

IN VITRO ANTIOXIDANT ACTIVITY OF ALCOHOLIC
EXTRACTS OF *WRIGHTIA TOMENTOSA*

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Summary

The objective of the present investigation is to assess the most potent antioxidant alcoholic extract from the leaf & bark of *Wrightia tomentosa*. The invitro-methods used to predict the antioxidant effect were DPPH radical scavenging assay, reducing power ability, Phospho molybdate method & the estimation of total phenolic and flavanoid contents using pyrocatechol & quercetin as standards. The % scavenging activity at different concentrations was determined and the IC₅₀ value of the extracts was compared with that of standard, ascorbic acid. The ethanolic bark extract gave an IC₅₀ value of 75.0 µg/ml when compared with leaf extract (IC₅₀ = 135.1 µg/ml). The reducing power was investigated by Fe³⁺ - Fe²⁺ transformation in the presence of extracts tested using Butylated hydroxy toluene as standard. The ethanol bark extract showed the highest reducing ability having an absorbance of 0.690 using 800µg/ml concentration at 700nm. The total antioxidant capacity by phospho molybdate method is expressed as α- tocopherol equivalents. Among the extracts tested, the ethanol bark extract contains 8.3 µg Vitamin E equivalent/ mg, which has apparently twice greater antioxidant capacity than the leaf extract (4.2 µg Vitamin E equivalent/mg). The content of total phenolics (9.1 µg Pyrocatechol equivalent/mg) and total flavanoids (20.0 µg Quercetin equivalent/mg) in the ethanol bark extract was considerably higher than the leaf extract. Based on the above results, the higher the flavanoid content, the higher the antioxidant capacity was very well observed with ethanolic bark extract. Hence the alcoholic extract of *Wrightia tomentosa* dried bark could be considered for preparation of nutraceuticals with potent antioxidant effect suitable for prevention of human disease.

Keywords: *Wrightia tomentosa*, Antioxidant activity, Ethanolic extract, Leaf, Bark.

Natural Products have been our single most successful source of medicines. Each plant is like a chemical factory capable of synthesizing unlimited number of highly complex and unusual chemical substances whose structures could otherwise escape the imagination forever. Although clinical trials and experiments involving whole animals are important in natural product screening but because of financial, ethical and time limitations, importance of invitro screening is gaining popularity [1].

Free radicals easily react with macro-molecules of crucial biological significance (DNA, lipids, protein) and destroy their structure and function what accelerates ageing and might lead to degenerative diseases, including cancer [2,3]. Certain portion of reactive oxygen species (ROS) is generated in normal human metabolism and the production rate is precisely controlled by specialized system of antioxidant defense [4]. This well-balanced ROS synthesis is impaired by inflammatory events, where activated macrophages and neutrophils upon contact with pro-inflammatory stimuli; release substantial amounts of aggressive oxygen and nitrogen-centered radicals [5].

Natural antioxidant defense system involves enzymes (superoxide dismutase, catalase, glutathione peroxidase), other proteins (albumin, ferritin, ceruloplasmin) and numerous smaller molecules (e.g. reduced glutathione, α -tocophrol, β -carotene, bilirubin, uric acid) of various modes of action. Antioxidant molecules counteract ROS and diminish their deleterious effects [6,7]. This protective barrier can be enhanced by the use of antioxidant micronutrient (vitamins C, E, β -carotene) and non-nutrient ingredients of edible plants, like polyphenols. Polyphenol subgroup of chemicals, flavanoids, is the extensively examined group of antioxidants [8,9].

Wrightia tomentosa Roem. & Schult, family Apocynaceae, is widely distributed at an altitude of 600m in the Himalayas. A novel isoflavone, wrightiadione isolated from the plant possess cytotoxic activity against murine P 388 lymphocytic leukemia cell line [10]. The root-barks are found to be useful in snake bite and scorpion-stings[11]. The butanol extract of the plant was reported to exhibit antimicrobial activity [12]. The ethanolic bark & leaf extract of *Wrightia tomentosa* possesses significant anti-allodynic effects [13] and antihyperglycemic activity [14] in streptozotocin induced diabetic rats. Hence, a study was designed to investigate the invitro antioxidant activity of ethanolic extract of various parts of the herb *Wrightia tomentosa* to establish the most potent antioxidant extract having therapeutic value.

Materials and Methods

I. Plant Material

Wrightia tomentosa was procured from the hills of yercaud forest, Salem district of Tamilnadu and authenticated by experts in the Research Department of Bharathidasan University, Tiruchirapalli, Tamilnadu, India.

II. Chemicals

All chemicals were analytical grade and chemicals required for all biochemical assays were obtained from Sigma Chemicals Co., USA.

