

Study of the inhibitory effect of Ethanolic extract of (*Quercus robur* ,*Cinnamomum zeylanicum* and *Thymus vulgaris*) on the growth of *Staphylococcus aureus* isolated from clinical mastitis in cow

Z. A. Hatem J. A. Sabeeh

Coll. of Vet. Med. /Unive. of Al- Qadisiyia

Abstract

The present study was designed to evaluate the inhibitory effects of three local plant ethanolic extracts (*Quercus robur* , *Cinnamomum zeylanicum* and *Thymus vulgaris*) against the growth of *Staphylococcus aureus* isolated from milk of cow infected with clinical mastitis in culture media by using of agar well diffusion method. For this purpose graduate concentrates for each extract (50 , 100 , 200 , 400)mg/ml prepared and tested .The result showed that the extract of *Quercus robur* was more effective followed by *Thymus vulgaris* and *Cinnamomum zeylanicum*.The statistical analysis by using ANOVA with LSD at level ($p<0.05$) showed that there was no significant differences between the effect of the studied concentrations of *Quercus robur* , *Cinnamomum zeylanicum* while for *Thymus vulgaris* we find that the concentration 100 mg/ml had a significant difference with the other studied concentration in inhibition of the growth of the tested bacteria. We also find that between the antibiotics the largest zone of inhibition was given by LOM followed by SPV,PI,NV,RA and CX.

Introduction

Medicinal plants are a source of great economic value all over the world. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of the country (1).Nowadays multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious disease. In addition to this problem, antibiotics are sometimes associated with adverse effects on the host including hypersensitivity, immune-suppression and allergic reactions. This situation forced scientists to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistance in bacteria of medical importance, there is a constant need for new and effective therapeutic agents. Therefore, there is a need to develop alternative antimicrobial drugs for the treatment of infectious diseases from medicinal plants (2). Antimicrobials of plant

origin have enormous therapeutic potential. They are effective in the treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. The beneficial medicinal effects of plant materials typically result from the combinations of secondary products present in the plant. In plants, these compounds are mostly secondary metabolites such as alkaloids, steroids, tannins, and phenol compounds, flavonoids , steroids, resins fatty acids gums which are capable of producing definite physiological action on body (3,4). Worldwide, economic losses due to mastitis have been estimated at \$35 billion (5). *Staphylococcus* spp. is the main causative agent of bovine mastitis, with higher prevalence in cases of clinical and subclinical manifestations (6). The most common treatment is based on intramammary infusion of antibacterial agents. However, cure rates obtained with

such drugs are not always effective, because it may determine the emergence of resistant bacteria (7) as well increase amounts of antibiotic residues in milk (6). Nevertheless, the treatment of bovine subclinical mastitis caused by *S. aureus* in the lactation can be economically unviable (7). In this context, this study aimed to: a) evaluate the *in vitro* antimicrobial activity of ethanolic extract of

(*Quercus robur* , *Cinnamomum zeylanicum* and *Thymus vulgaris*) against *Staphylococcus* spp.; b) compare the activity of these extracts against *Staphylococcus* spp. isolate with multiple profiles of susceptibility and resistance to (Lomefloxacin, Sparfloxacin, Novobiocin, , Pipemidic acid,, Rifampin, Cloxacillin) antibiotics.

Materials and Methods

1- Plant collection and preparation :

In this experiments we used three local medicinal plants include: *Quercus robur* , *Cinnamomum zeylanicum* and *Thymus vulgaris*. All these plants were obtained from the local market and identified by the national Iraqi institute for herbs, we take the fruits of the first plant , stem of the second plant and fruits of the third plant , then all the chosen parts of the above plants were subjected to aerial drying for two weeks, after drying of these parts we grinded it very well until it became as a fine powder . The Ethanolic extraction of the three plants were done by Harborn method (8) by using of Ethanol at a concentration (96%).

2- *Staphylococcus aureus* Isolates:

A-Sample collection :

Milk samples were collected in sterile tubes (2 tubes) for each sample one for California mastitis test (CMT) and another for bacteriological test) and a septic technique used for milk samples collection according to (9) .

