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Research Article

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Antibacterial and Anatomical Analysis of *Calotropis procera* (aiton) Dryand from Different Areas of District Mirpur, Azad JAMMU and Kashmir, Pakistan

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Abstract This work presents antibacterial potential and anatomical investigation of *Calotropis procera* (Aiton) Dryand. Populations from different areas of Mirpur (Azad Jammu & Kashmir), Pakistan. In the research, three different areas (viz: Jarikus, Khaliqabad and Jatlan) were selected for population sampling (leaf parts) having no symptom of disease. Leaf powder was macerated in two solvents and tested against four pathogenic bacterial strains (*Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa*). The methanolic extract depicted highest zone of inhibition (ZI) values with 14.67±1 mm against *Pseudomonas aeruginosa* (PA) while aqueous extract showed good ZI (14±1 mm) against this bacterium. Extract from Jatlan sample showed highest ZI values for methanolic fraction against *Staphylococcus aureus* and least effect against *Bacillus subtilis* (13±1 mm). Against *E. coli* methanol showed 14.7±0.8 mm and aqueous showed 11±0.5 mm zone of inhibition. Against *Pseudomonas aeruginosa* methanol showed 12±0.76 mm and aqueous showed 12.7±0.5 mm zone of inhibition. Among all three sampled areas, methanolic leaf extracts of Jatlan area showed very good ZI activity with 19±1 mm against *Staphylococcus aureus* followed by extracts of Khaliqabad samples with 14.67±1 mm against *Escherichia coli.* Among aqeous extracts leaves of Khaliqabad area presented highest antibacterial potential (14±1 mm) agaisnt *Pseudomonas aeruginosa.* It was observed that maximum zone of inhibitions were showed by methanolic extracts as compare to aqueous extracts. The maximum zone of inhibition in methanolic extracts of Jatlan was 18.7 mm among all areas. An anatomical analysis was made to explore leaf phenetics and its impact on concentration of phytochemicals with subsequent variation analysis in antibacterial potential. It was found that plant leaf samples of Khaliqabad had highest stomatal index (SI) 538 and same was found best antibacterial activity against various strains of bacteria. SI of Jatlan 408.7 followed by samples of Jarikus with SI values of 242.8. It was confirmed that there is direct and positive correlationship between SI and ZI of leaves of plants collected from different localities. These findings depict that leaves of *Calotropis procera* (Aiton) Dryand have good antibacterial activity confirming the past ethnomedicinal folklore applications of the plant and this result findings can be used as source of novel drug development from such herbal therapeutic products.

Keywords *Calotropis procera*; Mirpur; Jaatlan; Bhimber; Antibacterial; Stomatal Index

Introduction

Man has been relying on plants for food and medication from time immemorial and he has been using plants to cure different diseases in different recipes forms. Many plants of Pakistan are being explored for their ethnopharmacological potential in last past decade. *Calotropis procera* (Aiton) Dryand (CPAD) is a medicinal plant and it belongs to family Asclepiadaceae (the milkweed family), distributed largely throughout the tropics but also in

warm temperate climates [1]. CPAD has been used in many Ayurvedic formulations like Arkelavana. The medicinal potential of CPAD has been known as medicure tonic in various traditional medicinal systems of world. The leaves of CPAD are said to be valuable as an antidote for snake bite, sinus fistula, rheumatism, mumps, burn injuries, diarrhea, somatic, body pain, skin diseases [2] and jaundice curing [3]. About three drops of latex are put on the snake bitten area and pressed downward to bleed poison out [4]. In present, use of anti-venoms from plants is reported among many indigenous communities but there is need to search more plant based poison inhibitors [5]. The improvement of drug resistance in pathogens against commonly used antibiotics has required a search for new antimicrobial substances from other sources; including plants [6]. Anatomical and antimicrobial studies of same species also give indication to see variation in anatomy and antimicrobial difference due to some external factors such air or soil pollution and internal characters variations [7]. The aim of the present study to explore antibacterial potential of leaf samples of different sites of Mirpur and to find correlationship between anatomical features and antimicrobial activity of *Calotropis procera*.

