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Isolation and screening of endophytic actinomycetes producing antibacterial compound from *Citrus aurantifolia* Fruit

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ABSTRACT

Today more than 30,000 diseases are clinically described. Less than one third of these can be treated symptomatically and only a few can be cured. Actinomyctes is a potential producer of many antibiotics. Endophytic actinomycetes have been defined as that can be isolated from the disinfected surfaces of plant tissues or that can be extracted from within the plant that do not cause visible harm to the host It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, In our study we have isolated the endophytic actinomycetes from citrus fruit. The total 5 actinomycetes were isolated from citrus fruits (Citrus aurantifolia) using Starch Casein agar, Out of 5 actinomycetes strains, 2 actinomycetes showing antibacterial activity were recovered using Bennet agar media. The production of antibacterial compound was performed using L.B. Broth. Out of 2 actinomycetes strain only one show (CT1) shows the strong antibacterial activity and fermentation carried out using L.B. Broth for 15 days. After fermentation, extraction of the supernatant was carried out using solvent petroleum ether. The Antibacterial compound was recovered using TLC and Column Chromatography. The Rf value of the compound was found to be (0.6152). The structural study of the extracted compound was carried out by UV-spectroscopy, FT-IR. The antibacterial compound was effective against E. coli, S. typhi, K. pneumoniae, S. aureus bacteria some of them got resistance against some antibiotic drug.

Keywords: Endophytic actinomycetes, *Citrus aurantifolia*, Antibacterial activity, Structure elucidation.

INTRODUCTION

Endophytic actinomycetes have been defined as that can be isolated from the disinfected surfaces of plant tissues or that can be extracted from within the plant that do not cause visible harm to the host. They can promote the growth of many field crops by producing plant growth-promoting substances and by fixing nitrogen from the atmosphere and potential sources of novel natural products for exploitation in medicine, agriculture and industry. It is noteworthy that, of the nearly 300,000 plant species that exist on the earth, each individual plant is host to one or more endophytes.

There is a general call for new antibiotics, chemotherapeutic agents, and agrochemicals that are highly effective, possess low toxicity, and have a minor environmental impact. This search is driven by the development of resistance in infectious microorganisms (e.g., species of *Staphylococcus*, *Mycobacterium* and *Streptococcus*) to existing compounds and by the menacing presence of naturally resistant organisms. Most of the drug mainly produced by actinomycetes.

Name of Plants Consisting Endophytic Bacteria:

S.No.	NAME OF PLANTS
1	*Agropyron elongatum* (tall wheat grass)
2	*Agropyron intermedium* (intermediate wheat grass)
3	*Allium porrum*
4	*Amarylis belladonna*
5	*Amorpha canescens* (lead plant)
6	*Andropogon gerardi* (big blue stem)
7	*Andropogon scoparius* (little bluestem)
8	*Baptisia leucantha* (white false indigo)
9	*Betula pendula*
10	*Bouteloua curtipendula*(sideoats grama)
11	*Bouteloua gracilis* (blue grama)
12	*Brassica sps.*(mustard)
13	*Callirhoe involucrate* (purple poppy mallow)
14	*Calathea sp.*
15	*Calluna vulgaris*
16	*Camellia japonica*
17	*Dicanthelium oligosathes* (panicgrass)
18	*Euphorbia podperae* (leafy spurge)
19	*Euphorbia sp.*
20	*Festuca rubra*
21	*Fragaria vesca*
22	*Glycine max* (soyabean)
23	*Triticum aestivum* (wheat)
24	*Vicia villosa* (hairy vetch)

MATERIALS AND METHODS

Collection of sample:
Citrus aurantifolia fruits were collected from different regions of India

Surface sterilization:
Collected fruits first rinsed with sterilized water then plant parts were kept in sterile beaker consists of 70% ethanol for 3 min. After that the ethanol sterilized plant parts were kept in beaker consist of Sodium hypochlorite (5%) with tween 20 (0.1%) for 5 minutes. At last plant parts rinsed with sterilized water. Sterility checked and the surface sterilized plant parts were taken and crushed using sterile pestle and mortar and spread on the three of the media (Starch casein agar, YMA media, Albumin media) consisting of Nystatin 50 µg/ ml and kept at 28^0C for 3 weeks.

Isolation of actinomycetes producing antibacterial compound:
Isolated actinomycetes were streaked on solidified media at straight line and kept for incubation in incubator at 37^0C for nearly about 4 days. After completion of 4 days sensitive microbes streak at angle of 90^o and incubated at 37^oC for 24 hrs and observe zone of inhibition.

Identification of different actinomycetes:
Isolated actinomycetes were streaked on solidified ISP-media media in zigzag fashion and kept for incubation in incubator at 37^oC for about 24 hrs, and Biochemical test and Gram's staining also performed.

