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RESEARCH ARTICLE

Extraction and Identification of some metabolites produced by antagonistic apple plant bacteria *Brevibacterium halotolerans*

Hassan A. Ahmed*[1], Weaam Ebrahim[2,3], Peterson A. Mikhailovna[1], Birgit Henrich,[4] Peter Proksch[2]

1. Department of Microbiology and Plant Physiology, Faculty of Biology, Saratov State University, 83 Astrakhanskaya Street, 410012 Saratov, Russia
2. Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, 40225 Duesseldorf, Germany
3. Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, 35516 Mansoura, Egypt
4. Institute of Medical Microbiology and Hospital Hygiene, University Clinic of the Heinrich-Heine University, 40225 Duesseldorf, Germany

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Corresponding Author

Hassan A. Ahmed

Abstract

Many microorganisms are constantly selected by researchers for their antagonistic activity in *in vitro* or in plant screening systems *Brevibacterium halotolerans* strain have antagonistic activity against bacterial and fungal pathogens was isolated from 16 different apple plant stem samples from Golden delicious apple fields at Volga region, Saratov city, Russia. Bacterial secondary metabolites are believed to represent an important tool in chemical interaction and communication. Chemical metabolite analyses of *B. Halotolerans* indicated that all identified metabolites were likely responsible for the antagonistic activity and indicates that bioactivity may play an ecological role in these habitats. This isolate (SA87) morphologically and Molecular characterization performed by 16S rRNA gene sequence analysis and it was confirmed as *Brevibacterium halotolerans.* Among the secreted extract seven components, named Neurolenin B (1), Cyclopenin (2), Auronitol (3), Palitantin (4), Malformin A (5), Pestalotiopisorin A (6) and 2-hydroxy-3-methylbenzoic acid (7) were isolated from apple associated endophytic bacteria (SA87) cultivated in solid rice medium. Bacterial metabolites and their structures were elucidated by spectroscopic methods, including High performance liquid chromatography HPLC. And Liquid chromatography–mass spectrometry LC-MS

INTRODUCTION

After the initial description of the genus *Brevibacterium* by Breed (1953), the genus served as a depository for various Gram-positive rods later shown in chemotaxonomic studies to belong to different coryneform genera (Collins) 1992. The description of the genus was consequently emended to embrace only those species that correspond to the type species, *Brevibacterium* linens, in genus-specific characteristics (Collins et al., 1980).

B. halotolerans is an alkaliphilic bacterium (Takami and Horikoshi, 1999) that can grow well at pH 7–10.5 in saline environments. *B. halodurans* is well characterized physiologically, biochemically, and genetically (Horikoshi, 1999; Takami and Horikoshi, 1999). *B. halotolerans* It is able to inhibit the growth of both Gram-positive and Gram-negative pathogens such as *Staphylococcus aureus* (human pathogen) and *Vibrio anguillarum* (fish pathogen) (Gram, L et al., 2010 and Wietz, M. et al., 2010). This specific strain has been shown to produce a variety of secondary metabolites with distinct bioactivities (Mansson, M. et al., 2011). It produces holomycin, a known antibiotic (Wietz, M. et al., 2010), as well as a series of ngercheumicin analogues (Kjaerulff, L 2013) (Adachi, K. Kawabata, Y. Kasai, H. Katsuta, M. and Shizuri, Y. 13 September 2007, Japanese patent application JP 2007–230911 A) and most interestingly two novel cyclodepsipeptides named solonamide A and solonamide B (Mansson, M. et al., 2011).

Bioactive secondary metabolites represent a keystone in microbial interactions (Hibbing, et al. 2010), but also have biotechnological potential as antibiotics, biosurfactants, antiviral, or anticancer agents (Demain and Sanchez, 2009). Antagonistic bacteria from agriculture and marine environments (Nair and Simidu, 1987; Long and Azam, 2001; Gram, et al. 2010) are a still comparatively untapped resource of bioactive natural products. The structural diversity and broad activity spectrum of bioactive compounds (Debbab, et al. 2010) highlight the potential of novel discoveries among microorganisms, advising the investigation of largely unexplored environments. Antagonistic traits in bacteria have been mainly investigated in Antarctic strains, describing antimicrobial activities with *Actinobacteria* (O'Brien, et al. 2004, Lo Giudice, et al. 2007), bacilli and enterobacteria (Shekh, et al. 2010), and different *Gammaproteobacteria* (O'Brien, et al. 2004).

