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In vitro antimicrobial activity of clove extract against gram negative bacteria isolated from chickens

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***Abstract*---**Because of *Citrobacter freundii'* medical and economic value, as well as the fact that there are only a few local investigations on it, the study aimed to assess the anti-microbial activities of clove extracts (aqueous and ethanolic) and to evaluate the phytochemicals present in them, using the agar well diffusion method The antimicrobial activity was either weak or very strong with inhibition zones ranging from 10 to 21 mm in gram negative , in vitro results confirmed the efficacy of clove extract as natural antimicrobials and suggested the possibility of its use in treatment of such bacterial infections. The findings indicate that ,besides being safe and sensorially attractive, *Citrobacter freundii* has antimicrobial activity, which makes it a potential substitute for chemical food preservatives. avoiding healthy hazards of chemically antimicrobial agent applications. Ethanolic extract of clove had the highest inhibitory effect against *Citrobacter freundii* (21) mm ,whereas aqueous extract respectively was effective against it (20.) mm, The chemical constituents of clove seeds were analyzed by using gas chromatography-mass spectrometry (GC-MS). nineteen and twenty seven chemical constituents were identified based on GC-MS from clove extract(aqueous and ethanolic) . Major classes of compounds are sesquiterpenes, phenyl propanoid, oxygenated sesquiterpenes, and esters responsible for antimicrobial activity. Different compositions in major constituents were found between both extracts . Clove aqueous extract contained eugenol (27.84%) while ethanolic extract, (62.08%).

Research find that eugenol express significantly inhibitory effects on test bacteria and the mechanisms are associated with reducing adhesion and inhibiting the synthesis of biofilm and various virulence factors of these microorganisms. Clove extract are generally regarded as safe in vivo experiments . Susceptibility analysis to 15 drugs was performed by the diffusion method in agar Muller Hinton discs. The isolates showed a multi resistance for Cefpodoxine , Amoxyclav, Rifampin ,Ceftazidime ,Cefriaxone, Cefepem and Erythromycin, while all the isolates were intermediate to Tetracyclin and Ampicillin, also in this study showed higher sensitivity rate to the Norfloxacillin and Ciprofloxacin .Thereby, this study successfully demonstrated the possibilities of using Clove extracts as natural antimicrobials and it could be used as an effective antibacterial agent, alternative to the use of antibiotics. Results encourage the use of natural sources like some plants or some parts of plants to solve some problems done by bacteria activities that cause infection.

***Keywords*---**antimicrobial activity, *enterobacteriaceae*, *syzygium aromaticum*, antibiotic, chicken.

Introduction

The consumption of meat contaminated by foodborne pathogens leads to foodborne diseases and represent a huge public health problem nowadays in the developing countries (1). In the health care system, the traditional medicinal system based on the use of herbal treatments continues to play an important role. Medicinal plants have gained popularity in recent decades due to the idea that, as natural products (2), they have fewer adverse effects and better efficacy than their synthetic counterparts (3).Approximately 80% of the world's population currently uses traditional medicines as a key source of health care (4). Various herbal plants have anti-inflammatory, antimicrobial, spasmolytic, sedative, analgesic, and local anesthetic properties. They are utilized in embalmment, food preservation, and have anti-inflammatory, antimicrobial, spasmolytic, sedative, analgesic, and local anesthetic properties. (5).Phytoconstituents such as glycosides, saponins, flavonoids, steroids, tannins, alkaloids, terpenes, and others have been documented to exhibit pharmacological effects in many plant species (6). *Syzygium aromaticum*, often known as clove, is a dried flower bud from the Myrtaceae family that is native to Indonesia's Maluku islands but has recently been farmed in other locations throughout the world. (7). The presence of many chemical ingredients in high concentrations with antioxidant activity is ascribed to clove's efficient involvement in the suppression of several degenerative diseases (8). As a result, natural components such as plant extracts and spices have gotten increased attention as a substitute for artificial additives (9). Clove extract exhibits a wide range of pharmacological and biological activities such as antioxidant (10), anti-inflammatory (11) anticarcinogenic, and anti-viral effects (12,13),. Besides, some studies have reported antibacterial activity of essential oil from clove buds against several food-borne pathogens (14). This plant represents one of the richest sources of phenolic compounds such as eugenol which is the main bioactive ingredient (15) , eugenol acetate and gallic acid possesses great

