

Studies of Bioactive Metabolites from Endophytic Bacterial Isolate

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Abstract: The microbial source, variety of producing species, functions and an assortment of bioactivities of metabolites. The probable numbers of metabolites may be discovered in the future, the problems of dereplication of recently isolated compounds as well as the new trends and prediction of the research are also discussed. Ten isolates were isolated from different plant parts (root, stem, leaves) of *Adhathoda beddomei* (adosa), a medicinal plant. Among ten isolates, one isolate exhibited highest antimicrobial activity against gram positive and gram negative bacteria. Recognition 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 albican*. Minimum inhibitory concentration (MIC) of crude extract performed against microorganisms was determined. The controlling parameters the biosynthetic process of the antimicrobial agent formation including; different pH values, temperatures, incubation period, and different carbon source. NMR, FTIR, element and mass analysis of fractionated crude extract was carried out for functional group and experimental formula determination.

Keywords: Endophytic bacteria, *Adhathoda beddomei*, Antimicrobial activity, Minimum inhibitory concentration,



I. Introduction: An endophyte is an endosymbiont, often a bacterium or fungus that lives within a plant for at slightest part of its life cycle without causing any visible disease. Most plants are inhabited by bacteria both on the exterior and interior tissues [1, 2]. Endophytic bacteria colonize the tissues of their host plants and can be form a series of relationships including symbiotic, mutualistic, and trophobiotic. Recently, endophytic bacteria have gained consideration due to their interesting features related to plant growth and health. The bacteria are reported to increase highest nutrient availability, generate growth hormones, convey large stress tolerance, and support resistance to plant pathogens [3, 4]. Endophyte infected plants often grow more rapidly than non-infected plants due to the formation of metabolites [5,6]. These microbial metabolites showed activity in opposition to *E. faecalis*, *Pseudomonas aeruginosa*, *S. epidermidis* [7] Search for antimicrobial metabolites is progressing because pathogenic organisms are performance resistance against available antimicrobial agents [8]. As an effect, natural products research is always hunting for antimicrobial compounds which are the initial compounds for many man-made organic chemistry. In general, many antimicrobial compounds are presence in plants but to extract them in great scale we have to consume extra plants that are destructive to nature. Therefore substitute to move in the path of apart from chemical synthesis should be sought by targeting endophytic bacteria one of the most suitable resources for this purpose. Endophytic bacteria exist found in the roots, leaves, and stems of living tissues of different plants, generate a mutual relationship without indicating of any symptoms of disease [9]. Based on the ecological, climatically, morphologically and physiological factors the endophytic bacteria are live in plants such as geographical location, age, climate, shape-size and specificity of host tissue [10]. Endophytic bacteria have been widely investigated as source of variety of bioactive compounds [11]. Bioactive compounds show exciting and attractive properties such as antibacterial [12], antifungal [13], anticancer [14], antiprotozoal [15], antioxidant [16], antiviral, antimalarial [17], antitubercular [18], immunosuppressive, antidiabetic and antiviral [19]. These bioactive compounds could be mostly classified as Alkaloids, steroids, terpenoids, quinones, phenylpropanoids, isocoumarins, lignans, phenols and lactones etc [20]. These bioactive compounds

used for the healthcare purpose for human beings [21]. Several natural products produced by endophytic bacteria have exclusive structures and large bioactivities which applied in Pharmaceutical, industrial (food, beverage) and agricultural fields [22, 23]. In our current work, i have been isolated endophytic bacteria by *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 treatment of leprosy, Anti-inflammatory; skin diseases properties of plant extract have also been reported.

2. Materials and Method.

2.1 Selection of plant material: The following characteristics were taken into consideration in order

To isolate the endophytic bacteria from an *Adhathoda beddomei* medicinal plant [24]:-

1. Plants from a exceptional ecological environmental niche and growing in special habitats, Especially those with an unusual biology.
2. Plants that have an ethno botanical history, and are used for traditional medicines.
3. Plants those are endemic, having an unusual longevity.
4. Plants rising in field of great biodiversity.

For photochemical screening, about 5 Kg fresh plant materials (root parts) of *Adhathoda beddomei* were collected from Botanical garden of Banaras Hindu University (BHU) Varanasi (UP) India. Well aerial parts of plant were collected, placed in sterile plastic bags and immediately transfer aseptically condition to the laboratory and were used within 24 hrs. For microbiological works. Root parts samples were cut into about 1cm long pieces and then washed in running tap fresh water for 15 minutes to remove soil particles, unwanted microbes and adhered debris, then were washed in detergent (tween 20) and finally washed with distilled water.

