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Research Article

# Extraction and Identification of the Main Components of Cloves (*Syzygium aromaticum* L.) Oil Extract and its Antimicrobial Activity against Methicillin-resistant *Staphylococcus aureus* strain

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## Abstract

**Background:** Methicillin-resistant *Staphylococcus aureus* is widely recognized as a significant etiological agent responsible for infections around the world. One of the biggest problems in world health care is antibiotic resistance to the MRSA strain. The use of herbal medicines is one of the promising techniques for countering bacterial resistance to antibiotics.

**Objectives:** The study is designed to investigate the chemical composition of clove oil extract and its *in-vitro* antibacterial activities against MRSA.

**Methods:** The clove oil extract was obtained by using hydro-distillation by Clevenger apparatus. After that, phytochemical analysis was done to determine the secondary metabolites by Chromatography-Mass Spectrometry. *In-vitro* antimicrobial activity of clove oil extract against Methicillin-resistant *Staphylococcus aureus* was carried out by agar well diffusion method, the broth microdilution method, and *in-vitro* time-kill curve kinetic. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significant compare between means of results in this study.

**Results**: The results of this study revealed that the extraction percentage of the clove yielded 50%. The Chromatography-Mass Spectrometry results of the clove oil extract analysis showed that caryophyllen at 28.9%, Humulene at 21.6% and eugenol at 13.06% were the primary bioactive ingredients of the prepared extract. Furthermore, the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of clove oil extract against *Methicillin-resistant Staphylococcus aureus* were found to be 2.5µg/ml and 5.0 µg/ml respectively. Time killing curve of 2xMICs and 4xMICs of clove extract achieved the highest significant bactericidal effect (P≤0.05) in comparison to other concentrations.

**Conclusions:** The clove oil extract exhibited good *in-vitro* antibacterial properties and this can be attributed to the presence of phenolic compounds such as caryophyllene, humulene and eugenol.

**Keywords**: Antimicrobial, Caryophyllen, Clove extract, *Staphylococcus aureus* (MRSA), Time killing curve.

## Introduction

Multidrug-resistant bacteria causing infectious diseases were responsible for significant mortality, particularly in developing countries (1). High prevalence of methicillin-resistant *Staphylococcus aureus* strains (MRSA) was recorded in both health-care and community environments (2,3,4). To develop new drugs to treat multidrug-resistant pathogens, the search for novel antibacterial compounds, including herbal products, has increased quickly (5,6). Traditional treatments frequently use pharmaceuticals made from aromatic herbs to treat bacterial infections (7). Numerous investigations claim that utilizing essential oils can help to decrease antibiotic-resistant bacteria (8).

These oils have a wide range of biological and pharmacological effects and are very volatile, lipophilic, and hydrophobic (9).

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*Syzygium aromaticum* (family- *Myrtaceae*), more popularly known as clove, is indigenous to the Indonesian islands. Almost there is thirty compounds have been identified in Clove Essential Oil Composition **(**CEO), eugenol is the main component, composing at least half of its components. Caryophyllene, Eugenyl acetate, and humulene compose the remainder, which ranges from 10%–40%. The remainder is less than 10% which is considered minor elements(10). It is now grown all over the world as a flavoring ingredient, a medicine, and an ingredient in perfumes (11,12). Due to its antibacterial abilities, it is frequently used as a food preservative (13). Analgesic, antioxidant, anti-inflammatory, anesthetic, and insecticidal action has also been documented in addition to its antibacterial property (14) Additionally, the antibacterial activity of clove oil have been studied against a large number of multi-resistant *Staphylococcus* and oral pathogens(15). The

development of innovative antimicrobial treatment agents is necessary due to the high prevalence of bacterial strains that are resistant to many drugs. Thus, the goal of the current study was to determine the chemical composition as well as the *in-vitro* antibacterial effect of clove oil extracts against pathogenic MRSA bacterial strains.

