ANTI-BACTERIAL ACTIVITY OF *Oroxylum indicum* METHANOLIC LEAVES EXTRACT AGAINST GRAM NEGATIVE BACTERIA

by

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LIST OF ABBREVIATIONS

AMR – Anti-microbial resistance

CLSI – Clinical and Laboratory Standards Institute

DMSO – Dimethyl sulfoxide

MAC – MacConkey agar

MBC – Minimum bactericidal concentration

MDR – Multidrug-resistant

MHB – Mueller Hinton broth

MIC – Minimum inhibitory concentration

MPO – Myeloperoxidase

NA – Nutrient agar

UTI – Urinary tract infection

ABSTRAK

Pokok beko (*O. indicum*) digunakan sebagai ubat tradisional untuk merawat pelbagai penyakit dan ekstrak daunnya telah dilaporkan mempunyai ciri-ciri anti-mikrobial. Tujuan kajian ini adalah untuk menilai aktiviti '*in vitro*' anti-bakteria dalam ekstrak metanol daun beko terhadap spesies bakteria yang dipilih. Ekstrak metanol daun beko telah diuji untuk aktiviti anti-bakteria terhadap *E. coli*, *S. flexneri*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, *K. pneumoniae* dan *S.* Typhi. Aktiviti anti-bakteria telah dinilai oleh kaedah penyebaran telaga dan seterusnya kepekatan perencatan minimum (MIC) menggunakan teknik pencairan mikro bersiri berganda pada kepekatan antara 150 mg/mL kepada 1.17 mg/mL. Selepas 24 jam pengeraman, kepekatan minimum membunuh bakteria (MBC) akan ditentukan dengan mengsubkulturkan lubang yang tidak menunjukkan kekeruhan di atas agar nutrisi. Kehadiran metabolit sekunder yang berpotensi berada dalam ekstrak mentah telah diperiksa melalui ujian kualitatif fitokimia. Ekstrak metanol daun beko menunjukkan aktiviti anti-bakteria yang baik terutamanya *P. vulgaris* berbanding dengan spesies bakteria lain yang diuji. Ekstrak metanol daun beko juga boleh membunuh *P. vulgaris* dan *S. flexneri* (nisbah MBC / MIC 4.00). Flavonoid, tanin, triterpenoid dan steroid adalah metabolit sekunder utama dalam ekstrak mentah. Kajian ini mencadangkan bahawa ekstrak metanol daun beko mempunyai aktiviti anti-bakteria.

ABSTRACT

O. indicum is used as a traditional remedy of many ailments and its leave extract has been reported to have antimicrobial properties. The purpose of this study was to evaluate the *in vitro* anti-bacterial activity of the methanolic leaves extract of *O. indicum* against selected bacteria species. Methanolic leaves extract of *O. indicum* was tested for anti-bacterial activity against *E. coli*, *S. flexneri*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, *K. pneumoniae*, and *S.* Typhi. Anti-bacterial activities were evaluated by well-diffusion method and the Minimum Inhibitory Concentration (MIC) was determined using the two-fold serial broth microdilution technique of concentration ranging from 150 mg/mL to 1.17 mg/mL. After 24 hours of incubation, the Minimum Bactericidal Concentration (MBC) was determined by sub-culturing the wells which showed no turbidity onto Nutrient agar (NA). The presence of potential secondary metabolites in the crude extract was screened by phytochemical qualitative test. The methanolic leaves extract of *O. indicum* showed good anti-bacterial activity and *P. vulgaris* being the most susceptible among the tested bacteria species. The methanolic leaves extract of *O. indicum* also bactericidal to *P. vulgaris* and *S. flexneri* (MBC/MIC ratio of 4.00). Flavonoid, tannin, triterpenoid, and steroid were the major secondary metabolites of the crude extract. The study suggested that *O. indicum* methanolic leaves extract has promising anti-bacterial activity.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Bacterial infection can be divided into two categories which are nosocomial infection and community acquired infection. Nosocomial infection or hospital-acquired infections are caused by viral, bacterial, and fungal pathogens. On the other hand, community acquired infection is an infection occurred outside of a health care setting or an infection present on admission. Community-acquired infections are often distinguished from nosocomial diseases by the types of organisms that affect patients who are recovering from a disease or injury. Gram positive and Gram negative bacteria both can cause a disease to happen.

The problem arises when there is increase in the number of bacterial infection. Majority of the tested Gram negative bacteria species which are *E. coli*, *S. flexneri*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, *K. pneumoniae*, and *S.* Typhi can cause urinary tract infections (UTIs) to human being. The disease burden increase when the bacteria develop resistant toward the current antibiotic. Urinary tract infection (UTI) is one of the disease burdens in the society. According to Harvey *et al.* (2007), salmonellosis, shigellosis, and enterhemorrhagic *E. coli* had been reported as the notifiable diseases in United State in 2004. The examples of the current antibiotics are cephalosporin, tetracycline, and chloramphenicol. The Antibiotic resistance leads to higher medical costs, prolonged

hospital stays and increased mortality. An alternative treatment need to be developed to prevent antibiotic resistance that can increase disease burden.