potential for pharmaceutical (16) .Clove (*Syzygium aromaticum*) contain vitamin A (retinol), beta-carotene , vitamins K, B6, B1, and C (17)., Its extract is commonly used in the food industry because of its special aroma and natural safety. In addition, the extract from clove also exhibited strong antibacterial properties (18). So, it has become necessary to find out new antimicrobial agents (19). with growing concern of microbial resistance towards conventional preservatives, consumers tend to be suspicious of chemical additives and thus the exploration of naturally occurring antimicrobial for food preservations receives increasing attention. Infections caused by bacteria of the family *Enterobacteriaceae* are common in birds of the Passeriformes order; however, they are considered secondary, and the presence of predisposing factors is necessary to trigger diseases in birds .*C. freundii*. are facultative anaerobic, motile, gram-negative bacilli in the *Enterobacteriaceae* family that is widely distributed in environment and intestinal tracts of human and animals so these bacilli regarded as the environmental contaminants or harmless colonizers (19). *C.freundii*. are uncommon opportunistic nosocomial bacteria can cause urinary tract, hematologic, or neonatal infections (meningitis, sepsis, general bacteremia); intra-abdominal sepsis; brain abscesses; or pneumonia (20).Antibiotics are the mainstay tools in the management of infections. Disappointingly, a number of studies have reported inappropriate uses of antimicrobials as well as the emergence of antibiotic resistance in most parts of the world .Today, the emergence of multidrug-resistant (MDR) gram negative pathogens has become a major public health concern. Moreover, they negatively influence patients'outcomes and increase the length of hospital stays and healthcare costs (21).However, to the best of our knowledge, although the in vitro antimicrobial activity of clove extract has been reported earlier,very little is known about its antibacterial mechanism of action (22) . *C. freundii* is well-known for being resistant towards some antibiotics and for its production of several enterotoxins that cause many enteritis types and septicaemia (23), therefore, *C. freundii* was selected as the model organism to evaluate the antibacterial properties and mode of action of extract from clove seeds.

Method and materials
Samples collection

Cloacal samples were collected from 450 chicken randomly from local live broiler chickens were collected from three regions in Al - Diwaniyah province there were samples put in sterile tubes, and the samples were sent to the laboratories of the University of AL-Qadisiyah / College of Veterinary Medicine/ Laboratory of the Veterinary Public Health Department. . A total of samples were subjected to bacteriological examinations to give typical reactions of *Citrobacter freundii.* (24) . Using standard morphological and biochemical testing, the *Citrobacter* isolates were identified to the species level (25). The identification of isolates was confirmed by vitek2 compact system.

Antimicrobial Susceptibility Testing

The Kirby-Bauer disk diffusion method was used to test antimicrobial susceptibility (in Mueller-Hinton agar medium, Merck, Germany) according to the guidelines of the National Committee for Clinical Laboratory Standards (26) and

inhibition zone diameters were measured with millimetric ruler .The performed anti bio gram disks (Himedia laboratories, Mumbai, India) included ciprofloxacin (CIP, 10 mcg), Cefepiem (FEP,10 mcg), Norfloxacillin (NOR, 10 mcg), Amoxyclav (AMC, 30 mcg), Cefpodoxine (CPD, 30 mcg),penicillin G (P, 10 mcg), oxacillin (OXA, 5 mcg), amikacin(AMK, 10 mcg), cefotaxime (CTX, 30 mcg), ceftazidime (CAZ, 30 mcg), ceftriaxone (CRO,30 mcg), ceftazidime (CAZ,30mcg), Levofloxacin (LEV,5mcg), Azthromycin (AZM, 15 mcg), Tetracycline (TE, 10 mcg), Erythromycin (E, 60 mcg), ampicillin (AM, 25 mcg) and rifampin (RA, 5 mcg).

