DEVELOPMENT AND EVALUATION OF *CENTELLA ASIATICA* POWDER BY FREEZE DRYING: COMPARISON OF EXTRACTION METHODS ON YIELD AND HYGROSCOPICITY

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ABSTRAK

Teknik-teknik novel dan pembangunan kaedah terbaharu telah membuka peluang untuk menghasilkan ekstrak sebatian bioaktif semulajadi yang sangat tinggi di samping potensinya untuk merawat dan mencegah pelbagai penyakit. Kajian ini membandingkan peratusan dapatan serbuk *Centella asiatica* yang diperoleh melalui proses pengeringan beku yang diekstrak terlebih dahulu secara akueus melalui kaedah ekstrak konvensional (rebusan dan rendaman) dan kaedah bukan konvensional seperti pengekstrakan bantuan ultrabunyi. Kestabilan fizikal serbuk kemudiannya diuji melalui ujian higroskopi. Ujian ini menggunakan teknik analisis serapan gravimetrik dengan sedikit modifikasi. Setiap sampel serbuk melalui pra-rawatan pada 45 °C, 10% RH diikuti dengan proses ekuilibrasi pada 25 °C, 80% RH dan seterusnya dikategorikan mengikut European Pharmacopeia. Pengekstrakan bantuan ultrabunyi menghasilkan dapatan serbuk terbanyak (11.65%) diikuti oleh rendaman (10.48%) dan rebusan (10.03%). Setelah 24 jam dianalisa, ketiga-tiga sampel diklasifikasikan sebagai sangat higroskopi dengan peratusan serapan air melebihi 15%. Peratusan serapan air tertinggi adalah melalui rebusan (71.31%), diikuti oleh rendaman (71.28%) dan pengekstrakan bantuan ultrabunyi (68.71%).

ABSTRACT

Novel techniques and the development of new methods has provided an opportunity to obtain highly purified natural bioactive compound extracts with potential for the treatment and prevention of various diseases. This research compares the percentage yield of *Centella asiatica* obtained through freeze drying that was aqueous-extracted earlier by conventional extraction method (decoction and infusion) and an advanced non-conventional extraction method; ultrasound-assisted extraction (UAE).The physical stability of the powder obtained was then tested by hygroscopicity test. The studies were conducted using gravimetric sorption analysis method with slight modification. Each of the powder samples was subjected to the pretreatment at 45 °C, 10% RH followed by equilibrating at 25 °C, 80% RH and individually categorized as per European Pharmacopeia. UAE generated the highest powder yield (11.65%) followed by infusion (10.48%) and decoction (10.03%). After 24 hours of hygroscopicity analysis, all three samples were found to be very hygroscopic since their respective percentage of water sorption was more than 15%. The highest percentage of water sorption was by decoction (71.31%), followed by infusion (71.28%) and lastly UAE (68.71%).

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

cm	Centimetre
°C	Degree Celsius
G	Gram
hr	Hour
kcal	Kilo Calories
kg	Kilogram
kHz	Kilo Hertz
MHz	Mega Hertz
m/*m*	Mass per Mass
µg	Microgram
µm	Micrometre
mg	Milligram
ml	Millilitre
mm	Millimetre
mm Hg	Millimetre Mercury
mTorr	MilliTorr
mol	Mole
%	Percentage
w/v	Weight per Volume
w/w	Weight per Weight

LIST OF ABBREVIATIONS

AE	Ascorbic acid Equivalent
T_c	Collapse Temperature
E Ex	Ethanol Extract
Ph. Eur.	European Pharmacopeia
T_e	Eutectic Temperature
ES	Extractable Solids
GAE	Gallic Acid Equivalent
GAG	Glycosaminoglycan
M_{GSA}	Mass Gravimetric Sorption Analysis
M_{PT}	Mass Pre-treatment
PE	Pyrogallol Equivalent
QE	Quercetin Equivalent
RH	Relative Humidity
TE	Tannic acid Equivalent
TAC	Total Antioxidant Capacities
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TE	Trolox Equivalent
UAE	Ultrasound-Assisted Extraction
VH	Very Hygroscopic
H_2O Ex	Water Extract
XOI	Xanthine Oxidase Inhibitory

CHAPTER 1

INTRODUCTION

1.1 Background Study

Centella asiatica as shown in Figure 1.1, commonly known as centella, Asiatic pennywort or Gotu kola, is a herbaceous, frost-tender perennial plant belonging to the family Apiaceae. Easily grow and found in the most tropical and subtropical countries, *C. asiatica* is used for medicinal purposes (Singh and Singh, 2002). *C. asiatica* or Gotu kola should not be confused with kola nut as it does not contain any caffeine and has not been shown to have stimulant properties (Niamnuy et al., 2013). There are several important bioactive compounds present in *C. asiatica* such as triterpene saponins, phenolic compounds, vitamins, minerals, free amino acids, and polyacetylenic compounds (Niamnuy et al., 2013). All of these compounds are the major ingredients that are responsible for *C. asiatica* therapeutic effects.

Figure 1.1 *Centella Asiatica* (Source: Sudhakaran, 2017)

In the nineteenth century, *C. asiatica* and its extracts were incorporated into the Indian pharmacopoeia, wherein in addition to wound healing, it was recommended for various skin conditions treatment (Gohil, Patel and Gajjar, 2010; Gohil, Patel and Gajjar, 2012). *C. asiatica* extracts contain 70% triterpenic acids mainly two triterpene glycosides (asiaticoside and madecassoside) and corresponding aglycones (asiatic acid and madecassic acid) which exerts normalizing action on metabolism of connective tissues. It also enhances tissue integrity by stimulating glycosaminoglycan (GAG) synthesis without promoting excessive collagen synthesis or cell growth (Pizzorno, Murray and Joiner-Bey, 2016; Govarthanan et al., 2015). However, it is inconvenience for some users to consume it as it is. Thus, the *C. asiatica* plant is extracted before it is powdered and encapsulated to enhance its bioavailability, stability and shelf life (Rivas et al., 2017).

Extraction of botanicals and herbal sample is the crucial first step prior to analysis of bioactive compounds present in the preparations (Sasidharan et al., 2011). Various solvents can be used to extract the *C. asiatica* which includes methanol (Govarthanan et al., 2015), ethanol (Dewi and Maryani, 2015) and distilled water (Cheng et al., 2004). Different solvents will give different quantity of yield obtained. Due to unmatched availability in chemical diversity, pure compounds or standardized plant extracts provides unlimited opportunities for new drug discoveries (Cos et al., 2006). The extraction basic steps are pre-washing the sample, drying or freeze drying the plant material, grinding to obtain a homogenous sample and enhancing the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system. During the extract preparation, proper actions must be taken to assure that there is no loss, distortion or denaturation of potential active constituents of the plant samples (Sasidharan et al., 2011).

Drying is a common method of preservation, applied by removing the water from the plant. Convection drying and freeze drying are the two most popular drying methods. Conventional drying such as oven drying is a user-friendly and very low cost method compared to freeze drying, which is a lengthy procedure and requires higher cost (Vashisth, Singh and Pegg, 2011). Previous research showed that there are major quality (nutrient compound and colour) differences of the final product after conventional drying compared to freeze dried products (Wojdylo et al., 2014).

Freeze drying is conducted to remove the solvent in such a way that the sensitive molecular structure of the active substance in the plant is least disturbed (Alexeenko, Ganguly and Nail, 2009). It is more suitable for products sensitive to temperature or to high residual moisture content (Touzet et al., 2018).

1.2 Problem Statement

The raw, unprocessed *C. asiatica* plant have a shorter shelf life and physically unstable. The leaves are easily wilted upon exposure to sunlight and its bioactive components degraded due to oxidation which leads to physical changes of the plant. *C. asiatica* fresh plant is very likely susceptible towards microbial growth due to high moisture content especially in leaves and stems (Brinkhaus et al., 2000). Besides that, it may be inconvenient for patient to consume it raw since some of them may not like to eat vegetables or herbs. This study is conducted to overcome this problem by converting the *C. asiatica* into powders. A study shows that *C. asiatica* lost a high amount of phenolic compounds (total phenolic content (TPC) and total flavonoid content (TFC) when extracted with ethanol of concentration 80% and above (Chew et al., 2011). In addition, the safety consumption of oral drug with active ingredients extracted using alcohol solvents is probably the main issue to deal with (Man and Choo, 2018). In other aspect, conventional extraction methods (maceration, decoction, infusion etc.) are the most convenient and inexpensive as it does not require any advanced equipment (Easmin et al., 2014). However, commercial extraction methods such as microwave-assisted extraction, ultrasound-assisted extraction and super critical fluid extraction have been proposed to extract bioactive compounds from plants with higher recovery capacity (Caldas et al., 2018). Hence, the solvent as well as the extraction methods used must be optimized to obtain the highest yield of *C. asiatica* powder that is physically more stable.

1.3 Research Objective

The main research objective of this project is to develop the freeze-dried *C. asiatica* powder. Meanwhile, the individual research objectives would be:

1. To study the extraction method which generates the highest yield of powder.

2. To quantitatively measure the physical stability of *C.asiatica* powder obtained from different extraction methods by hygroscopicity test.

1.4 Scope of Study

In this study, *C. asiatica* was converted into powder by freeze drying method. Prior to the freeze drying process, the *C. asiatica* dried plant was extracted by using conventional extraction methods (decoction and infusion) as well as commercial extraction method (ultrasonic-assisted extraction). The purified extracted *C. asiatica* was obtained by filtration method. The freeze drying was selected as it least disturbs or denatures the sensitive molecular structure of the active substance in the plant (Alexeenko, Ganguly and Nail, 2009). The freeze drying process comprises mainly three stages which were the freezing, primary drying and secondary drying. *C. asiatica* powder obtained from different extraction methods was weighed to determine the yield. Physical stability of *C. asiatica* powder was analyzed through hygroscopicity test to determine which powder sample would absorb the lowest moisture and remain its properties.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction to *Centella Asiatica*

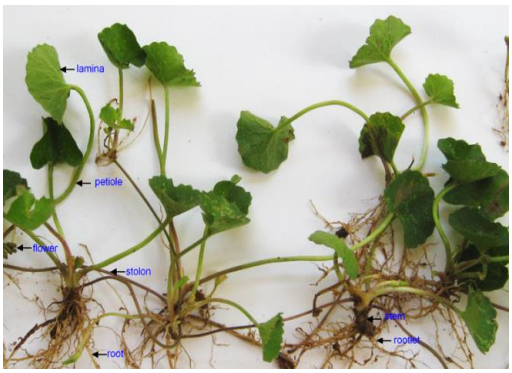
C. asiatica (Linn.) Urban is a prostrate, faintly aromatic, stoloniferous, perennial, creeping runner belongs to the family Apiaceae, is a tropical medicine native to Southeast Asian countries.The genus *Centella*, comprised about 40 species (Sudhakaran, 2017) commonly grow in most parts of the tropical or wet pantropical areas such as rice paddies, and also in rocky, higher elevations (Gohil, Patel and Gajjar, 2010). The generic name *Centella* is derived from the Greek. "Centum" means hundred which referring to profusely branched prostrate runner and specific epithet asiatica alludes to its native of the continent of Asia. This plant was originally described and published under the name *Hydrocotyle asiatica* by Carl Linnacus until it was reclassified as in the valid botanical systematics of *Centella asiatica* Urban. Its common names are Indian pennywort, and also water pennywort or marsh pennywort because of the preferred natural habitat. Since the plant is of pan-tropical distribution, it is known by an array of vernacular names in different regions of the world as; Fo-titieng (in Chinese); indischer Wassernabel (in German); tsubo-kusa (in Japanese); hydrocotyle asiatique (in French); idro cotile (in Italian); hierba de clavo (in Spanish) and gotukola in English ('gotukola' is originated from Sinhala language of Sri Lanka), pegaga (in Malaysia), daunkaki kuda (in Indonesia) and Brahmamanduki or Mandukaparni in Sanskrit. The species is native to India, China, Pakistan, Sri Lanka, Indonesia, Malaysia, and South Africa and found throughout the warmer, marshy to wet sandy areas on floodplains, and near streams, rivers or other watercourses (Sudhakaran, 2017). Locally known as 'Pegaga' (Tan, 2010), there are three Pegaga species identified by

Khatib et al. (2012) which are *Centella Asiatica*, *Hydrocotyle Bonariensis*, and *Hydrocotyle Sibthorpioides.*

2.1.1 Botanical Aspects and Macroscopic of *C. asiatica*, *H. bonariensis*, and *H. sibthorpioides*

According to Sudhakaran (2017), *C. asiatica* plant is herbaceous, slender, prostrate, creeping runner and attains a height up to 12-15 cm as shown in Figure 2.1 (a) and (b). A cluster of ascending petiolate leaves arising at each node of the stem and portrays of having long intermodal length as illustrated in Figure 2.1(c). Leaves are simple; lamina is rounded to reniform at the tip, petiole elongated, slightly pubescent on both surfaces. Stem with sheathing leaf bases consisted of 1-3 leaves at each node. The leaves are thin and soft, with palmate nerves, hairless or with only a few hairs, and measure about 2 to 5 cm in diameter (Brinkhaus et al., 2000). Lamina is broadly cordate at the base, coarsely toothed, crenate margins. Petiole is dorsi-ventrally differentiated and ventral side appeared furrowed. Stem is reddish, glabrous as shown in Figure 2.1(d) with long slender, horizontal stolons. Inflorescences: fasicled umbel, each umbel consisted of three purple or pink flowers as presented in Figure 2.1(e) and (f), borne on long peduncle (1 to 1.5 cm). Usually set 2-3 flowers side by side on short pedicel as in Figure 2.1(g). The petals are five, pink in color and often tinged with rose. Fruit: cremocarp, having the dimension of 3 to 4 mm long and 3 to 5 mm broad (broader than long), ellipsoid in shape with thickened pericarp. Flowering occurs in the month of April-June (Sudhakaran, 2017). Figure 2.1(a-g) presents *C. asiatica* plant as described.

