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Physiological Response, Antibacterial Activity and Cinnamaldehyde Production by a Marine *Streptomyces chartreusis*

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Fourteen actinomycete candidates were isolated from marine sediment (Mediterranean Sea, Alexandria, Egypt). The isolates were screened for their antagonistic activity against three Gram-positive (*Staphylococcu aureus, Bacillus cereus* and *Micrococcus luteus*) and three Gram-negative (*Aeromonas hydrophila, Pseudomonas aeruginosa* and *Proteus sp*) pathogenic bacteria. The most potent isolate was identified as *Streptomyces chartreusis* using 16S rRNA technology. Growth and antibacterial activity influencing parameters including culture medium, pH value and incubation temperature were all optimized using the procedures of response surface methodology. The antibacterial activity was increased with the decrease in the pH level from seven to five and with the increase of temperature from 20 to 30ºC. Maximum antibacterial activity (26.1 mm) was achieved after 4 days of incubation. A number of solvent systems have been experimented for the extraction of the bioactive compound(s). Ethyl acetate (EtOAc) crude extract showed highest activity against bacterial test strains with average inhibition zone diameter of 34.2 mm. The bio-toxicity of the crude extract showed a slight toxicity against the brine shrimp (*Artemia salina*). L.C$_{50}$ was achieved at 1023 mg/l. The bioactivity of crude extract was superior to the activity exerted by the tested antibiotics (Amikacin, Amoxicillin/ clavulanic acid; Chloramphenicol, Vancomycin, Erythromycin, Ciprofloxacin, Tetracycline). Based on data obtained by Fourier transform infrared spectrum (FT-IR) and gas chromatography - mass spectrum (GC-MS) and its library, the major compound was structurally identified as , cinnamaldehyde.

Keywords: *S. chartreusis*, antagonistic activity, GC-MS, FT-IR, bio-toxicity, Cinnamamldehyde.

Microbial diversity of the marine environment constitutes an infinite pool of diverse structures that remain a valuable source for innovative biotechnology to search for novel pharmaceutical compounds and to challenge the multiple resistant pathogenic microbes[1]. Sea floor has been reported as an eco-unique system with many forms of actinomycetes[2]. It appears that they are widely distributed either associated with higher living organisms[3] or free in the existing sediment[4,5].

Marine actinomycetes have different characteristics from their terrestrial counterpart; therefore, this might produce novel bioactive compounds[6]. Many of these active metabolites possess novel biological activities that can be used as a useful therapeutic agent[7]. Among the members of actinomycetes genus, *Streptomyces* is a dynamic producer of functional, bio-effective metabolites with broad medicinal range having tremendous applications[8-10]. *Streptomyces* spp. has

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been shown the ability to produce antimicrobial, insecticidal, antitumor, anti-parasitic, antiviral, antioxidant, and herbicidal compounds[11-16]. It is apparent that the marine environment is a potent source for finding new actinobacteria and new antibiotics or biologically active substances[17- 19].

Cinnamaldahyde is an aldehyde present as a major component of bark extract of cinnamon (*Cinnamomum zeylandicum*). The aforementioned molecules are classified by the United States Food and Drug Administration as generally regarded as safe[20]. Many applications were reported using cinnamaldehyde: as a flavor[21]; an agrichemical fungicide and insecticide[22]; and as antimicrobial agent[23]. Previous researches have reported the antimicrobial activity of cinnamaldehyde against various isolates of pathogens including Gram-positive (*S. aureus*, *B. subtilis*), and Gram-negative (*E. coli, E. aerogenes, P. vulgaris, P. aeruginosa, Vi. cholerae, V. parahaemolyticus* and *S. typhymurium*[24]. Kim *et al*[25] studied the reducing effect of cinnamaldehyde on the production of pyocyanin, the swarming motility, and the hemolytic activity of *P. aeruginosa*, and inhibit entero-hemorrhagic *E. coli* biofilm formation.

Therefore, this study aimed at the isolation of some potential local marine actinomycetes capable of producing biologically active agent(s) targeted against six locally isolated and identified pathogenic Gram-positive and Gram-negative test organisms.

MATERIALS AND METHODS

Study area and actinomycete isolation

Actinomycetes were isolated from eight different marine sites (Figure 1) from the Western to the Eastern coast of Mediterranean Sea (El-Agami, El-Dekhela, El-Mex, East Harbor, El- Shatby, Stanly, Sidi Bishr and Abu Quier). Briefly, water samples were collected in sterile blue cap glass bottles (250 ml) and the sediment in sterile plastic bags; kept in the icebox and transferred to the laboratory[26].

