

## ESTIMATION OF TOTAL PHENOLICS, FLAVONOIDS AND TANNIN CONTENTS AND EVALUATION OF *IN VITRO* ANTIOXIDANT PROPERTIES OF *CISSUS SETOSA* ROXB.

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### ABSTRACT

Estimation of certain phytochemical constituents and to evaluation of *in vitro* antioxidant activity of methanolic extracts of aerial parts of the medicinal herb, *Cissus setosa* were carried out. The study revealed that the total phenolics, tannins and flavonoids contents were 78mg GAE/g extract, 30mgTAE/g extract and 2mg RE/g extract respectively. The potential antioxidant activities for the extract by assaying reducing power activity and DPPH<sup>•</sup>, OH<sup>•</sup>, NO<sup>•</sup>, O<sub>2</sub><sup>•-</sup> scavenging activities and metal chelating activity indicate that *C. setosa* has strong biological activity and can be used as promising resource for manufacturing drugs with antioxidant property.

**Keywords:** *Cissus setosa*, Antioxidant activity, Total phenol, Flavonoids, Tannin.

### INTRODUCTION

Medicinal plants are the source for a wide variety of natural products among which the phenolics acids and flavonoids are very interesting for their antioxidant properties [1,2]. An antioxidant can be broadly defined as any substance that delays or inhibits oxidative damage to a target molecule [3]. It plays a major role in preventing oxidative damage, when it occurs it results in cancer, cardiovascular disease and diabetes in living beings [4]. Hence, research should focus on to improve the natural antioxidant from the natural resources.

*Cissus setosa* is a prostate herb belongs to the family, Vitaceae. It harbours many medicinal uses and as prescribed for various ailments in traditional medical practices of Tamil Nadu[5]. The macerated leaves are used as a poultice to promote suppuration and to aid in the extraction of the guinea worm [6]. Leaf extract is given to drink for 2 days to expel the intestinal worms [7] and used for washing cattle and vessels also [8]. The leaves of *C. setosa* along with the leaves of *Caryrattia pedata* are roasted and oiled and then applied to boils to bring about suppuration [9]. Despite these therapeutic uses, little or no scientific work has been carried out in this species so far. To address this lacuna, the present attempt was made to estimate the total phenolics, flavonoids and tannins contents and to the evaluate antioxidant activities of methanolic extract of *C. setosa* through various *in vitro* models.

### MATERIALS AND METHODS

#### Chemicals and reagents

2,2'-bipyridyl, 2,2-diphenyl-1-picryl-hydrazyl (DPPH<sup>•</sup>), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), ethylenediamine tetra acetic acid (EDTA), ferric chloride (FeCl<sub>3</sub>), ferrozine, ferrous ammonium sulfate, folin-ciocalteu reagent, nash reagent, griess reagent, gallic acid, tannic acid, polyvinylpolypyrrolidone (PVPP), potassium ferricyanide (K<sub>3</sub>Fe(CN)<sub>6</sub>), rutin, sodium carbonate, sodium nitrite, sodium hydroxide, aluminium chloride, sodium nitro prusside, trichloroacetic acid (TCA) and riboflavin were of analytical grades and purchased from Sigma Life Sciences, Mumbai, India.

#### Plant collection and extraction

The aerial parts of the study species, *C. setosa* were collected from Palani hills, Tamil Nadu, India. The plant materials were shade dried, pulverized and extracted with pure methanol by a soxhlet apparatus. The obtained extracts were filtered and concentrated in a rotary vacuum evaporator at 45°C under reduced pressure, at last the concentrated extract was stored at 4°C until use.

