Anti-angiogenic activity of Middle East medicinal plants of the Lamiaceae family

QASEM ABDALLAH[1], IBRAHIM AL-DEEB[2], AMMAR BADER[3], FAYEZ HAMAM[1],
KAMEL SALEH[4] and AMIN ABDULMAJID[2]

[1]Department of Pharmacology and Toxicology, College of Pharmacy, Taif University, Taif, Makkah 21974,
Saudi Arabia; [2]Department of Pharmacology, School of Pharmaceutical Sciences, University Sains Malaysia, Minden,
Penang 11800, Malaysia; [3]Department of Pharmacognosy, College of Pharmacy, Umm Al-Qura University, Makkah 21955;
[4]Department of Biology, Science College, King Khalid University, Abha, Asir 61413, Saudi Arabia

Abstract. Angiogenesis plays a crucial role in malignant tumor progression and development. The present study aimed to identify lead plants with selective anti-angiogenic properties. A total of 26 methanolic extracts obtained from 18 plants growing in Saudi Arabia and Jordan that belong to the Lamiaceae family were screened for their cytotoxic and anti-angiogenic activities using MTT and rat aortic ring assays, respectively. Four novel extracts of *Thymbra capitata* (L.) Cav., *Phlomis viscosa* Poir, *Salvia samuelssonii* Rech.f., and *Premna resinosa* (Hochst.) Schauer were identified for their selective anti-angiogenic effects. These extracts did not exhibit cytotoxic effects on human endothelial cells (EA.hy926) indicating the involvement of indirect anti-angiogenic mechanisms. The active extracts are potential candidates for further phytochemical and mechanistic studies.

Introduction

The cure for cancer remains elusive notably for the majority of the solid malignancies. Various challenges in treating cancers are linked to tumor progression and metastasis (1,2). The growth of solid malignances and their metastasis depends closely on adequate oxygen and nutrient supply, which ensures the formation of new blood vessels (angiogenesis) within the tumor that are vital for its growth (3). Therefore, anti-angiogenic agents may contribute in suppressing cancer growth by preventing nutrient and oxygen supply to the tumor. Plants have long been used as an important source of therapeutic agents against several diseases including cancer (4-8). While several natural products, such as taxanes and *Vinca* alkaloids are widely used in cancer treatment, the use of other plant products is limited to the support of the immune system and/or the increase in the anticancer effects of other anticancer drugs (4). The identification of plant extracts with anti-angiogenic activity and limited toxicity can potentiate the effects of currently used anticancer agents without increasing their side effects.

The targeting of tumor angiogenesis can be mediated via direct and indirect pathways (9,10). Direct anti-angiogenic agents exhibit their effects by inhibiting the growth and migration of endothelial cells. The targeting of endothelial cells within tumors by such direct anti-angiogenic agents may result in unwanted side effects due to their toxicity on endothelial cells found in other tissues. Indirect anti-angiogenic agents inhibit the neovascularization within solid tumors by suppressing the expression of proangiogenic factors, such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF). The mechanism of indirect anti-angiogenic agents may also involve the growth inhibition of mural cells (pericytes) embedded within the vascular basement membrane of blood micro-vessels. This in turn leads to inhibition of the formation, stabilization, remodeling and function of blood vessels (11). Previous findings have shown the direct and indirect mechanisms of anti-angiogenic agents (11-13).

In the present study, we screened 26 methanolic extracts of plants belonging to the Lamiaceae family for their cytotoxic and anti-angiogenic effects. Their mechanism of action with regard to their selective anti-angiogenic properties was further explored by evaluating their ability to inhibit the growth and migration of human endothelial cells.

Materials and methods

Chemicals and materials. Chemicals were purchased from Sigma-Aldrich; Merck KGaA, (Darmstadt, Germany). Cell culture media and supplements were obtained from Gibco; Thermo Fisher Scientific, Inc., (Waltham, MA, USA) and all sterile plastics used in cell culture and experiments were

Correspondence to: Dr Qasem Abdallah, Department of Pharmacology and Toxicology, College of Pharmacy, Taif University, 2657 Airport Road, Taif, Makkah 21974, Saudi Arabia
E-mail: qasem79@yahoo.com

obtained from Corning Incorporated, (Corning, NY, USA) unless specified otherwise.

