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Antimicrobial Activities and Cytotoxicity of *Sisymbrium irio* L Extract against Multi-Drug Resistant Bacteria (MDRB) and *Candida albicans*

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A B S T R A C T

The present study was conducted to evaluate antimicrobial and cytotoxicactivity of *Sisymbrium irio* L *extract* against MDRB (*Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Enterobacter cloacae* and *Pseudomonas aeruginosa*) and *Candida albicans*. Antibiotic sensitivity profile was performed using disc diffusion method. Cytotoxicity was measured against African Green Monkey Kidney (VERO) cell line using the colorimetric MTT assay. Antimicrobial activity of aqueous *Sisymbrium irio* L extract showed inhibition activity against tested organisms *Staphylococcus aureus* (17mm), *Enterococcus faecium* (22mm), *Klebsiella pneumoniae* (15mm), *Acinetobacter baumanni* (18mm), *Enterobacter cloacae* (17mm), *Pseudomonas aeruginosa* (15mm), and *Candida albicans* (21mm). The highest activity was observed against *Enterococcus faecium.* This inhibition activity was higher than most of antibiotics used in the study. The crude extract was purified using column chromatography and visualized under UV using Thin Layer Chromatography (TLC). The minimum inhibitory concentration (MIC) values of purified active compound ranged from 31.25 to 125µg/ml while the minimum bactericidal concentration (MBC)was two-fold higher than MIC ranged from 62.5 to 250µg/ml. Cytotoxic activity of purified active compound showed little toxicity withIC50 exceeding400µg/ml after 24hr of incubation. The purified compound was identified using UV, IR, ^{1}HNMR and mass spectroscopy. The analysis of data obtained indicated that it belongs to cyclo hexanone group.

Introduction

The emergence and spread of MDRB have substantially threatened the current antibacterial therapy. Infectious diseases caused by resistant microorganisms are associated with prolonged hospitalizations, increased cost, and greater risk for morbidity and mortality (Preeti *et al.*, 2016).

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, these bacteria include *Escherichia coli* (ESBL-EC), *Klebsiella pneumoniae* (ESBL-KP), carbapenem-resistant Enterobacteri-aceae, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, hospital acquired methicillin-resistant *Staphylococcus aureus* (MRSA), and *vancomycin resistant Enterococcus* (VRE). The main causes of

antibiotic resistance were abuse, inappropriate use of antibiotics in medical and agriculture uses (Gamal, 2015).

Due to the high cost of commercial antibiotics treatments in African countries necessitates the use of medicinal plants for treatment purposes (Swamy and Sinniah, 2015). For many years natural products have played an important role in healthcare and disease prevention. The old civilizations of North Africans have many written indicators for application of herbal medicine in prevention of various diseases (Phillipson, 2001). Nowadays, medicinal plants are registered as official medicines that are certified with all pharmacopoeias (Bahmani *et al.,* 2012). Medicinal plants play a major role in the development of pharmacological research and new drugs (Newman and Cragg, 2007). According to estimation of the World Health Organization (WHO), about 80% of people are still dependent on traditional herbal medications due to their low cost, easy accessibility and likely negligible side effects in comparison to allopathic medicines (Sandhya *et al.,* 2011). Due to the understanding of traditional medical practices for curing diseases has lead to the presence of many active drug molecules of plants and their derivatives in allopathic medicine (Swamy *et al.,* 2015). The discovery of new drugs is governed by natural plant-based compounds and their products, followed by synthetic chemical drugs. This has led towards increased global demand for herbal medicine in the modern era of natural medicine, leading to exploration and exploitation of all plant distribution in different ecological conditions for their different medicinal properties (Kumara *et al.,* 2012).

Medicinal plant based products have many advantages than synthetic chemicals compounds including but not limited to, decreased side effects, activity, efficacy, low cost and availability (Moorthy *et al.,* 2007). Thus, the aim of this study was to evaluate in vitro Antimicrobial and cytotoxic activity of *Sisymbrium irio* L extract against both multidrug resistant bacteria and *Candida albicans* isolated from clinical samples and elucidate the chemical composition of active purified compound through spectroscopic data analysis.

Materials and Methods

Multidrug resistant bacteria (MDRB)

Six multidrug resistant bacterial isolates (*Pseudomonas aeruginosa, Acinetobacter baumannii, Enterobacter cloacae, Enterococcus faecium, Klebsiella pneumoniae and Staphylococcus aureus*) were used in this study. These are local clinical isolates from Egyptian Hospital and identified by the research team from previously published paper (Moghannem *et al.,* 2016). *Candida albicans* was obtained from Regional center for Mycology & Biotechnology at Al-Azhar University, Nasr City, Cairo, Egypt.

