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In vitro antibacterial activity of *Quercus infectoria* gall extracts against multidrug resistant bacteria

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Abstract. Antimicrobial activities of plants have long been evaluated for their promising use as antimicrobial agent and in minimizing the unwanted resistance effects of microorganisms. The study was conducted to evaluate the antibacterial activity of *Quercus infectoria* gall crude extracts against multidrug resistant (MDR) bacteria *in vitro*. The screening test was determined by disc diffusion technique using sterile filter paper discs impregnated with 1 mg/disc (50 mg/ml) aqueous and ethanol extracts of *Q. infectoria* galls tested on five selected MDR bacterial strains. The minimum inhibitory concentration (MIC) was determined using the twofold serial micro dilution technique at concentration ranging from 5.00 mg/ml to 0.01 mg/ml. The minimum bactericidal concentration (MBC) was determined by sub culturing the microtitre wells showing no turbidity on the agar plate to obtain the MBC value. Both extracts showed substantial inhibitory effects against methicillin resistant coagulase negative *Staphylococcus* (MRCoNS) and methicillin resistant *Staphylococcus aureus* (MRSA). A slightly reduced inhibitory zone diameter was observed with MDR *Acinetobacter* sp. while no inhibitory effect was displayed among the extended spectrum beta lactamases (ESBL) *K. pneumoniae* and ESBL *E. coli* isolates. A significant difference in the zone sizes between both extracts was only observed in MRSA ($p < 0.05$). The MIC values ranged from 0.08 mg/ml to 0.63 mg/ml for aqueous and ethanol extracts against MRSA, MRCoNS and MDR *Acinetobacter* sp. while their MBC to MIC ratio values were 2 and less. The *Q. infectoria* gall extracts have shown very promising *in vitro* antibacterial activities and may be considered as a potentially good source of antimicrobial agent especially against MDR Gram positive bacteria.

INTRODUCTION

The emergence of MDR bacteria had become one of major public health concerns due to the increasing trend of infection incidences. Nosocomial and community acquired infections caused by these microorganisms are widely spread worldwide which often associated with extremely limited antibiotic treatment options, thus, leading to higher morbidity and mortality among the infected patients (Siegel *et al.*, 2007). Indiscriminate and widespread use of antimicrobial drugs for therapy or prophylaxis in the treatment of infectious diseases has led to the increased number of microbial resistance (Levy, 1998, 2002; Cowan, 1999; Engel, 2009).

Development of multidrug resistance in bacteria and non-availability of safe, cheap and effective antimicrobial agents necessitate a search for new antimicrobial substances from natural sources including plants. Of late, much attention was given to the search of new sources of antimicrobial agents especially from medicinal plants by the pharmaceutical and scientific figures. Thus, it would be beneficial to look for plant-derived antimicrobial compounds which are useful as an alternative strategy in the treatment of infections related to antimicrobial resistance bacteria. One of the potential plants that have been studied for their antibacterial properties is *Quercus infectoria*.

Quercus infectoria is grouped in the family Fagaceae. In Malaysia, the galls of *Q. infectoria* are known as "Manjakani" nut or seed. The galls have been reported to have antibacterial (Basri & Fan, 2005; Chusri & Voravuthikunchai, 2008; Vermani *et al.*, 2009; Basri *et al.*, 2012), antifungal (Yamunarani *et al.*, 2005), antiinflammatory (Kaur *et al.*, 2004), antioxidant (Kaur *et al.*, 2008) and wound healing properties (Umachigi *et al.*, 2008). It has also been used as local anaesthetic agents (Dar *et al.*, 1976) and in the post-partum period (Soon *et al.*, 2007). The galls contain about 50-70% of tannin, small amount of free gallic acid and ellagic acid which have shown to be the active compounds responsible for the antibacterial activity (Ikram & Nowshad, 1977; Kaur *et al.*, 2008).

Extracts from *Q. infectoria* galls have been reported to inhibit the growth of both Gram positive and Gram negative bacteria *in vitro* by interfering with cell division and altering the cell morphology (Suwalak & Voravuthikunchai, 2009; Sathirapathkul & Leela, 2011). Though previous report has determined the *in vitro* activity of *Q. infectoria* extracts against methicillin resistant *Staphylococcus aureus* (MRSA) (Chusri & Voravuthikunchai, 2008), however to the best of our knowledge, previous study on its activity against other commonly isolated MDR bacteria in hospital settings is still lacking. Thus, this study was conducted to evaluate the antibacterial activity of aqueous and ethanol gall extracts of *Q. infectoria* against few selected strains of MDR bacterial isolates.

