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Antimicrobial Activities of a Novel Marine *Streptomyces* sp. MMM2 Isolated from El- Arish coast, Egypt

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ABSTRACT

There is diversity and novelty among actinomycetes present in marine environments. Marine microbial resources may lead to the discovery of a new antimicrobial agent added to the commercially used ones. In this study, several marine Actinomycetes were isolated from the Red Sea (Berenice and Safaga) and Mediterranean Sea (Arish and Abou Quir), and screened for their ability to produce antimicrobial agents (s) against *Staphylococcus aureus*, *E.coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Edwardsiella tarda* using agar well diffusion. The most potent isolate identified as *Streptomyces* sp. MMM2 by the 16S rRNA sequence gene. Optimization of the antimicrobial products was performed by Plackett–Burman experimental design. Ethyl acetate, butanol, hexane, and acetone extracts were used for the screening. Anti-fungal activity of *Streptomyces* sp. MMM2 was also estimated. Chemical characterization of the ethyl acetate extract of *Streptomyces* sp. MMM2 was performed using GC-MS spectrophotometry, FTIR, Raman, and NMR. This analysis showed that effective compounds that had antimicrobial activities were hexadecanoic acid methyl ester, 9-octadecadienoic, octadecadienoic acid methyl ester, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, 9,12-octadecadienoyl chloride.

INTRODUCTION

Since ancient times and till now, people have used natural products extracted from plants, animals, microorganisms and marine organisms to get medicinal ingredients for different diseases **(Yuan *et al.*, 2016)**. Bioactive products obtained from natural sources are accepted by human compared to other chemical products. People realized that usage of chemical products in the long term will have a dangerous impact on their health. More than 500,000 natural bioactive compounds reported all over the world from biological sources, approximately 70,000 compounds were derived from microorganisms 29% of them were derived from Actinomycetes **(Subramani and Sipkema, 2019)**.

Over seventy percent of surface of the earth is covered by water and it is believed the first life origin was in the oceans (**Malash *et al.*, 2016)**. Marine microorganisms could

be a great opportunity to produce several bioactive products **(de la Calle, 2017**). Natural products produced from marine microbes have wide range of biological activities including antiparasitic, antitumor and antimicrobial activities (**Fremlin *et al.*, 2009)**. The reason for marine microorganisms production of biologically active compounds is to adapt to particular environmental conditions (**Penesyan *et al.*, 2010)**.

Marine Actinomycetes are great sources of novel bioactive compounds with many medical applications. Actinomycetes are priceless prokaryotes due to their competency to produce several bioactive compounds **(Subramanian *el al.*, 2017)**. Marine Actinomycetes have special characteristics vary from those isolated from soil therefore marine actinomycetes might produce alternative types of bioactive products (**Ameen *et al.*, 2021)**. Marine *Streptomyces* species are efficient in the production of secondary metabolites with biological activities including antibacterial **(Sivalingam *et al.*, 2019)**, antifungal **(Fadhilah *et al.*, 2021)**, anticancer **(Law *et al.*, 2020)**, anti-inflammatory **(Shin *et al.*, 2022)**, antiprotozoal (**Pagmadulam *et al.*, 2020)**, anti-malaria **(Kazmaier and junk, 2021)**, antiviral **(Manimaran *et al.*, 2021)** and antioxidant **(Abdel-Aziz *et al.*, 2019)**.

The main purpose of this work was to isolate Actinomycetes from sediments samples collected from Safaga and Berenice (Red sea), Arish and Abou Quir (Mediterranean Sea) to assess their antimicrobial activity against different pathogens and their chemical characterization of the probable effective compounds.

MATERIALS AND METHODS

1. Isolationof different marine actinomycetes:

Seventy sediment samples were collected from different parts of the Red Sea (20 samples from Safaga and 15 Berenice) and the Mediterranean Sea (20 from Arish and 15 from Abou Quir) as described by **Austin (1988)** during February 2019. From each sample, 5 g was dispersed in 50 ml sterilized sea water, from diluted samples a portion 0.1ml was used to inoculate plates prepared with seawater starch casein media for isolation different marine Actinomycetes. Inoculated plates were incubated at temperature 37 °C for 14 days. The obtained pure colonies were transferred to fresh slants and at 37°C incubator for 14 days. All slants were kept at 4°C for further investigations.

2. Screening for the antiantimicrobial activity of differen isolated Actinomycetes and identification of the most potent one:

The antimicrobial activity of different isolated Actinomycetes was carried out by using the agar well diffusion method (**Sharma *et al.*, 2013)**. Briefly Actinomycetes strains were individually inoculated into 50 ml of starch casein medium. The inoculated isolates were cultured in broth in a rotary shaker at 20 x g for 7 days at 37°C. The broth was centrifuged on two runs on 11269 x g for 15 minutes then 7826 x g 10 minutes to obtain cell free. Tested pathogens were *E.coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC

29212, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883 and *Edwardsiella tarda* (all these pathogens provided by the Microbiology Lab of NIOF, Egypt). Fifty µl of tested different pathogens at (McFarland=2) were seeded on nutrient agar and poured into petri dish (9 cm in diameter) and wells 10 mm in diameter were punched in the agar with a sterile tip, 100 µl of each extract were put in each well then incubated for 18-24 hour to observe zone of inhibition and measure diameter. The most potent isolate was identified. using microscopic examination and PCR detection using 16S rRNA sequencing molecular technique according to **Hamaki *et al.* (2005).** Sequencing was made on GATC Company, Germany by ABI 3730xl DNA sequencer using forward primer AGAGTTTGATCCTGGCTCAG and reverse primer GGTTACCTTGTTACGACTT **(Weisburg *et al.*, 1991**).

3. Different media effect on antimicrobial production:

Different media were recommended for production of the antimicrobial product from Actinomycetes **(Yaginuma *et al.*, 1981)**. Starch Nitrate medium, Oatmeal Nitrate medium, Starch casein agar and International Streptomyces Project (ISP) medium were used. This was carried out in 250ml conical flask using 50 ml of each culture medium inoculated with the most potent antimicrobial producer (3AR) incubated in shaker incubator at 20 x g and 37°C for 7 days to determine the best media for production of antimicrobial activity.

