EVALUATION OF THE MALE REPRODUCTIVE TOXICITY OF THE ANTHOCYANIN-RICH STANDARDIZED EXTRACT OF *Hibiscus sabdariffa* L. LOADED NIOSOMES IN SPRAGUE DAWLEY RATS

MOHAMAD LUQMAN HANAFI BIN GHAZALI

UNIVERSITI SAINS MALAYSIA

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by

MOHAMAD LUQMAN HANAFI BIN GHAZALI

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LIST OF SYMBOLS

$	Dollar
%	Percent
°C	Degree Celsius
µl	microliter
cc/ml	Cubic centimeter per mililiter
cm	centimeter
cm^2	Square centimeter
g	Grams
IU/L	International unit per liter
kg	Kilogram
m	Meter
mg/kg	miligram per kilogram
mg/ml	miligram per mililiter
ml	mililiter
ng/ml	nanogram per mililiter
nm	nanometer
nmol/l	nanomole per liter

LIST OF ABBREVIATION

AEHS-Nio	Aqueous Extract *Hibiscus sabdariffa* encapsulated in niosome
AIDS	Acquire Immunodeficiency Syndrome
ANOVA	Analysis of variance
ARASC	Animal Research and Service Centre
CVD	Cardiovascular Disease
Cy-3-glc	Cyanidin 3-glucoside
DART	Development and Reproductive Toxicant
DNA	Deoxyribonucleic Acid
FSH	Follicle-Stimulating Hormone
HIV	Human Immunodeficiency Virus
IACUC	Institutional Animal Care and Use Committee
IMR	Institute of Medical Research
IQR	Interquartile Range
LD_{50}	Median Lethal Dose
LH	Luteinizing Hormone
LUV	Larger Unilamellated Vesicle
MLV	Multilamellar Vesicle
ncRNA	Non-coding Ribonucleic Acid
NOAEL	No-Observed-Adverse-Effect-Level
OECD	Organisation for Economic Co-operation and Development
PCA	Protocatechuic Acid
Pg-3-glc	Pelargonidin-3-glucoside
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SD	Sprague Dawley
SDF	Soluble Dietary Fiber
Sdn. Bhd.	Sendirian Berhad

SEM	Standard Error Mean
SUV	Small Unilamellated Vesicle
UPM	Universiti Putra Malaysia
US	United State
WHO	World Health Organisation

PENILAIAN TOKSISITI FORMULASI NIOSOM EKSTRAK STANDARD *Hibiscus sabdariffa* L. KAYA ANTOSIANIN TERHADAP SISTEM REPRODUKTIF JANTAN DALAM TIKUS SPRAGUE DAWLEY

ABSTRAK

H. sabdariffa telah lama digunakan secara meluas untuk tujuan perubatan. Ekstrak akueus *H. sabdariffa* yang terkandung di dalam niosom (AEHS-Nio) telah diformulasi untuk meningkatkan keberkesanan ekstrak tumbuhan ini. Ia diberikan kepada empat puluh ekor tikus jantan Sprague Dawley pada empat dos yang berbeza 0 (kawalan), 250, 500, dan 1000 mg/kg/hari secara oral gavaj selama 63 hari untuk menilai potensi kesan AEHS-Nio terhadap sistem reproduktif tikus. Sejumlah besar parameter telah dinilai sepanjang tempoh rawatan yang terdiri daripada tiga fasa; sebelum mengawan, semasa mengawan dan selepas mengawan. Keputusan yang diperolehi menunjukkan kesan AEHS-Nio sedikit ketara terhadap sistem reproduktif tikus jantan bagi beberapa kumpulan dos. Tiada kematian dan tanda ketoksidan fizikal dikesan. Prestasi mengawan juga tidak terjejas. Walau bagaimanapun, disebabkan oleh kesan anti-obesiti yang dimiliki ekstrak *H. sabdariffa*, berat badan tikus mengalami perubahan. Akibatnya, berat organ reproduktif juga berbeza secara statistik. Tambahan lagi, AEHS-Nio meningkatkan aras testosteron dan kiraan sperma untuk kumpulan dos 250 dan 1000 mg/kg, manakala dos 500 mg/kg menunjukkan aras rendah bagi dua parameter tersebut. Kumpulan dos 500 mg/kg dikesan menunjukkan data yang tidak konsisten bagi sesetengah parameter berbanding kumpulan lain yang mungkin disebabkan oleh faktor luar, bukannya AEHS-Nio. Oleh itu, data ini mencadangkan bahawa AEHS-Nio tidak bersifat toksik terhadap sistem reproduktif tikus jantan. Namun, AEHS-Nio disarankan untuk dimakan dengan berhati-hati selagi dos yang

diambil tidak melebihi 250 mg/kg sehari. Risiko penggunaannya terhadap manusia juga perlu dianggarkan secara munasabah.