(a)



(b)

(c)

(d)

(e)

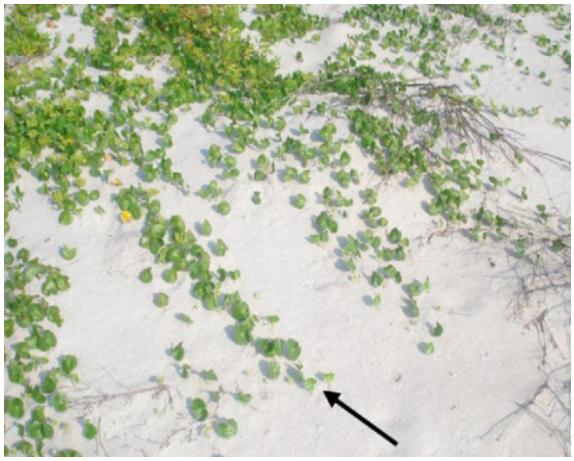
(f)

Figure 2.1 (a) *C. asiatica,* (b) Parts of *C.asiatica* , (c) *C. asiatica*: Leaf (stereo microscopic view x 1), (d) *C. asiatica*: Stem (Stereo microscopic view x 1), (e) *C. asiatica*: Inflorescence (stereo microscopic view x 1), (f) *C. asiatica*: Inflorescence (stereo microscopic view x 1) and (g) *C. asiatica*: Inflorescence (stereo microscopic view x 2). (Source: Sudhakaran, 2017)

(g)

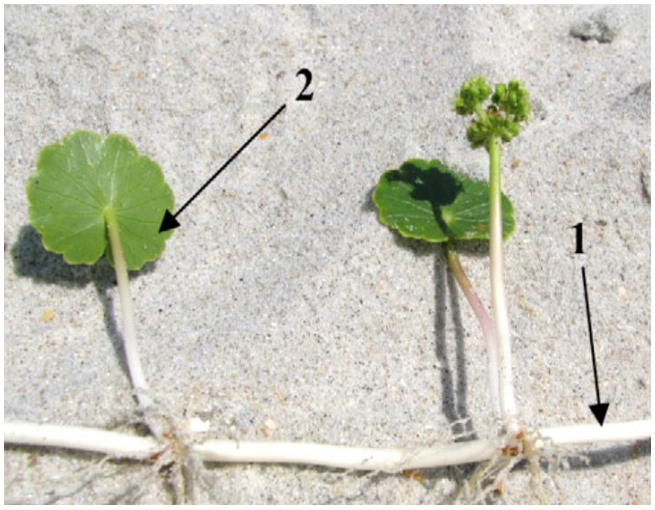
Figure 2.1 Continued

H. bonariensis (Apiaceae) Comm ex. Lam (large-leaf pennywort) as described by Joesting, Sprague and Smith (2012), is a clonal, perennial C3 herb indigenous to coastal sand dunes and moist, open sandy areas, from a northern limit of Virginia, USA to a southern limit of Chile. New leaves (ramets) are produced throughout the growing season, with leaf life spans ranging from two to eight weeks depending on location. Each ramet is composed of a single, circular broad leaf (~5–30 cm^2 area) supported by a stem with an independent, nodal root system. Inflorescences develop opposite of the leaf during new ramet production, and flower and seed production occurs continuously throughout the growing season. According to PlantNET (1992) and USDA-NRCS (n.d), lamina mostly 0.03–0.12 cm diameter, shallowly lobed, margins or lobes crenate. Its lamina is fleshy and glabrous. The petioles are mostly up to 15 cm long. The stipules are scarious and 2–4 mm long. Clones (genets) grow by producing alternating single-leaf ramets on branching rhizomes (lateral runners), and individual clones have been shown to consist of over 1500 interconnected ramets and cover over 100 m^2. (Joesting, Sprague and Smith, 2012). Figure 2.2 (a-c) presents *H. bonariensis* as decribed.



(a)

(b)



(c)

Figure 2.2 (a) *H. bonariensis* clone, showing individual ramets and lateral runner growth pattern. Youngest ramets are produced at the tip (indicated by arrow), (b) Individual *H. bonariensis* ramets showing leaves of typical orientations (leaf angles and azimuths) and (c) Single *H. bonariensis* runner (1) with individual ramet (2) consisting of a single broad leaf and individual root system. (Source: Joesting, Sprague and Smith, 2012).

H. sibthorpioides is native to eastern Asia in wet valleys, grassy areas, and stream banks. In North America it is reported from mostly isolated counties from Louisiana and Arkansas northeast to New Jersey and disjunct in California. It has been found in greenhouses, nurseries, lawns, sidewalks, and shorelines of brackish and fresh water (Atha, 2017). According to Lucidcentral (2018), the leaves are simple; round or reniform with small leafy outgrowth at the base as shown in Figure 2.3 (a) and (b). The leaf blade could range from 0.5-1.5 cm. Lamina is entire, crenate or lobed. Leaf edges are scalloped. The leaves are broad, alternate and palmate. In addition, it is often described as egg shaped. All of the leaves are hairless and the shoots grow above the ground. Its flowers could range around 1-1.5 mm with rounded cross section. It can be green or purple. Figure 2.3 (a-e) presents *H. sibthorpioides* as described.

(a) (b)

Figure 2.3 (a) *H. sibthorpioides*: Leaf shape, (b) Single *H. sibthorpioides* runner consisting of a single broad leaf, (c) Single *H. sibthorpioides* runner with individual root system, (d) *H. sibthorpioides*: Inflorescence (stereo microscopic view x 1) and (e) *H. sibthorpioides*: Inflorescence (stereo microscopic view x 2). (Source: Lucidcentral, 2018)

(c)

(d)

(e)

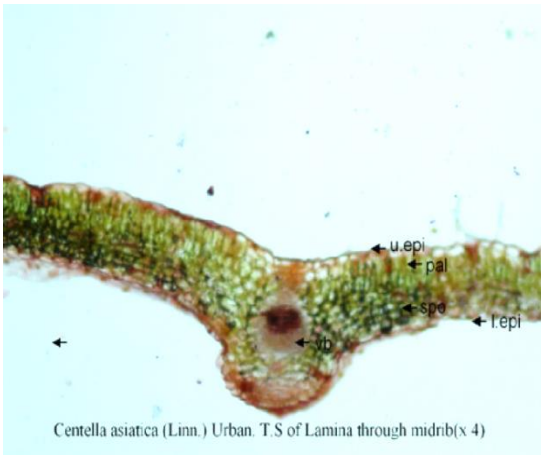
Figure 2.3 Continued

2.1.2 Microscopic Evaluation of *C. asiatica* Leaves

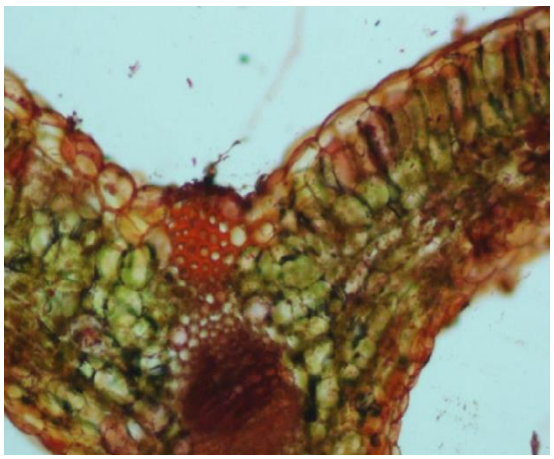
According to Sudhakaran (2017), *C. asiatica* leaves had an average length of about 3.5 cm and width of 1.5 cm as illustrated in Figure 2.1(c). Leaves have no characteristic taste and odour. Lamina was dorsiventrally differentiated and made of epidemics, mesophyll and vascular tissues as in Figure 2.4(a). Both epidermises were uniseriate, composed of compactly arranged rectangular cells with moderately striated outer walls.

Cuticle appeared either completely absent or poorly developed as shown in Figure 2.4(a) and (b). Non-cuticular striated epidermis which may facilitate the steady absorption of water from the surrounding. Some of the upper and lower epidermal cells located at the midrib portion were provided with uniseriate, trichomes. The presence of trichome in leaves may be considered as a land habitat; in general trichomes can play a role in the regulation of plant temperature, light reflection, defense against herbivores, and reduction of water loss. Beneath the abaxial epidermis contained a patch or band of sclerenchymatous tissues as illustrated in Figure 2.4(b) made of 4-5 layers, which may provide tensile strength to adaxial side of the mid vein.

Lamina was flat and much reduced in dimension compared to the midrib. The mid rib seemed to have a slight depression on the adaxial side with broadly semicircular on the abaxial side as in Figure 2.4(a). Midrib was composed of epidemics, collenchyma, mesophyll and vascular bundle. Lamina was dorsi-ventrally differentiated, with adaxial mesophyll having a compact palisade parenchyma with one layer of elongated, and barrel shaped cells. Mesophyll contained crystal idioblasts, both towards the adaxial and abaxial tissues of the midrib as in Figure 2.4(c) and (d). Cells of the mesophyll were found filled with plenty of chloroplasts as shown in Figure 2.4(c). The size of the individual palisade ranged from 158 to 663 μm in length with a mean length of about 367 μm. The spongy parenchyma composed of 3–4 layers of oval to rectangular cells, loosely arranged with wide intercellular spaces. They form about ¾ of total area of the midvein. A parenchymatous bundle sheath was encircled the vascular strand. Lamina also possessed vasular traces, but not differentiated into distinct metaxylem and protoxylem. The palisade ratio was found to be about 2. Figure 2.4 (a-d) presents microscopic evaluation of *C. asiatica* leaves as described.



(a)



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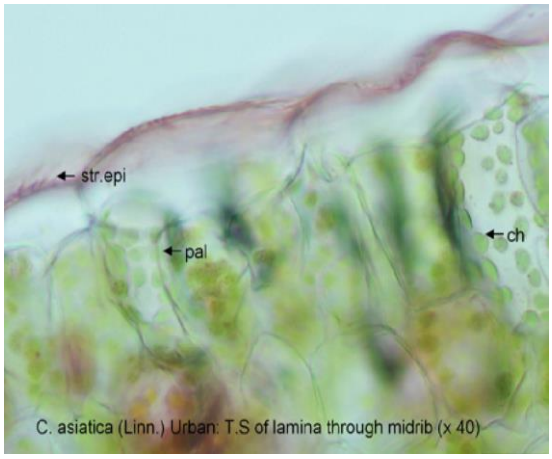

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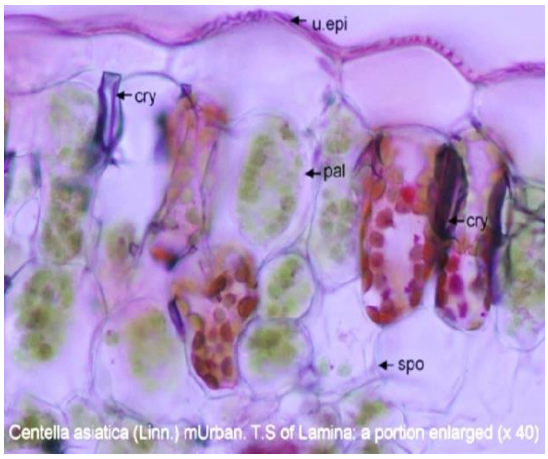


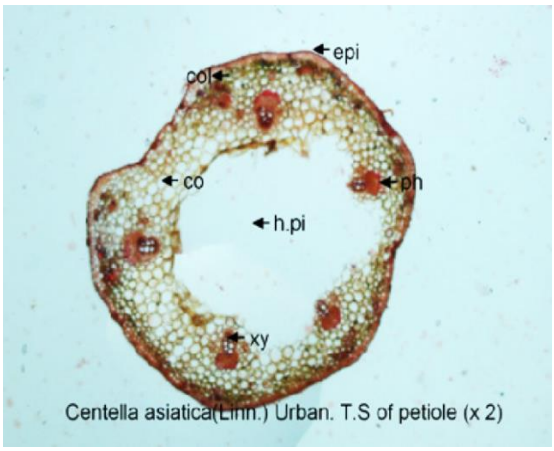
(d)

