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Total phenolic and flavonoid contents and *in vitro* antioxidant potential of methanolic extract of *Blepharis maderaspatensis* (L.) B. Heyne ex Roth. whole plant

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Abstract

Blepharis maderaspatensis (L.) B. Heyne ex Roth is a prostrate herb, distributed Tropical Africa to India in dry deciduous forests and also in the plains. Traditionally, the plant is used against bone fracture, wounds and cuts, boils, headache, scorpion bite and nervous disorders. This study was carried out to evaluate the chemical constituents and antioxidant potential of the *B. maderaspatensis* whole plant in order to scientifically validate its traditional claim. Phytochemical screening showed the presence of terpenoids, steroids, flavonoids, coumarins, glycosides and alkaloids. The total phenolic and flavonoid contents were measured using, the Folin-Ciocalteu reagent and aluminium chloride methods and are found to be 23.12 mg GAE/g and 9.87 mg QE/g, respectively. The antioxidant potential of methanolic extract was determined by DPPH radical scavenging, anti-lipid peroxidation, ABTS radical scavenging and NO radical scavenging assays. The total antioxidant capacity was evaluated by the phosphomolybdenum method. The extract showed potent DPPH, peroxide, ABTS and NO radicals scavenging activity with IC_{50} values 395.38, 142.53, 395.26 and 308.79 µg/ml, respectively, and the total antioxidant capacity found to be 20.78 mg AAE/g. The results obtained in the present study, point out that the methanolic extract of *B. maderaspatensis* is a potential source of natural free radical scavengers.

Key words : *Blepharis maderaspatensis* (L.) B. Heyne ex Roth, traditional use, phytochemical screening, free radicals, antioxidant potential

1. Introduction

Free radicals such as reactive oxygen (ROS) and nitrogen (RNS) species including peroxides, super-oxides, hydroxyl radicals and nitrous oxide are produced by the mitochondria, during normal oxidative phosphorylation (involves the tricarboxylic acid cycle and electron transport chain), as well as by numerous biochemical pathways, found both intra and extra cellularly. They have usually been associated with cellular damage, disease progression and ageing. There are multiple mechanisms in our body through which oxidative stresses are counteract by producing antioxidants through foods or other supplements either by naturally produced *in situ* or externally supplied (Pharm-Huy *et al.*, 2008). Antioxidant containing compounds are abundantly available in natural herbs that protect the cells, against the free radicals damage. The mechanism of antioxidant activity of plants leads call up to discover the phytoconstituents that are responsible *vis-a-vis* subsequent isolation of ascorbic acid from medicinal plants (Szent-Giorgvi, 1963). Since then, the antioxidant potential of plants has received a great deal of attention (Kasote *et al.*, 2015).

Blepharis maderaspatensis (L.) B. Heyne ex Roth. (Fam. Acanthaceae, Kew Record-2677397) is a prostrate herb, distributed Tropical Africa to India in dry deciduous forests and also in the plains. Hoorali, Kani and Paliyar tribals of southern Western Ghats region of Kerala and Tamil Nadu use this plant against bone fracture, wounds and cuts, boils, headache, scorpion bite and nervous disorders (Manjunatha *et al.*, 2004; Ignacimuthu *et al.*, 2006; Pullaiah, 2006; Ayyanar *et al.*, 2008; Kottaimuthu, 2008; Ayyanar and Ignacimuthu, 2009; Samuel and Andrews, 2010; Sandhya *et al.*, 2010; Subitha *et al.*, 2011; Pandikumar *et al.*, 2011; Suresh *et al.*, 2011; Subramanian *et al.*, 2011; Rani *et al.*, 2011; Arunachalam and Perimelazhagan, 2011; Bahekar *et al.*, 2012; Dhatchanamoorthy *et al.*, 2013). These informations prompted to the scientific validation of this plant for their future therapeutic applications.

2. Materials and Methods

2.1 Chemicals, glasswares and instruments

The chemicals (analytical grade) were purchased from Merck Life Science Private Limited, India; Sigma-Aldrich, India; HiMedia Laboratories, India; S D Fine-Chem Limited, India; Finar Limited, India and Nice Chemicals (P.) Ltd., India. Glasswares were purchased from Duran Group, Germany; Riviera Glass Private Limited, India and Borosil®, India.

