

Evaluation of antiangiogenic and antioxidant properties of *Parkia speciosa* Hassk extracts

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Abstract: *Parkia speciosa* Hassk is a traditional medicinal plant with strong antioxidant and hypoglycemic properties. This study aims to investigate the total phenolic content, antioxidant, cytotoxic and antiangiogenic effect of eight extracts from *P. speciosa* empty pods. The extracts were found to contain high levels of total phenols and demonstrated strong antioxidant effect in DPPH scavenging test. In rat aortic rings, *P. speciosa* extracts significantly inhibited the microvessel outgrowth from aortic tissue explants by more than 50%. The antiangiogenic activity was further confirmed by tube formation on matrigel matrix involving human endothelial cells. Cytotoxic effect was evaluated by XTT test on endothelial cells as a model of angiogenesis versus a panel of human cancer and normal cell lines. Basically the extracts did not show acute cytotoxicity. Morphology examination of endothelial cells indicated induction of autophagy characterized by formation of plenty of cytoplasmic vacuoles. The extracts were found to work by decreasing expression of vascular endothelial growth factor in endothelial cells.

Keywords: *Parkia speciosa*, angiogenesis, antioxidants, total phenols.

INTRODUCTION

Parkia speciosa Hassk is known as stink beans or petai (in Malay language). It is a tropical leguminous tree in the family of Leguminosae. It bears long, flat edible bean pods with bright green seeds the size and shape of almonds. *P. speciosa* is an edible legume believed by the locals to have medicinal properties including anti-diabetes, anti-hypertension and anti-bacterial primarily for treating urinary tract infections (Jamaluddin and Mohamed, 1993). Previous research on *P. speciosa* indicated hypoglycemic effect of chloroform extract from empty pods and of a pure compound called stigmast-4-en-one (Jamaluddin *et al.*, 1994a, 1994b, 1995). Other research carried on *P. speciosa* seeds reported the presence of antibacterial cyclic polysulfides (Susilo and Gmelin, 1982; Pandeya, 1972). Gan *et al.*, (2009) studied the physicochemical properties of pectin-like polysaccharides and reported antioxidative effect of *P. speciosa*. Recently, hemagglutinating activity of proteins from *P. speciosa* seeds was reported (Chankhamjon *et al.*, 2010).

Angiogenesis is a critical process in various physiological and pathological conditions including wound healing, growth and metastasis of solid tumors and chronic inflammatory diseases such as rheumatoid arthritis, proliferative diabetic retinopathy and psoriasis (Folkman, 1995; Pepper, 1997). Antioxidants have been reported to have potent antiangiogenic properties, these compounds

work by regulating angiogenesis modulators such as vascular endothelial growth factor (VEGF) (Lee *et al.*, 2006) or by changing the redox microenvironment of tumor vasculature (Mu *et al.*, 2007; Lee *et al.*, 2006).

A screening study carried out by our research group reported antiangiogenic effect of extracts from *P. speciosa* in the rat aortic rings model (Aisha *et al.*, 2009). The present study was undertaken to determine the total phenolic content, to study the free radical scavenging effect and to investigate the antiangiogenic properties of eight extracts from *P. speciosa*. Specifically, we focused on the effect on rat aortic vascular growth, tube formation involving endothelial cells on matrigel matrix, proliferation of endothelial cells, morphology changes of endothelial cells and to explore the mechanism of action by targeting expression of VEGF.

MATERIALS AND METHODS

Chemicals

Endothelial cell medium (ECM) supplied with endothelial cell growth supplements (ECGS) was obtained from ScienCell, USA. RPMI 1640, Dulbecco's Modified Eagle Medium (DMEM) and Minimum Essential Medium (MEM) and fetal bovine serum (FBS) were obtained from GIBCO, USA. Human VEGF assay kit was obtained from IBL, Japan. Folin-Ciocalteu reagent, gallic acid, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), sodium bicarbonate, penicillin/streptomycin (PS) solution, 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT), phenazine methosulfate (PMS),

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suramin, amphotericin B, aprotinin, 6-aminocaproic acid, L-glutamine, thrombin, and gentamicin were obtained from Sigma-Aldrich, USA. Fibrinogen was obtained from Calbiochem, USA. Matrigel matrix (10 mg/ml) was obtained from SABiosciences, USA. All solvents were of analytical grade and were obtained from Merck.

