EVALUATION OF FERTILITY AND TERATOGENICITY OF THE ANTHOCYANIN-RICH STANDARDIZED EXTRACT OF *Hibiscus sabdariffa* L. LOADED NIOSOMES IN FEMALE SPRAGUE DAWLEY RATS

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By

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	Degree Celsius
%	Percent
AEHS-Nio	Aqueous Extract *Hibiscus sabdariffa* L.- Niosome
ANOVA	Analysis of variance
ARASC	Animal Research and Service Centre
D	Diestrus
DCA	Drug Control Authority
E	Estrus
EIM	Ether injection method
FDA	Food and Drug Association
g	Gram
GMP	Good Manufacturing Practices
GUW	Gravid uterine weight
IQR	Interquartile range
JPMA	Japan Pharmaceutical Manufacturers Association
Kg	Kilogram
M	Metestrus
MBW	Maternal body weight
mg	milligram
ml	millilitre
n	Sample size
NPCB	National Pharmaceutical Control Bureau
OECD	Organization for Economic and Cooperation and Development
P	Proestrus
pc	Post-coitus
REV	Reverse phase evaporation method
ScCO2	Supercritical carbon dioxide fluid
SD	Sprague Dawley
TFH	Thin-film hydration method
USM	Universiti Sains Malaysia
WHO	World Health Organization

PENILAIAN FERTILITI DAN TERATOGENISITI FORMULASI NIOSOM EKSTRAK STANDARD *Hibiscus sabdariffa* L. KAYA ANTOSIANIN DALAM TIKUS BETINA SPRAGUE DAWLEY

ABSTRAK

Pelbagai tumbuhan perubatan seperti *Hibiscus sabdariffa* (rosel) telah mendapat perhatian dalam penyelidikan fitoterapi. Ekstrak *H. sabdariffa* yang dikapsulkan dalam niosom (AEHS-Nio) diformulasi untuk meningkatkan keberkesanan ekstrak tumbuhan ini. Kajian ini dijalankan untuk mengkaji kesan ekstrak AEHS-Nio terhadap kesuburan dan kesan teratogenik dalam empat puluh tikus betina Sprague Dawley. Empat dos AEHS-Nio yang berbeza iaitu 0 (kawalan), 250, 500 dan 1000 mg/kg/hari diberikan kepada tikus secara oral gavaj. Rawatan bermula dari tempoh sebelum mengawan dan berterusan sehingga hari ke-19 kehamilan. Sepanjang kajian ini, parameter reproduktif dinilai sehingga hari pengorbanan (hari ke-20 kehamilan). Keputusan yang diperolehi menunjukkan tidak ada perbezaan yang signifikan dalam kesihatan umum dan tingkah laku serta berat badan tikus sepanjang tempoh kajian. Di samping itu, purata tempoh kitaran estrus juga tidak terjejas secara statistik, walaupun beberapa ekor tikus menunjukkan kitaran yang tidak teratur. Selain itu, tidak ada perubahan yang signifikan pada indeks mengawan dan kehamilan, bilangan corpora lutea dan tapak implantasi, peratusan kehilangan pra-implantasi dan kematian pasca-implantasi dan berat organ reproduktif. Parameter fetus seperti bilangan fetus yang hidup, nisbah jantina dan berat fetus juga tidak dipengaruhi secara statistik oleh AEHS-Nio. Secara umumnya, tiada tanda kesan teratogenik yang diperhatikan kerana

tiada fetus yang menunjukkan malformasi kongenital. Kesimpulannya, kajian ini mencadangkan bahawa pemberian oral ekstrak AEHS-Nio sehingga 1000 mg/kg/hari tidak menunjukkan kesan ketoksikan yang signifikan terhadap kesuburan, kehamilan dan perkembangan fetus, tetapi mungkin mempengaruhi kitaran estrus tikus.

EVALUATION OF THE FERTILITY AND TERATOGENICITY OF THE ANTHOCYANIN-RICH STANDARDIZED EXTRACT OF *Hibiscus sabdariffa* L. LOADED NIOSOMES IN FEMALE SPRAGUE DAWLEY RATS

ABSTRACT

Various medicinal plants including *Hibiscus sabdariffa* (roselle) are gaining attention in phytotherapy research. Aqueous extract of *H. sabdariffa* encapsulated in niosome (AEHS-Nio) was formulated to improve the effectiveness of this plant extract. The present study was designed to investigate the possible fertility and teratogenicity of AEHS-Nio in forty female Sprague Dawley rats. The females were administered orally by gavage with AESH-Nio at four different dosages 0 (control), 250, 500 and 1000 mg/kg/day. Treatment began from pre-mating and continued through mating up to the 19^{th} day of pregnancy periods. Throughout this study, the reproductive parameters were evaluated until the day of sacrifice (day 20th of pregnancy). Results obtained revealed no significant differences in general health, behaviours and maternal body weights throughout the treatment period. Furthermore, the mean length of the oestrous cycle was not statistically affected, even though a few rats displayed irregular cycles. In addition, there were no significant differences in the mating and pregnancy indices, the number of corpora lutea and implantation sites, percentages of pre-implantation loss and post-implantation death and reproductive organ weights. Foetal parameters such as the number of live foetuses, sex ratio and body weight were also not statistically affected by AESH-Nio. Ultimately, there were no signs of teratogenicity observed since none of the foetuses exhibited congenital malformations. In conclusion,

