EXTRACTS OF *CERBERA ODOLLAM* FOR ANTI BACTERIA ACTIVITY AND EVALUATION ON SOME WOOD PRODUCT PROPERTIES AFTER IMPREGNATION

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2009

MOHD HAZIM BIN MOHAMAD AMINI 2009 MSc

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by

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Thesis submitted in fulfillment of requirements
for the degree of
Master of Science

October 2009

PENILAIAN TERHADAP AKTIVITI ANTI-BAKTERIA UNTUK EKSTRAK DARIPADA *CERBERA ODOLLAM* DAN PENILAIAN TERHADAP BEBERAPA CIRI-CIRI PRODUK KAYU SELEPAS DIIMPREG DENGAN EKSTRAK TERSEBUT

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MOHD HAZIM BIN MOHAMAD AMINI

Tesis yang diserahkan untuk
memenuhi keperluan bagi
Ijazah Sarjana Sains

Oktober 2009

ACKNOWLEDGEMENT

I would like to acknowledge to Ministry of Science, Technology and Innovation, Malaysia (MOSTI) for the Science Fund Grant 03-01-05SF0117 and Universiti Sains Malaysia for Fellowship Scheme awarded to me in order for me to complete this research. I would like to address my appreciation to Associate Professor Dr Rokiah Hashim as my supervisor for her helping hands and guidance, also thanks to Associate Professor Dr Othman Sulaiman, Dr Shaida Fariza Sulaiman from School of Biological Sciences of Universiti Sains Malaysia, Associate Professor Dr Faizah Abood from Universiti Putra Malaysia, Dr Fumio Kawamura from Japan International Research center for Agricultural Sciences (JIRCAS) and staff of Bio-resource, Paper and Coatings Technology Division, School of Industrial Technology, Universiti Sains Malaysia. Finally thanks to my parents for giving morale support to pursue my study.

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MUF – melamine-urea formaldehyde

PRF – phenol-resorcinol formaldehyde

AD – air dry weight

OD – oven dry weight

DMSO – Dimethylsulfoxide

EKSTRAK *CERBERA ODOLLAM* UNTUK AKTIVITI ANTI BAKTERIA DAN PENILAIAN TERHADAP SEBAHAGIAN SIFAT PRODUK KAYU SELEPAS IMPREGNASI

ABSTRAK

Projek ini mengkaji potensi kegunaan bagi *Cerbera odollam*. Keseluruhan kerja telah dibahagi kepada dua bahagian. Bahagian pertama ialah kajian tentang aktiviti antibakteria oleh ekstrak daripada bahagian berbeza *Cerbera odollam.* Bahagian kedua mengkaji ketahanan terhadap perosak, sifat – sifat fizikal dan mekanikal bod partikel yang diperbuat daripada partikel kayu yang telah dirawat dengan ekstrak metanolik. Sampel kayu padu dan sampel yang diimpreg dengan pengawet komersial juga diuji sebagai perbandingan.

Bagi kajian tentang aktiviti antibakteria, bahagian – bahagian pokok *Cerbera odollam* telah diekstrak dengan methanol. Ini diikuti pemecahan ekstrak kepada bahagian – bahagian yang larut n-heksana, etil asetat dan etanol. Aktiviti antibakteria pecahan ekstrak telah diuji terhadap kedua – dua bakteria jenis Gram-positif dan Gram-negatif iaitu dari spesis *Bacillus subtilis*, *Bacillus licheniformis, Escherichia coli* dan *Pseudomonas aeruginosa*. Bahagian larut n-heksana daripada bunga, buah, daun, kayu dan kulit serta bahagian larut etil asetat daripada kulit menunjukkan aktiviti antibakteria terhadap *Bacillus subtilis*. Bahagian larut n-heksana daripada daun dan kulit serta bahagian larut etil asetat daripada kayu menunjukkan aktiviti antibakteria terhadap *Bacillus licheniformis.* Semua ekstrak dan pecahan ekstrak tidak menunjukkan sebarang aktiviti antibakteria terhadap *Escherichia coli* dan

Pseudomonas aeruginosa. Ekstrak yang menghasilkan zon perencatan telah dianalisis menggunakan FTIR dan GC-TOFMS dan kumpulan kimia atau sebatian kimia yang berkemungkinan merupakan sebatian aktif telah dikesan. 1 – heksadekanol, 1 – undekanol, asid 9,12,15-oktadekatrienoik (linolenik), 1-undekanol dan 1-tridekanol adalah antara sebatian yang berkemungkinan adalah sebatian aktif untuk aktiviti antibakteria merujuk kepada kajian pengkaji terdahulu.

Ketahanan bod partikel dan sampel kayu padu yang telah diimpreg dengan ekstrak *Cerbera odollam* juga dikaji. Bahagian – bahagian *Cerbera odollam* telah diekstrak dengan metanol, dituras dan dikeringkan dengan mesin penyejat putaran. Ekstrak metanol yang diperolehi telah diimpreg ke dalam partikel kayu. Sampel kemudiannya didedahkan kepada kumbang perosak kayu dan juga ujian tanaman dalam tanah untuk melihat ketahanannya. Ujian kandungan formaldehid terbebas, ujian ikatan dalaman dan ujian pembengkakan dalam air juga dijalankan terhadap bod partikel. Keputusan eksperimen menunjukkan sampel kawalan bagi kayu padu menunjukkan kerosakan paling tinggi bagi ujian ketahanan kumbang perosak iaitu 12.47%. Mengimpreg ekstraktif ke dalam bod partikel mengurangkan sedikit kekuatan ikatan dalaman bod tetapi meningkatkan ketahanan terhadap serangan biologi semulajadi dalam ujian tanaman tanah. Keputusan eksperimen juga menunjukkan bod partikel yang dihasilkan menggunakan resin Melamin urea formaldehid dan diimpreg dengan sebarang ekstraktif melepasi piawaian P3 dan P4 oleh EN 312:2003 manakala bod partikel yang dihasilkan menggunakan Fenol resorsinol formaldehid hanya melepasi piawaian P4 dalam standard yang sama.

EXTRACTS OF *CERBERA ODOLLAM* FOR ANTI BACTERIA ACTIVITY AND EVALUATION ON SOME WOOD PRODUCT PROPERTIES AFTER IMPREGNATION

ABSTRACT

This study investigated the potential use of *Cerbera odollam*. The works carried out were divided into two parts. The first part is investigating the antibacterial activity of extracts from different parts of *Cerbera odollam*. The second part of the study investigated the decay resistance, physical and mechanical properties of particleboards made from wood particles that were impregnated with methanolic extracts of *Cerbera odollam*. Solid wood samples and samples treated with commercial preservative were also made as comparison.

