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Phytochemical Screening and *In vitro* Evaluation of Free Radical Scavenging Activity of *Dionysia revoluta* L.

Mohammad Ali Farboodniay Jahromi[1*], Mahmood Reza Moein[1,2], Hamed Etemadfard[1], Zahra Zebarjad[1], Fatemeh Farmani[1]

[1]Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.
[2]Demepartment of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

Dionysia revoluta L., a plant of *Primulaceae* family is used for treating ulcers and relieving pain in Iranian traditional system of medicine. The present study was aimed at preliminary phytochemical investigation and evaluation of antioxidant characteristics of *D. revoluta* L. ethanol extract and its various fractions. Total phenolic content was determined by Folin–Ciocalteu method. Evaluation of total flavonoid was carried out by the use of an aluminium chloride/sodium carbonate colorimetric procedure. Lipid peroxidation inhibitory effect of ethanol extract was studied and compared with that of butylated hydroxytoluene (BHT). Radical scavenging properties of ethanolic extract and various fractions were determined by 2,2-diphenyl-l-picryl-hydrazyl (DPPH) and nitric oxide methods. High phenolic and flavonoid contents and significant radical scavenging properties were detected for the ethyl, acetate and n-butanol fractions. Comparisons were made with known reference antioxidant compounds ascorbic acid, quercetin, and gallic acid. The radical scavenging effect of n-butanol fraction was the highest among all fractions. Acid hydrolysis of n-butanol fraction led to a significant enhancement in its phenolic and flavonoid contents and DPPH scavenging efficacy. The total phenolic content showed a good correlation with radical scavenging activity. The antioxidant activity found in the ethyl acetate and n-butanol fractions of *D. revoluta* L. may be attributed to the presence of flavonoids and other phenolic compounds. Among various chemical constituents of this plant, the concentration of flavonoids seems to prevail remarkably as indicated by thin layer chromatography of various fractions and diagnostic colour reactions. The results suggest that *D. revoluta* bears a remarkable radical scavenging and antioxidant activity and is worthy of further detailed phytochemical and antioxidant studies.

Keywords: Dionysia revoluta L., Free radical scavenging effect, Antioxidant, Flavonoid.

1. Introduction

Free radicals are reactive chemical species having an unpaired electron produced during aerobic cellular metabolism. Several other factors including radiation and smoking may also lead to free radical formation (1,2). These electron deficient species can cause oxidative damage to lipids, amino acids, proteins and nucleic acids (3). Experimental studies have so far corroborated that oxidative stress is among the major causative factors in the genesis of many chronic and degenerative diseases including atherosclerosis, diabetes mellitus and cancer (4). Antioxidants can protect the cells by neutralizing electrical charge and preventing free radicals from taking electrons from other molecules (5). Therefore, antioxidants have gained extensive interest in preventing diseases (6). The synthetic antioxidants like BHA (Butylated hydroxy anisol), BHT (Butylated hydroxy toluene) and gallic acid esters have been reported to cause liver damage and carcinogenesis in laboratory animals (7, 8). Hence, there is a continuous approach to substitute these compounds with the naturally occurring antioxidants and consequently lower the risk of diseases. In this trend, the phytopharmacological approaches have conclusively revealed the antioxidative and radical scavenging properties of many plant species (9,10). However, many studies have demonstrated that the major antioxidative and radical scavenging properties of various plant extracts, are due to their phyto-chemical contents such as flavonoids, isoflavones,

Corresponding Author: Mohammad Ali Farboodniay Jahromi
Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
E-mail: farboodnia@sums.ac.ir

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terpenoids, anthocyanins and coumarins. Among the major classes of phytoconstituents, flavonoids have a widespread distribution in the plant kingdom and have so far exhibited various types of activities. We have previously reported some biologically active flavonoids (11,12). This group of compounds have also shown extensive contribution in demonstration of antioxidant and radical scavenging properties (13,14). In continuation of our systematic phytochemical and biological screening for antioxidant plant species, our attention focused on *Dionysia revoluta* L. a small shrub of *Primula*cae family, endemic to Iran which is used for the treatment of ulcers, bruise and abdomen pain in Iranian traditional system of medicine (15). A recent report also declares the cytotoxic effect of another species in the genus, *Dionysia termeana* L. (16). The genus *Dionysia* which include 50 known species is nested in the family *Primulaceae*. A major character of *Dionysia* and *Primula* is considered to be production of waxy or farinose coatings rich in flavonoids on aerial parts including the floral region of the plant (17). These farina exudates consist of some unsubstituted and structurally rare types of flavones. This was earlier reported in hydrolysates of some *Primula* and *Dionysia* species (18,19). Based on etnobotanical uses, the present study was undertaken to investigate the antioxidant and free radical scavenging capabilities of *D. revoluta* L.

