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Analysis of phytochemicals and evaluation of phenolic contents and antioxidant activities of *Macrosolen parasiticus* L. (Dans.) leaves

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Abstract
The petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of *Macrosolen parasiticus* (Loranthaceae), a parasitic shrub growing on *Mangifera indica* were investigated for their phytochemical constituents, total phenolic content and antioxidant activities. Phytochemical screening of petroleum ether, chloroform, ethyl acetate and methanol extracts revealed the presence of alkaloids, flavonoids, saponins, tannins, phenolic compounds, sterols, terpenoids, glycosides, cardiac glycosides, carbohydrates, fixed oils and fats. Total phenolic content of petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of the plant were 18, 22, 32 and 37 µg of gallic acid equivalent in 100 µg of plant extract respectively. All the extracts showed promising antioxidant activity.

Keywords: *Macrosolen parasiticus*, phytochemical constituents, total phenolic content, antioxidant activity.

Introduction
India is known as the botanical garden of the world as it is the lead producer of medicinal plants [1]. It has 17,000 flowering plants, as a result India takes twelfth place in the mega-diversity countries in the world and has two hotspots- Eastern Himalayas and Western Ghats [2]. India has more than 2,500 medicinal plants many of which are yet to be investigated for their medicinal values [3]. From ancient times different parts of plants are used for the treatment and curing of various diseases [4] in the form of crude extracts or combination of many extracts. But now- a- days number of drugs derived from plants [5] are effective against many diseases and for this many of the chemical constituents present in the plants should be isolated. About 25 per cent of medicinal drugs in developing countries depend on the derivatives of plants [6] and are mainly used by rural peoples from many years [7].
Plants contain primary metabolites such as carbohydrates, proteins and peptides, in addition to this it also contain smaller molecules called secondary metabolites. These secondary metabolites such as tannins, saponins, flavonoids, phenolic compounds, glycosides, terpenoids, sterols, alkaloids, cardiac glycosides are responsible for the pharmacological activity of plants and has many biological activities. These are present in less quantity when compared to primary metabolite and they are derived from primary metabolite [8]. Plant derived antioxidants are important in research, dietary, cosmetic and pharmaceutical areas [9], nutraceutical and functional foods [10] as they are economic, safe and effective when compared to synthetic ones which are carcinogenic [11].
Today, 25- 50% pharmaceuticals are based on plants [12] and about 35,000 plant species are used [13]. These plants are used as a source of therapeutic agents due to lesser side effects [14]. Advancement in phytochemistry has led to the identification and isolation of compounds which are useful in the treatment of diseases [15]. Demand for plant- derived drugs are increased now- a- days, so the global- market rate is also increased [16].
Macrosolen parasiticus L. (Dans.), a parasitic shrub belonging to the family Loranthaceae has antioxidant activity and anticancer activity in their stem part [17-20]. So, the present study was undertaken to screen the phytochemical constituents and antioxidant activity of *Macrosolen parasiticus* leaves.

Materials and Methods

Collection of plant material

The fresh plant material (leaves) of *M. parasiticus* growing on *M. indica* was collected from Western Ghats of Karnataka, India. The plant was identified with the help of Flora of Presidency of Madras [21] and a voucher specimen is deposited in the Herbarium, Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore, Karnataka, India.

Preparation of the extracts

The fresh leaves of *M. parasiticus* was washed under running tap water, shade dried and powdered using wearing blender. 50 gm of dried leaf powder was filled in the thimble and successfully extracted with petroleum ether; chloroform; ethyl acetate and methanol using Soxhlet extractor. All the extracts collected were concentrated using rotary flash evaporator and stored at 4 °C in air tight vials and used for further studies [22].

Phytochemical Screening

The collected *M. parasiticus* leaves extracts were subjected to qualitative phytochemical screening for identification of various classes of active chemical constituents such as saponins, alkaloids, tannins, phenolic compounds, sterols, terpenoids, anthraquinones, flavonoids, cardiac glycosides, glycosides, carbohydrates, proteins, amino acids, fixed oils and fats using the method described by [23, 24].

Determination of Total Phenolics

Total phenolic in petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of *M. parasiticus* was determined by the method of Singleton *et al.* [25]. 20 µl of extract (5 mg/ ml) was mixed with 0.75 ml of 20% sodium carbonate solution and 0.25 ml of Folin- Ciocalteau reagent. The reaction mixture was allowed to stand in light for 3 min and incubated for 2 h in dark. The absorbance was measured at 765 nm using UV-Visible Spectrophotometer. Total phenolics were quantified by calibration curve obtained from measuring the absorbance of known concentrations of Gallic acid standard (0- 100 µg/ ml). The concentrations were expressed as µg of Gallic acid equivalents per ml.

