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Dandelion LeavesEthanolicExtract Induction Apoptosis on Primary Culture Human Cervical Cancer Stem Cells Through Up-regulation RARß2 Expression

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Abstract: Cervical cancer is the most common and the second mayor cause of cancer-related mortality gynecologyc malignancies in developing countries. Cancer stem cell (CSC) theory suggested, cancer is a stem cell diseases and related to the recurrence and drug resistant. Tumor suppressor gene RARß2 expression is reduced or undetectable inmany malignancy including cervical cancerwhich involved in multiple process carcinogenesis such as apoptosis signaling. The purpose of this study was to prove the anti tumor effect of DLEE (Dandelion leaves ethanolic extract) to Cervical stem cells and induce apoptosic through up-regulating the expression both gene and RARß2 protein. We conducted a control study using primary culture human cervical cancer stem cell treated with DLEE in various doses (6.25, 12.5, 25, 50, and 100 µg/mL) for 24 h. RARß2 gene confirmed by PCR technique and Agarose gel electrophoresis. The protein RARß2 expression were measured and confirmed by immunocytochemistry technique. The result showed the Significant ($p<0.001$) effect of the DLEE to induced apoptosis as 12.25, 16.25, and 23.75% after administration DEE with doses of 25, 50, and 100 µg/mL, respectively, compared with control value of 4.00%. And also havesignificant($p<0.00$) effect to induction expression of RARß2 gene as 89.136, 102.738, 124.459 after induced DEE with doses of 12.5, 25, and 50 µg/mL, respectively. The dose effective was 100 µg/mL that increased protein RARß2 expression as 14.00%.In Conclusion, DLEEproved to have antitumor effect againstto cervical cancer stem cells by inducing apoptosis through up-regulated tumor suppressor gene RARß2 expression and induction RARß2 protein expression in primary culture cervical cancer stem cells.
Keywords: Dandelion, apoptosis, RARß2 expression, cervical cancer, cancer stem cell.

Introduction

Cancer is among the most fatal diseases threatening human worldwide [1]. Cervical cancer is the second mayor cause of cancer mortality in woman worldwide and accounts for 250.000 deaths each year. Although several factors have been linked with cervical carcinogenesis, the molecular mechanism underlying cervical carcinogenesis are still poorly understood. In recent years, emerging evidence has suggested that tumorigenesis is dependent on a small subset of cells within the tumor, term cancer stem cells (CSCs). CSCs have been isolated from several human tumors that express marker for putative normal stem cells including : leukemia, breast brain, prostat, and ovarian cancer [2]. Recent years, have been isolated cervical cancer stem cells from cervical cancer cell-line (HeLa) and from primary carcinoma of the cervix [3,4].The standard

oncology treatments have incomplete and temporary effects that only shrink the tumor, and the tumor tends to relapse mainly due to the multiple resistant mechanisms existing in CSCs [5].

Current hypothesis suggest that tumor originate from cells carry out a process of malignant reprogramming driven by genetic and epigenetic alteration. Some experiments of epigenetic changes in cancer demonstrate the relevance that these have in cancer etiology. Alterations in DNA methylation and the other epigenetic alterations are mechanisms that directly contribute to tumorigenesis [6]. Expression of the retinoic acid receptor β2 (RAR-β2), a putative tumor suppressor gene, is reduced in various human cancers, including squamous cell carcinomas (SCC) of the uterine cervix. The mechanism of the inhibition of RAR-β2 expression remains obscure. Methylation of RAR-β2 gene could be responsible for this silencing in cervical SCC [7].

The aberrant regulation of apoptosis is essential for cancer initiation. Current cancer therapies mainly include gene therapy, immunotherapy, chemotherapy, and radiation therapy, these treatments primarily exert their anticancer effects by triggering cancer cell apoptosis [1]. Apoptosis is a fundamental process in the maintenance of multi cellular organisms and its regulation is commonly disrupted in human cancer [8].

The variety of health benefits associated with the used of Dandelion have been reported. Anti carcinogenic activities have been reported for the extract of Dandelion on various tumor such as mouse skin tumors, mammary carcinogenesis, and for cancer cell-line HL-60, B16 2F2 and HeP G2 [9], and inducing apoptosis drug-resistant human melanoma cells [10].

