Comparative Studies on the Antimicrobial Activity of Extracts of Chinese Medicinal Plant *Panax notoginseng* and Native Australian Plant *Eucalyptus globulus*

by

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List of Abbreviations

AAM	Antibiotic Medium no. 1 agar
AMR	Antimicrobial Resistance
CF	Cystic Fibrosis
CFU	Colony-Forming Unit
G	Grams
HPLC	High Performance Liquid Chromatography
MAE	Microwave-Assisted Extraction
MBC	Minimum Bactericidal Concentration
MDR-TB	Multidrug-Resistant Tuberculosis
mg	Milligrams
MIC	Minimum Inhibitory Concentration
mL	Millilitres
mm	Millimeters
MRSA	Methicillin-Resistant *Staphylococcus aureus*
OD	Optical Density
PPD	Protopanaxadiols
PPT	Protopanaxatriols
R_f	Retention Factor

SAE	Shaking-Assisted Extraction
TCM	Traditional Chinese Medicine
TFA	Trifluoroacetic Acid
TLC	Thin Layer Chromatography
TSA	Tryptone Soy Agar
TSB	Tryptone Soy Broth
w	Weight
v	Volume
VRE	Vancomycin-Resistant Enterococcus
µg	Micrograms
µl	Mirolitres
%	Percentage

Abstract

A number of plants have been used as an essential source of medicinal agents for centuries, such as remedies for human diseases, as they produce a diverse range of bioactive molecules. Today's modern drugs are predominantly obtained from semi-synthetic derivatives of natural products, while a small number are derived directly from natural plant sources used in traditional medicines. According to the World Health Organization (WHO), medicinal plants play a key role in health care; about 60-90% of the world's population depend traditional medicine. In addition, adverse side effects and high cost are commonly associated with synthetic antibiotics, such as allergic reactions, immunosuppression and hypersensitivity which are becoming increasingly major issues in treating infectious diseases due to long-term and persistent overuse. In the present scenario, researchers are increasingly turning their attention to develop these issues spawned research into utilising the large defence arsenal developed by plants for the development of novel better antibacterial drugs, which may be cheaper and less prone to elicit bacterial resistance. The *Eucalyptus globulus* leaves and *Panax notoginseng* roots were used in shaking-assisted extraction in 100% methanol while microwave-assisted extraction used water as a solvent. The antimicrobial activity of the *Eucalyptus globulus* at a concentration of 5 mg/mL and *Panax notoginseng* at a concentration of 10 mg/mL was evaluated using a well diffusion method against two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus pumilus*). Synergistic effects between crude plant extracts and the antibiotics Gentamycin and Streptomycin were also assessed using a well dilution method. The minimum inhibitory concentration (MIC) was also determined using dilutions. While higher yields were obtained with MAE (22.0%) compared to SAE (16.2%), SAE extracts had a higher antimicrobial and synergistic effect activity compared to MAE extracts. *Panax notoginseng*

failed to show any antimicrobial activity. The *E. globulus* extract does not show synergy with Gentamicin and Streptomycin. Biochemical compounds of the crude extracts exerting antimicrobial were determined using HPLC. In conclusion, shaking assisted extraction (SAE) of the native Australian plant *Eucalyptus globulus* could serve as an alternative source for the development of novel therapeutic drugs.

Declarations

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis.

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TINUSHA PARAMANANTHAN

15th July 2021

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Chapter 1: Introduction

1.1 Antimicrobial Resistance

Antimicrobial resistance (AMR) has emerged as one of the principal public health problems of the 21st century that threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria, fungi, parasites and viruses no longer susceptible to the common medicines used to treat them. The increasing global spread of multi- and pan-resistant bacteria known as "superbugs", which cause diseases that are resistant to existing antimicrobial treatments such as antibiotics (Marston et al., 2016). There have been several cases of antimicrobial resistance bacteria reported worldwide today such as Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant Enterococcus (VRE) and multidrug-resistant Tuberculosis (MDR-TB) (Dadgostar, 2019). However, as each decade passed, bacteria that could withstand multiple antibiotics became more common, leading to an increase in mortality, morbidity and healthcare costs (Prestinaci et al., 2015). Overuse of antimicrobial drugs has led to increased microbial resistance; especially in developing countries, more patients visit medical centers than ever. The intractable problem of antimicrobial resistance has led to; renewed interest in herbal products as sources of novel drugs to reduce or possibly eradicate the ever-increasing problems of the emergence of newer diseases (Dadgostar, 2019). Synthetic drugs are not readily available and expensive. It is therefore important to search for effective but low cost and reliable natural products with therapeutic properties (Roope et al., 2019).

1.2 Medicinal Plant

Recently, traditional medicinal plants have caught the interest of the pharmaceutical and scientific communities. Researchers are constantly exploring the world's resources for novel phytochemicals or their derivatives that could be used for the treatment of infectious diseases, particularly in light of the emergence of drug-resistant microorganisms and the need to produce

more effective antibiotics (Chandra et al., 2017). This begins with the isolation and identification and characterization of secondary metabolites found in plants and their use as active principles in medicinal preparations (Saranraj and Sivasakthi, 2014). Besides, the synergistic effects of extracts with antimicrobial potential in combination with antibiotics can provide effective therapy against drug-resistant bacteria. These synergistic combinations represent a largely untapped source of potential therapeutic drugs with novel and numerous mechanisms of action capable of overcoming antimicrobial resistance (Moussaoui and Alaoui, 2016).

There are various plants classified as an essential source of medicinal agents for centuries as remedies for human diseases and produce a diverse range of bioactive molecules. A huge number of modern drugs today are obtained from semi-synthetic derivatives of natural products or natural plant sources that are used in traditional medicines (Van Wyk and Wink, 2018). In addition, adverse side effects and high costs commonly associated with synthetic antibiotics such as allergic reactions, immunosuppression and hypersensitivity and are major issues in treating infectious diseases (Khan et al., 2013). According to the World Health Organization (WHO), medicinal plants a key role in health care; about 60-90% of the world's population depend on the use of traditional medicine. In the present scenario, researchers are increasingly turning their attention to medicine to develop better drugs against microbial infections and application of plant extract which cheaper, non-hazardous, cost-effective and easily available (Jamshidi-Kia et al., 2018).

1.2.1 *Panax notoginseng*

Panax notoginseng is a medicinal plant that has been cultivated for more than 400 years mainly grow in high mountain areas of China. It is a perennial herbaceous plant that belongs to the Araliaceae family. *P. notoginseng* is also known as Shanqi, Sanqi and Tianqi in East Asian countries. The Russian botanist Carl Meyer gave Asian Ginseng the botanical name genus

"Panax" which means a cure for all disease, "Noto" means "back, spine" and "ginseng" represents "essence of men" (Xue et al., 2019).

Ginsenosides or triterpene saponins, a group of secondary metabolites are considered the major bioactive compound found nearly exclusively in *Panax* species including *P. notoginseng*, *P. vietnamensis*, *P. ginseng*, *P. japonicas* and *P. quinquefolium*. Currently, more than 200 chemical constituents were isolated from *P.notoginseng* including saponins, flavonoids, saccharides, volatile oils, fatty acids, aliphatic acetylene hydrocarbon, amino acids, cyclopeptides, dencichine, polysaccharides and trace elements. However, the major components of *Panax notoginseng* are ginsenoside, notoginsenoside and gypenosides (Peng et al., 2018). Currently, more than 30 ginsenosides have been identified and isolated from leaves, rhizomes, rootlets, roots, fruit pedicels, flower buds and seeds. Ginsenosides are classified as dammarane tetracyclic triterpene and oleanane type pentacyclic triterpene, depending on their aglycone skeleton. Based on the sugar moieties attachment and hydroxylation pattern at C6, dammarane-type saponins, the dominant ginsenosides can be divided into protopanaxadiols (PPD), protopanaxatriols (PPT), or ocotillos (Fukuyama et al., 2012). Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, and Rd belong to the protopanaxadiol-type saponins. Protopanaxatriol group includes Re, Rf, Rg1, Rg2, Rh1, and F1 and notoginsenosides R1 and R2. Ro is classified as oleanane-group saponins and is not found in *P. notoginseng*. Oleanane-group saponins can be isolated from American ginseng and Asian ginseng (Zhang et al., 2017).

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Figure 1: The chemical structure of major active ingredients of *Panax notoginseng* (Zhang et al., 2017).

(Jang et al., 2016) studied extracts of ginseng stems, roots and leaves contain distinct ginsenosides profiles, which may exhibit various pharmacological activities. Several studies have been conducted on the pharmacological properties of ginseng extracts such as anti-aging, anti-diabetic, anti-allergic, lipid-lowering, anti-inflammatory and hypoglycaemia. Most pharmacologically active compounds are thought to be present in the root, which will be a focus in these studies.

Table 1: Some pharmacological effects on ginsenosides (Jang et al., 2016)

Compound	Pharmacological action
Rb1	Anti-obesity Angiogenesis inhibitor
Rc	Inhibit proliferation of breast cancer cells
Re	Anti-oxidant Anti-diabetic
Rg1	Suppresses oxidative stress
Rg2	Neuronal Ach inhibitor
Rg3	Inhibits proliferation of prostate cancer cells
Rh1	Anti-oxidant Anti-diabetic Anti-inflammatory
Rh2	Inhibit proliferation of breast cancer cells
F11	Assists memory improvement neuro-protective

1.2.2 *Eucalyptus globulus*

Eucalyptus globulus is known as "southern blue gum" is an aromatic tree belong to the Myrtaceae family, native to Australia. The genus eucalyptus compromising more than 700 species with over 500 containing volatile oil in their leaves. Traditionally, native Australian uses eucalyptus leaves for fungal infection and wound healing (Bachir and Benali, 2012).

Table 2: There are over 700 species of Eucalyptus. The major ones are enlisted below (Bachir and Benali, 2012)

Major Species of Eucalyptus	Major Species of Eucalyptus
Eucalyptus amygdalina	*Eucalyptus microtheca*
Eucalyptus australiana	*Eucalyptus nitens*
Eucalyptus botryoides	*Eucalyptus ovate*
Eucalyptus calophylla	*Eucalyptus pauciflora*
Eucalyptus camaldulensis	*Eucalyptus perriniana*
Eucalyptus citriodora.	*Eucalyptus pilularis*
Eucalyptus cladocalyx	*Eucalyptus polyanthemos*
Eucalyptus consideniana	*Eucalyptus polybractea*
Eucalyptus cypellocarpa.	*Eucalyptus populnea*
Eucalyptus dives	*Eucalyptus radiate*
Eucalyptus gigantean	*Eucalyptus regnans*
Eucalyptus globulus	*Eucalyptus risdonni*
Eucalyptus gomphocephala	*Eucalyptus robusta*
Eucalyptus grandis	*Eucalyptus rossi*
Eucalyptus gunnii	*Eucalyptus rostrata*
Eucalyptus incrassate	*Eucalyptus saligna*
Eucalyptus kino	*Eucalyptus sideroxylon*
Eucalyptus largeflorens	*Eucalyptus sieberiana*
Eucalyptus lesouefii	*Eucalyptus smithii*
Eucalyptus macrocarpa	*Eucalyptus tereticornis*
Eucalyptus macrorhyncha	*Eucalyptus tetrodonta*
Eucalyptus maculate	*Eucalyptus umbra*
Eucalyptus marginata	*Eucalyptus urophylla*
Eucalyptus melanophloia	*Eucalyptus viminalis*
Eucalyptus melliodora	*Eucalyptus wandoo*

The essential oil is used in the food industries, cosmetics, toiletries and pharmaceuticals. Different parts flower buds, leaves, mature fruits, seeds, roots and stems of *E. globulus* are nutritionally important due to rich source of phytochemical compound contain carboxylic acids, esters, ethers, aldehydes, alcohols, ketones, hydrocarbons along with sesquiterpenes and monoterpenes, flavonoids, propanoids, tannins and alkaloids (Luís et al., 2016). The main component of essential oil is 1,8-cineole eucalyptol aromadendrene, ß-pinene, globulol, pipertone, α-gurjunene, allo-aromadendrene and α-,ß-and γ-terpinen-4-ol, were found both in shoots and leaves; caproic acid, borneol, eudesmol, citral, myrecene, p-menthane, fenchone, myrtenol, α-terpineol, verbinone, glycine, cysteine glutamic acid, asparagine, threonine and ornithine were extracted from fruits; while sucrose and dextrin were extracted from honey and flowers (Salehi et al., 2019).

1.3 Test microorganisms

1.3.1 *Escherichia coli*

Escherichia coli is a type of bacterium that is found in the human body and can be pathogenic, non-pathogenic or commensal (Meng et al., 2012). It is one of the frequent causes of common bacterial infections such as urinary tract infection, kidney infection, cholangitis and cholecystitis (Kibret and Abera, 2011). The organism is clinically significant due to its cosmopolitan nature and ability to initiate, establish, and cause various types of diseases. The emergence of resistance in *Escherichia coli* due to the increased use of antimicrobial agents has led to the use of medicinal plant extracts against it (Kibret and Abera, 2011).

1.3.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic human pathogen linked to nosocomial infections in immunocompromised patients following burns or other severe trauma, underlying conditions such as diabetes, cystic fibrosis (CF) and cancer. It causes urinary tract infections, dermatitis, respiratory infections, bacteremia, soft tissue infections and other systemic infections (Breidenstein et al., 2011). *P. aeruginosa* is resistant to many antimicrobials, infections are generally difficult to treat, and this inherent resistance has long been linked to the outer membrane, a permeability-limited barrier (Musthafa et al., 2017).

