



Extraction, isolation, characterization, and antimicrobial study of a compound in the crude of Oak gall.

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ABSTRACT

Researchers reported studies on the compositions and biological activity of extracted oak galls. However, isolation and characterizing of bioactive compounds from the oak gall's extracts have become challenges. In this study, a new bioactive compound was isolated and characterized from extracted locally oak goal crude (EOGC). A novel mixture of (methanol 10% + acetonitrile 10% +water 80%) solvents was performed to fractionate the Soxhlet EOGC through a column chromatography technique. Thin Layer Chromatography (TLC) and High-Pressure Liquid Chromatography (HPLC) were utilized to study the constituents of the fractions. The isolated fractions were collected and tested against, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* ATCC 19404, *Escherichia coli* ATCC 8739, *Salmonella ebony* NTCT 6017 and *Candida albicans* ATCC 10231 utilizing the agar well diffusion method. Results demonstrated that only a single component fraction exhibited activity against (*S. aureus*, *M. leutues*, and *C. albicans*), in contrast, no detectable activity was observed against *E. coli* and *S. ebony*. Fourier Transform Infrared (FT-IR) and Mass Spectrometer techniques have been used to characterize the chemical composition of the compound. As a result, the new compound was postulated as **Chrysin-gallate** compound, which is considered a new bioactive chemical compound that may have a significant application as an antimicrobial and treat specific health issues.

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Keywords: Oak galls, Extraction, Isolation, Characterization, and biological activity.

1. Introduction

Natural sources of bioactive compounds have been utilized since ancient times, becoming a relevant complement to synthetic pharmacological drugs^[1, 2, 3, 4, 5]. Consequently, they have created a positive impact on the development of the human lifestyle. Nowadays, natural drugs offer the primary healthcare required for a large part of the inhabitants in developed countries. Furthermore, they have fascinated consideration in developed countries due to the dramatic increase in healthcare costs and global financial austerity^[6]. Virtually, half of the FDA-accepted chemical medicines for the curing of human health issues were either derived or inspired by natural products. Therefore, researchers always attempt to discover new natural medicines. Meanwhile, extraction, isolation, and characterization processes of bioactive constituents have become a challenge due to the complexity of the chemistry of natural products^[7,8,9,10,11].

Among the natural products, oak galls (*Quercus infectoria*) have

been widely used, due to containing various bioactive constituents, including tannin, gallic acid, syringic acid, ellagic acid, β -sitosterol, amentoflavone, hexamethyl ether, methyl betulate, hexagalloyglucose isocryptometrin, methyl oleanate, etc.^[12,13,14]. However, a lack of evidence can be seen to improve the molecular formula of these constituents.

Pharmacologically, it has been revealed that EOGC exhibits vigorous biological activities, such as ant larvicidal, anti-amoebic, anti-bacterial, antifungal, antiviral, anti-inflammatory, and antivenin^[15, 16, 17]. Basari et al. examined the mode of action of combining the extract of oak galls with vancomycin against Methicillin-Resistance *Staphylococcus aureus*. It was found that the ultrastructural and morphological changes in the bacteria. Shyma et al. also, reported a noticeable reduction in the population of both *Staphylococcus aureus* and *Candida albicans* after they had been exposed to the extracted oak galls^[18]. In addition, Dardmah et al. showed the positive impact of *Quercus infectoria* galls on healing open wounds in a streptozocin-induced diabetic BALB/c mouse model^[19].

Oak Gall belongs to the Fagaceae family and can be found as a tree or shrub, geographically it is distributed in various regions

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including Turkey (Anatolia), Syria, Iran, Greece, and the mountainous areas of the Kurdistan region in Iraq. The natural product is a multitude group with approximately 1400 species worldwide^[20] and has been introduced as one of the medicinal plants traditionally used by ancient society^[21,22].

The present study aims to further explore the chemical composition and biological activity of local EOGC.

2. Methods and materials

2.1 Collection and preparation of plants

The samples were collected in the (Qopi- Qarax) Sargma Mountain of the Kurdistan region. In the beginning, fresh Oak and oak galls were dried at room temperature, then powdered in by traditional way using mortar and pestle.

