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In vitro antioxidant and antitubercular activity of *Leucas marrubioides* Desf. root extracts

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ABSTRACT

Modern civilization is facing hundreds of disorders associated with free radicals. The natural antioxidants from non-edible plants are gaining importance to fight against these disorders. The intention of this study is to evaluate the petroleum ether, chloroform and methanol extracts of roots of *Leucas marrubioides* Desf. (Lamiaceae) for antioxidant and antitubercular activities. The antioxidant activity of the extracts has been evaluated using DPPH radical scavenging, reducing power and nitric oxide scavenging methods. The results of the study indicated that, methanol extract posses promising DPPH radical scavenging, reducing power and nitric oxide scavenging activity. The petroleum ether and chloroform extracts showed moderate antioxidant activity in all the three models. The antitubercular activity of all the extracts of *L. marrubioides* have been evaluated against *Mycobacterium tuberculosis* H_{73}Rv strain using Microplate Alamar Blue Assay (MABA). The activity was documented within MIC range of 0.2 to 100µg/ml. The results of MABA showed that petroleum ether extract exhibited excellent antitubercular activity. The chloroform extract is moderately active, whereas methanol extract is less active against *M. tuberculosis*. The present investigation suggests that *L. marrubioides* posses remarkable antioxidant and antitubercular activity.

INTRODUCTION

Plants are the important source of diverse range of bioactive principles. The revival of interests in plant derived drugs is mainly due to the current widespread belief that green medicine is safe and more dependable than expensive synthetic drugs, which have adverse side. In addition herbal remedies used in folk medicine provide an interesting and still largely unexplored source for the creation and development of new potential drugs effects (Chew *et al*., 2012; Jigna and Sumitra, 2006; Vinayaka *et al*., 2010). Majority of diseases/disorders are mainly linked to oxidative stress due to free radicals. Free radicals are fundamental to any biochemical process and represent an essential part of aerobic life and metabolism (Sangameswaran *et al*., 2009). The most common free radicals include super oxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxyl (ROO^-) radicals and reactive hydroxyl radicals (OH^-). The nitrogen derived free radicals are nitric oxide (NO_2) and peroxynitrate anion ($ONOO^-$). All these free radicals are known as reactive oxygen species (ROS), which are capable of reacting with lipids, nucleic acids, proteins, enzymes and other micro molecules resulting in cellular damage (Shivprasad *et al*., 2005).

Antioxidants have an important role to protect the human body against the damage caused by free radicals. However the use of synthetic antioxidants such as butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) has been restricted because of their reported toxic and carcinogenic effects (Buxiang and Fukuhara, 1997; Hirose *et al*., 1998). Furthermore, natural products of plant origin have been proposed as a potential source of natural antioxidants with strong activity, which is mainly due to the phenolic compounds like flavonoids, phenols, flavonols and pronanthocyaninidins and their consumption has contributed in the prevention of distractive process caused by oxidative stress (Sharma and Goyal, 2011).

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Tuberculosis (TB) is one of the leading infectious disease and health burden in the world (Dye *et al*., 1999). It has been estimated that, one third of world's population including 40% from India estimated to be infected with tuberculosis (Gupta *et al*., 2010). More than nine million new cases diagnosed and approximately two million people killed annually (Gizachew *et al.*, 2013). There are a number of new factors that make people more susceptible to tuberculosis infection worldwide, the important of which is Human Immunodeficiency Virus (HIV) infection and the corresponding development of AIDS. The association of tuberculosis with HIV infection is so dramatic that in some cases, nearly two third of the patients diagnosed with the tuberculosis are also HIV-1 seropositive (Ilango and Arunkumar, 2011).

Current tuberculosis treatment is a long course of combination of 3-4 antibiotic drugs, which have one or the other toxic side effects and led to poor patient compliance. Antitubercular drugs such as isoniazid (INH), rifampicin (RIF), pyrazinamide, ethambutol, streptomycin etc have been a mainstay in the treatment of tuberculosis (Panda *et al*., 2013). The global emergence of multidrug trsistance (MDR) and extensively drug resistant (XDR) strains of *Mycobacterium tuberculosis* and more recently the reports of totally drug resistant tuberculosis (Singh, 2007; Udwadia *et al*., 2012) has become a common phenomenon, which cause drugs to be ineffective.