Plant material and extraction

Commercially available fresh pods of *P. speciosa* were obtained from the local market in Balik Pulau, Pinang, Malaysia. The seeds were separated from the pods and the later were chopped and dried in oven at 45°C for 24 h.

Water extraction

250 ml distilled water was added to 50 g of pulverized material, extraction was performed at 50 °C in oven for 48 h. The extract was filtered and freeze dried at -50°C under reduced pressure.

N-hexane: methanol extraction

200 g pulverized pods were sequentially extracted with n-hexane and methanol. N-hexane extract was prepared by cold extraction method at room temperature for 24 h in 1 L n-hexane. The extract was filtered and the plant residue was dried and re-extracted in 1 L methanol at room temperature for further 24 h. The solvents were evaporated by rotavapor at 45°C.

Successive extraction of methanolic extract

8.275 g of methanolic extract was suspended in 200 ml distilled water with vigorous mixing on magnetic stirrer for 10 min. The suspension was partitioned sequentially (3 times × 100 ml) with n-hexane, chloroform and ethyl acetate using a separatory funnel. The organic layer was collected each time, and the pooled organic phases were dried by rotavapor at 45°C. After partition with ethyl acetate, precipitate was formed in the aqueous phase. The precipitate was collected by filtration, dissolved in methanol then dried at 45°C. The aqueous phase was dried at -50°C under vacuum. The extracts were saved at -20°C until used.

Thin layer chromatography

Thin layer chromatography (TLC) was performed using pre-coated plates with 0.25 mm layer of silica gel F60. The TLC chromatograms were developed using n-butanol: ethanol at 9:1 (v/v) as a mobile phase. The plates were dried and illuminated at 254 and 366 nm.

Determination of total phenols

Total phenols in *P. speciosa* extracts were determined by a colorimetric method as described by Lizcano *et al.*, (2010). Briefly, 100 µl of extracts (1 mg/ml in methanol) was added to 750 µl 1:10 diluted (in dd.H₂O) Folin-Ciocalteu phenol reagent. After 5 min incubation in the dark at room temperature, 750 µl sodium bicarbonate solution (60 g/l) was added and incubated at 30°C in the

dark for 90 min. Afterwards absorbance was measured at 725 nm. Gallic acid was used (5-80 µg) to construct the standard calibration curve. The results were expressed as gallic acid equivalents per 100 mg of extract (mg GAE/100mg).

DPPH scavenging effect

DPPH scavenging effect of extracts was investigated as described by Sharma and Bhat (2009). Briefly, stock solution of DPPH was prepared in methanol at 200 µM, and serial dilution of extracts was prepared in methanol to obtain (200, 100, 50, 25 and 12.5 µg/ml). DPPH reagent was added at final concentration of 50 µM and incubated at 30°C in the dark for 30 min. Consequently, absorbance was measured at 517 nm and DPPH scavenging activity was calculated using the formula $(1 - (\text{absorbance of samples} - \text{blank}) / (\text{absorbance of negative control} - \text{blank})) \times 100\%$. Median inhibitory concentration (IC₅₀s) were calculated from the dose response curves (n = 3). Gallic acid was used as a positive control and the solvent (methanol) was used as a negative control.

Cell culture

Human umbilical vein endothelial cells (HUVECs) (ScienCell, USA) were cultured in ECM containing 5% FBS, 1% PS and 1% ECGS. HCT 116 human colorectal carcinoma cell line (ATCC) was cultured in RPMI 1640 containing 10% FBS and 1% PS. MCF-7 human breast cancer cell line (ATCC) was cultured in DMEM containing 10% FBS and 1% PS. CCD-18CO human normal fibroblast cell line (ATCC) was cultured in DMEM containing 10% FBS and 1% PS. MCF-10A human normal epithelial cell line (ATCC) was cultured in MEM containing 10% FBS, 1% PS, 1% sodium pyruvate, 1% nonessential amino acids, 1% L-glutamine, 1% MEM vitamins, human insulin at 5 µg/ml and epidermal growth factor (EGF) at 100 ng/ml. All cells were propagated as monolayer at 37°C in an atmosphere of 5% CO₂.

Experimental animals

Ten (12-14 weeks old) Sprague Dawley male rats were obtained from animal unit facility of University Science Malaysia. The animals were kept for one week in transit animal unit (School of Pharmaceutical Sciences) to acclimatize with the new environment. The rats were kept in ventilated cages at 12 h light cycle with continuous supply of food and water. The animals were humanely euthanized by CO₂ and a midline incision was made into abdominal and thoracic cavities including splitting of the sternum, and thoracic aortas were collected. The experiments were performed according to guidelines of USM Animal Ethics Committee and had their approval, reference number USM/PPSF/50 (084) Jld.2.

