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Antioxidant and Antibacterial Activity of *Diospyros ebenum* Roxb. Leaf Extracts

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Abstract: The therapeutic properties of plants, including antioxidant and antibacterial activity, have been investigated. In the present study the petroleum ether, ethyl acetate, methanol, and aqueous extracts of *Diospyros ebenum* Roxb. were evaluated for DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging (antioxidant) activity and antibacterial activity. Antibacterial activity was determined against *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC29737, *Pseudomonas aeruginosa* ATCC27853, *Salmonella typhimurium* ATCC23564, and *Enterobacter aerogenes* ATCC13048 using the agar well diffusion method. Total phenolic and flavonoid content of these extracts was determined as the gallic acid and quercetin equivalent, respectively. Results show that the methanol extract was active against all 5 bacterial strains, whereas the aqueous extract was active against none. The methanol extract had the highest total phenolic content and DPPH free radical scavenging activity (IC_{50} = 20 µg/ml). The antioxidant and antibacterial potential of the methanol extract of *D. ebenum* observed in the present study indicates that most active constituents of this plant may be phenolics.

Key Words: *Diospyros ebenum*, antioxidant activity, antibacterial activity, total phenol content, flavonoid content

Diospyros ebenum Roxb. Yapraklarındaki Çözücü Özütlerin Antioksidant ve Antibakteriyel Aktiviteleri

Özet: Bitkilerin antioksidant ve anti-enfeksiyon gibi tedavi edici özellikleri, güçlü farmokolojik faaliyetlerinden dolayı, dünyadaki son bilimsel gelişmelerin ışığı altında araştırılmaktadır. Bu çalışmada, *Diospyros ebenum* Roxb. 'un petrol eteri, etil asetat ve su özütlerinin, DPPH (2,2-difenil-1-pikril-hidrazil) serbest radikal temizleme ve antibakteriyel aktiviteleri değerlendirilmiştir. Antibakteriyel aktivite, *Bacillus subtilis* ATCC6633, *Staphylococcus aureus* ATCC29737, *Pseudomonas aeruginosa* ATCC27853, *Salmonella typhimurium* ATCC23564 ve *Enterobacter aerogenes* ATCC13048 'e karşı agar kuyu difizyon metodu kullanılarak belirlenmiştir. Bu özütlerin toplam fenolik ve flavonoid içeriği sırasıyla gallik asit ve quercetin eşdeğeri olarak belirlenmiştir. Sonuç, su özütünün çalışılan bütün mikrobial suşlara karşı inaktif iken, metanol özütünün kullanılan beş bakteri suşuna karşı aktif olduğunu göstermiştir. Toplam fenolik içeriği ve DPPH serbest radikal temizleme aktivitesi metanol özütünde (IC_{50} = 20 µg/ml) daha fazla bulunmuştur. Bu çalışmada belirtilen *D. ebenum*'un metanol özütünün antioksidant ve antibakteriyel potansiyeli bu bitkinin aktif içeriğinin çoğunun fenolik bileşenler olabileceğini göstermektedir.

Anahtar Sözcükler: *Diospyros ebenum*, antioksidant aktivite, antibakteriyel aktivite, toplam fenol içeriği, flavonoid içeriği

Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources; much of this isolation was based on the uses of these agents in traditional medicine (1). The study of biologically active compounds from natural sources has always been of great interest to scientists looking for new sources of useful drugs for treating infectious diseases.

Oxidation processes are very important in all organisms. The uncontrolled production of oxygen free radicals and the unbalanced mechanism of antioxidant

protection results in cancer, diabetes, and coronary heart diseases (2-4). The antioxidant properties of plant extracts have been attributed to their polyphenolic content (5,6); therefore, plants containing high levels of polyphenols are of great importance as natural antioxidants. Many byproducts and wastes generated by the agro-industry contain polyphenols, which can be potentially used as food antioxidants and preventive agents against some diseases (7).

Infectious diseases caused by bacteria, fungi, viruses, and parasites remain a major threat to public health, despite tremendous progress in human medicine. Their impact is particularly great in developing countries because of the relative unavailability of medicines and the emergence of widespread drug resistance (8).

