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Evaluation of the inhibitory synergic effects of the Persian Gulf brittle star extract and taxol on ovarian cancer A2780cp

Baharara J.[1*]; Amini E.[2]

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Abstract

Paclitaxel is a current standard chemotherapeutic drug for ovarian cancer with several side effects. Recurrences of drug resistant clones have been considered the serious problem in the failure of chemotherapy. Medicinal marine natural products have been intensively proposed as diverse chemotherapeutic agents. Therefore there is an affinity to find efficient modality to overwhelm ovarian cancer chemo resistance complication. Here we examine whether brittle star extract as marine echinoderm natural resources can remarkably improve the cytotoxicity of paclitaxel in human ovarian cancer. MTT (dimethyl thiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) assay, PI (Propodium Iodide) assay, DAPI (4', 6-diamidino-2-phenylindole) staining, Acridine orange staining, caspase-3 and caspase-9 were performed to investigate cytotoxic effect. We found that a combination of sub-toxic concentrations of brittle star methanolic extract (lower than IC_{50}) can significantly enhance ovarian cell growth inhibition and intrinsic apoptosis pathways induced by paclitaxel. Consequently a combination of paclitaxel and brittle star extract may offer novel innovative strategies for ovarian cancer chemotherapy.

Keywords: Natural product, Paclitaxel, Apoptosis, Ovarian cancer

1- Department of Biology and Research Center for Animal Development Applied Biology, Mashhad Branch, Islamic Azad University, Mashhad, Iran
2- Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran
*Corresponding author's Email: Baharara78@gmail.com

Introduction

Ovarian cancer is a gynecological malignancy and one of the most leading causes of women mortality worldwide for which few etiological factors involved have been identified (Leung, 2013). There are several therapeutic strategies to ovarian cancer therapy; however the first line of treatment is a combination of surgery with platinum based chemotherapy (Kim *et al.*, 2013). Also, secondary cyto reductive surgery and combination chemotherapies such as carboplatin/paclitaxel and cisplatin/docetaxel have been proposed as main treatment options (Ingersoll *et al*., 2009). Chemotherapy has been used as a common therapeutic route due to the higher incidence of ovarian neoplastics in advanced stages III, and IV (Rutherford, 2013). Paclitaxel (taxol) as mainly plant derived agents are natural anticancer drugs, tubulin binding agents, antimitotic drugs and cell inducing apoptosis agents that are approved as anti-tumor drugs against ovarian cancer (Lee *et al*., 2012). However, acquisition of chemo resistance is the main obstacle of conventional ovarian cancer modality, therefore development of efficient alternatives against chemo resistance is appreciable in ovarian cancer treatment (Caruso and Tomao, 2013). Growing body of research have attempted to augment the worth of paclitaxel based therapy by the simultaneous administration of natural cytotoxic substances (Malik *et al*., 2011).

Natural products are rich sources of novel biomedicine compounds with numerous chemical diversity from terrestrial and marine ecosystems reflecting conservation of self-defense mechanism against predators, resisting environmental stress and acting as signaling molecules (Demain and Vaishnav, 2011). The safety, low toxicity and few side effects of natural products candidate them as chemotherapeutic drugs. Today, many anti-neoplastic drugs such as vincristine, vinblastine, irinotecan, etoposide and paclitaxel have been evolved from herbal resources (Fiona and Saunders, 2010).

Marine environments represent a rich source of efficient anti-cancer agents, so as in this regard, the application of marine natural products have received much attention due to their tremendous structural diversity in oncological approaches (Gerwick and Moore, 2012). Didemnin B, aplidine, bryostatn, citirabine, dolastatin and ecteinascidian (ET-743) are examples of marine derived agents that are being applied as anticancer leads (Haefner, 2003). Hence, introducing of valuable marine antitumor agents can suggest attractive insight to fighting cancer. One critical approach to discover new chemotherapeutic agents is the evaluation of anticancer effects of total natural extracts to obtain biomedicinal properties (Dhorajiya *et al*., 2012).

Brittle stars are extensive marine invertebrates and possess some bioactive metabolites, including glycosides, terpenes, naphtaquinone and cerebrosides which may be appreciable

in anti-cancer therapy (Weihong *et al*., 2004).

