

Growth Inhibition of Pathogenic Bacteria by Extract of *Quercus Infectoria* Galls

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Abstract—Extract of *Quercus infectoria* galls was evaluated for its antimicrobial activity against a wide variety of pathogenic bacteria such as *Escheria coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633. The antimicrobial activities of *Q. infectoria* extracts prepared from different solvents of varying polarity were examined and their efficacies were then compared by the disc diffusion method. Crude extracts of the solvents exhibited the antimicrobial effect to a different extent as seen in the varying diameters of the zones of inhibition. The antimicrobial activity of the methanol extract was found to be superior to all other extracts. Ethanol and aqueous extracts showed a strong though lower antimicrobial effect against all the tested organisms while chloroform and hexane extracts of *Q. infectoria* were found to be least active. Compared to the commercial antibiotics, all the extracts exhibited a good antimicrobial activity. All the gram-positive bacteria and gram-negative bacteria tested were susceptible to all aqueous and solvent extracts of *Q. infectoria* galls. The methanol extracts at different concentrations were then incubated with the three bacteria strains, and the minimum inhibitory concentrations (MICs) of each bacterial strain were determined. The MIC values of methanol extracts for *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 are 2.500, 1.25 and 0.625 mg ml⁻¹ respectively. Microscopic observation under scanning electron microscopy (SEM) revealed a sharp decline in bacterial population density for all three bacterial strains. The bacterial morphology of all the strains became more irregular in shape differing from their respective originally homogeneous forms.

Index Terms—antimicrobial activity, *Quercus infectoria* galls, pathogenic bacteria, the extracts

I. INTRODUCTION

Recently, there has been an increase in the number of reported poisoning outbreaks caused by food-borne pathogenic bacteria. The evolution of bacterial resistance to currently available antibiotics has necessitated the research for more novel antimicrobial compounds. Many local plants are cheap, readily available and widely used in traditional folk medicine since they produce a diverse range of bioactive compounds. In several occasions, the use of plant extracts is more preferable over the use of human-synthesized medicine.

Manuscript received on April 8, 2011. This work was supported by The National Research University Project of CHE and The Ratchadaphiseksomphot Endowment Fund (AM667A).

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Unlike human-synthesized medicine which normally consists of only a single bioactive compound, the extracts from plants may contain more than one bioactive ingredients which synergistically work against a particular disease. In addition, being from nature is normally perceived as safer and therefore, more acceptable by humans.

Antimicrobial, antifungal and antiviral activities are among the medicinally useful properties for which many plant extracts are employed. Particularly, the antimicrobial activity of such extracts has formed the basis of various applications, including raw and processed food preservation (antimicrobial cans or food preservatives), textile industries (antimicrobial dyes or antimicrobial cloths), pharmaceuticals (natural antibiotics), and alternative medicine (substitutes for human-synthesized drugs). Therefore, plants with possible antimicrobial activity should be tested against an appropriate microbial model to validate their effectiveness and to ascertain the parameters at which they best work.

Quercus infectoria Olivier (Fagaceae) is a small shrub mainly present in Greece, Asia Minor, Syria and Iran. It is locally cultivated for its valuable medicinal properties. The medicinal properties of the plant have been a subject of numerous investigations. In traditional folk medicine, the galls are extracted with hot water for use as a gargle to relieve inflamed tonsils or directly applied onto the inflamed skin to reduce swelling. Ethyl acetate extract of the galls has been reported to be very effective in killing mosquito larvae and is currently a promising candidate for the development of environmentally-friendly larvicide [1]. In addition, they have also been known to produce many bioactive compounds [2,3] with antibacterial [4], antifungal [5], antidiabetic [6], local anaesthetic [7], antiviral [8], and anti-inflammatory [9] activities. Moreover, they contain the mixture of tannin (60-70%) with antitumor activity [10], polyphenols with antivenom activity [11], gallagic acid, methyl gallate and ellagic acid with antioxidant activity [12,13]. Tannins are generally defined as water-soluble polyphenolic compounds ranging in molecular weight from 500 to 3000 Daltons and having the ability to precipitate proteins [14].