Figure 2.4 (a) *C. asiatica*: T.S. of lamina through midrib (x 4), (b) *C. asiatica*: T.S. of lamina through midrib (x 10), (c) *C. asiatica*: A portion of midrib is enlarged (x 40) and (d) *C. asiatica*: T.S. of lamina- a portion enlarged (x 40). (Source: Sudhakaran, 2017)

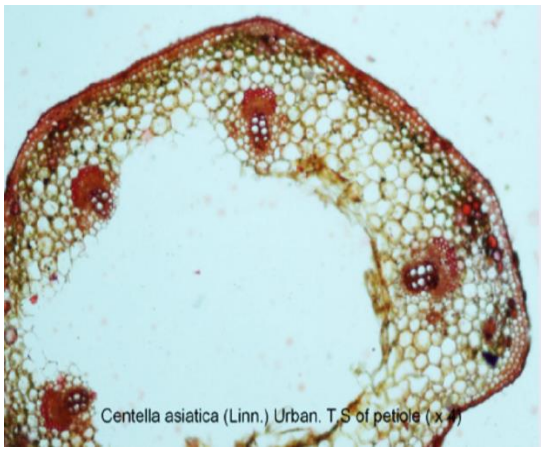
2.1.3 Microscopic Evaluation of *C. asiatica* Petiole

The petiole had dorsiventral differentiation, with a prominent groove or furrow on its ventral side as illustrated in Figure 2.1(c). Dorsal side of the petiole comprised of five sides (pentagonal shape), with a deeply notched groove on the ventral side as presented in Figure 2.5(a). A chlorenchyma zone consisting of 4-6 layers of cells was located beneath the epidermis, followed by a broad parenchymatous zone. The five free vascular bundles were aligned in the middle of the ground tissue; bundles appeared directed towards the five corners of the pentagon as shown in Figure 2.5(b). The vascular tissues form a continuous cylinder, with a band of periphloematic sclereids as in Figure 2.5(c) arches over each vascular cylinder. Vascular strands were separated from one another by wide areas of ground tissues. Xylem vessels were aligned in radial rows of two, with phloem on the adaxial side as in Figure2.5(c) (Sudhakaran, 2017).

The vascular cylinder appeared encircled by a parenchymatous bundle sheath, enclosing both the xylem and phloem. Crystal idioblasts as illustrated in Figure 2.5(c) were found embedded between the intervening walls of cortical parenchyma. The cross section of petiole had a prominent central hollow core of air canal or lacuna. The morphogenesis of arenchyma lacunae, may occur either by the process of the programmed cell death (Lysogeny) or schizogenic separations of the adhering cell of the original ground tissues (Sudhakaran, 2017). Figure 2.5 (a-c) presents microscopic evaluation of *C. asiatica* petiole as described.



(a)



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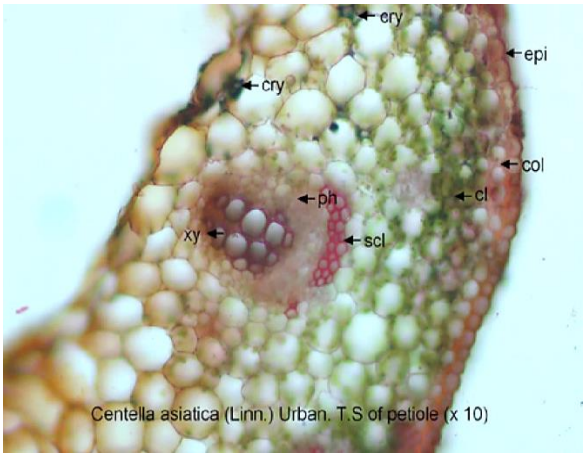

(c)

Figure 2.5 (a) *C. asiatica*: T.S. of petiole (× 2), (b) *C. asiatica*: T.S. of petiole (× 4) and (c) *C. asiatica*: a portion of petiole enlarged (×10). (Source: Sudhakaran, 2017)

The study on the macroscopic and microscopic details of all parts of the medicinal plants is important as their bioactive components can be obtained from the roots, stem, petioles, flowers or leaves (Zainol et al., 2008). Hence, for the assurance of safety and quality as well as the authentication of the extracts, this study is required (Sabaragamuwa, Perera and Fedrizzi, 2018). In addition, it helps us to determine the choice of an appropriate extraction method and solvent, which generates the highest yield and allowing the exhaustive extraction of bioactive constituents without their degradation, is crucial for the pharmacological efficacy of the final products (Pferschy-Wenzig and Bauer, 2015).

2.2 Extraction

Extraction is the transfer of one or more components of a biological feed from its source material into a fluid phase. The process is followed by separation of the fluid phase and recovery of the component(s) from the fluid (Llyod and Wyk, 2012). The extraction systems and detailed selection of suitable equipment are designated based on the objective of the process and physical properties of the material to be extracted as well as the yield obtained. The quality of the plant extract can be influenced by the selection of plant segments used as raw material of extraction, choice of solvent used during the extraction process and the process parameters of the extraction itself. The process parameters such as the polarity of solvent, extraction time, temperature and concentration of solvent and the geographical of plant origin may also influence the phytochemical constituents of the *C. asiatica* species.

2.3 Extraction Solvent

Various solvents can be used to extract the *C. asiatica* which includes ethanol (Dewi and Maryani, 2015) and distilled water (Cheng et al., 2004). Polarity index is a relative measure of the degree of interaction of the solvent with various polar test solutes. For extracting non-polar compounds like fats, oils and lipids non-polar solvents are used. Extraction of highly polar compounds like glycosides, sugars, amino acids, proteins and polysaccharides can be done with polar solvents such as ethanol and water. In the case of flavonoids, less polar ones such as isoflavones, flavanones, methylated flavones and flavonols are extracted with low polar solvents such as chloroform, dichloromethane, diethyl ether or ethyl acetate and the polar flavonoids and flavonoid glycosides are extracted with alcohols or aqueous alcohol mixtures. Generally the extraction of polar compounds can be done by using 50% alcohol or with 100% water.

Water is inexpensive and environmentally friendly. Water can be used as a universal solvent. In addition to extracting polar substances, water can also be used to extract less polar or borderline nonpolar substances, altering the polarity of water by adjusting the extraction temperature. The polarity of water at 250 ºC is the same as the polarity of methanol at 25 ºC. This implies that water can replace methanol from the

polarity perspective. Other than polarity reduction, viscosity, density and surface tension of water are also reduced at higher temperature.

Hence, mass transfer can be enhanced which simultaneously facilitate the extraction of polyphenols in water. Another method to reduce the surface tension is the addition of surfactant that significantly improves the solubility of polyphenols in water (Panja, 2017). Based on research done by Yahya, Attan and Abdul Wahab (2018), water extraction products mainly contains metals, ions, high hydrophilic compounds, and water-soluble proteins/enzymes, glycoproteins, peptides, amino acids, nucleotides, sugars, and polysaccharides. Among the viable solvents, distilled water is proven the safest extract solvent to be used. With no signs of toxicity but with effective extracting of compound comprising various pharmacological activity in the extracted fluid (Man and Choo, 2018), it provides the highest confidence level for patients to consume the drug.

Ethanol is the preferred solvent for the extraction of food grade isolates as it is less toxic compared to other alcohols (Oreopoulou, Tsimogiannis and Oreopoulou, n.d). Ethanol extraction product mainly contains high hydrophilic compounds, comprising of very polar neutral, acidic and basic compounds, sugar, amino acids, nucleotides as well as polysaccharides (Yahya, Attan and Abdul Wahab, 2018). Table 2.1 shows the comparison of antioxidant phytoconstituents obtained from 100% ethanol extract of *C.asiatica* and water extract of *C. asiatica* (Rahman et al., 2013).

Table 2.1 Antioxidant phytoconstituents of 100% ethanol extract of *C.asiatica* and water extract of *C. asiatica*

Antioxidant	**Content (µg/mg of extract)**	
Phytoconstituents	**100% E Ex**	**H_2O Ex**
Polyphenols (PE)	21.1±0.1	35.6±0.5
Flavonoids (QE)	9.3±0.3	11.7±0.2
Tannin (TE)	85.7±3.3	60.7±1.8
Vitamin C (AE)	12.5±0.7	13.3±0.4

Note: PE, Pyrogallol Equivalent; QE, Quercetin Equivalent; TE, Tannic acid Equivalent; AE, Ascorbic acid Equivalent; E Ex, Ethanol Extract; H_2O Ex, Water Extract.

According to Rahman et al. (2013), the polarity index value for 100% ethanol and water are 5.2 and 9, respectively. Extraction yield of both polyphenols and flavonoids increase as the polarity increases. The extraction yield for tannin was observed to reduce to a steady state with the increase of polarity. Extraction yield of vitamin C was found higher in higher solvent polarity.

In comparison, *C. asiatica* ethanol extract has the greatest amount of TPC (0.119 mg/100 g of extract in the forms of caffeoylquinic acid derivatives) and flavonoids (1.088 mg/100 g of extract), followed by *H. sibthorpioides* with TPC of 0.018 mg/100 g of extract and total flavonoids of 0.807 mg/100 g of extract, and *H. bonariensis* ethanol extracts with total flavonoids of 0.759 mg/100 g of extract. The research also proven that *C. asiatica* ethanol extracts inhibited 87.7% xanthine oxidase inhibitory (XOI) activity at the concentration of extract 200 μg/ml, while the two species of Hydrocotyle showed poor XOI activity (Maulidiani et al., 2014). Based on the stated analysis, it is proven that *C. asiatica* contains the highest amount of bioactive components compared to the other two 'Pegaga' species. Hence, it delivers the utmost efficiency and medical benefits to consumers.

Typically, bioactive compounds in herbal plants are present in low concentrations. Hence, it is crucial to develop more effective and selective extraction methods for the recovery of the desired bioactive compounds from the herb materials. Traditional organic solvent-based extraction often suffers from low extraction yields, long extraction times, and residual toxic organic solvents in final products. The residual solvents are problematic since the residual toxic organic solvents in extracts can deteriorate the quality of the extracts and can cause serious health problems when the extracts are taken into the human body (Rahman et al., 2013). Hence, it is more practical and safer for consumer if *C. asiatica* is extracted using water (Man and Choo, 2018). As extraction solvent has been selected, the most appropriate extraction method should be carefully selected considering its practicality, efficacy and time consumption that is appropriate to conduct the extraction of *C. asiatica*.

2.4 Extraction Methods

Extracts from plant tissues contain a myriad of bioactive compounds that are highly valuable to the neutraceutical and pharmaceutical fields (Yahya, Attan, and Abdul Wahab, 2018). The extraction process is a crucial first step before analysis of herbal plants can be carried out. Moreover, it is to ensure that the active ingredients within the plants are not lost or destroyed during preparation. Extraction process allows the desired components in the plant material to be isolated and characterized (Sasidharan et al., 2011). Conventional extraction methods such as maceration, decoction (Vongsak et al., 2013) and Soxhlet extraction (Wang and Weller, 2006) are the most common methods for medicinal plant extraction. However, commercial extraction methods such as microwave-assisted extraction (Caldas et al., 2018), ultrasound-assisted extraction (Chemat et al., 2016) and super critical fluid extraction (Wang and Weller, 2006) have been proposed to extract bioactive compounds from plants with higher recovery capacity (Caldas et al., 2018).

2.4.1 Decoction

Decoction is an extraction technique where sample will be extracted in boiling distilled water (100 ºC). This can be achieved by applying direct heat during extraction of the plant sample (Gohil, Patel and Gajjar, 2012). The most important effect in elevating the extraction temperature is the weakening of hydrogen bonds, resulting in water with a lower dielectric constant. The elevation also tends to promote solubility, as the thermal kinetic energy rises. Increasing the temperature will also facilitate analyte diffusion. Furthermore, higher temperature will reduce interactions between analytes and the sample matrix by disrupting intermolecular forces such as Van der Waals forces, hydrogen bonding, and dipole attractions. Higher temperature also decreases the viscosity of water, thus enabling better penetration of matrix particles. Finally, elevating the temperature will decrease the surface tension, allowing the water better “wet” the sample matrix (Llyod and Wyk, 2012).

2.4.2 Infusion

According to Cittan, Altuntas and Celik (2018), people would mostly prefer the hot water infusion to extract the medicinal properties in herbal plants. The infusion extraction mechanism is similar as decoction only that it is not conducted in a boiling temperature (100 ºC). According to Dent et al., (2013), the highest yield of *C. asiatica* is obtained at the temperature of 60 °C. In addition, it is proven that the extraction temperatures ranging from 45 °C to 60 °C exhibited a relatively high antioxidant activity (Ruenroengklin et al., 2008). These previous studies had suggested that better extraction efficacy of *C. asiatica* can be achieved if temperature lower than 100 °C is applied. Table 2.2 summarizes the TPC and TAC of herbal plant extracts obtained by infusion and ultrasound-assisted extraction (UAE).

