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The Antibacterial Effect of Phytosterols Isolated from *Echinops heterophyllus* in Comparison with MEBO® and Standard Antimicrobial Agents.

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Abstract:

This study investigated the types of phytosterols that exists in the aerial and root parts of *Echinops heterophyllus* and explored the antibacterial effect of these phytosterols against some pathogenic bacteria in comparison with Moist Exposed Wound Ointment (MEBO®) and gentamicin**.**Plant was extracted and phytosterols were isolated and purified from the crude extract of *Echinops heterophyllus* by using chemical method. Thin Layer Chromatography (TLC) detected the presence of steroids in the aerial and roots part and gas chromatography-mass spectrophotometer GC/MS analysis was used to identify some of these compounds and among them were beta-sitosterol and stigmasterol. Standard dilutions of the phytosterols isolated from *Echinops heterophyllus* were made from 10-100%; its antibacterial effect had been examined by seeded agar method against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The effect of these phytosetrols against *Staphylococcus aureus* was more potent than against *Pseudomonas aeruginosa.*

The minimum inhibitory concentration (MIC) was seventy mg/ml and the minimum bactericidal concentration (MBC) was eighty five mg/ml. This study illustrates that sitosterol isolated from *Echinops heterophyllus* has a greater inhibitory effect on the test bacteria than gentamicine and closer results to MEBO®. It can be concluded in this study is a good step to show the types of phytosterols isolated from *Echinops heterophyllus* and the antibacterial effect of these phytosterols in *vitro* against *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

Key Words: *Echinops heterophyllus, Phytosterols and antibacterial agent.*

تأثير المضاد البكتيري للستيرويدات البنباتية المعزولة من *Echinops* heterophyllus مقارنة مع ®MEBO والأدوية القياسية المضادة للجراثيم

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الخلاصة:

أجريت هذه التجربة لدراسة أنواع الستيرويدات النباتية الموجودة في الجزء الهوائي والجذور لنبتة *Echinops* heterophyllus وتقييم تأثير هذه الستيرويدات النباتية في نمو الجراثيم بالمقارنة مع دهن ميبو ®MEBO والجنتامايسين.

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تم إستخلاص وتنقية الستيرويدات بالطرق الكيمياوية، وتم تطبيق تقنية كروماتوكرافيا الطبقة الرقيقة TLC للكشف عن وجود الستيرويدات في الجزء الهوائي والجذور، كما تم استخدام تحليل GC/MS لتحديد هوية هذه الستيرويدات والتي من بينها كانت beta-sitosterol وstigmasterol .

حضرت تخافيف قياسية 10-100 بالمائة وأختبرت بلستخدام طبق الأكار المزروع بجرثومة المكورات العنقودية الذهبية وجرثومة الزوائف الزنجارية. وجد أن تأثير هذه الستيرويدات ضد جرثومة المكورات العنقودية الذهبية أكثر من جرثومة الزوائف الزنجارية. لقد وجد أن التركيز المثبط الأدنى هو 70 ملغم/مل والتركيز القاتل الادنى هو 85 ملغم/مل.

أثبتت هذه الدراسة أن المركبات الستيرويدية المستخلصة من *Echinops heterophyllus* لها تأثير مثبط أكبر من الجنتمايسن على الجراثيم المستخدمة في التجربة ، لكن كان تأثيرها مقارب للـ MEBO® .

تعد هذه الدراسة خطوة مهمة في تسليط الضوء على انواع الستيرويدات في هذه النبتة وأهمية النبتة كمضاد حيوي ضد جرثومة المكورات العقودية الذهبية وجرثومة الزوائف الزنجارية.

Introduction:

Moist Exposed Wound Ointment (MEBO®) is the basis of MEBT (Moist Exposed Burn Therapy). It has been popularized two decades ago and used to treat burned wounds. The main active ingredient is Phytosterols. Bacteria are the most common pathogen found in burn wounds. Organisms originate from the patient's own skin, gut and respiratory flora, as well as from contact with contaminated health care environments and workers [1, 2]. Gram-positive bacteria are the first to colonize burns, followed immediately by gram-negative bacteria [3]. The main bacterial species that infect wounds are methicillin-resistant *Staphy-lococcus aureus* (MRSA) and *Pseudomonas areuginosas*. These two species have proven particularly difficult to treat because they possess a large number of virulence factors and antimicrobial resistance genes [4].