Due to its myriad of useful medicinal properties mentioned above, there is a further incentive to find out more about the potential of this plant as an antimicrobial agent. Hence, this study was designed to assess the effectiveness of different solvent extracts of *Q. infectoria* galls toward the growth inhibition of gram-positive and gram-negative bacteria. We also investigated the effect of the extracts on the morphological changes of the test pathogenic bacteria under scanning electron microscope.

II. MATERIALS AND METHODS

A. Test microorganisms

Tested pathogenic bacteria comprised *Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6633, and *Staphylococcus aureus* ATCC 25923. The bacteria were maintained by subculturing periodically on nutrient agar and were preserved at 4 °C prior to use.

B. Culture media

The nutrient agar was prepared by dissolving 5 g peptone, 1.5 g beef extract, 1.5 g yeast extract, 5 g NaCl and 20 g agar in 1000 ml distilled water, boiling the mixture and adjusting its pH value to between 6.4–6.8. The nutrient mixture was then sterilized by autoclaving at 15 psi pressure (121 °C) for 20 min. Nutrient agar was prepared by pouring the nutrient mixture to the same thickness on sterilized petri plates. The test bacteria were then grown overnight at 37 °C, 120 rpm in 10 ml nutrient broth. This broth was used for seeding the bacteria onto the agar plates.

C. Plant materials

The galls of *Q. infectoria* were obtained commercially from a Thai traditional drug store. All samples were washed with distilled water, cut into small pieces and dried at 60 °C overnight. They were crushed with a mechanical mortar into fine powder before the extraction (Fig 1).

D. Extraction conditions

Extracts of dried plant materials were prepared by using solvents of varying polarity. The dried plant materials of 5 g each were extracted by maceration in different solvents (25 ml) for 5 days at room temperature in a dark place. The solvents used were methanol, ethanol, hexane, chloroform, and distilled water. Following the solvent maceration, the extract was filtered through Whatman filter paper IV. 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. The extracts were stored at -20 °C and freshly dissolved in 10 % dimethyl sulfoxide (DMSO, Merck, Germany) before use. The corresponding concentration was expressed in term of mg of extract per ml of solvent (mg ml⁻¹).

E. Antimicrobial assay

Antimicrobial activity was tested by the disc diffusion method. Small discs of filter paper (diameter 6.0 mm) were impregnated with 50 µl of different extracts and placed on top of the seeded media of the three bacterial strains. The antibacterial assay plates were incubated at 37 °C for 24 h and the diameters of the zones of clearing were noted. In addition, the discs of the standard antibiotics; tetracycline (10 µg per disc) and kanamycin (10 µg per disc) were also experimented separately which would serve as positive antibacterial controls. For this study, the diameter of the zone of inhibition around each disc (disc diameter included) was taken as a measure of the antibacterial activity. The diameters of the zones of inhibition by the samples were then compared with the diameters of the zones of inhibition produced by the standard antibiotic discs. Each experiment was carried out in triplicate and the mean diameter of the inhibition zones was recorded.

F. Minimum inhibitory concentration (MIC)

MICs of the extracts were determined by the broth dilution method using serial dilution of the plant extracts as described previously by Evans *et al.* [15]. Briefly, the test bacteria were prepared in nutrient broth and incubated at 37 °C for 24 h. After that, the cultures' concentrations were adjusted with sterilized saline to bring the optical density at 660 nm to 0.04. Serial two-fold dilutions of the extracts were prepared in nutrient broth with concentrations ranging from 5.0000 to 0.0049 mg/ml. The 1 ml of each serially diluted extract was separately added to the tubes containing an equal volume of the inoculum (1 ml). All the tubes (total volume of 2 ml) were then incubated at 37 °C for 24 h.