### Quantitative estimations

#### Determination of total phenolics and tannins

The total phenolic content was determined according to the method described by Siddhuraju and Becker [10]. 10µL extract was taken in test tubes and made up to 1 mL with distilled water. Then 0.5 mL of Folin-Ciocalteu phenol reagent (1:1 with water) and 2.5 mL of sodium carbonate solution (20%) were added sequentially in each tube. Soon after vortexing the reaction mixture, the test tubes were placed in dark for 40 min and then read at 725 nm against the reagent blank. The analysis was performed in triplicate and the results were expressed as gallic acid equivalents.

Using the same extract, the tannin content was estimated after treatment with polyvinylpolypyrrolidone (PVPP) [11]. 100µg/mL of PVPP was taken in test tube and to this 1 mL distilled water and then 1 mL sample extract were added. The content was vortexed and kept in the test tube at 4°C for 4h. Then the sample was centrifuged (3000 rpm for 10 min) and the supernatant was collected. This supernatant has only simple phenolics other than tannins (the tannins would have been precipitated along with the PVPP). The free phenolic content of the supernatant was measured and expressed as tannic acid equivalents. From the above results, the tannin content of the sample was calculated as follows:

$$\text{Tannin (\%)} = \text{Total phenolics (\%)} - \text{Non-tannin phenolics (\%)}$$

#### Determination of total flavonoid content

The flavonoid content was determined by following the slightly modified colorimetry method described previously by Zhishen *et al.* [12]. A 0.5mL aliquot was mixed with 2mL of distilled water and subsequently with 0.15mL of 5% NaNO<sub>2</sub> solution. After 6 min, 0.15 mL of 10% AlCl<sub>3</sub> solution was added and allowed to stand for 6 min, and then 2mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5mL, and then it was thoroughly mixed and allowed to stand for another 15min. Absorbance of the mixture was determined at 510 nm and the results were expressed as rutin equivalent.

### *In vitro* antioxidant activities

#### Reducing power

The reducing power of methanolic extract of the study species was determined by the method described by Siddhuraju *et al.*[13]. The extract (200-1000µg) was mixed with 5 mL of 0.2M phosphate buffer (pH 6.6). To this, 5 mL of 1% potassium ferricyanide solution was added and the mixture was incubated at 50°C for 20 min. After incubation, 5mL of 10% TCA was added. The content was then

centrifuged at 1000 rpm for 10 min. The upper layer of the supernatant (5mL) was mixed with 5mL of distilled water and 0.5mL of 0.1% ferric chloride. The absorbance of the reaction mixture was read spectroscopically at 700 nm.

#### Free radical scavenging activity on DPPH•

The antioxidant activity was determined in terms of hydrogen donating or radical scavenging ability, using the stable radical DPPH, according to the method of Blois [14]. The sample extracts at various concentrations (200-1000 µg) were taken separately and the volume was adjusted to 100 µL with methanol. 5 mL of 0.1 mM methanolic solution of DPPH• was added and allowed to stand for 20 min at 27°C. The absorbance of the sample was measured at 517 nm. Percentage radical scavenging activity of the sample was calculated as follows:

$$\% \text{ DPPH radical scavenging activity} = (\text{Control OD} - \text{Sample OD} / \text{Control OD}) \times 100 \dots\dots (\text{Equ 1})$$

The sample concentration providing 50% inhibition (IC<sub>50</sub>) under the assay condition was calculated from the graph of inhibition percentage against sample concentration.

#### Hydroxyl radical scavenging activity

The scavenging activity of the methanolic extract of *C. setosa* on hydroxyl radical was measured according to the method of Klein *et al.* [15]. Different concentrations of the extract (200-1000 µg) were added with 1mL of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 mL of EDTA solution (0.018%), and 1mL of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 mL of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in a water bath. After incubation the reaction was terminated by the addition of 1mL of ice-cold TCA (17.5% w/v). 3mL of Nash reagent (75.0g of ammonium acetate, 3 mL of glacial acetic acid, and 2 mL of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the colour formed was measured spectroscopically at 412 nm against reagent blank. The per cent hydroxyl radical scavenging activity was calculated as per equation 1.

