IJBPAS, September, 2020, 9(9): 2169-2190

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

BIOLOGICAL SIGNIFICANCE AND ANTITUMOR ACTIVITIES OF METABOLITES INCORPORATING EXOPOLYSACCHARIDES RECOVERED FROM *Rhizobium* sp.

SOLYMAN E[1], EL-GENDY AO[2*], AHMED RR[3], ZAHRAN HH[1] AND AHMAD MS[1]

1: Botany and Microbiology Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt

2: Microbiology and Immunology Department, Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt

3: Zoology Department, Faculty of Science, Beni-Suef University, Beni-Suef, Egypt

*** Corresponding Author: E Mail: ahmed.elgendy@pharm.bsu.edu.eg; Phone: +201223476015**

Received 19th Jan. 2020; Revised 24th Feb. 2020; Accepted 26th March 2020; Available online 1st Sept. 2020

https://doi.org/10.31032/IJBPAS/2020/9.9.5175

ABSTRACT

Rhizobium is a Gram positive bacterium equipped with many beneficial roles to humans and plants such as symbiotic behaviors, combating different pathogens and many other effects. For such importance, there is an urgent need to isolate and explore new *Rhizobium* sp. for their biological activities especially having antitumor activities and in the same time to influence broad symbiotic activities being able to grow and tolerate harsh environmental and industrial conditions like that existing in Egypt. A number of 35*Rhizobium* sp. were isolated and allowed to grow under different stress conditions including high salt and different pH. The maximum tolerance capability was recorded in two strains; ES-BM9 and ES-CS4.PCR amplification and sequencing of the 16S rRNA gene of ES-BM9 and ES-CS4 was performed and they were identified as *Rhizobium leguminosarum* and *Rhizobium pusense,* respectively. Fermentation and extraction of metabolites containing exopolysaccharide (EPS) were managed and evaluated for their cytotoxicity against HepG-2, MCF-7 andCaco-2 cell lines. Extracts of ES-BM9 showed the highest cytotoxicity against all cell lines.LC-HRESIMS analysis revealed presence of several compounds with key biological applications.To the best of our knowledge, this is the first study to identify Octapeptin-A2, Fortimicin, Chalcomycin

and Lipopeptide being recovered from *Rhizobium* sp. and the first study to report antitumor activities from *Rhizobium* sp. isolated from Egypt.

Keywords: *Rhizobium*, Exopolysaccharides, Cytotoxicity, Stress response, Bioproducts

INTRODUCTION

Rhizobium is a genus of Gram-negative diazotrophic bacteria living in the soil and infecting legumes (plant host) forming root nodules that are responsible for nitrogen fixation from atmosphere into NH_3 which is considered the most useful form of nitrogen for the plant **[1]**.

In agricultural loci, about 80% of biologically fixed nitrogenarises from a symbiosis relationship between leguminous plants and *Rhizobium* sp. **[2]**. The annual reported nitrogen-value of legume symbioses is around 70 million tons **[3]**.

Isolation and selection of stress-tolerant *Rhizobium* strains may develop the plant growth by nodulation and nitrogen fixation in poor soil conditions **[4, 5]**. The use of chemical fertilizers is carried out excessive, and this becomes very expensive for farmers. Now, the use of microorganisms which can fixes atmospheric nitrogen is of great practical significance since it makes it possible to bridge the restrictions of chemical fertilization, which has resulted in undesirable stages of water pollution **[6]**.

Phosphorus (P) is the greatest limiting element for plant growth afterward nitrogen. A great number of strains of *Rhizobium* were capable to solubilize phosphorous in liquid culture **[7]**. The significance of this ability to solubilize phosphorus in plant growth by certain *Rhizobia* strains has been confirmed in chickpeas and barley **[8]**.

Rhizobia have the ability to produce siderophores that have a high affinity for Fe^{3+}, making iron obtainable to plants **[9]** and can also make a stable complex with heavy metals like Cd, Cu, Al, and so on and with radionuclides containing U and Np **[10]**. Therefore, plant inoculation by siderophore-producing bacteria safeguards them from stress produced by heavy metals and aids them absorb iron **[11]**.

*Rhizobia*can produce *Phytohormone*s that promote plant growth at small concentrations, and providing good defense against pathogens **[12, 13]**. Antagonism of pathogen populations and pest by *Rhizobium* sp. takes numerous methods where species are pathogens of bacteria, fungi, nematodes and/or parasitic plants **[14]**. Many studies have been reported the lignification and accumulation of toxic compounds against pathogen in pea roots after inoculation by *Rhizobium* strains **[15, 16]**. The presence of *Rhizobium* sp. could indirectly encourage the plant to activate its defense devices when challenged with a pathogen by the secretion of plant defense compounds as phenolics, flavonoids or other phytoalexins **[14]**.

In *Rhizobia*, surface polysaccharides have an essential role in symbiosis with legumes **[17]**. These sugars have a significant role in the recognition of microsymbionts (*Rhizobia*) to the macrosymbionts (legumes) in the early stages of infection and symbiosis [18-20]. In addition, it protects the cell against desiccation, acts as antimicrobial agents and facilitates the adhesion of bacteria to different solid surfaces **[21, 22]**. Environmental factors (e.g., salt, temperature, etc.) impose negative effects on the biosynthesis of these surface polysaccharides **[23-25]**. These effects could be an obstacle inhibiting the symbiotic process between *Rhizobia* and the legumes **[24, 26, 27]**. Many chemical analysis of numerous bacterial and rhizobial exopolysaccharides (EXP) have been done **[28-30]**. As well as the biological key role of rhizobial polysaccharides in the symbiotic process, they might have a biotechnological and medical role **[29, 31]**. Antitumor activity of rhizobial exopolysaccharides has been previously reported **[29]**. In this study, different *Rhizobium* sp. have been isolated and characterized for their potential antitumor activities in addition to their abilities to produce various significant metabolites endorsed in improving plant growth and productivity, especially those grown in salty and pH disturbed soil environment in Egypt.

MATERIALS AND METHODS

Sampling area

Root nodules were collected from three species of crop legumes (*Vicia faba, Cicer arietinum, Trifolium resupinatum*) obtained from Beni-Suef Governorate, Egypt (29°06'14"N, 31°07'39"E). Also, one species of wild herb legumes (*Vicia faba*) was obtained from Minia governorate, Egypt (28°03'30.1"N, 30°45'09.4"E).

Samples preparation and isolation of *Rhizobium* sp.

Legumes were carefully uprooted and the root system was washed under running water to remove adhesive soil particles and healthy intact pink nodules were selected. Surface sterilization of root nodules was performed with 3-5% hydrogen peroxide (h_2O_2) and washed recurrently by sterile distilled water. Finally, they were washed in 70% ethyl alcohol followed by washing with sterile distilled water **[32]**. These prepared nodules were transferred to a culture tube half filled with sterile water and crashed with sterile glass rods to obtain a milky suspension. Serial dilution was performed followed by streaking on yeast extract mannitol (YEM) agar (0.4 g/L yeast extract, 10 g/L mannitol, 0.2 g/L $MgSO_4$,0.5 g/L K2HPO4, and 9 g/L agar, 0.1 g/L NaCl, pH 7.0) added with Congo red (25 µg /mL) and incubated for 2 to 3 days at 28°C **[32]**. A Gram stain technique was applied to identify collected *Rhizobium* isolates. All suspected isolates were stored in 40% glycerol at – 80ºC.

Three reference *Rhizobium* strains were graciously provided by the Agricultural Research Centre, Giza, Egypt **(Table 1)**. These reference strains were used as controls to detect validity of culture media and suitability of the used conditions for isolation of *Rhizobium* sp.

Tolerance of *Rhizobium* isolates to different stress environments

All isolated *Rhizobium* sp. were allowed to grow in YEM media under different stress conditions including 1%, 2%, 3% and 4% NaCl, and different adjusted pH (4, 6 and 8). The availability of each isolate to grow under these conditions was recorded.

