

Antibacterial activity of *Syzygium aromaticum* against *Klebsiella pneumoniae* and *Staphylococcus aureus* isolated from urinary tract of dogs

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Abstract

This study was conducted to investigate the antibacterial efficacy of *Syzygium aromaticum* extracts, (clove) is one of the traditional plants that have been used to prevent urinary tract infection. Our study showed the antibacterial effectiveness of the extract of *Syzygium aromaticum* and reduced bacterial resistance resulting from the misuse of the antibiotic. Our extract has been used to prevent UTI because the pharmacological activities are attributed to plant constituents such as flavonoids, alkaloids, glycosides, saponins, terpenes, and other active ingredients. Cloves were extracted by hydroalcoholic extract (ethanol), which yield a reddish-brown plant extract powder with 20%. It was observed that the hydroalcoholic extract (ethanol) of cloves against *Staphylococcus aureus* appeared at a concentration of 6.25 mg/ml, and increased until reached concentrations of 200; 400 and 800 mg/ml resulting in a zone of inhibition (16.3, 17.5, 20.7mm), respectively, while inhibition was recorded against *Klebsiella pneumoniae* at a concentration of 50 mg/ml. At a concentration of 400 and 800 mg/ml, an increase in inhibition was observed for the zone (18.1, 22.3mm). It was inferred that the hydroalcoholic extract (ethanol) of cloves has a higher antibacterial effect against *Staphylococcus aureus* because the zones of inhibition began with lower concentrations compared to *Klebsiella pneumoniae* because of their virulence factors in addition to bacterial resistance.

Keywords: Clove, *Syzygium aromaticum*, *S. aureus*, *K.pneumonia*, Urinary Tract Infection (UTI)

نشاط المضاد البكتيري لمستخلص القرنفل ضد المكورات العنقودية الذهبية والكلبسيلا الرئوية المعزولة من الجهاز البولي في الكلاب

الخلاصة

أجريت هذه الدراسة للتحقق من الفعالية المضادة للبكتيريا لمستخلص، القرنفل وهي من النباتات التقليدية التي تم استخدامها للوقاية من عدوى المسالك البولية. أظهرت دراستنا فاعلية المستخلصات المضادة للبكتيريا للقرنفل وتقليل المقاومة البكتيرية الناتجة عن سوء استخدام المضاد الحيوي. يعد (القرنفل) أحد النباتات التقليدية التي تم استخدامها للوقاية من التهاب المسالك البولية لأن الأنشطة الدوائية تُنسب إلى مكونات نباتية مثل الفلافونويد والقلويدات والجليكوسيدات والصابونين والتربينات والمكونات النشطة الأخرى. تم استخلاص واستخراج القرنفل بواسطة الإيثانول المائي الكحولي أعطت محصول مسحوق نباتي مستخلص بني مائل إلى الحمرة وبنسبة 20%. تم إجراء الدراسة التجريبية المستخدمة في بحثنا الفعالية المضادة للبكتيريا لمستخلص القرنفل ضد المكورات العنقودية الذهبية والكلبسيلا الرئوية لتحديد أفضل التراكيز القاتلة للبكتيريا. لوحظ أن المستخلص الكحولي المائي للقرنفل ضد المكورة العنقودية الذهبية في بدا عند التركيز 6,25 مجم / مل وأوضحت التراكيز 200 و400 و800 مجم / مل أعطى منطقة تثبيط (16,3؛ 17,5؛ 20,7) على التوالي بينما سجلت ضد الكلبسيلا الرئوية تثبيطاً عند التركيز 50 مجم / مل ولوحظ عند التركيز 400 و800 مجم / مل زيادة تثبيط للمنطقة (18,1؛ 22,3). تم الاستدلال بان المستخلص الكحولي المائي للقرنفل له تأثير مضاد للبكتيريا المكورات العنقودية الذهبية اعلى لان مناطق التثبيط بدأت بتراكيز اقل بالمقارنة من البكتريا الكلبسيلا الرئوية لما لها من عوامل ضراوة اضافة الى المقاومة البكتيرية .

Introduction:

The use of herbal treatments in the traditional medical system continues to be a significant part of the healthcare system. (1).

Syzygium aromaticum, was important in medicine because of its antibacterial, antioxidant, anti-cancer, anti-inflammatory, and anti-diabetic properties (2). The discovery of novel antimicrobial therapeutic agents is required due to the high incidence of bacterial strains that are drug-resistant, so the extracts from *Syzygium aromaticum* have also been observed to limit the spread of infection (3).