The isolation process was carried out using the pour plate technique. 0.1ml of each water sample and 1g sediment in 30ml sterile sea water were applied on starch– nitrate agar and then incubated at 30°C for 72 h. Slants containing pure cultures were stored at 4°C until further use. The macroscopic examination of actinomycetes' colonies was then observed.

Bacterial test organisms

Three Gram-negative bacteria: *A. hydrophila, P. aeruginosa,, Proteus* sp. and three Gram-positive bacteria*: B. cereus, M. luteus; S. aureus* were kindly provided by Medical Microbiology Laboratory, Faculty of Science, Alexandria University, Alexandria, Egypt.

Antibacterial potential of the isolates

Screening for the antibacterial potential was carried using disk-diffusion assay method according to Kirby-Bauer[27] to test for the inhibitory ability of isolated marine actinomycete isolates against the tested pathogens. Starch-nitrate liquid medium was used for maintaining growth condition of the isolates and to support the secondary metabolite production.

One ml of pre-cultured tested bacteria (OD_{550} = 1.0) was mixed well using 25 ml of sterile and molten Muller-Hinton agar. Sterile discs of 6 mm diameter were immersed in the culture supernatant and placed on the seeded Muller-Hinton agar plates. All plates were incubating at 30°C for 24-48h[28]. The most active marine isolate, which showed the highest inhibition zone diameter, was used for further investigation.

Identification of the potent actinomycete isolate

Isolate number EH1; showed the most potent antibacterial activity, was identified using Genomic DNA extraction performed by Gene JET Genomic DNA Purification Kit (Thermo Scientific-Sigma Aldrich, Germany) according to the manufacturer's instructions.

PCR conditions

PCR amplification was carried out using the following primers: F: 52 - AGA GTT TGA TCC TGG CTC AG 32 and R: 52 - GGT TAC CTT GTT ACG ACT T 32 . The programmed PCR was initiated by first cycle denaturation (95°C for 10 min), then 35 cycles of denaturation (95°C for 30s), followed by annealing (65°C for 60 s), extension (72°C for 1.5 min), and a final extension (72°C for 10 min). The PCR products were electrophoresed on 2.5% agarose gels to confirm successful PCR amplification for the sample. Sequencing to the PCR product on GATC Company by using ABI 3730xl DNA sequencer for forward and reverse primers was performed[29]. 16S rRNA gene was sequenced on both strands by dideoxy chain

termination method according to Sanger *et al*[30]. The 16S rRNA gene sequence for the PCR product was obtained using Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems). The sequence data have been deposited in the GenBank database under the accession number LC066678. Blast program (www.ncbi.nlm.nih.gov/blst) was used to assess the DNA similarities. Multiple sequence alignment and molecular phylogeny were performed using BioEdit software[31]. The phylogenetic tree was displayed using the TREE VIEW program[32].

Growth Description using different culture media

Five culture media namely; nutrient broth, starch nitrate, oat meal, yeast-malt and saline liquid media, have been used to trace the growth profile and to describe mycelial balls appearance and antibacterial activity response. The culture media were sterilized by autoclaving at 121°C for 20 minutes. Each medium was inoculated with $1x10^6$ CFU/ml then incubated at 30°C under shaking condition for 72 hrs.

Influence of the environmental conditions on the antibacterial activity

Effect of medium, pH value and temperature variation on the exhibited bioactivity was evaluated using 100 mL of culture broth. The effect of the three different pH values (6, 7 and 8) and three chosen temperatures (25, 30 and 35°C) were investigated considering each culture medium. The bioactivity profile was then recorded. The optimal levels of the above selected variables were evaluated using response surface methodology technique according to the procedures described by Box and Wilson[33]. For maximum productivity, culture broth medium was inoculated (one ml containing $1x10^6$ CFU/ml) and incubated for zero to 216 hrs. Samples were collected regularly every 24 hrs and tested against test strains. For growth determination, the optical density was also recorded at the wavelength of 550 nm.

Extraction of the active compound(s)

Five solvent systems namely, butanol, diethyl ether, ethyl acetate, hexane and petroleum-ether, were individually applied for the extraction purpose. The organic layer was separated and concentrated under vacuum using rotary evaporator (STUART, RE300DB) to obtain the sticky brown crude substance. Fifteen liters of 4-day culture filtrate of *Streptomyces chartreusis* were extracted from the supernatant using 1:1 (v/v) supernatant: EtOAc. The overall crude extract was amounted to 1 gm. The crude extract was then tested using disc- diffusion assay method[28].