2.2 Soxhlet extraction

Approximately 20 grams of powdered plant sample was added into a thimble, meanwhile, 350ml of methanol (95%) was added into a 500 mL round bottom flask of Soxhlet apparatus. Carefully, the system was gradually heated, the mixture was heated gradually to 60 °C, and the extraction was set for 2 hours and 20 minutes. Finally, the solvent was removed from the yield using a rotary evaporator (IKA; Germany) system at 45-48 centigrade/pressure 500 mmHg. All the steps were repeated using 95% Ethanol, water, n-Hexane, Ethyl acetate, chloroform, and dichloromethane as the extraction solvent.

2.3 Flash column chromatography

By using column chromatography, the oak gall extract was separated into its constituents. Firstly, the column packed with silica gel (0.03-0.2mm) (Roth: CAS No.7631-86-9, Germany) by using a mixture of solvents were (methanol 10% + acetonitrile 10% +water 80%), and after choosing this solvent among various solvents and their mixtures. Then pure nitrogen gas was used to push the eluent through the column gently. Finally, the fractions were collected using clean test tubes.

2.4 Rotary evaporator

These fractions were dried by rotary evaporator and freeze-dried (Labfrezze; FDIOR) and stored in a sterile bottle at (-38 centigrade) and pressure (17 Pascal) until used.

2.5 Antimicrobial activity of the oak gall extract (Bacteria and cultures and agar well diffusion methods)

The antimicrobial activity of the crude plant-extracted was investigated utilizing the agar well diffusion technique. The tested standard strains obtained from the Biology Department/College of Science/ University of Sulaymaniyah included; *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Salmonella ebony* NTCT 6017, *Micrococcus luteus* and *Candida albicans* ATCC 10231, were cultured in nutrient broth to prepare a standard optical density for each pathogen (0.08 OD₆₀₀). Each tested microbe was seeded on freshly prepared Muller Hinton agar plates (each containing 25 mL of the media), and wells with 6.0 diameter were punched in the plate using a sterile Cork borer. Then an amount of 80 µl of the crude (2mg/mL) was pipetted into the wells, which were incubated for

24 hours at 37 °C.^[23,24,25]. The inhibition zone around the wells has been considered an antimicrobial activity of tested microorganisms.

2.6 Characterization techniques

2.6.1 Thin Layer Chromatographic Analysis

This fraction was added to the TLC plate after collecting various fractions to count the number of biologically active substances. Approximately, 20 µl fractionated solution of oak gall was applied on a plate of TLC, then the plate was added to the mixture of acetonitrile: methanol, and water with a ratio of (10;10;80%). The TLC was kept at UV 254 and 366 nm, and the results were investigated. The spots were interpreted, and an individual Rf value for each spot was measured, and compared with standard reference compounds, when they were run in the same respective solvent techniques.

2.6.2 HPLC Study

HPLC (Thermo Fischer, Ultimate 3000) was used to study the extracted fractions. It consists of an Agilent 1200 series preparative pump coupled with a UV diode array detector. RP-HPLC analysis of a portion of oak gall was performed using a Zorbax SB-C18 analytical column (4.6 150mm, five µm particle size, Agilent, Germany). The mobile phase and wavelength were optimized. A 0.2 µm syringe filter was rapidly used to filter the sample. 20µL was taken as injection volume. The mobile phases were utilized with a flow rate constant at 1mL/min as acetonitrile: methanol: water (10: 10: 80). The wavelength was set at 230 nm, and the same wavelength was selected for preparative HPLC, using a UV-diode array detector.

2.6.3 FTIR Analysis

Fourier-Transform Mid-Infrared (FTIR) analyses were investigated utilizing a Tensor 27 spectrometer Oak gall was taken using an FTIR-Perkin Elmer, Spectrophotometer. Measurements were performed at room temperature (20° C), and the spectra were recorded for the sample from 4000 to 400 cm⁻¹, using 1 cm⁻¹ as resolution.