EVALUATION OF THE MALE REPRODUCTIVE TOXICITY OF THE ANTHOCYANIN-RICH STANDARDIZED EXTRACT OF *Hibiscus sabdariffa* L. LOADED NIOSOMES IN SPRAGUE DAWLEY RATS

ABSTRACT

Hibiscus sabdariffa has long been widely utilised for medicinal purposes. Aqueous extract of *H. sabdariffa* encapsulated in niosome (AEHS-Nio) was formulated to improve the effectiveness of this plant extract. It was administered to forty male Sprague Dawley rats at four different dosages 0 (control), 250, 500, and 1000 mg/kg/day by oral gavage for 63 days to evaluate the potential effects of AESH-Nio on the male reproductive system. A substantial number of parameters were evaluated throughout the treatment period that was comprised of three phases; pre-mating, mating, and post-mating. Results obtained demonstrated that the effects of AEHS-Nio on the male rat reproductive system were slightly significant for certain doses. No mortality and any signs of physical toxicity were observed. The mating performance was also not affected. However, due to the established anti-obesity effects of *H. sabdariffa* extract, the bodyweight of rats were considerably changed. As a consequence, the reproductive organ weights were found to be statistically different. Furthermore, AEHS-Nio increased the testosterone levels and sperm counts of the 250 and 1000 mg/kg dose groups, while the 500 mg/kg dose group showed considerably low levels for both parameters. The 500 mg/kg dose group was detected to exhibit inconsistent data for certain parameters when compared to other groups, which might be caused by confounding factors instead of AEHS-Nio. Therefore, the current data suggest that AEHS-Nio did not toxic to the reproductive system of male rats. Nevertheless, AEHS-Nio should be consumed with caution as long as the dose

administered does not exceed 250 mg/kg of bodyweight a day.The human risk on male reproductive parameters associated with its use should also be reasonably estimated.

CHAPTER 1

INTRODUCTION

1.1 Study background

Herbs have been extensively utilised by human beings as medications for a long time ago. The first proof of the human consumption of plants for treatment dates from the Neanderthal period (Shipley and Kindscher, 2016). The most popular reason for people to opt for natural sources as the cure for certain diseases is that they are believed to have little to no side effects (Karimi et al., 2015).

Roselle or its scientific name *Hibiscus sabdariffa* Linn is an annual herbaceous shrub usually favored for its flower, leaves, and seeds to be used as the traditional medication. It contains abundant phytochemicals that are beneficial to human beings in the sense of treating diseases. The compounds found in this plant that has been reported are minerals, flavonoids acids, and vitamins (Salami and Afolayan, 2021). These contents have been described to possess pharmacological properties including antihypertensive (McKay et al., 2010), antihyperlipidemic (Mohagheghi et al, 2011), cardioprotective (Si et al., 2017), antioxidants (Wu et al., 2018), and anticancer (Akim et al., 2011) effects. Among all the contents, anthocyanin, which is in the group of flavonoids acid plays a vital role as the active ingredient for anti-cholesterol and antihyperlipidemic properties as well as serves as a cardioprotective agent. These therapeutic activities demonstrated by anthocyanin are very captivating and may help in treating the patient with cardiovascular disease (CVD) which emerges due to overaccumulation of lipid group as one of the main risk factors. CVD poses a great impact on public health as a burdening disease that globally scores the highest mortality (Nowbar et al., 2019).

The compound that has been reported to deliver various health benefits found in *H. sabdariffa* is anthocyanin. Anthocyanins give most fruits, flowers, and leaves blue, purple, and red colors. Consumption of anthocyanin-rich foods has been linked to a lower risk of cardiovascular disease (Mink et al., 2007; Cassidy et al., 2011; Jennings et al., 2012; McCullough et al., 2012) and cancer (Touvier et al., 2012; Zamora-Ros et al., 2012). Anthocyanins are well absorbed through the stomach (Milbury et al., 2002) as well as intestines (Cai et al., 2011). Anthocyanins such as cyanidin-3-glucoside and pelargonidin-3-glucoside can be orally ingested into the gastrointestinal wall in their intact state, undergo thorough first-pass metabolism, and enter the systemic circulation as metabolites (Fang, 2014).

In order to ensure that anthocyanin is still intact even in the circulation, we used niosome encapsulation in this present study. A niosome is a vesicle made of non-ionic surfactants, primarily produced by adding a nonionic surfactant and cholesterol as active ingredients (Moghassemi and Hadjizadeh, 2014). They have a bilayer structure and are structurally similar to liposomes. However, niosomes are considerably a better choice because both the physical and chemical stability of their formation ingredients and the non-ionic surfactants are more solid than lipids (Ge et al., 2019). It can entrap both hydrophilic and lipophilic drugs, either in an aqueous layer or in a lipid-based vesicular membrane (Arora, 2016). Niosome can potentially be a great help in tackling the issue of anthocyanins bioavailability. It can safely deliver the compound into the circulatory system in intact form.

As the bioavailability issues have been taken care of, natural products and traditional medicines such as *H. sabdariffa* would provide a good alternative and complement to conventional drugs. Nowadays, natural health products spring up like mushrooms after rain in the market with various health benefits which are sometimes

irrelevant and just an overclaim. These ambiguous products may cause health problems instead of curing the existing ones. Apparently, up to the moment, there are no established regulations for alternative medicinal products in Malaysia (Ismail et al., 2020). This makes those available in the market mixed with fake and overclaimed products. Once the product cause health problems such as kidney and liver diseases, distrust will arise among the consumers that ultimately may lead to the waste of great therapeutic potential of natural products.

