Inhibitory effect of *Labisia pumila* leaf extract on angiogenesis via down-regulation of vascular endothelial growth factor

Nozlena Abdul Samad1,2*, Muhammad Asif1, Yasser M Tabana1,3, Mohamed B Khadeer Ahmed1, Aman Shah Abdul Majid2, Amin Malik Shah Abdul Majid1

1EMAN Testing and Research Laboratorys, Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11900 Penang, 2Integrative Medicine Cluster, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, Bertam, Penang 13200, Malaysia, 3Faculty of Pharmaceutical Sciences, Government College University Faisalabad, 38000, Pakistan

*For correspondence: Email: nozlena@usm.my; aminmalikshah@gmail.com

Sent for review: 9 November 2017 Revised accepted: 11 May 2018

**Abstract**

**Purpose**: To investigate the anti-angiogenic activity of a methanol leaf extract of *Labisia pumila* (ME), and its bioactive water fraction (WF), using in vitro models.

**Methods**: The antioxidant activity and total phenolic contents of ME and WF were assessed using DPPH and Folin-Ciocalteu reagents. Antiproliferative effects of extracts towards human umbilical vein endothelial cells (HUVECs) were evaluated using MTT assay. Isolated rat aortic ring and matrigel tube formation assays were performed to assess the antiangiogenic potential of ME and its WF. Levels of VEGF protein in the cell lysates were measured using ELISA kit.

**Results**: Among all the extracts prepared, ME and its WF showed higher total phenolic contents and exhibited moderate antioxidant effects. Significant (p < 0.001) suppression of microvessels outgrowth with half-maximal concentration (IC50) values of 20 and 26 μg/mL for ME and WF, was observed in rat aortic ring assay. ME and its WF halted proliferation and tube formation capacity of HUVECs in vitro assays. Marked reduction in VEGF levels was observed in lysates of HUVECs treated with ME and its WF.

**Conclusion**: *Labisia pumila* leaf extract and its water fraction halted angiogenesis by blocking VEGF secretion leading to inhibition of endothelial cells proliferation and differentiation which is suggested to be due to its phenolic antioxidant contents.

**Keywords**: *Labisia pumila*, Anti-angiogenesis, Antioxidant, Tube formation, Rat aorta

---

**INTRODUCTION**

Angiogenesis, the growth of new blood vessels from available vascular network, is a critical process for the of many human diseases, including cancer [1]. Judah Folkman is credited as being the first to propose that angiogenesis is essential for tumorigenesis and allows tumors to spread from one organ to another; and that suppressing the process can be an effective
approach to stop progression of disease [2]. Angiogenesis provides a continuous supply of nutrients and oxygen to cancer cells, without which tumors cannot grow beyond 1 to 2 mm³, and may become necrotic or even apoptotic [3]. Targeting VEGF is believed to inhibit cancer growth as it controls the major steps involved in tumor angiogenesis [4,5]. Reports have shown that vascular cells under different pathological conditions generate reactive oxygen species (ROS), which can have a negative or a positive impact on angiogenesis depending upon their levels in a living system. High ROS levels can cause oxidative stress and cell death. On the other hand, in negligible concentrations ROS can enhance angiogenesis by facilitating multiplication and migration of endothelial cells [6].

Phytochemicals have drawn much attention as potential therapeutic agents against cancer due to their relatively low toxicity and worldwide acceptability [7,8]. By virtue of their antioxidant and free radical scavenging activity, polyphenols have received much interest from researchers seeking to develop anticancer, antiangiogenic and cardiovascular-disease medications [9].

*Labisia pumila* (Primulaceae) is a flowering plant native to Malaysia, where it is commonly known as Kacip Fatimah. It is one of five herbs whose commercialization was declared by the Malaysian government to be of national interest. Previous reports have demonstrated Kacip Fatimah’s antioxidant potential [10,11] and antiangiogenic effects [12]. However, an extensive review of literature reveals lack of bioactivity guided analysis of antiangiogenic activity of its leaf extracts.