2.6.4 Mass Spectrometry Analysis

Agilent Technologies 5975C mass spectrometer was utilized to study the chemical formula of the EOGC. The mass spectra obtained under electron ionization (EI) conditions have frequently been applied. All sorts of natural goods have been subjected to this ionization process. Nevertheless, due to the extensive internal energy spread carried by the first generated MC⁺ ions, which may conceal or suppress the MC⁺ ions and significant primary fragment ions carrying structural information, complicated fragmentations may occur during EI. Because of their strong polarity and poor volatility, polyphenolics present struggles for the analysis using EI.

3. Results and discussion

Previously, researchers reported the chemical compositions and antibacterial activities of EOGC. However, due to the complexity of the natural product's composition, the emergence of

fractionation, isolation, and identification of the ingredients of EOGC has become a significant challenge for researchers.

In this study, the oak gall samples were extracted, fractionated using a unique solvent mixture, isolated, and identified a novel chemical compound in its composition. In addition, the biological activity was studied for all of the fractions.

The finding indicated that a mixture of (methanol 10% + acetonitrile 10% + water 80%) is the most convenient mixture for separating the fractions among the solvents, regarding the study of constituents of the fractions, using TLC and HPLC techniques. As far as we know, there is no report on the use of this mixture as an eluent in literature.

TLC technique was used to analyze the composition of the fractions to isolate the bioactive compound from the others. Figure (1a) shows a TLC picture of one of the fractions; obviously, a single spot is seen; This may indicate a single

compound in the fraction, while the other fractions showed multiple spots with difficulty to make differentiating between some spots of some fractions.

To further explore, HPLC technique was utilized to investigate the chemical composition of the fractions. Figures 1b, 1c, and 1d represent HPLC chromatograms for the multi-component and single-component fractions, respectively. The figures confirmed that the mixture succeeded in isolating the crude composition. The peak at (Rt. = 5.6 min) in Figure 1d indicates a single component fraction, while peaks in Figure 1b and 1c (Rt = 2.0, 2.5, 3.0, 5.6, and 9.0 min) are assigned for at least five different compounds in the fraction. For the biological activity study, all of the fractions went under a solvent extraction process using the rotary evaporator technique to remove the solvents and having pure oily partitioned fractions

