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EXPLORATION OF ACTINOMYCETES ENDOPHYTICALLY ASSOCIATED WITH *PIPER NIGRUM* FOR POTENTIAL BIOACTIVITY

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ABSTRACT

Piper nigrum is well known for its metabolite richness. So endophytic microorganisms that reside within such environments can be expected to have promising biosynthetic potential. The current study identified three endophytic actinomycetes with broad bioactivity which can have applications in natural product related pharmacological research. The *Verrucosispora* sp identified in the study was found to have promising anticancer and antimicrobial activities and *Streptomyces* sp. was found to have antioxidant activity. The results obtained are supported by many previous reports and this suggests the isolates obtained in the study to have the possible presence of potential known or novel compounds with broad spectrum of activity.

Keywords: Endophytic actinomycetes, MTT assay, DPPH assay, 16S rDNA sequencing, *Streptomyces* sp., *Verrucosispora* sp.

INTRODUCTION

Black pepper (*Piper nigrum*), which belongs to the family *Piperaceae* is an ingredient of almost all important traditional medicines and is one of the ancient and important spice crops of India. The broad bioactivity of the plant is due to the presence of diverse metabolites including piperine, which is the principle ingredient of pepper (**Vijayakumar *et al.*, 2004**). There are many other terpenoids like pinene, sabinene, limonene, caryophyllene and linalool that contributes to the medicinal properties of the plant. These compounds can have varying pharmacological effects including anti-fertility (**Vladimirov *et al.*, 1991**), anti-inflammatory (**Lee *et al.*, 1984**), bioavailability of drugs (**Allameh *et al.*, 1992; Khajuria *et al.*, 2002**), stimulation of release of epinephrine (**Kawada *et al.*, 1988**) and elevation of levels of circulating thyroid hormones (**Tripathi & Tripathi, 1989**). Because of the presence of these kinds of diverse metabolites, the microbes that live inside the plant as endophytes can also expected to have same or similar or novel compounds with superior biological effects.

Endophytic microorganisms reside within the internal tissue of plants for whole or part of their life time without causing immediately overt negative effects (**Long *et al*., 2008**). As actinobacteria are known to have the capability to produce diverse range of secondary metabolites with antibiotic, antitumor and immunosuppressive properties, exploration of those actinomycetes of endophytic origin can have much applications (**Locci, 1989; Berdy, 2005**). There are many reports on the production of novel bioactive compounds by endophytic actinobacteria isolated from a wide range of plants like tomato, banana, wheat, and maize with promising antipathogenic activities (**Coombs & Franco, 2003; Castillo *et al.*, 2007**). However the actinomycetes which are endophytically associated with metabolically diverse plants like *P. nigrum* is least studied and can be a better source for metabolites with diverse biological activities with promising applications in drug development.

In the present study focus was made to isolate and identify endophytically associated actinomycetes from *P. nigrum* which resulted in the isolation of three distinct isolates. The isolates were identified by 16S rDNA sequence based method as PnA 1 (*Verrucosispora* sp.), PnA 2 (*Streptomyces* sp.) and PnA 3 (*Streptomyces clavatus*). These isolates were screened for the antibacterial, antioxidant and anticancer activities which showed its promising bioactivity. The diverse activity shown by the isolates can be due to the presence of various compound(s) with potential bioactivity.

