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ISOLATION AND SCREENING OF ENDOPHYTIC BACTERIA FROM *RICINUS COMMUNIS* FOR ANTIMICROBIAL ACTIVITY

Pranay Jain* and Priyanka Sharma

Department of Biotechnology Engineering, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra, Haryana, India.

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***Correspondence for Author**
Dr. Pranay Jain
Department of Biotechnology Engineering, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra, Haryana, India.

ABSTRACT

The present study deals with the isolation of endophytic bacteria from the leaves, stem and beans of *Ricinus communis* plant and its screening for antimicrobial activity against twelve test pathogens *viz. Streptococcus mutans* (MTCC 497), *Streptococcus pyogenes* (MTCC 1924), *Staphylococcus aureus* (MTCC 7443), *B. megaterim* (MTCC 428), *Bacillus subtilis* (MTCC 121), *Escherichia coli* (MTCC 40), *Pseudomonas fluorescens* (MTCC 1748), *Candida albicans* (MTCC 227) *Candida tropicalis* (MTCC 3421), *Candida glabrata* (MTCC 3814), *Alternaria solani* (MTCC 10690) and *Fusarium graminearum* (MTCC 2089). Out of twelve test pathogens, the endophytic bacterial isolate inhibited the activity of eight test pathogens. The minimum inhibitory concentration of ethyl acetate extract of antimicrobial metabolite ranged from 12.207µg/ml to 25 mg/ml.

KEYWORDS: Endophytic bacteria, pathogens, antimicrobial activity, minimum inhibitory concentration.

INTRODUCTION

Various microorganisms residing in the internal tissues of the plants remain asymptomatic for at least a part of their life cycle. Such microorganisms are referred to as “Endophytes”. Endophytes isolated from the different plants, especially, the medicinal plants with established medicinal values are being evaluated for the compounds with antimicrobial properties.[1]

Ricinus communis is native to India. This indigenous medicinal plant is a member of the family Euphorbiaceae. This plant is traditionally called as castor oil plant (Arandi). Some of the ethnobotonical uses of this plant includes antitumour, antioxidant, anti-implantation, antiasthamatic properties. Antinociceptive and bone regeneration activity were also found associated with the castor oil plant. The extracts of *Ricinus communis* contains alkaloid (ricinine) and glycoside which may be useful for various herbal formulation as anti-inflammatory, analgesic, antipyretic, cardiac tonic and antiasthamatic etc.[2]

The present study was designed to evaluate the antimicrobial activity of the endophytic bacteria isolated from the leaf, beans and stem of the castor oil plant.

MATERIAL AND METHODS

Sample collection

Fresh and asymptomatic leaves from the *Ricinus communis* were cut with a sterile scalpel and were carried immediately to the laboratory in a clean plastic bag. All the samples were collected from the campus of Kurukshetra University, Kurukshetra, Haryana.

Isolation, purification and maintenance of endophytic bacteria

The sample was surface sterilized and cut into pieces of 1×1 cm^2. The sample pieces were then plated on nutrient agar plates and incubated at 37˚C for 48 hrs. After 48 hrs the bacterial isolates obtained were sub cultured on fresh nutrient agar plates and again incubated at 37˚C for 48 hrs. The pure cultures were then transferred to the nutrient agar slants. The bacterial slants were maintained at 4˚C in a refrigerator until further use.[3, 4]

Procurement and maintenance of test pathogens

The various test pathogens were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, which included Gram positive bacteria, *Streptococcus mutans* (MTCC 497), *Staphylococcus aureus* (MTCC 7443), *Streptococcus pyogenes* (MTCC 1924), *B. megaterim* (MTCC 428) and *Bacillus subtilis* (MTCC 121); Gram negative bacteria *Escherichia coli* (MTCC 40) and *Pseudomonas fluorescens* (MTCC 1748); fungal pathogens *Candida albicans* (MTCC 227), *Candida glabrata* (MTCC 3814) and *Candida tropicalis* (MTCC 3421). Two phytopathogenic fungi Alternaria *solani* (MTCC 10690) and *Fusarium graminearum* (MTCC 2089) were also obtained. The slants of brain heart infusion agar were made to preserve the cultures. All the slants were kept at 4ºC in the refrigerator for further studies.

