



Recent Advances in an Antibacterial and Antifungal Natural Product

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ABSTRACT

Ten isolates were isolated from different plant parts (root, stem, leaves) of *Adhathoda beddomei* (adosa), a medicinal plant. Among ten isolates, one isolate exhibited strong antimicrobial activity against gram-positive and gram-negative bacteria. Identification of endophytes was done on the basis of morphological characteristics. The antimicrobial activity was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Penicillium chrysogenum*, *Lactobacillus lactis*, *Bacillus subtilis* and *Candida albicans*. Minimum inhibitory concentration (MIC) of crude extract performed against microorganisms was determined. The controlling parameters of the biosynthetic process of the antimicrobial agent formation including; different pH values, temperatures, incubation period, and different carbon source. Antioxidant activity tested. NMR, FTIR, elemental and mass analysis of fractionated crude extract was carried out for functional group and empirical formula determination. The present study explains that the endophytic bacteria from medicinal plants have prominent ability to produce antimicrobial agent against a large number of human as well as plant pathogenic microorganisms. Therefore this metabolite could be a lead molecule in the field of pharmaceutical industry. The current study demonstrates that extracts of endophytic bacterial isolated from *A. beddomei* have significant antioxidant and antibacterial property. Many chemicals which are not easily synthesized are highly antioxidant agents. Hence, this work will also serve as a good informative source for comprehensive studies on the chemistry and biology of the bioactive natural products produced by these endophytes.

Keywords: Endophytic bacteria, *Adhathoda beddomei*, Antimicrobial activity, Minimum inhibitory concentration, characterization of bioactive metabolite. Optimization parameter.

INTRODUCTION

There are millions of bacteria in the universe but endophytes bacteria have proved to be a boon for human beings. Endophytes bacteria are the bacteria that reside in the healthy part of the plant without any harm to the plants. It's not only significant for humans but also for plants. Most plants are colonized by bacteria both on the surface and internal tissues. An endophyte is a bacterial or fungal organism; the bacteria live whole or partial life in plants without causing disease¹⁻² and bacteria caters to many needs with the plants. Endophytic bacteria colonize with the healthy tissues of their deferent part of the host plants and can be established many relationship with plat including symbiotic, mutualistic, and trophobiotic. The bacteria have promotes a lot of nutrients for the plant, produce growth hormones, convey stress tolerance, and encourage resistance to plant pathogens³⁻⁴. Endophyte-Infected plants grow fast enough than non-infected plants due to the production of many deferent metabolites⁵⁻⁶ and mutualism relationship with the host plant. There are many endophytes bacteria that produce some metabolites which we call bioactive metabolites this contains anti-property quality. These microbial bioactive metabolites showed activity against *E. faecalis*, *Pseudomonas aeruginosa*, *S. epidermidis*⁷ Search for antimicrobial metabolites is progressing because pathogenic organisms are showing resistance against available antimicrobial agents⁸. As an effect, natural

products research is always hunting for antimicrobial compounds which are the starting compounds for many man-made organic chemistry. In general, many antimicrobial compounds are present in plants but to extract them in big scale we have to consume more plants that are destructive to nature. Endophytic bacteria exist mainly in roots, leaves, and stems of living tissues of different plants, make a mutual relationship without showing in fact any symptoms of disease⁹. Based on the ecological and physiological factors the endophytic bacteria are distributed in plants such as geographical location, age and specificity of host tissue¹⁰. Endophytic bacteria inhabit millions of characteristic ecological niches in many abnormal environments¹¹. Temperate plants are maximally studied for endophytic bacteria resource¹². Endophytic bacteria have been broadly investigated as source of diversity of bioactive compounds¹³. Bioactive compounds show fascinating and attractive properties such as antiprotozoal, anticancer¹⁴, antifungal¹⁵, antibacterial¹⁶, antioxidant¹⁷, antiviral¹⁸, antimalarial¹⁹, antitubercular²⁰, immunosuppressive and antidiabetic²¹. These microbial bioactive metabolite compounds could be mainly classified as an alkaloids, steroids, terpenoids, quinones, phenols, lignans, lactones, isocoumarins, and phenylpropanoids etc²²⁻²³. Several natural products produced by endophytic bacteria have exclusive structures and large bioactivities which applied in Pharmaceutical, industrial (food, beverage) and agricultural fields²⁴⁻²⁵. Approaches for development of



new antibiotics have been followed such as combinatory chemistry method but only a few large spectrum antibiotics are reported to be formed by the pharmaceutical industry at the present time²⁶. In our present work, we have isolated endophytic bacteria with *Adhathoda beddomei* and screened them for their antimicrobial potentials against human and plant pathogenic bacteria. The plant which was chosen as study material is diuretic, purgative. This is useful in the treatment of leprosy, anti-inflammation; skin diseases properties of plant extract have also been reported.

