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Bacteriological and Biochemical study for effect of phenolic extract of *Quercus* infectoria against some food-born pathogenic bacteria

KEYWORDS: Antibacterial, Phenolic extract, Quercus infectoria, HPLC, LD50

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ABSTRACT *Qualitative and quantitative detection of the active compounds in the phenolic extract of Quercus infectoria was conducted by using HPLC. Results showed it contains all of the following compound Tannic acid ،Isoquercetin ، Quercetin، Ferulic acid ، Rutin ، Coumaric acid ، Kaempferol ، Vanillic acid ، Sinapic acid ، Genstic acid) (.Inhibitory effectiveness was evaluated for different concentration of phenolic extract of Quercus infectoria include (0.039, 0.0781, o.156,0.312, 0.625,1.25,2.5,5,10 and 20) mg/ml separately against gram positive bacteria Bacillus subltilis and Staphylococcus aureus and three isolates of gram negative bacteria E.coli , Pseudomonas aeruginosa and Salmonella typhimurium by Monitoring this activity in the broth using absorbance measurements The results showed high inhibition effect against all bacterial isolates with the effect of concentration and genus of bacteria. The result of an acute toxicity on administration of the extract by oral route for 24 hours showed that the LD50 calculated for the extract was greater than 500 mg / kg.*

INTRODUCTION

Consumers nowadays demand minimally preserved foods for maximum nutrient retention, without the addition of chemical preservatives. On the other hand, foods need to be safe, with prolonged shelf-life **(1)**. An increasing interest in the use of natural antimicrobials as food preservatives has been recorded. Natural preparations from plants which contain phenolic compounds exhibit antimicrobial activity. Numerous extracts from plants have been tested for their antimicrobial properties against various food-borne microorganisms .Antimicrobials from plants can be used as an alternative to chemical preservatives in order to satisfy consumers 'demand for safe, convenient and wholesome food **(2)**.

Quercus infectoria Oliv (Family Fagaceae) is a small tree or shrub about 2 M high, with many spreading branches. The bark is slightly grey in color. The galls are collected for medicinal use before the escape of the insect and well dried. The surface of mature dry gall may be smooth and shining, as though varnished and chestnut brown, but more usually it is rough and of a greyish brown in color. When the galls are gathered at the correct stage, i.e. before the insect emerges, the inner tissue is soft, of a deep greenish yellow color, with a very astringent taste and slightly sweet aftertaste **(3).** The galls of *Qurercus infectoria* have also been pharmacologically documented to possess astringent, antibacterial, antifungal, larvicidal, antidiabetic,local anaesthetic,antiviral, and anti- inflammatory **(4).** In addition, *Q. infectoria* gall extract have potential anti-ulcer activity **(5)**. So the researchers gave great attention to the therapeutic use Pharmaceuticals extracted from medicinal herbs to several factors, including the efficiency, safety and economic feasibility.

MATERIAL AND METHOD

Preparation of phenolic extract of galls of *Quercus infectoria*

The plant of Quercus infectoria were purchase from the local market of Baghdad . The plant was grind or crushed to power (10g) which was used for phenols extract with acetic acid (40ml) using reflex condenser below 80C° for 8hours and let solution to cooled. Solution was separated by centrifugation and put below part in separation funnel and equilibrium of n-propane and NaCl for were added to saturation, this revealed two layers which contain phenolic compounds which was concentrated under reduced pressure by a rotary evaporation below 40 C° (6). A stock solution was prepared by dissolving 5 grams of the extract in 100 ml of dimethyl sulphoxide (DMSO) to obtain a final concentration of 50 mg/ml.

