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Research Article

EVALUATION OF *IN VITRO* ANTIOXIDANT ACTIVITY OF A MEDICINAL HERB, *WEDELIA CHINENSIS* (OSBECK) MERRILL.

REHANA BANU H[1]*, NAGARAJAN N[2]

[1]Department of Botany, PSGR Krishnammal College for Women, Coimbatore - 641 004, Tamil Nadu, India. [2]Department of Botany, Kongunadu Arts and Science College, Coimbatore - 641 029, Tamil Nadu, India. Email: rehanabanu@psgrkc.ac.in

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ABSTRACT

Objective: The objective is to evaluate the antioxidant activity of the methanolic leaf extract of *Wedelia chinensis*.

Methods: *In vitro* antioxidant activity was evaluated by studying 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, superoxide radical scavenging activity, ferric reducing ability of plasma (FRAP) scavenging activity, hydroxyl radical scavenging activity, metal chelating activity, and total antioxidant activity. Ascorbic acid, BHT, quercetin, and EDTA were used as standards for the experiments.

Results: Inhibition concentration$_{50}$ values observed for DPPH radical scavenging, superoxide radical scavenging, and hydroxyl radical scavenging activities were determined to be 62.5, 769.23, and 617.28 µg/ml, respectively and that of FRAP scavenging, metal chelating, and total antioxidant activities were found to be 343 µmol Fe (II)/g, 79.56 mg EDTA/g extract, and 398.17 mg ascorbic acid eq/g extract, respectively.

Conclusion: The results clearly indicate that methanolic leaf extract of the study species *W. chinensis* is effective in scavenging free radicals and has the potential to be a powerful antioxidant.

Keywords: *Wedelia chinensis*, *In vitro* antioxidant activity, Inhibition concentration$_{50}$, Methanolic leaf extract.

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INTRODUCTION

Free radicals may be defined as any chemical species that are capable of existing with one or more unpaired outer shell electrons. They are extremely reactive and generally highly unstable [1]. They are produced endogenously during cellular metabolisms in all forms of aerobic living system, in addition to exogenous sources such as environmental pollutants, drug, radiation, and pathogens [2]. The imbalance between production of reactive oxygen species (ROS) such as O_2^-, H_2O_2, OH^-, and ROO^- and the capacity of the normal detoxification system in favor of the oxidants leads to oxidative stress, which itself lead to cellular damage caused by the interaction of ROS with cellular constituents and several types of biological damages: DNA damage, carcinogenesis, and cellular degeneration [3,4]. Tissue damage resulting from oxidative stress has been implicated in the pathology of a number of disorder diseases such as cancer, inflammatory joint disease, cardiovascular diseases, arthritis, and cataract and could play a role in neurodegenerative diseases and aging processes [5,6].

Antioxidants are considered as possible protective agents providing protection of living organisms from damage caused by uncontrolled production of ROS and the concomitant lipid peroxidation, protein damage, and DNA-strand breakage [7,8]. Natural antioxidants have a wide range of biochemical activities, including direct or indirect scavenging of free radicals that are released during the normal metabolic process of oxidation such as reactive oxygen free radical species [9]. In the recent years, food scientists and nutrition specialists agree that antioxidant-enriched fruits and vegetables consumed daily contribute to the reduction in risks of certain diseases including cancer and cardiovascular diseases [10,11].

The plant species, *Wedelia chinensis* selected for the present investigation is a perennial herb of family Asteraceae, commonly known as "Pilabhamgara" or "Bhringraj" in Hindi, Wedelia in Chinese, and "Manjalkarisalanganni" in Tamil [12]. Conventionally, the fruits, leaves, and stem are used in child birth and in the treatment of bites and stings, fever, and infection. The leaves are used in the treatment of kidney dysfunction, cold, wounds, and amenorrhea [13]. However, information pertaining to the antioxidant properties of *W. chinensis* is meager. Hence, in the present study, the possible antioxidant activity of this plant was investigated in detail by employing six different *in vitro* antioxidant models.

