Original Article

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In Vitro Free Radical Scavenging Effect and Total Phenolic and Flavonoid Contents of 30 Iranian Plant Species

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Abstract

This research involved antioxidant screening and evaluation of total phenol and flavonoid contents of the ethanolic extracts from 30 Iranian plant species. Total phenol content was determined for each extract using the Folin-Ciocalteu method and total flavonoids was assessed by the Dowd method. A high phenol content was detected for *Loranthus grewinkii* (35.32±0.31 mg gallic acid equivalent (GAE)/g to dry plant), followed by *Pteropyrum olivieri*, *Phoenix dactylifera*, *Cercis griffithii*, and *Lippia citriodora*. On the other hand, relatively low levels of flavonoid content were detected for the tested plants except for *Pteropyrum olivieri* (14.53±0.13mg QE/g of dry plant). Free radical scavenging activity was determined using DPPH (1,1-diphenyl-2-picrylhydrazyl), NO (Nitric Oxide), and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) free radical assays. Ferric Reducing Antioxidant Power (FRAP) assay was conducted as a measure of antioxidant capacity. *L. grewinkii* was superior in DPPH, NO, and ABTS free radical inhibition. Extract of *P. olivieri* demonstrated a potent inhibitory activity against NO free radical compared to quercetin. Based on the overall antioxidant activity, *L. grewinkii* was determined as the strongest in terms of free radical scavenging effect. A positive correlation observed between phenolic content and the activity, while the flavonoids may have major contributions to manifestation of antioxidant activity in most of the investigated plant species.

Keywords: Antioxidant activity, Total flavonoid, Total phenol.

1. Introduction

Several physiological and pathological processes are involved in producing free radicals. Oxidation occurs in most living organisms during energy production, which fuels biological processes. However, increased free radical production can cause many degenerative diseases and contribute to aging (1). Most living organisms are protected against damage caused by free radicals by oxidative enzymes as well as by many synthetic chemical and natural compounds. However, the protective processes of biological systems can be insufficient to entirely prevent damage from free radicals (2). Oxidative stress is considered as the main cause of pathogenesis in various diseases, including atherosclerosis, alcoholic liver cirrhosis, and cancer (3, 4). Oxidative stress is initiated by reactive oxygen species (ROS), such as superoxide anion (O^{-2}), perhydroxy radical (HOO^{-}), and hydroxyl radical ($HO^{\bullet}$). These radicals are formed in a one-electron reduction process from molecular oxygen (O^2). ROS can easily initiate peroxidation of membrane lipids that causes cell membrane damage of phospholipids and lipoproteins by propagating a chain reaction cycle (5). Thus,

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antioxidant defense systems have coevolved with aerobic metabolism to counteract the oxidative damage from ROS. Most living species have an efficient defense system that prevents oxidative stress induced by ROS (6). Natural products with antioxidant activity may be used to help the human body to reduce oxidative damage (7). Many investigations have declared the antioxidant properties of plants and their protective role against various human diseases, such as diabetes, cancer, and aging (8, 9). In this respect, flavonoids and other polyphenolic compounds have received great attention as bioactive compounds (10). Accordingly, the present study was conducted on 30 plant species of Fars region, southern Iran, as a part of our continuous search for active antioxidant plants.

Based on the available ethnomedical information related to Iranian traditional medicine, most of these plants have been used to treat various types of diseases, including microbial infections, cancer, and diabetes (11). Pharmacological investigations conducted on some of these plant species have revealed their efficacy against microbial infections (12), hyperglycemia (13), cancer (14), and diabetes (15). The species under investigation in this study contain various classes of phytochemicals, such as flavonoids (16), alkaloids (17), triterpenes (18), and steroids (19) as the major constituents in addition to their volatile components. The biological activities reported for these plants and the types of their active constituents and traditional uses prompted us to study their antioxidant capacities. Based on this idea, the *in vitro* screening of DPPH scavenging effect and determination of total phenol and flavonoid content of thirty plant species of Fars region were undertaken in the present work.

