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RESEARCH PAPER

6-shogaol a Active Component from Ginger Inhibits Cell Proliferation and Induces Apoptosis through Inhibition of STAT-3 Translocation in Ovarian Cancer Cell Lines (A2780)

Ting Liang, Ying He, Yuhua Chang, and Xiantong Liu

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Abstract Ovarian cancer is predominant form of malignant which occupies 70 % cancer in women worldwide. Taxals, anthrocyclins and platinum based drugs are used as chemotherapeutic for treatment of ovarian cancer. These drugs were limited by drug resistance and it produces several toxicity. Therefore, we used natural drug which inhibits the cancer cell growth by modulating specific molecular mechanisms. This study, we evaluated the 6-shogaol a active component from ginger inhibits cell proliferation by modulation of JAK/STAT-3 signalling in ovarian cancer cell lines (A2780). In this study, we found that 6-shogaol induces cytotoxicity, ROS production and apoptosis in A2780 cell lines by time depending manner. Moreover, signal transducer and activating transcription-3 (STAT-3) has been consider as novel transcriptional factor which regulates apoptosis, cell proliferation and angiogensis. Inhibition of STAT-3 transcription considered as novel strategy to inhibiting ovarian cell growth. In this study, we found that 6-shogaol inhibits STAT-3 translocation there by inhibits the over expression of PCNA, cyclin-D1, Bcl-2 and decreased expression of Bax, caspase-9 and 3 in A2780 cell lines. This results concluded that 6-shogaol inhibits ovarian cell growth and induces apoptosis through inhibits the translocation STAT-3.

Ting Liang
Biomedical Isotope Research Center,School of Basic Medical Sciences, Shandong University, Jinan, Shandong Shandong 250012, China

Ying He
Department of Gynaecology and Obstetrics, Feixian People's Hospital, Linyi, Shandong 5221030, China

Yuhua Chang, Xiantong Liu*
Maternal and Child Health Care of Shandong Province, Jinan, Shandong 250014, China
Tel: +8615589905577
E-mail: sdliuxiantong@sina.com

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1. Introduction

Ovarian cancer is the fifth most gynecologic malignancy in women worldwide and it leads to causing cancer related deaths in women [1]. This cancer may occupy more deaths than other cancer of the female [2-4]. In 2007, report from national cancer institute, cancer stat facts during the year 2007–2013 the ovarian cancer 5- year relative survival rate was found to be 46.5% [5] . Moreover, approximately there are 22,240 new cases were cancer diagnosed in 2018. Amongst, 14,070 ovarian cancer patients were died in the United States. Ovarian cancer are often late detected and it has been progressly metastasize within the peritoneal cavity [6]. The treatment of ovarian cancer is associated with the surgery followed by taxanes and platinum based chemotherapy is the standard conventional methods for ovarian cancer patients [7]. Despite, several chemotherapeutic drugs have been used for treatment of ovarian cancer; it produces more toxicity in patients. Therefore, it is urgent to expand more successful conventional treatment strategies to overcome the chemotherapy mediated toxicity against ovarian patients.

Signal transducer and activator of transcription-3 (STAT-3) is an oncogenic transcription factor and it has been widely attracted as a target for treatment of ovarian cancer. Generally, the over expression of STAT3 needs to activated by interleukins and cytokines. The activated STAT-3 predominantly involved in multi tasking cellular processes such as proliferation, cell survival and apoptosis [8]. The pathogenesis of ovarian cancer, prominent expression of interleukin-6 (IL-6) can stimulates the activation of JAK/

STAT3 signaling [9]. In addition, the genes encoding JAK biomarker often mutated in proliferative neoplasm's, resulting to constitutive activation of JAK/STAT3 signaling. These activated STAT3 promotes cell proliferation by enhancing expression of key transcription genes associated to cancer cell proliferation which includes cyclin D1 related markers and PCNA which are highly involved in the regulation of the cell cycle [10]. Therefore, JAK-STAT3 signaling inhibition has been considered pivotal target cancer chemotherapy.