O. indicum also called as midnight horror tree and known as '*beko*' in Malaysia, belongs to the family Bignoniaceae (Gokhale & Bansal, 2006). It is a medium-sized tree and grows mainly near the rivers and in swampy areas in most Asian country like India, Sri Lanka, South China, Philippines and Malaysia. In Malaysia, the young leaves and fruits of *O. indicum* is consumed raw or cooked as vegetables (Zazali *et al.*, 2013). *O. indicum* can grow up to 12 meter height with enormous seed pods that hang down from bare branches. The long fruits curve downward with round seeds and brown color bark. The leaves are long and broad. The purple flowers are born in rainy season and fruit appears in December to March (Dev *et al.*, 2010).

Figure 1.1: *O. indicum*: (a) tree (b) leaves (c) flowers (d) wood bark (adapted from Harminder *et al.*, 2011)

Ethnopharmacological Uses

O. indicum also known as '*Sonapatha*' is an important herb in Ayurvedic medicine to treat various health disorders. The root bark and stem bark has anti-allergic properties which help in treating allergic disease, jaundice, asthma, sore throat, diarrhea, and dysentery. The bark in the form of decoction with weight 8 to 16 g is reported as normal dose (Dev *et al.*, 2010). Young shoots and unripe fruits are eaten as vegetables. The fruits are acric, sweet, and good in treating diseases of the heart and the throat, bronchitis, improves appetite, and useful in leucoderma.

The decoction of the bark is taken for curing gastric ulcer and a paste made of the bark powder is applied for mouth cancer, scabies and other skin diseases. The seeds are ground with fire-soot and the paste applied to the neck for quick relief of tonsil pain. Also, a paste made of the bark is applied to the wounds of animals to kill maggots. Decoction of the bark is given to animals for de-worming. The sword-like fruit or a branch of the plant is used by the farmers to kill crabs in wet paddy fields (Preety & Sharma, 2016). In Indian system of medicine the root, bark, stem and leaf are prescribed for snake bite.

Pharmacological Activities

According to Dev *et al.* (2010), the extract of *O. indicum* was found to have significant activities. For example the aqueous and alcoholic extracts of stem bark showed significant anti-inflammatory activity when tested in three different *in vitro* systems. The plant leaves also showed anti-hepatotoxic activity against diseased rats at a dose of 300 mg/kg body weight and ethanolic extract was found to be more effective compared to other extract. *O. indicum* may be an appropriate ant-helmintic against equine strongyles. The

nitrosated *O. indicum* fraction has genotoxic and cell proliferative activity in the pyloric mucosa of rat stomach *in vivo*.

Dev *et al.* (2010) also stated that it has potential sources of anti-cancer compounds since it showed toxicity on tumor cell lines tested. The immunomodulatory activity with anti-oxidant potential in the active fraction of n-butanol in root bark of *O. indicum* has been assessed. Rats were used to measure the immune responses to sheep red blood cells and delayed-type hypersensitivity reactions. Flavonoids present in *O. indicum* were found to be responsible for its gastro-protective activity.

According to Harminder *et al.* (2011), the crude ethyl acetate extract showed mild to moderate activity against fourteen pathogenic bacteria and seven pathogenic fungi. Other than that, the anti-ulcer activity could be linked to the presence of baicalein in the root bark of the plant. The ethanol extract possessed significant anti-oxidant activity in two *in vitro* models. The aqueous extract of *O. indicum* had a significant effect which give 64% inhibition of release of myeloperoxidase (MPO), tested for *in vitro* release of MPO from rat peritoneal leukocytes.

Problem statement

Bacterial infection is the source of the burden disease nowadays. Bacterial infection can be treated by antibiotic treatment such as cephalosporin, quinolone, tetracycline, chloramphenicol, and penicillin. However, some strain of bacteria has developed resistant toward some types of antibiotic. Antibiotic resistance will lead to higher medical costs, prolonged hospital stays, and increased mortality. Even if new medicines are developed, without behaviour change, antibiotic resistance will remain a major threat. Behaviour

changes must also include actions to reduce the spread of infections through vaccination, hand washing and good food hygiene (Ducel & Fabry, 2002).

There will be limited antibiotic treatment option. The alternative treatments should be developed to avoid this problem. There are side effects of using drugs to treat bacterial infections. The examples of the side effect of current drug are vomiting, diarrhea, and rash. Those are problems of using drugs. This study aims to observe the potential of leaves extract of *O. indicum* as antibacterial. This is important for better understanding of the mechanism and capability of the extract to kill the bacteria.

1.2 Objectives

1.2.1 General objective

- To evaluate the anti-microbial activity of *O. indicum* methanolic leaves extract against Gram negative bacteria.