Evaluation of antiangiogenic effect on rat aortic rings

Antiangiogenic properties of *P. speciosa* extracts were first investigated *in vitro* on rat aortic rings as described

by Brown *et al.*, (1996). Briefly, thoracic aortas were cleansed of adipose tissue and cross sectioned into thin rings of one millimeter thickness. One ring was placed in the center of each well of 48-well plate containing 500 µl M199 basal medium supplied with fibrinogen, aprotinin and L-glutamine at 3 mg/ml, 5 µg/ml, and 1 % wt/v respectively. Then 10 µl thrombin (50 NIH U/l) was added to each well. After 90 min incubation at 37°C, another 500 µl M199 medium containing FBS at 20% v/v, L-glutamine at 2 mM, aminocaproic acid at 1 mg/ml, amphotericin B at 2.5 µg/ml and gentamicin at 60 µg/ml, was added on top of the solidified bottom layer. *P. speciosa* extracts were included in the top layer medium at 100 µg/ml. After 4 days incubation at 37°C in 5% CO₂, the top layer medium was changed with fresh one containing the extracts. On day five the growth of sprouting blood vessels was quantified as previously reported (Nicosia *et al.*, 1997). In brief, the distance of growth of at least twenty growth points was measured in each ring. The results were presented as a mean percent inhibition of three independent experiments ± SD (n = 18). Dimethylsulfoxide (DMSO) was used as a negative control and suramin at 100 µg/ml was used as a positive control.

Evaluation of cytotoxic effect

Cytotoxic effect of *P. speciosa* extracts was evaluated using XTT assay as previously described (Jost *et al.*, 1992). HUVEC was used as a model cell line of angiogenesis versus HCT 116, MCF-7, CCD-18CO and MCF-10A cell lines. Cells were harvested by trypsinization and resuspended in 5 ml fresh medium. Then cell count was adjusted to 1.5×10^5 /ml and consequently 100 µl was seeded in each well and the cells were allowed to attach for overnight. Afterwards an additional 100 µl fresh medium containing extracts at 100 µg/ml was added and further incubated for 48 h. Viability of cells was then assessed by XTT assay as described by Aisha *et al.* (2011). Percent of inhibition was calculated using the formula $(1 - (\text{absorbance of samples} - \text{blank}) / (\text{absorbance of negative control} - \text{blank})) \times 100\%$ (n = 3).

Evaluation of antiangiogenic effect on matrigel matrix involving HUVECs

The ability of HUVECs to form capillary-like structures was investigated *in vitro* on matrigel matrix as indicated by (Arnaoutova and Kleinman, 2010). Briefly, cells were treated for overnight with methanol extract and water sub-extract at 100 µg/ml. On the day of experiment matrigel stock solution (10 mg/ml) was diluted at 1:2 v/v in ice cold serum-free ECM. Then 150 µl was added to each well of 48-well plate and allowed to solidify for 45 min at 37°C in 5% CO₂. Afterwards 0.5 ml ECM containing 2×10^5 pre-treated HUVECs were added to each well and incubated at 37°C in 5% CO₂. After 6 h the tube like structures was visualized by light microscopy at 4x magnification. The area occupied by the capillary like

structures was determined for each treatment by ScnImage software package (available online for free) and the results are presented as percent inhibition to untreated cells.

Determination of VEGF levels in HUVECs

Concentration of human VEGF 165 in HUVEC cell lysates was determined by human VEGF ELISA kit (IBL, Japan) according to manufacturer's instructions. The kit consists of anti-human VEGF-1 mouse IgG monoclonal antibody and a horseradish peroxidase conjugated secondary antibody and recombinant human VEGF 165 as a standard. HUVECs were seeded in 6-well plates at 1×10^6 in 3 ml of ECM. After overnight attachment, the cells were treated for 6 h with either methanol extract or water sub-extract at 100 µg/ml. Calibration curve of VEGF standard was prepared simultaneously with the samples, and concentration of VEGF-1 in cell lysates was determined by applying the linear regression equation, $y = 0.0021x + 0.0585$, $R^2 = 0.999$ (n = 3).

