

## Use of *Nigella sativa* Linn. Supercritical Carbon Dioxide Extract for Targeting the Angiogenesis Cascade

Baharetha HM<sup>1</sup>, Nassar ZD<sup>2</sup>, Ahamed MK<sup>3</sup>, Aisha AFA<sup>1</sup>, Alfadly SO<sup>4</sup>, Kadir MOA<sup>5</sup> and Majid AMSA<sup>1\*</sup>

<sup>1</sup>EMAN Testing and Research Laboratory, Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden 11800, Pulau Pinang, Malaysia

<sup>2</sup>School of Pharmacy, University of Queensland, Woolloongabba, QLD 4102, Australia

<sup>3</sup>EMAN Biodiscoveries Sdn. Bhd., Suite 126, Level 1, Eureka Complex, Universiti Sains Malaysia (USM) Campus, Minden-11800, Penang, Malaysia

<sup>4</sup>College of Medicine and Medical Sciences, Hadramout University, Mukalla, Yemen

<sup>5</sup>Department of Environmental Technology, School of Industrial Technology, Universiti Sains Malaysia, Minden 11800, Pulau Pinang, Malaysia

### Abstract

The aim of this study was to investigate the antiangiogenic effect of the *Nigella sativa* seeds extracts. Twelve extracts were prepared from the *N. sativa* seeds by supercritical carbon dioxide by varying extraction pressure and temperature. GC-MS analysis revealed the presence of 22 compounds, mainly thymoquinone (TQ), thymohydroquinone and androstane-3,17-diol. The extracts showed significant antioxidant activity, and cell viability study on human umbilical vein endothelial cells (HUVECs) indicated that two extracts (A3 and B3), prepared at higher temperature (60°C) and the lower pressures (2500 and 3000 psi), were the most potent anti-proliferative extracts; the median inhibitory concentrations were  $41.5 \pm 1.9$  and  $60.2 \pm 2.2$  µg/mL, respectively. The extracts down-regulated the expression of vascular endothelial growth factor (VEGF) in endothelial cells causing inhibition of various events of the angiogenesis cascade including; cell proliferation, endothelial cell migration and differentiation on matrigel and consequently inhibited tube formation. The extracts also showed significant inhibition of angiogenesis *in vivo* by inhibiting the vascularization of the chicken embryo chorioallantoic membrane (CAM). The antiangiogenic activity observed could be due to the anti-oxidant nature of the extract. This study highlights the importance of *N. sativa* SC-CO<sub>2</sub> oil as anti-angiogenic agent.

**Keywords:** *Nigella sativa*; Supercritical CO<sub>2</sub> extraction; Anti-angiogenesis; Antioxidant

### Introduction

Angiogenesis is vitally required for the physiological functions such as reproduction, vascularization and wound healing. However, when angiogenesis gets dysregulated, it leads to numerous lethal diseases such as cancer, ischemia, and macular degeneration, and chronic inflammation, infectious and immune disorders. Activation of endothelial cells is a prerequisite angiogenic factor, which induces angiogenesis by stimulating the genetic expression of a chief pro-angiogenic mitogen, vascular endothelial growth factor (VEGF) [1]. Endothelial cell proliferation, migration, differentiation and survival activities are mediated by binding of VEGF to its receptor VEGFR-2 through tyrosine kinase signalling pathway [2,3]. As there is a strong interdependence between angiogenesis and malignant pathologies, angiogenesis could be an ideal therapeutic target to deal with the angiogenesis-related disorders. Thus, there is an increasing demand to discover and develop the anti-angiogenic agents to curb the pathological angiogenesis.

Essential oils from medicinal plants have been reported to possess strong antiangiogenic properties and thus essential oils have been used to treat various human ailments caused due to the excessive angiogenesis [4,5]. The widespread use of plant essential oils has recently gathered an intense attention and focus in the basic research on essential oils as phytomedicine [6-8].

Supercritical fluid extraction (SFE) is a useful technique for extraction of volatile oils from natural sources [9]. SFE is preferable method for extraction because it is solvent free and prevents secondary reactions such as oxidation. Supercritical carbon dioxide (SC-CO<sub>2</sub>) is the most common supercritical fluid used in SFE technology; it is fast, non-explosive, non-toxic, inflammable, inert to solutes, environmentally safe and results in solvent-free extracts [10,11].

*Nigella sativa* Linn. (*N. sativa*) is a very common medicinal plant from the family Ranunculaceae, it is known as black seed [12]. It has been used traditionally as carminatives, condiments and appetizer. Black seeds have also been used for treatment of various diseases such as fever, jaundice, inflammation and [12,13]. Recent studies have shown that *N. sativa* possesses medicinally active essential oils with various pharmacological activities including anti-cancer and anti-metastasis [14], anti-cestodal [15], antibacterial [16], antiviral [17], anti-inflammatory [18], antinociceptive [19], and antioxidant [20] activities. *N. sativa* fixed oils have showed cytotoxic and immunopotentiating activities *in vitro* [21,22]. Supercritical CO<sub>2</sub> extracts of *N. sativa* have showed potent antibacterial activities [23], also it has resulted in selective anti-proliferative and induced apoptosis of breast cancer (MCF 7) cells [14].

The major chemical constituents reported in *N. sativa* volatile oil are thymoquinone, beta-elemene, nigellone, dithymoquinone and thymohydroquinone [24]. *N. sativa* fixed oil has been distinguished into saturated and unsaturated fatty acids. Saturated fatty acids include myristic, myristoleic, palmitic, palmitoleic, stearic, arachidic,

\*Corresponding author: Majid AMSA, EMAN Testing and Research Laboratory, Department of Pharmacology, School of Pharmaceutical Sciences, Universiti Sains Malaysia, Minden 11800, Pulau Pinang, Malaysia, Tel: +60124230842; Fax: +6046534582; E-mail: [aminmalikshah@gmail.com](mailto:aminmalikshah@gmail.com)

Received February 23, 2016; Accepted March 03, 2016; Published March 07, 2016

Citation: Baharetha HM, Nassar ZD, Ahamed MK, Aisha AFA, Alfadly SO, et al. (2016) Use of *Nigella sativa* Linn. Supercritical Carbon Dioxide Extract for Targeting the Angiogenesis Cascade. Med Aromat Plants 5: 241. doi:10.4172/2167-0412.1000241

Copyright: © 2016 Baharetha HM, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

arachidonic, behenic, lignoceric, and erucic acids. The unsaturated fatty acids contain oleic and linoleic acids [25-27]. Many other compounds have been identified in the oil, such as para-cymene [28], anthenole, carvacrol and 4-terpineol [29], carvone, cycloartenol, d-limonene, hederagenin, indole-3-acetic-acid, rutin and thymol [30].

In present study an attempt was made to optimize the different extraction parameters using supercritical CO<sub>2</sub> method to obtain a maximum yield of a fraction of *N. sativa* seed extract with potent anti-angiogenic activity. Further a series of investigations were conducted using human umbilical vein endothelial cells to elucidate the mechanism of action of the most effective fraction of SC-CO<sub>2</sub> extract of *N. sativa*. Finally, a chemometric correlation between the activity and the chemical composition of the active fraction was done using UV, FTIR and GC-MS analysis.

## Materials and Methods

### Pant material and reagents

Seeds of *N. sativa* were collected from Seiyun city in Yemen. Its herbarium voucher specimen number is (11221- *Nigella sativa*-22/3/2011) as authenticated by a Senior Botanist Mr. Shanmugan, School of Biological Sciences, Universiti Sains Malaysia, Penang. The seeds were washed and dried. The dried seeds were ground mechanically and stored in a desiccator. Supercritical carbon dioxide extractor (SFX-220 SFE system) was purchased from ISCO, USA. Infinite M200 PRO micro-plate reader was obtained from Tecan Group Ltd., Switzerland. Gas chromatograph HP 6890N (G1530N) (HP, China) which accompanied with HP 5973 (G2579A) quadrupole mass spectrometer (Agilent Technologies, USA). Commercial liquid carbon dioxide gas with purity of 99 g/kg in a gas cylinder at a temperature below (-5°C) was supplied locally from Malaysian Oxygen Company, Penang, Malaysia. Dimethyl sulfoxide (DMSO), gallic acid, thymoquinone (TQ), suramin and betulinic acid were obtained from Sigma-Aldrich, USA. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) and DPPH (2,2-diphenyl-1-picrylhydrazyl) reagents were purchased from Sigma-Aldrich, Germany. Matrigel™ (10 mg/mL) was obtained from BD Bioscience, USA. The growth medium M199 was obtained from Gibco/Life technology, UK.

### Cell line and cell culture

Human umbilical vein endothelial cell line (HUVECs) were purchased from American Type Culture Collection (ATCC), Rockville, MD, USA (Cat No. 8000). They were maintained in ECM medium, supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin solution and 1% endothelial cell growth factor supplements. ECM and its supplements were also purchased from (ScienCell, USA). Cell culture work was performed in sterilized conditions in a class II Biosafety cabinet and an incubator adjusted with 5% CO<sub>2</sub>-humidified atmosphere at 37°C.

### Experimental animals

Eight to twelve weeks old Sprague Dawley male rats were obtained from the animal house facility from the School of Pharmaceutical Sciences at Universiti Sains Malaysia. The animals were kept in a clean and quiet transit room with adequate lighting system, temperature and ventilation for few days for acclimatization before euthanasia. The animal work was approved by the Animal Ethics Committee of Universiti Sains Malaysia. The approval reference was USM/Animal Ethics Approval/2010/(59) (228) from Universiti Sains Malaysia.

