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Original Research Article

ANTIOXIDANT POTENTIAL AND TOTAL PHENOLIC CONTENT OF *ZANTHOXYLUM ALATUM* STEM BARK

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ABSTRACT

In recent years much attention has been devoted to natural antioxidant and their association with health benefits. Plants are the potential source of natural antioxidants. The aim of this study was to assess the antioxidant activity (*in vitro*) of four extracts from stem bark of *Zanthoxylum alatum* Roxb. and to determine phenolic content of all extracts. The best antioxidant potential and phenolic content were found in Petroleum ether and ethyl acetate extract. Comparative antioxidant potential was evaluated using DPPH, Nitric Oxide scavenging assay and ferric reducing power assay. Estimation of the total phenolic content was performed using Folin-Ciocalteu's method. Petroleum ether and ethyl acetate extract gave an IC_{50} of 85.16±1.05, 72.39±1.53 and 99.25±2.53, 94.81±2.56 for DPPH and Nitric oxide scavenging activities respectively. The petroleum ether and ethyl acetate extracts showed good reducing power with increasing concentration. Both extracts also gave the maximum percentage of phenolic content (equivalent to that of gallic acid) when compared to other extracts. All the antioxidant assays and phenolic content estimation studies revealed that petroleum ether and ethyl acetate extracts were the most promising and significant extract among petroleum ether, chloroform, ethyl acetate and methanol extracts.

Keywords: Antioxidant activity, DPPH assay, NO assay, *Zanthoxylum alatum,*

Running title: Antioxidant potential of *Zanthoxylum alatum* stem bark

INTRODUCTION

Reactive oxygen species (ROS) such as singlet oxygen, superoxide anion (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) are generated as byproducts of biological reactions and from exogenous factors [1]. Excess ROS, if not eliminated by antioxidant system, results in high levels of free radicals which causes oxidative stress [2]. Oxidative stress arising from free radicals is the basis of many diseases. Oxidative stress is associated with pathogenic mechanisms of many diseases such as cancer, diabetes, inflammatory diseases, atherosclerosis neurodegenerative diseases and aging processes [3, 4]. It is defined as an imbalance between oxidants and antioxidants in favor of the oxidants potentially leading to damage [5]. The curative effects of several medicinal plants are usually due to antioxidant phytochemicals present in it [6]. Halliwell and Gutteridge defined antioxidants as compounds that when present in low concentration in relation to the oxidant prevent or delay the oxidation of the substrate [7]. It has been considered that there is an inverse relationship between dietary intake of antioxidant rich foods and incidence of human diseases [6]. Plants are rich in phenolic substances, usually referred to as polyphenols. Due to its chemical structure, phenolic compounds have the ability of phenoxide ion delocalize. The phenoxide ion can lose a further electron to form the corresponding radical which can also delocalize. In reference to this property, phenolic compounds have radical scavenging and antioxidant activity [8].

Zanthoxylum alatum (ZA) also known as *Zanthoxylum armatum* belongs to family Rutaceae. It is a perennial shrub or a small tree upto 6 m height with dense glabrous foliage and straight prickles on stem. It is distributed in Himalayas from Kashmir to Bhutan upto 2100 m and in Khasia hills upto 1350 m [9]. ZA is used in traditional medicinal systems for number of disorders like cholera, diabetes, cough, diarrhea, fever, headache, microbial infections, toothache, anti-inflammatory and cancer. The bark of plant is reported to contain a bitter crystalline principal identical with berberine,

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Table 1 Amount (g) and % yield of extracts of *Hypericum ericoides*

volatile oil, phenolic compounds and resin [10]. The fruit contains about 1.5% of an essential oil consisting chiefly of 1-α-phellandrene with small amounts of linalool. Leaves yield an essential oil which has a carbonyl compound identified as methyl n-nonyl ketone. The ketone-free fraction contains linalyl acetate, sesquiterpene, hydrocarbon and tricosane. The roots yield the alkaloids dictamnine, magnoflorine, fagarine, skimmianine, xanthoplanine [11, 12]. Carpals of the plant contain xanthoxylin [10]. Various reported pharmacological activities of ZA in different parts are antiproliferative [13], antibacterial, antifungal and anthelmintic [14], anti-inflammatory [15], hepatoprotective [16, 17], larvicidal [18], Antispasmodic, antidiarrhoeal, bronchodialator and in cardiovascular disorders [19]. Antidysentric [20], piscicide [21], lousicidal potential [22], Cytotoxic [23].

