EXTRACTION OF BIOACTIVE COMPOUND FROM PULP AND ROOTS OF *Benincasa hispida* a.k.a. KUNDUR

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ABSTRACT

This research was about the extraction of bioactive compound in pulp and roots of *Benincasa hispida*. The objective was to investigate bioactive compound (riboflavin) presence in the pulp and roots of *Benincasa hispida* aka kundur. The pulp was grind to supernatant juices. The juices were dried under the sunlight. As for the roots, it was also be cut into small pieces after been dry under the sunlight. Both of the samples then were undergoing the soxhlet extractor. Three different types of solvents were used, that are ultra pure water, methanol, ethanol. Both methanol and ethanol was prepared with three different concentrations which are 30%, 40% and 50%. After the samples undergo the soxhlet extractor, the samples then was undergo the rotary evaporator for separation of water from solvent. For data analysis, High Performance Liquid Chromatography (HPLC) were used as it shows accuracy more than other analysis methods. The HPLC will be use with C18 column. The concentrations also were determined by using HPLC. The result showed that methanol (40%) extracted more compare to the other solvents. For the roots, it can be said that there is no riboflavin detected. This research has the potential to be commercialized if appropriate time was given to do for more research and findings whereby the extracts of riboflavin can be sold at a high price and be used in medicinal field and food industry.

ABSTRAK

Kajian ini bertujuan untuk mengekstrak kompaun bioaktif yang ada dalam pulpa dan akar *Benincasa hispida*. Tujuannya adalah untuk menyiasat kehadiran kompaun bioaktif (riboflavin) dalam pulpa dan akar *Benincasa hispida* atau dikenali sebagai kundur. Bagi pulpa, ia dikisar menjadi jus pekat. Selepas itu, jus dikeringkan di bawah sinaran cahaya matahari. Bagi akar pula, ia dipotong halus selepas dikeringkan di bawah sinaran matahari. Kedua-dua sampel ini kemudiannya menjalani pengekstrakan soxhlet. Terdapat tiga jenis pelarut yang digunakan iaitu air tulen, methanol, dan etanol. Metanol dan etanol disediakan dalam tiga kepekatan yang berbeza iaitu 30 %, 40% dan 50%. Selepas menjalani pengekstrakan soxhlet, sampel kemudiannya menjalani proses pemisahan diantara air dan pelarut menggunakan alat pengewap berputar. Bagi analisa data pula, High Performance Liquid Chromatography (HPLC) akan digunakan memandangkan ketepatannya adalah tinggi berbanding kaedah analisa yang lain. HPLC akan digunakan bersama kolum C-18. Kepekatan riboflavin (B2) akan ditentukan menggunakan HPLC. Keputusan yang diperolehi menunjukkan bahawa pelarut methanol (40%) dapat mengekstrak lebih banyak daripada pelarut yang lain. Bagi akar pula, keputusan ujian menunjukkan bahawa tiada riboflavin yang ada pada akar *Benincasa hispida*. Kajian ini mempunyai potensi untuk dikomersialkan jika diberi masa yang sesuai untuk lebih banyak penyelidikan dan pembangunan. Ekstrak boleh dijual pada harga yang tinggi dan boleh digunakan dalam bidang perubatan dan industri makanan.

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LIST OF ABBREVIATIONS

HPLC	High performance liquid chromatography
g	Gram
mg	Milligram
µm	Micronmeter
mAUs	milliabsorbance unit × second

1 INTRODUCTION

1.1 Introduction

Benincasa hispida is well known for its nutritional value among people around the globe. It can be called as winter melon or ash gourd in certain country. The fruit usually are used in food making such as soups and candy. Unlike the other melon, it doesn't taste sweet at all. The winter melon provides its consumer with many natural sugars, amino acids, organic acids, mineral elements and vitamins in its fruit.

Moreover, Aqilah *et al*. (2011) stated that "Its versatile utility as a nutritious vegetable, and folk medicine and functional food ingredient provoked us to compile a comprehensive review of this multipurpose fruit on the distribution and, nutritional and medicinal properties together with its phytochemicals". Recently, more researches were conducted in Malaysia and some other countries to find the potentials of winter melon as sources of food and medicine. Based on research conducted by Kumar and Vimalavathini (2004) reported kundur fruit as an important ingredient of "Kusmanda lehyam" (Ayurvedic medicine).

