

Identification of the antibacterial efficacy of ethanolic extracts and oils of some medicinal plants against the growth of *Escherichia coli* *in vitro*

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Abstract

The present study had thrown the light on the *in vitro* antimicrobial potential of the ethanolic extract of four local medicinal plants; *Terminalia chebula*, *Lawsonia inermis*, *Origanum vulgare*, *Thymus vulgaris*, and essential oils of five types of medicinal plants; *Elettaria cardamomum*, *Eugenia caryophyllus*, *Trigonella foenum-graecum*, *Linum usitatissimum*, *Brassica nigra* against the growth of pathogenic *Escherichia coli* (*E. coli*). The antibacterial activity was carried out by using agar well diffusion technique in Mueller-Hinton agar.

The results were obtained by measured the zone of inhibition (mm) around the well that could be exhibited by each plant extract and oil following incubation of bacterial plates and expressed as mean±Standard error (SE). Ethanolic extract of *Terminalia chebula* was possessed the strongest antibacterial effect among the tested plant extracts, followed by *Origanum vulgare* and *Lawsonia inermis* extracts. *Escherichia coli* was not affected by *Thymus vulgaris* extract. On the other hand, *Escherichia coli* was variably susceptible to tow of the used essential plant oils; *Elettaria cardamomum* and *Eugenia caryophyllus*, whereas the other three plant oils were not active any more.

Key words: ethanolic extracts, plant oils, *Escherichia coli*, *in vitro*

تشخيص الكفاءة المضادة للبكتريا لمستخلصات ايثانولية وزيتو بعض النباتات الطبية ضد نمو

جرثومة عصيات القولون في المختبر

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المستخلص

لقد سلطت الدراسة الحالية الضوء على الكفاءة المضادة للبكتريا مختبريا للمستخلصات الايثانولية لأربعة انواع من النباتات المحلية وهي الاهليلج والبردقوش والحنة والزعرتر وخمسة انواع من الزيوت النباتية الاساسية هي زيت الهيل وزيت القرنفل وزيت الحلبة وزيت بذور الكتان وزيت الخردل ضد نمو جرثومة عصيات القولون المرضية حيث تم قياس الكفاءة المضادة للبكتريا بواسطة طريقة الانتشار في الحفر المعمولة على سطح اكار المولر-هنتون. وقد تم اخذ النتائج من خلال قياس نطاق التثبيط الناتج من تأثير المستخلصات والزيوت النباتية والمتكون حول كل حفرة والذي عقب حضان كافة الاطباق المزروعة وقد تم التعبير عنها بشكل المعدل±الخطأ القياسي.

لقد اظهر المستخلص الايثانولي لنبات الاهليج التأثير الاقوى كمضاد بكتيري من بين باقي النباتات، عقبه في التأثير مستخلص البردقوش والحنة في حين لم يظهر مستخلص الزعتر اي تأثير يذكر ضد الجرثومة المختبرة. من جانب اخر فان عصيات القولون كانت حساسة لاثنين من الزيوت النباتية المستخدمة في الدراسة وهي زيت الهيل وزيت القرنفل في حين لم تؤثر على الاطلاق الزيوت الثلاثة المتبقية في نمو الجرثومة. الكلمات المفتاحية: المستخلصات الايثانولية، الزيوت النباتية، عصيات القولون، في المختبر

Introduction:

The identification of compounds with antimicrobial activity has gained increasing importance in recent times due to growing worldwide concern about the alarming increase in the rate of infection by antibiotic-resistant microorganisms (18). Bacteria are very adaptable organisms because of their very short generation time (as little as 15 to 20 minutes for some species under ideal conditions) and their propensity for sharing genetic information-even among different species of bacteria. The presence of an antibiotic may kill most of the bacteria in an environment but the resistant survivors can eventually re-establish themselves and pass their resistance genes on to their offspring and often to other species of bacteria. Both medical and veterinary uses of antibiotics have resulted in the appearance of resistant strains, which may cause disease that are difficult to treat (8, 28).

