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Antioxidant properties of several medicinal plants growing wild in northeastern Iran

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ABSTRACT

There are an increasing number of studies on pharmaceutical properties of medicinal plants growing wild in various climates of Iran. However, there is little information available concerning antioxidant activity of medicinal plants growing wild in northeastern Iran. Thus, the aim of this study was to evaluate antioxidant capacity of the methanolic extracts from leaves of seven Iranian medicinal plants collected from the northeast. Evaluations were made for total phenols and flavonoids, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity, reducing power, and lipid peroxidation inhibition. Overall, findings revealed that there were significant differences in antioxidant potencies amongst the tested plants. Among the plant samples, Berberis integerrima exhibited the highest DPPH inhibition activity ($p < 0.05$) followed by Mentha piperita and Berberis vulgaris, whereas Salvia officinalis and Foenciculum vulgare were found to be less effective DPPH scavengers ($p < 0.05$). Almost similar results were obtained in terms of reducing power, and total phenols and flavonoids. In lipid peroxidation assay, however, no plant sample reached 50% inhibitory concentration. Overall, B. integerrima exhibited a remarkable antioxidant potency in vitro. This result identifies this plant as a good candidate for further investigations.

Keywords: medicinal plants, north-eastern Iran, antioxidant activity, *Berberis integerrima*

INTRODUCTION

Living organisms are equipped with a defense system to neutralize free radicals and other reactive oxygen species (ROS). This defense system include enzymes such as catalases, superoxide dismutase, glutathione peroxidise, glutathione reductase; or compounds such as glutathione, vitamins E and C, etc. As long as free radicals are balanced by the body's antioxidative defense system, the body is in healthy conditions. However, depletion or loss of antioxidant levels may lead to free radical-caused oxidative stress. Oxidative stress can cause cellular and tissue damages, DNA mutation, cancer etc [1]. Besides, in the current world, the human body is significantly exposed to external sources of free radicals. Therefore, the body's antioxidative defense system might not be adequate to prevent oxidative-caused damages completely [2]. In this regard, antioxidant complements, or foods encompassing antioxidants, could assist the body's defense system and contribute to the reduction or neutralization of oxidative damages.

Medicinal plants used in the traditional medicine are well-known significant sources of natural antioxidants. Medicinal plants-derived natural antioxidants, which are in the form of raw extracts and/or chemical constituents, are very efficient to block the process of oxidation by neutralizing free radicals [3]. It is also commonly accepted that medicines taken from plant products are safer than their synthetic counterparts; however, the toxicity profile of most medicinal plants have not been comprehensively assessed [4].

Having a wide range of climates, over 7500 plant species grow wild in Iran, of which 10-15% is expected to be medicinal plants [5]. In addition, Iran has a well-demonstrated long history use of medicinal plants in the traditional

medicine. However, due to having a large variety of medicinal plants species, all of the major medicinal plants have not yet been evaluated for their medicinal properties, including antioxidant activity. A recent review has listed several Iranian medicinal plants that have been reported for their antioxidant activities [5]. According to this review and other articles reported in the literature, the aim of this study was to investigate the antioxidant activities of several medicinal plants growing wild in northeastern Iran, which have not been studied for their antioxidant properties thus far. Various chemical and biochemical assays were undertaken to investigate antioxidant potentialities of these plants.

MATERIALS AND METHODS

Chemicals and reagents

Reagents used in this study included butylated hydroxyanisole (BHA), trichloroacetic acid (TCA), and thiobarbituric acid (TBA), methanol (grade 99.9%), sodium dodecyl sulfate (SDS), hydrochloric acid (grade 37%), acetic acid (grade 99.8%), and 1-butanol (grade 99%), which were all purchased from Merck (Darmstadt, Germany). Other compounds included gallic acid (Fluka, Spain), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, Germany), folin-ciocaltue (Panreac, Spain), catechin (Fluka, USA), and ascorbic acid (Scharlab S.L. Sentmenat, Spain).

Plant extracts production

The following medicinal plants were collected during the year 2012 from Binalood Mountains (northeastern Iran), authenticated by specialists in the plant herbarium of Faculty of Agriculture, Ferdowsi University of Mashhad: *Mentha piperita*, *Berberis integerrima*, *Berberis vulgaris*, *Melissa officinalis*, *Artemisia absinthium*, *Salvia officinalis*, and *Foenciculum vulgare*. The leaves of the plants were air-dried at 25°C and powdered. The whole plant materials were extracted twice with methanol. The total crude plant extracts were collected and evaporated to dryness by an oven.