Comparing the potency of crude extract with some representative antibiotics

Seven antibiotics (µg/disc): Amikacin 30; Amoxicillin/clavulnic 10; Chloromphinicol 30; Vancomycin 30; Erythromycin 15; Ciprofloxacin 5; Tetracycline 30, were purchased from Sigma (Dorset, UK) and used to compare the inhibitory effect with the crude extract against tested bacteria according to the CLSI interpretative standards[34].

Bio-toxicity of the crude extract

Twenty milligrams of the crude extract were evaluated by *Artemia salina* lethality test according to the procedure described by Meyer *et al.*[35]. After 48 hrs, encysted eggs of the brine shrimp (20 nauplii) were added to each set of the vials containing ethyl acetate crude extract dissolved in 5% DMSO and filled to 10 mL total volume with artificial salt water. The used concentrations of the crude extract were: 100, 200, 500, 1000, 1500, 2000 and 4000 µg/ml and DMSO used as negative controls. After 24 hrs, the number of survivals was counted and the death percentage was then calculated. The 50% lethal concentration (LC_{50} value) and the standard error. Values were all calculated by Probit analysis.

$$\text{Mortality (\%)} = \frac{\text{(No. of original nauplii) - (no. of alive nauplii) x 100}}{\text{(No. of original nauplii)}}$$

Physicochemical characterization of the crude extract

Gas Chromatography-Mass Spectrum analysis

Sticky brown crude (1g) was dissolved in 10 ml of ethyl acetate and subjected to chromatographic analysis. The active ethyl acetate fraction was subjected to GC-MS analysis using a Hewlett Packard Mass Spectrometer (5890 series ' GC hyphenated with 5989). MS conditions were as follows: Detector mass spectrometer voltage 70eV with 300°C as a source temperature. The injector temperature was 240°C and the split less mode 0.5¼L injection. The HP 55% dimethyl-95% diphenylpolysiloxane non-polar column was used (length 30 cm x 0.25 mm *i.d.*, coating thickness film 0.25 ¼m). The oven was adjusted at 80°C for 1 min and initial time 1.5 min with 40°C ended by a final temperature of 300°C, and 4 min hold time where

the total run time was 15 min. The components were identified by comparing their retention times to those of authentic samples, as well as by comparing their mass spectra with those of Wiley 275 Library[36]. Quantitative data were obtained by the peak normalization technique using integrated FID response.

Fourier Transform Infrared analysis

Ethyl acetate crude was subjected to FTIR-spectroscopic analysis (Perkin Elmer, USA) in the mid IR region of 400–4000 cm-1 with 16 scan speed using KBr discs.

RESULTS AND DISCUSSION

Phylogeny of active isolates

Fourteen pure isolates were inoculated into starch-nitrate broth medium and incubated at 30°C for 72 hrs under shaking condition. They were all tested for their antibacterial activity, where, the maximum activity (14.8 mm) for EH1 isolate was recorded (Table 1). EH1 isolate as potent strain, was analysed for its 16S rRNA gene. The similarity percentage of this isolate accounted for 97%. The phylogenetic relationship showed that this strain very close to the type strain *Streptomyces charteusis* NBRC 12753 deposited in culture collection centre of National Board of Respiratory Care (Figure 2).

Growth Description using different culture media

Descriptive growth of *S. charteusis* using five culture media was represented in Table 2. Using starch nitrate medium, highest activities were recorded at 16.7 mm and 17mm against G-ve and G+ve, respectively (Figure 3). After the incubation period at 30°C for 72 hrs, mycelia balls were of variable sizes ranged from large to small in shape with varying homogenous to heterogeneous features. Balls showed white color to dark ball in the culture media. Similarly, growth, aerial mycelium color, substrate mycelium color and pigmentation pattern were very similar to those reported for moderately halotolerant *Streptomyces* strain JAJ13, which were described on eight culture media[37]. Also, Claverías *et al*[38] reported culture diversity of the actinobacteria from marine sediments using four different medium.

Optimization of the bioactivity using response surface methodology

Different pH values were experimented to reveal the growth pattern and antibacterial activity of the marine isolate (*S. charteusis*) against tested bacteria. Maximum activity was recorded at pH 6.0 of the nutrient broth medium, while the lowest activity was detected at pH 8.0. Starch- nitrate medium recorded the highest value for the

