Available online at www.ijpsdr.com

International Journal of Pharmaceutical Sciences and Drug Research
2017; 9(3): 113-117

Research Article

ISSN: 0975-248X
CODEN (USA): IJPSPP

Isolation of Endophytic Bacteria, Bioactive Compounds and Its Antiviral Activity against *Herpes Simplex Virus* Type -1

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ABSTRACT

Microbial community has been a major part in the drug industry. They are known for their effectiveness and do not cause any undesirable effects. Exploitation of bacteria could be fruitful for mankind. Bacteria play a major role in producing useful bioactive compounds. HSV-1 infection causes 80% of oral lesions. A majority of the population is infected by at least one herpes subtype of HSV-1 before adulthood. The infection in some cases does not produce any symptoms. Drugs for HSV-1 are becoming suppressive. In the present study, isolation of endophytic bacteria was carried out from medicinal plant. A grown bacterium was identified and confirmed using molecular 16S rRNA sequencing followed by extraction of bioactive compounds using solvents. Twelve bioactive compounds were then investigated for *in vitro* cytotoxicity assay and *in vitro* antiviral assay. Chloroform extract was found to be effective in inhibiting the virus.

Keywords: Endophytic bacteria, 16S rRNA, Cytotoxicity, Antiviral, Chloroform, Aqueous ethanol.

DOI: 10.25004/IJPSDR.2017.090303 **Int. J. Pharm. Sci. Drug Res. 2017; 9(3): 113-117**

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Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Received: 11 April, 2017; **Revised:** 21 April, 2017; **Accepted**: 23 April 2017; **Published:** 29 May 2017

INTRODUCTION

Endophytic bacteria are found in almost all plants. They usually live inside the plant tissues of the host plant which could be symbiotic, mutualistic, commensalistic and trophobiotic. Phyllosphere forms the basis for the majority of endophytes. [1] Plant-microbe interactions promote plant health and development. Endophytes have been defined as organisms that are able to colonize living plant tissues without harming the plant or gaining benefit other than securing residency. [2] They can be isolated from surface disinfested plant tissue or extracted from within the plant and do not visibly harm the plant. [3] The bacteria have been found in most plants, colonize the internal tissues and construct diverse relationships with their host plants. [4] They can promote plant growth and

yield and can act as biocontrol agents. They can also be beneficial to their host by producing a range of natural products that could be harnessed for potential use in medicine, agriculture or industry. In addition, it has been shown that they have the potential to remove soil contaminants by enhancing phytoremediation and may play a role in soil fertility through phosphate solubilization and nitrogen fixation.

The use of 16S rRNA gene sequencing for definitive microbial identifications requires a harmonious set of guidelines for interpretation of sequence data that needs to be implemented so that results from one study can be accurately compared to another. The usefulness of 16S rRNA gene sequencing as a tool in microbial identification is dependent upon two key elements, deposition of complete unambiguous nucleotide sequences into public or private databases and applying the correct "label" to each sequence. [5]

HSV-1 is usually associated with infections above the waist. In underdeveloped parts of the world, the prevalence of antibody to HSV -1 is greater than 90% by 2 years of age, which reflects crowded living conditions. The virus is present in oropharyngeal secretions, spreads by direct person to person contact and presumably requires salivary exchange or at least salivary contact with a susceptible body surface. The most common spread is mouth to mouth; however mouth to skin contact, resulting in herpetic whitlow or type 1 genital herpes infection does occur. When people encounter certain stresses, emotional or physical, the virus may reactivate and cause new sores and symptoms. The following factors may contribute to recurrence: stress, ultraviolet light, fever, fatigue, hormonal changes immune depression, and trauma to a site or a nerve region where previous HSV infection occurred.

Many therapies have been suggested for HSV infections. In virtually all cases, subsequent double – blind randomized trials revealed the treatments to be ineffective. Acyclovir (ACV) is the exception and is a most effective anti- HSV drug. [6] Viruses are becoming acyclovir resistant these days, so the aim of the study was to provide an effective solution against resistant viruses without weakening the immune system.

MATERIALS AND METHODS

Collection of Plant Materials

The leaves of *Argemone mexicana* without any spots were collected in sterile, labelled polythene bags and were processed within 24 hours. [7] The Plant was identified and authenticated in the Department of Botany, R.K.M Vivekananda College, Mylapore, Chennai-4.