Morphology studies of HUVECs

HUVECs were cultured on polylysine-coated glass cover slips and one cover slip was placed in each well of 12 well plates. 5×10^4 cells in 1 ml medium were added and allowed to attach for overnight. Then the medium was replaced with fresh one containing water sub-extract at 100 µg/ml. At specified intervals, the cells were visualized and photographed by light microscope supplemented with digital camera.

STATISTICAL ANALYSIS

The results are presented as mean ± SD. Differences between groups were analyzed either by student t-test or One way ANOVA and were considered significant at $P < 0.05$.

RESULTS

TLC analysis

The extracts were analyzed qualitatively on normal TLC and the results are displayed in fig. 1. The TLC plate shows different chromatograms of the various extracts of *P. speciosa*. When the plate was illuminated at 254 nm, only the front band and the material at the application points could be detected. On the contrary, illumination at 366 nm resulted in >10 bands such as in the ethyl acetate sub-extract.

Total phenol contents of *P. speciosa* extracts

Total phenol content of *P. speciosa* extracts is presented in table 1. The results are expressed as mg GAE/100mg of extracts. Total phenols content was in the range of 0.72 - 25.55 GAE. Amongst the extracts, water and n-hexane extracts showed the lowest levels, 0.72 ± 0.32 and $1.62 \pm$

0.04 GAE, respectively. Whilst water sub-extract showed the highest content, 25.55 ± 1.57 GAE.

DPPH scavenging effect

Antioxidant effect of *P. speciosa* extracts was determined by DPPH scavenging assay. Amongst the extracts, n-hexane extract showed the lowest free radical scavenging effect and methanol sub-extract exhibited the highest effect (table 1). It can be noticed from the results presented in table 1 that extracts with high total phenols content demonstrated high antioxidant capacity, and vice versa.

Antiangiogenic effect on rat aortic rings

Antiangiogenic efficacy of extracts was first investigated on rat aortic rings (fig. 2). Water and n-hexane extracts did not show significant inhibition of rat aortic microvessel outgrowth. On the other hand, methanolic extract and all its sub-extracts caused significant inhibition of aortic microvessel outgrowth by >50% (table 1). Analysis by One way ANOVA indicates no significant difference of antiangiogenic effect between methanolic extract and its sub-extracts ($P = 0.083$).

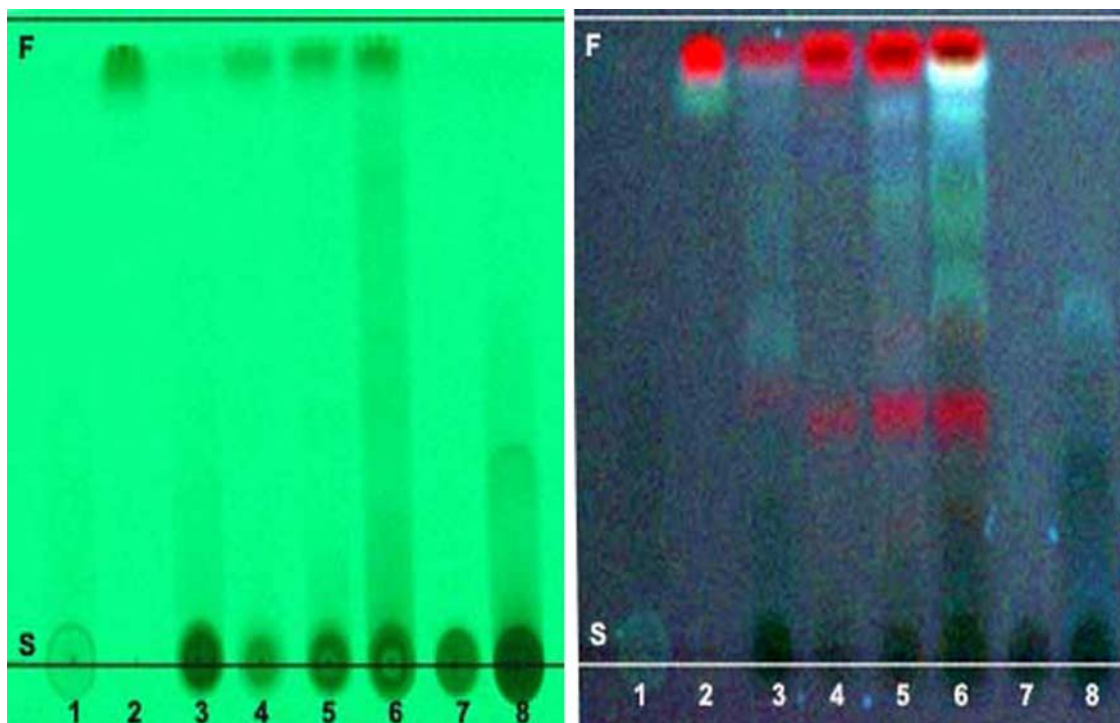


Fig. 1: TLC analysis of the extracts. (1) Aqueous extract, (2) n-hexane, (3) methanolic, (4) n-hexane sub-extract, (5) chloroform sub-extracts; (6) ethyl acetate sub-extract, (7) methanol sub-extract and (8) water sub-extract. Left at was illuminated at 254 nm and the right at 366 nm.

