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CONFLICT OF INTEREST
NONE DECLARED

Anti-Angiogenic Activities of the Standardized Methanol Extract of *Gynura Segetum* Leaf

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Abstract

Gynura segetum (family Compositae) is a cultivated species and can be found growing in the tropical regions of Indonesia and Malaysia. The plant is known for its use for the treatment of cancer, inflammation, diabetes, hypertension and skin afflictions. In the current study, the methanol extract of *Gynura segetum* leaf was standardized by high-performance liquid chromatographic (HPLC) analysis using seneciphylline and senecionine as marker compounds before assessment of anti-angiogenic activities. Anti-angiogenic activities of the standardized methanol extract of *Gynura segetum* leaf were examined by using *in vivo* chick embryo chorioallantoic membrane (CAM) assay, *ex vivo* rat aorta ring assay and *in vitro* assessment of the inhibitory effect on vascular endothelial growth factor (VEGF) activity. Additionally, the cytotoxicity effect of the standardized methanol extract was determined by MTT assay. The present study shows that non-cytotoxic methanol extract is an anti-angiogenic agent with inhibitory effects in both *in vivo* and *ex vivo* angiogenesis models, and the suppression of VEGF activity is the main mechanism of action involved in inhibiting the angiogenesis cascade. Inhibition of angiogenesis has been considered to be beneficial for the treatment and prevention of cancer and the present work suggests that methanol extract of *Gynura segetum*'s leaf could have the potential therapeutic benefits against cancer due to its anti-angiogenic effects.

Keywords: *Gynura segetum*, HPLC validation, anti-angiogenic, MTT, VEGF.

Introduction

Herbal medicine is the oldest form of health care and has been widely used in all cultures throughout history[1]. Despite the great advances observed in modern medicine in recent decades, plants still make an important contribution to health care[2]. The medicinal plants generally contain various bioactive constituents, which possess therapeutic properties or exert beneficial pharmacological effects. Attention is being focus on the investigation of efficacy of medicines plant due to their affordability and accessibility with minimal adverse effects[3].

Gynura segetum (family Compositae) has drawn a lot of attention due to its uses in traditional medicine. The plant is known for its traditional use for the treatment of cancer, inflammation, diabetes, and hypertension and skin afflictions[4]. Our preliminary phytochemical screening revealed the presence of alkaloids, terpenes, flavonoids, tannins and saponins in leaves of *G.segetum*[5]. The presence of phenolic compounds in the plants indicates that this plant may be anti-microbial agent and this agreed with our previous findings[6].

Previous studies of anti-inflammatory effects of the leaf extracts of *G.segetum* was carried out by using a modified version of the chicken chorioallantoic membrane (HET-CAM) assay and cotton pellet granuloma assay[7,8]. The methanol extract showed a significant good anti-inflammatory effect as compared with the anti-inflammatory drug indomethacin. The anti-inflammatory potential of methanol extract of *G. segetum's* leaf is mediated through the inhibition of pro-inflammatory cytokines and COX-2 enzyme activities .An inflammatory state can promote angiogenesis, and angiogenesis can facilitate chronic inflammation. Earlier study had demonstrated that the leaf extracts of *G. segetum* reduced the growth of blood vessels and exhibited the potent anti-angiogenic effect in chick embryo chorioallantoic membrane (CAM) model [9]. The anti-inflammatory activity of *G. segetum's* leaves seems to be closely linked with its anti-angiogenic activity.

In the present study, standardization of the methanol extract of *G. segetum*'s leaf by quantification of two marker compounds using a validated HPLC (high performance liquid chromatography) method was carried

out. The anti-angiogenic activities of the standardized methanol extract of *G. segetum*'s leaf and its probable mechanism of action were also investigated.

