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Isolation and Antibiotic Prospective of Endolithic Actinobacteria Inhabiting Diorite Rocks of South Sinai, Egypt

Hesham M. Abdulla [(1)], Sahar A. El-Shatoury [(1)#], Mohamed A. Abdel Rahman [(2)] and Nahla S. El-Shenawy [(2)]

[(1)]Botany Department, Faculty of Sciences, Suez Canal University, Ismailia, Egypt;
[(2)]Zoology Department, Faculty of Sciences, Suez Canal University, Ismailia, Egypt.

THIRTY-FOUR actinobacteria strains were recovered from diorite rocks of South Sinai, Egypt, using direct isolation. The predominant types were *Streptomyces* and *Nocardioides* (8 strains, each), followed by *Kitasatosporia* and *Nocardiopsis* (4 strains, each). Twenty-five strains were active against *Escherichia coli* NCMB 11943, two clinical cultures (*Staphylococcus aureus*, *Candida albicans*) and/or three solid tumor cell lines (colon C38, colon H-116, lung H-125 M).

One strain showed potent activity towards *S. aureus*, *C. albicans* and inhibited colon *HCT116* and liver *HepG2* carcinoma cell lines at IC_{50} 3.2 and 1.3µg/ml, respectively. Phylogenetic analysis indicated its identity as *Micromonospora citrea*, at 99.6% similarity. MALDI/TOF-MS analysis of its metabolic extract revealed 18 compound masses, molecular weight range of 585.52 to 669.64Da. Statistical general full factorial design indicated varying effects of four-carbon and three-nitrogen sources on its bioactivity. Starch and potassium nitrate at 1% w/v, as C- and N-sources, respectively, were significantly effective for enhancing the antitumor activity up to 1.3-fold (P = 0.007).

M. citrea extract revealed non-significant cytotoxic effects in rats, at 10mg/kg injection dose, every two days for two weeks. The kidney and liver functions were not significantly impaired, as indicated from results of serum enzymatic activities, urea, total protein and albumin levels. The strain was deposited at the Egyptian Microbial Culture Collection as *M. citrea* SP1 EMCC 1923 and was patent registered (Egyptian Patent Office, No. 655). These results indicate the importance of actinobacteria inhabiting arid rocks of Sinai for production of promising antibiotic leads.

Keywords: Endolithic actinomycetes, *Micromonospora citrea*, Antimicrobial activity, Antitumor activity, Hepatotoxicity.

Introduction

Endolithic (inside rock) microbial communities are rather widespread in cold and hot deserts, where they form a structured biofilm within the rock cracks. The endolithic environment is a stressed habitat characterized, particularly, by desiccation, nutrients scarcity, wide temperatures fluctuation and high UV irradiation (Walker, et al., 2005). Under such conditions, microbial production of unusual/novel metabolites and pigments is expected, possibly as means of adaptation for the harsh conditions (Tanaka et al., 2010). In this context, rocks can represent an important habitat of actinobacteria that foster novelty in drug discovery programmes.

The studies on endoliths were focused, mainly, on their role in the processes of weathering (Abdulla, 2009), mineral dissolution (Wang et al., 2011) and phylogeny (Horath & Bachofen, 2009); and only recently, their potential for production of bioactive compounds is being elucidated. The desert actinomycetes from Algeria, for example, showed the presence of strains related to *Streptomyces*, *Actinopolyspora, Saccharopolyspora* and *Nocardiopsis*; half of which were active against a panel of nine tested pathogenic microorganisms (Meklat et al., 2011). In their extensive review, Mohammadipanah & Wink (2015) reported the richness of desert habitats in actinobacteria; pointing to their high diversity, on the genetic level, for synthesizing novel new secondary metabolites. Genera of

#Corresponding author email: sahar_hassan@science.suez.edu.eg, Tel. 01222520880
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the actinobacteria, particularly, *Streptomyces*, represent the richest source of antimicrobials and antitumor compounds such as anthracyclines, peptides (Berdy, 2005) and others (Olano et al., 2009).

In the dry desert environments, endolithic microorganisms are most frequently investigated in the rock types originating from granite, sandstone, limestone, gypsum crusts, and to a less extent, diorite igneous rocks (Pedersen, 1997; Stivaletta et al., 2010; Wong et al., 2010; Tang et al., 2012 and Ivarsson et al., 2016). In this study, selective media and pretreatment methods were investigated for the recovery of culturable endolithic actinobacteria from the diorite igneous rocks of southern Sinai. Our aim was to illustrate the extent of actinobacteria presence in that rock type and to evaluate their potential for application as sources of antimicrobial and antitumor products.

Material and Methods

Site description and sample collection

Diorite rocks were sampled from Gabal Al-Monagah, World Heritage Site, Sinai (1854m Alt, 33°58´80"E, 28°33´70"N). The site physicochemical characteristics were: Moisture 2.13%, electric conductivity 0.03ds/m, pH 7.82, total nitrogen 0.19g/kg and organic matter 0.39% (El-Shatoury et al., 2013). Fifteen samples were collected from 1x1m sampling points within the rock sub-surface (1-8mm depth) using ethanol wipe chisel and kept in sterile polyethylene bags at 4°C. They were then surface sterilized and prepared for microbial investigation as described by Bhattacharjee & Joshi (2016).

Selective isolation of endolithic actinobacteria

Grinded rock samples were suspended in Ringer's solution and actinobacteria were recovered using three selective media, with modification by Abdulla (2009). These were: Starch casein (SC), 1/10SC (both with and without 20% rock extract; prepared from stock of the corresponding rock sample, 1g/ml distilled water) and MGA media (Baltz et al., 2010). The samples were processed in triplicates and all media were supplemented with 50µg/ml cycloheximide (antifungal), after sterilization. The actinobacteria were counted and purified for the following analysis, after incubation for 4 weeks at 28°C.

