SCREENING AND DEVELOPMENT OF HERBAL BASED ANTI-AGEING PRODUCT

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SCREENING AND DEVELOPMENT OF HERBAL BASED ANTI-AGEING PRODUCT

by

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LIST OF SYMBOLS, ABBREVIATION & NOMENCLATURE

$AlCl_3$	Aluminum chloride
BP	British Pharmacopeia
DE	Cumulative percentage release
DPPH	1,1-diphenyl-2-picrylhydrazyl
DSC	Differential scanning calorimeter
DW	Distilled water
FC	Folin Ciocalteu
FDA	United State Food and Drug Administration
FUdR	5-fluoro-2′-deoxyuridine
GAE	Gallic acid equivalent
HPLC-UV	High pressure liquid chromatography-ultraviolet detector
IC_{50}	Concentration giving 50% inhibition
L1	Larval stage 1
L3/4	Larval stage 3/4
LB	Luria Broth
LOD	Limit of detection
LOQ	Limit of quantification
N2	*C. elegans* strain, wild-type bristol
Na_2HPO_4	Disodium phosphate
$NaCO_3$	Sodium carbonate
NGM	Nematode growth medium
OP50	*E. coli* strain
QE	Quercetin equivalent
RH	Relative humidity

ROS	Reactive oxygen species
RPM	Rotation per minute
RSD	Relative standard deviation
Rt	Retention time
SD	Standard deviation
SEM	Standard error of mean
SGF	Simulated gastric fluid
TFC	Total flavonoid content
TPC	Total phenolic content
USP	United States Pharmacopeia
UV	Ultraviolet

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SARINGAN DAN PEMBANGUNAN PRODUK ANTI PENUAAN BERASASKAN HERBA

ABSTRAK

Saringan tumbuhan semulajadi seperti *M. oleifera*, *H. sabdariffa* dan *A. galanga* telah dijalankan untuk tujuan pembangunan produk anti-penuaan. Tumbuhan tersebut telah dilaporkan sebagai mempunyai sebatian bersifat antioksida, iaitu salah satu faktor terpenting bagi menunjukkan kesan anti-penuaan. Namun, hanya satu tumbuhan yang paling sesuai telah dipilih untuk dibangunkan. Satu kaedah pengekstrakan yang mudah dilakukan dengan etanol dan air sebelum analisis yang seterusnya. Daripada ujian antioksida, iaitu aktiviti hapus-sisa radikal, jumlah kandungan fenolik dan jumlah kandungan flavonoid ekstrak tumbuhan telah disiasat. Asai DPPH menunjukkan bahawa ekstrak akueus *H. sabdariffa* menunjukkan aktiviti merangkap radikal tertinggi dengan nilai IC_{50} 327.0 ± 0.1µg/ml dan diikuti oleh ekstrak hidroethanol *H. sabdariffa* dengan nilai IC_{50} 514.5 ± 0.1µg/ml dan ekstrak etanol *A. galanga* dengan nilai IC_{50} 531.5 ± 0.1µg/ml. Ekstrak hidroetanol *H. sabdariffa* menunjukkan kandungan fenolik tertinggi dengan 12.4 ± 0.9 mg bersamaan GAE/g dan kandungan flavonoid tertinggi dalam ekstrak etanol *M. oleifera* dengan 4.2 ± 0.5 mg bersamaan QE/g. Semua ekstrak kemudian diuji untuk kesan farmakologi dengan menggunakan *C. elegans* sebagai model *in vivo*. *C. elegans* telah dirawat dengan ekstrak tumbuhan pada kepekatan 1, 10, 100 dan 1000µg/ml. Ekstrak etanol *A. galanga* dan ekstrak hidroethanol *H. sabdariffa* dengan kepekatan 100µg/ml dan 1000µg/ml meningkatkan jangka hayat *C. elegans* kepada 21.0 ± 0.5 hari dan 21.1 ± 0.3 hari (20 hinggga 23%) berbanding kawalan. Kedua-dua ekstrak yang menunjukkan potensi ini kemudiannya dijadikan bentuk dos pepejal akhir menggunakan kaedah

granulasi basah. Beberapa eksipien telah dikaji bersama ekstrak dan granul *A. galanga* menggunakan kanji jagung telah dipilih kerana paling stabil dan serasi. Kandungan komponen aktif ekstrak *A. galanga*, kaempferol dan quercetin ditentukan dengan kaedah HPLC yang baru dihasilkan dan disahkan. Kaedah pelarutan menunjukkan bahawa kadar pelarutan telah meningkat sebanyak 60 hingga 80% dalam granul *A. galanga* berbanding ekstrak dan ia terbukti stabil sehingga enam bulan dalam keadaan penstoran yang panas dan lembab (30°C /75%).