Also, cyanobacteria from benthic mats produced multiple antimicrobial compounds (Biondi, et al. 2008). Structure-elucidated antibiotics include phenazines from *Pseudomonas* (Jayatilake, et al. 1996), aromatic nitro compounds from *Salegentibacter* (Al-Zereini, et al. 2007), and an angucyclinone from *Streptomyces* (Bruntner, et al. 2005). The present study aimed at the isolation of antagonistic bacteria and bioactive secondary metabolites from apple tree environments, a so far virtually unexplored habitat. We report the isolation of eleven bioactive isolates from apple tree, samples to the understanding of antagonistic traits among bacteria, and suggest a potential ecological role of the antibiotic compounds.

MATERIALS AND METHODS

Collection of environmental samples

Samples from apple Plant (*Malus domestica*) were collected in 2014 from different areas in Saratov, Russian Federation. These specimens were identified by Dr. Alexandra Mikhailovna, Department of Plant Physiology and Microbiology, Faculty of Biology, Saratov State University. Small stem and leaf pieces were cut from the apple plants and placed in plastic bags after any excess moisture was removed. Every attempt was made to store the materials at 4° C until isolation procedures could be instituted.

Isolation of endophytic bacteria from Apple

After proper drying, infected plant materials, to isolate microorganisms we applying 10 sterile swabs to take inoculums from 10 plant material then added to sterile 10 test tubes containing 1 ml sterile physiological saline solution (0.90%), mix swab well in these amount of saline, 0.1 ml from mixture was streaked on the LB medium for bacteria and potato dextrose agar (PDA) media for fungi, and diluted to 1:10-2 and 1:1-4 and the plates were incubated at 28°C for 48-72 hrs. Bacterial and Fungal colonies that appeared frequently and looked morphologically different were randomly selected and purified. Each isolate was stored in slants and keeping in refrigerator at 4 ºC for next use.

Phenotypic Identification

The dominant pure colonies from the collected samples were picked up based on the color, colony morphology and identified by morphological, biochemical and physiological confirmation tests at genus level following standard procedure described in Bergey's manual of determination bacteriology (Krieg and Holt, 1984, and Staley, et al., 1989).

Identification of bacteria by sequencing the 16S rRNA gene fragment

DNA extraction

Bacterial DNA isolation has been achieved by using DNeasy® Mini Kit (QIAgen). The lyophilized bacterial cells were pulverized and disrupted with the help of glass beads. Then cell lysis was carried out by addition of lysis Buffer AP-1 and RNAse-A solution followed by incubation of the mixture at 65 ºC. The remaining detergent, The lysate was then transferred to a new tube. An adequate volume of ethanolic Buffer AP3/E was added to the lysate and the mixture was then applied to DNeasy Mini Spin Column. After centrifugation, the filtrate was discarded. The column was washed by addition of ethanolic Buffer AW followed by centrifugation. Another portion of Buffer AW was added to the column and centrifuged at maximum speed to dry the membrane in the column from residual ethanol. Bacterial DNA, which is incorporated into the membrane, was eluted by addition of Buffer AE directly to the membrane in the DNeasy column. The column was then incubated at room temperature for 5 minutes and then centrifuged to collect the filtrate, which was the fungal DNA dissolved in Buffer AE.

The amplification of DNA

After the isolation process, the isolated DNA was then amplified by Polymerase Chain Reaction (PCR). The PCR was carried out using HotStarTaq Master Mix Kit (QIAgen). The Master Mix contains HotStarTaq®DNA polymerase, PCR buffer (with MgCl2) and dNTPs. 16S F1(with base sequences AGAGTTTGATCNTGGYTCAG) and 16S R2 (with base sequences CCGTGAATTCHTTTRAGTTT) (Invitrogen), as primers, were mixed with Hotstar Taq Master Mix Kit and DNA template. Thus, each PCR reaction mixture contained 5- 10 ng of genomic DNA, 1 µM each of the primers 16S F1and 16S R2, and 1 U of Hot start Taq- Polymerase (Invitrogen) in a total volume of 50 µL. The mixture was then applied to the thermal cycler (BioRad) using the programmed PCR cycle as outlined below:

- Initial activation step in 95º C for 15 minutes to activate HotStarTaq® DNA polymerase
- Cycling steps which were repeated 35 times: Denaturing: 1 minute at 95º C, annealing: 1 minute at 56º C, extension: 1 minute at 72º C
- Final extension for 10 minutes in 72º C.

