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Research Article

Antimicrobial and antioxidant activity of *Embelia robusta*: a common adulterant in black pepper

Umair Ikram Dar*, Farooq Saleem, Saad Touqeer
Faculty of Pharmacy, The University of Lahore, Lahore, Pakistan

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ABSTRACT

The present study was aimed to investigate the phytochemical as well as the antibacterial, antifungal, antioxidant and irritant activities of fruit of Embelia robusta. Agar-well diffusion method was used for antimicrobial study whereas for antioxidant activity three tests namely, DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, β-Carotene assay and ascorbic acid content were performed. The results from antibacterial assay showed presence of antibacterial activity in all the three plant extracts (methanol, chloroform and n-hexane). Significant antioxidant activity was also found to be present and plant extracts showed no irritant effect. Alkaloids, glycosides, phenolics, tannins and flavonoids were also detected in the plant. The study shows that the plant possesses significant medicinal value.

Keywords: Extract, Antioxidant activity, Antibacterial activity, Irritant activity

INTRODUCTION:

Medicinal plants have a long history for use in different disease conditions. Different bioactive compounds have been isolated from plants which are now being prescribed by physicians all over the world (Fransworth *et al.,* 1985).

Embelia robusta belongs to the family Myrsinaceae. It grows as a large scandent shrub and is commonly found in India and Pakistan. It is also known as False Black pepper due to its great resemblance with black pepper (*Piper nigrum*) and it is frequently used as an adulterant. The plant has been traditionally used to treat intestinal parasites, infections of skin and respiratory tract, cancer, GIT problems and mental disorders (Nayak *et al.,* 2009; Pandey and Ojha, 2011). Recent studies have proved many of the traditional claims related to the medicinal activity of the plant. The plant has been studied for anthelmintic, anti-inflammatory and hepatoprotective activity (Bharadwaj*et al.,* 2013; Sambrekar *et al.,* 2010; Vite *et al.,* 2011). Various chemical compounds have been isolated from the plant such as vilangin, embelin, gallic acid, vanillic acid and salicylic acid (Nayak *et al.,* 2009; Rao and Venkateswarlu, 1961).

The aim of the present study is to investigate the antibacterial, antifungal, antioxidant and irritant activities of the plant.

***Corresponding Author:** *Umair Ikram Dar*,
Address: Faculty of Pharmacy, University of Lahore, Lahore, Pakistan
E-mail: *umairikramdar@yahoo.com*

MATERIALS AND METHODS:

Plant material

The dried fruit of *Embelia robusta* was purchased from local market in Lahore, Pakistan. The plant material was identified by Dr. Ajaib Choudhary, Department of Botany, Government College University, Lahore. The voucher number received for *E. robusta* was GC. Herb. Bot. 2281.

Preparation of extract

500g of dried fruit of *E. robusta* was pulverized in an iron pestle and mortar. Extraction was carried using Soxhlet apparatus. First extraction was carried out using 2.5 Liter of n-Hexane for 14

hours. The residue obtained was extracted using 2.5 Liter chloroform for 12 hours and finally using same amount of methanol for 19 hours. The extracts obtained were concentrated using rotary evaporator and finally air dried to obtain solid mass. The weight of dried extracts were 25, 50 and 49 grams respectively.

Chemicals

Methanol, chloroform, n-Hexane and DMSO (Dimethyl sulfoxide) were purchased from Merck, Germany. Culture media were purchased from Himedia, India. All other chemicals and reagents were of analytical grade.

Microorganisms tested

The standard strains of *Staphylococcus aureus* (ATCC25923), *Bacillus subtilis* (ATCC6633), *Staphyloococcus epidermidis* (ATCC35984), *Escheria coli* (ATCC25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used for antibacterial studies while standard strains of *Candida albicans* (ATCC 10231), *Aspergillus flavus* (ATCC 16404) and *Saccharomyces cerevisae* (ATCC 9763) were used for antifungal studies. The microbial cultures were provided by Saffron pharmaceuticals and Nova Med pharmaceuticals (Pvt.) Ltd. Pakistan.

