

Study of the Inhibitory Effect of *Thymus vulgaris* and *Eucalyptus camaldulensis* on *In vitro* Growth of *Listeria monocytogenes*

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Abstract:

The antibacterial activity of the *Thymus vulgaris* and *Eucalyptus camaldulensis* was studied against *Listeria monocytogenes*. The inhibiting activity was evaluated by the agar well diffusion method. Results showed that the extracts of both tested plants demonstrated antimicrobial activity against the tested organism but the efficiency of the extracts was significantly affected by the solvent used in the extraction as well as plant species and the concentration. The aqueous extracts appeared to be the highly effective extracts against the tested organism, it exhibited an inhibition zone 16 mm-24 mm for thyme and 15 mm-24 mm for eucalyptus in comparison with 21mm and 23 mm for ampicillin and ciprofloxacin respectively. The alcoholic extract of thyme had no antibacterial activity in concentration of 25 and 50 mg /ml. The results of this study suggest that the antilisterial properties of these plants may be further investigated to explore the possibility of using them in the treatment of listerial infection.

دراسة التأثير التثبيطي لنباتي الزعتر *Thymus vulgaris* واليوكالبتوز *Eucalyptus camaldulensis* على نمو بكتريا *Listeria monocytogenes* مختبريا

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الخلاصة:

تضمنت الدراسة الحالية الكشف عن التأثير التثبيطي لنباتي الزعتر *Thymus vulgaris* و اليوكالبتوز *Eucalyptus camaldulensis* على نمو بكتريا *Listeria monocytogenes* في الزجاج باستخدام طريقة الانتشار بحفر الاكار. بينت النتائج ان جميع المستخلصات تمتلك تأثيرا مضادا للبكتريا المستخدمة و إن كفاءة تلك المستخلصات اعتمدت بشكل كبير على نوع المذيب المستخدم في الاستخلاص (ماء أو ايثانول) و نوع النبات إضافة إلى تركيز المستخلص. اظهر المستخلص المائي أعلى فاعلية مثبطة لنمو البكتريا مقارنة بالمستخلص الكحولي، إذ أعطى نطاق تثبيط تراوحت أقطاره بين 16-24 ملم بالنسبة لنبات الزعتر، و 13-24 ملم لنبات اليوكالبتوز. قورنت نتائج فعالية المستخلصات النباتية بالمضادات الحيوية الامبيسيلين و السايبروفلوكساسين والتي أعطت مناطق تثبيط 21 و 23 ملم على التوالي، ولم يكن للمستخلص الكحولي

لنبات الزعتر بتركيزي 25 و 50 ملغم /مل أي تأثير تثبيطي. و من خلال نتائج هذه الدراسة يمكن الكشف عن امكانية استخدام نباتي الزعتر و اليوكالبتوز كعلاج للاصابات الناتجة عن هذه البكتيريا.

Introduction:

Listeria monocytogenes is a Gram-positive, non-spore forming, facultatively anaerobic bacterium. It expresses a β hemolysin which causes destruction of red blood cells⁽¹⁾. *Listeria* spp. are responsible for listeriosis, a serious infection caused by eating food contaminated with the bacterium, it is occure in humans and animals mainly sheep. Encephalitis is the most common form of the disease in ruminant animals. Intra-uterine infection result in abortion in sheep, cattle and women⁽²⁾. This bacterium also responsible for the severe food borne illness. It is primarily transmitted through various foods. The organisms can grow at 4°C which means that organism replication continues in refrigerated foods. Listeriosis has fatality rates exceeding even *Salmonella* and *Clostridium botulinum*.⁽³⁾

The most effective and commonly used drugs for treatment of listeriosis is penicillin ,however this drug has many adverse effects such as nausea, diarrhea, erythema and superinfection (including candidiasis).Although penicillin is still the most commonly reported allergy up to 20% of all patients have penicillin allergy⁽⁴⁾.In addition this antibiotic can easily loss of effectiveness by β -lactamase enzyme of different bacterial species which destroy the β -lactam ring. Thus, the search for alternative

antibacterial compounds with high activity, low toxicity, cheaper, and more effective is still a necessary goal.