Apoptosis is a process which is involved in the eukaryotic programmed cell death and it is characterized by distinctive morphological changes such as alteration of mitochondrion matrix, cellular fragmentation and shrinkage and chromatin remodeling resulting in apoptotic bodies formation [11]. These processes are highly regulated by pro apoptotic genes i.e Bax related proteins and anti-apoptotic proteins such as Bcl-2 proteins can be initiated apoptosis in cancer [12]. Therefore, stimulation of apoptosis through inhibition of STAT3 pathway could be an significant strategy for chemoprevention [13]. Several documents have shown to be constitutive activation of STAT3 has linked with activation of apoptosis through modulation of Bcl-2 and Bcl-xl protein expression [14]. Hence, inhibition of STAT3 has been considered as promising approach using naturally occurring small-molecule inhibitors to induce apoptosis for chemoprevention.

Zingiber officinale is otherwise known as ginger; an tremendously familiar food spice for cooking in worldwide and it is used to treat a swarm of ailments in Asia countries, traditionally in India and China, over the past 2500 years [15]. 6-shogaol is a dietary major active component of ginger and it has exhibits several health beneficial implications such as antioxidant, antitumorgensis, antiproliferative and antidiabetic activities [16]. Previously, induction of ROS linked oxidative damage and apoptosis in colorectal carcinoma cells were modulated by 6-shogaol [17]. Moreover, 6-shogaol significantly inhibited cyclooxygenase-2 and nitric oxide synthesis in phorbol 12-myristate 13- acetate (PMA) induced experimental model [18]. 6-shogaol mediated JAK-STAT3 signaling associated ovarian cancer cell inhibition was still not yet studied. Therefore, we sought to investigate the role of 6-shogaol inhibits cell proliferation by modulation of JAK/STAT-3 signalling in ovarian cancer cell lines (A2780).

2. Materials and Methods

2.1. Chemicals

Minimum essential medium (MEM), fetal bovine serum (FBS) phosphate buffered saline (PBS), 0.25% trypsin EDTA, penicillin and streptomycin mixture were obtained from Lonza biosciences India. 6-shogaol, 2,7-diacetyl dichlorofluorescein (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Ethidium Bromide (EtBr Acridine Orange (AO) were purchased from Sigma, St. Louis, USA. The following monoclonal primary antibodies IL-6, JAK-1, STAT-3, Bcl-2, Bax, PCNA, cyclin-D1, β-actin, and secondary conjugated anti-mouse were purchased from Santacurz. Solvents and fine chemicals relvent to this work used analytical and molecular grade.

2.2. Cell culture

The ovarian cancer cell lines (A2780) was purchased from NCCS, Pune, India. Human dermal fibroblast cells (HDF) were purchased from Thermo scientific, USA. Cells were placed to cultured in MEM and maintained at 37ºC in a humidified atmosphere containing 5% CO_2 and 95% air environment.

2.3. MTT assay

The cytotoxic role of 6-shogaol against ovarian cancer A2780 cell lines and normal HDF cells were evaluated by MTTassay [19]. A2780 and HDF cells were seeded in microtiter plates at a 5000-10000 cells/well in a final volume of 100 µl with MEM medium then it was incubated for 24 h. After 24 h incubation. Cells were exposed to different concentration of 6-shogaol and it was incubated for 24 and 48 h respectively, Then the MTT solution (100 µg) was added to all the wells and it incubates for 4 h at 37ºC. Then the MTT reagent was removed and 100 µl of DMSO was added to to dissolve the purple formazan crystals. The plate was read at 570 nm in a ELISA plate reader (Robonic, India).

2.4. Measurement of intracellular ROS generation

6-shogaol mediated ROS production in ovarian cancer cell was detected by using DCFH-DA staining [20]. In brief, A2780 cells were seeded (1×10^6 cells/well) in 6-well plate; after hervesting cells were treated with 6- shogaol at different incubation time and kept in a CO_2 incubator for 24 h and 48 h respectively. After an appropriate incubation time, 5 µg DCFH-DA was added to well plate and it was incubated for 30-45 min in dark environment. Fluorescent intensity for DCFH-DA was measured with excitation and emission filters set at 485 ± 10 and 530 ± 12.5 nm, respectively (Multimode reader, Tecan).

2.5. Acridine orange/ethidium bromide staining

6-shogaol mediated apoptotic morphological changes in ovarian cancer cell was detected by using double staining methods used as acridine orange/ethidium bromide staining. Briefly, 1×10^5 cells were plated in 6-well plate, then cultured cells were treated with different incubation of 6-shogaol.

