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Original Article

IN VITRO ANTIOXIDANT CAPACITY OF THE FERN, *DRYNARIA QUERCIFOLIA* (L) SM., RHIZOME EXTRACT

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ABSTRACT

Objective: The study was aimed to evaluate polyphenolic composition and antioxidant properties of methanol extract of rhizome of *Drynaria quercifolia* (L.) Sm.

Methods: In this study, assessment of total phenolic and flavonoid contents were performed using Foiln-Ciocalteu and Aluminium chloride methods respectively. Antioxidant capacity of crude methanolic extract of rhizome of *D. quercifolia* were examined using established 1,1 – diphenyl – 2-picrylhydrazyl stable free radical (DPPH•) assay, hydroxyl ion radicals ($^{\bullet}OH$), nitric oxide (NO), hydrogen peroxide (H_2O_2) and 2, 2'-Azinobis (3-ethylbenzothiazoline sulphonic acid) ABTS•+ scavenging assays.

Results: The extract yielded total phenolic content (TP) of 240 ± 0.01 mg gallic acid equivalents (GAE)/100g of fresh mass (FM) and total flavonoid content (TF) of 150 ± 0.02 mg quercetin equivalents (QE)/100g of fresh mass (FM). The extract of *D. quercifolia* rhizome exhibited remarkable scavenging capacity towards DPPH• (EC_{50} 18.54 ±0.70 µg mL^{-1}), $^{\bullet}OH$ (EC_{50} 37.60±0.41 µg mL^{-1}) NO (EC_{50} 42.40 µg mL^{-1}), H_2O_2 (EC_{50} 32.80±1.89 µg mL^{-1}) and ABTS•+ (EC_{50} 29.80 ±0.70 µg mL^{-1}). The antioxidant capacities of the extract were comparable and stronger than that of the antioxidant standard, butyl hydroxy toluene (BHT). The findings were also comparable with antioxidant properties of other medicinal ferns which are discussed in the study.

Conclusion: Significant and positive correlations (R^2 0.999-0.862) were observed between polyphenolic contents and the antioxidant capacities, indicating that the phenolics were major contributors of the antioxidant property. Thus, the methanol extract from the rhizome of *D. quercifolia* indicated the existence of strong antioxidants and it can be used as tonic.

Keywords: Medicinal fern, *Drynaria quercifolia*, Antioxidant activity, Phenolics, Flavonoids.

INTRODUCTION

Free radicals including reactive oxygen species (ROS) induce oxidative damage to biomolecules and have been implicated with variety of chronic diseases including cancer, diabetes, atherosclerosis, neurodegenerative disorders and arthritis [1]. Natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds becomes important [2] (Halliwell, 1994). Epidemiological studies have indicated the relationship between the plant antioxidants and reduction of chronic diseases [3,4]. These benefits are thought to result from the antioxidant components of plant origin, vitamins, flavonoids, and carotenoids [5,6]. The studies in recent years have shown that polyphenols in plants scavenge active oxygen species and effectively prevent oxidative cell damage [7,8,9,10]. On search for antioxidants from medicinal ferns, several investigations were performed to identify new sources of antioxidant.

Drynaria quercifolia(L.) Sm., belonging to the family Drynariaceae, is used in typhoid fever, phthiriasis, dyspepsia, cholera, cough, arthralgia, cephalalgia, diarrhea, chronic jaundice, foul ulcers, inflammation and also used to treat skin diseases [11,12,13].

Different extracts of *D. quercifolia* were screened for their antibacterial activity against several bacterial pathogens [14]. The compounds friedelin, epifriedelinol, amyrin, β-sitosterol, β-sitosterol-3-β-D-glucopyranoside, naringin have been isolated from the rhizome of *D. quercifolia* [13].

Despite several studies on medicinal ferns on their antioxidant properties, a little work has been conducted on antioxidant activity of *D. quercifolia*. Hence, the present study encompasses phenolic composition and antioxidant capacity using established assay procedures.