Production of antibacterial compound using starch casein broth and L.B. broth: The 3 strains of actinomycetes CT1, CT2, CT3, producing antibacterial compound were inoculated into L.B. broth and Starch casein broth and kept for incubation at 37^oC for the production of antibacterial compound for 18 days.

Antibacterial activity of isolates checking through cylinder plate method:
After incubation of 7 days of fermentation of L.B.broth and Starch Casein Broth antibacterial activity was observed from 7^{th} day of fermentation by taking isolates and concentrated up to 10 times at 45^oC by using cylinder plate method against some gram +ve and some gram –ve bacteria, this antibacterial activity checking procedure continued upto 18^{th} day.

Extraction of antibacterial compound using different solvents:
Broth taken at the end of 15^{th} day and centrifuged at 10,000 rpm for 20 min to separate the mycelial biomass, the supernatant was obtained separated by filtration using Whatman filter paper. Certain solvents used for extraction of antibacterial compound like butanol, n-hexane, ethyl acetate**,** petroleum ether**,** chloroform, ethanol (1:1) ratio. Supernatant mixture was agitated for 45 min. with homogenizer and the solvent was separated from broth by

separating funnel, Solvent present in the broth was separated by centrifugation at 5000 rpm for 15 min to remove traces of fermentive broth. All extracts obtained through this method were assayed for antibacterial study against different microbes using respective solvents as control by agar well diffusion method.

Purification of antibacterial compound:
Purification of the compound was performed using TLC and Column chromatography using n-butanol: acetic acid: water (2:1:1) mobile phase or eluent.

Identification of antibacterial compound:
The structure elucidation of the compound was performed by using UV, FT-IR.

RESULTS AND DISCUSSION

Actinomycetes isolates:
There were 7 types of the actinomycetes were isolated (given code) from different parts of the species of Citrus plant (*Citrus aurantifolia)* on the three of nutrient media given in table no.1-3 and out of 7 actinomycetes.

Isolation of the antibacterial Compound producing actinomycetes:
Total *7* actinomycetes showed the production of antibacterial compound on Bennet agar and CT1 showing strong antibacterial activity was selected for further study.

Identification and biochemical test:
The growth ISP-media was shown in table no.4

Production of antibacterial compound:
One actinomycetes isolated from *Citrus aurantifolia* twig (CT1) was selected for the production of antibacterial because that showing higher activity on the Bennet agar shown in figure no.1. After that CT1 shows the production of antibacterial activity in L.B. Broth media and antibacterial activity was checked through after concentration of broth at 45^{o}C upto 10 times against some gram +ve and gram –ve microbes.

Extraction of antibacterial compound:
At last after extraction of the L.B. Broth (using petroleum ether showed good antibacterial activity) the brown gummy like substance obtained.

Purification of antibacterial compound:
After Purification of the antibacterial compound using TLC and Column chromatography { n-butanol: acetic acid :water (2:1:1) mobile phase or eluent.},whitish brown powder was obtained which showed good antibacterial activity shown in table no. 4.

Media (plant parts)	Colour of Colony	Code
Starch Casein agar (*Citrus aurantifolia* twigs)	Yellow Colony	CT1
	Dark Yellow Colony	CT2
	Milky White Colony	CT3
	Red Colony	CT4
	Creamy white Colony	CT5

Table no.1. The actinomycetes isolated from *Citrus aurantifolia* twigs on the Starch Casein media.

Media(plant parts)	Colour of Colony	Code
YMA media (*Citrus aurantifolia* Twigs)	Brown Colony	YMT1
	Light White Colony	YMT2

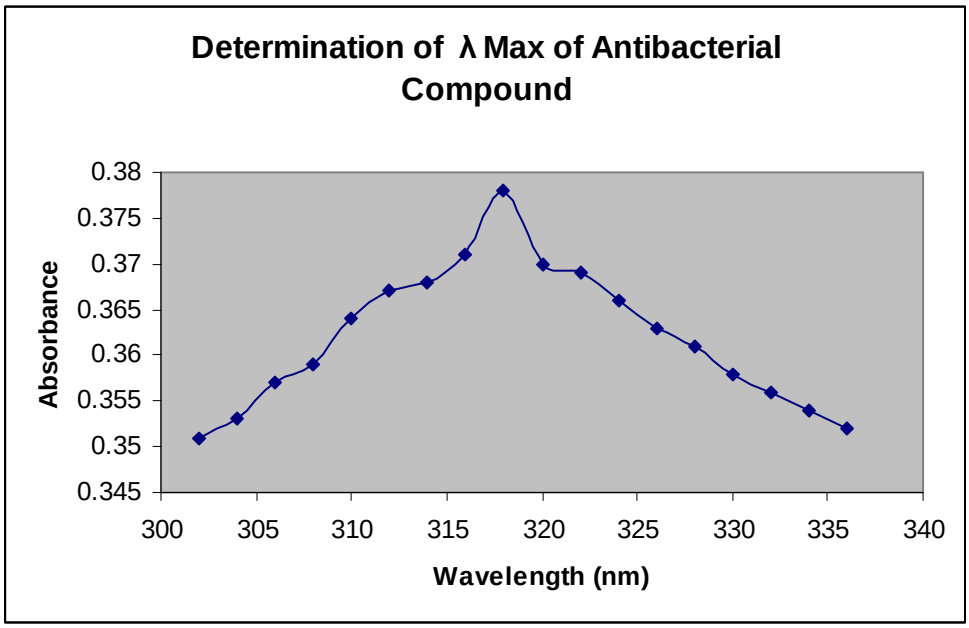
Table no.2. The actinomycetes isolated from Citrus *aurantifolia* twigs on the YMA media.

