PROTECTIVE EFFECTS OF *Kaempferia parviflora* AGAINST FENITROTHION-INDUCED MALE REPRODUCTIVE TOXICITY IN MICE

by

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ABSTRACT

PROTECTIVE EFFECTS OF *Kaempferia parviflora* AGAINST FENITROTHION-INDUCED MALE REPRODUCTIVE TOXICITY IN MICE

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Long-term exposure to fenitrothion (FNT), an agricultural pesticide, has been linked to male reproductive toxicity, including reduced sperm count and testosterone levels. However, specific reproductive parameters in FNT-induced male mice remain underexplored. *Kaempferia parviflora* (black ginger), traditionally used for male fertility enhancement, has shown promising results in rat models. Still, its protective effects against FNT-induced toxicity in mice have not been established. Thus, this study aimed to evaluate the antioxidant potential of *K. parviflora* rhizome extract and its protective effect against FNT-induced reproductive toxicity in male mice.

Firstly, the *K. parviflora* rhizome was extracted using three solvents, namely, ethanol, hexane, and water (aqueous). The extraction yield, total phenolic and flavonoid contents of the three extracts were tested. This was followed by an assessment of antioxidant activity via DPPH radical scavenging activity. The plant extract that possess the most prominent antioxidant activity was selected for phytochemical screening, GC-MS analysis and *in vivo* study.

A total of 24 male ICR mice (4-weeks-old) were randomly assigned into four groups (n = 6/group): control (EVOO), FNT-treated (20 mg/kg bw), KPE-treated (50 mg/kg bw) and FNT + KPE co-treated in the *in vivo* study. Treatments were administered orally once daily for 28 days. Three of the mice from each treatment group were paired with females to assess mating success, duration and further post-parturition parameters in terms of litter size and pups' survival rate. The remaining three males were subjected to the evaluation of body weight, mortality rate, relative testis weight, sperm concentration, motility, viability, testosterone level, lipid peroxidation and testicular histology assessment.

The *in vitro* results showed that aqueous extract of *K. parviflora* has the highest extraction yield, while ethanolic extract demonstrated highest phenolic content and hexane extract exhibited the highest flavonoid content. Meanwhile, the ethanolic extract (KPE) showed the best DPPH scavenging activity among other extracts. It is also revealed that all phytochemicals present in KPE except anthraquinone, while 8 peak compounds were detected in GC-MS analysis. The *in vivo* results showed that all mice experienced weight gain throughout the treatment period, with no mortality observed. FNT exposure significantly increased lipid peroxidation and impaired reproductive parameters. Mice treated with KPE treatment showed the highest testosterone levels, while mice subjected to co-treatment of KPE and FNT significantly enhanced sperm quality, testicular morphology and reduced mating duration compared to mice subjected

to FNT alone. However, assessment of relative testis weight, mating success or post-parturition outcomes between the four treatment groups did not show any significant difference ($p \geq 0.05$).

In conclusion, KPE demonstrated strong antioxidant activity and partial protective effects against FNT-induced reproductive toxicity.

Keywords: *Kaempferia parviflora*; Endocrine disruptor; Fenitrothion; Reproduction; Male fertility

Subject Area: QP(901)-(981) Experimental pharmacology

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(2-ethylbenzthiazoline-5-sulphonic acid)
ACE2	Angiotensin-converting enzyme 2
AChE	Acetylcholinesterase
$AlCl_3$	Aluminium chloride
Alpk:Apf	Alpha kinase 1: Antiperinuclear factor
ANOVA	Analysis of variance
BPA	Bisphenol A
bw	Body weight
C57BL/6N	C57 black 6
CB2	Cabin 2
CD1	Cluster of differentiation 1
CYP450	Cytochromes P450
DMF	5,7-dimethoxyflavone
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSCF	Dwass, Steel, Critchlow-Fligner
dw	Dry weight
e.g.	Exempli gratia
EC_{50}	Half maximal effective concentration
EDC	Endocrine disrupting chemical
et al.	et alia
EtOH	Ethanol

EVOO	Extra virgin olive oil
FNT	Fenitrothion
FRAP	Ferric reducing antioxidant power
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GAE	Gallic acid equivalents
GC-MS	Gas chromatography-Mass spectrometry
H and E staining	Haematoxylin and eosin staining
H_2O	Water (Aqueous)
H_3PO_4	Phosphoric acid
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HDMF	5-hydroxy-3,7-dimethoxyflavone
HFD	High fat diet
HIV-1	Human immunodeficiency virus type 1
HMF	5-hydroxy-7-methoxyflavone
HPLC	High performance liquid chromatography
HPG	Hypothalamic-pituitary-gonadal
HTMF	5-hydroxy-3,7,3',4'-tetramethoxyflavone
IC_{50}	Half maximal inhibited concentration
ICR	Institute of Cancer Research
IUPAC	International Union of Pure and Applied Chemistry
KPE	*Kaempferia parviflora* extract
KPE + FNT	Co-administration between *K. parviflora* extract and fenitrothion

LD_{50}	Lethal dose which kills 50% of test population
LH	Luteinizing hormone
LSD	Least significant difference
MMP-1	Matrix metalloproteinase-1
mRNA	Messenger ribonucleic acid
Na_2HPO_4	di-Sodium hydrogen phosphate
NaH_2PO_4	Sodium dihydrogen phosphate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
NO	Nitric oxide scavenging activity
NSY	Nagoya-Shibata-Yasuda
OMTs	*O*-methyltransferases
p	*p*-value (probability value)
PBS	Phosphate saline buffer
PMF	3,5,7,3',4'-pentamethoxyflavone
PPE	Personal protective equipment
ppm	Parts per million
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
QE	Quercetin equivalents
R^2	Pearson's correlation coefficient
RBD	Receptor-binding domain
ROS	Reactive oxidative species
RT	Retention time
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2

SD	Sprague-Dawley
SE	Standard error
SERC	Scientific and Ethical Review Committee
sPLA2	Phospholipase A2
STZ	Streptozotocin
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TMF	5,7,4'-trimethoxyflavone
TNF-α	Tumor necrosis factor-alpha
TRF	Tocotrienol-rich fraction
TSOD	Tsumura Suzuki obese diabetic
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

1.1 Research background

Kaempferia parviflora or black ginger, is a native sub-tropical plant in northeast of Thailand and southwest of Laos (Sharma and Gupta, 2013). Thai folks have been using *K. parviflora* rhizomes as natural folk medicine as they contain essential phytochemicals which possess anti-inflammation, anti-cancer and male aphrodisiac enhancing properties (Toda, et al., 2016; Chen, et al, 2018). *In vivo* studies also reported that *K. parviflora* extract (KPE) improves sperm concentration, enhanced testosterone level and courtship behaviour in murine model (Chaturapanich, et al., 2012; Lert-Amornpat, Maketon, and Fungfuang, 2017). Clinical studies also showed that KPE supplements could significantly enhanced muscle strength and aerobic endurance in elderly individuals as well as reducing physiological anxiety and stress in adults (Wattanathorn, et al., 2012; Eungpinichpong, et al, 2018). A study by Al-Rawaf, Gabr, and Alghadir (2021) demonstrated the effectiveness of KPE in improving sperm quantity and quality in sub-fertile Wistar rats. However, there is a lack of study investigating the co-administration of KPE with known endocrine-disrupting chemicals that induce male reproductive toxicity in mice.

The widely used organophosphate pesticide, fenitrothion (FNT), is a man-made endocrine-disrupting chemical that imposes adversed health risks in human, especially through occupational exposure. (Vermeire, MacPhail and Waters, 2003; Elhalwagy, Darwish and Zaher, 2008; Jain and Singh, 2022). Studies have shown that FNT exposure have caused severe damages to testis, kidneys, lungs, liver tissues and disrupt the proper function of nervous system in several animal models (Ishimatsu, et al., 1988; Al. Sahhaf, 2006; Abdel-Ghany, et al., 2016). In male rats model, FNT impairs sperm quality by disrupting hormones and spermatogenesis (Sohoni, et al., 2001; Yusoff, et al., 2020). It also generates reactive oxygen species (ROS), causing oxidative stress (Saber, Abd El-Aziz and Ali, 2015), which causes deoxyribonucleic acid (DNA) fragmentation and mutations in sperm cells, as observed in rats (Taib, et al., 2014). These damages may further reduce sperm quality and affect future progeny (Yusoff, et al., 2021). If exposure to FNT is unavoidable, pharmacological interventions such as clomiphene citrate and aromatase inhibitors (exempli gratia (e.g.), anastrozole, letrozole) have been developed to treat male infertility (Herzog et al., 2020; Yang, Li, and Li, 2022). However, these medications may potentially disrupt the hypothalamic-pituitary*gonadal (HPG) axis under long-term exposure, leading to mood changes, reduced libido, sexual dysfunction, and gynecomastia in men (Gallicchio, Calhoun and Helzlsouer, 2017; Huijiben, et al., 2022). As an alternative, naturopathic approaches, such as natural herbal supplements, may offer a promising strategy to mitigate FNT-induced male infertility with fewer side effects.

Previous studies have been focused on evaluating the effectiveness of various supplements or herb such as vitamin C, selenium, green tea and *Nigella sativa* extracts to mitigate the various negative effects of FNT (Korany and Ezzat, 2011; Milošević, et al., 2018). For example, quercetin, a natural flavonoid with high antioxidative properties, has been shown to effectively ameliorate the antiandrogenic and infertility effects induced by FNT (Saber, Abd El-Aziz and Ali, 2015). However, quercetin could potentially induce adverse effects on target organ (nephrotoxicity) while there is no reported toxicity observed on the administration of KPE even at a high dose (2000 mg/kg body weight (bw)) in rats (Chivapat, et al., 2004; Andres, et al., 2018; Yoshino, et al., 2019). Thus, this study aims to investigate the protective effects of KPE against FNT-induced male reproductive toxicity in mice.

1.2 Problem Statement

KPE has demonstrated various biological activities, particularly its antioxidant and aphrodisiac properties, in animal models (mainly rat models). However, no established studies have explored the co-administration of KPE with known endocrine-disrupting chemicals that induce male reproductive toxicity in mice. FNT, an organophosphate pesticide, is one such endocrine disruptor known to impair male reproductive health in numerous animal studies. Although several supplements and herbal remedies have been evaluated for their protective effects, limitations such as toxicity at higher dosages and the risks associated with prolonged exposure have hindered their effectiveness. Therefore, the potential of KPE to mitigate FNT-induced reproductive toxicity remains largely unknown and represents a novel area of investigation.

1.3 Objectives

To address problem mentioned above, this study was design with the main objective is to investigate the protective effects of KPE against FNT-induced male reproductive toxicity in mice. The specific objectives include:

1. Identifying the solvents (water, ethanol or hexane) that best preserve the antioxidant activities of *K. parviflora* extracts by quantifying the extraction yield, total phenolic and flavonoid contents, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity in each solvent.
2. Characterizing the phytochemical compounds of KPE by using phytochemical profiling and gas chromatography-mass spectrometry (GC-MS) analysis.
3. Assessing the effect of FNT and/or KPE on male mice weight, mortality and relative testis weight.
4. Evaluating the lipid peroxidation level in male mice serum after FNT and/or KPE administration.
5. Studying the effect of FNT and/or KPE on sperm quantity and quality, testosterone level, and histomorphometry of testis in treated male mice.
6. Evaluating the effect of FNT and/or KPE on mating success rate, mating duration in treated male mice, followed by post-parturition evaluation such as pups' litter size and survival rate.

CHAPTER 2

LITERATURE REVIEW

2.1 Male Reproductive System

2.1.1 Introduction to Male Reproductive System

The male reproductive system is a complex network of organs and tissues responsible for producing, nurturing, and delivering spermatozoa for reproduction (Picut and Plumlee, 2024). Key components include the testes, epididymis, vas deferens, seminal vesicles, prostate gland, and penis (Figure 2.1). Among these, the testes are the primary reproductive organs, tasked with the dual role of sperm production (spermatogenesis) and hormone secretion, particularly testosterone (Creasy and Chapin, 2013).

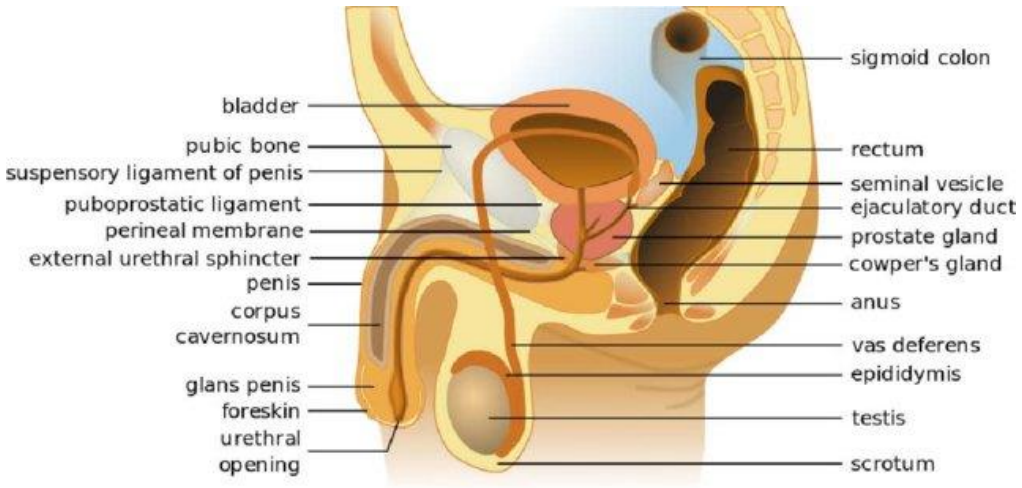


Figure 2.1: Male reproductive system (Adopted by Arango, 2014).

The testes are in the scrotum, which provides a cooler environment essential for optimal sperm production. Histologically, the testes are composed of seminiferous tubules, where germ cell development occurs, and interstitial (Leydig) cells, which synthesize testosterone, as shown in Figure 2.2 (Picut and Plumlee, 2024). Spermatogenesis, occurring within the seminiferous tubules, is a tightly regulated process essential for male fertility (Knoblaugh, et al., 2023).