Collection of plant materials and preparation of aqueous extract

Plant material was collected from Bahariya Oasis, Egypt [N 28.25 23 E 28.55 51.1] during March 2015 (Figure 1). The identification of plant was done by Dr. El-Baraa Mohammed El-Saied Ph.D. of plant ecology, Botany and Microbiology Department, Faculty of Science, Al_Azhar University".

Plant material was subjected to extraction process summarized in the following steps; first; aerial part of plant was washed with distilled water and then dried by air out of sun reach followed by crushing into powder form. From the dry powder; 15gm was soaked in

150ml of distilled water and then incubated at room temperature for 48hr under shaking 120rpm/min (New Bruunswick Scietific [Edison, N.J, USA]). Then, the crude extract was obtained by centrifugation at 3000rpm (Sigma 2K15) for 10 minutes at 25°C. Then, the clear supernatant was obtained and water was removed using rotary evaporator (Heidolph, 2001).

Crude extract was prepared for antimicrobial assay through dissolving 100mg/ml in distilled water and centrifuged at 10,000rpm to remove the solid residues and stored in refrigerator at 4°C for the next step.

Antimicrobial assay

Antibacterial and antifungal activity was evaluated according to (Perez *et al.,* 1990). The molten Muller Hinton Agar was inoculated with 100µl of test organisms (1.0 X 10^8 CFU/ml) and poured into the sterilized Petri plate (15cm). For disc diffusion method, the paper disc (7mm) was saturated with 100µl of crude extract, allowed to air dry and plated on the surface of seeded agar plate. The plates were incubated for 24hr at 37^{o}C. After incubation; the inhibition zone diameter was measured in millimeter (mm).The experiment was repeated three times.

Purification of the crude extract by column chromatography

The purification of crude extract was performed according to (Masud *et al.,* 2012) with modification of (Moghannem *et al.,* 2016).

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

MIC of purified compound was performed according to (Al-Fatimi *et al.,* 2007). Stock solution of 1000µg/ml was prepared and then two fold dilution was performed until 15.6µg/ml in 96 well flat bottom plate (Sigma-Aldrich, St. Louis, MO,USA) and then 100µl of pathogenic bacteria (1.0 X 10^8 CFU/ml) was added. Ampicillin was used as reference antibiotic. Solvent control 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). The experiment was repeated three times. After incubation; the bacteria was cultured on nutrient agar plate and incubated for 24hr to determine bactericidal effect. The experiment was carried out in triplicate.

Cytotoxicity screening

Cell Line: cell line used during this study was obtained from Tissue Culture Laboratory of Holding Company for Biological Product and Vaccine – VACSERA, Dokky, Agouza, Giza, Egypt.

The Vero cell line was initiated from kidney of a normal adult African green monkey on March 27th, 1962, by Yasummura and Kawakita at the Chiba University, Japan American Public Health Association, 1992). Vero cells were maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2 raM), penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were cultured at 37°C in a humidified 5% CO_2 incubator.

Cytotoxicity assay

The purified fraction of *Sisymbrium irio* L was tested for *in vitro* cytotoxicity, using Vero cells by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Yasumura and Kawakita, 1963). Briefly, 100 µl of media (RMPI 1640) was added into each

of the 96-well plates from row B to row G (triplicate). Then, 100 µl of diluted fraction was added in row A and row *B.* Starting from row B the 200 µl of solution (100 µl drug + 100 µl media) were mixed and 100 µl from row B were added into next row (row C) by using micropipette and a serial dilution was done up to row G. Finally, excessive 100 µl from row G were discarded. The final volume for each well was 100 µl. The cultured Vero/MCF-7 cells were harvested by trypsinization, pooled in a 50ml vial. Then, the cells were plated at a density of 1×10^6 cells/ml cells/well (100 µl) into 96-well micro-titer plates from row B to row G. Finally, 200 µl of cells were added in row H as a control. Each sample was replicated 3 times and the cells were incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µl of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under a microscope. Flowingly, the medium together with MTT (190 µl) were aspirated off the wells, DMSO (100 µl) was added and the plates shaken for 5 min. The absorbance (abs) for each well was measured at 540 nm in a micro-titre plate reader (Mosmann, 1983) and the percentage cell viability (CV) was calculated manually using the formula:

$$CV = \frac{\text{Average abs. of duplicate fraction well}}{\text{Average abs. of control well}} \text{ X } 100\ \%$$

A dose-response curve were plotted to enable the calculation of the concentrations that kill 50% of the Vero cells (IC50).

