QUALITATIVE PHYTOCHEMICAL ANALYSIS ON PSIDIUM GUAJAVA LEAVES EXTRACTS AND ITS INHIBITION EFFECTS ON HUMAN OSTEOSARCOMA CELLS

by

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CERTIFICATE

This is to certify that the dissertation entitled "**Qualitative Phytochemical Analysis On Psidium Guajava Leaves Extracts And Its Inhibition Effects On Human Osteosarcoma Cells**" is the bonafide record of research work done by Ms Nor Farhana bt Ruslan during the period from July 2008 to October 2008 under my supervision.

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LIST OF ABBREVIATIONS

HCl	Hydrochloric acid
FeCl3	Ferric chloride
KI	Potassium iodide
H2SO4	Sulphuric acid

LIST OF SYMBOLS

ml	mililiter
ug/ml	microgram/ mililiter
mg	miligram
g	gram

ABSTRAK

Dunia perubatan moden mula menerima penggunaan elemen tumbuh-tumbuhan dan usur botani dalam mencari penawar kepada sesuatu penyakit. Namun begitu, pengesahan secara saintifik masih diperlukan untuk membuktikan yang sesuatu tumbuhan tersebut benar- benar berpotensi merawat penyakit yang tertentu. Salah satu contoh tumbuhan yang dipercayai mempunyai kepentingan perubatan ialah *Psidium guajava* atau lebih dikenali sebagai jambu batu. Daun jambu batu sangat terkenal dan digunakan secara meluas dalam bidang perubatan tradisional. Salah satu kebaikan daun jambu batu yang menjadi perhatian adalah potensi daun jambu untuk merencat pertumbuhan sel kanser. Objektif penyelidikan ini adalah untuk membuat analisa fitokimia kualitatif ke atas ekstrak daun jambu (ekstrak metanol, petroleum eter dan air) dan juga menentukan kepekatan IC_{50} (rencatan ke atas 50 % populasi sel) ekstrak terhadap sel kanser tulang manusia (CRL 1543). Sampel daun *Psidium guajava* diperolehi dari salah satu kediaman penduduk tempatan di negeri Kelantan. Hasil ujian fitokimia menunjukkan ekstrak methanol dan air daun jambu mengandungi alkaloid, tannin dan saponin. Ekstrak petroleum eter pula hanya mengandungi alkaloid dan terpenoids. Ekstrak metanol digunakan untuk ujian ke atas sel, dan keputusan ujian menunjukkan ekstrak tersebut mampu merencat pertumbuhan sel kanser tulang (CRL 1543) dan kepekatan IC_{50} adalah dalam linkungan 50 ug/ml (nilai $p <$ 0.05).

ABSTRACT

The world of modern medicine is now beginning to accept the use of botanicals once they are scientifically validated. One example is the *Psidium guajava* or commonly known as guava. The leaves of *Psidium guajava* (PG) is very well known for its vast traditional uses and purposes. One of the areas in concern is the potential chemopreventive activities. The objective of this study is to perform qualitative phytochemical evaluation on the extracts of PG leaves (methanol extract, petroleum ether extract and water extract) and also to determine the inhibition concentration (IC_{50}) of the extract on the osteosarcoma cell lines (CRL 1543). Leaves collected from one of the local household in the Kelantan state is processed, and extracted using three different types of solvents, namely petroleum ether (PE), methanol and water. The qualitative phytochemical analysis done on the extracts showed that the methanol and water extracts contain alkaloids, tannins and saponins. The petroleum ether (PE) extract on the other hand contains only alkaloids and terpenoid. The methanolic extract showed significant growth reduction activity in CRL 1543 and the IC_{50} concentration of the methanolic extract on CRL 1543 is approximately 50 ug/ml (p value < 0.05)

