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Determination of total antioxidant activity of *Malaxis rheedii* Sw (Orchidaceae)

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Abstract
Malaxis genus is distributed throughout the world. *Malaxis rheedii* sw is a rare, terrestrial, endangered and medicinal orchid. In the study, investigated antioxidant activities of different solvent extract from the *M. rheedei* whole plant part. The whole plant extract of *M. rheedei* were screened for various antioxidant assays viz., DPPH, $ABTS^{\bullet+}$ and ferrous ion chelating assay. Higher levels of chelating ability for ferrous ions was observed in the crude methanol and ethyl acetate extract of *M. rheedei*. Petroleum ether extract also exhibited maximum antioxidant activity in DPPH activity and $ABTS^{\bullet+}$ activity. The results suggest that *M. rheedei* has promising antioxidant activity and could serve as potential source of natural antioxidants.

Keywords: *Malaxis rheedii* Sw, Orchidaceae, Diphenyl-1-picryl hydrazyl (DPPH) method, $ABTS^{\bullet+}$ radical cation scavenging activity and Chelating effects on ferrous ions

1. Introduction
Antioxidants are widely used as ingredients in dietary supplements and are exploited to maintain health and prevent oxidative stress-mediated disease such as cancer, atherosclerosis, diabetics, inflammation and ageing, malaria, rheumatoid arthritis, neuro-degerative disorders has been recently understood more (Vasconcelos *et al.,* 2007) [8]. Mainly phenol and flavanoid compounds, believed to have more antioxidant activity than vitamin C, E and B-carotene is rapidly gaining attention (Olajuyigbe and Anthony, 2011) [4]. Natural products could be potential drugs for humans or livestock species and also these products and their analogues can act as intermediates for synthesis of useful drugs (Abeer *et al.,* 2014) [1]. Synthetic antioxidants such as Butylated Hydroxytoluene (BHT) and Butylated Hydroxyanisole (BHA) have recently been reported to be dangerous for human health. Thus; many antioxidants have been isolated from different plant materials and search for effective, non-toxic natural compounds with antioxidant activity that has been intensified (Gupta and Sharma, 2006) [3]. Therefore, looking for new, local sources of natural antioxidants.
In India orchids have been used in medicinal treatment since vedic period. The first record of Indian orchid *Malaxis rhedei* used in ayurvedic medicine is discussed in 'Charaka Samhita', a classic ancient Indian medicinal treatise written by Charaka in Sanskrit, a few thousand years ago (Sahaya *et al.,* 2013) [6]. In Ayurveda "Ashtawarga" a group of eight drugs is used for preparation of tonic such as "Chyavanprash" and consists of four orchid species out of which *M. rheedei* is also among them (Gowhar *et al.,* 2013) [2]. *Balavaidyas* of north Kerala are mostly folk practitioners from *peruvannan* community. They are mostly consulted for treating ailments of children such as bronchitis, epilepsy, etc. One of the main components of the medicines is *Pachilaperumal* (*M. rheedi*) (Unnikrishnan E, 2004) [7]. Similarly the whole plant part of *M. rheedei* is used by *Kattunayaka* tribes in Nilambur area, Malappuram district at Kerala. They are used *M. rheedei* Sw. (Orchidaceae) is one of the best medicines for against snake poisons, fever, joint pain and burns (Renjini *et al.,* 2016) [5]. Therefore the objective of this study was to analyse antioxidant capacities of various extracts (petroleum ether, chloroform, ethyl acetate and methanol) of *M. rheedei* (whole plant) using antioxidant testing DPPH, $ABTS^{\bullet+}$ radical cation scavenging activity and Chelating effects on ferrous ions assays.

2. Materials and Method
2.1 Preparation of Plant Extract
The fresh whole plant parts of *M. rheedei* were washed with tap water and shade dried for two month and powdered coarsely. Then they were finely powdered mechanically using pulverizer

and passed through 40 mesh sieve and stored in airtight containers. About 250g of powdered aerial and root were extracted in soxhlet apparatus with petroleum ether, chloroform, ethyl acetate and methanol. The extract was dried under reduced pressure at low temperature (40-50 °C). The last traces of the solvent were removed under vacuum drier and the solid mass obtained was stored at 4 °C until further use.

2.2 Diphenyl-1-picryl hydrazyl (DPPH) method

2.2.1 Principle

The absorption of the deep violet DPPH solution is measured at 550nm, after which absorption decreases due to decolourisation to a yellow-white colour, in the event of reduction. This decrease in absorption is stoichiometric according to the degree of reduction.