A number of organisms, including *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Proteus mirabilis*, *Staphylococcus saprophyticus*, and others, can cause urinary tract infections (UTIs), a serious public health hazard in animals and human (4,5).

Bacterial urinary tract infections (UTIs) are among the most significant veterinary medical justifications for the use of antibiotics, and they lead to the emergence of antibiotic resistance (6,7). In fact, lower urinary tract symptoms, together with pee cytological findings, are subsequently followed by the urine culture test, thus empiric antibacterial therapies and phytochemicals as herbal medicine are frequently used in the presence of UTI clinical signs (8).

According to pharmacological research, are clove that consider the main sources of phenolic molecules, including hidroxibenzoic acids,

flavonoids, hidroxiphenyl propens, hidroxicinamic acids, and eugenol (C₁₀H₁₂O₂), the main bioactive molecule, as well as eugenol, gallic acid derivatives like hidrolizable tannins that are present in significant amounts in the fresh plant and extract (9,10).

The experimental study used in our research were the antibacterial activity of *S. aromaticum* extracts against *Staphylococcus aureus* and *klebsiella pneumoniae* to determine the best bactericidal concentrations.

2. Materials and Methods:

2.1. Preparation of Plant extracts

The herbarium at the University of Fallujah's Veterinary Medicine College was applied to identify clove buds that was taken from the local market in Fallujah, Iraq. Using hydroalcoholic ethanol 70% as solvents, dried after being cleaned with sterilized distilled water. To create a uniform powder, the buds were mechanically ground in a mortar. 500 ml of solvent were used to soak 150 g of clove powder before incubated for 72 hours at 25 C with a magnetic stirrer. Extracts were centrifuged at a speed of 5000 rpm for 10 min, Clear filtrates are produced by filtering via Whatman filter paper, and the plant residues were discarded. In order to concentrate the extracts, the solvents were ultimately evaporated using a rotatory evaporator.

The extracts were kept at 4 C until use, and the following formula was used to estimate the extraction yields:

The formula for calculating the Percentage

of the extract yield is $(R/S)*100$, where

R referred to the extract residue weight and **S** referred to the weight of the raw sample.

2.2. Bacteriological Examination

2.2.1. Bacterial isolates

Samples that used in our study taken from dogs suffering from urinary tract infection and the isolation and identification in laboratory of clinical pathology in collage of veterinary medicine \ University of Fallujah. Urine samples were collected from 9 dogs. The samples collected from different breed and from 2 to 7 years old. Five dogs were female and four dogs were male. The samples were examined immediately after collection during 5 h for determine bacterial Urinary tract infection in dogs.

2.2.2. Culturing

All bacterial culturing on nutrient agar by spreading 0.1 ml on agar then incubated at 37 °C up to 24-48 hrs. After incubation time was spent, colonies had been observed, and based on morphological characters (shape, color, and size) the suspected colonies of *K. pneumonia* and *Staphylococcus aureus*, were re-inoculated on MacConkey and Selective medium Eosin Methylene Blue Agar (EMB Agar) for isolation and identification of *K. pneumonia* and cultured also on blood agar to obtain blood hemolysis pattern and *Staphylococcus aureus* that cultured on media manitol salt agar (11).

2.2.3. Antibacterial assay

By using the macro dilution methods were carried out in sterilized tube .All tube were filled

with 2 ml of Mueller Hinton broth then 2 mL of *S. aromaticum* extracts were added to the first tube (serial dilutions were done by passing 2 mL of *S. aromaticum* extracts all tube to extract different concentration (6.25, 12.5, 25, 50, 100, 200, 400, 800 mg/2mL).Then, 0.1mL of the relevant standardized inoculum ($1.5*10^8$ cfu/ml of *bacteria*) was put to each tube. For the negative control (left without inoculation by bacteria), while positive inoculated by bacteria without added extracted. Then incubated **for 22 h at 35 degrees Celsius**. The visual turbidity of the tubes was noted, both before and after incubation and finally to confirm the MIC and MBC value make subcultures On Mueller-Hinton agar plates, the inoculated were of the MIC concentration and the two additional consecutive concentrations. Finally, the plates were placed in the incubator for 48 hours at 35 C and then later we start counting the bacteria depending on the growth rate from 30-300 (CFU)/ml and above 300 or below 30 is excluded (12,13).

