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Original article

The phytochemical screening and antiangiogenic activity of audthan al himar (*Moricandia sinaica* Boiss.) extracts in zebrafish embryos and human umbilical vein endothelial cellsMuhammad Farooq^{a,*}, Fahad A. Nasr^b, Nawaf D. Almoutiri^a, Nouf Al-yahya^a,
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ABSTRACT

Natural antiangiogenic products interact with multiple molecular angiogenic pathways and possesses comparably less toxicities than chemically synthesized anti-angiogenic compounds. There is a need to screen varieties of medicinal plants to identify more sources of antiangiogenic compounds, as anticancer drugs with less side effects. This study was designed to explore the antiangiogenic property and cytotoxicity of *Moricandia sinaica* (Boiss.) in zebrafish embryos, as well as its anti-migratory potential in human umbilical vein endothelial cells (HUVECs). Gas chromatography coupled with mass spectrophotometry was performed in order to identify bioactive molecules present in the stem and leaves part of *Moricandia sinaica*.

The methanol extract of the stem and leaves inhibited the formation of the inter-segmental, sub-intestinal vein, and dorsal longitudinal anastomotic veins formation in zebrafish embryos in a dose-dependent manner. It did not show cytotoxicity at lower concentration in HUVECs; however, it significantly ($p < 0.001$) obstructed HUVEC migration at sub-lethal concentrations after 24 and 48 h of treatment. More than 40 phytochemicals were identified from the extract using GC-MS.

Our study demonstrates that leaves and stem extract of *Moricandia sinaica*, exhibits a strong antiangiogenic activity and slight developmental toxicity in zebrafish embryos, and no cytotoxicity in HUVECs. The crude extract warrants further research to elucidate the anticancer potential of *M. sinaica*.

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1. Introduction

Herbs or medicinal plants serve as a source of biologically active molecules and have been used as therapeutic remedies in folk medicines for centuries (Atanasov et al., 2015). The herbal components are applied to treat diverse levels of human diseases, ranging from common cold to the complex lethal diseases, such as cancer in human. The significance of natural products can be judged by

the fact that the market for such products, presumably will reach to almost US\$115 billion in 2020 (Bob, 2017).

Normal tissue growth and regeneration benefits by angiogenesis. Angiogenesis is an important biological process involved in blood vessel formation. Its deficiency, as well as excess, can lead to several diseases like ischemic tissue injury, cardiac failure, cancer, diabetic retinopathy, and rheumatoid arthritis (Bisht et al., 2010). The therapeutic intervention using angiogenic inhibitors has shown promising results in solid tumors (Miletic et al., 2009). Recently, several natural products have been successfully applied in preclinical trials to target angiogenesis (Fu et al., 2015; Shanmugam et al., 2017). The advantage of natural antiangiogenic products is that they are more effective due to interactions with multiple angiogenic and apoptotic pathways and possessing less side effects, as compared to chemically synthesized angiogenic molecules which (Yehya et al., 2017).

Target-oriented biological screening of medicinal plants in suitable animal models is required at most. The availability of various

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transgenic lines in zebrafish has revolutionized the biological and high-throughput screenings due to the comparatively smaller size and transparency of embryos (Parng et al., 2002). A transgenic line Tg (fli1:EGFP) is characterized with the integration of a Friend leukemia integration 1 transcription factor (FLI1) together with enhanced green fluorescent protein (EGFP), resulting in ubiquitous EGFP expression in all the blood vessels of the embryo. The time lapse imaging of developing vasculature in these transgenic embryos has revealed sprouting of inter-segmental blood vessels (ISV), sub intestinal vein (SIV), and dorsal longitudinal anastomotic blood vessel (DLAV) from already existing vasculature (Lawson and Weinstein, 2002). Therefore, in zebrafish embryos, ISV, SIV, and DLAV are being considered angiogenic blood vessels. The zebrafish transgenic line Tg (fli1:EGFP) is routinely used as drug screening animal model in order to assess the antiangiogenic activity of chemically-synthesized small molecules or natural products (Tian et al., 2017; Zhang et al., 2018).