Phenotypic identification

Thirty-four strains were recovered, purified on SC agar plates and maintained as spore suspensions in 20% glycerol at -20°C. Identification to the generic level was according to standard microscopic and chemotaxonomic methods (Goodfellow et al., 2012), as follows:

Microscopic characteristics

Cultures were grown on cover slips at 28°C, using ISP4 medium. Examination of developed mycelia was performed under Axio Imager 2 Zeiss microscope at 3, 7 and 14 days.

Chemotaxonomic characterization

Mycelia were obtained from liquid cultures in TSB baffled flasks at 28°C and 100rpm shaking for 5–7 days; then processed as follows;

Diaminopimelic acid (DAP) isomers in whole-cell HCl hydrolysate was performed on thin-layer chromatography (TLC) cellulose-precoated plastic sheets (Merck 5577), using the developing solvent system: Methanol – water -10N HCl - pyridine (80: 17.5: 2.5: 10, v/v) and visualization after spraying with ninhydrin.

Mycolic acids were analysed in acidified mycelia saponificates, using TLC silica gel-precoated aluminium sheets (Merck 1.05553) and the developing solvent system: Petroleum ether-diethyl ether-glacial acetic acid (90: 10: 1, v/v). The presence of mycolic acids was visualized after exposure to iodine vapor.

Whole-cell sugar pattern was detected in mycelia hydrolysed by sulphuric acid, after neutralization with saturated barium hydroxide. The samples were run on TLC cellulose-precoated plastic sheets (Merck 5577) in the developing solvent system: n-butanol-water-pyridine (10: 6: 6: 1, v/v). Visualization was performed after spraying with acid aniline phthalate.

Fermentation and organic metabolites extraction

Spore suspensions were inoculated into 30ml SC broth at 28°C, 100rpm for 7-10 days. The crude metabolites were extracted with ethyl acetate (1:1 v/v) for three successive times, with vigorous shaking to thirty minutes, to suspend the organic molecules in the solvent. The solvent layers were combined and concentrated under vacuum using rotary evaporator (HS2005S-N-Hahn Shin Scientific Co.); then re-dissolved in ethanol and

DMSO, at concentration 1mg/ml, for antimicrobial and antitumor screenings, respectively.

Antimicrobial activity of metabolic extracts

The organic metabolic extracts were tested using disc technique (De Beer & Sherwood, 1945), applying 100µg per disk, against one reference bacterial strain (*Escherichia coli* NCMB 11943) and two human pathogen clinical cultures (*Staphylococcus aureus* and *Candida albicans*). The bacterial and fungal strains were grown on nutrient agar and potato dextrose agar, respectively. Results were scored after incubation at 37°C for 24h for bacteria and at 30°C for 48h for *Candida* sp. The experiment was performed in triplicate. Negative controls (ethanol loaded discs) were included.

Antitumor activity against Ehrlich ascite carcinoma (EAC) cells

In vitro screening was performed using EAC cells withdrawn from carcenoma-induced female Swiss albino mice. The metabolic extracts were added to EAC cells ($12\pm0.2 \times 10^6$ cells/ml phosphate buffer) to give a final concentration 100µg/ml and incubated at 37°C for 2h. The total number of viable and apoptotic cells were counted using a hemocytometer according to the method of Mc et al. (1957). Briefly, 2µl of 50% trypan blue was added to 18µl of the treated EAC cells and apoptotic cells were stained blue. Extracts exhibiting ≥ 55 % EAC cell death was considered to have antitumor activity.

In vitro solid tumor selectivity

Metabolic extracts from the EAC active strains were investigated for selective inhibition of solid tumors, using disk diffusion soft agar colony formation assay. For this assay, leukemia cells (L1210), normal cells (CFU-GM) and three investigated solid tumor cells lines were plated in soft agar. The metabolic extracts, 15µg in DMSO, were placed on a 6.5mm filter paper disk, which was then placed on top of the soft agar containing the tumor cells (Valeriote et al., 2002). The deferential cytotoxicity was expressed by observing a zone deferential between any solid tumor cells (colon C38, colon H-116, lung H-125 M) and either leukemia cells (L1210) or normal cells (CFU-GM). The diameter of the filter disc was arbitrary taken as 200 units. Extracts showing inhibition ≥ 300 zone unit (zu) were considered promising. Metabolic extracts that showed a difference ≥ 250 zu between solid tumor and normal or leukemia cells were considered as solid tumor selective extracts.

Phylogenetic identification of Micromonospora SP1 EMCC 1923

Micromonospora SP1 strain that showed potent solid tumor selectivity and promising inhibitory effect towards *S. aureus* was subjected to 16S rRNA gene sequencing. It was grown in starch casein broth at 28°C, 120rpm, for 4-7 days until mycelial growth was obtained before sporulation stage. One hundred mg of harvested mycelia were washed in saline solution (NaCl 0.085%). DNA extraction was carried out using the EZ-10 Spin Column Plant PCR kit (Bio Basic Inc, Canada) according to the manufacturer's instructions. The 16S rRNA gene of the strains was amplified in 20µl reactions using the universal bacterial primers 27F-AGAGTTTGATCMTG GCTCAG and 1492R-GGTTACC TTGTTACGACTT. The DNA sequencing was carried out by Macrogen Labs Ltd., Korea. Amplification and sequencing conditions were performed as previously described (Hall et al., 1999). The sequences obtained were compared with those in the GenBank database using the BLAST search program. The sequences homology with those sequences in the GenBank database was analysed using DNASTAR Lasergene 11 software (DNASTAR, Madison, WI, USA) and the evolutionary tree was constructed using the neighbour-joining method (Saitou & Nei, 1987).

Influence of C and N sources on M. EMCC 1923 bioactivity

Four carbon-sources (starch, glucose, D-fructose and mannitol, at 1% w/v) and three nitrogen-sources (potassium nitrate, yeast extract and soya bean meal l, at 1% w/v) were investigated for enhancing the production of bioactive metabolites from *M.* EMCC 1923. The statistical general full factorial design (Minitab 15 Statistical Software) was used. Accordingly, twelve combinations for the C- and N- sources were considered in duplicates (Table 1). Fifty millilitres of the basal medium M56 (Demain et al., 1999) were amended with the C- and N-sources at the defined combinations, inoculated with 20µl spore suspension ($2x10^8$cfu/ml) of the strain and incubated at 28˚C, 100rpm for 10 days. Solvent extraction was performed and the extracts were evaluated for antimicrobial and carcinoma inhibitory effects as described above. The statistical significance of the results was assessed

by univariate analysis of variance (ANOVA). Experimental settings that gave maximum active metabolites production were applied for the following experiments.

