Partial purification of tannins from *Quercus infectoria* galls and the study of its effect on some isolated skin pathogenic microorganisms.

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Abstract

Five methods were used for the extraction of tannins from *Quercus infectoria* galls. Results revealed that extraction with acetone (70%) was the best.

Biochemical detection showed the existence of tannins and glycosides in the gall extract, with no alkaloids and essential oils.

As regards biological test, the gall extract was very efficient in inhibiting the growth of *Staphylococcus aureus, Streptococcus pyogens, Pseudomonas auroginosa* and the dermatophytic fungi: *Trichophyton mentagrophytes*.

Minimal inhibitory concentrations (MIC) were 5 and 10 mg/ml for *S. aureus* and *P. auroginosa*, respectively.

A partial purification for the above- mentioned extract was conducted. The purification steps included extraction with acetone (70%), extraction with ethanol (95%) and adsorption chromatography with Sephadex LH-20.

"التنقية الجزئية للتانينات من أعفاص نبات البلوط *Quercus in fectoria* ودراسة تأثيرها على بعض الأحياء المجهرية الجلدية الممرضة "

الخلاصة

أستخدمت خمس طرائق لأستخلاص التانينات من أعفاص نبات البلوط *Quercus in fectoria* وتبين أن الأستخلاص بالأسيتون (70%) كان أفضل هذه الطرق . وتمخضت نتائج الكشوفات الكيميائية التمهيدية لمستخلص الأعفاص عن احتوائه على التانينات والجلايكوسيدات وعدم احتوائه على الكلويدات والزيوت الأساسية .

أما اختيار الفعالية البايولوجية فقد أظهر كفاءة عالية للمستخلص في تثبيط نمو كل من بكتريا *Staphylococcus aureus* ، *Streptococcus pyogens* ، *Pseudomonas aeroginosa*، والفطر الجلدي *Trichophyton mentagrophytes* .

وبلغت قيم التركيز المثبط الأدنى (5 و10) ملغم / مل لكل من بكتريا *S . aureus* و *P. auroginosa* ، على التوالي .أجريت تنقيه جزئيه للمستخلص المذكور وإشتملت خطوات التنقيه على الأستخلاص بالأسيتون (70 %) فالاستخلاص بالأيثانول (95 %) واخيراً كروماتوغرافي الأدمصاص باستخدام الهلام Sephadex LH - 20 .

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Introduction

Although a great advance in the synthetic drugs industry was performed, medical plants return to take their role in the modern drugs industry to the extent that 60% of the produced drugs were from plant origin.

Phenolic compounds which spread in most plant tissues (1) have a bactericidal, viricidal and fungicidal effect by destroying cell membrane and proteins.

Tannins are polymeric phenolic compounds with multiple hydroxyl groups. They are soluble in water and alcohol but insoluble in the organic solvents such as ether, benzene and chloroform. They are distinguished by small molecular weights ranging from (500-3000dalton). (1).

Quercus infectoria is a good source for galls production (2). Galls are small tumors with spherical or conical shape, produced in high quantities and contain tannins (60% from its weight) (3). Galls are formed as a result of the plant infection by some insects. The insect lays its eggs in the plant bud in order to begin its life cycle, during that the inner cells of the bud becomes bigger and all starch converts to tannins (4).

Recently, tannins received more and more attention; it has been recorded that the consumption of drinks containing tannins relieves different pathogens (5). In addition to the fact, tannins play an important role in many human physiological activities such as enhancing the macrophages (6) and finally the possibility of using this type of compounds as antimicrobial agents.

The purpose of this study is to detect some of the active compounds in the *Q. infectoria* galls, to purify tannins from galls extracts as well as to test the biological activity against some isolated skin pathogenic microorganisms.

Materials and methods

Tannins extraction from *Q. infectoria* galls:

Galls were obtained from local market and milled to get fine powder. Five extraction methods were used, including (ethanol 70%, acetone 70%, cold water, hot water at 95 ºC and boiled water). The extraction process included mixing 20 gm of gall powder with 400 ml from each solvent mentioned above. As regards extraction conditions were carried out as follows;

1- In the case of the first three solvents, the extraction mixture was put in flasks (1L) and incubated in shaker water bath at 40 ºC for 24 hrs.

2- Hot water: water at 95 ºC was added to a beaker contained the galls powder, mixed well and left to cool for 30 min with gentle shaking at intervals.

3- Boiled water: the galls powder was added to a beaker contained boiled water, boiling was allowed to proceed for 30 min with gentle shaking at intervals.