The genus *Moricandia* (Brassicaceae) includes eight species. Some species from this genus are used as traditional medicine. One species from this genus (*Moricandia sinaica*) is indigenous to Saudi Arabia, known locally as audthan al himar (due to typical shape of leaves resembling donkey's ear (Fig. 1). *Moricandia sinaica* (Boiss.) is an annual herb, which is distributed throughout Saudi Arabia, having medicinal application in folk medicine (Arif et al., 2010). The biological activities, phytochemical contents, and bio-active compounds of this plant are largely not known. Taking into account, the medical value, and pharmacological properties of the other species in this family, the *Moricandia sinaica* (Boiss.) was explored by extracting the crude extract from the roots, stem, leaves, and shoots and were tested for potential antiangiogenic activity in zebrafish embryos.

The antiangiogenic property of *M. sinaica* was studied in zebrafish embryos and in human umbilical vein endothelial cells (HUVEC) *in vitro*. Cell proliferation assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was performed to determine the cytotoxicity in HUVEC. Gas chromatography spectrophotometry (GC-MS) analysis was conducted to identify the biologically active molecules present in crude extract of *M. sinaica*.

2. Material and methods

2.1. Collection of plant

The plants were collected in flowering season from the outskirts of Riyadh district, Kingdom of Saudi Arabia. Jacob Thomas

Pandalayil, Department of Botany and Microbiology, College of Science, King Saud University has identified the plant. The voucher sample (MFQKSU-063/19) was submitted in King Saud University Herbarium.

2.2. Preparation of crude extract

The plants were washed thoroughly with distilled water to remove dirt and dust. The plants were cut into aerial parts and roots, and were dried under shade for several days. Dried roots, stem, leaves and shoots were grounded to powder. The crude extraction of dried parts of *M. sinaica* was obtained by adding the powder (100 g) in a soxhlet apparatus. Extraction was done in solvents of variable polarities, i.e. *n*-hexane (*n*-Hex), chloroform (CHCl₃), ethyl acetate (C₄H₈O₂) and methanol.

2.3. Cytotoxicity testing in human umbilical vein endothelial cell line

To determine the cytotoxic of different plant parts of *M. sinaica* on endothelial cells, human umbilical vein endothelial cells were used and cytotoxicity was analyzed by MTT cell proliferation assay. Human umbilical vein endothelial cells (HUVEC) were used to assess the cytotoxicity by MTT cell proliferation assay. Briefly, 1×10^5 normal cells were plated in 24-well tissue culture plates. After 24 h of incubation at 37 °C in 5% CO₂, the cells were treated with various concentrations of extracts for 48 h. At the end of treatment period, 100 μL MTT (5 mg/mL) was added to each well, and incubated for 4 h at 37 °C in 5% CO₂. One mL of acidified isopropanol (0.01 N HCL) was added to dissolve the formazan crystals by shaking on orbital shaker for 15 min at 25 °C. The absorbance at 490 nm was detected using microplate reader (BioTek, USA).

2.4. Cell migration assay of HUVECs *in vitro*

The anti-migratory effect of the *M. sinaica* methanol leaf extract was assayed using *in vitro* cell migration on HUVECs. Briefly, 1×10^5 cells/mL were seeded in 6-well plates for 24 h. Cells were then washed with PBS and sterile 200 μL tip was used to make a scratch. The cells were treated with 100 μg/mL of extract and cell migration was observed at 0, 24, and 48 h). The images were captured by inverted microscope (Leica, Germany), equipped with MC-170 HD digital camera.



Fig. 1. Aerial view of plant *Moricandia sinaica* (Boiss.).

2.5. Evaluation of anti-angiogenic activity in zebrafish embryos

2.5.1. Animals

Zebrafish transgenic line Tg (fli1:EGFP) was purchased at the zebrafish international resource center (ZIRC, university of Oregon, Oregon USA). The fish are maintained in animal facility at Bioproducts research chair, department of zoology, College of Science, King Saud University Saudi Arabia.) The zebrafish embryos used in this study were less than 5 days post fertilization (dpf), and hence are exempted to take the approval from the institutional committee ethical for the user and care of laboratory animals as stated by (Strahle et al., 2012).