Thymus vulgaris:(thyme) a member of the family Lamiaceae, is a small shrubby plant with a strong spicy taste, which grows in several regions of the world⁽⁵⁾.This plant is widely used as flavoring agents in food processing and in medicine for its expectorant, antiseptic and antifungal properties⁽⁶⁾.The main constituents of thyme include thymol, carvacrol and flavonoids often thought to have strong inhibition activity against both Gram-positive and Gram-negative bacteria such as *Clostridium botulinum*, *Escherichia coli* and *Salmonella typhimurium*^(7,8).

Eucalyptus camaldulensis: Is a diverse genus of trees in the family *Myrtaceae*,it is extensively cultivated in many countries including Iraq. This spp. is a tall, evergreen, fast growing tree, and usually grows to 45 meters in height⁽⁹⁾. Eucalyptus contains a many compounds that make it active in infections. Perhaps the most famous one is eucalyptol .Leaf extracts of eucalyptus have been used as expectorant, decongestant, pain relieving and food additives. Research data has demonstrated that the extracts exhibited good antibacterial activity against *Streptococcus pyogenes*, *Staphylococcus aureus*,

Haemophilus influenzae, and *Klebsiella pneumonia* ⁽¹⁰⁾. Anti fungal and antiviral properties of eucalyptus were also reported ⁽¹¹⁾.

The emergence of bacterial resistance to the currently available antimicrobial drugs necessitates further research in the discovery of new safe and effective antibacterial agents. The aim of this study is to in vitro evaluate the antibacterial activity of *T. vulgaris* and *E. camaldulensis* extracts against *L.monocytogenese*.

Material and Methods:

Plant materials

T. vulgaris leaves were obtained from traditional shops, while the leaves of *E. camaldulensis* were collected directly from the gardens tree. The plants were dried in the shade, Exposure to sunlight was avoided to prevent the loss of active components. The dried materials were ground to fine powders by electric grinder.

Extracts preparation

One hundred grams of powdered plant material were mixed with one liter of solvents (water for aqueous extraction and ethanol for alcoholic extraction). The mixture were kept at room temperature for 24 hours with continuous mixing by magnetic stirrer. This mixture was filtered through cloths and further extraction of the residue was repeated 3-5 times until a clear colorless supernatant extraction liquid obtained indicating that no more extraction from the plant

material was possible. The extracts were again filtered using Whatmann No.1 filter paper. The filtrates were dried until a constant dry weight of each extract was obtained. The extracts were sterilized by 0.22 μ m Millipore filter. The residues were stored at 4°C for further use.

Microorganism

The tested organism was obtained from Department of Microbiology, College of Veterinary Medicine ,University of Al-Qadisiya.The bacteria was identified by classical methods for its cellular, cultural and biochemical characteristics ⁽¹²⁾.

Antibacterial activity of the plants extracts

The antibacterial activity of the extracts was determined using the agar well diffusion method ⁽¹³⁾. Muller Hinton Media (Accumix) was used for this purpose. The prepared culture plates were inoculated with strain of bacteria using streaking plate method (a sterile cotton swab soaked in broth culture media its turbidity reach to 0.5 McFarland standard was used for streaking). Equidistant wells on the surface of the agar were done in the plates by 6mm cork bearer. The wells were filled with 0.1mL of extract concentration (25, 50, 75, 100 mg/ml) under aseptic conditions. Ampicillin (30 μ g) and ciprofloxacin (25 μ g) were used as positive control. Multiple plates were done for each of the extract (four replications). The plates were then maintained at room temperature for 2 hours allowing for

diffusion of the solution. All plates were then incubated at 37°C for 18 hours and the zones of inhibition were calculated by measuring the diameter of the inhibition zone around the well (in mm). The readings were taken in three different fixed directions and the average values were tabulated.

Result:

Table 1 and 2 shows the antibacterial activities of aqueous and ethanolic extracts prepared from *T. vulgaris* and *E. camaldulensis*. It was found that all prepared extracts from tested plants showed statistically antibacterial activity against *Listeria monocytogenes* except the ethanolic extract from *Thymus vulgaris* which had no effect at low concentration .