Plant extraction

Extraction for plant seed were done according to (27). Antimicrobial activity of Clove extracts using Different Concentrations of extracts were assessed against *C.freundii* . The extract were further analyzed using GC-MS. Analysis. The inoculums of the *Citrobacter freundii* were carried out using the colony suspension method described by (28). The bacteria strains and isolates were cultured in nutrient agar over night at 37°C. Identical colonies from the culture were suspended in sterile saline. Bacterial culture was diluted in sterile saline solution by using McFarland standard 0.5 to make 1.5 $_{x}$10^8 CFU/ml colony forming units per/ml (29). The antimicrobial activity of different extracts of *Syzygium aromaticum* (aquatic and alcoholic) against *Citrobacter freundii* was evaluated using well diffusion assay (30). Bacterial strain were maintained in nutrient agar plates and sub cultured at regular intervals. For evaluating antibacterial efficacy of extracts. The wells (6 mm) diameter were made in the Mullar- Hintton agar plate using a cork borer. Stock solution of each plant extract was prepared at a concentration of 200 mg/mL in different plant extract, 150 mg/mL ,100, mg/mL and 50 mg/mL. About 100 µL of different concentrations of plant solvent extracts were added with sterile syringe into the wells and allowed to diffuse at room temperature for 30 minutes .Control experiments comprising inoculums without plant extract were set up. The plates were incubated at 37 °C for 24 hours and the diameter of the inhibition zone (mm) was measured.

Statistical Analysis

All data of the experimental design were presented as mean± SEM. To compare between groups, multiple comparisons were performed using one-way ANOVA followed by the least significant difference (LSD) as a post hoc test. The 0.05 level of probability was regarded as the criterion for significance. All statistical analyses were performed with help of statistical package for social sciences (SPSS) for windows version 25 (SPSS Inc., Chicago Ill) (31)

Results and discussion

The worldwide increase and spread of infections caused by multidrug-resistant (MDR) Gram-negative bacteria of human and animal origin is a significant global public health burden in recent decades (32) . *Enterobacteriaceae* are common bacteria and usually associated with diferent types of community- and hospital-acquired infections and sometimes even animal infections (33) . After incubation at 37°C for 24 hours, all samples were cultured on S.S. agar for first isolation; numerous types of bacterial isolates developed on S.S. agar, including small pale

flattened colonies with black centers due to their ability to create H2S on S.S agar**.**, Because both *Citrobacter* and *Salmonella* are H2S, these colonies were subcultured on MacConkey, XLD, and EMB to distinguish *Citrobacter* from *Salmonella. Citrobacter* is a lactose fermenter on MacConkey agar and appears as pink colonies, whereas *Salmonella* appears as pale colonies (non lactose fermenter) on XLD. *Citrobacte*r colonies were yellow, while *Salmonella* colonies were red with a black center. Lactose fermenter (pink) on MacConkey, yellow colonies on XLD, and brown colonies on EMB were identified as *Citrobacter* after an incubation time. Gram stain was used to analyze the microscopic characteristics of Gram negative bacilli in order to validate the main identification. Figure (3).