1.3.3 *Staphylococcus aureus*

Staphylococcus aureus is a gram- positive bacteria that causes infections of the skin and soft tissues, food poisoning, and toxic shocks. It is a pathogen of great concern due to its intrinsic virulence and its ability to cause a wide range of life-threatening diseases, as well as its ability to adapt to different environmental conditions (Taylor and Unakal, 2017). The increased use of antimicrobials against *Staphylococcus aureus* has resulted in resistance, necessitating the development of new antimicrobial agents. Medicinal plant extracts have demonstrated antimicrobial activity against a wide range of fungal and bacterial pathogens. Several studies

have shown that some edible plant extracts have antimicrobial activity against *Staphylococcus aureus* (Snowden et al., 2014).

1.3.4 *Bacillus pumilus*

Bacillus pumilus is a species of *Bacillus*, is an aerobic or facultative anaerobic, gram-positive *bacillus*, which can produce spores (Zhu et al., 2018). It is widely distributed in soil, air plant surface and root system (Peng et al., 2013). Although *Bacillus pumilus* produces antibacterial substances, very little research has been studied to isolate these substances (Reiss et al., 2011).

1.4 Aims and objectives of study

1.4.1 Aim

To compare the antimicrobial activities of two plant extracts, the Traditional Chinese Medicine (TCM) plant *Panax notoginseng* and the native Australian plant *Eucalyptus globulus*, to lay the foundation to develop novel sources of antimicrobials to widen the arsenal to combat infectious diseases.

1.4.2 Objectives of study

1. To evaluate the antimicrobial activity of crude plant extracts of *Eucalyptus globulus* and *Panax notoginseng* against selected pathogenic bacteria.
2. To characterize the chemical composition of the native Australian plant *Eucalyptus globulus.*
3. To determine the MIC of *Eucalyptus globulus* plant extracts on the test bacteria.
4. To study the synergy interaction of combination plant crude extracts of *Eucalyptus globulus* and antibiotics (Gentamycin and Streptomycin).

1.5 Hypothesis

The Australian native plant *Eucalyptus globulus* extracts contain compounds that are more effective at inhibiting *Escherichia coli*, *Bacillus pumilus*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* than the Chinese plant extracts *Panax notoginseng.*

The Australia native plant *Eucalyptus globulus* does not show much effect on the minimal inhibitory concentration (MIC) of both Gentamicin and Streptomycin.

Chapter 2: Materials and methods

2.1 Materials

2.1.1 Plant materials

The leaves of *Eucalyptus globulus* were collected from trees growing on the Flinders University campus. The root powder of *Panax notoginseng* from China were purchased online from eBay Australia.

2.1.2 Bacteria

Pathogenic strains of *Escherichia coli* and *Staphylococcus aureus* were kindly provided by Dr Liu Fei Tan from Medical Biotechnology at College of Medicine and Public Health, Flinders University while *Bacillus pumilus* and *Pseudomonas aeruginosa* were obtained from SA Pathology at Flinders Medical Centre. The organisms were maintained on Tryptone Soy Agar at 4℃ for further experiments.

2.1.3 Equipment used

Blender (BlENDTECH XPRESS), Orbital mixer incubator (ADELAB SCIENTIFIC, RATEX), Rotary Evaporator (BUCHI B-300 BASE), Microwave (START SYNTH), Centrifuge Eppendorf 5804, Nylon syringe filter 0.22 µm and Virtis freeze drier and High-Performance Liquid Chromatography (HPLC). All the equipment purchased from the local suppliers in Adelaide, South Australia.

2.1.4 Reagents used

100% methanol (SIGMA-ALDRICH), chloroform (SIGMA-ALDRICH), 96 well microplates, Vancomycin (SIGMA), Colistin (SIGMA), Gentamicin (SIGMA), Streptomycin (SIGMA) and trifluoroacetic acid (TFA) (SIGMA). Some reagents were purchased from local suppliers, unless stated, such as Tryptone Soy Broth (OXOID), Tryptone Soy Agar (OXOID), Antibiotic

Medium No. 1 Seed Agar (OXOID) purchased from Basingstoke, Hampshire, England and TLC aluminium sheets 20 x 20 cm Silica gel 60 (MERCK) from Darmstadt, Germany

2.2 Methods

2.2.1 Preparation of plant samples

The collected leaves were washed under running tap water to remove the dust. The plant materials were dried at room temperature for two weeks and blended with the help of a blender in a fume hood. The powder was stored in a cool and dry place in an airtight container till further use.

2.2.2 Shaking-assisted extraction (SAE)

Two grams (2 g) of each plant part *E. globulus* (leaves) and *P. notoginseng* (roots) was weighed and placed into each of two conical flasks containing 80 mL of methanol. The plant material mixture was placed on an orbital mixer incubator for 24 h at room temperature (Jegal et al., 2019). Each plant extract was centrifuged at 4500 rpm for 20 minutes and filtered using a 0.22 µm syringe filter. The solvent was evaporated to dryness in a round bottom flask using a rotary evaporator at 60 ℃. The dry extracts were weighed and stored in a vial and refrigerated at 4 ℃.

2.2.3 Microwave-assisted extraction (MAE)

Two different extraction procedures were carried out on the plant samples:

Eucalyptus globulus

Two grams (2 g) of eucalyptus powder was weighed and placed in a 250 mL bottom round flask. The extracts were obtained using 80 mL of distilled water as a solvent with microwave radiation power of 150 W and the temperature at 55 ℃ for 20 minutes (Tran et al., 2020). The eucalyptus extract was then centrifuged at 4500 rpm for 20 minutes and filtered using a syringe filter. The samples were kept in a freezer at -80 ℃ overnight and placed in a freeze dryer for

two days to concentrate the water extracts. The dry extract was weighed and stored in a centrifuge tube at -20 ℃.

Panax notoginseng

Two grams (2 g) of ginseng powder was weighed and placed in a 250 mL bottom round flask. The extracts were obtained using 80 mL of distilled water as a solvent with microwave radiation power of 150 W and the temperature at 100 ℃ for 20 minutes (Jo et al., 2014). The eucalyptus extract was then centrifuged at 4500 rpm for 20 minutes and filtered using a syringe filter. The samples were kept in a freezer at -80 ℃ for overnight and placed in a freeze dryer for two days to concentrate the water extracts. The dry extract was weighed and stored in a centrifuge tube at -20℃.

The yield of extraction was quantified using:

$$\text{Percentage of yield (\%)} = \frac{\text{weight of dry extracts}}{\text{weight of dry plants}} \text{ X } 100$$

2.2.4 Thin layer chromatography

The silica gel plates were cut in 10 cm x 20 cm width and marked in spots with a pencil about 1 cm from the edge. Ten microliters of extracts were separately applied on the silica gel plate as the stationary phase. The solvents system containing a mixture of chloroform: methanol: water (65:35:10 v/v) was separated using a separating funnel to obtain the upper phase and lower phase. The lower phase solvent as the mobile phase was poured into the tank and Whatsman no.1 filter paper was placed in the TLC plate for 30 minutes to saturate. The silica gel plates were placed into a closed tank until the solvent reaches the top of the silica gel plate. The plates were removed and subsequently allowed to dry. After drying, the spot on the plates was visualized under the UV illuminator box at the wavelength of 254 nm and 365 nm (Sidana

et al., 2012). The retention factor (R_f) values were calculated for each spot using the following formula:

$$R_f = \frac{\text{distance travelled by the sample}}{\text{distance travelled by the solvent front}}$$

2.2.5 Preparation for Antimicrobial assay

Four isolated colonies of each bacterial strain (*Escherichia* coli, Staphylococcus *aureus*, *Bacillus pumilus* and *Pseudomonas aeruginosa*) were inoculated in 5 mL of Tryptone Soy Broth (TSB) using a sterile loop. The cultures were incubated at 37 ℃ overnight. The growth of each culture was monitored using a spectrophotometer at 600 nm. 1 ml of each overnight culture was diluted with the sterile TSB to obtain an OD of 0.2 using a spectrophotometer at 600 nm. Antibiotic medium no. 1 agar (AAM) were prepared for 40 ml in different dilutions of 1%, 2% and 4% of each culture. The wells were created in each petri plate using a 6mm cork borer. 50 µl of each plant extracts of *E. globulus* (5 mg/mL, w/v) and *P. notoginseng* (10 mg/mL, w/v) was tested followed by two antibiotics solution which was colistin (40 µg/mL) and vancomycin (40 µg/mL) as the positive control, methanol and water used as control were also tested. The plates were incubated at 37 ℃ for 24 hours and the diameter inhibition zones were measured in millimetres (mm) to determine the effectiveness of the extract against each organism (De Zoysa et al., 2019).

2.2.6 Bioautographic antimicrobial method

The thin layer chromatography was carried out mentioned in section plates and developed in the three eluent systems mentioned in section 2.2.4. The developed TLC plates with corresponding spots were placed aseptically onto the seeded Antibiotic medium no. 1 agar (AAM) plate with the cultures for 30 minutes to allow the extracts to diffuse into the agar. The wells were created in the agar at the side of the TLC plate and 40 µl of extracts were inoculated

into the wells. The TLC plates were removed and incubated for 24 hours at 37 ℃ (Horváth et al., 2013). Clear zones were observed inhibition of the bacteria used by the compound present at that Rf on the chromatogram.

2.2.7 Methanol extracts dilutions of plant extracts

Each plant extracts (5 mg/mL) of *E. globulus* and 10 mg/mL of *P. notoginseng* were diluted into different dilutions using 100% methanol solvent as shown in Table 3 and Table 4. 1 mg/ml of colistin and vancomycin were used as the positive control. The wells were created in each petri plate using a 6 mm cork borer. The plates were incubated at 37 ℃ for 24 hours and the inhibition zones were measured in millimetres (mm) to determine the effectiveness of the extract against each organism.

Table 3: The methanol extracts dilution of *E. globulus* from SAE and MAE.

Ratio	Eucalyptus (5 mg/mL) (µl)	Methanol (100%) (µl)
1:1	200	200
1:2	100	200
1:3	100	300
2:1	200	100
3:1	300	100

Table 4: The methanol extracts dilution of *P. notoginseng* from SAE and MAE.

Ratio	Ginseng (10 mg/mL) (µl)	Methanol (100%) (µl)
1:1	200	200
1:2	100	200
1:3	100	300
2:1	200	100
3:1	300	100

2.2.8 Antibiotics Serial Dilution

A stock solution of 1 mg/mL of each antibiotic (gentamicin and streptomycin) was added to 1 ml of distilled water. After mixing, each antibiotic was transferred into 1 ml of the from the first tube to the second tube and this was continued until the ninth tube. The wells were created in each petri plate using a 6 mm cork borer. Each dilution was tested into each culture and the plates were incubated at 37 ℃ for 24 hours and the inhibition zones were measured in millimetres (mm) to determine the identify similar concentration at which plant extract can be combined for synergy interaction.

2.2.9 Combination Assay of Plant Extracts and Antibiotics

After 24 hours incubation, the methanol extracts dilution of plant extract (*E. globulus*) and the antibiotics serial dilution was determined the best zones of inhibition depending on the cultures. The plant extracts were mixed with the antibiotic and were tested into each culture and the plates were incubated at 37 ℃ for 24 hours and the inhibition zones were measured in millimetres (mm) to evaluate the synergy interaction (Ncube et al., 2012).

2.3.0 The minimum inhibitory concentration (MIC) determination

2.3.1 Enunerating Bacteria

The overnight culture of each organism was grown in duplicate in 5 mL TSB and the OD was recorded separately at 600 nm. Ten sterile tubes with 270 µl sterile TSB in each was prepared. 30 µl of overnight culture was added to the first tube and mixed well. From the first tube of 30 µl was transferred to the second tube and this were continued until the twentieth tube. Tryptone Soy Agar plates per culture were taken and a grid was drawn. The cultures were placed from the twentieth tube added 2 x 10 µl was placed on the top line on the grid then followed by each tube until the third tube. The plates were incubated at 37 ºC without inverting them for 24 hours and the number of colonies of each culture was determined.

2.3.2 Antibiotics

A stock solution of 5 mg/mL of gentamicin and streptomycin were prepared. In the 96 well plate, 270 µl of sterile TSB was added into first well and the other nine wells were added into 150 µl. 30 µl of 5mg/mL stock of antibiotic was added into the first well and mixed. 150 µl from the first well transferred into the second well and continued until the tenth well. 30 µl of $5X10^5$ of organism was added to each well (Rekha et al., 2018). 150 µl of sterile TSB was added into each and the plates were incubated overnight at 37 ℃. TSB and the organism were used as a control.

2.3.3 Plant extracts

The plant extracts of 5 mg/mL (*E. globulus*) were infused into 10 discs each since it does not dissolve well into sterile TSB. So, the disc soaked into 1 ml sterile distilled water for two hours. 300 µl of the extract infused with water was transferred into 1.5 ml of sterile TSB in a tube as a first dilution. In the 96 well plate, 150 µl of sterile TSB was added into the ten wells in a row. From the first well, 150µl was added to the third well and continued until the tenth well. 30 µl of $5X10^5$ of each organism was added to each well and 150 µl of sterile TSB was added into each well again (Madikizela et al., 2013). The plates were incubated overnight at 37 ℃. TSB and the organism were used as a control.

