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Novel serial extraction method for antibacterial and antifungal evaluations of the entire *Eryngium campestre* L. plant from Jerusalem/ Palestine

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ABSTRACT

It is noteworthy that the medications isolated directly or indirectly from phytogetic products until the recent time play a primary part in the discovery of drugs. The huge utilizations of antimicrobial agents in medicine have caused directly the development of antibiotics resistant pathogens in various infectious diseases areas, urging the detection for new and effective antimicrobial drugs. This study will be the first of its kind which is designed to evaluate antibacterial and antifungal activities and to estimate exhaustive extraction yields of the aqueous and organic extracts of *Eryngium campestre* L. plant. Extraction yields estimated by using serial exhaustive extraction non thermo-reactive procedures and well diffusion method were used to evaluate antibacterial and antifungal activities of aqueous and organic extracts of the Field Eryngo entire plant (*E. campestre*) while the minimum inhibitory concentration and minimum bactericidal-fungicidal concentration were determined by the serial dilution method. The aqueous extract showed antimicrobial activity by using well diffusion method against all gram-positive bacteria with the greatest activity against *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. In addition to that the results showed antibacterial activity against one of the gram-negative (*Pseudomonas aeruginosa*) and against *Candida albicans* a fungi. The entire *Eryngium campestre* L. plant has antimicrobial activity, and we recommend exhaustive serial extraction method to get high concentration of the phytochemical yields with potential activity.

Keywords: *Eryngium campestre*, Serial extraction, Antimicrobial

INTRODUCTION

The plant kingdom represents an endless reservoir of the physiological, pharmacological and biological active phytochemical compounds with various chemical structures. These compounds have preventive or protective properties against various diseases. Herbal medicines are the oldest and the most widely used system of medicines which can heal every illness known to the humans if the scientists pool the folk knowledge from several traditions [1]. The lack of the effective and safe antibacterial and antifungal compounds leads to conversion of studies towards new scientific strategies such as reconnaissance of folk ethnopharmacological knowledge for the expansion of efficient and safe antibacterial and antifungal compounds [2]. Plants ingredients are an important and endless source of numerous secondary metabolic phytochemical compounds which can be isolated for therapeutic purposes. These secondary metabolites phytochemicals founded in small quantities in subterranean and aerial plants parts, include the tannins, steroids, glycosides, alkaloids, anthraquinones, flavonoids, isopenoids, and many others [3] .

Antimicrobial agents widespread from plant sources have always been of great interest to scientists working on health threatening infectious diseases. Over the past decade there has been an explosion of interest in the antibacterial and antifungal activities of the herbal products [4]. The genus *Eryngium*, belonging to the subfamily Saniculoidea of Apiaceae family, was represented by 317 accepted taxonomy worldwide [5].

Eryngium campestre L. plant has other names such as hundred headed thistle, Field Eryngo, Watling street thistle and Dane weed. The plant is glaucous perennial pale green herbaceous flowering plant growing to 60cm. It grows in dry rough grassland near coast, roadsides and waste places in the South Western areas of Asia, North Africa, Holland, Britain, Germany and North America. The plant leaves are basal 5-20cm long, leathery, subcordate at base stalked, tough, coriaceous, clasping the stem, cauline-sessile, pinnately divided with broad spiny-margined semiamplexicaul base. The flowers are hermaphrodite pale blue or white which form in numerous pedunculate, ovoid capitula, rigid teeth with narrow erect petals which is excurrent as a stout spine (Fig. 1, Fig. 2) [6-8].



