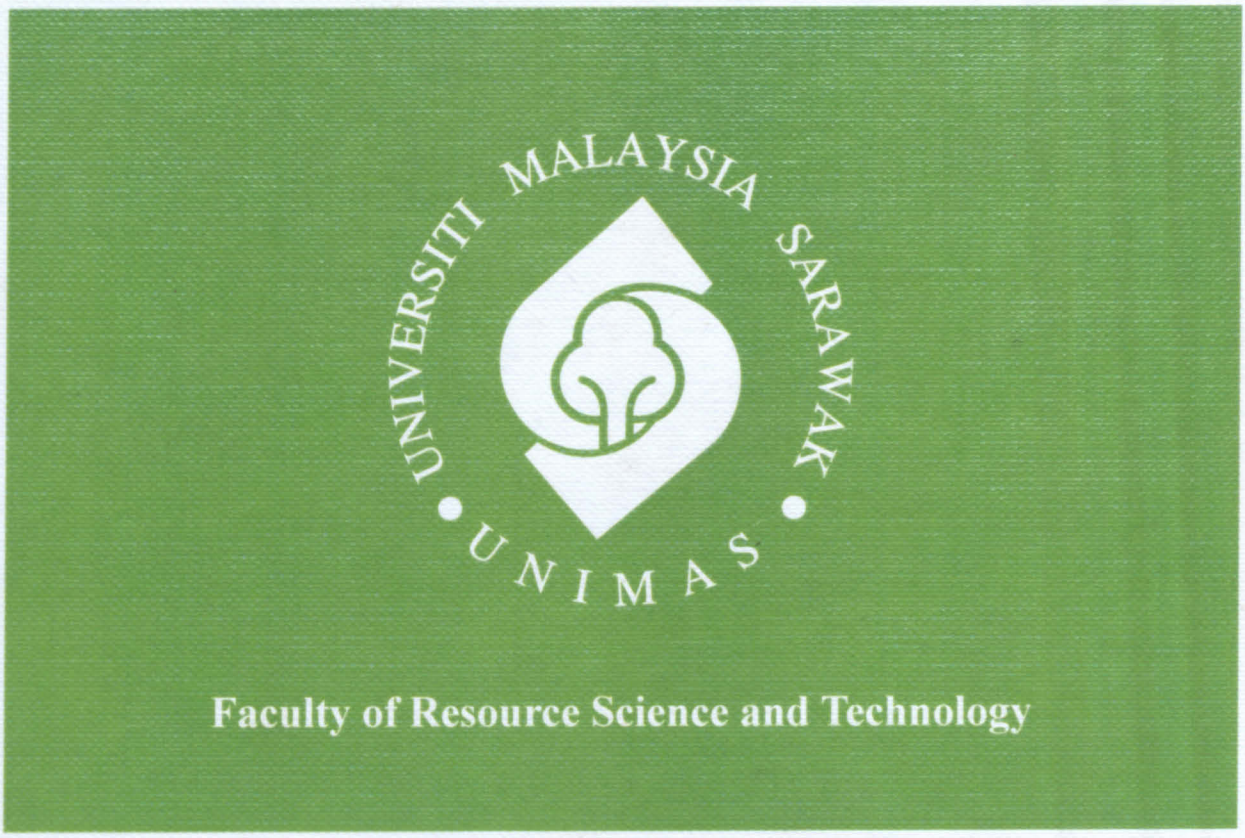

Screening of Antibacterial Activity of *Durio zibethinus* Murr. Leaves Extract

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Bachelor of Science with Honours
(Resource Biotechnology)
2018

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Screening of Antibacterial Activity of *Durio zibethinus* Murr. Leaves Extract

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The dissertation is submitted in partial fulfillment of requirement for degree of Bachelor Science with Honours in Resource Biotechnology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2018

