OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2018.

Research Article
Bactericidal Activity, Isolation and Identification of Most Active Compound from 20 Plants used in Traditional Mexican Medicine Against Multidrug-Resistant Bacteria

[1]C. Rivas-Morales, [2]V.M. Rivas-Galindo, [3]J. Rodríguez-Rodríguez, [1]S.A. Galindo-Rodríguez, [1]C. Leos-Rivas and [1]D.G. García-Hernández

[1]Universidad Autónoma de Nuevo León, Facultad de Ciencias Biológicas, Laboratorio de Química Analítica, México
[2]Universidad Autónoma de Nuevo León, Facultad de Medicina, Departamento de Química Analítica, México
[3]Tecnologico de Monterrey, Escuela de Medicina y Ciencias de la Salud

Abstract

Background and Objective: Plants are used in Mexico as traditional medicine for the treatment of diverse illnesses such as stomach pain, fever, diarrhea, insomnia, flu and other respiratory diseases. Twenty were selected to determine their bactericidal activity. The aim of this study was the isolation of molecules from plants used in Mexican traditional medicine. **Materials and Methods:** Using chromatographic procedures, the responsible bactericidal molecules from rosemary was extracted and then identified by spectroscopic analysis IR, ^{1}H NMR, ^{13}C NMR, DEPT, HSQC and GC-MS. Measures of central tendency were determined by statistical analysis. **Results:** Ten of these plants showed bactericidal activity against multidrug-resistant bacteria. This biological activity was reported for *Carya illinoensis* against *Pseudomonas aeruginosa*, also for *Equisetum robustum*, *Stevia rebaudiana* and *Castela texana* against Methicillin resistant *Staphylococcus aureus* (MRSA). The methanolic extract of *Rosmarinus officinalis* (rosemary) showed important bactericidal activity against MRSA (ATCC BAA-44) and clinically isolated MRSA. **Conclusion:** Rosemary's bactericidal molecules were isolated and then identified as a mixture of betulinic, oleanolic and ursolic acid (MIC = 725 μg mL^{-1}).

Key words: *Rosmarinus officinalis*, multidrug-resistance, MRSA, thin layer chromatography, flash chromatography, ^{1}H NMR, ^{13}C NMR, triterpenic acids

Received: Accepted: Published:

Citation: C. Rivas-Morales, V.M. Rivas-Galindo, J. Rodríguez-Rodríguez, S.A. Galindo-Rodríguez, C. Leos-Rivas and D.G. García-Hernández, 2018. Bactericidal activity, isolation and identification of most active compound from 20 plants used in traditional Mexican medicine against multidrug-resistant bacteria. Int. J. Pharmacol., CC: CC-CC.

Corresponding Author: David Gilberto García Hernández, Laboratorio de Química Analítica Unidad B. Av. Pedro de Alba s/n, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, 64455 San Nicolás de los Garza, Nuevo León, México Tel: (+52) 8115301578

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

An important prerequisite for the success of primary health care is the availability and use of suitable drugs. Plants have always been a common source of drugs, either in their traditional preparations or as pure active principles[1].

Current research in drug discovery from medicinal plants involves a multifaceted approach that combines botanical, phytochemical, biological and molecular techniques. Medicinal plant drug discovery continues providing new and important leads to various pharmacological targets. Although, research of medicinal plants is an important source of new drug leads, numerous challenges are encountered including the procurement of plant materials, the selection and implementation of appropriate high-throughput screening bioassays and the scale-up of active compounds[2].

Antibiotic resistance is the organism's acquired capacity to resist the effects of a chemotherapeutic agent to which it is usually sensitive[3]. This is due to bacterial chromosome mutations, plasmids or transposons which can transfer some resistance to many microorganism species faster than the new drugs developed for treatment[4-6].

Antibiotic resistance is an important public health problem in the world mainly in underdeveloped countries where their inadequate use has caused the development of multidrug-resistant bacteria. Resistant strains are highly disseminated due to the inefficient infrastructure of the public health system and incorrect control of infection treatments. The group of antibiotics most often used is β-lactams, which includes penicillin, cephalosporins, cephamycins, carbapenems and monobactamics. These antibiotics have low toxicity levels and a broad sprectrum[7,8].

The high incidence of infectious disease due to Enterobacteriaceae as well as the rise of multidrug-resistant strains are the main elements for current and future medical problems. Beta-lactam antibiotics have a structure similar to the binding sites of bacterial substratum, this similarity can deactivate some proteins required for cell wall peptidoglycan synthesis[9].

The spread of drug resistant pathogens is one of the most serious threats to successful treatment of microbial diseases. The plants extracts have evoked interest as sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases[10-13].

The aim of this study is the isolation of bioactive molecules from plants and their application in multidrug-resistant bacteria.

MATERIALS AND METHODS

This study was carried in Laboratorio de Química Analítica, Facultad de Ciencias Biológicas, UANL, México, with the collaboration of Tecnologico de Monterrey, México and Departamento de Química Analítica, Facultad de Medicina, UANL, México (January, 2015-December, 2016).

Plant material: Twenty plants used in traditional Mexican medicine (Table 1) were obtained commercially by PACALLI®.

Test organisms: Twelve strains were used (Table 2), 6 multi drug resistant bacteria (ATCC) and another six clinically isolated bacteria (CI) obtained from the Department of Microbiology of the Hospital Universitario Dr. José Eleuterio Gonzalez, Universidad Autónoma de Nuevo León, México.

Extraction: Dry and ground plant material (30 g) was extracted in a Soxhlet with 500 mL methanol (CTR Scientific, Monterrey, Mexico) to exhaust the extraction. Then, the solution was evaporated to dryness under reduced pressure using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) at 40°C. Later, its extraction percentage was calculated and the material was stored at room temperature in amber vials until its use[14].

Antibacterial assay: Twenty methanol extracts were screened for antibacterial activity using the disk diffusion method, which is normally used as a preliminary test to select the most efficient extracts[12]. These were performed using the M26-A protocol[15] with different culture media as a modification[16] An 18 h culture at 37°C in 3 mL of C. Rivas broth was performed. The cultures were adjusted to approximately 10^6 CFU mL^{-1} by the McFarland turbidity method. One hundred microliters of these suspensions were spread over the plates containing C. Rivas agar (Patent number 9810892) using a sterile Driglasky spreader in order to achieve uniform microbial growth.

The methanol extracts were dissolved in methanol (CTR Scientific) at a final concentration of 50 mg mL^{-1}. Under aseptic conditions, empty sterilized discs (Whatman No. 5, 6 mm diameter) were impregnated with 20 µL of the extracts and placed on the agar surface. The paper disc was moistened with methanol and placed on the seeded Petri dish as a negative vehicle control. Petri dishes were incubated at 37°C for 24 h. After incubation, the inhibition zone was measured with a rule.

