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Characterization of natural bioactive compounds produced by isolated bacteria from compost of aromatic plants

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Abstract

Aims:

This study aimed to highlight the importance of compost from aromatic plants as a stunning source for several bio active compounds generated from their inhabited

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thermophilic bacteria. Some of the isolated compounds could have a potential role in the treatment of microbial infections.

Methods and Results:

A total of forty different thermophilic bacteria were isolated from compost samples during their thermophilic stage. These isolates were tested for their antimicrobial capabilities against different Gram-positive and negative bacteria using agar diffusion and double layer agar methods. The potential isolates were further identified based on morphological, biochemical and 16S rRNA gene sequencing methods. They were subjected to submerged state fermentation and the total crude metabolites were recovered using ethyl acetate (EtOAc) extraction. All bioactive metabolites were identified using liquid chromatography coupled with high resolution mass spectrometry (LC-HRMS). It was observed that two out of forty isolates were remarkably active against Gram positive bacteria. These isolates were genetically identified as *Bacillus* species and their different active metabolites were characterized in the EtOAc extracts using LC-HRMS.

Conclusion:

LC-HRMS analysis of EtOAc extracts revealed the presence of active metabolites that are responsible for antimicrobial activities.

Significance and Impact of Study:

To the best of our knowledge, this is the first time to identify bioactive antimicrobial metabolites from retrieved compost microorganisms in Egypt. So, compost could be a beneficial area for research as a reliable and continuous natural source for different uncountable communities of bacteria.

Keywords: Compost, aromatic plants, thermophilic bacteria, bioactive natural compounds.

1. Introduction

Green biotechnology is a promising branch of biotechnology concerned with agricultural processes, food and microorganisms that grow under specific environmental conditions. (Dubey 2014) Its research has been expanded during the recent decades to focus on recycling and bio-degradation as renewable sources of energy to gain the maximum benefit from natural wastes. (Jouraiphy et al. 2005)

Composting is regarded as one of the most important bio-recycling, a bio-degradation process which bio-transforms hard, complex organic matters into more simple products to be easily mineralized and metabolized by microorganisms. (Bernal et al. 2009)

A humus like-substance "compost" which is the final product of composting procedures is involved in a lot of important applications such as soil restoration to preserve soil and plant parameters, and good fertilizer to reduce soil-borne diseases. (Martínez-Blanco et al. 2013)

For adequate composting, microbial activity should be enhanced by suitable carbon nitrogen ratio, good aeration, and good moisture content. (Reynnells 2013)

Also, the presence of residues from aromatic oil extraction processes of aromatic plants enhance composting more than organic matters. (Lemus and Lau 2002) Green compost from aromatic plants is a good example for successful composting process as shown in (Table 1).

During composting process, thermophilic stage seems to be the most vital step which is characterized by highly natural metabolic temperature above 50°C and may even reach 80°C resulting in degradative effects of microorganisms to simplify hard substances. (Waksman et al. 1939)

Recently, thermophilic bacteria have gained researchers' interest not only due to their unusual growth at high temperature, but also as excellent source for more thermostable biocatalyst with important role in industrial application such as bioorganic synthesis and biosensors. (Kristjansson 1991)

This study aimed to highlight the importance of compost from aromatic plants as a stunning source for several bioactive compounds generated from their inhabited thermophilic bacteria. Some of the identified compounds could have a potential role in the treatment of microbial infections.

2. Materials and Methods

2.1. Sampling area and sample collection

The samples of compost were collected from Beni-Suef district at depth of 10 – 15 cm from the core of compost where most of the microbial activities take place. Most of the bacterial populations were concentrated at approximately more than 55°C with a moisture content of 50 - 60%. Compost sample (approx. 500 g) were collected using a sterile spatula in clean, dry and sterile polythene bags before transportation to laboratory to be stored at 55°C for further studies.

2.2. Isolation of bacteria

Isolation and enumeration of bacteria were performed by using serial dilution agar plate technique (Aneja 2003, Ahmad et al. 2017) using Trypton Soya Agar (Oxoid, London, UK) and the plates were incubated at 55°C for 7 d. Culture growth characters of isolated bacteria were observed by inoculating bacteria on the different growth media, as: Nutrient agar (Difco, Chicago, IL, USA), Starch nitrate agar (Difco, Chicago, IL, USA), International Streptomyce Project ISP Medium No. 4 (Inorganic

Salt Starch Agar) (Himedia, Mumbai, India) and ISP Medium No. 5 (Glycerol Asparagine Agar Base) (Himedia, Mumbai, India).

