

The effect of antibiotics with *citrus limon* extract on local bacterial isolates

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Abstract:

Over three quarter of the world's population is using herbal medicines with an increasing trend globally. the aim of this study was investigation of antibacterial activity of dried black lemon extract that have been extracted with 96% ethanol alone and in combination with some antibiotics (Ampiciline (AM(10 mcg)), Gentamycine (CN(10 mcg)) , Amoxiline (AX(10mcg)) , Erythromycine (E(15 mcg)) and Tetracycline (TE(30)) against local bacterial isolates *Escherichia coli*, *Staphylococcus aureus* , *Protius mirabilis*, *Serratia marcescens* and *Pseudomonas aeruginosa* . The results have shown a good antibacterial activity of the dried black lemon extract against all of the selected bacteria except *Protius mirabilis* .this extract helped in increasing the antibacterial activity of some antibiotics and decrease or not affect on other antibiotics when they tested on bacteria. The results of this study showed that ethanolic extracts of dried black lemon can be introduced as an alternative to chemical antimicrobial drugs, wider investigation is required.

Introduction:

The resistance to antibiotics is an increasing problem although the pharmacological industries have produced a number of new antibiotics in the last three decades [1]. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents [2].To overcome this problem, researchers concentrate their study to find out new drug from plants [3].According to the World Health Organization (WHO) definition, a medicinal plant, is the one that can be used for therapeutic purposes and or its compounds are used as a pioneer in the synthesis of semi-synthetic chemical drugs [4] . Fruits have the ability to reduce the risk of several chronic diseases including cancer, the protective nature of the fruits is due to the presence of phytoconstituents like poly phenolic compounds [5].

Citrus fruits belong to six genera (*Fortunella*, *Eremocitrus*, *Clymendia*, *Poncirus*, *Microcitrus* and *Citrus*), which are native to the tropical and subtropical regions of Asia, but the major commercial fruits belong to genus *Citrus*.The genus *Citrus* includes several important fruits such as oranges, mandarins, lime, lemons and grape fruits [6].

Citrus fruits is reported for enormous number of biological activities such as anti-cancer, anti-diarrheal, antibacterial, antifungal, antiviral insecticidal and antioxidant, Some oils have been used in cancer treatment [7], [8] , [9] .

Due to the importance of this plant, we tried to determine the antibacterial effect of black dried lemon extract on local bacteria, and its effect associated with some popular antibiotics .

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a- Citrus limon (fresh)



b- Citrus limon (dry)

Material and methods:

1-Bacterial isolates:

Five different clinical microbial isolates (Gram positive, Gram negative bacteria) were isolated and identified by using conventional biochemical tests and Api system [10], and cultivated in pure culture, at Biotechnology division laboratory/applied science department/ University of technology /Baghdad. These bacteria included: (*Escherichia coli*, *Staphylococcus aureus*, *Protius mirabilis*, *Serratia marcescens*, *Pseudomonas aeruginosa*)

2-Extraction of black dried lemon:

Black dried lemon was purchased from the local market of Baghdad and has been crushed roughly. The Soxhlet extraction method has been used [11], by adding 100 gram of black dried lemon with 300 ml of 96% ethanol for 7 hours. The extraction solution was filtered and the solvent was removed using rotary evaporator at 45 °C to obtain the crude extract, and then it was kept in sterile bottle at 4°C. The resulted deposit was dissolved in distilled water to prepare the doses in the following steps.

3-Agar dilution method (MIC determination):

Series dilutions of black dried lemon extract was prepared ranging from (100, 50, 25, 12.5 and 6.5 mg.ml⁻¹) and added to the nutrient agar. After solidification of the media, the plates were inoculated with bacterial suspension then incubated at 37 °C for 24 hours. MICs were determined as the lowest concentration of dried black lemon extract that inhibit the growth of each bacteria on the agar plates. The presence of one or two colonies was disregarded [12].

4-Antibacterial activity:

Five dilutions of black dried lemon extract have been prepared with concentrations (100, 50, 25, 12.5 and 6.25 mg.ml⁻¹) by using distilled water. The screening of antibacterial activity of these dilutions was performed on Muller Hinton agar media, by the using agar well diffusion method. Wells of 6 mm diameter and 5 mm depth were made on the solid agar using a sterile glass borer [13]. Approximately 20µl of each dilution was loaded into wells were made in the spread plate culture of each bacterial isolates. All of the plates of the tested bacteria were then allowed to incubate at 37°C for overnight. The diameters of the inhibition zones were measured by millimeter (mm).

