Document heading doi: 10.21276/apjhs.2019.6.2.2 Research Article

Assessment of the In-vitro antioxidant activity of the alcoholic root extract/fractions of *Wrightia Tinctoria* Roxb.

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Received: 18-01-2019 / Revised: 20-02-2019 / Accepted: 20-03-2019

Abstract

Various parts of *Wrightia Tinctoria* Roxb have been utilized broadly in conventional Indian medication for treatment of anthelmintic, antidiarrhoeal, antidysenteric, astringent, febrifuge, seminal weakness and as an aphrodisiac. Current research on *Wrightia Tinctoria* Roxbis directed towards finding naturally-occurring antioxidants of plant origin that provided efficacy by added substance or synergistic exercises since cell reinforcements from plant root are basic to avoid the movement of free radical interceded issue. Antioxidant activity of methanolic *Wrightia Tinctoria* Roxbroot extract/fractiondiscover by utilizing distinctive in-vitro models. It includes Free radical scavenging activity of 1,1-Diphenyl-2-picryl-hydrazil (DPPH), ABTS radical scavenging activity**,** total antioxidant capacity**,** o-phenanthroline assay/Iron chelating activity. Plant contains a lot number of Phenolic compounds and Flavonoids. Plant shows significant antioxidant activity. The ponder was directed to investigate the utilization of root separate/portions of *W. Tinctoria*

Key words: *Wrightia Tinctoria* Roxb, Antioxidant, DPPH

Introduction

Wrightia tinctoria R. Br., (Apocynaceae) (W. tinctoria), a small deciduous tree is also known by different names, e.g. Sweet indrajao, Ivory tree, Easter tree and Pala indigoplant (English). In India, it is locally recognized by its differentvernacular names, the most commonly used ones are Indrajava, Svetkutaja, Krsnkutaja (Sanskrit), Kalakuada (Marathi) and Mitha indrajau (Hindi)[1]. It is widely used in Ayurvedic system of medicine for its various uses. The seeds are claimed to be useful as anthelmintic, antidiarrhoeal, antidysenteric, astringent, febrifuge, seminal weakness and as an aphrodisiac. The leaves and bark (decoction) are used, as febrifuge, in toothache, stomachic and tonic in bowel complaints. The bark is used as an antidysenteric, especially useful in piles, to treat skin diseases and biliousness in Ayurvedia[2].

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The bark of this plant is also used[3], as an anti-pyretic[4], anti-dysenteric, anti-diarrheal- and anti-hemorrhagic[5] agents, and as an antidote for snake poison[6]. Seeds of this plant are also used as an aphrodisiac[7]. In view of the reported severe health hazards of estrogen, such as increased risk of endometrial hyperplasia and carcinoma[8,9], breast cancer[10], and thromboembolic diseases[11]. A large number of natural products showing promising anti-fertility activity in preliminary studies could not be pursued due to their associated estrogen-agonistic activity[12].

The consumption of many vegetable has been associated with a lower risk of degenerative diseases, which depends on oxidative stress namely atherosclerosis, cancers, diabetes, Alzheimer's disease[13,14]. A great interest has been given to naturally occurring antioxidants, which may play important roles in inhibiting both free radicals and oxidative chain-reactions within tissues and membranes[15]. Therefore, screening plant materials on the basis of their antioxidant potency seems to be of central importance in order to identify extracts or

fractions possessing the ability either in scavenging both free radicals and chain reactions initiation or in binding with catalysts of the oxidative reactions, such as some metal[16]. From the viewpoint of their high antioxidant potency, the consumption at high scale of many plants have been recommended, therefore, the evaluation of antioxidant activities of extracts and fractions is considered as an important step prior to the isolation of antioxidant phytochemicals they contain[17].