Determination of total phenolic content

Total phenolic content was determined through a Folin-Ciocalteu colorimetric method, modified from Mayur et al. [6]. In brief, the plant extract solution (10 µL) was mixed with 100 µL of Folin-Ciocalteu's in a 96-well plate. After five minutes, 80 µL of a 15% sodium carbonate solution was added to the mixture and then kept in the dark for 30 minutes, after which the absorbance was read at 725 nm using a Nanodrop apparatus (Epoch-Biotek, USA). Gallic acid was used to generate the standard curve, and the reduction of Folin-Ciocalteu reagent by the samples was expressed as mg of Gallic acid equivalents (GAE) per g of extract.

Determination of total flavonoid content

Flavonoid contents were determined by a modified method described by Barros et al. [7]. The plant extract (10 $mg.ml^{-1}$) was mixed with 312.5 µL of distilled water and 18.7 µL of a 5% sodium nitrite solution, and after five minutes 37.5 µL of 10% aluminum chloride solution was added to the mixture. After six minutes, 125 µL of 1 M sodium hydroxide and 68.7 µL of distilled water were added to the mixture. The solution was properly mixed and 125 µL of the mixture was dispensed into each well and absorbance was then read at 510 nm. Catechin (4.9 $\mu g.ml^{-1}$ up to 2500 $\mu g.ml^{-1}$) was used to construct the standard curve and the results were expressed at mg Catechin equivalents per mL of plant extract.

DPPH scavenging activity assay

Inhibition of diphenyl-2-picrylhydrazyl (DPPH) radicals by the tested plant extracts were measured according to a method reported by Arbaayah and Kalsom [8], with minor modifications. A mixture consisting of an extract solution at different concentrations (50 µL) and the methanolic solution of the DPPH reagent (50 µL) was loaded into each well of a 96-well plate. The mixture was left to stand for 30 minutes in the dark, and then the absorption was measured at 517 nm using the Nanodrop apparatus. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: % RSA =
(Ac–As/Ac) ×100; where RSA: radical scavenging activity; Ac: absorbance of the negative control; As: absorbance of the plant sample or ascorbic acid.

Reducing power

The reducing power of the plant extracts was estimated by a basic method of Oyaizu [9]. Various concentrations of the extracts (500 µL) were mixed with 500 µL of sodium phosphate buffer (200 mM, pH 6.6) and 500 µL ml of 1% potassium ferricyanide. Then, the mixture was incubated at 50°C for 20 minutes. In order to stop the reaction, 500 µL of 10% trichloroacetic acid was added to the mixture followed by centrifugation at 3000 rpm for 10 minutes. The supernatant (500 µL) was mixed with 500 µL of deionized water and 100 µL of 0.1% ferric chloride solution and the mixture was left to stand for 10 minutes. The absorbance was then measured at 700 nm. A higher absorbance indicated a higher reducing power. BHA was used as a positive control for comparison.

Lipid peroxidation inhibition assay

The lipid peroxidation inhibition assay was determined according to a method described by Ruberto and Baratta [10] with slight modifications. One gram of fresh egg yolk homogenate (10%, in ice-cold 1.15% KCl, w/v) was centrifuged at 2500 g for 15 minutes. Then, 500 µL of the supernatant, 100 µL of each plant sample, 50 µL of 10 µM FeSO4, and 50 µL of 100 µM TCA were mixed and made up to one mL with sterile distilled water. The reaction mixture was incubated at 37°C for 30 minutes. The following controls were also taken instead of the plant sample: BHA (as the reference control) and sterile distilled water (as the fully oxidized control). The reaction was stopped by addition of 50 µL of TCA (20%, pH 3.5), 1.5 mL of TBA (0.8%, w/v, in 1.1% SDS, w/v) and 1.5 ml of acetic acid (20%, w/v, pH 3.5), followed by heating at 100°C for 60 minutes. After cooling, 5.0 ml of 1-butanol was added to each tube to stabilize the color, followed by centrifugation at 2500g for 15 minutes. The absorbance of the upper layer was taken at 532 nm. Inhibition percentage was determined through the following formula: Inhibition ratio (%) = (A-Al)/A × 100; where A was the absorbance value of the fully oxidized and A1 was the absorbance value of the sample.

