

Isolation and identification of eugenol from *Syzygium aromaticum* L. flower bud oil: An antimicrobial study

Firas A. Al-Bayati , Muthanna J. Mohammed

Department of Biology, College of Education, University of Mosul, Mosul, Iraq

(Received 14 / 3 / 2010 , Accepted 23 / 5 / 2010)

Abstract

The present study was conducted to isolate the most important bioactive compound from *Syzygium aromaticum* L. (Myrtaceae) flower buds. The plant essential oil was extracted via steam distillation. Eugenol was separated using a separating funnel and detected on TLC plates in comparison with standard eugenol that served as positive control. Moreover, FTIR spectrometry and HPLC analysis were used to confirm the purity and identity of eugenol. The isolated material was investigated for its antimicrobial activity against six selected pathogenic microorganisms: *Staphylococcus aureus*, *Bacillus cereus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhimurium* and the yeast *Candida albicans*. Eugenol at different concentrations (1:1, 1:5, 1:10 and 1:20 v/v) was active against all tested microorganisms and the highest inhibitory effect was observed against *B. cereus* (zone of inhibition: 26.7 mm) using the disc diffusion method. The minimal inhibitory concentration (MIC) of eugenol was determined using a broth microdilution method in 96-well microtiter plates. MIC values ranged from 7.8 to 62.5 µg/ml. The highest MIC value (7.8 µg/ml) was observed against *B. cereus*, while *S. aureus* and *P. vulgaris* ranked next (MIC 15.6 and 31.2 µg/ml respectively) followed by *P. aeruginosa* and *S. typhimurium* (MIC 62.5 µg/ml). Moreover, eugenol observed good antifungal activity against the yeast *C. albicans* (zone of inhibition range: 8.5–18.3mm; MIC: 62.5 µg/ml).

Introduction

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines. Higher plants, as sources of medicinal compounds, have continued to play a dominant role in the maintenance of human health since ancient times [1].

Syzygium aromaticum (L.) or *Eugenia caryophyllata* (Spreng) commonly known as clove, is the aromatic dried flower buds of a plant in the family Myrtaceae. Cloves are native to Indonesia and used as a spice in cuisine all over the world. Clove oil is frequently used in perfumery and medicine, but the largest part by far is used in flavorings. The dried flower buds of clove are an oriental drug, which has been used as vermifuge, an antibacterial agent, and to treat toothaches[2, 3].

Eugenol is the major component of clove essential oil (79%). Eugenol C₁₀H₁₂O₂, a phenolic compound clear to pale yellow oily liquid extracted from certain essential oils especially from clove oil, nutmeg, cinnamon, and bay leaf. Eugenol acts as an antioxidant on oleaginous foods, as an anticarminative, antispasmodic, antiseptic in pharmacy, and also as an antimicrobial agent[4, 5, 6].

In recent years a large number of essential oils and their constituents have been investigated for their antimicrobial properties against bacteria and fungi. There is vast diversity among aromatic and medicinal plants. Different chemotypes of the same

species may grow in the same place and produce different oils with different activity[7]. Thus, we report here the isolation, identification and purification of eugenol using different spectral techniques, and its inhibitory effect against the growth of some selected pathogenic bacteria.

Materials and Methods

All the practical parts (except for HPLC analysis) of the present study was accomplished in the Department of Biology, College of Education, University of Mosul during 2010.

Chemicals

Hexane, sodium hydroxide NaOH, hydrochloric acid HCl, chloroform CHCl₃, vanillin-H₂SO₄, acetonitrile, ammonium phosphate, phosphoric acid and dimethylsulfoxide "DMSO" were supplied from BDH Analar (England). Resazurin indicator tablet obtained from Thompson and Capper Ltd (England). Standard eugenol 99% purity, molecular weight 164.20, mp -12-10°C, bp 254°C, molecular formula C₁₀H₁₂O₂ was obtained from Sigma-Aldrich chemical com.

