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Research Article

A Comparison of efficiency of (AMP) and its derivative (AMPAA) against some pathogenic bacteria

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Abstract

Eugenol (4-allyl-2-methoxyphenol, AMP), is a well known a biologically active phenolic component and essential oil from *Eugenia caryophyllata*, which widely used of Eugenol as an antiseptic and analgesic in dental care, so it is active against oral bacteria associated with dental caries and periodontal disease as well as previous studies have shown the effect of Eugenol antifungal; anti-carcinogenic; anti-allergic; anti-mutagenic activity; antioxidant and insecticidal properties, therefore it can be used in preparation of various food as a flavouring agent and cosmetic.

This study aimed to synthesize derivative new medical material 2-(4-allyl-2-methoxyphenoxy) acetic acid (AMPAA) from eugenol (4-allyl-2-methoxyphenol) (AMP) and investigate the antimicrobial activities of both AMP and derivative component (AMPAA), The minimum inhibitory concentration (MIC); minimum bactericidal concentration (MBC) and sensitivity against six pathogenic bacterial isolates: *Streptococcus. pyogens; Enterococcus. faecalis; Escherichia. coli ; Klebsiella pneumoniae; Bacillus. subtilis* and *Proteus. mirabilis* with study compare the efficiency for both AMP and AMPAA on the same bacterial isolate obtained from Iraqi hospitals.

In this present study synthesize new medical material (AMPAA) from AMP by reacting sodium eugenate with sodium chloroacetic acid and prepare soluble water from eugenol and its derivative AMPAAto scanning the antimicrobial efficacy against some pathogenic bacteria isolatesby two common methods; well diffusion and broth dilution methods.

Results of the present study show all bacterial isolates were sensitive to both AMP and AMPAA in low concentration except *K. pneumoniae*, also 10.0 and 5.0 µg/mL of AMP and AMPAA as MBC for bacterial isolates except *K. pneumoniae* and *P. mirabilis* that's meaning the new derivative compound AMPAA has more efficacy on six bacterial isolate than eugenol (AMP).

By using Well diffusion method all bacterial isolates were sensitive to both (AMP and AMPAA) in low concentration, but *K. pneumoniae* was killed in same concentration, so all isolates have been killed at concentrations between (10 - 50 µg/mL) of new derivative compound (AMPAA), at the same concentration of AMP were killed for *K. pneumoniae* and *P. mirabilis*) ,as well as all isolates have varying degrees of sensitivity towards both (AMP and AMPAA), whereas all isolates where more sensitive to AMPAA than AMP.

In conclusion, all bacterial isolate were sensitive to both AMP and AMPAA in low concentration except *K. pneumoniae*, also 10.0 and 5.0 µg/mL of AMP and AMPAA respectively conceder as MBC for bacterial isolate except *K. pneumoniae* and *P. mirabilis*, that killed in these concentration,so at the same concentration of AMP and AMPAA bacterial isolates were sensitivity by well diffusion method, whilst its killed by broth method, that's consulate broth method was best than diffusion method.

Keywords: AMP, Eugenol, 4-allyl-2-methoxyphenol, AMPAA, 2-(4-allyl-2-methoxyphenoxy) acetic acid.

الخلاصة

زيت القرنفل (4 الأليل-2-ميثوكس فينول(AMP)، من مركبات الفينولية المعروف بفعاليتة البيولوجيا و هو من الزيوت المشتقة من القرنفل ، والتي تستخدم على نطاق واسع كمطهر ومسكن للعناية بالأسنان، كلذلك فهو فعال ضد البكتريا التي ترافق تسوس الأسنان وأمراض اللثة، وهناك دراسات سابقة حول تأثيرها كمضاد للفطريات و مضاد سرطاني, مضاد للحساسية؛ مضادة للعوامل المطفرة. ولة خصائص مضادة للأكسدة وقاتل للحشرات (1)، وبالتالي فإنه يمكن أن تستخدم في إعداد الطعام المختلفة كمادة منكهة وفي مستحضرات التجميل(2).