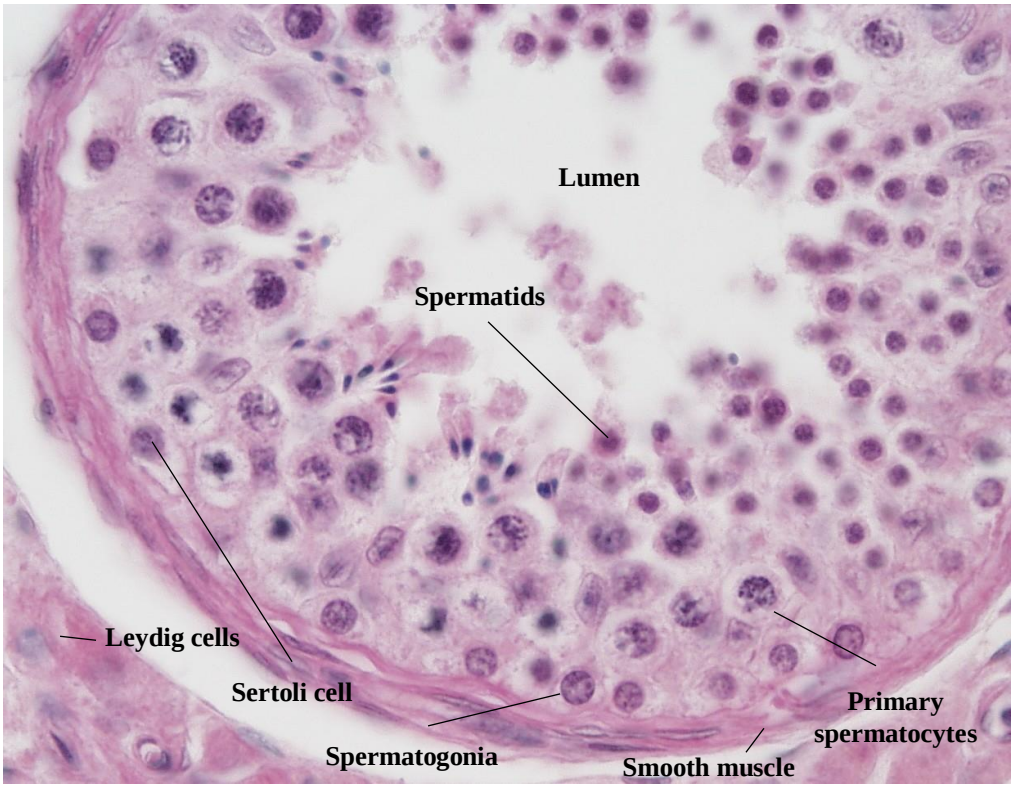


Figure 2.2: Histology of testicular tissue (Adopted from UNSW Embryology 2023).

2.1.2 Spermatogenesis and Spermiogenesis

Spermatogenesis is the process by which diploid spermatogonia differentiate into haploid spermatozoa. It is a continuous and complex process that can be broadly divided into three phases: mitotic proliferation of spermatogonia, meiotic division of spermatocytes, and the transformation of spermatids into mature spermatozoa through spermiogenesis (Nishimura and L'Hernault, 2017).

Spermatogenesis begins with spermatogonia, which are stem cells located at the basal membrane of seminiferous tubules. These undergo mitotic division to either self-renew or differentiate into primary spermatocytes (Chu and Shakes, 2012). This proliferation is regulated by Sertoli cells and endocrine signals such as follicle-stimulating hormone (FSH) and testosterone (Carreau and Hess, 2010). Primary spermatocytes undergo meiosis I to form secondary spermatocytes, which subsequently enter meiosis II to generate haploid spermatids. This reductional division ensures genetic diversity and halving of the chromosome number, essential for sexual reproduction (Sharma and Agarwal, 2011).

Spermiogenesis refers to the final phase of spermatogenesis, wherein haploid round spermatids undergo extensive morphological and biochemical changes to become mature, motile spermatozoa (Nishimura and L'Hernault,

2017; Knoblaugh, et al., 2023). Despite no cell division occurring during this phase, it involves critical remodelling processes including, nuclear condensation and elongation, acrosome formation from Golgi apparatus, flagellum development and cytoplasmic shedding (Sofikitis, et al., 2008). These transformations are tightly regulated by Sertoli cells, which provide structural and nutritional support throughout the process (Johnson, Thompson Jr and Varner, 2008). Furthermore, hormonal regulation, particularly by testosterone, is indispensable for the completion of spermiogenesis (Figure 2.3).

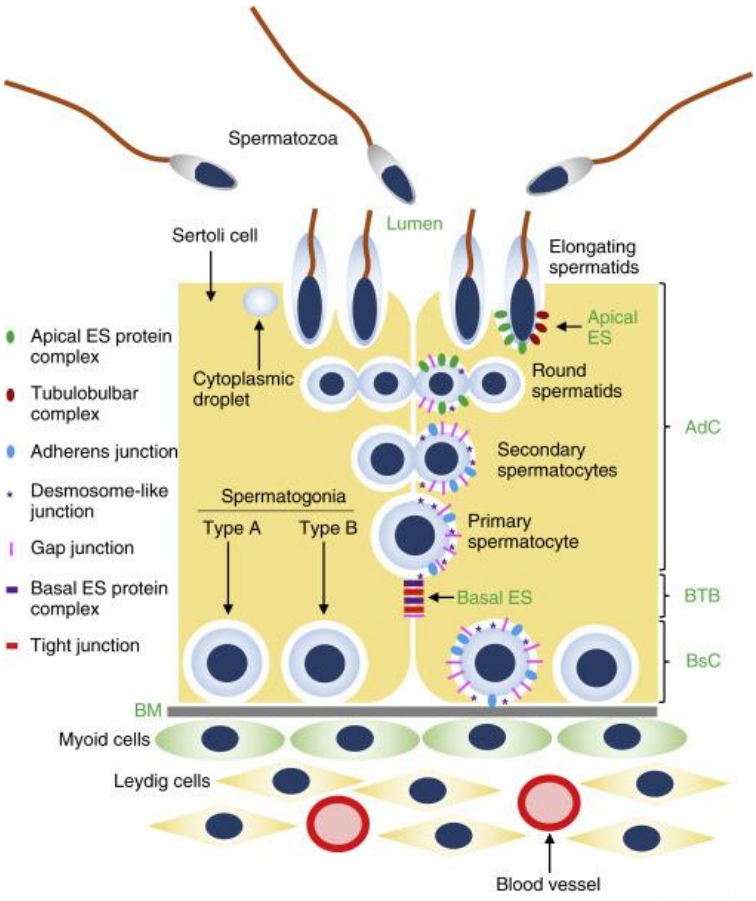


Figure 2.3: Spermatogenesis and spermiogenesis (Adopted by Nishimura and L'Hernault, 2017).

2.1.3 Hormonal Regulation of Spermatogenesis

The HPG axis governs the regulation of spermatogenesis. Gonadotropin-releasing hormone from the hypothalamus stimulates the anterior pituitary to release FSH and luteinizing hormone (LH) (Figure 2.4). LH acts on Leydig cells to produce testosterone, while FSH directly stimulates Sertoli cells, promoting the environment required for germ cell development (Corradi, Corradi and Greene, 2016). A delicate feedback loop involving inhibin and testosterone maintains hormonal balance (Maroto, et al., 2025).

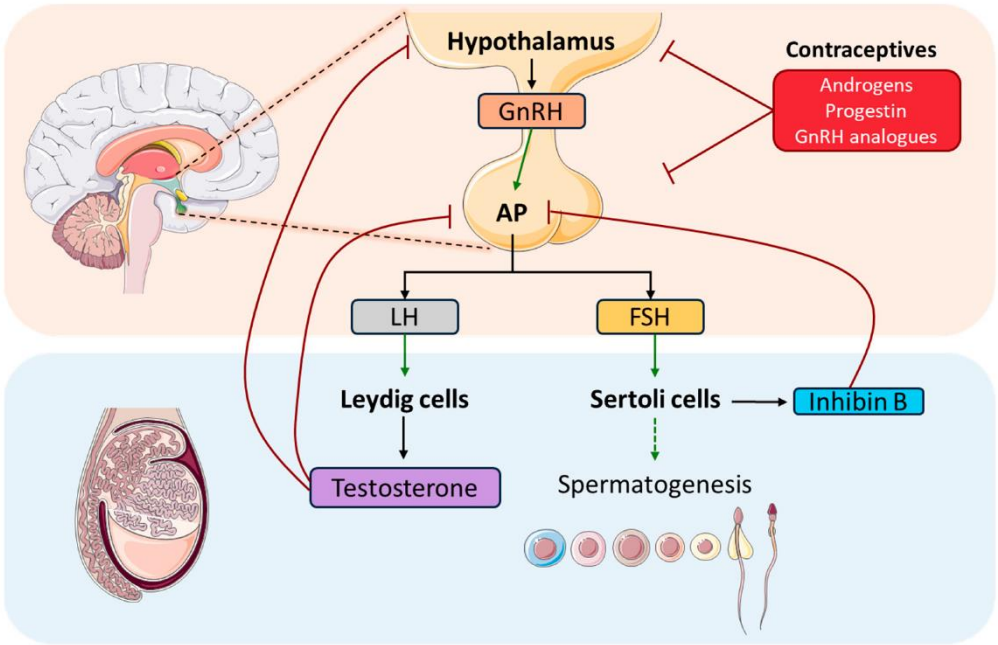


Figure 2.4: Hormonal regulation of spermatogenesis (Adopted by Maroto, et al., 2025).

2.1.4 Clinical Relevance and Pathophysiology

Infertility is characterized by the inability to achieve pregnancy after an extended duration of unprotected sexual intercourse (Palermo, et al., 2017). There are at least 186 million individuals worldwide currently troubled by was affected by infertility and at least 15% of couples worldwide suffered from difficulty in conceiving (Forti and Krausz, 1998; World Health Organisation, 2021). Male infertility contributes to 40-50% of infertility cases, while the highest rates of infertile men can be found in Eastern Europe and Africa, ranging from 8% to 12% (Agarwal, et al., 2015; Kumar and Singh, 2015).

According to Babakhanzadeh, et al. (2020), there are still no clear or justified aetiology in 20% (12 million) to 30% (24 million) of male infertility cases. There are several factors which may lead to infertility, namely stress, psychological mental disorder, abusive smoking, excessive alcohol consumption and chronic exposure to environmental toxicants (Harlev, et al., 2015; Rooney and Domar, 2018; Szkodziak, Krzyżanowski and Szkodziak, 2020; Finelli, Mottola and Agarwal, 2021). Meanwhile, other studies suggested that factors such as testicular injury, side effect of medical drugs (ranitidine, cimetidine), varicocele, hernia, herpes, semen abnormalities and sexual disorder also contribute to male infertility, as shown in Figure 2.5 (Mahboubi, et al., 2014; Jafari, et al., 2021).

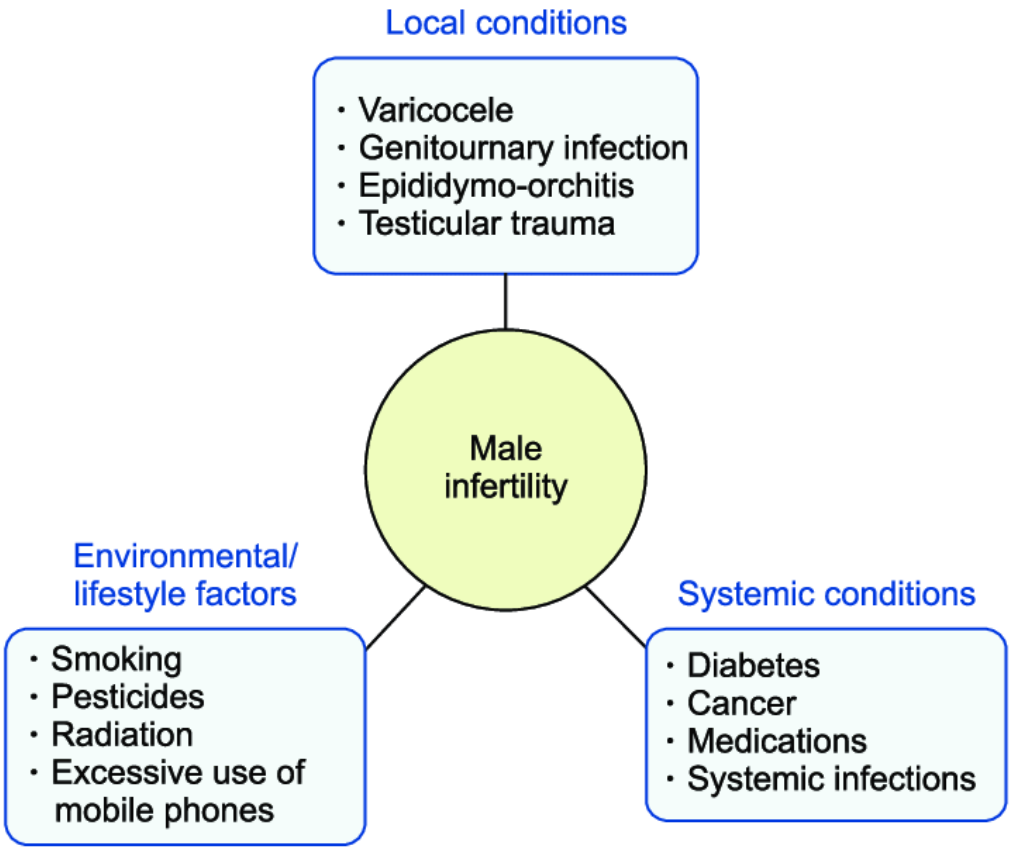


Figure 2.5: Key factors associated with male infertility (Adopted by Agarwal, et al., 2019).

Meanwhile, the disruptions in spermatogenesis can lead to male infertility. Factors such as hormonal imbalances, genetic mutations, toxicant exposures, varicocele, and infections can negatively affect the efficiency of sperm production (Agarwal et al., 2015). Conditions that are associated with male infertility include low sperm concentration, reduced sperm motility, abnormal sperm morphology, decreased testosterone levels, and a reduced sexual drive towards the opposite sex (Iwamoto, Nozawa, and Yoshiike, 2007). Furthermore, histological evaluations of male infertility frequently reveal structural and cellular abnormalities, such as damaged or degenerating spermatozoa, disruption of the seminiferous epithelium, vacuolization and

detachment of Sertoli cells, hypertrophy or atrophy of Leydig cells, and thickening of the basement membrane (Bretveld, et al., 2007). In more severe cases, there may be tubular hyalinization, germ cell aplasia (Sertoli cell-only syndrome), and interstitial fibrosis, which collectively contribute to impaired spermatogenesis and reduced fertility potential (Cerilli, Kuang and Rogers, 2010).

Ultimately, male infertility is a multifactorial disorder involving hormonal, structural, environmental, genetic, and behavioral components. Understanding its underlying pathophysiology is crucial for accurate diagnosis, targeted therapy, and improving reproductive outcomes.

