



Antimicrobial effects of *Quercus infectoria* gall extract on the cell morphology of *Streptococcus agalactiae*, *Proteus vulgaris* and *Candida albicans*

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ABSTRACT

Aims: *Quercus infectoria* (QI) gall extract is known to have broad spectrum anti-microbial activity *in vitro*. However, its mechanism of microbial growth inhibition is not well understood. The objectives of this study were to determine the antimicrobial effect of methanolic QI gall extract on bacteria and yeast and changes to their cell morphology.

Methodology and results: The minimum inhibitory concentrations (MICs) of methanolic QI gall extract against *Streptococcus agalactiae* (ATCC 13813), *Proteus vulgaris* (ATCC 49312) and *Candida albicans* (ATCC 10231) were determined using two fold serial microdilution technique at concentrations ranging from 0.01 mg/mL to 5.00 mg/mL (for bacteria) and 0.02 mg/mL to 12.00 mg/mL (for yeast). Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by subculturing the broth from the microtitre wells which showed no apparent growth or turbidity onto the nutrient agar plates. The scanning electron microscope (SEM) was used to analyze the morphology of bacteria and yeast cells treated with 1× MIC and 4× MIC of the extract. Substantial antimicrobial activity was observed against ATCC strains of *S. agalactiae*, *P. vulgaris* and *C. albicans* in this study. The MBC/MFC to MIC ratio (≤ 4) indicated the methanolic QI gall extract was bactericidal and fungicidal against all the tested strains. Changes to the cell morphology were more obvious at higher extract concentration (4× MIC).

Conclusion, significance and impact of study: This study showed that QI gall extract has antimicrobial effects on the bacterial and yeast cell morphology and thus, provides scientific information suggesting its possible antimicrobial mechanisms on the cell wall and membrane integrity.

Keywords: *Quercus infectoria* gall extract, antimicrobial activity, cell morphology, scanning electron microscope (SEM)

INTRODUCTION

Medicinal plants have been used in traditional treatment in various parts of the world. Some 80% of the Asian and African populations depend on traditional medicine for primary health care (World Health Organization 2008). The natural products and their derivatives have continued to be the most significant sources in the development of new pharmaceutical agents for treatment of various ailments

The nut gall of *Quercus infectoria* (QI) or locally known as "manjakani" is one of the most popular natural sources used in traditional medicines in Asia and the plant is mainly found in Asia Minor, Turkey, Syria, Persia, Cyprus, Greece and Iran (Basri *et al.*, 2011; Satirathkul and Leela, 2011). Its use is common among Malaysian women and claimed to be highly beneficial in the postpartum care to restore the elasticity of the uterine or vaginal wall as well as to treat vaginal discharge and related infections (Soon and Hasni, 2005). The plant extract has also been pharmacologically documented as anti-diabetic, anti-

bacterial, anti-viral, anti-fungal, larvicidal, anti-inflammatory, anti-amoebic, anti-pyretic, anti-parkinsonian, astringent, anti-tremorine, local anaesthetic and wound healing (Basri *et al.*, 2011; Rao *et al.*, 2013). The chemical constituents of the QI gall comprise a large amount of tannin and small amounts of free gallic acids, ellagic acid and synergic acid (Sucilathangam *et al.*, 2012; Vaidya *et al.*, 2013).

The QI gall extract is well known to have a broad spectrum of activity against Gram-positive bacteria, Gram-negative bacteria and yeast. The methanolic extract of the galls was reported to have higher potency in inhibiting the bacterial and *Candida* growth as compared to ethanol, water and other organic solvents extracts such as hexane and chloroform (Rao *et al.*, 2013; Baharuddin *et al.*, 2015). The inhibitory effect of the gall extract against microbial growth is generally dependent upon the structure of microbial cell wall. A Gram-positive bacterium has a thick, multilayered cell wall consisting mainly of peptidoglycan

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surrounding the cytoplasmic membrane while the cell wall components in the Gram-negative bacterium are more complex. The Gram-negative bacteria possess a second outer membrane external to the peptidoglycan layer which composed of lipopolysaccharide. The outer membrane confers important properties on the Gram-negative organisms in which it protects the peptidoglycan from the effect of lysozyme and impedes the ingress of many antibiotics (Barer, 2007). Yeasts however have cell walls made of thick polysaccharide consisting of polymers of mannose (mannoproteins), polymers of glucose (β -glucan) and polymers of N-acetylglucosamine (chitin) (Aguilar-Uscanga and Francois, 2003). These structures are unique to the bacteria and yeast in providing intact cellular morphology and can be an excellent protection from the activity of antimicrobial drugs.