Determination of the extractable compounds percentage:

Extracts were filtered using filter paper (Whatman No. 1) and dried using incubator at 37 ºC. After complete drying, extract weighted to calculate the extractable compounds percentage.

Biochemical detections:

The following biochemical detections were used: Drangendroff for alkaloids (7); ferric chloride 1% (8) and lead acetate 1% (9) for tannins; Fehling for glycosides (8) and Liberman-Berchard (8) for essential oils. pH was measured by pH-meter.

Biological activity:

Antibacterial activity:

Four isolates of skin pathogenic bacteria were obtained from bacteriology laboratory at Al- Husaini hospital/ Karbala. The isolates were: *Staphylococcocus aureus, Streptococcus*

pyogens, Escherichia coli and *Pseudomonas auroginosa*. They were activated on nutrient broth and incubated at 37 ºC for 18 hrs.

Agar well diffusion method (10) was used to test antibacterial activity . 0.1 ml of the activated bacteria was spreaded on Muller Hinton agar and left 2 hrs at 4ºC . Then 0.05 ml of galls extract was added to well (6 mm diameter) , left to diffuse and incubated at 37 ºC for 24 hrs .

Determination of minimium inhibitory concentration (MIC):

S. aureus and *P. auroginosa* were chosen to determine (MIC) by using the following concentrations of acetonic galls extract: 25, 20, 15, 10, 7, 5, 3 and 1 mg/ml.

Antifungal activity:

Trichophyton mentagrophytes was obtained from department of biology, college of science, university of Karbala. The fungus was activated on Sabouraud's dextrose agar at 28 ºC for 10 days. Agar dilution method (11) was used to study the effect of aqueous, ethanolic and acetonic galls extracts on *T. mentagrophytes* growth at concentrations 10, 8 and 5 mg/ml. The fungus was then grown at 28 ºC for 2 weeks. Two types of controls were included (positive by using clotrimazole and negative without any antifungal).

Partial purification of tannins from *Q. infectoria* galls extract:

Two indicators were used to follow purification steps of tannins. Firstly, by testing antibacterial activity against *S. aureus* and secondly, by the quantitative determination of tannins using Folin-Dennis method with some modification (1). Purification steps were as follows:

- **Extraction with acetone 70 %:** This was achieved by adding 20 ml of acetone 70% to 1 gm of galls powder. Extraction conditions were as described above. The mixture was filtered and the filtrate was dried to be ready to the subsequent purification step.
- **Extraction with ethanol 95%:** The dried acetone extract obtained from the first step was extracted with 3 volumes of ethanol 95%.
- **Adsorption chromatography on Sephadex LH-20:** The adsorption step was carried out according to the method described in (12) which was successful in separating tannins from non tanninc phenols. A coloum of sephadex LH-20 (8X3.6 cm) was prepared in ethanol 95%. After addition of sample to the top of the coloum, the purification step was achieved by two stages: firstly, wash, the coloum was washed with ethanol 95% and the unadsorbed compounds were collected in fractions. This could be confirmed when absorbance at 280 nm approaches to zero. Secondly, Elution, the adsorbed compounds which consist tannins were eluted by solvent changing, acetone 70% was used for this purpose.

Separation of tannins by thin layer chromatography (TLC):

Silica gel plates (20X20 cm) were used in the present study. Tannins solution which was obtained from the adsorption step, tannic acid and gallic acid were injected on silica gel plates using the separation mixture tolune-acetone-formic acid, 60: 60: 10 (v/v/v) (12).
Plates were sprayed with spray mixture described by (12). Finaly, Rf was calculated to the formed spots.

Results and Discussion

Tannins Extraction:

It seemed that extraction with acetone gave the highest percentage of extractable compounds (70%) in comparison with other methods used in this study, while extraction with cold water gave the minimal percentage (47.4 %) (Table 1).

Results indicate that organic solvents are more efficient in extraction than water. It has shown also the role of temperature in the extraction, since using boiled water gave 67.9% in comparison with hot and cold water which were (61 and 47.4%), respectively (table 1). According to these results extraction with acetone 70% was chosen as the best one.

Biochemical Detection:

The galls extract gave positive reactions with ferric chloride and lead acetate indicating the presence of tannins. This result was in agreement with previous studies which ensured that galls are good source of tannic acid (13), for this reason, tannins received our attention and occupied the main line in our study. Results showed also positive reactions with Fehling indicating the presence of glycosides, while it showed negative reactions with Drangendroff and Liberman-Berchard indicating the absence of alkaloids and essential oils, respectively.

The aqueous galls extract was acidic where pH value was 4.2.

Most plants contain many active drug compounds and the detection of these compounds lead to the possibility of utilizing them in controlling the human and animal pathogens caused by bacteria, fungi and viruses (14).

