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Phytochemistry and Free radical scavenging activity of Wattakaka volubilis (Linn. f.) Benth ex. Hook f. (Asclepiadaceae)- A rare and threatened medicinal plant.

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Abstact: The aim of the study is to investigate the antioxidant activity of various extracts of *Wattakaka volubilis* in different *in vitro* models. Methanol extracts of *Wattakaka volubilis* (whole plant) and it has showed significant dose dependent antioxidant activity, direct relationship between activity and concentration of extract. The extracts showed an effective free radical scavenging activity towards the total antioxidant activity, DPPH assay and superoxide radical scavenging assay with IC50 values of 31.69, 25.62, 36.46 µg/mL respectively. At 1000 µg/mL, in total antioxidant assay, DPPH radical scavenging assay and superoxide radical scavenging assay, it showed maximum inhibition of 88.33, 62.29, and 33.98% respectively. These results clearly indicate that *W. volubilis* is effective in scavenging free radicals and has potential to be powerful antioxidant ability.
Key words: *Wattakaka volubilis*, total antioxidant, DPPH, Superoxide radical scavenging activity.

INTRODUCTION

Free radicals and reactive oxygen species are by products in aerobic organism and also significant interest among scientists in the past decade. It has been reported that they could induce cellular damage and might be involved in several human diseases including cancer, arteriosclerosis, diabetic mellitus, hypertension, ischemia, reperfusion injury of many tissues, central nervous system injury, gastritis, cancer, AIDS and in aging processes [1,2,3,4]. Free radicals are known to have an important role in stimulation of phagocytosis, induction of drug detoxification pathways and stimulation of signal transduction pathways.

Therefore, antioxidants with free radical scavenging activities of medicinal plants may have great relevance in the prevention diseases and therapeutic properties. Phytoconstituents like flavonoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity [5, 6]. Generally, medicinal plants could be a potential source of natural antioxidants [7]. The present study focused on free radical scavenging potential of the whole plant of *Wattakaka volubilis* by different *in vitro* methods viz. total antioxidant assay, DPPH activity and superoxide radical scavenging assay and

also evaluated the phytochemical constituents by qualitative and quantitative methods.

MATERIALS AND METHODS

The whole plant of *Wattakaka volubilis* was collected from Velliangiri hills, Coimbatore district Tamilnadu (India) and authenticated by a taxonomist, M. Murugaesan, SACON, Coimbatore. The collected material was washed thoroughly, shade dried and powdered coarsely. The powder obtained (250 g) was extracted successively with petroleum ether, ethyl acetate, chloroform, ethanol and methanol in a soxhlet apparatus for 18-20 hrs. The extract was concentrated using rotary flash evaporator and preserved at 4˚C in air tight container. The extract also was subjected to qualitative and quantitative chemical tests for the identification of various phytoconstituents followed by the method of Trease and Evans [8], Harborne[9] and Sofowora[10].

TOTAL ANTIOXIDANT CAPACITY

To 1 ml of the various extract of *Wattakaka volubilis* of different concentrations (125, 250, 500, and 1000 µg/mL,) was treated with 1ml of reagent solution (0.6M sulphuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate) in eppendrof tube. Capped tubes were incubated in thermal block at 95˚c for 90 min. After cooling to room temperature, the absorbance was measured at 695nm against blank. Ascorbic acid was used as reference compound [11].

DIPHENYL-1-PICRYL HYDRAZYL (DPPH) METHOD

Various concentrations of sample plant extracts (125, 250, 500, and 1000 µg/mL) were mixed with 1 mL of DPPH solution (0.2 mM/mL methanol). The mixture was shacked vigorously and left to stand in the dark until stable absorption values were obtained. The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. DPPH scavenging effect was calculated as percentage of DPPH discoloration using the equation: % scavenging effect = [(ADPPH -AS)/ ADPPH] × 100, where AS was the absorbance of the solution when the sample extract had been added at a particular level and ADPPH was the absorbance of the DPPH solution. The extract concentration providing 50% inhibition (IC50), was calculated from the graph of scavenging effect percentage against extract concentration. Ascorbic acid was used as reference compound [12].