Statistical analysis

All the assays were carried out in triplicate and each experiment was independently repeated at least three times, through which means and standard deviations (SD) were generated. The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD Test with $\alpha = 0.05$. The analyses were conducted using GraphPad Prism version 6.

RESULTS

DPPH radical scavenging activity

The DPPH assay is based on the assumption that an antioxidant serves as a hydrogen donor and thus reduces (decolorizes) DPPH free radicals (the color turns from purple to yellow). This assay is well-known as a basic, quick tool to evaluate antioxidant activity of putative antioxidants. Thus, the antioxidant potency of a compound is relative to the loss of DPPH free radicals (DPPH scavenging) that can be quantified through a decrease in the maximum absorption of DPPH at 570 nm.

In this study, results showed that all samples had significant levels of radical scavenging activity in a dose-dependent manner (Fig. 1). The DPPH-derived EC_{50} values of the plant extracts are also illustrated in Table 1. All samples inhibited DPPH over 90% at concentrations 2.5-5 mg/mL, results that were not significantly different from those of the positive control ($p>0.05$). The inhibition percentages remained about 90% at concentration 1.25 mg/mL except those of *S. officinalis* and *F. vulgare* ($p < 0.05$).

Amongst the plant samples, *B. integerrima* and *Mentha piperita* were found to be the most potent DPPH scavengers, as they could inhibit DPPH free radicals up to 90% at low concentrations (even at 620 µg/mL), compared to the rest ($p < 0.05$). At the minimum tested concentration (7 µg/mL), *B. integerrima* showed a DPPH scavenging activity of 36%, whereas the figures for *S. officinalis* and *F. vulgare* were 5 and 0.75%, respectively ($p < 0.05$) (Fig. 1). Compatible with these data, the DPPH-EC_{50} values of *B. integerrima* and *Mentha. piperita* were significantly lower than the rest ($p < 0.05$) (Table 1), while DPPH-derived EC_{50}s of *S. officinalis* and *F. vulgare* were significantly higher than the rest ($p < 0.05$).

Reducing power

Reducing capability of a putative antioxidant can be assessed using its ability to convert Fe^{3+} to Fe^{2+}. Intensity of Perl's Prussian blue caused by this reduction is measured at 700 nm; a higher absorbance indicates higher reducing power. Then, reducing power of the compound can be contributed to its antioxidant potency. The reducing power values of the plant extracts tested in this study are illustrated in Fig. 2. Findings revealed that the values of reducing power of the plant extracts were functions of their concentrations. At concentration one mg/mL, *B. integerrima* and *Mentha piperita* had reducing power values of over 2 that were not significantly different from that of BHA (as the positive control). At this concentration, *B. vulgaris* showed a remarkable reducing power (over 2.8) that was significantly greater than those of the positive control as well as the rest of the plant samples ($p < 0.05$). At lower concentrations, reducing power values of the plant extracts exhibited more similarities to each other (Fig. 2). In agreement with these results, EC_{50} values derived from the reducing power assay (Table 1) showed that *B. vulgaris* had the minimum EC_{50} that was significantly lower than the rest of the plant extracts ($p < 0.05$) and similar to that of the positive control. Following *B. vulgaris*, three plant extracts *B. integerrima*, *Mentha piperita*, and *Mellisa officinalis* exhibited the absorbance of 0.5 at concentrations around 140-170 µg/mL which were significantly lower than the rest ($p < 0.05$). The highest values of reducing power-derived EC_{50} were seen in *S. officinalis* and *F. vulgare* (Table 1). Taken together, *B. vulgaris* was the best in terms of reducing power, followed by *B. integerrima* and *Mentha piperita*.