SCREENING AND DEVELOPMENT OF HERBAL BASED ANTI-AGEING PRODUCT

ABSTRACT

Screening of natural plants such as *M. oleifera*, *H. sabdariffa* and *A. galanga* was conducted in view of developing an anti-ageing product. Those plants have been reported to contain compounds with antioxidant properties which is one of the most important factors that impart anti-ageing effect. However, only one plant which proved to be the most suitable and promising was chosen. A simple extraction method was carried out with ethanol and water before further analysis. Antioxidant assays, namely radical scavenging activity, total phenolic content and total flavonoid content of the plant extracts were investigated. DPPH assay indicated that aqueous extract of *H. sabdariffa* showed the highest radical scavenging activity with IC_{50} of 327.0 ± 0.1 µg/ml and followed by hydroethanolic extract of *H. sabdariffa* with IC_{50} of 514.5 ± 0.1 µg/ml and ethanolic extract of *A. galanga* with IC_{50} of 531.5 ± 0.1 µg/ml. Hydroethanolic extract of *H. sabdariffa* showed highest phenolic content with 12.4 ± 0.9 mg GAE/g equivalent and total flavonoid content was highest in ethanolic extract of *M. oleifera* with 4.2 ± 0.5 mg QE/g equivalent. All the extracts were then tested for their pharmacological effect by using *C. elegans* as the *in vivo* model. The nematodes were treated with plant extracts at the concentation of 1, 10, 100 and 1000µg/ml. Ethanolic extract of *A. galanga* and hydroethanolic extract of *H. sabdariffa* with the concentration of 100 µg/ml and 1000µg/ml increased the mean lifespan to 21.0 ± 0.5 days and 21.1 ± 0.3 days, which were by 20 to 23% respectively compared to control. Both promising extracts were developed into a final solid dosage form using wet granulation method. Several excipients were tested with the extract and the developed

A. galanga granules using corn starch as the excipient was the most stable and compatible. It was then quantified for the active components, kaempferol and quercetin with newly developed and validated HPLC method. Dissolution studies shows that the dissolution rate was enhanced by 60 to 80 % in *A. galanga* granules compared to the extract. The granules also proved to be stable up to six months in hot and humid storage condition (30°C/75% RH).

CHAPTER 1

INTRODUCTION

1.1 General Issues on Ageing

Ageing is a natural process that is related with changes in the usual biological, physiological, psychological and behavioral process. It is an essential mechanism upon reaching the golden age, where the structural and functional changes pile up in an organism because of the transition of time. The differences that can be observed due to ageing is that reduced peak fertility and physiological functions until death. According to English Oxford Living Dictionaries, ageing means the process of growing old or the process of change in the properties of a material occurring over a period, either spontaneously or through deliberate action. According to Steves et al., (2012), at a biological level, ageing is associated with the gradual accumulation of a wide variety of molecular and cellular damage. Then those damages lead to a gradual failing in physiological abilities and senses functionality which then provides higher exposure to disease occurrence.

According to a report in Star News dated 27th February 2019, Malaysia's Healthcare National Key Economic Area Committee estimated that the country will reach ageing nation status in just 10 years, with more than 15% of the population being aged 60 and over by then. Malaysia is not alone in facing the prospect of having an ageing population as a United Nations report issued in 2002 states that by 2050, the number of older persons in the world will exceed the number of young for the first time in history. According to the Population and Demographic division of the Department of Statistics Malaysia, the number of people aged 65 years and over in

Malaysia has increased steadily since the 1970s, and it is projected the number will triple from 2.0 million today to more than 6.0 million by 2040. Even though much smaller in total size, the number of people ages 80 years and over is projected to grow more than four folds from 0.3 million today to nearly 1.4 million by 2040 (Wan Ibrahim et al., 2017). Although for statistical reasons there are clear definitions of ageing and aged nation but specifically for each individual, ageing is a process that creeps up in a very slow and subtle manner.

It is imperative to elucidate the ageing process as there are some opinions saying that ageing process or the normal ageing is a manifestation of the regular biological changes which happen according to age level and is not afflicted by any external consequences. While on the other hand, other opinions state that the process of ageing is said to be greatly dominated by external factors such as environmental factors, style of living and illness state (WHO, 2001). Ageing is a very intricate multiplex process and although much research has been done in order to identify its causes, it is still questionable. This is because there are many theories pinpointed explaining the root of ageing process. Elements that dominate ageing is still uncertain although current molecular biology and genetics study are at an advanced level. Davidovic et al., (2010) claimed that all the theories proposed previously for ageing mechanism are still not satisfying.

Several studies performed previously by researchers have reported that ageing to be influenced by genetics, lifestyle, malnutrition, *in utero* exposure and environmental risks. In terms of genetics, ageing is a progression of numerous circumstances which are inclined by hereditary factors. Those factors are intricate due

to the complexity of the mechanism itself as there is variation amongst people and even among tissues in the body (Rodriguez-Rodero et al., 2011). According to Passarino, De Rango, & Montesanto (2016), studies done previously proved that around 25% of the variation in each human's lifespan is caused by genetic components. Adding to this, recently, epigenetic studies show that both genetic background and lifestyle can either be a biomarker of the ageing quality or influence the rate of ageing (Passarino et al., 2016). In certain cases, ageing process is accelerated more than the normal condition according to the chronological age due to an unhealthy lifestyle.

