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Anti-Angiogenic and Anti Oxidant Properties of *Orthosiphon stamineus* Benth. Methanolic Leaves Extract

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Abstract: Angiogenesis is a process by which new blood vessels are formed from the pre-existing blood vessel. *Orthosiphon stamineus* Benth. OS has been used as a medicinal herb for many centuries. Due to the presence of high level of anti-oxidants and phenolic content compounds in OS and the effect of anti-oxidants and phenolic compounds being anti-angiogenic, the perturbation of new blood vessels ability of OS was tested. Dry powdered leaves of the OS plant were extracted with Petroleum Ether (PE), chloroform (CE), methanol (ME) and water (WE) by using sequential cold maceration method. The ME of OS has the highest anti-angiogenic activity (93.28±1.24%) in the rat aortic assay followed by CE (85.55±1.64%), PE (51.54±4.12%) and WE (50.22±1.23%) in descending order of reactivity. The methanol extract was also found to have potent anti-oxidant activity in the1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity assay. The IC_{50} value was measured to be 0.286 mg mL^{-1}. The total phenolic content of 1 mg mL^{-1} of methanol extract was equal to 38.27%.

Key words: Anti-angiogenesis, *Orthosiphon stamineus* Benth., cancer, anti-oxidant

INTRODUCTION

Orthosiphon Stamineus Benth. (Synonym: *Ocimum arstitum B1*, *Orthosiphon arstatus* (Blum). Local name in Malaysia: misai kucing, from the family Lamiacease is a shrub, which grows to a height of 1.5 m. OS has been used as a medicinal herb for many centuries. OS is well known in many countries in South East Asia such as Malaysia, Indonesia and Thailand, for treating kidney aliments and bladder related diseases (Jaganth and Ng, 2000). In Europe it is commonly referred as kidney tea and in France it is called moustaches de chat (Jaganth and Ng, 2000).

Articles have proved that OS contain several active components such as terpenoids and polyphenols (Tezuka *et al*., 2002). Most of the therapeutic effect and health benefit ascribed mainly to its polyphenolic contents (Akowuah *et al*., 2004). Akowuah *et al*. (2004) showed the presence of Rosmarinic acid (RA), sinensetin (SEN), eupatorin (EUP) in the leaves of OS.

Angiogenesis is a process of new blood vessel formation from pre-existing one, regulated by a variety of endogenous cytokines (Robert *et al*., 2003). This process plays a vital role in the growth and metastasis of tumors and several chronic inflammatory diseases including rheumatoid arthritis and proliferative diabetic retinopathy (Folkman, 1995; Beaux *et al*., 1999). Anti-angiogenic therapies, aimed at halting new blood vessel growth are being developed to treat these conditions. Recently the concept of using anti-angiogenic agents with conventional chemotherapy has been materialised in the clinical setting with the approval of the drug Avastin for the treatment of metastatic colon cancer (Tezuka *et al*., 2002). Such approach in treatment strategy may be ideal with natural products that exhibit anti-angiogenic activity using similar treatment regime.

The first stage of angiogenesis process involves the activation of endothelial cells by a series of growth factors which initiates the angiogenesis event. Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumor necrosis factor alpha (TNF-α) and

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interleukin-8 (IL-8) are the major players in the angiogenesis pathway (Carmeliet *et al*., 1996; Kerbel, 2000). The second major stage of the angiogenesis development is the enzymatic degradation of the endothelial basement membrane lining followed by the detachment of these cells from adhesion proteins that migrate into new locality (Peter and Rakesh, 2000).

A number of antioxidants such as rosmarinic acid, betulinic acid have been found to have potent anti-angiogenic properties (Matsubara, 1999). Others have also been cited including quercetin, myricetin and luteolin (Fotsis *et al*., 1997; Mukherjee *et al*., 1999; Eengelmann *et al*., 2002; Kim, 2003; Tan *et al*., 2003). The manner by which these compounds work in inhibiting angiogenesis stems from either direct interaction of key angiogenic receptors or by changing the redox microenvironment of the tumor vasculature (Bagchi *et al*., 2004). As betulinic acid, quercetin and rosmarinic acid has been found in high amount in OS, the thing that ergs us to investigate its anti-angiogenic activities of the crude extracts.