◦-Antibiotic Sensitivity test :

The sensitivity test was carried out by using Disk agar diffusion method according to (Baron & Finegold, 1990) [14], as follows:

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Bacterial inoculums have been prepared by taking few pure bacterial colonies and dissolved in normal saline, the turbidity of the bacterial inoculums should be compared with that of the recommended turbidity (No. 0.5) standard McFarland tube (10^6 cfu/ml). 0.1 ml from this suspension was spread on Muller-Hinton agar medium plates and let to dry at room temperature. Five antibiotics (Ampiciline (AM(10 mcg)), Gentamycine (CN(10 mcg)), Amoxiline (AX(10 mcg)), Erythromycine (E(15 mcg)) and Tetracycline (TE(30)) were used, by placing the disks of antibiotics on the inoculated plate and pressed into the agar with a sterile forceps. Then the plates were incubated at 37 °C for 24 hours in an inverted position. The results have been read after incubation. The diameters of the complete zone of inhibition were noted and measured by a millimeter (mm).

1-Synergism of dried black lemon extract with antibiotics:

A suspension of the tested microorganisms was uniformly swabbed on agar. Sterile blank discs were individually impregnated with different concentration of extracts (according to MICs results) and placed onto the inoculated agar plates. The plates were incubated at 37 °C for 24 h. The antimicrobial activity was measured by measuring diameter of the resulting zone of inhibition against the tested organisms [15].

Results and discussion:

1- Results MICs of dried black lemon extract against tested bacteria:

Table 1: MICs of dried black lemon extract against tested bacteria:

Bacteria	Concentration of dried lemon extract (mg.ml ⁻¹)					
	100	50	25	12.5	6.25	MICs
<i>Escherichia coli</i>	-	-	±	+	++	25
<i>Staphylococcus aureus</i>	-	-	-	±	+	12.5
<i>Protius mirabilis</i>	±	+	+	+	+	100
<i>Serratia marcescens</i>	-	-	-	±	+	12.5
<i>Pseudomonas aeruginosa</i>	-	-	-	±	+	12.5

(-) :no growth, (±) :minimum growth ,(+) : moderate growth ,(++) :maximum growth.

2- Results of dried black lemon extract dilutions against bacteria:

The extract of dried black lemon has shown good antibacterial activity against all of the selected bacteria that have been used in this research with one exception (*Protius mirabilis*) which did not affected by all dilutions of dried black lemon extract.

Staphylococcus aureus was the most bacteria that have been affected by this extract, the inhibition zone was 29 mm with dilution 1, and this was the biggest inhibition zone among the other bacteria. The results of dried black lemon extract dilutions against bacteria have been listed in the table 2, F. 1:

Table 2: results of dried black lemon extract dilutions against bacteria

Bacteria	Dilution1 (m.m)	Dilution2 (m.m)	Dilution3 (m.m)	Dilution4 (m.m)	Dilution5 (m.m)
<i>Escherichia coli</i>	20	18	13	-	-
<i>Staphylococcus aureus</i>	29	23	17	12	-
<i>Protius mirabilis</i>	-	-	-	-	-
<i>Serratia marcescens</i>	23	18	15	12	-
<i>Pseudomonas aeruginosa</i>	25	20	18	13	-

Dilution1,2,3,4 and 5 are (100,50,25,12.5 and 6.25 mg.ml⁻¹ respectively).