Metabolite activity of M. EMCC 1923 towards human cell lines

The metabolic extract was investigated against six human cell lines, by colorimetric cytotoxicity assay. The cell lines were: U251 (brain tumor), HepG2 (liver carcinoma), MCF7 (breast carcinoma) H460 (lung carcinoma), HeLa (Cervix carcinoma) and HCT116 (colon carcinoma). The cell lines were plated as cell monolayer in 96-multiwell plate (10^4cells/well) for 24h, then serial concentrations of the metabolic extract (0, 1, 2.5, 5 and 10µg/ml in DMSO) were added, in triplicate. After 48h of incubation, colour measurement using ELISA reader was performed after Sulfo-Rhodamine–B staining. The relation between survival fraction and extract concentration was calculated to get and LC_{50} was calculated.

Mass fingerprinting of M. EMCC 1923 metabolic extract

The extract, in triplicate, was desalted using Sep-Pak cartridges (Waters, Elstree, UK) followed by eluting the cartridges with 80% acetonitrile (ACN)/0.05% Trifluoroacetic acid (TFA). Samples of 2.4mg protein were prepared using a saturated (10mg/ml) solution of a-cyano-4-hydroxycinnamic acid (α-CHCA: Sigma-Aldrich) in 60% ACN/0.3% TFA. The samples were dissolved in matrix solution (10µL) and 0.5µL were spotted on a MALDI plate. Matrix Adsorbed Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI/TOF-MS) analysis was performed using Laser TOF LT1500 mass spectrometer (Scientific Analysis Instruments (SAI) Ltd., Manchester, UK), under the control of Laser TOF (SAI) software. Data were recorded in the mass range of m/z 100–5,000 and three spectra were obtained for each sample.

In vivo cytotoxicity of M. EMCC 1923 metabolic extract

Animal housing and experimental design: Male Fischer 344 rats (weight 110 to 120g) were used in this experiment, following the National Research Council guidelines for animal experimentation (2011). Animals were allowed to acclimate to the housing conditions for one week. They were provided with tap water *ad libitum* and basal diet during the study; laboratory conditions were maintained at 12h light/dark schedule and 23±3°C. Animals were assigned to 5 rats/group and observed twice daily for general wholesomeness and care.

LD_{50} of the metabolic extract was assayed by the standard method in rat. Briefly, three doses of the crude extract (50, 100, 500mg/kg) dissolved in normal saline were injected i.p. into each of 5-rats group, compared to a fourth group receiving normal saline as a control. Results were calculated for 96h (96-hrsLD_{50}). For cytotoxicity assay, two groups received i.p. injections of 10 and 100mg/kg of the metabolic extract in normal saline at 2-days intervals for 14 days. The control group received 0.1ml of normal saline. At the end of this period, blood samples were collected - retro-orbital - under light ether anesthesia. Sera were separated by centrifugation and stored at 4°C, for biochemical analysis.

Serum biochemical analysis

Four liver enzyme activities: alanine aminotransferase, aspartate aminotransferase (AST), alkaline phsphatase (ALP) and lactate dehydrogenase were determined according to the manufacture instruction of commercial kits (Human Gesellschaft for Biochemical und Diagnostics MBH, Germany). Total cholesterol and triglyceride (TG) were measured, spectrophotometrically, as described by Bucolo & David (1973). Protein content was measured according to Bradford (1976), using bovine serum albumin as a standard and albumin was determined using the commercial kits (Human Gesellschaft, MBH, Germany). Uric acid as non-enzymatic antioxidant was determined at 546nm using colorimetric method of Fossati et al. (1980). All results were expressed as means ± SE. Data were analyzed by Student t-test and $P \leq 0.05$ was considered as level of significance.

Results

Isolation and chemotaxonomy of actinobacteria from diorite rocks

SC medium amended with 20% rock extract, gave the highest counts (averaged 5.7 x10^3cfu/g), compared to the diluted 1/10 SC and MGA media which gave counts between 0.2-4.0 x 10^3cfu/g. Results indicated that actinobacteria recovery using rock extract-amended media was significantly higher than that on the conventional media ($P < 0.5$).

Thirty-four morphologically different strains were recovered from the fifteen sampling sites; of which twenty-eight were assigned into six generic groups (Fig. 1), based on phenotypic and chemotaxonomic criteria. Twenty strains contained L-DAP in their cell hydrolysate and were tentatively identified as *Streptomyces*, *Nocardioides* and *Kitasatosporia*, based on their microscopic characteristics; strains related to the later genus also contained m-DAP isomer in their cell hydrolysate. Eight strains contained m-DAP and were assigned to the genera *Nocardiopsis*, *Micromonospora* and *Actinomadura* based on their phenotypic and chemotaxonomic characterization (Table S1, supplementary material). While, six strains were unidentified, based on the followed standard methods. The predominant types in diorite rocks were *Streptomyces* and *Nocardioides* (23%, each). The unknowns represented 18%; that may indicate novel taxa.

Antimicrobial activity of the strains

Among the 34 strains, thirty of which were active against at least one of the tested microbes (88%). High activity was recorded against the reference *E. coli* NCMB 11943 strain and the clinical yeast culture (21 and 23 extracts, respectively); activity was comparably lower against the clinical *S. aureus* culture (8 extracts). Interestingly, crude extracts from the strains *Streptomyces* SP9 and *Nocardioides* SP13 showed potent activity towards *E. coli* (Table 1).

Antitumor activity and solid tumor selectivity

Only five extracts showed potent activity against Ehrlich Ascite Carcinoma, EAC (Table 1). These extracts were selected for investigation of solid tumor selectivity against murine and human cell lines. The highest effective solid tumor selectivity was recorded for *M.* EMCC 1923, towards colon tumor cell lines of mice and human, at both full and quarter concentrations (Table 2).