MATERIAL AND METHODS

Material

The stem bark of ZA was collected from the local areas of Tehri (Garwal), Uttrakhand, India and authenticated from NISCAIR, New Delhi (Ref. NISCAIR/RHMD/Consult/2013/2233/14). Plant drug was shade dried (<40° C), coarsely powdered and stored in air tight container.

Chemicals and reagents

All solvents used were of analytical grade and purchased from Rankem (Deejay Corporation, Jalandhar). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and L-ascorbic acid, Gallic acid were purchased from Sigma Chemical Co. (USA). Potassium ferricyanide, trichloroacetic acid (TCA) and ferric chloride, Aluminium chloride, **potassium acetate** were purchased from CDH, New Delhi. Sulphanilic acid and napthylelene diamine dichloride were purchased from Rankem, New Delhi. **Folin Ciocalteu's reagent** were procured from E-merck (India) Ltd, Mumbai.

Extraction of plant material

Successive solvent extraction scheme was used for the preparation of different extracts. The stem bark was crushed to coarse powder and extracted with Petroleum ether (40-60ºC) using Soxhlet's apparatus for 3 days. The extract was filtered through Whatmann filter paper and concentrated with rota-evaporator and transferred to a pre-weighed china dish and dried in a vacuum dessicator. The marc obtained was then air-dried and used for further extraction with chloroform followed by ethyl acetate and methanol. The dried extracts were placed in desiccators for further studies.

Phytochemical screening

Petroleum ether, chloroform, ethyl acetate and methanol extracts were subjected to phytochemical analysis for checking the presence of carbohydrates, alkaloids, glycosides, proteins, saponins, phenolic compounds, flavonoids, steroids and triterpenoids, etc. [24].

In vitro antioxidant studies

All the extracts were tested for their free radical scavenging property using different *in vitro* models. All experiments were performed thrice and their results averaged. L-Ascorbic acid was used as standard control in each experiment. Results were expressed in IC_{50} values.

DPPH radical scavenging activity [25]

DPPH radical scavenging activity was performed according to the method of Blois, 1958 with some modifications. The 0.1 mM solution of DPPH in methanol (22.2 mg in 1000 ml) was freshly prepared. Different concentrations of extract and standards (25-400 µg/ml) were added at an equal volume to methanolic solution of DPPH. After 30 min at room temperature, the absorbance was recorded at 517 nm. Radical scavenging activity was calculated by the following formula (formula 1)

% inhibition = $A_{control} - A_{test} / A_{control} \times 100$

where, $A_{control}$ = Absorbance of control reaction and A_{test} = Absorbance of samples of extracts.

Nitric oxide radical scavenging activity [26]

Nitric oxide radical scavenging activity was performed according to the method of Garrat, 1964 with some modifications. 2 ml of 10 mM sodium nitroprusside in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of extract at various concentrations (25-400 µg/ml) and the mixture was incubated at 25°C for 150 min From the incubated mixture 0.5 ml was taken out and added into 1.0 ml sulfanilic acid reagent (33% in 20% glacial acetic acid) and incubated at room temperature for 5 min. finally, 1.0 ml naphthylethylenediamine dihydrochloride (0.1% w/v) was mixed and incubated at room temperature for 30 min before measuring the absorbance at 540 nm. The nitric oxide radicals scavenging activity was calculated by the formula 1.

Reducing power assay [27]

Reducing power assay was performed according to the method of Oyaizu, 1986 with some modifications. The extract (0.75 ml) at various concentrations (25-400 µg/ml) was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium ferricyanide (1%, w/v) followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloracetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1% w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Estimation of total phenolic content

Gallic acid standard solution was prepared by dissolving 10 mg in 10 ml water to make 1 mg/ml solution. The total concentration of phenolic compounds in the extracts was determined using a series of gallic acid standard solutions (20-100 µg/ml) as described by Singleton and Rossi, 1965) but with some modifications. The extract (10 mg) was dissolved in 10 ml of water to prepare 1mg/ml solution of extract. Each extract solution (0.1 ml) was mixed with 2 ml of a 2% (w/v) sodium carbonate solution and vortexed vigorously. The same procedure was also applied to the standard solutions of gallic acid. After 3 min, 0.1 ml of 50% Folin-Ciocalteu's phenol reagent was added and each mixture was vortexed again. The absorbance at 760 nm of each mixture was measured after incubation for 30 min at room temperature and the concentration of the sample solution was determined from the calibration curve. Results were expressed as milligrams of total phenolics content per grams of extract as gallic acid equivalents (GAE) [28].

