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ANTI OXIDANT ACTIVITY, PHENOLIC AND FLAVONOID CONTENTS OF *Wrightia tinctoria* LEAVES

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ABSTRACT: *Wrightia tinctoria* (Roxb) R. Br. (Apocyanaceae) is widely distributed throughout India. Literature review of the plant reports that its bark is having wound healing, antibacterial, anti nociceptive and post coital interceptive activity. Antibacterial property has been reported in *Wrightia tinctoria* seeds. Studies on flowers proven to posses anti-inflammatory activity. Hence this study was intended to evaluate the antioxidant activity of the leaves of by beta- carotene bleaching method and nitric oxide radical scavenging method. Ethyl acetate fraction showed significant antioxidant activity.

KEYWORDS: - *Wrightia tinctoria*, Anti oxidant, Medicinal plant, Flavonoid, Free radical

1. INTRODUCTION

Antioxidants or inhibitors of oxidation are the compounds which retard or prevent oxidation and prolong the activity of oxidizable matter. The reactive oxygen species in the body such a superoxide anion ($O_2^{\cdot-}$), singlet oxygen ($O_2^{\cdot}$), hydroxyl radical (OH^{-}) and hydrogen peroxide (H_2O_2) tend to react with electrons of other molecules of the body and various enzyme systems; with the result the molecule as well as enzyme system of the body is damaged which may contribute to various disease conditions such as cancer, ischaemia, ageing, rheumatoid arthritis etc (1). As a result of this much attention has been focused on the use of antioxidants. A great no: of aromatic and other medicinal plants contain chemical compounds such as phenolics that exhibit antioxidant properties (2).

The objective of this study were to investigate the total phenolic and flavonoid content and the free radical scavenging activity of various fractions of total ethanolic extract and aqueous extract of Wrightia tinctoria leaves. The β-carotene bleaching method and nitric oxide radical scavenging method were used to determine the antioxidant activity.

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2. MATERIALS AND METHODS

2.1. Plant material

The fresh leaves of *Wrightia tinctoria* (Roxb) R.Br were collected from Palai, Kerala, India and authenticated by Mr. Joby Paul, Botanist, Environmental Sciences, M. G University, Kottayam. The collected leaves were dried in shade and used for the study.

2.2.1. Extraction

Dried leaves were powdered and 300g of the powder was exhaustively extracted with ethanol in a Soxhlet extractor. The extract was concentrated to a solid residue and the yield was calculated (3).

2.2.2 Fractionation of the extract

The total ethanolic extract was fractionated using the solvents in the order of increasing polarity like petroleum ether, benzene, chloroform and ethyl acetate. Each extracts were concentrated to a solid residue and the yield was calculated (3, 4).

2.2.3. Preparation of aqueous extract

The marc obtained after ethanolic extraction was subjected to aqueous extraction by reflux method.

The extract was concentrated to a solid residue and the yield was calculated (3,4).

2.3. Chemicals

Gallic acid, Follin Ciocalteau reagent (2 N was diluted to 1: 10 ml with distilled water), 7.5% Sodium carbonate, Rutin, 5% sodium nitrite, 10% Aluminium chloride, 1 M Sodium hydroxide, Beta carotene, Linoleic acid, Tween 20, Propyl gallate, Sodium nitroprusside (10 mM), Phosphate buffer saline, Sulphanilic acid reagent (0.33% sulphanilic acid dissolved in 20% GAA), 1-Naphthylamine, Quercetin, Methanol, Chloroform, Distilled water. All the chemicals used including solvents, were of analytical grade.

2.4. Estimation of Total Phenolic Content

Total phenolic content was estimated by Folin-Ciocalteu method using Gallic acid as standard (5, 6). 1 ml of each extracts (1000µg/ml) were separately mixed with 5 ml of Folin Ciocalteu reagent (1:10 diluted with distilled water) and left for 3 minutes and finally 4 ml of 7.5% sodium carbonate solution was added. A reagent blank was also prepared using 1 ml of distilled water instead of gallic acid. These test tubes were kept for 2 hours at room temperature away from strong light. A dark blue colour was developed, which was measured at 765 nm using UV/ VIS spectrophotometer. The standard curve was prepared by 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml solutions of gallic acid in methanol:water (50:50 v/v). Total phenolic content is expresses as gallic acid equivalent.