Surface sterilization: Surface sterilization was determined [25] with some modifications to remove epiphytes. Samples were deep 2 times in 70% ethanol for four minutes and deep twice in 2-4% solution of sodium hypochlorite for three minutes and again immersed for 1 minute in 60% ethanol. Finally, rinse samples 5-8 times with sterile water for 10 minutes and wash 2times in sterile distilled water for 8 min to remove surface sterilization agents with further drying in sterilized paper in a laminar flow hood.

Sterility check: To authenticate that the plant surfaces were effectively decontaminated 1ml aliquots of the sterile distilled water that was used in the last confirmation of surface sterilization procedures were plated onto nutrient agar medium and incubated at 37 °C for 48 hrs. Bacterial growth was observed after 48 hrs. Also, surface sterilized segments were rolled on nutrient agar plates, incubated at 37 °C for 48 hrs, and checked for possible microbial growth [26]. After complete surface sterilization, roots were cleaved aseptically into small pieces of 1.0 cm length and aseptically transferred to sterilized petri dishes containing (g/L): yeast extract 3.0, malt extract 3.0 peptone 5.0, dextrose 1.0 and agar 2.5%. The pH of medium was adjusted to 7.0. To check fungal growth inoculation medium was supplemented with kinokitazole (150 µg/mL). The petri dishes were incubated at 37°C until the outcomes of endophytes were recognised. Hyphal tips originating from segments were transferred to Petri dishes containing NAM medium devoid of antibiotics. Each isolate was then grown and examined to make certain that it was originated from a single organism. All bacteria present were isolated; sub cultured, and kept on NAM for further Identification.

Purification, selection and preservation of endophytic bacterial isolates: After incubation, number of aerobic heterotrophic bacteria was recorded as colony-forming units (CFUs). And choice of colonies was under taken based on the variation in macro-morphology characteristics, results not shown. Colonies were purified/ separated through repetitively re-streaking on nutrient agar medium. Isolates were preserved on slants with fresh nutrient agar medium covered with mineral oil at 4 °C for further use.

Preliminary characterization of endophytic bacteria: Phenotypic characteristics such as microscopic characterization of gram reaction was determined according to method described [27], Endospore staining was completed by the Schaeffer-Fulton process and motility was confirmed with the Hanging Drop Slide for all isolates using a Trinocular phase contrast compound microscope.

Fermentation conditions: Fermentation takes place according to [28] with some modification. It performed in 1 L Erlenmeyer flasks containing 500 ml of several media were tested (nutrient broth, nutrient broth supplemented with 2.5% glucose and enrichment medium (12 glucose, 3 NaNO₃, 0.5 KH₂PO₄, 2.5 KCl, 0.1 MgSO₄, 0.05 FeSO₄, 25 peptone, 15 beef extract in g/500 ml distilled water). The cultures were incubated at 37 °C with 120 rpm agitation. After 72 Hrs. the culture was harvested by centrifugation for 10 min at 10000 rpm (16000x g, Centrifuge plc series). The supernatants (extra cellular culture filtrates) were collected with identical volumes of ethyl acetate in an extrication funnel by shaking energetically for 20 minutes. After separation, the organic part was concentrated by Rotary Evaporator at 38 °C. The consequential crude extract was dissolved in 1-5 ml methanol and stored at 28 °C. The bacterial pellet was washed 2-3 times with sterile filtered saline solution. Extraction of washed cells was carried out with methanol overnight at 4 °C for three times. The organic phase was dried as filtrate ethyl acetate extract and stored at 28 °C. The residues were redissolved in dimethyl sulphoxide (DMSO) for subsequent spectrometric analysis.

Antimicrobial activity assay: Antimicrobial activity of the crude extract was tested by the disk diffusion method [29] 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 for the night with 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 and 48 hrs). All experiments were repeated three times to eliminate data error.