Calculation

$$\text{Total phenolic contents (\%)} = GAE \times V \times D \times 10^{-6} \times 100/W$$

Where, GAE - Gallic acid equivalent (µg/ml), V - Total volume of sample (ml),
D - Dilution factor, W - Sample weight (g) -

Statistical analysis

All analysis was done in triplicate and results are expressed as mean ± S.D. IC_{50} values were determined by interpolations.

RESULTS

Phytochemical screening

Phytochemical screening of different plant extracts are shown in **Table 1**

Table 1: Phytochemical screening of different plant extracts

Class of compounds	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Methanol extract
Carbohydrate	-	-	-	+
Glycoside	-	-	+	+
Proteins	-	-	-	-
Steroids and triterpenoids	+	-	-	-
Phenolic compounds	+	+	+	+
Flavonoids	-	-	+	+
Alkaloids	+	-	-	+
Saponins	-	-	-	-

+ indicates presence, - indicates absence

Antioxidant activity

In present study we have evaluated the antioxidant activity of different extracts of *Zanthoxylum alatum* stem bark. Various concentrations of extracts ranging from (25-400 µg/ml) were tested for their antioxidant activity in different *in vitro* models.

Among all the extracts tested for *in vitro* antioxidant activity using DPPH, nitric oxide radical scavenging activity and reducing power assay; petroleum ether and ethyl acetate extract has shown maximum antioxidant activity with IC_{50} values of 85.16±1.05 and 99.25±2.53 in DPPH assay and 72.39±1.53 and 94.81±2.56 in Nitric oxide radical scavenging assay **(Table 2).** The antioxidant activity increased with increasing concentration in all the models. The percentage inhibition of standard and extracts in various models DPPH and nitric oxide scavenging assay as shown in **Figure 1 & 2**. Reducing power of extracts was concentration dependent. Petroleum ether and ethyl acetate extract has maximum reducing power amongst other plant extracts as observed in **Table 3**. Higher absorbance indicates more reducing power.

Figure 1: DPPH radical scavenging effect of Ascorbic acid and various extracts of ZA stem Bark

Extract	DPPH	NO
Ascorbic acid	57.30±1.90	42.8±2.61
Petroleum ether	85.16±1.05	72.39±1.53
Ethyl acetate	99.25±2.53	94.81±2.56
Methanolic	149.26±3.00	176.11±3.69
Chloroform	287.38±2.16	303.24±3.74