After the incubation, the treated cells were washed with ice-cold PBS, then stained with 20 μL of acridine orange/ethidium bromide (10 μL/mg AO and 10 μL/mg EtBr) solution at 37°C for 30 min. The stained apoptotic and viable cells were observed by fluorescent microscope.

2.6. Western blot analysis

Western blot analysis was carried out for PCNA, CyclinD1, Bcl-2, IL-10, JAK-1, STAT3 and Bax protein expressions in 6-shogaol treated ovarian cancer cells. The obtained results were normalized to house keeping marker β-actin expression. Following the protein estimation, the treated test samples were separated using 10 % SDS-PAGE gel electrophoresis and the proteins transferred to PVDF membrane and treated with appropriate primary and secondary antibodies to detect the interest of protein markers as per the method described previously [20] (Britto *et al*., 2017).

2.7. Caspase-9 and 3 activity assay

The caspase-9 and 3 activities were measured by caspase test kit following the manufacturer's instruction (Invitrogen). In brief, the ovarian cancer cells were plated in 6-well plate and treated with 6-shogaol and keep incubation for 24 h and 48 h respectively at CO_2 incubator. Then the cells were allowed to treat with caspase-9 & 3 assay reagents and it incubated for 2 h in dark room. Then, the absorbance for caspase-9 & 3 were measured using a microplate autoreader at 400 or 405 nm.

2.8. Statistical examination

All experiments were carried out in three independent experiments and the results were expressed as the mean ± standard deviation (Mean ± SD) by using with one-way analysis of variance (ANOVA). Values of $P < 0.05$ were indicative of significant differences.

3. Results and Discussion

American cancer society estimated that the ovarian cancer has occupied the fifth rank in cancer deaths among women worldwide [21]. The epidemiological report stated that ovarian cancer mainly developing in older women and half of the women's are diagnosed with ovarian cancer are 63 years or older [22]. The most effective treatment method for advanced ovarian cancer contributes maximum efficacy to minimize the tumor burden by surgery followed chemotherapy drugs such as carboplatin and paclitaxel etc.,. These drugs eliminate highly tumor cells even it can produce significant cytotoxic and numerous toxicity on normal cells [23]. Therefore, natural based phytochemicals have been involved beneficial to health and promotes the

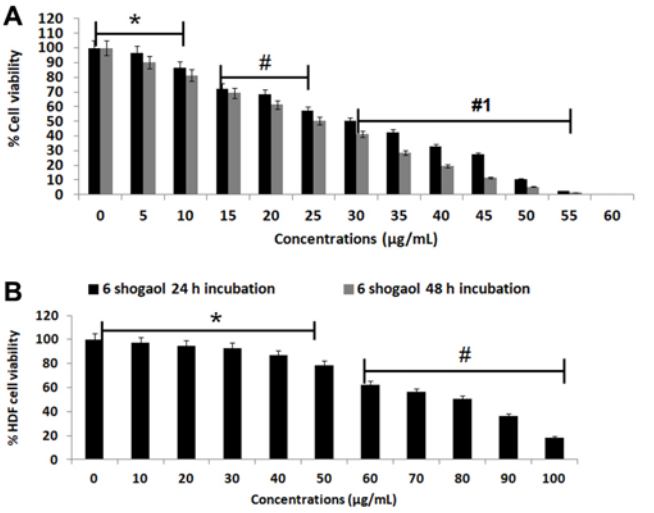

Fig. 1. The cytotoxicity effect of 6-shogaol on ovarian cancer A2780 and normal HDF cell lines measured by MTT assay. (A) The cytotoxic effect of 6-shogaol against ovarian cancer A2780 cell lines. (B) The cytotoxic effect of of 6-shogaol against normal human dermal fibroblasts (HDF). The statistical analysis was carried out using one way ANOVA. Values are represented mean ± S.D. of three experiments. $P < 0.05$ was significantly different from the control sample.

inhibition of tumor cell growth through the modulation specific mechanisms. In this present work, we stated for the first time, 6-shogaol inhibits ovarian cancer cell proliferation by inhibits of STAT-3 translocation in A2780 ovarian cancer cells.

