IN VITRO ANTIOXIDATIVE EFFECTS OF STEM BARK FROM *ZANTHOXYLUM NITIDUM* (ROXB.) DC (RUTACEAE)

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Summary

Abstract: *Zanthoxylum nitidum* (Roxb.) DC (Rutaceae), is a morphologically variable plant species occurring in South-East Asian countries and in Australia. In India the plant is traditionally used for several medicinal purposes. In the present investigation, the methanol extract of stem bark of *Zanthoxylum nitidum* (ZNME) was evaluated for its antioxidant properties by 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging assay and *in vitro* lipid peroxidation induced by the Fe^{2+}- ascorbate system in rat liver homogenate. In DPPH radical scavenging assay, the ZNME demonstrated marked and dose dependent free radical scavenging effect and the mean inhibitory concentration (IC_{50}) of the ZNME was found to be 77.8 µg/ml, while the ascorbic acid (reference) exhibited 43.7 µg/ml. The ZNME effectively inhibited the lipid peroxidation in a dose related manner showing the IC_{50} value of 297.9 µg/ml, whereas the quercetin (reference) showed 46.64 µg/ml. These findings reveal that the ZNME has remarkable *in vitro* antioxidative actions in the tested models.

Key-words: *Zanthoxylum nitidum,* bark, radical scavenging, lipid peroxidation.

Introduction

There are increasing evidences for the participation of free radicals in the etiology of various diseases like cancer, diabetes, cardiovascular diseases, autoimmune disorders, neurodegenerative diseases, ageing etc. A free radical is defined as any atom or molecule possessing unpaired electrons. Antioxidants are agents which scavenge the free radicals and prevent the damage caused by reactive oxygen species. Oxygen free radicals are formed in tissue cells by various endogenous and exogenous causes such as metabolism, chemicals, and ionizing radiation. Approximately 5% of oxygen gets univalently reduced to oxygen derived free radicals like superoxide, hydrogen peroxide, hydroxyl and nitric oxide radicals. All these free radicals are known as reactive oxygen species (ROS), exert oxidative stress to the cells. When the generation of ROS overtakes the antioxidant defense of the cells the free radicals start attacking cellular proteins, lipids and carbohydrates leading to the pathogenesis of many disorders including arthritis and connective tissue disorders, liver disorders, neurodegenerative disorders, cardiovascular disorders, diabetes, chronic inflammation, mutagenesis, carcinogenesis and in the process of ageing (1,2). Free radicals also cause DNA strand breaks and chromosome deletions and rearrangements leading to genetic damage. Antioxidants provide protection for living organisms from damage caused by uncontrolled production of reactive oxygen species (ROS) and the concomitant lipid peroxidation, protein damage and DNA strand breaking (3). Antioxidants natural or synthetic, play an important role in inhibiting and scavenging free radicals (ROS), thus preventing the aforesaid chronic degenerative diseases (2).

Traditional medicine worldwide is being re-evaluated by extensive research on different plant species and their therapeutic principles. Plants produce several secondary metabolites serving as antioxidants to control the oxidative stress caused by sunbeams and oxygen, they can represent a source of new compounds with antioxidant activity.

Zanthoxylum nitidum (Roxb.) DC (Rutaceae), called *Tez-mui* or *Tezamool* in Assamese, is a morphologically variable plant species occurring in South-East Asian countries and in Australia (4). In India it grows as a large prickly shrub particularly in North-East India (Sikkim, Assam and Nagaland states). In India the plant is traditionally used for various medicinal purposes. The root is used in toothache, stomachache, fever, rheumatism, paresis, boils and as an insecticide and piscicide. The fruit is used in the treatment of stomachache, cough, colic, vomiting, diarrhoea, and paresis and as an aromatic, stimulant and piscicide. The small branches, seeds and stem bark are prescribed in fever, diarrhea and cholera (5-7). Previously the authors have reported essential oil composition of fruits and leaves, antibacterial and pharmacognostic studies of stem bark and root, anti-nociceptive activity of stem bark, anti-inflammatory and antioxidant activities of root of *Z. nitidum* from India (8-13). There are no reports of antioxidant investigations carried out on *Z. nitidum* stem bark. The present work therefore, attempts to report the preliminary results of studies on *in vitro* antioxidative effects of *Z. nitidum* bark.

