In vitro antimicrobial and cytotoxic activity of *Tamarix dioica* Roxb. leaves

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Abstract: Medicinal plants have been in use for thousands of years for treatment of different types of diseases and a large inventory of useful drugs has been isolated from them. The aim of our study is to investigate a new and effective plant possessing antimicrobial activity. The crude extracts of *Tamarix dioica* Roxb. leaves, as well as fractions of the extract in various solvents, were investigated against standard strains of bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, *Shigella flexneri*, and fungi *Candida glabrata*, *Aspergillus niger*, *Trichophyton rubrum*, *Candida tropicalis*, and *Aspergillus fumigatus*. For in vitro antibacterial and antifungal studies of *T. dioica* extracts, the agar well diffusion technique was used. For cytotoxic study, brine shrimp (*Artemia salina*) larvae were used. Results of the crude extract and some fractionated samples showed significant antifungal properties, but low antibacterial response and negligible cytotoxic activity. It was recognized that *Tamarix dioica* has the potential for future development of new antifungal drugs/medicine.

Key words: *Tamarix dioica*, antifungal, antibacterial, cytotoxic activity

1. Introduction

Herbal medicines represent one of the most important fields of traditional medicine throughout the world. They play a significant role in the management of medical illnesses, such as digitalis, which contains cardiac glycosides. Cardiac glycoside is commonly used for the treatment of heart failure (1). Modern physicians are still prescribing many drugs of botanical origins. Local communities in the world are using about 10% of medicinal plants for the treatment of various infections, out of which only 1% have been recognized by scientists. Plants are a rich source of secondary metabolites such as flavonoids, tannins, and alkaloids, which have antimicrobial properties. Different types of medicinal plants are used for treatment of infectious diseases due to their lesser side effects and low toxicity (2).

The family Tamaricaceae consists of 60 different species, which are also commonly called salt cedars (3). Tamaricaceae are evergreen shrubs or trees growing from 1 to 18 m in height. Leaves are scale-like, on branched slender-type stems, with flowers that may be pink or white. The genus *Tamarix* is fed on by more than 250 species of invertebrates, as well as camels and livestock. Although *Tamarix* attains the stature of a small tree, it has a deep tap-root that may extend 30 m or more below ground (4).

*T. dioica* (Tamaricaceae), with the local name of jhau, is an evergreen shrub or small tree with reddish bark, vagnate leaves, and purple flowers. The tree is native to Pakistan, Afghanistan, Iran, India, Bangladesh, Bhutan, Kashmir, Nepal, and Myanmar. In Pakistan it is found throughout the country and is abundantly found in the Khyber Pakhtunkhwa (KPK) and Sindh provinces. The leaves of *T. dioica* are used as a carminative, a diuretic, and for the treatment of hepatic and splenic inflammation; the plant is also used as an astringent (5,6) for symptoms such as leucorrhea. The purpose of this study is to evaluate the in vitro antimicrobial and cytotoxic activities of *T. dioica* leaf extracts in different solvents.

2. Materials and methods

2.1. Collection and identification of plant material

Fresh and disease-free leaves of *T. dioica* were collected from the district of Dera Ismail Khan, KPK Province, Pakistan. The taxonomic identification of the plant material was confirmed by a taxonomist from the Department of Pharmacognosy, Faculty of Pharmacy, Gomal University, Dera Ismail Khan, KPK, Pakistan. The voucher herbarium specimen for *T. dioica* was coded FP 035. The collected material was washed 3 times with running tap water and once in sterile water before it was dried at room

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temperature, and after drying the material was ground with an electric grinder to be turned into powder form. Finally, it was packed in a plastic bag for further use.

2.2. Extraction procedures
A sample (500 g) of the powdered material was macerated at room temperature in an ethanol solvent with an ethanol-to-water ratio of 80:20. After 10 days the supernatants were filtered using Whatman filter paper No. 1 (Sargent-Welch, USA). The filtrate was then concentrated in a rotary vacuum evaporator at 40 °C to remove the ethanol. A dark brown semisolid crude extract (40 g) was obtained and then stored at 4 °C until further use (6).

