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Screening of Antiangiogenic Activity of Some Tropical Plants by Rat Aorta Ring Assay

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Abstract: Angiogenesis is essential for the growth and metastasis of most solid malignancies. Accordingly, tumor angiogenesis is an important pharmacological target for cancer prevention and treatment. This study was conducted to study antiangiogenic activity of some tropical plants. Seven plants were extracted successively with n-hexane and methanol to prepare 18 extracts and antiangiogenic properties of the extracts were studied by rat aorta ring assay. Nine extracts showed more than 50% inhibition of the blood vessels outgrowth from the primary tissue explants. The MTT assay was used to study the cytotoxic activities of extracts with more than 50% inhibition. The Human Umbilical Vein Endothelial Cells was used as a test cell line versus two human colon cancer cell lines HCT-116 and HT-29, two human breast cancer cell lines MCF-7 and T47D and one hepatocarcinoma cell line HepG-2. The selectivity index on Human Umbilical Vein Endothelial Cells was calculated. The selectivity index results indicated antiangiogenic activities of three plants *Parkia speciosa*, *Syzygium campanulatum* and *Sandoricum koetjape*. The results presented in this research article make these plants good candidates for further studies to purify the active compounds and to study *in vivo* antiangiogenic activity.

Key words: Angiogenesis, rat aorta ring assay, tropical plants

INTRODUCTION

Angiogenesis i.e., the sprouting of new blood vessels from pre-existing vessels is an essential feature of tissue remodeling associated with at least wound healing, solid tumor development, proliferative retinopathies, atherosclerosis and rheumatoid arthritis (Folkman, 1995). Tumor angiogenesis is the consequence of an angiogenic imbalance in which proangiogenic factors predominate over antiangiogenic factors (Giordano and Johnson, 2001; Udagawa *et al*., 2002). Furthermore, angiogenesis is essential for growth and metastasis of most solid malignancies and Vascular Endothelial Growth Factor-A (VEGF-A) is believed to be critical for tumor angiogenesis (Folkman, 1990, 1995).

Different *in vivo* and *in vitro* assays have been used so far in order to understand the development of the vascular system and to screen angiogenic activators and inhibitors. Commonly used *in vivo* models of angiogenesis include the chorioallantoic membrane of the chick embryo (CAM) assay, the rabbit cornea, the hamster cheek pouch and the Matrigell implant assay (Passaniti *et al*., 1992; McMahon *et al*., 2001). Despite the relevance of these *in vivo* assays, systemic inflammatory reactions can lead to interferences that hamper their use for the study of angiogenesis regulation. *In vitro* cultures of isolated endothelial cells are also useful to study the formation of micro vessels but the role smooth muscle cells, pericytes and fibroblasts are not taken into account in these models. Nicosia and Ottinetti (1990) demonstrated that rat aorta rings reproducibly generate micro vessel outgrowths in fibrin or collagen gels and provide a sensitive assay for the study of angiogenic agonists and antagonists in a chemically Defined environment.

Anti-angiogenic drugs work by inhibiting the synthesis of new blood vessels supplying tumor cells with the required nutrients and oxygen aiming to delay both primary and metastatic tumor growth while overcoming the inherent cytotoxicities of classical chemotherapies. Previous reports

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of Avastin; a monoclonal anti-body for VEGF; and fluorouracil-based combination therapy showed a significant improvement in survival of patients with metastatic colorectal carcinoma (Hurwitz *et al*., 2004). Consequently, tumor angiogenesis can be considered important pharmacological target for cancer prevention and treatment (Eatock *et al*., 2000; Pfeffer *et al*., 2003; Scappaticci, 2003).

The most deadly aspect of cancer is its ability to spread or metastasize, cancers which have metastasized usually indicate a later stage of the disease and treatment becomes more complicated with poorer outcomes. Metastasis most commonly occurs by ways of the bloodstream or the lymphatic system. Compelling data implicate angiogenesis and tumor-associated neovascularization as a central pathogenic step in the process of tumor growth, invasion and metastasis. Subsequently, it was shown that a significant correlation existed between the degree of tumor angiogenesis (micro vessel density) and survival in patients presenting with lymph node-negative breast carcinoma (Bosari *et al*., 1992).