MATERIALS AND METHODS

Preparation of rhizome extract

The rhizome of *D. quercifolia* was collected from Konni forest of Pathanamthitta district of Kerala State, India during the month of June 2009 and identified by Professor R. Gopalan, department of Biotechnology, Karpagam University, Coimbatore. A voucher specimen has been deposited at the herbarium. Fifty grams of each powder was extracted in soxhlet apparatus with methanol (250 ml). The extract, thus collected, was evaporated to dryness using rotary flash evaporator (Buchi type, Switzerland) under reduced pressure at less than 40^0 C. The crude methanolic extract was used for assessing the antioxidant capacity.

Total phenolic content (TPC)

The content of total phenolics was determined by using Folin-Ciocalteu method [15]. An aliquot of the sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of folin-ciocalteu reagent was added. After 3 minutes 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath for exactly one minute. It was then cooled and the absorbance was measured at 650nm using spectrophotometer (Elico Scanning mini spec SL 177, India) against the reagent blank. The results were presented as Mean (n=3) Standard Deviation (±) and expressed as Gallic acid equivalent (mg QE /g).

Total flavonoid content (TFC)

The content of total flavonoid in *D. quercifolia* rhizome extract was estimated based on the method previously described [16]. A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was added to 0.5 ml of sample solution. After one hour at room temperature, the absorbance was measured at 420 nm with UV-Visible spec (Elico, India) Extract

samples were evaluated at a final concentration of 0.1 mg/ml. Total flavonoid content were calculated as quercetin equivalent (mg/g).

Measurement of *in vitro* antioxidant activity

DPPH· radical quenching activity

The determination of DPPH· stable radical scavenging activity of methanol extract was based on the method as described previously [17]. Briefly, one millilitre of aliquots of the extract and standards (20-100 µg mL^{-1}) was added to MeOH solution of DPPH· (5 mL, 0.1 mM) and vortexed. After 20 min reaction at 25°C, the absorbance was measured at 517 nm against a blank in a UV-Vis spectrophotometer. BHT and ascorbic acid were used for comparison. The percentage quenching of DPPH· was calculated as follows: Inhibition of DPPH· (%) = 1-$Sample_{517nm}$/$Control_{517nm}$x100, where, $Sample_{517nm}$ was absorbance of the sample and $Control_{517nm}$ was absorbance of control. The results were expressed as EC_{50}, which means the concentration at which DPPH· radicals were quenched by 50%.

Reducing capacity (RC)

The ferric reducing power of the bark extract was quantified according to the method of Oyaizu [18]. Various concentrations of the extracts (20-100 µg mL^{-1}) were prepared. To all the extracts in test tubes 2.5 ml of sodium phosphate buffer followed by 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$] solution was added. The contents were vortexed well and then incubated at 50° C for 20 minutes. After incubation, 2.5 ml of 10% trichloroacetic acid (TCA) was added to all the tubes and centrifugation was carried out at 3000 g for 10 minutes. Afterwards, to 5ml of the supernatant, 5 ml of deionized water was added. To this about 1 ml of 1% ferric chloride was added to each test tube and incubated at 35°C for 10 minutes. The absorbance was read at 700 nm. Mean values were obtained from triplicate experiments. The reducing power of the extract was linearly proportional to the concentration of the sample. Increased absorbance of the reaction mixture indicated increased reducing power. Butylated hydroxy toluene (BHT) was taken as reference standard.

Scavenging capacity towards hydroxyl ion (˙OH) radicals

The ˙OH scavenging activity of the BME was determined according to the method described previously [19]. Different concentrations (20-100 µg mL^{-1}) of extract were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1.0 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90° C for 15 min in a water bath. The reaction was then terminated by the addition of 1.0 ml of ice-cold TCA (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the color formed was measured at 412 nm against reagent blank. BHT was considered as the reference standard. The hydroxyl radical scavenging activity is calculated by the following formula: HRSA (%) = 1-(difference in absorbance of sample/difference in absorbance of blank) × 100.