Instruments used in this study are pulveriser (Lakshmi Machine Works Limited, India); heating mantle, water bath, hot air oven (Beston Industries, India); rotary vacuum evaporator (IKA® RV10, Germany), weighing balance (Shimadzu Corporation, Japan), UV-Vis spectrophotometer (Agilent Technologies, U.S.A.), homogenizer, centrifuge (Remi Lab World, India), pH meter (Elico Ltd., India), and deep freezer (Haier, China).

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2.2 Collection and preparation of plant material

The whole plant of *B. maderaspatensis* (BM) was collected from Aruvikkara Gramapanchayat (8.5678° N, 77.0189° E), a Western Ghats region of Thiruvananthapuram District of Kerala State, India. The plant material was authenticated by plant taxonomist of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, India and a voucher specimen (TBGT 57063, dated 13/02/2012) was deposited in the herbarium repository of the Institute.

The fresh plant material of *B. maderaspatensis* (BM) collected was thoroughly washed in tap water, shade dried, powdered and was stored in air tight container. The powdered plant material (100 g) was extracted with methanol (BM-M), using a Soxhlet apparatus for 24 h. The extract was filtered and concentrated under reduced pressure in a rotary vacuum evaporator at 40-45^{o}C. The dry extract was stored at 0-4°C until further use. The percentage yield obtained of the extract was 7.13%, expressed in terms of air dried weight of the plant material.

2.3 Qualitative phytochemical analysis

The phytochemical analysis of the plant extract was carried out with standard protocols described by Harborne (1998) and Trease and Evans (2002).

2.4 Quantitative phytochemical analysis

2.4.1 *Estimation of total phenols*

Total phenolic content (TPC) of BM-M extract was determined according to method described by Lachman *et al*. (2000). 0.5 ml of each extract, 2.5 ml Folin-Ciocalteu reagent, 2 ml of 7.5% (w/v) sodium carbonate (Na_2CO_3) were mixed. The mixture was incubated at room temperature for 30 min. The absorbance was read, using UV-Vis spectrophotometer at 743 nm. Each analysis was performed in triplicates and the values were expressed in Mean ± SD. The results were expressed as mg GAE (gallic acid equivalents)/g dry extract.

2.4.2 *Estimation of total flavonoids*

The total flavonoids of BM-M extract was estimated by colorimetric method using aluminium chloride reagent (Chang *et al.*, 2002). The plant extract (0.5 ml) was mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride hexahydrate ($AlCl_3.6H_2O$), 0.1 ml of 1 M potassium acetate (CH_3CO_2K), and 2.8 ml of distilled water. After incubation at room temperature for 30 min., the absorbance of the solution was measured at 415 nm. Each analysis was performed in triplicates and the values were expressed in Mean ± S.D. The results were expressed as mg QE (quercetin equivalents)/g dry extract.

2.5 *In vitro* antioxidant activity

2.5.1 *Total antioxidant capacity*

The total antioxidant capacity (TAC) of BM-M extract was determined by phosphomolybdate assay, using ascorbic acid as the standard (Umamaheswari and Chatterjee, 2008). An aliquot of 0.1 ml of sample solution was mixed with 1 ml of reagent solution, containing 0.6 M sulphuric acid (H_2SO_4), 28 mM sodium phosphate (NaH_2PO_4) and 4 mM ammonium molybdate [$(NH_4)_2MoO_4$]. The tubes were capped and incubated in water bath at 95^{0}C for 90 min. After the samples were cooled to room temperature, the absorbance of the mixture was measured at 765 nm against a blank containing 1 ml of reagent solution and appropriate volume of the solvent. The antioxidant activity was expressed as ascorbic acid equivalent (mg AAE/g extract) which served as a positive control. The antioxidant capacity was estimated using the following formula:

$$\% \text{ of antioxidant capacity} = [(A_0 - A_1) / A_0] \times 100$$

where, A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of sample.

2.5.2 DPPH *radical scavenging activity*

The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of BM-M extract was determined by the method described by Gulcin (2010). 1 ml of methanolic extract at different concentrations was mixed with 3 ml of 0.1 mM solution of DPPH in methanol. After incubation at room temperature for 30 min. in the dark condition, the absorbance of the mixture was read at 517 nm. The control was prepared as above without sample. Ascorbic acid was used as an antioxidant standard. The percentage of free radical scavenging activity from the sample was calculated according to the following formula:

$$\% \text{ of DPPH radical scavenging} = [(A_0 - A_1) / A_0] \times 100$$

where, A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of sample.