In order to investigate the antibacterial activity, methanol extracts from different part of *Cerbera odollam* was prepared. Then they were further fractionated using *n*-hexane, ethyl acetate and ethanol. Obtained fractions were tested against both Gram-positive and Gram-negative bacteria of *Bacillus subtilis*, *Bacillus licheniformis, Escherichia coli* and *Pseudomonas aeruginosa* for their antibacterial activity. n-Hexane soluble parts from flower, fruit, leaf, wood, bark and ethyl acetate soluble part from bark showed antibacterial activity against *Bacillus subtilis*. n-Hexane soluble parts from leaf and bark and ethyl acetate soluble parts from wood showed antibacterial activity against *Bacillus licheniformis.* All of the extracts and fractions showed no antibacterial activity against *Escherichia coli* and *Pseudomonas aeruginosa.* Extracts showing inhibition zones were analyzed using FTIR and GC-

TOFMS and possible active compounds were detected. 1 – hexadecanol, 1 – undecanol, 9,12,15-octadecatrienoic (linolenic) acid, 1-undecanol and 1-tridecanol were among the possible active compounds for antibacterial activity determined by reviewing the work of previous researchers.

Cerbera odollam-impregnated particleboards and solid wood samples were prepared. *Cerbera odollam* were extracted using methanol, filtered and dried using rotary evaporator for storage. Methanolic extracts that were obtained were impregnated into wood particles. Samples were tested by exposure to wood-boring beetles and also undergone soil burial test to see their decay resistance. Particleboards were also tested for their formaldehyde release content, internal bonding and thickness swelling properties. Wood-boring beetles resistance test showed that control sample of solid wood have the highest degradation with 12.47% decay. The particleboards were less susceptible to the attack mainly because the adhesives used in board making process do have some toxic effect on the insects. Impregnation of extractives into the particleboards only slightly reduced their internal bonding strength but increased their resistance to natural biological attack in soil burial test. The results showed that thickness swelling and internal bond strength for Melamine urea formaldehyde particleboards impregnated with any extracts passed the requirement for both P3 and P4 of EN 312:2003 while Phenol resorcinol formaldehyde particleboards only passed P4 of the standard.

1.0 Introduction

There are many tree species that are very poisonous to living organisms such as *Atractylis gummifera* (bird-lime or blue thistle), *Blighia sapida* (ackee tree), *Thevetia peruviana* (yellow oleander), *Colchicum autumnale* (meadow saffron), *Oenanthe crocata* (hemlock water dropwort), *Taxus baccata* (English or common yew), *Narcissus pseudonarcissus* (daffodil), *Cerbera manghas* (pink-eyed cerbera or sea mango) *and Cerbera odollam* (yellow-eyed cerbera). These trees could cause death to human being by ingestion (Eddleston and Persson, 2003; Gaillard et al., 2004). Some of the trees usually planted intentionally along the road side or park as shade tree. The potential use of these tree species should be explored especially as disinfectant and preservative as they could be obtained abundantly.

1.1 Problems

Cellulose, hemicelluloses and lignin are the materials that builds up a polymeric material called wood. Wood is widely used as furniture and other structural usage mainly because of its renewable factor. Compared to other sources like metal, wood has the advantage that it can be obtained over and over again by reforestation of the logged areas. Versatility, durability and aesthetical value of wood besides its excellent strength properties and workability make it the most popular building material in the world. With many encouraging properties such as low density, low thermal expansion and renewable, it is extensively used for indoor and outdoor structural purposes (Pandey, 1999; Williams, 2004).

The main problem of wood for building material is it is a biodegradable material and Schultz (2002) reported that wood can be degraded by many types of organisms such as fungi and insects. Lignin always acts as physical barrier to enzymatic decomposition of cellulose and hemicelluloses in wood but this barrier can be destroyed mechanically by insects and marine borers, biochemically by white- and soft-rot fungi, and possibly by small non-enzyme catalysts in the case of brown-rot fungi (Kirk and Cowling, 1984). Fungi causes serious strength loss while strength loss due to insect attack is proportional to the amount of wood removed. Bacteria also play a role in wood degradation where they affect wood permeability, attack wood structure, or work synergistically with other bacteria and soft-rot fungi to predispose wood to fungal attack (Clausen, 1995).

Wood needed to be preserved to prolong their shelf life. The preservatives that are used to protect wood from biological attack could be a problem to the environment. Wood preservatives can be described as pesticides that protect wood from fungi, insects and other biological agent attack. These dangerous materials are badly affecting human health and the environment and consideration in making decision to use or not to use these preservatives had to be made wisely. Copper chrome arsenic (CCA) had been long widely used as wood preservative to prevent decay by fungi and insects (Yamamoto and Hong, 1988; Kartal and Clausen, 2001). In theory, CCA can be considered as safe because the chemicals are believed to bind strongly to the wood and do not leach out. However in practise, many studies showed that the chemicals do leach out and can gather on the ground underneath or transferred by direct hand contact. Improper disposal of treated wood will also create problem. Burning CCA-treated wood products down will release a large amount of

arsenic into the air which is very hazardous to health. Direct or indirect breathing or ingestion of wood ash from burnt CCA-treated wood has caused fatalities in animals and serious poisonings in humans. Besides that there is a possibility that the harmful preservatives are absorbed into the soil and come contact with underground water source and affecting people who use it. More environmental friendly agent should be developed to replace the conventional preservatives (Dickey, 2008).

Earlier researches have shown that without the use of artificial chemicals, biologically resistant boards still could be produced. Extractives from certain tropical hardwood timber species had shown potential use in wood protection. Different from artificial wood preservatives, organic based preservative obtained from natural source is believed easier to be detoxify by normal biological processes (Onuorah, 2000).

New types of wood preservative that is less harmful to the environment need to be developed. This study investigates the potential use of extracts of *Cerbera odollam*. No study has yet been conducted on the suitability of extracts from different parts of *Cerbera odollam* as antibacterial agent and wood preservative.

1.2 Objectives

The objectives of this study are:

a) To study the antibacterial activity of extracts of *Cerbera odollam*.

b) To evaluate the chemical composition of *Cerbera odollam* after extraction process.

c) To study the influence of the extracts on the properties of particleboards and solid wood.

CHAPTER 2

2.0 Evaluation of antibacterial activity of extracts of *Cerbera odollam*

2.1 Introduction

This experiment investigated the antibacterial activity of *Cerbera odollam*. Extractives from the different parts of biomass components of *Cerbera odollam* were exposed to bacteria to determine its antimicrobial activity. Four types of bacteria were used in this study that includes *Bacillus subtilis* (ATCC 21332), *Bacillus licheniformis* (ATCC 14580), *Escherichia coli* (*ATCC 25922*) and *Pseudomonas aeruginosa* (*ATCC 27853*).

