Comparison of ABTS, DPPH, Phosphomolybdenum Assays for Estimating Antioxidant Activity and Phenolic Compounds in Five Different Plant Extracts

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Abstract
Many plants and their constituents (carotenoids, flavonoids and other phenolic compounds), action to inhibit the free radical by the use of antioxidant activity, helping to a better efficiency of nutrients utilization. The purpose of this study was to investigate the antioxidant activity and phenolic content of five different plants, known for their antioxidant potential. Total phenols were determined by Folin-Ciocalteu method, with few modifications. The antioxidant capacity was evaluated by DPPH, ABTS radical-scavenging activity and phosphomolybdenum method. The results showed that the highest antioxidant activity was obtained for walnut leaves in terms of ABTS evaluation and for bilberry leaves by DPPH and phosphomolybdenum methods. In the present study, peppermint, walnut and bilberry leaves proved to have an important antioxidant activity and also a total phenolic content.

Keywords: ABTS, DPPH, phosphomolybdenum, polyphenols, plants

INTRODUCTION
In the last decades, it has been intensively studied natural alternatives to synthetic antioxidants and the effects of antioxidative substances obtained from natural sources. Antioxidants can be synthesized in vivo or taken as dietary antioxidants, but it is known that plants are a good source of antioxidants, Kasote et al., (2015). Many active components of herbs and spices can prevent lipid peroxidation having action against free radicals or through activation of antioxidant enzymes, helping to a better efficiency of nutrients utilization, Frankič et al., (2009).

The antioxidant potential of plants has been found to be a promising method of countering the undesirable effects of oxidative stress. The most important naturally occurring plant substances showing antioxidant activity are phenolic substances (carotenoids, flavonoids and other phenolic compounds, phenolic acids, hydrolysable tannins) (Balasundram et al., 2006). In this study, we considered five plants rich in phenolic compounds: marigold, walnut leaves, peppermint, bilberry leaves and sesame seeds. Bilberry (Vaccinium myrtillus L.) leaf and fruit contain a variety of antioxidant that prevent free radicals from causing oxidative damage in cells. These benefits take place thanks to presence of catechin tannins, plant chemicals that are both astringent and anti-inflammatory, according to Riihinen et al., (2008). For marigold (Calendula officinalis) it has been reported to contain a variety of phytochemicals, including carotenoids, terpenoids, flavonoids and other constituents, showing some important biological activities like wound healing, immuno-stimulant, spasmonic and spasmylytic, hepatoprotective, anti-bacterial and anti-fungal, antioxidant, Jan et al., (2017). Peppermint
(Mentha longifolia) is an herb with a wide range of pharmacological properties such as antimicrobial, gastrointestinal, and nervous system effects and used for treatment of various diseases such as headaches and digestive disorders, Mikaili et al., (2013). The walnut (Juglans Regia) leaves have tannins, phenolic compounds and other healing compounds. Walnuts are good source of flavonoids, sterols, pectic substances, phenolic acids and related polyphenols. Leaves have been used mostly in traditional medicines as antimicrobial, antihelminthic, astringent, keratolytic, antidiarrhoeal, hypoglycaemic, depurative, tonic, carminative (Taha and Al-wadaan, 2011). Sesame (Sesamum indicum L.) seeds are rich in oil, protein, dietary fiber, and micronutrients such as minerals, lignans, tocopherol, and phytosterol. Available data indicate that natural plant products have beneficial effects on human and animal health, notably in relation to their antioxidant activity, Lee et al., (2016). For this purpose several methods were developed for measuring the total antioxidant capacity of plants, like total antioxidant capacity (TAC), free radical scavenging of DPPH radical, oxygen radical absorbance capacity (ORAC), free radical scavenging of ABTS radical, phosphomolybdenum method, Altemimi et al., (2017).

The objective of this study was to assess the antioxidant activity and phenolic content of some plants, known for their potential of countering the undesirable effects of oxidative stress by four different analytical methods.