2.4 Minimum Inhibitory Concentration (MIC):

MIC of bacterial isolate was determined by dilution methods [30]. The MIC values of crude were tested and the result indicated that bacterial extract visibly showed antibacterial property against gram-positive as well as gram-negatives bacteria. The antibacterial activity of bioactive compound produced by isolate is equivalent with chloramphenicol as standard antibiotic. The extract obtained from isolate had MIC values, 120 µg/ml for *Escherichia coli*, 220 µg/ml for *Pseudomonas aeruginosa*, 250 µg/ml *Staphylococcus aureus*, 350 µg/ml *Penicillium chrysogenum*, 420 µg/ml *Lactobacillus lactis*, 550 µg/ml *Bacillus subtilis* and *Candida albican*. This isolate could be excellent entrant for advance studies of their antibacterial bioactive compounds.

3. Result:

3.1 SEM Analysis:

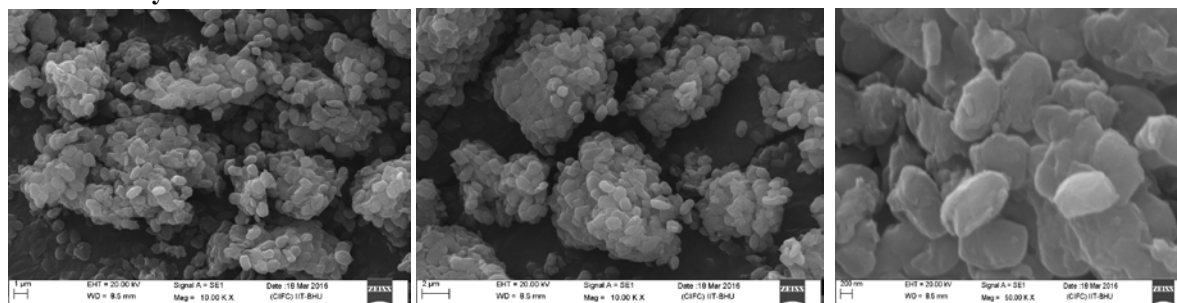
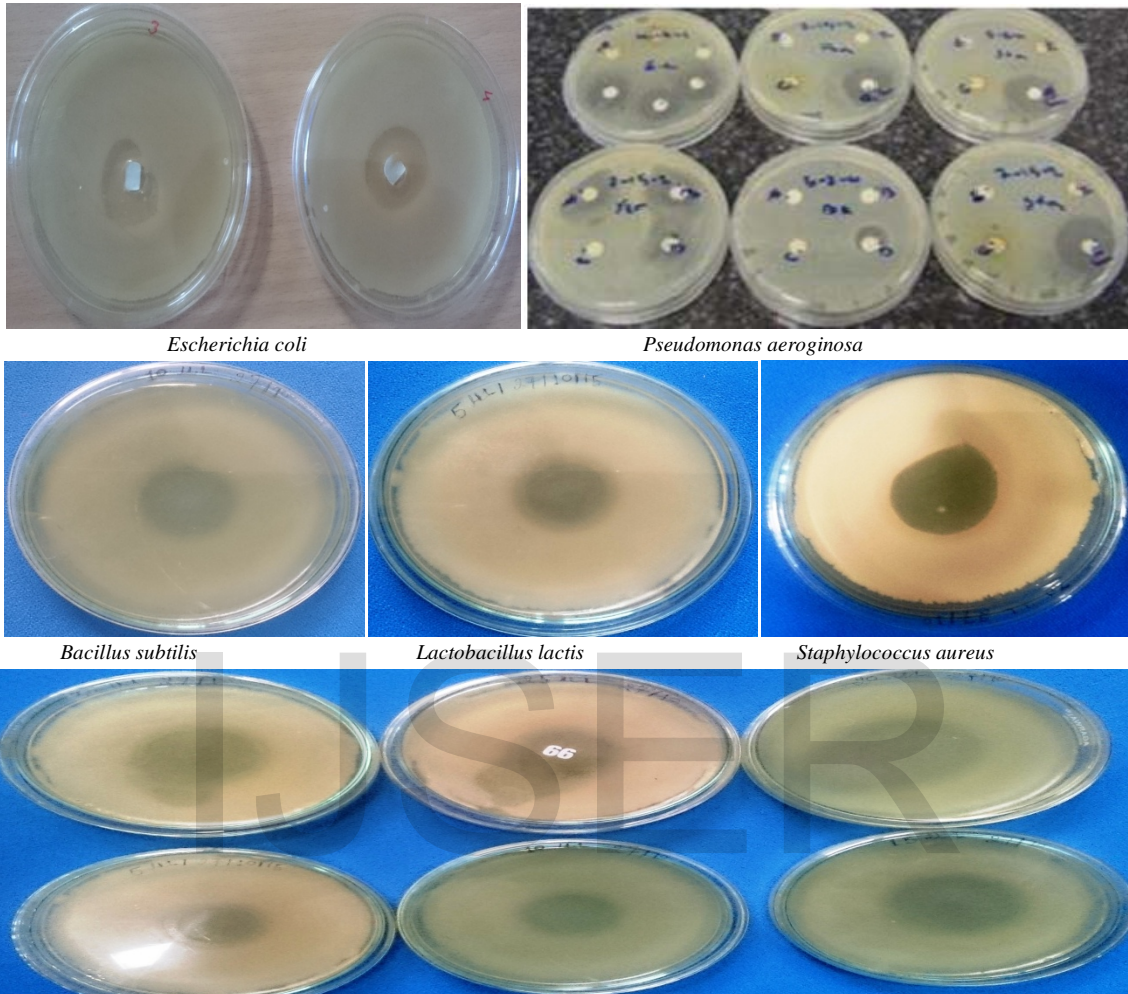