CHAPTER 1: INTRODUCTION

1.1 Medicinal Plants

The use of plants, plant extracts or pure chemicals isolated from natural products to treat disease is a therapeutic modality, which has stood the test of time even if much of the science behind such therapy is still in its infancy. Indeed today many pharmacological classes of drugs include a natural product prototype. morphine, digoxin, quinine, atropine, reserpine, physostigmine, pilocarpine, vincristine, vinblastine, artimesinin and taxol are only a few examples of what medicinal plants have given us in the past. Some active principles have limited application in modem medicine but are invaluable as pharmacological "tools" for evaluating the mode of action of other drugs or investigation of basic physiological function. Some examples are atropine, muscarine, nicotine, cocaine, reserpine, yohimbine and himbacine.

The modern medicine is now beginning to accept the use of botanicals once they are scientifically validated. However, the number of plants that still have not been studied for the content of biologically active compounds is significantly vast. In recent years, there has been an explosion of interest regarding plants and their medicinal value. In 1994, the Dietary Health and Education Act was passed which allowed any substance which can be found naturally to be sold as a “dietary supplement” regardless of its concentration or potential hazards. Since then, interest in medicinal plants has increased dramatically worldwide. However, unfortunately many people and physicians are not

aware of the potential hazard and toxicity of many plant species, thus risking their own health. So, regarding this, numerous studies must be done to investigate the true characteristic of each potential and known medicinal plant before they are commercialized for general use.

1.2 Osteosarcoma

Osteosarcoma is also known as osteogenic sarcoma. It is one of the most common types of malignant cancer that develops in bone. Like osteoblasts of normal bone, the cells that form this cancer can produce bone matrix. However, the malignant bone tissue of an osteosarcoma is not as strong and rigid as that of the normal bones. Like other cancers, osteosarcomas can spread beyond the bone into nearby tissues such as muscle, tendons, and fat. Cancer cells from osteosarcoma can also sometimes break away from the main tumor site and spread via the bloodstream to other bones, or to the lungs or other internal organs (metastasis). Current standard treatment is to use neoadjuvant chemotherapy which is actually the chemotherapy given before surgery, followed by surgical resection. The percentage of tumor cell necrosis (cell death) seen in the tumor after surgery gives an idea of the prognosis and also lets the oncologist know if the chemotherapy regime should be altered after surgery.

Standard therapy for osteosarcoma is a combination of limb-salvage orthopedic surgery when possible (or amputation in some cases) and a combination of high dose methotrexate with leucovorin rescue, intra-arterial cisplatin, adriamycin, ifosfamide

with mesna, etoposide, muramyl tri-peptite (MTP). Ifosfamide can be used as an adjuvant treatment if the necrosis rate is low. However, despite the high rate of success for chemotherapy in treating osteosarcoma, the side effects are critical and had worsened the quality of life of the patients. Thus, the potential effect of the PG leaves extract in inhibiting or suppressing the proliferation of osteosarcoma cells may lead to the development of better way of treating this cancer.

1.3 Psidium guajava

1.3.1 Botanical Aspect

Psidium guajava is commonly known as guava. It belongs to the Myrtacaea family. Common names are numerous, depending on the country and language spoken in that country. These include guayaba in Spanish-speaking countries, goiaba in Brazil, jambu batu in Malay, koejawel for Africaans and amaruud in India. Guava is a common shade tree or shrub in door-yard gardens in the tropics. It provides shade while the guava fruits are eaten fresh and made into drinks, ice cream, and preserves. There are many cultivation varieties but within the average of 10 meters in height and produce lemon-sized fruits (see Figure 2). The tree is easily identified by its distinctive thin, smooth, copper-colored bark that flakes off, showing a greenish layer beneath. This plant is considered as native to tropical America, probably from southern Mexico south to South America, but its distribution greatly extended through cultivation.