Materials and Methods

Plant leaf samples of *Calotropis procera* (Aiton) Dryand were selected from three sites of district Mirpur of Azad Jammu and Kashmir, Pakistan. Sampling sites were chosen on basis of their microclimate and soil texture with having different impact on morphology of plants. The healthy leaf samples were collected in triplicate form and immediately transferred to lab for shadow drying and subsequent processing. The plant samples were also collected for preparation of herbaria for future reference and these were deposited in the herbarium of department of Botany, Mirpur University of Science and Technology (MUST) Bhimber Campus Bhimber Azad Kashmir.

Extraction: The dried leaves were powdered and processed for extraction dipping in solvents (methanol and water for seven days in subsequent pattern). The extract was vacuum dried and then dissolved for further testing.Maceration was the process used to divide the different ingredient and component of the plants in the form of crude extract. All samples were macerated by two solvents. Maceration was done with methanol and distilled water. In this process each weighted crushed medicinal plant 10g was dissolved in 100 ml of solvent. These plant extracts were kept at room temperature for 7 days . After that each plant extract was filtered with what man filter paper. Filtered paper extract was kept to dry at room temperature for 2 days, then plant's extract were preserved for further process [6, 8].

Test Organisms: Four pathogenic bacteria *viz*: *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli* and *Pseudomonas aeruginosa* were obtained from Deptt of Biotechnology (MUST) Mirpur for analysis of experiment. The strains were sub-cultured on nutrient agar and incubation was done at 37 ˚C for 24 hours before use.

Antibacterial Analysis: The culture plates seeded with test organisms were allowed to solidify and punched with sterile cork borer (0.4 mm diameter) to make open wells. The open wells were filled with 100 µl of the extract. The plates were incubated at 37˚C for 48 hours. The zone of inhibition (ZI) was measured for all solvent extracts against four strains of bacteria. ZI values were observed by naked eye or using microscope and measured in millimeter (mm) following [9].

***Leaf Epidermal Anatomy*:** For anatomical studies fresh leaves of plant were washed and air shortly dried. The leaves were boiled in lactic acid and peeled off irrelevant materials and epidermal parts were observed for analysis [10]. The distributions of stomata on lower surfaces of the leaf were studied by removing the peels of the leaf from the lower epidermis and observing under microscope. The count of the number of stomata and epidermal cells in the microscopic field were taken and the stomatal index of each surface of the leaf was calculated using the following formula:

$$\text{Stomatal Index (SI)} = \frac{\text{No. of stomata}}{\text{Cell per unit area + No. of Stomata}} \times 100$$

Results and Discussion

In present research antimicrobial and anatomical analysis of *Calotropis procera* (Aiton) Dryand have been conducted from Mirpur (Azad Jammu & Kashmir). Different areas have been selected in order to show climatic

variation and or any other factors on anatomy and phytochemicals concentration. The areas which have been selected for study are Jarikus, Khaliqabad and Jatlan. Remarkable variations have been observed during antibacterial and anatomical research of leaf of *Calotropis procera* different areas of Mirpur (Azad Kashmir).

As plants are the best indicator of pollution and other environmental fluctuations. Those plants that possess high medicinal values, endure a lot abiotic stresses from the environment and then these have impact on morphology and internal features of plants as well and plants physiology do behave accordingly.

