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IN VITRO ANTIOXIDANT AND FREE RADICAL SCAVENGING ACTIVITIES OF VARIOUS SOLVENT EXTRACTS OF *MARSDENIA BRUNONIANA*

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ABSTRACT

In the present study, the antioxidant and free radical scavenging activities of various solvent extracts of leaves of *Marsdenia Brunoniana* was evaluated by different *in vitro* antioxidant assay models. The plant extracts exhibited strong antioxidant and radical scavenging activity on ABTS radical cation, DPPH free radical, hydrogen peroxide, superoxide radical and hydroxyl radical. The leaf extracts showed beneficial activity in total reducing power assay. The antioxidant and free radical scavenging properties of the extracts were comparable to standard antioxidants such as ascorbic acid and rutin. The extracts had esssential phenol and flavonoid contents. The antioxidant and radical scavenging activity of the plant extracts may be due to the rich amount of phenols and flavonoids. Therefore, the plant could be considered as a very good antioxidant source with strong therapeutic efficacy.

Key words: *Marsdenia brunoniana*, Antioxidant, Free radical scavenging activity, Flavonoids, Total phenol content, Total flavonoid content.

INTRODUCTION

A free radical (FR) can be defined as a chemical species possessing an unpaired electron. FR can be positively charged negatively charged or electrically neutral. When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to a number of physiological disorders. Oxidative damage to cellular biomolecules such as lipids, proteins and DNA is thought to play a crucial role in the incidence of several chronic diseases. Flavonoids are a group of polyphenolic compounds found abundantly in the plant kingdom. Interest in the possible health benefits of flavonoids and other polyphenolic compounds has increased in recent years owing to their potent antioxidant and free-radical scavenging activities. The effects of free radicals on human beings are closely related to toxicity, disease and aging. Most living species have an efficient defense system to protect themselves against the oxidative stress induced by Reactive Oxygen Species (ROS). Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense and different human diseases including cancer, atherosclerosis and the aging process. The antioxidants can interfere with the oxidation process by reacting with free radicals, chelating free catalytic metals and also by acting as oxygen scavengers (Habibur *et al.,* 2013).

Antioxidants are chemical substances that donate an electron to the free radical and convert it to a harmless molecule. They may reduce the energy of the free radical or suppress radical formation or break chain propogation or repair damage and reconstitute membranes.

Recent investigations suggest that the plant origin antioxidants with free-radical scavenging properties may have great therapeutic value and

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importance. Antioxidants are vital substances which provide protection to living organisms from damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage and DNA strand breaking (Ghosal *et al.,* 1996; Ozsoy *et al.,* 2008). Several anti-inflammatory, antinecrotic, neuroprotective, chemopreventive and hepatoprotective drugs have recently been shown to have free radical scavenging mechanism as part of their activity (Lin and Huang, 2000; Repetto and Llesuy, 2002). There is an increased interest in natural antioxidants present in medicinal and dietary plants, which might help to prevent oxidative damage (Silva *et al.,* 2005).

Marsdenia brunoniana is a plant of the genus *Marsdenia* belongs to Asclepiadaceae family. It is a rare medicinal twining shrub found in Tamil Nadu and Karnataka states of Peninsular India (Natarajan, 2004). It has long been used by tribes and native medical practitioners to treat various chronic disorders including diabetes. Literature review revealed that no phytochemical and pharmacological studies have been carried out in this plant. Based on these details, the present study is aimed to evaluate the antioxidant and free radical scavenging potential of various solvent extracts of leaves of *Marsdenia brunoniana* using various *in vitro* assay models.

MATERIALS AND METHODS

Chemicals

2,2'-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Sigma Aldrich Co., St. Louis, USA. Rutin and p–nitroso dimethyl aniline (*p*–NDA) were obtained from Acros Organics, New Jersy, USA. Ascorbic acid and nitro blue tetrazolium (NBT) were obtained from S.D. Fine Chem, Ltd., Biosar, India. 2- Deoxy–D-ribose was from Hi–Media Laboratories Ltd., Mumbai. All other chemical used were of analytical grade.

