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In vitro Anti-oxidant and Anti-cancer Activity of Methanolic Extract from *Sanchezia speciosa* Leaves

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Abstract: *Sanchezia speciosa*, is a bushy shrub from Acanthaceae family which commonly grows in tropical areas of South and Central America. In this study, we employed MTT assay to test the cytotoxicity of that methanolic fraction of *S. speciosa* leaves on MCF-7 human breast cancer, SK-MEL-5 human malignant melanoma and human umbilical vein endothelial cells, HUVEC cells. The extract showed highest activity on MCF-7 and moderate cytotoxicity towards SK-MEL-5. In contrast, the extract demonstrated lowest cell growth inhibition activity on HUVEC cells, indicating better selectivity compare to standard drug, doxorubicin. In addition, we also performed ORAC assay to determine the radical scavenging capacity of methanolic extract of *S. speciosa* leaves. The extract exhibited nearly similar anti-oxidant activity as quercetin, suggesting *S. speciosa* leaves as a potential source of natural anti-oxidant. To the best of our knowledge, this is the first report on anti-oxidant and cytotoxic activity of *S. speciosa*.

Key words: *Sanchezia speciosa,* MCF-7, SK-MEL-5, HUVEC, MTT, ORAC

INTRODUCTION

Breast cancer is a heterogeneous disease that has become a global public health problem in the recent years. The incidence of breast cancer has undergone an upward trend during the recent decades at an annual rate of 3.1% (Forouzanfar *et al*., 2011). The number of new cases has increased from 1.38 million women in 2008 (Ferlay *et al*., 2010) to more than 1.6 million in 2010 (Forouzanfar *et al*., 2011). Breast cancer has been reported as the most usual cause of cancer deaths in adult women in 2008 (16% of all cancer deaths) (WHO, 2008). In 2007, 3.242 female breast cancer cases were reported in Malaysia, accounted for 18.1% of reported cancer cases and 32.1% of female cases (Ariffin and Nor Saleha, 2011). Breast cancer cell lines are applied by many investigators to screen and characterize new therapeutics and among these cells, MCF-7 is the most extensively used model of estrogen positive breast cancer cell line. MCF-7 cell line was originally established using a pleural effusion from a metastatic breast cancer patient, at the Michigan Cancer Foundation in 1973 (Soule *et al*., 1973) and then these cells were distributed globally to be used in various investigations.

Anti-oxidants are compounds that prevent damage to cell structures caused by chemical reactions involving free radicals. Many synthetic anti-oxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyl hydroquinone (TBHQ) have been produced since the beginning of their application in food. However, majority of investigators tend to replace them with natural anti-oxidants, due to the serious side effects of these synthetic anti-oxidants (Van Esch, 1986; Grice, 1988; Miller *et al*., 2000). Prior to the era of modern medicine, medicinal plants and herbs have been extensively used for centuries as the main source of remedies against most ailments. There is a general desire among modern medicine researchers to utilize pharmaceutical plants as sources of novel drugs.

Sanchezia speciosa, commonly known as shrubby whitevein, is a semiwoody evergreen shrub with smooth bright green or purple stems, large variegated leaves and colorful flowers. Scientific synonym for this plant is *S. nobilis*. It is a member of Acanthaceae plant family, occurring in the lowlands of tropical South and Central America, mostly in Peru and Equador (Clay and Hubbard, 1977). *S. speciosa* is usually cultivated as an ornamental

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plant in shrub borders and hedges throughout the tropics and in botanical gardens of temperate areas due to its large, colorful bracts, foliage and flowers and sometimes even colorful leaves (Leonard and Smith, 1964). In this study, we evaluated the anti-cancer effect of methanolic extract from *S. speciosa* on MCF-7, SK-MEL-5 and HUVEC cells. In addition, we used ORAC (oxygen radical antioxidant capacity) assay to evaluate the anti-oxidant potential of *S. speciosa*.

MATERIALS AND METHODS

Plant material: The leaves of *S. speciosa* were obtained from Seri Subah Agrofarm, Negeri Sembilan, Malaysia and verified by their botanist. The plant leaves specimen was labelled as SS1 and kept at Department of Pharmacology, Faculty of Medicine, University of Malaya.