these findings suggest that the oral administration of AEHS-Nio up to 1000 mg/kg/day did not pose any significant toxicity on the fertility and teratogenicity but slightly affect the oestrous cycle in rats.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Herbal medicines, either in the forms of mixed food, beverages, or raw material, are used worldwide since ancient times to improve general health and treat various diseases (World Health Organisation, 2021). According to Msomi and Mthokozisi, 2017), the use of herbal products is growing substantially as approximately 80% of the world's population are resorting to these products for basic health care needs. This shows that natural medicines are well accepted in both developed and developing countries. Generally, herbal medicinal products have long been considered safe (Moreira et al., 2014). However, the products may cause adverse effects due to toxic constituents (George, 2011) or possible diverse phytochemicals interactions (Lee and Bae, 2017). Moreover, most herbal medicines remain untested and their uses are either poorly monitored or not even monitored at all (Ekor, 2013).

Some countries like China have produced definitive guidelines for alternative medicine and food products, such as toxicity testing (Standard National Food Safety, 2014). According to the guidelines, three types of food must undergo toxicological tests in China including functional health food, new food raw material, and new food additives. Currently, in Malaysia, there are no specific guidelines for alternative medicines produced from herbal or natural ingredients. Nevertheless, the Malaysian Ministry of Health is well aware of the issue and seeks to develop new guidelines that

cover clinical trials of alternative medicine (Shah, 2017). This action should be put forward to enhance public trust and health towards herbal usage in the future.

Hibiscus sabdariffa, popularly known as roselle, is an annual shrub plant that originated from West Africa and was cultivated in Malaysia around the 1990s (Osman et al., 2011). Currently, *H. sabdariffa* is widely commercialised as food, soft drink, and supplement in the market due to its promising therapeutic values (Salami and Afolayan, 2020; Wu et al., 2018). This plant contains nutritional and medicinal properties, including vitamin A, thiamine, carbohydrates, calcium, iron and protein (Khan et al., 2017). It is also rich in natural antioxidants, including anthocyanins and vitamin C. Most anthocyanin pigments can be found in the flowers of *H. sabdariffa* (Cisse et al., 2009). Anthocyanin has various pharmacological benefits, such as can lower blood pressure, inhibit tumour formation, prevent diabetes, and improve heart disease (Kaur, 2015). Despite its medicinal values, anthocyanin is known to be very unstable. Its stability is affected by multiple factors like pH, temperature, light, and storage. In order to overcome this problem, it is therefore, the anthocyanin-rich standardised extract of *H. sabdariffa* L. loaded niosomes (AEHS-Nio) was formulated to be utilised throughout this present study.

Numerous researches has been conducted investigating the pharmacological activities of *H. sabdariffa*. According to Arsad et al. (2013), no toxic effect was observed on animals receiving oral administration of 15 g/kg calyces extract of this plant in an acute experiment. Njinga et al. (2020) further reported no toxic effect in Wistar rats on the sub-acute (28 days) administration of the plant extract at doses of 125, 250 and 500 mg/kg/day. Moreover, Sireeratawong et al. (2013) reported that

calyces extract of *H. sabdariffa* gavaged orally at 50, 100, and 200 mg/kg daily for 270 days did not cause chronic toxicities in both male and female rats.

Although this herb has shown promising potential as herbal medicine, there is a lack of studies on the effects of *H. sabdariffa* extracts on fertility and teratogenicity *in vivo*. Information related to the risks of fertility and teratogenicity of any substance, particularly herbal products, must be investigated. Teratogenicity occurs due to teratogens which can cause defects to the embryo or foetus. Teratogens can be categorised into four types which are physical agents, chemicals and drugs, infection, and metabolic conditions. The adverse effects of teratogens include abortions, low birth weight, congenital disabilities, lactation disorders, premature birth, et cetera (Kour, 2016; Sorelle et al., 2019). According to WHO (2014), congenital disabilities were responsible for 270,000 of the 3.1 million newborn fatalities. Hence, this study was conducted to evaluate the potential effects of AEHS-Nio on fertility and teratogenicity of female Sprague Dawley rats. The evidence from this research could provide scientific information on the safety profiles of this herb particularly on female reproductive health and would increase awareness of the use of herbal products during pregnancy.

1.2 Problem statement

The use of herbs as traditional medicine is well-established since ancient times. Despite the rapid development of synthetic drugs in the 21st century, the utilisation of traditional medication is continuously escalating due to the adverse effects of synthetic drugs reported to be higher than the toxicities of herbal products (Msomi and Mthokozisi, 2017).

Apart from this, people also turn to traditional medicines when their illnesses cannot be cured by the costly modern therapies (Al-Taweel and Perveen, 2019). Furthermore, traditional medicines are more accessible and affordable particularly in rural areas and developing countries. Although most people well accept the benefits of this plant, the information on its appropriate dosages, efficacy, safety, and mechanisms of action are still poorly understood (Rason et al., 2018). Moreover, a study from Carvajal-Zarrabal et al. (2012) found a lack of standardisation in terms of chemical components of the materials from traditional plants including *H. sabdariffa*. Therefore, this study was conducted with the main aim to gather the safety profiles of this herb prior to be commercially developed as herbal medicinal product.