B-Bacterial culture and identification:

All milk samples from subclinical mastitis cases which gave a positive reaction with (CMT) were submitted to centrifugation at 3000 rpm / 15 minute, and the precipitate was cultured on :Blood Agar ,Nutrient Agar and MacConky Agar ,all the Petri plate that contain this agars were incubated at 37 C° for 24 - 48hrs .Diagnosis depend on morphological character & cultural character , then followed by examination with gram stain, after that the colonies were subcultured on selective media and

differential media according to the type of isolated bacteria then incubated at 37 C° for 24 – 48 hrs. The biochemical test used to diagnosis of *staphylococcus spp.* were include:

- Catalase Test , Oxidase test , Coagulase Test , Urease Test , Heamolysis on blood agar , Gelatin Liquefaction Test (Gelatinase) , Voges – Proskauer Test , Nitrate reduction Test , Sugar Fermentation Test (Mannitol , Lactose , Mannose , Xylose ,Trehalose , Sucrose , Maltose) according to the method of (9,10,11) .

Production of pigment in Mannitol salt agar and in (Staph 110 media) (LAB –U.K) MAST STAPH™: (Mast Group Ltd , USA) API Staph (biomerioux, France).

3- Antibiotics

In this study we use (6) antibiotics to compare their antibacterial effect with that of the medicinal plants ethanolic extracts due to their broad spectrum activity and these antibiotics include: Lomefloxacin(LOM) 10 mcg ,Sparfloxacin (SPX) 5 mcg ,Novobiocin (NV) 30 mcg ,Pipemidic acid (PI) 20 mcg, Rifampin(RA) 5 mcg, Cloxacillin (CX) .(Bioanalyse)® .

4- Serial dilutions :

For each of the tested medicinal plants we had been made a serial dilution to study the effect of the plants in inhibition the growth of *Staphylococcus spp.* at a different concentrations and select the most effective concentration of the plant extract depending on the zone of inhibition of growth that been given by each concentration , we started with a concentration (400)mg/ml (prepared by add 10 ml from Ethanol 96% to 4 gm of

the plant extract) and the second concentration is 200 mg/ml(prepared by taking 4ml from the first dilution and we add 4 ml of ethanol 96% to it ,the third dilution is 100 mg/ml (prepared by taking 2ml from the second dilution and add 2 ml of ethanol 96% to it) ,the fourth dilution is 50%(made by taking 2 ml from the third dilution and add 2 ml of ethanol 96 % to it). These serial dilution was decided depending on clinical trails.

5-Sensitivity test study:

After preparation of all the medicinal plants ethanolic extracts and activation of the pathogenic bacteria in the nutrient broth , the Mueller Hinton Agar (HIMEDIA –Mumbai-India) was prepared by dissolve 38 gm from the agar powder in 1000 ml of distilled water in a flask and shaking it well to dissolve the agar and then we start heating it by a Benzen burner in attempt to complete dissolving of all the agar powder and after that the agar was sterilize by using of autoclave at 15 IP for 15 minutes . After preparation of Mueller Hinton agar we poured it in to Petri plates and after solidification of the agar we made

4 wells (5 mm diameter) in each one of the Petri plates except those of the antibiotics discs , 13 Petri plates containing Mueller Hinton agar were used in this study {(9 plates for the medicinal plant extract study :3 plates for each extract) and (4 Petri plates for antibiotics: 2 plates for each four antibiotics)}. This study was done by taking swap from the test tube that contain the bacterial suspension and inoculated it on the Petri plates that contain the Mueller Hinton agar, and then we added 0.1 ml of each concentration of each plant extract on its own plates and we applied the chosen antibiotics discs in it is plates.After complete applying of all the medicinal plants extracts concentrations and the Antibiotics we incubated all the Petri plates at 37°C for 24 hours .The sensitivity of microorganisms towards the plants extracts was screened by following the agar well –diffusion method .The zone of inhibition (diameter in mm) in triplicates was measured and the mean value (μ) was tabulated (12) .

