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The anti-angiogenic activity of Artemisia herba-alba's essential oil and its relation with the harvest period

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Abstract

Artemisia herba-alba is widely used in traditional medicine to treat diabetes, bronchitis, diarrhoea, hypertension and neuralgias. The aim of this study is to investigate the essential oil extracted from the aerial part of this plant and at two different harvest periods (May and December 2012) to evaluate a possible cytotoxic and anti-angiogenic activity. At first the *Artemisia herba-alba* essential oils were analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC/MS). Then the HUVECs (human umbilical vein endothelial cells) were treated with various concentrations of essential oil (10, 20, 40 and 80 µl). We have noted a chemical variation of the essential oils extracted from the aerial parts of *Artemisia Herba-alba* in relation to seasonal changes. The essential oils extracted from the plants collected in May (seeds stage) and those of December (floral stage) showed a dose- and time-dependent inhibitory effect on cell viability. The essential oil of the floral stage showed more cytotoxic effect on human umbilical vein endothelial cells (HUVEC) than those of the seeds stage which displayed potent anti-angiogenic activity; it did not cause cytotoxicity of HUVECs but disrupted endothelial tube formation. The variation on the essential oils chemical composition due to the harvest period has different effects on HUVEC. The essential oil from the seeds showed an anti-angiogenic activity without a cytotoxic effect which is very important feature in the research of anti-angiogenic agents.

Keywords: Anti-angiogenic activity; *Artemisia herba-alba*; chemical composition; Essential oil; HUVECs.
Abbreviations: ANOVA_ Analysis of Variance; ART_ Artesunate; ATCC_ American Type Cell Collection; DHA_ Dihydroartiminisin; DMSO_ dimethyl sulfoxide; EBM-2_ endothelial cell basal medium-2; ECGS_ endothelial cell growth supplement; FID_ flame ionization detector; GC_ Gas chromatography; GC/MD_ Gas chromatography/mass spectrometry; HUVECs_ human umbilical vein endothelial cells; MTT_ 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS_ Phosphate Buffer Saline; SPSS_ Statistical Package for Social Sciences.

Introduction

Artemisia herba-alba (Asteraceae) is a greenish-silver perennial dwarf shrub growing in arid and semi-arid climates. *Artemisia herba-alba*, also known as white wormwood, is a perennial shrub of the *Artemisia* genus that grows commonly on the steppes of the Mediterranean regions in northern Africa, Western Asia, Southwestern Europe, in the Arabian Peninsula and in the Saharan Maghreb xeric steppes. *Artemisia* has been a productive genus in the search for new biologically active compounds. Phytochemical investigations have proven that this genus is rich in terpenoids, flavonoids, coumarins, acetylenes, caffeoylquinic acids and sterols and it has been shown that *Artemisia* has multiple beneficial bioactivities such as antimalarial, anti-viral, anti-tumor, anti-pyretic, anti-hemorhagic, anti-coagulant, anti-anginal, anti-oxidant, anti-hepatitis, anti-ulcerogenic, antispasmodic and anti-complementary activities (Tilaoui et al., 2011). Plants of the genus *Artemisia* (Asteraceae family) have been used in folk medicine by many cultures since ancient times. Herbal infusions from these species have been used as analgesic, antibacterial, antispasmodic, and haemostatic agents (Tilaoui et al., 2011; Abou El-Hamed et al., 2010). *Artemisia herba-alba* is also used in the traditional medicine of the northern Badia region of Jordan, in the form of a decoction against fever, menstrual and nervous problems (Abad et al., 2012). In Tunisia, *Artemisia herba-alba* can be located in the mountains around Jebel Oust to the South of the country (Nabli, 1989). It have been widely used in folk and traditional medicine to treat diabetes, bronchitis, diarrhea, hypertension and neuralgias (Marrif et al., 1995). *Artemisia herba-alba* was reported as a traditional remedy of enteritis and various intestinal disturbances among the Bedouins in the Negev desert (Friedman et al., 1986). The essential oil of this specie was known for its therapeutic disinfectant, anthelminthic and antispasmodic virtues (Hatimi et al., 2001). In fact essential oil showed antibacterial activity (Yashphe et al., 1979) as well as, antispasmodic activity on rabbits (Yashphe et al., 1987). A wide range of plants contains compounds with angiogenesis modulating properties (Fan et al., 2006). Angiogenesis, the growth of neovessels from existing

