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Isolation and partial purification of erythromycin from alkaliphilic *Streptomyces werraensis* isolated from Rajkot, India

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ABSTRACT

An alkaliphilic actinomycete, BCI-1, was isolated from soil samples collected from Saurashtra University campus, Gujarat. Isolated strain was identified as *Streptomyces werraensis* based on morphological, biochemical and phylogenetic analysis. Maximum antibiotic production was obtained in media containing sucrose 2%, Yeast extract 1.5%, and NaCl 2.5% at pH 9.0 for 7 days at 30 °C. Maximum inhibitory compound was produced at pH 9 and at 30 °C. FTIR revealed imine, amine, alkane (C=C) of aromatic ring and p-di substituted benzene, whereas HPLC analysis of partially purified compound and library search confirmed 95% peaks matches with erythromycin. Chloroform extracted isolated compound showed MIC values 1 μg/ml against *Bacillus subtilis*, ≤0.5 μg/ml against *Staphylococcus aureus*, ≤0.5 μg/ml against *Escherichia coli* and 2.0 μg/ml against *Serretia* GSD2 sp., which is more effective in comparison to ehtylacetate and methanol extracted compounds. The study holds significance as only few alkaliphilic actinomycetes have been explored for their antimicrobial potential.

1. Introduction

Among the antibiotic producing microbes, the class Actinobacteria represents a broad range of valuable and prominent sources of pharmaceutically active metabolites. Actinomycetes are responsible for the production of about half of the discovered secondary metabolites [1], notably antibiotics [2], antitumour agents [3], immunosuppressive agents [4] and enzymes [5]. Each actinomycetes strain has probably genetic potential for producing 10–20 secondary metabolites [6]. Terrestrial actinomycetes are one of the abundant sources of secondary metabolites and the vast majority of these compounds are derived from the single genus *Streptomyces*. *Streptomyces* are distributed widely in terrestrial and marine habitats [7] and are of commercial interest due to their unique capacity to produce novel metabolites. The genus *Streptomyces* was classified under the family *Streptomycetaceae*, which includes Gram-positive aerobic members of the order Actinomycetales and suborder *Streptomycineae* within the new class Actinobacteria [8,9]. They produce approximately 75% of commercially and medically useful antibiotics and 60% of antibiotics used in agriculture [10]. Major types of antibiotics produced by *Streptomyces* are aminoglycosides, anthracyclins, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, and tetracyclines [11].

In spite of the availability of new antimicrobial products, the development of new antimicrobial agents, preferably naturally occurring with novel mechanisms of action, is an urgent therapeutic need with increase in drug resistant pathogens, and the magnitude at which these pathogens are transmitted among people. Even though much work on the terrestrial actinomycetes is done but still especially soil remains the richest versatile source for new and clinically important antibiotics [12].

In view of the above, in the present study, we have described the morphological, biochemical and phylogenetic characteristics of isolated alkaliphilic strain *Streptomyces werraensis*. Strain was further explored for production of antimicrobial compounds.

2. Methods

2.1. Sample collection and isolation of microorganism

Soil sample was collected from the Saurashtra University campus, Rajkot, Gujarat, India. 1 g soil was suspended in 9 ml of sterile

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double distilled water. Diluted aliquots (0.1 ml) of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were spread on the isolation plates containing starch caseinagar, oatmeal agar and actinomycetes isolation agar (Himedia, Mumbai) containing combination of penicillin and chloramphenicol. Plates were incubated at 28 °C for 7–14 days. Stock culture of isolated strain was preserved in 15% glycerol (v/v) at 4 °C.

2.2. Physiological, biochemical and cultural characteristics

Morphological, biochemical and cultural characteristics of the isolated strain was studied as described in Bergey's manual. Carbohydrate utilization was determined by growth on carbon utilization medium supplemented with 1% carbon sources at 30 °C. Temperature range for growth was determined on actinomycete isolation agar by growing at different temperatures (10, 15, 20, 30, 37, 42 and 50 °C). Hydrolysis of starch was evaluated on starch agar media. Reduction of nitrate was determined and all cultural characteristics were recorded after six days of incubation.

