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Analysis of Total Proteins, Polysaccharides and Glycosaponins Contents of *Orthosiphon stamineus* Benth. In Spray and Freeze Dried Methanol: Water(1:1) extract and its Contribution to Cytotoxic and Antiangiogenic Activities.

Siddiqui M.J.[1]*, Hafizoh S. N.[1], Ismail Z.[1]Sahib H.B.[2], Helal M.H.S., and Majid A.M.S. Abdul

[1]*Department of Pharmaceutical Chemistry*
[2]*Department of Physiology and Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden, P.Penang -11800, Malaysia.*

* **Corresponding author:** *Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Universiti Sains Malaysia, P.Penang 11800, Malaysia. Phone and Fax No +6046563443, siddiquijamshed@hotmail.com.*

ABSTRACT

Orthosiphon stamineus (OS), Benth, (Lamiaceae), popularly known as Java tea, is a useful traditional medicinal herb cultivated largely in Malaysia and in Indonesia. This study was conducted to investigate the primary metabolic contents, cytotoxicity and antiangiogenic activity of freeze dried and spray dried extracts of the OS methanol: water (1:1). Primary metabolic contents were evaluated for total proteins, total polysaccharides and glycosaponins ($P<0.05$). Freeze dried extract showed high content of proteins and glycosaponins while contents of polysaccharides were high in spray dried extracts. Cytotoxicity was detected by MTT assay using MDA-MB-231 breast cancer cell line and anti angiogenesis assay was performed on rat aorta. Both extract showed moderate cytotoxic activity using extract in high and medium concentration. Freeze dried extract showed strong antiangiogenic activity while spray dried extract showed no activity. A significant change in total proteins of freeze dried extract may be the reason for strong antiangiogenic activity.

Keywords: Antiangiogenesis, Proteins, Polysaccharides, Glycosaponins, Cytotoxicity, *Orthosiphon stamineus*

INTRODUCTION

Ever since evidence emerged that growth of malignant tumors could be slowed or even prevented by cutting off their blood supply the search for antiangiogenic agents has widened and now includes natural compounds and agents derived from natural products (1). *Orthosiphon stamineus* (OS), Benth, (Lamiaceae) is one of the most useful traditional medicinal herbs cultivated in South East Asia for the treatment of eruptive fever, epilepsy, gallstone, hepatitis, rheumatism, hypertension, syphilis and renal calculus. In Malaysia, it is known as kumis kuching or Cat's whisker and is used as tea to improve health and for the treatment of kidney, bladder inflammation, gout and diabetes (2–3). *O. stamineus* contains several phytochemical active constituents, such as triterpenoids, diterpenoids, polyphenols (lipophilic flavonoid and phenolic acids) and sterols (4–10). Previous research suggests the pharmacological effects of *O. stamineus* being diuretic, antioxidant as well as the alleviation of hyperglycemia and improves lipid profile in diabetic rats. These properties have partially been attributed to the polyphenolic compounds such as flavones, sinensetin and 3'-hydroxy-5, 6, 7, 4'-tetramethoxyflavone (11–16). Presence of high levels of flavonoids, triterpenoids and caffeic acid derivatives

in methanol and water extract may contribute to its hypouricemic activity via their antioxidant properties (17). Antioxidants such as rosmarinic acid, sinensetin and betulinic acid have been shown to contribute to the antiangiogenic activity of methanol extract of *O. stamineus* leaves (18). Acute toxicity LD_{50} was estimated to be >5000mg/kg body weight for standardized *O. stamineus* extract (19).

