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# Evaluation of Antiangiogenic, Cytotoxic and Antioxidant Effects of *Syzygium aromaticum* L. Extracts

[1]Abdalrahim F.A. Aisha, [1]Zeyad D. Nassar, [1]Mohammad J. Siddiqui, [2]Khalid M. Abu-Salah, [2]Salman A. Alrokayan, [1]Zhari Ismail and [1]Amin Malik Shah Abdul Majid
[1]School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden 11800, Pulau Pinang, Malaysia
[2]The Chair of Cancer Targeting and Treatment, Department of Biochemistry and King Abdulla Institute for Nanotechnology, King Saud University, P.O. Box 2455, 11451, Riyadh, Saudi Arabia

*Corresponding Author: Dr. Amin Malik Shah Abdul Majid, School of Pharmaceutical Sciences, Minden 11800, Pulau Pinang, Malaysia Tel: +6046534582 Fax: +6046534582*

## ABSTRACT

This study aims to investigate the total phenolics content, antioxidant, antiangiogenic and cytotoxic effects of three extracts from the leaves, stem and bark of *Syzygium aromaticum* L (SA). The motivation behind this study was to highlight the importance of other parts of SA than the flower buds as botanical sources of bioactive materials. The total phenolics were determined in a colorimetric method using gallic acid as a standard, the antioxidant effects were studied in DPPH assay, antiangiogenic effects were investigated *in vitro* on rat aortic rings and the cytotoxic properties were evaluated in XTT assay on endothelial cells, breast and colon cancer cells. The extracts were found to contain high levels of total phenolics with strong antioxidant activity. Significant inhibition of the blood vessels outgrowth was also obtained in the angiogenesis model. Cytotoxicity testing on three cell lines indicated high cytotoxic effects with the highest activity obtained on the estrogen dependent breast cancer cells. The presented results illustrate the importance of SA leaves, stems and bark as good sources of phenolics-rich extracts. Further studies are required to investigate the antiestrogenic and the potential anti breast cancer activity of SA extracts especially the stem extract and to identify and purify the active constituents.

**Key words:** *Syzygium aromaticum* L., rat aortic rings, DPPH, MCF 7 and HCT 116, total phenolics

## INTRODUCTION

*Syzygium aromaticum* L. Merr. and Perry (Myrtaceae). Is an evergreen tree that can reach 10-20 m in height (Trease and Evans, 1972). The tree is cultivated in most tropical countries including Malaysia, Indonesia, Zanzibar, India and Sri Lanka. The essential oil obtained from the dried flower buds (clove) has been used since the ancient times in traditional medicine, perfume industry and in flavorings (Zheng *et al*., 1992). Traditional uses of clove oil include treating burns, dental care to relieve pain and to treat infections of the gums when used at high concentrations (Prashar *et al.,* 2006) and to treat respiratory and digestive aliments (Banerjee *et al.,* 2006). Phytochemical studies showed the main constituents of the clove essential oil including eugenol, eugenol acetate, caryophyllene and sesquiterpenes (Zheng *et al*., 1992; Chaieb *et al*., 2007). The presence of the pentacyclic triterpenes oleanolic acid, betulinic acid and urosolic acid was also

reported in clove extracts (Jäger *et al.,* 2009). Several research groups reported remarkable biological properties of SA including anticarcinogenic (Zheng *et al.*, 1992), antimutagenic (Miyazawa and Hisama, 2001) aphrodisiac (Tajuddin *et al.,* 2004), mosquito repellent (Trongtokit *et al.,* 2005), cytotoxicity (Prashar *et al.,* 2006), antimicrobial (Chaieb *et al.,* 2007), chemopreventive for lung cancer (Banerjee *et al.,* 2006), antioxidant (Ogata *et al.,* 2000; Gulcin *et al.*, 2010), antiinflammatory effect (Darshan and Doreswamy, 2004) and enhancement of gemcitabine cytotoxic effect on human cervical cancer cells (Hussain *et al.,* 2009). However the majority of studies focused on the biological activities of the clove essential oil. Accordingly, this study was undertaken to investigate some biological properties including antiangiogenic, antioxidant and cytotoxic effects and to determine the total phenol contents of different extracts from the leaves, bark and stem of SA. The motivation behind this study was to highlight the importance of other parts of SA than the flower buds as new, cheaper and more abundant botanical sources of bioactive materials.

