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ANTIBACTERIAL AND ANTIOXIDANT POTENTIAL OF STEM BARK EXTRACT OF *BOMBAX CEIBA* COLLECTED LOCALLY FROM SOUTH PUNJAB AREA OF PAKISTAN

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Abstract

Background: Ethnic medication systems have been used extensively by humans since their origin. Now a day, in the developing countries these systems are being used due to their effectiveness and affordability. Especially inhabitants of rural areas still rely on these systems as first line of treatment against diseased conditions. Till now, majority of medicines is derived from the natural origin particularly plants owing to their little side effects and cost effectiveness.
Materials and Methods: In the present work, we evaluated antibacterial and antioxidant activity of methanolic extract of *Bombax ceiba* stem bark. Total phenolic and flavonoid contents were also assessed in the extract. The antioxidant capacity was determined by DPPH, Nitric Oxide scavenging and reducing power activity. For antibacterial activity, Gram-negative (*Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi*) and Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) were used.
Results: Phenolic content was 133.21±1.56 μg GAE/mg of extract while Flavonoid content was 997.93±2.14 μg QE/mg. Plant extract demonstrated strong antiradical activity with EC_{50} 18.78 ± 0.69 and 23.62 ± 1.99μg/ml for Nitric Oxide and DPPH assay respectively while EC_{50} in case of reducing power activity was 139.4 ± 0.98μg/ml. Plant extract displayed inhibitory effect against microbial growth with *S. typhi* as the most resistant strain and *Staphylococcus aureus* the most sensitive one.
Conclusion: This study revealed that *Bombax ceiba* of local origin has broad spectrum antibacterial activity and it can also provide defense against oxidative stress.

Key words: antibacterial, antioxidant, DPPH, Nitric oxide, *Bombax ceiba*,

Introduction

For several years, plants remained in use as a source of conventional medicine for the cure of many diseased conditions. Majority of these plants contain good amount of phytochemicals having strong antioxidant activities and can be used for medicinal purposes (Razali et al., 2008). Ailments due to microbial infections are the foremost cause of death throughout the globe because of the multidrug resistant bacteria. These resistant microbes are posing threat to the Immuno-compromised patients by crippling them or by taking their lives in the developing countries (Kaneria and Chanda 2013). Microbial resistance to currently used antibiotics has indicated an increasingly important and serious international problem. The susceptibility of certain bacterial and fungal strains to different antimicrobial agents has been reduced thereby leading to the need of development of new entities from plants (Naz et al., 2015). New drugs can be developed from the natural source of plants to use against the diseases caused by microorganisms (Ruban and Gajalakshmi 2012) because the plants are inexpensive and safer substitute of antimicrobial agents (Kaneria and Chanda 2013).

Bombax ceiba that belongs to the family *Bombacaceae*, is recognized with the common names of silk cotton tree and semal. It is considered as a noteworthy medicinal plant in Indian tropical and subtropical regions and is also found in many countries including Pakistan. Indian traditional systems of medicine such as Ayurveda, Siddha and Unani describe its use for the treatment of many diseases such as sexual incapacity, to stop bleeding from wounds and against vaginal infections (Anandarajagopal et al., 2013).

Oxidative stress caused by free radicals and pro-oxidants can be managed by the use of antioxidants (Mandade et al., 2011). Free radicals are highly unstable molecules due to unpaired electrons in their structure causing significant damage to the stable compounds by taking out electrons from these compounds to attain stability (Garg et al., 2012). Singlet oxygen that is being formed within the tissues of the living organisms e.g. superoxide, hydroxyl and hydrogen peroxide is extremely reactive causing potential harm to the stable compounds. These highly reactive species are neutralized by different enzymatic antioxidants like superoxide dismutase, glutathione peroxidases and catalases. The

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imbalance between the antioxidants and pro-oxidants due to excessive formation of these unstable radicals, damages cell structures including genetic material and fatty constituents leading to increased threat of more than 30 various ailments (Mandade et al., 2011) such as malignancy, diabetes, osteoarthritis and other degenerative illnesses in humans (Bhaskar et al., 2011). Antioxidants can stabilize free radicals by donating hydrogen or electron to them thereby decreasing the risks of oxidative stress. Antiradicals are the compounds that can control the reactive species. It is proposed to employ more than one method for antioxidant activity to evaluate possible mechanisms of action of substances with antioxidant potential (Reihani et al., 2012). Antioxidants are largely used to prevent oxidation of pharmaceuticals, cosmetics and food products. There is an increased interest to find new antioxidants from plants to replace the synthetic ones (Zhang et al., 2010).

