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In vitro Antioxidant Activity and Phytochemical Analysis of Stem Bark of *Balanites roxburghii* Planch

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Abstract: The antioxidant activity and phytochemical analysis of aqueous and ethanolic extracts of medicinal plant *Balanites roxburghii* were investigated. The antioxidant activity of aqueous and ethanolic extracts from bark of *Balanites roxburghii* was evaluated by various *in vitro* antioxidant assays. The results indicate that both the extracts clearly have strong antioxidant effects. Both extracts exhibited significant antioxidant activity in DPPH, Nitric oxide and Hydroxyl radical induced in-vitro assay methods. The phytochemical analysis revealed the presence of alkaloids, saponins, tannins, flavonoids, phenolic compound, gum and mucilage in varying concentration. The antioxidant activity may be attributed to flavonoids and phenolics present in aqueous and ethanolic extracts of *Balanites roxburghii*. Thus it could be concluded that the aqueous and ethanolic extracts of *Balanites roxburghii* possess significant antioxidant property.

Key words: *Balanites roxburghii* · Antioxidant activity · Phytochemical analysis · DPPH

INTRODUCTION

Reactive oxygen species (ROS) including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide are often generated as byproducts of biological reactions or from exogenous factors [1]. *In-vivo*, some of these ROS play a positive role such as energy production, phagocytosis, regulation of cell growth and intercellular signaling or synthesis of biologically important compounds [2]. However, ROS may also be very damaging; they can attack lipids in cell membranes and also attack DNA, inducing oxidations that cause membrane damage such as membrane lipid peroxidation and a decrease in membrane fluidity and also cause DNA mutation leading to cancer [3, 4]. A potent scavenger of these species may serve as a possible preventive intervention for free radical-mediated diseases [5]. Recent studies showed that a number of plant products including polyphenolic substances (e.g., flavonoids and tannins) and various plant or herb extracts exert antioxidant actions [6-10]. However, the potential health benefits of *Balanites roxburghii* bark extracts have not been studied to date. The objectives of this study were to evaluate the antioxidant activity of stem bark of plant *Balanites roxburghii.*

Balanites roxburghii Planch. (Simarubaceae) locally known as Hingota, is one of the most common but neglected wild plant species of the dry land areas of India. Traditionally it is used as emetic, anthelmintic, anti-fungal, purgative, cathartic, colic, in whooping cough, skin diseases and dog bite. According to Ayurveda, bark is anthelmintic, spasmolytic, used in cough and skin diseases. Leaf is anthelmintic whereas root is emetic. Fruits are used in treatment of whooping cough and in skin diseases. The paste of bark is prepared and applied externally on the affected part of the body [11]. The whole plant is used in treatment of snake-bite. Seeds are used as expectorant (given in the treatment of cough) and colic [12]. Kernel is used in skin diseases and burns [13]. Roots and fruits contain 0.2-2.2 % and 0.3-3.8 % diosgenin (used in contraceptives), respectively. The steroids (sapogenin) are employed in the synthesis of drug including sex hormones and oral contraceptives. In case of pain and swelling, the bark of plants is used by traditional healers. The plant *Balanites roxburghii* having antifertility efficacy [14] and anti-inflammatory activity [15]. *Balanites roxburghii* which contains steroidal saponins has spermicidal, cardiovascular, molluscidal properties [16]. *Balanites roxburghii* pericarp extract show contraceptive efficacy in male mice [17]. Stem bark of *Balanites roxburghii* showed antimicrobial activity and anti-asthmatic activity [18, 19].

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Exhaustive literature survey indicated that systematic pharmacological work has not been done so far on this plant. Hence, this plant was selected to find its antimicrobial activity.