### Supercritical carbon dioxide extraction (SC-CO<sub>2</sub>)

Twelve extracts were prepared using the SFX-220 SFE system, by varying extraction pressure (2500, 3000, 4500 and 6000 psi) and temperature (32, 45 and 60°C) for 60 min dynamic according to the SC-CO<sub>2</sub> extraction method as described before [14]. The extracts were coded as (A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2 and D3) and percentage yield was calculated.

### Anti-proliferation assay

MTT assay [31] was used to evaluate the anti-proliferative effect of the SC-CO<sub>2</sub> extracts of *N. sativa*. Briefly, HUVECs were treated with various concentrations (5 to 100 µg/mL) of the extracts for 48 h. Betulinic acid and thymoquinone were used as positive controls. DMSO (0.1%) was used as the negative control. After 48 h MTT was added and the optical densities (OD) were measured at 570 nm and reference was 620 nm. The results were presented as mean ± SEM percent inhibition of cell proliferation.

### Rat aortic ring assay

This assay was carried out as previously described Goodwin with minor modifications [32]. Thoracic aortas were removed from euthanized male rats, rinsed with serum free medium and cleaned from fibroadipose tissues. The aortas were cross sectioned into small rings (approximately 1 mm thickness) and seeded individually in 48-wells plate in 300 µL serum free M199 media containing 3 mg/mL fibrinogen and 5 mg/mL aprotinin. Ten microliters of thrombin (50 NIH U/mL in 1% bovine serum albumin in 0.15 M NaCl) was added into each well and incubated at 37°C for 90 min to solidify. A second layer (M 199 medium supplemented with 20% HIFBS, 0.1% ε-aminocaproic acid, 1% L-Glutamine, 2.5 µg/mL amphotericin B, and 60 µg/mL gentamicin) was added into each well (300 µL/well). Various concentrations (25-250 µg/mL) were used for calculating IC<sub>50</sub> of the active extracts (A3, B3 C3 and D3). Suramin (100 µg/mL) and thymoquinone (5-50 µg/mL) were used as positive controls whereas, 0.1% DMSO was used as a negative control. On day four, the medium was replaced with a fresh one containing the test materials. On day five, aortic rings were photographed using EVOS f1 digital microscope (Advanced Microscopy Group, USA) (40X magnification) and subsequently the length of blood vessels outgrowth from the primary tissue explants was measured using Leica Quin software.

The inhibition of blood vessels formation was calculated using the formula

% blood vessels inhibition =  $(1 - (A_0/A_Z)) \times 100$ , Where; A<sub>0</sub>=distance of blood vessels growth in treated rings in µm, A=distance of blood vessels growth in the control in µm.

The results are presented as mean percent inhibition ± SD, (n=8). % inhibition was plotted against the concentrations and IC<sub>50</sub> was calculated.

The significant difference between the micro vessels out growth in treated versus untreated aortic rings was calculated using Student's t test. Based on the results of this assay, the most active extracts, A3 and B3 were chosen for the subsequent investigations for the anti-angiogenic property.

### Evaluation of antiangiogenic properties of A3 and B3

**Colony formation assay:** The assay was performed according to Franken et al. [33], HUVEC cells were cultured in 6 well plate using ECM medium (500 cell/mL) for 12 h. The cells were treated with 10,

20, 40 and 60 µg/mL of the extracts A3 and B3. Betulinic acid (20 µg/mL) and DMSO (0.1%) were used as positive and negative controls, respectively. The treatment was carried out for 48 h by incubating the plates in incubator with 5% CO<sub>2</sub>-humidified atmosphere at 37°C. The cells were maintained by replenishing the old medium with fresh ECM for every 3 to 4 days until sufficient number of colonies (≥ 50 cells) were recorded in the negative control group (usually 9-10 days). Eventually, the colonies were fixed, stained with 0.2% crystal violet and counted under stereomicroscope. Percentage of plating efficiency (PE%) and percentage of surviving fraction (SF%) was calculated.

**Endothelial cell migration assay:** The assay was conducted as described by Ref. [34]. The HUVECs were seeded in ECM and incubated for 48 h. The monolayer was scratched with a sterile 200 µL micropipette tip and then washed with PBS to remove the unattached cells and to smoothen the edges of the scratch. ECM (2 mL) with low serum concentration (2.0%) was added to each well followed by addition of two different concentrations (20 and 40 µg/mL) of A3 and B3. Whereas, 0.1% DMSO and betulinic acid (20 µg/mL) were used as the negative and positive controls, respectively. For each test group, 10 microscopic fields per well were photographed at 0, 12 and 24 h using AMG EVOS fl inverted microscope. The width of the wounded area was measured using Leica Quin software. The percent inhibition of cell migration was then calculated relative to wound closure at zero time using the formula:

$$\% \text{ wound closure} = (1 - \frac{\text{width at the indicated times (h)}}{\text{width at zero time}}) \times 100\%$$

The results are displayed as average ± SD, (n=6).

**Tube formation assay:** Inhibitory effect of A3 and B3 on tube-like networks of HUVECs on matrigel matrix was investigated as previously described [35]. In brief, a layer of matrigel (150 µL/well) was added to 48 well plate and allowed to solidify for 45 min in an incubator at 37°C and 5% CO<sub>2</sub>. Then, HUVECs were seeded onto the matrigel-coated plate and treated with three concentrations (20, 40 and 60 µg/mL) of A3 and B3 for 24 h. The plate was incubated in CO<sub>2</sub> incubator at 37°C and 5% CO<sub>2</sub>. Betulinic acid (10 µg/mL) and (0.1%) DMSO was used as positive and negative controls, respectively. Finally, the tubular networks in the wells were photographed using AMG EVOS fl inverted microscope and the network length and width was quantified [36].

**Measuring of VEGF expression:** Vascular endothelial growth factor (VEGF) activity was measured using human VEGF-165 ELISA kit (Raybio, USA) according to the manufacturing instructions. HUVECs were treated over night with the extracts A3 (20 µg/mL) and B3 (20 µg/mL). DMSO (0.1%) was used as a negative control. The concentration of VEGF in the HUVECs was calculated from the regression equation as pg/mL of cell lysates.

**Chick embryo chorioallantoic membrane (CAM) assay:** Inhibitory effect of A3 and B3 was investigated *in vivo* using fertilized chicken embryo [37]. Fertile eggs were cleaned and kept horizontally with rotation for 3-5 days at 37°C in a humidified incubator to ensure formation of chorioallantoic membrane (CAM) vessel network. Subsequently, a small window was made gently with sterilized seizer on the upper face of the eggshell. About 5 mL of albumin was aspirated carefully and the eggs were incubated horizontally to allow the CAM to detach from the shell. The SC-CO<sub>2</sub> extracts A3 and B3 were prepared in agarose 1.2% discs at 100 µg/disc and applied directly onto the CAM. Discs containing the vehicle only (1.2% agarose) were used as negative control, while 50 µg/disc of Suramin was used as a positive control). The opening was covered with sterilised surgical tape and the embryos

were incubated for 48 h. At the end, the CAMs at the disk area were photographed under a dissecting microscope and blood vessels in each CAM were counted. The results are presented as a mean blood vessel count per CAM ± SD, (n=6).

### Phytochemical analysis

The twelve extracts were analysed using FTIR, UV-Vis spectrophotometry, total phenolic and flavonoid contents. These assays and their results were published in our previous article [14].

### GC-MS

GC-MS analysis of the extracts was carried out on a gas chromatograph HP 6890N (G1530N) which was accompanied with HP 5973 (G2579A) quadrupole mass spectrometer, at 70 eV. Non polar capillary column HP-5MS 19091S-433 was used as a stationary phase. The initial oven temperature was 70°C (two min) and then increased to 285°C at a rate of 20°C/min. The total run time was 32.75 min. Helium flow rate was 1.2 mL/min. The source temperature was 230°C while the quadrupole temperature was 150°C. Then, mass spectrometer recorded mass spectra and mass/charge ratios (*m/z*) of molecular ions and compared them to the referenced data of NIST02 library of MSD ChemStation Data analysis application [38].

### Statistical analysis

The results were expressed as means ± SD. They were analysed statistically using SPSS 16.0 package by One-way ANOVA test. Significant level was considered as (0.05). Correlation was calculated by bivariate, 2-taild pearson test; R<sup>2</sup> values were calculated by linear regression test.

### Results

The dried powder of *N. sativa* seeds were extracted with SC-CO<sub>2</sub> extraction method. Totally twelve (12) extracts (A1, A2, A3, B1, B2, B3, C1, C2, C3, D1, D2 and D3) were resulted from different experimental conditions in accordance with the full factorial design with variables such as extraction time, pressure and temperature. The details of the extracts are given in Table 1. All the extracts were first screened for anti-angiogenic property using anti-proliferative assay on HUVECs and anti-neovascularization assay on rat aortic explants. Figure 1 illustrates the GCMS chromatograms.

Extract	SC-CO <sub>2</sub> extraction parameters	
	Pressure (psi)	Temperature (°C)
A1	2500	32
A2	2500	45
A3	2500	60
B1	3000	32
B2	3000	45
B3	3000	60
C1	4500	32
C2	4500	45
C3	4500	60
D1	6000	32
D2	6000	45
t	6000	60

**Table 1:** Codes of the SC-CO<sub>2</sub> extracts of *N. sativa* according to the extraction parameters of pressure and temperature.