Preliminary Screening for antimicrobial activity

The bacterial isolates were inoculated in nutrient broth and incubated at 37˚C for 48 hrs. The nutrient broths was then centrifuged and the supernatant was collected. The antimicrobial activity was then evaluated with agar well diffusion method. The inoculums of different test pathogens were adjusted according to 0.5 McFarland standards. 100µl of test pathogen was spread aseptically using cotton Swabs on the surface of Muller Hilton agar plates. Wells of about 6.0 mm diameter were aseptically punched in the agar plates using a sterile cock borer. 250µl of the supernatant obtained after centrifugation was transferred into each well. Plates were then kept in laminar Air flow for 30 minutes for pre-diffusion of metabolite to occur and then incubated at 37˚C for 24 hours. All the experiments were performed in triplicates and after 24 hours, the diameter of resulting zone of inhibition were measured in terms of millimeter (mm.) using a Hi-media zone scale.[3]

Solvent extraction and Determination of Minimal inhibitory concentration

The solvent extraction of the centrifuged broth showing the antimicrobial activity was performed using ethyl acetate. The ethyl acetate extract was dried and two-fold dilutions were prepared in DMSO (Dimethyl Sulphoxide). The minimal inhibitory concentration was determined using agar well diffusion method.[5, 6]

Determination of relative percentage inhibition

The relative percentage inhibition with respect to positive control was calculated by applying the following formula:[7]

Relative percentage inhibition of the test extract = $[100(X-Y))/(Z-Y)]$

Where, X = Total area of zone of inhibition of the test extract

Y = Total area of zone of inhibition of the solvent

Z = Total area of zone of inhibition of the standard drug

Total area of zone of inhibition was calculated according to area = πr^2, where, r = radius of the Zone of inhibition.

RESULTS AND DISCUSSION

Nature has healing properties. Plants have been viewed as a potential source of antimicrobial agents. Antimicobial compounds isolated from the microorganisms inhabiting the internal tissues of the plants are relatively new and unexplored area of research.[8] In the present study, an endophytic bacterium isolate obtained from *Ricinus communis* was screened for its antimicrobial activity against ten human and two plant pathogens by agar well diffusion

method. The presence of growth inhibition zones indicates the presence of antimicrobial agent in the filtrates of the endophytic bacterial strain. The centrifuged broth culture of the endophytic bacteria showed antagonism against seven of the twelve test pathogens. Both the gram negative test pathogens that were included in the study were inhibited by the endophytic bacteria while the antimicrobial activity of the endophytic bacteria was found against only two of the five gram positive test pathogens under examination. The values of zone of inhibition ranged from as high as 20±1.20 mm diameter against *Alternaria solani* (MTCC 10690) to as low as 11±0.33 mm diameter against *Bacillus subtilis* (MTCC 121).

Table1. Antimicrobial activity of an endophytic bacterial isolate.

Code of the sample	Sample part	Zones of inhibition											
		Test pathogens											
		Bacteria							Fungi				
		Gram-positive					Gram-negative		human pathogens			Plant pathogens	
		Bm	Bs	Sa	Sm	Sp	Ec	Pf	Ca	Cg	Ct	As	Fg
RIC. C. 4 LT & B V Crude extract		NZ	11±0.33 mm dia.	NZ	NZ	12±0.33 mm dia.	18±0.57 mm dia.	12±0.89 mm dia.	18±0.33 mm dia.	NZ	13±0.33 mm dia.	20±1.20 mm dia.	NZ
Positive control		48	35	NZ	39	38	40	50	NZ	NZ	NZ	NZ	NZ
Relative percentage inhibition		0	31.43	0	0	31.58	45	24	∞	0	∞	∞	0
Minimal inhibitory concentration													
RIC. C. 4 LT & B V Ethyl acetate exract		24.414 µg/ml	390.625 µg/ml	NZ	3.125 mg/ml	97.65 µg/ml	12.207 µg/ml	25 mg/ml	25 mg/ml	NZ	25 mg/ml	25 mg/ml	1.562 mg/ml

