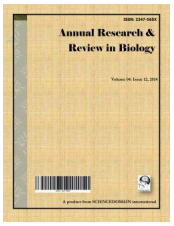

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Phylogenic Characteristics of a Unique Antagonistic *Micromonospora* Sp. Rc5 to *S. aureus* Isolated from Sinai Desert of Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. Authors NAA, ST, AA and EMHW designed the study and wrote the protocol. Author DHA managed the lab work of the study. Author AA managed the paper organization. Authors DHA and AA wrote the first draft of the manuscript. Authors DHA and AA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A distinct strain named *Micromonospora* sp. Rc5 was isolated from Sinai desert of Egypt and recorded high antagonistic activities against some food and bloodborne pathogens. Morphological and chemotaxonomy characterization confirmed that this isolate belongs to genus *Micromonospora*. Sequencing of partial 16S rDNA and BLASTN showed that isolate Rc5 is identical to *Micromonospora haikouensis* (99%) but with low bootstrap value in NJ phylogenetic tree. Comprehensive optimization of several growth factors was performed including initial pH, incubation periods, and different sources of carbon and nitrogen. The highest yield of antimicrobial agent production was obtained after 8 days of incubation at 30°C, pH 6.0, 3 x 10^5CFU/ml in soya bean meal broth media with agitation of 150 rpm. A dramatic proportional decrease occurred with

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0.3, 0.6, 0.9 µg active fraction /ml against *Staphylococcus aureus* culture and reached to complete inhibition at a minimum inhibitory concentration of (1.5 µg /ml). The physicochemical characterization of the purified fraction was identified as phthalate derivative. Our results indicated that Rc5 generated potential antimicrobial compounds against foodborne pathogens and may combat resistant strains of *Staphylococcus aureus*.

Keywords: Micromonospora; S. aureus; phthalate derivatives; 16SrRNA gene analysis.

1. INTRODUCTION

In 2015, World Health Organization (WHO) declared the occurrence of antimicrobial resistance everywhere in the world as a great challenge to public health [1]. *S. aureus* is associated with life-threatening diseases, such as pneumonia, meningitis, bacteremia, osteomyelitis, endocarditis, toxic shock syndrome (TSS), and sepsis [2,3,4]. The occurrence of Multi-drug-resistant *Staphylococcus aureus* has become overwhelming in Egyptian healthcare sectors against oxacillin [5] carbapenem [6] methicillin and erythromycin, co-trimoxazole, clindamycin and doxycycline [7].

Over the years, the treatment of suspected *S. aureus* infection has been complicated due to the resistance to multiple drug classes [8]. Exploring novel antibiotics that would compact earlier infection has become inevitability vital. Actinomycetes are among the promising sources of alternative antibiotics [9]. New approaches for exploring alternative antibiotic requires new approaches for screening less exploited rare Actinomycetes rather than well studied and repeated strains [10,11,12,5]. Rare actinomycetes isolated by conventional methods such as morphological, chemotaxonomic require further molecular identification [8,12]. 16S rRNA gene sequence analysis is considered as an efficient method for specific identification of actinomycetes on the genus level and it clearly distinguishes the phylogenetic analytical differences between the newly identified isolates and other related strains [13]. Rare actinomycetes produce many bioactive secondary metabolites [14]. Additionally, the genome sequences of rare actinomycetes have received much attention as important sources for bioactive compounds production [15,16]. *Micromonospora* is a rare genus belonged to family *Micromonosporaceae* with different well-known sources of diverse groups of antibiotics such as aminoglycoside antibiotics, gentamicin and netamicin [17]. They are gram-positive, spore-forming, generally aerobic, and form a branched mycelium; they are saprotrophic in soil and water.

A number of 183 rare actinomycetes strains were isolated in our laboratory from different Egyptian governorates (Giza, Qualubiya, Alexandria, Assuit and Sinai) with the aid of selective humic acid-vitamin agar media as described by [18]. Our present work studied the taxonomy of a *Micromonospora* sp. Rc5 using morphological, chemotaxonomy and phylogenetic analysis. In addition, various growth conditions were studies in order to reach to highest antimicrobial yield against Foodborne and bloodborne pathogen such as *Staphylococcus aureus* ATCC 6538. Antibiotic fractions were also analyzed with the use mass spectroscopy, Infrared spectra (IR) and ^{1}H, ^{13}C Nuclear Magnetic Resonance (NMR).

2. MATERIALS AND METHODS

2.1 Microorganism and Routine Cultivation

Rc5 strain was selectively isolated from Sinai desert using rehydration and centrifugation treatment [19]. A soil sample (0.5 g) was placed in a beaker, which was then softly flooded with 50 ml of 10 mM phosphate buffer. The vessel was roughly covered with aluminum foil, and incubated statically at 30°C. Eight milliliters of the flooding solution was then transferred into a screw-cap test tube (16.5 × 105 mm) and centrifuged (37°C, 20 min, 100 RPM) in a swinging bucket rotor. After settling for 30 min, an aliquot of 1 ml of the supernatant containing zoospores was serially diluted using 9 ml sterile water tubes, and then 0.1 ml inoculum of each serial dilutions were cultured on both starch casein and humic acid vitamin agar plates [20] for 20 days at 30°C. Colonies were selectively picked up and purified on starch casein agar plates containing cycloheximide (50 mg l^{-1}) to suppress fungal growth [21]. For antagonistic studies, cultivating of Rc5 was conducted with some modifications [22]. Rc5 Spore suspension was grown into 35 ml of soya bean meal broth

media for 8 days at 30°C with or without shaking at 150 RPM. The result spore suspension was lypholized at the Mycological center, Assiut University, Assiut, Egypt and stored at -20°C for the future studies. Test bacteria were routinely cultivated overnight in nutrient broth at 37°C and obtained from Ain Shams Specialized Hospital and the Microbial Resources Center (MIRCEN) at the Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Test bacteria, including Food and bloodborne pathogens (*Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 10145, *Klebsiella pneumonia* CCM 4415, *Streptococcus mutans* ATCC 25175, *Escherichia coli* O157:H7 ATCC 51659 and *Salmonella enterica* ATCC 25566) were used in this study.

2.2 Agar Well Diffusion Method

A preliminary screening of Rc5 antagonistic activities was evaluated using agar well diffusion method. Rc5 spore suspensions were prepared as stated above and used for inoculation. Cell-free supernatant was obtained by centrifugation for 5 min at 37°C with speed 100 RPM. An aliquot of (250 µl) was added to each well in nutrient agar Petri dishes containing 0.5Mcfarland of tested bacterial spores [23]. Petri dishes were then incubated for 24 hrs at 37°C and then the inhibition zone areas were recorded [24]. All tests and experiments were made in duplicates and the potent isolate was subjected to further study.