Table 2.2 TPC and TAC of herbal plant (*Tilia cordata*) extracts obtained by infusion and UAE

Extraction technique	**TPCs (mg GAE/g dry sample)**	**TACs (mg TE/g dry sample)**
Infusion	58.86 ± 21.51	82.99 ± 13.13
UAE	111.84 ± 13.99	197.52 ± 12.69

Note: UAE, Ultrasound-assisted Extraction; TPCs, Total Phenolic Contents; TACs, Total Antioxidant Capacities; GAE, Gallic Acid Equivalent; TE, Trolox Equivalent

Based on Table 2.2, yield of TPC and TAC obtained by UAE is higher than infusion. However, it can be seen that infusion is comparatively effective with approximately 50% yield as compared to the phenolic compounds obtained by UAE.

2.4.3 Ultrasound-assisted Extraction (UAE)

Various non-conventional extraction techniques including UAE have been developed for the extraction of nutraceuticals from plants in order to shorten the extraction time, reduce the solvent consumption, maximize the extraction yield, and enhance the extracts quality (Wang and Weller, 2006; Cong-Cong et al., 2017). UAE according to Easmin et al. (2014) is where sound waves at more than 20 kHz (up to 100MHz) pass into the solvent and the better extraction efficiency is related to the acoustic cavitation. When the ultrasound intensity is sufficiently high, the expansion cycle can create cavities or micro-bubbles in the liquid. These cavitation phenomena will lead to high shear forces in the media (Chemat et al., 2016). Once formed, energy from the sound waves will be absorbed by the bubbles and it will grow during the expansion cycles and recompress during the compression cycle. Bubbles may start another rarefaction cycle or collapse leading to shock waves having extreme conditions of pressure and temperature. Hence, the implosion of cavitation bubbles can hit the surface of the solid matrix resulting in micro-jetting which generates several effects including surface peeling, erosion and particle breakdown and eventually disintegrate the cells causing release of the desired compounds (Chemat et al., 2016; Easmin et al., 2014). Figure 2.6 presents the mechanism of UAE method.

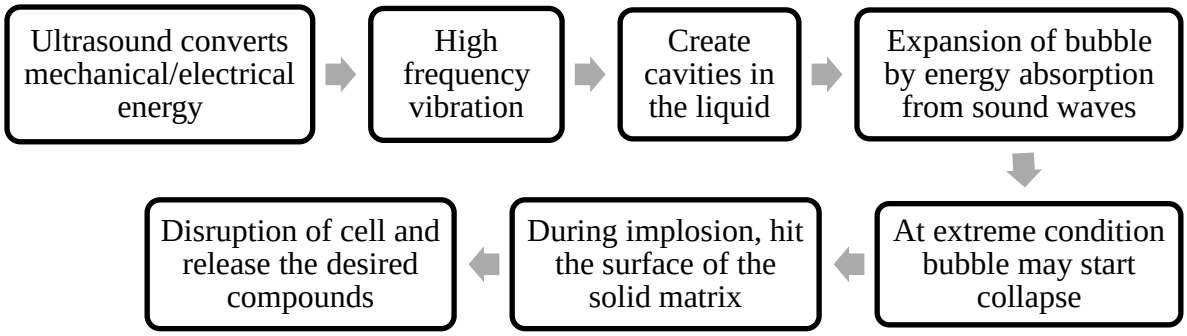

Figure 2.6 Mechanism of UAE method

2.5 Freeze Drying

2.5.1 History and Development

The actual freeze-drying process was first tested and used in 1890 in Leipzig, Germany. It became of practical important during the World War II, when the Canadian Red Cross had to make weekly deliveries of up to 2000 units of human blood plasma. Around the same time, R.I.N. Greaves, from University of Cambridge began the development of more advanced equipment, and later employed it in first commercial production of antibiotics. During 1950s, freeze-drying began to be routinely used by the food and drug industries. Although the freeze drying had been declined during the second half of the 20^{th} century, the appearance of probiotics and other "nutroceutical" products regenerated interest in the freeze drying of bacterial cultures. Even more, ambitious attempts have included the long-term stabilization of freeze-dried mammalian blood cells, embryos, spermatozoa and even organs (Franks, 2008).

2.5.2 Characteristics of Freeze Drying

According to Belachew et al. (2015), freeze drying process comprises of mainly two steps; (1) frozen of the product, and (2) the product is dried by direct sublimation of the ice under reduced pressure. In freeze drying , there are two main drying characteristics of the sample product; **(**i) virtual absence of air during processing: the low processing temperature and the absence of air prevent deterioration due to oxidation or chemical modification of the product and **(**ii) drying at temperature lower than ambient temperature: products that decompose or undergo changes in structure, texture, appearance, and/or flavor as a consequence of high temperature can be dried under vacuum with least damage.

Freeze drying comprises mainly of two stages: freezing and drying. In a way to obtain a product with minimal ice crystals and in an amorphous state, freezing must be very rapid. The drying process involves lowering the pressure to enable ice sublimation. Figure 2.7 and Figure 2.8 present the phase diagram of water and the freeze drying steps, respectively.

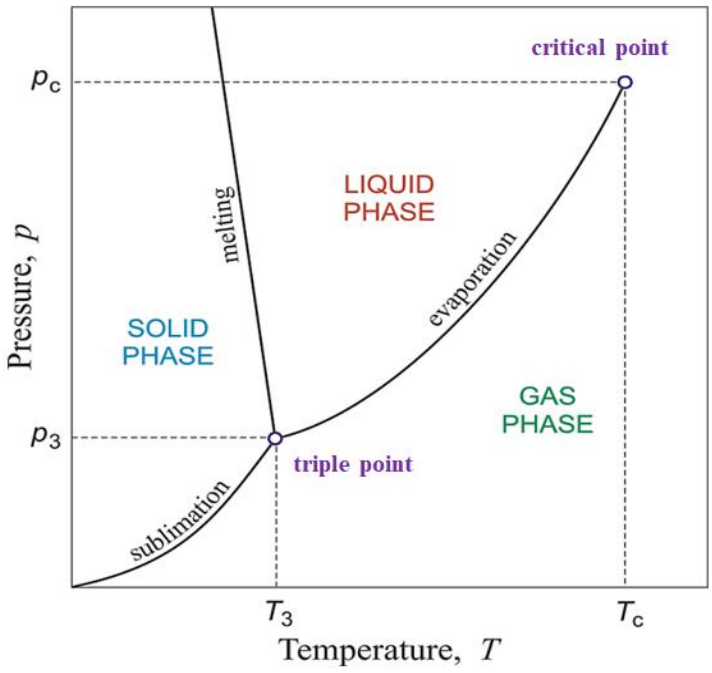

Figure 2.7 Phase diagram of water. (Source: Belachew et al., 2015)

At the triple point (0.0098°C and 4.58mm Hg), ice, water and water vapor coexist in equilibrium. Freeze drying takes place below the triple point, where water passes from solid phase directly to the vapor phase.

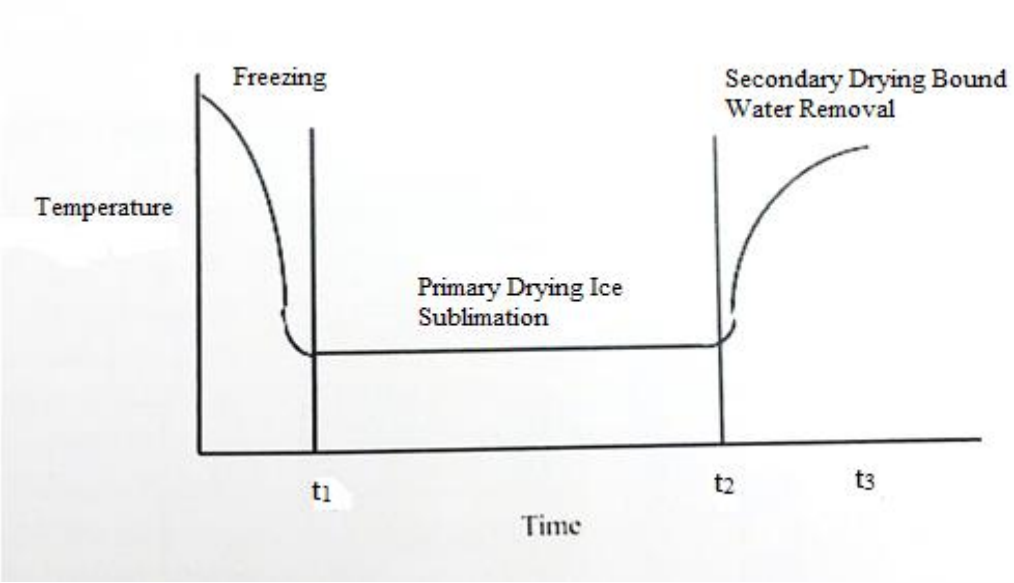

Figure 2.8 Freeze drying steps. (Source: Belachew et al., 2015)

Freeze-drying considers three important design variables which include; (1) vacuum inside the chamber, (2) radiant energy flux applied to the food, and (3) condenser temperature. The initial drying rate is high due to little resistance to either heat or mass flux. However, as the drying proceeds, buildup of a resistive layer on the frozen material slows down the rate. The dry layer surrounding the product serves as insulation material, affecting the heat transfer to the ice front. Also, the mass transfer from the ice front is decreased as thickness of the dry layer is increased. This is due to reduction in the diffusion process from the sublimation interface to the product surface (Belachew et al., 2015).

Freeze drying has been an interesting method used for product preservation which inhibits microbial spoilage and enzyme activity and, therefore, elongate the shelf life of sample product (De Bruijn et al., 2016; Belachew et al., 2015; Prosapio, Norton and De Marco, 2017). By skipping the liquid phase through sublimation, cell structure of product is better maintained and product quality is higher than other drying methods (Aramouni and Deschenes, 2015). In addition, the highest yield of the product can be obtained from the herb which was dried at low temperature (1.1%) and the lowest from that dried in the laboratory oven (0.6%) (Stanisavljevic et al., 2010).

2.5.3 Eutectic/Collapse Temperature

Eutectic temperature is the temperature at which all areas of concentrated solute are frozen. It is an intimate physical mixture of two or more crystalline solids that melts as a single pure compound. This is very important in freeze drying because it represents the maximum allowable product temperature during primary drying. If the product exceeds the eutectic temperature (T_e), drying takes place from liquid instead of the solid. Thus, the plant product will collapse. Collapse may occur if certain operation variables are not well set (Ratti, 2001). This phenomenon also occurs when the solid matrix of the foodstuff can no longer support its own weight, leading to drastic structural changes shown as a marked decrease in volume, increase in stickiness of dry powders, loss of porosity, etc. (Ratti, 2001; Levi and Karel, 1995). Freeze-drying under sub-optimal conditions can also lead to collapse (Levi and Karel, 1995).

2.5.4 Primary Drying

After pre-freezing the product, conditions must be established in which removal of ice from the frozen product via sublimation is achievable, resulting in a dry, structurally intact product. This requires very careful control of the two parameters, temperature and pressure, involved in the freeze drying system. In primary drying, the rate of sublimation of ice from a frozen product depends upon the difference in vapor pressure of the product compared to the vapor pressure of the ice collector. Molecules migrate from a higher pressure sample to a lower pressure area. Since vapor pressure is related to temperature, it is necessary that the product temperature is warmer than the cold trap (ice collector) temperature (Labconco, 2004). According to Karathanos, Anglea and Karel (1996), -55 °C is the ideal temperature for earlier stage of freezing as there is no collapse had occurred on plant sample. However, plant sample collapsed 20% during freeze drying at -45 °C and 50% at -28 °C during primary drying.

2.5.5 Secondary Drying

After primary freeze drying is completed, and all ice has sublimed, bound moisture is still present in the product. The product appears dry, but the residual moisture content may be as high as 7-8%. In a way to reduce the residual moisture content to optimum values, it is necessary to continue drying at the warmer temperature. This process is called isothermal desorption as the bound water is desorbed from the product.

Secondary drying is normally continued at a product temperature higher than ambient but compatible with the sensitivity of the product. All other conditions, such as pressure and collector temperature, remain the same. Because the process involves desorption, the vacuum should be as low as possible (no elevated pressure) and the collector temperature as cold as can be attained. Secondary drying is usually carried out for approximately 1/3 to 1/2 the time required for primary drying (Labconco, 2004).