4. Optimization the Production of the Antimicrobial product:

The Plackett Burman experimental design is fractional factorial design was applied by **plackett and Burman (1946**). Seven independent variables representing components of starch casein medium plus some of physiological factors were screened in nine combinations (Table 1). The design of Plackett-Burman was organized in to low level (-1), basal medium (0) and high level (+1). The factors tested were starch (5g, 10g, and 15g), casein (0.15g, 0.3g, 0.45g), KNO_3 (1g, 2g, 3g), K_2PO_4 (1g, 2g, 3g), pH (6, 7, 8), inoculum size (1 ml, 2 ml, 3 ml), temperature (30°C, 37°C, 44°C) (Table 2).

The main effect of each individual variable was calculated by the following equation where is Exj= (Mi+ – Mi-) / N where Exj is the variable main effect, Mi+ (high antimicrobial activities), Mi- (low antimicrobial activities), and N is the number of trials divided by 2 according to **El-Sersy (2012)**.

Microsoft Excel was used to calculate Statistical t-values for two samples assuming unequal variances to know significant variables according to **Hassan *et al.* (2017).**

Table 1: Plackett-Burman experiment on seven variables of starch casein medium.

Trial	starch	casein	KNO3	K2PO4	PH	Inoculum size	temp
1	1	-1	-1	1	-1	1	1
2	1	1	-1	-1	1	-1	1
3	1	1	1	-1	-1	1	-1
4	-1	1	1	1	-1	-1	1
5	1	-1	1	1	1	-1	-1
6	-1	1	-1	1	1	1	-1
7	-1	-1	1	-1	1	1	1
8	-1	-1	-1	-1	-1	-1	-1
9	0	0	0	0	0	0	0

Numbers in the table refere to low level (-1), basal level (0) and high level (+1) of each variable of starch casein medium.

Table 2: Factors examined as independent variables affecting the production of antimicrobial activity.

Variable	Low (-1)	Basal (0)	High (+1)
Starch(g/l)	5	10	15
casein(g/l)	0.15	0.3	0.45
KNO3(g/l)	1	2	3
KPO4(g/l)	1	2	3
pH	6	7	8
Inoculum size (ml)	1	2	3
Temp (°C)	30	37	44

5. Extraction of Antimicrobial products using different solvents:

The most potent antimicrobial producer (3AR) cultured on the optimized starch casein medium was centrifuged and the supernatant was extracted using equal amount of ethyl acetate, butanol, hexan and acetone (1:1 vol/vol) according to **Parthasarathi *et al.* (2012)**. The extract was completely dried using a rotary evaporator then the antimicrobial activity was estimated to detect best solvent to be used.

6. Minimum inhibition concentration (MIC) of ethyl acetate extract 3AR:

By using *Staphylococcus aureus* ATCC 25923 MICs of crude extract of ethyl acetate of 3AR was determined using a broth dilution method **(EUCAST, 2003; Wiegand *et al.*, 2008).**

7. Antifungal activity of crude extract of ethyl acetate of 3AR:

By using Agar dilution methods **(Souza *et al.*, 2002)**, the crude extract of 3AR at three concentrations 375, 750.1000 µg /ml were mixed with glucose peptone agar medium inoculated with the fungal strains *Aspergillus niger* GAD13 , *Aspergillus flavus, and*

Geotrichum candidum (three fungi kindly provided by dr/ Ahamed Gad, NIOF, Egypt) . All inoculated plates were incubated at 30 °C for three to seven days.

8. Chemical characterization of ethyl acetate extract of 3AR:

For the chemical characterization of the antimicrobial of ethyl acetate extract of 3AR several analysis were used as Gas Chromatography-mass Spectrophotometer (GC-Ms) according to **Sharma *et al.* (2016),** Fourier-transform infrared spectroscopy (FTIR) according to **Govindarajan *et al.* (2014)**, Raman spectroscopy according to **Miyaoka *et al.* (2014)** and Nuclear magnetic resonance (NMR) according to **Aouiche *et al.* (2014)**.

9. Comparison between crude extract of *Streptomyces* sp. MMM2 and commercial antibiotics:

The antibacterial activity of Streptomyces MMM2 (50 µg / well) was compared to different commercial antibiotic using Antibiotic sensitivity test to compare efficacy of antibacterial extracted from Streptomyces sp. MMM2 with commercial antibiotics against *E.coli* ATCC 8739, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 13883 and *Edwardsiella tarda* , sensitivity test was done according to **Bauer (1966**). Antibiotics used were tetracycline (TE 30 µg / disc), cefatoxime (CTX 30 / disc), ciprofloxacin (CIP 5µg / disc) and piperacillin (PIP 100 µg / disc).

RESULTS

1. Antimicrobial activity of different isolated Actinomycetes:

The result showed that seven Actinomycetes isolates have antimicrobial activity against *Enterococcus faecalis*, eleven isolates antimicrobial activity against *Staphylococcus aureus* ATCC 25923, two isolates have antimicrobial activity against *E.coli* ATCC 8739, eight isolates have antimicrobial activity against *Pseudomonas aeruginosa* ATCC 9027, six isolates have antimicrobial activity against *Edwardsiella tarda*, thirteen isolates have antimicrobial activity against *Klebsiella pneumonia*e ATCC 13883 and eleven isolates have antimicrobial activity against *Bacillus subtili*s ATCC 6633 (Table 3 and Fig. 1).The result showed that 3AR was the most potent antimicrobial producer, it was isolated from Arish and had antimicrobial activity against all tested bacterial. The Microscopic examination of 3AR showed that 3AR was a Gram positive filamentous threadlike in shape (Fig. 2).