The plant *Calotropis procera*'s leaf has been tested for antibacterial potential in this analysis. In first part of experiment, the plants' leaves were extracted in two solvents viz: methanol and water. There were four bacteria: *Bacillus subtilis*, *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa* used for this experimental trial. The zone of inhibition (ZI) of leaf extract of *Calotropis procera* against *Bacillus subtilis*, *Staphylococcus aureus*, *E. coli* and *Pseudomonas aeruginosa* was determined. The ZI was determined for three population samples and record of each was tabulated with geographic names separately (Tables 1-3). Methanolic extracts of Khaliqabad area samples showedhighest ZI (14.67±1 mm) agaisnt *Escherichia coli* and least ZI 12±1mm against *Pseudomonas aeruginosa.* Similar type of research reports have been published in past by Kareem *et al*., [9] and our results are inline with his conclusions. The aqueous showed 12.34±0.58 mm zone of inhibition against *Bacillis subtilis*. Against *Staphylococcus aureus* methanolic extract showed 14.3±1 mm and aqueous showed 12±1 mm zone of inhibition. Against *E. coli* methanolic extract shows 14.7±1 mm and aqueous shows 13±1 mm zone of inhibition. Against *Pseudomonas aeruginosa* methanolic extract shows 12±1 mm and aqueous shows 14±1 mm zone of inhibition. These findings are in coincidence with previous work of Ajaib and other researchers on *Calotropis procera* and other local plants of Azad Kashmir [11].

Table 1: Antibacterial Activity (Zone of Inhibition) of Leaf Extracts of *Calotropis procera* (Aiton) Dryand Collected from Khaliqabad Site

		Zone of Inhibition (mm)			
Sr. No.	**Solvents**	***Bacillus subtilis***	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***
1	Methanol	12±1	14.34±1	14.67±1	12±1
2	Aqueous	12.34±0.58	12±1	13±1	14±1

The leaf samples of Jatlan site extracted in methanol and water solvents do depicted that highest ZI (19±1 mm) was found for methanolic extract agaisnt *Staphylococcus aureus*, followed by ZI (18.7±0.58 mm) agaisnt *Pseudomonas aeruginosa*. In second solvent it was found that water extract had best ZI with 13±1 mm against *E. coli*. Similar findings were reported by past worker like Basu*et al*., in 2009 [12].

Table 2: Antibacterial Activity (Zone of Inhibition) of Leaf Extracts of *Calotropis procera* (Aiton) Dryand collected from Jatlan Site

		Zone of inhibition (mm)			
Sr. No.	**Solvents**	**Bacillus subtilis**	**Staphylococcus aureus**	**Escherichia coli.**	**Pseudomonas aeruginosa**
1	Methanol	13±1	19±1	15.3±0.58	18.7±0.58
2	Aqueous	10.7±0.58	11±1	13±1	11±1

The samples collected from Jarikus area were macerated in methanol and water for extraction. It was found that highest values of inhibition (14.7±0.58 mm) of bacterial growth was for methanol fraction against *Escherichia coli* and least readings (12±1 mm) were for *Bacillus subtilis.* In aqueous extract, maximum ZI was found to be 12.7±1.52 mm against *Pseudomonas aeruginosa* and least for *Bacillus subtilis* with 10.3±0.58 mm values.

Table 3: Antibacterial Activity (Zone of Inhibition) of Leaf Extracts of *Calotropis procera* (Aiton) Dryand Collected from Jarikus Site

		Zone of inhibition (mm)			
Sr. No.	**Solvents**	***Bacillus subtilis***	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***
1	Methanol	12±1	14.3±0.58	14.7±0.58	12±1
2	Aqueous	10.3±0.58	12±1	11±1	12.7±1.52