We conducted trial of true experimental study with the *post test control group design*. In this study, we have investigated the effect of DLEE on primary culture cervical cancer stem cells (CCSCs). It is the first examination to evaluate the effect of DLEE on CCSCs. We suggest that DLEE have potential effect anticancer by induction of tumor suppressor gene RARß2 expression and induction apoptosis on CCSCs.

Material and Methods

Reagents:

FBS, penicillin/streptomcyne, collagenase 0.6% (Gibco, USA), trypsin 0,25% (Amresco, USA), DMEM-T12 (Gibco, USA), bFGF (Pepro Tech, USA), L-glutamin (Sigma), B27 (Gibco), FITC anti CD44 dan PE-cys-anti CD34 (eBioscence), ISOCGEN-LS (Wako), chloroform (Merk), isopropanol (Merk) , ethanol 70% (Merk), DEPC, MgCl2 (Merk), 10XRT-buffer, dNTP, RNasin, AMV RT enzyme, oligo-dT primer, green Ge-Taq, primer forward and reserve (mTOR c1, p70SGK, and GADPH), nuclease free water, gel agarosa, TBE buffer, EtBr, H2O2, dH2O, DAB (Diamino Benzidine), meyer Hematoxilen,peroxidase-K, Tunel fragmented DNA labelling, peroxidase solution, leaves extract etanolic Taraxacum officinale.

Instruments:

well plate (Grenier-Germany), FAC Scann (Bechman), Centrufuge, sof-ware/INH, PCR tube, microwave, electrophoresis chamber, UV transiluminator, light microscope, camera canon Fowershot g12, flowcytometer.

Experimental methods

Dandelion extraction:

The Dandelion leaves were collected from B2P2TO2T (Bale Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional) Malang, East Java. The fresh leaves of Dandelionwere transported to the laboratory, washed with water to remove all traces and dust, then dried in the shad 25-30°C for one week, with continuous overtum to prevent mould weighed, ground in a mortar and pestle, placed in airtight bottles and stored in disscator. Air dried leaves were suspended with ethanol 70% (v/v) and left for 24 h at 35°C with continuous stiring in shaking incubator. Then the mixture was filtered with filter paper. The filtrate was centrifuged for 10 min at 2500 rpm [12].

CSCs cultureand treatment.

This studies were approved by Ethics Committee of Indonesian Central Naval Hospital Surabaya. Tumor specimens were obtain from 4 patients cervical cancer (squamous cell epidermoid carcinoma, staged III/FIGO criteria). Tumor were washed with 1X CPBS containing penicillin/streptomycin (10.000 U/ml and 10.000mg/ml, respectively), mechanically dissociated and subjected to enzymatic digestion. The tissue fragments were incubated at 37°C for 3 h in 0.6 collagenase (Gibco, USA), and then for 10 min in 0.25% trypsin (Amresco, USA). The resulting tumor cells were plated at $1X10^6$ cells/well (88-well plate, Grenier, Germany) in serum-free DMEM-F-12 (Gibco), supplemented with 10ng/ml basic fibroblast growth factor (bFGF, Pepro Tech, USA), 20 ng/ml epidermal growth factor (EGF, PeproTech, USA), 5µg/ml insulin (Sigma, USA), 1mM L-glutamine (Sigma,USA), 2% B27 (Gibco), and penicillin/streptomycin (10.000 U/ml and 10.000mg/ml, respectively). The cells incubated for 24 h. Cancer stem cells grown in these conditions to form non-adherent spherical clusters cells*('boll-spheres')*. The cells were treated with DLEE at various doses 6.25, 12.5, 25, 50, 100µg/ml for 24 h.

CSCs marker analysis (Flowcytometry).

By using a FACScan (Beckman), the cell markers were distinctly evaluated on cells obtained from spheres. FITC-anti CD34, PE-cy5-anti CD44 from eBioscience. Staining was done according to the instructions of the manufacture.For staining of membrane antigens (CD44 and CD34) , unfixed cells were allowed to recover in fress medium for 1 h at 37°C in gentle gitation after dissociation. Cells that are in tubes that contain as many as 10 cells were washed with phosphate buffered saline (PBS) and then shaken slowly. Washed the cells 2 times. Last sediment plus 100 mL of PBS and plus anti-CD44-FITC (1: 100) and anti-CD34-PE (1: 100). Cells were incubated with antibody for 30 min at room temperature. The sample was diluted to 200 mL and read by flowcytometer (FACS Callibur, BD).