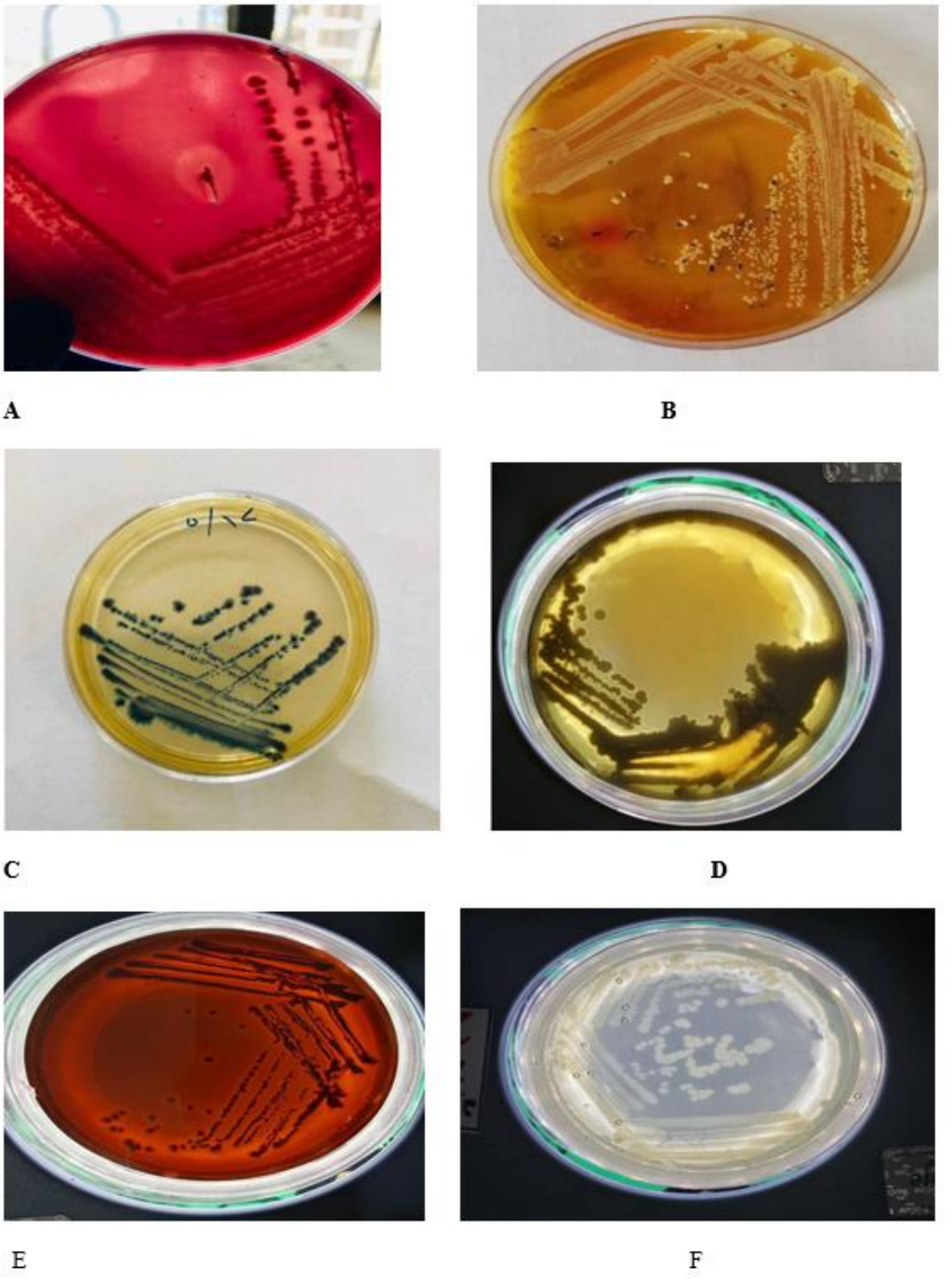

Figure (1) Different selective and differential media cultured with *C. freundii.* after incubation at 37°C for 24 hr..

A. MacConkey agar with small pink (Lactose fermenter) colonies.
B. S.S. agar Pale colonies with black center.
C. Metallic blue colonies on Chromogenic agar.

D. Yellow colonies on XLD agar.
E. Brown colonies on EMB
F. convex opaque gray color colonies on nutrient agar .

Conventional biochemical test, specific biochemical tests were used for the detection genus of bacteria , methods analytical profile index (API20 E) test to identify positive growth of C. *freundii* .Accordingly the profile of color changes was 3644772 which is characteristic of C. *freundii* bacteria. The use of Analytical Profile Index (API20 E) test to indentify *Citrobacter freundii* bacteria is shown in figure (2). Our results concerning the use of Analytical Profile Index (API20 E) are in agreement with (34 ,35). To confirm the primary identification Gram stain was performed to examine the microscopic properties which were gram negative bacilli, light microscopical examination, was used to distinguish Gram-positive bacteria from Gram-negative bacteria.

Figure (2): *Citrobacter freundii* bacteria are identified using the API20 E test (profil: 3644772)

Gram-positive bacteria have distinct cell walls than their Gram-negative relatives. Gram-positive walls have a thick peptidoglycan layer linked with teichoic acids, whereas Gram-negative walls have a thin peptidoglycan layer associated with lipoprotein lipopolysaccharide (36). Our results were similar to (37) in that *Citrobacter freundii* bacteria appears as long rod shaped .