Fig.01

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

3.2 Antimicrobial activity:



The antimicrobial activity was experienced against *B.subtilis* with different concentration (2 to 12 µl left to right).
Fig.02

3.3 FTIR Analysis:

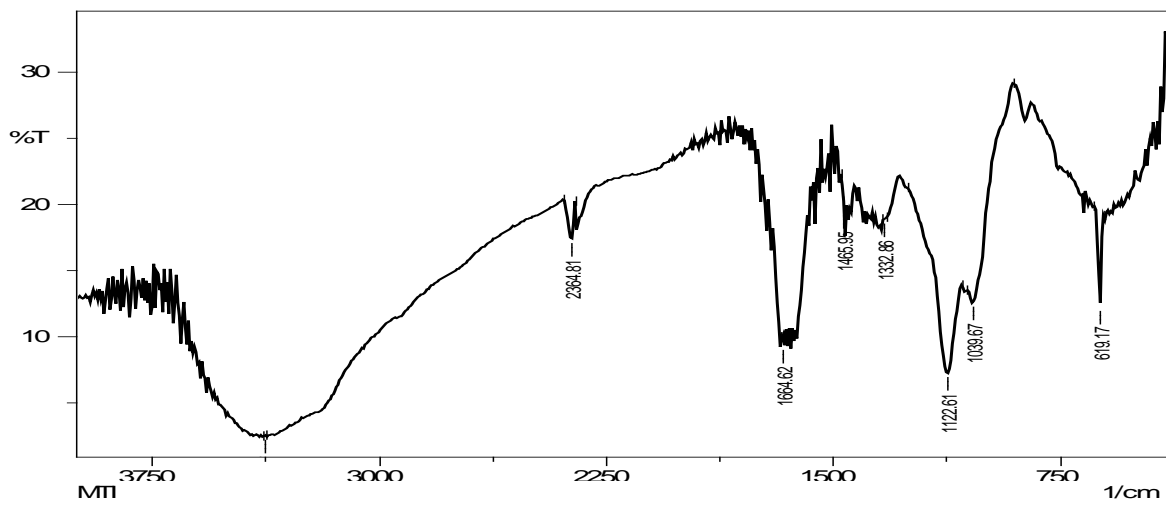
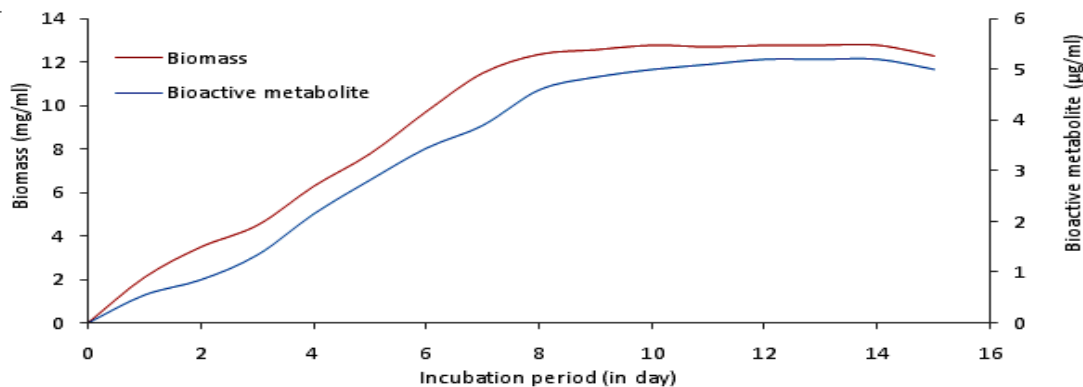