Animals used

Adult Rabbits of local breed from either sex were purchased from local market and were kept under standard conditions (12 hours dark and 12 hours light period and temperature 25 ± 2^{o}C) in wooden cages in animal house. They were provided free access to green vegetables and water *ad libitum.* The study protocol was approved by the ethical committee of The Faculty of Pharmacy, The University of Lahore, Pakistan.

Preliminary phytochemical study

Different tests for the detection of alkaloids, glycosides, flavonoids, saponins, tannins and steroids etc. were performed according to the methods described by Ramaan and Trease and Evans (Raaman, 2006; Trease and Evans, 1983).

Antibacterial assay

The antibacterial activity was studied using agar-well diffusion assay (Aqil and Ahmad, 2003; Rojas *et al.,* 2003). Sterilized nutrient agar media

Table 1: Preliminary phytochemical study

Chemical class	Test	ERHE	ERCE	ERME
Alkaloids	Mayer's test	-	+	+
	Wagner's test	-	+	+
	Hager's test	-	-	-
Glycosides	Legal's test	+	++	+
Phenolics and Tannins	Ferric chloride	+	++	+++
	Lead acetate	-	-	++
Flavonoids	Alkaline reagent	-	+	++
Saponins	Frothing test	-	-	-

ERHE, *E. robusta* n-Hexane Extract; **ERCE**, *E. robusta* Chloroform Extract;
ERME, *E. robusta* Methanolic Extract; "+" indicates presence whereas "-" indicates absence

was mixed with 100µl of the inoculum and was poured into each petridish. The culture media was allowed to solidify. Using sterilized cup-borer, wells of 8mm diameter were created at equal distances. Standard solutions of ampicillin and amikacin 1mg/ml were prepared in DMSO. The extracts to be tested were dissolved in DMSO to obtain 5mg/ml, 50mg/ml and 100mg/ml. 100µl of each standard drug solution, the three concentrations of each plant extract and blank solvent (DMSO) were added aseptically to each well and incubated for a period of 24h at 37^{o}C. The zone of inhibition was determined. The experiment was carried out in triplicate.

Antifungal assay

The antifungal assay was also carried by well diffusion method (Aqil and Ahmad, 2003; Rojas *et al.*, 2003). Sterilized Sabouraud Dextrose Agar (SDA) media was mixed with 1ml of the fungal suspension in normal saline and poured into petridish. The media was allowed to solidify and wells of 8mm diameter were created. 100µl of standard drug solution (terbinafine 1mg/ml), the three concentrations of each plant extract and blank solvent (DMSO) were added aseptically to each well and incubated for a week at room temperature. The zone of inhibition was determined. The experiment was carried out in triplicate.

Antioxidant assay

DPPH

Different concentrations (250µg/ml, 500µg/ml, and 1000µg/ml) of samples were mixed with 3ml of 0.1mM methanolic solution of DPPH. The mixture was then shaken vigorously for 5minutes and allowed to stand for 1h at room temperature. The absorbance of the solution was measured at 517nm using UV spectrophotometer with methanol as blank. The decrease in absorbance showed increased scavenging activity. BHT (Butylated hydroxytoluene) was used as a standard (Siddiqui *et al.*, 2011) Table 3 is showing antioxidant activity by DPPH method.