## Materials and Methods

**Collection of plant:** The origin of clove *(S.aromaticum*) utilized in this study is from a local market in Baghdad, Iraq, and classified by the National Center For Herbal Medicine and Al-Razi Center for Medical Herbs. Clove buds were cleaned with 5% sodium hypochlorite solution (NaOCl), rinsed three times with distilled water, and then kept to dry. Using a mechanical mortar, the dried plant was ground into powder.

**Extraction of clove oil (*Syzigyum aromaticum)*:** Clove oil was extracted from dry buds using hydro-distillation by Clevenger apparatus. After drying with sunlight, it was placed in a 1000 ml glass flask then 500 ml of distilled water was added. The flask was connected to the apparatus and operated for three hours. The flask's temperature was first raised to about 80°C and then gradually increased to 100°C. The oil was collected after isolating the water, and to increase the water disposal a little of anhydrous sodium sulfate was used (16).

**Extraction percentage yield of essential Clove oil** The collected cloves sample was used to extract the essential oil by hydrodistillation method. The extraction yield has been accomplished by the equation below (17):

Oil yield % =Volume of oil extracted *100/weight of the sample

**GC-MS analysis of *S. aromaticum* Oil extract*:*** To identify the active components of clove extraction, Gas chromatography-mass spectrometry (GC-MS) was used to study the phytochemicals of the clove *(S. aromaticum)* extracts. By injecting 1 L of the sample (0.1% in absolute methanol) and operating in scan mode on the GC/MS Thermo Trace GC Ultra / TSQ Quantum GC-MS, the GC/MS analysis was carried out. Using an Agilent HP- 5ms Ultra Ineit capillary column (30 m 0.25 m film thickness), the phytochemical investigation was conducted. The rates of the four ramps were as follows: ramp 1 was 60 °C hold to 3 min, ramp 2 was 60 °C to 180 °C hold for 7 min, ramp 3 was 180 °C to 280 °C holding to 8 min, and Ramp 4 was 280 °C holding to 3 min. The following describes the operation's conditions: The carrier gas was helium, with a 99.99% purity, and the injector and detector were 250 °C hot. Comparing the results of the GC-MS analysis with the reference retention time and spectral mass data from the NIST database allowed the chemical components of the clove bud extract to be identified.

**Bacteriological Examination**

**Test Organism:** The Methicillin resistance *staphylococcus aureus* (MRSA) was obtained from the Physiology, Biochemistry, and Pharmacology Department/ College of Veterinary Medicine /University of Baghdad.

**Activation and Maintenance of MRSA**: Ten mL of brain heart infusion agar slants were used to activate bacterial cultures in screw-capped tubes and then placed in the incubator at 37 °C for 24 hrs. For maintenance of isolates, the bacterium was cultured on brain heart infusion agar and kept at 4°C, then the bacterium was activated every 14 days. These bacteria were established for microscopic morphological, cultural, and biochemical studies (18).

**Preparation of standard bacterial suspension:** By comparing to the Standard McFarland solution (0.5), the quantity of MRSA bacteria in each milliliter of the stock suspension was standardized (18). Briefly, bacterial suspension equivalent to 0.5 McFarland ($1.5 \times 10^8$ CFU /ml) was arranged from overnight bacterial culture. The absorbance of this index was 0.136 as noted by the spectrophotometer at a wavelength of 450 nanometers. About 0.1 ml of the prepared bacterial suspension was diluted in 14.9 ml of Mueller-Hinton broth and incubated at 37 ºC for 1 hr. to obtain $10^6$ CFU/ml bacterial suspensions to prepare bacterial suspension for the time-kill curve kinetic assay in order to bring MRSA bacteria to the logarithmic phase of bacterial growth (19).