1.3 Scope of study

The present toxicity study investigated the potential effects of the herbal extract on the female reproductive system by adapting procedures from the internationally accepted guideline which is the Organisation for Economic Co-operation and Development No. 422 (OECD, 2015). This comprehensive guideline covers observation on the effects of a test substance on both male and female reproductive systems, including mating behaviour, gonadal function, conception, development of the conceptus, as well as parturition (OECD, 2015).

The administration of AEHS-Nio to female animals in this study was conducted via oral route by gavaging to represent similar conditions as human consumption. The rats were treated at three crucial phases: pre-mating, mating, and pregnancy. During the pre-mating period, the design of the study was to monitor any adverse effect of herbal treatment towards oestrous cyclicity, general health and

behaviour of rats. The subsequent mating phase was to observe the capability of mating, mating index as well as the duration of successful mating of rats. Further, the latter pregnancy phase was designed to evaluate the possible fertility and teratogenic effects of the herb on dams and foetuses respectively (Sooi and Keng, 2013). In this study, three different dose levels (250, 500 and 1000 mg/kg) were chosen based on the previous experiment that corresponded to the requirement for low, medium and high doses for toxicity study (Rason et al., 2018).

1.4 Study objectives

1.4.1 General objective

The general objectives of the present study were to investigate the possible fertility and teratogenic effects of AEHS-Nio in female Sprague Dawley rats.

1.4.2 Specific objectives

The specific objectives of this study are listed as follows:

i. To examine the effects of AEHS-Nio on general health and behavioural changes of rats.
ii. To investigate the effects of AEHS-Nio on the oestrous cycle of rats.
iii. To investigate the mating capability and establishment of pregnancy in rats.
iv. To assess the effects of AEHS-Nio on fertility and pregnancy parameters of rats.
v. To determine the teratogenic potential of AEHS-Nio on rat foetuses.
vi. To examine the effects of AEHS-Nio on foetal parameters of rat foetuses.

1.5 Hypothesis

This study hypothesised that AEHS-Nio showed no significant effect on the reproductive system of female Sprague Dawley rats.

1.6 Significance of study

This toxicity study was conducted since there is limited information on the potential effects of *H. sabdariffa* on the female reproductive system. Moreover, the data collected from this study is expected to establish a relevant safety profile that can reduce the concern on its possible toxicity towards pregnant females and their foetuses. Apart from that, this study can also provide adequate information in understanding the risks related to herbal consumption and guarantee the safety of this herb on the female reproductive system. Last but not least, by having a toxicity study on this herb, it is hoped that it can increase trust and confidence among the public.

CHAPTER 2

LITERATURE REVIEW

2.1 Herbal medicine

2.1.1 Overview of herbal medicine

Herbal medicine is defined as the practice of using plant material for medicinal purposes. It also refers to herbs, herbal materials, herbal preparations and finished herbal products comprising plant parts, active ingredients, plant materials or a combination. According to WHO (2019) some countries define herbal medicine as natural organic and inorganic active components that are not derived from the plants such as mineral materials and animals. Generally, herbal medicine can be categorised as food, dietary supplements, cosmetics and herbal products. Over decades, herbs played a crucial role in health promotion and cure illnesses. Several drugs that are derived from plants have played a main role as therapeutic agents, such as aspirin (*Salix* spp L.), vinca alkaloids (*Catharanthus roseus* (L.) G. Don) and taxol (*Taxus baccata* L.) (Pelkonen et al., 2017). It is approved that herbal medicine is well accepted in the health field.

Many historical civilizations, such as Mesopotamia, Indian Ayurveda, ancient traditional Chinese medicine, and Greek Unani medicine, have documented the evidence for the use of herbs in the treatment of various illnesses. Based on the fossil records, plants have been used as medicines for at least 60,000 years (Shi et al., 2010). There are roughly 200,000 natural products of plant origin and several established plant-based drugs that eventually improve health.

To date, many people still rely on herbs to cure their illness due to several reasons, such as they reside in a rural area where modern medicine is limited. Moreover, they may live in communities where herbal medicine has better acceptability compared to modern medicine. Some of them possibly prefer herbal medicine as they believe it is safer and produces fewer side effects. Moreover, they may decide to avoid using modern health facilities as they believe these are costly, unsafe or untrustworthy (Graz et al., 2011; Ifeoma and Oluwakanyinsola, 2013).

2.1.2 Safety of herbal medicine

Herbs can be dangerous in many ways. However, many people choose herbs over conventional medicines, believing that 'natural' is safer than synthetic substances. This problem is causing concern about the safety and efficacy of the herbs. The dose of constituents administered plays a huge part in medicinal herbs; as Paracelsus said, 'all substances are poisons, there is none which is not'. This reflects that the right dose differentiates a poison and a remedy (Deshpande, 2002). A substance that is non-toxic at a low dose can be toxic at a high dose, whereas a substance that is highly toxic at a high dose can be regarded as safe at a low dose (Hill, 1997). Therefore, the toxicity of any substance is determined by the dose used. Apart from an overdose, false identification of medicinal plants, inaccuracies in the use of herbal medicines by both healthcare practitioners and users, and overuse and usage over long periods, even at moderate doses, can resulting adverse effects. Moreover, toxicity issues with medicinal plants may occur due to a lack of quality assurance and non-compliance with good manufacturing practice standards (Kumar, 2019).

