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Orthosiphon stamineus Benth. Methanolic Extract Enhances the Anti-Proliferative Effects of Tamoxifen on Human Hormone Dependent Breast Cancer

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Abstract: Estrogen Receptor (ER+) antagonist, Tamoxifen (TMX), is widely used in the treatment of the hormone responsive breast cancer. However, the common occurrence of resistance after prolonged treatment of TMX hampers its effectiveness. *Orthosiphon stamineus* Benth. (OS) is a common herb found in South East Asia and is used traditionally to treat various types of ailments. The aim of this study was to determine whether the methanolic extract of *Orthosiphon stamineus* Benth. (MEOS), that had been proven in previous study to act as anti-angiogenic agents, enhance the anticancer efficacy of ER+ antagonists. In this study methanolic extract of (MEOS) was treated to MCF-7 hormone sensitive breast cancer cell line with the addition of TMX. MEOS showed no significant cytotoxic effect towards MCF-7 when used alone, however when combined with TMX, the anti proliferative activity of the combination increased five fold higher when compared to the anti-proliferative activity of singly treated TMX. The result suggests that MEOS synergistically enhance the activity of TMX against hormone responsive breast cancer cells *in vitro* and may prove to be useful for the treatment of metastatic breast cancer.

Key words: *Orthosiphon stamineus* Benth., cancer, synergistic activity, Tamoxifen, estrogen receptor antagonist

INTRODUCTION

Over 60% of breast cancer cases are Estrogen Receptor (ER+) positive which is highly dependent on estrogen for growth. Tamoxifen (TMX) is a potent antagonist of ER and has been approved to be the first line anti-estrogen therapy since the early 1970s (Jordan, 1993). However, most tamoxifen sensitive breast cancer cases succumb to tamoxifen resistance leading to poor prognosis (Lee *et al*., 2008). The resistance that is developed is mainly due to cross-talk between ER and type 1 tyrosine kinase receptors, including Epidermal Growth Factor Receptor (EGFR) and human epidermal growth factor receptor 2 (HER2). These two cytokines are found to be up-regulated during tamoxifen treatment leading to further progression of breast cancer (Abukhadeir *et al*., 2008). In addition, up-regulation of bcl-2 and cycloxigenase-2 (COX-2), which are key players in tumor development, also contribute to tamoxifen resistance (Tari *et al*., 2005).

Orthosiphon stamineus Benth. (OS) is widely used in many countries in South East Asia such as Malaysia, Indonesia and Thailand, for treating kidney ailments and bladder related diseases (Jaganth and Ng, 2000). The OS contains several active components such as terpenoids and polyphenols (Tezuka *et al*., 2002). Most of the therapeutic effects and health benefits of OS is mainly contributed by its polyphenolic contents. Akowuah *et al*. (2004) reported the presence of Rosmarinic acid (RA), Sinensetin (SEN) and Eupatorin (EUP) in the leaves of OS. Recently, methanolic extract of OS was found to have potent anti-angiogenic activity *in vitro* (Sahib *et al*., 2008). However, the exact mechanism is not known but molecular modeling data and immunohistochemical analysis reveal that this activity occurs due to direct inhibition of the VEGF receptor (Unpublished data). Previous studies have shown that VEGFR is present on the surface of hormone sensitive breast cancer cell line MCF-7 (Amin *et al*., 2000). Recent clinical outcome of a number of cancer therapy employing co-administration of antiangiogenic agents with classical chemotherapeutic agent such as TMX, have shown to be of significant success (Lee *et al*., 2008). This study aims to follow similar treatment model for hormone dependent breast

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cancer but by using an antiangiogenic plant extract with TMX instead as an alternative approach.

MATERIALS AND METHODS

Extraction: The leaves of OS were collected from the white flowered variety of the OS species. Leaf specimen was labeled and annotated with the date of collection and the plantation site. A voucher specimen number (11009) was deposited at the herbarium of School of Biology, Universiti Sains Malaysia. The plant was oven-dried at 40°C and the leaves were separated and grounded into fine powder. The powdered material (360 g) was extracted sequentially by adding 30 g in each flask with 200 mL of petroleum ether with continuous shaking for 8 h. The residue was dried and extracted successively with chloroform, methanol and water. The extracts were concentrated under vacuum at 40°C and freeze dried under vacuum. The methanol extract of *Orthosiphon stamineus* Benth. (MEOS) was kept in desiccator at room temperature for further studies.

Cell culture: MCF-7; an ER+ human breast cancer cell line; was purchased from American type cell culture (ATCC, USA) and was maintained in Dulbeccos Modified Eagle (DMEM) medium (Invitrogen, Carlsbad, CA, USA), supplemented with 100 U mL^{-1} Penicillin, 100 μg mL^{-1} streptomycin and 10% heat inactivated fetal calf serum (HIFCS) (Sigma-Aldrich, Germany). Cells were incubated at 37°C in a humidified atmosphere in 5% CO_2. Cells were sub-cultured twice weekly.