UNIVERSITI MALAYSIA SARAWAK

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LIST OF ABBREVIATION

B.cereus	*Bacillus cereus*
DMSO	Dimethyl sulfoxide
D.zibethinus Murr.	*Durio zibethinus* Murr.
E.coli	*Escherichia coli*
mm	Millimetre
µm	Micrometre
µl	Microliter
P.aeruginosa	*Pseudomonas aeruginosa*
SD	Standard Deviation
S.aureus	*Staphylococcus aureus*

Screening of Antibacterial Activity of *Durio zibethinus* Murr. Leaves

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ABSTRACT

Local durian or *Durio zibethinus* Murr. (*D.zibethinus* Murr) is a large edible fruit that currently well known in Indonesia, Philiphine, Malaysia and Southern Thailand. *D.zibethinus* Murr. is the species under Bombacaceae family. Many studies has been done towards the fruit flesh, its skin and the seed of this tree for the antibacterial activity. However, there are lacking on studies on leaves although the leaves was abundantly found. The present study, *D.zibethinus* Murr. leaves extract has been prepared using three different solvent which are ethanol, methanol and acetone and tested against two gram positive bacteria (*S.aureus* and *B.cereus*) and two gram negative bacteria (*E.coli* and *P.aeruginosa*) by using agar well diffusion method in order to looks for antibacterial activity. Different concentration of stock solution was prepared which are 62.5 mg/ml, 125 mg/ml, 250 mg/ml and 500 mg/ml using 10% DMSO. Gentamicin has been choosen as positive control and 10% DMSO as negative control. The inhibition zone were exhibitely found on gram positive bacteria from the *D.zibethinus* Murr. leaves extract. The diameter of inhibition zone were measured in milimeter (mm). Therefore, from the result obtained, further research on *D.zibethinus* Murr. Leaves should be conducted due to its potential in inhibit the gram positive bacteria and has high potential in medical application.

Key words: *Durio zibethinus* Murr. , leaves extract, zone of inhibition, agar well diffusion.

ABSTRAK

Durian kampung atau *Durio zibethinus Murr. (D.zibethinus Murr) merupakan buah yang besar yang boleh dimakan yang kini sangat dikenali di Indonesia, Filipina, Malaysia dan Selatan Thailand. D.zibethinus Murr. ialah spesis di bawah famili Bombacaceae. Banyak kajian telah dijalankan terhadap isi buah durian, kulitnya dan biji durian untuk aktiviti antibakteria. Namun, kajian terhadap daun sangat kurang walaupun daunnya senang ditemui. Dalam kajian ini, ekstrak daun D.zibethinus Murr. disediakan dengan menggunakan tiga jenis pelarut iaitu etanol, metanol dan aceton dan diuji keatas dua gram positif bakteria (S.aureus dan B.cereus) dan dua gram negatif bakteria (E.coli dan P.aeruginosa) untuk melihat aktiviti antibakteria menggunakan kaedah penyebaran agar. Pelbagai kepekatan larutan telah disediakan iaitu 62.5 mg/ml, 125 mg/ml, 250 mg/ml dan 500 mg/ml dengan menggunakan DMSO 10%. Gentamicin telah dipilih sebagai kawalan positif dan DMSO 10% sebagai kawalan negatif. Zon inhibisi telah didapati pada gram positif bakteria dari ekstrak daun D.zibethinus Murr.. Diameter zon inhibisi diukur dalam mililiter (mm). Oleh itu, dari hasil yang diperolehi, pemyelidikan lanjut mengenai daun D.zibethinus Murr. perlu dijalankan kerana potensinya dalam inhibisi gram positif bakteria dan mempunyai potensi tinggi dalam aplikasi perubatan.*

Kata kunci: *Durio zibethinus Murr., ekstrak daun, zon inhibisi, kaedah penyebaran agar.*

1.0 Introduction

Durio zibethinus Murr. *(D.zibethinus)* or durian fruit is a good source of carbohydrates and also contains significant amounts of protein and vitamins B and C. Its rich pulp is eaten raw, cooked as a vegetable, frozen or dried for later use. Indonesians ferment the pulp for a side dish or mix the fleshy arils with rice and sugar to produce a local dish, lempong. The seeds can be boiled or roasted and used as confections. Each part of durian from the fruit leaves and root can be used and well known has been used as traditional remedies.