تهدف هذه الدراسة إلى تصنيع مادة طبية جديدة 2- (4-الأليل-2-ميثوفينوكيس-حمض الخليك (AMPAA) مشتقة من الأوجينول (4 الأليل-2-AMP) والتحقق من نشاطها كمضاد ميكروبي للمادتين AMP و المشتقة (AMPAA)، من خلال دراسة الحد الأدنى للتركيز المثبط (MIC)؛ و الحد الأدنى من التركيز القاتل للبكتريا (MBC) وحساسيه تجاة ستة العزلات

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البكتيرية المسببة للأمراض, مع دراسة مقارنة لكفاءة كلا المادتين AMP وAMPAA على نفس العزلات البكتيرية التي تم الحصول عليها من المستشفيات العراقية.
هذه الدراسة تم اشتقاق مادة طبية جديدة (AMPAA) من AMP عن طريق تفاعل eugenate الصوديوم مع حامض الكلوروأسيتيك الصوديوم وتحضير محلول مائي من المادة الاولية و المادة الثانية الجديدة التي اشتقت منها, لها قابلية للذوبان, وقد تم دراسة فعاليتهما كمضاد ميكروبي ضد بعض أنواع البكتيريا المسببة للأمراض بطريقتي (الانتشار بالحفر والتخفيف المتسلسل).
وقد اظهرت نتائج الدراسة الحالية كل العزلات البكتيرية كانت حساسة لكلا المادتين AMP وAMPAA في تركيز منخفض عدا بكتريا *K. pneumoniae*، وقد وجد ان تركيزي 10.0 و 5.0 ميكروغرام / مل من AMP وAMPAA على التوالي اعتبر كتركيز ادنى قاتل للبكتريا (MBC) لكل العزلات عدا بكتريا *K. pneumoniae*و *P. mirabilis* والتي تم قتلهما في هذين التركيزين، وهذا يعني أن المركب المشتق الجديد AMPAA كان اكثر فعالية على عزلات الدراسة من الـ (AMP).
باستخدام طريقة الانتشار كانت كل العزلات البكتيرية حساسة لكلا الماتين (AMP وAMPAA) في تركيز منخفض، ولكنها بنفس التركيز قد قتلت بكتريا *K. pneumoniae* ، بالاضافة لذلك فان كل عزلات الدراسة قد قتلت في تراكيز تراوحت بين (10-50 ميكروغرام / مل) للمركب الطبي الجديد المشتق (AMPAA)، غير انه في نفس التركيز من AMP قتلت فقط عزلات بكتريا *K. pneumoniae*و *P. mirabilis*, في حين اعتبرت هذة التراكيز كـMBC للعزلات الباقية المتضمنه *S. pyogenes*, *E. faecalis, B. subtilis* and *E. coli* وكذلك كل العزلات لها درجات متفاوتة من الحساسية تجاه كل من (AMP وAMPAA).
اظهرت نتائج الدراسة ان كل عزلات البكتيريا كانت حساسة لكلا المادتين AMP وAMPAA في تركيز منخفض عدا *K. pneumoniae* ، وأيضا كان تركيزي 10.0 و 5.0 ميكروغرام / مل من AMP وAMPAA على التوالي كادنى تركيز قاتل للبكتريا لعزلات عدا *K. pneumoniae*و *P. mirabilis* حيث تم قتلهما بهذه التراكيز, وان طريقة التخفيف كانت أفضل من طريقة الانتشار ، وكذلك مركب مشتق جديد (AMPAA) هي أكثر تأثير على ستة عزلات بكتيرية من الأوجينول (AMP)، لذلك نوصي بدراسات مستقبلية قد يفتح طريقا جديدا في استخدام المركبات AMPAA وAMP كمضادات حيوية جديدة ضد البكتريا المرضية.

Introduction

Increasing resistance of pathogenic bacteria to many antibiotics, multidrug resistace as well as conventional chemicals were prompting the scientists to search alternative or novel sources such as plants extract and their derivatives as essential oils, that playing important role as bactericidal and bacteriostatic [3]. These activities of plants are extract and their derivatives due to have high level of phenolic derivatives [4]. Thus Eugenol, 4-allyl-2-methoxyphenol (AMP) is main constituent of essential oil obtained from commonly consumed spices such as *Eugenia caryophyllata* or *Syzygiumaromaticum* (clove), molecular formula of Eugenol is $C_{10}H_{12}O_2$ with has molecular weight (164.21), as dark yellow viscous oily liquid with a strong clove flavor in normal temperatures, so its slightly soluble in water as well as easily dissolved in organic solvents [5]. This component has widely applied in dentistry, anesthetics, analgesics, anti-inflammatory agents and flavouring agents. Eugenolhas been used as antibacterial against *Escherichia coli,* antihelicobacter and antiproliferative. So, in dentistry, it is employed as an antiseptic, disinfectant and also widely used as an analgesic, as well as in cosmetic and food products as flavoring, antimicrobial, and antioxidant agent [6].

