# THE EFFECTS OF *Quercus infectoria* GALL EXTRACT ON THE MORPHOLOGY OF GRAM-POSITIVE BACTERIA, GRAM-NEGATIVE BACTERIA AND YEAST

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By

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**CERTIFICATE**

This is to certify that the dissertation entitled "The Effects of *Quercus infectoria* Gall Extract on the Morphology of Gram-Positive Bacteria, Gram-Negative Bacteria and Yeast" is the bonafide record of research work done by Ms NorHaswani binti Abd Rahman during the period from September 2013 to May 2014 under my supervision.

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# TABLE OF CONTENTS


| List of Content | Page |
|---|---|
| CERTIFICATE | i |
| ACKNOWLEDGEMENTS | ii |
| TABLE OF CONTENTS | iii |
| LIST OF TABLES | vi |
| LIST OF FIGURES | vii |
| LIST OF PLATES | viii |
| LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS | ix |
| ABSTRAK | xi |
| ABSTRACT | xiii |
| CHAPTER 1. INTRODUCTION | 1 |
| 1.1 Background of Study | 1 |
| 1.2 Rationale of Study | 3 |
| 1.3 Objectives of Study | 3 |
| CHAPTER 2. LITERATURE REVIEW | 5 |
| 2.1 *Quercus infectoria* Gall | 5 |
| 2.2 Medicinal Plants Extraction and Phytochemical Properties | 6 |
| 2.3 Microorganisms | 8 |

2.3.1 *Streptococcus agalactiae* 10

2.3.2 *Proteus vulgaris* 11

2.3.3 *Candida albicans* 13

2.4 Antimicrobial Activity of *Q. infectoria* Gall Extract 14

2.4.1 Scanning Electron Microscope 16

CHAPTER 3. METHODOLOGY 18

3.1 Study Design 18

3.2 Equipment and Instrument 18

3.3 Materials 19

3.4 Plant Materials and Preparation of Crude Extract 21

3.5 Microorganisms 21

3.6 Screening of Antimicrobial Activity 22

3.7 Determination of MIC and MBC 23

3.8 Morphological Observations under Scanning Electron Microscope (SEM) 24

3.9 Statistical Analysis 25

CHAPTER 4. RESULTS 26

4.1 Disc Diffusion Test 26

4.2 Minimum Inhibitory Concentration (MIC) 31

4.3 Minimum Bactericidal Concentration (MBC) and Minimum Fungicidal Concentration (MFC) 34

4.4 Scanning Electron Microscope 37

CHAPTER 5. DISCUSSION 41

CHAPTER 6. CONCLUSIONS 47

6.1 General Conclusion 47

6.2 Limitation 48

6.3 Recommendation/ future studies 48

REFERENCES 49

APPENDICES 59

Appendix 1: Preparation of agar and broth media 59

Appendix 2: Research output 65

**List of Tables** **Page**

Table 2.1: Comparison of cell wall characteristics of Gram-positive bacteria, Gram-negative bacteria and yeast 9

Table 3.1: Equipments and instruments 18

Table 3.2: Materials 19

Table 4.1: The mean diameter of inhibition zones using different concentrations (1 mg/disc (20 mg/ml), 2 mg/disc (40 mg/ml) and 5 mg/disc (100 mg/ml)) of methanol *Q. infectoria* gall extract against tested bacteria and yeast 26

Table 4.2: MIC values of *Q. infectoria* gall extract against tested Gram-positive bacteria and Gram-negative bacteria 31

Table 4.3: MIC value of *Q. infectoria* gall extract against tested yeast 32

Table 4.4: MBC values of *Q. infectoria* gall extract against tested Gram-positive bacteria and Gram-negative bacteria. 34

Table 4.5: MFC value of *Q. infectoria* gall extract against tested yeast 35

Table 4.6: Summary of MIC and MBC or MFC values of *Q. infectoria* gall extract against tested Gram-positive bacteria, Gram-negative bacteria and yeast 36

**List of Figures** **Page**

Figure 2.1: Galls of *Q. infectoria* 5

Figure 4.1: Comparison of the mean inhibition zone diameter of different extract concentrations against *S. agalactiae* (a), *P. vulgaris* (b) and *C. albicans* (c) 27

Figure 4.2: Comparison of the mean inhibition zone diameter of 1 mg/disc extract against *S. agalactiae*, *P. vulgaris* and *C. albicans* 28

Figure 4.3: Scanning electron microscope images of *S. agalactiae* ATCC 13813 treated with the *Q. infectoria* gall extract at 12 h 38

Figure 4.4: Scanning electron microscope images of *P. vulgaris* ATCC 49312 treated with the *Q. infectoria* gall extract at 12 h 39

Figure 4.5: Scanning electron microscope images of *C. albicans* ATCC 10231 treated with the *Q. infectoria* gall extract at 24 h 40

**List of Plates**

Plate 4.1: Disc Diffusion test of 1 mg/disc (20 mg/ml), 2 mg/disc (40 mg/ml) and 5 mg/disc (100 mg/ml) extract from gall of *Q. infectoria* against Gram-positive bacteria, Gram-negative bacteria and yeast. *S. agalactiae* (A), *P. vulgaris* (B), *C. albicans* (C) 30