Apoptosis (Tunel Assay)

Slides were washed using PBS pH 7.4 and incubated using 20ug / ml proteinase-K for 15 min at 37°C. Wash using PBS pH 7.4 three times, each dive 5 min. Incubatied in 3% H2O2 for 15 min and then washed with PBS pH 7.4 three times, each 5 min. Incubated with fragmented DNA Tunel labeling(In Situ Death Detection Kit, POD) for 60 min at 37°C. Wash using PBS pH 7.4 three times, each 5 min. Incubated with peroxidase solution for 40 min at 37°C. Washed using PBS pH 7.4 three times, each 5 min. Added a substrate for peroxidase (DAB - Diamino Benzidine) for 20 min at room temperature. Washed with PBS pH 7.4 and counterstain with Mayer hematoxilen for 10 min, rinsed with tap water and washed with dH_2O drain and close the coverglass. Then observed under a light microscope with 400x magnification, apoptotic cells are shown fragmented cell and brown nucleus and calculated the percentage of cells undergoing apoptosis per 100 cells.

Analysis RARß2 gene expression (PCR)

RNA extraction. DNA/RNA extraction performed on each sample group, where 100uL sample (cell culture that has been scrap) in 1.5 mL tubes were frozen at -80 ° and placed metal block (-80 °). Added 250 µL ISOGEN-LS, and do homogenizing for 3 min. Incubated for 5 min at room temperature. Added 50 µL chloroform and do vortek, 30 seconds. Incubated for 10 min at room temperature. Centrifugated for 15 min at 12000 rpm, 4 °C. Take 150µL supernatant and place in a 1.5 mL tube and add 150uL cold iso-propanol, and do inversion. Incubated for 5 min at room temperature. Centrifugated for 10 min at 120 rpm, 4 °C. Discard supernatant, and add 200 µL cold ethanol and inverted and done tapping occasionally. Centrifugated for 10 min at 120 rpm, 4 °C. dispose of supernatant and add 200 µL ethanol 70%, inverted and done tapping occasionally. Centrifugated for 5 min at 7500rpm, 4 °C. Discard supernatant and drain the remaining water, and added 0.01% DEPC treated cold water. Save RNA at -40°C, until conduct for RT PCR

RT reaction (reverse transcriptase reaction). Reverse transcriptase is used to transform RNA strand into cDNA (RT reaction), according to the instructions on the leaflet material (Biorad inc). 2µg sample placed on the sample PCR tubes, incubation at 95 °C for 5 min (performed on PCR machine). Fast centrifugation, and are placed on ice. Add 4 µL MgCl2, 2µL 10XRT buffer, 2 µL dNTP, 0.5 µL RNasin, 0.6 µL AMV RT enzyme, 1 µL oligo-dT primers and added to 20 µL DEPC treated water. Incubated in PCR machine at 42 °C for 15 min, continue to 95°C for 5 min. And incubated 5 min on ice. And store at -20 °C until use for PCR.

PCR (Polymerse Chain Reaction). cDNA was synthesized from total RNA using iScript cDNA synthesis kit (Bio-Rad Lab) and subsequently use for the amplification of RARß2 promoter. The following primers were used for the gene expression of 360 bp fragmen from RARß2 gene forward 5' CTACACTGCGAGTCCGTCTT-3' and reverse 5'-CAGAGCTGGTGCTCTGTGTT-3'. GADPH was also amplified as an internal control with the primers, forward 5′-ACCACAGTCCATGCCATCAC-3′ and reverse 5′-TCCACCACCCTGTTGCTGTA-3′. Taken 2 µL RT reaction product in the PCR tube, add 12.5 µL green Go-Taq, 1nm forward and reverse primer (mTORC1, p70S6K and GAPDH as a control) and added nuclease free water up to 25 µL. PCR was performed in 3 cycle where, phase I with 1 cycle at 94 °C for 5 min, phase II with 30 cycles, consisting of 94 °C for 20 seconds, 55 °C for 20 seconds and 30 seconds 72 °C and stage III with 1 cycle at 72 °C for 10 min. Test results were observed in the form of bands seen RARβ2 RNA as measured by imaging sofwere / NIH.