Material and Method

Collection of Plant material

The plant material (root of W. *Tinctoria* Roxb.) was collected from University of Rajasthan (Jaipur, Rajasthan, India) in the month of September and authenticated by Herbarium department, University of Rajasthan, Jaipur, Rajasthan, India. The plant was deposited in the herbarium at Department of Botany (University of Rajasthan, Jaipur, Rajasthan, India).

Preparation of extract

Alcoholic extract: - The coarsely powdered (1000 g) oven-dried root of WT was extracted with alcohol by using soxhlet apparatus for 20 h. After completion of extraction, the solvent was removed by distillation and concentrated. The yield obtained was 18.8 % w/v.

Fractionation of crud extract

Fractionation of alcoholic extract Completely dried ethanolic extract was suspended in distilled water and extracted successively and exhaustively with solvents of increasing polarity like petroleum ether, dichloromethane, n-butanol, ethyl acetate. Each fraction was concentrated using rotary evaporator (Rotavapor, R-210, BUCHI Laborte, Switzerland) and stored in vacuum desiccator. The percentage yield of various extracts/fractions was Alcohol (WTA) [6.21% w/v], petroleum ether (WTP) [2.91 % w/v], dichloromethane (WTD) [7.56 % w/v], n-butanol (WTB) [18.54 % w/v] and ethyl acetate (WTE)[14.23% w/v]

Aqueous extract

1000 g of the coarsely powdered root of *W. tinctoria* was extracted by chloroform water (1:99) by cold maceration process for 7 days. After completion of extraction, the marc was filtered through muslin cloth and concentrated. The yield of WTW was obtained 18.3 %.

Qualitative determination of the chemical constituents

Presence of alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, phlobatannins, tannins and terpenoids in the extract and various fractions was confirmed individually by following standard procedures.

Test for alkaloids

Mixture of methanol extract of WT extract/faraction and its various derived fractions (0.4 g) in 8 ml of 1% HCl was warmed on water bath. After filtration 2 ml filtrate from the extract and each fraction was allowed to react with few drops of potassium mercuric iodide and with potassium bismuth, separately. Turbidity or precipitation formation was considered as a confirmation for presence of alkaloids.

Test for saponins

The criterion of oil emulsion formation of saponins was used for the screening of saponins [22]. Briefly, extract and various fractions (20 mg) suspended in 20 ml of distilled water and boiled for 5 min. In 10 ml of the above filtrate 5 ml of distilled water was added and mixed well to develop the froth. Development of emulsion after mixing the froth with olive oil confirmed the existence of saponins.

Test for terpenoids

Briefly, 2 ml of chloroform was mixed with 5 ml (1 mg/ml) of each sample in a test tube then 3 ml of concentrated H_2SO_4 was added to develop the color. Exhibition of reddish brown coloration at the interface confirmed the presence of terpenoids.

Test for anthraquinones

To a volume of 6 ml of 1% HCl, 200 mg of each sample was added separately and boiled. Benzene (5 ml) was mixed with the filtrate and after separation of benzene layer 2 ml of 10% ammonia solution was lowered. Development of pink, violet or red color in the ammonical phase indicated the existence of anthraquinones[18].

Cardiac glycosides determination

An aliquot of 5 ml of ethanol extract of *W. tinctoria* root and its various fractions (10 mg/ml in methanol) were added in the sequence of glacial acetic acid (2 ml) and $FeCl_3$ solution (one drop). Concentrated H_2SO_4 (1 ml) was added and the formation of brown ring at the interface confirmed the presence of cardiac glycosides.

Test for coumarins

In a vial having 300 mg/ml of the extract and each fraction was plugged with filter paper dipped in 1 N NaOH and boiled in a boiling water bath for few minutes. Yellow fluorescence of filter paper under UV light confirmed the presence of coumarins.

Test for phlobatannins

An amount of 80 mg of the extract and various fractions was boiled in 1% HCl. Development of red precipitate indicated the existence of phlobatannins[19].

