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Full Length Research Paper

Studies on antioxidant and antiradical activities of *Dolichos biflorus* seed extract

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The antioxidant potential of 70% methanolic extract of *Dolichos biflorus* seeds was assessed using different tests including total antioxidant activity, hydroxyl radical scavenging, superoxide radical scavenging, nitric oxide radical scavenging, hypochlorous acid scavenging, reducing power and lipid peroxidation. The total phenolic and flavonoid contents were also evaluated. The extract was found to be an antioxidant with a trolox equivalent antioxidant capacity (TEAC) value 0.28 ± 0.006, the extract has shown its scavenging activity for different radicals and 184.55 ± 7.08, 114.14 ± 6.85, 74.77 ± 1.73, 287.9 ± 8.56 µg/ml were determined as IC_{50} value for hydroxyl, superoxide, nitric oxide and hypochlorous acid respectively. The extract has shown moderate inhibition of lipid peroxidation with IC_{50} 128.63 ± 3.44 µg/ml. It was also observed that the plant extract (100 mg) has 44.67 ± 0.004 mg/ml gallic acid equivalent phenolic and 72.0 ± 0.008 mg/ml quercetin equivalent flavonoid content. The present results demonstrate *D. biflorus* seeds as a potential source of natural antioxidant.

Key words: Antioxidant, *Dolichos biflorus*, flavonoid content, free radical, phenolic content, TEAC.

INTRODUCTION

It has been proposed that free radicals are essential part of aerobic life and modulate various physiological functions (Halliwell, 1991). Normally they are generated at a low level but, sometimes, their excessive generation may disrupt the body's antioxidant system which may lead to "oxidative stress". This situation contributes to a variety of disorders including cancer, coronary heart disease, atherosclerosis, neurodegenerative disorders, diabetes, lung disease and inflammatory diseases (Marnett, 2000; Braca et al., 2002). However, antioxidants can prevent the free radical generation by reacting with them, chelating catalytic metals and also by playing as oxygen scavengers. Although the development of some synthetic antioxidants in the past few years has flourished, they are not yet widely used as therapeutic agents due to their possible toxicity. As a result of which the development of natural antioxidant has now drawn the attention of scientific community and different kinds of plant material have already been reported as natural antioxidant (Thirugnanasampandan et al., 2009; Mmbengwa et al., 2009).

Dolichos biflorus Linn., Syn. *Dolichos uniflorus* (Family - Fabaceae) is a branched, sub-erect and downing herb, native to most parts of India and is found up to altitudes of 1000 m. It is a fast growing annual vine with trifoliate leaves and brown, flat, curved pods filled with seeds (Sastri, 1969). The seeds can be cooked and eaten. In *Ayurveda*, the seed is used in the treatment of piles, pain, constipation, wounds, urinary calculi, cough, edema, asthma etc. The soup prepared from seeds is also beneficial in enlarged liver and spleen. The seeds of *D. biflorus* have been reported to show antilithiatic (Garimella et al., 2001), antihepatotoxic (Laskar et al., 1998) and hypolipidemic (Muthu et al., 2005) activity and involved in lowering the level of blood sugar and total cholesterol (Pant et al., 1968). Two *Ayurvedic* preparations (Pattanaik et al., 2003; Rao et al., 1999), having *D. biflorus* as an ingredient, have shown their antinephrotoxic and free radical scavenging activity. The present study is aimed to evaluate the antioxidant and free

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radical scavenging activity of a 70% methanol extract of *D. biflorus* seeds.

MATERIALS AND METHODS

Chemicals

2,2′-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was obtained from Roche diagnostics, Mannheim, Germany. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Fluka, Buchs, Switzerland. Potassium persulfate ($K_2S_2O_8$), 2-deoxy-2-ribose, mannitol, sodium nitroprusside (SNP) and quercetin were obtained from Sisco Research Laboratories Pvt. Ltd, Mumbai, India. Folin-ciocalteu reagent and butylated hydroxytoluene (BHT) were obtained from Merck, Mumbai, India. Curcumin was obtained from MP Biomedicals, France. catalase was obtained from HiMedia Laboratories Pvt. Ltd, Mumbai, India. Thiobarbituric acid (TBA) was obtained from Loba Chemie, Mumbai, India.