Therefore, the present work was undertaken to investigate effect of *Labisia pumila* leaf extracts towards different stages of angiogenesis using *in vitro* and *ex vivo* models. Moreover, active extract and its most potent fraction was chemically characterized using different techniques.

**EXPERIMENTAL**

**Collection of plant material**

*Labisia pumila* leaves were procured from a local herbal supplier in July 2016. The plant was authenticated by a taxonomist at the School of Biological Sciences, Universiti Sains Malaysia (USM), Pulau Pinang, Malaysia. A voucher sample (no. 11007) was deposited at the herbarium of the same school for future reference.

**Extraction and bioactivity-guided fractionation**

The leaves were washed with water to remove traces of dirt and foreign matter. They were dried at 40 ºC overnight and ground into coarse powder. Then, approximately 500 g of ground leaves was successively extracted with solvents of varying polarity to produce the following extracts: petroleum ether (PE), chloroform (CE), methanol (ME) and water (WE). All the extracts were filtered; and the resulting filtrates were concentrated *in vacuo* (−760 mmHg) by using a rotary evaporator (Heidolph Laborota 4000-efficient, Germany). The extracts obtained were thick semisolid masses, which were kept in air tight containers and stored at -20 ºC until further experimentation. The extracts were screened to detect those with antiangiogenic activity; and the most active extract was fractionated using solvents of varying polarity to produce three fractions: n-hexane (n-Hex), ethyl acetate (EA) and water fraction (WF) [13].

**Characterization of active extract and its fraction**

The most active extract (ME) and its bioactive fraction (WF) were chemically characterized using following identification techniques: Fourier Transform Infrared Spectroscopy (FTIR), Gas Chromatography/Mass Spectrometry (GC/MS) and Time-of-Flight Liquid Chromatography/Mass Spectrometry (TOF LC/MS). TOF LC/MS analyses of the most active extract and its bioactive fraction were performed using Agilent 6220 Accurate-Mass TOF System. Generated data was analyzed using the Agilent Mass Hunter Qualitative Software Package (Agilent Technologies); and further searches were conducted using the METLIN Personalized Database (Agilent Technologies). The search aimed to match the actual mass and molecular formula observed in the TOF LC/MS analysis with theoretical values stored in the METLIN database.

**Evaluation of DPPH scavenging activity**

In a 96-well plate, 100 µL/well was added of each test sample, along with a DPPH (200 µmol/L) methanol solution. The plate was incubated for 30 min at 22 ± 2 ºC in the absence of light. DPPH absorbance values were measured at a wavelength of 517 nm and half maximal inhibitory concentration (IC50), defined as concentration needed of a test substance to cause a 50% reduction of DPPH radicals was calculated. Ascorbic acid was used as a reference standard.
Determination of total phenolic content

In an eppendorf tube, 10 µL Folin–Ciocalteu reagent was mixed with 30 µL Na₂CO₃ solution and 2 µL standard/test sample (extract/fraction). Distilled water was added to complete the total volume to 200 µL. After thorough mixing, the tubes were allowed to stand for 2 h at 22 ± 2 °C in the absence of light. Absorbance values were measured at a 765-nm wavelength in triplicate. All values were reported as the micrograms of gallic acid equivalent to the dry extract/fraction in milligrams.

In vitro anti-angiogenic activity

Ex-vivo rat aortic ring assay

Thoracic aortae were harvested from male Sprague Dawley rats (10–12 weeks old) and cut into 1-mm thick rings. The rings were placed into the well of a 48-well plate. Three hundred microliters of M199 media containing fibrinogen (3 mg/mL), aprotinin (5 µg/mL) and 1% L-glutamine; were added per well. Next, 10 µL of thrombin (50 NIH U/mL in 0.15 M NaCl) was added to each well. The plate was kept in a humidified incubator for 30 min at 37°C. Different solutions were prepared for each test sample in a second layer of the M199 media containing 1% L-glutamine, fungizone (2.5 µg/mL), gentamycin (60 µg/mL), 0.1% 6-aminocaproic acid and 20% heat inactivated foetal bovine serum. The solutions had different concentrations; and 300 µL of each was added per well. Suramin and 1% DMSO were added into the media to respectively serve as the positive and negative controls. Then, the plate was returned to the incubator and kept there for 72 h.