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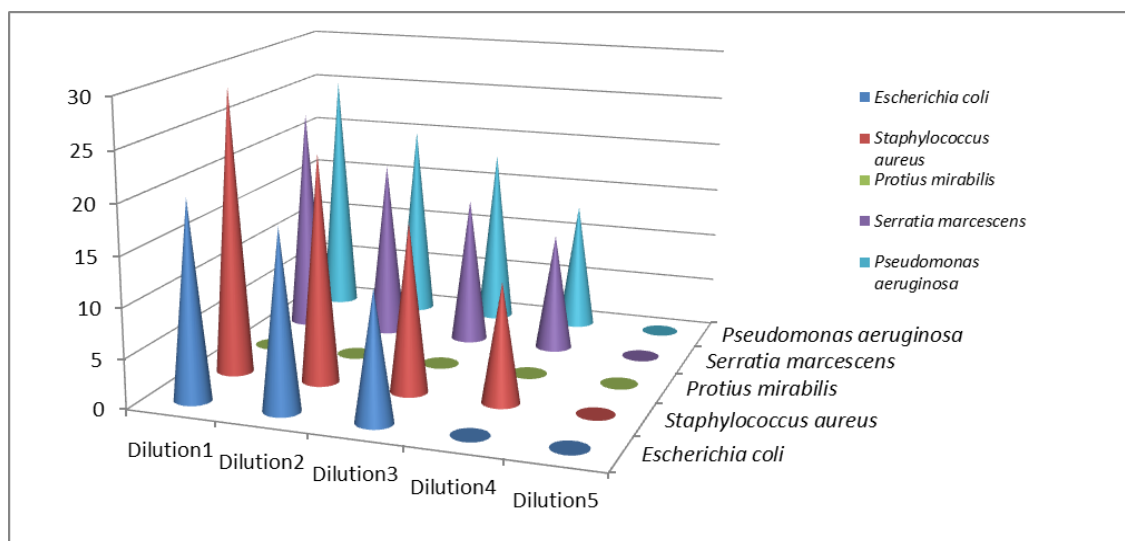


Figure 1: results of dried black lemon extract dilutions against bacteria

3- Results of sensitivity test of antibiotics against the isolated bacteria:

The results of the sensitivity test have shown that AX (25) was the most effective antibiotic on *Escherichia coli* and *Protius mirabilis*., E(15) was the most effective antibiotic on *Staphylococcus aureus*., while CN(10) was the most effective antibiotic on *Serratia marcescens* and *Pseudomonas aeruginosa*.as shown in table 3, F.2.

Table 3: results of sensitivity test of antibiotics against the isolated bacteria

Bacteria	AX (25)	CN(10)	AM(10)	E(15)	TE(30)
<i>Escherichia coli</i>	20	10	-	-	15
<i>Staphylococcus aureus</i>	15	25	13	31	26
<i>Protius mirabilis</i>	22	19	-	-	8
<i>Serratia marcescens</i>	-	22	10	-	9
<i>Pseudomonas aeruginosa</i>	-	12	-	-	-

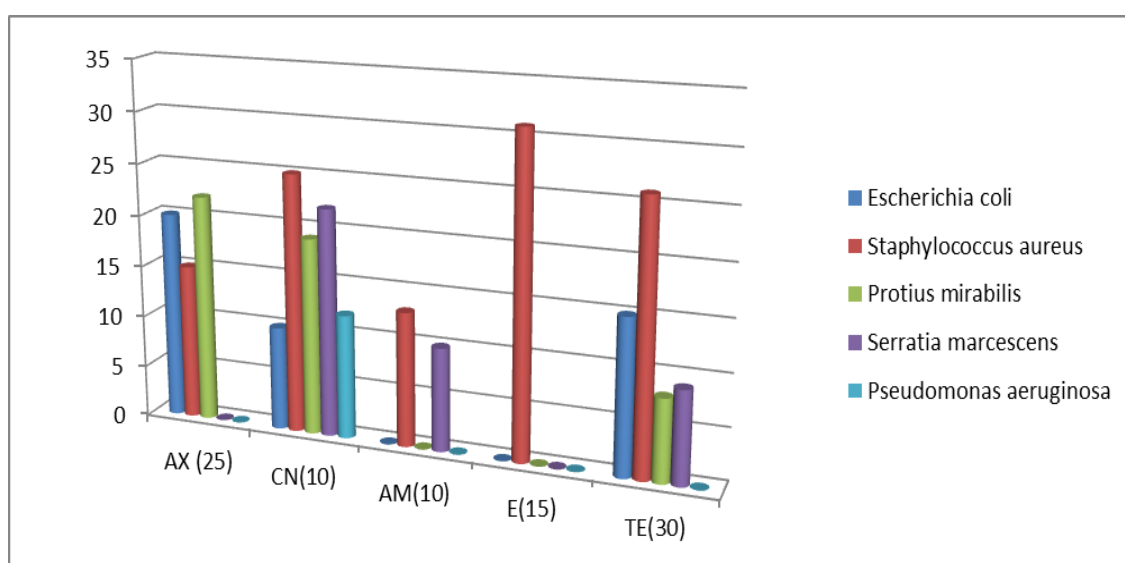


Figure 2: results of sensitivity test of antibiotics against the isolated bacteria

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4: results of the mixture of dried black lemon extract with antibiotics:

The impregnated antibiotics with dried black lemon extract have been tested on the bacterial isolates, and the inhibition zone have been detected in the table4, F.3 .