In Malaysia, there are a few legislations, such as the Sale of Drug Act 1952, Poison Act 1952, Control of Drug and Cosmetic Regulations 1984, Advertisement and Sale Act 1956 and Protection of Wild Life Act 1972 regarding herbal products and supplements. These regulations are applied to all forms of drug sales, including those of online and offline. For instance, the Sale of Drugs Act of 1952 and the Control of Drugs and Cosmetics Regulations of 1984 engage in marketed drugs and address the responsibilities of several parties as well as the manufacturer of the registered products and notified cosmetics. Meanwhile, traditional and herbal remedies advertisement must comply with the Medicines (Advertisement and Sale) Act of 1956. In 2002, the government developed the National Health Policy of Traditional and Complementary Medicine, to provide security and complementary practices and products.

Herbal medicines also need to fulfill the standard of the Malaysian Health Ministry's Drug Control Authority (DCA) that focus on establishing particular limits for heavy metals, contamination of microorganism, adulterants, excluding the herbs with harmful effect, and adhering Good Manufacturing Practices (GMP). However, some local manufacturers register herbal products as food supplements instead of pharmaceutical products because the registration criteria for the food supplement are less strict than those for pharmaceutical products.

The National Pharmaceutical Control Bureau (NPCB) as the secretariat of DCA, is in charge of the safety and quality of traditional products. NPCB is responsible for performing a suitable analysis to ensure heavy metal levels do not exceed the approved limit. The traditional products that are properly registered and pass the analysis test will be given a registration number and hologram. Following that, post-

marketing surveillance via sample testing of registered products needs to be conducted to make sure the products comply with their safety quality.

At present, there are no specific guidelines for complementary and alternative medicine. However, the Malaysian Ministry of Health is well informed of this issue and trying to set up new regulations that cover clinical trials for alternative medicine (Shah, 2017).

2.2 *Hibiscus sabdariffa*

2.2.1 Botanical description

Hibiscus sabdariffa, also known as roselle, belongs to the Malvaceae family which has over 300 species found in tropical and subtropical worldwide. It is an annual herbaceous, bast fibre-bearing plant, and this subspecies is the most commercially important member of the genus of *Hibiscus* in the production of fibre. According to Khan et al. (2017) although this plant can grow the best in porous soil, it still can survive to various types of soil in a warmer climate. Currently, it is cultivated in Africa, America, Egypt, India, Malaysia, Mexico, Sudan, Thailand, Philippines, Vietnam, and Taiwan (Chewonarin et al., 1999; Saheb Ali et al., 2019). There is a massive debate among researchers over the origin of *H. sabdariffa.* Cobley (1976) reported that *H. sabdariffa* is a native plant of West Africa, then it was exported to other areas such as Asia and America. However, some researchers suggested that the *H. sabdariffa* originated from India (Mat Isa et al.,1985).

2.2.2 Botanical characteristics

H. sabdariffa is a bushy, erect and herbaceous subshrub that can grow up to seven to eight feet tall. It has smooth, cylindrical and red stems. The leaves alternate with 7.5 to 12.5cm long, have long or short petioles, and are green with reddish veins. The flowers, borne singly in the leaf axils, have 12.5 cm in width, have bright white to pale yellow flowers and they eventually turn pink as they wither.

H. sabdariffa also produces calyx, which in red colour pointed pods, and its function is to protect the plant. The calyx is edible and can be used as drinks (Islam, 2019). It requires four to eight months of growth with night-time temperatures of at least 20°C, 13 hours of sunlight, and a monthly rainfall of 130–250 mm during the first few months to avoid premature flowering. Da-Costa-Rocha et al. (2014) stated that the seed stock, local growing conditions, time of harvest, post-harvest handling and the drying step influence the quality of *H. sabdariffa*.

2.2.3 Phytochemical and composition

Phytochemicals refer to non-nutrient bioactive compounds found in plant parts such as leaves, barks, flowers, and stems **(**Figure 2.1**)** (Obouayeba et al., 2014**).** *H. sabdariffa* is primarily grown for its calyx. There are three kinds of calyx which are red, green, and dark red. Generally, the red calyxes are the most widely utilised, and this type can be distinguished by their concentration of anthocyanin. The examples of anthocyanin are Delphinidin 3-Sambubioside and Cyanidin 3-Sambubioside (Cahlíková et al., 2015).

Moreover, *H. sabdariffa* is also rich in minerals, amino acids, carotene, organic acids, vitamin C and total sugar in the calyx, seeds and leaves (Salami and Afolayan, 2020). Thakuria et al. (2018) reported that numerous metabolites had been extracted and identified from *H. sabdariffa*, such as flavonoids, triterpenoids, steroids, alkaloids and anthocyanidins. The nutrient content of the various sections of *H. sabdariffa* per 100 g is shown in Table 2.1**.**

