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EVALUATION OF TOTAL PHENOLICS, FLAVONOID CONTENTS AND *IN-VITRO* ANTIOXIDANT PROPERTIES OF *CATHARANTHUS PUSILLUS* (APOCYNACEAE)

P. Y. Nithya [1], S. M. Jelastin Kala [2] and V. R. Mohan [*3]

Department of Chemistry [1], A.P.C. College for women, Thoothukudi, Tamilnadu, India.
Department of Chemistry [2], St. Xavier's College, Palaymkottai Tamilnadu, India.
Ethnopharmacology Unit [3], Research Department of Botany, V.O. Chidambaram College, Tuticorin-628008, Tamilnadu, India.

Keywords:

Ethanol, *Catharanthus pusillus*, DPPH, antioxidant, phenol, flavonoid

Correspondence to Author:
V. R. Mohan

Ethnopharmacology Unit,
Research Department of Botany,
V.O. Chidambaram College,
Tuticorin - 628008, Tamilnadu, India

Email: vrmohanvoc@gmail.com

ABSTRACT: Objective: Present study was aimed to evaluate the total phenolic, flavonoid content and *in vitro* antioxidant activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of whole plant of *Catharanthus pusillus*. **Methods:** Antioxidant activity have been tested using various antioxidant model systems *viz.*, DPPH, hydroxyl, superoxide, ABTS and reducing power. **Results:** Methanol extract of *Catharanthus pusillus* is found to possess higher DPPH, hydroxyl, superoxide radical scavenging activity, while ethyl acetate extract is found to possess higher ABTS radical cation scavenging activity. Methanol extract of whole plant of *Catharanthus pusillus* shows the highest reducing ability. **Conclusion:** The study indicates the significant free radical scavenging potential of *Catharanthus pusillus* whole plant which can be experimented for the treatment of various free radicals mediated aliments.

INTRODUCTION: The World Health Organization (WHO) has reported that approximately 80% of the World's population currently used as herbal medicine. The herbal drugs with easily accessible liquid such as water, milk to safe and reliable for human beings [1, 2]. Medicinal plants are of great importance to the health of individuals and communities in general. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds [3, 4].

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Antioxidant is a chemical compound or substance that inhibits oxidation. They are found in many foods. They work to keep our cells healthy by protecting them from damage by free radicals (molecules responsible for aging, tissue damage, and some disease). Oxidation results from everyday body functions such as breathing or walking, but certain processed and fatty foods, toxic substances, and sunlight can increase its effects. Antioxidants help repair damaged cells, which can prevent diseases, including cancer. A diet rich in a variety of plant-based foods provides all of the antioxidants the body needs. Research shows that vitamins, minerals, and phytochemicals from whole foods interact to boost their disease-fighting effects[5].

Catharanthus pusillus belonging to family Apocynaceae is known with various names in India and all over the world. It is widely used as various treatments of diseases and traditionally used as

herbal medicine [6]. The roots, leaves and latex of these plants are used to treat skin and liver diseases, leprosy, dysentery, worms, ulcers, tumor and ear aches. The leaf powder of *C.pusillus* were mixed with coconut oil and used for treat the antidandruff activity and also used to kill the lice [7].

The main purpose of this study was to evaluate total phenolic, flavonoids contents and *in vitro* antioxidant activity in whole plant of *C.pusillus.* However there is no information available on antioxidant activity of this plant.

MATERIALS AND METHOD:

Collection of plant material:

The whole plant of *C.pusillus* were collected from Pechiparai, Kanayakumari District, Tamil Nadu. With the help of local flora, voucher specimens were identified and preserved in the Ethnopharmacology unit, Research department of Botany, V.O. Chidambaram College, Thoothukudi, Tamil Nadu for further references.

Prepation of plant Extract:

The dried whole plant of *Catharanthus pusillus* were powdered in a Wiley mill.100 gm of whole plant powder was packed in a soxhlet apparatus and extracted with petroleum ether, benzene, ethyl acetate, methanol and ethanol. All these extacts were concentrated in a rotary evaporator. The concentrated extracts were used for antioxidant activity, methanol extract was used for the estimation of total phenolics and flavonoids.

