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The Efficacy of *Taraxacum officinale* Leaves Extract in Regulate Apoptosis, RARß2 gene and Sox2 expression on Primary Culture Human Cervical Cancer Stem Cells.

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Abstract: According to the cancer stem cell (CSC) theory, cancer is a stem cell diseases and CSCs have the potential for infinite proliferation, and play a crucial role in carcinogenesis, metastasis, recurrence, and drug resistant. Sox2 is one of the stem cell marker and recent studies have demostrated that Sox2 was abnormally expressed and related to drug resistance and relaps. Therefore, target therapy of CSCs is very important in cancer management. Dandelion (Taraxacum officinale) is well-known a folk medicine as anti cancer. Our study investigated the effects obtained from Dandelion leave on human cervical cancer stem cells (CCSCs). Cells were treated with Leaves Ethanolic Extract Taraxacum officinale (TO) ranging from 6.25 to 100 µg/mL for 24 hours. Significant apoptosis effect of TO were observed at 25, 50, and 100 µg/mL (12.25, 16.25, and 23.75%) compared with control value 4.00% ($p<0.001$), and this induction showed in a dose-dependent manner. The effective dose of TO to decreased Sox2 expression is 100 mg/mL (3.75%) compared with control value 12.00% ($p<0.001$), meanwhile, the effective doses of TO inducing expression of RARß2 gene are at 12.5, 25, 50, and 100 mg/mL (89.136, 102.738, 124.459, and 64.109) compared with control 54.246 ($p<0.001$) . Our findings indicated that TO is an effective induction apoptosis cervical cancer stem cell through down-regulation Sox2 expression and increased RARß2 gene expression.

Keywords: Taraxacum officinale, apoptosis, Sox2, methylation RARß2, cervical cancer, cancer stem cell.

Introduction

Cervical cancer is the second most common gynecologic cancer after breast cancer, and this malignancy is the most important causes of mortality with 250.000 deaths each year worldwide [1]. Most cases of clinical early-stage disease (stage IA1-IIA, according to the FIGO) are treated and cure with either

hysterectomy of primary radiotherapy. In spite of therapeutic mesures such as neoadjuvant chemotherapy and radiotherapy, the overall recurrence rate within 1 years upon the completion of therapy is 50%, and 75-80% within 2 years in the patients. Local recurrence in the pelvic cavity accounts for 70%, and distant metastasis for the remaining 30% [2]. Although traditional surgery, radiotherapy and combined treatments have obtained good results for early-stage cervical squamous cell carcinoma, the results from patiens who have been treated, which is an advanced and recurrent cervical cancer, are not favorable. The five-year survival rate is 30-50% for patient with stage III cervical carcinoma and only 5-15% for patients with stage IV disease. The five-year survival rate for patients with local recurrence and distant metastasis is upto 10% [3].

According to the CSCs hypothesis suggests that tumor masses may arise from a single cancer cell with stem-like properties [4]. So that, cancer is a stem cell disease [2]. CSCs have the potential for infinite proliferation, and play a crucial role in the initiation and progress of tumorigenesis. 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]. CSCs have been isolated from several human tumors that express marker for putative normal stem cells, including leukemia, breast brain, prostat, and ovarian cancer [3]. Recent years have been isolated cancer stem-like side population in HeLa a human cervical carcinoma cell line (expressed CD44 and CD133), the other experiment from primary carcinoma of the cervix uteri which cells are positive for surface marker CD44 but negative for CD34 and CD105 and express embryonic and adult stemness-related gene (Oct-4, Piwil2, C-myc, Start3 and Sox2) [2,6].