## MATERIALS AND METHODS

**Chemicals:** Endothelial cell medium (ECM) supplied with endothelial cell growth supplements (ECGS) was obtained from ScienCell, USA. RPMI 1640 and DMEM cell culture media and fetal bovine serum (FBS) were obtained from GIBCO, USA. Betulinic acid (BA), gallic acid (GA), Folin-Ciocalteau reagent, 1,1-diphenyl-2-pircryldrazyl (DPPH), sodium bicarbonate, penicillin/streptomycin (PS) solution, XTT reagent with phenazine methosulfate (PMS), amphotericin B, aprotinin, 6-aminocaproic acid, L-glutamine, thrombin and gentamicin were obtained from Sigma-Aldrich, USA. Fibrinogen was obtained from Calbiochem, USA. Solvents of either HPLC or analytical grade were obtained from Merck.

**Plant material:** Leaves, bark and stems of SA were collected from the botanical garden of School of Pharmaceutical Sciences, USM, during December 2009. The plant was authenticated by Prof. Dr. Zhari Ismail, School of Pharmaceutical Sciences. The plant was dried at room temperature for one week and grinded into fine powder by an electric grinder.

**Cell culture:** HUVECs human umbilical vein endothelial cells (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. MCF7 human hormone dependent breast cancer cell line (ATCC) was cultured in DMEM containing 10% FBS and 1% PS. All cell lines were propagated as monolayer at 37°C in an atmosphere of 5% $CO_2$.

**Extraction:** Ten gram of the dried powder was extracted exhaustively in 100 mL methanol for 24 h by Soxhlet extraction. Methanol was evaporated by rotavapor (Eyela, Japan) at 45°C under reduced pressure and the dried extracts were saved in air-tight containers until use. The respective percent yield was calculated with reference to the dried plant material.

**Determination of total phenols:** Total phenol content of SA extracts was determined by a colorimetric assay based on procedures described by Lizcano *et al.* (2010). Basically, 100 μL of the extracts at 1 mg $mL^{-1}$ in methanol was added to 750 μL of 1:10 diluted (in dd.$H_2O$) Folin-Ciocalteau phenol reagent. After 5 min incubation in the dark at room temperature, 750 μL of sodium bicarbonate solution at 60 mg $mL^{-1}$ was added and incubated in the dark for 90 min at 30°C. The absorbance was taken at 725 nm. Gallic acid was used at 5-80 μg $mL^{-1}$ to construct the standard calibration curve and the results were expressed as gallic acid equivalents (GAE) /100 mg (n = 6).

**DPPH antioxidant assay:** DPPH scavenging activity of the SA extracts was studied based on the procedure described by Sharma and Bhat (2009). Briefly, stock solution of DPPH was prepared in methanol at 200 μM. Serial dilutions of the extracts were prepared in methanol to obtain concentrations of 200, 100, 50, 25 and 12.5 μg $mL^{-1}$. DPPH reagent was added at final concentration of 50 μM and incubated in the dark for 30 min at 30°C and absorbance was measured at 517 nm. DPPH scavenging activity of the extracts was calculated using the formula (1-(absorbance of samples-blank)/(absorbance of negative control-blank)) X 100%. The results were expressed as mean of the median inhibitory concentration ($IC_{50}$)±SD. GA was used as a positive control and methanol was used as a negative control.