Given the lack of scientific information to confer the utilization of marine natural fauna in the ovarian cancer treatment and loss of adequate knowledge related to biomedical properties of ophiuroidae prompted us to evaluate the cytotoxic effect of the Persian Gulf brittle star (*Ophiocoma erinaceus*) as a frequent marine echinoderm in the Persian Gulf.

Materials and methods

Chemicals and reagents

A2780CP human ovarian cancer cells were purchased from NCBI (National Cell Bank of Iran). RPMI 1640 Medium, FBS (Fetal Bovine Serum), trypsin-EDTA and antibiotic (Penicillin-Streptomycin) were obtained from Gibco-USA. Taxol (paclitaxel) was purchased from Sigma-USA. MTT (3-[4,5- dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) and DAPI was prepared from Applichem (USA). PI (propodium iodide) and Acridine orange was obtained from Sigma (USA). Caspase-3 and caspase-9 colorimetric assay kits were purchased from Abcam (England). Specimens of the brittle star (bs, *Ophiocoma erinaceus*) were obtained from rocky intertidal flats of the Persian Gulf waters. Methanol was purchased from Merck (Germany). This experiment was performed at Research Center of Applied Biology of Mashhad Branch of the Islamic Azad University in 2013.

Preparation of brittle star methanol extract

The collected specimens of *Ophiocoma erinaceus* brittle star obtained from rocky intertidal flats of Qeshm Island were washed and stored at −80°C. For extract preparation, brittle star samples (about 20 g) were dried, girded and soaked in 200 mL methanol. Then, the extract was constantly stirred (72 h) to room temperature, filtered through an 11 µm Whatman filter, concentrated under a vacuum evaporator and stored in −20°C. To prepare the stock solution (100 mg/mL) a concentrated extract was dissolved in DMSO. For use in the experiments, the stock was diluted with serum-free cell culture medium (RPMI 1640) to reduce cytotoxicity of DMSO, so that the final concentrations of DMSO did not exceed 0.05%.

Cell line

A2780CP (human epithelial ovarian carcinoma) was purchased from the Pasture Institute of Tehran, Iran and were grown in RPMI medium supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator containing 5% CO_2 at 37 °C.

MTT assay

Cytotoxic effect of the methanolic brittle star extract and taxol on A2780cp cancer cells was evaluated by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The cancer cells were plated at a concentration of 10^4 cells/well in 96-well plates and adhered overnight. Then, the medium was removed and

incubated with different concentrations of methanol extract brittle star (0, 25, 50, 100, 200 µg/mL) and taxol (0, 10, 20, 40 μgmL^{-1}). After treatment, 20 µL of MTT stock solution (5 $mgmL^{-1}$ in PBS) was added to each well and the plates were further incubated for 4hr at 37°C. Then MTT solution was discarded and100 µL of DMSO was added to each well and the absorbance was measured at 570 nm using micro plate reader (Epoch, USA).

Cell morphological observation

In order to investigate the cytotoxic effects of brittle star methanolic extract and taxol on cell morphology, A2780cp cells were plated in 24 well culture plates (10^5 cells/well) in RPMI 1640 supplemented with 10% FBS. After 24 h adherence of cells, treatment was performed at a defined concentration. After 24 h, the cells were examined under inverted microscope (Bio Photonic, Brazil).

Acridine orange/ propodium iodide staining and DAPI staining

The cultured cells in 6 well plates were treated with defined concentrations of brittle star methanolic extract and taxol for 24 h, harvested and stained with 10 µL (100 μgmL^{-1} Acridine orange and 100µg/mL propodium iodide mixture). For DAPI staining, A2780cp cells were cultured on a coverslip, and then incubated with desired concentrations of brittle star methanolic extract and taxol for 24 h. Then the cells were fixed with methanol for 5 min and exposed to 100 µg/mL DAPI for 15 min at 37°C in the dark. Eventually, cells were visualized under a fluorescence microscope.

Analysis of cell death

To find out the apoptotic effect of methanol extract, brittle star and taxol cultivated A2780cp cells were incubated with medium containing concentrations of brittle star extract and taxol overnight. Then the cells were washed with PBS and resuspended with propidium iodide (PI; Sigma) containing 0.1% sodium citrate plus 0.1% Triton X100 at 37°C for 30 min and then placed at 4°C in the dark for 10 min and the apoptosis analysis was evaluated using a FACScan laser flow cytometer (FACSCalibur, Becton Dickinson, USA).