Table 3: Antimicrobial activity of isolated actinomycetes against different bacteria

code	Site	E.f	S.a	E.c	P.a	E.t	K.p	B.s
23A	Abou Quir	12	17	0	0	0	18	17
25A	Abou Quir	13	16	0	0	0	18	16
29A	Abou Quir	0	0	0	0	0	0	0
42A	Abou Quir	0	0	0	0	0	0	0
47A	Abou Quir	13	19	0	10	0	19	18
48A	Abou Quir	0	0	0	0	0	0	0
52A	Abou Quir	15	21	0	14	12	13	14
11A	Abou Quir	0	0	0	0	0	16	22
26A	Abou Quir	14	14	0	15	14	15	15
33A	Abou Quir	0	0	0	0	0	0	13
34A	Abou Quir	0	16	0	0	0	14	17
38A	Abou Quir	0	0	0	0	0	0	11
16A	Abou Quir	0	0	0	0	0	0	0
24A	Abou Quir	0	0	0	0	0	0	0
14A	Abou Quir	0	0	0	0	18	0	0
17A	Abou Quir	0	0	0	0	0	0	0
19A	Abou Quir	0	0	0	0	0	11	0
31A	Abou Quir	0	0	0	0	0	0	0
32A	Abou Quir	0	0	0	0	0	0	0
36A	Abou Quir	0	0	0	15	0	0	0
13A	Abou Quir	0	15	14	18	0	0	13
21A	Abou Quir	0	0	0	24	21	0	0
30A	Abou Quir	0	0	0	0	0	0	0
4AR	Arish	0	30	0	0	0	15	20
2AR	Arish	0	0	0	0	0	2	23
40AR	Arish	0	0	0	0	0	22	22
60AR	Arish	0	0	0	0	0	0	0
3AR	Arish	20	24	23	20	15	17	16
1AR	Arish	0	0	0	0	0	0	0
70AR	Arish	0	0	0	0	0	0	0
18AR	Arish	0	0	0	0	0	0	0
45AR	Arish	0	28	0	0	20	15	0
10S	Safaga	0	0	0	0	0	15	20
20S	Safaga	0	0	0	0	0	0	0
35S	Safaga	0	0	0	0	0	0	0
39S	Safaga	0	0	0	0	0	0	0
8S	Safaga	15	0	0	0	0	0	0
43S	Safaga	0	0	0	0	0	0	0
44S	Safaga	0	0	0	0	0	0	0
22S	Safaga	0	0	0	0	0	0	0
46S	Safaga	0	0	0	0	0	0	0
62S	Safaga	0	0	0	0	0	0	0
9B	Berenice	0	0	0	0	0	0	13
7B	Berenice	0	0	0	0	0	0	0

12B	Berenice	0	0	0	0	0	0	0
80B	Berenice	0	0	0	0	0	0	0
6B	Berenice	0	0	0	0	0	0	0
15B	Berenice	0	0	0	0	0	0	0
60B	Berenice	0	0	0	15	0	0	0
61B	Berenice	0	0	0	0	0	0	0

E.f (*E. faecalis*), S.a (*S. aureus*), E.c (*E. coli*), P.a (*P. aeruginosa*), E.t (*E. tarda*), K.p (*K. pneumonia*) and B.s (*B. subtilis*), Numbers in the table refere to inhibition zones in mm obtained by isolated Actinomycetes.

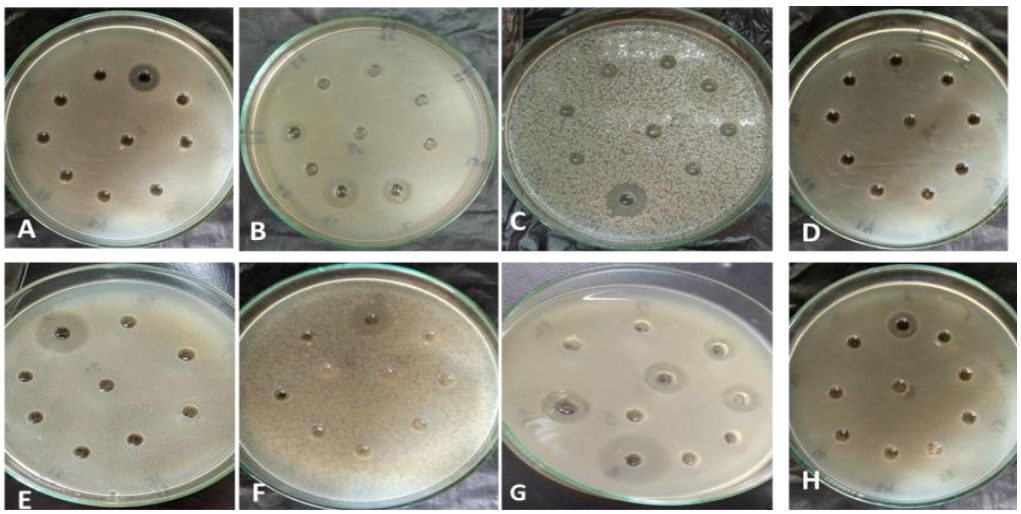

Fig. 1: Antimicrobial activity of different Actinomycetes isolates against different bacteria, *E. coli* (A, H), *K. pneumonia*e (B), *Edwardsiella tarda* (C), *Enterococcus faecalis* (D) and *Staphylococcus aureus* (E, G).

Fig. 2: 3AR a Gram positive threadlike shape under the light Microscope oil-immersion lens (100 X).

2. Genotypic Characterization:

The actinomycetes isolate 3AR that was isolated from sediment samples collected from Arish was identified using the 16S rRNA. It was found to be the most potent antimicrobial producer and was identified as *Streptomyces* sp. MMM2. The GenBank accession number for the sequence of *Streptomyces* sp. MMM2 is MZ397921.

3. Effect of Different Culture Media:

It was observed that starch casein medium gave inhibition zones of 20, 24, 23, 20, 15, 18, 15 mm, Starch nitrate medium gave 20, 23.17, 14, 0, 15, 12 mm, Shofan medium gave 12, 20, 22, 20, 15, 13 mm and ISP medium gave 20, 22, 20,12, 19, 17, 14 mm against *E.faecalis*, *S.aureus*, *E.coli*, *P.aeruginosa*, *E.tarda*, *K.pneumoniae*, *B.subtilis* , respectively (Fig. 3).