Cell culture: The human breast cancer cell line, MCF-7 was purchased from cell lines service (300273; Eppelheim, Germany), Human Umbilical Vein Endothelial Cells (HUVEC) cells was obtained from ScienCell Research Laboratories (Carlsbad, CA) and human malignant melanoma cells, SK-MEL-5 was acquired from American Type Culture Collection (ATCC, Manassas, VA). MCF-7 and SK-MEL-5 cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Inc, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO), 1% penicillin and streptomycin. HUVEC cells were grown in Endothelial Cell Medium (ECM, ScienCell Research Laboratories, Carlsbad, CA) supplemented with 5% heat-inactivated Fetal Bovine Serum (FBS, ScienCell Research Laboratories, Carlsbad, CA), 1% (ECGS, ScienCell Research Laboratories, Carlsbad, CA), 1% penicillin and streptomycin (P/S solution, ScienCell Research Laboratories, Carlsbad, CA). Cells were cultured in tissue culture flasks (Corning, USA) and were kept in incubator at 37°C in a humidified atmosphere with 5% CO_2. For experimental purposes, cells in exponential growth phase (approximately 70-80% confluency) were used.

Extraction: The fresh leaves of *S. speciosa* were cleaned, dried and powdered coarsely. The powder (100 g) was macerated with MeOH (3×300 mL) for 72 h. The resultant extract was filtered and dried under vacuum to yield 247 mg of the extract. Then the dried fractions were kept at -20°C until further use.

MTT cell viability assay: The influence of *S. speciosa* methanolic leaves extract of was determined by MTT assay (Mosmann, 1983). MCF-7, SK-MEL-5 and HUVEC cells were treated for different lengths of time including 24, 48 and 72 h. On the first day, 1.0×10^4 cells were seeded into a 96-well plate for 24 h incubation assay while 7.0×10^3 and 5.0×10^3 cells were seeded for 48 and 72 h incubation assay, respectively. It was followed by overnight incubation of the seeded cells at 37°C in 5% CO_2. On the next day, the cells were treated with a two-fold dilution series of six concentrations of methanolic extract from *S. speciosa* leaves and then they were incubated at 37°C in 5% CO_2, based on the certain time period of each plate (24, 48, 72 h). MTT solution (4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazoliumbromide) was added at 2 mg mL^{-1} and after 2 h of incubation at 37°C in 5% CO_2, DMSO was added to dissolve the formazan christals. The plates were then read in Chameleon multitechnology microplate reader (Hidex, Turku, Finland) at 570 nm absorbance. The cell viability percentage after exposure to *S. speciosa* leaves extract for 24, 48 and 72 h was calculated by previously described method (Mosmann, 1983; Looi *et al*., 2011). The ratio of the absorbance of treated cells to the absorbance of DMSO-treated control cells was determined as cell viability (percentage). The concentration of *S. speciosa* leaves extract which is required to reduce the absorbance of treated cells to 50% of the DMSO-treated control cells was defined as IC_{50}.

ORAC-antioxidant activity assay

Chemicals: Fluorescein sodium salt, AAPH (2, 2'-Azobis (2-methylpropionamidine) dihydrochloride), quercetin dehydrate and trolox ((±)-6-Hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma-Aldrich.

Oxygen Radical Antioxidant Capacity (ORAC) assay was done based on procedures previously described with slight modifications (Zulueta *et al*., 2009; Arya *et al*., 2013). Compounds were diluted to final concentration of 100 μg mL^{-1}, with total reaction volume of 200 μL. The assay was performed in a 96-well black microplate, with 25 μL of samples, standard (trolox), blank (solvent/PBS) or positive control (quercetin). Subsequently, 150 μL of working fluorescein solution was added to each well of assay plate. The plate was incubated at 37°C for at least 5 min. Twenty five microlitter of AAPH working solution was then added to the wells, making up total volume of 200 μL. Fluorescence was recorded with excitation wavelength of 485 nm and emission wavelength of 538 nm. Data were collected every 2 min for duration of 2 h and were analyzed by calculating the differences of area under fluorescence decay curve (AUC) of samples and blank. The values were expressed as Trolox Equivalent (TE).

Statistical analysis: Experimental values were expressed as the Means±Standard Deviation (SD) of the number of experiments indicated in the legends. GraphPad Prism 5 software was employed for the analysis of variance (ANOVA). Statistical significance was defined when $p<0.05$.

RESULTS AND DISCUSSION

The cytotoxic effect of *S. speciosa* methanolic extract on cell viability was determined by MTT assay on MCF-7, SK-MEL-5 and HUVEC cells. The MTT assay was done in different time points to have a better view of the cell growth pattern after treatment with *S. speciosa* methanolic extract. We treated the cells with different concentrations of doxorubicin, a cancer chemotherapy drug as positive control. No significant cell inhibitory effect was observed in DMSO (solvent)-treated samples. As shown in Table 1, the plant extract induced the highest cell growth inhibition activity on MCF-7 cells. However, its activity on SK-MEL-5 was moderate and demonstrated lowest cytotoxic effect on HUVEC normal endothelial cells. In contrast, doxorubicin exhibited no selectivity against cancer cell-lines or normal endothelial HUVEC cells.