Test for flavonoids

Mixture of methanol extract and various fractions of WT root were prepared by adding 50 mg of each sample to 100 ml of distilled water and filtered. An aliquot of 5 ml of dilute ammonia solution was mixed with 10 ml of the filtrate. Appearance of yellow coloration by addition of few drops of concentrated sulfuric acid indicated the presence of flavonoid.

Test for tannins

A mixture was prepared by mixing 50 mg of methanol extract and each fraction in 20 ml of distilled water and boiled. Appearance of brownish green or blue-black coloration after mixing few drops of 0.1% $FeCl_3$ confirmed the existence of tannins[20].

Determination of in-vitro scavenging activities

DPPH radical scavenging activity

The free radical scavenging activity of the extract and fractions was evaluated using the stable DPPH free radical. One mL of 0.1 mM DPPH solution in methanol was added to 1.0 mL of standard and extract/fractions solution at different concentrations. The mixture was incubated for 20 min and the absorbance recorded at 517 nm. Ascorbic acid was used as positive control. DPPH radical scavenging activity was calculated using the formula:

Percent scavenging = ((Ao – At)/Ao) × 100; where Ao =Absorbance of control (without extract) and At = Absorbance of sample. All determinations were carried out in triplicate[21,22].

ABTS radical scavenging activity

ABTS free radical was generated by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate. The mixture was allowed to stand for 15 h in dark at room temperature. ABTS solution was diluted with methanol to obtain the absorbance of 0.7 ± 0.2 units at 750 nm. The standard/extract solutions were prepared at different concentrations in methanol and 20 µL of test solutions were added to 180 µL of ABTS free radical solution. The absorbance was measured after 20 minutes incubation at 750 nm. Ascorbic acid was used as positive control. The ABTS free radical scavenging activity was calculated using the formula:

Percent scavenging = ((Ao – At)/Ao)× 100; where Ao = Absorbance of control (without extract) and At = Absorbance of sample[22]. All the tests were performed in triplicate.

Total antioxidant capacity

The total antioxidant capacities of the extract and fractions of *W. tinctoria* root were determined using phosphomolybdenum method. Briefly, 0.1 mL of standard/ extract solution was mixed with 0.3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) and incubated at 95°C for 90 min. The mixture was cooled down to room temperature and absorbance recorded at 695 nm. The blank solution contained all the reagents except the test sample. Ascorbic acid was used to plot the standard curve. The results were expressed as ascorbic acid equivalents[23]. All the tests were performed in triplicate.

o-Phenanthroline assay/Iron chelating activity

The 1, l0-Phenanthroline-iron (III) reagent was prepared by mixing 0.198 g of l, l0-phenanthroline monohydrate, 2 mL of 1 M hydrochloric acid and 0.16 g of ferric ammonium sulphate in 100 mL water. Briefly, 0.2 mL standard/extracts were mixed with 0.2 mL 1, l0- phenanthroline-iron (III) reagent, 0.6 mL methanol and 4 mL water. The solutions were incubated at 50°C for 30 min and absorbance read at 510 nm. Ascorbic acid was used as positive control. A higher absorbance indicated higher iron chelating activity

Percentage scavenging was calculated by using the following formula:

Percent scavenging = ((At – Ao)/At) × 100; where Ao = Absorbance of control (without extract) and At = Absorbance of sample[24,25]. All the tests were performed in triplicate.