Scavenging capacity towards nitric oxide (NO)

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be estimated by the use of Griess Illosvoy reaction [20]. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide. The reaction mixture (3 ml) containing SNP (10 mM, 2 ml), phosphate buffer saline (0.5 ml) and the rhizome extract at different concentrations and standards (20-100 µg mL^{-1}) were incubated at 25º C for 150 min. After incubation, 0.5 ml of the incubated solution containing nitrite was pipetted and mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then, 1 ml of N-1-naphthyl ethylene diamine dihydrochloride was added, mixed and allowed to stand for 30 min at 25º C. The absorbance of pink coloured chromophore formed during diazotization was measured at 540 nm in a UV-Vis spectrophotometer. BHT was used for comparison. The percentage scavenging of NO was calculated as follows: Inhibition of NO (%) = 1-$Sample_{540nm}$/$Control_{540nm}$x100, where, $Sample_{540nm}$ was absorbance of the sample and $Control_{540nm}$ was absorbance of control.

Hydrogen peroxide (H_2O_2) scavenging activity

The H_2O_2 scavenging activity of the extract was determined by the method of [21] Ruch *et al*. The extract at different concentration (20-100 µg mL^{-1}) was dissolved in 3.4 ml of 0.1 M phosphate buffer pH 7.4) and mixed with 600 µl of 43 mM solution of H_2O_2. The absorbance value of the reaction mixture was recorded at 230 nm and BHT was considered as the standard. The percentage scavenging of NO was calculated as follows: Inhibition of NO (%) = 1-$Sample_{230nm}$/$Control_{230nm}$x100, where, $Sample_{230nm}$ was absorbance of the sample and $Control_{230nm}$ was absorbance of control.

2, 2′-Azinobis (3-ethylbenzothiazoline sulphonic acid) ($ABTS^{\cdot+}$) cation decolorization test

The ABTS•+ radical cation was produced by oxidising ABTS•+ with potassium persulfate ($K_2S_4O_8$) [22] with minor changes. The ABTS•+ solution (7 mM) was oxidized with $K_2S_4O_5$ (2.4 mM) for 12 h at room temperature in the dark. The ABTS•+ solution was then diluted by mixing 990 µl ABTS•+ solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 at 734 nm. Fruit extract and BHT of various concentrations (20-100 µg mL^{-1}) were allowed to react with 1.0 ml of the ABTS•+ solution and the absorbance was measured at 734 nm after 7 min. BHT was used for comparison. Percentage inhibition was calculated as $ABTS^{\cdot+}$ radical scavenging activity (%): 1-$Sample_{734nm}$/$Control_{734nm}$x100, where, $Sample_{734nm}$ was absorbance of the sample and $Control_{734nm}$ was absorbance of control.

Statistical analysis of data

The experimental data were expressed as mean values ± SD of at least three independent measurements. Linear regression analysis was used to calculate the efficient concentration (EC_{50}) values. One way analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) were carried out. The *P* values of less than 0.05 were adopted as statistically significant.

RESULTS AND DISCUSSION

In the present study, the total phenolic and flavanoid content of the methanol extract of rhizome of *D. quercifolia* was measured. We observed high contents of phenolics and flavonoids in the rhizome extract. Total phenolic content of extract was found to be 240 mg /100g of gallic acid equivalent (GAE). The flavonoid content of *D. quercifolia* was found to be 150 mg /100g of quercetin equivalent. The polyphenolic contents are comparable to that of medicinal ferns studied previously [23].

Table 1: EC_{50} values of *D. quercifolia* methanol extract and standard BHT

Assay model	Extract	BHT
	(µg/ml)	
DPPH radical scavenging activity	18.54 ±0.70a	10.00 ±0.65a
Hydroxyl radical scavenging activity	37.60±0.41d	41.50±2.23b
Nitric oxide scavenging activity	42.40 ±1.45c	33.50±2.12c
Hydrogen peroxide activity	32.80±1.89e	43.87±2.02e
$ABTS^{\cdot+}$ radical scavenging activity	29.80 ±0.70b	41.50 ±0.08b

EC_{50} values of *D. quercifolia* methanol extract and standard butylated hydroxyl toluene (BHT). Each value in the table was obtained by calculating the average of three experiments ± standard deviation (*n* = 3). Values in a column with different superscripts indicate significantly different at $P < 0.05$.

