EXTRACTION OF GLYCOSIDES FROM THE MIXTURE OF CASSIA ALATA STEMS AND LEAVES

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ABSTRACT

The main objective of this research is to separate *anthraquinone glycosides* from the mixture of *cassia alata* stems and leaves. Besides it is also to find the kinetic of *anthraquinone glycosides* separation from the mixture of *cassia alata* stems and leaves using ultrasound extraction method. The mixtures of *Cassia alata* stems and leaves were dried in open shade. Dried *Cassia alata* will be milled to 60 mesh powder using mortar. The mixtures of *Cassia alata* stems and leaves powder will be stored at ambient temperature. The mixture of *Cassia alata* stems and leave powder (500 mg) was dissolved with 50 ml of different solvent-water ratio (20:80, 40:60, 60:40, 80:20 and 100:0, v/v) in an ultrasonicator bath. Time was used for sonication are 10, 20, 30, 40 and 50 minutes using ultrasonic cleaning bath (JAC ultrasonic type 1505) which nominal power is 250 W dimension 300x150x150 mm), frequency of 40 kHz and volume of 5.7 L. The extraction process was conducted at five different temperatures which is 20, 30, 40, 50 and 60 ^{0}C. The sample was allowed to cool at room temperature.Then at vacuum filter, dry the filter disk in the oven at 103°C to 105°C for 1 hour, cool in a desiccator and weigh. After that, rotary evaporator was used to record distillate temperature and measure the volume of collected product from receiving vessel. Combined all the collected distillate in a container and at the end of the experiment, measure its overall volume and composition. The volume and composition of the concentrate was determined. High Performance Chromatography (HPLC) was used to analyze the samples. A sample was injected into the Eurospher 100-5 NH_2 column. The samples was evaluated at a flow rate of 1 mL/min using a solvent system of acetonitrile:water (80:20, v/v). From the result we can conclude that the mixture of cassia alata leaves and stems has been successfully extract the anthraquinone glycosides. The extraction process has been affected by the variation of parameter such as by extraction time, extraction temperature, extraction power and ratio of solvent to water. The optimum yield can be extract from the mixture of cassia alata stem and leaves was found by the extraction time of 30 min, extraction temperature of 40 ^{0}C and ratio of solvent to water of 80:20 (v/v).

ABSTRAK

Objektif utama kajian ini adalah untuk memisahkan glikosida anthraquinone daripada campuran tangkai dan daun pokok gelenggang (cassia alata). Selain itu juga,bertujuan untuk mencari kinetik bagi pengasingan glikosida anthraquinone daripada campuran campuran tangkai dan daun pokok gelenggang dengan menggunakan kaedah pengekstrakan ultrasound. Campuran tangkai dan daun pokok gelenggang yang telah dikeringkan di bawah ruang terbuka. Kemudian,daun gelengang tersebut ditumbuk dan digiling mengunakan lesung batu. Serbuk yang terhasil daripada campuran tangkai dan daun gelengang disimpan pada suhu bilik. Campuran serbuk yang terhasil daripada campuran tangkai dan daun gelengang (500 mg) dilarutkan ke dalam 50 ml air dengan nisbah yang berbeza (20:80, 40:60, 60:40, 80:20 dan 100:0, v / v) dalam ultrasonicator.Masa yang diperuntukkan untuk proses perlarutan adalah 10, 20, 30, 40 dan 50 minit dengan menggunakan pembersihan ultrasonik (jenis ultrasonik JAC 1505) yang kuasa nominal ialah 250 W Dimensi 300x150x150 mm), kekerapan 40 kHz dan jumlah sebanyak 5.7 L. Proses pengekstrakan dilakukan dengan mengunakan suhu yang berbeza iaitu 20, 30, 40, 50 dan 60 ^{0}C. Kemudian sampel disejukkan pada suhu persekitaran. Kemudian pada penapis vakum, cakera penapis dikeringkan di dalam ketuhar pada suhu 103 ° C hingga 105 ° C selama 1 jam, sejuk di dalam pengering dan ditimbang. Selepas itu, penyejat putar telah digunakan untuk merekod suhu sulingan dan jumlah produk yang terkumpul dihitung. Gabungkan semua sulingan yang terkumpul dalam bekas dan pada akhir eksperimen jumlah keseluruhan dan komposisi diukur. High Perfomance Chromatography (HPLC) digunakan untuk menganalisis sampel. Sampel disuntik ke dalam Eurospher 100-5 NH2, sampel akan dijalankan pada kadar aliran 1 mL / min dengan menggunakan sistem pelarut acetonitrile:air (80:20, v / v). Daripada keputusan yang diperolehi,sy berjaya untuk mengestrakkan glikosida anthraquinone daripada campuran tangkai dan daun pokok gelengang. Proses pengekstrakkan juga dipengaruhi oleh beberapa sebab iaitu seperti masa,suhu,kuasa dan nisbah pelarut terhadap air 80:20 (v/v)..

