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Original Article

Antioxidant Activities and Thin Layer Chromatographic Analysis of Aqueous Extract of Tubers of *Drynaria quercifolia* (L).J.Sm

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ABSTRACT

Objectives: The aim of the study was to investigate phytochemical constituents, free radical scavenging activities and antimicrobial activities of the aqueous extract of tubers of *Drynaria quercifolia*. **Materials & Methods:** The qualitative and quantitative analyses were carried out for the aqueous extract using standard procedures. The antioxidant activities were carried out by DPPH free radical, $ABTS^{\bullet+}$ radical cation, $OH^{\bullet}$ radical, $NO^{\bullet}$ radical scavenging assays as well as Fe^{3+} reducing power and phosphomolybdenum reduction assays. **Results:** The tubers of *D. quercifolia* in aqueous extract showed good radical scavenging as well as reducing power activities. The major phytochemicals such as phenols and flavonoids were estimated as 4.29±1.23 and 24.56±0.86 µg/mL respectively. **Conclusion:** The aqueous extract of tubers of *D. quercifolia* possesses significant antioxidant activities.

Key words: *Drynaria quercifolia*, Phenols, Flavonoids, Antioxidant, Dot-blot assay.

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INTRODUCTION

Drynaria quercifolia belongs to the family Polypodiceae, which is popularly known as Attukal kizhangu is classified in the botanical division Pteridophyta, order Polypodiales.[1] It is commonly found in Bangladesh, India, Pakistan, North America, and Africa. Antioxidants are the substances which inhibit oxidation, and have the ability to remove the potentially damaging oxidizing agents in a living organism. Optimisation of antioxidants from Nelumbo seeds[2] and coumarin derivatives as potential antioxidant agents[3] manifest the potential of antioxidants having an therapeutic potential in treating various diseases. The presence of phytochemicals in plants are able to prevent the oxidative damage to the human cells which can even cause cancer. It is important to know the antioxidant activities of phytocompounds responsible for inhibiting radicals. In this study, the free radical scavenging activities of the extracts of *D. quercifolia is* analysed. Free radicals initiate the oxidative stress and damage the healthy cells and DNA along with lipid peroxidation. This damages can contribute to ageing, atherosclerosis, cancer, cardiovascular diseases, and inflammatory diseases.[4]

MATERIALS AND METHODS

Plant material

The rhizomes of *Drynaria quercifolia* are creeping and densely covered in brown scales with nested leaves having sterile and fertile fronds. The fertile fronds have a longer stalk growing about 100 cm in length than the sterile frond growing only 20-40 cm in length. The rhizome was associated with water-borne hyphomycetes that may be present as endophytes or epiphytes.[5] It has been highly found in Eastern Ghats of India. The tuber extract have potentially high antibacterial activity.[6]

Extraction

The dried tubers were cut into small pieces and extracted with hot distilled water using a pressure cookware. The hot aqueous extract was cooled and filtered using filter paper. Water was completely removed at 50°C hot plate apparatus. Finally brown gummy residue was obtained and was used for antioxidant activities as well as thin layer chromatographic (TLC) analysis.

Determination of total phenol

Folin-Ciocalteau reagent method was used to estimate total phenolic compounds (Spanos, and Wrosltad, 1990) with slight modifications. One mL of methanol extract (1mg/mL) of aerial parts of C. viscosa was mixed with 1 mL of Folin Ciocalteau reagent (1:10 diluted with distilled water). After 5 min, 1 mL of aqueous solution of Na_2CO_3 (20%) was added. The mixture was then allowed to stand for 30 min incubation in dark at room temperature. The absorbance was measured by UV-VIS spectrophotometer at 765 nm. The total phenolic content was expressed in terms of Gallic acid.