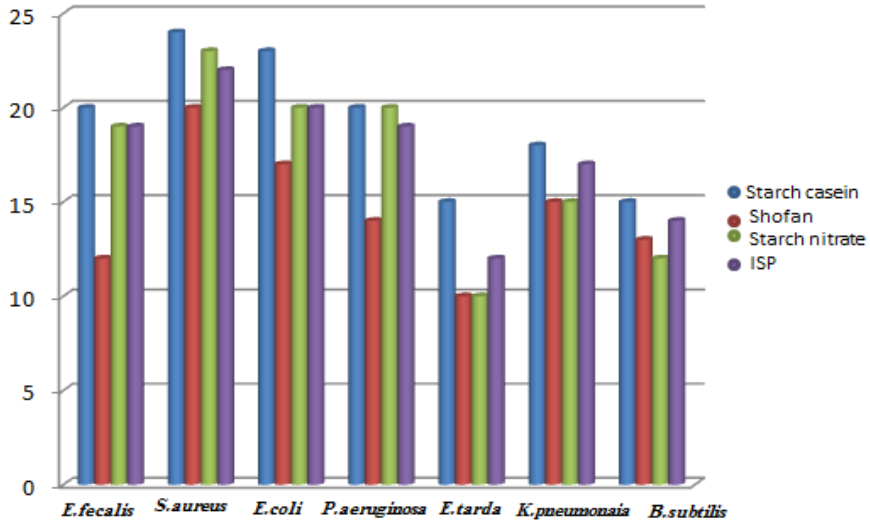

Fig. 3: Effect of different media on the production of antimicrobial activity by using different media as starch casein medium, starch nitrate medium, shofan medium and international Streptomyces medium.

4. Optimization of Antimicrobial Agent(s) Production using Plackett-Burman Design:

The main effect of each variable of antimicrobial activity of *Streptomyces* sp. MMM2 was estimated and presented graphically in (Fig. 4 and Table 4). It was shown that high level of starch, $K2PO_4$, inoculum size and yeast extract affected positively the antimicrobial activity. While the use of the low levels of Casein, KNO3, PH and Temperature resulted in increasing the antimicrobial activity. The results indicated the best antimicrobial activity can be obtained through this optimized medium composed of: 15 g starch, 0.15 g casein, 1 g KNO_3, 3 g K_2PO_4, 3 ml inoculum size, 6 pH and 37°C. Statistical analyses of the results showed that variations in temperature were the most significant variable on antimicrobial activity (Table 4).

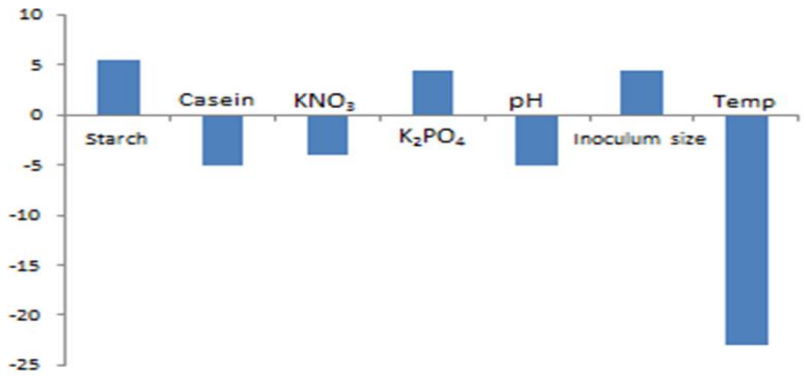

Fig. 4: The main effect of starch, casein, KNO3, K2PO4, inoculum size, pH and Temperature on the anti-microbial activity of Streptomyces sp. MMM2.

Table 4: Statistical analysis for Plackett Burman experment for the antimicrobial production from Streptomyces sp. MMM2.

variable	Main effect	T value
Starch	5.5	1.9
casein	-5	1.9
KNO3	-4	1.9
KPO4	4.5	1.9
pH	-5	1.9
Inoculum size	4.5	1.9
Temperture	-23	2.4

Significant of t-value at 5% is (2.45), at 10% level (1.94), at 20% level (1.37). Standard t-values are obtained from statistical methods **(Snedecor and Cochran, 1989).**

5. Antimicrobial Activity of Streptomyces sp. MMM2 using different solvents:

The obtained inhibition zones by ethyl acetate extract was better than supernatant (cell free) without any extraction, butanol extract and hexan extracts where aceton extract showed no activity (Table 5 and Fig. 5).

Table 5: Antimicrobial Activity of Streptomyces sp. MMM2 using different solvents.

Solvent	E.f	S.a	E.c	p.a	E.t	k.p	B.s
Ethyl acetate	39	45	40	40	37	37	45
Butanol	35	40	35	32	29	29	40
Hexan	27	24	22	0	20	0	28
Aceton	0	0	0	0	0	0	0
Supernatant	28	30	30	25	19	19	35

E.f (*E. faecalis*), S.a (*S.aureus*), E.c (*E. coli*), P.a (*P. aeruginosa*), E.t (*E. tarda*), K.p (*K. pneumonia*) and B.s (*B. subtilis*), Numbers of the table refered to inhibition zones in mm obtained by *Streptomyces* sp. MMM2.

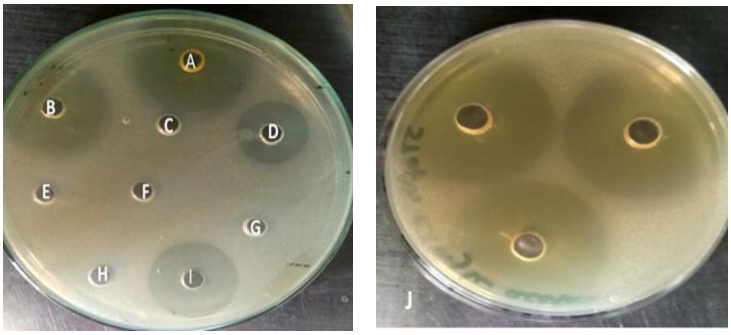

Fig. 5: Antimicrobial Activity of *Streptomyces* sp. MMM2 using different solvents (A) Ethyl acetate, (B) Butanol, (C) Aceton, (D) Supernatant (cell free), (E) Aqueous part of Ethyl acetate extraction, (F) Aqueous part of butanol extraction, (G) Aqueous part of aceton extraction, (H)Aqueous part of hexan extraction, (I) supernatant and (J) Ethyl acetate extract of *Streptomyces* sp. MMM2 in a separate plate.