2. Materials and methods

2.1 Plant material

The aerial parts of the plant were collected from Sepidar mountain in Meimand area of Fars state, Iran in May 2012. The taxonomic authentication was accomplished by the Department of Pharmacognosy, Faculty of Pharmacy, Shiraz University of Medical Sciences. A voucher specimen was deposited in the departmental herbarium under the code N6/MR /2012.

2.2 Chemicals

Sodium nitroprusside (SNP), ascorbic acid, gallic acid, ferric chloride, potassium ferricyanide, Folin-Ciocalteu, Griess reagent and aluminum TLC pre-coated silica gel 60 F254 plates were obtained from Merck, Darmstadt, Germany. DPPH (1,1-diphenyl, 2-picryl hydrazyl), BHT (butylated hydroxy toluene), linoleic acid and quercetin were purchased from Sigma Aldrich Chemical Co USA. All other chemicals and solvents used were of analytical grade from Merck.

2.3. Extraction method

The whole plant of *D. revoluta* was air dried, and the pulverized plant material (1.5 kg) was exhaustively extracted with 96% ethanol for 5 hours in a soxhlet apparatus. The ethanolic extract was filtered and the solvent was removed under reduced pressure on a rotary evaporator at 50 °C to afford a dark gummy residue as ethanol extract (120 g). The extract was suspended in water and further fractionated successively with petroleum ether, chloroform, ethyl acetate and n-butanol in a liquid-liquid extractors. The individual fractions were washed with water in a separatory funnel, and the solvent, was evaporated to dryness at 50°C under reduced pressure. The resulted extract and its various fractions were stored at -20°C before phytochemical and antioxidative analyses. Some physical characteristics of the plant extract and various fractions are shown in Table 1. The prepared extract and fractions were used for determination of total phenolic content and total flavonoids and for the assessment of their antioxidant and radical scavenging capacities.

2.4. Qualitative phytochemical inspection

Phytochemical screening was conducted to detect various classes of chemical constituents in the extract and fractions of *Dionysia revoluta* L. using the procedures described previously for qualitative

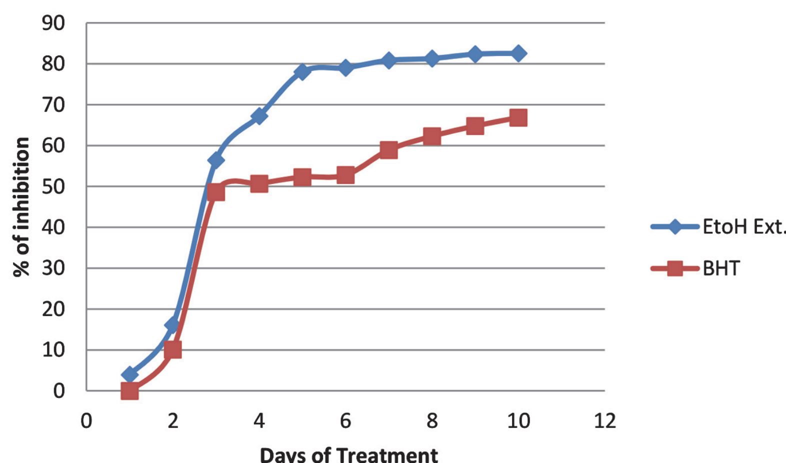

Figure 1. Lipid peroxidation inhibitory activity of *D. revoluta* L. ethanol extract and BHT.

Table 1. Physical characteristics and yield of extract/ fractions of the aerial parts of ***Dionysia revoluta*** L.

Extract/Fractions	Colour	Weight (g)	Yield%
EtOH Extract	Dark brown	120.9	6.07
Petroleum ether	Dark brown	27.11	1.36
$CHCl_3$	Dark brown	32.17	1.61
EtOAc	Dark brown	5.67	0.20
BuOH	Dark brown	8.18	0.40

phytochemical screening (20). The results of these diagnostic tests are presented in Table 2.