In addition to these antioxidant properties, it protects neurons in culture from toxic events. It has activities of anti-convulsive and hypothermic agent [7]. Zinc oxide-eugenol materials have been developed for utilization in number of dental applications; they are most widely employed as antibacterial and palliative agent in treatment of the lesions [8].

antiseptic, disinfectant and also widely used as an analgesic, as well as in cosmetic and food products as flavoring, antimicrobial, and antioxidant agent [Hattori *et al.,* 1986].

So eugenol has limit to stabilization and dispersion in aqueous food systems, which causing increasing concentration required for antimicrobial functions, which may lead to phase separation and negatively affect the quality of food [9].

A derivative of eugenol was prepared by reacting sodium eugenate with sodium chloroacetic acid to give, 2-(4-allyl-2-methoxyphenoxy)acetic acid(AMPAA), this derivative was characterized by the available elemental analysis,UV-visible, Infrared absorption spectrophotometry, ^{1}H and ^{13}C NMR spectrometric techniques[10].

Materials and Methods

A- Material:

Sodium hydroxide, Eugenol**,** sodium chloroacetate from BDH, UK, Mueller-Hinton agar (Difco)**,** and 95 % methanol from Merck, Germany.

B- Instrument:

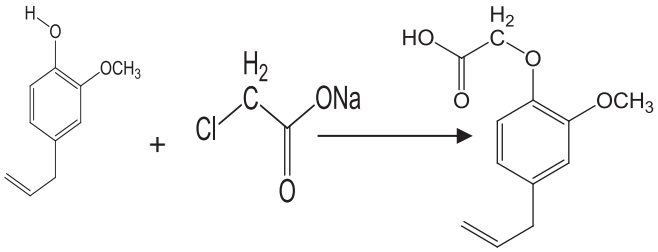
Melting points were determined with Stuart Scientific melting point SMP_1, England melting

point apparatus. The electronic absorption spectra were recorded in 95 % methanol by using Varian 100 Conc. UV-visible Spectrophotometer with 1.0cm quartz cell immediately after preparing the solutions in range 200-800 nm. Infrared spectra of the compounds were carried out by using KBr pellets in the range of 400-4000 cm^{-1} on Fourier transform Infrared spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on 300 MHz Bruker DMK-500 NMR Spectrometer by employing TMS as internal standard. Elemental analysis of C, H, and N of complexes were determined by micro analytical methods by Carlo-Erbamicroanalyser (Shimadzu model 8300).

C-P**reparation of 2-(4-allyl-2-methoxyphenoxy) acetic acid (AMPAA):**

Eugenol (5.0 g, 30 mmol) dissolved in a solution of NaOH (1.4 g., 33 mmol) in 75 mL 95 % ethanol until a clear solution was obtained. The solution was treated drop wise while stirring with a solution of sodium chloroacetate (3.85, 33 mmol) in 30 mL distilled water. The mixture was refluxed for three hour, and cooled to ambient temperature. A solution of 1:1 hydrochloric acid was added slowly until the pH of the solution was below 2.0. Glossy needles of the product were formed after induction time of about 5.0 minutes, and the mixture was left overnight at 4ºC. The crystals were filtered over ceramic media, washed with 1:1 of ethanol-diethyl ether mixture, and then dried at 60ºC for 3 hours to give a solid crystals m. p 71.5°C, 4.42 gm (65%) of the total product. Found: C, 64.54, H, 6.40 (Theoretical: C, 64.84; H, 6.35). UV-visible λ_{max} (H_2O): 280 nm, FT-IR (KBr disk): 1597 cm^{-1}(m, Ar); 1250-1280 (s, Ar-O-CH_2); 3100-3500 (s, COOH), 1650-1650 (m, C=C); 3000-3100 (m, Ar stretch.).1H NMR (300 MHz, $CDCl_3$): □ 3.25 (2H, d, J = 6.2 Hz, CH_2); 3.9 (3H, s, OCH_3); 5.09 (2H, m, =CH_2); 5.50 (2H, s, CH_2-O); 5.91 (1H, m, =CH-); 6.6 - 6.90 (3H, aromatic protons).