Measurement of caspase-3 and -9 activity assay

Colorimetric caspase-3 and -9 assay kits were utilized for the measurement of enzymatic activities on the basis of cleavage of p-nitroaniline (pNA) from labeled substrate DEVD-pNA and p-nitroanilide (pNA) from labeled substrate LEHD-*p*-NA by active enzyme. Briefly, 3×10^6 plated cells were incubated for 24h with appropriate concentrations of brittle star methanolic extract and taxol. Then, treated cells were trypsinized and incubated with 300µL lysis buffer for 10 min on ice and centrifuged at 4C to obtain a rich supernatant from cytosolic protein content. The lysate was examined for measurement of caspase 3 and caspase 9 activities according to the

manufacture's protocol. Absorbance of chromophore p-NA was determined spectrophotometerically at 405 nm (Epoch, USA).

Statistical analysis

The statistical analysis carried out by SPSS software, ANOVA, and Student's t test. All results were expressed as mean±SE and the level of $p \leq 0.05$ was considered significant.

Results

Morphological observations under treatment with bs extract and taxol The morphology of A2780cp cells were studied under inverted microscopy. The photomicrographs showed that ovarian cancer cells indicated typical characteristics of apoptosis in response to bs methanolic extract and paclitaxel treatment in a dose dependent manner. Meanwhile, bs extract did not induce significant cytotoxicity on A2780cp cells under exposure with 5% DMSO. The figures were taken from IC_{50} concentration (Fig. 1).

Bs extract enhances the cytotoxic effect of paclitaxel on ovarian cancer cells

The cell cytotoxicity of combination treatment of bs extract and taxol was evaluated by MTT assay.

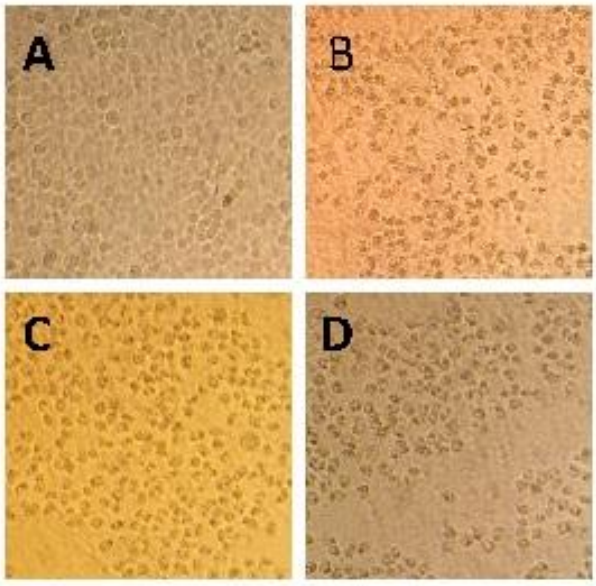

Figure1: Effects of bs extract and taxol on morphology of A2780cp ovarian cancer cells. Images were taken with 400 magnifications. A) Control (without treatment) B) bs methanolic extract (50 µgmL^{-1}) C) taxol (20 µgmL^{-1}) D) synergism effects of bs extract (25 µgmL^{-1}) and taxol (10 µgmL^{-1}). All concentrations were diluted with medium.

As exhibited in Fig. 2, bs extract and paclitaxel noticeably attenuated the cell viability of A2780cp cancer cells in a dose dependent manner.