2.5. Thin-layer chromatography

Thin-layer chromatography (TLC) was employed in this study as a rapid analytical and diagnostic tool for detecting phytochemical contents of ethanolic extract and various fractions of *Dionysia*. To reach proper chromatographic resolution, different solvent systems with polar, semipolar and nonpolar characteristics were used. The silica gel thin layer chromatograms were developed in suitable solvents and were inspected under the UV light at 254 and 366 nm. To detect the presence of various classes of phytochemicals, the spots were visualized by spraying with specific reagents and were thoroughly analysed (21).

2.6. TLC-DPPH assay

Thin layer chromatograms of ethanolic extract were developed in the solvents of various polarities as described above. The chromatograms were then dried and sprayed with 0.2% DPPH solution in methanol. The presence of antioxidant compounds was detected by yellow spots against a purple background (22).

2.7. Determination of total phenolics

The total phenol content was determined in the ethanolic extract and various fractions, using a previously reported method (23). Proper dilutions of the extracts were oxidized with 2.5 ml of 10% Folin–Ciocalteu reagent (v/v) and neutralized by 2.0 ml of 7.5% sodium carbonate. The reaction mixture was incubated for 40 min at 45 °C and the absorbance was measured at 765 nm in the spectrophotometer. The total phenol content was then calculated using gallic acid as a reference standard.

2.8. Determination of total flavonoid content

Aluminum chloride colorimetric method was employed for determination of total flavonoids (24). All samples including EtOH extract and individual fractions of the plant material [0.5 ml of 1:10 g. ml^{-1}] in methanol was separately mixed 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. The samples were then kept at room temperature for 30 min. The 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 solution at concentrations of 12.5 to 100 µg/ml in methanol. Experiments were performed in triplicates and results were recorded as mean±SEM.

2.9. Acid hydrolysis

Acid hydrolysis was carried out according to a previously reported procedure (25). Ten ml of 0.5 N HCl in 50% aqueous methanol was added to 1.0 g of butanol fraction of *D. revoluta* in a round bottomed flask. The reaction mixture was refluxed for 2 h at 90°C. After cooling, the solution was filtered and centrifuged. The resulted supernatant was then

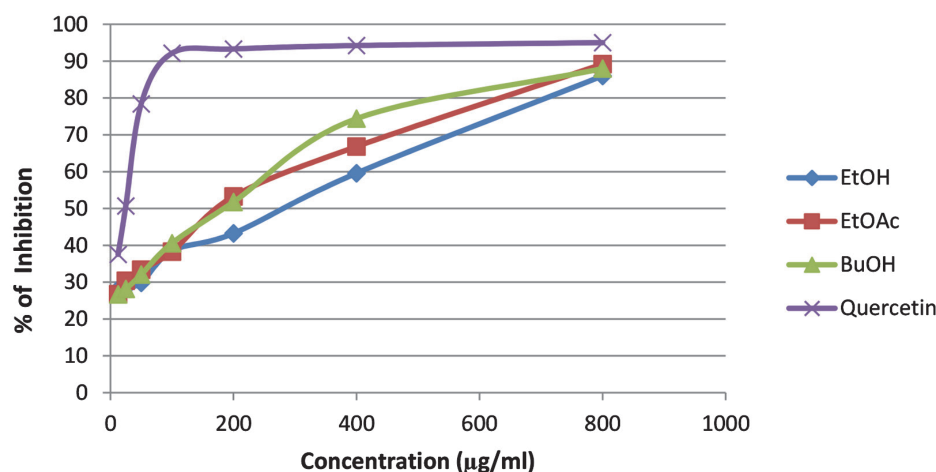

Figure 2. DPPH scavenging effect of extract/fractions of *D. revoluta* L. and standard quercetin.

Table 2. Reagent tests with ethanolic extract of *Dionysia revoluta* L.

Types of Tests	Phytochemical constituents	EtOH Extract
Ferric Chloride	Phenols	+
Vanillin HCl	Flavonols	+
Frothing	Saponins	-
Anisaldehyde / H_2SO_4	Phenyl propanoids	+
Shinoda	Flavonoids	+
Zinc HCl	Flavonoids	+
Draggendorff	Alkaloids	-
Mayer	Alkaloids	-
Wagner	Alkaloids	-
Salkowski	Terpenoids	+
Liebermann	Steroids	+
Vanillin / H_2SO_4	Terpenoids	+
DPPH	Antioxidant Activity	+

extracted three times with ethyl acetate (30 ml). The combined ethyl acetate layer was then washed with distilled water and the solvent was removed under reduced pressure at 45°C to afford a gummy residue as the hydrolysis product.