Results and discussion

Mastitis in cattle is caused primarily by bacteria that invade the udder, multiply, and produce toxins that are harmful to the mammary gland (13). A part from causing colossal economic losses, mastitis also posses the risk for the transmission of zoonotic diseases like tuberculosis, brucellosis, leptospirosis and streptococcal sore throat to human beings (14). It can be associated with health risks to consumers, especially those related to the presence of zoonotic pathogens and antimicrobial drug residues in milk (15). *Staph.aureus* is considered one of the major pathogens that cause clinical mastitis(16), . In our study we try to experiment the effect of selected medicinal plants in comparison to Antibiotics on *Staphylococcus aureus* isolated from milk of cow infected with clinical mastitis growing *invitro* in a Muller Hinton Agar by using of agar well diffusion

method.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 it is effect(17),The antibacterial activity of Oak(*Quercus robur*), Cinnamon(*Cinnamomum zeylanicum*) and Thyme (*Thymus vulgaris*) extracts were tested against *Staphylococcus aureus invitro* and the results are listed in (Table 1),(Fig. 1). It was found that ethanolic extract of Oak was the most effective antibacterial with a zone of inhibition as followed according to the studied concentrations (400,200,100,50 mg/ml) (33.555±0.929, 29.0±0.333, 29.222±0.222, 30.555±1.986) mg/ml respectively (Fig. 2) , followed by the effect of ethanolic extract of Thyme which gave a zone of inhibition according to the

studied concentrations (24.111±0.351, 25.333±0.6, 28.777±0.64, 20.556±1.684) mg/ml respectively (Fig.4), then followed by the inhibition zones gave by Cinnamon (25.333±0.288, 25.444±0.242, 24.0±0.5, 18.666±0.2350 mg/ml respectively(Fig.3) . The antibiotics used in this study also showed an inhibition to the growth of *S. aureus* invitro with an inhibition zones as follow: (LOM 29.44±0.41,SPX 21.55±0.44, PI 20.66±0.37, NV19±0.4,RA 11.11±0.26 , CX 0±0) table (2) . The result showed that there were no significant differences ($P < 0.05$) between the result obtained by the different concentrations that had been used for each of Oak and Cinnamon while there was a significant differences ($P < 0.05$) between the concentrations(400,200,50 mg/ml) and 100 mg/ml of Thyme .The major antimicrobial components of the medicinal plants in the study include Tannins and Quercetin for Oak ,also Tannins and Cinnamaldehyde for Cinnamon that had been reported to have an inhibition effect to the growth of *S.aureus*(18) and for Thyme it contain Caffeic acid ,tannins and thymol (19) , tannins can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (20).*S. aureus* (grampositive) being more susceptible to plant tannin extracts(21,22), and the mechanisms of action in the growth inhibition of bacteria are involved, such as destabilization of cytoplasmic and plasma membranes, inhibition of extracellular microbial enzymes and metabolisms, and deprivation of the substrate required for microbial growth (22; 23; 24).The site (s) and number of hydroxyl (-OH) groups on the tannins are also thought to be related to their relative toxicity to microorganisms, with increased hydroxylation resulting in increased toxicity (25,26).The medicinal plants like Cinnamon are being used traditionally for the treatment of

inflammation, cough, toothache, antiseptics expectorant, and some fungal infection like candidiasis. The ethanol extracts of Cinnamon showed notable antibacterial activity against Gram positive bacteria(27).The antibacterial activity has been attributed to the presence of some active constituents in the extracts. Studies suggested that the antibacterial ctivity of Cinnamon was probably due to their major component, cinnamaldehyde and their properties could be multiple. An important characteristic of plant extracts and their components is their hydrophobicity, which enable them to partition the lipids of the bacterial cell membrane and mitochondria, disturbing the cell structures and rendering them more permeable. Extensive leakage from bacterial cells or the exit of critical molecules and ions will lead to death.(28).The ethanolic extract of Thyme contains many phytochemicals substances including terpenoids, tannins and polyphenolic compounds as well as flavonoids. The different components diffusing at different rates may have been responsible for the varying zones of inhibition obtained in against the susceptible microorganisms. This plant is assumed to have compounds which have a potential antimicrobial activity its important compounds are flavonoids. Flavonoid's activity is probably due to their ability to complex with extra cellular and soluble proteins and to complex with bacterial cell walls and lipophilic flavonoids may also disrupt bacterial membranes. If the components could be separated and tested further we might find use as individual antibacterial agent against Gram-positive infections (29).The result showed that the inhibition zones produced by Oak was ranged between (29.22 -30.55)mm which is close to the result of (30)how showed in his study that Oak gave an inhibition zone ranged from (15-23mm) according to the studied concentrations ,also the result produced by Thyme was ranged between