Collection and Extraction

The leaves of *M. brunoniana* was collected from Sirumalai Hills, near Dindugal, Tamilnadu in the month of January 2012 and the authenticity of the plant was confirmed from Botanical survey of India, Coimbatore. The shade dried coarse powder of *M.brunoniana* (1.5 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. The residue obtained after extraction with ethanol was extracted with water by cold maceration process for 72 h. The extractive values and nature of the extracts of *M. brunoniana* were tabulated.

Preliminary phytochemical screening

Prepared plant extracts of *M. brunoniana* were analyzed for the presence of various phytochemical constituents employing standard procedures (Wagner *et al.,* 1984). Conventional protocol for detecting the presence of steroids, alkaloids, tannins, flavonoids, glycosides, etc., was used.

Preparation of test and standard solutions

The plant extracts of *M. brunoniana* and the standard antioxidants (ascorbic acid and rutin) were dissolved in distilled dimethyl sulfoxide (DMSO separately and used for the *in vitro* antioxidant assays except the hydrogen peroxide method because it interferes with the method. For hydrogen peroxide method, the extracts and the standards were dissolved in distilled methanol and used. The stock solutions were serially diluted with the respective solvents to obtain the lower concentrations.

In vitro antioxidant activity

Various extracts of *M. brunoniana* was tested for their *in vitro* antioxidant activity using the standard methods. In all these methods, a particular concentration of the extracts or standard solution was used which gave a final concentration of 1000-15.625 µg/ml after all the reagents were added. Absorbance was measured against a blank solution containing the extract or standards, but without the reagents. A control test was performed without the extract or standards. Percentage scavenging and IC_{50} values were calculated.

ABTS radical scavenging activity

In a final volume of 1 ml, the reaction mixture comprised 950 µl of ABTS* solution and 50 µl of the plant extracts at various concentrations. The reaction mixture was homogenized and incubated for 20 min. Absorbances of these solutions were measured spectrophotometrically at 734 nm (Re *et al.,* 1999).

DPPH radical scavenging activity

The DPPH assay method depends on the reduction of purple DPPH to a yellow colored diphenyl picrylhydrazine and the remaining DPPH which showed maximum absorption at 517 nm was measured. About 2 ml of various concentrations of the plant extracts or standards were added to 2 ml of DPPH solution (0.1 mM, 2 ml). After 20 min of incubation at 37 °C in the dark, the absorbance was recorded at 517 nm (Shirwaikar *et al.,* 2006).

Superoxide radical scavenging activity by alkaline DMSO method

In this method, superoxide radical is generated by the addition of sodium hydroxide to air saturated dimethyl sulfoxide. The generated superoxide remains stable in solution, which reduces nitroblue tetrazolium (NBT) into formazan dye at room temperature and that can be measured at 560 nm. Briefly, 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 extracts 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 (Elizabeth and Rao, 1990).

Hydrogen peroxide radical scavenging method

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. To 1 ml of various concentrations of extracts or standard in methanol was added to 2 ml of hydrogen peroxide (20 mM) in phosphate buffer saline. After 10 min the absorbance was measured at 230 nm (Jayaprakasha *et al.,* 2004).

Hydroxyl radical scavenging activity by *p*-NDA method

Various concentration of the extracts or standards in 0.5 ml of distilled DMSO were added to a solution mixture containing 0.5 ml of ferric chloride (0.1 mM), 0.5 ml of EDTA (0.1 mM), 0.5 ml of ascorbic acid (0.1 mM), 0.5 ml of hydrogen peroxide (2 mM) and 0.5 ml of *p*-NDA (0.01 mM) in phosphate buffer (pH 7.4, 20 mM) to produce a final volume of 3 ml. Absorbance was measured spectrophotometrically at 440 nm
qualitative analysis report data is displayed in Table 2.

ABTS and DPPH Radical Scavenging Assay

ABTS and DPPH radical scavenging activity of various solvent extracts of *M. brunoniana* are shown in Table 3. The extracts showed potent radical scavenging activity in concentration dependent manner. The various extracts of the leaf of *M. brunoniana* exhibited good radical scavenging activity against the tested models. Among the extracts tested ethanol extract showed potent activity when compared to other extracts. The results obtained were comparable with the standards used.