2.10. Evaluation of *in vitro* antioxidant activity

2.10.1. DPPH radical scavenging assay

The free radical scavenging ability of *D. revoluta* ethanolic extract, fractions and ascorbic acid as a standard were evaluated against DPPH by a previously reported procedure (26). Two hundred µl of a 100 mM methanolic solution of DPPH was mixed with 20 µl of proper dilutions of extract and fractions (6.25-3200 µg/ml). The mixture was left in the dark for 30 min and the absorbance was measured at 490 nm using an ELX 800 microplate reader, Biotek, USA. A sample containing 20 µl of methanol and 200 µl of DPPH solution served as control while the blank contained equal amounts of extract/fractions in methanol. DPPH free radical scavenging effect was then calculated with respect to reference standard which contained all the reagents without the test sample.

$\% \text{ Inhibition} = 100 - A_{test} - A_{blank}/A_{control} \times 100$

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.

2.10.2. Nitric oxide scavenging assay

Nitric oxide (NO) was generated from sodium nitroprusside (SNP) and was measured by the Griess reagent. SNP in aqueous solution 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 (27). SNP (10 mM) in phosphate buffer saline (pH =7.4) was mixed with different concentrations of extract/fractions (6.25-3200 µg/ml) in ethanol and water and incubated at 25°C for 150 min. Each sample were further reacted with 100 µl of Griess reagent. The absorbance of the chromophores formed was read at 542 nm and referred to the absorbance of ascorbic acid, used as a positive control treated in the same way with 50 µl. Blank contained 50 µl of extract/fractions while a sample containing 50 µl SNP, 50 µl ethanol and 100 µl Griess reagent taken as control. Each assay was performed in triplicate.

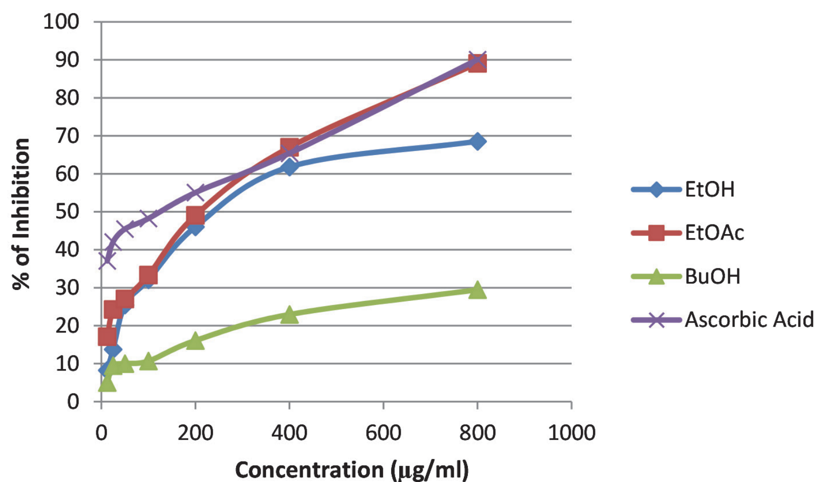

Figure 3. Nitric oxide scavenging effect of extract/eractions of *D. revoluta* L. and standard ascorbic acid.

Table 3. Total phenolic and flavonoid contents of extract/fractions of *Dionysia revoluta* La.

Extract/Fraction	Phenolic content (mg GAE/g extract)[b]	Total flavonoids (mg QE/g extract)[c]
EtOH Ext.	176.44±3.23	59.35±3.61
Petrol. ether	45.56±1.01	4.79±0.19
$CHCl_3$	113.83±7.97	63.19±5.43
EtOAC	181.22±2.00	74.41±4.49
BuOH	249.26±4.53	154.38±9.11
BuOH (Hydrolysed)	329.05±1.56	468±16.01

[a]Values expressed are mean±SD of three parallel measurements (p<0.05); [b]GAE. Gallic acid equivalent; [c]QE. Quercetin equivalent

$$\% \text{ Nitric Oxide scavenged} = \frac{A_{control} - A_{test}}{A_{control}} \times 100$$

Where, $A_{control} = A_{Absorbance}$ of control.