Plant material and essential oil extraction

S. aromaticum flower buds were obtained commercially from a local market in Mosul city, Nineveh province, Iraq on January 2010, and identified at College of Agriculture and Forestry, University of Mosul. The buds were dried at room temperature, powdered and submitted to steam distillation in a Clevenger type apparatus for 3 h. The resulted oil was dried over anhydrous sodium sulfate Na₂SO₄ then stored at 2-4°C until use.

Isolation of eugenol

1.0 ml of clove oil was transferred to a 125 ml separatory funnel and dissolved in 50 ml of hexane. 25 ml of 5% NaOH was added. The funnel was shaken vigorously and the layers were separated. The aqueous phase was drained into a 125 ml Erlenmeyer flask and the hexane phase was extracted a second time with 10 ml of 5% NaOH. The aqueous phase was drained from the separatory funnel into the 125 ml Erlenmeyer flask containing the first extract and the organic phase was transferred into another 125 ml Erlenmeyer flask. A 10 ml of 6M hydrochloric acid HCl was added to the flask containing the aqueous base extracts and then it was transferred to the separatory funnel and extracted with hexane (20 ml). Two layers were resulted, aqueous layer which was drained and eugenol in the organic layer [8].

Characterization of eugenol

Thin-layer chromatography (TLC)

Thin-layer chromatography (TLC) for eugenol was accomplished in the department of Biology, College of Education. Eugenol was dissolved in appropriate solvent, applied to silica gel plates, Merck (Germany) 20×20 cm, 0.25 mm in thickness and developed using the solvent system n-Hexane: chloroform CHCl₃ (3:2) v/v. The

separated zones were visualized spraying with vanillin- H_2SO_4 . Standard eugenol served as positive control.

FTIR studies

The IR spectrum of eugenol was recorded in the College of Education, Department of Chemistry, University of Mosul, using a computerized Tensor 27 FTIR spectrometer (Bruker Co., Germany) in the range of 400–4000 cm^{-1} by the KBr pellet technique.

High-performance liquid chromatography (HPLC)

HPLC analysis was performed in the Department of Chemistry, College of Science, University of Mosul, using a Shimadzo LC 2010 HPLC system (Japan), equipped with a Shimadzo LC 2010 UV–VIS detector with a thermostatted flow cell and two selectable wavelengths of 190–370 nm or 371–600 nm. The detector signal was recorded on a Shimadzo LC 2010 integrator. The column used was a C18 block heating-type Shim-pack VP-ODS (4.6 mm i.d. $\times$ 150 mm long) with a particle size of 5 μm . The following conditions were used for the analysis of eugenol. The eluent consisted of 50% acetonitrile, 50mM ammonium phosphate and 0.05% phosphoric acid, pH 3.1. The eluent was degassed once before use. The flow rate of the pump was 1 ml/min, sample volume injected was 20 μl and absorption was monitored at 280 nm.

Antimicrobial activity

Microbial cultures

Five strains of bacteria and one yeast were used as test microorganisms. The bacterial strains included Gram positive *Staphylococcus aureus*, *Bacillus cereus*; Gram-negative *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*; and the yeast *Candida albicans*. All microorganisms were clinical isolates, obtained from the Microbiology Laboratory at Department of Basic Science, College of Nursing, University of Mosul, Iraq.

Inoculum preparation

Nutrient broth and Sabouraud dextrose agar (SDA) were used for growing and diluting the microorganism suspensions. Bacterial strains were grown to exponential phase in nutrient broth at 37°C for 18 h and adjusted to a final density of 10^8 cfu/ml by diluting fresh cultures and comparison to McFarland density. *C. albicans* was aseptically inoculated on petri dishes containing autoclaved, cooled, and settled SDA medium. The petri dishes were incubated at 31°C for 48 h to give white round colonies against a yellowish background. These were aseptically sub-cultured on SDA slants. The yeast colonies from SDA slants were suspended in sterilized 0.9% sodium chloride solution (normal saline), which was compared with McFarland solution. According to the manufacturer's directions, 1 ml of yeast suspension in normal saline was added to 9 ml of sterile medium and kept at 45°C to give a concentration of 2×10^7 cells/ml.