Diospyros ebenum Roxb. belongs to the family Ebenaceae. This plant is distributed throughout India in deciduous forests. Terminal buds are absent and branchlet tips sometimes form a spine. The leaves are alternate, occasionally slightly translucent, dotted or with gland pits. The fruits of this plant are edible; the bark is astringent (9) and its decoction is used to treat diarrhea and dyspepsia. The leaves are diuretic laxative, carminative, and styptic. The dried flowers are used to treat urinary and skin infections.

The aim of the present study was to assess the in vitro antioxidant and antibacterial activity of different solvent extracts of *Diospyros ebenum* leaves.

Materials and Methods

Collection of Plant Material

The leaves of *Diospyros ebenum* Roxb. were collected from Rajkot, Gujarat, India in September 2007 and identified by Dr. N.K. Thakrar, Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India (voucher specimen no. PSN430). The leaves were thoroughly washed with tap water, air dried, homogenized to a fine powder, and stored in airtight bottles.

Extraction

The dried *D. ebenum* leaf powder was extracted successively in petroleum ether, ethyl acetate, methanol, and distilled water via the cold percolation method (10). Ten grams of dried *D. ebenum* leaf powder was placed in 100 ml of solvent in a conical flask, plugged with cotton wool, and then kept on a rotary shaker at 190-220 rpm for 24 h. Then the extract was filtered with 8 layers of muslin cloth, and the residue was dried and used for extraction in another solvent. While the filtrate was centrifuged at 5000 g for 10 min, the supernatant was collected, and the solvent was evaporated. The dried extract of each solvent was stored at 4 °C in airtight bottles. Extraction was performed in triplicate and the mean values are presented.

Total Phenol determination

Total phenolic content of the extracts was determined according to the Folin-Ciocalteu reagent method (11), with some modification. Plant extract (1 ml) was mixed with Folin-Ciocalteu reagent (0.1 ml, 1 N) and allowed to stand for 15 min. Then 5 ml of saturated Na_2CO_3 was added. The mixtures were allowed to stand for 30 min at room temperature and total phenolic content was determined spectrophotometrically at 760 nm. A calibration curve was made by preparing gallic acid (10 to 100 µg ml^{1}) solution in distilled water. Total phenol values are expressed in terms of the gallic acid equivalent (mg g^{-1} of extracted compound).

Flavonoid Determination

The aluminum chloride colorimetric method (12), with some modification, was used to determine flavonoid content. Plant extract (1 ml) in methanol was mixed with 1 ml of methanol, 0.5 ml of aluminum chloride (1.2%), and 0.5 ml of potassium acetate (120 mM). The mixture was allowed to stand for 30 min at room temperature and the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was made by preparing a quercetin (5 to 60 µg ml^{1}) solution in methanol. Flavonoid content is expressed in terms of the quercetin equivalent (mg g^{-1} of extracted compound).

DPPH Free Radical Scavenging Activity

The free radical scavenging activity of the *D. ebenum* extracts was measured using the modified method of McCune and Johns (13). Various concentrations of the stock solution (1 ml) were mixed with freshly prepared DPPH (0.3 mM, 1 ml) in methanol to produce a final DPPH concentration of 0.1 mM. The mixture was vigorously shaken and left to stand for 10 min in the dark, and its absorbance was measured at 517 nm. The concentration of each sample required for 50% scavenging of DPPH free radicals (IC_{50}) were determined

graphically by plotting the percentage of DPPH decrease as a function of the sample concentration:

$$\% \text{ DPPH radical scavenging} = [(B - A)/B] \times 100$$

where B is the absorbance of the blank (DPPH plus methanol) and A is the absorbance of the sample (DPPH, methanol plus sample).

Test Microorganisms

The bacterial strains used were identified strains obtained from the National Chemical Laboratory (NCL), Pune, India. In all, 2 gram-positive (*Bacillus subtilis* ATCC6633 and *Staphylococcus aureus* ATCC29737) and 3 gram-negative (*Pseudomonas aeruginosa* ATCC27853, *Salmonella typhimurium* ATCC23564, and *Enterobacter aerogenes* ATCC13048) bacterial strains were studied.