1.3.2 Use in traditional medicine

Its medicinal usage has been reported in most of the indigenous system of medicines all over the world. In Mexico, the uses are widely distributed. Commonly roots, bark, leaves and immature fruits, are used in the treatment of gastroenteritis, diarrhoea and dysentery. Leaves are applied on wounds, ulcers and for rheumatic pain, while they are chewed to relieve toothache. People there also used the decoction of the leaves to cure cough and to treat digestive problems associated with severe diarrhoea (Heinrich *et al.,* 1998). There is also a reported uses of the leaf of PG being used traditionally in South African folk medicine to manage, control, and treat a plethora of human ailments, including diabetes mellitus and hypertension (Ojewole, 2005). The use of guava leaves in the Chinese medicinal culture is also very common. They used leaves to treat diarrhoea which is also used as an antiseptic (Texeira *et al.,* 2003).

Figure 1: *Psidium guajava* tree

http://aquat1.ifas.ufl.edu/images/psigua/psigua5.gif

Figure 2: *Psidium guajava* fruit

http://www.plantcare.com/oldSite/httpdocs/images/namedImages/Guava.jpg

CHAPTER 2: LITERATURE REVIEW

2.1 Plant Phytochemical

2.1.1 Secondary Metabolites

Secondary metabolites are chemicals produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other primary functions. These chemicals are extremely diverse; many thousands have been identified in several major classes. Each plant family, genus, and species produces a characteristic mix of these chemicals, and they can sometimes be used as taxonomic characters in classifying plants. Humans use some of these compounds as medicines, flavourings, or recreational drugs.

Secondary metabolites can be classified on the basis of chemical structure (for example, having rings or containing a sugar), composition (containing nitrogen or not), their solubility in various solvents, or the pathway by which they are synthesized (e.g., phenylpropanoid, which produces tannins). A simple classification includes three main groups: the terpenes which are made from mevalonic acid, composed almost entirely of carbon and hydrogen, phenolics which are made from simple sugars, containing benzene rings, hydrogen, and oxygen, and nitrogen-containing compounds (extremely diverse, may also contain sulfur)

2.1.2 Alkaloids

Among all elements found in the plants, alkaloids are the most powerful as well as very effective. It is one of the large groups of nitrogenous substances found naturally in plants. They are usually very bitter and although the plant may be poisonous, many have extracts that are pharmacologically active. Other common alkaloids include quinine, caffeine, nicotine, strychnine and serotonin. Many alkaloids, though poisons, have physiological effects that make them useful as medicines. For example, curarine, found in the deadly extract curare, is a powerful muscle relaxant; atropine is used to dilate the pupils of the eyes; and physostigmine is a specific for certain muscular diseases. Narcotic alkaloids used in medicine include morphine and codeine for the relief of pain and cocaine as a local anesthetic. There is also a report on the plant alkaloid cryptolepine (isolated from the traditional Ayurvedic medicinal plant *Sida cordifolia*) which induces p21WAF1/CIP1 (potent inhibitor of cyclin-dependent kinases, in a p53-independent manner) and cell cycle arrest in the human osteosarcoma cell line (Matsui *et al.,* 2007).

2.1.3 Tannins and Phenolic Compounds

Tannins are polyphenols that are obtained from various parts of different plants belonging to multiple species. It is found in abundance in the tree bark, wood, fruit, fruitpod, leaves, and roots and also in plant gall. Tannins are usually found in large quantities in the bark of trees where they act as a barrier for micro-organisms

like bacteria and fungi and protect the tree. Tannins can be classified into two broad groups - hydrolysable tannins and condensed tannins. Hydrolysable tannins are basically derived from simple phenolic acids like gallic acid or ellagic acid. Condensed tannins are basically flavonoid dyes formed through bio-synthesis of flavins and catechins. There is a study reporting that gallic acid isolated from *T. arjuna* showed strong growth inhibition of P388, OVCAR-3, SF-295, A498, NCI-H460, KM20L2 and SK-MEL-5 cells (Pettit *et al.,* 1996)