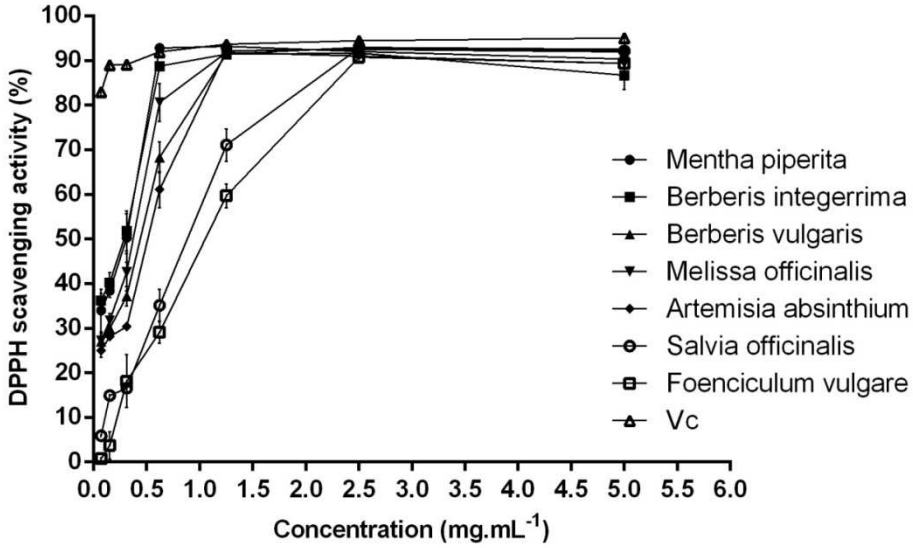

Figure 1. Dose-response curves of DPPH scavenging activity in the plants. Values represent reduction of DPPH radicals determined by measuring the absorption at 517 nm. Each experiment was independently repeated as least three times from which standard deviations have been derived. Vc stands for ascorbic acid that served as a positive control.

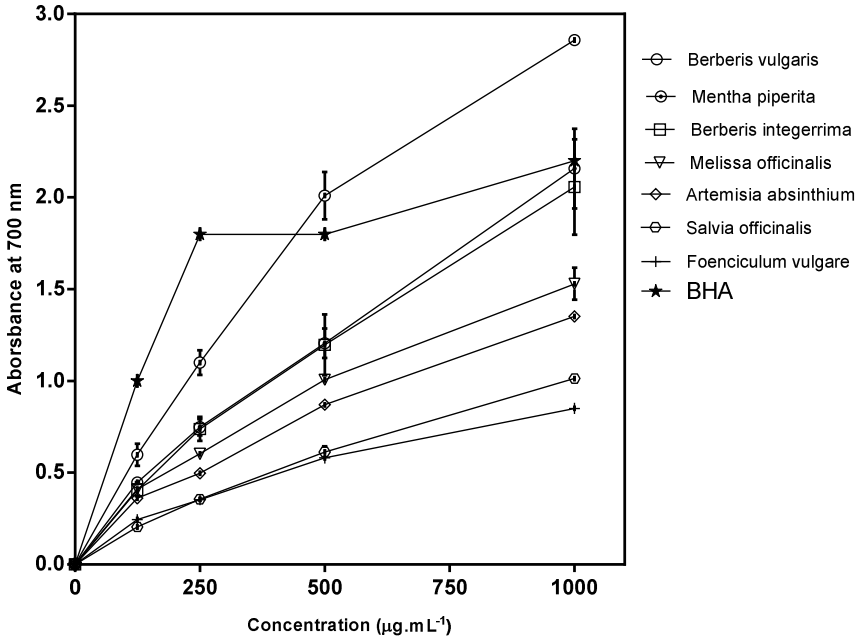

Figure 2. Reducing power of the tested plant samples. Reduction of Fe^{3+} to Fe^{2+} by the plant extracts (at various concentrations) changed the intensity of Perl's Prussian blue that was measured at 700 nm. Each experiment was independently repeated as least three times from which standard deviations have been derived. BHA served as the positive control.

Inhibition of lipid peroxidation

Effect of the plant extracts on lipid peroxidation was measured by a modified TBARS method using egg yolk homogenates as lipid rich media. Amounts of decrease in the pinkish-red color developed by TBA-MDA complex were attributed to inhibitory effects of the extracts on lipid peroxidation. The findings showed that no noticeable color change was occurred at concentrations below 40 mg/ml^{-1} (data not shown). Therefore, the lipid peroxidation assay was performed using the plant extracts at 40 $mg.ml^{-1}$. Overall, no lipid peroxidation inhibition above 50% was seen among the plant samples (Fig. 3, Table 1). Among the plants, *Melisa officinalis* showed highest lipid peroxidation (around 46%) that was significantly greater than the rest ($p < 0.05$). There was no significant difference in lipid peroxidation inhibition among the rest of the plant samples (Fig. 3).

