Antioxidative, Antiproliferative and Antiangiogenic Activities of *Nigella sativa* L. pulp, a Waste Material Remaining from Oil Production

Yağ Çıkarma İşlemi Sonrasında Atık Materyal Olarak Ortaya Çıkan Çörek Otu Posasının Anti-oksidan, Anti-proliferatif ve Anti-anjiyojenik Özellikleri

Research Article

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ABSTRACT

The aim of this study is to investigate the biological activities such as antioxidative, antimetastatic and antiangiogenic properties of the *Nigella sativa* L. (NS) pulp, which is a waste material of the oil production process. Following the investigation of the characteristic properties of NS pulp extract with UV-visible (UV-vis), fourier-transform infrared (FTIR) and gas chromatography-mass spectroscopy (GC-MS) measurements, its antioxidative capacity was determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Trolox equivalent antioxidant capacity (TEAC) assays. Antiproliferative effects of the pulp extract were tested on both cancerous and non-cancerous cells to examine whether the effects are specific to cell types. Antiangiogenic tests were performed via chick chorioallantoic membrane (CAM) assay. The results showed that the NS pulp extract still maintained its radical scavenging effects and reduced the proliferation of cancer cells more efficiently compared to non-cancerous cells. Furthermore, CAM assays demonstrated that the pulp extract effectively limited vascular endothelial growth factor (VEGF)-stimulated vascularization and development. NS pulp might be used for the suppression of the metastatic cell populations and for the treatment of the diseases particularly like cancer, which progress with pathological angiogenesis. This study highlights that such waste materials from plants can be recycled with various uses in many fields.

Key Words
Nigella sativa, cancer treatment, antiangiogenic activity, chorioallantoic membrane assay.

ÖZ

Bu çalışmanın amacı, yağ çıkarma işlemi sonrasında atık materyal olarak ortaya çıkan çörek otu (*Nigella sativa* L.-NS) posasının anti-oksidan, anti-metastatik ve anti-anjiyojenik özelliklerinden yola çıkarak biyolojik etkinliklerini incelemektir. NS posasından çıkarılan özütün UV-görünür bölge (UV-vis), Fourier Dönüşümlü Kızılötesi (FTIR) ve gaz kromatografisi-kütle spektrometresi (GC-MS) ölçümleriyle karakteristiksel özellikleri incelendikten sonra 2,2-difenil- 1-pikrilhidrazil (DPPH) ve Trolox eşdeğer antioksidan kapasitesi (TEAC) yöntemleri kullanılarak anti-oksidatif kapasitesi tayin edilmiştir. Posa özütünün anti-proliferatif etkileri, etkilerin hücre tipine özgü olup olmadığını incelemek amacıyla hem kanser hem de kanser olmayan hücreler üzerinde test edilmiştir. Anti-anjiyojenik testler civciv korioallantoik membran testi (CAM) aracılığıyla gerçekleştirilmiştir. Sonuçlar NS posa ekstratının radikal süpürücü etkilerini halen muhafaza ettiğini ve normal hücreler ile karşılaştırıldığında kanser hücre proliferasyonunu daha etkin bir biçimde düşürdüğünü göstermiştir. Ayrıca CAM testleri NS posa özütünün VEGF uyarımlı vaskülarizasyon ve gelişimini etkin bir biçimde sınırladığını göstermiştir. NS posa özütü metastatik hücre topluluklarının baskılanmasında ve kanser başta olmak üzere patolojik anjiyogenez ile seyreden hastalıkların tedavisinde faydalı sonuçlar üretebilir. Bu çalışma bitkisel kaynaklı atık maddelerin birçok alanda çok çeşitli amaçlar doğrultusunda kullanılmak üzere geri dönüştürülebileceğine dikkat çekmektedir.

Anahtar Kelimeler
Çörek otu, kanser tedavisi, anti-anjiyojenik aktivite, korioallantoik membran testi.

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INTRODUCTION

Plant-based therapies, also called phytotherapy, have been in use since ancient times to relieve various diseases and disorders. The effects of natural compounds from plant extracts on diseases are still an intensive research area. In this area, which has gained great popularity nowadays, many plant extracts such as *Nigella sativa* L. (NS), *Allium sativum, Curcuma longa, Cinnamomum zeylanicum, Syzygium aromaticum* have been studied for their radical scavenging, antioxidant, antibacterial and anticancerogenic properties. Among medicinal plants, NS also known as 'black cumin or seed', is an annual flowering plant belonging to *Ranunculaceae* family which grows in the Middle East, Western Europe, Eastern and Central Asia [1]. NS is a species that draws attention with its therapeutic effect from medical perspective as well as being used as food and spice [2-5]. NS seed is added into tea, coffee or bread and used in the production of the canned goods. Its ground seeds can be mixed with honey or can be tossed in the salads. NS seed is used for many pharmaceutical purposes beyond its uses in food. NS has been used in the Middle and the Far East as a natural medicine for more than 2000 years [6]. NS falls into the category of herbs that are believed to possess a healing trait in Islamic communities (known as "Habbat al-Sauda", a remedy of every disease) [6-7]. NS, stated as the healing black seed in the Bible, is natively used plant, which pharaohs have declared to accompany eternity, Hippocrates and Dioscorides referred to NS as "Melanthion" in their drug recipes [6]. NS is called as "Kalonji" and "Karayal" in south and south-west Asia, as 'Panacea' in old Latin and as "Hak Jung Chou" in China[6,8]. NS has been used as a folk medicine to treat intestinal, respiratory, gastric disorders, and to support immune and circulatory system as well as to maintain general well-being [7]. It is useful to regulate hypertension and to remedy bacterial diseases [9]. By mixing with other ingredients, it is also used to remove bad-breath odor and mouth-watering as well as being used for the treatment of diarrhea, dyspepsia, indigestion, burping[10]. Many phytochemical and bioactivity studies have been conducted on NS in addition to its remarkable traditional uses [9].The fixed oil in its seeds is particularly rich in polyunsaturated fatty acids, contributing to its antioxidant [11-14], antiallergic [14-15], anti-inflammatory [14-16], immune modulating [14], antibacterial [17-19], antiviral [14], antitumoral [14,20-21], antidiabetic [17,20,22], and hepatoprotective [17,23] effects, as well as, contributing to its effects on the cardiovascular- hematological [20,24-25], and gastrointestinal systems [20].