2.5.2. Embryo treatment

The embryos were obtained by pairwise mating of adult fish. The healthy and fertilized eggs were washed with distilled water, and 30 embryos were put in single sterile glass petri dishes of size 60 × 15 mm (NORMAX cat # 505854, Portugal). The serial dilution (10–700 µg/ml) of each crude extract was prepared in 5 ml of embryo medium (distilled water supplemented with 0.04% sea salt and 0.001% methylene blue) and added to each petri dish. The embryos were exposed to the extracts for three days. The extracts were replaced daily with freshly prepared embryo water containing the desired dilution of the extract.

The antiangiogenic potential of the plant was calculated by observing the effect of plants on angiogenesis blood vessels formation in live transgenic zebrafish embryos. The number of angiogenic blood vessels in untreated and treated embryos were counted at two developmental stages one at 48hpf and second at 72hpf. Five to eight embryos were used for each calculation. In order to calculate the blood vessels, the images of the control and treated embryos were taken using fluorescent microscope, and number of blood vessels were calculated using the image J software.

2.6. Gas chromatography-mass spectroscopy

Elmer Clarus 600 gas chromatograph which was linked to a mass spectrometer (Turbomass, PerkinElmer, Inc., Waltham, MA, USA) was used to perform the gas chromatography-mass spectroscopy. The compounds were identified by comparing the spectra with compound libraries at National Institute of Standard and Technology and WILEY.

2.7. Statistics

The IC₅₀ values in case of cytotoxicity and LC₅₀ values for zebrafish toxicity were calculated by probit analysis and also free available online program (<http://www.ic50.tk/>).

2.8. Microscopy

The image were snapped using Zeiss inverted microscope “Zeiss Observer D1 AX10 using ZEN software provided by Zeiss (Germany).

3. Results

3.1. The crude extracts yield in solvents of various polarities

The yield of various crude extracts from roots, stem, leaves, and shoots of *M. sinaica* shown in Fig. 2. Among all the solvents, the methanol crude extracts had maximum yield from all the parts of the plant. The highest yield of methanol crude extract was obtained from the stem part (17.5%), followed by shoot (11.5%),

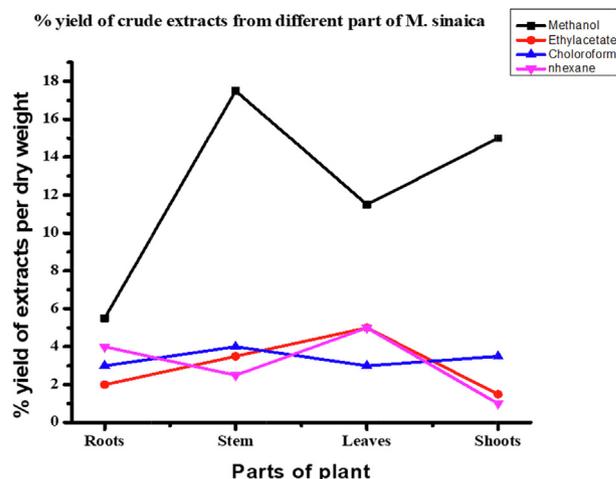


Fig. 2. The graphical representation of the yield of different extract prepared from roots, stem, leaves, and shoots.

and leaves (11.5%). The other solvents produced variable patterns of yield as presented in Fig. 2.

3.2. Evaluation of in vivo developmental toxicity of *M. sinaica* in zebrafish embryos

The crude extracts prepared from the roots, stem, leaves, and shoots did not produce severe level of developmental toxicity in treated zebrafish embryos. As shown in Fig. 3, the embryonic development of embryos that were treated with crude extracts was not affected, and the developmental and phenotype of the treated embryos was almost similar to control embryos at 72 h post fertilization (hpf).

3.3. The crude extract of leaves and stem of *M. sinaica* inhibited angiogenic blood vessels formation in zebrafish embryos on dose-dependent manner

The effective antiangiogenic concentration was determined experimentally by treating the zebrafish Tg (fli1:EGFP) embryos with serial dilution of each of the extract, and an effective antiangiogenic dose was a concentration at which significant level of inhibition of angiogenic blood vessels was observed with minimum level of toxicities in exposed embryos. As shown in Fig. 4, the roots and shoots of *M. sinaica* were completely inactive and did not alter the formation of angiogenic blood vessels in treated embryos. The dose–response effect has shown that at 40 µg/ml, methanol crude extract of leaves inhibited ≥70% of ISV, DLAV and 100% of SIV, while the effective antiangiogenic concentration for methanol crude stem extract was 50.31 µg/ml of *M. sinaica* in zebrafish embryos.