Taxonomic confirmation and documentation of M. EMCC 1923

The 16S rDNA sequence analysis of the potent *Micromonospora* strain indicated its relatedness to *M. citrea* strain 1-7 at 99.6% similarity level (Fig. 2); the sequence was submitted to the Gene Bank in NCBI (http://www.ncbi.nlm.nih.gov/genbank) with the accession number KM668040. The strain has been deposited in the Egyptian Microbial Culture Collection (EMCC) under the code *M. citrea* SP1 EMCC 1923; and all of its related intellectual properties has been patent registered at the Egyptian Patent Office, patent No. 655 (El-Shatoury, 2016) .

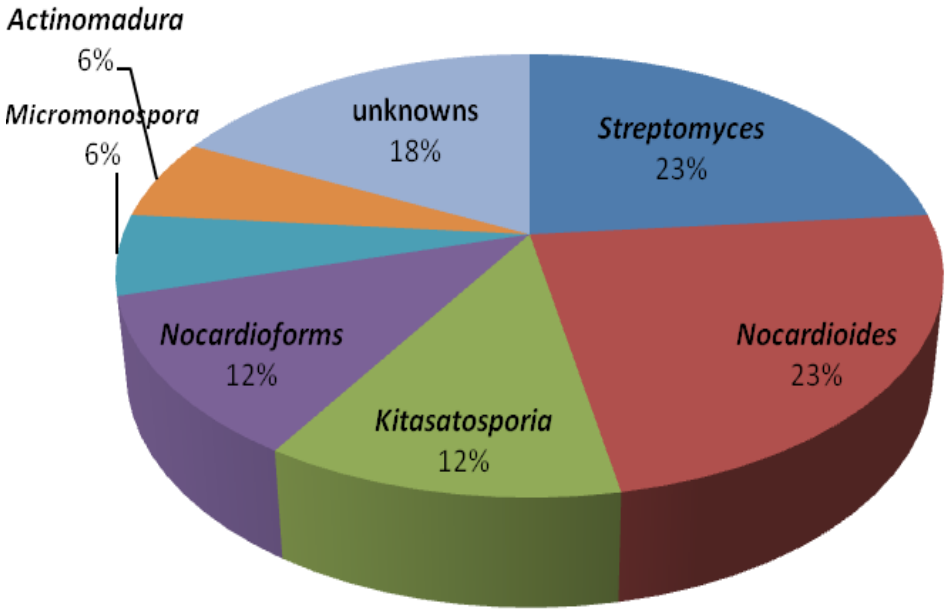

Fig. 1. Relative proportion of actinobacteria genera in diorite rocks.

TABLE 1. **Antimicrobial and antitumor activities of selected strains.**

Isolate	Antimicrobial activity[a] Inhibition zone diameter, cm (mean ± SE)			Antitumor activity[a] EAC inhibition, % (avg ± SD)[b]
	E. coli NCMB 11943	S. aureus	C. albicans	
Micromonospora SP1	-ve	0.8 ±0.05	0.7 ±0.1	**82.0** ±5.1
Streptomyces SP2	0.8 ±0.2	0.9 ±0.1	0.85 ±0.2	**61.5** ±3.1
S. SP3	0.75 ±0.15	-ve	0.9 ± 0.1	32.6 ±1.2
S. SP4	0.9 ±0.1	-ve	**1.05** ±0.15	54.8 ±5.1
S. SP5	0.7 ±0.1	-ve	0.95 ±0.35	16.7 ±6.0
S. SP6	0.9 ±0	0.54 ±0.1	0.95 ±0.2	-ve
S. SP7	0.85 ±0.1	0.6 ±0.2	0.8 ±0.1	-ve
S. SP8	0.85 ±0.1	-ve	0.95 ±0.15	45.2 ±5.1
S. SP9	**1.2** ±0.1	-ve	0.85 ±0.1	20.8 ±1.4
Nocardioides SP10	0.85 ±0.1	-ve	**1.65** ±0.15	53.5 ±4.0
N. SP11	0.85 ±0.1	-ve	0.75 ±0.2	-ve
N. SP12	-ve	0.6 ±0.2	0.6 ±0.2	**88.7** ±2.6
N. SP13	**1.1** ±0.1	0.67 ±0.11	**1.15** ±0.25	-ve
N. SP14	0.7 ±0.1	-ve	**1.3** ±0.15	-ve
N. SP15	0.8 ±0.1	-ve	0.85 ±0.15	44.2 ±4.5
N. SP16	0.85 ±0.1	-ve	0.75 ±0.1	20.0 ±2.3
N. SP17	0.8 ±0.1	-ve	**1.05** ±0.4	33.5 ±7.6
N. SP18	0.9 ±0.1	-ve	**1.15** ±0.3	-ve
Actinomadura SP19	0.9 ±0.2	-ve	**1.5** ±0.1	-ve
A. SP20	0.85 ± 0.1	-ve	0.9 ±0.15	-ve
Kitasatosporia SP21	0.73 ±0.07	-ve	0.9 ±0.1	-ve
K. SP22	0.83 ±0.2	-ve	**1.15** ±0.1	-ve
Unidentified SP23	-ve	0.58 ±0.02	-ve	**79.8** ±9.0
Unidentified SP24	-ve	-ve	-ve	**55.5** ±13.2
Unidentified SP26	0.7 ±0.1	0.5 ±0.1	**1.05** ±0.3	52.0 ±8.7

Data are presented as mean ± SE (n=3).
[a]-ve: no activity; high antimicrobial (> 1.0 cm) and antitumor (> 55 EAC inhibition %), activities are highlighted in grey.

TABLE 2. Solid tumor selectivity of the metabolic extracts from selected strains against murine and human cell lines.

