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Determination of antioxidant potential of different solvent extracts of a medicinal orchid *Malaxis rheedii* (sw.) Szlach

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Abstract
Orchidaceae is a family in the plant kingdom comprising morphologically and physiologically diverse plant species. Many orchid species were included in various medicinal preparations of ayurveda, Malaxis rheedii is one among them. The plant *Malaxis rheedii* was collected from Payyanur of Kannur district and soxhlet extraction was done with different solvents in increasing order of their polarity. DPPH radical scavenging activity shows that maximum scavenging activity in aqueous extract and least in petroleum ether extract. While calculating the total antioxidant activity by Phosphomolybdenum complex formation maximum activity obtained in petroleum ether extract. The assessment of reducing power of the plant shows that all the concentrations show maximum activity in ethanol extract. These results suggest that the plant *Malaxis rheedii* can constitute a source of natural compounds with antioxidant ability.

Keywords: medicinal plant, antioxidant, dpph, phosphomolybdenum

Introduction
Orchidaceae is a family in the plant kingdom comprising morphologically and physiologically diverse plant species. Orchidaceae is the largest family of flowering plants in the world after Asteraceae. The family consists of about 779 genera and 22,500 species (Mabberley, 2008) [10]. They live in diverse habitats from colder region to hot deserts with variously modified vegetative and floral structures. They adapt with the environment by living as an epiphyte (growing on trees or shrubs), saprophyte (growing on dead and decaying matter) and terrestrials (growing on ground). They are blessed with beautiful and long lasting flowers. In India 1331 species of orchid plants belonging to 186 genera were officially recorded (Misra, 2007) [12].
Many orchid species were included in various medicinal preparations of ayurveda. Four medicinal plants of the family Orchidaceae, *Malaxis muscifera, Malaxis acuminata, Habenaria intermedia* and *Habenaria edgeworthi* were the main ingredients of Chayvanprasa and they are included as a special classification in ayurveda called Astavarga. Another medicinal orchid *Dendrobium macraei* is included as a source of Jivanti (Singh & Duggal, 2009) [19].
The studies on orchid plants have shown that they are rich in important biochemicals such as carbohydrates, Flavonoids, alkaloids and glycosides. A large number of chemical compounds like alkaloids, bibenzyl derivatives, phenolic compounds, Flavonoids, phenanthrenes and terpenoids were recently isolated from the orchid plants. The Concentrates and metabolites isolated from these plants, especially from blooms and leaves, have variable phytochemical, pharmacological and antimicrobial properties. The plants were found to have antimicrobial, anti-diabetic, anti-inflammatory, diuretic, antirheumatic, anticarcinogenic, hypoglycemic, anti-convulsive, relaxation, neuroprotective, and antivirus properties. A large number of orchids have been used for treatment of several diseases, thus, different investigations have been undertaken to provide scientific proof to justify the medicinal use of various plants in the treatment of various ailments (Bulpitt *et al*., 2007) [4].
Free radicals are created by losing an electron and donating them during normal cellular functions in the body and under certain particular environmental conditions. The free radicals created, looking for another molecule and withdraw or donate an electron by creating a chain reaction that can able to damage hundreds of molecules in our body. Antioxidants help to halt this chain reaction and some antioxidants themselves act as free radicals and they donate electron to neutralize the dangerous free radicals. Which cause cellular damage by lipid membrane peroxidation, cellular denaturation of proteins, disrupting cellular functions and breakdown of DNA strands (Chanda *et al*., 2011) [5]. Another antioxidants work opposite to the molecules that form free radicals, destroying them before they can begin effect that leads to oxidative damage (Matill, 1947) [11]. The antioxidant activity of the plants may come from secondary metabolites like flavonoid and Phenolic compounds. Antioxidants and scavenging agents associated with oxidative damage and they act against free radicals (Norhazana *et al*., 2012) [14].