MATERIALS AND METHODS

Isolation of endophytic actinomycetes from *Piper nigrum*

Stem pieces of *Piper nigrum* plants collected from Navajyothi Sree Karunakara Guru Research Centre for Ayurveda and Siddha, Uzhavoor, Kottayam were used as source material for the isolation of endophytic actinomycetes. The collected material was washed and cut into segments of 2-3 cm long and the surface sterilization was conducted as per the methods reported by **Jasim *et al.* (2013)**. The plant material was surface sterilized with 2% sodium hypochlorite for 10 min. and washed with sterilized distilled water. Further, they were treated with 70% alcohol for one min. followed by wash with sterilized distilled water. For sterility check, 0.1 ml aliquot from the final wash was inoculated to ISP 3, Glycerol Arginine agar (ISP 5), ISP 6 and Low Nutrient Mineral Salt (LNMS) agar amended with 50µg/mL nystatin and nalidixic acid to prevent fungal and bacterial growth. The surface sterilized stem pieces were placed on the above media with three replicates. The inoculated plates were incubated at room temperature for 5 to 7 days. The individual colonies from each plate was selected and sub-cultured further on respective media used for isolation. Those batches of experiments where the bacterial growth, if any present, in the control plate were completely discarded. In such cases the entire experiment was repeated by modifying the sterilization procedures. The colonies with different morphological characters were selected, purified and used for further studies.

Identification and characterization of the isolates

The isolates were identified and characterized by using 16S rDNA sequencing. For this, the DNA was isolated from all the strains. For the DNA isolation, the isolates were inoculated separately into 20 mL of Tryptone Yeast Extract Broth ($g.L^{-1}$ - Casein enzymatic hydrolysate 6g, Yeast Extract 3g, pH 7.2 ±0.2) and incubated for 7 days in a shaking incubator. After incubation the biomass of the isolates were harvested by centrifuging the culture at 8000 rpm for 10 min at 4^0C and the genomic DNA was isolated using genomic DNA isolation kit (Chromous Biotech). The presence of DNA in the eluted sample was confirmed by using 0.8% (w/v) agarose gel and it was used as the template for amplifying the 16S rDNA. The primers used for the PCR amplification were 27F (5'-AgA gTT TgA TCM Tgg CTC Ag-3') and 1492r (5'-TAC ggY TAC CTT gTT ACg ACT T-3') (**Coombs & Franco, 2003**). PCR was carried out in a 50 µL reaction volume containing 50ng of genomic DNA, 20picomoles of each primer (both forward and reverse), 1.25 units of Taq DNA polymerase (Bangalore Genei), 200µM of each dNTPs and 1X PCR buffer. PCR was carried out for 35 cycles in a Mycycler™ (Bio-Rad, USA) with the initial denaturation at 94^0C for 5 min, cyclic

denaturation at 94^{0}C for 30 sec, annealing at 58^{0}C for 30 sec and extension at 72^{0}C for 2 min with a final extension of 7 min at 72^{0}C. The amplified PCR product was visualized by electrophoresis in 1.2% (wt/vol) agarose gel incorporated with 2µl of ethidium bromide (10mg/ml) for confirmation of amplification. The PCR product was then gel purified and was used for sequencing. Sequencing PCR was carried out using the Big Dye Terminator Sequence Reaction Ready Mix (Applied Biosystem). The sequencing product was purified and sequence run was carried out in the DNA sequencer ABI 310 Genetic Analyzer. The sequence data obtained was further analyzed by BLAST (**Zhang *et al.*, 2000**). For the phylogenetic analysis, the related sequences were retrieved from NCBI. The selected sequences were first aligned with ClustalW program. The aligned data was boot strap replicates (**Tamura *et al.*, 2011**).

Fermentation and extraction of the crude metabolites

The isolates were grown in Tryptone yeast extract broth for 7 days in a shaking incubator. After incubation, the culture supernatant was obtained by centrifugation at 10000rpm for 10 minutes. The fermented broth was then acidified to pH 2 with 1N HCl. The acidified broth was then extracted twice with ethyl acetate and the solvent phase was collected. The solvent phase was then subjected to evaporation at 45^{0}C under vacuum in a rotary evaporator. The residual powder was reconstituted in 1.5mL methanol. Uninoculated broth extracted and evaporated and resuspended in methanol served as the control.