Echinops heterophyllus is from the daisy family *Asteraceae* is an Iraqi plant found mainly in the northern parts of Iraq especially in Erbil and Sulaimani. In Hanara village and surrounding area in Wadi Bastora and shaklawa in Erbil governorate, the plant is called (Shakroka). The term (Shakroka) is come from that the circle-like part of the plant, before getting harder in the late spring, is eaten and the taste is sweet, therefore, it is called shakroka. Shakr means sugar, Shakroka sweet like sugar in the local Iraqi language [5].

This plant is found to have MEBO®'s same magical ingredient "phytosterols" mainly sitosterol. Phyto-sterols are structurally related to cholesterol but differ from cholesterol in the structure of the side chain [6]. They consist of a steroid skeleton with a hydroxyl group attached to the C-3 atom of the A-ring and analiphatic side chain attached to the C-17 atom of the D-ring. Sterols have a double bond, typically between C-5 and C-6 of the sterol moiety, whereas this bond is saturated in phyto-stanols.

The most common phytosterols are sitosterol (3β-stigmast-5-en-3ol), sitostanol (3β,5α-stigmastan-3-ol), campesterol (3β-ergost-5-en-3-ol), campestanol (3β,5α-ergostan-3-ol), stigmasterol (3β-stigmasta-5,22-dien-3-ol) and brassicasterol (3β-ergosta-5,22-dien-3-ol).

Phytosterols are high melting powders; phytosterol esters are chemically stable materials, having comparable chemical and physical properties to edible fats and oils. Phytosterols are insoluble in water, but soluble in non-polar solvents, such as hexane, iso-octane and two propanol. The esters are also soluble in vegetable fats and oils [3,7].

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Sitosterols are white, waxy powders with a characteristic odor. They are hydrophobic and soluble in alcohols [8]. It is widely distributed in the plant kingdom and found in *Nigella sativa*, *Serenoa repens* (saw palmetto), *Pygeum africanum*, sea-buckthorn, wolfberries, *Mirabilis jalapa, Cannabis sativa*, *Urtica dioica* and *Wrightia tinctoria* [9, 10]. Therefore, this study was designed to investigate the existence of phytosterols mainly sitosterols in Iraqi *Echinops heterophyllus* and their types with different chromatography methods and to explore the antibacterial action and the ability of purified and isolated sitosterols from this plant and compare it with MEBO® and gentamicin.

Materials and Methods:

Plant Material:

The whole plant of *Echinops heterophyllus* was collected from a small village named Nazali, in the Kurdistan region of Iraq. The plant was authenticated by Dr. Abdul-hussien Alkhait an Iraqi specialist in plant taxonomy.Aerial parts and roots were collected during the months of May and June and were washed, dried at room temperature in the shade, then milled mechanically and weighed.

Phytochemical study:

1 - **Preliminary phytochemical screening of phytosterols** [11]:

Chemical tests were carried out using ethanol extract of *Echinops heterophyllus*

A- Liebermann-Burchard test: extract 3ml was treated with chloroform, acetic anhydride and drops of sulphuric acid was added. The formation of a dark pink indicates the presence of steroids.

B- H_2SO_4 test: the development of a greenish color was considered as indication of the persistence of steroids, when 2 ml of the ethanol extract was treated with sulphuric and acetic acids.

2 - **Extraction and fractionation of sitosterols:**

120, 200, 500 grams of grinded aerial parts and roots separately were defatted with hexane for 24 hours then allowed to dry at room temperature. Then, the defatted plant material was extracted with (1.250, 3 and 2) L of 80% ethanol in a Soxhlet apparatus until complete exhaustion. The alcoholic extract was evaporated to dryness under reduced pressure at a temperature not exceeding 40°C to give a dark greenish residue to be the crude fraction. Later, four different fractions were obtained according to Harborn's guide to modern techniques of plant analysis [11]. Afterward, fraction 4 was examined using Thin Layer Chrom-atography Techniques using three different mobile phases [12, 13].