Fig.1: *Eryngium campestre* L. Adapted from J R Crellin 2007

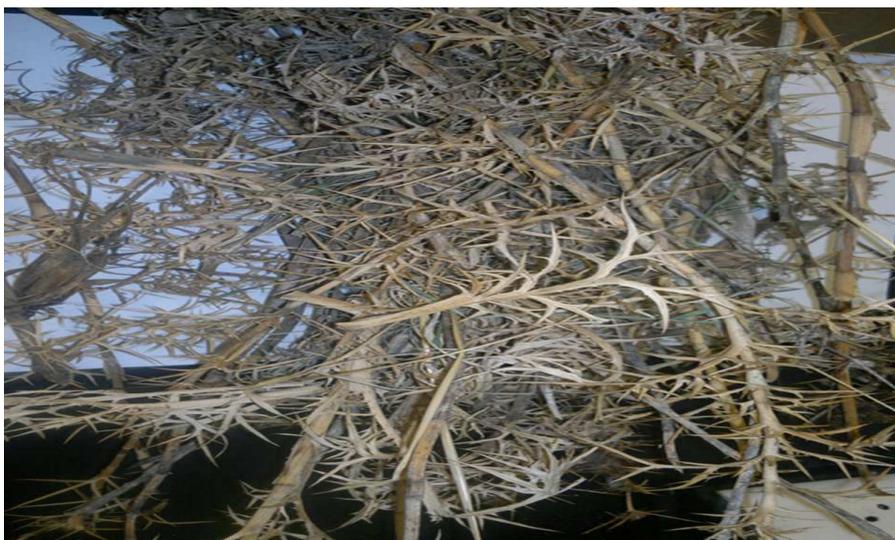


Fig. 2: *Eryngium campestre* L. dry plant of (our sample)

Eryngium campestre (Field Eryngo) plant contain a mixture of volatile oils as α -Pinene, β -Pinene, Germacrene D, curcumene, limonene, myrcene, linalool and farnesene also it contains triterpenoid saponins furanocoumarins, Pyranocoumarins, Monoterpene glycosides cyclohexenol type, Caffeic acid ester (Chlorogenic acid, Rosmarinic acid), Oligosaccharides and flavonol glycosides [5, 9-14].

Eryngium campestre L. roots used in the folk medicine for treatment of bladder stones, dropsy, skin disorders, diuretic, urinary tract infections, jaundice abdominal colic, delirium, whooping cough also used for promoting menstrual discharge as decoction (1-2 table spoons of the roots powder added to a cup of water, simmer 1 minute and stand for 4-10 minutes administrated 2-3 times daily), as well as its infusion used as appetizer, stimulant, antitussive and aphrodisiac [15, 16].

EXPERIMENTAL SECTION

Collection of plant materials:

The plants materials *Eryngium campestre* L. was collected by Prof. M. Abu-hadid from Jerusalem area between March and April 2012, and October 2012, and the voucher specimens have been deposited at the Herbarium of "Pharmacognosy Laboratory, An-Najah National University, Nablus, Palestine. (voucher specimen number is: Pharm-PCT-963) [17].

Serial exhaustive extraction of *Eryngium campestre* L.

The first extraction

The entire *Eryngium campestre* plants were dried in the shade for about 2 weeks, at room temperature, until they became completely dry. Then 25 gram of the whole plants were obtained and cut into small pieces, then powdered in a mechanical grinder. The 25 gram of the powdered plants, were suspended in 50 ml n-hexane which is cheap, relatively safe, largely unreactive, and easily evaporated non-polar (hydrophobic) solvent, and 250 ml of 50% ethanol in triple distilled water (to ensure sterility) in a bottle, with continuous shaking (200 round per minute) at 25°C for 72 hours in the Shaking Incubator. After that, the mixture was filtered by Whitman's No.1 filter paper using the Buchner funnel. The plant materials that had been accumulated on the filter paper were re-extracted again (2nd aqueous extraction).

The liquid filtrate was separated by separatory funnel into 2 phases: lower phase which has higher density (aqueous phase) and upper phase which has lower density (organic phase). The aqueous phase was collected first and kept in a volumetric flask at room temperature till the next step (obtaining the powder of aqueous extract). The organic phase was collected second and placed in a pre-weighed glass beaker, which was placed in the hood at room temperature in order to evaporate the solvent (n-hexane), and to obtain the organic extract. The beaker with the organic extract was weighed again after evaporation; the weight of the organic extract was determined by calculating the difference of the weights. Then it was dissolved in dimethyl sulfoxide (DMSO), which is one of the most powerful organic solvents [18], the extract was dissolved at 100 mg/ml concentration and was kept in a sterile brown bottle at -4°C in the refrigerator till further use.