There are many elements contains in the fruit consists of vitamins, amino acids, minerals, organic acids, including bioactive compound. Bioactive compound can be described as elements that are exists in natural plants that are used for medicinal purposes. Extraction of bioactive compounds from medicinal plants has been permitted the demonstration of their physiological activity by medicinal researcher (Asghari *et al.*, 2011).

The main goal of the study is to extract the riboflavin from root and pulp of *Benincasa hispida* in order to investigate the properties of the bioactive compound. Thus, to achieve the objective, High Performance Liquid Chromatography (HPLC) needs to be applied. Besides that, different solvents that are methanol, ethanol and water were used in order to determine, which solvent and which concentration can extract more riboflavin from *Benincasa hispida.*

1.2 Motivation

In this modern age, many researches have been done to fruits that have potential to be commercialized. In this context, *Benincasa hispida* has high potential due to its various nutritional values. Thus, it is best that this fruits was fully utilized to its maximum potential. For example, the mass production of sunquick (orange juice) that contained a mass amount of vitamin C. Riboflavin or vitamin B2 are often been used as supplements food. Recommendations for daily riboflavin intake increase with pregnancy and lactation to 1.4 mg and 1.6 mg, respectively. For infants, the RDA is 0.3-0.4 mg/day and for children it is 0.6-0.9 mg/day (Gropper *et. al*, 2009).

1.3 Problem statement

Benincasa hispida has many uses in food and medicinal industry. Many countries have been actively to mass produce the fruit includes Malaysia. Thus, the government is trying to exploit the benefits of the fruit. There have been many researches done on *Benincasa hispida* by using the pulp of the fruits. However, not much research has been done to the roots. There are possibilities of new discoveries can be found. Besides that, this research also can be use in order to find another alternatives source of food supplement.

1.4 Objectives

To investigate bioactive compound presence in the pulp and roots of *Benincasa hispida* a.k.a. kundur.

1.5 Scope of this research

There are many solvents that can be used to extract bioactive compounds. However, the common types of solvents used are methanol, ethanol and water. Thus, this project is to study:

I. The effects of different types of solvent used for extraction.

II. The effects of solvent concentration for extraction

2 LITERATURE REVIEW

2.1 Benincasa Hispida

Benincasa hispida or its local name such as Kundur (Malay), Bhuru Kolu or Safed Kolu (Gujarati), Petha (Hindi), Kushmanda (Sanskrit), dōngguā (Chinese), Fak kio (Thailand), Calabaza china or Calabaza blanca (Spanish), Kondol (Philippines) and Bleego (Indonesian), and winter melon (English). Some prominent cucurbit family members are gourd, melon, cucumber, squash and pumpkin (Robinson *et al*, 1999). The plants were generally cultivated due to the edible fruits. Winter melon is a fast growing, long season and warm climate vegetable. Initially, it is cultivated in Southeast Asia and now is widely grown in East Asia as well as South Asia. There are four recognized cultivars of winter melon fruit, namely, unridged winter melon, ridged winter melon, fuzzy gourd and wax gourd (Walter *et al.,* 1989). However, in Malaysia there is only two cultivars as mention by Aqilah. *et al.*, 2011.

2.1.1 Characteristic and physical properties

The fruit of *Benincasa hispida* may grow up to 80cm in length depend on the medium, temperature and enviromental factor. The size of the fruit is large and the flesh is white to pale green in color and spongy. The immature fruit has hairs that cover the body and the fruit will slowly losses it hairs as it matures and develop a waxy coating. Hairs that cover the ovary and young fruit are lost as it matures. Fruit of modern cultivars are up to 2 meters (m) long and 45 kilograms (kg) (Kendrick *et al*., 2006). One of the unique characteristics of kundur, if there is no injury to the fruits in that it can be stored for many months, even for a full year in dry and cool atmospheres (Morton, 1971). The plant was categorized based on its kingdom, family, and its genus as shown in table 2.1.