S1s= Chloroform: Methanol (10:1)

S2s= Hexane: Ethylacetate (1:1)

S3s= Chloroform: Ethylacetate (4:1)

3 - **Identification and characterization of the isolated sitosterols by GC-MS spectrophotometry:**

GC-MS-QP 2010 Ultra Shimadzu instrument model: AOC-2 Oi, column (30 mx 0.25µm) from vertical chromatography Co., LTd. was used. Mobile phase: Helium ''He'' gas (99.99 purity), flow speed was 0.8ml/ min, split ratio was 10:1, sample temperature: 280°C. Column temperature from 240°C and rose up to 265°C at the rising speed of 10 C°/Min, and remained at 265°C for 40 Min. Ionization mode was EI+. Electron energy was 70eV. Interface temp was 250°C. Ion source temp was 200°C, detection voltage was 350V, sample lading: 0.5µl [12-13].

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Antibacterial study:

Preparation of Standard dilutions of sitosterol from *Echinops heterophyllus*:

Dilutions were prepared through mixing the desired concentrations (10%-100%) of the plant extract and complete them with nutrient broth. Final volumes were completed to 10ml.

Bacterial culture:

Bacterial stock cultures previously isolated from skin infections of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were prepared. Bacteria was activated , then each type of bacteria was transferred into four sterilized tubes containing brain agar infusion broth then incubated for 24-72Hrs at 37°C. Total bacterial count was measured using spectrophotometer; the percentage of light transmittance was 26% at a wave length of 580 nanometer while the light transmittance was 100% for the nutrient broth used to prepare the bacteria [14-15].

In *vitro* experiment

Identification of the experimental bacteria:

Studying the culture properties using mannitol salt agar to distinguish *Staphylococcus aureus* bacteria, while macConkey agar was used to distinguish *Pseudomonas aeruginosa*. [16]. Also, the microscopic properties were studied using gram stains. Also, a number of biochemical tests were including gelatinase test , Oxidase test, Catalase test and finally the pigment production test.

Antibacterial effect of sitosterols from *Echinops heterophyllus:*

Sensitivity test was conducted through seeded agar method [17]. 0.6ml of the bacteria stock (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) was seeded in to 100ml of nutrient agar at 45°C in a final concentration of 10^6 CFU/ ml and poured into a depth of 4mm into sterile petri-dishes. Then, three wells if 6mm diameter were made on the surface of each agar plate and finally these wells were filled with different concentrations of the plant sitosterol from 10-100%. These plates were incubated at 37 °C for 24 hours; the presence of zones of inhibition was regarded as the presence of antimicrobial activity. Results were determined by measuring the diameter of these inhibition zones with a ruler [18]. In addition, Moist Exposed Wound Ointment (MEBO®) was used in comparison with sitosterol of *Echinops heterophyllus*. Standard dilutions of (MEBO®) were prepared from 10-100% using standard ethylene glycol solution which is inactive against microorganisms [19], same method was used for gentamicin and tested for antibacterial activity.

Examination of the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) of sitosterols from *Echinops heterophyllus* :

As stock solutions for sitosterol of *Echinops heterophyllus* were diluted to concentrations of(2.5,5,10,30,40,50 and 60%) with nutrient broth, and then 1ml of *Staphylococcus aureus* suspension in phosphate buffer saline. Tubes were completed to a volume of 5ml through adding nutrient broth, the plant Final concentrations were (0.5, 1, 2, 6, 8, 10 and 20) % respectively. After manual shaking for few minutes, the tubes were incubated for 18hours at a temperature of 37°C. The growth of the bacteria was determined visually.

The (MIC) is represented by the first clear tube while the (MBC) is that which when cultured on nutrient agar and incubated for 27 hours at 37°C shows the absence of bacterial growth [20-21].

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Results:

Phytochemical study:

The results of the phytochemical screening of the areal part and root extract (table-1), showed the presence of a number of plant steroids.

Stigmasterol and beta- sterol standards have very closer R_f value and they appeared as a single spot match with spots of both standards in three different developing solvent systems (S1s, S2s, S3s).

Table-1: R_f values of steroids (stigmasterol and β-sitosterol) obtained from different plant parts and their standards in different mobile phases of Thin Layer Chromatography (TLC).