In addition, the problem posed by the high cost, adulteration and increasing toxic side effects of antibacterials coupled with their inadequacy in diseases treatment found more especially in the developing countries cannot be over emphasized (51). However, there has also been a high interest in the research for natural products from herbs to discover new antimicrobial agents in the last three decades and in recent times (34, 41).

More so, many of these plants have been known to synthesize active secondary metabolites such as phenolic compounds found in essential oils (24, 17, 19) with established potent antimicrobial activities which indeed was formed the basis for their applications in some pharmaceuticals, alternative medicines and natural therapies (43, 31). Santos *et al.*, 1995 (49) remarked the World Health Organization has indeed recognized medicinal plants as the best source for obtaining a variety of synthetic drugs. No doubts, some studies have identified and isolated the main active ingredients in the plants responsible for this antimicrobial activity.

The purpose of this study was to investigate the antimicrobial efficacy of ethanolic extract of some medicinal herbs and some essential plant oils against the growth of pathogenic isolates of *Escherichia coli* in order to prove the folkloric claims.

Materials and methods:

Plant materials: Fruits of *Terminalia chebula*, leaves of *Origanum vulgare*, leaves of *Lawsonia inermis* and fruits of *Thymus vulgaris* had been dried and well grinded to be used in this study besides essential oils of five types of medicinal plants; *Elettaria cardamomum* (Cardamom oil, HEMANI-IRP), *Eugenia caryophyllus* (Clove oil, HEMANI-IRP), *Trigonella foenum-graecum* (Fenugreek oil, EMAD-Musel), *Linum usitatissimum* (Flax seed oil, Ashams for oil-ROI), and *Brassica nigra* (Mustard oil,

HEMANI-IRP). All these plant materials and oils were purchased from the local market, at Al-Qadissiyah province, and then they were identified and purified at the National Iraqi Institute for Herbs, Baghdad, Iraq.

Preparation of ethanolic extracts : Ethanolic extracts were accomplished according to the method of Le Grand *et al.*, 1988 (30). Briefly, 50 gm of each powdered plant sample was mixed with 250 ml of 96% ethanol. The mixture was kept for 2-5 days in tightly sealed containers at room temperature and shaken several times daily. This mixture was filtered through filter paper to remove the coarse plant materials. Further extraction of the residue was repeated 3-5 times until a clear supernatant extraction liquid was obtained. The filtrates of each tested plant were evaporated to dryness using a rotary evaporator at 40°C. The final dried samples were weighed and stored at -20°C until preparation of stock solutions at a concentration of 400 mg/ml that they were done by diluting 2 gm of each dry extract with 5 ml of 96% ethanol (3)

Antibiotics: Five standard antibiotics had been chosen according to their broad-spectrum activity used as positive control against the test microorganism (*Escherichia coli*), they include: CIP 5 (Ciprofloxacin-5 mcg), C30 (Chloramphenicol-30 mcg), CN 10 (Gentamicin-10 mcg), T30 (Oxytetracycline-30 mcg), CE 30 (Cephadrine-30 mcg) (Bioanalyse)[®].

***Escherichia coli* isolates :** Standard strains of *Escherichia coli* (gram-negative bacteria) were obtained from the Laboratory of Microbiology at the College of Veterinary Medicine, Al-Qadisiyah University and they were identified and confirmed at the Central Laboratory of Health, Baghdad, Iraq.