2.3. Antibacterial screening

Isolated colonies were tested for their antibacterial activities by agar diffusion method (Valgas et al. 2007), and double layer agar method. (Fujita et al. 2007; Dundar et al. 2015; AbdEl-Mogheith et al. 2017)

Against Gram-positive bacteria (*Staphylococcus aureus* ATCC 44330, *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 70080 and *Mycobacterium phlei* ATCC 11758), Gram-negative bacteria (*Escherichia coli* ATCC 35218 and *Pseudomonas aeruginosa* ATCC 27853).

Briefly, agar diffusion method was done by the following steps: each indicator microorganisms was incubated overnight in brain heart infusion broth (Oxoid, London, UK) at 37 °C, adjusted to 0.5 McFarland standard and streaked by sterilized cotton swab for three times over a 20 ml Muller Hinton agar plates (Oxoid, London, UK). Cups of 5 mm diameter were prepared by Wassermann tubes to be filled with 50 µl of isolated cell-free suspension. Ampicillin (10 mg ml^{-1}) was also tested as a positive control. The system was incubated at 37 °C for 24 h.

The double layer agar method depended on spot inoculation of pure bacterial colonies on a solid nutrient agar plates (Difco, Chicago, IL, USA) and incubated at 30 °C for 5 d. A 5 ml of semisolid Trypton Soya Agar (Difco, Chicago, IL, USA) was inoculated with 100 ml of overnight culture of the indicator microorganisms and were poured on spotted plates, and incubated at 37 °C for 24 h. Antibacterial activities were observed by measuring diameter of inhibition zones.

2.4. Production of crude extracts using submerged state fermentation

Submerged state fermentation method was performed to produce crude extracts from isolates which showed antibacterial activities. In a 500 mL Erlenmeyer flask, 100 mL of bacterial broth was inoculated with 1 mL of sub-cultured bacterial broth at 55°C and kept for 14 d in a shaker incubator at 200 rpm. After that, this 100 mL of bacterial culture was used to inoculate 1000 mL of sterilized bacterial broth and re-incubated in shaker incubator for 14 d. The cell free culture supernatant was prepared by centrifugation at 8000 rpm for 10 min.

The culture extract was obtained by adding equal volumes of ethyl acetate (1:1 v/v), and shacked twice vigorously for 1 h. By using a separating funnel, the ethyl acetate (EtOAc) extract was collected and evaporated at 60°C in a water bath by rotary vacuum evaporator at 100 r min^{-1} to remove solvent and to get crude extracts (Ilic et al. 2007).

2.5. Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) was determined by broth micro dilution method (Bakr et al. 2016, Nanasombat and Teckchuen 2009) against different strains of bacteria as *(Staphylococcus aureus ATCC44330, Enterococcus faecalis*, *Mycobacterium pheli* ATCC 11758, *Bacillus subtilis* ATCC 6633, and *Escherichia coli* ATCC 35218) using the following procedures: in flat-bottomed 96-well micro plates, 100 µL of Muller-Hinton broth were added into each well followed by addition of 100 µL aliquot of EtOAc extracts to reach the concentration of (500 $mg.mL^{-1}$). Then, a successive 2-fold serial dilution was adapted until well number 10. A 100 µL of inoculum suspension was used to inoculate all wells including well number 11 that represent a positive control. The bacterial inoculum was prepared by adjustment of

turbidity to 0.5 McFarland standard and was further diluted to obtain the final inoculum concentration of 10^6 CFU mL^{-1}.

After duplication of each experiment, the plates were incubated at 37°C for 48 h. The MIC was recorded as the lowest concentration of antimicrobial agent that completely inhibits growth.

2.6. Antimicrobial susceptibility testing (AST)

Bacterial isolates were emulsified in 0.45% saline to the equivalent of a 0.5 McFarland turbidity standard to form a suspension used for VITEK 2 AST.