Genomic DNA extraction and purification

Genomic DNA extraction was prepared according to **[33, 34]** with certain modifications. Briefly, a 1.5 mL of isolate growing on yeast extract mannitol broth was centrifuged for 10 min at 5,000 *g*, the supernatant was discarded, and the pellets were resuspended in 200 µL spheroblast buffer (10% sucrose, 25 mM Tris pH 8.4, 25 mM EDTA pH 8.0, 2 mg/ml lysozyme and 0.4 mg/ mlRNase A), vortexed and incubated at 37ºC for 10 min until cell lysis happened. Then, 50 µL of 5% SDS (lysis buffer 1) and 5 M NaCl (lysis buffer2) were added, mixed and incubated at 65ºC for 5 min. A 100 µL neutralizing buffer (60 mL 5M potassium acetate, 11.5 mL glacial acetic acid, and 28.5 mL dH_2O) was formerly added and placed on ice for 5 min before centrifugation at 13,000 *g* at 4ºC for 15 min. The supernatant (approximately 400 µL) was transferred to a new tube, mixed with equal volume of isopropanol, left for 5 min at room temperature and centrifuged at 13,000 *g* at room temperature for 15 min to precipitate the DNA. The resultant pellet was washed with 70% ethanol by centrifugation at 13,000 *g* at room temperature for 5 min. The last pellet was air-dried and re-suspended in 50 µL 1 × TE buffer pH 8 and kept in the refrigerator at 4ºC.

PCR amplification and sequencing of bacterial 16S rRNA

PCR amplification of the 16S rRNA of two *Rhizobium*sp. was performed using universal forward primer (27F 5'-AGAGTTTGATCCTGGCTCAG-3') and universal reverse primer (1492R 5'-GGTTACCTTGTTACGACTT-3'). PCR mixture was done in a 50 µL of PCR reaction volume using 10 µL of 5x buffer, 500 ng genomic DNA, 10 mM dNTPs mixture, 2.5 units of Taq DNA polymerase enzyme and 1 µL of both forward and reverse primers. The PCR program included template denaturation at 94°C (3 min), followed by 34 cycles of denaturing at 94°C (30 s), annealing at 56°C (30 s), and extension at 72°C (60 s), and followed by completion of DNA synthesis at 72°C (5 min). Primers were removed from the final PCR product before sequencing using QIAquick PCR purification kit (QIAGEN, Germany). The PCR product of interest was detected and purified by agarose gel electrophoresis using 1% (w/v) agarose gel with reference to 1 kbp DNA ladder. DNA was sequenced using the ABI Prism BigDye terminator sequencing ready reaction kit version 3.1 and analyzed with the ABI Prism 3100 generic analyzer.

Sequence manipulation and phylogenetic analysis

The Blast tool in National Center for Biotechnology Information (NCBI) was used to compare the good quality sequences to the GenBank database to identify the closest related species which have highly similar sequences to the amplified ones. Finally, the multiple sequence alignment of the amplified sequences and the closely related sequences from NCBI was carried out followed by phylogenetic analysis using MEGA7 software **[35]**.

Fermentation and extraction of exopolysaccharide (EPS)

Bacterial fermentation of both BM9 and CS11 isolates were done by inoculating 1% overnight cultured bacteria 250 mL YEM broth in 500 mL Erlenmeyer flask and incubated on rotary shaker incubator at 160 *g* for 2 days at 30°C. Bacterial broth was centrifuged at 8000 *g* for 10 minutes at 4°C and pellets were discarded. Cold 96% ethanol was added to the supernatant at 3:1 (v/v) ethanol: supernatant ratio to precipitate the EPS **[36]**. The mixture was refrigerated at 4ºC for 24 h. Afterward the refrigeration period, the samples were centrifuged again (10,000 *g*, 4ºC, 30 min) to separate the precipitated EPS from the solvent. The precipitated EPS was washed few times by ethanol, and ethanol was evaporated using vacuum evaporator.

In vitro anti-tumor cytotoxicity

The extracted exopolysaccharides of two selected *Rhizobium* isolates were assessed for their cytotoxicity by tissue culture technique. Hepatocellular carcinoma cell line (HepG-2), human breast carcinoma cell line (MCF-7) and colorectal adenocarcinoma cell line (Caco-2) were gotten from the Pharmacology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Egypt. Cells were maintained in DMEM medium with 10% fetal calf serum, sodium pyruvate, 100 U/ml penicillin and 100 mg/ ml streptomycin at 37°C and 5% CO_2 till the cytotoxicity bioassay was carried out. The potential cytotoxicity was tested using the method of **[37]**. Briefly, 100 cells/well were plated onto 96-well dishes overnight earlier the treatment with the tested EPS extracts to allow the attachment of cells to the wall of the plate. Diverse concentrations of each tested EPS extract (0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.65, 1.3, 2.6, 5.25, 10.5, 21 and 42 mg/ml) were added to the cell monolayer and triple wells were used for each individual dose. Monolayer cells were incubated with the tested agent(s) for 48 h at 37°C and 5% CO_2. At the end of the incubation period, the cells were fixed and stained with sulforhodamine B dissolved in acetic acid. Unbound stain was removed by washing four times with 1% acetic acid and the protein-bound dye was extracted with Tris-EDTA buffer. The absorbance was measured in an ELISA reader at 570 nm. The relation between surviving fraction and compound concentration was plotted to get the survival curve of each tumor cell line. The concentration of an agent which causes a 50% growth inhibition (IC50), for each tested agent using each cell line was achieved from the survival curve **[37]**.

LC-HRESIMS profiling of the metabolites formed by selected *Rhizobium* isolates

Major metabolites of two selected *Rhizobium* isolates were characterized using LC-HRESIMS spectrometric technique. It is composed of Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA autosampler and Accela Pump). The following conditions were used: capillary voltage 45 V, capillary temperature 260°C, and auxiliary gas flow rate 10–20 arbitrary units, sheath gas flow rate 40–50 arbitrary units, spray voltage 4.5 kV, mass range 100– 2000 amu (maximum resolution 30000). For LC/MS; Waters SunFire C18 RP analytical HPLC column (5 μm, 4.6 x 150 mm) using a gradient of MeOH in H_2O containing 0.01% formic acid as eluent (0–100% over 30 min) at a flow rate 1 ml/min (Marine Biodiscovery Centre, Chemistry Department, Aberdeen University). Metabolites molecular formulas were deducted by exact mass of eluted peaks, and theses formulas were searched in Dictionary of Natural Products, CRC press, online version, for matching chemical structures.

RESULTS

Isolation and characterization of the isolated bacterial colonies

A total of 35 pure isolates of Gram-negative bacteria of *Rhizobium*sp. were recovered from root nodules of leguminous from different locations in Beni-Suef and Menia Governorate, Egypt and were isolated by means of serial dilution method followed by inoculation on YEM agar media. The colonies colors were varied with milky white, creamy white and creamy yellow on YEM media, and were either opaque or translucent. Most of the isolates had an complete colony margin and had a mucoid texture because of the secretion of excess EPSs. All *Rhizobium* isolates showed a Gram negative pink rod appearance after applying Gram stain technique.

Tolerance of *Rhizobium* isolates to different stress conditions

All *Rhizobium* isolates along with the reference strains were evaluated for their ability to grow in different stress conditions. In order to investigate the ability of *Rhizobium* isolates to tolerate wide concentration range of NaCl, different YEM agar plates supplemented with 1, 2, 3 or 4% NaCl were streaked with a loopful of freshly cultured *Rhizobium* isolates. After incubating the plates for 3-5 days at 28°C, growth was recorded as positive (visible growth) or negative (no growth) **[38]** as shown in **(Table 2)**. A YEM agar plate with 0.01% NaCl was used as a control for positive growing of these isolates.

The ability of these isolates to grow under different pH values (4, 6, 7 and 8) were investigated by inoculation in YEM broth medium with different pH adjusted using 1M HCl and 1M NaOH. After 3-5 days of incubation, growth was recorded by visual observation compared to control treatments incubated at pH 7 as shown in **(Table 2)**.