This study was conducted to analyse the antiangiogenesis properties of 18 extracts prepared from seven plants; *Punica granatum* (Punicaceae), *Parkia speciosa* (Fabaceae), *Syzygium campanulatum* (Myrtaceae), *Delonix regia* (Fabaceae), *Cassia fistula* (Fabaceae), *Sandoricum koetjape* (Meliaceae) and *Garcinia mangostana* (Guttiferae). Two methods were used the rat aorta ring assay and antiproliferative activity on isolated cultures of endothelial cells by MTT assay. Nine extracts showed more than 50% inhibition of the blood vessels outgrowth by rat aorta ring assay. This research article reported antiangiogenesis properties of three extracts prepared from *S. campanulatum*, *P. speciosa* and *S. koetjape*.

MATERIALS AND METHODS

Plant material: Plant material was collected during the period of August-September 2008 except *P. granatum* which was collected during summer 2007 from Nablus city of Palestine. *C. fistula*, *Delonix regia*, *S. koetjape* and *S. campanulatum* were collected from the main campus of University Sains Malaysia (USM), Penang, Malaysia. These plants were previously authenticated and labeled by USM. *G. mangostana* and *P. speciosa* were collected from Balik Pulau, Penang, Malaysia.

Preparation of extracts: Collected fresh plant material was dried in oven at 50°C for 24 h. The plant material was successively extracted with n-hexane and methanol. N-hexane extracts were prepared by adding 250 mL n-hexane to 50 g of the pulverized plant material, extraction was carried out at room temperature for 24 h with intermittent shaking. The extracts were filtered and concentrated at 45°C under vacuum by rotary evaporator (Buchi, USA) and further dried overnight at 45°C. The plant residues were re-extracted with methanol for further 24 h and were processed as mentioned above. Stock solutions of the extracts were prepared at 20 mg mL^{-1} in 100% dimethyl sulfoxide (DMSO). The DMSO stock solutions as well as DMSO were diluted with cell culture medium, so the highest DMSO concentration exposed to the cells was 0.5% v/v.

Experimental animals: Twelve to forteen weeks old Sprague Dawley male rats were obtained from the animal house facility of USM and were kept for one week in transient animal house (School of Pharmaceutical Sciences) before doing the experiment. The animals were kept in well ventilated cages at 12 h light cycle with food and water provided all the time, the bedding was changed every other day. All experimental work was done according to the guidelines of USM ethical committee and had their approval, Reference number USM/PPSF/50 (084) Jld.2.

Cell lines and cell culture: Human Umbilical Vein Endothelial Cell line HUVEC; Catalogue number (8000) was purchased from ScienCell, USA. Human colorectal adenocarcinoma cell line HT-29; Catalogue number (HTB-38), human colorectal carcinoma cell line HCT-116; Catalogue number (CCL-247); human hormone sensitive and invasive breast cancer cell line MCF-7; Catalogue number (HTB-22), human hormone sensitive early stage breast cancer cell line; Catalogue number (HTB-133) and human hepatocarcinoma cell line HePG-2; Catalogue number (HB-8065) were purchased from ATCC, USA.

Cell culture reagents were purchased from Gibco, USA: RPMI 1640 medium; Catalogue number (A10491-01), Dulbecco's Modified Eagle Medium (DMEM); Catalogue number (21063-045), M199 medium; Catalogue number (31100-035), Foetal Bovine Serum (FBS); Catalogue number (10100139), Penicillin/Streptomycin (PS) solution; Catalogue number (15140163). The following reagents were purchased from Sigma, Germany: MTT reagent; Catalogue number (M5655), Human Insulin; Catalogue number (I9278), Suramin; Catalogue number (S2671), Amphotericin B; Catalogue number (A2942), Dimethyl sulfoxide (DMSO); Catalogue number (D4540), Aprotinin; Catalogue number (A1153), 6-Aminocaproic acid; Catalogue number (A7824), L-glutamine; Catalogue number (G3126); Thrombin; Catalogue number (T6634) and Gentamicin; Catalogue number (G1272). Fibrinogen;

Catalogue number (341573) was purchased from Calbiochem, USA. Endothelial Cell Medium (ECM) with Endothelial Cell Growth Supplements (ECGS); Catalogue number (1001) were purchased from ScienCell, USA. Tissue culture plates were purchase from NUNC, Denmark.