Materials and Methods

Chemicals

All the chemicals and solvents were of analytical grade, unless otherwise specified were obtained from Merck (Germany) and R&M chemical (U.K).Seneciphylline,senecionine,semaxanib (SU5416) and suraminwere purchased from Sigma Chemical Co (St. Louis, MO). Earles' salt (M199) medium, fibrinogen and foetal bovine serum were obtained from Bio-Diagnostics (Petaling Jaya, Selangor, Malaysia). Aprotinin, L-glutamine, thrombin, sodium chloride, gentamycin, 6-aminocaproic acid,MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), 5-Fluorouracil and rosmarinic acid were from Sigma-Aldrich (Subang Jaya, Selangor, Malaysia). Human colon carcinoma cell line (HCT116) and human colon fibroblast cell line CCD-18Co was obtained from the American tissue culture collection (Rockvill, MD, USA).

Plant material and preparation of extract

The leaves of *G.segetum* were collected from JabatanPertanianRelau, Penang. A voucher specimen (No.11013) has been deposited at the herbarium of School of Biological Sciences, UniversitiSains Malaysia. The dried powdered leaves (500g) were extracted successively with solvents of increasing polarity, namely petroleum ether (60-80°C), chloroform and methanol by using soxhlet apparatus. Each extract was then filtered and evaporated in a rotary evaporator and stored in a refrigerator until use. The percentage of yield of petroleum ether, chloroform and methanol extracts was 6.21%, 7.40% and 10.43%, respectively. The methanol extract which was previously evaluated [7,8] for its anti-inflammatory activity was selected for standardization and further investigation for its anti-angiogeniceffects and mechanisms of action.

Development and validation of HPLC method

Stock standard solutions of seneciphylline and senecionine were prepared in HPLC grade methanol (1 mg/mL) and stored in a refrigerator at 4°C until use. A series of working standard solutions (0.1953-50 µg/mL) were freshly prepared by diluting with HPLC grade methanol before injection. The test sample was prepared by dissolving an accurately weighed of methanol extract (10 mg) in 1ml of HPLC grade methanol and the resultant solution was sonicated. Both standard and sample solutions were filtered through a 0.45 µm syringe filter prior to HPLC analysis.HPLC analysis was performed on Agilent Technologies series 1100 system equipped with UV detector and a Multi Technique HP ChemStation (Agilent Technologies; Waldbronn, Germany). An Agilent ZORBAX Eclipse plus C18 (250 mm x 4.6 mm i.d., 5 µm particle) was used with the following analytical conditions: a mobile phase of 0.1% phosphoric acid in water: methanol (60:40 v/v), a flow rate of 1 mL/min and the signals detected at 210 nm. The column temperature was 24°C and the injection volume was 20 µL. The proposed method of seneciphylline and senecionine in the methanol extract was simultaneously validated according to the ICH (International conference on the Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use) guidelines (ICH, 2002).

In vivo CAM assay

Anti-angiogenic effect of methanol extract of *G. segetum*'s leaf was evaluated by using chick embryo chorioallantoic membrane (CAM) assay as described in the previous study [10]. The methanol extract and positive control (SU5416) were dissolved in 0.05% (v/v) DMSO and tested for anti-angiogenic activity at a concentration range of 12.5 to 100 µg per disc. CAMs treated with 10 µL of DMSO 0.05% served as blank control.

Fertilized chicken eggs were incubated in an incubator (Incucell, Germany) for 3 days at 37°C under a constant relative humidity of 80%. On day-3, eggs were swabbed with 70% alcohol under a laminar flow hood and about 2 mL of albumin was withdrawn through the pointer end of the egg in order to allow detachment of the developing CAM from the egg shell. A window was then cut in the shell and served as a portal access for the CAM. The window was closed with a cellophane tape and the eggs were returned to the incubator, keeping them horizontally with the window uppermost, until sample application on day 5. On day-5, digital images of the CAM before treatment were captured using a digital camera (Sony Cyber-shot DSC-T33). Filter paper discs with the test substances were placed directly using microsurgical forceps over a blood vessel on the growing CAM at day-5 incubation under sterile conditions and second digital image was taken. The eggs were closed with the cellophane tape and returned to the incubator. The final evaluations were carried out on day-7 of development. On day-7, the filter paper disc was gently discarded from CAM and the images of treated CAMs were captured. Each set of experiment was performed using 6 eggs (per test sample) and the experiment triplicated.The anti-angiogenic effects of the test samples were processed using the Image J software (version 1.48, with plug in). Analysis and quantification of each treated CAM were made through the captured images. Images at high magnification and in good focus

were used. The results were expressed as a percentage inhibition of blood vessel growth and processed statistically.