DNA sequencing for the selected isolates and phylogenetic analysis:

The partial 16S rRNA gene sequencing of both isolates ES-BM9 and ES-CS4 revealed similarity 98.7% with *Rhizobium leguminosarum* and99.2% with *Rhizobium pusense* respectively, according to BLAST tool at NCBI GenBank as shown in **(Table 3)**

The resulted sequence was aligned to 21 of the closely related *Rhizobium*sp. by retrieving their sequences from the NCBI GenBank database and assembled in MEGA7 software for phylogenetic analysis using the Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2-parameter method. The achieved phylogenetic tree **(Figure 1)** confirmed the similarity of both ES-BM9 and ES-CS4 isolates to *Rhizobium leguminosarum* and *Rhizobium pusense* with a similarity matrix bootstrap value of 96 and 78 respectively.

The resulted nucleotide sequences were deposited in Genbank under accession numbers MN460363 and MN460364.

In vitro anti-tumor cytotoxicity

The extracted exopolysaccharides of two selected *Rhizobium* isolates; *Rhizobium leguminosarum* ES-BM9 and *Rhizobium pusense* ES-CS4 were evaluated for their antiproliferative cytotoxicity using tissue culture technique against three selected cell lines; hepatocellular carcinoma cell line (HepG-2), human breast carcinoma cell line (MCF-7) and colorectal adenocarcinoma cell line (Caco-2).

Only ES-BM9 isolate showed strong anti-tumor cytotoxicity against all tested cell lines when compared with that of ES-CS4 isolate at which the survival fractions were pointedly reduced as the concentration increased **(Figure 2)**. The concentrations that cause a 50% growth inhibition (IC50) were calculated and revealed that the lowest recorded IC50 was against HepG-2 with 4.255 mg/mlas shown in **(Table 4)** and **(Figure 3)**.

LC-HRESIMS profiling of the metabolites

LC-HRESIMS screening revealed the presence of diverse compounds belonging to antimicrobials, antitumors, siderophores, exopolysaccharides and other biologically significant metabolites as referred in **(Table 5** and **6) (Figure 4** and **5)**. These isolated compounds were determined and compared to earlier isolated compounds using different libraries databases.

Table 1: Reference *Rhizobium* sp. used as controls during isolation steps.

Host Legumes	Rhizobium sp.	Strain
Viciae faba (Faba bean)	*R. leguminosarum bv. Viceae*	ICARDA 441
Trifolium alexandrinum (Berseem clover)	*R. leguminosarum bv. Trifolii*	ARC 103
Cicer arietinum (Chickpea)	*Mesorhizobium ciceri*	ICARDA 36

Table 2: Growth response of different *Rhizobium* isolates under different stress conditions of NaCl and pH

Rhizobium isolate	Growth at different salt concentration				Growth at different pH		
	NaCl 1%	NaCl 2%	NaCl 3%	NaCl 4%	pH 4	pH 6	pH 8
ES-BM2	-	-	-	-	+	+	+
ES-BM3	-	-	-	-	+	+	+
ES-BM5	-	-	-	-	+	+	+
ES-BM6	-	-	-	-	-	-	+
ES-BM7	-	-	-	-	+	+	+
ES-BM8	-	-	-	-	+	+	+
ES-BM9	+	+	+	+	+	+	+
ES-BM10	-	-	-	-	+	+	+
ES-BS1	+	+	+	-	+	+	+
ES-BS2	+	+	-	-	+	+	+
ES-BS4	+	-	-	-	+	+	+
ES-BS5	+	+	-	-	+	+	+
ES-BS6	+	+	-	-	+	+	+
ES-BS7	-	-	-	-	-	+	+
ES-BS8	-	-	-	-	+	+	+
ES-BS9	+	-	-	-	-	-	+
ES-BS10	+	-	-	-	+	+	+
ES-CS1	+	+	+	-	+	+	+
ES-CS2	+	+	+	-	+	+	+
ES-CS4	+	+	+	+	+	+	+
ES-CS5	+	+	-	-	+	+	+
ES-CS6	+	+	+	-	+	+	+
ES-CS7	-	-	-	-	-	-	+
ES-CS8	+	+	+	-	+	+	+
ES-CS9	+	+	-	-	-	-	+
ES-CS10	+	+	+	-	+	+	+
ES-TM1	+	-	-	-	+	+	+
ES-TM2	+	+	+	-	+	+	+
ES-TM3	+	+	+	-	+	+	+
ES-TM4	-	-	-	-	+	+	+
ES-TM5	+	+	+	-	-	+	+
ES-TM6	+	-	-	-	+	+	+
ES-TM8	-	-	-	-	+	+	+
ES-TM9	-	-	-	-	+	+	+
ES-TM1O	+	-	-	-	+	+	+

Table 3: Genotypic characterization of selected *Rhizobium* isolates.

Accession number	Obtained sequence size	NCBI BLAST result	Max score	Total score	Query cover	*E* value	Max identification
MN460363	1375	*Rhizobium leguminosarum bv. viciae* USDA 2370 16S ribosomal RNA, partial sequence	2447	2447	100%	0.0	98.70%
MN460364	1304	*Rhizobium pusense* strain NRCPB10 16S ribosomal RNA, partial sequence	2355	2355	100%	0.0	99.23%

Table 4: Estimated IC50 (mg/ml) of ES-BM9 against different cell lines

Cell Line	HepG2	MCF7	Caco2
IC50 mg/ml	4.255	7.655	8.699

Table 5: LC-HRESIMS analysis for extracts from *Rhizobium leguminosarum* ES-BM9 with deduced molecular formulas and suggested identified compounds

Accurate mass	Molecular formula	Tentative identification
206.0812	$C_{11}H_{11}NO_3$	Indole-3-lactic acid
228.1593	$C_{12}H_{21}NO_3$	N-Octanoyl-L-homoserine lactone
312.2532	$C_{18}H_{33}NO_3$	N-Tetradecanoyl-L-homoserine lactone
411.3983	$C_{30}H_{50}$	Hopene-B
429.4092	$C_{30}H_{52}O$	Gammaceran-3b-ol
813.28784	$C_{37}H_{50}FeN_5O_{12}$	Exochelin siderophore
1010.7085	$C_{48}H_{91}N_{13}O_{10}$	Octapeptin-A2
1185.5586	$C_{60}H_{84}N_2O_{22}$	No hits

Table 6: LC-HRESIMS analysis for extracts from *Rhizobium pusense* ES-CS4 with deduced molecular formulas and suggested identified compounds

Accurate mass	Molecular formula	Tentative identification
297.2424	$C_{18}H_{32}O_3$	Rhizobialide
338.2687	$C_{20}H_{35}NO_3$	Palmitoleyl homoserine lactone
347.2288	$C_{15}H_{30}N_4O_5$	Fortimicin KG aminoglycoside
378.1872	$C_{15}H_{27}N_3O_8$	Rhizobactin
407.2023	$C_{17}H_{30}N_2O_9$	5-Acetamido-3,5,7,9-tetradeoxy-7-(3-hydroxybutyramido)-L-glycero-L-manno-nonulosonic acid homopolysaccharide
531.3025	$C_{24}H_{42}N_4O_9$	Rhizobactin-1021
649.3402	$C_{27}H_{48}N_6O_{12}$	Vicibactin-7101
701.3743	$C_{35}H_{56}O_{14}$	Chalcomycin
711.1917	$C_{35}H_{34}O_{16}$	No hits
775.3720	$C_{33}H_{54}N_6O_{15}$	Vicibactin
887.5663	$C_{48}H_{79}N_5O_{10}$	Mycobactin (18) siderophore
921.5723	$C_{54}H_{80}O_{12}$	No hits
994.6435	$C_{50}H_{87}N_7O_{13}$	Lipopeptide NO
1049.7221	$C_{54}H_{96}N_8O_{12}$	Lichenysin-G9a
1131.4746	$C_{47}H_{78}N_4O_{25}S$	Ac-NodRm-1 (nodulation factor)
1217.7910	$C_{52}H_{85}N_{11}O_{22}$	AF-011A1 lipopeptide
1254.0678	$C_{75}H_{144}O_{13}$	No Hits
1341.9687	$C_{82}H_{132}O_{14}$	No hits
1501.7395	$C_{66}H_{112}N_6O_{32}$	Lipo-chitin oligosaccharide