2.3. In vitro screening of isolates for antimicrobial activity

Pure isolates were spot inoculated on actinomycetes isolation agar medium (Hi-Media, Mumbai) and plates were incubated at 30 °C for six days followed by inversion for 40 min over chloroform in fumehood. Colonies were then covered with a 0.6% agar layer of nutrient agar medium (for bacteria), previously seeded with two Gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and two Gram negative strains (*Escherichia coli* and *Serretia* sp.) to evaluate antimicrobial activity.

2.4. Phylogenetic analysis

The 16SrRNA gene was amplified with primers forward (5′-GAGTTTGATCC TGGCTCA-3′) and reverse (5′-ACGGCTACCTTGTTACGACTT-3′). Amplified PCR product was sequenced and nucleotide sequence was matched using BLAST program. Phylogenetic tree was constructed using neighbor-joining method [13]. Sequence of the isolate was submitted to GenBank (Accession ID: JQ964039).

2.5. Submerged fermentation

Seed culture media for submerged fermentation with following composition (g/l) was used: soybean meal 30, glucose 10, glycerol 10, $(NH_4)_2HPO_4$ 1, $(NH_4)_2SO_4$ 3.5, $CaCO_3$ 5.10% of inoculum was added in 100 ml production media with composition: (g/l): sucrose 35, yeast extract 15.0, NaCl 4, KH_2PO_4 3, K_2HPO_4 2 and $MnSO_4$ 1. Inoculated cultures were grown in a rotary shaker at 200 rpm at 30 °C for seven days. Biomass was separated by centrifugation and filter sterilized supernatant was used for extracellular antimicrobial activity. 100 μl of supernatant of each isolate was administrated in each well. Plates were incubated at 37 °C and zone of inhibition was measured after 24 h of incubation. Optimization of carbon and nitrogen sources *i.e.* glucose, starch, lactose, sucrose, galactose, fructose, maltose and xylose were added as individual carbon sources in production media at 1% concentration. Casein, yeast extract, peptone, soya bean meal, NH_4Cl, NH_4NO_3, $NaNO_3$ and urea were provided separately as a nitrogen sources into the production medium.

2.6. Extraction and partial purification of antimicrobial metabolites

Biomass was separated from growth medium by centrifugation at 4000 rpm for 10 min. Crude antimicrobial compound produced in culture was extracted through manual shaking with equal volume of chloroform or ethyl acetate or methanol in a separating funnel. The filtered supernatant was extracted by chloroform in ratio of 1:1 (v/v). The yellow colored residual crude active compound was purified by thin layer chromatography (TLC) in a running solvent system of methanol and chloroform. Two fractions with different R_f values recovered from TLC plates were dissolved in 10% Dimethylsulfoxide (DMSO) and bioassayed against the test microorganisms. Purification of this crude compound was carried out in column chromatography technique on silica gel (MerckLtd. India) using chloroform-methanol (Rankem Ltd. India) gradient (11:3) as running solvent system. Extract were collected and characterized by FTIR and HPLC analysis.

2.7. Antimicrobial testing activity

Antimicrobial activity of isolated active metabolite was studied by agar well diffusion method. Freshly grown colonies of bacterial strains were inoculated into 25 ml of nutrient broth (NB, Hi-media) in a shaking water bath for 4–6 h until turbidity reached to 0.5O.D. (660 nm). Final inoculum was adjusted to 5×10^8 CFUml^{-1} to each agar plate. The plates were incubated at 37 °C and the zones of inhibition were measured after 24 h. Pure solvent served as a control.

2.8. HPLC and FTIR analysis of antibiotic sample

Extracted purified antibiotic fractions were characterized by HPLC (High Performance Liquid chromatography) and FTIR (Fourier Transform infrared resonance) chromatography. HPLC of bioactive metabolite was determined at 215 nm with mobile phase of Acetonitrile-Methanol-0.2 M Ammonium acetate-Water (45:10:10:35) in C18 column. FTIR spectra of the purified antibiotic fractions were analyzed after homogenization of the sample with KBR. The FTIR spectra were recorded on SHIMADZU AUX 220 spectrometer in the range of 4000–400 cm^{-1}.