Cell lines and cell culture conditions. The human colorectal adenocarcinoma cell lines (HT-29, Caco-2 and DLD-1) were a kind gift from Dr A. Aljada (Department of Basic Medical Sciences, College of Medicine, King Saud Bin Abdulaziz University for Health Sciences, Riyadh, KSA). The human endothelial cell line (EA.hy926) was obtained from the American Type Culture Collection (Manassas, VA, USA). The colorectal adenocarcinoma cell lines were routinely maintained in advanced RPMI-1640 medium (Reduced Serum Medium, cat no. 12633-012, Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 4 mM L-glutamine, 4% fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin. EA.hy926 cells were routinely maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 mM sodium pyruvate, 2 mM L-glutamine, 10% FBS and 100 U/ml penicillin-streptomycin. All the cells were propagated in humidified atmosphere at 37°C supplemented with 5% CO_2.

Plant sample collection and extraction. A total of 18 plants belonging to the Lamiaceae family were collected from different locations in Saudi Arabia and Jordan and identified by analytical chemistry techniques. A voucher specimen of each plant was deposited to the Herbarium of Pharmacognosy Laboratory at the College of Pharmacy of the Umm Al-Qura University (Table I). The plants were air-dried in the shade at room temperature and then grinded finely. A total of 2 g of each plant were extracted with 20 ml methanol under sonication for 8 min and kept for 24 h at room temperature prior to filtration. The filtrates were dried in glass petri dishes under ventilation. Finally, the crude extracts were dissolved in Dimethylsulfoxide (DMSO) in order to produce a solution with a final concentration of 200 μg/ml. The extracts were kept at -20°C until further use.

In vitro cytotoxic assay on colorectal cancer cells. HT-29 cells (2,000 cells/well) were seeded in 96-well plates and left to adhere overnight at 37°C in a humidified incubator with 5% CO_2. The cells were then exposed for 96 h to complete cell culture medium containing plant extracts (100 μg/ml). The final concentration of DMSO was 0.05% and each plate also contained blank wells, containing only medium and no cells, and control wells containing untreated cells. After the exposure time course, the MTT colorimetric assay was performed as previously described by Mosmann (14), with minor changes. Thus, the supernatant was replaced with 200 μl/well of cell culture medium containing 0.5 μg/ml MTT and incubated for 4 h. The insoluble formazan was dissolved by 150 μl DMSO per well and the absorbance was measured at 540 nm (Spectramax® plus 384; Molecular Devices, LLC, Sunnyvale, CA, USA). The average absorbance in the control wells corresponded to 100% survival and the cell viability percentage following 100 μg/ml of the plant extract exposure was determined. All the extracts that induced <50% growth inhibition at a concentration of 100 μg/ml, were considered as non-cytotoxic and were selected for the direct anti-angiogenic screening.

Rat aortic ring assay. The procedure was performed as described by Brown *et al* (15), with minor modifications. Female Wistar rats (12-14 weeks old) were obtained from the animal house facility of the College of Pharmacy of Taif University. The rats were housed under standard animal facility conditions and were provided free access to food and water. The rats were maintained in a 12-h light/dark cycle at 25±2°C with relative humidity of 50-60%. The *in vivo* protocol was in accordance with the animal welfare guidelines adopted by the Taif University and was approved by the Medical Ethics Committee of the Department of Pharmacology and Toxicology of the College of Pharmacy of Taif University. The rats were humanely euthanized by neck dislocation under anesthesia with ketamine/xylazine that was administered IP (70 μg/kg ketamine and 10 μg/ml xylazine). The thoracic aorta was harvested, cleaned from fibro-adipose tissue and residual blood clots, and placed into M199 medium in order to maintain its integrity. The thoracic aorta was cut into rings of 1 mm in length and each ring/well was explanted into a 48-well plate preloaded with M199 medium (300 μl/well) containing fibrinogen (3 μg/ml) and aprotinin (5 μg/ml). In order to form the lower layer, 10 μl of thrombin (50 U/ml in normal saline: bovine serum albumin) was added and the layer was left to solidify for 1.5 h at 37°C in a 5% CO_2 supplemented humidified incubator. The upper layer M199 media (300 μl/well) consisted of 20% FBS, 2 mM L-glutamine, 0.1% (w/v) aminocaproic acid and 1% penicillin streptomycin and amphotericin B antibiotics (Lonza Group, Ltd., Basel, Switzerland). The plant extracts were added to the top layer at a concentration of 100 μg/ml, while suramine 100 μg/ml was used as a positive control. The concentration range of 50-25 μg/ml was used only for those extracts that inhibited the formation of the new vascularization at 100 μg/ml by 50% or more compared with the untreated control. The treatment of the extracts and the control samples was held in independent duplicates. Subsequently, the plates were restored into a humidified incubator (37°C supplemented with 5% CO_2) for seven days. On day four, the media of the upper layer were changed with M199 media containing similar treatments and concentrations. After the completion of the treatment course, the formation of micro-vessels was evaluated using phase contrast microscopy following the method developed by Nicosia *et al* (16). The measurement of the distance of the micro-vessel outgrowth was analyzed using the ImageJ 1.51n® software (National Institute of Health, Bethesda, MA, USA).