**Antiangiogenic effects on rat aortic rings:** Antiangiogenic effect of SA extracts was investigated *in vitro* on rat aortic rings according to the protocol reported by Brown *et al.* (1996) and according to our previous publications (Aisha *et al.*, 2009a, b). Briefly, the cleansed thoracic aortas were cross sectioned into thin rings of about one millimeter thickness. One ring was placed in the center of each well of 48-well plate containing 500 μL of M199 basal medium supplied with fibrinogen, aprotinin and L-glutamine at 3 mg $mL^{-1}$, 5 μg $mL^{-1}$ and 1% wt/v, respectively. Then 10 μL thrombin (50 NIH U $L^{-1}$) was added to each well. After 90 min incubation at 37°C, another 500 μL M199 medium supplied with; FBS at 20% v/v, L-glutamine at 2 mM, aminocaproic acid at 1 mg $mL^{-1}$, amphotericin B at 2.5 μg $mL^{-1}$ and gentamicin at 60 μg $mL^{-1}$ was added on top of the solidified bottom layer. SA extracts were also included in the top layer medium at 100 μg $mL^{-1}$. After 4 days incubation at 37°C in 5% $CO_2$, the top layer medium was replaced with fresh one containing the extracts. Afterwards, the magnitude of the growth of sprouting microvessels was quantified on day five according to technique reported by Nicosia *et al.* (1997). Briefly, the distance of growth of at least twenty points was measured for each ring. The results of three independent experiments were presented as mean percent inhibition to the vehicle (DMSO)±SD (n = 18). BA at 10 μg $mL^{-1}$ was used as a positive control and the vehicle (DMSO) was used as a negative control.

**Cytotoxicity testing:** Cytotoxic properties of SA extracts were evaluated using 2,3-bis [2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) assay according to Francoeur and Assalian (1996) and according to Aisha *et al.* (2009b). 70-80% confluent cells were harvested by trypsinization and were resuspended in 5 mL cell culture medium. Cell count was adjusted to $1.5\times10^5$ $mL^{-1}$ and 100 μL was seeded in each well of 96-well plate. After overnight incubation, additional 100 μL fresh medium containing the extracts at 100, 50 and 25 μg $mL^{-1}$ was added and further incubated for 48 h. Then viability of cells was assessed as the following; the old culture medium was replaced with 200 μL fresh one containing XTT reagent at 100 μg $mL^{-1}$ and PMS at 1 μg $mL^{-1}$ and was further incubated at 37°C in $CO_2$ incubator for 4 h. Optical density of reduced XTT reagent was measured at 450 nm by a microplate reader (Thermolab Systems 354, Finland). The percent inhibition was calculated using the formula (1-(absorbance of samples-blank)/(absorbance of negative control-blank)) × 100%. The experiment was repeated three times and the results are presented as a mean percent inhibition±SD.

**Experimental animals:** Four (12-14 weeks old) Sprague Dawley male rats were obtained from the animal unit facility of University Science Malaysia. The animals were kept for one week in the 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.

After euthanizing the animals by $CO_2$, a midline incision was made into the abdominal and thoracic cavities including splitting of the sternum and the thoracic aortas were collected. The experiment was performed according to guidelines of USM Animal Ethics Committee and had their approval, reference USM/PPSF/50 (084) Jld.2.

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

## RESULTS AND DISCUSSION

The dried leaves of SA led to the highest percent yield which was 12%. The percent yield of the stem and bark extracts was 9 and 8%, respectively.

**Total phenolics content:** Total phenolics content of SA extracts was calculated by applying the linear regression equation ($y = 0.0414x + 0.0807$, $R^2 = 0.9988$) of GA calibration curve. The results are presented as average of GAE (mg/100 mg) of three independent experiments±SD. The bark of SA was the richest in total phenols with 45±3 mg GAE/100 mg. The total phenolics content of the stem and the leaves was 37±2 and 31±1 mg GAE/100 mg, respectively (Table 1). Plant-derived phenolics represent good sources of natural antioxidants. Accumulating chemical, biochemical, clinical and epidemiological evidence supports the chemoprotective effects of phenolics against oxidative stress-mediated disorders (Soobrattee *et al.,* 2005).