3. Results and discussion

Present study focuses on isolation of potent antibiotic producing alkaliphilic actinomycetes. Fifty actinomycetes strains were isolated from ten soil samples collected from the different places of Saurashtra University, Rajkot, Gujarat, India. Among the isolated pure strains, only one actinomycetes culture, BCI-1 was found to produce wide spectrum of antimicrobial activities (Gram-positive and Gram-negative bacteria). BCI-I was characterized by 16srRNA sequencing and identified as *S. werraensis*. In general, *Streptomyces* are primarily saprophytic and are best known microorganism from soils where they contribute significantly to the turnover of complex biopolymers and antibiotics [14]. The isolated culture BCI-1 inhibited none of fungal test organisms; however, isolate BCI-1 inhibited all four bacterial test organisms, suggesting a prokaryotic inhibitory preference.

IsolateBCI-1 was aerobic, Gram positive and showed aerial mycelia with sporangium (sporophore). The vegetative mycelium showed cream-light, brown color while the aerial mycelium showed light gray color. Culture on examination in light microscopy showed characteristics like flexuous sporophores arising from the aerial mycelium which fits to be in the genus *Streptomyces* [15]. Strain was mesophilic in nature and grows up to 40 °C, 2.5% NaCl concentration with pH 9 as optimum. Organism could utilize glucose, arabinose, mannitol, maltose and sucrose as the carbon source along with acid production; however, xylose, galactose and fructose were utilized without the production of acid. The physiological and biochemical characteristics of the strains (BCI-1) are shown in Table 1.

The 16S rRNA gene partial sequence of the isolate was compared with the nucleotide sequences of other *Streptomyces* strains

Table 1
Physiological and biochemical characteristics of strains *Streptomyces werraensis* (BCI-1).

Characteristics	*Streptomyces werraensis*(BCI-1)
Colony characteristics	Gram Positive, Aerial Mycelia
Pigmentation	Cream light brown color
Anaerobic growth	−−
pH	9.0
Sugar fermentation with acid production	
Glucose	++
Maltose	++
Sucrose	++
Mannitol	++
Sugar fermentation without acid production	
Xylose	−−
Galactose	−−
Fructose	−−
Triple sugar iron (TSI) agar	+ve
Starch hydrolysis	+ve
Lipid hydrolysis	−ve
Indole production	−ve
Methyl red	−ve
Voges–Proskauer (V–P)	−ve
Citrate utilization	−ve
Gelatin hydrolysis	+ve
Catalase	+ve
Phenyl alanine	−ve
Nitrate reduction	−ve
Dehydrogenase	−ve
Hemolysin production	+ve
Casein hydrolysis	+ve
Ammonia production	+ve

retrieved from the NCBI GenBank database and phylogenetic position of the strain was determined using the neighbor-joining method. The strain showed maximum homology (99%) with *Streptomyces spp. DRL 337*(NCBI Accession No. FJ853207). Therefore, on basis of the microscopic, macroscopic, biochemical, physiological and 16S rRNA gene sequence, the designated isolate BCI-1was found to be *S. werraensis* (JQ964039) of genus *Streptomyces*.

Results from TLC showed two fractions with different $R_f$values. The fraction with R_f value 0.385 and UV λ_{max} at 241.99 nm in chloroform exhibits antimicrobial activity against all the test microorganisms. The fraction with R_f value 0.256 and UV λ_{max} at 278 nm in ethyl acetate showed higher inhibition toward Gram positive organism compared to Gram negative organisms. The reason of different sensitivity between Gram-positive and Gram-negative bacteria could be ascribed to the morphological differences between these microorganisms [16]. For further studies, the broad spectrum active fraction collected from chloroform was characterized. Partial purification process was carried out through column chromatography packed with silica gel. The purified fraction was soluble in ethyl acetate, chloroform and DMSO whereas sparingly soluble in water.