MATERIALS AND METHODS

Preparation of plant extract: The leaves of OS were collected from the white flowered variety of the OS species. Leaves specimen was labeled and annotated with 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 powder form. The dried leaves powder (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 then dried and extracted successively with chloroform, methanol and water. The solvents used in the experiment have been purchased from Riedel-de Haën, Germany. Each extract was concentrated using a Büchi RE121 rotary evaporator (Büchi Labortechnik AG, Switzerland) under vacuum and freeze dried in a Hetovac VR-1 freeze dryer (Heto Lab. Equipment AS, Denmark). The lyophilized extract was then kept in desiccators at room temperature prior to the experiment.

Experimental animal: Aortic tissue samples were acquired from 12-14 weeks old male sprague dawley (SD) rats obtained from the animal house facility (School of Pharmaceutical Sciences, Universiti Sains Malaysia). All animals were sacrificed via., cervical dislocation under anesthesia by using diethyl ether (Riedel-de Haën, Germany). All experimental procedures were done according to the guide line of USM ethical committee and had their approval.

Rat aorta assay anti-angiogenesis experiment: The angiogenesis assay used in this method is according to that developed by Brown *et al*. (1996) with slight modification. Freshly excised thoracic tissues were rinsed with hanks balanced salt solution containing 2.5 μg mL^{-1} amphotricin B (Sigma-Aldrich, Germany). The tissue specimens were then cleaned of periadventitial fibro adipose material and residual blood clots. This was then cut into 1 mm thick aortic ring segments under a dissecting Motic SMZ 143 microscope (Motic®, Taiwan).

The assay was performed in a 48-well tissue culture plate (Nunclon™, Denmark). Five hundred microliter of 3 mg mL^{-1} fibrinogen (Calbiochem, USA) in serum free M199 growth medium (Gibco®, USA) was added to each well with 5 mg mL^{-1} of aprotinin (Sigma-Aldrich, Germany) to prevent fibrinolysis of the vessel fragments. Each tissue section was placed in the center of the well and 15 μL of thrombin (50NIH U mL^{-1}) (Sigma-Aldrich, Germany) in 0.15 M NaCl. Bovine plasma (Sigma-Aldrich, Germany) was added to the well and mixed rapidly with fibrinogen. Immediately after embedding the vessel fragment in the fibrin gels, 0.5 mL of medium M 199 supplemented with 20% heat inactivated fetal calf serum (Gibco®, USA), 0.1% ε-aminocaproic acid (Sigma-Aldrich, Germany), 1% L-glutamine (Sigma-Aldrich, Germany), 1% amphotricin (Sigma-Aldrich, Germany), 0.6% gentamycin (Sigma-Aldrich, Germany) was added to each well. 100 μg mL^{-1} of the test substance was added to the complete growth medium and each treatment was performed in six replicates. Control cultures received medium without the test substance. The sample extract was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) and diluted in M199 growth medium to make 1% effective DMSO concentration. Vessels were cultured at 37°C in 5% CO_2 in a humidified CB150 incubator (Binder, Germany) for 5 days. Fresh medium was added on day four of the experiment. Suramin, a well recognized antiangiogenic agent was purchased from (Sigma-Aldrich, Germany) was used as a positive control. The extent of blood vessel growth was quantified under 40X magnification using an inverted Olympus LH 50A microscope (Olympus, Japan) on day five of the procedure with the aid of a (Lieca CCD, Japan) camera and (Lieca QWin) software packages interfaced with an Intel Pentium 4 desktop computer. The magnitude of blood vessel growth inhibition was determined according to the technique developed by Nicosia *et al*. (1997). Briefly, the length of the tiny blood vessel outgrowths from the primary ex-plant was measured. The data is represented as Mean±SD deviation (SDE). The percentage of blood vessels inhibition was determined according to the following formulae:

$$\text{Blood vessels inhibition} = 1 - (A_0/A) \times 100$$

Where:
A_0 = Distance of blood vessels growth
A = Distance of blood vessels growth in the control

Dose response relationship: Serial dilution of ME was made by diluting the OS ME with M199 growth medium to make eight consecutive solutions of OS with concentration value of 100, 50, 25, 12.5 and 6.25 μg mL^{-1}. The IC_{50} value of the ME anti-angiogenic activity was determined by using linear regression equation, the experiment has been repeated three times six replicate per concentration. Blood vessels inhibitions were quantified at day five.

