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IN VITRO ANTIOXIDANT ACTIVITIES OF VARIOUS EXTRACTS OF *DRYNARIA QUERCIFOLIA, (L.) J.Smith*

B.PARGAVI[1]* AND T. SIVAKUMAR [2]

1Research and Development Centre, Bharathiar University, Coimbatore-641 046, Tamil Nadu, India
2Department of Microbiology, Kanchi Shri Krishna College of Arts and Science, Kilambi, Kanchipuram-631551, Tamil Nadu, India

*Corresponding author e-mail: pargavi@yahoo.com

ABSTRACT
Drynaria quercifolia, (L.) J.Smith a non-flowering group of plant found to be growing in rain forest of Western Ghats. The rhizome paste is applied for treatment of diarrhea, typhoid, cholera, chronic jaundice, fever,headache and skin diseases. In this present study the bioactive compounds from *Drynaria quercifolia* was extracted using different solvents –hexane, chloroform, ethyl acetate, ethanol and water.The extracts were then examined for antioxidant activity by ABTS methd,DPPH method,superoxide radical scavenging method, hydrogen peroxide scavenging method and hydroxyl scavenging method. The results obtained from various free radicals scavenging systems reveal that various extracts of *D.quercifolia* have significant antioxidant activity.

Keywords: *Drynaria quercifolia*,, rhizome extract, antioxidant activity.

Introduction

The medicinal plants that reside in natural areas have received increasing scientific and commercial attention in recent years. Worldwide,between 50,000 and 80,000 flowering plants are used medicinally(IUCN Species Survival Commission,2007;Marinelli,2005).Of these,at least 15,000 may face extinction due to overharvesting and habitat loss.

Medicinal plants are the most important source of life saving drugs for the majority of world's population.In the United States ,of the top 150 prescription drugs, at least 118 are based on natural sources:74 percent come from plants,18 percent from fungi,5 percent from bacteria,and 3 percent from vertebrate species such as snakes or frogs(Ecology Society of America,1997).

Plant –derived anti- cancer drugs such as taxol,first isolated from the Pacific yew save at least30,000 lives per year in United States (Daily,1997).Drugs to fight life threatening diseases such as diabetes,HIV and Diarrhea are being developed from plants from African countries such as Egypt,Somalia,Libya, and Gambia (Barnett,2006).

Over centuries cultures around the world have learned how to use plants to fight illness and maintain health.In China,medicinal plant use goes back at least 4,000 years,and healers have used more than 5,000 plant species(Tuxhill;1999).More than 80 percent of South Asia's 1.4 billion people have no access to modern health care; they rely instead on traditional medicines using native species.

Several plants are used in folk medicine.Among them ferns are also used in different traditional medicinal systems of India.Ferns play an important role in folklore medicine. A systematic survey of medicinal use of fern has been scarcely undertaken. Chopra and his colleagues (1933) and Kirtikar and his colleagues(1975) worked on 44 and 27 species of ferns respectively and reported on the medicinal uses of these Pteridophytic plants.Medicinal uses of fern species were also described by Nadkarni(1954) and Nayar(1959).May (1978) published a detailed review of various ferns and their medicinal values.The antibacterial activity of some ferns has been studied(Kumar and Kaushik,1999;Parihar and Bohra,2000).Based on the information ,the fern *D.quercifolia* was selected to evaluate its antimicrobial activity.

Drynaria quercifolia,a non-flowering group of plant is being used by the tribals against skin diseases.It is found to be growing in rain forest of Western Ghats of Maharashtra.The rhizome paste is applied for treatment of diarrhea,typhoid,cholera,chronic jaundice, fever,headache and skin diseases.Whole plant is anthelmintic,expectorant and tonic(Viswanathan,1995; and Chopra RN, Nayar SL,Chopra IC,1956). An epiphytic fern *Drynaria quercifolia*, commonly called Oak leak Fern, is used in medicinal system by different groups of people to treat various kinds of health problems including urinary tract infection(Sen & Ghosh,2011). It is also used in the treatment of chest disease, cough, hectic fever, dyspepsia, loss of appetite, chronic jaundice and cutaneous infections (Khare,2007). Tribals in kalakad, Mundanthurai Tiger Reserve India, use this rhizome of this fern to cure rheumatism (Sutha *et al*, 2010).The rhizome of this fern is one of the twelve ingredients of a drug to treat cancer (Saetung *et al*,2005).