The global now facing the challenges where the microbes or pathogen is able to cause the antimicrobial resistance from the foodborne illness. An increasing Antimicrobial resistance (AMR) has resulted in morbidity and mortality from treatment failures and increased health care costs (Bagul and Sivakumar, 2016). A lot of study has been done to every part of *D.zibethinus* Murr. for the studies antimicrobial agent that may contain in *D.zibethinus* Murr. However, the studies were lacking in leaves part of *D.zibethinus* Murr. although the durian leaf are abundant and easily obtained.

In order to carry out this study, the solvent that will be used later in the plant extraction plays an important role. The comparison will be done between methanol, ethanol and acetone extraction. The reason choosing these three solvent is because of their polar and non-polar characteristic and thus this will influence the antimicrobial activity as these solvents are able to dissolved the bioactive contain in the *Durio zibethinus* Murr.leaves which is responsible for the antimicrobial activity.

Most researcher used methanol as the boiling point methanol is 64.7°C compare to ethanol that have higher boiling point of 78.4°C. Even though methanol boiling point is lower than ethanol, methanol is said to be more toxic than ethanol and both solvent is polar. For acetone solvent, the boiling point is 56°C and also the polar solvent. These three

solvent is all polar solvent and have the high evaporation rate and if states the polar solvent in ascending order, the lowest polar solvent was acetone, methanol and followed by ethanol which is the most polar solvent.

Therefore, this study were conducted in order to extract *the D.zibethinus* Murr.leaves using the ethanol, methanol and acetone solvent by using the maceration method. Besides, this study also conducted in order to evaluate the antimicrobial activity from the solvent extract of *D.zibethinus* Murr.leaves by using the agar well diffusion antibacterial screening method by looking the zone of inhibition that produces by the leaves extraction.

2.0 Literature review

2.1 *Durio zibethinus* Murr. leaves

Durian *(Durio zibethinus)* belongs to the genus Durio and the family Bombacaceae. There are 30 recognised Durio species, at least nine of which produce edible fruit. Although there are 30 known species, *Durio zibethinus (D. zibethinus)* is the most prized as a major Southeast Asian food crop according to Teh *et al.,* (2017). This fruit were widely known and revered in south-east Asia as the "king of fruits" as it has the unique characteristic which is distinctive for its large size, unique odour, and formidable thorn-covered husk.

According to Stuart (2005), the characteristic of durian leaves are dark green, smooth and shiny above, cinnamon-colored (gold colored look alike) and scaly beneath, elliptic to oblong, about 15- 25 cm long and 5 - 9 cm wide. Based on writing by Hai (2016) the young leaves and shoots of the durian are occasionally cooked as greens. Aside from can be used as greens, the durian leaves traditionally was used for traditional remedies. The leaf juice can be used to treat fever according to Brown (1997). Furthermore, as has been reviewed by Lim (2012), the leaf also has been employed in medicinal bath for people with jaundice.