1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity: The free radical scavenging activity of the ME was measured by DPPH scavenging activity as described by Oktay *et al*. (2003). Briefly, 1 mL of 0.1 mM solution of DPPH in methanol was added to 2 mL of the methanol extract. 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625 and 0.007813 mg mL^{-1} of the methanol extract were prepared through serial dilution. The absorbance reading was measured at 517 nm by spectrophotometer (Hitachi U-2000, Japan) after 30 min in triplicate. Percentage reduction of DPPH (Q) was calculated according to the formula below:

$$Q = 100(A_0 - A_C)\ A_0$$

Where:
A_0 = Absorbance of control
A_C = Absorbance of the batches of methanol extract and its fractions, DPPH after 30 min incubation

Total phenolic contents: Total soluble phenolic in samples were determined with Folin-Ciocalteu reagent according to the method of Slinkard and Singleton (1977) with gallic acid as standard. Hundred microliter of different concentration of gallic acid (Sigma, USA) and 100 μL of extracts were pipetted in different test tube, respectively. After that 2 mL of distilled water was added into each test tube. Then 200 μL of Folin-Ciocalteu reagent was added into the test tubes and mixed thoroughly. After 3 min, 1 mL of sodium carbonate (BDH, UK) (15%) was added; the mixture was allowed to stand for 2 h with intermittent shaking (24-26°C). The absorbance was measured at 760 nm with spectrophotometer (Hitachi U-2000, Japan). The concentration of total phenolic compounds in the mixture determined as mg of gallic acid equivalent by using an equation Y = 3096x+0.0288 that obtained from standard gallic acid graph (Yam *et al*., 2006).

Statistical analysis: All data is expressed as Mean±SDE. The statistical analysis of the data was carried out by using one-way ANOVA, followed by the Tamhans, Tukey post hoch test. The differences between the means are considered significant at the 5% confidence level. The median inhibit concentration (IC_{50}) value was analysed by linear regression equation.

RESULTS

Rat aorta angiogenesis assay

Blood vessels growth inhibition after administration of various crude extracts of *O. stamineus* Benth: A dose of 100 μg mL^{-1} was administered from ME, PE, CE and WE. Extracts on rat aorta embedded in complete growth medium of M199 treated with PE, CE, WE and ME significantly inhibited blood vessels growth at day five $p<0.05$. From these four extracts, ME extract was found to have relatively stronger anti-angiogenic activity in comparison to the other extracts (Fig. 1). The 100 μg mL^{-1} ME gave the highest level of inhibition of 93.28±1.24% followed by 85.55±1.64% for CE. PE showed 51.54±4.12% of inhibition level while WE gave a value of 50.22±1.23%. The level of inhibition for the PE is, however; insignificant when compared to the WE $p>0.05$. As ME gave the highest level of activity it was selected as a candidate