The AST-P515 card used for Gram-positive bacteria containing benzylpenicillin, clindamycin, erythromycin, fusidic acid, gentamycin, kanamycin, lincomycin, minocycline, nitrofurantoin, norfloxacin, ofloxacin, oxacillin, pristinamycin, rifampicin, teicoplanin, tetracycline, tobramycin, trimethoprim/sulfamethoxazole, and vancomycin was filled with bacterial suspension, sealed and loaded into the VITEK 2 system. Readings were taken continuously every 15 min.

Susceptibility testing was performed following the National Committee for Clinical Laboratory Standards (CLSI) guidelines explicitly (Doern et al. 2001).

2.7. Characterization of isolated bacterial colonies

The isolated colonies of bacteria were characterized by the following methods:

2.7.1. Morphological and biochemical examination

Macroscopic methods were used by observation of isolated microorganisms for their color, shape, size, media pigmentation, consistency, aerial and substrate mycelia. Microscopic methods as Gram staining, aerial hyphae, spore surface, spore mass and cover slip culture method were observed.

Different biochemical tests were performed for characterization of isolated bacteria as: catalase, oxidase, citrate utilization, nitrate reduction, starch hydrolysis, urea

hydrolysis, Esculin hydrolysis, acid production from sugar and NaCl tolerance by using VITEK 2 system bioMérieux, Inc. Hazelwood, MO, USA.

2.7.2. DNA isolation, purification and PCR sequencing

The isolated bacteria were grown at 25°C for 5 d and genomic DNA was isolated as mentioned in (Birri et al. 2012, AbdEl-Mogheith et al. 2017, El-Gendy et al. 2013), and stored at 4°C. The 16S rRNA gene amplification was performed using the following primers:

StrepF: 5.-AAACTYAAAKGAATTGACCG-3

StrepR: 3-CCGTCAATTCMTTTRAGTTT -5

PCR was carried out in a 50 µL reactions volume containing 1000 ng of extracted genomic DNA, 10 mM dNTPs mixture, 1 µL of 10 pmol μL^{-1} (20 uM of each primer), 2.5 units of Phusion DNA polymerase enzyme (Finnzymes) and 10 µL 5X reaction buffer. PCR thermocycler initiated by denaturation step at 94°C (3 min), then 35 cycles of denaturation at 94°C (30 s), followed by annealing at 56°C (30 s), extension at 72°C (60 s), and finally completion of DNA synthesis at 72°C (5 min) and hold at 4°C.

The amplified DNA was purified using (DNA Clean & Concentrator TM -25, United States) according to manufacturer's instructions, then analyzed by agarose gel electrophoresis in 1.5% (w/v) with reference to 1 kbp DNA ladder (Fermentas, Helsinki, Finland) and stained with ethidium bromide. Also, DNA concentration was measured by NanoDrop-ND1000-Spectrophotometer (Thermo Scientific, Wilmington, USA). Finally, in order to assess DNA similarity degree, the BLAST program was employed. MEGA 7 software was used to evaluate multiple sequence alignment and molecular phylogeny.

2.8. LC/HRMS analysis and dereplication

Natural metabolites were analyzed by liquid chromatography/mass spectrometry using a Thermo Instruments MS system (LTQ XL/LTQ Orbitrap Discovery) coupled to a Thermo Instruments HPLC system (Accela PDA detector, Accela PDA auto sampler, and Accela pump) TUV Rheinland of North America Inc.).

The following conditions were applied:Capillary temperature 260°C, capillary voltage 45V, sheath gas flow rates 40–50 arbitrary units, auxiliary gas flow rate 10-20 arbitrary units, mass range 100-2000 amu (maximum resolution 30 000), spray voltage 4.5KV. 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.

3. Results

3.1. Bacterial isolation

Forty compost samples were collected from different places, pure colonies of bacteria were isolated using serial dilution method followed by inoculation on different types of media showing different morphological appearance.

3.2. Antibacterial screening and MIC determination

The two isolates encoded (ID- MG649382) and (ID-MG649383) showed antibacterial activity against *Staphylococcus aureus* ATCC4*4330, Enterococcus faecalis* ATCC 70080 and *Mycobacterium phlei* ATCC 11758 when growing under both static and shaking conditions as indicated in (Table 2).