Superoxide and Hydrogen peroxide radical Scavenging Activity

Superoxide radical scavenging activity of leaf extracts of *M. brunoniana* were assessed by alkaline DMSO method. The plant extracts moderately inhibit the superoxide radical generation. In hydrogen peroxide (Jayaprakasha *et al.,* 2004).

Total reducing power assay

To 1 ml of the plant extracts were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1 % potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 min. After incubation period, 2.5 ml of 10% trichloroacetic acid was added and the reaction mixture was centrifuged at 1000 rpm for 10 min. The upper 2.5 ml layer was mixed with 2.5 ml of deionized water and 0.5 ml of ferric chloride and thoroughly mixed. The absorbance was measured spectrophotometrically at 700 nm. A higher absorbance indicates a higher reducing power (Oyaizu, 1986).

RESULTS

Preliminary Qualitative Analysis

Colour, nature and extractive yields of prepared extracts of *M. brunoniana* are presented in Table 1. Ethanol and water extracts showed highest extractive yield when compared to other solvents. Ethyl acetate gives moderate extractive yield. Preliminary qualitative analyses of the leaf extracts of *M. brunoniana* were undergone for the detection of various phytochemical constituents. The plant extract showed the presence of a variety of phytochemicals. Hexane extracts showed the presence of phytosterols whereas chloroform extract contains phytosterols and alkaloids. Ethyl acetate extract showed the presence of phenolic and flavonoid compounds. Ethanol extract showed the presence of majority of the phytochemicals present in the plant. Aqueous extract contains more polar compounds like carbohydrates, proteins, tannins and glycosides. Terpenoids, saponins and gums and mucilages were found to be absent in all the prepared extracts. The preliminary radical scavenging assay, the extracts were found to be equipotent with ascorbic acid but less potent when compared to rutin. The values were tabulated in Table 4.

Hydroxyl radical Scavenging Assay

Hydroxyl radical scavenging activity of leaf extracts of *M. brunoniana* was measured by p-NDA method. In this method, the ethanol and aqueous extracts showed potent activity when compared to standards used. The IC_{50} values were presented in Table 5.

Total Reducing Power Assay

In this assay model, an increase in absorbance was observed when the concentration of extracts increased. This indicates the antioxidant potential of the prepared plant extracts. Among the prepared extracts, ethanol extract showed potent antioxidant activity and the results obtained were comparable with the standards used. The results obtained were displayed in Fig.1.

Table 1. Color, nature and extractive yields of various extracts of *Marsdenia brunoniana*

Name of the Extract	Colour	Nature	Yield (%)
Hexane	Dark green	Sticky semisolid	2.6
Chloroform	Dark greenish yellow	Solid	1.5
Ethyl acetate	Greenish yellow	Solid	6.3
Ethanol	Yellowish brown	Solid	8.6
water	Brown	Solid	10.4

Table 2. Preliminary Phytochemical Studies of various extracts of *Marsdenia brunoniana*

Phytochemical constituents	Name of the Extract				
	Hexane	Chloroform	Ethyl acetate	Ethanol	Water
Carbohydrates	+	+	+	+	+
Phytosterols	+	-	-	+	-
Alkaloids	-	+	-	-	-
Glycosides	-	-	-	+	+
Terpenoids	-	-	-	-	-
Proteins & aminoacids	-	-	-	+	+
Saponins	-	-	-	-	-
Tannins	-	-	-	+	+
Phenolic compounds	-	-	+	+	+
Flavonoids	-	-	+	+	+
Fixed oils & Fats	+	-	-	-	-
Gums & Mucilages	-	-	-	-	-

(+) Presence (-) Absence

Table 3. Effect of the leaf extracts of *Marsdenia brunoniana* on ABTS and DPPH Method