MIC assay: The minimum inhibitory concentration (MIC) was determined based on the previous screening. Only the

Table 1: Traditional medicine plants selected for this study

Scientific names	Common names	Scientific names	Common names
Apium graveolens L.	Celery	*Carya illinoensis*	Pecan nut
Castela texana	Goat-bush	*Passiflora incarnata*	Passion flowers
Equisetum robustum A. Br	Horsetail	*Arctostaphylos pungens*	Manzanita
Amphipterygium adstringens	Cuachalalate	*Rosmarinus officinalis*	Rosemary
Eucalyptus globulus	Eucalyptus	*Tilia platyphyllos*	Lind
Larrea tridentata	Creosote-bush	*Valeriana ceratophylla*	Valerian
Verbascum thapsus	Mullein	*Cymbopogon citratus*	Lemon grass
Stevia rebaudiana	Stevia	*Casimiroa edulis*	White sapote
Foeniculum vulgare	Fennel	*Phalaris canariensis*	Canary seed
Matricaria recutita	Chamomile	*Azadirachta indica*	Neem tree

Table 2: Clinically important bacteria in this study

Gram-negative	Strains	Gram-positive	Strains
Escherichia coli	ATCC 25922	*Staphylococcus aureus MRSA*	ATCC BAA-44
E. coli	CI	*S. aureus*	CI
Pseudomonas aeruginosa	ATCC 27853	*Enterococcus faecalis*	ATCC 29212
P. aeruginosa	CI	*E. faecalis*	CI
Acinetobacter baumannii	ATCC 15308		
A. baumannii	CI		
Klebsiella pneumoniae	ATCC 700603		
K. pneumoniae	CI		

CI: Clinical isolate

most active extract was tested according to the M07-10 document in a 96 well microplate[17]. These were tested from 2900-45.31 μg mL^{-1} concentrations. Inhibition of bacterial growth in a well containing the test extract was judged by comparison with the growth control well. The MICs were determined as the lowest concentration of extract inhibiting visible growth of each organism in the well.

Thin layer chromatography (TLC): The extract with the highest bactericidal activity was analyzed by TLC. The TLC plate (TLC silica gel 60, Fluka, 25×75 mm, without UV indicator) was developed in benzene/acetone (9:1), was sprayed with cobalt chloride/sulfuric acid (2, 19% v/v) and visualized by heating to calculate the retention factor (Rf). Ursolic acid standard used as a control reference due to its presence in the plant.

Bioautography test: The contact bioautography method was used for assaying antibacterial activity[18]. TLC plates were developed as mentioned previously. In order to sterilize the plates, they were allowed to dry inside the Petri dish in a laminar flow hood with UV light for 30 min. Then, C. Rivas agar was cut with a sterilized spatula and deposited on the TLC plate. Thereafter, 100 μL of a standardized bacterial solution was spread over the agar layer using a sterile Driglasky spreader in order to achieve uniform microbial growth. Plates were incubated at 37°C for 24 h and then the inhibitions zones on the agar layer were observed, which indicated the presence of antimicrobial compounds.

Table 3: Column chromatography separation from methanolic rosemary extract

Fractions	Proportion	mg
1	Hx	180.3
2	Hx:Ac 9:1	198.4
3	Hx:Ac 8:2	633.1
4	Hx:Ac 7:3	598.0
5	Hx:Ac 6:4	360.1
6	Hx:Ac 1:1	396.6
7	Ac	483.7
8	Ac:MeOH 1:1	291.0
9	MeOH	370.5

100 mL per fraction was recovered. The material was measured in miligrams

Flash chromatography: Flash chromatography was performed using an Isolera One 2.0.8 device (Biotage, Charlotte, NC, USA). The sample (173.2 mg) was charged in a SNAP Ultra 10 g cartridge and eluted automatically via Isolera Spektra (Biotage) software. Hexane and ethyl acetate were performed as eluents. The fractions obtained were monitored with lambda-all UV detection mode (254, 280 nm).

Silica gel chromatography: Silica gel 60G (Sigma Aldrich, 63-200 mesh) was packed into an open column (28 mm diameter and 330 mm length). The sample (3.5 g) was also adsorbed in silica. Then an eluent gradient system, which consisted of hexane/acetone/methanol was prepared (Table 3). One hundred milliliters of each system was collected as a fraction, then this was dried under reduced pressure using a rotary evaporator (Buchi Rotavapor, Buchi Labortechnik, Essen, Germany) at 40°C for antimicrobial activity tests.

Table 4: Antibacterial activity of extracts and halo inhibition growth (mm)

Plant extracts	*S. aureus* MRSA ATTC BAA-44	*S. aureus* MRSA CI	*P. aeruginosa* ATCC 27835	*A. baumannii* CI
Rosemary	14 ± 0.115	10 ± 0.058	–	–
Horsetail	12 ± 0.058	06 ± 0.115	–	09 ± 0.05
Eucalyptus	12 ± 0.058	–	–	–
Goat-bush	11 ± 0.100	06 ± 0.115	–	–
Neem tree	10 ± 0.100	–	–	–
Stevia	08 ± 0.115	–	–	–
Creosote-bush	–	07 ± 0.058	–	–
Pecan nut	–	–	10 ± 0.012	–
Lemon grass	–	–	–	15 ± 0.023
Cuachalalate	–	–	–	09 ± 0.115

CI: Clinical isolate, ±Standard Deviation, n = 3, Not applicable, Halo was measured in mm, Negative data is not shown

Infrared spectroscopy: The infrared spectroscopic assay (IR) was performed in FT-IR Frontier spectrometer (Perkin Elmer, Tempe, AZ, USA). A 10 mg sample was dissolved in acetone and put into a universal attenuated total reflectance device (UATR). Measurements were made in triplicate taking 64 scans in the range of 400-4000 cm^{-1} and analyzed with Spectrum V10.4.00.0190 software (Perkin Elmer).

Nuclear magnetic resonance (NMR) spectroscopy: For NMR analysis, 10 mg of the isolated compound was dissolved in acetone-d6 with 0.3% of TMS as zero reference. The NMR spectra were recorded at 25°C using a Bruker Avance III HD 400 spectrometer (Bruker Corp., Billerica, MA) equipped with gradients and a 5 mm multinuclear probe at a base frequency of 100 MHz for ^{13}C and 400 MHz for ^{1}H. The spectrogram was analyzed via Topspin 3.0 software (Bruker Corp.).

Gas chromatography-mass spectrometry (GC-MS): Coupled gas chromatography-mass spectrometry (GC-MS) analysis was performed with an Agilent GC 6890 coupled to an MSD 5973N (Agilent Technologies Inc., Santa Clara, CA, USA), under the following instrumental conditions: HP-5 capillary column (30 m×0.25 mm×0.25 μm) with oven temperature programmed at 70°C for 2 min, then increased to 200 at 10°C min^{-1}, after that 200-320 at 10°C min^{-1}. Total run time: 37 min, injector temperature: 270°C, injector type: Split 1:20, detector temperature: 250°C, carrier gas: Helium (1 mL min^{-1}). MS conditions: Acquisition mode: Scan (m/z 30-650).

Statistical analysis: Measures of central tendency were performed in triplicate and mean value was calculated using Social Sciences (SPSS) software (version 17.0 for Windows, SPSS Inc., Chicago IL) the results were expressed as mean±SD (Table 4).