Screening the extract for antimicrobial activity

For the screening of antimicrobial activity, agar well diffusion method was used. A lawn culture of pathogenic bacteria (*Bacillus subtilis* (MTCC121), *Salmonella typhi* (MTCC734), *Salmonella paratyphi* (MTCC735), *Staphylococcus aureus* (MTCC96) and *Vibrio cholera* (MTCC3906) was made by using the inocula prepared by adjusting the turbidity of the suspension to match the 0.5 McFarland standard on Muller Hinton agar using a sterile cotton swab. After swabbing, wells were punched on the plates and 60µL of the corresponding extracts were added. The plates were incubated overnight at 37^{0}C and observed for zone of inhibition. The zone of inhibition was measured using a zone measuring scale.

Screening the extracts for antioxidant property

The antioxidant activity of the extracts was assessed on the basis of the radical scavenging effect by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) by modified method. The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid (5mg.mL^{-1}) prepared in methanol was used as standard. 150µL of DPPH (0.002%) prepared in methanol was mixed with varying concentration (5-50µL) of samples and standard was made up to 50µL using methanol. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using BIORAD micro-titre plate reader. Methanol with DPPH solution (0.002%) was used as blank. The optical density was recorded and % inhibition was calculated by using the following formula. Graph was plotted using the % inhibition values against the concentration and 50% inhibition (IC_{50}) for each samples were calculated.

$$\%\textbf{inhibition} = \frac{\textbf{Absorbance of control} - \textbf{Absorbance of Test}}{\textbf{Absorbance of control}} \times \textbf{100}$$

Screening the extracts for anticancer property.

HCT-15 colon carcinoma cell lines purchased from NCCS Pune maintained in Dulbecco's modified eagle's media (DMEM) and grown to confluency at 37^{0}C and 5% CO_2 was used for screening the anticancer property of the extracts. The cells were trypsinized by using 500µL of 0.025% (w/v) Trypsin in PBS for 2 minutes and aseptically transferred to a microtitre plate and incubated. After attaining 80% cell confluency, 5µL of sample extracts were added to corresponding wells and incubated for 24 hours. The anti-proliferative effect of crude extracts was determined by MTT cell viability assay as described by **Mosmann (1983)**. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in phosphate buffered saline at 5 mg/mL and was sterilized using filtration. After incubation the plates were washed with PBS and 200 µL of sterile MTT reagent was added to the wells and was incubated at 37^{0}C for 4 h in a CO_2 incubator. After incubation the optical density was measured at 540 nm for the formation of formazan during cell respiration using a microtitre plate reader.

$$\%\textbf{viability} = \frac{\textbf{Absorbance of test}}{\textbf{Absorbance of blank}} \times \textbf{100}$$

Statistical analysis

The results were analysed using statistical software Origin 7 (Northampton, MA, USA) by oneway ANOVA. *Post hoc* multiple comparison test was used to determine the significant difference among the experiments. The data is presented as mean±SD from three different experiments. $p < 0.05$ was considered significant (**Rihua *et al.*, 2011**).

RESULTS

Isolation of endophytic actinomycetes

Seven days incubation of surface sterilized stem pieces of *P. nigrum* resulted in the isolation of three morphologically distinct endophytic actinomycetes on various media. The isolates thus obtained were designated as PnA 1, PnA2 and PnA 3. Due to the absence of microbial growth on the representive control plate which contained the final wash of the surface sterilized material, the isolates obtained can be confirmed as endophytes. The obtained isolates were purified and subcultured in ISP 5 (Glycerol Asparagine Agar Base) medium for further analysis.

Identification of the endophytic actinomycetes

The PCR amplification of the 16S rDNA of the isolated endophytic actinomycetes was confirmed by agarose gel electrophoresis in which formation of band of approximately 1500 bp was observed. The 16S rDNA sequences obtained by the sequencing were submitted to NCBI under the accession number mentioned in **Table 1**. The obtained sequences were subjected to similarity analysis using NCBI BLAST which showed the isolate PnA 1 and PnA 2 to have 100% identity to *Verrucosispora* sp. (FJ999581) and *Streptomyces* sp. (HQ992748) respectively. However the isolate PnA 3 showed 99% similarity towards *Streptomyces carpaticus* (HQ711933). The phylogenetic analysis which was conducted using the sequence obtained along with the sequence retrieved from NCBI confirmed the BLAST result by distinct clustering of the isolates **(Figure 1)**.