2.6 Hygroscopicity

Hygroscopicity is the ability of a material to absorb or adsorb moisture from surrounding atmosphere. It can also be defined as a measure of interaction between water and a material (Allada et al., 2016). Water, according to (Chen, 2009), when take up by pharmaceutical solids, could alter their physicochemical properties and adversely affect the product performance. Physical implications such as poor dissolution, crystallization of formulation components, variation in water content in the final product, and powder caking may occur. In other aspect, chemical reactions such as hydrolysis and oxidation could also be accelerated by water sorption. In order to minimize downstream development risk, compounds with low hygroscopicity are desired during the drug development. A solid, according to Newman, Reutzel-Edens, and Zografi (2008), depending on the rate and extent of water uptake with changes in RH, may be ranked according to its hygroscopicity level as per Callahan et al. (1982). However, as reported by (Allada et al., 2016), a conventional and simpler method for determining hygroscopicity level is prescribed in European Pharmacopeia (Ph. Eur.). This categorization method is the most widely used in the pharmaceutical industry, due to its ease and cost effectiveness compared to viable advanced techniques. The Ph. Eur. method categorization relies on the amount of mass gain, when a material is equilibrated at 25°C ± 1°C and 80% ± 2% RH for 24 h. Unfortunately, the Ph. Eur. method does not prescribe any sample pre-treatment and the study starts with some amount of moisture already present with the material being studied, because the initial weighing happens in laboratory environment which is usually maintained at about 60% RH. Due to the initial moisture, for any given material, the amount of mass gain as Ph. Eur. method will be always lesser than the maximum amount of moisture that the materials can up-take when exposed to 25°C, 80% RH right from its dried state (Allada et al., 2016). Hence, pre-treatment on samples before undergoing Ph. Eur. method is recommended to obtain more accurate hygroscopicity result. Table 2.3 summarizes hygroscopicity categorization as per Callahan et al. (1982) and Ph. Eur.

Table 2.3 Hygroscopicity categorization as per Callahan et al. (1982) and Ph. Eur.

Categorization	**Criteria per Callahan et al.**	**Criteria per Ph. Eur.**
Non-hygroscopic	Class I: essentially no moisture increase below 90% RH; less than 20% increase in moisture content above 90% RH in 1 week	0–0.012% (w/w)
Slightly hygroscopic	Class II: essentially no moisture increase below 80% RH; less than 40% (w/w) increase in moisture content above 80% RH in 1 week	0.2–2% (w/w)
Moderately hygroscopic	Class III: moisture content does not increase >5% (w/w) below 60% RH; less than 50% (w/w) increase in moisture content above 80% RH in 1 week	2–15% (w/w)
Very hygroscopic	Class IV: moisture content will increase as low as 40–50% RH; greater than 20% (w/w) increase in moisture content above 90% RH in 1 week	>15% (w/w)

CHAPTER 3

METHODOLOGY

Extraction which includes decoction, infusion and ultrasound-assisted extraction (UAE) are the main processes that were carried out to obtain the *Centella Asiatica* aqueous extract. The outcome from these extraction methods was purified, freeze dried and the powder was analyzed based on the yield and its hygroscopicity under a specified condition. Figure 3.1 summarizes the entire process.

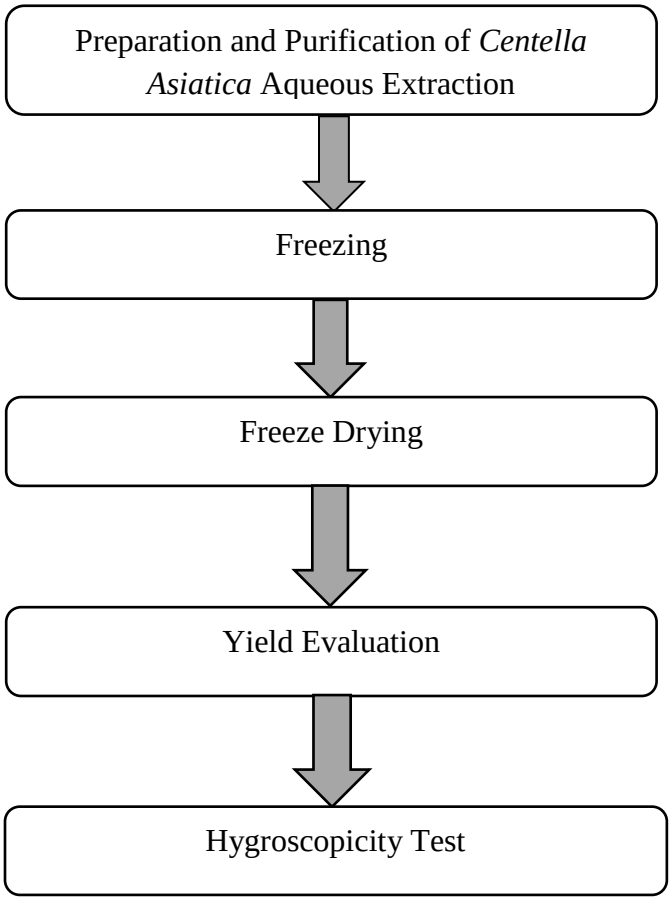

Figure 3.1 Flowchart of the entire process

3.1 Material/Chemical

The materials used in this study are listed in Table 3.1.

Table 3.1 Material list and quantity

Material	Source / Supplier	Quantity
Centella Asiatica plant	Wholesale market, Kuantan	7 kg
Whatman No.1 filter paper	Whatman	4 boxes
Parafilm	Bemis	1 roll

3.2 Equipment

The equipment used in this study are summarized in Table 3.2.

Table 3.2 Equipment list

Equipment	Brand	Purpose/Usage
Ultrasonic water bath	Bandelin Sonoren Digitec	Extracting *C. asiatica* by ultrasound-assisted extraction method
Freeze Dryer	VirTis BenchTop Pro	Freeze drying the purified *C. asiatica* sample
Refrigerator	Thermo Fisher Scientific-906	Freezing of *C. asiatica* sample

3.3 Moisture Content of Fresh *C. asiatica*

The fresh *C. asiatica* plant was oven dried at 50 °C. The percentage of mass loss of *C. asiatica* was recorded until a constant mass was achieved. The percentage of mass loss of the *C. asiatica* powder was calculated using Equation 3.1.

$$\% \text{ Moisture Content} = \frac{\text{Initial Mass} - \text{Final Mass}}{\text{Initial Mass}} \times 100 \quad (3.1)$$

3.4 Preparation of *C. asiatica* Aqueous Extract

3.4.1 Plant Materials

The fresh *C. asiatica* plant was oven dried at 50 °C, relative humidity 20%, and coarsely ground with grinder. Ground plant sample was stored in a sealed container at room temperature prior to extraction.

Several extraction methods were performed using the same solid-solvent ratio (1:10 w/v) with distilled water as solvent (Vongsak et al., 2013). Conventional solid-solvent extraction (decoction and infusion), and the use of emerging technology (ultrasound-assisted extraction) were conducted and described as in 3.4.2, 3.4.3 and 3.4.4.

3.4.2 Decoction

By using ratio 1:10 (w/v), 10 g of coarsely ground dried *C. asiatica* was extracted in a 100 ml boiling distilled water for 10 minutes under reflux (Cheng et al., 2004; Dent et al., 2013). Once extraction completed, the liquid was strained and the plant was pressed to collect the liquid residues. The extract was filtrated through Whatman No 1 filter paper with a pore size of 11 µm and concentrated to half of its volume using rotary evaporator. The extract was stored in chiller to prevent microbial contaminate on until further use. Figure 3.2 represents decoction of *C. asiatica.*

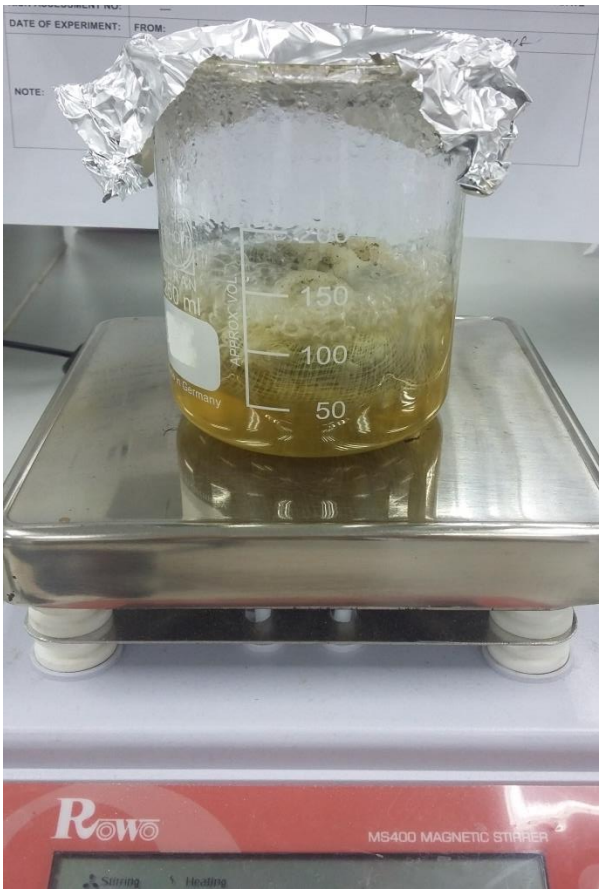

Figure 3.2 Decoction of *C. asiatica*

3.4.3 Infusion

By using solid-liquid ratio (1:10 w/v), 10 g of dried *C. asiatica* was added to 100 ml of distilled water. The initial temperature of distilled water was 98 ºC (Katalinic et al., 2006). Infusion was left to stay at room temperature without additional heating for 10 minutes. Once completed, the extract was filtrated through Whatman No 1 filter paper and concentrated to half of its volume using rotary evaporator. The extract was stored in a chiller to prevent microbial contamination until further use. Figure 3.3 represents infusion of *C. asiatica.*

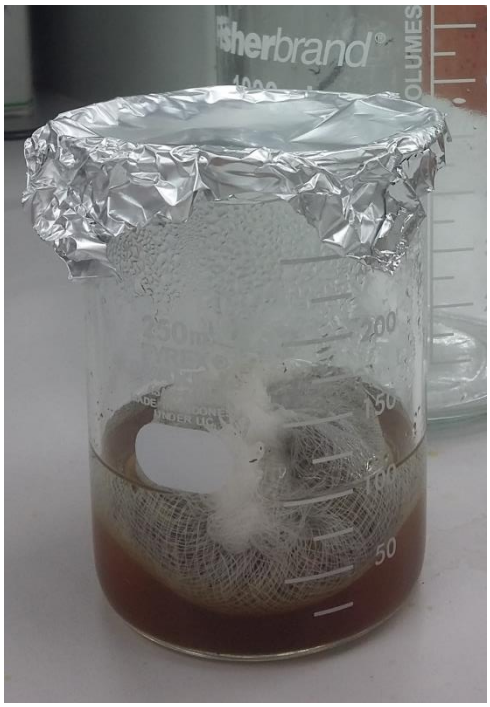

Figure 3.3 Infusion of *C. asiatica*

3.4.4 Ultrasound –assisted Extraction (UAE)

The UAE was carried out using an ultrasonic water bath that was preheated to 80 ºC (Castro-López et al., 2017). Then by using ratio 1:10 (w/v), the beaker containing 10 g dried *C. asiatica* in 100 ml distilled water was placed inside the water bath. Extraction was carried out for 10 minutes under high sonication setting. Once completed, the beaker was removed from the ultrasonic water bath. It was filtrated and concentrated to half of its initial volume using rotary evaporator. The extract was stored in a chiller to prevent microbial contamination until further use. Figure 3.4 represents an ultrasound-assisted extraction of *C. asiatica*.

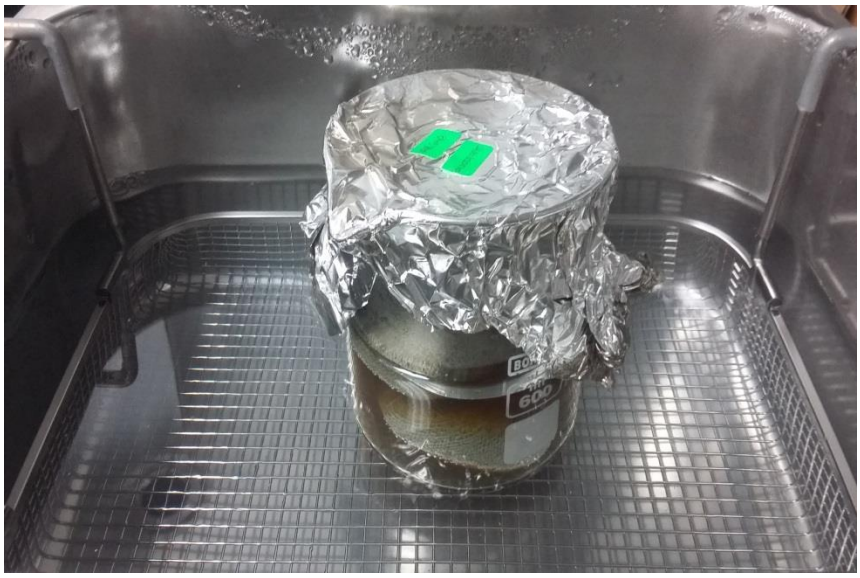

Figure 3.4 Ultrasound-assisted extraction of *C. asiatica*

3.5 Basic Operation of Freeze Dryer

The condenser must be kept clean, dry and empty before preceding the operation. The drain line was checked for residual moisture, which can cause slow vacuum pump-down. It was important to ensure that the plastic quick-connect drain fitting was not inserted into the drain fitting receptacle on the front of the unit. The product was pre-frozen at -40 °C. During pre-freezing of samples in a storage freezer, product were filled to half of the total capacity of the container and glassware should be positioned at a 30° to a 45° angle to increase surface area and reduce stress on the glass to prevent breakage. Next, all connections and ports were secured and the Quickseal valve was closed. The AUTO button was pressed and the system was allowed to proceed through the freeze-drying process using the controller's settings. The critical system parameters were ensured to stay within the acceptable ranges. The condenser temperature and vacuum pressure values displayed on the System Status screen appear as green or red, providing an easy status check. Periodically, the condenser was checked for ice build-up and defrosted as needed. The product was observed to determine the completion of drying stage. Low moisture content may be achieved by allowing the product to continue to dry for several hours. Once drying stage was completed, the vacuum and refrigeration system were disabled simultaneously by

pressing AUTO button. All products were removed and DEFR button was pressed to enable condenser defrost. The defrost function allowed ice to be lifted out of the condenser without fully melting. A drain line was connected to a plastic quick-connect fitting, which was then inserted into the drain receptacle on the front of the unit. To open the drain, the fitting was pushed into the drain receptacle. To close the drain line, the small grey release button on the top of the receptacle was pressed. The fitting popped out. Once the ice has melted away from the condenser, the ice was removed. The condenser was thoroughly cleaned, and rinsed with a mild detergent or baking soda solution (to neutralize acids). The defrost system was turned off automatically after one hour or when the condenser reaches 60 °C (VirTis SP Scientific, 2014). Figure 3.5 presents freeze drying process via VirTis BenchTop Pro with Omnitronics-8L.