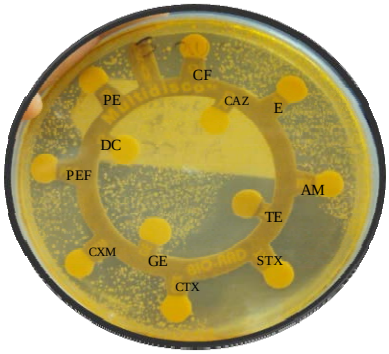


Fig. 1: Antimicrobial susceptibility test against *S. aureus* Rosenbach ATCC BAA-44 [S: Sensitive, R: Resistant], ampicillin (AM) = R, cephalothin (CF) = R, cefotaxime (CTX) = R, ceftazidime (CAZ) = R, cefuroxime (CXM) = R, dicloxacillin (DC) = R, erythromycin (E) = R, gentamicin (GE) = R, pefloxacin (PEF) = R, penicillin (PE) = R, trimethoprim+ sulfamethoxazole (SXT) = S, tetracycline (TE) = S

RESULTS

The extraction percentage of chamomile produced the highest percentage (58.82% p/p), while canary seed the lowest (3.42% p/p).

At first, the antimicrobial susceptibility to MRSA BAA-44 was tested, only trimethoprim/sulfamethoxazole and tetracycline were effective against this microorganism. Then bactericidal activity was performed (Fig. 1) and rosemary extract presented the greatest activity against both strains (ATCC and CI) so, the decision was to continue this study only with this extract. The activities of the other extracts are shown in Table 4.

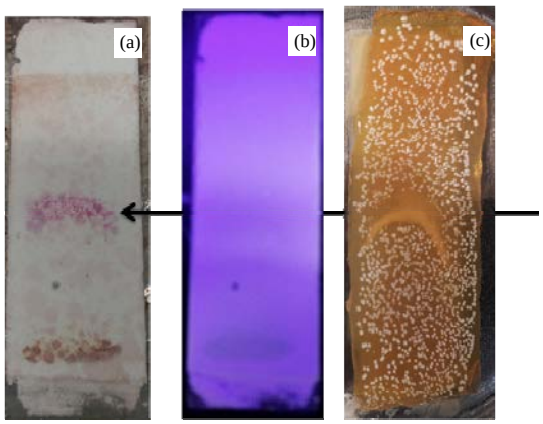


Fig. 2(a-c): (a) TLC rosemary extract revealed with $CoCl_2$, (b) TLC rosemary extract revealed with UV light, (c) Bioautography assay against *S. aureus* Rosenbach ATCC BAA-44. The black arrow shows the Rf 0.42 which developed the inhibition area in build c

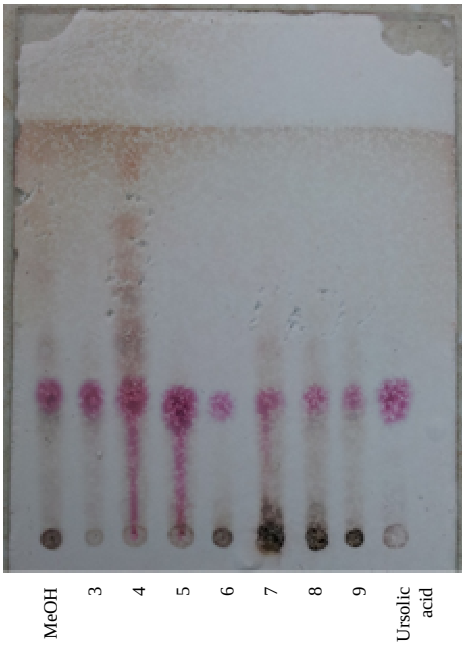
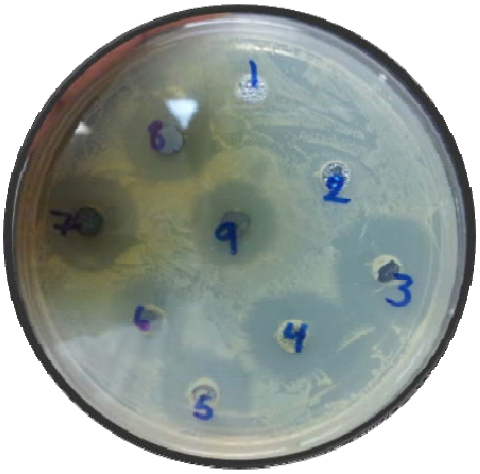


Fig. 3: Thin layer chromatography from fractions obtained from rosemary methanolic extract by column chromatography. Lane 1: Complete methanolic extract (MeOH), lanes 2-8: Obtained fractions and lane 9: Ursolic acid as a reference control. Rf = near 0.42

In order to separate rosemary extract, we decided to perform a TLC in which one had an Rf = 0.42, so we decided to use this system for performing the bioautography assay. Fig. 2 shows the results from the eluate system. The inhibition growth area was located on the Rf point.



Fraction	Inhibition growth halo (mm±SD)
1	0.0±NA
2	0.0±NA
3	19.7±1.53
4	16.5±1.00
5	11.0±2.08
6	08.0±0.58
7	13.0±2.52
8	13.5±0.58
9	14.5±0.58

Fig. 4: Antimicrobial test from fractions 3-9 from rosemary methanolic extract against *S. aureus* Rosenbach ATCC BAA-44 in C. Rivas agar, n = 3, NA: Not applicable, SD: Standard deviation

Column chromatography produced 9 fractions described in Table 3. The highest percent recovery was corresponded to fraction 3, while the lowest corresponded to fraction 1. These fractions were performed again in TLC and the results are shown in Fig. 3. Fractions 3-9 were displaced near the same Rf.

Fractions 3-9 were tested for antibacterial properties. Fig. 4 shows the results. Fraction 3 was more active than the others. Once the TLC and antibacterial results were obtained, we decided to join some fractions according to these results. It was concluded joining fractions 4, 5 and 7-9. A sample of fractions 4 and 5 (630 mg) were dissolved in acetone and then cooled at -20°C for 72 h. The precipitate (white powder, 300 mg) was recovered by filtration.