Sensitivity test: Inhibition of bacterial growth was tested by using the agar well diffusion method (46) . *Escherichia coli* isolates were subcultured in nutrient broth (HIMEDIA Laboratories, Mumbai-India) which was prepared by dissolving 13 gm of nutrient broth in 1000 ml of distilled water, shaken well and heated for several minutes using water bath at a temperature of 80°C to ensure complete dissolving, then sterilized for 15 minutes at 15 lb pressure in an autoclave. Nutrient broth media was later poured into 4 sterile test tubes at average of 10 ml of media for each tube. After that, several colonies of each of the tested bacteria were suspended with the help of sterile cotton swab into the tubes of nutrient broth. After mixing well, all the tubes were incubated at 37 C ° for 24 hours to produce bacterial suspensions revealed by the presence of turbidity . The turbidity of the culture was compared with 0.5 McFarland Nephelometer standard to get 150 x 10⁶ CFU/ml (47) . On the other hand Mueller Hinton Agar (HIMEDIA Laboratories, Mumbai-India) which is a growth media used for testing antibiotics and the chosen plant extracts and oils susceptibility of the test microorganisms was prepared by dissolving 38 gm of Mueller Hinton agar in 1000 ml distilled water, shaken, heated, and sterilized by autoclave in a similar way to the preparation of nutrient broth. This media was poured aseptically at 45°C into sterilized Petri plates (two plates were used for each plant extract and oil besides 2 plates for the antibiotic discs, so that the final number of Petri plates used in this study was twenty plates). After complete solidification, 4 uniform wells of 5 mm diameter were bored into each plate with sterile un-drawn Pasteur pipette and plugs

were removed with sterile tips (19) (with exception of those plates used for antibiotic study). A sterile cotton swab was dipped into the bacterial suspension produced by *Escherichia coli* to be inoculated on the Mueller-Hinton agar surface by streaking of the swab over its. Finally and after the inoculums were dried , 0.1 ml of each plant stock solution was dropped into the wells of its inoculated plates besides 0.1 ml of 96% ethanol which considered as a negative control was dropped in the central well on the same extract plates. As well as one disc of each antibiotic control was placed with the aid of sterile forceps over the surface of its own plates (so that 5 different discs of antibiotics were applied over each plate). All plates were incubated at 37 C⁰ for 24 hours. Zone of inhibition around each well measured in mm with the ruler (34) . The values were given as mean \pm SE and the data were analyzed by ANOVA test with least significant differences (LSD) at significant level of P<0.05 by using SPSS (Version 10) (45) .

Results and Discussion:

Ethanollic extract of *Terminalia chebula* exhibited maximum inhibitory activity (at p<0.05) against the tested bacteria with mean diameter of zone of inhibition; **40.11 \pm 0.77** mm, followed by **19 \pm 0.6** mm for *Origanum vulgare*, and **17.11 \pm 0.26** mm for *Lawsonia inermis* which represented the lowest value among the positive results of ethanolic herbal extracts (table:1; figures: 1, 2, 3). Whereas *Escherichia coli* isolates were resistant to *Thymus vulgaris* extract.

Table (1): Inhibition zones (mm) of the used ethanolic extracts of medicinal plants against the growth of *Escherichia coli* in vitro .

Ethanolic extracts	Inhibition zones (mm)
<i>Terminalia chebula</i>	40.11\pm0.77 a
<i>Origanum vulgare</i>	19\pm0.6 b
<i>Lawsonia inermis</i>	17.11\pm0.26 c
<i>Thymus vulgaris</i>	0\pm0 d

❖ Different small letters mean significant changes for vertical values at level (p<0.05).