SCAVENGING OF SUPEROXIDE RADICAL BY RIBOFLAVIN-NBT-SYSTEM

The method involves generation of superoxide radical of riboflavin and its detection by nitrite formation from hydroxylamine hydrochloride. The nitrite reacts with sulphanilic acid to produce a diazonium compound, which subsequently reacts with naphtylamine to produce a red azo compound whose absorbance is measured at 543 nm. Pipetted 1.4 ml aliquot of the reaction mixture (20 mM L-Methionine 1% (v/v) Triton X-100, 10 mM hydroxylamine hydrochloride and 50 µM EDTA) in a test tube. To 1 ml of the various extract of *Wattakaka volubilis* of different concentrations (125, 250, 500, and 1000 µg/mL,) was added followed by preincubation at 37^{o}C for 5 min. 80 µl of 50 µM riboflavin was added and the tubes were exposed for 10 min. The control tube contained equal amount of 50 mM phosphate buffer instead of sample. The sample and its respective control were run together. At the end of the exposure time, 0.1 ml of Greiss reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride) was added to each tube and the absorbance of the colour formed was measured at 543 nm. One unit of enzyme activity was defined as the amount of SOD capable of inhibiting 50% of nitrite formation under assay condition. Ascorbic acid was used as reference compound [13].

RESULT AND DISCUSSION

The results of qualitative phytochemical analysis of *Wattakaka volubilis* leaf and stem samples are represented in Table 1. The results showed the presence of phytochemical constituents such as alkaloids, glycosides, carbohydrates, cardiac glycosides, phenolic compounds, flavonoids, steroids, tannins, phlobatannins, anthraquinones, phenols, sugars, saponins, and resins. Quantitative estimation of phytochemical constituents of *W. volubilis* are represented in Table 2. The results showed the phytochemical constituents such as alkaloids, tannins, saponins, total phenol and proanthrocyanides respectively. The result of extractive value shows higher percentage in the methanol followed by chloroform, petroleum ether, and aqueous solvent. The qualitative and quantitative phytochemical studies of *W. volubilis* has revolved the presence of alkaloids, flavonoids, terpenoids, saponins, catachol, tannins and phenols in stem and leaf powder in various solvents.

Table 1. Qualitative analysis of *Wattakaka volubilis*

S. No	Phytochemicals	Different solvents used				
		Petroleum ether	Chloroform	Ethanol	Methanol	Aqueous
1	**Alkaloids** a)Mayer's test	-	-	-	+	-
	b)Wagner's test	-	-	-	+	-
	c)Dragendroff's test	-	-	+	++	++
2	**Flavonoids** a)Shinoda test	-	+	+	+	-
	b)sulphuric acid test	-	-	-	+	-
	c)Ferric chloride test	-	-	-	-	-
	d)Sodium hydroxide test	-	-	-	-	-
3	**Terpenoids** Salkowski test	-	-	+	++	+
4	**Cardiac Glycosides** Keller-Killani test	-	-	+	++	+
5	**Phenol** Ferric chloride test	-	+	+	++	-
6	**Sterols** a)Salkowaski test	-	+	+	++	-
	b)Liberman-Burchard's test	+	+	+	++	+
7	**Saponin** Foam test	-	-	-	-	-
8	**Catachol** Erlich's test	-	-	-	+	-
9	**Anthraquinones**	-	-	-	+	-
10	**Tannins** Braemer's test	-	-	-	+	-
11	**Phlobatannins**	-	-	-	+	-
12	**Resin**	-	+	+	++	+

Table 2. Quantitative analysis of leaf and stem samples of *Wattakaka volubilis*

S.No	Phytochemicals	Plantparts	mg/gm
1	Alkaloids	Leaf	0.023
		Stem	0.016
2	Flavonoids	Leaf	0.023
		Stem	0.011
3	Saponins	Leaf	0.004
		Stem	0.005
4	Tannins	Leaf	0.026
		Stem	0.017
5	Total Phenol	Leaf	0.063
		Stem	0.085
6	Proanthocyanidines	Leaf	0.003
		Stem	0.001

Table 3. Total antioxidant activity of *Wattakaka volubilis* using various solvent extracts.

Concentration (µg/ml)	% of activity(±SEM)					
	Different solvent of *W. volubilis*			Standard (Ascorbic acid) of *W. volubilis*		
	Methonal	Ethanol	Chloroform	Methonal	Ethanol	Chloroform
125	41.19±0.15	48.71 ± 0.65	37.74±0.97	32.56±0.87	33.72±0.63	33.83±0.21
250	63.54±0.39	66.39 ± 0.76	59.27±0.18	46.66±0.83	49.33±0.76	39.83±0.76
500	76.16±0.76	74.40± 0.23	78.86±0.42	63.66±0.36	61.48±0.87	56.96±0.43
1000	87.14±0.33	88.33 ±0.86	83.72±0.26	73.69±0.87	76.23±0.78	74.75±0.95
IC 50	**19.59**	**31.69**	**25.96**	**26.27**	**26.39**	**28.33**