The MIC values were interpreted as the highest dilution (lowest concentration) of the sample, which showed clear fluid with no development of turbidity. Solvent blanks and positive controls were also included. All tests were performed in triplicate.

G. Morphological observations under scanning electron microscope (SEM)

Cells of each strain at a logarithmic phase in nutrient agar were treated with the extracts for 12 h. The bacterial cells treated with 10 % DMSO were used as control. The cells were collected by centrifugation and washed with the sodium phosphate buffer. Following that, the samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, at 4°C overnight, and postfixed in 1% osmium tetroxide in the phosphate buffer for 1 h at room temperature before processing for the observations under scanning electron microscope.

III. RESULT AND DISCUSSION

A. Yield of extracts

The solvents with an increasing order of polarity were used for the extraction of *Q. infectoria* galls. The percentage yields of the extracts for *Q. infectoria* were: 52% (methanol), 42% (ethanol), 30% (water), 2% (hexane), and 2% (chloroform) as shown in Fig. 2. Methanol extracted the most materials from the plant followed by ethanol and water. The extracts of chloroform and hexane yielded the lowest amount.



(a)

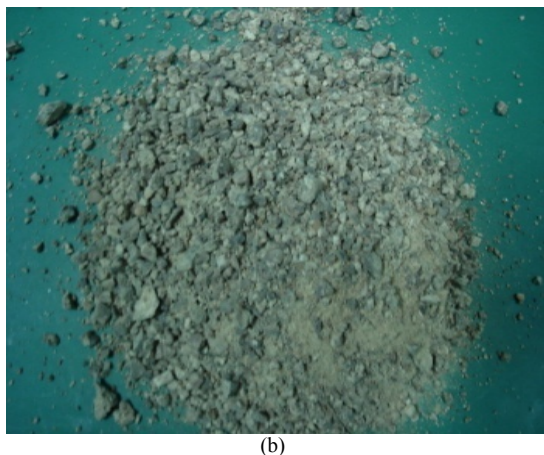


Figure 1. Characteristic of *Quercus infectoria* Olivier galls: dried samples (a) and powder (b)

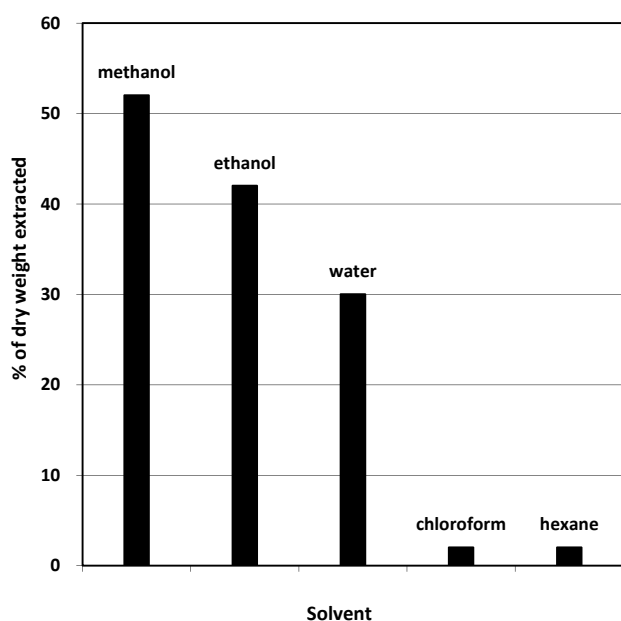


Figure 2. Percentage of 5 g dried material extracted after extractions with 25 ml of methanol, ethanol, water, chloroform and hexane.

