



Original article

Antimicrobial activity and biosynthesis of nanoparticles by endophytic bacterium inhabiting *Coffea arabica* L.

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ARTICLE INFO

ABSTRACT

Article history:

Received 23 October 2012

Accepted 18 November 2012

Available online 30 November 2012

Keywords:

Endophytes

Silver nanoparticles antimicrobial

Metabolites

The interface between endophytes and nanomaterials is a relatively new and unexplored area the present study evaluates screening of bacterial endophytes from surfaced sterilized leaf and stem segments of agro economical plant *Coffea arabica* L. towards synthesis of silver nanoparticles and antimicrobial metabolites. Among thirty two endophytes isolated nine isolates exhibited antimicrobial activity among which one bacterium was capable of extracellular synthesis of silver nanoparticles upon evaluation of supernatant with 1 mM of silver nitrate, biosynthesis of silver nanoparticles were assessed by UV-Visible Spectroscopy and the bacterium was capable of secreting antimicrobial secondary metabolites upon crude ethyl acetate extract evaluated for antimicrobial activity against panel of both gram positive and gram negative as well as phytopathogenic fungi. Partial characterization was carried out via bioautographic technique with *Rf* value 0.3 and 0.6 exhibiting antimicrobial activity against MRSA strain. Further studies in this area will be promising enough for molecular characterization of endophytic bacterium and chemical profiling of antimicrobial metabolites at the same time physiochemical characterization of nanoparticles will be valuable to reveal the size and shape.

1. Introduction

Interference of endophytes and nanomaterial is a new area of science with few studies has been already reported endophytes in production of nanoparticles. improved scientific knowledge and implementation of new technologies has unfolds interaction of nano-revolution with biological entities and the role of microbes in bio and green synthesis of nanoparticles seem to have drawn unequivocal attention with a view of reformulating the novel strategies as alternatives for conventional methods for the synthesis of nanoparticles which are bound with various implications such as expensive costs, toxicity risks on health from environmental contaminants, etc., (Syed baker and Satish, 2012). The present study envisions the bacterial endophytic flora of *Coffee arabica* L. towards synthesis of nanoparticles and production of antimicrobial metabolites against a panel of significant human and phytopathogenic microorganisms. The discovery of natural products is a multidisciplinary aspect which involves isolation, structural elucidation and establishing the biosynthetic pathway of the secondary metabolites with various bioactive compounds emerging from natural resource are of great therapeutic value hence there is an upsurge to haunt new ecological niches for potential sources of natural bioactive agents as renewable, eco-friendly and easily obtainable (Liu *et al.*, 2001). Once such source forms endophytic plethora, these endophytes harbors in unique biological niches of plants hence research on endophytes have expanded rapidly since recent past as it mimic plant chemistry and secret almost similar novel metabolite which are more bioactive than host and these bioactive metabolites have a broad range of biological activities and could be the starting materials for pharmaceuticals or novel lead structures for the development of pharmaceutical or agrochemical products (Schulz *et al.*, 2002). Endophytes can be defined as microorganism inhabiting in plants forming a symbiotic relation with its host without causing any inherent negative effects (Syed Baker *et al.*, 2012a).