Table 1: Extraction, total phenolic content, antioxidant activity, and inhibition of rat aortic vascular outgrowth induced by treatment with *P. speciosa* extracts. Results are presented as average $\pm$ SD.

Extract	Yield (wt/wt %)	Total phenol (GAE/100 mg)	DPPH IC ₅₀ (μ g/ml)	Rat aortic rings (% Inhibition)
Water	5.0	1.6 ± 0.04	357 ± 27	24 ± 13
N-hexane	0.2	0.7 ± 0.3	1181 ± 99	5 ± 13
Methanol	7.0	16.5 ± 0.3	53 ± 1.0	65 ± 8.0
N-hexane ^a	0.6	9.0 ± 0.3	72 ± 0.1	58 ± 8.0
Chloroform ^b	8.0	14.6 ± 0.4	44 ± 2.0	58 ± 11
Ethyl acetate ^c	5.0	14.8 ± 0.7	41 ± 2.0	64 ± 13
Methanol ^d	5.0	25.5 ± 1.6	26 ± 3.0	72 ± 8.0
Water ^e	54	13.4 ± 0.2	105 ± 8.0	60 ± 11

^{a-e}; refers to sub-extracts of methanolic extract.

Cytotoxic effect

Cytotoxicity of *P. speciosa* extracts was evaluated on HUVECs versus a panel of normal and cancer cell lines. The study was performed at the same concentration used in rat aortic rings i.e. 100 µg/ml. Methanolic extract did not show significant cytotoxic effect on all tested cell lines. Amongst methanol sub-extracts, those of n-hexane and water were not cytotoxic on all tested cell lines. The chloroform sub-extract exhibited selective cytotoxicity on HUVECs with 50% inhibition, and ethyl acetate sub-extract demonstrated selective cytotoxicity on the hormone sensitive breast cancer cells, MCF-7. Methanolic sub-extract was the most cytotoxic but selective on HUVECs however with some cytotoxic effects (<50% inhibition) on other cell lines (table 2).

P. speciosa extracts inhibit tube formation on matrigel matrix

Antiangiogenic effect of methanolic extract and the water sub-extract was studied in tube formation on matrigel

matrix involving HUVECs. Methanol extract and water sub-extract were selected as sample extracts of *P. speciosa* because they exhibited >50% inhibition in rat aortic rings and were not cytotoxic on all tested cell lines. The occupied area by the capillary like structures formed by endothelial cells was measured as mentioned previously. Then the percent of inhibition was calculated for each treatment. Methanolic extract and the water sub-extract showed significant but equivalent results with $58 \pm 7\%$ and $58 \pm 6\%$ inhibition, respectively. Suramin at 100 µg/ml showed >80% inhibition (fig. 3).

Light microscopy study of HUVECs

P. speciosa extracts caused characteristic morphology changes in HUVECs. Treatment of HUVECs with *P. speciosa* extracts resulted in the formation of cytoplasmic vacuoles (fig. 4). The vacuoles were more abundant upon treatment with water sub-extract. The number and the size of vacuoles increased more and more with treatment time without affecting viability of cells as indicated by cell