Characterization and identification of active purified fraction

Spectroscopic analysis of purified active fraction was performed according to (David, 2000) including; ultraviolet (UV (160A-Shimadzu), Infrared IR (Matson Satellite 113 spectrometer), ^{1}HNMR (various Mercury - 300BB/MHz NMR spectrometer) and Mass Spectrum (Direct Inlet part DI-50 to mass analyzer in Shimadzu GC-MS-QP5050 Thermo Scientific Prop). All spectroscopic analysis were performed at Micro analytical unit-FOPCU Cairo University.

Results and Discussion

Antimicrobial activity of plant extract

Egypt is one of main countries for diversity of the genus *Sisimbryum irio* family Cruciferae (Brassicaceae) the distribution of *Sisimbryum irio* in the Nile Delta, region includes the Nile valley, Nile Faiyum, the western and eastern Mediterranean regions, and the Isthmic Desert (Northern Sinia) (Hanaa, 2014).

Antimicrobial activity of *Sisymbrium irio* L extract in Egypt has poor review and studies. Therefore, the aim of this study was to evaluate and identify the antimicrobial and cytotoxic potential of this plant extract against MDRB.

Sisymbrium irio L aqueous extract was active against all tested pathogenic microbial isolates as shown in table 1 and figure 2. Maximum of inhibition activity (22mm) was observed against *Enterococcus faecium. Sisymbrium irio* L has several biological activities including; treatment of coughs and chest congestion, to relieve rheumatism, to detoxify the liver and the spleen, and to reduce swelling and clean wounds. It has analgesic, antipyretic and antimicrobial effects (Bailey and Danin, 1981).

Shabnam *et al.*, 2015 reported that the antibacterial activity of polarity based extract of *Sisymbrium irio* was active to inhibit the growth of majority of the pathogenic bacterial strains. n-Hexane extract of leaves of

Sisymbrium irio inhibited the growth of *K. pneumonia* and *S. epidermidis*. While seed showed marked inhibition against *P. aeruginosa* and *S. epidermidis*.

Ethyl acetate fraction of leaves was active against the bacterial strains of *E. coli*, *K. pneumonia* and *P. aeruginosa*. These are consistent with our results that have been obtained from antimicrobial assay because our study has been performed on the aerial part of plant.

The crude extract was fractionated into 120 fractions (each fraction five ml) and the active fractions start to appear from fraction 26 until 35 as shown in figure 3 were the mobile phase was cyclohexane.

MIC values of active purified compound were ranged between 31.25 to 125µg/ml while MBC ranged from 62.5 to 250µg/ml as shown in table 2. Also the result indicated that Gram-negative bacteria have high resistant than Gram-positive bacteria. This is due to the highly hydrophobic outer membrane that acts as permeability barrier mainly for hydrophilic compounds (Stavri *et al.*, 2007; Doughari and Manzara, 2008).

Starr and Engleberg (2006) demonstrate the maximum antibacterial activity was shown by the n-hexane and ethyl acetate fractions against the Gram positive bacteria *Streptococcus pyogenes* and the Gram negative bacteria *Salmonella enteritidis* (21.3–21.7 mm and 21.0–22.3 mm, diameter of zone of inhibition, respectively). *S. pyogenesis* the most common bacterial cause of pharyngitis, impetigo and serious skin infections involving deep layers such as erysipelas and cellulitis, while Salmonellais one of the major diarrhea-causing bacteria (Smith and Bayles, 2007). Furthermore, the n-hexane exhibited high activity against the food poisoning bacteria *C. perfringens*, while n-hexane fractions was moderately active against *S. aureus*, the most frequent cause of human skin and soft tissue abscesses (Lowy, 1991). All these results are consistent with our purified active fractions that appears with Hexane solvent layer during column chromatography purification.

