

Effect of leaf and stem extract of *Rhazya stricta*, calotropis, paper mulberry and ricinus on control of bacterial blight of pomegranate

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Abstract

Pomegranate blight is a serious disease caused by the bacterium *Xanthomonas axonopodis* pv. *punicae*. The aim of this study was to evaluate the invitro activity of leaf and stem extracts of four medicinal plants, *Rhazya stricta*, *Calotropis procera*, *Morus alba* (black moss tree) and *Ricinus communis*, against bacterial blight pathogen. The extracts were prepared using solvent extraction and tested for infectivity using disk diffusion. The results showed that all the plant extracts showed different anti-microbial activities. Among the tested extracts, *R. stricta* and *C. procera* showed the

highest inhibitory effects on bacterial growth. These findings suggest that these plant extracts could be potential sources of natural antimicrobial agents for the management of bacterial blight of pomegranate. Further research is needed to evaluate the efficacy of these extracts under field conditions and to identify the bioactive compounds responsible for their antibacterial activity.

Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit, belonging to the smallest botanical family Punicaceae.¹ Because of its great health and nutritional properties, it is very popular among consumers. It is among the first five domesticated edible fruits (pomegranate, date palm, fig, olive and grapes). The plant was cultivated as early as 5000 BC.² It has been mentioned multiple times in the Holy Quran with the name of Al-Ruman. It is extensively cultivated in Afghanistan, Iran, China, India, Turkey, Egypt, USA, Pakistan and Mediterranean countries (Spain, Morocco, Turkey, Tunisia).³ India is the world's leading producer followed by China, Iran, Afghanistan, USA, Israel etc. Turkey has the largest export share, followed by Egypt, India, and Spain.⁴ Pomegranates may be planted up to 1000 meters above sea level. It shows good resistance to drought, salt stress, iron chlorosis, and active calcium.⁵ It can withstand temperatures as low as -10°C and as high as 45°C, although the finest fruit is produced around 38°C. It has been declared a super fruit globally due to the presence of vital compounds in it. Apart from the table consumption of pomegranate arils, the flowers, fruit rind and vegetative parts of pomegranate are also good source of secondary metabolites such as phenolic compounds, anti-oxidants, tannins, dyes, alkaloids, protein, carbohydrates, vitamins, minerals, dietary fiber, bioactive compounds and some other phyto-chemicals as compared to other fruits.⁵ The edible portion of this fruit has up to 68% moisture contents, phosphorus (70 mg/100 g), proteins (1.6%), calcium (10 mg/100 g), iron (0.3 mg/10g), riboflavin (0.10 mg/100 g) and vitamin C (16 mg/100 g) of fruit weight. Pomegranates are rich in antioxidants, antibacterial, antifungal properties, and hence touted as a "super food".⁶

Regarding the medicinal properties of pomegranate, the fruit has extensively been used as a traditional remedy against acidosis, dysentery, microbial infections, diarrhea, helminthes infection, hemorrhage and respiratory pathologies. Its seeds have also been shown to contain the estrogenic compounds, estrone and estradiol. Furthermore, the dried pericarp and the juice of the fruit are considered beneficial for treatment of colic, colitis, menorrhagia, oxyuriasis, headache, diuretic, acne, piles, allergic dermatitis, and treatment of oral diseases.⁷ Recent studies have shown new scientific investigations for the traditional uses of this fruit. Modern science has found that pomegranates can help protect your heart and may even prevent cancer. Pakistan is the 12th largest producer of pomegranates. However, its cultivated area and production have

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Key words: *Rhazya stricta*, calotropis, paper mulberry, Ricinus, bacteria, pomegranate.

Contributions: all the authors made a substantive intellectual contribution. All the authors have read and approved the final version of the manuscript and agreed to be held accountable for all aspects of the work.

Conflict of interest: the authors declare no potential conflict of interest.

Funding: none.

Ethics approval and consent to participate: not applicable.

Informed consent: not applicable.

Patient's consent for publication: not applicable.