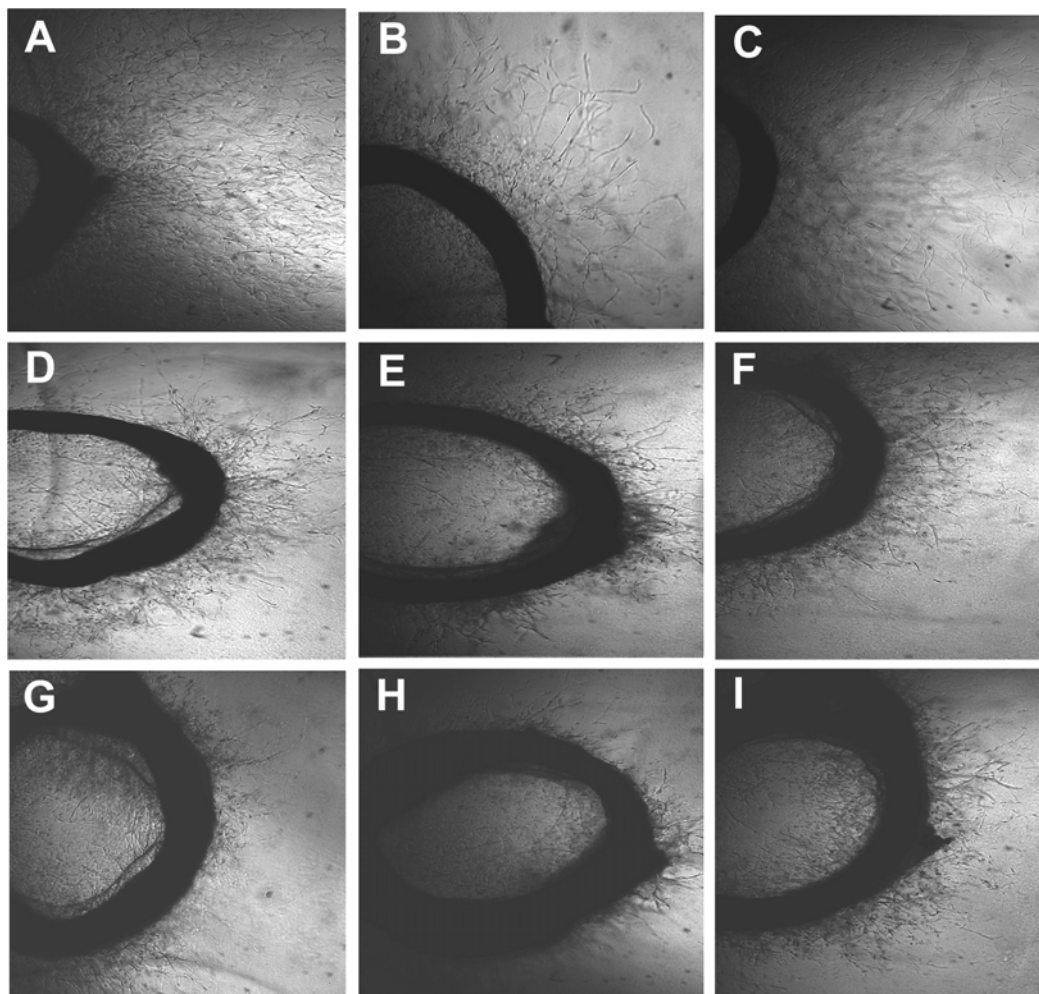


Fig. 2: *In vitro* antiangiogenic effects of *P. speciosa* extracts on rat aortic rings. (A) Untreated, (B) water extract, (C) n-hexane extract, (D) methanolic extract, and the methanolic sub-extracts; (E) n-hexane, (F) chloroform, (G) ethyl acetate, (H) methanol, (I) water.

viability testing after 48 h treatment. Previous reports said that the presence of these vacuoles is a marker of autophagy which indicates starvation of endothelial cells as a result of nutritional deprivation which is essential to maintain viability of cells (Nakatogawa and Ohsumi, 2008).

Effect on VEGF expression

In order to explore the mechanism of action of *P. speciosa* extracts, we studied the effect of methanolic extract and the water sub-extract on expression of VEGF 165 in HUVECs. Treatment of HUVECs with *P. extracts* caused significant reduction in VEGF levels compare to untreated cells. VEGF concentration in HUVECs treated with methanolic extract and the water sub-extract were 36 ± 2.2 and 45 ± 1.2 pg/ml, respectively. The concentration of VEGF in untreated cells was 51 ± 1.6 pg/ml. Student t-

test analysis showed significant difference between both methanol extract and water sub-extract when compared to untreated cells ($P = 0.001$ and 0.013 , respectively).