Antioxidant property:

Free radicals or ROS (Reactive oxygen species) are highly unstable molecules or molecular fragments that contain one or more unpaired electrons in their outer orbit which contributes to its high reactivity, as the unpaired electrons can receive/donate electrons from/to the neighbouring substance. Sometime excessive free radicals are generated at lower antioxidant levels leading to oxidative stresses causing diabetes, arthritis, cardiovascular, cancer, neurologic and other disorders. Antioxidants are the substances that counteract free radicals and their harmful activity (Sies , 1997)

Natural antioxidant mechanisms can be inefficient, hence dietary intake of antioxidant compounds becomes important(Halliwell,1994).The synthetic antioxidants are sometimes associated with adverse effect which has encouraged many to exploit the biological as they are natural, cost effective and has lesser side effects (Chellaram et al., 2009)Epidemiological studies have indicated the relationship between the plant antioxidants and reduction of chronic diseases(Halliwell ,1991;Lieu ,2003).These benefits are thought to result from the antioxidant components of plant origin, vitamins, flavonoids and carotenoids (Rice-Evans et al.,1997: Pietta, 2000).

Materials & Methods

Description of the Plant:

1)Drynaria quercifolia(L.)J.Smith:

This Plant belongs to Pteridophyta and family Polypodiaceae. It is an epiphytic fern. The rhizome is thick, fleshy covered with small brown coloured hairs. The plant has small sterile fronds and long stalked fertile fronds. The rhizome is used as medicine by the tribals.

Plant Collection and Processing:

1) The Rhizome of *Drynaria quercifolia* was collected from Kollimalai, Namakkal District, Tamilnadu. The plant is identified as Drynaria quercifolia.(J.)Sm using the Herbarium Specimen at Ranipat Herbarium (RPT), St.Joseph College, Trichirapalli.
   The hairs were removed and the rhizome was washed with sterile distilled water. It was cut into small pieces and air-dried in shade. The dried material was powdered using grinder and stored in air tight containers.

Extraction

The shade dried coarse powders of *D. hamiltonii* and *D. quercifolia* (2.5 kg) was extracted with various solvents by increasing order of polarity viz. Hexane, chloroform, ethyl acetate and ethanol by using soxhlet extractor for 72 h. After completion of extraction, each extract was filtered, concentrated to dryness in a rotavapor under reduced pressure and controlled temperature (40-50 °C). The residues were then stored in vacuum dessicator. 500 g of the each plant material was extracted with distilled water by cold maceration process for 72 h to get the aqueous extract. The extractive values and nature of the extracts of *D. hamiltonii and D. quercifolia* were tabulated. The extraction scheme is depicted in Fig.1.