Ex vivo rat aortic ring assay

The rat aortic rings assay of methanol extract was performed according to the standard protocol of Brown *et al.* [11] with minor modifications. Briefly, thoracic aorta was isolated out, rinsed with serum free media, cleaned off the adipose tissue and cut into thin rings of approximately 1 mm thickness. Aortic rings were seeded individually in 48-wells plate in 300 µL serum free M199 media containing fibrinogen (3 mg/mL), aprotinin (5 mg/mL) and L-glutamine (1% v/v). Thrombin (10 µL at 50 NIH U/mL in 0.15M NaCl) was then added in each well and incubated for 90 min at 37°C. Next, various concentrations of methanol extract (50, 100, and 200 mg/mL) were mixed with a second layer made of 300 µL M199 medium containing FBS (20% v/v), 1% L-glutamine, 6-aminocaproic acid (1 mg/mL), amphotericin B (2.5 mg/mL), and gentamicin (60 mg/mL) were added into each well and incubated at 37°C. Suramin (100 mg/mL) and 1% DMSO were used as positive and negative control respectively.

After 4 days of incubation, the medium on top layer was replaced with freshly prepared medium containing methanol extract. On day 5, aortic rings were photographed by using an AMG EVOS f1 inverted microscope (40X magnification). The outgrowth of the sprouting blood vessels was quantified using Leica QWin imaging software (Grand Island, NY, USA). The distance of growth between at least 20 growth points was measured in each ring. The results were presented as the mean percent inhibition of six independent experiments.

Cytotoxicity

The cytotoxicity of the standardized methanol extract of *G. segetum*'s leaf was evaluated on human colon carcinoma cell line, HCT116 and human colon fibroblast cell line, CCD-18Co (normal cells) by using a modified MTT assay [12]. The percentage of inhibition against cell line was calculated and the dose response curve was drawn. The results were expressed as a percentage inhibition and represented as mean ± SD (n=6). The median inhibitory concentration (IC_{50}) of methanol extract was determined from dose response curve.

Determination of VEGF concentration

The VEGF concentration was determined by using a human VEGF-165 ELISA kit (CUSABIO, China) according to the manufacturer's instructions. HCT116 cells (1 X 106 cells/ mL) were seeded in a 6 well cell culture plate and incubated for 24 hours. After an overnight incubation, the cells were then treated with positive control (rosmarinic acid, 100 µg/mL) and methanol extract (50, 100, 200 and 400 µg/mL) for 12 hours and cell lysates were prepared using cell lysis buffer provided with the kit. The calibration curve of VEGF was prepared at the same time. The concentration of VEGF in cell lysates was quantified by applying the VEGF linear regression equation, y= 0.0002x + 0.0262, r2 =0.9908 (n=6).

Statistical analysis

Significant differences between the treatment and control groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison tests (SPSS version 20). P values less than 0.05 was considered to be significant.