2.1.4 Saponins

The name saponin is derived from the Latin word 'sapo', which means the plant that consists of frothing agent when diluted in aqueous solution. Saponins comprise of polycyclic aglycones. The sapogenin or the aglycone part is either a triterpene or steroid. Saponins are basically phytochemicals which are found in most of the vegetables, beans and herbs. Saponins result in the lysis of the blood cells (haemolysis) like all detergents, and are therefore highly toxic. Saponins base are the basic of many arrow poisons. Saponins also have always been toxic to cold-blooded creatures like snake and fish. The most noticeable effect of saponins is on the respiratory system, by reflex stimulation of the stomach wall brought about by a stimulating expectoration. There is an emetic effect when saponins are taken in bulk: elimination on the portions of the stomach gets promoted due to their detergent action. Intake of sub-emetic dosages, sublimates the emetic action to a reflex-stimulating expectoration. It is well known ipecacuanha or ipecac, and lobelia

(*Lobelia inflata*). One example of an emetic-expectorant with a saponin constituent is squill or scientifically known as *Urginea maritima*.

2.1.5 Anthraquinones

Many natural pigments are derivatives of anthraquinone. Anthraquinones are more likely to be present in the plants as glycosides owing to the variety of sugar contents and this enhances the range of the compound. Pharmacological researches have proved that the effects of anthraquinones are reliant on the occurrence of burning up of acid and hence they are consumed as glycosides. In this case, the function of the bowel vegetation has also been involved. It has been proved that sennosides are basically diluted to sennidins in a phased manned through sennidin-8-monoglucosides and later diminished to an active purgative known as rheinanthrone. Anthraquinones have a laxative effect on the body, but are generally not recommended for regular use due to concerns about the risk of habit-forming dependence and possible adverse side effects, including a higher risk for colorectal cancer (Siegers *et al.,* 1993).

2.1.6 Flavonoids

Flavonoid can be found in all parts of the plants, including flowers, leaves, fruits and others. Flavonoids are polyphenolic compounds that are ubiquitous in nature and are categorized, according to chemical structure, into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones. Over 4,000

flavonoids have been identified, many of which occur in fruits, vegetables and beverages such as tea, coffee, beer, wine and fruit drinks. Flavonoids have been reported to have antioxidant and antitumor promoting effects (Kook *et al.*, 2007). The flavonoid quercetin, which is abundant in foods like onions and apples, is important because it acts as a building block for many other flavonoids. In a study conducted by Zhang and co- researchers in 2003, quercetin derived from the guava leaves extract is reported to show significant antidiarrhoeal activity on the contraction of guinea pig ileum *in vitro* and the peristaltic motion of mouse small intestine, and reduced the permeability of abdominal capillaries.

2.1.7 Terpenoids

Terpenoids or also called the isoprenoids is a subclass of the prenyllipids (terpenes, prenylquinones, and sterols), representing the oldest group of small molecular products synthesized by plants and are probably the most widespread group of natural products. Terpenoids can also be described as modified terpenes, where methyl groups are moved or removed, or oxygen atoms added. They are universally present in small amounts in living organisms, where they play numerous vital roles in plant physiology as well as important functions in all cellular membranes. They defend many species of plants, animals and microorganisms against predators, pathogens and competitors, and they are involved in conveying messages to conspecifics and mutualists regarding the presence of food, mates and enemies (Gershenzon and Dudareva, 2007)