2.3.4 Combination of Plant Extracts and Antibiotics

After 24 hours of incubation, the plant extract (*E. globulus*) and the antibiotics serial dilution was determined the MIC values depend on the cultures. The MIC value of gentamicin and streptomycin were prepared with different concentrations (k, 0.5k, 0.25k, 0.125k, 0.0625k, 0.03125k). K represented the MIC value of each antibiotic against each tested organism. The MIC value of plant extracts was prepared with different concentrations (4z, 2z, z, 0.5z). Z represented as the MIC value of plant extracts against each tested organism. 150 µl of plant extracts was added into seven well in a row and 150 µl of each antibiotic was added into seven

in a column (Rekha et al., 2018). 30 µl of each organism was added into each well and the plates were incubated at 37 ℃ for 24 hours. TSB and the organism were used as a control.

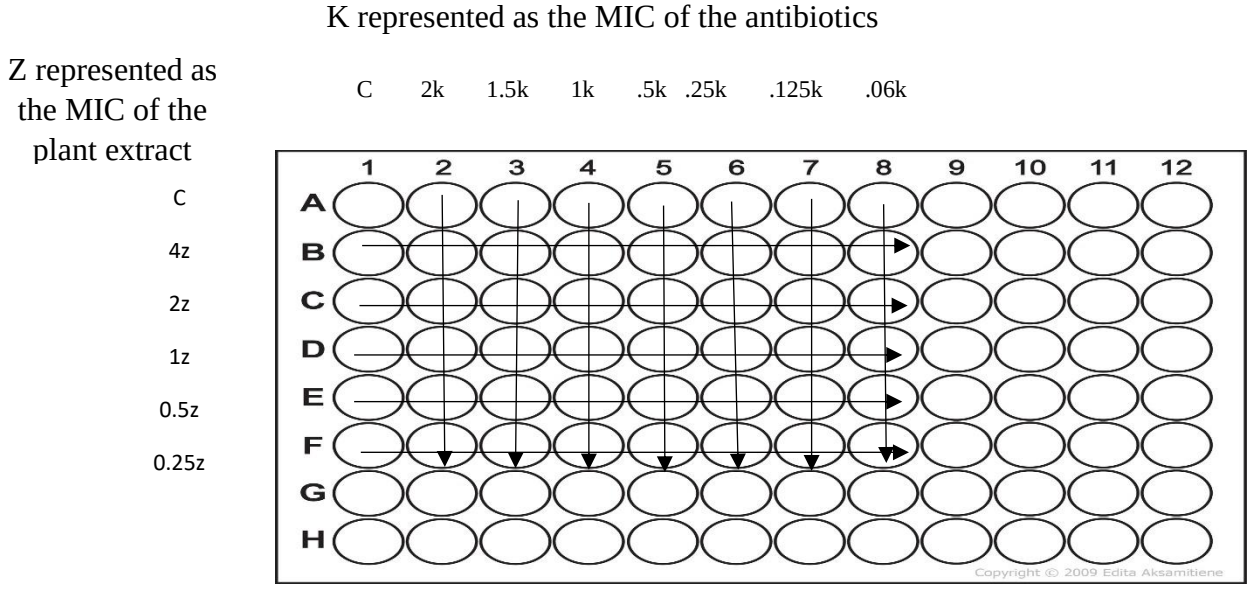


Figure 2:The combination of plant extract (*E. globulus*) and the antibiotics (Gentamicin and Streptomycin)

2.4.0 HPLC analyses

Two plant samples concentration of 5 mg/mL were used in chromatographic analyses were contained an auto-sampler, a column temperature controller and an ultraviolet detector coupled with an analytical workstation and C-18 column. The detection wavelength was set at 203 nm and the column temperature was at 30 °C. Water: trifluoroacetic acid (A, 100:0.01, v/v) and acetonitrile: trifluoroacetic acid (B, 100:0.01, v/v) used as mobile phase at room temperature conditions (25 ºC) and the 10 µl were directly injected in 1 mL/min flow rate. The identification of the individual compounds was carefully performed depend on the retention time and the total run time was 80 minutes (Qabaha et al., 2016).

Chapter 3: Results

3.1 Yields of extract

The preparation of extracts of *E. globulus* and *P. notoginseng* was performed using two types of extraction. The yield of *E. globulus* and *P. notoginseng* obtained from microwave assisted extraction using water solvent showed higher crude extracts of 22% and 17.5% compared to shaking assisted extraction using methanol 100% as solvent. The yields obtained for two type extracts are shown in Table 3.

Table 5: Extraction yields of the studied plants.

	Yield (%)	
Plants	**Shaking assisted extraction**	**Microwave assisted extraction**
E. globulus	16.2	22
P. notoginseng	8.2	17.5

3.2 Thin layer chromatography

TLC profiling of *E. globulus* and *P. notoginseng* from two different extracts were shown in Figure 3 and Figure 4.

3.2.1 Shaking assisted extraction

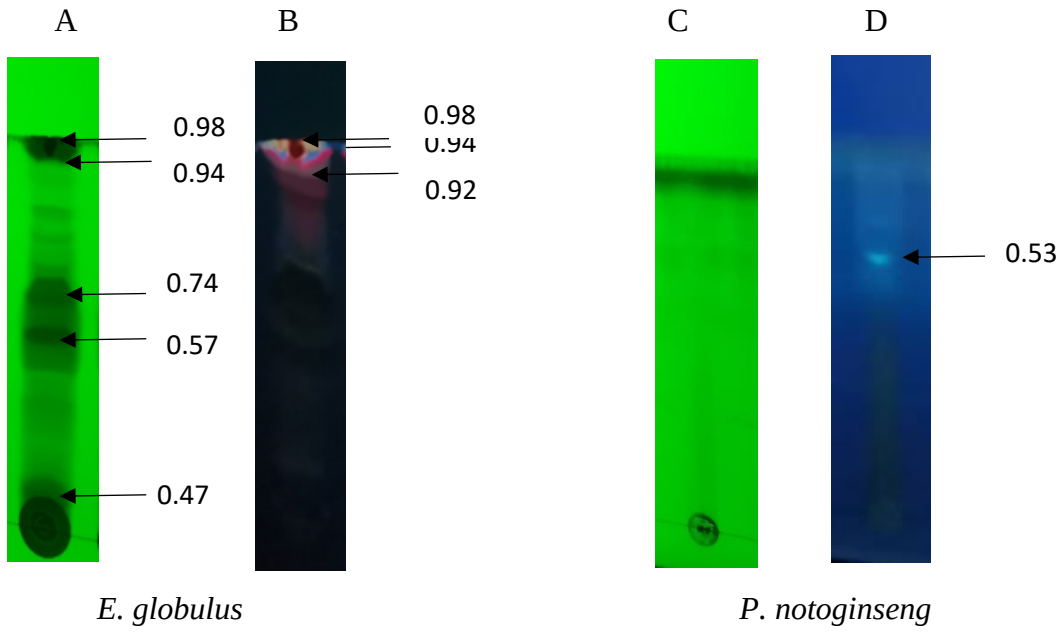


Figure 3: TLC profile of *E. globulus* and *P. notoginseng* from shaking-assisted extraction. Chloroform: methanol: water, lower phase (65:35:10 v/v) was used as solvent system to separate the compounds present in the extracts. Viewed under UV light 254 nm (A and C) and 365 nm (B and D). R_f values are also indicated.

3.2.2 Microwave assisted extraction

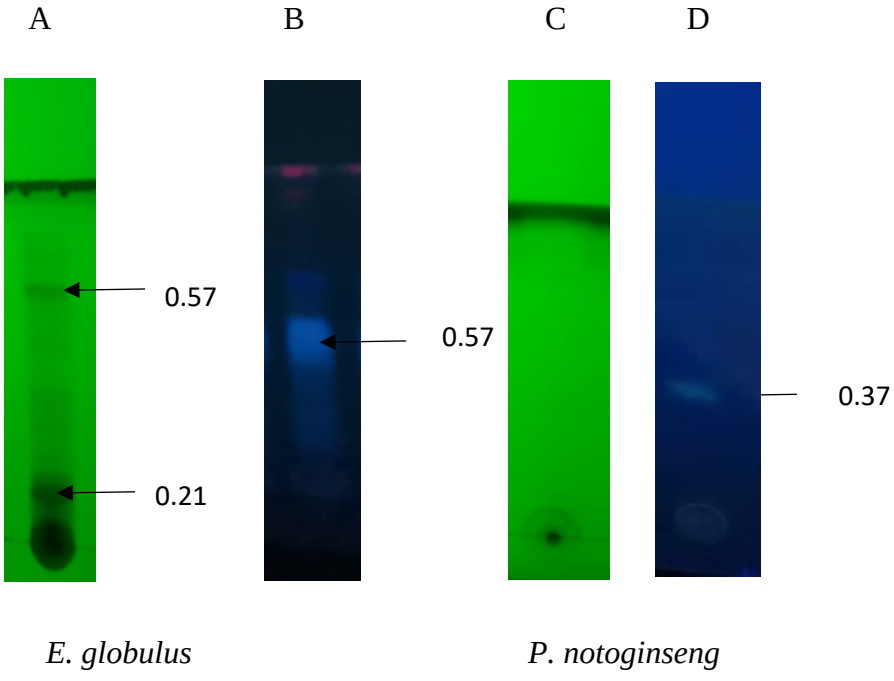


Figure 4: TLC profile of *E. globulus* and *P. notoginseng* from the microwave-assisted extraction. Chloroform: methanol: water, lower phases (65:35:10 v/v) was used as solvent system to separate the compounds present in the extracts. Viewed under UV light 254 nm (A and C) and 365 nm (B and D). R_f value are also indicated.

3.3 Antimicrobial activity of *E. globulus* and *P. notoginseng*

The antimicrobial activity of the crude extracts was determined based on the diameter of clear inhibition zone depending on different dilutions of inoculum using the well diffusion method as described in chapter 2 (section 2.2.5). The *E. globulus* and *P. notoginseng* crude extracts from microwave-assisted extraction (MAE) was re-constituted into 100 % methanol at 10 mg/ml concentration to determine the antimicrobial activity. The diameter of zone inhibition of *E. coli* showed 2% *P. aeruginosa* showed 4%, *S. aureus* showed 2% and *B. pumilus* showed 2% for both shaking assisted extraction (SAE) and microwave assisted extraction (MAE) to determine the standardize of the cultures. However, *P. notoginseng* showed weak inhibition effect on the growth of each organism because no inhibition zone was observed. The results are given in Table 6 for *E. globulus* and *P. notoginseng.*

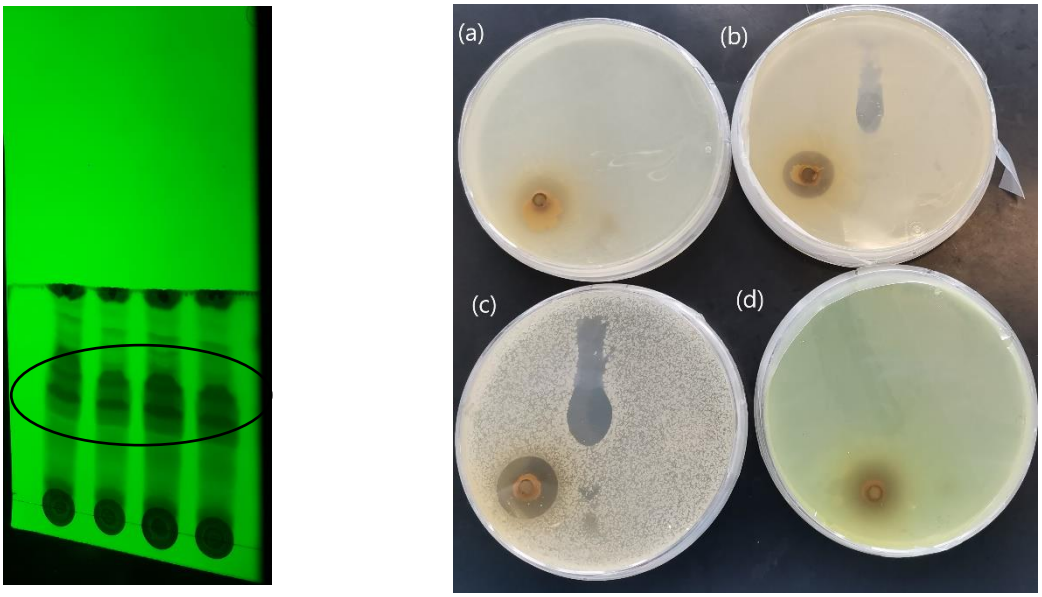
Table 6: Antimicrobial activity [zone of inhibition(mm)] of shaking-assisted extraction and microwave-assisted extraction of *E. globulus* leaves and *P. notoginseng* roots against Gram-negative bacteria (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (*S. aureus* and *B. pumilus*)

Zone of inhibition (mm)												
Extracts	***E. coli***			***P. aeruginosa***			***S. aureus***			***B. pumilus***		
	1%	2%	4%	1%	2%	4%	1%	2%	4%	1%	2%	4%
SAE												
EG (5 mg/ml)	14	14	16	14	17	13	9	13	9	11	13	9
PN (10 mg/ml)	-	-	-	-	-	-	-	-	-	-	-	-
MAE												
EG (5 mg/ml)	13	14	18	9	13	14	14	13	12	17	13	13
PN (10 mg/ml)	-	-	-	-	-	-	-	-	-	-	-	-
Methanol (100%)	-	-	-	-	-	-	-	-	-	-	-	-
Water	-	-	-	-	-	-	-	-	-	-	-	-
Colistin (1mg/ml)	12	16	14	14	14	12	-	-	-	-	-	-
Vancomycin (1mg/ml)	-	-	-	-	-	-	16	12	13	16	13	15

Key: EG: *E. globulus*, PN: *P. notoginseng*, -: no inhibition zones, SAE: Shaking assisted extraction, MAE: Microwave assisted extraction.