2.3 Morphological and Chemotaxonomic Analysis

Morphological and chemotaxonomic characterization was examined as a primary identification of the Rc5 isolate according to Bergey's Manual of Systematic Bacteriology, Vol. 4 [25]. Morphological characteristics including presence and color of aerial mycelium, the color of substrate mycelium and presence of experiments production or not. Growth of Rc5 was examined by naked eyes after 8 days of culturing at 30°C in different media; glycerol arginine agar, soya bean agar, oatmeal agar, glucose- yeast extract agar, czapeks agar, nutrient agar and starch casein agar [26]. Slide culture technique was prepared by inserting sterilized glass slides diagonally at the center of a Petri dish containing a thick layer of starch casein agar. Then, Rc5 was inoculated once at the inner interface between the slide and media in one direction. Petri dishes were incubated for 6, 8 days at 30°C [21] and the growth of aerial mycelium was examined under light microscopy, Labo, USA. Chemotaxonomic characterization of Diaminopimelic acid (DAP) isomers in the cell wall hydrolysate were examined as follows: Cells were harvested from 8-days culture of Rc5 in starch casein broth by filtration using filter paper (Whatman) and allowed to dry. Dried cells (3 mg) were mixed with 1 ml of 6 N HCl in clean Eppendorf tubes. After one hour in the autoclave at 121°C, 3 µl of each sample was added to cellulose paper chromatogram (Whatman - number 1) loaded with 1 µl of 0.01 M DL-DAP (Sigma). Cellulose paper chromatogram was placed in a closed glass container containing MeOH: H_2O: 6N HCl: Pyridine at a ratio (80:26:4:10, v/v)" and then left for 3 hrs to allow the mobile phase solvents to interact with the stationary phase. Spots were obviously visualized after spraying the chromatogram with 0.2% ninhydrin in water-saturated n-butanol follow by drying in the oven at 100ºC for 5 min [27].

2.4 16S-rRNA Genes Amplification

Extraction of genomic DNA was performed with the use of Promega Wizard® Genomic DNA Purification Kit and PCR analysis of 16S rRNA gene as described by [28]. Universal Primers pairs PA/F (5'- AGAGTTTGATCCTGGCTCAG-3') [24] and 517/R (5'-ATTACCGCGGCTGCTGG-3') [20] were used to amplify 500 bp. Amplification was conducted using thermal cycler (Applied biosystem 337) with initial denaturation at 94°C for 5 min and then 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C, and finally 10 min at 72°C. The negative control contained all components of the mixture except the DNA template. Five microliters of the PCR product was subjected to Gel electrophoresis procedure (1% agarose gels and stained with 50mg/ml of ethidium bromide) was conducted in 0.2MTris base, 0.1Msodium acetate, and 0.01 M Na_2EDTA, pH 7.8 for 30 min at 90 V. A UV transilluminator (Bio-Rad Laboratories, Hercules, CA) was used to capture the digital images.

2.5 Gene Sequencing and Phylogenetic Analysis

16S-rRNA genes sequencing was conducted using (DYEynamic ET Terminator Cycle Sequencing Kit, Amersham Pharmacia Biotech.), and genetic sequence analyzer(ABI 3130) at The Animal Health Research Institute, El Dokki,

Egypt. The 16S rDNA sequences were deposited at the GenBank database (http://blast.ncbi.nlm.nih.gov/) in order to display the closest matches of known species to the 16S rDNA sequences and phylogenetic tree construction was then conducted**.** NCBI database at (https://blast.ncbi.nlm.nih.gov) was used to compare 16S rRNA gene sequences of isolate Rc5 with other 16S rRNA sequences. Phylogenetic analysis was conducted with the use of CLUSTAL W program [29] and a phylogenetic tree was constructed using the neighbor-joining method using the Molecular Evolutionary Genetics Analysis (MEGA) software version 7 [29].

2.6 Optimization of Growth Conditions

The yield of antimicrobial obtained from Actinomycetes is greatly influenced by the growth conditions**.**The maximum yields of the antimicrobial agent were obtained from an inoculum of 3 x 10^5CFU/ml (equivalent to 1.0 ml spore suspension) of isolate Rc5 previously cultured into 35 ml of soya bean meal broth media for 8 days at 30°C with shaking at 150 RPM. So, this inoculum concentration was used in all experiments [30]. In order to maximize the yield of antimicrobial agents produced by isolate Rc5, a comprehensive optimization of the following growth factors studied: initial pH, incubation periods, and different sources of carbon and nitrogen. Soya bean meal broth media (35 ml) was inoculated with 1 ml spore suspension of 8-days grown a culture of Rc5 and subjected to various incubation periods (2, 4, 6, 8, 10 and 12 days) in shaking incubator (150 RPM) at 30°C. The initial pH of the starch casein broth was varied (4, 5, 6, 7, 8 and 9 pH values) using 0.1 M NaOH or 0.1 M HC1 for adjustments. The inoculation source was 1.0 ml spore suspension of 8-days grown a culture of Rc5 and incubated for 8 days (150 RPM) at 30°C. Carbon sources were investigated using D-glucose, D-fructose, sucrose, D-mannitol, lactose, maltose, starch and molasses. The ability to utilize nitrogen by isolate Rc5 was recorded with the use of ammonium nitrate, ammonium persulphate, casein, yeast extract, peptone, malt and soybean meal. Each of the previously mentioned carbon or nitrogen sources was replaced in soya bean meal broth media by the value 1% (w/v). A volume of 1.0 ml spore suspension of 8-days grown culture was added to each flask and incubated for 8-days (150 RPM) at 30°C. Evaluation of antimicrobial activities was conducted against *S. aureus* using agar well diffusion method as previously stated [24].

2.7 Isolation and Purification of Antimicrobial Products

Spore suspensions of isolate Rc5 were added to 800 ml soya bean meal broth media at a ratio of (1% V/V) and incubated for 8 days at 30°C under shaking conditions (150 RPM). Cell-free supernatant of isolate Rc5 was filtered through filter paper (Whatman) and tested against *S. aureus* via agar well diffusion method as previously stated. Bioactive components were extracted from the total cell-free supernatant using chloroform-ethylacetate (1:1 v/v) in a separating funnel. Three times extracts were conducted and then the organic and aqueous layers were tested against the *S. aureus* by agar well diffusion method as previously described. The mixture was then evaporated at 37°C in a rotatory evaporator under vacuum until dry. The crude residue was dissolved in 3 ml of chloroform for future use. The crude was then purified by loading 5.0 µl on aluminum silica gel Thin Layer Chromatography plate (TLC) with a dimension of (20 x 20 cm, Merck). TLC plates were inserted inside a glass jar containing chloroform and methanol (4:1 v/v) for 3 hrs at 30°C . The active substances were revealed on silica gel TLC plates and retention factor (RF) value was measured. Each fraction was scratched, eluted with 50 µl chloroform and antimicrobial activity was assessed again with agar well diffusion method against *S. aureus* ATCC 6538. Control wells were filled with chloroform solution and served as control.