Table 2.1 Classification of Kundur (*Benincasa hispida*)

Kingdom	*Plantae – Plants*
Sub-kingdom	*Tracheobionta – Vascular plants*
Superdivision	*Spermatophyta – Seed plants*
Division	*Magnoliophyta – Flowering plants*
Class	*Magnoliopsida – Dicotyledons*
Family	*Cucurbitaceae – Cucumber family*
Genus	*Benincas Savi – benincasa*
Species	*Benincasa hispida (Thunb.) Cogn. -waxgourd*

Source: U.S Department of Agriculture

The plants belonging to this family are frost-sensitive, drought-tolerant, and intolerant towet and poorly drained soils (Whitaker *et al.*, 1950). Thus, most of the fruit grown in Southeast Asia, East Asia and South Asia.The fruit were called as wax gourd because of the physical appearance of the fruit that has waxy coating. Figure 2.1 shows the pictures of winter melon.

Figure 2.1: *Benincasa hispida*

2.1.2 Food

The fruit serve as many purposes to mankind, mainly as foods and medicines.. Usually the fruit were serves as main dish. In China, this fruit is available during winter and typically that is how the fruit got its Chinese name, Winter Melon. Kundur is commonly used as a vegetable to make soup, suitable for pickling, curries, preserves, sweetened and even candied. In chinese cuisine, the melons are used with the addition of pork or beef to make winter melon soup. After the food were ready, the melon were scooped out, carved by scrapping off the waxy coating. In addition, the melon also were used in making candy or called táng dōng guā. The candy was commonly eaten during New Year Festivals with moon cake made from kundur. Meanwhile, in India, the melon was the main ingredient of curry and candy called petha. The fruits are consumed as baked, fried, boiled, pickled or candied/preserved (Robinson *et al.*, 1999). Meanwhile, fried seeds of the kundur can be eaten as a delicacy and young leaves as well as flower buds steamed are consumed as vegetables

2.2 Medicinal properties and health benefits

The kundur fruit has many benifits to human in terms of medicinal value. The fruits supply its consumer with abundant nutrients for healthy human body. In Ayurvedic remedies, kundur fruit were used to treat kidney stones while its seed will be boiled together with milk and it is believe to increase sperm count and improve the sperm locomotion. There are also other uses of *Benincasa hispida* fruit such as anti-cancer. In addition, the authors indicated that the fruit may provide protective effects against the development of atherosclerosis and also exhibits anticarcinogenic effects in vivo (Aqilah. *et al.*, 2011). In Ayurveda, *B*enincasa *hispida* is recommended for management of peptic ulcer, hemorrhages from internal organs, epilepsy and other nervous disorders (Warier *et al.*, 1994). Acid neutralizing and ulcer healing activities of *Benincasa hispida* has also been described (CSIR, 2000). Other than that, kundur also can treat diarrheal and obesity. This has been prove by Aqilah. *et al.* (2011) as he stated that a number of medicinal properties such as anti-diarrheal, anti-obesity, anti-ulcer, and antioxidant and diuretic have been ascribed to this fruit of high economic value.

Nowadays, there have been a lot of researches have been conduct on the effects of *Benincasa hispida* on the peptic ulcers. In Ayurveda, *Benincasa hispida* is recommended for management of peptic ulcer, hemorrhages from internal organs, epilepsy and other nervous disorders (Warier, 1994; Sharma, 1984). Acid neutralizing and ulcer healing activities of *Benincasa hispida* has also been described (CSIR, 2000). In earlier studies, the fresh juice of *Benincasa hispida* showed significant anti-inflammatory activity in cotton pellet granuloma and carrageenan induced edema in rats (Grover *et al.*, 1994). In addition, some researchers have undergone further research on how to fully use the antioxidant presence in *Benincasa hispida*. In Korea, kundur is used to treat diabetes and diuresis diseases. The researchers have conducted a research in 2005 about the Anti-angiogenic effect of the seed extract of *Benincasa hispida* Cogniaux. Based on their research, they concluded that the results obtained from two assays indicate that the fraction of *Benincasa hispida* seed extract has inhibitory effects on bFGF-induced angiogenesis. The angiogenesis can be defined as the physiological process involving the growth of new blood vessels from pre-existing vessels (Moon *et al.* 2008). Table 2.2 show summaries of medicinal and pharmacological properties of different parts of Kundur fruit done by Aqilah *et al* (2011).