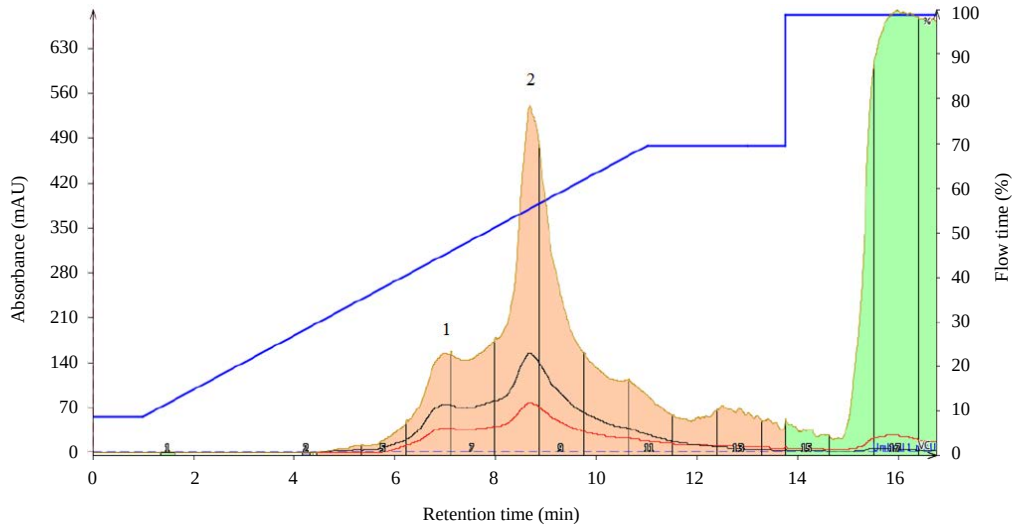


Fig. 5: Isolera one (Biotage) report. Mode: Lambda all. Two peaks were observed named isolated 1 and isolated 2 (1 and 2, respectively) through the flow time from automated flash chromatography

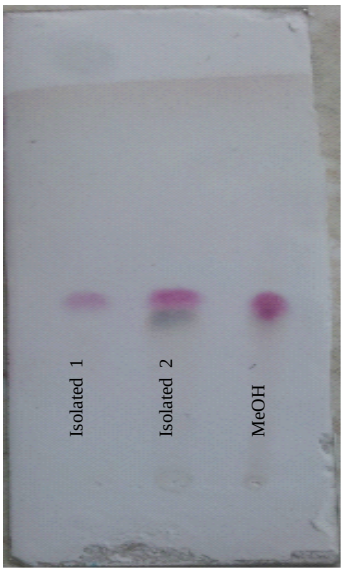


Fig. 6: Thin layer chromatography collected fractions from rosemary methanolic extract by flash chromatography revealed with $CoCl_2$. Lane 1: Isolated 1, Lane 2: Isolated 2, Lane 3: Methanolic extract as a control (MeOH)

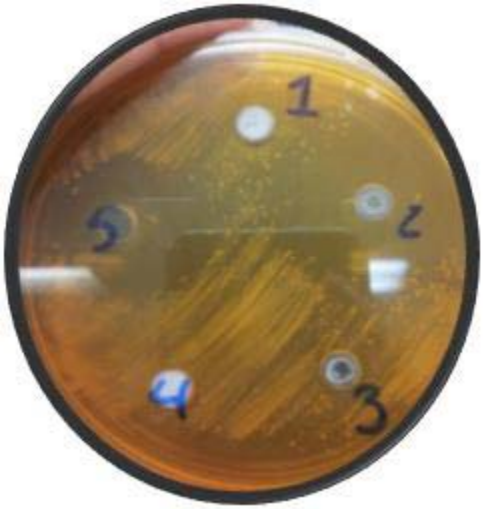


Fig. 7: Antibacterial test from partitions to isolated 2 chloroform and methanol soluble partitions from fractions 4 and 5 chloroform, acetone and methanol soluble (1) Chloroform soluble compound 2, (2) Methanol soluble compound 2, (3) Methanol soluble fractions 4 and 5, (4) chloroform soluble fractions 4 and 5, (5) acetone soluble fractions 4 and 5

From this precipitate, a sample (173.2 mg) was evaluated by flash chromatography. The Isolera Spektra software determines the flow for eluents. Figure 5 presents the report from the device. It was observed that two peaks in all detection modes that were programmed, materials collected before and after were discarded. Table 5 also shows recovered material. These peaks were named "isolated 1" and "isolated 2", respectively. Once these fractions were recovered, they were tested with TLC and the antibacterial test was applied as shown in Fig. 6 and 7. The TLC from compound 2 presents a dark point just under the reference Rf. This is the reason to

partition this compound into a soluble fraction in chloroform and methanol and test these partitions for antibacterial activity. The result showed that the methanol soluble fraction was responsible for its antibacterial activity. Likewise, the union of fractions 4-5 of rosemary extract was partitioned in chloroform, acetone and methanol and the acetone soluble partition had activity.

Table 5: Collected material for automated flash chromatography from the joined fractions 4 and 5 from rosemary methanolic extract

Fractions	mg	Recovery (%)
Impurity 1	0.9	0.52
Isolated 1	58.1	33.54
Isolated 2	85.1	49.13
Impurity 2	19.6	11.32

Flash chromatography was performed on a ISOLERA ONE (Biotage) device

The MIC for isolated 2 against *S. aureus* Rosenbach ATTC BAA-44 was 725 μg mL^{-1}.

The obtained fraction (isolated 2) had the following characteristics: IR are show in Fig. 8. ^{1}H NMR (acetone-d6), ^{13}C NMR are show in Fig. 9 and 10, respectively. Two-dimensional HSQC test it's showed in Fig. 11. The GC-MS (Retention time = 32.67 min) are show in Fig. 12. According to interpretation and literature results, it was concluded that a mixture of betulinic acid, ursolic acid and oleanolic acid was isolated.

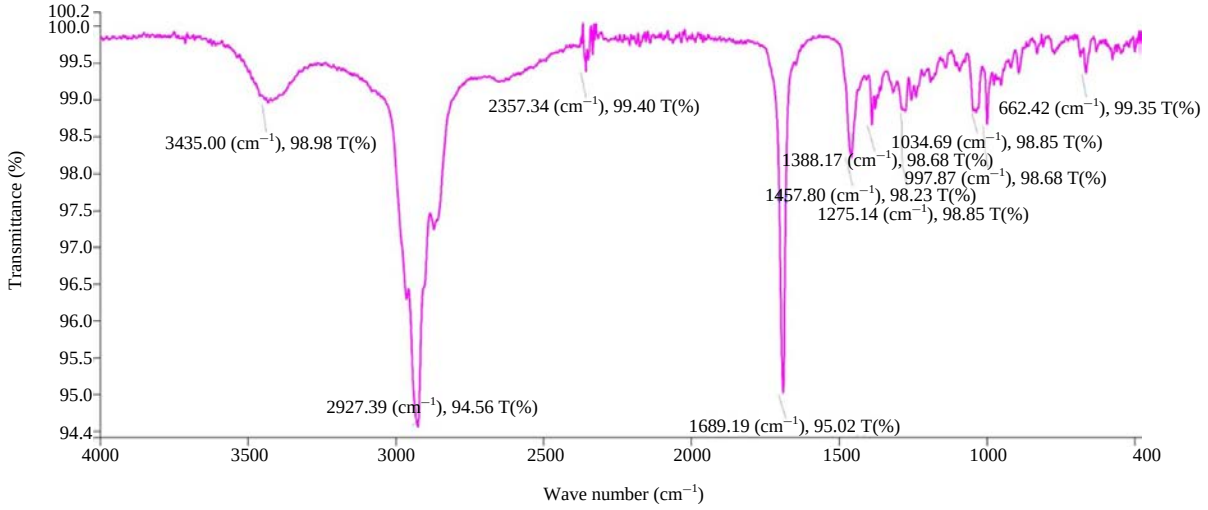


Fig. 8: IR spectrogram from methanol soluble partition to isolated 2 from rosemary methanolic extract