MIC values were ranged from 15.6 - 62.5 µg mL^{-1} as shown in **(**Table 3).

3.3. Characterization of isolated bacterial colonies

3.3.1. Morphological and biochemical examination

The isolated bacteria were Gram positive filamentous bacteria with spiral spores, smooth indented surface of the spores. Aerial mycelia were also observed to be grown

on glycerol–asparagine agar medium (ISP-5) and inorganic salts starch agar medium (ISP-4). Obvious differential characters of bacterial colonies were observed during isolation on various types of media as shown in (Table 4). The isolates exhibited good growth on nutrient agar medium, glycerol–asparagine agar medium (ISP-5) and inorganic salts starch agar medium (ISP-4), moderate growth on trypton soya agar medium (TSA) and poor growth on starch nitrate agar medium. Aerial and substrate mycelium were varied from light gray to dark gray during cultivation on different media with different diffusible pigments.

Biochemical tests also were performed using VITEK 2 system and results were indicated in (Table S1).

3.2.3. DNA isolation, purification and PCR sequencing

16rRNA gene amplification, sequencing and homology search using BLAST program revealed that the sample encoded (ID- MG649382) has a 100% similarity with *Bacillus coagulans* while that encoded (ID- MG649383) has a 98% similarity with *Bacillus smithii* as showed in (Table 5).

The resulted sequences were aligned to the closest species and assembled in MEGA 7 software for phylogenetic analysis using the Neighbor-Joining method and the evolutionary distances were computed using the Kimura 2- parameter method as shown in (Figure 1).

The resulted nucleotide sequences were deposited in Genbank under accession numbers MG649382 and MG649383, respectively.

3.4. Antimicrobial susceptibility testing

MICs of antimicrobial agents utilized by VITEK 2 system showed that both isolates were resistant to benzyl penicillin, oxacillin, tetracycline and sensitive to gentamycin, ciprofloxacin, levofloxacin, moxifloxacin, trimethoprim/sulfamethoxazole.

For the isolate ID- MG649382, it was resistant to erythromycin, clindamycin, and intermediate to quinupristin/dalfopristin, vancomycin, nitrofurantoin and rifampicin while the isolate ID- MG649 383 was sensitive to erythromycin, clindamycin, vancomycin, nitrofurantoin and rifampicin as shown in (Table S2).

3.5. LC-HRMS analysis and dereplication

LC-HRMS screening revealed the presence of few and diverse compounds belonging to alkaloids, polyketides, and steroids as referred in (Table 6) and (Figure S1).

These compounds were determined and compared to previous isolated compounds using different libraries databases.

4. Discussion

Owing to their important role in the treatment of infectious disorders, the second main cause of death, natural products grasped a great attention over the course of several decades especially from microorganisms as it much more renewable, readily and reproducible source than plants or animals. (Lam 2007)

Recycling and bio-degradation become important issues for research as vital branch of (Green Biotechnology) during recent centuries especially composting process which include bio-transformation of solid wastes to more bio-degradable materials by thermophilic compost populations of bacteria and fungi (Tuomela et al. 2000).

In this study, we have isolated forty samples from aromatic plants compost through thermophilic stage. It was observed that two samples showed antimicrobial activities against Gram positive bacteria (*Staphylococcus aureus* ATCC44330, *Enterococcus faecalis* ATCC 70080, and *Mycobacterium pheli* (Desai and Dhala 1967).

Phenotypic characterization of the isolated microorganisms was carried out by morphological examination and biochemical testing to observe that isolated bacteria which significantly resemble *Actinomycetes* on spiral spores, smooth indented surface

of the spores, filamentous growth, chalky appearance, an earthy soil smell, and strong adherence to medium on ISP4 media. Although biochemical tests were performed by VITEK 2 system bioMérieux, both isolates could not be completely identified. However, genetic characterization showed that isolates were similar to *Bacillus coagulants* and *Bacillus smithii*.

Obvious variations in results of isolates characterization supposed that major micro-organism (*Bacillus*) mask the presence of minor micro-organism (*Actinomycetes)* or one sequence could be favored over another during PCR (Partanen et al. 2010). So, further research for advanced characterization methods might be needed.

