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PHYTOCHEMICAL EVALUATION AND *IN VITRO* FREE RADICAL SCAVENGING ACTIVITY OF SUCCESSIVE WHOLE PLANT EXTRACT OF ORCHID *COTTONIA PEDUNCULARIS*

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ABSTRACT
Cottonia peduncularis, the only flowering plant species of monotypic genus Cottonia, found in Western ghats of India and in Srilanka. The orchid have high medicinal importance but there are no reports on Cottonia peduncularis so the present investigation was designed to evaluate its phytochemical constituents and free radical scavenging activity of whole plant successive extracts. The phytochemical secondary metabolite screening of extracts revealed the presence of flavonoids and phenols. Based on the quantitative estimation studies it revealed that extracts have a good amount of flavonoids in methanol extract and phenols in acetone extract. Successive different solvent extracts were subjected to free radical scavenging activity studies, screened with very good free radical scavenging activity and IC_{50} value with acetone extract for DPPH assay and methanol extract showing highest scavenging activity and IC_{50} value for ABTS cation radical decolorization assay. The present investigation shows that Cottonia peduncularis has reservoir of flavonoids and phenols which can be used in development of therapeutic phytomedicine for the therapy and treatments.

KEY WORDS
Cottonia peduncularis, phytochemical, free radical scavenging activity, extracts, phytomedicine.

INTRODUCTION
Plants are the natural source of bioactive components. These bioactive components in plants are produced as secondary metabolites such as alkaloids, carbohydrates, flavonoids, tannins and phenols. Orchids are the most beautiful creation of the nature, consisting a group of flowering plants which are known from ancient times (Naganada *et al.* 2013). Orchids are economically important for their ornamental value but many orchids are used in traditional medicine treatment as a remedy for several ailments. Chinese Materia Medica stated that *Dendrobium* is used as a tonic, analgesic and anti-inflammatory substance, *Vanila planifolia* used in treating hysteria and other types of fever. *Dendrobium nobile* is used in freshly cut wounds for healing and *Eulophia noda* tubers are used for blood purification (Sahaya *et al*., 2012). In India also orchids have been used in medicinal treatment since vedic period. 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 *Flickingeria nodosa* is also among them (Kaushik, 1983).

Antioxidants may be defined as radical scavengers which protect the human body against free radicals that may cause anaemia, asthma, arthritis (Shwetha *et al.*, 2011). Antioxidants inhibit the oxidation of other molecules. Oxidation is chemical reaction that transfers electron or hydrogen from a substance to an

oxidising agent. Oxidation reaction produces free radicals, in turn these radical can start chain reaction. Antioxidant terminates the chain reaction by removing free radicals intermediates and inhibit other oxidation reaction. Based on the recent literature few orchids like *Flickingeria nodosa* (Dalz.) Seidenf. (Chhajed *et al.,* 2008, Nagananda *et al.,* 2013) and *Dendrobium aqueum* Lindl (Sourav *et al*., 2012) have shown the presence of good antioxidant activity; these are mainly due to presence of secondary metabolite like phenols, flavonoids, alkaloids and others which plays an important role in neutralising free radicals activity (Nagananda *et al.,* 2013) Currently, there is a growing interest towards natural antioxidants of plant resources. *Cottonia peduncularis*, the only flowering plant species of monotypic genus *Cottonia*, found in Western Ghats and is endemic to peninsular India and Srilanka. It is an epiphytic orchid that grows on the upper branches of trees. The flowers are clustered at the tip of the branched spike during March –June (**Fig.1, 2**). This thin spike can be almost 2 feet in length (Anand rao, 1998, Pridgeon *et al.,* 1999). There are no reports on the phytochemical profile and free radical scavenging activity studies on *Cottonia peduncularis* . So the present investigation was started with an aim to evaluate the phytochemical constituents and *in vitro* free radical scavenging activity of successive extracts of orchid *Cottonia peduncularis.*

Fig.1: Plant collected from natural habitat and maintained in the green house.

