



Research Article

COMPARATIVE EVALUATION OF ABTS, DPPH, FRAP, NITRIC OXIDE ASSAYS FOR ANTIOXIDANT POTENTIAL, PHENOLIC & FLAVONOID CONTENT OF *EHRETIA ACUMINATA* R. BR. BARK

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ABSTRACT

Barks extracts of traditionally important medicinal plant, *Ehretia acuminata* R. Br. was screened for their free radical scavenging properties using ascorbic acid (A.A) and gallic acid (G.A) as standard antioxidant. Free radical scavenging activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), Ferric oxide reducing assay (FRAP) and nitric oxide assay. There were positive correlations between antioxidant activity assessed by ABTS, DPPH, FRAP & Nitric oxide, between Total Phenolic and flavonoids content with all methods using five extract Petroleum ether (P.E), chloroform (CH), ethyl acetate (E.A), ethanol (EOL) & water (WTR). The overall antioxidant activity of bark was the strongest in ethyl acetate and weakest in water extract. The IC₅₀ of the ethyl acetate extracts ranged between 22 and 140 µg/ml and that of ascorbic acid was 28 µg/ml. The study reveals that the consumption of this plant would exert several beneficial effects by virtue of their antioxidant activity.

KEY WORDS: DPPH, ABTS, FRAP, Nitric oxide, Total antioxidant capacity, Total phenolics, Flavonoids.

INTRODUCTION

Reactive oxygen species (ROS) like superoxide anion, singlet oxygen, hydrogen peroxide (H₂O₂) and hydroxyl radical are mostly generated as byproducts of biological reactions¹. These reactive species exert oxidative damage by reacting with nearly every molecule found in living cells and also with DNA². Excess ROS, if not eliminated by antioxidant system, result in high levels of free radicals and lipid peroxides which bring forth the pathogenesis diseases like atherosclerosis, diabetes, carcinogenesis, cataract, ageing etc^{3,4}. All aerobic organisms have antioxidant defence systems to setoff harmful effects caused by free radicals. Antioxidants can be found naturally in foods⁵. A majority of antioxidants naturally present in foods is because of phenolic and flavonoid structures. The natural phenolic antioxidants usually act as reducing agents, terminate the free radical chain reaction by removing the same, absorb light in the ultraviolet region (100-400 nm), and chelate transition metals, thus inhibit oxidation reactions by itself being oxidized and also forbid the production of oxidative damage⁶. Although It is well-known that flavonoids can neutralize different types of oxidizing species including hydroxyl radical, superoxide anion or peroxy radicals. They may also work as quenchers of singlet oxygen⁷. Total flavonoids, obtained significant antioxidant activity and inhibited the lipid peroxidation caused by H₂O₂, O₂ and UV irradiation⁸. In addition, antioxidants are added to nutrients to prevent deterioration in their smell, taste and colour. butylated hydroxytoluene (BHT), Butylated hydroxyanisole (BHA) and propyl gallate (PG) can be included in this group, which are commonly known synthetic antioxidants⁹. The high cost of natural antioxidants has led to more use of synthetic antioxidants. However, studies conducted subsequently have demonstrated that synthetic antioxidants have toxic effects and consequently, restrictions have been enforced on their use. Therefore, researchers have focused on plants derived natural

antioxidants^{10,11}. These natural antioxidants usually come from a diet rich in fruits and vegetables or they are carried in creams and topically applied¹². Plant extracts with antioxidant potential raise great interest in the phytocosmetic field as they present molecules that could inactivate ROS restoring skin homeostasis thus preventing erythema and premature aging of the skin.