Extract at different concentration (6.25, 12.5, 25, 50, 100, 200, 400, 800 mg/mL) was prepare on peptone water detect the antibacterial efficacy of *S. aromaticum* extracts concentration against (*S. aureus*) and (*K. pneumonia*). then by using streaking methods to inoculated and preparation the bacterial inoculum of approximately 10^5 (CFU)/ml which is derived from the equation formula for calculating a dilution is $(C1)(V1) = (C2)(V2)$ 1.5×10^8 colony forming unit (CFU)/ml (equivalent to 0.5

MacFarland) and finally incubated in appropriate conditions for 18 h at 37 °C. (14).

The inoculum was added to culture media that had been prepared according to usual procedures. For bacteria, this was MHA agar. A sterile hollow punch was used to create 6 mm-diameter wells concentrically in the agar after the medium had been inoculated. 50 µL of extract at various concentrations were flowed into each well (15).

For two hours, plates were kept in the refrigerator at 4 C to allow the extracts to diffuse through the medium. Finally, the plates were incubated at 35 C for 48 hours, and the suppressive zone diameters were estimated using a Vernier caliper (13).

The lowest dose of clove extract that still shows antibacterial effectiveness is known as the minimum inhibitory concentration (MIC). Since clove hydroethaolic *S. aromaticum* extract had the best antibacterial effectiveness, MIC was determined in it (16).

3. Result and discussion:

3.1. Extraction of *Syzygium aromaticum* :

Due to its high polarity compared to other organic solvents (petroleum ether, dichloromethane, acetone, and ethanol), which have varying polarities of 0.1, 3.1, 5.1, and 5.2, respectively, all active phytochemicals were extracted using ethanol as an organic solvent (17).

Syzygium aromaticum was extracted using hydroalcoholic ethanol to obtain a reddish brown

tinted extract as shown in figure (1) with a 20% plant powder yield percentage, which was estimated using the formula:

$$\begin{aligned} \text{yield of the extract (\%)} &= \text{weight of extract (gm)} / \text{weight of } S.\textit{aromaticum} \text{ (gm)} \times 100 \text{ (18).} \\ &= 30 \text{ (gm)} / 150 \text{ (gm)} \times 100 \\ &= 20\% \end{aligned}$$

This outcome is comparable to that of (19), who discovered that the extract's percentage yield recovery was assessed to be 18.2% (w/w).



3.2. Bacteriological Examination

3.2.1. Blood Agar Hemolysis Pattern

In the results of basic culturing on agar, *S. aureus* individual colonies have a distinct border and are round, convex, and 1-4 mm in diameter. Zones typically surround *S. aureus* colonies on blood agar plates in figure (2A). which is the typical β-hemolytic phenotype While they seemed as large, mucoid, transparent, alpha-hemolytic greenish-white around the colonies of *K. pneumonia* on Blood agar plates on figure (2B).



Figure (2): Shape of colonies and types of hemolytic for *Staphylococcus aureus* (A) and *K. pneumoniae* (B) on Blood agar

It is well known that *K. pneumoniae* and *S. aureus* routinely grow on blood agar, just like the majority of bacteria, based on the conventional steps of bacterial isolation; the most notable characteristic is the pattern of analysis because the blood agar functions as an enriched and differential medium at the same time (20).

There are three hemolytic patterns; partial (α), complete (β) or no hemolysis (γ) of bacterial colonies that can be produced on blood agar.

S. aureus markedly appear β -hemolytic due to hemolysin which consider 1 of most important virulence factors of *S. aureus* and causes and showing the typical β -hemolytic phenotype which is called complete hemolytic phenotype as well (21).

Figure (2B) shows a clear characteristic Alpha-hemolysis zone around the colonies of *K. pneumoniae* which is in agreement, and its attributed chiefly to the hemolytic activity of both α and β Hemolysins (21,22).

3.2.2. Cultured on selective and differential medium:

Colonies of *K. pneumoniae* are pink to purple in color without green metallic sheen. This

appear on the Eosin Methylene Blue (EMB) agar which is considered as a selective medium as in Figure (3A).(23).

S. aureus is isolated and identified from clinical and non-clinical materials using Mannitol Salt Agar (MSA), a selective and differentiating medium. Other Staphylococci create little pink or red colonies with no change in the medium's color, but *Staphylococcus aureus* produces yellow colonies with yellow zones. Mannitol can be fermented by an organism, and if it does, an acidic byproduct is produced, changing the phenol red in the agar yellow. Figure (3B) (22).