Availability of data and materials: all data generated or analyzed during this study are included in this published article.

Received: 19 March 2025.

Accepted: 2 September 2025.

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Infectious Diseases and Herbal Medicine 2025; 6:653

doi:10.4081/idhm.2025.653

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been steadily declining since 2000. The total area of pomegranate fruit production in Pakistan during 2010 was 12952 ha with annual production 49997 tons (6.97 tons/Ha) which got decreased to 9434 ha during 2015 with annual production 42641 tons (6.76 tons/Ha). Currently, the fruit is produced on 7330 hectares in Pakistan, with an output of 37.6 thousand tons per year. Baluchistan province contributes the maximum (72%), followed by Punjab (21%) and Khyber Pakhtunkhwa (3%). In Punjab, various districts like Rahim Yar Khan, Bahawalpur, Multan, Khanewal, and Vehari account for up to 80% of the total production of Punjab.⁸

Pomegranate production is associated with many problems like long dry spells, non-availability of suitable varieties, climate changes, nutritional deficiencies, physiological disorders, post-harvest losses, pests and diseases. Apart from some important fungal diseases of pomegranate, the fruit crop is severely attacked by bacterial blight (*Xanthomonas axonopodis* pv. *punicae*). To manage biotic diseases caused by both fungi and bacteria, 25-30% of the total input is consumed. The disease was noticed for the first time in India in 1952 while in Pakistan it was reported in 1995. Symptoms were characterized by dark brown, angular to irregular shaped spots on leaves and fruits, canker on stems, branches and trunks, and split trunks. Bacterial Blight (BB) caused by *Xanthomonas axonopodis* pv. *punicae* (*Xap*) is a major problem in pomegranate cultivation, leading to extensive yield losses of up to 60-80%. In recent years, total crop production has been severely compromised due to BB infection.⁹ Benagi and Kumar (2009)¹⁰ recorded annual yield loss as high as 1.8 lakh tons during 2007–2008 accounting for revenue loss of about Rs. 200 crores in India. BB disease was a minor threat when it was first reported initially, but now it has become an epidemic infecting all the major areas of pomegranate cultivation and hampering production in India.⁹ The disease has also been reported from Turkey, and South Africa.¹¹⁻¹³ Being environment-friendly, and in many cases a good alternate to chemicals, plant extracts are used for their antagonistic activity against several plant pathogens. Some of the plants which have been demonstrated to be effective against various plant pathogens, include *Azadirachta indica*, *Osmium sanctum*, *Allium sativum*, *Curcuma longa*, *Withaniacoagulans*, *W. somnifera*, *Adhatodavasicca*, *Xanthium stromarium*, *Rhazya stricta* etc. We also wanted to explore some of these plants such as *Rhazya stricta*, calotropis, ricinus, and paper mulberry, to control BB of pomegranate to achieve the following objectives.

Materials and Methods

Collection and isolation of diseased pomegranate fruits

A visit to the pomegranate orchard of ARI Tarnab, Peshawar was made to collect the infected fruit and leaves samples. The fruits and leaves showing typical symptoms like yellowish brown spots, discoloration, minute water-soaked lesions, cankers lesions and irregular yellow and brown patches were collected. The collected samples were transported back to the Plant Pathology Laboratory, The University of Agriculture, Peshawar. All the samples were stored properly for further processing to isolate the pathogen involved in causing the disease in pomegranate. To isolate the pathogen, the infected portion (1 cm) was removed from the fruit and leaves and was surface sterilized using mercuric chloride solution (0.1%) for one minute followed by washing thrice with sterile saline water. After washing, the sample was crushed with the help of sterilized scalpel to get a suspension in a sterile test tube containing 3 ml of sterilized saline water. The suspension

was serially diluted and streak onto sterilized Petri dishes containing nutrient agar media. Ten inoculated plates were incubated at 30°C for 72 hours. After the incubation, plates were carefully observed to identify the colonies of *Xcp* based on typical colony morphological characters, (light, yellow-colored bacterial colonies resembling *Xanthomonas* sp.) followed by streaking of the pure culture on the NA plates. Streaked plates were incubated at 28°C for 48 h for the multiplication of isolated bacteria. Identification and characterization of the bacterial blight pathogen was carried out by subjecting the bacterial isolates to various biochemical tests, such as potassium hydroxide (KOH) solubility test, and catalase tests and pathogenicity test.