Agarose gell electrophoresis

Weigh 0.32 g agarose and dissolved in 40ml 0.5M TBE buffer, place it in the microwave and heated 95 ° C for 1 menit, add 0.5µL EtBr and shake until mixed evenly. Pour the agarose mold plate, refrigerate up to 2 hours and placed in a horizontal electrophoresis chamber. Pour 250ml 0.5M TBE buffer. Insert the sample and running at 100 V, until the loading buffer touches the lower boundary electrophoresis plate. Gel is placed on UV transiluminator and photographed using Canon PowerShot G12, with black-white (BW) and macro mode.

Semi-quantitative analysis of cDNA expression with Image-J

Open the images from the agarose gel image-J program, create a pattern encircling bands for each DNA sample column. With the analysis tool on the main menu, do the analysis on each pattern that has formed around the ribbon of DNA. Analysis of each ribbon is done by simply sliding the circular pattern that is formed, thus ensuring that the area pattern for each band is the same measurable. Results of the analysis will appear as the average number of area and density of DNA. Do tabulation of density of each DNA for each agarose gel. Normalization of data, for subtracting the value that occurs when the gel with ethidium bromide participate in colour by reducing the value of the sample to the column without sample (H2O).

Result and Discussion

Cancer is one of the leading causes of death and globally the numbers of cases of cancer are increasing gradually [12]. Based on poorly prognosis of cancer patients and roles of cancer stem cells in cancer recurrent and chemotherapy and radiotherapy resistance are mayor indication that there are serious need for a more effective and non-toxic alternative to the conventionally available forms chemotherapy, radiotherapy and surgical prosedures. Natural products not only as sources of nourishment, but also for their therapeutic benefits, it is therefore necessary to study the vast array of natural products as non-toxic and less expensive alternatives for the treatment of cancer. Dandelion have been reported as anticancer effect. Apoptosis is a necessary modes of programmed cell death, are important mechanisms, which cells utilize for the maintain of cellular homeostasis [13]. In this study, we demonstrate theefficacy of Dandelion Leaves Extract in inducing apoptosis and regulate expression of tumor suppressor gene RARß2 in primary culture of human cervical cancer stem cells.

Boll-sphere and surfacemarkers (CD44 +, CD34-).

In this study, the group of cells which are growth in the specific stem cell medium to form non-attached cells, usually called boll-sphere (Fig. 1A). The CCSCs expressed surface marker CD44+ 98.16% and negative for CD34 (Fig. 1C), it is almost the same of cervical cancer cell-line (HeLa) expressed CD44+ 99.56% and negative for CD34 (Fig. 1B).Recent study, have been isolated a cancer stem-like cells from primary carcinoma of the cervix uteri which was expressed the cancer stem cell marker positive for membrane antigens CD44 and cytoplasmic antigens CK17, and demonstrating the expression of embryonic and adult stemness–related genes (Oct-4, Piwil2, C-myc, Stat3 and Sox2), but negative for CD34 and CD105. Analysis CD34- and CD105- are to exluded the possibility of haemopoietic and mesenchymal stem cell. CD44+ was univocally expressed in tumor-initiating cells of epithelial origin, and CK17+ as a marker of the HPV target cell-cervical reserve (stem) cell, which give rise to metaplasia [3]. Based on this data, we convinced the primary sphere culture which isolated from human cervical cancer are CCSCs.