Antioxidant Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) Scavenging Activity

The free radical scavenging capacity of petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of *M. parasiticus* was determined by DPPH (2,2-diphenyl-1-picrylhydrazyl) method [26]. The reaction mixture contained 5 µl of plant extract and 95 µl of DPPH (300 µM) in methanol. Different concentrations (100-1000 µg/ ml) of test sample were prepared, while the concentration of DPPH remained same. These reaction mixtures were incubated at 37º C for 30 min and the absorbance was measured at 517 nm. Per cent Radical Scavenging Activity (RSA) upon sample treatment was determined by comparison with a methanol treated control. All the determinations were performed in triplicates. Ascorbic acid was used as positive control. The per cent RSA was calculated using the formula:

$$\%\text{DPPH Scavenging Activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Nitric Oxide Scavenging Activity

Nitric oxide radical scavenging activity of petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of *M. parasiticus* was carried out as described by Marcocci *et al.* [27] with slight modifications. 1 ml of sodium nitroprusside (5 mM) in 0.5 M phosphate buffer was mixed with different concentrations (100- 1000 µg/ ml) of the plant extracts and they were incubated at 25º C for 150 min and the absorbance was measured at 546 nm. Ascorbic acid was used as positive control. The capability to scavenge NO radical was calculated using the formula:

$$\%\text{ NO scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Hydrogen Peroxide Scavenging Activity

Hydrogen peroxide scavenging activity of petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of *M. parasiticus* was carried out as described by Czochra and Widensk [28]. Hydrogen peroxide (40 mM) solution was prepared in phosphate buffer (pH 7.4) and 0.6 ml of hydrogen peroxide solution was mixed with different concentrations of (100-1000 µg/ ml) of the plant extracts (2.4 ml) and they were incubated at 10 min and the absorbance was measured at 230 nm. Ascorbic acid was used as positive control. The per cent hydrogen peroxide scavenging activity was calculated using the formula:

$$\%\ H_2O_2\text{ scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

Total antioxidant capacity by Phosphomolybdenum Method

Total antioxidant capacity of petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of *M. parasiticus* was evaluated by phosphomolybdenum method according to Prieto *et al.* [29]. 0.3 ml of plant extracts of different concentrations (100-1000 µg/ ml) was mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). These reaction mixtures were incubated at 95º C for 90 min and the absorbance was measured at 695 nm. The total antioxidant capacity was expressed as equivalents of ascorbic acid (mg of AA/ g of plant extract).

Reducing Power Assay

Reducing power estimation of petroleum ether, chloroform, ethyl acetate and methanol leaves extracts of *M. parasiticus* was carried out as described by Nagulendran *et al.* [30] with slight modifications. 0.75 ml of *M. parasiticus* leaves extract solution (1 mg/ ml) was mixed with 0.75 ml of 0.2 M phosphate buffer (pH 6.6) and 0.75 ml of 1% potassium ferricyanide and incubated at 50º C for 20 min. Then, 0.75 ml of 10% trichloroacetic acid was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 1.5 ml of the supernatant solution was mixed with 1.5 ml of distilled water and 0.5 ml of 0.1% $FeCl_3$. Absorbance was measured at 700 nm in UV-Visible Spectrophotometer using phosphate buffer as blank and butylated hydroxyl toluene (BHT) as standard. The experiments were performed in triplicates.

Statistical Analysis

Data from three replicates were analyzed for each experiment and analysis of variance (ANOVA) using SPSS Inc. 16.0. Significant effects of treatments were determined by F values ($P \leq 0.05$). Treatment means were separated by Tukey's Honestly Significant Differences (HSD) test.

Results

Phytochemical Screening

The methanol extract of *M. parasiticus* showed the presence of saponins, alkaloids, tannins, phenolic compounds, terpenoids, flavonoids, glycosides, cardiac glycosides, carbohydrates, fixed oils and fats. Ethyl acetate extract contained only saponins, alkaloids, terpenoids, glycosides and cardiac glycosides while sterols and glycosides were present only in petroleum ether and chloroform extracts (Table 1).

Table 1: Qualitative Phytochemical Analysis of *M. parasiticus* extracts

Phytochemical compounds	Extracts			
	Petroleum ether	Chloroform	Ethyl acetate	Methanol
Saponins				
a. Foam Test	--	--	++	++
Alkaloids				
a. Wagner's Test	--	--	++	++
b. Dragendorff's Test	--	--	++	++
c. Mayer's Test	--	--	--	++
d. Hager's Test	--	--	--	++
Tannins and Phenolic compounds				
a. Ferric chloride Test	--	--	--	++
b. Gelatin Test	--	--	--	--
Sterols				
a. Libermann-Burchard's Test	++	++	--	--
Terpenoids				
a. Libermann-Burchard's Test	--	--	++	++
Anthraquinones	--	--	--	--
Flavonoids				
a. Ferric Chloride Test	--	--	--	++
b. Alkaline reagent Test	--	--	--	++
Cardiac glycosides				
a. Keller-Killani Test	--	--	++	++
Glycosides				
a. Borntrager's Test	--	--	--	++
b. Baljet Test	++	++	++	++
Proteins and Amino acids				
a. Ninhydrin Test	--	--	--	--
b. Biuret Test	--	--	--	--
Carbohydrates				
a. Benedict's Test	--	--	--	++
b. Fehling's Test	--	--	--	++
Fixed Oils and Fats				
a. Saponification Test	--	--	--	++