Biological activity

Antibacterial activity

Results summarized in Table (2) showed that the galls extract was efficient in inhibiting the growth of G^{+ve} than G^{-ve} bacteria. It has also shown that ethanolic extract 70% was most effective on *S. aureus* with an inhibition zone (16 mm) . This may be due to the ethanol ability to extract both water soluble and alcohol soluble compounds (15). These results were in agreement with the result which has been reported that alcoholic extract of *Q. persica* bark was active against *S. aureus* with an antimicrobial equivalence (6.6 µg/ml) (16).

Acetonic and boiled water extracts were similar in their effect on *S. pyogens* with an inhibition zone (15.7 mm) in comparison with gentamicin (18 mm), while acetone extract showed the best effect on *P. auroginosa* (15 mm) . None of the five extracts exhibits any inhibition to *E. coli*. This may be due to the nature of the outer cell wall of G^{-ve} that contains autobarrier (lipopolysaccharide) combined with multiple proteins which prevent many harmful materials to enter the cell, as well as eliminating these materials out of the cell before they reach the level which enable them to inhibit protein synthesis (17).

No inhibition to *E. coli* was observed by using aqueous and alcoholic extracts of *Q. persica* bark (16).

The minimal inhibitory concentration (MIC) for the acetonic extract on *S. aureus* and *P. auroginosa* was studied. Table (3) illustrates the results of this study.

It has shown that antibacterial activity decreases with the decrease in the galls extract concentration. Maximum concentration used in this study inhibited *S. aureus* and *P. auroginosa* with 17 and 13.5 mm, respectively. No inhibition has been noticed at concentrations 3 mg/ml for *S. aureus* and 7 mg/ml for *P. auroginosa.* Therefore, it can be concluded that 5 and 10 mg/ml was the (MIC) for *S. aureus* and *P. auroginosa,* respectively.

Antifungal activity:

Antifungal activity of ethanolic, acetonic and aqueous galls extracts are illustrated in Table (4).

Acetonic galls extract gave highest inhibition percentage (100%) in all concentrations used in this study in comparison with clotrimazole (standard). Although ethanolic and aqueous extracts gave complete inhibition (100%) at concentrations 10 and 8 mg/ml, they gave less inhibition percentage at 5 mg/ml which were 95.9 and 94.5% for ethanolic and aqueous extracts, respectively. Galls extracts antifungal activity may be due to the presence of active compounds such as tannins and glycosides that could inhibit enzymes and carrier proteins located in the cell membrane (18). One of the previous studies mentioned that the isolated tannins from most medical plants have anti-dermatophytic activity (19). *T. mentagrophytes* (used in this study) was also inhibited by *Myrtus communis* L. extracts (20).

Partial purification of tannins from galls extract:

Purification procedure of tannins from galls extracts is summarized in scheme (1). Because extraction with acetone 70% gave the best results related to the extractable compounds percentage and biological activity, it was chosen to be the first step in tannins purification. The extract obtained from this step was called crude acetonic extract. This extract exhibited antibacterial activity against *S. aureus*, as well as gave positive reactions with Folin-Dennis, a test used for the quantitative determination of tannins. Yield obtained from this purification step was 70%. Extraction is the most common methods in materials purification (21).

Dissolving the crude acetonic extract in ethanol and centrifuging resulted in a good purification step by removing the precipitated compounds, while filtrate which contains tannins exhibited antibacterial activity against *S. aureus* and positive reactions with Folin-Dennis. This step was necessary to make the extract ready to the subsequent purification step. Yield obtained was 19.6%.

Generally, extraction with solvent is a fast method, this property made it very useful especially when the extracted compounds are unstable (22).

Sephadex LH-20 sorbs tannins in alcohol, and releases them in aqueous acetone (12), for this reason, this type of Sephadex was used in the present study. The first stage in this purification step (wash) was important to remove all unadsorbed compounds. This was confirmed by monitoring absorbance at 280 nm until no longer changing and is near base line. Then the ethanol washes were discarded. Wash was followed by eluting the coloum with acetone 70% . After collecting 40 fractions
(3 ml to each fraction) eluting was stopped. Results revealed that there was an antibacterial activity against *S. aureus* in the fractions 8-28 accompanied with positive reactions with Folin-Dennis in the same fractions (figure 1). It has been noticed that increasing in tannin concentration was paralleled with the increasing in the inhibition zone. This purification step resulted in highly purified galls extract with yield 14.7%.