Equimolecular quantities of eugenol and sodium chloroacetate react readily to produce the acetic acid derivative of eugenol according to the following equation: The product of 2-(4-allyl-2-methoxyphenoxy) acetic acid was identified with available analytical technique; elemental analysis for C, H, UV-visible, Infrared absorption spectrophotometry, ^{1}H and ^{13}C NMR spectrometric techniques.



Eugenol (AMP) chloroacetate

2-(4-allyl-2-methoxyphenoxy)acetic acid(AMPA)

D- **Bacterial strains:**

Test pathogenic bacteria used in this study were stock cultures of standard and local isolates obtained from Al-Yarmuk Teaching Hospital, Baghdad medical City Teaching Laboratories, and Health Center Laboratories in Baghdad. Bacterial isolate as gram-positive bacteria include: *S. pyogens*, *E. faecalis*, *B. subtilis* andGram-negative bacteria as: *E. coli, K. pneumoniae* and *P. mirabilis.* Cultures of these bacteria were grown in Mueller-Hinton broth at 37°C and maintained on slants of nutrient agar at 4°C. The isolates were identified and checked for their purity on the basis of the following characteristics: morphology of the colonies by microscopical examinations; Gram's stain [11] morphological feature on culture media, and biochemical tests [12] as well as Vietk system.

Antimicrobial activity assay:

Antimicrobial activity was measured by two methods; well diffusion method [13] and broth dilution method [14]. In the well diffusion method, a sterile 8 mm diameter stainless steel borer cylinders were used to make wells in plates of Mueller-Hinton agar, which was spreaded superficial with 100 µL of bacteria at logarithmic plan at a density adjusted to a 0.5 McFarland turbidity standards (10^{8} CFU/ mL). The wells were filled with 100 µL of sterilized eugenol (AMP) or its derivative solution 2-(4-allyl-2-methoxyphenoxy) acetic acid (AMPAA) solution at working concentrations of 50 µg/mL. The following dilution were prepared and used for broth methods; 0.1, 0.5, 1.0, 5.0, 10.0, and 25.050

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μg/mL. plates were then incubated for 24 hr at 37°C. The results were recorded by measuring zones of growth inhibition (in mm).

Broth dilution method procedure was used to measure Minimal inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of the test solutions. In these experiments, 0.4mL of a suspension containing 1×10^8 CFU/ mL was added to 3.6 mL of susceptibility test broth containing serial two fold dilutions of eugenol and its derivative 2-(4-allyl-2-methoxyphenoxy) acetic-acid (AMPA) in glass test tubes. All tubes were incubated at 37°C for 24 hr before being read, the MIC was considered the lowest concentration of the sample that prevented growth. MBC_s were determined by subculturing, 10 □L from each negative tube and from the positive growth control, MBCs were defined as lowest concentration yielding negative subcultures or only one colony. All samples were examined in duplicate in three separate experiments.

Results and Discussion

By using Broth method the MIC and MBC of AMPA and AMPAA against six pathogenic bacteria were presented in Table 1 and Table 2, all bacterial isolate were sensitive to both AMP and AMPAA at concentration 0.1 μg/mL except *K. pneumoniae* was killed at same concentration, as well as all bacterial isolates were sensitive to 0.5 μg/mL whilst this concentration concider as MIC_s for two isolate : *K. pneumoniae* and *P. mirabilis.* So based on these results all bacterial isolate were killed at concentration range 10.0 to 50.0 μg/mL of AMPAA and same results for same concentration of AMP except *S. pyogenes*, *E. faecalis, B. subtilis* that MBC in 10 μg/mL, as well as the concentration (10.0 and 5.0) μg/mL of AMP and AMPAA respectively conceder as MBC for bacterial isolate except *K. pneumoniae* and *P. mirabilis* that killed in these concentration are showed in table (1) meaning the new derivative compound (AMPAA) are more effect on six bacterial isolate than eugenol (AMP).