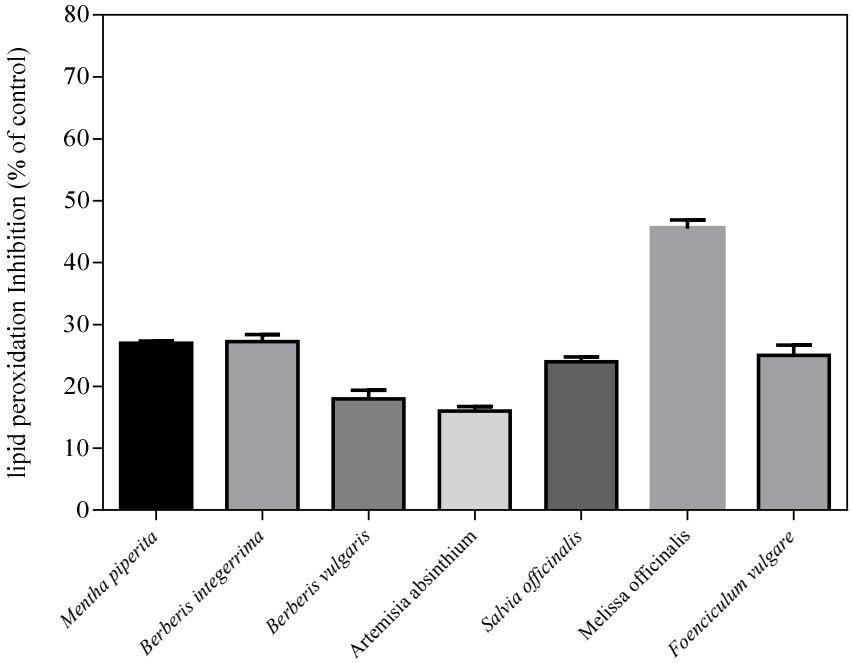

Figure 3. Inhibition of lipid peroxidation induced by $FeSO_4$ in egg yolk by the plant extracts. Amounts of decrease in the pinkish-red color developed by TBA-MDA complex were attributed to inhibitory effects of the plant on lipid peroxidation, as compared to fully oxidized control. Each experiment was independently repeated as least three times from which standard deviations have been derived.

Estimation of total phenolic contents (TPC)

Phenolic content of the plant samples was quantitatively measured using Folin–Ciocalteu assay method adapted in 96-well plate. Thus, TPC of each sample was measured and expressed as mg gallic acid equivalent per one g extract (Table 1). Amongst the plants, *B. integerrima* was found to possess the highest TPC with over 167 mg GAE.g^{-1}e, which was significantly different from the rest of the plant sample ($p < 0.05$). Following *B. integerrima*, two plants *Mentha piperita* and *B. vulgaris* had the high TPC compared to the rest. *F. vulgare* was seen to have the lowest TPC (around 13 mg GAE.g^{-1}e) among the tested plant samples (Table 1).

Estimation of total flavonoid contents (TFC)

Flavonoid contents were quantitatively measured through an $AlCl_3$ method adapted in 96-well plate, expressed as mg catechin equivalent per one g extract. Similar to the findings obtained with TPC, the highest and lowest amounts of TFC belonged to *B. integerrima* and *F. vulgare* with around 73 and 12 mg CE.g^{-1}e, respectively (Table 1).

Table 1. Biomolecules (phenolics and flavonoids) and antioxidant activity EC_{50} values obtained for Iranian medicinal plants

Plant	DPPH inhibition (mg. ml^{-1})	Reducing power (mg. ml^{-1})	TBARS (mg. ml^{-1})	TPC (mg GAE.g^{-1}e)	TFC (mg CE.g^{-1}e)
Mentha piperita	0.23 ± 0.06^{a}	0.14 ± 0.0005^{b}	40 >	76.8 ± 1.4^{a}	35.16 ± 0.9^{a}
Berberis integerrima	0.21 ± 0.02^{a}	0.14 ± 0.02^{b}	40 >	167.3 ± 3.5^{b}	72.8 ± 3.1^{b}
Berberis vulgaris	0.45 ± 0.03^{b}	0.08 ± 0.02^{a}	40 >	71.28 ± 4.2^{a}	22.6 ± 0.8^{c}
Melissa officinalis	0.4 ± 0.07^{b}	0.17 ± 0.008^{b}	40 >	43 ± 2.2^{c}	42 ± 1.1^{d}
Artemisia absinthium	0.56 ± 0.02^{b}	0.24 ± 0.01^{c}	40 >	34.47 ± 1.9^{d}	33.5 ± 2.2^{a}
Salvia officinalis	0.92 ± 0.04^{c}	0.42 ± 0.02^{d}	40 >	24.6 ± 0.9^{e}	23.6 ± 0.7^{c}
Foenciculum vulgare	1.06 ± 0.01^{c}	0.45 ± 0.03^{d}	40 >	13.4 ± 0.8^{f}	11.8 ± 0.7^{e}