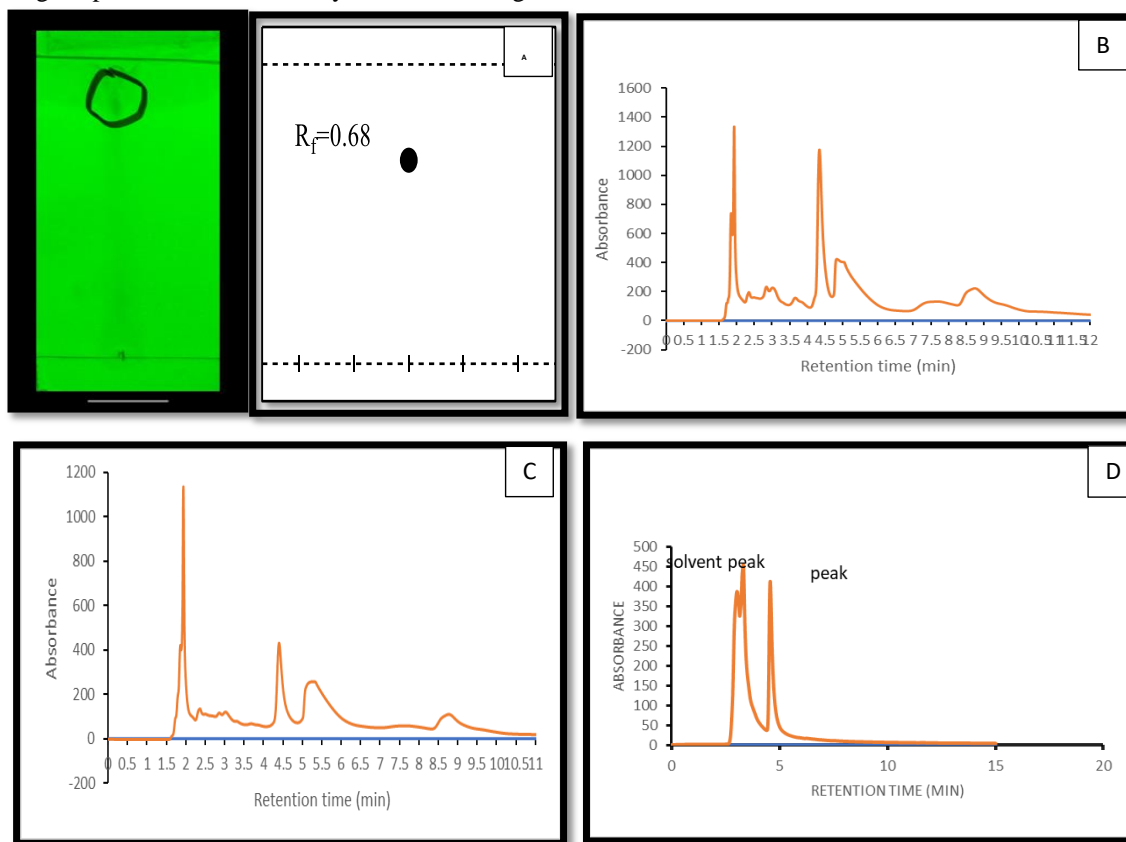


Figure 1: (A) shows TLC for the isolated fraction. (B) shows HPLC Chromatogram for one of the isolated fractions, shows more than one peak (C) Shows HPLC Chromatogram showing more than one peak (C) Preparative HPLC Chromatogram showing only one peak beside the solvent peak. Source: prepared by authors (2023).

3.1 Antibacterial activity of oak extracts after purification

For the biological activity study, all of the fractions went under a solvent extraction process using the rotary evaporator technique, to remove the solvents and having pure oily partitioned fractions. Antibacterial activity of all pure oily partitioned fraction products of the EOGC was carried out against two bacterial strains and a fungus. As luck would have it, the fraction with the single component showed antibacterial effectiveness, while the others were negative. The positive fraction is explained by its ability to pass through the bacterial cell wall up to the internal membrane,

interfere they are destroyed as a function of the cell's metabolism^[17,26].

In Gram-positive bacteria such as (*Staphylococcus sp* and *Micrococcus luteus*), the activity of isolated compounds is rapid, as shown in Figures 2 A and 2B. However, in Gram-negative bacteria such as (*Escherichia coli* and *Salmonella ebony*), it is slower shown in Figures 2D and 2E. This means pure Oak does not have biological activity against these bacteria. In addition, a large inhibition zone can be seen in Figure (2C); this indicates that the compound has a robust antifungal property when cultured against (*candida albicans*) fungus.