2.3. Fractionation
A crude extract (35 g) was suspended in 250 mL of distilled water and subsequently partitioned with petroleum ether (3 × 100 mL), chloroform (3 × 100 mL), carbon tetrachloride (3 × 100 mL), ethyl acetate (3 × 100 mL), and methanol (3 × 100 mL). The resulting fractions were concentrated by rotary vacuum evaporator and dried to obtain fractions of petroleum ether (5 g), chloroform (6 g), carbon tetrachloride (5.5 g), ethyl acetate (4 g), methanol (8 g), and water (6 g), respectively. The fractionated solid masses of all the solvents were stored at 4 °C for further processing (7).

2.4. Test organisms
For antibacterial studies, 5 strains of gram-negative bacteria (Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Shigella flexneri, and Salmonella typhi) and 2 strains of gram-positive bacteria (Bacillus subtilis and Staphylococcus aureus) were used. Candida glabrata, Aspergillus niger, Trichophyton rubrum, Candida tropicalis, and Aspergillus fumigatus were used for antifungal studies. All microorganisms were obtained from the Department of Microbiology, Gomal University, Dera Ismail Khan, KPK, Pakistan. Bacteria and fungi cultures were maintained in their appropriate growth medium at 4 °C throughout the study and used as stock cultures (7,8).

2.5. In vitro antibacterial bioassay
For assay of the antibacterial activity of the extracts, the agar well diffusion technique was used. Briefly, wells of uniform diameter (approximately 6 mm) were made in solidified sterile nutrient agar media contained in 9-cm sterile petri dishes using a sterile metallic borer. Bacteria inoculums that were 8 h old and contained 10^4 to 10^6 colony forming units per milliliter (CFU/mL) were spread on the surface of the nutrient agar media with the help of a sterile cotton swab. The entire surface of the growth media within the plate was streaked 3 times with the swab. On each streaking, the plate was turned at 60°. Different solvent extracts of T. dioica were then dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 10 mg/mL and sterilized by filtration with 0.45-µm Millipore filters. Thereafter, 0.1 mL of each test sample was added to designated wells. Two wells supplemented with imipenem and DMSO served as positive and negative controls, respectively. To allow diffusion of the samples, the plates were left at room temperature for 30 min and then incubated facing upward at 37 °C for 24 h. The antibacterial activity of the extracts was determined by measuring the zone of inhibition against the test organisms and calculated by the percentage of the antibacterial activities of each extract with reference to the positive control (6,9). All tests were performed in triplicate. The percentage activities of each sample were calculated using the following formula:

\[
\text{Activity percentage} = \frac{\text{Treatment growth inhibition (mm) - control growth inhibition (mm)}}{100} \times 100,
\]

where treatment growth inhibition (mm) is the zone of inhibition (mm) in the test sample and control growth inhibition is the zone of inhibition (mm) in the standard drug (positive control).

2.6. Antifungal activity
For antifungal study, each extract/fraction was resuspended in DMSO at a concentration of 10 mg/mL and then stored in a refrigerator until further used. All test samples were evaluated by means of the agar well diffusion technique. For the growth of fungus, Sabouraud dextrose agar (Difco) was used, containing a high concentration of glucose (40%). Sabouraud dextrose agar was prepared by mixing dextrose and distilled water and autoclaved at 121 °C for 15 min, and then 20 mL of the specified growth media, molten at 45 °C, was aseptically transferred into sterile petri dishes with a diameter of 9 cm. Each dish was inoculated by removing a 4-mm-diameter piece of inoculums from the 7-day-old culture of fungus. To record the number of colonies, fungi were suspended in saline. Counting was done by hemocytometer cell counting chamber (10). Once the media became hard, 6-mm wells were bored using a sterile cork borer; then 0.1 mL of each test solution with a final concentration of 10 mg/mL was placed in the designated wells and all plates were incubated for 72 h at 29 °C (11,12).