Table 1 Identification of the endophytic actinobacterial isolates from *Piper nigrum* by 16S rDNA sequence similarity method.

Isolate name	Media used	Accession number	Similarity (%)	Closest match with accession number	Identified as
PnA 1	LNMS	KC193251	100	*Verrucosispora* sp. (FJ999581)	*Verrucosispora* sp.
PnA 2	ISP3	KC193252	100	*Streptomyces* sp. (HQ992748)	*Streptomyces* sp.
PnA 3	ISP6	KC193253	99	*Streptomyces carpaticus* (HQ711933)	*Streptomyces carpaticus*

Screening the extract for antimicrobial activity

The fermented broth of the isolates and the control were extracted, dried and reconstituted in methanol and was used for screening its antimicrobial activity. From the results, *Verrucosispora* sp. was found to inhibit the growth of all the five pathogenic bacteria with a zone diameter of 15 to 20mm. At the same time, *Streptomyces* sp. was found to have inhibitory effects on four pathogens namely *Bacillus subtilis, Salmonella typhi, Staphylococcus aureus* and *Vibrio cholerae*. However *Streptomyces carpaticus* had an inhibition against *Bacillus subtilis, Staphylococcus aureus* and *Vibrio cholerae* only (Table 2).

Table 2 Antimicrobial activity of actinomycetes endophytically associated with *P. nigrum*

Isolates	Test organism (inhibition zone in mm)				
	S. aureus	*V. cholerae*	*B. subtilis*	*S. para typhi*	*S. typhi*
PnA 1	17	20	19	15	21
PnA 2	13	15	16	0	14
PnA 3	12	16	12	0	0

