A case study on in vitro investigations of the potent biological activities of wheat germ and black cumin seed oil

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Abstract: The objectives of this study were to investigate the potential biological activities of black cumin seed oil (BCSO) and wheat germ oil (WGO) on different cell lines. Initially, commercially available BCSO and WGO obtained by supercritical carbon dioxide extraction were analyzed in terms of tocopherol, aliphatic alcohols, and thymoquinone content via HPLC and GC analysis. Cell free antioxidant activities and total phenolic content of both oils were detected by DPPH assay and Folin-Ciocalteu method, respectively. As well as the DPPH assay, the protective effect against reactive oxygen species (ROS) was determined by microscopic observation of ROS generation in NIH-3T3 cells with or without oil samples by using an oxidation-sensitive fluorescent dye, H2DCFDA. Cytotoxicity was assessed using an MTT assay. In the case of BCSO, after exposing cells to 0.025–1.0 mg/mL and 1.0–100 µg/mL concentrations for 24 h, the IC50 values of BCSO were 0.58, 0.51, 0.47, and 0.36 mg/mL for NIH-3T3, A549, U87, and HeLa cells, respectively. On the other hand, concentrations of WGO lower than 0.1 mg/mL did not cause a decrease in cell viability for all cell lines. Apoptotic and necrotic rates of these cell lines were investigated via flow cytometry. BCSO also exhibited proliferative efficacy for NIH-3T3 cells.

Key words: Black cumin seed oil, wheat germ oil, cell culture investigations

1. Introduction
Oils are very important in the food industry and essential for human nutrition. Solvent extraction is a general method for extraction of oils from vegetable substance. Supercritical fluid extraction (SFE) is an alternative to the usual methods and has many advantages over them. It is applicable at low temperature, and allows easy separation with environmentally friendly fluids in a short time.1,2 Carbon dioxide (CO2) is the most frequently used supercritical solvent in the food and pharmaceutical industries due to its low toxicity, good safety, cheapness, and low critical temperature and pressure.3,4 Use of CO2 as a supercritical fluid for extraction of wheat germ oil (WGO) and black cumin seed oil (BCSO) has previously been reported by several research groups.5–7

Reactive oxygen species (ROS), which are formed under physiological conditions such as in the electron

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transport system, cause cell damage. Vitamins E, A, C, and \( \beta \)-carotene are natural antioxidants against ROS.\(^8\) Natural antioxidant sources are advantageous in comparison with synthetic antioxidants. Wheat germ is one of the richest natural sources of alpha tocopherol, possessing vitamin E activity and a number of health benefits. Vitamin E protects the body from oxidative processes as a result of ROS damage. Paranich and coworkers reported that oral implementation of WGO saturates the body of rats with vitamin E and WGO intake results in a change in the intensity of lipid peroxidation processes.\(^9\) Vitamin E can be given as a nutritional supplement to reduce oxidative stress. Bansal et al. investigated the protective role of vitamin E pre-treatment on \( N \)-nitrosodiethylamine; it reduced the degree of oxidative stress and induced lipid peroxidation and antioxidant enzyme activities in rat liver.\(^10\) Furthermore, wheat and WGO are good sources of policosanol, which is the common name that refers to a mixture of long chain (20–36 carbon) aliphatic primary alcohols.\(^11\) The mixture contains mainly docosanol (C22), tetracosanol (C24), hexacosanol (C26), octacosanol (C28), and triacontanol (C30). These compounds have been reported to inhibit lipid peroxidation and to improve protection of both the lipid and the protein moieties of lipoproteins against lipid peroxidation.\(^12\) Additionally, 1-triacontanol was found to be a plant growth promoter and an anti-inflammatory substance,\(^13,14\) whereas 1-octacosanol exhibited ergogenic, neurological, and antioxidant properties.\(^15\)

Black cumin seed (BCS) is one of the most investigated herbs among various medicinal plants. It has a wide spectrum of therapeutic potential such as anti-inflammatory,\(^16\) antitumor,\(^17,18\) antimicrobial,\(^19\) antifungal,\(^20\) and antioxidant effects.\(^21\) Thymoquinone (TQ) is a major component of BCS and has therapeutic activities. Several papers have reported that TQ was able to repress different carcinomas such as liver, colorectal, breast, and prostate carcinoma.\(^22\) Moreover, many studies reported that BCS and TQ have an antioxidant effect due to radical scavenging activity.\(^23,24\)

Here, we described a combination of various assays to evaluate the protective effect of WGO and BCSO extracts on oxidative damage using supercritical \( \text{CO}_2 \) as an extraction fluid. Chromatographic analyses as well as cell culture assays were successfully applied and this could be a promising route for the evaluation of biological activities of different functional foods depending of their content.