Afterwards, the upper layer was removed and a fresh layer was added, with the same quantities of extract/fraction, suramin and DMSO. Lastly, after 48 h of incubation, the aorta rings were examined under an inverted Olympus Light Microscope at 4× magnification. The growth of blood vessels was quantified by using the LeicaQwin Computerized Imaging Software. The experiment was repeated three times; and the inhibition (V) of vascularization was estimated based on the using Eq 1 [5].

\[
V(\%) = \frac{1 - (D_S/D_C)}{100} \text{ .................. (1)}
\]

where \(D_S\) = distance of growth for the sample in µm and \(D_C\) = distance of growth in the control in µm. The experiment was conducted according to the international guidelines [14,15].

Experimental animals

Healthy adult female Sprague Dawley (SD) rats (225–250 g) were used in the rat aortic ring assay. 8–10 week old healthy male SD rats (200–230 g). The rats were obtained from the Animal House Facility, Universiti Sains Malaysia. All experiments were performed according to international guidelines on animal experimentation. All efforts were made to minimize the suffering and the number of animals used [16-18]. The study protocol was approved by the Animal Ethics Committee, USM [approval reference number: 2015/ (97) (713)].

Determination of endothelial cell proliferation

The anti-proliferative effects of L. pumila active extract and its most active fraction; were evaluated in an MTT assay using human umbilical vein endothelial cells (HUVECs) as previously described [18]. Briefly, HUVECs were placed in a 96-well plate with a population average of 1 \( \times \) 10⁵ cells per well. The plate was then kept in a humidified CO₂ incubator for 24 h. The cells were pre-treated with different concentrations of the extract/fraction for 48 h before 20 µL of MTT ((3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide) were added to each well. The plate was returned to the incubator and kept for 4 h. Then, the used media was discarded from the wells before adding 100 µL of a DMSO solution. Lastly, absorbance values were obtained at a 570-nm wavelength. A reading at the wavelength of 620 nm was taken to serve as a reference. Measurements were performed in triplicate. All values were reported as the mean ± standard deviation (SD). The percentage of cell growth inhibition was estimated using the following equation Eq 2:

\[
\% \text{ inhibition} = \frac{1 - (A_S - A_B)/(A_V - A_B))}{100} \text{ ...... (2)}
\]

where \(A_S\) = the absorbance of sample, \(A_B\) = the absorbance of blank and \(A_V\) = the absorbance of vehicle.

Tube formation assay

The extract/fraction’s ability to prevent HUVECs from forming tube-like structures; was investigated using a well-established protocol in our laboratory [19,20]. In short, 150 µL of diluted matrigel was added to each well of a 48-well plate. To allow for polymerization to occur, the plate was placed on ice for 45 min. Next, pre-treated HUVECs were harvested and seeded onto the matrigel-coated plate. Media containing 1 % DMSO and suramin (10 µg/mL) respectively
served as the negative and positive controls. The plate was then kept in a CO\textsubscript{2} incubator for 6 h 37 \textdegree C. Lastly, the cells were visualized using an inverted microscope at 4\times magnification. Network length was quantified using the ImageJ software. The results were reported as the means ± SD of triplicate estimations of the percentage of inhibition.

Assessment of VEGF expression in HUVECs

A human VEGF-165 ELISA kit was used to estimate VEGF levels in HUVEC lysates. The assay was performed as per the instructions of the manufacturer (Raybio, USA). Briefly, HUVECs were treated for 24 h with different serial concentrations of the extract/fraction, ranging from 6.25µg/mL to100 µg/mL. They were then lysed by applying the corresponding buffer accompanying the kit. Thereafter, VEGF levels were estimated in the lysates through log regression using the standard calibration curve. The assay was performed in triplicate [19].