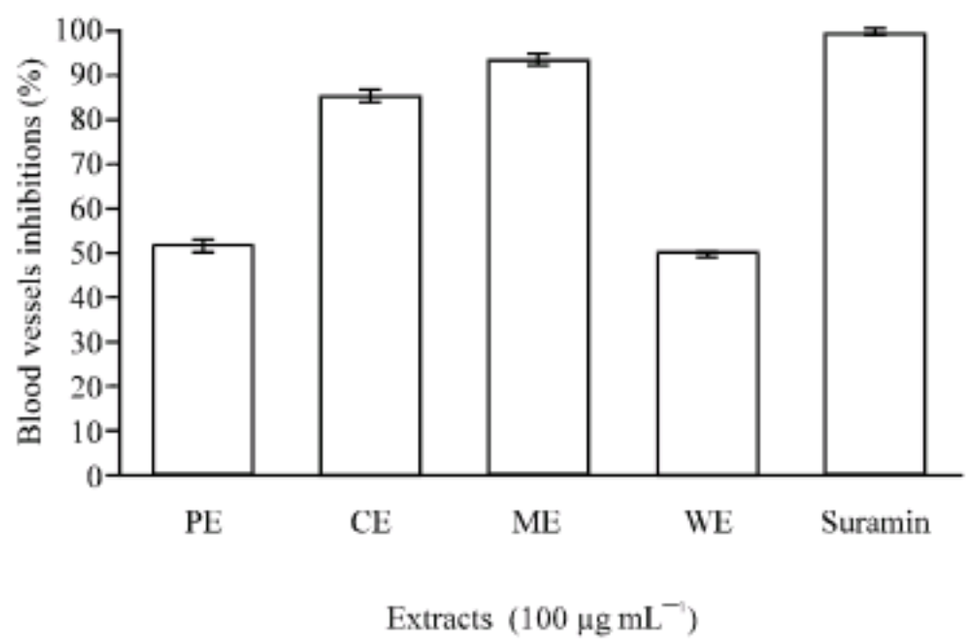

Fig. 1: The effect of administration of 100 μg mL^{-1} of different OS extracts: PE, CE, ME, WE and suramin as a positive control on rat aorta. Results are expressed as mean percentage changes in blood vessels growth on rat aorta±SDE. Suramin acts as a positive control. The experiment was repeated three times, n = 6, ME shows the highest inhibitory activity on new blood vessel development when compared to PE, CE and WE ($p<0.05$)