Test for phenols

Two (2) ml extract were taken into water and warmed at 45-50 C°. Then 2 ml of 3% FeCl3 was added. Formation of green or blue color will indicate the presence of phenols**(7).**

Sample preparation for HPLC analysis

10 g of sample was weighed, and then dissolved in 10ml HPLC methanol, the sample was shaking and agitated in ultrasonic bath for 10 minutes, then concentration by evaporating the solvent with a stream of liquid N2 until reach nearly 0.5 ml, then add some mobile phase to reach 1 ml. Then 20µl were injected on HPLC column. The concentration for each compound were quantitatively determined by comparison the peak area of the standard with that of the samples. The phenolic compounds separated on fast liquid chromatographic under optimum condition. Column:3 µm particle size (50 X 4.6 mm I.D) C-18 column, mobile phase: phosphate buffer 0.01%: acetic acid : methanol:water (0.01:40:60).Flow rate 1.4 ml/min, detection UV set at 264 nm.

Microorganisms

The bacterial isolates used in this study were *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Salmonella typhimurium and Bacillus subtilis* .All the bacterial isolates were grown and maintained on nutrient agar slants. Each bacterial strain was suspended in Mueller Hinton broth and incubated at 37C for 18 h. Mueller Hinton agar was used for testing antibacterial activity.

Determination of minimum inhibitory concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that inhibits the growth of a microorganism after 18-24 h. The extracts that showed antibacterial activity were subjected to the serial broth dilution technique to determine their minimum inhibitory concentration. Briefly, the stock solutions of the extracts were subjected to two-fold serial dilution in the Muller-Hinton broth to obtain concentrations from 0.039 mg/ml to 20 mg/ml (8). Standard DMSO was placed as controls. A 10 µl of 10 8 (Cfu/ml) bacterial cultures were added to the tubes and were incubated at 37° C for 18 h. the absorbance of broth suspension was measured at 620 nm with spectrophotometer. The minimum concentration of the extracts that showed no detectable

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growth was taken as the minimum inhibitory concentration.

Acute toxicity of extracts
The method adapted from **(9)** was used for acute toxicity assessment on both sex mice NMRI, weighing 35 ± 5.6 g. During the test, they were handled humanely according to the international ethical committee on animal handling. Five groups receiving five different concentrations (2000, 1000, 500, 250 and 125 mg / kg body weight) and one untreated control group that received distilled water. For this, animals of each group were receiving a specific dose of the extract to be tested and distilled water by oral route using intragastric syringe. Then, all mice were observed systematically during 72 hours ,the number of mice died was recorded and used in the calculation of the acute toxicity value (LD50).

Statistical analysis
Data obtained in the study were statistically analyzed using Analysis of Variance (ANOVA) and means were separated using Fisher's Least Significant Difference (LSD) at both 1 and 5% levels of significance **(10).**

RESULT AND DISCUSSION
The use of medicinal plants plays a vital role in covering the basic health needs in developing countries and these plants may offer a new source of antibacterial agents with significant potential activity against infective microorganism. Recently, much attention has been directed toward isolation of active compounds with biological response available in plant. Many reports of natural antimicrobial agent of plant origin have been published and their importance in health, food and preventive medicine has been well documented.

The existing antibiotics are become resistant infectious disease and urgent need is to diverse new antimicrobial compound with diverse chemical structure and novel mechanism of action of newly diagnosed and re-emerging infectious disease. The present study deals with extraction, and antibacterial activity of phenolic extraction of *Qurercus infectoria*. The results detection using *high- -performance liquid chromatography* that the retention time standard for each compound (Tannic acid ،Isoquercetin ، Quercetin، Ferulic acid ، Rutin ، Coumaric acid ، Kaempferol ، Vanillic acid ، Sinapic acid ، Genstic acid) was (0.84 ،2.37، 3.25، 4.10 ، 5.18 ، 6.18 ، 7.09، 8.01، 8.93 ، 9.85) minute respectively Table (1).The Fig. (1) includes the total components phenolic extract that appears contains all of (Tannic acid ،Isoquercetin ، Quercetin، Ferulic acid ، Rutin ، Coumaric acid ، Kaempferol ، Vanillic acid ، Sinapic acid ، Genstic acid) compared with standard compounds Fig(2). Tannic acid has the highest concentration followed by Isoquercetin and quercetin (863.776, 25.864, 47.012) mg/ml, respectively Table (2). The concentration of other phenolic compounds ranged from 19.757 to 1.151 mg/ml. Genstic acid was the lowest phenol compound 1.151 mg/ml.