Percentage scavenging was calculated using the following formula:

% age scavenging = [Abs (control) – Abs (sample) / Abs (control)] x 100

Beta Carotene

Antioxidant activity by β-Carotene method was carried out using the procedure by Taga *et al.*, 1984. For the assay, 1 ml of β-carotene solution (1 mg/ml in distilled water) was mixed with 0.02 ml of linoleic acid and 0.2 ml of Tween 20. The mixture was mixed with 0.2 ml of methanol as a control. In the same mixture BHT was added for standard and in case of test samples, various fractions were added all at a concentration of 1mg/ml. The mixtures were evaporated at room temperature and50 ml distilled water was added and finally shaken to form a liposome solution. Thermal auto-oxidation was induced in the samples at 50°C and after 2h the absorbance was measured at 470nm by taking reading at a 10min interval. Antioxidant activity coefficient (AAC) was calculated by the following formula:

$AAC = A_{s(120)} - A_{c(120)} / A_{c(0)} - A_{c(120)} \times 1000$

Where$A_{s(120)}$ and $A_{c(120)}$are the absorbance of the antioxidant mixtureand control respectively at t=120 min. $A_{c(0)}$ is the absorbance of the control at t=0 min whereas $A_{c(120)}$ is the absorbance of the control at t=120 min (Mallet et al., 1994). Table 4

Table 2: Antibacterial activity

Sample/ Standard	Conc. (mg/ml)	Zone of inhibition in mm (mean ± S.D)				
		Bacillus subtilis (ATCC-6633)	*Staphylococcus aureus (ATCC-25923)*	*Staphylococcus epidermidis (ATCC-35984)*	*Escherichia Coli (ATCC-25922)*	*Pseudomonas aeruginosa (ATCC-27853)*
Ampicillin	1	34.47±.61	35.62±.91	32.53±1.2	36.90±.52	32.29±.41
Amikacin	1	25.62±.54	21.36±.47	23.15±.05	20.40±.46	20.46±.66
	5	-	-	-	11.11±.07	-
ERHE	50	-	-	-	11.22±.09	-
	100	-	-	-	12.44±.23	-
	5	10.67±.46	-	-	10.16±.04	-
ERCE	50	11.33±.06	-	-	13.33±.29	-
	100	12.37±.1	-	-	14.25±.04	-
	5	14.64±.12	9.33±.28	12.99±.14	10.50±.08	11.53±.02
ERME	50	14.88±.09	9.49±.01	13.76±.22	11.13±.11	11.65±.11
	100	15.75±.24	10.04±.16	14±.05	11.23±.15	12.03±.05

ERHE, *E. robusta* n-Hexane Extract; **ERCE**, *E. robusta* Chloroform Extract; **ERME**, *E. robusta* Methanolic Extract

is showing antioxidant activity by β-Carotene method.

Ascorbic acid content

Ascorbic acid content was determined by titrimetric method described by Barakat *et al.*, 1973. The plant powder, 20 g was taken and extracted with 6% TCA (Trichloroacetic acid) / EDTA (Ethylenediaminetetraaceticacid) extractant solution for 30 min with continuous shaking. It was then centrifuged at 3000 rpm for 20 min and the supernatant layer obtained was separated by decantation. 20 ml of this liquid was used for titration (in conical flask) with 10ml of 10% potassium iodide and 1% starch solution as indicator. The resultant mixture was titrated against 0.01N copper sulfate until the appearance of bluish end point.

Blank reagent was carried out in which 20 ml of extractant solution was used having no contents from plant material. The ascorbic acid content was calculated with help of following formula:

Ascorbic acid mg / 100 g of sample = (100 / W) x (V_f / V_a) x 0.88 x T

Where;

W = Weight of plant sample used, V_f = Total volume of supernatant liquid, V_a = Total volume of extract titrated, T = Titre value less blank.

Irritant activity

10mg of each plant extract was dissolved in 10ml of acetone to prepare sample solutions (w/v). The rabbits were distributed randomly into 3 groups (n=3). Group I received 10µl of n-Hexane extract while group II and III received chloroform and methanol extract respectively. The inner surface of rabbit's ear was shaved. One ear served as control (for 10µl of acetone) while the other served as

Table 3: Antioxidant activity by DPPH method

Sample/ Standard	Conc. (mg/ml)	%age Scavenging
Ascorbic acid	5	61.70
ERHE	1	57.13
ERCE	1	56.25
ERME	1	58.34

Table 4: Antioxidant activity by β-Carotene method

Sample/Standard	Conc. (mg/ml)	AAC
BHA	1	264.9
ERHE	1	66.23
ERCE	1	107.66
ERME	1	149

ERHE, *E. robusta* n-Hexane Extract; **ERCE**, *E. robusta* Chloroform Extract; **ERME**, *E. robusta* Methanolic Extract; **AAC**, antioxidant activity coefficient

experimental (for 10µl of extract). The rabbits were checked for any redness of ear at intervals of 15min for 4hours and at 24th and 48th hour for chronic response. Irritancies on rabbit ears were recorded according to the scale of Hacker (Hacker, 1971).