Tannins separation by TLC

TLC was carried out to detect whether the tannins obtained from the last step of purification return to tannic or gallic acid. Rf values for tannins, tannic acid and gallic acid were 0.07, 0.06 and 0.28 , respectively, indicating that tannins separated in this study may be tannic acid. This result ensured previous study in which reported that *Q. infectoria* galls are good source of tannic acid (13).

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Table (1): Extractable compounds percentage from galls extracts.

Extraction method	Dry weight %
Ethanol 70%	65.5
Acetone70%	70
Cold water	47.4
Hot water at 95 º C	61
Boiled water	67.9

Table (2): Effect of *Q. infectoria* galls extract on some isolated skin pathogenic bacteria.

Extraction method	Inhibition zone (mm)			
	S. aureus	*S. pyogens*	*E. coli*	*P. auroginosa*
Ethanol 70%	16 ± 0.5	15.4 ± 0.6	0.0	13 ± 1.0
Acetone70%	12.8 ± 1.0	15.7 ± 1.2	0.0	15 ± 0.0
Cold water	12.2 ± 1.6	14.7 ± 1.2	0.0	13.3 ± 2.1
Hot water at 95 º C	12 ± 1.3	15.2 ± 2.4	0.0	12.7 ± 1.6
Boiled water	11.7 ± 1.6	15.7 ± 4.2	0.0	12.3 ± 3.1
Gentamicin (10 µg/ml)	20 ± 0.5	18 ± 1.7	12 ± 1.7	18 ± 0.9

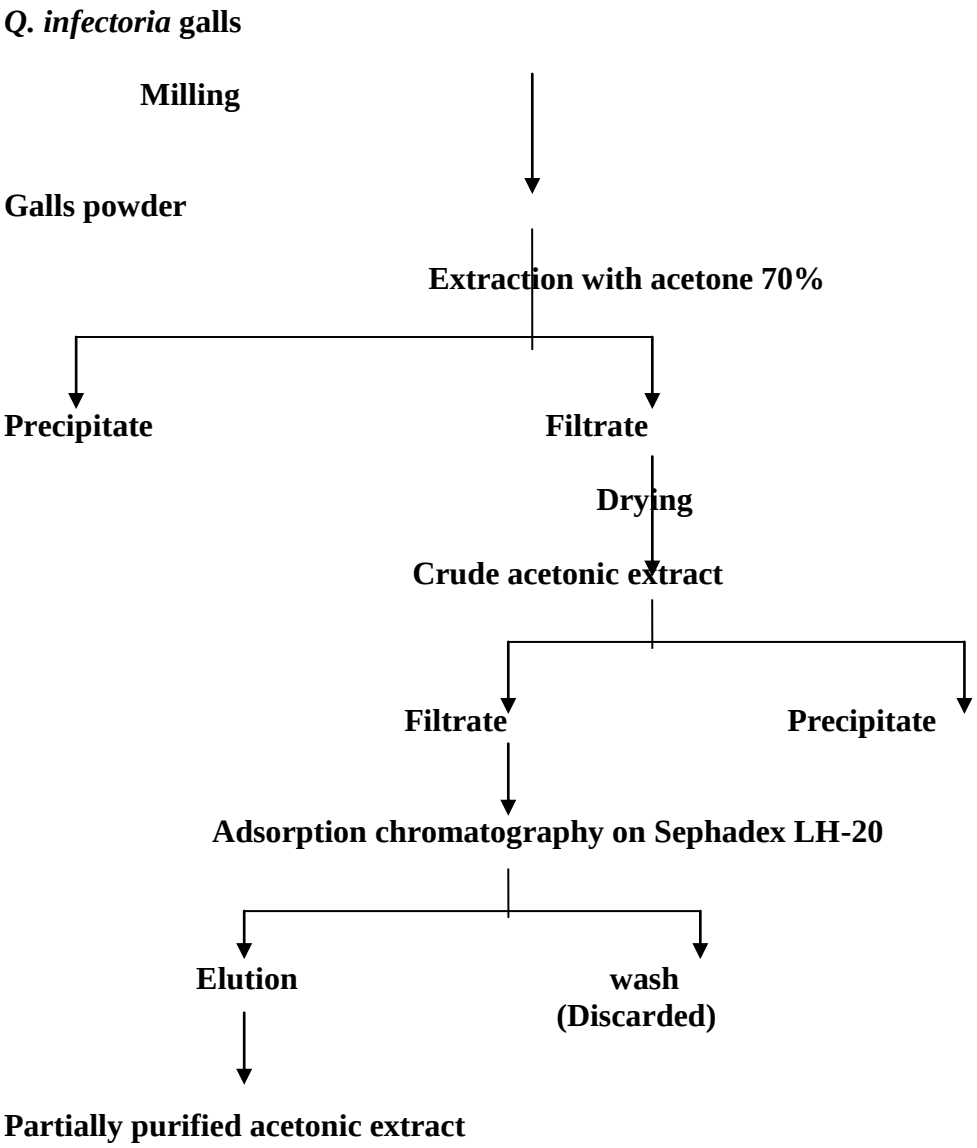
Table (3): Acetonic galls extract MIC for *S. aureus* and *P aurogenosa*.