2.8 Physico-chemical Characteristics of Antimicrobial Compounds

Active fraction obtained from TLC thin layer chromatography was subjected to Liquid Chromatography-Mass Spectrometry (LC-MS) conducted in the Selected Ion Monitoring (SIR) mode and High-Performance Liquid Chromatography (HPLC) conducted in the Multiple Reaction Monitoring (MRM) mode at AGERI center, Cairo, Egypt. The mass spectrum of the active molecules in methanol was obtained by using mass spectrophotometer API 2000 (PE Sciex Applied Biosystems, Foster City, CA, USA) at the Central Laboratory Services, National Research Centre in Cairo, Egypt. Additional measures were also performed: Infrared spectra (IR) of the active molecule recorded in chloroform using potassium bromide disks with

Perkin-Elmer Infrared 127B Spectrophotometer. ^{1}H, ^{13}C Nuclear Magnetic Resonance (NMR) analysis of pure fraction was recorded on a Varian 500 MHZ NMR spectrometer with tetra-methyl silane (TMS) as an internal standard, in Microbiology laboratory, Cairo University, Egypt.

2.9 Minimum Inhibitory Concentration (MIC)

Antimicrobial activities of bioactive metabolites were investigated via Minimum inhibitory concentration (MIC) with the use of Tube Dilution Assay [31]. Different concentrations of purified active fraction (0.3, 0.6, 0.9, 1.5, 3, 6, 12 and 24) µg/ml, eluted in (100 µl) chloroform were each added to 5 ml nutrient broth tubes, vortexed and then inoculated with equal amount (10 µl) of overnight culture of *S. aureus* ATCC 6538 (A_{600} of 0.164). Broth cultures were incubated at 37°C for 24 hrs followed by measuring the absorbance at 600 nm. The purified active fraction was replaced by 100 µl chloroform and serve as a control.

3. RESULTS

3.1 Potential Antimicrobial Activities of Isolate Rc5

Our initial investigation showed that pronounced antimicrobial activities of Rc5 isolate against Gram-positive (*Staphylococcus aureus* ATCC 6538 and *Streptococcus mutans* ATCC 25175) and Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 10145*,* and *Escherichia coli* O157:H7 ATCC 51659 and *Klebsiella pneumonia* CCM 4415).

3.2 Morphological and Chemotaxonomic Characterization

The cultivation of Rc5 for 8 days at 30°C showed leathery texture (Table 1) on 7 different media (glycerol arginine agar, soya bean agar, oatmeal agar, glucose- yeast extract agar, czapeks agar, nutrient agar and starch casein agar). Substrate mycelium illustrated a slight color variation (Table 1) with different culture media; orange to olive (glycerol arginine agar, soya bean agar, oatmeal agar, and czapeks agar), orange to brown (glucose- yeast extract agar and starch casein agar) and orange (nutrient agar). Moreover, single spores were observed on substrate mycelium. Results are shown (Fig. 1) Chemotaxonomic analysis indicated that isolate Rc5 contained meso, the diaminopimelic acid in the cell wall.

3.3 Molecular Characterization

Partial 16S rRNA genes (gamma region) of Rc5 were successfully amplified and produced amplicons of 500-bp fragments of 16S rRNA gene using PA and 517R primers as illustrated in the digital images of agarose gel captured by UV transilluminator (Bio-Rad Laboratories, Hercules, CA) (Fig. 2).

3.4 16S rRNA Genes Sequence Analysis and Phylogenetic Tree

16S rRNA gene sequencing has been recognized as an essential tool for identification of actinomycetes [32]. Purified amplicons of 500-bp fragments of 16S rRNA gene were sequenced and the partial 16S rRNA gene sequences were submitted in Gen-bank under accession number (KY817857).

GATACGCAGCTTACCTGCAAGTCGAGCGGAA
AGGCCCTTCGGGGTACTCGAGCGGCGAACG
GGTGAGTAACACGTGAGCAACCTGCCCTAG
GCTTTGGGATAACCCTCGGAAACGGGGGCT
AATACCGAATAGGACCTTCCCCCGCATGGGG
TTGGGTGGAAAGTTTTTCGGCCTGGGATGGG
CTCGCGGCCTATCAGCTTGTTGGTGGGGTG
ATGGCCTACCAAGGCGACGACGGGTAGCCG
GCCTGAGAGGGCGACCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGCACAATGGGCGGA
AGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCAGC
AGGGACGAAGCGCAAGTGACGGTACCTGCA
GAAGAAGCGCCGGCCAACTACGTGCCAGCA
CCGCCGGTAAAA.

Phylogenetic analysis of 16S rRNA *Micromonospora* gene was performed in order to elucidate the affiliation between Rc5 isolate and closely related *Micromonospora* species. The nucleotide sequences of *Micromonospora* strains exist in NCBI GenBank database were compared with 16S rRNA gene sequence of isolate Rc5. It showed that *Micromonospora haikouensis* strain 232617 was the closest match to isolate Rc5 with blast identity (99) %. This high blast identity shows that PCR product was almost fully sequenced. Phylogenetic analysis was conducted by comparing 16S rRNA gene sequence of the strain Rc5 with other nucleotide sequences from closely related *Micromonospora* strains using the neighbor-joining method. NJ

Table 1. The morphological characteristics of isolate Rc5 cultured at 30°C for 14 days

Culture media	Rc5			
	Texture	Aerial mycelium	Substrate mycelium	Soluble pigment
Glycerol arginine agar	Leathery	None	Orange- olive	None
Soya bean agar	Leathery	None	Orange-olive	None
Oat meal agar	Leathery	None	Orange-olive	None
Glucose- yeast extract agar	Leathery	None	Orange-Brown	None
Czapeks agar	Leathery	None	Orange-olive	None
Nutrient agar	Leathery	None	Orange-olive	None
Starch casein agar	Leathery	None	Orange	None

