INVESTIGATION OF *Smilax myosotiflora* METHANOL EXTRACT (SMME) ON ITS SAFETY PROFILE, MECHANISM OF PENILE ERECTION AND CHEMICAL CONSTITUENTS AS A MALE RAT APHRODISIAC

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by

RASMAIZATUL AKMA BINTI ROSDI

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Lots of love and thank you.

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LIST OF SYMBOLS

α	Alpha
*	Asterisk
β	Beta
Δ	Greek capital letter delta
°	Degree/ masculine ordinal indicator
δ	Delta
>	Greater than/ modifier letter right arrowhead
∞	Infinity
<	Less than
≤	Less than or equal to
µ	Micro
/	Per/ Solidus
%	Percent
±	Plus-minus
σ	Sigma
~	Tilde
X or x	Times

LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
AST	Aspartate aminotransferase
AOAC	Association of Official Agricultural Chemists
AOW	Absolute organ weight
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
Actβ	Actin-Beta
As	Arsenic
BAP	Benzylaminopurine
BSLT	Brine shrimp lethality test
bp	Base pair
C	Celsius
CCT	Corpus cavernosum tissue
CREB1	cAMP-response element binding protein 1
Ca	Calcium
Cd	Cadmium
Cl	Chloride
Cu	Copper
Cr	Chromium
Ct	Cycle of threshold
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
DCA	Drug Control Authority
DEHP	Bis(2-ethylhexyl) phthalate
DHEA	Dehydroepiandrosterone
DPPH	2,2-Diphenyl-1-picryl-hydrazyl
ED	Erectile dysfunction
FBC	Full blood count
FDA	Food and Drug Administration
FRIM	Forest Research Institute Malaysia
FSH	Follicle-stimulating hormone

dH_2O	Distilled water
GC-MS	Gas chromatography-mass spectrometry
GHS	Globally Harmonized System
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
GnRH	Gonadotropin-releasing hormone
g	Gram
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HNO_3	Nitric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
H_3BO_3	Boric acid
H&E	Hematoxylin and Eosin
ICP-MS	Inductively coupled plasma-mass spectrometry
IC_{50}	Median inhibition concentration
IL	Intromissions latency
IF	Intromission. frequency
JAKOA	Jabatan Kemajuan Orang Asli
K	Potassium
k	Kilo
kDa	Kilo Dalton
L	Liter
LC-MS/QTOF	Liquid chromatography-mass spectrometry/ Quadrupole Time-of-Flight
LC_{50}	Median lethality concentration
LDL	Low-density lipoprotein
LD_{50}	Median lethality dose
LH	Luteinizing hormone
Ltd.	Limited
MF	Mounts frequency
ML	Mount latency
MSB	Male sexual behavior
MSD	Male sexual dysfunction
Mg	Magnesium

$MgCl_2$	Magnesium chloride
m	meter
min	Minute/ minimum
mA	Milliamperes
N	Normality
NADPH	Nicotinamide adenine dinucleotide phosphate
NAION	Non-arteritis anterior ischemic optic neuropathy
NOS3	Nitric oxide synthase 3
NO	Nitric oxide
NPRA	National Pharmaceutical Regulatory Agency
NTC	Non-template control
Na	Sodium
NaOH	Sodium hydroxide
Na_2HPO_4	Sodium phosphate dibasic anhydrous
$Na_2HPO_4{}^*H_2O$	Sodium phosphate monobasic monohydrate
Ni	Nickel
n	nano
ns	Not stated in the study
n	Number of subjects
OECD	Organization for Economic Co-operation and Development
P	Phosphorus
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PDE2	Phosphodiesterase 2
PDE5	Phosphodiesterase 5
PEI	Penile erection index
Pb	Plumbum
pH	Power of hydrogen
ppm	Part per million
RAF	Residual aqueous fraction
RO	Reverse osmosis
ROW	Relative organ weight
RT-qPCR	Quantitative reverse transcription PCR
rpm	Rotations per minute
rt	retention time

S	Sulfur
SD	Standard deviation
SEA	Southeast Asia
SMME	*S. myosotiflora* methanol extract
SOP	Standard operation procedure
Sb	Antimony
sec	Second
TDF	Total dietary fiber
TIC	Total ion chromatogram
TPC	Total phenolic content
UPMS	Science Lab Management Unit
USA	United State of America
USM	Universiti Sains Malaysia
V	Volts
XEM	Sexually experienced male
X/XOD	Xanthine/xanthine oxidase
5’AMP	5′-adenosine monophosphate

LIST OF APPENDICES

PENYELIDIKAN TERHADAP EKSTRAK METANOL *Smilax myosotiflora* (SMME) KE ATAS PROFIL KESELAMATAN, MEKANISME EREKSI PENIS DAN KANDUNGAN KIMIANYA SEBAGAI AFRODISIAK TIKUS JANTAN

ABSTRAK

Smilax myosotiflora atau 'ubi jaga' merupakan tumbuhan popular dalam perubatan tradisional Malaysia sebagai ubatan afrodisiak lelaki. Kajian saintifik membuktikan bahawa ia mempunyai peptida yang dapat meningkatkan penghasilan testosteron sementara ekstrak metanolnya telah meninggikan tahap testosteron dan tingkahlaku seksual tikus jantan secara signifikan. Walau bagaimanapun, data terhadap kesan toksik, mekanisme tindakan dan kandungan bioaktif tumbuhan ini masih belum diketahui. Oleh itu, kajian ini bertujuan menyelidik ekstrak metanol *S. myosotiflora* (SMME) terhadap profil keselamatan, mekanisme ereksi penis dan kandungan kimianya sebagai afrodisiak tikus jantan. Makronutrien, mikronutrien dan ion logam berat *S. myosotiflora* dikaji melalui analisis proksimat dan spektrometri jisim plasma bergabung secara induktif (ICP-MS) untuk profil keselamatan pohon. Kesan ketoksikan SMME dinilai melalui kajian ketoksikan *in vivo* 'Limit Test' akut dan subakut berdasarkan panduan Organization of Economic Co-Operation and Development (OECD) no. 425 dan 407. Kajian keberkesanan dijalankan melalui ujian *in vivo* Dos Berulang 90-Hari ke atas tikus jantan berpengalaman seksual (XEM) (Kawalan-pelarut, SMME-800mg/kg, Viagra-5mg/kg) diikuti ujian tingkah laku seksual lelaki (MSB) dan analisis pengekspresan gen bagi menentukan mekanisme ereksi penis. Bagi kompaun kimia, ekstrak telah difraksinasi menggunakan kaedah fraksinasi cecair-cecair sebelum dianalisa melalui kromatografi gas-spektrometri massa (GC-MS) dan kromatografi cecair-spektrometri massa/kuadrupel 'time-of-