B. Antimicrobial assay

The antimicrobial activity of five solvent extracts of *Q. infectoria* galls was studied by the disc diffusion method and the results are shown (Table 1, Fig. 3-5). All the solvent extracts showed a significant inhibitory activity against all pathogenic bacterial strains. Methanol, ethanol and aqueous extracts exhibited a higher antibacterial activity than chloroform and hexane extracts did and produced inhibition zones ranging from 19.0 to 25.3 mm in diameter at the concentration of 5 mg ml⁻¹. Methanol extract displayed an excellent activity against gram-positive *B. subtilis* ATCC 6633 (25.3 mm), *S. aureus* ATCC 25923 (22.0 mm) and gram-negative *E. coli* ATCC 25922 (20.3 mm). Ethanol extracts showed a strong though a little bit lower activity against gram-positive *B. subtilis* ATCC 6633 (24.0 mm), *S. aureus* ATCC 25923 (20.0 mm) and gram-negative *E. coli* ATCC 25922 (19.0 mm). Similarly, aqueous extracts also showed a strong activity against gram-positive *B. subtilis* ATCC 6633 (21.0 mm), *S. aureus* ATCC 25923 (18.0 mm) and gram-negative *E. coli* ATCC 25922 (15.7 mm). However,

chloroform and hexane extracts only displayed a mild to moderate activity against all of the tested bacteria. Chloroform extracts were found to be active against gram-positive *B. subtilis* ATCC 6633 (13.0 mm), *S. aureus* ATCC 25923 (12.7 mm) and gram-negative *E. coli* ATCC 25922 (10.3 mm). Finally, hexane extracts were found to be active against gram positive *B. subtilis* ATCC 6633 (12.0 mm), *S. aureus* ATCC 25923 (11.3 mm) and gram-negative *E. coli* ATCC 25922 (10.0 mm). The results were compared with those of tetracycline, and kanamycin as standard antibiotics. On overall consideration, the antimicrobial activities of the alcoholic extracts were higher as compared to those of other less polar extracts. This may imply that the bioactive molecules responsible for the antimicrobial action should be more hydrophilic in nature. In addition, a higher antibacterial activity was also observed against gram-positive bacteria.

TABLE 1. ANTIBACTERIAL ACTIVITIES OF DIFFERENT EXTRACTS OF *Q. INFECTORIA* GALLS

Solvent	Diameter of inhibition zone (mm) \pm S.D. ^a		
	<i>E. coli</i> ATCC 25922	<i>S. aerus</i> ATCC 25923	<i>B. subtilis</i> ATCC 6633
Methanol	20.3 \pm 0.8	22.0 \pm 0.7	25.3 \pm 0.4
Ethanol	19.0 \pm 0.7	20.0 \pm 0.7	24.0 \pm 0.7
Distilled water	15.7 \pm 0.4	18.0 \pm 0.7	21.0 \pm 0.7
Chloroform	10.3 \pm 0.9	12.7 \pm 0.4	13.0 \pm 0.7
Hexane	10.0 \pm 0.7	11.3 \pm 0.8	12.0 \pm 0.7
Tetracycline	19.0 \pm 0.6	28.9 \pm 0.4	20.5 \pm 0.6
Kanamycin	18.6 \pm 0.8	18.7 \pm 0.5	26.3 \pm 0.5

^a Mean value of three determinations, each from a different plate

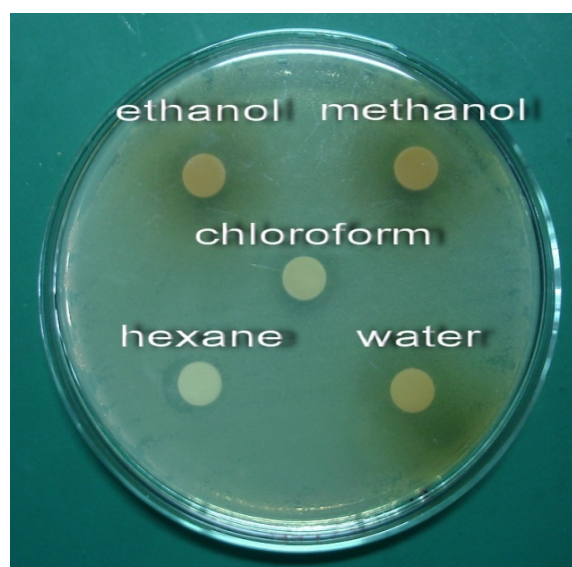


Figure 3. Antimicrobial activity of the extracts on *E. coli* ATCC 25922: Disc diffusion test for the effect of *Q. infectoria* against *E. coli* ATCC 25922 grown on nutrient agar medium. 1. ethanol; 2. methanol; 3. chloroform; 4. hexane; 5. water.