Two wells were supplemented with amphotericin B and DMSO as positive and negative controls, respectively. The humidity in the incubation room was maintained between 40% and 50%. The antifungal activity was determined by measuring the clear zone of growth inhibition in millimeters with reference to the positive control (13). The concentration of amphotericin B used as the standard drug was 0.2 mg/mL. All tests were carried out in triplicate.

2.7. Determination of minimum inhibitory concentration
Minimum inhibitory concentrations (MICs) of T. dioica extracts were determined based on a broth microdilution method in 96-microwell plates. Those extracts that showed significant antimicrobial activity against the test organisms were subjected to MIC testing. Crude extract and methanol, chloroform, and water fractions were
reconstituted in 5% DMSO, having the final concentration of 10 mg/mL. Two-fold serial dilutions of the extracts were then prepared in a sterile Mueller-Hinton broth to achieve a decreasing concentration ranging from 10 to 0.312 mg/mL (14). Each dilution was supplemented with 100 µL of the standardized inoculums for bacteria at 1 × 10^6 CFU/mL and 1 × 10^7 cell/mL for yeast. Broth wells containing standard drugs imipenem (in serial dilutions of 10 to 0.312 µg/mL) and amphotericin B (in serial dilutions of 0.2 to 0.00625 mg/mL) for antibacterial and antifungal study, respectively, were used as a positive control, while wells containing bacterial and fungal suspensions in sterile Mueller-Hinton broth (without plant extract) were used as negative controls. For bacteria, the microwell plates were incubated at 37 °C for 24 h, and for fungi at 28 °C for 48 h (15). After incubation, the MIC values were taken as the minimum concentration of the extracts in the well of the microwell plate that inhibited the growth of the test organisms when compared with the control (16). Both experiments were performed according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-A (17). The whole experiment was repeated 2 times.

2.8. Minimum bactericidal and fungicidal concentrations

The minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial agents that will prevent the growth of microorganisms after subculturing onto antibiotic-free growth media. For determining the MBC, a 100-µL aliquot was taken from the tube showing MIC and placed on an antibiotic-free Mueller-Hinton agar plate, spread thoroughly over the plate, and incubated at 37 °C for 24 h. After 24 h the plates were examined for the growth of bacteria to determine the concentration of the extract. The highest dilution (lowest concentration) that showed no single bacterial colony on solid medium was taken as the MBC (18).

To determine the minimum fungicidal concentration, a 100-µL aliquot was taken from the tubes that showed no growth, subcultured on Sabouraud dextrose agar plates that were free of antifungal drugs, and incubated at 29 °C for 72 h. After 72 h the plates were examined for the growth of colonies. Minimum fungicidal concentration was determined to be the test sample (extract) with the lowest concentration showing no visible growth on subculturing (19). Both of the above experiments were repeated twice.

2.9. In vitro cytotoxic bioassay

2.9.1. Hatching of brine shrimp eggs

Brine shrimp larvae have long been used for cytotoxic study because there is a positive correlation between brine shrimp and human cytotoxicity. Brine shrimp larvae are most commonly used to prescreen compounds for the potentiality of tumor activity and to predict pesticide activities in response to a broad range of chemical and pharmacological compounds. A rectangular dish (22 × 32 × 10 cm) was half-filled with a filtered brine solution of sea water prepared by dissolving 38 g/L sea salt (Sigma Chemical Co., UK) in distilled water. A plastic sieve (No. 20) was clamped to the dish to form 2 unequal compartments. The brine shrimp eggs (100 mg) were sprinkled into the larger portion of the dish and covered with aluminum foil, while the smaller portion of the dish remained open and exposed to light. The eggs were incubated to hatch for 48 h at 37 °C (7,20).

2.9.2. Preparation of stock solutions (crude extract/fractions) for brine shrimp testing

From each extract, 20 mg was dissolved in 2 mL of methanol separately, then packed into sterile glass vials of 5-mL capacity and used as stock solutions when required. From each stock solution we then transferred 5 µL, 50 µL, and 500 µL to the experimental vials (3 vials/concentration), making the final concentrations of the above doses equivalent to 0.01, 0.1, and 1.00 mg/mL, respectively. All of these vials were kept at room temperature for the evaporation of organic solvents.