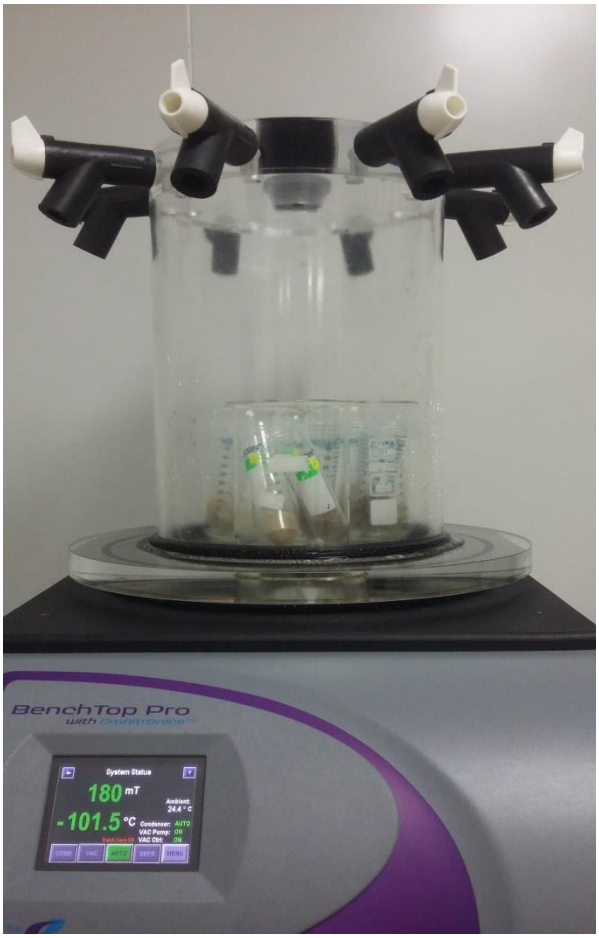

Figure 3.5 Freeze drying process via VirTis BenchTop Pro with Omnitronics-8L

3.6 Freezing of *C. asiatica* Extract

The *C. asiatica* aqueous extract was transferred into beakers, each containing same volume of the extract. These tubes were placed in the laboratory refrigerator overnight at a temperature of -80 °C (Zainol et al., 2009).

3.7 Primary Drying of *C. asiatica* Extract

The frozen *C. asiatica* extract was subjected to sublimation by primary freezing. The frozen extract was transferred to the condenser-type freeze-dryer. The temperature (approx.± -100 °C) and pressure (approx. ± 180 mTorr) for the primary drying process were automatically set up by the equipment. The process began when the status bar turned from red to green. The drying process continues for a minimum of 16 hours (Plengmuankhae and Tantitadapitak, 2015). The temperature and pressure remained constant throughout the process to ensure that there was no other factor that would affect the powder yield and quality.

3.8 Secondary Drying of *C. asiatica* Extract

Secondary drying began simultaneously after the primary drying stage completed. In the secondary drying stage, the extracted *C. asiatica* temperature was elevated to 35 °C (Zainol et al., 2008). The temperature must be slightly lower than the collapse temperature (T_c) of plant sample. Any further increase of temperature beyond the T_c will cause the solute material melts, preventing any structure from forming after the solvent has been removed. The vacuum pressure was kept constant as in primary drying automatically by the equipment. The secondary drying stage continued for 8 hours to complete the entire freezing cycle. This was because secondary drying usually carried out approximately half of the time required for primary drying (Labconco, 2004). The process was completed once the status bar displayed a change from red to green and the product appeared dryness where there was no powder residue at the inner surface of the beaker.

3.9 Yield of *C. asiatica* Powder

Powder obtained from decoction, infusion and ultrasound-assisted extraction was weighed. The percentage of yield obtained was calculated using Equation 3.2.

$$\%\ \text{Yield of } C.asiatica = \frac{\text{Yield obtained (g)}}{\text{Raw C.asiatica (g)}} \times 100 \quad (3.2)$$

3.10 Determination of Extractable Solids

Extractable solids were determined according to a method described previously by Ngo et al. (2017) with slight modification. A 10 ml of the extract was put in a centrifuge tube and then placed in freeze dryer to remove all moisture. Extractable solids (ES) were calculated using Equation 3.3.

$$\%\text{ES} = \frac{\text{Mass of 10ml extract after drying (g)}}{10} \times 100 \quad (3.3)$$

3.11 Hygroscopicity Study

Hygroscopicity of samples was determined according to a gravimetric sorption analysis (GSA) method described by Allada et al. (2016) with slight modification. Post-freeze dried, a 5 g of *C. asiatica* powder samples were subjected to three steps, which were: (Step I) Equilibration at 25 °C, 60% RH (normal laboratory condition), (Step II) pre-treatment step which was drying at 45 °C, 10% RH (the lowest RH as per compatibility of the stability chamber) until a constant mass was achieved and (Step III) Equilibration 25 °C, 80% RH for 24 hours. Step III is the measurement criterion equivalent to that of European Pharmacopeia method. Initial mass loss during pretreatment step was calculated using Equation 3.4. Total mass gain (Sorption from its dried state) of each sample was calculated using Equation 3.5. Hygroscopicity categorization of the powder samples were done as per Ph. Eur.

$$M_{PT} = \frac{M_i - M_{min}}{M_i} \times 100 \quad (3.4)$$

Where,

M_{PT} = Mass loss during pre-treatment step

M_i = Initial mass of the material

M_{min} = Minimum mass that was attained, during drying, in the pre-treatment step

$$M_{GSA} = \frac{M_{max} - M_{min}}{M_{min}} \times 100 \quad (3.5)$$

Where,

M_{GSA} = Total mass gain using GSA approach

M_{min} = Minimum mass that was attained, during drying, in the pre-treatment step

M_{max} = Maximum mass that was attained, during sorption step, at 25 °C, 80% RH

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Moisture content of fresh *C. asiatica* plant

The moisture content study on fresh *C. asiatica* was conducted to estimate the time taken needed to sufficiently dry the plant in a specific condition (50 °C, 20% RH). Drying was carried out until equilibrium moisture content was achieved. This was to avoid microbial or fungal growth on the plant sample during storage prior to extraction (Lemus-Mondaca, 2018). Hence, the lower the moisture content in the plant material, the more stable it will be for a prolonged storage period. The percentage of moisture loss by fresh *C. asiatica* was presented by the curve in Figure 4.1. The values presented were the mean of three replications for each fresh plant sample ± standard deviation. Data variation was analyzed by one-way ANOVA for comparisons. The values were significantly different at $p = 7.64365 \times 10^{-53}$.

From the curve shown in Figure 4.1, there was significant loss of moisture from hr 0 to hr 1 (44.45 ± 0.44% *m/m*). The drying process initially started with the removal of moisture on the plant surfaces. Hence, explained the significant moisture loss. From hr 1, the plant sample gradually loss more moisture up to 91.41 ± 0.48% *m/m* at hr 7. At hr 8 (92.43 ± 0.45% *m/m*), moisture content in the plant lost at a constant rate until hr 30. The *C. asiatica* plant material was found to lose its moisture content to a maximum of 93 ± 0.70% *m/m* after 30 hours of oven-dried, left only ±0.7% moisture in the plant structure. From this study, it was found that the *C. asiatica* plant can be oven-dried at 50 °C, 20% RH for a minimum of 7 hours to achieve moisture content in the plant of lower than 10%. This will not only sustain the stability of the plant material and prolong the plant

shelf life, but to dry it at a low temperature with the lowest relative humidity possible will also help preserving the green color of the plant, polyphenol contents and antioxidant activity of *C. asiatica*.

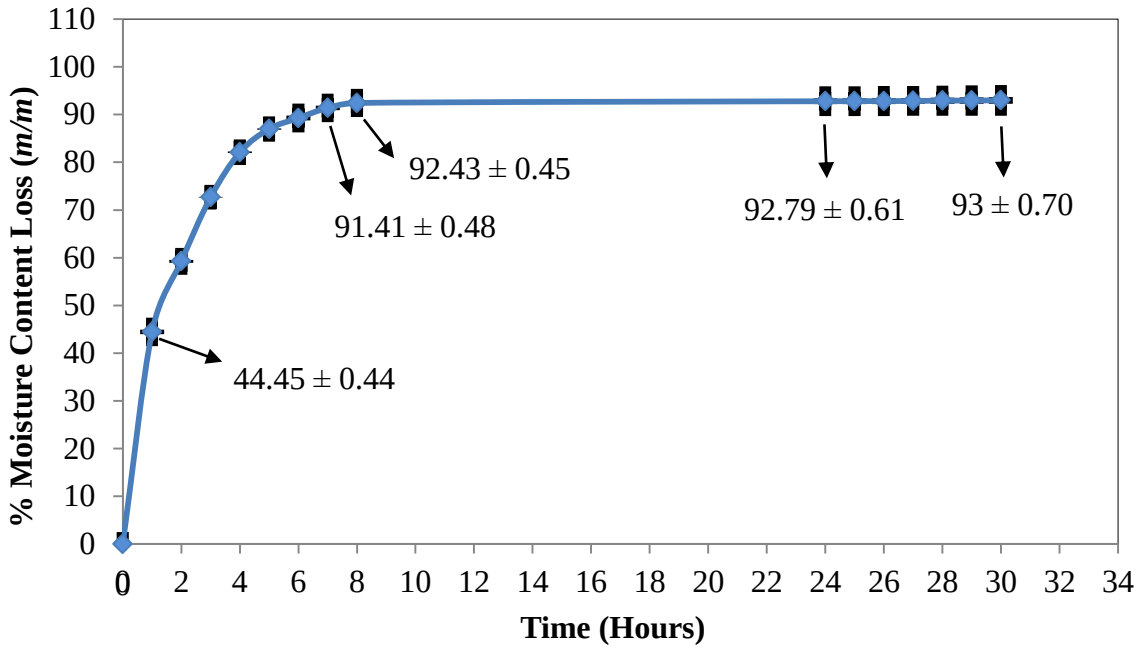

Figure 4.1 Curve of percentage moisture content loss on fresh *C. asiatica* during drying treatment in 30 hours. *The values presented were the mean of three replications for each fresh plant sample ± standard deviation. Data variation was analyzed by one-way ANOVA for comparisons. Values were significantly different at p< 0.05.*

4.2 Yield of *C. asiatica* powder

Post-freeze dry of all extracts, the powder obtained was weighed to determine the yield generated by each extraction methods. The outcomes were presented in Figure 4.2.

A 46.3 g of dried *C. asiatica* was subjected to three extraction methods and then freeze dried to obtain a powder yield. From the Figure 4.2, it was found that ultrasound-assisted extraction generated the highest yield percentage of 11.65% (g /46.3 g), followed by infusion with 10.48% (g /46.3 g) and decoction with 10.03% (g/ 46.3 g).