METHODOLOGY

Study Design

The current preliminary study was designed to obtain data on the *in vitro* antibacterial activity of *Q. infectoria* gall extracts towards selected MDR bacterial strains.

Plant materials and preparation of crude extracts

The galls of *Q. infectoria* used in this study were obtained from the local market in Kota Bharu, and identified based on its physical characteristics as described previously by Soon *et al.* (2007). The dried galls obtained were crushed to small pieces using pestle and mortar and powdered in an electric grinder prior to extraction. Two hundred grams of the dried gall powder was immersed in 1000 ml of distilled water and ethanol in the ratio of 1:5 and incubated in water bath at 50°C for 24 hours. The solution was stirred regularly to ensure complete mixing. The mixture was then filtered and concentrated under reduced pressure by using a rotary evaporator. For aqueous crude extract preparation, the resulting concentrate was finally pounded to freeze-dryness whereas for ethanol crude extract preparation, the pellet was put under fume hood to remove excess ethanol (Srivastava *et al.*, 2007). The extracts were stored in a sterile container at -20°C until further use. Prior to use, the extracts were freshly dissolved in 10% dimethyl sulfoxide (DMSO, Merck, Germany) to a final concentration of 50 mg/ml for disc diffusion test and 100 mg/ml for broth dilution microtiter plate assay.

Bacterial strains

Pure culture of five clinical isolates (one isolate from each bacterial species), namely methicillin resistant *Staphylococcus aureus* (MRSA), methicillin resistant coagulase negative *Staphylococcus* (MRCoNS), multidrug resistant *Acinetobacter* sp. (MDR *Acinetobacter* sp.), extended spectrum beta lactamase *Escherichia coli* (ESBL *E. coli*) and extended spectrum beta lactamase *Klebsiella pneumoniae* (ESBL *K. pneumoniae*) were obtained through the courtesy of stock culture unit in the Medical Microbiology and Parasitology Laboratory, School of Medical Sciences, USM. Each isolate was identified based on the standard biochemical tests and the resistance to different antimicrobial agents was determined using the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations. Ethical approval for the collection of these clinical isolates in the study was obtained from the Human Research Ethics Committee, USM (USMKK/PPP/JEPeM [257.4.(3.2)]).

Staphylococcus aureus ATCC 25923 and MRSA ATCC 43300 were used as reference strains. The bacteria were grown and maintained by sub-culturing periodically on the sheep blood agar or MacConkey agar media at 37ºC for 18-24 hours. The bacterial suspension of each test strain was freshly prepared at concentration of 10^8 cells per ml or McFarland equivalent of 0.5 for the disc diffusion assay and broth microdilution technique.

Screening of antibacterial activity

A modified disc diffusion method based on Clinical and Laboratory Standards Institute (CLSI) guidelines (2012) was performed to screen for the antibacterial activity. Sterile filter paper discs (Whatman No. 1, 6 mm) were impregnated with 20 µl of each of the extracts (50 mg/ml) to give the final concentration of 1 mg/disc. The discs were left to dry in the fume hood overnight. Mueller Hinton agar was used as the media for the test microorganisms. The bacterial inoculum was spread evenly onto the surface of the Mueller Hinton agar using a sterile cotton swab followed by placing the impregnated discs onto the inoculated agar surface. Three replicates of each extract were assayed and the mean values were then referred. Discs containing sterile distilled water served as negative control. Vancomycin (30 µg) and imipenem (10 µg) discs were used as positive control for the Gram positive and ESBL Gram negative bacteria respectively. Colistin (10 µg) disc was used as positive control for MDR *Acinetobacter* sp. All plates were incubated at 37ºC for 24 hours. The antibacterial activity was interpreted based on the diameter of clearing zone measured to the nearest millimeter (mm) surrounding the disc indicating growth inhibition.