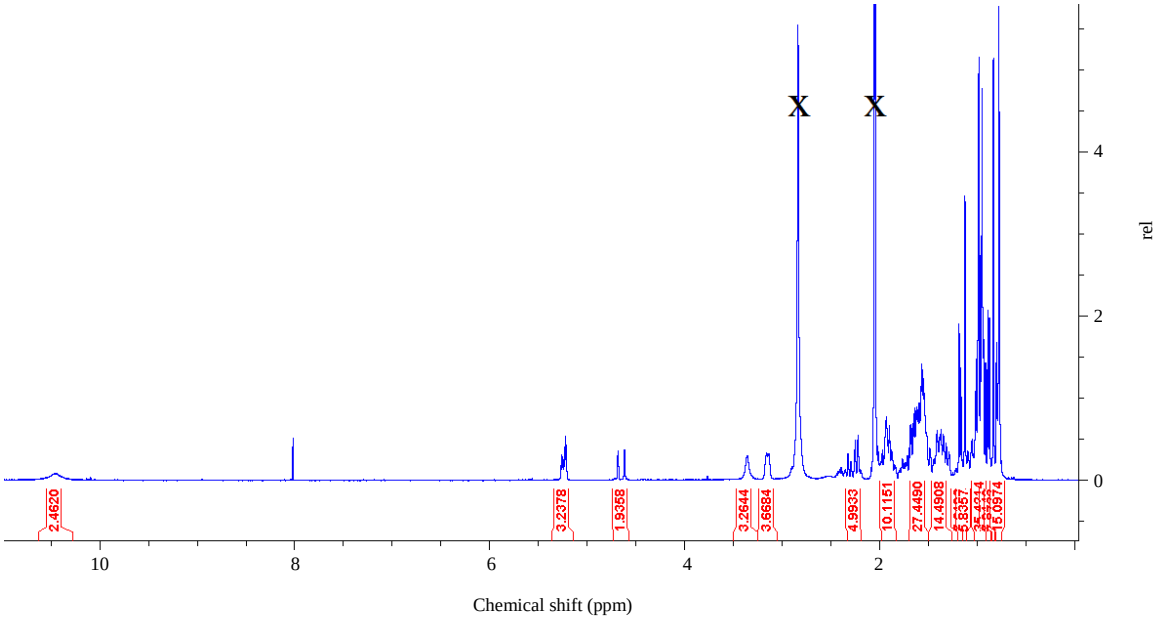


Fig. 9: ^{1}H NMR from isolated 2 from rosemary methanolic extract. X represents the dissolvent signals. Acetone d-6 was used as a dissolvent

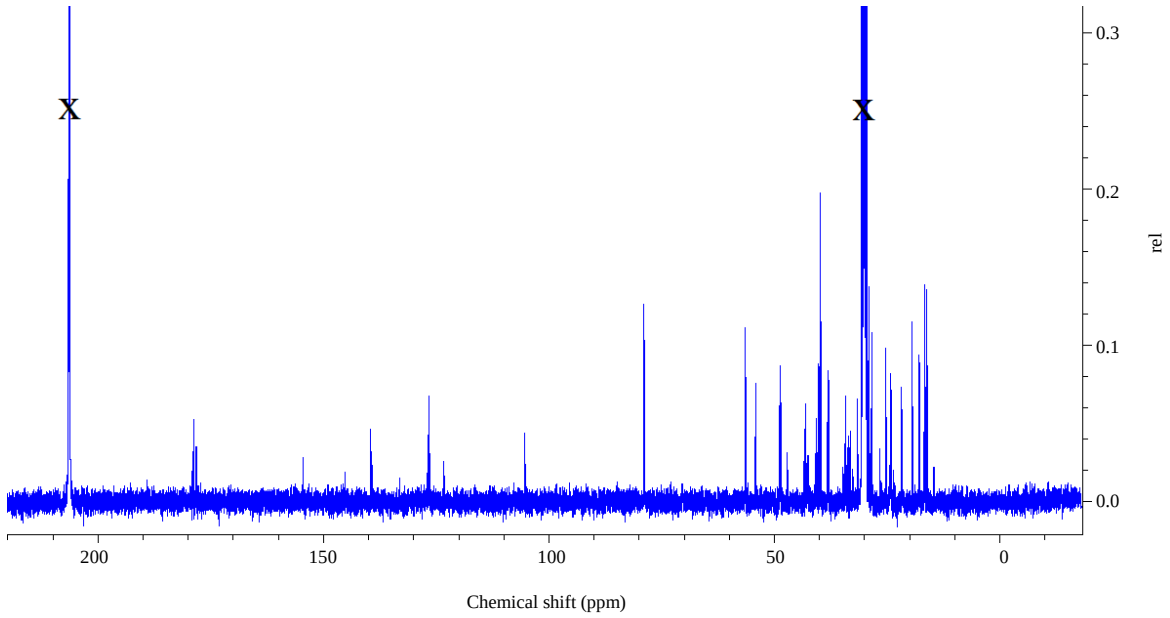


Fig. 10: ^{13}C NMR from isolated 2 from rosemary methanolic extract. X represents the dissolvent signals. Acetone d-6 was used as a dissolvent

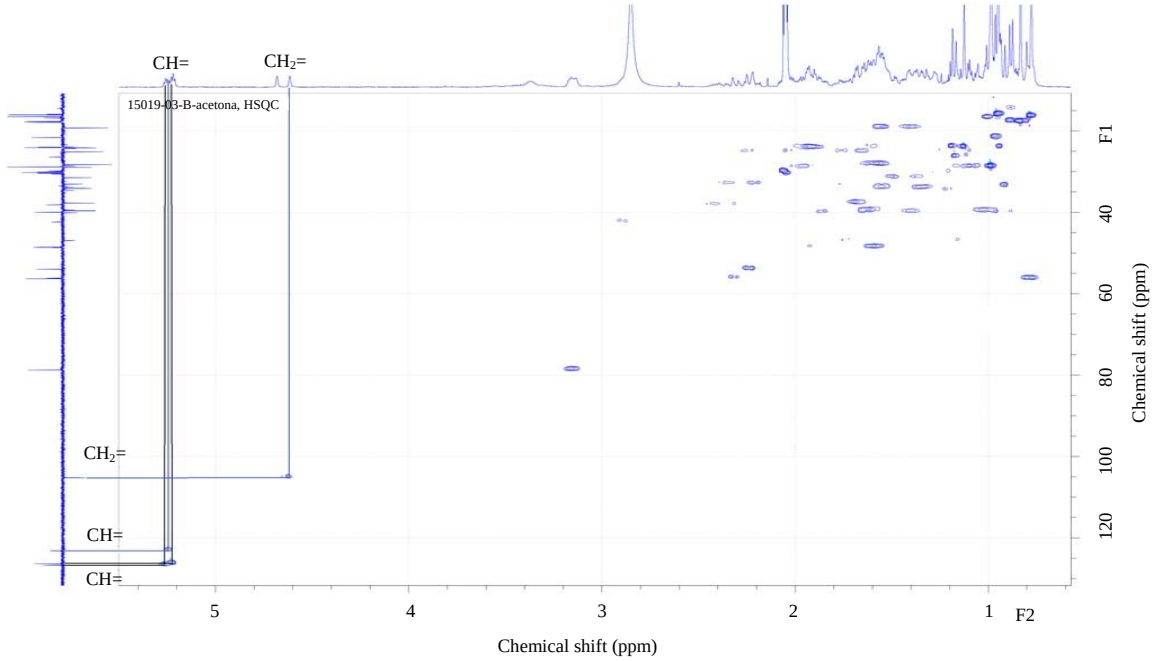


Fig. 11: HSQC test. The double bond signals from the three different carbons and five hydrogens are shown in their coupling signals by red lines. Sample of isolated 2 from rosemary methanolic extract