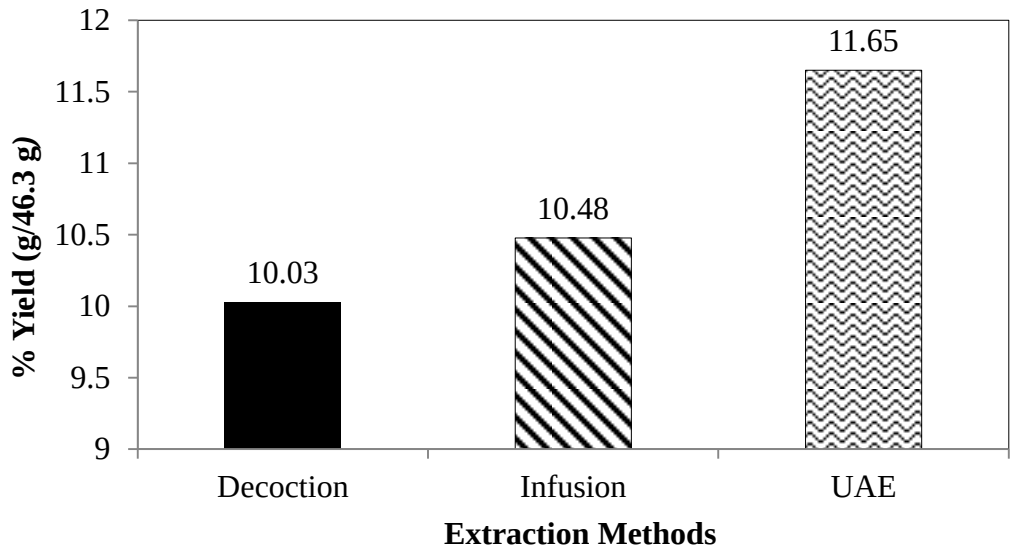

Figure 4.2 Crude powders obtained post-freeze dry of 46.3 g dried *C. asiatica* in each extraction methods presented in % yield (g/46.3 g)

The decoction and infusion were much simpler, more convenient and less costly in terms of instrumentation. However, UAE promoted extraction efficacy by passing sound waves into the solvent that eventually producing acoustic cavitation. These cavitation phenomena had led to high shear forces in the media. Once formed, energy from the sound waves were absorbed by the bubbles and it grew during the expansion cycles and recompressed during the compression cycle. Bubbles may start another rarefaction cycle or collapse which led to shock waves having extreme conditions of pressure and temperature. Hence, the implosion of cavitation bubbles hit the surface of the solid matrix resulting in micro-jetting which causes the swelling and hydration and so causing an enlargement in the pores of the cell wall. Due to this, the plant cells were highly disrupted. It also generated several other effects including surface peeling, erosion and particle breakdown (Chemat et al., 2016; Easmin et al., 2014; Oreopoulou, Tsimogiannis and Oreopoulou, n.d). Eventually disintegration of the plant cells causing released of the desired compounds into the solvent. Due to this mechanism, it enhanced the UAE efficacy in extraction even though it was carried out in a lower temperature as compared to decoction and infusion.

The infusion extraction mechanism was similar as decoction, only that it was not conducted in a boiling temperature (100 ºC). In the study, *C. asiatica* was proven to be extracted better in a lower temperature as the percentage yield obtained by infusion (10.48%) was higher than decoction (10.03%). Theoretically, the higher extraction temperature, the more compounds can be extracted from the plant materials (Llyod and Wyk, 2012). The increase of extraction temperature led to higher permeability of cell walls, higher solubility of the phenolic compounds, and higher heat and mass transfer phenomena through the plant matrix (Oreopoulou, Tsimogiannis and Oreopoulou, n.d). Therefore, an increase in extraction rate and yield can be observed. However, degradation of some plant compounds may be induced due to extraction at a very high temperature which may be the reason why decoction obtained the least yield. At the same time, the reason yield obtained by infusion (initially at 98 ºC, without any additional heating) and decoction (100 ºC) were lower than UAE (80 ºC water bath) can also be justified. Hence, the higher extraction temperature applied, the more compounds in *C. asiatica* were degraded and failed to be extracted which therefore explained the outcome. Besides that, certain compounds may also oxidize at higher temperatures. Oreopoulou, Tsimogiannis and Oreopoulou (n.d) stated that the oxidation of plant compounds can be accelerated in the presence of water. Since water was used as the extraction solvent, therefore oxidation of certain compounds in the *C. asiatica* plant may be as well facilitated.

The percentage of extraction yield was in correlation with the results obtained in extractable solids test. In the extractable solids test, each tube contained 10 ml of extracts. After freeze dried, the mass of crude powder obtained was weighed. Referring to Table 4.1, it was proven that an advanced technology like UAE was capable to generate the highest crude powder of 12.57 ± 0.01% (g/ 10 ml) followed by infusion with 11.99 ± 0.41% (g/ 10 ml) and lastly decoction with 11.20 ± 0.57% (g/ 10ml). Hence, supported the percentage yield results obtained earlier.

4.3 Extractable solids

The percentage of extractable solids by all extract samples were calculated and presented in Table 4.1. The results were in correlation with the yield outcome. The values presented in Table 4.1 were the mean of three replications for each extraction methods ± standard deviation. Data variation was analyzed by one-way ANOVA for comparisons. The values were significantly different at $p = 0.016775$.

Table 4.1 Percentage of extractable solids obtained from 10 ml extracts from each extraction methods presented as % (g/ 10 ml)

Methods	**%ES (g/10ml)**
Decoction	11.1967 ± 0.5687
Infusion	11.9900 ± 0.4060
UAE	12.5667 ± 0.0115

Note: UAE, Ultrasound-assisted extraction; ES, Extractable Solids. The values presented are the mean of three replications for each extraction methods ± standard deviation. Data variation was analyzed by one-way ANOVA for comparisons. Values are significantly different at $p < 0.05$.

4.4 Hygroscopicity study

Hygroscopicity study was conducted to measure the amount of moisture or water that was absorbed by the powder samples. Four climatic zones can be distinguished for the purpose of worldwide stability testing, as follows: (Zone I) Temperate, (Zone II) subtropical, with possible high humidity, (Zone III) hot/dry and (Zone IV) hot/humid. Malaysia located in Zone IV (SADC, 2004). According to Malaysia Meteorological Department (n.d), the humidity in Malaysia is very high, with monthly averages varying from 3% to 15% in any region of the country. The average relative humidity varies from a low 84% in February to a high of 88% in November in Peninsular Malaysia. During rainy season, city like Kuala Lumpur can reach a very high relative humidity of 98%. Therefore justified the reason why Ph. Eur. (equilibrium at 25 °C, 80% RH) was selected as the guideline for this test.

The steps involved were: (Step I) Equilibration at 25 °C, 60% RH which mimic the ambient in laboratory, (Step II) pre-treatment step which was drying at 45 °C, 10% RH (the lowest RH as per compatibility of the stability chamber) until a constant mass was achieved as presented in Table 4.2. Step II also known as dehydration step whereby all moisture was removed from the sample. (Step III) Equilibration 25 °C, 80% RH for 24 hours which was also known as the hydration step by exposing the samples to certain degree of ambient moisture. Basically, it tested the physical stability of the *C. asiatica* powder samples to remain in its powder morphology. From the results obtained, the powder samples will be categorized as per Ph. Eur. as shown in Table 2.3.

According to Pyper (1985), there were three ways of classifying the states of moisture in solids: (1) operational, (2) energetic, and (3) structural. *Operational* was a simple classification of the moisture as "bound" or "free". In using such a simple definition, it was necessary to specify the conditions. For example, an operational definition of free moisture would be "that moisture which can be removed by heating the substance at 110 °C for one hour in air at atmospheric pressure". *Energetic* was defined as the interaction of the water with the material (or some part of the material) which can be overcome by energy and the water can be removed to a specified standard state (gaseous non-associated water vapor at ambient temperature). For an example, in this classification scheme, the various states of moisture in solids would be described as "water bound with X kcal mol-'". Lastly, *Structural* defined as the water that was bound by physical forces or water bound by chemical bonds. This classification scheme is summarized in Figure 4.3. In this study, since the parameters of dehydration (Step II) was set at 45 °C and 10% RH and the moisture loss and gain were evaluated by the physical observation of the solid surface hence instantaneously the moisture in the solids samples was classified as operational and structural.

Table 4.2 Mass losses during pre-treatment of powders obtained from different extraction methods presented in % (*m/m*)

Time (Hours)	**% M_{PT} loss (*m/m*)**		
	Decoction	**Infusion**	**UAE**
0	0	0	0
0.25	0	1.6	0.2
0.5	0.2	1.6	0.4
0.75	0.2	1.8	0.6
1	0.4	2	0.8
1.5	0.4	2.2	1
2	0.4	2.2	1
2.5	0.6	2.4	1
3	1	3	1.8
3.5	1	3.2	2
4	1	3.2	2.2
4.5	1	3.2	2.2
5	1	3.2	2.2

Note: M_{PT}, Mass Pre-treatment; UAE, Ultrasound-assisted extraction

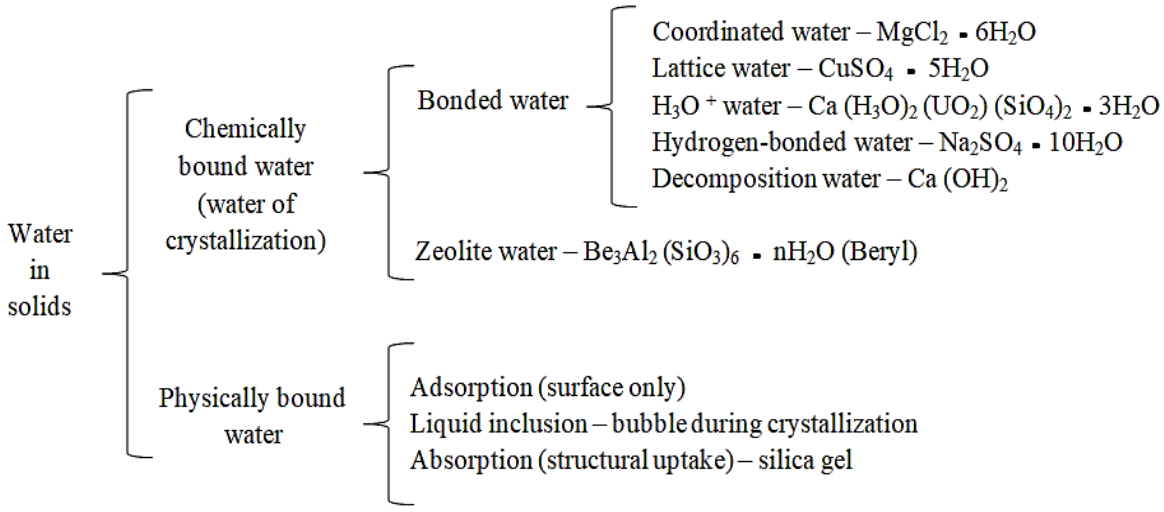

Figure 4.3 Structural classification of water in solids (Source: Pyper, 1985)

As presented in Table 4.2, in the first 15 minutes (0.25 hr) of the test, percentage of moisture loss inclined drastically by infusion powder (1.6% *m/m*), followed by a slight increased by UAE powder (0.2% *m/m*) and 0% (*m/m*) increased by decoction powder. Decoction powder only started to lose moisture after hr 0.25.

From the first 15 minutes result, the moisture on the surface of infusion powder was higher compared to the other powder samples. Infusion powder lost a lot of moisture during the first 15 minutes most probably due to insufficiently dried by 96 hours of freeze drying or due to high rate of moisture adsorption on the solid surface during Step I which was the exposure to normal laboratory environment. From hr 0.25 to hr 2.5, it can be observed that infusion powder (1.6 – 2.4% *m/m*) and UAE powder (0.2 – 1.0% *m/m*) proportionally lost more moisture while decoction powder was at much slower rate (0 – 0.6% *m/m*). Starting from hr 3, all three powder samples started to reach constant percentage of moisture loss and constant powder mass which indicated that all moisture that either absorbed or adsorbed on the powder due to Step I had been removed. After 5 hours in dehydration step (Step II) at 45 °C with 10% RH, the highest total moisture loss was by infusion powder (3.2 % *m/m*) followed by UAE powder (2.2% *m/m*) and decoction powder (1.0% *m/m*). At hr 5, the constant mass achieved by all powder samples were taken respectively as an initial mass before they were subjected to Step III which was the water sorption test. The percentage of mass gained by the powder of each extraction methods were calculated and presented in Table 4.3.

From Table 4.3, it can be observed that all three powder samples gained more than 15% of moisture after only 45 minutes (hr 0.75) of exposure. From there, the samples continuously increasing in mass which indicated the rising of moisture absorbed. After 24 hours of exposure to 25 °C with 80% RH (Step III), the highest percentage of mass gained (M_{GSA}) which indicated the total moisture sorption was by decoction powder (71.31% *m/m*) followed by infusion powder (71.28% *m/m*) and lastly UAE (68.71% *m/m*). Even on hr 24, all powder samples were still on peak and the curve did not reach constant which indicated that all powder samples were still continuously absorbing the ambient moisture even after hr 24. Referring to this result, categorization as per Ph. Eur. can be done. All three powder samples were found to be very hygroscopic (VH) as summarized in Table 4.4.

