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Research Article

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In vitro free radical scavenging activity of *Wattakaka volublis* leaf extract

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ABSTRACT

Many oxidative stress related diseases like cancer, diabetes, neurodegenerative disorders and cardiovascular diseases are occurring as a result of accumulation of free radicals in the body. Lots of researches are going on worldwide towards finding natural antioxidants from plant source. In the present study the antioxidant activity of methanolic extract of Wattakaka volublis leaf was investigated using various in vitro assays. 1,1-diphyenyl -2-picryl –hydrazyl,(DPPH), superoxide, nitric oxide and hydroxyl radical scavenging activity of the extract was determined at different concentrations and compared with standard antioxidants, such as BHT(butylated hydroxyl toluene), quercitin, 1- ascorbic acid. DDPH radical, superoxide, hydroxyl radical and nitric oxide radical scavenging effects of the extracts at different concentrations were evaluated following standard methods. The radical scavenging activity was concentration dependent, DDPH showing maximum percent inhibition(83.44%) at 200mg/ml . Similarly, hydroxyl, superoxide and nitric oxide scavenging potential also at their maximum at higher concentrations. superoxide radical inhibition was 80.04% at 200 mg/ml and 70.54% inhibition of nitric oxide was noted at 200 mg/ml. Hydroxyl scavenging effect was 86.96% at 200 mg/ml. The results indicated that methanolic extract of Wattakaka volubilis possess good natural antioxidant may be used to control some dangerous diseases.

Keywords: *Wattakaka volublis*, DPPH, Superoxide, Nitric oxide, hydroxyl radical scavenging activity**.**

INTRODUCTION

Free radicals and related species have attracted a great deal of interests in recent years. Free radicals are chemical species possessing un unpaired electron formed by the loss of a single electron from a normal molecule.[1]About 5% or more of the oxygen inhaled is converted to reactive oxygen species (ROS) such as O_2, H_2O_2, and OH by univalent reduction of O_2 [2] When oxygen is partially reduced it becomes activated and readily reacts with a variety of biomolecules by the addition of one , two or four electron to oxygen leading to formation of ROS such as superoxide anions, hydrogen peroxide, and hydroxyl radical, malondehyde and nitric oxide. Oxidative stress is essentially a regulated process, the equilibrium between the oxidative and antioxidative capacities. Under non-stressful conditions the Antioxidant defense system provides adequate protection against active oxygen and free radicals[3]. Super oxide anion radical, hydrogen peroxide and hydroxyl radical are the major reactive oxygen species generated during oxidative stress [4].

Antioxidants are protect living organisms from damage caused by uncontrolled production of (ROS) and the concomitant lipid peroxidation, protein damage and DNA strand breaking. Current interest focused on the potential role of antioxidant in the treatment and prevention of atherosclerosis, heart failure, neurodegenerative disorders, aging , cancer, diabetes mellitus and several others diseases.[5] These ROS cause destructive and

irreversible damage to the components of a cell, such as lipids, proteins and DNA. Although normal cells posses antioxidant defense systems, ROS produced in the cells induces diseases such as cancer and aging.[6] Exogenous chemical and endogenous metabolic processes in the human body or in the food system might produce very effective ROS, which are capable of oxidizing biomolecules, resulting in tissue damage and cell death. When the mechanism of antioxidant protection becomes unbalanced by endogenous factors, it results in inflammation, diabetes, genotoxicity, cancer and accelerating aging.[7]

Therefore, antioxidants that scavenge reactive oxygen species (ROS) and reactive nitrogen species (RNS) may be of major importance in preventing the onset and the progression of oxidative pathologies and provide protection to foods. It has been reported that there is an inverse relationship between the antioxidative status and occurrence of human diseases. Phyto constituents like flavanoids and phenolic acids, commonly found in plants have been reported to have multiple biological effects, including antioxidant activity.[8,9] Generally, medicinal plants could be a potential source of natural antioxidants[10] *Wattakaka volublis* (Asclepiadaceae) known as cotton milk plant and green wax flower.[11] It is distributed throughout the hotter parts of India. It is used in the treatment of various ailments since ancient times.[12,13] The literature survey revealed that among the various saponins obtained from the leaves of *Wattakaka volublis* two compounds are active against Ehrlich's ascites carcinoma cell line.[14,15] The present study an attempt has been made find out the *in vitro* free radical scavenging activity of *W. volubilis* leaves extracts.