Estimation of total phenolic content:

Total phenolic content was estimated using Folin-Ciocalteau reagent based assay as previously described [8] with little modification. To 1mL of each extract (100µg/mL) in methanol, 5mL of Folin-Ciocalteau reagent (diluted ten-fold) and 4mL (75g/L) of Na_2CO_3 were added. The mixture was allowed to stand at 20ºC for 30min and the absorbance of the developed colour was recorded at 765nm using UV-VIS spectrophotometer. 1mL aliquots of 20, 40, 60, 80, 100 µg/mL methanolic gallic acid solutions were used as standard for calibration curve. The absorbance of solution was compared with gallic acid calibration curve. The total phenolic content was expressed as gallic acid equivalents (GAE g/100g dry weight of extract).

Estimation of flavonoids:

The flavonoid content was determined according to Eom *et al* [9]. An aliquot of 0.5ml of sample (1mg/mL) was mixed with 0.1mL of 10% aluminium chloride and 0.1mL of potassium acetate (1M). In this mixture, 4.3ml of 80% methanol was added to make 5mL volume. This mixture was vortexed and the absorbance was measured spectrophotometrically at 415nm. The value of optical density was used to calculate the total flavonoid content present in the sample.

DPPH radical scavenging activity:

The DPPH is a stable free radical and is widely used to assess the radical scavenging activity of antioxidant component. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen donating antioxidant due to the formation of the non radical form DPPH-H.

The free radical scavenging activity of all the extracts was evaluated by 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) according to the previously reported method [10]. Briefly, an 0.1mM solution of DPPH in methanol was prepared, and 1mL of this solution was added to 3 mL of the solution of all extracts at different concentration (50,100,200,400 &800µg/mL).

The mixtures were shaken vigorously and allowed to stand at room temperature for 30 minutes. Then the absorbance was measured at 517 nm using a UV-VIS spectrophotometer (Genesys 10S UV: Thermo electron corporation). Ascorbic acid was used as the reference. Lower absorbance values of reaction mixture indicate higher free radical scavenging activity. The capability to scavenging the DPPH radical was calculated by using the following formula.

DPPH scavenging effect (% inhibition) = $\{(A_0 - A_1)/A_0)*100\}$

Where, A_0 is the absorbance of the control reaction, and A_1 is the absorbance in presence of all of the extract samples and reference. All the tests were performed in triplicates and the results were averaged

Hydroxyl radical scavenging activity:
The scavenging capacity for hydroxyl radical was measured according to the modified method of Halliwell [11]. Stock solutions of EDTA (1mM), $FeCl_3$ (10mM), Ascorbic Acid (1mM), H_2O_2 (10mM) and Deoxyribose (10 mM) were prepared in distilled deionized water. The assay was performed by adding 0.1mL EDTA , 0.01mL of $FeCl_3$**,**0.1mL H_2O_2, 0.36mL of deoxyribose, 1.0mL of the extract of different concentration (50,100,200,400 &800µg/mL) dissolved in distilled water,0.33mL of phosphate buffer (50mM, pH 7.9), 0.1mL of ascorbic acid in sequence. The mixture was then incubated at 37^0C for 1 hour. 1.0mL portion of the incubated mixture was mixed with 1.0mL of 10%TCA and 1.0mL of 0.5% TBA (in 0.025M NaOH containing 0.025% BHA) to develop the pink chromogen measured at 532nm. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Superoxide radical scavenging activity:
The superoxide anion scavenging activity was measured as described by Srinivasan *et al* [12]. The superoxide anion radicals were generated in 3.0 ml of Tris – HCL buffer (16 mM, P^H 8.0), containing 0.5 mL of NBT (0.3mM), 0.5 mL NADH (0.936mM) solution, 1.0 mL extract of different concentration (50,100,200,400 & 800µg/mL), and 0.5 mL Tris – HCl buffer (16mM, P^H 8.0). The reaction was started by adding 0.5 mL PMS solution (0.12mM) to the mixture, incubated at 25^oC for 5 min and the absorbance was measured at 560 nm against a blank sample, ascorbic acid. The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula

Antioxidant activity by radical cation (abts. +):
ABTS assay was based on the slightly modified method of Huang *et al* [13]. ABTS radical cation (ABTS+) was produced by reacting 7mM ABTS solution with 2.45 mM potassium persulphate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS + Solution were diluted with ethanol to an absorbance of 0.70+0.02 at 734 nm. After addition of sample or trolox standard to 3.9 mL of diluted ABTS+ solution, absorbance was measured at 734 nm by Genesys 10S UV-VIS (Thermo scientific) exactly after 6 minutes. Results were expressed as trolox equivalent antioxidant capacity (TEAC). The percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Reducing power:
The reducing power of the extract was determined by the method of Kumar and Hemalatha [14]. 1.0 mL of solution containing 50,100,200,400 &800µg/mL of extract was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH6.6) and potassium ferricyanide (5.0 mL, 1.0%): The mixture was incubated at 50^oC for 20 minutes. Then 5mL of 10% trichloroacetic acid was added and centrifuged at 980 g (10 minutes at 5^oC) in a refrigerator centrifuge. The upper layer of the solution (5.0 mL) was diluted with 5.0 mL of distilled water and ferric chloride and absorbance read at 700 nm. The experiment was performed thrice and results were averaged.

Statistical analysis:
Antioxidant activities like DPPH radical scavenging activity, hydroxyl radical scavenging activity, superoxide radical activity, ABTS radical cation scavenging activity and reducing powers were estimated in triplicate determinations. Data were analyzed using the statistical analysis system SPSS (SPSS software for windows release 17.5; SPSS Inc., Chicago IL, USA) Estimates of mean, standard error for aforesaid parameters were calculated.

RESULT:
Total phenolic content and total flavonoid content: The total phenolic content and total flavonoid content of the methanol extract of whole plant were found to be 0.621 A and 0.996 A.

DPPH radical scavenging activity:
DPPH radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extracts of whole plant *C. pusillus* was shown in **Fig.1**. The scavenging effect increases with the concentration of standard and plant extracts. Among the solvent tested methanol extract exhibited highest DPPH radical scavenging activity.

At 800µg/mL concentration methanol extract of *C. pusillus* whole plant possessed 113.96% scavenging activity a DPPH. IC_{50} values of methanol extract whole plant *C. pusillus* and standard ascorbic acid were 38.72µg/mL and 31.75µg/mL respectively. (**Table 1**)

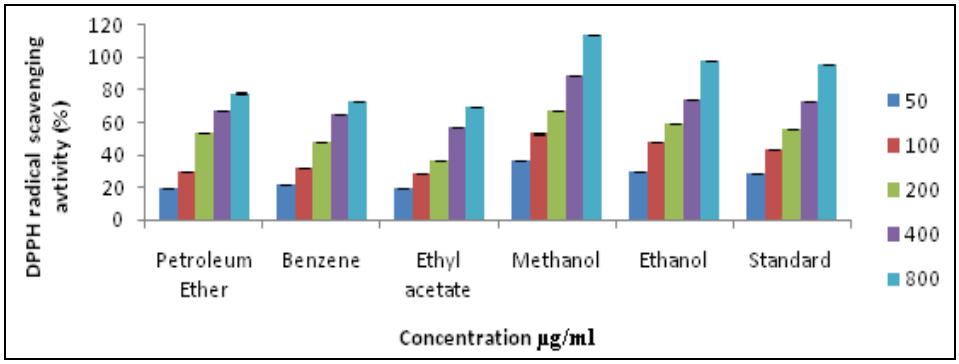

FIG. 1: DPPH RADICAL SCAVENGING ACTIVITY OF DIFFERENT EXTRACTS OF WHOLE PLANT OF *C .PUSILLUS*

Hydroxyl radical scavenging activity:

Hydroxyl radical scavenging activity of petroleum ether, benzene, ethyl acetate, methanol and ethanol extract of *C. pusillus* whole plant was shown in **Fig. 2**. Methanol extract showed very potent activity. At 800µg/ml concentration, methanol extract of *C.pusillus* whole plant possessed 131.16 % scavenging activity on hydroxyl radical. IC_{50} values of methanol extract of whole plant of *C. pusillus* and standard ascorbic acid were 31.22 µg/mL and 43.93µg/mL respectively.