Materials and Methods
Sample extraction
The whole plant of *Malaxis rheedii* was washed thoroughly in tap water, dried in shade for 10 to15 days and powdered in mechanical grinder and stored in airtight bottle for future use. The powder (20 gm) was extracted with petroleum ether, chloroform and ethanol in a soxhlet apparatus (3840; Borosil Glass works Ltd., Mumbai, India) in increasing order of their polarity. Finally the dried powder was macerated using water with constant stirring for 48 hours using the orbital shaker (Rivotek; Riviera Glass Pvt. Ltd., Mumbai, India) and the extract was filtered. The extracts were concentrated, dried and stored at -20ºC in the deep freezer (RQV- 300; plus, REMI electro technik Ltd., Thane, Maharashtra, India) for further analysis.

Extract Recovery Percentage

The amount of extract recovered after successive extraction was weighed and the percentage yield was calculated by the following formula,

$$\text{Extract Recovery Percentage} = \frac{\text{Amount of extract (g)}}{\text{Amount of plant sample (G)}} \text{X100}$$

In vitro Antioxidant Assays

DPPH Radical scavenging activity, (Braca *et al*. 1958).

The antioxidant activity of the extract was determined in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH**,** according to the method of Braca *et al*. (1958). Sample extracts at various concentrations were taken and the volume was adjusted to 100 µl with methanol. About 3 ml of a 0.004 % methanolic solution of DPPH was added to the aliquots of samples and standards (BHT and Rutin) and shaken vigorously. Negative control was prepared by adding 100 µl of methanol in 3 ml of methanolic DPPH solution. The tubes were allowed to stand for 30 minutes at 27°C. The absorbance of the samples and control were measured at 517 nm against the methanol blank. Radical scavenging activity of the samples was expressed as IC_{50} which is the concentration of the sample required to inhibit 50% of $DPPH^{\bullet}$ concentration.

Phosphomolybdenum assay, (Prieto *et al*., 1999) [16]

The antioxidant activity of samples was evaluated by the green Phosphomolybdenum complex formation according to the method of Prieto *et al*., (1999) [16]. An aliquot of 100 µl of samples and standards (BHT and Rutin) were taken into a series of test tubes and were made up to 300 µl with methanol. About 300 µl methanol taken in a test tube was considered as the blank. All the test tubes were added with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and vortex well to mix the contents. The mouth of the test tubes were covered with foil and incubated in a water bath at 95°C for 90 minutes. After the samples were cooled to room temperature, the absorbance of the mixture was measured at 695 nm against the reagent blank. Ascorbic acid was used as the reference standard and the results were expressed as milligrams of ascorbic acid equivalents per gram extract.

Reducing power assay, (Oyaizu, 1986) [15]

The antioxidant capacities of different extracts of samples were estimated according to the procedure described by Pulido *et al*., (2000). Prepared 0.2 M phosphate buffer with pH 6.6, 1% potassium ferricyanide solution and 10% Trichloro acetic acid solution. Taken the aliquots of the extract and made up to 1 ml with methanol. Added 5 ml of 0.2 M phosphate buffer and then added 5 ml potassium ferricyanide solution. Then incubate the mixture at 50^0c for 20 minutes. Cool rapidly the mixture and add 5 ml, 10% Trichloro acetic acid solution to the mixture. Centrifuged the reaction mixture at 3000 rpm for 10 minutes. Mix 5 ml of the upper layer of the solution with 5 ml of d.d H_2O. Added 1 ml ferric chloride solution and read absorbance at 700 nm. Increased absorbance indicates increased reducing power. BHT is used as standard for the test.

Results and Discussion

Extract yield percentage

Extraction of whole plant of *Malaxis rheedii* was carried out by soxhlet apparatus with non-polar to polar solvents (petroleum ether, chloroform and ethanol) and maceration with hot water. Percentage of extract yield is given in Table. 1. Extract yield percentage was maximum at ethanol extract and it was 4.02%

Table 1: Extract yield percentage

Solvent	Yield %			
	Petroleum ether	Chloroform	Ethanol	Water
	1	2.92	4.02	3.44

Antioxidant assay

DPPH Radical Scavenging Activity

DPPH (1, 1-diphenyl-2-picrylhydrazyl) analysis is one of the best-known, accurate, and frequently employed methods for evaluating antioxidant activity (Zhou, 2004) [20]. It is a stable free radical because of its spare electron delocalization over the whole molecule. The donation of H^+ to the DPPH radicals made a corresponding change from violet colour to pale yellow in the solution. The DPPH scavenging also made a proportionate decrease in its absorbance at 517 nm

