

Standardization of *Orthosiphon stamineus* Raw Material and Extracts for Anti-Uterine Fibroid

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ABSTRACT

This study aim to evaluate gravimetric parameters and physicochemical of raw materials of *Orthosiphon stamineus* in order to ascertain the quality of plant materials and achieved standardized material. Anti-uterine fibroid potential was investigated by cytotoxicity test on uterine leiomyosarcoma cell SK-UT-1. Gravimetric analysis showed that all parameters adhered to the standard according to Malaysian Herbal Monograph. Primary and secondary metabolites screening revealed that aqueous extract have highest total glycosaponins, total phenolics and total phospholipids with the amounts of 27.38%, 18.75% and 3.17%, respectively. Meanwhile, 50% ethanolic extract have highest total proteins, total polysaccharides and total flavonoid with amounts of 9.2%, 19.79%, 8.13%, respectively. Evaluation of cytotoxic potency showed that aqueous extract was more cytotoxic compared to 50% ethanolic extract with the IC₅₀ 18µg/mL and 34µg/mL, respectively. Mode of cell death analysis by AO/PI double staining substantiated apoptosis induction by water extract. The results of this study can serve as reference data for standardization of *O. stamineus* extracts for development as anti-uterine fibroid.

Keywords: *Orthosiphon stamineus*, SK-UT-1, Anti-uterine fibroid.

INTRODUCTION

Uterine fibroid is a comrade of cancer which composed of disordered smooth muscle cells buried in abundant quantities of extracellular matrix¹. In the age of 50s, more than 20% of women will develop fibroids. Modern treatment involving drug therapies and surgeries normally hampered with several side effects including nausea, vomiting and fever². *Orthosiphon stamineus* or Cat's Whiskers or "misai kucing" is distributed in Southeast Asia such as Malaysia, Indonesia, Thailand, Vietnam, and Myanmar. This plant has been traditionally used for the treatment of renal stones, arteriosclerosis, nephritis, diabetes and rheumatism because of hypersensitive, anti-inflammatory and diuretic properties³. Various bio-active compounds were identified and isolated from this plant which contributed to these medicinal properties including orthosiphonol, terpenoids, inositol, myo-inositol, carotenoids and saponins⁴. The recent surge of interest in *Orthosiphon stamineus* has led to the isolation of more than 50 components with different biological activities⁵. Current study investigates the potential of *O. stamineus* as anti-uterine fibrosis. Evaluation of standardization parameters were performed. Anti-uterine fibroid potential was evaluated using uterine leiomyomas (SK-UT-1) cell lines.

MATERIALS AND METHODS

Preparation of plant materials

Raw material of *Orthosiphon stamineus* was purchased from HERBAGUS Sdn. Bhd. A voucher specimen (no.

11009) was deposited at the herbarium of School of Biological Sciences, Universiti Sains Malaysia. Two hundred gram of the dried and ground leaves of *O. stamineus* were extracted with 50% ethanolic water (OSEW) and 100% water (OSW).

Gravimetric analysis of *Orthosiphon stamineus* leaves

Physicochemical tests of total foreign matter, total ash content, total insoluble ash, moisture content, water and ethanol extractive value (hot and cold) were determine according to protocols described in Malaysian Herbal Monograph (2009)⁶.

Primary and secondary metabolites screening of *O. stamineus* extracts

Total protein

Total protein analysis was performed based on the method described by Lowry *et al.* (1951)⁷. The reagents used in the analysis were freshly prepared; reagent A consists of 2% (w/v) sodium carbonate (Sigma- Aldrich, USA) added to 0.1 N sodium hydroxide (Sigma- Aldrich, USA) and reagent B was 0.5% (w/v) copper sulphate (Sigma- Aldrich, USA) in 1.0% potassium sodium tartrate (Sigma- Aldrich, USA). Bovine serum albumin (Sigma- Aldrich, USA) was prepared at 1 mg/mL as standard. The absorbance was measured at 600 nm. Total protein were calculated based serum albumin (n=3).

