Research Paper Biology

Identification of quantative chemical compounds of ethanolic extracts of *Quercus infectoria* and studies its inhibitory effect in some bacteria

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ABSTRACT

Phytochemical analysis of the extracts revealed the presence of antimicrobial active agents such as alkaloids, phenols, glycosides, flavonoids resins, saponins, and tannins and then were subjected to chemical compound analysis through qualitative High Performance Liquid Chromatography (HPLC) profiling, bioactivity property. The results from HPLC crude extract profile for gall of Quercus infectoria showed many peaks at the retention time between 0 to 8 minutes. HPLC analysis indicated presence of tannic acid, gallic acid, caffiec acid, chlorogenicn acid and cinnamic acid. The two types of bioactivity study are antibacterial and cytotoxicity assay. In antibacterial assay, five pathogenic bacteria, were selected and tested by using agar well diffusion method. The results showed that the ethanolic extracts at different concentrations inhibited the growth of all isolated bacteria. The concentration of 20mg/ml inhibited the isolate with highest diameter zone of inhibition ranging from 15mm to 28mm.. Cellular toxicity of plant extract was determined and was found have no a toxic effect against red blood cells of sheeps.

Keywords : Antibacterial, Ethanolic extract, *Quercus infectoria*, HPLC

INTRODUCTION

The most common bacteria causing food-borne illness are *Escherichia coli, Staphylococcus aureus, Salmonella sp., Salmonella typhimurium, Listeria monocytogenes, Clostridium botulinum, Vibrio vulnificus, Vibrio parahaemolyticus* and others **(1).**Now microorganisms have become resistance to many antibiotics due to increased use of drugs, which is decreasing efficiency of conventional medicines. So, it has become necessary to find out new antimicrobial agents. Recently, medicinal plants and their extracts have gained importance as potential antibacterial agents. Secondary metabolites of plants including tannins, flavonoids and alkaloids have been found to possess antimicrobial properties in vitro **(2(.**Therefore study aimed to investigate the inhibitory effectiveness of ethanolic extracts of *Quercus infectoria* on the growth of some food-born pathogenic bacteria. *Quercus infectoria* Oliv (Family Fagaceae) are an out growths formed on the young twigs of the dyer's oak, *Quercus infectoria* , as a result of the deposition of the eggs of the gall-wasp *Adleria gallae tinctoriae*Olivier. The galls are globular in shape and from 10 to 25 mm in diameter. They have a short, basal stalk and numerous rounded projections on the surface. The galls are collected for medicinal use before the escape of the insect and well dried. Keeping in view the medicinal important of the drug in Unani Medicine, an attempt has been made to review the available literature on its ethno-botany, traditional uses and pharmacological properties **(3).**

The plant tannins major source of tannic acid, which is used as an astringent, antibacterial(4), antifungal, antiviral, and anti- inflammatory. 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. Therefore study aimed to investigate the inhibitory effectiveness of ethanolic extracts of Quercus infectoria on the growth of some food-born pathogenic bacteria.

MATERIAL AND METHOD

Preparation of ethanolic extract of galls of Quercu infectoria

Dried galls of *Quercus infectoria* were purchased from the local market and identified by a botanist based on its physical characteristics. About 100 grams of the galls were crushed into small pieces in a mortar and pestle and then powdered in an electric grinder. Fifty grams of the gall powder was accurately weighed in an electronic balance, packed into a Soxhlet apparatus and extracted exhaustively using ethanol as the solvent. The obtained crude extract was evaporated at room temperature to get the dried residue of ethanolic extract of galls of *Quercus infectoria.* It was stored in a sterile bottle and preserved in desiccator until further use. The solvent was then distilled under reduced pressure in a rotary evaporator until it became completely dry. The weight of the solid residue was recorded and taken as yield of crude extracts. 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.

Microorganism

The test microorganisms used were *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. All of the test bacteria isolates was suspended in Mueller Hinton broth and incubated at 37C for 18 h. Mueller Hinton agar were used for testing antibacterial activity.

Phytochemical analysis of plant extracts

Ethanolic extract of galls of *Quercus infectoria* was subjected to preliminary phytochemical screening for the detection of various plant constituents **(6)**.

Test for Alkaloids

Wagner's test: A fraction of extract was treated with Wagner's test reagent (1.27 g of iodine and 2 g of potassium iodide in

100 ml of water) and observed for the formation of reddish brown color precipitate.

Test for Flavonoids
NaOH test: A small amount of extract was treated with aqueous NaOH and HCl, observed for the formation of yellow orange color.

H2SO4 test: A fraction of extract was treated with concentrated H2SO4 and observed for the formation of orange color.

Test for Tannins
Braymer's test: Few ml of extract was treated with 10% alcoholic ferric chloride solution and observed for formation of blue or greenish colour solution.

Test for Saponins
Foam test: A small amount of extract was shaken with water and observed for the formation of persistent foam.