#### Nitric oxide radical scavenging activity

The nitric oxide scavenging activity was measured according to the method of Sreejayan and Rao [16]. 3mL of 10mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) was mixed with different concentrations (200-1000 µg) of solvent extracts and incubated at room temperature for 150 min. After incubation time, 0.5 mL of Griess reagent (1% sulfanilamide, 0.1% naphthylethylene diamine dihydrochloride in 2% H<sub>3</sub>PO<sub>4</sub>) were added. The absorbance of the chromophore formed was read at 546 nm. Per cent radical scavenging activity of the sample was calculated as per equation 1.

#### Superoxide radical scavenging activity

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich [17]. The assay was based on the capacity of sample to inhibit farmazan formation by scavenging the superoxide radicals generated in riboflavin-light-NBT system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT and various concentrations (200-1000 µg) of sample extracts. Reaction was started by illuminating the reaction mixture with sample extract for 90 seconds. Immediately after illumination the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminium foil. Identical tubes with reaction mixture kept in dark served as blank. The percentage inhibition of superoxide anion generation was calculated as per the equation 1.

#### Metal chelating activity

The chelation of ferrous ions by the extract was estimated by the method of Dinis *et al.* [18]. Briefly, 50µL of 2mM FeCl<sub>2</sub> was added to sample. The reaction was initiated by the addition of 0.2 mL of 5mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the

solution was thereafter measured at 562 nm and the results were expressed as EDTA equivalent.

#### Statistical analysis

All the values were expressed as mean ± standard deviation (SD) of three determinations and subjected to one-way analysis of variance (ANOVA) followed by post hoc Duncan's multiple range test using SPSS (version 9, SPSS Inc., Chicago, USA). P<0.05 was chosen as the criterion for statistical significance.

## RESULTS AND DISCUSSION

#### Quantitative estimations

The contents of total phenols, flavonoids and tannins in the methanolic extract of aerial parts of the study species, *C. setosa* were found to be in considerable quantity (Fig. 1). The estimated quantity of these secondary metabolites were 78.54 mg gallic acid equivalent (GAE)/g extract, 30.62 mg tannic acid equivalent (TAE)/g extract and 2.43 mg rutin equivalent (RE)/g extract respectively. As the phenolic compounds are known as powerful chain breaking antioxidants and contribute its role directly for antioxidative action [19], its richness in the study species, *C. setosa* shows the species may be the reliable source of natural antioxidants. In addition, the higher phenolics content present in this species, may accounts for multiple biological effects like anticancer, antiproliferative, antimicrobial, wound healing and antibacterial activities [20].

#### In vitro antioxidant activity

The reducing abilities of extracts generally depends on the presence of reductones. The reductants (i.e. antioxidants) apparently reduces the Fe<sup>3+</sup> to its Fe<sup>2+</sup> form, which can be monitored by measuring the formation of Perl's Prussian blue at 700nm[21]. Several reports indicated that the reducing power of bioactive compounds was associated with antioxidant activity [22,23]. Fig. 2a and 2b shows the reductive capabilities of *C. setosa* and it is comparable to that of the standards, BHA and BHT.

The evaluation of antioxidant power by DPPH radical scavenging activity has been widely in use for different plant extracts and foods [24]. DPPH is a stable free radical, which changes its colour from violet to yellow upon reduction by the process of electron donation. In this study, the ability of samples to scavenge DPPH radical was determined on the basis of their concentration providing per cent scavenging effect. The IC<sub>50</sub> value of methanolic extract of the aerial parts of *C. setosa* was found to be 240µg/mL. However, it was not comparable to that of the positive standards, BHA and BHT.

Hydroxyl radical (OH•) is one of the potent reactive oxygen species in the biological system. It reacts with polyunsaturated fatty acid moieties of cell membrane, phospholipids and cause damage to cell [25]. The scavenging ability of *C. setosa* on OH• was determined to be higher (57.03%) in higher concentration of extract, 1000µg (Table 1). It indicates that the extracts possess the ability to scavenge this reactive oxygen species effectively. However, the IC<sub>50</sub> value (329.71µg/mL) obtained was not comparable with certain positive controls.

