



ANTIBACTERIAL ACTIVITY OF MEDICINAL PLANT (*CENTAURAEA CALCITRAPA*) AGAINST MULTI-DRUG RESISTANT BACTERIA (MDRB)

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ABSTRACT

The present study was conducted to evaluate the antibacterial activity of *Centauraea calcitrapa* against multidrug resistant bacteria (*Staphylococcus aureus*, *Enterococcus faecium*, *klebsiella pneumoniae*, *Acinetobacter baumannii*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*), purify and identify the active compounds. A sensitivity test was performed using disc diffusion method that resulted in the appearance of multiple drug resistance phenotypes of tested bacteria. Antibacterial activity of *Centauraea calcitrapa* showed that inhibition zone diameter against *Staphylococcus aureus* (23mm), *Enterococcus faecium* (17mm), *Klebsiella pneumoniae* (15mm), *Acinetobacter baumannii* (22mm), *Enterobacter cloacae* (20mm) and *Pseudomonas aeruginosa* (18mm). Highest activity was exhibited against *Staphylococcus aureus* and can be considered to be more effective than the most commercial antibiotics. The active compound was purified using column chromatography and visualized using thin layer chromatography (TLC). The Minimum inhibitory concentration (MIC) values of active purified compound ranged from 15.6 to 62.5µg/ml. Minimum bactericidal concentrations (MBC) values were two-fold higher than the corresponding MIC values ranged from 31.25 to 125µg/ml. The active purified compound was partial identified using UV, IR, ¹HNMR and mass spectroscopy. The analysis of data obtained indicated that; the purified active compound belongs to janerin group of chemical compounds. (*Centauraea calcitrapa*) is a new source for discovery of bio-active compound against (MDRB).

Keywords: Antibacterial activity, Multi-drug resistance bacteria, Clinical isolates, Medicinal plants, Plant extract.

Received: 1 June 2016/ Revised: 6 July 2016/ Accepted: 18 July 2016/ Published: 3 August 2016

Contribution/ Originality

This study is one of very few studies which have investigated the antibacterial activity of Centaurea plant (family Asteraceae) in Egypt against MDRB. Egypt is one of main countries for

diversity of this group of plants. Therefore, this study documents the antibacterial potential of *Centauraea calcitrapa* extract in Egypt against MDRB.

1. INTRODUCTION

In last decades, there is a remarkable increase in the emergence of multi-drug resistant (MDR) strains that represent risk factor to health and global drug discovery program. The main causes of antibiotics resistance were abuse, inappropriate use of antibiotics in medical and agriculture uses [1]. Evolution of multi drug resistance to antibiotics is greater cause of concern. The new generation of disease causing pathogens and mutations of existing microorganisms are responsible for human morbidity and mortality. Plants are known to be rich source of new active substance that may serve as a natural remedy against antibiotic resistant pathogens. Due to the high cost of traditional antimicrobial treatments in African countries necessitates the use of plant based medicine for treatment purposes [2]. For many of year's natural products have played an important role in health care and disease prevention. The old civilizations of the North Africans have many written indicators for using of herbal medicine in prevention of various diseases [3].

Since several decades, many herbal plants have been used as a source of natural medicines. Many of drugs have been obtained from medicinal plants. About 80 % of developing countries depend on medicinal plant based in their human primary health care [4]. Plants contain many bioactive compounds that have been used in different medicinal approach [5]. The majority of these bioactive compounds are secondary metabolites belong to groups of resins, fatty acids, tannins flavonoids, steroids, alkaloids and phenol compounds, etc. Many studies established that medicinal plants are sources of nutrient and non-nutrient compounds, many of which exhibition antioxidant and antimicrobial properties which can safeguard the human body against both pathogens and cellular oxidation reactions. Thus, it is very important to characterize the different types of medicinal plant compound for their antimicrobial activity and antioxidant [6]. Medicinal Plant derived products have many advantages than synthetic chemicals compounds such as decreased side effects, activity, less cost and availability [7]. Thus, the aim of this study was to evaluate, in vitro, antibacterial activity of *Centauraea calcitrapa* plant against multidrug resistant bacteria isolated from clinical samples and elucidation active purified compound by spectroscopic analysis.