Extracts/ Standards	IC_{50} (µg/ml)* by method	
	ABTS	DPPH
Hexane	>1000	>1000
Chloroform	101.67 ± 10.01	102.59 ± 9.96
Ethyl acetate	52.35 ± 2.20	28.78 ± 5.08
Ethanol	60.33 ± 5.88	30.61 ± 4.15
Water	79.03 ± 3.94	60.99 ± 4.37
Ascorbic Acid	13.63 ± 1.17	7.05 ± 0.76
Rutin	6.61 ± 0.64	8.11 ± 0.42

*Average of three determinations; Data are expressed as mean ± SEM

Table 4. Effect of Various Extracts of *Marsdenia brunoniana* on Superoxide Radical Scavenging and Hydrogen peroxide Scavenging Methods

Extracts/ Standards	IC_{50} (µg/ml)* by method	
	Superoxide radical scavenging	H_2O_2 radical scavenging
Hexane	>1000	>1000
Chloroform	216.7± 17.53	227.4 ±13.27
Ethyl acetate	134.17 ± 5.43	117.83 ± 11.58
Ethanol	91.88± 4.42	97.46 ±3.91
Water	107.74 ± 10.12	123.8 ± 5.95
Ascorbic Acid	187.53 ± 11.93	75.16 ± 3.78
Rutin	55.97 ± 2.21	32.56 ± 1.05

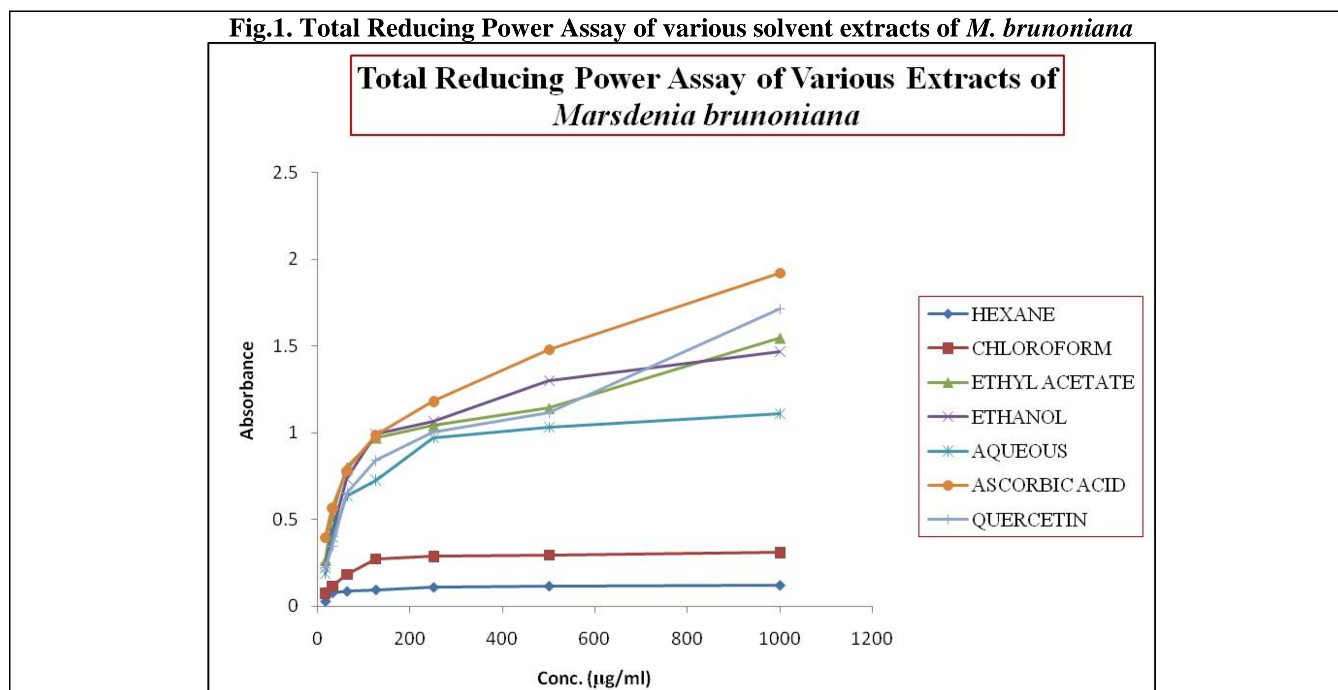
*Average of three determinations; Data are expressed as mean ± SEM