$A_{test} = A_{Absorbance}$ in the presence of extract/fraction.

2.10.3. Lipid peroxidation assay

Peroxide oxidation by ferric thiocyanate method

The antioxidant properties of *D. revoluta* L. butanolic fraction was determined by a previously described ferric thiocyanate method (28). The method was used to measure the amount of peroxides formed during initial stages of lipid oxidation. Crude ethanolic extract of *D. revoluta* (4 mg) was dissolved in 4 ml of 99.5% absolute ethanol. Distilled water (3.9 ml) was then added to 8.0 ml, 0.05 M phosphate buffer solution (pH 7.0) and 4.1 ml 2.51% linoleic acid. All sample solutions were kept in screw capped vials and placed in an oven at 40°C in the dark. From the day zero, the assay was performed every 24 hr for ten consecutive days. The mixture from each sample (0.1 ml) was used and to each sample, 9.7 ml 75% (v/v) aqueous ethanol, 0.1 ml 30% aqueous ammonium thiocyanate and 0.1 ml of 0.02 M ferrous chloride ($FeCl_2.4H_2O$) in 3.5% HCl were added. Precisely, 3 min after the addition of HCl, the absorbance of red colour formed was measured at 500 nm. The control and standard were subjected to the same procedure as the sample, except that for the control, only solvent was added and for the standard, sample was replaced with the same amount of BHT. All tests and analyses were carried out in triplicate and the results were averaged. The percentage of inhibition of lipid peroxidation was further calculated using the following equation:

$$\% \text{ inhibition} = A_{control} - A_{sample} / A_{control} \times 100$$

2.11. 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 Tukey post test and significant differences between controls and samples mean values determined using comparison test at a level of p<0.05.

3. Results

The potential need for developing new sources of natural antioxidants directed our attention toward *D. revoluta* L., an Iranian endemic plant which contributes its therapeutic properties as antiulcer and pain relieving traditionally (29). It was therefore, the objective of this work to investigate the anti-oxidative effect of this plant by evaluating the free radical scavenging capacity of its ethanolic extract and various other fractions and their total phenolic and flavonoid contents. Preliminary phytochemical analysis of ethanol extract of the aerial parts showed the presence of phenols, flavonoids, steroids and tri-terpenoids (Table 2). Careful inspection of thin layer chromatograms of ethanolic extract and fractions revealed the plant as a rich source of phenolics and flavonoids. Phenolic compounds are plant constituents mostly rich in hydroxyl groups. Flavonoids with the basic 2-phenylchromen-4-one structure constitute a large group of polyphenolic phytoconstituents with many interesting pharmacological properties including antioxidant effect (30).

3.1. Total phenolic and flavonoid contents of ethanolic extract and fractions

Total phenolic content were assessed by Folin-Ciocalteu method, and the results are reported with reference to a gallic acid standard curve ($y=0.00511x+0.02442$, $r^2=0.999$). Based on the results (Table 3), EtOAc and BuOH fractions had the highest total phenolic contents, with 181.22 and 249.26 mg GAE/g of fractions, respectively, while chloroform and petroleum ether fractions exhibited low phenolic contents (Table 3). Total flavonoid was determined by aluminum chloride method and the results presented

Table 4. DPPH radical scavenging characteristics of *D. revoluta* L. extract/fractions*

Extract/Fraction	DPPH Assay* IC_{50} (µg/ml)
EtOH Ext.	301±1.73
Petroleum ether	1763±10
$CHCl_3$	587±2.08
EtOAc	247±1.0
BuOH	252±0.57
BuOH (Hydrolysed)	124.28±0.4

*Values expressed are mean±SD of 3 parallel measurement (p<0.05).

Table 5. Percentage inhibition of nitric oxide radical by *D.revoluta* extract/fractions*

Ext./Fr. Conc(μg/ml)	EtOH	EtOAc	n-BuOH
25	13.76±4.04	24.29±7.05	9.46±2.78
100	32.11±6.54	33.33±4.21	10.69±5.73
400	61.80±3.99	66.97±6.70	22.95±4.19
800	68.55±5.96	89.097±3.89	29.47±7.83

*Values expressed are mean±SD of 3 parallel measurement ($p<0.05$)

as quercetin equivalents in mg/g of ethanolic extract and fractions, using a quercetin standard curve ($y = 0.03939x - 0.04486$, $r^2 = 0.999$). As shown in Table 3, total flavonoid content of EtOAc and BuOH fractions were found to be higher than EtOH extract and other fractions while the lowest flavonoid content was detected for petroleum ether fraction with 4.79 mg of quercetin equivalent/g of fraction. It may be concluded that the higher phenolic content in BuOH fraction is mainly due to the high concentration of flavonoids. This was also corroborated by the positive response to the relevant colour reagent observed for this fraction on thin-layer chromatogram.