Results

Phytochemical analysis

Phytochemical analysis of ethanol extract/fractions of *W. tinctorial* root indicated the existence of alkaloids, anthraquinones, cardiac glycosides, coumarins, flavonoids, saponins, phlobatannins, tannins and terpenoids. Cardiac glycosides, saponins, phlobatannins and terpenoids were not determined in pet. ether fraction while alkaloids, anthraquinones, cardiac glycosides, flavonoids and tannins were not detected in butanol fraction. Coumarins, saponins and terpenoids were not detected in ethyl acetate. cardiac glycosides, flavonoids, phlobatannins and terpenoids were not detected in dichloromethane fraction (table no.1)

Table No.1: Estimation of phytochemicals of extract and its fractions of *W. tinctorial* root

Phytochemical tests	Ethanol	Pet. Ether	Butanol	Ethyl Acetate	Dichloromethane	Aqueous
Alkaloids	+	+	-	+	+	+
Anthraquinones	+	+	-	+	+	+
Cardiacglycosides	+	-	-	+	-	+
Coumarins	+		+	-	+	+
Flavonoids	+	+	-	+	-	+
Saponins	+	-	+	-	+	+
Phlobatannins	+	-	+	+	-	+
Tannins	+	+	-	+	+	+
Terpenoids	+	-	+	-	+	+

DPPH radical scavenging activity

Scavenging of DPPH free radicals by different concentration of extract/fractions were evaluated. The extract/fractions showed concentration dependent radical scavenging activity.IC_{50} concentration of WTA,WTP,WTD,WTB, WTE and WTW were found to be 44.08, 17.73, 13.58, 8.94,12.13 and 16.86 µg/mL respectively **(Table 2 and Fig.1).**

Table 2: Effect of different extracts of *W. tinctoria*. on DPPH free radical scavenging activity

Concentration (µg/mL)	Percentage scavenging					
	WTA	WTP	WTD	WTB	WTE	WTW
6.25	39.24±0.00	35.71±0.00	44.62±0.00	45.45±0.00	44.19±0.00	43.31±0.00
12.5	45.86±0.00	46.27±0.00	50.00±0.00	55.28±0.00	50.34±0.00	51.02±0.00
25	48.20±0.00	49.30±0.00	54.14±0.00	56.10±0.00	56.89±0.00	53.25±0.00
50	51.35±0.00	65.05±0.00	59.32±0.00	64.18±0.00	62.30±0.00	54.43±0.00
100	53.25±0.00	75.51±0.01	62.30±0.00	69.62±0.00	66.82±0.00	56.36±0.00
200	56.10±0.00	96.51±0.29	72.93±0.00	70.49±0.00	75.51±0.00	60.00±0.00

All the values are in mean±SEM

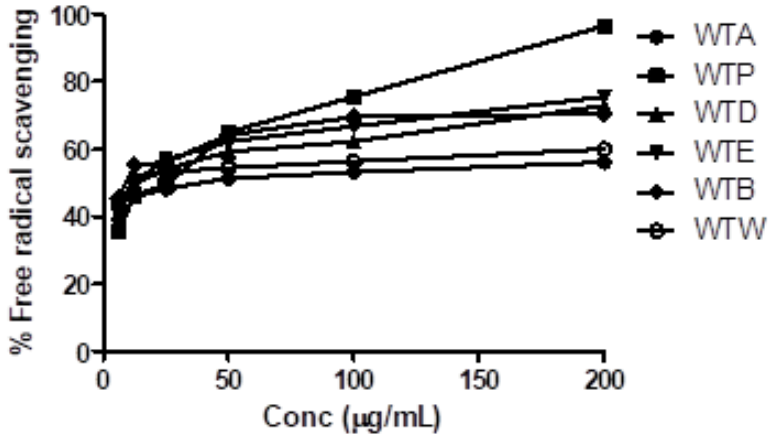

Fig. 1: Effect of different extracts of *W. tinctoria*. on DPPH free radical scavenging activity

ABTS radical scavenging activity

Distinctive Extract/portions demonstrated concentration dependent ABTS radical scavenging activity. IC_{50} concentration of WTA, WTP, WTD, WTB, WTE and WTW were found to be 7.208, 7.912, 5.505, 3.227, 6.198 and 4.584 µg/mL respectively **(Table 3 and Fig.2).**

Table 3: Effect of different extracts of *W. tinctoria*. on ABTS radical scavenging activity

