Extraction and Identification of A Flavonoid compound from Oak Plant(*Quercus infectoria Oliv.*) and study Of Its Antibacterial Activity, *in vitro*

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الخلاصة

تضمنت الدراسة الحالية عزل مركب فلافونيدي من نبات البلوط (*Quercus infectoria Oliv.*). باستخدام الكحول الاثيلي كمذيب، شخص الفلافونيد المعزول باستخدام طرق التشخيص الكيميائية المختلفة والتي شملت كروماتوغرافيا الطبقة الرقيقة (TLC) وطيف الأشعة تحت الحمراء (IR- spectrum)وطيف الأشعة البنفسجية (UV-visible) فضلا عن إجراء الكشوفات اللونية النوعية للمستخلص .

اختبرت فعلية المركب الفلافونيدي تجاه العزلات الجرثومية القياسية الموجبة لصبغة كرام (*Staphylococcus aureus* ATCC 25923) والسالبة لصبغة كرام(*Escherichia coli* ATCC 25922) وأظهرت النتائج قابلية المستخلص على تثبيط نمو كلا النوعين من العزلات ألا إن كفاءة المستخلص تجاه البكتريا الموجبة لصبغة كرام كانت أعلى منها تجاه البكتريا السالبة لصبغة كرام (31ملم و 19ملم على التوالي).

Abstact

A flavonoid compound was isolated from oak plant (*Quercus infectoria oliv.*) (Fagaceae) stem barks using ethanol alcohol as a solvent, the isolated compound was identified using conventional characterization methods, such as Thin Layer Chromatography(TLC),Ultraviolet-Visible (UV-Vis) and Fourier transform infrared spectroscopy(FT-IR), as well as, some biochemical assays using color reagents.

Antibacterial activity of extracted flavonoid was carried out against two types of reference strains bacteria: which are Gram positive bacteria (*Staphylococcus aureus* ATCC 25923) and Gram negative (*Escherichia coli* ATCC 25923), the results shows that the extract of oak barks has good a antibacterial growth activity in which gram positive bacteria are more susceptible to the oaks extract than Gram negative (31 mm and 19mm, respectively).

key words: Quercus infectoria, oak stem barks, flavonoid, Antibacterial activity.

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Introduction

The use of plant based medicines (local medicine) date back to (4000-5000) B.C. Furthermore according to WHO about 80% of world population depend on medicinal plant for their health care needs, and more than 30% of pharmaceutical preparations are based on plants[1,2].

Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, *i.e*. any part of the plant may contain active components[3].Plants contain different types of compounds such as resins, rubbers, gums, waxes, dyes, flavors, fragrances proteins, amino acids, bioactive peptides, phyto hormones, sugars, flavonoids, and bio pesticides [2,4].

Flavonoids are a group of about 4000 naturally occurring polyphenolic compounds, found universally in foods of plant origin. These are primarily recognized as the pigments responsible for the colors of leaves, especially in autumn[5]. Flavonoids (or bioflavonoids) (from the Latin word *flavus* meaning yellow), also collectively known as Vitamin P and citrin, are a class of plant secondary metabolites or yellow pigments having a structure similar to that of flavones. According to the IUPAC nomenclature, they can be classified into: *flavonoids*, (examples: quercetin, rutin), *isoflavonoids*, *neoflavonoids* (Fig. 1). The three flavonoid classes above are all ketone-containing compounds [6,7].