6. Minimum inhibition concentration (MIC) of antimicrobial of Streptomyces sp. MMM2:

MICs of ethyl acetate extract of *Streptomyces* sp. MMM2 using *staphylococcus aureus* were determined using the broth dilution method. Where MIC= 375 μg /ml.

7. Antifungal activity of antimicrobial of *Streptomyces* sp. MMM2:

Crude extract of ethyl acetate of *Streptomyces sp MMM2* decreased the growth of *Aspergillus niger, Aspergillus flavus, and Geotrichum candidum*as as shown in (Fig. 6).

8. Chemical characterization of Antimicrobial Extract of *Streptomyces* sp. MMM2:

Fourier-transform infrared spectroscopy (FTIR), Raman spectroscopy, gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy NMR were carried out to identify the chemical structure of antimicrobial extract of *Streptomyces sp. MMM2*. FTIR spectroscopic analyses revealed various chemical groups in the antimicrobial of *Streptomyces* sp. MMM2, as shown in (Fig. 7A). The peaks appearing at 3245.20 cm^{-1}, 3038.33 cm^{-1}, 2921.29cm^{-1}, 1725.06cm^{-1}, 1631.93$cm^{-1}$and 1537.11 cm^{-1} represent Alcohol/Phenol, Alkenyl, Carboxylic Acid, Carboxylic Acid, Amide and Aromatic, respectively. The Raman spectroscopy analysis (Fig. 7B) revealed the presence of three strong signal intensities with a very strong band at 1609.32 cm−1which corresponded to carboxylic acid, 1643.60 and 1398.32 Corresponding Carboxylic acid and Carboxylate salt, respectively. ^{1}H NMR spectrum of the isolated compounds showed the presence of one proton singlet at δ 9.97, the position and multiplicity of which was indicative of the (COOH) carboxylic groups (Fig. 7D). GC-MS analysis (Fig. 7C) revealed the presence of several compounds have antimicrobial activity such as hexadecanoic acid methyl ester, hexadecanoic acid, 9,12-octadecadienoic acid

methyl ester, 9-octadecenoic acid (Z)- methyl ester, octadecanoic acid methyl ester, 9-octadecenoic acid-Z, hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester and 9,12-octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl) ethyl ester. The structures of the chemical compounds are shown in (Fig. 8).

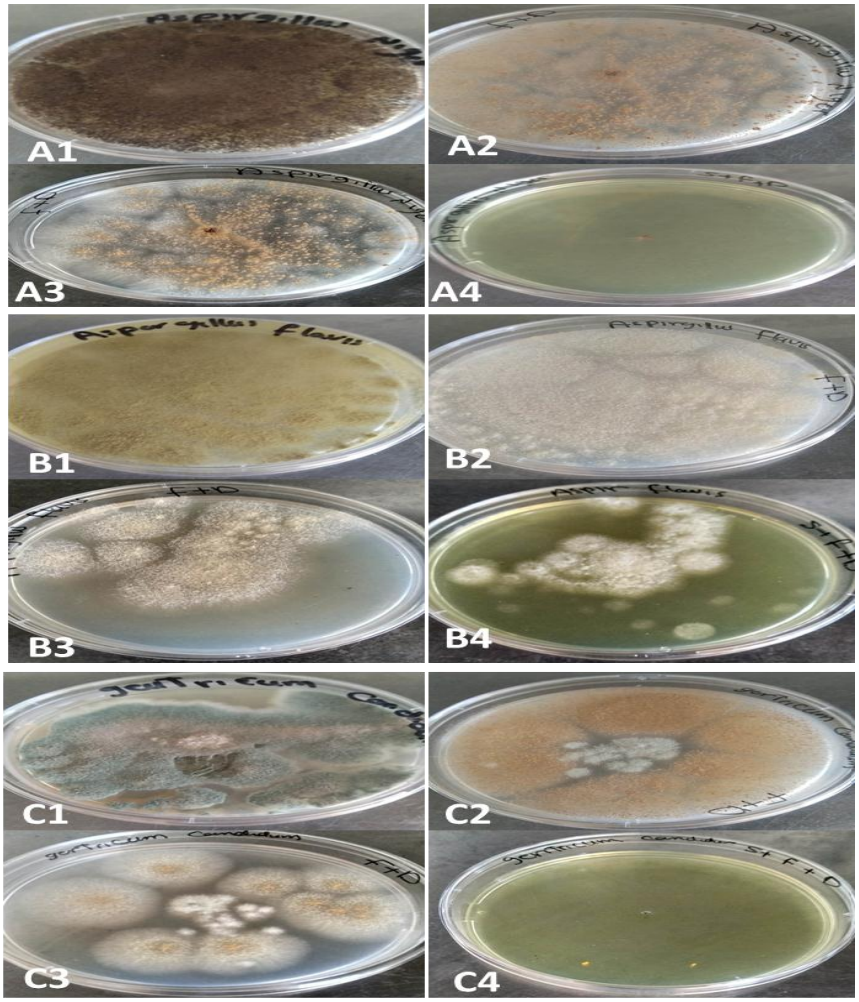

Fig. 6: Antifungal activity of crude extract of *Streptomyces* sp. MMM2 at different concentrations against *Aspergillus niger* (A1 = control, A2 = 375 µg/ml, A3 = 750 µg/ml and A4 = 1000 µg/ml), *Aspergillus flavus* (B1 = control, B2 = 375 µg/ml, B3 = 750 µg/ml and B4 = 1000 µg/ml) and *Geotrichum candidum* (C1 = control, C2 = 375µg /ml, C3 = 750 µg/ml and C4 = 1000 µg /ml).