Determination of MIC and MBC

The minimum inhibitory concentration (MIC) value of each extract was determined for bacterial strains which exhibited inhibitory zones. The assay was performed using twofold serial microdilution in the 96-well microtiter plate as described previously by CLSI (2012). Briefly, 100 µl of Mueller Hinton broth was pipetted into test and control wells (growth and sterility controls). Subsequently, twofold serial dilution of extracts was performed in the test wells giving rise to concentrations ranging from 5.00 mg/ml to 0.01 mg/ml. Then, 5 µl of the diluted bacterial suspensions (final inoculum of 10^5 bacteria per ml) were added to the wells and mixed thoroughly. The extracts in broth were used as negative control to ensure medium sterility while the bacterial suspensions in broth served as positive control to control the adequacy of the broth for bacterial growth. Each extract was assayed in triplicates. After an overnight incubation at 37ºC, the MIC values were taken as the lowest concentration of the extracts in the wells that showed no turbidity. Subsequently, the minimum bactericidal concentration (MBC) value was determined by sub-culturing the wells which showed no turbidity onto nutrient agar plate. The lowest concentration of extract showing no visible growth on the agar plate after an overnight incubation at 37ºC was considered as MBC value.

Statistical analysis

GraphPad Prism 6 software was used for data entry and statistical analysis. Independent *t*-test was used for statistical comparison of the mean values for inhibition zone diameter obtained from aqueous and ethanol extracts.

RESULTS

Screening of antibacterial activity

The antibacterial activity of both *Q. infectoria* aqueous and ethanol gall extracts against each isolate of MDR bacteria were tabulated (Table 1). Both extracts exhibited inhibitory effects against each MDR bacterial species tested except for ESBL entero-bacteriaceae (*E. coli* and *K. pneumonia*e). The size of inhibitory zones of aqueous extract against MRSA clinical isolate (Figure 1) was significantly higher ($P < 0.05$) as compared to those of ethanol extract. Both extracts however displayed relatively larger

zone sizes against MRCoNS compared to other bacterial strains tested. Overall, the inhibitory effects of both gall extracts were stronger against Gram positive MDR bacteria compared to the Gram negative bacteria at the extract concentration used.

Detemination of MIC and MBC values

The MIC and MBC values of the aqueous and ethanol extracts against MDR bacteria (MRSA, MRCoNS and *Acinetobacter* spp.) were shown in Table 2 and Table 3 respectively and summarized in Table 4. The MIC and MBC values of both extracts against each bacterium species (ranged from 0.08 to 0.63 mg/ml) correlated well to the screening test results.

DISCUSSION

MDR bacteria such as MRSA, MRCoNS, MDR *Acinetobacter* spp and ESBL enterobacteriaceae are among the most prevalent pathogens implicated in the occurrence of both nosocomial and community-acquired infections in Malaysia (Lim *et al.*, 2009; Kong *et al.*, 2011; Nurul Azirah *et al.*, 2011; Nazmul *et al.*, 2012). These isolates were studied because they have become increasingly recognized as agents of clinically significant infections that cause major concern in public health as they invoke tremendous financial burden as well as enhanced morbidity and mortality due to hard-to-treat systemic infections.

Table 1. Antibacterial activity of *Q. infectoria* gall extracts (1mg/disc) against MDR bacteria by disc diffusion test

Bacterial species	Inhibition zone diameter (mm ± SEM)†				*P value
	Aqueous extract	Ethanol extract	Positive control	Negative control	
MRSA ATCC 43300	*13.67 ± 0.33	*13.33 ± 0.33	18.50 ± 0.22	–	0.52
S. aureus ATCC 25923	*14.67 ± 0.33	*14.33 ± 0.33	17.33 ± 0.33	–	0.52
MRSA	*17.00 ± 0.00	*15.33 ± 0.33	15.00 ± 0.58	–	0.02
MRCoNS	*20.33 ± 0.33	*19.33 ± 0.33	14.67 ± 0.33	–	0.16
MDR *Acinetobacter* sp.	*12.67 ± 0.33	*12.67 ± 0.33	12.00 ± 0.00	–	0.52
ESBL *E. coli*	–	–	26.00 ± 0.00	–	
ESBL *K. pneumoniae*	–	–	25.00 ± 0.58	–	

† Mean value of three determinations, each from different plates
* Independent *t*-test
– No inhibition zone