Statistical analysis

Statistical analysis was carried out using version 19.0 of the Statistical Package for the Social Sciences (SPSS) program. Comparisons were performed between treatment groups with the nonparametric Kruskall-Wallis test. Linear regression and logarithmic equations were used to evaluate IC\textsubscript{50} values. All data was presented as the mean ± SD; and differences were considered significant at \( p < 0.05 \).

RESULTS

Characteristics of \textit{Labisia pumila} crude extract and its active fraction

ME was shown to be the most active extract of \textit{L. pumila} in terms of its potential to suppress angiogenesis. Its water fraction (WF) was later found to be the most active antiangiogenic fraction. Hence, both ME and WF were chemically characterized using FTIR, GC/MS, and TOF LC/MS techniques.

FTIR spectra revealed C-H stretching of an alkyl group at 2924.03 and 2925.94 cm\textsuperscript{-1} in both ME and WF, respectively. Moreover, indicating the presence of hydroxyl groups, both ME and WF exhibited O-H stretching patterns at 3422.76 and 3423.07 cm\textsuperscript{-1}, respectively. Accompanied by signs of C-O stretching, alcohol peaks were shown in both the extract and the fraction at 1072.39 cm\textsuperscript{-1} and 1052.64 cm\textsuperscript{-1}, respectively.

GC/MS analysis identified total of 13 volatile compounds in ME and WF collectively. As shown in Table 1, only the compounds with a similarity index of 80% or above were reported in this article. When searching against the METLIN database, two interesting major compounds were found to be present in both ME and WF: 4- phtalimidoglutaramic acid, and vitamin-D3. Other metabolites were also identified.

Table 1: GC/MS analysis of \textit{L. pumila} leaf methanol extract and its water fraction

<table>
<thead>
<tr>
<th>Extract/fraction</th>
<th>Compound</th>
<th>Quality</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME</td>
<td>Pentadecanoic acid 14 methyl, methyl ester</td>
<td>98</td>
</tr>
<tr>
<td>ME</td>
<td>N-Hexadecanoic acid</td>
<td>98</td>
</tr>
<tr>
<td>ME</td>
<td>9-Octadecanoic acid (Z) methyl ester</td>
<td>99</td>
</tr>
<tr>
<td>ME</td>
<td>7-Hexadecanoic acid, methyl ester, (Z)</td>
<td>87</td>
</tr>
<tr>
<td>ME</td>
<td>Hexadecanoic acid,2-hydroxy-1 (hydroxymethyl) ethyl ester</td>
<td>81</td>
</tr>
<tr>
<td>ME</td>
<td>1,2-Benzenedicarboxylic acid, discotyl ester</td>
<td>90</td>
</tr>
<tr>
<td>WF</td>
<td>Pentadecanoic acid 14 methyl, methyl ester</td>
<td>98</td>
</tr>
<tr>
<td>WF</td>
<td>N-Hexadecanoic acid</td>
<td>97</td>
</tr>
<tr>
<td>WF</td>
<td>Oleyl alcohol</td>
<td>86</td>
</tr>
<tr>
<td>WF</td>
<td>9,12, 15-Octadecanoic acid, methyl ester (Z, Z, Z)</td>
<td>99</td>
</tr>
<tr>
<td>WF</td>
<td>Phytol</td>
<td>90</td>
</tr>
<tr>
<td>WF</td>
<td>1,2-Benzisothiazole-3-(hexahydro-H-azepin-1-yl)-1,1 dioxide</td>
<td>91</td>
</tr>
<tr>
<td>WF</td>
<td>Hexadecanoic acid,2-hydroxy (hydroxymethyl) ethyl ester</td>
<td>91</td>
</tr>
</tbody>
</table>

ME: \textit{L. pumila} leaf methanol extract. WF: water fraction of ME

DPPH scavenging activity

ME and WF exhibited comparable DPPH scavenging activities as their IC\textsubscript{50} values were shown to be 402.10µg/mL and 406.01 µg/mL, respectively.