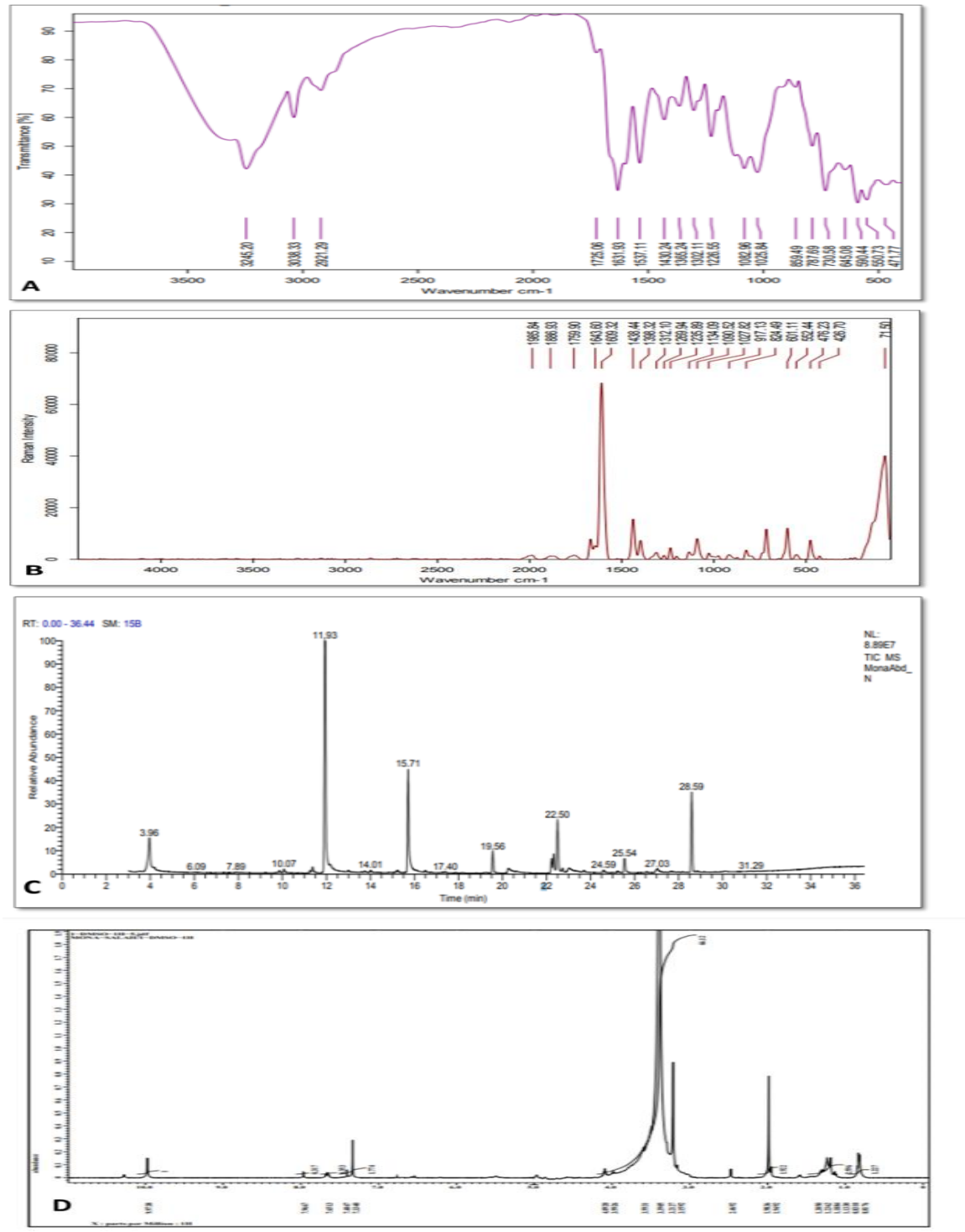

Fig. 7: Chemical characterization of ethyl acetate extract of *Streptomyces* sp. MMM2 (A) FTIR, (B) Raman spectroscopy, (C) GC-MS and (D) NMR analyses.