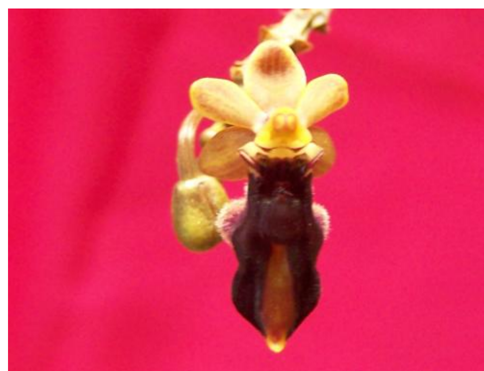

Fig. 2: Bloomed single flower.

MATERIALS AND METHODS

Preparation of the plant material: The plants were collected from natural habitat of Western Ghats and rinsed with distilled water to remove the dust particles. The water was removed by blotting over a filter paper. Then the plant materials were shed dried and powdered. Ten gram of powdered plant material was weighed and taken in muslin cloth and made into packets. The packets were used for successive extraction with soxhlet apparatus by using 4 different solvents namely petroleum ether, chloroform, acetone and methanol respectively.

Qualitative phytochemical screening:

The different qualitative chemical tests were performed for establishing phytochemical profile of hydro methanolic extracts obtained from cold and hot extractions. The tests for alkaloids, Saponins, Phytosterols, phenols, Tannins, glycosides, flavanoids were performed on all the extracts to detect various phytoconstituents present in them (Raaman, 2006, Nagananda *et al*., 2013).

Estimation of total flavonoid: The extracts were dissolved in DMSO and were estimated for total flavonoid content by aluminium chloride method (Zhishen *et al*, 1999) with absorbance measured at 510 nm with quercetin (100µg mL^{-1}) as the standard.

Estimation of total phenols: The extracts were dissolved in 5mLof distilled water and were estimated for total phenols by Folin-Ciocalteau reagent method (Sadasivam and Manickam, 1997) with absorbance measured at 650nm with catechol (50 µg mL-1) as the standard.

DPPH (1, 1-diphenyl-2-picrylhydrazyl) Free Radical Scavenging assay (Blois, 1958): Standard ascorbic acid and extracts (1 mg mL^{-1}) at various concentrations (10-50 µg) were taken and the volumes were adjusted to 100 µL with methanol. Five millilitre of a 0.1mM methanolic solution of DPPH was added and shaken

vigorously. The tubes were allowed to stand for 20min at 27°C. The absorbance of the sample was measured at 517nm. Methanol serves as a blank and the experiment was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula:

% DPPH radical scavenging activity = [(control OD – Sample OD)/ Control OD)] *100

ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolourization assay (Re *et al.*, 1999): ABTS radical cation ($ABTS^+$) was produced by reacting ABTS(7mM) with 2.45mM Ammonium persulfate and the mixture was allowed to stand in dark at room temperature for 12-16h before use. Standard ascorbic acid and sample extracts (1mg mL^{-1}) at various concentrations (10-50 µg) were taken and the volume was adjusted to 500µL with methanol and 500µL of methanol serves as blank. 300µL of ABTS solution was added; the final volume was made up 1mL with ethanol and incubated in dark for 30min at room temperature. The absorbance was read at 745nm and the experiment was performed in triplicate. Radial cation decolourization activity was expressed as the inhibition percentage of cation by the sample and was calculated using the formula:

% ABTS radical scavenging activity = [(control OD – Sample OD)/ Control OD)] *100.

IC_{50} value: IC_{50} value (concentration of sample required to scavenge 50% of free radical) were calculated from the regression equation, prepared from the concentration of the samples and percentage inhibition of free radical formation. Ascorbic acid was used as positive control and all tests were carried out in triplicate.

STATISTICAL ANALYSIS

The experiments were set up in a completely randomized design. All values obtained from the mean replicates to the variance and presented as mean± standard error (SE). Analysis of variance was conducted by two way ANOVA and the mean was compared by Tukey HSD test. All statistical analysis was performed at 1% significance level using IBM SPSS Statistics (version 20) by IBM.

RESULTS

Qualitative phytochemical screening: The different qualitative chemical tests were performed for establishing phytochemical profile of four extracts obtained from successive soxhlet extraction. Phytochemical screening was performed for four extracts which revealed the presence of flavonoids, phenols in different extracts (**Table 1**).

Qualitative estimation of phytochemicals: The quantitative estimation of phytochemicals, which were detected in phytochemical screening of *Cottonia peduncularis* revealed the presence of high phenol content (248.01µg/mL) was recorded in the acetone extract (**Fig.3**) (**Table-2**). High content of flavonoids (87.22µg/mL) was recorded in the methanol extract (**Fig. 4**) (**Table-2**).