Concentration (µg/ml)	Percentage scavenging					
	WTA	**WTP**	**WTD**	**WTB**	**WTE**	**WTW**
6.25	43.61±0.03	13.45±0.03	62.58±0.03	61.92±0.00	74.83±0.01	65.45±0.07
12.5	68.04±0.01	19.51±0.00	48.40±0.03	68.91±0.01	75.70±0.03	48.67±0.04
25	91.15±0.01	28.03±0.03	71.90±0.01	91.08±0.01	90.75±0.00	73.77±0.05
50	91.08±0.00	37.35±0.03	91.74±0.01	91.15±0.00	91.41±0.00	90.88±0.00
100	91.81±0.01	60.92±0.00	91.41±0.00	89.88±0.00	90.28±0.00	90.88±0.00
200	87.68±0.00	64.38±0.01	90.95±0.00	88.55±0.00	89.48±0.00	89.48±0.00

All the values are in mean±SEM

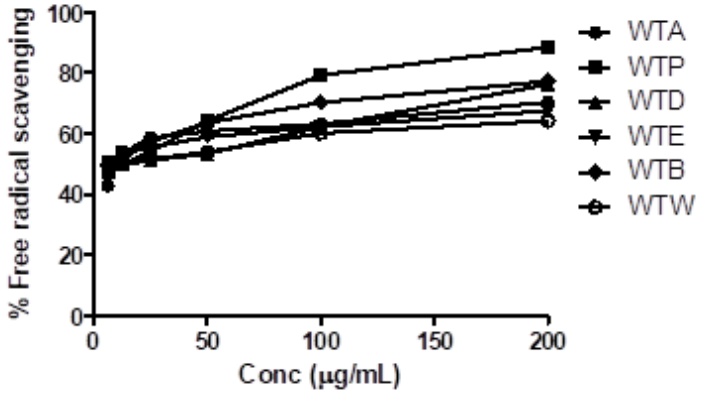

Fig. 2. Effect of different extracts of *W. tinctoria*. on ABTS radical scavenging Activity

O-Phenanthroline assay

Ferric ion reduction potential of the various extract/fractions were investigated. Extract/fractions exhibited concentration dependent response. The IC_{50} of WTA, WTP, WTD, WTB, WTE and WTW were found to be 10.95, 11.73, 14.14, 8.913, 6.366 and 10.63 respectively **(Table 4& Fig.3).**

Table 4: Effect of different extracts of *W. tinctoria*. on ferric ion reduction potential

Concentration (µg/ml)	Percentage scavenging					
	WTA	**WTP**	**WTD**	**WTB**	**WTE**	**WTW**
12.5	42.86±0.00	47.22±0.00	47.95±0.00	50.65±0.00	49.33±0.00	49.67±0.00
25	53.37±0.00	49.67±0.00	49.67±0.00	53.66±0.01	51.59±0.00	50.97±0.00
50	58.70±0.00	54.22±0.00	51.28±0.00	55.56±0.00	57.54±0.00	51.59±0.00
100	60.82±0.00	64.32±0.00	53.37±0.00	58.92±0.00	63.64±0.00	53.94±0.00
200	63.29±0.00	79.29±0.01	62.38±0.00	62.38±0.00	70.31±0.00	60.21±0.00
400	70.43±0.00	88.55±0.01	76.25±0.01	67.52±0.00	77.25±0.01	64.32±0.00