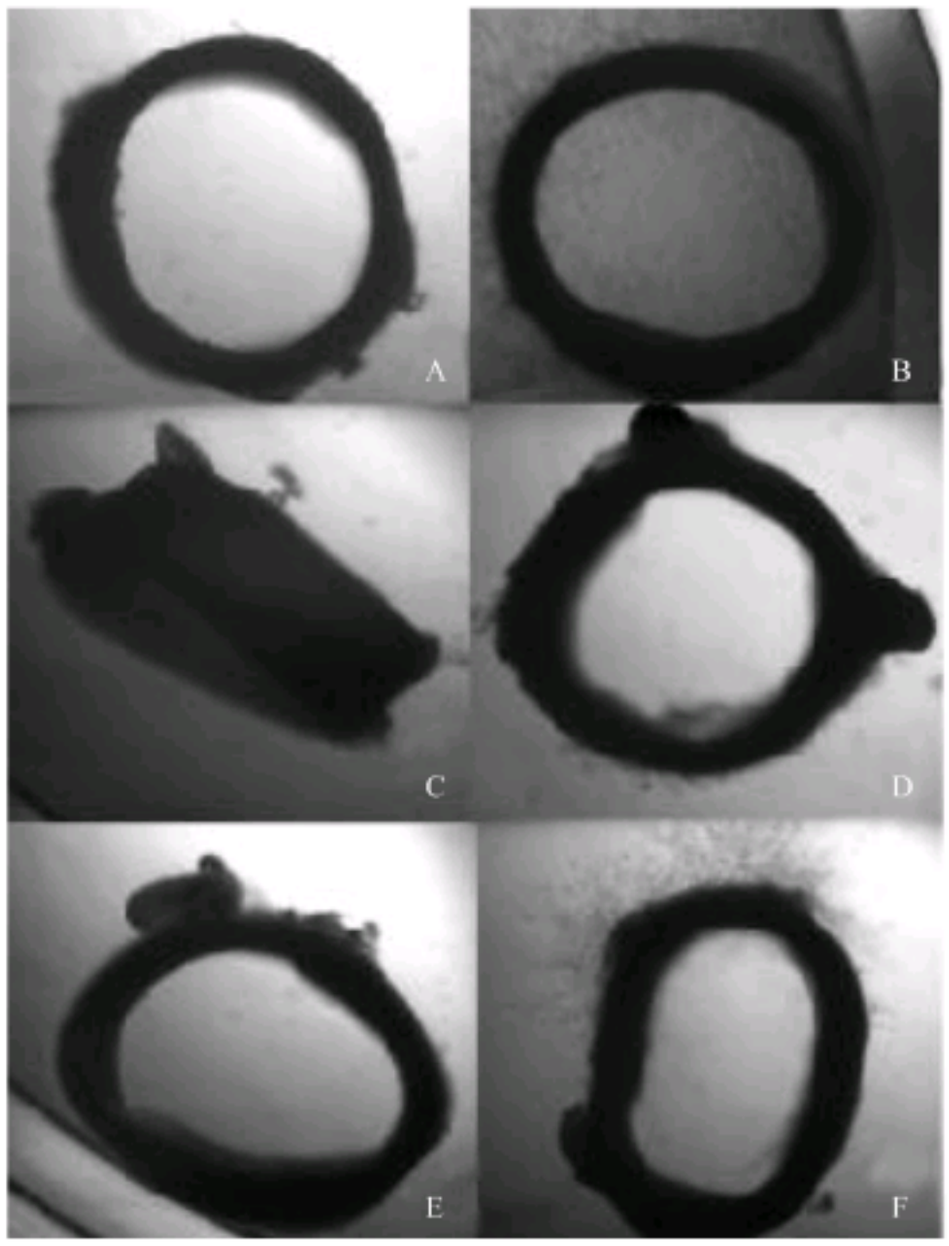

Fig. 2: Images of blood vessels growth displaying the various extent of antiangiogenic activity from ME, PE, CE and WE. The two controls consisting of suramin for the positive control and 1% DMSO for the negative control. A (suramin); B (negative control); C (PE); D (CE); E (ME) and F (WE)

extract for further investigation to determine its dose-response curve and IC_{50} value in an attempt to determine the optimum dose and free radical scavenging activity.

The results were obtained on day five of the procedure. The extent of angiogenesis inhibition was quantified by using Lieca QW in soft ware package. ME gave the highest level of anti-angiogenic activity in comparison to the negative control, $p<0.05$. While CE, PE and WE gave potent inhibition in comparison to the negative control, but at significantly lower level ($p<0.05$). Suramin was used as a positive control; 100 μg mL^{-1} from suramin inhibited the blood vessels growth by 100% after five days of culture (Fig. 2).

Eight different concentrations from ME were used as a serial dilution on rat aorta, ranging from 100, 50, 25, 12.5 and 6.25 μg mL^{-1} which gave inhibition value on the blood vessels growth of 99.99±8.13%, 69.80±4.5%, 51.02±3.17%, 48.53±3.05% and 46.66±2.91%, respectively ($p<0.05$). The assessment of blood vessel growth was