Toxicity testing is designed as one of the scientific approaches to examine specific endpoints for generating data concerning the adverse effects of any xenobiotics (Krewski et al., 2020). Data acquired from safety testing can improve the trustworthiness of products and help to regain trust among consumers. To break it down, toxicity testing of drugs and chemicals need to be addressed for its effects on acute, subacute, subchronic, chronic, reproductive and developmental toxicities (Brodniewicz and Grynkiewicz, 2010). To date, the *in vivo* general toxicity evaluation of *H. sabdariffa* has covered acute (Onyenekwe et al., 1999), subacute (Prometta et al., 2006), subchronic (Mahfudh & Ikarini, 2018), and chronic (Sireeratawong et al., 2013) through oral administrations with no significant negative impact in critical outcomes. Despite the availability of this research, it is still not enough to convince that *H. sabdariffa* is safe to be consumed as complementary medicine since the effect on the male reproductive toxicities has never been conducted. The urgency of addressing this matter is due to today's situation where men of the reproductive age are more vulnerable to diverse serious diseases (Harris et al., 1992; Rabaça et al., 2015). Although the infertility issue is not fatal, it may cause devastating effects in the future as generation progresses. This fact can be dated back to the tragedy of thalidomide which provides evidence on the importance of reproductive toxicity

studies. Thus, this present study was carried out by adapting the OECD guidelines No. 422 which is a combination of OECD test guidelines No. 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents) and No. 443 (Extended One-Generation Reproductive Toxicity Study).

1.2 Scope of the study

This study was conducted to assess the *in vivo* reproductive toxicity of *H.sabdariffa* L. in male Sprague Dawley rats. The *H. sabdariffa* extract-loaded niosome (AESH-Nio) was administered to experimental animals through an oral route by gavaging. The rats were treated for 63 days to observe the effects on various endpoints including bodyweight changes, general health and behavior, reproductive capability, reproductive organ weight, hormonal level, and sperm count. All of the study design, animal selection, and parameters observed were adapted from OECD No. 422 (OECD, 2015).

The 63 days treatment period was categorised into three phases which were pre-mating, mating, and post-mating. The pre-mating treatment was specified to observe the general physical and behavioral effects of rats when administrated with AEHS-Nio before mating. The subsequent mating period was to evaluate the effects of AESH-Nio treatment on the mating capability of male rats. The latter post-mating phase was to monitor the effects of repeated AESH-Nio dose administration on bodyweight, behaviour, reproductive organ weight, reproductive hormone, and sperm count since the reproductive system has its own feedback actions to oppose the adverse effect delivered by the test substance as immediately as two days following treatment (D'Cruz et al., 2010).

1.3 Study objectives

1.3.1 General Objective

To evaluate the potential reproductive toxicity of *H. sabdariffa* niosomal (AEHS-Nio) encapsulation in male Sprague Dawley rats.

1.3.2 Specific Objectives

The specific objectives of this study are outlined below:

1. To evaluate the effects of AEHS-Nio on general health and behavioral changes of male rats.
2. To assess the effects of AEHS-Nio on the capability of mating in male rats.
3. To evaluate the effects of AEHS-Nio on the sperm count of rats.
4. To determine the effects of AEHS-Nio on the reproductive organs of male rats.
5. To determine the impacts of AEHS-Nio on the reproductive hormonal concentrations in male rats.

1.4 Study hypotheses

AEHS-Nio leads to no significant effect on the reproductive system of male Sprague Dawley rats.

1.5 Significance of the study

The rapid emergence of herbal medicinal products that overclaimed a lot of health benefits without any scientific trial may pose harm to the consumers' health (Ismail et al., 2020). The reputation of natural products which is always thought to be

safe will be tarnished when the consumers attain diseases or adverse side effects from the unscreened products. This is such a waste as Malaysia's megadiversity has tremendous potential to be a hub in producing numerous herbal products that provide desirable therapeutic effects with minimum toxicity.

In order to restore consumer trust in these medicinal products, toxicity testing is highly recommended as being conducted to AESH-Nio in this present study. This experiment also has completed the set of toxicity testing conducted on AEHS. Scientific data gathered from a wide range of toxicity studies i.e. general to reproductive and developmental toxicities are of utmost importance to verify the safety profile of the AEHS. Ultimately, the findings of this study will help to boost the acceptability of *H.sabdariffa* product to be able for not just being marketed here in Malaysia but can go further even to countries with stringent food regulations like China and Australia whereby they have their specific guidelines for production and marketing of such products (Zhou et al., 2019).

CHAPTER 2

LITERATURE REVIEW

2.1 Herbal medicine

Herbal medicine or also widely known as phytomedicine, is used to treat diseases and enhance human health using plants for medical and therapeutic purposes. Herbal medications have been described by the World Health Organisation (WHO) as completed, labelled drugs containing active ingredients, aerial, underground parts, plant material, other plant substances, or combination (Parveen et al., 2015). People have been looking for medicinal products in nature from ancient times in search of a cure for their illness. The beginnings of the usage of therapeutic herbs were intuitive (Petrovska, 2012).

Over time, reasons were established for the use of particular plants to cure certain illnesses; the use of medicinal plants eventually dropped the empiric framework and was solely based on explanatory facts. Nevertheless, the birth of iatrochemistry in the 16th century drive the usage of plants as the source of therapy and prevention (Petrovska, 2012). Moreover, the decreased effectiveness of synthetic medicines and the growing contraindications of their use revitalise the expectation of natural medicinal products as an alternative approach. Herbal medicines such as anthroposophy, naturopathy,traditional Chinese, Ayurveda, and allopathy have been used by several medical systems such as in Mexico, Oman and South Africa (WHO, 2019).