Surface Sterilization of Plant Material

The specimens were washed under tap water and bisected into small pieces covering all sides. The bisected leaf pieces were then dipped in 70% ethanol for 5 seconds, the leaf pieces were then transferred to the sterile Petri dishes containing 4% Sodium hypochlorite (Naclo) and were dipped for 1 minute. The leaf pieces were rinsed in sterile water for 10 seconds and the excess moisture was blotted on a sterile filter paper. [8]

Isolation and identification of bacteria

The sterile leaves were crushed with sterile distilled water using mortar and pestle. The crushed leaves extract were spreaded in Petri dishes containing Nutrient Agar medium. [9] The sterile colonies were picked up and were carefully subcultured into fresh petriplates containing Nutrient agar medium. The isolate was identified using molecular 16S rRNA sequencing. An isolated bacterial colony was picked and suspended in 1ml of sterile water in a microfuge tube and was centrifuged for 1 minute at 10,000 rpm to remove the supernatant. 200µl of Insta Gene matrix was added to the pellet and incubated at 56°C for 15 minutes. It was vortexed at high speed for 10 seconds and the tube was placed in a 100°C heat block for 8 minutes. Finally, the contents were vortexed at high speed for 10 seconds and spun at 12,000 rpm for 2 minutes. In result, 20µl of the supernatant was used per 50µl PCR reaction. Universal primers gene fragment was amplified using MJ Research Peltier Thermal Cycler.

1µL of template DNA was added in 20µL of PCR reaction solution. 27F/1492R primers were used for bacteria, and then PCR reaction was performed with initial denaturation at 94°C for 2 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60 sec, and 72°C for 60 sec and final extension was at 72°C for 10 min. 1,400bp DNA fragments were amplified. *E. coli* genomic DNA was used as Positive control and a negative control in the PCR. Unincorporated PCR primers and dNTPs were removed from PCR products. The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Sequence data was aligned and analyzed for identifying the sample The 16s rRNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of the present sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment. MUSCLE 3.7 was used for multiple alignments of sequences. [10] The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise). [11] Finally, the program PhyML 3.0

aLRT was used for phylogeny analysis and HKY85 as Substitution model. PhyML was used to estimate maximum-likelihood phylogenies Tree Dyn 198.3 was used for tree rendering. [12]

Preparation of Bacterial extracts and separation of its compounds

Bacterial isolate was inoculated in an Erlenmeyer flask containing Nutrient broth and incubated for 72 hours. The fermentation flask was incubated at 110 rpm on a rotary shaker at room temperature for 7 days. [13] After fermentation the culture broth was filtered and the filtrates were added with solvents chloroform and aqueous ethanol separately in a ratio of 1:1. Chloroform added culture broth formed two layers; the solvent layer was separated using separating funnel and stored in sterile vials. Aqueous ethanol added culture broth was retained as aqueous extract.

GC-MS Analysis of Bioactive compounds

Bioactive metabolites were analyzed by Gas chromatography coupled mass spectrometry (GC-MS) to identify the compounds present. GC-MS analysis was performed in a JEOL GCMATE II GC-MS (Agilent Technologies 6890N Network GC system for gas chromatography). The column (HP5) was fused silica 50 m × 0.25 mm I.D. Analysis conditions were 20 min. at 100°C, 3 min at 235°C for column temperature, 240°C for injector temperature, helium was the carrier gas and split ratio was 5:4. The sample (1 µl) was evaporated in a split less injector at 300°C. Run time was 22 min. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were confirmed.

Cell line

In vitro cytotoxicity assay of bacterial secondary metabolites was carried out on monolayer cell culture made from Human Epithelial Type 2 (HEp-2) obtained from National Centre for Cell Sciences, Pune. Subculture of HEp-2 cell line was carried out in rectangular canted neck culture flasks using Eagle's minimum essential medium (MEM) (Sigma) with 5% v/v Foetal bovine serum (FBS) and Penicillin, Streptomycin, Amphotericin-B, L-Glutamine, Sodium bicarbonate. HEPES (2-[4-(2-Hydroxyethyl)1-piperzinyl ethane sulphonic acid) buffer was used. [14-15] Appropriate volume of CO_2 was passed into the medium till the pH reached the optimum range (i.e.) 7.1 - 7.5.