All cells were maintained at 37°C, 5% CO_2 and humidity in CO^2 incubator (Binder, Germany). HUVEC was propagated in ECM supplemented with 5% FBS, 1% PS and 1% ECGS. HT-29 and HCT-116 were propagated in RPMI-1640 cell culture medium supplemented with 10% FBS and 1% PS. T47D was propagated in RPMI-1640 supplemented with 10% FBS, 1% PS and human insulin at 5 μg mL^{-1}. MCF-7 was propagated in DMEM supplemented with 10% FBS and 1% PS. HepG-2 was propagated in MEM supplemented with 10% FBS and 1% PS. Cell culture work was done in sterile conditions using Class II biosafety cabinet (ESCO, USA)

Rat aorta ring assay: The assay was performed according to standard protocol of Brown *et al*. (1996) with minor modifications.

Preparation of aortic rings: The animals were humanely sacrificed via cervical dislocation under anesthesia with diethyl ether. A midline incision was made into the abdominal and thoracic cavities including splitting of the sternum. Thoracic aortas were excised, rinsed with serum free medium, cleaned of the fibroadipose tissue and were cross sectioned under dissecting microscope into thin rings of about 1 mL thickness.

Preparation of the tissue culture plates: Medium for the lower layer was prepared by adding fibrinogen and aprotinin at 3 mg mL^{-1} and 5 μg mL^{-1}, respectively to M199 basal m edium. The 300 μL was loaded in each well of 48-well plate and one aortic ring was seeded in each well. 10 μL of thrombin; prepared at 50 NIH U mL^{-1} in 0.15 M NaCl: bovine serum albumin; was added to each well and was allowed to solidify at 37°C in 5% CO_2 for 60-90 min.

The top layer medium was prepared by adding the following to M199 basal medium: FBS at 20% v/v, L-glutamine at 1%, aminocaproic acid at 0.1%, amphotericin B at 1% and gentamicin at 0.6%. The extracts were also added to top layer medium at 100 μg mL^{-1}. The tissues were incubated at 37°C in 5% CO_2 in a humidified incubator, on day four the top layer medium was changed with fresh medium prepared as previously mentioned with the plant extract added at 100 μg mL^{-1}. The DMSO was used as a negative control and suramine at 100 μg mL^{-1} was used as a positive control.

Quantification of the blood vessels outgrowth: The magnitude of blood vessel outgrowth was quantified according to the technique developed by Nicosia *et al.* (1997). Briefly, the distance of blood vessels outgrowing from the primary tissue ex-plants was measured on day five under the 4x magnification power of inverted light microscope supplied with Leica Quin computerized imaging system. The growth distance of at least twenty blood vessels per ring was measured; blood vessels were selected at regular intervals around the rings to reduce the bias. The experiment was performed in triplicates each replicate containing six rings and the results were presented as a mean percent inhibition to the negative control (n = 18)±Standard Deviation (SD). The following formula was used to calculate the percent of inhibition:

$$\text{Percent of blood vessel inhibition} = (1- (A_0/A))\times 100$$

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

Cytotoxicity assay: The MTT cytotoxicity assay was performed according to the method developed by Mosmann (1983) with minor modifications. Cells were seeded at 1.5×10^4 cells in each well of 96-well plate in 100 μL of fresh culture medium and were allowed to attach for overnight. The stock solutions of the extracts were diluted in cell culture medium to obtain 100 μg mL^{-1} and 100 μL was added to each well. After 48 h of treatment the medium was aspirated and the cells were washed once with sterile Phosphate Buffered Saline (PBS). The MTT solution prepared at 5 mg mL^{-1} in sterile PBS was added to each well at 10% v/v and was incubated at 37°C in 5% CO2 for 3 h. The water insoluble formazan salt was solublized with 200 μL DMSO/well. Absorbance was measured by Multiskan Ascent microplate reader (Thermolab Systems 354, Finland) at primary wave length of 570 nm and a reference wave length of 650 nm. Each plate contained the samples, negative control and blank. DMSO at less than 0.5% v/v was used as a negative control. The assay was performed in quadricates and the results are presented as a mean percent inhibition to the negative control±SD. The following formula was used to calculate the percent of inhibition:

$$\text{Percent of Inhibition} = (1 - (OD_0/OD))\times 100$$

Where:
OD_0 = Optical density of the samples
OD = Optical density of the negative control

Statistical analysis: All results are presented as Mean±SD.

All experimental work was carried out at pharmacology labs, School of Pharmaceutical Sciences, University Sains Malaysia during the period of July 2008 to July 2009.

RESULTS

Plant extraction: Two extracts were prepared from each plant, one by n-hexane and the other by methanol. The weight of each extract was reported and the results are presented as a wt/wt percent yield. Table 1 shows the list of plants used in this study. Each extract was given a number starting by AE-(A from the first letter of the first author's name; Abed and E from the first letter of Extract).

Rat aorta ring assay: This assay was performed as the primary assay to study the antiangiogenic properties the plant extracts. The assay was conducted in triplicates and each replicate contains six rings, the distance of the blood vessels outgrowth from the primary tissue ex-plants was measured on day five and the results are presented as a mean percent inhibition to the vehicle (DMSO)±SD. Plant extracts showing more than 50% inhibition of the blood vessels outgrowth were considered significant. Table 2 shows antiangiogenesis properties of plant extracts by rat aorta ring assay and Fig. 1 shows the rat aorta discs with more than 50% inhibition of blood vessels outgrowth. Out of 18 extracts, nine extracts showed more than 50% inhibition.

Cytotoxicity assay: The MTT assay was used to study cytotoxic activity of seven extracts with more than 50% inhibition of the blood vessels outgrowth by rat aorta ring assay. Six cell lines were used where HUVEC was used as a model cell line versus two colon cancer cell lines HCT-116 and HT-29, two breast cancer cell lines MCF-7 and T47D and one hepatocarcinoma cell line HepG2. The cells

Table 1: The extraction results, 18 extracts were prepared from seven plants; the results are presented as a wt/wt percent yield

Extract No.	Botanical name	Local name	Used part	Solvent	Yield (%)
AE-3	*P. granatum*	Pomegranate	Pericarp	n-hexane	0.01
AE-4	*P. granatum*	Pomegranate	Pericarp	Methanol	15
AE-5	*D. regia*	Flame of the forest	Flowers	n-hexane	1.08
AE-6	*D. regia*	Flame of the forest	Flowers	Methanol	1.5
AE-7	*P. speciosa*	Petai	Seeds	n-hexane	7.5
AE-8	*P. speciosa*	Petai	Seeds	Methanol	7.5
AE-9	*P. speciosa*	Petai	Fruit pulp	n-hexane	0.1
AE-10	*P. speciosa*	Petai	Fruit pulp	Methanol	2.3
AE-11	*D. regia*	Flame of the forest	Leaves	n-hexane	0.2
AE-12	*D. regia*	Flame of the forest	Leaves	Methanol	8.3
AE-13	*C. fistula*	Golden shower	Leaves	n-hexane	1.7
AE-14	*C. fistula*	Golden shower	Leaves	Methanol	4.3
AE-15	*S. campanulatum*	Kelat paya	Leaves	n-hexane	0.6
AE-16	*S. campanulatum*	Kelat paya	Leaves	Methanol	9.3
AE-17	*S. koetjape*	Sentul	Stem bark	n-hexane	5
AE-18	*S. koetjape*	Sentul	Stem bark	Methanol	5
AE-19	*G. mangostana*	Mangostin	Pericarp	n-hexane	1
AE-20	*G. mangostana*	Mangostin	Pericarp	Methanol	12