Disc diffusion assay

A modified agar diffusion method [9] was used to determine antimicrobial activity. Nutrient agar was inoculated with microbial cell suspension (200 μl in 2

ml medium) and poured into sterile petri dishes. Sterile filter paper discs 6 mm in diameter were impregnated with 20 μl of eugenol in different concentrations (1:1, 1:5, 1:10, 1:20 initially prepared by diluting in DMSO and sterilized by filtration through 0.45 μm Millipore filters), and placed on the inoculated agar surface. Standard 6 mm discs containing chloramphenicol 30 μg /disc and amphotericin B 10 μg /disc (Bioanalyse) were used as positive controls. The plates were incubated overnight at 37°C for 18–24 h. In contrast, *C. albicans* was incubated at 31°C for 48 h. and the diameter of any resulting zones of growth inhibition was measured (mm). Each experiment was tested in triplicate.

Micro-well dilution assay

Preparation of resazurin solution: The resazurin solution was prepared by dissolving a 270 mg tablet in 40 ml of sterile distilled water. A vortex mixer was used to ensure that it was a well-dissolved and homogeneous solution.

Preparation of the plates: The minimal inhibitory concentration (MIC) values of eugenol were determined based on a micro-well dilution method as previously described by [10], with modifications. A stock solution of eugenol was prepared in 10% DMSO and then serial two-fold dilutions were made in a concentration range from 7.8 to 1000 $\mu\text{g}/\text{ml}$. The 96-well plates were prepared by dispensing, into each well, 95 μl of nutrient broth, 100 μl of eugenol and 5 μl of the inoculants. The inoculums of microorganisms were prepared using 24 h. cultures and suspensions were adjusted to McFarland standard turbidity. The final volume in each well was 200 μl . A positive control containing a broad-spectrum antibiotic (chloramphenicol in a serial dilution of 1000–7.8 $\mu\text{g}/\text{ml}$) was included on each microplate. As an indicator of bacterial growth, 10 μl of resazurin solution was added to the wells. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37°C for 24 h. Color change was then assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of three values was calculated as the MIC for the test material.

As for *C. albicans*, a simple turbidity test [11] was used to determine the MIC value of eugenol. A volume of 0.1 ml from each serial dilution of eugenol concentrations (7.8–1000 $\mu\text{g}/\text{ml}$) was added into tubes containing 9.8 ml of sterile nutrient broth, and then the tubes were inoculated with 0.1 ml of yeast suspension and incubated at 31°C for 48 h. Amphotericin B (7.8–1000 $\mu\text{g}/\text{ml}$) was used as a positive control. The optical density was determined using a SERIES CECIL 1021, 1000 spectrophotometer at 630 nm. The MIC value was the lowest concentration of compound that showed no growth after 48 h of incubation in comparison with the control tube, which included 9.8 ml of nutrient broth and 0.1 ml of yeast suspension in addition to 0.1 ml of each compound concentration (unincubated).

Results

The present study was conducted to isolate the main bioactive compound from *S. aromaticum* flower bud oil. Eugenol was isolated from the extracted essential oil, and then detected on TLC plates in comparison with standard eugenol. A brown zone with a retention factor (R_f) value of 0.31 was identified as eugenol in comparison with standard eugenol that, had the same R_f value after spraying with vanillin-H₂SO₄. The FTIR spectrum confirmed the isolated compound from *S. aromaticum* as eugenol (Figure 1). Infrared spectrum showed significant peaks that, confirmed the purity of the isolated material.