In vitro cytotoxicity assay by MTT method

Assaying the toxicity of the bacterial extract on cell line was carried out in the Tissue Culture 96 well microtitre plates. [16] MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide was dissolved in Phosphate - buffered saline at 5 mg/ml. After the incubation period, 20µl of MTT solution was added to all wells and plates were incubated at 37°C at 5% CO_2 atmosphere for 4 hours. After 4 hours, 100µl of DMSO was added to wells and incubated for 10 minutes. The plates were read on a Microelisa reader, using a test wavelength of 540 nm. [17]

In vitro antiviral Assay

Monolayer of HEp-2 cells was grown in 96 well microtitre plates. 0.1 ml of $10^{-6.5}$/ml viral suspension of National Institute of Virology (NIV), Pune was diluted in 2% FBS MEM, and added to the wells of microtitre plates. 0.1 ml of 2% FBS maintenance medium alone was added into cell control. The 96 well microtitre plates for HSV-1 was incubated at 37°C in 5% CO_2 atmosphere for 90 minutes to facilitate adsorption of virus to the cell line. After 90 minutes, bacterial extracts were added to the wells and incubated for 96 hours.

RESULTS AND DISCUSSION

Endophytic bacteria isolated from *Argemone mexicana* leaf was identified using 16S rRNA sequencing. DNA was isolated and amplified for 16S rRNA region using Polymerase chain reaction. The amplified 16S rRNA gene fragments were about 1453 bp in length. The 16S rRNA genes were sequenced and compared with BLAST (NCBI). The bacterium was identified as *Micrococcus luteus*. The sequence submitted to the National Center for Biotechnology Information (NCBI) was given an accession number of KT732286.

GC-MS chromatogram of the chloroform extract showed 5 peaks indicating the presence of five Bacterial constituents. On comparison of the mass spectra of the constituents with the NIST library the five microbial constituents were characterized and identified as Phenol, 2, 4-bis (1, 1-dimethylethyl)-; Pyrrolo (1, 2-a) pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)-; 7, 9-Di-tert-butyl-1-oxaspiro(4, 5)deca-6, 9-diene-2, 8-dione; Propanol, 3-(1, 3, 5-trimethyl-2, 6-dioxocyclohexyl). Out of these Pyrrolo (1, 2-a) pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)- resulted twice (Table 1).

GC-MS chromatogram of the aqueous ethanol extract showed 6 peaks indicating the presence of six Bacterial constituents. On comparison of the mass spectra of the constituents with the NIST library the five microbial constituents were characterized and identified as 1H Pyrrolo [3, 4-c]pyridine-1, 3,(2H)-dione, 4-amino-6, 7-dimethyl-; Cyclopenta [c] pentalen-3 (3aH) one, 1, 2, 5a, 6, 7, 8-hexahydro-6, 6-dimethyl; 5Aminosalicyclic acid, 3-isobutyl-; Acetic acid, 17 (1, 5-dimethylhexyl)-10, 13-dimethyl-3-oxohexadecahydrocyclopenta[a] phenanthren-2-yl ester; 10-Myrtenone, 10-(9-anthryl)-3-(á-methylbenzylamino)-; Gamabufotalin (Table 2).

The maximum toxic free concentration of chloroform extract was 25µg/ml. The aqueous- ethanol extract was found to be non toxic at 50µg/ml (Table 3). The anti HSV-1 activity of chloroform extract resulted at 25µg/ml. The aqueous-ethanol extract showed anti HSV-1 activity at 50µg/ml. The Minimum inhibitory concentration of Acyclovir was exhibited at 25µg/ml (Table 4).

Table 1: Chloroform extract compounds of *Micrococcus luteus*

S. No	Retention Time	Chloroform extract compounds of *Micrococcus luteus*	Molecular Formula	Molecular Weight	Peak Area %
1.	11.57	Phenol,2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206.323	52.34
2.	15.73	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.272	13.89
3.	16.26	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O3$	276.370	6.48
4.	16.63	Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210.272	15.26
5.	16.86	Propanol,3-(1,3,5-trimethyl-2,6-dioxocyclohexyl)	$C_{12}H_{18}O_3$	210.270	12.01