The extract exhibited dose dependent DPPH quenching scavenging activity (92.78% at 100 µg mL^{-1}) towards DPPH with an EC_{50} value

of 18.54 ± 0.65 µg mL^{-1}. The activity is comparable with the standard butyl hydroxyl toluene (BHT) shown EC_{50} of 10.00 ±0.65 µg mL^{-1} (Table 1). DPPH• is a stable radical with a maximum absorption at 517 nm that can readily undergo scavenging by an antioxidant [24]. In the present study, the extract exhibited a concentration dependent antiradical activity by quenching DPPH radical. The activity observed in this study was higher than that of *Drynaria fortunei* (IC_{50} = >400) and other medicinal ferns from China [23]. The results observed in the present study was comparable with DPPH scavenging activity of the rhizome of *D. quercifolia* observed previously [25]. The DPPH quenching capacity of this fern is also more efficient than that of *Marsilea quadrifolia* [26].

Fig. 1. shows the reducing power of the methanolic rhizome extract and BHT using the potassium ferricyanide reduction method, The reducing power capacity of methanol extract *D. quercifolia* was found to possess OD value of 1.102 at 100 µg mL^{-1}, whereas the standard BHT possessed 1.21 at 100 µg mL^{-1} at 700 nm. The OD value of the extract was comparable with that of BHT. The highest value of reducing power indicates that some compounds in the extract are electron donors which would react with the free radicals. The increased reducing ability may be due to the formation of reductants which would react with free radicals to stabilize and terminate radical chain reaction and converting them to more stable products [27].

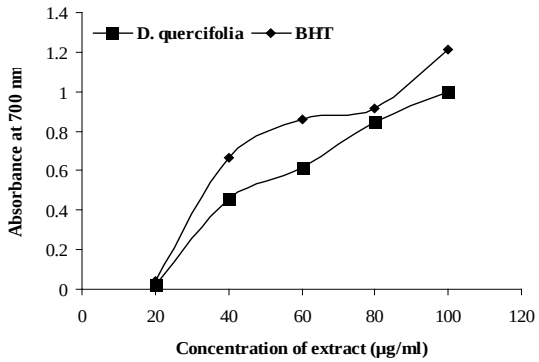

Fig. 1: Reducing capacity of rhizome extract of *D. quercifolia*

Hydroxyl radical (˙OH), the most reactive free radical has the capacity to conjugate with nucleotides in DNA, cause strand breakage, and lead to carcinogenesis, mutagenesis and cytotoxicity [28]. The methanol extract of *D. quercifolia* scavenged ˙OH radicals in dose dependent manner. At 100 µg mL^{-1} there was 87.1% inhibition of ˙OH radicals with an EC_{50} value of 37.60±0.41 µg mL^{-1}(Table 1). The result shows that *D. quercifolia* extract has significant response on the ˙OH radicals. The ˙OH scavenging activity indicates that the rhizome extract scavenged ˙OH radicals and percentage inhibition was proportional to the concentration of the extract. The extract of *D. quercifolia* exerted better activity than other medicinal ferns, *Selaginella involvens* (IC_{50} =170.2) and *S. wightii* (IC_{50} = 88.2) and lower activity than *S. delicatula* (IC_{50} =32.1) [29]. Extent of nitric oxide radical scavenged was determined by the decrease in intensity of pink coloured chromophore at 540 nm. Nitric oxide is a secondary messenger and plays an important role in the control of blood pressure. The toxicity of NO increases greatly when it reacts with superoxide radical, forming the highly reactive peroxynitrite anion (ONOO-) [30]. From the results obtained, it was found that rhizome extract of *D. quercifolia* inhibits nitrite formation by directly competing with oxygen in the reaction with nitric oxide. The extract of *D. quercifolia* inhibited NO species up to 90.1% at the concentration of 100 µg mL^{-1} against nitric oxide generation. EC_{50} value was found to be 42.40 ±1.45 µg mL^{-1} which was comparable with BHT as standard (Table 1).