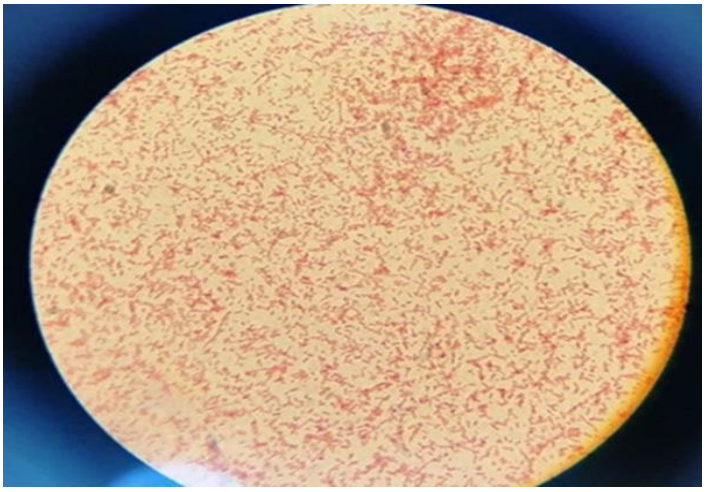

Figure (3): *Citrobacter freundii* isolates were discovered in a colony of the bacteria. Bacilli that are gram-negative have a long rod-like appearance.

Figure (4.) shows the antimicrobial activity of aqueous Clove seeds extract on the *C.freundii*, the extract were effective against bacteria . While, ethanolic clove seeds extract70% was more effective as compared to aqueous Clove seeds extract against test bacterial as shown in figure (5.)

Figure (4.) The inhibition zone (mm) against *Citrobacter freundii* bacteria by using different concentrations of aqueous Clove seeds extract using Muellar-Hinton agar. 9 (200mg/ml), 10 (150mg/ml), 11(100mg/ml) and 12 (50 mg/ml).

Figure (5) The inhibition zone (mm) against *Citrobacter freundii* bacteria by using different concentrations of ethanolic 70 % Clove seeds extract using Muellar-Hinton agar. 1 (200mg/ml) ,2 (150mg/ml), 3 (100mg/ml) and 4 (50mg/ml) .

The antimicrobial activity effect of Clove (aqueous and ethanolic 70%) extracts against *Citrobacter freundii* bacteria isolated from chicken feces were investigated using agar well diffusion method. It was observed that, the extracts showed a remarkable in vitro effectiveness against the test bacterial isolates at various concentrations (50, 100, 150 and 200) mg/ml as shown in table (1) .However, ethanolic extract was found to be more effective compared to the aqueous extract against *Citrobacter freundii* based on the zones diameters of growth inhibition. The differences in inhibition zone showed significant level at ($p \leq 0.05$) between the Clove extracts (aqueous and ethanolic) at concentrations of 50 mg / ml , the inhibition zones of bacteria increased with the increasing of the concentration of the extract ,the maximum zone of inhibition for Clove ethanolic extract (21±1) mm was shown at 200 mg/ml concentration while aqueous clove recorded (20 ± 2) mm at 200 mg/ml concentration, these results were in agreement with results of research (38 ,39) . It is found that the negative gram tested bacteria, were proportional in their response toward the treatments and shows a gradual increasing in inhibition zones with the rising of extract concentrations, these facts were in accord with some studies (40). Also this result is in line with other study which showed that clove extracts have the ability to effect on negative bacteria (41 ,42) , ethanol is often used as a solvent because of its relatively high solubility. It is also inert therefore reaction with other components could be avoided, similar work was done by (43) using Clove ethanolic extract. Their extract demonstrated antimicrobial activities against *C,freundii* .Medicinal plants are considered as a rich resource of ingredients which can be used in drug development and synthesis. The compounds found in plants are of many kinds, but most are in four major biochemical classes, alkaloids, glycosides, polyphenols, and terpenes. Some plants consider as important source of nutrition while others are recommended for their therapeutic values. In this study

According to (44) clove has vital role as a anti-microbial agent because of its high value for its therapeutic activity. Figure (6) showed the typical gas chromatogram in the GC-MS analysis of volatile compounds from clove seed extract. It was observed that 19 peaks in aqueous and Figure (7) showed 27 peaks in ethanolic clove had already well separated and measured by GC-MS under the optimized parameters, indicating that the optimized parameters can be used to analyze the volatile compounds from clove extract. The phenylpropene eugenol compound in clove is a well-known aromatic compound. Eugenol is the main necessary component of Clove that has significantly higher anti-microbial properties against micro-organisms eugenol. The hydrophobicity could break down the cellular lipid and damage the bacterial cell wall, resulting in cell lysis and leakage of intracellular fluids (45) ,these results agreement with (46), caryophylleneas β-caryophyllene was able to alter membrane permeability and integrity of bacteria, leading to membrane damage and intracellular content leakage, which eventually caused cell death (47).