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LIST OF ABBREVIATIONS

THP-Thai Herbal Pharmacopoeia

HPLC - High performance liquid chromatography

AIDS - Acquired immune deficiency syndrome

ASEAN- Association of Southeast Asian Nations

WHO-World Health Organization

CSE-Conventional solvent extraction

MAE-Microwave-assisted extraction

UAE-Ultrasound-assisted extraction

LIST OF ABBREVIATIONS

Ce = concentration in the liquid phase at equilibrium

Kobs = first order rate constant

t= is the extraction time

h= is the initial extraction rate

1 INTRODUCTION

1.1 Research Background

Senna alata (L.) Roxb. Or *Cassia alata* L. is a medicinal plant in the family Fabaceae which has been known in Thai language as Chumhetthet. The English names are Candelabra bush and Ringworm bush (Smitinand T. 2001).It is a native plant of South America and can be found widely in tropical region, up to 1500 m, on waste places often along ditches (Larsen K, Larsen SS, Vidal JE. 1984). In Indonesia, Philippines and Thailand, this plant can be found all over the countries, sometimes cultivated for medicinal purposes. After 3 months of planting, leaves are ready for harvest, but the best period for the best quality is about 6-7 months after planting. Fresh or dried leaflet of *C. alata* has been used as folk medicines in many countries for treatment of constipation, stomach pain, ringworm and skin diseases (Grosvenor PW, Gothard PK, Mc.William NC, Supriona A, Gray DO. 1995).

The leaf contains anthraquinones both aglycone and glycoside forms i.e. rhein, aloe-emodin, chrysophanol, glycosides of rhein, emodin, physcione and sennosides A, B, C, D7-10 while rhein is a major component investigated total anthraquinone glycosides content in the leaves of nine *Cassiae* i.e. *C. siamea, C. fistula, C. alata, C. surattensis* subsp. *surattensis, C. grandis, C. spectabilis, C. bakeriana, C. sophera* and *C. tora* collected in Summer, Winter and Rainy seasons from the Central areas of Thailand and found that the leaves of *C. alata* collected in Winter and Summer contain the highest amount (1.24% dry weight) of total anthraquinone glycosides. (Gritsanapan W, Phadungrakwitya R, Nualkeaw S, 2005).

We also found that most of *Cassia* leaf samples containing the maximum content of anthraquinone glycosides are the samples collected in Summer (March-June) and Winter (November-February) seasons. *C. alata* is one of the plants recommended to be used in primary health care in Thailand and has been listed in Thai traditional household drug list for laxative and antifungal drugs. At present, *C. alata* is included in the List of Herbal Medicinal Products A.D. 2006 of Thailand. According to the Standard of ASEAN Herbal Medicine4) and Thai Herbal Pharmacopoeia (THP),*C. alata* leaves

should contain not less than 0.5 and 1.0 % dry weight of total hydroxyanthracene derivatives calculated as rhein-8-glucoside, respectively. (Bruneton J. 1995)
The normal way of using *C. alata* for laxative is that 12 fresh or dried leaf lets are coarsely cut, boil with 2 glasses of water until 1 glass of decoction is obtained and strain into a glass. Take the whole decoction as a single dose when needed. Another way, macerate 1-2 teabags of 3 g of dried powdered leaves in a cup of boiling water for 2-5 minutes, and take the infusion at bed time (Sakulpanich A, Gritsanapan W. 2008).

Extraction method and extracting solvent are important for quantity and quality of the extracts. Appropiate extraction method for each plant should be investigated in order to promote the highest amount of active components. Thus, this study was conducted to find out the appropriate extraction method for *S. alata* leaves to promote the 80% ethanolic extract containing the maximum amount of total anthraquinones and to standardize the extracts of *C. alata* leaves collected from 10 different locations in the North, North-East, Central and South of Thailand (Supriona A, Gray DO. 1995).

In order to extract *anthraquinone glycosides* from the mixtures of *cassia alata* stems and leaves, we use ultrasound-assisted technique. Then, High Perfomance Liquid Chromatography(HPLC) will be use to analyzed the content. Extraction process is selected in this research because it is quite selective, effective, and able to separate *anthraquinone glycosides* from the mixtures of *cassia alata* stems and leaves. Actually, there are several technique to extract *anthraquinone glycosides* from the mixtures of *cassia alata* stems and leaves other than ultrasound-assisted technique which is conventional extraction method and microwave extraction method. Ultrasound-assisted extraction technique allows faster extraction, decreased uses of solvent and higher recovery. In term of yield,time and energy consumption it also more effective than other method.(Jaitak et.al, 2009). The process by which microwave energy is used to heat solvents in contact with solid samples and to partition compounds of interest from the sample into the solvent is known as ultrasound-assisted extraction method.(Hayat et.al, 2009).

Using this method can result in a yield increase in shorter time using less solvent at the same temperature. Choose a solvent in which their target analyte is soluble because more microwave energy can be absorb if use solvent with high dielectic constant. The

polarity of the solvent is very important in ultrasound-assisted technique.(Proestos and Komaitis, 2007). With the purpose to identify,quantify and purify the individual components of the mixture, High Perfomance Liquid Chromatography(HPLC) is used to seperate a mixtures of compound in analytical chemistry and biochemistry.