The representative images of Tg (fli1:EGFP) embryos at 72hpf showing the effect of extract of *M. sinaica* treatment on angiogenic blood vessels formation. A & B) The ISV and DLAVE of control embryos are intact and developed normally. The SIV 8–10 blood vessels on yolk region of control embryos is shown in C. The Tg (fli1:EGFP) embryos at 48hpf; treated with crude extract of stem are shown in D and E. The ISV are completely absent at the posterior trunk region shown by asterisk. The posterior trunk region of treated embryos has been shown at higher magnification in E. The severity of the abnormalities in angiogenesis blood vessels in treated embryos has been represented by different number: 1) ISV sprouted from dorsal aorta but did not grow and connect to DLAV. 2) ISV sprouted from dorsal aorta and has grown to middle of trunk

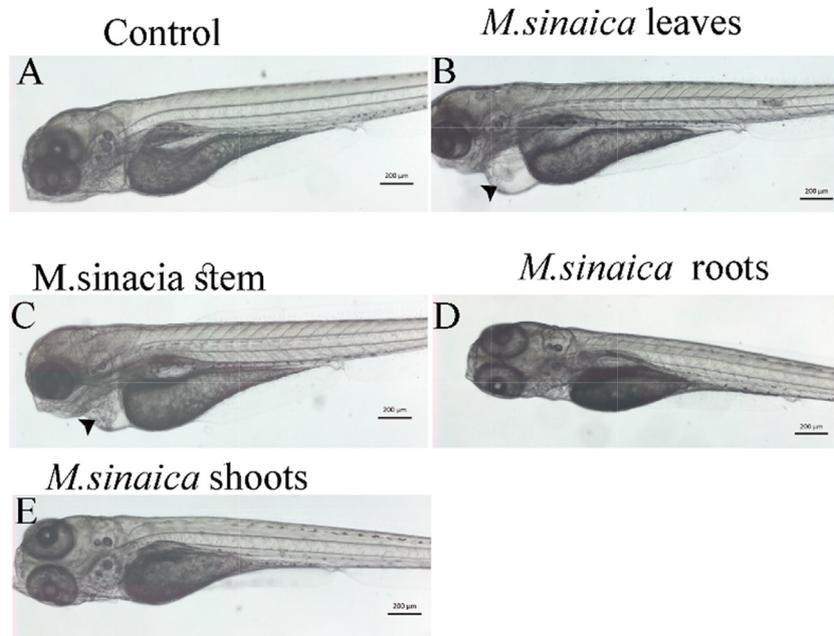


Fig. 3. Developmental toxicity of crude extract of *M. sinaica* parts in zebrafish embryos. The representative images of 72 hpf wild type zebrafish embryos Control (A) and treated with crude extract of leaves (B), stem (C), roots (D), and shoots (E) of *M. sinaica*. It is evident, that the treated embryos did not exhibit severe malformation except a mild cardiac edema by the leaves and stem extracts which could be due to absence of blood vessels.