MATERIALS AND METHODS

Collection of Plant Materials: The stem bark of *Balanites roxburghii* was collected from the roadside location of the village- Doniapura, Gormi, Bhind (M.P.) and was identified by Prof. J.R. Patel, Shri Ramnath Singh Mahavidyalaya (Pharmacy), Gormi, Bhind (M.P.). Plant material was collected in the month of June-2007 and preserved in herbarium of institution (voucher specimen no. J-52). The stem bark of the plant was separated, dried in shade and coarsely powdered with mechanical grinder.

Preparation of Extracts: Dried bark of *Balanites roxburghii* were powdered and added to distilled water and boiled on slow heat for 2 hr. It was then filtered through 8 layers of musilin cloth and centrifuged at 5000 rpm for 10 min. The supernatant was collected. This procedure was repeated twice. After 6 hr, the supernatant collected at an interval of every 2 hr was pooled together and concentrated to make the final volume one-fourth of the original volume. It was then autoclaved at 121°C and at 15 lbs pressure and stored at 4°C. For solvent extraction, the air-dried powder was taken in ethanol in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220 rpm for 24 hr. After 24 hr the supernatant was collected and the solvent was evaporated to make the final volume and stored at 4°C in airtight bottle.

Chemicals

- 1-diphenyl-2-picrylhydrazyl (DPPH) and Naphthylethylenediamine dihydrochloride was purchased from Sigma Chemical Co. All other reagents were used of analytical grade.

Phytochemical Screening: To test for alkaloids, about 0.5 g of the extract was stirred with 5 ml of 1% aqueous hydrochloric acid on a steam bath. A few drops of Dragendorff's reagent were used to treat 1 ml of the filterate. Turbidity or precipitation with this reagent was taken as evidence for the presence of alkaloids. Exact 0.5 g of the extract was dissolved in distilled water in a test tube. Frothing which persisted on warming was taken as preliminary evidence for saponins. Also, to test for presence of tannins, about 0.5 g of the extract was dissolved in distilled water and about 10 ml of bromine water added. Decolourization of bromine water indicated the presence of tannins. Borntrager's test was used for detecting the presence of anthraquinones. In this case 0.5 g of the plant extract was shaken with benzene layer separated and half of its own volume of 10% ammonia solution added. A pink, red or violet coloration in the ammoniacal phase indicated the presence of anthraquinone. The presence of cardiac glycosides was confirmed by Liberman's test, Salkowski test and Keller-Killani test.

Evaluation of Antioxidant Activity

Scavenging of DPPH Radical: This assay is based on the measurement of the scavenging ability of antioxidant test substances towards the stable radical. The free radical scavenging activity of aqueous and ethanolic extracts was examined *in vitro* using DPPH radical. The test extracts were treated with different concentrations from a maximum of 250 µg/ml to minimum of 4 µg/ml. The reaction mixture consisted of 1 ml of 0.1mM DPPH in ethanol, 0.95 ml of 0.05 M Tris-HCl buffer (pH 7.4), 1 ml of ethanol and 0.05 ml of the herbal extract. The absorbance of the mixture was measured at 517 nm exactly 30 sec after adding extract. The experiment was performed (in triplicate) and % of scavenging activity was calculated using the formula 100- [100/blank absorbance × sample absorbance].

Scavenging of Nitric Oxide: Sodium nitroprusside (5 µM) in standard phosphate buffer solution was incubated with different concentration of the test extracts dissolved in standard phosphate buffer (0.025M, pH 7.4) and the tubes were incubated at 25°C for 5 hr. After 5 hr, 0.5 ml of incubation solution was removed and diluted with 0.5 ml Griess reagent (prepared by mixing equal volume of 1% sulphanilamide in 2% phosphoric acid and 0.1% naphthylethylene diamine dihydrochloride in water). The absorbance of chromophore formed was read at 546 nm. The control experiment was also carried out in similar manner, using distilled water in the place of extracts. The experiment was performed (in triplicate) and % scavenging activity was calculated using the formula 100- [100/blank absorbance × sample absorbance]. The activity was compared with ascorbic acid, which was used as a standard antioxidant.