2. Materials and methods

2.1. Chemicals and reagents

Folin-Ciocalteu, ethanol 96%, methanol, aluminum chloride, sodium carbonate (Na_2CO_3), acetate buffer, ferric chloride (FeCl3.6H2O), hydrochloric acid, ammonium persulphate, and methanol were purchased from Merck (Darmstadt, Germany). Quercetin, Gallic acid, DPPH, ABTS, TPTZ (2,4,6-tripyridyl-s-triazine), and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and solvents used were of Merck analytical grade.

2.2. Plant materials

Plants selected for this investigation were collected from different locations in Fars province and authenticated by the plant taxonomist of the Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran. Voucher specimens of all plant species (MRCH-91-65-95) have been retained at the herbarium of Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

2.3. Preparation of extracts

Freshly collected plants (100 g) were minced and exhaustively extracted with ethanol 96% for 4.0 h using a Soxhlet apparatus. Extracts were filtered and solvent was removed from each sample by distillation under reduced pressure to afford ethanolic extract as a gummy residue. Samples of extracts were kept at -20 °C in the dark, prior to antioxidant screening.

2.4. Determination of total phenol

Total phenolic content of the spathe ethanolic extracts were determined using a Folin-Ciocalteu spectrophotometric method (20). To develop a standard calibration curve, 0.5 mL of gallic acid solutions of various concentrations, ranging from 6.25 to 200 mg/L, were mixed with 5 mL of 10% Foiln-Ciocalteu reagent and 4 ml of 1.0 M sodium carbonate solution. Proper dilutions of the extracts were oxidized with Folin-Ciocalteu reagent and neutralized by sodium carbonate solution as given for the standard. The absorbance of solution was recorded at 765 nm after 15 min against Folin-Ciocalteu reagent as blank using a PG instrument T90 UV spectrophotometer. Total phenolic content was expressed as mg of gallic acid equivalent (GAE)/g to dry plant material using the expression from the calibration curve (Y=0.00571x - 0.0843, R_2=0.9985). Where Y is the absorbance, and x is the gallic acid equivalent.

2.5. Estimation of total flavonoid

The aluminum chloride colorimetric meth-

od was employed for determination of total flavonoids (21). Each sample, including EtOH extract and individual fractions of plant material (0.5 ml of 1:10 g ml^{-1}) in methanol was mixed separately with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. Samples were then kept at room temperature for 30 minutes. Absorbance of the reaction mixture was measured at 415 nm with a double beam using UV-VIS spectrophotometer, T-90, PG Instrument, England. The calibration curve was generated using quercetin solutions at concentrations of 12.5 to 100 µg/ml in methanol. Experiments were performed in triplicate, and results were recorded as mean±SEM.

2.6. Evaluation of antioxidant activity

2.6.1. DPPH radical scavenging assay

The free radical scavenging ability of *D. revoluta* ethanol extract and fractions and ascorbic acid as the standard were evaluated against DPPH free radical by a previously reported procedure (22). 200 µl of a 100 mM methanolic solution of DPPH radical was mixed with 20 µl of appropriate dilutions of extract and fractions (6.25-3200 µg/ml). The mixture was left in the dark for 30 min, and absorbance was measured at 490 nm using an ELX 800 microplate reader of Biotek, USA. A sample containing 20 µl methanol and 200 µl DPPH solution served as the control, while the blank contained equal amounts of extract or fractions in methanol. The DPPH free radical scavenging activity was subsequently calculated with respect to the reference, which contained all reagents without the test sample.

% of Inhibition=100-Atest-Ablank/Acontrol×100

The IC_{50} value in µg/ml as the required amount of each sample to inhibit DPPH radical by 50% was also calculated. All tests were carried out in triplicate.