2.2 Literature review

2.2.1 Cerbera odollam

Grouped under kingdom of Plantae, Division of Magnoliophyta, Class of Magnoliopsida and Order of Gentianales, the *Cerbera odollam* tree is also known as *othalanga maram* in the Malayalam language used in Kerala, *kattu arali* in the adjacent state of Tamil Nadu, *famentana*, *kisopo*, *samanta* or *tangena* in Madagascar and *pong-pong*, *buta-buta* or *nyan* in southeast Asia (Gaillard et. al., 2004; Wikipedia, 2008). Belongs to the family of Apocynaceae, and originated from coastal area in India and south-east Asia (Chen and Steldt, 1942; Laphookhieo et. al., 2004). Reaching a height of around 8 to 15 meters, it grows wild in mangrove swamps and widely grown in park and gardens as shade tree. The latex from this tree may cause blindness and the fruit can results in death if eaten. This tree can be

described as small sized tree with a rounded crown (Tung, 2005). Figure 2.1 shows the *Cerbera odollam* tree.

Figure 2.1: The *Cerbera odollam* tree

The fruits are like apples or mangos. When dropped onto the ground, the outer layer of the fruit peeled off, exposing the thick fibrous inner layer that protects a seed inside as shown in Figure 2.2. The fruits of *Cerbera odollam* are large, round, smooth and green when young. It ripens to dull red when ripe and black when rotten. The seed contains non-siccative oil and are widely used as rat poison. In Burma, they use it for lighting, as a cosmetic or mixed with other oils as an insecticide or insect-repellent. In India the latex is known for its emetic, purgative and irritant effects (Gaillard et. al., 2004).

Figure 2.2: Cross section of young fruit of *Cerbera odollam* (left) and the mature fruit (center) and dried fruit (right).

The leaves are shiny with dark green colour. The leaf shape is simple, elliptical and spirally arranged along the branches. The flowers are bisexual and white in colour with a diameter of around 5 cm. The flowers occur in small cluster and releasing pleasant fragrance. The nearest tree similar to *Cerbera odollam* is the *Cerbera manghas*. The differences between these two species are the leaves of *Cerbera manghas* are smaller and glossier. The flower of *Cerbera manghas* is also smaller with orange pink eye, later turn reddish pink (Tung, 2005).

Gaillard et. al (2004), the *Cerbera odollam* tree is responsible for about 50% of plant poisoning cases and 10% of all poisoning cases in Kerala, India. The number of recorded fatalities from 1989 to 1999 from *Cerbera odollam* poisoning is 537 compared to 163 by yellow oleander in the same state. The number of fatalities from year 1989 to 1999 is shown in the Figure 2.3.

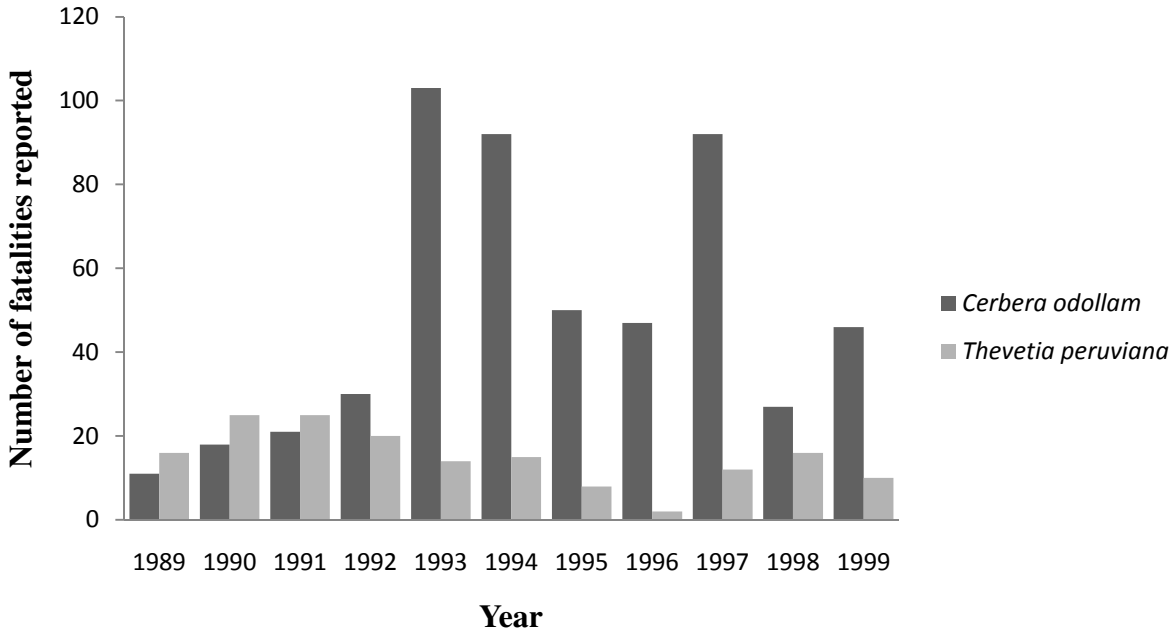

Figure 2.3: Number of fatalities from *Cerbera odollam* and *Thevetia peruviana* reported from 1989 to 1999 in the state of Kerala, India.

The ease of availability of the tree is determined as the main factor for choosing it as poison for suicide or homicide. People take the white fleshy kernel out from the fibrous husk of the seed and mash it before consuming it as a sweet to commit suicide. A few kernels are mixed with food containing plenty of chillies to cover the bitter taste of the poison for homicide. Death is likely to occur 3 to 6 hours after ingestion (Gaillard et. al., 2004).

Chemical properties of some parts of the tree had been already done by several previous researchers. Laphookhieo et. al (2004) has study on cytotoxic cardenolide glycoside from the seeds of *Cerbera odollam*. They have isolated a cardenolide glycoside, 3*β*-*O*-(2'-*O*-acetyl-l- thevetosyl)-15(14→8)-*abeo*-5*β*-(8*R*)-14-oxo-card-20(22)-enolide (2'-*O*-acetyl cerleaside A), from a methylene chloride extract of the seeds of *Cerbera odollam*, besides four known compounds that are cerleaside A, 17*α*-neriifolin, 17*β*- neriifolin and cerberin. The compound was

obtained in white solid with molecular formula of $C_{32}H_{46}O_9$. All compounds except cerleaside A showed cytotoxic activities against oral human epidermoid carcinoma, human breast cancer cell and human small cell lung cancer (NCI-H187).