These components could be responsible for the antibacterial activity .High amounts of tannin present in the galls of *Q. infectoria* at 52.85% this accords with brought by **(4)** confirmed it contains tannin (50-70) %. **(11)** reported that the Polyphenols such as tannic acid and quercetin, present in *Q. infectoria*. As well as it contains p-coumarin, vanillic acid and kaempferol **(12**).The phenolic extracts of *Q. infectoria* showed inhibitory activity against all the five food associated bacteria in which showed the result of MIC of the phenolic extract from the gall of *Q. infectoria* against *B. subltilis* and *Staph. aureus* which were 2.5-5 mg/ml ,respectively Table (3) while MIC for, *E.coli* , *Ps. aeruginosa* and *S. typhimurium* was 10 mg/ml(Fig. 3).

TABLE-1
Retention time record for standard compound

Compound	Retention time (minute)	Area of standard
Tannic acid	0.84	20201
Isoquercetin	2.37	83974
Quercetin	3.25	36150
Ferulic acid	4.10	69583
Rutin	5.18	29684
Coumaric acid	6.18	19118
Kaempferol	7.09	51929
Vanillic acid	8.01	59547
Sinapic acid	8.93	65448
Genstic acid	9.85	69241

TABLE -2

Compound	Concentration (ppm)	Percentage %
Tannic acid	863775.93	52.85
Isoquercetin	25863.96	6.58
Quercetin	47012.10	5.15
Ferulic acid	19756.80	4.16
Rutin	45235.65	4.07
Coumaric acid	69231.88	4.00
Kaempferol	19663.87	3.09
Vanillinc acid	16990.36	3.06
Sinapic acid	9802.82	1.94
Genstic acid	1150.87	0.24

Concentrations and percentages the phenolic compounds in the extract of galls of *Quercus infectoria*

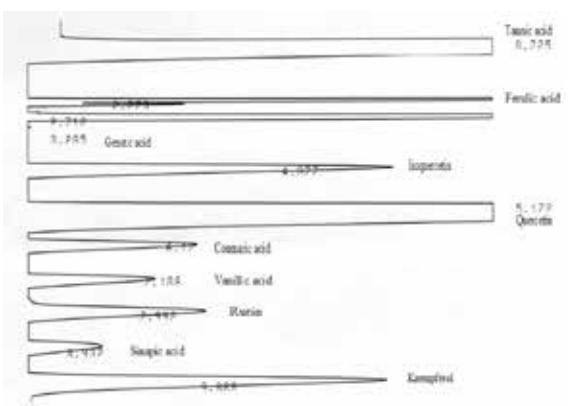

Figure 1: chromatography for the components of the total phenolic extract of f *Quercus infectoria* **using HPLC technique at wave length 264 nanometer.**

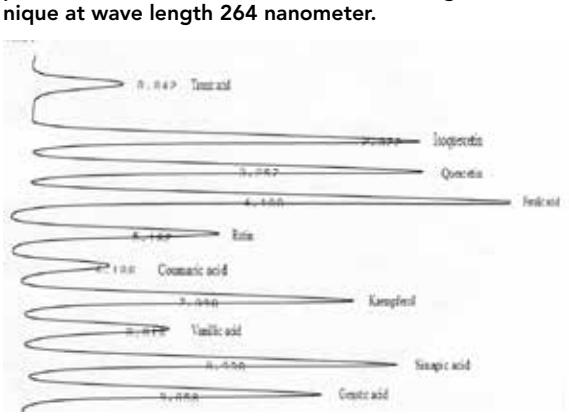

Figure 2: chromatography for the standard compounds

The results indicated that the tested phenolic extracts showed antibacterial activity towards the Gram-positive bacteria and

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Gram-negative. **(13)** reported that *Q. infectoria* galls possess antibacterial activity against *E.coli*, *Staph. aureus* and *B. subtilis*. In addition, **(14)** showed that galls had higher antimicrobial activity against methicillin resistant *Staph. aureus*. **(15)** recorded activity of ethanolic extracts of galls against *E. coli* where MIC was 1200 µg/ml. These values are less than those recorded in the current study. **(16)** recorded the MIC values of Q. infectoria extract against *Staph. aureus* to be 0.41 mg/ml and 6.25 mg/ml for *ps. aueroginosa*. The Fig. (4) Show The maximum percentage inhibition, 100% was observed at 10 and 20 mg/ml of extract concentration.