Test for Phenols
Ferric chloride test: The fraction of extract was treated with 5 % ferric chloride and observed for formation of deep blue or black color.

Test for Glycosides
The glycosides content of the extracts was determine by dissolving 10.0g of the extracts in 100ml of 50% H2SO4 in test tubes. The mixture was heated in boiling water for 15minutes, and 10ml of Fehling solution added, and the mixture boiled. A red precipitate in each extract tested, indicated the presence of glycosides.

Test for Resins
Two grams of the ethanolic extract was dissolved in 10ml of acetic anhydride. A drop of concentrated sulphuric acid was added. Appearance of purple color, which rapidly changed to violet, was indicative of the presence of resins.

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 N_2 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.

Screening of antibacterial activity by agar well diffusion
Suspension of micro-organisms was made in sterile normal saline and adjusted to 0.5 Macfarland standard (10^8 Cfu/ml) ،from the stock of 50 mg/ml extract, serial dilutions were made to (0.039, 0.0781, o.156,0.312, 0.625,1.25,2.5,5,10 and 20) mg/ml **(7)**. Each labeled medium plate was uniformly inoculated with a test organism by using a sterile cotton swab rolled in the suspension to streak the plate surface in a form that lawn growth can be observed. A sterile cork borer of 5mm diameter was used to make wells on the medium. 0.1ml of the various extract concentration were dropped into each, appropriate labeled well **(8).** The Mueller Hinton Agar plates were incubated at 37° C for 24 hours. Antimicrobial activity was determined by measuring the diameter of inhibition zones (mm) formed after incubation. 20 µl of DMSO were placed as controls **(7)**. All the tests were performed in triplicate.

Determination of cellular toxicity using sheep erythrocytes
The method described by **(9)** was employed to study cellular toxicity. Briefly, 10-fold serial dilutions of the extract were made in phosphate buffered saline. A total volume of 0.8 ml for each dilution was placed in an eppendorf tube. A negative control tube (containing saline only) and a positive control tube (containing saponin, 5 mg/ml) were also included in the analysis. Fresh sheep erythrocytes were added to each tube, to give a final volume of 1 ml. Solutions were incubated at 37 C° for 30 min and all tubes were centrifuged for 5 min and then observed for haemolysis. Complete haemolysis was indicated by a clear red solution without any deposit of erythrocytes. Haemolysis was also checked microscopically and presence or absence of intact RBCs.

RESULT
There has been an increasing consumer demand for foods free or with low, if any, added synthetic preservatives because synthetic preservatives could be toxic to humans **(10**). Concomitantly, consumers have also demanded for wholesome and safe food with long shelf lives. These requirements are often contradictory and have put pressure on the food industry for progressive removal of chemical preservatives and adoption of natural alternatives to obtain its goals concerning safe food with long shelf lives **(11).**The growing concern about food safety has recently led to the development of natural antimicrobials to control food borne pathogens and spoilage bacteria. Therefor the aim of this study is to evaluate the antibacterial efficacy of galls of *Quercus infectoria* extract.

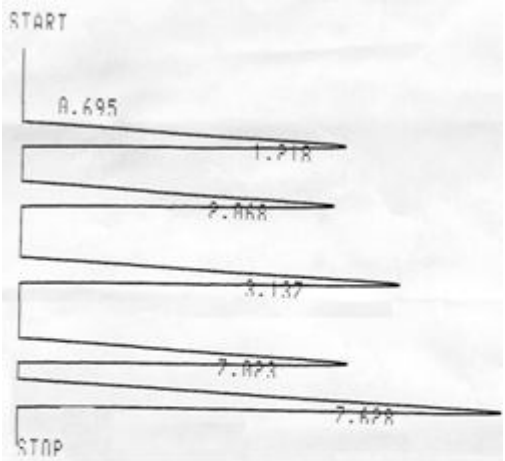
Phytochemical screening of ethanolic extract yielded positive results for tannins, phenols,saponins ,resins, glycosides and flavonoids (Table1). The same compounds were extracted by **(12).** Active compounds present in the crude ethanol extracts show the antibacterial activity with the dose dependant manner .The mechanisms thought to be responsible for phenolic toxicity to microorganism

TABLE-1-Phytochemical screening of Q.infectoria extract

Compound detection methods	Plant
	Q.infectoria
Alkaloids	+
Glycosides	+
Flavonoids	+
Tannins	+
Saponins	+
Resins	+
Phenols	+

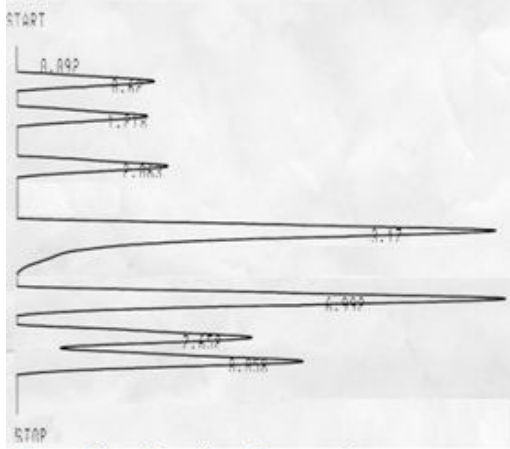
include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins**(13)**.Tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (**14**) and One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (**15**)). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, etc. They also complex with polysaccharide (**16**).