Sox2, one of the gene that maintains self-renewal of embryonic stem cells ant relates to the differentiation potential of these cells, is abnormaly expressed in various human tumor such as ovarian cancer, breast cancer, pancreatic cancer, lung squamous cell carcinoma, and gastrointestinal tumors [1]. Sox2 plays an early role in breast carcinogenesis and high expression my promote metastatic potential [7]. In the present study illustrated the expression of Sox2 in normal and pathologic cervical tissues, as well as cervical cancer cell lines. Sox expression does not shaw any association with the HPV infection. Sox2 expression in human cervix about 80% of CIN III or cervical squamous carcinoma, but it expressed in only 25% of normal cervix. Sox2 my play an important role in tumorigenesis because overexpression of Sox2 increased proliferation, clonogenicity, and tumorigenicity in cervical cancer, and these results suggest Sox2 is a possible therapeutic target molecule in cervical cancer [8].

Retinoids are active metabolite of vitamin A have been investigated extensively for their utility in cancer prevention and treatment such as leukemia, breast cancer. The limited treatment success observed to date in the prevention and treatment of solid tumors may relate to the frequent epigenetic silencing of RARß [9]. The leaves *Taraxacum officinale* possesses a higher content of ß-caroten as source vitamin A than carrot [10]. Plants of the genus *Taraxacum*, also known as dandelion, are members of the Asteraceae family, as anti carcinogenic activities of the aqueus root extrat of *Taraxacum japonicum* on mous skin tumors, inhibited spontaneous mammary carcinogenesis. In the present study, the aqueous extraxts of *Taraxacum officinale* were prepared from the matur leaves, flowers and roots, and investigated on tumor progression related processes such as proliferation and invasion. The crude axtract of dandelion leaf decreased the growth of MCF-7/AZ breast cancer cells, whereas the aqueous extracts of dandelion flower and root had no effect on the growth of the either cell line. Furthermore, root extract was found to block invasion of MCF7/AZ while dandelion leaf extract block the invasion of LNCaP prostat cancer cells [11]. *Taraxacum officinale* leaves ethanolic extract bave been reported as immunostimulatory agent for reducing side effect of doxorubicin in Sprague Dawley rats [12]. Chatterjee et al. have been reported the efficacy of dandelion root extract in inducing apoptosis in drug-resistant human melanoma cells, without toxicity to healthy cells [13].

We conducted trial of true experimental study with the post test only control group design investigating the effect of TO in regulating apoptosis, Sox2 expression, and expression RARß2 gene on primary culture CCSCs. In this study, we had investigated the effect of TO on primary culture human CCSCs which as isolated from 3th stadium of squamous cell cervical carcinoma patients. For human primary culture CCSCs, we have shown that TO has been effective in inducing apoptosis, inducing RARß2 gene expression, and down regulated Sox2 expression. Based on our results, we suggest that TO anticancer activities might contribute to cancer therapy for the future, however further studies are needed to isolate the active components and to elucidate the exact mechanism of action *in vitro* and *in vivo*.

Material and methods

Mayor reagents and instruments.

Setting: This experiment has been conducted in laboratory Physiology, Biochemistry and Central Biomedical Laboratory of Faculty Medicine, Brawijaya University, Malang, East Java, Indonesia.

Mayor reagents: FBS, penicillin/streptomcyne, collagenase 0.6% (Gibco, USA), trypsin 0,25% (Amresco, USA), DMEM-T12 (Gibco, USA), bFGF (Pepro Tech, USA), FITC anti CD44 dan PE-cys-anti CD44, anti Sox2, antigen membrane (CD34, CD44), rabbit polyclonal anti Sox2 (Lab Vision), DAB (Diamino Benzidine), meyer Hematoxilen, dH2O,, proteinase-K, Tunel fragmented DNA labelling, peroxidase solution, leaves extract etanolic *Taraxacum officinale* .

Mayor instruments: well plate (Grenier-Germany), FAC Scann (Bechman), Centrufuge, light microscope, camera canon Fowershot g12, flowcitometer.

Experimental methods.

Taraxacun officinale extraction:

The *Taraxacum officinale* leaves were collected from B2P2TO2T (Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional) Malang, east Java. The *Taraxacum officinale* leaves were transported to the laboratory, washed with water to remove all traces and dust, then dried in 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 hrs at 35°C with continuous stiring in shaking incudator. Then the mixture was filtered by filter paper. The filtrate was centrifuged for 10 min at 2500 rpm [10].