Acetonic extract (mg/ml)	Inhibition zone (mm)	
	S. aureus	*P. aurogenosa*
25	17 ± 0.8	13.5 ± 0.7
20	15.3 ± 0.6	13.0 ± 1.4
15	14.7 ± 0.6	11.0 ± 0.8
10	12.3 ± 0.5	7.5 ± 0.0
7	11.2 ± 0.5	0.0
5	8.3 ± 0.5	0.0
3	0.0	0.0
1	0.0	0.0

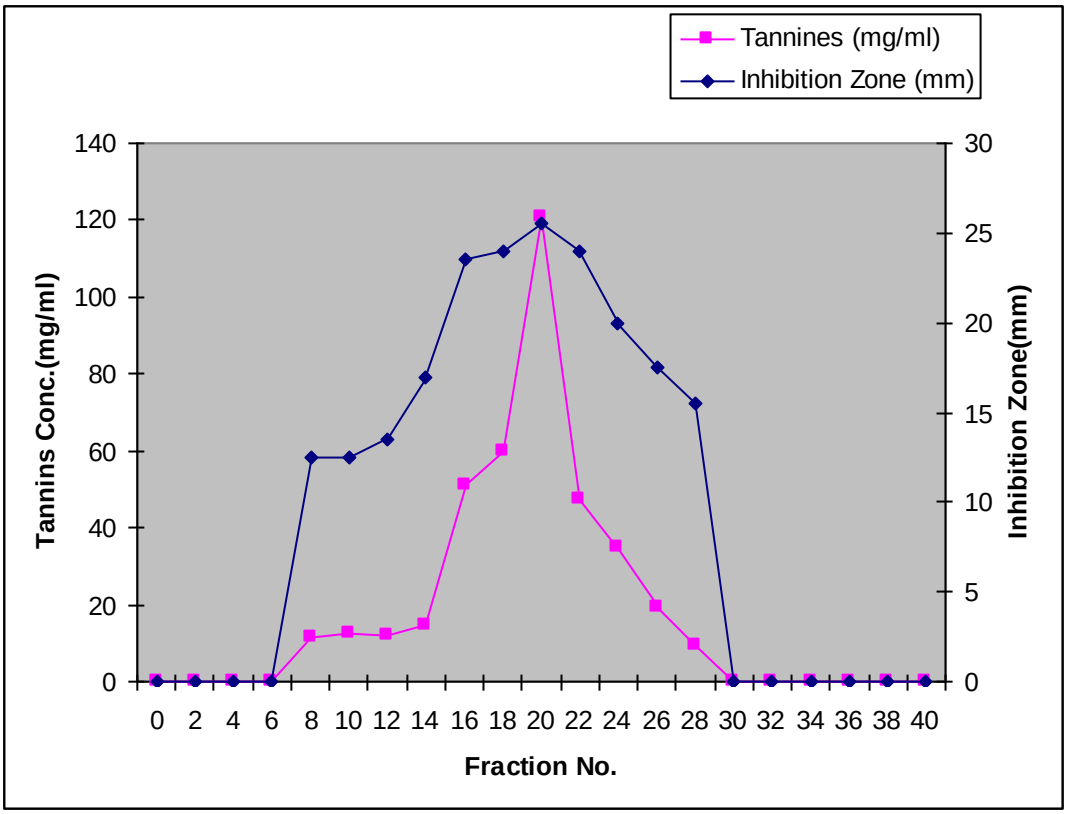
Table (4): Effect of ethanolic, acetonic and aqueous extracts on *T. mentagrophytes* growth.

Acetonic galls extract (mg/ml)	Fungus inhibition %		
	Ethanolic extract	Acetonic extract	Aqueous extract
10	100	100	100
8	100	100	100
5	95.9	100	94.5

Clotrimazole (2 mg/ml) 100%



Scheme (1): Purification procedure for tannins from *Q. infectoria* galls.



Figer (1) : Adsorption chromatography of tannins from *Quercus infectoria* galls on sephadex LH-20 . Elution was carried out with acetone 70% at a flow rate (0.8ml/min.) and a fraction volume (3ml).