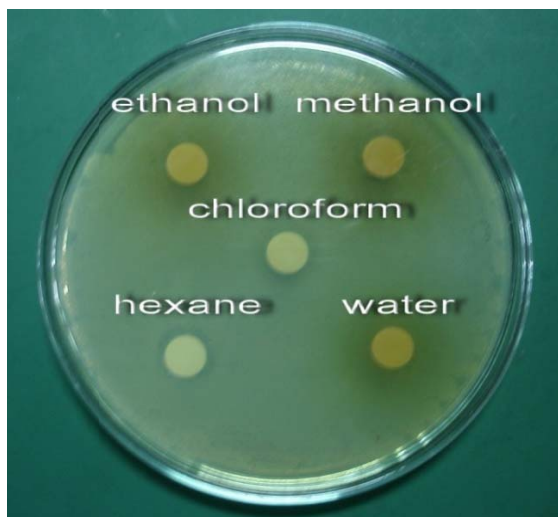


Figure 4. Antimicrobial activity of the extracts on *S. aureus* ATCC 25923: Disc diffusion test for the effect of *Q. infectoria* against *S. aureus* ATCC 25923 grown on nutrient agar medium. 1. ethanol; 2. methanol; 3. chloroform; 4. hexane; 5. water.

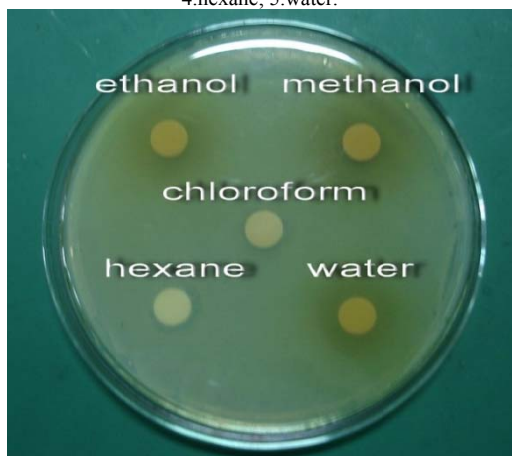


Figure 5. Antimicrobial activity of the extracts on *B. subtilis* ATCC 6633: Disc diffusion test for the effect of *Q. infectoria* against *B. subtilis* ATCC 6633 grown on nutrient agar medium. 1. ethanol; 2. methanol; 3. chloroform; 4. hexane; 5. water.

The results from this study demonstrate that the gram-positive bacteria like *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 are more susceptible to the extracts than gram-negative bacteria such as *E. coli* ATCC 25922 were. The reason behind the more resistant behavior of such gram-negative bacteria could be due to the lipopolysaccharide (LPS) layer in their outer membrane which acts as an extra barrier against the entry of the bioactive molecules. In addition, there is also a report from Ikigai H et al. that the more resistant behavior of gram-negative bacteria is actually partly pertaining to the highly negative charges on the LPS layer even though its exact mechanism is not yet understood [16]. High amounts of tannin present in the galls of *Q. infectoria* may suggest that tannin is the active compound responsible for the antibacterial activity in this study [17,18]. When considering the nature of tannin, it is a phenolic compound which is soluble in water and alcohol so it is hydrophilic in nature which is in correspondence to what we have predicted earlier about the nature of the bioactive molecules in the *Q. infectoria* extracts [16, 18]. Hence, it may be concluded that the active compounds in the extracts are likely to be hydrophilic tannins and that gram-positive bacteria are more

resistant to such compounds due to the LPS layer which forms an extra protective coating.

