Neuroprotective potential of the fruit (acorn) from *Quercus coccifera* L.

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Abstract: Neurodegenerative diseases such as Alzheimer disease and Parkinson disease are among the deadly disorders affecting the elderly population. Unfortunately, effective treatments and medications are still needed to combat these diseases. The ethanol extracts of the raw peeled acorns and acorn coffees from *Quercus coccifera* prepared by two different methods (boiled-roasted ground and roasted ground) were tested against acetylcholinesterase (AChE), butyrylcholinesterase (BChE), and tyrosinase (TYR), the enzymes associated with neurodegeneration. The acorns used in the present study were harvested from wild *Quercus coccifera* trees in Kilis Province, located in the Eastern Mediterranean region of Turkey in November 2016. Cholinesterase (ChE) and TYR inhibitory activity of the ethanol extracts prepared from raw material and two coffee samples were screened using an ELISA microtiter assay at 2 mg/mL stock concentration. Antioxidant effects of the extracts were also tested for their scavenging activity against DPPH, while total phenol and flavonoid quantities of the extracts were determined spectrophotometrically. Our results indicated that all of the extracts had notable AChE and BChE inhibition, while they were inactive against TYR. The most active extract against ChEs was prepared from the raw material, which led to 65.94 ± 3.59% and 85.04 ± 3.83% inhibition against AChE and BChE, respectively. Our data indicate that the fruit extract from *Quercus coccifera* is a source of promising ChE inhibitors and deserves future study.

Key words: Alzheimer disease, cholinesterase inhibition, neurodegeneration, *Quercus coccifera*

1. Introduction

Forests have played an important role for mankind for centuries. Humans depend on forests for survival, from the air we breathe to the wood we use. Besides providing habitats for animals and livelihoods for humans, forests also offer watershed protection, prevent soil erosion, and mitigate climate change (Çalışkan and Boydak, 2017; Kahveci, 2017; Razzaghi et al., 2017).

The genus *Quercus* (Fagaceae), known as “meşe” in Turkish, is represented by 32 taxa, 5 of which are endemic to Turkey, and it is vital for the Anatolian landscape (Güner et al., 2012). *Quercus* species are mostly used for production of furniture, agricultural instruments, and building materials, while these plants are also a source of fodder for animals (Özcan and Bayçu, 2005). Moreover, 26.34% of the forestland in Turkey consists of oak trees. Fruits (acorns) of this species have also been used as a coffee substitute like racahout, which is a spicy Turkish acorn drink similar to hot chocolate (Bainbridge, 1986; Rakic et al., 2006). *Q. coccifera* L. (kermes oak) is one of the evergreen species especially distributed in the Mediterranean phytogeographic region. Based on ethnobotanical studies, the use of different parts of kermes oak has been recorded against hemorrhoids, diabetes, diarrhea, and kidney stones as well as for its hypotensive, abortive, and wound-healing properties in Anatolian folk medicine (Agelet and Velles, 2003; Tuzlacı and Sadıkoğlu, 2007; Ugurlu and Secmen, 2008; Güneş et al., 2017). *Q. coccifera* has been so far reported to have several biological effects such as antiulcerogenic, antibacterial, antifungal, antihelmintic, and antioxidant activity (Alkofahi and Atta, 1999; Şöhretoğlu et al., 2007; Manolaraki et al., 2010; Buzayan and El-Garbulli, 2012; Genç et al., 2012). Additionally, previous studies have shown that *Q. coccifera* contains phenolic compounds such as tannins and flavonoids, besides tocopherols and fatty acids (Ito et al., 2002; Genç et al. 2012; Şöhretoğlu et al., 2014; Akcan et al., 2017). In our previous study, we analyzed minerals of the same samples and found that processing affected their mineral composition as well as heavy metal concentration, which were below acceptable limits (Sekeroglu et al., 2017).

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Alzheimer and Parkinson diseases are progressive neurodegenerative disorders, which are especially affecting the elderly population. Today, Parkinson disease affects approximately 10 million people worldwide, whereas Alzheimer disease is responsible for about 60%–70% of dementia cases (http://www.neurodegenerationresearch.eu/about/what/). Both of these diseases have no complete cure now, but drugs only for symptomatic treatment based on a cholinergic hypothesis are available clinically. The majority of commercially available medications that proved to be effective in cognitive-related disorders or were approved by the US Food and Drug Administration to treat symptoms are mostly plant-derived molecules (D’onofrio et al., 2017).