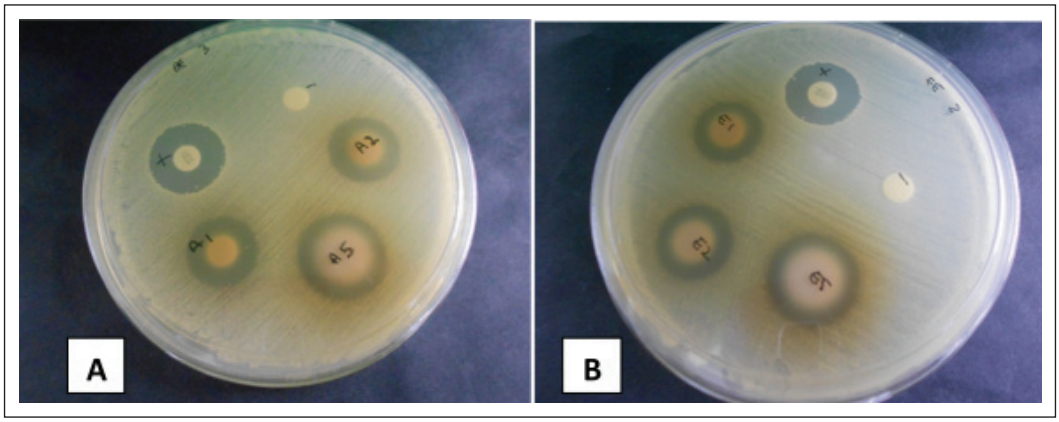

Figure 1. A replicate of the disc diffusion test showing inhibition zones of extracts (A. aquoeus; B. ethanol) and control discs on the agar plates inoculated with MRSA clinical isolate

Table 2. Determination of the MIC values of *Q. infectoria* gall extracts against MDR bacteria

Concentration (mg/ml)	MRSA		MRCoNS		MDR *Acinetobacter* sp.		Control	
	Aqueous	Ethanol	Aqueous	Ethanol	Aqueous	Ethanol	Pos	Neg
5.00	–	–	–	–	–	–	+	–
2.50	–	–	–	–	–	–	+	–
1.25	–	–	–	–	–	–	+	–
0.63	–	–	–	–	–	–	+	–
0.31	–	–	–	–	+	+	+	–
0.16	–	–	–	–	+	+	+	–
0.08	–	–	–	–	+	+	+	–
0.04	+	+	+	+	+	+	+	–
0.02	+	+	+	+	+	+	+	–
0.01	+	+	+	+	+	+	+	–

– Absence of growth (no turbidity), + Presence of growth (show turbidity), Positive control: bacterial suspensions in Mueller Hinton Broth (MHB), Negative control: extracts and broth only

Table 3. Determination of the MBC values of *Q. infectoria* gall extracts against MDR bacteria

Concentration (mg/ml)	MRSA		MRCoNS		MDR *Acinetobacter* sp.	
	Aqueous	Ethanol	Aqueous	Ethanol	Aqueous	Ethanol
5.00	–	–	–	–	–	–
2.50	–	–	–	–	–	–
1.25	–	–	–	–	–	–
0.63	–	–	–	–	–	–
0.31	–	–	–	–	ND	ND
0.16	–	–	–	–	ND	ND
0.08	+	ND	+	–	ND	ND
0.04	ND	ND	ND	ND	ND	ND

+ : Presence of bacterial growth
– : Absence of bacterial growth
ND : Not done because the microtiter well at the tested concentration showed the presence of bacterial growth (turbidity) as shown in Table 2

Table 4. Summary of MIC and MBC values of *Q. infectoria* gall extracts against MDR bacteria

Bacterial species	Extracts	MIC (mg/ml)	MBC (mg/ml)	MBC/MIC ratio
MRSA ATCC 43300	Aqueous	0.16	0.16	1
	Ethanol	0.08	0.08	1
S. aureus ATCC 25923	Aqueous	0.16	0.31	2
	Ethanol	0.08	0.16	2
MRSA	Aqueous	0.08	0.16	2
	Ethanol	0.16	0.16	1
MRCoNS	Aqueous	0.08	0.16	2
	Ethanol	0.08	0.08	1
MDR *Acinetobacter* sp.	Aqueous	0.63	0.63	1
	Ethanol	0.63	0.63	1

Various solvents are available and can be used to extract the active compounds from this plant. Aqueous solvent was used in this study because water is a universal solvent which was used traditionally to extract plant products with antimicrobial activity. Ethanolic extract of plants was reported to have high antimicrobial activity (Chusri & Voravuthikunchai, 2008; Satirapathkul & Leela, 2011) and relatively low toxicity to the test organisms (Eloff, 1998). Therefore we compared both extracts to establish their potential inhibitory effects on the MDR bacterial growth.

In this study, both aqueous and ethanolic gall extracts demonstrated antibacterial activity against MDR bacteria based on disc diffusion test results except for ESBL organisms. The screening test of the extracts against MRSA showed that aqueous extract produced significantly larger inhibition zone as compared to ethanolic extract. On the contrary, studies by Chusri & Voravuthikunchai (2008 & 2011) demonstrated higher inhibitory effect of ethanolic extract against MRSA as compared to aqueous extract.