Hydroxyl Radical Scavenging Activity: The Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extract for hydroxyl radicals generated from the Fe^{3+} / ascorbate/ EDTA/ H_2o_2 system. The reaction mixture contained deoxy ribose (2-8mM), $Fecl_3$ (0.1mM), EDTA (0.1mM), H_2o_2 (lmM), ascorbate (0.1mM), KH_2PO_4- KOH buffer (20mM, pH 7.4) and various concentrations (25-400 µm of extracts and std 10 to 80 µm /ml) of standard drug in a final volume of 1 ml. The reaction mixture was incubated for 1hr at 37°C, deoxyribose degradation was measured at 532 nm.

Statistical Analysis: The statistical analysis was carried out using one way analysis of variance (ANOVA) followed by Dunnet's t – test, P- values < 0.05 were considered as significant.

RESULTS

Phytochemistry of the Plant Extracts: Phytochemical analysis of both the extract revealed that carbohydrate, alkaloid, flavonoids, saponins, Tannins and phenolic compound are generally present in both the extracts. Glycosides, phytosterols, gum and mucilage were found only in aqueous extract and protein, fat and oils were found in ethanolic extract. Table 1 shows the phytochemical screening results of aqueous and ethanolic extracts of the plant *Balanites roxburghii* used in this study.

Table 1: Qualitative analysis of aqueous and ethanolic extracts of stem bark of *Balanites roxburghii*

S. No.	Chemical constituents	Aqueous extract	Ethanolic extract
1.	Alkaloids	+ve	+ve
2.	Carbohydrates	+ve	+ve
3.	Glycosides	+ve	-ve
4.	Saponins	+ve	+ve
5.	Phytosterols	+ve	-ve
6.	Proteins	-ve	+ve
7.	Flavonoids	+ve	+ve
8.	Fat and oils	-ve	+ve
9.	Tannins and phenolic compound	+ve	+ve
10.	Gum and Mucilage	+ve	-ve

+ve indicates presence of chemical constituents and –ve indicates absence of chemical constituents

Antioxidant Effect

DPPH Scavenging: The aqueous and ethanolic extracts of the *Balanites roxburghii* bark showed promising free radical scavenging effect of DPPH in a concentration dependant manner up to a concentration of 250 µg/ml. The ethanolic extract showed more scavenging activity than the aqueous extract. The reference standard ascorbic acid also demonstrated a significant radical scavenging potential in the concentration of 1 µg/ml. The DPPH radical inhibition (%) was 50.13, 52.12 and 78.12 for aqueous extract, ethanolic extract and ascorbic acid, respectively (Table 2).

Table 2: In vitro free radical scavenging effect of *Balanites roxburghii* bark extracts by DPPH method

	% Scavenging (Mean ± SEM) of triplicates						
Drug	4µg/ml	8µg/ml	15µg/ml	30µg/ml	60µg/ml	125µg/ml	250µg/ml
AEBR	21.35±0.002*	22.54±0.001*	23.34±0.001*	30.77±0.001*	37.4±0.001*	45.22±0.002*	50.13±0.002*
EEBR	25.02±0.002*	25.86±0.002*	27.85±0.001*	31.3±0.001*	42.44±0.002*	44.03±0.002*	52.12±0.002*
Vit-C	0.1µg/ml	0.2µg/ml	0.4µg/ml	0.6µg/ml	0.8µg/ml	1µg/ml	----------
	5.9±0.002	13.54±0.001*	29.51±0.001*	46.18±0.003*	62.15±0.001*	78.12±0.001*	----------

AEBR = Aqueous extract of *Balanites roxburghii*, EEBR = Ethanolic extract of *Balanites roxburghii*.* P < 0.001 compared to reagent blank

Table 3: In vitro free radical scavenging effect of *Balanites roxburghii* bark extracts by nitric oxide scavenging method