3.2. Inhibition of lipid peroxidation

Our primary antioxidant screening of ethanolic extract of *D. revoluta* revealed a significant degree of inhibition toward lipid peroxidation in comparison with BHT (Fig. 3).

3.3. DPPH radical scavenging effects

Antioxidant screening of ethanol extract and other fractions of *D. revoluta* were carried out by measuring their scavenging capacity against DPPH free radical, the results of which can be seen in Table 4 and Figure. 2. Ethanolic extract and EtOAc and BuOH fractions showed varying degrees of free radical scavenging properties as determined by DPPH method. Meanwhile, BuOH fraction showed the lowest IC_{50} value and thus stronger radical scavenging effect compared to other fractions and ethanolic extract. In this assay, quercetin was used as a reference standard (Table 4, Fig. 2). The results declared the plant as a suitable free radical scavenger and signifying its DPPH scavenging properties.

3.4. NO radical scavenging properties

Nitric oxide scavenging properties was also observed for EtOH extract and other fractions. Excessive NO generation in the human body is detrimental and is associated with various diseases and inflammatory conditions including arthritis, carcinomas, colitis and multiple sclerosis (31). Generally antiradical plant extracts or other NO scavengers inhibit nitrite formation by competing with oxygen in reaction with nitric oxide (32). The results of the present study revealed the nitric oxide scavenging properties of ethanolic extract, EtOAc and BuOH fractions of *D. revoluta* L .when compared with the standard quercetin (Table 5, Fig. 3).

3.5 Relationship between phenolic and flavonoid compositions and radical scavenging effects

Comparison of phenolic contents of total ethanolic extract and butanolic fraction was found to be significant ($p<0.001$). Also, phenolic composition of EtOAc and BuOH fractions showed the same level of significance ($p<0.001$). But the comparison between phenolic content in EtOH extract and EtOAc fraction found not to be significant. Among all *Dionysia* fractions, n-butanolic fraction with 249.26±4.53 mg GAE/g of extract had the highest content of phenolic compounds

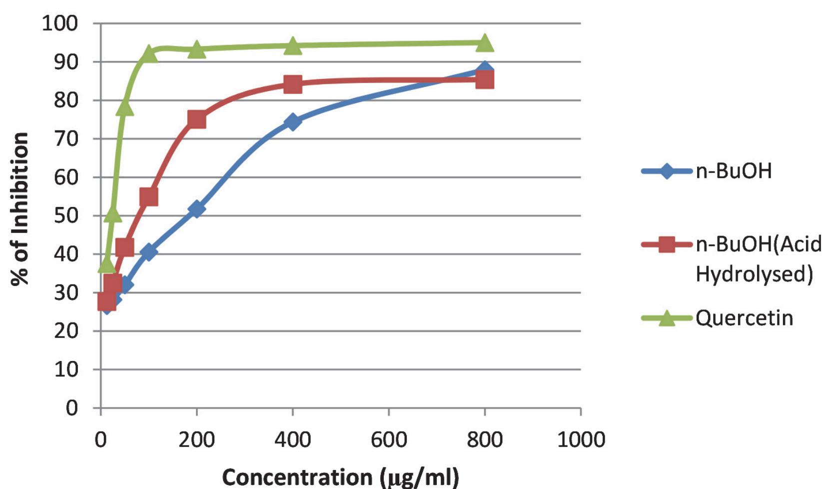

Figure 4. DPPH scavenging effect of n-butanol fraction and its acid hydrolysis product.

(Table 3). Total flavonoid content in EtOH extract and BuOH fraction were found to be comparable, significantly ($p<0.001$). The total flavonoid content of EtOH extract and various other fractions showed a similar profile as the total phenolic content.