Preparation of plant extract

The seeds of *D. biflorus* were purchased from local market and finely powdered. The powder (100 g) was stirred using a magnetic stirrer with a 7:3 mixture of methanol : water (500 ml) for 15 h, the mixture was then centrifuged at 2850 x g and the supernatant was decanted. The process was repeated again by adding the solvent with the precipitated pellet. The supernatants were collected and concentrated in a rotary evaporator. The concentrated extract was then lyophilized. The residue was stored at -20°C until use.

Total antioxidant activity

An improved $ABTS^{\cdot+}$ radical cation decolorisation assay was used to evaluate the antioxidant capacity of the sample in comparison to trolox standard (Hazra et al., 2008). ABTS solution was mixed with potassium persulfate and kept overnight to generate $ABTS^{\cdot+}$ radical cation. Then 10 µl sample solution was mixed with 1 ml $ABTS^{\cdot+}$ solution and the absorbance was measured at 734 nm. All experiments were repeated 6 times. The Trolox equivalent antioxidant capacity (TEAC) was determined by plotting the percentage inhibition of absorbance as a function of concentration of standard and sample.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging assay was performed by a standard method (Hazra et al., 2008). Hydroxyl radical was generated by the Fenton reaction using a Fe^{3+}-ascorbate-EDTA-H_2O_2 system. The assay quantifies the 2-deoxyribose degradation product, by its condensation with TBA. All tests were carried out 6 times. Mannitol was used as a reference compound. The percent inhibition was calculated by comparing the results of the test and blank solution.

Scavenging of superoxide radical

Measurements of superoxide anion scavenging activities of the sample and standard quercetin were done based on the reduction of NBT according to a previously described method (Hazra et al., 2008). The non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS/NADH) system was used to generate superoxide radicals. These radicals reduce nitro blue tetrazolium (NBT) into a purple colored formazan which was measured spectrophotometrically at 562 nm. All tests were performed 6 times.

Nitric oxide radical scavenging

Nitric oxide generated from sodium nitroprusside (SNP) interacts with oxygen to produce nitrite ions which give rise to a pink chromophore through Griess Illosvoy reaction (Hazra et al., 2008). The chromophore generated was measured spectrophotometrically at 540 nm against blank sample. All tests were performed 6 times. Curcumin was used as a standard.

Hypochlorous acid scavenging assay

According to a previously described method (Hazra et al., 2008), hypochlorous acid (HOCl) was prepared just before the experiment. The scavenging activity of the plant extract was evaluated by measuring the decrease in the absorbance of catalase at 404 nm. Ascorbic acid, a potent HOCl scavenger, was used as standard. All tests were performed 6 times.

Reducing power

The Fe^{3+}-reducing power of the extract was determined by a standard method (Hazra et al., 2008). All tests were performed 6 times. The reducing power of the plant extract is directly proportional to the absorbance of the reaction mixture at 700 nm. BHT was used as a reference compound.