The cytotoxicity potential of 6-shogaol against ovarian cancer cell lines was assessed by MTT based cytotoxicity assay. In this study we found that 6-shogaol induces significant cell death in concentration of drug and time depended manner (Fig. 1). IC 50 value of 6-shogaol was found to be 30 μg/mL for 24 h incubation; whereas 48 h incubation of 6-shogaol was found to be 25 μg/mL respectively. Based on this study, 25 μg/mL was used as optimum dose for further experiments. Previously, ginger inhibits SKOV-3 ovarian cancer cells proliferation after 72 h at the IC 50 concentration of 40 μM [24]. Similarly, several research groups has been proved numerous cancer cell lines. In addition, 6-shogaol was analyzed to investigate the cytotoxicity of normal human dermal fibroblasts (HDF). In this study, we found that 6-shogaol up to 50 μg/mL did not produced significant toxicity in HDF cells. Moreover, after 50 μg/mL concentration of 6-shogaol produces cytotoxicity in HDF cells. Previously, 6-shogaol did not produce cytotoxicity in the normal gingival fibroblast cells (HGF-1) and the minimum cytotoxicity was found at a concentration of 80 μM (Annamalai *et al*., 2016) [27]. Based on these result, 6-shogaol have strongest antioxidants in nature; therefore protects normal cells.

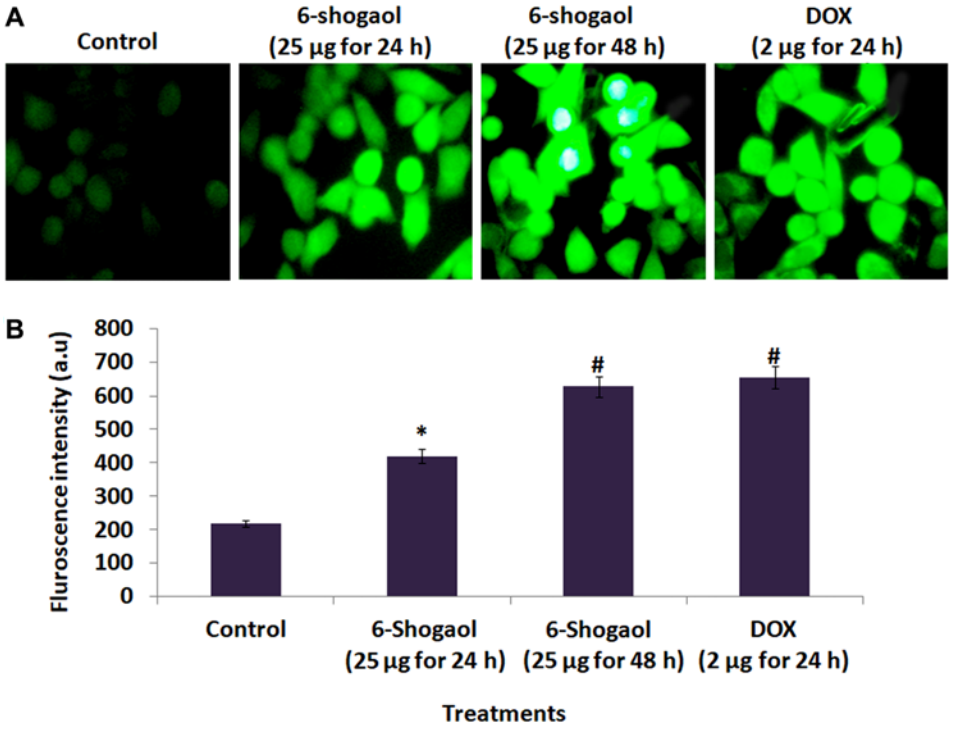

Fig. 2. The effect of 6-shogaol on intracellular ROS generation in ovarian cancer A2780 cell lines was evaluated by using DCFH-DA staining. (A) Fluroscence microscopic for 6-shogaol on intracellular ROS generation. (B) Percentage of ROS generation was detected by spectrofluorometer. DOX (2 µg/mL for 24 h incubation) has been used for standard chemotherapeutic drug for ovarian cancer cell lines. The statistical analysis was carried out using one way ANOVA. Values are represented mean ± S.D. of three experiments. $P < 0.05$ was significantly different from the control sample.