DISCUSSION

The aim of this study was the isolation of molecules from plants used in Mexican traditional medicine. The search for molecules in plants as new antimicrobial agents, today brings the traditions of indigenous peoples and their knowledge to the treatment of diseases with the most inexpensive medicine accessible to all. In this study, 20 plants were selected that are used around the world as spices, flavors and as principal ingredients to make infusions. Early

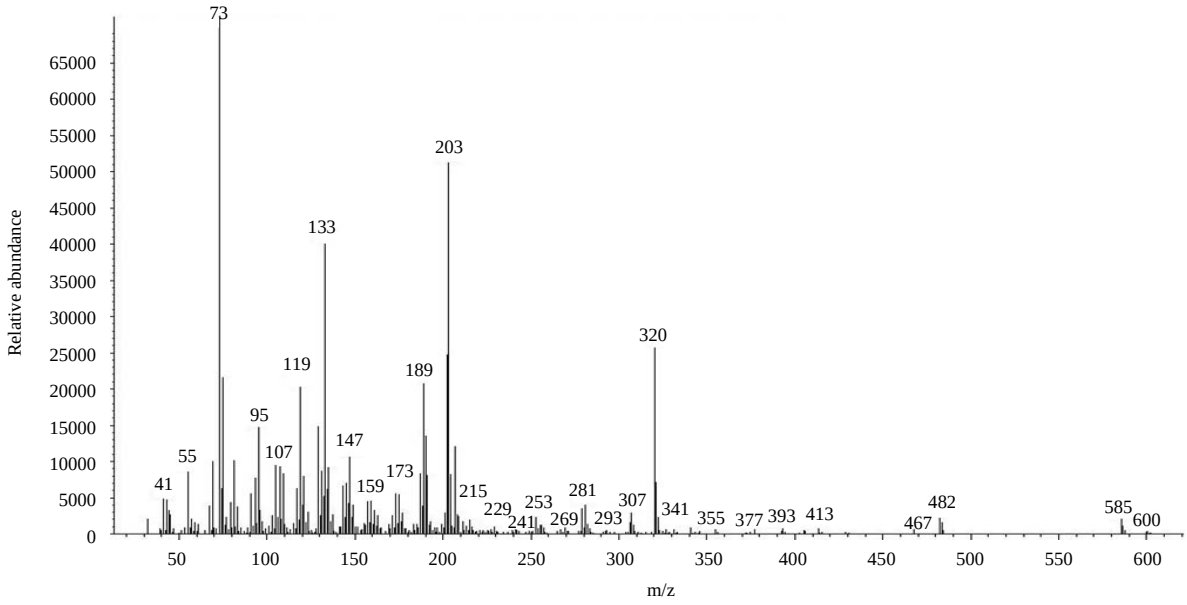


Fig. 12: Fragmentation pattern (MS) from isolated 2 from rosemary methanolic extract

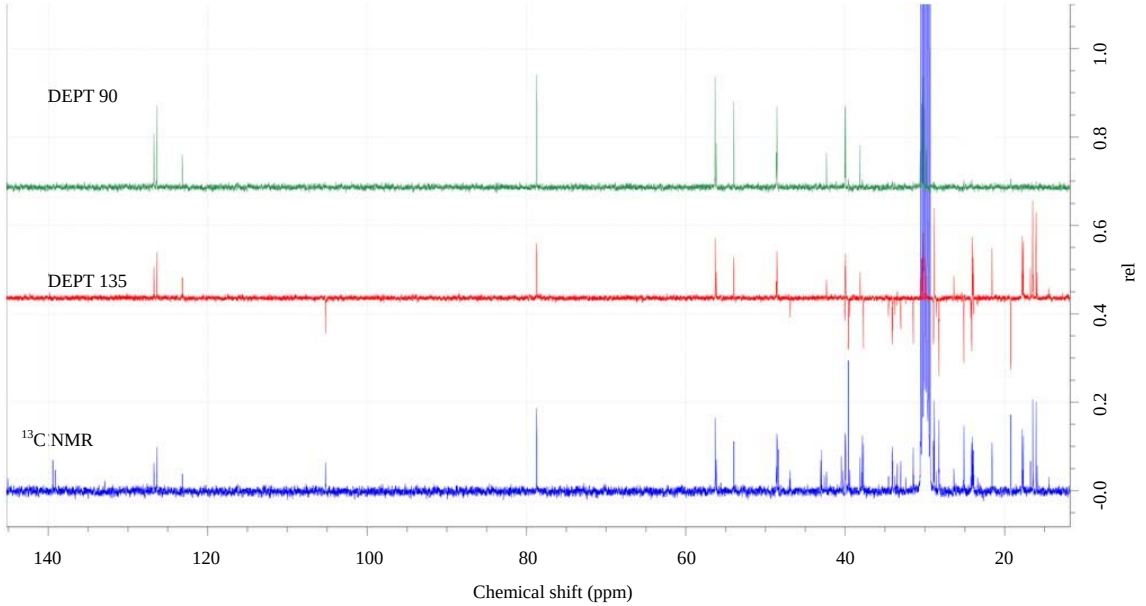


Fig. 13: DEPT (Distortionless Enhancement by Polarisation Transfer) 135 and 90 analyses from isolated 2 from rosemary methanolic extract

research of plants extracts report some importance or clinical of strains that are the etiologic agents of opportunist diseases. Oluwatuyi *et al.*[19] reported antibacterial activity against *S. aureus* Efflux (NorA) from chloroformic aerial parts of rosemary. Other plant extracts against multidrug resistant bacteria consisted of methanolics and essential oils[20,21]. Ten of the plants had antibacterial activity against reference ATCC and clinical isolate strains. The first antibacterial activities reported were for *C. illinoensis* (pecan nut) against *P. aeruginosa* ATCC-27853, *E. robustum* (horsetail), *S. rebaudiana* (Stevia) and *C. texana* (goat-bush) against *S. aureus* Rosenbach ATCC BAA-44 and *C. citratus* (lemon grass), horsetail, *A. adstringens* (cuachalalate) against *A. baumannii*. While *R. officinalis* (rosemary) showed major activity in both strains, the decision was to isolate the molecule(s) responsible for this property. Firstly, after column