Table 5. Effect of leaf *extracts of Marsdenia brunoniana* on Hydroxyl Radical Scavenging Assay

Extracts/ Standards	IC_{50} (µg/ml)*
	Hydroxyl Radical Scavenging (p-NDA method)
Hexane	>1000
Chloroform	211.07±6.67
Ethyl acetate	128.8 ±4.16
Ethanol	108.6 ± 3.7
Water	115.57 ± 2.31
Ascorbic Acid	182.5 ± 4.88
Rutin	44.1 ±4.4

*Average of three determinations; Data are expressed as mean ± SEM

Fig.1. Total Reducing Power Assay of various solvent extracts of *M. brunoniana*



DISCUSSION

The biochemistry of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radicals, and singlet oxygen is important in aerobic metabolism of the cell mostly reactive nitrogen species are well recognized for playing dual function as both dangerous and beneficial species. Overproduction of ROS from mitochondrial electron transport chain leakage or excessive stimulation of xanthine oxidase and other oxidative enzymes results in oxidative stress, a process that can be an important mediator of damage to cell structure and function, lipids, proteins, carbohydrates and DNA(Eboh Abraham., 2014).

In contrast, beneficial effects of ROS/RNS occur at very low concentrations and involve physiological roles in cellular responses in defense against infectious agents, gene expression, cellular growth, in the function of a number of cellular signaling pathways, hypoxia and respiratory burst. In the past and present years, progress has been made in the recognition and understanding of the roles of reactive oxygen species in many diseases. The body protects itself from the potential damages of reactive oxygen species, by utilizing antioxidant enzymes and non-antioxidant enzymes e.g. superoxide dismutases, glutathione peroxidases, glutathione reductase and catalase (Eboh Abraham., 2014).

Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant phytoconstituents of the plant materials act as radical scavengers and helps in converting the reactive free radicals to less reactive species. Natural antioxidants occur in all parts of plants. These antioxidants include carotenoids, vitamins, phenols, flavonoids, dietary glutathione, and endogenous metabolites. Plant-derived antioxidants have been shown to function as singlet and triplet oxygen quenchers, free radical scavengers,

peroxide decomposers, enzyme inhibitors, and synergists. The most current research on antioxidant action focuses on phenolic compounds such as flavonoids. Fruits and vegetables contain different antioxidant compounds, such as vitamin C, vitamin E and carotenoids, whose activities have been established in recent years. Flavonoids, tannins and other phenolic constituents.

The antioxidant assays used in this study measured the oxidative products at the early and final stages of oxidation. The antioxidant and free radical scavenging activity of the leaf extracts of *M. brunoniana* was investigated against various *in vitro* models. Since, free radicals are of different chemical entities, it is essential to test the extracts 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 rutin.

ABTS radical scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts, beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of ABTS • + for the estimation of antioxidant activity (Nenadis *et al.,* 2004). The extracts showed potent antioxidant activity in ABTS method which is comparable to the standard used. Here, the extracts radical scavenging activity showed a direct role of its phenolic compounds in free radical scavenging.

The electron donation ability of natural products can be measured by 2, 2 -diphenyl-1- picrylhydrazyl radical (DPPH) purple-coloured solution bleaching. The method is based on scavenging of DPPH through the addition of a radical species or antioxidant that decolourizes the DPPH solution. The degree of colour change is proportional to the concentration and potency of the antioxidants. A large decrease in the absorbance of the reaction mixture indicates significant free radical scavenging activity of the compound under test (Pratap Chandran *et al.,* 2013).

The experimental data of the extracts revealed that the extracts are likely to have the effects of scavenging free radicals. From the result we observe that a dose dependent relationship in the DPPH radical scavenging activity. The involvement of free radicals, especially their increased production, appears to be a feature of most of the human diseases including cardiovascular diseases and cancer (Deighton *et al.,* 2000). It has been found that cysteine, glutathione, ascorbic acid, tocopherols, flavonoids, tannins and aromatic amines reduce and decolorize the DPPH by their hydrogen donating ability. Flavonoids and phenolic compounds of leaf extracts of *M. brunoniana* are possibly involved in its radical scavenging activity.