Table also shows the popular names of the plants as well as the botanic names in addition to the extract numbers with the solvents used for the extraction

Table 2: The rat aorta ring assay results, the results are presented as a mean percent inhibition to the vehicle (DMSO)±D, (n = 18)

Extract No.	Botanic name	Inhibition (%)	SD
AE-3	*P. granatum*	15	8
AE-4	*P. granatum*	67	6
AE-5	*D. regia*	15	5
AE-6	*D. regia*	46	8
AE-7	*P. speciosa*	5	6
AE-8	*P. speciosa*	3	4
AE-9	*P. speciosa*	74	9
AE-10	*P. speciosa*	82	6
AE-11	*D. regia*	18	6
AE-12	*D. regia*	18	4
AE-13	*C. fistula*	8	5
AE-14	*C. fistula*	23	5
AE-15	*S. campanulatum*	66	4
AE-16	*S. campanulatum*	74	8
AE-17	*S. koetjape*	97	0.2
AE-18	*S. koetjape*	90	1
AE-19	*G. mangostana*	100	0.2
AE-20	*G. mangostana*	100	1

Nine extracts show >50% inhibition of the blood vessels outgrowth

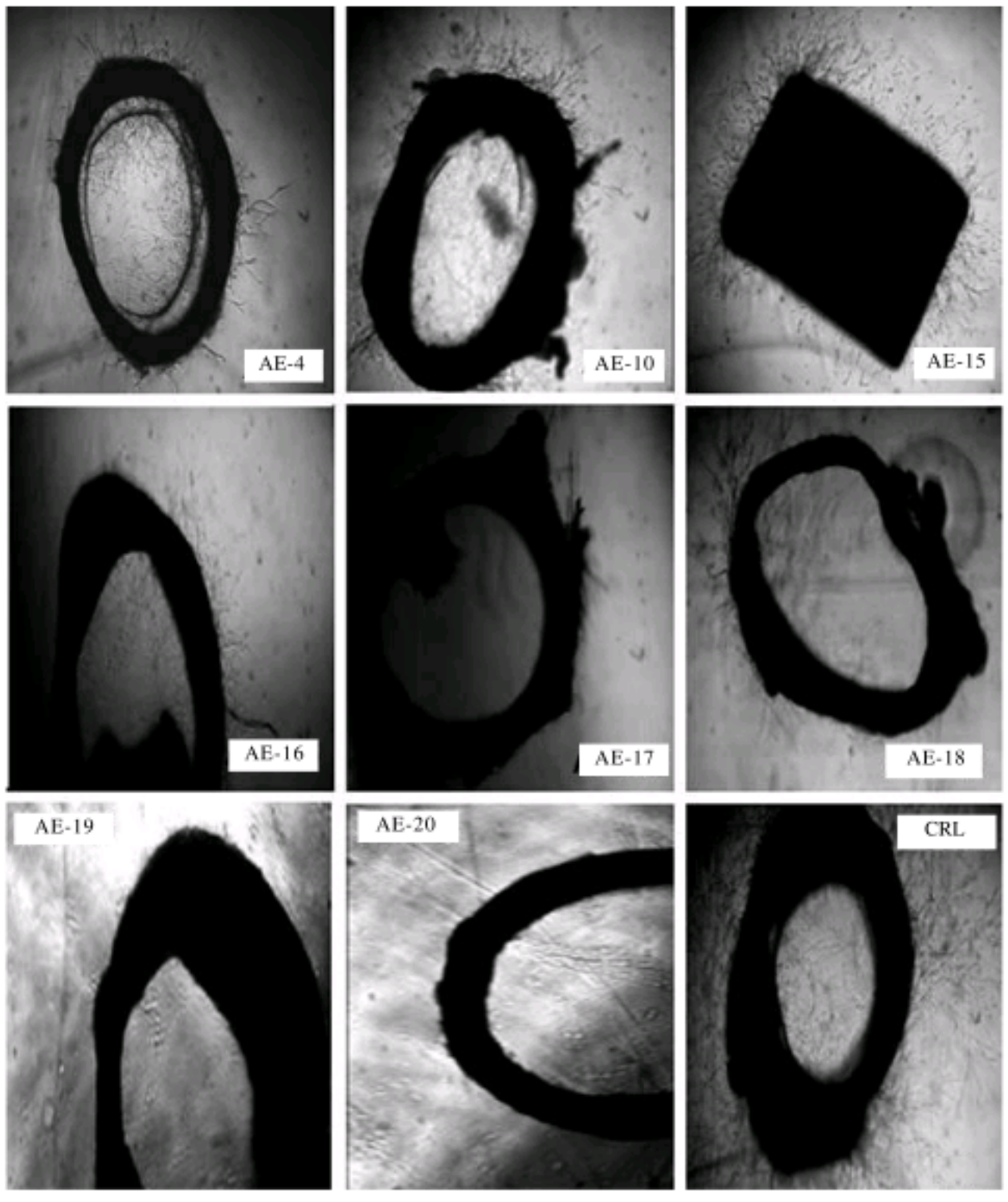

Fig. 1: Graphs show aortic rings with >50% inhibition of the blood vessels outgrowth versus the control (CRL). The Figure shows the differences in the distance of the blood vessels outgrowth between the control (CRL) and the treated tissues, the figure shows almost 100% inhibition by AE-17, AE-19 and AE-20. Figure shows also significant inhibition of the blood vessels outgrowth (>50%) by AE-4, AE-10, AE-15, AE-16 and AE-18 (Table 2)