In general, the long history of usage in treating diseases based on information gained over several decades has made herbal medicines deemed safe. A careful selection of medicinal plants for use has seldomly produced hazardous deaths in

various cultural environments. Although hundreds of people die each year, with allegedly "safe" over-the-counter herbal medicines, dead or hospitalisation cases are rarely reported or even if reported with minimal evidence. This shows that the National Poison Control Centers of the United States do not have complete databases for adverse reactions related to herbs (Mensah et al., 2019).

2.1.1 Current trends in the use of herbal medicine

In the majority of poor nations, herbal medicine continues to be popular, and its usage spreads quickly to the developed countries as well. Seventy percent of all French and German medical practitioners have been authorised for herbal medicines prescription regularly. In addition, there is an increasing number of people seeking herbal treatments (Khan and Ahmad, 2019). Globally, 80% of the population is predicted to use herbs, with rates of 95% in underdeveloped nations (Tilburt and Kaptchuk, 2008). In China, it is around 30–50% of the overall medication intakes are from traditional herbal medicines. Herbal medications provide almost 60% of the first-line home treatments in Mali, Ghana, Zambia, and Nigeria. It is thought that over 50% of the people have taken herbal medicines at least once in their lives in Europe, the Northern American continent, and other well-developed nations. Approximately, 70% of individuals infected by HIV/AIDS utilise herbal formulations in London, South Africa, and San Francisco (Khan and Ahmad, 2019).

In the U.S., 158 million adults are thought to utilise herbal remedies and their use is constantly increasing. Above all, now there are more than US$60 billion a year for the worldwide market for herbal medicines and it is gradually increasing (Robinson and Zhang, 2011; Gunjan et al., 2015). In addition, it is noteworthy that both

conventional and herbal medications are more likely to be used by adult populations. Since this group has a greater prevalence of chronic illnesses because of their long-lasting adverse effects, generally prohibit long-term usage of complicated conventional medicines. Herbal treatments on the other hand give a long-term therapy with little to no adverse effects.

2.1.2 Medicinal plants used in conventional medicine

For the synthesis of novel medicines, medicinal plants play a significant role. According to the WHO, about 25% of current medical products are first developed from traditional flora. Later on, there are many more synthetic analogues produced from plant-derived model compounds. WHO has officially acknowledged herbal medicines as a key ingredient in primary healthcare (Khan and Ahmad, 2019).

Plant-derived medicines have contributed to contemporary treatments revolutionarily. In the case of Hodgkins, choriocarcinomas, non-Hodgkin lymphomas, leukaemia, testicular and neck cancer, vinblastine from *Catharanthus rosesus* has been utilised effectively (Das and Sharangi, 2017). For metastatic ovarian cancer and lung cancer, Taxol extracted from *Taxus brevifolius* is used. In addition, dated back to 1953, a chemical called serpentine extracted from the *Rauwolfia serpentina* root is notable for hypertension therapy (Hasan et al., 2009). In 1950-70, over 100 novel plant-based medicines, including reseinnamine, reserpine, deserpidine, vincristine, and vinblastine are developed in the US pharmaceutical sector. New plant isolating medicines including nabilone, teniposide, E-guggulsterone, plaunotol, artemisinin, lectinan, Z-guggulsterone, and ginkgolides have been released from 1971 to 1990 (El-Awady et al., 2015). In addition, numerous additional plant-derived chemicals, including aspirin,

ephedrine, digoxin, quinine, colchicine, and atropine have been identified in different pharmacological studies throughout the last decades. In the pharmaceutical industry, several additional medicinal products of plant origin have been invented from 1991 to 1995 including paclitaxel, topotecan, gomishin, and irinotecan (Rungsung et al., 2015).

2.2 *Hibiscus sabdariffa* L.

2.2.1 Origin

H. sabdariffa is a part of the Malvaceae family popularly referred to as the "red-sorrel" or "roselle." It is a widely known medicinal plant with more than 300 species scattered around the world in tropical and subtropical climatic regions (Anel et al., 2016). Originally from India and Malaysia, it is widely grown and transported to Africa initially (Mahadevan and Kamboj, 2009). It is also grown in Sudan, Egypt, Mexico, Nigeria, Saudi Arabia, Taiwan, the West, and Central America (Chewonarin et al., 1999; Ismail et al., 2008).

It is a plant or a woody sub-shrub, growing annually or perennially, reaching 2-2.5 m tall. The leaves are thick, palmately lobed, and 8-15 cm long and glossy, cylindrical red stems are distributed alternatively (Mohamed et al., 2007). The blooms are subsidiary and have a diameter of 8-10 cm, white and pale yellow, and a dim fleshy calyx at the base of the petals of 1–2 cm in size, with a thickness of 3–3.5 cm, fleshy and bright red as the fruit matures. Approximately six months are necessary to mature. In the early rainy season of mid-April, *H. sabdariffa* is cultivated and picked for calyxes about 3 weeks before bloom (El Naim and Ahmed, 2010).

Figure. 2.1. The flowering stage of *H.sabdariffa*.

Figure. 2.2. Freshly harvested of *H.sabdariffa* calyces.

2.2.2 Uses of *H.sabdariffa*

In traditional medicine, several components of the *H. sabdariffa* plant have been used to cure common cold, toothaches, and headaches. It is reported that in Thai traditional medicine, this plant is utilised for the treatments of kidney and urine bladder stones (Maganha et al., 2010). In India, the tribes use *H. sabdariffa* historically as an

ethnic cuisine for the treatment of illnesses such as discomfort in urination and indigestion.