Cancer stem cell culture and treatment.

This studies had been 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), 2% B27 (Gibco), and penicillin/streptomycin (10.000 U/ml and 10.000mg/ml, respectively). Stem cells grown in these conditions usually form non-adherent spherical clusters (also named 'spheres'). The cells were treated with TO at various doses 6.25, 12.5, 25, 50, 100µg/ml for 24 hr, and RA at doses 13.3, 26.7, 53.3, 106.7, and 213.3 µg/mL. This study aimed at determining direct effect of TO exposure on primary culture cervical cancer stem cell to regulate apoptosis, Sox2 exprssion, and RARß2 gene expression.

CSCs marker analysis (Flowcytometry).

By using a FACScan (Beckman), the cell markers were distinctly evaluated on cells obtained from spheres. We used FITC-anti CD34, PE-cy5-anti CD44 from eBioscience, USA. 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 fresh medium for 1 h at 37°C in gentle gitation after dissociation. Cells that are in tubes which contain as many as 10^6 cells were washed with phosphate buffered saline (PBS) and then shaken slowly. Washed the cells to 2 times. Last sediment added 100 mL of PBS and anti-CD44-FITC (1: 100) and anti-CD34-PE (1: 100). Cells were incubated with antibody for 30 minutes at room temperature. The sample was diluted to 200 mL and read by FACSCallibur Flowcytometer (Beckman).

Apoptosis analysis

Apoptosis analyzed by Tunel assay (In Situ Cell Death Detection Kit, POD). Slides were washed using PBS pH 7.4 and incubated using 20ug / ml proteinase-K for 15 minutes at 37°C. Washed using PBS pH 7.4

three times, each dive 5 minutes. Incubated in 3% H_2O_2 for 15 min and then washed with PBS pH 7.4 three times, each 5 min. Incubated with fragmented DNA Tunel labeling for 60 minutes at 37°C. Washed 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, rinse with tap water and washed with dH2O drain and close the coverglass. Then observed under a light microscope with 400x magnification, apoptotic cells are shown in brown in the cell nucleus and calculated the percentage of cells undergoing apoptosis per 100 cells.

Analysis Sox2 expression (ICC).

Slides were washed with pH 7.4 PBS one time for 5 minutes. Blocking endogenous peroxide using a 3% H_2O_2 for 20 minutes. Washed using pH 7.4 PBS three times, diving 5 minutes. Blocking unspecific protein using 5% FBS containing 0.25% Triton X-100. Washed using pH 7.4 PBS three times, for 5 minutes. Incubated using rabbit polyclonal anti SOX-2 (LabVision), for 60 minutes.Washed using pH7.4 PBS three times, diving 5 minutes. Incubated using HRP conjugated anti-rabbit for 40 minutes. Washed using pH 7.4 PBS three times, diving 5 minutes. Dropped with DAB (Diamino Benzidine) and incubated for 10 min. Washed using pH7.4 PBS three times, diving 5 minutes. Washed using dH_20, diving 5 minutes. Counterstaining use Hematoxilen Mayer were incubated for 10 minutes and washed using tap water. Rinsed and dried using dH_2O aired. Mounting using entelan and cover with a cover glass. Observe under the light microscope and calculated the percentage of cells undergoing activation SOX2 is microscopically brown cell nucleus.

Analysis RARß2 gene expression (PCR)

RNA extraction.

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 °C and placed metal block (-80 °C). Added 250 µL ISOGEN-LS, and homogenizing for 3 min. Incubated for 5 min at room temperature. Added 50 µL chloroform and vortex, 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 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. Centrifugated for 10 min at 120 rpm, 4 °C. dispose of supernatant and add 200 µL ethanol 70%, and inverted. 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 done RT reaction.

RT reaction (reverse transcriptase).

Reverse transcriptase is used to transform RNA stran into cDNA (RT reaction), according to the instructions (Biorad inc). 2µg sample placed on the sample PCR tubes, incubated 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 PCR.

PCR (Polymerse Chain Reaction).