The second extraction

This extraction was only for the aqueous extract, the plant materials that accumulated on the filter paper after the first filtration were re-extracted again, by adding 250 ml of 50% ethanol in triple distilled water, with continuous shaking for 72 hours in the shaking incubator at 25°C as before. A second filtration for the mixture was done by using Whitman's No.1 filter paper on the Buchner funnel. The second aqueous phase was collected after filtration and kept in a volumetric flask at room temperature.

The rotary evaporator was used for 1 hour at 40°C to evaporate any leftover organic solvents from both aqueous phases obtained from the first and second extraction. Then both aqueous extracts were put separately in pre-weighed freeze dryer bottles and placed on the freeze dryer for 24 hours till they dried completely. Then the freeze dryer bottles were reweighed again, and the dry weight of both extracts was calculated. The dry aqueous extracts were dissolved (a concentration of 100 mg/ml) in 30% ethanol in phosphate buffered saline (PBS) which is a buffer solution that maintains constant pH [19]. Then the prepared solutions of the aqueous extracts were placed in amber bottles in refrigerator at -4°C till we used them for the biological test.

Antimicrobial assay**Microorganisms and control tests**

In vitro antimicrobial activities of the aqueous and organic extracts of *Eryngium campestre* L. were tested against five potentially human pathogenic bacterial strains, and against one fungus (yeast) (Table 1).

Table 1. The tested microorganisms and their sources
*ATCC American Type Culture Collection

The microorganism	Category	ATCC [*] reference no.
<i>Staphylococcus aureus</i>	Gram positive bacteria	6538P
<i>Staphylococcus epidermidis</i>	Gram positive bacteria	12228
<i>Bacillus subtilis</i>	Gram positive bacteria	6633
<i>Escherichia coli</i>	Gram negative bacteria	8739
<i>Pseudomonas aeruginosa</i>	Gram negative bacteria	9027
<i>Candida albicans</i>	Yeast	10231

Imipenem 10 µg/ml, a broad spectrum antibacterial antibiotic and nystatin antifungal drug were used as a positive control, and the solvents (30% ethanol in PBS for the aqueous extracts and dimethyl sulfoxide for the organic extract) were used as a negative control.

Preparation of the bacterial and Candida suspensions

The bacterial and the *Candida inocula* obtained from the ATCC were sub-cultured into prepared nutrients broth and incubated at 37°C for 24 hrs and standardized to 0.5 Mc-Farland Scale (10⁸ cfu/mL) [20].

Screening for antibacterial and anticandida activity of the plant extract

Well diffusion method was used for screening, by determining the zone of inhibition [21]. The prepared cell suspensions were seeded into prepared plates of Muller-Hinton agar. For each strain, 20 µl of the suspension was added on the surface of the plate, and then was spread by special spreading tool in all directions and around the agar margins to ensure even distribution. Wells were then bored into the plates of the seeded organism using sterile straw of 6 mm diameter. Wells were filled completely with the plant extracts (the 1st and 2nd aqueous and the organic) with 100 µl in each well. Then the plates were incubated at 37°C for 24 hours for the bacteria cultures, and 48 hours for the *Candida* cultures in an incubator. Controls were also set up in parallel, using the solvents as negative control and disks of broad spectrum antibiotic (Imipenem) as positive control. After the incubation, the plates were observed for inhibition zones, which were measured in millimeters. This procedure was carried out three times for confirmation except for the organic extract due to its low volume. All steps were performed in a sterile condition.