flight' (LC-MS/QTOF). Keputusan menunjukkan makronutrien, mikronutrien dan ion logam berat dalam *S. myosotiflora* berada dalam julat selamat. Tiada kesan toksik akut/subakut didapati kecuali peningkatan tahap testosteron dan penurunan aspartate aminotransferase (AST) secara signifikan dalam tikus jantan SMME serta kenaikan trigliserida yang signifikan dalam tikus betina SMME dalam ujian subakut. SMME tidak memberi efek dalam ujian Dos Berulang 90-Hari termasuk ujian MSB kecuali pengurangan urea yang signifikan dalam tikus XEM SMME berbanding kawalan dan pengurangan AST yang signifikan dalam tikus XEM SMME berbanding Viagra. Secara histologinya, tikus XEM SMME dilihat mempunyai densiti sperma yang lebih tinggi dalam kauda epididimisnya berbanding kumpulan-kumpulan lain. Untuk mekanisme ereksi penis, SMME berpotensi meningkatkan regulasi pengekspresan gen nitrik oksida sintase 3 (NOS3) serta menurunkan regulasi fosfodiesterase 5 (PDE5) dan 2 (PDE2) bagi menggalakkan relaksasi korpus kavernosum. Kompaun kimia utama dalam SMME adalah asid lemak, flavonoid, alkaloid dan terpenoid sementara stearamida dari fraksi baki akueus (RAF) berkemungkinan merupakan kompaun aktif afrodisiak lelaki. Kajian ini menunjukkan bahawa SMME adalah selamat, mempunyai potensi sebagai agen afrodisiak lelaki dan boleh dibangunkan sebagai ubat masalah disfungsi seksual lelaki (MSD) sekiranya lebih kajian dijalankan pada masa depan.

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ABSTRACT

Smilax myosotiflora or 'ubi jaga' is a popular plant in Malaysian traditional medicine as a male aphrodisiac. Scientific findings proved it possessed a peptide that increased testosterone production while the methanol extract has significantly elevated testosterone level and sexual behaviours in male rats. However, data on its toxicity effect, mechanism of action and bioactive compounds were still unknown. This study aims to investigate the *S. myosotiflora* methanol extract (SMME) on its safety profile, mechanism of penile erection and chemical constituents as a male rat aphrodisiac. *S. myosotiflora* were investigated for macronutrients, micronutrients and heavy metal ion through proximate analysis and inductively coupled plasma-mass spectrometry (ICP-MS) for the safety plant profile. Toxicity effects were evaluated through *in vivo* 'Limit Test' of acute and subacute toxicity tests using the Organization of Economic Co-Operation and Development (OECD) guidelines no. 425 and 407 accordingly. Repeated Dose 90-Day *in vivo* test was done on sexually experienced male (XEM) rats (Control-vehicle, SMME-800mg/kg, Viagra-5mg/kg) followed by male sexual behaviour (MSB) test and gene expression analysis to determine the mechanism of penile erection. For chemical constituents, the SMME was fractionated using liquid-liquid fractionation method prior to gas chromatography–mass spectrometry (GC-MS) and liquid chromatography–mass spectrometry/quadrupole time-of-flight (LC-MS/QTOF) analyses. Results showed that the macronutrients, macronutrients and heavy metal ion of *S. myosotiflora* were within safe limit. No acute/subacute toxicity

effects were noted except it significantly reduced aspartate aminotransferase (AST) and increased testosterone level in SMME-treated male rats while significantly increased triglycerides in SMME-treated female rats in the subacute test. The SMME also did not cause any effect in the 90 days treatment and MSB test except significantly reduced urea in the XEM SMME versus control rats and reduced AST in the XEM SMME versus Viagra rats. Histologically, the SMME-treated XEM rats were also have higher sperm density in their cauda epididymis than the other groups. For penile erection mechanism, SMME has potential to upregulate the expression of nitric oxide synthase 3 (NOS3) and downregulate the phosphodiesterase 5 (PDE5) and 2 (PDE2) to stimulate the corpus cavernosum relaxation. Main chemical compounds in the SMME were fatty acid, flavonoid, alkaloid and terpenoid while stearamide from residual aqueous fraction (RAF) was a putative male aphrodisiac active compound. The study revealed that SMME is safe, has potential as a male aphrodisiac and could be developed as a drug of the male sexual dysfunction (MSD) provided more studies are done in the future.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

In the Greek mythology, Aphrodite was referred to a daughter of Zeus which embodied the meaning of goddess of love, beauty, allure and procreation. Adapted from the word, an aphrodisiac is defined as the substance or component that is able to boost sexual desire or enjoyment (Owaba *et al.*, 2021). Today, its comprehension has been widened by the Food and Drug Administration (FDA) to include any materials which able to aid sexual function or improve sexual operations either in the male or female (Capogrosso *et al.*, 2021; U.S. Food and Drug Administration, 2023). The substance can be designated to any form which can increase sexual performance for example from foods, vitamins, beverages or natural/chemical compounds. Inability to perform in the intimate event may create huge problems where it emotionally and physiologically affects to the one and his/her partner (Hocaoglu, 2023). One may face depression, anxiety and low self-esteem disorders which are hardly encountered by physical symptoms (Traish, 2018; Hocaoglu, 2023). For a man, it is also an indicator that he may potentially have male sexual dysfunction (MSD) disorder, one of the most common health threats after heart and diabetes diseases among them (Andrea *et al.*, 2021; Capogrosso *et al.*, 2021).

It is estimated that more than 70% of men in the world are affected by the MSD regardless of their ages, ethnicities or cultural backgrounds (Kessler *et al.*, 2019). Therefore, a number of treatments have been developed in regard to the consistent growth of the cases. Unfortunately, many options including oral medications, invasive procedures and an emerging therapy, extracorporeal shockwave therapy (ESWT) caused significant drawbacks. The treatments are often very high at cost, not easily

accessible and associated with numerous serious side effects such as penis pain, headache, flushing, indigestion, swelling and urethra bleeding. Besides, even though various synthetic drugs, for instance, sildenafil (Viagra), papaverine, alprostadil, vardenafil or tadalafil are easily available in the market to overcome the problems, some of the adverse effects from the drugs including cost-consuming and serious adverse effects are yet unavoidable (Jain, 2019; Nimesh *et al.*, 2019). Patients with low blood pressure, severe liver/kidney disease, non-arteritis anterior ischemic optic neuropathy (NAION) or under the nitrate medications are not permitted to take such drugs due to the severe side effects which may cause to the organ systems failure (Capogrosso *et al.*, 2021; Hor *et al.*, 2022). While the ESWT offers a non-invasive alternative, its efficacy and long-term safety remain questionable.