Table 4: results of the mixture of dried black lemon extract with antibiotics

bacteria	Inhibition zone of Dried black Lemon extract mixed with antibiotics tested on bacteria (m.m)				
	extract+AX (25)	extract+CN (10)	extract+AM (10)	extract+E (15)	extract+TE (30)
<i>Escherichia coli</i>	-	8	8	-	18
<i>Staphylococcus aureus</i>	11	20	-	18	25
<i>Protius mirabilis</i>	12	16	13	12	18
<i>Serratia marcescens</i>	8	21	-	8	8
<i>Pseudomonas aeruginosa</i>	-	12	-	6	-

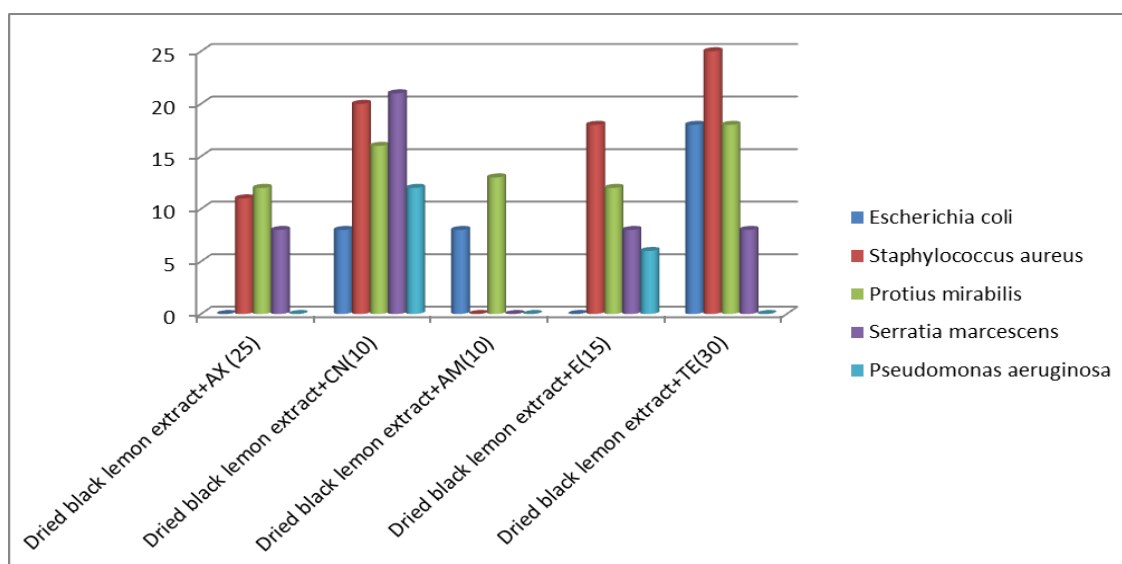


Figure 3: results of the mixture of dried black lemon extract with antibiotics.

Dried black lemon extract helped in increase the antibacterial activity of some antibiotics and decrease or not affect on other antibiotics when they tested on bacteria.

The combination of antibiotics with dried black lemon extract had different effects against tested bacteria (synergism ,antagonism and indifference).

Escherichia coli , was resistant to Tetracycline (TE(30)) but inhibition zone appeared (about 8 mm) when this antibiotic was mixed with dilution 3of dried black lemon extract. Ampiciline (AM(10 mcg)) ,the inhibition zone increased from 15 mm to 18 mm when it was mixed with the same dilution of dried black lemon extract. But this bacteria remain resistant to Erythromycine (E(15 mcg)) even after mixing with dried black lemon extract.

Protius mirabilis resist Ampiciline (AM(10 mcg)) and Erythromycine (E(15 mcg)), but inhibition zone appeared to them when they mixed with dilution 1 of dried black lemon extract , also the mixture of this extract with Tetracycline (TE(30 mcg)) led to increase the inhibition zone from 8 mm to 18 mm.

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In *Serratia marcescens* , the antibacterial activity of Amoxiline (AX(100mcg)) and Erythromycine (E(15 mcg))increased (from 0 mm to 8 mm) when they impregnated with dilution 4 of dried black lemon extract .