Table 3: Cytotoxicity results by MTT assay of seven extracts prepared from four plants. The results are presented and a mean percent inhibition to the negative control (DMSO)

	MCF-7		HCT-116		HUVEC		HT-29		Hep-G2		T47D	
Extract No.	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD	AV	SD
AE-10	11	2	10	6	56	8	17	4	9	2	8	4
AE-15	34	4	62	5	66	5	69	4	39	4	25	6
AE-16	11	5	32	8	47	3	57	2	59	3	50	1
AE-17	96	1	93	0.1	99	1	100	1	100	0.2	83	2
AE-18	27	6	46	6	18	6	1	3	1	4	36	4
AE-19	95	0.2	94	1	75	3	96	1	93	1	89	1
AE-20	98	0.1	100	1	91	2	99	0.2	97	1	98	0.4

Table shows nonselective cytotoxic activities of AE-17, AE-19 and AE-20. AE-10, AE-15 and AE-16 show selective cytotoxic activities at least against one cell line. AE-18 didn't show any cytotoxic activities against HUVEC. The precision of the results are presented as SD, (n = 4)

were treated with the extracts at 100 μg mL^{-1} for 48 h, the assay was performed in quadricates and the results are presented as a mean percent inhibition to the negative control±SD. Table 3 shows the cytotoxicity results by MTT assay.

Calculation of the Selectivity Index (SI): The selectivity index corresponds to the mean percent inhibition on HUVEC divided by the mean percent inhibition on other cell lines, SI were considered interesting for values>2. Table 4 shows the SI results of cytotoxicity testing.