Table.1 Antimicrobial activity of *Sisymbrium irio* aqueous crude extract against multidrug resistant bacteria and *Candida albicans*

Bacterial Strain	mean diameter of inhibition zone (mm)	Bacterial Strain	mean diameter of inhibition zone (mm)
Staphylococcus aureus	17	*Klebsiella pneumoniae*	15
Enterococcus faecium	22	*Enterobacter cloacae*	17
Acinetobacter baumanii	18	*S. aureus* ATCC 29213	16
Pseudomonas aeruginosa	15	*E. coli* ATCC 25922	21
Candida albicans	21		

Table.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) of active purified compound

Microbial Species	**MIC (µg/ml)**	**MBC (µg/ml)**
Staphylococcus aureus	62.5	125
Enterococcus faecium	31.25	62.5
Acinetobacterbaumanii	62.5	125
Pseudomonas aeruginosa	125	250
Enterobacter cloacae	62.5	125
Klebsiella pneumonia	125	250
S. aureus ATCC 29213	62.5	125
E. coli ATCC 25922	31.25	62.5
Candida albicans	62.5	125

Figure.1 *Sisymbrium irio* L plant

Figure.2 Antibacterial activity of *Sisymbrium irio* crude aqueous extract
C = crude extract, 1 = Amoxicillin/Clavulanic acid (AMC), 2 = Trimethoprime/Sulfomethoxazole(SXT), 3 =Vancomycin (VA), 4 = Erythromycin (E),G=Griseofulvin, CL=Clotrimazole, M=Miconazole, N=Nystatin

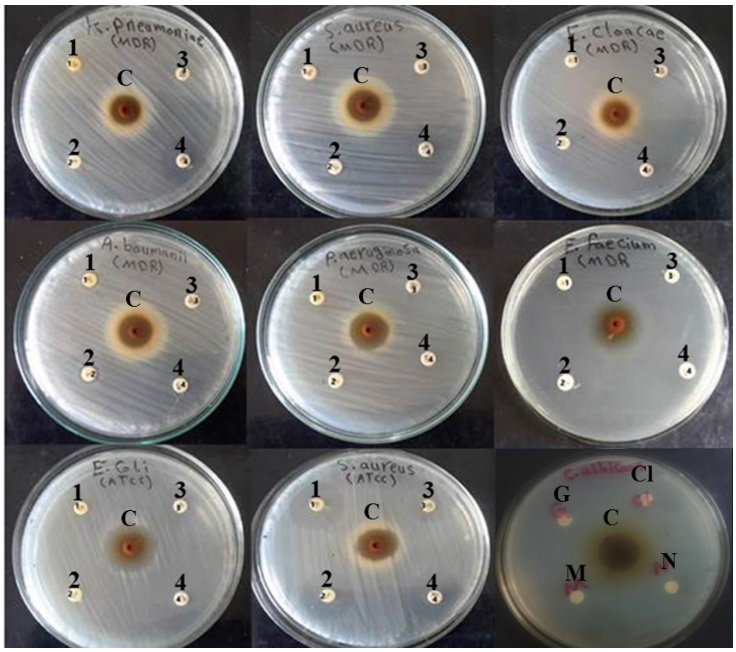


Figure.3 Antibacterial activity of collected fractions from column chromatography

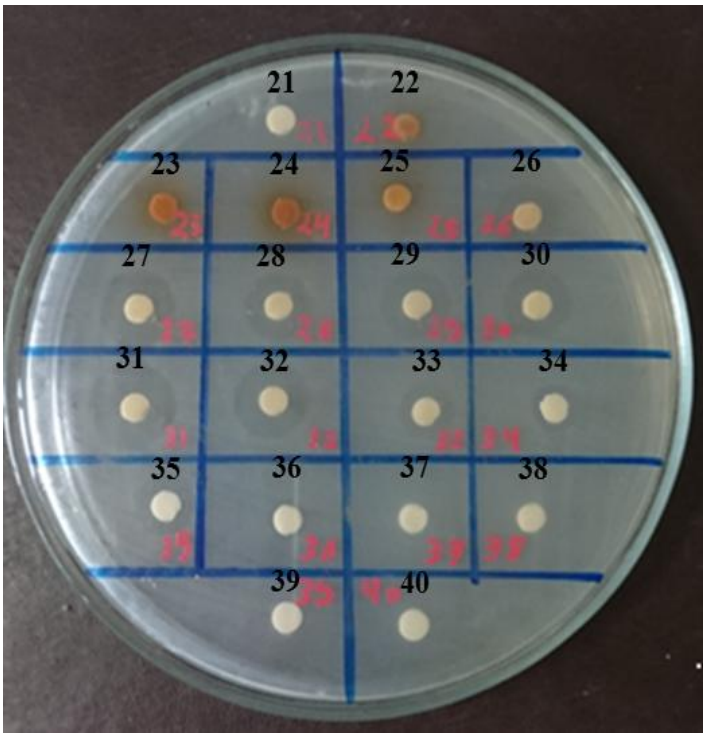


Figure.4 Correlation between antibacterial activity and cytotoxicity of purified compound after 24hr of incubation