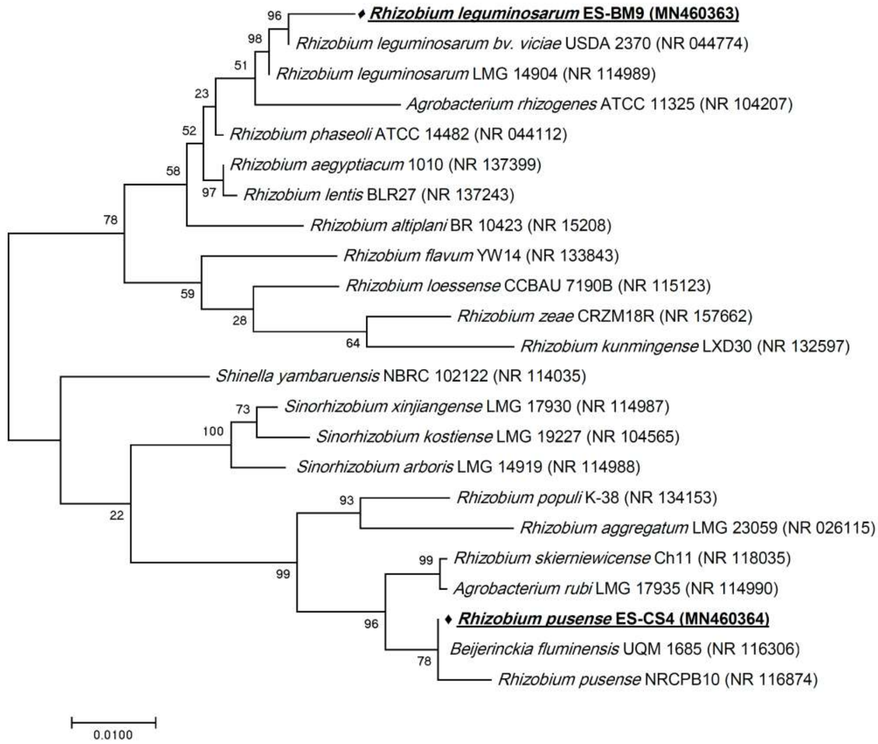
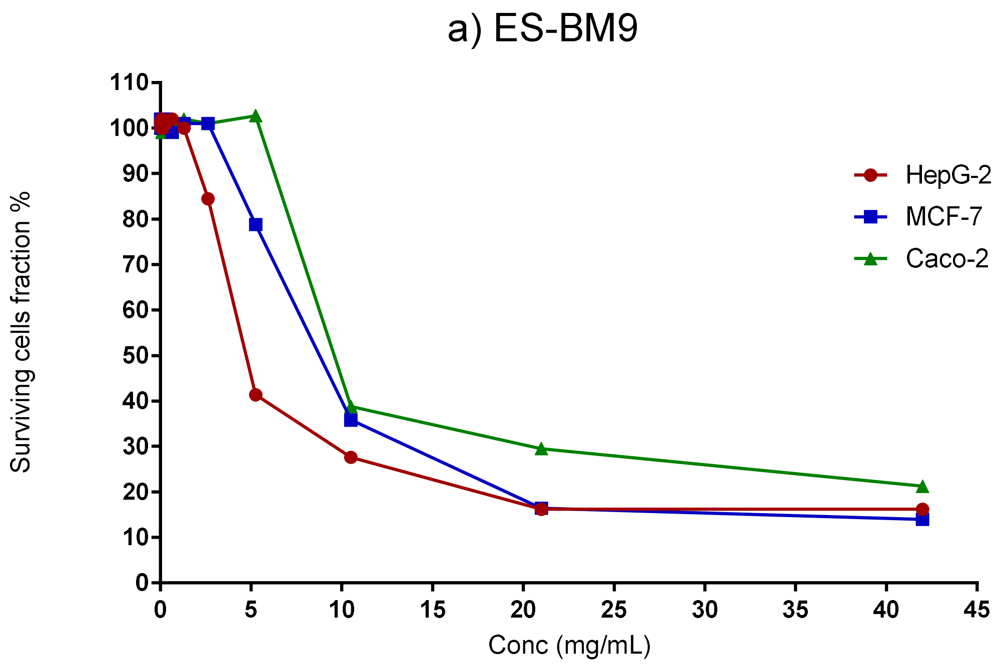


Figure 1: Phylogenetic tree of both *Rhizobium leguminosarum* ES-BM9 and *Rhizobium pusense* ES-CS4 isolates based on partial 16S rRNA gene sequences. The phylogenetic tree was inferred using the Neighbour-Joining method. The distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. Numbers at nodes indicate percentages of 1000 bootstrap resamplings, only values above 50% are shown. The analysis involved 23 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 [71]



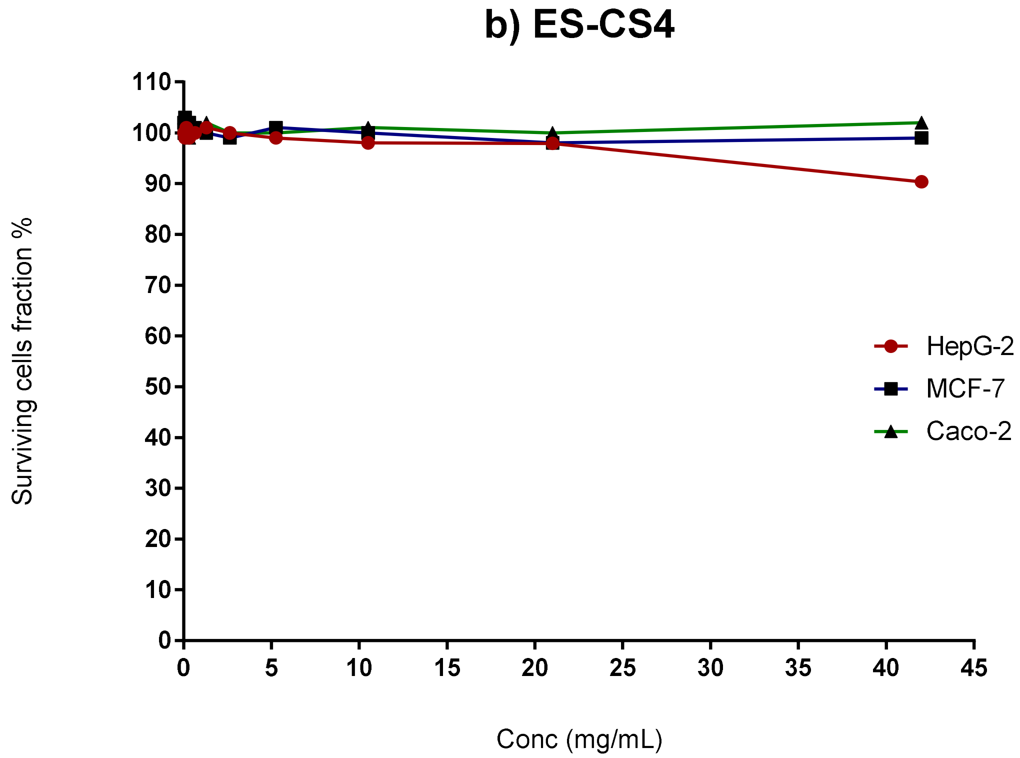


Figure 2: Antitumor survival curves of extracted exopolysaccharides of a) *Rhizobium leguminosarum*ES-BM9 and b) *Rhizobium pusense* ES-CS4 against HepG2, MCF7 and Caco2 cell lines, showing strong anti-tumor cytotoxicity in case of ES-BM9 compared to almost no activity in case of ES-CS4