EXPERIMENTAL SECTION

Collection of plant material

The *Wattakaka volublis* was collected from Tiruchirappalli, Tamil Nadu, India. The plant was identified and voucher specimen was deposited in the Rapinet Herbarium, St. Joseph's college, Tiruchirappalli.

Preparation of extract

The plant leaves were air dried and crushed to small piece using pestle and mortar then the powdered in an electric grinder. Dried and powedered plant material is extracted using soxhlet apparatus with methanol, ethanol solvent (50-60° C) for 72 hrs.

DPPH free radical scavenging activity

The ability of the extracts to annihilate the DPPH radical (1,1-diphenil-2-picrylhydrazyl) was determined followed the method of Harbone and Baxter.[16] Stock solution of leaf extracts was prepared to the concentration of 1mg/ml. 100µg of each extracts were added, at an equal volume, to methanolic solution of DPPH (0.1%). The reaction mixture is incubated for 30 min at room temperature; the absorbance was recorded at 517 nm. The experiment was repeated for three times. BHT was used as standard. The annihilation activity of free radicals was calculated in % inhibition.

% of Inhibition = (A of control – A of Test)/A of control * 100

Superoxide anion scavenging activity

The superoxide anions generated by phenazinmethosulfate (PMN)/ nicotinamid-adenin-dinucleotidphosphat, reduced form (NADPH) system, were detected by the reaction with 2,2'-di-p-nitrophenyl)-5,5'-diphenyl-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride (nitro blue tetrazolium – NBT). Stock solution of leaf extracts and Quercitin (standard) was prepared at a concentration of 1mg/ml. The reaction mixture contained 1ml of Nitro blue tetrazolium (NBT) solution (312 µM was prepared in phosphate buffer, pH-7.4), 1ml of Nicotinamide adenine dinucleotide (NAD) solution (936 µM was prepared in phosphate buffer, pH-7.4) and samples and standards at different concentration (25,50, 100,150 and 200mg/ml)) obtained from stock solution were added and Finally the reaction was accelerated by adding 100µl phenazinemethosulfate (PMS) solution (120 µM prepared in phosphate buffer, pH-7.4). The reaction was incubated at 25°C for 5 minutes and absorbance was measured at 560nm against the corresponding blank solutions.[17]

% of Inhibition = (A of control – A of Test)/A of control * 100

Nitric oxide scavenging activity

Sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide which interacts with oxygen to produce titrite ions that can be estimated using Griess reagent. Scavengers of nitric oxide compete with oxygen, leading to reduced production of nitrite ions. Stock solution of leaf extracts and ascorbic acid (standard) was prepared to the concentration of 1mg/ml. The reaction mixture contains 2 ml of sodium nitroprusside (10mM), 0.1ml of phosphate buffer saline and samples at different concentration (25,50, 100, 150 and 200mg/ml) or standard solution (25,50,100, 150and 200µg/ml) obtained from stock solution, were incubated at 25º C for 150 min. After incubation, 0.5ml of the reaction mixture was mixed with 1ml of sulphanilamide (1%) and allowed to stand for 5 min for completing diazotization. Then, 1ml of naphthylethylene diaminedihydrochloride (0.1% W/V) was added, mixed and allowed to stand for 30min at 25º C. A pink colored chromophore was formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions[18]

% of Inhibition = (A of control – A of Test)/A of control * 100

Hydroxyl radical scavenging (HO) assay:

The scavenging ability for hydroxyl radicals was measured as described below. [19] The reaction mixture (1ml) consisted of 100 µl of 2-deoxy-D-ribose (28 mM in 20 mM KH2PO4 –KOH buffer, pH 7.4), 1ml of methanol extract of Wattakaka vlubilis concentrations ranging from 25-200 mg/ml 200 µl EDTA (1.04 mM) and 200 µM FeCl3 (1:1 v/v), 100 µl of H2O2 (1mM) and 100µl of ascorbic acid (1mM) which was incubated at 37ºC for 1 h.1ml of thiobarbituric acid (1%) and 1ml of trichloroacetic acid (2.8%) were added and incubated at 100ºC for 20 min. After cooling, absorbance was measured at 532 nm, against a blank sample. Ascorbic acid used as positive controls.

% of Inhibition = (A of control – A of Test)/A of control * 100

Statistical Analysis

All data are expressed as mean ± S.D.