Results of this study demonstrated that the Gram- positive bacteria were more susceptible to the extracts than Gram-negative bacteria such as *E.coli* exhibited more resistant than *S. aureus and B. subtilis* when they were tested with *Q. infectoria* extract. The reason would be that lipopolysaccharide (LPS) layer of Gram-negative bacteria in outer membrane having high hydrophobicity and acts as a strong barrier against hydrophobic molecules. It can pass through cell wall of Gram-positive bacteria easier than the Gram-negative bacteria because cell wall of the Gram- positive contained peptidoglycan and lack of outer membrane **(17)**. The extract may interfere with staphylococcal enzymes **(18).** High amounts of tannin present in the galls of *Q. infectoria* implied that tannin is the active compound for the antibacterial activity in this study. Tannins are a group of polymeric phenolic substances characterized by antibacterial activity owing to inactivation of bacterial adhesions, cell envelope and transport proteins **(19).** In addition, tannin is potent inhibitors of microbial enzymes like protease **(20)**. Other studies showed that tannin inhibits the growth of both *E. coli* and *S. aureus* and has been attributed to a similar inhibitory action of the mechanism of tannin binding with the protein of the bacterial cell walls **(21).** On the other hand, the *Q. infectoria* have quercetin (**11**) which has **activity** against microbial **(22)**. **(23)** Showed that the quercetin markedly enhanced antibacterial activity against MRSA. The activity of quercetin has been at least partially attributed to the inhibition of DNA gyrase **(19).** Numerous studies have shown that kaempferol and some glycosides of kaempferol have anti-inflammatory and antimicrobial activity.**. (24)** recorded inhibitory activities of these polyphenols such as quercetin، vanillic acid ،ferulic acid and coumaric acid against all important pathogens including *E. coli Staph. aureus* and *ps. aeruginosa* and others.Use method logarithmic in determining the lethal dose of phenolic extract in laboratory animals using doses different of it where the results showed in Figure (4) and Table (4) that the dosage phenolic extract for laboratory animals by 0.125g/kg There has been no mortality either dose of 0.25g/kg has reached the mortality rate 12.5% and the percentage increased to reach 50% and 78.5% when animals that have been dosage by 0.5g/kg and 1g/kg, respectively, therefore the LD50 of this extract is greater than 500 mg / kg These results are comparable to those of **(25)** who obtained an LD50 of 0.75 g / kg with aqueous extract of *Q. infectoria* in mice by e subcutaneous administration.

TABLE- 3-
Antibacterial activity of *Quercus infectoria* **extract by two fold serial dilution method**

Concentration (mg / ml)	Bacterial species				
	Staph. aureus	E. coli	B. subtilis	S. typhimurium	Ps. aeruginosa
	Absorption spectrum at the wavelength of 260 nm ± standard error				
Control	b0.574± 0.013	a0.632± 0.014	c0.491± 0.014	c0.495± 0.012	b0.586± 0.013
0.039	b0.502± 0.023	a0.622± 0.028	c0.488± 0.012	d0.455± 0.016	bc0.497± 0.012
0.0781	b0.444± 0.024	a0.528± 0.026	c0.381± 0.012	c0.394± 0.012	b0.457± 0.015
0.156	b0.405± 0.013	a0.448± 0.023	c0.300± 0.014	c0.356± 0.012	b0.417± 0.021
0.312	b0.317± 0.032	a0.408± 0.023	d0.235± 0.011	c0.289± 0.013	b0.344± 0.023
0.625	b0.294± 0.021	a0.348± 0.012	d0.198± 0.014	c0.238± 0.012	c0.254± 0.025
1.25	c0.112± 0.031	a0.246± 0.011	d0.023± 0.021	b0.189± 0.032	a0.234± 0.021
2.5	c0.036± 0.021	a0.145± 0.027	d0.000± 0.00	ab0.132± 0.015	b0.101± 0.013
5	c0.000± 0.0	ab0.022± 0.012	c0.000± 0.00	a0.023± 0.012	b0.020± 0.013
10	0.000± 0.0	0.000± 0.0	0.000± 0.00	0.000± 0.0	0.000± 0.0
20	0.000± 0.0	0.000± 0.0	0.000± 0.00	0.000± 0.0	0.000± 0.0