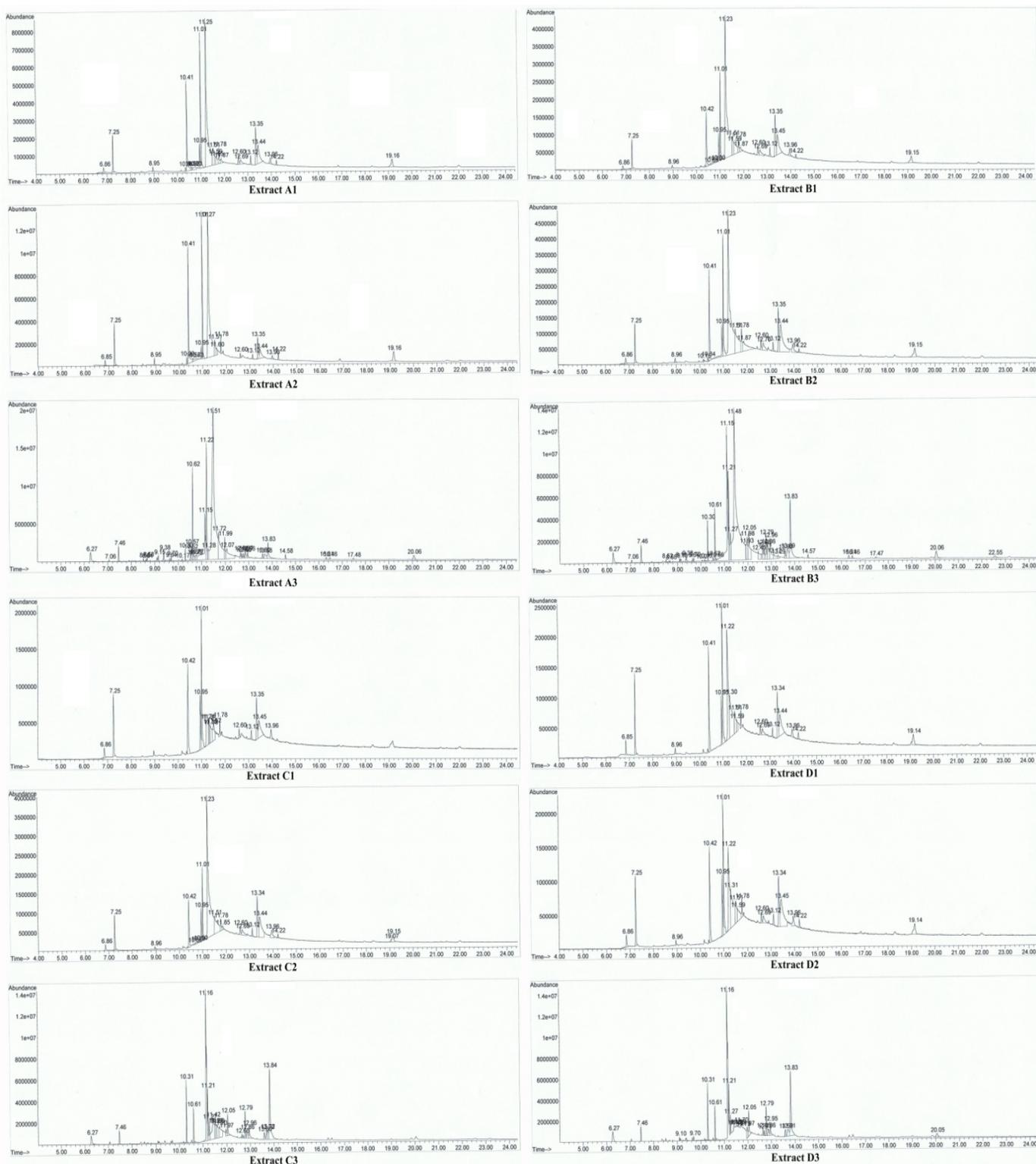


Figure 1: Gas chromatograms of the extracts.

### Rat aortic ring assay

Table 2 shows that A3 and B3 showed significant ( $P < 0.05$ ) antiangiogenic effect with  $IC_{50}$   $90.75 \pm 4.75$  and  $98.60 \pm 2.91$   $\mu\text{g/mL}$ , respectively whereas, C3 and D3 demonstrated a moderate activity with  $IC_{50}$   $109.40 \pm 3.53$  and  $125.44 \pm 7.93$   $\mu\text{g/mL}$ , respectively. The dose-response curves of the active extracts were depicted in Figure 2c.

Table 3 illustrates that at a concentration of  $100 \mu\text{g/mL}$ , the extracts A3, B3, C3 and D3 showed the more pronounced inhibitory effect on neovascularization in rat aortic explants. The percentage inhibition of A3, B3, C3 and D3 at  $100 \mu\text{g/mL}$  was  $54.38 \pm 5.17$ ,  $46.88 \pm 5.18$ ,  $49.10 \pm 6.32$  and  $40.27 \pm 8.35\%$ , respectively. However, TQ and suramin showed almost complete inhibition of sprouting of micro vessels from the aortic explants. The inhibitory effect of the test samples on the sprouting of micro-vessels from the aortic rings are depicted in Figure 2a and 2b.

The extracts, A3 and B3 were selected to elucidate the mechanism of action with a series of *in vitro* and *in vivo* antiangiogenic assays.

Extract	$IC_{50}$ ( $\mu\text{g/mL}$ )
A3	$90.75 \pm 4.75^*$
B3	$98.60 \pm 2.91^*$
C3	$109.40 \pm 3.53$
D3	$125.44 \pm 7.93$
TQ	$12.69 \pm 1.44^*$

\* $P < 0.05$  in relation to each other.

**Table 2:**  $IC_{50}$ s ( $\mu\text{g/mL}$ ) of A3, B3, C3, D3 and TQ on inhibition of neovascularization (rat aortic ring assay).

Extract	$EC_{50}$ on DPPH (mg/mL)	% of HUVECs cell viability (MTT)	% of inhibition on rat aortic ring assay
A1	$1.93 \pm 0.05$	$89.77 \pm 4.39^*$	$14.48 \pm 4.31$
A2	$2.11 \pm 0.13$	$67.86 \pm 3.43^*$	$21.74 \pm 6.99$
A3	$4.47 \pm 0.16$	$(-0.22) \pm 0.70^*$	$54.38 \pm 5.17^*$
B1	$1.86 \pm 0.02$	$101.03 \pm 2.31$	$12.68 \pm 4.79$
B2	$1.90 \pm 0.04$	$112.69 \pm 0.55$	$20.42 \pm 4.37$
B3	$4.29 \pm 0.07$	$(-0.14) \pm 0.24^*$	$46.88 \pm 5.18^*$
C1	$1.58 \pm 0.04$	$106.24 \pm 1.64$	$13.71 \pm 4.33$
C2	$1.67 \pm 0.03$	$105.66 \pm 0.78$	$9.63 \pm 5.62$
C3	$4.13 \pm 0.09$	$103.18 \pm 0.82$	$49.10 \pm 6.32^*$
D1	$0.86 \pm 0.04$	$98.97 \pm 1.24$	$17.61 \pm 6.76$
D2	$1.50 \pm .04$	$85.23 \pm 1.14^*$	$26.16 \pm 8.33$
D3	$3.27 \pm 0.07$	$89.20 \pm 2.56^*$	$40.27 \pm 8.35^*$
TQ	$(17.46 \pm 1.40) \times 10^{-3}$	-	-
GA	$(1.08 \pm 0.01) \times 10^{-3}$	-	-
TQ (50 $\mu\text{g/mL}$ )	-	-	$100.04 \pm 3.73$
Suramin (100 $\mu\text{g/mL}$ )	-	-	$99.80 \pm 4.24$

\* $P < 0.05$  comparing to the negative control (MTT assay).  $P < 0.05$  in relation to the negative control,  $P < 0.05$  in relation to other extracts (Rat aortic ring assay).

**Table 3:** Activities of the extracts on DPPH free radical as  $EC_{50}$  (mg/mL), percentages of cell viability of HUVEC cells treated with  $100 \mu\text{g/mL}$  of the extracts, and the potencies of the extracts on inhibition of sprouting of microvessel growth on rat aortic ring assay after treatment with  $100 \mu\text{g/mL}$  of extract.

### Antioxidant activity

Effect of the SC- $\text{CO}_2$  extracts on DPPH scavenging effect is depicted in Table 3. Figure 3 illustrates the effect of different extraction pressure and temperature on the antioxidant effect.

### Cell viability assay

The SC- $\text{CO}_2$  extracts of *N. sativa* seeds were tested for their inhibitory effect on HUVECs proliferation using MTT assay. The extracts that were prepared at  $60^\circ\text{C}$  with different pressures A3 and B3 (2500 and 3000 psi, respectively) displayed most potent activity as compared to the other extracts tested. The extracts A3 and B3 inhibited proliferation of HUVECs completely at dose of  $100 \mu\text{g/mL}$ . The extracts A3 and B3 exhibited dose-dependent anti-proliferative effect with  $IC_{50}$   $41.50 \pm 1.92$  and  $60.21 \pm 2.19$   $\mu\text{g/mL}$ , respectively (Figure 4). The  $IC_{50}$ s of the positive controls, TQ and betulinic acid were  $2.39 \pm 0.18$  and  $3.73 \pm 0.20$   $\mu\text{g/mL}$ , respectively. The result obtained in MTT assay for the SC- $\text{CO}_2$  extracts of *N. sativa* seed is given in the Table 3.