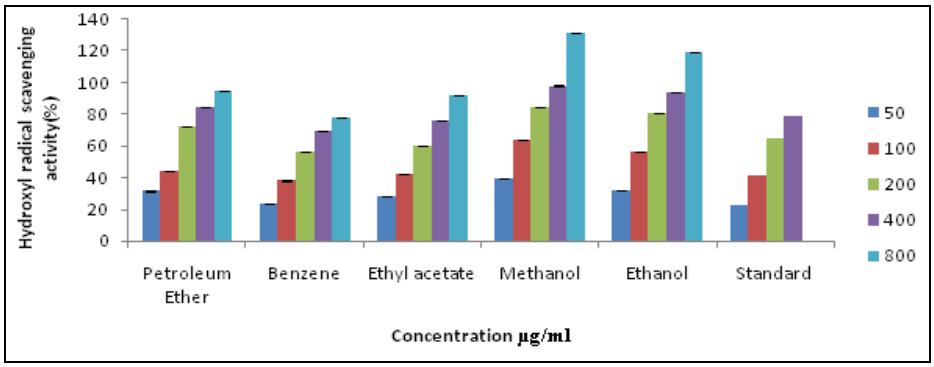

FIG. 2: HYDROXYL RADICAL SCAVENGING ACTIVITY OF DIFFERENT EXTRACTS OF WHOLE PLANT OF *C .PUSILLUS*

Superoxide radicial scavenging activity:

The different solvent extracts of *C. pusillus* whole plant extract were subjected to the superoxide radical scavenging activity and the results were shown in **Fig. 3**. It indicated that methanol extract of whole plant (800µg/ml) exhibited the maximum superoxide radical scavenging activity of 136.81%. The IC_{50} values of methanol extract and standard ascorbic acid were 38.55µg/mL and 31.75µg/mL respectively.

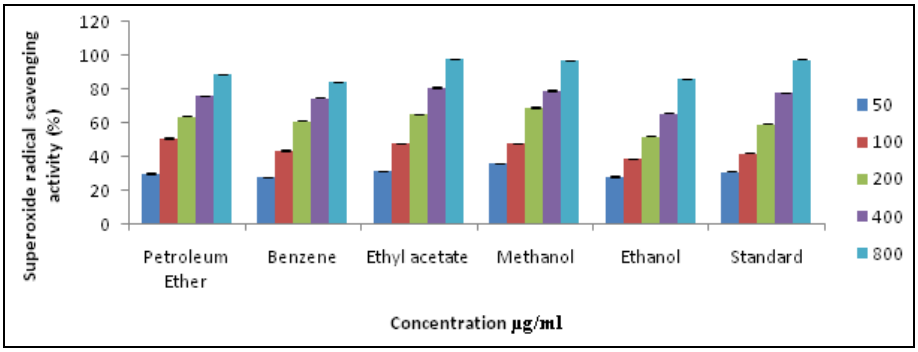

FIG. 3: SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF DIFFERENT EXTRACTS OF WHOLE PLANT OF *C.PUSILLUS*

ABTS radical cation Scavenging activity:

The *Catharanthus Pusillus* whole plant extract were subjected to the ABTS radical cation scavenging activity and the results were presented the **Fig.4**. The ethyl acetate extract exhibited potent ABTS radical cation scavenging activity in concentration dependent manner. At 800µg/mL concentration, *C.pusillus* whole plant possessed 98.33% scavenging activity in ABTS. The IC_{50} values of methanol extract and standard trolox were 39.63µg/mL and 37.64µg/mL respectively.