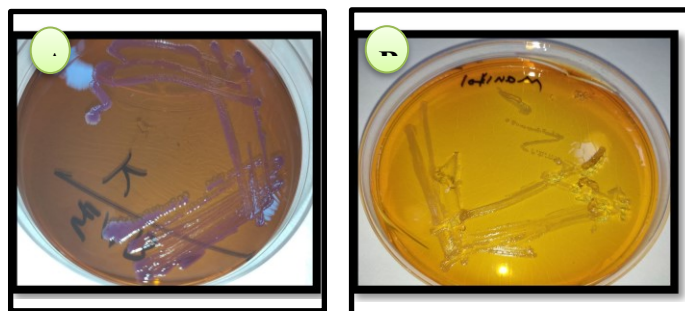


Figure (3): Shape of colonies Eosin Methylene Blue agar a for *K. pneumoniae* and

3.3. Antibacterial assay and detection of minimum inhibitory concentration:

The effectiveness of *S. aromaticum* extracts as antibacterial agents against *S. aureus* and *K.pneumoniae* different concentration of hydroethanolic extract are (6.25, 12.5, 25, 50, 100, 200, 400, 800 mg/2mL). Many methods were used to determined MIC and MBC and in our achievement by macro dilution are the MIC recorded 50mg/ml, 100mg/ml and MBC 100mg/ml and 200mg/ml against *S. aureus* and *K.pneumoniae* respectively. So that the Minimum Inhibitory Concentration (MIC) plays a key role

in the determination of an antibacterial potency (24).

Antibacterial efficacy and potency of *S. aromaticum* extracts against *S. aureus* recorded inhibition at concentration 6.25 mg/mL and the increase of zone inhibition when concentration in our pilot study used in our research till reach 200; 400 and 800 mg/mL recorded (16.3,17.5 and 20.7mm) respectively as mentioned in table (1).

While the antibacterial efficacy of *S. aromaticum* extracts against *K. Pneumonia* recorded inhibition at concentration 50 mg/mL and the increase of zone inhibition when concentration in our pilot study used in our research till reach 400 and 800 mg/mL recorded (18.1 , 22.3) respectively while disappear the zone on inhibition in (6.25,12.5 and 24 mg/mL) as mentioned in table (1) .

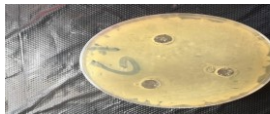
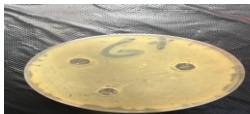



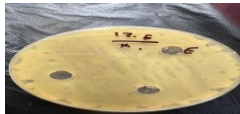




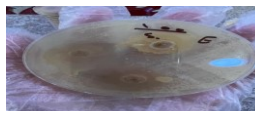






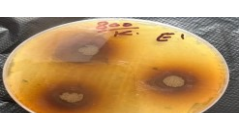
Determination of Bactericidal With the previously completed antibiotic susceptibility testing, the test for establishing the susceptibility curve was conducted for the extracts that gave a growth inhibition diameter of the seeds 12 mm (presumptive test). It was carried out utilizing the tube dilution technique. (15,25), A series of sequential geometric dilutions were made from a stock solution of 800 mg/mL extract, in accordance with the results of the pilot investigation.

According to (26), the current study showed that the *S. aromaticum* (hydroethanolic) extracts had suppressive zones (10-13mm) and a

potential anti-MRSA action, our study that recorded (16.3; 17.5 and 20.7) mm, for 200; 400 and 800 mg/mL concentration respectively.

The insult was explained as being due to the antibacterial activity of *S. aromaticum* extracts (clove extract) varied against all gram-negative uropathogen (27). Our achievement against *K.pneumonia* recorded inhibition at concentration 50 mg/mL and the increase of zone inhibition when concentration in our pilot study used in our research till reach 200; 400 and 800 mg/mL recorded (14.2; 18.1 ; 22.3) respectively while disappear the zone on inhibition in (6.25; 12.5 and 24 mg/mL) , So clove in terms of comparison and clarification of our results with other studies exhibited maximum activity against bacteria with 23.75 mm mean diameter of zone of inhibition (27). These outcomes were consistent with (28).s assessment of the antibacterial potency and efficacy of the *S. aromaticum* extract against MRSA isolates with inhibitory zones ranging from 19 to 23 mm. So that according to (29), the MIC values for clove methanolic extract against *S. aureus* and *K. pneumonia* strains were 48 and 70.31 mg/ml, respectively when it came to bacteria, extracts with inhibitory diameters < 12 mm were effective., MICs and MBCs were calculated. The ratio of MBC/MIC ≤ 1 (Bactericidal); MBC/MIC ≥ 2 (Bacterostatic) was used to determine the effects of the extracts on the bacteria (16). Our interpretations and results are similar against *S. aureus* and *K.pneumonia* and in terms of the idea with a difference in the method and the solute of the extract (29).