2.6.2. Nitric oxide assay

Sodium nitroprusside (SNP) 10 mM (in phosphate buffer pH 7.4, 20 mM) were prepared and 50 µL of that were mixed with 50 µL of a 400 µg/mL solution of the extracts. The mixture was incubated at 27 °C for 150 minutes. Then 100 µL griess reagent was added with shaking, and absorbance was measured at 542 nm immediately using Epoch microplate spectrophotometer (23). NO free radical scavenging activity was calculated using the following equation:

$$\text{Percentage of inhibition} = \frac{\text{Abs. control} - \text{Abs. test}}{\text{Abs. control}} \times 100$$

The Where blank composed of 50 µL solution of each extract, while control was made by mixing 50 µL SNP and 50 µL methanol without extract.

Each test was performed in triplicate and quercetin, a known standard antioxidant, was used as a positive control.

2.6.3. ABTS assay

$ABTS^+$ radical scavenging assay was conducted for each sample by application of a method presented before (24). $ABTS^+$ was freshly prepared by adding 5mL of Ammonium persulphate solution (4.9 mM) to 5 mL of ABTS solution (14 mM). The mixture was kept for 16 h in dark. Then the mixture was filtered and diluted in ethanol 96% (1:1) to achieve an absorbance of 0.7±0.02 in 734 nm. 950 µL of $ABTS^+$ radical solution was added to 50 µL of different concentrations of sample (6.25-500 µg/mL) and vortexed for 10 sec. The reaction mixture was stored at room temperature for 6 min, and level of absorbance was measured at 734 nm in a BioTek Epoch microplate reader. The control contained 950 µL of $ABTS^+$ and 50 µl ethanol 96%. Percentage inhibition was calculated by the following formula:

% inhibition=[$Abs._{test}$ / $Abs._{control}$]×100

2.6.4. FRAP assay

Ferric reducing antioxidant power of each sample was performed by a procedure that was described previously in the literature (25). Solution acetate buffer 300 mM PH 3.6, Ferric chloride (III) 20 mM and TPTZ 10 mM (in hydrochloric acid 40 mM) were prepared. Then FRAP solution was prepared with a combination of 10 (buffer solution): 1 ($FeCl_3$ solution):1 (TPTZ solution). 20 µL of extract was mixed with 180 µL of FRAP solution. Level of absorbance of the mixture was measured at 593 nm by a BioTek Epoch microplate spectrophotometer after 10 min incubation at 37 ºC. The control contained 20 µL methanol and 180 µL

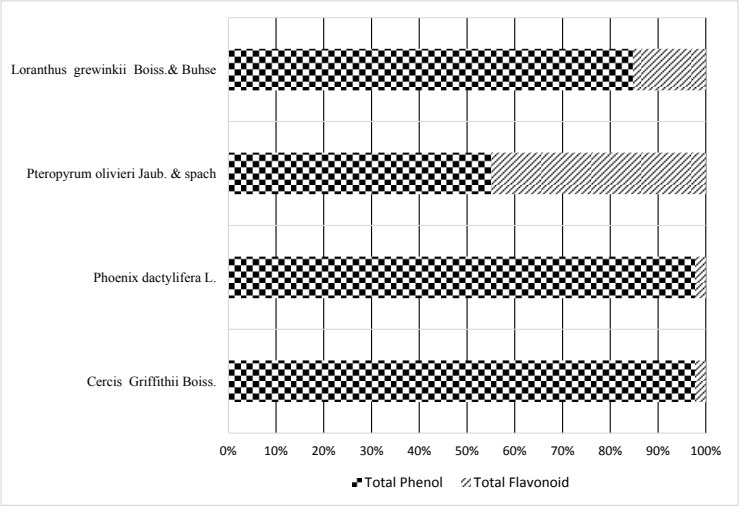

Figure 1. Proportional distribution of flavonoids among phenolics in active antioxidant plants.