Results

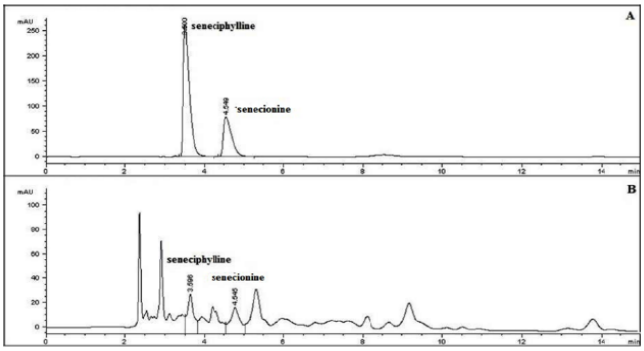

Figure 1- HPLC chromatogram of reference standards (**A**) and methanol extract of *Gynura segetum*'s leaf (**B**)

HPLC analysis of Gynura segetum's leaves

A simple HPLC method for qualitative analysis of the chromatographic profile of *G. segetum*'s leaves and validated for quantitative analysis of seneciphylline and senecionine was developed. The authenticity of seneciphylline and senecionine in methanol extract of *G. segetum*'s leaf is checked by running a standard injection of a mixture of methanol extract and standards (seneciphyllineand senecionine). The identification of seneciphylline and senecionine were performed by comparing the retention time with those of their respective standards. Figure 1 shows the HPLC chromatogram of methanol extract of *G. segetum*'s leaves and standards (seneciphylline and senecionine). The concentrations of seneciphylline and senecioninein the methanol extract were simultaneously quantified by a validated method. The correlation coefficients (R^2) for each standard were higher than 0.999, demonstrating an acceptable data fit to the regression line. The intra and inter-day precisions for the developed method were satisfied with acceptable coefficient of variation (CV <3%), which demonstrated good precision of the method. The recoveries of seneciphyllineand senecionine were found in the range of 98.16-101.25% and 98.14-101.08%, respectively. The high recovery values (>98%) associated with the low %CV (<5%) indicated a very good accuracy

of this method. According to the validated method, the content of seneciphylline and senecionine quantified in *G. segetum*'s leaves extract were found to be 7.13 ± 0.87 µg/mL and 16.07 ± 0.16 µg/mL, respectively.

In vivo CAM screening

In vivo anti-angiogenic activity of methanol extract of *G. segetum*'s leaf was examined by using chick embryo chorioallantoic membrane (CAM) assay. The growth of blood vessels focus on the treatment area under disc was quantified by using the Image J software suite and the results are summarized in Table1. The test samples were tested at four different concentrations (12.5, 25, 50 and 100 µg per disc) and the results demonstrated that there was a dose-dependent inhibition of blood vessel growth.

Test samples	Concentration (µg/mL)	Inhibition of blood vessel (%)	IC_{50} (µg per disc)
Positive control			
SU5416	12.5	17.2 ± 0.17	45.1
	25	34.7 ± 0.35	
	50	58.2 ± 0.33	
	100	66.1 ± 0.46	
Methanol extract	12.5	30.3 ± 0.29	28.9
	25	47.8 ± 0.18	
	50	66.9 ± 0.53	
	100	71.7 ± 0.41	

Table 1- CAM assay: The percent inhibition of blood vessel on CAM treated with SU5416 and methanol extracts of *Gynura segetum* leaf and their IC_{50} values.

The area of the CAM below disc containing DMSO 0.05% did not show changes in vascular density. The CAM treated with positive control (SU5416) resulted in a reduced number of blood vessels and capillary free area below the disc. SU5416 showed good anti-angiogenic effect and a CAMs treated with different concentrations of SU5416 are shown in Figure2A. It should be noted that the image result shown here are selected based on providing the most representative examples of the effects observed. However, the results did vary between egg and even within the treated area.

Methanol extract was highly significant (p<0.001) as compared to the positive control (SU5416) group with the lowest IC_{50} values (28.88 µg per disc). Methanol extract performed a consistent inhibition of blood vessel and the percentage of inhibition is higher than SU5416 at same concentration. The branching pattern of blood vessels below disc containing methanol extract was dramatically decreased, large pre-existing vessels was obviously reduced and capillary free area below the disc could be seen (Figure2B).