Values are mean inhibition zone (mm) ± S.D of three replicates

Legend: Sm: *Streptococcus mutans* (MTCC 497); Sa: *Staphylococcus aureus* (MTCC 7443); Sp: *Streptococcus pyogenes* (MTCC 1924); Bm: *B. megaterim* (MTCC 428); Bs: *Bacillus subtilis* (MTCC 121); Ec: *Escherichia coli* (MTCC 40); Pf: *Pseudomonas fluorescens* (MTCC 1748); Ca: *Candida albicans* (MTCC 227); Cg: *Candida glabrata* (MTCC 3814); Ct: *Candida tropicalis* (MTCC 3421); As: *Alternaria solani* (MTCC 10690); Fg: *Fusarium graminearum* (MTCC 2089); (-): No activity, positive control: fluconazole and ciprofloxacin.

The results showed that gram positive test bacteria were more resistant than the gram negative test bacteria. Hence, the secondary metabolite isolated from the endophytic bacteria showed inhibitory effects against a wide range of pathogens; Gram positive and Gram negative bacteria and unicellular and filamentous fungi. The relative percentage values ranged from zero to infinity. (Table 1)

The presence of bigger inhibition zones was indicative of either the higher concentration of antimicrobial agent or the good potential of the antimicrobial compound present against the

test pathogens. However, the presence of no inhibition zone nor smaller inhibition zones does not indicate the complete absence of antimicrobial activity or the lower antimicrobial activity against the test pathogen. The endophytic microorganisms which show low or lack of antagonistic activity may have active compounds in smaller amounts and/or the screened filtrate may yield better results after some purification. Concentrating the active principles and the removal of the contaminants could help in better estimation of the antimicrobial potential of the endophytic bacteria. The culture broth of the endophytic bacteria inhibited only seven test pathogens but when it was extracted with ethyl acetate and minimal inhibitory concentration was evaluated, it was found to exhibit antimicrobial activity against ten of the twelve test pathogens. It must be considered that the extracts which demonstrated no antagonistic activity against any test pathogens may have activity against any other pathogens which were not included in the study.[9]

The biological activities (MIC) of the antimicrobial agent indicates the broad spectrum action against the test pathogens where MIC of the gram positive test pathogens ranged from 24.414 µg/ml to 3.125 mg/ml and the MIC of both the gram negative bacteria was evaluated as 25 mg/ml. On the other hand, MIC values were much higher for the fungal pathogens; ranged from 1.562 mg/ml to 25 mg/ml. the Minimal inhibitory concentration value was almost constant for three of the fungal test pathogens. (Table 1)

The present study used standard antibiotic fluconazol as antifungal agent and ciprofloxacin as antibacterial agent. Neither the antibacterial drug ciprofloxacin nor the endophytic bacterial isolate could inhibit the growth of test pathogen *Staphylococcus aureus* (MTCC 7443). On the contrary, the endophytic isolate demonstrated much efficiency as antifungal agent as compared to the standard drug fluconazol which was unable to inhibit the any of the test pathogens (at a concentration of 1mg/ml). The endophytic bacteria inhibited the growth of two out of three opportunistic human pathogens and inhibited both the plant fungal pathogens. This highlights the possible potential of the endophytic bacteria in the biocontrol of the pathogenic fungi; *Alternaria solani* (MTCC 10690) and *Fusarium graminearum* (MTCC 2089) in agricultural fields. (Table 1)

CONCLUSION

The discovery of endophytes opened new doors for the drug development and drug discovery studies. Earlier, the researchers were largely dependent on the plant extracts for the isolation of bioactive compounds from natural source but now endophytes have emerged as a potent source for novel antimicrobial compounds. Endophytes derived from the indigenous

medicinal plants may be exploited for its pharmacological properties. There exists an ample of scope in investigations related to the role of endophytes in the therapeutic properties of the plants and *vice versa*. Extensive studies on the structural elucidation and on the mode of action of antibiotic compound needs to be performed in order to use the available resources with maximum benefits.

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