The growing incidences and demands for better therapeutic drugs for men sexual incompetence issue have led to the scientific discoveries of natural compounds from aphrodisiac plants, organisms or microbes as the alternatives. The natural chemical constituent received a great deal and global attention by scientists because they are cheaper, more accessible, lesser toxic, no physical suffer and safer than the synthetic drugs or other conventional clinical treatments (Wattanathorn *et al.*, 2019; Niazi *et al.*, 2022). According to an extensive systematic review and the world ethnobotanical data, there were 300-500 herbal species identified to possess aphrodisiac attribution and potentially act as the remedies of the male or female sexual dysfunctions (Ramgir *et al.*, 2022; Kyarimpa *et al.*, 2023). Some of the documented aphrodisiac plants were *Boesenbergia rotunda* (Ongwisespaiboon & Jiraungkoorskul, 2017), *Eurycoma longifolia* (Farag *et al.*, 2022) and *Aloe barbadensis* (Erhabor & Idu, 2017). The plants may act diversely in treating sexual disorder either by stimulate the libido, elevate the production of nitric oxide (NO), increase the erection, decrease the

serum prolactin level or so forth (Niazi *et al.*, 2022; Adeli *et al.*, 2023; Alrumaihi *et al.*, 2024).

In Malaysia, other than *E. longifolia*, the long jack, the old folks and indigenous communities have orally practiced *Smilax myosotiflora* as one of their libido enhancer therapies (Rosdi *et al.*, 2022). *S. myosotiflora* which most recognized as 'ubi jaga' by locals, is a creeping plant that wildly grow in the forests of Peninsula Malaysia, southern Thailand and throughout tropical climate regions in Southeast Asia (SEA). Over the years, *S. myosotiflora* has been seen to be one of the most effective local aphrodisiacs which also has potential to counteract the MSD problem after numerous scientific findings on the plant and the related issue were disclosed (Rosdi *et al.*, 2022). Apparently, *S. myosotiflora* was found to have a comparable peak of protein with *E. longifolia*, *Rafflesia sp*. and *Labisia pumila* which responsible to increase the expression of testosterone level, the 4.3kDa peptide (Osman *et al*., 2007). In previous studies, the *S. myosotiflora* methanol extract (SMME) displayed significant aphrodisiac activities through rat model tests (Hoon, Leng & Kiyoshi, 2005; Wan, Ahmad & Sul'ain, 2013, 2016; Wan Yaacob, Sul'ain & Syed Sahil Jamalullail, 2014). This plant can be a promising drug source due to its therapeutic effects and as an alternative treatment for one of the most common male issues, the MSD provided more studies could be done to delve into deeper and affirm its potentials.

1.2 Problem statement

It is such a great public interest on the topic of aphrodisiacs where many people are seeking for them to enhance their sexual experiences. However, although the aphrodisiac plants have been used for centuries to help in enhancing sexual performance, the efficacy and safety of these remedies have not yet been fully explored. Natural products or herbal drugs that often pronounced as 'safe' and derived various pharmacological effects might be therapeutic on one side but could be toxic on the other side (Gaston *et al.*, 2020). Besides, Lüde *et al*. (2016) have listed the top ten of herbal plants that have most frequently been reported to cause the adverse effects including *Passiflora incarnata* and *Panax ginseng* which were also traditionally consumed for male aphrodisiac effects. While the synthetic drugs have documented its restrictions and safety data in treating sexual dysfunction, the detrimental effect of the aphrodisiac herbal plants still need more to be investigated. Till to date, the safety profile and toxicological effects of *S. myosotiflora* are still scarce. The findings also are necessary to establish the safety data of *S. myosotiflora* other than its therapeutic study in order to support and prove the capability of the plant as a male aphrodisiac plant.

Several reports have recognized the aphrodisiac potentials of *S. myosotiflora* tubers extracts through *in vivo* and *in vitro* tests (Osman *et al*., 2007; Wan *et al*., 2016). Previously, the SMME has significantly stimulated sexual behaviours in rats by increasing the penile erection index (PEI), reduced mount and intromission latency and improved copulatory rate (Hoon, Leng & Kiyoshi, 2005; Wan Yaacob, Sul'ain & Syed Sahil Jamalullail, 2014). However, despite all those, there has been no investigation on the plant onto the underlying mechanism of action in the male reproductive system. No finding on the influence of *S. myosotiflora* on mediators of

penile erection such nitric oxide synthase 3 (NOS3), phosphodiesterases 5 (PDE5) and 2 (PDE2) either regulated directly or indirectly in the particular pathway. For men, sexual performance and satisfaction are integral elements in cementing their masculinity and virility. Hence, the lack of knowledge on the mechanism of action would limit the ability to utilize the use of the plant, predict and develop new alternative in the male sexual problems. Therefore, it is important to research more and understand the influence of *S. myosotiflora* in the mechanism of action such as in penile erection in order to gain deeper comprehension on the plant's role as a sexual booster and become an alternative treatment to improve the male sexual problem.

Many drugs have been developed based on the natural products or the extracts which particularly rich with hundreds bioactive compounds for various therapeutic activities. However, due to the complex mixture of the biochemical constituents in plant, it is barely difficult to analyse and ascertain particular compounds that possess specific therapeutic properties. Earlier, aurones which are useful in the treatments of prostatic cancer, prostatomegaly, masculinism and breast cancer was detected abundantly in the *S. myosotiflora* (George *et al.*, 2010). However, the possible compounds with aphrodisiac activity in the *S. myosotiflora* plant have not yet been found. Likewise, the chemical constituents of the SMME were not also yet well determined. The identification of the detected compounds from the plant extract could be then interpreted accordingly and more doors on the *S. myosotiflora*-drug development will be wide opened in the future.

Aligned with the up-trend research and applications of medicinal plants to treat numerous medicinal issues and diseases, the toxicology studies supposed to come along in order to ensure the safety used of the plant especially when it relates to the reproductive organ system. Therefore, safety and toxicity evaluations are prerequisite

to ensure that individuals can make informed decisions on the use of aphrodisiacs and help to promote sexual health and wellbeing. Furthermore, to support their extensive use in procreation and drug discovery, the mechanism of action and chemical profile should also be devoted. By addressing and investigating these concerns, researchers will be able to identify the interest compounds with specific therapeutic effects and mechanism and utilize the information to develop new drug candidates including as a male sexual enhancer. Figure 1.1 illustrated the research questions that brought up to the scopes of this study.