FRAP solution without extract. A blank was used containing only sample and solvent. Quercetin was used as antioxidant standard and positive control. Level of absorbance was compared among tested samples to a $FeSO_4$ standard curve and values were expressed as Ferrous Equivalent (FE), the concentration of extract that gave the same absorbance as 1 mmol ferrous ion. An antioxidant agent or extract causes reduction of Fe^{3+} to Fe^{2+} ion leading to an increase in the absorption at 593 nm due to formation of a blue complex (Fe^{2+}/TPTZ). All tests were carried out in triplicate.

2.7. Statistical analysis

IC_{50} values were calculated by linear regression. Data were expressed as Mean±SD. Statistical differences between treatments were identified using one-way ANOVA followed by the Tukey post-test and significant difference was determined between controls and mean values of samples using comparison test at the level of $p<0.05$.

3. Results and discussion

3.1. Total phenolic and flavonoid contents

In the present study, total phenolics and flavonoids of ethanolic extracts from thirty plant species were determined. The results of total phenolic assessment were reported as mg gallic acid equivalent GAE/g of extracts (Table 1) with reference to a gallic acid standard curve, y=0.00511 x+0.02442, r_2=0.999. Based on obtained results, *L. grewinkii* Boiss. & Buhse ethanol extract revealed the highest amount of phenolic content (35.32±0.31 mg GAE/g of dry plant) (Table 1). Total flavonoid content was presented as mg quercetin equivalents (QE)/g of extract using a quercetin standard curve, y=0.03939x -0.04486, r_2=0.999. As shown in Table 1, the highest flavonoid content was revealed in Pteropyrum olivieri extract (14.53±0.13 mg QE/g of extract). The proportional distribution of phenol and flavonoids among the active plant extract has been depicted in Figure 1.

3.2. DPPH radical scavenging activity

Plant extracts including *Loranthus grewinkii*, *Pteropyrum olivieri*, *Phoenix dactylifera*, and *Cercis griffithii* ranked as the top four most active DPPH radical scavenging extracts, exhibited strong activity with IC_{50} values 80±0.57, 83±0.57, 92.96±1.40, and 131.057 µg/mL respectively (Table 1). As evidenced by the results *Loranthus grewinkii* extract was found to be the most active extract against DPPH free radical (Figure 2).

The results of DPPH assay prompted us to further assess the effectiveness of these four plant extracts against other free radicals, such as NO and ABTS, and their ferric reducing antioxidant power (FRAP). The IC_{50} value was determined for each extract and compared with that of standard quercetin (Table 2).

3.3. NO scavenging activity

In nitric oxide assay aqueous solution of

Table 1. Total phenol, flavonoid, and DPPH assay of 30 Iranian medicinal plant species.