Table 4: Selectivity index of seven extracts on HUVEC, extracts with SI>2 are highlighted

Extract No.	HUVEC/MCF-7	HUVEC/HCT-116	HUVEC/HT-29	HUVEC/HepG2	HUVEC/T47D
AE-10	5.2	5.5	3.3	5.9	7.0
AE-15	1.9	1.1	1.0	1.7	2.6
AE-16	4.1	1.5	0.8	0.8	0.9
AE-17	1.0	1.1	1.0	1.0	1.2
AE-18	0.7	0.4	NC*	NC*	0.5
AE-19	0.8	0.8	0.8	0.8	0.8
AE-20	0.9	0.9	0.9	0.9	0.9

*SI was not calculated because the extract is not cytotoxic to HUVEC. The SI was calculated as the mean percent inhibition against HUVEC divided by the mean percent inhibition to other cell lines. AE-10 was the most selective, with SI >3 against all tested cell lines. AE-15 and AE-16 also show some selectivity on HUVEC at least against one cell line

DISCUSSION

N-hexane: methanol extraction method was used in order to obtain the hydrophobic constituents in n-hexane extracts whereas the hydrophilic and the intermediated hydrophobic compounds will be obtained in methanol extracts. Two assays were used in this study the rat aorta ring assay and MTT cytotoxicity assay. Rat aorta ring assay is considered to come closest to simulating *in vivo* situations because it includes nonendothelial cells beside the endothelial cells and the endothelial cells themselves are not preselected by passaging and thus are not in the proliferation state at the time of explanation and more representative of the real life (Robert *et al*., 2003). The assay is based on the ability of the aortic wall to produce neo-vessels in bio-matrix gels after mechanical injury or angiogenic factor stimulation. MTT assay provides a simple method for determination of live cell number in order to assess the rate of cell proliferation and to screen cytotoxic agents. The assay measures cell's viability based on mitochondrial activity in living cells to reduce MTT to water-insoluble formazan crystals, the intensity of the colour positively correlates with the number of living cells.

Nine extracts prepared from *G. mangostana*, *S. koetjape*, *P. speciosa*, *S. campanulatum* and *P. granatum* showed more than 50% inhibition of the blood vessels outgrowth (Table 2). Extracts prepared from *G. mangostana* and *S. koetjape* were the most potent, however the SI results suggested nonselective cytotoxic properties. Extracts of *P. speciosa*, *S. campanulatum* and *P. granatum* showed the most promising results, although it was less effective than *G. mangostana* but the SI results on HUVEC suggested true antiangiogenic properties, SI results indicated these extracts are more effective against HUVEC than the other cell lines, based on cytotoxicity results we can conclude that inhibition of the blood vessels outgrowth by the rat aorta ring assay can be explained by the selective cytotoxic activity induced by *P. speciosa*, *S. campanulatum* and *P. granatum* extracts. MTT assay was performed to verify rat aorta ring assay results, because all cytotoxic agents are antiangiogenic but not the reverse. HUVEC was used as a model cell line for angiogenesis because it forms the internal lining of the new blood vessels and five cancer cell lines were used as control and the cytotoxicity SI toward HUVEC was calculated (Table 4); the higher the SI the more likely of true antiangiogenic activity. SI results shown in this research article suggest true antiangiogenic properties of *P. speciosa* methanol extract with SI>3, *S. campanulatum* extracts and *S. koetjape* methanol extract with SI>2 for at least one cell line. N-hexane extract of *S. koetjape* and both extracts of *G. mangostana* were the most effective by rat aorta ring assay but they are also the most effective cytotoxic extracts. *S. koetjape* was previously reported by our research group to have cytotoxic and antiangiogenic properties (Aisha *et al*., 2009) and *G. mangostana* was reported to contain high levels of cytotoxic xanthones such as alpha-mangostin (Matsumoto *et al*., 2003). Further fractionation and purification of the crude extracts is required in order to obtain more selective and effective compounds. The cytotoxic properties of *P. granatum methanolic* extract was not studied because this plant was previously reported to have antiangiogenic properties both *in vitro* and *in vivo* (Toi *et al*., 2003) and it was used in this study as a control. Cytotoxic properties of n-hexane extract of *Parkia speciosa* wasn't also studied because methanol extract showed more interesting results in terms of the percent yield and solubility.

In summary, this is the first time to report antiangiogenic properties of *P. speciosa*, *S. campanulatum* and *S. koetjape*. The results shown in this research article make these plants good candidates for future studies. The next steps will be purification and studying the mode of action and *in vivo* antiangiogenesis.

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