The percentages of inhibition of micro-vessel formation were compared with non-treated control samples and were presented as mean ± standard deviation (SD). All the extracts that showed 50% or higher (≥50%) inhibition of micro-vessel formation at 100 μg/ml, were considered potentially active and were selected for further evaluation.

Scratch assay of endothelial EA.hy926 cells. This assay was used to evaluate the effectiveness of selected plant extracts to prevent the movement and growth of endothelial cells following induction of a scratch. The scratch was performed manually on a monolayer of cells as described by Liang *et al* (17). Subsequently, EA.hy926 cells (4x10⁵ cells) were seeded into a 6-well plate. The cells were incubated in a 5% CO_2 supplemented humidified incubator at 37°C for 48 h until they reached

90% confluency and/or higher. A straight line in the middle of the monolayer was scratched using a sterile 200 μl-pipette tip. The supernatant was discarded, and the monolayer was rinsed with PBS twice before the addition of extracts at concentrations of 100, 50 or 25 μg/ml in DMEM (2 ml). The control cells were treated with DMSO (0.05% in DMEM). A total of five images of different areas were taken for each scratch using an inverted light microscope at 12 and 24 h (Leica Microsystems GmbH, Wetzlar, Germany). The magnification used was x50. The area of the scratch in each image was analyzed using ImageJ 1.51n (National Institute of Health, Bethesda) software. The resulted area of scratched monolayer at each time point was expressed as the percentage of inhibition of migration compared with the mean area of the scratch at 0 h. All experiments were performed in independent triplicates and the results were expressed as mean ± SD.

Growth inhibition assay on EA.hy926 cell line. EA.hy926 cells (1.5x10^3 cells/well) were seeded in a 96-well plate and left to adhere overnight at 37˚C in a humidified incubator with 5% CO$_2$. The cells were exposed to plant extracts at a different concentration range (200-12.5 μg/ml) for 96 h. The final concentration of DMSO was 0.1% or less and each plate also included blank wells, which contained only medium and no cells and control wells, which contained untreated cells. Following the completion of the exposure time course, the MTT colorimetric assay was performed as described above. The average absorbance in the control wells was considered as 100% survival and the IC$_{50}$ values were defined as the extract concentrations that inhibited the cell growth by 50% after 96 h exposure. The results were obtained from three independent experiments and expressed as mean ± SD.

Statistical analysis. All data were analyzed using SPSS v.22.0 (IBM Corp., Armonk, NY, USA). The inhibition assay data were compared using one way ANOVA, and the significant differences were evaluated at a confidence interval of 95% and P<0.05. In addition, the Tukey post hoc statistical test was employed in order to produce pairwise comparisons among treatments.

Results

Cytotoxic activity. In the present study, 26 extracts were obtained from 18 plants of the Lamiaceae family and were evaluated for their antiproliferative activities against HT-29 colorectal cancer cells (Table I). The antiproliferative activity test of the extracts was performed with the aim to exclude those extracts with potential cytotoxic effects. Therefore, extracts which caused 50% growth inhibition or higher at a concentration of 100 μg/ml, were considered potentially cytotoxic. The non-cytotoxic effects of the selected 16 plant extracts were confirmed on two other colorectal cancer cell lines, and finally they were screened for their anti-angiogenic activity on rat aortic rings (Table I).