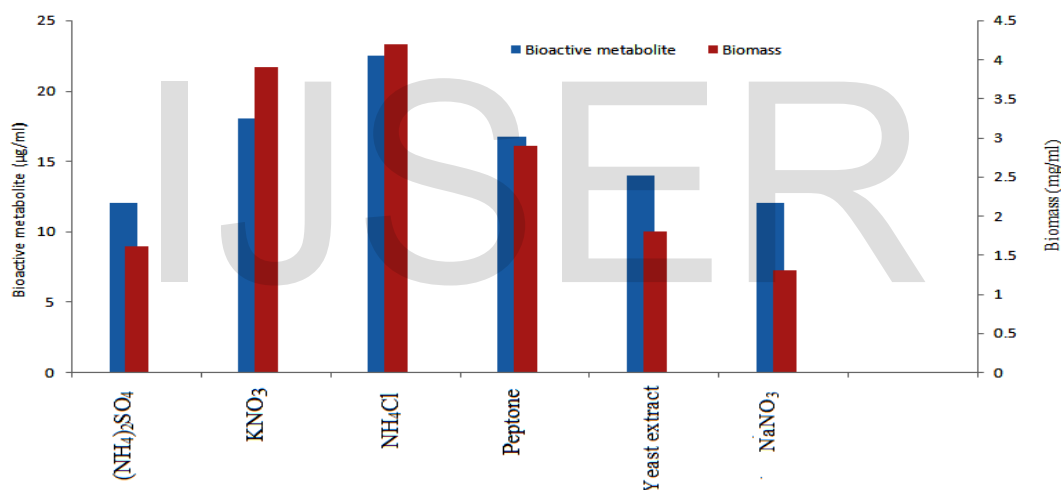
Fig.03

The infrared (IR) spectrum specify characteristic band corresponding to 07 peaks. Stretching at 2364.81cm^{-1} denotes chance of presence of alkynes group. Stretching 1122.61 and 1039.61cm^{-1} indicates the presence of aliphatic amines group. Stretching at 1664.62cm^{-1} denotes possibility of existence of alkenes group. A strong stretching was observed at 1464.86cm^{-1} showing presence of alkynes. . Stretching 1332.86cm^{-1} denotes possibility of presence of aromatic amines. And last stretching (619.17cm^{-1}) is indicate generally presence of alkynes.