2.2 Black ginger – *Kaempferia parviflora*

2.2.1 Morphology

Kaempferia parviflora Wall. ex-Baker or black ginger, Krachaidum in Thai, "cekur hitam" in Malay is a subtropical plant found abundantly in North-eastern Thailand and South-western Laos (Sharma and Gupta, 2013). It belongs to the family of Zingiberaceae, a family of ginger flowering plants that has the similar properties of sympodial branched rhizomes and the axillary buds of the plant are protected by scale leaves (Tomlinson, 1956; Kumar, et al., 2013). Zingiberaceae plant family are known for its medicinal properties, including Alpinia, Amomum, Curcuma, Elettaria, Jedychium, Kaempferia and Zingiber genera (Prabhu, Thomas and Sabu, 2010). Morphologically, Kaempferia genus have a very short stem, making *K. parviflora* an understory plants with a dark purple fleshy rhizome (Figure 2.6) and white inconspicuous flowers with violet labellum (Kumar, et al., 2013; Labrooy, et al., 2016). They have a long dormancy period of five to six months and a slow regeneration through rhizome, making them scarce in Malaysia's plantation despite the high market demand (Techaprasan, et al., 2010; Noor Camellia, 2012).

Figure 2.6: *Kaempferia parviflora* Wall. ex-Baker (Adopted from Jeong, et al., 2016).

2.2.2 Phytochemical Composition of *K. parviflora*

The rhizomes of *K. parviflora* contain major sources of methoxyflavones. Flavonoid and phenolic compounds can be found abundantly in a 10-months-old rhizomes of *K. parviflora* and studies reported that these compounds can be effectively extracted with ethanol (Kitwetchar, et al., 2020; Sitthichai, et al., 2022). The identified methoxyflavones found in the rhizomes of *K. parviflora* are listed in Table 2.1 (Azuma, Tanaka and Kikuzaki, 2008; Okabe, et al., 2014). The typical chemical structure of methoxyflavone is shown in Figure 2.7. Among the methoxyflavone derivatives identified, three high-yield fractions have been isolated via high-performance liquid chromatography (HPLC), namely 3,5,7,3',4'-pentamethoxyflavone (PMF), 5,7,4'-trimethoxyflavone (TMF) and 5,7-dimethoxyflavone (DMF) (Nakao, et al., 2011). These bioactive compounds have since become the focus of numerous studies investigating their antioxidative properties and potential therapeutic

applications, particularly from *K. parviflora* ethanolic extracts (Nakana, et al., 2014; Chen, et al., 2018). However, there were also studies on other phytochemicals such as alkaloids, saponins, tannins and phenolic glycosides (kaempferiaosides A and B) found in the rhizome of *K. parviflora* have also been reported (Chaipech, et al., 2012; Mishra and Sharma, 2021).

Table 2.1: IUPAC nomenclature of methoxyflavones identified in *K. parviflora* by Chen, et al. (2018) with the reference of methoxyflavone chemical structure on Figure 2.2.

No.	Methoxyflavones	Substitution position				
		3	5	7	3'	4'
1	3,5,7,3',4'-pentamethoxyflavone	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$
2	3,4',5,7-tetramethoxyflavone	$-OCH_3$	$-OCH_3$	$-OCH_3$		$-OCH_3$
3	3',4',5,7- tetramethoxyflavone		$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$
4	4',5,7- trimethoxyflavone		$-OCH_3$	$-OCH_3$		$-OCH_3$
5	3,5,7-trimethoxyflavone	$-OCH_3$	$-OCH_3$	$-OCH_3$		
6	5,7-dimethoxyflavone		$-OCH_3$	$-OCH_3$		
7	3,5-dihydroxy-7,3',4'-trimethoxyflavone	-OH	-OH	$-OCH_3$	$-OCH_3$	$-OCH_3$
8	5,3'-dihydroxy-3,7,4'-trimethoxyflavone	$-OCH_3$	-OH	$-OCH_3$	-OH	$-OCH_3$
9	5,4'-dihydroxy-7-methoxyflavone		-OH	$-OCH_3$		-OH
10	5-hydroxy-3,7,3',4'-tetramethoxyflavone	$-OCH_3$	-OH	$-OCH_3$	$-OCH_3$	$-OCH_3$
11	5-hydroxy-7,3',4'-trimethoxyflavone		-OH	$-OCH_3$	$-OCH_3$	$-OCH_3$
12	5-hydroxy-3,7,4'-trimethoxyflavone	$-OCH_3$	-OH	$-OCH_3$		$-OCH_3$
13	5-hydroxy-3,7-dimethoxyflavone	$-OCH_3$	-OH	$-OCH_3$		
14	5-hydroxy-7,4'-dimethoxyflavone		-OH	$-OCH_3$		$-OCH_3$
15	5-hydroxy-7-methoxyflavone		-OH	$-OCH_3$		
16	4'-hydroxy-5,7-dimethoxyflavone		$-OCH_3$	$-OCH_3$		-OH

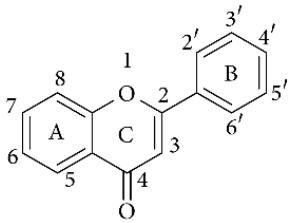


Figure 2.7: Chemical structure of a typical methoxyflavone which consists of a benzopyran-4-one "A+C" group (functional groups substitution at 3, 5 and 7 carbon positions), an attached aromatic ring B group (functional group substitution at 3' and 4' carbon positions).

2.2.3 Essential Biological Activities of *K. parviflora*

The rhizomes of *K. parviflora* and many Zingiberaceae families are known to possess essential biological activities such as antioxidant, anti-cancer, anti-inflammatory, anti-microbial, anti-cholinesterase, anti-emetic, cardioprotective and aphrodisiac properties (Saokaew, et al., 2017; Alolga, et al., 2022; Ballester, et al., 2023). In the subsequent sub-chapter, antioxidant activity, and other bioactivities of *K. parviflora* will be the review.

2.2.3.1 Antioxidant Activity of *K. parviflora*

Oxidative stress is defined as the level of imbalance between both ROS and antioxidants which causes stress in the biological system (Frijhoff, et al., 2015). This imbalance results from a deficiency in both antioxidant compounds and endogenous antioxidant enzymes, which compromises the body's ability to counteract oxidative stress (Scalbert, et al., 2005; Ďuračková, 2010). Such an inability to manage oxidative stress leads to elevated risks of developing diseases, including cardiovascular disorders, neurodegenerative conditions, and cancer (Galle, 2001; Bahorun, et al., 2006; Giacco and Brownlee, 2010).

Highly ROS are found to be capable of oxidizing and damaging biological molecules such as carbohydrates, proteins, lipids and DNA (Young

and Woodside, 2001). These ROS includes superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), oxygen singlet ($^1O_2$), hydrogen peroxide (H_2O_2), peroxynitrite radical ($NO_3^{\bullet -}$), hypochlorite ion (^-OCl) and nitric oxide radical ($^{\bullet}NO$) (Lobo, et al., 2010). The formation of free radical in the body can be induced externally (ultraviolet rays, environmental toxicants etc) and internally by body reaction (enzymatic reaction, peroxisomes, inflammation etc) (Bannister, Bannister and Rotilio, 1987; Liu, et al., 1999). These exposures will then cause the body to quickly response by releasing antioxidative enzymes (catalase, superoxide dismutase, glutathione reductase) or utilizing non-enzymatic compounds (ascorbic acid, tocopherols, melatonin) to neutralize the free radicals and prevent them from causing oxidative damages, as shown in Figure 2.8 (Bagchi and Puri, 1998; Herrera and Barbas, 2001; Padayatty, et al., 2003; Chelikani, Fita and Loewen, 2004).

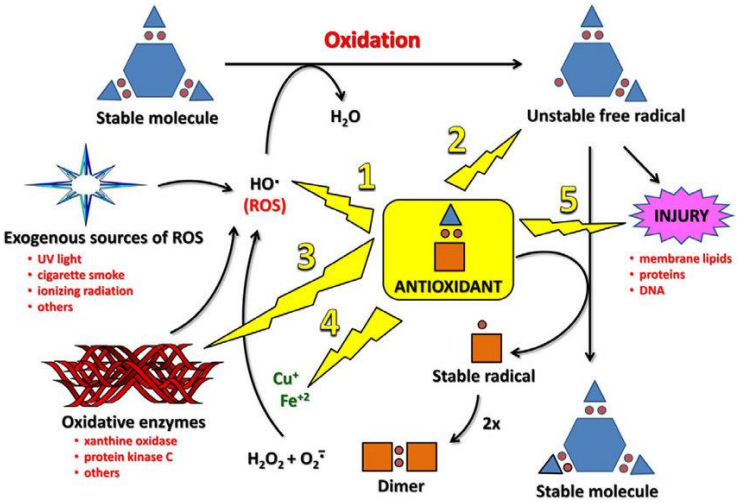


Figure 2.8: The roles of antioxidants. (1) Scavenge ROS. (2) Promote stability of free radicals. (3) Inhibit oxidative enzymes. (4) Chelate transition metals. (5) Repair damaged tissues and promote wound healing. (Adopted by de Oliveria and Batista, 2017).

Many studies have reported on the relationship between oxidative stress and diseases such as cancer, chronic obstructive pulmonary disease, cardiovascular and neurological diseases, rheumatoid arthritis, kidney diseases, diabetes and infertility (Halliwell, 2001; MacNee, 2001; Mahajan and Tandon, 2004; Tremellen, 2008; Ruder, Hartman and Goldman, 2009). Thus, researchers have been investigating on natural herbs as a potential antioxidant which can be used for alternative naturopathy treatment (Kaczor, 2006).

Antioxidant activities and capabilities can be determined via free radical scavenging activity assays. These assays include DPPH scavenging activity assay, 2,2'-azinobis-(3-ethylbenzothiazonline-6-sulfonic acid) (ABTS) scavenging activity assay and ferric reducing antioxidant power (FRAP) assay (Cerretani and Bendini, 2010; Shivembe and Ojinnaka, 2017). They measure the formation of the reduced state of DPPH, ABTS and ferric ion (FRAP) by the presence of antioxidant compounds (Kedare and Singh, 2011). It was then quantified via ultraviolet-visible light spectrophotometer at specified wavelengths of 517 nm (DPPH), 734 nm (ABTS) and 593 nm (FRAP) (Re, et al., 1999; Sarla, et al., 2011).

There were several studies reported on the antioxidant activities of *K. parviflora*. Lee, et al. (2018) reported that approximately 90% of ABTS free radicals was removed with 0.20 mg/mL of *K. parviflora* ethanolic extract (KPE) treatment while at least 0.40 mg/mL of KPE was needed to remove half of the

DPPH radicals. In addition, Choi, Kim, and Yook (2018) found that aqueous extract of *K. parviflora* demonstrated antioxidant activity with an IC_{50} value of 9.16 mg/mL in DPPH assay, 13.24 mg/mL in ABTS assay and give FRAP value of 0.22 mM in the concentration of 1 mg/mL. Furthermore, they also shown that both ethyl acetate and ethanolic extracts possessed higher antioxidant activities shown in DPPH assay, with the IC_{50} values of 0.38 mg/mL and 0.48 mg/mL, respectively, when compared to hexane (3.62 mg/mL) and aqueous extracts of *K. parviflora*. The findings suggest that semi-polar solvents like ethanol and ethyl acetate are most effective for extracting bioactive compounds from the rhizomes of *K. parviflora*. In the meantime, Mishra and Sharma (2021) found that KPE possess higher antioxidant activities as compared to the ethanol extracts of *K. galanga* and *K. pulchra*. Specifically, KPE showed lower IC_{50} values in both DPPH (34.69 μg/mL) and NO (34.69 μg/mL) assays compared to *K. galanga* (DPPH: 58.79 μg/mL, NO: 90.36 μg/mL) and *K. pulchra* (DPPH: 83.01 μg/mL, NO: 106.50 μg/mL). In general, the study above revealed that KPE has the highest antioxidative activities among tested species in *Kaempferia* genera.

2.2.3.2 General Effects of *K. parviflora* towards Male Reproductive System

The rhizome of *K. parviflora* has been used as folk medicine to boost male libido and sexual performance in Thailand. Many studies had investigated the properties of KPE in male aphrodisiac effect by using murine model. Table 2.2 summarises various *in vivo* studies using KPE.

Table 2.2: List of *in vivo* studies using KPE.

KPE (dosage in mg/kg bw)	Duration of treatment	Rodent used	Observation	References
Ethanolic extract (60, 120, 240)	30 and 60 days	Male Wistar rats	Enhanced courtship behaviour was observed in all treatment groups.	Sudwan, et al. (2006)
Ethanolic, hexane and aqueous extract (70)	3 – 5 weeks	Male Sprague-Dawley (SD) rats	Only ethanolic KP extract reduced mount and ejaculatory latencies, and enhanced blood flow to the testis of male rats.	Chaturapanich, et al. (2008)
Ethanolic extract (70)	4 weeks	Male SD rats	Promoted courtship performance in male rats	Chaturapanich, et al. (2012)
Ethanolic extract (140, 280, 420)	6 weeks	Male Streptozotocin (STZ)-induced diabetic Wistar rats	Serum testosterone elevated and improved testicular histological structure were observed in all treatment groups.	Fungfuang, Lert-Amornpat, and Maketon (2016)
Water-soluble extract (140, 280, 420)	6 weeks	Male Streptozotocin (STZ)-induced diabetic Wistar rats	Enhanced overall courtship behaviours and chances of ejaculation in all treated groups. Higher sperm count, motility, viability and testosterone level were observed.	Lert-Amornpat, Maketon, and Fungfuang (2017)
Ethanolic extract (140, 280, 420)	6 weeks	Male Wistar rats	Increased in the number of sperm count, motility and viability in all treated sub-infertile rats.	Al-Rawaf, Gabr and Alghadir (2021)

From the above listed murine studies, it can be summarized that *K. parviflora* has male sexual boosting ability by vasodilative action of the blood flow towards reproductive organs and not by the stimulating action of the testosterone production (Trisomboon, 2009). Interestingly, there are also relevant studies conducted to determine the aphrodisiac effect of *K. parviflora* on various animal model. Authaida, et al. (2024) demonstrated that feeding Thai native roosters with 200 mg/kg bw KPE for 14 days improved the sperm motility, progressive motility, and viability of the treated roosters. Similarly, the experimental boar which received 1% of *K. parviflora* ethanolic extract showed a significant increase in semen volume as compared to testosterone injected boar group (Kupittayanant, et al., 2007). The above findings could be observed in other animal after treated with *K. parviflora* ethanolic extract.