**Measurement of Antimicrobial Activity of clove oil extract:**

**Agar well diffusion method:** The agar well diffusion method was carried out to evaluate the antibacterial activity of *S.aromaticum* extract against MRSA according to **(**20). Standardized bacterial suspension ($1.5\times10^8$cfu/ml) of *S. aureus* was carefully mixed with sterile Mueller Hinton agar. Twenty-five ml of this agar was dispersed into sterile Petri dishes and left for 10 minutes at room temperature to dry, and six mm. diameter wells were bored in the agar. The *S.aromaticum* extract was reconstituted in distilled water to a concentration from5µg/ml to 0.312µg/ml and then 100µl was added to wells. The plates were incubated at 37 C° for 24 hours after allowing the extract to diffuse into the agar at room temperature. Three plates were made for each concentration with negative control using buffer phosphate and the strength of the clove oil extract was dictated by measuring the inhibition zone diameter around every well against the tested bacteria and used as positive bioactivity compared with negative control. Standard error and mean were calculated.

**Microtiter plate Dilution**: Clove oil extract (10 mg/ml) was made in Mueller-Hinton broth, from this broth, two folds dilution was downgraded from 40 µg for clove oil in U- shape (200 µl well capacity) 96 well micro-titer plate. Each well was inoculated with 100 µl of $1.5\times10^8$ CFU/ml *S. aureus* and

incubated at 37ºC for 24hrs. For colorimetric identification of bacterial growth, 15µl of 0.125% triphenyl tetrazolium chloride dye (TTC) which was used as an indicator of cellular viability was added to each well of the test and re-incubated for two hours to determine MIC by observing whether or not the red color that results from the reductions of TTC (colorless) to formazan (red) develops (21). While the minimum bactericidal concentration (MBC) was determined by subculturing 50 µl from the well that showed no apparent growth (clear) onto fresh nutrient agar plates. After the incubation period, if there was no growth this concentration was taken as MBC which is considered the lowest concentration of extracts that kill the bacteria (22).

**Time Kill Curve Kinetic Assay*:*** The time-kill curve assay of clove extract against MRSA bacteria was done according to the procedure described by (19). Briefly, bacterial suspension was prepared as mentioned before to obtain $10^6$ CFU/ml bacterial suspensions, and the Clove extract had been dissolved in Mueller-Hinton broth to prepare 10 mg/ml stock solution. After that, clove extract concentrations from 4x MIC to 0.25x MIC were prepared. Bacterial colonies were calculated at 0, 1, 2, 4, 6, and 24 hr. through the incubation time by making serial dilutions and spreading of 20 µl of each dilution on Mueller-Hinton agar plate (triplicate); colonies range 30-300 CFU/plate was accepted (19).

The trapezoidal method was used to estimate the area under the time-kill curve of the concentration of clove extract as below (23):

$$AUC_{kill} = \sum \left( \frac{\log(Cn) + \log(Cn+1)}{2} . \Delta t \right)$$

Where the $AUC_{kill}$ is the area under the killing curve of clove extract, log (Cn) is the logarithm of a specific concentration at a specific time, log (Cn+1) is the logarithm of the next concentration while (Δ t) resembles the difference between their times.

**Statistical Analysis:** The Statistical Analysis System- SAS program was used to detect the effect of different factors on study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to significantly compare between means in this study.

**The authentication of the plant:** The clove that utilized in this study was classified by the National Center For Herbal Medicine and Al-Razi Center for Medical Herbs as *Syzygium aromaticum* L. family of *Myrtaceae* to be used as oil extract in this study.

**Extraction of essential Clove oil:** The collected cloves sample was used to extract the essential oil by hydrodistillation method. The oil obtained was yellow in color as show in (Figure 1) with the highest extraction yield of 50%.