Chen and Steldt (1942) have isolated cerberin and other active substances that may be present in the kernel of the nuts, and conducted various pharmacological experiments using it. Stock solutions of 0.1% cerberin in 38% alcohol by volume and 0.2% cerberoside in 19% alcohol were prepared for animal experiments. When tested on frogs, adequate doses of whether cerberin or cerberoside injected into the frog's lymph sac, typical systolic arrest of the ventricle could be easily observed at the end of an hour. By perfusion into the inferior vena cava of the frogs, cerberin in the concentrations of 1:250 000 and 1:125 000, induced systolic standstill within 40 minutes, preceded by slowing of the heart rate and later *A-V* block. A concentration of 1: 500 000 produced a decrease of diastole and sinus rhythm, and appearance of premature beats.

Chen and Steldt (1942) had also done the experiments on cats. Cats injected by sufficient amount of cerberin or cerberoside caused a marked rise of blood pressure, arrhythmia, and sudden circulatory collapse. Vomiting uniformly took place when non-anesthetized pigeons and cats were given appropriate doses of cerberin or cerberoside. When applied to isolated rabbits' intestines and guinea pigs' uteri, both glycosides produced stimulation. At the end of the experiments they concluded that cerberin can be isolated from both the oil and the defatted kernels of *Cerbera odollam* nuts. Isolated glycoside is similar to but not identical with cerberin

has been isolated, to which the name of cerberoside has been proposed and cerberin is much more potent on the heart than cerberoside.

Hiên et al. (1991) studied the immediate and delayed toxicity of the leaves of *Cerbera odollam* against mice. Dried leaves of *Cerbera odollam* were extracted at room temperature in a percolator using 95% ethanol for 12 hours. Extracts were vortexed and filtered to eliminate resins and chlorophyll and dried up. Extracts were diluted in isotonic saline for pharmacological studies in proportion of 1 ml isotonic saline to 1 g of leaf powder. In the toxicological study, most of the mice treated with the extract died without any sign of seizure or convulsion (body shakes rapidly and uncontrollably due to person's muscles contract and relax repeatedly). At the right dosage, the extract slowed down the spontaneous motility and lengthens the reaction time for the mice to thermal pain.

In an other research on *Cerbera odollam*, Rahman et al. (1993) conducted a study on the effect of *Cerbera odollam's* seed against Instar IV larvae of *Culex quinquefasciatus* and *Aedes aegypti.* Seeds of *Cerbera odollam* were extracted with methanol and further fractioned using n-hexane, diethyl ether, chloroform and ethyl acetate. Residue from the fractionation process is water soluble. Water and n-butanol mixture in ratio of 50:50 was poured to the residue and shake. Two layers of solutions were formed and then separated to gain aqueous and n-butanol fractions. Fractionated extracts were diluted in dimethyl sulfoxide (DMSO) and tested against the larvae. Results from the study found that n-butanol soluble fraction was the most effective fraction in killing the larvae of both mosquito species.

2.2.2 Wood extractives

Wood extractives are chemical compounds that could be extracted from wood using polar and non-polar solvents. Usually wood extractives are 1 – 20% of wood. Extractive content generally decreases with the increment in tree height and mostly found in heartwood. Wood extractives influence the utilisation of the wood such as affect the durability of wood, colour, allergic on human, contribution to wood density and staining and corrosion on wood processing machine (Walker, 2006).

They include both lipophilic and hydrophilic compounds. Some extractives such as fats provide the energy source for the wood cell, while lower terpenoids, resin acids and phenolic substances gives wood a protection against microbiological or insect damage. The term resin is usually used for lipophilic extractives. Resin acids can be found in resin canals while fats and waxes are located in the ray parenchyma cells. Phenolic extractives are present in heartwood and the bark (Sjöström, 1993).

2.2.3 Bacteria

One of the purposes of wood preservative is to protect wood from bacterial decay. Bacteria fall under the category of prokaryotic microorganisms. The basic shapes of bacteria are sphere (coccus), rod (bacillus) and the curved rod (vibrio) as shown in Figure 2.4. In bacteria, sometimes cell division is not followed by separation of the daughter cell. The daughter cell remains attached to parent cell, creating a multicellular structure. The motility of bacteria could be by flagella,

gliding or axial filaments. Some bacteria called myxobacteria have the characteristic of forming fruiting bodies which is a special multicellular structure. Under suitable conditions such as lack of nutrient, a swarm of vegetative cells aggregate and form a fruiting body. Other type of bacteria is the actinomycetes, a large group of filamentous bacteria that shape just like a fungi. It also produces spores and can be easily obtained from the soil. Actinomycetes such as from the genus Streptomycetes produce antibiotics which is important in pharmaceutical industry (Wilkinson, 1986). Streptomyces species are saprophytic bacteria that decompose organic matter, especially polymers such as lignocellulose, starch, and chitin (Crawford, 1993).

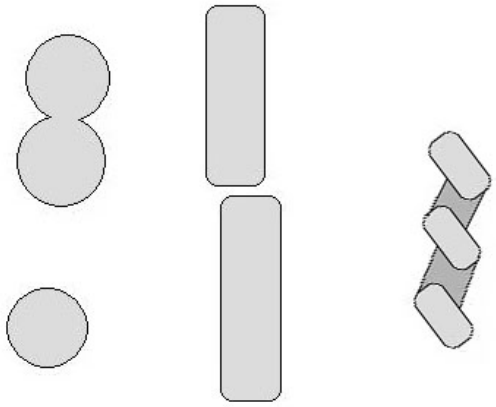

Figure 2.4: Coccus (left), bacillus (center) and vibrio (right) shape of bacteria

2.2.3 (a) Antibacterial agents

There are many types of microbicides. Microbicides could be described as any materials that kill microorganisms. Microbicides belongs to biocide which is a more general term which comprises among others microbicides, molluscicides, acaricides, insecticides, herbicides, rodenticides etc. Antibacterial agent is a microbicides. There are many types of chemical groups that could be a possible microbicides including alcohols, aldehydes, aldehyde releasing compounds, phenol derivatives, acids, acid esters, amides, carbamates, dibenzamidines, pyridine

derivatives, azoles, heterocyclic N, S compounds, compounds with activated halogen atoms, surface-active agents, organometallic compounds and oxidizing agents (Paulus, 2004).