2. MATERIALS AND METHODS

2.1. Collection of Clinical Specimens

Specimens used in this study were collected from clinical specimens include on, abscess, tooth, sputum, urine, ear, eye, nose. tonsils, wound and throat swabs during period from March to October 2015 from Damanhour National Medical Institute (DNMI) and Damanhour Hospital Fever(DHF), Egypt. One hundred and forty bacterial isolates were isolated from collection specimens (85 isolate from (DNMI) and 55 isolate from (DHF).

2.2. Bacterial Strains

The bacterial isolates were identified through morphological, physiology, conventional biochemical tests and confirmed with Automated Identification Biomerieux Vitek 2 System [8].

2.3. Antibiotics Susceptibility Testing

Susceptibility to antibiotics was assessed using the Kirby-Bauer disc diffusion technique. The results were read according to [Clinical and Laboratory Standards Institute\(CLSI\)](#) [9]. The panel of antibiotics used included: (tetracycline, vancomycin, cefuroxime, clindamycin, metronidazole, rifampicin, amikacin, gentamycin, neomycin, nalidixic acid, amoxicillin/clavulanic acid trimethoprim/Sulfomethoxazole, ciprofloxacin, bacitracin, chloramphenicol, kanamycin, erythromycin, rifampin and penicillin). The suspension of the test organism in nutrient broth was matched with 0.5 McFarland turbidity standards to give a concentration of 1.0×10^8 CFU/ml; while the inoculated plates were incubated at 35°C for 18 – 24 hours. The degree of susceptibility of the bacterial isolates to each antibiotic was determined. Multidrug resistance (MDR) means resistant to at least 3 families of antibiotics tested. All antibiotic discs were purchased from Oxoid. UK.

2.4. Culture Preparation

Each bacterial strain was inoculated in Muller Hinton broth and incubated at 37°C overnight for activation. Bacterial count was adjusted to 1.0×10^8 CFU/ml. The molten Muller Hinton Agar plates (15 cm) were seeded with 100µl of bacterial suspension [10].

2.5. Collection of Plant Materials and Preparation of Methanolic Extract

plant used in this work was collected and identified by Dr. El-Baraa Mohammed El-Saied “lecturer of plant ecology, Botany and Microbiology, Faculty of Science, Al_Azhar University” from Bahariya Oasis, Egypt [N 28.21 20.6 E 28.51 47.8]). Plant leaves were subjected for extraction process summarized in the following steps; first plant leaves are washed with distilled water and then dried by air out of sun reach followed by crushing into powder form. From the dry powder 15 gm was soaked in 150ml of methanol (Sigma-Aldrich Co) and then incubated at room temperature for 72hr under shaking (120rpm) (NEW BRUNSWICK SCIENTIFIC [EDISON,N.J,U.S.A]). After incubation between solvent and plant leaves, the crude extract was obtained by centrifugation at 3000rpm (SIGMA 2K15) for 10 minutes at 25°C then the solvent was removed using rotary evaporator (Heidolph VV2001). Crude extract was prepared for antibacterial assay through dissolving 100mg/ml in 1% dimethylsulphoxide (DMSO) (Sigma-Aldrich Co) centrifuged at 10,000rpm to remove the solid residues. The obtained solutions were stored in refrigerator at 4°C for the next studies. Solvent control was prepared under the same condition.



Fig-1. Centaurea calcitrapa plant

2.6. Antibacterial Assay

Antibacterial activity was evaluated according to [Perez, et al. \[11\]](#). The molten Muller Hinton Agar was inoculated with the 100 μ l of inoculums (1.0×10^8 CFU/ml) and poured into the sterilized petri plate (15 cm). For disc diffusion method, the paper disc (7mm) was saturated with 100 μ l of crude extract, allowed to air dry and plated on the surface of seeded agar plate. The plates were incubated for 24hr at 37°C. After bacteria grow the inhibition zone was determined by measuring the diameter of inhibition zone in millimeter (mm) and using paper disc contain solvent only as control for each bacterial species. The experiment was repeated three times.