The 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 amplification of 390 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 gel 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 minutes, 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).

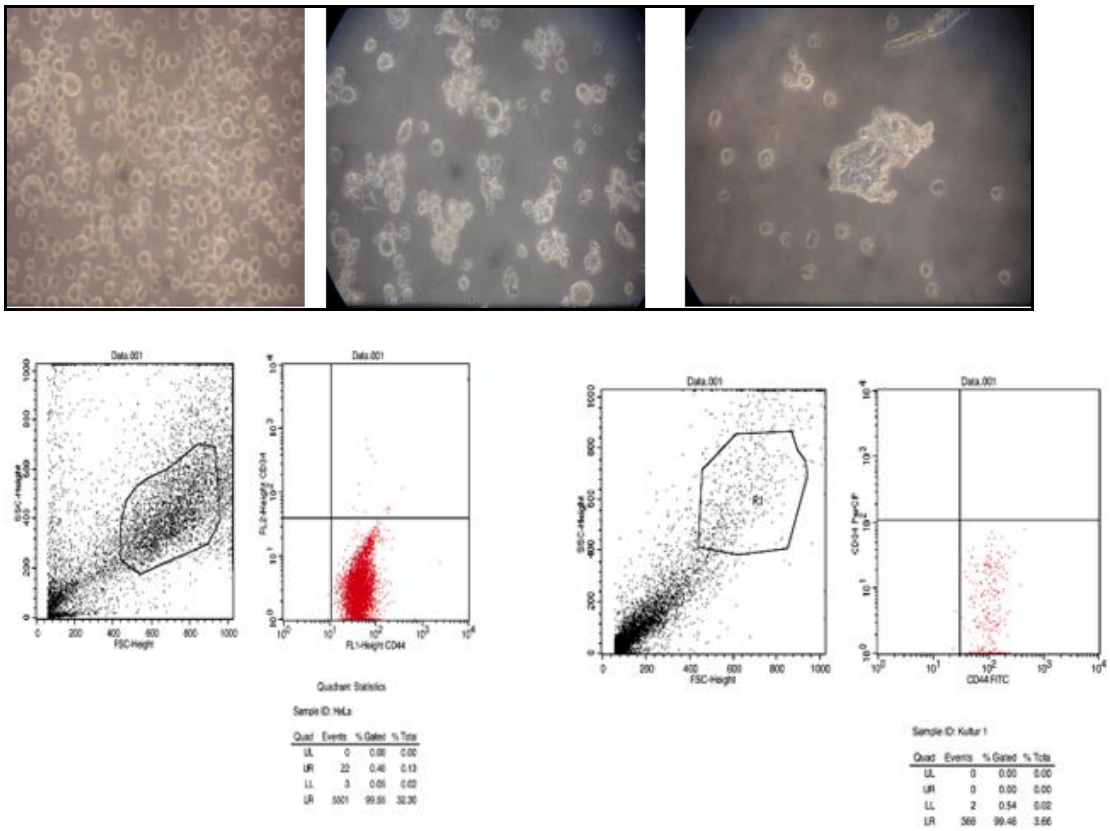
Statistic analysis

Data analysis was performed by descriptive and inferential. Descriptive analysis is done by calculating the mean and standard deviation, and minimum and maximum values for each variable in each group. Data presented in tables and diagrams. Inferential analysis was conducted by the dependent variable data comparisons between treatment groups. Statistic analysis for apoptosis and Sox2 performed one-way ANOVA, followed by Tukey HSD, and expression of gene RARß2 perform Brown-Forsythe, followed by Games-Howell.

Results and Discussion

Cancer is a mayor public health burden in both developed and developing countries. Every year, milions of people are diagnosed with cancer, leading to death. According to the American Cancer Sosiety, deaths arising fron cancer constitute 2-3% of the annual deaths recorded worldwide. Thus cancer kills about 3500 million people annually all over the world [Prakash et al., 2013]. In women cervical cancer is the second most common gynecologic malignancy after breast cancer. This malignancy is one of the most important causes of mortality, with 250.000 deaths each year worldwide [1]. Historical perspective has been clear that only a minority of cells from most hematologic malignancies and solid tumor are clonogenic in vitro and in vivo. The minority group of cells called tumor stem cells [15]. CSCs have been isolated from several tumors such as leukemia, breast, brain, prostate, ovarian cancer [3].

