Wild-growing Rosa heckeliana Tratt.: phenolic constituents with cytotoxic and antioxidative properties

Nursen ÇORUH 1,* , Nizamettin ÖZDOĞAN 2
1 Department of Chemistry, Faculty of Arts and Science, Middle East Technical University, Ankara, Turkey
2 Department of Environmental Engineering, Faculty of Engineering, Bülent Ecevit University, Zonguldak, Turkey

Abstract: Rosa heckeliana, as one of the wild-growing species of the family Rosacea, has a wide use in the folk medicine; however, scientifically there is very little known about it. Our objective was to examine the antioxidative and antiproliferative properties of Rosa heckeliana root extract and its phenolics. The phenolic constituents, namely catechin, caffeic acid, and ellagic acid, were obtained by the methods of extraction, fractionation, and purification through column chromatography. The DPPH and ABTS radical scavenging assays and total flavonoid content analysis were employed as the current antioxidant methods over the crude extract and fractionated parts. Among the extracts/fractions, the ethyl acetate fraction exhibited the highest amount of flavonoid content (4.58 ± 0.018 µg CE/mg of extract). The ethyl acetate fraction also displayed high antioxidative properties for DPPH (EC50 value: 2.78 ± 0.01 µg/mL) and ABTS scavenging capacities (586 ± 6.64 µM trolox as TEAC value). Additionally, the antiproliferative properties of the crude extract and the ethyl acetate fraction and its isolated constituents were evaluated for two breast cancer cell lines (MCF-7 and MDA-MB-231) using the XTT method. Acetate fraction over the cells of MCF-7 and MDA-MB-231 resulted in ED50 values of 61.18 ± 0.99 µg/mL and 62.54 ± 2.01 µg/mL, respectively. The isolated phenolic constituents were twice as effective as the ethyl acetate fraction on both MCF-7 and MDA-MB-231 cells.

Key words: Rosa heckeliana, antioxidant, cytotoxicity, breast cancer cell lines

1. Introduction

Ever since the beginning of civilization, in every culture, plants have been utilized for medicinal treatments. In the late 19th century, it became important to isolate and identify the active ingredients of those plants. Since then, plant-originated chemical substances have been used as drug sources, as well as their synthesized derivatives. From 1983 to 1994, 39% of the newly approved drugs were of natural origin as the natural products themselves or their semisynthetic derivatives, as well as synthetic products imitating those natural molecules (Cragg et al., 1997). The global market of natural products is growing exponentially parallel to synthetic drugs. The estimated number of plant species on the planet is about 422,000 (Bramwell, 2002). However, only about 6% of those have been surveyed for biological activity, and of those only 15% have been characterized for phytochemicals (Verpoorte, 2000). The assessments of the therapeutic functions of those plants have led to the discovery of several clinically applicable drugs (digoxin, digitoxin, morphine, etc.) Clarification of the structure of active components laid the steps for the synthesis of compounds with higher efficacy and lower antagonistic effects (metformin, nabilone, oxycodone, etc.) (Daniel and Norman, 2001). As the knowledge in the discipline advanced, scientists became more interested in the phytocompounds with high potentials. Therefore, the isolation, purification, and identification of the natural compounds mainly responsible for the pharmacological action have accelerated (Fabricant and Farnsworth, 2001). Subsequently, progress was inevitable for the investigation of the antioxidant, antiinflammatory, antimicrobial, anticancer, and antimutagenic effects of many plant-originated compounds.

Rosa heckeliana as a member of the family Rosaceae has been frequently utilized in folk medicine. However, the curative properties of this plant are still waiting to be explored by researchers. Recently, the authors reported the identification and quantification of phenolic constituents of R. heckeliana root extract (Çoruh and Özdoğan, 2015). Therefore, this study aimed to determine the antioxidative and antiproliferative properties of the roots of R. heckeliana.
2. Materials and methods

2.1. Plant materials
The roots of *R. heckeliana* were collected from the East Anatolia Region of Turkey at altitudes of 1300–2900 m. The roots were picked in the late spring of 2011 and 2012. Collected plant samples were pressed according to herbarium rules. The plant materials were identified and donated by Professor Fevzi Özgökçe, Department of Biology of Yüzüncü Yıl University, Van, Turkey. The voucher specimen was deposited in the herbarium of Yüzüncü Yıl University (voucher specimen number F5474).

2.2. Chemicals and apparatus
Chromatography-grade petroleum ether, chloroform, ethyl acetate, and n-butanol were purchased from Merck (Darmstadt, Germany) for the fractionation processes. Standard compounds such as catechin, caffeic acid, and ellagic acid were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Column chromatographic separations were performed by using silica gel 60F254 using 0.25-mm aluminum coated plates purchased from Merck. Sephadex LH-20 (particle size: 25–100 µm) was bought from Pharmacia (Stockholm, Sweden). Dimethyl sulphoxide (DMSO) was purchased from Merck (Darmstadt, Germany). Roswell Park Memorial Institute (RPMI 1640) medium, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) modified, and fetal calf serum (heat-inactivated) were purchased from Biochrom Ltd. (Cambridge, UK). L-Glutamine, gentamicin, sterile Dulbecco’s phosphate buffered saline (sPBS), sodium 3,3’-{1-[(phenylamino) carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), phenazine methosulfate, and trypan blue were purchased from Biochrom Ltd. (Cambridge, UK). L-Glutamine, gentamicin, sterile Dulbecco’s phosphate buffered saline (sPBS), sodium 3,3’-{1-[(phenylamino) carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), phenazine methosulfate, and trypan blue were purchased from Biochrom Ltd. (Cambridge, UK). L-Glutamine, gentamicin, sterile Dulbecco’s phosphate buffered saline (sPBS), sodium 3,3’-{1-[(phenylamino) carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT), phenazine methosulfate, and trypan blue were purchased from Biochrom Ltd. (Cambridge, UK).

2.3. Extraction and fractionation
Roots of *R. heckeliana* (500 g) were ground to obtain a particle size of 2–4 mm with a Waring blender at the highest speed for at least 3 min. The ground root samples (120 g) were mixed in methanol in a ratio of 1 to 10 (w/v) and shook in the oval-shaker (180 rpm, 25 °C) for 24 h. Mixtures were then filtered to separate the filtrate from its residues using a rough filter paper. This procedure was repeated three times to increase the efficiency of the extraction process with the addition of further methanol over the plant residues (Çoruh and Özdoğan, 2015). The filtrates were collected repeatedly and, using a rotary evaporator, were brought to complete dryness (at 40 °C under vacuum). When the filtrates came to complete dryness for a crude extract then it was proceeded to the fractionation process. The crude extract was redissolved in a total solution of 500 mL made of methanol/water in a 7:3 ratio (v/v). After dissolution in water/methanol the crude extract was partitioned into the successive solvents of increasing polarity: petroleum ether, chloroform, ethyl acetate, and saturated n-butanol.