2.2.3 (b) Bacteria association with wood decay

Little attention has been given on the effect of bacteria on wood decaying process compared to degradation by fungi. It is an advantage for bacteria that has the capability to colonize wood under conditions that are usually not suitable to most fungi such as waterlogging and low oxygen content. Besides that, they also can live in rotten wood and wood from wide range of environment, including treated and untreated wood. It can be said that bacteria is the most present organism among all wood-inhabiting microorganisms. Clausen (1996) classified these bacteria into 4 groups:

- Bacteria that affect the permeability of wood towards liquid but does not change the strength of the wood
- Group of bacteria that may affect the strength properties of the wood by attacking the cell walls
- Type of bacteria that work synergistically with other bacteria to destroy wood
- Group of passive colonizers that may be antagonists to other bacterial populations.

The usual effect of bacterial degradation of wood polymer is increment in porosity of wood (Wilcox, 1970). Permeability of wood is known to occur because of water storage. *Bacillus polymyxa* is a species that confirmed to have effect on the permeability of wood. This bacteria is able to hydrolyse pectin and hemicelluloses. By removing the ray cell contents and destroying the walls of parenchyma cells, it increases the permeability of wood. Knuth and Mc Coy (1961) as reported by Greaves (1971) was able to increased the porosity of *Pinus ponderosa* sapwood using bacteria. They also tried to improve the permeability of *P. radiate* heartwood specimen using *B. subtilis*, *P. fluorescens* and a mixed bacterial population but appeared to be unsuccessful. Many reasons could be used in explaining this situation. Since the penetration of the bacteria is limited to the outer 1 cm of the wood, it shows that the motility of bacteria is an important factor involved in bacterial decaying process in wood where too much bacterial slime produced on the surfaces of the specimen could also be the factor that prevents adequate liquid penetration.

Bacteria that belong to this group affect the wood in many ways. First is through the utilization of cell contents. In the same way, pit chambers are often colonized by bacteria where the margo structure is usually cleared. Pit structure is showed in Figure 2.5. The second way is through breakdown of ray parenchyma cell walls. The attack on ray parenchyma cell walls was found, including a progressive attack on crystalline cellulose. The ray cells contain much nutrient supply so there is a rapid growth of microorganisms inside. After consuming the contents, the bacteria will continue attacking the parenchyma cell walls that are rich in cellulose and increases the permeability of wood (Wilcox, 1970).

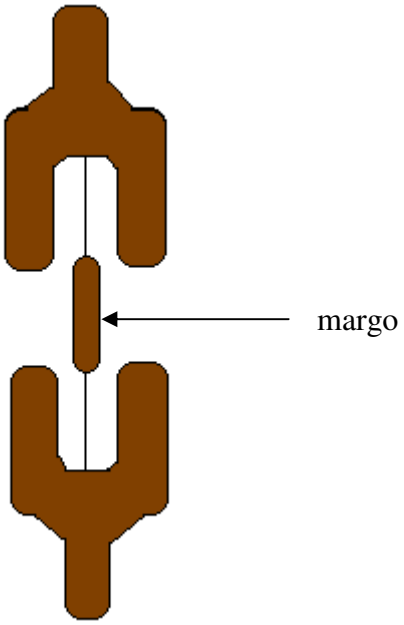

Figure 2.5: Pit structure of wood

The third way is by non-destructive attack of the secondary cell walls of rays. It is a submicroscopic attack where under normal light the cell walls looks like unaffected. When examined under polarized light, cell wall structure shows that the bacteria are affecting the regular crystalline structure of the cellulose microfibrils by advance diffusion of cellulolytic causing the hydrolysis of cellulose enzymes. The fourth way is by attacking the pit structure of the wood which usually happens in wood that is stored in water. Attacks on the borders, margo or torus increases the wood permeability to moisture and liquid (Greaves, 1971).

The second group of bacteria attacks wood structure. Bacterial decaying process in wood is slower compared to attack by fungi but in some cases especially involving lot of moisture, bacteria had showed rapid decaying process where severe tracheid destruction was observed in *P. radiata* cooling tower slats after only three years in service (Greaves, 1968). As stated before, motility is an important factor to bacterial decay. Bacteria moved from cell to cell where the walls and pit floors are

assumed to be penetrable by the microorganism. Water movements inside the wood provide an alternative way of transportation for bacteria like in a process of partial drying and rewetting of wood, bacteria will spread throughout the wood cells (Greaves, 1971). There is a group of bacteria called myxobacteria. It is also called 'slime bacteria' and lives mainly in the soil. This group of bacteria is so flexible that they have the ability to creep and glide over a substrate. The myxospores are resistant to dehydration and freezing enables them to survive in harsh environments such as deserts and arctic tundra. They are known to be cellulolytic and found abundantly in rotting wood (Shimkets, 1990).

The cell wall damage can be seen in three different forms. First is erosion or lysis through where the decay does not progress beyond the middle lamella which have two types:

- Shallow or surface erosion that happen usually at tertiary lamella (S_3) of the cell wall. Size of the erosion is a few times wider than the bacterium producing them and often done by bacilli.
- Deep lysis throughs where the damage progress from lumen in secondary cell wall layers and sometimes it is deeper into the middle lamella. It could be determined by narrow through, steep sided and a little wider than the bacteria producing it and usually done by cocci.

Second form is wall pitting. It happened when each bacterial cell in the colony which is in contact with the wall produces a small eroded pit on the surface of

the lumen and the third form of damage is attack of cellulose structure in the secondary cell wall (Greaves, 1971).

The third group of bacteria works by association with other microbes. Structural and permeability changes may predispose wood to further bacterial and fungal attack. Pectinases from bacteria attacks the membrane of the bordered pit and degrades the pit membrane (Liese and Bauch, 1967). Besides that, dead bacterial cells may provide a valuable source of nitrogen which is an important nutrient for fungi. Some bacteria also have the capsules or slime layers inside them which provide as sugar reserves for the growth of fungi (Clausen, 1996).

The fourth group is the passive wood-inhabiting bacteria. The bacteria under this group are classified due to their antagonistic or inhibitory effect on other members of wood microflora (microscopic plants). Some bacteria are able to produce antibiotics which have fungicidal properties. Vasilev (1968) as reported by Greaves (1971) had proven that bacterium isolated from ponded pine sapwood can be a wood protector against *Ceratocystis, Pullularia pullulans, Trichosporium tingens, Discula pinicola* and *Corticium evolvens*. Some highly antagonistic actinomycetes were isolated from fence posts and assayed together with *Bacillus cereus, Bacillus subtilis, Pseudomonas aeruginosa* and *Alcaligenes faecalis* as an inhibitor for wood-decaying basidiomycetes and soft rot fungi. Decay capacity test were conducted using the sapwood of *E. regnans* and *P. radiata* showed that the white rot fungus *Trametes versicolor* was inhibited by *Streptomyces violaceoniger* and *Bacillus cereus* (Greaves, 1971).