III. Extraction:

The leaves and bark of *Wrightia tomentosa* were dried at room temperature and reduced to a coarse powder. The powdered materials (leaves and bark) were subjected to qualitative tests for the identification of various phyto constituents like alkaloids, glycosides, steroids, terpenoids and flavanoids. Then the powder was subjected to soxhlet extraction with benzene, chloroform and alcohol (90%) and water separately for 72 hours at a temperature of 50-60°C. The extracts were concentrated and the solvent was completely removed. They were freeze dried and stored in the vacuum desiccator. Further, the ethanolic extracts of leaf and bark were used for the *in vitro* antioxidant studies.

IV. Methodology:

1. DPPH radical scavenging assay

The free radical scavenging activity of the extracts was measured *in vitro* by 1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay (15). About 0.3 mM solution of DPPH in 100% ethanol was prepared and 1 ml of this solution was added to 3 ml of the extract dissolved in ethanol at different concentrations (25-400 µg/ml). The mixture was shaken and allowed to stand at room temperature for 30 min and the absorbance was measured at 517 nm using a spectrophotometer. The % scavenging activity at different concentrations was determined and the IC₅₀ value of the extracts was compared with that of ascorbic acid, which was used as the standard.

2. Reducing power ability

The reducing power was investigated by the Fe³⁺-Fe²⁺ transformation in the presence of the extracts as described by Fejes *et al.*, (16). The Fe²⁺ can be monitored by measuring the formation of Perl's Prussian blue at 700 nm. One ml of the extract (50-800 µg/ml), 2.5 ml of phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were incubated at 50°C for 30 min and 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged for 10 min at 3000g. About 2.5 ml of the supernatant was diluted with 2.5 ml of water and is shaken with 0.5 ml of freshly prepared 0.1% ferric chloride. The absorbance was measured at 700 nm. Butylated hydroxy toluene (50-800 µg/ml) was used as the standard. All tests were performed in triplicate and the graph was plotted with the average of the three determinations.

3. Phosphomolybdate method

The total antioxidant capacity of the extract was determined with phosphomolybdenum using α-tocopherol as the standard (17). An aliquot of 0.1ml of the extracts (1mg) solution was combined with 1.0 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had cooled to room temperature, the absorbance was measured at 695 nm against the blank using an UV spectrophotometer. The blank solution contained 1.0 ml of reagent solution and the appropriate volume of the same solvent used for the sample and it was incubated under same conditions as rest of the sample. The total antioxidant capacity was expressed as µg equivalents of α-tocopherol by using the standard tocopherol graph.

4. Estimation of total phenolic content

Total soluble phenolics of the extract were determined with Folin-Ciocalteu reagent using pyrocatechol as the standard (**18**). An aliquot of 0.1 ml suspension of 1 mg of the extracts in water was totally transferred to a 100 ml Erlenmeyer flask and the final volume was adjusted to 46 ml by the addition of distilled water. Folin-Ciocalteu reagent (1 ml) was added to this mixture, followed by 3 ml of 2% sodium carbonate 3 min later. Subsequently, the mixture was shaken for 2 h at room temperature and the absorbance was measured at 760 nm. The concentration of total phenolic compounds in the extracts was determined as μg pyrocatechol equivalent by using the standard pyrocatechol graph.

5. Estimation of total flavonoid content

Total soluble flavonoid content of the extracts was determined with aluminium nitrate using quercetin as the standard (**19**). One mg of the extract was added to 1ml of 80 % ethanol. An aliquot of 0.5 ml was added to test tubes containing 0.1 ml of 10 % aluminium nitrate, 0.1 ml of 1 M potassium acetate and 4.3 ml of 80 % ethanol. The absorbance of the supernatant was measured at 415 nm after incubation at room temperature for 40 min. The total flavonoid content in the extracts was determined as μg quercetin equivalent by using the standard quercetin graph.

Results

Phytochemical Investigations

Preliminary phytochemical tests of the ethanolic leaf extract shows the presence of alkaloids whereas the ethanolic bark extract reveals the presence of flavonoids as predominant active constituent.

DPPH assay

Both the leaf and bark extract demonstrated H-donor activity. The highest DPPH radical scavenging activity was detected in the ethanol bark extract with the IC_{50} value of 75.0 $\mu\text{g}/\text{ml}$ (Table 1). These activities are less than that of ascorbic acid.

Reducing power ability

Table 2 shows the reductive capabilities of the extract when compared to the standard, BHT. The reducing power increased with increasing amount of the extract. The ethanol bark extract showed the highest reducing ability ($A=0.690$ at a conc. of 800 $\mu\text{g}/\text{ml}$) than the leaf extract. However, the activity was less than the standard, BHT.