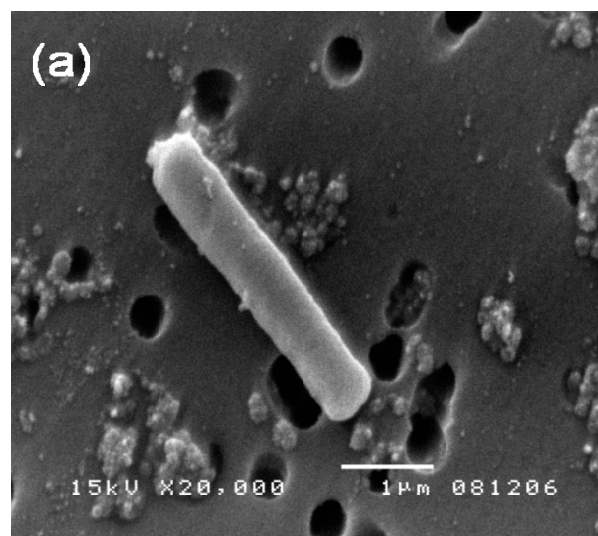
C. Minimum Inhibition concentration (MIC)

Table 2 indicates the minimum concentrations of the methanol extracts required to completely inhibit the growth of the three bacterial pathogens. The relative growth of each microorganism after 24 h of incubation in the presence of different concentrations of *Q. infectoria* extracts was compared to the control. Methanol extract of *Q. infectoria* suppresses the growth of *E. coli* ATCC 25922 at the MIC of 2.500 mg ml⁻¹ while the growth of *S. aerus* ATCC 25923 is affected at the MIC of 1.250 mg ml⁻¹. The most susceptible bacterial strain in the study is *B. subtilis* ATCC 6633 which requires only the MIC of 0.625 mg ml⁻¹ to inhibit their growth.

TABLE 2. MICs OF DIFFERENT EXTRACTS OF *Q. INFECTORIA* GALLS

Solvent extract	MICs of solvent extracts (mg/ml) ^a				
	<i>E. coli</i> ATCC 25922	<i>S. aerus</i> ATCC 25923	<i>B. subtilis</i> ATCC 6633	Positive Control	Negative Control
5.0000	-	-	-	+	-
2.5000	-	-	-	+	-
1.2500	+	-	-	+	-
0.6250	+	+	-	+	-
0.3125	+	+	+	+	-
0.1563	+	+	+	+	-
0.0781	+	+	+	+	-
0.0391	+	+	+	+	-
0.0195	+	+	+	+	-
0.0098	+	+	+	+	-
0.0049	+	+	+	+	-

^a - Absence of growth, Positive Control: Bacterial suspension and saline; + Presence of growth, Negative Control: Extracts and broth



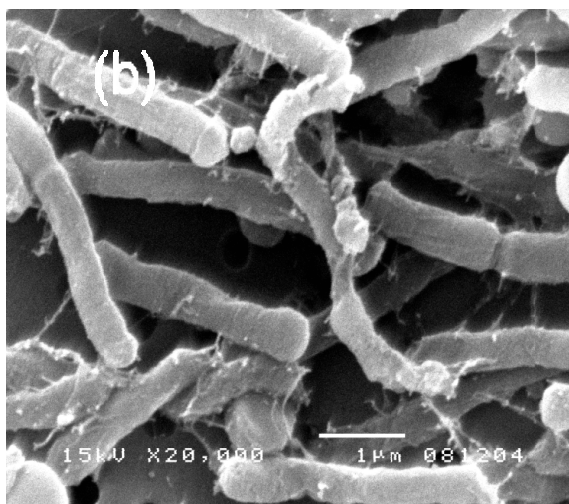


Figure 6. Scanning electron microscope images of *E. coli* ATCC 25922 with the methanol extract of *Q. infectoria* at 24 h. Untreated bacterial cells were typically rod-shaped and the cell surfaces were relatively smooth (a). The cells treated with the extract at 5 mg ml⁻¹ (b).