The majority of NS-related studies in the literature have been conducted with the seeds, which have been obtained from the plant grown in the countries, where it is widely consumed or with the seed oil, which is easily available in the market. We have also noticed that the phytochemical studies have mainly focused on seeds and the other parts of the plant have not been investigated sufficiently yet. Nevertheless, it is possible to detect significant amounts of several important compounds like vitamins and proteins or their solid substances in the remaining seedcake (pulp) after the oil or extract production process. In fact, this pulp can be used in several products. They are used in the production of food additives for forage, herbal additives, soil fertilizers, cosmetics, pharmaceuticals and products in several other sectors. Therefore, unlike the studies in the literature, this study aims to investigate the phytochemical and biological activities of the extract, which is obtained from the pulp produced as a by-product of the oil production and to compare our results with the findings in the literature obtained from the seed extract and oil. This also enables to determine the potential of the pulp extract for the use as food and as medical substances. For this purpose, we have tried to investigate the radical scavenging and antioxidative capacity of the NS pulp extract and to demonstrate the relationship between the antioxidative effect and chemical compounds in the extract with fourier transform infrared (FTIR) and ultraviolet visible (UV-vis) spectroscopy measurements. In order to investigate the biological activities of the NS pulp extract, antiproliferation and antiangiogenesis tests have been performed. We have evaluated the antiproliferative effect of the NS pulp extract on both cancerous and non-cancerous cells *in vitro* in a comparative manner. In addition, the antiangiogenic properties of the pulp extract have been investigated on *in ovo* and *ex ovo* chick chorioallantoic membrane (CAM) model via vascular endothelial growth factor

(VEGF)-stimulated vascularization. This study, which enables us to compare pulp extract of NS with its seed extract or seed oil, has a guiding role regarding the further studies on biological activity of other pulp extracts.

MATERIALS and METHODS

Preparation of NS Pulp Extract

NS extracts are generally used for their oil obtained by the cold press process of the seeds. At the end of this stage, the remaining pulp is discarded as waste. However, NS pulp also still contains considerable amounts of therapeutic components. In this study, the NS pulp was purchased a local herbal market. Sample was kept under sunshade for drying. The dried sample was grounded thoroughly to powder form. 50g of powder was boiled into 1000 mL sterile distilled water for 5 min and then filtered through Whatman's No.1 filter paper. The extract was kept at 4 ºC in sterile tubes covered by aluminium foil for further experiments. The samples were later evaporated under reduced pressure using a rotary evaporator (Labconco) for FTIR analysis.

UV-vis Spectroscopy

UV-Vis spectroscopic study was carried out using a Shimadzu UVmini-1240 UV-vis spectrophotometer. Samples were scanned at the wavelength range from 300 to 600 nm.

FTIR Instrumentation and Spectral Measurement

FTIR experiment was carried out to determine the biomolecules present in the seed extract. FTIR spectra of NS pulp extract were recorded at room temperature (ca. 22°C) using a Nicolet Avatar 380 spectrometer (Thermo Electron Inc., San Jose, CA) scanning over the frequency range of 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}. The peaks obtained were analysed by using a standard IR spectra table.

GC-MS Analysis of Extract

Fatty acid composition of NS pulp extract was investigated by GC-MS analysis. NS pulp extract was dissolved in methanol, vortexed vigorously and then filtered. The fatty acid methyl esters were analyzed using an Agilent Technologies 5975C VL MSD with a triple-axis detector system and an Agilent 7890A GC system. The column was 5MS (60m x 0.25 mm ID, 0.25 nm film thickness). Carrier gas was helium (1.5 mL/min). GC oven temperature was programmed as 5 min at 50°C initially, then heated up to 220°C at the rate of 2°C min (total 90 min). One microliter sample was injected into the system. Injection mode: was adjusted to split mode (20:1). Injector temperature was set at 250°C. Transfer line temperature was set at 280°C. Mass spectrum was operated at 70 eV. Mass range was 70 to 250 m/z. GC Detector or flame ionization detection (FID) was set at 210°C. In order to obtain the same elution order with GC-MS, the column outlet was split into two, one for FID and the other for MS detector.

pH Measurement

Since the acid base balance is extremely important, the pH of the materials to be used must be determined. Prepared extract pH was measured with ISOLAB magnetic mixer and pH / ORP / temperature meter.

Trolox Equivalent Antioxidant Capacity (TEAC)

In accordance with Marinova and Batchvarov [26]'s methods, Trolox standard was prepared at concentrations of 0.5 ppm, 1 ppm, 1.5 ppm, 2 ppm, 2.5 ppm and plotted against % reduction values. Subsequently, % reduction value of sample was determined as the result of absorbance readings and then the trolox concentration value over the graph was calculated. For the spectrophotometric assay, the absorbance was determined at 517 nm.

Radical Scavenging Activity by DPPH Assay

This assay was carried out as described by Kirby and Schmidt [27], Sarikurkcu, *et al*. [28-29] with some alterations suggested by Sanda, *et al*. [30]. 0.5 ml of the methanolic sample extracts and commercial antioxidant butylated hydroxytoluene (BHT) in the methanol was mixed with 3 mL 6.10^{-5} M of a methanol solution of 2, 2-Diphenyl-1-picrylhydrazyl (DPPH). Then the mixture was incubated for 30 min in the dark at room temperature. Following to incubation period, the absorbance of mixture was measured at 517 nm with a spectrophotometer (Beckman Coulter DU 730 UV-Vis Spectrophotometer). Inhibition of

free radical DPPH in percent (I %) was calculated according to equation given below.

$$I(\%) = 100 \times (A_{blank} - A_{sample})/A_{blank} \quad \text{(Equation 1)}$$

Where A_{blank} indicates absorbance of the control, whereas A_{sample} represents absorbance of sample. BHT was considered as the standard antioxidant used for positive control. Results were expressed by calculating IC_{50} values obtained from regression curve. The lower the IC_{50} values point toward the higher antioxidant activity.

