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INHIBITION OF CYSTEINE PROTEASES AND ANTIBACTERIAL ACTIVITY OF RHIZOSPHERE-MICROBIAL ISOLATES OF *JUSTICIA WYNAADENSIS*

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ABSTRACT

Proteases are proteolytic enzymes secreted by most pathogenic microorganisms during their mode of entry into the host system. Many pathogenic bacteria such as *Staphylococcus aureus* produce proteases during infection and due to their emergence as antibiotic resistance, pose problems and threat for efficient control of bacterial diseases. To control such pathogenicity, mediated by the secretion of proteases, protease inhibitor of microbial origin from medicinal plant, *Justicia wynaadensis* plant-rhizosphere microorganisms were screened, to check their possible role in inhibiting the proteolytic activity and antibacterial effect against the pathogenic organisms such as *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas sp, and Escherichia coli.* Broth dilution assays were performed to check antibacterial activity.

KEYWORDS: Cysteine proteases, Inhibition, *Justicia wynaadensis*, Rhizosphere-bacterial isolates, Antibacterial, Broth dilution.

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INTRODUCTION

Medicinal plant-rhizosphere soil microorganisms constitute one of the largest reservoirs of biological diversity and are crucial to the functioning and for incorporating medicinal values. The region of soil surrounding and including the plant root is of crucial importance for plant health and nutrition. It has a high level of microbial activity, particularly because of nutrients secreted by plant roots in the form of soluble exudates as amino acids, organic acids and other photosynthates. It is a habitat for a vast interactive community of rhizotrophic microorganisms whose activities largely determine the physico-chemical properties of the rhizosphere soil. Many bacteria are intimately associated with plant roots. Several antimicrobial proteins derived from the rhizospheric soil inhibit growth of pathogens isolated from various sources[1]. *Justicia wynaadensis*, belonging to Acanthaceae family, is reported to be endemic in South India. A survey in and around the region with exclusive low temperature and unique weather condition of the Kodagu region and with more information among the local populace revealed that, the plant locally called *Maddhuthoppu* is believed to acquire the medicinal property during the Hindu calendar month of *Kataka* or *Adi* (July to August). The plant is believed to have maximum medicinal property when harvested on the 18th of this month (first week of August). The juice from the stem and leaves of this plant is extracted either by soaking in water or boiling in water. The deep purple colored extract thus obtained is consumed, generally as a sweet dish by the local community. This traditional practice is believed to keep the people healthy throughout the year. The total phenolic and flavonoid content has been investigated. The catalase and peroxidase activity revealed in fresh leaves could contribute to anti- oxidant activity of this plant[2, 3]. Further potential beneficial properties of flavonoids include antiviral, antiallergic, antiplatelet, anti inflammatory and antitumor effects have been investigated[4]. In the present study, prospecting the protease inhibitor in the microbial isolates of the rhizosphere soil-associated with the medicinal plant *Justicia wynaadensis,* prominently and exclusively grown in the study region is considered. Protease inhibitor plays essential role in biological systems regulating proteolytic processes and in defence mechanisms against insects and other pathogenic microorganisms. Several studies on protease inhibitors were published with the aim of investigating enzyme mechanisms of controlling disease and pathological processes[5, 6]. Protease inhibitors regulate the proteases which catalyze the hydrolysis of the peptide bonds forming the primary structure of protein irreversibly[7]. Proteases are expressed in varied amounts in organisms according to their physiological role in the given environment, [8-10]. With relevance to this, the pathogenic microorganisms are no exception. They specifically express and secrete proteases during their entry into host organisms, during infection, they find their way hydrolyzing the proteins, modulating the endogenous protease inhibitors leading to pathogenesis [11, 12]. At this point, there is a need to identify protease inhibitors from various reliable resources. One such resource is the microbial resources that are indigenous to a particular ecological niche. Henceforth in our attempt, in the isolation and identification of bacterial and fungal isolates from *Justicia wynaadensis*-rhizosphere soil, with cysteine protease inhibition activity by cell free culture extract of the isolates were performed. Concomitant antibacterial activity against the test pathogenic organisms viz., *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas sp, and Escherichia coli* were performed by broth dilution assay using the cell free culture extract of the isolates.

MATERIALS AND METHODS

All the chemicals and reagents used were of high grade obtained from Himedia chemicals. Cysteine protease and iodoacetamide are of analytical grade from Sigma chemicals, Bangalore India. The standard pathogenic microorganisms were isolated and maintained

in the laboratory of the Post graduate Department of Studies and Research in Microbiology, Mangalore University, Chikka Aluvara.