Besides that, there were several studies investigating the aphrodisiac effect and physical fitness upon consumption of KPE in human volunteers. Wannanon, et al. (2012) reported a significant increment of penile circumference and length measurements in either erected or flaccid state on healthy elder male participants after treated with 90 mg of KPE capsules for 8 weeks continuously. Stein, et al. (2018) also reported on the improvement of erectile function and sexual intercourse satisfaction on middle-aged men with mild erectile dysfunction after supplemented with 100 mg of *K. parviflora* supplement (KaempMax™) for 30 days. Another study also showed the improved muscle strength, physical endurance and speed on male adolescent athletes after taking 360 mg of KPE supplement daily continuously for 12 weeks

(Sripanidkulchai, et al., 2022). A recent comprehensive review on the biological activities of *Kaempferia* species highlighted that, to date, only *K. parviflora* has been extensively studied for its potential as a male aphrodisiac enhancer by researchers (Elshamy, et al., 2019; Pham, Nguyen, and Nguyen, 2021; Singh, et al., 2023).

2.2.3.3 Other Bioactivities Properties of *K. parviflora*

Apart from its well-known antioxidant and male aphrodisiac properties, there were also studies conducted on the rhizome of *K. parviflora* to explore the anti-inflammation, anti-allergy, anti-cancer and antimicrobial properties of *K. parviflora*.

The methoxyflavone derivatives found in the rhizome of *K. parviflora* has shown its anti-inflammation, anti-allergy and anti-cancer properties. A study also reported that *K. parviflora* crude extract has significantly reduced *Helicobacter pylori*-induced interleukin (*IL*)-8 secretion and gastric adenocarcinoma cells viability, which implying on its potential cure against *H. pylori*-induced inflammation and gastric cancer (Nemidkanam, et al., 2020). Furthermore, a study also reported on the potent anticancer activity of KPE against HeLa cells (cervical cancer cell line) by inducing apoptotic cell death (Potikanond, et al., 2017). Sitthichai, et al. (2022) reported KPE gel-cream effectively treated on severe *acne vulgaris* inflammation. Horigone, et al. (2014)

has further proven the effectiveness of *K. parviflora* in anti-inflammatory property by the observed result of a significant suppressed mRNA expression of inflammatory mediator gene in basophilic leukemia cells of a rat after treating with the isolated DMF and TMF from *K. parviflora*. On the other hand, DMF, TMF, and PMF from KPE has shown to prevent age-related inflammatory effect towards epidermal skin by stabilizing epidermal-dermal thickness, which suggest that KPE has anti-aging properties (Klinngam, et al., 2022). Similarly, Nakata, et al. (2014) found that PMF and TMF also possessed anti-glycation activity which prevent the progression of inflammatory reactions. Interestingly, 5-hydroxy-3,7,3',4'-tetramethoxyflavone (HTMF), a derivative methoxyflavone compound found in *K. parviflora* was reported to exhibit the highest anti-allergy activity in RBL-2H3 (basophilic leukemia cell) cell line by inhibiting antigen-induced beta-hexosaminidase secretion which acts as a marker of cell degranulation (Tewtrakul, Subhadhirasakul and Kummee, 2008). Thus, *K. parviflora* has shown potential to serve as natural herbs with its anti-inflammation, anti-allergy and anti-cancer properties.

Meanwhile, KPE has been reported as antimicrobial properties against bacteria, fungus and virus by several studies. Research suggests KPE may be a useful anti-acne agent due to its antimicrobial activity against *Propionibacterium acnes* and *Cutibacterium acnes* (Jin and Lee, 2018; Sitthichai, et al., 2022). Jeong, et al. (2016) also showed the effectiveness of ethanolic extract of *K. parviflora* on inhibiting the growth of both *Cronobacter spp.* and *Escherichia coli*. In the meantime, a study has shown that 3,5,7-

trimethoxyflavone, one of bioactive compounds found in KPE, has showed potent anti-fungal activity against *Trichophyton mentagrophytes, Microsporum gypseum* and *Trichophyton rubrum* (Kummee, Tewtrakul and Subhadhirasakul, 2008). This suggests that KPE could effectively control against specific fungal species. Furthermore, Sookkongwaree, et al. (2006) found that three bioactive compounds isolated from KPE which are 5-hydroxy-7-methoxyflavone (HMF) and 5-hydroxy-3,7-dimethoxyflavone (HDMF) and DMF. They possess antiviral properties which suppresses the multiplication of hepatitis C virus (HCV), human immunodeficiency virus type 1 (HIV-1) as well as human cytomegalovirus (HCMV). KPE also showed antiviral activity against SARS-CoV-2, potentially by preventing the interaction between the virus's receptor-binding domain (RBD) and the human angiotensin-converting enzyme 2 (ACE2) receptor (Supian, et al., 2024). In general, these findings suggested that KPE contains bioactive compounds with antimicrobial properties against viral, fungal and bacterial infections. This supports the potential use of *K. parviflora* as a therapeutic agent for various infectious diseases.

In addition, KPE also possesses the ability of weight reduction and lowering the risk of obesity-induced metabolic diseases. Studies showed that KPE significantly supressed weight gain and visceral fat accumulation in both Tsumura Suzuki obese diabetic (TSOD) and Nagoya-Shibata-Yasuda (NSY) diabetic mice (Shimada, et al., 2011a, Ochiai, et al., 2019). In the meantime, KPE also significantly reduced insulin resistance and glucose intolerance on TSOD mice (Shimada, et al., 2011b; Okabe, et al., 2014). Furthermore, recent

study by Lee, et al. (2023) observed that 8 weeks of KPE administration reduces serum glucose, triglycerides, cholesterol level in the high fat diet (HFD)-induced obese C57 black 6 (C57BL/6N). Interestingly, Shimada, et al. (2011) discovered PMF in KPE has obesity-preventive properties through pancreatic lipase inhibition in TSOD mice. Relevant findings also reported on the discovery of PMF in supressing the accumulation of subcutaneous and visceral fat layers as well as reducing in bw in TSOD mice (Akase, et al., 2010; Hidaka, et al., 2017). Therefore, this makes KPE a promising therapy for managing obesity. In summary, the findings above would strongly imply that KPE could serve as an alternative anti-obesity and anti-diabetic naturopathy for obese or type II diabetes mellitus individuals.

Furthermore, several studies also investigated the mechanisms of action of bioactive compounds found in the rhizome of *K. parviflora.* Two methoxyflavone derivatives isolated from *K. parviflora,* PMF and 3',4',5,7-tetramethoxyflavone possess xanthine oxidase inhibition property which can reduce the risk of hyperuricemia (Nakao, et al., 2011; Julianti, Bakar and Wikantyasning, 2022). In another study, the pharmacokinetics interaction between *K. parviflora* and cytochromes P450 conducted by Mekjaruskul, Jay and Sripanidkulchai (2012a) showed that the methoxyflavones interact with the isoforms of the mouse hepatic cytochromes P450 (CYP450) which enhances the activities of CYP1A1, CYP1A2, CYP2B, and CYP2E1 enzymes. The above finding could alert on the usage of KPE as potential modulator of CYP450 in liver as they also reported that the highest concentration of methoxyflavone

derivatives of KPE found in the liver of male rats after administration (Mekjaruskul, Jay and Sripanidkulchai, 2012b).

Interestingly, 5,3'-dihydroxy-3,7,4'-trimethoxyflavone, isolated from *K. parviflora* exhibited hepatoprotective properties by effectively inhibiting the cytotoxicity activities of *D*-galactosamine (Chaipech, et al., 2012). Another study by Phung, et al. (2021) demonstrated that *K. parviflora* possessed both ameliorative and protective effects against tumor necrosis factor-alpha (TNF-α)-induced damage in human dermal fibroblasts. The methoxyflavones from *K. parviflora* played a critical role in mitigating oxidative stress by reducing ROS levels induced by TNF-α. Additionally, they also inhibited the activity of matrix metalloproteinase-1 (MMP-1), thereby preserving the skin structural integrity.

In conclusion, *K. parviflora* possess various bioactivities properties that are beneficial to human health, emphasizing it on the use as alternative drug or medication in the future. Table 2.3 shows the summarized biological activities of *K. parviflora* reported by previous findings.

Table 2.3: Biological activities of *K. parviflora*.

List of biological activities	References
Antioxidant / Free radical scavenging activity	Choi, Kim Yook (2018), Lee, et al. (2018) Mishra and Sharma (2021)
Male aphrodisiac activity promotion	Sudwan, et al. (2006), Chaturapanich, et al. (2008), Fungfuang, Left-Amornpat and Maketon (2016), Left-Amornpat, Maketon and Fungfuang (2017), Stein, et al. (2018), Al-Rawaf, Gabr and Alghadir (20121)
Anti-inflammatory activity	Horigome, et al. (2014), Nakata, et al. (2014)
Antimicrobial activity	Jeong, et al. (2016), Jin and Lee (2018), Sitthichai, et al. (2022)
Antiviral activity	Sookkongwaree, et al. (2006), Supian, et al. (2024)
Anticancer activity	Potikanond, et al. (2017), Nemidkanam, et al. (2020)
Adipogenesis / Inhibitory activity in lipogenesis	Akase, et al. (2010), Shimada, et al. (2011a), Hidaka, et al. (2017), Ochiai, et al. (2019), Lee, et al. (2023)
Anti-acne agent	Sitthichai, et al. (2022)
Physical activity and endurance booster	Sripanidkulchai, et al. (2022)

2.2.4 Toxicity of KPE

Acute toxicity study of *K. parviflora* via oral administration in murine model, revealed that its acute toxicity LD_{50} value is greater than 13.3 g/kg bw (Chivapat, et al., 2004). Additionally, a single dose of *K. parviflora* ethanolic extract (KPE) at 2000 mg/kg bw in rats showed no sign of adverse effects on bw, physical condition or organs damage (Chivapat, et al., 2010). In chronic toxicity studies of KPE, high dose of 1000 mg/kg bw KPE administration did not show adverse effect on the mating behaviour, reproductive hormones,

reproductive and non-reproductive organ weights in the adult Wistar-Imamichi male rats (Trisomboon, et al., 2007; 2008). However, in another study by Chivapat, et al. (2010) showed a decreased bw, relative organ weights (heart, liver, stomach, kidney) and haematological values (eosinophil, basophil) in 500 mg/kg bw KPE treated rats. Regarding the findings above, there is no significant pathological changes observed upon histology evaluation of visceral organs.

Yoshino, et al. (2019) also stated that there was an increased in salivation behaviour observed in sub-chronic 249 mg/kg bw KPE-treated rats and slight elevation of platelet count in low dose of KPE (25 mg/kg bw) treated rats. Despite that, there are no detectable findings on mutagenic effect and hematotoxicity in SD rats after 90 days of 25 mg/kg bw, 125 mg/kg bw and 249 mg/kg bw of KPE administration. Interestingly, Sudwan, et al. (2006) observed the hypertrophy of vacuolar cell at the rat liver when treated with 240 mg/kg bw KPE for 60 days, but no observable hematological changes on all doses of KPE in treated SD mice.

Additionally, Eungpinichpong, et al. (2018) revealed that the consumption of 360 mg of *K. parviflora* rhizome for 14 days in 80 volunteers aged between 24-46 years did not exhibit negative physical and physiological effect. In return, the volunteers showed a positive impact of reduction on physiological and psychological stress as shown in stress index and stress variable scoring system. Meanwhile, a study by Stein, et al. (2018) reported no

side effects in older men who were supplemented with a 100 mg dose of *K. parviflora* capsules daily for 30 days. Instead, the supplementation significantly improved erectile function and intercourse satisfaction in middle-aged and older men who self-reported to have mild erectile dysfunction. Based on the above studies, we can deduce that *K. parviflora* highly safe for oral consumption. However, more clinical trials are still needed on the long-term safety of *K. parviflora* consumption and its potential interactions with medications such as drugs.

2.3 Environmental Toxicant – Fenitrothion (FNT)

FNT or its IUPAC name as *O*,*O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate is a type of organophosphate compound with its chemical structure (Figure 2.9). FNT appears as a yellow-brownish oily liquid at room temperature and it possesses a distinct phenol-like odour (Pohanish, 2015). It is highly soluble in most volatile solvent, but it has low solubility of 38 mg/L in water (Samadi-Maybodi and Nikou, 2021).

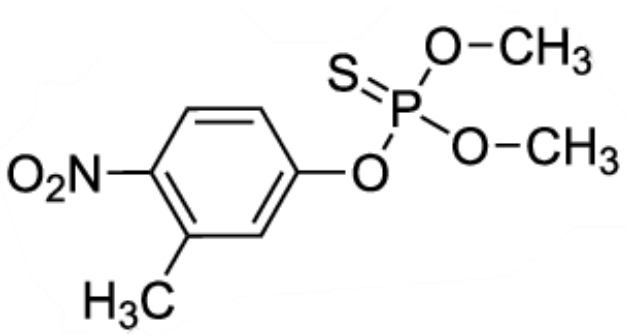


Figure 2.9: FNT (Adopted from Cruickshank, et al., 2013).

2.3.1 Industrial Application of FNT and its Environment Impacts

FNT is known for its agricultural usage as organophosphorus pesticide. Furthermore, FNT is also used for indoor mosquito control, predatory bugs (tiger bug) removal in ponds and locust pest control (World Health Organization, 2001; Espinoza-Navarro, Ponce-LaRosa and Bustos-Obregón, 2017; Shahjahan, et al, 2021; Story and French, 2023). FNT works by inhibiting acetylcholinesterase (AChE), an important enzyme which breaks down

acetylcholine at the synapses of nerve cells, and thus affecting the insect's nervous system (Talesa, 2001; Chen, et al., 2023). The excessive acetylcholine accumulation at synapse causes ongoing signal transmission and prolonged muscle contraction which leads to the death of insect (Fukuto, 1990).

Due to the high demand of insecticide worldwide, there were around 15000 to 20000 tons of FNT being produced annually (Trinh, et al., 2021). Excessive utilization of insect-pest control product has resulted in numerous adverse effects and burden towards the environment (Pathak, et al., 2022). Studies have revealed that FNT could adversely affect freshwater species, marine species and also beneficial insects such as pollinators and parasitoids (Kevan, 1975a; Sabater and Carrasco, 2001; Islam, et al., 2019). This could further lead to the potential disruption of food chain in freshwater or seawater and reduction of food crops as some flowering plants are pollinator-dependent (Kevan, 1975b; Salam, et al., 2015; Rahman, et al., 2020).

Firstly, FNT can be absorbed into the soil and it will be readily degraded by microbes found in soil, converting it into a less harmful residues (Spillner, et al. 1979; Roy, et al., 1996). However, these FNT residues in soil can disruot the microbial balance in soil and most biochemical cycling processes (Durand and Barcelo, 1992a). The prolonged usage of FNT can lead to the accumulation in the soil, and thus affecting microbial population, plant growth and the overall ecosystem sustainability (Durand and Barcelo, 1992b).