Plate 4.2: Determination of MIC values of *Q. infectoria* gall extract against *P. vulgaris* (upper side) and *S. agalactiae* (down side). Arrows showed the MIC values of methanol extract against *P. vulgaris* and *S. agalactiae* were 0.63 mg/ml 33

Plate 4.3: Determination of MIC value of *Q. infectoria* gall extract against *C. albicans*. An arrow showed the MIC value of methanol extract against *C. albicans* was 0.75 mg/ml 33

## LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

| | |
|---|---|
| - | To/Until |
| % | Percentage |
| / | Over to |
| = | Equal to |
| > | More than |
| α | Alpha |
| °C | Degree Celcius |
| μl | Microliter |
| ml | Millilitre |
| L | Liter |
| m | Meter |
| mm | Millimeter |
| μg | Microgram |
| mg | Milligram |
| g | Gram |
| MHA | Mueller Hinton agar |
| MHB | Mueller Hinton broth |
| RPMI | Roswell Park Memorial Institute |
| ANOVA | Analysis of variance |
| ATCC | American Type Culture Collection |
| CAMP | Christie Atkins Munch-Petersen |

| | |
|---|---|
| CLSI | Clinical and Laboratory Standards Institute |
| MBC | Minimum bactericidal concentration |
| MFC | Minimum fungicidal concentration |
| MIC | Minimum inhibitory concentration |
| FEG | Field emission gun |
| IBM | International Business Machines |
| SEM | Scanning electron microscope |
| SPSS | Statistical Package for the Social Sciences |
| UTI | Urinary tract infection |
| WHO | World Health Organization |

# ABSTRAK

## KESAN EKSTRAK MANJAKANI KE ATAS MORFOLOGI BAKTERIA GRAM NEGATIF, BAKTERIA GRAM-POSITIF DAN KULAT

Ekstrak manjakani telah dikenalpasti memiliki aktiviti yang meluas terhadap bakteria Gram-positif, bakteria Gram-negatif dan kulat. Walau bagaimanapun, kajian-kajian dalam mengenal pasti dan membandingkan mekanisma antimikrob fitokimia didalam ekstrak manjakani masih lagi terhad dan tidak banyak diterbitkan. Tujuan kajian ini adalah untuk menentukan aktiviti antimikrob daripada ekstrak methanol manjakani terhadap bakteria Gram-positif, bakteria Gram-negatif dan kulat, serta untuk mengkaji kesan ekstrak manjakani ke atas morfologi sel mikroorganisma yang diuji. Aktiviti antimikrob ekstrak manjakani ini telah diperiksa menggunakan kaedah cakera resapan keatas bakteria Gram-positif (*S. agalactiae*), bakteria Gram-negatif (*P. vulgaris*) dan kulat (*C. albicans*). Kepekatan minimum halangan (MIC) telah ditentukan menggunakan teknik siri dua kali ganda micropencairan pada kepekatan antara 0.01 mg/ml dan 5.00 mg/ml (untuk bakteria) dan antara 0.02 mg/ml dan 12.00 mg/ml (untuk kulat). Kepekatan minimum bakterisidal (MBC) dan kepekatan minimum fungisidal (MFC) telah ditentukan oleh subkultur plat telaga yang menunjukkan tiada pertumbuhan yang jelas di atas plat agar. Kemudian, perubahan morfologi sel-sel yang diuji (1xMIC dan 4xMIC) diperhatikan di bawah mikroskop imbasan elektron (SEM). Satu aktiviti antimikrob yang lebih tinggi ditunjukkan terhadap *C. albicans* dalam kajian ini. Berdasarkan nisbah MBC kepada MIC atau MFC kepada MIC, ekstrak ini dianggap sebagai bakterisidal dan fungisidal terhadap

mikroorganisma yang diuji. Perubahan morfologi telah diperhatikan pada setiap sel yang diuji, tetapi perubahan yang lebih ketara dapat dilihat pada 4xMIC di bawah SEM. Kajian awal ini menyediakan maklumat saintifik untuk kesan ekstrak manjakani terhadap morfologi bakteria dan kulat, di mana ia mencadangkan mekanisma antimikrob pada dinding sel dan keutuhan membran.

# ABSTRACT


## THE EFFECTS OF *Quercus infectoria* GALL EXTRACT ON THE MORPHOLOGY OF GRAM-POSITIVE BACTERIA, GRAM-NEGATIVE BACTERIA AND YEAST

The gall extract from *Q. infectoria* is well known to have a broad spectrum of activity against Gram-positive bacteria, Gram-negative bacteria and yeast. However, studies on identifying and comparing the antimicrobial mechanisms of phytochemicals in the *Q. infectoria* gall extract are still limited and not well established. The aims of this study were to determine the antimicrobial activity of methanolic *Q. infectoria* gall extract against Gram-positive, Gram-negative and yeast strains, and also to study the effects of the gall extract on the cell morphology of the tested strains. Antimicrobial activity of gall extract was screened using disc diffusion method against Gram-positive bacteria (*S. agalactiae*), Gram-negative bacteria (*P. vulgaris*) and yeast (*C. albicans*). Minimum inhibitory concentration (MIC) was determined using twofold serial microdilution technique at concentrations ranging between 0.01 mg/mL and 5.00 mg/mL (for bacteria) and between 0.02 mg/ml and 12.00 mg/ml (for yeast). Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were determined by subculturing the wells plate which showed no apparent growth or turbidity onto the agar plates. Morphological changes of treated cells (1xMIC and 4xMIC) were then observed under scanning electron microscope (SEM). A higher antimicrobial activity was observed against *C. albicans* in this study. Based on the MBC to MIC ratio or MFC to MIC ratio, the

extract was considered as bactericidal and fungicidal against the tested strains. Morphological changes were observed in all treated cells but more distinct at 4xMIC under SEM. This preliminary study provides scientific information to the effect of gall extract on the bacterial and fungal morphology suggesting its antimicrobial mechanism on the cell wall and membrane integrities.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