RESULTS AND DISCUSSION

DPPH Radical scavenging activity:

Natural antioxidants have been proved to be effective protectors of body from the adverse effects of free radicals caused oxidative stress *In Vitro* DPPH Radical Scavenging activity DPPH is an easy, rapid and sensitive method for the antioxidant screening of plant extracts. The present study investigated the scavenging activity of methanol extract of *Wattakaka volubilis* leaves, and expressed in percentage of inhibition of DPPH free radicals using BHT as standard reference compound. Methanol extract of Wattakaka volubilis showed significant free radical scavenging activity generated by DPPH. Scavenging activity was observed from 25 mg/ml to 200 mg/ml (42%,61.7%, 75.9%, 82.1 % and 83.4%). Since more than 50% of DPPH radical inhibition is considered to be significant, the inhibition was observed from 50 mg/ml. BHA showed strong free radical scavenging activity at all concentrations **(Table 1).**

DPPH is characterized as stable free radicals by virtue of the delocalization of the spare electron where the molecule as a whole, so that the molecule do not dimerise, as would be the case with most other free radicals. The delocalization gives rise to the deep violet color, characterized by an absorption band (517 nm) in methanol solution. When a solution of DPPH is mixed with a substance of H donor, it gets reduced into non radical state (Diphenyl picryl hydrazine). Hence, the significant decrease in free radical can be attributed to the scavenging ability of Methanolic extract of Wattakaka volubilis leaves. 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 inhibition of deleterious free radical mediated chain reactions in susceptible matrices eg: biological membranes[20].

Superoxide anion radical scavenging activity:

Superoxide radical is a well known precursor for formation of more harmful reactive substances in the body. The major risk of the superoxide generation is related to its interaction with the nitric oxide to form peroxynitrite, a potent oxidant that causes nitrosative stress. Decrease in the absorbance of the superoxide anion with MEWV is noted in the present study, indicating effective scavenging potential comparable to the Quercitin control. (**fig**.**1**) shows the inhibition of superoxide ions by MEWV and Quercitin at concentrations ranging from 25 mg/ml to

200mg/ml. Maximum inhibition of 72.04% and was noted with *Wattakaka volubilis* whereas Quercetin showed 80.05%.

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 situation [21]. Super oxide radicals one generated during the normal physiological process mainly in mitochondria. Although superoxide anions is by itself a week oxidant, it gives rise to the power full and dangerous hydroxyl radicals as well as singlet oxygen both of which contribute to the oxidative stress. Therefore super oxide radical scavenging by a antioxidant has physiological implications [22].

Nitric oxide radical scavenging activity

Nitric oxide is another free radical generated in human cells. Though associated with many regulatory functions, excess production would be detrimental to the body system as it readily reacts with oxygen to produce stable products of nitrate and\ nitrites. Nitic oxide inhibition by the *Wattakaka volubilis* has been shown in **Table** .**2** The study shows a maximum inhibition of 54.18% at 200mg/ml concentration when compared to ascorbic acid control(50.18%).

NO is a potent pleoiotropic inhibitor of physiological process , such as smooth muscle relaxation, neural signaling , inhibition of platelet aggregation and regulation of cell mediated toxicity. It is a diffusible free radical that plays many roles as an effectors molecule in diverse biological systems including neuronal messenger, vasodilation and antimicrobial and antitumour activities [23].

Hydroxyl radical scavenging activity

Hydroxyl radicals are the major active oxygen species that cause lipid oxidation and enormous biological damage.The percentage of hydroxyl radical scavenging activity of the Methanolic extract of Wattakaka volubilis increased with increasing concentration as given in **Fig**:**2**. At 25mg/ml to200 mg/ml concentration the activity was over to 22.29% as against 94.2% inhibition exhibited by ascorbic acid respectively at this concentration. The scavenging effects of methanolic extracts from *Wattakaka volubilis* and its antlers showed 6.66% and 50% at 20 mg/ml respectively.

