-AST serum levels. The right lobe of livers were cut off and soaked into formalin 10% for microscopic examination. The results showed that flavonoids, polyphenols and saponins had been identified in H. tiliaceus leaves infuse. The degree of liver cells necrosis caused by toxic dose of paracetamol reduced relatively significant in the treatment groups which given H. tiliaceus leaves infuse. In conclusion, Waru (Hibiscus tiliaceus) leaves infusion at dose 500-1000 mg/kg of body weight decreased hepatotoxicity of paracetamol.

Key words: hepatoprotective, Hibiscus tiliaceus, paracetamol

INTRODUCTION

Liver as the vital organ that has main function in the chemical substance metabolism. Liver may change the chemical structure of lipophilic element to become the hydrophilic to be excreted through urine and bile. If the regeneration ability of liver cells has been lost or reserved power has less than liver resistance, it can cause the liver damage permanently that is caused by the virus, bacteria or alcohol and medicines consumption.

The medical therapy to the liver disease like cirrhosis, fat degeneration or chronic hepatitis still cause the problems because of the greater risk of side effect than its advantages (Luper, 1998). One of the drug toxicity is hepatotoxicity that is the condition that describes the liver damage caused by the drugs usages.

The paracetamol usage as an analgesic-antipyretic within the excess doses in long duration can cause liver cells damage consistently because paracetamol metabolism in liver become reactive metabolit N-acetyl-p-benzoquinon imine (NAPQI) as hepatotoxic (Vandenberghe, 1996). Parkinson (2001) explains that the formation of paracetamol toxic metabolites by cytochrome P-450 cause centrilobular necrosis in liver.

Patients with liver disease requires an effective treatment, the minimum risk of side effects or toxicity, and drug prices that more affordable because of the longer treatment. The use of herbal (plants) as a Complementary and Alternative Medicine (CAM) beside of modern medicine for curing the liver inflammation based on the pathogenesis as lipid lowering agent, antioxidant, antiinflammation, immunomodulator-imunostimulansia, liver cell regeneration agents, bile secretion and hypocholesterole (Lelosutan, 2008).

Narender et al. (2009) reported that one of the plants whose antiinflammation and antitoxic effects is Waru tree (Hibiscus tiliaceus). Hibiscus tiliaceus leaves have the highest antioxidant activity
compared to other *Hibiscus* species. Empirically *Hibiscus tiliaceus* leaves has efficacy for reducing inflammation, expectorants, antitoxics, and diuretics. Chan *et al.* (2001) have isolated chemical compounds in the stem bark of *Hibiscus tiliaceus* as scopoletin, hibiscusin, vanilic acid, P-hydroxybenzoic acid, syringic, a mixture of β-sitosterol and stigmasterol, that was reported having anticancer activity in P-388 cells and HT-29 cells in vitro with IC<sub>50</sub> values lower than 4 g/ml.

*Hibiscus tiliaceus* leaves contain chemicals compounds such as saponins, flavonoids, and phenolic compounds whose effects as antioxidant, but has not been widely explored as hepatoprotector. Therefore, it is necessary testing to determine the hepatoprotective effects of *Hibiscus tiliaceus* leaves infusion in high dose paracetamol induced hepatotoxic rats by measuring ALT-AST serum levels, liver histopathology observation, and also identifying the chemical compounds of *Hibiscus tiliaceus* leaves.

**METHODOLOGY**

**Preparation of *Hibiscus tiliaceus* leaves infusion**

A total of 4 grams of *Hibiscus tiliaceus* leaves powder was added with 100 ml of water and an additional of extra water 2 times of the material weight (8 ml) in a special pan to make infusion. Then heated over a water bath for 15 minutes, calculated from the temperature in the pot at 90° C, and stirring occasionally. Infusion was filtrated while still hot. To fulfill the water shortage, add boiling water through the residu to obtain the desired volume (DepKes, 1995).

**Identification of the chemical compounds**

Qualitative testing of the chemical compounds in *Hibiscus tiliaceus* leaves infusion done with chemical reagents for flavonoids, polyphenols and saponins (Harborne, 1996).

**Experimental design**

In this experiment, a total of 25 rats were used and divided randomly into 5 groups of 5 rats each as follows:

Group I were induced high dose paracetamol orally (2 g kg<sup>-1</sup> bodyweight). Group II were normal rats treated with distilled water orally (2.5 ml once daily). Group III, IV, V were treated with Hibiscus leaves infusion orally (250, 500 and 1000 mg kg<sup>-1</sup> bodyweight) once a day and then were induced paracetamol (2 g kg<sup>-1</sup> bodyweight) on the 6<sup>th</sup> day.

**Blood sampling and analysis**

The blood sample is taken through the sinus orbitalis on the 8<sup>th</sup> day (48 hours after all of the treatment stopped), then conducted a number of reaction with the reagen (Diasys, Germany) to estimated the ALT-AST serum levels by using UV spectrophotometer.

**Liver histopathological assessment**

After making liver tissue preparat and staining permanent preparat with Haematoxylin-Eosin, next liver preparat were observed under the light microscope. Liver tissues *that was observed were* the central vein, the structure of liver cells, nucleus and sinusoid. Observation of the liver tissue is performed directly and expressed descriptively.

**RESULTS AND DISCUSSIONS**

The chemical reaction to determine the type of chemical compounds of *Hibiscus tiliaceus* leaves infusion whose potential effect as hepatoprotective had given positive result for qualitative test of flavonoids, polifenols, and saponins. According to Kumar *et al.* (2008), *Hibiscus tiliaceus* leaves contain the chemical compounds of saponins and flavonoids, also have at least five phenolic compounds.

According to Yuningsih research (2003), the ALT and AST serum levels was estimated 48 hours after the treatment stopped, because at the time paracetamol elevated the serum ALT-AST maximally. The results (Table I) showed that the treatment of *Hibiscus tiliaceus* leaves infusion with lower doses (250 mg kg<sup>-1</sup> bodyweight) had the highest activity in reducing ALT-serum (58.36%) and only reducing 28.58% AST-serum levels. The treatment with *Hibiscus tiliaceus* leaves infusion at dose 500 mg kg<sup>-1</sup> bodyweight (Table II) reduced 56.22% ALT-serum and 32% of AST. The treatment of *Hibiscus tiliaceus*
leaves infusion at the highest doses (1000 mg kg\(^{-1}\) bodyweight) just little reduced ALT-serum (7.40\%) but occured the highest activity in reducing AST-serum levels (47.62\%).

The results of liver histopathologic examination showed changes at the cellular level (Fig 1). Table III showed that groups were induced single dose paracetamol orally (2 g kg\(^{-1}\) bodyweight) observed necrosis. Most of the normal liver cells are showed in the normal groups and pre-treatment groups with *Hibiscus tiliaceus* leaves infusion at dose 500-1000 mg kg\(^{-1}\) bodyweight, but at lower dose infusion (250 mg kg\(^{-1}\) bodyweight) was occured congestion and vacuolar degeneration.

Table I. ALT serum levels in the pre-treatment of Hibiscus leaves infusion compared with paracetamol induced (2 g kg\(^{-1}\) bodyweight) and normal groups (2.5 ml distilled water)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>ALT serum levels</th>
<th>% difference of (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(X \pm SE) U/l</td>
<td>I</td>
</tr>
<tr>
<td>I</td>
<td>paracetamol induced</td>
<td>62.82 ± 6.32</td>
<td>+ 105.70(^s)</td>
</tr>
<tr>
<td>II</td>
<td>normal groups</td>
<td>30.54 ± 5.12</td>
<td>- 51.38(^s)</td>
</tr>
<tr>
<td>III</td>
<td>250 mg kg(^{-1}) BW infusion</td>
<td>26.16 ± 5.46</td>
<td>- 58.36(^s) - 14.34(^ns)</td>
</tr>
<tr>
<td>IV</td>
<td>500 mg kg(^{-1}) BW infusion</td>
<td>27.50 ± 4.59</td>
<td>- 56.22(^s) - 9.95(^ns)</td>
</tr>
<tr>
<td>V</td>
<td>1000 mg kg(^{-1}) BW infusion</td>
<td>58.17 ± 10.46</td>
<td>- 7.40(^ns) + 90.47(^ns)</td>
</tr>
</tbody>
</table>

\(^a\) ns = not significant differences (p>0.05); \(s\) = significant differences (p<0.05)

CONCLUSION

*Hibiscus tiliaceus* leaves infusion contains flavonoids, polyphenols and saponins compounds. *Hibiscus tiliaceus* leaves infusion at dosing interval 500-1000 mg kg\(^{-1}\) bodyweight showed the hepatoprotective effect in single dose paracetamol induced hepatotoxic rats.

Figure 1. Liver histopathology observation (400x microscope magnification).
Table II. AST serum levels in the pre-treatment of Hibiscus leaves infusion compared with paracetamol induced (2 g kg\(^{-1}\) bodyweight) and normal groups (2.5 ml distilled water)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>AST serum levels</th>
<th>% difference of ( a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>X ± SE (U/l)</td>
<td>I</td>
</tr>
<tr>
<td>I</td>
<td>paracetamol induced</td>
<td>102 ± 8,20</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>normal groups</td>
<td>76.34 ± 13.95</td>
<td>-25.16(^{ns})</td>
</tr>
<tr>
<td>III</td>
<td>250 mg kg(^{-1}) BW infusion</td>
<td>72.85 ± 6.74</td>
<td>-28.58(^{ns})</td>
</tr>
<tr>
<td>IV</td>
<td>500 mg kg(^{-1}) BW infusion</td>
<td>69.36 ± 3.44</td>
<td>-32(^{ns})</td>
</tr>
<tr>
<td>V</td>
<td>1000 mg kg(^{-1}) BW infusion</td>
<td>53.43 ± 10.74</td>
<td>-47.62(^{ns})</td>
</tr>
</tbody>
</table>

\( a \) ns = not significant differences (p>0.05); s = significant differences (p<0.05)

Table III. Liver histopathological assessment in the pre-treatment of Hibiscus leaves infusion compared with paracetamol induced (2 g kg\(^{-1}\) bodyweight) and normal groups (2.5 ml distilled water)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatment</th>
<th>Level of Liver Damage (% animals subject)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>normal</td>
</tr>
<tr>
<td>I</td>
<td>paracetamol induced</td>
<td>67</td>
</tr>
<tr>
<td>II</td>
<td>normal groups</td>
<td>67</td>
</tr>
<tr>
<td>III</td>
<td>250 mg kg(^{-1}) BW infusion</td>
<td>67</td>
</tr>
<tr>
<td>IV</td>
<td>500 mg kg(^{-1}) BW infusion</td>
<td>67</td>
</tr>
<tr>
<td>V</td>
<td>1000 mg kg(^{-1}) BW infusion</td>
<td>67</td>
</tr>
</tbody>
</table>

REFERENCES


ISOLATION OF ANTIBACTERIAL COMPOUND FROM MARINE SPONGE *Stylissa carteri* AGAINST GINGER BACTERIAL PATHOGEN (*Ralstonia solanacearum*)

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**ABSTRACT**

Chemical investigation of marine sponge *Stylissa carteri* from West Sumatera Indonesia has been performed. This study was aimed to isolate and test its antibacterial activity against ginger bacterial pathogen (*Ralstonia solanacearum*). Isolation procedure was performed by using chromatography method. Antibacterial activity of the isolate was tested against *Ralstonia solanacearum* by using agar diffusion method. This study yielded two isolated compound that are T and N compounds. Both of them showed Minimum Inhibitory Concentration (MIC) of 0.1% against *Ralstonia solanacearum*.