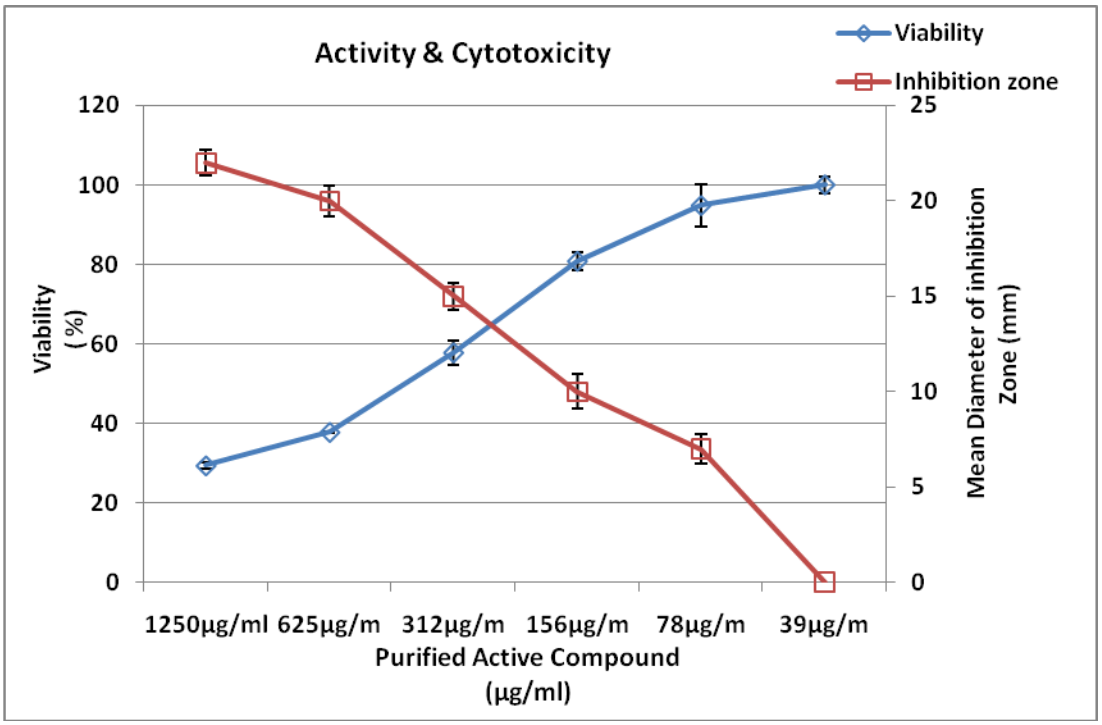


Figure.5 UV spectrum of purified active compound

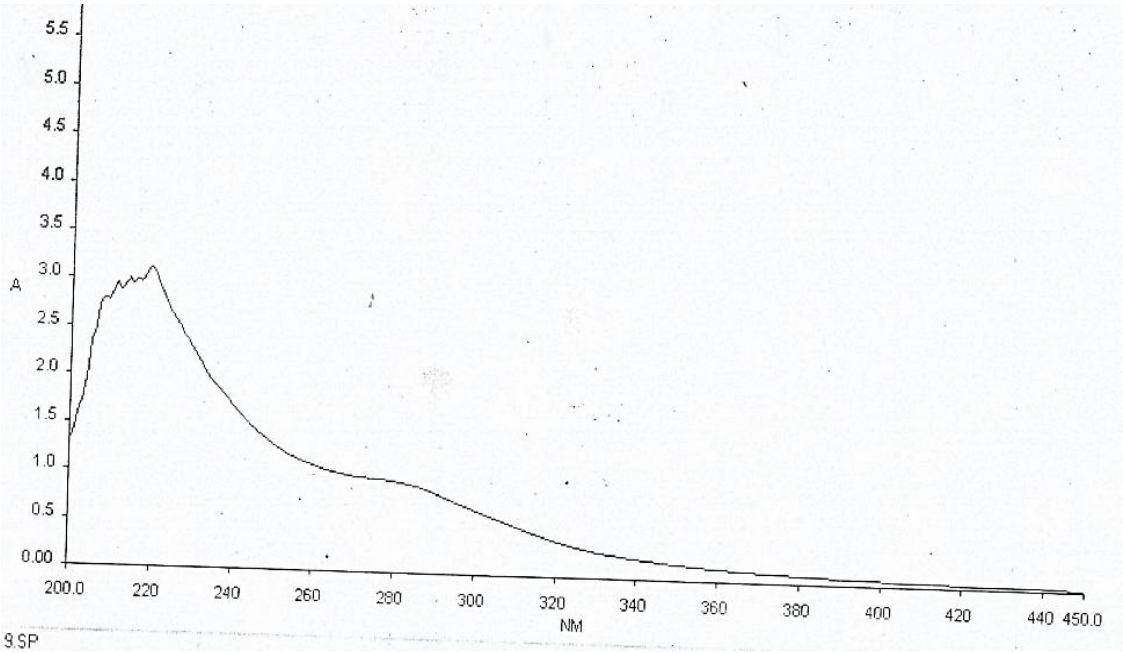