Table 1. Bioactivity pattern of the isolated marine actinomycetes

Location	Code	IsolateInhibition zone diameter (mm) against					
		Gram- negative bacteria			Gram -positive bacteria		
		A. hydrophila	*Pseudomonas aeruginosa*	*Proteus* sp	*Staphylococcu aureus*	*Bacillus cereus*	*Micrococcus luteus*
Abu-Quier	AQ1	-	6	-	-	-	1.2
	AQ2	-	-	-	-	-	-
Sidi Bishr	SB	6	-	-	6	-	2.4
Stanley	ST	-	-	-	-	8	1.6
El-Shatby	SH1	15	-	-	13	14	8.4
	SH2	-	-	-	-	-	-
East harbor	EH1	14	18	10	17	15	14.8
	EH2	10	16	8	14	14	12.4
El-Mex	MX	11	9	8	-	-	5.6
El-Dekhela	DK1	11	11	-	8	9	7.8
	DK2	11	11	-	10	8	8
El-Agami	AG1	8	8	-	-	9	5
	AG2	8	8	-	7	7	6
	AG3	8	-	8	-	-	3.2

(-)=No inhibition zone

bioactivity (21.8 mm) followed by nutrient broth (20.5 mm) at lower pH value (at pH 6.0). Saline medium gave the lowest activities at all pH levels (Data not shown). EH1 isolate grown well in the nutrient broth at 30^{0}C and exhibited the highest antibacterial activity (22 mm) followed by starch-nitrate medium (19 mm) at 30^{0}C. Lower activities were recorded for oatmeal medium at different temperature levels (ranged from 10-12 mm), wheras, no activity was detected when saline medium was used (Data not shown).

Comparing to other marine isolates, Kannan *et al*.[39] studied the optimized condition on marine *Streptomyces* sp. PVRK-1 as anti-MRSA metabolite production showing maximum activity at neutral pH and 37^{o}C. In addition, maximum bioactivity was reported to be produced by marine *Streptomyces* strain at 30- 40°C and at pH range 5 – 7 and for three active *Streptomyces* strains at pH range (4-7) and at temperatures range 20-30°C[40]. On the other hand, ptimization of growth conditions for champacyclin biosynthesis by *Streptomyces* strain C42 were maintained at 28 °C[41]. In addition, temperature range for secondary metabolites from a marine *Streptomyces* sp. VITBRK2 was reported between 25-37°C and pH range of 6-8[42].

The 3D response surface response (Figure 4) indicated the optimum ranges of the variables for the maximal activities. It was found that the bioactivity increased with the decrease of pH value (7 ~ 5) and with the increase of the temperature level between 20 ~ 30^{o}C. It was clearly

Table 2. Growth description of *S. charteusis* using five culture media

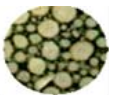
Medium	Growth Description	Appearance
Nutrient broth	Balls are quite large in size, heterogeneous with a clear supernatant; darkened by elapsed time and by rising the temperature	
Starch-nitrate broth	Small in size and homogenous white balls with a clear supernatant	
Oat-Meal broth	Very Small size , Homogenous balls with a colored (Orange) supernatant	
Yeast –Malt broth	Large Size, heterogeneous Balls and a brown colored supernatant	
Saline broth	Extremely heavy Small size, homogenous balls	

Table 3. Bioactivity of *Streptomyces charteusis* against the six tested bacteria using different solvents

Solvent	Inhibition Zone Diameter (mm) against						
	Gram-negative bacteria			Gram-positive bacteria			
	A. hydrophila	*Pseudomonas aeruginosa*	*Proteus* sp	*Staphylococcu aureus*	*Bacillus cereus*	*Micrococcus luteus*	AVG
Hexane	-	-	-	-	-	-	0
Ethyl acetate	32	40	31	30	35	37	34.2
Diethyl-Ether	-	-	-	-	-	-	0
Butanol	10	7	7	6	6	6	7
Petroleum ether	8	6	6	-	9	6	5.8

shown that nutrient broth is the growth and activity supportive medium for EH1isolate.

Incubation period for maximum antibacterial activity

The antibacterial activity was measured at 0 time and after 24, 48, 72, 96, 120, 144, 168,192 and 216 hr using disc diffusion assay. A gradual increase in the inhibition zone diameters was observed from 24 to 72 hr reaching its maximum value 26.1 mm at 92 hr against the tested bacteria. After the fourth day, a gradual decrease of the antibacterial activity was observed. No activity was evidently detected after day nine of incubation (Figure 5). Optical density (O.D) reading for the 4-day culture of EH1 isolate was 1.5, showing its most active secondary metabolite harvest at stationary phase of growth. Similar to our results, the maximum inhibition zones of 32, 26 and 25 mm were recorded by the end of the fifth day of incubation for three *Streptomyces* isolates[40]. Recently, anti-MRSA activity of *Streptomyces* sp. SUK 25 showed for the 7 -day culture, O.D reading at 0.143[43].