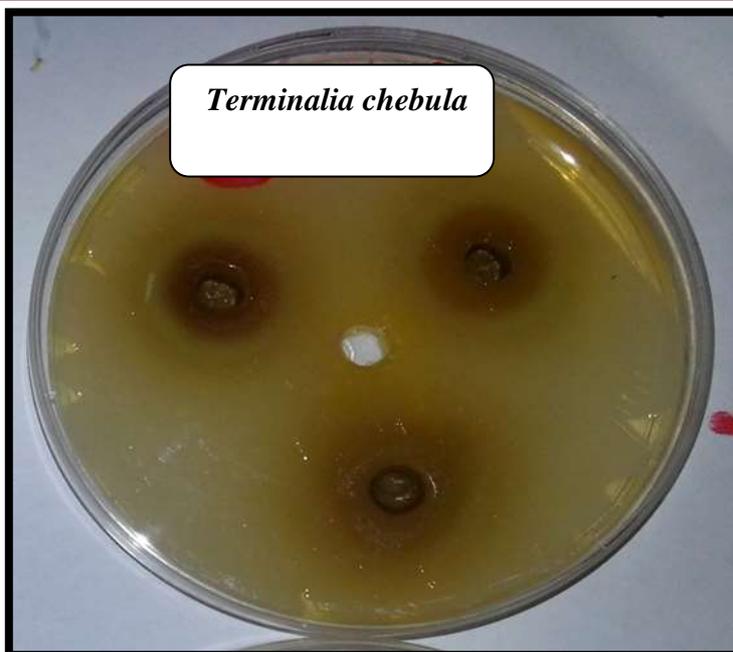


Figure (1): Inhibition zones of *Escherichia coli* growth on Mueller-Hinton agar produced by ethanolic extract of *Terminalia chebula*, the peripheral three wells contained the extract at a concentrations of 400 mg/ml, whereas the central well contained 0.1 ml of 96% ethanol .

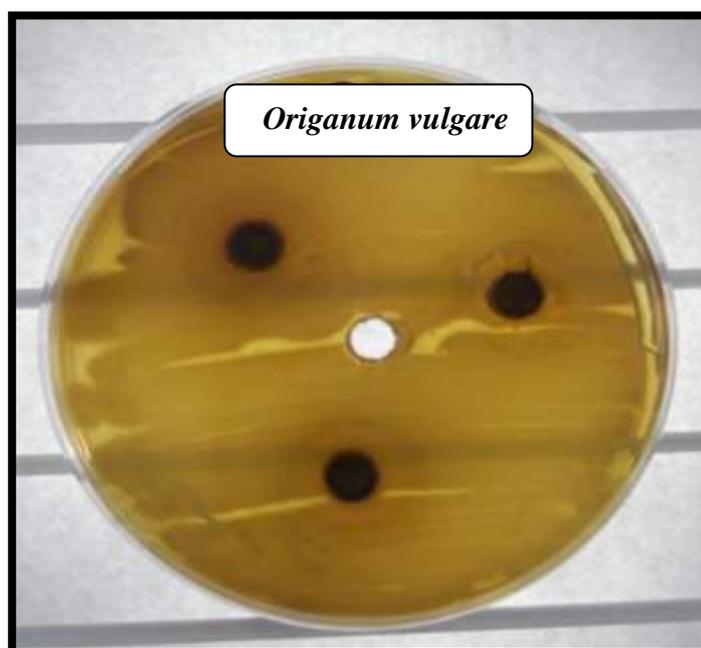


Figure (2): Inhibition zones of *Escherichia coli* growth on Mueller-Hinton agar produced by ethanolic extract of *Origanum vulgare*, the peripheral three wells contained the extract at a concentrations of 400 mg/ml, whereas the central well contained 0.1 ml of 96% ethanol.



Figure (3): Inhibition zones of *Escherichia coli* growth on Mueller-Hinton agar produced by ethanolic extract of *Lawsonia inermis*, the peripheral three wells contained the extract at a concentrations of 400 mg/ml, whereas the central well contained 0.1 ml of 96% ethanol.

On the other hand, the essential oil of *Elettaria cardamomum* showed strong antimicrobial activity (at $p < 0.05$) against *Escherichia coli* on agar plate surface with mean diameter of zone of inhibition; 28.55 ± 0.97 mm, while the essential oil of *Eugenia caryophyllus* revealed moderate inhibitory effect against the tested bacteria with mean diameter of zone of inhibition ; 17.88 ± 0.45 mm . Essential oils of *Trigonella foenum-graecum*, *Linum usitatissimum*, and *Brassica nigra* were showed negative results against the growth of *Escherichia coli* (table:2; figures: 4, 5) .

Table (2): Inhibition zones (mm) of the used essential oils of medicinal plants against the growth of *Escherichia coli in vitro* .

Essential oils	Inhibition zones (mm)
<i>Elettaria cardamomum</i>	28.55 ± 0.97 a
<i>Eugenia caryophyllus</i>	17.88 ± 0.45 b
<i>Trigonella foenum-graecum</i>	0 ± 0 c
<i>Linum usitatissimum</i>	0 ± 0 c
<i>Brassica nigra</i>	0 ± 0 c

❖ Different small letters mean significant changes for vertical values at level ($p < 0.05$) .