**Key words:** *Stylissa carteri*, *Ralstonia solanacearum*, Antibacterial, Marine sponge, Isolation

**INTRODUCTION**

Indonesia as the world’s largest archipelagic country has a vast sea territory. Containing marine biodiversity such as plants, animals, and other marine biota. Marine sponge is one of the marine life that contains the most bioactive and interesting chemical compounds. Sponges (phylum Porifera) are the multicellular animals (metazoa) that represent an important constitutive group of the coral reef fauna with a widerange of species and are considered as interesting target to screen for antimicrobial substances for many reasons. In fact these animals are frequently exposed to intense predation and tissue infection by microorganisms. Despite being sessile and soft-bodied, sponges appear to be predated only by selected groups of marine animals, such as several fishes, turtles, sea urchins and sea stars, some nudibranches, some kinds of gastropoda and flat worms (Touati, 2007). Sponges compete for space on the substrate with other sessile organisms such as corals and algae. This mechanism is not well-known but supposed to be chemical (Van Soest, *et al.*, 2006). They are known to produce a large number and diversity of bioactive secondary metabolites. Until now, more than 5000 different compounds have been isolated from about 500 species of sponges (Rivai, *et al.* 2005). Up to 800 antibiotic have been isolated from marine sponges (Touati, 2007). The first report of antimicrobial activity of sponge extract was by Nigrelli *et al.* (Newbold, 1999). Since then, there has been a growing number of antibacterial extracts reported from marine sponges. The chemical nature of metabolites isolated from marine sponges has been extensively reviewed by several authors and are studied as interesting target to screen for antimicrobial substances for many reasons. More recently, several marine secondary metabolites have entered human clinical trials or are in preclinical development. Other marine natural products have provided chemical templates for the possible development of new classes of human therapeutics or have proven to be important tools or probes for the molecular study of complex biochemical and cellular processes (Edrada, *et al.*, 2000).

Research of Bergmann in the early 1950’s against *Cryptotethia crypta* sea sponge is the first step in promoting the study of bioactive compounds from marine biota. Genus *Stylissa* has some interesting chemical content. Based on the literature search are reported, such as debromostevensine and debromohymenin (Eder, 1999), carteramine A (Kobayashi, 2007), stylissazoale A-C (Patel, 2010) and latonduines A and B (Linton, 2003). From the literature search are reported, bioactivity of carteramine A as inhibitors of neutrophil chemotaxis (Kobayashi, 2007). Marine sponge known to be a place to live some kind of bacteria that number reached 40% of sponge biomass. Symbiosis that occurs between marine sponge bacteria by causing the organism as one of the marine life that have a greater antibacterial potency compared with other terrestrial and marine organisms.

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**Isolation of antibacterial………..**

*Ralstonia solanacearum* (Smith) is an important soilborne bacterial plant pathogen with a worldwide distribution and a wide host range of more than 200 species in 50 families. Some of its economically important plant hosts include tomato, potato, eggplant, pepper, tobacco, banana, chilli, peanut, and ginger (Lemessa, 2007). In Indonesia *R. solanacearum* is an important disease of ginger (Rostiana, 2005) and its importance is increasing from time to time. To date, no effective control method has been developed for this wilt disease. Plant breeding, field sanitation, crop rotation, and use of bactericides have met with only limited success (Ciampi-Panno, et al., 1989). Although disease resistance is an important component of integrated disease management, it is generally agreed that breeding for resistance is not completely effective, producing only modest gains and often lacking stability and/or durability. Furthermore, the high variability of strains of *R. solanacearum* combined with the influence of environmental factors on host–pathogen interactions (Lemessa, 2007), often restricts the expression of resistance to specific regions. Consequently, biological control of pathogens is gaining great importance worldwide. To investigate for proper biocontrol agents and to obtain antibacterial antagonists from marine sponges, a vast survey was performed.

**METHODOLOGY**

**Material**

The marine sponge was collected from the coral reef at a depth of around 4 - 6m in Mandeh Island of West Sumatera, and the sample was stored in methanol immediately after collection. The specimen was identified by Dr. Nicole. J. de. Voogd (Zoological Museum of the University of Amsterdam, Netherlands). The voucher specimens are also deposited at the Laboratory of Sumatran Biota, Pharmacy Faculty, Andalas University, Indonesia. *Ralstonia solanacearum* obtained from the microbiology laboratory of Faculty of Agriculture, University of Andalas.

**Extraction and isolation**

Sponge (2000g, wet wt.) was homogenized and then extracted with MeOH in Erlenmeyer at room temperature. The concentrated total extract was subjected to liquid-liquid partitioning resulting in n-hexane, and ethyl acetate soluble fraction.

**Secondary metabolites testing**

The presence of secondary metabolites was performed based on modified Simes et.al. method for alkaloid, terpenoids/steroids, phenolics and saponins.

**Antibacterial Activity Assay**

The assay was carried out using plate diffusion agar and dilution method. Antibacterial activity was determined against strains of *R. solanacearum*. The bacteria was grown in Nutrient Broth medium and incubated on 35-37°C for 24 hours. Twenty milliliters Mueller Hinton agar inoculated bacteria were introduced into sterile petri dishes. Paper disc (6 mm of diameter) were impregnated 20 µl of extracts/isolated substance deposited on petri dish after solvent evaporation. In the same plate, it was also deposited paper disc containing Streptomycin sulfate as a comparison or positive control and negative control was DMSO. The plate was then incubated for 24 h at 35–37°C before reading the inhibition zone diameter. The inhibition zone diameter around the paper disc correlates with susceptibility of the tested extracts. Further correlations using zone diameter allowed the designation of an organism as “susceptible”, or “resistant” to concentrations of the extract.

**RESULTS AND DISCUSSION**

The methanol extract of marine sponge *Stylissa carteri* gave positive test with Liebermann Bourchard’reagent indicating that the sponge contained steroid/terpenoid, phenolic and saponin. The result of secondary metabolites testing showed in Table 1.
Table 1. Secondary metabolites testing of marine sponge *Stylissa carteri*

<table>
<thead>
<tr>
<th>No</th>
<th>Secondary Metabolite</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Alkaloid</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>Terpen/steroid</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Phenolic</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Saponin</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2. Antibacterial activity of the marine sponge extracts.

<table>
<thead>
<tr>
<th></th>
<th>Chloramphenicol</th>
<th>Inhibition Zone diameter (mm)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Inhibition Zone diameter (mm)</td>
<td>5 %</td>
<td>3 %</td>
<td>1 %</td>
</tr>
<tr>
<td>Crude Extract</td>
<td>25 mm</td>
<td>12</td>
<td>11</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>n-Hexane Fraction</td>
<td>24 mm</td>
<td>12</td>
<td>11</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate Fraction</td>
<td>21 mm</td>
<td>13</td>
<td>12</td>
<td>11</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Antibacterial activity of the compound N and T.

<table>
<thead>
<tr>
<th>Concentration (%)</th>
<th>Inhibition Zone diameter (mm) of compound N</th>
<th>Inhibition Zone diameter (mm) of compound T</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>0.5</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>0.25</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.025</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.01</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Fractionation of the methanol extract of marine sponge *Stylissa carteri* with *n*-hexane, and ethyl acetate, gave *n*-hexane, and ethylacetate fractions. Each fraction was concentrated under reduced pressure and their antibacterial activities against *R. solanacearum* were tested *in vitro* using diffusion agar method. Ethyl acetate fraction shown a moderate antibacterial activity compared to the *n*-hexane and ethyl acetate fractions. (Table 2).

The ethyl acetate fraction was fractionated by silica gel chromatography, TLC and recrystallization. The fractions having the activity were collected. Finally, 2 active compounds were isolated as white amorf. Based on data from chemical reaction with Lieberman Bourchard, ultraviolet and infrared spectra suggest that compounds are belong to terpenoid group. The work to elucidate its structure is still in progress.

The activity of isolated compounds were determined by agar dilution method against four species of bacteria. Its antibacterial activity was evaluated by MIC (Minimum Inhibitory Concentration) and these values are presented in Table 3. Both of compound N and T showed antibacterial activity with MIC of 0.1% against *R. solanacearum*. This result indicates that marine sponges remain an interesting source of new antibacterial metabolites with better activity then some antibiotics. This is not surprising because the sponge *Stylissa carteri* belonging to this genus possess a wide variety of compound with different biological activities. Thus during the identification of this indicates that marine sponges remain an interesting source of new antibacterial metabolites with better activity then some antibiotics.
CONCLUSION

The marine sponge *Stylissa carteri* are potential source of antibacterial agents. The isolated compound from this sponge had an ability to inhibit the growth of *R. solanacearum* with MIC value of 0.1% using plate diffusion agar method.

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SYNTHESIS HEPTANTETRA-BENZYLcatechin AND TOXICITY TESTS ON BRINE SHRIMP LETHALITY TEST (BSLT)

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2 Department of Chemistry- FMIPA, University of Indonesia (UI), Depok, Indonesia

ABSTRACT

Catechin is a flavonoid compound that has potential to be made as a drug candidate. This compound has biological activity as antioxidants and also inhibit the growth of cancer cells. In this research tetrabenzylcatechin as the lead compound was modified by esterification of the (-3) OH group by heptanoat to form alkoxy catechin and tested for it biological activity. The purpose of the synthesis is to increase the activity. This synthesis produced heptatetrabenzylcatechin compound with the yield is 57.60%. The toxicity tests on Artemia salina Leach showed that heptantetra-benzylcatechin compound is toxic with a LC50 value of 60.26 ppm.

Key words: Catechin, flavonoid, tetrabenzylcatechin and heptatetrabenzyl catechin.