In *Pseudomonas aeruginosa* , the inhibition zone of Erythromycine (E(15 mcg)) appeared (about 6mm) when it impregnated with dilution 4 of dried black lemon extract. The inhibition zone to Ampiciline (AM(100 mcg)) remained the same 12 mm, while the resistant to other antibiotics did not change.

On the other hand, in all of our bacterial isolates, the inhibition zones of the rest antibiotics decreased when they mixed with this extract,as in *Staphylococcus aureus*.

Our results shows that dried black lemon extract could be helpful to overcome the resistance problem to antibiotics, also this extract have good antibacterial properties and can be considered as natural antibiotic.

Previous reports showed that fruits and vegetable plants extracts have antifungal activity more than antibacterial activity, also ,they are more effective against Gram positive bacteria more than Gram-negative [16], [17], [18] .but in our results (as in table 3),The dried black lemon extract showed good antibacterial activity against both of Gram positive and Gram negative bacteria.

This finding agreed with Hayes and Markovic (2002) [19] who investigated the antimicrobial properties of lemon and found that lemon possesses significant antimicrobial activity against *S. aureus*, *Klebsiella*, *Escherichia coli*, *P. aeruginosa* and other. Also Al-Ani *et al* (2009) [20], mentioned that *C. limon* have good bacterial inhibition against *S. aureus*, *P. aeruginosa* and *P. vulgaris*. And this funding agreed with our results.

There was different susceptibilities to this extract between the bacteria that have been tested, and this due to the bacterial genus and species [8] . Hindi and Chabuck (21) mentioned that there was no significant differences between *C. limon* (peel) and *C. limon* (dry) extract on microbial isolates, and this facilitates using it as an antibacterial .Other studies mentioned that the daily usage of pure hand gel which contain *C. limon* in its ingredients is helpful to reduce microbial growth [22] .

Many studies referred that the activity of dried black lemon extract related to the antiplasmid agent property and antimiotic property[23] . it's important to notice that during the detection of antimicrobial susceptibility to different plants extracts, the solubility and the rate of diffusion in agar medium or its volatilization effect on the diameter of the inhibition zone of these extracts, therefore the results could be affected[24].

The plant medicines may be beneficial but are not completely harmless, they can be used in the treatment of infectious diseases caused by resistant microbes. The synergistic effect from the association of antibiotic with plant extracts against resistant bacteria leads to new choices for the treatment of infectious diseases. This effect enables the use of the respective antibiotic when it is no longer effective by itself during therapeutic treatment.

Other study mentioned that citrus fruit peel extract had a better inhibitory activity than synthetic compounds when it was tested against the common pathogens of the gastrointestinal tract [25].

The results of our study showed that dried lemon extract had broad spectrum of antibacterial activity , and it could be used as alternative of antibiotics ,therefore pharmacological tests are necessary to isolate and characterize the its active compounds

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 , and it should be investigated in-vivo to better understanding of its safety efficiency and properties.