Angiogenesis is a process of new blood vessel development. It depends mainly on locomotion, proliferation and tube formation by capillary endothelial cells (20). During angiogenesis, endothelial cells emerge from their quiescent state and can proliferate rapidly but for brief duration. Angiogenesis plays an important role in the diseases of ocular neovascularisation, arthritis, skin diseases and tumors and until recently been difficult to suppress therapeutically (21). Therefore, the fundamental goal of all antiangiogenic therapy is to return foci of proliferating microvessels to their normal resting state, and to prevent their growth (22). Single antiangiogenic agents have limited efficacy. Natural products contain a range of complex organic chemicals that may have synergistic activity. They may inhibit angiogenesis by interacting with multiple pathways and by acting in other ways that can affect cell signalling, the apoptotic pathways, and the interaction of cancer cells with the immune system (23). A number of antioxidants molecules such as rosmarinic acid, sinensetin, betulinic acid, quercetin, myricetin and luteolin can cause direct interaction of key angiogenic receptors or by changing the redox microenvironment of the tumor vasculature hence perturbing the angiogenesis event (24–27). Angiogenic activity in *O. stamineus* extract may be constituted by the presence of antioxidant molecules.

The aim of the present study is to investigate the freeze and spray dried methanol: water (1:1) extract of *O. stamineus* for total contents of proteins, polysaccharides glycosaponins and to evaluate their cytotoxicity and antiangiogenic activity. The current study attempts to compare between freeze and spray dried methanol: water (1:1) extract of *O. Stamineus* in terms of their pharmacological activity particularly in inhibiting angiogenesis process.

MATERIALS AND METHODS

Extraction

Orthosiphon stamineus leaves cultivated and propagated under controlled conditions with the joint venture of USM-UNIMAP at Titi Tinggi, Perlis, Malaysia. Extraction of leaves was carried out at CEPP, UTM to obtain the crude extract after extraction process using methanol: water (1:1). The crude extract was dried using spray dried and freeze dried techniques. Voucher specimens of the plant materials were deposited at Bilik Herba, School of Pharmaceutical Sciences, Universiti Sains Malaysia.

Chemicals and instrument

Chemicals and solvents were of analytical grade, which include methanol (Merck), ethanol (Merck), water (Universiti Sains Malaysia), DMSO (Sigma Aldrich), Bovine Serum Albumin Fraction V (Sigma Aldrich), Glucose (Sigma Aldrich), and Folin- Ciocalteau reagent (Sigma Aldrich). MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide, Sigma, USA], MDA-MB-231 from ATCC catalogue number (HTB-26), DMEM cell culture medium was from Gibco, USA. Lambda 45 UV/VIS Spectrometer (Perkin Elmer) instrument was used for study.

Experimental animal

Male Sprague Dawley (SD) rats were chosen in their 10th week age and were obtained from the animal house (School of Pharmaceutical Sciences, Universiti Sains Malaysia). All animals were subjected to cervical dislocation under anaesthesia by using diethyl ether. Experiments were performed according to the guidelines of Universiti Sains Malaysia Ethical Committee with prior approval.

Estimation of total proteins

Total protein estimation was performed by a method described by Lowery (28). Fifty milligram extract was mixed with 10 ml distilled water in a centrifuge tube and vortex for two minutes. Tube was further centrifuged for 10 min at 2700rpm. Supernatant was used for analysis. A volume of 0.05 ml supernatant was pipette out into a test tube, make it up to 1ml with distilled water, 3 ml of reagent C added, which was prepared by mixing 50 ml of reagent A (2% sodium carbonate in 0.1N sodium hydroxide) and 1 ml of reagent B (0.5% copper sulphate in 1% potassium sodium tartrate). Further add 0.2 ml of Folin- Ciocalteau reagent and tube was incubated for 30 min at room temperature. Bovine Serum Albumin (Fraction V) was used as a reference standard in a range of 10-250 ug/ml. All the samples and standards were prepared in triplicates and absorbance was measured at 600nm against a blank having all the reagents except the sample. Total proteins were calculated from linear regression equation, obtained from the standard curve.