Measuring the minimum inhibitory concentration (MIC)

The MIC is the lowest concentration of an antimicrobial that inhibits the growth of a microorganism after 18-24 hours [22]. Serial broth dilution technique was used to determine the MIC for all the test microorganisms [23], even those with negative results (exhibit no inhibition) in the well diffusion method. A set of 7 tubes were prepared for each microorganism, 750 µl of nutrient broth was added in all the tubes, then 200 µl of the aqueous extract of *Eryngium campestre* L. (Its concentration 100 mg/ml) was added in the first tube by the micropipette and was mixed well. Then, from the solution of the first tube, 200 µl were transferred to the second tube, mixed well. Then, 200 µl of the solution in the second tube were transferred to the third, then from the third to the fourth and so on, until the last tube, the 200 µl were discarded. Finally 50 µl of bacterial/candida suspension standardized to 0.5 Mc-Farland Scale (10⁸ cfu/mL) was added to all the tubes after the dilution was done. The extract's concentration in the first tube was 20 mg/ml, and five times dilution was carried out.

Negative control tubes were prepared, by using 30% ethanol in PBS instead of the plant extract as a negative control, and tubes containing broth and suspensions as positive control. The tubes were incubated at 37°C for 24 hours for the bacteria, and 48 hours for the *Candida*. After the incubation, the clear tubes (exhibit inhibitory action) were observed for each microorganism, and the last clear tube from each set was considered as the MIC. This test was repeated two times for confirmation, and all the steps were carried out under sterile conditions, by working near Bunsen flame, and sterilizing instruments in the autoclave.

Minimum bactericidal/ Fungicidal concentration (MBC/MFC)

The MBC/MFC of the plant extracts, which is the minimum concentration that is required to kills the bacteria/fungi [22], were tested after the results of the MIC. The tubes of the MIC that showed no growth (no turbidity) of the microbes were sub-cultured into nutrient agar plates and incubated at 37°C for 24 hours for the bacteria, and 48 hours for the *Candida*. The concentration of the extract that did not show any colony growth was labeled as the MBC/MFC.

RESULTS AND DISCUSSION**Well Diffusion Method and Zone of Inhibition**

In the well diffusion method, the antibacterial and antifungal activities of *E. campestre* plant were screened, in comparison with the antibacterial drug (Imipenem) and antifungal drug (Nystatin). Using the first aqueous extract of the stock solution (100 mg/ml), the diameter of inhibition zone (DIZ) was the greatest for the gram-positive *Bacillus subtilis* with a diameter of (18 mm), which is less than the DIZ of Imipenem (46 mm) as shown in (Tables 2, 3) and (Fig. 3). For the other two gram-positive bacteria *Staphylococcus aureus* and *Staphylococcus epidermidis*, the DIZ were 12 mm and 8 mm, which are less than the DIZ of Imipenem (46 mm) and (32 mm) respectively.

Table 2. The diameter of the inhibition zone results for aqueous and organic extracts of *E. campestre* using 100 microlitre of the stock concentration (100 mg/ml)

Plant Extract	E. C	P.A	S.A	S.E	B.S	C.A
1 st aqueous ext.	NI*	6 mm	12 mm	8 mm	18 mm	NI*
2 nd aqueous ext.	NI*	NI*	6 mm	NI*	14 mm	NI*
Organic ext.	12 mm	NI*	10 mm	8 mm	10 mm	NI*
Imipenem positive control	36 mm	26 mm	46 mm	32 mm	46 mm	NI*
Nystatin Positive Control	NI*	NI*	NI*	NI*	NI*	20 mm
30% ethanol PBS Negative Control	NI*	NI*	NI*	NI*	NI*	NI*
Dimethyl Sulfoxide Negative Control	NI*	NI*	NI*	NI*	NI*	NI*

Notes: B.S- Bacillus subtilis, S.A- Staphylococcus aureus, S.E- Staphylococcus epidermidis, E.C- Escherichia coli, P.A- Pseudomonas aeruginosa, C.A- Candida albicans. NI: no inhibition