2.1.8 Phytochemical Study

The leaves of PG have been reported to contain essential oil with the main components being α-pinene, β-pinene, limonene, menthol, terpenyl acetate, isopropyl alcohol, longicyclene, caryophyllene, β-bisabolene, cineol, caryophyllene oxide, β-copanene, farnesene, humulene, selinene, cardinene and curcumene (Li *et al.*, 1999). Also have been isolated from the guava leaves is guavanoic acid, guavacoumaric acid, 2α-hydroxyursolic acid, jacoumaric acid, isoneriucoumaric acid, asiatic acid, ilelatifol d and β-sitosterol-3-*O*-β-d-glucopyranoside (Begum *et al.*, 2002). In another study, flavonoids and saponins combined with oleanolic acid have been isolated from the leaves (Arima and Danno, 2002). In addition, the leaves also contain triterpenic acids as well as flavonoids, avicularin and 3-l-4-pyranoside with a strong antibacterial action, fixed oil, resin, tannin, and a number of other fixed substances such as fat, cellulose, tannin, chlorophyll and mineral salts (Nadkarni and Nadkarni, 1999).

Phytochemical studies on other parts of guava also have been done. Nadkarni and Nadkarni (1999) showed that the bark contains tannin, resin and crystals of calcium oxalate. The seeds contain phenolic and flavonoid compounds including quercetin-3-*O*-β-d-(2″-*O*-galloyl-glucoside)-4′-*O*-vinylpropionate. Some isolated compounds are reported to be toxic on cells (Salib and Michael, 2004).

2.2 Biological Activity

Previously, numerous studies have been done on the guava plant to fully understand its medical capability.

2.2.1 Leaves

Antidiarrhoeal is one of its well- known properties. In a study conducted by Zhang and co- researchers in 2003, quercetin (plant- derived flavonoid) derived from the leaves extract showed significant antidiarrhoeal activity on the contraction of guinea pig ileum *in vitro* and the peristaltic motion of mouse small intestine, and reduced the permeability of abdominal capillaries. It is also reported that the asiatic acid, also extracted from the leaves, showed dose-dependent (10–500 μg/ml) spasmolytic activity in spontaneously contracting isolated rabbit jejunum preparations (Conde *et al.*, 2003). Methanol extract from leaves (8 μg/ml) of *Psidium guajava* showed activity against simian (SA-11) rotavirus (93.8% inhibition) (Goncalves *et al.*, 2005). Another reported antidiabetic effect of the leaves extract is by the study of Oh and co- researchers in 2005 where injection of the leaves extract had significantly lowered the blood glucose level in *Lepr db*/*Lepr db* mice, which are homozygous for the diabetes spontaneous mutation.

Another potential biological activity of PG is the antimicrobial activity. The inhibitory effects of aqueous and alcoholic extracts of the PG (root as well as leaves) on the growth of *Staphylococcus aureus*, *Streptococcus mutans*,

Pseudomonas aeruginosa, *Salmonella enteritidis*, *Bacillus cereus*, *Proteus* spp., *Shigella* spp. and *Escherichia coli*, causal agent of intestinal infections in humans were examined using the in vitro agar well diffusion method (Chah *et al.*, 2006). In a study by Jaiarj and co- researchers in 1999, other than reported antimicrobial activity against *S. aureus* and β-streptococcus group A, the leaves extract also showed significant anticough activity evaluated in rats and guinea pigs.

There are also many reports on antioxidant activity of the PG leaves extract. A study by Chen and Yen in 2007 showed that the leaves extract posses antioxidant activity and free radical- scavenging capacity. In another study, the total phenolic content in the leaves extract was determined spectrophotometrically according to Folin–Ciocalteu's phenol method and calculated as gallic acid equivalent (GAE). A remarkably high total phenolic content 575.3 ± 15.5 were obtained. The antioxidant activity of lyophilized leaf extracts was then determined using free radical DPPH (2,2-diphenyl-1-picrylhydryzyl) scavenging. The results obtained showed that guava leaf extracts comprise effective potential source of natural antioxidants. (Qian and Nihorimbere, 2004).