Strong antibacterial activities of both extracts were observed not only against MRSA but also MRCoNS and relatively weak inhibitory effects against MDR *Acinetobacter* sp. in which the inhibition zone diameters were all larger than the positive controls (Table 1). No antibacterial activity against the ESBL group of gram negative bacteria was detected in the disc diffusion test probably due to the need of method standardization among different researchers for the *in vitro* screening assay. MIC and MBC values could probably be obtained for the ESBL producers if tested further as indicated in previous study by Suwalak & Voravuthikunchai (2009).

Previous reports stated that alcoholic solvents tend to possess a higher antibacterial activity compared to aqueous extracts (Parekh *et al.*, 2005; Satirapathkul & Leela, 2011). In this study, the MIC values of the extracts against the tested MDR bacteria ranged from 0.08 mg/ml to 0.63 mg/ml which correlated well with the results obtained using disc diffusion method. Both extracts showed similar MIC values against each MDR bacteria tested except for MRSA in which the MIC value for the aqueous extract (0.08 mg/ml) was relatively lower than the ethanolic extract (0.16 mg/ml).

On overall consideration, the antimicrobial activities of alcoholic and water extracts were higher as compared to those of less polar extracts such as hexane and chloroform (Satirapathkul & Leela, 2011). This may imply that the bioactive molecules responsible for the antimicrobial action could be more hydrophilic in nature. The similarity in the antimicrobial activity of both aqueous and ethanolic extracts suggests that these extracts might have high tannin content. Furthermore tannin in *Q. infectoria* galls which could be the principle antibacterial compound is soluble in water, alcohol and acetone.

Based on the size of inhibition zones and MIC values obtained in this study, it was found that both aqueous and ethanolic crude extracts of *Q. infectoria* gall inhibited Gram positive bacteria relatively better than the Gram negative bacteria. These findings corresponded with previous study reported by Basri & Fan (2005) and Basri *et al.* (2012) which regarded the difference in cell wall composition as one of the attributing factors. Gram positive bacteria contain peptidoglycans on their cell wall and lack an outer membrane. Meanwhile, the presence of the outer membrane in the Gram negative bacteria which is composed of complex hydrophobic lipopolysaccharides acts as a relatively stronger barrier in preventing morphologic alteration of the cell wall (Satirapathkul & Leela, 2011).

The phytochemicals in *Q. infectoria* gall extracts may inhibit the bacteria by different mechanisms from the currently used antibiotics and probably add to the clinical value in the treatment of resistant microbial strains. The antimicrobial properties shown are mainly due to the presence of tannin which is the major constituent present in *Q. infectoria* (Dar *et al.*, 1976; Ikram & Nowshad, 2009). Tannin is a hydrophilic compound usually extracted from aqueous and hydrophilic organic solvent and inhibits microbial growth by forming complex

molecules with microbial enzymes as well as membranes of microorganisms, altering the bacterial metabolism by inhibition of oxidative phosphorylation and reducing iron concentration through precipitation with various nitrogen containing groups of protein (Scalbert, 1991). There was a report on MRSA and staphylococci treated with *Q. infectoria* extract which resulted in reduced ability of cell survival due to significant loss of tolerance to the variation in osmotic pressure but not because of direct cell lysis of the bacteria (Chusri & Voravuthikunchai, 2011).

MBC is one of various *in vitro* microbiological parameters used to determine the bactericidal activity of antimicrobial agents. Microbiological definition of bactericidal activity has been taken arbitrarily as a ratio of MBC to MIC of 4 or less (Pankey & Sabath, 2004). In this study, the MBC values of the extracts ranged from 0.08 mg/ml to 0.63 mg/ml. The MBC/MIC ratio of both extracts against MRSA, MRCoNS and MDR *Acinetobacter* sp. were 2 and less. These findings indicated that, both extracts might be regarded bactericidal for all tested MDR bacteria. Previous study by Basri & Fan (2005) reported similar findings when the extracts were tested against *S. aureus* and *S. typhimurium*.

The results of this study suggest the potential use of *Q. infectoria* gall extracts as one of the effective phytotherapeutic agents in the treatment of MDR bacterial infections. More in-depth studies to identify the bioactive compound(s) responsible for the antibacterial properties of *Q. infectoria* gall extracts against MDR bacteria, testing their effectiveness and toxicity *in vivo* are warranted in the near future.

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