Hydroxyl radical is very reactive and can be generated in biological cells through the Fenton reaction The potential scavenging abilities of phenolic substances might be due to the active hydrogen donor ability of hydroxyl substitution. Similarly, high molecular weight and the proximity of many aromatic rings and hydroxyl groups are more important for the free radical scavenging by specific functional groups [23] .The hydroxyl radical produced in the biological systems attack the sugar of DNA base causing sugar fragmentation, base loss and DNA strand breakage [24]. In the *in vitro* system hydroxyl scavenging activity was determined by studying the competition between deoxyribose and the extracts of *W.volubilis* for the hydroxyl radicals generated from Fe3+-ascorbate-EDTA-H2O2 system. The degradation of deoxyribose to TBARS by the hydroxyl radicals in the system were markedly reduced by the methanolic extract of *W.volubilis.* The potential hydroxyl radical scavenging activity of methanolic extract of *W.volubilis* may be due to its active hydrogen donating ability.

Leaves of medicinal plants are common ingredients of many folk and herbal medicines [25.26] and leaf extract of a number of medicinal plants have been reported to possess pharmacological activity[27]and[28]. The present study reveals that dried leaf extract of wattakaka volubilis possesses phytochemical and in vitro antioxidant activity. Different parts of *W.volubilis* plant enjoy considerable reputation for their various medicinal uses . The leaf paste is used to clear boils .Plant paste is mixed with hot milk and taken for urinary troubles. Leaf juice is inhaled to stop sneezing . The alcoholic extract of the plant is widely used in India as a traditional medicine for boils and absesses. The alcoholic extract of the plant is also reported to show activity on the central nervous system, as well as anticancer activity against sarcoma 180 in mice [29] 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[30] the mechanisms of action of flavonoids are through scavenging or chelating process [31] . Hence, the significant decrease in free radical can be attributed to the scavenging ability of *W. volubilis* leaves.

Radical scavenging activity of BHT and *Wattakaka volubilis* leaves extract on DPPH free radical. Scavenging activity is expressed as percentage of inhibition of DPPH free radical. 50% and above inhibition DPPH radical is considered as significant for scavenging activity.

Table 1: DPPH radical scavenging activity of W.volubilis

Concentration	% Inhibition of DPPH free Radical	
	BHT (µg/ml)	Methanol extract of Watakaka volubilis (mg/ml)
25	62.2 ± 1.9	42± 2.2
50	80.6 ± 2.6	61.7 ± 3.5
100	93.3 ± 2.4	75.9 ± 2.7
150	95.6 ± 1.8	82.1 ± 2.3
200	96.4 ±1.4	83.4 ± 2.5