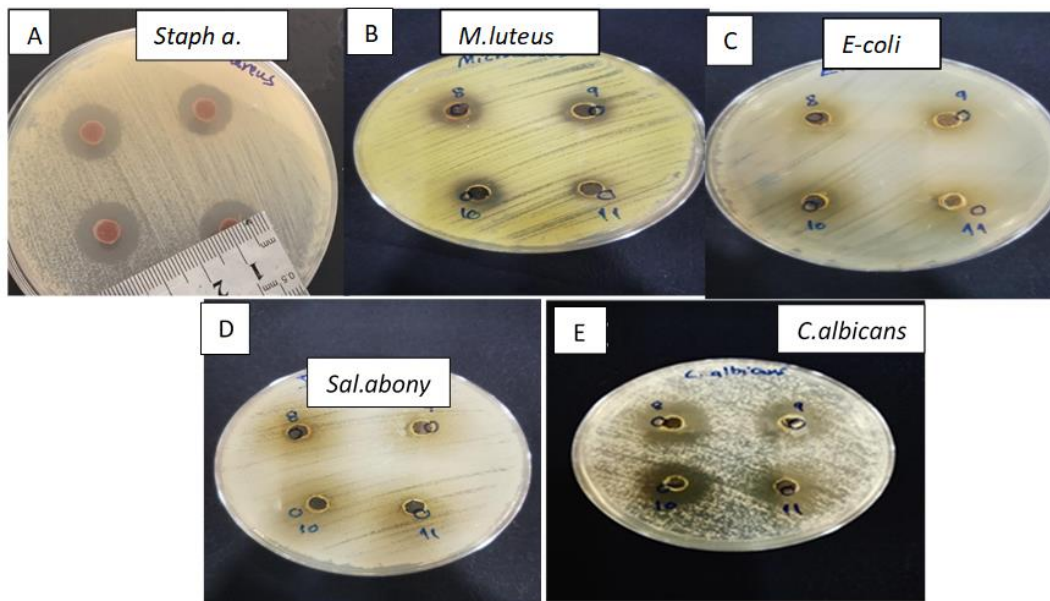


Figure 2: Antibacterial activities of the single component of EOGC Inhibition zone for Staph au. (A) for Micrococcus sp (B), for E-coli (C) and for Salmonella (D), and for Candida albicans is (E).

3.2 Characterization of the extracted bioactive compound

The chemical structure of the new bioactive compound was characterized using FTIR, Mass-spectroscopy, and HPLC techniques. Figure 3 shows the FT-IR spectrum of the compound that exhibits different bands. The peak at (3410 cm^{-1}) is a good indication of O-H stretching vibration, and the band at (2924 cm^{-1}) is assigned to stretching vibrations of the aliphatic C-H groups such as ($-\text{CH}_3$, $-\text{CH}_2$, and C-H in the aromatic ring) groups.

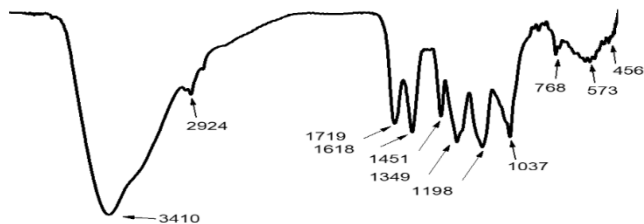


Figure 3: Shows FT- IR spectrum of the bioactive compound.

While C=C, the C-H and O-H bonds in the plane deformation are indicated by the band at (1037 cm^{-1}). A band represents the C=C stretching bond at 1618 cm^{-1} . In addition, C=O group stretching vibration is represented by (1719 cm^{-1}), 1198 cm^{-1} is assigned to C-O stretching in phenol, and (1451 cm^{-1}) expresses the presence of hydroxyl ($-\text{OH}$) bending of the phenol group. Stretching of C-C- with C-H deformation vibration and CH_2 rocking vibration were characterized by ($573\text{-}768\text{ cm}^{-1}$).

On the same line, a Mass Spectrometer was used to interpret the molecular formula of the compound further. Figure 4 depicts the compound's disintegration with embedded main structural fragments. It indicates that the compound's molecular ion (M^+) is approximately 404 m/z . Besides the FT-IR data, the most probability of the postulated molecular formula is Chrysin-gallate (flavonoid-gallic acid), which has a molecular mass of 406 g/mole . Flavonoids and gallic acid compounds in the composition of the EOGC have been reported by researchers [27].