Fig. 1: *Leucas marrubioides* Desf.

Leucas marrubioides Desf. belongs to Lamiaceae family (Figure 1). It is an aromatic herbaceous plant growing about a meter height and widely distributed in India, Srilanka and Indonesia. It is used in traditional medicine for the treatment of pain, wound, chronic skin eruptions, inflammation and asthama. The lives are used for the treatment of cobra venom. Moreover, no scientific reports on biological activity of the plant have been reported.

Thus the scientific screening of the plant for various biological activities will prove the medicinal efficacy of the plant. The objective of this study is to investigate the antioxidant activity of crude extracts from roots of *L. marrubioides* through DPPH scavenging, nitric oxide scavenging and reducing power method. In addition the antitubercular activity of the crude extracts against *Mycobacterium tuberculosis* H_{37} Rv-ATCC 27294 was also examined by Microplate Alamar Blue Assay (MABA) technique.

MATERIALS AND METHODS

Collection and Identification of Plant Material

L. marrubioides was collected in Tunga river basin of Central Western Ghats of Karnataka. The plant was authenticated in Dept. of studies and research in Applied Botany, Jnana Sahyadri, Shankaraghatta and voucher specimen (KU/SD/TI/135) was deposited in the department for future reference.

Extraction of plant material

The roots of *L. marrubioides* were washed thoroughly 2-3 times with running tap water and once with sterile water. The material was shade dried, coarsely powdered and used for extraction. Weighed amount (500g) of the material was successively extracted using solvents of varying polarity namely, petroleum ether (pet-ether; 60-80^{0}C), chloroform and methanol using soxhlet extractor. Each extraction was carried out nearly 48 cycles. The extracts were filtered and concentrated using rotary flash evaporator under reduced pressure and at controlled temperature. The extracts obtained were dried, packed and stored at 4^{0}C in refrigerator (Gowrish *et al*., 2013).

Phytochemical analysis

All the extracts were subjected to preliminary phytochemical analysis using standard procedure to identify the various phytoconstituents (Mallikarjuna *et al*., 2007; George *et al*., 2010).

Test for Tannins

Small quantity (0.5g) of the extract was stirred with 10 ml of distilled water and filtered. 5% ferric chloride reagent was added to the filtrate. A Blue-black precipitate indicates the presence of tannin.

Test for Saponins

Small quantity (0.5g) of the extract was dissolved with 5 ml of distilled water and filtered. Persistent frothing observed when the filtrate was shaken vigorously indicates the presence of saponins.

Test for Terpenoids

Small quantity (0.5g) of extract was dissolved with 5 ml of chloroform and filtered. 10 drops of acetic anhydride was added to the filtrate followed by two drops of concentrated acid. Presence of pink colour at the interphase was an indication of the presence of terpenoids.

Test for Flavonoids

Few pieces of magnesium metal were added to extract solution and concentrated hydrochloric acid was carefully added. The formation of orange or crimson colour indicates presence of flavonoids.

Test for Glycosides

Small quantity (0.5g) of the extract was dissolved in 2 ml of chloroform. Concentrated sulphuric acid was carefully added to form a lower layer. A reddish-brown coloration at the interphase indicates the presence of a steroidal ring of glycoside.

Test for Alkaloids

5ml of 1% aqueous hydrochloric acid was added to Small quantity of the extract and warmed in a steam bath while stirring. It was filtered and the filtrate was used to test for alkaloid. i) 1 ml of the filtrate was treated with a few drops of Dragendorff's reagent. Formation of a reddish -brown turbid dispersion or precipitate shows the presence of alkaloids. ii) 1 ml of the filtrate was treated with a few drops of Mayer's reagent. Formation of creamy turbid dispersion shows the presence of alkaloids.

***In-vitro* Antioxidant Activity**

The free radical scavenging activity of the pet-ether, chloroform and methanol extract of the roots of *L. marrubioides* was determined using various *in-vitro* assays namely 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging, nitric oxide scavenging and reducing power method.