2.5.3 *Antilipid peroxidation activity*

The antilipid peroxidation of BM-M extract was studied *in vitro* by the method described by Kimuya *et al*. (1981). 0.5 gm of rat liver tissue was homogenised with 10 ml of 150 mM KCl-Tris-HCl buffer (pH 7.2). 0.25 ml of liver homogenate was incubated in Tris-HCl buffer (pH 7.2) with 0.1mM ascorbic acid (AA), 4 mM ferrous chloride ($FeCl_2$) and 0.05 ml of various concentrations of BM-M extract at 37^{0}C for 1 h in capped tubes. Further, 0.1 N HCl, 0.2 ml of 9.8 % sodium dodecyl sulphate (SDS), 0.9 ml of distilled water and 2 ml of 0.6 % thiobarbituric acid (TBA) were added to each tube and shaken vigorously. The tubes were placed in a boiling water bath at 100^{0}C for 30 min and cooled. The flocculent precipitate was removed by adding 5 ml of n-butanol and centrifuged at 3000 rpm for 20 min. The absorbance of the supernatant was measured at 532 nm.

2.5.4 ABTS *radical scavenging activity*

The ABTS [2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid)] radical scavenging activity of BM-M extract was performed by the method described by Re *et al*. (1999). The ABTS radical was generated through the reaction of the ABTS stock solution 7 mM with 140 mM of potassium persulphate ($K_2S_2O_8$) leaving it in the dark at room temperature for 16 h before use. This solution was suitably diluted with alcohol to yield an absorbance of 0.70 ± 0.05 at 734 nm. Then, 1 ml of ABTS radical solution was added 3 ml of plant extract solution in alcohol at different concentrations. After 6 min, the percentage inhibition was calculated by spectrophotometric reading at 734 nm relative to a blank absorbance. Ascorbic acid was used as reference standard.

$$\% \text{ of ABTS radical scavenging} = [(A_0 - A_1) / A_0] \times 100$$

where, A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of sample.

2.5.5 *Nitric oxide radical scavenging activity*

The NO radical scavenging potential of BM-M was substantiated by method described by Sreejayan and Rao (1997). 3 ml of 10 mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations of the sample extracts and incubated at room temperature for 150 min. After incubation, 0.5 ml of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2% H_3PO_4) was added. The absorbance was recorded at 546 nm against reagent blank. Ascorbic acid was used as a positive control. The percentage of free radical scavenging activity from the sample was calculated according to the formula:

$$\% \text{ of NO radical scavenging} = [(A_0 - A_1) / A_0] \times 100$$

where, A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of sample.

2.6 Statistical analysis

Results were expressed as Mean ± SD. Linear regression analysis was carried out for standards to calculate total phenolic and flavonoid content and IC_{50} was analysed using non-linear regression.

3. Results and Discussion

3.1 Qualitative phytochemical analysis

Phytochemical screening of BM-M showed the presence of terpenoids, steroids, flavonoids, coumarins, glycosides and alkaloids.

3.2 Quantitative phytochemical analysis

Many studies have shown that polyphenols contribute significantly to the antioxidant capacity of medicinal plants. The activity of phenolic compounds is due to their ability to scavenge free radicals, donate hydrogen atoms or electron, or chelate metal cations (Afanas'ev *et al.*, 1989; Amarowicz *et al.*, 2004). It is known that only flavonoids with a certain structure, particularly hydroxyl position in the molecule can act as proton donor and show radical scavenging activity (Nickavar *et al.*, 2007; Wojdylo *et al.*, 2007). In the present study, the total phenolic and flavonoid content of the BM-M was estimated, using the Folin-Ciocalteu reagent and aluminium chloride methods, respectively. The total phenolic and flavonoid content of BM-M were found to be 23.12 mg GAE/g and 9.87 mg QE/g, respectively. The standard curve used for calculation was represented in Figures 1 and 2.