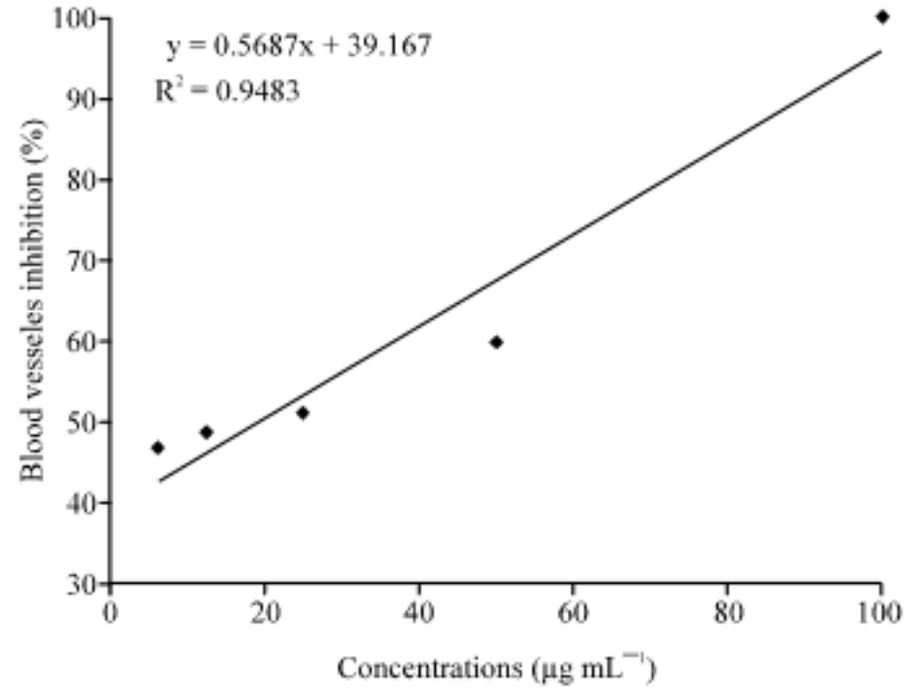

Fig. 3: The figure show the dose response curve of *Orthosiphon stamineus* Benth. ME on rat aorta. The serial dilutions of 200, 100, 75, 50, 25, 12.5, 6.25 and 3.125 μg mL^{-1} of the ME showing significant dose dependant inhibitions $p<0.05$ (n = 3). The percentage of blood vessels inhibition is represented as Mean±SDE

quantified 5 days after drug treatment. The experiment was repeated twice and the IC_{50} value was 19.05 μg mL^{-1} as determined from the linear regression equation Y = 0.5687x+39.167 (Fig. 3).

Antioxidant activity of the methanol extract: The methanol extract of OS on DPPH scavenging activity IC_{50} value was calculated according to the formula Y = 138.74x+10.241. Where, Y is the percentage of scavenging and X is the concentrations of the most biological active extract. The IC_{50} value of Y is fixed as 50. Hence, the IC_{50} for the methanol extract of OS is determined to be 0.286 mg mL^{-1}. This was achieved from experimental data of serially diluted ME of the following concentrations of 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156 and 0.0078 mg mL^{-1}. The experiment was carried out in triplicate using morin and rutin as positive control with their IC_{50} value being 0.384 and 0.0158 mg mL^{-1}, respectively. ME of OS showed a similar potency in its antioxidant activity to that of the morin positive control with the value measured to be at 0.286 mg mL^{-1}.

Total phenolic content: The total phenol content in the methanol extract was determined as gallic acid equivalent (GAE) by using an equation obtained from the reference curve (Y = 3096x+0.0288). 1 mg mL^{-1} of methanol extract in 80% ethanol gave 0.459 of absorbance. The 1 mg mL^{-1} of methanol extract was equal to 38.27%.

DISCUSSION

This study aimed to investigate the anti-angiogenic activity of OS in different extracts; ME gave the most active extract in comparison to other extracts and as the angiogenesis process can be happened by more than one pathway, total phenolic content and its anti-oxidant activity has been tested to fine the most likely mechanisms and to make this point the start point for further study. Antioxidants are well-known to have potent anti-angiogenic activity. Amongst those that have been identified includes vitamin C, vitamin D, vitamin E, vitamin A, betulinic acid, rosmarinic acid, 3-hydroxyflavone, 3',4'-dihydroxyflavone, 2',3'-dihydroxyflavone. In this study, we found that (ME) of OS leaves extract gave the most potent anti-angiogenic activity as shown in the rat aortic arch ring assay. Its potency in inhibiting new blood vessel development could be contributed to its significant anti-oxidant behavior as shown in the DPPH scavenging assay. This perhaps may result in a decrease in free radicals present which are known to activate the hypoxia responsive element gene. The latter acts as a trigger for VEGF, a key cytokine in angiogenesis activation (Goodwin, 2007). Moreover, one of the potent angiogenic agents is TGF α; anti-oxidant agents have the ability to inhibit TGF α expression as one aspect of their inhibition of angiogenesis (Shklar, 1998). The presence of significantly high phenolic contents in OS may possibly be presumed to possess a big role in contributing to its anti-angiogenic potentials via its down-regulating activity on nitrous oxide (NO) production. Which may be partly responsible for the pharmacological efficacy of several folkloric medicines (Tsuda *et al*., 2004).

Further study is required to confirm weather the inhibitory effect on angiogenesis is due to perturbation on the other angiogenesis pathways, standardization for the crude extract should be done to identify the most likely compounds which might have this activity.

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