DPPH Radical Scavenging Activity

The antioxidant activity of the plant extracts namely pet-ether, chloroform, methanol and the standard was assessed on the basis of radical scavenging effect of stable DPPH free radical (Gowrish *et al*., 2013). The solution of DPPH (0.3mM) was prepared in methanol and 1ml of this solution was added to 3ml of crude extracts dissolved in methanol at different concentrations (400, 800, 1200, 1600µg/ml). The mixture was kept at room temperature for 30 minutes and the absorbance was measured at 517nm using a spectrophotometer (ELICO, SL159). The percentage scavenging activity at different concentrations was determined and the IC_{50} value of the fractions was compared with that of gallic acid, which was used as the standard. All the tests were performed in triplicates and percentage inhibition was calculated using the formula:

Percentage inhibition= $\{(A_{Control} - A_{Test}) / A_{Control}\} \times 100$,

where $A_{Control}$ is the absorbance of control (without extract) and A_{Test} is the absorbance with extracts.

Nitric Oxide Scavenging Activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by Griess reaction (Gowrish *et al*., 2013). The sodium nitroprusside (3ml of 100mM) in phosphate buffer (0.5ml) was added to 1ml of different concentrations (100, 200, 300, 400, 500 and 600µg/ml) of extract and standard. The resulting solution was then incubated at 25^0C for 60 minutes. Griess reagent (5ml, 1% sulphanilamide, 0.1% naphthyl ethylene diamine dihydrochloride, 2% H_3PO_4) was then added to incubated sample. The absorbance of the chromophore formed during the diazotization of nitrate with sulphanilamide and with subsequent coupling with naphthyl ethylene diamine dihydrochloride was measured at 546nm using spectrophotometer (ELICO, SL159).

The same reaction mixture without plant extracts, but with 0.5M phosphate buffer served as control. Vitamin C was used as standard. All the tests were performed in triplicates. The percentage inhibition of nitric oxide generated was calculated using the formula:

Percentage inhibition= $\{(A_{Control} - A_{Test})/ A_{Control}\} \times 100$,

where $A_{Control}$ is the absorbance of control (without extract) and A_{Test} is the absorbance in presence of extracts.

Reducing Power Activity

The extracts (1ml) of various concentrations (250, 500, 750, 1000 and 1250µg/ml) was mixed with phosphate buffer (2.5ml, 0.2M, pH 6.6) and 1% potassium ferricyanide (2.5ml). The mixture was incubated at 50^0C for 20 minutes. Aliquot of trichloroacetic acid (2.5ml, 10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 minutes. The upper layer of the solution (2.5ml) was mixed with distilled water (2.5ml) and freshly prepared ferric chloride solution (0.5ml, 0.1%). The absorbance was measured at 700nm spectrophotometrically (ELICO, SL159). Gallic acid was used as standard and all the tests were performed in triplicates. The increased absorbance of the reaction mixture indicated increased reducing power (Gowrish *et al*., 2013).

Antitubercular Activity

The antitubercular activity of crude extracts was assessed against *M.tuberculosis* using Microplate Alamar Blue Assay (MABA) (Munna *et al*., 2014). This methodology is nontoxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. Sterile deionised water (200µl) is added to all outer perimeter wells of sterile 96 well plates to minimum evaporation of medium in the test wells during incubation. The 96 well plates received 100µl of Middle brook 7H9 broth and serial dilution of compounds was made directly on plate.

The final extracts concentration tested were 100 to 0.2µg/ml of DMSO. plates were covered and sealed with parafilm and incubated at 37^0C for five days. After this time, 25µl of freshly prepared 1:1 mixture of alamar bue reagent and 10% tween 80 was added to the plate and incubated for 24 hours. A blue color in the well was interpreted as no bacterial growth and pink color was scored as growth. The Minimum Inhibitory Concentration (MIC) was defined as the lowest extract concentration, which prevented the color change from blue to pink. Pyrazinamide and streptomycin were used as standard antibacterial drugs.

Statistical analysis

All the data were presented as Mean±SEM. The Statistical Analysis was carried out by one way analysis of variance (ANOVA). P values <0.05 were considered as

significant. The IC_{50} values were obtained by linear regression method of plot using Microsoft Excel 2007 software.

RESULTS AND DISCUSSION

Phytochemical Screening

Plants are the important source of diverse range of bioactive principles. This is due to the fact that, plants have an almost limitless ability to synthesize chemical compounds of therapeutic value. The most important bioactive compounds of plants are alkaloids, flavonoids, steroids, terpinoids, tannins and phenolic compounds (Nisa *et al.*, 2011; Edeoga *et al.*, 2005). These compounds are synthesized by primary or secondary metabolism of the plants. Preliminary phytochemical screening of the crude extracts of *L. marrubioides* revealed the presence of various phytochemical constituents as shown in Table 1.