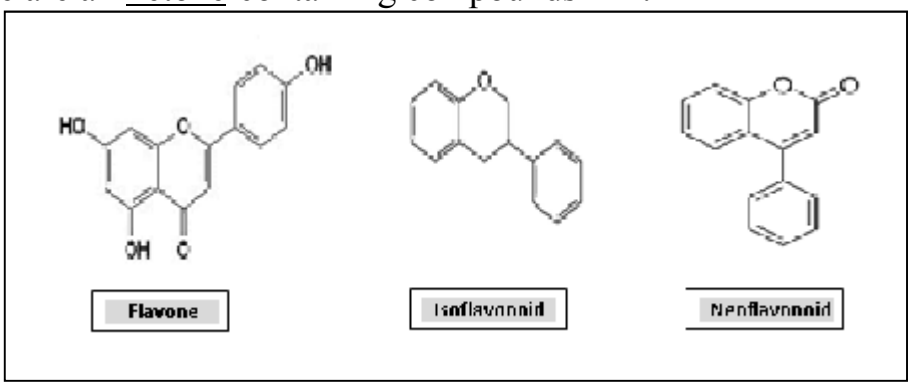


Figure (1): ***Flavonoid groups structures.***

Flavonoids are also important for human health. Like vitamins, these compounds are not produced endogenously by the body and must be supplied either through the diet or nutritional supplements[8].These flavonoids display a remarkable array of biochemical and pharmacological actions *viz*., antiinflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic activities [9,10,11,12].

Flavonoids are widely distributed in barks, fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers as well as tea and red wine [13]. More than 2000 flavonoids have been reported among woody and non-woody plants[14]. Presence of flavonoids has been reported from many plant species like *Lycium barbarum* [15], *Passiflora plamer* [16], *Cassia angustifolia* [17], *Jatropa curcas* L. [7] and *Quercus infectoria* [18].

Oaks are one of the important trees, distributed in many regions of temperate zone in the world. They are source of raw materials, for some useful products to human race [19]. The species of oak, the (Quercus genus), are classified under the family Fagaceae. Four species of oak (***Q. aegilop, Q. infectoria, Q. libani and Q. Marcantherea***) are grown in the Iraqi Kurdistan Forest[20].

Oaks contain about (25-28) chemical compounds. These include tannic acid, egallic acid, ellagic acid, monoterpens, p-coumarin, vanillic acid, tolune and kaempferol [21]. These Quercus species contain secondary products such as poly phenolic compounds; tannins are ellagic acids which are considered to be a great importance in medicinal, pharmaceutical, antimicrobial and anti disease [13,22].

Quercus infectoria oliv. is well-known since ancient times. Early study showed that as part of postpartum care, the Arabs, Persians, Indians, Malays and Chinese have traditionally used *Q, infectoria oliv*. after childbirth to treat vaginal discharge and related postpartum infections [23,24,25]. Therefore, the present study was aimed to isolate and characterize of a flavonoid compound from local medical oak plant, as well as to evaluate the biological activity against bacterial growth *in vitro*.

Materials and Methods

Plant Material:

Quercus infectoria Oliv. (Fagaceae) barks sample used in this study was obtained from the local markets and identified based on its physical characteristics. The plant samples were air dried in shadow, crushed to small pieces using pestle and mortar and finely powdered in an electric grinder. Plant powder was then used for the extraction procedure.

Flavonoid extraction methods:

Flavonoid extract was prepared by immersing 100 gm of dried material of *Q. infectoria* in 500 ml ethyl alcohol (100%) for 24 hrs at room temperature using magnetic stirrer [7,26,27].

The mixture was then filtered using Whatman No. 1 filter papers and the process was repeated using the remaining residue with 300 ml ethanol alcohol to ensure the complete extraction in each time. The two filtrates were added and treated with 100 ml lead acetate (1%) for 4 hrs for precipitation. The mixture was filtered , then a mixture of 250 ml acetone and 30 ml of concentrated HCl was added to the precipitate, and filtered. The resulting pellet was finally lyophilized (Freeze- dried) at -50^{o}C under vacuum for 12 hrs. The extract was dissolved in ethyl alcohol, the extraction process was repeated for 1 hr, filtered to produce red filtrate .Finely, the filtrate was placed in a clean and dry Petri dish away from light at room temperature until deep red-brown powdered was obtained.

Phytochemical Screening

The dry extracts were subjected to various chemical tests in order to detect the presence of different phytoconstituents [28,29]:

[1] flavonoids: to detect the flavonoid in plant extract, the addition of KOH (1%) to alcoholic extract led to the formation of yellow color and this was a result of the presence of flavonoid.

[2] Phenolic compound: detected by a portion of the aqueous filtrate of each plant extract, 5ml was added to (1-2) drops of 1% of ferric chloride. A blue-green indicated the presence of phenolic compounds.

[3] Double bind test: using KMnO4 reagent, brown solution formed indicate the presence of double bond.