The leaves extract also has been reported to have the ability of inhibiting the growth of many cancer cell lines. An aqueous extract of PG leaves inhibited the viability of the brain- derived prostate cancer cell line (DU-145) in a dose-dependent manner. At 1.0 mg/ml, the extract reduced the viability of PCa DU-145 (the androgen independent PCa cells) to 36.1% and 3.6%, respectively after 48 hours and 72 hours of incubations (Chen *et al.*, 2007). Essential oil extracted

from the guava leaves was highly effective in reducing the growth of human mouth epidermal carcinoma (KB) and murine leukemia (P388) cell lines when they were treated with different concentrations of the oil ranging from 0.019 mg/ml to 4.962 mg/ml. Guava leaf oil showed the highest anti-proliferative activity with an IC_{50} value of 0.0379 mg/ml (four times more potent than vincristine) on P388 cell lines (Manosroi *et al.*, 2006). A chemopreventive effect was also demonstrated in another study of a methanol leaf extract on mice-induced cancer inoculated with B16 melanoma cells. A significant decrease in the incidence and average number of animals with cancer was found compared to the control group. These findings suggest that the aqueous extracts of guava leaves are efficacious for the prevention of tumour development by depressing Tr cells and subsequently shifting to Th1 cells (Seo *et al.*, 2005)

2.2.2 Other parts

Several studies have also been done on the other parts of the guava plant, which include the seed, fruit, stem and root. In one study, galactose-specific lectin in guava fruit was shown to bind to *Escherichia coli* (a common diarrhoea-causing organism), preventing its adhesion to the intestinal wall and thus preventing infection resulting diarrhoea (Coutiño *et al.*, 2001). In another study, it was observed that methanolic extract from fruit ripe have fungicidal action against *Arthrinium sacchari* M001 and *Chaetomium funicola* M002 strains (Sato *et al.*, 2000). Garcia and co- researchers in 2000 has studied the extracts of 14 plants used in the traditional medicine of Mexico and were evaluated for their effects on the growth,

spore formation, and enterotoxin production of *Clostridium perfringens* type A. The extracts of *Psidium guajava* L., were one the most effective inhibitors of growth, spore formation, and enterotoxin production. No enterotoxins were detected when extracts were added to the media at less than the MIC (minimum inhibition concentration) for growth.

2.2.3 Clinical and Animal study

There are also several animal studies and clinical trials done on investigating the potential medicinal purpose of the guava leaves. Mukhtar and co- researchers (2006) have evaluated the antihyperglycaemic activity of the ethanol extract obtained from the stem and bark of PG on blood glucose levels of normal, alloxan-induced hyperglycaemic rats and normal glucose loaded rats. Belemtougri and co-researchers in 2006 have reported that the guava leaves extract showed a decrease of caffeine induced calcium release in a dose dependent manner from sarcoplasmic reticulum of rat skeletal muscle cells. A randomized, double-blinded, clinical study performed by Lozoya and co- researchers in 2002 have been done in order to evaluate the safety and efficacy of a phytodrug developed from the guava leaves extract, standardized in its content of quercetin. The drugs are orally administered to a group of adult patients with acute diarrheic disease. Capsules containing 500 mg of the product were administered to 50 patients every 8 hours for 3 days. Results obtained showed that the used guava product decreased the duration of abdominal pain in these patients. A study by Chowororo and Ojewole (2008) showed that graded concentrations of the leaves extract (0.25–4.0 mg/ml) caused concentration-

dependent, initial brief but significant (p <0.05) rises of the basal tones and amplitudes of pendular, rhythmic contractions, followed by secondary pronounced, longer-lasting and significant (p <0.05–0.001) inhibitions of contractile amplitudes of the isolated portal veins, suggesting possible usage as phytomedicine in the management of arterial hypertension.