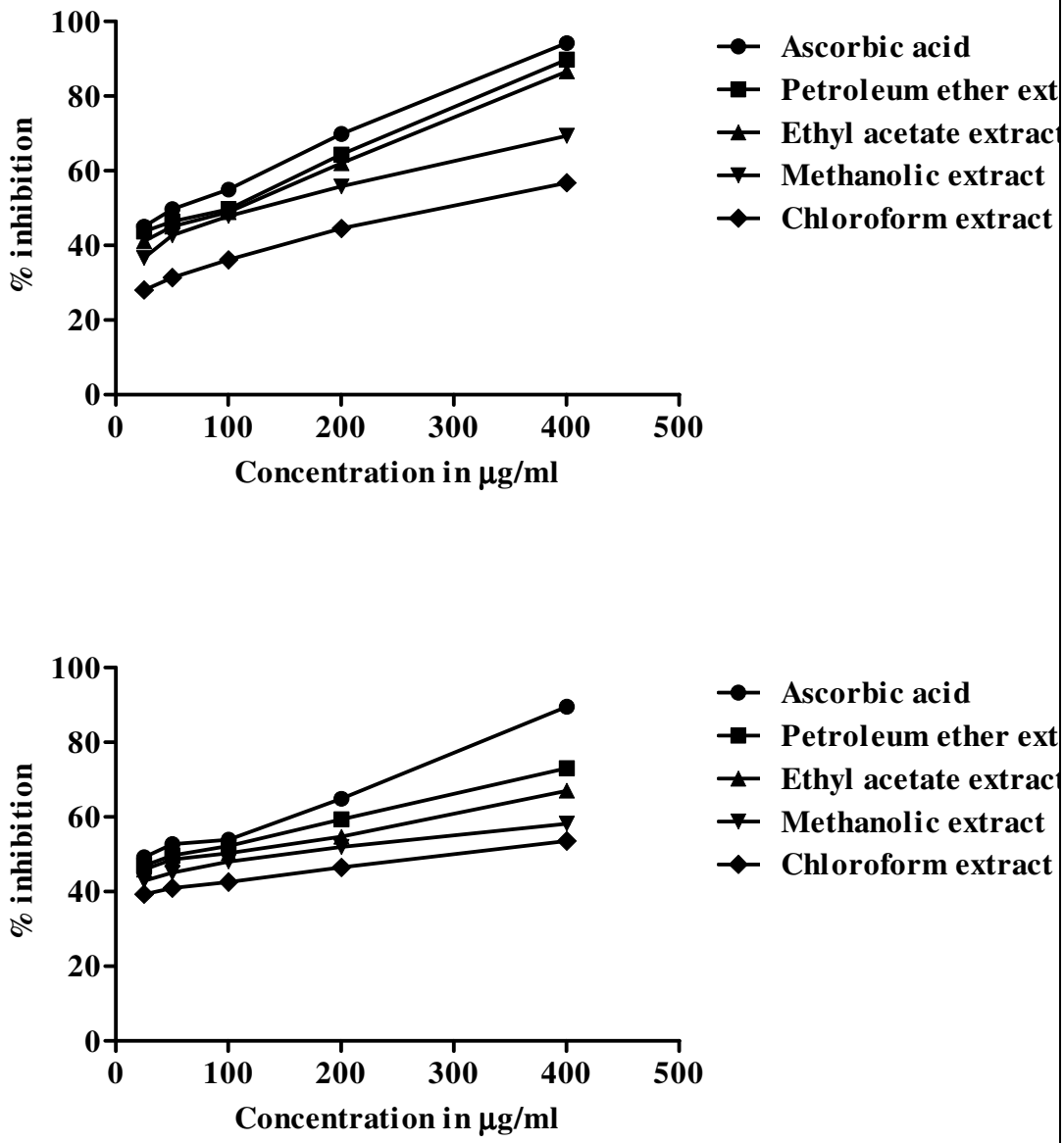

Figure 2: Nitric oxide scavenging activity of Ascorbic acid and various extracts of ZA stem Bark

Conc. (µg/ml)	Absorbance				
	Ascorbic acid	Petroleum ether extract	Ethyl acetate extract	Methanolic extract	Chloroform extract
25	0.127 ± 0.005	0.092 ± 0.006	0.062 ± 0.007	0.046 ± 0.007	0.022 ± 0.004
50	0.212 ± 0.008	0.196 ± 0.002	0.128 ± 0.008	0.098 ± 0.001	0.047 ± 0.005
100	0.467 ± 0.006	0.367 ± 0.006	0.317 ± 0.008	0.147 ± 0.007	0.102 ± 0.002
200	0.897 ± 0.005	0.669 ± 0.009	0.569 ± 0.009	0.299 ± 0.006	0.229 ± 0.005
400	2.121 ± 0.007	1.537 ± 0.004	1.287 ± 0.009	0.602 ± 0.008	0.409 ± 0.006

Total phenolic estimation

The standard ascorbic acid and extracts were determined for its total phenolic content on the basis of its gallic acid equivalent Folin-Ciocalteu phenol assay. The amount of gallic acid equivalent was determined from the calculation of calibration curve of gallic acid **(Figure 3)**. The maximum phenolic content was found in petroleum ether extract and ethyl acetate extract (5.12% and 4.36 mg/g GAE resp.). The results are reported in **Table 4.**

Thus the above results exhibited that the petroleum ether extract is the most active whereas the chloroform extract is the lowest one.