METHODS

Plant material and extraction

W. chinensis (Osbeck) Merrill leaves were collected from Attakatti Hills, India. The plant material was taxonomically identified by Dr. V. Balasubramanian, Associate Professor, Department of Botany, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India, and was deposited at the college herbarium for future reference. For extraction, about 50 g of the shade dried and powdered leaf material was taken. The powdered material was transferred into 250 ml quick fit flask and extracted in the Soxhlet extractor for 48 h [14,15] using of organic solvents, namely, petroleum ether, chloroform, ethyl acetate, and methanol separately according to the increasing polarity of the solvents. The extract was filtered over Whatman no. 1 filter paper, and the filtrate was concentrated under reduced pressure to pasty mass [16] for further studies.

Chemicals

All the chemicals used in the work were purchased from HiMedia Pvt., Ltd., Mumbai. The chemicals used were of analytical grade.

Determination of antioxidant activity

The antioxidant activity was evaluated by six methods which are as follows:

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH assay measures hydrogen atom (or one electron) donating activity and hence provides a measure of free-radical scavenging antioxidant activity. Free-radical scavenging capacity was evaluated on

the basis of the scavenging activity of DPPH by measuring the reduction of absorbance at 517 nm. The method was carried out as described by Brand-Williams *et al.* [17]. Crude methanol extract of *W. chinensis* was redissolved in methanol, and various concentrations (10, 50, 100, 500, and 2000 μg/mL) of extract were prepared. The assay mixture contained in a total volume of 1 mL consists of 500 μL of the extract, 125 μL of freshly prepared DPPH solution (1mM in methanol), and 375 μL of solvent (methanol). The contents were mixed vigorously in a vortex mixer for 10 s and incubated at room temperature in the dark (wrapped with aluminum foil) for 30 min. The absorbance was read at 517 nm using a spectrophotometer. In each experiment, the tested sample alone in methanol was used as blank while the DPPH solution alone in methanol was used as control. All experiments were carried out in triplicate. Ascorbic acid was used as standard. The results were expressed as percentage inhibition.

The DPPH radical scavenging activity was calculated using the formula:

% inhibition = [(A control – A sample)/A control]×100

where A control and A sample are the absorbance values of the control and test sample, respectively.

Superoxide radical scavenging activity

Superoxide radical generated from the photoreduction of riboflavin was detected by nitro blue tetrazolium reduction method Beauchamp and Fridovich [18]. Each 3 ml of reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 2 lM riboflavin, 100 lM EDTA, NBT (75 lM), and 1 ml of sample solution. The tubes were illuminated under incandescent lamp for 15 min. The production of blue formazan was followed by monitoring the increase in absorbance at 560 nm after 10 min of illumination from a fluorescent lamp. The inhibition of superoxide radical generation was determined by comparing the absorbance values of the control (phosphate buffer as blank) with that of the treatments. BHT and quercetin were used as reference materials.

The superoxide radical scavenging activity was calculated using the formula:

% inhibition = [(A control – A sample)/A control]×100

where A control and A sample are the absorbance values of the control and test sample, respectively.

Ferric reducing ability of plasma (FRAP) scavenging activity

The ferric reducing capacity of the extract was measured based on the ferric reducing activity assay (FRAP) [19]. FRAP reagent was prepared by mixing 2.5 mL of solution ferric trichloride hexahydrate (20 mM), 2.5 mL of solution (2,4,6-tripyridyl-s-triazine) TPTZ (10 mM in 40 mM of hydrochloric acid), and 25 mL of acetate buffer 0.3 M (pH 3.6) and incubating at 37°C. The extract was dissolved in methanol to yield a final concentration (w/v) of 1 mg/mL. For each analysis, 30 μL of methanolic solution was added to 180 μL of distilled water and 1.8 mL of FRAP solution. The increase of absorbance was recorded at 593 nm, during 30 min at 37°C. The standard curve was constructed using $FeSO_4.7H_20$ solution, and the results were expressed as μmol Fe (II)/g dry weight of plant material.

Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity was determined according to the method of Klein *et al* [20]. Various concentrations of plant extract in methanol were placed in different test tubes and evaporated. One ml of iron–EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA (0.018%), and 1 ml of DMSO (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) were added to these tubes, and the reaction was initiated by adding 0.5 ml of 0.22% ascorbic acid. Test tubes were capped tightly and heated on a water bath at 80–90°C for 15 min. The reaction was terminated by the addition of ice-cold trichloroacetic acid (17.5% w/v). Three milliliters of Nash reagent (75.0 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetylacetone were mixed and raised to 1 L with distilled water) was added to all the tubes, and the tubes were allowed to stand at room temperature for 15 min for color development. Intensity of the yellow color formed was measured at 412 nm against reagent blank. Quercetin and BHT were used as reference standards. Percentage hydroxyl radical scavenging was calculated by the following formula:

Hydroxyl radical scavenging activity (%) = 1 - (difference in absorbance of sample/difference in absorbance of blank)×100

Metal chelating activity

The chelation of ferrous ions by the plant extract and standards was estimated by the method of Dinis *et al.* [21]. Aliquots (1 ml) of the plant extract dissolved in the same solvents at concentrations of 0 (control), 1, 2.5, and 5 mg/ml were separately added to 2.8 ml of distilled water, followed by mixing with 50 μl of 2 mM $FeCl_2.4H_2O$ and 150 μl of 5 mM ferrozine. The mixtures were then shaken vigorously and left standing at room temperature for 10 min. Absorbance levels of the solutions were measured using a spectrophotometer at 562 nm. All tests and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine - Fe^{2+} complex formation was calculated using the formula given below:

Chelating activity (%) = [(A0–A1)/A0]×100

Where A0 is the absorbance of the control and A1 is the absorbance in the presence of the plant extract. EDTA was used as a reference compound.

Total antioxidant activity

Total antioxidant capacity of extract was estimated as described by Prieto *et al.* [22]. An aliquot of 0.1 ml of extract was mixed with 1 ml of phosphomolybdenum reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate) and 1.8 ml of distilled water in an Eppendorf tube. The tubes were capped and incubated at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance of each was measured at 695 nm against a reagent blank. Gallic acid was used as standard (0.02–0.1 mg/ml), and transport accident commission was estimated as mg GAE/g dried extract from calibration curve given by equation y=0.006 x+0.102 (R^2=0.93).

Inhibition concentration$_{50}$ (IC_{50})

IC_{50} was introduced and interpreted by Brand-Williams *et al.* [17]. The discoloration of sample was plotted against the sample concentration to calculate the IC_{50} value. It is defined as the amount of sample necessary to decrease the absorbance of the radical by 50%.

RESULTS

The level of free-radical scavenging activity through DPPH method, superoxide radical scavenging activity, and hydroxyl radical scavenging activity of methanol leaf extract of *W. chinensis* is depicted in Tables 1-3.

DPPH radical scavenging activity

The assay of the scavenging of 1,1-diphenyl 2-picrylhydrazyl (DPPH) radical is widely used as the model system to investigate the scavenging activities of several natural compounds such as crude mixtures or solvent extract of plants [23]. The principle of DPPH assay is that the antioxidant reacts with the stable free-radical DPPH (deep violet color) and converts it to DPPH with a yellow color. The degree of discoloration indicates the scavenging potential of the sample antioxidant [24], resulting in a decrease in absorbance at 517 nm.

The methanolic extract of *W. chinensis* quenched DPPH radicals in a dose-dependent manner. The percentage of scavenging effect on the

Table 1: DPPH scavenging efficiency of methanol leaf extract of *W. chinensis*

Concentration (µg/ml)	% DPPH inhibition			
	Extract	IC_{50} (µg/ml)	Ascorbic acid	IC_{50} (µg/ml)
1000	77.25		98.01	
500	70.23		97.20	
250	69.52		96.3	
125	65.23		90.34	
62.5	52.85	31.25	89.32	3.906
31.25	49.56		83.56	
15.625	45.23		77.40	
7.8125	34.58		71.45	
3.906	23.63		68.32	
Cell control	100		100	

DPPH: 1,1-diphenyl-2-picrylhydrazyl, IC_{50}: Inhibition concentration50, *W. chinensis: Wedelia chinensis*

Table 2: Superoxide radical scavenging activity of methanol extract of *W. chinensis*