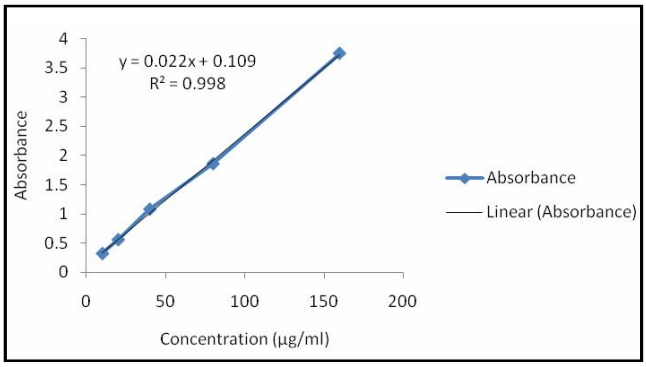

Figure 1: Total phenolic content

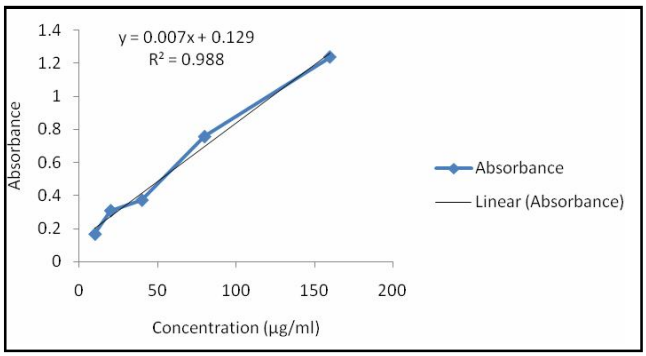

Figure 2: Total flavonoid content

3.3 *In vitro* antioxidant activity

Free radicals are known to play a specific role in a wide variety of pathological manifestations. Antioxidants fight against free radicals and protect us from various diseases. They exert their action either by scavenging the reactive oxygen species or protecting the antioxidant defence mechanisms (Umamaheswari and Chatterjee, 2008). In the present investigation, various concentrations (100, 200, 400 µg/ml) of BM-M were tested for antioxidant activities in different *in vitro* methods such as total antioxidant capacity, DPPH radical scavenging, anti-lipid peroxidation, ABTS radical scavenging and nitric oxide radical scavenging activities. The results showed that the BM-M showed potent antioxidant activity (Table 1 and Figures 3-7) and IC_{50} value was also calculated (Table 2). The results also indicated that free radical scavenging activity of the extract in all the methods are in a dose dependent manner. Percentage of inhibition of reference standard used in each assay is depicted in Figure 8.

Table 1: Percentage of activity of different concentrations of BM-M

Concentration of plant BM-M (µg/ml)	Activity (%)			
	DPPH	LP (Inhibition of MDA)	ABTS	NO
100	23.17 ± 0.005	39.31 ± 0.007	17.51 ± 0.01	32.58 ± 0.004
200	32.46 ± 0.008	65.59 ± 0.003	25.64 ± 0.01	37.93 ± 0.005
400	50.76 ± 0.002	70.14 ± 0.008	51.57 ± 0.002	59.28 ± 0.01