chromatography, the white amorphous powder that was separated had a coincident with Ghias Uddin *et al.*[22], who isolated betulinic acid from a methanol extraction of *Grewia optiva* and Chung *et al.*[23] from *Callicarpa farinose*, who tested betulinic acid against MRSA and MSSA strains. The IR data according to Kovac-Besovic *et al.*[24] is very similar with an O-H stretching signal of 3484 cm^{-1} (3435 cm^{-1} observed), C = O stretching signal of 1686 cm^{-1} (1689 cm^{-1} observed), C-OH stretching signal of 1432 cm^{-1} (1457 cm^{-1} observed). These signals were referenced to an organic acid. In the search of references of organic acids in rosemary, three principal acids were found: Ursolic acid, oleanolic acid and betulinic acid, all of which were triterpenic compounds. Their differences were defined by the positions of their unsaturations and some substituents[25,26] (Fig. 12). For the ^{13}C NMR spectrogram, the signals were coupled as pentacyclic triterpene compounds due to their COOH signals (172-180 ppm)[27]. Fifty-nine signals were observable, the DEPT analyses (135 and 90) revealed 13 CH_3, 19 CH_2, 13 CH and 14 did not show carbons signals (Fig. 13). The HSQC revealed the 105.17 ppm (^{13}C NMR, bottom signal) that was coupled with the 4.65 and 4.7 ppm (^{1}H NMR) as a = CH_2, the 122 ppm (^{13}C NMR, up signal) was coupled with 5.23 ppm (^{1}H) as a = CH and the 125 ppm (^{13}C NMR, up signal) was coupled with 5.25 ppm (^{1}H NMR) as a = CH, corresponding to carbons number 29 from betulinic acid, 12 from oleanolic acid and 12 from ursolic acid (Fig. 11) as according to Seebacher *et al.*[28]. For ^{1}H NMR a comparison was made with the literature, Peng *et al.*[29] and Adesanwo *et al.*[30]. Derivatization was performed with BSA (N,O-bis (trimethylsilyl) acetamide), which replaces the active proton in -OH, -COOH[31,32], in the chemical structures of pentacyclic triterpenes of the ursane, oleane and lupane families, the COOH position is carbon 28 and the OH position is carbon 3[33], for this reason the silyl derivative contains two trialkylsilyl groups (146.296 g/mol). Ursolic, oleanolic and betulinic acid present a molecular weight of 456.70 g/mol as a consequence of the loss of two protons, with the addition of trialkylsilyl groups, the molecular weight from this derivative results in 600.982 g/mol[34] (Fig. 12).

The pentacyclic triterpenes mixture showed an MIC = 725 μg mL^{-1}, according to Wang *et al.*[35], who reported an MIC>128 μg mL^{-1} for the same microorganism, this information is important since it can use an objective dose in the treatment of opportunist diseases caused by *S. aureus* Rosenbach, also, if combine another molecule that is present in the plants it can improve new therapy or use it as a coadjuvant to a synergistic molecule. In addition, the betulinic acid has these reported activities: Apoptotic and anticancer. Some derivatives are protease inhibitors (against HIV)[36-39]. Now this study provides a method for isolating this beneficial molecule.

CONCLUSION

In conclusion, the antimicrobial activity of methanolic extracts from 20 plants used in traditional Mexican medicine was tested against multidrug-resistant bacteria. Half of these showed biological activity. For the first time *Carya illinoensis* against *Pseudomonas aeruginosa* and also *Equisetum robustum*, *Stevia rebaudiana* and *Castela texana* against *S. aureus* Rosenbach was reported. A mixture conformed by betulinic, ursolic and oleanolic acids (MIC 725 μg mL^{-1}) was isolated from *Rosmarinus officinalis* which showed major activity against *S. aureus* Rosenbach as an ATTC reference strain (BAA-44) as well as against clinical isolates.

SIGNIFICANCE STATEMENT

This study establishes the antibacterial activity of *Carya illinoensis*, *Equisetum robustum*, *Stevia rebaudiana* and *Castela texana* against multidrug-resistant bacteria. In addition, the isolation of a mixture compound by triterpenic acids from *Rosmarinus officinalis*, which has activity against ATCC reference and clinically isolated bacteria, identified by chromatography and spectroscopic methods is reported. This study help the researchers to discover critical areas of new sources of antibacterial molecules and alternative treatments for multidrug resistant bacteria which many researchers are not able to explore. Thus, a new theory on plant extracts and their molecules as antimicrobial agents may be reached.

ACKNOWLEDGMENT

This project was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT, México) under registration number 377431.

REFERENCES

1. Farnsworth, N.R., O. Akerele, A.S. Bingel, D.D. Soejarto and Z. Guo, 1985. Medicinal plants in therapy. Bull. World Health Organiz., 63: 965-981.
2. Balunas, M.J. and A.D. Kinghorn, 2005. Drug discovery from medicinal plants. Life Sci., 78: 431-441.