2.3 Materials and methods

Antibacterial activity assays of the extractives were carried out by the disc diffusion assay method on agar medium (Dash et al., 2005). Figure 2.6 shows an overall step for antibacterial testing.

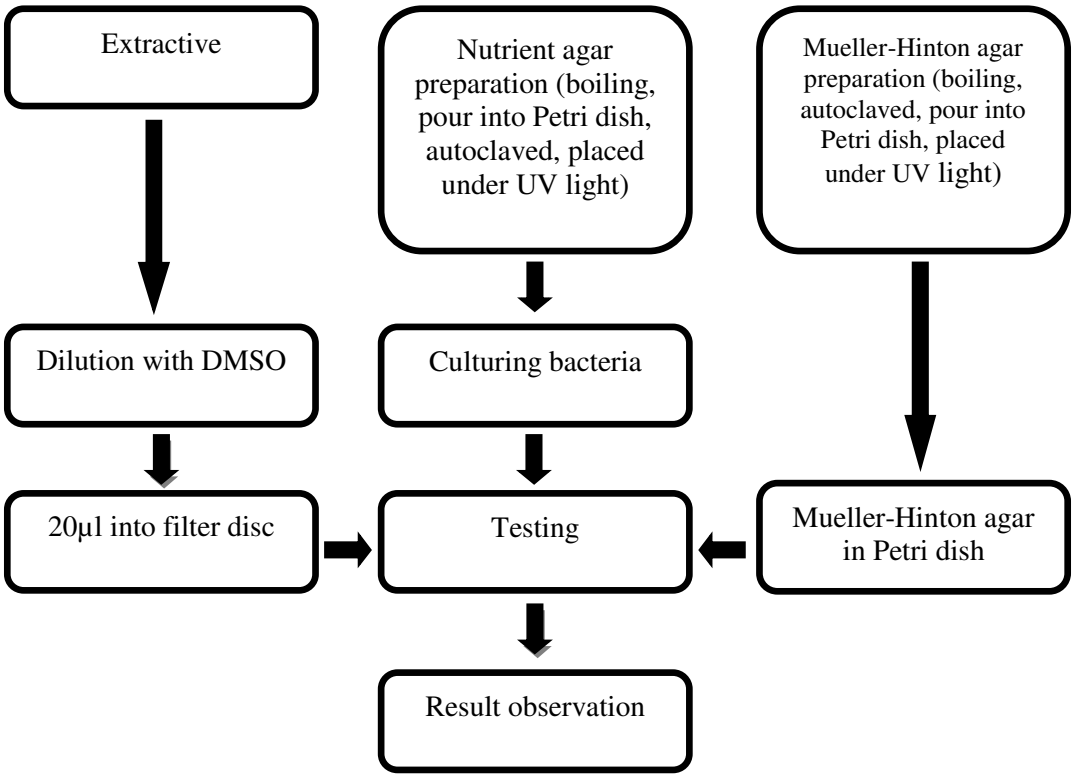

Figure 2.6: Antibacterial assay

2.3.1 Sample preparation

Before conducting antibacterial assay, samples were initially prepared. Samples of different parts of *Cerbera odollam* were collected around Universiti Sains Malaysia, Penang. Figure 2.7 shows parts of the tree that had been taken. Samples were chopped into smaller pieces and cooled in the freezer at -20^0C followed by freeze drying at the temperature of -40^0C and pressure under 0.3 mBar.

After drying, samples were pulverized using grinding machine. Samples were stored in plastic bag or closed container for further step.

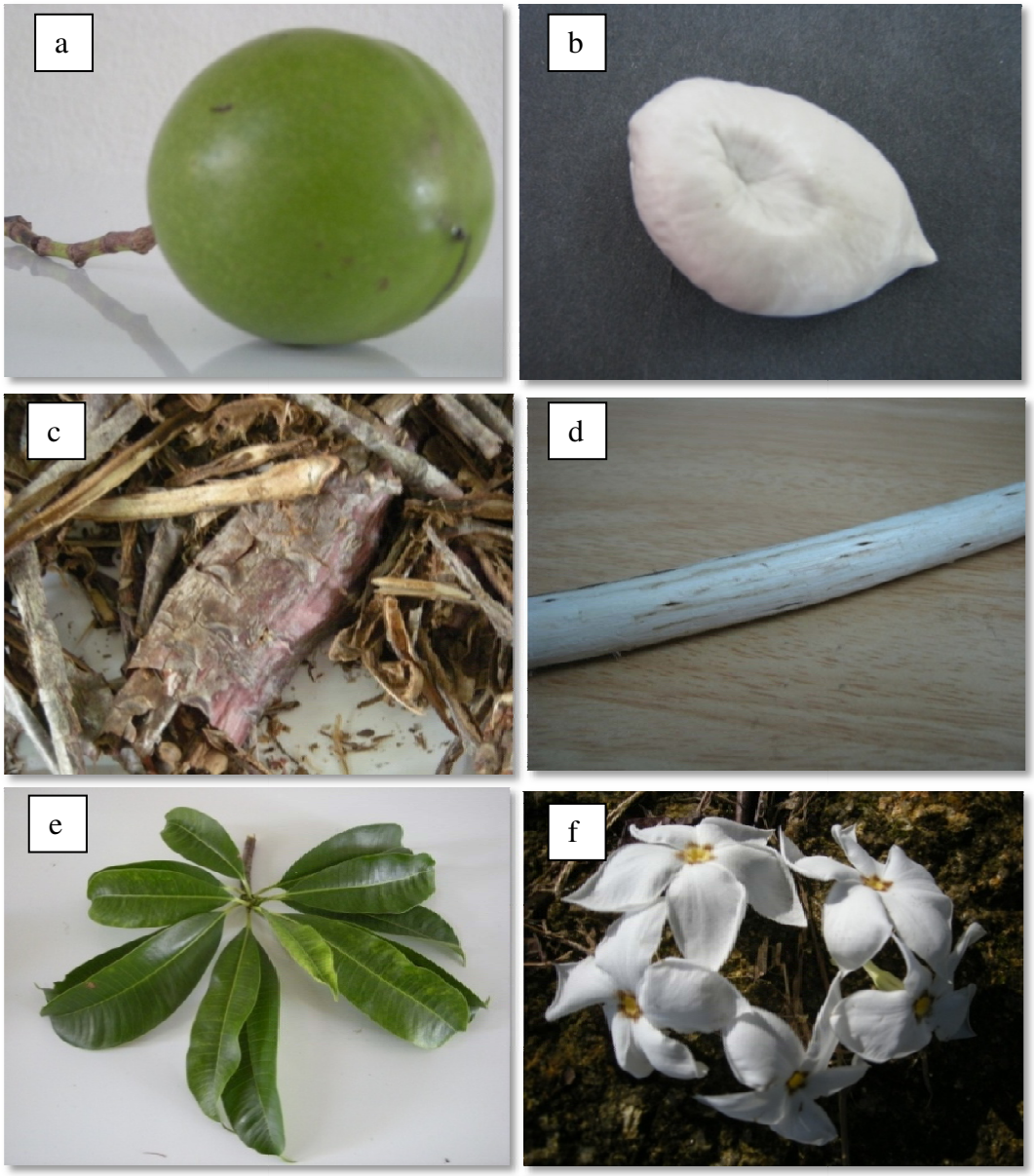

Figure 2.7: Different parts of *Cerbera odollam*, fruit (a), seed (b), bark (c), wood (d), leaf (e) and flower (f)