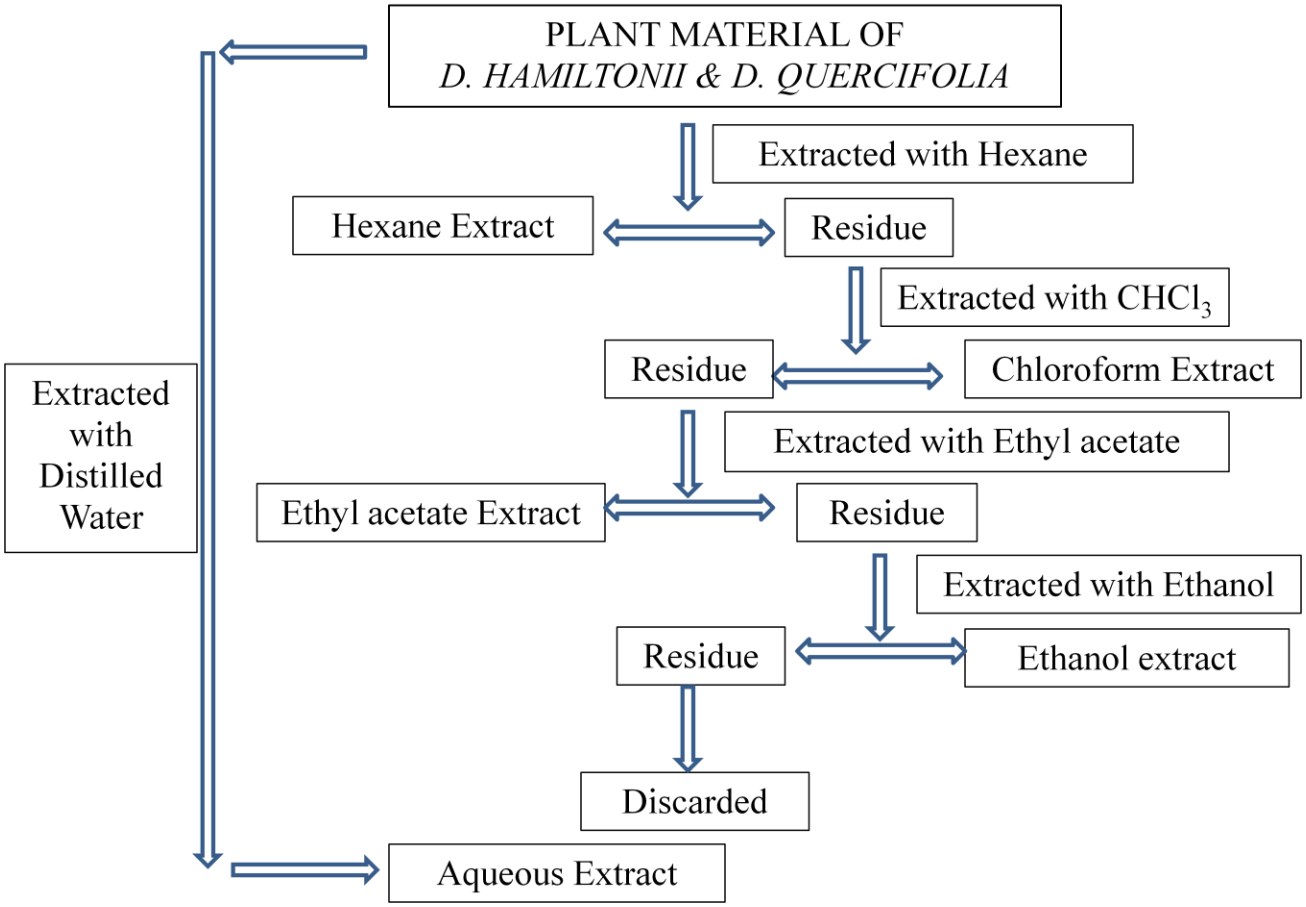

Figure 1 Schematic representation of the extraction process

***In vitro* Antioxidant activity**

The *in vitro* methods are based on inhibition. Samples are added to a free radical-generating system, inhibition of the free radical action is measured and this inhibition is related to antioxidant activity of the sample. Methods vary greatly as to the generated radical, the reproducibility of the generation process, and the endpoint that is used for the determination.

Even though *in vitro* methods provide a useful indication of antioxidant activities, data obtained from *in vitro* methods are difficult to apply to biological systems and do not necessarily

predict a similar *in vivo* antioxidant activity. Important is that all methods developed have strengths and limitations and a single measurement of antioxidant capacity usually is not sufficient. A number of different methods may be necessary to adequately assess *in vitro* antioxidant activity of a specific compound or antioxidant capacity of a biological fluid.

Because of the vast utility of the antioxidants in the treatment of various diseases discussed above and based on the phytochemical constituents present in the various extracts of *D. hamiltonii* and *D. quercifolia,* the antioxidant potentials of the extracts were studied using standard methods. The final concentration of the extracts and standard solutions used were 1000, 500, 250, 125, 62.5, 31.25 and 15.6256 μg/ml. The absorbance was measured spectrophotometrically against the corresponding blank solutions. The percentage inhibition was calculated by using the following formula.

$$\text{Radical scavenging activity (\%)} = \left[\frac{\text{OD control - OD sample}}{\text{OD control}} \right] \text{X } 100$$

IC_{50}, which is the concentration of the sample required to scavenge 50% of free radicals was calculated.

1. Scavenging of ABTS radical cation

ABTS assay is relatively recent one, which involves a more drastic radical, chemically produced and, is often used for screening complex antioxidant mixture such as plant extracts, beverages and biological fluids. The solubility in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of $ABTS^{\bullet+}$ for the estimation of the antioxidant activity (Nenadis,2004) .