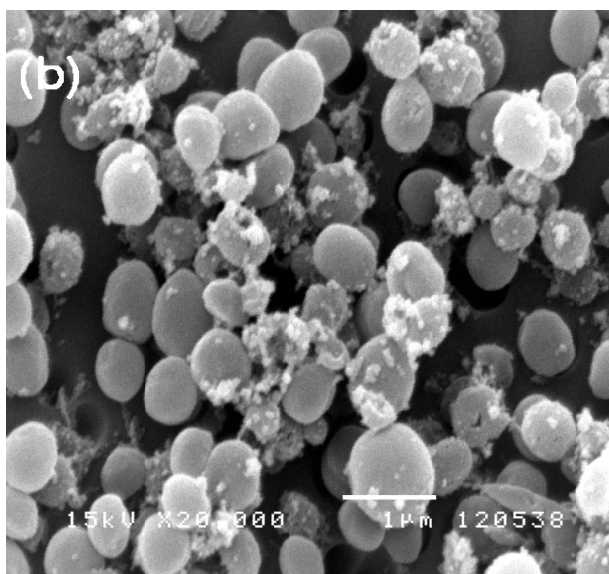
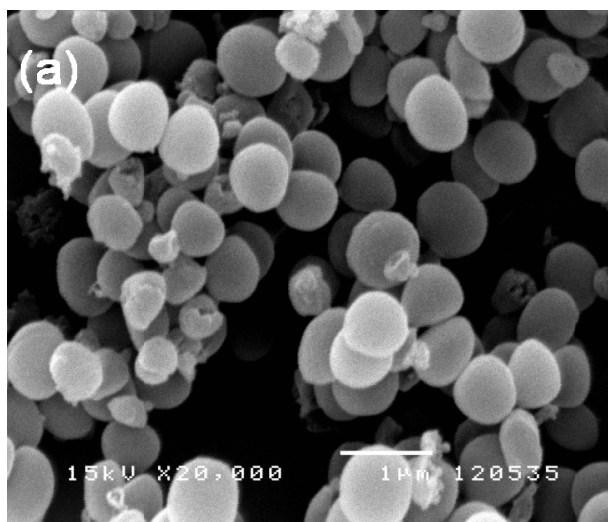


Figure 7. Scanning electron microscope images of *S. aureus* ATCC 25923 with the methanol extract of *Q. infectoria* at 24 h. Untreated bacterial cells were typically spherical in shape (a). The cells treated with the extract at 5 mg ml⁻¹ (b).

The effects of the methanol extract on the surface morphology of *E. coli* ATCC 25922 were generally similar. Untreated organisms (Fig. 6a) appeared rod-shaped. Exposure to the extract resulted in only occasional morphologic defects characterized by tubular outpouchings from the cell wall (Fig. 6b).

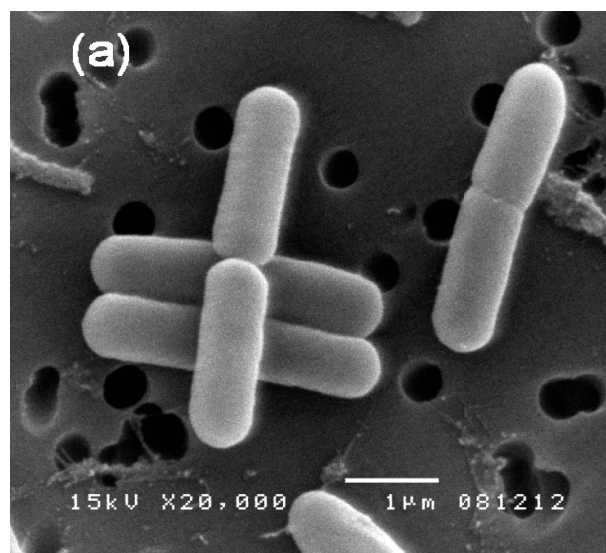
The effects of the methanol extract on the surface morphology of *S. aureus* ATCC 25923 during its logarithmic phase of growth were similar under the experimental conditions utilized. Untreated staphylococci (Fig. 7a) appeared to be smooth and spherical in grapelike clusters. Exposure to the extract resulted in the appearance of small bleb-like structures on the surface of some cells; irregular spherical structures lying free or appearing to extrude from cells were also observed (Fig. 7b).