We success isolated cancer stem cell from squamous cell cervical carcinoma which expressed CD44+ and CD34-. The groups of cells which are growth in the specific stem cell medium to form non-attached cell, usually called boll-sphere (Fig.1). By flow cytometric analysis, these cell expressed cancer stem cell marker CD44+ 98.33% and negative for CD34 (Fig.1B1), compared with HeLa cell expressed CD44 99.56% and negative for CD34 (Fig. 1B2). Present studies has been isolated cancer stem cells which are expressed CD44+, CD133+ from HeLa cell line [*3*], Brcp1+ from HeLa cell line [2], CD44+ CK17+ but negative for CD34 and CD105, and stem ness genes (Oct-4, Piwil2, C-myc, Stat3, and Sox2) from primary carcinoma of the cervix uteri [*6*]. Based on this data, we convinced the primary sphere culture which isolated from human cervical cancer are cervical cancer stem cells.



Figures 1: Ball-sphere of primary culture human cervical cancer stem cells (observed under a light microscope with magnification A1-100X, A2-200X, (A3-400X). Flowcytometry: Analysis Flowcytometry CD44 and CD34: (B1) cells HeLa expressed CD44+ (99.55%), CD34-(0%), (B2) primary culture cervical cancer stem cells expressed CD44+(99.46%), CD34-(0%).

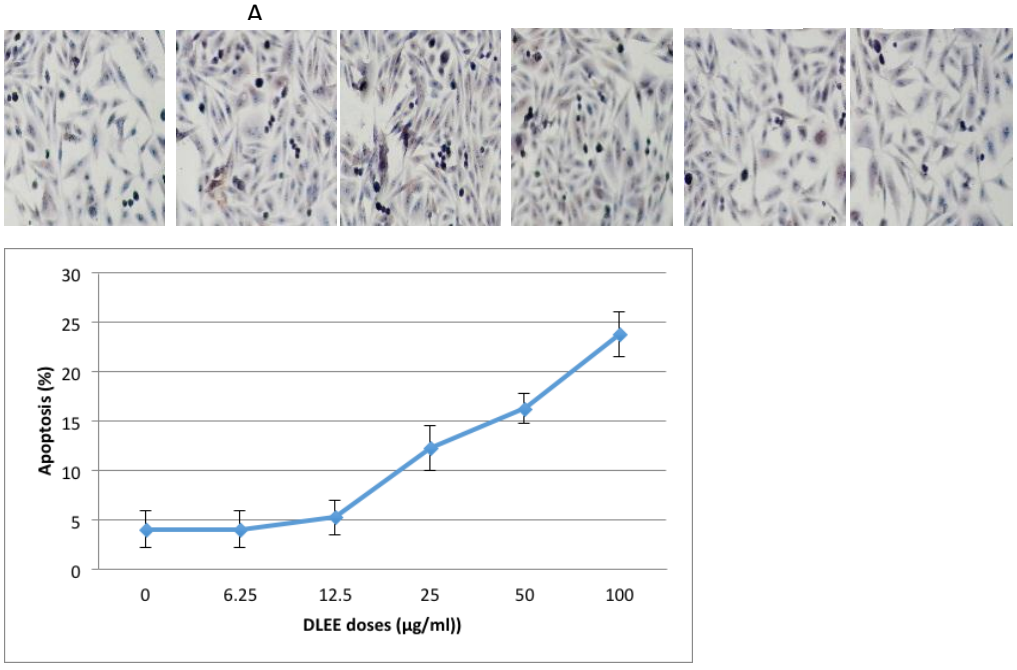


Figure 2: Apoptosis effect of TO (Tunel assay) on primary culture human cervical cancer stem cells. Apoptosis cells :: apoptotic cells (fragmented and brown nuclear). K=control, A-E= apoptosis cells treated to TO each dose 6.25, 12.5, 25, 50, and 100 µg/mL; Curve on the average % apoptosis effect (per 100 cells microscope field). TO exposure (black line) 4, 5.25, 12.25, 16.25, and 23.75%. A significant increasion in the cell apoptosis was seen with TO treatment of concentrations of 25, 50, and 100 µg/mL (12.25, 16.25, and 23.75%) of the cells population compare with control value 4.00% ($p<0.001$