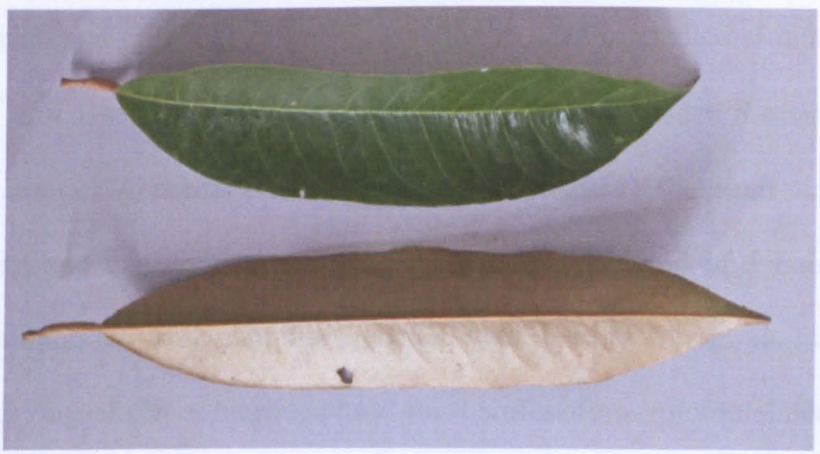

Figure 2.1 : *Durio zibethinus* Murr. leaves.

Apart of can be used to treat the jaundice, the *D.zibethinus* Murr. leaves that have chlorophyll and fibre, which indeed contained in almost all kinds of leafy trees and lush green. One of the most important benefits of chlorophyll and fibre is can help you lose weight. The leaves can be manipulated as a type of durian stir fry and mix it with other ingredients to help lose weight and maintain the health of your body. The leaves of the durian is believed to have a high fibre content thus good for digestion.

Previously, the study has been done on antifungal screening and wound healing effects of *D.zibethinus Murr.* seeds extract. Other than that, there also the study that has been conducted to determine the activity of the methanolic crude extracts collected from the seeds and rinds of native durian (*D.zibethinus*) against hospital isolates of *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (*S.aureus*) by Duoza *et.al*, 2012. Not only that, the fresh fruit pulp of *D.zibenthinus Linn.* was successively extracted by using various solvents like petroleum ether, chloroform, ethyl acetate and aqueous alcohol in other to study the biologically active constituents present in the fruits.

2.2 Plant sample

2.2.1 Fresh versus dried sample

Both fresh and dried sample of flower can be used in medicinal plant studies. But, in this study, dried sample is preferred as we need to consider the time needed for experimental design. According to Sulaiman *et al.,* (2011) the limit time of interval between harvest and experimental work at the maximum period of 3 hours to maintain freshness of samples, as fresh samples are fragile and tend to deteriorate faster than dried samples. The drying of spices has been used for disinfestations,microbial decontamination, and long-term preservation (Schweiggert *et al.*, 2007). Therefore, used the dried sample was more preferred compared to fresh sample.

There are several method for drying the plant sample such as shade dried, dried by sun and oven drying and freeze-drying (lyophilisation) that widely used for drying the plant samples. Shade dried is the method where the plant samples was dried under sunlight exposure with a good air ventilation as has been mentioned by Syahrin (2013). The plant sample that dried using the shade dried were dried at least 3-5 days depend on the plant samples. This method was the standard method that has been said prevent the destroy of phytochemical that responsible for the antimicrobial compound according to Chigurupati *et al.*, (2017).

Other than that, oven drying also the known method that has been apply to dried the plant sample. Oven-drying is a method that uses thermal energy to remove moisture from the samples. This sample preparation is considered as one of the easiest and rapid thermal processing that can preserved phytochemicals. However, oven- or microwave-drying treatments led to significant reductions in antioxidant property with microwave-drying causing the highest decrease in total polyphenols, radical scavenging activity, and ferric-reducing antioxidant power antioxidant capacity (Lim and Murtijaya, 2007). According to Tomaino *et al.*, (2005), intense and/or prolonged thermal treatment can cause significant loss of active compound in plant sample as a result of the heat instability of compounds.

Last but not least, freeze drying or lyophilisation method. Freeze-drying is a method base on the principle of sublimation where the solid was changed into gas phase without entering the liquid phase. The plant sample need to be frozen at -80°C to -20°C prior to lyophilisation in order to solidify any liquid that present in the samples. Freeze-drying yielded to higher level of phenolic contents compared to air-dying as most of the phytochemicals are preserved using this method. However, freeze-drying is a complex and expensive methods of drying compared to the others method. Thus, the use is restricted to delicate, heat-sensitive materials of high value.