Values are the Mean ± SD, n=3

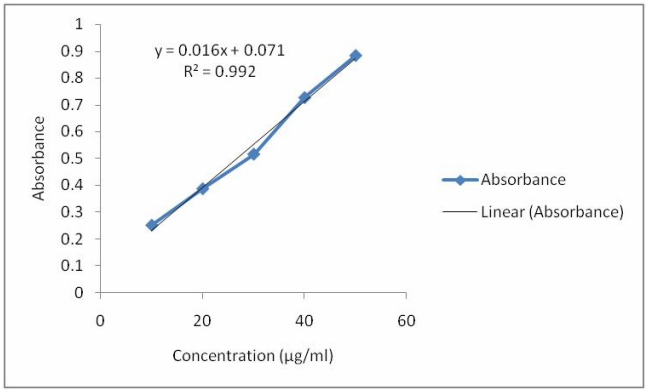

Figure 3: Total antioxidant capacity

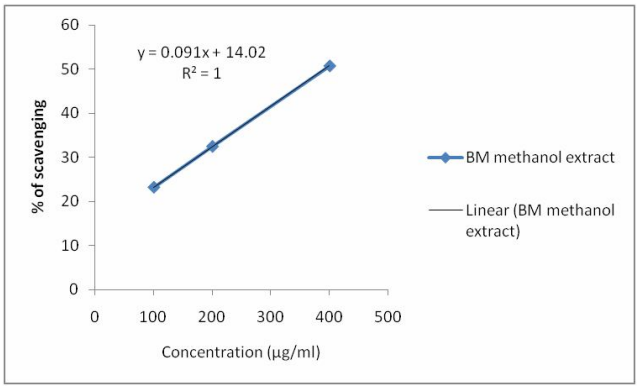

Figure 4: DPPH radical scavenging activity

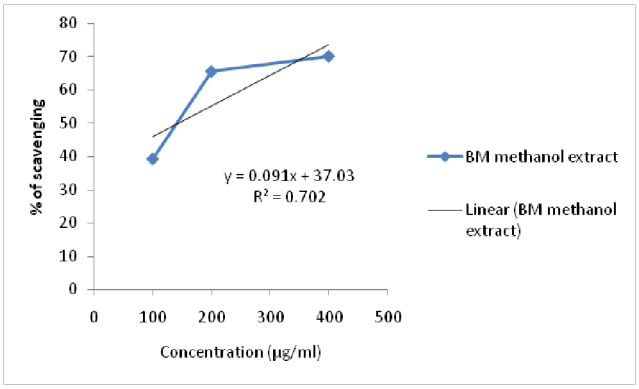

Figure 5: Antilipid peroxidation activity

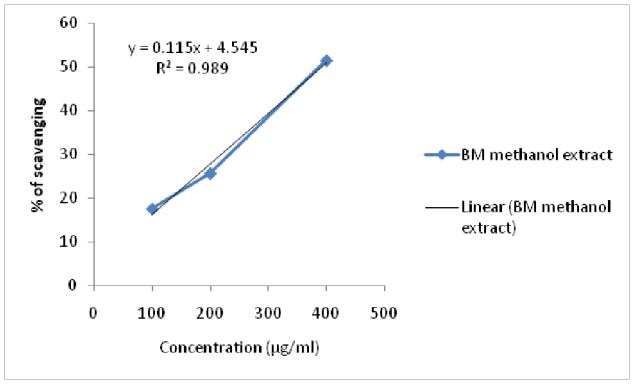

Figure 6: ABTS radical scavenging activity

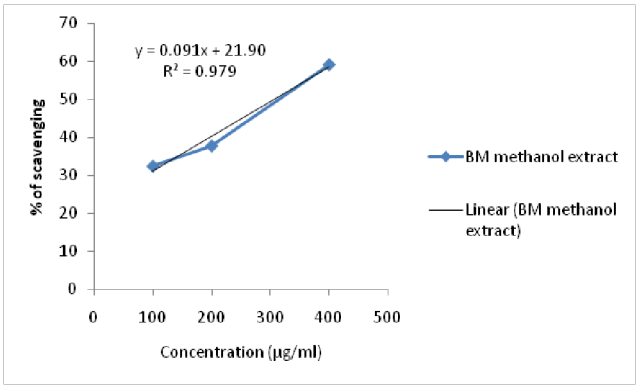

Figure 7: NO radical scavenging activity

Table 2: IC_{50} value obtained in each assay

Method	DPPH	LP	ABTS	NO
Value of BM-M	395.38	142.53	395.26	308.79
Value of standard	9.67	-	11.76	11.23

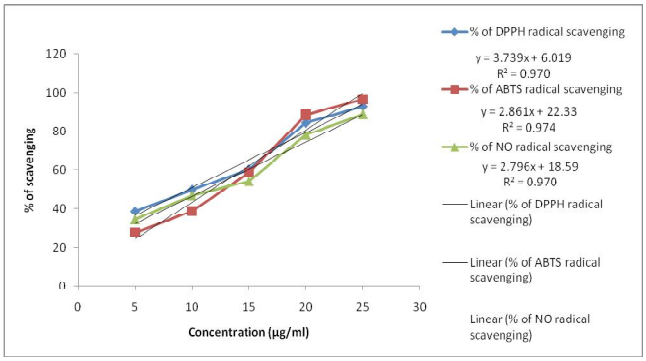

Figure 8: Percentage of radical scavenging by ascorbic acid standard

The total antioxidant capacity (TAC) of BM-M was evaluated by the phosphomolybdenum method, which is dependent on the capacity of BM-M to reduce Mo (VI) to Mo (V), resulting in the formation green phosphate/Mo (V) complex at acidic pH. TAC is expressed in ascorbic acid equivalents (AAE) per gram of plant extract and was calculated, using the standard curve of ascorbic acid ($y = 0.016x + 0.071$; $R^2 = 0.992$). It was observed that the extract possesses significant TAC of 20.78 mg AAE/ g.