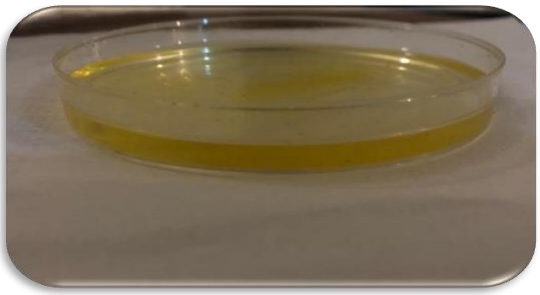

**Figure1: Clove oil extract**

**Results:**

**Gas chromatography-mass spectrometry (GC-MS):** Seventeen peaks related to separate components were obtained from gas chromatography–mass spectrometry (GC/MS) in (*S. aromaticum)* extract by hydrodistillation method (Figure 2).

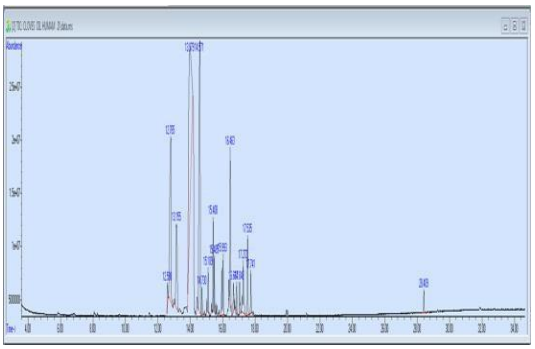

**Figure 2: Gas chromatogram of clove oil extract**

The main constituents were caryophyllen with 28.9%, eugenol 13.06%, and Humulene 21.6% from the GC-MS database. Additionally, other compounds were listed in (Table 1).

**Table 1: Phytochemical analysis of *S. aromaticum* essential oil.**

| Compounds | Retention time | Percentage of total % |
|---|---|---|
| Caryophyllene | 13.9 | 28.9 |
| Humulene | 14.5 | 21.6 |
| Eugenol | 12.7 | 13.06 |

**Antibacterial activity of clove oil extract**

**Agar well diffusion method**: Different concentrations of clove oil extract were used in agar well diffusion methods, resulting in different sizes of inhibition zones against methicillin resistance *Staphylococcus aureus*. The sizes of inhibition zones were different according to the concentration of the Clove oil extract. Results indicated that MRSA bacteria were sensitive significantly ($P<0.05$) to clove oil extract in a concentration-dependent manner 5, 2.5 ,1.25,0.625, and 0.312 µg/ml. Increasing the diameter measurement of the zone of inhibition in MRSA growth was proportionally related to clove oil extract concentrations, (Table 2 and Figure 3).

**Table 2: Antibacterial activity of Clove oil extract in different concentrations against MRSA (measured as the diameter of the inhibitory zone in millimeters).**

| Groups | Con.(µg/ml) | | | | |
|---|---|---|---|---|---|
| Conc. | 5µg/ml | 2.5µg/ml | 1.25µg/ml | 0.625µg/ml | 0.312µg/ml |
| Clove oil extract | 18.0±0.75 A a | 15.0±0.58 B a | 13.0 ±0.61 BC a | 11.0 ±0.52 C a | 9.0 ±0.38 C a |
| Buffer phosphate | 0.0±0.0 A b | 0.0±0.0 A b | 0.0±0.0 A b | 0.0±0.0 A b | 0.0±0.0 A b |
| LSD value | 3.337* | | | | |

- **Values represent mean ± S.E**
- **Different capital letters mean significant *(P<0.05)* results between different concentrations.**
- **Different small letters mean significant *(P< 0.05)* results between buffer and clove oil extract.**
- ***LSD: least significant difference**