In this study, we showed that *S. speciosa* methanolic extract inhibited MCF-7 cell growth in a time and concentration dependant manner (Fig. 1). HUVEC cells were used to determine the level of selectivity of the extract on normal and cancer cells. As shown in Table 1, the IC_{50} value of the extract for HUVEC cells in each time point was at least 3 fold higher than MCF-7 cells. Higher selectivity is crucial to avoid side effects and ensure the other non-target cells are not/less affected in order to gain optimal therapeutic effect. As many scientists have turned to natural product for anti-cancer drug discovery research, methanolic extract of *S. speciosa* leaves may contained anti-cancer compounds worthy of further development (e.g., bio-assay guided isolation).

ORAC-antioxidant activity assay: ORAC assay has been widely used for measuring anti-oxidant capacity as it is the only assay which involves the use of peroxyl radical as pro-oxidant and quantify activity via., Area Under Curve (AUC) technique (Prior and Cao, 1999). In our experiment, quercetin was used as the standard for comparison of antioxidant activity. The assay performed showed that methanol extract of *S. speciosa* leaves exhibited comparable anti-oxidant capacity with the standard quercetin (Table 2).

Many diseases such as cancer and atherosclerosis had been reported to be complicated by excessive production of free radicals and Reactive Oxygen Species (ROS) (Machlin and Bendich, 1987; Aruoma, 1998). Thus, natural products which contain anti-oxidant property is useful to alleviate oxidative stress induced by ROS and possess therapeutic value in treating these chronic diseases mentioned above. To serve this aim, we performed ORAC assay to determine the radical scavenging capacity of methanolic fraction of *S. speciosa* leaves. The principle of ORAC assay is on the capacity of compounds tested to compete with the probe in scavenging generated radicals, thus inhibiting the decay of fluorescent probe (Takashima *et al*., 2012). From our results, methanol extract of *S. speciosa* leaves possess nearly similar anti-oxidant activity as the standard (quercetin), suggesting *S. speciosa* leaves as a potential source natural anti-oxidant. Anti-oxidants developed from natural product are safer compare to synthetic anti-oxidant which may have many unwanted side effects.

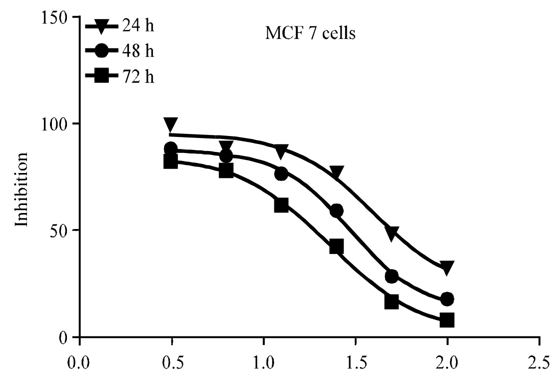

Fig. 1: Dose-dependent inhibition of MCF-7 cells proliferation treated with methanolic extract of *S. speciosa* leaves for different lengths of time (24, 48 and 72 h)

Table 1: Cytotoxic activity of *S. speciosa* methanolic fraction on MCF-7, SK-MEL-5 and HUVEC cells in terms of IC_{50}

Treatment period (h)	IC_{50} ($\mu g\ mL^{-1}$) on MCF-7 cells		IC_{50} ($\mu g\ mL^{-1}$) on SK-MEL-5 cells		IC_{50} ($\mu g\ mL^{-1}$) on Huvec cells	
	S. speciosa	Doxorubicin	*S. speciosa*	Doxorubicin	*S. speciosa*	Doxorubicin
24	39.83±1.59	2.27±0.31	77.90±8.21	8.83±2.43	>100	9.46±1.79
48	29.62±0.44	2.11±0.17	69.33±6.91	7.21±1.28	97.26±4.68	8.73±1.14
72	23.20±1.18	1.93±0.12	62.56±5.32	7.95±0.92	91.15±2.83	8.29±1.37

Table 2: Anti-oxidant capacity of compounds by ORAC method

Compound	μM TE/100 (μg mL^{-1})
S. speciosa	55.77±1.73
Quercetin	63.07±0.93

CONCLUSION

The methanol fraction of *S. speciosa* leaves exhibited significant anti-oxidant activity, comparable to the standard drug, quercetin. The extract also indicated highest cytotoxicity against human breast cancer cells, MCF-7, in comparison with human umbilical vein endothelial, HUVEC and SK-MEL-5 human malignant melanoma cells. To the best of our knowledge, this is the first report on anti-oxidant and cytotoxic activity of *S. speciosa* extracts.

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