Total polysaccharides

Total polysaccharides analysis was performed using phenol-sulphuric method as described by (Bhatti *et al.*, 2013)⁸. Ten mg of extract were dissolved in 100 mL of

Table 1: Gravimetric analysis of *O. stamineus* raw materials.

Sample	Amount
Foreign matter (%)	Not more than 2%
Total ash (%)	7.43 ± 0.15
Acid insoluble ash (%)	1.25 ± 0.04
Water soluble ash (%)	5.33 ± 0.25
Loss on drying (%)	5.63 ± 0.40
Water extractive value (cold method)	20.5 ± 0.97
Water extractive value (hot method)	24.76 ± 1.70
Ethanol extractive value (cold method)	6.41 ± 0.67

Table 2: Analysis of primary and secondary metabolites of OSW and OSEW extracts.

Test (% w/w)	OSW	OSEW
Total proteins	9.2 ± 1.11	7.94 ± 1.70
Total polysaccharides	19.79 ± 0.34	6.16 ± 0.07
Total glycosaponins	21.43 ± 0.58	27.38 ± 3.70
Total flavonoids	8.13 ± 0.41	3.36 ± 0.44
Total phenolics	14.06 ± 2.86	18.75 ± 1.49
Total phospholipids	0.83 ± 0.06	3.17 ± 0.56

distilled water. From this, 1 mL of the solution was taken into a test tube, then 1 mL of 50% phenol reagent (Sigma-Aldrich, USA) and 5 mL of concentrated H₂SO₄ (Merck, USA) were added and mixed. The mixture was incubated for 10 min at room temperature. The absorbance was measured at 488 nm against blank having all the reagents except the sample, and glucose (Sigma-Aldrich, USA) as the standards in the range of 10 – 100 µL. The calibration curve was constructed and the total polysaccharides were calculated by taking mean of 3 replicates and total polysaccharides were expressed as average percentage ± SD.

Total glycosaponins

Glycosaponins were determined using gravimetric method. One gram of extract was mixed with 50 mL of methanol (Merck, USA) in 250 mL round bottom flask and refluxed for 30 min and filtered. After three cycles of reflux, the filtrate was concentrated using rotary evaporator to approximately 10 mL. Subsequently, 50 mL acetone (Merck, USA) was added drop wise to the extract and the glycosaponins were precipitated at the bottom of weighed Falcon tube. The tube was centrifuged at 2700 rpm for 10 min; precipitate was collected and dried in oven at 100°C until constant weight. Total glycosaponins was calculated as percentage of precipitated weight over sample weight.

Total flavonoids

Total flavonoids determination was conducted using aluminium chloride method as described by Kale et al. (2010)⁹. Five mg of *O. stamineus* extracts were dissolved in 1 mL of methanol. 500 µL of extracts were pipetted into tubes and mixed with 1.5 mL methanol. Next, 100 µL of

10% AlCl₃ and 100 µL of 1 M potassium acetate were added and mixed with the extracts. The samples were incubated at room temperature for 30 minutes. The absorbance was measured at 415 nm against blank that consisted of all reagents and samples except AlCl₃. Total flavonoids were calculated based quercetin (Sigma-Aldrich, USA) standard curve and expressed as average percentage ± SD

Total phenolics

Five mg/mL of extract was prepared in methanol and 100 µL of Folin-Ciocalteu phenol reagent. The samples were incubated for 5 min in dark condition and 200 µL of 20% sodium carbonate was added. The mixtures were further incubated for 60 min at 30°C in dark condition and absorbance was measured at 750 nm. Total phenolics were calculated based gallic acid (Sigma-Aldrich, USA) standard curve and expressed as average percentage ± SD.

Total phospholipids

Total phospholipids were determined according to method proposed by Stewart, 1980¹⁰. Twenty seven grams of ferric chloride (Sigma-Aldrich, USA) and 30 g of ammonium thiocyanate (Sigma-Aldrich, USA) were dissolved in 1 L of distilled water as the test reagent. Approximately 2 mg of extracts were weighed and dissolved in 2 mL chloroform. One mL of the thiocyanate reagent was added, vortex for 1 min and centrifuged at 2700 rpm for 5 min. The absorbance of lower layer was measured at 488 nm. Total phospholipids were calculated by taking means of 3 replicates and total phospholipids were expressed as average percentage ± SD.