The prominent bands were around: (3522) cm^{-1} which corresponds to hydroxyl group; (1432–1612) cm^{-1} corresponds to aromatic ring; (1035–1149) cm^{-1} attributes to (C–O) bond (etheric); (2853) cm^{-1} ascribes to (C–H) of (C=C) bond; (1638) cm^{-1} corresponds (C=C) bond, and (2927) cm^{-1} corresponds to (C–H) of methyl group. Moreover, eugenol was analyzed using the HPLC system (Figure 2) and identified by comparing its retention time (t_R) and UV spectra with that of the standard compound. The retention time 8–9 min and UV spectra of the isolated compound on HPLC were completely identical to that of standard eugenol.

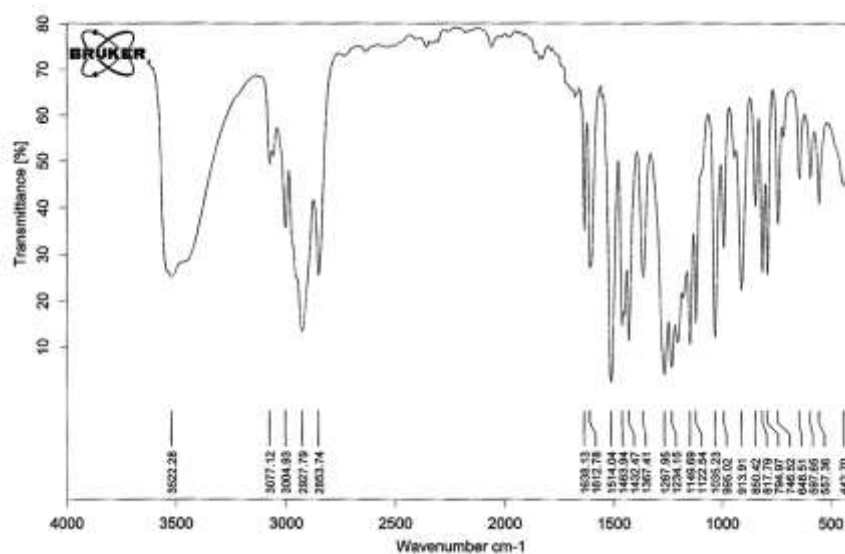


Figure (1): FTIR spectra of eugenol isolated from *S. aromaticum* flower bud oil.

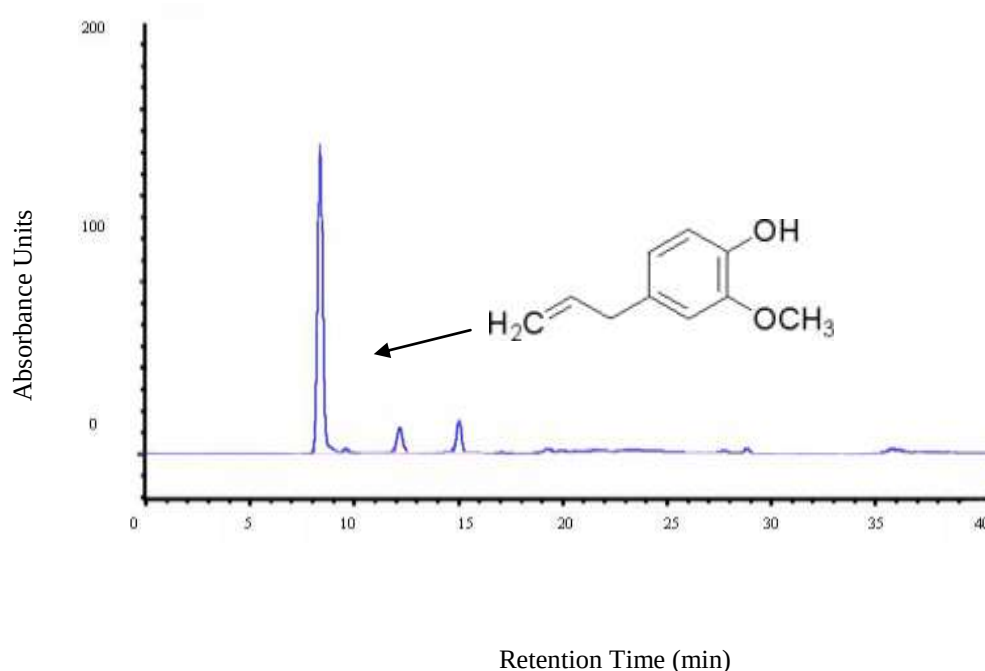


Figure (2): HPLC chromatogram of eugenol isolated from *S. aromaticum* flower bud oil.