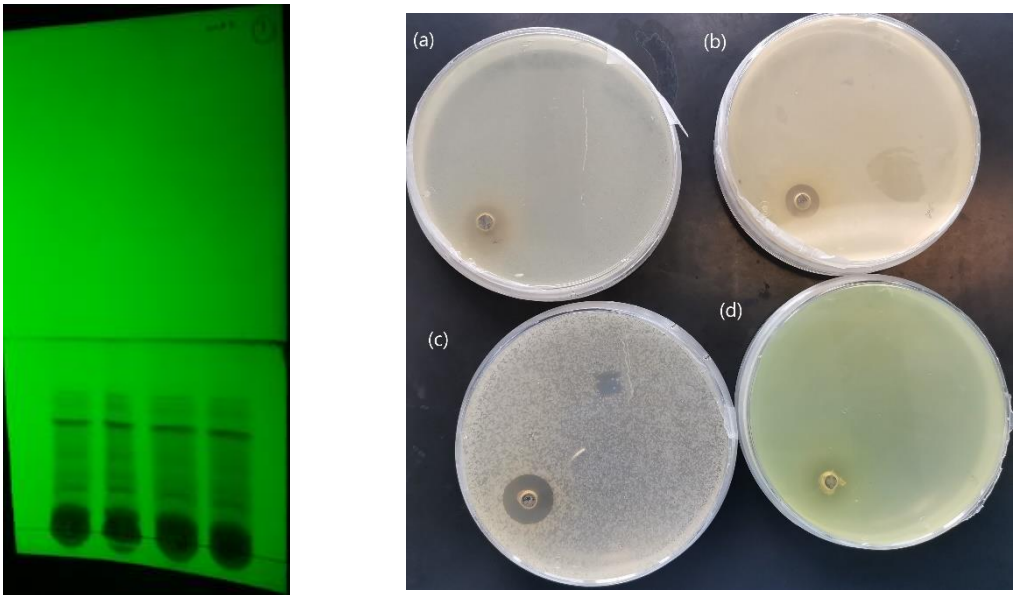
3.4 TLC-Bioautography

In the bioautography assays, the antimicrobial activity of the compounds separated on TLC was determined in Figure 5 and Figure 6. Significant of antimicrobial activity against *S. aureus* and *B. pumilus* was demonstrated the presence of the compounds on the agar in shaking assisted extraction (SAE), as evident by the significant clear zone of inhibition shown in Figure 5 (b) and (c). In Figure 6, there were no presence of compound shown in microwave assisted extraction (MAE) for all the organisms but there was clear zone inhibition shown in (b) and (c).



A B

Figure 5: [A]: TLC plate of *E. globulus* were viewed under UV light 254 nm. The spots were marked which presence the active compound. [B]: The bioautography of *E. globulus* shaking-assisted extraction (SAE) tested on (a) *E. coli*, (b) *S. aureus*, (c) *B. pumilus* and (d) *P. aeruginosa*



A B

Figure 6: [A]: TLC plate of *E. globulus* viewed under UV light 254 nm. [B]: The bioautography of *E. globulus* microwave-assisted extraction (MAE) tested on (a*) E. coli*, (b) *S. aureus*, (c) *B. pumilus* and (d) *P. aeruginosa*

3.5 Methanol extracts dilutions of plant extracts

3.5.1 *E. globulus*

The investigation of the antimicrobial activity of the methanol extracts dilution was performed using the agar well diffusion method where the zone of inhibition was measured. Figure 7 (a) showed the plant methanol extracts dilution of 1:3 formed largest zone of inhibition of 24 mm and 21 mm against *B. pumilus* and *S. aureus* compared to other methanol extracts dilution. *E. coli* showed inhibition zone of 12 mm and *P. aeruginosa* showed inhibition zone of 13 mm in methanol extracts dilution of 3:1.

Figure 7 (b) showed the plant methanol extracts dilution of 1:1 and 1:3 formed similar zone of inhibition of 9 mm against *E. coli. P. aeruginosa* showed largest zone of inhibition of 12 mm in methanol extracts dilution. *S. aureus* showed inhibition zone of 8 mm in dilution of 2:1 and *B. pumilus* showed inhibition zone of 12 mm in both dilution of 2:1 and 3:1. The results are given in Figure 5 for *E. globulus* in shaking assisted extraction (SAE) and microwave assisted extraction (MAE).

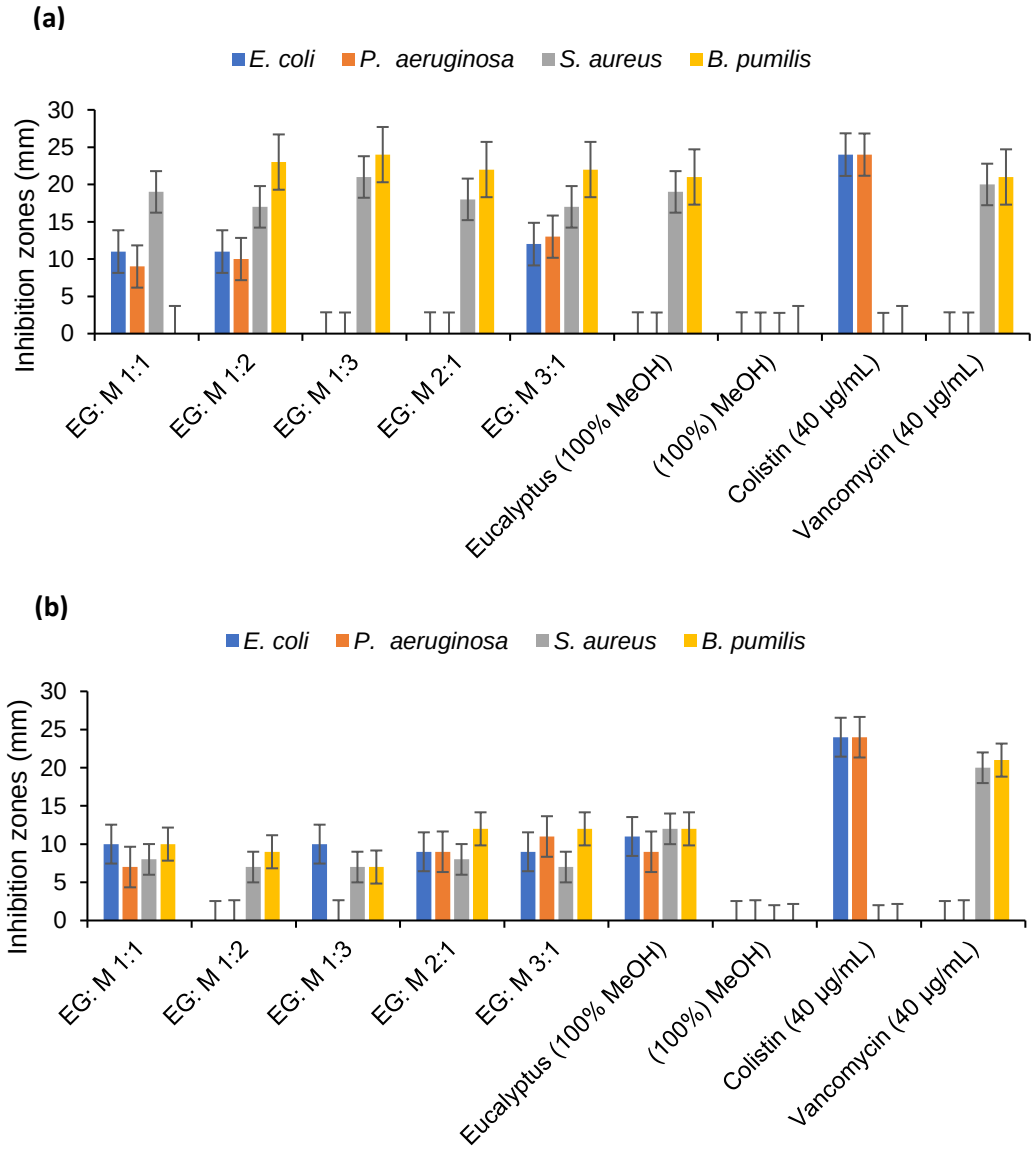


Figure 7: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. There is a significant difference [zone of inhibition (mm)] of methanol extracts dilution of *E. coli*, *P. aeruginosa*, *S. aureus* and *B. pumilus* ($\rho < 0.05$). EG: *E. globulus*, M or MeOH: 100% Methanol

3.5.2 *P. notoginseng*

The methanol extracts dilution of *P. notoginseng* showed no inhibition zone tested against *E. coli*, *S. aureus*, *B. pumilus* and *P. aeruginosa* was observed. The results are given in Figure 8

for *P. notoginseng* in shaking-assisted extraction (SAE) and microwave-assisted extraction (MAE).

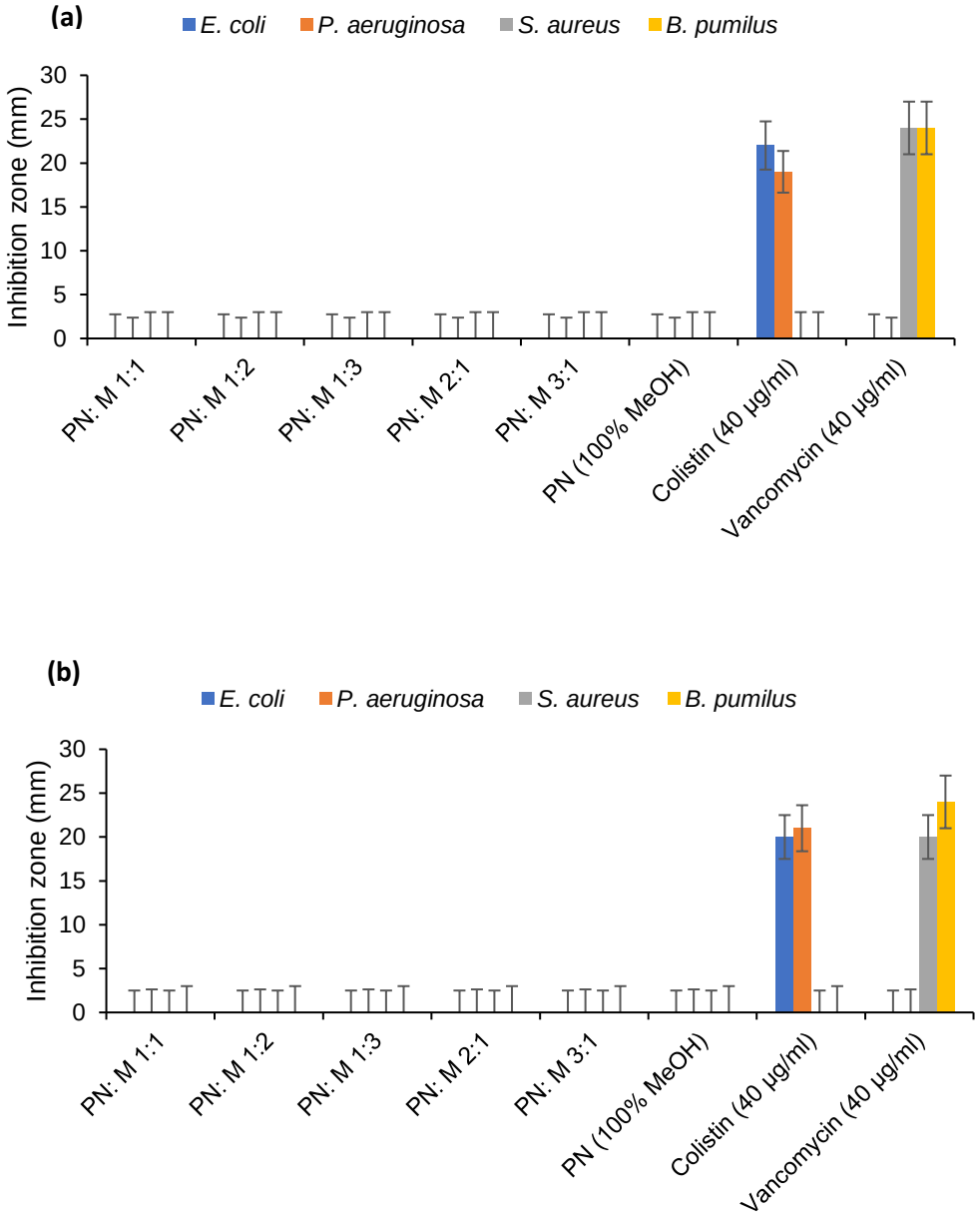


Figure 8: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. There is a significant difference [zone of inhibition (mm)] of methanol extracts dilution of *E. coli*, *P. aeruginosa*, *S. aureus* and *B. pumilus* ($\rho < 0.05$). PN: *P. notoginseng*, M or MeOH: 100% Methanol

3.6 Antibiotic serial dilution

The potential antimicrobial activity was determined based on the diameter of clear inhibition zone depending on different antibiotic serial dilution was performed using the agar well diffusion method where the zone of inhibition was measured in Table 7. The antibiotic concentration for gentamicin at the 16 µg/mL showed inhibition zone of 12 mm against *E. coli* and *P. aeruginosa. S. aureus* and *B. pumilus* showed inhibition zone of 12 mm and 13 mm for gentamicin concentration at the 4 µg/mL. The antibiotic concentration for streptomycin at the 16 µg/mL showed inhibition zone of 12 mm, 11 mm and 15 mm against *E. coli*, *S. aureus* and *B. pumilus*. *P. aeruginosa* showed inhibition zone of 13 mm for streptomycin concentration at the 64 µg/mL.