In this work, antibacterial and antioxidant activity of stem bark extract of *B. ceiba* grown wildly in the south Punjab was carried out to ascertain its medicinal value as is regarded by the indigenous practitioners.

Materials and Methods

Chemicals and Reagents

Folin–Ciocalteu's reagent, garlic acid, quercetin, sodium nitroprusside, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), sulphanilamide and ascorbic acid were acquired from Sigma–Aldrich (St. Louis, USA). Sodium carbonate, sodium nitrite, aluminium chloride, sodium hydroxide, ferric chloride, potassium ferricyanide, trichloroacetic acid, and analytical grade methanol were procured from the Merck (Darmstadt, Germany). Nutrient broth and nutrient agar were purchased from oxoid ltd. Ciprofloxacin was gifted by pharmedic. Distilled water was prepared in our lab.

Plant Material

The plant material was collected locally from Bahawalpur in the month of June. The identification of *Bombax ceiba* was done by Cholistan Institute of Desert Studies (CIDS), The Islamia University of Bahawalpur, Bahawalpur, Pakistan. Voucher specimen (No. 3524/CIDS/IUB) was deposited in the herbarium of Cholistan Institute of Desert Studies (CIDS).

Method of Extraction

The collected plant material was dried in the shade and powdered by the use of a grinder after removing the dust and dirt from it by tap water. The powdered material (100 g) was soaked in methanol for 3 days with infrequent shaking. Then it was passed through several layers of muslin cloth and then through the filter paper. After repeating the process three times collective filtrate was concentrated by the use of a rotary evaporator (Heidolph Laborota 4000 efficient, Germany) at low temperature (40 °C) and reduced pressure. For further use extract was kept at 8 °C.

Test bacteria and Antibacterial activity

The bacterial strains used in the study were *Pseudomonas aeruginosa* (ATCC 9087), *Staphylococcus aureus* (ATCC 6538), *Salmonella typhi, Bacillus subtilis and Escherichia coli*. S. typhi, B. subtilis and E. coil were the clinical isolates obtained from the pathology department of Quaid-e-Azam medical college Bahawalpur. These strains were identified by staining and morphological characteristics. Overnight kept culture of bacterial strain was used in the study and extract was dissolved in DMSO to made different concentrations i.e. 15 mg/ml, 30 mg/ml, 60 mg/ml and 120 mg/ml. Antibacterial activity of the extract was evaluated by agar well diffusion method by applying standard inoculums (0.5 MacFarland turbidity standards, 10^6 CFU/mL) of each strain with the help of sterilized cotton swab. Three equidistant wells were made through 6 mm diameter sterile cork borer in germ-free freshly prepared plates and then these plates were kept at 37 °C for 24 hours after adding 10 µL of plant extract. The diameter of zone of inhibition was measured in millimeters (mm) (Naz et al., 2015). Ciprofloxacin was used as standard. All the tests were done in triplicate and mean ± S.D. were calculated using MS Excel.

Determination of total phenolic content (TPC)

Total phenolic content (TPC) of the plant extract was determined by Folin-Ciocalteu reagent using the procedure of Ismail et al. (2004) after minor alterations. In the analysis, concentration of methanolic solution of the extract used was 5 mg/ml. The extract samples (20 µl) were mixed with Folin-Ciocalteu reagent (90 µl that was 10 times diluted with distilled water) in 96 well microplate. This mixture was kept at 25 °C for 5 minutes afterwards Na_2CO_3 (90 µl, 6% w/v) was added to make final volume 200 µl. Phenolic contents were determined at 725 nm after 30 minutes. The mean values of three

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experiments were obtained. The calibration line was constructed by using the standard solution of garlic acid by the same process. Phenolic contents in the extract were expressed in terms of garlic acid equivalent (µg of GAE/mg of extract) after reading the concentration of phenolic (µg/ml) from the calibration line and calculated using C = cV/m

Where C is the phenolic compounds total content in µg GAE/mg dry extract, c the concentration of gallic acid obtained from the calibration curve (µg/mL), V the volume of extract (ml) and m is the weight (mg) of extract.