Oxidative stress is an irregularity reaction between the induction and an elimination of reactive oxygen species which leads to damage to macromolecules [25]. Production ROS associated to the cell death and cell survival of cancer cells and moderate levels of ROS production can activate prosurvival through oxidative in activation of cancer cells [26]. Over production ROS involved to depletion of antioxidants and enhace the lipid peroxidation that resulted in oxidative stress. In our result, increased ROS levels were observed 6-shogaol treated ovarian cancer cells in a time dependent manner (Fig. 2). More importantly, 48 h incubation of 6-shogaol was shown to be more pronounced ROS production in ovarian cancer A2780 cell lines. In this study, doxorubicin (DOX) was used to standard chemotherapeutic drug for ovarian cancer. We found that DOX (2 µg/mL for 24 h incubation) showed significant ROS generation in ovarian cancer A2780 cell lines.

Annamalai *et al*., 2016 [27] have reported redox-active transition metals and or phenolic compounds especially 6-shogaol can act as pro-oxidant through Fenton and Fentonlike reactions by involving in redox-cycling and stumilating the production of hydroxyl radicals when cells are cancerous condition. We also observed that 6-shogaol can act as pro-oxidant and induces ROS mediated oxidative stress in ovarian cancer cells. Previously, 6-shogaol induces ROS and oxidative stress mediated apoptosis in Hep-2 cells [27].

6-shogaol mediated apoptotic features and morphological changes were studied by double staing with ethidium bromide and acridine orange. Control ovarian cancer A2780 cell lines showed there is no apoptotic cells by observing acridine orange stained green cells. In contrast, treatment of 6-shogaol induces apoptotic features such as membrane blubbing, nuclear fragmentation in ovarian cancer A2780 cell lines (Fig. 3). More importantly, 48 h incubation of 6-shogaol was shown to be highly apoptotic cells in ovarian cancer A2780 cell lines. The standard drug DOX (2 µg/mL) showed significant apoptotic cells in ovarian cancer A2780 cell lines.

Ovarian cancer cells are needs high level of reactive oxygen species (ROS) for their proliferation and angiogenesis [28]. Cyclin-D1 and PCNA are important proliferative marker which participates ovarian cancer cell proliferation [29]. There are several transcription factors such as NF-κB, STAT-3 and AP-1 etc., have regulated the expression of cyclin-D1 and PCNA in several cancer cell lines [30]. In this study, we found that control ovarian cancer cells are highly expressed in PCNA and cyclin-D1 (Fig. 4). Conversely, treatment of 6-shogaol inhibits the over expression of in