2.3.2 Extraction for antibacterial assay

The extraction procedure was conducted based on earlier work of Kawamura et al. (2004) with modification (Figure 2.8). Weight of the sample was taken. Sample was placed inside a round bottom flask and filled with methanol until the difference of sample surface level and methanol level is around 1 cm. Samples were extracted using methanol for 1, 2 and 3 hours with heat by direct extraction. Methanol is used to take out as much as possible extractives from the samples. The resulting liquid was filtered into a measuring cylinder. The solution was shaked vigorously using hands. Extract solution in the measuring cylinder was poured into a round flask and dried using a rotary evaporator. The solution was dried until only small amount of solvent is left. Extractive was transferred to a beaker, dried and the weight of extractives was taken. Previous steps were repeated using water as solvent with the methanol extracted residue as the sample. Hot water extracts were recovered using freeze drier to remove excess water. Extraction process is shown in Figure 2.9.

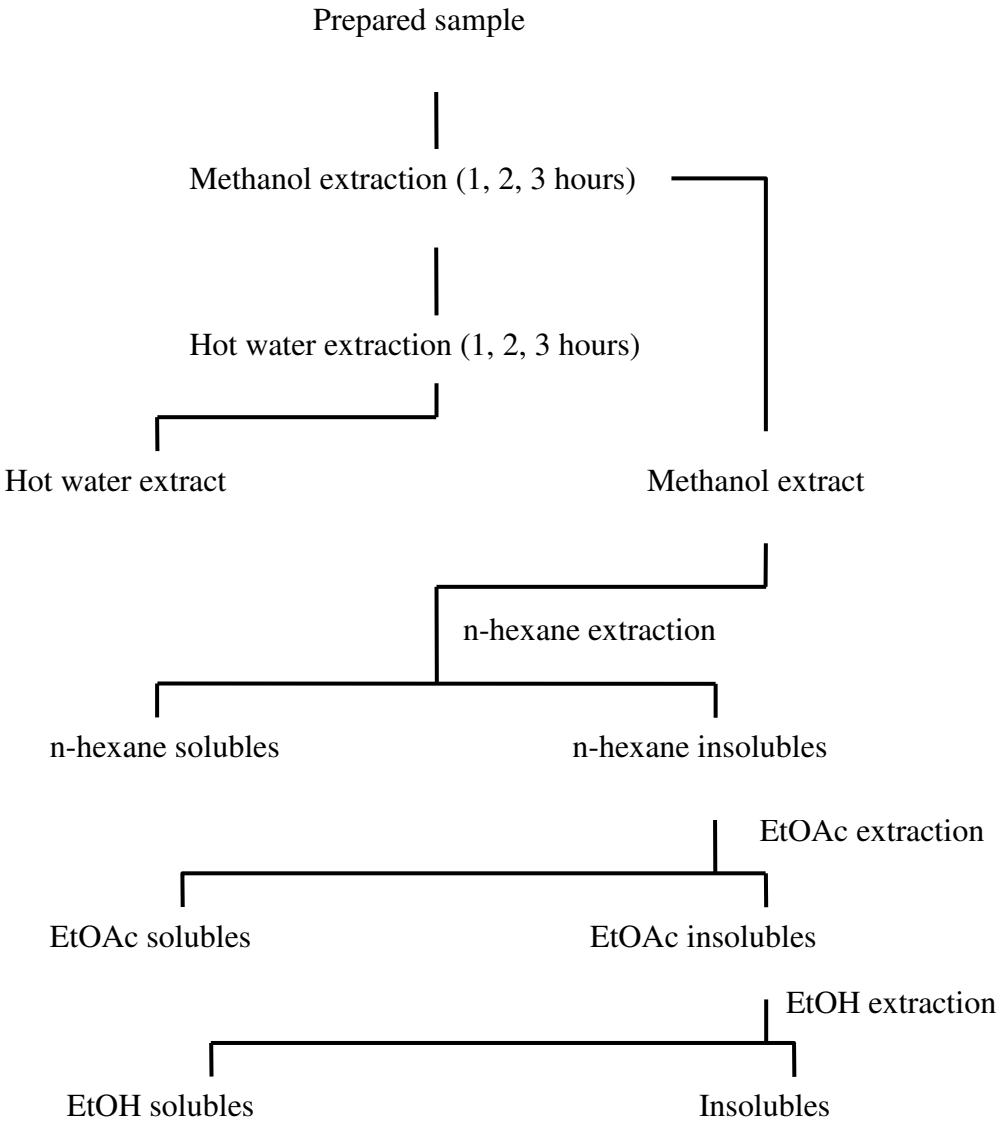

Figure 2.8: Extraction and fractionation procedures of samples.