Determination of flavonoid content (TFC)

The amount of total flavonoid content (TFC) in the plant extract was ascertained by employing the procedure of Lim et al. (2007). In 96-well microplate that already contains 20 µl of extract (5 mg / ml), 80 µl of deionized water was added. After this 16 µl of 5% $NaNO_2$ solution was added in this mixture and incubated for 6 minutes at 25 °C. After 6 minutes, 16 µl of 10% $AlCl_3$ and 68 µl of 4% NaOH solution were poured into the wells of microplate, making the total volume to 200 µl. Absorbance of the samples were taken at 510 nm after keeping the samples for 20 minutes at room temperature. The mean values of three experiments were noted. The calibration line was constructed with standard solution of quercetin using the same process. Flavonoid contents in the extract were expressed in terms of quercetin equivalent (µg of QE/mg of extract) after reading the concentration of flavonoid (µg/ml) from the calibration line.

Reducing Power Activity

Reducing power activity of the extract was established as done by Singhal et al. (2011) with slight modification. In the 96-well microplate, 25 µl of test sample and 25 µl of phosphate buffer (pH 7.2) were taken and mixed. After the addition of 50 µl of 1% potassium ferricyanide solution, this blend was kept at 50 °C for 10 minutes. Then 25 µl of 10% trichloroacetic acid (w/v) solution along with 100 µl distilled water was mixed with it. Finally, 25 µl of freshly prepared 0.2% ferric chloride ($FeCl_3$) solution was added into the mixture and absorbance was taken at 700 nm by using microplate reader. Quercetin was used as standard. To find EC_{50} value, test solution was used at various concentrations. Ferric reducing power activity was measured by equation (1)

Increase in reduction (%) = [(As - Ac) / Ac] × 100 (1)

Where A_c = Absorbance of Control and A_s = Absorbance of sample

DPPH Assay

Antiradical activity of the extract against DPPH was done using the technique of Baylac and Racine (2003). Ten µl of test solution and 90 µl of 100 μM methanolic DPPH solutions were added in 96-wells plate making the final volume 100 µl. After mixing completely, the contents were stored at 37 °C for half an hour. Microplate reader (Synergy HT BioTek® USA) was used to take the absorbance at 517 nm. Standard antioxidant in this study was quercetin. EC_{50} values were determined by using several dilutions i.e. 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.015 Mm. Data obtained was calculated by using Ez-fit software. The decrease in absorbance shows increased antiradical activity that was calculated by equation (2)

Inhibition (%) = $[(A_c - A_s) / A_c] \times 100$ (2)

Where A_c = Absorbance of Control and A_s = Absorbance of sample

Nitric oxide scavenging Activity

Nitric oxide scavenging activity of the extract was assessed by the process described by Okolie et al. (2014) with necessary amendments. Test compound (5 µl) was mixed with 45 µl methanol and 150 µl sodium nitroprusside solution. Then absorbance was taken at 540 nm after placing the mixture at 37 °C for 2 hours. After 10 min of Griess reagent (50 µl) addition, the absorbance was observed at 540 nm. The mean value of absorbance was obtained after three readings. To compare the antioxidant potential quercetin was used as the standard. Lower absorbance values depicted higher nitric oxide scavenging. To evaluate the activity equation (2) was used.

Statistical Analysis

All results were taken in triplicate and expressed as mean ± standard deviation. Data was analyzed by analysis of variance (ANOVA) to determine any significant differences between the means at the confidence level of 5% using SPSS (version 21.0).

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Results

Antibacterial activity of methanolic extract of stem bark of *B. ceiba* was performed by agar well diffusion method. Plant extract showed significant activity ($p < 0.05$) against both Gram positive and Gram negative bacteria in dose dependent manner as shown in **Table 1**. The results showed that *Salmonella typhi* with a diameter of 19 mm of zone of inhibition was the most resistant microbe against the tested extract among the selected microbial strains while *Staphylococcus aureus* was most susceptible to the extract with 24 mm zone of inhibition. It was observed that the extract was active against Gram positive strains as well as Gram negative strains ($p > 0.05$) even at lower concentration. Plant extract depicted inhibitory affect against all the tested strains in a dose dependent manner that with increase in concentration the inhibitory effect against bacterial growth was increased and maximum inhibition was observed at highest concentration used in all the cases (**Table 1)**. The order of sensitivity from highest to least was S. aureus > E. coli > P. aeruginosa > B. subtilis > S. typhi.