2. Results and discussion

2.1. Quantification of thymoquinone, tocopherol, and aliphatic alcohols

TQ, tocopherols, and aliphatic alcohols of the BCSO and WGO were analyzed by HPLC-DAD, HPLC-FLD, and GC-FID systems. The quantitative results of the TQ, tocopherols, and aliphatic alcohols for WGO and BCSO are given in Tables 1 and 2, respectively. Alpha tocopherols and aliphatic alcohols were active ingredients of WGO and TQ was the main component of BCSO.

2.2. Assays for total phenolic contents and DPPH radical scavenging activity

A comparative study on the antioxidant properties of WGO and BCSO was performed. The antioxidant capacity of seed oil extracts was determined by evaluating total phenolic contents and 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity.

The absorbance values of the WGO and BCSO, reacted with Folin–Ciocalteu reagent, were compared with those of the standard solutions of gallic acid equivalents (GAE) described in the Experimental section. Results of the colorimetric analysis of total phenolics were estimated as WGO 3.41 \( \pm 0.19 \) (mg GAE/g oil extract) and BCSO 1.95 \( \pm 0.03 \) (mg GAE/g oil extract). The high phenolic content of the WGO may be responsible for
Table 1. Tocopherol and aliphatic alcohol compositions of WGO.

<table>
<thead>
<tr>
<th>Component/oil</th>
<th>Wheat germ oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha tocopherol (µg/g)</td>
<td>2482.5</td>
</tr>
<tr>
<td>Beta tocopherol (µg/g)</td>
<td>958.5</td>
</tr>
<tr>
<td>Gamma tocopherol (µg/g)</td>
<td>76.6</td>
</tr>
<tr>
<td><strong>Total tocopherol (µg/g)</strong></td>
<td><strong>3517</strong></td>
</tr>
<tr>
<td>Docosanol (C22) mg/kg</td>
<td>8.4</td>
</tr>
<tr>
<td>Tetracosanol (C24) mg/kg</td>
<td>8.0</td>
</tr>
<tr>
<td>Hexacosanol (C26) mg/kg</td>
<td>63.7</td>
</tr>
<tr>
<td>Octacosanol (C28) mg/kg</td>
<td>12.3</td>
</tr>
<tr>
<td><strong>Total alcohols mg/kg</strong></td>
<td><strong>92.4</strong></td>
</tr>
</tbody>
</table>

Table 2. TQ content and tocopherol composition of BCSO.

<table>
<thead>
<tr>
<th>Component/oil</th>
<th>Black cumin seed oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymoquinone (µg/g)</td>
<td>4478</td>
</tr>
<tr>
<td>Alpha tocopherol (µg/g)</td>
<td>35.9</td>
</tr>
<tr>
<td>Beta tocopherol (µg/g)</td>
<td>0</td>
</tr>
<tr>
<td>Gamma tocopherol (µg/g)</td>
<td>202.1</td>
</tr>
<tr>
<td><strong>Total tocopherol (µg/g)</strong></td>
<td><strong>238</strong></td>
</tr>
</tbody>
</table>

the antioxidant activity of WGO. Some reports have indicated that there is a positive correlation between total phenolic compounds and free radical scavenging potential. In addition, the health benefit of natural products is dependent on the contents of their phenolic compounds. Hence, WGO may be recommended as a source of plant-based antioxidants that are healthy alternatives to synthetic antioxidants. These antioxidants may protect the cells against the effects of free radicals as well as lipid peroxidation of food products.