By using well diffusion method all bacterial isolates were sensitive to both AMP and AMPAA in concentration 0.1 μg/mL except *K. pneumoniae* was killed so all bacterial isolate were sensitive to 0.5 μg/mL whilst this concentration were MIC_s for two isolate (*K. pneumoniae* and *P. mirabilis*). In addition, current results showed all isolates have been killed concentrations of between (10-50) (μg/mL) of new derivative compound (AMPAA), at the same concentration of AMP were killed for *K. pneumoniae* and *P. mirabilis*) whilst considered as MBC for other isolate such as *S. pyogenes*, *E. faecalis, B. subtilis* and *E. coli* in Table 2.

Results summarized in both tables 1 and 2 show that all isolates have varying degrees of sensitivity towards both AMP and AMPAA, however at the same time all isolates were more sensitive to AMPAA than AMP, as well as results in both table 1 and 2 appearance at the same concentration of AMP and AMPAA showed sensitive bacteria by well diffusion method, whilst it was killed by broth method, that's consulate broth method is best than diffusion method.

The results of Minimum inhibitory concentration (MIC) showed that *S. pyogenes*, *E. faecalis, B. subtilis* and *E. coli* were more sensitive to inhibition of eugenol(AMP) and (AMPAA) at concentration between (0.1 to 1.0) (μg/mL) (Table 1) while its killed in both concentration 25, 50 (μg/mL).

These results of current study is fully compatible with Thosar, *et al*. (2013) who showed that AMP active at the lowest concentration against many bacteria as *S. aureus*MIC (0.4 μl/mL) while 1 μl/ml of MIC for both *E. coli* and *E. facelis* [15]. So Walsh *et al.,* showed in his study the MIC and MBC of eugenol against the bacteria tested have been proved to be as low concentration [16]. The determination of MIC and MBC of eugenol was necessary to induce death cells at ranging concentrations of these agents.

Also these results of this study shown all isolates have varying degrees of sensitivity towards both (AMP and AMPAA), this difference in sensitivity were study by many researchers as Bennis*et al.* ,(2001) who showed in his study the eugenol (AMP) exerts causing different envelope damage ,this difference may be explained by the fact that the envelopes of gram negative bacteria and gram positive bacteria do not have the same structure [17], so eugenol effect on lipid of cell membrane for both Gram negative positive pathogenic bacteria [18].

As well as Burt has shown in his studied the mechanical effect of eugenol in degraded bacterial cell wall, damages plasma membrane and membrane protein [8 Burt, 2004], because AMP containing phenol compound which disruption bacterial membrane and causing high leakage of pro-

tein content [14 ; 16 and 18], so the hydrophobicity of eugenol is play as important factor of antibacterial activity via separate lipid from mitochondria and cell membrane causing change in structure as well as increasing the penetrability of the cell membrane [5].

This study was the first time in the world and in Iraq which derivative 2-(4-allyl-2-methoxyphenoxy) acetic-acid (AMPA) by the researcher from Eugenol (AMP) which its known composition and chemical and their effects, so the researcher has found necessary of experimental study on some pathogenic bacteria and comparison effect with AMP.

Conclusions

In conclusion, all pathogenic bacterial isolate were sensitive to both (AMP and AMPAA) in low concentration except *K. pneumoniae*, so (10.0 and 5.0) µg/mL of AMP and AMPAA, respectively considered as Minimum Bactericidal Concentration (MBC) for bacterial isolate except *K. pneumoniae* and *P. mirabilis,* that killed in these concentration, that meaning the new derivative compound (AMPAA) are more effect on six bacterial isolate than eugenol (AMP).

By using well diffusion method all bacterial isolate were sensitive to both (AMP and AMPAA) in low concentration, but *K. pneumoniae* was killed in same concentration. So all isolates had killed a concentration of between (10-50 µg/mL) of new derivative compound (AMPAA), In the same concentration of AMP were killed for *K. pneumonia andP. mirabilis*) although considered as MBC for other isolate as *S. pyogenes*, *E. faecalis, B. subtilis* and *E. coli,* whilst all isolates have varying degrees of sensitivity towards both AMP and AMPAA, But at the same time all isolate showed more sensitive to AMPAA than AMP. So at the same concentration of AMP and AMPAA showed sensitivity bacteria by well diffusion method, but it's killed by broth method, that's consulate broth method was best than diffusion method.

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