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Determination of total flavonoid

The total flavonoid content of methanol extract of aerial parts of C. viscosa was determined using aluminium chloride colorimetric method with slight modification as described by Liu *et al*., 2007. One mL of extract (1mg/mL) was mixed with 0.5 mL of 5% sodium nitrite and incubated for 5 min at RT. Then, 0.5 mL 10% aluminium chloride solution was added and after 5 min incubation at RT, 1 mL of NaOH solution (1 M) was added. The total volume was made up to 5 mL with distilled water. Absorbance was measured at 510 nm using spectrophotometer. The result was expressed as quercetin equivalent (µg/mg of dry mass), which is a common reference compound. The phytocompounds like flavonoids and phenolics exhibit various biological activities, the most important being the antioxidant activity.[7]

Antioxidant activities

Screening of antioxidant activity by dot-blot DPPH staining method

Drops of DPPH (0.4 mM) solution in methanol were loaded onto a 5 cm × 5 cm TLC plate (silica gel 60 F254; Merck) in each column and allowed to dry for 2 min. The first row of TLC plate was considered as control, containing only DPPH. Aqueous extract of tubers of *Dynaria quercifolia* of various concentrations was carefully loaded onto the DPPH spot in a second row. The third row of TLC plate was considered as standard reference, where ascorbic acid was carefully loaded onto the DPPH spot. The staining of the silica plate was based on the procedure of Soler- Rivas *et al*.,.[8] Stained silica gel layer revealed a purple background with yellow to white spots at the location where radical scavenging capacity observed. The intensity of disappearance of purple colour depends upon the amount and nature of radical scavenger[9] present in the tubers of *Drynaria quercifolia* in aqueous extract

DPPH radical scavenging activity

The DPPH radical scavenging activity was carried out according to the method of Raman *et al*.,.[10] 1 mL of 0.1 mM DPPH solution in methanol was mixed with 1mL of various concentrations of aqueous extract in methanol (20-120 µg/mL). Mixer of 1mL and 1mL DPPH solutions were used as control. The reaction was carried out in triplicate and the decrease in absorbance was measured at 517 nm after 30 minutes in dark using UV-Vis spectrophotometer. Ascorbic acid was used as reference standard. The inhibition % was calculated using the following formula.

$$\%\ \text{of DPPH radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

ABTS$^{\bullet+}$ radical cation scavenging activity

The antioxidant capacity was estimated in terms of the ABTS$^{\bullet+}$ radical cation scavenging activity following the procedure described by Delgado-Andrade *et al.*[11] Briefly, 7 mM ABTS stock solution by reacting with 2.45 mM potassium persulfate and the mixture was left to stand in the dark at room temperature for 12-16 h before use. The ABTS solution (stable for 2 days) was diluted with 5 mM phosphate-buffered saline (pH 7.4) to an absorbance at 734 nm of 0.70±0.02. To various concentrations (5-30 µg/mL) of aqueous extract of tubers of *D. quercifolia*, 1 mL of diluted ABTS$^{\bullet+}$ solution was added and after 10 min, the absorbance was measured at 734 nm. Ascorbic acid was used as reference standard. The ABTS $^{\bullet+}$ radical cation scavenging activity was expressed as

$$\%\ \text{of ABTS}^{\bullet+}\ \text{radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined using standard protocol.[12] Various concentrations (10-60 µg/mL) of the aqueous extract of tubers of *D. quercifolia* were taken in different test tubes and evaporated to dryness. One milliliter of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA (0.018%), and 1mL of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5 mL of 0.22% ascorbic acid. The test tubes were capped tightly and heated on a water bath at 80- 90°C for about 15mins. The reaction was completed by the addition of 1mL of ice-cold TCA solution (17.5% w/v). One milliliters of Nash reagent (18.75 g of ammonium acetate, 0.75 mL of glacial acetic acid and 0.5 mL of acetyl acetone were mixed and raised to 250 mL with distilled water) was added to all the tubes and left at room temperature for about 15 min for color development. The intensity of the yellow color was measured at 412 nm against reagent blank. Ascorbic acid was used as reference standard. The percentage of hydroxyl radical scavenging was calculated by the following formula

$$\%\ \text{of OH}\bullet\ \text{radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Nitric oxide radical scavenging activity