Growth medium supplementation with different carbon and nitrogen sources showed better antibiotic production. The strain *S. werraensis* was cultivated in fermentation medium supplemented with various carbon and nitrogen sources and their effect on growth as well as antimicrobial activity was studied. The strain was able to grow in all the tested carbon sources with maximum antibiotic production in medium supplemented with sucrose (Table 2). The result shows that antibiotic production was higher in medium having sucrose (3.5%) as carbon source. The antibiotic production is largely influenced by nature of carbon and nitrogen sources as reported by Vilches and group [17]. The growth as well as antibiotic production decreases with either increase or decrease of sucrose concentration. Our result are similar to that of bioactive metabolite production using reported *Streptomyces tanashiensis* strain A2D by Singh et al. [18] where sucrose supported the production of

Table 2
Effect of different carbon and nitrogen sources on antibiotic production.

Media components	Media pH after fermentation	Antibacterial activity (Zone in mm)	
		S. aureus	*E. coli*
Production Media(PM)	9.0 ± 0.3	0.0	0.0
Carbon sources			
PM + Glucose	8.9 ± 0.23	9 ± 1.0	10 ± 2
PM + Starch	8.7 ± 0.3	11 ± 2.0	11 ± 2.0
PM + Lactose	9.2 ± 0.2	10 ± 1.0	11 ± 2.0
PM + Sucrose	9.0 ± 0.05	21 ± 2.0	20 ± 2.0
PM + Galactose	8.6 ± 0.2	15 ± 1.0	14 ± 2.0
PM + Fructose	8.8 ± 0.3	17 ± 2.0	15 ± 2.0
PM + Maltose	9.12 ± 0.05	12 ± 1.0	10 ± 1.0
PM + Xylose	9.23 ± 0.05	8 ± 2.0	9 ± 2.0
PM + Glycerol	8.6 ± 0.1	10 ± 2.0	9 ± 2.0
PM + Glycerol + Sucrose	9.0 ± 0.2	15 ± 2.0	13 ± 1.0
Nitrogen sources			
Casein	8.8 ± 0.15	9 ± 2.0	5 ± 1.0
Yeast extract	9.0 ± 0.25	24.0 ± 2.0	22.0 ± 2.0
Peptone	9.1 ± 0.3	11 ± 2.0	10 ± 2.0
Soya bean meal	9.1 ± 0.2	13 ± 2.0	12 ± 2.0
NH_4Cl	8.5 ± 0.05	8 ± 1.0	6 ± 1.0
NH_4NO_3	8.4 ± 0.1	7 ± 2.0	7 ±2.0
$NaNO_3$	8.6 ± 0.3	5 ± 1.0	4 ± 1.0
Urea	8.6 ± 0.2	8 ± 1.0	8 ± 1.0

bioactive metabolites. The production started during mid-stationary phase that confirmed the compound to be a secondary metabolite in nature. In the present study glucose does not support the production of antibacterial compounds, which was in