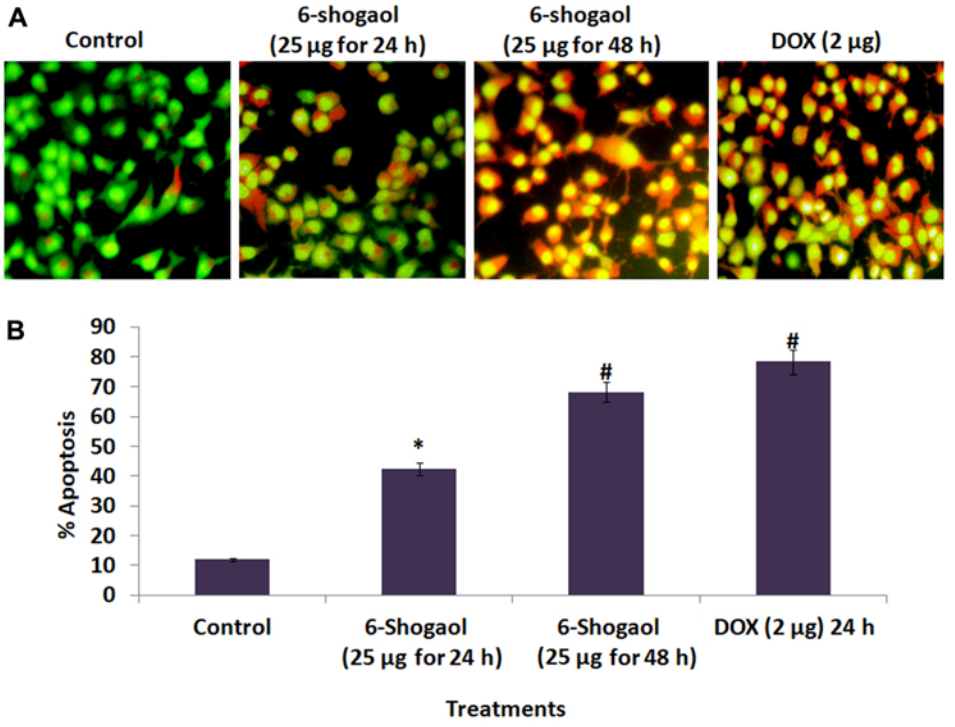

Fig. 3. 6-shogaol on apoptotic morphological changes was analayzed by dual staining (AO/EtBr). (A) Microscopical images shows that control cells shoes AO stained cells and 6-shogaol and DOX treated cells showed EtBr stained cells. (B) Bar diagram shows percentage apoptotic cells were calculated. DOX (2 µg/mL for 24 h incubation) has been used for standard chemotherapeutic drug for ovarian cancer cell lines. The statistical analysis was carried out using one way ANOVA. Values are represented mean ± S.D. of three experiments. $P < 0.05$ was significantly different from the control sample.

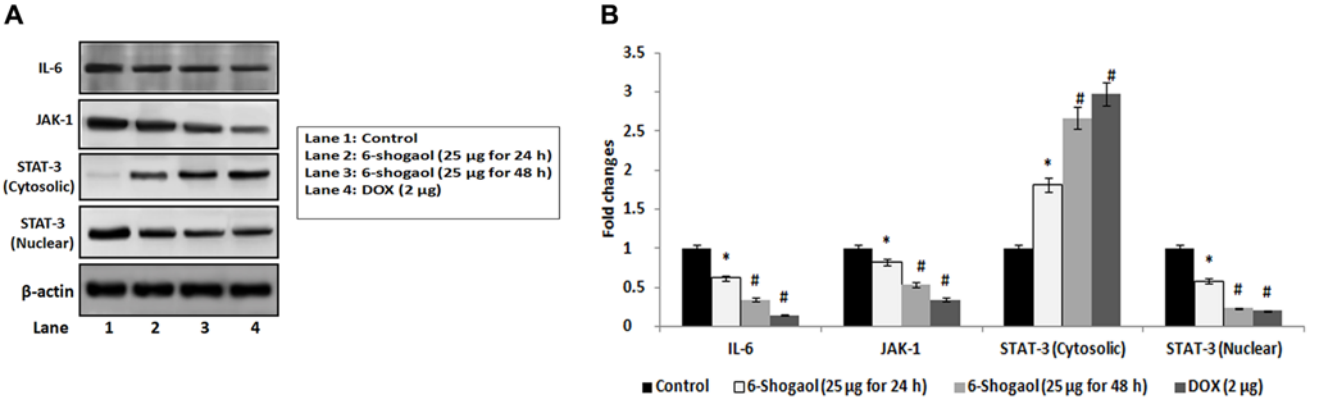

Fig. 4. 6-shogaol inhibits PCNA and cyclin-D1 proliferative markers in ovarian cancer A2780 cell lines. (A) Western blot images for PCNA and cyclin-D1 proliferative markers in ovarian cancer A2780 cell lines. Lane 1: Control; Lane 2: 6-shogaol 25. (B) Densitometric data was analyzed the interest of proteins normalized to respective β-actin loading control. There are three independend experiments were done.

PCNA and cyclin-D1 in A2780 cell lines. Moreover, we noticed that 6-shogoal 48 h incubation highly inhibited the over expression of PCNA and cyclin-D1 when comparing the activity of standard drug DOX. Natural phytochemicals resveratrol have been documented that downregulates cyclin D1/Cdk4, PCNA in colon cancer cells [31].