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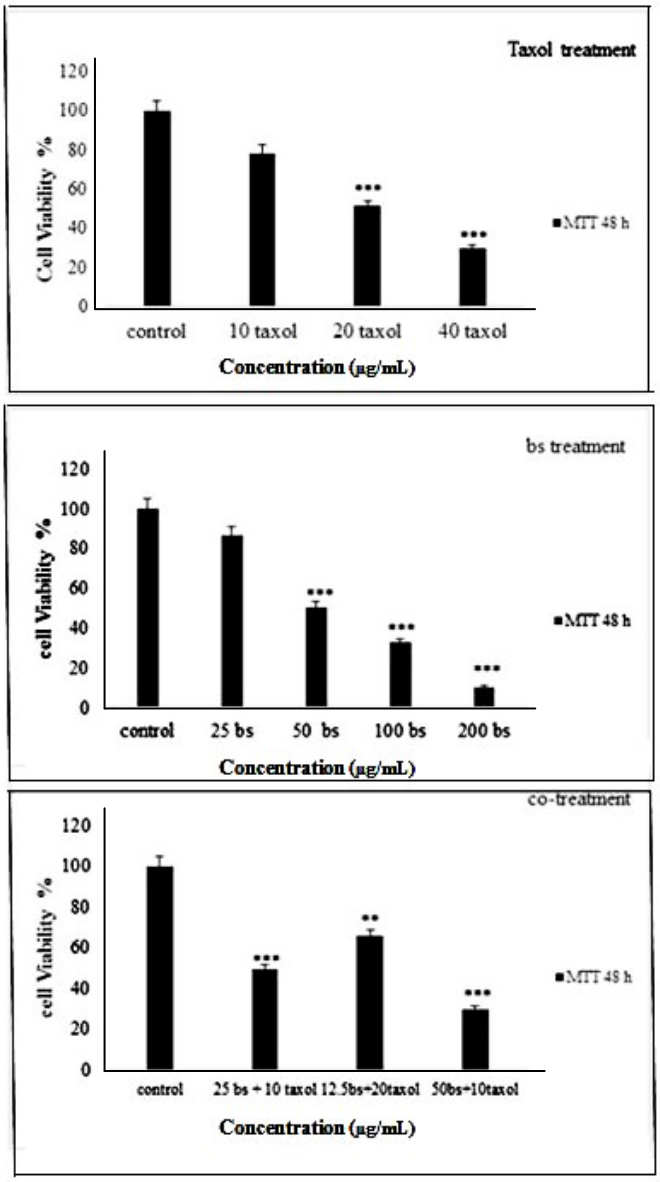

Figure 2: The effect of Brittle star alcoholic extract, taxol and co-treatment on cell viability of A2780cp cell line at 48h treatment period as compared to untreated cells using MTT assay. Data represented as Mean±SEM. ***p<0.001 were considered significant between experimental groups and control.

The cell survival was 50.5, 51.1 and 49.4 following treatment with 50 µgmL^{-1} bs, 20 µg/mL taxol and 10 µgmL^{-1} taxol and 25 µg/mL bs (synergistic treatment) for 48h, respectively. IC_{50} value was determined as 49.2 µgmL^{-1} (bs treatment) and 18.4 µg/mL (taxol treatment) (Fig. 2).

Apoptosis detection by DAPI staining

Apoptosis is programmed cell suicide that is distinguished by multiple morphological alterations such as chromatin condensation, nuclear fragmentation, cytoplasmic blebbing and apaptosome formation. DAPI is a fluorescence stain that creates

fluorescent complex with double strand DNA that is applied for apoptotic detection. As shown in Fig. 3, ovarian cancer cells treated with bs extract or taxol or combination treatment with lower concentration of IC_{50} of each alone, have revealed chromatin gathering that is considered one of the main features of apoptosis (Fig. 3).

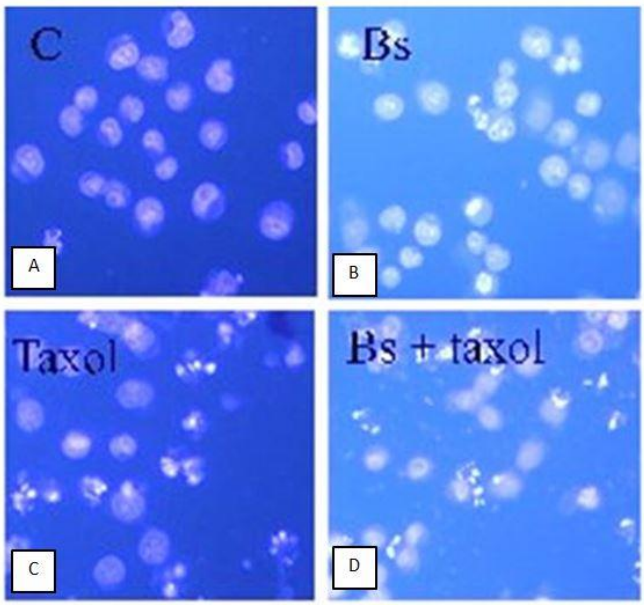

Figure 3: Fluorescence micrographs of bs extract and taxol on A2780cp cells that indicated nuclear fragmentation. A) untreated cells, B) 50µgmL^{-1} Bs extract treatment, C) 20 µgmL^{-1} paclitaxel treatment, D) combination treatment of Bs extract and taxol.