Table 1: Phytochemical screening of four extracts of *Cottonia peduncularis*

Phytochemical Screening	PE	C	A	M
Alkaloids				
Mayer's	-	-	-	-
Wagners	-	-	-	-
Hager's	-	-	-	-
Dragendroff's	-	-	-	-
Saponins				
Foam Test	-	-	-	-
Phytosterols				
Liebemann-Burchards	-	-	-	-
Phenols				
Ferric chloride	+	+	+	+
FC reagent	+	+	+	+
Flavonoids	+	+	+	+
Glycosides	-	-	-	-

Note: PE-Peroleum ether, C: Chloroform, A: Acetone, M: Mehanol

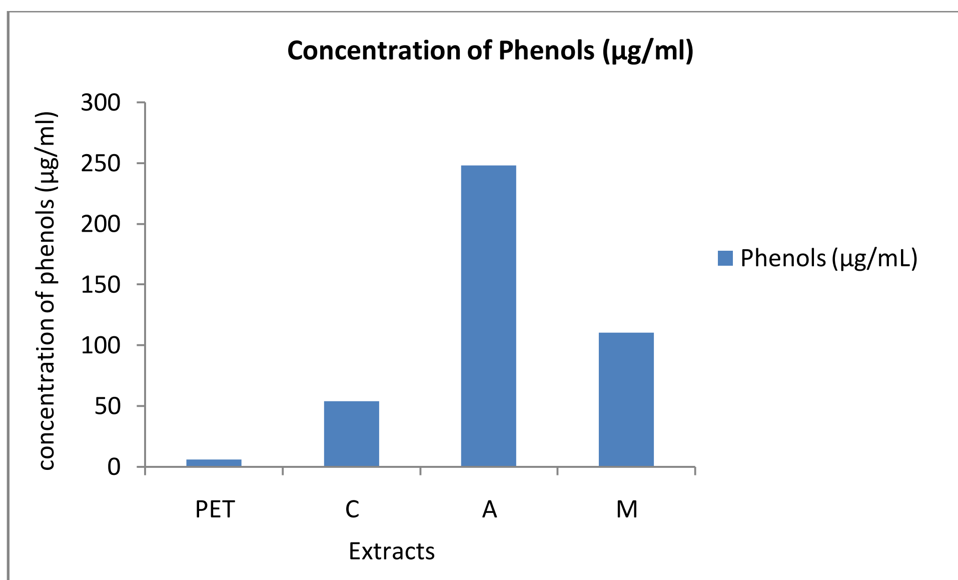

Fig. 3: Concentration of Phenols (µg/mL) present in different extracts.

Table 2: Quantitative estimation of phytochemicals

Extracts	Phenols (µg/ml) X*± SE	Flavonoid (µg/ml) X*± SE
PE	5.73±0.034	47.00±0.017
C	54.00±0.080	76.87±0.014
A	248.01±0.127	54.16±0.012
M	110.62±0.046	87.22±0.028

Note: * - Mean of 3 replications, **SE**- Standard Error.