Antiangiogenetic activity. The extracts that caused higher than and/or equal to 50% inhibition in micro-vessel formation compared with untreated aortic rings were considered potentially antiangiogenic. The inhibition of micro-vessel formation in rat
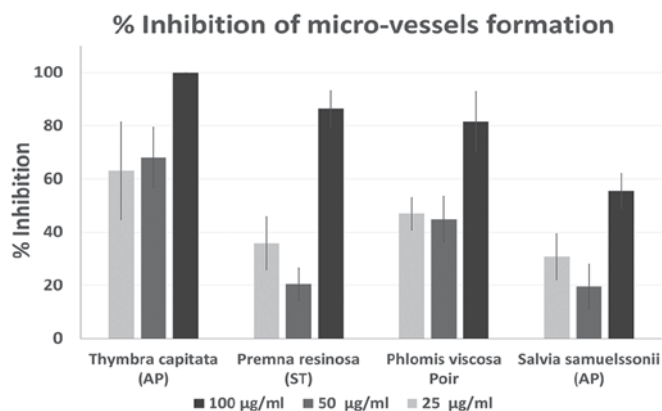

Figure 1. Percentage inhibition of micro-vessel formation in rat aortic ring assay is expressed as mean ± SD following exposure to the extract at 100, 50 and 25 μg/ml. Percentage inhibition of micro-vessel formation in treated groups was calculated in comparison to corresponding untreated (negative) control. Inhibition was statistically significant compared to the control for all extracts at the concentrations tested (P<0.05). No significant differences were detected between percentage of inhibition for 25 and 50 μg/ml doses. AP, arial parts; ST, stems; SD, standard deviation.
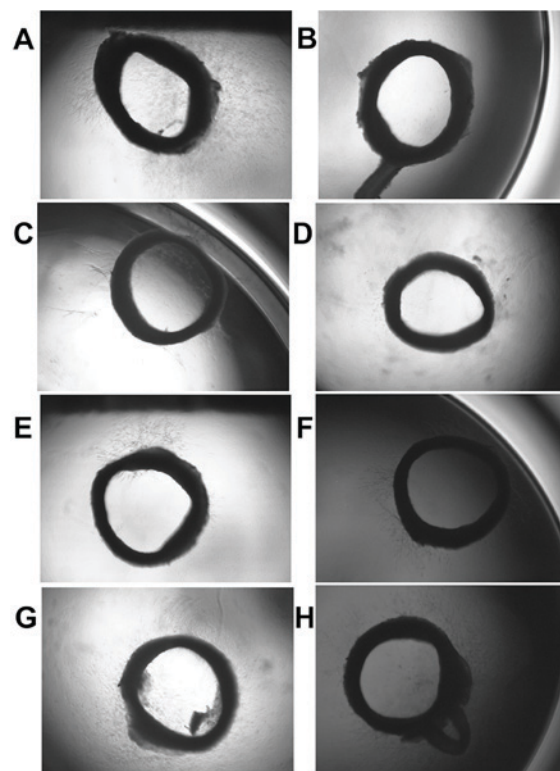

Figure 2. Effect of selected plant extracts on rat aortic ring assay. (A) Negative control (0.05% DMSO), (E) Positive control (100 μg/ml Suramine), (B, C and D) 100, 50 and 25 μg/ml of *Thymbra capitate* (arial parts), (F) 100 μg/ml of *Salvia samuelssonii* (arial parts), (G) 100 μg/ml of *Eremostachys laciniata*, (H) 100 μg/ml of *Moluccella laevis*. DMSO, dimethylsulfoxide.

aortic rings was analyzed at a concentration of 100 μg/ml of extract (Table I). A total of six plants, namely *Teucrium polium*, *Thymbra capitata*, *Phlomis viscosa* Poir, *Salvia samuelssonii*, *Salvia syriaca*, and *Premna resinosa* were proven to have selective anti-angiogenic activity (Table I). These plants indicated no cytotoxic effects on cancer cells, although they significantly

Table I. Methanolic extracts of the plants screened for cytotoxicity in MTT test and anti-angiogenic activity in rat aortic assay.