2.5. Estimation of Total Flavonoid Content

Total Flavonoid content was estimated by aluminium chloride colorimetric method (5, 7). 1 ml of each extracts (1000µg/ml) were separately mixed with 0.3 ml of sodium nitrite. After 5 minutes 0.3 ml of 10% Aluminium chloride solution was added. At the 6th minute 2 ml 1 M sodium hydroxide solution was added. And total volume was made up to 10 ml with distilled water. A blank was prepared by adding the entire component except Aluminium chloride solution, wherein 0.3 ml distilled water was used in place of Aluminium chloride. These solutions were mixed well and the absorbance was measured against a blank at 510 nm using UV/ VIS spectrophotometer. The standard curve was prepared by 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml solutions of rutin in methanol. Total flavonoid content is expresses as rutin equivalent.

2.6. Beta Carotene Bleaching Method

10 mg of Propyl gallate dissolved in 0.2 ml of ethanol was used as the standard and a control containing 0.2 ml of methanol was used for the assay (5). To the standard and control 5 ml of beta carotene emulsion was added. The tubes were gently shaken and placed at 45°C in a water bath for 60 minutes. The absorbance of the standard, sample and control was measured at 470 nm using UV/ Visible spectrophotometer against a blank consisting of an emulsion without beta carotene.

Samples included the total ethanolic extract of the leaves and their chloroform, ethyl acetate and aqueous extract. 10 mg of each extract were dissolved in 0.2 ml of ethanol and preceded in the same manner as standard.

The measurement was carried out at initial time (t = 0) and successively at 30 and 60 minutes. The antioxidant activity was measured in terms of successful bleaching of Beta carotene using the equation.

$$AA = [1- A_0 - A_t / A_0° - A_t°] \times 100$$

Where A_0 and $A_0°$ are the absorbance measured at the initial incubation time for standard/ sample and control respectively. While A_t and $A_t°$ are the absorbance measured in the standard/ sample and control respectively at t = 0 minute and t = 60 minute.

2.7. Nitric Oxide Radical Scavenging method(5)

The standard solution was Quercetin prepared in methanol (100µg/ ml). The reaction mixture (3 ml) containing sodium nitropruside (2 ml), phosphate buffer saline (0.5 ml) and standard solution (0.5 ml) was incubated at 25° C for 2.5 hours. After incubation 0.5 ml of the reaction mixture containing nitrate

was pippeted out and mixed with 1ml of sulphanilic acid reagent and allowed to stand for 5 minutes for completing diazotization. Then 1ml of 1-naphthylamine was added mixed and allowed to stand for 30 minutes at room temperature. A pink colour was formed in diffused light. A control was also prepared by the above solutions without standard. The absorbance of the solution was measured at 540 nm against the corresponding blank solution.

Different extracts of *Wrightia tinctoria* (100µg/ ml) were prepared in phosphate buffer saline and preceded in the same manner as standard. The percentage inhibition was calculated for the samples and standard using the following equation

$$I\% = Ac - At / Ac \times 100$$

Where, Ac is the absorbance of the control and At is the absorbance of sample/ standard.

2.8. Statistical analysis

Data obtained are expressed as mean ±SEM. significant differences in means of samples were evaluated by one way analysis of variance (ANOVA).

3. RESULTS AND DISCUSSION

3.1. Estimation of total phenolics

The standard graph of gallic acid is shown in Table 1 and Figure 1 .The amount of phenolics in the total ethanol extract, its different fractions and aqueous extract are shown in Table 2 and Figure 2. The total phenolics content was found to be maximum in ethyl acetate fraction followed by total ethanolic extract compared to other fractions.

3.2. Estimation of total flavonoids

The standard graph of rutin is shown in Table 3 and Figure 3. The amount of flavonoids in total ethanolic extract, its different fractions and aqueous extract are shown in Table 4 and Figure 4. The total flavonoid content was found to be highest in ethyl acetate fraction followed by total ethanol extract compared to other fractions.