Estimation of total polysaccharides

To a centrifuge tube 0.2g sample was added and dissolved with 7 ml hot ethanol (80%) just to remove sugars. The sample tube was vortex for 2 minutes and then centrifuged at 2700 rpm for 10 minutes. Repeat the process till washing of residue shows no colour with anthrone reagent. The residue was then dried on a water bath and extracted with each 5ml of water and 25% HCl at 0?C for 25minutes. Sample tube was centrifuged at 2700 rpm for 10 minutes and the supernatant was collected in a 100 ml volumetric flask. Repeat the extraction this time with10 ml 25% HCl and collected supernatant again into a same 100 ml volumetric flask and made it up to 100 ml with distilled water. Pipette out 0.1ml of supernatant to a test tube and make it up to 1ml with distilled water. To it add 4ml of anthrone reagent and test tube was heated for 8 minutes in boiling water bath. Sample tubes were cooled rapidly while keeping on ice and the intensity of green colour was measured at 630 nm against a blank having all the reagents; except sample. Reference standard solutions of glucose were prepared from 20, 40, 60,100 and 200 μg/ml and treated in the same way. All samples and standards were prepared in triplicates. Concentration of the glucose was calculated from the linear regression equation obtained from the standard curve. Starch contents were calculated by multiplying the glucose contents obtained from standard curve with factor 0.9.

Estimation of total glycosaponins

One gram extract was dissolved in 50 ml methanol, refluxed for 30 min and then filtered. This process was repeated twice. Filtrate was concentrated to 10 ml using rotary evaporator and the saponins were precipitated by adding extract drop wise to 50 ml acetone in tarred beaker.

Precipitate was dried in oven at 100°C to constant weight and calculation was done by following formula: Glycosaponins = (Weight of precipitate / Weight of sample)×100

Cytotoxicity Assay

Cell proliferation assay

The cell was cultured in DMEM growth medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

Cell proliferation assay for attached cells

MTT viability assay was performed with slight modification as described by Mosmann (29). In brief, cells were seeded at 5000 cell density per well for each 96-well plates in 180μl medium. After an overnight incubation, extracts MKP/UTM/FD and MKP/UTM/SD were added into each well using the stock solution to make the final concentration 100 and 25μg/ml. These two concentrations were used only for screening purpose. The untreated cells received only DMSO as a negative control. DMSO was serially diluted at concentrations ranging from 1-0.03%. All the cells were treated for 48hours. The experiment was repeated twice with four replicates for each concentration. MTT was first prepared as a stock solution in 5mg/ml of phosphate buffer saline solution. At the end of the treatment period (48hrs), 20μl of MTT stock solution was added to each well. After 4h incubation at 37°C, the medium was removed and 200μl of DMSO was added to each well to dissolve the formazon crystal. After 1 min of shaking, the optical density was measured using a micro-plate reader (Multiscan Ascent) at 570nm for absorbance and 650nm as reference filter. The percent of inhibition was calculated according to the following formula:

$$\text{Percent of inhibition} = (1-(A_O/A)) \times 100$$

Where: A_O is the absorbance of the drug-treated cells; A is the absorbance of the DMSO treated cells

Anti-angiogenic assay

The angiogenesis assay was performed by slight modification of the method by Brown et al (30). Thoracic tissues were rinsed with hanks balanced salt solution containing 2.5μg/ml amphotricin B. The tissue specimens were then debrided of adipose material and blood clots. After that it was cut into 1-2mm thick aortic ring segments under a dissecting Motic SMZ 143 microscope (Motic, Taiwan). Two types of media were prepared. The first layer contained L-Glutamine, Fibrinogen dissolved in M 199 media. The second layer contained Gentamycin, Fungizon, dissolved in same media. SD rats were chosen in their 10th week age to perform the rat anti-angiogenesis assay. These rings were seeded in 48-well plate which contained 300μl of the first layer media. Then 10ml thrombin was added and incubated for 1.5hours. The OS freeze and spray dried extracts were dissolved in the second layer media and then added on the wells. DMSO was used as negative control at 1% concentration. Only one concentration was used which was 100mg/ml and repeated in six wells. After 7 days the extension of the blood vessels was measured for each extract and compared with negative control. The magnitude of blood vessel growth inhibition was determined as per the technique developed by Nicosia et al (31). Lengths of the tiny blood vessel outgrowths were measured with Mean±SD deviation for representing the data.