No.	Plants Name	Part used	Code	Survival following 100 μg/ml exposure ±SD (%)			Inhibition of micro-vessel formation (%)
				HT-29	Caco-2	DID-1	Rat aortic ring
1	*Ballota undulata* (Sieber ex Fresen.) Benth.	AP	UQU-JO-33	94.14±6.92	106.28±6.58	85.47±4.99	42.67±1.78
2	*Eremostachys laciniata* (L.) Bunge	AP	UQU-JO-39	98.55±3.24	115.02±7.09	99.43±1.87	32.09±4.34
3	*Moluccella laevis* L.	AP	UQU-JO-20	93.62±4.64	104.8±1.34	89.31±4.28	2.5±5.12
4	*Moluccella laevis* L.	RO	UQU-JO-20	73.88±2.52	116.89±6.41	78.59±1.81	-78±8.64
5	*Phlomis kurdica* Rech.f.	AP	UQU-JO-27	93.43±8.08	109.95±3.41	96.35±5.16	35.76±8.24
6	*Phlomis syriaca* Boiss.	AP	UQU-JO-69	54.48±0.52	99.30±4.03	72.27±1.87	45.99±30.79
7	*Phlomis viscosa* Poir.	AP	UQU-JO-31	68.35±15.66	75.35±3.47	64.94±5.30	81.46±22.7
8	*Premna resinosa* (Hochst.) Schauer	ST	UQU-SA-109	56.26±2.72	110.10±2.02	82.02±4.97	86.45±16.67
9	*Premna resinosa* (Hochst.) Schauer	LE	UQU-SA-109	22.90±1.53	ND	ND	ND
10	*Salvia aegyptiaca* L.	AP	UQU-SA-107	15.34±2.79	ND	ND	ND
11	*Salvia ceratophylla* L.	AP	UQU-JO-49	46.32±8.55	ND	ND	ND
12	*Salvia ceratophylla* L.	RO	UQU-JO-49	39.07±14.85	ND	ND	ND
13	*Salvia deserti* Decne.	AP	UQU-SA-112	27.11±3.85	ND	ND	ND
14	*Salvia dominica* L.	AP	UQU-JO-42	9.58±2.78	ND	ND	ND
15	*Salvia hierosolymitana* Boiss	RO	UQU-JO-28	101.57±0.81	98.04±3.06	89.37±3.55	44±6.13
16	*Salvia hierosolymitana* Boiss	AP	UQU-JO-28	98.68±5.24	104.18±3.30	84.56±4.17	-25±14.28
17	*Salvia multicaulis* Vahl.	AP	UQU-JO-47	63.82±7.11	95.64±4.24	88.83±3.81	46.24±5.39
18	*Salvia multicaulis* Vahl.	RO	UQU-JO-47	18.53±4.04	ND	ND	ND
19	*Salvia palaestina* Benth.	AP	UQU-JO-209	82.00±2.79	85.71±2.52	97.80±2.58	-12±5.42
20	*Salvia samuelssonii* Rech.f.	AP	UQU-JO-40	96.48±2.40	97.08±3.10	96.84±1.53	55.37±12.93
21	*Salvia samuelssonii* Rech.f.	RO	UQU-JO-40	13.40±2.26	ND	ND	ND
22	*Salvia syriaca* L.	RO	UQU-JO 48	70.31±7.13	88.89±6.33	71.52±1.95	57.43±10.85
23	*Teucrium polium* L.	AP	UQU-JO-21	52.14±2.80	91.46±5.12	96.01±4.45	94.95±7.14
24	*Teucrium polium* L.	RO	UQU-JO-21	16.52±5.13	ND	ND	ND
25	*Thymbra capitata* (L.) Cav.	AP	UQU-JO 23	86.63±1.14	83.95±2.66	87.93±4.63	100±0.0
26	*Thymbra capitata* (L.) Cav.	RO	UQU-JO 23	10.98±0.25	ND	ND	ND