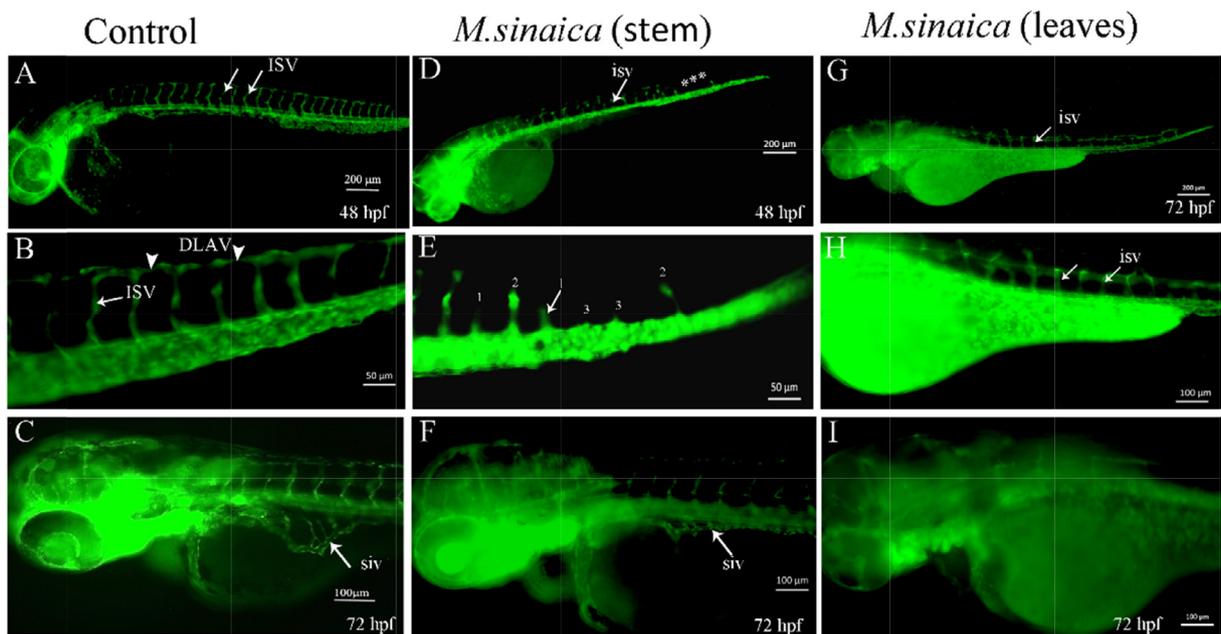


Fig. 4. The crude extract of leaves and stem of *M. sinaica* inhibited angiogenic blood vessels formation in zebrafish embryos on dose-dependent manner.

region. **3)** Complete absence of ISV. The Tg (fli:EGFP) embryos which were treated with leaves extract had severe malformation in angiogenic blood vessels at 48hpf (G&H). The ISVs in 100% of treated embryos were not intact and most of them hardly reached to middle trunk, and DLAV is completely absent.

The image F shows the SIV of transgenic zebrafish embryos treated with stem extract. The SIV formed in treated embryos however, did not grow into basket like structure that could be due to inhibition of migration of endothelial cells. The yolk region of the

transgenic zebrafish embryos treated with leaves extract is shown in I), The SIV in treated embryos are completely absent.

3.4. The crude extract of leaves and stem of *M. sinaica* showed weaker level of cytotoxicity in HUVECs

MTT cell proliferation assay has showed that *M. sinaica* exhibited a weaker level of cytotoxicity in HUVECs. The crude extracts prepared from roots, leaves, and flowers were completely inactive

even at maximum concentration (700 $\mu\text{g/ml}$). The crude methanol extract of leaves showed weak level of cytotoxicity with IC_{50} values of 246.30 $\mu\text{g/ml}$ in HUVECs and crude extract of stem had IC_{50} values of 348.78 $\mu\text{g/ml}$.

3.5. The methanol leaves extract perturbed the migration of HUVECs

Treatment of HUVECs with crude methanol extract of leaves significantly inhibited cellular migration of HUVECs in a time-dependent manner. As shown in Fig. 5, the control untreated HUVECs almost covered the scratched area after 48 h, whereas the migratory ability of HUVECs treated with 100 $\mu\text{g/ml}$ of the leaves extract of *M. sinaica* was significantly reduced ($p < 0.01$ and $p < 0.001$, 24 h and 48 h, respectively).

The migration assay showing the control and crude leaves extract treated HUVECs (A) and graphical representation of distance covered (B). The pictures at 0 h are soon after scratch is made. The control HUVEC cells migrated and covered around 38% of scratched area after 24 h and around 75% of scratched area after 48 h, whereas the treated cells had covered around 13% of scratched area after 24 h and the migration potential of treated cells remained same after 48 h of treatment.