Table 1 Preparation of standard graph

Concentration of Gallic acid in µg/ml	Absorbance at 750nm
10	0.105
20	0.23
30	0.301
40	0.394
50	0.519
60	0.623
70	0.716
80	0.828
90	0.924
100	0.988

Figure 1 Standard graph of gallic acid

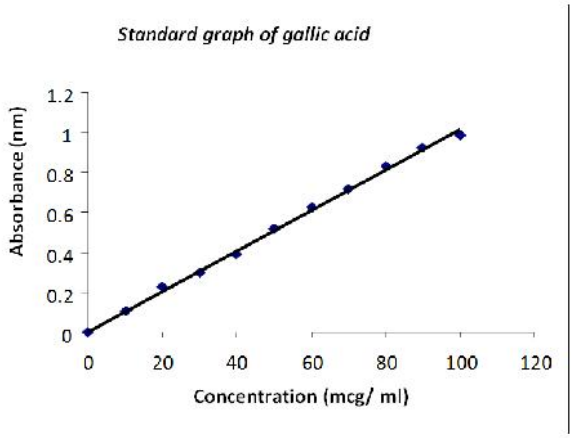
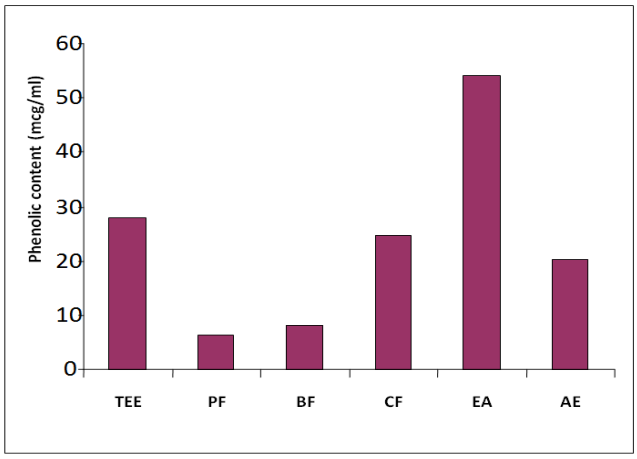


Table 2 Estimation of total phenolics in samples

Name of sample	Absorbance (750nm)	Total phenolics (in Gallic acid equivalents)(µg/ml)
TEE	0.287	28.13
PEF	0.065	6.3
BF	0.083	8.14
CF	0.253	24.80
EAF	0.5518	54.09
AE	0.207	20.29

TEE: Total ethanolic extract; PEF: petroleum ether fraction; BF: benzene fraction; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

Figure 2 Total phenolics in various fractions



TEE: Total ethanolic extract; PEF: petroleum ether fraction; BF: benzene fraction; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

Table 3 Preparation of standard graph

SI No:	Concentration of Rutin (µg/ml)	Absorbance 510 nm
1.	10	0.028
2.	20	0.049
3.	30	0.080
4.	40	0.107
5	50	0.131
6	60	0.158
7	70	0.191
8	80	0.218
9	90	0.246
10	100	0.273