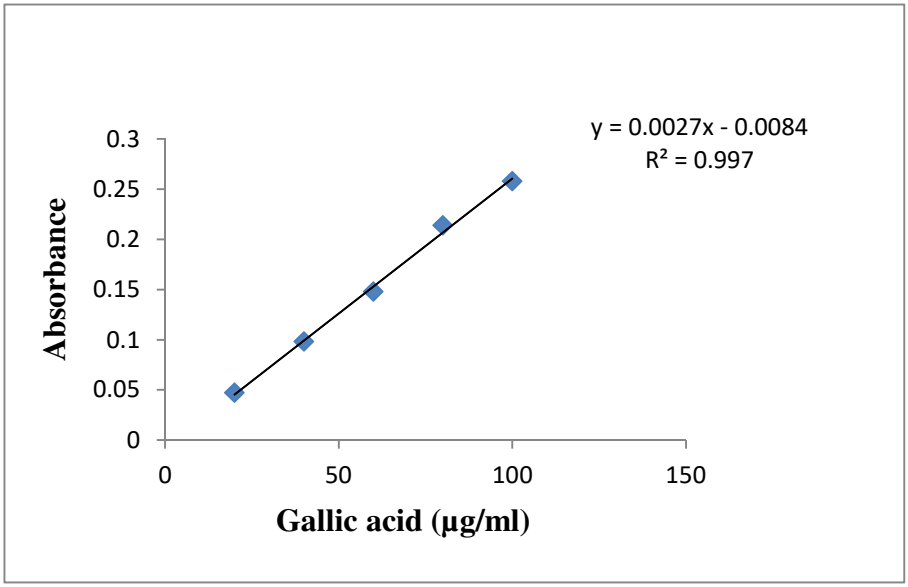

Table 3: Reductive ability of Standard and various extracts of ZA stem Bark

Extract	Phenolic content mg/g gallic acid equivalents (GAE)
Petroleum ether extract	5.12
Chloroform extract	0.74
Ethyl acetate extract	4.36
Methanolic extract	1.64

DISCUSSION

Natural antioxidants that are present in plants are responsible for inhibiting or preventing the harmful consequences of oxidative stress. Plants contains free radical scavengers like polyphenols, flavonoids and phenolic compounds [29]. These compounds have shown to have antioxidant activity. In the present paper, we have evaluated the free radical scavenger activity of different extracts of *Zanthoxylum alatum* stem bark.

There are number of assay designed to measure overall antioxidant activity/reducing potential, as an indication of total capacity of plants to withstand free radical stress [30].

DPPH is very convenient for the screening of number of samples of different polarity. The measurement of the scavenging of DPPH radical allows to determine the intrinsic ability of substance to donate hydrogen atom or electrons to this reactive species in a homogenous system. Methanolic DPPH solution get reduced because of the presence of antioxidant substances having hydrogen-donating groups such as phenols and flavonoid compounds due to the formation of non radical DPPH-H form [31].

Nitric oxide is a diffusible free radical that plays an important role as an effectors molecule in diverse biological systems including neuronal messenger, vasodilatation and antitumor activities [32]. Although, NO is involved in host defence, over production of these radical contributes to the pathogenesis of various diseases [33]. The petroleum ether extract and ethyl acetate extract significantly inhibited NO in a dose dependent manner with the IC_{50} being 72.39±1.53 and 94.81±2.56 µg/ml as compared with the standards ascorbic acid having the IC_{50} values of 42.8±2.61 µg/ml. The results indicated that both petroleum ether and ethyl acetate extract contain the compounds which are

able to inhibit NO and act as an antioxidant.

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action [34]. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe^{3+} to Fe^{2+} by donating an electron [35]. Being good electron donors, phenolic compounds shows the reducing power and have ability to convert ferric ion to ferrous ion by donating an electron (20). Increasing absorbance at 700 nm indicates an increase in reductive ability [36].

In our study, the different extracts of ZA showed high total antioxidant and DPPH activity, NO and ferrous iron reducing capacities. Considerable correlations were observed between DPPH and NO scavenging, reducing power and total phenols. Antioxidant capacity may be associated with a high phenol content as Mansouri et al. 2005 reported that most of the antioxidant activity of plants is derived from phenols [37]. Structurally, phenols comprise an aromatic ring having one or more hydroxyl groups. The antioxidant activity of this type of molecule is due to their ability to scavenge free radicals, donate hydrogen atoms or electrons or chelate metal cations [38]. Previously stem bark of ZA has been reported to contain phenolic compounds [39].

In the present study, the antioxidant activity of petroleum ether and ethyl acetate extracts may be attributed to the combined effects of the phenolic compounds and the results are in full agreement with previous studies on other plants [40, 41]**.**

CONCLUSION

Amongst all the extracts, petroleum ether and ethyl acetate extracts of ZA showed potent antioxidant potential as determined by different procedures. Presence of phenolic compounds in the extracts confirmed their utility as potent antioxidant agent.

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