Materials and Methods

Plant material
The fully mature entire plants of *Z. nitidum* were collected during the month of November 2007 from Dibrugarh district of Assam state, India. The species was identified by Dr. S. J. Phukan, taxonomist, from Botanical Survey of India, Eastern Circle, Shillong, India, and a voucher specimen (No. DUPS-06-003) was deposited in Department of Pharmaceutical Sciences, Dibrugarh University, Dibrugarh 786004, India, for future reference. Just after collection, all the prickles were removed from the stems and branches carefully by using a sharp knife, without harming the bark. Then the barks were peeled off from the shoots. Then the stem barks were shade dried at temperature 21-24°C and ground into coarse powder with a mechanical grinder and stored in air-tight container.
Preparation of extract
Powdered plant material (250 g) was macerated with 400 ml of methanol at 21-24°C temperature for 2 days with frequent shaking. After 2 days, the extracts were filtered and to the marc part 300 ml of the methanol was added and allowed to stand for next 2 days at same temperature for second time maceration (re-maceration) and after two days, again filtered similarly. The combined filtrates (extract) were evaporated *in vacuo* at 40 ºC and the dry extract thus obtained (ZNME, yield 11.7 % w/w) was stored in a refrigerator for further use. Preliminary phytochemical studies were performed on ZNME as per reported method (14).
Drugs and chemicals
1,1- diphenyl-2-picryl hydrazyl (DPPH), thiobarbituric acid and L-ascorbic acid were from Sigma Chemical Co., USA; quercetin was from Aldrich Chemical Co., UK. All other chemicals, reagents and solvents were of analytical grade available commercially.
Animals
Male adult Wistar albino rats of weighing 150-180 g were obtained from the animal supplier (Rita Ghosh & Co., Kolkata, India). The animals were grouped in polyacrylic cages (38 cm × 23 cm × 10 cm) with not more than four animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C, dark and light cycle 14/10 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment.

Evaluation of *in vitro* antioxidant activity
Free radical scavenging activity by DPPH: The antioxidant property of ZNME was determined on the basis of their scavenging activity of stable 1,1- diphenyl-2-picryl hydrazyl (DPPH) free radical. 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3 ml of the solutions of ZNME in methanol at different concentrations (25-200 µg/ml). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Then their absorbances were measured at 517 nm on UV-visible spectrophotometer (Genesys 10 UV: Thermo Electron Corporation). Ascorbic acid was used as the reference material. The lower absorbance values of reaction mixture indicated higher free radical scavenging activity. The results were expressed as percentage of inhibition at different concentrations and IC_{50} was determined. The IC_{50} (concentration producing 50%

inhibition) value denotes the concentration of the sample (in µg/ml) required to scavenge 50 % of the DPPH free radicals (15,16). The percentage scavenging activity was calculated by using the following formula,

DPPH scavenging activity (% inhibition) = $[(A_0 – A_1 / A_0) \times 100]$

Where A_0 is the absorbance of the control reaction (containing all reagents except the test extract or reference) and A_1 is the absorbance of the sample at different concentrations . All the tests were performed in triplicate and the results were averaged.

Determination of lipid peroxidation inhibitory activity in rat liver homogenates

Tissue sample preparation: The normal rats as mentioned under the subheading 'animals' were sacrificed by cervical dislocation and the liver was excised and perfused *in vitro* with ice-cold normal saline (0.9 % w/v). The tissues were then homogenized at a concentration of 10 % w/v in 1.15 % w/v KCl solution and centrifuged at 1200 *g* at 4 ºC for 10 mins. The supernatant was collected which was again centrifuged at 10000 *g* at - 4ºC for 10 mins. The supernatant was taken and stored at -20 ºC for use in the study (17).