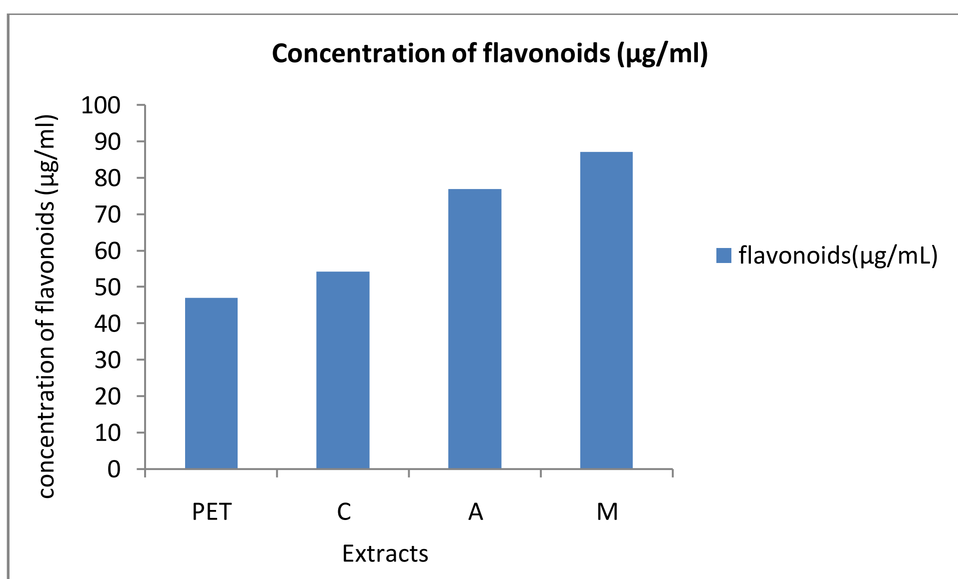

Fig. 4: Concentration of flavonoids (µg/mL) present in different extracts.

DPPH free radical scavenging activity assay: Free radical scavenging potential of extracts at different concentration was tested by DPPH method. Highest percentage of scavenging activity and IC_{50} value were found to be 53.18% and 26.79µg/mL respectively for acetone extract. The standard ascorbic acid showed the percentage of scavenging activity and IC_{50} values as 70.27% and 15.03µg/mL (**Fig.5)** (**Table 3**).

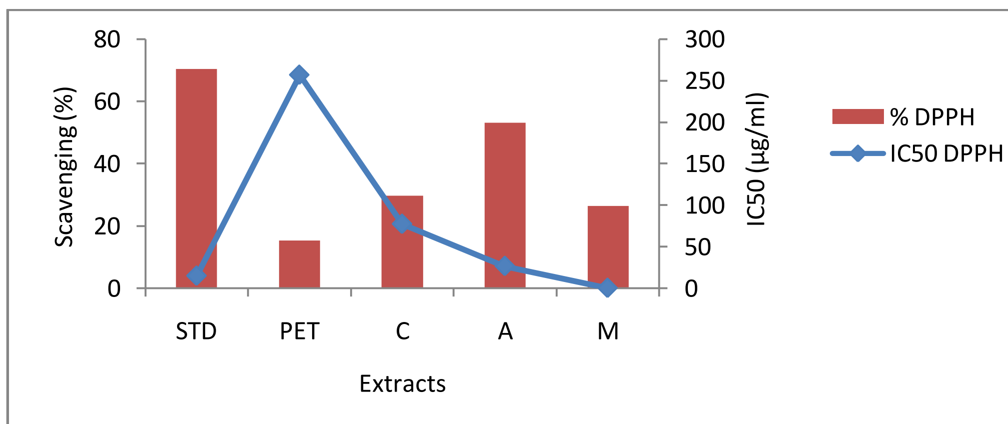

Fig. 5: DPPH scavenging activity of different extracts and its IC_{50} (µg/mL)

Table 3: Percentage of scavenging activity and IC_{50} value of different extracts against different assays

Extracts	DPPH% of Scavenging	ABTS% of Scavenging	DPPH IC_{50} (µg mL^{-1})	ABTS IC_{50} (µg mL^{-1})
STD	70.27[a]	52.082[a]	15.03[a]	55.66[c]
PE	15.35[e]	24.32[d]	257[e]	204.9[e]
C	29.83[c]	44.94[b]	77.51[c]	62.1[d]
A	53.18[b]	36.31[c]	26.79[b]	46.78[b]
M	26.45[d]	52.02[a]	86.29[d]	27.73[a]

Note: Mean of 15 replicate. Mean values with different superscripts ([a, b, c, d, e, f, g]) differ significantly at P<0.01 by Tukey (HSD) test

ABTS free radical scavenging activity assay:
Radical cation decolourisation activity of extracts at different concentrations was tested by ABTS method. Highest percentage of scavenging activity and IC_{50} value were found to be 52.02% and 27.73µg/mL respectively for methanol extract. The standard ascorbic acid showed the percentage of scavenging activity and IC_{50} values as 52.082% and 55.66µg/mL. (**Table 3**) (**Fig. 6**)

