ANTIOXIDANT AND FREE RADICAL SCAVENGING PROPERTY OF METHANOL EXTRACT OF *BLUMEA LANCEOLARIA* LEAF IN DIFFERENT *IN VITRO* MODELS

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Summary

The defatted methanol extract of *Blumea lanceolaria* (MEBL) was evaluated for its antioxidant property in various *invitro* systems. DPPH radical scavenging, nitric oxide radical scavenging, hydroxyl radical scavenging, lipid peroxidation inhibition, reductive ability and estimation of total phenolics were evaluated for this study. The methanol extract of *Blumea lanceolaria* exhibited significant DPPH radical scavenging property with an IC_{50} of 45.79µg/ml whereas the IC_{50} value of the standard drug L-ascorbic acid was found to be 13.76µg/ml. The methanol extract also exhibited significant antilipid peroxidative effect in Fe^{2+}–ascorbate system. The IC_{50} value of the extract was found to be 62.48µg/ml, where as the standard drug exhibited 32.64µg/ml. The extract significantly scavenged the hydroxyl radicals by Fe^{3+}–ascorbate system generated through the Fenton's reaction and it has also significant reducing ability at IC_{50} of 31.78µg/ml. The extract also exhibited significant nitric oxide radical scavenging property at IC_{50} of 80.85µg/ml. In the estimation of total phenolics was found that one milligram of the extract contains 59.03µg equivalent of pyrocatechol. Thus from the present investigation it can be concluded that the defatted methanol extract of *Blumea lanceolaria* exhibited significant antioxidant property in various *invitro* system.

Key words: *Blumea lanceolaria*; anti oxidant; free radicals.

Running Title: Antioxidant property of MEBL *in vitro* models

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Introduction

Reactive oxygen and reactive nitrogen species such as superoxide anion, hydrogen peroxide, hydroxyl radical and nitric oxide have been widely implicated in the pathogenicity of several degenerative diseases such as ulcer, liver cirrhosis, atherosclerosis, Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and cancer [1, 2]. Although most of the living species has an efficient system to protect themselves against the oxidative stress induced by reactive oxygen species, epidemiological evidence indicates that the consumption of foodstuffs, herbals enriched with antioxidant phytonutrients such as flavonoids, phenolic acids, tannins, alkaloids, chlorophyll derivatives, amino acids, carotenoids, tocopherols and ascorbic acid are found to be beneficial in the treatment of pathological diseases due to oxidative stress [3]. Several studies indicated that diets enriched with antioxidant phytonutrients are found to be beneficial in the treatment of various ailments that arise due to reactive oxygen species.

The plant *Blumea lanceolaria* (Roxb) belongs to Compositae family. The other *Blumea* species are also available in different states of India. The pharmacological investigations have been reported about this plant. The leaf extract of different *Blumea* species showed the presence of sesquiterpene lactones; isoalantolactone derivatives, essential oil, glycoside [4] and acetylenic thiophenes [5] but there is no significant reported work on *Blumea lanceolaria*. Since the plant was used traditionally, the present investigation was aimed at evaluating the antioxidant potential of the methanol extract of *Blumea lanceolaria* in various *in vitro* assays such as DPPH radical scavenging assay, lipid peroxidation inhibition assay, hydroxyl radical scavenging assay and nitric oxide scavenging assay.

Materials and Methods

Plant material

The leaf of the plant *Blumea lanceolaria* were collected from West Bengal, India and identified by Botanical survey of India, Shibpur, Kolkata, West Bengal, India and the voucher specimen (UMU-01) has been preserved in our research laboratory for future reference. The leaf of the plant were dried under shade and powdered with a mechanical grinder. The powdered plant material was then passed through sieve # 40 and stored in an airtight container for future use.