Determination of the appropriate solvent(s) for extraction of the active agent(s)

The results represented in Table 3 showing that ethyl acetate solvent was selected for the extraction procedures where the yielded extract obtaining showed the highest average zone of inhibition; 34.2 mm (Figure 6). However, the other

Fig. 1. Sampling locations for the isolation of marine actinomycetes

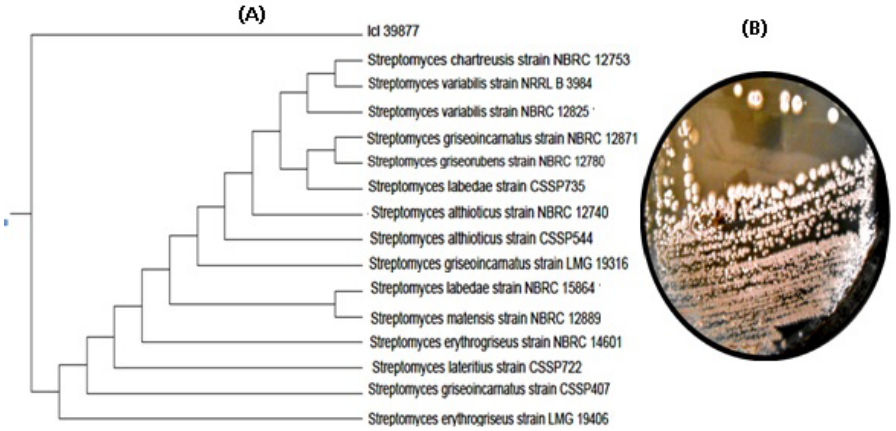


Fig. 2. Phylogenetic tree based on the similarity pattern for the 16S rRNA genes of the marine isolate EH1 identified as *Streptomyces charteusis* (A), Culture pattern for the isolate EH1 grown on starch-nitrate agar (B)

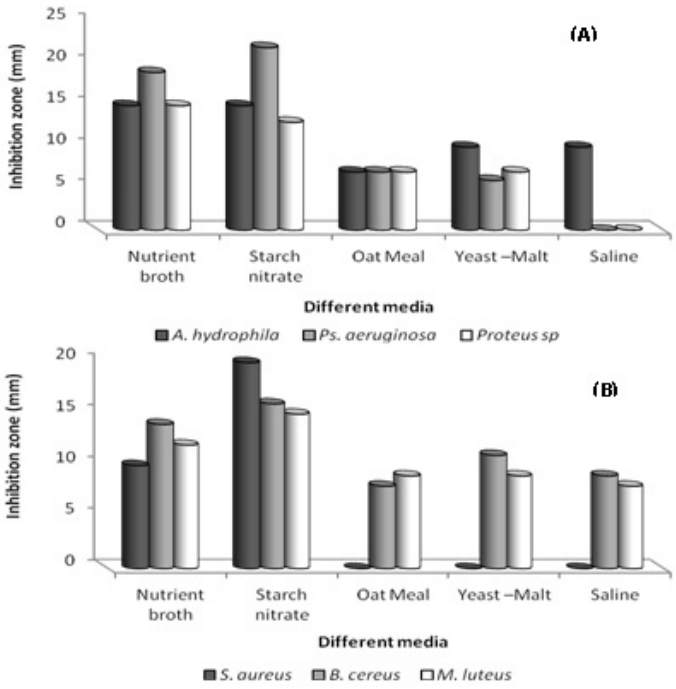


Fig. 3. Bioactivity of *Streptomyces chartreusis* at different culture media against (A) Gram Negative bacteria (B) Gram positive bacteria