Preliriminary antioxidant screening of ethanolic extract by thin layer chromatography and development of yellow spots on spraying the chromatograms with DPPH solution led to the detection of DPPH inhibitory properties of this extract (Table 2). Further results of lipid peroxidation assay with EtOH extract of *D. revoluta* showed a promising inhibitory effect when compared to BHT (Fig.1). These observations led us to verify the inhibitory capacity of extract and various fractions toward other free radical species. A further inspection of the results of DPPH assays clearly showed significant differences between EtOAc and BuOH fractions with EtOH extract ($p<0.001$) (Table 4). The n–butanolic fraction was found to be superior in manifestation of DPPH radical scavenging activity (Table 4, Fig. 2). In nitric oxide scavenging assay (Table 5), ethyl acetate and n–butanolic fractions showed similar levels of significance with ethanolic extract ($p<0.001$). Comparison of the IC_{50} values obtained for these fractions indicated ethyl acetate to be more potent than n-butanol fraction in demonstration of NO scavenging effect (Table 5). Thus, it may be postulated that a group of compounds in ethyl acetate fraction exert their antioxidant effects through inhibition of nitric oxide radicals while the active constituents of n-butanol fraction exhibit antioxidant characteristics by inhibition of DPPH free radical.

4. Discussion

The data obtained in this study exhibited a good harmony between antioxidant capacity and polyphenolic contents of *D. revoluta* extract and fractions. Our results were found to be in agreement with earlier reports that indicated the involvement of polyphenolics in the manifestation of antioxidant activity by herbal extracts (33,34). As can be seen from the results, the EtOAc and BuOH fractions which showed high phenol and flavonoid content exhibited higher degrees of effectiveness in free radical inhibition whereas other fractions with low polyphenolic components, were devoid of radical quenching properties. But comparison of free radical scavenging potency of the above two fractions indicated a higher DPPH radical scavenging capacity of n-butanol fraction while EtOAc surpassed n-BuOH fraction in demonstration of nitric oxide inhibitory properties.These differences between the two fractions in type and mode of free radical inhibition may point to the possibility that the more polar constituents may involve in DPPH scavenging while the EtOAc fraction containing compounds of lower polarity shows a higher nitric oxide inhibitory properties. Thus the prominent antioxidant activity of n-BuOH fraction may be attributed to the high content of total phenolics and particularly flavonoids. However, the contribution from other antioxidant constituents and their synergistic effects cannot be neglected. The results of previous studies have also indicated high flavonoid content in several *Dionysia* species. They reported the isolation of five distinctive chemical markers including 3′,4′-dihydroxyflavone, chionanthin, hirsutin and gossypetin in addition to quercetin and kampferol (19).

Following the lead of remarkable antioxidant effect of *Dionysia* n-butanol fraction, we prompted to investigate the effect of acid hydrolysis and the possible improvement of free radical scavenging potency of this fraction on acid treatment. So far, several studies have reported the effect of acid hydrolysis on antioxidant efficacy of herbal extracts. These studies have declared considerable improvement in the activity of hydrolysed products (35,36).

Based on our results, acid hydrolysis of n-butanol fraction significantly enhanced the concentration of phenolics and flavonoids (Table 3) and the protective capacity of hydrolysed product against DPPH radical (Table 4 Fig. 4). It may be concluded that cleavage of glycosidic linkages in glycosylated and methylated flavonoids and so the rise in phenolic groups would make the n-butanol fraction more potent participant in redox type of reactions (37). The bioactivity may therefore be attributed to the aglycone moiety of flavonoid glycosides and more specific to the catechol structure in the aglycones (38). Therefore, the free hydroxyl groups confer scavenging ability to these groups of plant constituents (39,40).

5. Conclusion

As evidenced by the results obtained in the present study, it is concluded that EtOAc and BuOH fractions of *D. revoluta* L. which contain appreciable amounts of phenolics and flavonoids, exhibit high antioxidant and free radical scavenging activities.We also conclude that acid hydrolysis reaction led to a remarkable increase in the content of phenol and flavonoids and significantly improved the antioxidant efficacy of n-butanol fraction. Furthermore, the results clearly indicated that this plant is a rich source of natural antioxidant compounds which might be helpful in preventing the progress of various deletorious free radical reactions and oxidative stress. But the components responsible for the antioxidative activity are currently unclear. Thus, further investigation is needed to isolate and identify the chemical constituents responsible for the activity.

6. Acknowledgement

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Conflict of Interest

None declared

7. References

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