(20.55-28.77) mm which was closed to the results of (31) how tested the effect of *Thymus daenensis* on a number of bacteria include *S.aureus* and gave an inhibition zone ranged between (19-20)mm in studied concentration it was also agree with the results of (32,33,34,35,36) advanced studies used *Thymus* spp. extracts as antimicrobial agents depend on presence of both thyme essential oil and thymol. Also, these studies suggested use of thyme as an antibiotic. the result of Cinnamon which was gave a zone of inhibition of the growth of the tested bacteria ranged from (18.66-25.44) mm also agree with the result of (37) who used *Cinnamomum Zeylanicum* effect against *S.aureus* and the inhibition zone was about 10 mm for the minimal bactericidal concentration 10 mg/ml which indicated that increasing the concentration of the plant extract will lead to increase the inhibition

zone as indicated by our study, our result for Cinnamon was also close to the result of (38) who found that the ethanolic extract of Cinnamon was active against *Staphylococcus aureus* strains with zones of inhibition ranging from (10.0 - 11.4)mm.As a conclusion we find in our study that each of the used medicinal plants ethanolic extracts (Oak, Cinnamon and thyme) had antibacterial effect against *S.aureus* isolated from milk of cows infected with clinical mastitis with a significant differences between their effect ($P<0.05$) while there was no clear effect to the studied concentration of the used medicinal plants extract on it is inhibition effect on the tested bacteria except for Thyme that showed significant differences ($P<0.05$) between the inhibition effect of the studied concentrations of the plant on the tested bacteria.

Table (1): The antibacterial activity of the ethanolic extract of (*Quercus robur*, *Cinnamum vulgaris*, *Thymus vulgaris*) on *S.aureus*

Plants extract	Concentrations(mg/ml)			
	400	200	100	50
<i>Quercus robur</i>	33.555±0.929 aA	29.0±0.333 bA	29.222±0.222 cA	30.555±1.986 dA
<i>Cinnamum vulgaris</i>	25.333±0.288 aB	25.444±0.242 bB	24.0±0.5 cB	18.666±0.235 dB
<i>Thymus vulgaris</i>	24.111±0.351 aC	25.333±0.6 bC	28.777±0.64 cB	20.556±1.684 dC

*Values were expressed as means ± standard error

*Values with different capital letters are significant differences vertically at ($p < 0.05$).

*Values with different small letters are significant differences horizontally at (p

Table (2): The antibiotic effect against *S.aureus*

Antibiotics	Inhibition zone(mm)
LOM	29.44±0.41
SPX	21.55±0.44
PI	20.66±0.37
NV	19±0.4
RA	11.11±0.26
CX	0±0