Antibacterial Assay

A loop full of each strain was inoculated in 25 ml of nutrient broth in a conical flask and then incubated at room temperature on a rotary shaker for 24 h in order to activate the test bacteria. The final cellular concentration was 1×10^8 cfu/ml. Muller Hinton agar (Hi Media) was used to determine antibacterial susceptibility. Bacterial assays were performed using the agar well diffusion method (14). Media and test bacterial cultures were poured into petri dishes (Hi-Media). Each test strain (200 µl) was inoculated into the media when the temperature was 40-42 °C. Care was taken to ensure proper homogenization. After media were solidified a well was made in the plates with the help of a cup-borer (8.5 mm). The well was filled with 100 µl of extract (500 µg well $^{-1}$) and the plates were incubated overnight at 37 °C. Bacterial growth was determined according to the diameter of the zone of inhibition. The experiments were performed 3 times and mean values are presented. For each bacterial strain, controls were maintained, in which pure solvent (DMSO) was used instead of the extract. The results of all the solvent extracts were compared with the standard antibiotics amikacin (30 µg) and piperacillin (100 µg) (Hi Media).

Statistical Analysis

All the experiments were performed in triplicate and results are presented as mean ± SEM (standard error mean).

Results and Discussion

The use of medicinal plants plays a vital role in meeting basic health requirements in developing countries. These plants may be a new source of antibacterial, antifungal, and antiviral agents with significant activity against infective microorganisms (15,16).

In the present study the extractive yield of *D. ebenum* leaf varied among the different solvents used. The methanol extract had the highest extractive yield (12.37%) and the ethyl acetate extract had the lowest (2.70%). Parekh et al. (17) and Vaghasiya and Chanda (10) reported similar results.

A number of studies have focused on the biological activity of phenolic compounds, which are potential antioxidants and free radical scavengers (18,19). In the present study total phenolic content was highest in the methanol extract and lowest in the petroleum ether extract, whereas flavonoid content was highest in the petroleum ether extract and lowest in the aqueous extract (Figure 1).

Various assays are used to test antioxidant activity, but the most widely used methods are those that involve generation of free radical species that are then neutralized by antioxidant compounds (20,21). The DPPH radical is commonly used as a substrate to evaluate antioxidant activity; it is a stable free radical that can accept an electron or hydrogen radical to become a stable molecule.

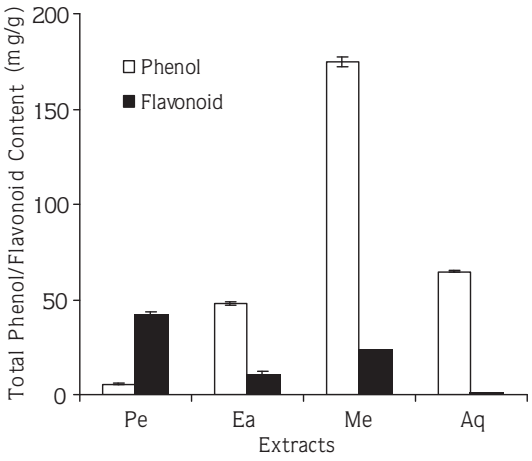

Figure 1. Total phenol and flavonoid content of the solvent extracts of *Diospyros ebenum* leaf. Pe: Petroleum ether extract; Ea: ethyl acetate extract; Me: methanol extract; Aq: aqueous extract. Values are expressed as mean ± SEM, n = 3.

Reductions in the DPPH radical induced by antioxidants was determined by the decrease in its absorbance at 517 nm. The concentration of a sample at which the inhibition percentage reaches 50% is its IC_{50} value. The IC_{50} value is negatively related to antioxidant activity, as it expresses the amount of antioxidant needed to decrease its radical concentration by 50%. The lower the IC_{50} value, the higher the antioxidant activity of a tested sample.

In the present study the methanol extract had the highest antioxidant activity (IC_{50} = 20 µg/ml). This activity was very near to that shown by the standard (ascorbic acid, IC_{50} = 11.4 µg/ml), while the petroleum ether extract had the lowest antioxidant activity (IC_{50} = 145 µg/ml) (Figure 2). Liu and Ng (22), and Siriwardhana et al. (23) reported a high correlation between DPPH radical scavenging activity and total polyphenolic content. In the present study the methanol extract of *D. ebenum* had the highest phenolic content, as well as the highest DPPH free radical scavenging activity.