Preparation of extract and standard solutions:

Accurately weighed 13.5 mg of each of the extracts and standards, ascorbic acid and rutin and dissolved separately in 2 ml of freshly distilled DMSO. These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions.

Procedure:

ABTS (54.8 mg, 2 mM) was dissolved in 50 ml of distilled water and potassium persulphate (0.3 ml, 17 mM,) was added. The reaction mixture was left to stand at room temperature overnight in dark before usage. To 0.2 ml of various concentrations of the extracts/standards added 1.0 ml of freshly distilled DMSO and 0.16 ml of ABTS solution to make a final volume of 1.36 ml. After 20 min, absorbance was measured spectrophotometrically at 734 nm[(Re et al,1995).

2. DPPH Assay

The DPPH free radical is reduced to a corresponding hydrazine when it reacts with hydrogen donors. The DPPH radical is purple in colour and upon reaction with hydrogen donor's changes to yellow in colour. It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in ethanol or methanol and the decrease in absorbance was measured at 490 nm.

Reagents

2,2-Diphenly 1-picryl hydrazyl solution (DPPH, 100 μM): Accurately weighed 22 mg of DPPH was dissolved in 100 ml of methanol. From this stock solution, 18 ml was diluted to 100 ml with methanol to obtain 100 μM DPPH solution.

Preparation of extract solutions

Accurately weighed 21 mg of the extract was dissolved in 1 ml of freshly distilled DMSO separately to obtain solutions of 21 mg/ml concentration. These solutions were serially diluted separately to obtain the lower concentrations.

Procedure

The assay was carried out in a 96 well microtitre plate. To 200 μl of DPPH solution, 10 μl of each of the extract or standard solution was added separately in wells of the microtitre

plate. The plates were incubated at 37 ^{o}C for 30 min and the absorbance of each solution was measured at 490 nm (Jayaprakasha,2004), using ELISA reader.

3. Scavenging of hydroxyl radical by p-NDA method

Hydroxyl radical is measured by the inhibition of p-NDA bleaching by hydroxyl radical. Hydroxyl radical is generated through Fenton reaction. In this reaction, iron-EDTA complex reacts with hydrogen peroxide in presence of ascorbic acid to generate hydroxyl radical, which can bleach p-NDA specifically. Hydroxyl radical scavenger shows scavenging activity by inhibition of bleaching and percentage of scavenging was measured as absorbance at 440 nm.

Preparation of extract and standard solutions:

Accurately weighed 30 mg of the extracts and standards, ascorbic acid, rutin and BHA and dissolved separately in 5 ml of freshly distilled DMSO. These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions.

Procedure:

To a reaction mixture containing ferric chloride (0.5 ml, 0.1 mM), EDTA (0.5 ml, 0.1 mM), ascorbic acid (0.1 mM, 0.5 ml), hydrogen peroxide (2 mM, 0.5 ml) and p–NDA (0.01 mM, 0.5 ml) in phosphate buffer (pH 7.4, 20 mM), added various concentrations of extract or standard (0.5 ml) to give a final volume of 3 ml. Sample blank was prepared by adding 0.5 ml sample and 2.5 ml of phosphate buffer. Absorbances of these solutions were measured at 440 nm(Elizabeth and Rao,1990).

3. 1. Scavenging of hydroxyl radical by deoxyribose method

The sugar deoxyribose (2–deoxy–D–ribose) is degraded on exposure to hydroxyl radical generated by irradiation or by Fenton systems. The resulting complex mixture of products is heated under acid conditions, malondialdehyde (MDA) is formed and may be detected by its ability to react with thiobarbituric acid (TBA) to form a pink chromogen(Barry et al,1987).

Preparation of extract and standard solutions:

Accurately weighed 16 mg of each extracts and the standard BHA and separately dissolved in 2 ml of freshly distilled DMSO. These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions.

Procedure:

To the reaction mixture containing deoxyribose (0.2 ml, 3 mM), ferric chloride (0.2 ml, 0.1 mM), EDTA (0.2 ml, 0.1 mM), ascorbic acid (0.2 ml, 0.1 mM) and hydrogen peroxide (0.2 ml, 2 mM) in phosphate buffer (pH, 7.4, 20 mM), added 0.2 ml of various concentrations of extract or standard in freshly distilled DMSO to give a total volume of 1.2 ml. The solutions were then incubated for 30 min at 37 °C. After incubation, ice–cold trichloro acetic acid (0.2 ml, 15% w/v) and thiobarbituric acid (0.2 ml, 1% w/v), in 0.25 N HCl were added. The reaction mixture was kept in a boiling water bath for 30 min, cooled and the absorbance was measured at 532 nm(Halliwell and Gutteridge,2007).

