

RESEARCH ARTICLE

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Orthosiphon stamineus Extracts Inhibits Proliferation and Induces Apoptosis in Uterine Fibroid Cells

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Abstract

Objectives: The effects of water and 50% ethanolic-water extracts of *Orthosiphon stamineus* Benth (OS) on cell proliferation and apoptotic activity against uterine leiomyosarcoma (SK-UT-1) cells were investigated. **Methods:** Anti-proliferation effect was evaluated through cell cycle analysis whereas apoptotic activity was determined via screening and quantifying using fluorescence microscopy and flow cytometric analysis, respectively. The effect of extracts on molecular mechanism was studied using real-time reverse transcription polymerase chain reaction and Western blotting. **Results:** Cell cycle flow cytometric analysis showed the induction of cell cycle arrests were behaves in a p53-independent manner. The examination using fluorescence microscopy and Annexin V flow cytometry revealed the presence of morphological features of apoptotic bodies. Downregulation of anti-apoptotic gene (Bcl-2) supports the apoptotic activity of OS extracts although poorly induce PARP-1 cleavage in Western blot analysis. The extracts also inhibit the SK-UT-1 growth by suppressing VEGF-A, TGF- β 1 and PCNA genes, which involved in angiogenesis and cell proliferation. **Conclusion:** This study demonstrates that *O. stamineus* extracts are able to inhibit proliferation and induced apoptosis of uterine fibroid cells and is worth further investigation.

Keywords: *Orthosiphon stamineus* Benth- SK-UT-1 cells- apoptosis- growth factor

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Introduction

Uterine fibroid also known as leiomyoma or leiomyomata are benign tumors which made up of smooth muscle cells in abundant quantities of extracellular matrix (Bulun, 2013). Several growth factors including transforming growth factors (TGF- β 1), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) reported regulate this fibroid. About 25% of women in USA diagnosed with uterine fibroid clinically (Stewart, 2001). On the other hand, women with uterine leiomyosarcoma (ULMS) (cancerous) showed similar symptoms as benign uterine fibroid patient including excessive menstrual bleeding, abdominal pain, pelvic pressure and reproductive dysfunction (Stewart, 2001; Eskander et al., 2012). ULMS has poor prognosis even after early stage diagnosis with recurrence rates about 53%-71% (Eskander et al., 2012). The treatments for the fibroid mainly based on surgeries involving hysterectomy and abdominal myomectomy (Stewart, 2001). New treatment strategies focusing on a combination of cytotoxic agents results in limited response rate despite considerable toxicity (Amant et al., 2003). Therefore, safe, economical and effective alternative

treatments are in needs. In order to find new potential drugs for uterine fibroid treatment, a screening for biologically active extracts against uterine fibroid cells is required to assess for their pharmacological qualities.

Orthosiphon stamineus Benth (Misai Kucing) (OS), a herb originated from Southeast Asia has been traditionally used for diabetes, gallstone, jaundice and menstrual disorder remedies (Rao et al., 2014). Extensive biological properties such as a diuretic, anti-inflammatory, anti-oxidant, anti-angiogenic and hepatoprotective have been reported (Ahamed Basheer and Abdul Majid, 2010). OS also showed an anti-proliferative effect against many cancer cell lines including HeLa, PC3, HCT116, HL-60 and HepG2 cells. In addition, we have also reported the cytotoxic effect of *O. stamineus* standardized extracts against SK-UT-1 cells (Halim et al., 2017). Phytochemical studies also revealed the presence of abundant bioactive compounds including terpenoid, phenolic, flavonoid, saponin, essential oil and organic acids in this plant (Maheswari et al., 2015). In particular, terpenoid compound was shown to be effective against cancer and inflammation due to their ability to block the NF- κ B activation, inhibit I κ B α degradation and phosphorylation, induce intrinsic apoptosis (targeting Bcl2), inhibit

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proliferation, invasion, metastasis, angiogenesis, blocked cell cycle progression in G1 phase (Yadav et al., 2010), and inducing PARP-1 cleavage (Mishra et al., 2016). Phenolic also reported to exhibit anti-oxidant activity, anti-proliferative activity, induce G2/M cell cycle arrest, induce apoptosis in addition to increase the level of p53, Bax and stimulate the cleavage of PARP-1 (Akowuah et al., 2004; Doleckova et al., 2012; Estévez et al., 2014). Rosmarinic acid and betulinic acid were reported to possess antioxidant activity which believed contribute to anti-angiogenesis activity of this plant (Sahib et al., 2009). Apart from that, this plant also deprived of chronic toxicity, genotoxicity, nephrotoxicity and hepatotoxicity (Al-Suede et al., 2014), thus suitable for the further studies. In continuation to our report (Halim et al., 2017), here we present our finding on the effect of *O. stamineus* standardized extracts on proliferation and apoptosis of uterine fibroid cell line, SK-UT-1.