Total phenolic content

The total phenolic content of 1 mg of ME was found to be equivalent to 22µg of gallic acid. WF,
however, exhibited a rather decreased phenolic content equivalent to 18 µg of gallic acid only.

**Ex-vivo rat aortic ring**

The antiangiogenic activity of PE, CE, ME and WE was evaluated. ME was shown to inhibit blood vessel outgrowth significantly ($p < 0.001$) as compared with the negative control. It was, thus, considered to be the most active extract. When applied on rat aorta rings at various concentrations, ME was found to have an IC$_{50}$ value of 20.22 µg/mL only (Figure 1).

![Figure 1](image)

**Figure 1:** Antiangiogenic screening of *L. pumila* crude extracts using rat aorta rings. (A) Petroleum ether extract, (B) chloroform extract, (C) methanol extract, (D) water extract, (E) negative control, (F) suramin.

The anti-angiogenic activity of ME fractions (n-Hex, EA and WF) was assessed at the concentration of 100 µg/mL. The water fraction, WF was found to be the most active; and its effects were further evaluated at different concentrations to calculate its IC$_{50}$ value. Compared with the control, WF significantly ($p < 0.001$) inhibited blood vessel outgrowth (IC$_{50}$ = 26 µg/mL) in a dose-dependent manner (Figure 2).

![Figure 2](image)

**Figure 2:** Antiangiogenic effects of WF. (A) suramin, (B) WF, (C) negative control. WF: water fraction of *L. pumila* methanol extract, ME.

**Anti-proliferative effects of ME and WF**

The anti-proliferative effects of ME and WF were investigated in an MTT assay involving the use of HUVECs. ME and WF were shown to effectively inhibit HUVEC proliferation, with their IC$_{50}$ values being 45 ± 1.80µg/mL and 42 ± 4.80 µg/mL, respectively.

**Plate 3:** Inhibitory effects of ME and WF against HUVEC tube formation. (A) ME, (B) WF, (C) negative control. ME: *L. pumila* methanol extract. WF: water fraction of ME.

**Effects of ME and WF on VEGF expression**

ME and WF treatments were shown to cause significant ($p < 0.05$) decreases in VEGF expression inHUVECs, as compared with the control. At IC$_{50}$ concentrations, VEGF levels in ME- and WF-treated cells were 11 ± 0.22 pg/mL and 9 ± 0.07 pg/mL, respectively. The control (untreated cells), however, maintained a VEGF expression level of 21 ± 0.17 pg/mL.

**DISCUSSION**

In the past century, a staggering number of drugs were developed from plant sources. Medicinal herbs contain many bioactive compounds with potential therapeutic effects. In physiological conditions, low concentrations of ROS contribute to protecting the cells from infectious agents, and regulating cell growth. Some ROS may even be involved in intercellular and intracellular signaling, and the production of key biological molecules, like the steroidal hormones. High levels of ROS, on the other hand, can result in the onset of a great many diseases, including cancer.

Antioxidants are chemical compounds that act to preserve the integrity of biological systems. They are characterized by high potency as minute quantities may be sufficient to prevent oxidative-
stress injuries. Many dietary antioxidants with antiangiogenic activity have been shown in the past to be potential chemopreventive agents [22-25]. DPPH radicals are normally used to evaluate the free-radical sequestering capacity of an antioxidant compound. In this work, a DPPH assay was used to assess the antioxidant potential of ME and WF. The findings showed that they exhibited moderate antioxidant activity.