In our ongoing studies for finding new cholinesterase (ChE) and TYR inhibitors of natural origin, we recently explored the neuroprotective properties of several commercial brands of Turkish coffee sold in Turkey and Northern Cyprus (Aslan Erdem et al., 2016), a number of traditional herbal coffees consumed in Turkey such as terebinth coffee (Orhan et al., 2012), and some other herbal coffees prepared from the different parts of carob, black cumin, dates, and tumble thistle. Our previous results revealed that date samples (Phoenix dactylifera) exerted the highest acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition among the tested extracts (Sekeroglu et al., 2012), and taking these promising results into consideration, we have now decided to screen the neuroprotective potential of acorns and coffees from Q. coccifera.

2. Materials and methods
2.1. Plant material
The fruits of Quercus coccifera L. were collected from Kilis (Turkey) in November 2016 and were identified by the second author. A voucher specimen was preserved in the herbarium of Kilis 7 Aralık University (Kilis, Turkey).

2.2. Preparation of the coffee samples and extracts
The plant materials were air-dried at room temperature and peeled. Acorn coffees were prepared with two different methods: boiled, roasted, and then ground; and only roasted and then ground. The raw materials were ground in a mechanical grinder. The coffee samples and ground raw materials were weighed accurately in a digital balance (Shimadzu, Japan) and extracted with ethanol (80%) for 2 days. The ethanol phases were filtered and removed using a rotary evaporator (Buchi, Switzerland) until dryness to give the crude extracts. The extract yields (w/w%) are given in the Table.

2.3. Enzyme inhibition assays
2.3.1. AChE and BChE inhibitory activity
AChE and BChE inhibitory activity of the samples was measured by the slightly modified spectrophotometric method of Ellman et al. (1961). Electric eel AChE (EC 3.1.1.7, Sigma, St. Louis, MO, USA) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma) were employed as substrates of the reaction. 5,5’-Dithiobis(2-nitrobenzoic)acid (DTNB, Sigma) was used for the measurement of the anticholinesterase activity. All reagents, conditions, and calculations were the same as described in our previous publication (Senol et al., 2016). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. The experiments were done in quadruplicate. Galanthamine hydrobromide (Sigma) was used as the reference drug.

2.3.2. TYR inhibitory activity
TYR (EC 1.14.18.1, mushroom tyrosinase, Sigma) inhibitory activity of the samples was determined spectrophotometrically by the slightly modified method of Masamoto et al. (1980) with L-DOPA (Sigma) as the substrate (Lee et al., 2009). All reagents and conditions were the same as described in our previous publication (Aslan

<table>
<thead>
<tr>
<th>Raw material</th>
<th>Inhibition against AChE (%)</th>
<th>Inhibition against BChE (%)</th>
<th>Inhibition against TYR (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.17</td>
<td>65.94 ± 3.59</td>
<td>85.04 ± 3.83</td>
<td>10.13 ± 3.22</td>
</tr>
<tr>
<td>1.03</td>
<td>30.22 ± 3.13</td>
<td>47.66 ± 3.13</td>
<td>4.84 ± 0.75</td>
</tr>
<tr>
<td>1.30</td>
<td>39.91 ± 2.51</td>
<td>57.40 ± 3.23</td>
<td>2.95 ± 1.93</td>
</tr>
<tr>
<td>97.12</td>
<td>86.77 ± 2.86</td>
<td>83.97 ± 1.68</td>
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<thead>
<tr>
<th>Yield % (w/w)</th>
<th>Inhibition against AChE (Inhibition% ± SD), 200 µg/mL</th>
<th>Inhibition against BChE (Inhibition% ± SD), 200 µg/mL</th>
<th>Inhibition against TYR (Inhibition% ± SD), 133.33 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw material</td>
<td>5.17</td>
<td>65.94 ± 3.59</td>
<td>85.04 ± 3.83</td>
</tr>
<tr>
<td>Roasted</td>
<td>1.03</td>
<td>30.22 ± 3.13</td>
<td>47.66 ± 3.13</td>
</tr>
<tr>
<td>Boiled-roasted</td>
<td>1.30</td>
<td>39.91 ± 2.51</td>
<td>57.40 ± 3.23</td>
</tr>
<tr>
<td>Reference</td>
<td>97.12</td>
<td>86.77 ± 2.86</td>
<td>83.97 ± 1.68</td>
</tr>
</tbody>
</table>