Materials and Methods

Standardized *Orthosiphon stamineus* leaves extracts

Orthosiphon stamineus Benth was purchased in Herbagus Sdn Bhd, Penang, Malaysia. A voucher specimen (no. 11009) was deposited at the herbarium of School of Biological Sciences, Universiti Sains Malaysia. The leaves were pulverized into a fine powder using an electric grinder (Restch, Germany). Two hundred grams of the dried and ground leaves of *O. stamineus* was extracted with 50% ethanolic water (OEW) and was macerated with water (OSW) repeatedly for 3 days. Extracts were filtered and concentrated using rotary evaporator and kept in -20°C. The standardized extracts were prepared as previously reported by Halim et al., (2017).

Cell lines

Uterine leiomyosarcoma (SK-UT-1) cells were purchased from American Type Culture Collection (ATCC). Cells were grown in RPMI 1640 media, supplemented with 10% Fetal Bovine Serum and 1% antibiotics Penicillin/Streptomycin and maintained in an incubator at 37°C with 5% CO₂ in a humidified atmosphere. The media was changed every 2 days.

Cell cycle analysis

The effect of *O. stamineus* extracts on the cell cycle distribution was studied using BD CycleTest™ Plus DNA Kit (BD Bioscience). The SK-UT-1 cells (1x10⁵ cells/well) in 6 well-plates were treated with IC₅₀ values (72 h incubation) reported by Halim et al., (2017) i.e. OSW: 18 µg/mL, OEW: 34 µg/mL, Etoposide: 1 µg/mL (1.69 µM) and EGCG: 22 µg/mL (47.99 µM), and incubated for 24, 48 and 72 h at 37°C in 5% CO₂. Cells were harvested following manufacturer's protocol and analyzed by flow cytometer (CytoFLEX, Beckman Coulter) using the CytoExpert software. Epigallocatechin-3-gallate (EGCG) and Etoposide (ETOP) were served as the positive control.

Acridine Orange/Propidium Iodide double staining assay

AO/PI double staining assay was carried out as described by Tajudin et al., (2012) with modification.

Briefly, cells were seeded overnight in 6-well plate and treated with extracts at their IC₅₀ concentration. After 24, 48, and 72 h incubation, cells were washed two times with 1x phosphate buffer saline (PBS) and trypsinized. The cells were centrifuged at 300 x g, 4°C for 15 min and pellets were stained with 50 µL (1:1) of acridine orange/propidium iodide (10 µg/mL). A volume of 10 µL of stained cells was pipetted onto a glass slide and observed under fluorescence microscope (Nikon TE2000-U, Nikon, Japan).

Annexin V-FITC/Propidium Iodide assay

The number of apoptotic cell death induced by *O. stamineus* was measured BD Pharmingen™ Annexin V-FITC Apoptosis Detection Kit I (BD Bioscience). Briefly, SK-UT-1 cells were treated using IC₅₀ value for 24, 48 and 72 h. The cells were harvested following manufacturer's protocols and analyzed by flow cytometer (CytoFLEX) using the CytoExpert software within 1h. 10,000 events were recorded. Percentage of viable, apoptosis and necrosis cells were determined.