Table 2.2 Medicinal and pharmacological properties of different parts of Kundur fruit.

Part	Medicinal and pharmacological properties	References
Pulp	Anti-inflammatory, anti-ulcer, anti-depressant, anti-histaminic, antioxidant, anti-compulsive, anti-diarrheal and anti-obesity activities; beneficial effects in allergic inflammation, insanity and epilepsy; preventive and curative effects in nervous disorder, intestinal worms, jaundice, diabetic, leucorrhoea, stomach and bile problems; potential uses as diuretic, laxative, aphrodisiac, clearing heat and detoxificant;	Morton (1971), Ramesh, Gayathri, Appa Rao, Prabhakar, and Seshagiri Rao (1989), Grover and Rathi (1994), Mingyu *et al.,* (1995), Kumar and Ramu (2002), Huang *et al.,* (2004), Kumar and Vimalavathini (2004), Mathad *et al.,* (2005), Raveendra Retnam and Martin (2006), Roy *et al.,* (2007),
Seed	Anti-angiogenic, anti-tumor, antioxidant, antinociceptive, and anti-pyretic activities; soporific potential, and beneficial effects for brain and liver; used for the treatment of syphilis, cardiovascular diseases, inhibition of angiotensin converting enzyme (ACE).	Choi, Lee, and Kim (2003), Huang *et al.,* (2004), Lee *et al.*, (2005), Raveendra Retnam and Martin (2006), Qadrie *et al.,* (2009).
Peel	Antioxidant activity; inhibition of angiotensin converting enzyme (ACE)	Huang *et al.,* (2004).

Source: (Aqilah *et al.,* 2010)

2.3 Nutrition compound

2.3.1 Composition

The *Benincasa hispida* fruits contains many compounds such as carbohydrate, amino acids, volatile compounds, miscellanaeous compounds, minerals and vitamins. In the pulp, most of the vitamin presence is vitamin C. Table 2.3 shows the tabulated amounts of composition that present in mature kundur fruits.

Table 2.3: Compositions of mature kundur fruit

Compounds	Amount (g /100 g of edible portion)
Moisture	94.50
Protein	0.50
Carbohydrate	4.00
Fiber	0.50
Fat	0.20
Ash	0.30

Source: (Aqilah *et al.,* 2010)

In table 2.3, it shows that in the fruits are mostly made up from water followed by carbohydrate. The least component that exist in kundur fruits are ash followed by fat. The amount of protein and fiber are same which is 0.5 gram per 100 gram edible portion of the fruits. Table 2.4 shows amounts of vitamins contain in the *Benincasa hispida* fruits.

Table 2.4: Vitamins contains in *Benincasa hispida* fruits

Vitamins	Amounts (mg/ 100 g of edible portion)
Vitamin C	68.00
Thiamin	0.02
Riboflavin	0.31
Niacin	0.20

Source: (Aqilah *et al.,* 2010)

In table 2.4, vitamin C show the highest amounts in the fruits which is 68 milligram per 100 grams of edible portion of the fruits followed by riboflavin which is 0.30 milligrams. The niacin only shows 0.20 milligrams while the thiamin which the least shows 0.02 milligrams.

Besides that, there are also minerals such as potassium (K) and calcium (Ca) which are major in percentage compare to othe minerals. MacWillian (2005) have emphasized that both of these minerals (K and Ca) play a very beneficial role in maintaining electrolytic balance of body fluid as well as in contributing to alkalinizing the body. For amino acids, the result from Aqilah. *et al.* (2011), show that total protein and free amino acids are present in high amounts in seed, with 5714.017 mg/100 g fresh weight and 264.366 mg/100 g fresh weight, respectively.