Toxicological studies have shown that stevioside does not have mutagenic, teratogenic or carcinogenic effects. Likewise, allergic reactions have not been observed when it is used as a sweetener.(Lemus-Mondaca et.al, 2011).The purpose of this study is to determine the *anthraquinone glycosides* extracted from the mixtures of *cassia alata* stems and leaves which can give advantages to country to produce a new product from agriculture herbs. Another purpose is to determine weather the mixtures of *cassia alata* stems and leaves can increase yield of *anthraquinone glycosides* (Wandee Gritsanapan and Peeranuch Mangmeesri) or not.

1.2 Problem Statement

In this research, ultrasound-assisted extraction technique was used to get the yields in the leaves of Cassia alata. Cassia alata L. is a coarse, erect, branched shrub, which is 1.5 to 3.0 m in height, leaves are pinnate with leaflets 16 to 28 cm, oblong, 5 to 15 cm in length, and broad but rounded at the apex (Quisumbing 1978). Its flowers are yellow, borne either at the ends of the stems or axils of the leaves. The seeds are small and enclosed in pods. The pod is straight, dark brown or nearly black. On both sides of the pods, there is a wing that runs the length of the pod. The pod contains 50 to 60 flattened triangular seeds.

Most studies on C. alata were focused on its antimicrobial, antifungal, analgesic, antimutagenic, mutagenic, and anti-inflammatory properties. Only a few studies were done on its antitumor and antiangiogenic potential. The extract of C. alata has been reported to possess some medicinal value, for example, the leaves have been reported to have a laxative effect and are also used against ringworm, scabies, ulcers and other skin diseases (Seaforth, 1962). Decoction of flowers, bark and wood are also reported to treat skin diseases such as pruritis, eczema, itching, bronchitis and asthma (Kirtikar and Basu, 1975).

In Malaysia, especially among the traditional medicine practitioners the *Cassia alata* leaf extract was also used to treat ringworm infection and in order to get a maximum effect, a little amount of lime was sometimes mixed with the extract. Besides that, a previous investigation revealed that water extract from C. alata leaves contained potential antifungal agent against Candidia albicans and antibacterial agent against Escherichia coli for the treatment of opportunistic infections in patients afflicted with Acquired Immunodeficiency Syndrome (AIDS). These results were comparable to commercial antifungal drug amphotericin B and antibiotic chlorampenicol (Crockett et al., 1992).

1.3 Objective

There are three objectives to conduct this research:

a) To separate *anthraquinone glycosides* from the mixture of *cassia alata* stems and leaves.

b) To find the kinetic of *anthraquinone glycosides* separation from the mixture of *cassia alata* stems and leaves using ultrasound extraction method.

c) To study the effects of various parameter on extraction yield and extraction kinetic.

1.4 Scope

1.4.1) Separation of *anthraquinone glycosides* from the mixture of *cassia alata* stems and leaves.

1.4.2) Observation the effect of some parameters (time,temperature and percentage of solvent in water) to the separation of *anthraquinone glycosides* from the mixture of *cassia alata* stems and leaves.

1.4.3) Analyzing extraction processes that using ultrasound extraction method.

1.4.4) Kinetic model of separation of *anthraquinone glycosides* from the mixture of *cassia alata* stems and leaves.

1.5 Rationale and Significance

In this experiment, *anthraquinone glycosides* (Wandee Gritsanapan and Peeranuch Mangmeesri) will be extracted from the mixture of *cassia alata* stems and leaves. The research of production of *anthraquinone glycosides* from the mixture of *cassia alata* stems and leaves have been made in order to use the whole plant of *cassia alata* instead of it leaves only. Before this, many researchers have been made to extract *anthraquinone glycosides* (Wandee Gritsanapan and Peeranuch Mangmeesri) from it leaves. Most studies on C. alata were focused on its antimicrobial, antifungal, analgesic, antimutagenic, mutagenic, and anti-inflammatory properties. Only a few studies were done on its antitumor and antiangiogenic potential.

2 LITERATURE REVIEW

2.1 Anthraquinone glycosides in the mixtures of cassia alata leaves and stems and the applications.

Traditional medicine is the oldest method of curing diseases and infections and various plants have been used in different parts of the world to treat human diseases and infections. According to the World Health Organization (WHO, 2002), traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world. In recent years, however, medicinal plants have represented a primary health source for the pharmaceutical industry (Ajose, 2007).

The leaf contains anthraquinones both aglycone and glycoside forms i.e. rhein, aloe-emodin, chrysophanol, glycosides of rhein, emodin, physcione and sennosides A, B, C, D7-10 while rhein is a major component investigated total anthraquinone glycosides (Wandee Gritsanapan and Peeranuch Mangmeesri) content in the leaves of nine *Cassiae* i.e. *C. siamea, C. fistula, C. alata, C. surattensis* subsp. *surattensis, C. grandis, C. spectabilis, C. bakeriana, C. sophera* and *C. tora* collected in Summer, Winter and Rainy seasons from the Central areas of Thailand and found that the leaves of *C. alata* collected in Winter and Summer contain the highest amount (1.24% dry weight) of total anthraquinone glycosides. (Gritsanapan W, Phadungrakwitya R, Nualkeaw S, 2005).