STAT3 is an oncogenic transcription factor, activated by various up-stream positive genes such as tyrosine kinases and interleukins and interferon's that can negatively regulates the activation of STAT3 and can participate transcriptional role several proliferative genes [32]. In the present study, for the first time we demonstrated for 6-

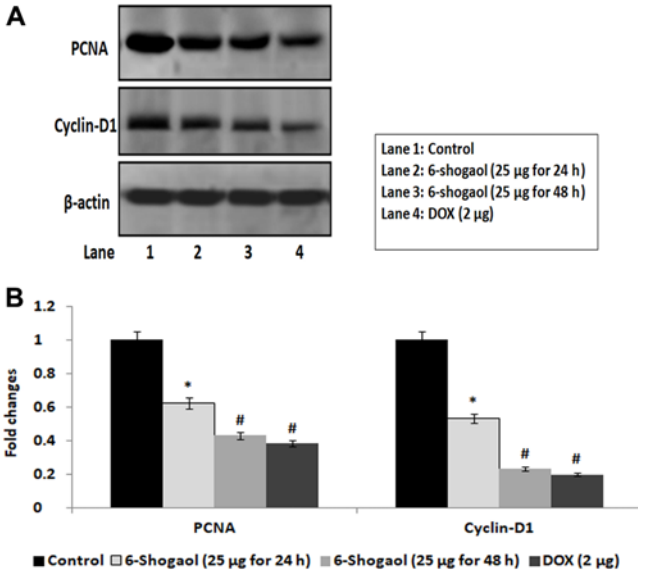

Fig. 5. 6-shogaol inhibits JAK/STAT-3 translocation in ovarian cancer A2780 cell lines. (A) Western blot images for IL-6, JAK1 and STAT-3 in ovarian cancer A2780 cell lines. (B) Densitometric data was analyzed the interest of proteins normalized to respective β-actin loading control. There are three independend experiments were done.

shogal inhibits STAT3 activation in ovarian cancer cells. Interleukin-6 (IL-6) and janus kinase-1 (JAK-1) is an initiating agent for activating translocation of STAT-3 resulted in proliferation, angiogenesis and apoptosis [33]. In this present work, first we found that 6-shogaol inhibits the over expression of IL-6 and JAK-1 in ovarian cancer A2780 cell lines when compared to control A2780 cell lines (Fig. 5). Moreover, translocation of STAT-3 was analyzed by the cytosolic and nuclear fraction. In this work also we found that control A2780 cell lines highly expressed in nuclear STAT-3; decreased expression of cytosolic STAT-3. In contrast, treatment of 6-shogaol significantly decreased protein expression of nuclear STAT-3; increased cytosolic STAT-3 in A2780 cell lines. Moreover, we noticed that 6-shogoal 48 h incubation highly inhibited the translocation of STAT-3 when comparing the activity of standard drug DOX.Previously, caffeic acid inhibits IL-6, JAK and STAT-3 translocation thereby prevents skin cancer [34]. Moreover, natural compounds inhibits STAT3 could enhance the efficacy of doxorubicin in lung and breast cancer [35].

The cells having ability to revert the apoptotic program has been considered as one of the remarkable mechanisms for the expansion of cancers [36]. The programmed cell deaths in eukaryotes are known to be an apoptosis which is closely regulated by several transcriptional factors. 6-shogaol treatment induces apoptosis in ovarian cancer cells by observing that increased expression of Bax ,caspases and decreased expression of Bcl-2 when compare to