Cell viability assay: The cells viability was evaluated by Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma, Aldrich, Germany) assay. Cells were seeded in 96-well plat (nunclon™, Denmark) at a concentration 1×10^4/well, passage 8 and incubated at 37°C for 24 h. Then MEOS was added by dissolving the extract with dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) and dilute the mixture with the medium to make the final DMSO concentration 1%. MEOS was incubated with the cells for 48 h and then 20 μL from 5 mg mL^{-1} of MTT (Sigma-Aldrich, Germany) was added on each well and incubated for 5 h, at 37°C and 5% CO_2. The medium was aspirated and 200 μL from DMSO added to dissolve the fromazon crystal that is formed. The spectrophotometrical absorbance of the samples were measured by the micro-plate reader (Hitachi U-2000, Japan), with measurement wave length of 570 nm and reference wave length of 650 nm. DMSO at 1% v/v, was used as a negative control and the results were presented as a mean percent of inhibition to the negative control±SD. Each experiment was performed in quadreplicate. The inhibitory effect was calculated according to the following equation:

$$\text{Cell growth inhibition (\%)} = (1-(A_0/A)) \times 100$$

Where:
A_0 = Absorbance of the samples
A = Absorbance of the negative control

The additive inhibitory effect of agents a and b was calculated as following (Koren *et al*., 2000):

$$CA_{ab} = 100\times[1-(1-D_a/100)\times(1-D_b/100)]$$

where, CA_{ab} is calculated additive inhibitory effect of combinations, D_a, D_b and $D_{a,b}$ are the measured inhibitory effect of each agent alone and in combination.

The growth inhibitory effect of combination treatment was determined as following:

- Synergistic effect $D_{a,b}>CA_{ab}$
- Additive effect $D_{a,b} = CA_{ab}$
- Antagonist effect $D_a<CA_{ab}$

RESULTS

The effect of combined treatment of Tamoxifen (TMX) and MEOS, of O. Stamineus, exert an enhanced anti-cancer effect on breast cancer cells. Cell viability was examined by MTT assay on human positive estrogenic receptors MCF-7 breast cancer cell. Cells were treated with MEOS. Figure 1 shows the inhibitory effect of different concentrations of MEOS, on the MCF-7 cell line after 48 h of treatment. The IC_{50} were 45.39 μg mL^{-1}. This value has been calculated through the linear regression equation for MEOS:

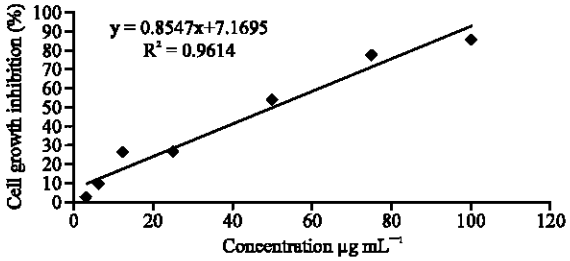

Fig. 1: The inhibitory effect of MEOS, on MCF-7 breast cancer cell after 48 h of treatment, cell viability was measured by MTT assay; cells were treated with serial dilution of MEOS, data are represented as Mean±SD, n = 4, experiment repeated twice

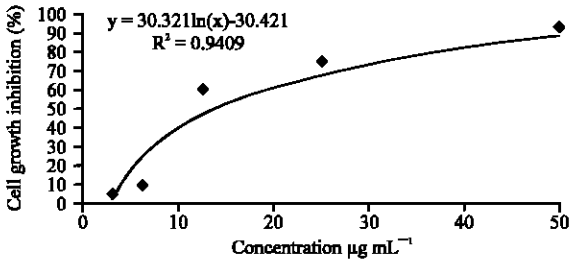

Fig. 2: The inhibitory effect of Tamoxifen, on MCF-7 breast cancer cell after 48 h of treatment. Cell viability was measured by MTT assay; cells were treated with serial dilution of tamoxifen. The data are represented as Mean±SD, n = 4. The experiment was repeated twice

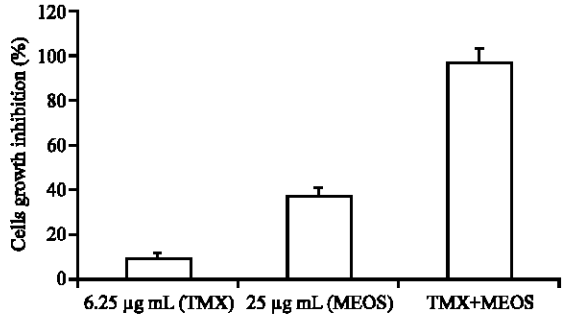

Fig. 3: The effect of combined treatment of tamoxifen with the sub-effective dose of MEOS. The bar chart shows the inhibitory activity of individual dose of Tamoxifen and MEOS on MCF-7 cancer cell line after 48 h, n = 4. The experiment was repeated twice and the % of inhibition is represented as Mean±SD. Cell viability was measured by employing MTT assay

$$Y = 0.854X+7.1696$$

where, Y is percentage of cells proliferation inhibition and X is concentration.