In the Mexican culture, treatment of hypertension and some other diseases is done by employing the benefits of *H. sabdariffa* from its calyces and leaves concoctions (Da-Costa-Rocha et al., 2014). The extract of calyces combined with salt is useful for the healing of diarrhoea and human dysentery. It is usually believed to help in postpartum cases such as waist pain and other gynaecological disorder. The calyx (Sudan tea) infusion is used to alleviate cough and digestive disorder (Cisse, Dornier, et al., 2009; Cisse, Vaillant, et al., 2009) and also to reduce body temperature (Da-Costa-Rocha et al., 2014). The beverages are also consumed for hepatitis, fever, hypercholesterolemia, high blood pressure, antispasmodics, and antibacterial agents (C.-C. Chen et al., 2005; Khalid et al., 2012).

2.2.3 Nutritional constituents

H. sabdariffa contains carbohydrates, dietary fibres, proteins, vitamins, minerals, and bioactive substances. The relative dry weight composition for calyces of *H. sabdariffa*, which comprise of protein (7.51%), fat (0.46%) carbohydrates (69.62%), fibre (11.17%), and ash (11.24%) has been previously recorded (Abou-Arab et al., 2011). However, another study revealed that the most often identified component in the flower of this plant is dietary fibre (33.9%), which is high in insoluble (85.6%) and soluble dietary fibre (SDF) (14.4%) (Sáyago-Ayerdi et al., 2007). This SDF is connected with polyphenols that have the action of antioxidants in the colon.

Vitamins like niacin, riboflavin, and ascorbic acid are abundant in *H. sabdariffa* calyces (Mohammed, 2019). Niacin and pyridoxin are detected to such a

significant extent (Luvonga et al., 2012). It also contains a high content of minerals such as iron, calcium, magnesium, and potassium. Recently, it is found that calyces are rich in polyphenols, notably in relatively hydrophilic antioxidants such as delphinidin-3-glucoside, sambubioside, and cyanidin-3-sambubioside (Jabeur et al., 2017; Sinela et al., 2017). Moreover, the organic acids such as tartaric acid, malic acid, polyphenolic acid, and citric acid are found plentiful in the calyces. Hence, *H. sabdariffa* calyces is potentially an excellent source to be utilised in formulating functional nutraceutical products.

2.2.4 Anthocyanin

The foundation of many therapeutic agents originates from the plant's secondary metabolisms. Calyces are an intriguing source of possible bioactive compounds having antibacterial, anti-inflammatory, antidiabetic, anticarcinogenic antioxidant, hypocholesterolemic, and antihypertensive properties (Riaz and Chopra, 2018). Several scientific researchers have shown that polyphenols and flavonoids are abundant in *H. sabdariffa* calyces, which increase the nutritional benefits of the plant since these compounds are connected to its antioxidant properties (Riaz and Chopra, 2018).

Anthocyanins belong to the phytochemical flavonoid group. The molecules of anthocyanin are prone to degradation. Its stability relies on the pH, temperature, enzyme presence, light, and structure, other flavonoids, and phenolic acids (Idham et al., 2012).

Two main compounds of anthocyanins (delphinidin-3-sambubioside and cyanidin-3-sambubioside), and 2 minor compounds (dolphinidin-3-glucoside and

cyanidin-3-glucoside) are identified in *H. sabdariffa* calyces in several studies Gradinaru et al., 2003; B. H. Ali et al., 2005). Total anthocyanin in dry weight sample contained in *H. sabdariffa* including cyanidin 3-glucoside, (6.22 mg/g), delphinedin-3-o-sambubioside (7.03 mg/g), delphinidin-3-o glucoside (1.54 mg/g), and cyanidin-3-o-sambubioside (4.40mg/g) have been reported earlier by Jabeur et al. (2017).

2.2.5 Bioavailability of anthocyanin

Despite the molecular dimensions and forms of sugar or acylates linked, anthocyanins can be absorbed intact (Stalmach et al., 2012). Glycone, sugar content, and the acylated groups impact the rate and amount of absorption of anthocyanins (Tian et al., 2006). For complex anthocyanins, the degree of absorption may be reduced (Fang, 2014). After ingestion of rich anthocyanin food, the peak plasma concentration is achieved within 0.5–2 hours. After intake of berries or grapes in human trials, the maximum plasma levels of the total anthocyanins are 1-100 nmol/l (Prior and Wu, 2006). The anthocyanin ingested is immediately removed from the bloodstream.

The systemic bioavailability of anthocyanins in animal research is shown to be just 0.26–1.8% when intravenous doses are used as a benchmark (Ichiyanagi et al., 2006; Marczylo et al., 2009). It is calculated that fewer than 0.1% of intact anthocyanin discharged from urine is found in humans (Fang, 2014). This shows that anthocyanins are subjected to significant body metabolism before excretion into the urine.

However, the systemic bioavailability of intact anthocyanins is still not likely the best approach to assess the level of anthocyanin absorption (Fang, 2014). In the disposition of certain anthocyanins such as pelargonidin-3-glucoside (Pg-3-glc) and

cyanidin-3-glucoside (Cy-3-glc), the first-pass-metabolism plays a significant role. Following consumption of anthocyanin-containing fruit, high levels of plasma containing phenolic acid metabolites have been observed (Fang, 2014). From the intake of blood orange juice and black raspberries, between 30% and 44% of consumed Cy-3-glc was detected as protocatechuic acid (PCA) in human plasma (Vitaglione et al., 2007; W. Chen et al., 2012). After 71 mg of Cy-3-glc in humans has been discovered, it is revealed that the maximum concentration of PCA was around 0.5 mM (Vitaglione et al., 2007). Since PCA may be absorbed after oral intake (Fang, 2014), the phenolic acids are proposed to be formed before being absorbed within the gastrointestinal lumen (Guo et al., 2008; Russell et al., 2009; W. Chen et al., 2012).