Table (1) Antimicrobial activity effect of plant aqueous and ethanolic 70% extracts against *Citrobacter freundii* isolated from chicken feces on Muellar- Hinton.

	Average zone of inhibition (mm) at different concentrations (mg/ml) mean±SE			
Plant extracts	**50**	**100**	**150**	**200**
Aqueous clove	**10±1Ba**	**17 ±1A b**	**19 ± 1Ac**	**20 ± 2 Ac**
Ethanolic clove	**14±1.2 Aa**	**16 ± 1 Ab**	**19 ±1.2 Ac**	**21 ±1 Ad**
LSD0.05 =1.218				

Different lowercase letters mean significance for horizontal comparisons.($p \leq 0.05$)
Different uppercase letters mean significance for vertical comparisons. .($p \leq 0.05$)
. Results showed that inhibition diameters of antibiotic varied ($p \leq 0.05$) ,table (2) depicts the in vitro susceptibility patterns of the isolated *Citrobacter freundii* to fifteen different antimicrobial agents. According to Clinical and Laboratory Standards Institute (CLSI) handbook 2020 (48), *Citrobacter freundii* isolates in this study showed higher susceptibility rate to the Norfloxacillin and Ciprofloxacin (10 mcg) ,the highest inhibition zone of diameter for both were 40 mm as compared to others against the test bacteria, these results agreement with (49). All the *Citrobacter* isolates were found to be resistant to Cefpodoxine , Amoxyclav, Rifampin ,Ceftazidime ,Cefriaxone, Cefepem and Erythromycin, the result of this

study is agreement with the (50) in Tikrit, Iraq, that he demonstrated that the 33% of isolates were resistant to ceftazidime and these results agree with (51) who found that the *C.freundii* was resistance for Erythromycin, while all the isolates were intermediate to Tetracyclin and Ampicillin. The results are in agreement with the study by (52) in Iraq , and this study is not agreement with the study in China , by (53). In Pakistan (54) proved that there were (100%) isolates of *Citrobacter freundii* were resistant to Amoxicillin/clavulanic acid antibiotic. The *Citrobacter freundii* phenotypically having ability to resist aminoglycosides antibiotics due to many mechanisms like reducing uptake or decreasing cell permeability by a transport defect or membrane impermeabilization this mechanism is chromosomally mediated and results in cross reactivity to all aminoglycosides (55). The differences in in resistance rate is may be due to environment that influence on the bacterial strains (56,57).

Table (2) Antibiotic sensitivities of the bacteria isolated from the broiler chicken.

Antibiotics	**Concentration (mcg)**	**Zone inhibition (mm)**	**Susceptibility**
Norfloxacillin	10	40± 4.32 A	S
Cefpodoxine	30	28± 3.39 B	R
Levofloxacillin	5	35± 3.39 A	S
Ciprofloxacin	10	40± 4.96A	S
Cefotaxime	30	27± 3.08 B	S
Cephalothin	30	27± 2.91 B	S
Amikacin	10	21± 3.39 B C	S
Tetracyclin	10	15± 3.39CE	I
Amoxyclav	30	0± 0 D	R
Rifampin	5	0± 0 D	R
Ceftazidime	30	0± 0 D	R
Cefriaxone	30	15± 1.61CE	R
Cefepem	10	10± 1.14 E	R
Erythromycin	60	0± 0 D	R
Azthromycin	15	25± 1.41 B	S

Ampicillin	25	16± 2.58 CE	I
LSD**0.05** 7.73			

S: susceptible, R: resistant, I: intermediate, capital letters were used to show significant difference among different antibiotics in vertical line at ($p \leq 0.05$).