Determination of total phenolic contents

The total soluble phenolic contents in the freeze and spray dried extract methanolic: water (1:1) of *O. stamineus* leaves were determined by using Folin-Ciocalteau reagent and gallic acid (3, 4, 5-trihydroxybenzoic acid) as a standard according to the method of Slinkard and Singleton (32). A solution of 4mg/ml of the freeze and spray dried extracts in methanol and solutions of 0.0625, 0.125, 0.25, 0.5, 1, and 2 mg/ml of gallic acid in methanol were prepared. 20µl of the extracts and each concentration of gallic acid solution was pipette out in separate test tubes followed by the addition of 1.58 ml of distilled water and 100µl of 2N Folin-Ciocalteau reagent. Subsequently, the test tubes were mixed thoroughly. After 10 minutes 300µl of 20% sodium carbonate solution were added. The mixture was then allowed to stand for 2 hours with intermittent shaking. The absorbance of solutions was measured at 765 nm with a Lambda 45 spectrophotometer.

The concentrations of total phenolic compounds in the extracts were determined as milligram of gallic acid equivalent by using and equation which was obtained from standard gallic acid graph.

Determination of total flavonoid content

The total flavonoid contents in the freeze and spray dried extract methanol: water (1:1) of *O. stamineus* leaves were determined by using aluminium chloride colorimetric method with quercetin as standard (33-35). A solution of 4mg/ml of quercetin in methanol was prepared. 500µl of extracts and each concentration of quercetin solution was pipette out in separate test tubes followed by the addition of 0.1ml of 10% (w/v) aluminium chloride solution, 0.1ml of 1M potassium acetate solution, 1.5ml of methanol and 2.8ml of distilled water. The test tubes were thoroughly mixed and after incubation at room temperature for 30 minutes, the absorbance of the reaction mixture was measured at 415 nm with Perkin Lambda 45 spectrophotometer. The amount of 10% aluminium chloride was substituted by the same amount of distilled water in a blank. The concentration of total flavonoid contents in the extracts were determined as micrograms of quercetin equivalent by using an equation that was obtained from the standard quercetin graph.

Statistical analysis

All the samples and standards were performed in triplicates and results were expressed as mean ± SD. P values <0.05 was considered significant.

RESULTS AND DISCUSSION

The freeze and spray dried extracts of *O. stamineus* Benth. Leaves were investigated for primary metabolites. Total protein contents were estimated using linear regression equation ($Y = 4.7989e^{02} + 2.0434e^{03} X$) was obtained from calibration curve of standard Bovine Serum Albumin (fraction v). Total polysaccharides were estimated by linear regression equation ($Y = 4.0423e^{02} + 6.4936e^{03} X$), which was obtained from the standard curve of Glucose. Total glycosaponins were estimated by gravimetric assay. The results of total primary contents are shown in Table 1, which indicate the percentage contents of primary metabolites in freeze and spray dried extracts.

The contents of proteins and glycosaponins were higher in freeze dried extract as compared to spray dried extracts. In the case of polysaccharides, spray dried extract were higher in contents as compared to freeze dried extract. This suggests that freeze dried extract is a better choice over spray dried for antiangiogenic activity. Freeze dried extract of *O. stamineus* methanol: water (1:1) is rich in total protein contents and total glycosaponins while spray dried extract is rich in total polysaccharides. Thermal decomposition may be the reason for lowering antiangiogenic activity as the total protein and glycosaponins is less in spray dried extract.

Figure 1 shows that freeze and spray dried extracts have strong cytotoxic activity on MDA-MB-231 breast cancer cell line, which may be due to presence of some cytotoxic compounds present in the extracts.