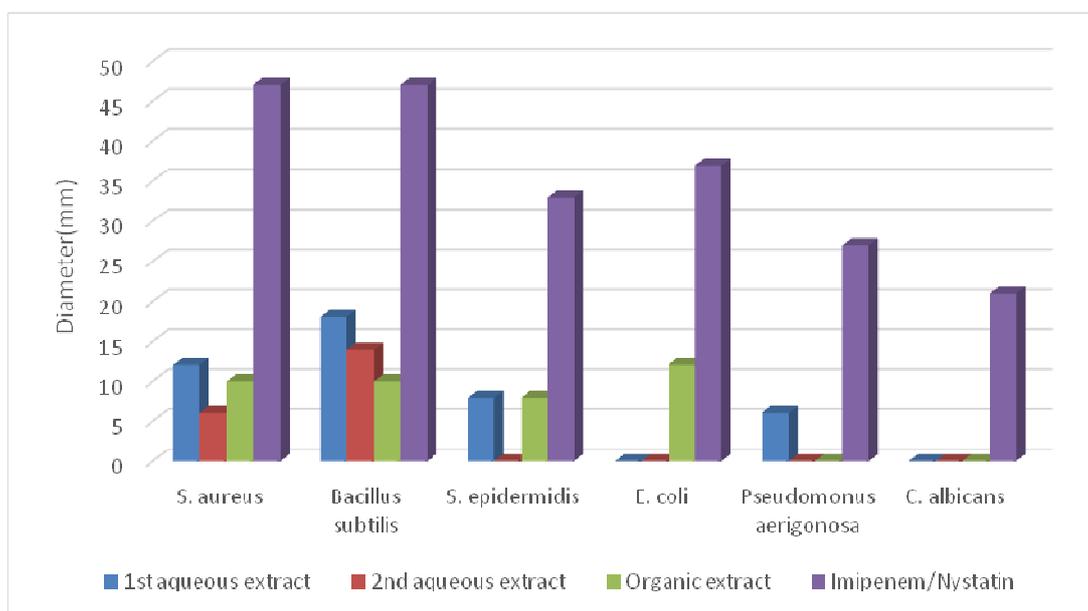


Fig. 3: Comparison between antimicrobial activity of the aqueous and organic extracts of *E. campestre* and the standard antibiotic (Imipenem), and antifungal (Nystatin)

Using the well diffusion screening method there were no inhibition zone for the gram-negative *E. coli* neither for the fungus *Candida* while the DIZ of Imipenem against *E. coli* was (36 mm) and the DIZ of Nystatin against *Candida*

was (20 mm). However, the well diffusion screening method showed inhibition of the gram-negative *Pseudomonas aeruginosa* with DIZ of (6 mm), while the Imipenem DIZ was (26 mm).

For the second aqueous extract of the same stock solution, the screening showed antibacterial activity against the following two gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus* with DIZ of (14 mm) and (6 mm) respectively comparing to the Imipenem DIZ of (47 mm) for both.

In the aqueous extract screening, the control was the solvent of (30% ethanol in PBS PH 7.4) which showed no inhibition to make sure that the extract itself killed the bacteria and not the solvent.

Regarding the screening of organic extract, it showed inhibition of the three gram positive bacteria *Bacillus subtilis*, *S. aureus* and *S. epidermidis* with DIZ (10 mm), (10 mm) and (8 mm) respectively and one gram negative bacteria *E. coli* which had the greatest DIZ of (12 mm) as shown in (table 2) and (Fig. 3). The control for the organic extract was DMSO (dimethyl sulfoxide), which showed no inhibition to make sure that the extract itself that killed the bacteria and not the solvent.

Minimum inhibitory concentration (MIC)

The serial dilution method showed more specific results of antimicrobial activity of *E. campestre* extracts in comparison with the well diffusion screening technique. At beginning, MIC is achieved using the stock concentration of (100 mg/ml) with amount of 100 microliter, which showed activity against two gram-positive bacteria *Bacillus subtilis* and *S. aureus*, and against *Candida albicans* as shown in (Table 3), but when using the stock concentration (50 mg/ml) and a higher amount of 400 microliter the MIC showed activity against all microorganisms including gram positive and gram negative as well as against *Candida albicans* as shown in (Table 4).