MATERIALS AND METHOD

Isolation of endophytic bacteria

The bacteria were isolated from *Adhathoda beddomei* and maintained on nutrient agar slant as the stock culture at 10⁰ C. Root and stem samples of *Adhathoda beddomei* were washed by running tap water for 15 min followed by fifth times washing with distilled water to reduce the concentration of microorganisms from the Piece of a root. The surface treatment was completed by adopting the methodology of Petrini et al. (1992), and the effectiveness of surface sterilization was checked following to the method of Schulz et al²⁷. To eliminate surface microorganisms, stem and root were dipped in 70% ethanol for 2–4 min and stem and root also were dipped in an aqueous solution of sodium hypochlorite (4%) for 2–4 min and then washed with 70% ethanol for the 30s. The tissues were washed three times with sterile double distilled water and allowed to surface dry in a sterile environment. The samples of root and tissue were cut into small pieces of 4x4 cm² with the sterile knife. To confirm absolute sterilization, a small volume of most recent washing water was inoculated into Petri dishes containing peptone 5 (g/l), sodium chloride 1.0(g/l) and agar 2.5%, malt extract 5 (g/l), pH of the medium was maintained to 7.5. After surface sterilization, roots were cut aseptically into small pieces of 0.5 cm length and aseptically transferred to sterilized Petri dishes containing (g/L): beef extract 3.0, malt extract 3.0 peptone 5.0, dextrose 10 and agar 2.5%. The pH of the medium was adjusted to 7.5. To make sure bacterial growth inoculation medium was supplemented with kinokitzazole (150µg/mL). The Petri dishes were incubated at 37°C until the outcomes of endophytes were discerned.

Preparation of endophytic bacterial extract

The endophytic bacterial culture was inoculated in 10 ml of nutrient broth for 24 hrs. 2% of inoculums was added to 500 ml beaker containing 300 ml of nutrient broth and incubated for 5 days. After 5 days, it was centrifuged and the supernatant was collected. The microbial supernatant was subjected to solvent-extraction, using chloroform in a separating funnel and solvent was evaporated to find the endophytes bacterial extract.

Antimicrobial activity assay

Antimicrobial activity of the crude extract of all 04 isolates was tested by the disk diffusion method²⁸ against four human pathogenic bacteria; *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Escherichia coli*. Human pathogenic bacteria were purchased from the Institute of Microbial Technology (IMTECH), Chandigarh, India. Nutrient agar plates were inoculated overnight with the culture of each bacterial suspension. The inoculated organisms were evenly smeared with sterile glass spreader. Bacterial plates were then incubated at 36±1 °C for 24hrs and fungal plates were incubated at 28 °C for 48 hrs. The zone of inhibition was recorded after the incubation period 24 hrs. All experiments were repeated three times to eliminate data error²⁸.

Minimum Inhibitory Concentration (MIC)

MIC of the bacterial isolate was determined by dilution methods 41. The MIC values of crude were tested and the result indicated that bacterial extract clearly showed antibacterial property against gram-positive and gram-negative bacteria. The antibacterial activity of bioactive compound produced by isolate is comparable with chloramphenicol as the standard antibiotic. The extract obtained from isolate had MIC values, 200 µg/ml of *Escherichia coli*, 250 µg/ml for *Pseudomonas aeruginosa*, 300 µg/ml *Staphylococcus aureus*, 350 µg/ml *Penicillium chrysogenum*, 400 µg/ml *Lactobacillus lactis*, 450 µg/ml *Bacillus subtilis* and *Candida Albicans*. This isolates could be a potential source for advance studies of their antibacterial bioactive compounds.