Even though many studies have reported on the antimicrobial properties of various types of plant extracts, the understanding of their mechanisms of cell growth inhibition and cellular changes are however limited and have not been firmly established (Basri *et al.*, 2011; Satirapathkul and Leela, 2011; Jeyaseelan *et al.*, 2012). Thus information related to the morphology or microstructure of the cell is useful in characterizing the type of changes that occur inside the cell prior to treatments by antimicrobial agents (Suwalak and Voravuthikunchai, 2009). Hence it would be beneficial to compare the effects of the gall extract on the microbial cell morphology, in order to correlate morphological changes of microorganisms with the mechanisms of antibacterial and anticandidal activity that causes biochemical alterations within cells. This might be helpful for better understanding why some drugs are only effective against certain microorganisms. Therefore, this paper reports the antimicrobial effect and cellular morphological changes on selected bacteria and yeast following treatment with QI gall methanolic extract.

MATERIALS AND METHODS

Plant materials and preparation of crude extract

The galls of QI were obtained from a local herb store in Kota Bharu, Kelantan, Malaysia and identified based on their morphological and organoleptic characteristics as described by Shrestha *et al.* (2014). Methanol extraction of the gall was carried out by immersing 50 g of the dried gall powder in a flask containing 250 mL of methanol and placed in water bath at 50 °C for 72 h. The mixture was then filtered using Whatman No. 1 filter paper and the filtrate was concentrated under reduced pressure by using rotary evaporator. The pellet was pounded to dryness under air-dryer to form powdered crude extract and stored at -20 °C prior to use. The crude extract powder was freshly dissolved in sterile distilled water to a final concentration of 10 mg/mL (for bacteria) and 48 mg/mL (for yeast) for use in minimum inhibitory concentration (MIC) assay.

Bacterial and fungal strains

The microorganisms used in this study were *S. agalactiae* (ATCC 13813), *P. vulgaris* (ATCC 49312) and *C. albicans* (ATCC 10231). The bacterial strains were grown and maintained by subculturing onto blood agar at 37 °C for 24 h and the yeast was grown and maintained on Sabouraud Dextrose agar media at 35 °C for 24 h. Fresh suspension of each test strain was prepared at a concentration of 10^8 cells per mL (bacteria) and 10^6 cells per mL (yeast) corresponding to 0.5 McFarland prior to microdilution assay.

Determination of MIC and MBC/MFC

The procedures described for determination of MIC and MBC values were according to the standard antimicrobial susceptibility method provided by Clinical and Laboratory Standards Institute (CLSI, 2012). The MIC of the methanolic gall extract was determined using the twofold serial microdilution method in Mueller-Hinton broth (MHB) and was performed in the 96-well microtiter plate. The final concentration ranged from 5 to 0.01 mg/mL for bacteria and 12 to 0.02 mg/mL for yeast. As for *S. agalactiae*, cation-adjusted MHB (CAMHB) supplemented with 2.5% (v/v) lysed horse blood (LHB) was used instead.

One hundred microliter of MHB or CAMHB + LHB was pipetted into wells 1 to 11, while 200 μ L of the broth was pipetted into well 12. One hundred microliter of the tested extract was added into well 1. Subsequently, two-fold serial microdilution from well 1 to 10 was performed. Then, 5 μ L of diluted bacterial inoculum and 100 μ L fungal suspension containing 10^5 bacteria/mL and 0.5×10^3 yeast/mL respectively were added into wells 1 to 11 and mixed thoroughly. Each strain was assayed in triplicate. The bacterial or yeast suspensions were used as positive control (well 11) and extract in broth was used as negative control (well 12). The growth was indicated by turbidity of the wells in the microtiter plate. The MIC values were taken as the lowest concentration of the extract in the wells of the microtiter plate that showed no turbidity at 37 °C for bacteria and at 35 °C for yeast after 24 h of incubation. The MIC value obtained by each strain was used in the subsequent extract treatment of the microorganisms prior to the SEM analysis.

The minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) values were determined by sub-culturing the wells plate which showed no apparent growth onto the agar plates. The lowest concentration which showed no visible growth on sub-cultured agar after an overnight incubation at 37 °C for bacteria and at 35 °C for yeast was considered as MBC or MFC value (Basri *et al.*, 2011).

Cell morphology analysis by scanning electron microscope (SEM)

The method of sample processing was adopted from Suwalak and Voravuthikunchai (2009) and IMF (2007). Cells at a logarithmic phase in MHB or CAMHB + LHB (for

bacteria) and Roswell Park Memorial Institute (RPMI) (for yeast) were treated with 1x MIC and 4x MIC of QI galls extract for 12 h (for bacteria) and 24 h (for yeast). The bacterial and fungal cells treated with 1% (v/v) DMSO were used as negative control. The samples were centrifuged at 1000-2000 xg for 15 min and the pellets were fixed with McDowell-Trump's fixative prepared in 0.1 M phosphate buffer (pH 7.2) overnight. The centrifugation was repeated two times. The pellet was then post fixed in 1% osmium tetroxide prepared in phosphate buffer for 1 h at room temperature. After centrifugation, the pellet was resuspended in distilled water before performing the dehydration process for 10 minutes each with 50%, 75%, 95% and 100% ethanol (2x) and hexamethyldisilazane (HMDS) (2x). The cells were allowed to dry in a desiccator at room temperature. The dried cells were then mounted onto the SEM stub with double-sided carbon tape and

coated with gold using sputter coater. The samples were then examined under SEM (Quanta 450 FEG).

RESULTS

MIC and MBC/MFC values of methanolic QI gall extract

The MIC and MBC/MFC values of QI gall extract against *S. agalactiae*, *P. vulgaris* and *C. albicans* strains are shown in Table 1 and Table 2. The QI gall methanolic extract inhibited the bacterial strains at a lower concentration compared to the yeast. *P. vulgaris* demonstrated the lowest MIC (0.63 mg/mL) and MBC (0.63 mg/mL) values. In addition, the gall extract exhibited MBC/MIC ratio of ≤ 4 against all tested strain which was considered as bactericidal and fungicidal.

Table 1: The MIC and MBC values of methanolic *Q. infectoria* gall extract against *S. agalactiae* and *P. vulgaris*.

		Concentration (mg/mL)	5.00	2.50	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01
<i>S. agalactiae</i>	MIC		-	-	-	-	+	+	+	+	+	+
	MBC		-	-	+	+	+	+	ND	ND	ND	ND
<i>P. vulgaris</i>	MIC		-	-	-	-	+	+	+	+	+	+
	MBC		-	-	-	-	+	+	ND	ND	ND	ND

+, Presence of bacterial growth; -, Absence of bacterial growth; ND, Not done

Table 2: The MIC and MFC values of methanolic *Q. infectoria* gall extract against *C. albicans*.

Concentration (mg/mL)	12.00	6.00	3.00	1.50	0.75	0.38	0.19	0.09	0.05	0.02
MIC	-	-	-	-	-	+	+	+	+	+
MFC	-	-	-	+	+	+	+	ND	ND	ND

+, Presence of bacterial growth; -, Absence of bacterial growth; ND, Not done

Cell morphology analysis by scanning electron microscope (SEM)

The effects of the QI gall extract on the cellular morphology of the Gram-positive bacteria, Gram-negative bacteria and yeast during their logarithmic growth phase as observed under the SEM are shown in Figures 1 to 3. The untreated *S. agalactiae* cells were spherical in shape with smooth surface (Figure 1A). Following the exposure of the bacterial cells to the extract at 1x MIC showed no cell morphological changes (Figure 1B). When the bacterial cells were treated with the extract at 4x MIC, no significant change in shape and size were observed except for the minimal roughening of the surface of the *S. agalactiae* cells (Figure 1C).