1.2.2 Specific objectives

1) To determine the percentage yield of *O. indicum* methanolic leaves extract by cold extraction method.
2) To determine the presence of secondary metabolites of *O. indicum* methanolic leaves extract.
3) To screen for anti-microbial activity of *O. indicum* methanolic leaves extract using well diffusion method.

4) To determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values of *O. indicum* methanolic leaves extract against Gram negative bacteria.

1.3 Hypothesis

Null hypothesis (H_o): *O. indicum* methanolic leaves extract shows no anti-microbial activity against Gram negative bacteria.

Alternative hypothesis (H_a): *O. indicum* methanolic leaves extract shows anti-microbial activity against Gram negative bacteria.

1.4 Significance of study

In Malaysia, young leaves and fruits of *O. indicum* are consumed raw or cooked as vegetables. *O. indicum* have been applied traditionally to treat stomach ache, rheumatism, jaundice. It possesses some biological activities such as anti-ulcer, anti-cancer and anti-inflammatory properties. It is edible and inexpensive to get *O. indicum*. Some strain of bacteria has developed resistance toward some types of antibiotic but none of the study reported the anti-microbial activity and resistance towards *O. indicum*. Information on anti-microbial activity of *O. indicum* is scarced. No extend research regarding the benefits of *O. indicum*.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This literature review explains about the most common and worldwide health problem related with bacteria that affects the people worldwide and how '*beko*' or *O. indicum* can be used as antibacterial agent in reducing bacterial infections. It may sometimes be more efficient than the current antibiotics which have its own side effects.

2.2 Bacterial infection

Bacterial is an etiological factor for nosocomial infection and community acquired infection. Hospital acquired infection can be defined as an infection that is acquired in a hospital or other health care facility. Hospital acquired infection includes the infection among staff in the hospital and the infections acquired in the hospital but appearing after discharge (Ducel & Fabry, 2002).

Meanwhile, community acquired infection is the infection that occurred outside of a health care setting or an infection present on admission. There is one way to distinguish between the community acquired infection with the hospital acquired infection by the types of organisms and diseases that affect patients who are recovering from a disease or injury. *Haemophilus influenzae* or *Streptococcus pneumoniae* are the common strains that lead to community acquired respiratory infection. These strains are usually more sensitive towards

antibiotic compared to other strains. There are Gram negative and Gram positive bacteria that can cause bacterial infections in both community acquired infection and hospital acquired infection.

2.2.1 Gram negative bacteria

The bacteria cell envelope is composed of a complex multilayered structure which functions as a protection to the organism from the unsuitable environment. Gram negative bacteria are made up a thin peptidoglycan cell wall and outer layers containing lipopolysaccharide. Meanwhile, Gram positive bacteria are surrounded by the layers of peptidoglycan which is thicker than in the Gram negative bacteria and lack the outer layer containing lipopolysaccharide. There are long anionic polymers which is called as teichoic acids that threading through the layers of peptidoglycan. The cell wall of Gram negative bacteria is very tough to withstand extreme pressure up to approximately 3 atm, temperature and pH (Silhavy *et al.*, 2010). The cell wall structure of Gram negative bacteria is shown in Figure 2.1.

The Gram staining method could distinguish between the Gram negative and Gram positive bacteria with different response. Because of the structure of the Gram negative bacteria which composed of lipid-rich outer membrane with thin layer of peptidoglycan, the alcohol decolorizing step of Gram staining washes the primary stain which is crystal violet from the cells and the secondary stain which is carbol fuchsin or saffranin colors the bacteria red (Beveridge, 1999).

Infection by Gram negative bacteria showed highly efficient at up-regulating or acquiring genes that code for mechanisms of antibiotic drug resistance (Anton *et al.*, 2010).

They often use multiple mechanisms against the same antibiotic or by using a single mechanism to affect multiple antibiotics.