Figure.6 IR analysis of active purified compound

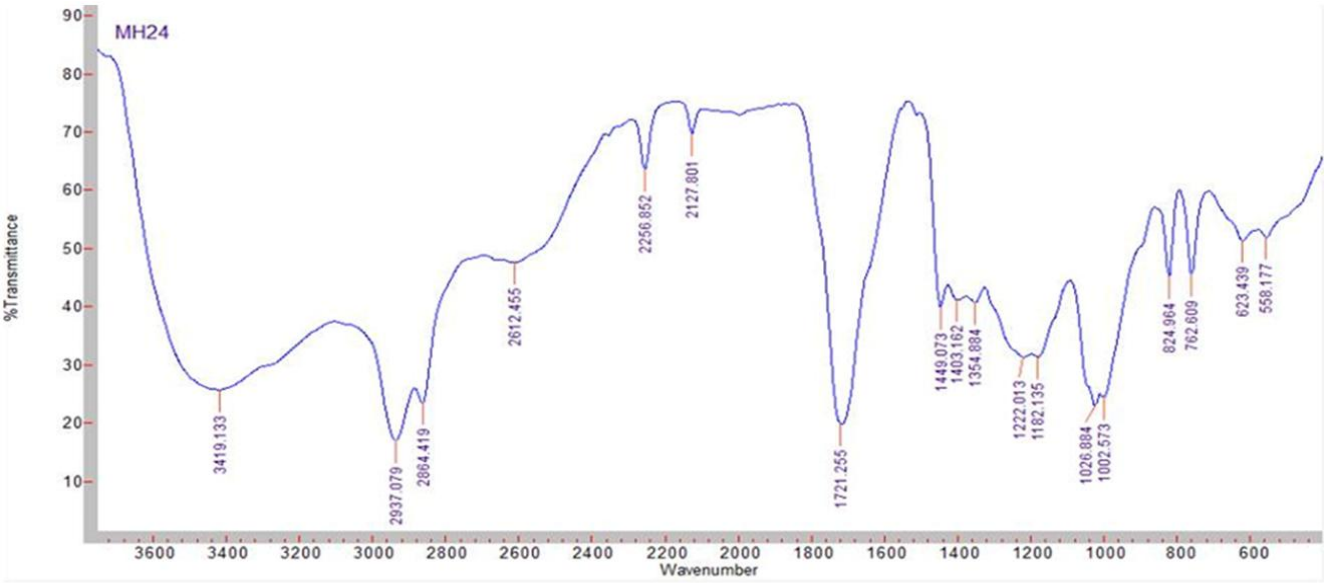


Figure.7 ^{1}H NMR of active purified compound

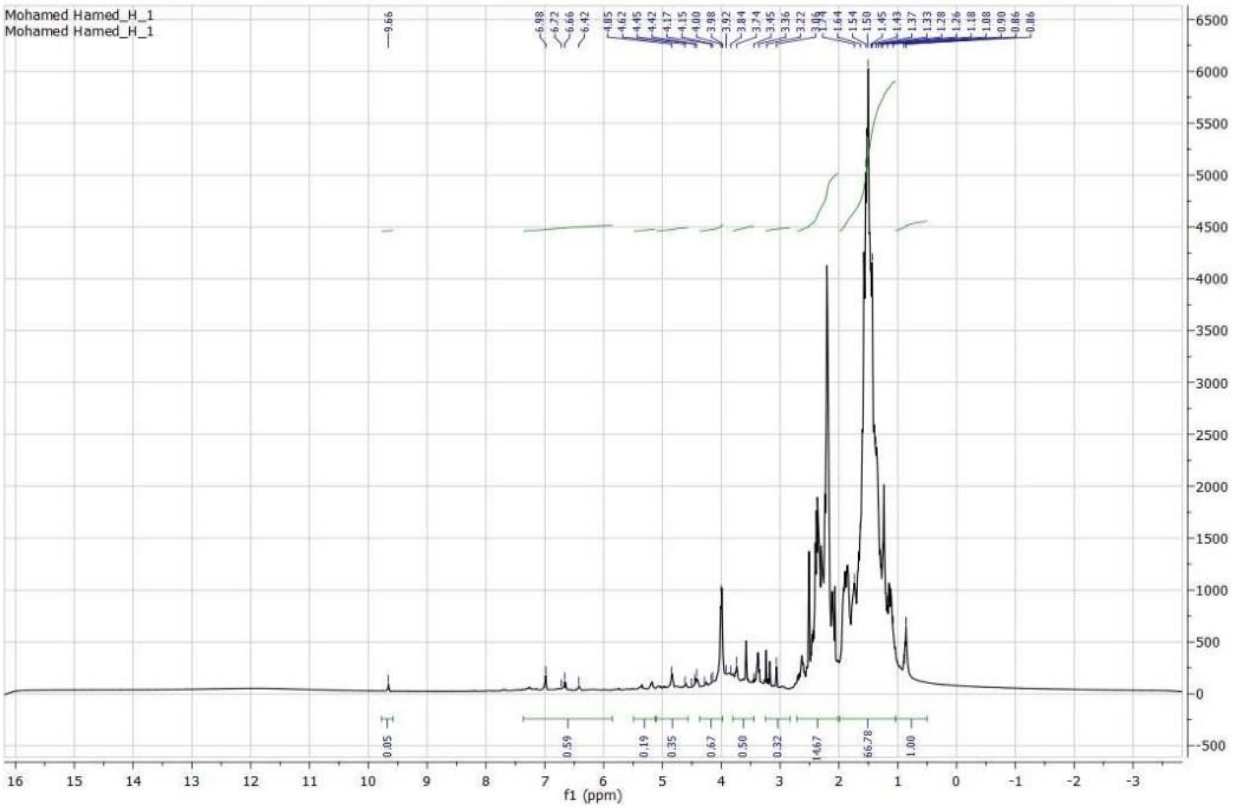