Plant secondary metabolites, especially phenolic and flavonoid phytochemicals, can help maintain homeostasis by influencing a large number of physiological processes, such as the ones accompanied by free-radical production [26]. Compounds of the sort often have anti-mutagenic and anti-carcinogenic properties associated with their potent antioxidant activity [27]. Both ME and WF were shown to have comparable total phenolic contents. This suggested that the anti-angiogenic activity displayed by both was largely due to the presence of some bioactive phenolic phytochemical components [28,29]. Extracts rich with phenolic compounds had previously been shown to exert remarkable antiangiogenic effects [30].

The rat aorta ring assay is widely used as a model to screen antiangiogenic agents because it enables the study of all the key steps of angiogenesis in an environment of multiple cell types. Moreover, unlike pre-selected cells that are not in a proliferative state, endothelial cells within an aortic explant give a true representation of the real events of angiogenesis [31]. Initial screening of four crude extracts of L. pumila leaves (PE, CE, ME and WE) using the ring assay revealed that ME was the best candidate for further mechanistic studies. Bioactivity-guided fractionation of ME resulted in three fractions (n-Hex, EAF and WF), among which WF had the most potent anti-angiogenic activity.

Both ME and WF caused significant inhibition of vascularization. Our findings indicated that the antiangiogenic effects of L. pumila were particularly due to the presence of certain active, polar compounds in its extract. To confirm the observations made with the aortic ring assay and elucidate possible treatment targets, a battery of in vitro tests was performed. Factors with a negative influence on vascularization processes, can be identified using the tube formation assay [32]. This assay offers an invaluable technique to monitor endothelial cells as they develop capillary-like structures on a basal membrane matrix. In this article, both ME and WF were shown to inhibit endothelial cell tube formation in a statistically significant manner ($p < 0.05$). Hence, the results of the assay supported the data obtained earlier in the rat aorta ring assay. Interestingly, suramin, the standard drug, was shown to cause a 95%± 6.00% inhibition of tube formation.

VEGF, an essential cytokine for the angiogenesis cascade, plays central roles in the propagation, relocation and persistence of endothelial cells [33-35]. In the present work, both ME, L. pumila most active extract, and its aqueous fraction, WF were shown to significantly ($p<0.05$) decrease VEGF expression. Considering that VEGF overexpression can lead to metastasis and enhanced tumorigenesis, our findings indicated that VEGF down-regulation was one of the mechanisms underlying the anti-angiogenic activity of L. pumila. Further research is warranted to investigate the possibility of utilizing our extracts as novel sources of VEGF inhibitory agents.

**CONCLUSION**

L. pumila shows significant antioxidant properties and bioactive phenolic phytochemicals. The methanol extract demonstrated good anti-angiogenic activity. As hypothesized, the tests revealed that both the extract and its fraction exerted inhibitory effects against VEGF expression—a hitherto unknown mechanism by which L. pumila suppressed angiogenesis. Overall, L. pumila is potentially a good source of suitable agents or adjuncts to existing therapeutics used in the treatment of cancer, psoriasis, rheumatoid arthritis and diabetic retinopathy.

**DECLARATIONS**

**Acknowledgement**

Muhammad Asif would like to acknowledge having received the USM Fellowship award from the Institute of Postgraduate Studies at Universiti Sains Malaysia. The authors acknowledge financial support received from Universiti Sains Malaysia under the following grant: (RUT 1001/PFARMASI/851001). The kind efforts of Mr. Shanmugan A/P Vellosamy of the School of Biological Sciences, USM, in identifying the plant were highly appreciated.

**Conflict of interest**

The authors declare that no conflict of interest is associated with this study.

_Trop J Pharm Res, June 2018; 17(6): 1092_
Contribution of authors

We declare that this work was done by the authors named in this study; and all liabilities pertaining to claims related to the content of this article shall be borne by the authors. Noziena Abdul Samad and Muhammad Asif contributed equally in this work.

REFERENCES

28. Duarte-Almeida JM, Santos RJ, Genoveses M, Lajolo FM. Avaliação da atividade antioxidante utilizando sistema β-caroteno/ácido linoléico e método de sequestro de
radicais DPPH. Ciênc Tecnol Aliment 2006; 26: 446-452.