Next, according to Cheng, Bohr, & Cabo (2010) malnutrition and accelerated ageing also can be closely related as many studies proved that nutritional deficiency or excess contributes to the ageing process. Nutrition is one of the important factors that influence the prevention of disease occurrence and aids in achieving a healthy ageing at the same time. Nutrition can be divided into two which are macronutrients and micronutrients. Macronutrients are those foods that are consumed to provide the body with adequate number of calories and energy while micronutrients are those foods that help in maintaining good body function for an optimal level of health. Macronutrients are usually consumed in a larger quantity compared to micronutrients, however based on several studies obtained throughout the years show that an increase in micronutrients intake and decrease in macronutrients intake would successfully lead to healthy ageing (Barzilai & Bartke, 2009; Cox & Mattison, 2009 ; Piper & Bartke, 2008; Masoro, 2005).

Furthermore, another major root cause of ageing is due to high exposure to environmental risks. According to Karol, (2009), ageing is a genetically-regulated

process that is responsive to environmental influences; those agents present in indoor and outdoor environments and also in the diet affect the ageing process. Free radicals, which are not only generated internally in our body system but also through external sources like environmental pollution, toxic metals, cigarette smoke, and pesticides, would add damage to our body system (Aseervatham et al., 2013).

Free radicals or oxidants are those atoms or molecules that haves an imbalanced electron in its valence shell and lead to that electron's random reactivity which then disrupt millions of nearby cells to replace their missing electron in order to become stable. Generally, there are two types of free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) which are responsible for alteration or disruption of redox status in the body (Phaniendra, Jestadi, & Periyasamy, 2015). They are produced from both endogenous and exogenous sources and in an excess production, oxidative and nitrosative stresses are developed especially in a condition of antioxidants deficiency. Free radicals that are commonly produced endogenously are by metabolic and enzymatic reactions in the body while exogenously is as mentioned previously such as ultraviolet light, chemical pollutants, radiation, toxin and smoke.

When there is accumulation of free radicals, it causes an imbalance between free radicals and antioxidants. Similar to the situation of free radicals' production, antioxidant defense also functions in the same way where it is being produced endogenously and exogenously. A healthy phenomenon is when all the oxidants and antioxidants are in the state of equilibrium. Antioxidants are substances which inhibit or delay oxidation of a substrate while present in minute amounts (Fusco et al., 2007).

This condition of imbalance is known as oxidative stress, a condition where there is too much reactive oxygen that is unstable, and they tend to attach to the other normal cells and tissues where this situation disturbs the normal function of the body system.

Although there are many theories of the biological causes of ageing with different mechanisms, yet a common theory that has been hypothesized with ageing is the free radical theory of ageing. Back in 1945, chemist Denham Harman proposed that ageing was caused by reactive molecules called free radicals that build up in the body and cause cellular damage which then leads to ageing. As a result, they come out with molecules that neutralise free radicals and antioxidants that are also good for human health.

Table 1.1 List of ROS produced during metabolism (Source: Phaniendra, Jestadi, & Periyasamy, 2015)

Free Radicals	**Symbol**	**Half life**	**Source**
Superoxide	O_2^{*-}	10^{-6} s	Formed through enzymatic reaction, autoxidation reaction and electron transfer reaction (Michelson et al, 1977)
Hydroxyl	OH^*	10^{-10} s	Neutral form of hydroxide ion and highly reactive free radical (Bedwell et al, 1989)
Alkoxyl radical	RO^*	10^{-6}	Alkoxyl radicals ($RO^{\cdot}$) are versatile intermediates which not only play a pivotal role in many biological processes, but also are key chemical species in a wide variety of organic transformations (Majetich & Wheless, 1995)
Peroxyl radical	ROO^*	17s	Derived from oxygen in living systems and its simplest form is generated by the protonation of superoxide (De Grey, 2002)

Hydrogen peroxide	H_2O_2	Stable	Formed *in vivo* in a dismutation reaction activated by superoxide dismutase (SOD) (Phaniendra et al, 2014)
Singlet oxygen	$^1O_2$	10^{-6} s	Meta-stable state of molecular oxygen and it is a very toxic oxygen species which is also extremely reactive (Hojo et al, 2000)
Ozone	O_3	s	Produced *in vivo* by antibody catalyzed by water oxidation pathway which is vital in the process of inflammation (Lerner and Eschenmoser, 2003)
Organic peroxide	ROOH	Stable	
Hypochlorous acid	HOCl	Stable (min)	Formed by neutrophils which are activated at the location of inflammation through enzymatic reaction of hydrogen peroxide and chloride and the enzyme involved is myeloperoxidase (Winterbourn and Kettle, 2000)