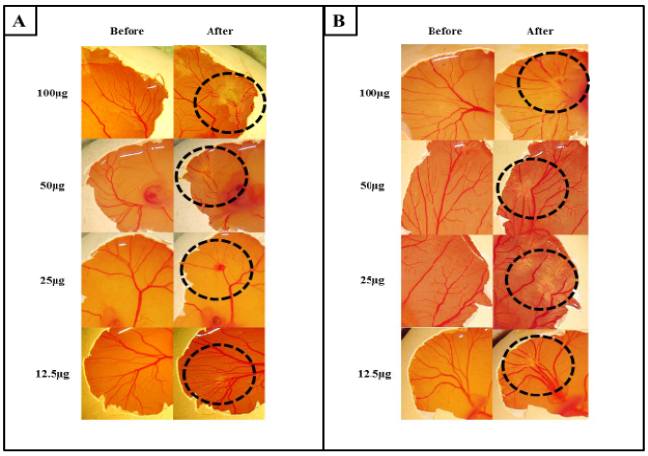

Figure 2: Images of CAM treated with 12.5-100 µg positive control, SU5416 (**A**) and methanol extract of *Gynura segetum* leaf (**B**). Dotted circles identify the anti-angiogenic effect.

Ex vivo rat aortic ring assay

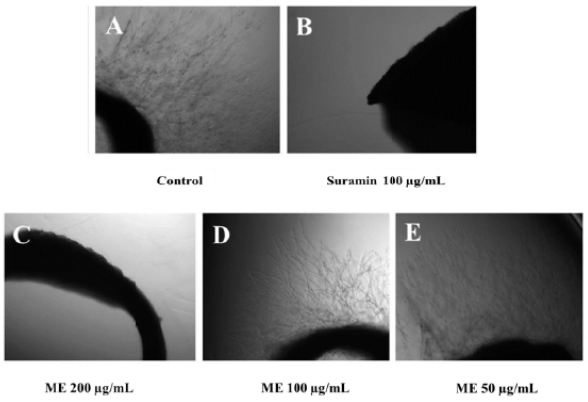

Figure 3: Rat aortic ring assay: The effect of different concentrations 50-200 µg/mL of methanol extract of *Gynura segetum* leaf and suramin on the microvessels outgrowth in rat aorta rings. **A**) Control rings are shown; **B**) suramin (100 µg/mL) as positive control; **C**, **D** and **E**) Rings treated with 200, 100 and 50 µg/mL of methanol extract.

The anti-angiogenic effect of methanol extract of *G. segetum* leaf was investigated using the *ex vivo* rat aortic ring model at three concentrations: 50, 100 and 200 µg/mL. The anti-angiogenic activity was quantified by measuring the blood vessels sprouting area around the aortas exposed to the various concentrations of methanol extract and the positive control (suramin). The results demonstrated that there was a dose-dependent inhibition of microvessels outgrowth. The methanol extract exhibited significant (p<0.01) inhibitory effects at 200 µg/mL and the percentage inhibition was more than 95%. The IC_{50} of methanol extract was calculated from dose response curve and found to be 104.11 µg/mL. At 100 µg/mL, suramin showed almost 100% inhibitions on the microvessels outgrowth. Methanol extract (200 µg/mL) did not show any significant difference as compared to suramin (100 µg/mL) (p>0.05). However, the inhibitory effect of methanol extract at 100 µg/mL was drastically dropped became less than 50%. Figure3A shows the prominent growth of vasculature in the control (untreated

group). Figure3B-E show the inhibition of the microvessels outgrowth in rat aorta rings by suramin and methanol extracts (50, 100 and 200 µg/mL).

Cytotoxicity

The cytotoxicity of standardized methanol extract of *G. segetum*'s leaf was determined by MTT cell proliferation assay and 5-fluorouracil was used as a positive control. Human colon carcinoma cell line, HCT116 and human colon fibroblast cell line, CCD-18Co (normal cells) were exposed to different concentration of methanol extracts. The median inhibitory concentration (IC_{50}) value for 5-fluorouracil were found to 6.89 µg/mL and 20.24 µg/mL in HCT116 cell and CCD-18Co cell, respectively.Results showed that only high concentration of methanol extract exhibited very low cytotoxicity against HCT 116 colon cancer cells (Table 2).