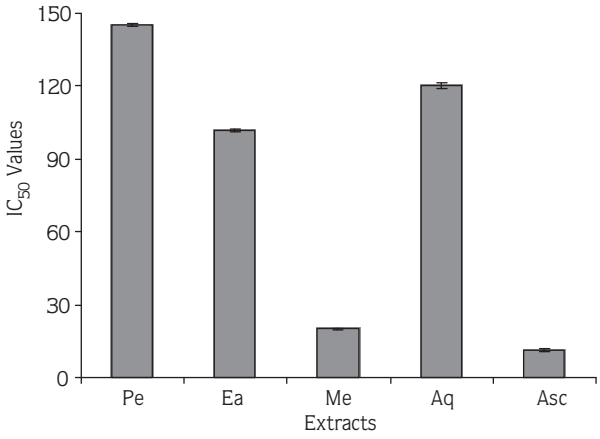

Figure 2. DPPH free radical scavenging activity (IC_{50} values) of the solvent extracts of *Diospyros ebenum* leaf. Pe: Petroleum ether extract; Ea: ethyl acetate extract; Me: methanol extract; Aq: aqueous extract; Asc: ascorbic acid.
Values are expressed as mean ± SEM, n = 3.

The past 3 decades have seen a dramatic increase in resistance to antimicrobial agents, leading to the repeated use of antibiotics and insufficient disease control (24). Due to the increasing prevalence of antibiotic-resistant pathogens in hospitals and homes, a deliberate search is in progress for alternative treatments to combat further spread of antibiotic-resistant pathogens (25). The aqueous extract tested in the present study did not have any activity against the bacterial strains studied. The methanolic extract demonstrated activity against all 5 bacterial strains and a large zone of inhibition against *P. aeruginosa* (Figure 3). The ethyl acetate extract showed

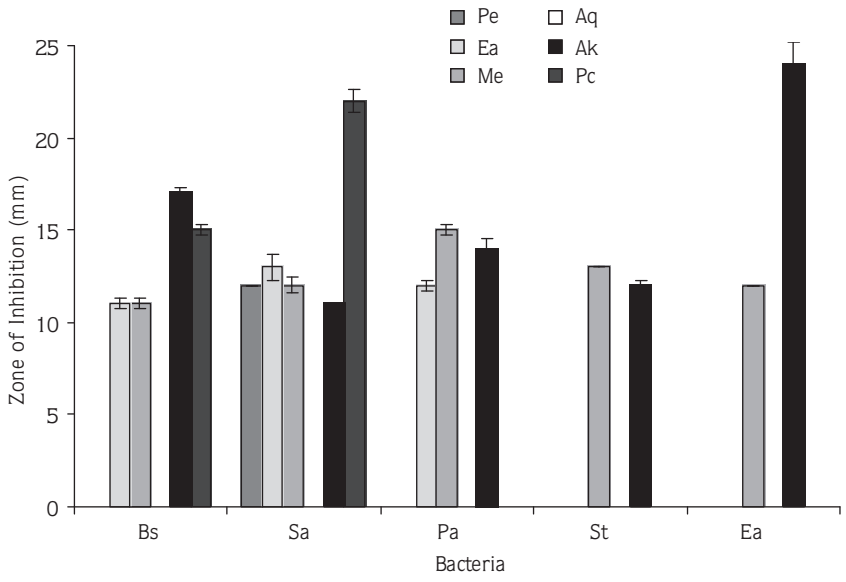

Figure 3. Antibacterial activity of the solvent extracts of *Diospyros ebenum* leaf. Bs: *Bacillus subtilis*; Sa: *Staphylococcus aureus*; Pa: *Pseudomonas aeruginosa*; St: *Salmonella typhimurium*; Ea: *Enterobacter aerogenes.* Pe: Petroleum ether extract; Ea: ethyl acetate extract; Me: methanol extract; Aq: aqueous extract; Ak: amikacin; Pc: piperacillin. Values are expressed as mean ± SEM, n = 3.

activity against 3 bacterial strains—*B. subtilis, S. aureus*, and *P. aeruginosa*—while the petroleum ether extract showed activity only against *S. aureus*. The standard antibiotic piperacillin showed activity only against the gram-positive bacteria. All the extracts, except the aqueous extract, were more potent against *S. aureus* than was the standard amikacin. The methanol extract was more active against *P. aeruginosa* and *S. typhimurium* than was amikacin.

Many studies have shown that natural antioxidants in plants are closely related to their biofunctionality, such as the reduction of chronic diseases and inhibition of pathogenic bacterial growth, which are often associated with the termination of free radical propagation in biological systems (26).

The results of the present study show that the methanol extract of *D. ebenum* leaf contained a high total phenolics level, and is a good source of antioxidant as well as antibacterial agents; therefore, it can be considered potentially useful for medicinal application.

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