Secondly, dissolved FNT penetrates deeply into the soil and flow to the groundwater which causes water contamination (Pionke and Glotfelty, 1989). These accumulated FNT undergoes photodegradation in natural water and affects the overall aquatic ecosystem (Molla, et al., 2018). Furthermore, FNT acts as AChE inhibitor which inhibit most bioactivities function of aquatic microorganisms (phytoplankton and zooplankton etc.) (Sabater and Carrasco, 2001; Rahman, et al, 2020). Meanwhile, it also acts as endocrine disruptor which induced toxicity towards bigger aquatic species such as fishes and invertebrates (Zeid and Khalil, 2014; Salam, et al., 2015).

Lastly, FNT residues can remain or being absorbed by the plant crops which later consumed as food by human and animals. There were studies reported on the findings of FNT residues in food and animal crops such as wheat, tomatoes, green beans and rice (Takimoto, Ooshima and Miyamoto, 1978; Khani, Imani and Larijani, 2011; Malhat, 2012; Malhat, et al., 2017). Interestingly, a study reported that by administering FNT at a dose of 2 mg/kg bw to hens for 7 consecutive days, it resulted in detectable FNT residue level of 0.055 parts per million (ppm) in eggs (Kazumasa, Yoshinori and Junshi, 1979). Though minimal penetration of FNT is found in the rice and wheat grain, some FNT residues can be removed through milling and cooking (Hagstrum and Subramanyam, 2006). However, extensive usage of FNT in agricultural crops can lead to the bioaccumulation of FNT residues in food crops, which can lead to a greater risk of exposure to human and environmental.

FNT are slightly volatile compound which requires full personal protective equipment (PPE) while handling the chemical (Jepson, et al., 2020). Mosquito spray containing FNT can persist in air for a short period of time before it is completely degraded by ultraviolet (UV) upon sunlight exposure (Krzymien, 1982; Katsumata, et al., 2010). Though FNT was reported to be low in mammalian toxicity, but it still possessed a great concern when an individual chronically exposed at high amount of atmospheric FNT through inhalation and contact exposure through skin, commonly seen in agriculture workers (Wang, Naito and Nakajima, 2012).

The extensive use of FNT has raised concerns about its potential routes of exposure. FNT can be readily absorbed through ingestion of contaminated food and drinking water, as well as via inhalation during pesticide application (Kamanyire and Karalliedde, 2004; Elhalwagy, Darwish, and Zaher, 2008; Tudi, et al., 2022). A study conducted by Hollingworth, Metcalf, and Fukuto (1967) demonstrated that FNT is rapidly metabolized and excreted, with more than 90% of the administered dose excreted through the urine and faeces within 72 hours post administration in mice model. The primary metabolites identified in the excreted waste include demethyl FNT (Metabolite 1), 3-methyl-4-nitrophenol (Metabolite 3), dimethylphosphorothioic acid (Metabolite 5), *O*,*O*-dimethyl phosphate (Metabolite 6) and demethyl fenitrooxon (Metabolite 8) as shown in Figure 2.10 (Hollingworth, Metcalf, and Fukuto, 1967b; Miyamoto, J., Mihara, K., and Hosokawa, 1976).

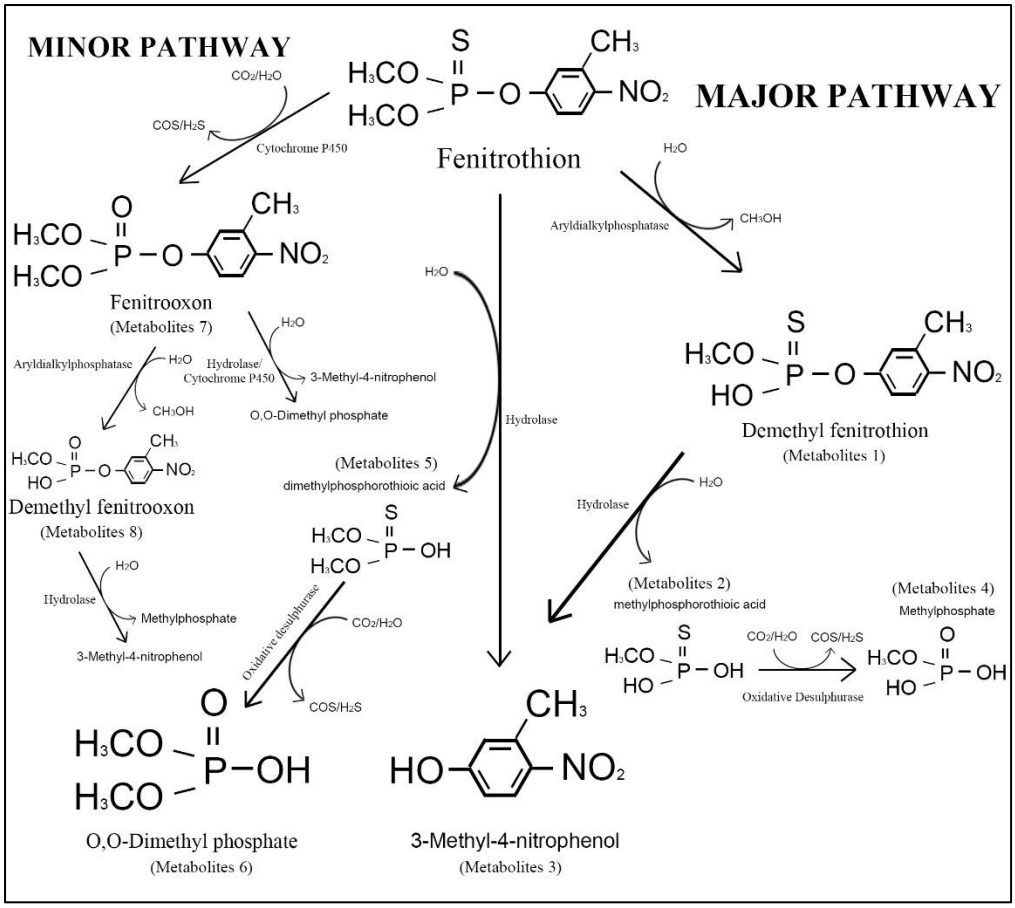


Figure 2.10: Metabolic pathways of FNT (Ugaki, et al., 1985; World Health Organization, 1992).

Some of the metabolites of FNT exhibit toxic effects on the body. For instance, studies have demonstrated that fenitrooxon, a reactive metabolite of FNT, inhibits AChE activity and induces oxidative damage in spermatozoa of the rats (Miyamoto, et al., 1978; Choi, Roche, and Caquet, 2000; Sarabia, Maurer, and Bustos-Obregón, 2009). Meanwhile, 3-methyl-4-nitrophenol was reported for impairing testicular function and reducing plasma testosterone level in rats by its endocrine disrupting effect (Bhushan, et al., 2000; Yue, et al., 2011).

In general, the extensive usage of FNT could lead to environmental pollution and caused severe adverse effects towards human health and ecosystem. It is highly recommended either on reducing utilization of FNT and other pesticides or finding an alternative solution to suppress or ameliorate the adverse effect of these pesticides' exposure on human body.

2.3.2 Effects of FNT on Male Reproduction

Jepson, et al. (2020) have revealed that FNT as a high-risk exposed pesticides towards human health and environment. It acts as an endocrine disrupting chemical (EDC) that acts on endocannabinoid system of the male reproductive organs, which causes damage to the testicular tissue structure of the testis and induces spermatotoxicity (Ito, et al., 2014; Taib, et al., 2014). Saber, Abd El-Aziz and Ali (2015) found that FNT exposure significantly reduces serum testosterone and LH in experimental rats, this further verify its endocrine disrupting properties. Tamura, et al. (2001) also stated that FNT as a competitive androgen receptor antagonist, it actively binds onto the androgen receptors and disrupts the overall endocannabinoid system of the male reproductive organs.

A study by Taib, et al. (2013) reported that FNT reduced sperm quality, sperm quantity and induced testicular damage on experimental rodent at 20 mg/kg oral gavage dose of FNT for 28 days. Miyake, et al. (2018) also reported

that FNT induced epididymal phospholipidosis by inhibiting phospholipase A2 secretion (sPLA2), thus leading to the induction of cytoplasmic vacuolation in epithelial cells. Meanwhile, FNT causes spermatotoxicity by inducing oxidative stress towards spermatocytes, which leads to the lower chances of mating behaviour and successful pregnancy (Yusoff, et al., 2020). FNT-treatment on fertile male SD rats lead to higher percentage of abnormal sperm and sperm DNA fragmentation, thus reducing the fertilization capacity and outcomes of pregnancy (Yusoff, et al., 2021).

2.3.3 Other Adverse Effects of FNT towards Human and Living Species

As an AChE inhibitor, FNT are known to exert neurotoxic effects by cholinergic overstimulation, neurotransmission disruption and causes neuropathological changes (Ahmed and Zaki, 2009). Besides that, they are also known to induce toxicity towards liver, kidney, lung and brain in murine model (Trottier, et al., 1980; Abdel-Ghany, et al., 2016).

Several studies reported that FNT have caused the liver damage (elevated serum markers of alanine transaminase, aspartate aminotransferase and alkaline phosphatase), disruption of kidney function (increased serum creatinine, uric acid and urea) and damaged in the brain neurotransmitter (elevated serum AChE, serotonin, glutamate and gamma-aminobutyric acid (GABA)) (Trottier, et al., 1980; Abdel-Ghany, et al., 2016). Afshar, et al. (2008)

also reported adverse changes in liver and kidney of rats treated with 100 mg/kg bw of FNT. The histopathology analysis of the liver tissue showed infiltration of leucocytes in portal area and mild necrosis in hepatocytes. Whereas significant medulla haemorrhage, degenerated tubular epithelium and dilated tubular were observed in the kidney tissues. FNT was also reported on inducing oxidative stress, genotoxicity, hepatopathy inflammation, nephropathy injury, and encephalopathy (Diab, et al., 2012b; Galal, et al., 2019).

Table 2.4: Harmful effects of FNT.

List of harmful effect	References
Oxidative stress (high malondialdehyde (MDA) level)	Budin, et al. (2012), Donia, et al. (2018)
Male infertility impairment (low sperm count, motility, testosterone level, testicular damages)	Taib, et al. (2013), Ito, et al. (2014), Taib, et al. (2014), Saber, et al. (2016), Donia, et al. (2018), Miyake, et al. (2018), Yusoff, et al. (2020; 2021)
Inhibition of AChE	Trottier, et al. (1980), Ahmed and Zaki (2009), Abdel-Ghany, et al. (2016)
Liver toxicity	Trottier, et al. (1980), Afshar, et al (2008), Diab, et al. (2012b), Abdel-Ghany, et al. (2016)
Kidney toxicity	Afshar, et al. (2008), Diab, et al. (2012b), Budin, et al. (2013), Abdel-Ghany, et al. (2016)

2.3.4 Ameliorating Therapy on FNT-induced Adverse Effects in Murine Model

Due the hazardous effects of FNT exposure towards human health, studies were carried out using animal model to determine the restorative and/or preventive approaches towards the impairment caused by FNT. Tocotrienol-rich fraction (TRF) was proposed as potential compound that can ameliorate

impairment due to FNT exposure. TRFs have gained significant attention due to their potent antioxidant and anti-inflammatory properties (Muid, et al., 2013). Interestingly, several studies showed that TRF co-administration ameliorate pancreas, liver and kidney damages caused by FNT in SD rats (Budin, et al., 2012; 2013; Jayusman, et al., 2017). This could be due to high antioxidative activity exhibit by TRFs protect the testicular cells from oxidative damages. Improvement in male fertility were also observed with significant elevation of sperm quality and quantity in male SD rats (Taib, et al., 2014).

Meanwhile, green tea extract was also reported to ameliorate the adverse effects induced by FNT in animal model. Elhalwagy, Darwish and Zaher (2008) have shown the prophylactic effect of green tea extracts in protecting liver and kidney from pathological damages with the significant reduction of the targeted markers (alanine aminotransferase (ALT), aspartate transferase (AST), albumin and urea) in FNT-treated albino rats. It can be due to the polyphenol in green tea extracts possessed strong antioxidant properties which capable of suppressing the oxidative stress induced by FNT in the liver and kidney (Tahoun, Mohammed and Donia, 2018). In another study, green tea extract was reported to have restorative effects on FNT-induced damages of the lung, brain and testis in rats (Donia, et al., 2018).

Besides that, natural supplement such as vitamin C, selenium and quercetin were also able to mitigate the induced adverse effects in animal

studies. Saber, Abd El-Aziz and Ali (2015) reported that the co-administration of quercetin and FNT have showed significant reduced damaged in the testicular tissues with an improvement of sperm quality and quantity in male SD rats. At the same time, vitamin C and selenium have also significantly reduce lipid peroxidation level and oxidative stress level besides repairing of damaged tissues in rats due to FNT administration (Donia, et al., 2018; Milošević, et al., 2018; Tahoun, et al., 2018). Besides that, vitamin E were also reported to exhibit mild ameliorating effect towards FNT-induced oxidative damage with the observed restoration of damaged liver and kidney tissues in albino rats (Abdel Reheim, et al., 2008).

From the overall findings above, TRF, green tea extracts, quercetin, vitamin C, vitamin E and selenium are exhibiting promising protective effects against FNT-induced toxicity. However, more investigation is still required on the underlying mechanisms and clinical relevance of supplementation of the selected bioactive compounds as part of the alternative therapeutic strategies for the mitigation of FNT-induced impairment.

Interestingly, the findings suggest a notable research gap regarding the interaction between FNT toxicity and the potential protective effects of KPE. FNT toxicity is hypothesized to act through multiple mechanistic pathways, primarily involving the generation of ROS, disruption of endocrine signaling pathways and impairment of androgen receptor activity. These effects

collectively result in oxidative damage to sperm cells, testicular inflammation and impaired spermatogenesis. Alternatively, the KPE, rich in methoxyflavones, is proposed to counteract these effects via its strong antioxidative properties, scavenging ROS and enhancing testosterone level by inducing androgenic signalling activity. Therefore, *K. parviflora* may play a critical role in protecting against FNT-induced male reproductive toxicity. This hypothesis sets a foundation in the present study to investigate the protective effects of *K. parviflora* against FNT-induced male reproductive toxicity (Figure 2.11).