Nitric oxide generated from aqueous sodium nitroprusside (SNP) solution interacts with oxygen to produce nitrite ions at physiological pH, which may be quantified and determined according to Griess Illosvoy reaction.[13] The reaction mixture contained 1 mL of 10 mM SNP in 0.5 M phosphate buffer (pH 7.4) and 1 mL of various concentrations (10–60 µg/mL) of the aqueous extract of tubers of *D. quercifolia*. After incubation for 60 min at 37°C, Griess reagent (0.1%) napthyl ethylenediamine dihydrochloride in water and 1% sulfanilamide in 5% H_3PO_4) was added. The pink chromophore generated during diazotization of nitrite ions with sulfanilamide and subsequent coupling with napthyl ethylenediamine dihydrochloride were measured spectrophotometrically at 540 nm. Ascorbic acid was used as a positive control. The percentage of nitric oxide radical scavenging activity was calculated by the following formula

$$\%\ \text{of nitric oxide radical inhibition} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

Reducing power assay

The reducing power of tubers of *Drynaria quercifolia* in aqueous extract was evaluated according to the method of Ravisankar *et al.*[14] Different concentrations of aqueous extract of tubers of *D. quercifolia* (20–120 µg/mL) were mixed with 1 mL of 0.2 M phosphate buffer (pH 6.6) and 1 mL of 1% $K_3Fe(CN)_6$. This mixture was incubated at 50°C for 20 min. After, 1 mL of 10% TCA was added and centrifuged at 3000 rpm for 10 min. The upper layer of the solution was mixed with 0.5 mL of $FeCl_3$ (0.1%) solution and the absorbance was measured at 700 nm using a spectrophotometer. Ascorbic acid was used as the standard reference.

Phosphomolybdenum reduction assay

The total antioxidant capacity was measured by spectrophotometeric method of Prieto *et al*.,.[15] At different concentration, aqueous extract of tubers of *D. quercifolia* (20–120 µg/mL) was combined with 1mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate). The tubes were incubated at 95°C for 90 min in a water bath. After, the mixture was cooled to room temperature and the absorbance was read at 695 nm. Ascorbic acid was used as the standard reference.

RESULTS AND DISCUSSION

Medicinal plants are an important source of practical and inexpensive new drugs.[16] Oxidative stress has been implicated in the pathology of many diseases such as inflammatory conditions, cancer, diabetes and the aging. This study was made to evaluate polyphenolic content and antioxidant activity. Phenolics are composed of one or more aromatic rings bearing one or more hydroxyl groups and are therefore potentially able to quench free radicals by forming stabilized phenoxyl radicals.[17]

Dot-blot assay for rapid radical scavenging activity

The results of dot-blot assay showed coloured spots where the aliquots of aqueous extract of barks of *Drynaria quercifolia* were placed in row. The purple zone on the plate indicates no (free radical scavenging) antioxidant activity and the yellow zone indicates antioxidant activity. The more intense the yellow colour, the greater the antioxidant activity (Figure 1). The result indicates that the aqueous extract of tubers of *D. quercifolia* have significant antioxidant activity when compared to standard ascorbic acid.

Total phenol and flavonoid

The phenolic and flavonoid compounds quantified in the extracts seemed to be responsible for the antioxidant activity. Phenolic acids, flavonoids are the most commonly found polyphenolic compounds in plant extracts.[18] The antioxidant activity of phenolics plays an important role in absorption or neutralization of free radicals.[19] Polyphenols also enhance the level of cellular antioxidative system and induce the cytochrome P-450 resulting in detoxifying the activity of carcinogens intracellularly.[20] Total phenol content was 4.295±1.23 µg/mL of GAE and flavonoid content was 24.564±0.86 µg/mL of QE in the aqueous extract of tubers of *D.quercifolia*, as shown in Table 1. These investigations provide a comprehensive profile of the antioxidant activity of extracts of plants with respect to their phenols and flavonoids content.