Table (1): The effect of aqueous and alcoholic extracts of *Thymus vulgaris* leaves against the growth of *Listeria monocytogenes*.

Concentration mg/ml	Inhibition zone (mm)	
	Aqueous extract	Ethanolic extract
25	16 ± 0.00	0.00
50	19 ± 0.45	0.00
75	24 ± 0.00	12.33 ± 0.33
100	24 ± 0.00	17.33 ± 0.42

Table (2): The effect of aqueous and alcoholic extracts of *Eucalyptus*

camaldulensis leaves against the growth of *Listeria monocytogenes*.

Concentration mg/ml	Inhibition zone (mm)	
	Aqueous extract	Ethanolic extract
25	13 ± 0.45	12 ± 0.00
50	17 ± 0.00	15.67 ± 0.33
75	21.67 ± 0.42	19.5 ± 0.22
100	24 ± 0.45	23 ± 0.00

Table(3) : Inhibition zones (mm) produced by *Listeria monocytogenes* against ampicillin and ciprofloxacin

Ampicillin	21 ±0.45
Ciprofloxacin	23±0.68

Results presented in Table 1 and 2 show that the aqueous extract of *T. vulgaris* was the most active agents against the test organism ,it exhibited an inhibition zone 16,19 and 24 mm in diameter in the concentration 25,50,75 and 100 mg/ml respectively (Figure 2), while the aqueous extract of *E. camaldulensis* appeared to be the second-most effective agent in that it produced zone of inhibition ranged from 13 to 24 mm.

The different ethanolic extracts varied in their antibacterial efficiency, moreover the growth of *L. monocytogenes* was not inhibited at low concentrations (25 and 50 mg/ml)

of ethanolic extract from *T. vulgaris*, and it had the least inhibitory activity.