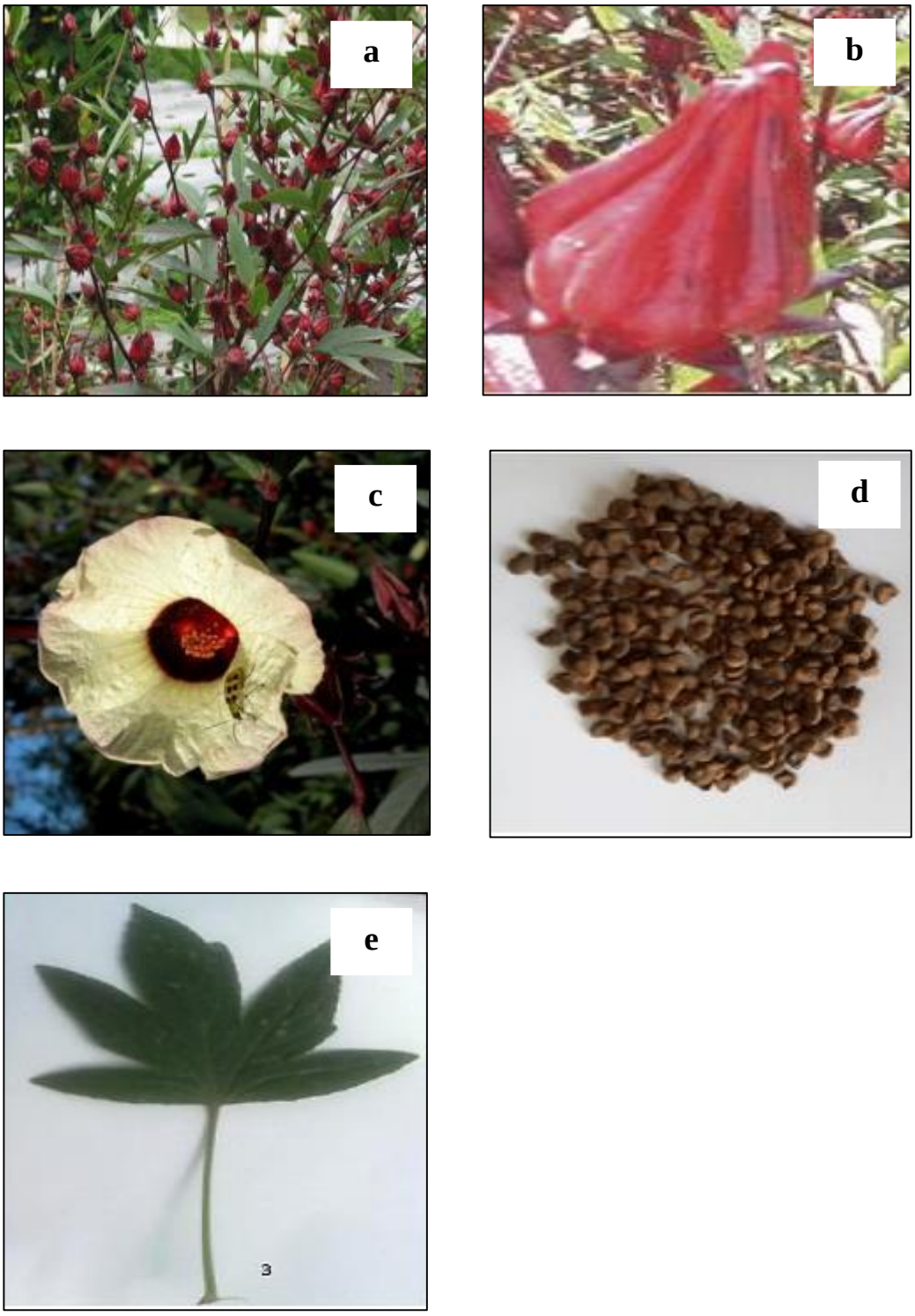

Figure 2.1 Various parts of *H. sabdariffa*; a) plant; b) calyx, c) flower, d) seed, e) leaves (Lim, 2014; Al-Okbi et al., 2017; Mohamed et al., 2012)

Table 2.1 Nutrient content of the different sections of *H. sabdariffa* per 100 g (Augustburger et al., 2002).

Nutrients	Calyxes	Seeds	Leaves
Protein (g)	2	28.9	3.5
Carbohydrates (g)	10.2	25.5	8.7
Fat (g)	0.1	21.4	0.3
Vitamin A (I. E)	-	-	1000
Thiamine (mg)	0.05	0.1	0.2
Riboflavin (mg)	0.07	0.15	0.4
Niacin (mg)	0.06	1.5	1.4
Vitamin C (mg)	17	9	2.3
Calcium (mg)	150	350	240
Iron (mg)	3	9	5

2.2.4 Biological activities

2.2.4.1 Antimicrobial

Research has shown that some Hibiscus species, including *H. sabdariffa* have been proven to possess therapeutic values (Obouayeba et al., 2014). Tolulope (2007) reported that aqueous methanolic extract of *H. sabdariffa* contains saponins, flavonoids, cardiac glycosides, and alkaloids that shown antibacterial activities against *Staphylococcus aureus, Clostridium sporogenes, Bacillus strearothermophilus, Micrococcus luteus, Serratia mascences, Escherichia coli, Klebsiella pneumoniae, Bacillus cereus, and Pseudomonas fluorescence*. Moreover, Higginbotham et al. (2014), suggested that *H. sabdariffa* extract was efficient to treat clinical samples and advised that extracts could be used as promising antimicrobials in foods against *Salmonella Typhimurium*, *E. coli* and *Listeria monocytogenes.*

2.2.4.2 Antioxidant

The dried calyx that has natural pigments demonstrated antioxidant activity and liver protection. Based on previous studies conducted by Wang et al. (2000), it was found that the concentrations of 0.10 mg/ml and 0.20 mg/ml of *H. sabdariffa* extract could decrease the leakage of lactate dehydrogenase, the formation of malondialdehyde and serum levels of hepatic enzyme markers (alanine and aspartate aminotransferase) as well as reduced oxidative liver damage. Apart from that, anthocyanins and protocatechuic acid of *H. sabdariffa* have been found to comprise high antioxidant levels (Lee et al., 2002) and antitumor effects (Chang et al., 2005; Lin, 2005).