Table 1: Antibacterial activity of stem bark extract of *Bombax ceiba* and standard

Bacterial strain	Zone of inhibition (mm)				
	150 µg/well	300 µg/well	600 µg/well	1200 µg/well	Ciprofloxacin 20µg/well
B. subtilis (+)	11 ± 0.57	15 ± 1.52	17 ± 0.57	21 ± 0.57	36 ± 0.57
S. aureus (+)	10 ± 0.57	15 ± 0.57	17 ± 0.57	24 ± 1.52	34 ± 1
E. coli (-)	13 ± 1	16 ± 0.0	18 ± 1.15	23 ± 1	38 ± 0.57
P. aeruginosa (-)	12 ± 0.57	16 ± 0.0	20 ± 0.0	23 ± 1.73	39 ± 1.52
S. typhi (-)	11 ± 1.52	15 ± 1.52	18 ± 0.57	19 ± 0.0	31 ± 1.73

Each result is presented here as mean ± standard deviation (n = 3)
(+) mean Gram Positive and (-) mean Gram negative

It is reported in the literature that plants are good stabilizers of free radicals and oxidants due to rich source of phenolic and flavonoid contents (Yen et al., 2001)**.** Therefore, the amount of total phenolic and flavonoids in bark extract of *Bombax ceiba* was determined in this study. Total phenolic contents as detected by Folin-Ciocalteu reagent were found to be 133.21±1.56 μg GAE/mg of extract. Total flavonoid contents were 997.93±2.14 μg QE/mg of extract as assessed by $AlCl_3$ colorimetric assay as shown in **Table 2**.

In the current work bark extract of *Bombax ceiba* indicated significant antioxidant activity ($p < 0.05$) by the employed three methods namely free Radical scavenging activity by DPPH, Nitric Oxide and reducing power activity. It was found that activity of extract was augmented when the concentration of the extract was increased. Extract showed 90% scavenging of DPPH radical at concentration of 100 µg / ml while that of standard showed 92% inhibition at the same concentration (**Table 2**). The EC_{50} value for extract was found to be 23.62 ± 1.99 µg / ml. The concentration at which the absorbance is 0.5 or the inhibition rate is 50% is the EC_{50} value.

Substances with reducing capacity change ferricyanide (Fe^{3+}) to ferrocyanide (Fe^{2+}), that upon reaction with ferric chloride make a complex that have highest absorption at 700 nm (Singhal et al. 2011). Test solution that was initially yellow in color becomes greenish or bluish depending on the reducing power of the compound. In our study, reducing potential was dose dependent with the reducing ability of the extract was 82.57 ± 1.05% while that of quercetin was 98.12 ± 0.89%. It showed that the extract of *B. ceiba*, as electron donor, could end radical chain reactions by transforming free radicals to more stable products. Reducing power ability of a compound can additionally serve as an important sign of potential antioxidant activity. The results as shown in **Table 2** suggest that the bark extract possessed a significant electron donating capacity.

Griess reagent can be used to detect nitrite ions produced by the reaction of nitric oxide (produced by Sodium nitroprusside in aqueous solution at biological pH) with oxygen. The formation of nitrite ions is abridged due to scavenging of nitric oxide by the antioxidant compounds (Razali et al., 2008). Plant extract exhibited potent nitric oxide radical scavenging potential. Furthermore, increase in scavenging ability of the extract was observed with increasing concentration and highest inhibition of 98% was seen at 100 µg / ml as shown in **Table 2**.

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Table 2: Antioxidant activity, Total phenolic and flavonoid contents of *Bombax ceiba* extract

	B. ceiba Extract		Quercetin		Total phenolic and Flavonoid content	
	Inhibition (%)	EC_{50} (µg/ml)	Inhibition (%)	EC_{50} (µg/ml)	TPC	TFC
Nitric oxide	98 ± 2.22	18.78±0.69	96.34±1.12	6.01±1.13	133.21±1.56 µg GAE/mg	997.93±2.14 µg QE/mg
DPPH	90.03±1.50	23.62±1.99	92.12±0.49	16.18±0.24		
RPA	82.57±1.05	139.4±0.98	98.16±0.89	91.16±0.78		

Values represented are means ± SD (n= 3), RPA = reducing power activity
GAE = gallic acid equivalent, QE =quercetin equivalent

Discussion

Worldwide spread of the resistant microorganisms causing infections that are difficult to treat requires novel substances that can cope with the changing chemistry of microorganisms. There are several studies that depict the antimicrobial and insecticidal properties of the numerous plant extracts. S. aureus is the source of severe community acquired infections and there is an increase in Methicillin Resistant S. aureus infections in many countries of the world (Seyyednejad et al., 2010). Different bacterial strains were used in the study based on the disease-causing potential in humans and including both gram-negative and gram-positive bacterial classes. *B. ceiba* stem bark methanolic extract showed good antibacterial activity against the bacterial strains used in the study. This might be due to the phenolic and flavonoid compounds that are present in good amount in the extract. These compounds account for the antibacterial activity of the plant extracts against different strains of microbes by forming complexes with extracellular and soluble proteins and with bacterial cell wall (Ruban and Gajalakshmi 2012). Rani and Khullar (2004) reported antibacterial activity of stem bark extract of *B. ceiba* against multi drug resistant *Salmonella typhi*. Wang and Huang (2005) found that *B. ceiba* root ethanolic extract has activity against H. pylori when they were screening 50 different traditionally used Taiwanese plants. Islam et al. (2011) found significant activity by hexane extract of root of *B. ceiba* against *Pseudomonas aeruginosa* (12mm zone of inhibition). Our findings are in agreement with these studies.