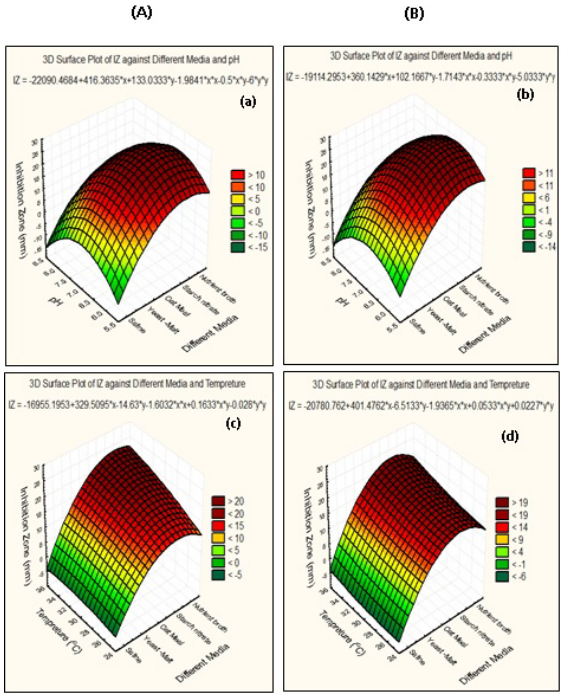


Fig. 4. 3D response surfaces for the bioactivity of *S. charteusis* against Gram-negative **(column A)** and Gram-positive **(column B)** bacteria. Each figure shows the effect of two variables on the production of cinnamaldhyde, **(a, b)** the effects of different media (*X*) and different pH (*Y*); **(c, d)** the effects of different media (*X*) and different temperature (*Y*)

solvent showed low or no antimicrobial activity against the six pathogens. Comparing with our results, antimicrobial activity of ethyl acetate extract metabolites produced by *Streptomyces bangladeshiensis* was 9.25 mm [44]. Nandhini and Selvam[19] reported that maximum activity of diisodecyl ether extractable compounds from marine *Streptomyces* sp. was 24.6 mm. In addition, antagonistic activities of ethyl acetate extract of *Streptomyces* sp. VITBRK2 against drug resistant MRSA and VRE strains were 16.25 and 21 mm, respectively[42]. Recently, Junaidah *et al*[43] recorded an average of inhibition zone 23.7 mm as anti-MARSA using ethyl acetate crude extract from *Streptomyces* sp. SUK 25.

Biotoxicity of different crude extracts concentrations using Artemia salina

The biotoxicity experiment was carried out to detect the toxicity of different EtOAc crude concentrations (ranging from 100 to 4000 µg/ml) using *Artemia salina* as biomarker. The mortality percent was calculated. The results indicated that the crude had a low toxicity effect with maximum crude concentration ranged from 1000 to 1500 µg/ml after 24hrs. Where, the LC_{50} of this crude (the concentration at which 50% of the biomarker individuals died) was estimated from the best-fit line obtained by using the probit analysis method. It was found to be 3.01 or 1023 ¼g crude/ml after 24h using the brine shrimp (*Artemia salina*) (Figure 7).

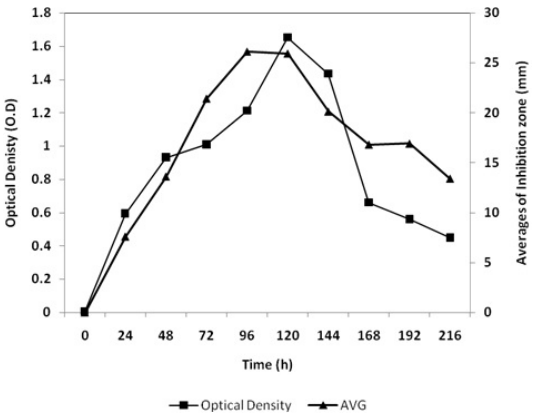


Fig. 5. Average of bioactivity and optical density of EH1 isolate at interval time of incubation period

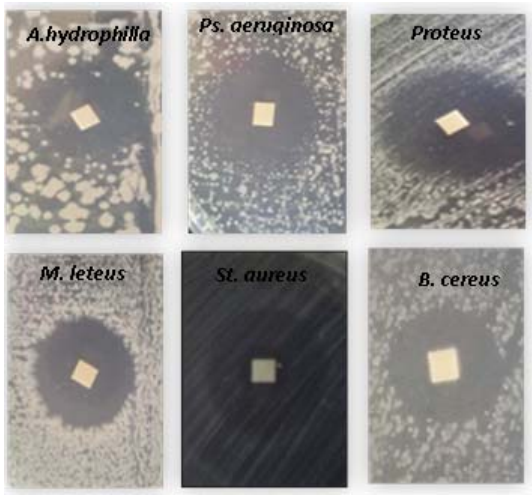


Fig. 6. Antibacterial activity of ethyl acetate extract against Gram-positive and Gram-negative tested bacteria using disc-diffusion method