Medicinal plants have been used in traditional treatment in various parts of the world. In some Asian and African countries, 80% of the populations depend on traditional medicine for primary health care (WHO, 2008). According to statistics from the Ministry of Health, there were about 7,282 traditional and Chinese medicine practitioners in Malaysia (Pillay, 2006). The natural products and their derivatives have continued to be the most significant sources for development of new pharmaceutical agents.

Interest in the search for novel, safe and effective antimicrobial compounds has been triggered by the development of microorganisms resistance to antibiotics and the emergence of new infectious diseases (Jeyaseelan *et al.*, 2012). Although certain studies have been carried out on antimicrobial properties of certain types of plant, the mechanisms of inhibition in the cells of microorganisms by the plant extract are still poorly understood.

The nut gall of *Quercus infectoria* (Fagaceae) is one of the potential plant that has been pharmacologically documented for its antimicrobial activities. The gall is also known as "manjakani" in Malaysia, and it is one of the most popular natural sources used in traditional medicines in Asia (Basri *et al.*, 2012). *Q. infectoria* is a small shrub tree which can grow up to 2.0 m high. It is mainly found in Asia Minor,

Turkey, Syria, Persia, Cyprus, Greece and Iran (Leela and Satirapathkul, 2011; Basri *et al.*, 2012). The chemical constituents of the gall comprise a large amount of tannins (50-70%) and small amounts of free gallic acids (2-4%), ellagic acid and synergic acid (Sucilathangam *et al.*, 2012). The gall has also been pharmacologically documented as antidiabetic, antibacterial, antiviral, antifungal, larvicidal, anti-inflammatory, antiamoebic, antipyretic, antiparkinsonian, astringent, antitremorine, local anaesthetic and wound healing (Basri *et al.*, 2012; Rao *et al.*, 2013).

The gall extract from *Q. infectoria* is well known to have a broad spectrum of activity against Gram-positive bacteria, Gram-negative bacteria and yeast. Further understanding on the mechanisms of action may help researchers on the development of antimicrobial agents which attack cell functions of target microorganisms. Besides, 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 (Suwalak and Voravuthikunchai, 2009).

Hence, it would be beneficial to compare the effects of the gall extract on morphology between Gram-positive bacteria, Gram-negative bacteria and yeast, in order to correlate morphologic changes of microorganisms with the mechanisms of antibacterial and anticandidal activity that causes biochemical alternations occurring within the cells. This would also give a better understanding why some drugs are effective against certain microorganisms only.

## 1.2 Rationale of Study

Previous studies mostly compared the effect of *Q. infectoria* extract between certain Gram-positive bacteria and Gram-negative bacteria only. Although a number of studies have been carried out on antibacterial and anticandidal properties of different types of *Q. infectoria* extracts, to the best of our knowledge, no past research has been conducted to compare the effects of the gall extract on the morphology of the Gram-positive bacteria, Gram-negative bacteria and yeast.

Besides, studies identifying and comparing the mechanisms of phytochemicals in *Q. infectoria* gall extract against bacteria and yeasts are still limited and not well established. In addition, the mechanisms of inhibition in the cells of microorganisms by the gall extract are still poorly understood.

## 1.3 Objectives of Study

To study the effects of methanolic *Q. infectoria* gall extract on the morphology of a Gram-positive bacteria, a Gram-negative bacteria and a yeast.

Specific objectives:

1. To determine the antimicrobial activity of methanolic *Q. infectoria* gall extract against *Streptococcus agalactiae*, *Proteus vulgaris* and *Candida albicans*.

2. To compare the effects of methanolic *Q. infectoria* gall extract on the morphology of *S. agalactiae*, *P. vulgaris* and *C. albicans* using scanning electron microscope (SEM).

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 *Quercus infectoria* Gall

The gall of *Q. infectoria* Olivier (Fagaceae) is locally known as "manjakani", and it is one of the most popular natural sources used in traditional medicines in Asia (Basri *et al.*, 2012). *Q. infectoria* is a small shrub tree mainly found in Asia Minor, Turkey, Syria, Persia, Cyprus, Greece and Iran (Leela and Satirapathkul, 2011; Basri *et al.*, 2012). The gall arises on the young branches of this tree as a result of gall-wasp *Adleria gallae-tinctoria* attack (Sucilathangam *et al.*, 2012).