++ : Present; -- : Absent

Determination of Total Phenolics

The total phenolic content in the petroleum ether, chloroform, ethyl acetate and methanol extracts of *M. parasiticus* was determined as Gallic Acid Equivalent (GAE). The petroleum ether, chloroform, ethyl acetate and methanol extracts showed total phenolic content of about 18, 22, 32 and 37µg of GAE in 100 µg of plant extract respectively.

Antioxidant activity

DPPH, Nitric Oxide and Hydrogen Peroxide Scavenging Activity

The various extracts of *M. parasiticus* was subjected for DPPH activity and it revealed that methanol (202 µg/ ml) > ethyl acetate (216 µg/ ml) > chloroform (233 µg/ ml) > petroleum ether (249 µg/ ml). In nitric oxide and hydrogen peroxide activity, among the extracts the highest activity was shown by chloroform extract (115 and 199 µg/ ml) followed by methanol (124 and 211 µg/ ml), ethyl acetate (226 and 298 µg/ ml) and petroleum ether (305 and 317 µg/ ml) respectively. The control ascorbic acid showed 50% inhibition at 40 µg/ ml, 24 µg/ ml and 27 µg/ ml concentration in DPPH, nitric oxide and hydrogen peroxide activity (Fig. 1, 2 and 3).

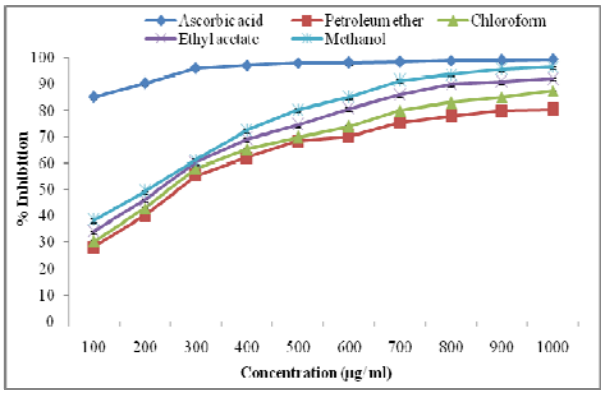

Fig 1: DPPH scavenging activity of different solent extracts of *M. parasiticus*. Values are mean of three independent replicates. ± indicate Standard Error

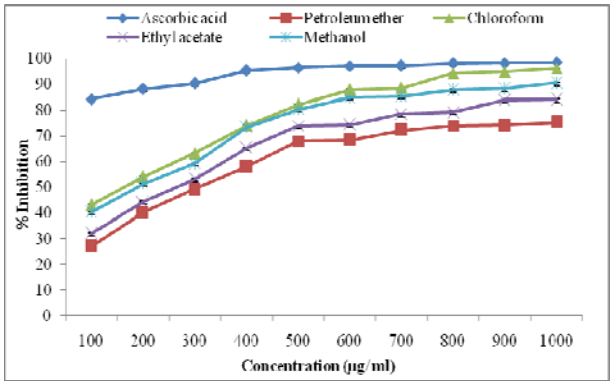

Fig 2: Nitric oxide scavenging activity of different solvent extracts of *M. parasiticus*. Values are mean of three independent replicates. ± indicate Standard Error

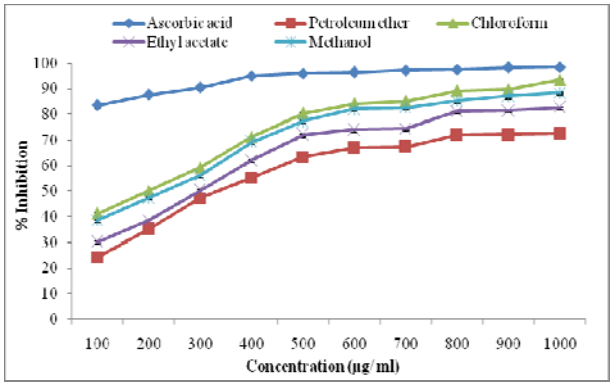

Fig 3: Hydrogen peroxide scavenging activity of different solvent extracts of *M. parasiticus*. Values are mean of three independent replicates. ± indicate Standard Error

Total antioxidant capacity by Phosphomolybdenum Method and Reducing Power Assay

The total antioxidant capacity and reducing power ability of *M. parasiticus* extracts was compared with standard ascorbic acid and BHT respectively. An increase in absorbance at 695 nm and 700 nm indicated the total antioxidant capacity and reducing power ability of the extracts. Among all the extracts, methanol extract showed good total antioxidant capacity and reducing power ability with an increase in absorbance with respect to increase in the concentration. Ethyl acetate, chloroform and petroleum ether extracts showed least total antioxidant capacity and reducing power ability (Fig. 4 and 5).