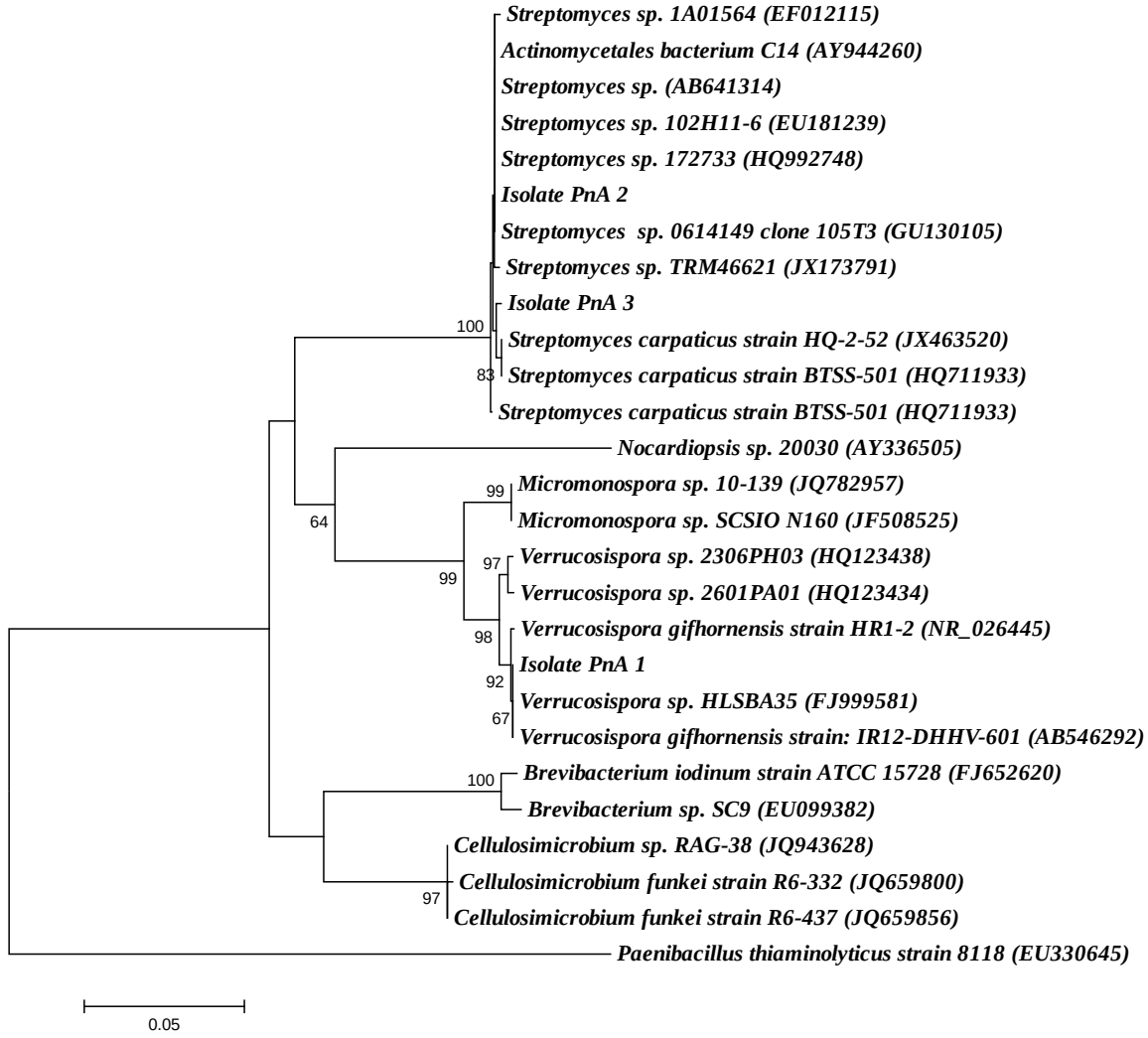

Figure 1 The phylogenetic analysis of partial 16S rDNA sequences of the endophytic actinomycetes isolated from *Piper nigrum* along with sequences from NCBI using MEGA 5 with neighbor joining method using 1000 bootstrap replicates.

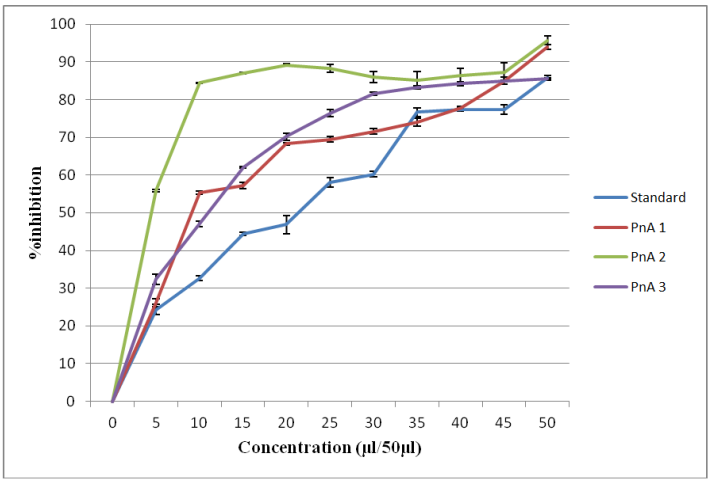

Figure 2 Graph showing the average % inhibition when analyzed for antioxidant activity of the crude methanolic extracts of the endophytic actinomycetes from *Piper nigrum* and standard at different concentration. Data presented as mean ± SD of three separate experiments.

Screening the extracts for antioxidant property

The screening of antioxidant activity was performed by DPPH assay. The % inhibition of the samples was calculated based on the formula absorbance obtained from the blank and the test. When this was plotted on a graph, the isolate PnA 2 showed maximum activity by getting the highest percentage of about 85% activity at around 10µL of the crude methanolic extract. In the case of extracts of other isolates (PnA 1 and PnA 3) and standard (5mg/mL ascorbic acid) similar percentage of inhibition was observed at 50µL sample (**Figure 2**). The results of IC_{50} clearly indicate the antioxidant capacity of isolate PnA 2 which needed only a very low concentration to attain the 50% inhibition which is very interesting (**Figure 3**). The mean and standard deviation of the 50% inhibition was summarized as table 3.