Viability of HT-29, Caco-2 and DLD-1 cells at 100 μg/ml of exposure is expressed as mean ± SD. The inhibition of the micro-vessel formation at 100 μg/ml is expressed as mean ± SD. Suramine was used at 100 μg/ml as a positive control and the mean percentage of inhibition was 63.39±2.41. SD, standard deviation, ND, not determined; AP, arial parts; RO, roots; ST, stems; LE, leaves; JO, Jordan; SA, Saudi Arabia.
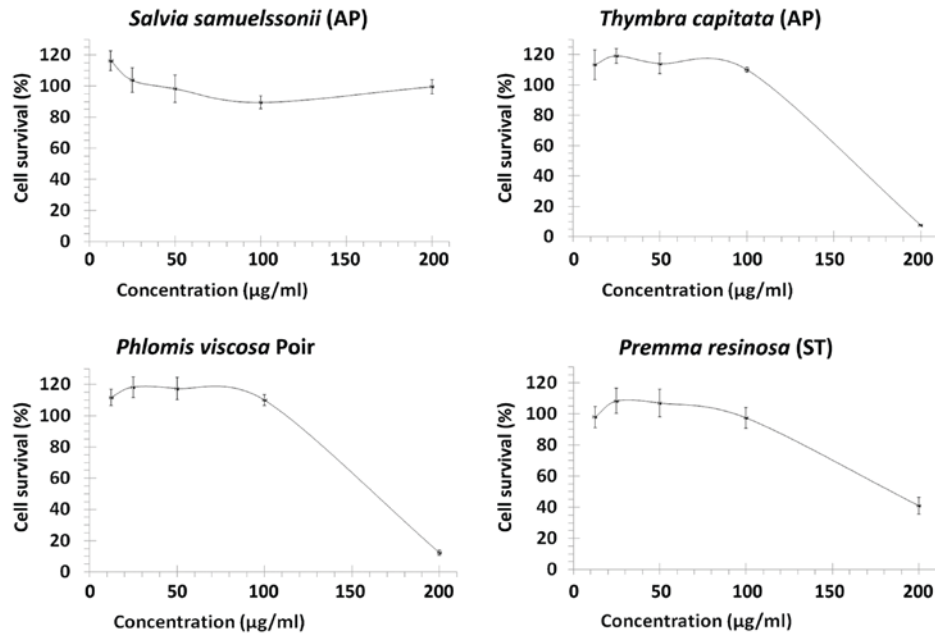
Figure 3. Growth inhibition assay on EA.hy926 cells after 96 h exposure. IC_{50} values for *Thymbra capitata*, *Phlomis viscosa* Poir, *Salvia samuelssonii* and *Premna resinosa* were 158.70±12.12, 161.42±10.91, > 200 and 184.14±8.2 μg/ml respectively. Negative control was 0.1% DMSO. The results are expressed as mean ± SD of three independent experiments. AP, arial parts; ST, stems; DMSO, dimethylsulfoxide; SD, standard deviation.
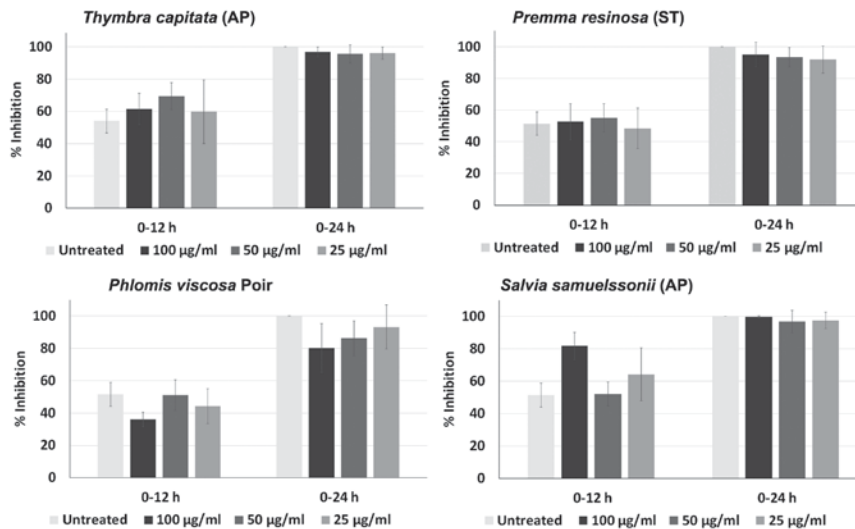