Morphological characterization of apoptosis by AO/PI staining

To examine apoptotic induction under treatment with bs extract and paclitaxel, the A2780cp cells were stained with Acridine orange/propodium iodide. This method recognizes alive, apoptotic and necrotic cells so that green color, bright green color, orang color and red color are characterized as live, early apoptotic, late apoptotic and necrotic cells, respectively. As exhibited in figure 4, treated ovarian cancer cells displayed augment of cell permeability compared with untreated cells. Therefore, anti proliferative effects of bs extract and paclitaxel would be attributed with apoptosis induction of ovarian cancer cells (Fig. 4).

Flow cytometry analysis for apoptosis detection

PI staining to evaluate cell membrane integrity of treated ovarian cancer cells with paclitaxel or bs extract or synergic effects indicated sub-G1 peak demonstrated in apoptotic cells following DNA fragmentation. Flow cytometry histogram of treated cells revealed apoptosis cell death in cell induced cytotoxicity (Fig. 5).

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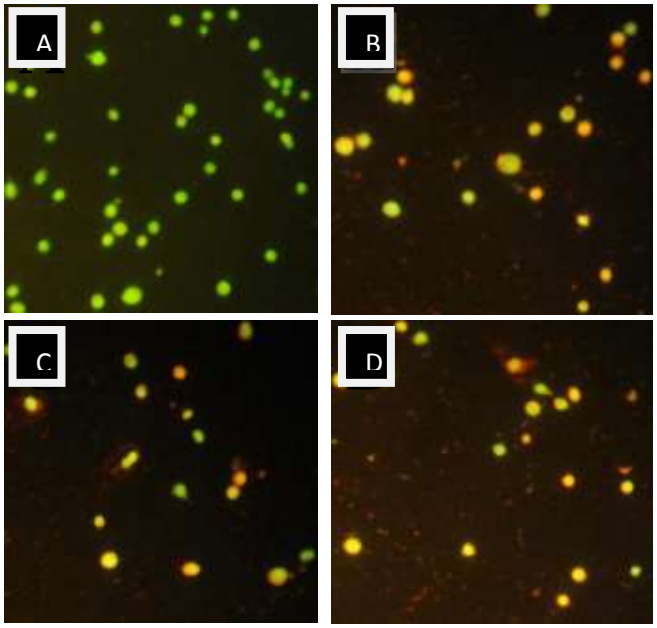

Figure 4: Fluorescence images of acridine orange / propodium iodide stained ovarian cancer cells treated with bs extract and paclitaxel. A) Control group with normal structure, B) treatment with IC_{50} of bs extract, C) treatment with taxol and D) synergism treatment with paclitaxel and bs extract.

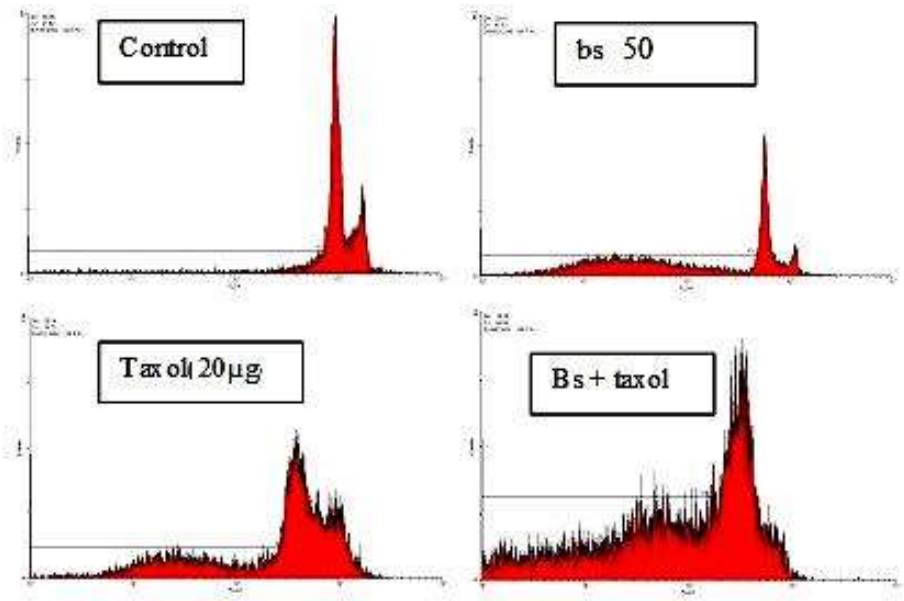

Figure 5: Apoptotic effect of Brittle star alcoholic extract and paclitaxel treatment of the ovarian cancer cells by flow cytometry analysis. Flow cytometry histogram of treated A2780cp cells indicated sub-G1 peak as hallmark of apoptotic cell death.