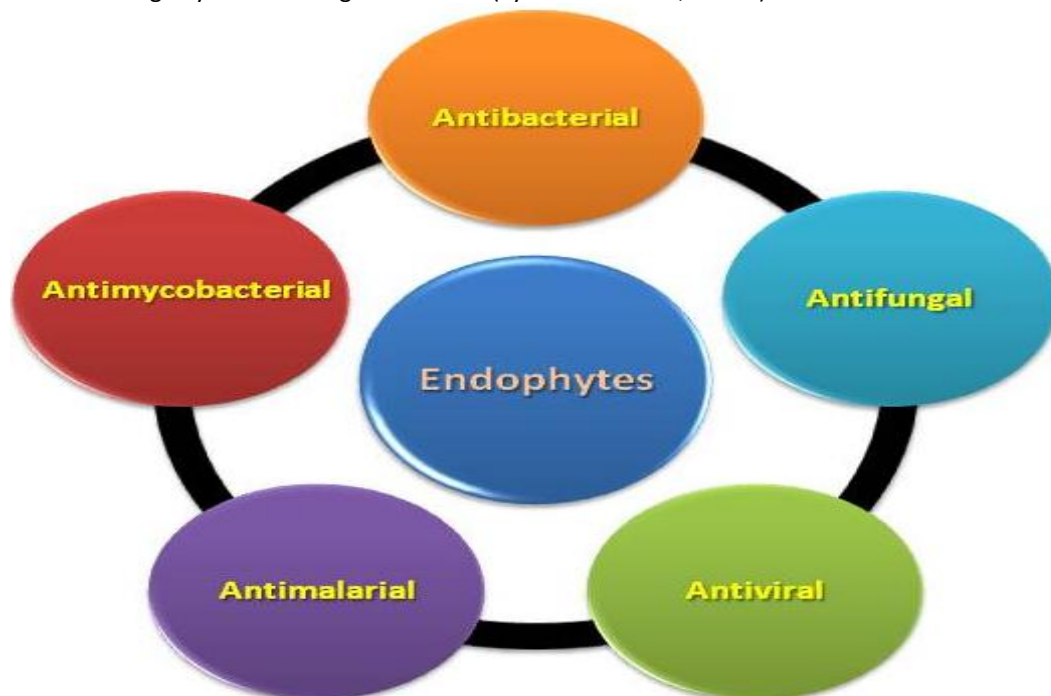


Fig. 1. Endophytic plethora as source of antimicrobial metabolites.

Endophytes presuming as immense important biological entities forming rich source of valuable bioactive compounds, among which endophytic plethora are known to secrete antimicrobial agents such as antibacterial,

antifungal, antiviral, antimalarial and antimycobacterial etc. (Fig. 1). At the same time yet an unimaginable role of endophytic microorganisms are in synthesis of nanoparticles. Thus present study employ endophytes from *Coffea arabica* L. as an agro economical plant has generated attention due to which an interest to study the microorganism associated with *Coffea arabica* L. has been expressed in various studies due to increase the coffee yield and prevent the disease associated with coffee plants (Vega *et al.*, 2005). Thus the selected plant became the area of interest in the present study.

2. Materials and methods

2.1. Sample collection

Plant material such as stem and leaves were collected during the growing season of *Coffea arabica* L. from 10 healthy mature plants per site at two different geographical locations from southern parts of India. The materials were collected in a sterilized polybags and transported to laboratory within two hours before processing. Collected plant materials were thoroughly washed under running tap water then were immersed in a double distilled water containing 50 µg/ml of cycloheximide for 60 mins (minutes) to suppress the growth of fungal endophytes.

2.2. Surface sterilization

Stem and leaves were subjected to surface sterilization under aseptic condition by sequential steps of immersing in 3.15% sodium hypochlorite for five minutes and then followed by ethanol 70% for thirty seconds later outer tissue of surface sterilized stem and leaves were excise with sterilized scalpel, into 0.5-1.0 cm tissue blocks and placed onto nutrient agar supplemented with 250 µg/ml of cycloheximide and incubated till bacterial endophytic colonies are visible (Zin *et al.*, 2010 and Webster *et al.*, 2001). To confirm the surface disinfestations process was successful and to verify no biological contamination from the surface during the surface sterilization protocol, sterility checks were carried out for each step to monitor the effectiveness by impressions were taken onto nutrient agar and 0.1 ml of final rinse water was plated out on nutrient plates were maintained as control.

2.3. Preliminary screening of endophytic bacteria for synthesis of nanoparticles

The colonies emerging from surfaced sterilized leaves and stem segments of *Coffea arabica* L. were evaluated for biosynthesis of nanoparticle by culturing the isolates onto nutrient agar supplemented with 2 mM silver nitrate. Colonies capable of growing in the media supplemented with silver nitrate were cultured at large scale by inoculating the actively growing isolates onto 500ml nutrient broth and incubated onto rotary incubator shaker at 32 °C for 72 hours. Later the culture broth was centrifuged at 8000 rpm at 4 °C for 20 minutes to separate biomass and supernatant later the supernatant was assessed for production of silver nanoparticles by challenging the supernatant with 1 mM of silver nitrate with 50ml of supernatant and 10 ml of silver nitrate and incubated on rotary incubator at 25 °C with 180 rpm. Samples were drawn periodically and monitored at UV-Visible spectrophotometer for primary confirmation of silver nanoparticles synthesis.