Ehretia acuminata, which is commonly known as Koda, is one of the oldest spices of genus *Ehretia*. Nowadays, it is used in Chinese herbal medicine¹³. Traditionally, it has been used fever, sores on tongue, dysentery and many more health ailments^{14,15}. Some species of the *Ehretia* genus are used for medical purposes. However, many species include phytochemicals alkaloids^{16,17}, flavonoids^{18,19,20}, fatty acids²¹, which are responsible for antioxidant^{22,23}, anti-inflammatory^{24,25}, anti-allergic^{26,27}, antibacterial^{28,29} activities in different species like *E. laevis*, *E. tinifolia*, *E. microphylla*, *E. longiflora*, *E. obtusifolia*.

The aim of the present research is comparative study of antioxidant activity by ABTS, DPPH, FRAP, reducing capacity of Nitric oxide, total phenolic content and flavonoid content from *Ehretia acuminata* bark extracts by different solvent systems. Moreover, Ascorbic acid and gallic acid which are synthetic antioxidant, was evaluated as a positive control and compared with all extracts of the plant.

MATERIAL AND METHODS

Plant materials

E. acuminata was collected from pantnagar (daytime air temperature, 26–27.9 °C) in Udham singh nagar district of Uttarakhand in Kumaun region of India in the month of April 2017 and authenticated from Botanical survey of India (BSI) Dehradun (Voucher specimen number 117138 05/2017). Bark were dried for 15–20 days under shade until bark seem brittle or

ready for grinding and stored at 15 °C, were subjected to grinding in a laboratory grinder and stored at 4 °C.

Chemicals and instruments

Folin ciocalteu reagent (Merck), the stable free radical 2, 2'-Diphenyl-1-picryl hydrazyl (DPPH) (Sigma), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), ascorbic acid (AA) (Merck) and gallic acid (GA) (Loba chemie), All other chemicals used were of analytical grade. UV-VIS Spectrophotometer 118 (Systronic).

Preparation of crude plant extract

Plants material was collected from kumaun region Uttarakhand. Plant material consisting of Bark was also collected & dried. The dried bark materials were powdered using a grinder. The extraction was done at room temperature. About 100g of dried, ground plant materials were soaked in different solvents (600 ml) according to increasing order of polarity (petroleum ether, chloroform, ethyl acetate, ethanol, water) for 5-8 days separately. The soaked material was stirred every 20 hours using a sterilized glass rod. The final extracts were passed through Whatman filter paper No.1. The filtrates obtained were concentrated under vacuum on a rotary evaporator at 42 °C & stored at 4°C for further use. The stock solution of different extracts (5 mg/ml) was prepared by dissolving a known amount of dry extract in 98% methanol. The working solutions (25, 50, 75, 100, 250, 500 & 1000 µg/ml) of the extracts were prepared from the stock solution using suitable dilution.

ANTIOXIDANT PROPERTIES

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) assay

Radical scavenging activity of the *E. acuminata* extracts was determined by DPPH essentially as described by Blois (1958) with some modification³⁰. The extracts of different concentrations and Ascorbic acid were taken in different test tubes. The volume was adjusted to 100 µL by adding MeOH. Five milliliter of 0.1 mM methanol solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at 27°C for 30 min. The control was prepared as the same without any extract. The changes in the absorbance of the prepared samples were measured at 512 nm. Radical scavenging activity was estimated as the inhibition percentage and was calculated using the following formula,

$$\text{Radical scavenging activity \%} = \frac{(\text{OD of blank} - \text{OD of sample})}{\text{OD of blank}} \times 100.$$

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay

The total antioxidant activity was determined by ABTS radical cation scavenging assay by the method of Re et al. (1999)³¹. ABTS radical cation was produced by ABTS (stable radical) aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h. Prior to assay, ABTS solution was diluted in ethanol (1:89 v/v) to give an absorbance of 0.700± 0.02 at 734 nm.

Triplicates of 10 µL samples (1 mg/ mL of respective organic solvents) and Ascorbic acid (concentration 0–15 µM) were added to 1 mL of diluted ABTS solution. The reaction mixture was

incubated at 30°C exactly for 30 min and the absorbance was measured at 734 nm against the ethanol (blank).