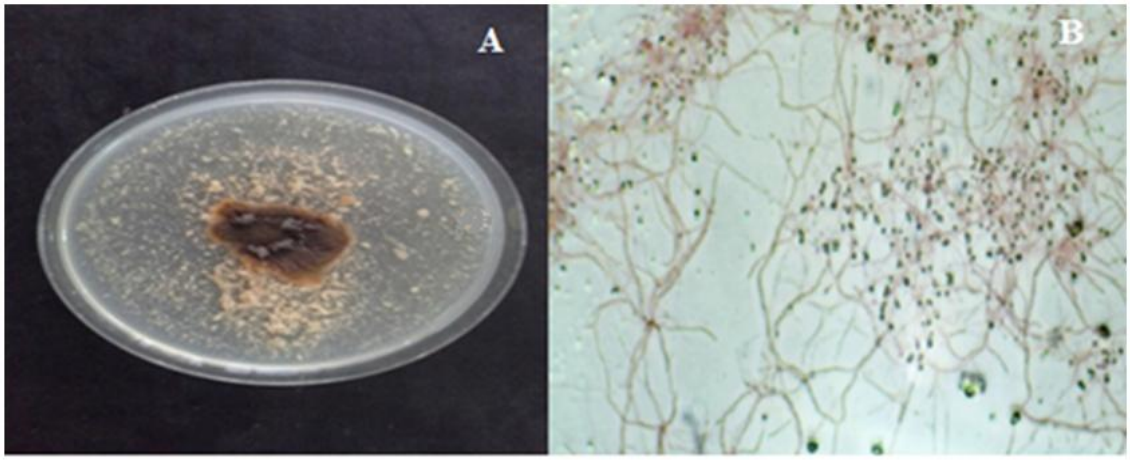

Fig. 1. Morphological identification of isolate Rc5. (A) Macroscopic characteristics of isolate Rc5, (B) Microscopic examination of isolate Rc5 using Slide culture technique

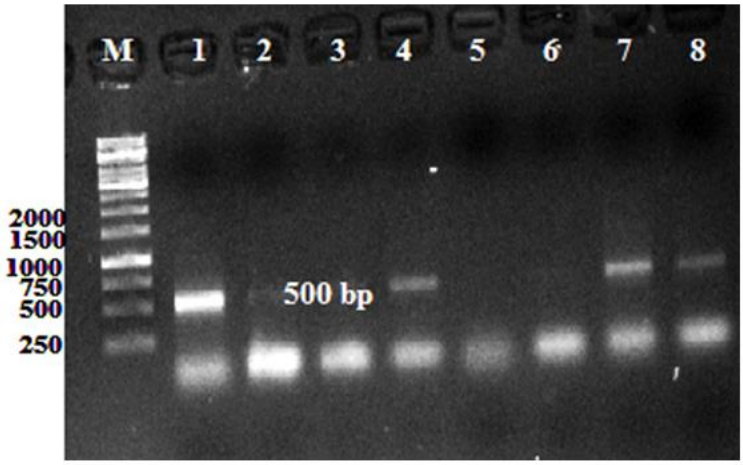

Fig. 2. Gel electrophoresis of agarose gel illustrating the amplification of partial 16SrRNA genes of isolate Rc5

Phylogenetic tree consisted of one main clade where isolate Rc5 was amongst other *Micromonospora* strains in the same clade. This ensures that isolate Rc5 belonged to genus *Micromonospora*. Similar 16S rRNA gene sequence belonged to *Micromonospora*

haikouensis strain 232617 and *Micromonospora carbonaceae* strain SZ2 found to fall under the same category. It is of interest to mention that, isolate Rc5 had low bootstrap (28%) even with the closest matches. Results are shown in the Fig. 3. Based on morphological and chemotaxonomic criteria along with 16S rRNA gene sequencing confirmed that strain Rc5 isolated from Egyptian soil was identified belongs to genus *Micromonospora* in Family *Micromonosporaceae* and identified as *Micromonospora* sp. Rc5.

3.5 Optimization of Growth Condition

Our data showed that optimization of growth conditions led to the production of highest yield of the antimicrobial agent. Soya bean meal broth was used for growing up *Micromonospora* sp.*Rc5* at 30°C by varying the fooling variables: cell density, pH value, incubation period, carbon source and nitrogen source were. The highest inhibition zoning of antimicrobial substances (27 mm) was obtained at a pH value of 6.0 (Fig. 4A). The largest zone was achieved after 8 days (19 mm) of incubation and then gradually declined as shown in (Fig. 4B). Various levels of antimicrobial activities were observed with different carbon sources as follows: glucose with the highest inhibition zone diameter (19 mm), followed by molasses, sucrose and fructose (Fig. 5). Lactose, maltose, mannitol and starch did not show any antimicrobial activities. This study proved that soya bean meal was the most favorable nitrogen source for the antimicrobial productivity reporting (19 mm) diameter. Another nitrogen source such as casein, ammonium nitrate, ammonium sulfate, recorded very low antimicrobial activities. No antimicrobial production was recorded on using peptone, malt and yeast extract in the culture media as shown in (Fig. 5).

3.6 Partial Characterization of Potential Antimicrobial Compounds

Various bioactive metabolites are generated from antibiotic-producing Actinomycetes [33]. Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) are known as efficient methods for the purification of bioactive components [33]. Four different bioactive fractions were obtained using TLC Purification of *Micromonospora* sp.*Rc5* extracted culture broth. All fractions were scratched from TLC plates, eluted in chloroform and the antimicrobial activities of each fraction were assayed against *S. aureus* ATCC 6538 as a test organism using agar well diffusion method. These fractions were easily differentiated by their distinguished colors; fraction 1 was faint pink (RF=0.81), fraction 2 was faint violet (RF=0.85), fraction 3 (RF=0.95) was faint brown while fraction 4 (RF=1) was deep brown (Fig. 6). Results showed that, only fraction 4 (RF=1) exhibited the antimicrobial activity as shown in (Fig. 6).

The molecular weights of the active fractions were analyzed and detected using LC mass scanning (Fig. 7A). Scanning spectrum showed the presence of several MW 472, 391, 360 and 279. The LC/MS analysis was conducted in the Selected Ion Monitoring (SIR) mode to separate the compounds present in this extract and identify their number. It also identified whether all detected molecular weights were different compounds or obtained from one compound with a parent molecule of MW 472, and their daughter's ions of 391, 360 and 279. HPLC analysis was conducted in the multiple reaction monitoring (MRM) modes to detect each of the daughter ions (391, 390 and 279) in relation to the parent molecule (MW 472). HPLC chromatogram showed the presence of the same peak at retention time 2.5 min and detectable parent molecule (MW 472) with all daughter ions (Fig. 7B). This result confirmed the presence of one pure active compound with a molecular weight 472 that can be fragmented into ions of MWs of 391, 360 and 279. The mass spectrum showed a fragment at 167m/z which is corresponding to dicarbonyl phenyl moiety and another one at 279m/z which is corresponding to phthalate moiety. The MW of 472m/z present in a very small intensity (Fig. 8). IR spectrum showed a strong signal at 1632 cm-1, which detects the presence of carbonyl group, in addition to the signals at wave number 2950 cm-1 and 2920 cm-1 which detect the C-H groups (Fig. 9). ^{1}H-NMR showed signals at 7.4 and 7.6 ppm that confirm the presence of aromatic ring (Fig. 10). The ^{13}C-NMR spectrum showed characteristic signals for the aromatic ring at ppm 128, 129 and 131 (Fig. 10). Based on the previous analysis, we confirm that the antimicrobial compound produced by isolate Rc5 is belonging to the phthalate derivatives. Further investigations are required to identify the actual structure of this molecule include the 2D-NMR analysis COSY, HSQC and HMBC.