2.4. Primary screening of endophytic bacterial isolates for antimicrobial activity

The preliminary screening of endophytic bacterial colonies emerging from surface sterilized segment of stem and leaves were subjected to primary screening for antimicrobial activity by dual culture method for fungi and agar over lay method for bacteria where in endophytic isolate were point-inoculated and incubated for 3-days. Later colonies were inactivated by inverting the upper lid with 1-5ml chloroform for 40 mins. The inactivated colonies were overlaid with 5 ml of sloppy soft agar with 0.65% nutrient agar that had been inoculated with the test organisms. Zones of inhibition around the colonies were recorded after 24 hrs at 30 °C. Endophytic bacteria exhibiting activity were further subjected to scale production to extract the antimicrobial metabolites (Hayakava *et al.*, 2004 and Syed Baker *et al.*, 2012b).

2.5. Optimization for large scale production

Endophytic colonies exhibiting activity against the test pathogens in preliminary screening were produced at large scale via fermentation by inoculating the actively growing colonies onto mother culture flask containing nutrient broth and then at mid log phase the culture broth as aseptically transferred into ten liters fermenter under optimized parameter with neutral pH and 32 °C for 72 hours.

2.6. Extraction of secondary metabolites

After the fermentation process the fermented broth was harvested by centrifugation to separate the supernatant for solvent extraction process with ethyl acetate as a solvent. Equal volume of supernatant and ethyl acetate with ratio 1:1 was pooled in a separating funnel and shaken vigorously and allowed to settle. Organic phase was separated and collected the procedure was repeated 3 times to pool the organic phase. Later the extracted solvent was flash evaporated and residue obtained was dry to obtain the crude extract for further antimicrobial studies.

2.7. Evaluation of antimicrobial activity

2.7.1. Disc diffusion assay

Antibacterial activity was evaluated using the disc diffusion assay with Pre-warmed Mueller-Hinton agar plates seeded with 10^6 CFU (colony forming unit) suspensions of test bacteria. Crude ethyl acetate extract dissolved in ethyl acetate (1 mg/ml) and 20 μ l extract was impregnated onto sterile paper discs (6 mm diameter) and placed onto the surface of inoculated agar plates. Plates were incubated at 37 °C for 24 hrs. Antibacterial activity was expressed as the diameter of the inhibition zone in mm (millimeter) produced by the extracts across the disc. Ethyl acetate was used as negative control and gentamicin standard disc were used as positive control (Radii *et al.*, 2011). Antibacterial activity was determined with zone of inhibition in mm excluding the disk with extracts. All data were analyzed using SPS Software.

2.7.2. Bioautography

The antimicrobial activities of the crude ethyl acetate extract were detected using Thin Liquid Chromatography bioautography agar-overlay assay wherein crude extract was subjected to thin liquid chromatography technique solvent system bearing 1:1 ethyl acetate and hexane later the separated bands onto the TLC plates were covered with 1-2 mm layer of soft medium BHI (Brain Heart Infusion agar 0.6%) containing 0.1 % (w/v) 2, 3, 5 triphenyltetrazolium chloride (tetrazolium red) with previous inoculated test MRSA at a final concentration of 10^7 CFU/ml. The plates were placed in a sterile tray, sealed to prevent the thin agar layer from drying, and incubated at 37 °C for 24 hrs the plates were run in duplicate (Mattana *et al.*, 2010).

3. Results

The present investigation resulted in isolation of thirty two bacterial endophytes from surface sterilized tissue of stem and leaves of *Coffea arabica* L. During the surface sterilization protocol employed resulted in elimination of epiphytic microorganism on stems and leaves, addition of cycloheximide suppressed the growth of fungi which resulted in only isolation of endophytic bacteria with myriad morphological characteristic. All the isolates were individually evaluated for biosynthesis of nanoparticles and antimicrobial activity among which nine isolates expressed antimicrobial activity but one isolate exhibited both antimicrobial activity against all the test pathogens and was capable of extracellular synthesis of silver nanoparticles which is was subject of interest in the present investigation.

3.1. Biosynthesis of nanoparticles

When supernatant of the isolate upon challenging with 1mM silver nitrate solution resulted in production of silver nanoparticles with the preliminary confirmed by change in color of colorless solution of silver nitrate to brownish color further confirmation was carried out by spectroscopic technique with peaks between 410 to 440 nm as displayed in figure-2.