Western blot

The cells were treated with the IC₅₀ value of extracts and incubated for 24, 48, and 72 h. The old media was discarded and the cells were washed twice with ice-cold PBS prior to lyse in 10 mM Tris pH 6.8, 4% of sodium dodecyl sulfate (SDS), 20% of glycerol and distilled water. The lysate was stored at -20°C until used. Protein concentration was determined using BSA assay (DC Assay, BioRad). 1mg of protein was separated using 12% SDS-PAGE and blotted using PVDF membrane (BioRad). The membranes were blocked for 1h with 5% non-fat milk in PBS-Tween 20 (0.1%). The membranes were washed with PBST and milk containing primary antibody (1:500 of PARP-1, IκBα, Cyclin B1 and 1:1,000 GAPDH) was added and incubated for 30 min at room temperature before incubating at 4°C overnight. After that, the membranes were incubated for 2 h at room temperature prior to addition of secondary antibody. The membrane of p53 was blocked with 5% non-fat milk PBST in 4°C overnight. Later primary antibody (1:500 of p53) was added and incubated for 1 h in 4°C and further incubated at room temperature for another 1h. Then, membranes were washed and conjugated with secondary antibody (1:1,000 Rabbit IgG antibody (HRP) GTX213110-01 and Mouse IgG antibody (HRP) GTX213111-01) for 1h before washed and viewed using ECL (Advansta, Matrioux). PARP-1 (sc-7150), IκBα (sc-371), cyclin B1 (sc-245), p53 (sc-6243) and GAPDH (sc-25778).

Real-time Reverse transcription polymerase chain reaction (qRT-PCR)

Cells were treated using IC₅₀ values for 30 min. Total RNA was extracted using TRIzol reagent (Invitrogen) following manufacturer's protocol. The cDNA was synthesized using Maxima First Strand cDNA synthesis kit for qRT-PCR (Thermo Scientific) following manufacturer's protocol. The cDNA was then amplified using TaqMan® Gene Expression Master Mix (Applied Biosystem) following manufacturer's protocols using Rotor-Gene 6000 (Corbett Research). The parameter

was set up as follows; 95°C for 15 s and 60°C for 60 s. The expression of the genes of interest was monitored and compared using the relative method. GAPDH gene was used as internal control. The IDs for Taqman Gene Expression Assays (Applied Biosystem) used in this study were GAPDH: Hs02758991_g1, Bax: Hs00180269_m1, Bcl2: Hs00608023_m1, TGF- β 1: Hs00998133_m1 and PCNA: Hs00427214_g1.

Statistical Analysis

All experiments were carried out for three times with 3 replicates and values are reported as means \pm standard deviation (S.D.). Data were analyzed using One Way ANOVA and differences were considered statistically significant at the level of $p < 0.05$.

Results

The effect of OS on cell cycle

Uncontrolled cell proliferation is the indicator of cancer. Figure 1 and Figure 2 showed the representative histograms of SK-UT-1 cells distribution and its percentage after exposed to different treatments for 24,

48 and 72h, respectively. In comparison with the untreated group, we found that OSW-treated cells were significantly arrested at G0/G1 at 24h and both G0 and G0/G1 after 48 and 72h ($p < 0.001$). This extract also showed a slight increase in cells population in G2/M phase throughout the incubation although not significantly different with the untreated group. OSEW-treated cells on other hand showed significant G0/G1 cell cycle arrest ($p < 0.001$) at 24h, G0 ($p < 0.001$), G0/G1 ($p < 0.01$) and G2/M ($p < 0.001$) during 48h, and G0 ($p < 0.001$) and G2/M (not significant) after 72h. Cells treated with ETOP showed induction of G2/M arrest at 24h, both G0 and G2/M ($p < 0.001$) phase after 48 and 72h. However, EGCG-treated cells were found to significantly induced G2/M arrest at 24h, G0 and G2/M at 48h, and G0 ($p < 0.001$) and G2/M (not significant) after 72h. This finding was supported by Chakrabarty et al., (2015) which reported the induction of G2/M arrest by EGCG in HeLa cells after 24h incubation, although Du et al., (2012) reported the induction of G1 arrest in HCT116 cells after 48h treatment. Several studies including Siddiqui et al., (2015), Schonn et al., (2010), Jamil et al., (2015) and Groschel and Bushman (2005) have also supported the induction of G2/M arrest by ETOP.