- Different letters mean a significant difference ($p < 0.05$) for the comparison between the columns
- Results represent the rates for five replicates ± standard error

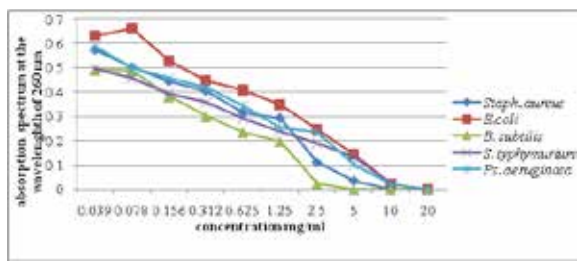

Figure 3: Effect of phenolic extract of the *Quercus infectoria* **on the growth food-born pathogenic bacteria**

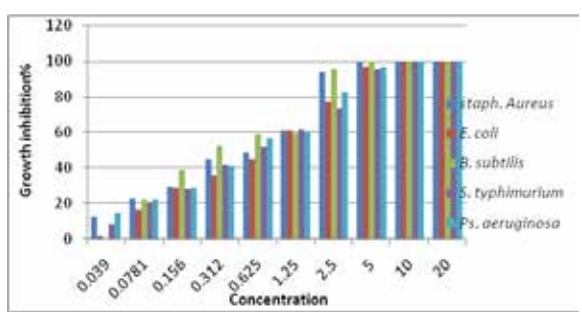

Figure (4): Inhibitory effect of phenolic extract of galls of *Quercus infectoria* **against food-born pathogenic bacteria**

TABLE-4
Mortality rate of *Quercus infectoria* **extract**

Quercus infectoria Dose gm/kg	Number of mice	Number of life mice	Number of dead mice	Accumulated No.		Total No.	Percent of mortality %
				life	dead		
2	8	0	8	0	20	20	100
1	8	1	7	1	12	13	78.50
0.5	8	4	4	5	5	10	50.00
0.25	8	7	1	12	1	13	12.50
0.125	8	8	0	20	0	20	0.00

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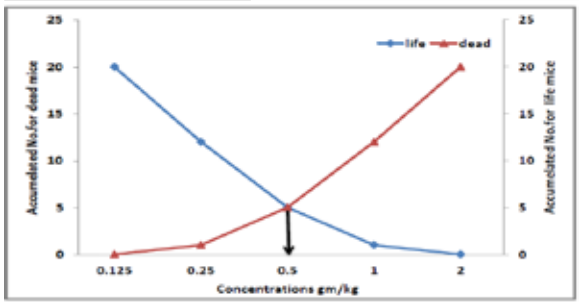

Figure 5: Calculation of the LD50 of Extract of *Q. infectoria*

CASE STUDY

The study aimed to investigate the inhibitory effectiveness of phenolic extracts of *Quercus infectoria* on the growth of some food-born pathogenic bacteria, and detection of the active compounds in the phenolic extract of *Quercus infectoria***.** In addition to calculation of the acute toxicity value (LD50).

CONCLUSIONS

The results of present study supports the traditional usage of plant and *Q. infectoria* plant extracts which posses compounds with antibacterial properties. Activity that the extract could be used as food additives to certain foods to reduce or eliminate food-borne bacterial pathogens and food spoilage bacteria.

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