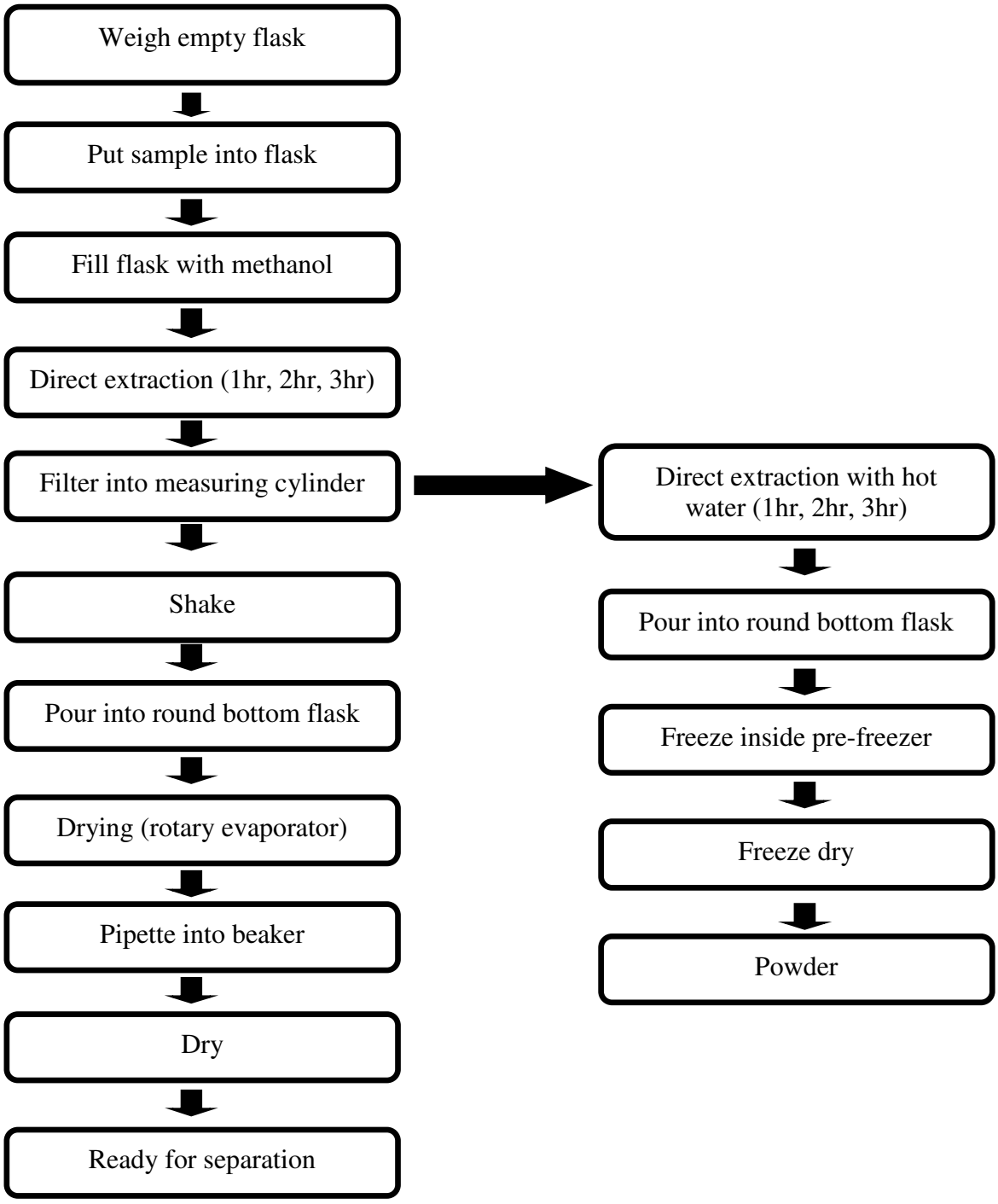

Figure 2.9: Extraction process

2.3.3 Separation (or fractionation) process

Separation process used is shown in Figure 2.10 employing the method by Kawamura et al. (2004) with slight modification by further separation of ethyl acetate insoluble part using ethanol. Extractives were separated by three different solvents. A small amount of n-hexane was poured into the beaker containing the extractive from the methanol extraction and stirred. The extracted solution was filtered using cotton wool into a round bottom flask. The process was repeated several times until no colour changes was observed in the solvent. Colour changes show that there is still extractives could be dissolved with that solvent. The extractive was dried using rotary evaporator and carefully pipetted into the sampling bottle. The extractive was oven dried at 50^0C to remove excess solvent without damaging the extractives. The weight of extractive was determined based on oven dry weight. The insoluble left in the beaker was also weighed. Process of separation will be repeated using ethyl acetate and ethanol in sequence. Fifty miligrams of every extractive was diluted in 1ml dimethylsulfoxide (DMSO). Diluted extractives were pipetted onto filter discs for later use in antibacterial assay.

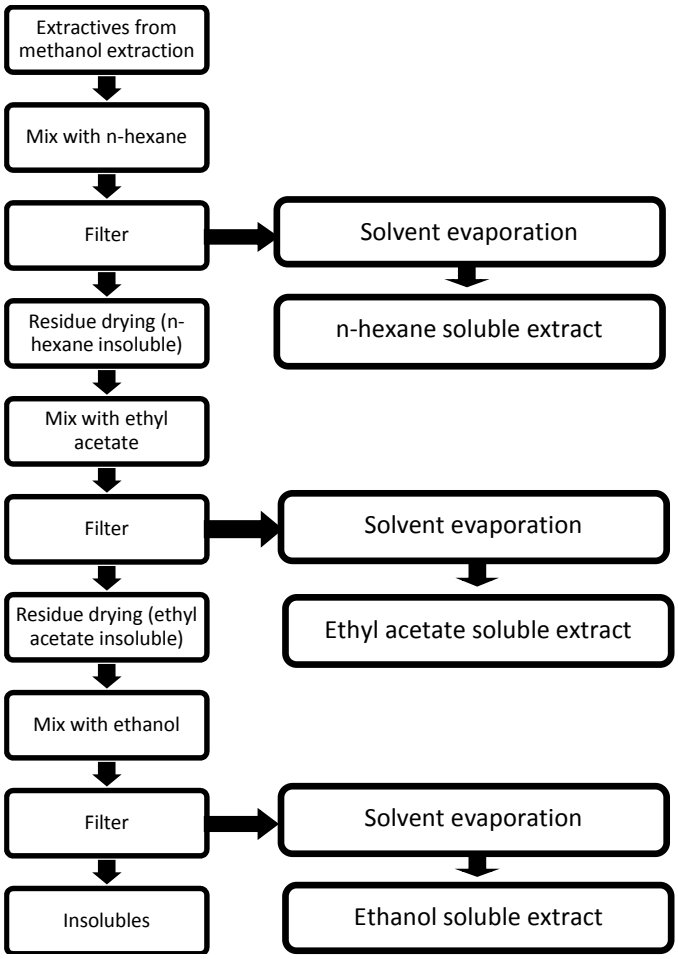

Figure 2.10: Separation process

2.3.4 Moisture content determination

Moisture content of different parts of *Cerbera odollam*, extracts and fractions were determined using oven-drying method (Hon and Shiraishi, 2001). Samples were weighed, placed into the oven at 105±3^{0}C for 24 hours and weighed again. Process was repeated until the oven dry weight is constant. Moisture content (MC) was calculated using the formula;

$$Moisture\ content, \% = \frac{Air\ dry\ weight - Oven\ dry\ weight}{Air\ dry\ weight}\ X\ 100$$