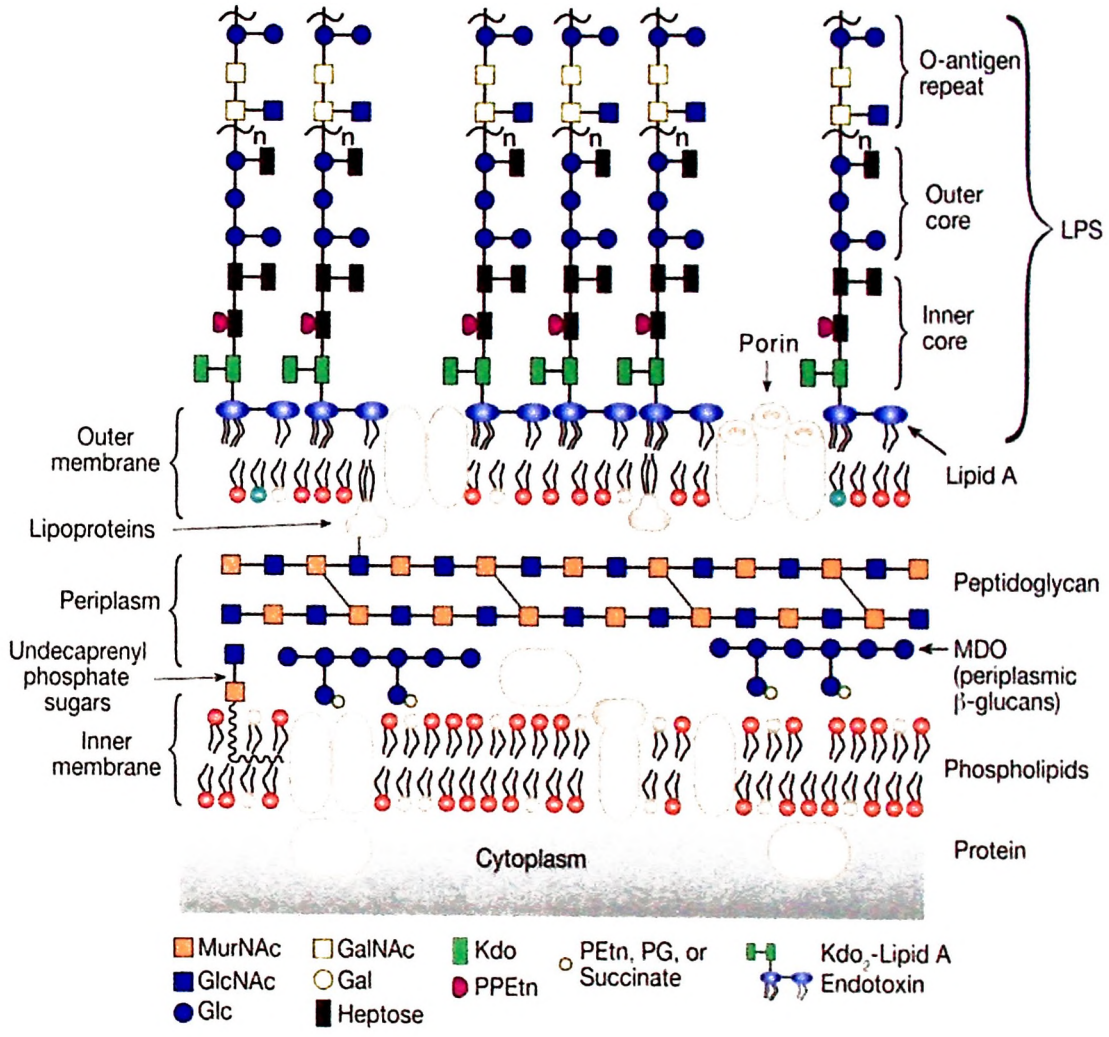

Figure 2.1: The cell wall of Gram-negative bacteria (adapted from Kessel, n.d)

2.2.2 Prevalence

Gram negative bacterial infection is the most common disease that affects the people worldwide. Gram negative bacterial infection can affect people with any ages. Nor *et al.* (2015) revealed that bacteria present in children with urinary tract infection at tertiary hospital in Malaysia. Urinary tract infection (UTI) is a common bacterial infection presenting to the paediatric services worldwide, (Figure 2.2).

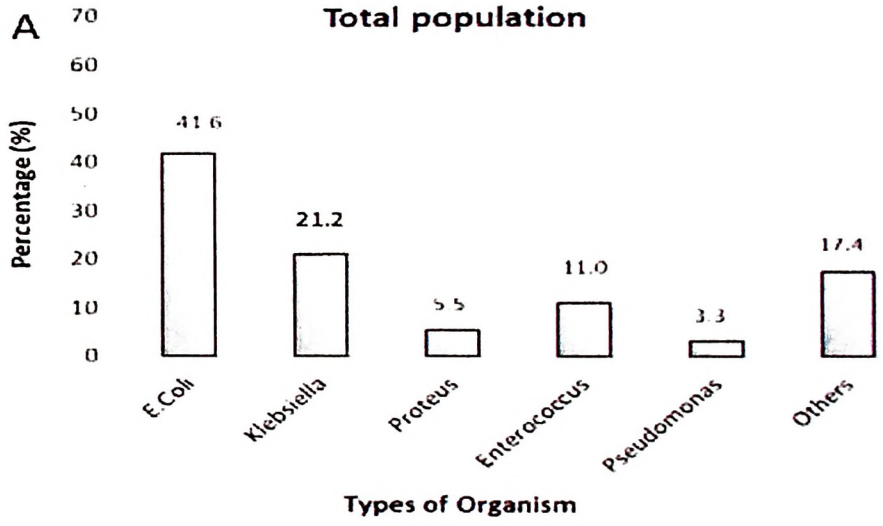

Figure 2.2: The percentage of the types of organism for the total population (adapted from Nor *et al.*, 2015)

The percentage of *E. coli* infection was the highest with 41.6% and followed by *Klebsiella* 21.2%, others 17.4%, *Enterococcus* 11.0%, *Proteus* 5.5%, and *Pseudomonas* 3.3%.