The isolated compound was investigated for its antimicrobial activity against five bacterial species and one yeast. The initial screening of antibacterial activity of eugenol was assayed *in vitro* by the agar diffusion method using four concentrations (1:1, 1:5, 1:10, 1:20). All eugenol concentrations were active against all tested

bacteria (Table1). The highest inhibitory effect was observed against *B. cereus* (zone of inhibition: 26.7mm) using the concentration (1:1), while the weakest activity was demonstrated against *P.aeruginosa* (zone of inhibition: 8.0mm) using the concentration (1:20).

Table (1): Antimicrobial activity of eugenol isolated from *S. aromaticum* flower bud oil.

Microorganisms	Zone of inhibition (mm)					
	Eugenol concentrations				Control	
	1:1	1:5	1:10	1:20	C	A
<i>S. aureus</i>	25.2	23.5	19.4	13.9	23.4	N.T
<i>B. cereus</i>	26.7	24.4	20.5	15.7	24.5	N.T
<i>P. vulgaris</i>	24.4	21.5	18.2	12.5	15.5	N.T
<i>P. aeruginosa</i>	19.8	16.9	12.5	8.0	00.0	N.T
<i>S. typhimurium</i>	20.5	17.8	13.5	9.1	16.1	N.T
<i>C. albicans</i>	18.3	14.5	11.8	8.5	N.T	10.2

C: Chloramphenicol (30 µg/disc), A: Amphotericin B (10 µg/disc), N.T: Not tested

In view of the results obtained by the disc diffusion method, the minimal inhibitory concentration MIC of eugenol was determined by broth microdilution assay (Table 2). The highest MIC value (7.8 µg/ml) was observed against *B. cereus*, while *S. aureus* and *P. vulgaris* ranked next (MIC 15.6 and 31.2 µg/ml

respectively) followed by *P. aeruginosa* and *S. typhimurium* (MIC 62.5 µg/ml). Moreover, eugenol observed good antifungal activity against the yeast *C. albicans* (zone of inhibition range: 8.5–18.3mm; MIC: 62.5 µg/ml).

Table (2): Minimum inhibitory concentration (MIC) of eugenol isolated from *S. aromaticum* flower bud oil.

Microorganisms	MIC values (µg/ml)		
	Eugenol	Control	
		C	A
<i>S. aureus</i>	15.6	7.8	N.T
<i>B. cereus</i>	7.8	7.8	N.T
<i>P. vulgaris</i>	31.2	15.6	N.T
<i>P. aeruginosa</i>	62.5	00.0	N.T
<i>S. typhimurium</i>	62.5	15.6	N.T
<i>C. albicans</i>	62.5	N.T	7.8

C: Chloramphenicol, A: Amphotericin B, N.T: Not tested

The standard drug chloramphenicol was active against all reference bacteria except *P.aeruginosa* (zone of inhibition range: 15.5–24.5mm; MIC range: 15.6–7.8 µg/ml). In addition, amphotericin B demonstrated good antifungal activity against *C.albicans* (zone of inhibition: 10.2mm; MIC: 7.8 µg/ml).

Discussion

In the last decades, the essential oils and various extracts of plants have been of great interest as they have been the sources of natural products. They have been screened for their potential uses as alternative remedies for the treatment of many infectious diseases and the preservation of the foods from the toxic effects of the oxidants. Particularly, the antimicrobial activities of plant oils and extracts have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [12].