Anatomical Studies of Leaf of *Calotropis procera*

In second part of this experiment leaf anatomy of the selected plant was determined. The leaf epidermis of leaf was separated and observed under microscope for calculation of stomatal index (SI) of lower epidermis. All the plants samples contained variety of stomatal index. Generally tetracytic as well as paracytic stomata were observed in leaf epidermis of same plant from all three zones of sampling sites. *Calotropis procera* of Khaliqabad contained highest stomatal index with 538 values and this might be due to high water content in soil of the area due to water penetration and seepage from Mangla dam banks and nearby canal outlets [Table 4-6; Fig 1-3). Leaves of *Calotropis procera* of Jatlan area contained stomatal index of 408.7 that might be be due to sandy soil land texture and inflow of canal from this area yet makes it second good place for growth of sampled plant. There was least number of stomata was found in samples of populations of Jarikus area and its was value of SI was 242.8 which depictes that this area is sandy with gravel in it and it contains comparatively less water reservoir under soil as compared with other two sampling sites. In another parameter surveyed, it was found that number of stomata per unit area in *Calotropis procera* leaf from different areas were also different. The number of stomata per unit area in *Calotropis procera* of Jatlan were 39, followed byKhaliqabad area with 38 value and last one was area of Jarikus with 21 value for stomata per unit area (Fig 1, 2). This proves that number stomata vary due to land composition, hydrological table in soil and climatic factors changes. These type of research findings have been reported by previous workers [13, 14], who claimed that environmental disturbances do change the morphology and physiology of plants of any area.

In other parameter, the size of stomata in different areas of *Calotropis* was also different. The size of stomata in *Calotropis procera* leaf for samples of Jarikus was 0.328 whereas the stomata size of *Calotropis procera* of Jatlan and Jarikus were 1.16 and 0.6, respectively (Fig 3) and these values prove that if there is scarcity of water in an area then stomata size is less as shown in above case. Epidermal cells of leaf also showed variation in size and largest size of epidermal cell was 0.660 whereas small size of epidermal cell was 0.133 (Figs 1,2,3). These changes in SI of plant are also demonstrated by previous researchers where they were of opine that pollution and other climatic stresses do impart impact of anatomy and phytochemical profiling of plants [15]. Our research findings also do support the idea and views of above cited researcher.

Leaf epidermal abaxial surface of *Calotropis procera*(Aiton) Dryand. of Jarikus: Size oflarge epidermal cell was 0.1 µm , Size of small epidermal cell was 0.06 µm , Stomatal size was 0.3 µm , No. of stomata were 21 and Stomatal index was 242.8 mm.

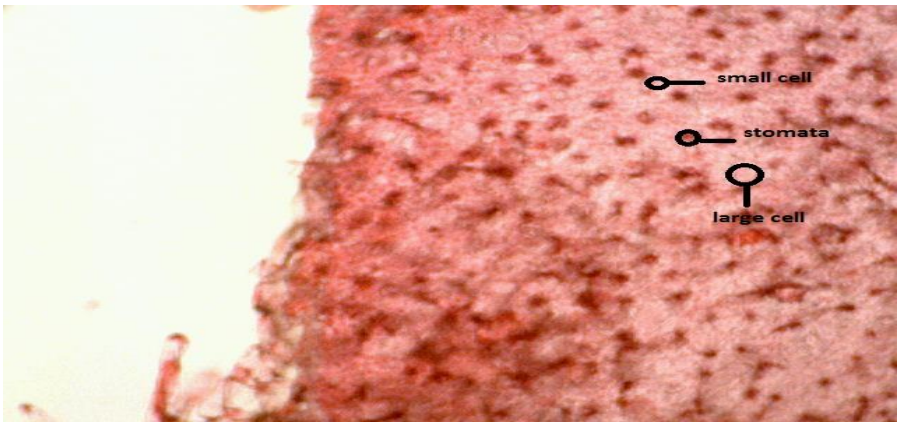

Figure 1: Leaf epidermal abaxial surface of Calotropis procera (Aiton) Dryand. of Jarikus

Table 4: Leaf Epidermis Profile of *Calotropis procera* from Samples of Jarikus

Calotropis procera	*Large cell (µm)*	*Small cell (µm)*	*Stomata size (µm)*	*No. of Stomata*	*Stomata per unit area (mm²)*	*Stomatal/ index (mm)*
	0.1	0.06	0.3	21	9.47	242.8

Leaf epidermal abaxial surface of *Calotropis procera* (Aiton) Dryand of Jatlan: Size of Large epidermal was 0.6 µm, size of small epidermal cell was 0.2 µm, stomatal size was 1.1 µm,no. of stomata were 39 and stomatal index was 408.7 mm.