Table 3 Antioxidant activity of the endophytic actinomycetes isolated from *Piper nigrum*. Data is presented as mean ± SD of three separate experiments.

Sample	Name of sample	Antioxidant activity (50% Inhibition)
Standard	Ascorbic acid	21.3±1.5
PnA 1	*Verrucosispora* sp.	9.5±0.5
PnA 2	*Streptomyces* sp.	5.2±0.2
PnA 3	*Streptomyces carpaticus*	10.4±0.4

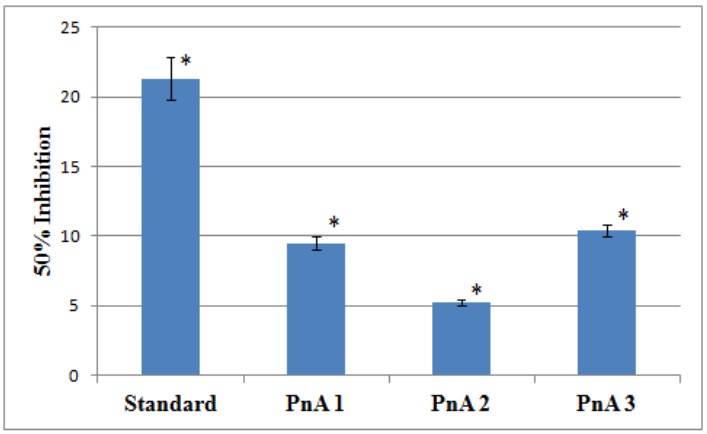

Figure 3 Graph showing the 50% inhibition when analyzed for antioxidant activity of the crude methanolic extracts of the endophytic actinomycetes from *Piper nigrum* when compared to the standard. Data represented as mean ± SD of three separate experiments. *p < 0.05.

Screening of the extracts for anticancer property

The screening of the antiproliferative activity of the extracts was performed on HCT-15 colon carcinoma cell lines by MTT assay. All the three isolates obtained were screened and all had promising antiproliferative activity. Among the isolates, extracts from PnA 1 showed the higher activity by getting the lowest % viability of about 48% when the cells were treated with 5µL of the methanolic extract. The % viability obtained is summarized as **Table 4** and based on the graph it is clear that the extracts from all the isolates showed significant antiproliferative effect when compared to the control (extract from uninoculated ISP 1 broth) (**Figure 4**). The obtained results were checked for its statistical significance using ANOVA and confirmed that p<0.05.

Table 4 Anticancer activity of the endophytic actinomycetes isolated from *Piper nigrum*. Data is presented as mean ± SD of three separate experiments.

Sample	Name of sample	Anticancer activity (% Viability)
Control	Uninoculated ISP 1 medium	98.36±1.64
PnA 1	*Verrucosispora* sp.	48.36±2.13
PnA 2	*Streptomyces* sp.	50±2.17
PnA 3	*Streptomyces carpaticus*	55.46±2.50

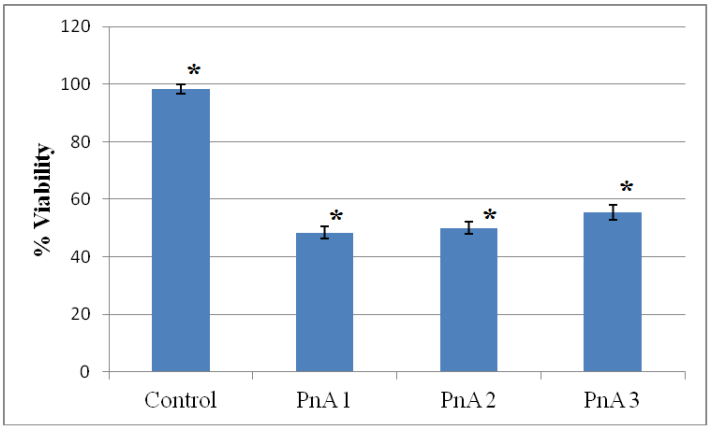

Figure 4 Graph showing the % viability of the cancer cells when analysed for anticancer activity of the crude methanolic extracts of the endophytic actinomycetes from *Piper nigrum* when compared to the standard. Data represented as mean ± SD of three separate experiments. *p < 0.05.