Plant Species	Family	Part used	Yield of Ext.(%)	Total Phenol*	Total Flavonoid**	DPPH Assay (IC_{50} µg/mL)
Loranthus grewinkii Boiss.& Buhse	Loranthaceae	Aerial parts	9.78	35.32±0.31	5.39±0.12	80.00±0.57
Jaub. & Spach	Polygonaceae	Aerial parts	10.08	32.35±0.35	14.53±0.13	83.00±0.57
Phoenix dactylifera L.	Palmaceae	Bark	8.96	22.32±0.46	0.50±0.05	92.96±1.40
Cercis griffithii Boiss.	Caesalpinaceae	Seed	5.3	18.71±0.43	0.43±0.01	131.00±0.57
Lippia citriodora H. B. *et* K.	Verbenaceae	Leaf	7.23	13.53±0.19	4.85±0.01	>200
Stachys lavandulifolia Vahl.	Labiatae	Aerial parts	11.6	12.51±0.01	6.12±0.09	>200
Celtis caucasica Willd.	Ulmaceae	Leaf	9.05	9.13±0.03	2.21±0.00	>200
Lonicera nummulariifolia Jaub.& SP.	Caprifoliaceae	Leaf	16.97	9.00±0.17	7.38±0.09	>200
Smyrnium cordifolium Boiss.	Umbelliferae	Aerial parts	10.36	8.55±0.36	4.71±0.03	>200
Anthemis odontostephana Boiss.	Compositae	whole plant	6.88	8.16±0.06	5.85±0.10	>200
Tecomella undulata (Roxb.)Seem	Bignoniaceae	Leaf	9.90	7.72±0.2	3.23±0.01	>200
Lepidium draba L.	Cruciferae	Leaf	15.8	7.69±0.08	5.61±.012	>200
Biebersteinia multifida DC.	Geraniaceae	Aerial parts	9.83	5.76±0.05	4.74± 0.07	>200
Colutea persica Boiss.	Papilionaceae	Aerial parts	5.07	5.70±0.05	0.20±0.01	>200
Echinophora cinerea (Boiss.)	Umbelliferae	Aerial parts	9.82	4.71±0.05	2.78±0.10	>200
Rosa damascena Mill.	Rosaceae	Flower	6.03	4.08±0.00	0.66±0.00	>200
Viola modesta Fenzl	Violaceaee	Aerial parts	5.90	3.89±0.07	2.02±0.02	>200
Glaucium oxylolobum Boiss. & Buhse	Papaveraceae	Aerial parts	4.89	3.82±0.02	2.25±0.06	>200
Silene chlorifolia Sm.	Caryophyllaceae	Aerial parts	6.18	3.77±0.06	1.88±0.06	>200
Parietaria judaica L.	Urticaceae	Aerial parts	4.2	3.36±0.00	2.35±0.06	>200
Oliveria decumbens Vent.	Umbelliferae	Stem	3.90	3.07±0.03	1.80±0.02	>200
Mesostemma kotschyanum (Fenzl)	Caryophyllaceae	Aerial parts	10.22	2.98±0.03	1.31±0.05	>200
Acer monspessulanum L.	Aceraceae	Fruit	2.98	2.75±0.00	0.62±0.01	>200
Dorema aucheri Boiss.	Umbelliferae	Whole plants	5.30	2.57±0.01	0.67±0.01	>200
Onosma platyphyllum H.Riedl.	Boraginaceae	Aerial parts	6.04	2.08±0.06	1.06±0.05	>200
Astragalus merdonalis L.	Papilionaceae	Aerial parts	4.77	1.85±0.01	1.22±0.05	>200
Centaurea depressa M.B.	Compositae	Leaf	4.94	1.44±0.01	0.68±0.02	>200
Trichodesma incanum (Bge.) A. DC .	Boraginaceae	Aerial parts	2.9	1.24±0.01	0.97±0.06	>200
Micromeria persica Boiss.	Labiatae	Aerial parts	0.84	0.67±0.04	0.12±0.02	>200
Quercetin				-	-	26.51±0.06

*mg GAE/g of dry plant **mg QE/g of dry plant N.D.= Not Detected

SNP at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by Griess Reagent. Scavengers of NO compete with oxygen leading to reduced production of NO (26). *P. olivieri* extract with IC_{50} value 24.59±1.10 µg/mL showed strongest inhibition towards nitric oxide free radical followed by *L. grewinkii* extract (IC_{50}=57.24±1.37 µg/mL). Both the extracts surpassed quercetin (68.73±1.59 µg/mL) in NO radical scavenging activity. *P. dactylifera* and *C. griffithii* extracts, respectively, with IC_{50} values 121.22±1.12 and 142.26±1.24 µg/mL revealed weaker degrees of inhibition towards NO radical.

3.4. ABTS scavenging activity

Based on the results of the present study, the inhibitory properties of extracts against