3.6. Evaluation of biologically active molecules containing in the stem and leaves of *M. sinaica*

As the methanol crude extract of leaves and methanol extracts of stem have shown antiangiogenic activity in zebrafish embryos, we, next, sought to identify the molecules, which are represented in both types of the extracts. In order to find out the diversity of bioactive molecules presented in both type of extracts, GC–MS analysis was performed. Twenty-one compounds were identified in methanol crude extract of leaves. The identified compounds with chemical formula, molecular weight, retention time, and percentage of the covered areas are summarized in Tables 1 and 2, while the GC–MS chromatogram of leaves and stem are shown in Figs. 6 and 7 respectively.

The compound “3-pentenenitrile” was identified as major compound in the methanol extract of leaves of *M. sinaica* covering 30%

of the chromatogram. The second major compound was hexadecanoic acid in the methanol extract of leaves, which composes around 23%, and third major compound was D-allose (16%).

4. Discussion

Many of currently used pharmacological agents are either the secondary metabolites or pure compounds, derived from medicinal plants. The majority of Saudi Arab peninsula is composed of arid land. The vegetation consists of mostly xerophytic plants and herbs. Moreover, the flora of Saudi Arabia is very diverse, representing a mixture of indigenous species and flora from Asian, African, and Mediterranean regions (Rahman et al., 2004). However, most of the local medicinal plant species have not been scientifically studied, and biologically active phytochemicals are not known.

The genus *Moricandia* (*Brassicaceae* family) includes eight species. Some species from this genus are used as traditional medicine. Leaves and stems of *M. arvensis* (*L.*) are used in the treatment of syphilis and scorbout (Skandranj et al., 2017). The lipase inhibitory activity from the crude extract of aerial parts of *M. arvensis* (*L.*) has been shown by (Marrelli et al., 2018). The leaf and root extracts of *M. arvensis* have been shown to elicit a protection effect against H_2O_2 -induced genotoxicity in human lymphoblast cell line and several phytochemicals have been identified from the aerial parts of *M. arvensis* (Bennett et al., 2004; Tatsuzawa et al., 2015).

M. sinaica is a perennial herb however; the phytochemical contents and biological properties of this plant have not been investigated prior to this study. The methanol extract of the stem and leaves inhibited the formation and growth of the intersegmental, sub-intestinal vein and dorsal longitudinal anastomotic veins in a dose-dependent manner. The *in vitro* endothelial cell migration assay performed in HUVECs has revealed that methanol extract of the leaves interfered with the migration of HUVECs. The MTT cell proliferation assay has shown that the methanol extract of leaves of *M. sinaica* inhibited the cell proliferation of HUVECs with IC_{50} values more than 170 $\mu\text{g/ml}$, whereas significant reduction in migration of HUVEC was observed at concentration of 100 $\mu\text{g/ml}$. Hence, it is quite evident from a result that the antian-