Purification of PCR products and DNA sequencing

The PCR product was purified using 2% Agarose-Gel-Electrophoresis at 75 V for 60 minutes in TBE buffer. The agarose gel was then stained using 1% ethidium bromide. A 500 bp stained DNA fragment was then excised from the agarose gel. The next step of PCR product purification was performed using Perfectprep® Gel Cleanup Kit (Eppendorf). The binding buffer was mixed to the PCR product and incubated at 50º C for 10 minutes in an eppendorf thermomixer at 1000 rpm. The mixture was mixed with a volume of isopropanol and then centrifuged. The filtrate was discarded and the column was washed with wash buffer twice followed by centrifugation.

Amplified Bacterial DNA (PCR product), which was incorporated into the column, was eluted by addition of elution buffer or molecular biology grade water to the center of the column. The column was then centrifuged to collect the filtrate, which was the bacterial DNA dissolved in elution buffer. The amplified bacterial DNA was then submitted for sequencing by a commercial service (Prof. Birgit Henrich Institute of Medical Microbiology and Hospital Hygiene Heinrich-Heine-University Moorenstr. 5 / Bdg. 22.21 40225 Duesseldorf) and the base sequence was compared with publicly available databases such as GenBank with the help of Blast-Algorithmus.

***In vitro* screening of isolates for antagonism**

Many bacteria with the capacity of colonizing plants utilize the nutrients and most of them might even actively switch from root, stem and leaf surface to endophytic lifestyles Figure 1. This bacteria comprise several well characterized species of *Bacillus* and *Pseudomonas* and a number of metabolites, particularly lipopeptides synthesized by non-ribosomal peptide synthesases. Bacterial isolate was screened *in vitro* against *Fusarium tricinctum* (A) and *Tricoderma harzianum* (B) Figure 2. By applying a dual culture technique, one 6-mm diameter of *Bacillus* agar plug was placed on the edge of PDA medium in a Petri dish with 11 cm diameter. Simultaneously, a 5 mm mycelial plug cut from the edge of a 7 day-old culture of the fungal strain was placed at the another side of the plate. After 7 days at 28°C the inhibitory effect on fungal growth was evaluated. All *in vitro* antagonism assays were made in triplicate.

Extraction of secondary metabolites

Mass growth of pure bacteria as well as isolation and identification of secondary metabolites was carried out by transferring fresh bacterial culture into Erlenmeyer flasks (1L) containing rice medium, 100 g rice and 100 ml water. The culture was then incubated at room temperature (no shaking) for 21 days. 250 mL EtOAc were added to the culture and left overnight. Culture media were then cut in pieces to allow complete extraction and left for 3–5 days. Then filtration was done followed by repeated extraction with EtOAc and MeOH till exhaustion. The combined EtOAc phases were washed with distilled water and then taken to dryness. The dry residues obtained from EtOAc and MeOH extracts were partitioned between *n*-hexane and 90% MeOH. The stationary phase consists of porous beads (Sephadex LH-20). Size exclusion chromatography involves separations based on molecular size of compounds being analyzed. The larger compounds will be excluded from the interior of the bead and thus will elute first. The smaller compounds will be allowed to enter the beads and elute according to their ability to exit from the small sized pores they were internalized through. Elution was performed using 100% MeOH.

Thin layer chromatography (TLC)

TLC was performed on pre-coated TLC plates with silica gel 60 F254 (layer thickness 0.2 mm, E. Merck, Darmstadt, Germany) with the following eluents: For polar compounds EtOAc:MeOH:H2O (30:5:4, 30:6:5 and 30:7:6), For semi-polar compounds DCM:MeOH (95:5, 90:10, 85:15, 80:20 and 70:30) DCM:MeOH:EtOAc (90:10:5 and 80:20:10) For non-polar compounds *n*-Hexane:EtOAc (95:5, 90:10, 85:15, 80:20 and 70:30) *n*-Hexane:MeOH (95:5 and 90:10) TLC on reversed phase RP18 F254 (layer thickness 0.25 mm, Merck, Darmstadt, Germany) was used for polar substances and using the different solvent systems of MeOH:H_2O (90:10, 80:20, 70:30 and 60:40). The band separation on TLC was detected under UV lamp at 254 and 366 nm, followed by spraying the TLC plates with anisaldehyde/H_2SO_4 or vaniline/H_2SO_4 reagent and subsequent heating at 110 °C.