Total protein and free amino acids are the lowest in amount in the pulp, having concentrations of 216.400 and 92.549 mg/100 g fresh weight, respectively. The amino acid that presence in the pulp of *Benincasa hispida* are Ornithine, Aspartate, Threonine, Serine, Glutamate, Proline, Glycine, Alanine, Cysteine, Valine, Isoleucine, Leucine, Tyrosine, Phenylalanine, Lysine, Histidine, Arginine and, γ-Aminobutyric acid. As for polysaccharides, the most abundant in winter melon are arabinigalactants. In another study Mazumder *et al.* (2004) reported that the alcohol insoluble residues from Kundur fruit contain high amounts of homogalacturonan and β- (1→4)-D-galactan together with a small amount of acidic arabinan. Furthermore, there are also volatile compounds that presence in winter melon. There are 18 identified volatile compounds using GC/MS with 2-aminohexanoic acid, 2-amino-3-cyano-propanoic acid, 2- aminobutanoic acid, 2-amino-4-hexenoic acid and 3-cyclohexenyl-1-glycine being considered as unusual substances (Mingyu *et.al*, 1995).

2.3.2 Riboflavin

In this project, the target bioactive compound is riboflavin or known as vitamin B2. Riboflavin can be found at high percentage in the pulp of benincasa hispida. The name of riboflavin comes from ribose which is reducing sugar. The reduced form, which occurs in metabolism along with the oxidized form, is colourless. Table Riboflavin is best known visually as the vitamin which imparts the orange colour to solid B-vitamin preparations, the yellow colour to vitamin supplement solutions, and the unusual fluorescent-yellow colour to the urine of persons who supplement with high-dose B-complex preparations. Table 2.3 shows the properties of riboflavin. While figure 2.2 shows the structural diagram of riboflavin.

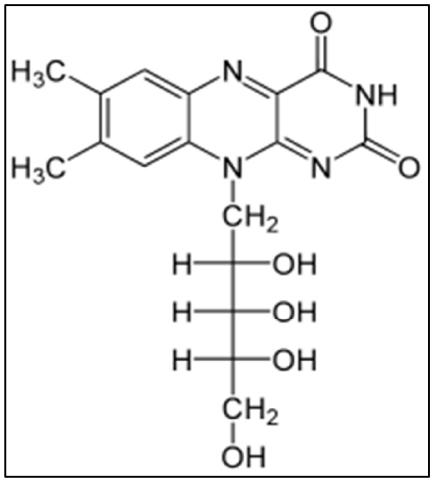

Figure 2.2: Structural formula for riboflavin (Stoker, 2013)

Table 2.3: Properties of riboflavin

Molecular formula	$C_{17}H_{20}N_4O_6$
Molar mass (g / mol)	376.36
Appearance	Orange crystals
Acidity (pKa)	9.888
Basicty	4.109

Source: (Stargrove *et al*., 2008)

Riboflavin are needed in human body as it play roles in energy metabolism, and for the metabolism of fats, ketone bodies, carbohydrates, and proteins. It is also used as an orange-red food colour additive. Other than that, riboflavin also plays role in secretion of protein in human culture Karoline *et al*. (2006) have stressed that Riboflavin deficiency impairs the oxidative folding and subsequent secretion of proteins in human cell cultures. In addition, riboflavin often used in the production of baby foods, breakfast cereals, pastas, sauces, processed cheese, fruit drinks, vitamin-enriched milk products, and some energy drinks.

2.4 Extraction

The extraction process is done by using soxhlet extraction method. The appratus was divide into four different section namely, condenser tube, extraction tube, solvent flask and heating mantle. The sample was insert into a thimble which the was insert into the extraction tube. Figure 2.3 shows the diagram of soxhlet extractor.

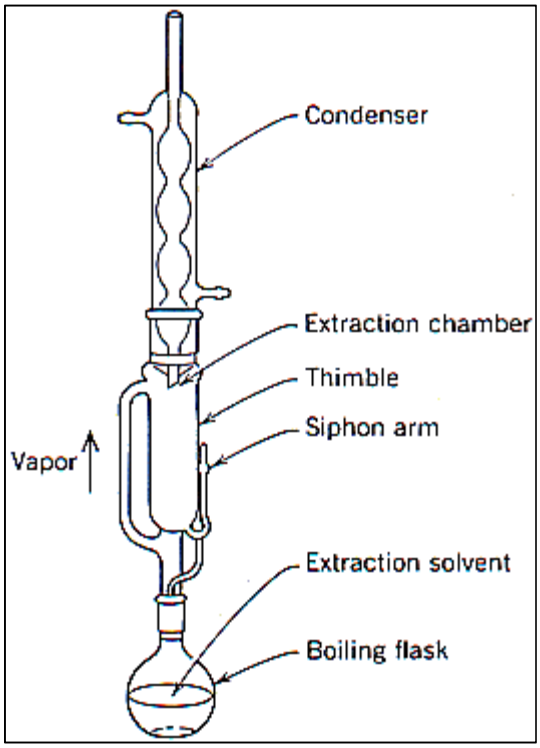

Figure 2.3: Soxhlet extractor diagram

2.4.1 Solvent

In this projects, three different solvents are used which are methanol, ethanol and water.