RESULTS AND DISCUSSION:

The results of preliminary phytochemical study are given in Table 1. The study shows the presence of alkaloids in the chloroform and methanol extract but in low quantities. Glycosides were also found to be present in the n-Hexane and chloroform extract. Phenolic compounds and tannins were present in all three solvent extracts while flavonoids were absent in the n-Hexane extract. Saponins were absent in the plant.

Antibacterial activity was also found to be present in the plant. ERHE (*E. robusta* n-Hexane Extract) was active against only *E. coli* (12.44±.23 at conc. of 100mg/ml) whereas ERCE (*E. robusta* chloroform extract) showed activity against *E. coli* as well as *B. subtilis*. The activity of ERCE against *E. coli* was lower (12.37±.1 at highest conc.) as compared to *B. subtilis* (14.25±.04). ERME (*E. robusta* methanolic extract) showed the broadest spectrum and large zone of inhibitions. The highest activity of ERME was against *B. subtilis* where it produced a zone of inhibition of 15.75±.24 at 100mg/ml and then against *S. epidermidis* (14±.05 at 100mg/ml). There was no considerable variation in the activities when the three concentrations i.e. 5, 50 and 100mg/ml of any extract against any strain were observed. The results showed presence of significant antibacterial activity in the plant. The superior activity of the methanolic fraction may be due to the ability of methanol to extract a wide range of chemical compounds from the plant material. The antibacterial activity of the standard compounds is given in Table 2.

The plant was also studied for antifungal activity against *C. albicans, A. flavus and S. cerevisae*. No activity was found to be present. The antifungal activity of the standard drug terbinafine (1mg/ml) for *C. albicans, A. flavus* and *S. cerevisae* was 55.37, 44.34 and 28.55mm respectively.

Antioxidant activity was tested by three different techniques namely, DPPH free radical scavenging assay, β-Carotene method and ascorbic acid content. In case of DPPH assay the activities of all three samples were found to be very close to each other. The percentage scavenging of ERHE was 57.13% whereas that of ERCE and ERME were 56.25 and 58.34 respectively. The activities of all three samples were comparable to that of standard drug ascorbic acid (61.70% scavenging).

The antioxidant activity coefficient (AAC) calculated in case of beta carotene assay was found to be highest in ERME (49) whereas in ERCE and ERHE was 107.66 and 66.23 respectively. The AAC of standard drug Butylated hydroxyanisole (BHA) was 264.9. The methanolic extract of the plant possessed significant antioxidant activity.

Ascorbic acid content was found by a special extraction technique. Instead of coarse powder, fine powder was used to extract ascorbic acid and its quantity was determined titrimetrically. Ascorbic acid content was found to be 10.22 mg/100g of the sample.

The irritant effect of the three samples studied showed no redness in the rabbit ears in both acute and chronic studies. This shows that the plant possess no irritant effect on the epidermal layer.

CONCLUSION:

The increasing resistance of pathogens towards conventional antimicrobial agents has increased the need to explore new and safer drugs. Natural products, if included in routine diet, continuously provide protection from diseases especially infections due to presence of variety of biologically active compounds in them. Our study proved the high therapeutic value of the plant. The plant seeds though used as adulterant in black pepper thereby reducing the flavor but still provide some benefit to its consumer. The presence of antibacterial and antioxidant activity also demand further studies in order to isolate the active principles involved in this medicinal effect.

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