KOH test (3%)

Two drops of potassium hydroxide were placed on a glass slide. A colony of culture was picked up from the medium with the help of inoculating needle and mixed with KOH drops for 10 seconds. After thorough mixing the needle was raised 0.5 to 2 cm to confirm the formation of thread.

Catalase test

Using a sterile inoculating loop, small amount of culture was collected from a well isolated 18- to 24-hours incubated colony and was placed on the slide. A drop of 3% H₂O₂ was transferred onto the bacterial culture using a dropper. On mixing the culture and hydrogen peroxide. The mixture was observed for bubble formation (O₂ + water = bubbles) against a dark background that enhances readability.

Pathogenicity test

Three plants were selected to perform the pathogenicity test. Care was taken to select the newly matured plants with no disease on it. Leaves and fruits were selected, and injuries were made with the help of sterile needle. Leaves and fruits were inoculated with fresh bacterial culture using a swab. Two injuries were made for negative control without inoculating it with the bacterial culture.

Lower surface of leaves was pricked with sterilized needle at multiple (5-10 numbers) points. Bacterial inoculum (2x10⁸ cfu/ml population) was placed on the pricked area by placing a drop of cell suspension and using the cotton swab, presoaked in inoculums, it was rubbed to facilitate inoculation. The inoculated leaves and fruits were covered with plastic bags after placing a moist tissue with these to provide humid conditions for easy growth and inoculation of the bacteria. Observation was made for the symptoms after 24 and 48 hrs. The organism was re-isolated from artificially inoculated plant to get the organism having similar characteristics to one that was used during the initial inoculations satisfying all the conditions required to establish that the disease was bacterial borne and was caused by the pathogen *Xanthomonas axonopodis* pv. *punicae*.

Preparation of plants extracts

Previously reported plants (*Rhazya stricta*, calotropis, paper mulberry, ricinus) were selected for screening against the isolated pathogen. The extracts were obtained by soaking 10 g of each powdered sample (leaf, stem) in 100 ml of sterile distilled water. The suspension was left for 24 hrs with constant stirring using a magnetic stirrer. After 24 hrs, the suspension was filtered through muslin cloth and was stored at 4°C till its use. Four concentrations were made before using the extract to have final concentrations of 25%, 50%, 75% and 100%, using the sterile distilled water, for final application.

Antimicrobial activities of the plant extracts

Antimicrobial activities of the extracts at different concentrations were tested using agar diffusion test by making wells in the nutrient agar plate with the help of cork borer already inoculated with the pure bacterial culture. Wells of 2 mm diameter was prepared (using a cork borer) each properly labeled with the concentrations used and plant extracts (100 uL) were filled in wells. Streptomycin (2 mg/mL) was used as positive control. Inoculated plates were incubated at 30°C for 24 hrs and after incubation the inhibition zones were measured and compared with that of positive control. Each experiment was repeated two times.

Results

Isolation and purification

The isolates of *X. axonopodis pv. punicae* with pale yellow, mucoid, circular colonies were obtained on NA medium. These isolates were obtained from samples collected from the ARI TARNAB peshawar. The isolates of *X. axonopodis pv. punicae* were purified from a single pure colony and used for further studies.

KOH test

Formation of slime threads or loop is an indication of being gram-negative because gram negative bacteria have relatively fragile cell walls, bounded by an outer membrane. *The X. axonopodis pv. punicae* were showed to form mucoid thread after added KOH and found positive test.

Catalase test

Catalase enzyme mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. A small amount of bacterial inoculum was mixed with few drops of hydrogen peroxide solution (3%) and was observed for the rapid production of oxygen bubbles. The lack of catalase is evident by a lack of or weak bubble production. Catalytic activities of *X. axonopodis pv. punicae* were found positive, as vigorous oxygen bubble formation was observed within a minute when culture was mixed with H_2O_2 .