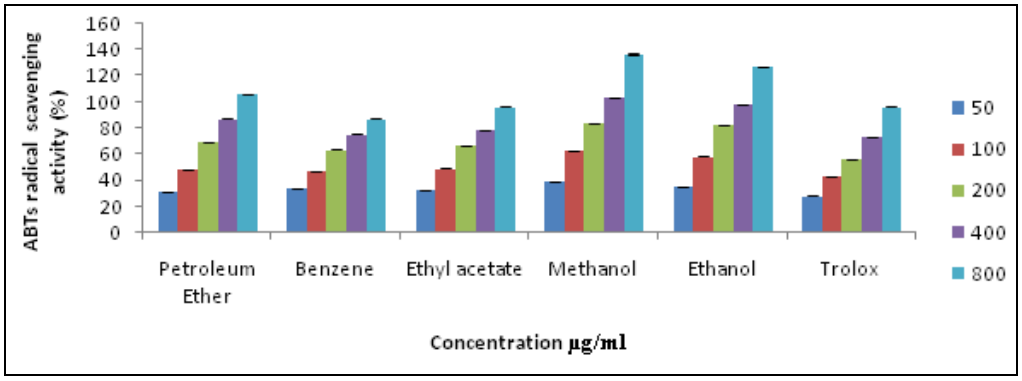

FIG. 4: ABTS RADICAL SCAVENGING ACTIVITY OF DIFFERENT EXTRACTS OF WHOLE PLANT OF *C. PUSILLUS*

Reducing Power:

Fig.5 shows the reducing ability of different solvent extracts of *C. pusillus* whole plant compared to ascorbic acid. Absorbance of the solution was increased when the concentration increased. A highest absorbance indicated a higher reducing power. Among the solvent tested, methanol extract exhibited higher reducing activity. (0.593 OD).

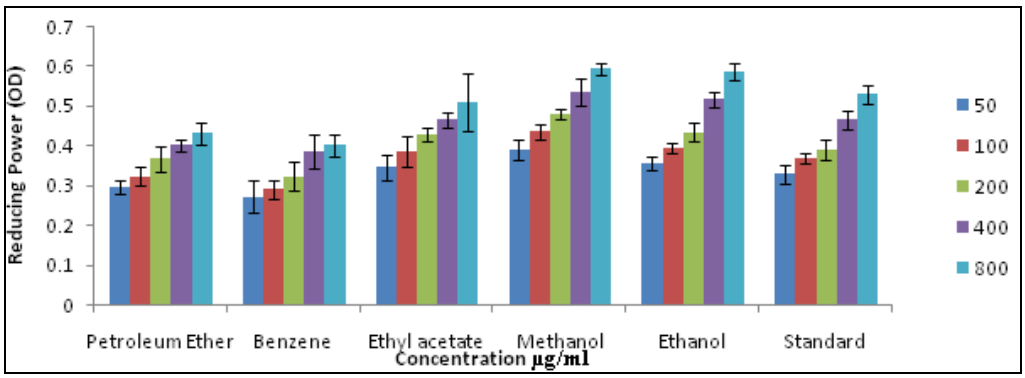

FIG. 5: REDUCING POWER OF DIFFERENT EXTRACTS OF WHOLE PLANT OF *C. PUSILLUS*

TABLE1: IC_{50} VALUES OF DIFFERENT SOLVENT EXTRACTS OF WHOLE PLANT OF *C. PUSILLUS*

Solvent	IC_{50} (µg/mL)			
	DPPH	Hydroxyl	Superoxide	ABTS
Petroleum ether	28.83	34.86	35.24	34.55
Benzene	26.88	26.54	30.88	31.05
Ethyl acetate	25.19	32.39	32.36	39.11
Methanol	38.72	43.93	38.55	39.63
Ethanol	32.98	39.65	37.92	32.86
Ascorbic acid	31.75	31.22	31.75	-
Trolox	-	-	-	37.84