2.4.1.1 Methanol

The first solvent is methanol which was bought from sigma alderich. The purity of methanol used must be higher 99.8%. This is to ensure the validity of the research. The commercial grade A of methanol is nowadays sufficiently pure for practically all synthetic uses and for its use as a solvent, except for very specific requirements (Marcus *et al*. 1985). Methanol has electron-pair donation capabilities, not to mention that methanol is an amphoteric solvent. Thus, it has similar properties as water. Other than that, methanol is capable of solvating both cations and anions, and although its dielectric constant (relative permittivity) is modest (32.7 at 25 00), in comparison with water (78.5 at 25 00), it supports the ionic dissociation of most electrolytes (Marcus *et al.* 1985). These properties have made methanol a very extensively utilized and studied solvent.

2.4.1.2 Ethanol

The second solvent is ethanol which was bought from sigma alderich. The purity of ethanol used must be higher 99.8%. This is to ensure the validity of the research. Since ethanol is the same class as methanol (alcohol). It has only a modest dielectric constant (relative permittivity), 24.55 at 25° C, hence most electrolytes, when not too dilute, will be extensively ion-paired in it (Marcus *et al*. 1985).

2.4.1.3 Water

The third solvents that were used are ultra pure water. Ultra pure water was used since HPLC cannot detect normal tap water. Other than that, it was to ensure the validity of the research.

2.4.2 Soxhlet extraction

The soxhlet extractor are one of the conventional extractor as it were commonly used for extraction. It is one of the oldest method and most widely used approaches for conventional extraction of solid samples. The Soxhlet procedure remains the most exhaustive extraction technique, and today it is still widely used (Anderson, 2004). It has its own advantages and disadvantages. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. This technique is particularly useful in cases when the pure compound is partially soluble in a solvent and the impurity is not soluble in that solvent and vice versa. Also the working principle of mechanism is so simple that we can obtain more desired compounds without any difficulty. As for the disadvantages, this method needs a long process. Other than that, the extractor has poor extraction of polar lipids and large volumes of solvent are needed. Soxhlet extraction has the fundamental drawback of being slow and requiring a high volume of organic solvents that must later be eliminated (Crespo *et. al*, 2004).

2.5 Separation process

In this process, rotary evaporator is used to separate solvents from water and extracts. The rotary evaporator used the principle of differences in boiling points in order to separates the liquid. A rotary evaporator is a special device, which utilizes low heat and high vacuum in order to remove the solvents (Ledgard, 2003). Figure 2.4 shows the diagram of rotary evaporator.