2.9.3. Cytotoxic study against brine shrimp

From the brine shrimp solution, 2 mL was transferred to all of the vials (3 vials for each concentration and 1 vial each for positive and negative control). The negative control was left blank without any test compound, containing only brine shrimp solution (artificial sea salt solution), while the positive control contained brine solution plus the standard reference drug etoposide (Lianyungang Guiyuan Chem Pharm Co., China). After 48 h, the larvae (nauplii) were collected using a 150-mm Pasteur pipette (John Poulten Ltd., UK) from the open side of the rectangular dish and 10 shrimp were transferred to each vial, including the positive and negative controls. To all of these vials, more brine solution was added to increase the volume to 5 mL. The vials were incubated at 27 °C for 24 h under illumination. After 24 h the survivors were counted by using a dissection microscope, and the percentage mortality (M%) of each dose was calculated as compared with the control (6,20).

In this bioassay, the mortality of brine shrimp incubated in the test solution was recorded. Each experiment was repeated 2 times.

2.10 Statistical analyses

Median lethal dose (LC₅₀) was determined by using the probit analysis method with 95% confidence interval (21).

3. Results and discussion

Medicinal plants play a very important role in meeting the health needs in developing countries and may offer a new source of antibacterial and antifungal drugs. Over the past 20 years, there has been an increased interest
in the evaluation of natural products as sources of new antimicrobial, insecticidal, and phytotoxic agents (22,23).

*T. dioica* deserves more attention due to its biological activities. To investigate the antibacterial potential of the crude extract and fractionated samples of *T. dioica* leaves mentioned in Section 2 of this paper, the plant was screened against 5 strains of gram-negative and 2 strains of gram-positive bacteria by applying agar well diffusion techniques (24,25). The results indicate that crude extract and methanol fractions possessed moderate antibacterial activities of 41% and 44.82% against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, respectively, compared with the standard drug imipenem, while chloroform, ethyl acetate, and carbon tetrachloride extracts showed negligible activities against the standard strains of bacteria as mentioned above (Table 1; Figure 1). An inhibition zone of 18 mm or greater was considered to be significant antimicrobial activity. The results outlined in Table 2 summarize the antifungal studies of all the above-mentioned extracts, namely that the crude extract, and methanol and water fractions exerted significant activities at variable degrees against one or more of the tested fungi. Crude extract showed significant activity against *Candida glabrata* and *Trichophyton rubrum* and low activity against *Aspergillus fumigatus*. Chloroform fraction showed significant activity against *Candida glabrata* and *Trichophyton rubrum* and low activity against *Aspergillus fumigatus*. Methanol fraction showed moderate and significant activities against *Candida glabrata* and *Aspergillus fumigatus*, respectively. Water fraction showed significant activity against *Candida glabrata*, *Trichophyton rubrum*, and *Aspergillus fumigatus* (Table 2). Crude extract showed 75.86% antifungal activity against *Trichophyton rubrum* and 82.75% against *Aspergillus fumigatus* compared with the standard drug amphotericin B, while chloroform, ethanol, and water fractions showed 79%, 72%, and 76%, respectively, against the above-mentioned 3 types of fungi (Table 2; Figure 2). Parmar et al. studied the phytochemistry of *T. dioica* and isolated 2 flavones, 5,2',4'-trihydroxy-6,7,8-trimethoxyflavone (tamadone) and 5,7,2'-trihydroxy-6,4'-dimethoxyflavone (tamaridone), from the aerial parts of *T. dioica*. They isolated hexaicosyl-p-coumarate; gardenins A, B, C, and E; nevadensin A; and apigenin (26). Furthermore, they reported that gardenin B exhibited antiviral activity, which strongly supports our antimicrobial study. Table 3 displays results of the brine shrimp lethality bioassay to evaluate the cytotoxic activity of various extracts in vitro with doses of 0.01, 0.1, and 1.00 mg/mL, respectively. None of the extracts showed cytotoxicity.