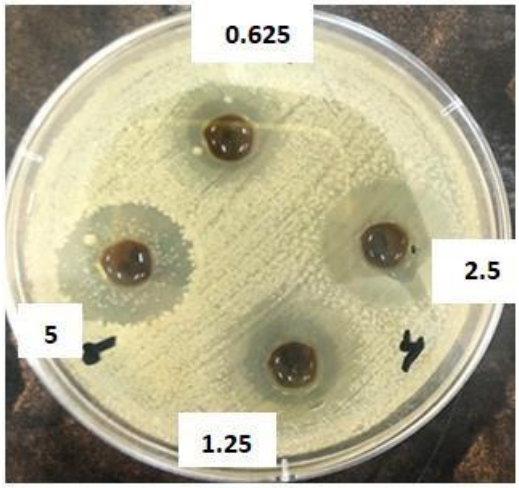


**Figure 3: Susceptibility of MRSA strain to different concentrations of clove extract**

**Microtiter plate Dilution:** The Minimum Inhibitory Concentration plays a key role in the determination of an antibacterial potency (24). The microdilution assay was used to determine MIC. The results showed that the concentration of 2.5 µg/ml of clove oil was effective in preventing MRSA from growing, this concentration was shown to have a positive value that inhibited the growth and 5.0 µg/ml of clove oil extract killed MRSA so they were considered as the (MBC). MBC value was tested in micro-dilution assay by sub-culturing 50 µl from the well that showed no apparent growth (clear), if there was no growth this concentration was taken as MBC.

**Time Kill Curve Kinetic Assay:** The time-kill curve kinetics of clove oil extract is based on the highest MIC recorded from the micro-dilution assay which was 2.5µg/ml for the MRSA strain. The concentrations used *in vitro* study were 0.25x MIC, 0.5x MIC, 1x MIC, 2x MICs, and 4x MICs.

The clove oil extract at a concentration of (4×MIC, 2×MIC) showed significant killing activities both at 6 and 24 hrs. respectively by reducing ≥ 3 log10 of the total number of CFU/ml in comparison to other concentrations used as shown in (Figure 4).

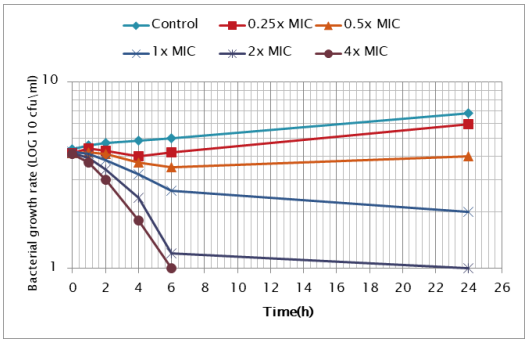


**Figure 4: Time kill curve kinetics of clove extract against MRSA**

The area under the time of killing curve of clove extract was calculated and compared to the control inoculum growth rate, and the difference in the area under the curve values among different treatments was set as an endpoint whereas the lowest area under the curve refers to the highest bactericidal effect as reported in the (Table 3). The results showed that both 2xMICs and 4xMICs achieved the highest significant bactericidal effect (*P≤0.05)* in comparison to other treatments. The 1xMIC concentration achieved a purely bacteriostatic effect (*P≤ 0.05*) in comparison to all concentrations and control groups; both 0.5xMIC and 0.25xMIC failed to achieve a significant bacteriostatic or bactericidal effect *(P≥0.05*) in comparison to 1xMIC, 2xMIC and 4xMIC.

**Table 3: Area under the time-kill curve of clove extract against MRSA (h*log 10 CFU/ml).**

| Antibacterial | Control | 0.25x MIC | 0.5xMIC | 1xMIC | 2xMIC | 4Xmic |
|---|---|---|---|---|---|---|
| Clove extract MIC=2.5µg\ml | 125.3 ±0.31 A | 111.5 ±0.3 A | 86.81 ±0.30 B | 57.22 ±0.19 C | 29.87 ±0.5 D | 17.25 ±0.9 D |
| SD | | | | 13.3* | | |

- **Values represent mean ± S.E**
- **Different capital letters denoted a significant difference (*p≤0.05*) among the groups.**
- ***** LSD: least significant difference**