MATERIALS AND METHODS

Plant material

All medicinal plants used were obtained from local pharmacies, dried, grounded and packed. The choice of the plants investigated was based on their use in the traditional medicine (bilberry leaves, walnut leaves, mentha leaves, sesame seeds, marigold flowers).

Plant extracts

The methanol extract was obtained by extracting 1 g of dried plant powder in 10 mL methanol and kept on a rotary shaker for 24 h, in the dark. The extract was centrifuged at 1500 g for 10 min and the supernatant was considered for analysis.

Content of Total Polyphenols

The total phenol content of all plants extracts was measured spectrophotometrically according to the Folin-Cioalteu’s method, as described by (Astill et al., 2001) with slight modifications. Briefly, the extract samples (0.5 mL of different dilutions) were mixed with 0.5 mL FolinCioalteu reagent, 7 mL water and was homogenized. The solution was kept at room temperature for 3 min before adding 2 mL of sodium carbonate solution 20%. After an hour in the dark the absorbance was measured at 732 nm against a blank (solution with no extract added). Calibration curve of Gallic acid was used to determine total phenol compounds, and the results were reported as mg gallic acid equivalents per gram sample (mg GAE/ g).

Total Antioxidant Capacity (TAC) by phosphomolybdenum method

The total antioxidant capacity of the plant extracts was evaluated by the method of Prieto et al., (2010). An aliquot of 0.2 mL of the sample solution was mixed with 4 mL of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). For the blank, 0.2 mL ethanol was mixed with 4 mL of the reagent. The absorbance of the test sample was measured at 695 nm. The results were reported as mmol eq ascorbic acid /Kg sample.

ABTS free radical scavenging assay

The scavenging potential of plant extracts against ABTS radical was determined using the method described by Qwele et al., (2013). The radical was generated by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in the same ratio and allowed to react in the dark for 12 h at room temperature. This solution was further diluted with ethanol to adjust its absorbance to 0.70 at 734 nm. The diluted ABTS+ solution (6 mL) was reacted with 40 μL of the supernatant followed by measuring the absorbance at 734 nm (Jasco V-530, Japan Servo Co.Ltd., Japan) using ethanol as blank. The scavenging activity of sample against ABTS radical was expressed using a standard calibration curve constructed by plotting percentage inhibition against concentration of trolox. The results were reported as mmol eq trolox/Kg sample.

DPPH free radical scavenging assay

DPPH radical-scavenging activity of the supplements was estimated by the method of Qwele et al., (2013). A volume of 2 mL of 0.2 mM DPPH prepared in methanol was added to 400 μL supernatant and 1.6 mL distilled water. The mixture was vortexed and left to stand at room
temperature for 30 min. A tube containing 2 mL of methanol and 2 mL of DPPH solution was used as control. The absorbance of the solution was measured at 517 nm using a spectrophotometer (Jasco V-530, Japan Servo Co.Ltd., Japan). The scavenging activity of sample against DPPH radical was expressed using a standard calibration curve constructed by plotting percentage inhibition against concentration of trolox. The results were reported as mmol eq trolox/Kg sample.

Statistical analysis
All analyses were performed in triplicate, with data expressed as mean ± standard deviation. Pearson’s correlation was used to analyze the relationships between polyphenol content and antioxidant activity measured by three different methods.

RESULTS AND DISCUSSIONS
Table 1 presents the results obtained for total polyphenol content and antioxidant activity (determination methods used: ABTS ([2,2’- azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid)]), DPPH (2,2-Diphenyl-1-picrylhydrazyl, total antioxidant capacity (TAC) by phosphomolybdenum method), for the five considered plants.

The results showed that the highest antioxidant activity was obtained for walnut leaves in terms of ABTS evaluation and for bilberry leaves by DPPH and phosphomolybdenum methods. Also, important results were obtained for marigold by DPPH and phosphomolybdenum methods. Sesame seeds have shown the lowest antioxidant capacity resulted from all methods used. Results reported in scientific literature showed values of 302.7% DPPH inhibition for bilberry leaves (Martz et al., 2010) and 3.47 g/L DPPH for mentha (Mkaddem et al., 2009). In terms of ABTS evaluation, mentha was considered to have important values (195 mg/L Mkaddem et al., 2009 and 153 mg/L Nickavar et al., 2010).