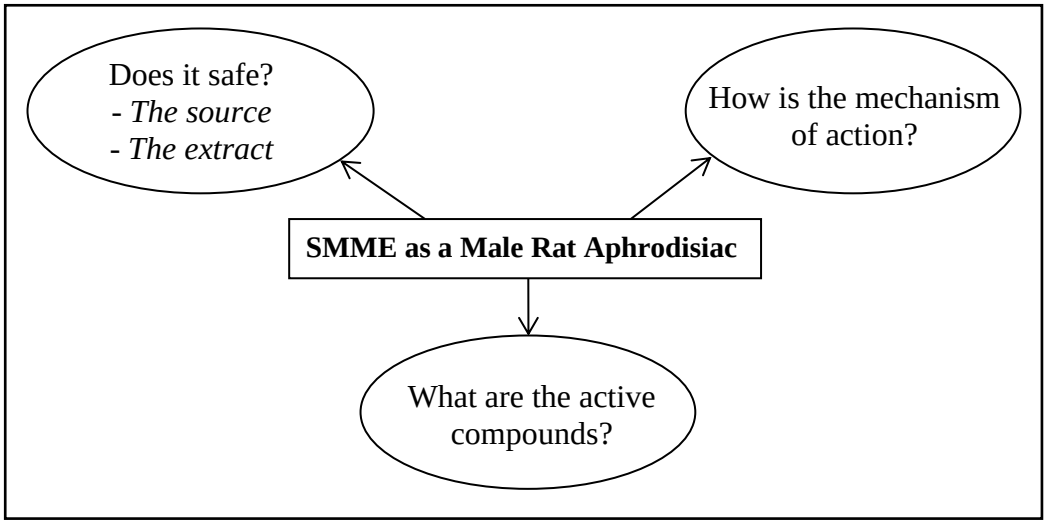

Figure 1.1 The inquisitions that brought to the scope of the study.

1.3 Justification and significant of the study

Countless numbers of men in the whole world have consumed the aphrodisiac-plant-based which was claimed to be natural and safe in order to improve their sexual intimacy. In order to ensure the safety usage and preventing the likelihood that a disease or other negative health outcome would occur, toxicology study is required to provide the essential information and knowledge that can be used by the researchers, regulatory agencies and the committee prior to the drug assessment. Toxicology studies on natural medicinal plants including the *S. myosotiflora* are critical to

ascertain its safety since many fatal side effects from plants have been reported worldwide. Herbal plant which committed with the adverse effects through the experimental studies may also act similarly to the human. In the recent years, there has been a noticeable upward trend in the toxicology studies, as reflected in a scientific search through PubMed engine, indicating a rising concern on the safety of natural products (Figure 1.2). Therefore, safety investigations of the *S. myosotiflora* extract and its source should be performed in order to determine its safety profile as a natural aphrodisiac agent. Safety data is essential for protecting public health and preventing harmful effects from the exposure of toxins especially when it relates to the reproductive system.

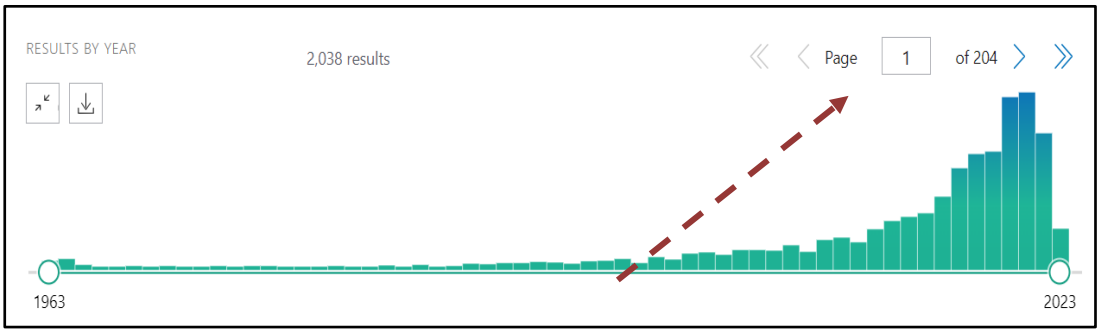

Figure 1.2 An upward trend in the toxicology studies using combined keywords and Boolean search of "toxicology AND natural product" from PubMed search engine accessed on 16th April2023.

Apparently, poor sexual performance and infertility become the significant factors to cause disintegration of many families and yet an unravelled issue in various parts of the world (Enema *et al.*, 2018). Furthermore, it is such an imperative concern for male to ensure and maintain their respect, prestige and honour in the eyes of their life partners (Kingsley Ogemdi, 2019). Herbal plants with aphrodisiac potentials have increased the demand in medicinal plants screening that deal and rectify with the male sexual incompetence. A lot of scientific findings have clearly reported that aphrodisiac plants intake also able to treat patients of MSD generally since they consisted of

heterogenous mechanisms in enhancing the sensuality and intimacy (Gu, Zhou & Wang, 2018; Leonti & Casu, 2018). The plants may act directly in the mechanism of action of male sexual functions including the penile erection event. But some of aphrodisiac plants helped in relieving oxidative stress which indirectly help to maintain the performance in the sexual course (Mahmudul Hasan, Tamanna & Haque, 2018; Goel & Maurya, 2020). Moreover, the available synthetic drugs for instances sildenafil, tadalafil and vardenafil are developed to overcome the problem by inhibiting the PDE5 enzyme in the erectile tissue of the penis to sustain the erection (ElHady *et al.*, 2023). Therefore, determining the mechanism of action in male sexual function namely the penile erection by *S. myosotiflora* extract will help to comprehend the biological mechanisms underlying sexual desire and arousal which can lead to the development of a new *S. myosotiflora* evidence-based treatments for sexual dysfunction problem.

Medicinal plants are historically valuable as the sources of chemical constituents for many pharmacological potentials. In the recent decades, plants have gained a broad attention among researchers for new drugs discovery or innovations in order to manage the increasing cases of many critical health issues (Rocchetti *et al.*, 2019). The plants consist of various combinations chemical compounds, for instances alkaloids, flavonoids, terpenoids and phenolics at different amounts which construct multiple functions in the mechanism of actions on the particular therapeutics events. Meantime, eurycomanone that extracted from *E. longifolia* and yohimbine from *Pausinystalia yohimbe* are the two examples of secondary metabolites responsible for the aphrodisiac activities and eventually had been developed as sexual enhancer drugs (Farag *et al.*, 2022; Niazi *et al.*, 2022). Ergo, study on *S. myosotiflora* chemical profile can be the door opener to a new drug discovery for male aphrodisiac and other potential

pharmacology activity related to the plant. Inevitably, fractionation on *S. myosotiflora* extract is the essential step to identify the phytochemicals in the plant in order to develop the plant chemical profile.