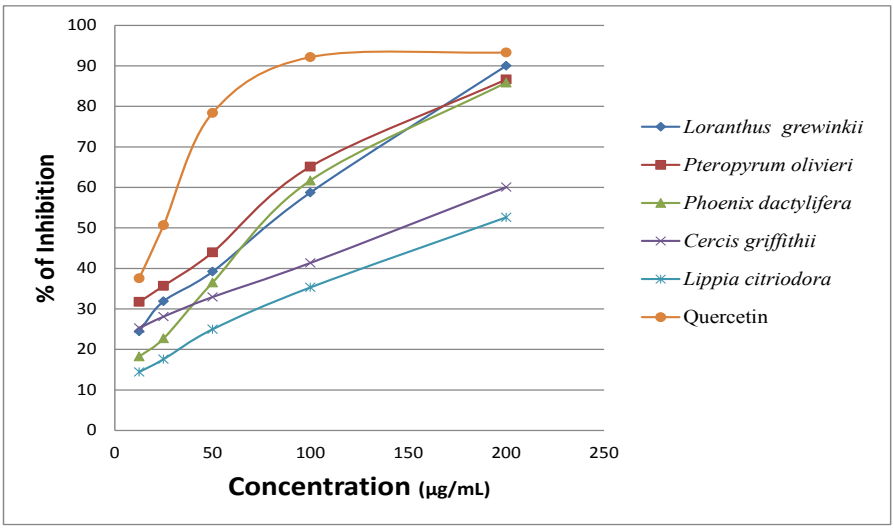

Figure 2. DPPH radical inhibitory effect of active plant extracts and quercetin.

$ABTS^+$ radical in terms of IC_{50} values, ranged from 63.00±0.50 µg/mL for highly active *P. dactylifera* to 468±1.10 µg/mL for the *P. olivieri* extract (Table 2). These values indicated that all four extracts except *P. olivieri* exhibited significant inhibition against $ABTS^+$ free radical. The efficient $ABTS^+$ scavenging properties of *P. dactylifera* may be mainly attributed to the phenolic constituents and not to the flavonoids, as the flavonoid proportion was found to be very low in the extract (0.50±0.05 mg QE/g). Therefore, a noteworthy correlation were found between phenol content and $ABTS^+$ free radical inhibition declared by this extract. Weaker inhibition of $ABTS^+$ was observed by *L. grewinkii* (73.00±0.00 µg/mL) and *C. griffithii* (90.00±0.57 µg/mL) extracts (Table 2). The results may reflect the capacity of phenolic components of these extracts to donate electrons or hydrogen atoms to inactivate this radical cation.

3.5. Ferric reducing antioxidant power

FRAP, ferric reducing antioxidant power, is a useful analytical tool for the assessment of antioxidants by measuring their oxidation-reduction potential. The ethanolic extracts of four plants displayed reducing abilities in comparison to quercetin, indicating the presence of compounds with potential reducing capacity in the extracts. In FRAP assay, antioxidant agents or extracts can reduce the ferric-tripyridyl triazine (Fe^{+3}-TPTZ) complex formed during this test to an intense blue ferrous form (Fe^{+2}-TPTZ) at low pH conditions. Ferrous (Fe_2^+) is capable of generating free radicals from peroxide, which is implicated in many diseases. Therefore, reduction of Fe_2^+ levels in the Fenton reaction would protect against oxidative damage and hence the reducing ability of an extract or compound may serve as a significant indicator of its potential antioxidant activity (27). Compari-

Table 2. Plant species demonstrated free radical inhibition and ferric reducing capacity.

Plant Species/ Std.	DPPH	NO	ABTS	FRAP (mM)
		(IC_{50} µg/mL)		
Loranthus grewinkii Boiss.& Buhse	80.00±0.57	57.24±1.37	73.00±0.004	5.20±1.00
Pteropyrum olivieri Jaub. & spach	83.00±0.57	24.59±1.10	468.00±1.10	3.20±1.00
Phoenix dactylifera L.	92.96±1.40	121.22±1.12	63.00±0.50	1.51±0.50
Cercis griffithii Boiss.	131.00±0.57	142.26±1.24	90.00±0.57	3.60±0.01
Quercetin	26.51±0.06	68.73±1.59	25.64±0.02	8.69±0.03

son of data obtained from other antioxidant assays showed that the free radical scavenging ability of extracts may be partly due to their iron chelating capacity. This interpretation gained support from the previous studies reporting the second position for *P. Dactylifera* fruit extract in demonstration of antioxidant activity among 28 fruits using FRAP assay (28). The FRAP values of the four active plant extracts varied from 1.51±0.50 to 5.20±1.0 mM/dry weight of extract and expressed as ferrous equivalent (FE) antioxidant power (Table 2). According to the results, *L. grewinkii* extract exhibited the strongest reducing ability (5.20±1.0 mM FE) among all extracts (Table 2).