Extraction

The air-dried powdered plant material (2 kg) was defatted with petroleum ether (60-80°C) in a Soxhlet extraction apparatus. The defatted plant material was successively extracted with chloroform and methanol. The solvents were completely removed under reduced pressure to obtain a dry mass. The yields of the chloroform and methanol extracts were found to be 5.6% and 8.3% w/w, respectively. The extracts were stored in a vacuum desiccator for further use. The methanol extract was used for the present investigations. Preliminary phytochemical investigation of the methanol extract indicated the presence of steroids, triterpenoids saponins and flavonoids.

Chemicals

All chemicals and solvents were obtained from SRL, Mumbai, India and were of analytical grade. 1, 1-Diphenyl-2-picryl hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Other chemicals used were sodium nitroprusside, sulphanilamide, O-Phosphoric acid, Napthyl ethylene diamine dihydrochloride, 2-Deoxy-D-ribose, Hydrogen peroxide, Ascorbic acid, Ferric chloride ($FeCl_3$), Ferrous sulphate ($FeSO_4$), Trichloroacetic acid (TCA), Thiobarbituric acid (TBA), Potassium chloride (KCl), Potassium ferricyanide ($K_3Fe\ [CN]_6$), Ethylene diamine tetra acetic acid

(EDTA), Tris- Hydrochloride buffer and Folin-Ciocalteu's phenol reagent (FCR) used were of analytical grade.

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay

The antioxidant activity of the MEBL was assessed using the DPPH free radical method [6]. Briefly 10 µl of the various concentrations of the extract (from 160 µg to 10 µg) or standard drug ascorbic acid (50µg to 10µg) was added to 200 µl of DPPH in methanol (100 µM). After incubation at 37^0C for 30 min bleaching of the DPPH was measured at 490 nm. The corresponding blank reading was also taken and the remaining DPPH was calculated. The antioxidant activity was expressed as IC_{50} (concentration in µg is required to scavenge 50% DPPH free radical) calculated from the dose inhibition curve (y=26.668Ln(x)-51.984).

Nitric oxide radical scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide (NO), which interacts with oxygen to produce nitrite ions that can be estimated with Griess reagent [7, 8 & 9]. The reaction mixture containing 2ml of (10mM) sodium nitroprusside, 0.5 ml of phosphate buffer and various concentrations (from 160 µg to 10 µg) of the extract (0.5 ml) were incubated at 25^0C for 150 min. IC_{50} value was estimated as concentration of sample required to scavenge 50% nitric oxide radical from the dose inhibition curve (y=21.48Ln(x)-44.354).

Hydroxyl radical scavenging activity

Hydroxyl radical scavenging was carried out by measuring the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+}/ascorbate/EDTA/H_2O_2 system. The attack of the hydroxyl radical on deoxyribose leads to the formation of

TBARS, which was measured at 532 nm [10]. Reaction mixture (1 ml) containing deoxyribose (2.8 mM), $FeCI_3$ (0.1 mM), EDTA (0.1 mM), ascorbic acid (0.1 mM), H_2O_2 (1 mM), phosphate buffer (20 mM, pH 7.4) and various concentrations of the methanol extract (from 160 µg to 10 µg) of *Blumea lanceolaria* were incubated at 37° C for one hour. After the incubation period TBARS thus formed was measured as mentioned earlier (Ohkawa et al., 1979). The IC_{50} of the extract was from the dose inhibition curve (y=24.063Ln(x)-33.23).

Fe^{2+}–ascorbate induced lipid peroxidation

Lipid peroxidation induced by Fe^{2+}–ascorbate system in rat liver homogenate was estimated by the method of Ohkawa et al., 1979 [11]. Reaction mixture (0.5 ml) containing rat liver homogenate 0.1 ml (25 % w/v in Tris–HCl buffer (20 mM, pH 7.0); 0.1ml of KCl (30 mM); 0.1ml of $FeSO_4 \cdot 6H_2O$ (0.16 mM); 0.1ml of ascorbic acid (0.06 mM) and 0.1 ml of various concentrations (from 160 µg to 10 µg of the extract) of *Blumea lanceolaria* were incubated at 37° C for one hour. After the incubation period, reaction mixture was treated with 0.2 ml SDS (8.1%); 1.5 ml TBA (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml by distilled water and then kept in a water bath at 95–100 °C for 30 minutes. After cooling, 1.0 ml of distilled water and 5.0 ml of *n*-butanol and pyridine mixture (15:1, v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The organic layer was removed and its absorbance was measured at 532 nm. The IC_{50} of the extract was from the dose inhibition curve (y=22.122Ln(x)-41.472).