Lipid peroxidation inhibition assay

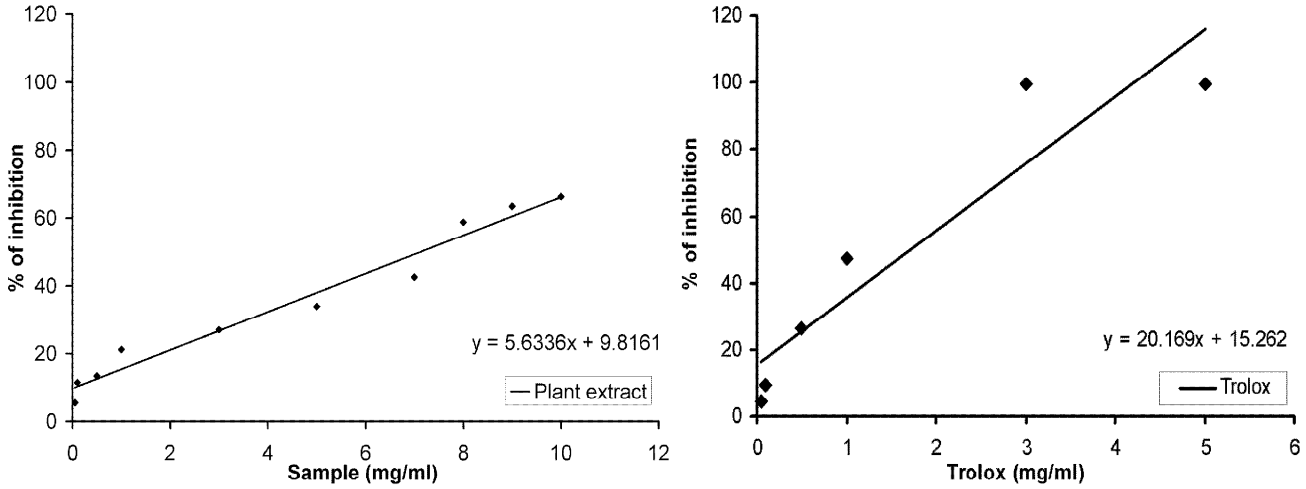
The inhibition of lipid peroxidation was assessed according to the method of Kizil et al. (2008), with slight modification. Brain homogenate was prepared by centrifuging Swiss albino mice brain (20 ± 2 g) with 50 mM phosphate buffer (pH 7.4) and 120 mM KCl, at 3000 rpm for 10 min. A 100 µl aliquot of the supernatant homogenate was mixed with plant extract of various concentrations (2.5 - 25 µg/ml), followed by addition of 0.1 mM $FeSO_4$ and 0.1 mM ascorbic acid, each of 100 µl and incubated for 1 hr at 37°C. 500 µl 28% TCA was used to stop the reaction and then 380 µl 2% TBA was added with heating at 95°C for 30 min, to generate the colour. Then, the samples were cooled on ice, centrifuged at 8000 rpm for 2 min and the absorbance of the supernatant was taken at 532 nm. All tests were repeated 6 times. Trolox was used as the standard.

Assay of total phenolic content

The amount of total phenolics present in the plant seed extract was determined using Folin-Ciocalteu (FC) reagent by a formerly reported method (Hazra et al., 2008). A gallic acid standard curve was used to measure the phenolic content.

Assay of total flavonoid content

The amount of total flavonoids was determined with aluminium chloride ($AlCl_3$) according to a known method (Hazra et al., 2008). The flavonoid content was calculated from quercetin standard curve.



(a) (b)

Figure 1. Total antioxidant activity of plant extract and trolox. Effect of (a) *Dolichos biflorus* extract and (b) reference compound trolox on decolorization of ABTS radical cation. The percentage inhibition was plotted against the concentration of sample. All data are expressed as mean ± S.D. (n=6).

Statistical analysis

All data were reported as the mean ± SD of 6 measurements. The statistical analysis was performed by KyPlot version 2.0 beta 15 (32 bit). The IC_{50} values were calculated by the formula, Y = 100*A1/(X + A1) where A1 = IC_{50}, Y = response (Y = 100% when X = 0), X = inhibitory concentration. The IC_{50} values were compared by paired t test. $p < 0.05$ was considered significant.

RESULTS

Total antioxidant activity

The total antioxidant activity of the extract was calculated based on the decolorization of the $ABTS^{+}$. The results of the sample and standard, expressed as percentage inhibition of absorbance, are shown in Figure 1(a) and (b), respectively. The TEAC value of the extract was found to be 0.28 ± 0.06.