Figure 2.1: Galls of *Q. infectoria*

(Source:https://www.herbalveda.co.uk/index.php?dispatch=products.view&product_id=30375) accessed on 17th March 2014

*Q. infectoria* gall was reported to contain ellagic acid, tannins, sitosterol, methyl betulate, and methyl oleanolate. However, the chemical constituents of the gall have been reported to comprise a large amount of tannins (50-70%) and small amounts of free gallic acids (2-4%), ellagic acid and synergic acid (Sucilathangam *et al.*, 2012). Based on past studies, the gall of *Q. infectoria* has also been pharmacologically documented to possess activities such as antidiabetic, antibacterial, antiviral, antifungal, larvicidal, anti-inflammatory, antiamoebic, antipyretic, antiparkinsonian, astringent, antitremorine, local anaesthetic and wound healing (Basri *et al.*, 2012; Rao *et al.*, 2013).

## 2.2 Medicinal Plants Extraction and Phytochemical Properties

Solvent extraction is a technique that is usually used for the isolation of plant antimicrobial compounds. However, the extract yields of the plant materials for antimicrobial activities are depend on the nature of the extracting solvent. This is due to the presence of different compounds with various chemical characteristics and polarities that may or may not be soluble in a particular solvent (Sultana *et al.*, 2009).

Previous study showed that acetone was a better extractor, followed by methanol, ethanol, and water (Downey and Hanlin, 2010). Acetone was claimed as a useful solvent because it dissolves wide range of active plants components, volatility, miscibility with polar and non-polar solvents and has a low toxicity to test microorganisms (Eloff, 1998).

Tannin is a major compound in *Q. infectoria* with high concentration in the gall (Hassan, 2011). Tannin is the active compound that is responsible for the antimicrobial activity and is soluble in water, alcohol and acetone and gives precipitates with protein (Basri and Fan, 2005; Soon *et al.*, 2007; Leela and Satirapipathkul, 2011; Basri *et al.*, 2012). Tannin has also been shown in previous reports, especially from plant extracts to exhibit antimicrobial activity (Soon *et al.*, 2007).

Phytochemicals are a large group of plant-derived chemical compounds which can be divided into several classes: phenolics or polyphenols (flavonoids, phenolic acids, tannins, stilbenes, coumarins and lignans), carotenoids, alkaloids, sterols, terpenes and fibre (Scalbert *et al.*, 2005). Preliminary phytochemical screening shown that the various extracts of the *Q. infectoria* gall contains certain components such as saponins, alkaloids, tannins, glycosides, triterpenes, sterols, phenolic compounds, carbohydrates, and flavonoids (Shrestha *et al.*, 2014). Other phytochemicals such as amentoflavone, hexamethyl ether, iso-cryptomerin and beta-sitosterol have also been isolated from the gall (Khare, 2004). Phytochemicals produced by plants are usually used to protect themselves (Scalbert *et al.*, 2005), but certain molecules have antimicrobial properties and can be a great significance in therapeutic treatments (Gobalakrishnan *et al.*, 2013).

Polyphenols are aromatic compounds and secondary plant metabolites. Among the aromatic compounds, flavonoids and tannins are the most common molecules found in plant organs (Luthar, 1992). As the gall contain large amounts of tannin, this shows that tannin might be the active compound responsible for the antimicrobial activity.

## 2.3 Microorganisms

A Gram-positive bacterium is a class of bacteria that has a thick, multilayered cell wall consisting mainly of peptidoglycan surrounding the cytoplasmic membrane. Other components such as teichoic acids, lipoteichoic acids and complex polysaccharides may also be present in the Gram-positive cell wall (Navarre and Schneewind, 1999).

A Gram-negative bacterium has a complex cell wall components compared to Gram-positive cell walls. There are two layers external to the cytoplasmic membrane and a thin peptidoglycan layer next to the cytoplasmic membrane. A Gram-negative bacterium has a unique component, which is the outer membrane next to the peptidoglycan layer. The area between the external surface of the cytoplasmic membrane and the internal surface of the outer membrane is referred to as the periplasmic space, which contains a variety of hydrolytic enzymes and components of the sugar transport systems and other binding. Different from the Gram-positive bacteria, there are no teichoic or lipoteichoic acids in the Gram-negative cell wall (Le Brun *et al.*, 2013).

Yeasts are unicellular fungi which usually appear as oval cells. The yeasts are primarily made up of budding cells (blastoconidia). Some of these yeasts produce pseudohyphae and true hyphae. The yeast cell wall is made of three main groups of thick polysaccharide: polymers of mannose (mannoproteins), polymers of glucose (b-glucan) and polymers of N-acetylglucosamine (chitin) (Aguilar-Uscanga and Francois, 2003).

Table 2.1: Comparison of cell wall characteristics of Gram-positive bacteria, Gram-negative bacteria and yeast

| **Images** | | | |
|---|---|---|---|
| **Microorganisms** | Gram-negative bacteria | Gram-positive bacteria | Yeast |
| **Cell wall characteristics** | Have a thin cell wall, thick peptidoglycan layer, periplasmic space, outer membrane and high lipopolysaccharide | Have a thick cell wall, thin peptidoglycan layer and teichoic acid | Consist polymers of mannose (mannoproteins), polymers of glucose (b-glucan) and polymers of N-acetylglucosamine (chitin) |

(Adapted from Parija, 2009)

### 2.3.1 *Streptococcus agalactiae*

*Streptococcus agalactiae* is a beta-hemolytic Gram-positive streptococci which is also known as Group B streptococci (GBS). The Lancefield Group B carbohydrate (GBC) is a peptidoglycan-anchored antigen that have a thick cell wall which acts as an exoskeleton to prevent any mechanical stresses and to maintain the cell shape of the bacteria (Caliot *et al*, 2012).