Fresh or dried leaflet of *C. alata* has been used as folk medicines in many countries for treatment of constipation, stomach pain, ringworm and skin diseases (Grosvenor PW, Gothard PK, Mc.William NC, Supriona A, Gray DO. 1995).

2.2 Ultrasound-assisted extraction technique.

Extraction is one of the methods used to obtain contain anthraquinones both aglycone and glycoside forms from the mixture of cassia alata stems and leaves. There are various extraction technique to extract *anthraquinone glycosides* (Wandee Gritsanapan and Peeranuch Mangmeesri) from the mixture of cassia alata stems and leaves such as

Conventional solvent extraction (CSE), microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE).

Ultrasound-assisted extraction (UAE) is one of the new extraction method to extract *anthraquinone glycosides* from the mixture of cassia alata stems and leaves. This method has been developed to reduce the extraction time and improve the extraction yield (Goula, 2012). UAE has lower energy consumption, lower consumption of solvents, higher extraction efficiency and higher level of automation and is preferable to CSE (Ying et.al, 2011). Increasing extraction yield, reducing solvent usage, economizing power consumption and shortening extraction time also the advantages of UAE (Zou et.al, 2009).

Ultrasound-assisted extraction method also used to extract many other materials not only can be applied to extract *anthraquinone glycosides* from *cassia alata* leaves, stems or flowers. For example, this method also used to extract *anthocyanins* from grape skins. The extraction of *anthocyanins* from grape skins using ultrasound extraction method was carried out under different extraction conditions. (Liazid et.al, 2010).

2.3 Other method to extract anthraquinone glycosides from cassia alata leaves.

Up to now, several extraction techniques have been reported for the extraction of *anthraquinone glycosides* (Wandee Gritsanapan and Peeranuch Mangmeesri) from *cassia alata* plants other than ultrasound-assisted extraction technique like conventional extraction technique and microwave-assisted extraction technique. Among all method microwave-assisted extraction method afforded highest yield of *anthraquinone glycosides* but Microwave energy in MAE is a non-ionising radiation. The radiation causes motion of molecule and rotation of dipoles to heat solvents to promote targeted compounds to move from the sample matrix into the solvent.

However, the radiation does not induce changes in molecular structure (Ying et.al, 2011). Solvent extraction method also can be used to extract *anthraquinone glycosides* from *cassia alata* leaves. The powdered sample will be extracted in ethanol using

Erlenmeyer flasks in a shaking hot-water bath for 30min at 70^0C. The extractor volume was 100mL, thus it was filled with about 30g of ground *cassia alata* leaves. The independent variables were temperature, pressure and co-solvent ratio. (Erkucuk et.al, 2009).

2.4 Kinetic model

The solid–liquid extraction process can be considered as the reverse of an adsorption operation, therefore the bases of the adsorption kinetic equations can be applied to solid–liquid extraction and the second-order law was found to give the best fits for the extraction rate. The general second- order model can be written as (Goula, 2012).

$$\frac{dCt}{dt} = k.(Ce - Ct)$$

where k is the second-order extraction rate constant (L/g min), Ce is the equilibrium concentration of anthraquinone glycosides in the liquid extract (g/L) (extraction capacity), and Ct is the cassia alata concentration (g/L) in the liquid extract at a given extraction time t. The integrated rate law for a second-order extraction under the boundary conditions t = 0 to t and Ct = 0 to Ct, can be written as an Eq. (1) or a linearized Eq. (2) (Goula, 2012).

$$Ct = \frac{k.t.Ce^2}{1+k.t.Ce} \qquad (1)$$

Or

$$\frac{t}{Ct} = \frac{1}{kCe^2} + \frac{t}{Ce} = \frac{1}{h} + \frac{1}{Ce} \qquad (2)$$

where h is the initial extraction rate (g/L min) when t approaches 0:

$$h = k.Ce^2$$

3 METHODOLOGY

3.1 Overall Flow Chart Of Research Methodology

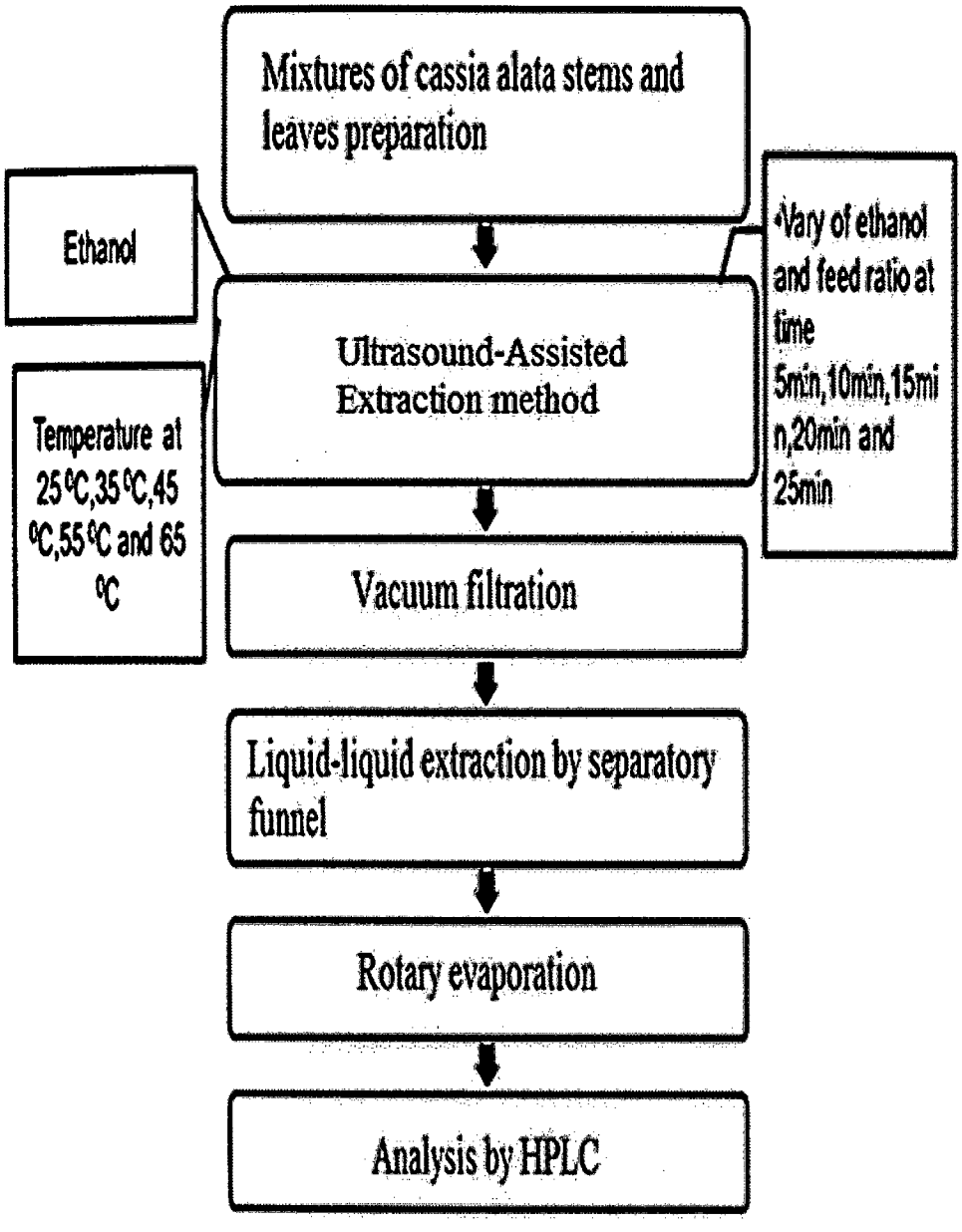


Figure 3.1: Overall Flow Chart for Extraction of Cassia Alata

3.2 *Materials*

i. The mixtures of *cassia alata* stems and leaves.

ii. Water

iii. Ethanol

iv. Acetonitrile

3.3 *Apparatus*

i. Beaker

ii. Measuring cylinder

iii. Rotary Evaporator

iv. Ultrasound

v. High Performance Liquid Chromatography (HPLC)

vi. Mortar

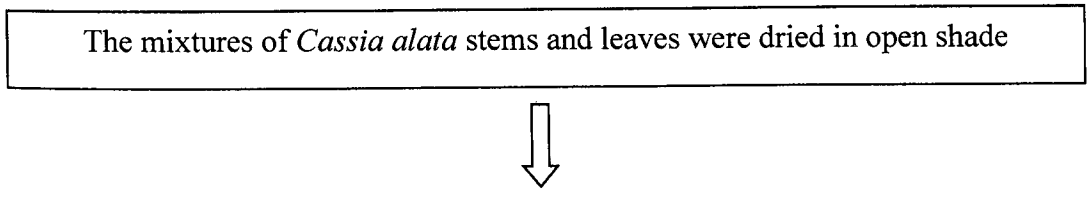
vii. Vacuum filter

viii. Separating funnel

3.4 *Experimental Procedures*

3.4.1 *The Mixture of Cassia Alata Stems and Leaves*

The mixtures of *Cassia alata* stems and leaves were dried in open shade. After that they will be milled to 60 mesh powder using a mortar and then store at ambient temperature.