Reductive ability

The reducing power of MEBL was determined according to the method of Oyaizu, 1986 [12]. Various concentrations (from 12 mg to 2 mg) of extract in 2.5 ml of phosphate buffer (pH 6.6) were mixed with 2.5 ml potassium ferricyanide

(1%). The mixture was incubated at 50° for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000g for 10 min. A portion of the supernatant (2.5 ml) was taken and 2.5 ml of water and 0.5 ml of $FeCl_3$ (0.1%) were added to this portion. Absorbance was measured at 700 nm. Absorbance increases with the increase in reducing power.

Determination of total phenolic compounds:

The content of total phenolic compounds in MEBL was determined by using Folin-Ciocalteu's phenol reagent (FCR), and the absorbance at 760 nm was determined according to method of Slinkard and Singleton [13]. The content was expressed as equivalent of pyrocatechol (µg) by using the following equation, which was obtained from a standard pyrocatechol graph.

Absorbance = 0.001×pyrocatechol (µg) + 0.0033

Results

The defatted methanol extract of *Blumea lanceolaria* was evaluated for antioxidant property in various *in vitro* assay systems. The methanol extract of *Blumea lanceolaria* exhibited significant DPPH radical scavenging property with an IC_{50} 45.79µg/ml of whereas the IC_{50} value of the standard drug L-ascorbic acid was found to be 13.76µg/ml. The DDPH radical scavenging property of the methanol extract of *Blumea lanceolaria* is shown in figure 1 and table 1. The methanol extract also exhibited significant antilipid peroxidative activity in Fe^{2+}–ascorbate system (Fig.3). The extract significantly inhibited the formation of MDA and the results are illustrated in table 2.

Further, the extract also effectively scavenged the hydroxyl radicals generated by using Fenton's reaction and nitric oxide. The results are summarized in table 1 and figure 3. The percentage inhibition in various models *viz.* DPPH, nitric oxide, hydroxy radical and lipid peroxidation, is shown in table.1 where from IC_{50} values were found to be 45.79, 80.85, 31.79 and 62.48µg/ml respectively. The percentage of total phenolics was calculated from the calibration curve of

pyrocatechol plotted and total phenolics are 58.3 µg equivalent amount of pyrocatechol. The methanol extract *B. lanceolaria* showed good antioxidant activity in all *in vitro* free radical scavenging models when compared with different standards such as ascorbic acid and butylated hydroxy toluene (BHT). The reducing power of MEBL was also dose dependent and shown in Figure 4.

Table 1. IC_{50} values of methanol extract of *Blumea lanceolaria* on various free radical scavenging assay.

In vitro *assays*	**IC_{50} value of MEBL** (µg/ml)	**IC_{50} value of ascorbic acid** (µg/ml)
DPPH radical scavenging assay	45.79	13.76
Nitric oxide radical scavenging assay	80.85	36.71
Hydroxyl radical scavenging assay	31.79	12.32
Lipid peroxidation inhibition assay	62.48	32.64

Fig.1. Effect of methanol extract of *Blumea lanceolaria* in DPPH radical scavenging assay