From antimicrobial sensitivity test, it was observed that both isolates were sensitive to gentamicin, ciprofloxacin, levofloxacin, moxifloxacin and trimethoprim/sulfamethoxazole, while resistant to benzyl penicillin, oxacillin and tetracycline.

In fact, natural products became is currently regarded as the future solution for antimicrobial resistance which is the most dangerous healthcare problem in the 21st century. (Alanis 2005) Phytochemical screening for antimicrobial metabolites was performed by LC–HRMS which is considered one of the most reliable techniques since the last decade of 20th century. (Niessen 1999) LC-HRMS analysis of the EtOAc extracts of these isolates indicated the presence of bioactive compounds which could be responsible for such antimicrobial activates, such as cyclo (L-Leu-L-Pro), cyclo (L-4-OH-Leu-L-Pro), 1,6-dimethoxyphenazine, oncorhynoclide, and other compounds that showed no hits which could be of great interest to be followed as they were not isolated from nature before. Further investigation of theses fractions on large-scale fermentation, fractionation, isolation, and structure elucidation are needed.

These identified compounds were previously mentioned in researches which indicated antibacterial activities of diketopiprazines (Martins and Carvalho 2007), oncorhyncolide (Needham et al. 1991), di-(2-ethylhexyl) phthalate (Habib and Karim 2009) and 24-Hydroxy-12-oleanene-3, 22-dione (Shai et al. 2008).

High temperature during fermentation process could justify the limited number of bioactive compounds produced.

Antibiotic resistance has become a critical worldwide problem with significant increase during the last years (Levy 2002). Therefore the need to discover unique and new antibiotics is considered to be an urgent purpose for scientific researches. Mainly, antibiotics from natural source which gained great interest nowadays especially after approval of their roles for treating human diseases (Chin et al. 2006).

Overall, our study approved that compost, as a new environmental source, has antibacterial activity against Gram positive bacteria which is the first time to be reported in Egypt.

5. Author contributions

Microbiological and molecular biology experiments were performed by AE-G and EE-G. HPLC/HRMS and data manipulation and characterization of chemical structures were elucidated and by HH and MR.

Finally manuscript was drafted and revised for the final approval by all authors.

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7 Footnotes

1-(www.ncbi.nlm.nih.gov/blst).

8 Conflict of interest

No conflict of interest declared.

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Figure captions

Figure 1 Phylogenetic tree of MG649382 and MG649383 isolates based on partial 16S rRNA gene sequences. The phylogenetic tree was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The distances were computed using the Kimura 2-parameter method (Kimura 1980) and are in the units of the number of base substitutions per site. Numbers at nodes indicate percentages of 1000 bootstrap re-samplings, only values above 50% are shown. The analysis involved 25 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 (Kumar et al. 2016).

Tables:

Table 1 Comparison between two different types of compost from different sources at the same conditions; (Source; Soil, Water and Environment Research Institute "SWERI", Egypt).

Nile compost (green compost)	Type of analysis	Al Obour compost (animal manure/green compost)
25	Moisture Content	20
7.24	pH	8.32
5.95	Electrical Conductivity (EC)	4.85
1.41	Total Nitrogen (N)	1.09
131	Ammonium Nitrogen (NH_4-N)	177
56	Nitrate Nitrogen (NO_3-N)	56
41	Organic Matter	27.26
21.08	Ash	72.74
1:14.95	C: N ratio	1:14.50
1.77	Phosphorus (P)	2.25
0.65	Potassium (K)	0.28

Table 2 Antimicrobial activities represented in zones of inhibition (mm) in both shaking and static conditions.

Inhibition zones (mm)				
Isolate	Condition	*Staphylococcus .aures* ATCC44330	*Enterococcus faecalis*	*Mycobacterium pheli*
ID- MG649382	Shaking	18	30	13
ID- MG649383	Shaking	26	34	26
ID- MG649382	Static	16	26	28
ID- MG649383	Static	18	26	11

Table 3 MIC of crude extracts represented in concentrations µg ml^{-1}

MIC (µg ml^{-1})			
Isolate	*Staphylococcus aureus* ATCC44330	*Enterococcus faecalis*	*Mycobacterium pheli*
ID- MG649382	62.5	15.6	15.6
ID- MG649383	125	15.6	31.25