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive species (Halliwell and Gutteridge, 2007). The superoxide radical is known to be produced *in vivo* and can result in the formation of hydrogen peroxide via dismutation reaction. Moreover, the conversion of superoxide and hydrogen peroxide into more reactive species. The extracts are found to be an efficient scavenger of superoxide radical generated in alkaline DMSO system. The result clearly indicates that the plant extracts have a noticeable effect as scavenging superoxide radical.

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 defense in cell system. Polyphenols have also been shown to protect mammalian cells from damage induced by hydrogen peroxide, especially compounds with the orthohydroxy phenolic compounds like quercetin, gallic acid, caffeic acid and catechin (Nakayama, 1994). Therefore, the phenolic compounds of the leaf extracts of *Marsdenia brunoniana* may probably be involved in scavenging hydrogen peroxide.

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 leaf and bark extracts of *M. brunoniana* was assessed by the inhibition of p-NDA bleaching method and deoxyribose degradation method. In p-NDA method, the 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. The extracts show potent scavenging activity by inhibition of bleaching of p-NDA. The scavenging activity may be due to the presence of various phytochemicals including polyphenols and flavonoids in the extracts.

In the measurement of the reducing ability, it has been investigated from the Fe^{3+} - Fe^{2+} transformation. Fe^{3+} reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action and can be strongly correlated with other antioxidant properties (Dorman *et al.,* 2003). The reducing properties of the plant extracts are generally associated with the presence of reductones, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom (Gordon, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. The data obtained in the present study suggest that it is likely to contribute significantly towards the observed antioxidant effects. However, the antioxidant activity has been attributed by various mechanisms, like

prevention of chain initiation, binding of transition metal ion catalysts, prevention of continued hydrogen abstraction, reductive capacity, radical scavenging activity and decomposition of peroxides. Like the antioxidant activity, the reducing power of the extracts increases with increasing concentration.

The systemic literature collection, pertaining to this investigation indicates that the plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavengers. Therefore, it is necessary to determine the total amount of phenols and flavonoids in the plant extracts chosen for the study. Flavonoids are the most diverse and widespread group of natural compounds and are proposed to be the most important natural phenolics. These compounds possess a broad spectrum of chemical and biological activities including radical scavenging activity. The extracts contains considerable amount of total flavonoids and phenols. Previous literatures showed that high phenol and flavonoid content increases the antioxidant activity (Holasova *et al.,* 2002) and there is a linear relation between the phenol and flavonoid contents and antioxidant activity (Gheldof and Engeseth, 2002).

Phenolic compounds are commonly found in both edible and medicinal plants and they have been reported to have various biological effects including antioxidant activity. The antioxidant activities of phenolic compounds is mainly due to their redox properties, which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Osawa, 1994). The leaf extracts of *Marsdenia brunoniana* showed strong antioxidant activity in various *in vitro* systems tested. The antioxidant effect of *M. brunoniana* is may be due to the phenolic compounds present in it. To our knowledge this is the first report on the antioxidant and radical scavenging potential of *Marsdenia brunoniana.*

CONCLUSION

The results from various free radicals scavenging systems reveal that various solvent leaf extracts of *Marsdenia brunoniana* have significant antioxidant activity. The extracts are found to have different levels of antioxidant activity in all the methods tested. IC_{50} values obtained were comparable with that of the standards used, that is, ascorbic acid and rutin. Since, free radicals are of different chemical entities, it is essential to test the extracts against many free radicals to prove their antioxidant activity. Hence, a large number of *in vitro* methods were used for the screening. However, the difference in the activity in extracts may be due to the different chemical entities of the free radicals and the diverse chemical nature of the extracts. According to this study, a significant and linear relationship was found between the antioxidant activity, total phenol and flavonoid contents, indicating that these compounds could be major contributors to antioxidant activity. Further studies are in progress for the isolation and identification of phytochemical compounds and to ensure the most important medicinal properties of the plant *in vivo* in our laboratory to correlate with its antioxidant activity.

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CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

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