2.4. Determination of antioxidant capacity
DPPH and ABTS methods were selected for the determination of antioxidant capacity. Those methods are frequently used due to their sensitivity, reproducibility, and simplicity.

2.4.1. Free radical scavenging by DPPH method
The protocol of Blois (1958), with minor modifications, was applied for the DPPH method. DPPH radical scavenging capacity was determined by monitoring the decrease in absorbance at 517 nm due to the depletion of the DPPH radical in the reaction mixture 15 min after the beginning of the reaction period. Quercetin was used as a phenolic standard of the positive control. The results were expressed in percentage of radical scavenging activity [RSA (%)] and calculated as:

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

where \(A_0\) is the absorbance of the control (ethanol only) and \(A_1\) is the absorbance of the samples prepared in ethanol. The fifty percent effective concentration (EC\(_{50}\)) values were calculated by constructing graphics of radical scavenging (\%) versus extract concentration (in log).

2.4.2. Free radical scavenging by ABTS method
The ABTS method depends on scavenging of the ABTS\(^*\) cationic radical. In this method, the trolox is utilized as a standard, and antioxidant capacity is defined as the samples’ cationic radical scavenging ability that is in turn expressed as the trolox equivalency of the antioxidant concentration (TEAC) (Rice-Evans and Miller, 1997).

The generation of the radical (ABTS\(^*\)) involves the reaction between ABTS and potassium persulfate, resulting in the cationic radical with maximum absorbance at the wavelength of 734 nm. The scavenging reaction starts by the addition of an antioxidant substance into the solution of the generated ABTS radical. Recordings of the decrease in the absorbance (734 nm) began 5 min after the start. The calculation of RSA (\%) is realized by plotting absorbance values (734 nm) versus the concentration of...
the antioxidant sample. The standard compound trolox, used as an antioxidant reference, is utilized for the plotting of a calibration curve. Subsequently, the RSA (%) is plotted against the concentration of trolox as well as that of the samples. Next, using the constructed RSA curves, a linear regression equation is determined for trolox and samples separately. Consequently, by dividing the slope value of each sample curve by that of trolox curve, TEAC values were calculated for each sample as the trolox equivalents in µmol/g.

### 2.4.3. Determination of total flavonoid content

Determination of total flavonoid content was accomplished by a slightly modified version of the method of Zhishen et al. (1999). A volume of 0.2 mL of the various concentrations of catechin used as a standard was diluted with water and 0.075 mL of 5% NaNO₂ was added and mixed. After 5 min, 0.15 mL of 10% AlCl₃ was mixed in. Six minutes later 0.5 mL of 1.0 M NaOH was added and the solution was diluted to 3.0 mL with water. Immediately after mixing of the solution, the catechin concentration was monitored by its absorbance at 510 nm. The same procedure was followed for extract/fraction solutions. Total flavonoid content was calculated as catechin equivalence obtained from the concentration curve plots of catechin and the samples. The experiments were carried out in duplicates and repeated three times.

### 2.5. Isolation of phenolic constituent by silica gel and Sephadex LH-20

In this study, the isolation and purification of phenolic compounds were performed by liquid column chromatography using silica gel 60 and a Sephadex LH-20 as the adsorbent materials.

#### 2.5.1. Silica gel 60 column preparation

In the first step of isolation, a sufficient amount of dry silica gel 60 was added to a chloroform and methanol mixture at a 3:1 (v/v) ratio, and the suspension was poured into a glass column (3.5 × 110 cm) for the silica column preparation. The mobile phase was applied as a gradient, starting with a 9:1 ratio of the chloroform and methanol mixture and continued at ratios of 8:2, 7:3, 6:4, and 1:1, finalized by 100% methanol in volumes of 100 mL. Additional silica gel glass columns (2 × 60 cm) were set for the further purification of the fractions collected from the previous purification steps, as necessary. Subsequently, the adsorbent (silica gel 60) was suspended in an adequate amount of chloroform, ethyl acetate, and methanol at a ratio of 3:1:0.75 (v/v), used for equilibration. The same isocratic mixture was also used for the elution system.

#### 2.5.2. Sephadex LH-20 column preparation

About 50 g of dry Sephadex LH-20 was soaked overnight in an ample amount of methanol. The glass column (2 × 60 cm) was established by Sephadex-methanol suspension. The 100% methanol was also used for eluting. TLC with the silica gel 60 F₂₅₄ 0.25-mm coated aluminum plates was employed for identification of the solvent mixture to be used as the mobile phase, before each step of the silica gel and Sephadex column preparations of the isolation and purification procedures. TLC was also a useful tool for following the fluorescent compounds reflected as fluorescent spots under UV radiation (254 and 360 nm).

#### 2.6. In vitro cytotoxicity assay against human breast cancer cells

The cytotoxic effects of crude extract, ethyl acetate fraction, and isolated compounds in MCF-7 and MDA-MB-231 cells were investigated by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. In brief, MCF-7 and MDA-MB-231 cells (about 10,000 cells/well) were seeded into 96-well plates and left for 24 h at 37 °C and 5% CO₂, in order to let them attach and grow. After 24 h, the medium was changed, 50 µL of fresh complete medium was added, and cells were treated with 50 µL of extract/fraction/isolated compounds at varying concentrations (0, 20, 50, 100, and 150 µg/mL) and DMSO at a concentration of 1% and were left for 24 h of incubation. When the reaction time was completed, 100 µL of phenazine methosulfate was added to 5 mL of XTT reagent before use, and 50 µL of this XTT solution was added to the cells cultivated already in a 96-well plate for 24 h, which were then incubated for 5 h in a CO₂ incubator at 37 °C. The cell viability or the proliferation effect of the extracts (in percentage) was determined by monitoring the optical density at 415 nm using an ELISA plate reader. The cell viability was calculated from the absorptive differences due to the treated concentrations of extract/fraction/isolated compounds in the presence and absence of the cultivated cells, divided by the control of solvent (DMSO) absorbance according to the presence and absence of the untreated cells, as follows:

$$\text{Cell viability (%) } = \left( \frac{[\text{OD}]_{\text{avg}} \text{ of X conc with cells) } - [\text{OD}]_{\text{avg}} \text{ of X conc without cells) }}{[\text{OD}]_{\text{avg}} \text{ of DMSO with cells) } - [\text{OD}]_{\text{avg}} \text{ of DMSO without cells) }} \times 100 \right)$$

X: concentration of the fractions or isolated compounds

[OD]avg: Optical density (in average)

DMSO: Dimethyl sulfoxide

The results were expressed in terms of percentage of cellular viability, calculated using the cell viability (%) equation, taking the relevant controls into account. Statistical analysis of three independent experiments performed in quadruplicate and calculation of effective dose of 50 percent (ED₅₀) from a dose response curve were done using Microsoft Excel.