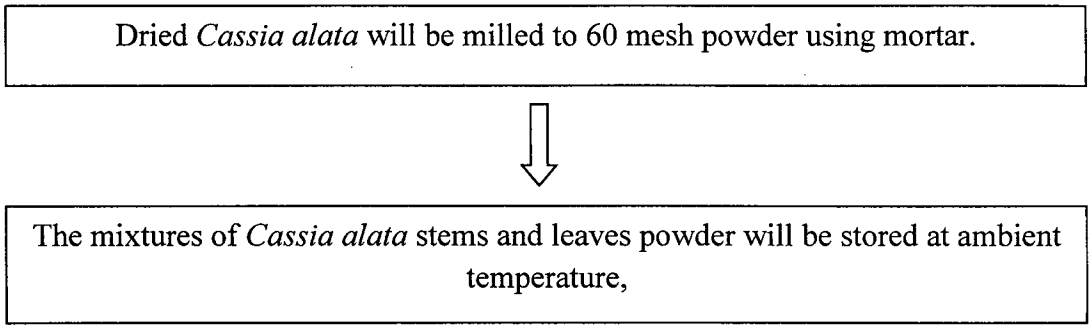
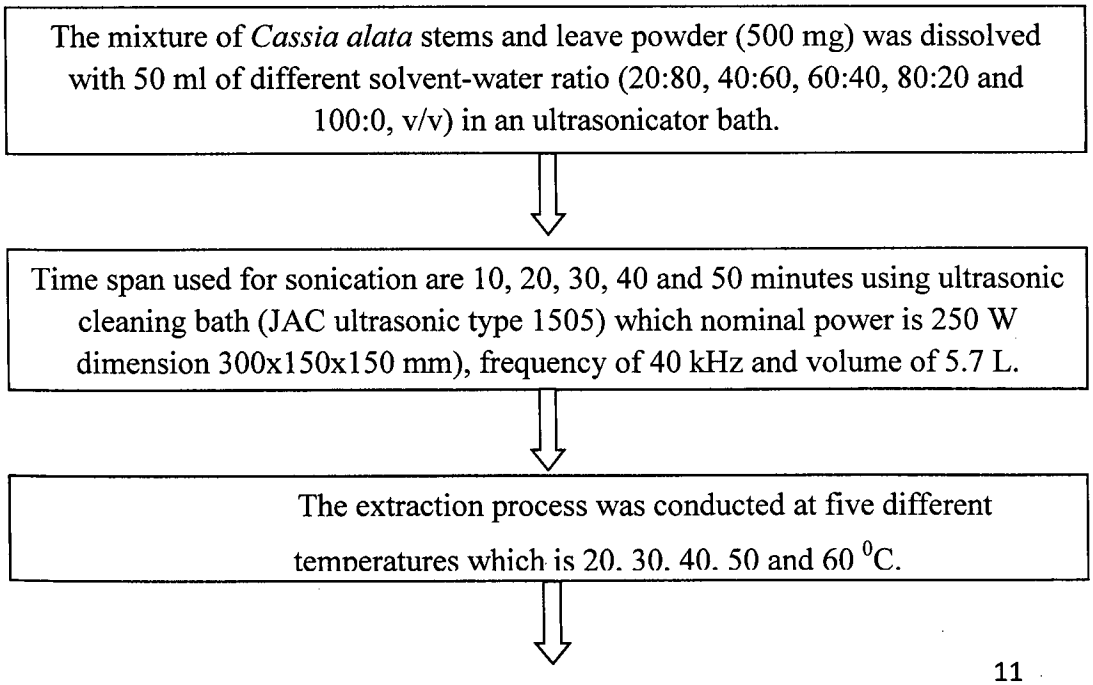


Figure 3.2: Flow diagram for the preparation of the mixtures of *Cassia alata* stems and leaves

3.4.2 Ultrasound-Assisted Extraction

The mixture of *Cassia alata* stems and leave powder (500 mg) was dissolved with 50 ml of different solvent-water ratio (20:80, 40:60, 60:40, 80:20 and 100:0, v/v) in an ultrasonicator bath. Time span used for sonication are 10, 20, 30, 40 and 50 minutes using ultrasonic cleaning bath (JAC ultrasonic type 1505) which nominal power is 250 W dimension 300x150x150 mm), frequency of 40 kHz and volume of 5.7 L. The extraction process was conducted at five different temperatures which is 20, 30, 40, 50 and 60 ^{0}C. Then the sample will be allowed to cool at room temperature.



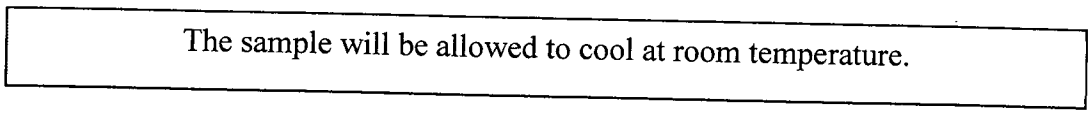


Figure 3.3: Flow diagram for ultrasound-assisted extraction technique.