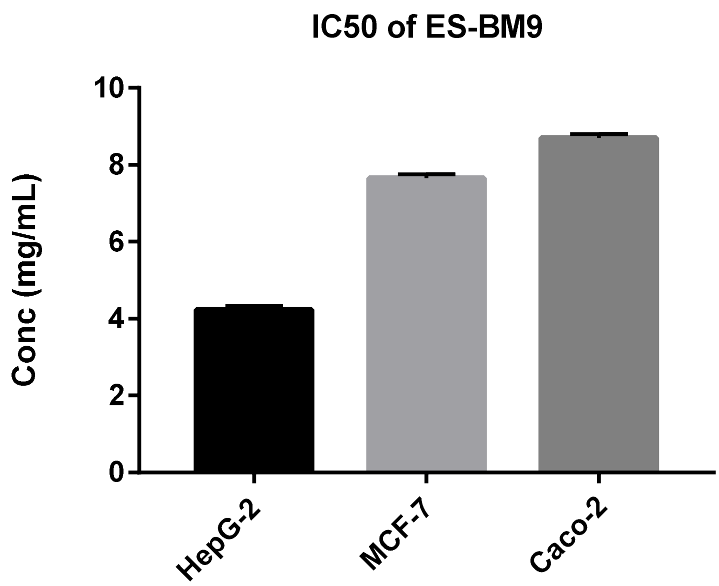


Figure 3: Different IC50 of exopolysaccharide extract of ES-BM9 isolate against HepG2, MCF7 and Caco2 cell lines

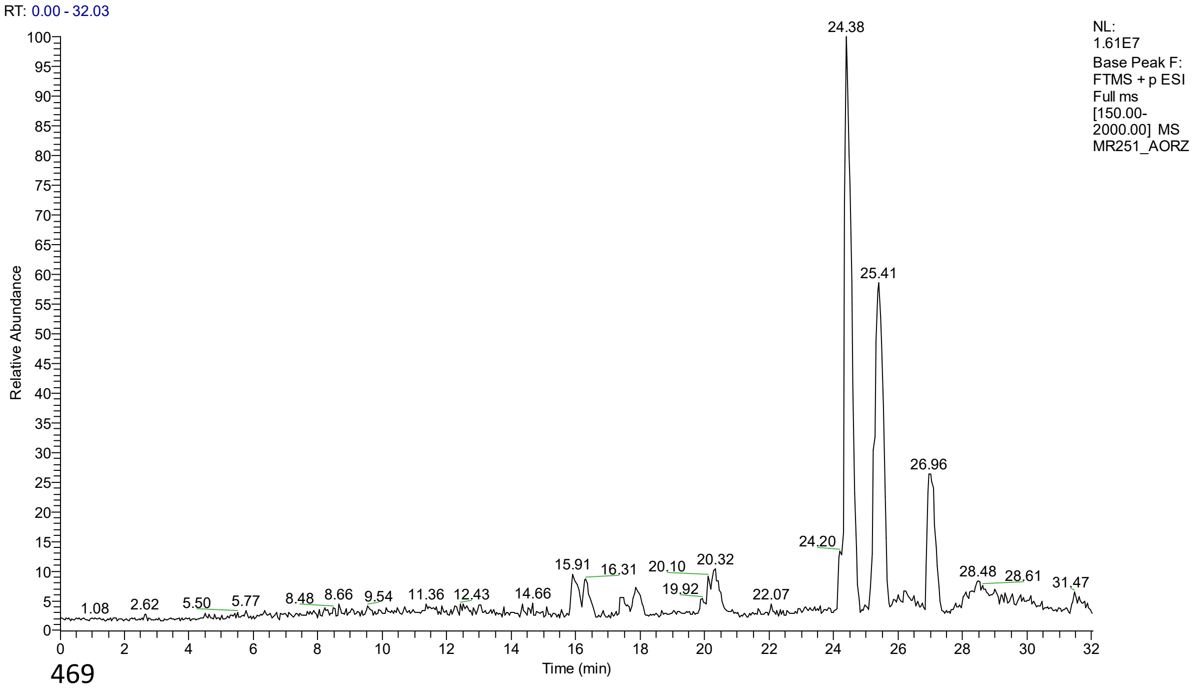


Figure 4: HPLC chromatogram of extract from *Rhizobium leguminosarum* ES-BM9 indicating peaks that are characteristic for metabolites explained previously in table 5

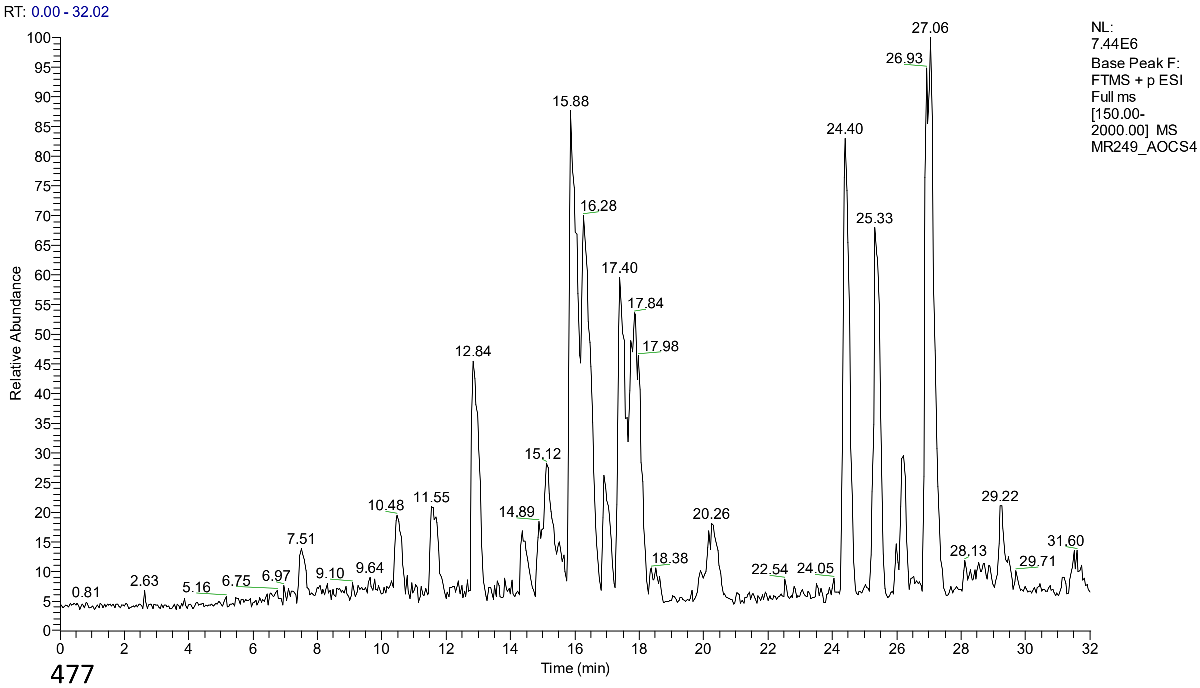


Figure 5: HPLC chromatogram of extract from *Rhizobium pusense* ES-CS4 indicating peaks that are characteristic for presence of exopolysaccharides and other compounds which were explained previously in table 6

DISCUSSION

*Rhizobium*is the bacteria responsible for nitrogen fixation through symbiosis with legumes **[1]**. It also secret compounds (EPS) which play important role in many biological mechanisms **[39]**. Microbial polysaccharides are used widely in numerous industrial and pharmacological applications because of their unique physical and rheological properties **[40]**. Interestingly several exopolysaccharides (EPS) products have revealed functional biological activities once used as anti-tumor, antiviral, anti-coagulant and/or immunomodulating agent **[41, 42]**.

In saline soils inoculation of crops and trees with tolerant symbiotic strains of *Rhizobium* help the plants in tolerating stress through improved nutrition **[43]**. Previous studies were done to find salt tolerant isolates of *Rhizobium* which have great symbiotic performance with NaCl concentration ranging from 0% to 9.5%. Also, many studies has been done for selecting effective strains of *Rhizobium* which have the ability to survive in some extreme conditions (salt stress) found in Egypt **[44]**. Susceptibility was recorded as positive and negative result **[45]**. Acid pH restricts the persistence of *Rhizobia* strains in many soil, nodulation and nitrogen fixation. Several studies were searching for strains that can resist pH 4.00 up to pH 7.00 **[46]**. In the recent study, different *Rhizobium* sp. were found to resist and tolerate wide range of salinity and different pH conditions especially the two strains ES-BM9 and ES-CS4.