Fig:1 Superoxide radical scavenging activity of MEWV

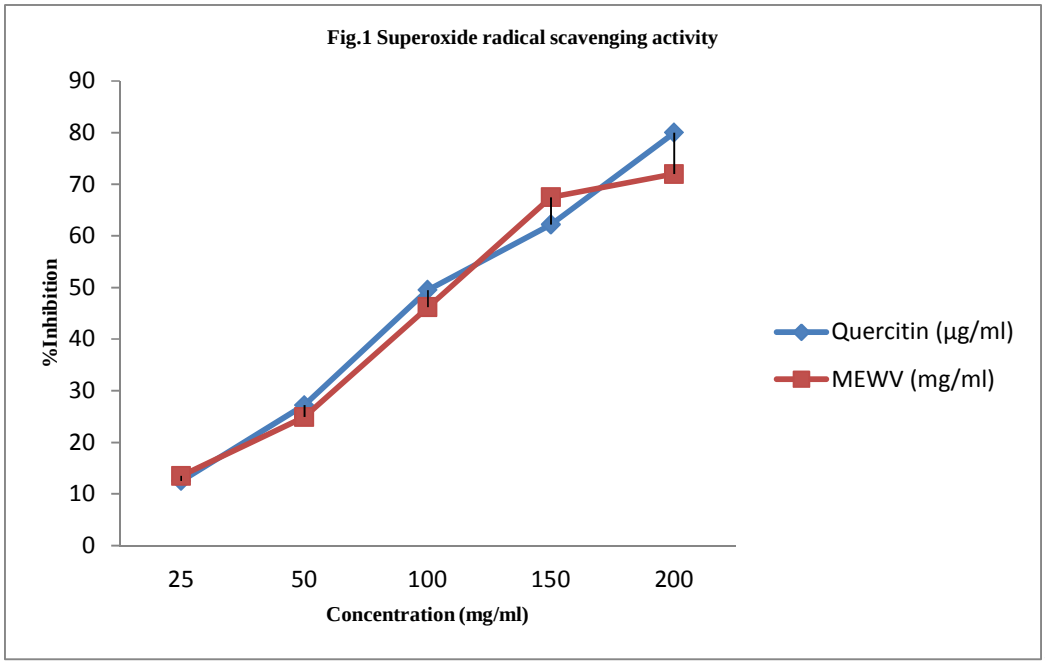
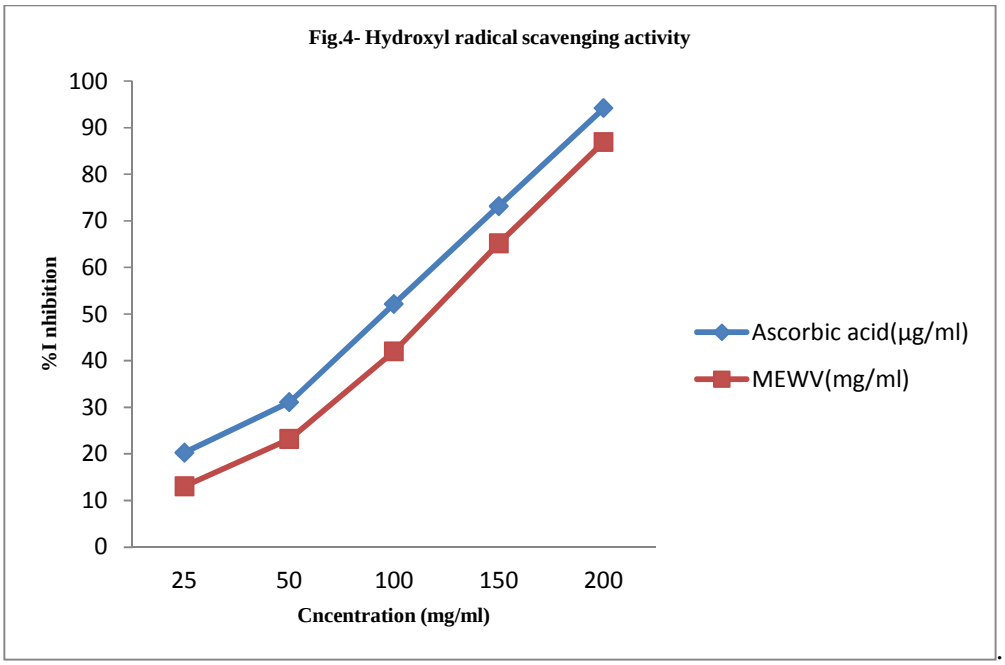


Table 2: Nitric oxide radical scavenging activity of methanolic extract Wattakaka volubilis leaf

Concentration	% Inhibition of Nitric oxide radical scavenging activity	
	Ascorbic acid(µg/ml)	MEWV(mg/ml)
25	9.09±1.2	6.66±1.3
50	17.39±2.4	15.71±2.5
100	48.09±1.5	40.30±1.2
150	54.10±2.6	50.81±3.5
200	74.18±2.9	70.54±1.8

Fig:2 Hydroxyl radical scavenging activity of Methanolic extract of Wattakaka volubilis leaf



CONCLUSION

The present study were to investigated the *in vitro* antioxidant activity of *methanol extract of Wattakaka volubilis* through the DPPH radical scavenging, superoxide anion radical scavenging, hydroxide radical scavenging, nitric oxide scavenging activity. The results of the present study showed that MEWV possesses strong antioxidant activity tested by various radical scavenging activities. The antioxidant potential of MEWV directly proportional to concentrations (i.e. increase the concentration of MEWV is increase the scavenging activity). This work has gathered experimental evidence on the MEWV as natural antioxidant for its capacity to scavenge reactive oxygen/nitrogen species/free radicals and protect cells/organism from oxidative damage and thus could be an effective against oxidative stress. In addition, the MEWV reported to contain a noticeable amount of total phenols which plays a major role in controlling antioxidants. Thus, it can be concluded that MEWV 200mg/kg showed better result when compared to other concentrations. MEWV can be used as an accessible plant antioxidants with consequent health benefits. Overall, the *Wattakaka volubilis* are a source of natural antioxidants that can be important in disease prevention and health preservation. However, the *in vivo* safety of methanol extract of *Wattaka volubilis* needs to be thoroughly investigated in experimental models prior to its possible application as an antioxidant ingredient, either in animal feeds or in human health foods.

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