By investigating those issues, better understanding on the advantages and disadvantages of utilizing medicinal plants to boost male sexual level can be obtain in order to help the individuals making the informed decisions for their sexual health. It will also provide brighter future for overcoming one of the man's most common health threats in the world, the MSD. As researchers, the studies of *S. myosotiflora* on its safety profile, mechanism of action and chemical compositions will expectantly contribute to a new knowledge of the aphrodisiac plants and the male sexual health.

1.4 Objectives of the study

The overall objectives of this study are such below.

1.4.1 General objective

To study the safety profile, mechanism of action and chemical constituents of SMME as a male aphrodisiac agent.

1.4.2 Specific objectives

1. To investigate the safety profile of *S. myosotiflora* plant through nutrition and heavy metal toxicant analyses.
2. To evaluate the toxicity effects of SMME in acute and subacute tests *in vivo* based on the OECD guidelines no. 425 and 407 accordingly.

3. To determine the expression of NOS3, PDE5, PDE2 and CREB1 in the mechanism of action in penile erection after SMME treatment.
4. To analyse and identify the chemical constituents of SMME using GC-MS and LC-MS/QTOF that contribute to its potential aphrodisiac activity.

1.5 Hypothesis

SMME is safe and causes no toxicity effect to the rats in the *in vivo* tests. It able to significantly upregulate the expression of gene NOS3 and downregulate the expression of PDE5 in the mechanism of action of penile erection. The polar fractions of SMME consists of biochemical compounds that are responsible to the aphrodisiac effect in the male rats.

1.6 Conceptual framework of the study

This study was based on the integration of pharmacological, toxicological, molecular and phytochemical aspects of *S. myosotiflora* as a potential male rat aphrodisiac. The framework in Figure 1.6 addressed the safety profile of the plant and its methanol extract by requiring the toxicological assessments *in vitro* and *in vivo*. The extract of the plant was assumed able to contribute through specific molecular pathways that lead to its aphrodisiac properties and the mechanism of penile erection in rats. This study also incorporated the analysis of chemical constituents of SMME to identify possible active compounds, linking their presence of the aphrodisiac effects and safety data. Overall, this conceptual framework guided the investigation of SMME from the view of safety, mechanism of action and chemistry for its application as a male rat aphrodisiac. In order to achieve the output of study, related experiments were designed and developed based on the objectives of particular aspects to prove the call.

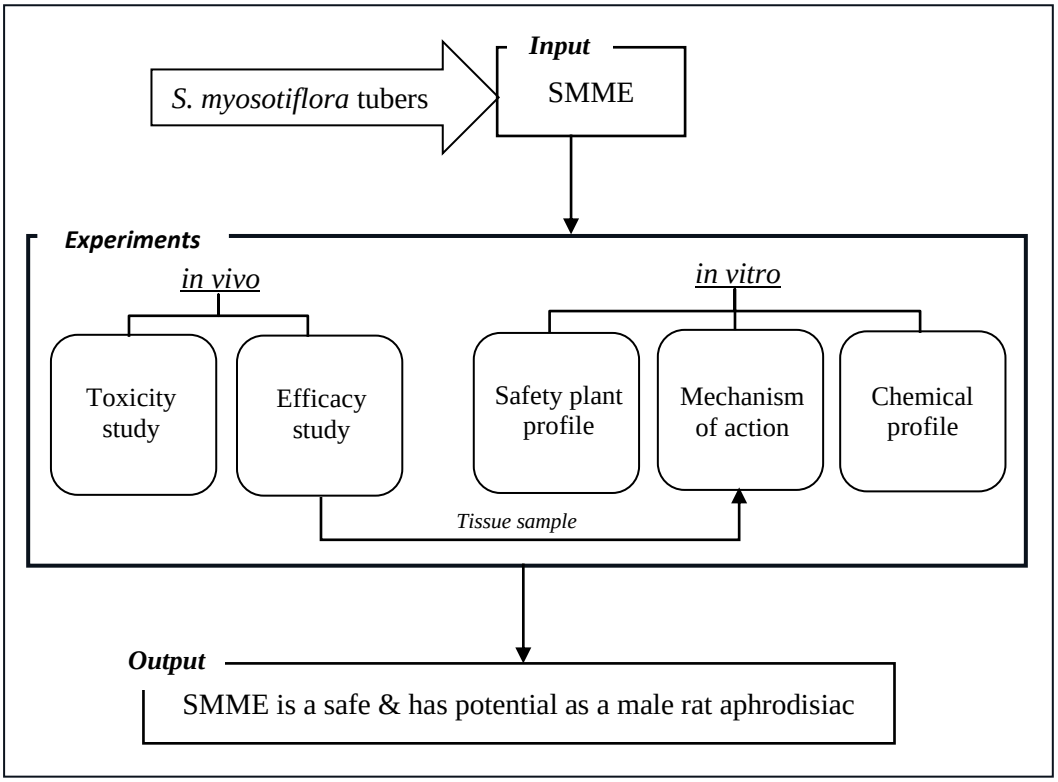

Figure 1.3 Conceptual framework of the study. *Abbreviation*: SMME - *S. myosotiflora* methanol extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Plants have become the basis of traditional medicine systems since thousands of years ago. The traditional medicine refers to the ancient, cultural-based healthcare practice where sometimes with unique approach in treating or preventing health problems or illnesses (Dasgupta, 2023). Today, current data illustrated that up to 80% of the populations use the traditional/complementary medicine globally (Tangkiatkumjai, Boardman & Walker, 2020). The WHO have recognized herbal medicines as the valuable resources and emphasized the need to establish a systematic inventory data of medicinal plants for implementation in modern regulatory standards and the adoption on good manufacturing practices (Wang *et al.*, 2023). Through rigorous scientific investigations and research, many traditional remedies have been scientifically proved their effectiveness and these have contributed hugely to the evolution of modern medicine today (World Health Organisation, 2019).