High pressure liquid chromatography (HPLC)

Analytical HPLC was used to identify the distribution of peaks either from extracts or fractions, as well as to evaluate the purity of isolated compounds. The solvent gradient used started with MeOH:nanopure H_2O (10:90), adjusted to pH 2 with phosphoric acid, and reached to 100 % MeOH in 35 min. The autosampler injected 20 µL sample. All peaks were detected by UV-VIS photodiode array detector. In some cases, special programs were used. HPLC instrument consists of the pump, the detector, the injector, the separation column and the reservoir of mobile phase. The separation column (125 × 2 mm, ID) was pre-filled with Eurospher-100 C18 (5 µm), with integrated pre-column (Knauer, Berlin, Germany). LC/UV system specifications are described as follows: Pump Dionex P580A LPG, Detector Dionex Photodiode Array Detector UVD 340S, Column thermostat STH 585, Autosampler ASI-100T,

HPLC Program Chromeleon (V. 6.3), Column Knauer (125 × 4 mm, ID), pre-packed with Eurosphere 100-5 C18, with integrated pre-column.

RESULTS AND DISCUSSION

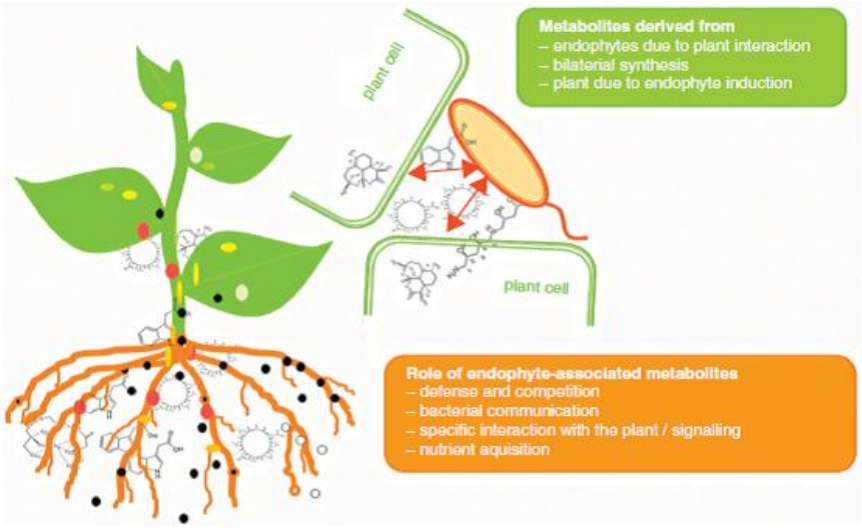


Figure 1. Schematic overview showing the different types of plant-endophyte interactions leading to the synthesis of metabolites

(A) (B)

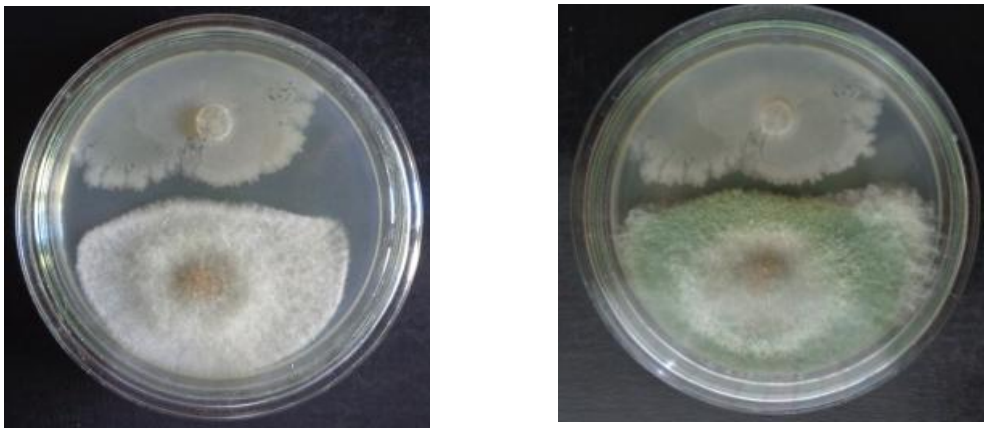


Figure 2. Inhibition of *F. tricinctum* (A) and *T. harzianum,* (B) growth with *B. halotolerans (SA87)*