2.2.4.3 Anticancerous

Anthocyanins have been demonstrated to induce apoptosis in cancer cells, particularly in HL-60 cells (Lin et al., 2017). The anti-oxidative activity of anthocyanins was assessed by their effects on LDL oxidation in a cell-free system and anti-apoptotic properties in RAW264.7 cells (Semaming et al., 2015). The study found that anthocyanins from *H. sabdariffa* can be used as a chemopreventive agent by inhibiting LDL oxidation and oxLDL-mediated macrophage apoptosis. Moreover, the inhibitory effect of protocatechuic acid on tumour promotion in mouse skin showed that protocatechuic acid could be used as a cancer chemopreventive agent against tumour (Tseng et al., 1998).

Anthocyanins isolated from *H. sabdariffa* petals were also inhibited cell growth and cause apoptosis in human HL-60 promyelocytic leukaemia cells (Chang et al., 2005). The extract also decreased cell growth in MCF-7 breast cancer cells and had anticancer effects in human stomach adenocarcinoma (Zainal et al., 2016).

2.2.4.4 Antihypertension

Studies on the efficacy of *H. sabdariffa* aqueous extract in hypertensive patients found a significant reduction of pressure difference in both systolic and diastolic compared to the control group (Haji Faraji and Haji Tarkhani, 1999). Tea of calyces also can reduce systolic blood pressure by 11.2% and diastolic blood pressure by 10.7%. The aqueous extract of *H. sabdariffa* petals demonstrated antihypertensive and cardioprotective effects in the rats. Infusion also was reported to significantly reduce systolic and diastolic pressure in hypertensive and normotensive rats (Ngamjarus et al., 2009). The effectiveness and tolerance of a standardised extract of

H. sabdariffa were studied in patients with mild to moderate hypertension, revealing a 10% reduction in systolic and diastolic blood pressure (Herrera-Arellano et al., 2004).

2.2.5 Anthocyanin and its functional properties

Anthocyanin is a natural water-soluble pigment derived from a flavonoid found in the phenolic compounds (Da-Costa-Rocha et al., 2014). The research reported that the anthocyanins such as pelargonidin, peonidin, cyaniding, malvidin, petunidin and delphinidin are often found in the fruits and vegetables like grapes, raspberries, roselle and purple cabbage (Brooks et al., 2019). There are two types of anthocyanins found in the *H. sabdariffa* extract, which are delphinidin-3-O-sambubioside and cyaniding-3-O-sambubioside (Obouayeba et al., 2014).

Apart from satisfies hunger, it also has anti-oxidant and bioactive properties associated with health benefits such as anti-diabetic, anti-inflammatory, and anti-cancer effects (Mozaffari-Khosravi et al., 2009; Wu et al., 2018; Yang et al., 2010). Moreover, anthocyanin-rich plant materials are currently labelled as functional materials because they have high levels of secondary plant metabolites and comprise a high percentage of the human diet.

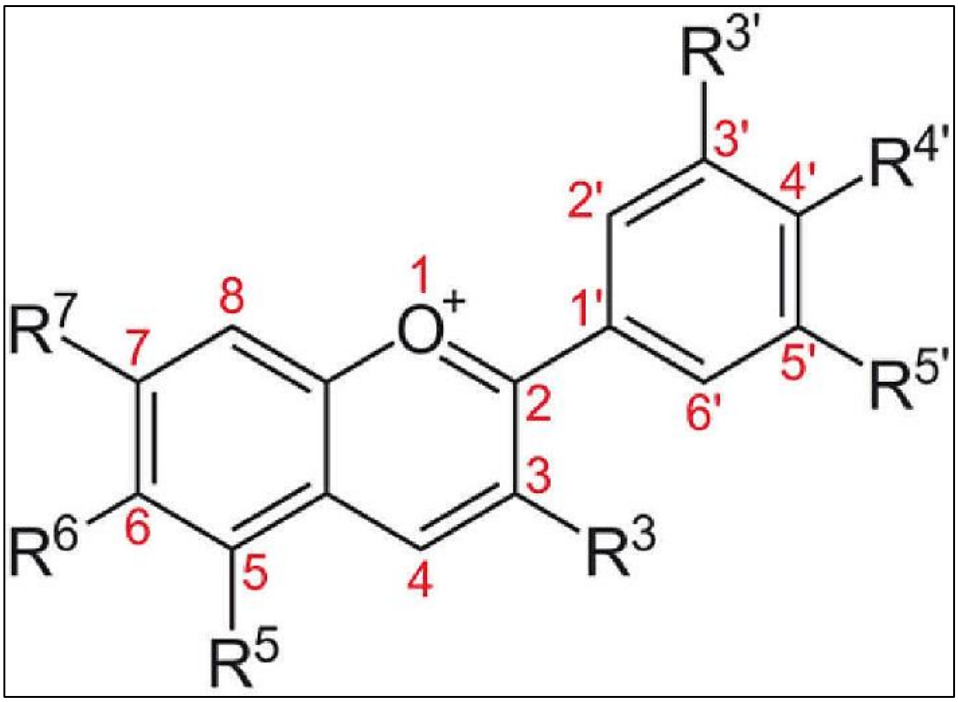

Figure 2.2 Basic structure of anthocyanin (Khoo et al., 2017)