1.2 Free Radical Theory of Ageing

Free radical theory of ageing states that human beings or even all living organisms age due to accumulation of free radicals over time (Yang & Hekimi, 2010). Free radical theory does discuss other radicals too such as hydrogen peroxide (H_2O_2) and peroxynitrite ($OONO^-$) as it is not only concerned about superoxide (O_2^-). Hypothesis of free radical theory of ageing supports that it is a common process which is affected by both hereditary and environmental aspects. Those free radicals as a product of oxygen derivatives are associated with cell and tissue damages (Fusco et al., 2007). After several years, free radical theory was developed to not only focus on ageing process but age-related diseases as well (Harman, 2009). Since then, many others have advanced the field and scientific understanding of ageing, oxidative stress, cell signaling pathways, cellular redox, telomere shortening, and more. Figure 1.1 shows the chronological order of the development of Free Radical Theory of Ageing.

Moses Gomberg (1900)

Comes out with the idea of the presence of organic free radical in living system

Gershman (1954)

Initiated "free radical theory of oxygen toxicity". According to these theory, oxygen toxicity is because of its capability of free radical formation due to its ability to donate electron to other substances and become reactive, which means when the amount of oxygen is higher in body the chances of free radical formation is also higher. (Lobo et al., 2010)

Denham Harman (1956)

Proposed "free radical theory of ageing", it enhances the effect of free radicals in the process of ageing as there are opinions of free radicals unstability and how it could ever exist in living systems (Harman, 1956). The reason was to investigate the relationship between lifespan, metabolic rate and also consumption of oxygen and convinced that free radicals such as oxygen formed throughout regular respiration would incite collective damage and results in organism's functional loss or even death. (Harman, 1956; Speakman & Selman, 2011)

Denham Harman (1972)

Altered initial theory of free radicals where he suggested that ROS which are being formed in the mitochondria deteriorate proteins and lipids and as the result of this situation, mutation occurs and leads to upsurge of ROS generation which further intensify the free radicals' amount (Jang & Remmen, 2009).

Figure 1.1 Chronological order of the development of Free Radical Theory of Ageing

1.3 Concept of Oxidative Stress and Antioxidants

According to Liebert & Harman (2003), alternatives should be found in order to stop or prevent the activities of the free radicals as they are the enhancer of ageing process. These include changes due the effect of ageing and disease occurrence. The body has several defence mechanisms to counteract excess level of free radicals and the primary one are antioxidants. Antioxidants are molecules that assist in neutralizing the excess free radicals and protect against toxicity induced cell death and disease prevention. Antioxidants with beneficial values function through distinguishable ways and in different sections but still maintain their main function as scavengers of free radicals. Some of the mechanisms are :

- Direct free radicals neutralization
- Helps in reduction of peroxide concentration
- Repairs oxidized membranes
- Decreases ROS production by iron quench
- ROS neutralization through lipid metabolism, short-chain free fatty acids and cholesteryl esters.

Although antioxidants are naturally available within our body, it is still not balanced with the increased amount of free radicals that were present in the environment. Hence it is vital for the body to maintain the healthy balance of free radicals and antioxidants to prevent oxidative stress and keep cells functioning by consuming more antioxidants rich food in daily diet. Antioxidants are highly found in natural resources such as vegetables, fruits, nuts, seeds, coco, tea and whole grain. According to Aseervatham et al., (2013), the use of herbal products could be a better option to meet the objective of finding a suitable treatment for reducing the free radicals generated from environmental

and physiological factors. Supporting this, Narayanaswamy & Balakrishnan, (2011), also mentioned that many herbs contain antioxidant compounds which protects the cells against the damaging effects of reactive oxygen species (ROS). Medicinal plants contain high amounts of phenolic and flavonoids and have been associated with their antioxidant activities that play a role in the prevention of the development of age-related disease, particularly those caused by oxidative stress (Azwanida, 2015). Therefore, in present study, three plants of interest have been selected to study their antioxidant and anti-ageing properties.

1.4 Plants of Interest

Medicinal plants contain phytochemicals, which produce definite physiological actions on the human body (Akinmoladun, Ibukun, Obuotor, & Farombi, 2007). Phytochemicals are natural bioactive compounds that are found in plants such as vegetables, fruits, medicinal plants, flowers, leaves and roots. Phytochemicals can be divided into two components of primary and secondary. Primary components include common sugars, amino acids, proteins and chlorophyll while secondary components include alkaloids, terpenes, phenolic compounds, flavonoids and tannins (Krishnaiah et al., 2011).