Table 7: Antimicrobial assay [zone of inhibition(mm)] of antibiotic: gentamicin and streptomycin (1mg/mL) dilution series

Zone of inhibition (mm)				
Concentration of antibiotic (µg/mL)	***E. coli***	***P. aeruginosa***	***S. aureus***	***B. pumilus***
Gentamicin				
1000	24	21	24	24
500	23	20	24	22
125	21	19	23	21
64	19	16	19	19
32	16	16	17	17
16	14	14	15	16
8	12	12	13	15
4	11	11	12	13
Streptomycin				
1000	20	18	18	24
500	18	17	17	24
125	16	14	14	21
64	16	13	14	18
32	14	11	12	17
16	12	9	11	15
8	10	9	9	13
4	10	7	9	13

3.7 Evaluation of Antimicrobial Synergy Interaction

3.7.1 Gentamicin

The investigation of the antimicrobial synergistic effect with the combination assay of plant extracts and gentamicin was performed using the agar well diffusion method where the zone of inhibition was measured. According to Figure 9 (a), synergistic effect was observed at a ratio of 3:1 with gentamicin against *B. pumilus*. However, synergistic effect of gentamicin with plant extract against *E. coli*, *P. aeruginosa* and *S. auerus* showed mostly less or no effect. Figure 9 (b), less synergistic effect was observed against each tested organism.

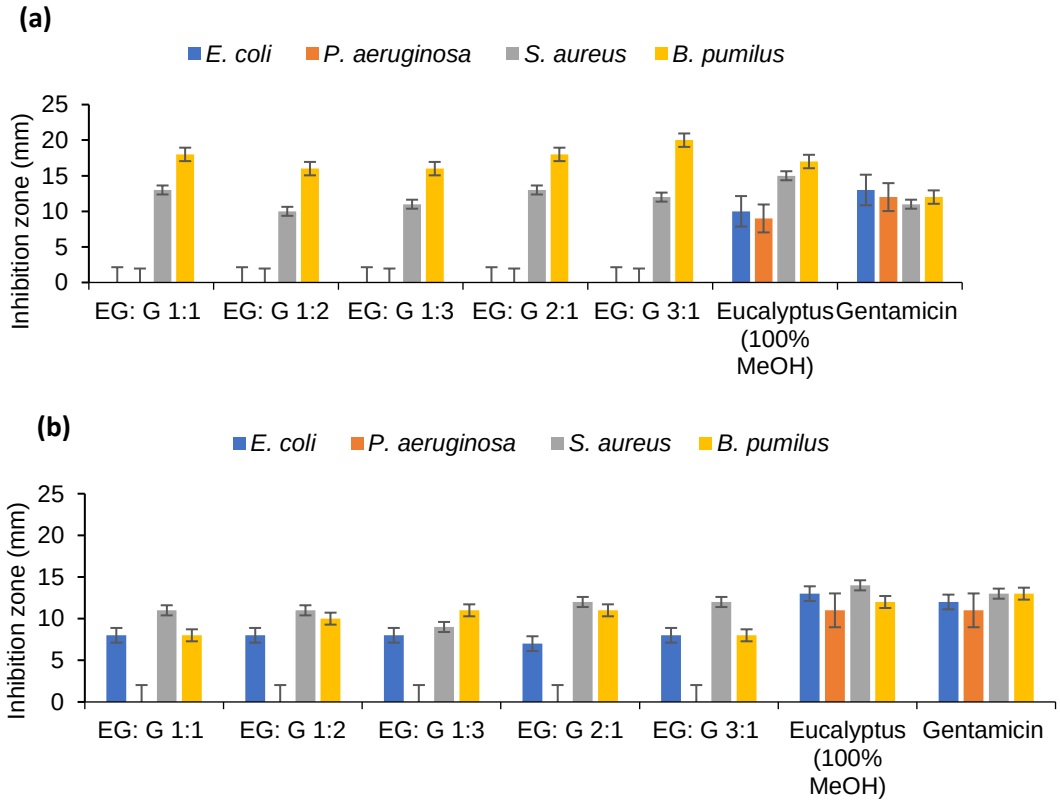


Figure 9: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. There is a significant difference [zone of inhibition (mm)] of combination of *E. globulus* and gentamicin (16 µg/mL and 4 µg/mL) of *E. coli, P. aeruginosa, S. aureus* and *B. pumilus* ($\rho < 0.05$). EG: *E. globulus*, G: Gentamicin and MeOH: Methanol 100%

3.7.2 Streptomycin

The evaluation of the antimicrobial synergistic effect with the combination assay of plant extracts and streptomycin was performed using the agar well diffusion method where the zone of inhibition was measured. Based on the Figure 10 (a), the synergistic effect was observed at a ratio of 3:1 with streptomycin against *E. coli*. Whereas, *B. pumilus* showed synergy interaction at a ratio of 1:1, 1:2, 1:3 and 3:1. Synergistic effect of streptomycin with plant extract against *P. aeruginosa* and *S. aureus* showed mostly less or no effect. Figure 10 (b), less synergy effect was observed on *E. coli* while at ratio of 3:1 was observed on *P. aeruginosa. S. aureus* showed synergy effect at a ratio of 3:1 while *B. pumilus* showed at a ratio of 1:1, 1:2 and 1:3.

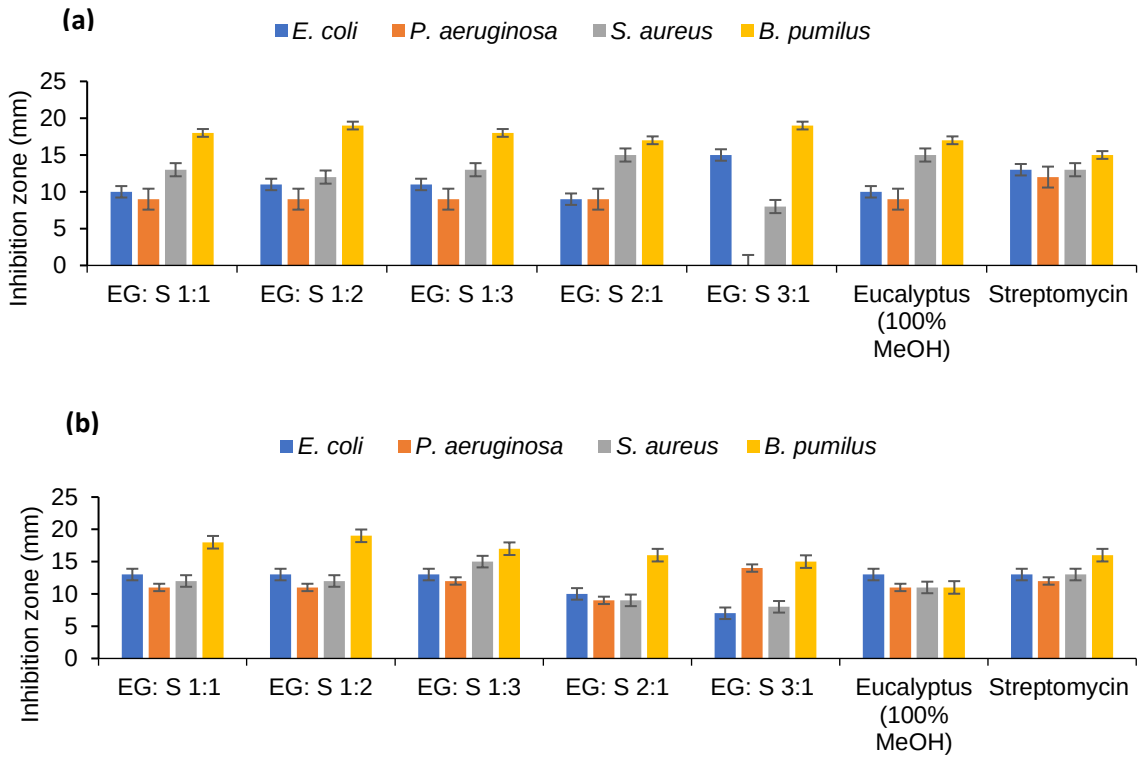


Figure 10: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. There is a significant difference [zone of inhibition (mm)] of combination of *E. globulus* and

streptomycin (64 µg/mL and 16 µg/mL) of *E. coli*, *P. aeruginosa*, *S. aureus* and *B. pumilus* ($\rho < 0.05$). EG: E. globulus, S: Streptomycin and MeOH: Methanol 100%

3.8 Evaluation of Minimum Inhibitory Concentration (MIC)

3.8.1 Antibiotics and Plant extracts

The analysis of MIC and MBC of antibiotic and plant extracts were performed using the Tryptone Soy Broth (TSB) dilution method with doubling serial dilution. The result of the minimum inhibitory concentration test indicated different levels of MICs depending on the bacterial strain being tested. The concentration of the plant extracts used was 20 mg/ml and the antibiotics were used at concentration of 5 mg/mL. In shaking-assisted extraction, both gentamicin and streptomycin showed the same MIC values of 20 mg/mL against *S. aureus.* However, MIC value for microwave-assisted extraction (MAE) in both antibiotics showed no effect. The results summary in Table 8 and Table 9 displays the MIC and MBC values for the antibiotics and plant extracts against each organism.

Table 8: MIC and MBC for gentamicin (5 mg/mL) and streptomycin (5 mg/mL)

		Concentration (µg/ml)			
Antibiotics		*E. coli*	*P. aeruginosa*	*S. aureus*	*B. pumilus*
Gentamicin	MIC	8	16	2	4
	MBC	16	16	4	8
Streptomycin	MIC	120	120	60	60
	MBC	250	250	120	120

Key: MIC- minimum inhibitory concentration and MBC- minimum bactericidal concentration.

Table 9: MIC and MBC for plant extracts (20 mg/mL)

		Concentration (mg/mL)			
Plant extracts (SAE)		*E. coli*	*P. aeruginosa*	*S. aureus*	*B. pumilus*
Gentamicin	MIC	-	-	20	-
	MBC	-	-	-	-
Streptomycin	MIC	-	-	20	-
	MBC	-	-	-	-
MAE		-	-	-	-
Gentamicin	MIC	-	-	-	-
	MBC	-	-	-	-
Streptomycin	MIC	-	-	-	-
	MBC	-	-	-	-

Key: MIC- minimum inhibitory concentration, MBC- minimum bactericidal concentration, - - no activity.

3.8.2 Combination of Plant extracts and Antibiotics

3.8.2.1 Gentamicin

The effectiveness of the combination of extracts using the concentration of 20 mg/ml and gentamicin of 2 µg based on Table 8 and Table 9 on tested bacterial strains was determined by measuring the minimum inhibitory concentration (MIC). Figure 11 (a) showed it inhibited growth with minimum concentration range of 4 µg to 2 µg (column 2 and 3). Minimum inhibitory concentration (MIC) in Figure 11 (b) did not showed inhibition of bacterial growth in each column with different concentrations of 4 µg to 3 µg.

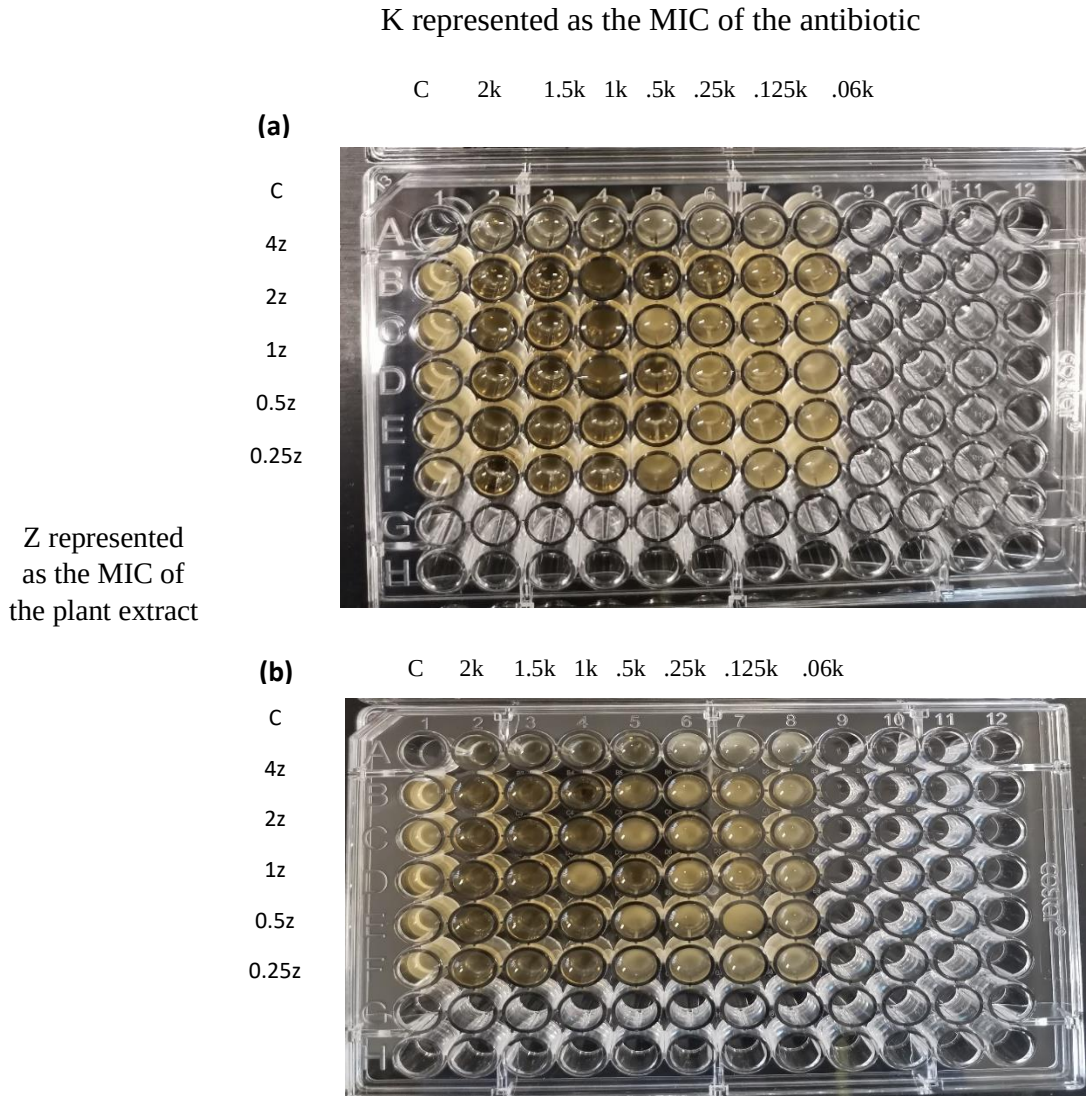


Figure 11: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. Both plant extracts and gentamicin were prepared with different concentrations as described (Chapter 2, section 2.3.4) on *S. aureus*

3.8.2.2 Streptomycin

The effectiveness of the combination of extracts using the concentration of 20 mg/ml and streptomycin of 60 µg based on Table 8 and Table 9 on tested bacterial strains was determined by measuring the minimum inhibitory concentration (MIC). The MIC value of both combinations did not showed inhibition of bacterial growth in each column with different concentrations of 120 µg to 3.75 µg.