DISCUSSION: The antioxidant properties of the different solvent extracts of *C. pusillus* whole plant were significantly corroboralated by the phytochemical constituents of the extracts. In general the phenolic compound are also found in many plants, These compounds composes of an aromatic ring bearing one or more hydroxyl groups and their structures may range from that of a simple phenolic molecule to a complex high-molecular weight polymer [15]. The antioxidant activity of

phenolic compounds depends on their structure, in particular the number and positions of the hydroxyl groups and the nature of substitutions on the aromatic rings. Antioxidant activity of phenolic compounds is based on their ability to donate hydrogen atoms to free radicals.

Flavonoids are important secondary metabolites of plant modulating lipid peroxidation involved in atherogenesis, thrombosis and carcinogenesis. It has been confirmed that pharmacological effects of flavonoids is correlating with their antioxidant activities [16]. Free radicals and other reactive species are thought to play an important role in many human diseases. Radical scavenging activities are very important due to the deleterious role of free radicals in biological systems. Many secondary metabolites which include flavonoids, phenolic compounds etc serve as sources of antioxidants and do scavenging activity [17]. The presence of high phenolic and flavonoid content in the methanol extract of *C. pusillus* whole plant has contributed directly to the antioxidant activity by neutralizing the free radicals.

In the present study, the different solvent extracts of whole plant *C.pusillus* possess free radical scavenging activity with different *in vitro* models viz DPPH, hydroxyl , superoxide and ABTS radical cation scavenging activity.

The DPPH test provided information on the reactivity of test compounds with a stable free radical. Because of its odd electron, DPPH gives a strong absorption band at 517nm in visible spectroscopy. The efficacies of antioxidants are often associated with their ability to scavenge stable free radicals [18]. In the DPPH assay, the methanol extract of *C.pusillus* exhibited high scavenging activity (113.96%). A higher DPPH radical scavenging activity is associated with the lower IC_{50} value (38.72µg/ml). Hydroxyl radical is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane phospholipids and causes damage to cell [19]. IC_{50} values of methanol extract of whole plant of *C. pusillus* and *pusillus* whole plant found to be contain a noticeable amount of total phenolics and flavonoids which plays a major role in controlling standard ascorbic acid were 31.22 µg/mL and 43.93µg/mL respectively. The hydroxyl radical scavenging activity may be due to the presence of various phytochemicals including polyphenols and flavonoids in *C. pusillus* whole plant extract.

Although superoxide anion is a weak oxidant, it gives rise to generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress [20]. Numerous biological reactions generate superoxide anions which are highly toxic species. Therefore, whole plant *C. pusillus* was undertaken to test whether it has scavenging activity of superoxide anions. The IC_{50} values of methanol extract and standard ascorbic acid were 38.55 µg/mL and 31.75µg/mL respectively. The results clearly indicate that the *C. pusillus* whole plant extracts have a noticeable effect as scavenging superoxide radical.

The decolorization of ABTs radical reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to inactivate this radical species. The ABTs radical cation is generated from the reaction of ABTs with potassium persulfate overnight in water [21]. The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical. The IC_{50} values of ethyl acetate extract and standard ascorbic acid were 39.63µg/mL and 37.64µg/ml respectively.

The reducing capacity of a compound may serve as an important indicator of its potential antioxidants activity [22] .The reducing activity of a compound generally depends on the presence of reductases which have been exhibited antioxidant potential by breaking the free radical chain, donating a hydrogen atom [23]. The reducing capacity of *C. pusillus* whole plant is a significant indicator of this potential antioxidant activity.

CONCLUSION: The methanol extracts of *C. pusillus* whole plant showed strong antioxidant activity of inhibiting DPPH, hydroxyl and superoxide scavenging activity when compared with standard ascorbic acid. In addition, the *C.* antioxidants. Although the antioxidant activities found *in vitro* experiments were only indicative of the potential health benefit, these results remain

important as the first step in screening antioxidant activity of *C. pusillus* whole plant.

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