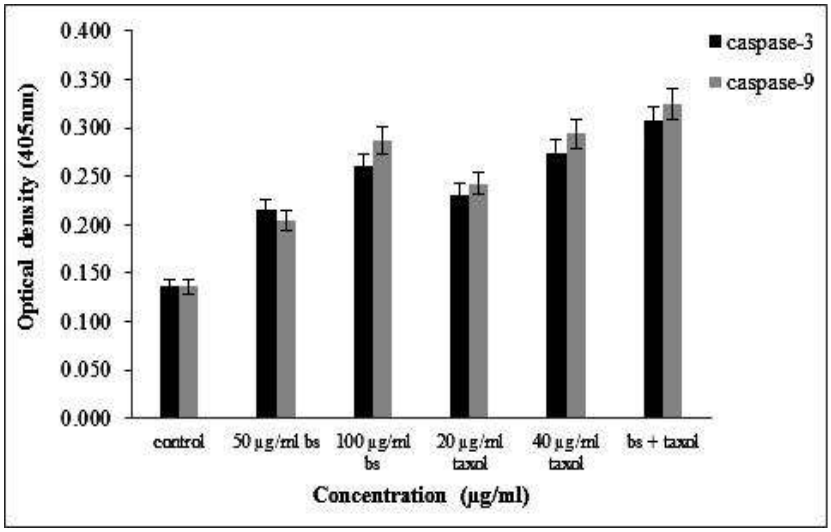

Figure 6: Bs alcoholic extract and paclitaxel induced activation of caspase-3 and caspase-9 in A2780cp cells. $p<0.05$ were considered significant between experimental groups and control.

Discussion

Standard chemotherapeutic strategies for the treatment of ovarian cancer are comprised of paclitaxel and platinum based drug combinations (Zhu *et al*., 2005). Taxol is a diterpene ester and apoptosis inducing drug that is currently utilized as a drug in ovarian cancer (Meshkini and Yazdanparast, 2012). Significant attention has been recently concentrated to distinguish naturally occurring anti-carcinogenic metabolites from marine organisms but investigation on the therapeutic effect of brittle star is less evident (Blunt *et al*., 2012). In the present study, we aimed to compare the anti-cancer effects of brittle star methanolic extract with common chemotherapeutic drug paclitaxel. As revealed in our data, brittle star methanolic extract exhibited a dose dependent cytotoxic effect against human ovarian A2780CP cells and enhanced chemo sensitivity of A2780CP ovarian cancer cells synergistically with paclitaxel. Importantly, the combination of brittle star methanolic extract and taxol induced apoptosis cell death which can be an appropriate adjuvant for ovarian cancer therapy.

Substantial evidence exhibits that natural products possess considerable cytotoxicity against ovarian cancer cells (Zhu *et al.*, 2005). For example, Shosaikoto is a mixture of seven plant extracts (Bupleurum root, Pinellia tuber, Scutellaria root, Jujube fruit, Gingseng root, Glycyrrhiza root, and Ginger rhizome) that have been utilized as treatment options against ovarian cancer in Chinese traditional medicine (Silva *et al*., 2003). Methanol extracts from the root bark of *Hymenocardia acida*, Methanol extracts from the stem bark of *Mangifera indica* , Methanol extracts from the leaves of *Sida rhombifolia*, and the aqueous extract of

Cinnamomum cassia have been considered potent medicinal herbal extract inhibitors of ovarian neoplasia (Silva *et al*., 2003). The literature survey identified many chemically defined compounds isolated from nature in different stages of investigations for ovarian cancer treatment of which the most promising agents attributed to alkaloids (Kumaran *et al*., 2009).