2.2.6 Existing toxicity data on *H. sabdariffa*

Traditionally, *H. sabdariffa* is a very popular plant used for its medicinal purposes. Over the years, the possible adverse effect of *H. sabdariffa* has been a captivating topic of research. There are several studies conducted that have added informative data on the safety profiles of this plant for acute (Onyenekwe et al., 1999), subacute (Prommetta et al., 2006), subchronic (Mahfudh and Ikarini, 2018), and chronic (Sireeratawong et al., 2013) toxicity experiments. All data show that *H. sabdariffa* is entirely safe to be consumed even for a long period but precaution should be made if the concentration exceeds the limit of 1000 mg/kg bodyweight (Prommetta et al., 2006).

2.3 Niosome

Niosome is referred to as a non-surfactant liposome. Cholesterol inclusion as a formulation forms most of the niosome's structure. There may also be the employment of other excipients. The capacity of the niosome is higher than in prior emulsion preparation. The materials utilised to produce niosome make them physically similar to liposomes in which they have a bilayer structure. Nevertheless, niosome provides many additional benefits compared to liposomal products. Niosome dimensions are nanometric and tiny. The particle size varies between 10 and 100 nm. A common niosome vesicle would comprise of a non-ionic surfactant such as Span-60, which is generally stabilised by the addition of cholesterol, and a limited number of anionic surfactants like diacetyl phosphate, which also helps to stabilise the vesicles (Gharbavi et al., 2018).

The niosomes are categorised by the number of bilayers, for example, multilamellar vesicle (MLV) and small unilamellar vesicles (SUV) or according to dimensions like larger unilamellated vesicles (LUV) and SUV. Several types of niosomes are MLVs (size ≥0.05 μm), LUVs (size ≥0.10 μm), and SUVs (size =0.025–0.05 μm) (More et al., 2018).

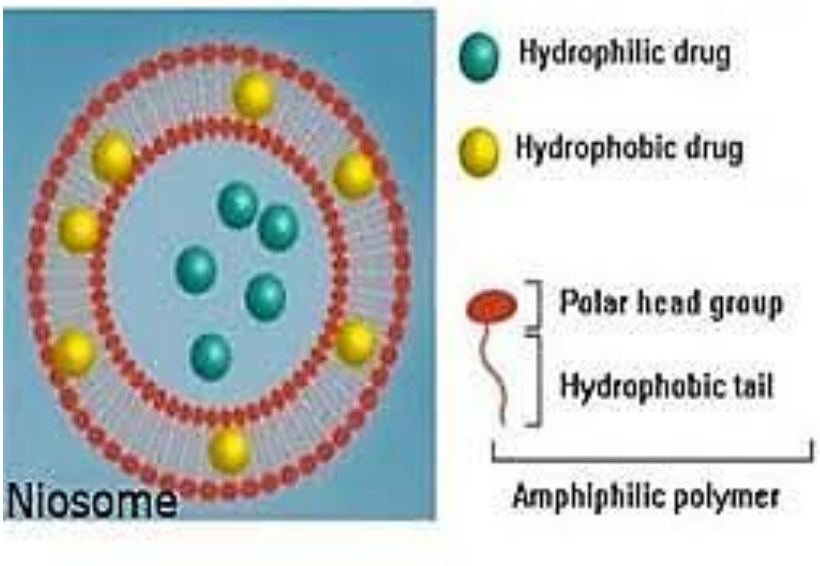

Figure. 2.3. Structure of niosome (Maurya et al., 2021).

2.3.1 Strengths and limitations of niosome

The advantages of using niosome as a drug delivery system rely on its vesicles that serve as depots, releasing the drug in a regulated way. They are osmotic and robust, and also improve the stability of the medication being held. The therapeutic performance of drugs molecules can be improved by delaying circulatory clearance, shielding the drugs from the physiological activity, and minimising impacts on target cells. The substances utilised are biologically degradable, biocompatible, and non-immunogenic. They boost the oral bioavailability and dermal penetration of poorly-absorbed medicines. It can be carried out by mouth, parenteral or topical routes to the site of action. No specific conditions are required for the handling and storage of surfactants. Due to the unique hydrophilic, amphiphilic, and lipophilic moieties architecture, they may therefore accept therapeutic molecules with various solubilities (More et al., 2018).

Ultimately, the benefits of niosome may provide a solution to the bioavailability issue posed by anthocyanin since niosome may aid in delivering the intact anthocyanin into the bloodstream. Niosomes, on the other hand, have physical stability issues. During storage dispersion, niosomes are vulnerable to agglomeration, fusion, drug leakage, or hydrolysis of encapsulated medicines. Furthermore, sterilising the niosomes requires a tremendous amount of work. Heat sterilisation and membrane filtration are not appropriate for niosomes (Bhardwaj et al., 2020).