EC50, the effective concentration at which DPPH radicals were scavenged by 50%, or the absorbance was 0.5 for reducing power, obtained by interpolation from linear regression analysis.

Values are expressed as mean ± standard deviation, generated through at least three independent experiments.

TPC; total phenolic contents are expressed as mg gallic acid equivalents (GAEs) per one g of extract (e); TFC; total flavonoid contents are expressed as mg catechin equivalents (CEs) per one g of extract (e).

Means that do not share a letter are determined as significantly different ($p<0.05$).

DISCUSSION

In this study, several Iranian wild-growing medicinal plants were examined for their antioxidant potency through various chemical and biochemical methods. Although all the methanolic plant extracts showed degrees of antioxidant activity, there were considerable differences among the plants. Medicinal plants have long been used in the folk medicine of Iran. In addition, there are various climates and a large number of wild-growing plants in the country. Thus, it is not surprising that there are a vast number of studies undertaken to investigate antioxidant activity and other pharmaceutical properties of Iranian medicinal plants [5]. However, factors related to seasonal abundance, region, ecology and topography may affect pharmaceutical properties (including antioxidant activity) of medicinal plants belonging to the same species. Thus, the current study focused on some medicinal plants growing in northeastern Iran, for which no study has been undertaken to investigate antioxidant activity.

In this study, *B. integerrima* and *Mentha piperita* exhibited the highest DPPH inhibitory activity among the plants, whilst *S. officinalis* and *F. vulgare* were significantly less effective than the rest. Almost similar results were obtained in terms of reducing power so that *B. vulgaris*, *B. integerrima* and *Mentha piperita* had the highest values, while *S. officinalis* and *F. vulgare* were less effective ones. In agreement with these findings, *B. integerrima* was also found to possess the highest TPC and TFC. As opposed to the data obtained with the chemical assays, the tested plants were found to exert moderate or slight inhibition of lipid peroxidation. These findings are supported by Barros et al. [7], where they stated that the antioxidant activities generated by chemical assays were greater than those of biochemical assays such as lipid peroxidation.

To the best of our knowledge, this is the first report (at least in English) on antioxidant potency of leaves of *B. integerrima* from Iran, while a report from Turkey has demonstrated antioxidant activity of methanolic extracts of leaves and fruits of this plant [11]. However, the DPPH-EC_{50} reported for the Turkish *B. integerrima* sample was much less effective than those reported in present study. In addition, total phenol and flavonoid contents as well as reducing power were not reported for the Turkish *B. integerrima*.

Despite a lack of information on in vitro antioxidant activity of Iranian *B. integerrima*, other important pharmaceutical properties of this plant have well been demonstrated. *B. integerrima* is a shrub belonging to Berberidaceae that grows wild in most of the regions in Iran, particularly in the northeast. Due to having a variety of secondary metabolites, many medicinal properties have been demonstrated for *B. integerrima*, including antioxidant, anti-inflamatory, anticancer, hepatoprotective, hypoglycemic and hypolipidemic activities. Recently, it has been demonstrated that aqueous extracts of *B. integerrima* root had preventive effects on liver damage and oxidative stress induced by diabetes mellitus in rats [12].

CONCLUSION

In addition to those pharmaceutical properties of *B. integerrima* reported in the literature, this research showed that leaves of this plant may possess considerable antioxidant activities compared to the rest of the medicinal plants as well as BHA and ascorbic acid (as positive controls). Thus, further research may be warranted to study active compounds of *B. integerrima* that confer the antioxidant activity. The findings presented here might have implications in the population diet and disease prevention through diet.

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