3.4.3 Vacuum Filter

Firstly, dry the filter disk in the oven at 103°C to 105°C for 1 hour, then cool in desiccators. After that Pipette 50 mL of water sample onto center of filter disk in a Buchner flask, using gentle suction (under vacuum). Next step is assemble filtering apparatus and filter then begin suction. Wet the filter with a small volume of distilled water to seat it. Lastly, wash filter with three successive 10 mL volumes of distilled water, allowing complete drainage between washings, and continue suction for about 3 min after filtration is complete.

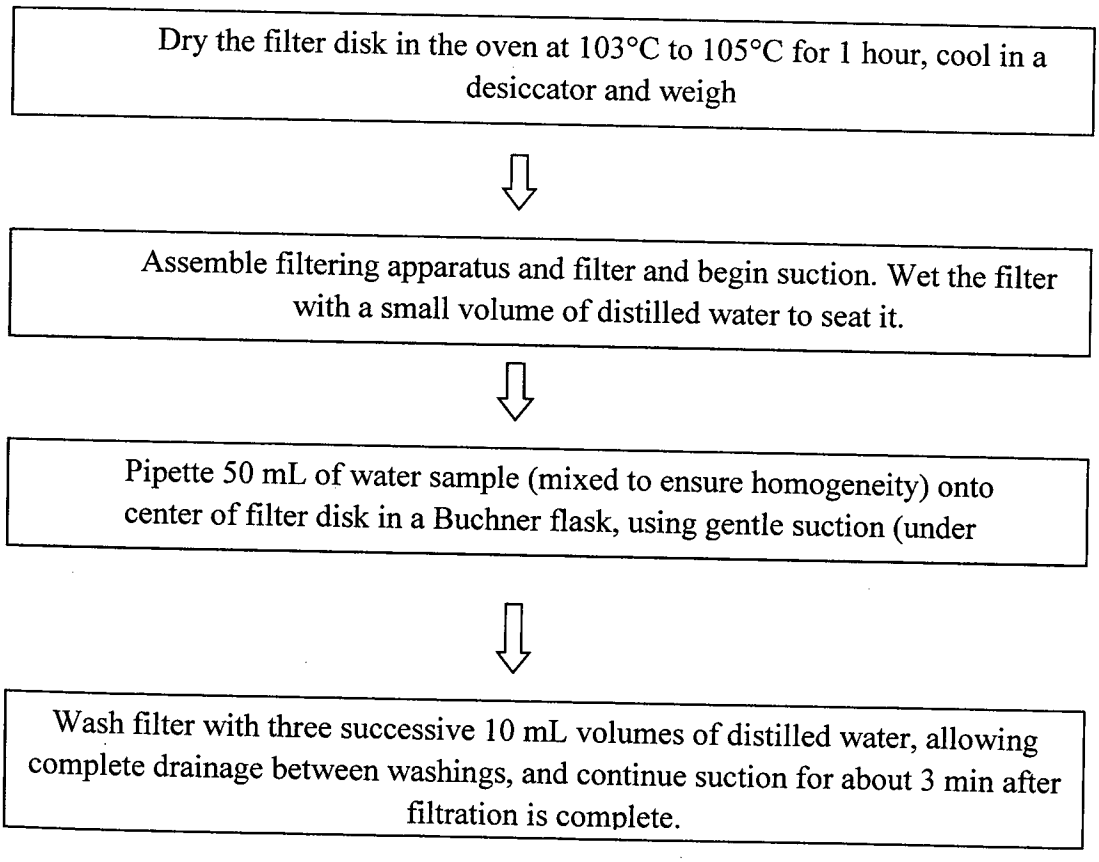


Figure 3.4: Flow diagram for vacuum filter process.

3.4.4 Rotary Evaporator

First step, perform general start up procedures. Then start the timer once liquid starts to enter receiving vessel B2. After that Every 5 min record distillate temperature and measure the volume of collected product from receiving vessel B2. Next, Combined all the collected distillate in a container and at the end of the experiment, measure its overall volume and composition. Lastly, wait for the concentrate in rotary sphere B1 to cool down before collection. Determine the volume and composition of the concentrate.