	% Scavenging (Mean ± SEM) of triplicates						
Drug	4µg/ml	8µg/ml	15µg/ml	30µg/ml	60µg/ml	125µg/ml	250µg/ml
AEBR	1.13±0.002	6.02±0.001*	6.49±0.003*	7.53±0.003*	9.03±0.004*	14.68±0.001*	22.22±0.002*
EEBR	42.61±0.002*	42.71±0.002*	42.9±0.005*	43.19±0.001*	43.77±0.001*	46.28±0.002*	47.92±0.002*
Vit-C	0.1µg/ml	0.2µg/ml	0.4µg/ml	0.6µg/ml	0.8µg/ml	1µg/ml	----------
	3.14±0.001	13.36±0.002*	31.28±0.001*	40.33±0.001*	61.47±0.004*	75.23±0.002*	----------

AEBR = Aqueous extract of *Balanites roxburghii*, EEBR = Ethanolic extract of *Balanites roxburghii*.* P < 0.001 compared to reagent blank.

Table 4: In vitro free radical scavenging effect of *Balanites roxburghii* bark extracts by hydroxyl radical scavenging method

	% Scavenging (Mean ± SEM)				
Drug	25µg/ml	50µg/ml	100µg/ml	200µg/ml	400µg/ml
AEBR	15.81±1.12	27.42±1.11	32.69±0.76	45.21±1.25	56.30±0.84
EEBR	16.23±1.05	28.26±0.90	40.51±0.73	56.74±0.76	70.25±0.54
Vit-C	10µg/ml	20µg/ml	40µg/ml	60µg/ml	80µg/ml
	25.83±0.91	39.25±0.84	54.21±0.81	66.24±0.97	77.03±0.45

AEBR = Aqueous extract of *Balanites roxburghii*, EEBR = Ethanolic extract of *Balanites roxburghii*.Values are (Mean ± SEM) of six replicates

Nitric Oxide Scavenging: The aqueous and ethanolic extracts of stem bark of *Balanites roxburghii* showed significant free radical scavenging action against nitric oxide (NO) induced release of free radicals at the concentration 250 µg/ml, showing 22.22% and 47.92% of NO inhibition, respectively. Ascorbic acid was used as reference standard. The % inhibition is shown in Table 3.

Hydroxyl Radical Scavenging: The aqueous and ethanolic extracts significantly scavenged the hydroxyl radicals generated by the EDTA/H_2O_2 system, when compared with that of Ascorbic acid. The percentage scavenging of OH radicals by the aqueous and ethanolic extract was increased in a dose dependant manner. The standard vitamin-C also showed scavenging effect (Table 4).

DISCUSSION

Reactive Oxygen species (ROS) generated endogenously or exogenously are associated with the pathogenesis of various diseases such as atherosclerosis, diabetes, cancer, arthritis and aging process. Thus antioxidants which can scavenge ROS are expected to improve these disorders. The free radical scavenging activity of the extracts was evaluated based on the ability to scavenge the synthetic DPPH. This assay provided useful information on the reactivity of the compounds with stable free radicals, because of the odd number of electrons. DPPH shows a strong absorption band at 517 nm in visible spectrum (deep violet colour). As the electron became paired of in the presence of free radical scavenging, the absorption vanishes and the resulting discoloration stoichiometrically coincides with respect to the number of electrons taken up. The bleaching of DPPH absorption is representative of the capacity of the test drugs to scavenge free radicals independently. Hydroxyl radical is the principal contributor for tissue injury. The formation of Hydroxyl radical from fenton reaction was quantified using 2, deoxy-D-ribose degradation. The aqueous and ethanolic extract inhibited hydroxyl scavenging activity. Sodium nitroprusside serves as a chief source of free radicals. The absorbance of the chromophore formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with napthylethylene diamine is used as the marker for NO scavenging activity. The chromophore formation was not complete in the presence of aqueous and ethanolic extract of *Balanites roxburghii* stem bark, which scavenges the NO thus formed from the sodium nitroprusside and hence the absorbance decreases as the concentration of both the extracts increases in a dose dependent manner.

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