**Free radicals scavenging activities of SA extracts:** Antioxidant activities of SA extracts were determined by DPPH scavenging assay. $IC_{50}$ values of three independent experiments were calculated from the regression equations of the dose response curves of extracts; leaves ($y = 33.703\text{Ln}(x) - 76.407$), stem ($y = 29.754\text{Ln}(x) - 51.571$) and bark ($y = 27.647\text{Ln}(x) - 40.589$). Amongst SA extracts, the bark extract showed the highest scavenging activity of DPPH with $IC_{50}$ 26±1.5 μg $mL^{-1}$. Lower activities of stem and leaves extracts were obtained with $IC_{50}$ values 30±1.5 and 43±2.5 μg $mL^{-1}$, respectively. GA was used as a positive control with $IC_{50}$ 3.5±0.5 μg $mL^{-1}$ (Table 1). Phenolics antioxidants can delay or inhibit the oxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions mainly from their free radical scavenging and metal chelating properties as well as their effects on cell signaling pathways and on gene expression (Soobrattee *et al.,* 2005; Ma *et al.,* 2003). Phenolic antioxidants were reported to exhibit anti-inflammatory, anticarcinogenic and antidiabetic activities in animals (Ma *et al.,* 2003). Furthermore, antioxidants have been reported to have potent antiangiogenic properties either by regulating angiogenesis modulators such as vascular endothelial growth factor (VEGF)

Table 1: Extraction, total phenolic contents and antioxidant effects of SA extracts

| Extracts or compounds | Extraction yield (wt/wt %) | Phenolic contents GAE (mg/100 mg) | DPPH $IC_{50}$ (μg $mL^{-1}$) |
|---|---|---|---|
| Bark | 8 | 45±3 | 26±1.5 |
| Leaves | 12 | 31±1 | 43±2.5 |
| Stems | 9 | 37±2 | 30±1.6 |
| BA | -- | -- | -- |
| GA | -- | -- | 3.4±0.05 |

BA: Refers to betulinic acid, GA: Refers to gallic acid

(Lee *et al.,* 2006), or by changing the redox microenvironment of tumor vasculature (Lee *et al.,* 2006; Mu *et al.,* 2007).

**Antiangiogenic effects of SA extracts:** Antiangiogenic properties of SA extracts were investigated *in vitro* on rat aortic rings. The percent inhibition of the sprouting blood vessels was calculated from three independent experiments±SD. The sprouting blood vessels continued to grow from the aortic rings treated with the vehicle (Fig. 1a). On the contrary, the aortic rings treated with BA or SA extracts showed significant retardation of the sprouting blood vessels (Fig. 1b-e). Amongst the extracts, the leaves extract was superior with percent inhibition 53±6%, likewise the extract from the stem showed 49±3.4%. The bark extract showed a bit lower activity with percent inhibition 43±6.5%. BA at 10 μg $mL^{-1}$ showed 37±5.5% inhibition of sprouting blood vessels. Statistical analysis by One way ANOVA shows significant difference between antiangiogenic effect