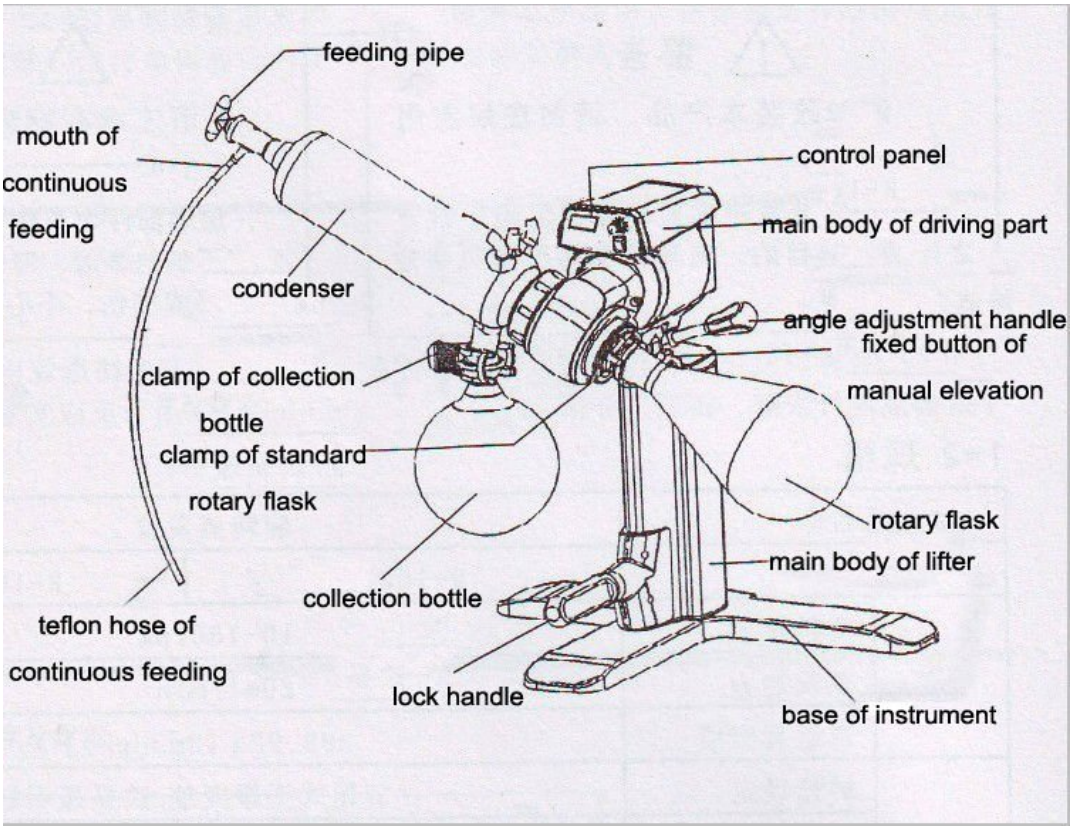

Figure 2.4: Rotary evaporator.

2.6 Analysis

2.6.1 High Performance Liquid Chromatography (HPLC)

After the extractions have been done, analysis is needed in order to confirm whether the results of the research have been achieved or not. Thus, high-performance liquid chromatography (HPLC) method will be used. High-performance liquid chromatography (HPLC) is a form of liquid chromatography to separate compounds that are dissolved in solution. Progress in analytical chemistry during the last decade made high pressure liquid chromatography (HPLC) the prevalent separation technique (Parvez *et al*., 1988). The types of HPLC column that are used need to precise in order to get an accurate reading. The column are sensitive, thus only certain compound can be read by certain column. The column might be damaged if the wrong column were used.