Compound	S_{1s}	S_{2s}	S_{3s}
Stigmasterol standard	0.75	0.8	0.83
β-Sitosterol standard	0.73	0.85	0.88
Steroid isolated from aerial Part	0.75	Upper spot 0.93 Lower spot 0.8	Upper spot 0.89 Lower spot 0.88
Steroid isolated from root	0.75	Upper spot 0.93 Lower spot 0.79	one spot 0.86

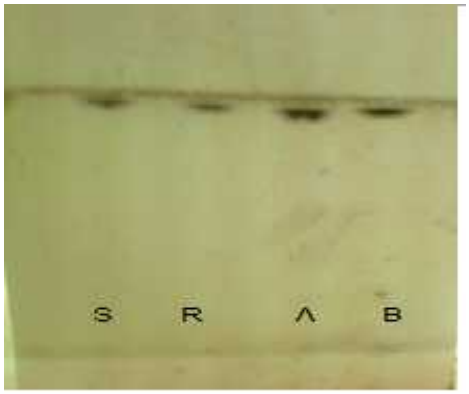

Figure-1: TLC of fraction four (F-4) for different *Echinops heterophyllus* parts (aerial parts, roots) using silica gel GF_{254nm} as adsorbent and S_{1s} as a mobile phase. Visualization by Liebermann-Bur chard spray reagent, followed by heating for 10 mints at 105 ˚C.
S=Stigmasterol standard. B=Beta-sitosterol standard. A=Aerial parts. R=Root part.

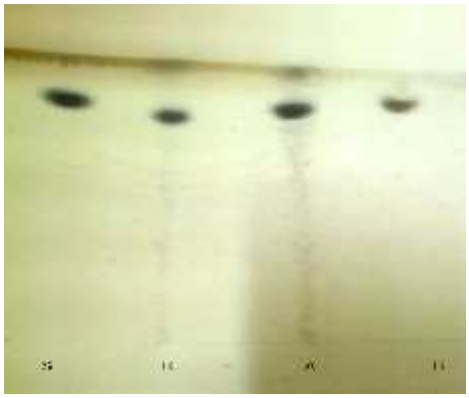

Figure-2: TLC of fraction four (F-4) for different *Echinops heterophyllus* parts (aerial parts, roots) using silica gel GF_{254nm} as adsorbent and S_{2s} as a mobile phase. Visualization by Liebermann-Bur chard spray reagent followed by heating for 10 mints at 105 ˚C.
S=Stigmasterol standard. B=Beta-sitosterol standard. A=Aerial parts. R=Roots.

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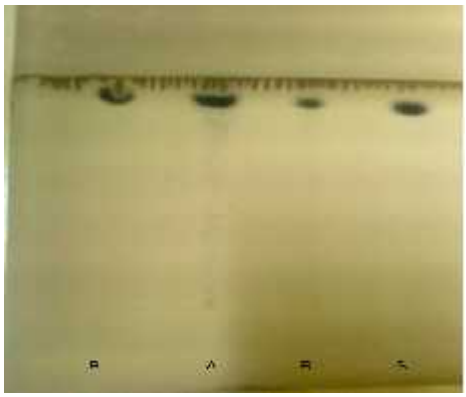

Figure-3: TLC of fraction four (F-4) for different *Echinops heterophyllus* parts (aerial parts, roots) using silica gel GF_{254nm} as adsorbent and S_{3s} as a mobile phase. Visualization by Liebermann-Bur chard spray reagent, followed by heating for 10 mints at 105 ˚C.
S=Stigmasterol standard. B=Beta-sitosterol standard. A=Aerial parts. R=Roots.

Identification of steroids by gas chromatography–mass spectrometry (GC-MS) analysis:

The GC-MS spectrum of aerial plant parts (figures 4 and 5) exhibited a prominent molecular ion peak at m/z 413 $[M]^+$ that correspond to molecular formula of stigmasterol (C29H48O). Ion peaks were also observed at m/z 380, 352, 303, 300,271,213, 199, 133, 97, 83, 43. In addition to ion peak at m/z 415 $[M]^+$ that correspond to molecular formula of β-sitosterol (C29H50O) and other prominent peak appeared at m/z 330 which is characteristic for sterols with C5-C6 double bond.

The GC-MS spectrum of plant roots (figures 6 and 7) exhibited the same results obtained from the aerial parts (i.e. a prominent molecular ion peak at m/z 413 $[M]^+$ that correspond to molecular formula of stigmasterol and other peak at m/z 415 $[M]^+$ that correspond to molecular formula of β-sitosterol with a fragmentation pattern characteristic for sterols.