*S. agalactiae* is part of the normal bacterial flora colonizing the gastrointestinal tract and genitourinary tract in the human body and it usually does not cause any symptoms. In some cases, however, the bacteria can be dangerous and cause various infections in neonates and young infants such as congenital pneumonia, sepsis or meningitis; in pregnant women such as urinary tract infection, chorioamnionitis, early postpartum endometritis, postcesarean section febrile morbidity; in adult with underlying diseases or certain chronic medical conditions such as diabetes, cardiovascular disease, congestive heart failure, obesity, and cancer (Hall *et al.*, 1992).

Since the early 1970s, GBS has been the leading pathogen causing serious perinatal infection in the USA and most developed countries (Zaleznik *et al.*, 2000). The Centers for Disease Control and Prevention (CDC) estimates that in 2010, GBS has caused about 1,000 cases of early-onset invasive disease per year (Verani *et al.*, 2010).

Early-onset infections are acquired vertically through exposure to GBS from the vagina of a colonized woman (Verani *et al.*, 2010). Maternal factors that increase the risk for early-onset disease include gestational age <37 weeks, longer duration of

membrane rupture, intra-amniotic infection, young maternal age, black race, fever during labor, multiple births, and low maternal levels of GBS-specific anticapsular antibody (Hall *et al.*, 1992; Verani *et al.*, 2010). As a result of prevention efforts such as intravenous intrapartum antibiotic prophylaxis and GBS vaccines, GBS cases reduced over the past 15 years, from 1.7 cases per 1,000 live births in the early 1990s to 0.34–0.37 cases per 1,000 live births in 2010 (Verani *et al.*, 2010).

The CAMP test is used for identification of *S. agalactiae*, since it is the only beta-hemolytic streptococci which gives a positive CAMP test. This test detects an extracellular protein produced by the bacteria that acts synergistically with the beta hemolysin produced by *S. aureus* to induce enhanced hemolysis of sheep RBCs. A known strain of *S. aureus* (ATCC 25923) is streaked down the centre of the sheep blood agar plate. Test inoculum is streaked at a 90° angle, perpendicular to *S. aureus* streak without touching it. A known Group B streptococci may also be streaked as a positive control or a known Group A streptococci as a negative control. The plate is incubated overnight at 35 - 37°C. An arrowhead-shaped area between the junction of growth of *S. aureus* and Group B streptococci indicates a positive result (Cheesbrough, 2006).

### 2.3.2 *Proteus vulgaris*

The genus *Proteus* is classified as the enteric bacteria. *P. vulgaris* is a short Gram-negative rod, motile, do non-spore producing (Herter and Broeck, 1911), facultative anaerobes and can usually be found in soil, intestinal tract of humans and animal, water and fecal matter (Talaiekhozani *et al.*, 2013). *Proteus* is an

opportunistic pathogen to human and is known to cause urinary tract infections and wound infection (Talaiekhozani *et al.*, 2013).

Individuals with compromised immune system and in long-term care facilities are easily infected with the bacteria. Patients with recurrent infections, long-term catheterization, structural abnormalities of the urinary tract, and those whose infections were acquired in the hospital are more susceptible to infection caused by *Proteus* (Struble *et al.*, 2013).

The helpful way to reduce transmission of nosocomial organisms is for all hospital personnel to wash their hands meticulously after each patient contact (Guentzel, 1996). Oral quinolone is usually used to treat individuals infected with the bacteria. Drugs such as trimethoprim/ sulfamethoxazole (TMP/ SMZ) can be used to treat uncomplicated urinary tract infections (UTIs) in women, while ceftriaxone, gentamicin and cephalosporin can be used to treat acute uncomplicated pyelonephritis and complicated UTIs in both men and women (Guentzel, 1996; Struble, 2013).

For laboratory identification test, *P. vulgaris* is a motile organism, produce hydrogen sulfide ($H_2S$) and indole positive in a sulfide indole motility (SIM) test. *P. vulgaris* also ferments glucose and sucrose in the fermentation test. The fermentation occurred is correlated with the yellow color of slant and butt in triple sugar iron (TSI) test. The yellow color of butt also indicates the bacteria are facultative anaerobes. Besides, *P. vulgaris* is known to give positive result for methyl red test and urease production, and can test positive or negative for citrate (Cheesbrough, 2006).

### 2.3.3 *Candida albicans*

*Candida albicans* is a diploid fungus that exists as yeast, pseudohyphal, and hyphal cells (Whiteway and Bachewich, 2007). The fungal is usually present on the skin, vagina, oral cavity, intestinal tract and rectum (Cao *et al.*, 2013). The fungus can also travel through the blood stream and affect the throat, intestines, and heart valves. *C. albicans* is a harmless commensal (Berman and Sudbery, 2002). However, this opportunist can cause some infections related with venous catheters, urinary catheters, and some other implanted devices, when the immune system is weakened and bacterial flora are eliminated (Kojic and Darouiche, 2004; Pukkila-Worley *et al.*, 2011).