Cell Lines

Mouse fibroblastic (L929) and lung adenocarcinoma (A549) cell lines were cultured and grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented by 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Sigma-Aldrich, Germany). The cells were maintained at 37°C under a humidified atmosphere with 5% CO_2. The medium was refreshed two or three times each week.

MTT Assay

The cytotoxic effect and the antiproliferative activity of NS pulp extract were measured *in vitro* on A549 and L929 using with the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay described by Mosmann [31]. Cells ($5x10^3$ cells/well) were separately seeded onto 96-well microplates and incubated at 37°C in a humidified 5% CO_2 atmosphere. On the second day, 200 μL fresh medium containing with different concentrations (0.1-1000 μg/mL) of pulp extract was added. The samples were then incubated for an additional 24h. After 24h of incubation, the medium was removed and 100 μL of serum-free medium containing 5 mg/mL of MTT was added to each well. MTT was converted by intact mitochondrial reductase and precipitated as blue crystals during a 4h contact period. The medium was then removed carefully and 100 μL of isopropyl alcohol was added to each well to solubilize formazan. The plates were agitated for 10 min and absorbance was measured with a plate-reading spectrophotometer at 570 nm (Biochrom EZ Read 400). Absorbance of the treated cells was compared with that of the control, and cells exposed only to normal medium were considered 100% viable. The percentage of cell viability was calculated using the following formula:

$$\text{Cell Viability}(\%) = (\text{Optical Density of Treated Cells}/\text{Optical Density of Control Cells}) \times 100 \quad \text{(Equation 2)}$$

L929 fibroblastic cell line from mouse, which was not tumorigenic, served as controls in order to analyse toxicity of NS pulp extract on normal cells.

CAM Assay

The Atabey type fertilized chicken eggs were incubated at 37°C, at constant humidity. In order to reveal the developing vascularized chorioallantois, on day 3 of incubation, the eggs were removed from the incubator and cleaned with 70% alcohol and a hole is drilled through the pointed pole of the shell. In order to initiate a pathological vascularization condition, 10 ng/mL $VEGF_{165}$ was injected with an insulin syringe on the embryos. After 24h, 100 μL of NS pulp extract at different concentrations (10, 100, and 500 μg/mL) was applied via micropipettes. Then, the holes were covered with cellophane tape. After one day of incubation, the top cover of the eggs was broken. The CAM arterious branches in each treatment group were photographed using Leica stereomicroscope (Leica M205C) fitted with a camera system (Leica DCF295, Heerbrug, Switzerland) and counted by Wimasis online analysis tool (Wimasis GmbH, Germany). The antiproliferative effect of the NS pulp extract was determined as relative numbers of arteriosus branches. The assay was performed three times to ensure reproducibility.

Statistical Analysis

All experiments were performed in triplicate (n=3). The variance analysis was performed to compare means with $p<0.05$ and $p<0.01$ indicating statistical significance. All statistical analyses were performed using IBM SPSS (ver. 21).

RESULTS and DISCUSSION

In the present study, the UV-vis absorption spectrum recorded from NS pulp extract showed an absorption peak at 290 nm attributed to the surface plasmon resonance band (SPR) (Figure 1).

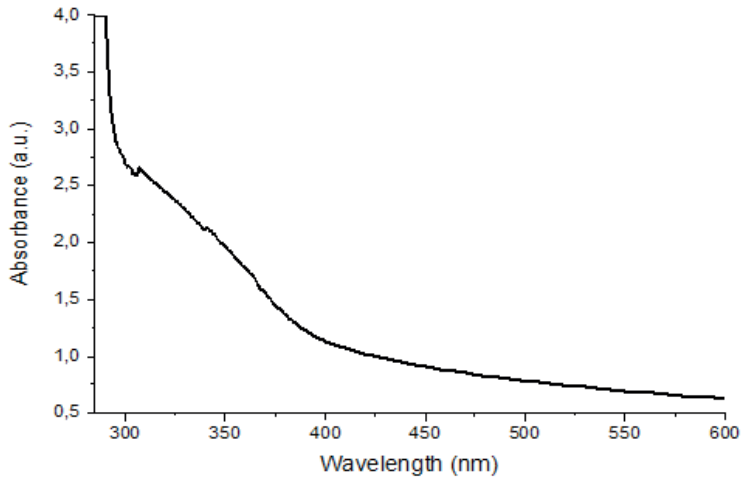

Figure 1. UV-Vis scan of *Nigella sativa* pulp extract. The absorbance measurements were recorded at wavelengths between 300 and 600 nm.

This result is in agreement with the previous findings. Velho-Pereira, *et al.* [32] reported that the macerated extract from the aqueous seed of NS showed the absorbance peak at 287 nm. We also observed the peaks in the UV range corresponding to 300 and 350 nm, which may be attributed to the presence of proteins in the solution released by the NS pulp extract. The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups present within a molecule. Thanks to providing information about fingerprint characteristics of samples and being broadly applicable to the samples, FTIR has a prominent place in pharmaceutical analysis. FTIR measurement was carried out to identify possible biomolecules or bio functional groups of NS pulp extract.