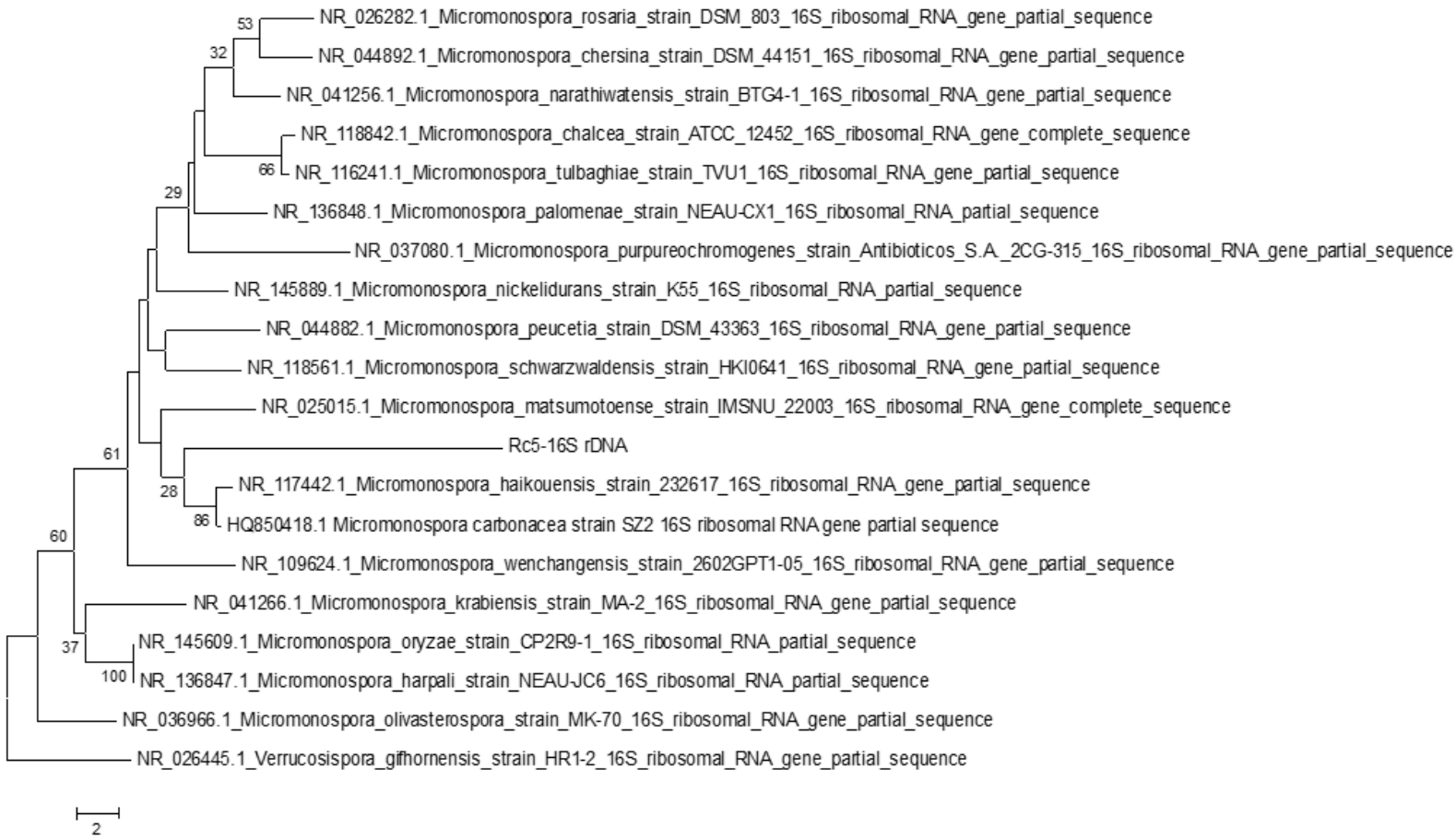

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences from *Micromonospora* sp.Rc5 and other *Micromonospora* species (The numbers beside the branches indicate the percentage bootstrap value of 1000 replicates)

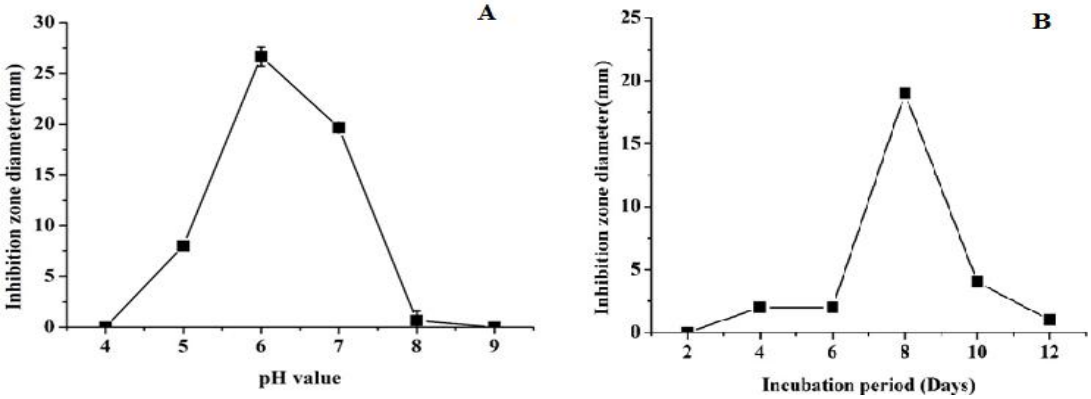

Fig. 4. Factors affecting the antimicrobial activities of *Micromonospora* sp.Rc5 against *S.aureus.* (A). Effect of incubation time and (B) Effect of pH values on antimicrobial agent production

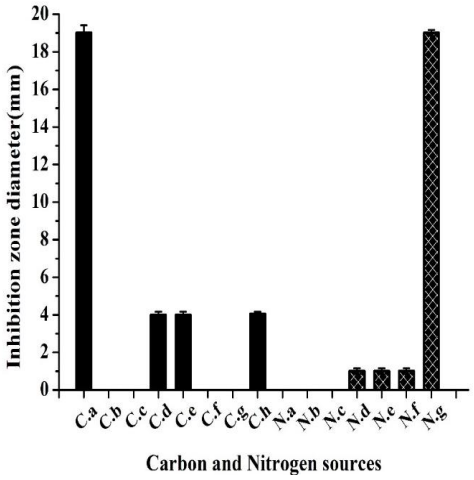

Fig. 5. Effects of different sources of carbon and nitrogen on antimicrobial activities of *Micromonospora* sp.Rc5 against *S. aureus*

3.7 Biological Evaluation of Active Fraction of Isolate Rc5

Our data indicated that the bioactive compound generated by Rc5 was highly effective as an antibiotic for the inhibition of the growth of *S. aureus*. A strong inhibition occurred when purified active fractions (0.3, 0.6, 0.9, 1.5, 3, 6, 12 and 24) µg/ml were tested against *S. aureus* in Tube Dilution Assay (Fig. 12). A dramatic proportional decrease occurred with 0.3, 0.6, 0.9 µg active fraction /ml broth of *S. aureus* culture and then complete inhibition at a Minimum inhibitory concentration (MIC) of (1.5 µg /ml). This shows that antimicrobial compound produced by the Rc5 is extremely active and we recommend future structural elucidation for this compound as a great antimicrobial candidate for antimicrobial resistance in Egypt and worldwide.