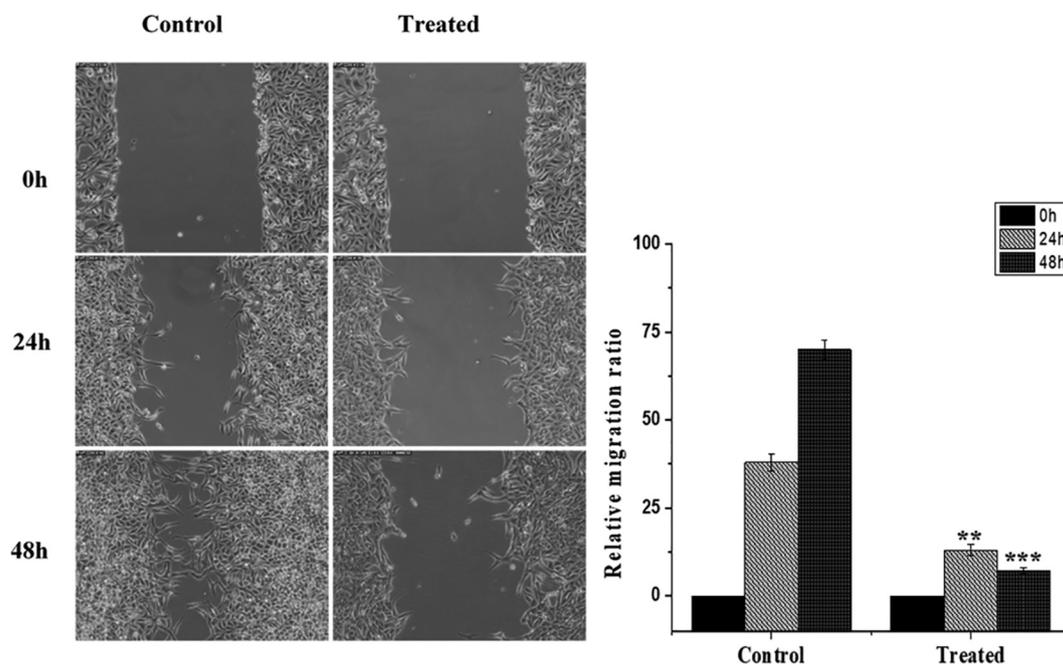


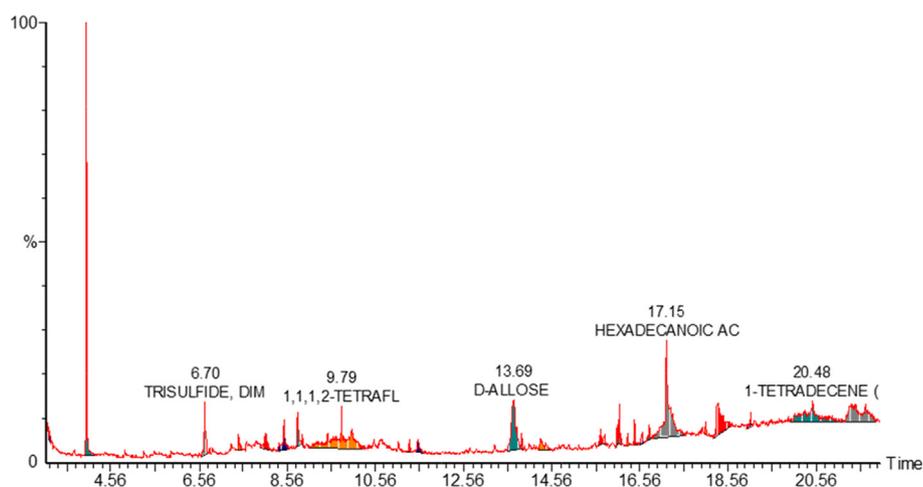
Fig. 5. The methanol leaves extract perturbed the migration of HUVECs.

Table 1
Phytochemical contents present in the leaves extract of *M. sinaica*.

Compound name	Chemical formula	MW (g/mol)	RT	Area %
3-Pentenenitrile	C5H7N	81.12	4.00	28.950
L-Proline	C5H9NO2	115.13	7.45	4.170
[1,3]Diazepane-2,4-dione	C5H8N2O2	128.13	8.06	3.890
4,5-Epithiovaleronitrile	C5H7NS	113.18	8.48	1.150
2,3-Dihydro-3,5-dihydroxy-6-methyl	C6H8O4	144.12	8.79	3.310
3,7,9-trimethyl-6-dodecanone	C15H24	204.35	8.90	0.580
1-Undecanol	C11H24O	172.31	11.34	0.410
2-Pyrrolidinone	C4H7NO	85.1	11.53	5.100
D-Allose	C6H12O6	180.16	13.69	15.470
3-Hexadecene	C16H32	224.42	13.87	0.320
D-Mannoheptulose	C7H14O7	210.18	14.31	1.650
8-Azaxanthine	C4H3N5O2	153.1	15.67	2.960
1-Heptadecene	C17H34	238.5	15.74	0.260
Neophytadiene	C20H38	278.5	16.10	0.760
7-Methylbicyclo[4.1.0]heptane	C8H14	110.2	16.28	0.290
3,7,11,15-tetramethyl-2-hexadecene	C20H40	280.5	16.43	0.580
5-Methyl-2-(1-methyl)-cyclohexanol	C10H18O	154.25	16.59	0.390
Methyl ester of heptadecanoic acid	C18H36	284.47	16.78	0.620
Hexadecanoic acid	C16H29O2	256.43	17.15	22.670
3,7,11,15-tetramethyl-2-hexadecen-1-ol	C20H40O	296.5	18.06	0.580
9-Octadecenoic acid	C18H34O2	282.5	18.33	4.090