DPPH radical scavenging activity

The ability of aqueous extract of tubers of *D. quercifolia* to scavenge free radicals formed was assessed using 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH). This was compared with a standard (ascorbic acid). Aqueous extract of tubers of *D.quercifolia* demonstrated high capacity for scavenging free radicals as shown by the data by reducing the stable DPPH (1,1-diphenyl-2-picrylhydrazyl) radical to the yellow coloured 1,1-diphenyl-2-picrylhydrazine and this capacity increases with increasing concentration as reported earlier by Huang DJ *et al*.,.[21] The maximum percentage of DPPH radical scavenging activity was 55.11±5.27% at 120 µg/mL concentration as shown in Table 2 and Figure 2. It was compared with the standard ascorbic acid (70.95±4.96/120 µg/mL) and the IC_{50} of DPPH radical scavenging activity was 105.78 µg/mL concentration. The scavenging ability of the aqueous extract of tubers of *D.quercifolia* may be due to its bio compositions such as phenolic acids and flavonoid. The radical scavenging activities of the extracts were determined by using DPPH a stable free radical at 517 nm. 1,1 -diphenyl-2-picrylhydrazyl is a nitrogen-centred free radical, color of which changes from violet to yellow on reduction by donation of H or e^- by the aqueous extract of tubers of *D.quercifolia*

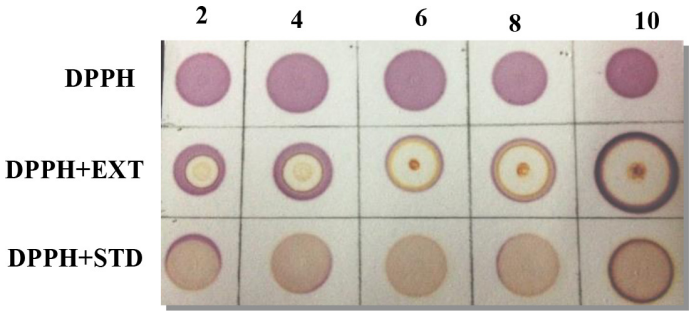

Figure 1: Dot-blot assay of aqueous extract of D. quercifolia in DPPH Radical scavenging activity. DPPH - 1, 1 Diphenyl-2-picryl hydrazyl STD - Standard (Ascorbic acid).

Table 1: Total phenol and flavonoid present in aqueous exracts of tubers of Drynaria quercifolia

Phytochemicals	Value (µg/mL)
Phenols	4.295±1.23
Flavonoids	24.564±0.86

ABTS$^{\bullet+}$ radical scavenging activity

In the total antioxidant activity, ABTS$^{\bullet+}$ is a blue chromophore produced by the reaction between ABTS and potassium persulfate and in the presence of the plant extract or trolox, preformed cation radical gets reduced and the remaining radical cation concentration after reaction with antioxidant compound was then quantified.[22] The maximum percentage of ABTS$^{\bullet+}$ radical cation scavenging activity was 54.91±5.55% at 30 µg/mL concentration as shown in Table 3 and Figure 2. It was compared with standard (52.41±3.66/30 µg/mL) ascorbic acid. The IC_{50} of ABTS$^{\bullet+}$ radical cation scavenging activity was 27.32 µg/mL concentration.

Hydroxyl (OH•) radical scavenging activity

Scavenging of hydroxyl radical is an important antioxidant activity because of very high reactivity of the OH radical which enables it to react with a wide range of molecules found in living cells, such as sugars, amino acids, lipids and nucleotides. The hydroxyl radical is an extremely reactive free radical formed in biological systems and has been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells.[23] The maximum percentage of OH$^{\bullet}$ radical scavenging activity was 85.91±5.16% at 60 µg/mL concentration as shown in Table 4 and Figure 2. It was compared with standard (97.67±6.83/60 µg/mL) ascorbic acid. The IC_{50} of OH$^{\bullet}$ radical scavenging activity was 26.54 µg/mL concentration.

Nitric oxide (NO•) radical scavenging activity

In this spectrophotometric method, the absorbance of chromophore formed during the diazotization of the nitrile with sulphanilamide and the subsequent coupling with naphthyethylenediamine dihydrochloride was measured. NO, being a potent pleiotropic mediator in physiological processes and a diffusible free radical in the pathological conditions, reacts with superoxide anion and form a potentially cytotoxic molecule, the peroxynitrite (ONOO).[24] Its protonated form, peroxynitrous acid (ONOOH), is a very strong oxidant. The main route of damage is the nitration or hydroxylation of aromatic compounds, particularly tyrosine. Under physiological conditions, peroxynitrite also forms anadduct with carbon dioxide dissolved in body fluid and responsible for oxidative damage of proteins in living systems. The maximum NO$^{\bullet}$ radical scavenging activity was 52.57±3.67% at 60 µg/mL concentration as shown in Table 4 and Figure 2. It was compared with standard (62.85±4.39/60 µg/mL) ascorbic acid. The IC_{50} of NO$^{\bullet}$ radical scavenging activity was 57.07 µg/mL concentration.