2.3 Chemicals and reagents

- DPPH solution: 22mg of DPPH (2,2-diphenyl-1-picryl hydrazyl) was accurately weighed and dissolved in methanol. The volume was made up to 100ml. From this stock solution, 18ml was taken and diluted to 100ml using methanol to obtain 100µM DPPH solutions.
- Standard solution: 105mg of ascorbic acid was weighed separately and dissolved in 5.0ml DMSO to get 21mg/ml solution. This was serially diluted with DMSO to get lower dilutions.
- DMSO.
- Methanol.

2.4 Procedure

Various concentrations of sample were mixed with 1ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH (0.2mM). The mixture was shaken vigorously and left to stand for 30 min, and the absorbance was measured at 517nm. The percentage inhibition was calculated according to the formula:

$$\% \text{ of inhibition} = (A_0 - A_1)/ A_0 \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance of the sample.

2.5 $ABTS^{\bullet+}$ radical cation scavenging activity (Re *et al.*, 1999)

2.6 Principle

$ABTS^{\bullet+}$ decolourization assay involves the generation of the $ABTS^{\bullet+}$ chromophore by the oxidation of $ABTS^{\bullet+}$ with ammonium per sulphate. It is applicable for both hydrophilic and lipophilic compounds. The scavenging activity of the plant extracts on $ABTS^{\bullet+}$ radical cation were measured at 734nm.

2.7 Reagents

- 7mM $ABTS^{\bullet+}$
- 2.45mM ammonium persulphate
- Ethanol
- $ABTS^{\bullet+}$ solution: 7mM $ABTS^{\bullet+}$ was mixed with 2.45mM ammonium persulphate and the mixture was allowed to stand in dark at room temperature for 12-16 hrs before use. $ABTS^{\bullet+}$ solution was diluted to an absorbance of 0.7±0.05 with ethanol at 734nm.

2.8 Procedure

The reaction was initiated by the addition of 1ml of diluted $ABTS^{\bullet+}$ to 10µl of different concentration of methanolic extract of sample and 10µl of methanol as control. The absorbance was read at 734nm and the percentage inhibition was calculated by the following equation.

$$\text{Inhibition } (\%) = (A_0 - A_1)/ A_0 \times 100$$

Where A_0 is the absorbance of control, A_1 is the absorbance of test compound.

2.9 Chelating effects on ferrous ions/metal chelating activity

2.9.1 Principle

Iron (II) chelating activity was measured by the inhibition of the formation of iron (II) ferrozine complex after preincubation of the samples. The Fe^{2+} was monitored by measuring the formation of ferrous iron-ferrozine complex against methanol blanks at 562nm.

2.9.2 Reagents

- 2mM Ferric chloride
- Methanol
- 5mM Ferrozine

2.9.3 Procedure

The reaction mixture contained 1ml of various concentrations of the extract, 0.1ml of 2mM ferric chloride and 3.7ml of methanol. The control contained all the reaction reagents except sample. The reaction was initiated by the addition of 0.2ml of 5mM ferrozine. After 10 mins at room temperature the absorbance of the mixture was determined at 562nm against blank. A lower absorbance of the reaction mixture was indicated as higher Fe^{2+} chelating ability. The capacity to chelate the ferrous ion was calculated by

$$\text{Chelation } (\%) = (A_0 - A_1)/ A_0 \times 100$$

Where, A_0 was the absorbance of the control and A_1 of the mixture containing the extract or the absorbance of a standard solution. IC_{50} value (µg extract/ml) is the effective concentration at which ferrous ions were chelated by 50%. EDTA was used as standard in the present experiment.

3. Result and Discussion

Different extracts like petroleum ether, chloroform, ethyl acetate and methanol extracts of the whole plant part of *M. rheedei* was evaluated for its antioxidant activity on different *in vitro* models like DPPH, $ABTS^{\bullet+}$ and ferrous ion chelating assay.

3.1 DPPH radical scavenging activity

The percentage of scavenging effect on the DPPH radical was accelerated with the increase in the concentrations of the extract from 50-250µg/ml. The petroleum ether extract showed 26.44, 34.6, 46.17, 51.41 and 73.96% scavenging activity. Chloroform extract showed 23.24, 37.80, 61.05, 62.19, 67.04% scavenging activity. Ethyl acetate showed 55.16, 73.24, 73.76, 74.89 and 76.54% scavenging activity. Methanol extract showed 33.16, 34.19, 45.55, 68.8 and 74.48% scavenging activity. Ethyl

acetate extract exhibited the maximum antioxidant activity while methanol and petroleum ether extracts exhibited moderate activity. Comparatively least activity was exhibited in chloroform extract. The result confirmed that the whole plant extract of *M. rheedei* has exhibited the highest ability to quench the DPPH radical (Table 1).