2.7. Column Chromatography Purification of the Crude Extract:

Column chromatography purification was performed according to [Masud, et al. \[12\]](#) with some modification as following; The glass column (1.1cm diameter and length 55cm) was packed with silica gel (Kieselgel 60, mesh 70-230). The sample was loaded to the column through slowly adding of 1.5gm of concentrated crude extract (dissolved in 4ml methanol) to the surface of silica gel and then The column was eluted with the following sequence of solvent (100 ml from each solvent) (Hexane, Petroleum ether, Benzene, Toluene, Diethyl ether, Chloroform, Ethyl acetate, Acetic acid, Isopropyl alcohol, Acetone, Ethanol, Methanol, water) All solvent are (Sigma-Aldrich Co) mixtures of solvent increasing polarity. Column fractions (5ml each) were collected and labeled for testing purity using TLC (FLUKA Batch 001011 Fluka Art.NR.:60778), antibacterial activity to select the active fractions was tested and finally characterize and identify the active purified fraction.

2.8. Characterization and Identification of Active Purified Fractions

The active purified fractions was characterized using the following spectroscopic analysis, ultraviolet (UV (160A-Shimadzu), Infrared IR (Matson Satellite 113 spectrometer), ¹HNR (various Mercury -300BB/MHz NMR spectrometer), Mass Spectrum (Direct Inlet part DI-50 to mass analyzer in Shimadzu GC-MS-QP5050 Thermo Scientific Prop) this spectroscopic analysis was performed according to David [13].

2.9. Determination of Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC)

MIC of active purified compound was performed according to AL-Fatimi, et al. [14] were 1000µg/ml was serially diluted two fold until 15.6 µg/ml in 96 well plate (polystyrene flat-bottom) (Sigma-Aldrich, St. Louis, MO,USA) and then 100µl of pathogenic bacteria (1.0x10⁸ CFU/ml) were added. Ampicillin was used as reference antibiotic. Solvent control DMSO was used as the negative control. The absorbance was measured at the start of incubation and also at the end (after 24hr) using ELISA plate reader (Bio kinetic Reader EL 350, Bio-Tek TM Instruments, Winooski, VT, USA). MIC was determined and the experiment was repeated three times. After incubation; bacterial cells were cultured on nutrient agar plate in incubated 24hr for determine bactericidal effect. The experiment was carried out in triplicate.

3. RESULTS AND DISCUSSION

3.1. Antibacterial Activity of Plant Extract

Egypt is one of main countries for diversity of the genus *Centaurea* plant (family Asteraceae) [15]. Large numbers of this group of plants are used in folk medicine such as *Centaurea cyanus*, *Centaurea behen*, *Centaurea calcitrapa* [16, 17] this family of plants were known to have antibacterial activity such as *C. thessala* and *C. attica* [18] *Centaurea diffuses* [19] *Centaurea nicolai* [20] *Centaurea sessilis* and *Centaurea armena* [21] *Centaurea solstitialis* ssp. *solstitialis* [17] whereas the antimicrobial activity of (*Centaurea calcitrapa*) which is endemic in Egypt has poor review and studies. Therefore, the aim of the present work was to evaluate the antibacterial potential of the leaf plant extracts against multi drug resistant. The bacterial isolates from clinical specimens were identity by morphological, physiology, conventional biochemical tests and confirmed with Automated Identification Biomerieux Vitek 2 System. After identification bacterial isolates subjected to antibiotics sensitivity test. The results revealed that six bacterial isolates belong to *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterobacter cloacae*, *Enterococcus faecium*, *klebsiella pneumonia* and *Staphylococcus aureus* are mildrug resistant table [1]. The multidrug resistant isolates represented incidence, especially the emergence of resistance to most antibiotics tested indicates that designing a surveillance system for antimicrobial resistance in Egypt and the introduction of integrated guidelines for the appropriate use of antibiotics are urgently needed.