2.3 Osteosarcoma

Osteosarcoma is a primary malignant bone tumor that typically affects children and young adults and is an extremely aggressive disease that is associated with a high degree of lung metastases. Many studies have been done to see potential plant that can be developed into some kind of chemopreventive agent for this cancer, and cancer in general. In one preclinical study (Walters *et al.*, 2008), the data showed that curcumin exhibit potent cytotoxic effect on the osteosarcoma cell lines by mediating apoptosis process. In another study, it was reported that a flavonoid-rich herbal medicine that has long been used in Korea inhibited the proliferation and induced apoptosis in human osteosarcoma (HOS) cells (Kook *et al.*, 2007). There is also a report on the methanolic extract of *Terminalia chebula* fruit which significantly decreased the cell viability, inhibited cell proliferation, and induced cell death in a dose dependent manner in the human osteosarcoma cell lines (Saleem *et al.*, 2007). Matsui and co- reserchers (2007) reported that the plant alkaloid cryptolepine (isolated from the traditional Ayurvedic medicinal plant *Sida cordifolia*) induces p21WAF1/CIP1 and cell cycle arrest in a human osteosarcoma

cell line. So far, there are no reported studies on the effect of PG leaves extract on the proliferation of the human osteosarcoma cell lines.

CHAPTER 3

3.1 Objective of Study

The objective of this study is to perform qualitative phytochemical evaluation on three extracts of PG leaves which are the methanol extract, petroleum ether extract and water extract, and also to determine the inhibition concentration (IC_{50}) of the extract on the human osteosarcoma cell lines (CRL 1543).

3.2 Importance of Study

Recent treatment regiment for patients with osteosarcoma is surgery and chemotherapy. However, both cause the depletion of the quality of life of the patients. Therefore, the potential effect of the guava leaves extract in inhibiting or suppressing the proliferation of osteosarcoma cells may lead to the development of better way of treating this cancer.

CHAPTER 4: MATERIALS AND METHODOLOGY

4.1 Preparation of Plants

The leaves of *Psidium guajava* can be easily obtained because it is cultivated in most of the local household in Kelantan. The leaves are collected from one of the local household in the state. After leaves collection, the leaves are washed thoroughly and dried in sun shade for few days until they were completely dry. After that, the dried leaves are grinded using grinder into fine powdery form. The powdered dried leaves are then divided into three parts, for each type of solvents, and weighed.

4.2 Extraction of Plants

4.2.1 Petroleum Ether (PE) Extraction

First, the soxhlet apparatus is set up. Then, the powdery leaves are filled into the thimbles. The thimble is then loaded into the main chamber of the extractor and placed onto the round bottom flask containing the extraction solvent, PE. The volume for the solvents must be at least three to four times of the amount of solid material to be extracted. The heating mantle is turned on up to 40°C to heat up the PE to reflux. The warm PE produced filled the chamber containing the solid material. Desired compound in the solid material will dissolve with the warm PE.

When the chamber is almost full, it is automatically emptied using the siphon side arm. The process is repeated for a fix of 3 days time (as suggested by the laboratory assistant) until the PE is clearly transparent. The resulting extract solution is the concentrated using the rotary evaporator, followed by evaporating it in room temperature for few days (*de vacuo*) until the PE is completely dried. The semi-solid, paste- like extract is then kept in sterile container in 4°C.

4.2.2 Methanol Extraction

The soxhlet apparatus is set up. Then, the powdery leaves are filled into the thimbles. The thimble is then loaded into the main chamber of the extractor and placed onto the round bottom flask containing the extraction solvent,methanol. The volume for the solvents must be at least three to four times of the amount of solid material to be extracted. The heating mantle is turned on up to 60°C to heat up the methanol to reflux. The warm methanol produced filled the chamber containing the solid material. Desired compound in the solid material will dissolve with the warm methanol. When the chamber is almost full, it is automatically emptied using the siphon side arm. The process is repeated for a fix of 3 days time until the methanol is clearly transparent. The resulting extract solution is the concentrated using the rotary evaporator, followed by evaporating it in room temperature for few days (*de vacuo*) until the methanol is completely dried. The semi- solid, paste- like extract is then kept in sterile container in 4°C.