Table 1: Phytoconstituents detected in the root extracts of *L. marrubioides.*

Phytochemical Test	Pet-ether Extract	Chloroform Extract	Ethanol Extract
Alkaloids	+	+	-
Flavonoids	-	+	+
Terpenoids	+	+	+
Tannins	-	+	+
Saponins	-	-	-
Glycosides	-	-	+

'+' Present; '-' Absent

In-vitro Antioxidant Activity

Free radicals are involved in the development of degenerative diseases. They have also been implicated in the pathogenesis of diabetes, liver damage, nephrotoxicity, inflammation, cancer, cardiovascular disorders, neurological disorders and in the process of ageing. To protect these free radical induced damage, antioxidants are most popular agents that interact and synergistically neutralize the reactive species. Hence there has been an increased interest in food industry as well as in the preventive medicine in the development of "natural antioxidants" from plant materials (Max, 1987; Rekha *et., al* 2012).

DPPH Radical Scavenging Activity

DPPH is one of the free radicals widely used for testing preliminary radical scavenging activity of plant extracts (Jamuna *et al.*, 2012; Chougule *et al.*, 2012). DPPH is a stable nitrogen centered free radical, the color of which changes from violet to yellow upon reduction by either the process of hydrogen or electron donation. The substances which are able to perform this reaction can be considered as antioxidants[24] and hence they are radical scavengers. Figure 2 shows the DPPH radical scavenging activity of pet-ether, chloroform and methanol extracts as well as standard antioxidant gallic acid. The methanol extract showed scavenging activity close to gallic acid, where as pet-ether and chloroform extracts showed a weak radical scavenging activity. The IC_{50} values for DPPH radical scavenging activity for gallic acid, methanol, pet-ether and chloroform extracts are 245. 3±5.75, 280.9±4.67, 1240±15.24, 1580±16.21µg/ml respectively.

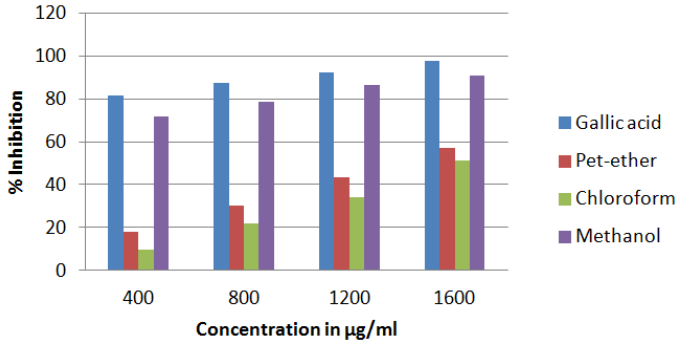

Fig. 2: DPPH radical scavenging activity of extracts of *L. marrubioides.*

Nitric Oxide Radical Scavenging Activity

The nitric oxide assay is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which reacts with oxygen to produce nitrite ions that can be estimated by Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduce production of nitrite ions (Ebrahimzadeh *et al.*, 2011). The percentage inhibition was increased with increase in concentration of extracts. Figure 3 shows the nitric oxide scavenging activity of standard (Vitamin C) and samples tested. Among the tested samples, methanol extract has potent nitric oxide scavenging activity as compared to the standard. The IC_{50} value was found to be 335.33±6.02µg/ml for methanol extract and 235.3±4.17µg/ml for Vitamin C. The pet-ether extract showed moderate scavenging activity with IC_{50} value 580±7.03µg/ml whereas chloroform extract showed weak nitric oxide radical scavenging activity with IC_{50} values >600µg/ml.