1.4.1 *Moringa oleifera* (Leaves)

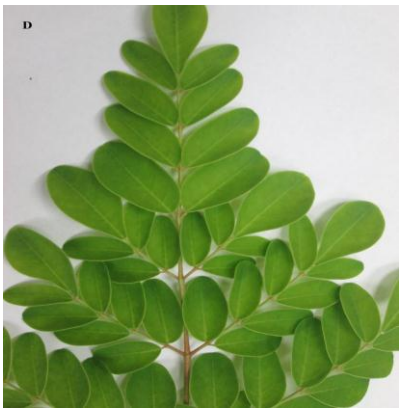


Figure 1.2 *Moringa oleifera* leaves

The origin of *M. oleifera* is sub-Himalayan regions of North West India, but it can also be widely found in countries such as Africa, Arabia, South East Asia, the Pacific, Caribbean Islands and South America (Sreelatha, 2009). The kingdom and order of *M. oleifera* is Plantae and Brassicales respectively. It belongs to the family of Moringacae and one of the species which is vastly harvested and become familiar in many places in tropics (Fahey et al., 2009). Moringaceae family have 12-14 species and it all belongs to the genus Moringa (Sanchez-Machado et al.,2010). *M. oleifera* also do have some other names for instance, drumstick tree, 'horse radish tree' and kelor tree (Anwar & Bhanger, 2003). The leaves are bipinnate or more commonly tripinnate, up to 45-60 cm long with 4-6 pairs of pinnae, in alternate and spirally arrangements on the twigs, leaflets are finely hairy and almost hairless on the upper surface with red-tinged mid veins, the entire margins are rounded or blunt-pointed at the apex and short-pointed at the base (Lim, 2013; Foidl, Makkar, & Becker, 2001). All the parts of *M. oleifera* like the roots, bark, leaves, flower and seeds have been credited for their large-scale assortment of nutritional and therapeutic uses (Mbikay, 2012).

M. oleifera is a plant which has many uses such as functional food for a specific activity, water material clearance and biofuel production. Traditionally, *M. oleifera* leaves give benefits in several treatments such as headaches, haemorrhoids, fevers, noise and throat inflammation, bronchitis, infections of eye and ear. It was also to overcome the lack of vitamin C (Marrufo et al., 2013) as the leaves have been proclaimed by Mukunzi et al., (2011) to contain more vitamin A and C compared to carrots and oranges. Furthermore, the leaves are also consumed together in soup and salads preparation while the leaf juice helps in controlling glycaemia and applied to swollen glands (Marrufo et al., 2013). Moreover, *M. oleifera* have been also reported to have certain medicinal benefits such as antihypertensive, anticancer, antitumor, anti-inflammatory, diuretic properties, antihepatotoxic, antifertility, antiurolithiatic and analgesic activities (Asare et al., 2012). Prior studies on phytochemicals reported that *M. oleifera* leaves consist of compounds such as glucosinolate glycosides and complex flavonoids which plays the role as anti-atherosclerotic, antioxidative and anti-diabetic (Fahey et al., 2009; Chumark et al., 2008; Ndong et al., 2007). Other than that, according to Vongsak, Sithisarn, & Gritsanapan (2014), three major components in the leaf ethanolic extracts of *M. oleifera* are crypto-chlorogenic acid, isoquercetin and astragalin while according to Singh et al., (2014), β-sitosterol, quercetin and kaempferol are present in the leaf ethanolic extract which contributed to the antioxidant and hepatoprotective activity.

1.4.2 *Hibiscus sabdariffa* (Calyx)

Figure 1.3 *Hibiscus sabdariffa* calyx

H. sabdariffa or also commonly known as roselle does have other names too such as asam susur, asam paya or Ribena Malaysia as it very much tastes like cranberries (Mohd-Esa et al., 2010). *H. sabdariffa* is a plant under the category of tropical or subtropical from West-africa, India and Malaysia (Liuqing et al., 2016) and it is broadly cultivated in several tropic areas such as Caribbean, Central America, India, Africa, Brazil, Australia, Hawaii, Florida and Philippines (Mahadevan, Shivali, & Kamboj, 2009). It can grow up to 5-7 feet in height, with lobed narrow leaves and the stems are reddish green in color. The main edible part is the fleshy sepal, called a calyx which surrounds the seed boll in the flower. Each size of the calyx varies but ranges from ½ to 1 ½ inches in diameter (James, 1994). Almost all parts of *H. sabdariffa* such as leaves, seed, fruits and roots are being used but the most famous and utilized is the calyx part. Its belongs to the kingdom of plantae and order of Malvales. It is a herbaceous plant that belongs to the family of Malvaceae and the genus of Hibiscus L. (Rosemallow) which are grown ultimately for its flower but the seeds and leaves also do have several good qualities in terms of medical benefits (Eltayeb & Hamade, 2014). Its blooming leaves and delicate stem are consumed in raw form as salads and chutney other than playing the role of seasoning in certain Malaysian dishes (Mohd-Esa et al., 2010).