3.2 Ferrous ion chelating assay

The metal chelating activity was determined and it increases with the increase in the concentration of extract from 50-250µg/ml. The percentage of inhibition of the metal chelation varied from 5.91% (50 µg/ml) to 30.64% (250 µg/ml) in petroleum ether whole plant part extract. In the case of chloroform , ethyl acetate and methanol extract the percentage of inhibition of the metal chelation was varying from 3.49% (50 µg/ml) to 24.73% (250 µg/ml), 2.06% (50 µg/ml) to 69.62 (250 µg/ml) and 22.04% (50 µg/ml) to 45.69% (250 µg/ml), respectively (Table 2). Ethyl acetate extract exhibited the maximum antioxidant activity while methanol and petroleum ether extracts exhibited moderate activity. Comparatively least activity was exhibited in chloroform extract. Therefore it is apparent that the whole plant part extract of *M. rheedei* exhibited excellent chelating ability for ferrous ions and might afford protection against oxidative damage.

3.3 $ABTS^{\bullet+}$ radical scavenging activity

$ABTS^{\bullet+}$ assay measures the relative antioxidant ability to scavenge the radical ABTS. The $ABTS^{\bullet+}$ may be generated through all enzymic or a chemical oxidation reaction. This method involves the scavenging of secondary radicals. Different solvent extracts like petroleum ether, chloroform, ethyl acetate and methanol extracts of the whole plant part of *M. rheedei* were taken to examined for antioxidant activity. Petroleum ether extract exhibited the maximum antioxidant activity while chloroform and ethyl acetate extracts exhibited moderate activity. Comparatively least activity was exhibited in methanolic extract (Table 3). From the results obtained for DPPH, $ABTS^{\bullet+}$ and ferrous ion chelating assay the plant *M. rheedei* can be considered as a potential radical scavenging activity.

Table 1: DPPH scavenging activity of different solvent extracts of *M. rheedei.*

S. No.	Solvents	Concentration (µg/ml)	% of inhibition	IC_{50} Value (µg/ml)
1	Petroleum ether	50	26.44	170.06
		100	34.6	
		150	46.17	
		200	51.41	
		250	73.96	
2	Chloroform	50	23.24	159.23
		100	37.80	
		150	61.05	
		200	62.19	
		250	67.04	
3	Ethyl acetate	50	55.16	124.68
		100	73.24	
		150	73.76	
		200	74.89	
		250	76.54	
4	Methanol	50	33.16	155.27
		100	34.19	
		150	45.55	
		200	68.8	
		250	74.48	

Table 2: Effect of ferrous iron chelation in various extracts of *M. rheedei*.

S. No	Solvents	Concentration	% of inhibition
1	Petroleum ether	50	5.91
		100	8.06
		150	18.81
		200	27..95
		250	30.64
2	Chloroform	50	3.49
		100	9.13
		150	16.39
		200	22.84
		250	24.73
3	Ethyl acetate	50	2.06
		100	36.55
		150	40.05
		200	41.12
		250	69.62
4	Methanol	50	22.04
		100	35.21
		150	40.70
		200	43.54
		250	45.69

Table 3: Effect on $ABTS^{\bullet+}$ scavenging activity in various extracts of *M. rheedei*.

Solvents	Concentration(µl)	% of inhibition
Petroleum ether	50	5872.5±10.1
Chloroform	50	5616±21.1
Ethyl acetate	50	5781.3±10.1
Methanol	50	4789.1±10.1

4. Conclusion

In the present study, higher levels of DPPH activity and chelating ability for ferrous ions was observed in the ethyl acetate and methanolic crude extract of *M. rheedei* which might be due to the presence of biomolecules with pronounced antioxidant activity. $ABTS^{\bullet+}$ radical scavenging activity also accelerated with the increase in the concentrations of the extract from 50-250µg/ml. Petroleum ether extract also exhibited maximum antioxidant activity. Accordingly, it is suggested that the antioxidant ability of *M. rheedei* would be beneficial in protecting against the adverse effects of oxidative damage.

5. Reference

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