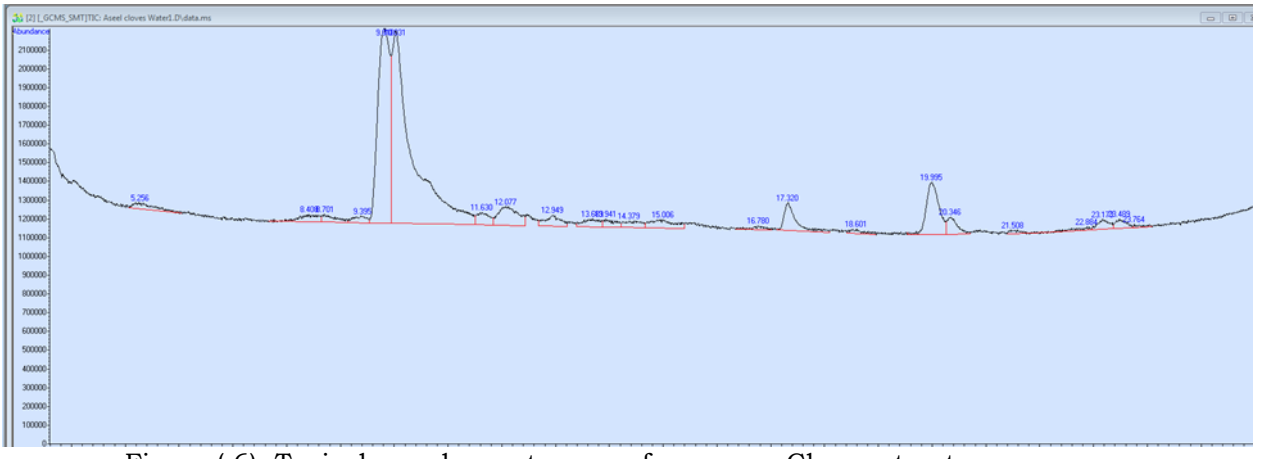

Figure (6). Typical gas chromatogram of aqueous Clove extract

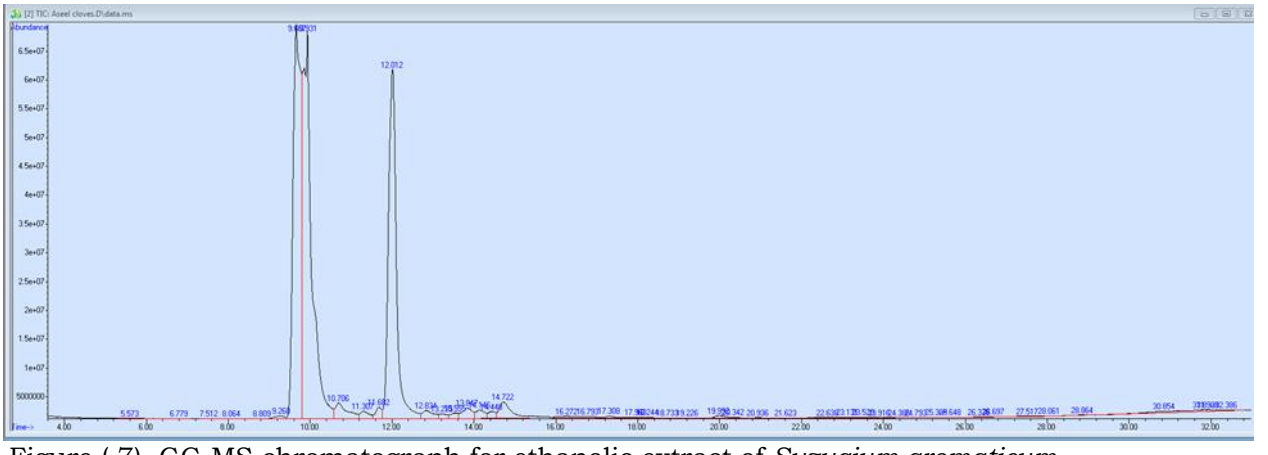

Figure (7). GC-MS chromatograph for ethanolic extract of *Syzygium aromaticum* .

Conclusion

It could be concluded that clove may be used as a major source for the isolation of natural eugenol, a phenolic compound that has been shown to possess antimicrobial properties. This research shows that clove extracts (aqueous and ethanolic) have the ability to inhibit *Citrobacter freundii* in-vitro. Further research is needed on the antibacterial effectiveness of clove extracts against *C. freundii* by

using different methods. Besides, further research is also needed on the antimicrobial effectiveness of clove extract against another microorganisms. Synergistic effect of combined clove extract with other herb is probably also needs to be investigated. 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.

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