Actinobacteria strain (metabolic extract concentration)		Zone Unit (zu)[a]					
		Murine (mouse) cell line			Human cell line		
		L1210	C38	CFU-GM	H125	H116	CFU-GM
Micromonospora SP1	(full strength)	200	**400**	**150**	0	**350**	**100**
	(1/4 strength)	0	**350**	**100**	0	**350**	**100**
Streptomyces SP2	(full strength)	550	500	600	**800**	600	**500**
	(1/4 strength)	0	0	NT[b]	200	0	150
Nocardioides SP12	(full strength)	200	**400**	**150**	50	100	NT
	(1/4 strength)	0	250	100	0	0	NT
unidentified SP23	(full strength)	200	**400**	**150**	0	0	NT
	(1/4 strength)	0	**400**	**150**	0	0	NT
unidentified SP24	(full strength)	200	**400**	**150**	100	50	NT
	(1/4 strength)	0	400	200	0	0	NT

[a] A difference in zones between tumor cells and normal (CFU-GM) cells ≥ 250 zu defines solid tumor selective extracts; these are indicated in bold. Zone unit of <300 with tumor cell line was taken as the extract is of low activity.
[b] NT: normal cell lines were not tested as the zone units for the corresponding tumor cell lines were < 250 zu.
[Murine cell lines: L1210 (lymphocytic leukemia); C38 (colon adenocarcinoma); Human cell lines: H-116 (colon tumor); H-125 M (lung non-small cell carcinoma); CFU-GM (normal bone marrow)]

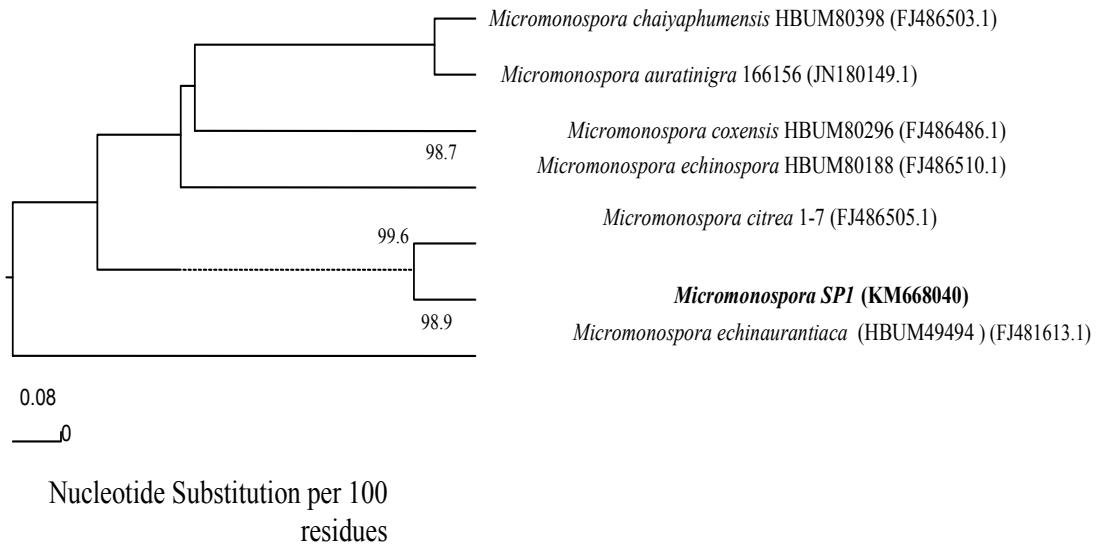

Fig. 2. Phylogenetic tree of *Micromonospora citrea* SP1 EMCC 1923, based on a neighbour-joining analysis of 16SrDNA sequences.

Antitumor activity on human cell lines

The metabolic extract derived from *M.* EMCC 1923 was investigated on six malignant human cell lines; among which HEPG2 (liver carcinoma cell line) and HCT116 (colon carcinoma cell line) were effectively inhibited at IC_{50} of 1.3 and 3.2µg/ml, respectively. The surviving fractions of cell lines against the different concentrations (1-10µg/ml) of SP1 metabolic extract are shown in Fig. 3A and B.

Mass fingerprinting

The molecular composition of the metabolic extract from *M.* EMCC 1923 was analysed using, MALDI/TOF-MS. Fig 4A and B reveals the detection of 175 molecular masses. Eighty-eight molecules, representing 50% of the extract, were related to the masses between 100-300Da. While 18 compound masses (11% of the extract) had molecular weight 585.52 – 669.64Da. Details of mass fingerprint data are provided in Table S2 (supplementary material). The UV spectrum of the extract showed two absorption peaks λmax at 235 and 278nm.

Influence of C and N sources on the bioactivity

M. EMCC 1923 was grown on different combinations of C and N sources, applying a general full factorial design experiment (Table 3). ANOVA analysis indicated non-significant effect ($P > 0.05$) of the investigated sources on antimicrobial activity (Fig. 5A), although significantly affected antitumor activity ($P < 0.02$). Interestingly, a significant interaction between starch (C-source) and potassium nitrate (N-source) combination for antitumor activity ($P = 0.007$) has enhanced the antitumor activity by 1.2 to 1.3-fold (Fig. 5B, green line). Accordingly, their use in a basal medium was applied in the subsequent investigations.

Cytotoxicity of the metabolic extract on rat model

The injection of *M.* EMCC 1923 metabolic extract, up to 500mg/kg, didn't cause lethal effects in experimental male rats for 96h. Hepatotoxicity was monitored in male rats receiving 10 and 100mg/kg injection dose, every two days for two weeks. The quantitative serum analysis of ALP, ALT, AST and LDH activities are shown in Table 4. Results indicated non-significant changes in the liver parameters for the group that has received the low extract dose. While, rats treated with the high dose (100mg/kg) exhibited significant adverse effect on liver; indicated from an increase of 19.3% and 35% in AST and ALT, respectively, compared to control. Significant increase in cholesterol was only observed in the high-dose group. While, the triglycerides increased significantly in both groups ($P \leq 0.05$), compared to control. In both groups, the levels of total protein were not statistically different from controls. Similarly, no statistically significant changes were noted for albumin and uric acid.