Characterization and identification of selected antagonists strain

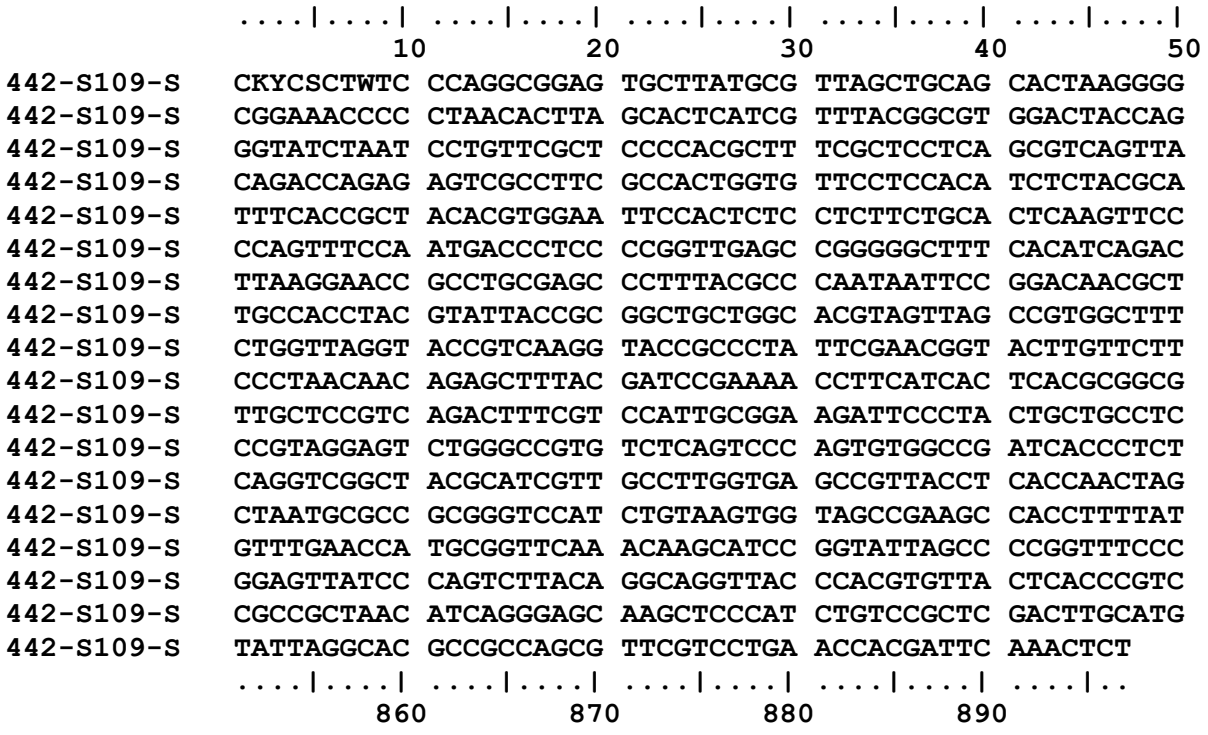
Bacterial identity was determined using morphological and biochemical characteristics as motility, aerobic growth, Gram stain, catalase, oxidase, etc. **Table 1**. To confirm the identity of these antagonistic isolate 16s rDNA gene was sequenced and subjected to BLAST searches. The isolate was identified as belonging to *B. halotolerans* **Figure 2.** Endophytic bacteria are found in virtually every plant on earth (Ryan, et al., 2008). Knowledge on the diversity of endophytic bacteria is important for both ecological and biotechnological studies. The organisms that reside in the living tissues of host plants form a variety of relationships ranging from symbiotic to pathogenic (Chen, et al., 2011). Endophytes may contribute to their host plants by producing a plethora of substances that provide protection, and ultimately have survival value, to the plants (Soca-chafre , et al., 2011). Ultimately, these compounds, once isolated and characterized, may also have potential uses in modern medicine, agriculture, and in various industries.