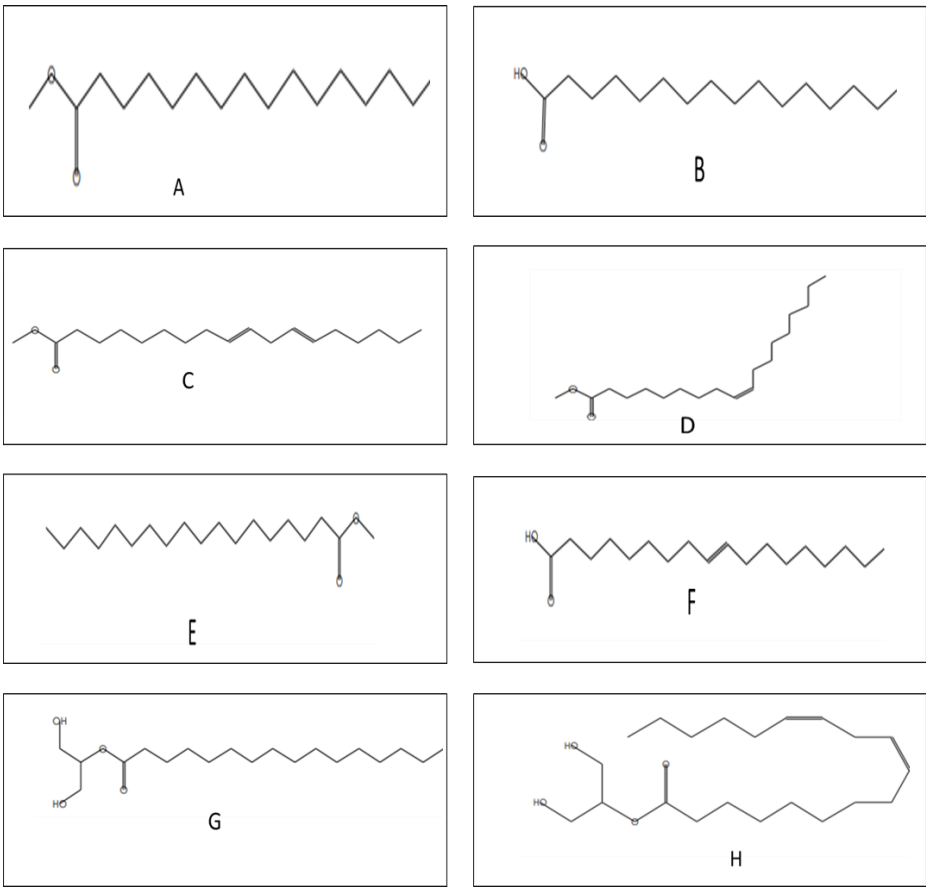

Fig. 8: Chemical structure of antimicrobial compounds detected by GC-MS Hexadecanoic acid, methyl ester Formula (A), Hexadecanoic acid (B), 9,12-Octadecadienoic acid, methyl ester, (E,E) (C), 9-Octadecenoic acid (Z)-, methyl ester (D), Octadecanoic acid, methyl ester (E), 9-octadecenoic acid (z)-(F), Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester (G) and 9,12-Octadecadienoic acid (Z,Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester (F).

9. Antibiotic sensitivity test: Antibiotic sensitivity test were performed by using commercial antibiotics and the results are shown in (Table 6 and Fig. 9).

Table 6: antimicrobial activity of streptomyces sp. MMM2 and commercial antibiotics against tested pathogen

bacteria	SME (30 µg)	CIP (5 µg)	CTX (30 µg)	TE (30 µg)	PIP (100 µg)
E.f	39± 0.20	17± 0.11	14± 0.07	36± 0.20	13± 0.21
S.a	45±0.22	24 ± 0.13	24± 0.13	22± 0.18	20± 0.22
E.c	40±0.17	35± 0.11	25± 0.11	20± 0.13	18± 0.13
P.a	40±0.13	27 ± 0.15	22± 0.11	15± 0.14	20± 0.14
E.t	37±0.11	24± 0.12	23± 0.13	19± 0.03	24± 0.024
K.p	37±0.06	20± 0.04	11± 017	21± 0.23	12± 0.17
B.s	45±0.05	20± 0.03	11± 0.18	22± 0.23	12± 0.18

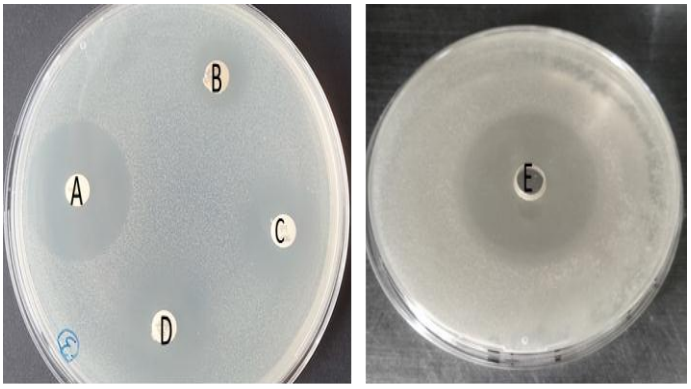

Fig. 9: antimicrobial test against E.coli where is (A) ciprofloxaxine 5 µg, (B) tetracycline 30 µg, (C) piperacillin 100 µg, (D) cefatoxime 30 µg and (E) crude extracted of *streptomyces* sp. MMM2 50 µg.

DISCUSSION

In the present study, 50 marine Actinomycetes isolates were screened against *Staphylococcus aureus* ATCC 25923, *E.*coli ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumonia* ATCC 13883 and *Edwardsiella tarda* using Agar well diffusion method. It was found that 3AR that isolated from Arish was the most potent antimicrobial producer against all tested bacteria. The 3AR isolate was identified as *Streptomyces* sp. MMM2 using the 16s rRNA gene. Similarly, many authors used *Streptomyces* species for producing several pharmaceutical products since they have several biological activities as antibacterial **(Zhang *et al.*, 2018; Al-Dhabi *et al.*, 2020),** antifungal **(Karpiński *et al.*, 2019; Karim *et al.*, 2021),** antitumor **(Sivalingam *et al.*, 2019),** anti-inflammatory **(Elaiyaraja *et al.*, 2018 ; Shin *et al.*, 2022)**, antviral **(Yi *et al.*, 2022)** as well as many other agents such as enzyme inhibitors **(Siddharth and Vittal, 2018).**

Several natural compounds have been discovered from the marine Actinomycetes, especially *Streptomyces* species. *Streptomyces* is a potent genus for making novel pharmaceutical compounds **(Sivalingam *et al.*, 2019)**. Mediomycins A and Clethramycin were isolated from *Streptomyces mediocidicus* ATCC23936 and *Streptomyces malaysiensis* DSM4137, respectively showed a wide activity of antifungal activity **(Sun *et al.*, 2018)**. Polyketides have many important bioactive compounds used in pharmaceutical industries **(Demain, 2014)**. *Streptomyces* produced many biologically active polyketides, including antibiotics, immunosuppressants, antiparasitics and antitumor agents **(Chater, 2006)**. Examples of polyketides are Pikromycin produced by *Streptomyces venezuelae* had antibacterial activity (**Xue and Sherman, 2001)**, Nystatin produced by *Streptomyces noursei* had antifungal activity **(Brautaset *et al.*, 2000**) and Avermectin produced by *Streptomyces* had antiparasitic activity **(Campbell *et al.*, 1983)**.

Mitomycin C naturally synthesized by *Streptomyces caespitosus* is a potent antineoplastic antibiotic for the treatment of tumors **(Yang *et al.*, 2019**). Protease inhibitor (PISC-2002) produced by *Streptomyces chromofuscus* has antiviral activity against influenza virus A/Rostock/34 (H7N7) **(Angelova *et al.*, 2006)**. *Streptomyces* sp. produced pimprinine that have antimicrobial activities, anticonvulsant activity and antiviral activity against Enterovirus 71 (EV71) **(Wei et *al.*, 2014)**. lipomycin and Saphenic acid have anti-inflammatory activity were obtained from marine Actinomycetes **(Manivasagana *et al.*, 2014)**. the anti-inflammatory metabolites cyclomarin A and C produced by *Streptomyces arenicola* was proved to have anti-malaria and anti-tuberculosis activity **(Barbie and Kazmaier, 2016)**.