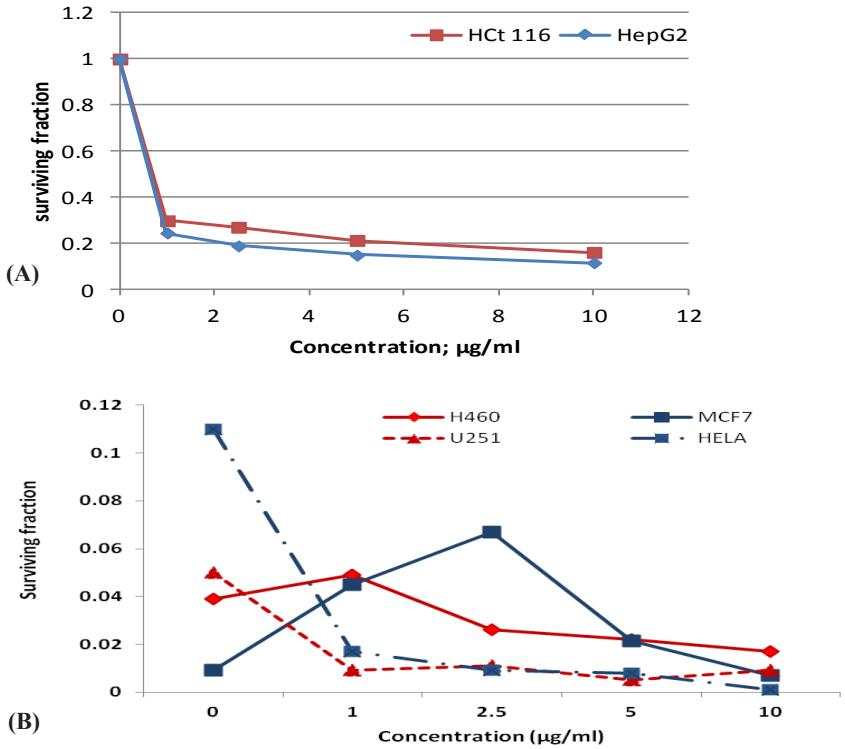

Fig. 3. Potential antitumor activities of M. EMCC 1923 metabolic extract against liver HepG2 and colon HCT116 carcinoma cell lines (A) and Negative effect on carcinoma cell lines: U251 (brain tumor), H460 (lung carcinoma), HELA (Cervix carcinoma) and MCF7 (breast carcinoma) (B).

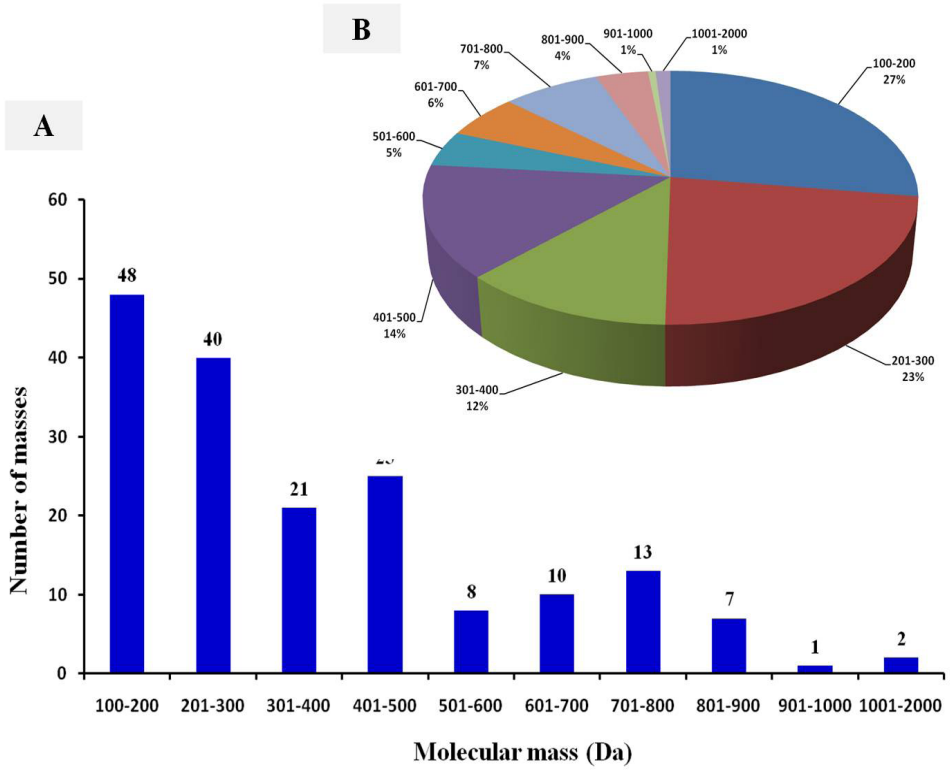

Fig. 4. Distribution of molecular masses (A) and relative proportion of each category (B) of the metabolic extract derived from M. EMCC 1923.

TABLE 3. Experimental design showing factors' levels effect on bioactivity of *M.* EMCC 1923.

Standard order	Run order	C-sources (1%)	N-sources (1%)	Antimicrobial activity [a] (*S. aureus* inhibition, mm)	Antitumor activity [a] (EAC inhibition, %)
8	1	Starch	Potassium nitrate	0.80	82
1	2	Glucose	Yeast extract	1.00	65
6	3	D-Fructose	Soya bean meal	0.51	64.1
5	4	D-Fructose	Potassium nitrate	0.51	69.5
3	5	Glucose	Soya bean meal	0.51	70
4	6	D-Fructose	Yeast extract	0.73	64.5
10	7	Mannitol	Yeast extract	0.51	64.9
2	8	Glucose	Potassium nitrate	0.85	79.1
9	9	Starch	Soya bean meal	0.95	75.3
7	10	Starch	Yeast extract	0.83	69.5
12	11	Mannitol	Soya bean meal	0.75	63
11	12	Mannitol	Potassium nitrate	0.75	65.1

[a] The highest bioactivity is highlighted in grey.