Table 3. MIC values of *E. campestre* aqueous extract using the stock concentration of (100 mg/ml) with amount of 100 microliter. (From the dry plant sample)

Microorganism	E.C	P.A	S.A	S.E	B.S	C.A
MIC	NI*	NI*	10 mg/ml	NI*	1 mg/ml	10 mg/ml

Notes: B.S- *Bacillus subtilis*, S.A- *Staphylococcus aureus*, S.E- *Staphylococcus epidermidis*, E.C- *Escherichia coli*, P.A- *Pseudomonas aeruginosa*, C.A- *Candida albicans*, MIC: minimum inhibitory concentration, NI: no inhibition, Needs >100microliter.

Table 4. MIC values of *E. campestre* aqueous extract using the stock concentration of (50 mg/ml) with amount of 400 microliter. (From the green fresh plant sample)

Microorganism	E.C	P.A	S.A	S.E	B.S	C.A
MIC	20 mg/ml	2 mg/ml	0.2 mg/ml	2 mg/ml	0.2 mg/ml	0.02 mg/ml

Notes: B.S- *Bacillus subtilis*, S.A- *Staphylococcus aureus*, S.E- *Staphylococcus epidermidis*, E.C- *Escherichia coli*, P.A- *Pseudomonas aeruginosa*, C.A- *Candida albicans*, MIC: minimum inhibitory concentration.

Minimum bactericidal-fungicidal concentration (MBC-MFC) evaluations

Our plant showed bactericidal activity against the three gram-positive bacteria, *Bacillus subtilis*, *S. aureus* and *S. epidermidis*, as well as against one gram negative bacteria (*Pseudomonas aeruginosa*); it had also fungicidal activity against *Candida albicans*. All activity was at the same concentration of the extract 20 mg/ml, which is prepared from the stock solution of 50 mg/ml with amount of 400 microliter as shown in (Fig. 4) (No bactericidal activity against *E. coli*).

In Our study, *E. campestre* showed broad spectrum activity against both gram positive and gram negative microorganisms, but more activity against gram positive bacteria mainly *Bacillus subtilis*, which had the greatest DIZ in the first and the second extracts of (18 mm) and (14 mm) with activity 39.1% and 30.4% in comparison with Imipenem respectively. The activity against gram negative microorganisms is less because such resistance could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism [24].

Staphylococcus epidermidis had a first extract activity with the second extract being not active which may be due to that all the active material had dissolved in the first extract, and this confirms that the extraction method is exhaustive.