INTRODUCTION

The flavonoids are very large and important group of polyphenolic natural products which are united by their derivation from aromatic heterocycle, flavone (Harbone J. B., 1986). Catechin one of the typical flavonoids, widely distributed in plant kingdom (Rice 1996; Huang, 1997). The use of catechins in the field of health have been widely tested in both human and animals so the potential to be developed as drug candidates. Catechins have a pharmacological effect is to prevent cancer and cardiovascular disease, anti tumor, anti-mutagenic, anti-bacterial, and antioxidant (Park, KD, et al, 2008). The use of catechins in the health sector has been widely tested both on humans and animals so that the potential to be developed as drug candidates.

The compounds of this class of catechins can be enhanced by making the biological activity of derivatives-synthesized derivatives. Some derived of catechine active as antibacterial and also inhibit the growth of cancer cells. Hayes, et al. 2006 reported that with a derived class of compounds to synthesize catechins namely the addition of propyl groups on atoms C-8 into a compound C 8-propyl catechin Gallat known to have higher biological activity than native catechin compounds. Gallat catechin derivative compounds obtained by reaction of hydroxy group on the protection of rings A and B basic framework catechin (Fig. 1). catechins by reacting with benzyl chloride to obtain compounds tetrabenzil-catechin. The second reaction is an esterification reaction of hydroxy group at C-3 with heptanoat acid.

The benzyl ether was chosen as the protecting group because of its deprotection under neutral condition, acid or base, sensitive protecting group needed because to be avoided of possible racemization (Basab Roy, 2008). The Present study to assigned to increase the activity of catechin to synthesis of catechin protected benzyl eter to esterification of OH of C-3 and toxicity tests on Artemia salina Leach.

![Figure 1. (-)-epicatechin and (+)-catechin](image-url)
**METHODOLOGY**

**Material**

Catechin

**Synthesis**

The mixture of compound tetra-benzylcatechin (77 mg or 0.4046 mM), heptanoic acid (40 mg or 3094 mM), DCC (83 mg 0.1999 mM), DMAP (0.074 mg or 0.6069 mM) and 1 mL toluene after stirring for 3 days at 72 – 78 °C. Reaction mixture was diluted with ethyl acetate and washed water and brine (NaOH 1 N, HCl 10%, NaCl 2%, MgSO 4). The organic layer was dried over Na2SO4 and the solvent was evaporated in vacuo to yield mixture. The mixture was purified by column chromatography used n-hexane – ethyl acetate (95:5 v/v) as eluent. The pure compound was identified by spectroscopic, 1H and 13C NMR and FT-IR.

**Bioassay BSLT**

**Hatching the brine shrimp**

Brine shrimp egg (*Artemia salina*, Sanders™ Great Salt Sea. Brine Shrimp Company L.C. USA) were hatched in sea water. The two compartment plastic chamber with several holes on the divider was used for hatching the eggs were sprinkled in to the larger compartment which was darker, while the smaller compartment was illuminated. After 48 hours incubation at room temperature (25 – 29°C), nauplii (larvae) were collected by pipette from the light side whereas their shell were left in the other side.

The procedure for BSLT was modified from the assay describe by Pisutthanan (2004). Ten milligrams of the sample were made up to 2 mg/mL in sea water except for the water insoluble compounds which were dissolve in DMSO 50 µL prior to adding sea water. Serial dilution were made in the wells of 96 well micro plates, in triplicate in 120 µL sea water, control wells with DMSO were included in each experiment. A suspension nauplii containing 10 – 15 organisms (100 µL) was added to each well. The plates were covered and incubated at room temperature (25 – 29 °C) for 24 hours. Plate were then examined under the binocular stereomicros.

![Figure 2. Heptantetra-benzyl catechin](image1)

**Figure 2.** Heptantetra-benzyl catechin

![Figure 3. Mechanism of Esterification with DCC](image2)

**Figure 3.** Mechanism of Esterification with DCC
RESULTS AND DISCUSSIONS

Benzyl ether is a popular protecting group of phenolic hydroxyl groups in organic synthesis, because this ether is stable under basic or mildly acidic conditions but is readily cleavage by hydrogenolysis (Kawamoto H., 1996). The reaction was stirred at 72-78°C for 3 days. After the reaction is complete, the sample was added water and extracted with EtOAc 3 times. Organic phase is then
washed with 1 N NaOH, 10% HCl to obtain pH 7. Then into the organic phase was added anhydrous 2% NaCl solution to dissolve the salt formed and anhydrous MgSO4 to remove the remains of the water and on evaporation. Results obtained was purified using column chromatography with a ratio of mobile phase hexane: EtOAc (v/v, 95:5) to obtain pure compound heptantetra-benzilkatekin (0.053 g, 0.068 mmol; 57.60%). Preliminary analysis indicated the occurrence of the esterification reaction by thin layer chromatography (TLC) where different spots obtained with the initial compound with Rf = 0.51 in the mobile phase hexane: EtOAc (v/v, 9:1). Test the esterification reaction product using a spectrophotometer FT-IR absorption showed a loss of hydroxy groups in the region 3600-3300 cm\(^{-1}\) because it was tersubtitusi by ester groups. At 2983 cm\(^{-1}\) region are the CH stretching vibrations of aliphatic CH\(_3\) methyl. In the area of 2937 cm\(^{-1}\) shows the CH stretching vibration of methylene group. Strong absorption at 1741 cm\(^{-1}\) region showed the carbonyl absorption of aliphatic ketones, carboxylic acids, and esters also show a strong absorption C=O stretching vibrations in the region 1870-1540 cm\(^{-1}\). Absorption is also relatively constant at the same position, the intensity is high on compounds having C=O group and not disturbed by the presence of other functional groups. The characteristics of this led to a very specific uptake of the carbonyl group is very easily interpreted using FT-IR spectrophotometer. In the compound-benzipilepikatekin heptantetra an increase of carbonyl absorption wavelength of the 1741 cm\(^{-1}\). This is due to the influence of the carbonyl environment that can raise or lower the absorption frequency of the group C=O. Stretching vibrations of C=C aromatic group shown in the asymmetric 1450 cm\(^{-1}\). 1373 cm\(^{-1}\) region is asymmetric CH bending vibration. In the area of 1240 cm\(^{-1}\) are the asymmetric stretching vibration of the benzyl ether group COC. Absorption of CO cyclic ether group-6 ring is shown in the 1097 cm\(^{-1}\). 1047 cm\(^{-1}\) region is a region of symmetric stretching vibrations of C-O of primary alcohol. While there is a finger print on the bending frequency of the C=C group in the region 937-846 cm\(^{-1}\) and CH bending frequencies in the 634-609 cm\(^{-1}\).

Tabel 2. Chemical shifts (\(\delta\)) \(^{13}\)C data of heptatetribenzyl catechin

<table>
<thead>
<tr>
<th>Position of C</th>
<th>Chemical shifts ((\delta))</th>
<th>Position of C</th>
<th>Chemical shifts ((\delta))</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>78,5</td>
<td>1’</td>
<td>131,5</td>
</tr>
<tr>
<td>3</td>
<td>68,9</td>
<td>2’</td>
<td>94,5</td>
</tr>
<tr>
<td>4</td>
<td>28,8</td>
<td>3’</td>
<td>149,1</td>
</tr>
<tr>
<td>5</td>
<td>155,0</td>
<td>4’</td>
<td>149,0</td>
</tr>
<tr>
<td>6</td>
<td>101,6</td>
<td>5’</td>
<td>114,0</td>
</tr>
<tr>
<td>7</td>
<td>159,0</td>
<td>6’</td>
<td>120,1</td>
</tr>
<tr>
<td>8</td>
<td>93,9</td>
<td>CH(_2)</td>
<td>70,1 ; 70,1</td>
</tr>
<tr>
<td>9</td>
<td>157,8</td>
<td>CH(_2)</td>
<td>71,4 ; 71,4</td>
</tr>
<tr>
<td>10</td>
<td>115,0</td>
<td>CH phenyl</td>
<td>137,1 ; 137,1 ; 137,2 ; 137,3</td>
</tr>
<tr>
<td>Phenyl aromatic</td>
<td>128,7 - 127,4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results of measurements of \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(^1\)H NMR spectrum showed peaks chemical shifts (\(\delta\)), can be seen in Table 1 and 13 C NMR measurement results can be seen in Table 2. The presence of aromatic groups benzyl aromatic ring is shown in the chemical shifts (\(\delta\)) = 7, 33 to 7.43 ppm, the aromatic CH group of ring B at 6.97 ppm and 6.88 ppm. And the chemical shifts CH aromatic of the ring A shown in area (\(\delta\)) 6.23 to 6.26 ppm. The existence of aliphatic CH\(_2\) group attached to the ring C is shown in the \(\delta\) range from 1.17 -2.87 ppm and a methyl (triplet) from aliphatic group contained in the region \(\delta = 0.84\) ppm. he data of NMR chemical shifts of proton (1H) is also supported by the carbon shift data (Table.2). From the data obtained so-benzilkatekin heptantetra compounds from the esterification reaction of tetra-benzilkatekin been formed.
CONCLUSION
1. The result of heptantetra-benzylcatechin from esterification of OH group of C-3 on tetra-benzylcatechin is 57.60%.
2. Toxicity tests on larva shrimp on Artemia salina L. showed the level of toxicity of compound tetra-benzylcatechin with LC₅₀ value is 60.26 ppm.

ACKNOWLEDGEMENT
This research was funded from government (DIPA 2011) through the Research Center for Chemistry - LIPI Project.

REFERENCES
MUCOLYTIC ACTIVITY OF ETHANOLIC EXTRACT OF RED HIBISCUS FLOWERS (Hibiscus rosa-sinensis L.) ON BOVINE INTESTINE MUCUS BY IN VITRO METHOD

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Departement of Pharmaceutics, Faculty of Pharmacy, Gadjah Mada University, Indonesia

ABSTRACT

Hibiscus flowers (Hibiscus rosa-sinensis L.) has been used in the treatment of productive cough. This study was aimed to find out the mucolytic activity of ethanolic extract of hibiscus flowers on the bovine intestine mucus by in vitro method, and to know the concentration of the extract has activity comparable with asetilsistein 0.1%. Ethanolic extract of hibiscus flowers was made by maceration method using Petroleum Eter and followed with 70% ethanol. Concentration of ethanolic extract used were 1.00%, 1.25% and 1.50%. Each extract concentration was added to pH 7-20%-mucus-phosphate buffer solution. As a negative control was 20%-mucus-phosphate buffer solution, and 0.1% acetylcysteine in 20%-mucus-phosphate buffer solution as a positive control. In each treatment was added by tween 80. The viscosity of each treatment was measured with Ostwald viscometer. The reduction of viscosity value to the viscosity of negative control was analyzed using Kolmogorov-Smirnov, followed by ANAVA, and Tukey test with 95% confidence level. Tukey test results showed: mucolytic activity of 1.25% and 1.5% ethanolic extract based on decreasing viscosity of the mucus, were equal to 0.1% acetylcysteine (p > 0.05); mucolytic activity of 1.00% ethanolic extract had no significant difference to the negative controls (p > 0.05); 1.25% and 1.50% ethanolic extracts had significant difference to the negative control (p <0.05) and had no significant difference to the positive control (p > 0.05). Ethanolic extract 1.00% of Hibiscus rosa-sinensis flower has no mucolitic effect, while 1.25% and 1.50% ethanolic extract have effect equal to 0.10% acetylcysteine by in vitro method.