Figure 3 Standard graph of rutin

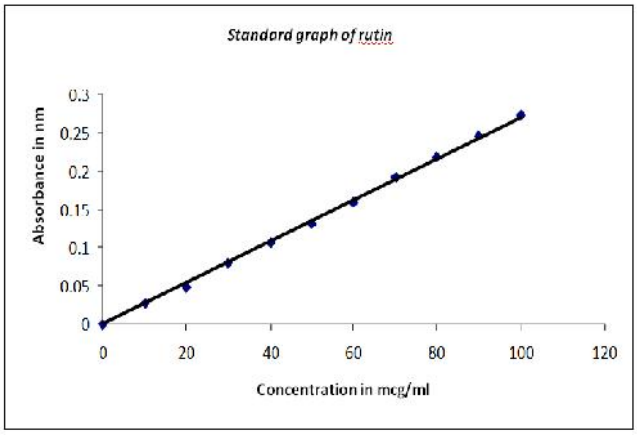
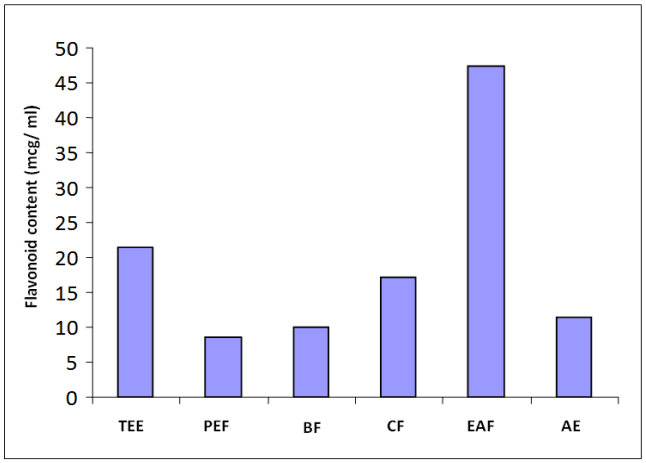


Table 4 Estimation of total flavonoids in samples

Name of sample	Mean Absorbance at 510 nm	Total flavonoids (in rutin equivalents)
TEE	0.058	21.48
PEF	0.023	8.52
BF	0.027	10
CF	0.046	17.04
EAF	0.128	47.41
AE	0.010	11.48

TEE: Total ethanolic extract; PEF: petroleum ether fraction; BF: benzene fraction; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

Figure 4 Total flavonoid content of various fractions



3.3. β- carotene bleaching method

In the *in vitro* anti oxidant activity study using beta carotene bleaching method, all the fractions showed anti oxidant activity. Ethyl acetate fraction showed more activity compared to other fractions (Table 5 and Figure 5).

3.4. Nitric oxide radical scavenging method

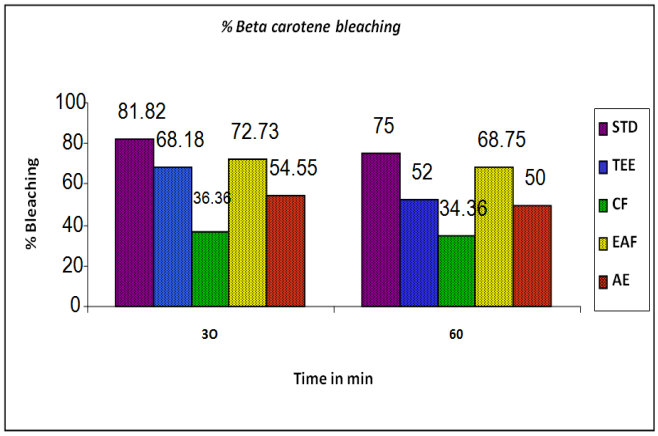
In the *in vitro* anti oxidant activity study using nitric oxide radical scavenging assay, total ethanolic extract, its chloroform fraction, ethyl acetate fraction and aqueous extract were used. All the fractions showed anti oxidant activity. Ethyl acetate fraction showed more activity compared to other fractions. (Table 6 and Figure 6)

Table 5 β Carotene bleaching method

Name of sample	Absorbance 470 nm			% β-carotene bleaching	
	0 time	30 minutes	60 minutes	30 min	60 min
CTRL	0.169 ±0.0023	0.147 ±0.0029	0.137 ± 0.0012	-	-
STD	0.222 ± 0.0020	0.218 ± 0.0018	0.214 ± 0.0010	81.82	75.00
TEE	0.589 ± 0.0020	0.582 ± 0.0020	0.573 ± 0.0020	68.18	52.00
CF	0.671± 0.0017	0.660 ± 0.0062	0.655 ± 0.0026	36.36	34.36
EAF	0.558 ± 0.0030	0.552± 0.0032	0.548 ± 0.0023	72.73	68.75
AE	0.676 ± 0.0021	0.662 ± 0.0050	0.655 ± 0.0021	54.55	52.00