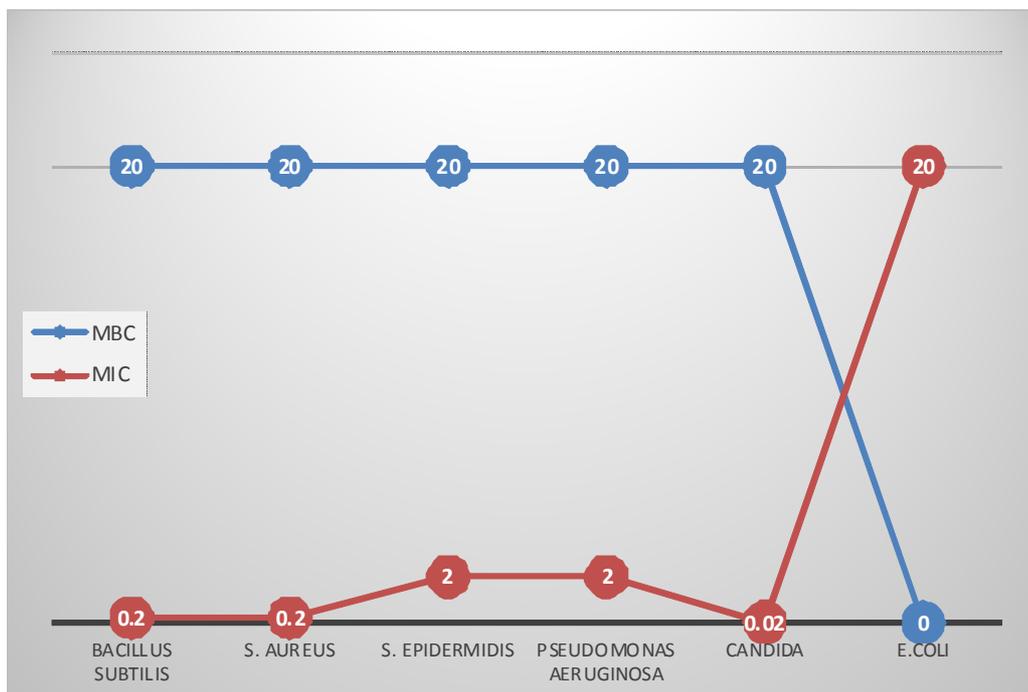


Fig. 4: Comparison between MBC concentrations (mg/ml) and MIC concentrations (mg/ml) of *E. campestre* aqueous extract against different Microorganisms using the stock 50 mg/ml

E. campestre plant also showed antifungal activity against *Candida albicans*. This may have a different mechanism of action than antibacterial activity because the antifungal action mainly targets either the formation or the function of ergosterol, an important component of the fungal cell membrane, while antibacterial activity works by inhibiting steps important for the formation of peptidoglycan, the essential component of the bacterial cell wall [25].

Infections caused by *Pseudomonas aeruginosa*, especially those with multi-drug resistance, are among the most difficult infections to treat with conventional antibiotics. In our study, the growth of *Pseudomonas aeruginosa* was inhibited by *E. campestre* extract at (MIC 2 mg/ml) and killed at (MBC 20 mg/ml) of the stock concentration (50 mg/ml) (Fig. 5). It seems very likely, therefore, that the antibacterial compound extracted from *E. campestre* may inhibit bacteria by a different mechanism than that of currently used antibiotics and may have therapeutic value as an antibacterial agent against multi-drug resistant bacterial strains [26].

The organic extract showed broad-spectrum antimicrobial activity, it affected gram positive and gram negative bacteria, with maximum diameter zone of inhibition reached 12 mm (against *E. coli*).

Plant extracts and essential oils may exhibit different modes of action against bacterial strains, such as interference with the phospholipids bilayer of the cell membrane, which has consequently an increased permeability, loss of cellular constituents, damage of the enzymes involved in the production of cellular energy, alteration of the synthesis of structural components and destruction or inactivation of genetic material. In general, the mechanism of action involves disturbance of the cytoplasmic membrane disrupting the proton motive force, electron flow, active transport and coagulation of cell contents [27].

The organic extract showed greater activity against *E. coli* this may be because the main active ingredient is a non-polar hydrophobic compound [28]. The diameter of inhibition zone of aqueous and organic extracts is less than the diameter of inhibition zone of the Imipenem or Nystatin, this is not surprising because standard antibiotics are well refined industrial products so there is no doubt their activity will be more compared to crude extracts [29].