Known as a delicacy and for its medicinal benefits, it is widely used in tropical areas for several reasons (Obouayeba et al., 2014). Jams, jellies, sauces and wines are several examples of goods created with *H. sabdariffa*. Compared to its benefits in the food category, it is believed to have much more gain in the field of pharmaceuticals (Alaga et al., 2014). Moreover, various parts have been exploited in avoidance of diseases for instance cardiovascular disease, liver disease, fever and also hypertension (Jafarian et al., 2014). Calyces are the most vital part of roselle as there are many studies focusing on its good effects such as being a treasure of phenolic compounds (Al-Hashimi, 2012) and anthocyanins (Prenesti et al., 2007). Those anthocyanins can increase the health benefits because it is a good point of supply of not only antioxidants but also as a native food colourant (Chumsri, Sirichote, & Itharat, 2008).

1.4.3 *Alpinia galanga* (Leaves)

Figure 1.4 *Alpinia galanga* leaves

A. galanga is one of the member of Zingiberaceae family. Zingiberaceae is a big family which consists of about 1200 species that belongs to 49 genera and the one most widely studied and famous is the *Alpinia* genus (Wong et al., 2009). It is from the kingdom of Plantae and order of Zingiberales. Its other name is greater galangal and the rhizomatous herb is found in several parts of India and throughout Southeast Asia (Rao et al., 2010). *A. galanga* is also known as Java galangal and Siamese ginger in English and this species is very much related to lesser galangal species such as *A.officinarum* Hance and *A.calcarata* Rosc as they have the much or less same properties and the way of using in culinary art and also medicine (Ravindran et al.,2012). The distribution places of *A. galanga* are Indonesia, China, Saudi Arabia, Malaysia, Egypt and Sri Lanka and it mainly grows in sunny places, forest and brushwoods (Lim & Lim, 2016). Its rhizomes are branched, subterete, about 3-5 cm in diameter, fibrous, hard,aromatic and its colour is shiny pink, greenish, red or in pale yellow. Its leaves alternate in two rows with suborbicular ligule and contains 1 cm long hairy petiole. The leave blade is oblong lanceolate, 25-60 long by 6-15 cm wide and it is glabrous or abaxially pubescent with base attenuate. Other than that, the flower of *A. galanga* is yellow-white to greenish

white and fragrant with tubular calyx and corolla. However those properties of *A. galanga* species may vary according to places and its agro-ecological and climate situations.

Galangal had been reported to give antimicrobial effects (Hamad et al.,2016). Moreover, *A. galanga* has been used in traditional ways to treat eczema, bronchitis, coryza, morbili, pityriasis versicolor, otitis interna, gastritis, ulcers and cholera (Raviraja Shetty & Monisha, 2015). It is also a highly beneficial plant in the sector of medical and pharmacological due to the chemical constituents present within it (Raviraja Shetty & Monisha, 2015). All different parts of these plants have their own benefits that somehow help not only in daily routine works such as cooking but also in terms of medical benefits. According to Menon (2006), generally this plant had been helped to give the effect of antibacterial,antispasmodic,bile stimulant, catarrh, cleansing effect other than being used as a folk medicine for detoxification, digestive orders and vascular disorders. Its rhizomes are used as cooking spices and as a source of essential oils (Raviraja Shetty & Monisha, 2015) other than taken as a supplement by women during the period of ailment, illness and confinement and also as a carminative to overcome flatulence. Several chemical constituents or compounds that are abundantly found in the rhizomes especially the one in the form of essential oils. As reported by Raviraja Shetty and Monisha (2015), rhizome also contains flavonoids, some of which have been identified as kaempferol, kaempferide, galangin, alpinin and quercetin and several other compounds. Moreover *A. galanga* also provides a safe and sound antimicrobial system for natural drug products development and this involves the whole plant (Rao et al., 2010). The extract of *A. galanga* leaf proved to have antimicrobial effect and can be used as food preservatives other than being used in the development

of new drugs (Beula Rani et al., 2016). Moreover, previous studies performed highlights the benefit of the leaf part as a good natural antioxidant due to its high antioxidant activity due to the high amount of flavones and flavonols in the leaf compared to the rhizome as the leaf part is more exposed to the sunlight (Wong et al., 2009).

1.5 Anti-ageing products

There is a growing interest all over the world to identify medicinal plants for their therapeutic uses. One of the examples of therapeutic uses highlighted in present study is as antioxidants. According to Peng (2011), nutraceuticals rich in antioxidants have the potential to be competent anti-ageing candidate compounds. As free radical reactions are promoters of the ageing process, it implies that any compound that inhibits them should be able to reduce the rate of ageing process. Antioxidants are the molecules that reduce the chance of ageing by diminishing or maintaining the level of oxidants with or without free radical activity. Therefore, it can be assumed that antioxidants would be a suitable anti ageing agent. Supporting this, Berger.,(2005) and Fusco et al.,(2007) stated that nutritional antioxidants act through different mechanisms but are mainly as free radical scavengers. Moreover, recently, the role of herbal drugs, herbal products and certain phytochemicals in the control of ageing has been reported (Mishra et al., 2011; Kapoor et al., 2009).