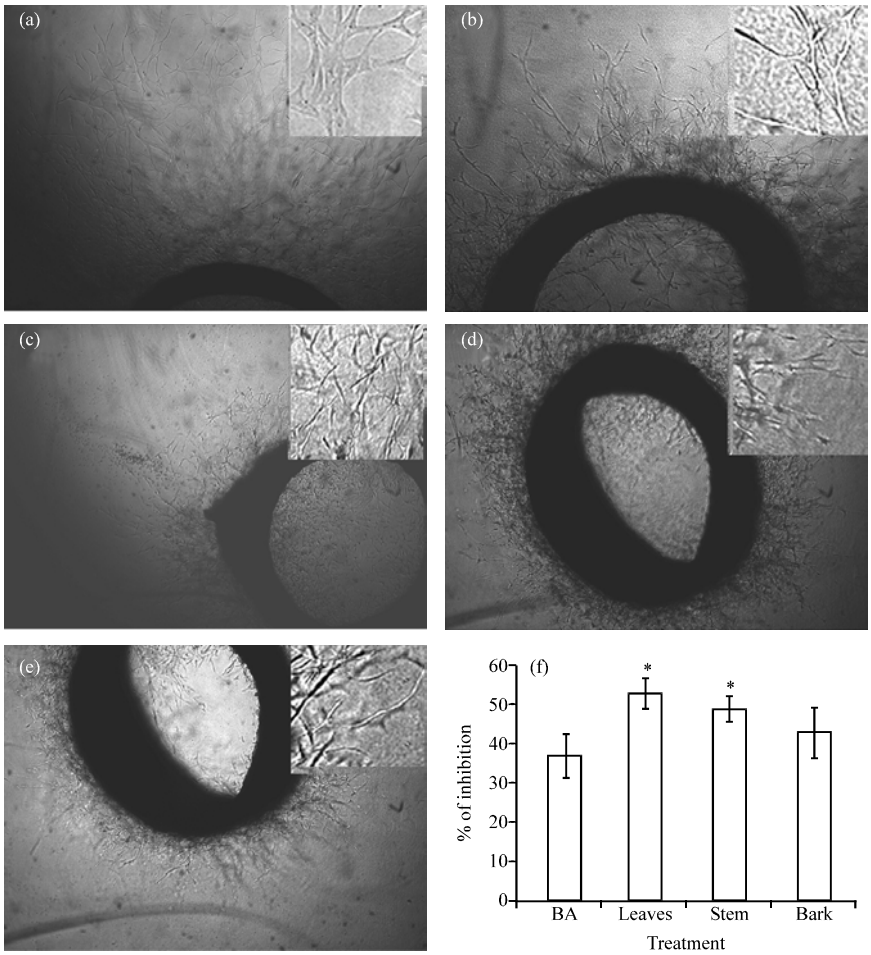


Fig. 1: *In vitro* inhibition of the vascular outgrowth of rat aortic explants by *S. aromaticum* extracts; untreated ring (a), treated with BA at 10 μg $mL^{-1}$ (b), treated with the bark extract (c), treated with leaves extract (d), treated with the stem extract (e) and a bar chart showing the percent inhibition of the vascular outgrowth relatively to the untreated rings (f). (*) indicates significant difference of antiangiogenic effect of extracts when compared with BA, $p<0.05$. The upper right corner is magnified portion of the microvessels (4x)

Table 2: Cytotoxic effect of SA extracts on colorectal carcinoma (HCT 116), breast cancer (MCF7) and the endothelial (HUVEC) cell lines

| Cell lines | Concentration (μg mL$^{-1}$) | Bark (% inhibition±SD) | Leaves (% inhibition±SD) | Stem (% inhibition±SD) |
|---|---|---|---|---|
| HUVEC | 100 | 32±4 | 82±5 | 77±1 |
| | 50 | 12±3 | 41±8 | 25±7 |
| | 25 | 6±1 | 25±0.1 | 14±3 |
| | BA (10 μg mL$^{-1}$) | 87±1 | | |
| MCF-7 | 100 | 99±0.1 | 96±0.1 | 98±1 |
| | 50 | 96±1 | 67±2 | 95±4 |
| | 25 | 28±4 | 32±1 | 49±1 |
| | BA (10 μg mL$^{-1}$) | 18±2 | | |
| HCT 116 | 100 | 49±1 | 84±1 | 72±1 |
| | 50 | 16±8 | 77±1 | 18±2 |
| | 25 | 5±1 | 66±1 | 8±5 |
| | BA (10 μg mL$^{-1}$) | 77±1 | | |

BA: Refers to betulinic acid

of leaves and stem extracts when compared to BA, p-values 0.00 and 0.003, respectively. Antiangiogenic effect of the bark extract at 100 μg mL$^{-1}$ was equivalent to BA at 10 μg mL$^{-1}$, p = 0.163. 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). Inhibition of angiogenesis which was suggested by Judah (Folkman, 1971) is now considered to be one of the most promising strategies leading to the development of new antineoplastic therapies (Folkman, 1971).