Therefore, the shade dried method was choosen for drying method of *D.zibethinus* Murr. leaves after comparing the others method limitation.

2.2.2 Grinded versus powdered sample

Lowering particle size increases surface contact between samples and extraction solvents. Grinding resulted in coarse smaller samples meanwhile, powdered samples will produce more homogenized and smaller particle, that leading to better surface contact with extraction solvents. This particular pre-preparation is important, as for efficient extraction to occur. the solvent must make contact with the target analytes and particle size smaller than 0.5 mm is ideal for efficient extraction. This particular size of particle was mentioned in Sulaiman *et al.,* (2011), preparing vegetable samples that was ground to 400 μm (0.4 mm) in size. Conventional mortar and pestle or electric blenders and mills are commonly used to reduce particle size of sample.

2.3 Leaves extraction method

Extraction is the process where the medicinally active portions of plant were separate using selective solvents through the several standard procedures. According to Azwanida (2015), the main purpose of extraction is to separate the soluble plant metabolites and leaving behind the insoluble cellular marc or unwanted water or residue that may interrupt the analysis later. There are several methods that widely used for the extraction of plant soluble metabolites such as maceration, infusion, decoction and soxhlet extraction.

Maceration is one of conventional method that widely used and adopted in medicinal plants research. Maceration involved soaking plant materials (coarse or powdered) in a stoppered container with a solvent and allowed to stand at room temperature for a period of minimum 3 days with frequent agitation (Handa *et al.,* 2008).

Through this process, the plants cell wall intended to soften and break and release the soluble phytochemicals.

Infusion and decoction uses the same principle as maceration which are both are soaked in cold or boiled water. However, the maceration period for infusion is shorter as the sample are boiled in specified volume of water for a defined time for decoction compare to standard maceration that does not apply any heat. Besides, decoction is only suitable for extracting heat-stable compounds, hard plants materials and usually resulted in more oil-soluble compounds compared to maceration and infusion (Azwanida, 2015).

Next method that can be used are Soxhlet extraction or also known as hot continuous extraction. In this method, finely ground sample is placed in a porous bag or "thimble" made from a strong filter paper or cellulose, which is place, is in thimble chamber of the Soxhlet apparatus. Extraction solvents is heated in the bottom flask, vaporizes into the sample thimble, condenses in the condenser and drip back. When the liquid content reaches the siphon arm, the liquid contents emptied into the bottom flask again and the process is continued.

However, the extraction method that will be used later is maceration. This method has been chosen as this technique is the most suitable, easiest and simple method. Although the infusion method has a shorter period than maceration, through the maceration process it believed can break the cell wall and release the soluble phytochemicals. For soxhlet method, although it was the best method for extraction, this method requires a smaller quantity of solvent compared to maceration (Handa *et al.*, 2008). Furthermore, the chances being exposed to hazardous and flammable liquid organic solvents, with potential toxic emissions during extraction also very high.

2.4 Antimicrobial screening activity

2.4.1 Diffusion method

Antimicrobial screening activity or antimicrobial susceptibility testing can be used for drug discovery, epidemiology and prediction of therapeutic outcome (Balouri *et al.*, 2016). There are two major method in antimicrobial screening activity which are diffusion method and dilution method. However, there are several bioassay that are commonly used and well known such as disk diffusion and well diffusion under diffusion method and agar dilution and broth dilution under dilution method.

Disk diffusion method is the most commonly used method for screening the antimicrobial property among Malaysian plants. This method refers to the diffusion of an antimicrobial agent of a specified concentration from disks, tablets or strips, into the solid culture medium that has been seeded with the selected inoculum isolated in a pure culture (OIE, 2012). This method is based on the determination of an inhibition zone proportional to the bacterial susceptibility to the antimicrobial present in the disk.