Staphylococcus aureus cause the majority of Gram positive septicemias which usually complicate infections of skin, soft tissues, lungs, bones, joint and cause more than 80 % of cases of acute septic arthritis and possess a wide range of potential virulence factors, including hemolysins, lipases, proteases, hyduronidase, nucleases and enterotoxins [22, 23]. *Pseudomonas aeruginosa* is represented major nosocomial pathogens because of their ubiquitous nature and ability to colonize and survive in hospital reservoirs and because of their role in causing infections in immunocompromised and critically ill patients. *P. aeruginosa* shows a high level of intrinsic resistance to antibiotics and an ability to become even more drug resistant [24]. *klebsiella pneumoniae* is the second most common uropathogen and important aspects of *Klebsiella* associated infection is the emergence of multi-drug resistance (MDR) [25]. Enterococci emerged in the 1970s and 1980s as leading causes of antibiotic-resistant infection of the bloodstream, urinary tract, surgical wounds and contributing to 10,000 to 25,000 deaths per year [26] *Enterobacter* is well adapted to cause nosocomial infections, as it is ubiquitous in the environment and can survive on skin, dry surfaces as well as replicate in contaminated fluids and increasing in frequency particularly in intensive care units [27]. In December 2005, reported that five extremely resistant isolates of *A baumannii* this number was a dramatic increase. Most multidrug resistant *A. baumannii* outbreaks occur in critical care settings and involve resistance to multiple classes of antimicrobial agents [28].

Table-1. Susceptibility results of six bacterial isolates to antibiotics

AB Code No	Cefuroxime 30 µg/ml	Metronidazole 5 µg/ml	Amoxicillin/clavulanic acid 30 µg/ml	Tetracycline 30 µg/ml	Vancomycin 30 µg/ml	Rifampin 5 µg/ml	Clindamycin 2 µg/ml	Trimethoprim/Sulfometh oxazole 25 µg/ml	Ciprofloxacin 5 µg/ml	Penicillin 10 µg/ml	Gentamycin 10 µg/ml	Chloramphenicol 30 µg/ml	Bacitracin 10 µg/ml	Erythromycin 15 µg/ml	Rifampicin 30 µg/ml	Kanamycin 30 µg/ml	Amikacin 30 µg/ml	Neomycin 30 µg/ml	Nalidixic acid 30 µg/ml
Sym bol	C X M	M E T	A M C	T E	V A	R A	D A	S X T	C I P	P	C N	C	B	E	R F	K	A K	N	N A
19	S	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	R	S	R
24	R	R	R	I	R	R	R	R	S	R	S	R	R	R	R	S	I	S	S
60	S	R	R	R	R	R	R	R	S	R	S	R	R	R	R	R	S	S	S
74	R	R	R	R	R	R	R	R	R	R	R	R	R	R	I	R	R	R	R
219	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R
302	S	R	R	R	R	R	R	R	S	R	R	R	R	R	R	S	R	S	R

19 = *Enterococcus faecium*, 24 = *Enterobacter cloacae*, 60 = *Pseudomonas aeruginosa*, 74 = *Acinetobacter baumannii*, 219 = *Staphylococcus aureus* and 302 = *klebsiella pneumoniae*. *Centaurea calcitrapa* extract was active against all assayed bacteria isolates multidrug resistant. Maximum of inhibition activity (23mm) was observed against *Staphylococcus aureus* as shown in table (2) and figure (2). Several Biological studies on

Centauraea sp. had revealed significant activities such as anti-inflammatory, anti-platelet, anti-pyretic, analgesic, wound healing, anti-plasmodial, cytotoxic, anti-proteasomal, anti-ulcerogenic, anti-oxidant, anti-bacterial, anti-fungal, hepatoprotective, and allelochemical activities [29, 30] showed that the methanol and ethyl acetate extracts of *Centauraea* sp. inhibited the growth of thirteen bacteria: *Salmonella enteritidis*, *Pseudomonas aeruginosa* (NRRL B-23), *Morganella morgana*, *Escherichia coli* (ATCC 35218) (isolated from human urine), *Klebsiella pneumonia* (ATCC 27736), *Yersinia enterocolitica* (RSKK 1501), *Proteus vulgaris* (RSKK 96026), *Micrococcus luteus* (NRRL B-4375), *Staphylococcus aureus*(ATCC 25923), *S. aureus*(ATCC 12598), *Listeria monocytogenes* *Bacillus cereus* (RSKK 863) and *B. subtilis* (ATCC 6633).