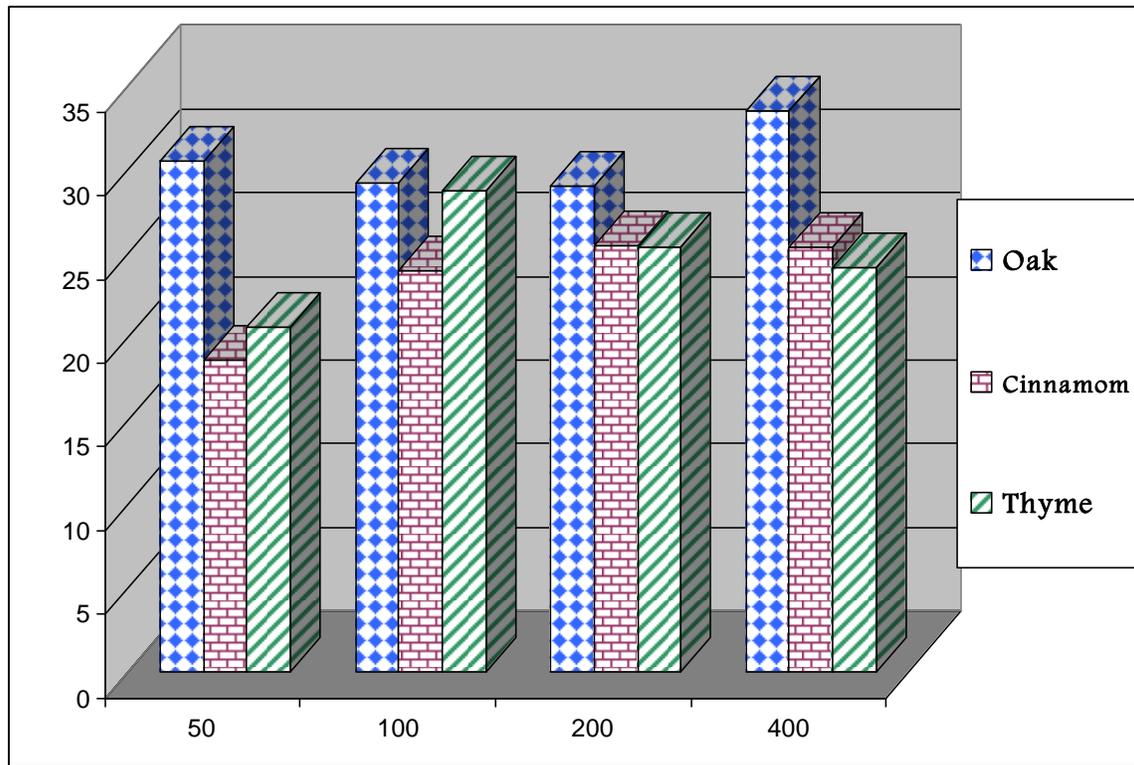


Figure (1): Inhibition zones of *Staphylococcus aureus* exhibited by the ethanolic extracts of the tested plants.

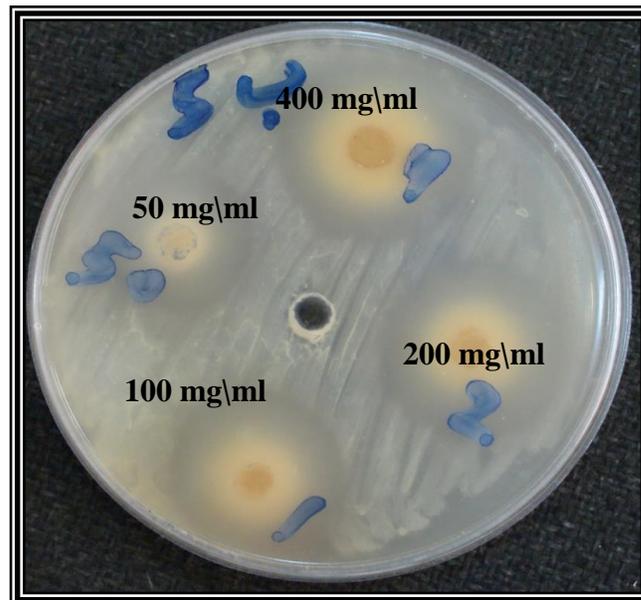


Figure (2): Inhibition zones of *Staphylococcus aureus* growth on Mueller-Hinton agar produced by ethanolic extract of *Quercus rubur*, the peripheral four wells contained extract concentrations (50, 100, 200, 400 mg/ml) where as the central well contained 0.1 ml of 96% ethanol.

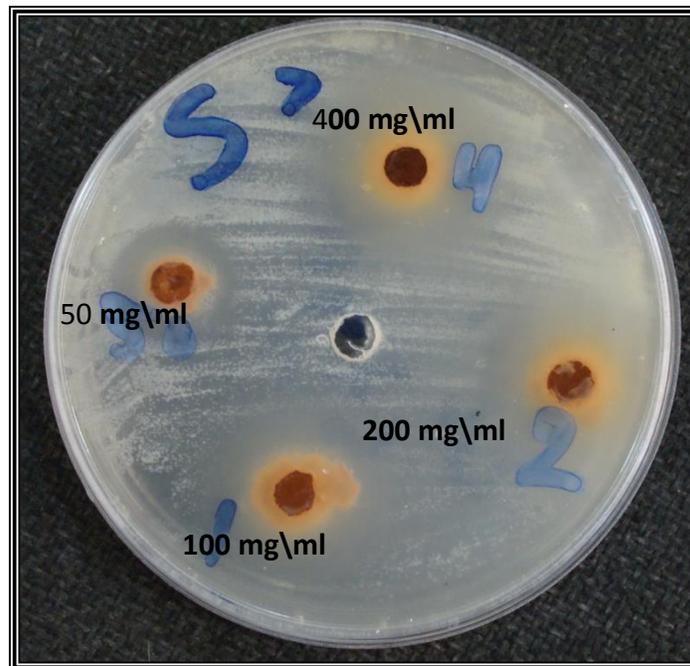


Figure (3): Inhibition zones of *Staphylococcus aureus* growth on Mueller-Hinton agar produced by ethanolic extract of *Cinnamomum zeylanicum* , the peripheral four wells contained extract concentrations (50, 100, 200, 400 mg/ml) where as the central well contained 0.1 ml of 96% ethanol .