Antioxidant assay

The free radical activity was tested as bleaching of stable 1,1-diphenyl -2-picrylhydrazyl radical (DPPH). The endophytic bacterial metabolite extract was diluted to take concentrations of 300, 200, 150, 80, 40, 20, 10, 5 µl/ml. 100 ml of 0.002% DPPH solution was prepared in 80% methanol. Diluted samples (1 ml each) were mixed with 2 ml of a methanolic solution of DPPH. The solution of DPPH and each part was shaken well and kept in dark at the controlled temperature (26-28°C) for 1 hrs. Later than incubation changes in colour was obtained at 517 nm. A mixture of 2 ml of 80% methanol was used as a blank. Control sample enclosed all the reagents except the extract. 2 ml of DPPH solution and 1 ml of methanol was taking as control²⁹ Percentage of inhibition was determined by the following equation, (I- inhibition)

$$\% I = \frac{[\text{absorbance of control} - \text{absorbance of fraction}]}{\text{absorbance of control}} \times 100$$

Determination of total phenolic compounds

The phenolic compounds were determined by using the Folin– Ciocateau reagent solution. The reaction mixture was prepared by mixing of 0.5 ml of microbial extract, 1 ml of methanolic solution, 2.5 ml of 10% Folin–Ciocalteu's reagent, 2.5 ml of 7.5% NaHCO₃ in water. Blank solution



was prepared by adding 0.5 ml of methanol and 2.5 ml of 10% Folin–Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of NaHCO_3 . The samples were incubated in a thermostat at 45°C for 45 min. The absorbance was determined by using spectrophotometer at 765 nm. The experiment was performed three times for each analysis and the mean value of data was obtained²⁹.

RESULTS AND DISCUSSION

Antimicrobial activity

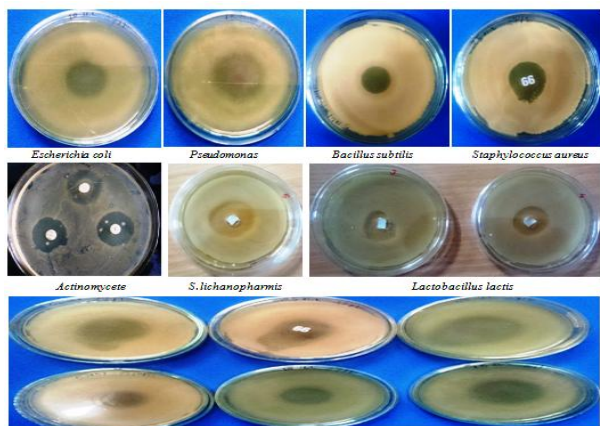


Figure 1: The antimicrobial and antifungal activity was tested against different microbes.

The antimicrobial and antifungal activity was tested against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Penicillium chrysogenum*, *Lactobacillus lactis*, *Bacillus subtilis* and *Candida Albicans*. Minimum inhibitory concentration (MIC) of the crude extract against performed microorganisms was determined. The antimicrobial activity was experienced against *B. subtilis* with different concentration (2 to 12 μl left to right), (Figure 01)

Scanning electron micrograph

Scanning electron micrograph of the endophytic bacteria growing on nutrient agar medium showing spore shape is between cocci and rod (coccobacillus) and spore surfaces slightly rough. Bacterial cell mass is grey, slightly black and size of spore is 8.5 μm . (Figure 02)

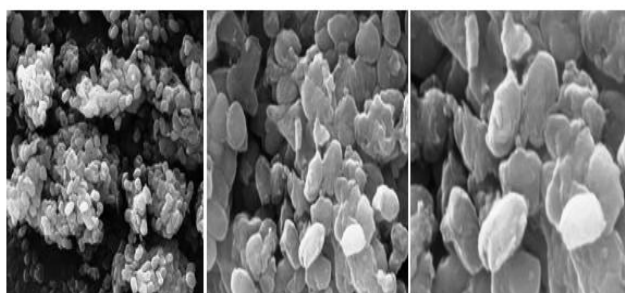


Figure 2: Scanning electron micrograph of the endophytic bacteria isolates.

Evaluation of antioxidant activity of the endophytic bacterial extract

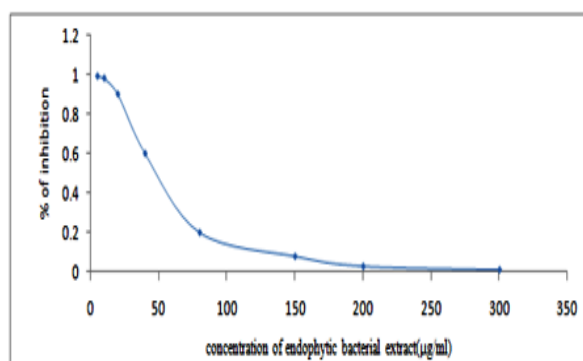


Figure 3: Evaluation of antioxidant activity of the endophytic bacterial extract.