Many antitumor drugs as cyclophosphamide has powerful effect against tumor cells but it has bad side effects causing suppression of immunologic function and leading to atrophy of the spleen and thymus **[47]**. So, many efforts are currently established to search for more effective anti-tumor substances with minimal side effects especially from natural sources **[48]**.

In the current study, the extracted metabolites containing exopolysaccharides of *Rhizobium leguminosarum* ES-BM9 showed strong anti-tumor cytotoxicity against all tested cell lines. The concentrations that cause a 50% growth inhibition (IC50) were calculated and revealed that the lowest recorded IC50 was against HepG-2 with 4.255 mg/ml and it was 7.655 and 8.699 mg/mlin case of MCF-7 and Caco-2 respectively.

Previous studies have been done and indicated that the polysaccharide from *Rhizobium* (REPS) an attractive chemoprevention agent for the inhibition or treatment of tumors due to of its enormously low clinical toxicity and immunomodulation compared with that of chemical drugs, which propose a potential therapeutic role for REPS as a drug or as an adjuvant for the treatment of tumors **[49]**.

Some previous studies showed antitumor activities of REPS against sarcoma 180 (S-180), hepatoma 22 (H-22), and Ehrlich ascites carcinoma (EAC) **[49]**. Others showed similar effects against sarcoma 180 (S-180) and hepatocarcinoma (HepG-2) **[47]**.

LC-HRESIMS is a highly sensitive and effective technique in the dereplication of natural products through providing accurate masses and molecular formulae which are valued information for dereplication, being obtainable in the most databases of natural products and independent on the source, the sample preparation and the measurement conditions **[50]**. LC-HRESIMS overcomes the danger of false positive identification that was considered as the most common risk of LC-MS approach as it has the advantage of supplying certain information about structures of natural products **[50]**.

In the current study, this technique was applied to collect accurate information about all metabolites containing exopolysaccharides of two selected *Rhizobium* bacteria; ES-BM9 and ES-CS4. LC-HRESIMS analysis revealed the presence of some compounds that were determined by comparison with the aid of different libraries databases as displayed in **(Table 5** and **6) (Figure 4** and **5)**. Some identified metabolites were known with their potential effects on the symbiosis and has many important applications in biotechnology. Other identified metabolites were not yet explored and should be investigated intensively in the future for putative biological activities.

In the current study, the LC-HRESIMS analysis for extracts from *Rhizobium leguminosarum* ES-BM9 revealed presence of many significant metabolites such as indole-3-lactic acid, N-Octanoyl-L-homoserine lactone, N-Tetradecanoyl-L-homoserine lactone, Hopene-B, Gammaceran-3b-ol, Exochelin siderophore and Octapeptin-A2. Surprisingly and to the best of our knowledge, this is the first report to identify Octapeptin-A2 recovered from extracts of *Rhizbium* sp. Octapeptin-A2 was firstly known as lipopeptides produced from *Bacillus* sp. and *Paenibacillus* sp. with potent antimicrobial activities **[51]**. It was reported that the phytohormone indole-3-aectic acid (IAA) not only could promote plant growth and remediate fluoranthene-contaminated soil **[52]** but also had shown anticancer activity **[53]**. Also, N-octanoyl-l-homoserine lactone was identified and was used as autoinducer in the transcription of some microorganisms **[54]**. Exochelin siderophore is essential in limiting iron condition for biofilm formation in many pathogenic bacteria which form structured biofilm communities at liquid - air interfaces and on solid surfaces **[55]**. Also plant inoculation by siderophore producing bacteria protects them from stress caused by heavy metals and helps them in absorbing iron **[14]**. It was reported that siderophores could have potential anti-cancer effects **[56]**. N-tetradecanyol-l-homoserine lactone and acylated-homoserine lactones (acyl-HSLs) are common quorum-sensing signals that regulate a diverse range of target functions, which are often involved in host interactions and has a role in the development of sessile microbial populations, called microbial biofilms **[57]**. So, this could help in colonization of Gram-negative bacteria as *Rhizobium* sp. with the plant host in the process of symbiosis.

The LC-HRESIMS analysis for extracts from *Rhizobium pusense* ES-CS4revealed presence of many significant metabolites and exopolysaccharides as shown in **(Table 5)**. Rhizobialide was previously known as an antibacterial sterolactone produced by *Mesorhzobium* sp. inside roots of *glycyrrhiza uralensis* **[58]**. Fortimicin kg aminoglycoside was discovered in 1977 as an aminoglycoside antibiotics produced from fermentation broth of *Miccromonspor Olivasterospora* cs-26 **[59]**. Rhizobactin-1021 is an important metabolite that was identified as siderophore isolated from *Rhizobium meliloti* 1021 and had high affinity for Fe^{+3}, making iron available for plants and it could affect the ability of the bacteria to fix nitrogen [60]. The Vicibactin is a cyclic trihydroxymate siderophore produced previously by *Rhizobium leguminosarum bv. Viciae*, which promote iron uptake by iron starved cell **[61]**, also this metabolite is responsible of alleviation of aluminum toxicity to some species as *Rhizobium leguminosarum bv. Viciae* **[62]**. Chalcomycin was known as a macrolide antibiotic from the marine isolate *Streptomyces* sp. B7062. **[63]**, with antimicrobial activities against *Staphylococcus* sp. and *Streptococcus pyogenes* **[64]**. Mycobactin (18) siderophore was identified in 2013 as novel siderophore export system for iron utilization which was necessary for virulence of *Mycobacterium tuberculosis* **[65]**. Lipopeptide NO had many uses as inducer for cell-mediated HIV immune response in sero-negative volunteers **[66]** and was acting as antifungal agent affecting *Aspergillus fumigatus* and *Candida albicans* **[67]**. Ac-nodrm-1 is a nodulating factor which elicits root hair deformation specifically *on Rhizobium meliloti* – alfalfa plant symbiosis **[68]**. Lipochitin oligosacchride (LCO) is an example of exopolysaccharide identified in this study. It was known as causative agent of the formation of root nodules in leguminous plants **[69]**, it also decrease auxin transport ability in *Vicia sativa* subsp. *nigra* roots

[70] To the best of our knowledge, this is the first study to report the production of Fortimicin, Chalcomycin and Lipopeptide NO from *Rhizobium* sp.

Overall, the following study shed the light on *Rhizobium* that are still considered a significant source for expressing many natural compounds and exopolysaccharides with great importance as natural antitumor, in addition to other beneficial symbiotic effects for plants. *Rhizobium* strains resistant to high salt and different pH conditions are with importance to be used as supplement in poor soil areas. To the best of our knowledge, this is the first time to identify Octapeptin-A2, Fortimicin, Chalcomycin and Lipopeptide NO being recovered from *Rhizobium* sp.

CONCLUSION

Isolation and identification of stress tolerant straines of rhzobium sp. from the natural samples collected from Beni-Suef and Minia govern ate ,Egypt.

The stress tolerant bacterial isolate rhzobium sp.ES-BM9 and ES-CS4 is a promising bacterial strain which can survive in stress conditions found commonly in Egypt.

These two strains can be used as inoculants for improving the productivity in similar areas.

Detection of substances with great benefits ,did not recovered from rhzobium sp. before.

Reporting of antitumor activity of extracted metabolites containing EXP isolated from rhizobium.

Rhizobiums are still considered a significant source for expressing many nature, safe and cheap byproducts and exopolysaccharides with great importance for plants, surrounding environment and humans.

Author contributions

Microbiological and molecular biology experiments were performed by AOE and HFZ. HPLC/HRMS and data manipulation and characterization of chemical structures were elucidated by ES and MSA. Antitumor experiments were conducted by RRA. Finally manuscript was drafted and revised for the final approval by all authors.