4.2.3 Water Extraction

First, the soxhlet apparatus is set up. Then, the powdery leaves are filled into the thimbles. The thimble is then loaded into the main chamber of the extractor and placed onto the round bottom flask containing the extraction solvent, water. The volume for the solvents must be at least three to four times of the amount of solid material to be extracted. The heating mantle is turned on up to the highest level to heat up the water to reflux. The warm water produced filled the chamber containing the solid material. Desired compound in the solid material will dissolve with the warm water. When the chamber is almost full, it is automatically emptied using the siphon side arm. The process is repeated for a fix of 3 days time until the water is clearly transparent. The resulting extract solution is then subjected to the freeze drier and turned to powder. The powder is then kept in sterile container in 4°C.

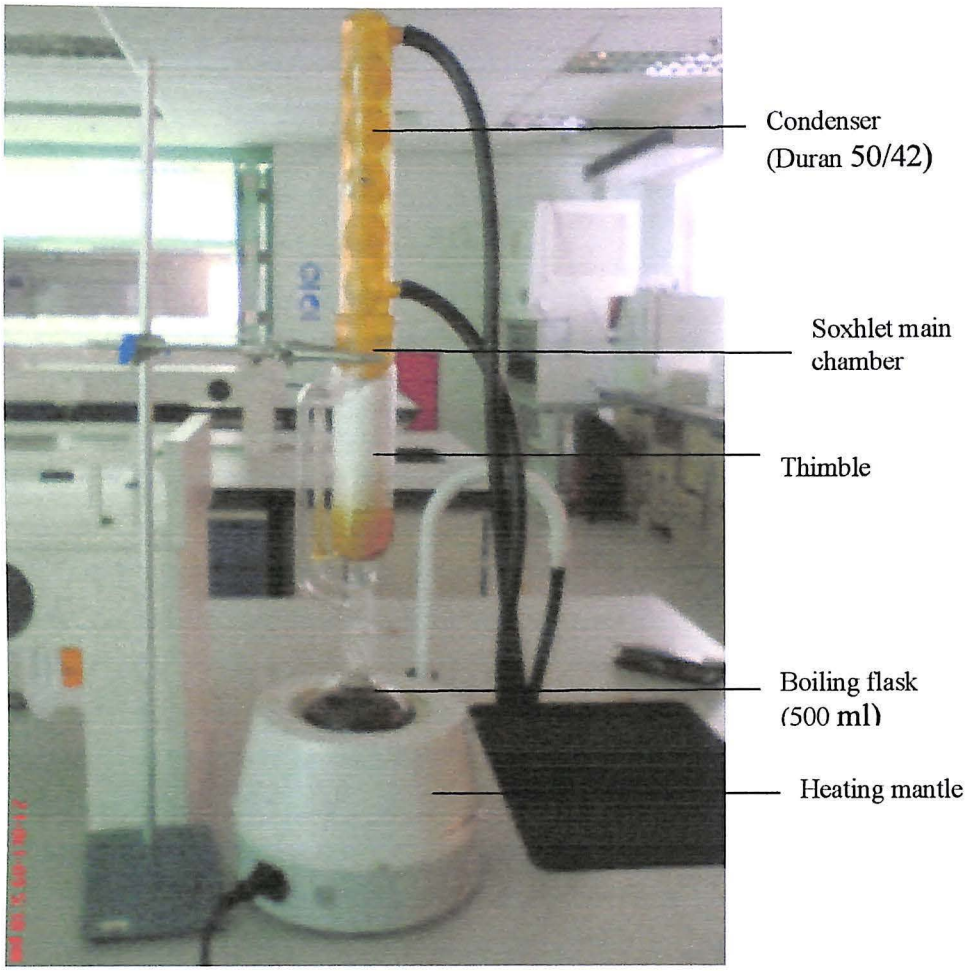

Figure 3: The set up of the Soxhlet apparatus

Figure 4: Rotary evaporator used to concentrate the extracts