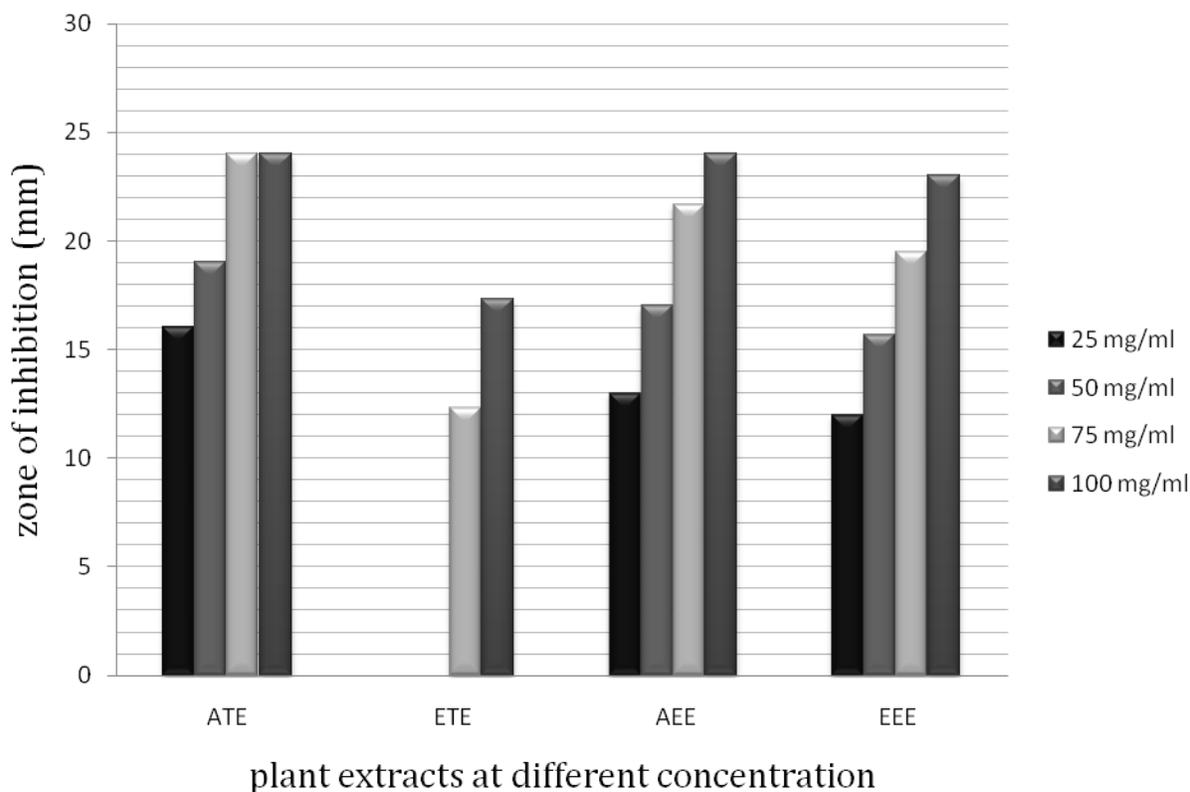


Figure (1) :Antibacterial activity of plant extracts against *Listeria monocytogene*. (ATE represents aqueous thyme extract; ETE represent ethanolic thyme extract; AEE represent aqueous eucalyptus extract and EEE represent ethanolic eucalyptus extract).

Figure (1) show that there was a significant differences ($P < 0.05$) in antimicrobial activity between different extracts according to the type of plant ,type of extract(aqueous or alcoholic) and the concentration of extract. There is a significant correlation between concentrations of

tested plants extracts and the mean of inhibition zone of listeria .

In comparison with positive control (ampicillin and ciprofloxacin were used in this study ,they exhibited an inhibition zone 21 and 23 mm ,respectively),there was no significant differences between the aqueous extract from *E. camaldulensis* (75 mg/ml) and ampicillin ,also the average zone size for ethanolic extract from the same plant at100mg/ml was identical to the average zone size obtained with ciprofloxacin. This indicating that tested plants and tested antibiotics had the same antibacterial effects. Moreover the antimicrobial

activities of the aqueous extract of *T.vulgaris* (at 75 and 100 mg/ml) and *E. camaldulensis* (at 100 mg/ml) against *L.monocytogenes* were significantly greater than those produced by these antibiotics. These

results suggest that the antilisterial properties of thyme and eucalyptus may be further investigated to explore the possibility of using them in the treatment of Listeria infection.