Table 2: Aqueous Ethanol extract compounds of *Micrococcus luteus*

S. No	Retention Time	Aqueous Ethanol extract compounds of *Micrococcus luteus*	Molecular Formula	Molecular Weight	Peak Area %
1.	4.62	1H Pyrrolo[3,4-c]pyridine-1,3,(2H)-dione,4-amino-6,7-dimethyl-	$C_9H_9N_3O_2$	191.186	23.44
2.	12.15	Cyclopenta[c]pentalen-3(3aH) one,1,2,5a,6,7,8-hexahydro-6,6-dimethyl-	$C_3H_{18}O$	190.281	31.21
3.	12.6	5Aminosalicyclic acid,3-isobutyl-	$C_{11}H_{15}NO_3$	209.241	3.94
4.	14.27	Acetic acid,17 (1,5-dimethylhexyl)-10,13-dimethyl-3-oxohexadecahydrocyclopenta[a]phenanthren-2-yl ester	$C_{29}H_{48}O_3$	444.689	7.96
5.	15.47	10-Myrtenone,10-(9-anthryl)-3-(á-methylbenzylamino)-	$C_{32}H_{31}NO$	445.594	22.00
6.	19.15	Gamabufotalin	$C_{24}H_{34}O_5$	402.52	11.42

Table 3: Cytotoxicity Profile of *Micrococcus luteus* extracts

S. No.	Concentrations (µg/ml)	Cytotoxicity		Cell Control	Acyclovir
		Chloroform	Aqueous Ethanol		
1.	100	+	+	-	+
2.	50	+	-	-	+
3.	25	-	-	-	-
4.	12.5	-	-	-	-
5.	6.25	-	-	-	-
6.	3.12	-	-	-	-
7.	1.56	-	-	-	-

(+) → Presence of cytotoxicity; **(–)** → Absence of cytotoxicity

Table 4: Antiviral activity of *Micrococcus luteus* extracts

S. No.	Concentrations (µg/ml)	Cytopathic Effect		Virus Control	Cell Control	Acyclovir
		Chloroform	Aqueous Ethanol			
1.	100	NP	NP	NP	NP	NP
2.	50	NP	+/-	+	-	NP
3.	25	+/-	+	+	-	-
4.	12.5	+	+	+	-	+
5.	6.25	+	+	+	-	+
6.	3.12	+	+	+	-	+
7.	1.56	+	+	+	-	+

(+) → Presence of cytopathic effect, **(–)** → Absence of cytopathic effect, (+/-) → 50% of cytopathic effect, NP → Not performed

Phenol, 2, 4-bis (1, 1-dimethylethyl) - had resulted in a retention time of 11.57, whereas the same compound had resulted in a retention time of 16.68 from *Alternaria* sp. This compound had been reported to have antimicrobial activity. [18] Chloroform extract of *Micrococcus luteus* resulted a compound Pyrrolo [1, 2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), the same compound was obtained from ethyl acetate extract of *Micrococcus luteus* and reported to be having good anti cancerous activity. [19] 7, 9-Di-tert-butyl-1-oxaspiro (4, 5) deca-6, 9-diene-2, 8-dione had been reported in ethyl acetate extract of *Euphorbia pulcherrima* plant as flavonoids. [20] Chloroform and aqueous ethanol extracts of endophytic bacterial compounds proved to be effective in inhibiting the virus. Separation of single compounds would promote the present study to next level.

ACKNOWLEDGEMENTS

We are honoured to thank Secretary and Principal Ramakrishna Mission Vivekananda College (Autonomous), Chennai, India for providing all facilities. We specially thank Department of Science and Technology (DST) and the Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Chennai, India for GC-MS studies and validation of the results. We are thankful to National Centre for Cell Science (NCCS), Pune, India for providing Cell lines. We acknowledge the help of Dr. S. Rajarajan, Vice Chancellor, SRM University, Sonepat, Haryana, India and Dr. S. Sasikala, P.G & Research Dept. of Microbiology and Biotechnology, Presidency College (Autonomous), Chennai, India in carrying out antiviral assay.

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HOW TO CITE THIS ARTICLE: Anantha Padmanabhan S, John Wyson W, Marimuthu S, Saravanan P, Anand D. Isolation of Endophytic Bacteria, Bioactive Compounds and Its Antiviral Activity against *Herpes Simplex Virus* Type -1. Int. J. Pharm. Sci. Drug Res. 2017; 9(3): 113-117. **DOI: 10.25004/IJPSDR.2017.090303**