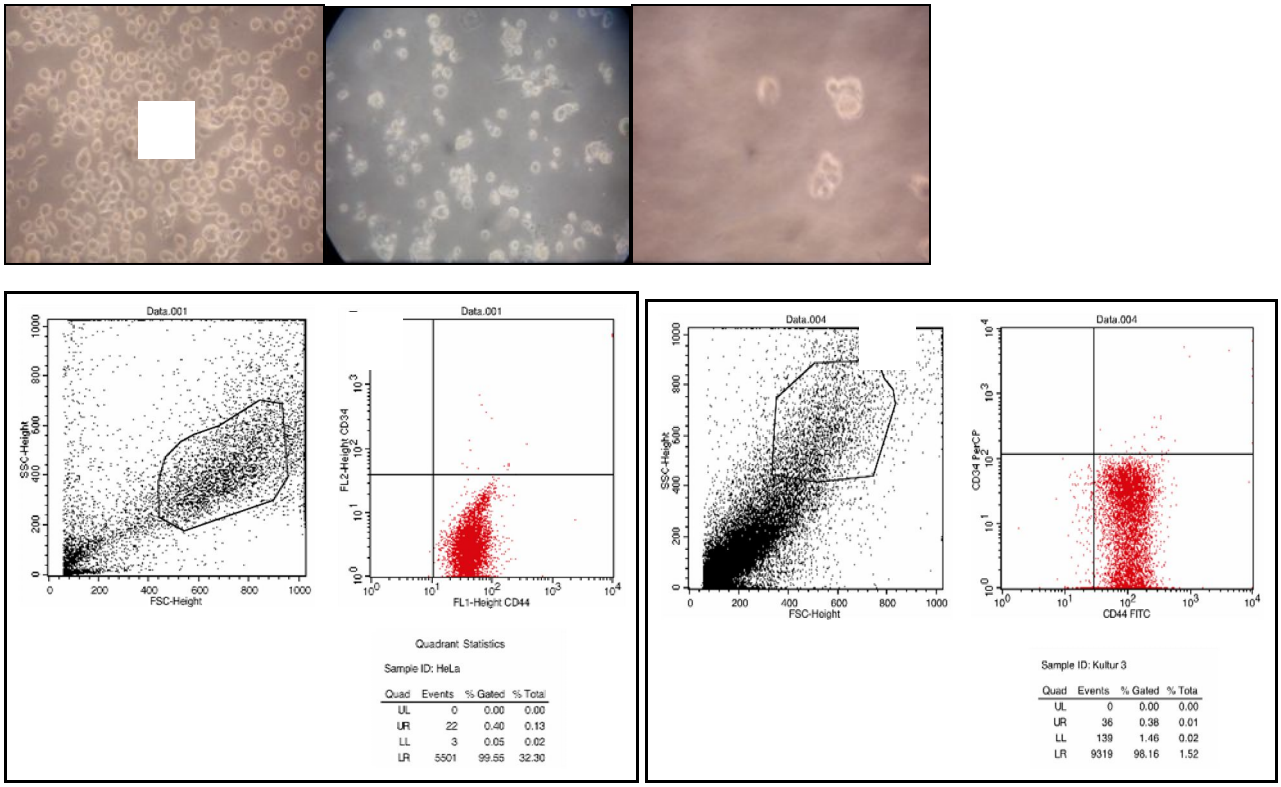

Figure 1: Sphere culture cervical cancer stem cells and CD44CD34 expression. (A) Boll-sphere of primary culture cervical cancer stem cells (magnification A1-100X, A2-200X, A3-400X); (B) Analysis Flowcytometry CD44 and CD34 HeLacells expressed CD44+ (99.56%), CD34-(0%), C. primary culture cervical cancer stem cells expressed CD44+(98.16%, CD34-0%).