The inhibition % was represented by Mean±SD. Serial concentrations have been used for the crude extract. It showed dose dependant of inhibition as shown in Fig. 1. 100, 75, 50, 25, 12.5, 6.25, 3.125 and 1.625 µg mL^{-1} for the MEOS showed cells proliferation inhibition of 85.67±0.7, 77.56±4.6, 53.83±2.9, 27±3.6, 26.33±0.5, 10.71±1.9 and 1.89±3.8%, respectively.

Figure 2 shows the percentage of cell growth inhibition of Tamoxifen alone on MCF-7, serial dilution ranging from 50, 25, 12.5, 6.25 and 3.125 µg mL^{-1}, the percentage of cell line growth inhibitions were represented as Mean±SD, as follow, 99.92±3.9, 63.22±2.01, 52.43±1.8, 12.92±1.8 and 4.98±1.6%, respectively. The IC_{50} for the tamoxifen alone was calculated through the logarithmic regression equation Y = 30.77ln(X)-32.285 it was 13.46 µg mL^{-1}.

Figure 3 shows the results of combined treatment of (TMX) and MEOS resulted in synergistic inhibitory effect on MCF-7 cells. As 6.25 µg mL^{-1} from (TMX) and 25 µg mL^{-1} from MEOS, when used alone, induced only 9.34±2.3 and 36.86±3.4% of cell growth inhibitory effect, respectively, while combined use of same dose of (TMX) with MEOS resulted in an inhibitory effect of 97.55±2.43%, $p<0.05$ in compared to the calculated additive inhibitory effect of 42.4%.

DISCUSSION

Chemoprevention is widely accepted to suppress or prevent carcinogenesis, but treatment of cancer with chemotherapeutic agents at Maximal Tolerated Dose (MTD) is associated with deleterious side effects such as immunosuppression, cardiotoxicity, nephrotoxicity and hepatotoxicity. However, treatment of cancer with chemotherapeutic drugs at MTD has not shown to cause any significant increase in the cure rates (Hemaiswarya and Mukesh, 2006). In order to overcome the general toxic effects of chemotherapeutic compounds at MTD, combination of chemotherapeutic agents at low concentrations with other drugs including herbal preparations that have synergistic activity, may improve the outcome of chemotherapy (Shah and Schwartz, 2000).

In this study, there was significant synergistic activity between MEOS and TMX on human hormone dependent breast cancer cells (MCF-7). Sahib *et al.* (2008) have shown that MEOS has potent antiangiogenic activity *in vitro*. This multi-activity composing antiangiogenesis and synergistic activity may be due to the overall effect of active constituents present in the MEOS extract targeting multiple pathways that governs cancer cell survival.

Clinically, nearly all hormonal responsive breast cancer cases succumb to drug resistance (Lee *et al.*, 2008). Hence, a combined treatment regime which can enhance the efficacy of estrogen receptor antagonist such that of Tamoxifen, will be of great benefit. *Orthosiphon stamineus* Benth. (OS) contains high amount of tri-terpenoids and flavonoids (Gringauz, 1997). Many of these chemical components have been demonstrated to have potent antiangiogenic activity via direct inhibition of Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF), cyclooxigenase receptor (COX-2) and Epidermal Growth Factor Receptor (EGFR) (Krol *et al.*, 1995). These cytokines are amongst the key

players in the angiogenesis process. Betulinic acid, which exists in high quantity in OS (Akowuah *et al*., 2004), have been found to play important role as apoptotic inducer via the mitochondrial pathway, leading to cytochrome-c release and apoptotic cell death of cancer cells (Thurnher, 2003). This compound has also been demonstrated to have strong antiangiogenic response (Sahib *et al*., 2008). Another important component found in OS is rosmarinic acid. This compound has also been demonstrated to have potent antiangiogenic property (Sahib *et al*., 2008).

The synergistic effect of MEOS, observed in this study, could have been contributed by the presence of high amount of betulinic acid and rosmarinic acid in the extract. However, there are also other constituents present which may also play important role in the synergistic activity of OS extract with TMX when used against MCF-7 breast cancer cells.

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