Table-2. Antibacterial activity of methanolic crude extract of *Centauraea calcitrapa* against multidrug resistant bacteria and reference strains.

Bacterial Strain	Inhibition Zone Diameter (mm)	Bacterial Strain	Inhibition Zone Diameter (mm)
<i>Staphylococcus aureus</i>	23	<i>klebsiella pneumoniae</i>	15
<i>Enterococcus faecium</i>	17	<i>Enterobacter cloacae</i>	20
<i>Acinetobacter baumannii</i>	22	<i>S. aureus</i> ATCC 29213	18
<i>Pseudomonas aeruginosa</i>	18	<i>E. coli</i> ATCC 25922	20

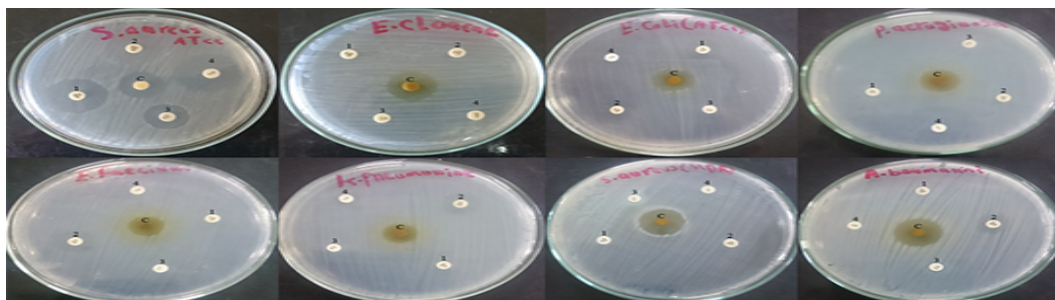


Figure-2. Antibacterial activity of crude methanolic extract obtained from *Centauraea calcitrapa* leaf, C is crude extract, 1 is Amoxicillin/Clavulanic acid (AMC), 2 is Trimethoprim/Sulfamethoxazole(SXT), 3 is Vancomycin (VA) and 4 is Erythromycin (E).

The crude extract was fractionated into 120 fractions (each fraction five ml) and the active fractions were start to appear starting from fraction 21 until 30 as shown in figure (3) were the mobile phase was cyclohexan.

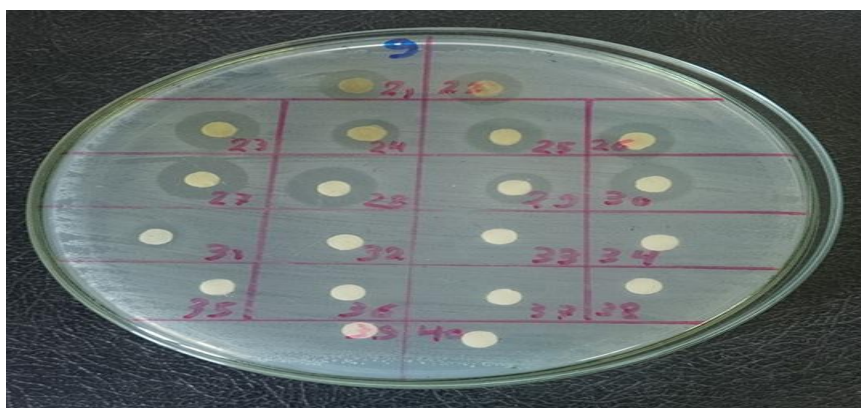


Figure-3. antibacterial activity of collected fractions obtained from column chromatography