vasculature, is essential for primary and metastatic tumor growth. Plants contain many active ingredients. The inhibition of angiogenesis is considered highly likely to prevent tumor growth or metastasis (Harris, 2003). It has been thought that *Artemisa herba-alba's* wide range effects might be due to its angiogenesis property. The angiogenesis process is related to many different metabolic diseases. The angiogenic activity can be considered at the first step of the cancer proliferation. So, the aim of this study is to determine the cytotoxic and anti-angiogenic activity of the essential oil extracted from the aerial part of *Artemisia herba-alba* and the effect of the harvest period on the essential oil composition, since there is no data in the literature concerning it. This is the first study testing the effect of *Artemisia herba-alba*'s essential oils extracted at two different stage of plant growth and its anti-angiogenic activity.

Results

The A.herba-alba's essential oil chemical composition

The chemical composition of the essential oil of *Artemisia herba-alba* determined by GC and GC–MS is presented in the table 1. The main compounds were α-thujone (43.00%) and cis-sabinol (21.65%) followed by β-thujone (11.53%), chrysanthenyl acetate (4.97%), 1,8-cineole (2.96%) and chrysanthenone (1.47%). The major compound is the thujone for both essential oils. This is similar to some samples collected in the southern of Tunisia (Akrout et al., 2010; Haouari and Ferchichi, 2009) but for our samples the second important amount is the cis-sabinol. The presence of this compound can be explained by the monoterpene metabolism according to Dehal SS and Croteau R (Dehal, 1987) since cis-sabinol is the result of hydroxylation of monoterpene oleine sabinene (Karp, 1987).

Cytotoxicity action of A. herba-alba essential oil on HUVEC

The cytotoxicity effect of the essential oils (seeds and floral stages) was measured by an MTT assay. HUVEC cells were incubated for 24, 48 and 72 h with different concentrations of each compound and concentration response curves were then derived (Fig.1). The two essential oils treatment displayed a concentration and time-dependent decrease on cell viability (Fig.1). The essential oil of floral stage (Fig.1.C) showed more cytotoxic effect on HUVEC than the one of the seeds stage (Fig.1.B).

The A. herba-alba's essential oil Anti-angiogenic activity in vitro

HUVEC cells, when seeded onto Matrigel, form capillary-like tube structure and an anti-angiogenic effect can be mediated by the interference with this morphogenetic capacity. In the control group, HUVECs on a Matrigel substratum displayed high mortality and differentiated into well-defined network-like structures within 12 h. At 100 µM concentration of Suramin there is no-cytotoxicity at the first day but there is a significant inhibition of tube formation (Fig.2). At the same time, our results revealed that the essential oil of the seeds stage, at a concentration of (80 µM) can inhibit capillary vessel formation (Fig.2) and exhibited non-cytotoxic feature (Fig. 1) in cultured HUVECs. On the other hand at the same concentration of (80 µM), the essential oil of floral stage did not show inhibitory effect on tube formation (Fig. 2).