❖ Similar small letters mean non-significant changes for vertical values at level ($p < 0.05$).

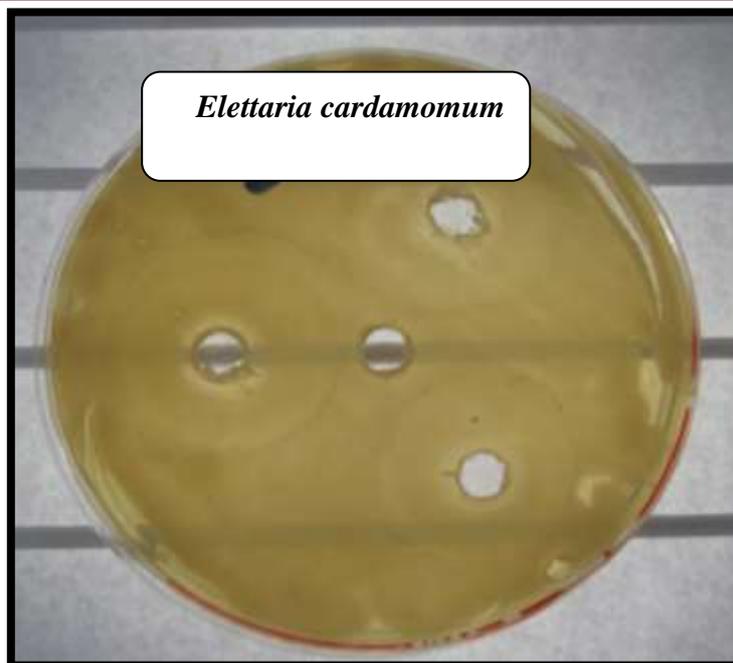


Figure (4): Inhibition zones of *Escherichia coli* growth on Mueller-Hinton agar produced by essential oil of *Elettaria cardamomum*, the peripheral three wells contained the plant oil, whereas the central well contained 0.1 ml of 96% ethanol .



Figure (5): Inhibition zones of *Escherichia coli* growth on Mueller-Hinton agar produced by essential oil of *Eugenia caryophyllus*, the peripheral three wells contained plant oil, whereas the central well contained 0.1 ml of 96% ethanol.

The present study had also been depended on the use of five standard antibiotics as a positive control for test microorganism (Table 3).

CIP5 (ciprofloxacin) was the strongest among the used antibiotics, it produced significant inhibitory effect (at $p < 0.05$) against the growth of *Escherichia coli* with mean diameter of zone of inhibition; **24.33±0.88** mm. C30 (chloramphenicol) produced positive results against the above bacteria with mean diameter of zone of inhibition; **21.66±0.33** mm. CN 10 (gentamicin) and T30 (oxytetracycline) were revealed equal significant inhibitory results (at $p < 0.05$) against the test microorganism with value of; **20.33±0.33** mm for both. Finally CE 30 (cephradine) also it had been used in the present study, but it exhibited negative antimicrobial activity against isolates of *Escherichia coli*.

Table (3): Inhibition zones (mm) of the standard antibiotics against the growth of *Escherichia coli* in vitro .

Antibiotics	Inhibition zones (mm)
CIP 5	24.33±0.88 a
C 30	21.66±0.33 b
CN 10	20.33±0.33 c
T 30	20.33±0.33 c
CE 30	0±0 d

- ❖ Different small letters mean significant changes for vertical values at level ($p < 0.05$).
- ❖ Similar small letters mean non-significant changes for vertical values at level ($p < 0.05$).

The present study was designed to obtain preliminary information on the antimicrobial effect of some medicinal plants on pathogenic *Escherichia coli*. The hole plate diffusion technique was preferred to be used in this study since it was found to be better than the disc diffusion technique, also it provided more suitable conditions for the microbial growth (20, 55)

On the other hand, Agar-based methods are attractive because of their simplicity and low cost, in addition to that it may help to detect if there any resistance from the yeast or the bacteria to any drug , medicinal plants or agents that may be used to study their effect (55)

The present study had showed different degrees of inhibitory effect produced by the plant extracts and oils on the growth of the tested microorganism.