Malaxis rheedii showed Maximum DPPH radical scavenging activity in aqueous extract and it was 24.12 µg/ml. IC_{50} value was 79.37 µg/ml in petroleum ether extract, 25.56 µg/ml in chloroform extract and 79.37 µg/ml in ethanol extract of the plant *M. rheedii*. DPPH radical scavenging activity of different solvent extracts of *Malaxis rheedii* were shown in table 2 and the graph showing increases in percentage inhibition shown in figure 1. Aqueous and chloroform extracts of the plant showed highest DPPH scavenging activity. Percentage of inhibition increases with increase in concentration. The lowest IC_{50} indicates the strongest ability of the extracts to act as DPPH radicals scavengers. While comparing the results with total phenolic and flavonoid content in the plant, it has been shown that water, chloroform and ethanol extract shows highest amount of total phenolics and flavonoid content.

Table 2: DPPH radical scavenging activity of different solvent extracts of *Malaxis rheedii*

Sl. No	solvents	concentration	% of inhibition	IC_{50} (µg/ml)
1	Petroleum ether	50	6.03	147.49
		100	11.86	
		150	19.75	
		200	23.05	
		250	26.17	
2	Chloroform	50	16.13	25.56
		100	18.92	
		150	26.88	
		200	30.91	
		250	39.31	
3	Ethanol	50	9.14	79.37
		100	22.47	
		150	32.49	
		200	43.29	
		250	50.78	
4	Water	50	13.58	24.12
		100	19.75	
		150	31.31	
		200	32.88	
		250	41.08	

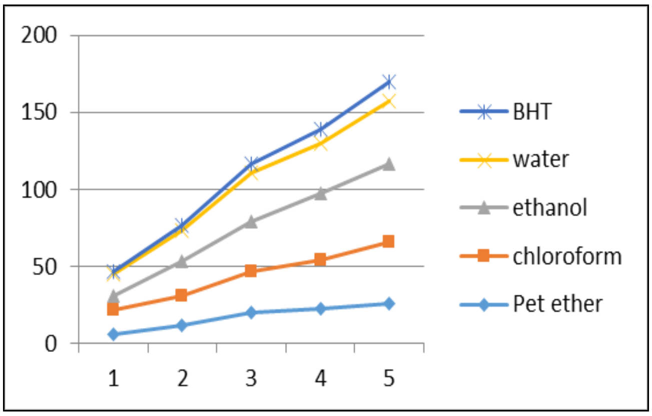

Fig 1: Graph showing percentage inhibition in different solvent extracts of *Malaxis rheedii*

Phosphomolybdenum assay

The basic principle to assess the antioxidant capacity through phosphomolybdenum assay includes the reduction of Mo (VI) to Mo (V) by the plant extract possessing antioxidant compounds. Phosphomolybdenum method evaluates the total antioxidant activity of the extracts and expressed as equivalents of Ascorbic acid (mg/g) at 300 µg/ml sample. While studying the Phosphomolybdenum complex formation in all the four extracts of the plant *Malaxis rheedii* maximum total antioxidant activity showed in petroleum ether extract and minimum in ethanol extract and it was 346.29±2.25 mg ascorbic acid equivalents /g sample and 268.15±.64 mg ascorbic acid equivalents /g sample respectively. Chloroform extract of *M. rheedii* showed an absorbance of 334.26±.32 mg ascorbic acid equivalents /g sample and aqueous extract showed 321.48±5.13 mg ascorbic acid equivalents /g sample.