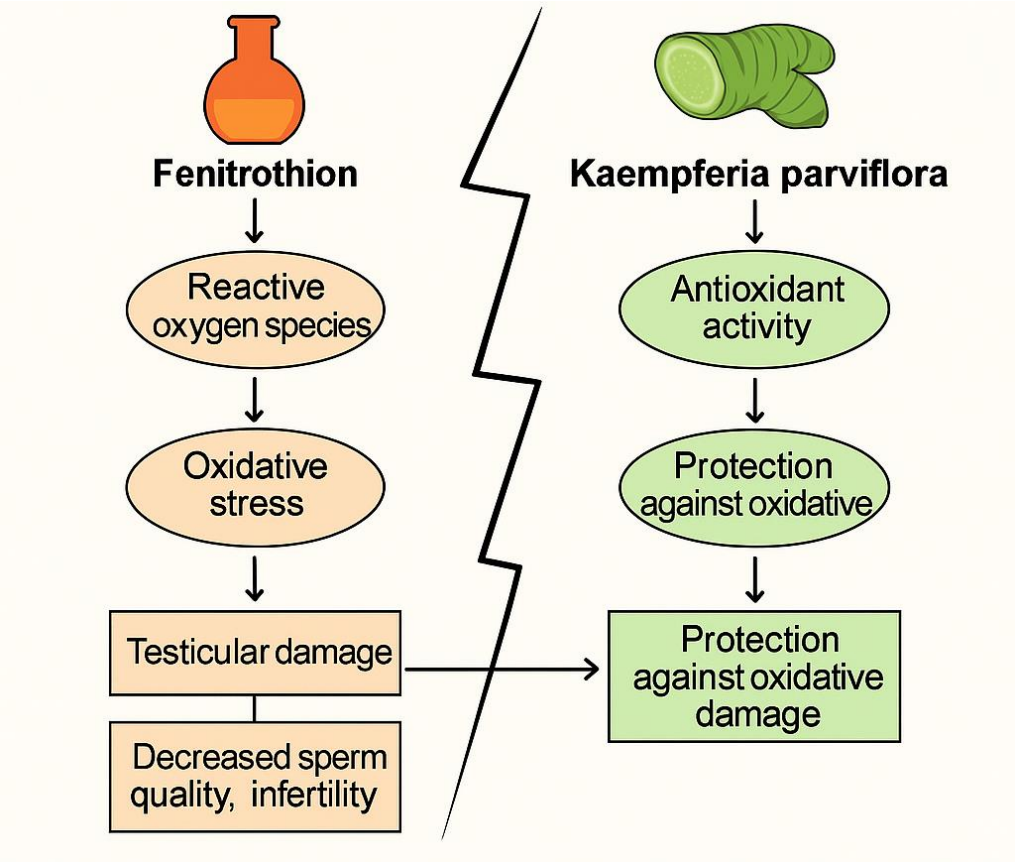


Figure 2.11: Proposed mechanistic pathways in FNT toxicity and counteractive effects of *K. parviflora*.

2.3.5 Other Environmental Toxicants towards Male Reproduction

Environmental toxicants are man-made chemical and physical agents which responsible for causing harm through environmental or occupational exposures. They can be classified as household consumables (bisphenol A (BPA) products, phthalates), pharmaceutical products (estradiol, diethylstilbestrol) and industrial chemicals (polychlorinated biphenyls, FNT, dioxins) (Jain and Singh, 2022). These toxicants can be absorbed by various routes, including ingestion, dermal absorption and inhalation which possibly induce adverse health effect under long term exposure (Kamanyire and Karalliedde, 2004; Tudi, et al., 2022).

Studies have shown that the exposure to environment toxicants such as pesticides, heavy metals and industrial chemicals could lead to increase of oxidative stress injury towards the male reproductive system (Sharma, et al, 1999; Agarwal, Prabakaran and Said, 2005; Tremellen, 2008). Environmental toxicants can act as EDCs, which are responsible for causing infertility, epigenetic alteration in cell, thus leading to cancer and other possible endocrine disorders (Pogribny and Rusyn, 2012; Maqbool, et al., 2016; Jain and Singh, 2022). EDCs mimic endogenous hormones, where they bind towards the receptors of the cell as shown in Figure 2.12 (Warner, et al, 2020). They cause interference in hormone signalling pathway, that causes the disturbances in reproductive development, immune and neuronal system as they bind to steroid hormone receptors (Lee, et al., 2012; Stamatian and Goidescu, 2016).

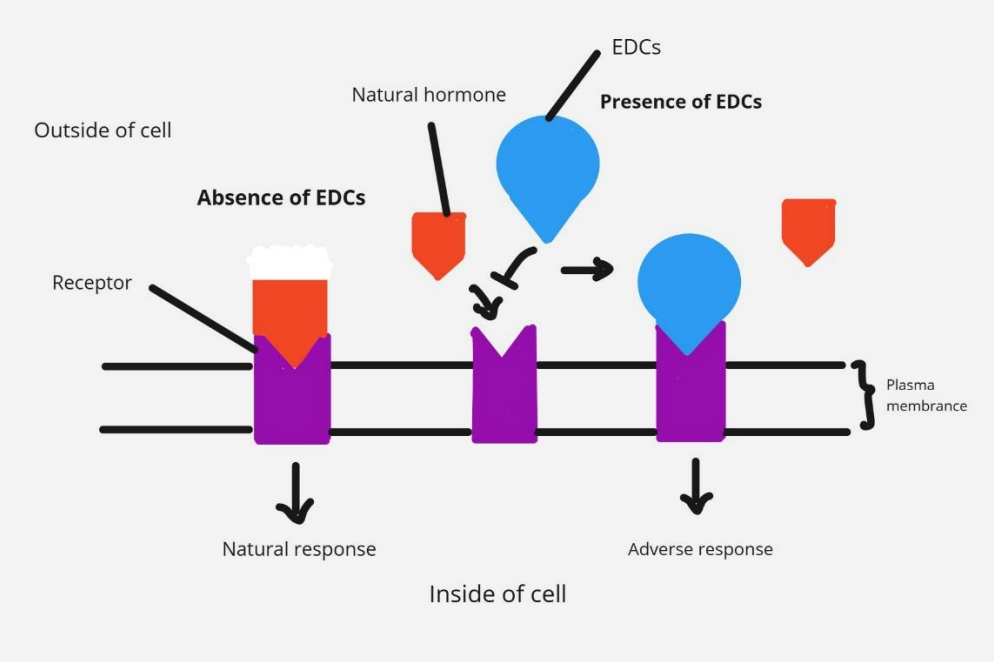


Figure 2.12: Mechanism of how EDCs worked in biological cells (modified from Stamatian and Goidescu, 2016).

Several studies have showed on the adverse effect of these environment toxicants towards male reproduction. BPA was reported to reduce the number of germ cells and induced apoptotic cell death in testicular tissue, leading to low sperm count in rats (Kaur, Saluja and Bandal, 2017). 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has also been proven on inducing oxidative stress in the mouse testis (Jin, et al., 2008). Cadmium, a type of heavy metal, was also reported capable of inducing necrosis in the testicular tissue of murine model (Parizek and Záhor, 1956; Chiquoine, 1964). Benzene hexachloride BHC, an organochlorine pesticide which was known better as lindane, has also been reported in affecting the gap and tight junction proteins in Sertoli cells of testicular tissue (Defamie, et al., 2001).

Due to the increasing usage of pesticides and formulation of pesticides in recent years, study found traces of pesticide residue in soil, sediment and ground-water bodies of agroecosystems (Estévez, et al., 2012; Vryzas, 2018). The adverse effects of chronic exposure to pesticide have led to several compilation, such as oxidative stress, epigenetic alteration in cells and hormonal dysregulation. It eventually leads to the alteration of the reproductive organ function and impaired fertility level in male and female (Sengupta and Banerjee, 2013; Singh, et al., 2017; Kalyabina, et al., 2021).

CHAPTER 3

MATERIALS AND METHODS

3.1 Overview of Research

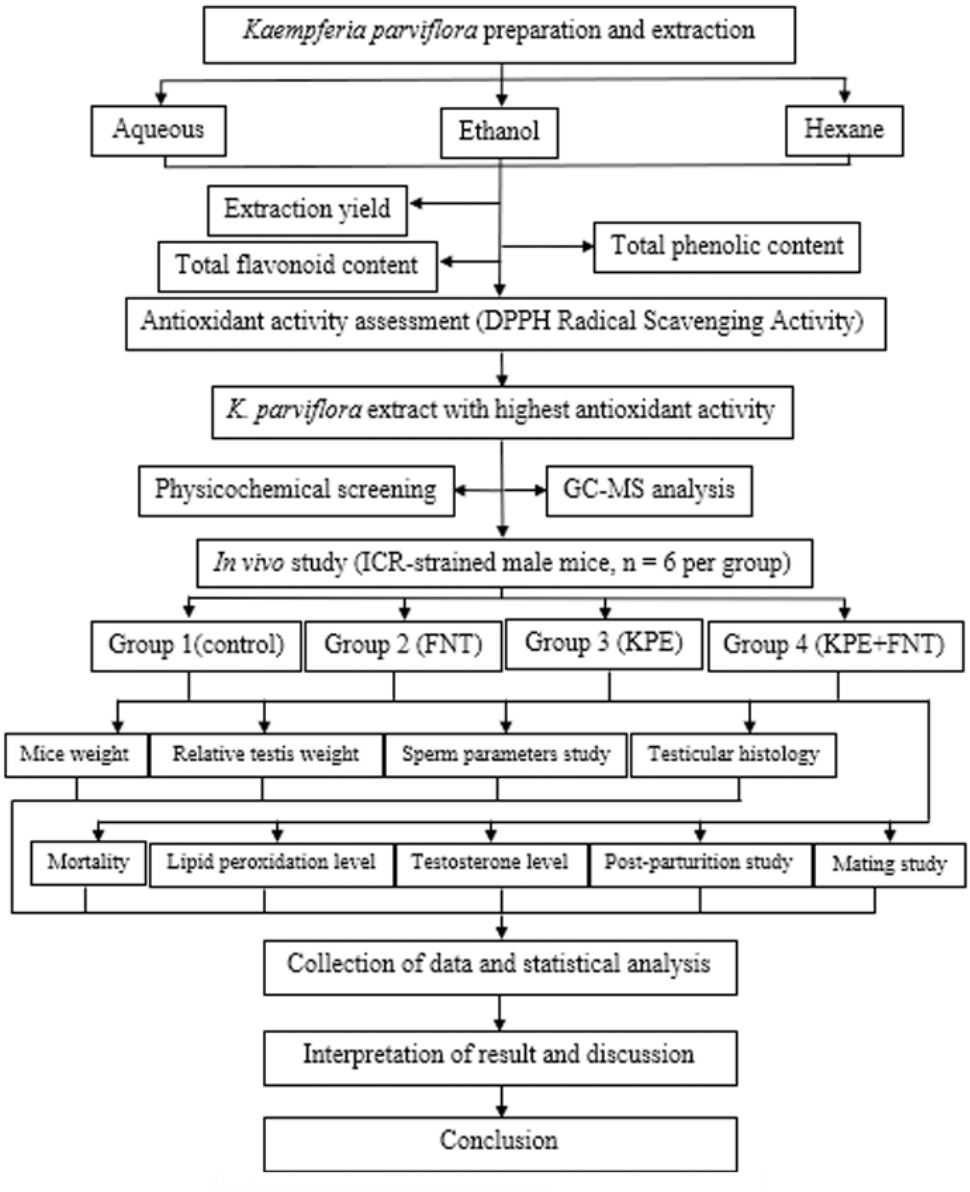


Figure 3.1: Overview of the study.

3.2 Extraction of *K. parviflora*, Antioxidant Qualitative and Quantitative Analysis

3.2.1 Collection of *K. parviflora* and Preparation of *K. parviflora* Extracts

Fresh rhizomes of Kaempferia parviflora (about 8 – 9 months old) were harvested from the Agricultural Park, Universiti Tunku Abdul Rahman, Kampar, Perak, Malaysia (GPS coordinates: 4.3418174, 101.13998) and the plant specimens were authenticated by Dr. Khairil Mahmud from the Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen was deposited at the Herbarium of the Institute of Bioscience, Universiti Putra Malaysia under the voucher number KM0212/25. The plants were washed, peeled and air-dried (3 to 4 days) (An, et al., 2016). The dried slices of the rhizomes were pulverized and macerated using either distilled water, ethanol or hexane (Sudwan, et al., 2006). The resulting extracts from the 3 different solvents were subsequently concentrated using a rotary evaporator and further dried via lyophilization (Figure 3.2) (Weerapol, et al., 2017). The extraction yield of each extract was determined as shown below (Takuathung, et al., 2021; Sitthichai, et al., 2022):

$$\text{Extraction yield (\%)} = \frac{\text{Weight of freeze-dried extract}}{\text{Weight of } \textit{K. parviflora} \text{ powder used for extraction}} \times 100\%$$

Figure 3.2: Freeze drying of *K. parviflora* extracts.

3.2.2 Total Phenolic Content (TPC)

The determination of total phenolic content followed the protocol outlined by McDonald, et al. (2001) with minor adjustments. The sample extracts were prepared at a concentration of 1 mg/mL by dissolved the powdered extracts in ethanol. Gallic acid standard was prepared in several concentrations (20 - 100 µg/mL). A volume of 20 µL of the standard and samples were prepared in 96-well-plate, with each well then pipetted with 100 µL of Folin–Ciocalteu reagent (10× dilution) and 80 µL of 1 M sodium carbonate (Figure 3.3). The 96-well-plate was incubated at 45°C for 30 minutes. Subsequently, the mixtures were allowed to cool for 10 minutes and the absorbance of each standard and samples were measured at 765 nm using a microplate reader. All tests were conducted in triplicate. A standard curve of absorbance was plotted and the determination of the total phenolic content of

the sample extract was expressed in milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

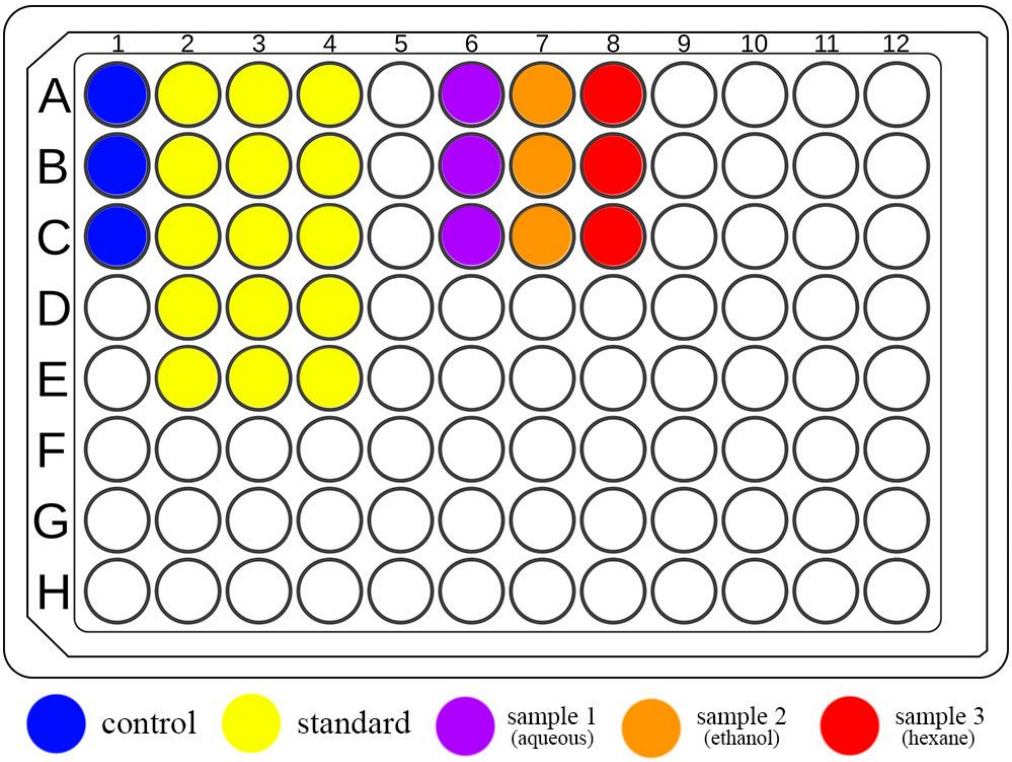


Figure 3.3: TPC assay conducted in 96 well plates.