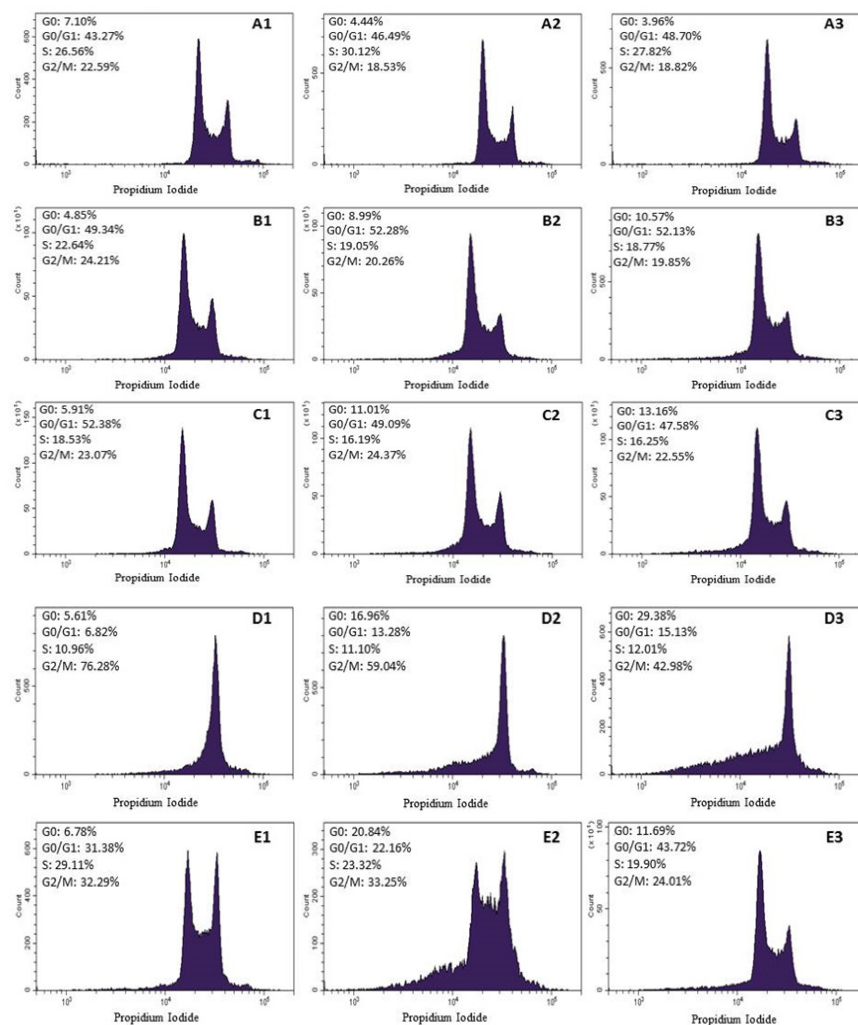


Figure 1. Cell Cycle Analysis of SK-UT-1 Cells Untreated (A1=24 h, A2=48h, A3=72h), treated with OSW (B1=24h, B2=48h, B3=72h), OSEW (C1=24h, C2=48h, C3=72h), ETOP (D1=24h, D2=48h, D3=72h), EGCG (E1=24h, E2=48h, E3=72h).