Figure 2 illustrates the infrared spectrum of NS pulp extract in the region from 4000 to 500 cm^{-1}. The OH stretching in Phenol group was observed at 3280.33 cm^{-1}. The CH_3 and CH_2 (C-H in $-CH_3$ and $-CH_2$)

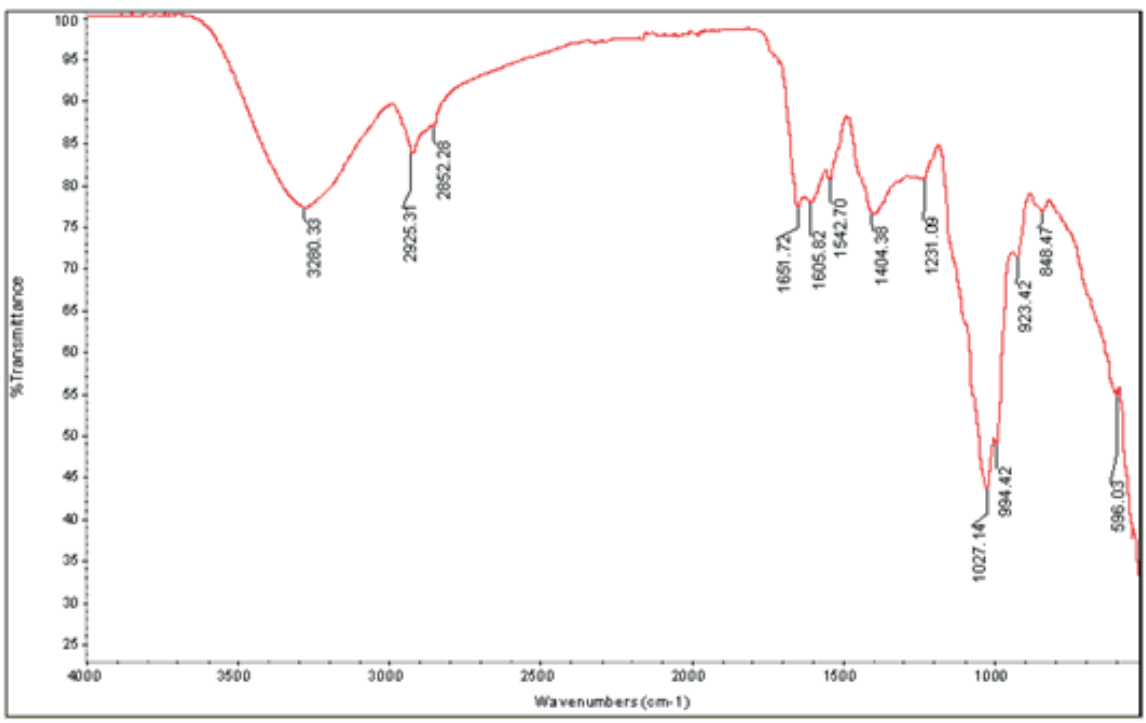

Figure 2. Representative FTIR spectra from dried *Nigella sativa* pulp extract. All wavenumber values are in cm^{-1}.

Table 1. FTIR spectrum of the *Nigella sativa* pulp extract depicts the related functional groups of transmittance bands of the corresponding wavelengths.

Wavelength range (cm^{-1})	NS pulp extract	Assignment	Possible Compounds
3500-3000	3280.33	Bonded –OH groups	Alcohols, Phenols, Acids
2900-2800	2925.31, 2852.28	CH stretching vibration (aliphatic) (CH_3 and CH_2)	Alkanes
1670-1640	1651.72	C=O carbonyl groups	Alkanes
1600-1300	1605.82, 1542.70, 1404.38	C=C stretching, carboxylic groups and N-H vibrations	Aromatics, Amines
1300-1200	1231.09	Carbonyl group	Ester
1150-950	1027.14, 994.42, 923.42, 848.47	C-H bending and C-N stretching	Aliphatic amines, Alkenes
800-500	596.03	C-X stretching	Alkyl halides

asymmetric stretching vibrations were found in the region of 2925 and 2852 cm^{-1}, respectively [33-35]. These characteristic bands indicate the presence of alkanes. The C=O (carbonyl) stretching band was observed at 1651.72 cm^{-1}. The C=C stretching band at 1600-1350 cm^{-1} could be attributed to the presence of aromatic compounds like freely water-soluble flavonoids in NS pulp extract. The bands between the wavenumbers of 1500 to 500 cm^{-1} (fingerprint regions) of the spectrum, for the control reflected the biochemical compositions, especially the moieties of carbohydrate, lipid, protein and polyphenols in NS pulp extract. The FTIR spectroscopy results showed that phenolic and aromatic compounds were present in the NS. which are consistent with the findings of the phytochemical analysis (Table 1). Also, pH value of the sample is measured as 6.27.

GC-MS results showed that the NS pulp extract contains a largely higher proportion of unsaturated fatty acids as compared to saturated oils. The major fatty acids of the pulp are linoleic acid methyl ester or methyl linoleate (28,2 %), linolelaidic acid (17,3%), 1-hexadecyne (17,3%), linoleic acid (16,3%), oleic acid (11,4%), myristic acid (6%), palmitic acid (1,5%) margaric acid (heptadecanoic acid) (1,2%). GC-MS chromatogram, area % and retention times of the compounds identified from pulp extract are given in Figure 3 and Table 2, respectively.

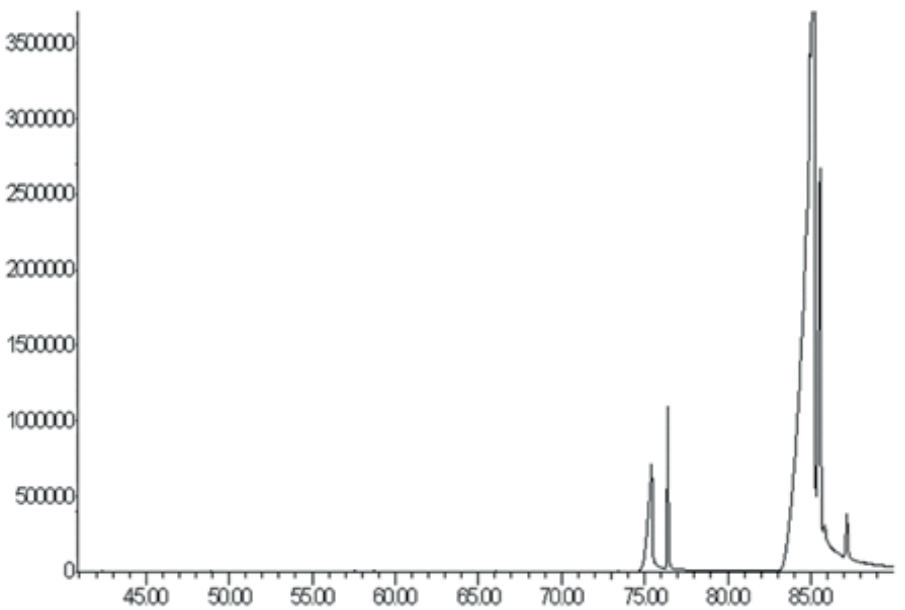

Figure 3. GC-MS chromatogram of main compounds in *Nigella sativa* pulp extract.