The major objective of anti-tumor therapy is finding bioactive substances which suppress the proliferation of ovarian tumor cells merely. Ecteinnascidin (ET-743) is a tetra hydro iso quinoline alkaloid extracted from the Carrabine tunicate applied as a potent drug against ovarian carcinoma xenografts as being more officious compared with cis-platinum. mycalamide A and B, heterocyclic compounds isolated from *Mycale* sp. and *Theonella* sp. sponges that are efficient against M5076 ovarian sarcoma (Valoti *et al*., 1998).

An in vitro study by Powell *et al* (2003) reported that a water extract of *Scutellaria barbatae* administrated to ovarian cancer cells, inhibited cancer cell growth and induced ovarian cancer cell apoptosis (Powell *et al*., 2003). Moreover, Rhode *et al* (2007) suggested that ginger (*Zingiber officinale* Rosc) possesses anti-carcinogenic properties against ovarian cancer with NF-ƙB suppression and VEGF, IL-8 diminishing (Rhode *et al*., 2007). Based on reports carried out by Ooi, *Physalis minima* whole extracts can induce apoptotic and autophagy cell death in Caov-3 ovarian cancer cells that are characterized by DNA fragmentation, analysis of mRNA expression of p53, c-myc, caspase-3 and ultra-structural changes in TEM analysis which can be valuable as new anti-cancer sources in ovarian cancer therapy (Leong *et al.*, 2010).

One of the major complications correlated with ovarian cancer therapy is drug resistance and the evasion of apoptosis accounting for crucial factors for anti-neoplastic drug resistance. It assumes that the combination of two or more drugs is more effective than a single agent therapy in the process of ovarian chemotherapy and overwhelming to ovarian cancer chemo-resistance (Wender *et al*., 2012). For instance the application of carboplatin with paclitaxel approved standard regimen for advanced or recurrent ovarian cancer. Zhang et al evaluated the co-administration of tetrandrine (a bis benzyl isoquinoline alkaloid, extracted from the root of the *Stephania tetrandrinerandra*) with cisplatin to overcome drug resistance in ovarian cancer treatment. Their findings indicated that the mixture of tetrandrine with cisplatin enhanced the rate of apoptosis and induced higher anti-tumor activities than each drug alone, consequently tetrandrine can utilize appropriate candidates as adjunct to improve ovarian cancer chemotherapy (Zhang *et al*., 2011).

Sun *et al*. (2011) examined the combination of perifosine (an oral alkyl phospholipid) with paclitaxel as a chemotherapeutic option for ovarian

cancer and concluded that the synergistical application of perifosine and paclitaxel with suppression of AKT/mTOR complex 1, elevation of reactive oxygen species generation and activation of AMPK, JNK, caspase3 increased sensitization of ovarian cancer cells to apoptosis, so this modality may be valuable in ovarian cancer therapeutics (Sun *et al*., 2011).

Tie et al designed an investigation on the anti-cancer effects of aqueous and organic Devil's club *Oplopanax horridus* root bark extracts on sensitive and resistant ovarian cancer cells and concluded that this plant extract possesses cytotoxic effects against cisplatin sensitive and resistant cancer lines and at low dosage induced apoptosis with S, G_2/M cell cycle arrest that can be proposed as an appropriate ovarian anti-cancer candidate (Tai *et al*., 2010).

Yu (2013) conducted an investigation on the anti-cancer properties of *Rauwolfia vomitoria* plant extract along with carboplatin to assess the efficacy of chemotherapy. Their results showed that *Rauwolfia vomitoria* extract inhibited ovarian cancer cells proliferation dose dependently with apoptosis induction. Besides, the combination of the extract with carboplatin was more effective because of diminished tumor size and ascite volume. Therefore, this array can be appreciable as a chemotherapeutic option (Yu *et al*., 2013).

A synergistic effect of whole extract with ovarian cancer drugs was offered by Tai and co-workers, who found that *Rosmarinus officinalis* extract can enhance the cytotoxicity of cisplatin on A2780 and A2780CP70 ovarian cancer cells by inducing apoptosis and altering the expression of apoptotic related genes. Therefore rosemary extract further exploited the discovery of an adjunct in ovarian cancer therapeutic (Tai *et al*., 2012).

In general, the outcomes of this experiment for the first time demonstrated that the methanolic brittle star extract exerts precious cytotoxic and apoptotic effects by increasing caspase-3 and caspase-9 activity and holds as a promising adjunct to improve the anti-tumor efficacy of paclitaxel in human ovarian cancer.

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