Graph 1 show that a decrease in concentration of the endophytic bacterial extracts, and its increased antioxidant activity. (Figure 08)

Determination of phenolic content in the endophytic bacterial extract

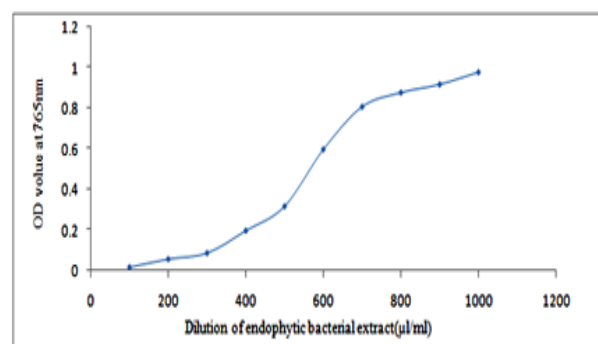


Figure 4: Determination of phenolic content in the endophytic bacterial extract.

The total phenolic concentration in the endophytic bacterial extract was determined

Spectroscopic analysis of compounds

Compound 1

We took the Black sticky compound in which the C (72.25%) H (8.93%) O (23.41%) is so much, molecular IUPAC Name (1R, 5S, 6R)-4-ethyl-hydroxy-1-(3-methylbut-2-en-1-yl)-3-[(1E)-prop-1-en-1-yl]-7-oxobicyclo [4.1.0] Hept-3-en-2-one. The $^1\text{H-NMR}$ data in $(\text{CD}_3)_2\text{CO} \backslash \text{D}_2\text{O}$ showed 5 doublet signals at $\delta = 3.890(\text{d})$, $3.823(\text{d})$, $3.063(\text{d})$, $3.906(\text{d})$ and $2.988(\text{d})$ ppm for H-13, H-14, H-18, H-19 and H-20 respectively, 3 triplet signal at $\delta = 3.198(\text{t})$, $4.232(\text{t})$ and $3.126(\text{t})$ ppm for H-8, H-9, AND H-10 and H-14 singlet signals at $\delta = 7.285$, 8.453 , 6.082 , 7.894 , 7.198 , 7.103 , 4.384 , 3.883 , 2.096 and 1.964 ppm for H-1', H-2, H-3, H-4, H-5, H-6, H-7, H-8, and H-9', respectively. This information was compared with those available in the literature. The structure was complete by determination of negative electrospray ionization mass spectrometry (ESI-MS) m/z , $[\text{M}+\text{H}]^+$ (100%), which is the M.wt. of (Z) -

13-(aminomethyl) -2- (2- hydroxyphenyl) pentadec-13-ene- 1, 8-diol (C₂₃H₃₉NO), and m/z 521 (30%), [M⁺] of the aglycone. Therefore, microbial was identified as (Z) - 13-(aminomethyl)-2-(2- hydroxyphenyl) pentadec-13-ene- 1, 8-diol (maïi et al., 2015).

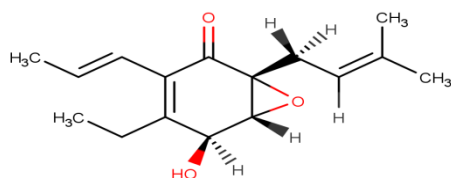


Figure 5: Molecular Structure of antimicrobial agent (compound 1).

Compound 1 was observed active against

This active compound works to kill many bacteria, whom it dies, *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Penicillium chrysogenum*, *Lactobacillus lactis*, and *Penicillin sp.*

Compound 2

We took the Black sticky compound in which the C (68.25%) H (11.93%) O (20.41% is so much, molecular IUPSC Name is 3-[[[2-[-(1-[-[4-(dihydroxyamino)-2-methylphenyl] carbomoul] eth-1-en-1-yl) carbamoyl] ethyl] carbamoul] methyl] amino} propanoic acid.