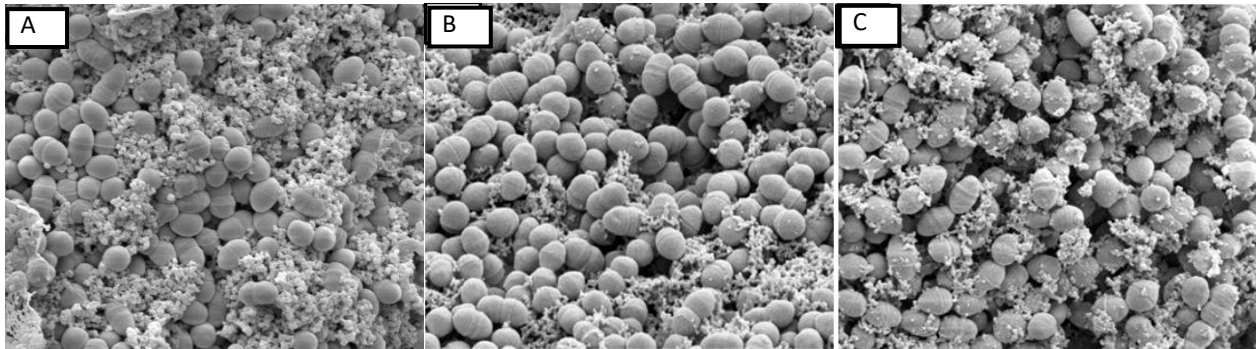
Untreated *P. vulgaris* cells appeared rod-shaped with the flagella (Figure 2A). The bacterial cells treated with the extract at 1x MIC demonstrated some lumpy surface, irregular shape and size (Figure 2B). Most bacterial cells treated with the extract at 4x MIC demonstrated loss of surface appendages (Figure 2C).

The untreated *C. albicans* cells were oval in shape with smooth cell surface (Figure 3A). When the fungal cells were exposed to the extract at 1x MIC, there were bleb-like structures on the surface of some cells and also some un-dividing cells were seen (Figure 3B). The yeast cells treated with the extract at 4x MIC showed cell wall defect, with formation of biconcave shape of the cells (Figure 3C).

DISCUSSION

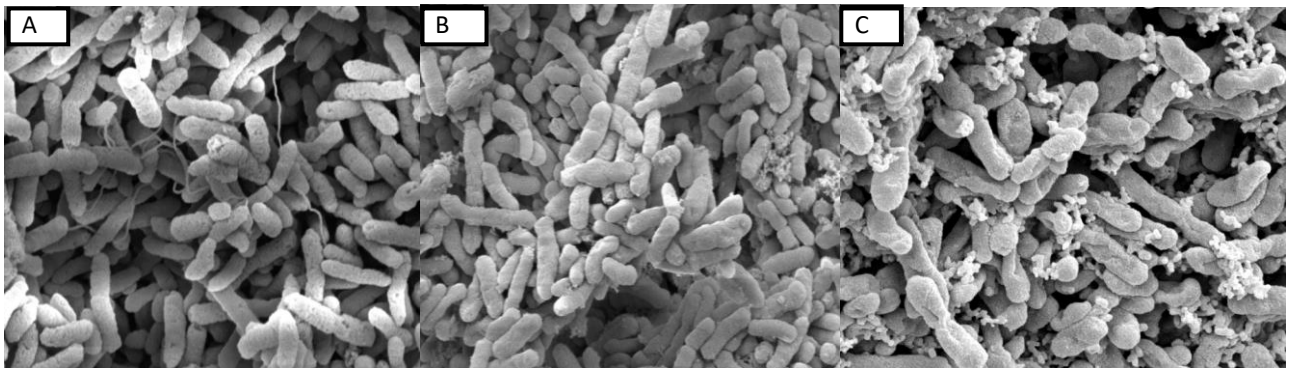
Phytochemicals from plant have received much attention in recent years as many antimicrobial screening studies have discovered new bioactive compounds that showed promising antimicrobials activities. The compounds are not only effective in controlling the growth of microorganism's *in-vitro*, but they also have therapeutic potentials against pathogenic bacteria, fungi and viruses (Sen and Batra, 2012).