(i) Isolation of bacteria/fungi

One gm of the rhizospheric soil sample was diluted in 9ml of sterile distilled water and serially diluted to 10^{-6}. The dilution of 10^{-4} was used for inoculation. 0.1 ml was inoculated on Nutrient agar medium with pH 7.0, for bacteria and Sabouraud media for fungal colonies. The obtained colonies were then isolated and cultured into different preservation tubes containing Nutrient media and Sabouraud media. A loopful of isolated bacterial and fungal pure culture was inoculated into the Nutrient broth and Sabouraud broth. The bacterial broths were incubated for 24-48 hrs at 37°C, while fungal cultures were incubated for 7 days in a rotary shaker.

(ii) Cysteine protease-inhibition assay

About 0.1 ml of Cysteine protease enzyme in 0.3 ml of phosphate buffer (pH 7.2, 0.01 M) was taken in a reaction tube. The reaction was followed by adding 0.5 ml of substrate casein, incubated at room temperature for 30 min. After incubation, 1.5 ml of 5 % TCA solution was added and centrifuged at 9000 rpm for 5 min. Absorbance was taken at 280 nm. Test reaction tubes were maintained with appropriate volume of 0.1 ml enzyme pretreated with 0.1 ml of cell free culture extract obtained from bacterial and fungal samples. The obtained values were compared with 0.05 ml cysteine protease inhibitor iodoacetamide (100mM) and percentage of protease inhibition was calculated.

(iii) Staining and biochemical tests

Gram staining was performed and the shapes of the bacteria were studied. Lacto-phenol cotton blue staining was performed to analyze the fungal mycelium. For bacteria, Motility, MR-VP, Carbohydrate utilization, Catalase, Urease, Starch hydrolysis, Hydrogen sulphide, Gelatin hydrolysis, and Citrate utilization were estimated for identification.

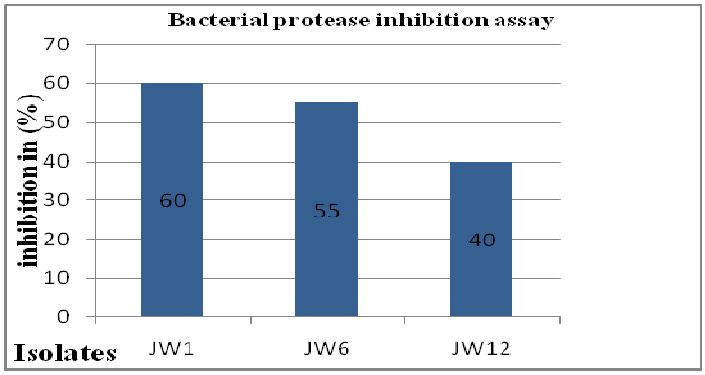
(iv)Antimicrobial activity by broth dilution method

The cell free culture extracts of each bacterial isolates showing positive for protease inhibition were examined for antibacterial activity against the test organisms viz., *Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas sp, and Escherichia coli.* Volumes ranging from 50-250 µl of the cell free culture extract were added into the culture medium with prior inoculation of the test organisms in a 20 ml broth culture. The growths of test organisms with or without cell free culture extract were recorded at 620 nm using *Labman* spectrophotometer.

RESULTS AND DISCUSSION

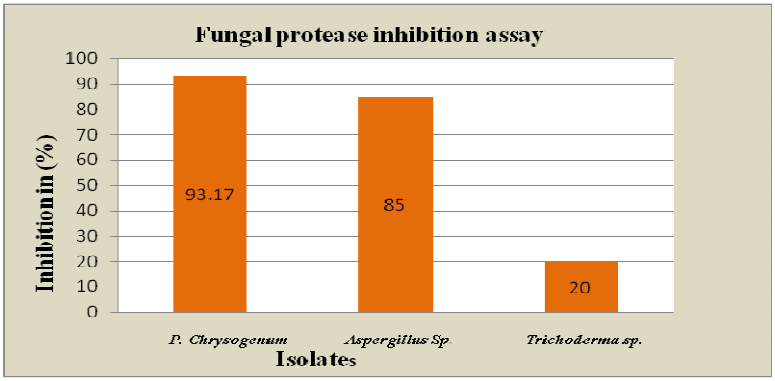
The enzyme assay for cysteine protease was carried out using the substrate casein incubated at 37°C for 30 min and the reaction was stopped by adding 5 % TCA. The product of the reaction was centrifuged and the hydrolyzed product was recorded as enzyme activity. The cell free extracts of the fungal isolates and bacterial isolates were checked for their protease inhibition activity by comparing the activity as percentage inhibition using 100mM iodoacetamide, an inhibitor of cysteine proteases in a reaction containing enzyme, buffer and incubation with the substrate. The bacterial isolates from *Justicia wynaadensis*-rhizosphere soil, prominent 15 colonies were selected and maintained. These isolates were cultured in nutrient broth and the cell free culture extract was examined for cysteine protease inhibition activity. Out of the fifteen bacterial isolates, the cell free culture extract of three bacterial colonies exhibited significant cysteine protease inhibition when assayed for enzyme activity. Initially the isolates were designated as JW 1 which showed an inhibition around 60 %, JW 6 showed 55 % inhibition and around 40 % inhibition was recorded for JW 12 isolate as shown in graph 1.