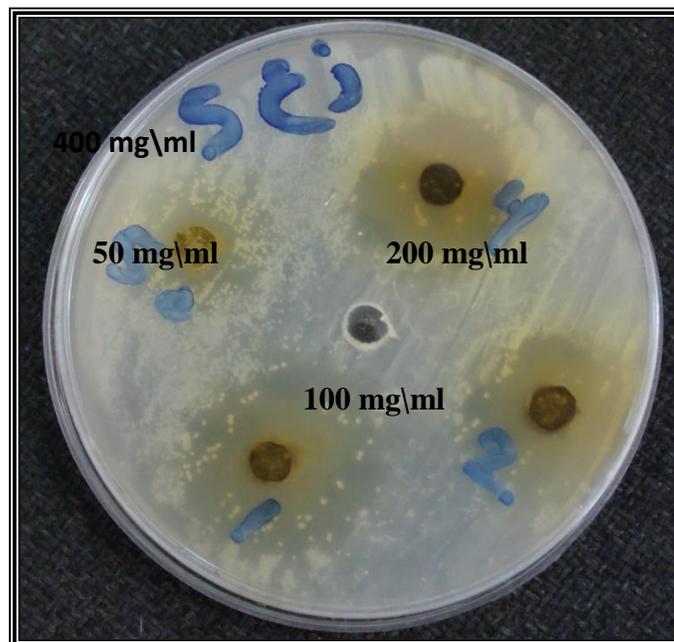


Figure (4): Inhibition zones of *Staphylococcus aureus* growth on Mueller-Hinton agar produced by ethanolic extract of *Thymus vulgaris* , the peripheral four wells contained extract concentrations (50, 100, 200, 400 mg/ml) where as the central well contained 0.1 ml of 96% ethanol .

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دراسة التأثير المثبط للمستخلص الأيثانولي لنباتات (البلوط ، الدارسين، الزعتر) على نمو جرثومة المكورات العنقودية الذهبية المعزولة من التهاب الضرع السريري في الأبقار

زينب عدنان حاتم جنان عبد الأمير صبيح
كلية الطب البيطري/ جامعة القادسية

الخلاصة

صممت هذه الدراسة لتقييم التأثير المثبط للمستخلص الأيثانولي لثلاثة من النباتات المحلية (البلوط، الدارسين و الزعتر) ضد نمو جرثومة المكورات العنقودية الذهبية المعزولة من حليب الأبقار المصابة بالتهاب الضرع السريري والمنمأة في الوسط الزرعي في المختبر باستخدام طريقة الانتشار في الوسط المغذي . ولهذا الغرض حضرت تراكيز متدرجة من كل من المستخلصات النباتية (400,200,100,50) ملغم/مل وتم اختبارها . اظهرت النتائج ان مستخلص البلوط كان هو الأكثر تأثيراً ثم يليه الزعتر ثم الدارسين.تم اجراء التحليل الإحصائي باستخدام اختبار تحليل التباين مع اقل فرق معنوي تحت مستوى احتمالية (05,0) حيث اظهرت النتائج انه لم يكن هناك فرق معنوي بين التراكيز المستخدمة لنباتي البلوط والدارسين بينما بالنسبة للزعتر وجد بأن هنالك فرق معنوي بين تركيز 100 ملغم/مل بالمقارنة مع التراكيز الأخرى لنفس النبات في تثبيط نمو الجرثومة المدروسة. كذلك وجد بأنه بين المضادات الحياتية التي تم استخدامها فان المضاد الحيوي لومفلوكسايين اعطى اكبر نطاق تثبيط لنمو البكتريا يليه سبارفلوكساسين ، نوفوبايوسين، بمبيدك اسيد ، ريفامبين وكلوكساسيلين .