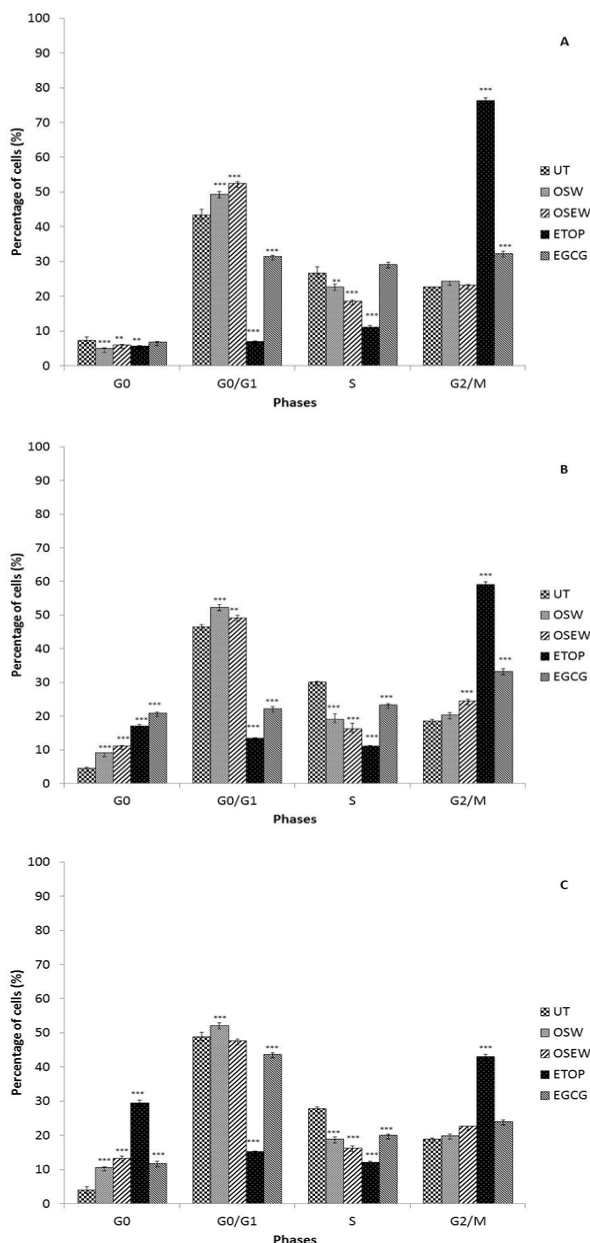


Figure 2. Cell Cycle Arrest of SK-UT-1 Cells after (A) 24h, (B) 48h, and (C) 72h Incubation. The values of the bars represented mean \pm S.D. of three replicates ($p < 0.05$). The [*] indicated the samples are slightly significant when $p < 0.05$, [**] indicated the samples are significant when $p < 0.01$ and [***] indicated that samples are highly significant when $p < 0.001$.

We further evaluate the effect of treatments onto two proteins involved in cell cycle i.e p53 and Cyclin B1. By comparing with untreated group, we found that OSW slightly increase p53 expression and lowered the expression of cyclin B1 protein in a time-dependent manner. Although OSEW showed no changes in p53 level expression but resulted in decreasing the Cyclin B1 protein expression. Yeh et al., (2015) have also observed a slight increase in p53 expression in SK-UT-1 cells after treated with genistein. Meanwhile, Etoposide and EGCG reduced p53 protein expression in a time-dependent manner and a slight reduction in Cyclin B1 expression. The decreased in p53 level in the untreated group might

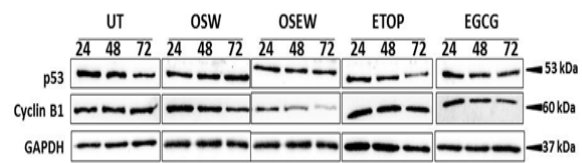


Figure 3. Immunoblotting of Cyclin B1 and p53 Protein Expression in SK-UT-1 Cells Normalized with GAPDH.

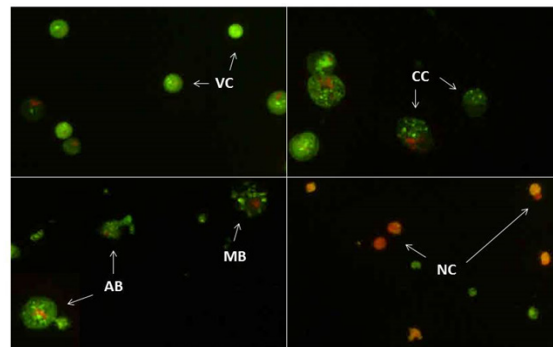


Figure 4. Representative Images of Morphological Changes in SK-UT-1 after Treatment. The cells were observed under fluorescence microscope. Viable cell (VC) with intact membrane appeared fluorescence green. Early apoptosis appeared as green-orange colour nuclei with condensed (CC) or fragmented chromatin. Late apoptosis appeared as orange to red colour with highly condensed or fragmented chromatin and apoptotic bodies (AB). Necrotic cells (NC) fluoresced orange to red without chromatin fragmentation.

due to the mutations which previously reported in most of all cancer cases (Li et al., 2012) including colon, stomach, breast, ovary, and esophagus cancers (Abdolmohammadi et al., 2009). However, the reason for the decrease of p53 expression in Etoposide and EGCG treated cells remain to be explored.

The effect of OS extracts on mode of cell death

Cells which undergo apoptosis were characterized by a series of morphological and biochemical alteration including cell shrinkage, membrane blebbing, nuclear condensation, DNA fragmentation, externalization of plasma membrane phosphatidylserine (PS) and activation of apoptotic caspase (Leppert et al., 2014). Screening of morphological changes of SK-UT-1 cells after double staining with Acridine Orange and Propidium Iodide (AO/PI) shows the presence of apoptotic bodies in treated cells (Figure 4). The quantification of apoptotic cell population carried out by flow cytometric analysis showed in Figure 5. Even after 72h incubation, apoptotic cell population was found to be less than 10% in OSW-, OSEW-, EGCG-treated cells, respectively. Etoposide-treated cells exhibited significant increase in apoptotic population with $15.45\% \pm 0.16$, $38.98\% \pm 0.40$ and $61.78\% \pm 0.23$ after 24, 48 and 72h, respectively. Thirty minutes incubation with OSW unable to upregulate the expression of Bax in SK-UT-1 cells but significantly downregulate the anti-apoptosis gene, Bcl2 (Figure 6). The expression of Bax was significantly induced by ETOP ($p < 0.05$) followed by EGCG and OSEW when compared