DISCUSSION

By occupying the unique chemical environment of host plants, the endophytic actinomycetes can expect to have much biosynthetic potential. In the present study, three actinomycetes were isolated and identified from surface sterilized stem portion of *Piper nigrum*. The endophytic actinomycetes were identified by PCR based method using 16S rDNA sequencing. The molecular identification of microorganisms by using 16S rDNA sequence based method is considered to be the most efficient and rapid method (**Clarridge, 2004**). The sequence similarity analysis using NCBI BLAST identified the isolates as *Verrucosispora* sp. (PnA 1), *Streptomyces* sp. (PnA 2) and *Streptomyces carpaticus* (PnA 3). The phylogenetic analysis conducted using MEGA5 also supported the result of the similarity analysis by forming distinct clustering of the isolates with related species (**Figure 1**). Eventhough only three isolates were obtained in the current study, they were found to have promising potential based on the available information from other members of the genus reported from other sources. The reports of **Houbo *et al.* (2012)** shows the isolation of novel strains of *Verrucosispora* sp endophytically associated with the marine plant *Thalassia hemprichii* (seagrass). Similarly many reports show the presence of novel biologically active *Streptomycetes* sp. associated with wide range of plants (**Castillo *et al.*, 2007**). Even some of them are known to have antiphytopathogenic properties as in the case of endophytic *Streptomyces* sp. with activity against *Colletotrichum musae* and *Fusarium oxysporum* (**Taechowisan *et al.*, 2005**). From these reports it can also be confirmed that the microorganisms of the same genus as obtained in the study are present as endophytic inhabitant of a wide range of plants, also previous reports of the same from *Piper nigrum* is very limited.

All the three organisms obtained were found to have potent antibacterial effect. The isolate PnA 1 which belongs to *Verrucosispora* sp. were found to be active against all the tested pathogens. *Streptomyces* sp. was found to inhibit *Bacillus subtilis, Staphylococcus aureus, Salmonella paratyphi* and *Vibrio cholerae* but *Streptomyces carpaticus* was inhibitory towards *Bacillus subtilis, Staphylococcus aureus* and *Vibrio cholera* only.

The genus *Verrucosispora* has gained much importance because of its ability to produce antibiotic abyssomicin which could inhibit p-aminobenzoic acid biosynthesis and also aminofuran proximicins (**Fiedler *et al.*, 2008; Riedlinger *et al.*, 2004**). There are also reports on *Verrucosispora* sp. from marine sources which has been explored for its ability to synthesise bioactive metabolites. But reports on exploration of endophytically associated *Verrucosispora* sp. is very limited. As the endophytic isolates live in a specific chemical environment, it may favour the isolates to develop biosynthetic machinery for the synthesis of still more interesting compounds possibly in a strain specific manner.

The studies of **Ghadin *et al.* (2008)** explain the high inhibitory effect of the extracts from *Streptomycetes* sp. towards both gram positive and negative organisms when compared with standard antibiotics that are in clinical practice. Also there are recent reports suggesting the synthesis of new antibiotics like alnumycin, munumbicins A to D, and coronamycins from endophytic *Streptomyces* sp. (**Qin *et al.*, 2009**). Some strains of *Streptomyces* sp. even have the ability to act on *Trypanosoma* sp., a protozoan that causes the sleeping sickness (**Zin *et al.*, 2011**). These reports can be intimation for the occurrence of still novel compounds in various unexplored endophytic *Streptomyces* sp. which may have the potential to replace even the drugs in practice.