Ferric reducing power assay

The ferric reducing ability of different extracts was estimated by the method of Pulido et al. (2000)³². The FRAP reagent was prepared by mixing 2.5 mL of 10 mM TPTZ in 40 mM HCl, 2.5 mL of 20 mM FeCl₃.6H₂O and 25 mL of 0.3 M acetate buffer (pH 3.6). 900 µL of FRAP reagent was mixed with 10 µL of aliquots of plant extracts (1 mg/mL of respective organic solvents) and incubated at 37°C. After incubation, ferric reducing ability of plant extracts was measured at 595 nm.

Nitric oxide radical scavenging assay

Nitric oxide was generated from sodium nitroprusside and measured by Griess reaction³³. Sodium nitroprusside (5 mM) in PBS (phosphate buffer saline) was incubated with different concentrations (25-200 µg/ml) of the extracts, dissolved in phosphate buffer (0.25 M, pH 7.4) and the tubes were incubated at 25°C for 5 h. Controls without the test compounds, but with equivalent amounts of buffer were conducted in identical manner. After 5 h 0.5 ml of Griess reagent (1% sulfanilamide, 2% O-phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance was measured at 546 nm.

Total Phenolic contents

The total phenolic contents in the bark extracts were estimated using Folin–Ciocalteu reagent³⁴ (Yu et al., 2003). In brief, the reaction mixture contained 50 µl of each plant extract, 250 µl of freshly prepared Folin–Ciocalteu reagent, 0.75 ml of 20% sodium carbonate, and 3 ml of pure water. After 2 h of reaction at ambient temperature, the absorbance at 765 nm was measured and used to calculate the phenolic contents using gallic acid as a standard.

Total flavonoid contents

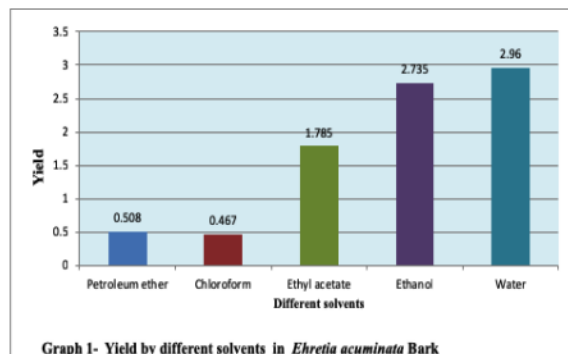
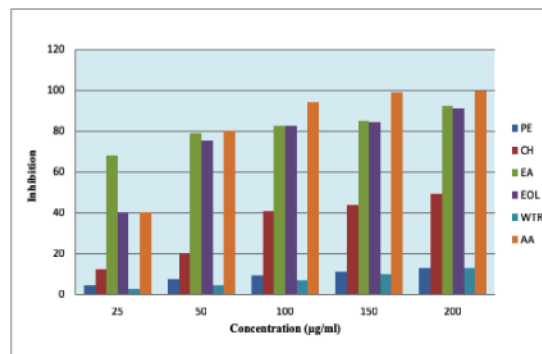
The total flavonoid content (TFC) of each extract was investigated using the aluminum chloride colorimetry method described by Chang et al with slight modifications³⁵. In brief, the extract sample was diluted with methanol until 100 mg/ mL. The calibration curve was prepared by diluting quercetin (QR) in methanol (100 mg/mL). The diluted extract or quercetin (2.0 mL) was mixed with 0.1 mL of 10% (w/v) aluminum chloride solution and 0.1 mL of 0.1 mM potassium acetate solution. The mixture was kept at room temperature for 30 minutes. Then the maximum absorbance of the mixture was measured at 415 nm using a UV-VIS spectrophotometer