Bacillus has been found to produce antifungal factors such as antifungal hydrolytic enzymes (Chang et al., 2007), spore-specific lipopeptides (Yao, et al., 2003), and fengysin (Lin et al., 1999). Furthermore, *Bacillus* strains are stable in soil as spores, and this property is advantageous for their use as biocontrol agents.

The screening of bacteria for antifungal activity against the Apple pathogen *F. circinatum* showed that all of them exhibited growth inhibition against the pathogen. All the strains arrested the mycelium growth at 1 cm or more of the fungal colony margin as we mentioned above Figure 2. The development of an inhibition halo was observed between the fungal colonies and the bacteria inoculum. This may be due to the production of bacterial metabolites that may diffuse in the culture medium and suppress the growth of *F. circinatum*. These results are consistent with those obtained by (Nourozian, et al., 2006) who evaluated the antagonist activity of different bacteria (*Bacillus*, *Pseudomonas*) against *F. graminearum*. They observed, in dual culture experiments, the formation of inhibition zones between bacteria and fungus. The micromorphology of mycelia in the interaction zone showed a change in hyphal mode development, exhibiting empty, vacuolated and swollen hypha and a different ramification pattern.

Table 1. Morphology and biochemical characteristics of *Brevibacterium halotolerans*

Test	Result
Gram stain	Positive
Cell morphology	Positive
Pigmentation	Negative
Motility	Positive
Anaerobic growth	Positive
Catalase activity	Positive
Oxidase activity	Positive
VP reaction	Negative
Citrate utilization	Negative
Nitrate reduction	Positive
Hydrolysis of: Casein	Positive
Gelatin	Positive
Starch	Positive
Acid production from : Glucose	Positive
Mannose	Positive
Arabinose	Negative
Xylose	Negative
Lactose	Negative
Mannito l	Negative
Sorbitol	Negative
Sucrose	Positive
Temperature**:** 10 °C	Negative
45 °C	Positive
Growth in**:** 6.5% NaCl	Positive
10% NaCl	Positive
15% NaCl	Negative
Growth in**:** 5 pH	Positive
6 pH	Positive
9 pH	Positive
10 pH	Positive



Figuer 2. Consensus sequence of *Brevibacterium halotolerans*

The bacterial isolates also analyzed in the dual culture experiments, beside several isolates demonstrated antagonistic effects against the mycelial growth of *Trichoderma harzianum*. The width of the inhibition zone produced in dual culture tests shows the degree of antagonism bacterial isolates have against target organisms, and has been used extensively as an in vitro test for preliminary screening of biological control agents (Desai, et al., 2002). Therefore, the width of the inhibition zones between the *Bacillus* isolate and the *Trichoderma* was a reliable measure and was used to rate the antagonistic effects of the *Bacillus* species (Figure. 2). The mode of antagonism generally observed with *Bacillus* sp. is antibiosis (Edwards, et al., 1994). This is supported by reports that most *Bacillus* sp. produces many antibiotics such as bacillomycin, fengycin, mycosubtilin and zwittermicin, which are all effective at suppressing growth of target pathogens in vitro and/or in situ (Pal and Gardener, 2006). This evidence allows the assumption that antibiotics are related to the formation of inhibition zones between the bacterial and the fungal isolates shown in this study.

Microbe–plant interactions are far from being fully understood. Nevertheless, more evidence shows plant-associated microorganisms provide substantial benefits to agriculture, industry, and the environment. In brief, this study determined that there are regional differences between microbial communities associated with apple plant. Most of the bacteria we examined had antagonistic activity. The results of this study revealed that *B. halotolerans* SA87 is the potential isolate capable of producing metabolites acting against different fungus and showing the potential use of endophytic bacteria for biocontrol to protect plants from fungal or bacterial diseases. Further studies are needed to separate and extract the active substances from these endophytic bacteria.

