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

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**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 supper critical 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 ± 1.9 and 60.2 ± 2.2 µg/mL, respectively. The extracts down-regulated the expression of vascular endothelial growth factor (VEGF) in endothelia 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-CO₂ oil as anti-angiogenic agent.

**Keywords:** *Nigella sativa*; Supercritical CO₂ extraction; Anti-angiogenesis; Antioxidant

**Introduction**

Angiogenesis is vitally required for the physiological functions such as reproduction, vasularization 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-CO₂) is the most common supercritical fluid used in SFE technology; it is fast, non-explosive, non-toxic, 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 CO₂ 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,

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


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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, hederaegenin, indole-3-acetic-acid, rutin and thymol [30].

In present study an attempt was made to optimize the different extraction parameters using supercritical CO2 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-CO2 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 sulphoxide (DMSO), gallic acid, thymoquinone (TQ), suramin and betulinic acid were obtained from Sigma-Aldrich, USA. MTT (3-(4,5-Dimethylthiazol-2-yl)- 2,5diphenyl tetrazolium bromide) and DPPH (2,2-diphenyl-1-picrylhydrazyl) reagents were purchased from Sigma-Aldrich, Germany. 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 medium 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% 1-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 IC50 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-(A0/A)Z) × 100, Where; A0=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 IC50 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

Colon 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,
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-tailed pearson test; R² values were calculated by linear regression test.

**Results**

The dried powder of *N. sativa* seeds were extracted with SC-CO₂ 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-neovascularization assay on rat aortic explants. Figure 1 illustrates the GCMS chromatograms.

<table>
<thead>
<tr>
<th>Extract</th>
<th>SC-CO₂ extraction parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pressure (psi)</td>
</tr>
<tr>
<td>A1</td>
<td>2500</td>
</tr>
<tr>
<td>A2</td>
<td>2500</td>
</tr>
<tr>
<td>A3</td>
<td>2500</td>
</tr>
<tr>
<td>B1</td>
<td>3000</td>
</tr>
<tr>
<td>B2</td>
<td>3000</td>
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<tr>
<td>B3</td>
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<tr>
<td>C1</td>
<td>4500</td>
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<tr>
<td>C2</td>
<td>4500</td>
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<tr>
<td>C3</td>
<td>4500</td>
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<tr>
<td>D1</td>
<td>6000</td>
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<tr>
<td>D2</td>
<td>6000</td>
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<td>t</td>
<td>6000</td>
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</tbody>
</table>

**Table 1**: Codes of the SC-CO₂ extracts of *N. sativa* according to the extraction parameters of pressure and temperature.
Figure 1: Gas chromatograms of the extracts.
Table 2 shows that A3 and B3 showed significant (P<0.05) antiangiogenic effect with IC$_{50}$ 90.75 ± 4.75 and 98.60 ± 2.91 µg/mL, respectively whereas, C3 and D3 demonstrated a moderate activity with IC$_{50}$ 109.40 ± 3.53 and 125.44 ± 7.93 µg/mL, respectively. The dose-response curves of the active extracts were depicted in Figure 2c.

Table 3 illustrates that at a concentration of 100 µg/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 µg/mL was 54.38 ± 5.17, 46.88 ± 5.18, 49.10 ± 6.32 and 40.27 ± 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.

<table>
<thead>
<tr>
<th>Extract</th>
<th>IC$_{50}$ (µg/mL)</th>
<th>% of migration</th>
<th>% of proliferation</th>
<th>% of plating efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>A3</td>
<td>90.75 ± 4.75</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B3</td>
<td>98.60 ± 2.91</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C3</td>
<td>109.40 ± 3.53</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D3</td>
<td>125.44 ± 7.93</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TQ</td>
<td>12.69 ± 1.44</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2: IC$_{50}$ (µg/mL) of A3, B3, C3 and D3 on inhibition of neovascularization (rat aortic ring assay).

The IC$_{50}$s (µg/mL) of A3, B3, C3, D3 and TQ on inhibition of neovascularization in rat aortic explants. The percentage inhibition of 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 µg/mL was 54.38 ± 5.17, 46.88 ± 5.18, 49.10 ± 6.32 and 40.27 ± 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.

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 µg/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 µg/mL of extract.