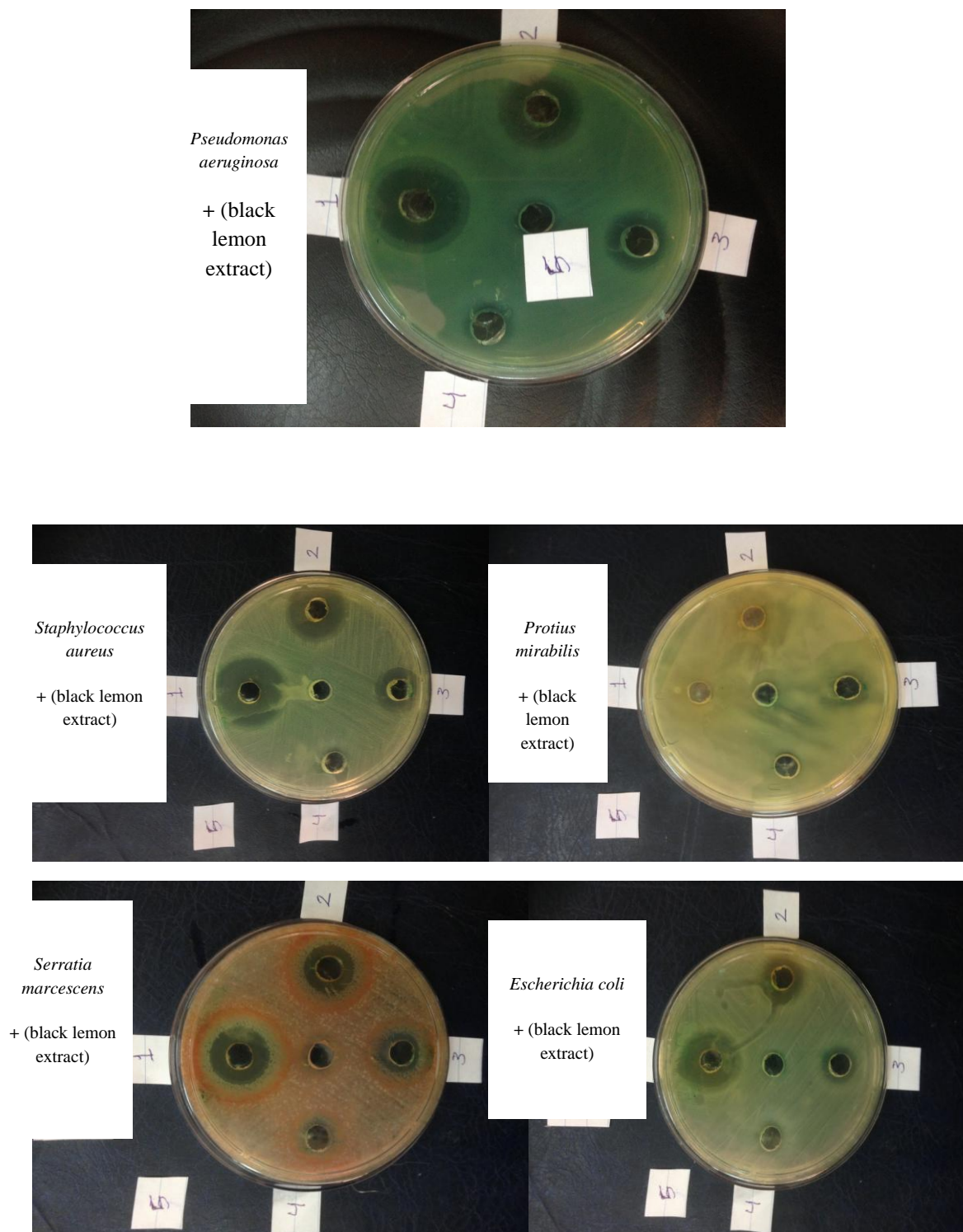


Figure 4: shows the results of dried black lemon extract dilutions(1,2,3,4 and 5) against bacteria(*Escherichia coli*, *Staphylococcus aureus*, *Protius mirabilis*, *Serratia marcescens* and *Pseudomonas aeruginosa*)

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Escherichia coli



Pseudomonas aeruginosa



Serratia marcescens



Protius mirabilis



Staphylococcus aureus



Figure 5: show the results of sensitivity test of antibiotics (AX(25), CN(10), AM(10), E(15)and TE(30)) against bacteria(*Escherichia coli*, *Staphylococcus aureus*, *Protius mirabilis*, *Serratia marcescens* and *Pseudomonas aeruginosa*)