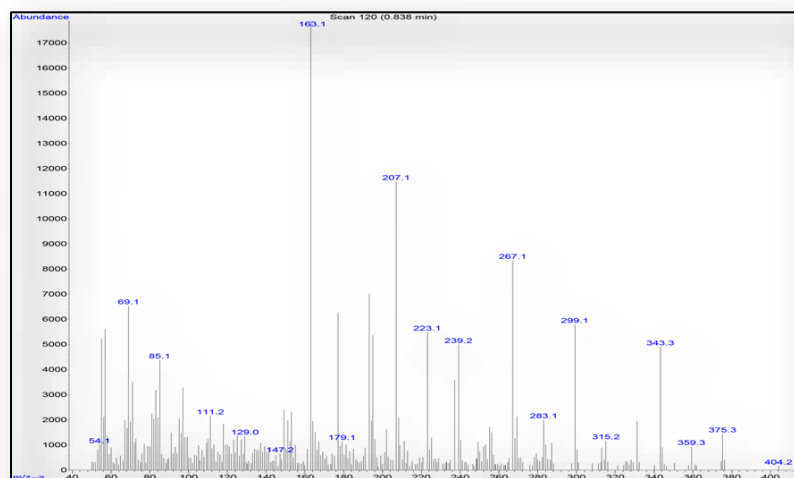


Figure 4: MS spectrograms of TIC peaks from oak galls extracted.

The product ions produced from the isolation and fragmentation of the primary producers are also shown in Figure 5, which can be used as more evidence to prove the postulated chemical formula of the compound. The base peak of this spectrum is observed at 163.1 m/z; it could be fragmenting number one in Figure 5, which is formed through the loss of gallate and methyl benzene ions from the precursor ion. In addition, peaks observed at m/z 129, 147.2, 281, 329, and 375 m/z represent approximately the fragment ions of 2, 3, 4, 5, and 6, respectively.

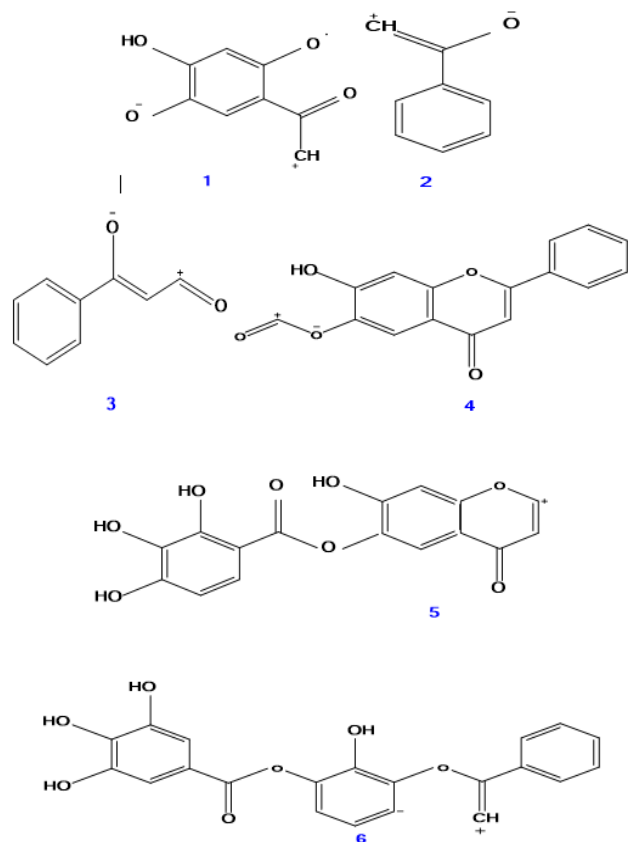


Figure 5: Fragment ion of Chrysin-gallate.

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Previously, researchers reported the presence of gallic acid, tannins, polyphenolic compounds, and phytochemicals, such as amentoflavone, in the non-fractionated extracted leave and oak gall crudes^[28]. Meanwhile, many attempts have been made to study the extracted oak galls' antibiotic, antioxidant, antiviral, and antipyretic properties. However, this study found a new bioactive compound (Chrysin-gallate) that was successfully isolated and characterized. The novel molecule will be studied more in our future works.

Conclusions

Another potential bioactive application of the extracted oak gall crude has been discovered. A fascinating point in this study is extracting, separating, and characterizing a new (novel) antibiotic compound (chrysin-gallate) of EOGC. Furthermore, the efficiency of the unique mixture of (methanol 10% + acetonitrile 10% +water 80%) solvents in isolating the crude components through the column chromatography techniques. FT-IR and Mass Spectrometer show a vital role in postulating the bioactive compound's chemical structure, which exhibits an apparent biological activity against two bacterial strains and a fungus. Further study will be recommended on the compound.

Conflict of interests

None

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