2.3 Niosomes

2.3.1 Overview of niosomes

Niosomes are potential drug carriers with a bilayer structure formed by a self-associated aqueous process of nonionic surfactants and cholesterol. They are biocompatible, biodegradable, and nonimmunogenic. In addition, they have a long shelf life, are highly stable and allow the drug delivery in a managed or sustained manner at the target site (Seleci et al., 2016).

The niosomes have been thoroughly researched as a drug carrier (Bini et al., 2012; Tavano et al., 2014). Different kinds of nonionic surfactants have been found in the niosomes, allowing numerous drugs to be trapped with a broad range of solubility (Sahoo et al., 2014). The efficacy of the drug delivery of niosomes depends on the composition, size, number of lamellae and surface charge of niosomes. There are many methods for preparing niosomes, including thin-film hydration method (TFH), ether injection method (EIM), reverse phase evaporation method (REV), microfluidisation method, supercritical carbon dioxide fluid (scCO2), proniosome, transmembrane pH gradient, heating method and the “bubble” method.

2.3.2 Structure and components of niosomes

Niosomes are spherical and have unilamellar and multilamellar structures (Figure 2.3). The bilayer consists of nonionic surfactants, with or without cholesterol and the agent causing the charge (Ge et al., 2019). Niosomes are formed using several surfactants at variable combinations and molar ratios (Sagar et al., 2007). Alkyl ethers,

alkyl glyceryl ethers, polyoxyethylene fatty acid esters and sorbitan fatty acid esters are examples of active surfactants (Kumar and Rajeshwarrao, 2011).

The addition of cholesterol to the bilayer keeps its rigidity, resulting in fewer leaky niosomes. In addition, the charge inducers can deliver charge to the vesicles, increase vesicle size, and improving the drug entrapment efficiency. Meanwhile, positive charge inducers (cetylpyridinium chloride and stearyl amine) and negative charge inducers (diacetyl phosphate, lipoamino acid, and dihexadecyl phosphate) can improve the stability of the vesicles (Nekkanti and Kalepu, 2015).

The nonionic surfactants in niosomes have hydrophilic end faces outward, which is towards the aqueous phase. In contrast, the hydrophobic end faces inward to each other to form a closed bilayer structure with an aqueous solution (Yeo et al., 2017). Consequently, niosomes have a closed bilayer structure with hydrophilic inner and outer surfaces and sandwiched lipophilic space between them (Gandhi et al., 2012). Moreover, an energy such as heat or physical agitation is needed to produce a closed bilayer structure. Apart from that, van der Waals and repulsive forces present among the surfactant molecules are essential in maintaining the vesicular structure. The component of vesicles such as type, size, concentration, composition, surface charge and volume can alter the properties of resultant niosomes (Diljyot, 2012; Karim et al., 2010). Based on the size of niosomes, they can be categorized into three groups which are small unilamellar vesicles (0.025–0.05mm), multilamellar vesicles (>0.05mm), and large unilamellar vesicles (>0.10mm).