The purified fraction (containing only one band under UV) was from 22 to 29 while fractions 21 and 30 was containing more than one band as shown in the following figure(4):

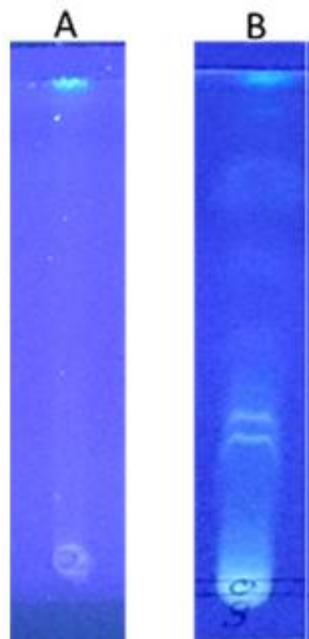


Figure-4. active fractions collected from column chromatography under UV were (A) containing only one band while (B) containing more than one band.

3.2. Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations

MIC values of active purified compound showed variation from one bacterial strain to another but MIC values was generally ranged between 15.6 to 62.5 $\mu\text{g/ml}$ as shown in table 3. MIC values of the ethyl acetate extracts from *Centauraea* sp. were determined as 250 $\mu\text{g/ml}$ for *Escherichia coli* and 62.5 $\mu\text{g/ml}$ for *Staphylococcus aureus* [30]. The results of the MBC indicated that *Centauraea calcitrapa* has bactericidal effect and MBC values were ranged from 31.25 to 125 $\mu\text{g/ml}$ as shown in table 3. Also the result indicated that Gram-negative bacteria have high resistant than Gram-positive bacteria and this is due to the highly hydrophobic outer membrane that act as permeability barrier mainly for hydrophilic compounds [31, 32].

Table-3. Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of active purified compound

Bacterial Species	MIC ($\mu\text{g/ml}$)	MBC ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i>	15.6	31.25
<i>Enterococcus faecium</i>	31.25	62.5
<i>Acinetobacter baumannii</i>	31.25	62.5
<i>Pseudomonas aeruginosa</i>	62.5	125

<i>Enterobacter cloacae</i>	31.25	62.5
<i>klebsiella pneumonia</i>	62.5	125
<i>S. aureus</i> ATCC 29213	15.6	31.25
<i>E. coli</i> ATCC 25922	15.6	31.25

3.3. Spectroscopic Analysis of Active Purified Compound

1- UV Spectroscopy

UV- visible spectrum of compound showed that λ max at 230.82nm and 340nm which indicate that $\pi \rightarrow \pi^*$ transition

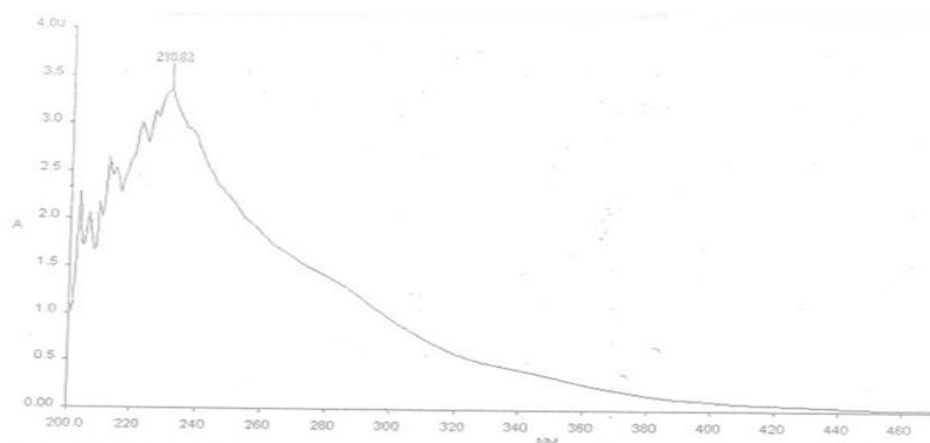


Figure-5. UV spectrum of purified active compound.