The ¹H-NMR data in (CD₃)₂O\ D₂O showed 2 doublet signals at δ= 7.25(s) and 6.60(s) ppm for H-1 and H-3 (J= 1.35 Hz), respectively, 1 quarded signal at δ= 3.89 ppm for H-8, and 2 singlet signals at δ= 7.24 and 4.8 ppm for H-2` and H-4`, respectively, indicating a substituted H-2 These data were compared with those available in the literature. 2 triplet signals at δ= 2.53 and 3.19 ppm for H-7` and H-9, The structure was confirmed by determination of negative electro spray ionization mass spectrometry (ESI-MS) m/z 412.4 da [M+H] (100%). These structure draw with chemdraw software. Which is 3-[[[2-[-(1-[-[4-(dihydroxyamino)-2-methylphenyl] carbomoul] eth-1-en-1-yl) carbamoyl] ethyl] carbamoul] methyl] amino} propanoic acid (C₁₈H₂₃N₅O₆), is 412.4da,

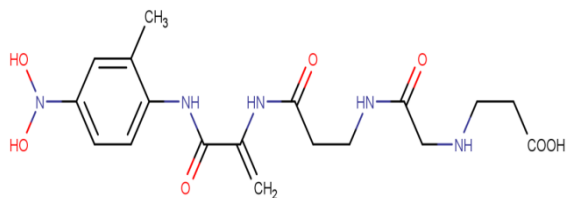


Figure 6: Molecular Structure of antimicrobial agent (compound 2).

Compound 2 was observed active against

This active compound works to kill many bacteria *E. coli*, *S. pneumonia*, *B. subtilis*, *S. hyicus*, *B. sphaericus*, *S. aureus*, and *pseudomonas aeruginosa*, *C. albicans*, *P. exigua*, *S. rolfisii*, *Sclerotinia*, *scleratiourm Fusarium sp.* and *Penicillin sp.*

Compound 3

We took the Black sticky compound in which the C (69.25%) H (10.93%) O (22.41% is so much, molecular IUPSC Name is 7-chloro-4, 6, 6'-trimethoxy-2'- methyl-3H-spiro [1-benzofuran-2, 1'- cyclohexan] -2- ene-3,4'-dione. The ¹H-NMR data in (CD₃)₂O\ D₂O showed 2 doublet signals at δ= 8.35(s) and 6.8(s) ppm for H-1 and H-3 (J= 1.35 Hz), respectively, 1 quarded signal at δ= 2.98 ppm for H-8, and 2 singlet signals at δ= 7.24 and 4.8 ppm for H-2` and H-4`, respectively, indicating a substituted H-2 These data were compared with those available in the literature. 2 triplet signals at δ= 3.56 and 4.16 ppm for H-7` and H-9, The structure was confirmed by determination of negative electro spray ionization mass spectrometry (ESI-MS) m/z 401.9 da [M+H] (100%).

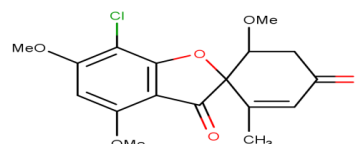


Figure 7: Molecular Structure of antimicrobial agent (compound 3).

Compound 3 was observed active against

This active compound works to kill many bacteria such as *B. Subtilis*, *Penicillium chrysogenum*, *C. Albicans*, *B. sphaericus*, *S. Aureus*.

CONCLUSION

These studies confirmed the occurrence and variety of culturable endophytes. In India, only countable number of the reports showed the diversity of endophytic bacteria and fungi from the medicinal plants. This work may be the first report on endophytic bacteria from *Adhathoda beddomei* form Uttar Pradesh state. There are many reports on the isolation of fungal endophytes from *Adhathoda beddomei* but there are only few reports on the isolation of endophytic bacteria from *Adhathoda beddomei* (Tiwari et al., 2013).

All the compounds isolated from crude metabolite showed broad spectrum antibacterial activity against Gram-positive and Gram-negative bacteria and plant pathogenic bacteria.

The present study explains that the endophytic bacteria from medicinal plants have prominent ability to produce antimicrobial agent against large number of human as well as plant pathogenic microorganisms. Therefore this metabolite could be a lead molecule in the field of pharmaceutical industry. The current study demonstrates that extracts of endophytic bacterial isolated from *A. beddomei* have significant antioxidant and antibacterial property. Many chemicals which are not easily synthesized are highly antioxidant agents. Hence, this work will also serve as a good informative source for comprehensive studies on the chemistry and biology of the bioactive natural products produced by these endophytes.

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