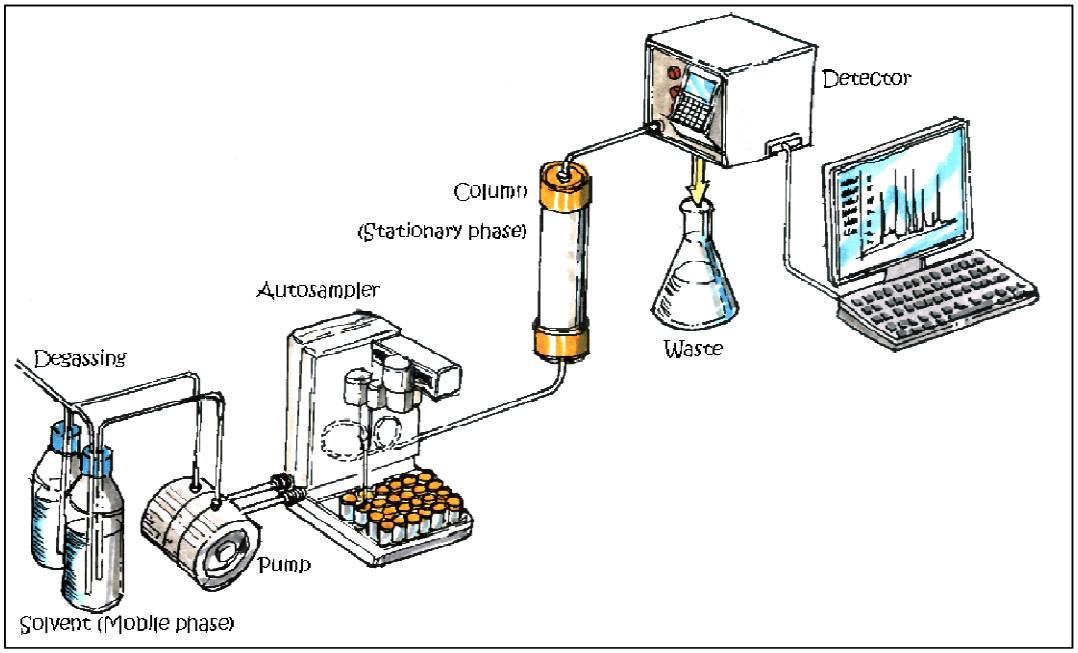

Figure 2.5: High Performance Liquid Chromatography

3 MATERIALS AND METHODS

3.1 Overview

This chapter describe the materials and methods employed for the extraction of bioactive compounds from pulp and roots of *Benincasa hispida*. A schematic structure of the whole process flow has been conducted and illustrated in figure 3.1

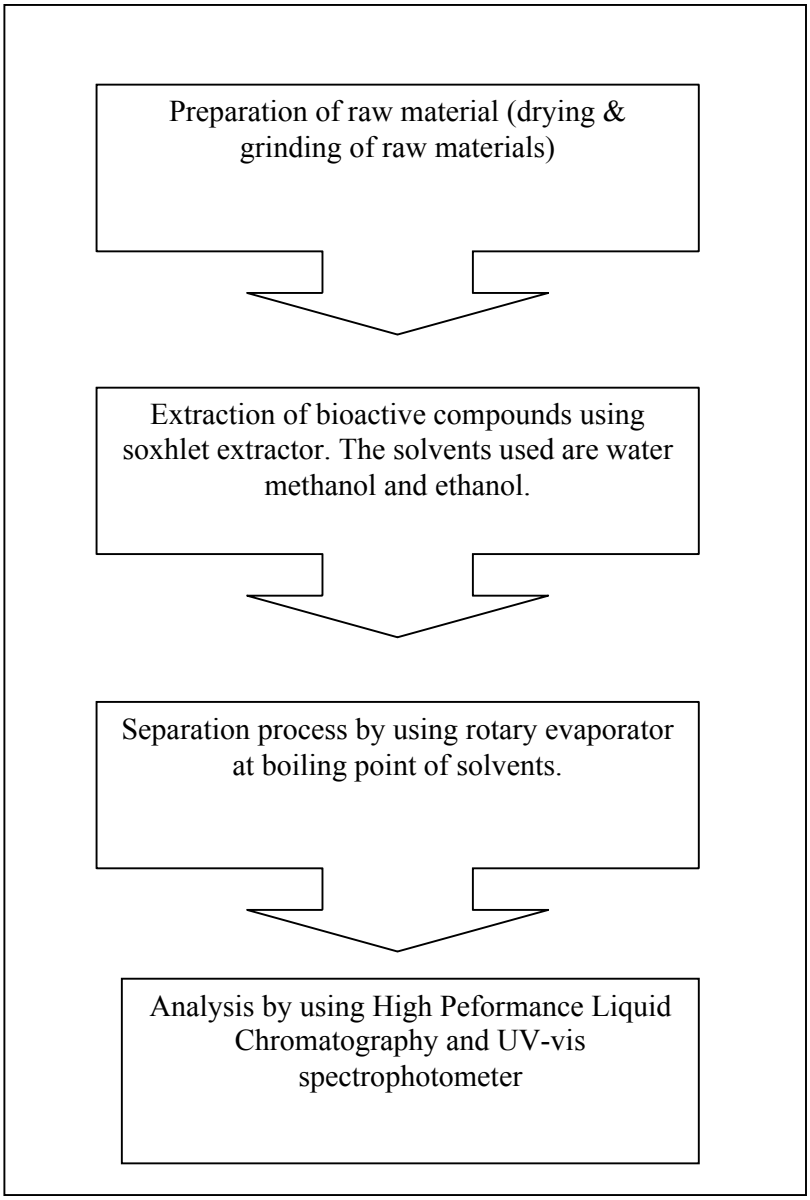

Figure 3.1: Process flow diagram