Our previous study on *T. dioica* leaf extract and fractionated samples showed no cytotoxic activities against brine shrimp larvae, which supports our present study (6). Those extracts that showed antibacterial and antifungal activities were further studied by determination of MIC, as well as MBC and minimal fungicidal concentration, with application of a serial 2-fold dilution method according to NCCLS document M27-A (17). Regarding the bacterial study, the MICs and MBCs of crude extract against *Klebsiella pneumonia* and *Pseudomonas aeruginosa* were 1.25 and 5.00 mg/mL and 2.5 and 10 mg/mL, respectively (Table 4).

### Table 1. In vitro antibacterial activity of various solvent extracts of *Tamarix dioica* Roxb. compared to the reference standard drug imipenem (10 µg/mL).

<table>
<thead>
<tr>
<th>Plant extracts, 10 mg/mL</th>
<th>E. coli</th>
<th>K. pneum</th>
<th>P. aerug</th>
<th>S. flex</th>
<th>S. typhi</th>
<th>B. sub</th>
<th>S. aur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>-</td>
<td>12 ± 1.5</td>
<td>10 ± 0.5</td>
<td>-</td>
<td>7 ± 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PE</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>-</td>
<td>5 ± 1</td>
<td>5 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>8 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EtOAc</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MeOH</td>
<td>7 ± 0.5</td>
<td>13 ± 0.5</td>
<td>9 ± 0.5</td>
<td>7 ± 0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Std. drug</td>
<td>25 ± 1</td>
<td>29 ± 0.5</td>
<td>30 ± 0.5</td>
<td>30 ± 0.5</td>
<td>32 ± 0.5</td>
<td>31 ± 0.5</td>
<td>26 ± 0.5</td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Zone: mean ± SD for n = 3.
In the case of fungal study, MICs and minimum fungicidal concentrations of crude extract, chloroform, methanol, and water fraction against *Candida glabrata* were 2.5, 0.625, 2.5, and 0.312 mg/mL and 5.00, 1.25, 5.00, and 0.625 mg/mL, respectively, as shown in Table 4. The above-mentioned 4 extracts showed significant fungicidal activity against *Trichophyton rubrum* and *Aspergillus fumigatus*, as shown in Table 4. Their MIC values were 0.312, 5.00, 0.625, and 0.625 mg/mL and minimum fungicidal concentration values were 0.625, 10.00, 1.25, and 0.625 mg/mL, respectively.

Table 2. In vitro antifungal activity of various solvent extracts of *Tamarix dioica* Roxb. compared to the reference standard amphotericin B (0.2 mg/mL).

<table>
<thead>
<tr>
<th>Plant extracts, 10 mg/mL</th>
<th>Diameter of zone of inhibition (mm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. glab</em></td>
<td><em>A. nig</em></td>
</tr>
<tr>
<td>Crude</td>
<td>10 ± 1</td>
<td>8 ± 0.5</td>
</tr>
<tr>
<td>PE</td>
<td>9 ± 0.5</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>CHCl&lt;sub&gt;3&lt;/sub&gt;</td>
<td>23 ± 0.5</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>CCl&lt;sub&gt;4&lt;/sub&gt;</td>
<td>8 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>EtOAc</td>
<td>7± 1</td>
<td>-</td>
</tr>
<tr>
<td>MeOH</td>
<td>12 ± 0.5</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>22 ± 1</td>
<td>7± 1.5</td>
</tr>
<tr>
<td>Std. drug</td>
<td>29 ± 0.5</td>
<td>30 ± 1</td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Zone: mean ± SD for n = 3.
and 1.25 mg/mL, respectively (Table 4). In both of these experiments, imipenem and amphotericin B were used as standard antibacterial and antifungal drugs, respectively, as mentioned in Section 2.

The present investigation clearly reveals the antifungal nature of *T. dioica*, and so the authors suggest that *T. dioica* leaves should be further investigated for isolation and characterization of active compounds and should be exploited in the management of diseases caused by the aforementioned fungi in human systems.

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**References**