3.4 Optimization condition:

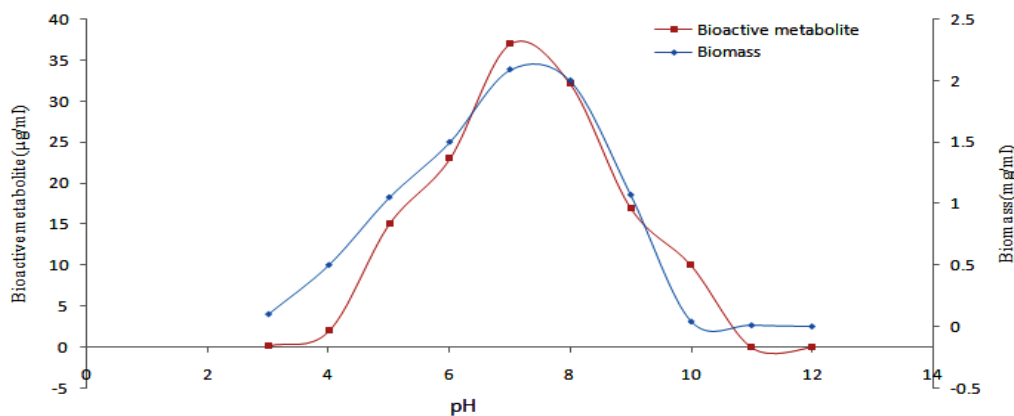


Graph.01



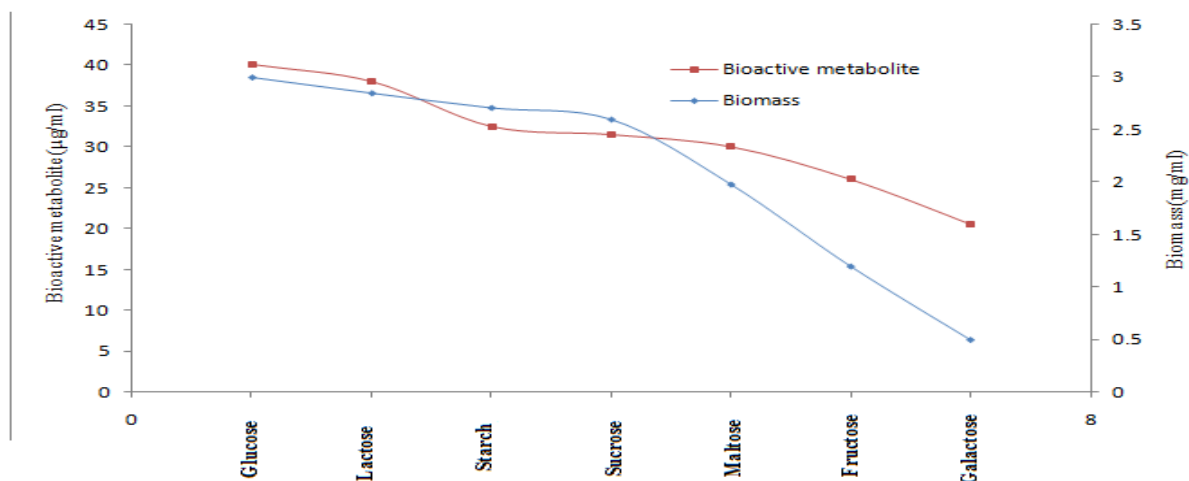
Graph.02

Maximum antimicrobial metabolite production could be recorded for an incubation period for 7 days and ammonium chloride was found to be the best nitrogen source for the antimicrobial metabolite production.



Graph.03

The biosynthesis of the antimicrobial metabolite agent reached its maximum yield in the production medium adjusted at pH 7.0.



Graph.04

Glucose was found to be the best carbon source for the antimicrobial agent production with concentration 2.5 g/100 ml.

3.3 NMR and Mass spectra:

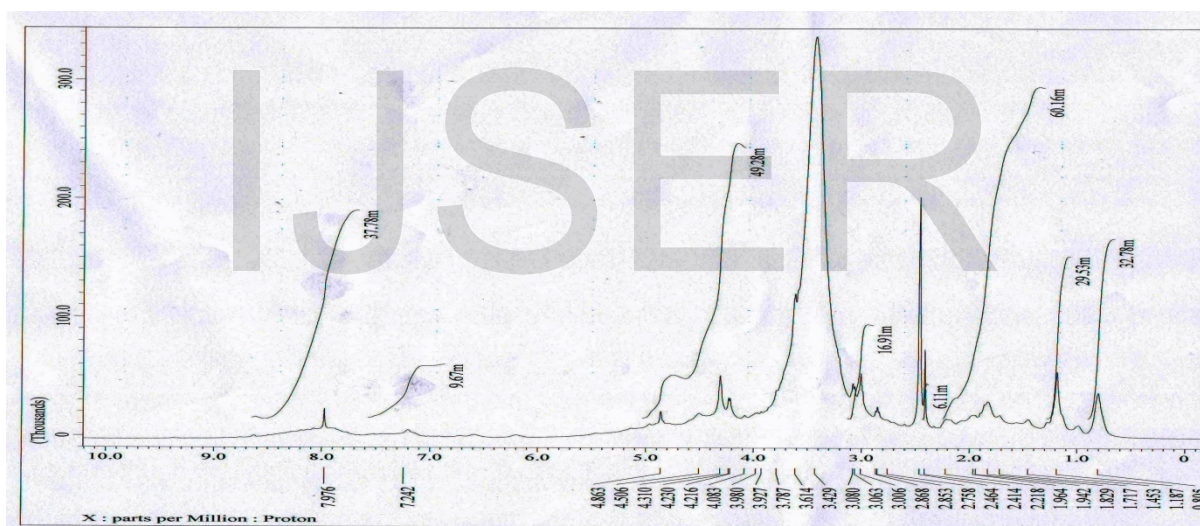


Fig.04

The Mass spectrum revealed that the molecular weight is 423.50. The NMR-Spectrum could be also determined.