Wounds are a significant cause of morbidity worldwide. Previous studies showed that for every million wound patients, at least 10,000 die from microbial infections (Wong *et al.*, 2015). They had conducted a study of the prevalence and antibiotic susceptibility of bacteria from acute and chronic wounds in Malaysian subjects, (Figure 2.3).

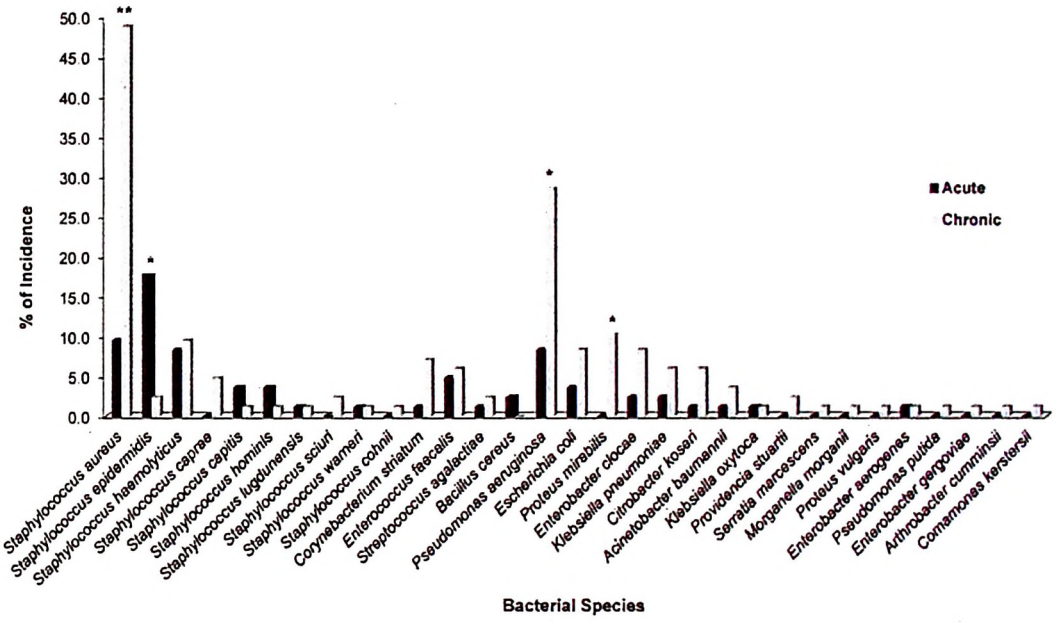

Figure 2.3: Prevalence of bacteria isolates in 84 chronic wounds and 84 acute wounds (adapted from Wong *et al.*, 2015)

It has been reported that the percentage of incidence for *Staphylococcus aureus* is the highest and followed by *P. aeruginosa* in chronic wounds while the percentage of incidence for *Staphylococcus epidemidis* is the highest in acute wounds. Gram negative bacteria showed many incidences of bacterial infections in acute and chronic wound. As examples are *E. coli*, *P. aeruginosa*, *P. mirabilis*, *K. pneumoniae*, and *P. vulgaris*.

A study had been conducted in Aseer region, Kingdom of Saudi Arabia related with hospital nosocomial infection and also community acquired infection, (Figure 2.4).

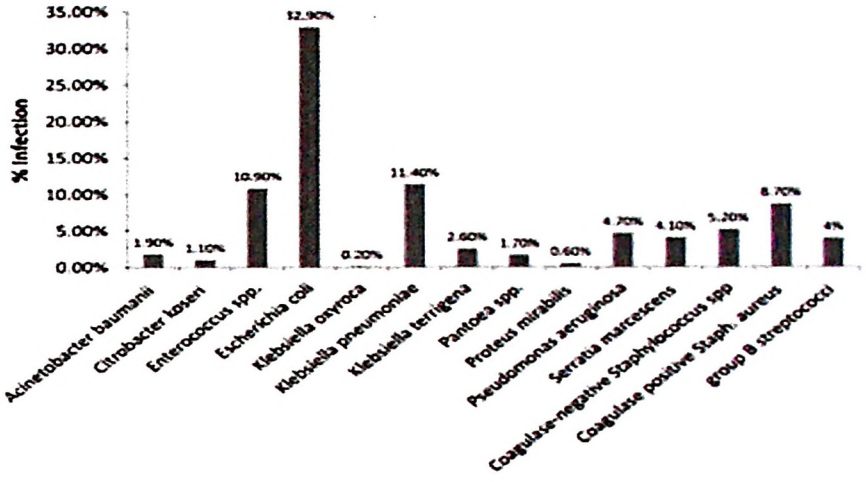

Figure 2.4: Recovery of various bacteria from patients in Aseer region in relation to *S. aureus* (adapted from Hamid *et al.*, 2011)

According to Hamid *et al.* (2011), The surgical site infection is highly attribute to the incidence of nosocomial infections among the surgical patients. These infections were observed in superficial wound infections, with minimal mortality rates. However it added a considerable cost to patient care, to necrotizing soft tissue infections that associated with prolonged hospitalization, significant healthcare expense and a high mortality rate.