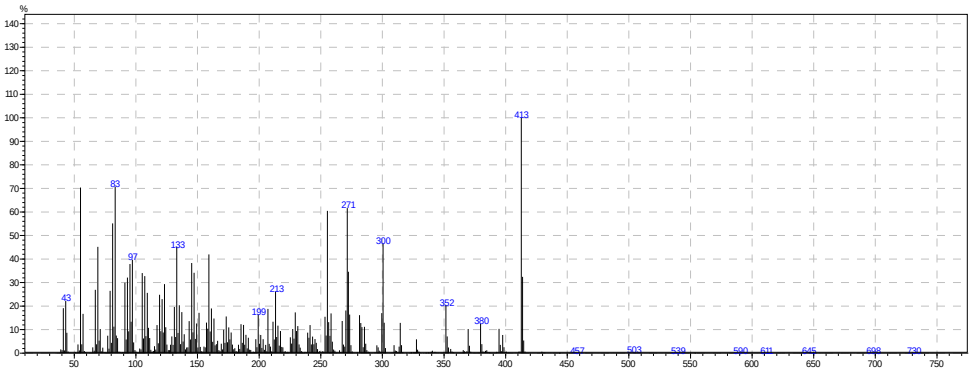

Figure-4: GC-MS analysis of aerial parts of *Echinops heterophyllus* that exhibited a prominent molecular ion peak at m/z 413.

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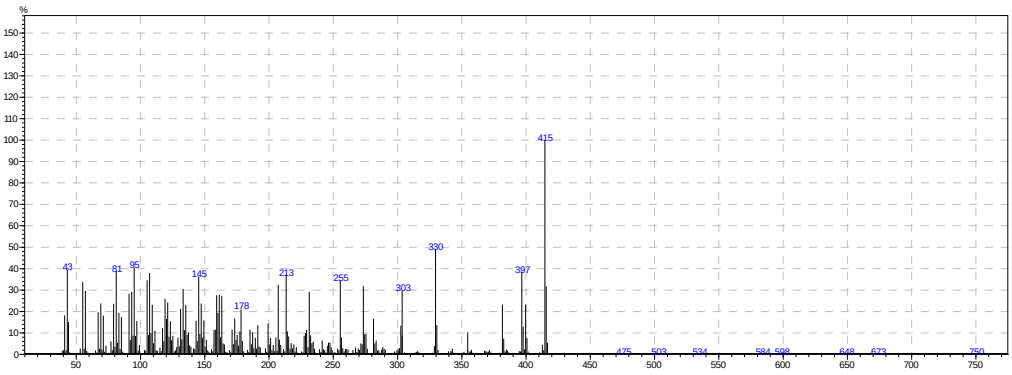

Figure-5: GC-MS analysis of aerial parts of *Echinops heterophyllus* that exhibited a prominent molecular ion peak at m/z 415

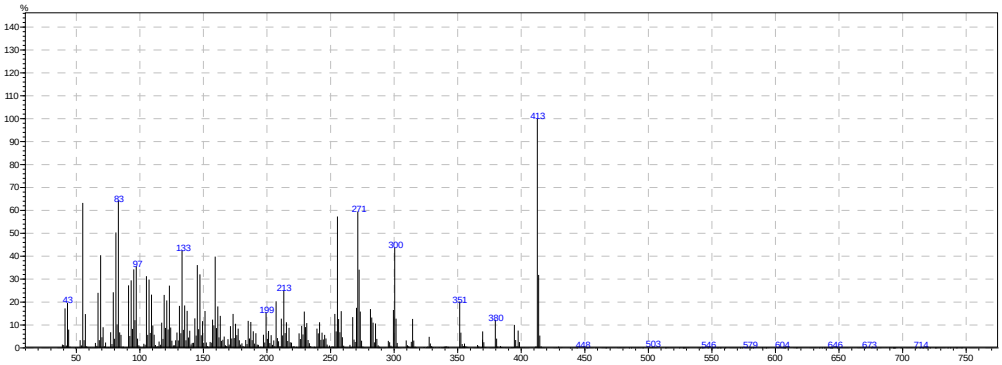

Figure-6: GC-MS analysis of root parts of *Echinops heterophyllus* that exhibited a prominent molecular ion peak at m/z 413.