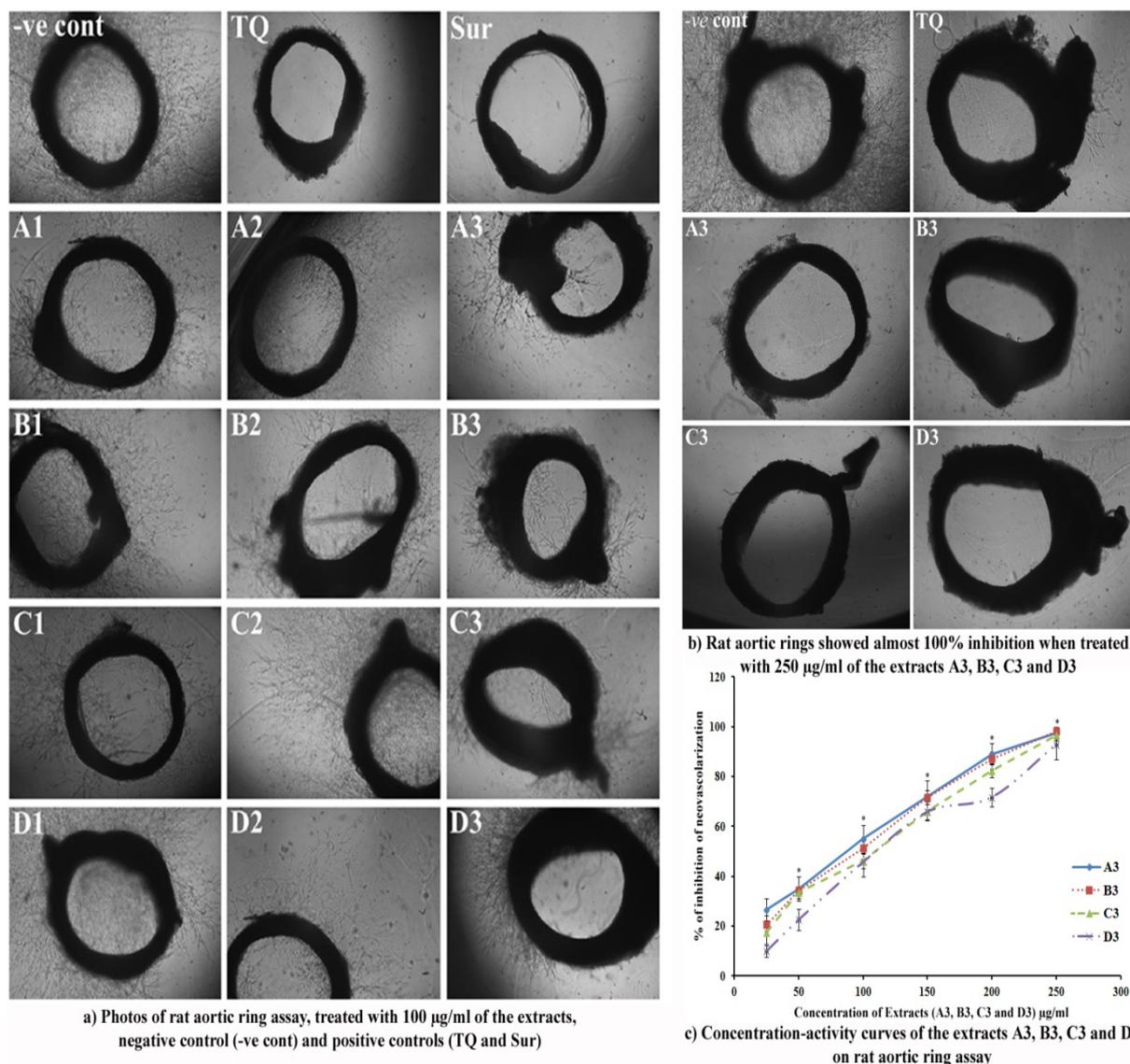
### Antiangiogenic properties of A3 and B3

**Colony formation assay:** The dose-dependent inhibitory effect of A3 and B3 on colony formation of HUVECs is shown in Figure 5a. Percentage of plating efficiency (PE) in vehicle (0.1% DMSO) treated cells was  $88 \pm 2.4\%$  which was drastically reduced by the treatment of A3 and B3 with PE  $34 \pm 1.7$  and  $21 \pm 3.1\%$ , respectively. The results showed that A3 displayed significantly higher inhibitory effect than B3. The surviving fraction (SF) was determined (Figure 5b) to be  $14.97 \pm 1.37$  and  $54.22 \pm 2.71\%$  after A3 and B3 treatment at the concentrations  $20 \mu\text{g/mL}$ , respectively (Figure 5b). These results can be compared with that of the standard reference, betulinic acid ( $20 \mu\text{g/mL}$ ) which produced the PE of  $11.13 \pm 0.82\%$ .

**HUVECs cell migration:** A3 and B3 exhibited an obvious inhibition of migration of HUVECs. Both the extracts demonstrated time and dose-dependent inhibitory effect on migration of HUVECs. The extracts produced significant inhibition in cell motility (Figure 5c) at the concentration lower than its  $IC_{50}$  on proliferation of HUVECs. The percent inhibition of cell migration for A3 ( $30 \mu\text{g/mL}$ ) calculated after 12 and 18 h was  $29.04 \pm 2.34$  and  $52.35 \pm 2.65\%$ , respectively ( $p < 0.05$ ). However, B3 resulted in  $46.42 \pm 1.79$  and  $41.88 \pm 1.95\%$  of inhibition at 12 and 18 h, respectively. Interestingly, at lower concentration ( $15 \mu\text{g/mL}$ ) A3 exhibited significant anti-migratory effect with ( $P < 0.05$ ). Figure 5d clearly depicts the comparative inhibitory effects of the extracts A3 and B3 on migration of endothelial cells with that of untreated cells.

**Inhibition of tube formation:** The extracts A3 and B3 showed strong abolishing effect on capillary tubules formation of HUVECs. At concentrations 40 and  $60 \mu\text{g/mL}$ , A3 produced  $27.06 \pm 2.78$  and  $48.29 \pm 0.87\%$  inhibition, respectively. Similarly, B3 exhibited significant inhibitory effect on tube formation with  $41.47 \pm 2.70$  and  $57.86 \pm 1.12\%$  at 40 and  $60 \mu\text{g/mL}$  concentration, respectively. The results can be compared with that of the positive control, betulinic acid as shown in Figure 6a and 6b.

**Inhibition of VEGF expression:** In human VEGF-165 ELISA assay, HUVECs were treated separately with  $20 \mu\text{g/mL}$  of each extract (A3 and B3) for 24 h treatment. The results showed remarkable inhibition of VEGF-165 expression in HUVECs by both the extracts. Content of VEGF-165 in vehicle (0.1% DMSO) treated cells was recorded up to  $252.45 \pm 7.34$   $\text{pg/mL}$ , which was drastically reduced to  $77.18 \pm 8.43$  and  $67.8 \pm 5.25$   $\text{pg/mL}$  after treatment with A3 and B3, respectively. Figure 6c illustrates the comparative effects of A3 and B3.



**Figure 2:** Effect of the extracts on rat aortic ring assay. a) Rings treated with 100 µg/mL of the extracts (A1 - D3), negative control (-ve cont) and positive controls (TQ and suramin (Sur)). b) Rat aortic rings showed almost 100% inhibition of neovascularization, treatment with 250 µg/mL of the most active extracts (A3, B3, C3 and D3), negative control (-ve cont) and positive control (TQ). c) Concentration-activity curves of the extracts (A3, B3, C3 and D3). \**P*<0.05.

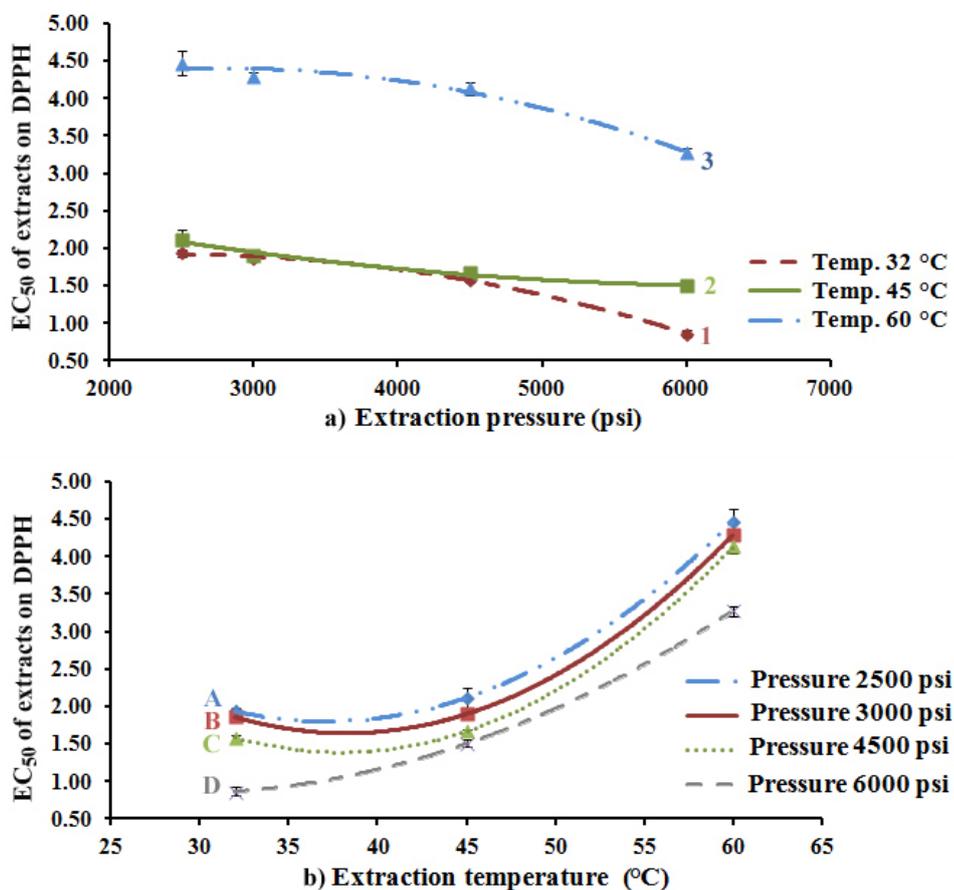
**CAM assay:** Treatment of fertilized chick embryo with A3 or B3 produced a significant deformed vascular architecture. The inhibitory percentage of 100 µg/disk of A3 was  $63.67 \pm 2.55\%$  which was significantly higher than that of B3 ( $51.75 \pm 3.68\%$ ). The positive control (50 µg/disk Suramin) showed  $63.47 \pm 4.24\%$  inhibitions. Figure 6d shows normal vasculature pattern in the vehicle (1.2% agarose) treated CAMs with prominent primary, secondary and tertiary vessels and dendritic branching pattern. Whereas, a distorted architecture in the vasculature can be seen clearly in the treated CAMs with the extracts (A3 and B3). The results of blood vessel count in treated CAMs showed that the treatment reduced the number of the blood vessels drastically.