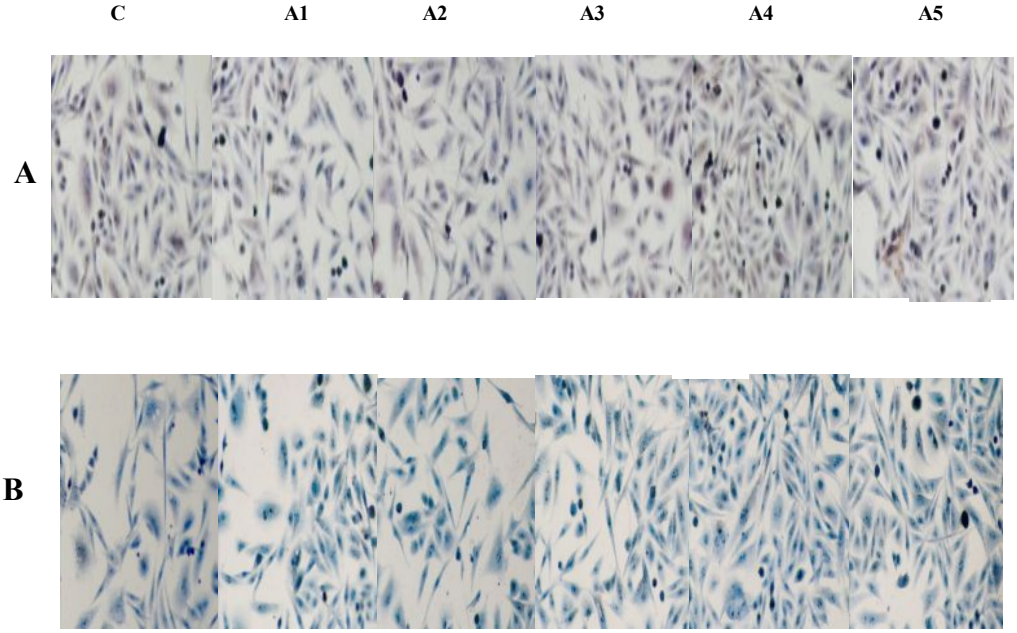

Figure 2: Apoptosis cells and cells expressed RARß2 protein. A. Apoptosis cells/Tunel (fragmented, dark nuclear) and B. Cells expressed RARß2 protein (dark nuclear): C=control, A1-5= apoptosis cells; B1-5 cell expressed RARß2 protein (ICC) exposed DLEE each dose 6.25, 12.5, 25, 50, and 100 µg/mL for 24 hr.

Apoptosis in the main target of cancer therapy. Apoptosis occurs in normal cells to maintain balance between cell proliferation and cell death. A deregulation of this balance due to modifications in the apoptotic pathway leads to different human diseases including cancer. Apoptosis resistance is one of the most important

hallmarks of cancer and some new therapeutical strategies focus on inducing cell death in cancer cells. Nevertheless, cell cancer are resistant to treatment inducing cell death because of different mechanisms, such as DNA mutations in gen coding for pro-apoptotic proteins, increases expression of anti-apoptotic proteins and/or pro-survival signals, or pro-apoptotic gen silencing mediated by DNA hypermethylation [14].

In this study we evaluated the apoptosis effect of DLEE on primary culture cervical cancer stem cells. Apoptosis analyzed by Tunel assay (fragmented and brown nucleus). The effect evaluated by calculate average percentage of apoptosis cells every 100 cells. When cells were exposed to various concentrations of DLEE ranging from 6.25 to 100 µg/ml for 24 h, DLEE shown apoptosis activity in a dose-dependent manner (4.00, 5.25, 12.25, 16.25, 23.75%). A significant effect increase in the apoptosis cells was seen at concentration 25, 50, and 100 mg/mL (12.25, 16.25, and 23.75%) compared with control value 4.00% ($p<0.001$) (Table 1). This result suggest that DLEE contain a bioactive component which are effective induced apoptosis CCSCs.

Tabel 1: Apoptosis of primary culture human cervical cancer stem cells treated with various doses of DLEE.

Group	N	Apoptosis				Anova (p)
		$\bar{x}$	SD	Minimum	Maksimum	
Control	4	4.00[a]	1.826	2	6	<0,001*
DLEE 6,25 µg/ml	4	4.00[a]	1.826	2	6	
DLEE 12,5 µg/ml	4	5.25[ab]	1.708	3	7	
DLEE 25 µg/ml	4	12.25[cd]	2.217	10	15	
DLEE 50 µg/ml	4	16.25de	1.500	15	18	
DLEE 100 µg/ml	4	23.75[f]	2.217	21	26	

* significant α=0,05; [abcdef]same superscript shows that there isn't any significant differences (Tukey's HSD).

Recent study have been reported the efficacy of Dandelion Root Extract (DRE) in inducing apoptosis in drug-resistant Human Melanoma cell-line. Treatment with 2.5 mg/mL DRE for 24 h resulted in up to 50% reduction in cell viability measured by WST-1 assay. The other effective effect of DRE in apoptosis processes through induction of caspase-8 activity at 2.5 mg/mL for 48 hours, decrease in the levels of Bcl2 expression [10]. The crude extract effect of dandelion on breast cancer cell line and prostate cancer cell line has been evaluated which the leaf extract (DLE) decreased the growth of breast cancer cell line in an ERK-dependent manner, and the root extract (DRE) was found to block invasion breast cancer cell line, while DLE block invasion of prostate cancer cell line. Inhibition of invasion was further evidenced by decreased phosphorylation levels of FAK and src as well as reduced activities of matrix metalloproteinases, MMP-2 and MMP-9 [9]. Induction of programmed cell death in three human CMML cell lines (identified by two main characteristics of apoptosis, nuclear condensation and externalization of the phosphatidylserine) reported by Ovadje et.al. The result indicate that DRE is able to efficiently and selectively induce apoptosis and autophagy (self-eating) in these cell line in a dose and time dependent manner, with no significant toxicity on non-cancerous peripheral blood mononuclear cells. More importantly, observed early activation of initiator caspase-8, with led to mitochondrial destabilization and the induction of autophagy, suggesting that DRE acts through the extrinsic pathway of apoptosis. The doses of DRE are 0.6, 1, 2.5, and 5 mg/mL for 48 hours [13].