**Cytotoxic effect of SA extracts:** Cytotoxic effect of SA extracts was evaluated by XTT assay on three cell lines. Cytotoxicity results might help to explain the antiangiogenic effect on the rat aortic rings. HUVEC was used as a model cell line of angiogenesis because it forms the internal lining of blood vessels and two other cell lines were used as control. The assay was performed at three concentration points 100, 50 and 25 μg mL$^{-1}$ and the results are presented as mean percent inhibition to untreated cells (Table 2). SA extracts exhibited moderate cytotoxic effect on HUVECs as significant inhibition was obtained only at 100 μg mL$^{-1}$ and lower concentrations of extracts were found noncytotoxic. Amongst the extracts, extract of leaves was the most potent and bark extract was the least cytotoxic on HUVECs. On MCF7 cells the extracts were found more cytotoxic with the highest activity obtained with stem extract which showed almost 50% inhibition at 25 μg mL$^{-1}$. Basically, the extracts were 2 to 5 times more cytotoxic on MCF7 cells than the other two cells with the exception of leaves extract on HCT 116 cells. On HCT 116 cells the leaves extract was the most potent with 66% inhibition at 25 μg mL$^{-1}$ and cytotoxic effect of stem and bark extracts was equivalent to that on HUVECs (Table 2). Taken together, the results presented in Table 2 indicate the breast cancer cell line MCF 7 the most sensitive to cytotoxic effects of SA extracts and HCT 116 cells the most sensitive to the leaves extract.

## CONCLUSIONS

In the present study, we investigated the total phenolics, antioxidant, antiangiogenic and cytotoxic properties of three extracts from the leaves, stems and bark of *S. aromaticum*. The extracts

were found to contain high levels of total phenols with strong free radical scavenging activity. A correlation analysis study showed negative correlation between total phenolics and the $IC_{50}$ values of DPPH scavenging activity ($r = -0.86$, $p = 0.003$, Pearson correlation, $n = 9$). It can be concluded that increasing the total phenols will decrease the $IC_{50}$ values, i.e., increasing the activity. Therefore the presented free radical scavenging effect of SA extracts can be explained due to total phenols. This result is consistent with previous results of other research groups where antioxidant effect of clove oil from SA flower buds was reported Ogata *et al.* (2000) and Gulcin *et al.* (2010). Then we investigated the antiangiogenic effect of the extracts on rat aortic rings. The extracts exhibited significant inhibition of the microvessel outgrowth of aortic tissue explants. In order to distinguish the antiangiogenic from cytotoxic effects, cytotoxicity of the extracts was evaluated on HUVECs, MCF7 and HCT 116 cells. The results indicate differential cytotoxic effects with the least cytotoxicity obtained on HUVECs. MCF 7 and HCT 116 were found more sensitive to cytotoxic effect of the extracts especially to the stem extract (MCF 7) and the leaves extract (HCT 116). Basically, the antiangiogenic effects of SA extracts might be explained due to presence of cytotoxic constituents in the extracts. The antiangiogenic and selective cytotoxic effect on estrogen dependent breast cancer cells is reported for the first time in this article and further studies are needed to explore the mechanism of action and to investigate the anti-estrogenic activity of SA extracts.

Previous research conducted on clove indicated the presence of constituents with cytotoxic, apoptotic and antiangiogenic effects including eugenol (Zheng *et al.*, 1992; Chaieb *et al.*, 2007), pentacyclic triterpenes (Jäger *et al.,* 2009) and sesquiterpenes (Zheng *et al.,* 1992). The pentacyclic triterpenes betulinic acid, urosolic acid and oleanolic acid are well known cytotoxic, apoptotic and antiangiogenic agents (Mukherjee *et al.,* 2004; Fulda, 2008; Karna *et al.,* 2010). The clove sesquiterpenes were previously reported to have anticarcinogenic effects (Zheng *et al.*, 1992). The antioxidant, antiangiogenic and cytotoxic effects of SA stem, bark and leaves extracts reported in this study are comparable to those previously reported from clove extracts and accordingly the results presented in this article might be explained on the basis of the chemical composition of clove. Finally, we report *S. aromaticum* leaves, stems and bark as good and cheaper sources of bioactive materials than the flower buds. Further investigations are required to illustrate the antiangiogenic, antiestrogenic and the chemical composition of *S. aromaticum* extracts.

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