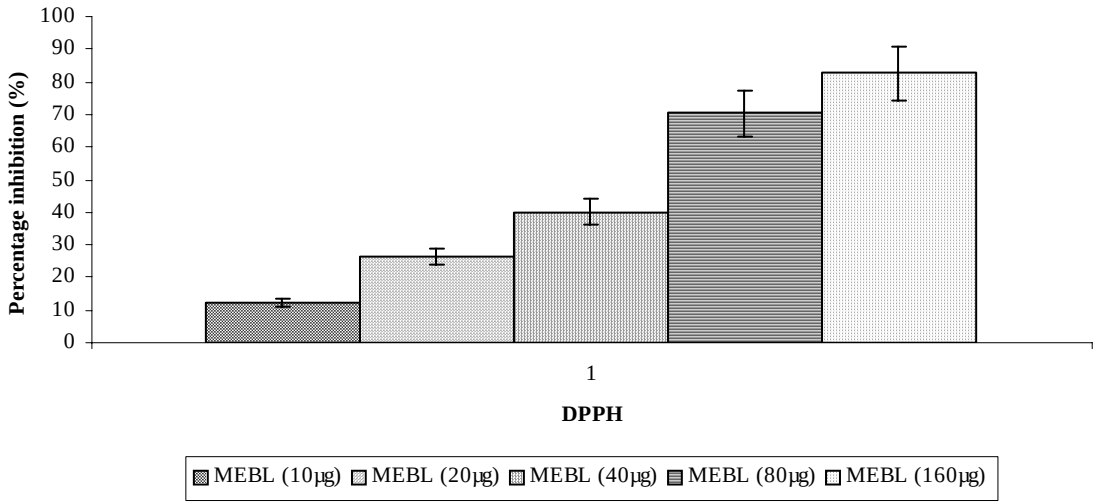


Fig.2. Effect of methanol extract of *Blumea lanceolaria* in nitric oxide radical scavenging assay

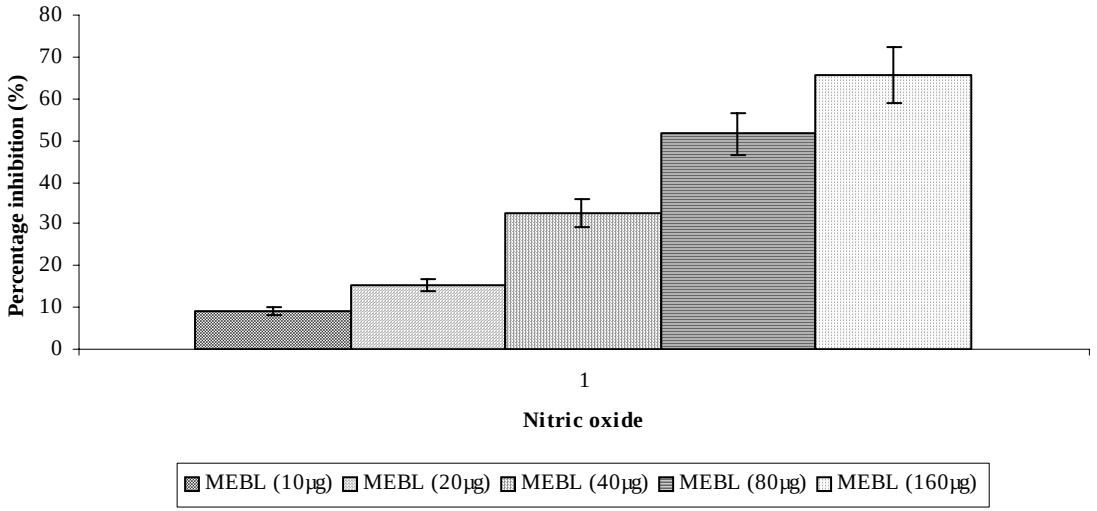


Fig.3. Percentage inhibition of methanol extract of *Blumea lanceolaria* on MDA level in Fe^{2+}-ascorbate and Fe^{3+}-ascorbate system.