A research had been conducted at the tertiary hospital in India about the surgical site infections. According to Sikka *et al.* (2012), the most frequently isolated organisms from surgical site were *E. coli* (58%), *K. pneumoniae* (9.9%), *S. aureus* (8.6%), *Enterobacter spp.* and *P. aeruginosa* (6.2% each), *Acinetobacter baumanii* (4.9%), *Citrobacter spp.* (3.7%) and *P. vulgaris* (2.5%).

2.3 Properties of *O. indicum* as traditional remedies

2.3.1 Chemical constituent of *O. indicum*

There are several chemical constituents have been reported from different parts of the plant (Padgilwar *et al.*, 2014) as shown in Table 2.1.

Table 2.1: Chemical constituents of different parts (adapted from Palgilwar et al., 2014)

Part	Chemical constituents
The leaves	Flavones, glycosides, baicalein, scutellarein anthraquinone and aloe-emodin
Root and stem	Oroxylin A, baicalein, chrysin, pterocarpan, rhodioside, p-hydroxyphenylethanols and cyclohexanols
Root bark	Bhrysin, baicalein, oroxylin A, dihydrobaicalein, β-sitosterol, iso-flavone and prunetin
Stem bark	Alkaloids, tannic acid, sitosterol and galactose
Seeds	Chrysin, baicalein, baicalein-7-O-glucoside, baicalein-7-O-diglucoside (Oroxylin B)
Fruits	Oroxylin A, chrysin and ursolic acid and aloe-emodin

The chemical structures of several important constituent such as bacelin, chrysin, scutellarein, antraquinone, aloe emodin, and oroxylin-A are important in this issue, (Figure 2.5). This medicinal plant is the unique source of various types of compounds having diverse chemical structure and nature. The active constituents of the plants are responsible for the pharmacological activities. Harminder *et al.* (2011) had stated that the flavonoids are known for their anti-inflammatory and anti-allergic effect. Chrysin is a flavone which has many biological activities such as anti-bacterial, anti-oxidant, anti-inflammatory, anti-allergic, and anti-cancer.

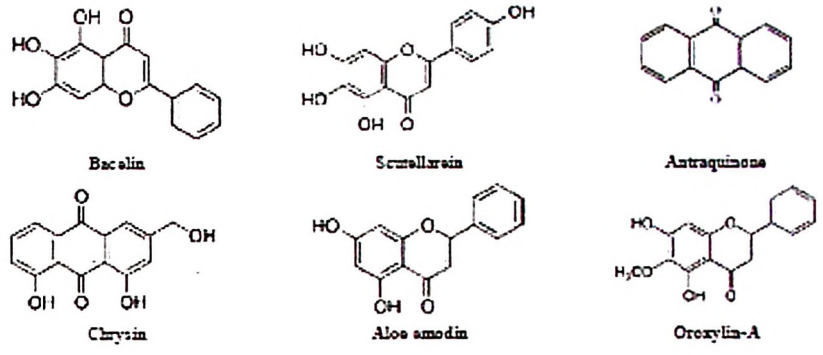

Figure 2.5: Chemical structures of important constituents (adapted from Palgilwar et al., 2014)

2.3.2 *O. indicum* as natural remedies

O. indicum or '*beko*', is used as a traditional remedy of many ailments. Since a century ago before the emergence of modern medicine, '*beko*' had been used as the natural remedies by many people to treat various health problems. For example, various parts of the plant is used to treat different ailments such as cancer, diarrhea, fever, ulcer and jaundice (Harminder *et al.*, 2011). *O. indicum* was also used in the treatment of cough, acute or chronic bronchitis, pharyngitis, pertussis and other respiratory disorders in the traditional Chinese medicine. Nowadays, the therapeutic effects of the *O. indicum* are widely studied as the alternative for the treatment of various health problems such as anti-proliferative, anti-microbial, anti-oxidant, hepatoprotective, and anti-inflammatory.

2.3.3 *O. indicum* as anti-microbial agent

There are many medicinal plants being studied as anti-microbial agent including *O. indicum*. A research conducted by Dubey *et al.* (2012) on antimicrobial activity of medicinal plants used by aborigines of Kalahandi, Orissa, India against multidrug resistant bacteria. In this study, there were 20 types of medicinal plant being used against 10 pathogenic bacteria which are *S. aureus*, *Acinetobacter sp.*, *Citrobacter freundii*, *Chromobacterium violeceum*, *E. coli*, *Klebsiella sp.*, *Proteus sp.*, *P. aeruginosa*, *S.* Typhi and *Vibrio cholera*. The bacteria were isolated from the clinical samples in 2 hospitals. Water and ethanolic extracts of the leaves and barks were prepared and the screening method is by agar-well diffusion method.