The present study showed that the best culture medium for *Streptomyces* sp. MMM2 is starch casein it gave inhibition zones 20, 24, 23, 20, 15, 18 and 15 mm against *E.fecalis, S.aureus, E.coli, p.aeruginosa, E.tarda, K.pneumonia*e, *B.subtilis,* respectively. In agreement with the results of this study, several studies showed variations in media compositions, temperature and pH having great impact on the secondary metabolism of *Streptomyces* sp. **(Thakur *et al.*, 2009; Tan *et al.*, 2020)**.

The Plackett-Burman design is an efficient technique for optimization of medium component and identify significant variables that enhance antimicrobial compound(s) production and to find out their probable optimal levels in a limited number of experiments **(El-Sharouny *et al.*, 2015; Malash *et al.*, 2016)**. Development of an efficient production of secondary metabolites by microorganisms requires examination of nutritional and environmental factors that play key roles in cell metabolism **(Kiranmayi *et al.*, 2011)**. In this study, response to plackett-Burmen design showed wide variation ranging of activity from 30 mm zone of inhibition to nil activity corresponding to combined effect of seven parameters. The results indicated high antimicrobial activity was got by optimized starch casein medium as indicated in (Table 4 and fig. 4). Statistical analysis revealed that temperature is the most significant variable similarly, many authors reported efficacy of temperature on antimicrobial production as **Narayana and Vijayalakshmi (2008); Ripa *et al.* (2009) and Oskay (2011)**.

Ethyl acetate extract was the best solvent for antimicrobial extraction followed by butanol then hexan while aceton didn't give any activity. Similarly several studies used the ethyl acetate as a solvent to extract antimicrobials from *Streptomyces* sp. **(Nandhini *et al.*, 2018 ; Khadayat *et al.*, 2020)**. In this study, it was found that crude ethyl acetate extract of *Streptomyces* sp. MMM2 had anti-fungal activity against *Aspergillus niger* , *Aspergillus flavus, and Geotrichum candidum*. Similarly several studies produced products with antifungal activities from marine *Streptomyces* (**Dharmaraj., 2010)**. Ikarugamycin derivatives produced by marine *Streptomyces zhaozhouensis* had antifungal activity against *A. fumigatus and C. albicans* **(Lacret *et al.*, 2014)**. Marine *Streptomyces sp. DC4-5* produced Iseolides A–C that showed antifungal activity against *Glomerella cingulata*, *Trichophyton rubrum* and *Candida albicans* **(Zhang *et al.*, 2020).**

By analysis, ethyl acetate extract of *Streptomyces* sp. MMM2 with Fourier Transform Infrared Spectroscopy revealed the presence of various functional groups as Alcohol/Phenol, Alkenyl, Carboxylic Acid, Amide and Aromatic. Fourier Transform Infrared Spectroscopy is a useful tool for identifying functional groups and therefore, can be used to identify some components of an unknown compounds **(Vasanthabharathi *et al.*, 2011)**. Functional groups obtained by FTIR analysis in this study were known to have antimicrobial properties as results were witnessed by **Sholkamy et *al.* (2020)** and **Chelliah et *al.* (2017)**. In agreement with the results obtained from FTIR analysis both Raman spectroscopy and ^{1}H NMR analysis revealed the presence carboxylic acid groups. Carboxylic acid derivatives have a growing importance in medicine **(Sirajuddin *et al.*, 2015)**. Many Carboxylic acid derivatives have antimicrobial activity **(Asahina *et al.*, 2005; Wang *et al.*, 2016)**, antifungal activity (**Yu *et al.*, 2009; Mert *et al.*, 2014**). Also GC-MS analysis found that there are several peaks corresponding to carboxylic acids derivatives as hexadecanoic acid methyl ester that had antibacterial activity against multidrug-resistant bacteria *Staphylococcus aureus* W35, *Pseudomonas aeruginosa* D31, *Klebsiella pneumoniae* DF30, and *K. pneumoniae* B40 **(Shaaban *et al.*, 2021)**. Hexadecanoic acid methyl ester had antimicrobial activity produced from *Streptomyces* species according to **Kawuri and Darmayasa (2019)** and **Boublenza *et al.* (2021)**. GC-MS revealed presence of hexadecanoic acid that had antimicrobial activities according to **Köse *et al.* (2007)** and **Djebbah *et al.* (2022)**. Hexadecanoic acid produced from Actinomycetes possesses potent antioxidant, anticancer and antimicrobial properties (**Srikesavan and Selvan, 2012**; **Narendhran *et al.*, 2014**). Also GC-MS analysis revealed presence of octadecadienoic acid derivatives. Several studies revealed that octadecadienoic acid produced from marine *Streptomyces* species had antimicrobial activity (**Manikandan *et al.*, 2019**; **Al-Dhabi *et al.*, 2020; Chakraborty *et al.*, 2022**).

CONCLUSION

Actinomycetes are known for production of many bioactive compounds. *Streptomyces* sp. MMM2 isolated from sediment from Arish, Egypt had the ability to produce antimicrobial agent against *E.coli, Pseudomonas Aeruginosa, Staphylococcus aureus, , Enterococcus faecalis, bacillus subtilis, Edwardsiella tarda* and *Klebsiella pneumoniae*, Extraction by ethyl acetate increased the antimicrobial activity. *Streptomyces* sp. MMM2 showed also antifungal activity against of *Aspergillus niger, Aspergillus flavus, and Geotrichum candidum*. Chemical analysis by GC- MS spectrophotometry, FTIR, Raman and NMR indicated the probable effective compounds as antimicrobial agent (s) according to the obtained data were carboxylic acid derivatives that known to have antimicrobial properties.. Bioactive compounds from *Streptomyces* sp. MMM2 may be useful in antibacterial production, as well as antifungal and in biotechnological applications.

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