[4] Aldehyde & keton test: using 2,4 dinitrophenyl hydrazine , in which the presence of yellow precipitate indicate that the extracted compound has aldehyde & keton groups.

Chemical Identification:

(1) Thin layer chromatography (TLC)

The crude extract was dissolved in ethanol and spotted on TLC plates (5x 20 cm) coated with silica gel .These plates were developed in chromatography chamber containing solvent mixture of (butanol, acetic acid and water (70:25:5, v/v/v) and let to stand for 1 hr. The developed plates were air dried and visualized under UV light.

The plates were then placed in a chamber saturated with ammonia vapours to observe the color of spot and plates were also placed in a chamber saturated with I_2 vapours to observe the color of spot . R_f values were calculated for isolated sample [26,30].

(2)* Structural analysis of major components*:

(i) Ultraviolet-Visible spectroscopy:

UV-Visible absorption in the range of (200-800)nm was recorded in Central laboratory- kufa university by Computerized thermospectronic model LR 115161(England), using ethanol as a solvent.

(ii) Infrared spectroscopy:

FT-IR spectrum was analyzed to fined the most important functional groups of flavonoid extract by KBr disk technique using FT-IR 8400S SIMADZU (Japan) in the central Lab./ Kufa university.

Antibacterial activity :

(i) Preparation of extract solution:

The extracts were dissolved in ethanol (100%) to a final concentration of 10 mg/ml for disc diffusion assay .

(ii) Bacterial Cultures:

The bacterial strains used in this study were *Staphylococcus aureus* ATCC 25923, , *Escherichia coli* ATCC 25922 obtained from Microbiology laboratory- College of Medicine / Kufa university. All the bacterial strains were grown and maintained on nutrient agar slants. The inoculum size of each test strain was 10^8 bacteria/ml for disc diffusion assay which was standardized by adjusting the optical density of the bacterial suspension (OD=620 nm).

(iii) Screening for antibacterial activity

The disc diffusion method was used to evaluate the antibacterial activity[31,32].

Mueller Hinton agar was prepared in the plates as the media for the test microorganisms. Sterile filter paper discs (Whatman No. 1mm) were impregnated with 100 µl of each of the extracts (10 mg/ml) and left to dry under the laminar flow cabinet overnight. The bacterial inoculum was spread evenly onto the surface of the Mueller Hinton agar plates using a sterile glass L-form rod before the extract discs were positioned on the inoculated agar surface. Each extract was assayed in triplicate. Sterile distilled water served as negative control. All the plates were incubated for 24 hr at 37° C. The antibacterial activity was interpreted from the size of the diameter of zone inhibition measured to the nearest millimeter (mm) as observed from the clear zones surrounding the discs.

Results and discussion

Extraction:

In the present study ,flavonoid extract was isolated from stem barks of *Qurecus infectoria oliv*. Fagaceae medicinal plant, using ethanol alcohol as a solvent, the extract has a deep brown color with crystal-like appearance with a good yield about 9.6 gm/dry material. Table (1) shows some physical and chemical properties of isolated extract.

Table (1): Some physical and chemical properties of isolated extract.

Test	Flavonoid
Description	Red-brown crystals
Melting point	(146-165) decomposition
Solubility test	Soluble in ethanol, methanol, ethyl acetate, DMSO. DMF,

	acetone, while insoluble in water
Yield (%)	9.5 %/ 100 gm dry weight

Results of the biochemical assays (Table 2) indicate that the isolated extract from oak plant was a flavonoid compound contain double bonds, keton group with phenolic hydroxyl groups within its structure .

Table (2): Results of preliminary quantitative tests.

Tests	Result	Color reaction
Flavonoid test	+ve	Yellow precipitate
Double bond test	+ve	Brown
Aldehyde& keton	+ve	Yellow precipitate
Phenol test	+ve	Green