The results of the antioxidant activity was highly interesting as the three isolates were having a distinct % inhibition pattern. From the % inhibition patterns it was clear that the isolate PnA 2 has the maximum activity by attaining 85% activity at around 10µL of the crude methanolic extract. Eventhough the extracts of other isolates (PnA 1 and PnA 3) and standard (5mg.mL^{-1} ascorbic acid) showed antioxidant property, they required a higher concentration of around 50µL for attaining a similar percentage of inhibition. This result was also supported by the IC_{50} result as the isolate PnA 2 showed a low concentration to attain the 50% inhibition. Free radicals and oxidants are toxic to the body as it has implications in the development of many human diseases including arthritis, inflammatory diseases, kidney diseases, cataracts, inflammatory bowel disease, colitis, lung dysfunction; pancreatitis; drug reactions, skin lesions, and aging (**Thenmozhi & Kannabiran, 2012**). Thus the identification of compounds having antioxidant properties are very important. A melanin pigment isolated from *Streptomyces* sp. with an excellent antioxidant property was reported by **Silambarasan *et al.* (2012)**. Many of the previous studies demonstrates the ability of *Streptomyces* to synthesise a wide range of compounds with antioxidant activity like isoflavonoids (**Komiyama *et al.*, 1989**), diphenazithionin (**Hosoya *et al.*, 1996**), dihydroherbimycin A (**Chang & Kim, 2007**), poly-saccharide (**He *et al.*, 2008**) and protocatechualdehyde (**Kim *et al.*, 2008**). These reports clearly supports the results obtained in the current study and provides a background indication that the isolates obtained in the study can be expected to have known or novel bioactive compounds with wide pharmacological applications.

When the Colon carcinoma cell line HCT-15 was treated with the extracts of the isolates, PnA 1 showed the higher activity by reducing the viability of the cancer cells to about 48% even when they were treated with 5µL of the methanolic extract. The extracts of the other two isolates also showed inhibitory property to the cells, but with a lower activity. Strains of *Verrucosispora* are the focus area for researchers as they are the source for a wide array of novel bioactive compounds. Reports suggest that Proximicins, a compound produced by a marine *Verrucosispora* sp. can have strong cytostatic effect towards various human tumor cell lines (**Fiedler *et al.*, 2008**). Also these organisms have gained much importance after the demonstration of its ability to synthesise diterpene gifhornenolones A and B by specific *Verrucosispora* sp. (**Shirai *et al.*, 2010**). The whole genome sequence of marine *Verrucosispora* sp. suggests the presence of around 23 biosynthetic gene clusters that encodes the production of known and predicted secondary metabolites (**Roh *et al.*, 2011**). This indicates that the isolate obtained in the study can be expected to have the ability to produce more diverse

metabolites which can be used as novel candidates for development of drugs with potential applications.

The assay on antiproliferative effects of Streptopyrrolidine and Daryamides isolated from marine *Streptomyces* on human colon carcinoma cell line HCT-116 confirms potent activity of the isolates. The assessment of cytotoxicity is very important as it is a crucial step in the identification of novel drugs which can be considered for clinical application (**Saurav & Kannabiran, 2011**). The potential of *Streptomyces* sp. for the production of bioactive compounds were discovered decades before. But the strain specific biosynthetic variations and endophyte specific biosynthetic potential acquired by them is least studied. This highlights the importance of screening actinomycetes present in a metabolite rich plant like *Piper nigrum*.

CONCLUSION

In the current study three endophytic actinomycetes were isolated from the stem region of *Piper nigrum*. All the three isolates were found to have antibacterial, antioxidant and anticancer activity. However the isolate PnA 1 identified as *Verrucosispora* sp was found to have higher anticancer and antimicrobial activity and PnA 2 identified as *Streptomyces* sp. showed excellent antioxidant activity. The obtained results are supported by many reports as several bioactive metabolites have been identified from related isolates.

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Conflict of interest statement: We declare that we have no conflict of interest.

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