Microbiological techniques that are commonly used to determine the bactericidal or fungicidal activity of



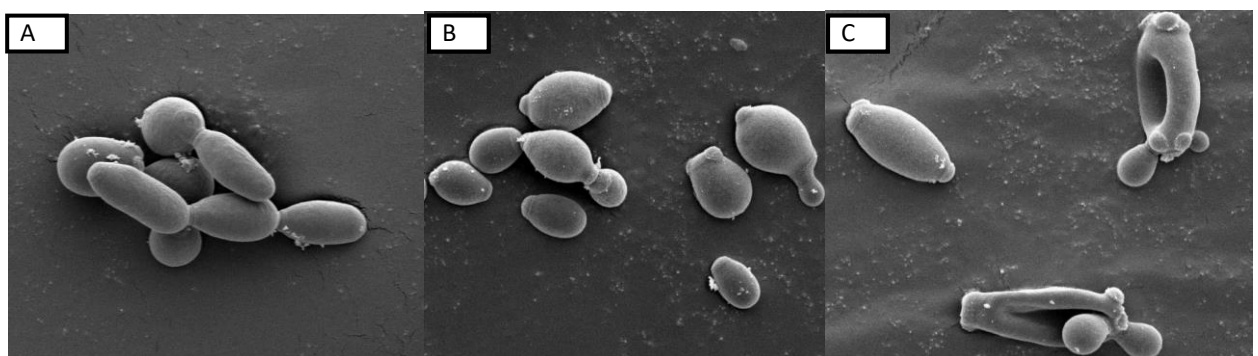
(Magnification: 30,000x)

Figure 1: Scanning electron micrograph of *S. agalactiae* (ATCC 13813) treated with the *Q. infectoria* gall extract at 12 h. A, Untreated bacterial cells were typical spherical shaped and the cell surfaces were relatively smooth; B, The cells treated with the extract at 1x MIC (0.63 mg/mL) and C, 4x MIC (2.50 mg/mL) showing minimal roughening of the cell surface.



[Magnification: 30,000x]

Figure 2: Scanning electron micrograph of *P. vulgaris* (ATCC 49312) treated with the *Q. infectoria* gall extract at 12 h. A, Untreated bacterial cells were typically rod-shaped with flagella; B, The cells treated with the extract at 1x MIC (0.63 mg/mL) and C, 4x MIC (2.50 mg/mL) showing lumpy surface and loss of flagella.



(Magnification: 15,000x)

Figure 3: Scanning electron micrograph of *C. albicans* (ATCC 10231) treated with the *Q. infectoria* gall extract at 12 h. A, Untreated bacterial cells were typical oval shaped and the cell surfaces were relatively smooth; B, The cells treated with the extract at 1x MIC (0.75 mg/mL) and C, 4x MIC (3.00 mg/mL) showing bleb-like structures and deformed cell shape.

antimicrobial agents include the minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) and time-kill curve (Pankey and Sabath, 2004; Meletiadiis *et al.*, 2007). In this study, both the MIC and MBC or MFC were conducted to determine the bioactivity effects of the gall extract. The antimicrobial activity can either be bacteriostatic/fungistatic or bactericidal/fungicidal. Bacteriostatic or fungistatic activity was defined as a ratio of MBC or MFC to MIC values of > 4 , while bactericidal or fungicidal activity was defined as a ratio of MBC or MFC to MIC of ≤ 4 (Pankey and Sabath, 2004; Meletiadiis *et al.*, 2007). Our study showed that the MBC to MIC ratio were 4 and 1 for *S. agalactiae* and *P. vulgaris* respectively while the MFC to MIC ratio for *C. albicans* was 4 (Table 2). The results indicated that the QI gall methanolic extract was bactericidal or fungicidal against the tested microorganisms. Similar findings were obtained from previous studies performed on the QI gall methanolic extract tested against urogenital pathogens (Nur Saeida *et al.*, 2014; Baharuddin *et al.*, 2015).