Pathogenicity test

The pathogenicity of *Xanthomonas axonopodis pv. punicae* was established on young and healthy plants of susceptible pomegranate. The plants treated with sterile distilled water served as control. Both inoculated and controlled plants were kept in polyhouse for development of typical symptoms of the diseases. The symptoms of the disease developed within 24 to 48 hrs after inoculation on leaves. First symptoms (after 24 hrs) appeared on lower sides of the leaves as irregular water-soaked spots. Later on, such spots appeared on the upper side of the leaves as well and were rough when touched, and were exhibiting yellow haloes around the lesions. Subsequently, the lesions turned brown in color and coalesced to form blighting appearance along with defoliation of the leaves. The control plants were free from such symptoms and no infection could be recorded. After regular and careful observations symptoms were of the bacterial blight were recorded in each treatment.

Plant extracts

Rhazya stricta, calotropis, paper mulberry, and ricinus exhibited antimicrobial activities against the bacterial pathogen in nutrient agar plate. The maximum zone of inhibition was shown by *Rhazya stricta* when 100% concentration of extract was used producing ZI measuring 23.8 mm after 48 hrs followed by *R. stricta* at 75% 22.2 mm after 24 hrs, 100% concentration of *R. stricta* after 24 hrs produced the zone inhibition measuring 22 mm. The streptomycin, being used as a positive control, produced the zone inhibition measuring 19.6 mm after 24 hrs and 20 mm after 48 hrs. There was no zone inhibition produced in case of paper mulberry used at 25% and *Ricinus* at 25% and 50%. The zone inhibition showed by the *Calotropis* used at 100 % measured 16.38 mm after 48hrs: which was also significant after the results of *R.stricta*.

Results

This research explored the effectiveness of natural extracts derived from four medicinal plants—*Rhazya stricta*, *Calotropis procera*, paper mulberry (*Morus alba*), and castor (*Ricinus communis*) in managing pomegranate bacterial blight, a serious disease caused by the bacterium *Xanthomonas axonopodis pv. Punicae* (Table 1).

The causative bacterium was isolated from diseased pomegranate samples obtained from an orchard in Tarnab, Peshawar.

Table 1. Efficacy of the different plant extracts.

Plant extract	Concentration	Time		Mean
		24 hrs	48 hrs	
<i>Calotropis</i>	25%	2.2 IJ	2.2 IJ	2.2 EF
	50%	6 GHI	9.74 FGH	7.87 D
	75%	11.4 EFG	14.46 CDEF	12.93 C
	100%	14.4 DEF	16.38 BCDE	15.39 C
<i>Rhazya stricta</i>	25%	4 HIJ	0 J	0
	50%	12.8 EF	19.4 ABCD	16.1 BC
	75%	22.2 AB	21.8 AB	22 A
	100%	22 AB	23.8 A	22.9 A
Paper mulberry	25%	0 J	0 J	0
	50%	1.8 IJ	0 J	0
	75%	1.4 IJ	1.6 IJ	0
	100%	4 HIJ	3.6 IJ	3.8 DEF
<i>Ricinus</i>	25%	0 J	0 J	0
	50%	0 J	0 J	0
	75%	2.2 IJ	0 J	0
	100%	4.6 HIJ	6.4 GHI	3.8 DEF
Control		19.6 ABCD	20 ABC	19.95AB
Mean		9.370 A	10.029 A	

Standard biochemical assays (KOH and catalase tests) and a pathogenicity test were used to confirm the identity and virulence of the isolated pathogen.

Leaf and stem extracts from each plant were prepared using water and evaluated at four different concentration levels (25% to 100%) for their ability to inhibit bacterial growth through an agar well diffusion assay. The antibiotic streptomycin served as a benchmark for comparison.