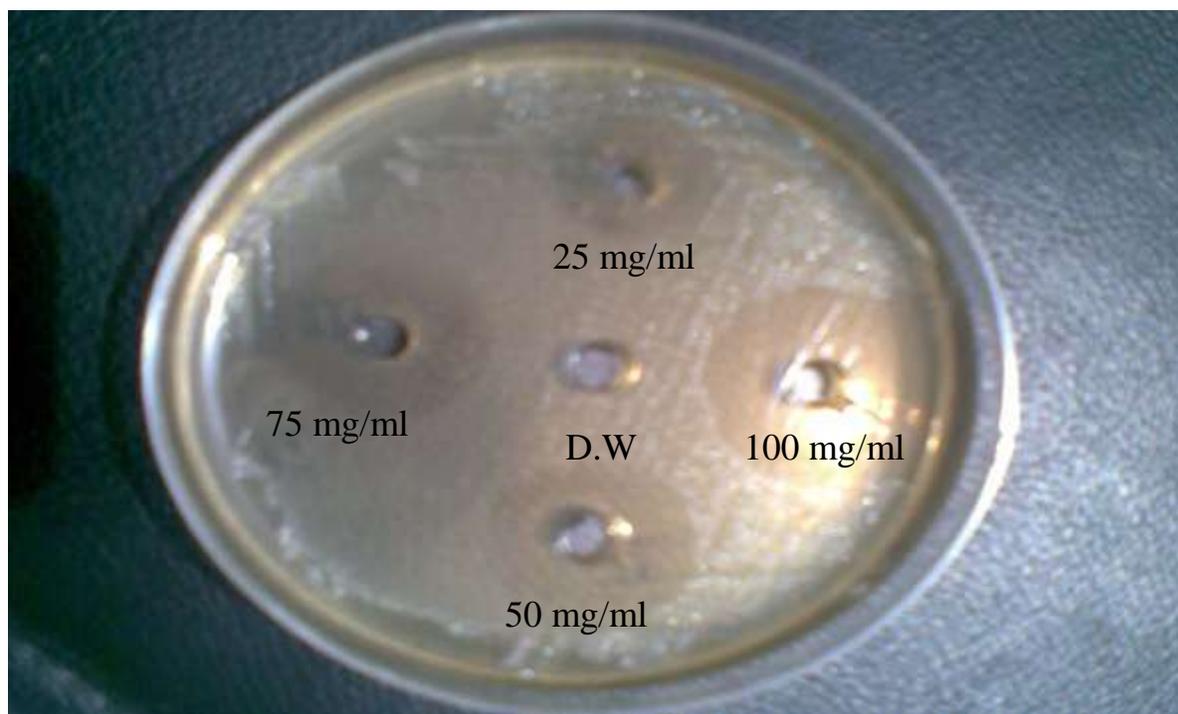


Figure (2) : Effect of aqueous thymus extract at different concentration on in vitro growth of *Listeria monocytogenes*.

Discussion:

Plants are important source of potentially useful structures for the development of new chemotherapy- eutic agents even against some antibiotic-resistant strains. The first step towards this goal is the *in vitro*

antibacterial activity assay ⁽¹⁴⁾. Many reports are available on the antiviral, antibacterial, antifungal and anti-inflammatory properties of plants ^(11,15). Some of these observations have helped in identifying the active principle responsible for such

activities and in the developing drugs for the therapeutic use. The present study was done to investigate the antibacterial effect of *T. vulgaris* and *E. camaldulensis* against one of the most important pathogenic bacteria. Results from the antibacterial tests demonstrated that both plant extracts had an excellent inhibitory effect. This supports and confirmed the findings of many authors^(16, 8, 17, 10).

Analysis of variance showed a significant difference ($p < 0.05$) in efficacy between antimicrobial agents which could have arisen as a result of genetic differences in the sensitivities of the isolates to the antimicrobial agents and differences in the modes of action of the antibiotics.

Extract and antibiotic had similar levels of antimicrobial activity against *L.monocytogenes* This was attributed to similarity in the mechanism of action of these plant and antibiotics. While some extracts showed greater antimicrobial activity than antibiotic especially ampicillin. These plants are active even against organisms that have become resistant to antibiotics.

The antimicrobial activity of the crude extract might be due to the presence of active compounds including alkaloid, quinines, tannins, flavonoides, saponins and iridoids⁽¹⁵⁾. The main target for these compound might be some important enzymes, bacterial cell wall or membrane. It has been shown that alkaloids are able to intercalate DNA, lipophilic compounds that might bind within or

internal to the cytoplasmic membrane⁽¹⁸⁾. Thyme extract possess active compounds that produce antibacterial activity in vitro like thymol (the main constituent of thyme 46.2%), carvacrol, tannin, saponin, triterpenic acids and flavonoides. the presence of aromatic nuclei containing a polar functional group, especially the thymol phenol group is the cause of exhibited a good antimicrobial properties⁽¹⁹⁾.

E. camaldulensis was effective against *L. monocytogenes*, the ability of these *Eucalyptus* extracts to inhibit the growth of bacteria is in agreement with previous reports^(11,20). This efficiency may be belong to the presence of antibacterial compound such as eucalyptol, cineol, tannins, kinoin, kinored, actechin, pyrocatechin and trichlosan. It has been reported that trichlosan (a compound present in the leaves structure) interacted with an enzyme in the fatty acid biosynthetic pathway and exhibited inhibitory activities against both Gram-positive and Gram-negative bacteria⁽²¹⁾.

The aqueous extract exhibited the most great effect, this may be due to absence of many active ingredients in ethanolic extract⁽²²⁾. however, In previous comparison of extraction yield in different extraction solvents (methanol, ethanol and water) the investigator found that the water had the highest effect of extraction yield⁽²³⁾. From the above studies, it is concluded that the traditional plants

may represent new sources of antimicrobial with stable, biologically active components that can establish a scientific base for the use of plants in modern medicine. However, the higher efficacy of thyme which have been recommended for usage in short-term storage of products but further studies are needed before these extracts can be applied to this purpose.

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