## Discussion

Clove oil extraction using hydro-distillation (water distillation) by Clevenger apparatus gave a bright yellow color oil and brown color extract with a typical clove oil smell. The extraction yield of clove extract was 50% as the amount of clove buds that were used in the current procedure was 200 g. However this outcome is nearly in line with the outcomes of the study of Ishaq and his colleagues(2019) who discovered that when clove bud powder was extracted using a soxhlet equipment, the yield of hexane extract was 48.84%
(25). Importantly, one of the reason of the resulting higher percentage of extraction may belong to the

reduction in the particle size as reported by Ratri and his colleagues(2020) as they suggested the percentage yield of clove extract is influenced by the cloves' particle size, with smaller clove particles producing higher extraction yields (26). However, the extraction procedure can also be influenced by several other factors, such as the quality and freshness of the clove buds, the temperature and pressure used during the distillation process, and the duration of the distillation process **(**27, 28).

To identify the active components of clove extraction, GC/MS was used and the main compounds of the *S. aromaticum* extract were caryophyllen, humulene, and eugenol. According to reports, these substances make up the majority of the clove buds' active ingredients which have a therapeutic use such as using as analgesic, antioxidant, anti-inflammatory, anesthetic, and antibacterial agents (29). In this study ground clove was used and found β-caryophyllene to be the most abundant since the volatile profile from whole buds showed a different pattern when compared to the volatile composition of ground clove. This is in agreement with Gaspar and his colleagues (2018) who approved that the most prevalent compound is β--caryophyllene. (49.31% concentration) as they compared compositions from whole and ground clove (30). However, other studies, have found a difference in the majority of compounds, such as the study of Lee (2009) who has shown that eugenol, β- caryophyllene, 2-propanone, and methylhydrazone are the main composites of *(S. aromaticum)*essential oil (31).

The anti-MRSA potential of clove oil extract has been reported, and the requirement to investigate bioactive components has been highlighted (32). The clove extract exerted the highest antimicrobial efficacy against the pathogenic bacterial strains (MRSA), which may be assigned to the high percentage of a phenolic compound of clove oil extract as presented in GC– MS results and this complies with the result of Alanazi and his colleagues (2022) who demonstrated the lowest concentration of clove oil that inhibit the growth of MRSA found to be 2.5 µL/Ml.(33).

The phytochemical constituents that are present in clove oil extract are responsible for the antibacterial activity. Eugenol is one of the bioactive components that may have considerably contributed to the antibacterial effects of clove oil (34). Humulene is another component that present in clove oil similar to caryophyllene with antibacterial properties (35). However, the minor components could potentially contribute by combining them with other main components to limit MRSA development. The oil's hydrophobic properties may also aid in interactions with the outer cytoplasmic membrane of MRSA, which impairs the integrity and functionality of the cell membrane (36).

Time killing-curve kinetic is a combined and extensive tool to assess both bacteriostatic and bactericidal effects of the antibiotics; it depends on the change in the logarithmic number of bacterial colonies through the defined chronological pattern (19). More accurate descriptions of antimicrobial activity are provided by a measure of bacterial killing (kill kinetics) than by the MIC, and it has also shown better sensitivity developments to physicians than disc diffusion methods (37, 38)

The current time-kill kinetic outcomes required a little more time to completely eradicate the bacteria when compared with the study conducted by Mandal and his colleagues (2011) who found that clove extract showed significant killing activities against MRSA, both at 3 and 6 hrs at a concentration of 256 µg/ml (1× MIC) (38). These differences are possibly caused by a variety of bacterial species used, the concentration of the antibacterial used, and the method used.

**Conclusion:** The clove oil extract at different concentrations had antimicrobial activity against MRSA bacteria. The GC-MS analysis of the clove oil showed the presence of 17 volatile components previously reported to possess antibacterial effects. Hence the antibacterial properties demonstrated by the clove oil extract can be attributed to the compounds identified caryophyllen, humulene, and eugenol. Therefore, clove oils may be used in the medicinal formulation of antimicrobial drugs.

**Authors' declaration:**

We confirm that all the Figures and Tables in the manuscript are mine/ ours. Besides, the Figures and images, which are not mine /ours, have been given permission for re-publication and attached to the manuscript. ethical consideration's approval-Ethical Clearance: The project was approved by the local ethical committee in the Collage of Veterinary Medicine/ University of Baghdad) according to the code number (1604 on 26-7-2023).