Concentration (µg/mL)	Inhibition (%)
400	28.58
200	21.27
100	15.52
50	11.00
25	7.80
12.5	0.90
6.25	-3.33

Table 2-Percentage inhibition of different concentration of methanol extract of *Gynura segetum* leaf on HCT 116 colon cancer cells

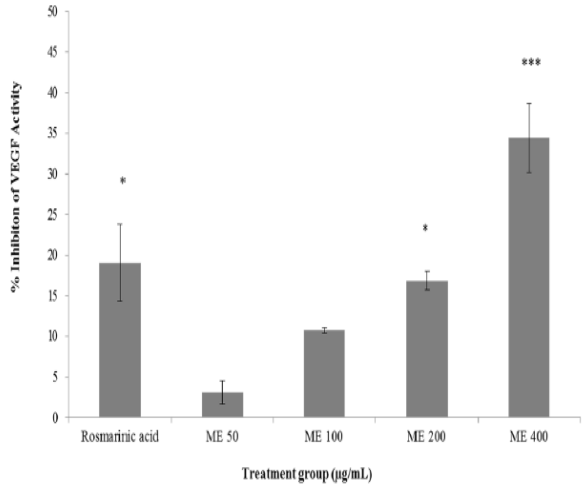

Figure 4 - Inhibitions on VEGF activity by different concentrations of methanol extract of *Gynura segetum*'s leaf and Rosmarinic acid (100 µg/mL). ME 400, ME 200, ME 100 and ME 50 indicate methanol extract in concentration of 400, 200, 100 and 50 ug/mL. Values are the mean ± SD (n=6); *** p<0.001 and * p<0.05 are significant differences as compared with the control group.

The median inhibitory concentration (IC_{50}) was calculated from dose response curve and the IC_{50} of methanol extract was no cytotoxic as the IC_{50} value was found to be 8.09 mg/mL (Figure 4). It is also been noted that methanol extract show no cytotoxic activity against the normal cell line tested.

Determination of VEGF concentration

The inhibitory effect of the extracts on VEGF activity in HCT116 cells was determined by quantifying VEGF 165 concentration in cell lysate. The results show that methanol extract of *G. segetum's* leaf treatment dose-dependently suppressed VEGF activity in HCT116 cells (Figure 5).Rosmarinic acid, which was used as a positive control showed 19.08 ± 4.73% inhibition on VEGF activity at 100 µg/mL. Methanol extract at 400 ug/mL showed highly significant inhibition on VEGF activity in HCT116 cells with 34.40 ± 4.27% (p<0.001) as compared with untreated cells. The VEGF concentration in cell lysates in cells treated with methanol extract at 200 µg/mL (2738.5 ± 38.2 pg/mL) was significantly (p<0.05) lower than untreated cells (3294.25 ± 21.57 pg/mL).

Discussion

Standardization of herbal medicines means confirmation of its identity and determination of its quality and purity[13]. Phytochemical standardization comprises all the possible information with regard to the chemical constituents present in herbal medicines. The phytochemical evaluations include preliminary screening for the presence of different chemical groups (e.g. alkaloids, flavonoids, saponins, triterpenic acids, tannins and etc.), quantification of chemical of interest and establishment of fingerprint profiles based upon single or multiple markers[14].

According to literature, seneciphyllineand senecionine are the main constituents of *G. segetum*'s plant[15; 16]. Therefore, seneciphylline and senecionine were selected as chemotaxonomic markers of *G. segetum*'s plant. The fingerprint profiles and quantification of the selected marker compounds (seneciphyllineand senecionine) were carried out in the present study by using HPLC. All analytical methods that are intended to be used for analyzing any chemical constituents in plant material will need to be validated [17]. In the present study, an improved and validated HPLC method for the quantification of alkaloids seneciphylline and senecionine in the *G. segetum*'s leaves standardized extract has been successfully developed. The developed method is rapid, selective, precise, accurate and with high sensitivity as compared to the reported method [15]. The results also provided reference information to ascertain the identity and to determine the quality, safety and reproducibility efficacy of *G. segetum*'s leaves.