Due to its simplicity and stability, and the short time required for analysis, the DPPH radical scavenging assay is a widely used method to test the antioxidant activity of samples. Basically, the antioxidant capacity of the samples determined by the DPPH free radical scavenging activity assay is the activity of quenching free radicals, or the H-donor capability of the antioxidant. Therefore, the antioxidant potentials can be determined through the discoloration of the mixture using a spectrophotometer. Figure 1 shows the DPPH radical scavenging activity of WGO and BCSO at various concentrations (2.0 to 16.0 mg/mL). As positive controls, BHA and BHT were also examined at 1.25–50 µg/mL concentrations. The DPPH assay of commercially available WGO and BCSO was also studied at 10–1000 µg/mL concentrations, but the expression of the obtained results was not meaningful. WGO showed the highest radical scavenging activity through all concentrations for the DPPH assay. BCSO showed slightly less radical scavenging activity. Significant differences in DPPH scavenging activities were observed between BCSO (35.3%) and WGO (91.3%) at a concentration of 6.0 mg/mL and BHT (51.3%) and BHA (83.9%) at a concentration of 10 µg/mL. The observed radical scavenging activity and total phenolic contents for WGO and BCSO are in good alignment. This correlation has been reported to be present in natural oils, which is very important for the oxidative stability of polyunsaturated fatty acids (PUFA). WGO and BCSO displayed lower radical scavenging activity than did BHA and BHT, the most commonly used synthetic antioxidants, as positive controls. However, in view of recent reports on the carcinogenicity and toxicity of these synthetic antioxidants, their application as a food additive is not encouraged.
Figure 1. DPPH radical scavenging activities of wheat germ oil (WGO) and black cumin seed oil (BCSO). Values are the mean ± SD of the data (n = 3).

2.3. Cytotoxicity

Previous studies indicated anticancer activity of BCS against different cell lines. Recently, Al-Sheddi et al. demonstrated that seed extract and seed oil of black cumin significantly reduced the cell viability of a human lung cancer cell line. In the present study the cytotoxicity of BCSO was assessed by MTT assay, after exposure of the cells to 1.0–100 μg/mL and 0.025–1.0 mg/mL BCSO for 24 h. Cytotoxicity data obtained from the MTT assays were extrapolated using the exponential equation \( y = 1 - \left( \frac{1}{1 + e^{ax}} \times (b - x) \right) \), where a is the slope, b is IC\(_{50}\) (50% inhibitory concentration), and x is the concentration of the sample. The IC\(_{50}\) values of BCSO were 0.58, 0.51, 0.47, and 0.36 mg/mL for NIH-3T3, A549, U87, and HeLa cells, respectively. The results showed that BCSO induced significant decreases in cell viability of cancer cells in a concentration-dependent manner (Figures 2A–2D). The highest cytotoxic effect was observed for HeLa cells. However, BCSO had less cytotoxic potential against normal NIH-3T3 cells. In the case of lower concentrations of BCSO a proliferative effect was observed on NIH-3T3 cells (Figure 3).

WGO had no significant effect on the cell viability of A549 and U87 cells. On the other hand, NIH-3T3 and HeLa cells showed a statistically significant (P < 0.001) decrease in cell viability at a concentration of 1.0 mg/mL WGO (Figure 4). The concentrations of WGO of 0.1 mg/mL and lower did not show a decrease in the cell viability of all cell lines.

2.4. Flow cytometry

TQ induces apoptosis in various human cancer cells, such as neoplastic keratinocytes, colorectal cancer cells, breast adenocarcinoma, and ovarian adenocarcinoma cells. As demonstrated in Figure 5A, treatment with BCSO induced a greater level of apoptosis (38.5%) and necrosis (34.2%) in A549 cells. Necrotic and apoptotic rates in 0.5 mg/mL BCSO-treated HeLa cells were 66.7% and 8.9%, respectively (Figure 5B), and 69.8% of U87 cells were detected as necrotic after incubation with BCSO (Figure 5C). The anticancer effect of BCSO can be
**Figure 2.** Dose-dependent cytotoxic effects of BCSO on viability of A549 (A), HeLa (B), U87 (C), and NIH-3T3 (D). Green dotted line represents point of significant cytotoxicity (70% viability). Values are the mean ± standard deviation of the data (n = 4).

**Figure 3.** Dose-dependent cytotoxic effects of BCSO and WGO on viability of NIH-3T3 cells. Each bar represents a mean ± SD (n = 4). Asterisks show significant differences from control values (*P < 0.05; **P < 0.01; ***P < 0.001).

**Figure 4.** Dose-dependent cytotoxic effects of WGO on viability of cell lines. Each bar represents a mean ± SD (n = 4). Asterisks show significant differences from control values (*P < 0.05; **P < 0.01; ***P < 0.001).
Figure 5. Apoptotic and necrotic cells were detected by flow cytometry using Annexin V and propidium iodide (PI) staining. A) A549 cells, B) HeLa cells, and C) U87 cells. Cells in the lower left quadrant (Annexin VFITC−/PI−) are viable, those in the lower right quadrant (Annexin VFITC+/PI−) are apoptotic, those in the upper left quadrants (Annexin VFITC−/PI+) are necrotic, and those in the upper right quadrants (Annexin VFITC+/PI+) are late apoptotic or necrotic.
ascribed to the content of BCSO with high amounts of TQ.\textsuperscript{18,37} WGO has no significant effects in A549 and U87 cells. Apoptotic and necrotic rates of these cell lines were the same as in nontreated cells. In the case of HeLa cells, WGO treatment induced a drop in apoptotic rates to 2.7%. These results are in good agreement with the cytotoxicity results.