2.4 Toxicity testing

The toxicity testing sometimes referred to as safety evaluation or toxicology tests, determines the degree to which a chemical of interest has a negative influence on the normal physiological activity of the organisms (Rovida et al., 2015). Toxicology testing is frequently performed for a certain chemical, method of exposure, exposure environment, exposure time, or for a specific organism of interest or a particular stage in developmental interest, by researchers that follow defined toxicological tests procedure. Tests for a chemical intended for human exposure are usually done during preclinical development. *In vitro* and *in vivo* research are performed for the determination in model organisms of tolerable exposure levels. When required, human toxicity testing is part of the consequent phase of research during a first-in-man study (Zuidmeer-Jongejan et al., 2015). The pharmaceutical industry, biotechnology firms, contract research institutions, or environmental scientists may be the ones responsible to execute toxicology testing.

2.4.1 Types of testing

The common types of animal toxicity testing for the evaluation of human health are acute toxicity (irritating/corrosive skin and eye, systemic acute toxicity), allergenicity (skin and respiratory sensitisation), repeated-dose toxicity, genotoxicity and mutagenicity, carcinogenicity, toxicity to developmental and reproduction, and biokinetics (also referred to as toxicokinetics or pharmacokinetics) (Parasuraman, 2011). In these instances, the protective objective is the human.

2.4.2 Repeated-dose toxicity testing

The main aim of repeated-dose toxicity studies is to classify the adverse toxicity of a substance for a certain period, up to its anticipated lifetime, resulting from repeated daily dosing and/or exposure usually from 3 weeks up to 2 years in animal studies. Effects include morphological changes, physiology, growth and lifespan, and clinical chemistry (Laroche et al., 2019).

For the quantitative risk evaluation of chemical compounds, repeated dose toxicity studies are necessary. The investigations include obtaining information on general toxicity features, toxicity to specific target tissues, dose-response relationship, responses to organism-formed toxic metabolites, retarded reactions, collective effects, and information on the effect's reversibility/irreversibility (Bae et al., 2021).

2.4.3 Reproductive and development toxicity testing

The main source for data on the effects of possible developmental and reproductive toxicants (DART) is from the traditional developmental and reproductive toxicity research in rats. Structural and functional changes that can influence

reproductive competency include reproductive toxicity (fertility, parturition, and lactation). Evaluation of the effects on reproductive damage is necessary including data on fertility, pregnancy, lactation, maternal as well as paternal behaviour. The assessments offer information on gonadal functions, oestrous cyclicity, mating behaviour, conception, parturition, lactation, weaning, and child growth and development (Hood, 2016).

Generally, developmental toxicity affects generations F or F2. Mortality, dysmorphogenesis (structural changes), growth change, and functional abnormalities are the four symptoms of developmental toxicity (Hood, 2016). Developmental toxicity deaths from conception to maturity can occur at any stage. Dysmorphogenic impacts are often considered as progeny deformities or variations to the skeleton or viscera of the offspring (Hansen, 2012). Changes in growth are typically viewed as delaying development, however, there may also be excessive growth or early maturity. Any permanent changes in normal physiological or biochemical operations might involve functional modifications (S. Wang et al., 2020).

The DART investigations aim to check whether a test substance can produce negative consequences on the male and female reproductive system or the developing foetus and to define the no observable adverse effects level (NOAEL) for the development and/or reproductive effect caused by the test substance. Developmental or reproductive NOAEL is the maximum dose of therapy studied and demonstrates no reproductive or developmental impact (Laufersweiler et al., 2012).

2.4.4 Male reproductive toxicity

Any substances can be assumed as toxic to the male reproductive system if they have the 8 key characteristics summarised from a study by Arzuaga et al., (2019). The first characteristic is changes in the growth, function, or death of germ cells. In general, the adult testis is less susceptible to toxicant-induced mutation than the immature gonad. However, modern treatment shows that exposure to toxic substances may disturb human spermatogenesis (Brennemann et al., 1997). Furthermore, new research suggests that endocrine disorders might imitate CatSper, a calcium channel found in human sperm cells that controls sperm motility and acrosomal exocytosis, with progesterone effects (Schiffer et al., 2014).

The second characteristic is the changes in the growth, functioning, or death of somatic cells. When exposed to harmful chemicals, somatic cells in various organ systems necessary for optimum reproductive outcomes have been shown to produce a detrimental influence on male reproductive systems. Somatic cells offer structural support and nutrition in normal conditions and control endocrine activities essential for adequate sperm and fertility (Wallig et al., 2017; Woldemeskel, 2017).

The proper development and operation of the male reproductive system include the gonadotropins, sex steroids, and thyroid hormones generated by the hypothalamic-pituitary-adrenal axis, the hypothalamic-pituitary-thyroid, and hypothalamic-pituitary-gonadal (HPG) axis (Dent et al., 2015). Some of the hormones heavily responsible for maintaining proper activities of the male reproductive system are follicular stimulating hormone (FSH), luteinizing hormone (LH), and testosterone. LH promotes testosterone synthesis from the testicular interstitial cells in men (Leydig cells). FSH stimulates testicular development and increases the synthesis of an androgen-binding protein by Sertoli cells, which are a component of the testicular

tubule required for the developing sperm cell to survive (Sacchi et al., 2018). This androgen-binding protein generates high local concentrations of testosterone around the sperm, which is required for proper spermatogenesis to develop. Sertoli cells produce inhibin, a polypeptide that may aid to control spermatogenesis locally when stimulated by androgens. Chemical changes in the reproductive hormones have been proven to have deleterious effects, including deformities and infertility throughout developing and sexually mature phases (Semet et al., 2017). Some substances can directly block the action of the steroid enzyme, reduce the expression of cells, or speed up metabolism (Hotchkiss et al., 2008).