Hydroxyl radical scavenging

Figure 2 shows the abilities of the extract and standard mannitol of scavenging hydroxyl radical. The IC_{50} values (Table 1) of the extract and standard in this assay were 184.55 ± 7.08 and 571.45 ± 20.12 µg/ml, respectively.

Superoxide radical scavenging

As illustrated in Figure 3, the plant extract has comparable ability to quench superoxide radicals to the reference compound quercetin and the IC_{50} values (Table 1) of the plant extract and quercetin were found to be 114.14 ± 6.85 and 60.5 ± 2.09 µg/ml, respectively.

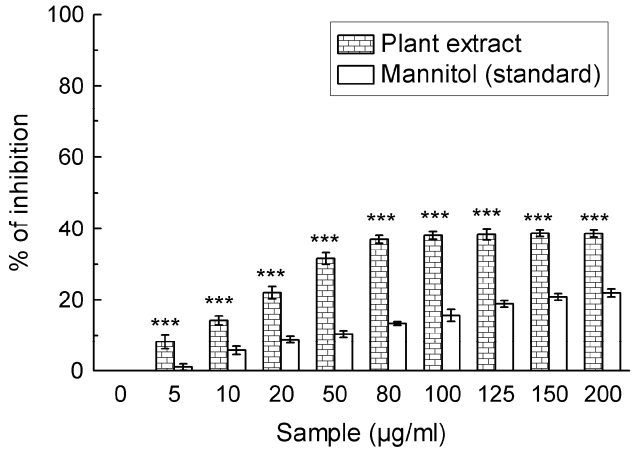


Figure 2. Hydroxyl radical scavenging activities of the *Dolichos biflorus* extract and the reference compound mannitol. The data represent the percentage inhibition of deoxyribose degradation. The results are mean ± S.D. of six parallel measurements. ***p < 0.001 vs 0 µg/ml.

Nitric oxide radical scavenging

D. biflorus extract also scavenges nitric oxide (Figure 4) with the IC_{50} value being 74.77 ± 1.73 µg/ml in comparison to the reference compound curcumin, which shows 90.82 ± 4.75 µg/ml as IC_{50} (Table 1).

Table 1. Radical scavenging and lipid peroxidation inhibition (IC50 values) of *D. biflorus* and reference compounds.

Activity	Extract/Reference	IC_{50} (µg/ml)
Hydroxyl radical ($OH^{\cdot}$) scavenging	*D. biflorus*	184.55 ± 7.08 (6)
	Mannitol	571.45 ± 20.12 (6) ***
Superoxide anion ($O_2^{\cdot-}$) scavenging	*D. biflorus*	114.14 ± 6.85(6)
	Quercetin	60.5 ± 2.09 (6) ***
Nitric oxide radical (NO) scavenging	*Dolichos biflorus*	74.77 ± 1.73 (6)
	Curcumin	90.82 ± 4.75 (6) ***
	Lipoic acid	0.04 ± 1.16 (6) *
Hypochlorous acid (HOCl) scavenging	*D. biflorus*	287.9 ± 8.56 (6)
	Ascorbic acid	235.95 ± 5.75 (6) NS
Lipid peroxidation inhibition	*D. biflorus*	128.63 ± 3.44 (6)
	Trolox	6.76 ± 0.17 (6) ***

Data are expressed as mean ± S.D. Data in parenthesis indicate number of independent assays. EDTA, ethylenediamine tetraacetic acid; NS, non significant; * $p < 0.05$; *** $p < 0.001$ vs *D. biflorus*.