**Conflicts of Interest**: None

**Funding:** None.

**Authors' contributions:**

Study conception & design: (Orooba MS). Literature search: (Mays Uday & Orooba MS). Data acquisition: (Mays Uday). Data analysis & interpretation: (Mays Uday & Orooba MS). Manuscript preparation: (Mays Uday & Orooba MS). Manuscript editing & review: (Mays Uday)

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# استخراج وتحديد المكونات الرئيسية لمستخلص زيت نبات القرنفل (*Syzygium Aromaticum L.*) وفعاليته المضادة للميكروبات ضد جرثومة المكورات العنقودية الذهبية المقاومة للميثيسيلن.

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**الخلاصة:**

**خلفية**: تُعرف بكتيريا المكورات العنقودية الذهبية المقاومة للميثيسيلن على نطاق واسع كعامل مسبب مهم مسؤول عن العدوى في جميع أنحاء العالم. وان واحدة من أكبر المشاكل في مجال الرعاية الصحية العالمية هي مقاومة المضادات الحياتيه لهذه البكتيريا.حيث يعد استخدام الأدوية العشبية إحدى التقنيات الواعدة لمواجهة مقاومة البكتيريا للمضادات الحياتية.

**الأهداف:** دراسة التركيب الكيميائي واختبارالنشاط المضاد للبكتيريا في المختبر لمستخلص زيت القرنفل ضد جرثومة المكورات العنقودية الذهبية المقاومة للميثيسيلن .

**الطريقة**: تم الحصول على مستخلص زيت القرنفل باستخدام التقطير المائي بواسطة جهاز Clevenger. بعد ذلك ، تم إجراء التحليل الكيميائي النباتي عن طريق التحليل الكروماتوجرافي-مطياف الكتلة. تم إجراء فحص لمضادات الميكروبات لزيت نبات القرنفل في المختبر باستخدام طريقة انتشار القرص المزدوج ، وطريقة التخفيف الدقيق ، وطريقة منحنى وقت القتل.تم استخدام اختبار اقل فرق معنوي – LSD(تحليل التباين-ANOVA) لاجراء مقارنه معنويه بين النتائج في هذه الدراسه.

**النتائج**: اظهرت هذه الدراسه ان نسبة استخلاص مستخلص القرنفل 50٪. وقد أظهر التحليل الكيميائي النباتي وجود مكونات مختلفة وبشكل رئيسي هي بنسبه28% Caryophyllen و 21.6%Humulene و13.06% Eugenol. حيث تبين ان المكورات العنقودية الذهبية كانت حساسة لمستخلص القرنفل بتركيزات مختلفة علاوة على ذلك ، وجد أن أقل تركيز مثبط (MIC) وأقل تركيز مبيد للجراثيم (MBC) كان 2.5 ميكروغرام / مل ، 5.0 ميكروغرام / مل على التوالي.وقد حقق منحنى وقت القتل من xMICs2 و xMICs4 لمستخلص القرنفل أعلى تأثير قاتل للجراثيم ($P \geq 0.05$) مقارنة بالتراكيز الأخرى.

**الاستنتاجات**: ظهر تحليل GC-MS لزيت القرنفل وجود 17 مكونًا متطايرًا في مستخلص القرنفل ويمكن أن تُعزى الخصائص المضادة للبكتيريا الجيدة التي أظهرها مستخلص زيت القرنفل ويعزى ذلك إلى المركبات الموجودة في الفينوليه مثل الكاريوفيلين , الهيمولين , والاوجينول.

**الكلمات المفتاحية:** المكورات العنقودية الذهبية المقاومة للميثيسيلن(MRSA), الكاريوفيلين, مضادات الميكروبات ، مستخلص القرنفل ، منحنى وقت القتل