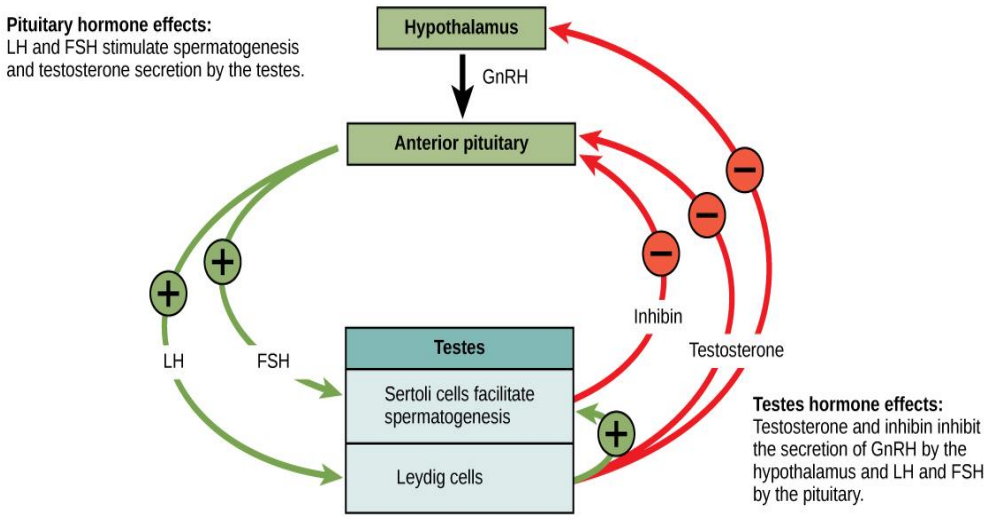

Figure. 2.4. A male hormonal feedback loop (Molnar and Gair, 2015).

Interfering substances for steroid and protein hormonal receptors may alter their normal action. This can be done in different ways such as the binding and activation of target cell receptors at the working site of a receiver and the blocking by endogenous hormones of normal activation, interference with normal interaction

between membrane and nuclear hormone receptors, or modification of co-activator or co-repressor recruitment for transcriptional complexes (Yeung et al., 2011).

Human data indicates that damage to DNA in sperm is linked to decreased fertility, poor embryonic quality, and loss of pregnancy (Ioannou et al., 2016). The intake of toxicants can disrupt the sperm DNA via direct methods, such as DNA breaking or DNA binding, or indirectly by oxidative stress induction (Delbès et al., 2010). Several endogenous contributing factors lead to sperm DNA damage, such as protamine insufficiency (by decreasing chromatin compaction in sperm) (González-Marín et al., 2012). Fully developed sperm has no DNA repair capability, thus the mature sperm that has been introduced to this toxicant cannot be repaired before conception (González-Marín et al., 2012). The toxicant exposure may also lead to sperm aneuploidy, which is the increase or disintegration of entire or parts of chromosome in the phase of gametogenesis. The incident of sperm aneuploidy was found to be linked to infertility, miscarriage, and foetal malformations (Ioannou et al., 2016).

Studies have demonstrated that some of the substances which cause endocrine interruption and other male reproductive toxicants affect the pattern of the DNA and histone methylation. It was revealed that the mechanism of action for these chemicals is based on epigenetic changes (Wu et al., 2015; Estill and Krawetz, 2016). Alteration of epigenetics in germ cells can potentially be passed to the descendants with transgenerational inheritance potential (Youngson and Whitelaw, 2008). The function of the sperm epigenetic in male fertility, embryo growth, and foetus wellness is becoming increasingly interesting. Histones are mainly replaced by specialised protamine during the maturation of sperm DNA to provide for a tightly compact packaging that will prevent sperm DNA from aberration (Carrell et al., 2012).

However, the preserved histones play an essential function in the development of the offspring as well as serving as a site for environmental reprogramming together with DNA methylation and non-coding RNA (ncRNA) (Siklenka et al., 2015; Belleau et al., 2018; Ben Maamar et al., 2018). Changes in the patterns of sperm DNA methylation are therefore proposed to have adverse effects on male fertility (Jenkins et al., 2016), embryo development (Aston et al., 2015), and susceptibility for progeny towards diseases (Jenkins et al., 2014).

An oversupply of ROS causes oxidative stress, which overpowers the antioxidant capacity of cells and tissues (Sabeti et al., 2016). Despite this, ROS are essential for optimal sperm activities including maturation and oocyte fertilisation. However excessive oxygen radical generation has been related to sperm abnormalities such as tail malformations and acrosome aberrations, increased DNA damage, and reduced sperm motility and survivability. (Sabeti et al., 2016). Moreover, male gamete are vulnerable to excessive oxygen radicals because they lack DNA repair mechanisms (Agarwal et al., 2014), have low antioxidant enzymes quantities (Sabeti et al., 2016), and contain high amounts of polyunsaturated fatty acids in the plasma membrane, which are candidates for lipid peroxidation (Agarwal et al., 2014).

A factor of male infertility is known to be caused by the inflammation of the male reproductive system induced by aetiological agent invasion, hormone disruptions, or exposure to dangerous substances in the environment (Fijak et al., 2018). Inflammatory reactions induced by chemicals are worsened by stimulated macrophages and lymphocytes, which generate several cytokines, chemokines, and growth factors found in human sperm. Elevated levels or generation of these cytokines has been linked to declines in sperm quantity, mobility, and survivability, as well as