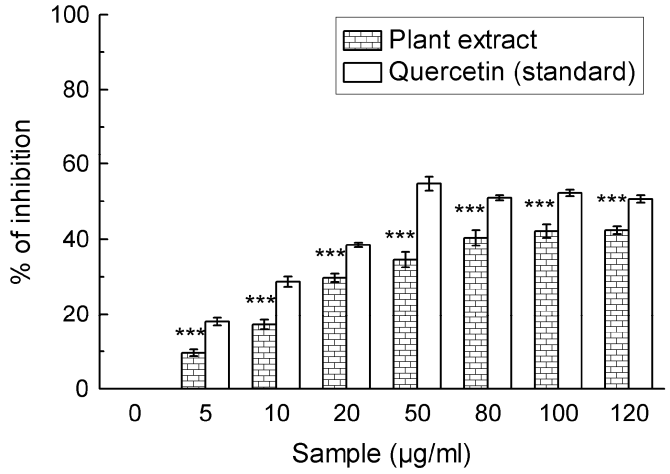


Figure 3. Scavenging effects of *D. biflorus* plant extract and the standard quercetin on superoxide radical. The data represent the percentage of superoxide radical inhibition. All data are expressed as mean ± S.D. (n=6). ***$p < 0.001$ vs 0 µg/ml.

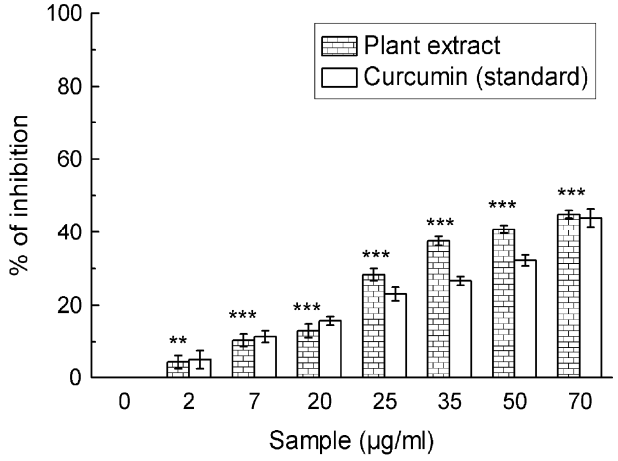


Figure 4. The nitric oxide radical scavenging activities of *Dolichos biflorus* extract and the standard curcumin. The data represent the percentage of nitric oxide inhibition. Each value represents mean ± S.D. (n=6). **$p < 0.01$ and ***$p < 0.001$ vs 0 µg/ml.

Hypochlorous acid scavenging

Figure 5 shows the dose-dependent hypochlorous acid scavenging activity of *D. biflorus* extract compared to that of standard. The calculated IC_{50} of the sample was 287.9 ± 8.56 µg/ml, which was higher than that of the standard compound ascorbic acid (IC_{50} = 235.95 ± 5.75 µg/ml) (Table 1).

Reducing power

As illustrated in Figure 6, Fe^{3+} was reduced to Fe^{2+} in the presence of *D. biflorus* extract and the reference compound BHT. At 0.2 mg/ml, the absorbances of the plant extract and BHT were 0.15 and 0.13, respectively, whereas the absorbances were 0.35 and 0.37, respectively at 0.4 mg/ml. This result indicates that the reductive capability of the extract is almost equivalent to standard BHT.

Lipid peroxidation inhibition

The IC_{50} values (Table 1) of the sample (128.63 ± 3.44 µg/ml) and the standard (6.76 ± 0.17 µg/ml) support the

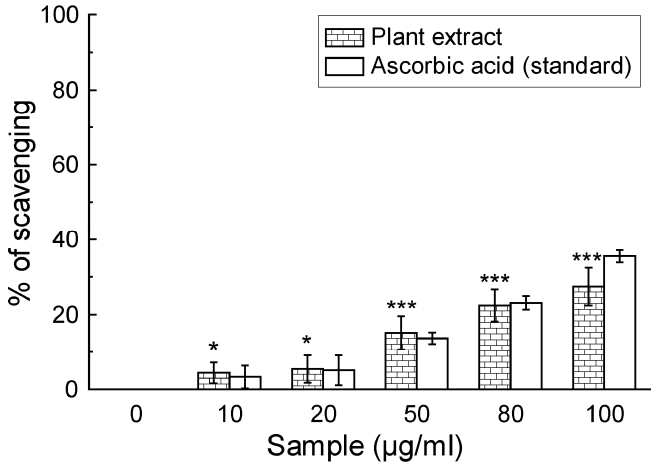


Figure 5. Hypochlorous acid scavenging activities of *Dolichos biflorus* plant extract and the standard ascorbic acid. All data are expressed as mean ± S.D. (n=6). *p < 0.05 and ***p < 0.001 vs 0 µg/ml.