Table 1: Total proteins, polysaccharides, glycosaponins, phenolics and flavonoids in *Orthosiphon stamineus* Benth. Leaves freeze and spray dried methanol: water (1:1) extracts

Extract	Total Proteins (%)	SD	Total Polysaccharides (%)	SD	Total Glycosaponins (%)	SD	Total Phenolic mg/g	Total Flavonoids mg/g
MKP/UTM/FD (Freeze dried)	39.7	±0.32	1.47	±0.86	38.3	±0.61	6.32	1.27
MKP/UTM/SD (Spray dried)	36.1	±0.41	2.11	±1.5	34.8	±0.97	6.38	1.13

Angiogenic activity comparison was made according to the length of vessel extensions. When full length vessels extension is seen, this means no inhibition activity was performed (Figure 2). The results were obtained on day seven of the procedure and distance were measured from the images of rat aorta (Figure 3). Study results shows that freeze dried extract gave the potent antiangiogenic activity, which is due to presence of number of antioxidants such as rosmarinic acid, sinensetin and other flavonoids(18). As per the previous findings *O. stamineus leaves* extracts also contains diterpenes and triterpenes especially betulinic acid, hydroxy betulinic acid and Oleanolic acid responsible for angiogenesis. Previous study highlighted the role of isolated polysaccharides from *Antrodia cinnamomea* in the suppression of angiogenesis (36). Further studies are required not only to identify the active ingredients present in proteins, polysaccharides and glycosaponins but to establish their structure activity relationship is the need of time.

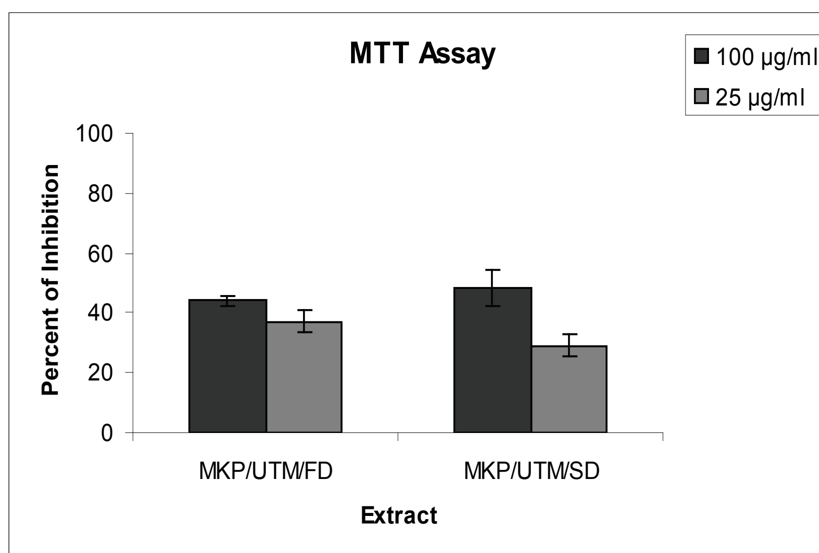

Figure 1: Bar chart showing cytotoxic properties of two *O. stamineus* freeze and spray dried extracts by MTT assay. Bars represent the percent of cell growth inhibition of MDA-MB-231 breast cancer cell line.