Ferric (Fe3+) reducing power activity

Studies were made on total reduction ability of Fe^{3+} to Fe^{2+} transformation in the presence of the tubers of *Drynaria quercifolia* in aqueous extract and found increasing in showing reduction ability in a

Table 2: DPPH, Ferric (Fe^{3+}) reducing power and phosphomolybdenum reduction of aqueous extract of tubers of *D. quercifolia*

Concentration	DPPH Assay % of inhibition	Fe^{3+} Reducing power Assay	Phosphomolybdenum Reducing Assay
20	13.07±0.91	0.038±0.002	0.135±0.009
40	16.12±1.12	0.15±0.010	0.187±0.013
60	28.10±1.96	0.197±0.013	0.267±0.018
80	33.55±2.34	0.383±0.026	0.344±0.024
100	47.27±3.30	0.481±0.033	0.394±0.027
120	55.11±5.27	0.582±0.040	0.441±0.030

Table 3: $ABTS^{\cdot+}$ radical cation scavenging activity of aqueous extract of tubers of *D. quercifolia*

Concentration (µg/mL)	% of inhibition
5	11.60±0.81
10	15.17±1.06
15	23.66±1.65
20	37.50±2.62
25	42.85±2.99
30	54.91±5.55

Table 4: Nitric oxide and hydroxy radical scavenging activity of aqueous extract of tubers of *Drynaria quercifolia*

Concentration (µg/mL)	Nitric oxide scavenging	hydroxy radical
10	10.94±0.76	19.82±1.38
20	14.59±1.02	39.13±2.73
30	30.81±2.15	56.52±3.95
40	34.86±2.44	75.65±5.29
50	43.10±3.01	83.65±5.85
60	52.57±3.67	85.91±5.16

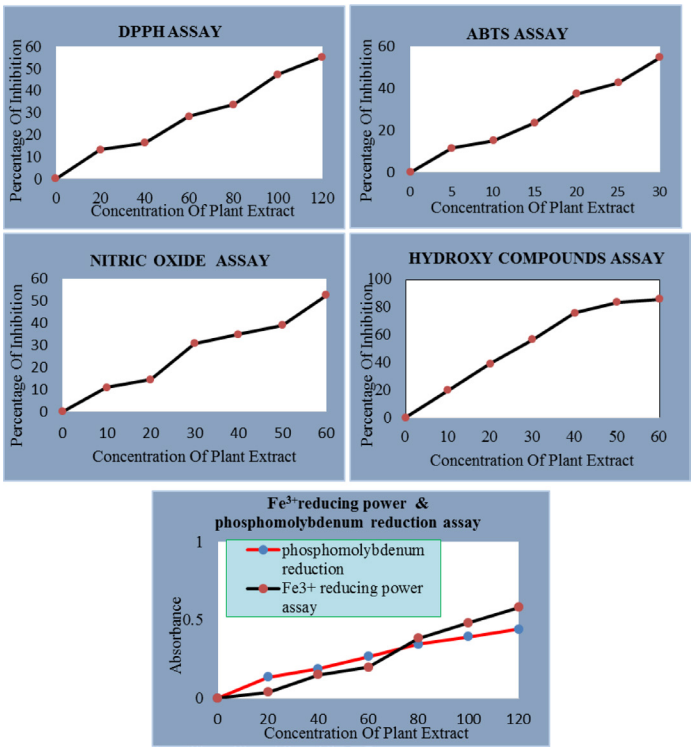

Figure 2: Graphical representation of antioxidant activities of aqueous extract of tubers of *Drynaria quercifolia*