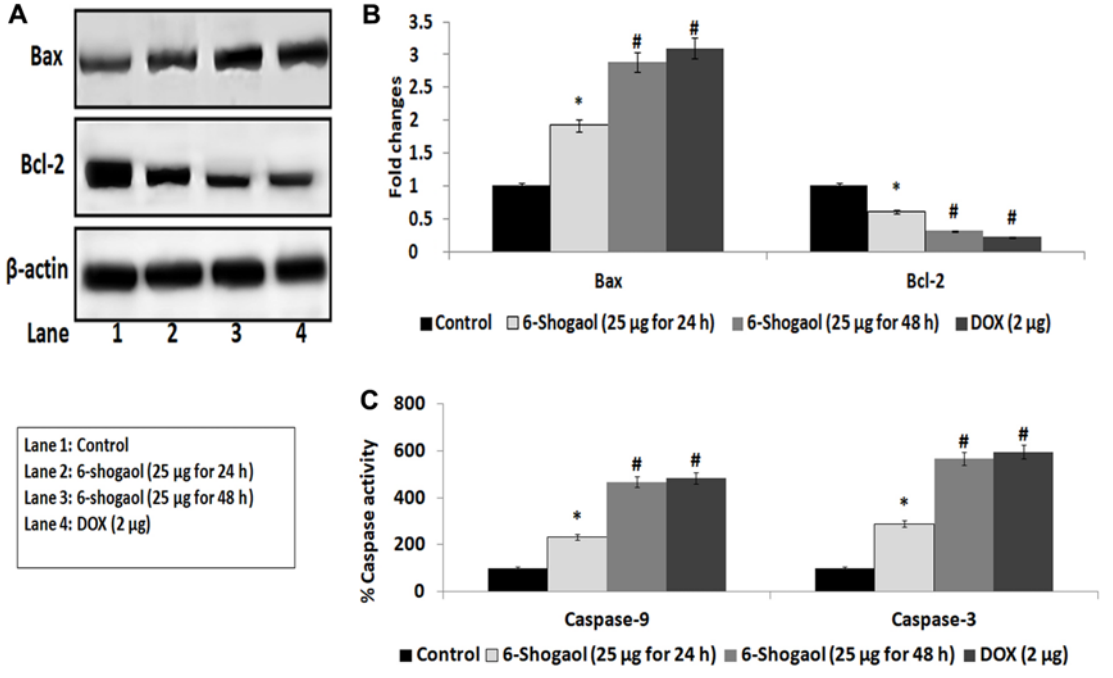

Fig. 6. 6-shogaol induces apoptotic protein expression in ovarian cancer A2780 cell lines. (A) Western blotting analysis for Bax and Bcl-2 protein expression (B) Densitometric data was analyzed the interest of proteins normalized to respective β-actin loading control. There are three independend experiments were done. (C) Colorimetric assay for caspase-9 & 3 activity expression in 6-shogaol treated ovarian cancer A2780 cell lines. The statistical analysis was carried out using one way ANOVA. Values are represented mean ± S.D. of three experiments. $P < 0.05$ was significantly different from the control sample.

control A2780 ovarian cancer cells (Fig. 6A & B). Bcl-2 belongs to upstream molecules, which is critically involved to inhibit the cell apoptosis. STAT-3 is a major transcriptional factor and it has been regulated to Bcl-2 expression. Activated STAT-3 was translocated in to nuclease and it can promote the over-expression of Bcl-2. Therefore Bcl-2 has identified as a potent suppressor of apoptosis [37].

First, we demonstrated the 6-shogaol mediated apoptotic features and morphological changes were studied by double staing with ethidium bromide and acridine orange. In this study, treatment of 6-shogaol induces apoptotic features such as membrane blubbing, nuclear fragmentation in ovarian cancer A2780 cell lines. Further, we found that 6-shogaol treatment induces Bax, caspase-9 and caspase-3 which are down-regulating molecules in the apoptotic pathway. Moreover, 6-shogaol inhibits Bcl-2 over-expression in ovarian cancer cells. Previously resveratrol have been induces apoptosis in human epidermoid carcinoma A431 cells by increases the expression of c through modulation of JAK-STAT signaling [28]. Consist with these report, our study also induces apoptosis by the regulation of STAT-3 signaling in ovarian cancer cells. In this study, for the first time 6-shogaol inhibits ovarian cancer cell growth through inhibiting STAT-3 translocation in ovarian cancer A2780 cell lines. However, 6-shogaol effectively induces apoptosis in ary human leukemia cells and leukemia xenografts by the modulation of eIF2α dependent caspase activation [38]. Moreover, 6-shogaol inhibits breast cancer cell invasion by reducing matrix metalloproteinase-9 and nuclear factor-κB activation [39]. Therefore, 6-shogaol exhibits several anticancer effects based on the different concentrations of efficacy.

4. Conclusion

In this study, we found that 6-shogaol induces cytotoxicity, ROS production and apoptosis in A2780 cell lines by time depending manner. Moreover, 6-shogaol inhibits STAT-3 translocation there by inhibits the over expression of PCNA, cyclin-D1, Bcl-2 and decreased expression of Bax, caspase-9 and 3 in A2780 cell lines. This result was concluded that 6-shogaol inhibits ovarian cell growth and induces apoptosis through inhibition of translocation STAT-3.

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