2.5. Protective effect against reactive oxygen species

The level of ROS generation in NIH-3T3 cells with/without BCSO and WGO was compared using an oxidation-sensitive fluorescent dye, H\textsubscript{2}DCFDA. Treatment of TBHP caused generation of intracellular ROS in NIH-3T3 cells that was evidenced by a large number of H\textsubscript{2}DCFDA positive cells (Figure 6A). In contrast, nontreated cells were all H\textsubscript{2}DCFDA negative (Figures 6B and 6C). On the other hand, BCSO and WGO both prevented ROS generation in TBHP-treated cells (Figures 6D-6G).

![Fluorescence microscopy images](image.png)

**Figure 6.** Fluorescence microscopy images of A) positive control: cells were treated with working solution of TBHP (carboxy-H\textsubscript{2}DCFDA staining), B) negative control: nontreated cells (DAPI staining), C) negative control: nontreated cells (carboxy-H\textsubscript{2}DCFDA staining), D) BCSO: cells were treated with working solution of TBHP and 0.1 mg/mL BCSO (DAPI staining), E) BCSO: cells were treated with working solution of TBHP and 0.1 mg/mL BCSO (carboxy-H\textsubscript{2}DCFDA staining), F) WGO: cells were treated with working solution of TBHP and 0.1 mg/mL WGO (DAPI staining), G) WGO: cells were treated with working solution of TBHP and 0.1 mg/mL WGO (carboxy-H\textsubscript{2}DCFDA staining).

2.6. Cell proliferation

In the CellTiter-Blue Cell Viability Assay the dye resazurin was used to observe the metabolic activity of cells. Viable cells are able to reduce resazurin to resoruflin, which is a highly fluorescent dye. The fluorescence signal is proportional to the number of metabolic active cells. The cell amount of NIH-3T3 increases in the case of treatment with BCSO and is comparable to the untreated cells. However, no changes were observed with WGO treatment on NIH-3T3 cells (Figure 7). Similar results were obtained by Benhaddou-Andaloussi et al.,
who investigated the effect of BCS extract on pancreatic β-cell proliferation. BCS ethanol extract induced proliferation of pancreatic β-cells.  

![Graph showing absorbance (570 nm/630 nm) vs. duration of cultivation (h) for different samples: Nontreated cells, BCSO, and WGO.](image)

Figure 7. Proliferative effect of BCSO and WGO on NIH-3T3 cells.

In summary, the content of tocopherol, aliphatic alcohols, and TQ in WGO and BCSO was analyzed by HPLC and GC. The DPPH radical scavenging assay and Folin–Ciocalteu method were used for assaying the cell free antioxidant activities and TPC of both oils, respectively. The anticancer effects of BCSO and WGO were examined by MTT assay and flow cytometry analyses. The protective effect against ROS was observed by fluorescence microscopy using the oxidation-sensitive fluorescent dye H2DCFDA in NIH-3T3 cells with/without oil samples. The obtained results indicated that BCSO and WGO both had antioxidant effects on NIH-3T3 cells because of containing high amounts of TQ and tocopherol, respectively. BCSO was more toxic to cancer cells in comparison with healthy cells and induced apoptosis and necrosis. Moreover, when used at lower concentrations of BCSO, a proliferative effect was observed on NIH-3T3 cells.

3. Experimental

3.1. Materials

BCSO and WGO obtained by supercritical CO₂ extraction (SCE) were kindly donated from TABIA (Turkey). Cell culture supplies including fetal calf serum (FCS Gold) and penicillin/streptomycin (P/S, 100 ×) were purchased from Biochrom AG. Dulbecco’s Modified Eagle’s medium (DMEM) and 4,6-diamino-2-phenylindol (DAPI) were purchased from Sigma-Aldrich. Phosphate buffer saline (PBS) was prepared with 137 mM sodium chloride, 2.7 mM potassium chloride, 10.1 mM disodium hydrogen phosphate, and 1.8 mM potassium dihydrogen phosphate, pH 7.4; all chemicals were also provided by Sigma Aldrich. Sodium dodecyl sulfate (SDS) was ordered from Applichem. All other chemicals were of analytical grade.