2- IR Spectroscopy

Infrared spectroscopy of purified active compound showed that δ 3395 cm^{-1} (OH stretching), 2937 for (CH aliphatic), 1653 for (C=O), 1448 for (CH₃&CH₂ bending) 1390 for (OH bending), 1208 for (CH bending), 1077 for (C-O-C), 881(OH-out of plane), 704 for (OH- out of plane).

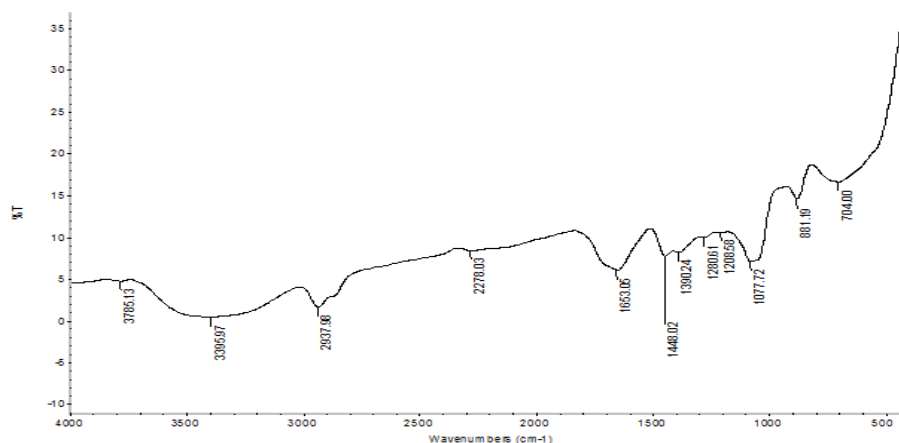


Figure-6. IR analysis of active purified compound.

3- ¹H NMR SPECTROSCOPY

The ¹H NMR Spectrum of compound showed in figure(C) which recorded in the DMSO d₆ which give 0.9 δ ppm indicate to CH₃, range of (1-2) ppm for CH₂ aliphatic, range(2-3) ppm CH, range (5.3,5.5) ppm for OH, range (7.3,7.4) ppm for hydrogen bond.

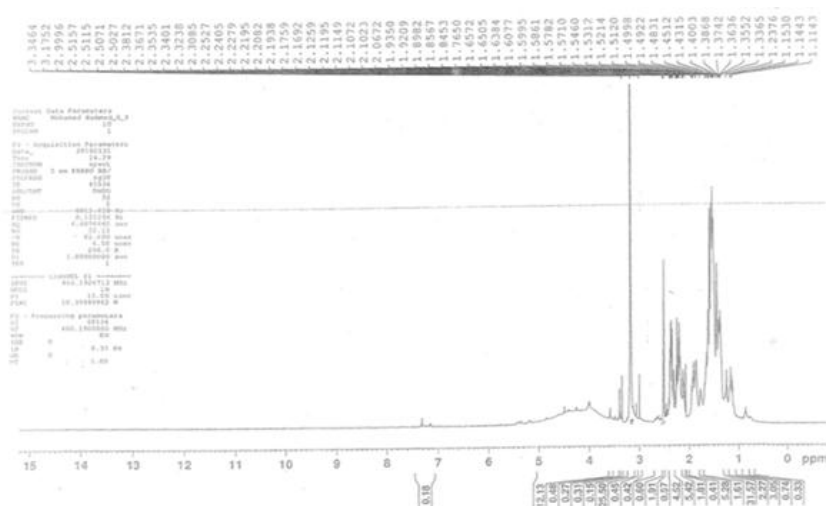


Figure-7. ¹H NMR of active purified compound

4- Mass Spectroscopy of Purified Active Compound

The mass spectrum (m/z) of purified active compound showed that Molecular ion peak at 364(m/z) and base peak at 57(m/z) which identical with suggested compound (janerin).