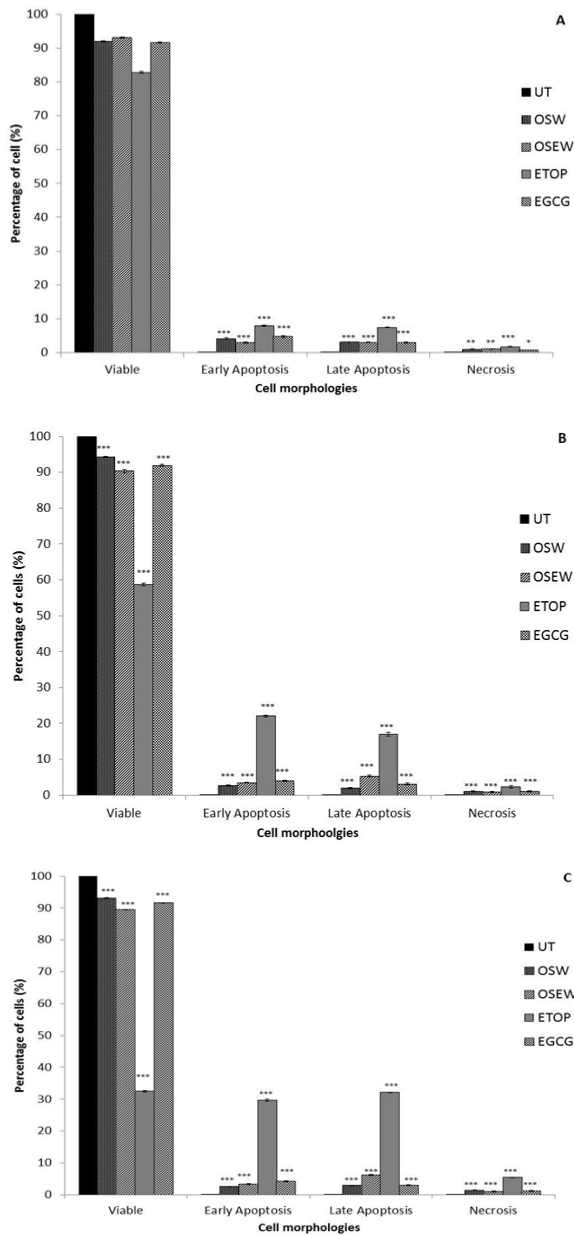


Figure 5. Percentage of Morphological Changes Induce after (A) 24h, (B) 48h, and (C) 72h Incubation. Data represent as mean \pm SD from three replicates ($p < 0.05$). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

with untreated group. Interestingly, the treatments with OSW, OSEW and EGCG which not significantly affect Bax expression were found significantly downregulate Bcl2 expression ($p < 0.01$).

Effect of OS on apoptotic and inflammation-related proteins

Poly (ADP) ribose polymerase (PARP-1) is a protein related to apoptosis. The cleavage of PARP-1 into 89 kDa fragment has become a hallmark of apoptosis (Solowey et al., 2014). However, PARP-1 also processed during necrosis revealing another major fragment of 50kDa (Gobeil et al., 2001). Treatment with OS extracts and compounds gave less intensity in PARP-1 expression compared to untreated (Figure 7). The present of both 89

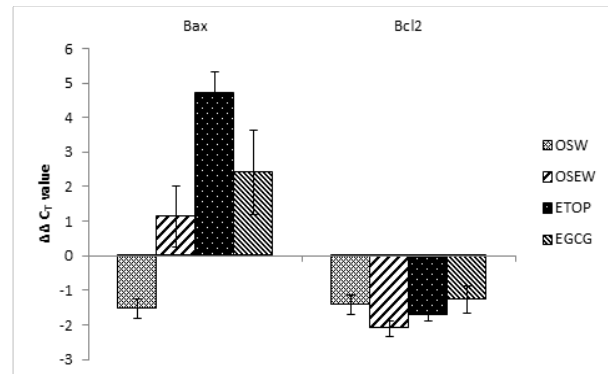


Figure 6. The Expression of Apoptosis-related Genes in SK-UT-1 Cells after Treated for 30min Using $\Delta\Delta C_t$ Value which Normalized Using GAPDH of Untreated Group. * $p < 0.05$, ** $p < 0.001$.