K represented as the MIC of the antibiotic

Z represented as the MIC of the plant extract

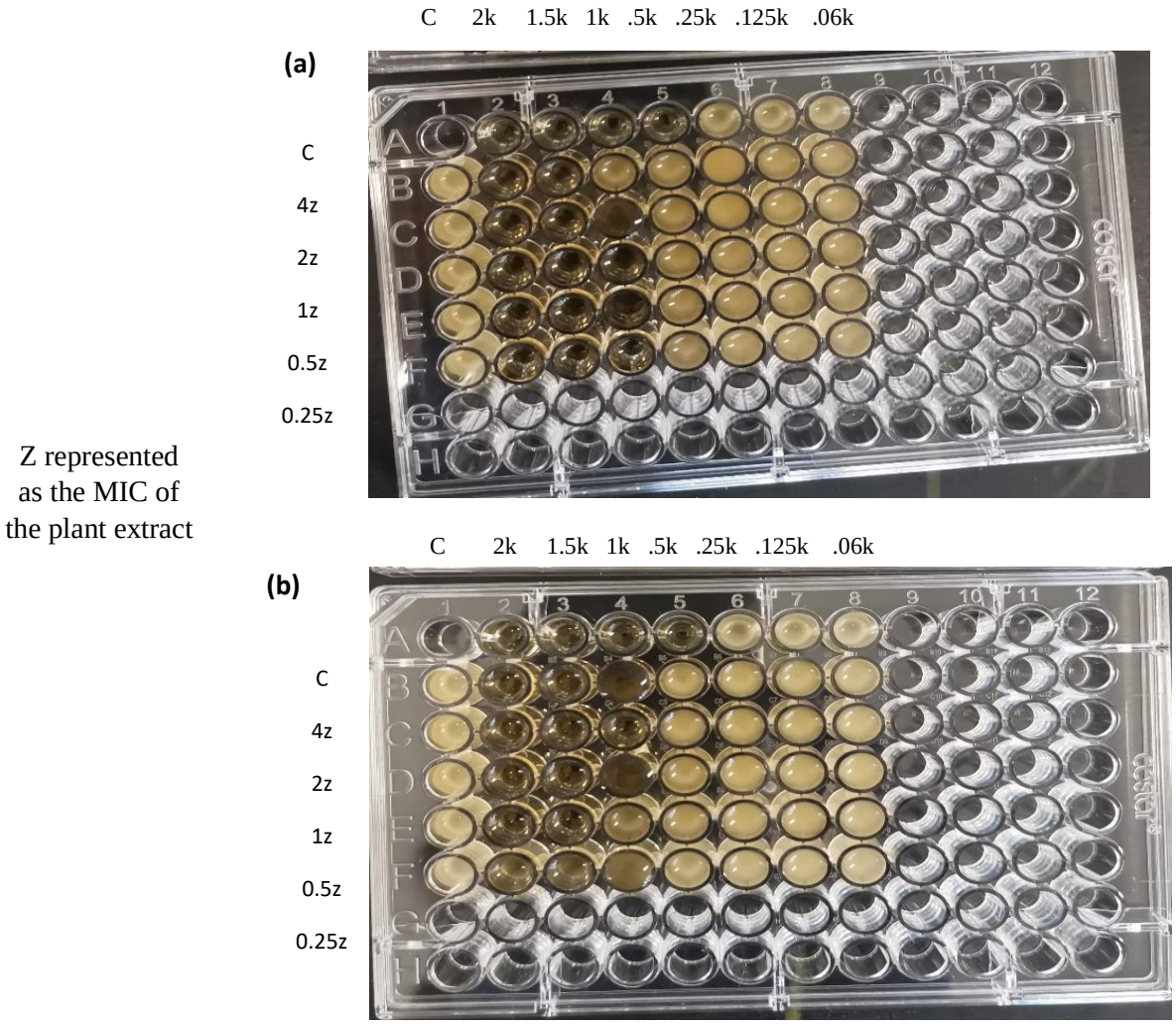


Figure 12: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. Both plant extracts and streptomycin were prepared with different concentrations as described (Chapter 2, section 2.3.4) on *S. aureus*

3.9 HPLC analyses

As an initial step in the separation process, analytical HPLC was performed on the methanolic extracts of *E. globulus* (5 mg/mL). The purpose of this study was to determine the complexity and the separation of the mixture of compounds found in these samples. The separations were conducted by injecting an equal amount of sample onto the C-18 analytical HPLC column. The solvent systems were used in this study for optimisation of the analytical HPLC parameters. Water: trifluoroacetic acid (A, 100:0.01, v/v) and acetonitrile: trifluoroacetic acid (B, 100:0.01, v/v) used as mobile phase at room temperature conditions (25 ºC) and 10 µl were directly injected in 1 ml/min flow rate. The chromatograms were recorded at 203 nm for 80 minutes. The chromatogram of analytical HPLC crude extract profile of *E. globulus* from two different extractions as shown Figure 13 and Figure 14 displayed no separation peaks achieved from 0 min to 45 minutes, in retention time. However, from visual observation, several peaks showed a better separation with sharp at different retention time in this study.

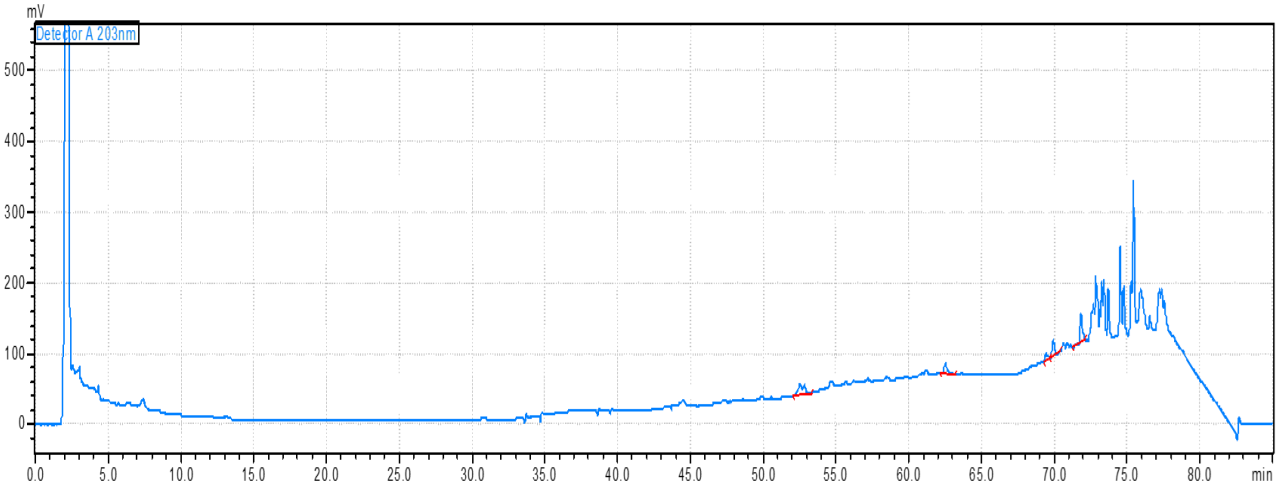


Figure 13: HPLC chromatogram of *E. globulus* from shaking-assisted extraction

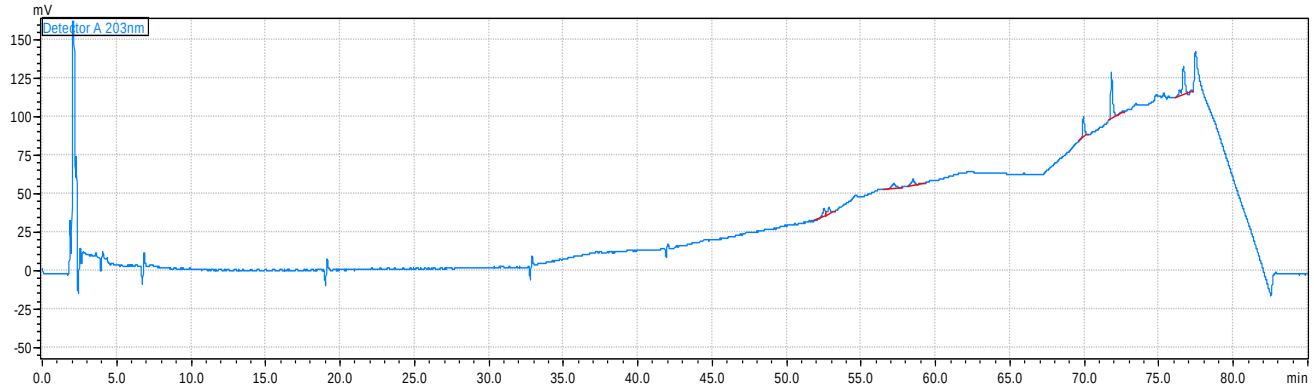


Figure 14: HPLC chromatogram of *E. globulus* from microwave-assisted extraction

Chapter 4: Discussion

Natural resources are now being sought as a source of antimicrobial agents which are becoming increasingly ineffective due to the development of resistance mechanisms. Secondary metabolites with complex interactions and varying modes of action are found in the complex plant-based products. The presence of numerous secondary metabolites with varying modes of action reduces the like-hood of bacteria developing resistance to the products (Obeidat et al., 2012). Plant-based antimicrobials bring greater therapeutic potential since they are beneficial in the treatment of infectious diseases while avoiding many of the adverse effects often associated with synthetic antimicrobials (Manandhar et al., 2019). With limited access to antibiotics especially in developing countries and an increase in multi-resistant bacteria worldwide, alternative treatments derived from plant-based products may be crucial (Silva and Fernandes Júnior, 2010).

4.1 Extraction of *E. globulus* and *P. notoginseng*

The preparation of medicinal plants for study purposes is initial and most important stage in achieving quality outcome of research. The selection of a suitable solvent, extraction methods, phytochemical screening method and identification techniques are all important steps in obtaining a high-quality bioactive molecule (Azmir et al., 2013).

The choice of extraction methods in this experiment was shaking-assisted extraction (SAE) and microwave-assisted extraction (MAE). In the shaking-assisted extraction (SAE) technique, the samples in the solvent were shaken at room temperature for 24 hours. This technique overcomes some of the drawbacks such as long extraction time, relatively high solvent consumption and the suitable for traditional extraction methods currently in use (Zhang et al., 2018). Modern or greener methods include microwave-assisted extraction (MAE) produce heat by interacting with polar molecules in the plant matrix, such as water and some organic components, through ionic conduction and mechanisms of dipole rotation. The mass and heat

transfer in the same direction resulting in a synergistic effect that enhances extraction and improve the yield of extraction (Qi et al., 2014). Microwave-assisted extraction (MAE) has offered some of the benefits such as shorter extraction time, higher selectivity and reduce organic solvent consumptions (Chaturvedi, 2018). Results obtained from Table 5 showed microwave-assisted extraction (MAE) using water solvent gave a higher yield and bioactivity than that obtained by the shaking-assisted extraction (SAE) using 100% methanol because the dissolution of the compounds depends on their relative solubility in the extraction solvent. During the extraction process, temperature plays an important key role which increases the yield of crude extracts(Chaturvedi, 2018).

The choice of the solvent is an important factor affecting the efficiency of extraction. The solvents should be with low heat evaporation, low toxicity, promote rapid physiological absorption and unlikely to cause the crude extracts to complex or dissociate (Pasquet et al., 2011).