Elimination of H_2O_2 is very important for antioxidant defense in cell or food systems since H_2O_2 may give rise to hydroxyl radicals which are toxic to cell [3]. The rhizome extract of *D. quercifolia* tested exhibited marked inhibitory capacity towards H_2O_2. Hydrogen peroxide decomposition activity was in a concentration dependent manner. The percentage inhibition of methanolic extract of *D. quercifolia* was 84.2% at 1000 µg mL^{-1} with an EC_{50} value of 32.80±1.89 µg mL^{-1}, where as the standard BHT was amounted to have 85.1% at 1000 µg mL^{-1}.(Table 1)

The decolourization of ABTS•+ radical also reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to deactivate the radical species [22]. The ability of a compound to scavenge ABTS•+ radical can demonstrate oxygen radical absorbance capacity. The methanolic extract of *D. quercifolia was* an effective scavenger of the ABTS•+ and was comparable to BHT. The extract expressed the maximum scavenging activity of 92.7% at 100 µg mL^{-1} (EC_{50} value 29.80 ±0.70) which was approximately equal to that of BHT (Table 1). The findings are considered to be noteworthy when compared to the results of previous studies on medicinal ferns [23].

Multiple Correlations

Correlation between the assays was tested to link the antioxidant capacity measured by different assays used with each other. The coefficient of correlation between different antioxidant parameters are presented in table 2, relevant regression equation that they posses curvilinear exist between TPC and other antioxidant parameters. TPC and TFC was well correlated (R^2 =0.999).Correlation between DPPH and TPC was found to have better correlation (R^2 = 0.961). A moderate correlation was found between TPC and NO scavenging activity (R^2=0.952). TFC and DPPH was found to have better correlation (R^2= 0.965). The correlation between reducing power and TFC has a moderate correlation (R^2= 0.969). ABTS•+ and DPPH was found to have low correlation (R^2 =0.879) and the better correlation was found between Hydrogen peroxide assay and Reducing power (R^2 =0.998) respectively. Nitric oxide radical activity scavenging activity was highly correlated with reducing power and ˙OH (R^2=0.906).The correlation between reducing power and ABTS•+ has a moderate correlation (R^2= 0.862) respectively. Many supportive reports emphasize the positive correlation between phenolic content and antioxidant efficacy [31,32]. A positive correlation between antioxidant activity and polyphenol content was found, suggesting that the antioxidant capacity of the plant extracts is due to a great extent to their polyphenols [33, 34]. Strong positive correlations between antioxidant activity and flavanoid and phenol contents suggests that the antioxidant capacity of the rhizome extract of *D. quercifolia* is due to a great extent of phytochemicals like flavonoids and other phenols.

Table 2: Linear correlation coefficients

	TP	TF	DPPH	NO	HR	RC	ABTS•+	H_2O_2
TP	1							
TF	0.999	1						
DPPH	0.961	0.965	1					
NO	0.952	0.952	0.921	1				
OH	0.960	0. 967	0.918	0.952	1			
RC	0.972	0.969	0.965	0.952	0.905	1		
ABTS•+	0.915	0.920	0.879	0.921	0.983	0.880	1	
H2O2	0.910	0.916	0.957	0.891	0.892	0.998	0.862	1

TP- Total phenol content; TF- Total flavonoid content; DPPH- diphenyl picryl hydrazyl; ABTS•+ - 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); NO- nitric oxide scavenging; OH- hydroxyl radical scavenging; RC- reducing capacity; H_2O_2 - hydrogen peroxide.

The findings obtained in the present study indicated that the antioxidant potential of *D. quercifolia* was in agreement with the previous works on antioxidant activity of other medicinal ferns. The study showed the significance of the medicinal ferns and also necessitated further *in vivo* and phytochemical investigations on the medicinally important ferns.

CONCLUSION

In conclusion, the results presented in this report indicated that *D. quercifolia* rhizome extract might be a potential source of natural antioxidant. However, further studies are necessary to isolate active principles responsible for the overall antioxidant activity of the extract. Based on the uses of ferns, studies could be tailored to illuminate the possible pharmacological activity.

CONFLICT OF INTERESTS

Declared None

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