197
2.7. Determination of morphological changes
Any morphological changes in the cells’ shape, level of adhesion, and any other alterations were observed by microscopy, using an inverted phase contrast microscope (magnification 400×; Olympus CKX 41) for 24 h.

2.8. Statistical analysis
All values were expressed as mean ± standard error by Microsoft Excel. The data were statistically analyzed by one-way ANOVA tests and P < 0.05 was the value at which the difference was considered statistically significant.

3. Results and discussion
3.1. Yield determination of extract and fractionation
The dried roots of *R. heckeliana* (120 g) yielded 20.12 g of dried crude methanol extract. Activity-guided, solvent-solvent fractionation was performed for the identification process of phenolic constituents, as explained in Section 2.3. The procedure of fractionation started with the dissolution of the dry crude extract (20.12 g) in 500 mL of a mixture of methanol and water (70:30, v/v). As soon as the extract dissolved in the mixture of methanol and water, it was sequentially partitioned in the solvents of petroleum ether, chloroform, ethyl acetate, and saturated *n*-butanol, respectively. Each step of respective fractionation yielded fractionated extracts of the petroleum ether, chloroform, ethyl acetate, saturated *n*-butanol, and aqueous phase as the remaining part in amounts of 0.622 g, 1.81 g, 1.52 g, 7.65 g, and 5.95 g, respectively. The calculated yields (%) of the crude and fractionated extracts of *R. heckeliana* roots are presented in Table 1.

Table 1. Extraction yield of the fractions of root extracts of *R. heckeliana*.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude methanol extract</td>
<td>16.8</td>
</tr>
<tr>
<td>Petroleum ether fraction</td>
<td>3.08</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td>9</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>7.4</td>
</tr>
<tr>
<td><em>n</em>-Butanol fraction</td>
<td>38</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>29.5</td>
</tr>
</tbody>
</table>

3.2. Antioxidant capacity
The fractionation process helps to successively concentrate the phenolic constituents of the total/crude extract in solvents of differing polarity, as well as separating them from the nonphenolic molecules. Therefore, each fractionated extract should be examined for their own amounts of antioxidant content. In that sense, it is important to apply the tests for antioxidant capacity to each fraction to distinguish the fraction(s) with the highest amount of antioxidant constituents.

3.2.1. DPPH radical scavenging capacity
The DPPH method is one of the most frequently applied methods to determine the radical scavenging capacity (antioxidant capacity) of the compounds as well as foods and extracts (Koleva et al., 2002). In this study, the free radical scavenging activity of the crude and fractionated extracts of *R. heckeliana* root was estimated using the DPPH radical. The DPPH radicals were allowed 15 min of reaction time for the completion of scavenging reactions using the specified extract concentrations. The decrease in the absorbance of DPPH radical was then recorded at 517 nm. RSA was calculated in percentage as described in Section 2, and the results are displayed in Figure 1. Most of

![Figure 1](image-url)
the extracts, as displayed in Figure 1, exhibited noticeable antioxidant capacities on DPPH radicals, and their EC$_{50}$ values were calculated from their RSA values as listed in Table 2. Among the listed extracts in Table 2, the ethyl acetate fraction revealed the highest radical scavenging capacity as indicated by its lowest EC$_{50}$ value (2.78 ± 0.018 µg/mL). On the other hand, the extract of the chloroform fractionation showed the lowest radical scavenging capacity with its EC$_{50}$ value of 82.64 ± 0.0422 µg/mL. A positive control was also used in this study; quercetin was a phenolic compound (EC$_{50}$ = 4.415 µg/mL).

To date, in the literature, we did not come across any scientific publications regarding *R. heckeliana* roots from the family Rosaceae and their antioxidant capacity. However, there were a number of articles published concerning other species of *Rosa* in the previous decades. Kumar et al. (2009) indicated the DPPH scavenging capacity of *R. burunonii*, *R. bourboniana*, and *R. damescana* with EC$_{50}$ values of 35.2 ± 0.69, 25.0 ± 0.71, and 21.4 ± 0.52 µg/mL, respectively. In another article, published by Ozsoy et al. (2013), the DPPH scavenging of the leaves and flowers of *R. horrida* was given in respective EC$_{50}$ values of 0.62 ± 0.031 and 0.61 ± 0.035 mg/mL. Another study on the radical scavenging capacity of flowers of *R. chinensis* determined the EC$_{50}$ value to be 21.3 µg/mL (Cai et al., 2005). A few years ago, the leaves of *R. agretis*, collected from Turkey, were evaluated for DPPH scavenging capacity, and the EC$_{50}$ value was given as 47.43 µg/mL (Bitis et al., 2010).

### 3.2.2. ABTS method

ABTS is another method used to estimate antioxidant capacity in this study. The ABTS assay, in principle, is similar to the scavenging method of DPPH. The scavenging reaction starts as soon as the antioxidant compound is added to the ABTS radical solution. The fall in the absorbance of the ABTS radical at 734 nm was noted 5 min after the start of the reaction. The RSA in percent is achieved from the plot of absorbance (734 nm) against the concentration of antioxidant samples. The standard compound trolox, used as an antioxidant reference, was utilized for the construction of the calibration curve at 734 nm. The TEAC values were calculated from the slopes of trolox versus sample curves. The ABTS scavenging capacities of the crude and fractionated extracts of *R. heckeliana* ranged from 9.64 ± 0.17 to 586 ± 6.64 µM, tabulated as TEAC values in Table 2. In the study of ABTS radical scavenging for the extracts covered here, the ethyl acetate fraction exhibited the highest radical scavenging capacity, as indicated by the highest TEAC value. Consequently, all of the results of ABTS radical scavenging tests were appropriately suitable with the outcomes of DPPH scavenging experiments (Table 2).