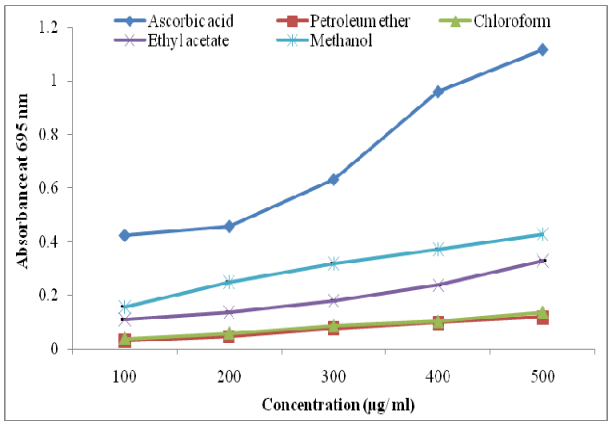

Fig 4: Total antioxidant capacity of different solvent extracts of *M. parasiticus*. Values are mean of three independent replicates. ± indicate Standard Error

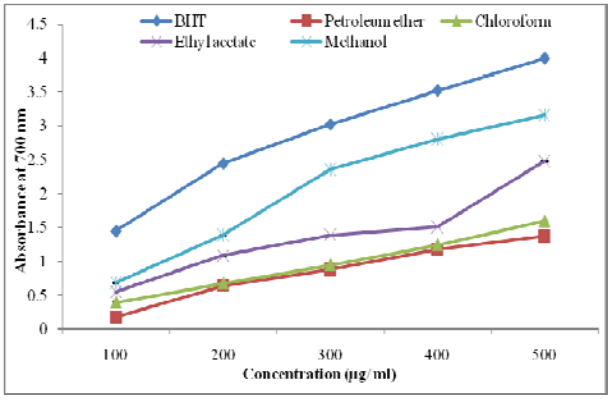

Fig 5: Reducing power assay of different solvent extracts of *M. parasiticus*. Values are mean of three independent replicates. ± indicate Standard Error

Discussion

The medicinal values of the plant is attributed due to the presence of phytochemical constituents such as cardiac glycosides, tannins, saponins, alkaloids, flavonoids and phenolic compounds which has antioxidant and antimicrobial activities [31]. Cardiac glycosides are used as stimulants for cardiac failure [32] and in the treatment of asthma [33]. Saponins have anti- inflammatory, antimicrobial, antidote, antifungal, antiyeast, antifeedant, anti high cholesterol properties, act as hypotensive and cardiac depressant agent and has cardiotonic and haemolytic effects [34]. Alkaloids posses antimicrobial, antiviral, anticancer and many pharmacological activities [35] whereas tannins have antiviral, antibacterial, antiparasitic, antiulcer, anti- inflammatory and antioxidant activities [36]. Flavonoids have many therapeutic properties such as antirheumatism, antihypertensive, antimicrobial, anticancer, anti- inflammatory, diuretic, antioxidant [37] and give protection against free radicals, allergies, hepatotoxins, ulcers, platelet aggregation and viruses [38].

Phenolic compounds have the ability to scavenge free radicals which induce oxidative stress and cause many human disorders such as rheumatoid arthritis, tissue injury, cancer, aging, Parkinson, Alzheimer and neurodegenerative diseases, hence they are responsible for antioxidant property [39]. The best method to estimate free radical scavenging activity is DPPH which is a stable free radical and it get neutralizes with antioxidants which donate hydrogen or electron [40]. Another indicator of antioxidant property is reducing power which is used to measure the ability of the extracts to reduce Fe^{3+} to Fe^{2+} [41]. Mistletoe *M. parasiticus* also showed the presence of phenolic content and good concentration dependent antioxidant activity which was also reported in many other mistletoes by Murali *et al*. [42]; Puneetha *et al*. [43,44] and Amruthesh and Puneetha [45].

Synthetic antioxidants such as BHT (Butylated Hydroxy Toluene), BHA (Butylated Hydroxy Anisole), TBHQ (Tertiary butylhydroquinone) and PG (Propyl Gallate) are used to reduce the risk of above mentioned diseases [46] but due to their toxicity they are responsible for carcinogenesis and liver damage [47]. Hence many of the researchers screened plants for their antioxidant activity [48].

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