4.2 Phytochemical screening

To research the various secondary metabolites in *E. globulus* and *P. notoginseng* extracts including saponins, tannins, flavonoids, alkaloids and propanoids which characterized by phytochemical screening. Separation by thin-layer chromatography (TLC) is a valuable technique in the organic laboratory. TLC method has some advantages of rapid, characterized the target compound and also convenient, whereas it needs standard and lack of uniqueness of bioactive compound (Choma and Jesionek, 2015). TLC is based on the use of two phases which is stationary phase, silica gel and mobile phase, the combination of organic solvents. It can be used to determine the substance purity and separate mixtures (Annegowda et al., 2013).The polarity of the solvent, solute and adsorbent are all important factors in determining a compound's mobility rate. This method helps in identifying various compound mixtures based on mobility differences (Horváth et al., 2013). Depending on the type of solvent used, the

polarity of the molecules can affect how far the spots travel. Polar molecules are more strongly attracted to polar solvents and will therefore move further if a polar solvent is used instead of a non-polar solvent (Friesen et al., 2015). In this study, the chloroform: methanol: water solvent system was made up in the ratio of 65:35:10 for the separation of the compound which used as standard method for polar and non- polar compounds. The selected solvent was done according to the order of increasing polarity, starting from chloroform the least polar to water with the highest polarity (Sidana et al., 2012). The leaves from *E. globulus* showed good separation compound from the shaking-assisted extraction (SAE) compared to microwave-assisted extraction (MAE) while the roots from *P. notoginseng* did not show any good separation in both extractions. The presence or absence of bioactive compound from the TLC results can be explained by the extraction process chosen.

Test microorganisms can be exposed to all compounds on a TLC plate after an extract has been separated, to identify an extract's active components. Based on Figure 5 and Figure 6, a bacterial culture is exposed to a TLC plate and the growth of microbial will occur everywhere except the areas with growth-inhibitory compounds. Although thin-layer chromatography was first used for antimicrobial assays on antibiotics, plant extracts are now commonly screened for antimicrobial compounds known as bioautography. The separation used do not reveal all compounds of the extract but identifies the zones of antimicrobial activity.

4.3 Antimicrobial Activity of the Plant Extracts

The rise of antimicrobial resistance to many available antimicrobial agents has implied the development of new drugs (Hwang and Gums, 2016). Drugs are also known to have side effects on the host such as hypersensitivity allergic reactions and immune suppression (Vadhana et al., 2015). The use of plant extracts to test for antimicrobial activity has been suggested as one of the ways of achieving this goal. The E. *globulus* and P. *notoginseng* in the study seem to have medicinal properties. This study evaluated the use of the plants in treating Gram-negative

bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus pumilus*) to test if they are effective or not when used singly and in combinations. Based on Table 6, the antimicrobial activity of the crude extracts was determined based on the diameter of the clear inhibition zone depending on different dilutions of inoculum (1%, 2% and 4%). The inoculum is required for the concentration of the expected organism as a standard test. The concentration of extracts and the size of the inoculum affected the growth of the organisms. Both shaking-assisted extraction (SAE) and microwave-assisted extraction (MAE) for *E. globulus* showed antimicrobial activity against *E. coli, P. aeruginosa, S. aureus* and *B. pumilus* which may reflect the antimicrobial activity of plant active ingredients that inhibit the growth of organisms. Different dilutions of methanol extracts were performed to understand the influence of the methanol solvent on the activity disregarding the concentration. The highest activity of crude extracts from the leaves was observed in *E. globulus* at 5 mg/mL inhibition zones of 24 mm in *B. pumilus*. Methanol is classified as a polar solvent, although not very polar as water. This explains that this solvent is water-miscible and will extract primarily ionic components from eucalyptus (Dos Santos Ferreira et al., 2016). Studies proved that the methanolic crude extracts were found to have potent antimicrobial activity compared to aqueous extracts against all the tested bacteria. The crude extracts of *E. globulus* from microwave-assisted extraction (MAE) using water solvent and was re-constituted in 100 % methanol showed less effective in antimicrobial activity against all tested bacteria. Phytochemical compounds of *E. globulus* contain carboxylic acids, esters, ethers, aldehydes, alcohols, ketones, hydrocarbons along sesquiterpenes and monoterpenes, flavonoids, propanoids, tannins and alkaloids might be inhibited the growth of organisms (Ishag et al., 2018). The leaves of *E. globulus* contain 1,8-cineole (cineole or eucalyptol) which are traditionally used as antipyretic remedies for the symptoms of respiratory problems such as flu, cold and sinus congestion, anti-inflammatory and analgesics (Kaur et al., 2019).

The roots from *P. notoginseng* at the concentration of 10 mg/ml did not show any antimicrobial activity against *E. coli, P. aeruginosa, S. aureus and B. pumilus* based on Table 6 and Figure 8 in shaking-assisted extraction (SAE) and microwave-assisted extraction (MAE). Previously studies were confirmed microwave-assisted extraction (MAE) showed some roots at a high temperature could change the structure of compounds especially ginsenoside or saponins resulting in antimicrobial activity against all the organisms (Zhao et al., 2019). Moreover, water is a better solvent compared to methanol which has cytotoxic nature and is also unsuitable for extraction for ginseng especially in the roots (Jegal et al., 2019). The ginsenoside in ginseng powder was found to be reduced by autoclaving, consistent with previous results of a study indicating that ginsenosides are not stable at high temperatures (Lee et al., 2016). However, the antimicrobial activity of ginseng is not active in this study.

This variation may cause the freshness of plant materials, age of the plant, time of harvesting of plant materials, physical conditions include temperature, water and light and drying method employed before the extraction process might affect the antibacterial activity of a plant extract (Na et al., 2004). In the field of natural product drug development, extraction is the critical stage in the preparation of plant potential therapeutic candidates (Lee et al., 2011). Modern methods of extraction will be more effective in advancing the development of potential antimicrobial activity (Vuong et al., 2015). Another factor that might cause is the solvent of the plant extract might affect the chemical properties and the chemical structures of the solute (Kalaivani et al., 2012).

Furthermore, the choice of drugs used in antimicrobial activity was colistin and vancomycin. Colistin is an effective antibiotic against the majority of multi-drug resistant Gram-negative bacteria including *Pseudomonas aeruginosa* and *Enterobacteriaceae* members such as *Escherichia coli*. It exhibits rapid, concentration-dependent bactericidal activity (Roberts et al., 2015). Colistin is a polycationic peptide and has lipophilic moieties and hydrophilic. By

displacing calcium and magnesium bacterial counter ions in the lipopolysaccharide, this region interacts with the bacterial outer membrane (Yahav et al., 2012). Vancomycin against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus pumilus* involves the alteration of the peptidoglycan synthesis pathway (Bruniera et al., 2015). The glycopeptide antibiotic inhibits the growth of bacteria by disrupting the polymerization of peptidoglycans in the bacterial cell wall (Raza et al., 2018). Gram-positive bacteria are more susceptible to many antimicrobial substances, including antibiotics as compared to Gram-negative bacteria which could be attributed to the protection provided by gram-negative bacterial cell wall outer membrane (Miller et al., 2016). The tough outer membrane most likely influences the uptake of the eucalyptus extract by Gram-negative bacteria (*E. coli* and *P. aeruginosa*) (Azzam, 2020). Following the literature, the root extracts from ginseng were active against Gram-negative bacteria (*E. coli* and *P. aeruginosa)* and Gram-positive bacteria (*S. aureus* and *B. pumilus*) (Kim and Yang, 2018).

4.4 Synergistic activity of Plants Extracts and Antibiotics

The evaluation of synergistic studies examines a combination of two or more drugs in the hopes of achieving an enhanced overall effect that is substantially greater than the sum of their parts. Combination therapy has recently gained widespread recognition, particularly in the field of infectious disease (Pezzani et al., 2019). Depending on the method of extraction, plant extracts demonstrated various synergistic abilities to inhibit the growth of microorganisms in this study. It is well known that one of the most effective ways for combating bacterial resistance is to restore antibiotic activity through the synergistic action of antimicrobial materials derived from natural and synthesised agents (Rai et al., 2017). There is already scientific proof that conventional antibiotics can be improved by interacting synergistically with plant-derived compounds. The advantages of synergistic interactions are reducing side effects, reduce to lower dosage in comparison to synthetic products, increased stability and bioavailability and

increased efficiency (Tiwari et al., 2005). However, a combination of two antimicrobial agents can be classified as additive, non-interactive or antagonistic (Moon et al., 2011).

The choice of the drug used in this study was gentamicin and streptomycin. Gentamicin and Streptomycin are groups as aminoglycoside antibiotics with broad bactericidal activity against gram-negative and Gram-positive bacteria. There are some limitations on the use of gentamicin and streptomycin due to their side effects, especially in nephrotoxicity. As a result, recognising the extent and severity of this complication can be useful in preventing or reducing it in gentamicin and streptomycin therapy (Said et al., 2016). Like other aminoglycosides, it acts to suppress protein synthesis by binding to bacterial ribosomes. Based on Figure 9 and Figure 10, the experiment was done to test for any effect with the combination of the plant extract and gentamicin and streptomycin at different dilutions. Synergistic evaluation in shaking-assisted extraction (SAE) is more effective compared to microwave-assisted extraction (MAE). The combination of plant extracts and both antibiotics showed the most synergy effect against *B. pumilus*. The organisms showed less or no effectiveness due to some crude extracts cause loss of intracellular components and damage to the cytoplasmic membrane of resistant organisms (Aiello et al., 2020).

Numerous *in vitro* studies have shown that plant extracts and antibiotics have synergistic effects, resulting in a reduction of minimum inhibitory concentration (MIC) (Gaudereto et al., 2020). The extracts and the concentration of antibiotics will then undergo serial dilution in to determine the minimum concentration which can inhibit a certain amount of bacteria. Determination of MIC of crude extracts of *E. globulus* Figure 11 (a) showed it inhibited growth with a minimum concentration range of 4 µg to 2 µg (columns 2 and 3). Among the other results in Figure 11 (b) and Figure 12, the *E. globulus* has been found to exhibit against *S. aureus* in high concentrations of MIC. This might be explained by the extraction method, strain to strain differences and differences in susceptibility testing conditions (Vieira et al., 2017).

4.5 Identification of compound

In the study of natural products, chromatography techniques such as HPLC are primarily used to identify the of active compounds relative to the impurities (Abdelkhalek et al., 2020). The C-18 column was used as a stationary phase while water: trifluoroacetic acid (A, 100:0.01, v/v) and acetonitrile: trifluoroacetic acid (B, 100:0.01, v/v) used as mobile phase s had a significant effect on the retention time and elution order as shown in Figure 13 and Figure 14. Based on the HPLC chromatogram, the methanolic extracts from shaking-assisted (SAE) and aqueous extracts which re-constitute in methanol from microwave-assisted extraction (MAE) displayed different types of an unknown compound. This unknown compound showed active antimicrobial activity. Extracts can then be made with different solvents to isolate and purify the active compounds that are responsible for bioactivity.

4.6 Project Limitation

The causes of projects limitation due to delays in supply or shortage of materials. The plant materials especially ginseng powder mainly from China takes a longer time for sourcing and transporting of the products. The ginseng crude extracts were not active in antimicrobial activity might be due to the loss of internal components in powder cause different extraction processes. The solvent used for extraction might not be effective for the target compound since water solvent only can be used for microwave-assisted in the laboratory.

Changes in bacterial growth patterns are caused by changes in the bacteria's environment. The growth rate of the cultures was inconsistent when performing the antimicrobial activity. Because conditions can be easily controlled in a lab, certain properties of bacteria and their growth media that determine growth patterns can be identified. In general, as environmental conditions weaken, a colony's growth pattern gets more complex.

Minimum inhibitory concentration MIC can vary greatly with minor changes in this study that can result in large variations of the MIC might be due to prolonged incubation. The principal of MIC was tedious which antibiotic and plant extracts preparation for each test, the possibility of errors in this study.

4.7 Conclusion

The present study has shown that medicinal plants have an antimicrobial effect against various bacteria. Among the two medicinal plants that were tested in this study, the Australian plant *E. globulus* has been demonstrated to have antimicrobial activity against both Gram-negative bacteria and Gram-positive bacteria especially in *B. pumilus*. The shaking-assisted extraction of *E. globulus* had the highest activity compared to microwave-assisted extraction. Furthermore, the presence of unknown compounds that were responsible for antimicrobial activity appears to be relatively stable since the results are shown. The MIC value of combination *E. globulus* and gentamicin were shown a synergy effect compared to streptomycin against *S. aureus*. However, *P. notoginseng* failed to show any antimicrobial activity.

4.8 Future directions

The future finding of this study can be based on evaluating toxicity studies of the effective plants to determine the safety indices of the extracts. Clinical trials should be conducted to investigate the efficacy of these plant extracts in the treatment of these infectious diseases. The plant extracts should be further analyzed to isolate the specific antimicrobial principles in them. Determine the activity of these plant extracts on the types of pathogenic bacteria to evaluate the synergistic activity of these medicinal plants with non-antibiotics.