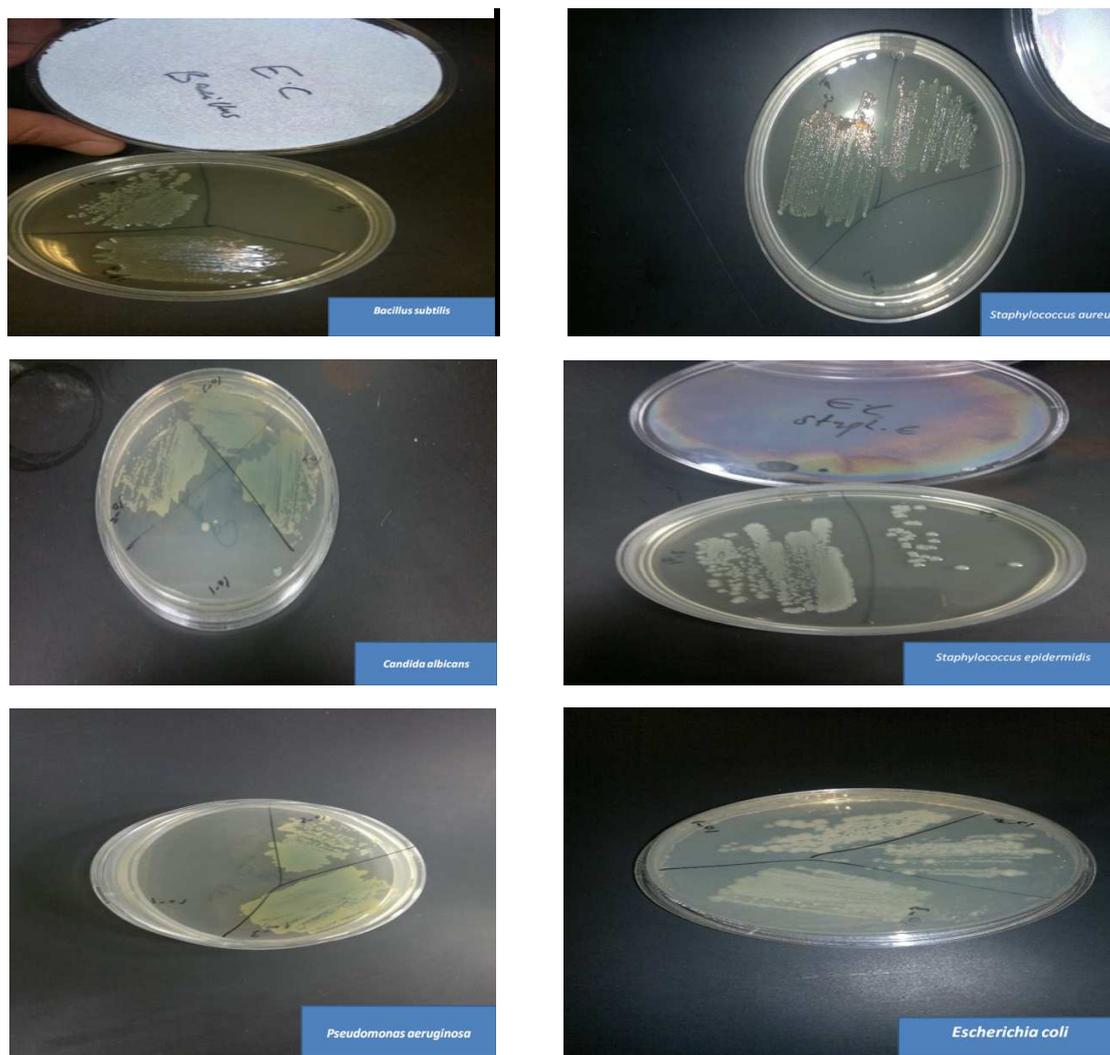


Fig. 5: MBC results of *E. campestre* aqueous extract at concentration 20 mg/ml against different microorganisms using the stock 50mg/ml

It is also important to mention that when increasing the amount and subsequently the concentration in MIC, a significant increase in the activity is noticed. The first time, 100 microliter (stock concentration 100 mg/ml of the dry plant extract) is used and only two gram positive (*Bacillus subtilis* and *Staphylococcus aureus*) and the *Candida albicans* were inhibited. However, when using 400 microliter (stock concentration 50 mg/ml of the green fresh plant extract) all bacteria, including gram positive and gram negative, as well as the *Candida albicans* were inhibited and the majority were even killed.

CONCLUSION

We can conclude from this study that *E. campestre* has antimicrobial activity, not only by inhibiting the growth of bacteria especially by using exhaustive extraction technique, it has also a bactericidal effect against both gram positive and gram negative bacteria in addition to this broad-spectrum activity it has antifungal activity against *Candida albicans*.

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