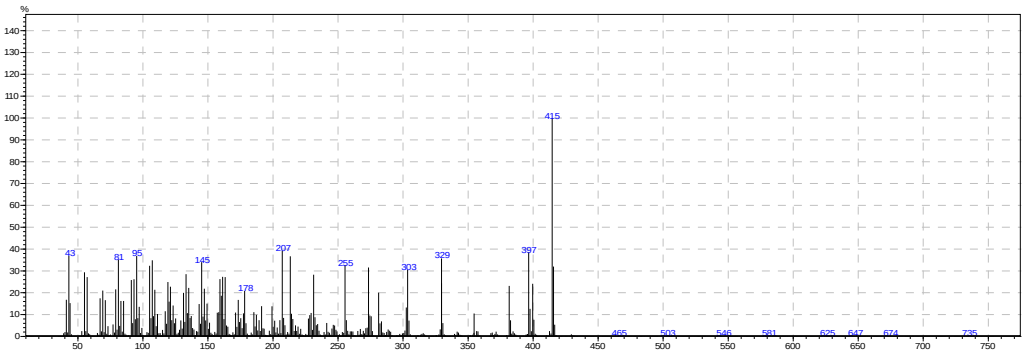

Figure-7: GC-MS analysis of root parts of *Echinops heterophyllus* that exhibited a prominent molecular ion peak at m/z 415.

In *vitro* experiment:

Bacterial growth, Microscopic and biochemical results:

Morphological examination of the bacteria showed two types of cells, G+ bacteria which were spherical in shape which resembles *Staph.aureus*, while the other type of cells were G- bacteria which were rod in shape, and both types of bacteria given positive result for Gatalase and

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Gelatinase test, the *Staph. aureus* given negative result for Oxidase test with golden color while the *Pseudo.aeruginosa* given a positive result for Oxidase test with green color.

Sensitivity Test:

Results showed that both sitosterols from *Echinops heterophyllus* and (MEBO) had inhibitory effect on the experiment bacteria as in the charts 1, 2, 3, 4 and 5. Figures 8 and 9.

The sensitivity of the previously mentioned bacteria gradually increased with the increase of the increase of concentrations. Results were verified in the following tables and figures.

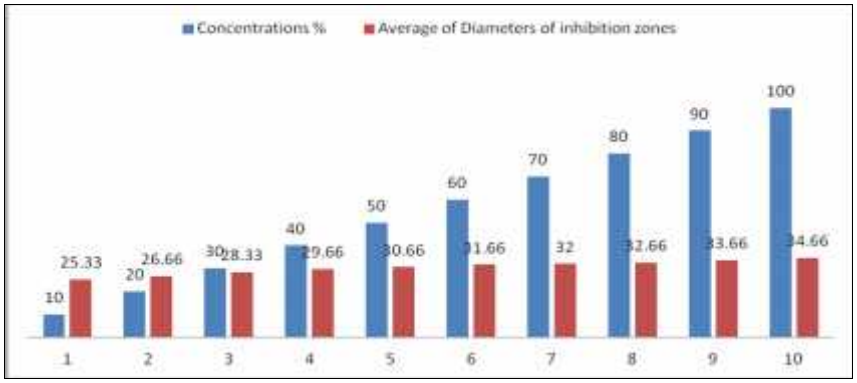

Chart-1: In *vitro* activity of sitosterols from *Echinops heterophyllus* against *Staph.aureus* measured by the diameter of inhibition zones against concentrations. Statistical analysis showed there was a significant difference between each concentration P<0.05.

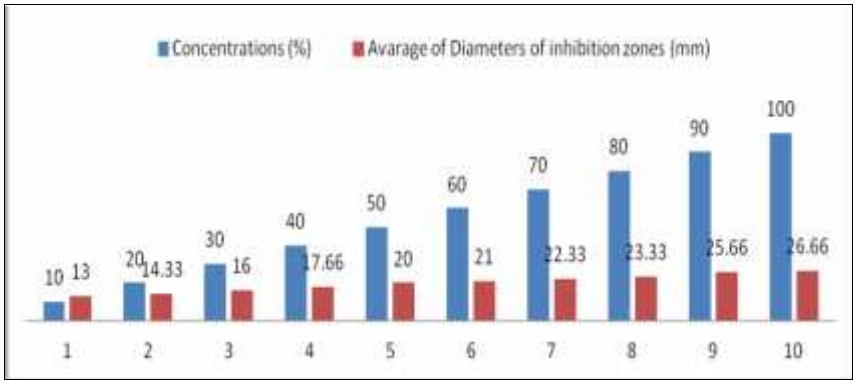

Chart-2: In *vitro* activity of sitosterols from *Echinops heterophyllus* against *Pseudo. aeruginosa* measured by the diameter of inhibition zones against concentrations. Statistical analysis showed there was a significant difference between each concentration P<0.05.