Keywords: Extract Ethanolic, Hibiscus flower, mucolytic, in vitro.

INTRODUCTION

Nowadays the traditional medical treatment is getting more widely published, both in Indonesia and in foreign countries. In Indonesia the traditional medical treatment has been used for a long time. It is supported by high level of variety of vegetables or biological resources, which also make Indonesia potential as one of traditional medicine producers. One vegetable which is abundant in Indonesia and a big potency as a medicine is hibiscus flower (Hibiscus rosa-sinensis L.). It has a restorative power to heal fever, cough, and oral ulceration (Syamsuhidayat dkk, 1991).

Many Indonesian people use hibiscus rosa sinensis flower as medicine to cure various diseases, and one of them is to drop out phlegm (mucus). Accordingly, it is needed to carry out scientific demonstration of the use of hibiscus rosa sinensis flower as the medicine of phlegm (mucus). To know the effect of phlegm (mucus), the simple in-vitro method can be used through the measurement of the effect of the medicine against viscid mucus of bovine (cow) intestine. For this experiment it is used 0,1% acetylcysteine as comparison. (Anonym, 1991)

Besides to identify whether there is mucous activity of the ethanol extract of hibiscus rosa sinensis flower or not, this research is also aimed to find out the concentration of ethanolic extract of hibiscus rosa sinensis flower which is parallel to acetylcysteine 0,1%. The next step is to carry out the chromatographic test of the thin layer to find out the compound found in hibiscus rosa sinensis flower. Hibiscus rosa sinensis flower plant contains flavonoid, saponin, polyfenol (Syamsuhidayat & Hutapea, 1991), Ca oxalat, peroxidase, fat, protein (Tampubolon, 1981), Cyanidyn diglucoside, hibicetin, bitter substance and mucus as well as taraxeryl acetate (Wijayakusuma, 2000).
METODOLOGY

Equipment

Oven (Memmert), blender (Philip), buchner (Pyrex®), vacuum, gelas ukur 500 ml (Pyrex®), fan, neraca analitik (Ohaus®), pHmeter (Hanna Instruments HI 8314 membrane pH meter), magnetic stirrer, hot plate thermolyne (IkaMag® RH), glass equipment (Pyrex®), waterbath (Digisystem Lab Instrumentals, Inc., model: DSB-1000ED), Ostwald viscometer (Pyrex®), Picnometer 10 ml (Pyrex®), thermometer, sonicator (Elma®), stopwatch, TLC, flacon, plate silica gel 60 F254 (Merck®), UV lamp λ254nm (Philip®) and λ366nm (Desaga®), capiler pipe.

Materials

Hibiscus rosa-sinensis flower from Graha Sabha Pramana, Gadjah Mada, Yogyakarta, Indonesia, Petroleum ether and ethanol 70% technic quality, bovine mucus intestine from Jl. Kaliurang, acetylcysteine pharmacetics quality from capsul Fluimucil®; tween 80, phosphat buffer pH 7 from NaOH (Merck®) analysis quality, KH2PO4 (Merck®) analysis quality, aquadest (distillate water), still phase silica gel 60 F254, moving phase etyl acetat-methanol (1:5 v/v), dragendorf reagent.

The Rundown of Research

Determination of plants

Plant determination was carried out in the Laboratory of Biological Pharmacy, Faculty of Pharmacy of Gajah Mada University, Yogyakarta.

Preparation of the Main Material

The hibiscus rosa sinensis flower is picked up from its plant from 7 a.m. to 8 a.m.. Then, the flower is cleaned using the clean flowing water, and then it is dried up under the sun for ten minutes, and then it is put into the oven under 40 to 50 Celcius degree.

Extraction

Extraction is done in two steps using the method of maserasi (separation the fiber from the liquid), that is the process of separation using petroleum ether and ethanol 70%. The separation process is done within 5 days for every dissolving. For the first three days, it is stirred. Then it is filtered; the essence is separated, and the fiber is put into the same dissolvent for two days without stirring. The fine liquid is evaporated using water bad (specific devise to extract liquid) to get some thick extract (Anonym, 1995).

Gathering mucus of cow intestine

The bovine (cow) intestine is cleaned from its content (food residue) by rubbing gently. Then the intestine is cut alongside it and the inside part is scraped slowly to gather the mucus. The mucus then stirred slowly until it is entirely homogenous, and then it is kept in the refrigerator under 4 C degree until it is ready for the test.

Production of phosphate buffer pH7 solution

Phosphate buffer pH7 solution is produced by mixing 50,0 ml of the solution of KH2PO4 0,2 M and 29,1 ml of NaOH O,2 N in aquadest (distillation water) until 200 ml. Then put some distillation water without CO2 up to 200 ml. Check the pH using pH meter.

Production of mucus phosphate buffer pH7 20% solution

Mucus solution 20% in phosphate buffer is produced by mixing mucus as much as 0,2 part of the total weight with phosphate buffer pH7 as much as 0,8 part of the total weight. Add solution 0,5% tween 80 from the total weight.

Production of solution of acetylcysteine 0,1%

The solution of acetylcysteine 0,1% is produced by weighing all the content of the capsule of Fluimucil which contains 231 mg acetylcystein. Half of the content of the capsule is dissolved in the solution of mucus phosphate buffer 20% by adding 0,5% tween 80.
**Production of test solution**

Test solution is produced in this way. Supposed test solution 1% is produced by mixing 0,15 g of thick extract of hibiscus rosa sinensis flower with 0,075 g tween 80, then it is dissolved in mucus phosphate buffer 20% until it reaches the weight 15 g. To make mucus phosphate buffer 20% homogenous with the extract, it is stirred with 40 seconds. The concentrate tween 80 which can be added to the test solution is only 0,5%

**Test of Mucolytic activity**

Before determining the viscosity, the test solution is incubated within 37 C degree for 30 minutes on the waterbath. The measurement of viscosity is done on waterbath using the following procedure (Anonym, 2009). As much as 10 ml test solution is put into Ostwald viscometer, set a pump and then the test solution is pumped until it passes the upper edge of Ostwald. Close the end of the pipe and remove it when the timer is ready. As positive control, put the solution of acetylcysteine 0,1% into the solution of mucus phosphate buffer 20%, and for negative control the solution of mucus 20% is used. The viscosity of every control is tested using the same procedure. The measurement is done three times for each test solution using the new citation for every repetition.

**Measurement of Density (Specific weight)**

The measurement of the density of the test solution is done by using picnometer. The test solution from the Ostwald viscometer is poured full into picnometer. Put the picnometer into warm water until it reaches 37 C degree, then close the edge of the tube of the picnometer. Before it is weighted, the picnometer has to be rubbed dry. The measurement of the density is done three times for every test solution using the new citation for every repetition.

**Identification of compound of hibiscus rosa sinensis flower with Thin Layer Chromatography (TLC)**

Some thick ethanol extract which is dissolved into ethanol 70% with certain concentration is prepared in flacon. Then the solution is splattered on still phase silica gel 60 F254 as much as three times. Then the TLC rubbed with motion phase of etil acetate- methanol (5:5 v/v). After the elusion process, TLC plate is then dried up from the motion phase in the open air. The spots on the TLC plate is observed under the lamp of UV 366 nm. To identify the alkaloid compound, the compound detection is done by spraying the TLC plate with spraying reagent dragendorf and it is heated for 5 to 10 minutes under 110 C degree in an oven. The color of the spots yielded by the process is observed under the exposing ray so that it is visible.

**Data Analysis**

**Analysis of mucolytic activity data**

The change of mucus viscosity after it is given some extract in various concentration determined by the Ostwald viscometer is counted by using the formula:

\[
\text{Viscosity} = \frac{(\text{rate of citation} \times \text{citation density}) \times \text{viscosity of aquadest}}{(\text{rate of aquadest} \times \text{density of aquadest})}
\]

Note:
- \( t \) = time needed for the citation to flow (in second)
- \( \text{density} \) = specific weight (gram/ml) (Turner & Hebborn, 1971)

In this research, distillation is used. Viscosity of aquadest (distillation water) under 37 C degree is yielded from the intra-polation viscosity of distillation water under 30 C degree and 40 C degree (Anonym, 1979). Picnometer is used to measure the specific weight of the test solution, and the test is done three times for every test solution with the citation which is always new. The special weight of test solution is found by comparing the mass of mucus solution (gram) with water volume (ml) which is measured using the same device and under the same temperature. After that, viscid(viscosity) data is analyzed using statistic test, that is Kolmogorov-Smirnov, continued by using test of ANAVA, and the
last it is tested using Tukey with the acceptability level of 95%. Overall statistic analysis is done using software SPSS 15.0.

**Analysis of TLC Data**

TLC data which has been gained is in the form of chromatogram that shows the existence or absence of alkaloid compound. The appearing spot are observed by its color under ultra violet ray, UV 366 nm and 254 nm. Then the value hRf spots that appear are counted. The identification of specific alkaloid compound is done by using spraying reagent dragendorf. The change of color that happens is observed under ultra violet ray.

**RESULT AND DISCUSSION**

**Determination of Plants**

Determination is done by matching the morphological characteristic of hibiscus plant found in books according to *Flora untuk Sekolah di Indonesia* by Steenis dkk. (1975). The result of the determination is hibiscus plants (*Hibiscus rosa-sinensis* L.).

**Extract Rendemen**

In this research, rendemen extract yielded is as much as 18,26%. This amount is yielded from 108,3 gram of hibiscus rosa sinensis flower powder which is extracted using ethanol 70%. As much as 19,78 gram of thick ethanolic extract is yielded, and its color is brownish red.

**Test of Mucolytic Activity by in Vitro**

The first data gained from the mucolytic activity test against the ethanolic extract of hibiscus rosa sinensis flower is the running time and the density. The result of the measurement of the running time and the density of ethanolic extract are shown in table I and table II.