Figure 4. Percentage inhibition of scratch healing in endothelial EA.hy926 monolayer cell culture over 24 h. Results were expressed as mean ± SD of three independent experiments following incubation with the extracts at 100, 50 and 25 μg/ml. AP, arial parts; ST, stems; SD, standard deviation.

inhibited the formation of micro-vessels (Table I). The results of *Teucrium polium* and *Salvia syriaca* were in agreement with those demonstrated previously by Zihlif *et al* (18), with the exception of the *Salvia syriaca*, extract origin, which was derived from the roots, instead of the leaves. The remaining four plants were further investigated for their role in the inhibition of micro-vessel formation at lower concentrations (50 and 25 μg/ml) as shown in Fig. 1.

A significant drop in the percentage of inhibition was noted at lower concentrations, however no significant differences were observed among 50 and 25 μg/ml. Furthermore, the highest efficacy was noted for *Thymbra capitata*. The repre-

sentative images of the effect of the selected plant extracts on rat aortic vessel outgrowth are shown in Fig. 2.

Antiproliferative and anti-migratory activities of plant extracts on human endothelial cells (EA.hy926). The exposure of the endothelial EA.hy926 cells to the four selected plant extracts indicated no significant change in their growth rate (Fig. 3). Similarly, no significant effects were observed with regard to the endothelial proliferation and migration (Fig. 4). The latter results strongly indicated that these extracts did not mediate their anti-angiogenic effects via direct mechanisms of action. Selective anti-angiogenic assays on these crude extracts that
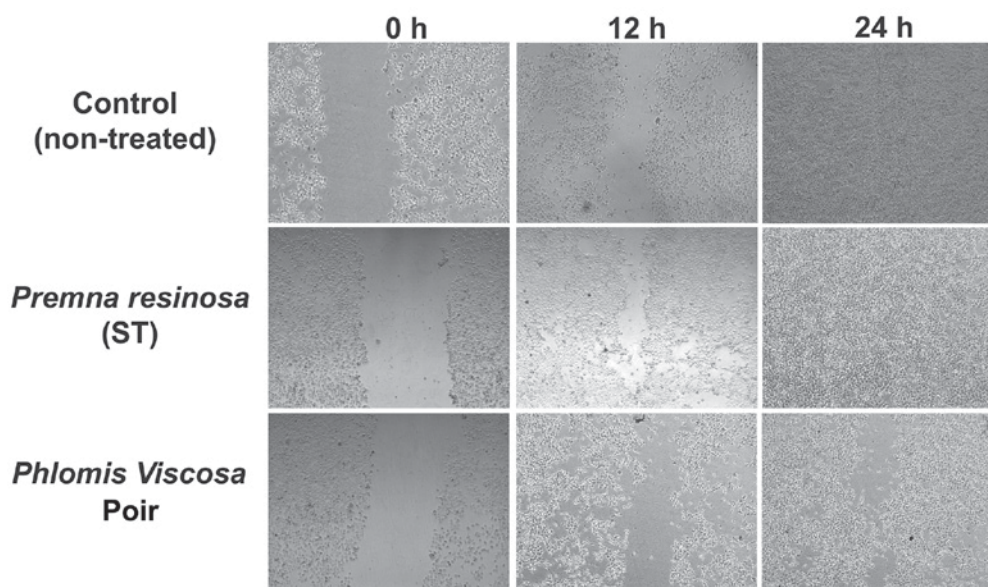
Figure 5. Scratch assay inhibition. A scratch was created and the cells were exposed to 100, 50 and 25 µg/ml of plant methanolic extracts for 24 h. Representative images captured in order to evaluate the effects of *Premna resinosa* and *Phlomis viscosa* Poir extracts on EA.hy926 endothelial cell proliferation and migration at 12 and 24 h using an inverted microscope at magnification, x50. The images were captured at 0, 12, and 24 h. ST, stems.

can be conducted in future studies, can aid the evaluation of their therapeutic effects as complementary medicine and/or the isolation of their pharmacologically active ingredients.