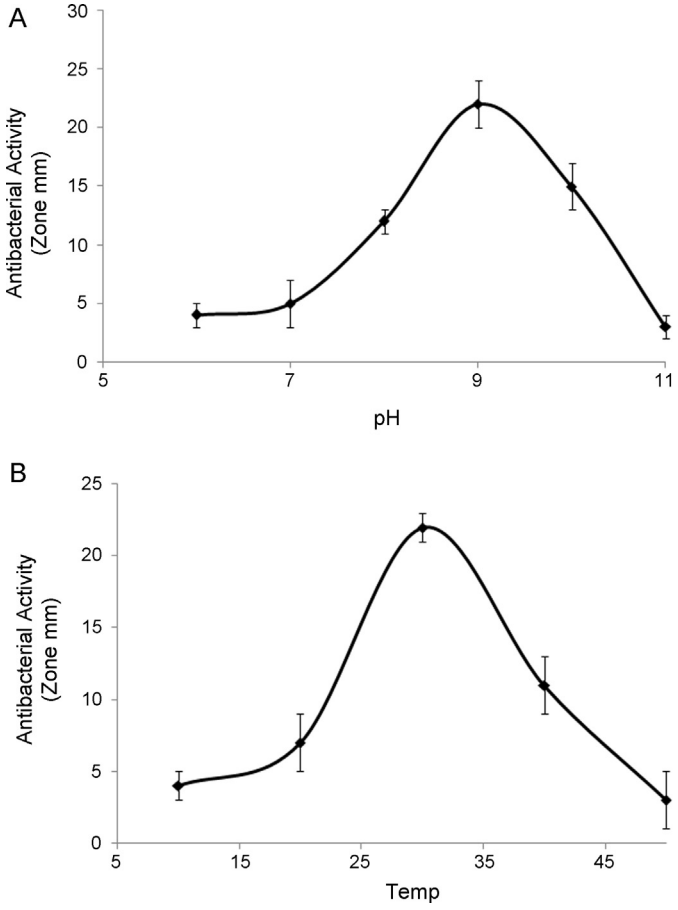

Fig. 1. (A) Effect of pH on bioactive metabolite production. (B) Effect of temperature on bioactive metabolite production.

Table 3
Minimum inhibitory concentration of crude extract.

Crude extract	MIC (μg/ml)			
	B. subtilis	*S. aureus*	*E. coli*	*Serretia* GSD2 sp.
Chloroform	3.0 μg/ml	1.0 μg/ml	≤0.5 μg/ml	2.0 μg/ml
Ethyl acetate	>20 μg/ml	7.5 μg/ml	5.0 μg/ml	>20 μg/ml
Methanol	>20 μg/ml	10 μg/ml	>20 μg/ml	>20 μg/ml

Table 4
Minimum Inhibitory Concentration of isolated compound.

Isolated compound	MIC (μg/ml)			
	B. subtilis	*S. aureus*	*E. coli*	*Serretia* GSD2 sp.
Chloroform	1 μg/ml	≤0.5 μg/ml	≤0.5 μg/ml	2.0 μg/ml
Ethyl acetate	15 μg/ml	5.0 μg/ml	2.5 μg/ml	>20 μg/ml
Methanol	>20 μg/ml	10.0 μg/ml	15.0 μg/ml	>20 μg/ml

contradiction with the previous reports in strains *Streptomyces sannanensis* strain RJT-1 [19], *Streptomyces kanamyceticus* M27 [20] where the glucose facilitates the production of secondary metabolites. The depleted growth in the glucose supplemented media was might be due to high concentration of glucose increases the cell growth and leads to inhibition of antimicrobial agent production and also repress the secondary metabolism [21,22]. Out of both organic and inorganic nitrogen sources, maximum antibiotic production was found in the medium consists of yeast extract (1.5%) as nitrogen source, our results are in lines with the previous reports of optimum antibiotic production using organic nitrogen sources for better yield [23,24].

S. werraensis strain grew and secreted antibiotic in range 2–5% NaCl with optimal concentration at 2.5%. However, further above 3% salt concentration, strain was grown without production of antibiotic. BCI-1 secreted the antibiotic in wide range of pH 6–9, while poor growth was evident at pH values below pH 6.0. The maximum growth as well as antimicrobial compound production was obtained at pH 9. The result strongly depicts the alkaline nature of organism which supports the previous reports [18,25,26]. The *S. werraensis* was found to be in mesophilic in nature as it shows narrow range of incubation temperature for relatively good growth and antibiotic production. *S. werraensis* secreted antibiotic after 7 days of incubation at 30 °C which was found optimum for maximum growth and antibiotic production (Fig. 1). It has been reported that the environmental factors like temperature, pH, salt concentration and incubation have profound influence on antibiotic production [27–29]. Production of antibiotic was found to be highest at pH 9, whereas at pH 10 antibiotic production was completely depleted. The results are comparable with some *Streptomyces* species recorded to produce antibiotics against bacteria, fungi and yeast at alkaline Ph [18]. The results are in contrast to the result reported using *Streptomyces* sp. ERI-3 for antimicrobial production [30]. Our findings supports fact that generally alkaline environment is more suitable for the growth of *Streptomyces* and thus production of antimicrobial compound [16]. Antibiotic production was optimum at 2.5% NaCl with in significant decrease at 3 and 4%. The strain of Saha and group reported that the antimicrobial potential of actinomycetes isolate grew in the presence of 20% (w/v) NaCl, while 5% salt concentration was found to be optimum for antibiotic production [31]. *S. werraensis* secreted the antibiotic with optimum temperature at 30 °C. This temperature range is reported as adequate for good production of secondary metabolites is narrow temperature range for example, 5–10 °C (Tables 3 and 4).