**Table I. Measurement of the flow time of samples**

<table>
<thead>
<tr>
<th>No</th>
<th>Flow time (second)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aquadest Negative control</td>
</tr>
<tr>
<td>1</td>
<td>14,37</td>
</tr>
<tr>
<td>2</td>
<td>14,26</td>
</tr>
<tr>
<td>3</td>
<td>14,31</td>
</tr>
<tr>
<td>Mean</td>
<td>14,31</td>
</tr>
<tr>
<td>SD</td>
<td>0,06</td>
</tr>
</tbody>
</table>

Note:EE = ethanolic extract

**Table II. The density of samples**

<table>
<thead>
<tr>
<th>No</th>
<th>density (gram/milliliter)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative control</td>
</tr>
<tr>
<td>1</td>
<td>1,0100</td>
</tr>
<tr>
<td>2</td>
<td>1,0124</td>
</tr>
<tr>
<td>3</td>
<td>1,0117</td>
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<tr>
<td>Mean</td>
<td>1,0114</td>
</tr>
<tr>
<td>SD</td>
<td>0,0012</td>
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</table>

Note:EE = ethanolic extract
The running time in the positive control and after the addition of ethanolic extract show that the running time is faster. Table III shows the data of viscosity solution of phosphate buffer mucus 20% after the addition of extract of ethanol hibiscus rosa sinensis flower.

Table III. The value of viscosity of samples by Ostwald viscotester

<table>
<thead>
<tr>
<th>No</th>
<th>Negative control</th>
<th>Positive control</th>
<th>EE 1,00%</th>
<th>EE 1,25%</th>
<th>EE 1,50%</th>
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</thead>
<tbody>
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<td>1,7165</td>
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<td>1,4089</td>
<td>1,3164</td>
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<td>2</td>
<td>1,8771</td>
<td>1,2743</td>
<td>1,3689</td>
<td>1,3197</td>
<td>1,3268</td>
</tr>
<tr>
<td>3</td>
<td>1,6538</td>
<td>1,3505</td>
<td>1,4663</td>
<td>1,3288</td>
<td>1,3002</td>
</tr>
<tr>
<td>Mean</td>
<td>1,8043</td>
<td>1,4471</td>
<td>1,4689</td>
<td>1,3525</td>
<td>1,3145</td>
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<tr>
<td>SD</td>
<td>0,13</td>
<td>0,24</td>
<td>0,10</td>
<td>0,05</td>
<td>0,01</td>
</tr>
</tbody>
</table>

Note: EE = ethanolic extract

It can be seen that there is decrease of solution viscosity after the addition of ethanolic extract of hibiscus rosa sinensis flower in various concentration. Besides in positive control, it is seen that there is decrease of mucus viscid solution. Acetylcysteine 0,1% is used as positive control which is dissolved in solution of phosphate buffer-mucus 20% by adding 0,5% tween 80.

Acetylcysteine is the derivation of natural sustain amino acid which is useful to dissolve thick phlegm mucus) by cutting of the disulfide in mucus so that the long chain of every mucous proteins is opened up, and is easily pulled out through coughing (Ganiswara, dkk., 2005; Tjay & Rahardja, 2003).

The addition of tween 80 is aimed to increase the dissolving extract in the solution of phosphate buffer-mucus 20%. Therefore, the addition of tween 80 as much as 0,5% is done to every test solution, both for negative control, positive control and for the treatment of ethanolic extract.

The decrease of viscid in mucus solution which is mixed with ethanolic extract with certain concentration shows that ethanolic extract of hibiscus rosa sinensis flower contains mucolytic activity. The data in table III shows that the more the concentration extract which is added to the mucus solution, the decrease of mucus viscosity is greater. Therefore it can be assumed that the higher the extract concentration which is added, the mucolytic activity of hibiscus rosa sinensis flower will be bigger.

Tabel IV. The result of Tukey test

<table>
<thead>
<tr>
<th>Variable</th>
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<th>EE 1,25%</th>
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<td>TBB</td>
<td>BB</td>
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<tr>
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<td>-</td>
<td>TBB</td>
<td>TBB</td>
<td>TBB</td>
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<tr>
<td>control</td>
<td></td>
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<td></td>
<td></td>
</tr>
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<td>EE 1%</td>
<td>TBB</td>
<td>-</td>
<td>TBB</td>
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<td>EE 1,5%</td>
<td>TBB</td>
<td>TBB</td>
<td>TBB</td>
<td>-</td>
</tr>
</tbody>
</table>

Note:  
BB = significant difference  
TBB = no significant difference

The result of the statistic analysis for the test of Kolmogorov-Smirnov states that the research data which is gained is normally distributed. Accordingly, one way ANAVA test can be done, which is then followed by the Tukey test with the acceptability of 95% to know the difference between one group treatment with another.
The result of the ANAVA output shows that the probability figure is 0.007 smaller than 0.05 (probability<0.05), which means that there is differences in the rate of decrease grade between the five treatments. The result of Tukey test with the acceptability of 95% can be seen in table IV.

Table IV shows that the decrease of viscous negative control doesn’t show significant difference with the ethanol extract 1% (p > 0.05), while against the positive control, the ethanol extract 1.25% and 1.5% shows that there is significant difference. Therefore it can be concluded that ethanolic extract 1% doesn’t show the presence of mucolytic activity, while with concentration 1.25% and 1.5%, it shows that there is mucolytic activity.

The decrease of viscosity in ethanol extract 1.25% and 1.5% to negative control shows that there is no significant difference. On the other hand, the decrease of viscous in ethanol extract 1.25% to ethanol extract 1.5% and vice versa, doesn’t show significant difference. It explains that mucous activity on ethanol extract 1.25% has equal value with mucolytic activity on ethanol 1.5%. Therefore it can concluded that ethanolic extract 1.25% and 1.5% have mucolytic activity which is equal to positive control (acetylcysteine 0.1%).

**Compound Identification**

Compound identification in ethanolic extract of hibiscus rosa sinensis flower was done using TLC method with appropriate system, that is as still phase, gel 60 F254 (Merck) is used, ethyl acetate-methanol (1:5 v/v) as moving phase, spot exposure using ultra violet lamp UV and dragendorf spraying reagent. The result of TLC can be seen in picture 1.

According to Wagner and Bladt (1996), alkaloid compound will yield spot which color is brown or orange under the ultraviolet ray when it is sprayed with dragendorf. The color which appears after the spraying is stable. Besides, some alkaloid will appear as spots which blur under UV 254 nm, while under UV 365 nm alkaloid will flourish blue, green-blue, or purple colors.

![Chromatogram identification of alkaloid by still phase silica gel 60 F254 (Merck®) and moving phase ethyl acetate-methanol (1:5 v/v): (a) UV 254 nm; (b) UV 366 nm; (c) visible after spraying with dragendorf and drying in the oven](image)

The result of Chromatogram after spraying with dragendorf shows purple spot under hRf 18.75. Therefore it can be concluded that ethanolic extract of hibiscus rosa sinensis flower contains alkaloid type compound.

**CONCLUSION**

Ethanolic extract 1.25% and 1.5% of red hibiscus rosa sinensis flower has mucolytic activity indicated by the decrease of viscosity of mucus solution.

Ethanolic extract 1.25% and 1.5% has mucolytic activity which is parallel to acetylcysteine 0.1%

Hibiscus rosa sinensis flower has alkaloid which can be used as identity compound (marker) from the plant.
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THE EFFECT OF ADMINISTRATION OF N-HEXANE EXTRACT OF KEMBANG BULAN \( [Tithonia diversifolia \ (Hemsley)\ A.\ Gray] \) LEAF TO ALLOXAN DIABETES MICE

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ABSTRACT

The usage of kembang bulan \( (Tithonia diversifolia \ [Hemsley] \ A. \ Gray) \) leaf as traditional medicine is aimed to reduce blood glucose concentration. This research was performed to study the effect of administration of n-hexane extract of kembang bulan leaf to the reduction of blood glucose concentration and the pancreas histopathology description of alloxan diabetes mice. The mice were separated into five groups each consisting 25 mice which were K1 (normal), K2 (negative control), K3 (positive control; chlorpropamide 0.032 g/kg body weight), K4 (n-hexane extract of kembang bulan leaf; 5.38 g/kg body weight dose), and K5 (n-hexane extract of kembang bulan leaf; 10.75 g/kg body weight dose). Diabetes induction was performed by injecting the alloxan tetrahydrate (70 mg/kg body weight dose) intravenously to all groups except normal group (K1). The administration of the sample was performed orally for 14 days after hyperglycemia condition was achieved which was 7 days after alloxan injection. The measurement of blood glucose concentration and the preparation of pancreas organ histopathology were performed on day 0 (hyperglycemia pre-induction), day 7 (hyperglycemia condition), day 21 (post-treatment), and day 28 (recovery period). The obtained result is that the hypoglycemic ability of chlorpropamide is 36%, the kembang bulan extract (5.38 g/kg body weight) is 24%, and kembang bulan extract (10.75 g/kg body weight) is 31%

**Key words:** Kembang bulan leaf extract, blood glucose concentration, pancreas β-cell, alloxan diabetes mice

INTRODUCTION

Diabetes mellitus (DM) is a disease caused by relative or absolute insulin deficiency with specific symptoms which are polyuria, polydipsia, and polyphagia. The blood glucose concentration of patient with DM exceeds 126 mg/dL (fasting) or 200 mg/dL (non-fasting). Based on the survey done by WHO, Indonesia stands on fourth position of the countries with highest number of diabetes mellitus patients in the world after India, China, and United States. Based on data from Depkes, the amount of diabetes mellitus inpatient and outpatient in hospitals stands on top of all endocrine diseases (Depkes RI, 2005)

METHODOLOGY

Materials

The used plant is kembang bulan leaf obtained from the housing in Cipinang Kebembem sub-district in East Jakarta and it has been determined by Herbarium Bogoriense Pusat Penelitian Biologi LIPI. The experimental animals used are 125 DDY-strain white mice aged 1-2 months obtained from Bagian Perhewanan Pusat Pengawasan Obat dan Makanan (PPOM) Central Jakarta.