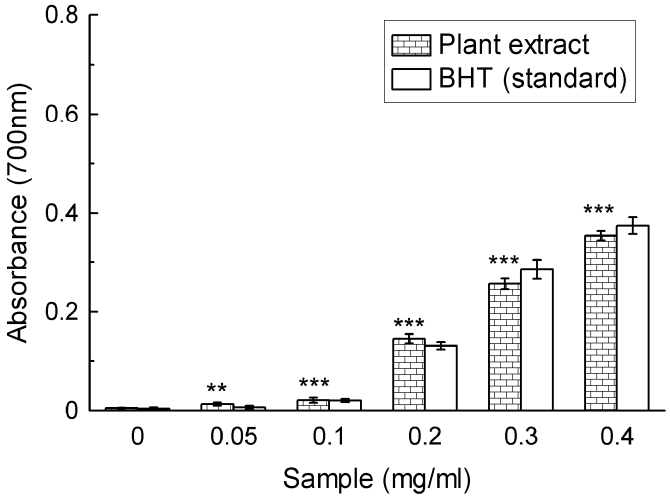


Figure 6. The reductive abilities of *Dolichos biflorus* extract and the standard BHT. The absorbance (A_{700}) was plotted against concentration of sample. Each value represents mean ± S.D. (n=6). **p < 0.01 and *** p < 0.001 vs 0 mg/ml.

fact that the inhibitory efficiency of the sample is poor compared to standard trolox. As shown in Figure 7, the increase in lipid peroxidation inhibition with increasing concentration of the sample reflects the antioxidant property of sample.

Determination of total phenolic and total flavonoid content

The presence of 44.67 ± 0.004 mg/ml gallic acid equivalent phenolic content and 72.0 ± 0.008 mg/ml quercetin equivalent flavonoid content per 100 mg plant extract supports *D. biflorus* as an affluent source of phenolic and flavonoid compounds.

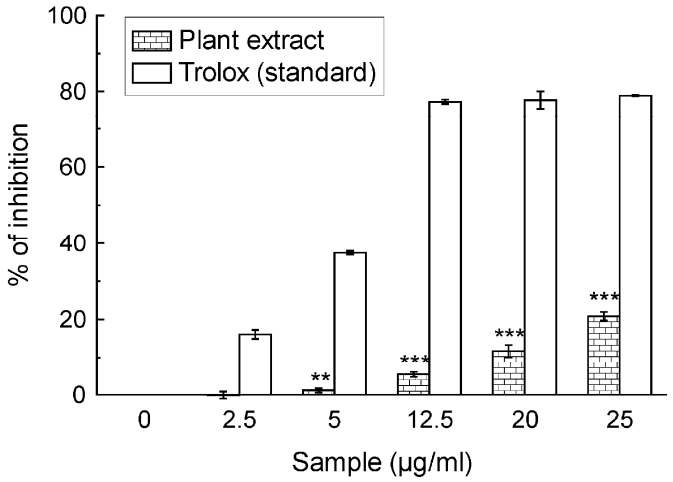


Figure 7. Inhibition of Lipid peroxidation by *Dolichos biflorus* extract and the standard trolox. The data is expressed as the % of lipid peroxidation inhibition of brain homogenate, induced by Fe^{2+}/ascorbic acid. Each value represents mean ± S.D. (n=6). **p < 0.01 and *** p < 0.001 vs 0 µg/ml.