The FT-IR spectrum of the partially purified antimicrobial compound produced by *S. werraensis*, showed 96% structural similarity with that of the Erythromycin A (screened form the Library match) (Fig. 2).

In HPLC analysis, two peaks were found to be merged with that of standard after merging the two chromatograms. Further test chromatogram was screened for the library match build in Shimadzu HPLC (Fig. 3a and b). On the basis of the standard erythromycin and standard build in library for identification of the antimicrobial agent, it could be stated that the antimicrobial compound is suggestive of being belonging to erythromycin antibiotic. For partial purification, separation of antibiotic has

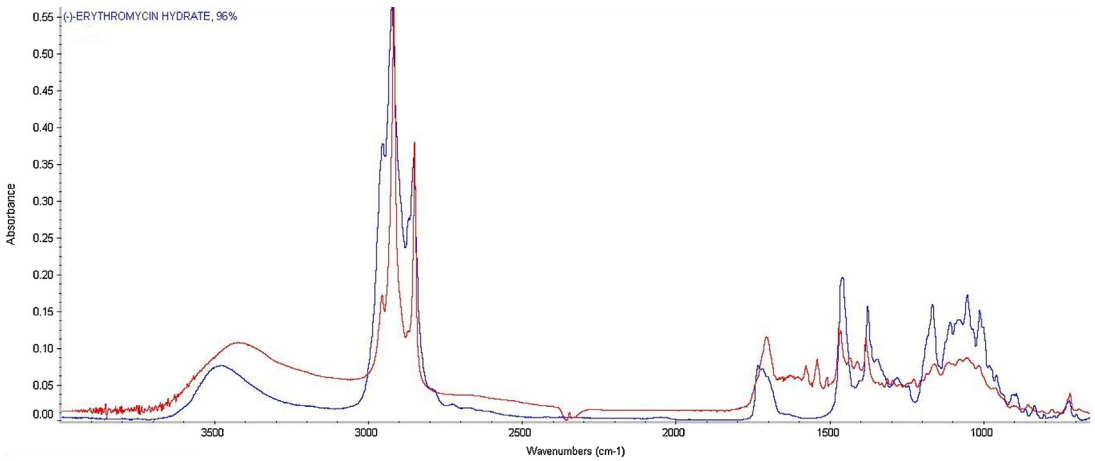

Fig. 2. FT-IR spectra of the partially purified bioactive compound showing 96% structural resemblance with that of erythromycin hydrate (Blue color – Erythromycin Std., Red color – Crude compound). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