**GC-MS:** The SC-CO<sub>2</sub> extract of *N. sativa* seeds prepared in the present study were analysed using GC-MS to specify and quantify the major chemical constituents present in them. Figure 1 depicts the

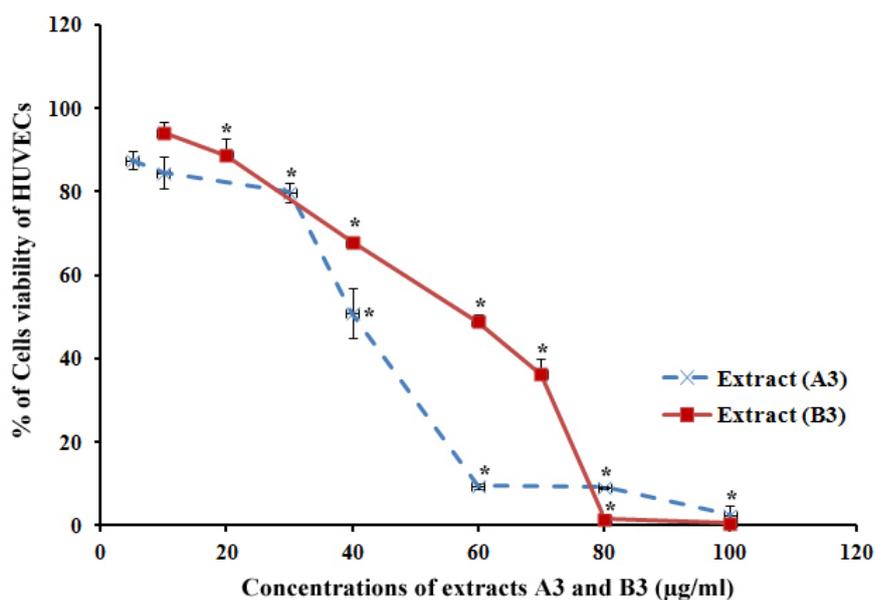
GC-MS chromatograms of the SC-CO<sub>2</sub> extracts. The quantified data presented in the Figure 1 that illustrates the composition and proportion of the major chemical constituents in the extracts. The parameters such as retention time, % area peak, molecular formula and molecular weight obtained from the GC-MS of the extracts are given in Table 4. Detailed characteristics of the peaks identified in the GC-MS were described in the Table 4. Further GC-MS analysis of active and inactive extracts depicted in Tables 5 and 6, which represent the compounds present in active extracts and the compounds commonly, found in all the extracts. Mass spectra for each peak in the chromatogram are given in the Supplementary Information.

## Discussion

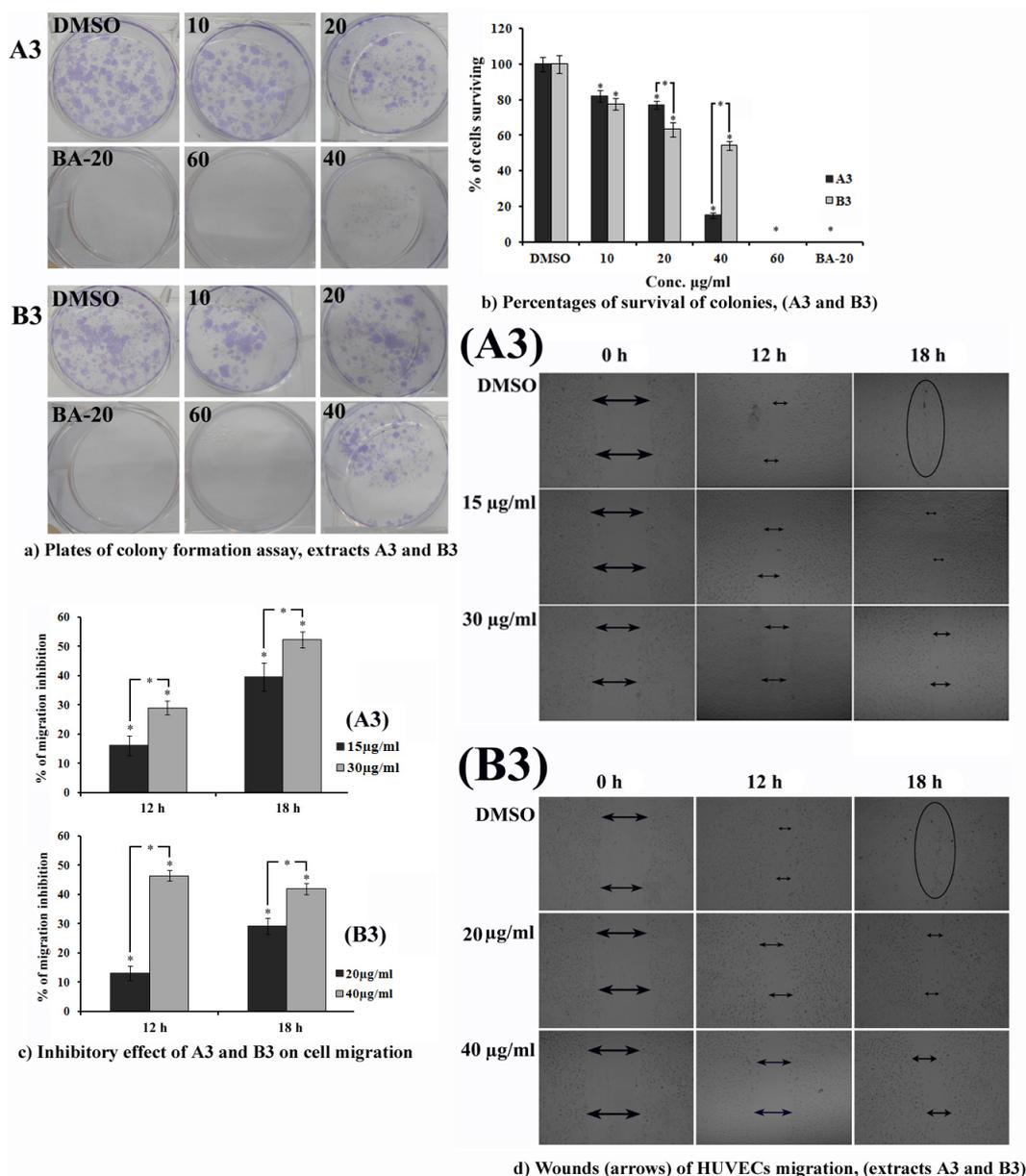
In the present study, an anti-angiogenic property of 12 SC-CO<sub>2</sub>



**Figure 3:** Correlation of the extraction pressure and temperature on DPPH scavenging activity of the extracts. a) EC<sub>50</sub> of the extracts on DPPH scavenging as a function of pressure (psi). b) EC<sub>50</sub> of the extracts on DPPH scavenging as a function of temperature (°C),  $P < 0.05$ .



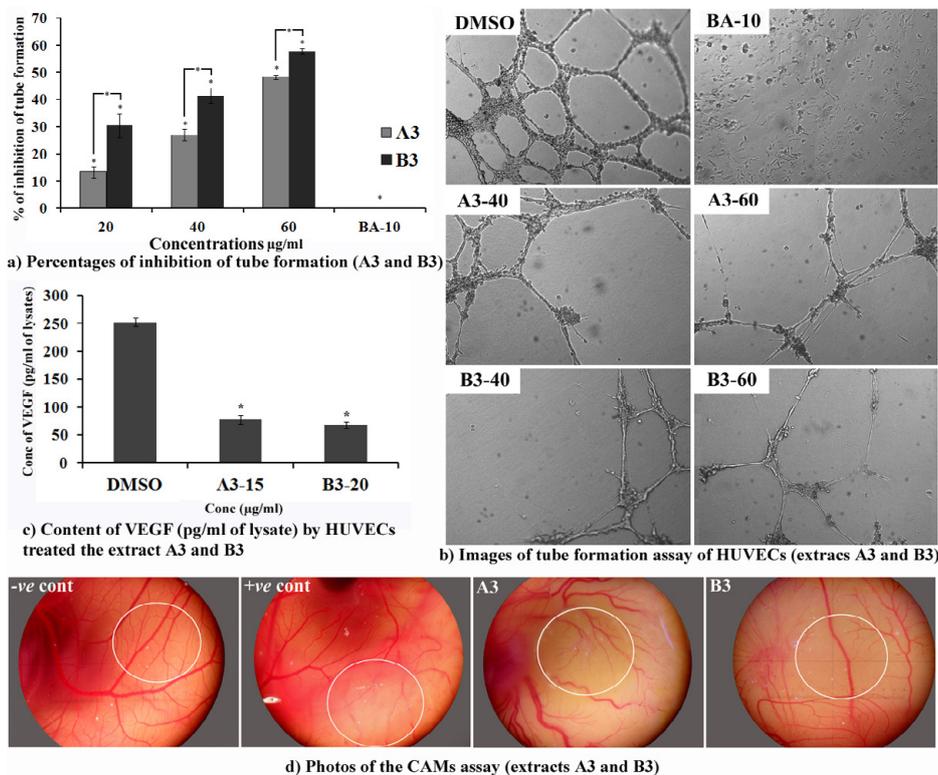
**Figure 4:** Concentration-activity curves of the extracts A3 and B3 on HUVEC cells, IC<sub>50</sub>=41.50 ± 1.92 and 60.21 ± 2.19 µg/mL, respectively, (MTT assay).