The fungi are detectable in gastrointestinal and genitourinary tracts of about 70% of humans and about 75% of women (Kabir *et al.*, 2012). Candidiasis is common in intensive care units and frequent in neonatal care units where mortality rates reach 45–49% (Pukkila-Worley *et al.*, 2011; Kabir *et al.*, 2012). Although other Candida species such as *Candida glabrata*, *Candida krusei*, *Candida dubliniensis*, *Candida parapsilosis*, and *Candida tropicalis* can also be recovered from infected individuals, *C. albicans* remains a major infectious fungal agent (Gozalbo *et al.*, 2004).

Several factors have contributed to the increase in fungal infections. The most important is immunocompromised inviduals due to mucosal or cutaneous barrier disruption, defects in the number and function of lymphocyte and granulocyte, metabolic dysfunction, and advanced age (Kojic and Darouiche, 2004; Pfaller and Diekema, 2007). Furthermore, increasing use of broad-spectrum antibiotics,

cytotoxic chemotherapies, and transplantation may increase the risk for opportunistic fungi (Pfaller and Diekema, 2007).

It was proven that the mucosal candidiasis and invasive candidiasis in neutropenic patients can be treated by antifungal prophylaxis (Pfaller and Diekema, 2007). Besides, topical administration of antifungal drugs such as clotrimazole, miconazole, nystatin, tioconazole or oral administration of drugs such as fluconazole and amphotericin B is also can be used to treat most candidal infection (Pfaller and Diekema, 2007).

The Germ Tube is a commonly used method for the identification of *C. albicans*. This test is specific for *C. albicans* since it is the only yeast that produces germ tubes. A small number of yeast cells obtained from an isolated colony is suspended in human or rabbit serum that is placed into a tube. The tube is incubated at 35°C for 2 to 3 hours before the serum is examined under a microscope. *C. albicans* will produce germ tubes while *C. tropicalis*, *C. parapsilosis* and *C. glabrata* will not (Cheesbrough, 2006).

### 2.4 Antimicrobial Activity of *Q. infectoria* Gall Extract

*Q. infectoria* gall extracts using acetone, methanol, ethanol and aqueous extracts exhibited a great antimicrobial activity towards the growth of microorganisms and have a broad spectrum of activity against Gram-positive bacteria, Gram-negative bacteria and yeast (Suwalak and Voravuthikunchai, 2009). The gall was also potent against some strains of Gram-positive bacteria and Gram-negative bacteria with certain MIC value (Basri and Fan, 2005; Leela and Satirapipathkul, 2011; Basri *et*

*al*., 2012). In 2011, Leela and Satirapipathkul reported that methanol extract of *Q. infectoria* gall inhibited the growth of *B. subtilis, S. aureus* and *E. coli* at the MIC of 0.625 mg/ml, 1.250 mg/ml and 2.500 mg/ml respectively.

In 2012, Hassan showed *Q. infectoria* gall extracts were active against yeasts, such as *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. famata*. In his studies, alcoholic extract results in higher zone inhibition of growth of *C. albicans* compared with zone inhibition of growth of *C. glabrata.* While aqueous (boiled distilled water) extract results in higher zone inhibition of growth of *C. glabrata* compared with zone inhibition of growth of *C. albicans*. However, aqueous (distilled water at 25°C) extract results in same zone inhibition of growth of *C. glabrata* with zone inhibition of growth of *C. albicans* (Hassan, 2011; Hassan, 2012).

Antimicrobial actions of tannins have been reported as bacteriostatic and bactericidal against certain harmful bacteria such as *A. hydrophilla, E. coli, Listeria, Pseudomonas, Salmonella, Staphylococcus* and *Streptococcus* (Chung *et al*., 1998; Banso and Adeyemo, 2007; Doss *et al*., 2009). Previous studies showed that there were several mechanisms in tannins that may be involved in growth inhibition. Inhibitory mechanisms of tannins are explained as a direct inhibition caused by interacting with membranes, cell walls and/or extracellular proteins (Scalbert, 1991; Doss *et al*., 2009). Plant polyphenols have the potential to form complex with polymers and minerals which suggested tannin toxicity and the inhibition of microbial enzymes (Bossi *et al*., 2007). Besides, tannins are able to form complex with metal ions and cause an indirect inhibition by making nutrients unavailable for bacteria (Akiyama *et al*., 2001).

Previous studies demonstrate visual effects of the *Q. infectoria* gall extract on the morphology or ultrastructural of treated microorganisms (Suwalak and Voravuthikunchai, 2009; Leela and Satirapipathkul, 2011). Under scanning electron microscope (SEM), the effects such as irregularity in shape, loss of surface appendages and cell collapse can be seen on the microbial morphology of several treated strains. With transmission electron micrographic (TEM), ultrastructural changes such the presence of vacuoles in the cytoplasm and loss of cell integrity due to the effect of the extract were observed. The observation under SEM and TEM may help to correlate morphologic changes of microorganisms with the mechanisms of antimicrobial activities that cause biochemical alternations occurring within the cells.

### 2.5 Scanning Electron Microscope (SEM)

Electron microscopy is one of the techniques that is able to show the structure of much smaller bacteria in greater or finer details in their natural environment (Kaláb *et al.*, 2008). The electron microscope uses a focused beam of high-energy electrons to generate high-resolution image of specimens that allow imaging and quantification of surface topographic features (El Abed *et al.*, n.d.). There are two types of electron microscope, SEM and TEM.