3.2. Antimicrobial evaluation

The preliminary screening of antimicrobial activity by dual culture inhibited the growth of test pathogen with perpendicular streak of isolate and agar over lay methods displayed clear zone in the lawn of test pathogenic bacteria laid over point inoculated endophytic isolate expressing the antimicrobial (Figure-3). The putative endophytic bacterium expressing significant activity against all the test pathogen upon fermentation and crude ethyl acetate extraction revealed antimicrobial activity via disc diffusion assay as zone of inhibition of test

pathogens across the ethyl acetate crude extract impregnated on the disc which was validated with the standard gentamycin.(table -1)

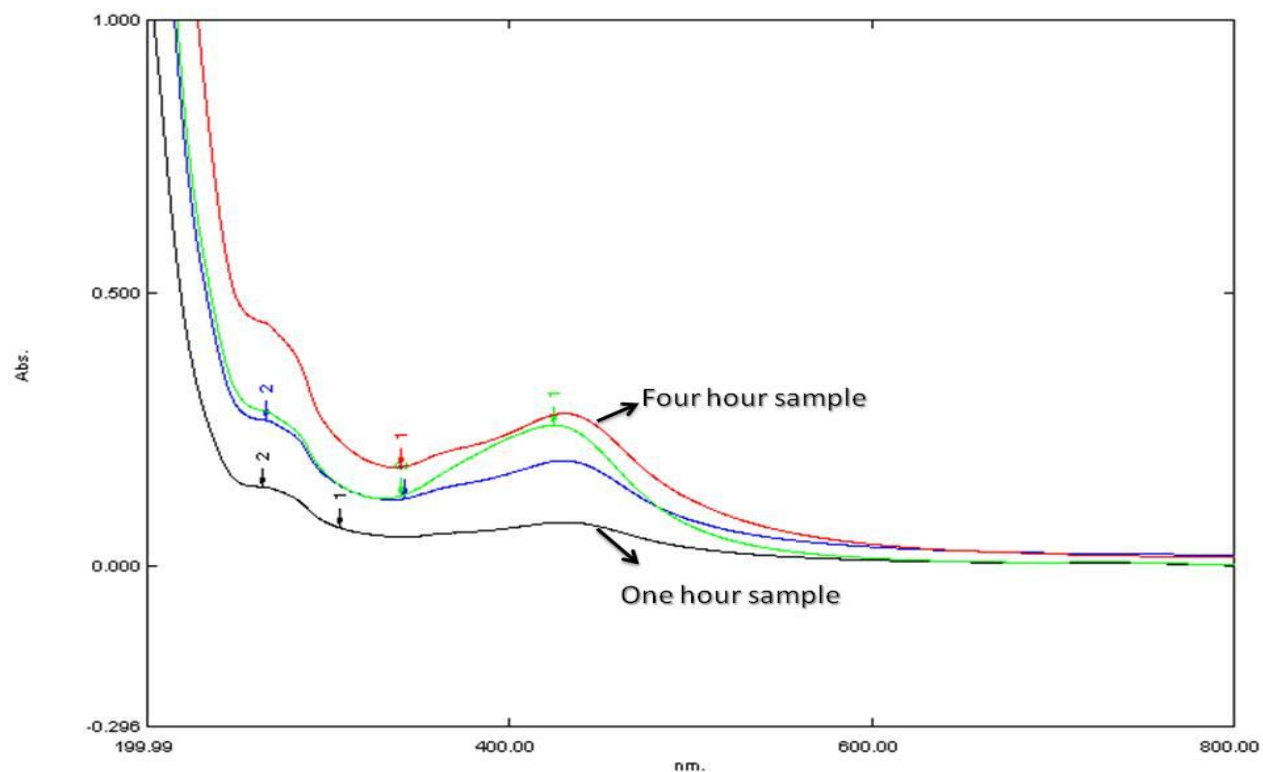


Fig. 2. Representing the spectral peak of synthesis of nanoparticles recorded at UV-Visible spectroscopy.

Table 1

Antibacterial activity measured as zone of inhibition at 50µl of ethyl acetate extracts and standard antibiotic Gentamycin on pathogenic bacteria.

| Test organism | Zone of Inhibition(mm) Crude extract | Zone of Inhibition(mm) Standard |
|--|---|------------------------------------|
| <i>Bacillus subtilis</i> (MTCC 121) | 28 ± 1.5 | 36 ± 1.5 |
| <i>Escherichia coli</i> (MTCC 7410) | 13 ± 1.5 | 28 ± 1.5 |
| <i>Proteus mirabilis</i> (MTCC 245) | 19 ± 1.5 | 30 ± 1.5 |
| <i>Shigella flexnerri</i> (MTCC 731) | 25 ± 1.5 | 33 ± 1.5 |
| <i>Staphylococcus aureus</i> (MTCC 7443) | 29 ± 1.5 | 37 ± 1.5 |
| <i>Xanthomonas campestris</i> (7908) | 12 ± 1.5 | 24 ± 1.5 |

Values are zone of inhibition in mean ± Standard error. Test cultures were procured from IMTECH-MTCC Chandigarh India.