Concerning *R. heckeliana*, there are no published studies in regards to the scavenging capacity available in literature. However, many other studies regarding the ABTS scavenging capacity of various other *Rosa* species were reported. The ABTS scavenging properties of *R. horrida* leaf and flower extracts were recently given as 1.47 ± 0.102 and 1.44 ± 0.110 mg/mL, respectively (Ozsoy et al., 2013). In another study the TEAC value of the flowers

### Table 2. Comparison of the antioxidant capacities of crude extracts/fractions of roots of *R. heckeliana*.

<table>
<thead>
<tr>
<th>Samples</th>
<th>DPPH EC$_{50}$ (µg/mL) ± SD</th>
<th>TEAC$_{ABTS}$ (µM trolox) ± SD</th>
<th>Total flavonoid CE (µg/mg) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>12.83 ± 0.21</td>
<td>49.82 ± 0.95</td>
<td>0.184 ± 0.0047</td>
</tr>
<tr>
<td>Petroleum ether fraction</td>
<td>&gt;250</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Chloroform fraction</td>
<td>82.64 ± 0.42</td>
<td>9.64 ± 0.17</td>
<td>0.097 ± 0.028</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>2.78 ± 0.01</td>
<td>586 ± 6.64</td>
<td>4.58 ± 0.125</td>
</tr>
<tr>
<td>Butanol fraction</td>
<td>25.31 ± 0.02</td>
<td>32.1 ± 0.58</td>
<td>0.208 ± 0.0061</td>
</tr>
<tr>
<td>Aqueous fraction</td>
<td>60.8 ± 1.24</td>
<td>16.0 ± 0.39</td>
<td>0.161 ± 0.0044</td>
</tr>
<tr>
<td>Quercetin</td>
<td>4.415 ± 0.118</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

EC$_{50}$: 50% of effective concentration (µg/mL).
CE: catechin equivalents (µg/mg of extract).
TEAC: trolox equivalence of antioxidant concentrations (µM trolox/mg of extract).
ND: not determined.
NA: not applicable.
Quercetin: positive control.
SD: standard deviation.
of *R. chinensis* was given 235.8 ± 16.7 mM/100 g by Cai et al. (2005). Another report found the TEAC values of *R. sempervirens* extracts to range from 1.1 to 10 µg/mL (Ghazghazi et al., 2012).

### 3.2.3. Total flavonoid content

Flavonoids, as a part of plant phenolics, are also considered as effective free radical scavengers and antioxidant compounds. Hence, in the present study, it was important to determine the amount of the total flavonoid contents of each of the extract/fractions. The total flavonoid content was determined with a UV-Vis spectrophotometer through the construction of a calibration curve using various concentrations of the standard catechin at a maximum wavelength of 510 nm. The results were calculated as catechin equivalents (CE). Total flavonoid contents of the crude extract and fractions are listed in Table 2.

The results showed that the flavonoid content analysis also indicated the same increasing order for the presently applied tests of antioxidant capacities. Accordingly, the flavonoid content analysis also indicated the same increasing order for the presently applied antioxidant tests, which was helpful to decrease the tremendous amount of work of searching the active constituents of the total extracts. For that reason, the tedious work of isolation and purification methods was found worthy to apply on the ethyl acetate fraction with the aim of determining the active constituents of the root extracts of *R. heckeliana*. Consequently, the silica gel 60 and Sephadex LH-20 columns were set for further purification of that fraction. The dried ethyl acetate fractionated extract (1.52 g) was first subjected to column chromatography separation using silica gel 60 as mentioned in Section 2.5. The loaded extract sample was eluted by solvent mixtures composed of CHCl₃ and MeOH (9:1, 8:2, 7:3, 6:4, and 1:1 ratios) and pure methanol was used as the final step of the gradient elution. The flow rate of the elution was fixed as 1 mL/min (10 mL per each tube). Thus, 212 tubes were collected and the tubes were pooled together, which led to 9 main pools of fractions (Fr.1–Fr.9) after monitoring by TLC as shown in Figure 2. The mobile phase mixture for silica gel was also determined after checking by TLC. Hence, a suitable mobile phase was established to be the chloroform and methanol 3:1 mixture. All of the fractions collected were freeze-dried after removing the organic solvent under vacuum (40 °C). Among the subfractions, fraction 2 (Fr.2: 273 mg), fraction 4 (Fr.4: 366 mg), and fraction 6 (Fr.6: 131 mg) exhibited stronger antioxidant activity while the other fractions showed little or no antioxidant activity. As a result, the active fractions were further chromatographed using another silica gel 60 as well as a Sephadex LH-20 column for the further isolation and purification of the active compounds.

As presented in Figure 2, fraction 2 (Fr.2) was subjected to a second elution process on another silica gel 60 column (2 × 60 cm), using an isocratic mobile phase of chloroform, ethyl acetate, and methanol (mixed at a ratio of 3:1:0.75), which yielded five more subfractions (Fr.2A–Fr.2E). Subfraction Fr.2C (99 mg), after testing by TLC, was further purified using a Sephadex LH-20 column eluted by pure methanol, and this resulted in a pure compound numbered as 1. Another fraction labeled Fr.4 (366 mg) for additional separation was loaded on the Sephadex LH-20 column and its pure methanol eluted fractions, through verification by TLC with the mobile phase of chloroform and ethyl acetate (0.5:4.5), were pooled into four subfractions. Subsequently, subfraction Fr.4B (137 mg) was chromatographed by the silica gel 60 column (70–230 mesh) and eluted using an isocratic mixture of chloroform, ethyl acetate, and methanol (3:1:0.75, v/v,v). Resulting fractions were pooled again after testing by TLC and thus compound 2 was obtained as the pure constituent
of fraction 4B. Previously pooled and dried Fr.6 was also exposed to another silica gel 60 column isolation by elution through an isocratic mobile phase composed of chloroform, ethyl acetate, and methanol (3:1:0.75, v/v), which then led to the purification of compound 3. At the end of the application of serial column chromatography isolation approaches, pure compounds of compound 1 (85 mg), compound 2 (178 mg), and compound 3 (72 mg) were gathered, as shown schematically in Figure 2, which represents the whole procedure of the extraction, fractionation, and isolation of the phenolic components of *R. heckeliana* roots.

3.4. Identification of the compounds purified from the ethyl acetate fraction of *R. heckeliana*

Chemical structures of the purified compounds obtained from the ethyl acetate fraction of *R. heckeliana* were identified by the data of proton \( (^1H) \) and carbon \( (^{13}C) \) NMR spectroscopy.

Compound 1: UV \( \lambda_{\text{max}} \) 253, 364 nm (in methanol).