Table 4.3 Mass gained during gravimetric sorption analysis method of powder samples obtained from different extraction methods presented in % (*m/m*)

Time (Hours)	**% M_{GSA} gain (*m/m*)**		
	Decoction	**Infusion**	**UAE**
0	0	0	0
0.25	5.6566	4.3388	4.4990
0.5	17.5758	15.7025	12.2699
0.75	20.4040	17.9752	14.3149
1	22.4242	19.8347	16.1554
1.25	23.6364	21.0744	17.3824
1.5	24.2424	21.4876	17.7914
1.75	24.6465	21.9008	18.4049
2	25.2525	22.7273	19.4274
2.5	25.8586	23.3471	20.0409
3	26.6667	24.1736	20.8589
3.5	26.8687	24.5868	21.2679
4	27.0707	25.0000	21.8814
5	27.4747	25.6198	22.2904
6	28.8889	26.2397	24.7444
7	29.2929	26.6529	25.1534
8	29.6970	26.8595	25.3579
21	64.4444	62.8099	56.2372
22	66.4646	65.2893	60.7362
23	69.0909	69.4215	67.0757
24	71.3131	71.2810	68.7117

Note: M_{GSA}, Mass Gravimetric Sorption Analysis; UAE, Ultrasound-assisted extraction

Table 4.4 *C. asiatica* powder categorization per Ph. Eur.

Methods	**Maximum % M_{GSA} gain (After 24 hours)**	**Categorization per Ph. Eur.**
Decoction	71.3131	VH
Infusion	71.2810	VH
UAE	68.7117	VH

Note: VH, Very Hygroscopic

From these findings, it was proven that *C. asiatica* was incompatible as solid dosage form due to its instability to remain in its dry solids state. This can be related to several reasons. One of it could probably due to the morphology and the anatomy of the *C. asiatica* fresh plant itself. *C. asiatica* is a creeping runner with thin leaves and glabrous roots that can easily grow in the presence of water. This is why the plant can easily be found as it flourishes extensively in paddy fields, river banks, streams and any wet surfaces (Sudhakaran, 2017). Due to the environment that *C .asiatica* grew in, the moisture content inside the plant structure itself is very high compared to woody plants. The total moisture in a fresh *C. asiatica* can be up to 93% as presented in Figure 4.1. This explains the difficulty to prepare the plant sample itself. Starting from preparing the extract, since the plant contains very high moisture, the fresh plant cannot be stored for too long. High moisture content in the plant making it very vulnerable and easily infected by microbial growth. Hence justifies why the plants were oven-dried. This was to prolong the shelf life, to obtain a more stable raw plant material and most importantly to prolong storage time prior to extraction process.

Once the extraction process completed *C. asiatica* extracts were subjected to freeze drying process. Woody plants like *Morus alba L.* (mulberry) or *Stevia rebaudiana* required only 6 hours and 10 hours respectively of freeze drying to completely remove the extraction solvent and obtaining the dry crude powder (Ma et al., 2018; Lemus-Mondaca et al., 2018) . However, it was different for *C. asiatica*. The *C. asiatica* concentrated extracts required at least 96 hours of freeze drying. Some batches required up to 192 hours to

completely remove the moisture. The structural moisture inside *C. asiatica* was too high making it very difficult to be dried. Longer freeze drying time was required instantaneously would use more energy and operating cost.

Once the crude powder was obtained, another problem occur which involved the powder samples handling. It was observed to be very hygroscopic that making it almost impossible to be evaluated. The powder seemed stable where it remained in dry solid forms during the pretreatment step (45 °C, 10% RH) in hygroscopicity study. However, once it was exposed to room ambient, instantly it started to appear sticky. The *C. asiatica* powder was proven to be very sensitive in the presence of any form of moisture. It tends to attract moisture from the air to achieve its stable state therefore was incompatible in solid dosage form. In order to still preserving the antioxidants and bioactive components in *C. asiatica*, other alternatives were suggested.

CHAPTER 5

CONCLUSION

5.1 Conclusion

Based on the results obtained, it can be concluded that UAE was the best extraction method. It generated the highest *C. asiatica* powder yield which was 11.65% (g /46.3 g), followed by infusion with 10.48% (g /46.3 g) and lastly decoction with 10.03% (g/46.3 g). The results were supported by extraction solids study which proven an equivalent outcome. These may be due to the UAE ability to produce sound waves that penetrates into the plant structure and causes acoustic cavitation which induces the plant particle breakdowns. The temperature selected for UAE (80°C) which was lower compared to infusion and decoction may also contributed to its efficiency to extract the highest desired compounds. From here it can be concluded that the higher extraction temperature applied on *C. asiatica* the more rapid its components will degrade. As for hygroscopicity test, basically the study was conducted to determine whether the *C. asiatica* plant was compatible and stable to be in solid dosage form. Unfortunately, the results obtained proven that all three powder samples were very hygroscopic with total percentage of moisture sorption of more than 15% as per Ph. Eur. Therefore, recommendations on how to overcome this matter were suggested in order to sustain the ability to prolong the *C. asiatica* shelf life and its stability as medicinal product.

5.2 Recommendation

One alternative to reduce degradation of the *C. asiatica* compound would be the selection of extraction solvent. According to Oreopoulou, Tsimogiannis and Oreopoulou (n.d), the oxidation of plant compounds can be accelerated in the presence of water. Hence, instead of using 100% distilled water as extraction solvent, it is recommended to either use 100% alcohol or alcohol-water mixture as the extraction solvent. The alcohol solvent can be removed easily by rotary evaporator leaving a minimal amount of water in the plant extracts. Therefore, reduces the possibility of rapid oxidation of the plant compounds and would probably shorten the time required for freeze drying of extracts.

Another way to protect the formulation from moisture is to use a tabletting excipient designed to absorb water in the place of the active ingredients (API) in order to prevent its degradation. Amongst such excipients is magnesium aluminium silicate, carboxymethylcellulose or calcium carbonate. All those excipients have porous structure and the ability to bound water. Hence, water-particle in API binding can be prevented. Next, other effective solution for protection of a finished drug product is through packaging. However, it does not exclude moisture uptake during multiple openings. Protecting the cores with a moisture barrier film is appropriate, since it also eliminates this problem. An ideal moisture barrier coating should exhibit low permeability to water vapor without compromising its dissolution functionality. For a film-coating to be able to protect the core, it must contain a hydrophobic ingredient. This ingredient can be the polymer itself or a hydrophobic plasticizer. The film coating range of excipients containing stearic acid that prevents the moisture from reaching the core of the dosage form can be applied on powder, pellets or tablets at various mass gains depending on the hygroscopicity of the formulation for the protection to be achieved (SEPPIC, n.d).

The last alternative is to prepare *C. asiatica* in a liquid dosage form. From the study, it was very difficult to process *C. asiatica* in solid dosage form. Not only that the final products were very unstable, but the longer time required in freeze drying will result in a more energy to be consumed and higher cost to be operated. Therefore, a much convenient, cheaper and faster method such as liquid dosage form is highly recommended in order to obtain *C. asiatica* product that is highly stable.

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APPENDIX A

Figure 1A *C. asiatica* fresh plant used for the research

Figure 2A Bandelin Sonoren Digitec ultrasonic water bath used to extract *C. asiatica*

Figure 3A Rotary evaporator used to concentrate the *C. asiatica* extracts volume

Figure 4A From left: Decoction, Infusion and UAE, 10 ml extracts filled in each tubes

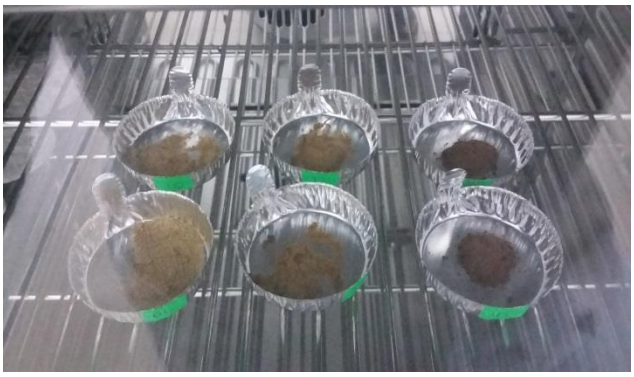

Figure 5A *C. asiatica* powder samples used for hygroscopicity test

Figure 6A After some time, the samples appeared sticky and some melted completely to water

APPENDIX B

Table 1B Percentage of moisture content loss (*m/m*) by fresh *C. asiatica* in 30 hours

Time (Hours)	**% Moisture content loss (*m/m*)**
0	0.0000 ± 0.0000
1	44.4528 ± 0.4421
2	59.2390 ± 0.2901
3	72.6730 ± 0.0663
4	82.1006 ± 0.1429
5	86.9811 ± 0.1321
6	89.2830 ± 0.4906
7	91.4088 ± 0.4778
8	92.4340 ± 0.4532
24	92.7925 ± 0.6123
25	92.8113 ± 0.6255
26	92.8365 ± 0.6150
27	92.8491 ± 0.6123
28	92.9245 ± 0.6429
29	92.9371 ± 0.6564
30	93.0000 ± 0.7027

The values presented are the mean of three replications for each extraction methods ± standard deviation. Data variation was analyzed by one-way ANOVA for comparisons. Values are significantly different at $p < 0.05$.

Table 2B Data collected for triplicate sampling on moisture content study of fresh *C. asiatica*

Time (Hour)	**Mass of sample (g)**			**% Moisture content loss (*m/m*)**			**Av % Moisture content loss**	**Standard deviation**
	A	**B**	**C**	**A**	**B**	**C**		
0	5.3	5.3	5.3	0.0000	0.0000	0.0000	0.0000	0.0000
1	2.959	2.917	2.956	44.1698	44.9623	44.2264	44.4528	0.4421
2	2.150	2.178	2.153	59.4340	58.9057	59.3774	59.2390	0.2901
3	1.445	1.452	1.448	72.7358	72.6038	72.6792	72.6730	0.0663
4	0.954	0.940	0.952	82.0000	82.2642	82.0377	82.1006	0.1429
5	0.687	0.685	0.698	87.0377	87.0755	86.8302	86.9811	0.1321
6	0.598	0.554	0.552	88.7170	89.5472	89.5849	89.2830	0.4906
7	0.460	0.428	0.478	91.3208	91.9245	90.9811	91.4088	0.4778
8	0.409	0.420	0.374	92.2830	92.0755	92.9434	92.4340	0.4532
24	0.355	0.418	0.373	93.3019	92.1132	92.9623	92.7925	0.6123
25	0.354	0.418	0.371	93.3208	92.1132	93.0000	92.8113	0.6255
26	0.353	0.416	0.37	93.3396	92.1509	93.0189	92.8365	0.6150

27	0.352	0.415	0.37	93.3585	92.1698	93.0189	92.8491	0.6123
28	0.351	0.414	0.36	93.3774	92.1887	93.2075	92.9245	0.6429
29	0.349	0.414	0.36	93.4151	92.1887	93.2075	92.9371	0.6564
30	0.349	0.414	0.35	93.4151	92.1887	93.3962	93.0000	0.7027

Table 3B Analysis of variance (ANOVA Single factor) – Moisture content study

SUMMARY

Groups	***Count***	***Sum***	***Average***	***Variance***
0	3	0	0	0
1	3	133.3585	44.45283019	0.195443218
2	3	177.717	59.23899371	0.08413433
3	3	218.0189	72.67295597	0.004390649
4	3	246.3019	82.10062893	0.020410585
5	3	260.9434	86.98113208	0.01744393
6	3	267.8491	89.28301887	0.240655037
7	3	274.2264	91.40880503	0.228313753
8	3	277.3019	92.43396226	0.205411178
24	3	278.3774	92.79245283	0.374866501
25	3	278.434	92.81132075	0.391242435
26	3	278.5094	92.83647799	0.378189154
27	3	278.5472	92.8490566	0.374866501
28	3	278.7736	92.9245283	0.413314347
29	3	278.8113	92.93710692	0.430876943
30	3	279	93	0.493770025

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	29176.53	15	1945.101673	8076.556687	7.64365E-53	1.99199
Within Groups	7.706657	32	0.240833037			
Total	29184.23	47				

Table 4B Crude powders obtained post-freeze dry of 46.3 g dried *C. asiatica* in each extraction methods presented in yield % (g/46.3 g)

Methods	**Total yield (g)**	**%Yield (g/46.3 g)**
Decoction	4.643	10.0281
Infusion	4.851	10.4773
UAE	5.394	11.6501

Note: UAE, Ultrasound-assisted extraction

Table 5B Data collected for triplicate sampling on extractable solids obtained from 10 ml extracts from each extraction methods presented as % (g/ 10 ml)

Extraction methods	**Mass (g)**			**%ES (g/10 ml)**			**Av % ES**	**Standard deviation**
	Tube 1	**Tube 2**	**Tube 3**	**Tube 1**	**Tube 2**	**Tube 3**		
Decoction	1.057	1.168	1.134	10.570	11.680	11.340	11.197	0.569
Infusion	1.155	1.207	1.235	11.550	12.070	12.350	11.990	0.406
UAE	1.256	1.256	1.258	12.560	12.560	12.580	12.567	0.012

Table 6B Analysis of variance (ANOVA Single factor) – Extractable solids study

SUMMARY

Groups	***Count***	***Sum***	***Average***	***Variance***
Decoction	3	33.59	11.19667	0.323433
Infusion	3	35.97	11.99	0.1648
UAE	3	37.7	12.56667	0.000133

ANOVA

Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Between Groups	2.838822	2	1.419411	8.719337	0.016775	5.143253
Within Groups	0.976733	6	0.162789			
Total	3.815556	8				