Concentration (µg/ml)	Superoxide radical scavenging activity					
	Extract	IC_{50} (µg/ml)	BHT	IC_{50} (µg/ml)	Quercetin	IC_{50} (µg/ml)
100	7.85±0.68		36.11±0.04		32.11±0.04	
200	14.76±0.32		60.42±0.02		49.42±0.02	
300	20.81±0.64	769.23	66.55±0.04	105	62.06±0.01	233
400	26.21±0.53		75.25±0.01		70.48±0.01	
500	31.49±0.63		90.33±0.01		86.42±0.02	

IC_{50}: Inhibition concentration50, *W. chinensis: Wedelia chinensis*

Table 3: Hydroxyl radical scavenging activity of methanol extract of *W. chinensis*

Concentration (µg/ml)	Hydroxyl radical scavenging activity					
	Extract	IC_{50} (µg/ml)	BHT	IC_{50} (µg/ml)	Quercetin	IC_{50} (µg/ml)
100	9.25±1.19		30.17±0.042		29.12±0.052	
200	16.51±0.45		46.25±0.048		45.18±0.011	
300	25.67±1.07	617.28	67.42±0.058	350	66.28±0.011	430
400	32.93±0.45		69.42±0.057		68.13±0.012	
500	39.40±0.59		83.38±0.020		82.22±0.013	

IC_{50}: Inhibition $concentration_{50}$, *W. chinensis: Wedelia chinensis*

DPPH radical was increased with the increase in the concentration of leaf extract. The IC_{50} values were found to be 62.5 µg/ml (plant extract) and 3.906 µg/ml (ascorbic acid), respectively (Table 1).

Superoxide anion scavenging activity

In the present study, superoxide radical reduced NBT to a blue colored Formosan that was measured at 560 nm [25]. The relative scavenging effects of the extract and the standards BHT and quercetin toward superoxide anion radicals are shown in Table 2. The plant extract showed moderate superoxide anion scavenging activity as compared to BHT and quercetin. The IC_{50} values were found to be 769.23 (plant extract), 105 (BHT), and 233 (quercetin). The percentage inhibition of superoxide radical of plant extract was 7.85% (100 µg/ml), 14.76% (200 µg/ml), 20.81% (300 µg/ml), 26.21% (400 µg/ml), and 31.49% (500 µg/ml). Therefore, the superoxide anion scavenging activity in increasing order was BHT>quercetin>plant extract.

FRAP radical scavenging activity

The FRAP radical scavenging activity of the extract was 343±1.7 µmol Fe (II)/g. According to the high IC value of the methanolic extract of *W. chinensis*, it could be considered that compounds in the methanolic extract are good electron donors and could terminate oxidation chin reactions by reducing the oxidized intermediates into the stable form [26].

Hydroxyl radical scavenging activity

Table 3 showed that the methanolic leaf extract of *W. chinensis* exhibited concentration-dependent scavenging activity against hydroxyl radicals generated in Fenton reaction. Percentage of inhibition of hydroxyl radical was highest in positive controls of BHT with 83.38% and quercetin with 82.22% when compared to plant extract with 39.40% at 500µg/ml concentration. The IC_{50} values of BHT, quercetin, and plant extract were 350 µg/ml, 430 µg/ml, and 617.28 µg/ml, respectively. The hydroxyl radical scavenging activity of the extract is due to the free-radical quenching activity of the extract, which can be attributed to the presence of polyphenolics in the extract.

Total antioxidant capacity by phosphomolybdenum method

Total antioxidant capacity by phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by antioxidant compounds and subsequent formation of a green phosphate - Mo(V) complex at acidic pH which has a maximum absorption at 695 nm [27]. In the present assay, the total antioxidant activity of the leaf methanolic extract of *W. chinensis* was measured and compared with BHT. According to the result, total antioxidant activity increased with increasing concentration. The total antioxidant activity of the extract was 398.17±3.44 mg ascorbic acid eq/g extract.

Metal chelating activity

The iron-chelating capacity measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion [28]. In this assay,

ferrozine can complex with ferrous ions, and in the presence of chelating agents, complex (red colored) formation is interrupted; as a result, the red color of the complex decreases [29]. The transition metal ion, Fe^{2+} possess the ability to move single electrons by virtue, of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals [30]. The formation of the ferrozine - Fe^{2+} complex is interrupted in the presence of methanol extract of *W. chinensis*, indicating the chelating activity with IC_{50} of 79.56±2.55 mg/g of EDTA. However, IC_{50} of standard was 76.71±1.25 g/ml.