4. DISCUSSION

In the present work, *Micromonospora* sp. strain Rc5 was isolated from Sinai desert of Egypt and tested against some food and bloodborne pathogens. Isolate Rc5 showed great inhibition activities against selected Gram-positive and Gram-negative pathogens. The identification process for isolate Rc5 was carried out according to Bergey's Manual of Systematic Bacteriology [25]. Morphological and chemotaxonomy characterization confirmed that this isolate belongs to genus *Micromonospora*. Sequencing of partial 16S rDNA and BLASTN showed that isolate Rc5 is identical to *Micromonospora haikouensis* (99%). However the NJ phylogenetic tree showed the low bootstrap value that means that it is a unique strain. *Micromonospora haikouensis* was firstly isolated from a composite mangrove sediment sample collected in Haikou, China [31]. It was also isolated from marine sediment at Bohai Bay, Dalian, China. This is the first study conducted on the identification of *Micromonospora haikouensis* isolated from Sinai desert and the evaluation of its antimicrobial potential against *S.aureus*. The optimum parameters for the highest yield of the antimicrobial agent were obtained after 8 days of culturing 3 x 10^5CFU/ml of *Micromonospora* sp.Rc5 in soya bean meal broth media with agitation (150 RPM) at 30^{o}C, pH 6.0. Nearly similar pH values 6.8 -7 was the optimum for the

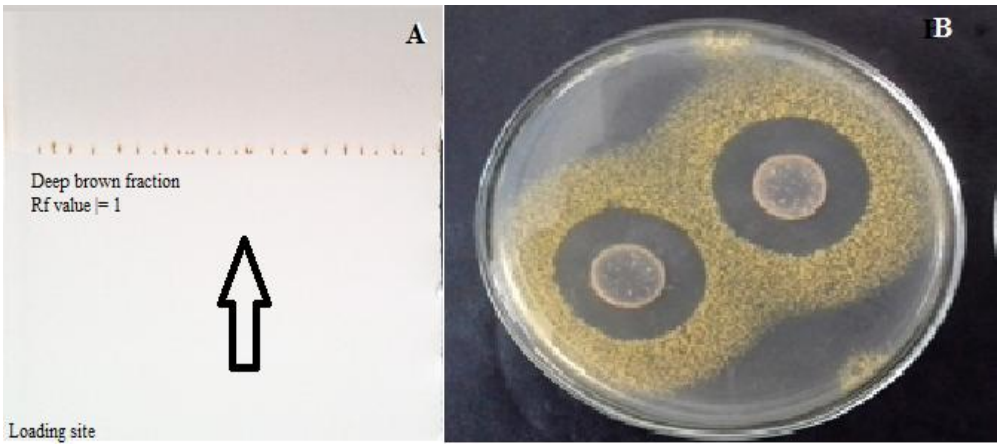

Fig. 6. TLC crude extract study. (A). Antimicrobial of crude extract on aluminum silica gel TLC plate and (B). Agar diffusion method of a brown fraction of *Micromonospora* sp.Rc5 against *S. aureus* agar diffusion method

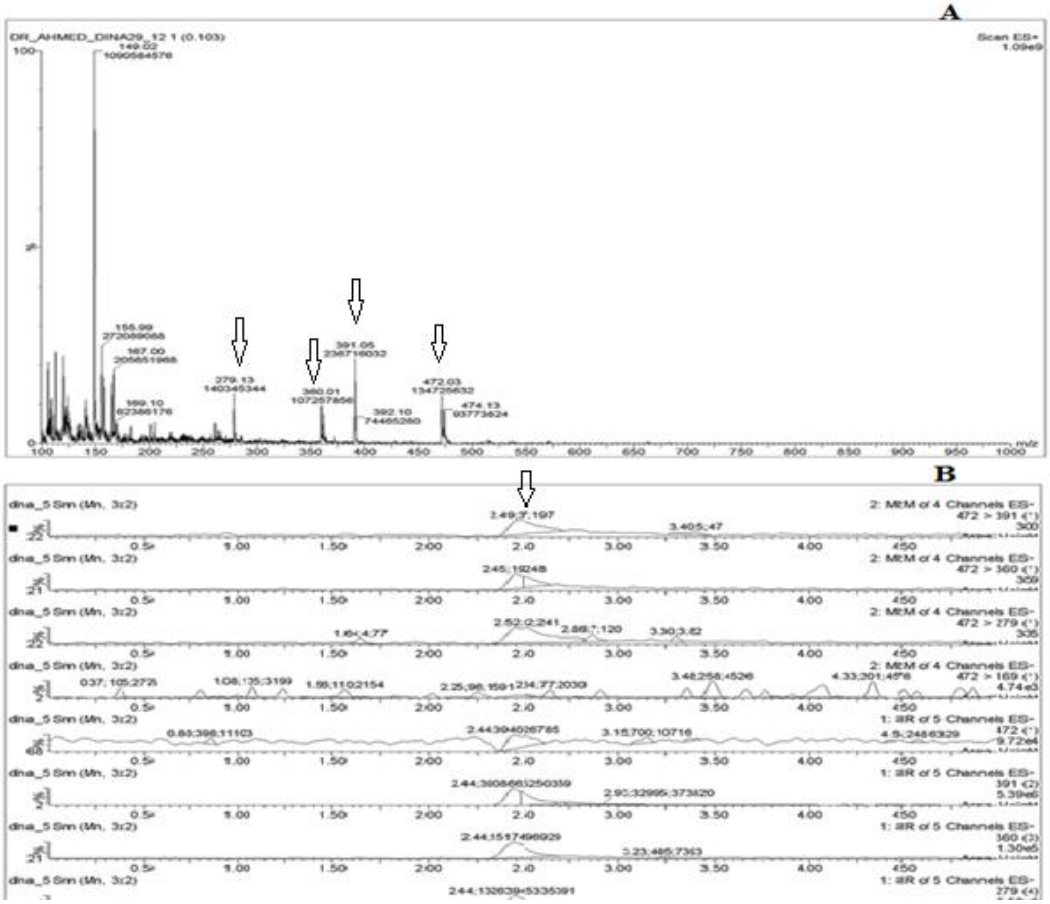

Fig. 7. (A) LC-MS/MS of brown band of *Micromonospora* sp.Rc5 dissolved in chloroform and (B) HPLC of brown band of *Micromonospora* sp.Rc5 dissolved in chloroform