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Appendix

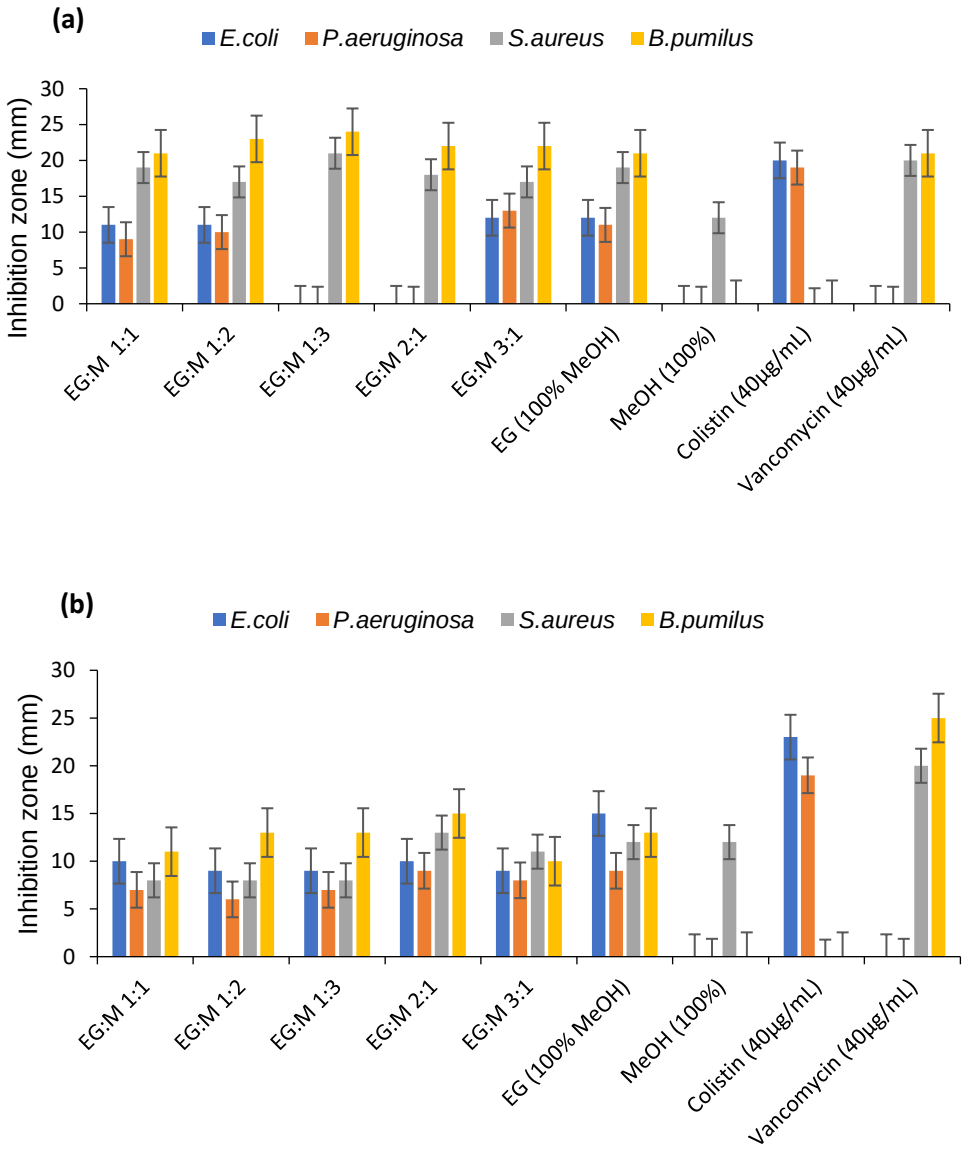

Figure 15: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. There is a significant difference [zone of inhibition (mm)] of methanol extracts dilution of *E. coli*, *P. aeruginosa*, *S. aureus* and *B. pumilus* ($\rho < 0.05$). EG: *E. globulus*, M or MeOH: 100% Methanol

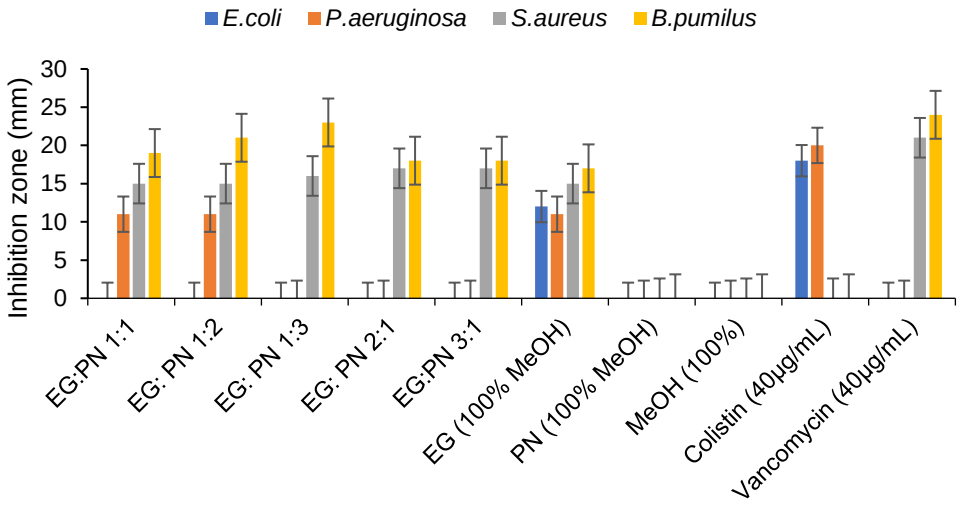

Figure 16: Combination of plant extracts (*E. globulus* and *P. notoginseng*). There is a significant difference [zone of inhibition (mm)] of methanol extracts dilution of *E. coli*, *P. aeruginosa*, *S. aureus* and *B. pumilus* ($\rho < 0.05$). EG: *E. globulus*, PN: *P. notoginseng*, M or MeOH: 100% Methanol

Table 10: Antimicrobial assay [zone of inhibition(mm)] of antibiotic: colistin and vancomycin (1mg/mL) dilution series

Zone of inhibition (mm)				
Concentration of Antibiotic (µg/mL)	***E. coli***	***P. aeruginosa***	***S. aureus***	***B. pumilus***
Colistin				
1000	22	19	0	0
500	20	18	0	0
125	18	15	0	0
64	16	14	0	0
32	13	12	0	0
16	11	10	0	0
Vancomycin				
1000	15	0	23	28
500	13	0	22	26
125	11	0	20	26
64	9	0	18	25
32	8	0	16	23
16	0	0	14	18
8	0	0	12	15

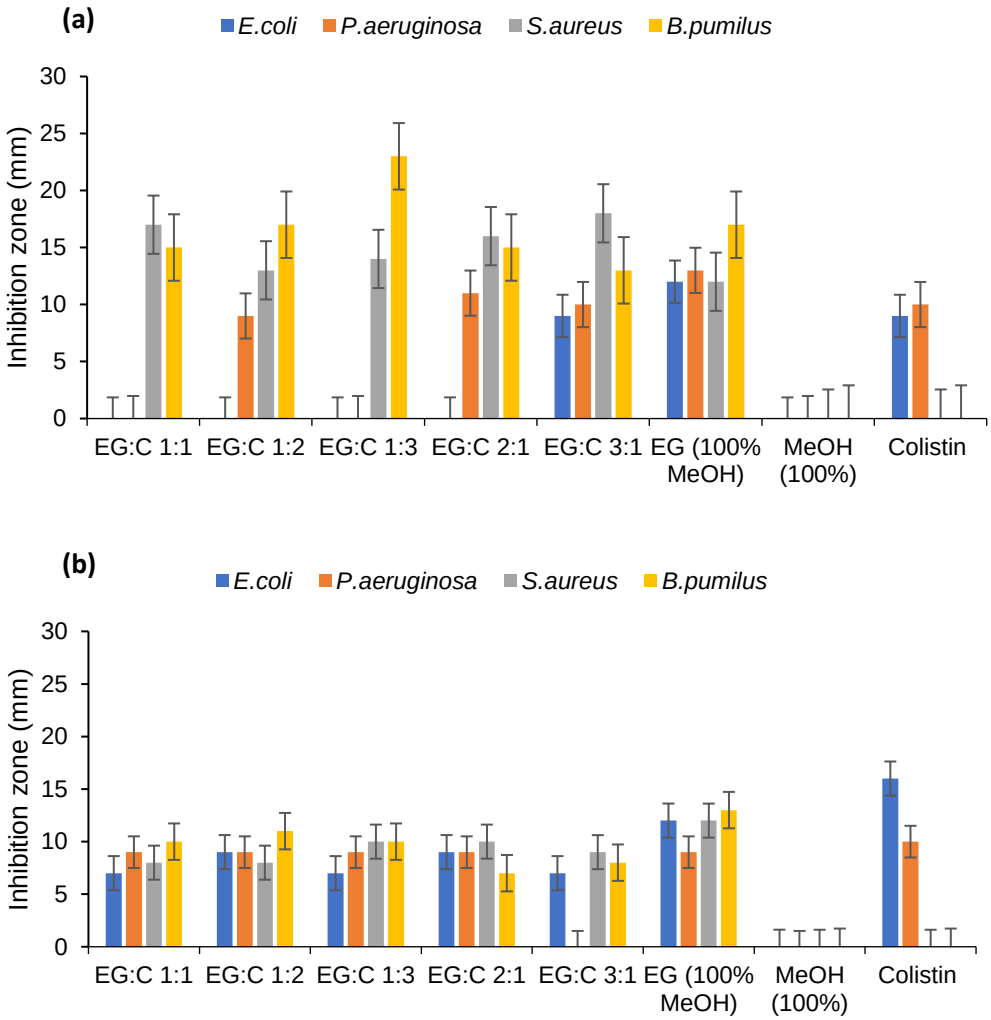

Figure 17: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. There is a significant difference [zone of inhibition (mm)] of combination of *E. globulus* and Colistin (16 µg/mL) of *E. coli, P. aeruginosa, S. aureus* and *B. pumilus* ($\rho < 0.05$). EG: *E. globulus*, C: Colistin and MeOH: Methanol 100%

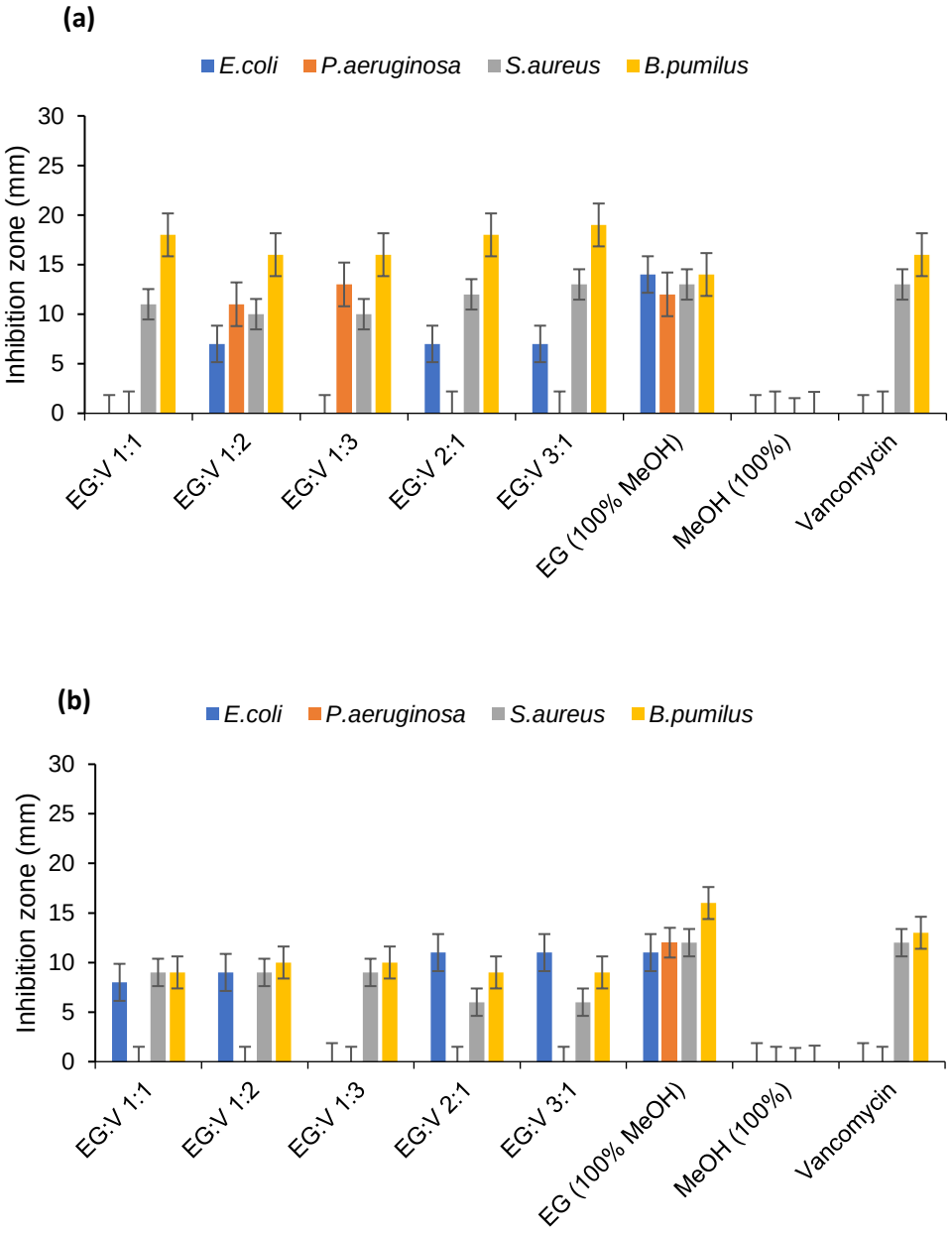

Figure 18: (a) Shaking-assisted extraction and (b) Microwave-assisted extraction. There is a significant difference [zone of inhibition (mm)] of combination of *E. globulus* and Vancomycin (500 µg/mL and 4 µg/mL) of *E. coli*, *P. aeruginosa*, *S. aureus* and *B. pumilus* ($\rho < 0.05$). EG: *E. globulus*, V: Vancomycin and MeOH: Methanol 100%