Discussion

In this study, essential oils extracted from leaves at tow phenological stages have been investigated for their cytotoxicity and anti-angiogenic activity on HUVEC. The essential oil of the floral stage showed more cytotoxicity effect on human umbilical vein endothelial cells (HUVEC) than those of the seeds stage which displayed potent anti-angiogenic activity. An anti-cancer cytotoxic effect of *Artemisia herba-alba* essential oil was reported by the work of Tilaoui et al. (2011). The genus *Artemisia* is widely used in many parts of the world either alone or in combination with other plants to treat a variety of diseases (Xie et al., 2008), such as hepatitis (Hong et al., 2004), fever and malaria (Klayman, 1985), rheumatoid arthritis (Wang et al., 2005), and asthma (Kim et al., 2006). The plant has medicinal properties such as anticholesteremic, antipyretic, antiseptic, antibacterial, cholagogue, diuretic, and vasodilator (Yeung, 1985). It is also used in earache and as a purgative (Singh et al., 2009). The essential oil of plant has also insecticidal activity (Negahban et al., 2006). In the United States *A. tripartita* is a native species that has been used in the treatment of colds, sore throats, tonsillitis, headaches, and wounds by Native Americans (Moerman, 1998). Other research showed that the essential-oils, ethanol–water and infusion extracts from *A. campestris* L., and the hexane and ethanol–water extracts from *T. hirsuta* can inhibit the human adenocarcinoma cell growth (Akrout et al., 2011) which is similar to our result since *A. Herba-alba*'s essential oils in our study stopped the capillary tube formation of HUVECs.
The strong and aromatic smell of some species of *Artemisia* genus is due mainly to high concentrations of volatile terpenes, constituents of their essential oils, especially in their leaves and flowers. Many studies have shown that *Artemisia* species display significant intraspecific variations in the terpene constituents of their essential oils which is in concordance with our results. According to our knowledge the quality and yield of the essential oils from the *Artemisia* species is influenced by the harvesting season (Abad et al., 2012). Indeed the climatic conditions and water availablility in the soil have an effect on the vegetal secondary metabolism and, consequently, alter the composition of essential oils, through the seasons of the year (Freire et al., 2006). The biological activities of the same plant collected at different periods of the year showed significant variation which could be attributed to changes in the amounts of the active compounds present in each plant at different seasons. This explains the differences of the essential oil activity in our tests. Changes in chemical composition could be explained in terms of a thermoregulatory action of the hydrophobic compounds which could protect the plant from desiccation. Furthermore polar solids (triterpenes, flavonoids, diterpene acids) present in the plants might act as a physical barrier to prevent water permeation and dehydration (Kamatou et al., 2008; Harbone et al., 1975). Based on this view, Ghanmi et al. (2010) reported that there are differences in the essential oil constituents of *Artemisia herba-alba* in the vegetative and flowering stages, we found that these differences exist also between the seeds and flowering stage.
The cytotoxic effect of the essential oils from seeds and floral stages on HUVEC cells was investigated by the MTT assay. MTT is a yellow water-soluble tetrazolium salt and viable cells are only able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium rings (Kilani et al., 2008). This method is used extensively to determine the cellular toxicity of natural and synthetic compounds. The essential oil of the two stages showed a

Table1. Major constituents of the essential oils of *A. Herba-alba* related to the harvest period. The results mention the percentage of each major component from the total essential oil composition.

N°	RI	Components	*A. herba-alba* EO component (%)	
			December (floral stage)	May (seed stage)
1	1032	α-pinene	0.24	0.31
2	1076	Camphene	0.10	0.13
3	1132	Sabinene	0.11	0.14
4	1213	1,8-cineole	1.13	2.96
5	1280	p-cymene	1.00	1.22
6	1312	*3-octen-2-one*	0.34	0.63
7	1430	α-thujone	36.82	43.00
8	1445	*Filifolone*	0.19	0.07
9	1451	β-thujone	10.73	11.53
10	1522	*Chrysanthenone*	0.48	1.47
11	1534	*Chrysanthenyl acetate*	6.50	4.97
12	1800	*Cis-sabinol*	27.01	21.65
Total identified			84.65	88.08

RI: Retention indices on a polar column.