TABLE 4. Effects of M. EMCC 1923 metabolic extract on male rat after treatment for two weeks

Parameters	10mg/kg	100mg/kg	Control
AST (IU/L)	133.75 ±2.17	157.50± 3.23 [a]	132.00 ±0.91
ALT (IU/L)	30.00± 1.87	51.50± 0.65 [a]	33.50 ±2.75
AST/ALT ratio	4.46	3.06	3.94
LDH (IU/L)	2277.50± 50.00	2377.50 ±36.37	2031.25 ±23.13
ALP (IU/L)	412.50± 3.04	427.75 ±5.72	411.00 ±4.14
Total cholesterol (mg/dL)	130.00 ±5.40	107.25± 4.92	123.75 ±2.39
Triglyceride (mg/dL)	69.00 ±3.89 [a]	65.50 ±2.40 [a]	50.00± 2.04
Total protein (mg/dL)	3.43 ±0.05	4.10± 0.04	3.28 ±0.05
Albumin (mg/dL)	3.08± 0.05	3.20± 0.04	3.20 ±0.04
Uric acid (mg/dL)	2.257 ±0.11	1.9 ±0.04	2.20 ±0.06

Data are presented as mean ± SE (n=5 for each group).
[a] Significant differences, as compared to control ($P \leq 0.05$), are highlighted in grey.
[ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase].

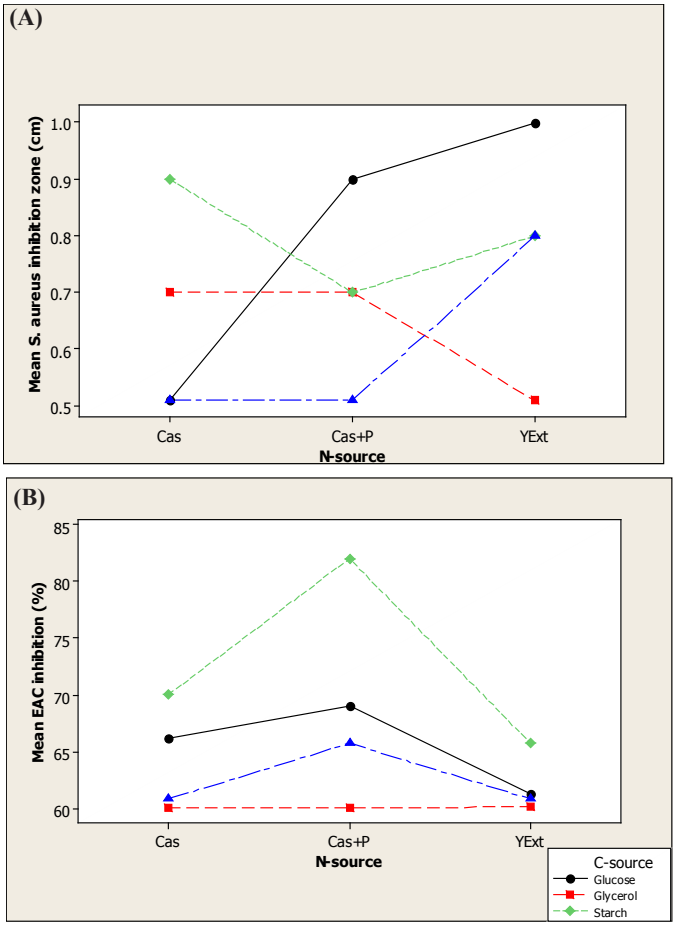

Fig. 5. Full interaction matrix of carbon and nitrogen sources for antimicrobial (A) and antitumor (B) activities of M. EMCC 1923 metabolic extract.

Discussion

In this study, endolithic actinobacteria has been isolated from diorite rocks of Sinai and investigated for antibiotic activities. Results indicated that 46% of the strains were assigned to *Streptomyces* and *Nocardioides*. These findings compare well with that observed in rocks collected from relevant arid deserts (Wong et al., 2010 and Tang et al., 2012). The predominance of *Streptomyces* and its related genera in such habitats was suggested to be due to their high adaptability to the desiccation, nutrients limitation and UV irradiation existing in the rock varnish. Additionally, it is well known that the strong cell wall, the capability of forming spores and the high GC-content of actinobacteria are all advantages under extreme environments (Cheeptham et al., 2013). Interestingly, about 18% of the recovered strains could not be identified using the standard chemotaxonomy techniques; thus they may represent novel taxa. The notable frequency of unknown actinobacteria in diorite rocks was consistent with Horath & Bachofen (2009), who proved broad genetic diversity and novelty of actinobacteria in dolomite rocks of the Swiss Alps using culture independent techniques. A remarkable number of the strains (88%, including 2 unidentified strains) showed antimicrobial and/or antitumor activities, indicating that the Egyptian rocks are under-explored habitats and can represent promising sources for actinobacteria. The recent publication reveals few reports which has described endolithic bioactive actinobacteria from the Egyptian rocks; examples are those by Hozzein et al. (2011) and Saleh et al. (2013).

Due to the solid tumor selectivity and promising antimicrobial activity of *M. citrea* SP1 EMCC 1923, it was selected for further investigation of the nutritional factors, chemical

nature and cytotoxicity of its metabolic extract. Species from genus *Micromonospora* are, particularly, important producers for diverse antibiotic metabolites; a comprehensive review on that topic is provided by (Tiwari & Gupta, 2012). The previous studies have, also, shown the importance of *M. citrea* metabolic products against a spectrum of Gram positive bacteria (Carter et al., 1990). Nevertheless, the current study provides the first recording of the effectiveness of *M. citrea* against hepatic carcinoma (HepG2) human cell lines. Although the UV and MALDI/TOF-MS analysis revealed 11% of the extract (18 compound masses) to be in the range of citreamicin group (Liu et al., 2012), further investigation is required to elucidate its chemical nature and assess the active fractions.