**Figure 5:** a) Plates of colony formation assay of HUVECs treated with A3, B3, negative control (DMSO) and positive control betulinic acid (BA-20). b) Survival percentages of colonies treated with A3, B3, negative control (DMSO) and betulinic acid (BA-20). c) Inhibitory effect of A3 and B3 on migration of endothelial cells. d) Wounds (arrows) of treated HUVECs with A3, B3 and negative control (DMSO) at magnification of 4X. \* $P < 0.05$ .

extracts of *N. sativa* seeds was evaluated. The anticancer effect of these extracts was measured by MTT test on five cancerous human cell lines: MCF 7, MDA-MB-231, HCT 116, Hep G2 and PC-3, as well as one normal human cell lines: CCD-18Co. It showed that the extract A3, which was extracted at the highest temperature (60°C) and the lowest pressure (2500 psi), was the most potent extract against breast cancer (MCF 7) with  $IC_{50}$  value of  $53.34 \pm 2.15 \mu\text{g/mL}$  as we reported previously [14]. The oil of *N. sativa* has showed significant anti-tumour potency [39,40]. Thymoquinone (TQ), the most active component of *N. sativa*, has resulted in an obvious anti-proliferative effect against HUVEC cell line. Also, it inhibited endothelial cell migration, tube formation and VEGF expression [41].

The anti-proliferative activity of the SC-CO<sub>2</sub> extracts against HUVECs was reported. It showed that two extracts which were extracted at the higher temperature (60°C) and the lower pressures, A3 (2500 psi) and B3 (3000 psi) represented the most potencies. A3 ( $IC_{50}$  of  $41.50 \pm 1.92 \mu\text{g/mL}$ ) showed stronger activity than B3 ( $IC_{50}$  of  $60.21 \pm 2.19 \mu\text{g/mL}$ ) as tested by MTT assay. Angiogenic activity of the 12 different extracts of SC-CO<sub>2</sub> of *N. sativa* revealed that four extracts (A3, B3, C3 and D3) significantly thwarted the micro vessel outgrowth as tested using *ex-vivo* isolated tissue assay. When the extraction pressure was increased, the level of antiangiogenic activity decreased. Closer inspection of the constituents present in these extracts indicates the presence of thymo-quinone and thymo-hydroquinone. The other eight



**Figure 6:** a) Percentages of inhibition of tube formation of HUVECs by A3, B3, betulinic acid (BA-10) and negative control (DMSO). b) Images of tube formation assay of HUVECs treated with A3, B3, betulinic acid (BA-10) and negative control (DMSO<0.5%), magnification was 4X. c) Content of VEGF (pg/mL of lysate) expressed by HUVECs treated with A3, B3 and negative control (DMSO). d) Photos of CAM assay after treatment (circles) with 100 µg/disk of A3 and B3. 50 µg/disk of Suramin (+ve cont) and untreated CAM (-ve cont). \*P<0.05.

Peak no.	RT min	Compounds	Molecular formula	Molecular weight
1	6.27	TQ	C <sub>10</sub> H <sub>12</sub> O <sub>2</sub>	164.08
2	6.86	(+)-α-longipinene (sesquiterpene)	C <sub>15</sub> H <sub>24</sub>	204.19
3	7.25	(+)-longifolene (sesquiterpene)	C <sub>15</sub> H <sub>24</sub>	204.19
4	8.36	Thymohydroquinone	C <sub>10</sub> H <sub>14</sub> O <sub>2</sub>	166.1
5	8.52	Cetane (hexadecane)	C <sub>16</sub> H <sub>34</sub>	226.27
6	9.1	Heptadecane	C <sub>17</sub> H <sub>36</sub>	240.28
7	9.13	Pristane (norphytane) (terpenoid)	C <sub>19</sub> H <sub>40</sub>	268.31
8	9.65	Octadecane	C <sub>18</sub> H <sub>38</sub>	254.3
9	9.7	Phytane	C <sub>20</sub> H <sub>42</sub>	282.33
10	10.17	Eicosane	C <sub>20</sub> H <sub>42</sub>	282.33
11	10.15	Methyl palmitate (metholene 2216)	C <sub>17</sub> H <sub>34</sub> O <sub>2</sub>	270.26
12	10.41	Squalene (triterpene)	C <sub>30</sub> H <sub>50</sub>	410.39
13	10.59	Palmitic acid (fatty acid)	C <sub>16</sub> H <sub>32</sub> O <sub>2</sub>	256.24
14	10.95	Methyl octadeca-8,11-dienoate	C <sub>19</sub> H <sub>34</sub> O <sub>2</sub>	294.26
15	11.28	Methyl stearate	C <sub>19</sub> H <sub>38</sub> O <sub>2</sub>	298.29
16	11.25	Linoleic acid (linolic acid) (fatty acid)	C <sub>18</sub> H <sub>32</sub> O <sub>2</sub>	280.24
17	11.51	9-Eicosyne	C <sub>20</sub> H <sub>38</sub>	278.3
18	11.78	Androstane-3,17-diol ((3α,5α)-androstane-3,17-diol) (neurosteroid)	C <sub>19</sub> H <sub>32</sub> O <sub>2</sub>	292.24
19	12.05	Methyl (11E,13E)-11,13-icosadienoate	C <sub>21</sub> H <sub>38</sub> O <sub>2</sub>	322.29
20	13.29	Hexacosane (cerane)	C <sub>26</sub> H <sub>54</sub>	366.42
21	13.82	2-Linoleoyl glycerol (monolinolein)	C <sub>21</sub> H <sub>38</sub> O <sub>4</sub>	354.28
22	14.22	trans-squalene (E,E,E,E-squalene)	C <sub>30</sub> H <sub>50</sub>	410.39

**Table 4:** GC-MS data of the extracts by comparison of experimental with referenced data of NIST02 library.

No.	Extract	Compound	% of Area
1	A3, B3, C3 and D3	TQ	1.20-2.10
2	A3, B3, C3 and D3	Cetane	0.14-0.24
3	A3, B3, C3 and D3	Octadecane	0.11-0.21
4	A3, B3, C3 and D3	Phytane	0.22-0.30
5	A3, B3, C3 and D3	Methyl stearate	1.13-2.30
6	A3 and B3	2-Linoleoyl glycerol	2.51-6.52
	C3 and D3	2-Linoleoyl glycerol	12.60-16.13
7	A3	Eicosane	0.60
8	B3	Hexacosane	0.26-0.38
9	B3	Thymohydroquinone	0.19
	C3 and D3	Thymohydroquinone	0.56-0.62
10	B3, C3 and D3	Heptadecane	0.14-0.25
11	B3, C3 and D3	Methyl (11E,13E)-11,13-icosadienoate	2.55-4.75
12	C3 and D3	Pristane	0.28-0.30

**Table 5:** Compounds that were contained in the most active extracts (A3, B3, C3 and D3).

extracts did not show promising anti-angiogenic effect and thymoquinone, thymo-hydroquinone was not detected.

The *in vitro* anti-angiogenic activities of A3 and B3 were evaluated by quantifying their inhibitory effect on endothelial cell proliferation, colonization, migration, tube formation and on VEGF expression.

No.	Extract	Compound	% of area
1	A3, B3, C3 and D3	(+)- $\alpha$ -longipinene	0.12-0.36
	A1, A2, B1, B2, C1, C2, D1 and D2	(+)- $\alpha$ -longipinene	0.32-1.51
2	A3, B3, C3 and D3	(+)-Longifolene	0.90-1.41
	A1, A2, B1, B2 and C2	(+)-Longifolene	1.77-2.95
	C1, D1 and D2	(+)-Longifolene	5.37-7.76
3	A3 and B3	Methyl palmitate	0.51-1.50
	C3 and D3	Methyl palmitate	3.29-4.38
	A1, A2 and B2	Methyl palmitate	0.11-0.21
4	A3	Palmitic acid	1.1
	A1 and A2	Palmitic acid	0.47-1.29
5	A3 and B3	Methyl octadeca-8,11-dienoate	7.86-13.45
	C3 and D3	Methyl octadeca-8,11-dienoate	21.26-21.55
	A1, A2, B1, B2, C1, C2, D1 and D2	Methyl octadeca-8,11-dienoate	1.65-6.17
6	All 12 extracts	Linoleic acid	33.97-44.13
7	C3 and D3	9-Eicosyne	2.44-6.01
	B1, C2, D1 and D2	9-Eicosyne	1.68-11.79
8	A3	Androstane-3,17-diol	1.92
	A1 and A2	Androstane-3,17-diol	2.59-2.86
9	A3 and B3	trans-squalene	0.24-0.34
	A2	trans-squalene	0.56
10	A2	Squalene	8.24