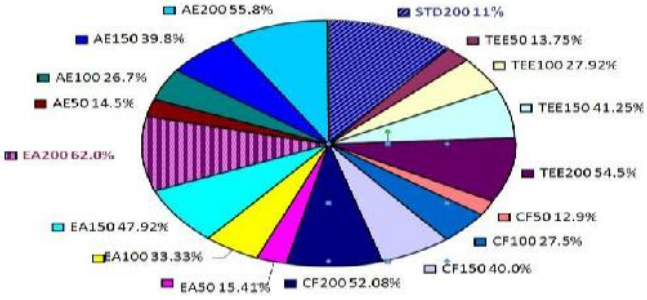
CTRL: Control; STD: Standard; TEE: Total ethanolic extract; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

Figure 5 Percentage β-carotene bleaching



STD: Standard; TEE: Total ethanolic extract; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

Figure 6 Nitric oxide radical scavenging method



STD: Standard; TEE: Total ethanolic extract; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

Table 6 Nitric oxide radical scavenging assay

Name of sample	Concentration of sample	Absorbance	% Inhibition
CTRL	-	0.240	-
STD	50	0.162 ± 0.012	32.50
	100	0.138 ± 0.011	42.50
	150	0.109 ± 0.015	54.92
	200	0.069 ± 0.011	71.25
TEE	50	0.207 ± 0.011	13.75
	100	0.173 ± 0.008	27.92
	150	0.136 ± 0.015	41.25
	200	0.109 ± 0.005	54.50
CF	50	0.209 ± 0.010	12.90
	100	0.174 ± 0.002	27.50
	150	0.144 ± 0.002	40.00
	200	0.115 ± 0.005	52.08
EAF	50	0.203 ± 0.008	15.41
	100	0.160 ± 0.008	33.33
	150	0.125± 0.004	47.92
	200	0.091± 0.013	62.00
AE	50	0.205 ± 0.009	14.50
	100	0.176 ± 0.005	26.70
	150	0.145 ± 0.007	39.80
	200	0.106 ± 0.009	55.80

CTRL: Control; STD: Standard; TEE: Total ethanolic extract; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

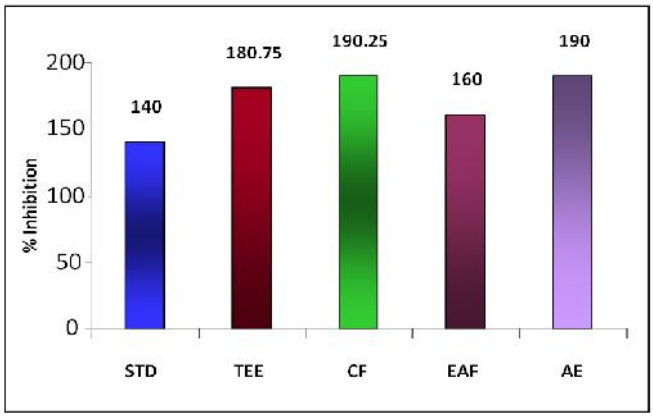
IC_{50} values of total ethanolic extract, its chloroform fraction, ethyl acetate fraction and aqueous extract in nitric oxide radical scavenging method is shown in Table 7 and Figure 7.

Table 7 IC_{50} values of different fractions

SNO	Samples	IC_{50} value (µg/ml)
1	Quercetin (STD)	140.00
2	TEE	180.75
3	CF	190.25
4	EAF	160.00
5	AE	190.00

TEE: Total ethanolic extract; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

Figure 7 IC_{50} values of different fractions



TEE: Total ethanolic extract; CF: Chloroform extract; EAF: Ethyl acetate fraction; AE: Aqueous extract of mark

4. CONCLUSION

Based on the results of present study, we conclude that the *Wrightia tinctoria* plant extract possesses strong anti oxidant potential. Total ethanolic extract and its various fractions showed significant antioxidant activity. But the activity was more with ethyl acetate fraction. However further studies are necessary examine underlying mechanisms of antioxidant effects and to isolate active compounds responsible for these pharmacological activities.

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