The results revealed a clear variation in antibacterial potency among the extracts (with a Least Significant Difference, LSD value of 0.05, and a critical value of 1.975). The most compelling activity was observed in *Rhazya stricta*, whose pure extract outperformed streptomycin by generating a larger inhibition zone. *Calotropis procera* also demonstrated notable antibacterial properties at higher concentrations. Conversely, the extracts from paper mulberry and castor plant showed minimal to no effect.

In conclusion, the findings indicate that *Rhazya stricta* and *Calotropis procera* contain potent antimicrobial agents and represent viable, natural candidates for controlling bacterial blight. The study suggests subsequent work should focus on isolating the active compounds and validating these results in field applications.

Discussion

Green plants are the major reservoirs of antimicrobial bioactive compounds which can be a major source for the development of natural pesticide. Therefore, many plants contain chemicals that may prove effective against bacteria, nematodes, and fungi. In our study, different plant parts and different doses of *Rhazya stricta* and *Calotropis* were tested to reveal their efficacy against *Xanthomonas axonopodis pv. punicae*. Very encouraging results were obtained especially in case of *R. stricta*.

Rhazya stricta Decne. is an important medicinal species that is rich in alkaloids containing anticancer alkaloids as well. Over 100 alkaloids have been isolated from *R. stricta*, but the pharmacological activities are known for only a few of these compounds. Very few ethno botanical studies of *R. stricta* have been done, and there is a dire need for further such studies in various regions of South Asia and in Middle Eastern countries where the species grows.¹⁴

Calotropis procera is also a valuable medicinal plant that contains many valuable phytochemicals such as glycosides (mostly cardenolides), flavonoids, triterpenes, alkaloids, steroids, saponins, proteins and enzymes.¹⁵

The presence of bioactive compounds is known to exhibit medicinal as well as physiological activities.¹⁶ Flavonoids are phenolic compounds and are reported to be synthesized by the plant in response of pathogen attack. They have the ability to make complexes with extracellular and soluble proteins. Flavonoids have also been reported to coagulate bacterial cell proteins and to affect enzymes involved in synthesis of essential amino acids.¹⁷

Tannins have anti-microbial properties due to their basic character. Tannins react with proline rich proteins and form stable water-soluble compounds. They also kill bacteria by directly damaging their cell membrane.¹⁸ Tannins bind to adhesins so that the bacteria cannot attach to the surface of the host and in this way, they remain unable to cause infection.¹⁹

Saponin is a broad group and has been classified into various categories on the basis of its function. Steroidal saponins are reported to inhibit bacterial growth, because they react with membrane sterol and stop membrane function which leads to inhibition of cell growth.²⁰ Alkaloids are also a vast group. Some alkaloids

inhibit important enzymes such as topoisomerase. Other alkaloids such as bisindole monoterpene alkaloids, act as DNA intercalating agent which sometimes result in certain kind of poisoning.²¹

Terpenoids are reported to inhibit bacterial growth by denaturation of proteins or by acting as dehydrating agents. Terpenoids act upon the phospholipid bilayers of the cell, due to which different processes like electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions are affected and finally membrane disruption occurs which results in bacterial growth inhibition.²²

Conclusions

Extracts from *Rhazya stricta* and *Calotropis procera* demonstrated strong antibacterial properties against the pathogen responsible for pomegranate BB during *in vitro* testing. The concentrated extract from *Rhazya stricta* inhibited bacterial growth more effectively than the antibiotic streptomycin. In contrast, preparations from paper mulberry and castor plant exhibited minimal antibacterial activity. The observed inhibition is likely due to natural bioactive components, including alkaloids and flavonoids, present in the effective plant species. These findings suggest that *R. stricta* and *C. procera* could serve as effective biological alternatives to conventional antibacterial treatments.

Recommendations

Further investigation should evaluate the performance of these plant extracts under actual field conditions. Development of commercially viable formulations derived from these plant extracts should be pursued. Comprehensive studies regarding the environmental safety and non-target effects of these botanical treatments are recommended. Integration of these natural treatments into comprehensive crop management programs should be considered.

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