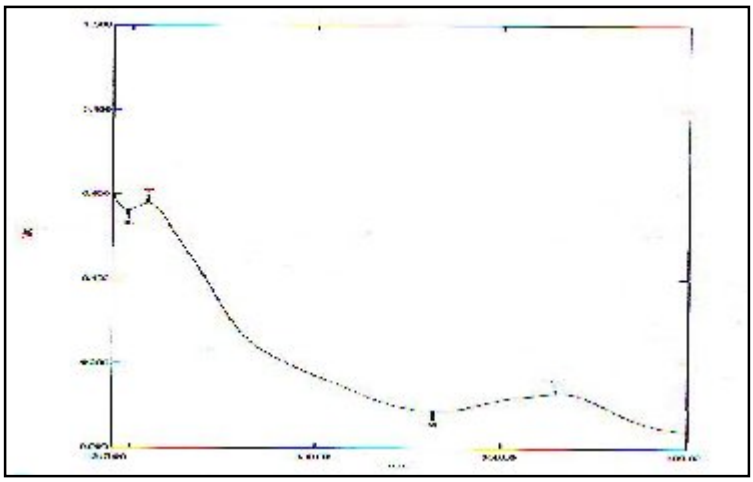
The preparation of the extract by TLC using butanol: acetic acid : water (70: 25 :5 v/v/v) as developing solvent gave one major component of which had R_f value of (0.82).

Table(3): TLC and R_f values of flavonoid extract.

Developing solvent	Reagents	Spot(s)	Rf value
butanol: acetic acid : water (70: 25 :5 v/v/v)	Naked eye	1	0.82 (yellow)
	UV360 nm	1	0.82(fluorescent)
	I_2 & ammonia vapours	1	0.82 (brown)

When the plates were placed in chamber saturated with ammonia and I_2 vapours , it showed deep yellow- brown spot, while when the plates developed under UV-light showed fluorescent spot .R_f value (0.82). Results of TLC (Table 3) which presence as one single spot indicate that the isolated compound was single pure compound .

The UV-visible spectrum (Fig.1),shows two peaks of maximum absorption at (254 nm) and (364nm) due to $\pi \rightarrow \pi^*$ transition which is the characteristic of unsaturated double bond, the visible spectrum also shows max absorption at λmax= 453 nm due to the $n \rightarrow \pi^*$ transition due to the presence of pairs electrons.



Figure(1): UV-Visible of the purified flavonoid compound from Q. infectoria plant.

Table (4) and Figure (2) shows the most important absorption peaks of functional groups belonged to the IR- spectrum of isolated flavonoid compound which .From IR- spectrum , we can concluded that the isolated compound has aromatic structure contain phenolic hydroxyl groups, ether, and carbonyl groups within its structure.

Table (4): The functional groups of the purified flavonoid compound from IR-spectrum.

Wavenumber (cm^{-1})	Band shape	Band	Functional group
3500-3200	Broad	O-H	Stretching of phenolic-OH
1348	Broad	O-H	Bending of phenolic -OH
1612	Medium, broad	C=O	Stretching of ketone carbonyl
1620	Strong, sharp	C=C	Stretching of olfenic C=C
1039	Sharp	C-O-C	Stretching of ether

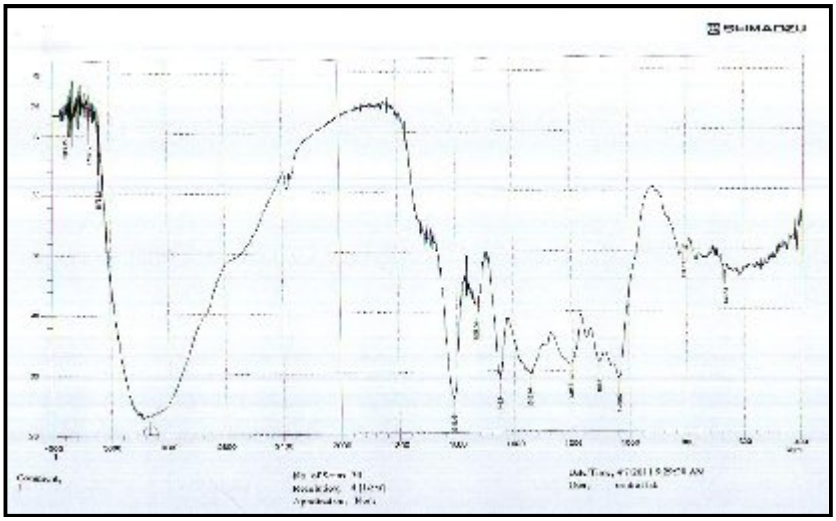


Figure (2): Infrared spectrum of the purified flavonoid compound .