Table (1): Microbiological assay Show pictures and mean of Zone inhibition (mm) of *S. aromaticum* extracts against *S. aureus* and *K. pneumonia* after incubation (24 hours).

Concentration of extract mg/mL	<i>S. aureus</i>	Mean of Zone inhibition (mm)	<i>K. pneumonia</i>	Mean of Zone inhibition (mm)
Control positive		No inhibition		No inhibition
6.25		7.4		No inhibition
12.5		9.7		No inhibition
25		11.6		No inhibition
50		13.1		8.6
100		15.7		10
200		16.3		14.2
400		17.5		18.1
800		20.7		22.3

The growth inhibition diameters of the active extracts (hydroethanolic and ethanolic) range from 12 to 17 mm; these diameters are less than those of Gentamicin antibiotic, the drug employed as a reference. The ethanolic extract's MICs and MBCs are identical, and as $MBC = 2MIC$, MBC/MIC must equal 2, indicating that it has bacteriostatic action. On the clinical strain and the standard strain, the hydroethanolic extract has bacteriostatic and bactericidal effects. The two strains were both susceptible to the hydroethanolic extract (30).

With inhibitory diameters between 13 and 19 mm, extracts significantly inhibited *P. aeruginosa* and *K. pneumonia*, which is *P. aeruginosa*'s clinical equivalent. With inhibition diameters larger than those of the gentamycin as reference medication, the ethanolic extract was more effective (31). The ethanol extract has a larger MIC on the clinical strain, but otherwise the MICs are the same. Beyond the ethanolic extract, where the MIC is equal to the MBC on the reference strain, the $MBC = 2MIC$ (32). Some authors have demonstrated the presence of phytochemical substances in the fruits of *S. aromaticum*, including alkaloids, tannins, flavonoids, saponosides, anthocyanins, terpenes, coumarins, steroids, and reducing chemicals (33).

The present study shows that the hydroethanolic extracts of *S. aromaticum* tested have significant bactericidal activity, one or more

of these phytochemical substances, acting either separately or in combination to provide the antibiotic activity shown with the aqueous, hydroethanolic, and ethanolic extracts, would be the cause of the antimicrobial activity. The antimicrobial effects that have been noticed may be due to eugenol, a phytochemical component mostly found in cloves and already known for its antibacterial activities (34,35). In reality, it is known that the alkaloids, saponosides, flavonoids, and tannins in the plants have a therapeutic effect against a number of pathogenic agents: *E. coli*, *P. aeruginosa*, *C. albicans*, and *S. aureus* (35, 36).

The bactericidal and bacteriostatic actions of the aqueous, hydroethanolic, and ethanolic extracts of *S. aromaticum* on the examined microorganisms could be partially explained by these mechanisms of action of tannins and flavonoids (37).

The mode of action of tannins is complexations, either with enzymes, bacterial substrates, metal ions, or its impact on the bacterial cell membrane (38, 39). Tannins have been shown to inhibit the growth of *S. aureus* and *P. aeruginosa* (40). Flavonoids cause membrane lysis, which results in cell death (38, 41). our study is agreement with (56) who reported that clove ability to inhibit the growth of MRSA was studied through *in vitro* and *in vivo* studies. Zone diameter Value (mm) used to indicate susceptible, intermediate and resistance bacteria (42). and according to zone diameter (mm) ≥ 20 the MIC value (mg/ml) ≤ 4 consider Susceptible

; zone diameter (mm) 15-19 the MIC value (mg/ml) 8-16 consider Intermediate ; if the zone diameter (mm) ≤ 14 the MIC value (mg/ml) ≥ 32 consider Resistant Susceptible breakpoint is 4mg/ml or 20 mm. Resistant breakpoint is 32 mg/ml or 14 mm. (42).

4. Conclusion:

Our achievement confirmed the *S. aromaticum* extracts' strong antibacterial activity against various pathogenic bacterial strains that cause urinary tract infections. The antibacterial activity of clove hydroethanolic extract was highest against *Klebsiella pneumoniae* and *Staphylococcus aureus* isolated from urinary tract infection. Clove extracts' potential antibacterial effectiveness confirmed their potential for use in the creation of new antimicrobial agents. Also, can be used as a combination with novel antibiotics due to safety of photochemical extract , less drug interaction , and It has been proven to have an effect when mixed with antibiotics, as it reduces bacterial resistance.

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Conflicts of interest

There are no conflicts of interest

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