Figure.8 Mass spectroscopy of active purified compound

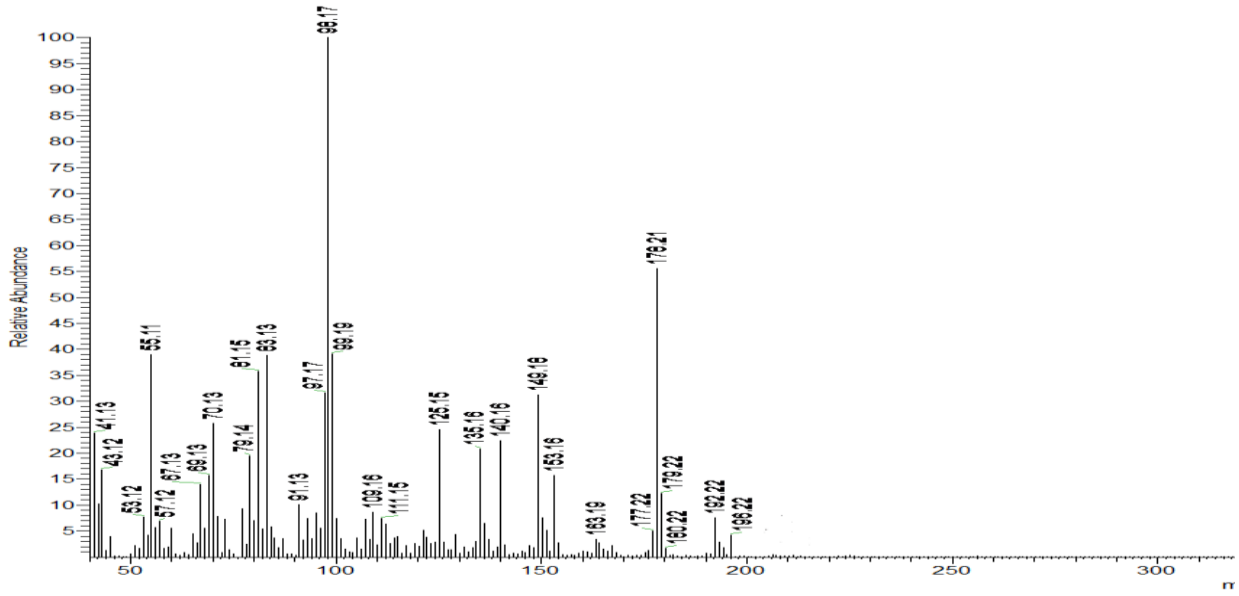
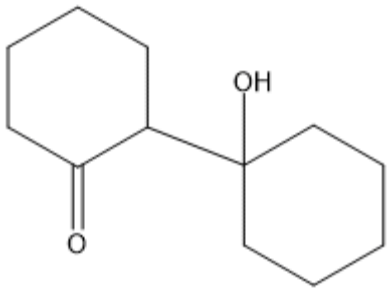