Dandelion extract has thus far been used in traditional medicine for aliments ranging from digestive disorders to complex disorders such as uterine, breast and lung tumours [13]. Leaves, flowers and root extracts of *Taraxacum officinale* was investigated against tumor progression related processes such as invasion and proliferation. Among al the extracts, leave extract of *Taraxacum officinale* reduced the grouth of breast cancer cells [14]. Other repot shown that rich flavons as luteolon and luteolin 7-glucoside exist in the dandelion, which contributed to noted in vitro antioxidant and anticancer activity. These flavones are also showed the biological activity without introducing cytotoxicity and anti proliferatife effects of dandelion flower extract were investigated on SK-OV-3 cells [16]. In this study proved the ethanolic extract of the *Taraxacum officinale* leaves as effective anticancer through induction apoptosis, suppression Sox2, and induction RARß2 genes expression by demethylation effects on primary culture cervical cancer stem cells.

Apoptosis in the main target of cancer therapy. Apoptosis is regarded as an active and organiszed form cell death, trigered in response to physiologic or pathologic stimuli. It is characterized by condensation and fragmentation of the chromatin accompanied by intranucleosomal DNA clavage, caspase activation, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, and formation of the so-called "apoptosis bodies"[17].

In this study we evaluated the apoptosis effect of TO on primary culture cervical cancer stem cells. Apoptosis analyzed by Tunel assay (fragmented and brown nucleus, Fig.1). The effect evaluated by calculate average percentage of apoptosis cells every 100 cells in microscope field. When cells were exposed to various concentrations of TO ranging from 6.25 to 100 µg/ml for 24 hr, TO shown apoptosis activity in a dose-dependent manner. Effect TO induced apoptosis each 4.00, 5.25, 12.25, 16.25, 23.75%. A significant effect increase in the cell apoptosis was seen with TO treatment at concentration 25, 50, and 100 mg/mL (12.25, 16.25, and 23.75%) compared with control value 4.00% (Anova, $p<0.001$).

In the present study, the Dandelion flower ethanol extract (DFE) effect to induced apoptosis by activation of p53 investigated in SK-OV-3 cells, which demonstrated significant cell cycle arrest and sub-G0 DNA fragmentation. Activation of p53 in response to DNA damage led to cell cycle arrest and inhibition of cell proliferation. Moreover, DFE treatment resulted in an increase in p21 expression, a dawn stream target of p53, could inhibit the G1-S phase transition and result in G1 phase cell cycle arrest. Modulation of BCl-2 and Bax was observed after exposure to DFE in dose-dependent manner. Members of Bcl-2 family of proteins are critical regulators of apoptotic pathway, controlling mitochondrial permeability and cytochrome-c expression [16].