RARβ2 tumor suppressor gene is a member of the super family of receptors that regulate thyiroid-steroid regulation of transcription in the nucleus of cells, affects the growth and differentiation of normal cells, pre-malignant and malignant cells [15]. Many studies have demonstrated that among these receptors, loss of RARß2 is the most common and the loss is progressive in premalignant and malignant tissue and cells including cervical cancer [16]. RAR-β2 gene has been shown decreased even disappear in various types of human cancer, including squamous cell carcinomas (SCC) of the uterine cervix, carcinoma of the head and neck, breast cancer [6]. Silencing RARß2 mRNA expression in a number of malignant tumors, including lung carcinoma cell lines, squamous cell carcinoma of the head and neck, and breast cancer may be an important event in tumorigenesis. One study showed that the RARß2 gene, as well as the tumor suppressor gene p53, can inhibit focus formation induced by oncogenes. RARß2 gene expression in breast cancer decreased along with the increase in cancer stage, even in grade III had not expressed [17]. From study of cervical cancer and cervical cancer cell lines, there is a significant decrease RAR-β2 mRNA compared to normal tissue. ?? About 40% of cervical SCC shown decrease of RARß2 mRNA and all of them showed methylation of the promoter. The RARß2 gene from non-neoplastic cervical tissue was mostly unmethylated and expressed [7]. The

mechanisms underlying the loss of RARß2 gene expression are not fully understud. Multiple studies have been focused on decoding the genetic and epigenetic mechanisms responsible for the acquisition of stemness features and CSC genesis. Genetic and epigenetic changes would provide survival adventages in CSC supopulation and contribute to tumor initiation capability and tumor progression. Hypermethylation of some tumor suppressor genes, that drive oncogenesis at early stages, was already present in the CSCs [6].

Many studies showed that loss of RARß2 expression in cancer caused by chromosome 3p deletion, decreased levels of co-activators, the presence of co-repressors. Nevertheless, many recent studies shown that loss of RARß2 gene results from epigenetics silencing by methylation of cytosine-phospho-guanosine (CpG) islands in the promoter region of the gene [16].

Tabel 2: RARß2 protein expression of primary culture human cervical cancer stem cells treated with various doses of DLEE.

Group	n	RARb2 protein expression (ICC)				Anova (p)
		$\bar{x}$	SD	Minimum	Maksimum	
Control	4	3.25[a]	1.893	2	6	<0,001*
DLEE 6,25 µg/ml	4	3.25[a]	1.893	2	6	
DLEE 12,5 µg/ml	4	4.50[a]	1.732	2	6	
DLEE 25 µg/ml	4	5.50[ab]	2.517	2	8	
DLEE 50 µg/ml	4	9.50[bc]	1.291	8	11	
DLEE 100 µg/ml	4	14.00[cd]	3.367	10	18	

* significant α=0,05; [abcdef]same superscript shows that there isn't any significant differences (Tukey's HSD).