In vitro cytotoxicity assay against anti-uterine fibroids cell line SK-UT-1

Cell lines

Uterine leiomyoma's cell lines SK-UT-1 and normal fibroblast, NIH/3T3 were purchased from the American Type Culture Collection (ATCC). The cells were maintained in RPMI-1640 supplemented with 10% of fetal bovine serum with 1% penicillin/streptomycin at 37°C in 5% CO₂. Growing media was changed every other day.

Cell viability assay

Cell viability was assessed by MTT assays using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide. Briefly, SK-UT-1 and NIH/3T3 cells were seeded in a 96-well plate (1x10⁵ cells / well). After twenty-four hours of incubation, the cells were treated with *O. stamineus* extract at various concentrations (µg/mL). The cells were incubated for seventy-two (72) hours and then 20 µL MTT reagent was added and incubated for an additional four (4) hours at 37°C. Etoposide and epigallocatechin gallate (EGCG) have been used as the positive control.

Acridine Orange and Propidium Iodide staining (AO/PI)

The cells were treated with water extract at IC₅₀ concentration and incubated for 24h and 48h. After each time point, the cells were harvested, stained with 10µL of acridine orange/ propidium iodide and observed under fluorescence microscope.

RESULTS AND DISCUSSION

Gravimetric analysis of *O. stamineus* leaves

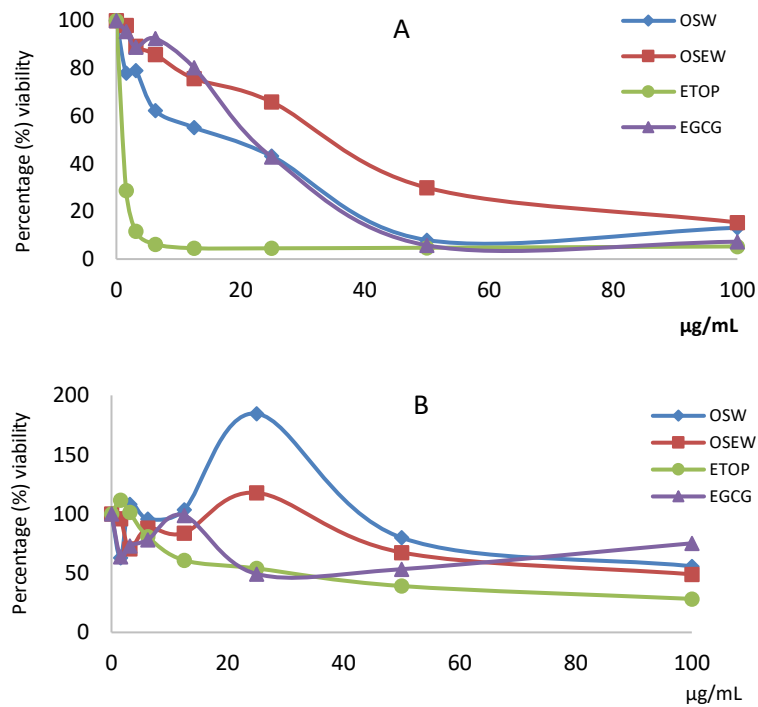


Figure 1: Cytotoxicity of *O. stamineus* extracts evaluated using MTT assay against (A) SK-UT-1 cells (B) NIH/3T3 cells for 72H incubation.