HPLC analysis of the bacterial extract

The bacterial extract of *B. Halotolerans* is shown in Figure 3. From two different Sephadex fractions, the peaks of the HPLC- chromatogram were matched with the reference compound available in the database by UV-Visible spectrum. The peaks in the chromatogram having the same UV-Visible spectrum and retention time with that of the reference compound was identified and named. In the UV-Visible spectra various peaks observed corresponded to different compounds (Fig.4). Peak at 26.6 min. (A) represents Neurolenin B, 27.6 min. (B) 2-Hydroxy-3-methylbenzoic acid, 28.3 min. (C) Cyclopenin, 34.9 min. (D) Auereonitol, 38.6 min. (E) Palitantin, 20.7 min. (F) Malformin A and Peak at 30 min. (G) corresponds to Gamahorin derivative compound. All compounds identified are listed in Table 2.

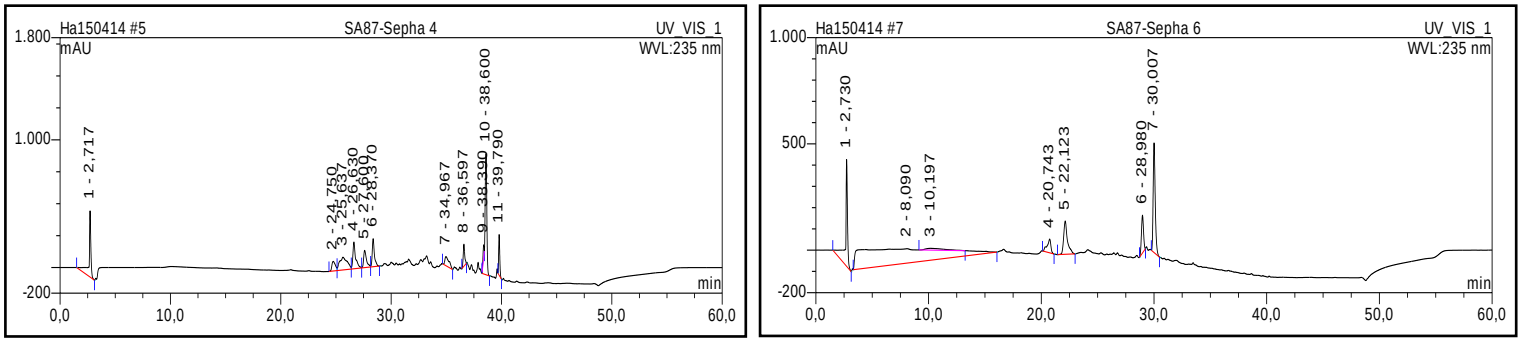


Figure 3. The HPLC chromatogram of *B. Halotolerans* (SA87) strain

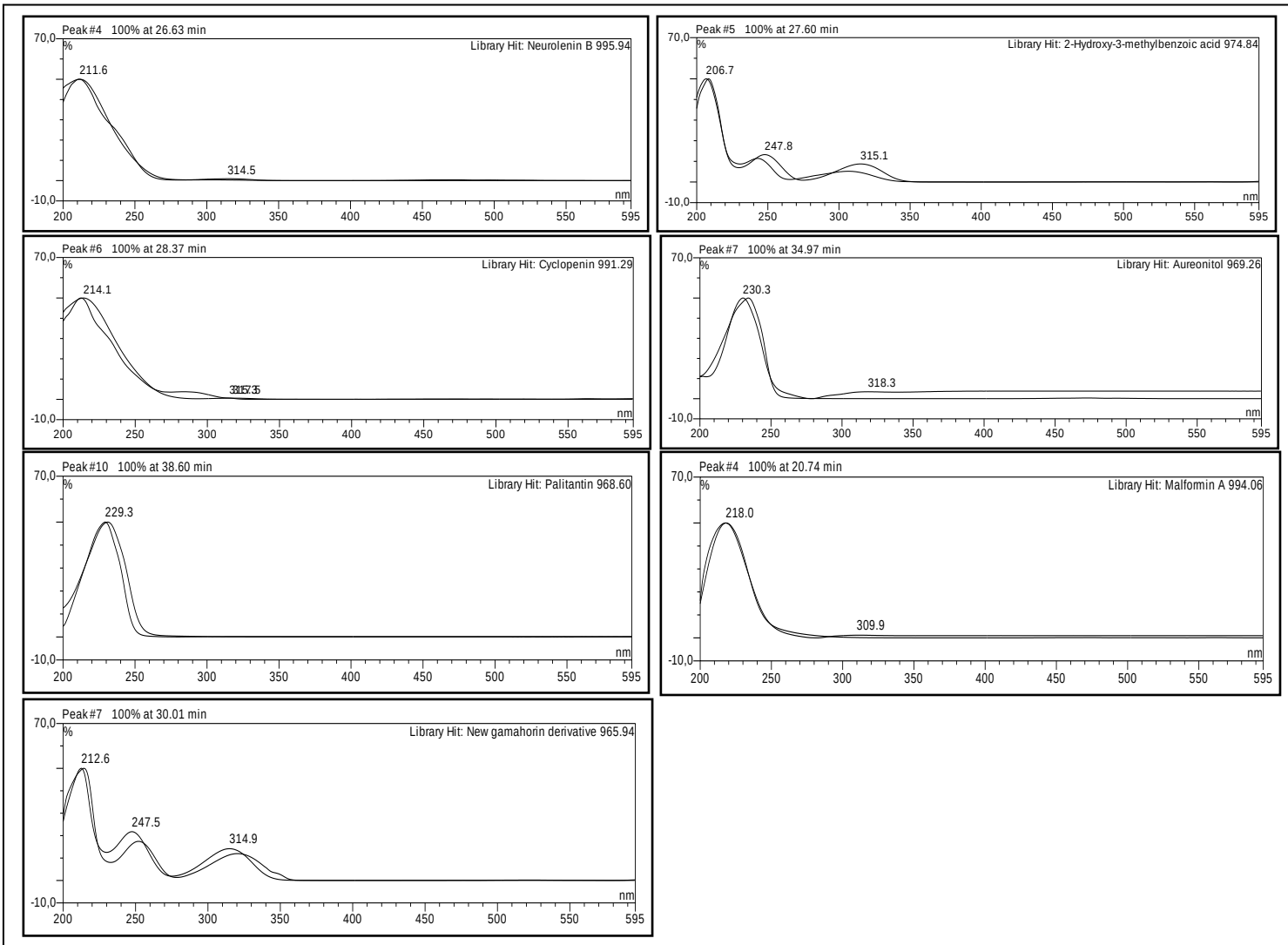
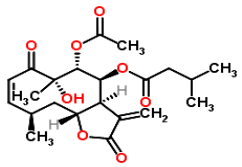
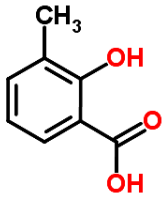