*Standard deviation, †final concentration, ‡galanthamine hydrobromide - 100 µg/mL, §kojic acid - 133.33 µg/mL.
The assays were performed in a 96-well microplate using an ELISA microplate reader (VersaMax Molecular Devices, USA) by measuring their absorbance at 492 nm. Results were compared with a control (DMSO) and kojic acid (Sigma) was used as the reference. The percentage TYR inhibition (I%) was calculated as follows:

\[ I\% = \frac{(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}})}{\text{Absorbance}_{\text{control}}} \times 100. \]

2.4. Antioxidant activity by DPPH radical-scavenging assay

The stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity of the samples was determined by the modified method of Hatano et al. (1988). The samples and references dissolved in ethanol (75%) were mixed with DPPH solution (Barros et al., 2007). Quercetin (Sigma) was employed as the reference. Inhibition of DPPH in percentage (I%) was calculated as given below:

\[ I\% = \left(\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100, \]

where \( A_{\text{blank}} \) is the absorbance of the control reaction (containing ethanol instead of test sample), and \( A_{\text{sample}} \) is the absorbance of the samples/reference. Analyses were run in triplicate and the results were expressed as average values with standard deviation.

2.5. Determination of total phenol and flavonoid contents

Phenolic compounds in total were determined in accordance with the slightly modified Folin–Ciocalteu method (Singleton and Rossi, 1965; Orhan et al., 2009). Absorption was measured at 760 nm using a 96-well microplate reader (VersaMax Molecular Devices). Total flavonoid content of the extracts was calculated by aluminum chloride colorimetric method (Woisky and Salatino, 1998). A number of dilutions of quercetin were obtained to prepare a calibration curve. Absorbance of the reaction mixtures was measured at a wavelength of 415 nm using a 96-well microplate reader (VersaMax Molecular Devices). The total phenol and flavonoid contents of the extracts were expressed as gallic acid and quercetin equivalents (mg g\(^{-1}\) extract), respectively.

3. Results

ChE and TYR inhibitory activity of the ethanol extracts of the raw-peeled acorns and acorn coffees prepared by two different methods (boiled-roasted ground and roasted ground) tested at 200 µg/mL final concentration are summarized in the Table. The extracts were not active against TYR, whereas they had prominent AChE and BChE inhibition. The most active extract against ChEs was prepared from the raw material, which exerted 65.94 ± 3.59% and 85.04 ± 3.83% inhibition against AChE and BChE, respectively.

The extracts were screened against DPPH for their possible radical-scavenging activity at 2000 µg/mL stock concentration. Among the tested extracts, the highest DPPH scavenging activity was caused by the ethanol extract of the raw material (91.06 ± 0.22%), followed by the ethanol extract of boiled-roasted and only roasted coffee samples (90.46 ± 0.16% and 84.09 ± 1.18%, respectively). Total phenol and flavonoid quantities of the extracts were determined according to the equations as \( y = 3.5049x + 0.1012 \) (\( r^2 = 0.9996 \)) and \( y = 4.2992x + 0.0797 \) (\( r^2 = 0.9999 \)), respectively. As summarized in the Figure, the ethanol extract prepared from the raw material possessed the highest amount of total phenol (166.45 ± 1.01 mg/g extract as gallic acid equivalent). The highest total

<table>
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<th>DPPH</th>
<th>Flavonoid content</th>
<th>Phenolic content</th>
</tr>
</thead>
<tbody>
<tr>
<td>raw material</td>
<td>91.06</td>
<td>2.24</td>
<td>166.45</td>
</tr>
<tr>
<td>roasted</td>
<td>84.09</td>
<td>8.93</td>
<td>58.32</td>
</tr>
<tr>
<td>boiled+roasted</td>
<td>90.46</td>
<td>3.12</td>
<td>15.54</td>
</tr>
<tr>
<td>Quercetin</td>
<td>90.78</td>
<td></td>
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</table>
flavonoid quantity was observed in the ethanol extract of the roasted coffee sample (8.93 ± 1.07 mg/g extract, as quercetin equivalent).