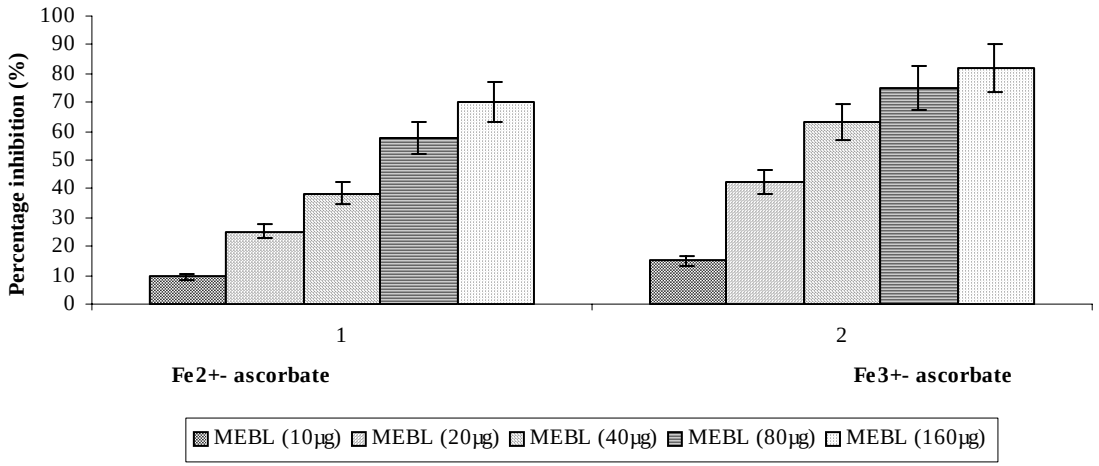
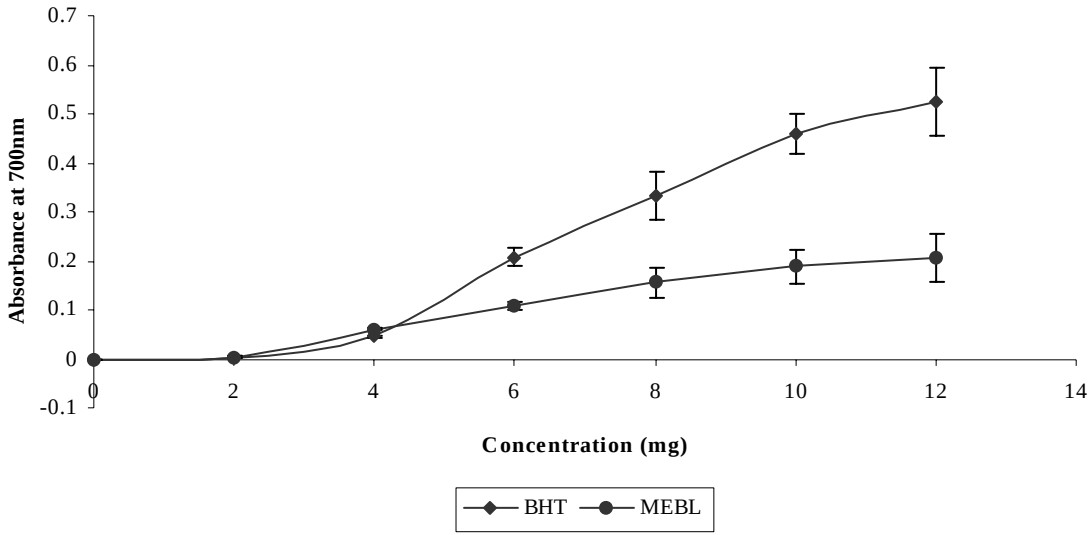


Fig. 4. Reducing ability of methanol extract of *Blumea lanceolaria*



Discussion

Reactive species such as hydroxyl radicals and nitric oxide radicals have been widely implicated in the pathology of several diseases. Removal of these reactive oxygen species or suppression of their generation is an efficient way in controlling these diseases.