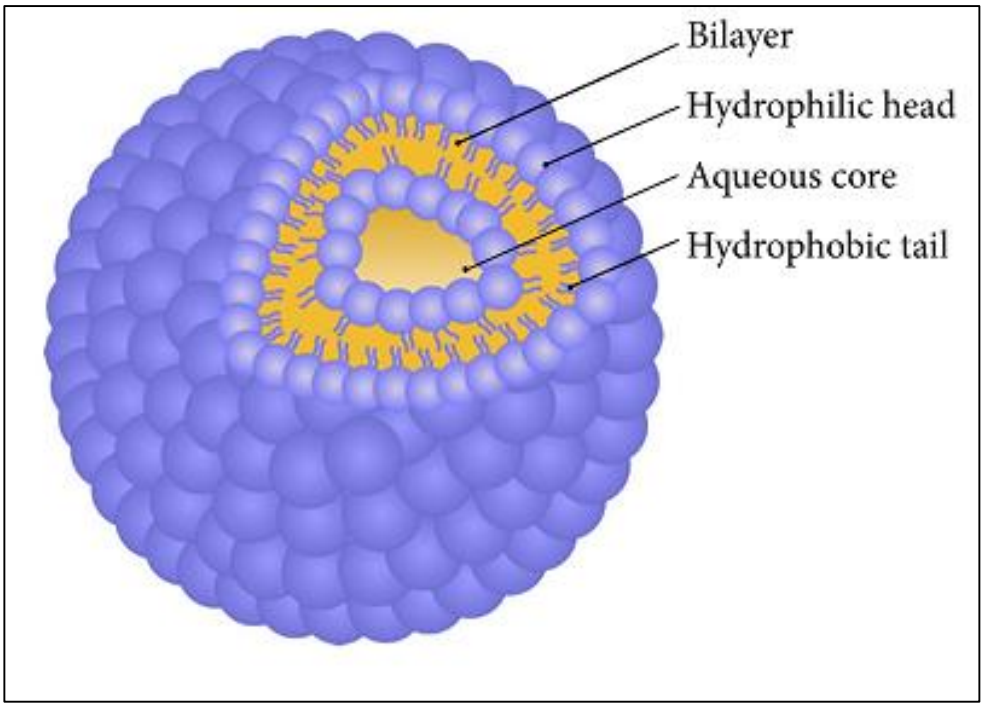

Figure 2.3 The structure of niosome (Seleci et al., 2016)

2.3.3 Niosomes as drug carriers

Numerous papers reported that niosomes are highly promising carriers that can deliver various pharmacological and diagnostic agents. They have good biocompatibility and low toxicity due to their non-ionic nature. The distinctive niosomal structure facilitated the development of effective new drug delivery systems to load both hydrophilic and lipophilic drugs (Chen et al., 2019). Hydrophilic and lipophilic drugs are captured into an aqueous core and membrane bilayer of niosomes, respectively. Currently, chemotherapy is the cancer treatment. However, efficacy of anticancer drug is limited due to low penetration into tumour tissue and have adverse effects on the cells. The use of niosomes as a novel drug delivery system is one of the ways to overcome this problem. The efficiency and specificity of cellular targeting of niosomal drug delivery systems can be improved by active targeting for tumour therapy employing a ligand coupled to the niosomal surface that could be actively absorbed. For instance, receptor-mediated endocytosis. Small molecules and macromolecular targeting ligands can be attached to niosomal surfaces to facilitate cell-specific targeting (Shah et al., 2020).

2.3.4 Strengths and limitations of niosomes in drug carriers

One of the major strengths of niosomes is that niosomes can act as an agent to deliver the drug in a gradual and controlled manner. Niosomes also contribute to stabilising the drug for a more extended storages period compared to liposomes. Niosomes have low toxicity and are highly compatible within biological systems due to biocompatible, biodegradable, and non-immunogenic nonionic surfactants. Furthermore, niosomes have many administration routes such as intravenous, oral et cetera. The niosomes also increase the oral bioavailability of drugs with low absorption

by improving the permeability of the drug into the skin. Niosomes are also an excellent way to deliver many drugs to targeted organs. They are easily altered due to the functional groups on their hydrophilic heads, and its surfactant does not need special conditions (Khoee and Yaghoobian, 2017).

On the other hand, niosomes have some limitations, such as a lack of research on the toxicity of niosomes. Most of the study is focusing on the toxicity effect of some surfactants only. Niosomes also have problems with physical stability. Furthermore, niosomes have a risk for aggregation, fusion, drug leakage or hydrolysis of encapsulated drugs during storage dispersion. Niosomes require much effort to be sterilised, and they are also not suitable for heat sterilisation and membrane filtration. Therefore, further research needs to be conducted in order to manufacture commercially niosomal preparations (Mehta and Jindal, 2013).

2.4 Reproductive and developmental toxicology

Toxicology is a study of an adverse effect of substances on humans, animals, and other living organisms, either by accident or with intention (Wexler, 2005). Toxicology also includes physical phenomena, such as radiation of various kinds and noise. Toxicology can be considered as a single molecular event that begins with exposure, continuing through distribution and metabolism, and lastly the interaction with cellular macromolecules and the expression of a toxic. It is a multidisciplinary science closely interrelated with other branches of sciences such as biology, pharmacology, medicine, chemistry, genetics, economics and even law (Hodgson, 2004; Radenkova–Saeva, 2008).

The issues related to reproductive and developmental toxicology have been known for ages, especially since the thalidomide tragedy. From 1957 to 1961, many pregnant women over the world consumed thalidomide for morning sickness. From this event, over 10,000 children were born with congenital disabilities, mainly phocomelia and amelia, after expose to thalidomide during the first trimester of gestation. They also suffer from anomalies that involved the eyes, ears and nervous system (Miller and Stromland, 1999).

Currently, thalidomide drugs are available in the market, and they are used to treat leprosy, Crohn's disease, HIV, multiple myeloma, and vascular disorders. However, this drug still cannot be prescribed for pregnant women or those trying to get pregnant. Since the thalidomide tragedy, the field of reproductive toxicology has been growing because of the public concern on the effect of environmental pollutants on the reproductive system (Hutz et al., 2006). Reproductive toxicology involves studying toxic or adverse effects of chemicals on sexual function and fertility, either the male or females and developmental toxicity in the offspring. Developmental toxicity can be defined as the detrimental effects induced during pregnancy, or as a result of parental exposure manifested at any period in the organism's life span (UNECE, 2013).

Following the thalidomide tragedy, the efforts of drug safety have increased around the world. Despite over 80,000 compounds currently on the market, only 200 of them have been assessed for toxicity and safety. Since the late 1940s, developmental and reproductive toxicity testing (DART) in animals is compulsory for the developing a new drug intended for humans. Approval from regulatory agencies such as the US