3.2.3 Total Flavonoid Content (TFC)

Total flavonoid content assay was performed following the protocol described by Jimoh, Afolayan and Lewu (2019) with adjustments. The TFC colorimetric reagent was prepared by combining 0.6 mL of 5% sodium nitrite with 8 mL of distilled water, 0.6 mL of 10% aluminium chloride, and 4 mL of 4% sodium hydroxide. The sample extracts were prepared at a concentration of 1 mg/mL by dissolved the powdered extracts in ethanol. Quercetin standard was

prepared in several concentrations (400 - 2000 µg/mL). A volume of 20 µL of the standard and samples were prepared in 96-well-plate, with each well then pipetted with 132 µL of TFC reagent and 48 µL of distilled water. The 96-well-plate was incubated at 40°C for 20 minutes (Figure 3.4). Subsequently, the mixture was allowed to cool for 10 minutes and the absorbances of each standard and samples were measured at 430 nm using a microplate reader. All tests were performed in triplicate. A standard curve of absorbance was plotted and the determination of the total flavonoid content of the sample extract was expressed in milligrams of quercetin equivalent per gram of dw (mg QE/g dw).

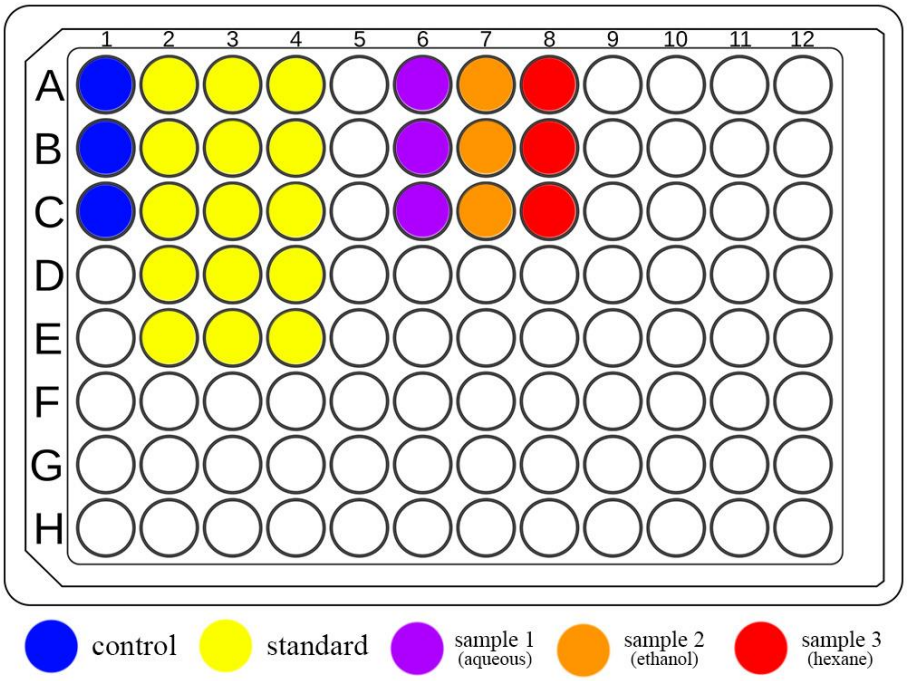


Figure 3.4: TFC assay conducted in 96 well plates.

3.2.4 DPPH Radical Scavenging Activity Assay

The DPPH assay method was adapted from Prieto (2012) with adjustments. Initially, a 0.2 mM DPPH solution was prepared by dissolving 39.4 mg of DPPH in 500 ml of ethanol. Ascorbic acid served as the standard, with fixed concentrations (2 to 10 µg/mL), while the stock sample extract was prepared at a concentration of 10 mg/mL. Subsequent dilutions of the sample were performed in a 96-multiwell plate, with a total volume of 100 µL, alongside the specified concentrations of ascorbic acid, each in 100 µL. The solution was added into specified wells as shown in Figure 3.5. Later, a volume of 100 µL of 0.2 mM DPPH solution was added into all wells. The 96-well-plate was then incubated in dark for 30 minutes . The absorbance readings of both the standard and samples were taken at 517 nm using a microplate reader. The assay was conducted in triplicate, and the percentage of DPPH scavenging activity was determined using the formula provided below (Mishra and Sharma, 2021):

$$\text{DPPH scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100\%$$

Subsequently, the half effective concentration for inhibiting DPPH free radicals (EC_{50}) was determined by plotting the graph of DPPH scavenging activity against the concentration of the tested standards and samples.

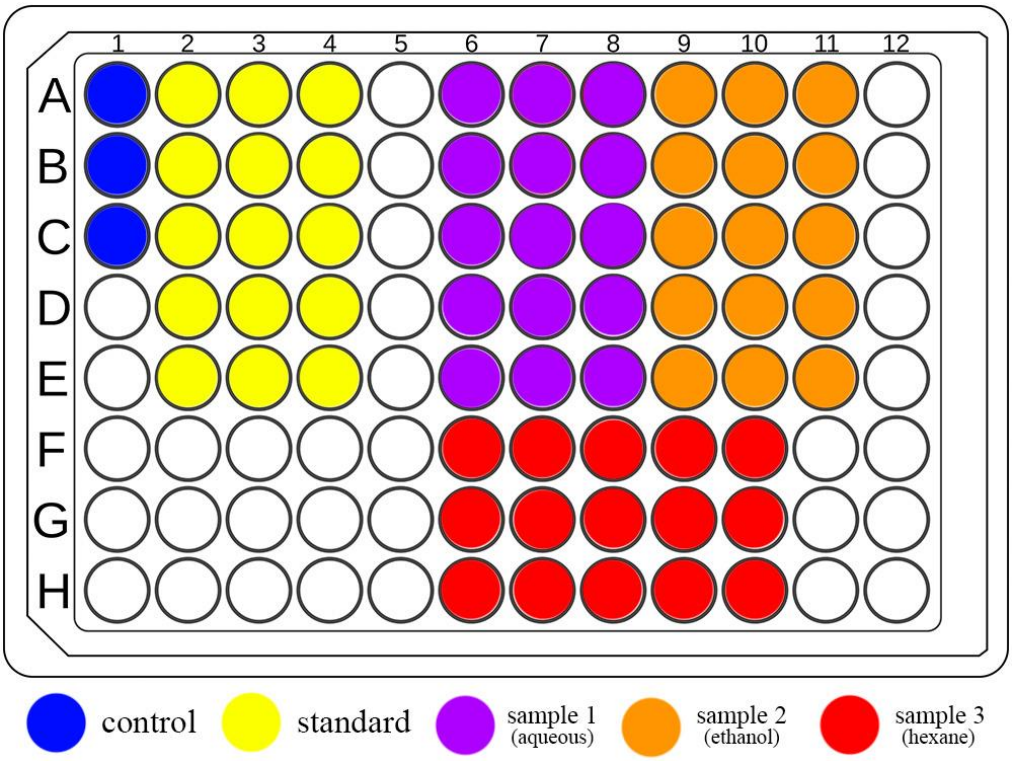


Figure 3.5: DPPH assay conducted in 96 well plates.

3.2.5 Physicochemical Properties of KPE

After the selection of the KPE with the best antioxidative properties, the list physicochemical properties within the KPE were evaluated as described in Table 3.1.

Table 3.1: **Types of tests to evaluate physicochemical properties of KPE.**

Test	Method	If present (+)	References
Tannins	A volume of 2 mL of 5% ferric chloride was mixed with 2 mL of KPE.	Yellow brown precipitates present.	Parekh and Chanda (2007)
Saponins	A volume of 5 mL KPE was shaken vigorously with the addition of few drops olive oil.	Emulsion forms.	Anto, et al. (2016)

Alkaloids	A volume of 2 mL KPE was mixed with 1.5 mL of 1% hydrochloric acid and heated for 1 minute. The warm mixture was added with of 6 drops Wagner's reagent and gently mixed.	Formation of reddish-brown precipitates.	Mushtaq, et al. (2014)
Anthraquinones	A volume of 1 mL KPE was boiled together with 10 mL of concentrated sulphuric acid. The solution was filtered while hot and mixed vigorously with 5 mL of chloroform. After the solution have formed double layer, the bottom layer of the solvent was extracted and mixed with 1 mL of diluted ammonia.	Pink-red layer forms at ammonia lower layer.	Baoduy, et al. (2015)
Cardiac glycosides	Two millilitres of KPE were mixed with 1 mL of concentrated glacial acetic acid, followed by the addition of 2 drops of ferric chloride and 1 mL of concentrated sulphuric acid.	Brown ring interface forms above the solution.	Trease and Evans (1989)
Terpenes	A volume of 2 mL KPE was shaken vigorously after the addition of 2 mL of concentrated glacial acetic anhydride and 5 mL chloroform. Later, the mixture was added slowly with 3 drops of concentrated sulphuric acid.	Reddish-brown layer forms on top of the solution.	Harborne (1984)
Volatile oil	A volume of 0.5 mL diluted sodium hydroxide was added into 2 mL of KPE. Then, the solution was mixed. Later, three drops of diluted hydrochloric acid were gently added into the mixture.	Precipitates form below the solution.	Anto, et al. (2016)
Phenolics	One millilitre of 1% ferric chloride was mixed with 2 mL KPE.	Solution turns into blue-green solution.	Mushtaq, et al. (2014)
Flavonoids	Small chunk zinc metals were dissolved in 1 mL of concentrated hydrochloric acid. The solution was then mixed with 2 mL of KPE.	Solution turns into pink-red/magenta-red solution.	Parekh and Chanda (2007)

3.2.6 GC-MS Analysis

GC-MS analysis was performed to identity the major phytochemical compounds in KPE (Pitakpawasutthi, Palanuvej and Ruangrungsi, 2018). The instrument was set up according to the conditions below:

Column: BPX fuse silica column, 30 × 0.25 mm, 0.25 µm

Oven temperature: 60°C - 240°C (3°C/min)

Carrier gas flow: Helium (1 mL/min)

Injection: 10 µL sample (1 mL KPE sample in 100 mL GC grade methanol)

Electron impact positive mode: 70 electron volts

The eluted *m/z* constituents from the peak were analysed by using NIST02 Mass Spectral library and matching the mass spectra with the retention time of the constituents. The identified results were presented according to the retention time, name of the identified constituents and the abundancy of the constituents.

3.3 *In vivo* Mice Study

3.3.1 Mice Husbandry

All animal handling and experimental procedures in this study were approved by the UTAR Scientific and Ethical Review Committee (SERC) under the reference number U/SERC/254/2022. The ICR-strain mice were purchased from Universiti Kebangsaan Malaysia Animal House, Selangor, Malaysia and they were housed in Cabin 2 (CB2), Animal Containment Unit, Agriculture Park Gate A, UTAR Kampar Campus, Perak, Malaysia. The animal containment unit was maintained at 23 ± 3°C, with regulated air ventilation and a controlled 12 hours of light and 12 hours of dark cycle. Pelleted mice chow from Gold Coin Feedmills Pte. Ltd and clean water were given to mice *ad libitum*.

3.3.2 Experimental Design

The ICR mouse was selected as *in vivo* experimental model due to its high fertility and rapid growth rate (Eaton, et al., 1980). These characteristics make it ideal for studies where reproductive health, developmental changes, or treatment effects need to be assessed within a shorter experimental timeframe (Johnson, 2012). A total of 24 male ICR-strained mice (4-weeks-old, 30 ± 5 g) were assigned randomly into 4 different treatment groups (6 mice per treatment group) which is shown in Table 3.2 below:

Table 3.2: Descriptions of treatments given to each treatment group of mice.

Treatment group	Description of treatments
Control group (CTL group)	5 mL/kg/day bw extra virgin olive oil (EVOO) twice
FNT treatment group (FNT group)	20 mg/kg/day bw FNT, followed by 5 mL/kg/day bw EVOO
KPE treatment group (KPE group)	50 mg/kg/day bw KPE, followed by 5 mL/kg/day bw EVOO
Co-administered treatment group (KPE + FNT group)	50 mg/kg/day bw KPE, followed by 20 mg/kg/day FNT

The dosages of FNT (20 mg/kg bw) and KPE (50 mg/kg bw) were selected for in vivo administration. The 20 mg/kg bw dose of FNT represents one over fifty (1/50) of its LD_{50} in mice and corresponds to a low dose previously reported to induce spermatotoxicity in rats (World Health Organization, 1992; Yusoff, et al., 2020). Meanwhile, the 50 mg/kg bw dose of KPE was chosen based on studies demonstrating its ability to ameliorate cimetidine-induced testicular toxicity in mice without observable adverse effects (Luangpirom and Komnont, 2011; Yoshino, et al., 2019). All administrations were done daily by oral gavage route (Figure 3.6) for 28 consecutive days alongside the record of mice weight (Ng, et al., 2021). All mice were observed daily throughout the treatment period for any signs of illness, distress, or mortality. At the end of the treatment, the mortality rate was calculated based on the number of deaths recorded in each group.