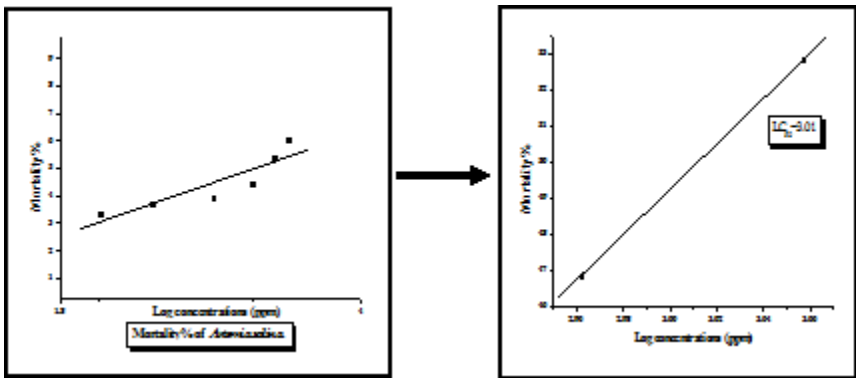


Fig. 7. Biotoxicity of the crude extract showing LC_{50} value against *Artemia salina*

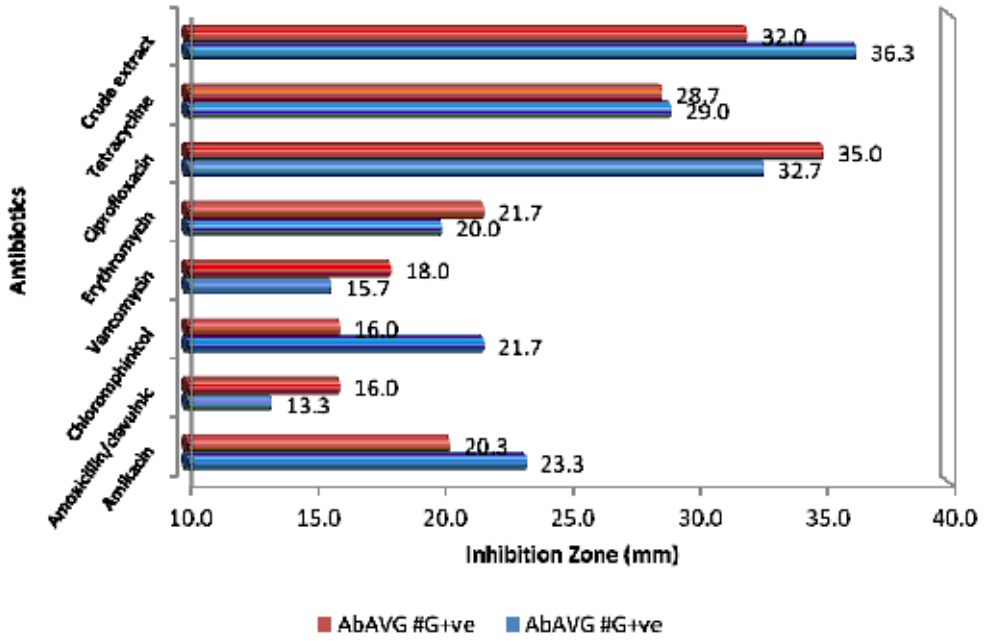


Fig. 8. Antibiotics sensitivity profile compared with the crude extract activity against Gram-positive and Gram-negative bacteria