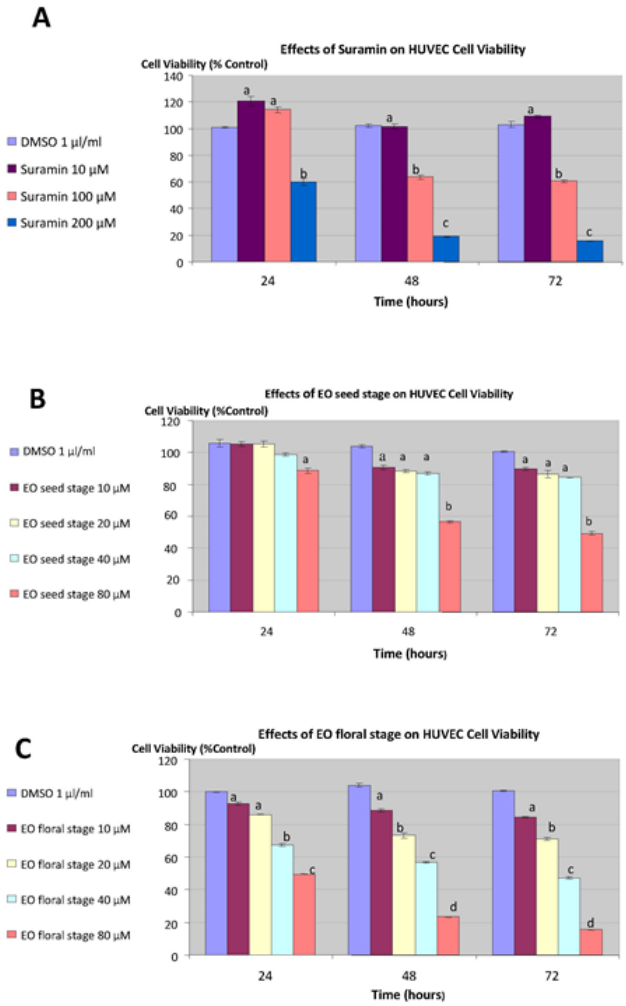

Fig 1. Inhibitory effect of *Artemisia herba-alba* essential oil (EO) from seeds stage (B), floral stage (c) and *Suramin* (A) on cell proliferation of HUVECs. Cells were incubated with various concentrations (10-20-40 and 80 µM) of EO seed stage (Fig1.B) (Supplementary Table 1), EO floral stage (Fig1.C) and *Suramin* (Fig1.A) for 24-48 and 72 hours of treatment, and cell viability was measured by MTT cell proliferation assay. The results are expressed as the mean_SD. Letters Indicates significant difference from the control group by the Tukey test ($p \leq 0.05$). Different letters for each essential oil concentration at different time are statistically different.