DISCUSSION

The antioxidant potential of *D. biflorus* (100% methanolic extract of whole plant) has also been evaluated in high fat diet (HFD) fed rabbits by Muthu et al. (2006). The study has been carried out to assess the antioxidant effect of *D. biflorus* by measuring the level of antioxidant enzyme (superoxide dismutase and catalase) and reduced glutathione in HFD induced oxidative stress. This study shows promising results. But free radicals also have significant impact on body's antioxidant defense system and they are capable of generating oxidative stress. So, in the present study, the antioxidant potential of *D. biflorus* (70% methanolic extract of seed) was evaluated by examining the different free radical scavenging and total antioxidant activity.

The total antioxidant activity of the extract was calculated based on the decolorization of the $ABTS^{\cdot+}$ produced by reaction of ABTS with potassium persulfate, and measured spectrophotometrically at 734 nm. The addition of the plant extract and trolox convert this preformed radical cation to ABTS and suppress the absorbance in a concentration dependant manner. The obtained TEAC value reflects the antioxidant potency of the extract.

Hydroxyl radical is one of the most detrimental free radicals formed in biological systems and causes

enormous damage on biomolecules of the living cells (Halliwell, 1991). In course of the Fenton reaction, hydroxyl radicals are formed that cause 2-deoxy-2-ribose damage and generate malondialdehyde (MDA) like product. As the *D. biflorus* extract or standard mannitol is added to the reaction mixture the hydroxyl radicals are scavenged and thereby sugar damage can be blocked. The results indicate that the plant extract is a better hydroxyl radical scavenger than standard mannitol.

Superoxide anion is also another harmful reactive oxygen species as it damages cellular components in biological systems. PMS-NADH coupling reaction accelerates the yield of superoxide radicals from dissolved oxygen. These superoxide radicals can be measured by its ability to reduce NBT. The results suggest that the plant extract is a superoxide radical scavenger but efficiency is low compared to standard quercetin.

The chronic emergence of nitric oxide radical is linked with various carcinomas and inflammatory conditions including juvenile diabetes, multiple sclerosis, arthritis and ulcerative colitis (Tylor et al., 1997). The nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract directly competes with oxygen to react with nitric oxide and thereby inhibits nitrite formation. The present study proved that the nitric oxide scavenging activity of the extract is better than the standard curcumin.

At the sites of inflammation, the neutrophil enzyme myeloperoxidase oxidizes Cl^- ions and produces another harmful ROS, hypochlorous acid (Aruoma et al., 1989). The inhibition of catalase deactivation in the presence of plant extract indicates its HOCl scavenging activity. So, from the results, it is anticipated that *D. biflorus* is an efficient HOCl scavenger but not as good as ascorbic acid.

In the process of lipid peroxidation, the iron catalysed generation of ferry-perferryl complex or hydroxyl radicals accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. The highly reactive hydroxyl radical reacts with polyunsaturated fatty acid moieties of cell membrane and yield carbonyl products like malondialdehyde (MDA), which generate a pink chromogen with TBA. With addition of the sample extract or standard, the generation of MDA is reduced, hence indicating the ability of the sample, although less than the standard, to inhibit lipid peroxidation. The results from their reducing abilities also corroborate the interpretation.

Phenolic and flavonoid compounds are very important plant constituents because of their reducing power, scavenging activity and metal chelating property (Rice-Evans et al., 1995; Kessler et al., 2003). The absorption of these compounds rich diet has remarkable effects on human nutrition and health. As depicted earlier, the plant extract has got quite remarkable phenolic and flavonoid content, thus marking it up as a potent antioxidant and free radical scavenger.

Conclusion

In conclusion, the present study provides the evidence that the 70% methanolic extract of *D. biflorus* seeds, which contains high amount of flavonoid and phenolic contents, shows potential antioxidant and free radical scavenging activity. These *in vitro* assays demonstrate that this plant extract is an important source of natural antioxidant, which might be preventive against oxidative stresses. Therefore, further studies should be carried out to isolate active principles having antioxidant property. Currently, the evaluation of *in vivo* antioxidant activity of this extract is in progress.

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