Transcription factor Sox2 is located on chromosome 3q26.3 plays an important role for the properties of stem cells such as differentiation, self-renewal and proliferation [8]. Sox2 protein impacted many processes of cancer cells such as maintenance of stemnes, proliferation ad growth, maintenance of tumorigenicity, tumorigenesis, invasion, cellular migration, metastasis, aupoptosis and chemoresistance. In clinics, high levels of Sox2 are correlated with poor prognosis and increased proliferation of cancer stem cells [18]. Sox2 is marker for cervical cancer stem cells (SiHa and C33A) [4]. Increases cervical cancer stage followed by increased expression of Sox2 (25% of normal cervix; 83.3% of CIN III and invasive cervical cancer); and expression in stage II and III significantly increased compared to stage I. Sox2-overexpressing in cervical cancer cells had increased proliferation, clonogenicity, and tumorigenicity in vitro and in vivo [8]. Increased Sox2 expression in breast cancer begun early stage and happened earlier than other transcription factors such as Nanog and Oct4 (7). Sox2 is a key regulatory gene of CSCs and by reduced SOCE activity plays an important role in apoptosis-resistant properties of prostat cancer [19]. Decreases of dawn stream of Sox2 such as survivin and p27 can induction apoptosis and Sox2 gene amplification followed the low life expectancy of cancer patients [17], it proved to be involved in the development of cervical cancer and the others (8,18), invasion and metastasis of cancer cells [8,18]. Reduction Sox2 expression in neural stem cells, followed by a decrease in cell proliferation, differentiation and induction of apoptosis [20], and suppressed the growth and metastasis of gastric cancer [21].

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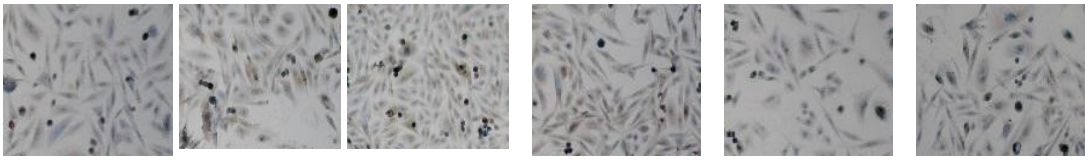

**Figure 3: Sox2 expression of primary culture human cervical cancer stem cells treated with various doses of TO and RA. *Cells expressed Sox2* : K=control, A-E= expression Sox2 cells treated to TO each dose 6.25, 12.5, 25, 50, and 100 µg/mL; **

This is the first study to evaluation Sox2 effect of TO on primary culture cervical cancer stem cells. The primary culture cell we treated with various doses of TO from 6.25 to 100 µg/ml. Sox2 expression analyzed by immunocytochemistry technic (ICC shown brown nucleus of the cell, Fig.3). The effect evaluated by calculate average percentage of cell expressed Sox2 every 100 cells in microscope field. When cells were exposed to various concentration of TO ranging from 6.25 to 100 µg/mL for 24 hr, TO showed reduced Sox2 expression in a dose-dependent manner (12.00, 10.50, 7.75, 8.00, and 3.75%). A significant decrease Sox2 expression was seen with TO treatment at concentrations 100 mg/mL (3.75%), compared with control value 12.00% ($p<0.001$).

The term "epigenetic" refers to modifications in the gene expression caused by heritable, but potentially reversible, changes in DNA methylation and chromatin structure [22]. Cancer is a multistep process in which genetic and epigenetic errors accumulate and transform a normal cell into an invasive or metastatic tumor cell. Altered DNA methylation patterns can change the expression of cancer-associated genes [23]. In solid tumor, RARß expression is frequently lost in primary tumors and their metastasis compared to adjacent non-cancerous tissues, and many studies extensive evidence that RARß2 is silenced in cancer [9]. Expression of the RARß2, a putative tumor suppressor gene, is reduced in various human cancer, including squamous cell carcinomas of the uterine cervix. Recent studies has been shown that the retinoic acid can induce an increases of RARß2 expression in carcinoma cell line including cervical carcinoma cell lines]24].

In this study we examined whether the demethylation effect of TO could be responsible for increases of RARß2 gene expression in primary culture squamous cervical cancer stem cells (Fig.4). This is the first study to evaluation Sox2 effect of TO on primary culture cervical cancer stem cells. Demethylation effect analysis by evaluate the expression of RARß2 genes (CRT-PCR technic). When cells were exposed to various concentrations of TO ranging from 6.25 to 100 µg/ml for 24 hr, TO shown increases relative expression RARß2 gene (60.149, 89.136, 102.738, 124.459, and 64.109, Fig 5). A significant effect increase in the relative expression RARß2 gene was seen with TO treatment at concentration 12.5, 25, and 50 mg/mL (89.136, 102.738, and 124.459) compared with control value 54.246 ($p<0.001$).