**Table 6:** Compounds that were found in all the extracts commonly.

These assays provide a valuable data for anti-angiogenesis assessment [37,42]. Endothelial cells tend to be in contact with their neighbouring cells. These population characteristics lead to formation of colonies and enhance angiogenesis [43]. A3 and B3 inhibited colony formation by HUVECs in dose-response manner. This confirms the cytotoxic effect of the two extracts on HUVECs at higher concentration, whereas the lower concentrations showed cytostatic effect. The higher concentrations of both extracts resulted in irreversible inhibition of the cells viability. A3 showed stronger activity than B3 especially at 60 and 40  $\mu$ g/mL. Endothelial cell migration and invasion are two integrated pathways required for normal embryonic morphogenesis, tissue regeneration in adults and they play important role in cancer development and tumour growth and neovascularization [44,45]. Migration signalling pathway is mediated by many interlinked positive feedback loops, such as Rho family GTPases, phosphoinositide 3-kinases, integrins, angiostatins and endostatins [46,47]. A3 and B3 elucidated potent suppression of HUVEC cell migration, especially after 18 h, where the untreated cells (DMSO) caused total closure of the scratch wounds. Previous study that looked into the effect of TQ reported inhibitory effect of the compound on endothelial cell migration [41]. *In vitro* tube formation assay demonstrates differentiation of HUVECs; it mimics *in vivo* model of angiogenesis [48]. The extracts A3 and B3 significantly altered the architectural capillary tube networks in dose-dependent manner. B3 showed more efficient inhibition of tube formation, where 60 and 40  $\mu$ g/mL of A3 resulted in  $48.29 \pm 0.87$  and  $27.06 \pm 2.78\%$  of inhibition, respectively. Where as, 60 and 40  $\mu$ g/mL of B3 resulted in  $57.86 \pm 1.12$  and  $41.47 \pm 2.70\%$  of inhibition. It has been reported that TQ resulted in a significant inhibition of endothelial cell tube formation [41]. Measuring of VEGF expression is one of the important methods for

evaluating angiogenesis inhibition. There are many isoforms of VEGF, such as VEGF-121, VEGF-165 and VEGF-189, but VEGF-165 plays the predominant effective role in angiogenesis mechanism, its expression is regulated by hypoxic condition of tumour [49,50]. In this study, A3 and B3 stopped angiogenesis cascade, by counteracting VEGF-165 expression significantly. The inhibitory effect of these two extracts on VEGF expression pathway leads to down-regulation of hypoxia [51,52]. The inhibition of VEGF expression prevents binding of HIF-1 with HREs, so it antagonizes hypoxic conditions of tumour [53,54]. This activity of A3 and B3 might be due to their content of TQ, which has showed significant inhibition of VEGF expression [41].

GC-MC data showed that the 12 SC-CO<sub>2</sub> extracts of *N. sativa* seeds resulted in clear differences in their constituents. This explained the reported variation in antiangiogenic activities of the extracts. GC-MS qualitative analysis resulted in semi-quantitative identification of twenty-two compounds in the extracts. The most active extracts (A3, B3, C3 and D3) contained many compounds that were detected only in these active extracts at different ratios. They include TQ, cetane, octadecane, phytane, methyl stearate and 2-linoleoyl glycerol. Eicosane was detected in the extract A3 and hexacosane in B3. Three other compounds (thymohydroquinone, heptadecane and methyl (11E,13E)-11,13-icosadienoate) were obviously reported in the extracts B3, C3 and D3. In addition, the extracts C3 and D3 exhibited presence of pristane. Previous studies reported various bioactivities of these compounds, such as cytotoxic and antitumour potential of TQ [21,41], thymohydroquinone [55,56] and pristane [57].

Many studies have revealed the contribution of antioxidants in cancer prevention as apoptosis inducers or as antiangiogenic agents [58,59]. TQ has showed a good scavenging activity for superoxide radical [60]. The results of antioxidant activities of the extracts showed that the most active extract was D1 ( $EC_{50} = 0.86 \pm 0.04$  mg/mL); while, the least activity was recorded by the extract A3 ( $EC_{50} = 4.47 \pm 0.16$  mg/mL). The extraction pressure showed a positive correlation with the antioxidant activity significantly. On the other hand, there is a significant negative correlation between the extraction temperature and antioxidant activity. Antioxidant activity of the natural extracts is linked to the presence of phenolic constituents [60,61]. It is suggested that phenolic content has anticancer activity [62-64]. These extracts showed good content of polyphenolics and total flavonoids content as published in our article [14].

The extracts A3 and B3 resulted in a significant inhibition of sprouting of new blood vessels of CAM assay. A3 has significantly higher level of activity (percentage of inhibition was  $63.67 \pm 2.55\%$ ) than B3 ( $51.75 \pm 3.68\%$ ) ( $P < 0.05$ ). However the level of TQ in A3 is not significantly different to B3. This suggests that the other compounds present in A3 may also contribute to its *in vitro* and *in vivo* antiangiogenic activity. Compounds such as eicosane, palmitic acid and androstane-3,17-diol were present in A3 but absent in B3. The effect of eicosane on angiogenesis has not been thoroughly investigated however palmitic acid has been found to increase angiogenesis by up-regulating IL8 [65], hence may not have contributed towards the antiangiogenic activity by A3. The other compound present is androstane-3,17-diol, where the related compound androsten-3,17-diol has been showed to have anti-inflammatory response by up-regulating IL-4 and IFN- $\gamma$ . IL-4 and IFN- $\gamma$  are potent inhibitors of angiogenesis [66,67]. Therefore it is probable that the additional potency in antiangiogenic activity by A3 may be due to the androstane-3,17-diol. Thymoquinone, an active principle of black seeds has resulted in antitumour effects [55] and suppressed proliferation, migration, invasion and tube formation of

HUVECs. In addition, TQ restricted neovascularization on rat aortic ring assay [41].

## Conclusion

In this study, the oil of *N. sativa* seeds was extracted using a SC-CO<sub>2</sub> extraction method, which was developed to target angiogenesis cascade. Angiogenesis plays important role in tumorigenesis. The study reveals that SC-CO<sub>2</sub> extraction can be useful to produce antiangiogenic extracts from *N. sativa* seeds. It is reported that the antiangiogenesis potency of this extract is highly dependent on extraction temperature and pressure. The higher temperature (60°C) and lower pressure (2500 and 3000 psi) produced the most potent antiangiogenic extracts against HUVECs. TQ is the key compound that has an important role in inhibiting angiogenesis. Another compound androstane-3,17-diol, is suspected to have acted synergistically with TQ to cause selective anticancer activity of the A3 extract. The extracts inhibited angiogenesis *in vitro* and *in vivo* by disrupting endothelial cell migration and tube formation by down-regulation of VEGF expression. It caused selective cytostatic activity against endothelial cells. The antiangiogenic activity could be due to the anti-oxidant nature of the extracts.

## Acknowledgements

We would like to thank Universiti Sains Malaysia for supporting this study by a research university team (RUT) grant number 1001/PFARMASI/851001 and the postgraduate student research grant (1001/PFARMASI/834052). Our thanks to the Institute of Health Sciences in Seiyun – Hadramaut and Ministry of Health in Yemen for awarding the financial scholarship to support this study.