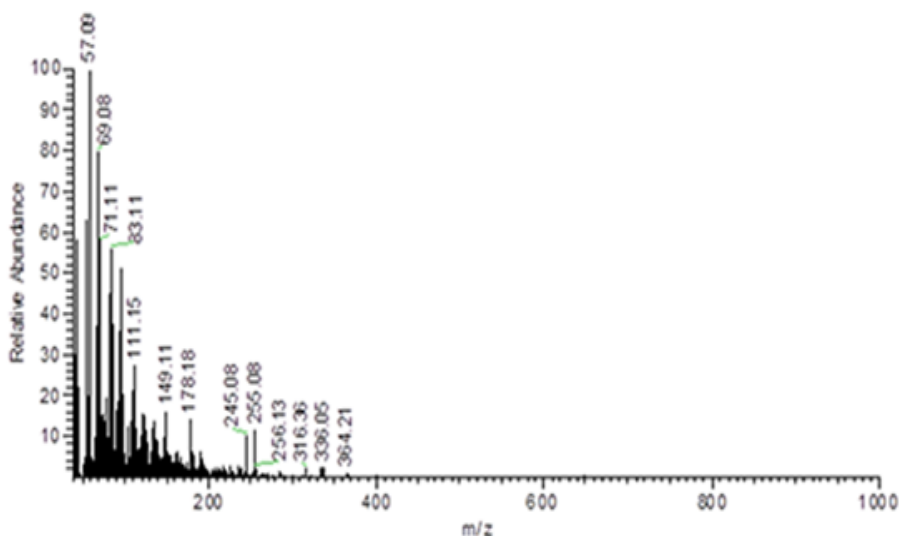


Figure-8. Mass spectroscopy of active purified compound

Comparison of data obtained from spectroscopic analysis and data library from mass spectroscopy suggested that the purified active compound may be janerin group compounds. This group of chemicals well known to have antibacterial activity [33].

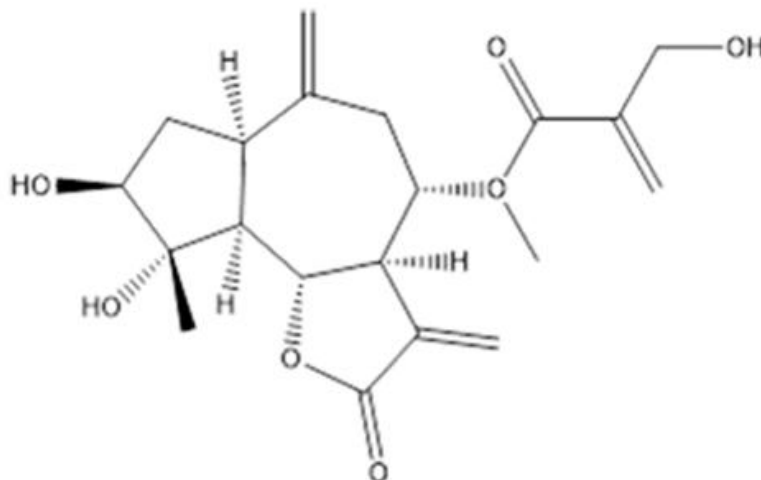


Figure-9. suggested structure of active purified compound (Janerin).

This chemicals group belongs to quinolone group of chemicals that well known to have antibacterial activity targeting DNA gyrase and its inhibition by sets off complex series of events which ultimately causes bacteria to die [34].

Centauraea calcitrapa extract has potential antibacterial action against multi-drug resistant bacterial pathogen. Such as investigation on natural products to cure diseases may create an alternative source of promising medicines. This study might open the possibilities of finding new clinically effective herbal remedy against multi-drug resistant bacterial pathogen.

Funding: This study received no specific financial support.

Competing Interests: The authors declare that they have no competing interests.

Contributors/Acknowledgement: All authors contributed equally to the conception and design of the study. The authors acknowledge to Dr. El-Baraa Mohammed El-Saied "lecturer of plant ecology, Botany and Microbiology, Faculty of Science, Al_Azhar University" for the identification of plant *Centauraea calcitrapa*.

Conflict of Interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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