The QI gall extracts is well known to have broad spectrum antimicrobial activities. Previous studies have shown that *Staphylococcus* spp. are more susceptible towards both methanolic and aqueous QI gall extracts compared to the Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Basri and Fan, 2005; Satirapathkul and Leela, 2011; Nur Saeida *et al.*, 2014). The difference in the susceptibility towards the QI gall extracts was due to the difference in the composition of the bacterial cell wall. The Gram-positive bacteria have a thick peptidoglycan layer, which acts as an exoskeleton that guarantee resistance to mechanical stresses and maintenance of cell shape (Caliot *et al.*, 2012). Lipopolysaccharide (LPS) layer in the outer membrane of Gram-negative bacteria is believed to contribute to the difference in the susceptibility, since the layer acts as an extra barrier that protects the bacteria (Barer, 2007; Caliot *et al.*, 2012). In our study however, *S. agalactiae* was relatively less susceptible towards the methanolic extract of QI gall compared to *P. vulgaris* based on the MBC/MIC ratio and SEM findings.

It was observed that the treated *S. agalactiae* cells at $1\times$ MIC showed negligible morphological changes in the structure and the surface, while the cells only showed roughening of the surface at $4\times$ MIC. The Lancefield Group B carbohydrate (GBC) present in the peptidoglycan is an essential component of the cell wall of *S. agalactiae*. The peptidoglycan-anchored glycopolymer are required for the maintenance of normal growth and proper cell division thus it might be possible that GBC affects the sensitivity of *S. agalactiae* towards the QI gall extract (Caliot *et al.*, 2012). Several other mechanisms which confer its resistance to the antimicrobial agents include the efflux pumps and ribosomal modification (De Mouy *et al.*, 2001; Fitoussi *et al.*, 2001). This strategy might also affect the respond of *S. agalactiae* towards the gall extract. Therefore, higher concentration of the extract is necessary to increase its inhibitory effect and the bacterium sensitivity. Morphological abnormality such as

cell lumpiness and loss of appendages was clearly observed on the treated *P. vulgaris* cells rendering its inactivity, defective cell wall and subsequent cell death. Absence of peptidoglycan may cause the bacteria to succumb to the large osmotic pressure differences across the cytoplasmic membrane and lyse.

This study also reports some evidence to the effects of QI gall extract on cell morphology of yeast at different concentrations. The effects of the QI gall methanolic extract on the morphology of *C. albicans* appeared destructive as observed under the SEM with the presence of the un-dividing cells, bleb-like structures on the cell surface and biconcave form. Similar to the effect on *P. vulgaris* cells described above, the extract may interfere with the fungal cell wall synthesis and cell division.

High total tannin content in the QI gall extract suggests it may be responsible for the antimicrobial properties against several microorganisms (Darogha, 2009; Hassan, 2011). The source of QI galls used in this study were as those used in the previous studies (Nur Saeida *et al.*, 2014; Baharuddin *et al.*, 2015) and they have been tested phytochemically for the presence of major polyphenols namely 1,2,3-benzenetriol or pyrogallol. The hydroxyl groups and alpha-beta double bonds in the phenolic compound contributes to the activities of killing or inhibiting the microbial growth by interfering with the cell wall or cell membrane biosynthesis (Akiyama *et al.*, 2001). This is indicated in this study by which the mechanisms of antimicrobial activity may be related to the ability of tannins to weaken the membrane of the treated cells (Suwalak and Voravuthikunchai, 2009).

The mechanisms of microbial growth inhibition of tannins were not only in a direct way by interacting with membranes, the tannins are also able to form complex with metal ions which results in an indirect inhibition causing nutrients unavailable for the treated bacteria (Akiyama *et al.*, 2001). In this case, tannins might inhibit the growth of *S. agalactiae* in an indirect way instead of interacting with the membranes, cell walls and/or extracellular protein.

However, further studies are required to improve the findings by comparing the effects of methanolic QI gall extract on the morphology of Gram-positive bacteria, Gram-negative bacteria and yeast using TEM. Information of ultrastructural changes inside the cells is also useful to give a better understanding of correlation between ultrastructural changes and the mechanisms of antibacterial and anticandidal activity that causes biochemical alternations occurring within the cells. Besides, further knowledge on the mechanisms of action may help in the rational development of antimicrobial agents that target specific part of cell functions.

In conclusion, this study showed that QI gall extract has promising antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and yeast. Considerable extent of morphological changes was observed in all microorganisms (except *S. agalactiae*) and is concentration-dependent. QI gall methanolic extract may exert antimicrobial effect towards the tested microorganisms in several other mechanisms.

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