Micromonospora EMCC 1923 was grown on different carbon and nitrogen sources to investigate the nutrients effect on the bioactivity. The results revealed significant dependence of the antitumor activity on the medium composition, although the antimicrobial activity was not significantly affected. Previous studies have confirmed that the nature of C- and N-sources can greatly affect bioactivity in different actinobacteria (Tiwari & Gupta, 2012). Among the investigated C- and N-sources in this study, there was a significant interaction effect between starch and potassium nitrate ($P = 0.007$), resulting in enhancing antitumor activity up to 1.3-fold. This is in good agreement with the earlier work on *M. echinospora*, in which gentamicin production was mainly affected by starch and potassium salt concentrations (Himabindu et al., 2006). Studies have, also, indicated that the type of nitrogen sources in the production medium control a number of pathways involved in secondary metabolism and may affect enzymes that synthesize precursors for the production of antibiotics (Navone et al., 2014). Our finding suggests that investigation of other economic C- and N-sources may remarkably enhance the bioactivity and feasibility for future application.

Investigations on *M*. EMCC 1923 metabolic extract on animal model revealed non-significant toxicity effect of the low dose 10mg/kg. Though, the higher dose significantly affected serum ALT, AST and LDH levels. The ratio of serum AST to ALT can be used to differentiate liver damage from other organ damage. In the present study, AST/ALT ratio was > 1 in both groups, indicating low hepatotoxicity of the extract in rats receiving dose up to 100mg/kg at 2-days intervals for two weeks. The measurements of ALP confirmed a low hepatotoxicity of the extract; because its levels did not differ significantly from the control. ALP is linked to cell membranes, particularly, in hepatocytes. It is, primarily, a marker of hepatobiliary effects and cholestasis (Ramaiah, 2007). Also, the levels of uric acid did not show significant changes in all groups; possibly indicating either no liver injury due to free radical stress or unchanged uric acid levels by inability of excretion. Uric acid is the end product of the catabolism of tissue nucleic acid, i.e. purine bases metabolism (Carr & Frei, 1999). It is suggested that uric acid can act, together with glutathione, as non-enzymatic antioxidant to offer a significant protection against oxygen free radical-induced liver injury (Shrestha et al., 2012). Overall, *M*. EMCC 1923 metabolic extract had low hepatotoxicity, which makes it promising for detailed investigation.

In conclusion, this study evidences promising taxonomic diversity and bioactivity of actinobacteria which colonized the diorite rocks. The metabolic extract derived from *Micromonospora citrea* SP1 EMCC 1923 represented a potential hepatocellular carcinoma inhibitor. It showed non-significant effect, at 10mg/kg dose, on the measured biochemical parameters in animal model. Results indicated that amendment of basal medium with starch and potassium nitrate (1% w/v), as sole C- and N-sources, respectively, significantly interacted and affected antitumor production by *M.* EMCC 1923. This investigation illustrates the antimicrobial and antitumor potential of endoliths from the Egyptian rocks; and represent a part of an ongoing extensive study on diversity, ecological role and biotechnological applications of actinobacteria inhabiting rocks of South Sinai, Egypt.

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العزل وإمكانية التضاد الحيوي للأكتينوبكتريا المتعايشة داخل صخور الديوريت في جنوب سيناء، مصر

هشام عبدالله[1]، سحر الشطورى[1]، محمد عبدالرحمن[2] و نهلة الشناوى[2]
[1] قسم علم النّبات - كلية العلوم - جامعة قناة السّويس - الإسماعيلية - مصر و [2] قسم علم الحيوان - كلية العلوم - جامعة قناة السّويس - الإسماعيلية - مصر.

تم عزل أربع وثلاثين سلالة أكتينوبكتريا من صخور الديوريت في جنوب سيناء، مصر. وكانت الأنواع السائدة هي ستربتوميسيز و نوكارديويدس (8 سلالة، لكل منها)، وتلاها كيتاساتوسبوريا ونوكارديوبسيس (4 سلالات، لكل منها). أظهرت الدراسة نشاط متنوع من قبل 25 سلالة مضاد لبكتريا إيشيريشيا كولاي مرجعية ولعزلتين سريريتين من ستافيلوكوكاس أورياس وفطر كانديدا ألبيكانس، ولثلاث أنواع من الأورام السرطانية للقولون والرئة.

وقد أظهر مستخلص إحدى سلالات الميكرومونوسبورا نشاطاً ملحوظاً ضد العزلات السريرية، وسمية تخصصية مع الخلايا السرطانية البشرية للكبد HepG2 والقولون HCT116 بجرعة نصف مميتة 1.3 و 3.2 ميكروجرام/مل، على التوالى. وبإستخدام التحليل الجينى ثبت إنتماء السلالة للميكرونوسبورا سيتريا (99.6 % تماثل)، بالتحليل الطيفى الكمى ثبت أن مستخلصها يحتوى على 18 مركب في نطاق الوزن الجزيئي من 585.52 إلى 669.64 دالتون. وبتطبيق التصميم الإحصائي الكلي العام لدراسة تأثير أربعة مصادر كربونية و ثلاث مصادر نتروجينية لتعزيز النشاط الحيوي للسلالة، وجد أن النشا ونترات البوتاسيوم تأثيرا ملحوظاً يزيد إنتاجية مضادات الأورام إلى 1.3 مرة ($P = 0.007$).

وأوضحت تجارب السمية على الجرذان أن المستخلص الأيضى غير ضار على وظائف الكلى والكبد مقارنة بالمجموعة الضابطة من حيوانات التجارب. حيث أن مجموعات الفئران التى حقنت بجرعة 10 و 100 ملجم/كجم، جرعة كل يومين على التوالى لمدة أسبوعين أظهرت تغير غير معنوى فيما يخص تحاليل النشاط الإنزيمى الكلى، و مستوى اليوريا والكرياتينين والبروتين الكلى والزلال. وتم إيداعها في بنك الثروة الميكروبية المصري باسم *M. citrea* SP1 EMCC 1923 ، وتسجيلها براءة إختراع في مكتب البراءات المصرى (برقم 655). وتخلص الدراسة إلى أهمية البيئة الصخرية القاحلة في سيناء كمصدر متميز للأكتينوبكتريا ذات قدرات واعدة لإنتاج المضادات الحيوية.