1.6 Problem statement

On the basis of Free Radical Theory of Ageing, it is postulated that any substance with high antioxidant capacity can be a potential candidate for delaying the ageing (Peng et al., 2014). Therefore, nutraceuticals rich in antioxidants should slow down ageing and prolong lifespan. This postulation has stimulated enormous number of studies aimed at finding a relationship between levels of endogenous antioxidants and lifespan of various organisms on the effects of addition of exogenous antioxidants to the course of ageing and lifespan of model organisms (Sadowska-Bartosz & Bartosz, 2014). However, nutraceuticals are typically consumed as part of a regular human diet, and are usually present within foods at low and variable levels which makes it difficult to control the type and amount consumed by individuals (Mcclements et al., 2015). Furthermore, nutraceuticals consist of a wide variety of phytochemicals making it difficult to be characterized and according to Ogbonna et al., (2012), for many herbs the active constituents are not known and in such cases, products may be standardized on the content of certain marker compounds. However herbal medicines rarely meet this standard for several reasons, including the lack of scientific information about the acting pharmacological principles. Hence, the standardization of the correct dosage form of those potential herbal medicines will result in a safer integration of it in conventional medical practices other than improving the bioavailability of the constituents in the herbal medicine. In herbal formulation, it is a must to have all the related knowledge of that particular drug including all its organoleptic characters to phytoconstituents to pharmacological action to its standardization in respect to various parameters via various techniques (Shulammithi et al., 2016).

1.7 Scope of study

Hence, this study aimed to investigate the antioxidant and anti-ageing properties of plant extracts such as the leaves of *M. oleifera* and *A. galanga* and calyx of *H. sabdariffa*. They are common natural herbal materials which are available and famous in Malaysia for their respective benefits. The plants are extracted and studied for their antioxidant properties through DPPH, Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) studies. Then, the plant extracts are further evaluated for their anti-ageing properties by the performance of lifespan assay using *Caenorhabditis elegans* (*C. elegans*) as the animal model. Those plant extracts which showed good antioxidant effects and increased the lifespan of *C. elegans* are then formulated into an anti-ageing natural product in order to improve the stability and efficiency of the nutraceuticals.

1.8 Objectives of study

1) To produce ethanolic and aqueous extracts of *M. oleifera* leaves, *H. sabdariffa* calyx and *A. galanga* leaves and to determine their potential antioxidant activity through DPPH, Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) assays.

2) To evaluate the effect of ethanolic and aqueous extracts of *M. oleifera*, *H. sabdariffa* and *A. galanga* on the life span of *C. elegans.*

3) To formulate the selected plant extract with both antioxidant and anti-ageing properties into a suitable final solid oral dosage form of natural anti-ageing product which is stable, simple to fabricate and cost-effective.

CHAPTER 2

EXTRACTION METHODS AND ANALYSIS OF EXTRACTS

2.1 Introduction

In many consequences, antioxidants and anti-ageing were relatable as antioxidants help to inhibit free radical production which causes ageing and thus protect the cells from damage. According to Ames, Shigenaga, & Hagen (1993), antioxidants plays the role of controlling and reducing the oxidative damage in foods by putting off or suppressing the reactive oxygen species (ROS) oxidation products which then leads to the increment of the food's shelf-life and quality. Other than that, high content of phenolic and flavonoids in medicinal plants have also been associated with their antioxidant activities that helps in the prevention of the development of age-related disease, particularly the one caused by oxidative stress (Azwanida, 2015). The antioxidant activities of the plants can be evaluated through several antioxidant assays.

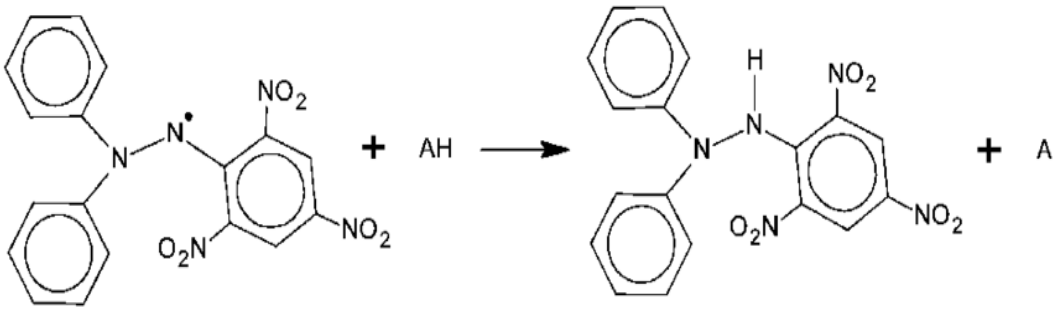
Plants of interest were extracted with suitable solvents and methods according to previous studies methodology. Proper measures must be taken to ensure that prospective active constituents are not lost, distorted or demolished during plant extraction and preparation especially if the plant has been chosen based on traditional uses (Fabricant & Farnsworth, 2001). In order to remove the phytochemicals from natural products, various solvent systems are accessible, and the choice of solvent relies mainly on the particular nature of the targeted bioactive compounds (Sasidharan et al., 2011).