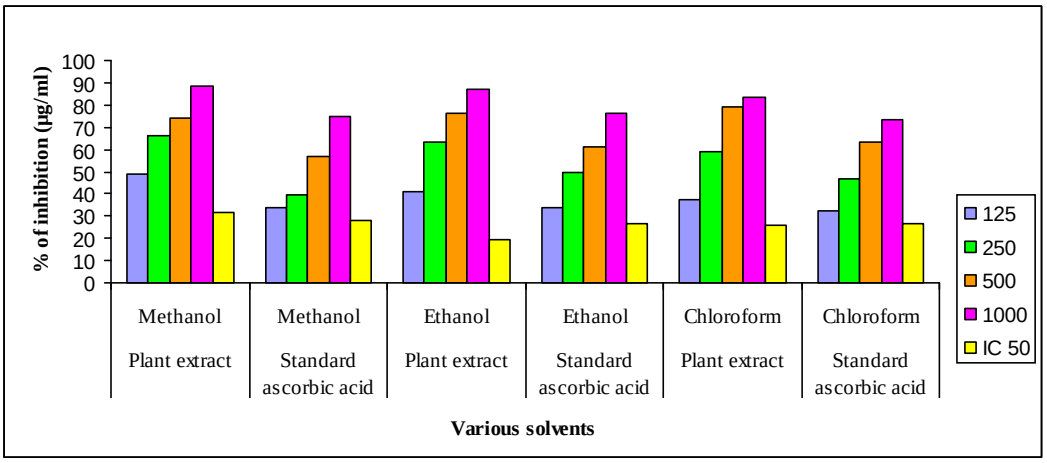

Fig 1. Effect of *W. volubilis* in different extracts on the percentage of Total antioxidant activity

Table 4. Effect *W. volubilis* extract in different solvent on the percentage of DPPH activity

Concentration (µg/ml)	% of activity(±SEM)					
	Different solvent of *W. volubilis*			Standard (Ascorbic acid) of *W. volubilis*		
	Methonal	Ethanol	Chloroform	Methonal	Ethanol	Chloroform
125	25.11±0.27	21.58±0.12	22.56±0.36	15.21±0.25	20.11±0.71	26.16±0.63
250	43.15±0.32	29.47±0.27	26.19±0.04	31.36±0.46	26.27±0.16	35.33±0.28
500	51.18±0.38	52.14±0.34	29.25±0.07	67.24±0.11	39.16±0.60	39.16±0.64
1000	61.25±0.31	62.29±0.29	35.93±0.53	78.66±0.55	43.11±0.31	43.27±0.24
IC 50	**19.83**	**25.62**	**24.21**	**19.54**	**20.67**	**21.22**

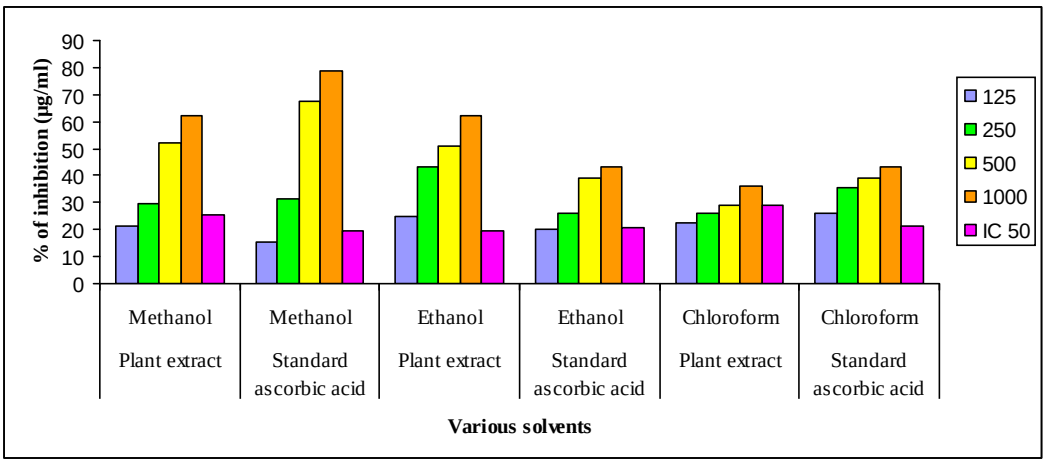

Fig 2. Effect of *W. volubilis* in different extracts on the percentage of DPPH activity

Table 5. Effect of *W. volubilis* extract in different solvent on the percentage of Superoxide dismutase activity