Disk diffusion is straightforward to perform, reproducible, and does not require expensive equipment. Disk-diffusion assay offers many advantages over other methods which is simplicity, low cost, the ability to test enormous numbers of microorganisms and antimicrobial agents, and the ease to interpret results provided (Balouiri *et al.*, 2016). Although it has a lot of advantages, disk diffusion still have its limitation. It was hard to find the suitable disk for putting the extract on and not too accurate that were need to repeat a lot of time for confirmation.

Besides disk diffusion method, agar well diffusion method also commonly used and well known in antimicrobial activity test. According to Balouiri *et al.*, (2016), agar well diffusion method is widely used in order to evaluate the antimicrobial activity of plants or microbial extracts. This method have the same concept with disk diffusion except it used

the agar well which are the hole (6-8 mm diameter) that are aseptically punched with a sterile cork borer or a tip, and then the antimicrobial agent or extract solution with a desired concentration and volume (20-100 µl) that has been introduced will diffused through the solid media.

Despite of a lot of sample extract could be introduced into the agar well for the screening of antibacterial activity, this method still have its own limitation which are the method may give inconsistent result according to Janssen *et al.,* (1987). Besides that, this method also requiring all tool that need to used to be aseptic as to avoid the direct contamination to the media that may affect the testing. This method was used in this study as it easy to carry out and the volume that need to be introduced are standardized and in the same quantity compared to disk diffusion method that are the volume of the extract may not adhere on the disk as it may evaporated during preparation of disk for testing.

3.0 Materials and Methods

The maceration method was applied in order to extract the medicinally active plant from *D. zibethinus* Murr. leaves and agar well diffusion method was used for the screening of antimicrobial activity. The procedure done in this study is summarized in Figure 3.1.