\(^1H\) NMR (400 MHz, DMSO-\( d_6 \)): \(^1H\)-NMR spectral peaks were at \( \delta \): 7.46 (2H, s, H-1 and H-6).

\(^{13}C\)-NMR (DMSO-\( d_6 \)): Carbon atoms peaks were detailed at \( \delta \): 159.4 (C-5 and C-10), 148.4 (C-3 and C-8), 140.9 (C-2 and C-7), 109.9 (C-4 and C-9). The spectral

![Figure 2. Schematic diagram of the isolation steps of pure active compounds from *R. heckeliana*.](image-url)
data were compatible with those of ellagic acid (Luo et al., 2009).

Compound 2: UV λ max 276 nm (in methanol).

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(^1\)H-NMR spectral peaks were obtained at δ: 6.84 (1H, d, \(J = 2.0\) Hz, H-2’), 6.77 (1H, dd, \(J = 8.1\) Hz, H-5’), 6.71 (1H, dd, \(J = 2.0, 8.1\) Hz, H-6’), 5.92 (1H, d, \(J = 2.4\) Hz, H-8), 5.85 (1H, d, \(J = 2.4\) Hz, H-6), 4.56 (1H, d, \(J = 8.0\) Hz, H-2), 3.97 (1H, dd, \(J = 8.0, 8.0, 4.8\) Hz, H-3), 2.85 (1H, dd, \(J = 4.8, 16.0\) Hz, H-4), 2.50 (1H, dd, \(J = 8.0, 16.0\) Hz, H-4).

\(^1\)C-NMR (100 MHz, DMSO-\(d_6\)): Peaks of carbons were at δ: 158.0 (C-9), 157.7 (C-5), 157.1 (C-7), 146.4 (C-3’), 146.4 (C-4’), 132.4 (C-1’), 120.2 (C-6’), 116.2 (C-5’), 115.4 (C-2’), 101.0 (C-10), 96.4 (C-6), 95.7 (C-8), 83.0 (C-2), 69.0 (C-3), 28.7 (C-4). The spectral data were in agreement with the literature data reported for catechin (Zhang et al., 2012).

Compound 3: UV λ max 325 nm (in methanol).

\(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \(^1\)H-NMR spectrum revealed peaks at δ: 7.42 (d, 1H, \(J = 15.8\) Hz), 7.02 (d, 1H, \(J = 2.1\) Hz), 6.95 (dd, 1H, \(J = 2.1, 8.1\) Hz), 6.75 (d, 1H, \(J = 8.1\) Hz), 6.17 (d, 1H, \(J = 15.8\) Hz).

\(^1\)C-NMR (100 MHz, DMSO-\(d_6\)): Carbon atom peaks were at δ: 168.1, 148.2, 145.4, 144.4, 125.6, 121.1, 115.7, 115.2, and 114.6. The above spectral data were consistent with the literature data (Wei et al., 2010) and compound 3 was identified as caffeic acid.

As consistently seen from the data given above and by estimations based on the literature (Luo et al., 2009; Wei et al., 2010; Zhang et al., 2012), isolated compounds 1, 2, and 3 were predicted as catechin, caffeic acid, and ellagic acid, respectively. Moreover, for the first time, the present study has documented the analysis of those active phenolic compounds purified from the roots of \(R.\) heckeliana. The phenolic constituents of \(R.\) heckeliana were not published previously; however, some of its compounds were quite comparable with those of other \(Rosa\) species (Sumere et al., 1993; Hvattum, 2002; Kumar et al., 2009). The compounds (1, 2, and 3) were confirmed by UV-Vis spectra. They exhibited UV-Vis absorption maxima at 254 nm and 364 nm for ellagic acid, 280 nm for catechin, and 240 nm and 324 nm for caffeic acid. Additionally, the purity of the isolated compounds was also verified by HPLC-DAD chromatography (Figure 3).

To our knowledge, the only publication in the literature was the previous report published by Çoruh and Özdoğan (2015) in regards to the phenolic constituents of \(R.\) heckeliana. Nevertheless, some reports on other \(Rosa\) species such as \(R.\) damascena, \(R.\) persica, and \(R.\) canina are available for the characterization of phenolic constituents. Kumar et al. determined 10 polyphenolic compounds, namely gallic acid, rutin, quercitrin, myricetin, quercetin, quercetin derivatives, galloyl, tannin, kaempferol, and flavonol glucosides, in the fresh flowers of \(R.\) bourboniana, \(R.\) brunonii, and \(R.\) damascena (Kumar et al., 2009). Consequently, in most of the analyzed species, the main phenolic compounds observed consisted of quercetin, kaempferol, and their glycosides (Veligolu and Mazza, 1991; Jassbi et al., 2003; Nowak and Tuzimski, 2005; Nowak and Gawlik-Dziki, 2007).

3.5. Investigation of cytotoxic properties of the extracts and isolated constituents of \(R.\) heckeliana

Antioxidant properties of many plants are also indicative of their cytotoxicity potential and might be effective on numerous cancer cell lines (Ju et al., 2004; Boivin et al., 2009). As we have discussed so far, the ethyl acetate fraction of \(R.\) heckeliana contains the highest active phytochemical content and has the highest antioxidant potential at the same time among the other fractions. Thus, the ethyl acetate fraction as well as the compounds isolated, catechin, caffeic acid, ellagic acid, and the crude extract for comparison, were all used for cytotoxic studies on the selected cancer cell lines.

3.6. Analysis of cell viability by XTT assay

Examining the cytotoxic properties of the compounds of interest against cancer cell lines is conceivable by pursuing cell viability tests like XTT, MTT, WST-1, and MMT. The XTT and MTT assays are colorimetric techniques that allow the quantitative determination of cell viability. They are based on the capability of viable cells to transform the XTT salt or the MTT salt into formazan dyes (Mossman, 1983; Scudiero et al., 1998). Our chosen test was the tetrazolium reduction assay using XTT in order to monitor the populations of the viable cells. Subsequently, the cell viabilities for human breast cancer cells were considered for those sensitive and insensitive to estrogen receptors, which were respectively the MCF-7 and MDA-MB-231 cell lines. The method of the XTT test was applicable via a rapid colorimetric assay using 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide salt. The cell viability was monitored through the absorption wavelength of 415 nm. Both of the cell lines were treated with the crude and the ethyl acetate extracts, as well as the isolated compounds (catechin, caffeic acid, ellagic acid) plus a standard compound of choice, catechin. The treatment was applied to the cells (MCF-7 and MDA-MB-231) in a dose-dependent manner and monitored at 415 nm. The cytotoxic effects of the treated extracts/compounds were expressed in ED\(_{50}\) calculated from dose-response plots. As a consequence, the lower the ED\(_{50}\) value is, the higher the cytotoxic effect.