Concentration (µg/ml)	% of activity(±SEM)					
	Different solvent of *W. volubilis*			Standard (Ascorbic acid) of *W. volubilis*		
	Methonal	Ethanol	Chloroform	Methonal	Ethanol	Chloroform
125	15.31±0.12	18.75±0.31	31.64±0.31	30.61±0.51	54.31±0.24	36.16±0.63
250	20.16±0.01	24.14±0.04	36.11±0.10	36.27±0.76	63.26±0.44	39.33±0.28
500	27.34±0.33	28.71±0.51	42.02±0.32	39.56±0.30	67.31±0.45	49.16±0.64
1000	29.19±0.05	33.98±0.01	49.32±0.02	49.14±0.61	79.45±0.54	53.27±0.24
IC 50	**24.67**	**36.46**	**33.68**	**21.03**	**29.67**	**23.66**

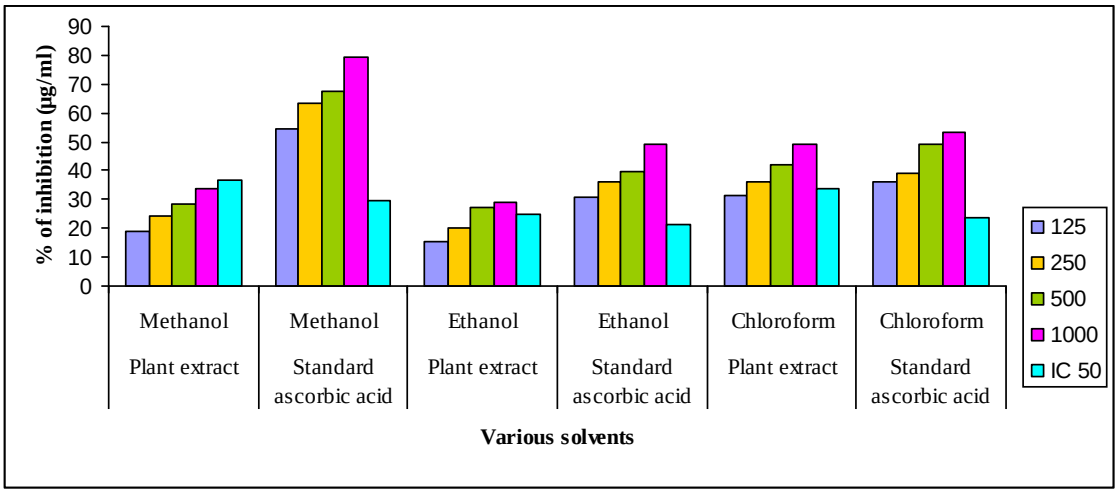

Fig 3. Effect of *W. volubilis* in different extracts on the percentage of Superoxide dismutase activity

The members of the family Asclepiadaceae is well known to Indian system of medicine since ancient times, and reported to contain several phytochemicals like alkaloids, sterols, tannins, terpenoids, flavonoids, wax and resins[14 15]. Many pregnane glycosides, flavonens, liteolin and flavone C are reported in *Caralluma* spp. [16] and the other species of *caralluma adscendens* contains saponin glycosides, caratubersides A and B and various boucersides. *C. adscendens* is a traditional food consumed in the form of pickle and vegetable and also eaten during famines[17] and also used in the treatment for diabetes[18] and an emergency food. *Caralluma* species have shown great anti-inflammatory[19] antiviral and antioxidant properties [20].

The phenolic compounds such as flavonoids, phenolic acids, tannins and steroids are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also posses diverse biological activities such as anti-careinogenic, anti-atherosclerotic and anti-inflammatory activities. These activities may be related to their antioxidant activity[21] . So far as phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical terminators, it was responsible to determine their total amount in the selected plant extract.

The effects of methanolic, ethanolic and chloroform extracts of *W. volubilis* was evaluated for its antioxidant activity on different *in vitro* models like DPPH radical scavenging activity, scavenging of Superoxide radical and total antioxidant activity in a concentration dependent manner. The antioxidant activities of *W. volubilis* plant extracts in different solvent systems using various models are summarized in the table IV. In the total antioxidant activity, IC_{50} value of *W. volubilis* in methanol, ethanol and chloroform extracts were found to be 31.69, 19.59 and 25.96 µg/ml, whereas the standard ascorbic acid showed with an IC_{50} values of 28.33, 26.39 and 26.27 µg/ml. The results indicated that the methanolic extract of *W. volubilis* has more total antioxidant activity (Table III and Fig I). For a long time, plant-derived antioxidants have been used to reduce the level of