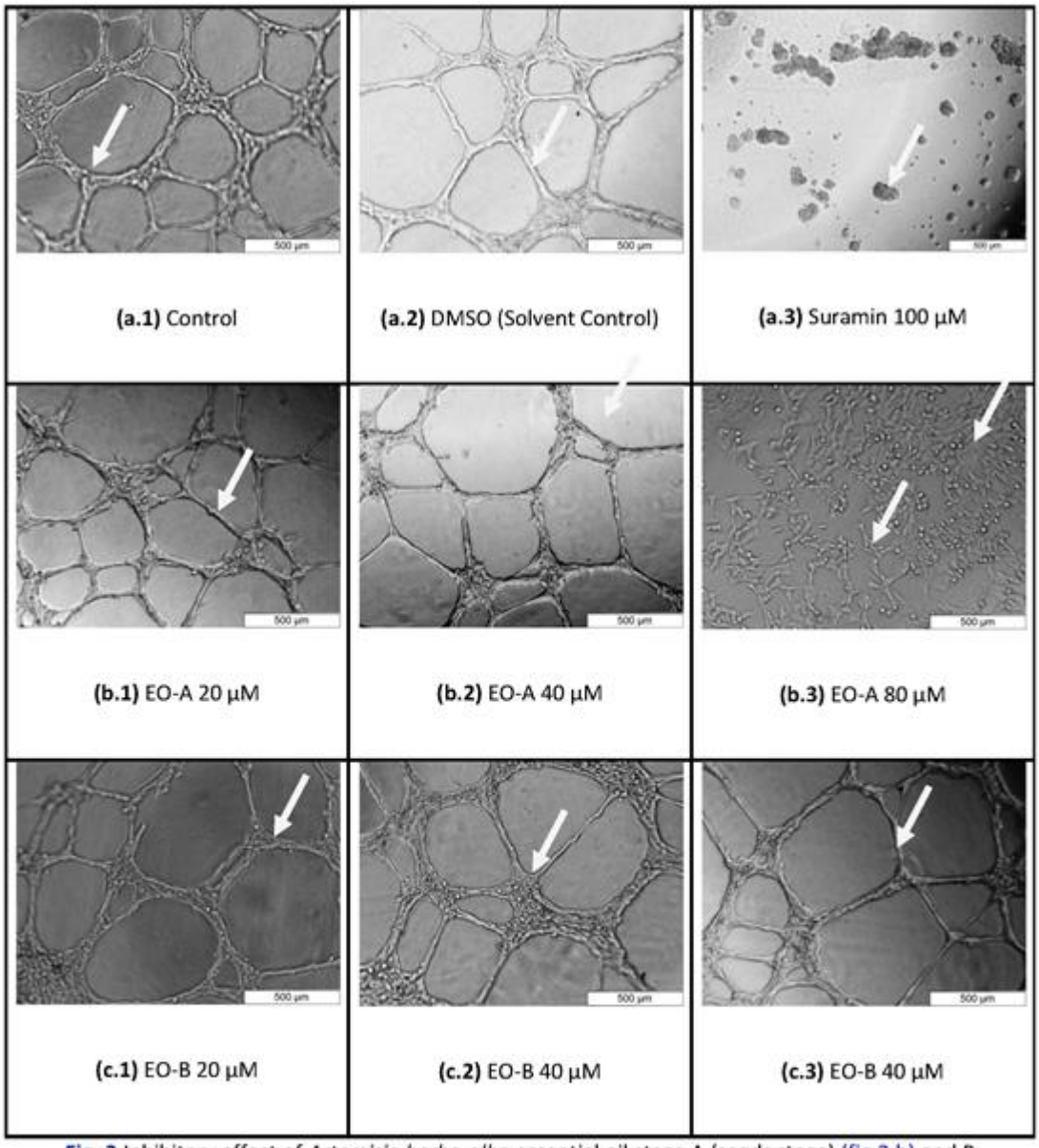

Fig. 2 Inhibitory effect of *Artemisia herba-alba* essential oil stage A (seeds stage) (fig.2.b) and B (floral stage)(fig.2.c) on tube formation of HUVEC. There is no tube formation in presence of suramin and 80 µM of EO A. HUVECs were cultured on matrigel matrix and incubated with various concentrations (20-40-80 µM) of EO A and EO B, and capillary-like tube formation was examined at 12 h after treatment. Suramin was used as a positive control (a.3), DMSO as negative control (a.2) and control cell (a.1). Images shown are representative of independent triplicate assays. Scale bar, 500 µm.

concentration and time-dependent inhibitory effect on cell viability. Although the essential oil of floral stage has high cytotoxic character, our suggestion is that this essential oil may be useful to combat cancer. Further research to know the apoptotic mechanism of inhibition of this compound is currently under progress in the laboratory of molecular biology at the Faculty of Sciences, Eskisehir Turkey. Angiogenesis has become one of the most exciting approaches in the development of cancer drugs (Cao, 2004). Many researchers have been trying to screen novel anti-angiogenic principles from various natural products. Although many papers have shown *Artemisia herba-alba* essential oil's different activities, there is no data about the effect of the harvest period on the angiogenic activity. We have addressed the anti-angiogenic activity by testing the essential oils (the two stages floral and seeds) compounds' effects on angiogenesis *in vitro* using HUVECs. Our present results showed that the essential oil of the seeds stage composition caused inhibition of the tube formation without cytotoxicity. In the drug discovery process, it is important to find not only the most active, but also the least toxic agent. Based on this data the essential oil of the seeds stage is a strong candidate as an anti-angiogenic drug area. Wu et al. (2005) reported that Artesunate (ART), a semi-synthetic