3. Madigan, M.T., J.M. Martinko, P.V. Dunlap and D.P. Clark, 2009. Brock Biology of Microorganisms. 12th Edn., Pearson Benjamin-Cummings, San Francisco, ISBN: 0-13-2232460-1, Pages: 592.
4. Kimpe, A., A. Decostere, A. Martel, L.A. Devriese and F. Haesebrouck, 2003. Phenotypic and genetic characterization of resistance against macrolides and lincosamides in *Streptococcus gallolyticus* strains isolated from pigeons and humans. Microb. Drug Resistance, 9: 35-38.
5. Salipante, S.J., M. Barlow and B.G. Hall, 2003. GeneHunter, a transposon tool for identification and isolation of cryptic antibiotic resistance genes. Antimicrob. Agents Chemother., 47: 3840-3845.
6. Shahid, M., A. Malik and Sheeba, 2003. Multidrug-resistant *Pseudomonas aeruginosa* strains harbouring R-plasmids and AmpC β-lactamases isolated from hospitalised burn patients in a tertiary care hospital of North India. FEMS Microbiol. Lett., 228: 181-186.
7. Mandell, G.L., J.E. Bennett and R. Dolin, 2002. Enfermedades Infecciosas: Principios y Practica. 5th Edn., Vol. 1. Medica Panamericana, Buenos Aires.
8. Martinez-Martinez, L., A. Pascual and G.A. Jacoby, 1998. Quinolone resistance from a transferable plasmid. Lancet, 351: 797-799.
9. Forero-Gomez, J., 2002. Beta lactamasas de espectro extendido en pediatria. Rev. Colomb. Pediatria, 37: 12-15.
10. CDC Department of Health and Human Services, 2005. Community-associated MRSA information for clinicians. CDC Department of Health and Human Service, Atlanta, GA.
11. Karvouniaris, M., D. Makris and E. Zakynthinos, 2010. Community-associated *Staphylococcus aureus* infections: Pneumonia. Microbiol. Res., 2: 15-21.
12. Prabuseenivasan, S., M. Jayakumar and S. Ignacimuthu, 2006. *In vitro* antibacterial activity of some plant essential oil. BMC Complement. Altern. Med., Vol. 6. 10.1186/1472-6882-6-39.
13. Silva, J., R. Gatica, C. Aguilar, Z. Becerra and U. Garza-Ramos *et al*., 2001. Outbreak of infection with extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* in a Mexican Hospital. J. Clin. Microbiol., 39: 3193-3196.
14. D'Sousa'Costa, C.O., P.R. Ribeiro, M.B. Loureiro, R.C. Simoes, R.D. de Castro and L.G. Fernandez, 2015. Phytochemical screening, antioxidant and antibacterial activities of extracts prepared from different tissues of *Schinus terebinthifolius* Raddi that occurs in the coast of Bahia, Brazil. Pharmacogn. Magaz., 11: 607-614.
15. NCCLS., 1999. Methods for determining bactericidal activity of antimicrobial agents; approved guideline. CLSI Document M26-A, Vol. 19, No. 18, Clinical and Laboratory Standards Institute, Wayne, PA., USA., September 1999.
16. Rivas-Morales, C., M. Salinas-Carmona, L. Galan-Wong and H. Medrano-Roldan, 2007. Operacion unitaria para la propagacion de *Nocardia brasiliensis* HUJEG-1 para la produccion de proteasas con potencial biotecnologico. Patente No. 252592 MPI, MX/07/11/2007.
17. CLSI., 2015. M07-A10: Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: Approved standard. Clinical and Laboratory Standars Institute, Wayne, PA., pp: 1-87.
18. Choma, I.M. and E.M. Grzelak, 2011. Bioautography detection in thin-layer chromatography. J. Chromatogr. A, 1218: 2684-2691.
19. Oluwatuyi, M., G.W. Kaatz and S. Gibbons, 2004. Antibacterial and resistance modifying activity of *Rosmarinus officinalis*. Phytochemistry, 65: 3249-3254.
20. Bocanegra-Garcia, V., M.D.R. Camacho-Corona, M. Ramirez-Cabrera, G. Rivera and E. Garza-Gonzalez, 2009. The bioactivity of plant extracts against representative bacterial pathogens of the lower respiratory tract. BMC Res. Notes, Vol. 2. 10.1186/1756-0500-2-95.
21. Tohidpour, A., M. Sattari, R. Omidbaigi, A. Yadegar and J. Nazemi, 2010. Antibacterial effect of essential oils from two medicinal plants against Methicillin-resistant *Staphylococcus aureus* (MRSA). Phytomedicine, 17: 142-145.
22. Ghias Uddin, Waliullah, B.S. Siddiqui, M. Alam, A. Sadat, A. Ahmad and Ala Uddin, 2011. Chemical constituents and phytotoxicity of solvent extracted fractions of stem bark of *Grewia optiva* Drummond ex burret. Middle-East. J. Sci. Res., 8: 85-91.
23. Chung, P.Y., L.Y. Chung and P. Navaratnam, 2014. Potential targets by pentacyclic triterpenoids from *Callicarpa farinosa* against methicillin-resistant and sensitive *Staphylococcus aureus*. Fitoterapia, 94: 48-54.
24. Kovac-Besovic, E., K. Duric, Z. Kalooera and E. Sofic, 2009. Identification and isolation of betulin, betulinic acid and lupeol from birch bark. Planta Med., 75: PJ133-PJ133.
25. Razborsek, M.I., D.B. Voncina, V. Dolecek and E. Voncina, 2007. Determination of major phenolic acids, phenolic diterpenes and triterpenes in rosemary (*Rosmarinus officinalis* L.) by gas chromatography and mass spectrometry. Acta Chim. Slovenica, 54: 60-67.
26. Fontanay, S., M. Grare, J. Mayer, C. Finance and R.E. Duval, 2008. Ursolic, oleanolic and betulinic acids: Antibacterial spectra and selectivity indexes. J. Ethnopharmacol., 120: 272-276.
27. Ayatollahi, A.M., M. Ghanadian, S. Afsharypour, O.M. Abdella, M. Mirzai and G. Askari, 2011. Pentacyclic triterpenes in *Euphorbia microsciadia* with their T-cell proliferation activity. Iran. J. Pharm. Res., 10: 287-294.

28. Seebacher, W., N. Simic, R. Weis, R. Saf and O. Kunert, 2003. Complete assignments of ^{1}H and ^{13}C NMR resonances of oleanolic acid, 18α-oleanolic acid, ursolic acid and their 11-oxo derivatives. Magn. Res. Chem., 41: 636-638.
29. Peng, C., G. Bodenhausen, S. Qiu, H.H. Fong, N.R. Farnsworth, S. Yuan and C. Zheng, 1998. Computer-assisted structure elucidation: Application of CISOC-SES to the resonance assignment and structure generation of betulinic acid. Magnet. Resonance Chem., 36: 267-278.
30. Adesanwo, J.K., O.O. Makinde and C.A. Obafemi, 2013. Phytochemical analysis and antioxidant activity of methanol extract and betulinic acid isolated from the roots of *Tetracera potatoria*. J. Pharm. Res., 6: 903-907.
31. Walton, D.R.M., 1979. Handbook of derivatives for chromatography. J. Organometallic Chem., 168: C47-C47.
32. Knapp, D.R., 1979. Handbook of Analytical Derivatization Reactions. John Wiley and Sons, New York, ISBN: 9780471034698, Pages: 741.
33. Jemmali, Z., A. Chartier, C. Dufresne and C. Elfakir, 2016. Optimization of the derivatization protocol of pentacyclic triterpenes prior to their gas chromatography-mass spectrometry analysis in plant e xtracts. Talanta, 147: 35-43.
34. Caligiani, A., G. Malavasi, G. Palla, A. Marseglia, M. Tognolini and R. Bruni, 2013. A simple GC-MS method for the screening of betulinic, corosolic, maslinic, oleanolic and ursolic acid contents in commercial botanicals used as food supplement ingredients. Food Chem., 136: 735-741.
35. Wang, C.M., H.T. Chen, Z.Y. Wu, Y.L. Jhan, C.L. Shyu and C.H. Chou, 2016. Antibacterial and synergistic activity of pentacyclic triterpenoids isolated from *Alstonia scholaris*. Molecules, Vol. 21. 10.3390/molecules21020139.
36. Eichenmuller, M., B. Hemmerlein, D. von Schweinitz and R. Kappler, 2010. Betulinic acid induces apoptosis and inhibits hedgehog signalling in rhabdomyosarcoma. Br. J. Cancer, 103: 43-51.
37. Ehrhardt, H., S. Fulda, M. Fuhrer, K.M. Debatin and I. Jeremias, 2004. Betulinic acid-induced apoptosis in leukemia cells. Leukemia, 18: 1406-1412.
38. Ahmad, F.B.H., M.G. Moghaddam, M. Basri and M.B. Abdul Rahman, 2010. Enzymatic synthesis of betulinic acid ester as an anticancer agent: Optimization study. Biocatal. Biotransformation, 28: 192-200.
39. Zhao, H., S.S. Holmes, G.A. Baker, S. Challa, H.S. Bose and Z. Song, 2012. Ionic derivatives of betulinic acid as novel HIV-1 protease inhibitors. J. Enzyme Inhib. Med. Chem., 27: 715-721.