Acknowledgement

The authors acknowledge department of Microbiology and Immunology, Faculty of pharmacy, Beni-Suef University for their continuous efforts to facilitate this work.

Conflict of Interest

No conflict of interest declared.

REFERENCES

[1] Aung TT, Tittabutr P, Boonkerd N, Herridge D, andTeaumroong N. 2013. Co-inoculation effects of Bradyrhizobium japonicum and Azospirillum sp. on competitive nodulation and rhizosphere eubacterial community structures of soybean under

rhizobia-established soil conditions. *African Journal of Biotechnology.*12.

[2] Vance CP. 1998. Legume symbiotic nitrogen fixation: agronomic aspects, pp. 509-530. *The rhizobiaceae*, Ed. Springer ,

[3] Brockwell J, Bottomley PJ, andThies JE. 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. *Plant and soil.***174:** 143-180.

[4] Zou N, Dart P, andMarcar N. 1995. Interaction of salinity and rhizobial strain on growth and N2-fixation by Acacia ampliceps. *Soil Biologyand Biochemistry.***27:** 409-413.

[5] Biswas S, Das RH, Sharma GL, andDas HR. 2008. Isolation and characterization of a novel cross-infective rhizobia from Sesbania aculeata (Dhaincha). *Current microbiology.***56:** 48-54.

[6] Sprent JI and Sprent P. 1990. Nitrogenfixing organisms: pure and applied aspects. *Nitrogen fixing organisms: pure and applied aspects*.

[7] Halder A and Chakrabartty P. 1993. Solubilization of inorganic phosphate byRhizobium. *Folia microbiologica.***38:** 325-330.

[8] Imen H, Neila A, Adnane B, ManelB, Mabrouk Y, Saidi M, andBouaziz S. 2015. Inoculation with phosphate solubilizing Mesorhizobium strains improves the performance of chickpea (*Cicer aritenium* L.) under phosphorus deficiency. *Journal of plant nutrition.***38:** 1656-1671.

[9] Rajkumar M, Ae N ,Prasad MNV, andFreitas H. 2010. Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. *Trends in biotechnology.***28:** 142-149.

[10] Neubauer U, Furrer G, Kayser A, andSchulin R. 2000. Siderophores, NTA, and citrate: potential soil amendments to enhance heavy metal mobility in phytoremediation. *International journal of phytoremediation.***2:** 353-368.

[11] Chabot R, Antoun H, andCescas MP. 1996. Growth promotion of maize and lettuce by phosphate-solubilizing Rhizobium leguminosarum biovar. phaseoli. *Plant and soil.***184:** 311-321.

[12] Dobbelaere S, Vanderleyden J, andOkon Y. 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical reviews in plant sciences.***22:** 107-149.

[13] Boiero L, Perrig D, Masciarelli O , Penna C, Cassán F, andLuna V. 2007. Phytohormone production by three strains of Bradyrhizobium japonicum and possible physiological and technological implications. *Applied microbiology and biotechnology.***74:** 874-880.

[14] Mabrouk Y, Hemissi I, Salem IB, Mejri S, Saidi M, andBelhadj O. 2018. Potential of rhizobia in improving

nitrogen fixation and yields of legumes. *Symbiosis.* 107.

[15] Mabrouk Y, Zourgui L, Sifi B, Delavault P, Simier P, andBelhadj O. 2007. Some compatible Rhizobium leguminosarum strainsin peas decrease infections when parasitised by Orobanche crenata. *Weed Research.***47:** 44-53.

[16] Mabrouk Y, Simier P, Arfaoui A, Sifi B, Delavault P, Zourgui L, andBelhadj O. 2007. Induction of phenolic compounds in pea (Pisum sativum L.) inoculated by Rhizobium leguminosarum and infected with Orobanche crenata. *Journal of Phytopathology.***155:** 728-734.

[17] Zahran HH. 2009. Enhancement of rhizobia–legumes symbioses and nitrogen fixation for crops productivity improvement, pp. 227-254. *Microbial strategiesfor crop improvement*, Ed. Springer ,

[18] Carlson RW, Forsberg LS, andKannenberg EL. 2010. Lipopolysaccharides in Rhizobium-legume symbioses. *Subcell Biochem.***53:** 339-386.

[19] Ivashina TVandKsenzenko VN. 2012. Exopolysaccharide biosynthesis in Rhizobium leguminosarum: from genes to functions. *The complex world of polysaccharides.* 99-127.

[20] Kelly SJ, Muszynski A, Kawaharada Y, Hubber AM, Sullivan JT, Sandal N, *et al.* 2013. Conditional requirement for exopolysaccharide in the Mesorhizobium-Lotus symbiosis *.Mol Plant Microbe Interact.***26:** 319-329.

[21] Leigh JAandCoplin DL. 1992. Exopolysaccharides in plant-bacterial interactions. *Annu Rev Microbiol.***46:** 307-346.

[22] Nicolaus B, Kambourova M, andOner ET. 2010. Exopolysaccharides from extremophiles: from fundamentals to biotechnology. *Environ Technol.***31:** 1145-1158.

[23] Janczarek M. 2011. Environmental signals and regulatory pathways that influence exopolysaccharide production in rhizobia. *Int J Mol Sci.***12:** 7898-7933.

[24] AKemad, M., and Khan, M.S. (2011). Functional aspects of plant growth promoting rhizobacteria: recent advancements. Insight Microbiol, 1(3), 39-54.

[25] Zahran H, Räsänen LA, KarsistoM, andLindström K. 1994. Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. *World Journal of Microbiology and Biotechnology.***10:** 100-105.

[26] Khan MS, Zaidi A, andMusarrat J. 2010. *Microbes for legume improvement*, pp. Ed. Springer.

[27] Zahran HH. 1999. Rhizobium-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. *Microbiol Mol Biol Rev.***63:** 968-989, table of contents.

[28] Freitas F, Alves VD, andReis MA. 2011. Advances in bacterial exopolysaccharides: from production to biotechnological applications. *Trends Biotechnol.***29:** 388-398.

[29] Zhao L, Chen Y, Ren S, Han Y, andCheng H. 2010. Studies on the chemical structure and antitumor activity of an exopolysaccharide from Rhizobium sp. N613. *Carbohydrate research.* **345:** 637-643.

[30] Zong A, Cao H, andWang F. 2012. Anticancer polysaccharides from natural resources: a review of recent research. *Carbohydr Polym.***90:** 1395-1410.

[31] Castellane TCL, Lemos MVF, andde Macedo Lemos EG. 2014. Evaluation of the biotechnological potential of Rhizobium tropici strains for exopolysaccharide production. *Carbohydrate polymers.***111:** 191-197.

[32] Vincent JM. 1970. A manual for the practical study of the root-nodule bacteria. *A manual for the practical study of the root-nodule bacteria*.

[33] Sinha S, Srivastava R, De Clercq E, andSingh RK. 2004. Synthesis and antiviral properties of arabino and ribonucleosides of 1, 3-dideazaadenine, 4-nitro-1, 3-dideazaadenine and diketopiperazine. *Nucleosides, Nucleotides and Nucleic Acids.***23:** 1815-1824.

[34] Dundar H, Brede DA, La Rosa SL, El-Gendy AO, Diep DB, andNes IF. 2015. The fsr quorum-sensing system and cognate gelatinase orchestrate the expression and processing of Proprotein EF_1097 into the mature antimicrobial peptide enterocin O16. *Journal of bacteriology.***197:** 2112-2121.

[35] Tamura K, Dudley J, Nei M, andKumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Molecular biology and evolution.***24:** 1596-1599.

[36] Breedveld M, Zevenhuizen L, andZehnder A. 1990. Osmotically induced oligo-and polysaccharide synthesis by Rhizobium meliloti SU-47. *Microbiology.***136:** 2511-2519.

[37] Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D*, et al.* 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *JNCI: Journal of the National Cancer Institute.***82:** 1107-1112.

[38] Moawad HandBeck D. 1991. Some characteristics of Rhizobium leguminosarum isolates from uninoculated field-grown lentil. *Soil biology and biochemistry.***23:** 933-937.