There has also been a long history of human's fascination discoveries across different cultures in seeking better and stronger sex drive and performance through the medicinal plants. The ancient literature texts like Ayurveda, Egyptian, Chinese and Roman civilizations had documented the human endless search of the substances which were known as the aphrodisiac either from plants, animals or creatures to enhance their sexual performances, desire and/or satisfaction or to treat sexual dysfunction (Bamola, Verma & Negi, 2018). Sexual relationship which characterized by a delicate balance of quality and frequency in the intimate encounters, is a major fundamental of reproduction involving conjugation, conception and procreation (Valsiner, 2020). It is also regarded as a fundamental human right and a measurement

of sexual health that contributed to the physical and psychological benefits including lowering stress, improving sleep and boosting the immune function (Coleman, Corona-Vargas & Ford, 2021). In contrast, sexual dysfunction encompasses the inability to attain normal sexual activity due to disturbances in sexual desire/performance or psychophysiological changes associated with the sexual response cycle in men or women (Briken *et al.*, 2020). It is a serious medical and social symptom that occurs in 10-52% of men and 25-63% of women (Feng *et al*., 2022). Hence, by enhancing the sexual behaviour or performance, it may increase the intimate relationship satisfaction and self-esteem in humans which brings to a healthier well-being and happier life.

A number of aphrodisiac plants that unravelled from the old claims which were pharmacologically proven to have beneficial effects regardless male or female sexual functions were now being marketed by pharmaceutical companies for its favourable benefits. The global aphrodisiac market volume in 2020 has emerged 4 billion dollars with the plant-based drugs as the major market player and it is expected to continuously grow in the years coming (Ismail & Aminyoto, 2018). Due to the high demands and benefits of the aphrodisiacs-natural based such as plants, more studies should be done in order to understand their safety profile and efficacy to the humans. Without the safety data, clear mechanisms of action and lack of knowledge to support the extensive use of sexual enhancer substances, the uses of these products might be risky to the human beings. Besides, with more clinical data in safety profile, exact mechanisms of action and the responsible bioactive compounds, more sexual problems also could be solved.

2.2 Male sexual problems

Throughout the history, findings on the male sexual related remedies have been a topic of great interest and cultural pursuit since the ancient times to the present days. Sexual performance is defined by the ability to maintain and achieve satisfactory of erection during the sexual intercourse. However, the aspect is often being influenced by a variety of psychological and physical factors such as mental stress, unhealthy lifestyle, chronic illnesses and drug toxicity which over the time will erode sexual response, satisfaction and lead to the relationship challenges and personal confidence of oneself as well as the partner (Hocaoglu, 2023). The downturn of erosion in men may also serve as an indicator to the MSD problem that currently have affected over 70% of men worldwide regardless of age, ethnicity or cultural background (Kessler *et al.*, 2019; Andrea *et al.*, 2021; Capogrosso *et al.*, 2021).

The prevalence of the MSD patients in Asia region was much higher with almost 95%, yet remained undiagnosed and untreated compared to the European counterparts due to their conservative cultural norms, society's stigma, lower socioeconomic conditions and lack of awareness (Irfan *et al.*, 2020). The MSD generally entails an alteration in sexual functions where its most related complaints were regarding erectile dysfunction (ED), hypoactive sexual desire disorder, anorgasmia or difficulties in reaching orgasm and premature or delayed ejaculation (Briken *et al.*, 2020; Irfan *et al.*, 2020). The ED which also termed as male impotence is defined as the persistent inability to achieve and maintain a rigid penile erection for satisfactory in the sexual intercourse (Sooriyamoorthy, Thushanth Leslie, 2023). Any atypical changes that affects smooth muscle tissues or corpus cavernosum, penile arteries, nerves, hormone levels, corporal endothelium or tunica albuginea could be the aetiology of the ED (Sooriyamoorthy & Thushanth Leslie, 2023).

In Malaysia, it was reported that 56.4% of the urban men were diagnosed with mild to severe ED (Arasalingam *et al.*, 2016). According to Ma et al. (2021), having repeated sexual relationship failure due to such erection problem was a significant cause which contribute to the infertility resulting to repercussion adverse effects on the emotional well-being, marital satisfaction and quality of the couple's life. A study also showed that the sexual disorder and low desire among female could be influenced by the male ED problem (Manurung & Rahardjo, 2023). Therefore, critical attention and investigation should be given on such issue for the betterment of sexual relationship and fertility in the couple's life.

2.3 Medicinal plants as male aphrodisiacs

With the continuous rise in sexual dissatisfaction issues and demands, men around the globe have putting hope on the nature for their quest in order to enhance their sexual desire, pleasure and performance. Earliest men had used stones, ritual dances and hunting ceremonies to exhibit their sexual powers before they went for more leisure and effective ways; the plants, foods or animals (Cvorovic & Coe, 2022). Decades later, throughout the regions and cultures worldwide, many herbal plants have anciently known to energize, vitalize and improve sexual functions and performances in men. And today, various herbal plants used in the folk medicines as aphrodisiac have been scientifically identified and uncovered their potential therapeutic effects. Looking back in the history, the traditional Chinese medicine had used the *P. ginseng* and *Boesenbergia rotunda* as the sex stimulants while *Lavandula bipinnata* was a popular Indian medicinal plant which has long been used as an aphrodisiac in Ayurvedic medicine (Dhaked, Nariya & Acharya, 2021). Meanwhile, through the manuscripts of medieval Persian medicine, onion was claimed to be one of the herbal plants which can enhance the male sexual functions (Nimrouzi, Jaladat & Zarshenas,

2020). Some of these plants were applied through various methods of administration including oral, topical, smoking and nasal applications with certain ways of extractions and preparations (Cvorovic & Coe, 2022). On the contrary, *E. longifolia*, *S. myosotiflora*, *Stemona tuberosa* and *Acalypha indica* were among the medicinal plants used by the old folks and native communities in Malaysia for the aphrodisiac effects (Bunawan & Baharum, 2015).

An extensive systematic review with the global ethnobotanical data has identified a spectrum of 300-500 herbal species showcasing aphrodisiac properties while others had underwent clinical trials for the approval or currently have research ongoing (Kyarimpa *et al.*, 2023). Till today, the search for natural aphrodisiac from such substances are intensifying probably due to its fewer side effects, less pain and cost, while the current conventional drugs and other types of medical treatments have limited efficacy, unpleasant side effects and contraindications in certain disease conditions (ElHady *et al.*, 2023). The aphrodisiac herbs are categorized based on the three distinct modes of action which were elevating libido, augmenting potency or intensifying sexual pleasure during the intimate encounters (Ramgir *et al.*, 2022; Kyarimpa *et al.*, 2023). More studies on the medicinal plants are prerequisite in order to explore more on its efficacy and mechanism as well the safety profile prior to the aphrodisiac drugs development.