production of gentamicin from *Micromonospora purpurea* [34] and LL-E19085α antibiotic production by *Micromonospora citrea* [35]. Less incubation periods (3 to 5 days) found to release

the highest antimicrobial agent production [34, 36], respectively. Carbon and nitrogen sources are vital components for the fermentation process. In our study, the addition of glucose and soya bean meal to *Micromonospora* sp.Rc5 culture media produced the highest antimicrobial agent yield. Similarly, soya bean meal was a favorable nitrogen source for antimicrobial production form *Micromonospora* sp. [34,37]. Other studies found that the best carbon sources for the maximum yield of antibiotic productivity were sucrose with *Micromonospora* sp. [34] and maltose with *Micromonospora echinospora* [36].

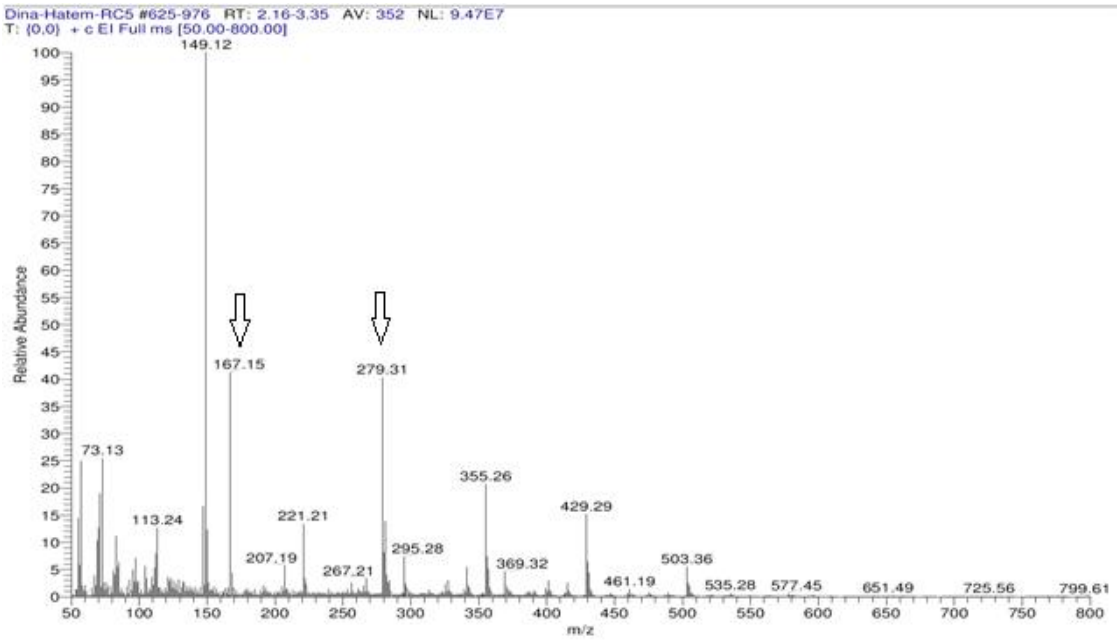

Fig. 8. Mass spectrum of the brown band of *Micromonospora* sp. Rc5

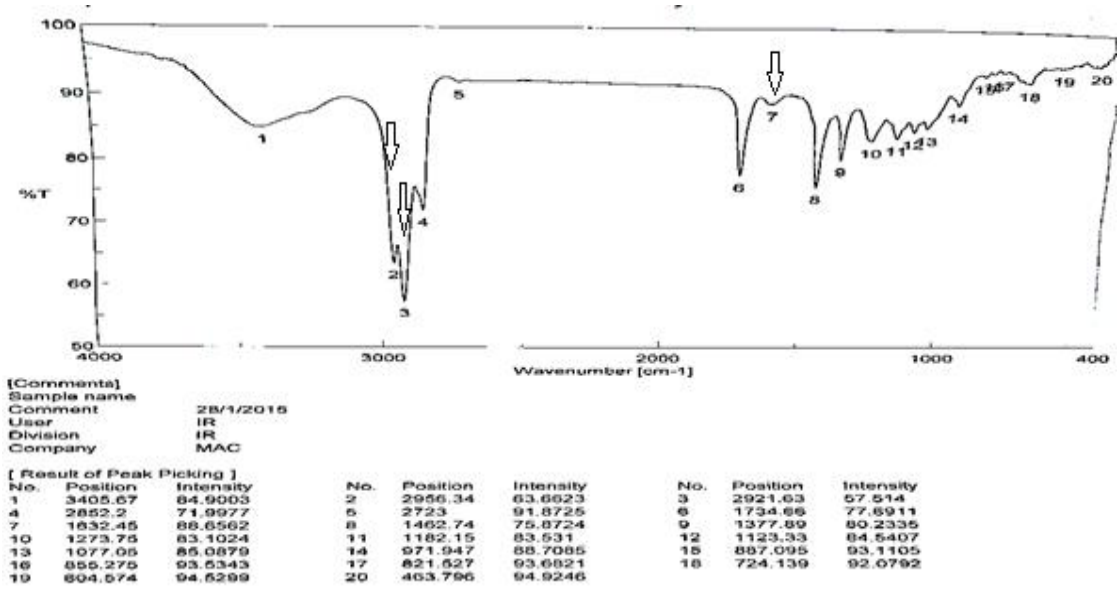

Fig. 9. IR analysis of the eluted brown fraction of *Micromonospora* sp. Rc5

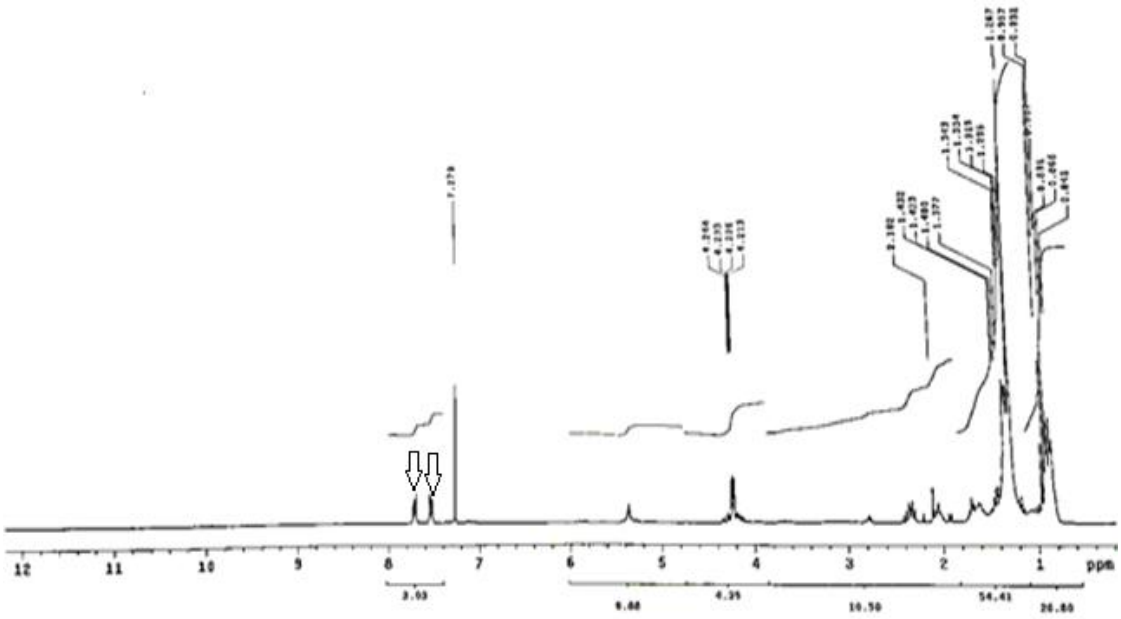

Fig. 10. ^{1}H-NMR of eluted brown fraction of *Micromonospora* sp. Rc5

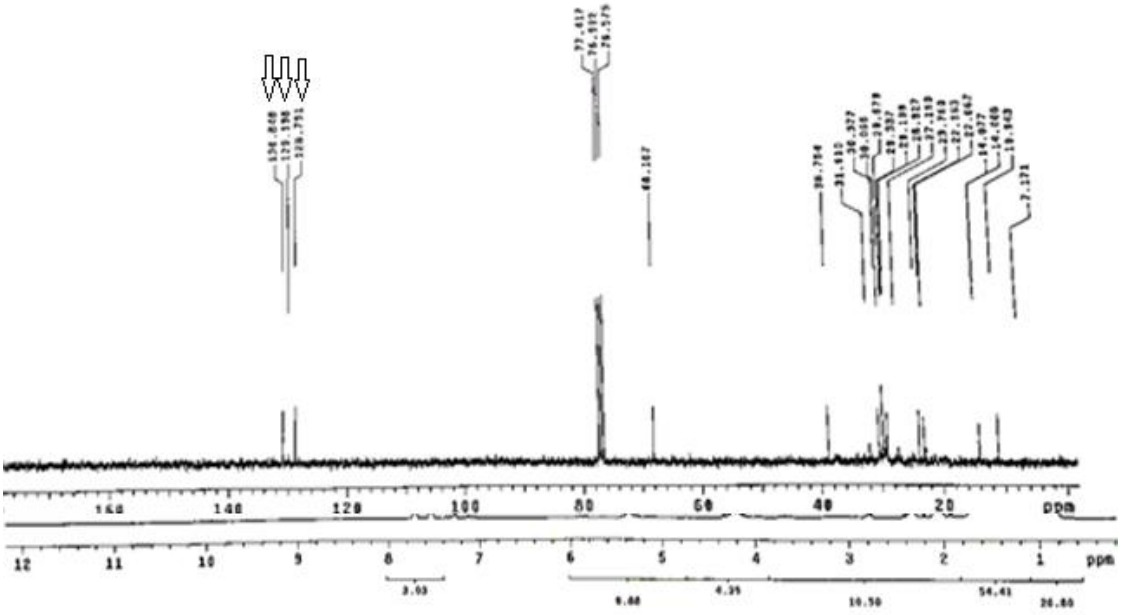

Fig. 11. ^{13}C-NMR of eluted brown fraction of *Micromonospora* sp. Rc5

The physicochemical characteristics of the purified fraction were identified as phthalate derivatives and showed antimicrobial activities against *S. aureus* ATCC 6538. Some existing published data showed that another strain of same species produced tetrocarcins with the phthalate core structure [38]. However, IR, ^{1}H, ^{13}C NMR patterns we