3.6.1. Application to estrogen receptor-sensitive breast cancer cells (MCF-7)

The studied crude and ethyl acetate fractionated extracts were applied to the MCF-7 cell line in a dose-dependent
Figure 3. a) RP-HPLC chromatogram of the isolated ellagic acid (compound 1), b) chromatogram of the isolated caffeic acid (compound 2), c) chromatogram of the isolated caffeic acid (compound 3) at 280 nm. Inlays: UV-Vis scans of the isolated compounds.
manner; as a result, a substantial effect of cytotoxicity was noticeable for all. Subsequently, the cytotoxic outcome was even more pronounced for the ethyl acetate extract, as shown in Table 3. The phenolic compounds isolated from the ethyl acetate fraction of *R. heckeliana*, including catechin, caffeic, and ellagic acid, as well as the standard compound catechin, were also examined against the MCF-7 cells as given in Table 3. Complete MCF-7 cell death (100%) was noticeable with the treatment of all the isolated phenolic compounds and the standard compound when applied at a concentration of 150 µg/mL, as is apparent in Table 3.

As tabulated in Table 3, the dose-dependent cell viability values and calculated ED₅₀ values of crude extract, ethyl acetate fraction, and isolated compounds including catechin, caffeic acid, and ellagic acid and standard catechin were given. The treatments were carried out at the determined concentrations of 10, 25, 50, 100, and 150 µg/mL for 24 h. The ED₅₀ value of *R. heckeliana* crude extract was 113.38 ± 11.38 µg/mL for 24 h of treatment. On the other hand, 24 h of treatment of the ethyl acetate fractionated extract against MCF-7 cells resulted in an ED₅₀ value of 61.18 ± 0.99 µg/mL. Thus, the ethyl acetate fraction was more effective than the crude extract. At the same time, the isolated compounds of the ethyl acetate fraction of *R. heckeliana* such as ellagic acid (ED₉₀ value: 30.26 ± 0.77 µg/mL), caffeic acid (ED 50 value: 32.70 ± 1.65 µg/mL), and isolated catechin (ED₉₀ value: 35.80 ± 2.19 µg/mL) were quite effective on the estrogen receptor-sensitive MCF-7 cells.

Friedman et al. (2007) reported that catechin was effective on MCF-7 breast cancer cells. Cytotoxicity tests were achieved by the application of the MTT assay and using catechin concentrations ranging from 50 to 400 µg/mL. In the given report, using catechin, a cytotoxic effect was realized against MCF-7 cells, obtaining about 20% cell death at the highest concentration of catechin (400 µg/mL). However, in our experiment, we obtained 60% cell death with 50 µg/mL catechin.

More recently, a study examined the cytotoxic effects of *Hedeoma drummondii* and its isolated compounds, caffeic acid, chlorogenic acid, and rosmarinic acid, on MCF-7 and HeLa cells by applying the WST-1 colorimetric assay. It was demonstrated that caffeic acid revealed the highest antiproliferation for both of the cancer cell lines studied with IC₅₀ values of 10.7 µg/mL and 8.7 µg/mL, respectively (Valdez et al., 2010). In the present study, however, for caffeic acid ED₅₀ (IC₅₀) was obtained as 32.70 µg/mL.

In another study, ellagic acid was examined for its antiproliferative effect on breast cancer cell lines (MCF-7 and MDA-MB-231); however, it was found effective only at higher concentrations (more than 250 µM for MCF-7) by applying the MTT assay (Kim et al., 2009). This is quite interesting, since we have observed almost 10% cell viability or 90% antiproliferative effect for ellagic acid, even at a concentration of 50 µg/mL.

### Table 3. XTT cell viability test against MCF-7 breast adenocarcinoma cell line by the application of *R. heckeliana* extracts.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>10 µg/mL ± SD</th>
<th>25 µg/mL ± SD</th>
<th>50 µg/mL ± SD</th>
<th>100 µg/mL ± SD</th>
<th>150 µg/mL ± SD</th>
<th>Effective dose ED₅₀ µg/mL ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>92.30 ± 3.18</td>
<td>86.67 ± 1.04</td>
<td>72.47 ± 6.86</td>
<td>52.80 ± 8.52</td>
<td>38.50 ± 4.07</td>
<td>113.38 ± 11.38</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>95.50 ± 1.42</td>
<td>85.97 ± 6.59</td>
<td>70.07 ± 7.60</td>
<td>28.90 ± 1.85</td>
<td>16.37 ± 7.09</td>
<td>61.18 ± 0.99</td>
</tr>
<tr>
<td>Isolated catechin</td>
<td>82.57 ± 5.32</td>
<td>71.53 ± 5.00</td>
<td>39.27 ± 2.67</td>
<td>17.37 ± 1.10</td>
<td>3.90 ± 1.05</td>
<td>35.80 ± 2.19</td>
</tr>
<tr>
<td>Isolated caffeic acid</td>
<td>90.00 ± 4.00</td>
<td>64.67 ± 2.52</td>
<td>24.33 ± 0.58</td>
<td>10.67 ± 0.58</td>
<td>8.33 ± 1.53</td>
<td>32.70 ± 1.20</td>
</tr>
<tr>
<td>Isolated ellagic acid</td>
<td>81.40 ± 3.75</td>
<td>61.73 ± 4.47</td>
<td>34.43 ± 2.51</td>
<td>9.16 ± 2.06</td>
<td>0.33 ± 0.58</td>
<td>30.26 ± 0.77</td>
</tr>
<tr>
<td>Standard catechin</td>
<td>94.40 ± 2.37</td>
<td>58.70 ± 1.69</td>
<td>30.30 ± 2.71</td>
<td>16.2 ± 1.45</td>
<td>3.80 ± 1.05</td>
<td>34.11 ± 1.65</td>
</tr>
</tbody>
</table>

Experimental values are given as mean ± standard deviation. Each value is the mean of quadruple measurements from three different experiments (n = 12).
crude extract and ethyl acetate fractions were calculated with values of 127.24 ± 2.88 and 62.54 ± 2.01 µg/mL, respectively (Table 4).

Cells were exposed to increasing concentrations of extracts (from 10 to 150 µg/mL) for 24 h. When the cytotoxic effects of isolated compounds and the standard catechin were determined against the cells, even at low concentrations (50 µg/mL) of both compounds there were significant decreases in cell viability. At higher concentrations of catechin, such as 100 µg/mL, for example, it was almost complete with more than 90% cell death (Table 4).