Discussion

Tumor angiogenesis is linked to poor prognosis and metastasis (19). Co-administration of herbal medicines with anti-angiogenic properties as complementary treatment may enhance the therapeutic outcomes of anticancer therapy (20). Various Lamiaceae plants (e.g., *Salvia* and *Phlomis* species) have long been used in folk medicine for the treatment of various diseases including cancer (5,21). For example, the diterpenoids isolated from the Lamiaceae plants demonstrated a wide range of pharmacological activities including antibacterial, antifungal, cytotoxic, cardiovascular and insecticidal activities. *Salvia triloba* (Lamiaceae) has demonstrated direct and indirect anti-angiogenic activities by inhibiting human umbilical vein endothelial cells (HUVECs) proliferation and expression of VEGF (18,22). The AIS-L1023 extract from the leaves of *Melissa officinalis* (Lamiaceae) also inhibited tube formation of HUVECs by lowering the level of VEGF (23).

The micro-vessel formation assay in the aortic ring occurs by the proliferation and migration of endothelial cells, in addition to pericyte recruitment (24). Therefore, inhibition of angiogenesis in the cultured aortic rings could be mediated via direct and indirect mechanisms. The mechanism of action of the anti-angiogenic agents is mediated via direct and indirect pathways (25). The direct pathway involves the growth inhibition of endothelial cells via cytotoxic effects and/or via the inhibition of endothelial VEGF receptors. The indirect anti-angiogenic pathway, however, involves more complicated mechanisms and is active in the tissues surrounding the endothelial cells, thus leading to diminished expression of proangiogenic factors (26).

In conclusion, the present study identified several plants of the Lamiaceae family with potential selective anti-angiogenic activity. Their effects on angiogenesis were not mediated via a direct mechanism of action. Their activity could be attributed partially to the known compound carvacrol, which is the main constituent of the essential oil of *Thymbra capitata* (27). In addition, other constituents such as flavonoids could play a synergistic role in the anti-angiogenic activity of these extracts (28). With regard to the *Salvia* species, the diterpenes that are present in this extract have been reported to have anti-angiogenic effect, such as tanshinone, which is the main constituent of *Salvia miltiorrhiza* (29). *Salvia samuelssonii* could be a good target for further phytochemical studies as it has not been investigated with regard to its anti-angiogenic activity, with the exception of its essential oil, which has been characterized and includes phenylpropanoids, monoterpenes, sesquiterpenes and diterpenes (30). *Phlomis viscosa* is rich in bioactive compounds including oleanolic derivatives, iridoid glycosides, megastigmane, lignan glucosides, neolignan and phenylethanoid glycosides. The latter class of natural compounds includes verbascoside, isoacteoside, forsythoside B, myricoside, and samioside (31,32). To the best of our knowledge, this is the first study that examined *Phlomis* species with regard to its anti-angiogenic effect. *Premna resinosa* is growing in Saudi Arabia and was recently investigated for its cytotoxic and antimicrobial activities (33). A previous study revealed the presence of several flavonoids with considerable bioactivity in this plant species (33). The extracts of *Salvia hierosolymitana* roots, *Phlomis syriaca* aerial parts and *Salvia multicaulis* aerial parts have been reported to have 50% of inhibition of micro-vessel formation. The activity of *Salvia multicaulis* was attributed to the presence of diterpenes and flavonoids (34,35). The organs of the investigated plants have shown considerable differences in their activities, and this is attributed to the chemical diversity in the plant's organs. In some cases both aerial parts

and roots have shown comparable effects, although they are phytochemically different, such as in the case of *Salvia syriaca* (36,37). Further studies are warranted to explore the possible indirect pathway of their anti-angiogenic mechanism and to identify their active constituents.

Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

QA planned, designed and conducted the present study. IA performed the cytotoxicity, rat aortic ring assay, scratch method and antiproliferative experiments. AM collected, identified and extracted the plants. KS and FH contributed in the scratch assay design and statistical analysis. AA contributed in rat aortic assay design, development and data analysis. All authors contributed to the scientific writing and the evaluation of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Medical Ethics Committee at the Department of Pharmacology and Toxicology of the College of Pharmacy of Taif University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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