Table 2. The main compounds determined by GC-MS in *Nigella sativa* pulp extract.

Pk#	Compound name	%area	Retention Time
1	Myristic Acid	5.601	75.434
2	Palmitic Acid	1.477	76.397
3	Margaric Acid	1.213	76.406
4	Linoleic Acid	16.292	84.428
5	1-Hexadecyne	17.335	84.766
6	Linolelaidic Acid	17.337	84.985
7	Methyl Linolelaidate	28.218	85.186
8	Oleic acid	11.431	85.559
9	Stearic Acid	0.630	87.178
10	Myristic Acid	0.425	87.195

Scavenging free radicals resulting from aerobic cellular reactions might reduce the oxidative stress level of cells, and so prevent the oxidation of biomolecules, which causes pathological conditions for many diseases such as cancer, cardiovascular disorders, neurodegenerative diseases and so on [36-39]. Total antioxidant activity analyses were determined according to the DPPH radical scavenging method. DPPH has been broadly accepted free radical to evaluate radical-scavenging activities of plant extracts [40-41]. To evaluate antioxidant capacity of NS pulp extract, its Trolox equivalents and IC_{50} values to scavenge DPPH were determined. TEAC values of pulp extract were calculated using a Trolox standard curve and were expressed as Trolox equivalents (mg TE /100 mL). TEAC values of pulp extracts were calculated as 8,75±0,06 TE mg/100 mL.

The antiradical activity of NS pulp extract and synthetic antioxidant BHT were measured by using DPPH based on their IC_{50} values, described as the amount of the antioxidant needed to inhibit 50% of DPPH existing in the test material. The assessment of radical-scavenging activity showed that NS pulp extract was able to eliminate free radicals.

The radical scavenging effect of NS pulp extract (IC_{50}=153.61±9.78 μg/ml) was found to be 4.5-fold less potent than the standard BHT (IC_{50}=34.06±0.38 μg/ml) (Table 3). However, all these results also indicated that NS pulp extract still preserves its antioxidant capacity and can be potentially used to scavenge free radicals. This can be considered to relate to the phenolic components particularly in the structure of pulp extract. The presence of flavonoids and some particular alkenes might also has an effect on antioxidant activity [42]. Sangeetha, *et al.* [42] reported that NS exhibited higher antioxidant activity than *Eugenia jambolana*, which contains quite similar compounds to NS, except some alkenes. Table 4 also shows that scavenging activity of pulp extract on DPPH enhanced linearly with increasing concentration.

In literature, NS shoots and roots have been reported to display 17-fold and 28-fold lower radical scavenging effect than that of BHT, respectively [43]. This suggests that the NS pulp extract has a higher antioxidant capacity when compared to its root and shoots. Singh, *et al.* [44] concluded that the essential oil and acetone extract of NS provide moderate and high antioxidant activity compared to synthetic antioxidants such as BHT and BHA. In another study, the seed extract of NS from India exhibited relatively lower scavenging effect with a IC_{50} value of 1240 μg ml^{-1} compared to our pulp extract [45]. Undoubtedly, one reason for these

Table 3. IC_{50} values of sample (*Nigella sativa* pulp extract) and standard (BHT).

	IC_{50} (μg/ml) *
Sample	153.61±9.78
BHT	34.06±0.38

*Mean of three parallel analysis ± S.D.

Table 4. DPPH scavenging activity of *Nigella sativa* pulp extract

Concentration (μg/ml)	Inhibiton (%)*
1000	71.57±0.36
500	60.06±1.04
250	51.23±1.32
125	49.71±0.18

*Mean of three parallel analysis ± S.D.

findings relates to the geographical area where NS is grown-in [46]. For this reason, it should be noted that pulp extracts might have different free radical scavenging effects depending on the region where NS is grown.

Kadam and Lele [47] revealed the characteristic LC-MS profile of NS pulp extracts and founded many phenolic contents such as Kaempferol, p-coumaroyl acid derivative and Thymol-O-sophoroside. The authors also reported that polyphenols extracted in NS pulp exhibited significant antioxidant and anti-inflammatory activity [47]. Similarly, Mariod, et al. [48] investigated antioxidant activities of crude methanolic extract and its different fractions with ethyl acetate, hexane and water respectively of NS pulp extract by using different techniques including DPPH, β-carotene bleaching and inhibition of corn oil oxidation assays. The results showed that NS pulp extract and their fractions successfully inhibited corn oil oxidation thanks to their phenolic contents. In another study, due to its rich polyphenolic compounds and antioxidant properties thereof, NS pulp extract was mixed with chitosan solutions at different v/v ratios to form films[49]. The polyphenolic extract of NS pulp contributed to substantial alterations in functional properties of chitosan films including water vapor permeability, crystallinity, thermal stability, tensile strength and elongation, antioxidant capacity etc. Thus, it was reported that these films obtained from NS pulp remaining after oil extraction can be used in food packaging or preservation and nutraceutical applications. In a study on calves, it was observed that the use of NS pulp as an additive in animal feeds resulted in an increase in the number of leukocytes and accordingly immune system-enhancement against various parasites [50]. Therefore, it is suggested that the use of NS pulp in animal feeds to strengthen the immune system and reduce the risk of diseases.