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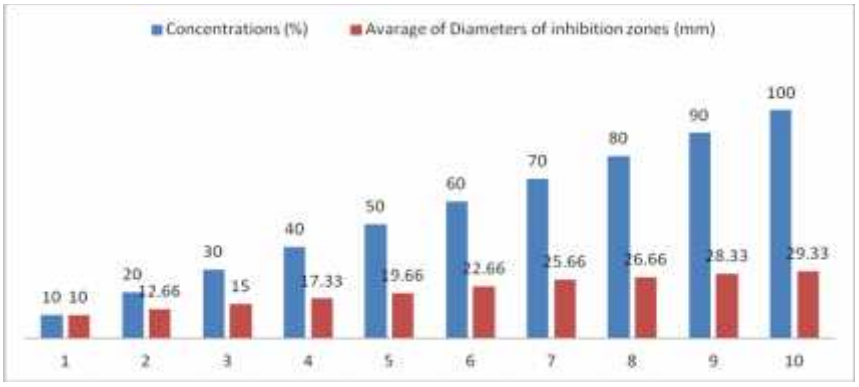

Chart-3: In *vitro* activity of MEBO® against *Staph. aureus* measured by diameter of inhibition zones against concentrations. Statistical analysis showed there was a significant difference between each concentration P<0.05.

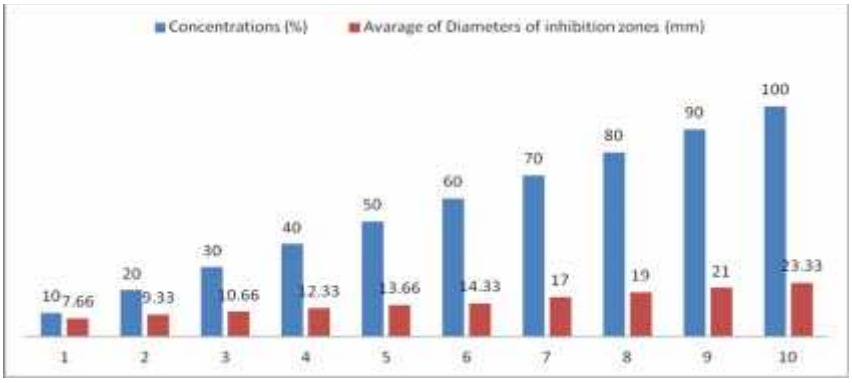

Chart-4: In *vitro* activity of MEBO® against *Pseudo. aeruginosa* measured by diameter of inhibition zones against concentrations. Statistical analysis showed there was a significant difference between each concentration P<0.05.

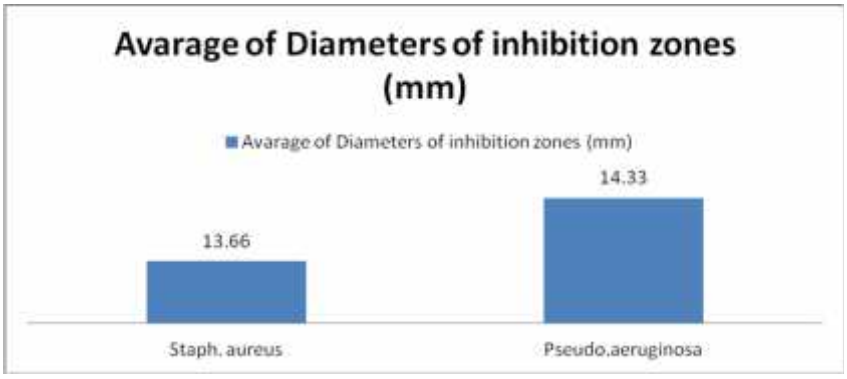

Chart-5: In *vitro* activity of gentamicin (30µg) against the test bacteria.

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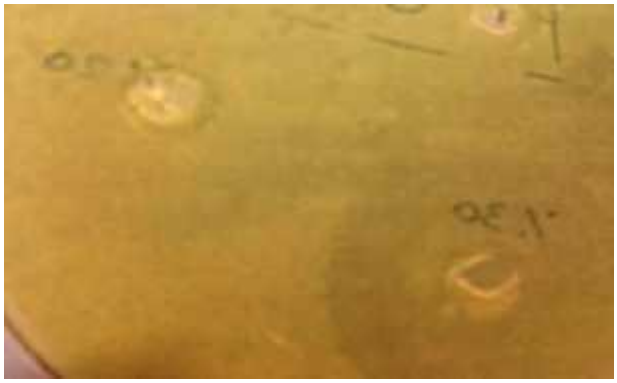

Figure-8: The activity of different concentrations of sitosterols from *Echinops heterophyllus* against *Staph.aureus* measured by the diameter of inhibition zones against concentrations.