Graph 1
Cysteine protease inhibition by bacterial isolates



When examined for fungal cell free culture extract for inhibition activity, out of the six fungal isolates isolated from *Justicia wynaadensis*-rhizosphere soil, three fungal isolates showed significant inhibition of the cysteine proteases (Graph 2). The fungal isolates were identified as *Penicillium chrysogenum, Aspergillus Sp., and Trichoderma Sp. Penicillium chrysogenum* showed around 93.17 % inhibition of cysteine protease activity followed by 85 % inhibition by *Aspergillus Sp.*, and around 20 % inhibition was exhibited by *Trichoderma Sp.*

Graph 2
Cysteine protease inhibition by fungal isolates



Further, the bacterial isolates which showed cysteine protease inhibition activity were examined for antimicrobial effect against the test organisms selected. The broth dilution assay was performed by incorporating an increasing cell free culture extract into the growth medium that was inoculated with the respective test organism. At the maximum growth time of the logarithmic phase of the respective culture, absorbance was recorded along with control bacterial growth. The Gram staining and biochemical test results, in comparison with the standard manual for bacterial identification, the bacterial isolates JW1, JW6 and JW12 were identified as *Bacillus megatarium, Bacillus subtilis*, *Bacillus cereus* respectively.

Table 1
Colony characteristics of the bacterial isolates

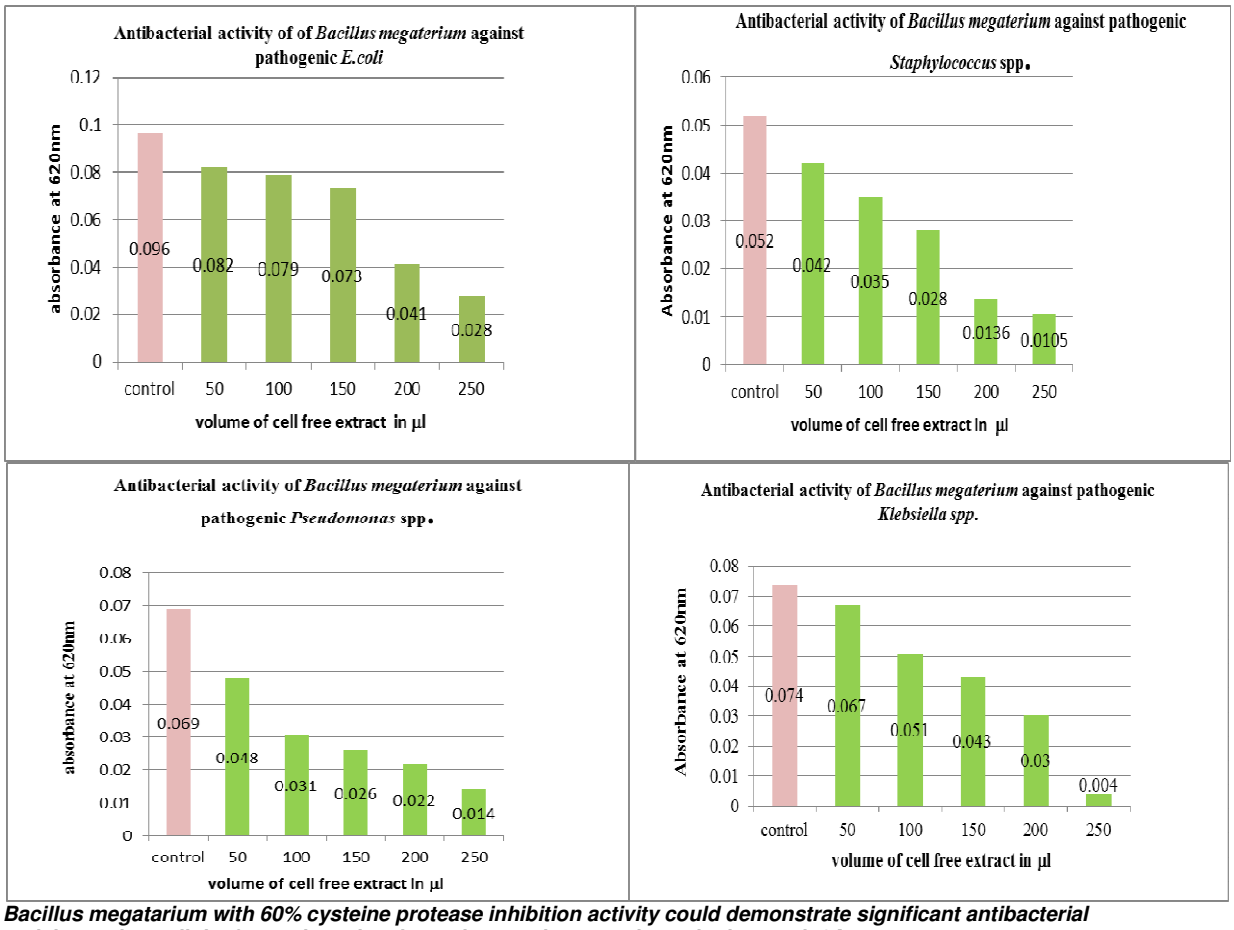
SL.NO	ISOLATE	COLONY MORPHOLOGY
1	JW1	Round, white, flat, opaque, dry, rough,
2	JW 6	Irregular, yellowish, flat, smooth, opaque.
3	JW12	Round, white, flat, slimy, transparent