derivative of artemisinin isolated from the traditional Chinese herb *Artemisia annua*, inhibited cell growth induced apoptosis in HUVEC. The study went on to say that ART may be clinically useful for the treatment of the diseases related to angiogenesis. Dihydroartiminisin (DHA), the active component of a Chinese herb (*Artemisia annua)*, has also been showed to inhibit proliferation of cancer *in vitro* (Wu et al., 2012). ART has anti-angiogenic and anti-tumoral effects (Chen et al., 2003; 2004). In our study the essential oils of *Artmisia herba-alba* showed similar effect to ART and DHA on HUVEC. The essential oil of the floral stage compounds may be an effective anti-angiogenic agent and similar to the suramin it has a cytotoxic effect on HUVEC cell viability. Suramin that inhibits angiogenesis was originally used in the 1920s to treat trypanosomiasis (Hawking, 1989). The clinical use of suramin has been limited by its toxicity (Meyers et al., 2000). *Artemisia* species could serve as a good source of lead compounds against trypanosomes (Nibret and Wink, 2010). The essential oil from the seeds stage compounds, possess a potent anti-angiogenic activity which might provide a pharmacological background for its traditional use as a treatment of inflammatory diseases and cancer (Khlifi, 2013).

Materials and Methods

Plant material

Aerial parts (leaves) of *Artemisia herb-alba* (Asteraceae) were collected on May (seeds stage) and December (flowering stage) 2012 in Maknessy the center of Tunisia. The identification of the species was realized by the Associate Professor Mouhiba Benasri, botanist at the University El Manar, Faculty of Sciences of Tunis using Flore de la Tunisie keys (Pottier-Alapetite, 1981). Samples were air-dried during 15 days in the laboratory at room temperature till the weight stay stable. About 200 g of sample was subjected to hydrodistillation for 3 h with 1000 ml of distilled water using a Clavenger apparatus (Singh et al., 2009). The oil obtained was separated from the distillate water and dried with anhydrous sodium sulphate then it was stored at 4 ◦C for further use in bioassay and composition determination.

Essential oil analyses by gas chromatography (GC)

GC analysis was performed by a HP 6890N GC system gas chromatograph fitted with a flame ionization detector (FID), using a HP Innowax column (60 m length, 0.25 mm id, 0.25 µm film thickness). The oven temperature was held initially at 60°C for 10 min after injection, then increased to 220 ◦C with 4◦C/min heating ramp for 10 min and increased to 240°C with 1°C/min heating ramp then held at this temperature for 10 minutes. The injector temperature was 250°C; the detector temperature was 250°C; the carrier gas: nitrogen (1.0 ml/min); sample manually injected: 0.1 µl. The relative amount of components in the oil was calculated by electronic integration of the FID peak areas.

Gas chromatography/mass spectrometry (GC/MS)

GC/MS is an analytical method that combines the features of gas-liquid chromatography and mass spectrometry to identify different substances within a test sample (Bjarke, 1999). The analysis was carried out using Shimadzu QP 5050A GC/MS with electron impact ionization (70 eV). A HP Innowax column (60 m, length, 0.25 mm id, 0.25 µm film thickness) was used. Oven Temperature Program: 60^{o}C 10 min.,4 ^{o}C/min. 220^{o}C 10 min., 1 ^{o}C/min. 240^{o}C 10 min. The carrier gas was helium. The mass range was 40-500 m/z. the column held initially at 60 ◦C for 10 min and after injection it increased to 220 ◦C with 4◦C/min heating ramp for 10 min and then increased to 240 ◦C with 1 ◦C/min heating ramp subsequently holding at this temperature for 10 minutes.