In vitro studies in this work showed that eugenol inhibited the growth of all tested bacteria. The zones of inhibition ranged from 8.0–26.7 mm and 8.5–18.3 mm in diameter against *C. albicans* using the disc diffusion method. Furthermore, MIC values ranged from 7.8–62.5 µg/ml against tested bacteria and 62.5 µg/ml against *C.*

albicans. Generally, the essential oils possessing strong antimicrobial properties against pathogens contain a high percentage of phenolic compounds such as carvacrol, eugenol and thymol[13,14]. It seems reasonable that their mechanism of action would therefore be similar to other phenolics; this is generally considered to be the disturbance of the cytoplasmic membrane, disrupting the proton motive force (PMF), electron flow, active transport and coagulation of cell contents[15]. Moreover, Sub-lethal concentrations of eugenol have been found to inhibit production of amylase and proteases by *B. cereus*. Cell wall deterioration and a high degree of cell lysis were also noted [16]. The hydroxyl group on eugenol is thought to bind to proteins, preventing enzyme action in *E. aerogenes* [17].

The Gram-positive bacterium *B. cereus* and *S. aureus* were more sensitive to eugenol than the Gram-negative bacterium. It has frequently been reported that Gram-positive bacteria are more susceptible to essential oils than Gram-negative bacteria[18]. The tolerance of Gram-negative bacteria to essential oils has been ascribed to the presence of a hydrophilic outer membrane that blocks the penetration of hydrophobic essential oils into target cell membrane.

Previous studies have reported that eugenol isolated from *Dianthus caryophyllus* grown in Iraq was unable to inhibit the growth of *P. aeruginosa* [19]. In contrast with the present study which showed good action of eugenol against *P. aeruginosa*. Different chemotypes of the same species may grow in the same place and produce different oils with different activity[7].

The standard drug chloramphenicol was active against all tested bacteria except *P.aeruginosa*. Several mechanisms of antimicrobial resistance are readily spread to a variety of bacterial genera. First, the organism may acquire genes encoding enzymes, such as β -lactamases, that

destroy the antibacterial agent before it can have an effect. In addition, bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect. Finally, bacteria may acquire several genes for a metabolic pathway which ultimately produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent, or bacteria may acquire mutations that limit access of antimicrobial agents to the intracellular target site via down-regulation of porin genes [20].

References

- 1- R. Nair, T. Kalariya, S. Chanda, Turk. J. Biol. 29 (2005) 41-47.
- 2- M. Miyazawa and M. Hisama, J. Agric. Food Chem. 51 (2003) 6413-6422.
- 3- H. Dorman, A. Figueiredo, J. Barroso, S. Deans, J. Essent. Oil Res. 12 (2000) 241-248.
- 4- R.S. Farag, A.Z.M.A. Badei, F.M. Hewedi, G.S.A. El-Baroty, J. Am. Oil Chem. Soc. 66 (1989) 792-799.
- 5- B.K. Jadhav, K.R. Khandelwal, A.R. Ketkar, S.S. Pisal, Drug Dev. Ind. Pharm. 30 (2004) 195-203.
- 6- B. Shan, Y. Cai, M. Sun, H. Corke, J. Afr. Food Chem. 53 (2005) 7749-7759.
- 7- D. Kalembe and A. Kunicka, Curr. Med. Chem. 10 (2003) 813-829.
- 8- M. Zakariya and F. Radeif, Virtual organic chemistry. Dar Alkutub for publishing. University of Mosul. 1981.
- 9- R.A. Mothana and U. Lindequist, J. Ethnopharmacol. 96 (2005) 177-181.
- 10- S.A. Sarker, L. Nahar, Y. Kumarasamy, Methods 42 (2007) 321-324.
- 11- F.A. Al-Bayati and K.D. Sulaiman, Turk. J. Biol. 32 (2008) 57-62.
- 12- M. Lis-Balchin and S.G. Deans, J. Appl. Bacteriol. 82 (1997) 759-762.
- 13- H.J. Dorman and S.G. Deans, J. Appl. Microbiol. 88 (2000) 308-316.
- 14- R.J. Lambert, P.N. Skandamis, P. Coote, G.E. Nychas, J. Appl. Microbiol. 91 (2001) 453-462.
- 15- J. Sikkema, J.A. De Bont, B. Poolman, Microbiol. Rev. 59 (1995) 201-222.
- 16- J. Thoroski, G. Blank, C. Biliaderis, J. Food Prot. 52 (1989) 399-403.
- 17- C.N. Wendakoon and M. Sakaguchi, J. Food Prot. 58 (1995) 280-283.
- 18- C.M. Mann, S.D. Cox, J.L. Markham, Lett. Appl. Microbiol. 30 (2000) 294-297.
- 19- M.J. Mohammed and F.A. Al-Bayati, Phytomedicine. 16 (2009) 632-637.
- 20- F. Tenover, Amer. J. Med. 119 (2006) 3-10.