Terminalia chebula is a well known plant used in ayurvedic traditional medicine for their effectiveness against wide range of diseases due to the advantage of the diversity of secondary metabolites responsible for their curing property. Despite the

existence of potent antibacterial agents, the appearance of resistant or multi-resistant strains imposes the need for a permanent search and development of new drugs (7).

The antibacterial activity of the crude extracts of *T. chebula* fruits was determined against pathogenic strains of *Escherichia coli*. The ethanol extract exhibited highest antibacterial activity in terms of zone of inhibition when compared to the other tested extracts. These observations may be attributed to two reasons; firstly, due to the nature of biologically active components (alkaloids, flavonoids, sterols, quinine, tannins etc.) which might be enhanced in the presence of ethanol (28). It has been documented that alkaloids, flavonoids and tannins are plants metabolites well known for their antimicrobial activity (54). Secondly, the stronger extraction capacity of ethanol could have produced a greater number of active constituents responsible for antibacterial activity.

The positive results of the inhibitory effects of *T. chebula* were similar to those recorded by (50) who found that *T. chebula* leaf galls has shown the better activity profile against gram positive and gram negative microorganisms. The above observations were also similar to those found by (53), they demonstrated the antibacterial activity of the dried fruit extract from *T. chebula* against urinary tract pathogens using disc method. In addition, they noticed that acetone and ethanol extracts showed good antibacterial activity against *Escherichia coli* strains than the cold and hot water extracts.

Ethanol extract of *Origanum vulgare* had revealed growth inhibitory activity against the test *Escherichia coli*. This result was in agreement with those found by (32) who used the hydroalcoholic extract of *Origanum vulgare* that showed the highest efficacy against *Escherichia coli*. This study demonstrates that the decoction could be used for antioxidant purposes, while the hydroalcoholic extract could be incorporated in formulations for antimicrobial features. Moreover, the use of infusion/decoction can avoid the toxic effects showed by oregano essential oil, widely reported for its antioxidant and antimicrobial properties.

Whereas *Origanum vulgare* (oregano) oils also show antimicrobial activity against a number of multi resistant pathogenic bacteria, especially gram negative bacteria including *Escherichia coli* that were more sensitive to oregano oils than gram positive bacteria (44, 16, 6)

The major constituents of the ethanolic *Origanum vulgare* extract that related to the cytotoxic, antioxidant, and antibacterial properties of the extract or to the overall extract biological activity had been searched fully by (15). Gas chromatography/mass spectroscopy analysis showed that the extract contained monoterpene hydrocarbons and phenolic compounds, the major ones being carvacrol and thymol and to a lesser extent p-cymene, 1-octacosanol, creosol, and phytol. The extract exhibited antimicrobial properties against Gram-positive and Gram-negative bacterial strains including clinical isolates which are mostly attributed to carvacrol and thymol.

Pathogenic strains of *Escherichia coli* was recorded to be moderately sensitive to the ethanolic extract of *Lawsonia inermis* leaves.

According to the study of (37) , phytochemical constituents of *Lawsonia inermis* exhibit antimicrobial activity only against gram positive bacteria while ineffective for gram negative bacteria. In our study, it was interested to note that *Lawsonia inermis* had antimicrobial activity against gram negative bacteria including *Escherichia coli*. The studies of (9) , (23) and (25) support our findings .

Alcoholic extract includes flavonol, alkaloids, tannins, sterols polyphenols etc . Main chemical constituents of *Lawsonia inermis* include ; Lawsone (2-hydroxynaphthoquinone) , mucilage , mannite , gallic acid and tannic acid . (22) (27) observed that the highest inhibitory effect was showed by ethyl acetate extract of fruit on *Escherichia coli* and *Bacillus subtilis*. The study demonstrated that the ethyl acetate and ethanol extracts of fruit and flower of *Lawsonia inermis* are potentially better source of antibacterial agents compared to leaf extracts of respective solvents .