Table 4 also shows the dose-dependent cytotoxic effect of the isolated caffeic acid on cell viability. The MDA-MB-231 cells were again treated with a concentration as low as 10 µg/mL of caffeic acid, whereby the cytotoxicity was quite low, but at increasing concentrations, the observed cytotoxicity increased proportionally.

When the cytotoxicity of the isolated ellagic acid was tested on MDA-MB-231 breast cancer cells, it was quite prominent when compared to all other extracts and isolated compounds so far, as shown in Table 4. Complete cell death (100%) was achieved by the application of the ellagic acid isolate at a concentration of 100 µg/mL. Consequently, when the ED_{50} values of those isolated compounds, ellagic acid, caffeic acid, and catechin, were calculated, it resulted in 30.27 ± 0.53 µg/mL, 36.94 ± 0.20 µg/mL, and 40.07 ± 2.17 µg/mL, respectively. As an obvious outcome, the ellagic acid was found more cytotoxic than caffeic acid and catechin. When the ellagic acid was confined to 10.5% of crude extract, it was highly effective as a cytotoxic component (Çoruh and Özdoğan, 2015). Comparing ED_{50} values of the isolated compounds with the extracts obtained from _R. heckeliana_ they were clearly more effective. Moreover, the magnitudes of ED_{50} values of the isolated catechin versus the commercial catechin were not statistically different, as shown in Table 4.

The results of the present study demonstrate that the crude extract of _R. heckeliana_ root displayed weak cytotoxic effects by inhibiting only about 40% of MCF-7 and MDA-MB-231 cell proliferation. On the other hand, the ethyl acetate fractionation extract was more cytotoxic than the crude extract. The cytotoxic responses observed in this study were not found to be dependent on estrogen receptor sensitivity; in other words, similar results were obtained for sensitive (MCF-7) and insensitive (MDA-MB-231) cell lines.

According to the criteria established by the U.S. National Cancer Institute (NCI), compounds with ED_{50} less than 30 µg/mL are considered as active, while ED_{50} between 30 µg/mL and 100 µg/mL was determined to be moderately active and ED_{50} higher than 100 µg/mL was considered inactive (Suffness and Pezzuto, 1990). In the results of the present study, the ethyl acetate fractionation extract displayed a noteworthy effect of cytotoxicity with an ED_{50} of less than 100 µg/mL, which indicates moderate cytotoxicity according to NCI criteria. Even though the extracts and the fractionated extracts of _R. heckeliana_ were not yet evaluated for their antiproliferative or cytotoxic effects against cancerous cell lines in any other studies so far, we tried to discuss and compare our work with similar standards or isolated compounds that were examined for the mentioned breast cancer cell lines.

Sartippour et al. (2001) reported that catechin in increasing concentrations (0–40 µg/mL) was effective on the viability of MDA-MB-231 cancer cells for 24 h of application. Percent cell proliferation was obtained at about 30% for the catechin concentration of 40 µg/mL.

**Table 4. Cytotoxic effect of _R. heckeliana_ extracts against MDA MB-231 breast cancer cell lines by XTT assay.**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MDA-MB-231</th>
<th>Effective dose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cell viability (%)</td>
<td>ED_{50} µg/mL ± SD</td>
</tr>
<tr>
<td></td>
<td>10 µg/mL ± SD</td>
<td>25 µg/mL ± SD</td>
</tr>
<tr>
<td>Crude extract</td>
<td>97.90 ± 0.44</td>
<td>82.70 ± 2.33</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td>90.00 ± 2.24</td>
<td>78.00 ± 3.89</td>
</tr>
<tr>
<td>Isolated catechin</td>
<td>85.50 ± 5.07</td>
<td>73.87 ± 2.5</td>
</tr>
<tr>
<td>Isolated caffeic acid</td>
<td>97.00 ± 1.78</td>
<td>71.87 ± 4.24</td>
</tr>
<tr>
<td>Isolated ellagic acid</td>
<td>81.00 ± 1.00</td>
<td>56.37 ± 1.11</td>
</tr>
<tr>
<td>Standard catechin</td>
<td>80.27 ± 2.79</td>
<td>78.2 ± 1.56</td>
</tr>
</tbody>
</table>

Experimental values are given as mean ± standard deviation. Each value is the mean of quadruple measurements from three different experiments (n = 12).
Approximately 35% inhibition was also obtained in our study for catechin, as seen in Table 4.

In the literature, caffeic acid exhibited an antiproliferative effect against MDA-MB-231 cells at a concentration of 50 µM for 72 h. Cell density was decreased and the cell viability was estimated by the MTT colorimetric assay (Gomes et al., 2003). In the experiments that we conducted on MDA-MB-231 cells it was for 24 h of growth; however, in the study by Gomes et al. (2003), cells were monitored for 72 h.

Kim et al. (2009) reported that MDA-MB-231 cells showed a significant antiproliferative effect with a dose-dependent pattern when exposed to different concentrations of ellagic acid for 24 h. Cell proliferation was calculated by MTT assay. At an ellagic acid concentration of 250 µM, the proliferation (% control) of the cells declined to approximately 30%. In another study, Diospyros lotus and one of its isolated compounds, namely ellagic acid, showed significant cytotoxic effects with a dose-dependent pattern against MDA-MB-231 cells via another colorimetric assay, MMT. A 50% cytotoxic concentration (CC$_{50}$) value of 35.84 µg/mL was determined (Rashed et al., 2012). That was quite similar to the result presented in Table 4 in our study.

### 3.7. Morphological alterations

The MCF-7 and MDA-MB-231 cell lines were grown in 24-well plates (2 × 10$^5$ cells/mL; 1 mL/well) or incubated with the desired (over or under the ED$_{50}$ value: 50, 100, and 150 µg/mL) crude extract/ethyl acetate fraction/isolated compound concentrations for cell viability tests. Any changes in cell shape or in the level of adhesion or any other alteration possibilities were photographed by using an inverted-phase contrast microscope for 24 h.

#### 3.7.1. MCF-7 cell line

Apparent changes in the cell morphology, with cell deformity and floatation by losing the adhesion to the surface of the 24-well plates, were monitored through 24 h of treatment with two different concentrations of extract/fraction/isolated compounds. As shown in the negative control or 1% DMSO-treated controls, the cells were normally round and smooth in shape and size with the ability of adhesion to the surface of the 24-well plates (Figures 4a and 4b).