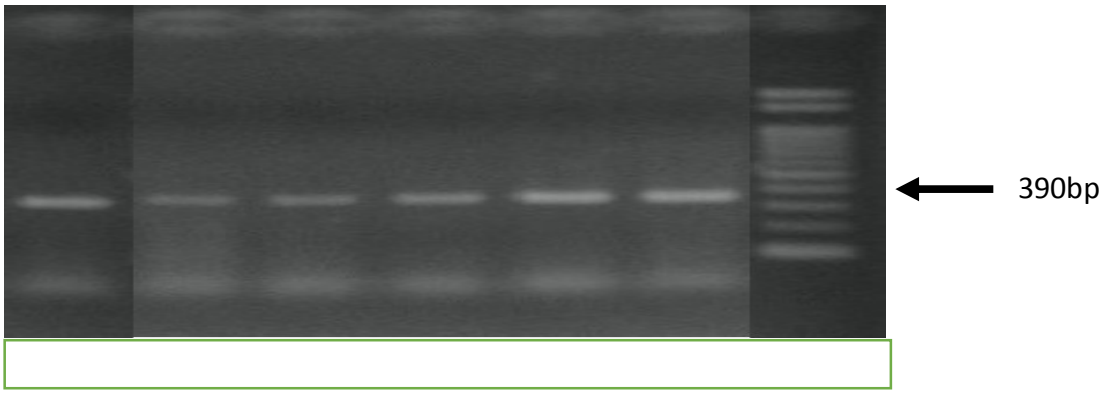

Figure 3: Electrophoresis gel agarosa RT- PCR RARß2 gene expression primary culture human cervical cancer stem cells.1=control, 2-6 = DLEE each doses 6.25, 12.5, 25, 50, and 100 µg/mL; M=Marker.

Tabel 3: RARß2 gene expression of primary culture human cervical cancer stem cells treated with various doses of DLEE.

Groups	n	RARβ2 gene expression (RTPCR)				Brown-Forsythe (p)
		$\bar{x}$	SD	Minimum	Maximum	
Control	4	54.246[a]	7.168	48.998	64.380	<0,001*
DLEE 6.25 µg/ml	4	60.149[a]	3.959	54.703	63.996	
DLEE 12.5 µg/ml	4	89.136[bc]	4.866	82.923	94.804	
DLEE 25 µg/ml	4	102.738[de]	6.376	96.632	111.537	
DLEE 50 µg/ml	4	124.459[fg]	2.683	120.826	127.224	
DLEE 100 µg/ml	4	64.109[ab]	11.278	55.019	80.004	

* significant α=0,05; [abcdef]same superscript shows that there isn't any significant differences (Gomez-Howell).

Dysregulated apoptosis is a mayor hallmark of cancer, and retinoic acid showed effect in induction of apoptosis in cancer by various mechanism. The most obvious role for retinoic acid in apoptotic regulation is via regulation of gene expression [18]. Recent studies have demonstrated that in many malignant cell lines and cancer cell the level of RARß2 is decreased or undetectable. A decrease of RARß2 mRNA level are also

observed in carcinomas of cervix [7].RARß2 has been well known for its tumor suppressive effects in epithelial cells and exogenous expression of the RARß2 gene can cause RA-dependent and RA-independent apoptosis and growth arrest [19]. Dandelion extract which are containing a lot of vitamin A has been very effective in inducing apoptosis [10]. In this study we evaluated the effect of DLEE regulate expression of RARß2 gene on CCSCs (Fig 3). When cells were exposed to various concentrations of DLEE ranging from 6.25 to 100 µg/ml for 24 hr, by RT-PCR analysis shown relative expression of RARß2 gene 60.149, 89.136, 102.738, 124.459, and 64.109 (Fig. 3 and Table 3). A significant effect increases expression RARß2 gene at concentration from 12.5 to 50 µg/mL (89.136, 102.738, 124.459) compared with control value 54.246 ($p<0.001$). DLEE seen a significant effect to induce RARß2 gene expression which are silenced in CCSCs. The functional product of RARß2 gene is protein RARß2. We evaluated protein RARß2 expression which as a functional product of the gene. When cells were exposed to various concentrations of DLEE ranging from 6.25 to 100 µg/ml for 24 hr, by ICC analysis shown the effect on expression of RARß2 protein each 3.25, 4.50, 5.50, 9.50, and 14 % compare with control value 3.25% (Table2). A significant effect increases expression RARß2 protein at concentration 50 and 100 µg/mL (9.50 and 14%) compare with control value 3.25% ($p<0.001$). It is well known, down-regulation RARß2 caused by hypermethylation and it can be up-regulated with retinoic acid. So, DLEE induction RARß2 expression through demethylation effect and followed by apoptosis CCSCs.

Conclusion

In conclusion, our data demonstrate that biologic active componentof DLEE act an effective antitumor effects which induction apoptosis throughup-regulated tumor suppressor gene RARß2 expression and induction RARß2 protein expression in primary culture cervical cancer stem cells. Futhermore, the results indicate that the anticancer active compounds of DLEE my be used in the development of new agents or drug to combat cervical cancer stem cell and to reduce the recurrent cancer.

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