oxidative stress within the human bodies [22] . Hydroxyl radicals are the most reactive of all the reduced forms of dioxygen and are believed to initial cell damage both *in vitro* and *in vivo* [23]. In the present study the antioxidant properties exhibited by *W. volubilis* are supported by the detection of plant phenols in the phenol test. Polyphenols (electron-rich compounds) having the ability to go into electron-donation reactions with oxidizing agents and still from stable species and thus inhibit or delay the oxidation of different biomolecules[24] . In addition, plant sterols such as β- sitosterols also found in *W. volubilis* have been found to inhibit lipid peroxidation under certain experimental conditions [25]. The ethanol extract was slightly more active among the other extract (62.25µg/ml). Among these solvent systems the ethanol extract had high DPPH radical scavenging activity, whereas the standard ascorbic acid showed IC_{50} value of 20.67µg/ml (Fig II). The result of DPPH-free radical scavenging assay suggested that the extracts are capable of scavenging free radicals via electron (or) hydrogen donating mechanisms and thus could be potent enough to prevent the initiation of deleterious free radical mediated chain reactions in susceptible matrices eg:- biological membranes[26] .

Superoxide radical scavenging activity in different solvent systems of *W. volubilis* extract had a more potent scavenger of superoxide anion and the results are presented in the table 2. The IC_{50} value of methanol, ethanol and chloroform of *W. volubilis* are 36.46, 24.67 and 33.68 µg/ml respectively. The standard ascorbic acid with an IC_{50} value of methanol was 29.67µg/ml shows high activity. The ethanol extract of *W. volubilis* has a more potent scavenger of superoxide radical activity when compare to the standard, ascorbic acid (Table V and Fig III). Superoxide anions are the most common free radicals *in vivo* and are generated in a variety of biological systems by either auto-oxidations processes or by enzymes. The concentration of superoxide anions increases under oxidative stress and related situations [27]. Superoxide radicals one generated during the normal physiological process mainly in mitochondria. Although superoxide anion is by itself a weak oxidant, it gives rise to the powerful and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to the oxidative stress. Therefore superoxide radical scavenging by antioxidants has physiological implications [28] .

VanderJagt *et al.,* [29] have carried out few antioxidant activities of *Ocimum tenuiflorum.* They have screened the leaf and stem extract separately for their free radical scavenging property using BHT and ascorbic acid as standard antioxidant. In the study DPPH free radical activity was assayed and it was shown that the leaf extract have more activity than the stem. According to Lukmanul Hakkim *et al.,* [30] *Ocimum gratissium* extract was the most potent scavenger (81.1%) while *O. americanum, O. minimum, O. citriodorum, O. kilimandscharicum, O. grandiflorum, O. lamiifolium and O. selloi* have significant lower scavenging activity. Weel *et al.,* [31] carried out their work of antioxidant activity of *Leucas aspera* root. In this study, the ethanolic extract of *L. aspera* root produce significant inhibition in acetic acid induced writhing in mice at the dose of 250 and 500mg/kg. The extract showed a significant free radical scavenging activity with an IC_{50} of 8 µg/ml. Mantle *et al.* [32] carried out the antioxidant activity of *Mellissa officinalis* leaves. In this study they evaluated the antioxidant activity of water extract and ethanolic extract of *Mellissa officinalis.* These two extracts evaluated for their radical scavenging activities by means of the DPPH and DMPO assays. Thus the study showed that the water extract have effective antioxidant and radical scavenging activities as compared to the ethanolic extract.

It has been reported that compounds such as flavonoids, which contain hydroxyls, are responsible for the radical scavenging effects of most plants [33]. The mechanisms of action of flavonoids are through scavenging or chelating process [34]. Phytochemical screening of the extract of the leaves and stems of *W. volubilis* revealed the presence of flavonoids, tannins, saponins, glycosides, alkaloids, steroids, and phenolic compounds. Phenolic compounds have been recognized as antioxidant agents, which act as free radical terminators [35] and have been known to show medicinal activity as well as exhibiting physiological functions [36] .

In conclusion, the results of the study clearly indicate that methanolic extract of *W. volubilis* possess powerful *in vitro* antioxidant activity. The encouraging results of *W. volubilis* with the various *in vitro* antioxidant tests proved the plant as a reducing agent and effectiveness as scavengers of superoxide free radicals. The overall antioxidant activity of *W. volubilis* might be attributed to its polyphenolic content and other phytochemical constituents. The plant merits further investigation in animal models to confirm its antioxidant activity *in vivo* and to isolate the active constituents, which may result in a modern drug from this plant.

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