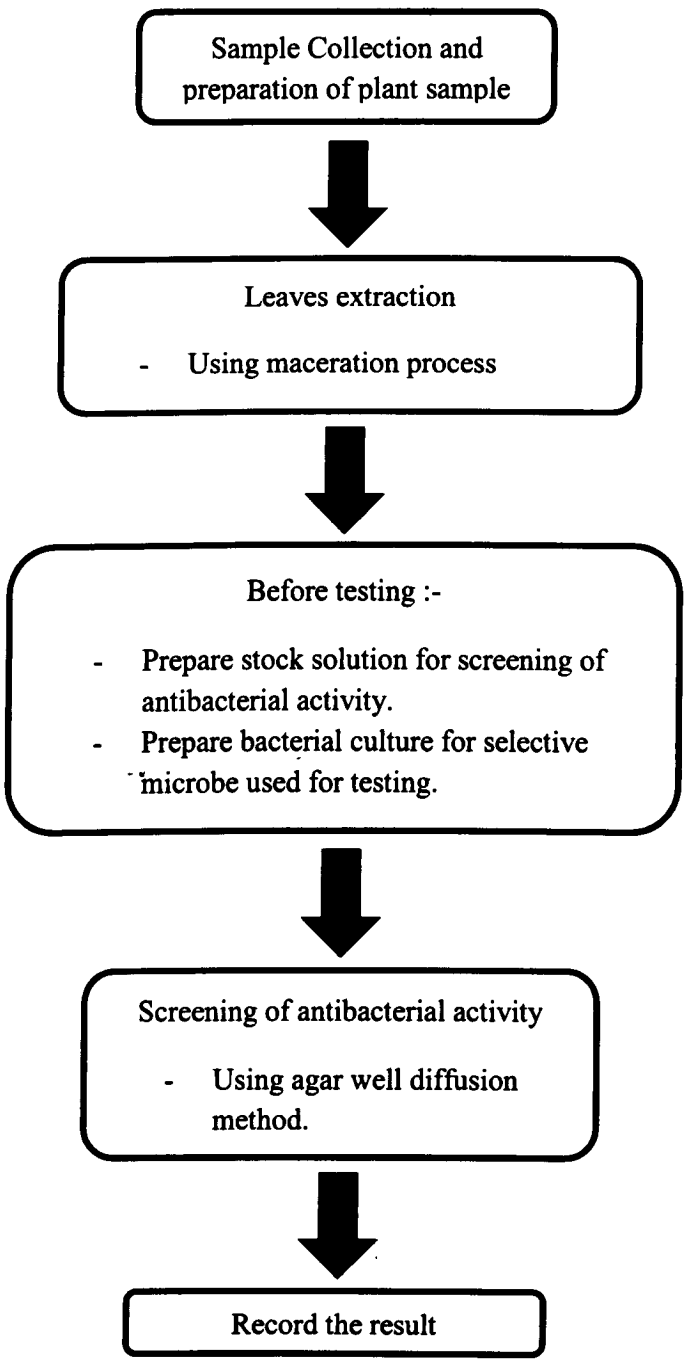

Figure 3.1: Overview of overall process done in this study

3.1 Sample collection and preparation of plant sample

The *D.zibethinus* Murr. Leaves was freshly collected from Encik Aran's house at Kampung Batu Kapor, Mentakab, Pahang. The leaves was gathered and thoroughly washed under running tap water and shade dried for not more than 4 days. After the leaves were fully dried, the leaves were grinded using the Narutron electrical blender until it become the fine powder. The fine powder was kept and stored in air tight plastic before used.

3.2 Preparation of leaves extracts

The ratio of 1:10 were used for the preparation of sample extract. The extraction done by using 3 different solvent which is ethanol, methanol and acetone. The maceration method was used for the preparation of the sample extraction. Fine powder sample that has been prepared was weighted out about 25 grams and added into 250 ml of each solvent in the conical flask. After added the sample in the solvent, the extraction was put on continuous agitation on rotary shaker at 120 rev/min for 72 hours. After 72 hours, the mixture was filter and the extract was collected and concentrated using rotary evaporator at 37°C- 45°C. The crude extract obtained then was stored in air tight container and kept in fridge at 4°C for further used.

3.3 Preparation of stock solution of leaves extracts

From the crude extract of ethanol, methanol and acetone, varying concentration of extraction was prepared and the extracts were diluted using the 10% Dimethyl sulfoxide (DMSO). The varying concentration that has been prepared was 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml.

3.4 Preparation of test bacteria

The extracts of *D. zibethinus* Murr. leaves were screened against four pathogenic bacterial clinical isolates. The microbial strain was obtained from Virology laboratory, FRST. The microbial strains used for the screening of antibacterial activity included two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) while another two strains are from Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*). The microbes was streaked on nutrient agar and incubate for 18-24 hours at 37°C. After incubation period, single colony taken using the sterilized inoculating loop and inoculate into 5 ml Muller Hinton broth and incubate for 18-24 hours at 37°C. The turbidity of the suspension was adjusted to match 0.5 McFarland standard using a spectrophotometer (Mini UV–Vis Spectrophotometer) at 625 nm, which corresponds to 2.4 x 10^8 cfu/ml according to Roy *et al.*, (2010).

3.5 Antibacterial activity screening

3.5.1 Agar well diffusion method

Muller Hinton agar was prepared and swabbed with different bacterial suspension before aseptically punched a well with a diameter of 6 mm on the plate. The total well that has been cut was 6 well included the positive and negative control. After swab the bacterial suspension, the plate was let it to dry about 30 minutes before punched the well. About 50 µl of each sample with different concentration (500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml) added into the well. For the negative control, 50 µl of 10% DMSO was added into the well while the positive control, 25 µl of 1 mg/ml gentamicin was added into the well. The plates were then incubated overnight at 37°C for allowing bacterial growth. After incubation, the diameter of the zones of inhibition were measured and tabulated for each test microbes.