Table 3: Phosphomolybdenum assay of different solvent extracts of *Malaxis rheedii*

Sample	**Absorbance** (mg/g)			
	Petroleum ether	**Chloroform**	**Ethanol**	**Water**
MR	346.29±2.25	334.26±.32	268.15±.64	321.48±5.13

Reducing power assay

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron (Ferreira, 2007). In this assay, the ability of extracts to reduce Fe^{3+} to Fe^{2+} was determined. The presence of antioxidants in the extracts resulted into reduction of the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}). In reducing power assay, antioxidants cause the reduction of the Fe^{3+} into Fe^{2+}, thereby changing the solution into various shades from green to blue, depending on the reducing power of the compounds (Shimada, 1992). Strong reducing agents, however, formed Perl's Prussian blue colour and absorbed at 700 nm. Figure 2 showed the reducing activities of various extracts of *Malaxis rheedii* in comparison with ascorbic acid as standard. The higher the absorbance of the reaction mixture, the higher would be the reducing power. Ethanol extract shows higher amount of electron donation. Reducing power of different extracts slightly increased with the concentration of the extract. Chloroform, petroleum ether and aqueous extracts showed reducing power capacity nearly same level. The reducing power of reference compound (Ascorbic acid) was found to be lower than all the tested extracts. While correlating these results to the total phenolic content of the plant, total phenolic content was highest in ethanol extract which could be responsible for the antioxidant activity.

The reducing power assay is often used to evaluate the ability of an antioxidant to donate an electron which is an important mechanism of phenolic antioxidant action (Mohamed *et al*, 2009) [13]. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts (Koleva, 2002; Benzie, 1999) [9, 1].

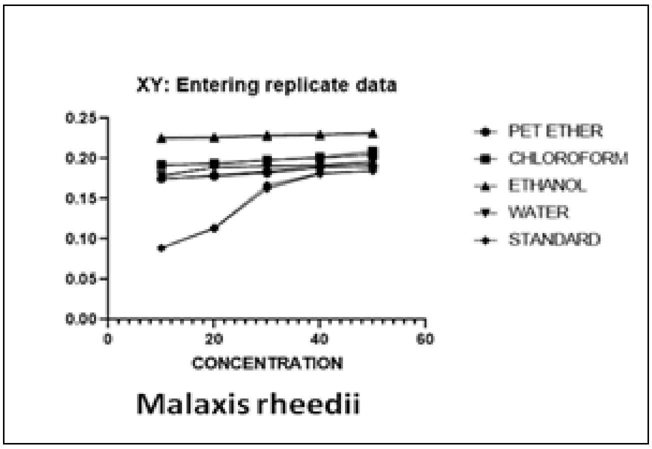

Fig 2: Reducing power assay different solvent extracts of *Malaxis rheedii*

Studies of antioxidant potential of medicinal orchid *Malaxis acuminata* by Bose *et al* and reported that a significantly higher antioxidant potential, DPPH, metal chelating, ABTS were observed in invitro derived and wild plants. The extract of entire plant of *Malaxis rheedei* were screened for different antioxidant assays *viz*., DPPH, ABTS•+ and ferrous ion chelating assay by Renjini Haridas *et al*., Higher levels of chelating ability for ferrous ions were observed in the crude methanol and ethyl acetate extract of *Malaxis rheedei*. Petroleum ether extract also shows maximum antioxidant activity in DPPH assay and ABTS•+ assay. The results suggest that the plant *Malaxis rheedii* has promising antioxidant potential and could serve as potential source of natural antioxidants.

Conclusion

The study is focused on the medicinal plant *Malaxis rheedii* of the family Orchidaceae. The plant is growing in sacred groves and local gardens of Payyanur region of Kannur district, Kerala, India and used for traditional healing practices. Orchidaceae family includes many plants having medicinal properties but most of them were not studied or exploited yet. To assess the antioxidant activity of a compound it is important to calculate the reducing power of a compound. Presence of reductone is related with the reducing power capacity of extract and one of the key mechanisms for action of antioxidants. In the plant *M. rheedii* all the concentrations show maximum activity in ethanol extract and least activity in Petroleum ether extract. Total antioxidant potential is calculated by Phosphomolybdenum assay and maximum activity in petroleum ether extract. In the case of DPPH radical scavenging assay aqueous extract of the plant shows higher activity than all other extracts. These results suggest that the plant *Malaxis rheedii* can constitute a source of natural compounds with antioxidant ability.

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