For *C. freundii*, ethanolic extracts *E. scaber* and *O. indicum* (leaf and bark) were effective, whereas for *E. coli*, water and ethanolic extracts of *B. monosperma*, *Cassia fistula* registered good antibacterial activities, whereas *Terminalia alata*, *A. cadamba*, *O. indicum* (leaf and bark) and *P. santalinus* (bark) showed moderate antibacterial activity. Chusri *et al.* (2012) also claimed that the ethanol extract of *O. indicum* from southern Thailand has anti-bacterial activity against skin and wound pathogen including *S. epidermidis*, methicillin-resistant *S. aureus*, *S. pyogenes*, multidrug-resistant (MDR) *E. coli*, MDR *P. aeruginosa*, and MDR *Acinetobacter baumannii*. In this research, only ethanol extracts was used and disc diffusion method for screening instead of agar-well diffusion method.

The extracts which showed inhibitory effect were further investigated for minimum inhibitory concentration (MIC) using the broth microdilution method. Results indicated that THR-SK004 and its medicinal components, *Metroxylon sagu* Rottb. and *O. indicum* Vent.

show good anti-bacterial activity with MIC values in the range of 30 to 1,000 µg/mL. According to Valgas *et al.* (2007), the well diffusion method give better sensibility compared to the disc diffusion method. The research also stated that the well diffusion method is less time consuming, simple, more convenient, and higher sensitivity than the disc diffusion method. In addition, the well diffusion method yielded larger growth inhibition zones since the sample diffuse easily across the medium.

Another study by Talari *et al.* (2013) which is to study the anti-bacterial activity of stem bark extracts of *O. indicum* against disease causing Gram negative and Gram positive bacteria. Anti-microbial activity of solvent extracts of stem bark of *O. indicum* has been studied to find out its activity against four important bacterial strains which are *Bacillus subtilis*, *B. cereus*, *Staphylococcus albus* and *S. aureus*. The anti-microbial activity of the stem bark extracts was done through well diffusion method and by measuring the inhibition zone around the disc. The results revealed that the aqueous extracts of *O. indicum* exhibited anti-microbial activity against all the microbes under study. The results provided evidence that the species *O. indicum* can be used as a potential source of anti-microbial agent.

A compound from stem bark of *O. indicum* has anti-bacterial activity and its MIC against antibiotic resistant bacteria (Das *et al.*, 2012). Bacterial strains used were *B. subtilis*, *E. coli* and *P. aeruginosa.* Ampicillin, Amikacin and Tetracycline were used as antibiotic standards. Minimum Inhibitory Concentration (MIC) of SDP_F38 was tested with Amikacin as standard. The result showed MIC was 8µg/mL and 16µg/mL for *E. coli* 468 and *P. aeruginosa* respectively.

Parekh *et al.* (2005) claimed that the methanol extracts were more potent than the aqueous extracts for all 12 plants studied. The anti-bacterial activity of aqueous and methanol extracts was determined by agar disk diffusion and agar well diffusion method. A

review stated that the *O. indicum* shows anti-microbial activity when compared with standard kanamycin disc (K-30 µg/disc) using the standard disc diffusion method (Harminder *et al.*, 2011).

2.4 Current anti-microbial drugs

According to (WHO, 2014), anti-microbial resistance (AMR) is an increasingly serious threat to global public health. AMR develops when a microorganism (bacteria, fungus, virus or parasite) no longer responds to a drug to which it was originally sensitive. This means that standard treatments no longer work. The infections are harder or impossible to control while the risk of the spread of infection to others is increased. In addition, the illness and hospital stays are prolonged, with added economic and social costs and the risk of death is greater. Anti-microbial resistance can occurred through mutation or gene transfer and the resistance genes encode various mechanisms which allow microorganisms to resist the inhibitory effects of specific antimicrobials (WHO, 2001).

Cars *et al.* (2008) stated that for many years, needs for anti-bacterial drugs were met by the pharmaceutical industry. But nowadays, the existing antibiotics are losing their effect, but development of new antibiotics is declining. The role of the patients as consumers is growing stronger. They need access to information and knowledge to reduce their expectations of antibiotics in self limiting infections, and doctors need new tools to help them justify their treatment decisions. Antibiotic-resistant pathogens are not more virulent than susceptible ones and the same numbers of resistant and susceptible bacterial cells are required to produce disease. But the resistant forms are harder to eliminate (Levy, 1998).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant source

The fresh leaves of *O. indicum* were obtained from markets in Kota Bharu, Kelantan. The leaves were identified based on their physical appearance of the tree and their leaves itself. The plant was identified as *O. indicum* and given Voucher No. USM-PPSK-004. The leaves were washed with distilled water in order to remove unnecessary materials. The leaves were dried in an oven with the temperature 55 - 60°C until dried. The completely dried leaves were grinded into powder form. The leaves powder was used for the preparation of methanol extract by cold extraction method.