The DPPH assay constitutes a quick and low cost method, which has frequently been used for the evaluation of the antioxidant potential of natural products (Molyneux, 2004). The DPPH antioxidant assay is based on the ability of DPPH, a stable free radical, to decolourize in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple colour (Sreejayan and Rao, 1996). The results of our study infer that the BM-M reduces the radical to the corresponding hydrazine when it reacts with the hydrogen ions released from the samples, which contain antioxidant principles. The highest percentage of DPPH radical scavenging activity of BM-M was 50.76 % at 400 µg/ ml (IC_{50} = 395.38 µg/ml). The IC_{50} value of the ascorbic acid standard is 9.67 µg/ml.

The free radical chain reaction is widely accepted as a common mechanism of lipid peroxidation. Radical scavengers may directly react with and quench peroxide radicals to terminate the peroxidation chain reactions (Ak and Gülçin, 2008), which are important in the pathogenesis of various diseases. The process of lipid peroxidation increases during inflammation and treatment with plant extracts found to inhibit lipid peroxidation process (Pandey *et al.*, 1994). $FeCl_2$-ascorbic acid mixture is known to cause lipid peroxidation in rat liver *in vitro* (Lin *et al.*, 1998). In the present investigation, $FeCl_2$-ascorbic acid treated liver homogenate showed a significant increase of malondialdehyde (MDA) when compared to normal control without $FeCl_2$-ascorbic acid. Treatment of $FeCl_2$-ascorbic acid stimulated liver homogenate with 400 ìg/ml dose of BM-M showed a decrease in MDA levels by 70.14 % (IC_{50} = 142.53 µg/ml).

The ABTS radical, generated from oxidation of ABTS by potassium persulfate, is presented as an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants (scavengers of aqueous phase radicals) and of chain breaking antioxidants (scavengers of lipid peroxyl radicals) (Leong and Shui, 2002). This assay is based on decolorization that occurs when the radical cation ABTS is reduced to ABTS'(2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). The highest percentage of ABTS radical scavenging activity of BM-M was 51.57 % at 400 µg/ml (IC_{50} = 395.26 µg/ml). Many researchers preferred trolox standard equivalents to express ABTS activity. Re *et al.* (1999) reported that ascorbic acid showed an ABTS activity of 0.99 as trolox equivalents (mM). Thus in our study the ascorbic acid calibration curve was chosen for presentation of results. The IC_{50} value of the ascorbic acid standard obtained is 11.76 µg/ml.

Nitric oxide is a potent pleiotropic inhibitor of physiological processes such as smooth muscle relaxation, neuronal signaling, inhibition of platelet aggregation and regulation of cell mediated toxicity (Hagerman *et al.*, 1998). Suppression of released NO may be partially attributed to direct NO scavenging, as the BM-M decreased the amount of nitrite generated from the decomposition of sodium nitroprusside solution. The highest percentage of NO radical scavenging activity of BM-M was 59.28 % at 400 µg/ml. The IC_{50} value of the BM-M extract is 308.79 µg/ml and of ascorbic acid standard is 11.23 µg/ml.

4. Conclusion

The present results suggest that the methanolic extract of *B. maderaspatensis* are apparently good free radical scavengers, especially of those of peroxy type and probably have the ability to inhibit autoxidation of lipids and, thus, it could be beneficial in the treatment of various diseases where lipid peroxidation is an important mechanism for pathogenesis. The free radical scavenging activity of the BM extract is of great significance for their traditional use against inflammation and associated diseases. Thus, it is concluded that, detailed chemical and pharmacological investigations of *B. maderaspatensis* can be helpful in finding desired bioactive principles and their molecular action mechanism.

Conflict of interest

We declare that we have no conflict of interest.

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