[39] Skorupska A, Janczarek M, Marczak M, Mazur A, andKról J. 2006. Rhizobial exopolysaccharides: genetic control and symbiotic functions. *Microbial cell factories.***5:** 7.

[40] Sutherland I. 2002. A sticky business. Microbial polysaccharides: current products and future trends. *Microbiology today.***29:** 70-71.

[41] Ruas-Madiedo P, Hugenholtz J, andZoon P. 2002. An overview of the functionality of exopolysaccharides produced by lactic acid bacteria. *International Dairy Journal*-163 :12. .171

[42] Pan DandMei X. 2010. Antioxidant activity of an exopolysaccharide purified from Lactococcus lactis subsp. lactis 12. *Carbohydrate polymers*.**80:** 908-914.

[43] Subba Rao NSandDommergues YR. 1998. *Microbial interactions in agriculture and forestry*, pp. Ed. Science Publishers, Inc.

[44] Zahran HH. 1992. Characterization of root-nodule bacteria indigenous in the salt-affected soils of Egypt by lipopolysaccharide, protein and plasmid profiles. *Journal of basic microbiology*.**32:** 279-287.

[45] Singh AK, Singh G, andBhat R. 2015. Effects of salt stress on cell surface properties and symbiotic performance of root nodulating bacteria. *UK J. Pharmaceut. Biosci*.**3:** 23-29.

[46] Graham PH, Draeger KJ, Ferrey ML, Conroy MJ, Hammer BE, Martinez E, *et al.* 19 .94Acid pH tolerance in strains of Rhizobium and Bradyrhizobium, and initial studies on the basis for acid tolerance of Rhizobium tropici UMR1899. *Canadian journal of microbiology*.**40:** 198-207.

[47] Ding G-B, Nie R-H, Lv L-H, Wei G-Q, andZhao L-Q. 2014. Preparation and biological evaluation of a novel selenium-containing exopolysaccharide from Rhizobium sp. N613. *Carbohydrate polymers*.**109:** 28-34.

[48] Ser H-L, Palanisamy UD, Yin W-F, Chan K-G, Goh B-H, andLee L-H. 2016. Streptomyces malaysiense sp. nov :.a novel Malaysian mangrove soil actinobacterium with antioxidative activity and cytotoxic potential against human cancer cell lines. *Scientific reports*.**6:** 24247.

[49] Zhao L, Chen Y, Ren S, Han Y, andCheng H. 2010. Studies on the chemical structure and antitumor activity of an exopolysaccharide from Rhizobium sp. N613. *Carbohydrate research*.**345:** 637-643.

[50] Konishi Y, Kiyota T, Draghici C, Gao J-M, Yeboah F, Acoca S*, et al.* 2007. Molecular formula analysis by an MS/MS/MS technique to expedite dereplication of natural products. *Analytical chemistry*.**79:** 1187-1197.

[51] Cochrane SAandVederas JC. 2016. Lipopeptides from Bacillus and Paenibacillus spp.: a gold mine of antibiotic candidates. *Medicinal Research Reviews*.**36:** 4-31.

[52] Li M, Guo R, Yu F, Chen X ,Zhao H, Li H, andWu J. 2018. Indole-3-acetic acid biosynthesis pathways in the plant-beneficial bacterium Arthrobacter pascens ZZ21. *International journal of molecular sciences*.**19:** 443.

[53] Kim SY, Ryu JS, Li H, Park W-J, Yun H-Y, Baek KJ*, et al.* 2010 .UVB-

activated indole-3-acetic acid induces apoptosis of PC-3 prostate cancer cells. *Anticancer research.***30:** 4607-4612.

[54] Kuo A, Callahan SM, andDunlap PV. 1996. Modulation of luminescence operon expression by N-octanoyl-L-homoserine lactone in ainS mutants of Vibrio fischeri. *Journal of Bacteriology.***178:** 971-976.

[55] Ojha AandHatfull GF. 2007. The role of iron in Mycobacterium smegmatis biofilm formation: the exochelin siderophore is essential in limiting iron conditions for biofilm formation but notfor planktonic growth. *Molecular Microbiology.***66:** 468-483.

[56] Miller MJ, Zhu H, Xu Y, Wu C, Walz AJ, Vergne A*, et al.* 2009. Utilization of microbial iron assimilation processes for the development of new antibiotics and inspiration for the design of newanticancer agents. *Biometals.***22:** 61.

[57] Fuqua CandGreenberg EP. 2002. Signalling: listening in on bacteria: acyl-homoserine lactone signalling. *Nature reviews Molecular cell biology.***3:** 685.

[58] Wei GH, Yang XY, Zhang JW, Gao JM, Ma YQ, Fu YY, andWangP. 2007. Rhizobialide: a new stearolactone produced by Mesorhizobium sp. CCNWGX022, a rhizobial endophyte from Glycyrrhiza uralensis. *Chemistry & biodiversity.***4:** 893-898.

[59] Nara T, Yamamoto M, Kawamoto I, Takayama K, Okachi R, Takasawa S, *et al.* 1977 .Fortimicins A and B, new Aminoglycoside antibiotics. *The Journal of antibiotics.* **30:** 533-540.

[60] Persmark M, Pittman P, Buyer JS, Schwyn B, Gill Jr PR, andNeilands J. 1993. Isolation and structure of rhizobactin 1021, a siderophore from the alfalfa symbiont Rhizobium meliloti 1021. *Journal of the American Chemical Society.***115:** 3950-3956.

[61] Dilworth MJ, Carson KC, Giles RG, Byrne LT, andGlenn AR. 1998. Rhizobium leguminosarum bv. viciae produces a novel cyclic trihydroxamate siderophore, vicibactin. *Microbiology.***144:** 781-791.

[62] Rogers NJ, Carson KC, Glenn AR, Dilworth MJ, Hughes MN, andPoole RK. 2001. Alleviation of aluminum toxicity to *Rhizobium leguminosarum* bv. viciae by the hydroxamate siderophore vicibactin. *Biometals.***14:** 59-66.

[63] Asolkar RN ,Maskey RP, Helmke E, andLaatsch H. 2002. Chalcomycin B, a new macrolide antibiotic from the marine isolate Streptomyces sp. B7064. *The Journal of antibiotics.***55:** 893-898.

[64] Jordan D. 1963. Effect of chalcomycin on protein synthesis by Staphylococcusaureus. *Canadian Journal of Microbiology.***9:** 129-132.

[65] Wells RM, Jones CM, Xi Z, Speer A, Danilchanka O, Doornbos KS*, et al.* 2013. Discovery of a siderophore export system essential for virulence of

Mycobacterium tuberculosis. *PLoS pathogens.***9:** e10031.20

[66] Pialoux G, Gahéry-Ségard H, Sermet S, Poncelet H, Fournier S, Gérard L*, et al.* 2001. Lipopeptides induce cell-mediated anti-HIV immune responses in seronegative volunteers. *Aids.***15:** 1239-1249.

[67] Kanasaki R, Abe F, Kobayashi M, Katsuoka M, Hashimoto M, Takase S*, et al.* 2006. FR220897 and FR220899, novel antifungal lipopeptides from Coleophoma empetri no. 14573. *The Journal of antibiotics.***59:** 149.

[68] Roche P, Lerouge P, Ponthus C, andPromé J. 1991. Structural determination of bacterial nodulation factors involved in the Rhizobium meliloti-alfalfa symbiosis. *Journal of Biological Chemistry.***266:** 10933-10940.

[69] Spaink HPandCarlson R. 1996. Regulation of plant morphogenesis by lipo-chitin oligosaccharides. *Critical Reviews in Plant Sciences.***15**.582-559 :

[70] Boot KJ, van Brussel AA, Tak T, Spaink HP, andKijne JW. 1999. Lipochitin oligosaccharides from Rhizobium leguminosarum bv. viciae reduce auxin transport capacity in Vicia sativa subsp. nigra roots. *Molecular plant-microbe interactions.***12:** 8.844-39

[71] Kumar S, Stecher G, andTamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular biology and evolution.* msw, 054.