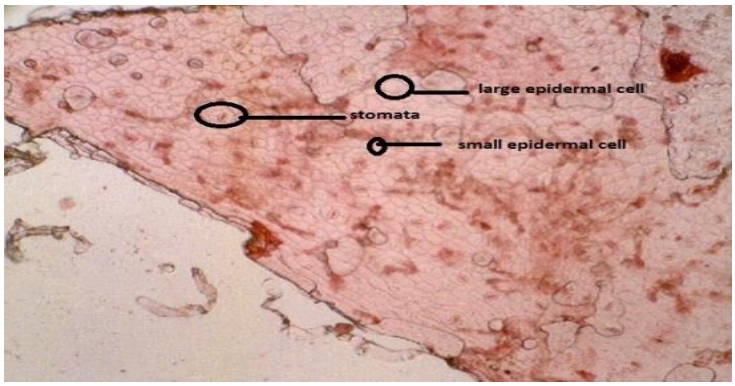

Figure 2: Leaf epidermal abaxial surface of Calotropis procera (Aiton) Dryand of Jatlan

Table 5: Leaf Epidermis Profile of*Calotropis procera* from Samples of Jatlan

Calotropis procera	Large cell (µm)	Small cell (µm)	Stomata size (µm)	No. of Stomata	Stomata per unit area (mm²)	Stomatal index (mm)
	0.6	0.255	1.1	39	10.55	408.7

Leaf epidermal abaxial surface of *Calotropis procera* (Aiton) Dryand of Khaliqabad: Size of large epidermal cell was 0.5 µm,size of small epidermal cell was 0.1 µm,stomatal size was 1.2 µmandnumber of stomata were 38 and stomatal index was 538 mm.

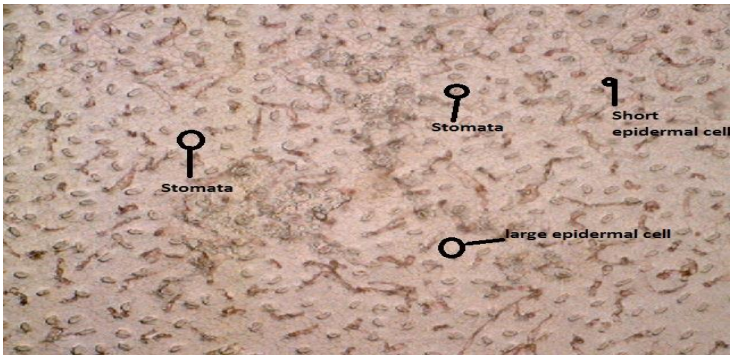

Figure 3: Leaf epidermal abaxial surface of Calotropis procera (Aiton) Dryand of Khaliqabad

Table 6: Leaf Epidermis Profile of *Calotropis procera* from Samples of Khaliqabad

Calotropis procera	Large cell (µm)	Small cell (µm)	Stomata size (µm)	No. of Stomata	Stomata per unit area (mm²)	Stomatal index (mm)
	0.5	0.1	1.2	38	7.6	538

Our research findings are inline with work of Mirta*et al*., [16] who said that environmental factors like temperature, water, height and light do exert impact on structure and morphology of organisms particularly plants, being sedentary. These findings that leaf epidermal features are variable from area to area are due to climatic impact on anatomy of leaf and then leaf is most active and the most sensitive part of plant. This was also supported by past work of Paliwal*et al*., [13] who provided details of stomatal changes and types due to climatic variations.

Conclusion

It is concluded that *Calotropis procera* is a good medicinal plants and it has good antibacterial potential and it can be used for drug development by pharmaceutical industries. In the other paradigm it was concluded that type of soil

and water content of area do have impact on plant morphology and physiology. It was finally concluded that phytomedicinal values of plants do change due to change in anatomy culminated into change by climatic variations.

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