This relatively high antioxidant capacity suggests that NS pulp extracts, which emerged as a waste product, might be potentially useful as plant fertilizer and animal food as well as food preservation component. NS pulp extract can also be used with different antioxidant components. As it is known, the vast majority of natural antioxidant constituents affect each other and synergistically create a broad range of antioxidative activities against free radicals [51]. Briefly, all these conclusions suggest us to consider about the use of pulp extract as a natural antioxidant.

Despite the increasing number of studies regarding the cellular aerobic conditions in recent years, the effect of oxidative stress on intracellular processes and structures has not yet been understood in all aspects. Recent studies have shown the importance of the balance between aerobes and anti-antioxidants. The modulation of aerobes' activities via antioxidants especially the balance between them enables reactive oxygen species (ROS) to undertake useful functions while preventing harmful effects of oxidative stress [52]. Amount of antioxidant agents used may impair their positive effects on the cell signaling [53]. It also should be noted that the excessive removal of ROS from the biological systems might be harmful for health. The effect observed may vary depending on the applied dose [54]. On the other hand, cell medium conditions might lead to enhance oxidative stress level on the cells, resulting in an artificial effect on the role of reactive oxygen species as well as antioxidants applied on the cultured cells. This leads us to act with suspicion towards whether some redox active ingredients (especially flavonoids and polyphenols) may exhibit antioxidant properties under *in vivo* conditions [52]. In literature, more studies comparing the effects of antioxidants on both *in vitro* and *in vivo* conditions are highly needed to capture the in-depth understanding of

the potential of one compound over another[55]. In addition, the use of different antioxidant assays is also highly critical [56]. This study contributes to the existing literature, thanks to being performed both *in vitro* and *in vivo* conditions as well as utilizing different antioxidant analysis methods.

Cancer is a disease that occurs resulting from continuous and uncontrolled proliferation of cells. Several *in vivo* and *in vitro* studies have demonstrated the anticancer effects of NS seeds and their active ingredients. In order to test antimetastatic properties of NS pulp extract, the effects of its different concentrations were studied on A549 and L929 cells for 24 h. When we looked at the effects on cell viability, there was no significant decrease in L929 mouse fibroblast cells after 24h of incubation period, whereas a decrease in cell viability was observed at 500 μg/mL in A549 adenocarcinoma cells, an extremely aggressive cancer cell line. Cell viability decreased by 65% on A549 cells at 1000 μg/mL (Figure 4).

Researches have recently shown that the active ingredients of NS seed like Thymoquinone in particular, has an inhibitory effect on the proliferation of cells in many types of cancer including breast and ovarian adenocarcinoma [57], colorectal cancer [58], neoplastic keratinocytes [59], human osteosarcoma [60], fibro sarcoma [61], lung carcinoma [62-63], prostate cancer [64]. Thymoquinone and dithymyquinone can inhibit cancer cell growth by triggering apoptosis while the cells are in G1 phase. The arrest of cell growth is achieved by increasing gene expression and protein expression of p53 and inhibiting the antiapoptotic Bcl-2 protein [58]. Briefly, thymoquinone is mainly considered to be responsible for the anticancer and antimetastatic activities of NS [65-68]. However, our GC-MS results revealed that thymoquinone, which is the main constituent of NS extracts and their volatile oils [69], was not found in NS pulp. Nevertheless, although NS related studies up to date have not focused on as much as thymoquinone, according to our results poly- and monounsaturated fatty acids, present in NS pulp extract have also anti-proliferative effects against cancer cells. The complexes formed with unsaturated fatty acids such as linoleic acid and oleic acid, were reported to cause cytotoxicity against different cancer types, induce apoptosis as well as restrict tumor growth and progression *in vivo* [70-73]. In addition, high-unsaturated fatty acid contents in NS pulp can be useful contribute considerable resistance to oxidative reactions on food storage in particular [74].

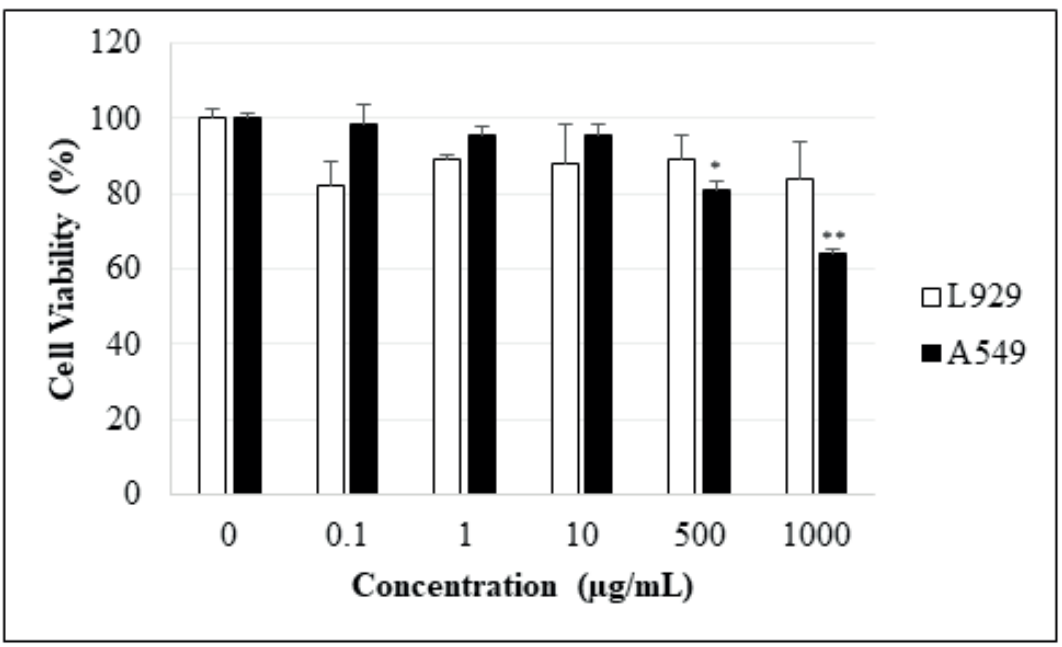

Figure 4. The effect of *Nigella sativa* pulp extract on both L929 and A549 cell viability. Cells were treated with the extract for 24h. Values are presented as mean ± SEM of three independent experiments. (*p<0.05, **p<0.01 vs Control).