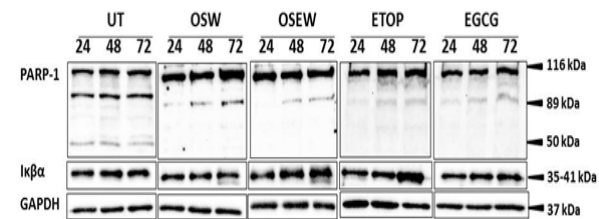


Figure 7. The Apoptosis and Anti-inflammatory Effect of Treatments on SK-UT-1 Cells for 24, 48 and 72 h Incubation.

kDa and 50 kDa PARP-1 protein fragment in untreated group might due to excessive or accumulation of DNA damage which might have force PARP-1 to consume more substrate (NAD⁺) which eventually result in an extremely low levels of NAD⁺/ATP causing cell to die rapidly through necrosis due to acute energy depletion (Strom and Helleday, 2012). It was therefore, suggested that the treatments might poorly induce PARP-1 cleavage but inhibit necrosis. Cells treated with OSW extract showed no changes in I κ B α intensity throughout the incubation time. Meanwhile, the intensity was increased in a time-dependent manner in OSEW- and Etoposide-treated cells. EGCG which displayed low intensity of I κ B α during 24h when compared with untreated was increased in intensity in a time-dependent manner.

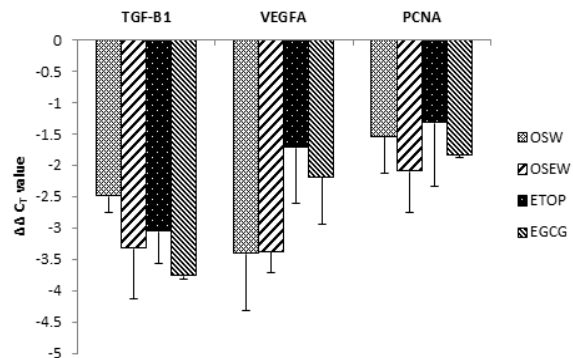


Figure 8. Anti-fibroid Activity of OS Extracts, ETOP and EGCG on SK-UT-1 Cells after 30min Incubation. Data showed no significance difference.

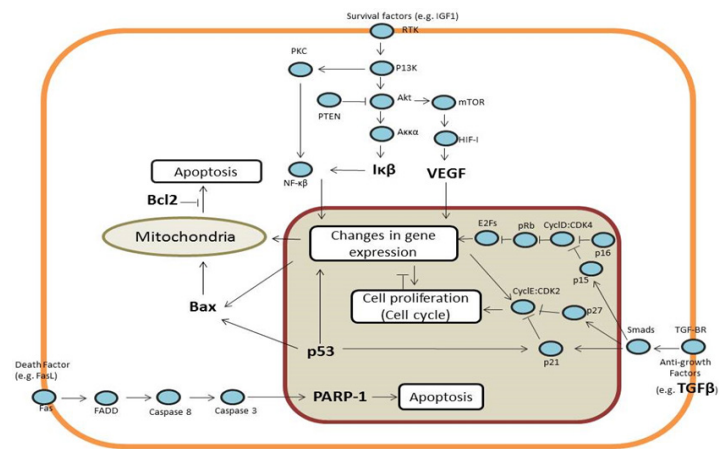


Figure 9. The Schematic Diagram Displayed the Relationship among All Tested Factors.

Effect of OS on the expression of growth factor-related genes

Tumor growth factor-beta 1 (TGF-β1), vascular endothelial growth factor (VEGF-A) and proliferating cell nuclear antigen (PCNA) are growth factors which regulate uterine fibrogenesis (Stewart, 2001). In comparison with the untreated group of respective gene, the effect of the treatments towards the expression of tumor growth factors was presented in Figure 8. Although not statistically significant as compared to the untreated group, all tumor growth factors were downregulated by all the treatments. TGF-β1 was strongly suppressed by EGCG followed by OSEW extracts. Meanwhile, suppression of VEGF-A was dominated by OS extracts followed by EGCG and Etoposide. Interestingly OSEW showed better inhibition effect for all these genes as compared to commercial drug Etoposide.