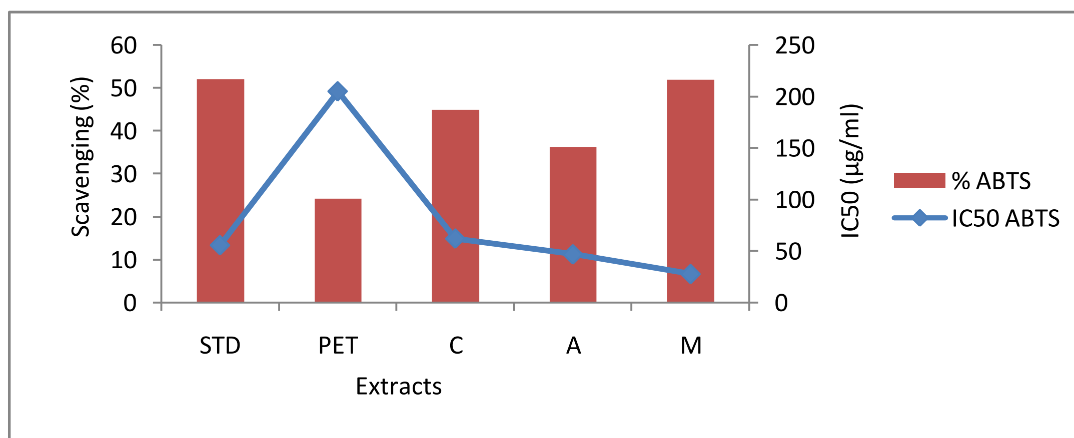

Fig. 6: ABTS scavenging activity of different extracts and its IC_{50} (µg/mL).

DISCUSSION

Nature is the source of medicinal agent and there are number of modern drugs have been isolated from natural sources. In the present investigation, successive soxhlet extraction was carried with different solvent (Petroleum ether, Chloroform, Acetone, Methanol) revealing the presence of flavonoids and phenols, showing to be the first report for phytochemical screening and free radical scavenging assay from different successive solvent extracts.

Free radicals are produced under certain environmental conditions and during normal cellular functions in the body. These molecules are losing an electron, by donating them. To neutralize this charge, free radicals try to withdraw an electron from, or donate an electron to, a neighbouring molecule. The newly created free radicals in turn, looks out for another molecule and withdraw or donate an electron, setting off a chain reaction that can damage hundreds of molecules. Antioxidant halts this chain reaction. Some antioxidants are themselves free radicals donating electron to stabilize and neutralize the dangerous free radicals which may cause cellular and subcellular damage by lipid membrane peroxidation, cellular denaturation of proteins and breakdown of DNA strands, disrupting cellular functions (Chanda *et al.*, 2011). Other antioxidants work against the molecules that form free radicals, destroying them before they can begin domino effect that leads to oxidative damage (Matill, 1947).

Phenolic the majority of the antioxidant activity may come from active substances such as flavonoid and Phenolic compounds are well known as Antioxidant and Scavenging agents against free radicals associated with oxidative damage (Norhazana *et al.*, 2012). The presence of these compounds such as Phenols and Flavonoids in *Cottonia peduncularis* extract may give credence to its local usage for the management of oxidative stress induced ailment. Flavonoids are the important secondary metabolites of the plant modulating lipid peroxidation involved in Thrombosis and Carcinogenesis (Mbaebie *et al.*, 2012).

Based on medicinal value and phytochemicals of the plants, antioxidant activity was evaluated from various extracts of *Cottonia peduncularis* by DPPH and ABTS free radical scavenging activity assay.

In DPPH free radical scavenging activity assay result showed that the Acetone extract of *Cottonia peduncularis* gives higher free radical scavenging activity followed by chloroform extract. Very less scavenging activity was seen in Methanol extract followed by Petroleum ether. In ABTS free radical scavenging activity assay result showed that the Methanol extract of *Cottonia peduncularis* gives higher free radical scavenging activity followed by Chloroform extract. Compare these both Methanol and Chloroform extract, Acetone and Petroleum ether extract showed very less free radical scavenging activity. The free radical scavenging activity increased as concentration increased in all the extracts which agree with the earlier findings (Thoudam Bhaigyabati, 2011).

CONCLUSION

In our present study we conclude that successive methanol and acetone extracts have potent antioxidant property than the petroleum and chloroform extract. The plant can be a source material to herbal drug industry. Plant derived phytochemicals have significant contribution to human health and that can be used for the development of therapeutic phytomedicines for the therapy and treatments.

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