4. Scavenging of superoxide radical by alkaline DMSO method

In alkaline DMSO method, superoxide radical is generated by the addition of sodium hydroxide to air saturated dimethyl sulfoxide (DMSO). The generated superoxide remains stable in solution, which reduces nitro blue tetrazolium into formazan dye at room temperature and that can be measured at 560 nm. Superoxide scavenger capable of reacting inhibits the formation of a *red dye formazan*

Preparation of extract and standard solutions:

Accurately weighed 14 mg of each extract and the standard, ascorbic acid rutin and BHA and dissolved separately in 3 ml of freshly distilled DMSO. These solutions were serially diluted with freshly distilled DMSO to obtain the lower dilutions.

Procedure:

To the reaction mixture containing 1 ml of alkaline DMSO (1 ml DMSO containing 5 mM NaOH in 0.1 ml water) and 0.3 ml of the extract in freshly distilled DMSO at various

concentrations, added 0.1 ml of NBT (1 mg/ml) to give a final volume of 1.4 ml. The absorbance was measured at 560 nm (Jayaprakasha,2004).

5. Scavenging of hydrogen peroxide

Hydrogen peroxide is generated *in vivo* by several oxidase enzymes. There is increasing evidence that hydrogen peroxide, either directly or indirectly via its reduction product hydroxyl radical ($OH^{\bullet}$) causes severe damage to biological systems. In this method, when a scavenger is incubated with hydrogen peroxide, the decay or loss of hydrogen peroxide can be measured spectrophotometrically at 230 nm(Jayaprakasha,2004).

Preparation of extract and standard solutions:

Accurately weighed 30 mg of the extracts and standards, ascorbic acid and rutin and dissolved separately in 10 ml of methanol. These solutions were serially diluted with methanol to obtain the lower dilutions.

Procedure:

A solution of hydrogen peroxide (20 mM) was prepared in phosphate buffered saline (PBS, pH 7.4). Various concentrations of 1 ml of the extract or standard in methanol were added to 2 ml of hydrogen peroxide solution in PBS. After 10 min, the absorbance was measured at 230 nm.

Table 1. Colour, Nature and Extractive Values of various Extracts of *Drynaria quercifolia.*

Name of the extract	Parameters observed		
	Colour	**Nature**	**Yield (%)**
Hexane	Dark green	Solid mass	1.8
Chloroform	Greenish yellow	Solid mass	1.3
Ethyl acetate	Yellowish green	Solid mass	2.9
Ethanol	Brownish yellow	Semi- solid mass	10.3
Water	Brown	Dried powder	10.6

Table 2. ABTS and DPPH Radical Scavenging Activities of Various Extracts of *Drynaria quercifolia*

Extracts/ Standards	**IC_{50} (µg/ml)* by method**	
	ABTS	DPPH
Hexane	>1000	>1000
Chloroform	98.2 ± 2.21	131.6± 11.7
Ethyl acetate	42.3 ± 1.26	36.1 ± 2.72
Ethanol	17.12 ± 1.26	22.6 ± 1.89
Water	93.2 ± 2.76	102.3 ± 6.32
Ascorbic Acid	13.21 ± 0.49	5.21 ± 0.45
Quercetin	1.22 ± 0.04	6.21 ± 0.63

*Average of three determinations, three replicates; Data were expressed as Mean ± SEM.

Table 3. Hydroxyl Radical Scavenging Activities of Various Extracts of *Drynaria quercifolia*

Extracts/ Standards	**IC_{50} (µg/ml)* by method**	
	Hydroxyl radical scavenging assay	
	P-NDA method	**Deoxyribose method**
Hexane	>1000	>1000
Chloroform	294.1± 13.4	368.6 ±12.3
Ethyl acetate	206.3 ± 10.3	272.8 ± 15.2
Ethanol	141.6 ± 11.2	216.2 ± 12.13

Water	294.6 ± 16.3	369.2 ± 12.8
Ascorbic Acid	>1000	-
Quercetin	>1000	27.4 ± 1.72

*Average of three determinations, three replicates; Data were expressed as Mean ± SEM.