3.1.2 Microorganisms

Seven bacterial strains which are *E. coli* (ATCC 25922), *S. flexneri*, *P. mirabilis* (ATCC 12453), *P. vulgaris* (ATCC 49132), *P. aeruginosa*. *K. pneumoniae* (ATCC 1706), and *S.* Typhi were obtained from the Microbiology Laboratory, School of Health Sciences, USM. The microorganisms were cultured and maintain by sub-culturing on the MacConkey agar (MAC) every five days to maintain growth and purity.

3.1.3 Chemicals

The extraction solvent, methanol, is used as a soaking agent in order to get the leaf extract. Dimethyl sulphoxide (DMSO) is used as a universal solvent to dissolve the leaf extract and also use as a negative control in antibacterial screening test.

3.2 General preparation

3.2.1 Preparation of 0.1% Dimethyl Sulphoxide (DMSO)

Table 3.1 below shows the components of 0.1% DMSO:

Table 3.1: 0.1% DMSO Components

Components	For 100 mL
100% DMSO	0.1 mL
Distilled water	99.9 mL

The 0.1% Dimethyl sulphoxide (DMSO) was used to dissolve the methanol crude leaf extract and as a negative control. 0.1 mL of DMSO was measured and then made up with distilled water until 100 mL in order to get 0.1% Dimethyl sulphoxide (DMSO) volume per volume (v/v).

3.2.2 Preparation of 70% Ethanol

Table 3.2 below shows the 70% ethanol components:

Table 3.2: 70% Ethanol Components

Components	For 100 mL
100% ethanol	70 mL
Distilled water	30 mL

The 70% ethanol was prepared by mixing 70 mL of absolute ethanol with 30 mL of distilled water. The mixture was mixed well and kept in a 100 mL Duran bottle. This 70% ethanol is used as a disinfectant.

3.2.3 Standard Antibiotic

The Imipenem Oxoid™ antibiotic discs were used in the screening test as a positive control. Ten Imipenem disc were immersed in 1 mL of 0.1% DMSO and mixed well by vortex.

3.3 Media Preparation

3.3.1 MacConkey Agar

The MacConkey agar was set up as follows:

Table 3.3: MacConkey Agar Components

Components	For 1 L
MacConkey Agar	51.5 g
Distilled water	1 L

The MacConkey agar powder was weighted for 51.5 g. Next, distilled water was added into the Schott bottle containing 51.5 g MacConkey agar powder to a final volume of 1 L. The mixture was mixed well by using the hot plate and magnetic stirrer. The media had completely dissolved when the mixture has clear appearance. The media was autoclaved at 15 psi, 121°C (250 F) for 15 minutes.

3.3.2 Nutrient Broth

The Nutrient broth was set up as follows:

Table 3.4: Nutrient Broth Components

Components	For 1 L
Nutrient Broth	13 g
Distilled water	1 L

The Nutrient broth powder was weighted for 13 g. Next, distilled water was added into the Schott bottle containing 13 g Nutrient broth powder to a final volume of 1 L. The mixture

was mixed well by shaking the bottle. Then, 10 mL of the nutrient broth was added into the universal bottle. The universal bottles were autoclaved at 15 psi, 121°C (250 F) for 15 minutes.

3.3.3 Nutrient Agar

The Nutrient agar was set up as follows:

Table 3.5: Nutrient Agar Components

Components	For 1 L
Nutrient Agar	28 g
Distilled water	1 L

The Nutrient agar powder was weighted for 28 g. Then, distilled water was added into the Schott bottle containing 28 g Nutrient agar powder to a final volume of 1 L. The mixture was mixed well by using the hot plate and magnetic stirrer. The media had completely dissolved when the mixture has clear appearance. The media was autoclaved at 15 psi, 121°C (250 F) for 15 minutes.

3.3.4 Semi Solid Nutrient Agar

The semi solid Nutrient agar was set up as follows:

Table 3.6: Semi Solid Nutrient Agar Components

Components	For 100 mL
Nutrient Agar	0.8 g
Distilled water	100 mL

The Nutrient agar powder was weighted for 0.8 g. Then, distilled water was added into the Schott bottle containing 0.8 g Nutrient agar powder to a final volume of 100 mL. The mixture was mixed well by using the hot plate and magnetic stirrer. The media had completely dissolved when the mixture has clear appearance. The media was autoclaved at 15 psi, 121°C (250 F) for 15 minutes.

3.4 Instrument and Appliances

For the methanol extraction, the rotary evaporator was used to concentrate the leaf extract. Other equipments that were used in this research are biosafety cabinet, incubator, grinder, electronic weighter, autoclave machine, Appendorf pipette, laboratory glassware and etc.

3.5 Method

3.5.1 Preparation of the crude extract

The *O. indicum* crude extract was prepared by the cold extraction method. Only one fraction of the *O. indicum* was obtained which is methanolic fraction. The methanolic fraction of the *O. indicum* crude extract was obtained by the rotary evaporator machine.