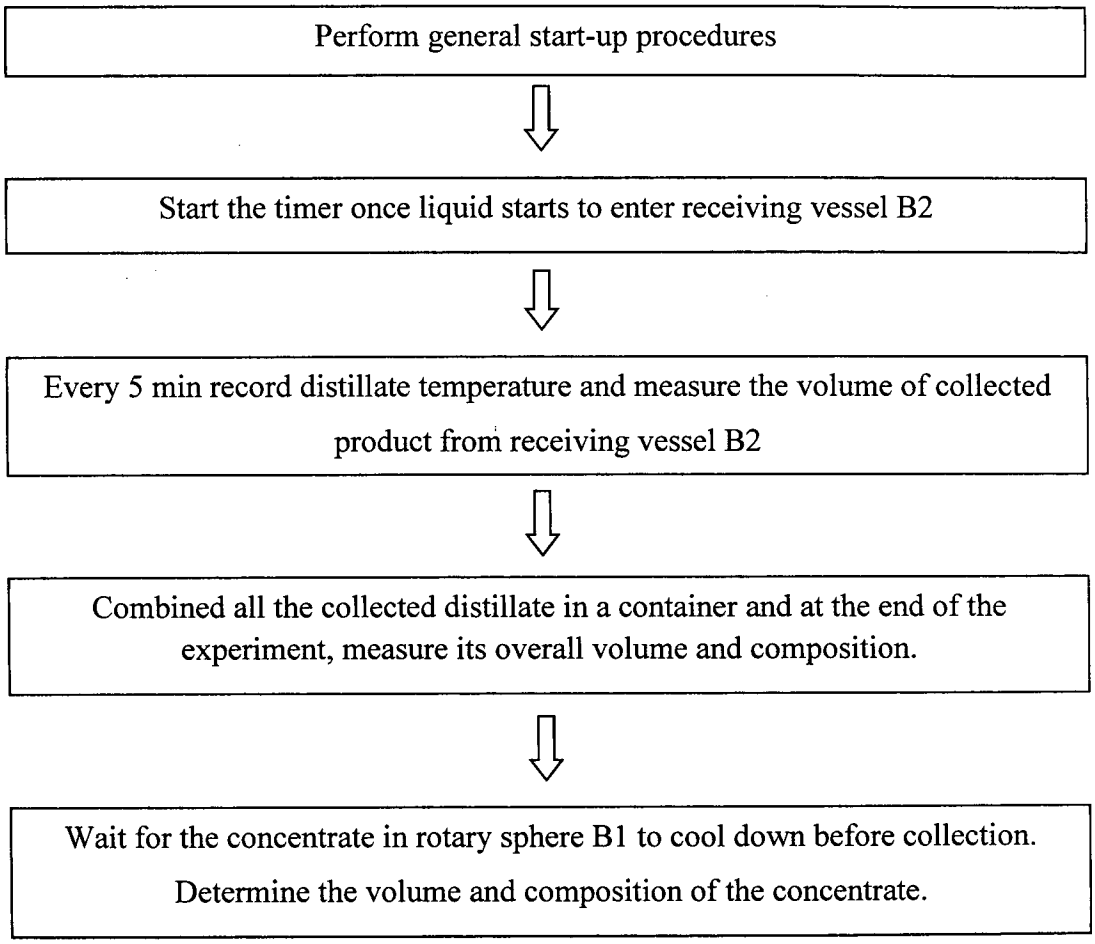


Figure 3.5: Flow diagram for rotary evaporator process

3.4.5 Analysis Using High Performance Chromatography (HPLC).

Samples will be injected into the Eurospher 100-5 NH_2 column and evaluate at a flowrate of 1 mL/min using a solvent system of acetonitrile:water (80:20, v/v). The column temperature was 35^0C throughout the experiment. Then, the result will be detected.

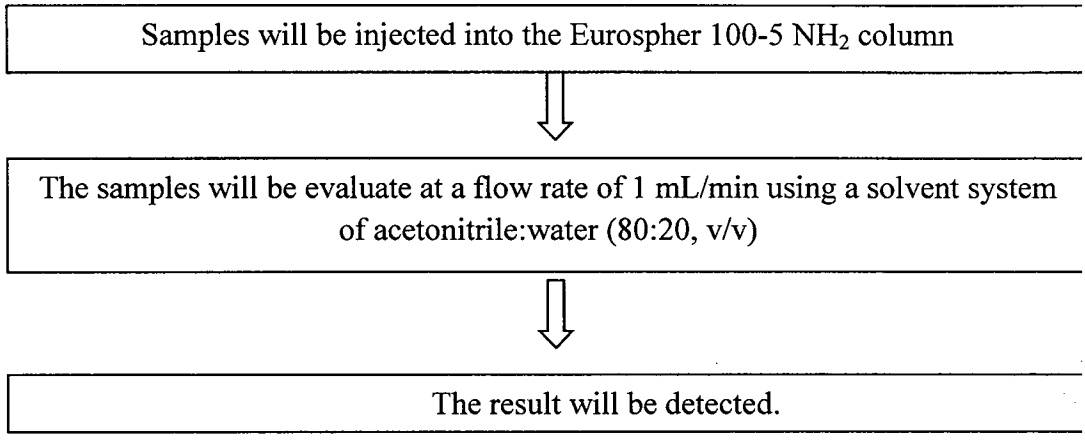


Figure 3.6: Flow diagram of the analysis using High Performance Chromatography (HPLC)

3.4.6 Analyzing Sample.

3.4.6.1 Preparation of Stock Solution

2.5mg of the standard solution were prepared by diluting with 80 % ethanol in water into 10 ml volumetric flask respectively in prior to sonicate them for 15 minutes.

3.4.6.2 Preparation of Standard Solution

Standard solutions are prepared before running the High Performance Liquid Chromatography (HPLC). Solutions are prepared by diluting 1mg/mL of stock solution

into a 10ml volumetric flask for concentration ranging from 0.001 to 0.1 mg/ml by using the equation.

$$M_1V_1 = M_2V_2$$