Table 2
Biochemical tests for bacterial isolates

Isolate	Motility	Urea	Citrate	Starch	Gelatin	Methyl Red test	Voges Proskauer test	Catalase	Indole production
JW1	+	_	+	+	+	_	_	+	_
JW6	+	_	+	+	+	_	_	+	_
JW12	-	+	+	+	+	_	+	+	_

The cell free culture extracts of *Bacillus megatarium*, *Bacillus subtilis*, *Bacillus cereus* showed significant antibacterial effect against *E coli*, *Staphylococcus aureus, Pseudomonas sp*, and *Klebsiella pneumoniae* as shown in the graphs (3A, 3B, 3C) for each increase in the cell free culture extracts versus the absorbance reading since increase in the absorbance is taken as a direct measure of the bacterial growth. At maximum phase of selected pathogenic bacterial growth, the absorbance recorded at 620 nm were obtained when not treated with cell free culture extract of *Bacillus megatarium, Bacillus subtilis* and *Bacillus cereus.* Pathogenic cultures were maintained in different culture tubes as control and treatment groups. In treatment groups the cell free culture extracts of isolated bacterial isolates were incorporated in different volumes to analyze their effect on growth inhibition of pathogenic organisms.

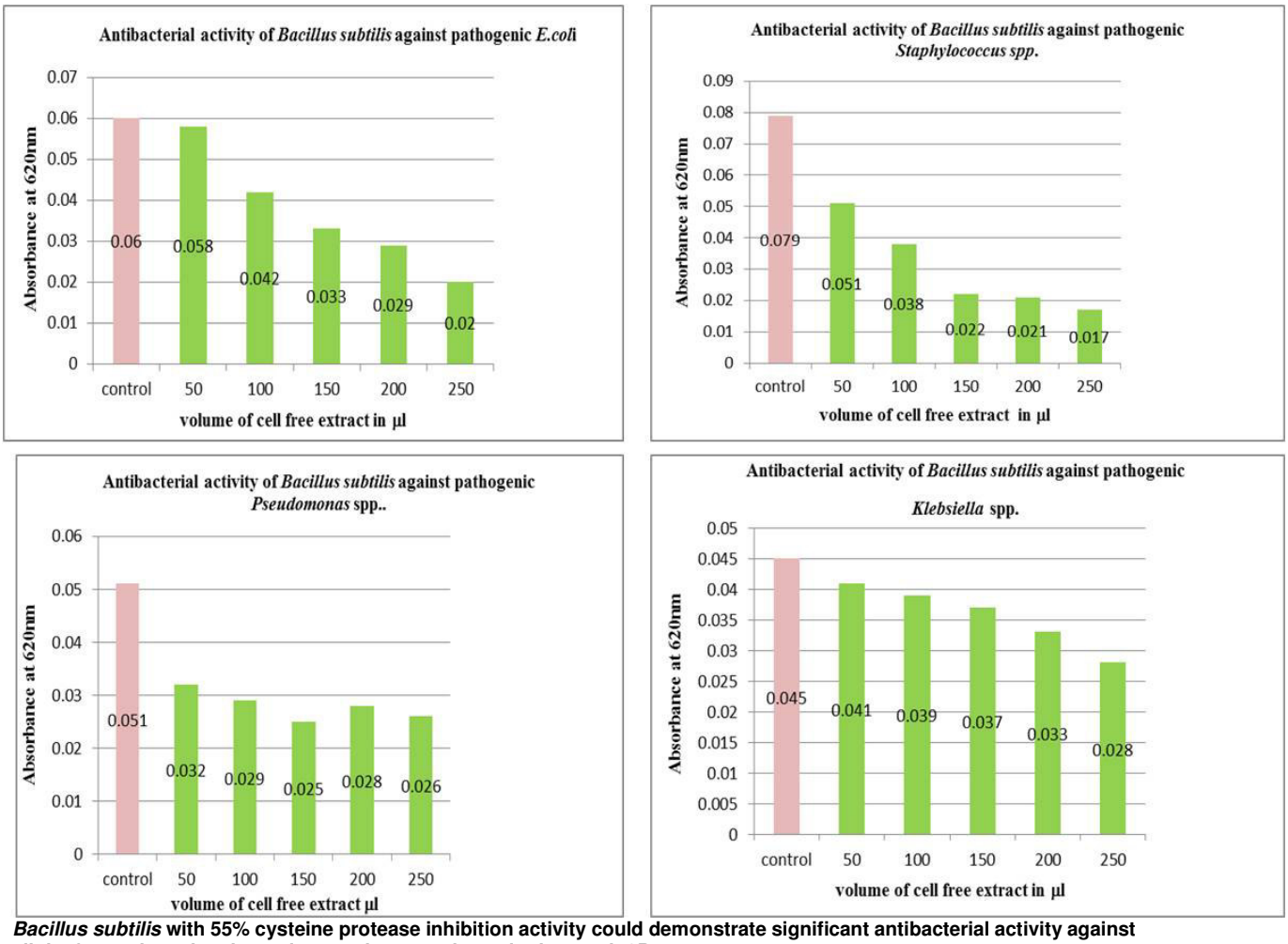
Graph 3A
Antibacterial activity of Bacillus megaterium against E.coli, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas spp.



Bacillus megatarium with 60% cysteine protease inhibition activity could demonstrate significant antibacterial activity against all the four selected pathogenic organisms as shown in the graph 3A.