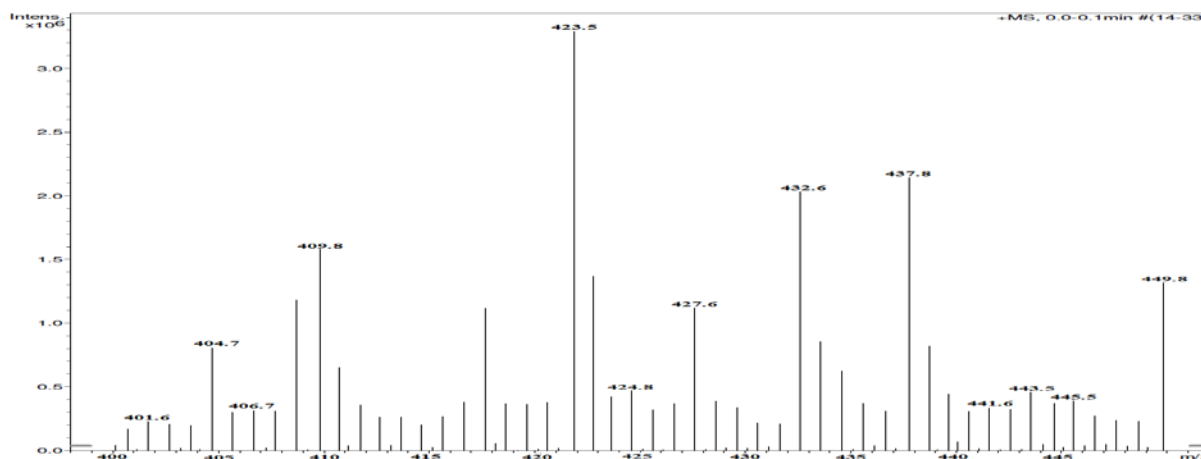


Fig.05

Discussion: The endophytic bacteria were isolated from *Adhathoda beddomei* (adosa) sample collected from Botanical garden of Banaras Hindu University (BHU) Varanasi (UP) India. The isolate was grown on starch-nitrate agar medium for investigating its potency to produce antimicrobial metabolite agents. The growth of the endophytic bacteria isolate exhibited antimicrobial activities against (Gram-positive and Gram-negative bacteria and unicellular fungi). For optimizing the biosynthesis of the antimicrobial agent from endophytic bacteria, altered cultural conditions such as pH, and incubation period, effect of different carbon, and nitrogen sources were deliberate. The highest biosynthesis was obtained at the end of an incubation period of seven days for the antimicrobial agent production [31]. Maximum yield of the antimicrobial agent produced at the end of an incubation temperature of 37 °C [32] and pH 7 (Graph.03). Data of the effect of different carbon (Graph.04) and nitrogen sources (Graph.02) on the optimum production of the antimicrobial metabolite indicated that endophytic bacteria require glucose and ammonium chloride at concentrations 2.5 g/100 ml; 0.5 g/100 ml, respectively [33, 34]. The compound is generously soluble in chloroform, Ethyl acetate, n-Butanol and water but insoluble in petroleum ether, and hexane. Infrared absorption spectrum indicated by 07 peaks. The Mass spectrum shown that the molecular weight is 423.50 (Fig.05) and NMR-spectrum (Fig.04) was determined. The MIC of bioactive metabolite was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against difference pathogenic, non pathogenic bacteria and fungus (Fig.02). Recognition of the antimicrobial agent according to recommended international keys indicated that the bioactive metabolite is suggestive of being likely belonging to Depsipeptide (Mikamycin) group (Vernamycin-An antibiotic) produced by endophytic bacteria.

5. Conclusion: The current study shows the present data focusing on obtaining microbial confined isolates which have the ability to produce innovative antimicrobial agent beside pathogenic microorganisms (Gram-positive as well as Gram-negative bacteria) and 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 important antibacterial and antifungal property. Many chemicals which are not simply synthesized are highly antifungal and antibacterial agents. Hence, this work will also provide as a good useful source for complete studies on the chemistry and biology of the bioactive natural products produced by these endophytes.

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