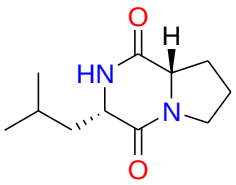
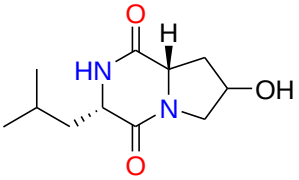
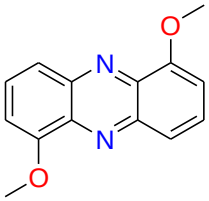
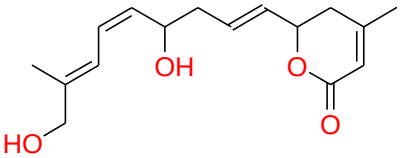
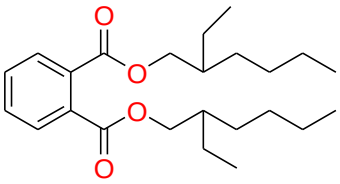
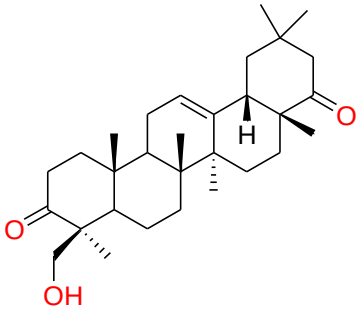
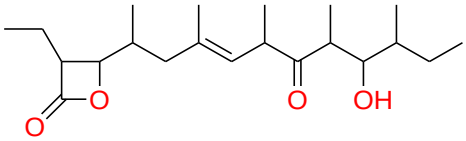
Table 4 Morphological characters of isolated bacteria on different media.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigments
1-Trypton Soya Agar	Moderate	Light gray	Light yellowish brown	moderate yellowish brown
2- Nutrient agar	Good	Dark gray	moderate yellowish brown	moderate yellowish white
3- Starch nitrate agar	Poor	Light gray	light brown	moderate yellowish brown
4-Glycerol–asparagine agar (ISP-5)	Good	Dark gray	light yellowish brown	yellowish green
5-Inorganic salts starch agar (ISP-4)	Good	Dark gray	moderate yellowish brown	yellowish green

Table 5 Genetic identification results of active isolates.

Accession number	Obtained Sequence size	NCBI BLAST result	Max score	Total score	Query cover	E value	Max Identification
MG649382	653 bp	*Bacillus coagulans* 2-6 strain 2-6 16S ribosomal RNA, complete sequence	1206	1206	100%	0.0	100%
MG649383	985 bp	*Bacillus smithii* strain NRS-173 16S ribosomal RNA gene, partial sequence	1533	1533	90%	0.0	98%

Table 6 Liquid chromatography/mass spectrometry data of *Bacillus* fractions with the suspected formula and suggested identified compounds.

No.	Rt	Mwt	Cf	Structure	Identification
1	4.85	211.14548	$C_{11}H_{18}O_2N_2$		Cyclo (L-Leu, L-Pro)
2	4.85	227.14044	$C_{11}H_{18}O_3N_2$		Cyclo (L-4-OH-Leu, L-Pro)
3	4.88	241.09877	$C_{14}H_{12}N_2O_2$		1,6-Dimethoxyphenazine
4	4.82	279.16101	$C_{16}H_{22}O_4$		oncorhyncolide
5	10.76	609.28333	$C_{27}H_{40}O_{10}N_6$		No hits
6	11.45	391.28607	$C_{24}H_{38}O_4$		di-(2-ethylhexyl) phthalate
7	13.00	455.35410	$C_{30}H_{46}O_3$		24-Hydroxy-12-oleanene-3,22-dione
8	15.58	353.27078	$C_{21}H_{36}O_4$		Ebelactone B

Abbreviations; Mwt, molecular weight; Rt, retention time, Cf compound formula

Supporting materials legends:

Fig S1 LC-MS charts of bioactive metabolites derived from ethyl acetate extracts of isolates

Table S1 Biochemical identification of isolates by VITEK 2

Table S2 Antimicrobial susceptibility results of isolates ID- MG649382 and ID-MG649383 against different antimicrobial agents.