Statistical analyses

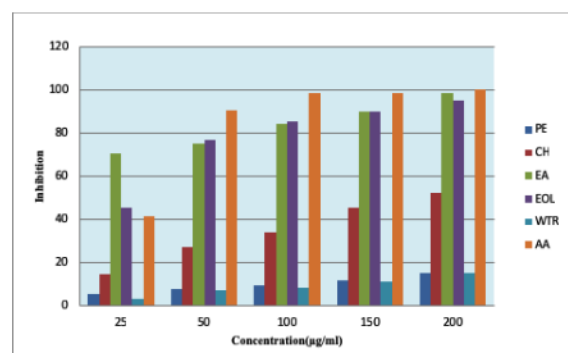
All the experiments were done in triplicates and the results were expressed as Mean± SD. The data were statistically analyzed using one way ANOVA followed by Duncan's test. Mean values were considered statistically significant when p> 0.05.

RESULT AND DISCUSSION

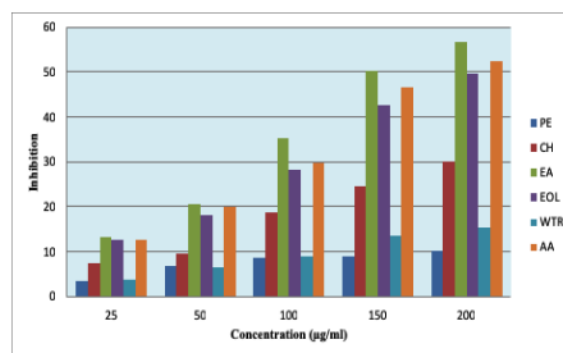
After the complete extraction of bark material by different solvents found different yield, nature and colour of plant extract. Highest yield found in Water (2.96gm) extract and least in chloroform (.467gm).


Graph 1 Yield by different solvents in *Ehretia acuminata* Bark


Graph 2- DPPH radical scavenging activity



Graph 3- ABTS free radical scavenging activity



Graph 4- Nitric oxide radical scavenging activity

Table 1- Ferric reducing antioxidant potential of *Ehretia acuminata* bark

FRAP antioxidant free radical scavenging activity			
S.no	Extract(µM/ml)	FRAP value	Ferric reducing antioxidant power
1.	P.E	.102	43.66
2.	CH	.895	232.64
3.	E.A	2.32	830.44
4.	EOL	2.15	820.91
5.	WTR	.106	76.24
6.	A.A	2.0	790.92

Table 2- Total Phenolic content of *Ehretia acuminata* bark.

Total Phenolic content of <i>E. acuminata</i> bark extract.		
S.no	Extract	Total Phenolic Content (mg GAE/100 gm dw)*
1.	P.E	32.421
2.	CH	210.325
3.	E.A	980.102
4.	EOL	840.267
5.	WTR	40.412

*Results are expressed as mean of 3 values $\pm$ standard deviation.

Table 3- Total Flavonoid content of *Ehretia acuminata* bark.

Total Flavonoid content of <i>E. acuminata</i> bark extract.		
S.no	Extract	Total Flavonoid Content (mg QRE/100 gm dw)*
1.	P.E	.02
2.	CH	.109
3.	E.A	.221
4.	EOL	.180
5.	WTR	-

*Results are expressed as mean of 3 values $\pm$ standard deviation.

Table 4- Correlation of IC₅₀ by different methods of *Ehretia acuminata* bark

IC ₅₀ in ABTS, DPPH, Nitric oxide radical scavenging activity.				
S.no	Extract	IC ₅₀ µg/ml (ABTS)	IC ₅₀ µg/ml (DPPH)	IC ₅₀ µg/ml (Nitric oxide)
1.	P.E	1220	1250	1305
2.	CH	190	220	360
3.	E.A	22	26	140
4.	EOL	30	34	195
5.	WTR	1520	1550	1625
6.	A.A	28	30	175

DPPH radical is a stable organic free radical with an absorption band at 512 nm. It loses this absorption when accepting an electron or a free radical species, which results in a visually noticeable discoloration from purple to yellow. It can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations³⁶. Graph 2 shows the DPPH scavenging activities of the extracts in a concentration-dependent manner. All extracts obtained by 100% methanol yielded the highest DPPH radical scavenging activity at concentrations ranging from 25 mg/mL to 200 mg/mL. However, at concentrations ranging from 200 mg/mL to 350 mg/mL, its DPPH radical scavenging activity is not significantly different in all of these extracts. All extracts obtained by using a pure organic solvent and ethyl acetate gave stronger radical scavenging capacity than that of the other extracts.