4. Discussion
The neuroprotective potential of phytochemicals has always attracted great attention from scientists due to their antioxidant and anti-inflammatory activities and their impacts on maintaining the brain's chemical stability by affecting the function of receptors (Kumar and Khanum, 2012).

Our results indicated that ethanol extract prepared from the raw material of kermes oak acorn displayed potent BChE and moderate AChE inhibitory activity and it also had higher inhibition values towards both enzymes than the coffee samples tested. According to Custodio et al. (2013), hexane, methanol, and hot water extracts prepared from Quercus suber and Quercus ilex acorns were screened for their ChE inhibitory effects. Similar to our results, the methanol extracts exerted ChE inhibitory activity and their AChE inhibitory activity was higher than BChE. They concluded that gallic acid, one of the main compounds of cork oak acorns, may be linked to the ChE inhibitory activity of the methanol extract. In another study, six different Quercus species from Korea were analyzed for their anti-Alzheimer activity using a passive avoidance test in mice. Gallic acid, (+)-catechin, and (-)-epicatechin were determined as the main constituents of the methanol extract prepared from Q. mongolica leaves and the same extract also inhibited AChE activity in scopolamine-induced memory impairment in mice (Nugroho et al., 2016). Based on our results, the raw material has been found to contain the highest total phenolic content. The total phenolic content of the extracts was decreased with roasting or boiling and then roasting processes. According to Schmitzer et al. (2011), no change was observed in the total phenolic contents with roasting of hazelnuts; nevertheless, it had a negative effect on individual phenolic compounds and catechin derivatives in particular. In another study, significant decrease in condensed tannins and gallic acid amount was observed with the roasting of Turkish hazelnut varieties (Alasalvar et al., 2010). A boiling process (cooking for 5 min) also led to a decrease or increase in total phenolic contents of some vegetables (Türkmen et al., 2005). On the contrary, the total flavonoid content of extracts was increased with a roasting process. In a temperature-dependent study on onion varieties, flavonoid content was shown to increase up to 120 °C, then decrease at 150 °C (Sharma et al., 2015). Roasting or boiling temperature and time seem to be important factors for total phenolic and flavonoid contents of different kinds of plant extracts and coffee samples.

Oxidative damage caused by free radical interaction with neural cells led to degeneration, while exogenous and endogenous antioxidants such as polyphenols, vitamin E, melatonin, or flavonoids could retard cell death (Uttara et al., 2009; Lalkovicova and Danielisova, 2016). One of the possible mechanisms of the impact of coffee on the risk for neurodegenerative diseases is the polyphenolic content of the coffee, which could prevent low-density lipoprotein oxidation and inhibit the development of cerebral arteriosclerosis (Wierzejska, 2017). The antioxidant activities of the extracts against DPPH radical were similar to each other and the results were not correlated with total phenolic content of the extracts. Previous studies concluded that antioxidant activity depends on the roasting time and coffee type, and the selected method is also a significant factor (Pokorna et al. 2015; Priftis et al., 2015).

Acorn coffee samples did not inhibit mushroom TYR, while the raw material has shown a weak inhibitory effect. Studies by different research groups disclosed that galls of Q. infectora, which also grows in Turkey, has significant inhibitory effects on mushroom TYR and also reduced intercellular melanin amount. Phenolic acids and flavonoids are the main compounds of galls, which have remarkable anti-TYR effects (Khazaeli et al., 2009; Sharififar et al., 2013; Jamshidzadeh et al., 2017).

In conclusion, our data indicate that the fruit extract from Q. coccifera appears to be a natural source having promising ChE inhibitory molecules, which is under further investigation by our group. Previous studies indicated that plant extracts are natural sources for phytochemicals that are important for human health (Kamiloglu et al., 2009; Tosun et al., 2009; Ercisli et al., 2012).

References


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