DISCUSSION

Antioxidants refer to a group of compounds that are able to delay or inhibit the oxidation of lipids or other biomolecules and thus prevent or repair the damage of the body cells that are caused by oxygen [31,32]. They work by preventing the formation of new free-radical species, converting existing free radicals into less harmful molecules and preventing radical-chained reactions [33]. In the present investigation, different antioxidant assays such as DPPH radical scavenging capacity, FRAP assay, metal chelating activity, superoxide radical scavenging activity, hydroxyl radical scavenging activity, and total antioxidant activity by phosphomolybdenum assay were employed to assess the antioxidant effect of the leaf extract of *W. chinensis*. From the study, it is evident that the extract of the study species possesses effective antioxidant activity which may be due to the presence of respective phytochemicals such as flavonoids and phenolics alkaloids, in this species [34].

DPPH radical scavenging is considered to be a good *in vitro* model widely used to assess antioxidant efficacy within a very short time [35-37]. The relatively stable organic radical, DPPH, has been widely used in the determination of antioxidant activity of single compounds as well as of different plant extracts [38].

The methanolic extract of *W. chinensis* reduced the DPPH radical in a dose-dependent manner. Similar trend of results was displayed in the methanolic leaf extracts of several members of Asteraceae family, namely, *Helichrysum chionophilum* [39], *Centaurea cyanus*, *Centaurea scabiosa* [40], *Centaurea calolepis*, *Centaurea cadmea* [41], *Vernonia amygdalina* [42], *Diospyros lotus* [43], *Hieracium pilosella* [44], *Chrysanthemum balsamita* [45], *Centaurea cheirolopha*, *Centaurea kurdica*, *Centaurea rigida* [46], *Helichrysum chasmolycicum* [47], *Hypericum hookerianum* [48], and *Stevia rebaudiana* [49].

Superoxide anions play important roles in the formation of ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins, and DNA [50]. The present study shows that *W. chinensis* possesses superoxide quenching ability. Based on the result, it appears that *W. chinensis* scavenges superoxide radicals by combining with superoxide radical ions to form stable radicals, thus terminating the radical chain reaction [51].

FRAP assay measures the changes in absorbance at 593 nm owing to the formation of blue colored Fe - tripyridyltriazine compound from the colorless oxidized Fe form by the action of electron-donating antioxidants [52]. In the present study, the ferric reducing antioxidant power was found to be high in methanolic extract of *W. chinensis*.

Hydroxyl radicals degrade the sugar deoxyribose (2-deoxy-D-ribose) through Fenton-type reactions leading to the production of complex products which can be estimated as malondialdehyde [53]. The methanolic extract showed minimum activity against hydroxyl radical when compared to that of controls BHA and quercetin.

Total antioxidant activities reflect the capacity of a non-enzymatic antioxidant defence system. In the phosphomolybdenum method, molybdenum VI (Mo^{6+}) is reduced to form a green phosphate/Mo^{5+} complex at acidic pHs. High absorbance values indicate that the sample possesses significant antioxidant activity. The method is utilized for the spectrophotometric quantitation of total antioxidant capacity and employs cost-effective reagents [22]. The total antioxidant activity of the extract increased with increasing concentration.

The Fe^{2+} chelating capacity of methanolic extract of *W. chinensis* was determined by measuring the iron–ferrozine complex. The chelating capacity of the extract was found to be higher.

CONCLUSION

The results of the present study indicate that the methanol leaf extract of *W. chinensis* has significant antioxidant activities which are comparable to that of the standard drugs such as ascorbic acid, BHT, quercetin, and EDTA. It may be related to the presence of phenolic compounds such as flavonoids and tannins because these compounds contain an aromatic hydroxyl moiety. Despite the higher IC_{50} values of all the six studied antioxidant assays than the standard drug, the leaf extract may be considered as an important source of material for the scavenging of radicals. The high antioxidant activities demonstrated by *W. chinensis* extract suggest that the plant leaves could be a source of potent antioxidant compounds.

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AUTHOR'S CONTRIBUTIONS

Both the authors have contributed well in the completion of the above research work.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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