Superoxide radical is known to be very harmful to cellular components as a precursor of more reactive oxidative species such as singlet oxygen and hydroxyl radicals. Furthermore, superoxide radical is considered to play an important role in the peroxidation of lipids [26,27]. In the present study, the inhibition of O<sub>2</sub><sup>••</sup> was found to be concentration dependent and the IC<sub>50</sub> value was 240µg/mL (Table 1).

Nitric oxide formed during their reduction with oxygen or with superoxide such as NO<sub>2</sub>, N<sub>2</sub>O<sub>4</sub>, N<sub>3</sub>O<sub>4</sub> is very reactive. These radicals are responsible for altering the structure and functional behavior of many cellular components. Nitric oxide is also implicated for inflammation, cancer and other pathological conditions [28]. The methanolic extract of aerial parts of *C. setosa* was reported to have higher NO• scavenging activity (46.94%) at the higher concentration of 1000 µg with the IC<sub>50</sub> value of 495.94µg/mL (Table 1).

Iron is an essential mineral for normal physiological activity of the

human body, but excess can cause cellular damage and injury. The ferrous ions are the most effective pro-oxidants in food systems, the good chelating effect would be beneficial and removal of free iron ion from circulation could be a promising approach to prevent oxidative stress induced disease [29]. The Fe<sup>2+</sup> chelating activity of

*C. setosa* shows potent chelating power of this species (76.49 mg EDTA equivalent/g extract) (Table 2).

Total phenol values were expressed as mg gallic acid/g extract; Tannin values were expressed as mg tannic acid/g extract; Total flavonoids values were expressed as mg rutin/g extract.

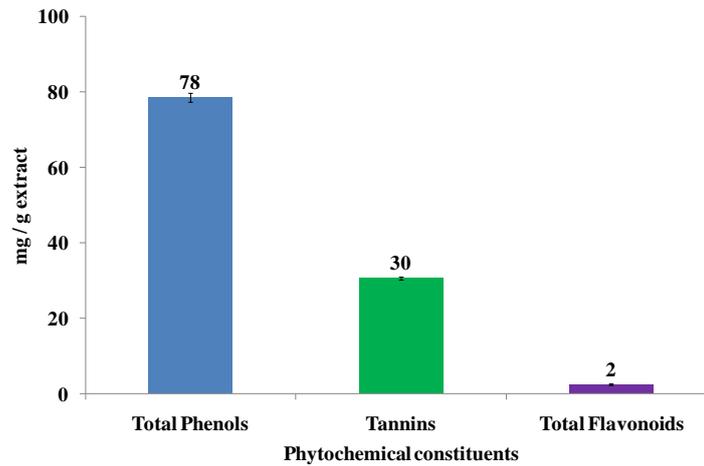


Fig 1: Total phenolics, tannins and flavonoids contents of *Cissus setosa*.

Values are mean ± standard deviation (SD) of three independent experiments.

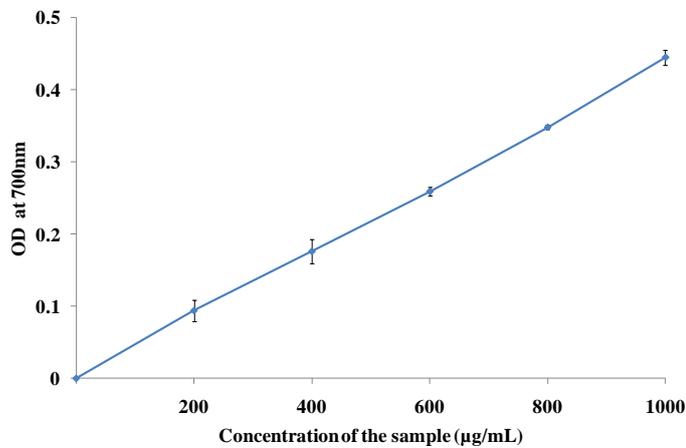


Fig. 2a: Reductive capability of methanolic extract of aerial parts of *Cissus setosa*