Plants with naphthoquinone contents such as Juglone , lawsone (active constituent of *Lawsonia inermis*) , and plumbagin display very significant pharmacological properties. They are cytotoxic , they have significant antibacterial, antifungal, antiviral, insecticidal, anti-inflammatory, and antipyretic properties. The mechanism of their effect is highly large and complex. They bind to DNA and inhibit the processes of replication, interact with numerous proteins (enzymes) and disturb cell and mitochondrial membranes, and interfere with electrons of the respiratory chain on mitochondrial membranes (1) .

Ethanol extract of *Thymus vulgaris* used in the present study showed no inhibitory activity on *Escherichia coli* growth over agar plate surface.

On the other hand, only essential oils of *Elettaria cardamomum* and *Eugenia caryophyllus* were produced positive antibacterial effect whereas the tested isolates of *Escherichia coli* resisted those of *Trigonella foenum-graecum*, *Linum usitatissimum*, and *Brassica nigra*.

Firstly, Cardamom seed oil that obtained naturally from dried ripe seeds of *Elettaria cardamomum* . Major components in the oil were 1,8-cineole, α -terpineol, dl-limonene, nerolidol, 4-terpineol, δ -terpineol, δ -3-carene, β -myrcene, germacrene D, α -terpinene and longifolenaldehyde (36). In this study, essential oil of cardamom seed displayed the highest antimicrobial activity on the tested microorganism. Present study was in agreement with (38) and (2). Antimicrobial characteristics of the herbs are due to various chemical compounds including volatile oils, alkaloids, tannins and lipids that are presented in their tissue (21). The inhibitory effect of cardamom detected in the present study may be due to the presence of the major component , γ -terpinene in its oil (38) .

Secondly, *Eugenia caryophyllus* which is the aromatic dried flower buds of a tree in the family Myrtaceae (52, 12). It is used as a carminative to increase hydrochloric acid in the stomach and to improve peristalsis (39). It is also used in dentistry where the essential oil of its was used as anodyne for dental emergencies (40). In addition it is antimutagenic (33), anti-inflammatory, antithrombotic (52), and antiparasitic (58). The essential oil extracted from the dried flower buds of *Eugenia caryophyllus* is used as a topical application to relieve pain and to promote healing (12).

Several constituents of *Eugenia caryophyllus* had been identified mainly eugenol, eugenyl acetate, β -caryophyllene, 2-heptanone (13), acetyl eugenol, α -humolene, methyl salicylate, isoeugenol, methyl eugenol (58), phenyl propranoides, dehydrodieugenol, trans-confireryl aldehyde, biflorin, kaempferol, rhamnocitrin, myricetin, gallic acid, ellagic acid and oleanolic acid (11). The antimicrobial activity of *Eugenia caryophyllus* had been studied by several authors (56). The spectrum of activity is fairly broad, with action against gram-positive and gram-negative rods and cocci, yeast and fungi (10). The present study had shown strong antimicrobial activity of *Eugenia caryophyllus* essential oil against *Escherichia coli*. Similar results were achieved by (48) that reported antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter aerogenes*. Our results also agreed with (4) in exhibiting antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*. Different results were achieved by (42) that revealed antimicrobial activity against *Staphylococcus aureus*, but no action against *Escherichia coli*. A possible explanation for these diverse results is the fact that *Eugenia caryophyllus* oil composition is variable depending on the region and season that it is collected (14). Consequently, the active compounds may not present in sufficient quantities or quality. The mechanism of antimicrobial action of *Eugenia caryophyllus* oil, though not completely understood, seems to be complex and may vary according to its composition. The compounds known to have antimicrobial action are mainly the flavonoids and cinamic acids (57).

Finally, tested bacteria was highly sensitive to ciprofloxacin that was the strongest among the other used antibiotics followed by Chloramphenicol. Furthermore, Gentamicin and Oxytetracycline were recorded equal efficacy against the *in vitro* growth of *Escherichia coli*, which clearly resisted the effect of Cephadrine discs. These findings were similar to those recorded by (35) and were identical to **KIRBY-BAUER** chart for Antibiotic Susceptibility (26).

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