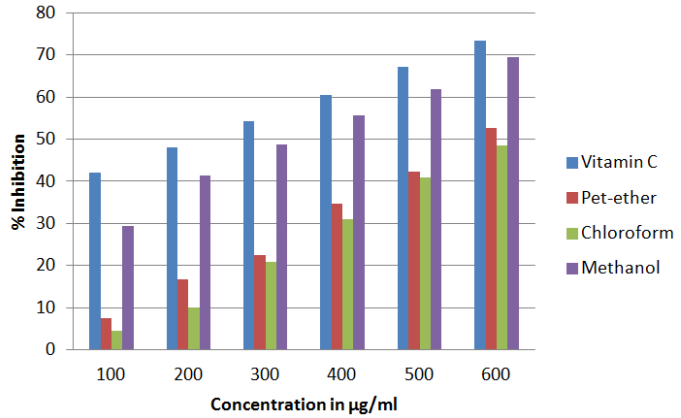

Fig. 3: Nitric oxide radical scavenging activity of extracts of *L. marrubioides*

Reducing Power Activity

Reducing power activity is often used to evaluate the ability of natural antioxidants to donate electrons, which would results in the reduction of Fe^{3+} to Fe^{2+} ion. This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and increase in absorbance could indicate an increase in reducing power (Umamaheswari and Chatterjee, 2008). The reducing power of various extracts and standard (gallic acid) are shown in Figure 4. Methanol extract showed a promising reducing ability when compared with standard gallic acid. The IC_{50} values

of gallic acid and methanol are 219.2.5±3.59µg/ml and 575.8±5.77µg/ml respectively. However pet-ether and chloroform extracts showed a weak reducing ability with IC_{50} values 1200.3±17.51µg/ml and 1475.6±18.51µg/ml respectively.

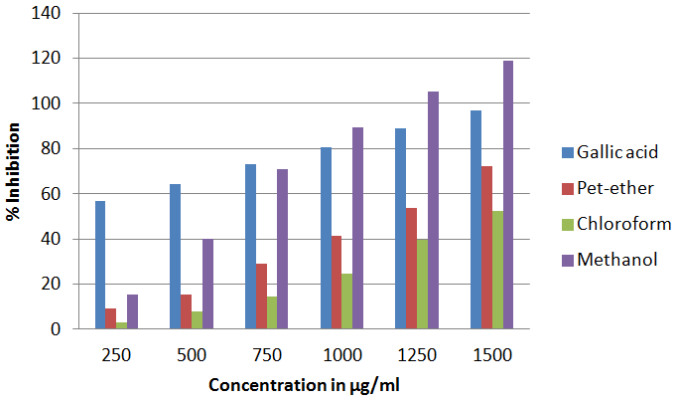

Fig. 4: Reducing power of extracts of *L. marrubioides* .

Antitubercular Acitvity

Natural products isolated from different plants have played an important role in the discovery of drugs against infectious diseases like tuberculosis. So far, few plants have been tested against mycobacteria and few plants which showed antitubercular activity are *Salvia hypargeia* (Ulubelen *et al*., 1998), *Euclea natalensis* (Lall and Meyer, 2001) etc. Now there is a need to discover and develop new, safe and herbal antituberculosis drugs particularly to target drug resistant and improve the treatment of chronic tuberculosis. In the present study, all the extracts were tested for their *in-vitro* antitubercular activity against *M. tuberculosis* by MABA with the use of Middle brook 7H9 broth and the MIC was determined. The MIC was defined as the lowest drug concentration, which prevented the color change from blue to pink. The MIC values of various extracts are presented in Table 2. The MIC of pet-ether, chloroform and methanol extracts were 12.5µg/ml, 50µg/ml and 100µg/ml and those of standard drugs pyrazinamide and streptomycin were 3.125µg/ml and 6.25µg/ml respectively. DMSO was not found to exhibit inhibitory activity. It was found that among the tested samples, pet ether extract was more active, whereas chloroform and methanol extracts were moderately active towards *M. tuberculosis* when compared to standard drugs.

Table 2: MIC of *L. marrubioides* root extracts against *M. Tuberculosis.*

Extracts	MIC (µg/ml)
Pet-ether	12.5
Chloroform	50
Methanol	100

CONCLUSION

The results of *in-vitro* antioxidant tests suggested that the methanol extract of *L. marrubioides* possess strong antioxidant activity analogous to standard antioxidant than pet-ether and chloroform extracts in all the three models. The overall antioxidant activity of these extracts might be attributed due to the presence of flavonoids, phenolics and other phytoconstituents. The pet-ether extract of the plant also exhibited promising antitubercular activity. However the compounds responsible for antioxidant and antitubercular activity are currently unclear. Therefore further investigation to be carried out to isolate and identify the bioactive compounds present in the root extract. Furthermore, the study will also need to be confirmed using *in-vivo* models.

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