(Values are represented as mean±SD (n=3, P < 0.05))

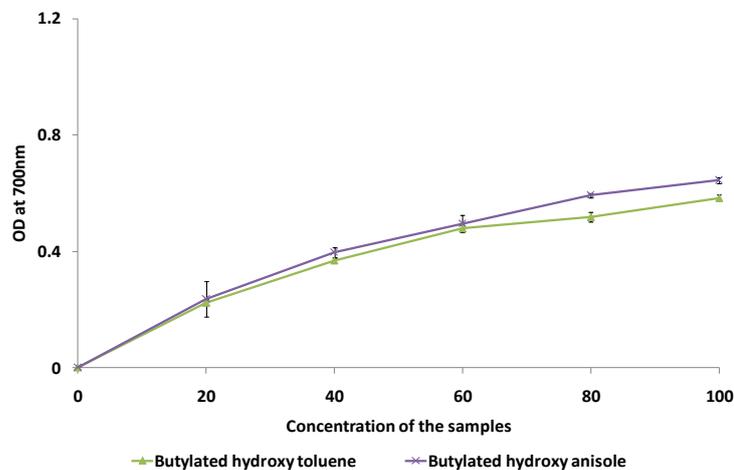


Fig . 2b: Reductive capability of standard antioxidants.

(Values are represented as mean±SD (n=3, P < 0.05))

Table 1: Radical scavenging ability of methanolic extract of aerial parts of *Cissus setosa*.

Concentration (µg) / Standards	DPPH <sup>•</sup>		OH <sup>•</sup>		O <sub>2</sub> <sup>•-</sup>		NO <sup>•</sup>	
	(%) activity	IC <sub>50</sub>	(%) activity	IC <sub>50</sub>	(%) activity	IC <sub>50</sub>	(%) activity	IC <sub>50</sub>
200	32.91 ± 0.28		5.29 ± 0.43		4.49 ± 0.25		2.43 ± 0.27	
400	44.04 ± 0.17		10.48 ± 0.57		27.91 ± 0.59		10.27 ± 0.27	
600	55.35 ± 0.27	240.01 ± 1.66	16.24 ± 0.59	329.71 ± 4.36	45.28 ± 0.40	240.40 ± 1.99	17.57 ± 0.27	495.94 ± 7.04
800	64.58 ± 0.13		30.12 ± 0.82		57.80 ± 0.42		34.23 ± 0.31	
1000	73.98 ± 0.10		57.03 ± 0.59		67.80 ± 0.33		46.94 ± 0.41	
BHA		21.4 ± 0.1		35.5 ± 1.9		-		52.7 ± 0.1
BHT		34.7 ± 0.3		45.6 ± 0.9		-		38.5 ± 1.0

Values are means of three independent analyses ± standard deviation (n = 3).

Table 2: Metal chelating ability of methanolic extract of aerial parts of *Cissus setosa*.

Sample	Metal chelating ability (mg EDTA equivalent/g extract)
<i>Cissus setosa</i>	76.49 ± 0.26

Values are means of three independent analyses ± standard deviation (n = 3).

## CONCLUSION

Evaluation of antioxidant properties of aerial parts of *C. setosa* in terms of scavenging activities of DPPH free radical, OH radical, NO radical and O<sub>2</sub><sup>•-</sup> radical proved the indigenous knowledge employed for healing various ailments by traditional health care practices in Tamil Nadu, India is most appropriate. Therefore, the species, *C. setosa* can be used as a potent source for the manufacturing of effective drugs.

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