Mode of action

**Preparation of n-hexane extract**

An amount of 300 grams of kembang bulan leaf powder is macerated with ethanol 70% for 3x24 hours. The filtrate obtained is then concentrated with vacuum rotary evaporator until thick extract is obtained. Then, the thick ethanol extract is part by n-hexane:water (1:1), the n-hexane extract obtained is then concentrated with vacuum rotary evaporator until thick n-hexane extract is obtained.
The mice are randomly divided into five groups which are group K1 (normal), K2 (negative control), K3 (positive control; chlorpropamide 0.032 g/kg bw), K4 (n-hexane extract of kembang bulan leaf; dose of 5.38 g/kg bw), and K5 (n-hexane extract of kembang bulan leaf; dose of 10.75 g/kg bw). Each group consists of 25 mice. The diabetes induction is performed by intravenously injecting alloxan tetrahydrate (dose of 70 mg/kg bw) to all groups except normal group (K-1). The administration of sample is performed orally for 14 days after hyperglycemia state achieved which is 7 days after alloxan injection. The measurement of blood glucose concentration and the preparation of pancreas histopathology are performed on day 0 (normal glucose concentration), day 7 (hyperglycemia state), day 14 (hyperglycemia glucose concentration after 7 days sample administration), day 21 (hyperglycemia glucose concentration after 14 days sample administration), and day 28 (recovery; glucose concentration after 7 days without sample administration). The observed parameters of kidney histopathology preparation are the amount of β-cell and the diameter of pancreas Langerhans islets. During the treatment, the physiological parameters observed are body weight, the amount of food-drink consumption, and excreted urine volume. The hypoglycemic potential of n-hexane extract (%) to decrease blood glucose concentration of alloxan diabetes mice is measured by comparing the difference between area under curve (AUC) of negative control group (K2) and treatment groups (K3, K4, K5) with negative control group (K2).

RESULTS AND DISCUSSIONS

The extraction of 300 grams of kembang bulan leaf results in 50 grams of ethanol extract. After being extractated by n-hexane:water (1:1), 8 grams of n-hexane extract is achieved. From the phytochemical screening, the compound groups of coumarine, steroid/triterpenoid, and essential oil are found.

Alloxan tetrahydrate is administered to induce the damage on β-cell pancreas in the experimental animal. The damage is the nuclear DNA fragmentation by the reactive compound of \( \text{H}_2\text{O}_2 \) or formed hydroxyl radical, the increase of polysynthetase (ADP-ribose) activity, the depletion of intracellular Nicotinamide Adenine Dinucleotide (NAD\(^+\)) and ends with the death of cell (Halliwell, 1999).

The result shows that the induction of alloxan tetrahydrate to mice (iv; 70 mg/kg bw) is able to increase blood glucose concentration 3 times higher of the normal value (>180 mg/dL) on day 7 (Figure 1). The hypoglycemic potential (Figure 2) of n-hexane extract (dose of 10,75 g/kg bw = 34%) is a bit lower than chlorpropamide (0,032 g/kg bw = 36%).

![Figure 1](image1.png)

Figure 1.Profile of blood glucose concentration of alloxan diabetes mice

The main symptoms of diabetic patients are polyuria (often urinating), polydipsia (often drinking), and polyphagia (often eating). In this research, the observed physiological changes of alloxan diabetes mice during treatment are the body weight development of mice (Figure 3), food consumption (Figure 4), drink (Figure 5), and excreted urine volume (Figure 6).
Figure 2. Hypoglicemic potential of chlorpropamide (K-3) and n-hexane fraction (K-4, K-5)

Figure 3. Body weight development

Figure 4. Profile food and Consumption
In hyperglycemia state (day 7), all alloxan diabetes mice groups show diabetic symptoms such as body weight decrease, food consumption increase, drink consumption increase, and also excreted urine volume.
The administration of sample (chlorpropamide or n-hexane extract), we find the recovery state of the diabetic mice physiological data towards normal value on the treatment mice groups with sample while the contrary on the groups without sample administration (K2).

On observation day 21, which is 14 days after sample administration, the blood glucose concentrations of alloxan diabetic mice are acquired which are 81,2 mg/dl (K3), 118,4 mg/dl and 98,0 mg/dl (K5); those glucose concentrations are still above normal. To achieve normal glucose concentration, it needs higher dose or lengthened administration period.

The insulin is produced by pancreatic β-cell and functions in the management of blood glucose concentration. The deficiency of secreted insulin may cause the increase of blood glucose concentration. In this research, the pancreatic cell damage caused by alloxan tetrahydrate is observed by looking at the pancreas histopathology preparation which includes parameters as β-cell amounts (Figure 7.) and diameter of Langerhans islets (Figure 8.).

The amounts of β-cell before alloxan induction (normal state) is 35 β-cells/Langerhans islets with diameter mean of 12 μm. The hyperglycemic state on day 7 is followed by the decrease of β-cell amounts of 80% and Langerhans islets diameter decrease of 50%. The administration of chlorpropamide and n-hexane extract for 14 days gradually recovers β-cell amounts and Langerhans islets diameter, although they have not been back to normal value yet.

CONCLUSION

The n-hexane extract of kembang bulan leaf (dose of 10,75 g/kg bw) is able to decrease blood glucose concentration of alloxan diabetes mice by 34% and it recovers the β-cell amounts and Langerhans islets diameter. The administration of n-hexane extract for 14 days has not decreased glucose concentration or recovered β-cell amounts or Langerhans islets as normally were.

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DISCUSSION

Metabolic Engineering Strategies for Optimization Of Medicinal And Aromatic Plants : Expectations And Realities
Oliver Kayser
Applying genetic and biotechnological techniques, like metabolic engineering and pathway optimization to increase productivity is the main focus of research. Because of some drawbacks with plant cell culture and isolated enzymes giving no sufficient high production for commercialization, research strategies shifted more to metabolic engeneering. Engineering a microorganism is proven as a valuable tool and concept have been transfered to plant science and opened new promising perspectives for improving plants and cell lines.

Q:
Prof. Soedarsono:
Enzim blocked in biosynthetic pathway, its blocked mechanism only qualitative or also quantitative? metabolic engineering that you make the target destination is predictable or still trial and error?
In increasing or decrease some of the metabolites, the fact is we need to give or maybe strange downregulation?
For edible vaccines that could be used for injection? Examples of small peptides (epitopes) that are expressed in plants, can it be taken?

A:
We doing qualitative not quantitative pathway blocked, so we see small/unsmall. On a quantitative theory can be done but in practice we do not do it we did a trial and error because there is no information about the genome, the steps taken in selecting and then check whether the pathway, including downregulation/upregulation after it matched with the problem
This test not only downregulation but we also practice upregulation so viewed from compounds for define the concept

Oliver:
Is a nice idea to take a part of the plant but i do not know what a market exists, the animal is difference because the system is more specific expression, maybe Mr. Andreas can answer..

Andreas:
there are programs in our company manufacture the edible film but to be made of injection this is dangerous because it entered into the system so that needs the regulation system

Regulation Needed On Traditional Medicines In Developing Countries From Plant Resources To Finished Product
Suwijiyo Pramono
The use of traditional medicines in developing country still trends to increase both in the daily life of the poeple and in industrial level. This is because the drugs are considered safer than chemical drugs. but the bad handling can cause the growth of microbes, aflatoxin and heavy metal contamination. The collaboration between the authority, farmers or collectors, research institution and industries will give optimal result in achieving sustainability, safety, efficacy and quality of traditional medicines.

Q:
how the regulation of traditional medicine? done checking whether the biological activity?
What do you suggest to manage medicinal plants so that its use does not cause damage?
the synthetic compound, could an industry do repacked in the form of traditional medicine? is there regulatory to act the problems?

A:
yes, the authority took the product on the market and then be checked biology, chemistry and pharmacology compared with the existing parameters. if there is a problem, the authority will contact the industry to give a warning or it could also pull the product from the market.

One of which is to Regulation of the use of endangered medicinal plants by the authority. authorities also perform control so that the use of plants is limited. limitation causes the high cost. but in general the industry is very sympathetic, although very difficult to manage them.

Ever found in herbal medicine "pegal linu" can cause not only the moon face but also because it contains dexametasone osteoporosis. the government examine the small shop that provides a "jamu". but because the small shop does not apply to pay after, then it is very unwise because it would harm small shops, the government followed up by asking the police to conduct checks on the industry.

Andreas Bernkop S
Non-Invasive Delivery Of Therapeutic Peptides : Barriers, Strategies And Future Trends
Resume :
many peptide therapeutics administered parenterally but these treatments tend to be preferred because it is difficult, painful, and sometimes dangerous. development of innovation needed to get a high bioavailability, among others, overcome barriers. Strategies overcome. strategies to overcome these barriers are based on auxiliary agents such as enzym inhibitors and permeation enhacers. The combination with advanced drug delivery system can result in formulations providing sufficient high peptide drug bioavaliblity after non-invasive administration.

Q :
1. Insanu : is posible if we using cycle peptide in your methode?
2. Oliver K : how to do to take your amino acid sequence to be prodrugs or new molecules and to design computer?
3. Sismindari: is it possible to create a formula with specific targets such as cancer?
A :
1. yes, we must differentiate the cyclic peptides that have conditioned the dimensions of enzymatic is eksopeptidase or aminopeptidase, of course you must have a program with exopeptidase and peptide cyclimation.
2. yes, the point is to do research to make simple compounds using amino acids. The problem is to consider the use of guidelines from the formulation, its more hydrofobic or not, where the binding site, etc, required privileges and compromise with the peptide contained.
3. yes, of course, possible to make a cancer drug target, but not exactly true because during the time outstanding not only attack specific targets, so it is necessary to design capable of attacking the cancer but provide protection to the other parts, and it may be produced

APORANEE CHAIYAKUM, MSc, BCP
1. Q : What is the reason that leadership is needed to be improved in clinical pharmacy skill?
   A : When we work as a team in the hospital, sometimes we need to give some intervention. And that intervention is needed to be following by another in our team. So we need some confident to influence your team. That confident which should be increased by improved our leadership skill.
2. Q: How is training of leadership skill implementation in pharmacy education?
   A: We try to do group discussion to pharmacy student. And for the clinical pharmacy we put in the ward so they have some responsibility to do some intervention with their team. The most important thing that should be remembered, we need to show what the clinical pharmacy is.
3. Q: Is there any financial impact (Salary) to the preceptors (hospital) which approved the training?
   A: To encourage training student, they need to study in real situations. And they will pay the pharmacist preceptor.

Genetic Polymorphisms of the Cytochrome P450 Subtypes CYP-2D6, CYP-2C9, and CYP-2C19 in Ethnic Makasar Population
ZULLIES IKAWATI
1. Q: What is benefit of the participant joined for being as subject in this research?
   A: They will know about their cytochrome characteristic (is there any mutation or not.)
2. Q: How if there is a mutation in their cytochrome?
   A: We can inform the participant about their metabolized and educated about which kind of drug or food that will alter some medicines if they are taking that medicines.
3. Q: How could we know that we are affected some mutation?
   A: It is too difficult if we want to see from our physical appearances.
4. Q: What is the most caused a mutation?
   A: Almost mutation can be caused by some heritage, another factor for example: cancer, influence from environment, cross marriage can caused some mutation.