R_f Value
1st spot - 0.46
2nd spot - 0.63
3rd spot – 0.70
4th spot – 0.85

Figure 3: Thin layer chromatography analysis of aqueous extract of tubers of *D. quercifolia*

dose dependent manner, with increasing concentrations. Increase in absorbance of the reaction mixture indicated increased reducing power. Since the reducing capacity of the aqueous extract of tubers of *Drynaria quercifolia* serve as a significant indicator of its potential antioxidant activity, the reducing ability was 0.582±0.040 at 120 µg/mL concentration as in Table 2 and Figure 2, which was compared with standard (0.359±0.02/120 µg/mL) ascorbic acid. The antioxidant activity has been reported to be concomitant with development of reducing power.[25]

Phosphomolybdenum reduction activity

The total antioxidant activity of aqueous extract of tubers of *D. quercifolia* was measured spectrophotometrically by the phophomolybdenum method, which is based on the reduction of Mo (VI) by the petroleum ether fraction and the subsequent formation of green phosphate/Mo (V) complex at acidic pH, with a maximum absorption 695 nm. It evaluates both water-soluble and fat-soluble antioxidants with a high absorbance value of the petroleum ether fraction indicated its strong antioxidant activity.[26] The maximum absorbance was 0.441±0.030 at 120 µg/mL concentration, as in Table 2 and Figure 2, which was compared with standard (0.359±0.02/120 µg/mL) ascorbic acid.

Thin layer chromatography

TLC analysis was carried out for aqueous extract of tubers of *D. quercifolia* by using Hexane:Ethyl acetate (0.2:1.8) solvent system. The separated

bands were visualized by UV light at 254 nm. The R_f values of the separated compounds were measured (Figure 3).

CONCLUSION

The replacement of synthetic with natural antioxidants may be advantageous. In the present study, aqueous extract of tubers of *D. quercifolia* tested with respect to their total phenolic and flavonoid content, antioxidant capacity and oxidative stability. Extractions were performed using the conventional method reflux and methanol as solvent. The existence of phenolic and flavonoid compounds was confirmed by the Folin-Ciocalteu and $AlCl_3$ methods. The antioxidant capacity was measured by DPPH free radical scavenging method was proven to be high. Finally, the results in this study indicate that the examined extract contains significant sources of antioxidants.

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CONFLICT OF INTEREST

There is no conflict of interest associated to the work done.

ABBREVIATIONS USED

DPPH$^{\bullet}$: 2,2-diphenyl-1-picrylhydrazyl radical, **NO$^{\bullet}$:** Nitric oxide radical, **$^{\bullet}$OH:** Hydroxyl radical, **$O^{2\bullet-}$:** Superoxide anion radical, **Fe^{2+}:** Ferrous ion, **IC_{50}:** Inhibitory concentration 50.

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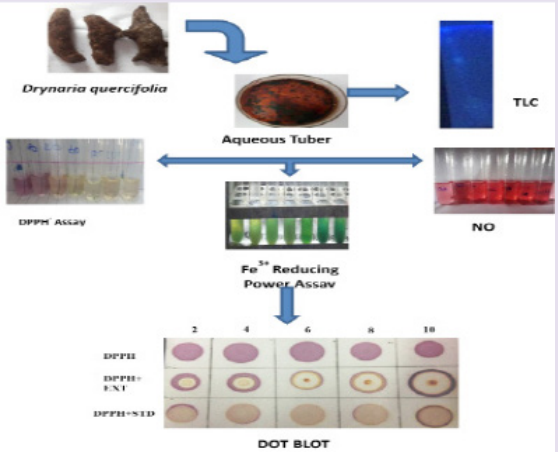
GRAPHICAL ABSTRACT



SUMMARY

- The aqueous extract of tubers of *Drynaria quercifolia* was prepared and analyzed for antioxidant activities such as, DPPH$^{\bullet}$, OH$^{\bullet}$,NO$^{\bullet}$ radical scavenging assays and Fe^{3+} reducing power as well as phosphomolybdenum reduction assay methods. The results revealed that the aqueous extract of *D. quercifolia* showed significant antioxidant activities. The radical scavenging activity was further confirmed by Dot-blot assay method where, the purple colour of DPPH changed to yellow upon reduction.

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