فصل وتشخيص الیوجینول من زيت البراعم الزهرية لنبات القرنفل *Syzygium aromaticum* L. : دراسة

الفعالية المضادة للأحياء المجهرية

فراس عباس البياتي ، مثنى جاسم محمد

كلية التربية ، قسم علوم الحياة ، جامعة الموصل ، الموصل ، العراق

(تاريخ الاستلام: ١٤ / ٣ / ٢٠١٠ ، تاريخ القبول: ٢٣ / ٥ / ٢٠١٩)

الملخص

تضمنت الدراسة الحالية فصل المركب الفعال الأهم من البراعم الزهرية لنبات القرنفل *Syzygium aromaticum* L. فصل الزيت الأساسي للنبات باستخدام جهاز التقطير البخاري، بعدها تم فصل مركب الیوجینول Eugenol باستخدام قمع الفصل وكشف عنه باستخدام طريقة الفصل بکروماتوغرافيا الطبقة الرقيقة TLC بالمقارنة مع عينة السيطرة (Eugenol). فضلا عن ذلك تم التأكد من نقاوتها وماهيتها باستخدام مطياف الأشعة تحت الحمراء FTIR وتحليل كروماتوغرافيا السائل عالي الكفاءة HPLC. تم التحري عن الفعالية البايولوجية للمركب المفصول ضد ستة أنواع مختارة من الأحياء المجهرية المرضية: *Salmonella typhimurium*، *Pseudomonas aeruginosa*، *Proteus vulgaris*، *Bacillus cereus*، *Staphylococcus aureus* والخميرة *Candida albicans*. أظهرت المادة المفصولة فعالية تثبيطية ضد كل الأحياء المجهرية قيد الدراسة عند التراكيز (١:١٠، ١:٥، ١:١ و ١:٢٠ v/v)، ولوحظ أعلى تثبيط ضد بكتريا *B. cereus* (قطر التثبيط: 26.7 ملم) باستخدام طريقة الانتشار بالأقراص. تم التحري عن التركيز المثبط الأدنى (MIC) للیوجینول باستخدام طريقة الـ Microdilution assay وبمعدل تركيز مثبط تراوح بين (7.8 – 62.5 مايكروغرام/مل). لوحظ أن أعلى قيمة تثبيط كانت ضد بكتريا *B. cereus* عند التركيز (7.8 مايكروغرام/مل)، في حين أن التركيز المثبط الأدنى لكل من *S. aureus* و *P. vulgaris* كانت عند (15.6 و 31.2 مايكروغرام/مل) على التوالي، تلتها *P. aeruginosa* و *S. typhimurium* بتركيز مثبط أدنى عند (62.5 مايكروغرام/مل). إضافة إلى ذلك، أظهر الیوجینول تأثيرا تثبيطيا جيدا ضد الخميرة *C. albicans* وبقطر تثبيط تراوح بين (8.5 – 13.3 ملم) وبتركيز مثبط أدنى عند 62.5 مايكروغرام/مل).