SEM as a specialized field of science that uses the electron microscope as a tool. It is engineered to visualize images and provide microanalysis data from all specimens, with or without processing (fixed, dehydrated, and dried). The specimen is placed in a small place of high vacuum chamber at a temperature several degrees higher than the freezing point of water. A low partial pressure of water vapor

provides ions at a concentration sufficient to neutralize electrons to prevent any charging artifacts on the observed specimens (Kaláb *et al.*, 2008).

The effects of *Q. infectoria* gall extract on Gram-positive bacteria and Gram-negative bacteria has been previously demonstrated. The antibacterial effects of the *Q. infectoria* extract were seen on Gram-positive bacteria and Gram-negative bacteria such as staphylococci and *E. coli.* From the SEM observations, appearance of small bleb-like structures on the surface of cells with irregular spherical structures appearing to extrude from cells were observed in staphylococci (Leela and Satirapipathkul, 2012). With *E. coli*, exposure with the extract resulted in morphological defects characterized by cell elongation, roughening on the surface (Suwalak and Voravuthikunchai, 2009) and tubular outpouchings from the cell wall (Leela and Satirapipathkul, 2012).

Visual information of SEM is very useful in determining any changes on the microstructure of the cell of microorganisms and help in identifying the types or changes that occur on the cell composition in response to the test compound (Suwalak and Voravuthikunchai, 2009).

# CHAPTER 3

# METHODOLOGY

## 3.1 Study Design

This was a preliminary experimental study to observe the effect of *Q. infectoria* gall extract on the morphology of Gram-positive bacteria, Gram-negative bacteria and yeast. The study was conducted in the School of Health Sciences, Universiti Sains Malaysia, Kubang Kerian from September 2013 until May 2014.

## 3.2 Equipments and Instruments

The equipments and instruments used in this study are listed below:

Table 3.1: Equipments and instruments

| No. | Item |
|---|---|
| 1 | Autoclave machine |
| 2 | Beaker |
| 3 | Biological Safety Cabinet (BSC) Level II |
| 4 | Bunsen burner |
| 5 | Centrifuge |
| 6 | Dessicator |
| 7 | FEI Quanta 450 Scanning Electron Microscope |

| | |
|---|---|
| **8** | Filter funnel |
| **9** | Freezer (-20°C) |
| **10** | Incubator |
| **11** | Magnetic stirrer |
| **12** | Nephelometer |
| **13** | Pipettes |
| **14** | Refrigerator (4°C) |
| **15** | Rotary evaporator |
| **16** | Schott Duran bottle |
| **17** | Sputter coater |
| **18** | Water bath |

### 3.3 Materials

The materials used in this study are listed below:

Table 3.2: Materials

| **No.** | **Item** |
|---|---|
| **1** | Amphotericin B (10 µg/disc) |
| **2** | Carbon tape |
| **3** | Centrifuge tubes |
| **4** | Columbia Sheep Blood agar (commercially prepared; Oxoid, Thermo Scientific) |
| **5** | Dimethyl sulfoxide (DMSO) |

| | |
|---|---|
| **6** | Ethanol (50%, 75%, 95%, 100%) |
| **7** | Forceps |
| **8** | Gentamicin (10 µg/disc) antibiotic disc (Oxoid, Thermo Scientific) |
| **9** | Gloves |
| **10** | Hexamethyldisilazane (HMDS) |
| **11** | Mac Conkey agar powder (Oxoid, Thermo Scientific) |
| **12** | McDowell-Trump fixative |
| **13** | Membrane filter |
| **14** | Methanol (100%) |
| **15** | Mueller Hinton agar powder (Oxoid, Thermo Scientific) |
| **16** | Mueller Hinton with 5% Sheep Blood agar (commercially prepared; Oxoid, Thermo Scientific) |
| **17** | Mueller Hinton broth powder (Oxoid, Thermo Scientific) |
| **18** | Ofloxacin (5 µg/disc) antibiotic disc (Oxoid, Thermo Scientific) |
| **19** | Osmium tetroxide |
| **20** | Petri dish |
| **21** | Parafilm Seal |
| **22** | Pipette tips |
| **23** | Phosphate buffer |
| **24** | SEM pin stub mount |
| **25** | Sterile 96-wells microtitre plate with lid (Greiner bio-one) |
| **26** | Sterile cotton swab |
| **27** | Sterile distilled water |
| **28** | Sterile filter paper discs (Whatman No. 1, 6 mm) |
| **29** | Wire loop |

### 3.4 Plant Materials and Preparation of Crude Extract

The galls of *Q. infectoria* were obtained from a local herb store at Kota Bharu, Kelantan, and identified based on their physical characteristics as described by Shrestha *et al.* (2014). The galls were crushed to small pieces using pestle and mortar and grinded in a macerator machine prior to extraction.

The powdered form of dried gall was extracted by using methanol solvent. In the ratio of 1:5, 50 g of the gall powders was immersed in 250 ml of methanol and incubated in the water bath for 72 hours at 50°C. The mixture was filtered using the Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure by using a rotary evaporator. In order to obtain a powdered crude extract, the resulting pallet was pounded to dryness under air-dryer and stored in a sterile container at -20°C until further use.