The Benefit of Curcuma Xanthorriza and Curcuma Domestica For Osteoarthritis Treatment
Dr. NYOMAN KERTIA, Sp.PD, KR
1. Q: There is a research which conclusion is chronic used of curcumin can cause liver damaged and it was so different with your project. Could you explain about it?
   A: There is a difference in dose curcumin. In that research, they use 10 g/day, and dose that we suggest to use is 15 mg/kg/weight
2. Q: Is the patient have the same baseline?
   A: We use 64 patient which divided into 2 groups and there is no different at baseline data at all the subjects.

Improving Drug Safety with The Use of Information Technology
Invited speaker: PAULINE SIEW MEI LAI
1. Q: How about the manual operation, is still use there?
   A: Although computerized are developing, the manual operation is still used too.
2. Q: How about the development of e-prescribing in the community pharmacy?
   A: Development of community pharmacist in Malaysia with e-prescribing still need a long time
3. Q: How do you monitor if there’s any compatibles material in TPN?
   A: Some compound which can make precipitation must be given an attention for example calcium phosphide, must be mixed at the end of preparation, but usually the computerized will give some alert.

Social Pharmacy Education and Research: The needs and Challenges
MOHAMED AZMI AHMAD HASSALI
1. Q: How do you face the challenge?
A: It is about how your product can give benefit to the social and thinking not just came to bargain the product, then work with generic industrial to develop generic medicine. And the most important thing is showing what you can do.

2. Q: What is social pharmacy competency?
   A: It needs knowledge about medical pluralism and need to understand and aware with culture for example herbs preparation as a food supplement etc.

**Marketing in Pharma**
Invited speaker: HARRY BAGYO

1. Q: Is there any marketing strategy, beside deal with the doctor?
   A: It is better if we have national insurance, so we can improve our rational medication too.

**The Profile of Prescription Services in Community Pharmacies in Surabaya: A Simulated Patient Method**
UMI ATHIJAH

1. Q: Based on your recommendation, could you give a reason why you recommendation to do research about pharmacist performance toward medicine information?
   A: In our result, we could give information about drug information service because pharmacist staff just could to do administration screening, if they want to give drug information, it needs supervision by pharmacist.

**Identification of Antibiotic Use in Hospitalized Chronic Renal Failure Patients**
VICTORIA YULITA FITRIANI

1. Q: Could you explain about the toxicity of using antibiotic?
   A: There is no laboratory data and symptom can show about toxicity. It might be toxicity when use chronically.

2. Q: Which antibiotic is the most toxic?
   A: There is no data report about the most toxic antibiotic.

**Improving Diabetic Patient’s Adherence to Treatment Program by Using CBIA-DM Strategy in Hospital-Based Patient Community**
TITIEN SIWI HARTAYU

1. Q: How do you collect data about adherence activities, whereas you just collect data about knowledge and attitude?
   A: The adherence was showed by increasing of practice score supported by the data of pill counting and blood glucose level.

**Health-Related Quality of Life in Type 2 Diabetes Outpatient with Insulin in RSUP DR SARDJITO Yogyakarta Hospital**
TRI MURTI ANDAYANI

1. Q: Is insulin in that study is given in the first time?
   A: Yes, the inclusion criteria in my study were patient whose blood glucose target not achieved for 3 month and switching in the therapy into insulin.

2. Q: Why did you compare short acting insulin and premixed insulin? As we know, short acting insulin is for reducing post prandial glucose and premix insulin is for reducing post prandial and basal glucose as well, I think that two insulin are not comparable because of totally different mode of action.
   A: It can be comparable because premixed insulin is also containing 30% of short acting insulin agent. We focused on the impact of insulin type 2 (health related quality of life).
History Taking Profile on Self Medication Services of Diarrhea Patients at Pharmacies in Surabaya
EKARINA RATNA HIMAWATI
Q : How can we make sure the research data was really taken from the sample respondents?
A : By recording the history taking conversation (the actor bring a tape recorder during the visit)
Q : Which data were collected from the pharmacist who recognized the simulated patient as a researcher?
A : We drop those data (reject) because if the pharmacist recognized the actor, the data was not valid.

Evaluation of Pharmacoepidemiology Course for Undergraduate Pharmacy Program in Malaysia
SHAFIE AZRUL ANWAR
Q : What is the strategy to avoid peer-group assessment or manipulate score?
A : The student have their individual task which is sent by email to the trainer course.

How Do Community Pharmacist Manage Their Medicines?
ANILA IMPIAN SUKORINI
Q : Is there any regulation about the new pharmacy establishment by the non-pharmacist?
A : There is no regulation cover it until now.
Q : How about the prescription drug when pharmacist decided to sold with cheaper price, in order to manage expired date where is getting closer?
A : The pharmacist usually get information deeply and give some counseling in order to assurance that patient's adherence will better.

Recent Progress On Biological Activities of Benzylidenecyclopentanon Analogues of Curcumin on Histamin – Mediated Allergy Inflammation
AGUNG ENDRO NUGROHO
Q : How is interaction model between curcumin and histamine receptor?
A : There is no report which can explain about it, but it might be has the same mechanism with PGV-0

Reduction of Blood Glucose Levels of Ethanolic Extract of Bungur (Largestroemia speciosa (L) pers) Leaves in Alloxan Induced Diabetic Rats
ANGELICA KRESNAMURTI
Q : Why do you choose alloxan for conditioning diabetic type 2 in rats? As we know, alloxan will destroy B-cell in pancreas and insulin deficiency will be happened. Deficiency of insulin is categorized in diabetic mellitus type 1.
A : Some research said that alloxan just destroy 50 % of all the B-Cells in pancreas, so alloxan can be used for conditioning the diabetic mellitus type 2.

Naringenin as Chemosensitizing Agent on Resistant Breast Cancer MCF-7 Cells
DYANINGTYAS DEWI PAMUNGKAS PUTRI
Q : How did you get the naringeni compound?
A : Buy from some industrial pharmacy
Q : How did you keep the cell resistance by doxorubicin?
A : Treat with 7 nM of doxorubicin and 25 nM of doxorubicin

Selectivity of Awar-Awar (Ficus septica burm f.) Ethanolic Extract to WIDR Cancer Cell Line
ERLINA RIVANTI
Q : Why did you use ethanol?
A : Because ethanol is the universal solvent that can be solveable with the compound.
Q : Why do you dry the compound?
A : Because by drying we can get bigger amount of the compound.

Ethanolic extract of Citrus Maxima Peels as the source of Phytiestrogens based on Increasing Uterine Weight and c-Myc Expression on Mammary Glands of Ovariectomized Sprague Dawley Female Rats
FIKRI AMALIA
Q : Is there another parameter to assess phytoestrogen activities?
A : Bone density will increase by giving estrogen
Q : Which formulation that can be appilicated for citrus maxima?
A : It will be simple in oral route.
Q : Is there any relation of antioxidant and phytoestrogen activities?
A : There is no relationship of both of them.

Molecular Dynamic Simulation of siRNA and modified siRNAs
Elisabeth Catherina W
Q : what can your research help to siRNA and what the benefit of it?
A : As we see that the wild type is not stable at on. siRNA we can change the pharmaceutical dosage form. There is many I did to stabilize for example by lock the nucleid. The ribose has hidroxy group. The hidroxy group can be bounded to another, it is mean lock nucleid acid. There is some modification of siRNA.

Lack of evidence for anti-migratory effect of neolignan activators of PPARγ on VSMC
Nanang fakhruddin
Q : Did you do western blot with PPARγ is here for make sure this is also inhibitor adipogenesis?
A : I did not do western blot in this case, but my friend in our laboratory did the experiment with magnolol because it very natural compound. My friend did the glucose uptake and this is positif and also western blot.
Q : Do you think you can explore this compound like from the plant that growth in Indonesia ?
A : Actually the story how we select this plant is not easy. First we make computer model with PPARγ and then we use natural product databases then we choose compound that can find to the receptor and we back to the nature with plant contain with this compound. we did also several modification with the brand of the structure. We try already well compound is magnolol modification. In Indonesia maybe we can start also with the ethnomedicine approach.

Hepatoprotective Effect of Waru (Hibiscus tiliaceus) leaves infusion in Paracetamol induced hepatotoxic Rats
Harwoko
Q : How many gram or mg if you calculated this for using in human?
A : I make infusion in 3 concentration 2%, 4% and 8% then i calculate it with the dose conversion. For 2% we calculate it 250 mg/Kg/BW, 4% is 500 mg/Kg/BW and 8% is 1000 mg/kg/BW. In this research I calculate the dose from human to rat but I didn't calculate from rat to human again.
Q : What kind of saponin had been identified in this research ?
A : In this investigation we use two pereaction, so we can't identified what kind of saponin of this. We use chemical reaction only foam reaction.
Cytotoxic Activity and Apoptosis Induction of Hydrocotyle Sibthorpioides in MCF-7 Cells
Agustina Setiawati

Q: The penetration problem that they will get the different activity, is it general or it just your hypothesis or have you done some like clarification of those penetration thing because maybe also the compound have different mechanism not only the penetration!
A: It just our assumption first. So we didn’t do what going on to the cell yet, just do further molecular research on yet. Maybe we can make sure what happen to the cell with other method like western blot or other.
Q: Is it same with Centella asiatica?
A: No, it is different. Firstly it is classified into one genus but after the research of some researcher the Centella asiatica is out from this one. The morphology of leaves is different.
Q: How about the stability of the compound and is this herbs also be traditional medicine had been used for the other?
A: Actually we did not use the compound or isolated compound from this herbs. We just took the extract. In traditional Indonesian medicine is usually used for antioxidant. There so many research about this herbs is into antioxidant activity of this herbs so I think for cytotoxic activity in Indonesian maybe no.