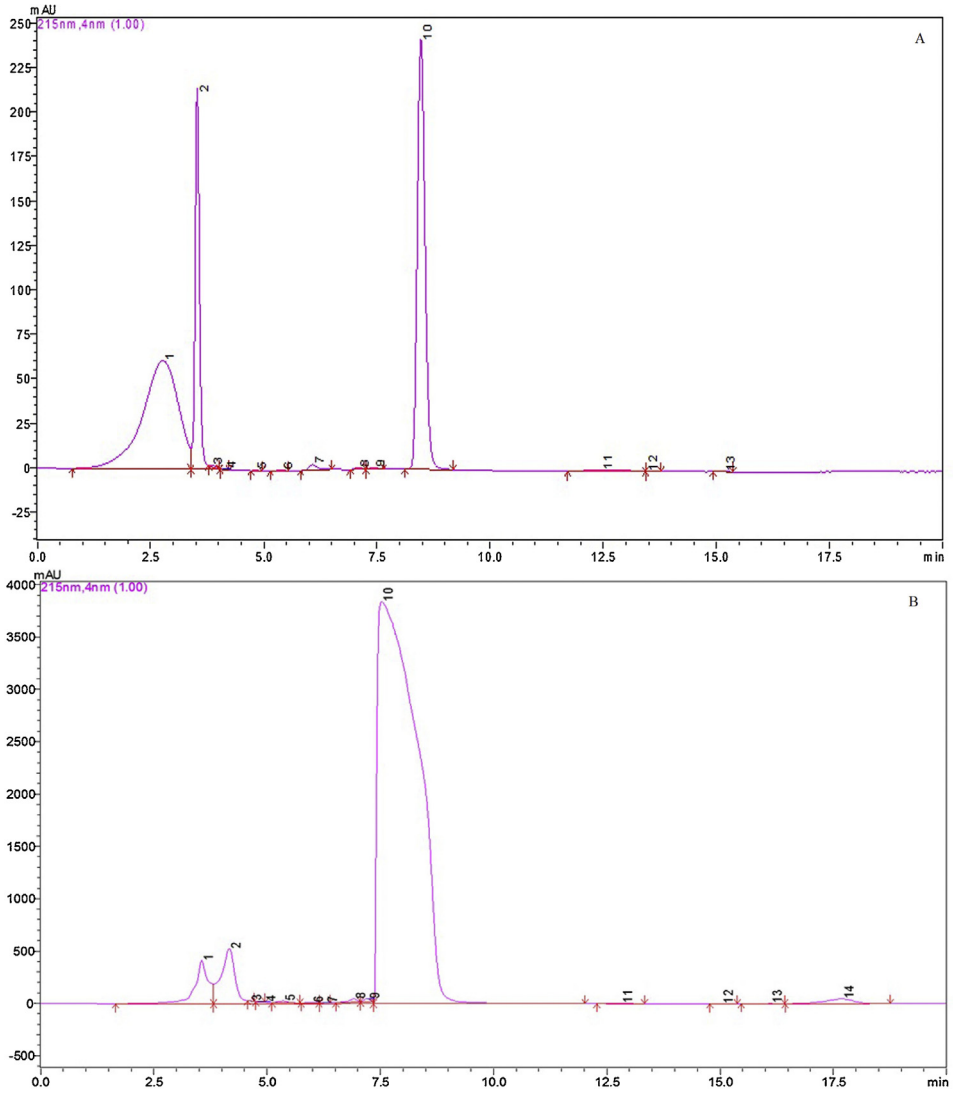

Fig. 3. (A) HPLC chromatogram of erythromycin. (B) HPLC chromatogram of the partially purified antibiotic.

been tried by thin-layer chromatography using a solvent system of chloroform and methanol (24:1, v/v) [32,33]. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol but sparingly soluble in water; similar results were recorded earlier [33,34]. The characterization of partial purified compounds was carried out by FTIR and HPLC analysis. Infrared spectra showed a primary imine function (3469–3451 cm^{-1}), amine function (3040, 673 cm^{-1}), alkane groups (2958–2853 cm^{-1}, 1466–1461 cm^{-1}) (C=C) of aromatic ring (1639–1495 cm^{-1}), p-di substituted benzene (831 and 801 cm^{-1}) and secondary alcohol function (3469–3451, 1370^{-1}, 408, 1192^{-1}, 198, 1040–1111 cm^{-1}). HPLC analysis showed confirmation through similar λ_{max} of standard, constructed library of reference standards by Shimadzu Inc. with isolated antibiotic, similar characterization of compound was reported earlier by many investigators [25,35].

Currently, increased resistant among pathogens against the available antimicrobial compounds, search of novel natural source for production of antimicrobial compounds is important. Present investigation highlights importance of media and cultural conditions for production of antimicrobial compound with its structural characterization.

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