Many reports have supported the use of natural antioxidant in order to reduce oxidative stress or slow down the development of free radical-related complications (29). In this context, numerous studies have documented free radical scavenging activity and antioxidant properties of plant products (30). The need to develop new sources of natural antioxidants directed our attention towards a group of plants, most of which have traditionally been used for their therapeutic properties in Iranian folk medicine.

Following the results of this study, *L. grewinkii* significantly inhibited three free radicals and showed prominent ferric reducing properties. The effectiveness of this extract may be attributed to the high phenolic content of the extract. Therefore, a significant correlation was observed between phenol content and DPPH free radical inhibition of this extract.

P.olivieri was found to be the second active free radical scavenging extract among the four tested extracts. This extract revealed significant effect against the tested free radicals except $ABTS^{+}$, while a good reducing capacity was detected for the extract. The free radical inhibition and more specific strong nitric oxide inhibitory properties of this extract may be well justified by the presence of both phenol and flavonoids, as the flavonoids forms a major proportion of phenolic content in this extract (Figure 1). It has already been reported that chronic expression of nitric oxide radical is associated with various inflammatory conditions, such as cancer, multiple sclerosis, coronary heart disease, and diabetes (31).

P. dactylifera extract was detected to be a significant scavenger of ABTS,but showed the lowest ferric reducing power. The biological behavior of this extract might be due to the specific phenol structures, such as acidic, methylated, or glycosylated forms as the extract was found to contain a negligible content of flavonoids. *C. griffithii* significantly inhibited $ABTS^{+}$ radical and demonstrated ferric reducing antioxidant power. The extract was detected to bear a very low distribution of flavonoids and thus the radical scavenging activity resides in the phenolic portion of this extract.

The relationship between phenol and flavonoid contents and free radical scavenging activity have so far been the subject of many studies describing the roles of both these groups of phytoconstituents in manifestation of free radical inhibition properties by herbal extracts (32, 33). Phenolic compounds exert their radical quenching effect mainly through their redox properties and hence act as singlet oxygen quenchers, hydrogen donators, and reducing agents and additionally have metal-chelating properties (34).

Thus, phenolic constituents of these plant extracts and to a lesser extent flavonoids may make a direct contribution to radical scavenging and antioxidative activity (35).

Compelling evidence indicates that flavonoids bear some structural characteristics, such as abundance of phenolic hydroxyl groups and more specifically the vicinal diol moiety, which contribute to their active biological behaviors, as well as manifestation of radical scavenging effects.

Although some of the 30 plant species screened in this study contained phenol and flavonoids, only four of the extracts declared preventive role against tested free radicals and shared their contribution in reducing properties. These active extracts merit further comprehensive chemical and pharmacological investigation in order to locate their active antioxidant constituents and be used properly in producing antioxidant products.

4. Conclusion

This study suggests that among the plant species tested in the present work, four of the extracts possessed significant DPPH scavenging

activity, which might be helpful in preventing or slowing down the symptoms and progression of various diseases attributed to oxidative stress. The results obtained here may also provide justification for the use of some of these plants in folk medicine. Differences among plant extracts in terms of their inhibitory effect against various types of free radicals may be attributed to the presence of phenolics of diverse structural features that may exert their inhibitory properties against various free radicals through different mechanisms of action. Additional experimental work must be conducted on fractionation of the active extracts in order to find the highest active components suitable for production of antioxidant supplemental products. Isolation and characterization of active component(s) of individual fractions may still remain an interesting task for future investigations.

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Conflict of interest

None declared.

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