The development of specific anti-angiogenic agents arises as an attractive therapeutic approach to treat cancer and other angiogenesis-dependent diseases [18]. Traditional herbal medicines have long been recognized as a rich source for discovering such agents. The selected and careful use of plant extracts may help in anti-angiogenic

therapy and thus, in cancer treatment [19]. In this study, we focus on the inhibition of angiogenesis and the possible underlying mechanism of action of the methanol extract of *G. segetum* leaves. Methanol extract of *G. segetum*'s leaf exhibited considerable potency in anti-angiogenic activity in both *in vivo* CAM assay and *ex vivo* rat aortic ring angiogenesis models. The inhibitory doses of methanol extract of *G. segetum* leaves was much lower than positive control SU5416 (Semaxanib) in CAM assay and suramin in rat aoric ring assay.As an angiogenesis inhibitor, SU5416 and suramin have been demonstrated to inhibit the VEGF-dependent endothelial cell proliferation activity and the activity exerted by fibroblast growth factors (FGFs) [20; 21; 22]. The *in vivo* CAM assay and *ex vivo* rat aortic ring assay confirmed the methanol extracts inhibit angiogenesis and act as a anti-angiogenic agent. However, the inhibitory effect can be due to nonselective cytotoxic or interference with the angiogenesis process[23]. Thus, the cytotoxicity of methanol extract of *G. segetum*'s leaf was determined by MTT assay. According to Lee and Houghton [24], a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value in carcinoma cells is less than 20 µg/mL, while it is less than 4 µg/mL for pure compounds. The present study showed that the IC_{50} value of methanol extract was found to be 8.09 mg/mL, indicated that methanol extract is noncytotoxic as the value are far below 20 µg/mL. Taken together, the results of the anti-angiogenic assay (CAM and rat aortic ring) and the MTT assay conclude that the anti-angiogenic effect of methanol extract is not due to non selective cytotoxicity, but interference with angiogenesis process. Therefore, the mechanism action of the anti-angiogenic activity of the methanol extract was determined.

Vascular endothelial growth factor (VEGF) is a potent proangiogenic agent that regulates all the key steps of the angiogenic process, including endothelial cell proliferation and migration[25]. Suppression of VEGF activity is therefore considered as a potential targets in treatment of angiogenesis dependent diseases. In the present study, the result showed that VEGF activity has been significantly reduced in methanol extract at 400 ug/mL (p<0.001), proposing that suppression of VEGF activity as one of the mechanisms of action involved in inhibiting the angiogenesis cascade.

Inhibition of angiogenesis has been considered to be beneficial for the treatment and prevention of chronic inflammation. Both inflammation and angiogenesis are exacerbated by increased production of chemokines/cytokines, growth factors, proteolytic enzymes, proteoglycans, lipid mediators and prostaglandins [26]. The hypothesis is that angiogenesis and chronic inflammation are codependent process, so inhibitors of angiogenesis are predicted to inhibit the chronic inflammatory responses. In our previous studies, methanol extract of *G. segetum* exhibited great anti-inflammatory effects (both *in vivo* and *in vitro*) which have proven that angiogenesis and inflammation are codependent process.

In conclusion, the results suggest that methanol extract of *G. segetum*'s leaf could be a potential anti-angiogenic agent that targets the VEGF signaling pathway and could have the prevention against cancer due to its anti-angiogenic effects. Further studies of the active constituents of the methanol extract of *G. segetum*'s leaf will be necessary in order to establish if they could be safely used as effective natural remedies.

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Conflict of interest

The authors have no conflicts of interest to declare.

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