3.2. Detection of aliphatic alcohols

The aliphatic alcohols method was introduced into the International Olive Oil Council trade standard. The method describes a procedure for determination of the aliphatic alcohols (C22, C24, C26, and C28) content of fatty substances. The aliphatic alcohols were analyzed by gas chromatography–flame ionization detector (GC-FID). The Agilent 7890B GC-FID system consisted of a G4513A auto-liquid sampler and HP-5 capillary
column (0.25 μm particle size, 0.25 mm internal diameter, and 30 m length). The temperature program for the GC was as follows: initial temperature, 180 °C held for 8 min, linear gradient of 5 °C min 260 °C held for 15 min. The injector temperature was 280 °C and injection was performed in the split mode (1:50). The injection volume was 1.0 μL. Hydrogen was used as carrier gas, 30 cm/s. The contents of each aliphatic alcohol, expressed in mg/1000 g fatty substance, were calculated according to the literature.39

3.3. Thymoquinone and tocopherol quantification

Quantification of the TQ in BCSO was achieved using an Agilent 1100 RP-HPLC system consisting of a gradient pump, a DAD detector, and a C18 column (Agilent Eclipse column XDB-C18 (5.0 μm particle size, 4.6 × 150 mm)). Quantitative analysis was based on the peak areas. Detection and quantification were carried out at 254 nm. The column temperature was 25 °C. The mobile phase consisted of methanol:water:methyl tert-butyl ether (46:42:12 v/v/v) with a flow rate of 1.0 mL/min. To detect TQ in the BCSO, 0.1 g of BCSO was dissolved in 10 mL of hexane, vortexed for 1 min, and filtered; then 20 μL was injected into the HPLC system. Tocopherols were determined according to the AOCS Lipid Library.40 Separation and quantification were carried out on an Agilent NP-HPLC using silica columns. The system used consisted of a gradient pump and an FLD detector with a LiChrosorb SI 60 column (5 μm, 4.6 × 150 mm). The excitation and emission wavelengths were 290 nm and 330 nm, respectively. Hexane:2-propanol (99:1) was used as mobile phase for α-tocopherol, β-tocopherol, γ-tocopherol, and δ-tocopherol at a flow rate of 0.8 mL/min. In the assay, 0.5 g of BCSO and WGO was dissolved in 10 mL of hexane, vortexed for 1 min, and filtered; then 20 μL was injected into the HPLC system at 25 °C.

3.4. DPPH radical scavenging assay

The antioxidant activities of WGO and BCSO were evaluated by DPPH radical scavenging assay, which was originally described by Blois41 with a slight modification by Guler et al.42 First 0.2 mM DPPH solution in ethanol was prepared and 1.0 mL of this solution was added to 1.0 mL of various concentrations (2.0 to 16.0 mg/mL) of WGO and BCSO samples dissolved in ethanol to be tested. The reference butylated hydroxyanisol (BHA) and butylated hydroxytoluene (BHT) dissolved in ethanol (final concentration were 1.25 to 50 μg/mL) were used as reference test materials. The test samples and reference solutions were added to different test tubes. After 30 min at room temperature in the dark, absorbance was measured at 517 nm by UV-Vis spectrophotometer (Thermo Fischer, EVO 60 Model, Madison, WI, USA). All tests were performed in triplicate. The scavenging activity (%) was calculated as follows:

\[
DPPH \text{ radical scavenging activity(\%)} = \left( \frac{A_0 - A_1}{A_0} \right) \times 100,
\]

where \(A_0\) was the integral intensity of the DPPH signal of the control sample and \(A_1\) was the integral intensity after the addition of the studied extract concentration to the control sample.