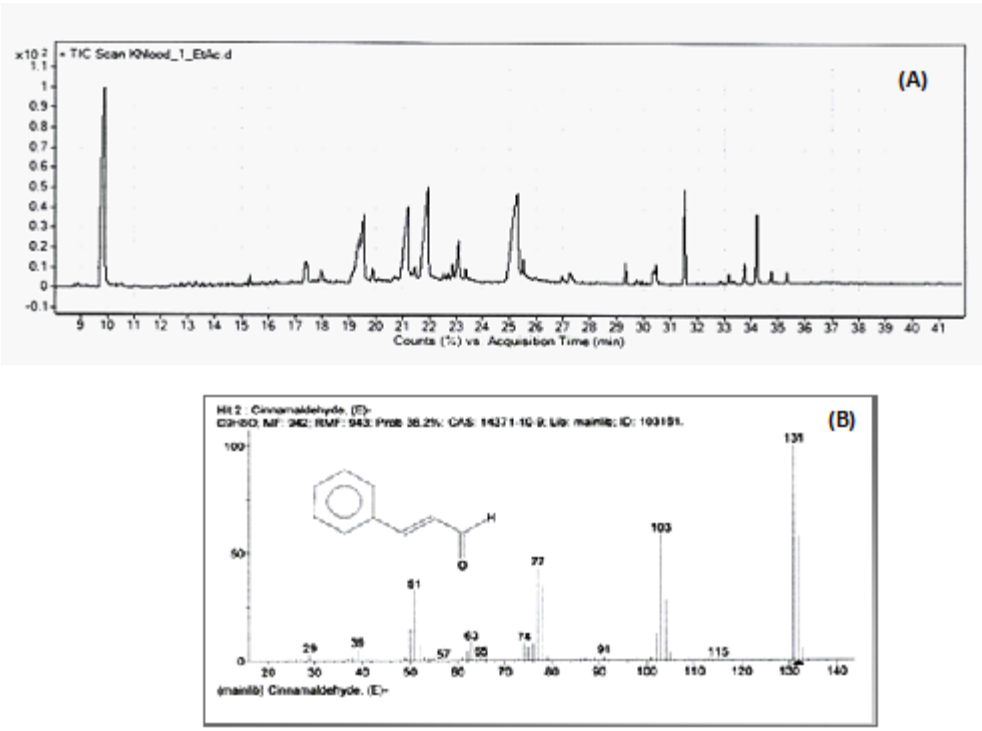


Fig. 9. Gas Chromatography-Mass Spectra showing the major peak in GC analysis (A) and identified compound "cinnamaldehyde" in Mass Spectral analysis (B)

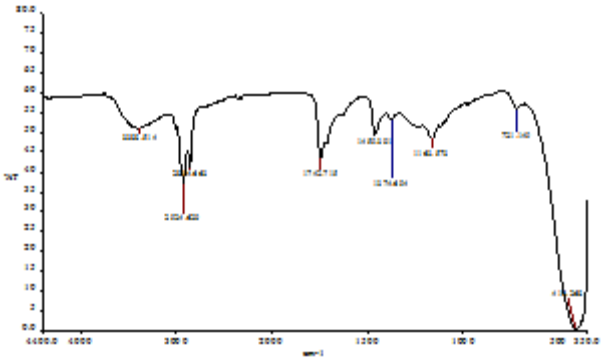


Fig. 10. FT-IR trace of cinnamaldehyde as a major product

Comparison of the active metabolite with some antibiotics representatives

Antibiotics currently used in the market were used to compare the potency of the active metabolite produced by the marine isolate *S. chartreusis* against the six pathogens. The pathogens were sensitive to ciprofloxacin (average zone of inhibition of 33.8 mm) and showed a resistance towards amoxicillin/clavulanic (15.7 mm). Active metabolite showed higher activities compared with the tested antibiotics with average inhibition zone 36.3 and 32 mm, against Gram-positive and Gram-negative bacteria, respectively (Figure 8). Previous report showed antibiotic activity of *Streptomyces* sp. IMD2703 and some commonly used aminolgycoside antibiotics against MRSA strains representing 16.3 mm compared with 5.1 mm[45]. Kumar *et al*.[46] showed the activity of the ethyl acetate extract from *Streptomyces* strain ASCA5 was recorded at 12.8 mm against Gram +ve and 11.6 mm against Gram –ve; lower than that obtained by the used antibiotics (25.8 and 28.1 mm, respectively). Junaidah *et al*.[43] showed EtOAc extract activity obtained from *Streptomyces*-SUK was 23.7 mm compared to vancomycin activity 15.5 mm.

Physicochemical characterization of the crude extract

A major sharp peak was observed at acquisition time 9.8 min with the highest count percentage $1x10^2$ on gas-chromatographic analysis. This major peak was identified (Figure 9) as cinnamaldehyde using mass spectrum and with the aid of its library. FT-IR spectrum of cinnamaldehyde showed the absorption band frequency at 3388.5cm^{-1} was strong (s) and broad (b), with the presence of O-H stretch, H-bonded for alcohol and phenol and 2924.6cm^{-1} medium band (m) indicated =C-H stretch for alkenes. The absorbance band at 1742.7 cm^{-1}(s) revealed the presence of C=O bond for aldehyde. Due to the influence of conjugation and aromatic ring, the peak is wider than the normal cases of aldehyde compound. A strong band at 1374.69 cm^{-1} indicated the presence of alkyl $–CH_3$ stretch. A strong absorption band at 721cm^{-1} indicated the presence of aromatic C=C (Figure 10). Ethyl acetate crude extract containing the major cinnamaldehyde represented the highest antibacterial activities averages against Gram-positive and Gram-negative test bacteria. Recent report by Adinew[47] who reported that cinnamaldehyde is the main constituent of the essential oil from the cinnamon bark using the same spectroscopic analysis, where extractable cinnamon oil showed an inhibitory effect against the Gram-positive bacteria *B. cereus*, *M. luteus*, *S. aureus* and *E. faecalis* and Gram-negative bacteria *A. faecalis*, *E. cloacae*, *E. coli*.

In conclusion, this work reports the isolation of a marine *S. chartreusis* showing the ability to produce cinnamaldehydes with antibacterial potential. This metabolite could be used in further investigation as a potential candidate for analytical purposes and /or further biotechnological applications.

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