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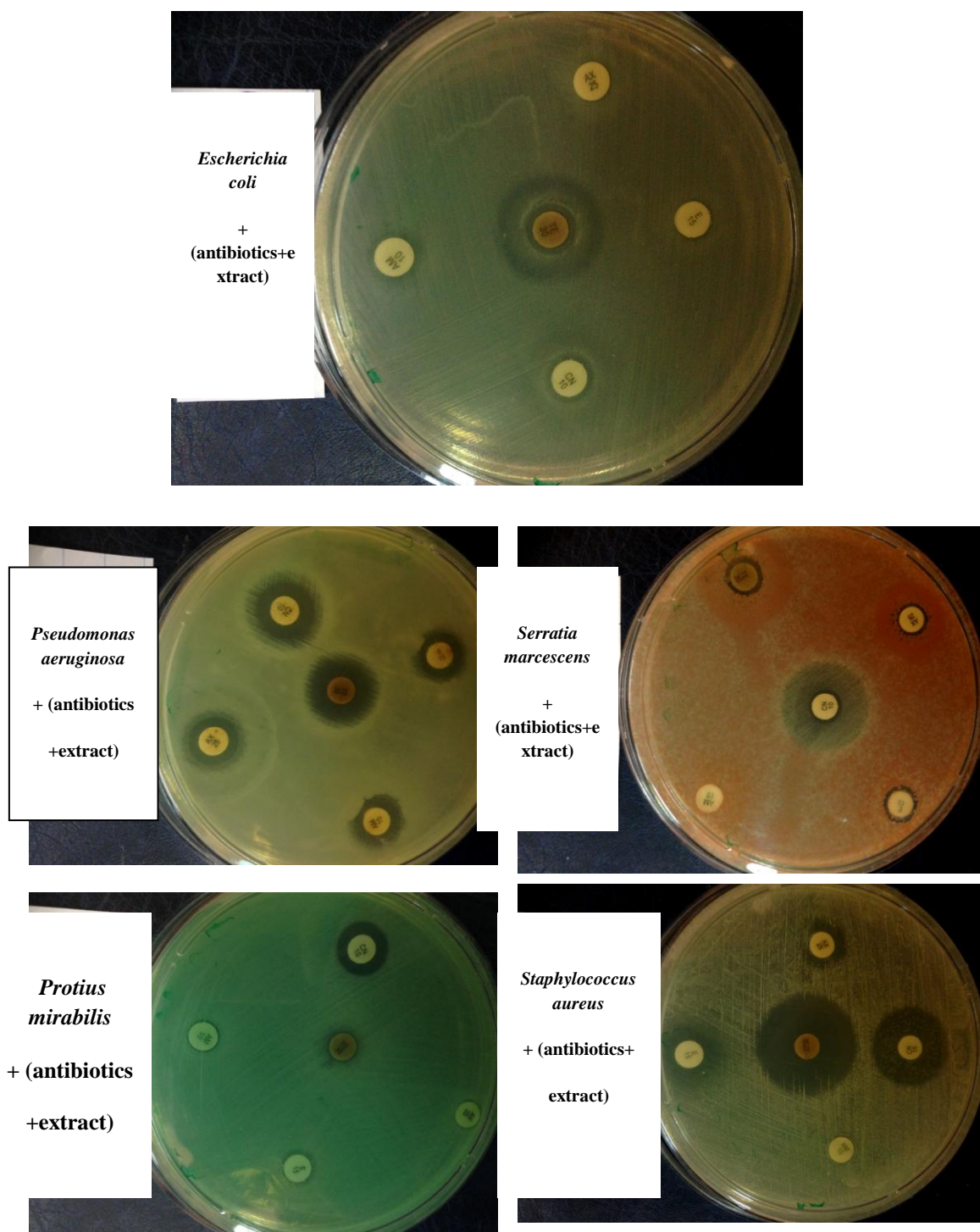


Figure 6: show the results of the mixture of dried black lemon extract with antibiotics against bacteria(*Escherichia coli*, *Staphylococcus aureus* , *Protius mirabilis*, *Serratia marcescens* and *Pseudomonas aeruginosa*).

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تأثير المضادات الحياتية مكملة بخلاصة الليمون الاسود على عزلات بكتيرية محلية

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العلوم التطبيقية

فرع التقنيات الاحيائية

الخلاصة:

نتيجة لتزايد الميل العالمي نحو استخدام الادوية المصنعة من النباتات والاعشاب , سعت هذه الدراسة لمعرفة تأثير مستخلص احدى النباتات الشائعة الاستخدام في العراق (نبات النومي بصرة),على عزلات بكتيرية معزولة محليا. تم اجراء الاستخلاص بأستعمال الكحول الايثيلي بتركيز ٩٦%, وتمت دراسة تأثيره لوحده تارة وممزوجا بمضادات حياتية تارة اخرى. شملت هذه المضادات :الامبسيلين (١٠ mcg), الجنتاميسين (١٠ mcg) , الاموكسيلين (٢٥ mcg) , الاريثرومايسين (١٥ mcg) و التتراسايكلين (٣٠ mcg) على بعض العزلات الموجبة والسالبة لصبغة غرام والمعزولة محليا وهي: *Serratia marcescens*, *Pseudomonas aeruginosa* , *Protius mirabilis* *Staphylococcus aureus* , *Escherichia coli*, اظهرت النتائج وجود فعالية مضادة للبكتريا لمستخلص النومي بصرة الكحولي على كل العزلات المستخدمة بأستثناء بكتريا. ساعد هذا المستخلص في زيادة الفعالية المضادة للبكتريا لبعض المضادات الحياتية , في حين قلل او لم *Protius mirabilis* يؤثر على باقي المضادات الحياتية الاخرى عندما فحصت على البكتيريا.