DISCUSSION

In the present study, we investigated the total phenolic content, antioxidant and the antiangiogenic properties of three extracts and five sub-extracts from *P. speciosa* empty pods. Methanolic extract and its sub-extracts showed high levels of total phenols with strong antioxidant activity and significant inhibition of vascular outgrowth from rat aortic tissue explants. The antiangiogenic effect was further confirmed on matrigel tube formation involving HUVECs. In order to make difference between results due to nonselective cytotoxicity from true antiangiogenic effects, we studied

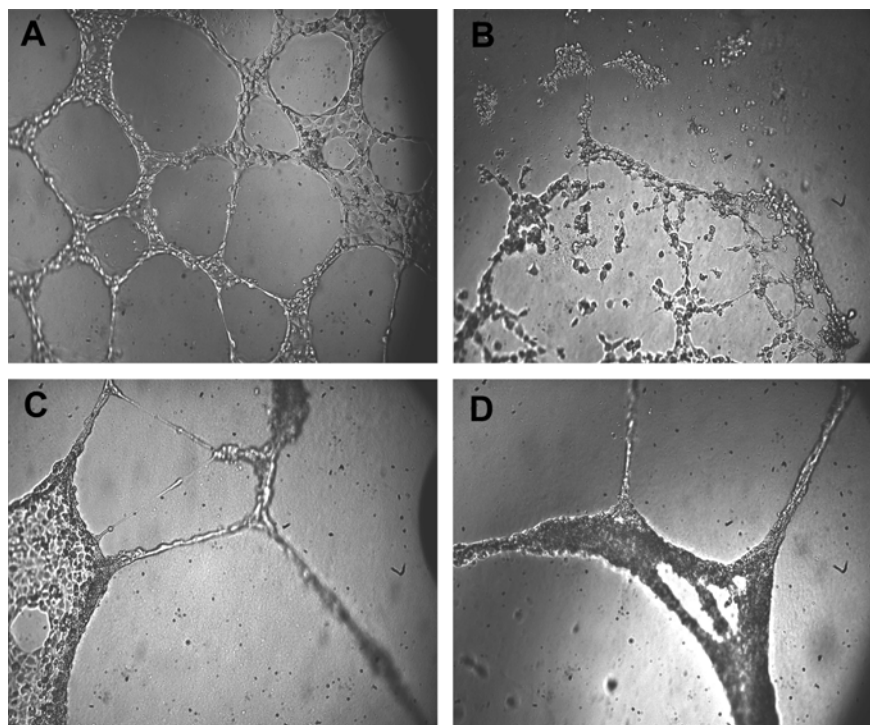


Fig. 3: Effect of the methanolic extract, and water sub-extract of *P. speciosa* on HUVECs ability to form capillary-like structures on matrigel matrix. (A) Untreated cells, (B) Suramin at 100 µg/ml, (C) Methanolic extract at 100 µg/ml, and (D) water sub-extract at 100 µg/ml.

Table 2: Cytotoxic effect of *P. speciosa* extracts. Results are presented as mean percentage inhibition $\pm$ SD.

Extract	HUVEC	MCF7	HCT116	CCD-18CO	MCF-10A
Methanol	0.0 ± 4.0	13 ± 7.0	21 ± 8.0	5.0 ± 4.0	8.0 ± 2.0
N-hexane ^a	0.0 ± 3.0	0.0 ± 1.0	16 ± 5.0	3.0 ± 3.0	7.0 ± 4.0
Chloroform ^b	50 ± 5.0	2.0 ± 2.0	13 ± 5.0	2.0 ± 2.0	12 ± 3.0
Ethyl acetate ^c	2.0 ± 4.0	56 ± 2.0	11 ± 1.0	5.0 ± 4.0	14 ± 2.0
Methanol ^d	86 ± 1.0	9.0 ± 1.0	41 ± 5.0	45 ± 5.0	41 ± 1.0
Water ^e	1.0 ± 3.0	3.0 ± 0.4	15 ± 3.0	3.0 ± 2.0	13 ± 3.0

^{a-e}; refers to sub-extracts of methanolic extract.