Table 2
Phytochemicals contents present in the stem extract of *M. sinaica*

Compound name	Chemical formula	MW (g/mol)	RT	Area %
3-Pentenenitrile	C5H7N	81.12	4.01	54.790
Piperidine	C5H11N	85.15	4.88	1.800
1,2-Cyclopentanediol	C5H10O2	102.13	5.30	1.450
Methyl beta-D-galactopyranoside	C7H14O6	194.18	6.88	1.190
2,3-Dihydro-3,5-dihydroxy-6-methyl-4 h-pyran-4-one	C6H8O4	144.12	8.81	3.660
1-Undecanol	C11H24O	172.31	11.36	0.990
L-Proline	C5H9NO2	115.13	11.54	11.200
D-Allose	C6H12O6	180.16	13.63	6.330
5-Eicosene	C20H40	280.5	13.88	1.650
1-Tetradecanol	C14H30O	214.39	15.75	1.220
9-Octadecenoic acid	C18H34O2	282.5	17.26	2.760
Dotriacontane	C32H66	450.9	19.28	7.670

**Fig. 6.** GC-MS chromatogram of the leaves extract of *M. sinaica*.

giogenic activity of leaves extract of *M. sinaica* was due to inhibition in the migration of endothelial cells rather than a toxicity. The migration of endothelial cells is a key step for the formation of angiogenic blood vessels (Lamallice et al., 2007).

The GC-MS data has demonstrated that the extracts of leaves and stem shared major biomolecules. The 3-pentenenitrile was

present with ratio of 29% in the extract of leaves, while it was found to be 54% in the extract of stem. The D-allose constitutes 6.33% of the extract of stem and 15% of leaves extract. The leaves also contained 23% hexadecanoic acid, which was not detected in the extract of stem and, therefore, could be the reason for the more potent angiogenic activity of leaves extract of *M. sinaica* as com-

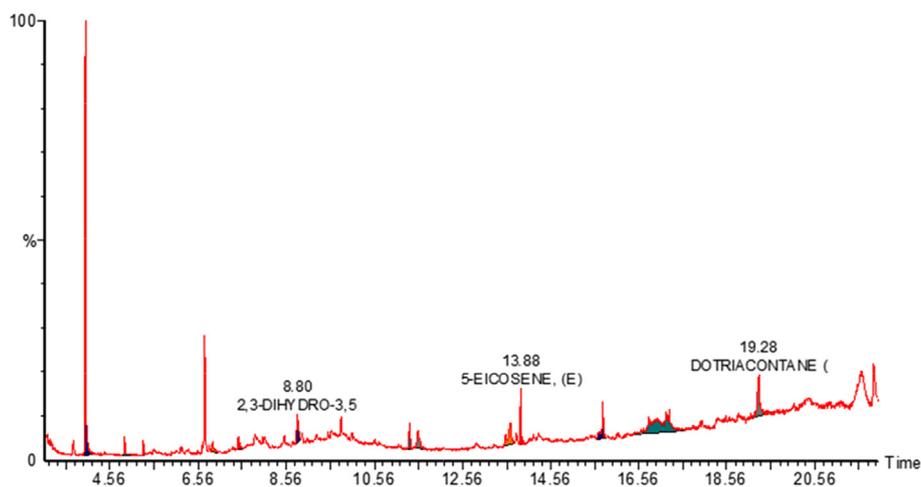


Fig. 7. GC-MS chromatogram of the stem extract of *M. sinaica*.

pared to stem extract. Hexadecanoic acid (palmitic acid) has been shown to inhibit angiogenesis via regulating the Hippo–Yes-associated protein (YAP) pathway (Laouirem et al., 2019).

These results clearly demonstrate that the inhibition of angiogenesis blood vessels in zebrafish embryos and inhibitory effect on the migration of HUVECs *in vitro* leaves extract of *M. sinaica* could be due to the antagonist activity of 3-pentenitrile, D-allose, and hexadecanoic acid on the regulatory proteins of angiogenesis.

5. Conclusion

The xerophytic plants of Saudi Arabia as *M. sinaica* possess valuable phytochemicals, which have the potential to be developed as an effective and safe antiangiogenic drugs to be used as a single therapy or in combination with other chemotherapy agents to treat cancer patients and cure the complications in diabetic retinopathy.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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