Media	CT1	CT2	CT3	CT4	CT5	YMT1	YMT2
ISP2	+	-	+	+	+	-	+
ISP4	-	-	-	+	-	+	+
ISP5	-	-	+	+	-	-	+
ISP6	+	-	+	+	+	-	+
ISP7	-	-	+	+	+	+	+

Table.no. 3. The growth on different types of actinomycetes strains on ISPmedia.

S.No.	Name of microorganisms	Zone of inhibition (mm)
1.	*E. coli* ATCC 8739	8.2 mm
2.	*S. typhi* ATCC 23564	7.3 mm
3.	*S. aureus* ATCC 29736	8.9 mm
4	*M. luteus ATCC11880*	0 mm
5.	*K. pneumoniae ATCC 10031*	6.2 mm
6.	*S. fecalis ATCC 8043*	0 mm
7.	*B. subtilis ATCC 6633*	7.0 mm
8.	*S. boydi ATCC 9207*	0 mm
9.	*P. mirabilis ATCC 2124*	0 mm

Table.no.4. The zone of inhibition against different microbes by the antibacterial compound.



Graph no.1 λ Max of the antibacterial compound.

Fig no.1 Actinomycetes (CT1) strain showing strong antibacterial activity against some bacteria.

Structural analysis of the antibacterial compound:

The structure elucidation of the compound was performed by using UV, FT-IR . The λ max of antibacterial compound was found 318 when taken in $CHCl_3$ shown in graph no.1.The IR of the compound were shown in figure no. 2. The I.R. spectra shows the presence of OH group, presence of aromatic ring and presence of NH_2 group.

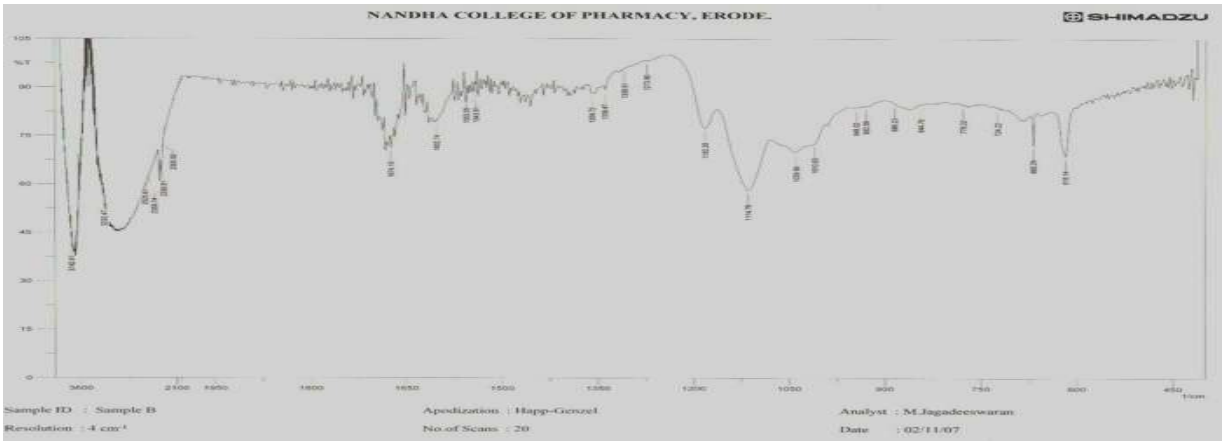


Fig no.2. I.R spectra of the antibacterial compound.

CONCLUSION

The actinomycetes were isolated from *Citrus aurantifolia* plant and the production of antibacterial compound was carried out by using L.B. broth which showed antibacterial activity against the some gram + and gram– bacteria (E.coli, S.typhi, S.aureus, B.subtilis, P.mirabilis, S.boydi) which have got resistance against some antibiotic so from our study it was concluded that antibacterial compound produced by endophytic actinomycetes isolated from *Citrus aurantifolia* will be useful for future

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