There are many studies in the literature on NS extract. However, they are mostly *in vitro* studies and the number of *in vivo* studies is rather limited. CAM assay is used as an *in vivo* model in many angiogenesis studies. In our study, NS pulp extract with different concentrations was tested on *in ovo* CAM model. During the analysis, total vessel density, total vessels network length, total branching point and total segment were investigated as indicators of the angiogenesis process. The data were generated by analysing three stereomicroscope images for each group with an online CAM analysis software (Figure 5). In the analysis process, especially newly formed vessels were identified or marked and then measured. The analysed form of CAM images in Figure 5A can be seen in Figure 6. In addition, the effect of the pulp extract on the VEGF-stimulated embryonal development was evaluated with an *ex ovo* model to support the results of the *in ovo* testing.

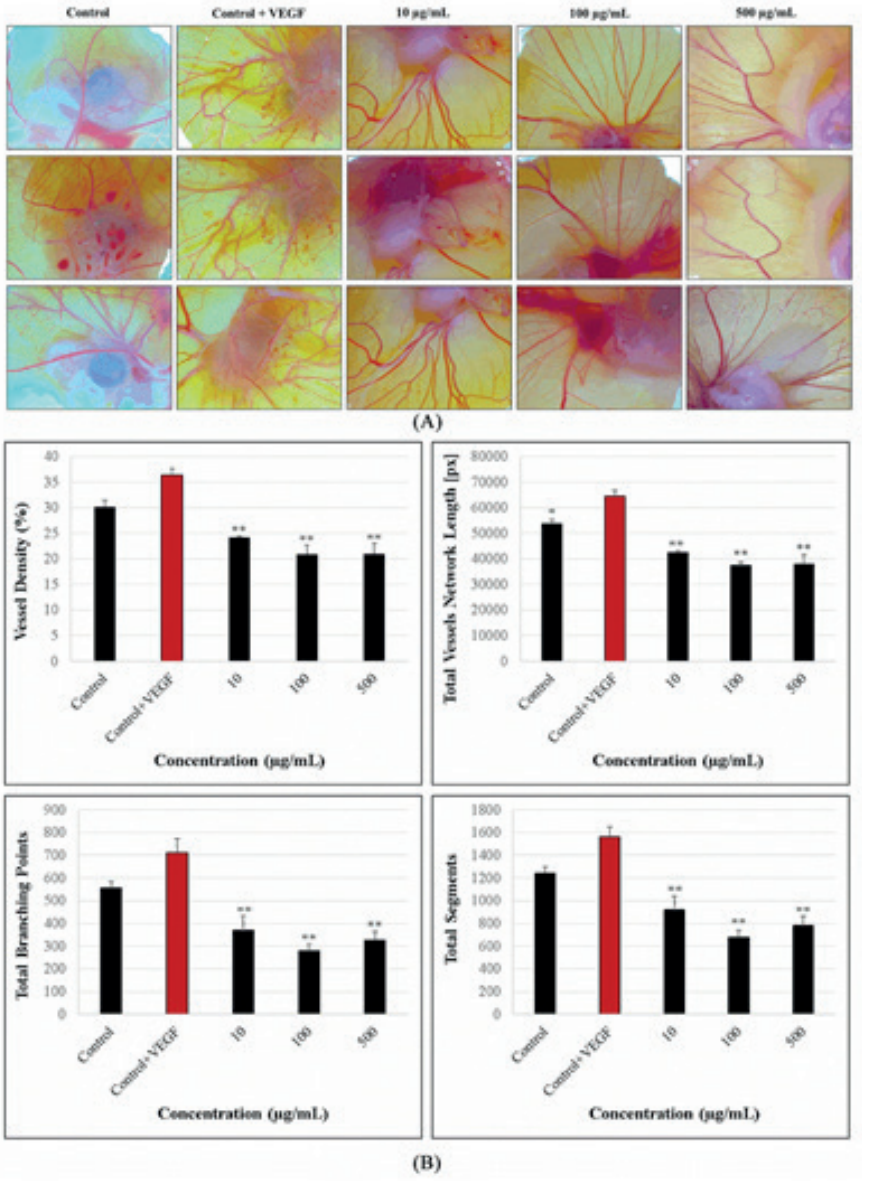

Figure 5. A.) Stereomicroscope images of VEGF-stimulated CAM vascularization treated with *Nigella sativa* pulp extract at different concentrations (10, 100 and 500 µg/mL). Scale bars, 1 mm. All groups except control were pre-incubated with 10 ng/mL VEGF for 24h. B.) The effects of *Nigella sativa* pulp extracts on VEGF-stimulated angiogenesis including vessel density (%), total vessels network length (px), total braching points (number) and total vessel segments (number) obtained from three stereomicroscope CAM images of each group. All treatment groups were pre-treated with 10 ng/mL $VEGF_{165}$ for 24h. All comparisons were made with respect to the 10 ng/mL VEGF group considered as the positive control. The values represent as means and standard error of triplicate experiments ($^{*}p<0.05$, $^{**}p<0.01$ vs Control+VEGF).