After 24 h of the treatment, the effects of the extracts on the morphological structures of the MCF-7 cells were observed (Figure 4). When the crude extracts were applied in concentrations of 100 µg/mL and 150 µg/mL, increased cell death was quite evident in latter (Figures 4c and 4d). However, for the concentrations of the ethyl acetate fraction (50 and 100 µg/mL), the level of adhesion decreased and some of the cells ended up with round shapes as an indication of the cell death (Figures 4e and 4f). Meanwhile, the effect of the ethyl acetate fraction was much more profound when compared to crude extract, even at the lower end of the concentration range selected.

Furthermore, the isolated active compounds of the ethyl acetate fraction were also examined individually for their influence on the morphology of the cell lines tested (Figures 5a–5b). The isolated compounds including catechin, caffeic acid, and ellagic acid showed a noticeable amount of morphological alterations of MCF-7 cells and treatment at concentrations of 50 and 100 µg/mL of those compounds showed a decrease in the adhesion level of cells when compared with the controls. At a concentration of 50 µg/mL, catechin inhibited cell growth and led to slight cell shrinkage (Figure 5c). However, in the presence of 100 µg/mL catechin, a higher amount of MCF-7 cells were rounded and deformed in shape (Figure 5d). The exposure of the MCF-7 cells to the isolated compounds including caffeic and ellagic acids at 50 µg/mL concentration for 24 h led to blebbing in the membranes of the cells as well as shrinkage and shape deformation (Figures 5e and 5g), as monitored by inverted-phase contrast microscope (400×). At the 100 µg/mL concentration of ellagic acid and caffeic acid, however, increasing numbers of cell deaths were realized (Figures 5f and 5g).

#### 3.7.2. MDA-MB-231 cell line

Control or DMSO-treated cells were regular in cell shape and size (Figures 6a and 6b). After 24 h of treatment of MDA-MB-231 cells with 100 µg/mL and 150 µg/mL of the crude extract, some of the cells exhibited a decrease in their adhesion levels as well as an observable change in cell morphology (Figures 6c and 6d) in a dose-dependent manner. The ethyl acetate fractionated extract was quite effective, at both the concentrations applied (50 and 100 µg/mL), in changing the morphology of the cells and with reduced confluence (Figures 6e and 6f).

After 24 h of treatment of MDA-MB-231 cells (Figures 7a–7h) with the isolated catechin concentration of 50 µg/mL, they showed a noticeable alteration in cell morphology as well as a decrease in adhesion levels (Figure 7c). When the higher concentration (100 µg/mL) of catechin was applied, the level of adhesion was even further reduced and nearly 90% of the cells were rounded, with a noticeable decrease in cell confluence (Figure 7d).

With treatment of the MDA-MB-231 cells for 24 h with the isolated caffeic acid (50 µg/mL), the cells began to round up and the level of adhesion declined (Figure 7e). At higher concentrations of caffeic acid (100 µg/mL), the observed effects were even more pronounced, e.g., more than half of the cells displayed rounding as an indication of cell death (Figure 7f).

When MDA-MB-231 cells were treated with isolated ellagic acid (50 µg/mL) for 24 h, more than half of the cells were dead and floating, along with an observed loss of cell confluence (Figure 7g). Subsequently, 24 h of treatment...
with the higher concentration of the isolated ellagic acid (100 µg/mL) resulted in a greater number of dead cells and an amplified amount of cell debris (Figure 7h). Neither the extract nor the treated isolated compounds showed a noticeable difference between the morphological changes of estrogen receptor-sensitive MCF and receptor-insensitive MDA cells.

In conclusion, *Rosa* is an important genus, extensively investigated by researchers due to its therapeutic properties in many diseases and for its long use in folk medicine. The purpose of this study was to determine the phytochemical constituents that were responsible for the noticeable antioxidant and cytotoxic capacities of *R. heckeliana* roots. *R. heckeliana* roots were extracted in methanol-water, followed by solvent-solvent fractionation. Each fractionated phase was analyzed for its phytochemical contents using the well-known radical scavenging methods of DPPH and ABTS. Among the tested fractions of *R. heckeliana*, the ethyl acetate fraction was found effective in both the DPPH and ABTS tests, indicating that the existing phenolic compounds were concentrated in this middle-polarity solvent. As supporting evidence of that, the total amount of flavonoid content was tested and it was also found highest in the ethyl acetate fraction.
Figure 5. Morphological alterations of MCF-7 cells after 24 h of treatment of the isolated compounds. a) Control; (b) in 1% DMSO; the effect of isolated catechin: c) 50 μg/mL, d) 100 μg/mL; the effect of isolated caffeic acid: e) 50 μg/mL, f) 100 μg/mL; the effect of isolated ellagic acid: g) 50 μg/mL and h) 100 μg/mL.
The ethyl acetate fraction was further separated into its pure constituents. Isolation of the bioactive contents of the ethyl acetate fraction provided three phenolic compounds as the main constituents, namely catechin, caffeic acid, and ellagic acid. Cytotoxicity studies were carried out against two lines of breast cancer cells, MCF-7 and MDA-MB-231, which are sensitive and insensitive to estrogen receptors, respectively. Crude extract, ethyl acetate fraction, isolated products, and a standard were applied at different concentrations to the feeding environment of the cell lines. Accordingly, isolated ellagic acid is considered as a moderately effective cytotoxic compound, with a 50 µg/mL ED₅₀ value for both MCF-7 and MDA-MB-231 cells.

Acknowledgments

We would like to express our gratitude to Professor Fevzi Özgökçe for his generous gift of the plant materials throughout this work. The authors gratefully acknowledge the financial support of this study provided by Middle East Technical University (BAP-08-11-DPT2011K121010).

Figure 6. Morphological changes of the MDA-MB-231 cells after crude extract and fraction with 24 h of treatment. a) Control, b) in 1% DMSO. The crude extract at concentrations of: c) 100 µg/mL, d) 150 µg/mL. The ethyl acetate fractionated extract at concentrations of: e) 100 µg/mL, f) 150 µg/mL.
Figure 7. Morphological alterations of MDA-MB-231 cells upon 24 h of treatment of the isolated compounds. a) Control; b) in 1% DMSO; treatment of catechin concentrations: c) 50 µg/mL, d) 100 µg/mL; treatment of caffeic acid concentrations: e) 50 µg/mL, f) 100 µg/mL; treatment of isolated ellagic acid concentrations: g) 50 µg/mL, h) 100 µg/mL.
References