3.5. Determination of total phenolic contents

The Folin–Ciocalteu method was used for the determination of the total phenolic content (TPC) of WGO and BCSO.43,44 TPCs in the oil extracts were determined by UV-Vis spectrophotometer, according to a calorimetric oxidation/reduction reaction. Folin–Ciocalteau reagent (2.0 N, 200 μL) was mixed with 1160 μL of distilled
water and 40 μL of oil extract. The reaction was carried out for 5 min in the dark. Then 600 μL of sodium carbonate (20%) was added. The reaction mixture was incubated at 40 °C for 30 min in a water bath and centrifuged at 2000 × g for 10 min. The supernatant absorbance was measured by a UV-Vis spectrophotometer at 765 nm. Gallic acid was used to plot the calibration curve for calculation. TPC was standardized against gallic acid (GA), and the results were expressed in mg GA equivalent (GAE)/g oil extract. Analyses were conducted in triplicate, and the TPCs were calculated by the following equation, which was obtained using a standard GA curve ($R^2 = 0.996$):

$$A_{760}\text{nm} = 0.0027 \times [\text{GAE}].$$

3.6. Cell culture studies
A549 (lung cancer cells), U87 (human glioblastoma cells), HeLa (cervical cancer cells), and NIH-3T3 (normal mouse fibroblast cells) cell lines were provided from the German Collection of Microorganisms and Cell Cultures (DSMZ). All cells were grown in DMEM containing 10% FCS and 1.0% P/S. The cells were cultivated in medium and incubated with samples and reagents at 37 °C in a humidified environment with 5.0% CO₂.

3.7. Cytotoxicity assay
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays were used to determine the dose-dependent cytotoxicity effect of oils on A549, U87, HeLa, and NIH-3T3 cells. Cells were seeded out in 96-well-tissue plates and cultivated for 3 days. Then the cells were washed once in PBS and treated with oils at 0.025, 0.05, 0.1, 0.25, 0.5, and 1.0 mg/mL concentrations and also 1.0, 5.0, 10, 25, 50, and 100 μg/mL for 24 h. Samples were removed and cells were incubated in 110 μL/well 10% MTT solution (5.0 mg/mL PBS) in medium for 4 h. Subsequently 100 μL of SDS solution (1.0 g of SDS in 10 mL of 0.01 M HCl) was added per well and after 24 h of incubation UV-Vis absorption was measured at 570 nm with 630 nm as reference wavelength using a microplate reader Model 680 (BioRad).

3.8. Flow cytometry analysis using annexin V and PI double staining
For flow cytometry studies the cells were harvested by accutase treatment, washed once in ice-cold PBS, and collected. Subsequently cells were treated for 2 h with 0.5 mg/mL BCSO and WGO and treated cells were tested using the Annexin V-FLUOS staining kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, cells were incubated in 100 μL of incubation buffer including 2.0 μL of Annexin fluorescein isothiocyanate (FITC) and 2.0 μL of propidium iodide for 15 min. The stained cells were then analyzed in a COULTER EPICS XL-MCL flow cytometer.

3.9. Protective effect against reactive oxygen species
The Image-iT LIVE Green Reactive Oxygen Species (ROS) Detection Kit (Invitrogen) was used to evaluate the protective effect of BCSO and WGO on NIH-3T3 cells. The assay is based on 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H2DCFDA), a reliable fluorogenic marker for ROS in live cells. NIH-3T3 cells were grown to confluence in 96-well plates and incubated for 90 min with 100 μM tert-butyl hydroperoxide (TBHP) working solution in the presence or absence of 0.1 mg/mL BCSO and WGO. After removing the samples, cells were treated with 25 μM carboxy-H2DCFDA solutions and were incubated for 30
min at 37 °C, protected from light. Cells were washed with PBS and DAPI fluoroshield was dropped on them. Finally images were taken using a fluorescence microscope, Olympus IX50, equipped with an Olympus SC30 camera.

3.10. Cell proliferation

In the cell proliferation assay, the untreated NIH-3T3 cells were applied as a control and a 4-fold determination was studied for each sample. BCSO and WGO at 100 µg/mL concentration were added to the wells in triplicate. Cells were cultivated for 3 h (time for adherence), 24 h, 48 h, and 72 h. At the end of each cultivation time, cells were treated with 20 µL of CellTiter-Blue Reagent per 100 µL of medium for 4 h. Fluorescence (544 nmEx/590 nmEm) was measured using the Fluoroskan Ascent fluorescence plate reader (Thermo Scientific).

3.11. Statistical analysis

All experiments were repeated 4 times. Statistical analysis was carried out with the GraphPad Prism 6 statistical software. One-way analysis of variance (ANOVA) was performed with Tukey’s test for multiple comparisons in statistical evaluation. The difference between two groups was considered to be significant when the P value was less than 0.05.

Acknowledgments

The authors thank TABIA for providing black cumin seed oil and wheat germ oil. The Konrad Adenauer Foundation is acknowledged for financial support of PhD student Didem Ağ Seleci. The authors also thank SEM (İzmir) for their support during the HPLC and GC analysis.

References