All the values are in mean±SEM

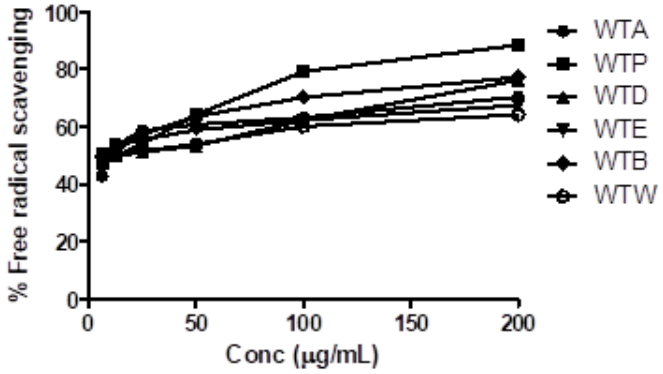

Fig.3. Effect of different extracts of *W. tinctoria*. on O-Phenanthroline assay

The reducing power of WT different extract/fractions displayed a concentration-dependent antioxidant activity there was increase in the absorbance with increase in concentration (**Fig.1**). It was observed that WT scavenged the radical in concentration dependent manner. The percent scavenging of DPPH radical, ABTS radicals by WT and ascorbic acid were found to be and respectively (**Table 2** and **3**).Ferric ions do not get chelated by o-Phenanthroline. However, ferrous ions readily get chelated by o-Phenanthroline. The absorbance of this complex is WT assured at 510 nm. The absorbance is directly proportional to the antioxidant activity of the test compound.The IC_{50} values of various extracts/fractions of WT and ascorbic acid shown in **Table 5** for the reduction of ferric ions in the o-Phenanthroline assay, free radical scavenging activity by DPPH radical and ABTS radical assay. Where it was observed WT possess strong antioxidant activity compared to ascorbic acidin ABTS radical assay.

Table 5: IC_{50} values of different extracts of *W. tinctoria*. in various *in vitro* antioxidant assays

Extracts/Fractions	IC_{50} values (µg/ml)		
	DPPH	ABTS	o-Phenanthroline
WTA	44.08	7.208	10.95
WTP	17.73	7.912	11.73
WTD	13.58	5.505	14.14
WTB	8.94	3.227	8.913
WTE	12.13	6.198	6.366
WTW	16.86	4.584	10.63
Asc.A	8.445	13.39	1.445

Discussion

The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides[26]. For the measurements of the reductive ability, it has been found that the Fe^{3+}-Fe^{2+} transformation occurred in the presence of extract/fractions samples which was postulated previously by Oyaizu[27] Tanaka et al. have observed a direct correlation between antioxidant activities and reducing power of certain plant extracts[28]. The reducing properties are generally associated with the presence of reductones,[29] which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[30].Reductions are also reported to react with certain precursors of peroxide, thus preventing peroxide formation. In this assay, depending on the reducing power of antioxidant compounds, the yellow color of the test solution changes into various shades of green and blue. Therefore, by measuring the formation of Perl's Prussian blue at 700 nm, we can monitor the Fe^{2+} concentration. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. WT indicates significant antioxidant activity. Phenolic compounds are known powerful chain breaking antioxidants,[31] important plant constituents because of their scavenging ability due to their hydroxyl groups and contribute directly to antioxidative action[32] Phenolic compounds are also effective hydrogen donors, which makes them good antioxidants[33]. It is suggested that polyphenolic compounds have inhibitory effects on mutagenesis and

carcinogenesis in humans, when ingested up to 1g daily with a diet rich in fruits and vegetables[34].2,2-diphenyl-1- picrylhydrazyl (DPPH) is a stable radical that has been used widely to evaluate the antioxidant activity of various natural products[35,36].In this study, all of the extracts tested possess radical scavenging activity. This activity was increased by increasing the concentration of the sample extract. Further research on this plant may be helpful to find out more potent antioxidant.

Conclusion

The present examination gives proof about nearness of much measure of flavonoid and phenolic content in the WT. It demonstrates potential cell reinforcement and free radical searching movement. These in-vitro assays show that plant extracts/fractions are significant wellsprings of common cancer prevention agents, which may be helpful as preventive specialists against oxidative stress. To clarify the prime wellspring of cell reinforcement properties further examinations ought to be done with isolate active principles.

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Conflict of Interest: None
Source of Support: Nil