Table 2. Reducing power ability of the extracts of *W.tomentosa*

Extract (Conc. µg/ml)	Absorbance at 700 nm				
	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	800 µg/ml
Ethanol-leaf extract	0.363±0.003	0.374±0.005	0.445±0.008	0.480±0.008	0.592±0.005
Ethanol-bark extract	0.492±0.002	0.517±0.004	0.580±0.002	0.612±0.023	0.690±0.002
BHT	0.060±0.002	0.713±0.007	0.839±0.005	0.911±0.006	1.190±0.006

*All values are expressed as mean± SEM of three parallel measurements.

Phosphomolybdate method

The phosphomolybdate method is quantitative, since the total antioxidant capacity is expressed as α -tocopherol equivalents. Among the extracts tested, the ethanol bark extract contains 8.3µg vitamin E equivalent/ mg (Table 3).

Total phenolic and flavonoid content

Total phenolic content was estimated by using Folin-Ciocalteu reagent. Total phenolic content of the extract was solvent dependent and expressed as µg pyrocatechol equivalent. The content of the total phenolics in the ethanol bark extract (9.1 µg pyrocatechol equivalent/mg) was higher than the leaf extract. The total flavonoid content in the extracts was expressed as µg quercetin equivalent. The ethanol bark extract showed highest amount of flavonoids (20.0 µg quercetin equivalent/mg) among the extracts tested (Table 3).

Table 3. Total antioxidant activity (phosphomolybdate method) and total phenolic and flavonoid contents of the extracts of *W.tomentosa*

<i>Wrightia tomentosa</i> Extract	Total antioxidant activity (µg vitamin E equivalent/ mg)	Total phenolic content (µg-pyrocatechol equivalent/mg)	Total flavonoid content (µg-quercetin equivalent/mg)
Ethanol-leaf extract	4.2±0.03	7.2±0.88	16.9±1.0
Ethanol-bark extract	8.3±0.33	9.1±0.88	20.03±1.0

* All values are expressed as mean± SEM of three parallel measurements.

Table 1a. DPPH radical scavenging activity of the extracts of *W.tomentosa*

Extract	% Inhibition					*IC ₅₀ (µg/ml)
	25 µg/ml	50 µg/ml	100 µg/ml	200 µg/ml	400 µg/ml	
Ethanol leaf extract	14.85	31.4	43.53	65.64	88.32	135.1
Ethanol bark extract	19.72	41.26	61.79	73.24	87.18	75.0

Table 1b. DPPH radical scavenging activity of the standard, ascorbic acid

Standard	% Inhibition					*IC ₅₀ (µg/ml)
	1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	
Ascorbic acid	42.98	52.72	65.64	81.97	95.46	3.1

* Values obtained from regression lines with 95% of confidence level. IC₅₀ is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%.

All values given are mean of triplicate experiments at S.D (5%) for the above tables.

Discussion

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxy radicals, hydroxyl radicals and peroxynitrite. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, and inflammation and neurodegenerative diseases. Many flavanoids may help to provide protection against these diseases by contributing, along with antioxidant vitamins and enzymes, to the total antioxidant defense system of the human body.

In this connection, the phytochemical results also indicate the predominant active constituent flavanoid is present in bark extract of ethanol rather than leaf extract of *Wrightia*

tomentosa. The results obtained from DPPH radical scavenging assay reveals that the ethanolic bark extract gave an IC₅₀ value of 75.0 µg/ml when compared with leaf extract (IC₅₀ value = 135.1 µg/ml). Further more, the results from reducing power ability, total antioxidant capacity, total phenolic content and total flavanoid content in comparison with corresponding standards, Butylated hydroxy toluene (BHT), α – tocopherol, pyrocatechol & quercetin also clearly demonstrates that the bark extract showed markedly high antioxidant activity than the leaf extract. (Table 1-3).

The protective role of flavanoids involves several mechanism of action: direct antioxidant effect, inhibition of enzymes of oxygen – reduction pathways and sequestration of transient metal cations [8,9,20].

Some Isoflavones and 13 coumarins act as antioxidants through the inhibition of enzyme aldose reductase of oxygen-reduction pathway [21]. Since the plant possess the isoflavone active constituent wrightiadiene, the ethanolic bark extract of *Wrightia tomentosa* exert their mechanism probably by modulating the enzyme aldose reductase action in mammals. Further investigation is needed in this aspect. The above research studies suggest that *Wrightia tomentosa* bark possess a good antioxidant property which supports the literature that it maintains good health by boosting the immune system and reducing inflammation and allergies.

Therefore, it can be concluded that the alcoholic extract of *W.tomentosa* dried bark could be considered for preparation of nutraceuticals with potent antioxidant activity suitable for prevention of human disease.

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