The extract was freshly dissolved in distilled water ($dH_2O$) to a final concentration of 20, 40 and 100 mg/ml for disc diffusion test and 10 and 48 mg/ml for MIC microtiter plate assay. The choice of dose concentration was based on our previous optimization. The solutions were sterilized through membrane filter sized 0.2 μm.

### 3.5 Microorganisms

The bacterial and fungal strains used in this study were *Streptococcus agalactiae* (ATCC 13813), *Proteus vulgaris* (ATCC 49312) and *Candida albicans* (ATCC

10231). All the bacterial and fungal strains were grown and maintained by subculturing on the blood agar, Mac Conkey agar and Sabouraud Dextrose agar media for Gram-positive bacteria, Gram-negative bacteria and yeast respectively. All agar media were incubated at 37°C (for bacteria) and 35°C (for yeast) for 24 hours. Fresh suspensions of each test strain were prepared at concentration $10^8$ cells per ml for bacteria and $10^6$ cells per ml for yeast prior to disc diffusion test and micro-dilution assay which were standardized by adjusting the optical density of the microbial suspension to a turbidity corresponding to 0.5 Mac Farland by using nephelometry (CLSI, 2012).

### 3.6 Screening of Antimicrobial Activity

Antimicrobial activity was tested by the disc diffusion method that was modified according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (2012). 50 µl of each extract concentration (20, 40, 100 mg/ml) were impregnated onto sterile filter paper discs (Whatman No. 1, 6 mm) to give a final concentration of 1, 2 and 5 mg/disc. The discs were left to dry in the fume hood overnight. The bacterial and fungal inoculums were spread evenly onto the surface of the Mueller Hinton agar (for *P. vulgaris* and *C. albicans*) and Mueller Hinton with 5% sheep's blood agar (for *S. agalactiae*) using sterile cotton swab before the impregnated discs were positioned on the seeded medium. The extract was tested against the bacteria and yeast in triplicate. Impregnated disc with sterile distilled water was used as negative control. Antimicrobial agents such as ofloxacin (5 µg/disc), gentamicin (10 µg/disc) and amphotericin B (10 µg/disc) were used as a positive control for *S. agalactiae*, *P. vulgaris* and *C. albicans* respectively. All plates were incubated at

37°C (for bacteria) and 35°C (for yeast) for 24 hours. The diameter of inhibitory or clearing zone around each of the disc was measured to the nearest millimeter (mm) to determine the antimicrobial activity.

### 3.7 Determination of MIC and MBC

The minimum inhibitory concentration (MIC) of the methanol extract was determined for *S. agalactiae, P. vulgaris* and *C. albicans* using the twofold serial microdilution method which was performed in the 96-well microtiter plate, at a final concentration ranging from 5 mg/ml to 0.01 mg/ml for bacteria and from 12 to 0.02 mg/ml for yeast. Initially, the microtiter plate was labeled and 100 µl of Mueller-Hinton broth was pipetted into wells 1-11, while 200 µl of Mueller-Hinton broth was pipette into well 12. 100 µl of the tested extract was added into well 1. Subsequently, twofold serial microdilution from well 1-10 was performed. Then, 5 µl of diluted bacterial inoculum and 100 µl fungal suspension containing of $10^5$ bacteria/ml and 0.5 x $10^3$ yeast/ml respectively were pipetted into wells 1-11 and mixed thoroughly (CLSI, 2012). Each strain of microorganisms was assayed in triplicate. The bacterial suspensions were used as positive control (well 11) and extract in broth was used as negative control (well 12). The MIC values were taken as the lowest concentration of the extract in the wells of the microtiter plate that showed no turbidity after 24 hours of incubation at 37°C for bacteria and 35°C for yeast. The growth of microorganism was indicated by turbidity of the wells in the microtiter plate.

The minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) values were determined by subculturing the wells plate which

showed no apparent growth onto the agar plates. The lowest concentration which showed no visible growth on subcultured agar after an overnight incubation on 37°C and 35°C for bacteria and yeast respectively was considered as MBC or MFC value (Basri *et al.*, 2012; CLSI, 2012).

### 3.8 Morphological Observations under Scanning Electron Microscope (SEM)

The method of sample processing was adopted from Dunlap and Adaskaveg (1997) and Suwalak and Voravuthikunchai (2009). Cells of each strain at a logarithmic phase in MHB (for bacteria) and RPMI (for yeast) were treated with 1x MIC and 4x of *Q. infectoria* galls extract for 12 hours (for bacteria) and 24 hours (for yeast). The bacterial and fungal cells treated with 1% DMSO were used as negative control. The samples were centrifuged at 1000 – 2000 g for 15 minutes and the pellet was fixed with McDowell-Trump fixative prepared in 0.1M phosphate buffer (pH 7.2) overnight. The centrifugation was repeated two times. Then, the pellet was postfixed in 1% osmium tetroxide prepared in phosphate buffer for 1 hour at room temperature. After centrifugation, the pellet was resuspended in distilled water before performing the dehydration process with 50% ethanol (10 minutes), 75% ethanol (10 minutes), 95% ethanol (10 minutes), 100% ethanol (10 minutes, 2x) and Hexamethyldisidalazane (HMDS) (10 minutes, 2x). The cells were allowed to dry in a dessicator 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).