Phenolic compounds are ubiquitous constituents of plants and their major sources in human diet are vegetables, fruit and various beverages. The polyphenols evaluation presented important values for mentha, walnut and bilberry leaves. Results from our study showed a wide variation in total polyphenol content of plant species tested: from sesame seeds to walnut leaves. Vinson and Cai, (2012) found values of 69.3 μmol catechin equivalents/g total polyphenols in walnut and Vishwanath et al. (2012) reported 2.88 mg gallic acid equivalents/g total polyphenols in sesame seeds.

By correlating the antioxidant capacity to phenolic compounds with antioxidant activity, it has been found that mentha has maximum values of phenolic compounds and concurrently, intense antioxidant activity.

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**Table 1. Total polyphenol content and antioxidant activity of selected plants**

<table>
<thead>
<tr>
<th></th>
<th>ABTS mmol eq trolox/Kg</th>
<th>DPPH mmol eq trolox/Kg</th>
<th>TAC mmol eq ascorbic acid/Kg</th>
<th>Total polyphenol content Mg GAE/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marigold</td>
<td>65.69</td>
<td>225.14</td>
<td>135.74</td>
<td>10.42</td>
</tr>
<tr>
<td>Mentha</td>
<td>285.74</td>
<td>228.02</td>
<td>105.57</td>
<td>71.95</td>
</tr>
<tr>
<td>Sesame seeds</td>
<td>31.77</td>
<td>72.51</td>
<td>28.82</td>
<td>5.61</td>
</tr>
<tr>
<td>Bilberry leaves</td>
<td>320.94</td>
<td>255.83</td>
<td>196.22</td>
<td>52.82</td>
</tr>
<tr>
<td>Walnut leaves</td>
<td>332.36</td>
<td>196.25</td>
<td>186.79</td>
<td>53.94</td>
</tr>
</tbody>
</table>

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**Table 2. Antioxidant methods determination and polyphenols content correlation**

<table>
<thead>
<tr>
<th></th>
<th>ABTS</th>
<th>DPPH</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH</td>
<td>0.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAC</td>
<td>0.75</td>
<td>0.81</td>
<td></td>
</tr>
<tr>
<td>Total polyphenols content</td>
<td>0.92</td>
<td>0.61</td>
<td>0.52</td>
</tr>
</tbody>
</table>
Table 2 presents the correlation of methods for determining the antioxidant capacity ABTS, DPPH, TAC and the total polyphenol content.

The correlation coefficients between values obtained by three antioxidant methods evaluation were between 0.6 and 0.8. The best correlation was between TAC and DPPH with a correlation of 0.81. Recently, Aliyu et al., (2012), found positive correlations between the determination of total antioxidant capacity by phosphomolybdenum method and DPPH assays. A good correlation is also between TAC and ABTS of 0.75. Table 2 highlighted that the ABTS radical-scavenging activity reflects the polyphenols antioxidant potential, the correlation having the higher coefficient. The results suggested that the phenolic compounds contributed significantly to the antioxidant capacity of studied plants. Similar results were also obtained by Karouet et al., (2005) who showed that the total phenolic compounds contents were highly correlated with the ABTS, R=0.94.

Phenolic compounds are considered to be the most important antioxidants of plant materials and their antioxidant activity is based on their ability to donate hydrogen atoms to free radicals (Sulaiman et al., 2011). Some authors showed correlation between total phenolic content and antioxidant activity, measured by different methods and others found no such relationship (Sulaiman et al., 2011). The low correlations might be explained that total antioxidant activity is not due to only one contributor, the presence of non-phenolic antioxidants (vitamin C, vitamin E and carotenoids) having accountable antioxidant activity.

CONCLUSION

In the present study, peppermint, walnut and bilberry leaves proved to have an important antioxidant activity determined by three different methods and also a total phenolic content. To confirm the beneficial effects of studied plants, further studies are necessary to establish their in vivo activity and bioavailability.

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REFERENCES