Estimation method: Lipid peroxidation induced by Fe^{2+}- ascorbate system in rat liver homogenate was estimated as thiobarbituric acid reactive substances (TBARS) by previously reported method[13.] The reaction mixture contained rat liver homogenate 0.1 ml in Tris-HCl buffer (40 mM, pH 7.0); KCl (30 mM); $FeSO_4(NH_4)_2SO_4$□$7H_2O$ (0.16 mM); sodium ascorbate (0.06 mM); and various concentrations of ZNME (10-1000 µg/ml) in a final volume of 0.5 ml. The reaction mixture was incubated at 37ºC for 1 h. After the incubation period, 0.4 ml was removed and treated with 0.2 ml sodium dodecyl sulphate (SDS, 8.1%); 1.5 ml thiobarbituric acid (TBA, 0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The total volume was made up to 4 ml with distilled water and then kept in a water bath at 95 to 100°C for 1 h. After cooling, 1.0 ml of distilled water and 5.0 ml of *n*-butanol and pyridine mixture (15: 1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The upper butanol-pyridine layer was removed and its absorbance at 532 nm was measured by using UV-visible spectrophotometer (Genesys 10 UV: Thermo Electron Corporation). Inhibition of lipid peroxidation was determined by comparing the absorbance of treatments with that of the control (18). Quercetin was used as the reference.

The inhibitory ratio of the test sample was evaluated by the following formula,

Percentage inhibition = A_c- $A_s/A_c \times 100$ %.

Where A_c is the absorbance of control (containing all reagents except the test extract) and A_s is the absorbance of the sample at different concentrations. All the tests were performed in triplicate and the results averaged. The IC_{50} value was also determined here.

Statistical analysis

The values were expressed as mean ± standard error of mean (SEM).

Results

Preliminary phytochemical studies on ZNME demonstrated the presence of true alkaloids, flavonoids, carbohydrates, proteins and amino acids. The antioxidant activity of

ZNME was evaluated by DPPH radical scavenging assay and the results are summarized in Table 1. The percentage of inhibition was found to be 62.47 % at the concentration of 200 µg/ml and 8.19 % at the concentration of 25 µg/ml. The 50% inhibitory concentration i.e. IC_{50} value of ZNME was 77.8 µg/ml. Ascorbic acid was used as reference and its IC_{50} value was found to be 43.7 µg/ml.

The antioxidant property of ZNME was also evaluated by the assessment of inhibition of malondialdehyde formation generated by Fe^{2+}- ascorbate in rat liver homogenate and the results are summarized in Table 2. The percentage of inhibition was 62.78 % and 25.57 % at the concentrations of 1000µg/ml and 10 µg/ml respectively. The IC_{50} value of ZNME was 297.9 µg/ml and that of quercetin (reference) was found to be 46.6 µg/ml.

Table 1. Free radical scavenging effect of ZNME on DPPH radical scavenging assay.

Test samples	**Percentage of inhibition***	**IC_{50} (µg/ml)**
Control	-	-
ZNME 25 µg/ml 50 µg/ml 100 µg/ml 200 µg/ml	 8.19 16.60 36.28 62.47	77.8
Ascorbic acid 50 µg/ml 100 µg/ml	 51.33 67.02	43.7

* Values are mean ± SEM (n = 3). SEM = Standard error of mean.

Table 2. Inhibitory effect of ZNME on Fe^{2+}- ascorbate induced lipid peroxidation in rat liver homogenates.

Test samples	**Percentage of inhibition***	**IC_{50} (µg/ml)**
Control	-	-
ZNME 10 µg/ml 100 µg/ml 1000 µg/ml	 25.57 34.88 62.78	297.9
Quercetin 10 µg/ml 100 µg/ml	 44.17 51.15	46.6

* Values are mean ± SEM (n = 3). SEM = Standard error of mean.