2.4 Safety profile of male aphrodisiac plants

Initially, the herbal medicines were generally perceived as safe and 'natural' owing to their historical usage. Nevertheless, certain herbal plants that underwent clinical trials have been terminated due to the lack of efficacy, unacceptable safety profile plants, herb-drug interactions, adverse effects or toxicant ion metal contamination (Wang *et al.*, 2023). As the popularity of these 'love potions' continues to grow, it becomes increasingly crucial to delve more into the safety profile of these plants. Two important aspects in the safety plant profile include the nutritional (macronutrients/micronutrients) contents and the presence of heavy ion metals. Study on the nutritional content is essential as it helps to understand and determine the impact of the plant to overall health benefits beyond their therapeutics effects (Rai *et al.*, 2019). Concurrently, examining heavy metal levels such as chromium, plumbum, arsenic and antimony, is a paramount importance since an excess of those substances can lead to severe health issues and toxicity (Kumar *et al.*, 2019).

In Malaysia, the Forest Research Institute Malaysia (FRIM) under the Ministry of Natural Resources, Environment and Climate Change, is an authorized body that responsible to ensure the raw materials used in their plant-based products are genuine and unadulterated, hence, no vague or inadequate labelling compositions during the product developments occurred (http://www.frim.gov.my). Another executive party, the Drug Control Authority (DCA), under the National Pharmaceutical Regulatory Agency (NPRA) which empowers by the Control of Drugs and Cosmetics Regulations 1984 is an authorized body in ensuring the safety and quality of the pharmaceuticals, health and herbal-based products including the aphrodisiac's before their presence in the market (http://www.npra.gov.my). Rigorous investigations, inspections and quality control tests are conducted by the agencies in order to ensure the safety and

capabilities of the plant-based products are at their best for the consumers. Investigating the plant/product safety is not only a matter of fundamental principle in the quality control of herbal medicines but also as a safety plant profile to safeguard the well-being for those who turn to these plants for the benefits associated with a crucial human function, the reproductive system.

To date, many researchers have conducted the investigations on the nutritional contents and heavy metal ion levels in various aphrodisiac plants (Enema *et al.*, 2018; Kingsley Ogemdi, 2019; Dube *et al.*, 2023). A natural aphrodisiac of Nigerians, *Beta vulgaris* was reported to possess high moisture content, an essential element in macronutrients that pro-long the shelf-life of the plant and preserve protoplasmic content from the microbial attacks (Iwuozor & Afiomah, 2020). Meanwhile, through the comparisons of three different aphrodisiac plants, the protein yield was detected almost double in *E. longifolia* compared to *Polyalthia bullata* and *S. tuberosa* but none consisted heavy metal ion in the plants (Ahmad *et al.*, 2023). The presence of protein in aphrodisiac plants is crucial where glycoprotein plays role in testosterone-boosting abilities (Qi *et al.*, 2019). Meantime, the assessment of impurities in the substance can be conducted through the ash content analysis and a study by Parvathy et al. (2021) revealed that *Mimosa pudica* flowers contained of less than 10% total ash demonstrating lower impurities in the plant. Investigating both elements of the safety profile; nutritional and heavy metal ion, establish a better understanding on aphrodisiac plants besides of promoting their safety and proper usage.

2.5 Toxicology studies of male aphrodisiac plants *in vivo*

In 2016, Lüde *et al*. had listed ten herbal plants that have been most reported to cause the adverse effects including two of the popular traditional male aphrodisiacs in the regions which were *P. incarnata* and *P. ginseng*. The perception of herbal plants or natural products as inherently 'safe' due to their natural origin may be misleading since their various therapeutic potentials are sometimes converge with the risk of toxicity effects (Gaston *et al.*, 2020). It is intolerably unaccepted for human consumption when the plant is ultimately effective in therapeutic events but causing the detrimental effects at the same time (Rosdi *et al.*, 2022). Notably, plants that appeared to induce adverse effects in the experimental studies may exhibit similar responses to the humans too (Prasathkumar *et al.*, 2021). Hence, the toxicology studies towards the medicinal plants are becoming critical and undeniable to ascertain their safety due to a growing number in the worldwide cases related to the unsafe and fatal side effects, thereby, people can ensure and look for safer and sustainable options to improve their sexual health.

The 'OECD Guidelines' which were developed by an international organisation known as Organisation for Economic Co-operation and Development (OECD), are the most internationally relevant and accepted guidelines that can be used by the governments, industrial players and scientific laboratories in order to assess the safety profile of the chemicals or substances (http://www.oecd.org). These sets of guidelines include the assessment of toxicity effects through the *in vivo* studies from a single or regular exposure of particular chemicals or substance at the different durations or administrations. Some of the perennial OECD guidelines are Acute Oral Toxicity – Up-and-Down-Procedure (No.425), Repeated Dose 28-day Oral Toxicity Study in Rodents (No.407), Repeated Dose 90-day Oral Toxicity Study in Rodents

(No.408), Two-Generation Reproduction Toxicity Study (No.416) and so forth. Alternatively, for the substances that are likely to cause low toxicity or nontoxic at the meantime the animal welfare becomes a big concern, the OECD had gazetted the 'Limit Test' where animals or rodents involved in the test were used at the minimal number, five and were tested using the highest dose of the substance following the procedure of the guideline adhered.

A lot of researchers had implemented the 'Limit Test' in their studies in order to determine the acute or subacute toxicity effects of the aphrodisiac plants or drugs for example *P. yohimbe* (Okwakpam *et al.*, 2023), *Gardenia aqualla* (Abdulhamid *et al.*, 2022), *Griffonia simplicifolia* (Nyarko *et al.*, 2019) and *M. Pruriens* (Saikarthik, Vijayakumar & Vijayaraghavan, 2016). Toxicology evaluations *in vivo* towards the aphrodisiac plants are utmost important as a preparation of the herbal products and to set its limitation of the plants consumptions. Moreover, the research findings especially in the context of sexual dysfunctions problems, emphasize the critical importance to establish the toxicological effect of the aphrodisiac plant or drugs before any recommendation to the patients could be made in order to prevent potentially severe adverse effects.