DPPH radical is a stable free radical, which has been widely used to evaluate the free radical scavenging effects of natural antioxidants [14]. In this assay MEBL reduced the DPPH and by the odd electron of the DPPH radical becomes paired off. Because of the odd electron DPPH radical is having the maximum absorbance at 517nm after getting the hydrogen by MEBL, the absorbance was reduced at 517nm [15, 16 & 17]. The results shown that the MEBL was exhibited significant DPPH radical scavenging property. In this assay the methanol extract exhibited effectively antioxidant property and IC_{50} value, which was lower than that L-ascorbic acid, a well-known antioxidant.

In order to determine if the extracts were capable of reducing *in vitro* oxidative stress, lipid peroxidation was assessed by means of an assay that determines the production of malondialdehyde and related compounds in rat liver homogenates. Malondialdehyde (MDA) is one of the major degradation products of lipid peroxidation, which has been extensively studied and measured as a marker for oxidative stress [18] Thiobarbituricacid reactive species (TBARS) are produced as by-products of lipid peroxidation that occurs in the hydrophobic core of biological membranes [19]. Therefore lipid peroxidation has been identified as one of the basic reactions involved in oxygen free radical induced cellular damages [20] ands plays an important role in the pathogenesis of several diseases. The methanol extract of *Blumea lanceolaria* prominently inhibited the formation of MDA and exhibited significant antilipid peroxidative effect.

The effect of the plant extracts on the inhibition of free radical mediated DNA-sugar damage was assessed by means of the iron (II)-dependent DNA damage assay. The Fenton reaction generates hydroxyl radicals (OH•), which degrade DNA deoxyribose, using Fe^{2+}salts as an important catalytic component [21]. Oxygen radicals may attack DNA either at

the sugar or the base, giving rise to a large number of products. Attack at a sugar ultimately leads to sugar fragmentation, base loss and strand break with a terminal fragmented sugar residue [22]. Addition of low concentrations of transition metal ions such as iron to DNA causes degradation of the sugar into malondialdehyde and other related compounds which form a chromogen with thiobarbituric acid (TBARS). The methanol extract of *Blumea lanceolaria* significantly scavenged the hydroxyl radical generated through the Fenton reaction. Hydroxyl radicals are the most reactive radicals that are produced via the Fenton's reaction in living systems. Theses radicals are mainly implicated in the pathology of several diseases such as Parkinson's disease, rheumatoid arthritis, and carcinogenesis.

Nitric oxide is generated from the amino acid L-arginine by vascular endothelial cells, phagocytes and certain cells in the brain. Nitric oxide reacts with superoxide and forms peroxynitrite radicals and is responsible for the inflammatory response by the release of prostaglandin. Some scientists believe that repeated infections throughout life cause an excessive production of NO, which, over time, diseases such as heart disease, Alzheimer's disease and diabetes. In the present study, nitric oxide was generated from sodium nitroprusside, which at physiological pH (7.4) liberates nitric acid. This nitric acid gets converted to nitrous acid and further forms nitrite ions on contact with air. The nitrite ions diazotize with sulphanilic acid and couple with naphthylethylenediamine forming pink colour, which can be measured at 546 nm. From the results, the extract was also found to be beneficial in scavenging the nitric oxide radicals. Thus from the present investigation it can be concluded that the defatted methanol extract of *Blumea lanceolaria* exhibited significant antioxidant property.

Various mechanisms, including reducing capacity, prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction and radical scavenging have been claimed to explain the antioxidant activities [23 & 24]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [25].

In this study, MEBL and ascorbic acid exhibited effective reducing capacity in increased with increasing concentration (Fig. 4). Earlier authors [26 & 27] have observed a direct correlation between antioxidant activities and reducing capacity of certain plant extracts. The reducing properties 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 [28](Gordon MH, 1990). Reductones are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. From the data of the reducing capacity suggested that MEBL can act as electron donors and can react with free radicals to convert them to more stable products and thereby terminate radical chain reactions.

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