## References

- Dietrich A, Gudermann T (2008) Another TRP to endothelial dysfunction: TRPM2 and endothelial permeability. *Circ Res* 102: 275-277.
- Dougher M, Terman BI (1999) Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. *Oncogene* 18: 1619-1627.
- Zeng H, Sanyal S, Mukhopadhyay D (2001) Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. *The Journal of biological chemistry*, 276: 32714-324719.
- Mitoshi M, Kuriyama I, Nakayama H, Miyazato H, Sugimoto K, et al. (2012) Effects of essential oils from herbal plants and citrus fruits on DNA polymerase inhibitory, cancer cell growth inhibitory, antiallergic, and antioxidant activities. *J Agric Food Chem*, 60: 11343-11350.
- Gautam N, Mantha AK, Mittal S (2014) Essential oils and their constituents as anticancer agents: a mechanistic view. *Biomed Res Int* 2014: 154106.
- Zu Y, Yu H, Liang L, Fu Y, Efferth T, et al. (2010) Activities of ten essential oils towards *Propionibacterium acnes* and PC-3, A-549 and MCF-7 cancer cells. *Molecules* 15: 3200-3210.
- Buckle J (1999) Use of aromatherapy as a complementary treatment for chronic pain. *Altern Ther Health Med* 5: 42-51.
- Sylvestre M, Pichette A, Longtin A, Nagau F, Legault J (2006) Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe. *J Ethnopharmacol* 103: 99-102.
- Reverchon E, De Marco I (2006) Supercritical fluid extraction and fractionation of natural matter. *The Journal of Supercritical Fluids* 38: 146-166.
- Brunner G (2005) Supercritical fluids: technology and application to food processing. *Journal of Food Engineering* 67: 21-33.
- Herrero M, Mendiola JA, Cifuentes A, Ibáñez E (2010) Supercritical fluid extraction: Recent advances and applications. *J Chromatogr A* 1217: 2495-2511.
- Malik S (1985) Studies on the chemical constituents of *Rhazya stricta* and *Nigella sativa*. Doctor of Philosophy, University of Karachi.
- Nadkarni KM (1976) *Indian Materia Medica*. 3rd edn. Mumbai: Popular Prakashan Pvt. Ltd.
- Baharetha HM, Nassar ZD, Aisha AF, Ahamed MB, Al-Suede FS, et al. (2013) Proapoptotic and antimetastatic properties of supercritical CO<sub>2</sub> extract of *Nigella sativa* Linn. against breast cancer cells. *J Med Food* 16: 1121-1130.
- Akhtar MS, Riffat S (1991) Field trial of *Saussurea lappa* roots against nematodes and *Nigella sativa* seeds against cestodes in children. *J Pak Med Assoc* 41: 185-187.
- Salman RA, Khan RA, Shukla I (2008) Antimicrobial activity of *Nigella sativa* Linn. seed oil against multi-drug resistant bacteria from clinical isolates. *Natural Product Radiance* 7: 10-14.
- Salem ML, Hossain MS (2000) Protective effect of black seed oil from *Nigella sativa* against murine cytomegalovirus infection. *Int J Immunopharmacol* 22: 729-40.
- Al-Ghamdi MS (2001) The anti-inflammatory, analgesic and antipyretic activity of *Nigella sativa*. *J Ethnopharmacol* 76: 45-48.
- Abdel-Fattah AM, Matsumoto K, Watanabe H (2000) Antinociceptive effects of *Nigella sativa* oil and its major component, thymoquinone, in mice. *Eur J Pharmacol* 400: 89-97.
- Ramadan MF, Kroh LW, Marsel JT (2003) Radical scavenging activity of black cumin (*Nigella sativa* L.), coriander (*Coriandrum sativum* L.), and niger (*Guizotia abyssinica* Cass.) crude seed oils and oil fractions. *Journal of Agricultural and Food Chemistry* 51: 6961-6969.
- Worthen DR, Ghosheh OA, Crooks PA (1998) The *in vitro* anti-tumor activity of some crude and purified components of blackseed, *Nigella sativa* L. *Anticancer Res* 18: 1527-1532.
- Swamy SM, Tan BK (2000) Cytotoxic and immunopotentiating effects of ethanolic extract of *Nigella sativa* L. seeds. *J Ethnopharmacol* 70: 1-7.
- Alhaj NA, Shamsudin MN, Zamri HF, Abdullah R (2008) Extraction of essential oil from *Nigella sativa* using supercritical carbon dioxide: study of antibacterial activity. *American Journal of Pharmacology and Toxicology* 3: 225-228.
- Edris AE (2009) Anti-cancer properties of *Nigella* spp. essential oils and their major constituents, thymoquinone and beta-elemene. *Curr Clin Pharmacol* 4: 43-46.
- Al-Naqeeb G, Ismail M, Al-Zubairi AS (2009) Fatty acid profile,  $\alpha$ -tocopherol content and total antioxidant activity of oil extracted from *Nigella sativa* seeds. *International Journal of Pharmacology* 5: 244-250.
- El-Dakhkhny M (1963) Studies on the chemical constitution of Egyptian *Nigella sativa* L. seeds. II) The Essential Oil. *Planta Med* 11: 465-470.
- Mahfouz M, Eldakhkhny M (1960) The isolation of a crystalline active principle from *Nigella sativa* L. seeds. *J Pharm Sci UAR* 49: 1-19.
- Hajhashemi V, Ghannadi A, Jafarabadi H (2004) Black cumin seed essential oil, as a potent analgesic and antiinflammatory drug. *Phytother Res* 18: 195-199.
- Burits M, Bucar F (2000) Antioxidant activity of *Nigella sativa* essential oil. *Phytother Res* 14: 323-328.
- Duke JA (2003) *CRC handbook of medicinal spices*, Boca Raton, FL, CRC Press.
- Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* 65: 55-63.
- Goodwin AM (2007) *In vitro* assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents. *Microvasc Res* 74: 172-183.
- Franken NA, Rodermond HM, Stap J, Haveman J, van Bree C (2006) Clonogenic assay of cells *in vitro*. *Nat Protoc* 1: 2315-2319.
- Liang CC, Park AY, Guan JL (2007) *In vitro* scratch assay: a convenient and inexpensive method for analysis of cell migration *in vitro*. *Nat Protoc* 2: 329-333.
- Ahamed MB, Aisha AF, Nassar ZD, Siddiqui JM, Ismail Z, et al. (2012) Cat's whiskers tea (*Orthosiphon stamineus*) extract inhibits growth of colon tumor in nude mice and angiogenesis in endothelial cells via suppressing VEGFR phosphorylation. *Nutr Cancer* 64: 89-99.
- Guidolin D, Albertin G, Ribatti D (2010) Exploring *in vitro* angiogenesis by image analysis and mathematical modeling. *Microscopy: Science, Technology, Applications and Education* 2: 876-884.

37. Nassar ZD, Aisha AF, Ahamed MB, Ismail Z, Abu-Salah KM, et al. (2011) Antiangiogenic properties of Koetjapic acid, a natural triterpene isolated from *Sandoricum koetjaoe* Merr. *Cancer Cell Int* 11: 12.
38. Kitson FG, Larsen BS, Mcewen CN (1996) *Gas chromatography and mass spectrometry: A practical guide*, San Diego, Academic Press.
39. Salomi MJ, Nair SC, Panikkar KR (1991) Inhibitory effects of *Nigella sativa* and saffron (*Crocus sativus*) on chemical carcinogenesis in mice. *Nutr Cancer* 16: 67-72.
40. Salomi NJ, Nair SC, Jayawardhanan KK, Varghese CD, Panikkar KR (1992) Antitumour principles from *Nigella sativa* seeds. *Cancer Lett* 63: 41-46.
41. Yi T, Cho SG, Yi Z, Pang X, Rodriguez M (2008) Thymoquinone inhibits tumor angiogenesis and tumor growth through suppressing AKT and extracellular signal-regulated kinase signaling pathways. *Molecular Cancer Therapeutics* 7: 1789-1796.
42. Wong ML, Prawira A, Kaye AH, Hovens CM (2009) Tumour angiogenesis: its mechanism and therapeutic implications in malignant gliomas. *J Clin Neurosci* 16: 1119-1130.
43. Critser PJ, Yoder MC (2010) Endothelial colony-forming cell role in neovascularization and tissue repair. *Curr Opin Organ Transplant* 15: 68-72.
44. Chen JX, Lawrence ML, Cunningham G, Christman BW, Meyrick B (2004) HSP90 and Akt modulate Ang-1-induced angiogenesis via NO in coronary artery endothelium. *J Appl Physiol* (1985) 96: 612-620.
45. Valster A, Tran NL, Nakada M, Berens ME, Chan AY, et al. (2005) Cell migration and invasion assays. *Methods* 37: 208-215.
46. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, et al. (2003) Cell migration: integrating signals from front to back. *Science* 302: 1704-1709.
47. Tonini T, Rossi F, Claudio PP (2003) Molecular basis of angiogenesis and cancer. *Oncogene* 22: 6549-6556.
48. Madri JA, Pratt BM, Tucker AM (1988) Phenotypic modulation of endothelial cells by transforming growth factor-beta depends upon the composition and organization of the extracellular matrix. *J Cell Biol* 106: 1375-1384.
49. Cheng SY, Nagane M, Huang HJS, Cavenee WK (1997) Intracerebral tumor-associated hemorrhage caused by overexpression of the vascular endothelial growth factor isoforms VEGF121 and VEGF165 but not VEGF189. *Proceedings of the National Academy of Sciences* 94: 12081-12087.
50. Ferrara N (2001) Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 280: C1358-1366.
51. Folkman J (2002) Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* 29: 15-18.
52. Shweiki D, Itin A, Soffer D, Keshet E (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359: 843-845.
53. Harris AL (2002) Hypoxia--a key regulatory factor in tumour growth. *Nature reviews. Cancer*, 2, 38-47.
54. Wenger RH (2002) Cellular adaptation to hypoxia: O<sub>2</sub>-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O<sub>2</sub>-regulated gene expression. *FASEB J* 16: 1151-1162.
55. Ivankovic S, Stojkovic R, Jukic M, Milos M, Milos M, et al. (2006) The antitumor activity of thymoquinone and thymohydroquinone in vitro and in vivo. *Exp Oncol* 28: 220-224.
56. Lee S, Ackland-Berglund C, Jones C (1992) The tumor promoter pristane activates transcription by a cAMP dependent mechanism. *Molecular and Cellular Biochemistry* 110: 75-81.
57. Collins AR (2005) Antioxidant intervention as a route to cancer prevention. *Eur J Cancer* 41: 1923-1930.
58. Thapa D, Ghosh R (2012) Antioxidants for prostate cancer chemoprevention: challenges and opportunities. *Biochem Pharmacol* 83: 1319-1330.
59. Nagi MN, Alam K, Badary OA, Al-Shabanah OA, Al-Sawaf HA (1999) Thymoquinone protects against carbon tetrachloride hepatotoxicity in mice via an antioxidant mechanism. *Biochem Mol Biol Int*, 47: 153-159.
60. Hatano T, Kagawa H, Yasuhara T, Okuda T (1988) Two new flavonoids and other constituents in licorice root: their relative astringency and radical scavenging effects. *Chem Pharm Bull (Tokyo)* 36: 2090-2097.
61. Gulcin I, Oktay M, Kufrevioglu OI, Aslan A (2002) Determination of antioxidant activity of lichen *Cetraria islandica* (L.) Ach. *J Ethnopharmacol* 79: 325-329.
62. Aisha AFA, Nassar ZD, Siddiqui MJ, Abu-Salah KM, Alrokayan, Ismail SA, et al. (2011) Evaluation of antiangiogenic, cytotoxic and antioxidant effects of *Syzygium aromaticum* L. extracts. *Asian J. Biol. Sci* 4: 282-290.
63. Hocman G (1988) Chemoprevention of cancer: phenolic antioxidants (BHT, BHA). *Int J Biochem* 20: 639-651.
64. Stagos D, Amoutzias GD, Matakos A, Spyrou A, Tsatsakis AM, et al. (2012) Chemoprevention of liver cancer by plant polyphenols. *Food Chem Toxicol* 50: 2155-2170.
65. Joshi-Barve S, Barve SS, Amancherla K, Gobejishvili L, Hill D, et al. (2007) Palmitic acid induces production of proinflammatory cytokine interleukin-8 from hepatocytes. *Hepatology* 46: 823-830.
66. Volpert OV, Fong T, Koch AE, Peterson JD, Waltenbaugh C, et al. (1998) Inhibition of angiogenesis by interleukin 4. *J Exp Med* 188: 1039-1046.
67. Hayakawa Y, Takeda K, Yagita H, Smyth MJ, Van Kaer L, et al. (2002) IFN-gamma-mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, alpha-galactosylceramide. *Blood* 100: 1728-1733.