Identification of the compounds

The components of the essential oils were identified by comparison of recorded mass spectra with those of a computer library (Wiley GC/MS Library) (McLafferty 1989). Alkanes were used as reference points in the calculation of relative retention indices (RI). Relative percentage amounts of the separated compounds were calculated from FID chromatograms (Adams, 2007).

In vitro Angiogenesis Bioassays

Cell culture

Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from the ATCC (American Type Cell Collection), which were incubated and grown in Nutrient Mixture F12 HAM medium supplemented with 20% heat-inactivated fetal calf serum, and endothelial cell growth supplement (ECGS, 0.05 mg/ml). The cells were cultured in an incubator in a humidified atmosphere (70%) at 37^{o}C, and 5% CO_2 (Bostancıoglu et al., 2013).

Cytotoxicity assay

The effects of the essential oil extracted at the seeds stage and the one at the floral stage were determined by using *in vitro* colorimetric MTT [3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cytotoxicity assay (Mossman, 1983). The stock solutions of the two essential oils were initially prepared in DMSO (Dimethyl sulfoxide), which were further diluted in fresh liquid medium. HUVEC cells (8 x 10^3 cells/well) were seeded into 96-well microtiter tissue culture plates (Techno Plastic Products AG) having a final volume of 100 µl. After 24 h of attachment, the culture medium was removed and the HUVEC cells were treated with different concentrations (10-80 µM) of the test samples. Eight replicate wells per concentration were used and repeated in triplicate at different intervals. Untreated medium control (blank) and solvent control (DMSO at a final concentration of 0.1%, v/v) were also assayed. After treatment with various concentrations of test samples for 24, 48 and 72 h, liquid media containing relevant samples from each well was replaced with 100 µl fresh medium containing MTT (0.5 mg/ml) dissolved in Phosphate Buffer Saline (PBS) respectively. The samples were then added to culture wells and incubated 2 h at 37^{o}C. The supernatant solution was afterwards removed, 100 µl/well DMSO (dimethyl sulfoxide) was added and samples were shaken for 5 min. Absorbance at 570 nm was measured with a microplate reader (Bio-Tek, ELX808IU, USA). Suramin initially dissolved in DMSO was used as positive control.

In vitro Matrigel tube formation assay

The assay was performed as previously described (Ouchi et al, 2004). For this assay the HUVECs, which were serum starved by culturing in endothelial cell basal medium-2 (EBM-2; Cambrex Bio Sciences) with 2% FBS for 4 h, were

used. The serum starved cells were plated at the density of 4 x 10^4 cells/well on Matrigel, which coated the wells of 96-well plates and were equilibrated with EBM-2 medium containing test samples. Suramin was used as positive control. After 12 h the endothelial cells-deriving like tube structures were visualized under an inverted microscope (Olympus IX71) and photographed (Olympus DP71 camera) at a magnification of 10x.

Statistical analysis

All extractions and analyses were conducted in triplicate. Data was expressed as means ± SD. The SPSS (Statistical Package for Social Sciences) software has been used for the statistical analyses of assessment of the MTT assay. Data were evaluated using one-way ANOVA (Analysis of Variance) followed by the Tukey test (supplementary Fig.1). A value of $p \leq 0.05$ was considered significant.

Conclusion

In conclusion, our present results found that there is a difference between the percentages of each compound in the two essential oils of *Artemisia herba-alba* related to the harvest period. The results of our research showed that there is a difference between the two essential oils on the HUVEC. This can be explained by the fact that some compounds of the essential oil during the seed stage have an anti-angiogenic activity without any cytotoxic effect. It might be due to the change in the chemical composition percentage. The fractionation of the essential oil will be necessary to determine which compounds have this activity and if it is the result of a synergy between different compounds.

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