Phytochemical analysis revealed that flavonoid contents were more than phenolic in the extract that we studied. Vaghasiya et al. (2011) found similar results in the methanolic extract of leaf of *B. ceiba*. So, our findings are in accordance with these results with the difference that they studied leaf extract of the plant. Zahan et al. (2013) reported that phenolic contents of 74.38±7.42 mg/g of garlic acid were detected in the methanolic extract of bark of *B. ceiba*. There are some studies that reported phenolic contents of the gum obtained from *B. ceiba* plant (Surveswaran et al., 2007).

Merely one method cannot entirely describe the antioxidant ability due to the number of factors that are influencing antioxidant activity. So, antioxidant capacity should be assessed by numerous test systems (Zengin et al. 2010). Primary antioxidants prevent oxidation by inhibiting the propagation of chain reaction or by quenching the free radicals while secondary antioxidants serve as metal chelating agents. Therefore, restoring primary antioxidants and stabilizing singlet oxygen. Consequently, numerous chemical-based tests that have their own applications have been developed and adjusted for the detection of antioxidant activities during the past decades (Yang et al., 2015). DPPH is the commonly and frequently adopted method for antioxidant activity determination of plant extracts (Yesmin et al. 2008). DPPH converts to a stable diamagnetic molecule by taking an electron or hydrogen atom. The purple colored alcoholic solution of DPPH radicals change color to yellow or becomes clear in the presence of a hydrogen-donating antioxidant which could be measured at 517 nm. The scavenging effect of the extract against DPPH free radical was comparable to that of quercetin that was used as standard in the study. Different studies state that *B. ceiba* contains many compounds including lupeol, β-sitosterol and shamimicin with potent antioxidant activities (You et al., 2003). So, these compounds could be responsible for the excellent scavenging activity of the extract. These effects are in accordance with the study conducted by Shyur et al. (2005). They used *B. ceiba* whole plant extract that showed higher scavenging activity against DPPH with IC_{50} value of 68 µg / ml. In another study Zahan et al. (2013) while studying the methanolic extract of bark of *B. ceiba* demonstrated good DPPH radical scavenging activity having IC_{50} 32.1 µg / ml.

Antioxidant activity has been suggested to be associated to reducing power (Zengin et al 2010). Plant extract is having remarkable reducing power in our study. Jain et al. (2011) described in their study that the methanolic extract of root demonstrated dose-dependent reduction from Fe^{3+} to Fe^{2+} with highest absorbance of 1.11 at 500 µg / ml. In another work

done by Zahan et al. (2013) on methanolic extract of bark of *B. ceiba* showed that the extract possesses adequate reducing activity.

Leafy and root vegetables contain large quantities of nitrite that is extremely toxic. The excessive ingestion of nitrites leads to negative effects like methemoglobinemia by the oxidation of hemoglobin (Kim et al., 2014). In this study the extract showed excellent scavenging of nitric oxide radicals. Phenolic compounds chiefly flavonoids are recognized not only as free radical scavengers but also active in stabilizing nitric oxide, peroxynitrite and reactive oxygen species during lipid peroxidation (Taira et al., 2015). Because extract is rich in flavonoid contents as shown in **Table 2**, it could be the reason for the excellent scavenging of nitric oxide radical. There is no study evaluating the nitric oxide scavenging activity of *B. ceiba* plant extract to best of our knowledge. So, this is the first report describing NO scavenging ability of the plant extract.

Conclusion

It can be concluded from the above findings that methanolic extract of bark of *B. ceiba* is good in phenolic and flavonoid contents. Extract exhibited great antioxidant activity against DPPH and NO radicals while it displayed good reducing power ability that was significant statistically ($p < 0.05$) implying as an efficient source of antioxidants. Extract also displayed broad spectrum of activity against the pathogens signifying its potential in the cure of infectious diseases caused by resilient microbes. These antioxidants can be used in pharmaceuticals and cosmeceuticals for their promising effects.

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