Figure.9 Suggested structure of active purified compound (2-(1-Hydroxycyclohexyl)cyclohexanone)



Cytotoxic activity

Cytotoxicity of purified compound against VERO cell line was studied at different concentrations. The variation of extract activity correlated to cytotoxicity was illustrated in figure 4.

The purified compound has little toxicity with IC50 exceeding 400µg/ml. It was obvious there is strong correlation between antimicrobial activity and cytotoxicity. With increasing concentration, both antimicrobial activity and toxicity increase.

According to the results shown in figure 4 demonstrated a dose-dependent cytotoxic effect against VERO cell line, the purified fraction was obtained from n-hexane layer that means these activities can be attributed, in part, to its hydrophobic character and their ability to penetrate cell membrane more easily (Shah *et al.*, 2014).

There is need for more attention towards research in the use of family Cruciferae for the treatment/prevention of cancers and development of safer and more effective therapeutic agents (Jolene and Michael, 2010). There is a few number of researches

that mention this family of plants has anticancer activities against different types of cancer cell lines including MCF-7 (breast cancer), colon cancer cells. Studies have also shown that apoptosis is the major cause for inhibition of proliferation of colon cancer cells by crucifers (Victor and Ronald, 2008). However, until now, very little information is available about the inhibition of HEp-2 cells by family Cruciferae.

The IC50 values of crude extract is high due to the synergistic effect of various compounds like indoles, isothiocyanates, anthocyanins, nitriles and oxazolidinethiones. Anthocyanins were reported to possess anticancer activity in various cancer types. Thus there is a clear indication of cytotoxicity by components of Cruciferae on laryngeal cancer cells HEp-2.

Spectroscopic analysis of active purified compound

UV spectroscopic

UV- visible spectrum of compound showed that λ max at 225nm which indicate that π→π* transition and 284 nm which indicate that n→π* transition (Fig. 5).

IR spectroscopy

Infrared spectroscopy of purified active compound showed that δ 3419 cm-1 (OH stretching), 2937 and 2864 for (CH aliphatic), 1721 for (C=O), 1449 for (CH and CH_2 bending), 1354 and 1403 for (OH bending), 1182 for (CH in plane bending), 1002 and 1026 for (CH_2 cyclohexane ring vibration) and 824 for (OH-out of plane) (Fig. 6).

^{I}H NMR spectroscopic

The ^{I}H NMR Spectrum of compound showed in figure 7 which recorded in the DMSO d_6 which give (1-2) δ ppm indicate to CH_2 aliphatic in cyclohexane ring, 2.35 δ ppm CH and 4.14 δ ppm for OH (Fig . 7).

Mass Spectroscopy of purified active compound

The mass spectrum (m/z) of purified active compound showed that molecular ion peak at 196(M^+) (m/z) and base beak at 98(m/z), 178(M^+-H_2O), 150[M^+-(H_2O+CO)] which identical with suggested compound (2-(1-Hydroxycyclohexyl) cyclohexanone) (Fig. 8).

Comparison of data obtained from spectroscopic analysis and data library from mass spectroscopy suggested that the purified active compound may be (2-(1-Hydroxycyclohexyl) cyclohexanone) group compounds (Fig. 9). This group of chemicals well known to has antibacterial activity (Raj *et al.,* 2003).

This chemical compound belongs to cyclohexanone group of chemicals that well known to have antibacterial activity (Niloofar and Ali, 2016).

It is concluded that *Sisymbrium irio* extract has potential antibacterial action against multi-drug resistant bacterial pathogen. Discovery of new active drugs from natural origin either to cure or treatment of diseases can create a new era of promising medicines. This study might open the possibilities of finding new clinically effective herbal remedy against multi-drug resistant bacterial pathogen.

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