Table 2.1 A brief summary of the experimental conditions for various methods of extraction for plants material (Source from Sasidharan et al, 2011)

	Soxhlet extraction	Sonication	Maceration
Common Solvents Used	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water	Methanol, ethanol, or mixture of alcohol and water
Temperature (°C)	Depending on solvent used	Can be heated	Room temperature
Time required	3–18 hour	1 hour	3-4 days
Volume of solvent required (ml)	150–200	50–100	Depending on the sample size

The antioxidant capacity of the plants involved were tested after the whole extraction and drying process. One of the simplest and widely used inexpensive method in order to determine the antioxidant ability of a particular natural source is by 1,1-diphenyl-2- picrylhydrazyl (DPPH) assay (Shekhar & Anju, 2014). DPPH is a stable free radical which has been used broadly in many researches to assess the radical scavenging activity of various plants. Several chemicals generally react with DPPH by H-atom donation or electron transfer and this reaction is often used for probing the antiradical or "antioxidant" ability of natural compounds. Upon accepting an electron or a hydrogen atom from a donor, it will become a stable diamagnetic molecule of DPPH, which is non-radical. When the antioxidant containing plant sources reacts with DPPH, which is a stable free radical, it becomes paired off in the presence of a hydrogen donor from the antioxidant and is reduced to the DPPH. As a consequence, the

absorbance decreased from the DPPH radical to the DPPH-H form and led in decolorization from purple to yellow colour. According to Shekhar & Anju, (2014), the more decolorization, the more is the reducing ability.



2,2'-diphenyl-1-picrylhydrazyl (purple colour)

2'-diphenyl-1-picrylhydrazyl (yellow colour)

Half-maximal inhibitory concentration (IC_{50}) was reported as the amount of antioxidant required to decrease the initial DPPH concentration by 50% (Do et al., 2014).

The investigation of the phenol and flavonoid content in the natural plant source is also crucial as they might serve as bioactive compounds that lead to the ability to scavenge those free radicals. Natural phenolic and flavonoid compounds are secondary plant metabolites which retain at least one hydroxyl group of an aromatic ring (Tungmunnithum et al., 2018). Phenolic compounds are excellent donors of electrons because they can directly contribute to antioxidant action by their hydroxyl groups (Bendary et al., 2013). In addition, some stimulate endogenous antioxidant molecules synthesis in the living cell (Cote et al.,2010). According to previous literature reports, phenolic compounds also display free radical inhibition, peroxide decomposition, metal inactivation and oxygen scavenging in biological systems in order to stop the burden of

oxidative stress (Babbar et al., 2015). Total phenolic content of the extracts was determined using Folin-Ciocalteu method. The Folin-Ciocalteu method is an electron transfer based assay, and gives reducing capacity which is expressed as phenolic content. Polyphenols in plant extracts respond with Folin-Ciocalteu to form a blue complex that can be quantified by spectrophotometry with visible light (Schofield et al.,2001). In general, the reaction provides accurate and specific data for several groups of phenolic compounds, as many compounds change color differently due to differences in unit mass and kinetics of reaction (Folin & Ciocalteau, 1927). Besides that, total flavonoid content was determined by aluminium chloride colorimetric assay ($AlCl_3$). The spectrophotometric test based on aluminium complex formation is one of the most frequently used methods for so called complete flavonoid determination, since the content of these compounds is deemed a significant parameter for the evaluation of food or medicinal plant samples (Pekal & Pyrzynska, 2014). This method involves the measurement of the flavonoid content of plant extracts from 410 to 430 nm after addition of $AlCl_3$ solution (Pekal & Pyrzynska, 2014).

In accordance with the extraction yields, the antioxidant capacity and the content of the phytochemicals such as phenolics and flavonoids varied amongst the extracts. Hence, the purpose for this part of study is to extract *M. oleifera*, *H. sabdariffa* and *A. galanga* with distilled water and ethanol in order to evaluate their antioxidant activity by performing DPPH, Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) assays.

2.2 Materials

The dried leaves of *M. oleifera* and *A. galanga*, and the dried calyces of *H. sabdariffa* were purchased from Herbagus Sdn. Bhd grinded form. All the plants were identified by Assoc. Prof Dr. Rahmad Zakaria, senior lecturer from School of Biological Science, Universiti Sains Malaysia (USM). Voucher specimens for *A. galanga* (Voucher number: 11841), *H. sabdariffa* (Voucher number: 11835) and *M. oleifera* (Voucher number: 11626) attached in the Appendix section. Ascorbic acid (Sigma-Aldrich, St., MO,USA) was supplied from Hovid Sdn, Bhd. (Ipoh, Perak). 1,1-diphenyl-2-picrylhydrazyl (DPPH) in powder form was purchased from Sigma-Aldrich (St.Louis, USA). 99.6% ethanol was bought from QRec^{TM} (Selangor, Malaysia)