ABTS⁺ scavenging assay is a widely accepted model to determine the total antioxidant activity. Among various solvent extracts of *E. acuminata*, the successive Soxhlet ethyl acetate extract possessed the highest ABTS⁺ scavenging activity, while the petroleum ether extract of fractionated ethanol extract showed the lowest ABTS radical cation scavenging activity. The total antioxidant activity of different extracts of whole plant was analyzed and shown in Graph 3.

The FRAP assay is determined by the ferric reducing ability of plant crude extracts. The successive Soxhlet ethyl acetate extract showed higher ferric reducing ability compared to ascorbic acid. The petroleum ether and water extract showed least ferric reducing ability.

Sodium nitroprusside generates nitric oxide radical in the presence of physiological buffer solution at 25°C. Nitric oxide reacted with Griess reagent and diazotization of nitrite with sulfanilamide and subsequent coupling with naphthylethylene diamine form color complex. Decrease in color intensity is directly proportional to nitric oxide radical scavenging. Ascorbic acid used as standard showed least colour (light pink) & WTR extract showed immediate dark colour.

Phenolic compounds are a class of antioxidant agents, which act as free radical terminators. Total phenols were measured by Folin-Ciocalteu reagent in terms of Gallic acid equivalent. The total phenolic in P.E, CH, E.A. EOL and WTR of *E. acuminata* was found to be 32.421, 210.325, 980.102, 840.267, 40.412 respectively. The compounds such as flavonoids and polyphenols, which contain hydroxyls, are responsible for the radical scavenging effect of plants. According to our study, the high contents of this Phytochemical in E.A extract of *E. acuminata* can explain its high radical scavenging activity.

Correlation of IC₅₀ in ABTS, DPPH, Nitric oxide method

The IC₅₀ of a compound is inversely related to its antioxidant capacity, as it expresses the amount of antioxidant required to decrease the ABTS, DPPH, Nitric oxide concentration by 50%, which is obtained by interpolation from a linear regression analysis³⁷. A lower IC₅₀ indicates a higher antioxidant activity of a compound. Table 4 shows the IC₅₀ values in the DPPH, ABTS, Nitric oxide radical scavenging activity assay of different extracts. It was found that the 100% ethyl acetate extract possesses the strongest radical activity (IC₅₀ 90 mg/mL) by all method. Phenolics were the main antioxidant components, and their total contents were directly proportional to their antioxidant activity³⁸. Result shows a correlation between total phenolic content, ABTS, DPPH and Nitric oxide antioxidant activity of the freeze-dried *E. acuminata* bark extracts. In this study, it was also observed that the TPC, TFC, and antioxidant activity of ethyl acetate extracts is higher than those of pure ethanol, water, and the other non polar solvents.

CONCLUSION

The DPPH, ABTS, FRAP, and nitric oxide assays gave comparable results for the antioxidant activity measured in all extracts of bark of *E. acuminata*. The ABTS technique showed high reproducibility, was simple, rapidly performed and had highest correlation with both ascorbic acid and total phenolics. Therefore, it would be an appropriate technique for determining antioxidant in *Ehretia acuminata* bark. Antioxidant activity in ethyl acetate extract is highest and in water extract is lowest compared to all other extracts. Ascorbic acid, flavonoids and phenolics are the major contributors to antioxidant activity *Ehretia acuminata*.

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