Graph 3B

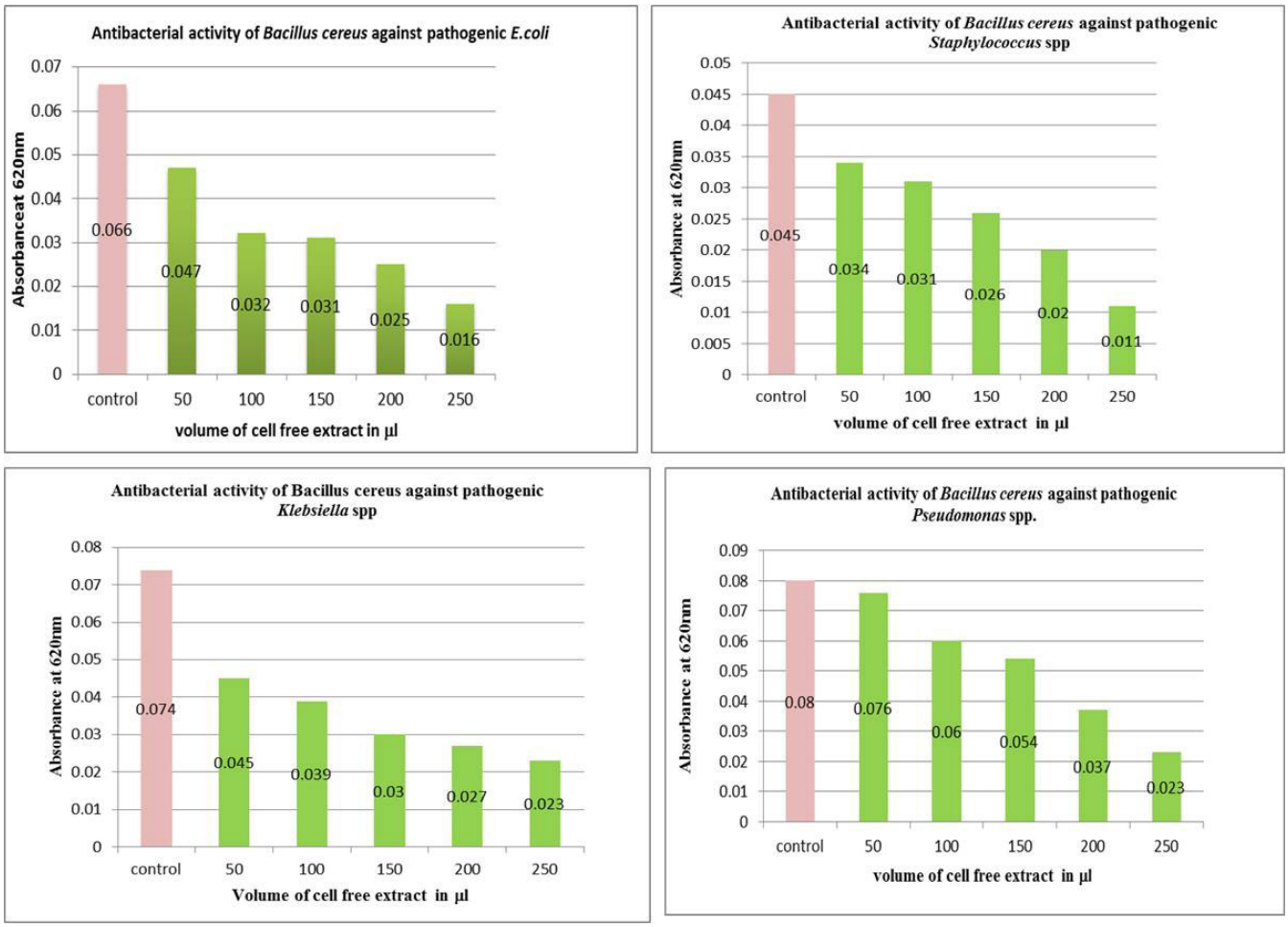
Antibacterial activity of Bacillus subtilis against E.coli, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas spp.



Bacillus subtilis **with 55% cysteine protease inhibition activity could demonstrate significant antibacterial activity against all the four selected pathogenic organisms as shown in the graph 3B.**

Graph 3C

Graphs showing antibacterial activity of Bacillus cereus against E.coli, Staphylococcus aureus, Klebsiella pneumoniae and Pseudomonas spp.



Bacillus cereus with 40% cysteine protease inhibition activity could demonstrate significant antibacterial activity against all the four selected pathogenic organisms as shown in the graph 3C.

Cysteine proteases are a class of protease enzymes expressed and produced by pathogenic bacteria such as *Staphylococcus aureus* and other pathogens [13]. These proteases are implicated during infection processes and required for establishing the pathogenic condition in the host organism. In this study, the cell free culture extracts of the bacterial and fungal isolates that were isolated from the rhizosphere region of the medicinal valued plant *Justicia wynaadensis* were analyzed for protease inhibition activity. Among the isolates, three bacterial and three fungal isolates exhibited cysteine protease inhibition. The bacterial isolates were identified as *Bacillus megatarium, Bacillus subtilis* and *Bacillus cereus.* The cell free culture extracts of these isolates were examined further for their antibacterial effect against the test organisms by broth dilution assay. The obtained results of cysteine protease inhibition activity and antibacterial activity can be correlated further with substantial evidence for inhibiting the protease activity that has been considered as one of the virulence factors during pathological conditions.

CONCLUSION

Microorganisms of rhizosphere soil of medicinal plants may prove to have immense capacity to carry out biochemical and physiological effect when appropriately applied to treat certain microbial infections. The

microbial isolates from the rhizosphere of the medicinal plant *Justicia wynaadensis* that has shown cysteine protease inhibition activity and antibacterial activity against the pathogenic bacteria offers significant scope for purification and characterization studies towards efficient practical applications.

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