Partial purification via bioautography assay, has found widespread application in the search for compounds bearing antimicrobial activity during which metabolites with antimicrobial activity will be displayed as clear inhibition zones on a red-colored. In present investigation test pathogen MRSA strain upon evaluation with bioautography assay resulted in two clear zone of inhibition at R_f value of 0.3, 0.6 indicated the separated band with antimicrobial compounds, on TLC chromatogram.

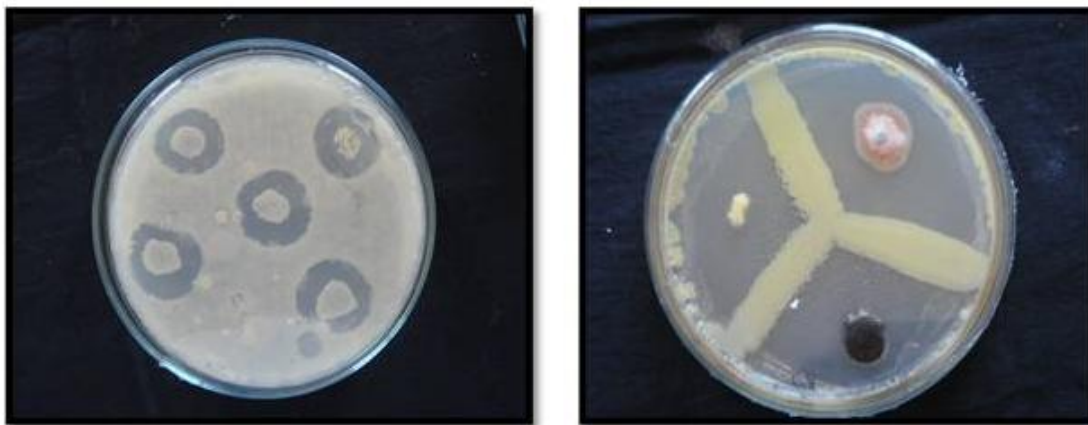


Fig. 3. Preliminary antimicrobial activity of bioactive isolate against test pathogens.

4. Discussion

As endophytes are presumed to have reported as feasible source of bioactive compounds and have become a subject of interest across the globe recent past due to its myriad activities in association with plants. Earlier reports confer of endophytes reported to synthesize nanoparticles, endophytic fungus *Aspergillus clavatus* be isolated from sterilized stem tissues of *Azadirachta indica* is reported to synthesize gold nanoparticles (Verma et al., 2010). Similarly *Penicillium* sp, isolated from the *Centellaasiatica* is reported to produce silver nanoparticles (Devi et al., 2012). Perusal of studies by far reports majority of endophytic fungi employed in synthesis of nanoparticles but in present study was devoted to screen bacterial endophytes and it is a preliminary study conferring the nanoparticles synthesis by isolated endophytic bacteria and further physicochemical characterization will be valuable in future to reveal the size and shape of synthesized nanoparticles. The present investigation crude ethyl acetate extract has expressed significant antimicrobial activity against the panel of pathogenic microorganisms including MRSA strain. The results are promising enough for further characterization of bioactive metabolite responsible for antimicrobial activity.

5. Conclusion

Conventional methods for nanoparticle synthesis are bound with various implications which have limited the use of conventionally synthesized nanoparticles in biomedical applications hence an accelerating haunt towards green chemistry principle for nanoparticle synthesis has expanded drastically the present study is a preliminary study confirming nanoparticle synthesis and evaluating the secondary metabolite of bioactive endophytic isolate for antimicrobial activity. Encouraging further study will be valuable enough to reveal any novel lead of pharmaceutical importance. At the same time further aim would be to bioconjugate the antimicrobial agent with nanoparticles to improve the efficacy the antimicrobial activity.

Acknowledgement

The authors are thankful to the University Grant Commission (UGC) for financial support and also would like to thank the Department of Studies in Microbiology, University of Mysore, for providing facilities.

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