Discussion

This study is the first to report the effect of OS extract on uterine fibroid cells. We evaluate apoptotic event and anti-proliferative effect of two OS standardized extracts against uterine leiomyosarcoma cells, SK-UT-1. Alongside with these extracts, etoposide and EGCG were used as positive control. Etoposide is a commercial drug for small cell lung, testicular and uterine cancer (Siddiqui et al., 2015) whereas EGCG is a green tea polyphenol which was reported to be effective against uterine leiomyoma cell in *in vitro* and *in vivo* study (Ozercan et al., 2008; Roshdy et al., 2013; Zhang et al., 2010).

In normal cell cycle, G1 phase is a phase where cells prepare for DNA synthesis. Whereas, at G2/M phase, cells were prepared for division or mitosis. G0 is another phase called resting stage in which cells undergo non-growing and non-proliferating state. However, in response to DNA damage, cycle of cells were arrested at checkpoints to provide time for DNA repair. Cyclin B1 is highly expressed during G2/M phase to promote cell mitosis by promoting the condensation of the chromosome, resolution of nuclear lamina and assembly of the mitotic spindle (Hsieh et al., 2015; Yu and Yao, 2008). It was

reported that Cyclin B1 absent during G0/G1 phase. Moreover, the decrease of this protein in response to p53 protein has been associated with G2/M arrest (Innocente et al., 1999). In contrast, we found the presence of Cyclin B1 protein in cells arrested at G1 phase as found in MOLT and T-7 cells (Shen et al., 2004). The low p53 protein expression as a result of OS treatment showing that the induction of cell cycle arrest and decreasing of Cyclin B1 protein were in a p53-independent manner. Several studies have also reported the occurrence of G0/G1 or G2/M arrest in the p53-independent manner (Suárez et al., 2003; Park et al., 2017; Clifford et al., 2003) and the involvement of ATM and ATR pathways in the cell cycle regulation (Jamil et al., 2015; Taylor and Stark, 2001).

Moreover, all treatments showed a significant increase in G0 phase after 48 and 72h, indicating the occurrence of cell death (Solowey et al., 2014). This data supporting the increase in the number of the apoptotic population in flow cytometry analysis. The induction of apoptosis by OS extracts might due to suppression of Bcl2 gene expression as reported by Fernandez-Cabezudo et al., (2013) rather than inducing PARP-1 89 kDa cleavage. Although displaying poor PARP-1 cleavage as compared to the untreated group, the treatments did indicated necrosis inhibition by increasing the intensity of IκBα protein expression which eventually inhibits the inflammation, angiogenesis and proliferation of tumor cells (Wu et al., 2015).

TGF-β1 which involved in cell growth, proliferation, angiogenesis and ECM production (Islam et al., 2016) has been found elevated in leiomyomata as compared with myometrium (Li, 2014). TGF-β1 also induced VEGF expression, an angiogenic inducer, secretion in human cytotrophoblast cells (Geng et al., 2013) and they also played role in oncogenesis of the stomach (Guan et al., 2009). Apart from that, TGF subtype, TGF-α has been reported to have a positive correlation with PCNA genes in uterine leiomyosarcoma of B6C2FI mice (Moore et al., 2000). Interestingly, our extracts have downregulated the expression of all these genes. The suppression of TGF-β1 by the treatments might as well result in downregulation of VEGF-A and PCNA gene expression. Since overexpression of NF-κB has

also been reported to be related to accumulation of PCNA (Zhao et al., 2016), we suggest that the downregulation of PCNA in the present study might play significant role in the suppression of NF- κ B through I κ B α stabilization as well as induce apoptosis in SK-UT-1 cells (Paunesku et al., 2001). Figure 9 shows the schematic diagram of the relationship between all tested factors.

In conclusion, in this present study, the findings demonstrate that OS extracts inhibited proliferation and induced apoptosis against SK-UT-1 cells through involvement of Bcl2/Bax signaling pathway with involvement of cell cycle arrest at G0/G1 phase and suppression of tumor growth-related genes. Therefore, OS extracts exhibit a potential anti-fibroid activity in SK-UT-1 cells and worth further investigation especially on the identification of bioactive compounds in the extracts.

Conflicts of interests

All authors have none to declare.

Acknowledgements

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