Hesperetin enhances cancer cell growth inhibition induced by doxorubicin on doxorubicin resistant MCF-7 Cell by Sarmoko and Study of Hesperidin as Preventive Resistance Agent in MCF-7 Breast Cancer Cells Line Resistant Doxorubicin
Rifky Febriansah

Q: Why the two research using MCF-7, is there any characteristic specific of MCF-7?
Q: Is this kind of treatment using hesperidin and hesperitin only suitable for the cell which overexpression the P-gP?
Q: Hesperetin is the aglycon of the hesperidin, why the hesperidin more potent than hesperetin?
A: We use MCF-7 as resistant model because according the previous study said that the MCF-7 cell resistant have a characteristic with the overexpression of P-gP. So we want to know about the mechanism is right or not and to prove on with mechanism is right or not. So from this pre-eliminary research, we think the MCF-7 resistant in this research is through in P-gP mechanism.
A: One of characteristic MCF-7 is overexpression P-gP and hesperetin or hesperidin can over take this problem, so we take this more just cell to our research.
A: Many target can cause resistant, P-gP is one of resistant gene by MDR. The other gene that related with resistance for example PCRP and MRP. PCRP is the center resistance protein which can expression ABC G2 protein. So P-gP is one of protein and targeted therapy for resistant problem. There are many target cause resistant and P-gP is one of them.
A: we also not yet get the reason where hesperidin more potent than hesperetin. For the possibility of the reason maybe it because hesperidin is a glycoside and hesperetin is aglycon. It different in structure. From this chemically, hisperidin more soluble water than hesperetin, so it can distribute more effective and can penetrate on cell more easy than hesperetin. In addition we know that flavonoid can over take resistant with 2 mechanism that is inhibit both of expression and activation of P-gP so flavonoid can dual mechanism inhibition.
Modulation of Macrophage Immune Responses of Extract Mixture of Betel Leaf (*Piper betle, l*), Gambier (*Uncaria gambier, roxb*) and Calcium Hydroxide on Phagocytic Cells of Mice
Muhammad yanis Musdja

Q: Are people still attractive to chew this the one that your formula because everyday they like to “menyinang” because the stimulatory effect of *Nicotiana tobacum* and *Areca cathecu*, it is not contain with true ingredient, whether is it still good taste?
A: Some people just use for chewing piper bettle, gambier and Calcium hidroxide or like this plant and some people like in India use Areca cathecu and tobacco. There are research by WHO that when we chew it more than twice everyday, it can cause mouth cancer. Therefore in India recommended to do not chew bettle, areca cathecu, and tobacco because tobacco and alkaloid like arecaidine and arecoline is synergistic same like benzopiren act. In our body the mechanism of arecaidine and arecoline same with benzopiren.

Validation of Mercury Analyzer Technique for Mercury Determination in Snake Fruit
Eka Noviana (Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia)

Q: Our campus is developing a kind of test kits of mercury. Is there another method that can be used? This method is considered expensive
A: There is a simpler method who is AAS (Atomic Absorption Spectrophometry). The method used in this study was similar AAS higher levels of sensitivity but because it is designed specifically for mercury analysis
Q: What is a critical stage in the preparation of the samples so obtained samples meet terms of purity?
A: For sample preparation is a good idea to do the elimination of impurities prior first. However, this tool is automatically able to separate the samples from polluter
Q: How the selectivity of these methods to other compounds or compound-compounds that affect?
A: Metode tersebut sudah sangat selektif terhadap merkuri. Untuk senyawa lain akan menghasilkan absorban yang lebih rendah

DSC in Fast Determination of Counterfeit Paracetamol
Ahmad Yusri Mohd Yusop (Department of Pharmacy, Faculty of Medicine, University of Malaya)

Q: Why choose paracetamol in research? what the reasons for the selection counterfeit paracetamol?
A: Paracetamol is commonly known to the public as an analgesic and antipyretic. At the time of sampling with simple defects there are 5 samples taken place in Malaysia. Only one who qualifies as a sample.
Q: How to analyze a compound suspected of paracetamol counterfeit?
A: Using HPLC, in original and not original testing

Simultaneous Determination of Caffeine and Nicotinamide in Energy Drinks by First-Order Derivative Spectrophotometry
Liliek Nurhidayati ((Faculty of Pharmacy, Pancasila University, Jakarta)

Q: In Table 2, the listed dose of nicotine and caffeine. Percent recovery is obtained from where and what the purpose of comparing between the initial material and its derivatives?
A: 20 mg dose of nicotine, caffeine 50 mg. Percent recovery is obtained by standard USP. Data processing using software to obtain the results of such comparisons.

Metal Chelating Activity of Rice Bran and Rice Husk
Kartini (Faculty of Pharmacy, University of Surabaya)

Q: Can you explain why used methal chelating activity method? Another method?
A : This method was chosen because many studies use these methods to antioxidants test. There are other methods to test the antioxidant such as DPPH method, another reduction reaction
Q : Correction to the conclusion. The statement said that the extracts have activity as inhibitors or as khelating metal?
A : Several compounds have activity as khelating metal so that it can be assumed to inhibit oxidation (antioxidants)

**Synthesis and Anticancer Activity of Antimycin A₃ Analogue**
Ade Arsianti (Department of Medical Chemistry, Faculty of Medicine, University of Indonesia, Indonesia)

Q : EC₅₀ values between analog 1 may increase double, what are the benefits?
A : Make these compounds is not easy. Require many steps. The profits earned on each step can produce more than one ring.
Q : How solubility of analogous compounds?
A : Analogue compounds are very water soluble. The solution is also very easy to recrystallized.

**Sterilization Heat Effect to Gel Base Physical Properties: Gelling Agent CMC Na and Ca Alginate Case Study**
Sri Hartati Yuliani (Faculty of Pharmacy Gadjah Mada University)

Q : Why in the sterilization process with a certain level of warming, a large effect on viscosity?
A : Research done by comparing the sterilization by heat and without heat. It aims to determine the effect of heat sterilization process pd to changes in viscosity. The result states that the wet heat a smaller effect than dry heat sterilization. The physical properties of gel time and the heat affected
Q : Considered compare between wet heat and dry heat
A : The presence of water causes hydrolysis and oxidation reactions. It did not occur in the formulation so that its influence can be ascertained only at the time and temperature.
Q : The use of CMC-Na and Ca-alginate in the formulation has a specific concentration for each of the sterilization process. Why choose these concentrations and for what reason?
A : Selection refers to excipients Handbook, which states that CMC-Na and Ca-alginate sterilized at a certain temperature and if they will be mixed into a gel base.

**Dissolution Profile of Acetaminophen Tablet and Ibuprofen Tablet With l–HPC 21, l–HPC 22, and Sodium Starch Glycolate as Disintegrant in Wet Granulation Method**
Yoga Windhu Wardhana (Faculty of Pharmacy, Universitas Padjadjaran)

Q : Method for making tablets using the technique of granulation or non granulation?
A : Using the method of granulation (extract granular) to control violence
Q : What is the difference which is reflected in the dissolution profile curve?
A : The curve shows that acetaminophen destroyed faster than ibuprofen

**In vitro Antibacterial Activity of Nigella sativa Seeds Against Streptococcus pyogenes**
Endang Dwi Wulansari (STIFAR “Yayasan Pharmasi” Semarang)

Q : What is the reason to continue research with a concentration below the previous? Why is the fraction of n-hexane is used to test the dilution method while n-hexane can not diffuse?
A : The method used in this study is diffusion rather than dilution. Fraction n-hexane can not be associated with the diffuse component of the compounds contained there in. Aiming to obtain the MIC so it needs to do research with a different concentration than before. Thus
it can be seen that the lowest concentration effective against the bacteria *Streptococcus pyogenes*.

**Q** : PEG used is a liquid with the diffusion method, how to influence PEG on the antibacterial properties?

**A** : PEG is non-polar and is used as a negative control. In the test does not inhibition zones obtained so that the PEG did not provide antibacterial activity against *Streptococcus pyogenes*.

**Fast Dissolving Tablet Formulation of Metoclopramide Hydrochloride by Addition of Collidon Cl-F as Superdisintegrant**

Dolih Gozali (Faculty of Pharmacy, Universitas Padjadjaran)

**Q** : Any test conducted on the formulation?

**A** : The formula uses a combination of two tests but did not succeed in the tests. It may be used other combinations.

**Q** : Which method is used in research?

**A** : At first using wet granulation first and then do direct compressed

**Ethanolic Extract of Secang (Caesalpinia sappan L.) Wood Performs as Chemosensitizing Agent on Resistant Breast Cancer MCF-7 Cells**

Rahmi Khamsita (Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta)

**Q** : The compound is contained in a secang that acts as an anticancer?

**A** : Brasilin

**Losartan Nanoparticle Formulation With Chitosan as a Carrier and In Vitro Transdermal Test**

Nuri Ari Efiana (Faculty of Pharmacy, Ahmad Dahlan University, Yogyakarta)

**Q** : Why use a factorial design is not SLD?

**A** : Factorial design is more simple.

**Effect of Sarang Semut Tuberous (Myrmecodia tuberosa (non jack) bl.) on TCD4+ and TCD8+ Cells Profile of Doxorubicin-Induced Sprague Dawley in Vivo**

Sumardi (Faculty of Pharmacy Gadjah Mada University)

**Q** : Why choose TCD4+ cells and TCD8+?

**A** : TCD4+ is part of the immune system that delivers the immune response. TCD8+ cytotoxic cells are used for.

**Estrogenic Effects of The Ethyl Acetate Fraction of Pachyrrhizus erosus (L.) urb. tubers on Blood Cholesterol Level and Bone in Ovariectomized Sprague Dawley Rats**

Fransiska Leviana (Faculty of Pharmacy Gadjah Mada University)

**Q** : The results showed estradiol have different effects on rats and humans. How do apply the results from rats to humans?

**A** : Rats and humans have different lipoapoprotein affinity to HDL. The evidence suggests a decrease LDL but showed no increase in HDL.

**Author : Noer Khasanah**

**Q** : In our screening the gene using a bacterium, is possible to use bacteria to produce metabolites standard because some bacteria insect?

**A** : if you have poliketide this is no problem but if not you can push the microorganism to produce halogenated compound you like by good precursor.
Author: Kumala Dewi

Question:
you do a lot of treatments to increase / decrease the content so why you save this product?
what different lighting effects to produce x in this study?

Answer:
This study carried out determinations Andrographolide so just check the other contents of secondary metabolites.
provide stress conditions for secondary metabolite is an adaptation of the plant during stress conditions.

Author: Maria Ja’afar

Q
to detect sildenafil, is there a limit of detection? how the specific characters?
whether the method can be used when there are two compounds?
Is it can be used for thermal characteristics?

A:
On this project used a variety percent sildenafil, so if we increase the sildenafil in the mix then the peak in the thermogram will also be the highest and dominant. so for the limit of detection if you ask about this actually seen from the quantity of sildenafil detection is similar to paracetamol.
This method is only used for qualitative screening, not for quantitative. In Malaysia is used as a fast scanning to see the active ingredient.
.yes, certainly can. In sildenafil used to detect the nature and characteristics but I do not show here.

Author: Wahyuning Setyani

Q:
how to determine the active site of receptor?
whether this research has been done until in vivo?
whether the results of this method can be said to be valid because this method uses small angstrom?

A:
active site of protein analog selected because the protein will bind to the receptor. Cavity no.1 of the PDB structure is too interacted, and with computers that have chosen a smaller energy.
Not yet, here only selected to give a smaller energy. where the less energy necessary given the predicted effects to be better.
I'm sorry, I am not aware whether requiring small / large angstroms, which I use as the primary parameters of the potential which is seen from moldock score / rerank score that most small

Tunggul Adi P

Q: How do you fix the compartment models?

A: Conform the human activity from the data so the data is obtained from the human population, here be increased to include an important factor. more factors including the models so we can expected this.