Discussion

The results of the present study indicate that the ZNME has effective degrees of *in vitro* antioxidant activity by the methods employed. The DPPH test provides information on the reactivity of test extract (ZNME) with a stable free radical. The stable DPPH radical model is a widely used, relatively quick method for the evaluation of free radical scavenging activity. The effects of antioxidants on DPPH radical scavenging is thought to be due to their hydrogen-donating ability. DPPH is a relatively stable nitrogen centred free radical containing an odd electron in its structure that can accept an electron or hydrogen radical to become a stable diamagnetic molecule and usually utilized for detection of radical scavenging activity (15). The absorption maximum of a stable DPPH radical in methanol is 517 nm. Because of its odd electron DPPH gives a strong absorption at 517 nm in the visible region (deep violet colour). The decrease in absorbance of DPPH radical is caused by antioxidants or suitable reducing agents, as the reaction between antioxidant molecules and radical progresses, results in the scavenging of the free radicals by hydrogen donation. As the electron becomes paired off in presence of a free radical scavenger, the absorption diminishes, thus the resulting decrease in absorbance is stoichiometric with respect to the number of electrons taken up (19). It is visually noticeable that as a change in color from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate the free radical scavenging activity of antioxidants (20). The ZNME exhibited marked and dose dependent free radical scavenging effect in DPPH radical scavenging assay showing the IC_{50} value 77.8 μg/ml.

Lipid peroxidation, an autocatalytic free radical chain propagating reaction, is known to be associated with pathological conditions of a cell. Lipid peroxidation is a complex process whereby polyunsaturated fatty acids of cellular membranes undergo reaction with reactive oxygen species to yield lipid hydro-perperoxides. Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydro-peroxides into peroxyl and alkoxyl radicals which eventually yield numerous carbonyl products such as malondialdehyde (MDA) known as thiobarbituric acid reactive substances (TBARS). This lipid peroxidation can be prevented either by reducing the formation of free radicals or by supplying the competitive substrate for unsaturated lipids in the membrane or by accelerating the repair mechanisms of damaged cell membrane. Several natural and synthetic antioxidants are used to prevent the lipid peroxidation (21,22).

The antioxidant activity of the ZNME was confirmed by evaluating the inhibition in production of malondialdehyde (MDA) and related carbonyl products (TBARS) that are produced as by products of lipid peroxidation induced by Fe^{2+}- ascorbate system in the biomembranes of rat liver homogenate. These carbonyl products (TBARS) are responsible for DNA damage, carcinogenesis, inflammatory, ageing and related degenerative diseases (23). The MDA reacts with thiobarbituric acid in specific reaction medium to produce a strong absorption at 532 nm. The ZNME effectively inhibited the lipid peroxidtion in a dose related manner exhibiting the IC_{50} value of 297.9 μg/ml. This activity is perhaps related to the H^+ ion donating capability of ZNME, which can scavenge the peroxyl radical to inhibition or termination of the peroxidation chain (16).

Preliminary phytochemical studies revealed the presence of true alkaloids, flavonoids, carbohydrates, proteins and amino acids in ZNME. The findings are consistent with our

previous phytochmical and planar chromnatographic studies on *Z. nitidum* bark (9). Polyphenolic compounds, especially flavonoids, are well known natural antioxidant principles commonly found in higher plants. The flavonoid content of ZNME could be responsible for its observed antioxidant activity.

Present preliminary investigation therefore, confirms remarkable *in vitro* antioxidative potential of ZNME in the tested models. The antioxidant property of *Z. nitidum* bark could substantiate, partly its traditional usage in North-Eastern India. Further studies are presently underway to ascertain its antioxidant potential in other models and to confirm the identity of bioactive principles responsible for these actions by the bark extract of *Z. nitidum.*

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