Flavonoids are water soluble polyphenolic molecules containing 15 carbon atoms. Flavonoids belong to the polyphenol family. Flavanoids can be visualized as two benzene rings which are joined together with a short three carbon chain. One of the carbons of the short chain is always connected to a carbon of one of the benzene rings, either directly or through an oxygen bridge, thereby forming a third middle ring, which can be five or six-membered. The flavonoids consist of 6 major subgroups: chalcone, flavone, flavonol, flavanone, anthocyanins and isoflavonoids. Together with carotenes, flavanoids are also responsible for the coloring of fruits, vegetables and herbs [33.34].

Antibacterial activity:

Table (5) and Figure (3) show the results of antibacterial activity of alcoholic extract from the barks of *Q. infectoria* against *Staphylococcus. aureus* and *Escherichia coli*, the alcoholic extract , however, displayed consistent antibacterial activity against both bacterial strain. The inhibition zone against *S. aureus* was higher (31) mm compared to the extract activity against *E. coli* (19)mm.

Our study showed that the extract from *Quercus infectoria* inhibited both tested bacteria, and show better inhibition for the Gram negative than Gram negative .Our findings were also

supported by other researchers who reported that crude powder of the *Q. infectoria* was found to be active against *S. aureus* [35,36].

Generally, plant extracts are usually more active against Gram positive bacteria than Gram negative bacteria [37] .The potent antibacterial activity of extracted flavonoid suggested that these extracts may have high total flavonoid content [38]. for phenols and phenolic compounds, an injury of membrane functions has been reported as a mechanism of action [39,40,41].

The presence of alcoholic groups(-OH) in the structure of the flavonoid increase the activity of the plant extract to inhibit the microbial growth, so, the alcoholic compounds and their derivatives are considered as antiseptic agents[42], which are changing the cell protein nature and increase the permeability of the cell membranes[43].

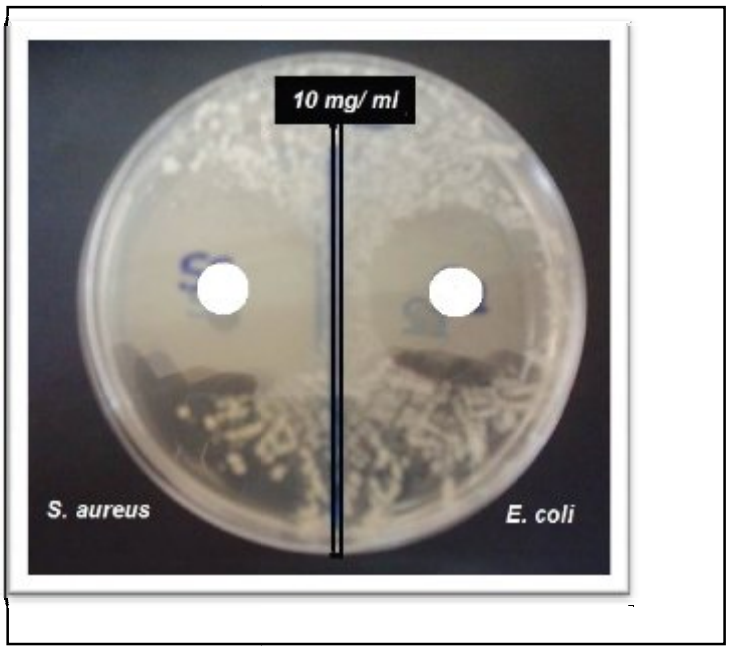


Figure (3) :The antibacterial activity of carotenoid pigment against Staphylococcus aureus and Escherichia coli bacteria.

Table(5): Antibacterial activity of Q. infectoria extract determined by disk diffusion assay.

Bacteria	Zone of inhibition (mm)
Staphylococcus aureus (ATCC 25923)	31
Escherichia coli (ATCC 25922)	19

Conclusion

In conclusion, the extract of *Q. infectoria* has high potential as antibacterial agent. This finding provides an insight into the usage of the bark of *Q. infectoria* in traditional treatment of wounds or burns associated with bacterial infections.

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