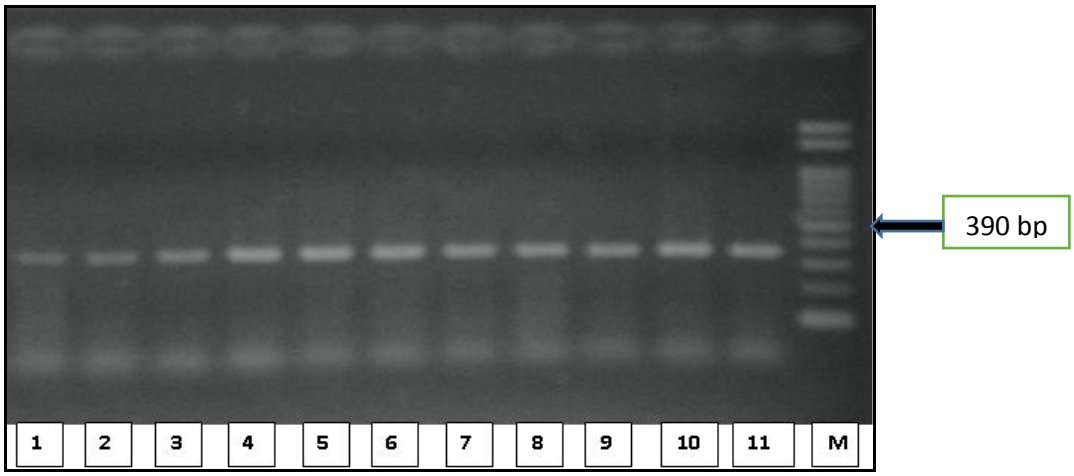

Figure 4: Electrophoresis gel agarosa RT-PCR RARß2 gene primary culture human cervical cancer stem cells. 1-5 = TO doses 6.25, 12.5, 25, 50, and 100 µg/mL; 7-10= RA doses 13.3, 26.7, 53.3, 106.7, and 213.µg/mL; 11= control; M=Marker.

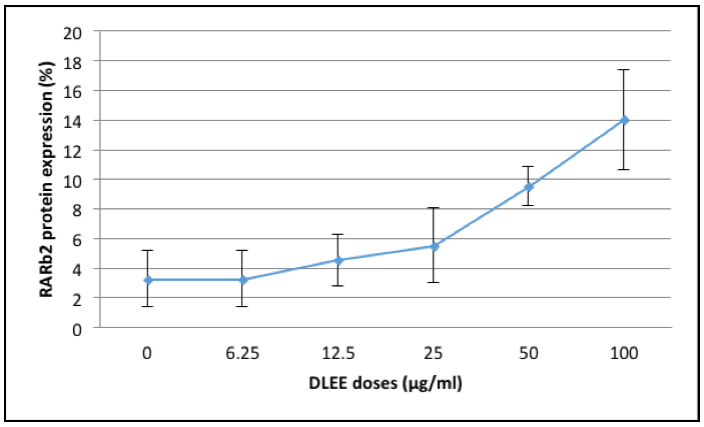

Figure 5. Curve of Relative expression RARß2 gene effect of TO on primary culture human cervical cancer stem cells. Cell were exposed for 24 hours to TO at various concentrations 6.25, 12.5, 25, 50, and 100 µg/mL shown effect 60.149, 89.136, 102.738, 124.459, and 64.109. A significant effect of RARß2 genes expression was seen with TO concentrations of 12.5, 25, 50, and 100 µg/mL (89.136, 102.738, 124.459, and 64.109) of the cells population compare with control value 54.246 (*Brown-Forsythe, p<0.001*). The effect of TO to increase RARß2 expression .

Conclusion

TO shown significant effect induction apoptosis on CCSCs through induction RARß2 gene expression and decrease Sox2 expression, TO is a potential anticancer for CCSCs. However, further are needed to isolate the active components and to alucidate the axact mechanism of action in vitro and in vivo.

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