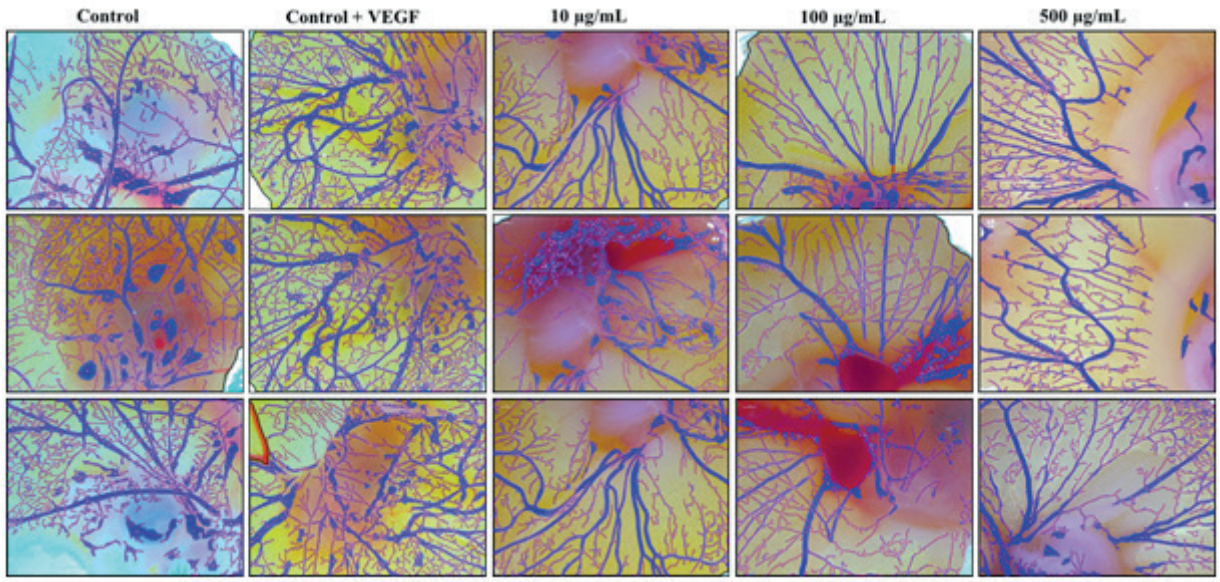

Figure 6. The stereomicroscope CAM images for each group after analysis.

The positive control group treated with 10 ng/mL $VEGF_{165}$ showed an increase in all angiogenesis indicators measured during the study compared to non-treated control group. After the treatment of 10 ng/mL $VEGF_{165}$ in all groups except the control group, NS pulp extract at different concentrations (10, 100, 500 µg/mL) was applied to the CAM model. When the total vascular density was taken into account, the group treated with 10 µg/mL NS pulp showed a decrease of approx. 33%, whereas the concentrations of 100 and 500 µg/mL induced a decrease of approx. 45%. Total vessel length decreased in all groups.

As an interesting result, total branching points and total segments were lower in the groups treated with 100 µg/mL than the groups treated with 500 µg/mL. In this study, it was shown in the CAM model that the NS pulp extract inhibited dose-dependently the formation of the blood vessel formation. For a more detailed investigation of the suppressive effect on the VEGF-stimulated angiogenesis of the NS pulp extract, we investigated the embryonic development

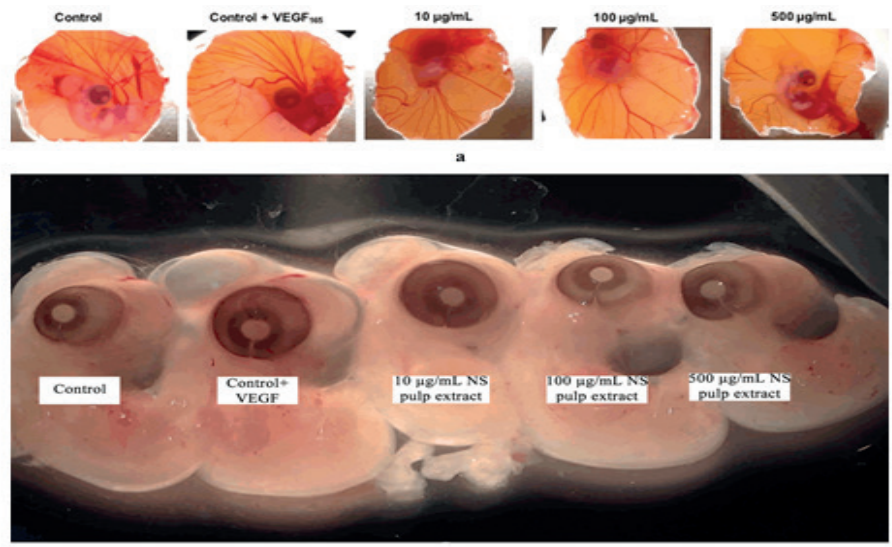

Figure 7. The restrictive effect of *Nigella sativa* pulp extract on VEGF-stimulated embryonic growth both *in ovo* (a) and *ex ovo* (b). All groups except control were pre-treated for 24h with 10 ng/mL $VEGF_{165}$.

of the fertile chick eggs exposed to the pulp extract. The results displayed that the NS pulp inhibited the VEGF-stimulated embryonic development under both *in ovo* and *ex ovo* conditions (Figure 7).

One of the underlying factors of NS's anticancer properties is its ability to inhibit some angiogenesis and metastasis-inducing cytokines. All these results showed that NS pulp extract might be used in diseases like cancer, which progress with pathological angiogenesis thanks to its antimetastatic and antiangiogenic properties and it might provide positive results in the prevention and treatment of many diseases thanks to its antioxidative activity, which enables the removal of the free radical compounds from the cells as well.

In the literature, all studies on NS have been carried out mainly with seed extract or seed oil. There are only a few studies on biological activity of NS pulp extract directly. This aspect, which is one of the distinguishing features of our study, will make an important contribution to the usage of a waste material and, thus pave the way for an environmentally friendly approach. This study shows that the pulp extract contains active constituents of NS even small quantities. It is desirable to show that the biological effects of the pulp can produce such remarkable results that it can be used for many different purposes in medicine, animal husbandry and so on, rather than decapitating it. The extract obtained from the pulp might be evaluated as a source for the phytotherapeutic activity studies in the future.

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Disclosure statement (Conflict of interest)

Author(s) declare that there are no conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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