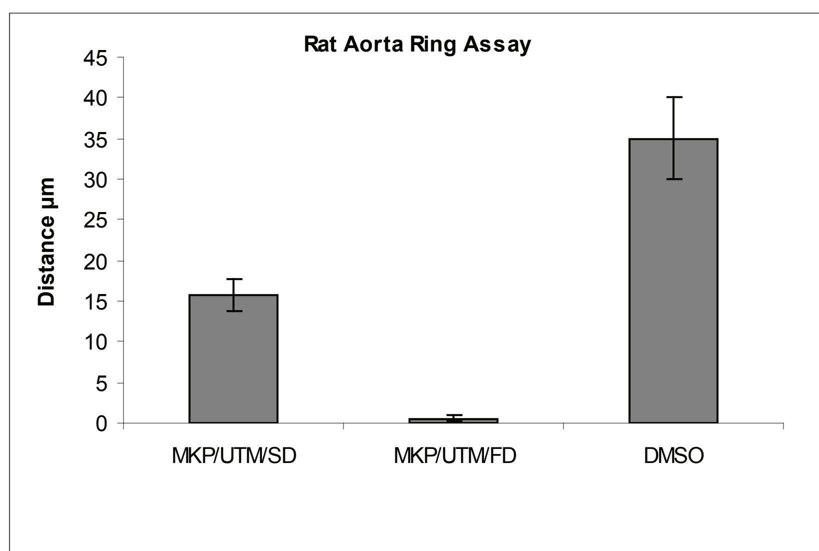

Figure 2: Bar chart showing anti-angiogenic properties of two *O. stamineus* freeze and spray dried extracts by rat aorta ring assay. The bars represent the average distance of blood vessel outgrowth from the aortic discs.

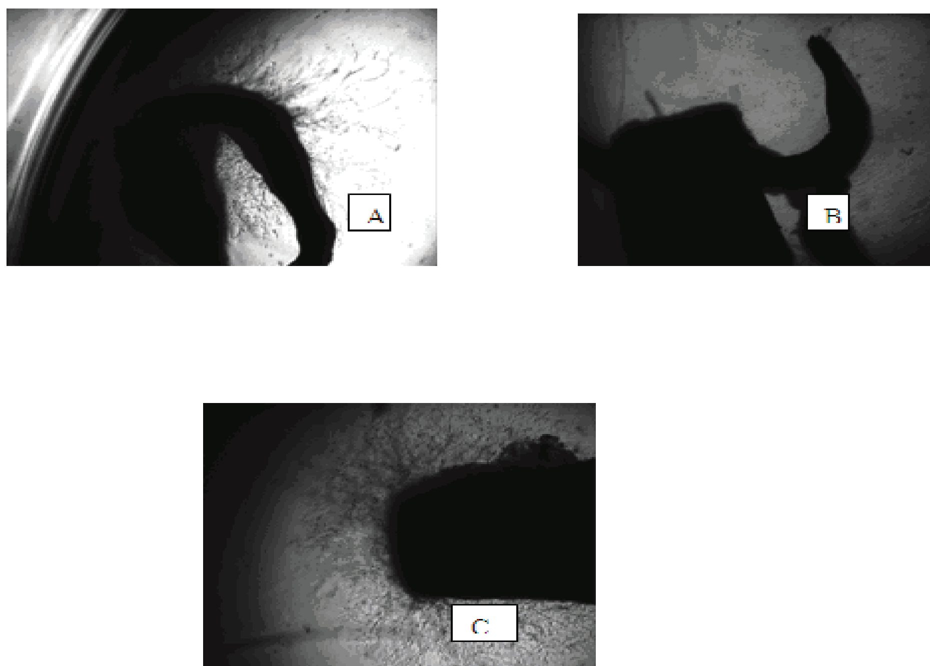

Figure 3: Images of blood vessels growth displaying the angiogenesis activity of *O. stamineus* extracts with 1% DMSO for the negative control; A (MKP/UTM/SD); B (MKP/UTM/FD); C (negative control).

This process can be used for rapid screening of natural products plant extractives so that cost and time can be saved. Further study is required to calculate the blood vessels inhibition quantitatively using different concentrations to get the IC_{50} values which can be determined by using linear regression equation repeated three times six replicate per concentration.

CONCLUSION

Freeze and spray dried methanol: water (1:1) extracts of *O. stamineus* consists of different contents of total proteins, total polysaccharides and total glycosaponins. Freeze dried extract is a better choice because it is rich in proteins and glycosaponins with strong angiogenic activity while spray dried extract does not exhibit antiangiogenic activity.

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