Figure-9: The activity of different concentrations of sitosterols from *Echinops heterophyllus* against *Pseudo. aeruginosa* measured by the diameter of inhibition zones against concentrations.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) of sitosterols from *Echinops heterophyllus* against *Staph.aureus:*

The Minimal Inhibitory Concentration (MIC) was 70 mg/ml while the Minimal Bactericidal Concentration (MBC) was 85 mg/ml.

Discussion:

The existence of sitosterol in *Echinops heterophyllus* and studying its antimicrobial activity in *vitro* was reported for the first time in this study.

In a previous study we were able to prove the wound healing activity of the crude extract of Iraqi *Echinops heterophyllus* in *vivo*. As it defeated all inflammatory signs and was able to protect the wound area from secondary wound infections [22]. β - sitoterols were proven to possess a strong anti-inflammatory properties which might be one of the reasons that the whole extract gave a stronger effect than the bioactive fractions[20].

Therefore, this study was designed to investigate the types of phytosterols present in this plant.

The result of TLC in different part of *Echinops* plant (aerial parts, roots) shows the existence of sitosterols in *Echinops heterophyllus*. Since, the TLC does not give

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a clear idea about the content of the steroidal compounds in fraction-4. Therefore, GC-MS was used to identify and clarify the steroidal compounds in these two parts.

The strong peaks at (m/z 271 and m/z 415) which appeared in GC-MS spectrum of aerial plant parts might attributed to the formation of carbocation by β bond cleavage of the side chain leading to the loss of C10H21 that corresponds to the M-141, which comes in agreement with reported values of the structure of stigmasterol [1, 13,23]. Also, there were another sharp peak appeared at m/z 330. This peak has the characteristic for sterols with C5-C6 double bond. Other peaks were also found in conformity with those reported for beta-sitosterol [6, 13, 23, 24].

The difference between stigmasterol and beta- sterol is the presence of C22=C23 double bond in the first one and C22_C23 single bond in the later one [25].

The results of Sensitivity test was carried out to determine the antibacterial activity of sitosterol isolated from *Echinops heterophyllus* against *Staph. aureus* and *Pseudo. aeruginosa*. These results where compared with MEBO and gentamicine. This antibacterial effect might be attributed to the existence of sitosterols. As Phytosterols are known to possess a strong antimicrobial activity [26].

The purpose of using different concentrations was to obtain a broad image of the effect of different concentrations on the growth of pathogenic bacteria and the proportion between the increases in concentrations with the increase of size of zones of inhibition, which gave a positive ascending result (Chart 1, 2 and Figure 8, 9) This result may refer to the activity of phytosterols. This comes in agreement with what Alkhayyat mentioned in his M.S.c thesis [17].

In the present study sitosterol from *Echinops heterophyllus* has possessed greatest inhibitory effect on *Staph.aureus* this might be regarded to the absent of the outside protective capsule, while, *Pseudo. aeruginosa* was more resistant to sitosterol isolated from *Echinops heterophyllus* might attributed to this bacteria contains an outside capsule to as protection from antimicrobial agents [27].

Results in Chart (1, 2, 3, 4 and 5) illustrates that sitosterol isolated from *Echinops heterophyllus* has a greater inhibitory effect on the test bacteria than gentamicine and closer results to MEBO®. This might be attributed to the high amounts of β-sitosterols in this plant which are known to possess a very high antibacterial effect and it is the key antibacterial ingredient for Mebo® [28].

Conclusion:

This study investigates the types of sitosterols exist in this plant. As β-sitosterol and stigmasterol, both appeared as main sterol components in the steroidal fraction of both aerial and root part of Iraqi *Echinops* species.

From the high peaks of the GC-MS results concluded the plant contains high quantities of β-sitosterol and stigmasterol. The microbial studies of the sitosterols phytochemical compounds showed the most antimicrobial properties indicating the potential of the discovery of novel drugs from plants.

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