**Antioxidant activity**

Effect of the SC-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-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°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 µg/mL. The extracts A3 and B3 exhibited dose-dependent anti-proliferative effect with IC$_{50}$ 41.50 ± 1.92 and 60.21 ± 2.19 µg/mL, respectively (Figure 4). The IC$_{50}$ of the positive controls, TQ and betulinic acid were 2.39 ± 0.18 and 3.73 ± 0.20 µg/mL, respectively. The result obtained in MTT assay for the SC-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 ± 2.4% which was drastically reduced by the treatment of A3 and B3 with PE 34 ± 1.7 and 21 ± 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 ± 1.37 and 54.22 ± 2.71% after A3 and B3 treatment at the concentrations 20 µg/mL, respectively (Figure 5b). These results can be compared with that of the standard reference, betulinic acid (20 µg/mL) which produced the PE of 11.13 ± 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 µg/mL) calculated after 12 and 18 h was 29.04 ± 2.34 and 52.35 ± 2.65%, respectively (p<0.05). However, B3 resulted in 46.42 ± 1.79 and 41.88 ± 1.95% of inhibition at 12 and 18 h, respectively. Interestingly, at lower concentration (15 µg/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 µg/mL, A3 produced 27.06 ± 2.78 and 48.29 ± 0.87% inhibition, respectively. Similarly, B3 exhibited significant inhibitory effect on tube formation with 41.47 ± 2.70 and 57.86 ± 1.12% at 40 and 60 µg/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 µg/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 ± 7.34 pg/mL, which was drastically reduced to 77.18 ± 8.43 and 67.8 ± 5.25 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 ± 2.55% which was significantly higher than that of B3 (51.75 ± 3.68%). The positive control (50 µg/disk Suramin) showed 63.47 ± 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-CO₂ 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-CO₂ 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-CO₂
Figure 3: Correlation of the extraction pressure and temperature on DPPH scavenging activity of the extracts. a) EC_{50} of the extracts on DPPH scavenging as a function of pressure (psi). b) EC_{50} 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_{50}=41.50 ± 1.92 and 60.21 ± 2.19 µg/mL, respectively, (MTT assay).
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 line: 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 IC50 value of 53.34 ± 2.15 µg/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, invasion, tube formation and VEGF expression [41].

The anti-proliferative activity of the SC-CO2 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 (IC50 of 41.50 ± 1.92 µg/mL) showed stronger activity than B3 (IC50 of 60.21 ± 2.19 µg/mL) as tested by MTT assay. Angiogenic activity of the 12 different extracts of SC-CO2 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

**Peak no.** | **RT min** | **Compounds** | **Molecular formula** | **Molecular weight** |
---|---|---|---|---|
1 | 6.27 | TQ | C10H12O2 | 164.08 |
2 | 6.86 | (+)-α-longipinene (sesquiterpene) | C15H24 | 204.19 |
3 | 7.25 | (+)-longifolene (sesquiterpene) | C15H24 | 204.19 |
4 | 8.36 | Thymohydroquinone | C10H14O2 | 166.1 |
5 | 8.52 | Cetane (hexadecane) | C16H34 | 226.27 |
6 | 9.1 | Heptadecane | C17H36 | 240.28 |
7 | 9.13 | Pristane (norphytane) (terpenoid) | C19H40 | 268.31 |
8 | 9.65 | Octadecane | C18H38 | 254.3 |
9 | 9.7 | Phytane | C20H42 | 282.33 |
10 | 10.17 | Eicosane | C20H42 | 282.33 |
11 | 10.15 | Methyl palmitate (metholene 2216) | C17H34O2 | 270.26 |
12 | 10.41 | Squalene (triterpene) | C30H50 | 410.39 |
13 | 10.59 | Palmitic acid (fatty acid) | C16H32O2 | 256.24 |
14 | 10.95 | Methyl octadeca-8,11-dienoate | C19H34O2 | 294.26 |
15 | 11.28 | Methyl stearate | C19H38O2 | 298.29 |
16 | 11.25 | Linoleic acid (linolic acid) (fatty acid) | C18H32O2 | 280.24 |
17 | 11.51 | 9-Eicosyne | C16H30 | 278.3 |
18 | 11.78 | Androstane-3,17-diol ((3α,5α)-androstane-3,17-diol) (neurosteroid) | C18H26O | 292.24 |
19 | 12.05 | Methyl (11E,13E)-11,13-icosadienoate | C20H32O2 | 322.29 |
20 | 13.29 | Hexacosane (cerane) | C26H52 | 366.42 |
21 | 13.82 | 2-Linoleoyl glycerol (monolinolein) | C19H38O4 | 354.28 |
22 | 14.22 | trans-squalene (E,E,E-squalene) | C40H72 | 410.39 |

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

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

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

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. Extracts did not show promising anti-angiogenic effect and thymoquinone, thymo-hydroquinone was not detected.
None
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$_2$ extraction method, which was developed to target angiogenesis cascade. Angiogenesis plays important role in tumourigenesis. The study reveals that SC-CO$_2$ 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 androstande-3,17-diol, is suspected to have acted synergistically with TQ to cause selective anticaner 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 Ministry of Health in Yemen for awarding the financial scholarship to support this study.

References