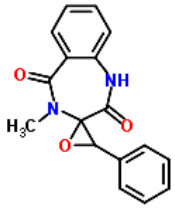
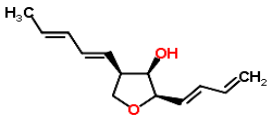
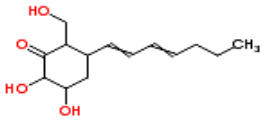
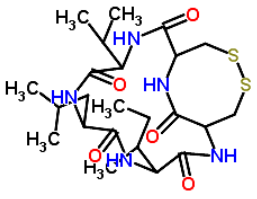


Figure 4. The HPLC chromatogram of *B. Halotolerans* (SA87) strain, Peak at 26.6 min. (A) represents Neurolenin B, 27.6 min. (B) 2-Hydroxy-3-methylbenzoic acid, 28.3 min. (C) Cyclopenin, 34.9 min. (D) Auereonitol, 38.6 min. (E) Palitantin, 20.7 min. (F) Malformin A and Peak at 30 min. (G) corresponds to Gamahorin derivative compound.

No.	Compound name	Chemical structure	Chemical formula	HPLC retention time
1	Neurolenin B		$C_{22}H_{30}O_8$	26.6 min.
2	2-Hydroxy-3-methylbenzoic acid		$C_8H_8O_3$	27.6 min.
3	Cyclopenin		$C_{17}H_{14}N_2O_3$	28.3 min.
4	Auereonitol		$C_{13}H_{18}O_2$	34.9 min.
5	Palitantin		$C_{14}H_{22}O_4$	38.6 min.
6	Malformin A		$C_{23}H_{39}N_5O_5S_2$	20.7 min.

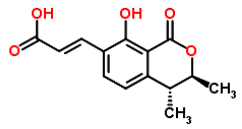
7	Pestalotiopisorin A		$C_{14}H_{14}O_5$	30 min.

Table 2. Characteristics of identified compounds

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