Ethanolic extract of galls of *Quercus infectoria* was subjected to *high- performance liquid chromatography* and the obtained records were superimposed on the retention time values of the extract. Tannin acid, Gallic acid, Caffeic acid, Chlorogenic and Cinnamic acid were used as standard. Figure (1) shows the chromatogram with retention time, area of the standards. Fig (1) shows the chromatogram of ethanolic extract of galls of *Quercus infectoria*, which was compared with the standards and found to contain Tannic acid, Gallic acid, Caffeic acid, Chlorogenic acid and Cinnamic acid . It has been reported by **(17).** Caffeic acid has the highest concentration followed by Chlorogenic acid (28.04, 24.39) µg/ml respectively Table (2) .This compound is an antimicrobial compound having wide spectra of antimicrobial. Caffeic acid and Chlorogenic acid a natural phenolic compound, is abundant in medicinal plants and possesses multiple biological effects such as anti-bacterial **(18,19).**



Time	Area of sample	Compound
1.218	41552	Tannic acid
2.868	41410	Gallic acid
3.137	46985	Caffeic acid
7.823	39811	Chlorogenic acid
7.628	57383	Cinnamic acid

Figure 1: HPLC chromatogram of standard compound



Time	Area of sample	Compound
1.218	32229	Tannic acid
2.863	33450	Gallic acid
3.17	105384	Caffeic acid
6.992	77672	Chlorogenic acid
7.652	48446	Cinnamic acid

Figure 2: HPLC chromatogram of extract of *Quercus infectoria* **at 264 nm**

TABLE-2-
Concentrations and percentages the compound of the extract of the gall of *Quercus infectoria*

The ethanolic extracts of *Q. infectoria* showed inhibitory activity against all the five food associated bacteria in which the diameter of zone of growth inhibition varied between 7 and 28 mm.(Table 3) showed highest diameter of zone of inhibition of 28mm against *B. subtilis* followed by *Staph. aureus* (23mm) and *E. coli* (18mm) Fig (3).**(20)** showed that galls had higher antimicrobial activity against methicillin resistant *Staph. aureus*. **(21,22,23)** reported that *Q. infectoria* galls possess antibacterial activity against *E.coli*, *Staph. aureus* and *B. subtilis* .The gram negative bacteria are more resistant than Gram-positive bacteria. Such resistance could be due to the permeability barrier provided by the cell wall or to the membrane accumulation mechanism **(24).**

Determination of cellular toxicity using sheep erythrocytes. The compounds showed no cellular toxicity as any hemolysis was observed.

Compound	Concentration (µg/ml)	Percentage %
Tannic acid	9.7	3.19
Gallic acid	10.1	3.39
Caffeic acid	28.04	10.42
Chlorogenic acid	24.39	7.68
Cinnamic acid	8.82	2.87

TABLE-3- Rate of inhibition zone diameters at different concentration of phenolic extract of *Quercus infectoria*

Bacterial species	Concentration mg/ml				
	1.25	2.5	5	10	20
Staph. aureus	0	B8.200± 0.176	CD9.30±0.133	B13.13± 0.104	B23.201± 0.180
E. coli	0	0	C7.230± 0.076	B14.120± 0.132	C18.211± 0.126
B. subtilis	A9.012±0.104	A17.105± 0.144	21.41±±0.133 0.382	A25.200± 0.275	A28.100± 0.153
S. typhimurium	0	0	C7.104±0.076	C11.3± 0.212	D15.310± 0.265
Ps. aeruginosa	0	0	C8.212± 0.101	C11.14± 0.131	Cd16.230±0.126

- different letters mean a significant difference (P <0.05) for the comparison between the lines

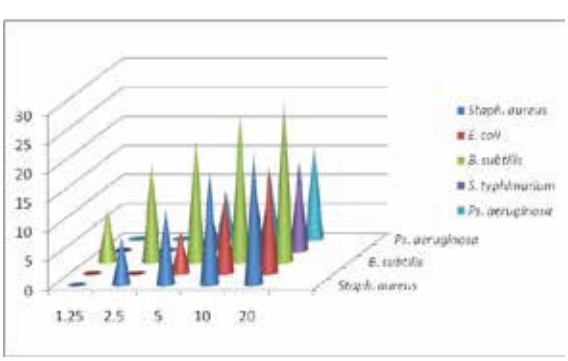


Figure 3: Antibacterial activity of *Q. infectoria* **extract against food-born pathogenic bacteria**

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