obtained is different from tetrocarcins N, O already produced by *Micromonospora haikouensis*. This can be a new analog, of tetrocarcins depends on strain variation. In addition, our strain showed higher antimicrobial activity against *S. aureus* than already recorded by tetrocarcins N, O. Tetrocarcins are important molecules that have antibacterial activity [10] and antitumor activity

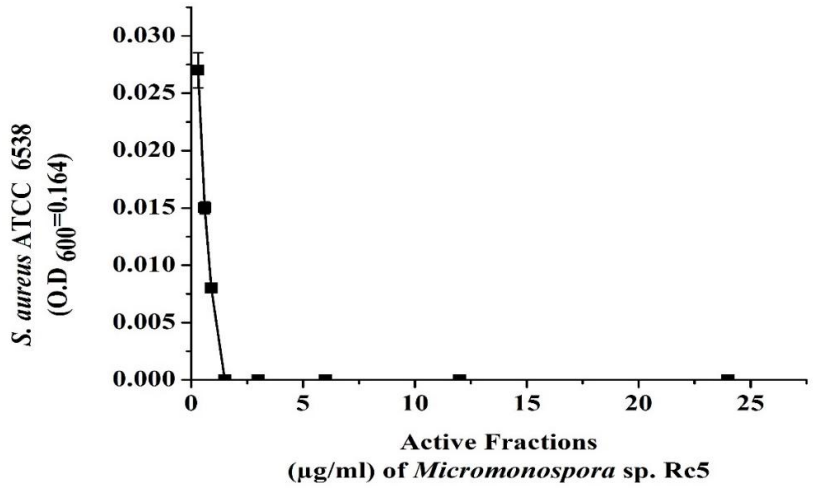

Fig. 12. The Minimum inhibitory concentration (MIC) of purified active fraction of *Micromonospora* sp. Rc5 was detected using tube Dilution Assay against *S. aureus*

against sarcoma 180, P388 leukemia, and B16 melanoma [39]. Further investigations are required for the complete structural elucidation of this interesting, highly active compound .This can be a promising product to control microbial drug resistance in Egypt.

5. CONCLUSION

This study concluded that *Micromonospora* sp. Rc5 was isolated from Sinai desert of Egypt and showed high antagonistic activities against some food and bloodborne pathogens. This was confirmed via Morphological and chemotaxonomy characterization and 16S rDNA sequencing. partial 16S rDNA Sequencing showed that isolate Rc5 is 99% identical to *Micromonospora haikouensis* but with low bootstrap value in NJ phylogenetic tree. The physicochemical characterization of the purified fraction was identified as phthalate derivative. A complete inhibition of *S. aureus* occurred at a minimum inhibitory concentration of (1.5 µg /ml).

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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