The effects of the methanol extract on the surface morphology of *B. subtilis* ATCC 6633 were also relatively similar. Untreated organism (Fig. 8a) appeared rod-shaped. Exposure of this organism to the extract resulted in morphological abnormality as seen in the formation of spherical globules and collapse in structures of the treated cells (Fig. 8b).

For all strains of bacteria tested, treatments with methanol extract of *Q. infectoria* clearly result in a sharp decline in bacterial population density observed under scanning electron micrographs. Morphology of the bacteria was also altered becoming more irregular and inhomogeneous in shape. Small degraded pieces of cellular debris were also observed in all micrographs following the treatment with the *Q. infectoria* extract.

D. Scanning electron microscope observation at 24 h

The effects of the methanol extracts of *Q. infectoria* galls on the surface morphology of gram-positive and gram-negative bacteria during its logarithmic phase of growth were shown in Fig. 6-8. The spectrum of antimicrobial activities on the surface morphology of individual cells and bacterial populations was visualized.



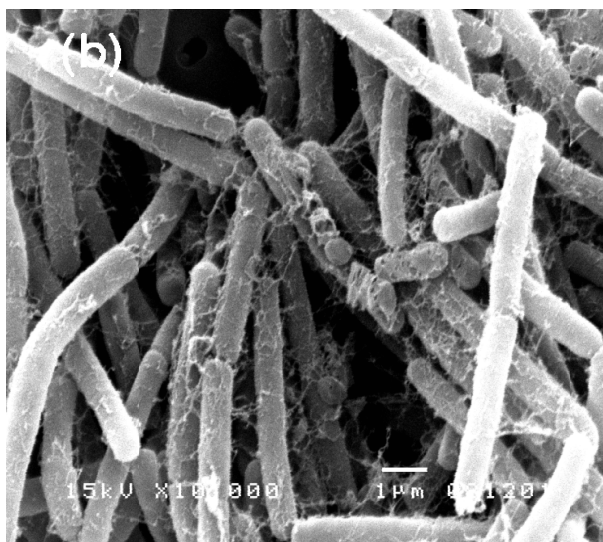


Figure 8. Scanning electron microscope images of *B. subtilis* ATCC 6633 with the methanol extract of *Q. infectoria* at 24 h. Untreated bacterial cells were typically spherical in shape (a). The cells treated with the extract at 5 mg ml⁻¹ (b).

IV. CONCLUSIONS

The results obtained from this study reveal that *Quercus infectoria* galls have an antimicrobial activity against gram-positive and gram-negative bacteria. In this study, it was observed that the potency of this medicinal plant was enhanced by the type of solvents used with methanol being the most effective solvent. This study corresponds with the results of other researchers who have observed that the alcoholic extracts of *Q. infectoria* were found to be more active against all bacteria ever studied till present. Hence, the active materials, most likely to be tannins, in *Q. infectoria* were probably be better dissolved in alcohol and water rather than in hexane or chloroform.

In addition, it was also found that all extracts from the galls inhibited the growth of gram-positive bacteria more effectively than they did on the gram-negative bacteria since the gram-negative bacteria possess an extra protective layer of LPS on the cell membrane which is highly negatively charged.

This study also demonstrates visually the spectrum of the effects of the methanol extract on the surface morphology of specific bacteria making them more irregular and inhomogeneous in shape. The more thorough investigation under scanning electron microscope may help to correlate these various morphologic forms with biochemical alternations occurring within the cell wall.

ACKNOWLEDGMENT

The authors are grateful to Associated Professor Dr. Chirakarn Muangnapoh for invaluable advice.

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