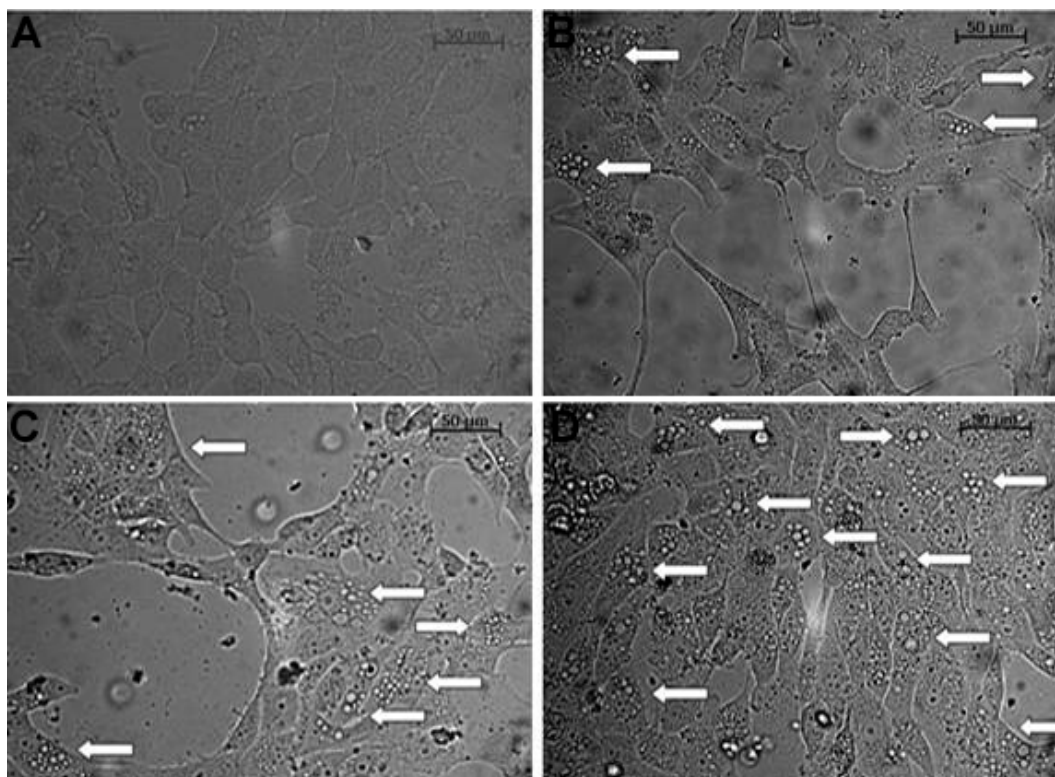


Fig. 4: Morphology observation of HUVECs by light microscopy (100X magnification). (A) Untreated cells, (B) After 4 h treatment, (C) After 10 h treatment, and (D) After 20 h treatment. The figure shows the development of cytoplasmic vacuoles (white arrows) in a time dependent manner.

the cytotoxicity of extracts with >50% inhibition in rat aortic rings on HUVEC versus a panel of human normal and cancer cell lines. The results indicate that the antiangiogenic effect of *P. speciosa* extracts was either due to selective cytotoxicity on HUVECs or due other unknown mechanism. The extracts caused the formation of plenty cytoplasmic vacuoles in HUVECs which might be the cause behind the inhibition of angiogenesis in rat aortic rings and in the matrigel matrix tube formation. Since the most promising antiangiogenic effects were obtained with methanolic extract and water sub-extract, the mechanism of action these two extracts was studied targeting the expression of VEGF in HUVECs. Significant reduction of VEGF expression was achieved with both treatments which suggest the down regulation of VEGF as the mechanism of action *P. speciosa* extracts. Due to unavailability of standard compounds of the plant, the extracts were only analyzed qualitatively on TLC at this stage, and our future plan is the purification of active compounds. By comparing the TLC chromatograms of the active extracts or sub-extracts with those without activity, it can be concluded that the activity is probably due to the unseparated material at the application points (fig. 1, spots 3-8). However, this conclusion is not decisive since we are dealing with crude extracts and the activity might be due to synergistic effects of more than one compound. Nevertheless, the reported TLC method

might provide some hints to be employed in the chromatographic separation of the active ingredients.

Previous research on *P. speciosa* reported the presence of several cyclic polysulfides with antibacterial activity (Gmelin *et al.*, 1981), thiazolidine-4-carboxylic acid with anticancer and hypoglycemic effects (Suvachittanont *et al.*, 1996), β -sitosterol and stigmasterol with hypoglycemic effect (Jamaluddin *et al.*, 1994b). Analysis by GC-MS of supercritical carbon dioxide extracts from *P. speciosa* indicated the presence of 77 compounds including propanoic acid, 3, 3'-thiobis - didodecyl ester, linoleic acid chloride, palmitic acid, linoleic acid, myristic acid, arachidonic acid, undecanoic acid and 2-Hexyl-1-decanol, terpenoids compounds of β -sitosterol and squalene, stigmasterol, lupeol and campesterol (Salman *et al.*, 2006). The antiangiogenic effect of *P. speciosa* extracts is reported for the first time in this article and more detailed research is required to identify and isolate the compounds behind this activity.

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