Table 4. Superoxide and Hydrogen peroxide Radical Scavenging Activities of Various Extracts of *Drynaria quercifolia*

Extracts/ Standards	**IC_{50} (µg/ml)* by method**	
	Superoxide radical scavenging	H_2O_2 radical scavenging
Hexane	>1000	>1000
Chloroform	292.8 ± 13.2	236.7± 11.4
Ethyl acetate	172.8± 11.2	132.6 ± 12.2
Ethanol	154.7± 9.48	107.1 ± 86.2
Water	188.1 ± 10.6	218.6 ± 10.8
Ascorbic Acid	>1000	167.4 ± 3.42
Quercetin	>1000	21.53 ± 1.32

*Average of three determinations, three replicates; Data were expressed as Mean ± SEM.

Result and Discussion

Various solvent extracts of *D.quercifolia* was prepared and the percentage yield was calculated(Table -1).Among the extracts ,maximum yield was obtained by using ethanol and water.

In the last two decades there has been an explosive interest in the role of oxygen free radicals,more generally known as "reactive oxygen species"(ROS) and of reactive nitrogen species'(RNS) in experimental and clinical medicine(Halliwell and Gutteridge ,2007).ROS/RNS are known to play a dual role in biological systems, since they can be either harmful or beneficial to living system(Valko et al, 2004).The antioxidant activity of various extracts of *D.quercifolia* was investigated against various in vitro models.Since free radicals are of different chemical entities,it is also essential to test the extract against many free radicals to prove their antioxidant activity.Hence ,a large number of in vitro methods were used for the screening.IC 50 values obtained were compared with the standards used,that is ,Ascorbic acid and Quercetin.

ABTS radical scavenging activity is relatively recent one,which involves a more drastic radical,chemically produced and is often used for screening complex antioxidants mixtures such as plant extracts,beverages and biological fluids.The ability in both the organic and aqueous media and the stability in a wide pHrange raised the interest in the use of ABTS for the estimation antioxidant activity.In the present study ,all the prepared extracts,except hexane,showed radical scavenging activity against ABTS radical. Ethanol and ethyl acetate showed potent activity against ABTS radical scavenging. The order of activity was as follows : Ethanol > Ethyl acetate > Water > Chloroform > Hexane. The results were displayed in table 2.

The DPPH is a stable free radical which has been accepted as a tool for estimating free radical scavenging activities of antioxidants (Sancehz-Moreno, 2002).Ethanol and ethyl acetate showed potent activity against DPPH radical scavenging method. The order of activity was as follows: Ethanol > Ethyl acetate > Water > Chloroform > Hexane. The results were displayed in table 2. Presence of flavonoids and phenolic compounds in the extracts are possibly involved in their antiradical activity.

Among the oxygen radicals, Hydroxyl radical is the most reactive and induces severe damage to adjacent biomolecules (Sakanaka et al, 2005). In the present study the Hydroxyl radical scavenging activity of various extracts was assessed by p-NDA method and deoxyribose degradation method. In this method the plant extracts showed potent Hydroxyl radical scavenging activity and is compared with standards used (table 3.). The scavenging activity may be due to the presence of various phytochemicals including polyphenols and flavonoids in the extracts.

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species (Halliwell and Gutteridge, 2007). Based on the IC_{50} values, Ethanol and ethyl acetate showed potent activity against Superoxide radical. The order of activity was as follows : Ethanol > Ethyl acetate > Water > Chloroform > Hexane. The results were displayed in table 4. Hydrogen peroxide itself is not very reactive but sometimes is toxic to cell because it may give rise to hydroxyl radical in the cells (Halliwell, 1991). Therefore removing of hydrogen peroxide is very important for antioxidant deffence in cell system. The phenolic and flavonoidal compounds of the extract may probably be involved in scavenging hydrogen peroxide.

In conclusion, the results obtained from various free radicals scavenging systems reveal that various extracts of *D.quercifolia* have significant antioxidant activity. The extracts are found to have different levels of antioxidant activity in all the methods tested. IC50 values obtained were comparable with that of the standards used, that is, ascorbic acid and Quercetin. Since, free radicals are of different chemical entities, it is essential to test the extracts against many free radicals to prove their antioxidant activity.

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