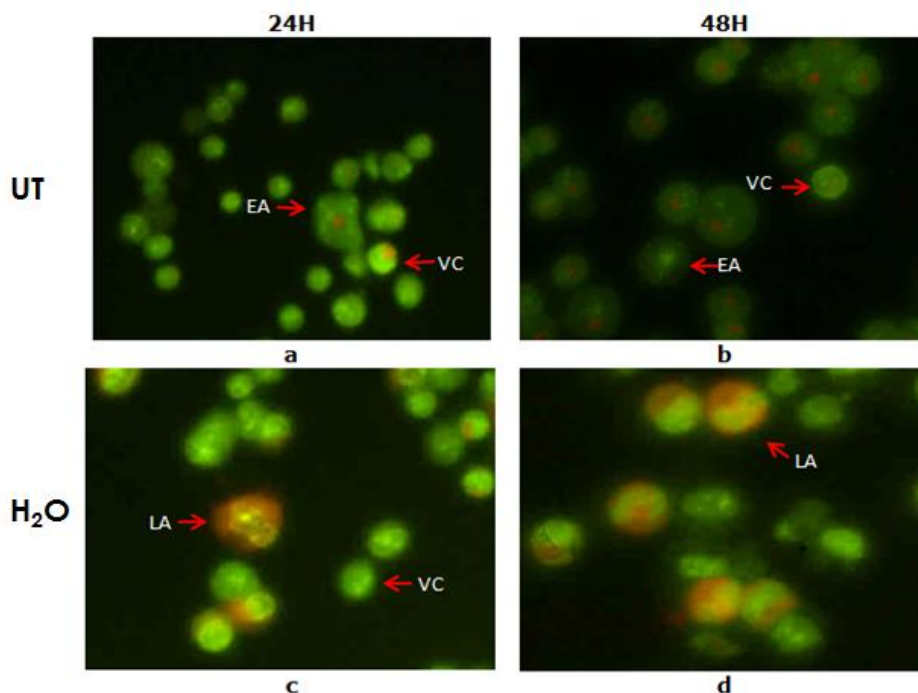


Figure 2: Morphological changes of SK-UT-1 cells treated with OS water at IC_{50} ($\mu\text{g/mL}$) for 24H and 48H after staining with acridine orange (AO) and propidium iodide (PI). (VC) viable cells, (EA) Early apoptosis, (LA) Late apoptosis and (NC) Necrosis.

In order to use *O. stamineus* powders and extracts for clinical application, standardization of physical and chemical content is important to ensure the quality and safety of the materials used. Table 1, shows gravimetric analysis where the quality of raw material met the standard of Malaysian Herbal Monograph (2009)⁶. According to the

Malaysian Herbal Monograph, the moisture content and total ash content must not exceed 9% and 10%, respectively. Then, acid and water insoluble ash must not exceed 8% for each. Hot extractive value for aqueous extract, cold extractive value of aqueous extract and 50%

ethanolic extract must not exceed 10%, 20% and 6%, respectively.

Metabolites screening of *O. stamineus* extracts

Metabolites content is crucial for the quality standard for plant material. Results showed that water extract contain more total protein, polysaccharides and flavonoids. Meanwhile, total glycosaponins, phenolic and phospholipids were occurring more in ethanol-water extract. Phytochemical screening is helpful to know the chemical constituents present in the herbal materials¹¹.

In vitro anti-uterine fibroids

The extracts or compounds were considered to be cytotoxic by American National Cancer Institute when IC_{50} is less than 30 μ g/ml¹². The result of cytotoxic effects against uterine leiomyosarcoma (SK-UT-1) cells demonstrated by etoposide shown to be very cytotoxic with IC_{50} of 1 μ g/mL, whereas OSW and EGCG to be less cytotoxic with IC_{50} of 18 μ g/mL and 22 μ g/mL, respectively. OSEW however showed to be non-toxic towards SK-UT-1 cells with 34 μ g/mL (Fig. 1A). We later tested against normal fibroblast cells (NIH/3T3) and the result showed that OSW, OSEW and EGCG have no cytotoxic effect against tested cells with IC_{50} more than 100 μ g/mL (Figure 1B). Etoposide on the other hand possess strong cytotoxic effect toward NIH/3T3 with IC_{50} of 8 μ g/mL. Assessment on the cell death revealed the present of apoptotic bodies (Figure 2) in all treatments in time dependent manner. Results of the cytotoxicity against SK-UT-1 shows that this plant is worth to be further investigated as anti-uterine fibroid.

CONCLUSION

The water extract of *Orthosiphon stamineus* show promising cytotoxicity effect against uterine fibroid cells. Physicochemical, phytochemical and biological data presented here would be an important data for standardization of *O. stamineus* toward development as anti-uterine fibroid botanical drug.

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