2.5.1 Acute toxicity studies of male aphrodisiac plants

The acute toxicity study is conducted to determine the short-term adverse effect of a substance after its single administration given to healthy young rodents and proceeded with 14 days observation after dosing (OECD, 2001). The acute toxicity test can be performed based on the OECD guidelines no. 425, 420 or 423 where the OECD Guidelines No. 425 was a revised guidelines of OECD Guideline no. 420 and 423, allowing the test substance to be classified according to Globally Harmonized System (GHS). The OECD also has provided guidance entitled 'Guidance Document

on Acute Oral Toxicity, Environmental Health and Safety Monograph Series on Testing and Assessment No 24' for researchers to facilitate their selection on an appropriate acute toxicity guideline in conducting the acute toxicity test (OECD, 2001). A few parameters were evaluated in the acute test including behaviour signs of toxicity, body weight gain, gross necropsy, organ weight and median lethality dose (LD_{50}), an estimated dosage of a substance that caused to 50% mortality in a population of tested animals. Any sign that presence or changes in the assessed parameters might indicate that the substance may relatively pose the same risks to other living organisms, including the humans (Erhirhie, Ihekwereme & Ilodigwe, 2018).

The published studies on the acute toxicity study done towards the aphrodisiacs plants or plant extracts demonstrated numerous findings on their toxicity effects. A traditional African aphrodisiac, *P. yohimbe* which administered at 5000mg/kg resulted in a complete mortality of the treated female rats (Okwakpam *et al.*, 2023). Elsewhere, the single intake of 2000mg/kg *Caesalpinia bonduc* ethanol extract has significantly reduced the glucose and creatinine levels in the treated rats (Sindete *et al.*, 2019). Another traditional aphrodisiac plant, *Parinari campestris*, displayed no sign of toxicity behaviour, no significant changes in any parameters and no mortality occurred in the acute toxicity tests, hence, they were pronounced as nontoxic and safe with the LD_{50} of the plant was estimated to be more than 2000mg/kg (Sundaram *et al.*, 2021). Meanwhile, the LD_{50} of *Cassia sieberiana* was found to be more than 5000mg/kg after no mortality or lethality toxicity effects occurred among its treated rats after a fortnight monitoring (Evenamede *et al.*, 2019).

2.5.2 Subacute toxicity studies of male aphrodisiac plants

The subacute toxicity test is intended to evaluate the toxicity of a substance after repeated administration of 2-4 weeks duration. To test for oral administration of subacute toxicity effect, the OECD guideline no. 407 (Repeated Dose 28-Day) was usually applied by the researchers. Some of the parameters examined in the subacute test are behavior signs of toxicity, food intake, body weight gain, gross necropsy, organ weight and hematology, biochemical and histopathology analyses which involved both sexes of the tested rodent species. Through this guideline, the reproductive organ toxicity effect may also be disclosed (OECD/OCDE, 2008b). A study done on an aphrodisiac plant extract of *Melandrii erba* at dose 1000mg/kg, revealed a significant increase of glucose and total protein in the treated female rats after its 28 days dispensation (Park *et al.*, 2016). Meanwhile, through the histopathology analysis on the liver of 1000mg/kg *Mesua ferrea* treated rats, mild lymphocytic infiltration and degeneration of hepatocytes episodes were observed (Shirsat *et al.*, 2022). On the other hand, despite of its used as a traditional aphrodisiac, *Massularia acuminata* extract was found to cause some disturbances such as rarefaction of spermatids, thinning of the tubular lumen and disorganization of the germ cells in the testes after its long-run usage, 28 days (Gbogbo *et al.*, 2021).

2.6 Physiology of penile erection

Penile erection or tumescence refers to the physiologic process when the penis becomes engorged with blood and caused to firming and enlarging the organ which usually in response to sexual arousal or sometimes spontaneously. Comprehension in anatomy of reproductive organs-related, the associated hormones and molecular pathways are required in order to understand the mechanism of male sexual behaviour (MSB) and develop the therapy of erectile dysfunction (Mirone *et al.*, 2023). The

primary parts that involved in this intricate process is the erectile tissue of the penis namely the corpus cavernosum tissue (CCT). Meanwhile, the crucial hormone androgen involved in this process is testosterone, a hormone that play role in maintaining the integrity of the erectile tissue and promoting sexual desire and performance (Panchatsharam, Durland & Zito, 2023). Balanced interplay in the gene expression and regulation are integral to coordinate the pathway of penile erection. This will emphasize the unique connection between reproductive physiology and sexual behaviour at the molecular level.

2.6.1 Morphology of male reproductive organs

Male reproductive system is the part of the male body that involve in sexual activity, fertility and reproduction (Knoblaugh *et al.*, 2021). It includes both the external genital such as the penis and testes, as well as the internal parts such as the epididymis, seminal vesicle, prostate gland and urethra as pictured in . Those structures are well-vascularized with glands and ducts such as vas deferens to support the formation, storage and ejaculation for fertilization and produce important androgens in male development and puberty. For the prerequisite sexual functional, the key parts of the male reproductive system are penis and testes (*Singular*: testis) (Knoblaugh *et al.*, 2021). The body of penis, called the glans, consists of three sensitive erectile (cavernous) tissues, two corpora cavernosa (*Singular*: cavernosum) that can fill with blood during sexual arousal to cause an erection and corpus spongiosum, to delivers semen or urine outside of the body via urethra (Crisler *et al.*, 2020). Meanwhile, the testes are a pair of oval-shaped glands which are located in sacs called scrotums hanging behind the shaft of penis. The organs contain seminiferous tubules where sperm production occurs and Leydig cells that secrete testosterone hormone (Gurung, Yetiskul & Jialal, 2024).

Another component in the male reproductive system is epididymis which located on top and bottom each testis, connecting the testis to the vas deferens. It is a long-coiled tube that collects sperm from the testes and provide a reserve of viable, motile sperm ready in the cauda until the sexual ejaculation happens. The epididymis supports male fertility and reproductive capacity by allowing sperm to gradually mature before they would enter the next duct on their way of potential fertilization (Crisler *et al.*, 2020). Whereby, seminal vesicles are a pair of glands that connect to the vas deferens near the prostate gland which produce fluid that mixes with sperm to create semen. They add critical nutrients and lubricants that help to protect, support and activate sperm during the sexual intercourse (Gurung, Yetiskul & Jialal, 2024). The vas deferens are two muscular tubes that transport mature sperm from the epididymis to the ejaculatory duct. They pass through the spermatic cord in the scrotum upwards into the pelvic region behind the bladder, contracting during sexual climax to propel sperm forward into the female reproductive tract to potentially achieve the fertilization (Crisler *et al.*, 2020). Prostate gland produces prostatic fluid, a key part of semen. It surrounds the first part of the urethra below the bladder. The prostate fluid protects and nourishes sperm, while also promoting sperm motility. It contributes to seminal volume and neutralizes the acidity of the female reproductive tract, improving the chances that sperm remain vigorous enough to reach the fallopian tubes (Gurung, Yetiskul & Jialal, 2024).