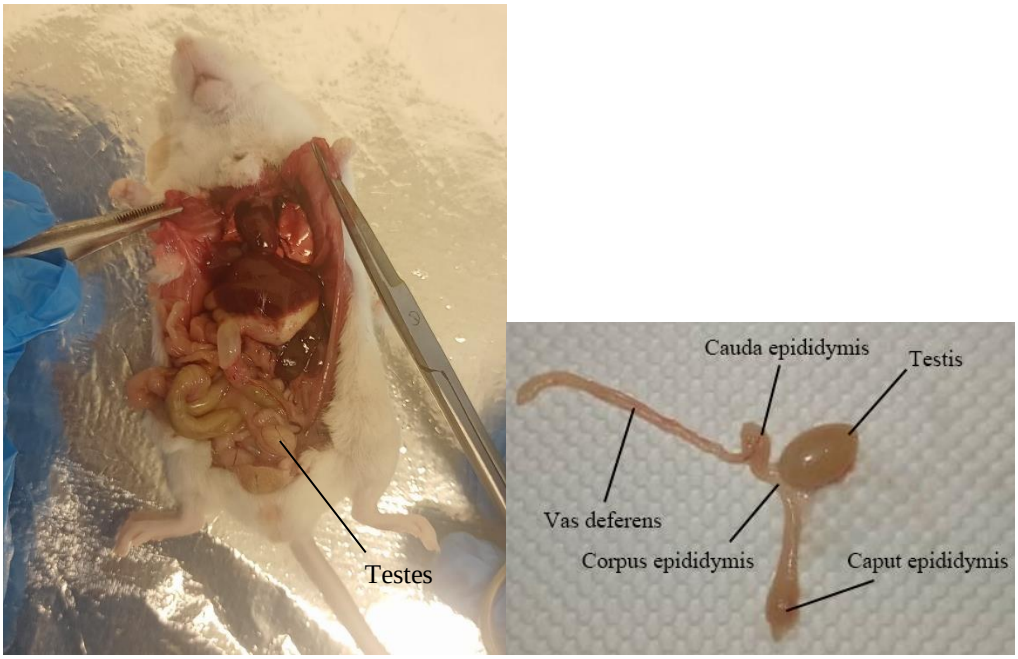
Figure 3.6: The administration of mice via oral gavage route, the needle used was sterile packaged and it was allowed for one time usage for each administration.

Meanwhile, the selected KPE was prepared fresh weekly by dissolving the freeze-dried KPE powder in a mixture of absolute ethanol and EVOO solution (1 to 9 ratio) (Figure 3.7). FNT vehicle was prepared weekly by dissolving FNT in EVOO as EVOO is refined olive oil which possesses low α-tocopherol content as compared to other potential vegetable oils (Harwood and Yaqoob, 2002; Psomladou, Tsimidou and Boukou, 2000). Both KPE and FNT vehicle were then stored in refrigerators and thawed at room temperature before use.

Figure 3.7: KPE dissolved in olive oil.

After 28 days of administration, a total of three male mice from each treatment group were euthanized via cervical dislocation. After euthanizing the male mice, the testes were carefully identified and dissected through a midline incision in the scrotal sac. The surrounding fat and connective tissues were gently removed to avoid damaging the organ (Figure 3.8). Each testis was blotted dry and weighed immediately using a precision balance to obtain its absolute weight (Sanghamitra, et al., 2008). The relative testis weight of mice was calculated using the following formula:

$$\text{Relative testis weight (\%)} = \frac{\text{Testis wet weight (g)}}{\text{Final bw (g)}} \times 100\%$$



(a) (b)

Figure 3.8: (a) Dissection of mouse, the target organs (cauda epididymis, testis) were identified and harvested for sperm parameters and histological assessment. (b) The male mouse reproductive system anatomy.

The collected testes were fixed in 10% neutral buffered formalin overnight for histology studies. Meanwhile, the cauda epididymis was extracted in PBS via epididymal puncture at 37°C for sperm parameter assessment (sperm concentration, progressive motility and viability). Blood was collected via cardiac puncture and stored in -20°C for lipid peroxidation level and testosterone measurement. The remaining 12 live male mice from the 4 treatment groups were subjected for mating and breeding assessments.

3.3.3 Lipid Peroxidation Level in Blood Serum Measurement

The measurement of lipid peroxidation level was performed using malondialdehyde (MDA) colorimetric assay kit from Elabscience®. Approximately 1 mL of blood sample was collected from each mouse in each treatment group. The blood samples were allowed to clot and were centrifuged to obtain serum for MDA assay. The standard curve of the assay was plotted with the following concentrations 5-40 µmol/L. The calculation of serum MDA levels was performed using the formula provided by the manufacturer shown below:

$$\text{MDA } (\mu\text{mol/L}) = \frac{\Delta A - b}{a} \times f$$

$\Delta A = A_{\text{sample}} - A_{\text{control}}$

b = intercept of standard curve

a = slope of standard curve

f: dilution factor of sample (for mice, f = 1)

3.3.4 Testosterone Level in Blood Serum Assessment

Blood serum collected from each mouse treatment group were used for testosterone level assessment, modified HPLC method according to Dikma® Technologies Inc. Testosterone HPLC grade, 98% purity was used as standard and prepared in the series of 20 µg/mL to 100 µg/mL by dissolving in 30% acetonitrile. Meanwhile, ten microlitres of serum sample was diluted to 100 µL using 30% acetonitrile. Blank was prepared by using 30% acetonitrile. A blank, standard and sample were filtered using 0.45 µm polyvinylidene fluoride or polyvinylidene difluoride (PVDF) syringe filter before running HPLC. The HPLC instrument was set up according to the conditions below (Table 3.3):

Table 3.3: **Setting up of mobile phase gradient flow.**

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
0.00	60	40	1.0
0.60	60	40	
2.40	40	60	
4.80	40	60	
7.20	30	70	
10.00	30	70	
11.00	60	40	
15.00	60	40	

Column: Diamonsil® C18 (2), 150 x 4.6 mm, 5 µm

Mobile phase (MP): (A) 30 mM Potassium phosphate dibasic, pH 8.0

(B) Acetonitrile : Methanol (60 : 40 ratio)

Column temperature: 30°C

Wavelength: 242 nm

Injection volume: 10 μL

The retention time of the identified testosterone peak from standard was used as reference for the determination of testosterone level in blood serum according to the specification (relative retention time: 0.95 – 1.05) while other specifications such as plate count (not less than 2000) and tailing factor (not more than 2.0) were assessed to check the effectiveness of the column used above. Lastly, the standard curve of the standard was plotted and the sample serum testosterone level was calculated in the formula given below (Purdon and Lehman-McKeeman, 1997):

$$\text{Serum Testosterone (ng/mL)} = (x/1000) \div \text{m} \times \text{f} \times 1000$$

where

$x/1000 = \text{sample peak area under curve}$

m = slope of standard curve

f = dilution factor of sample (f = 10)

3.3.5 Measurement of Sperm Parameters

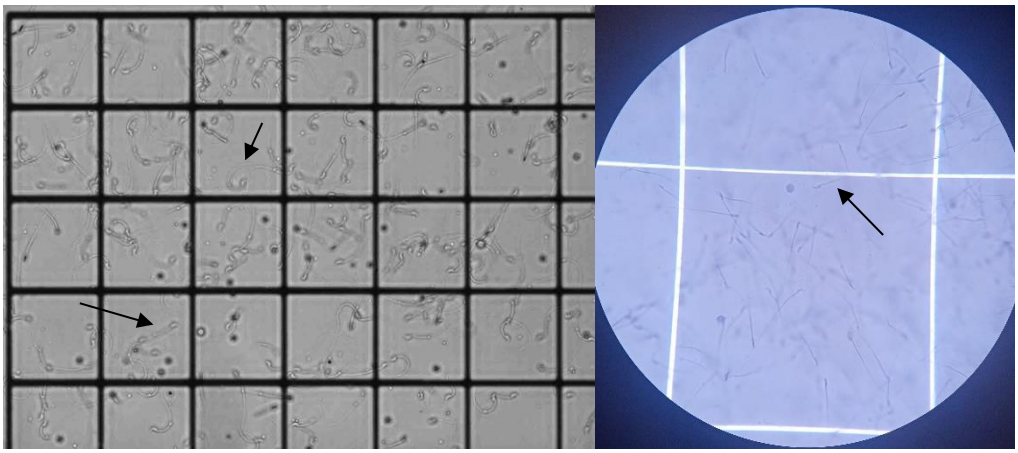
The fresh cauda epididymis harvested from euthanised mice was quickly immersed in 37°C of 100 µL PBS. The suspension was then stored at 37°C for a minute prior sperm evaluation. Ten microlitres of the suspension was then pipetted and placed onto a Makler counting chamber for sperm motility assessment as shown in Figure 3.9(a) (World Health Organisation, 2010). A total of 100 random sperm were evaluated according to the sperm motility patterns (Grade A: rapid motility, grade B: slow motility, grade C: non-progressive motility, grade D: immotile sperm) as described by Abdollahi, et al. (2021). The test was conducted in triplicate. Sperm motility was assessed based on the calculation below:

$$\text{Progressive sperm motility (\%)} = \frac{\text{Grade A + Grade B}}{100} \times 100\%$$

Meanwhile, a volume of 20 µL of the suspension was mixed with 10 µL 1% eosin and 10 µL 1% nigrosine. Ten microlitres of the mixture was then placed onto a Neubauer chamber and the mixture was allowed to flow throughout the chamber for a minute prior to sperm concentration and sperm viability as shown in Figure 3.9(b) (Mangoli, et al., 2013). A total of 200 sperm at random from the Neubauer chamber per sample were evaluated for sperm viability as per Figure 3.10. The test was conducted in triplicates. All calculations of the remaining sperm assessments are shown below:

$$\text{Sperm concentration} = \text{No. of sperm counted in a square box} \times 2 \text{ (dilution factor)} \times 10^5$$

$$\text{Sperm viability (\%)} = \frac{200 - \text{total dead sperm cell (pink-stained head sperm)}}{200} \times 100\%$$



(a) (b)

Figure 3.9: Sperm characteristics analysis using Makler counting chamber and Neubauer counting chamber. (a) Makler counting chamber (b) Neubauer counting chamber. Arrows indicate spermatozoa.

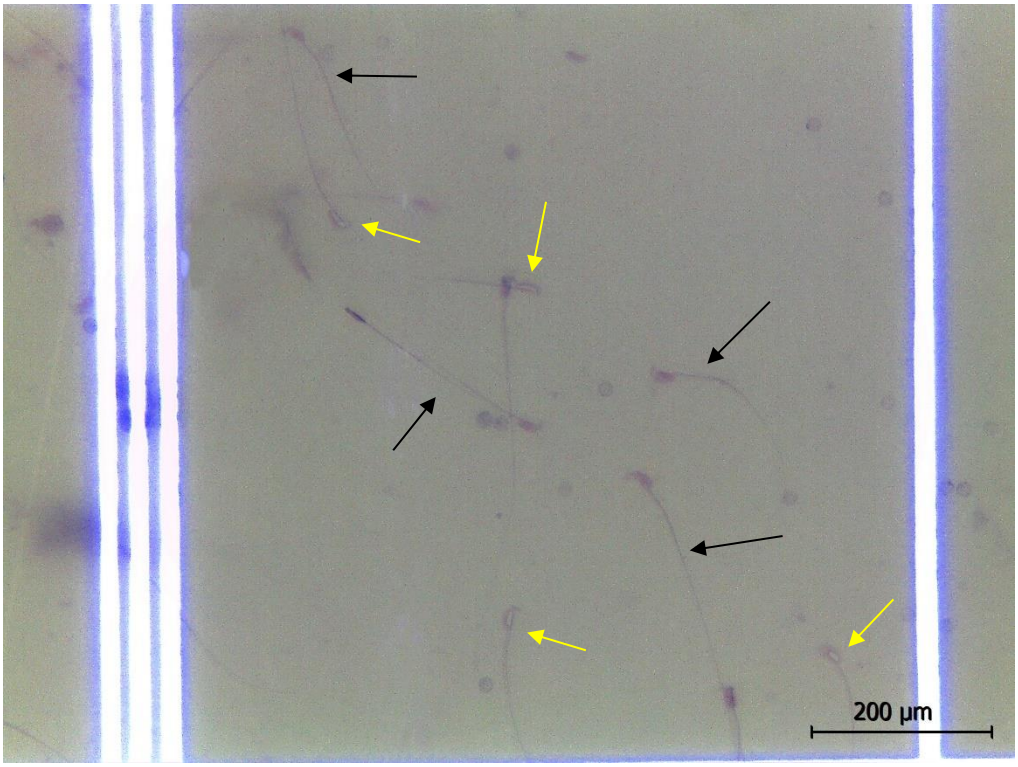


Figure 3.10: Sperm viability assessment under light microscope (400x). The sperm suspension which mixed with eosin-nigrosine staining consists of live sperm cells (yellow arrow, not stained-head), and dead sperm cells (black arrow, pink-stained head).

3.3.6 Histological Study

The testis of the euthanised mice was harvested and fixed in 10% NBF overnight. They were all placed in labelled cassettes before dehydrating in vacuum infiltration tissue processor. The processed organs were then transferred to tissue embedding module and all embedded in molten paraffin to form tissue block by appropriate metal mould size. The tissue block was solidified with the metal mould at 0°C. The solidified tissue blocks were then detached carefully from the metal mould and stored at room temperature.

The tissue blocks were transferred to rotary microtome and 5 µm of tissue section was obtained from each tissue blocks. The thin-sliced tissue section was allowed to float on 45°C water bath and desired section were fished onto uncoated glass slide. The excess water was drained from the glass slide and placed on 60°C slide dryer for drying and attachment to slide for a 1 hour. The glass slide was then slanted in 45°C oven overnight to remove excess paraffin before proceeding haematoxylin and eosin (H & E) staining steps.

3.3.7 Haematoxylin and Eosin Staining (H & E Staining)

The H and E staining was performed according to ST InfinityTM H&E Staining System (Leica) manufacturer's method. The staining was separated into different stages (Table 3.4).

Table 3.4: Haematoxylin and eosin staining stages.

Stage	Solvent/Solution	Time (min:sec)
Deparaffinization	Xylene	2:00 (3 times)
Rehydration	Absolute ethanol	1:00 (3 times)
	80% Ethanol	1:00
	ST HemaLast	1:00
	Running tap water	0:30
Nuclear staining and	ST Hematoxylin	5:00
blueing	Running tap water	2:00
	ST differentiator	2 dips

	Running tap water	1:00
	ST Bluing agent	1:00
	Running tap water	1:00
	70% Ethanol	1:00
Counterstaining	ST Eosin	3:00
Dehydration	95% Ethanol	2:00
	Absolute ethanol	2:00 (2 times)
Clearing	Xylene	2:00 (2 times)

After clearing stage, DPX mounting medium was used as glue to cover the stained tissue with coverslip. The DPX-mounted slide was left to dryn and viewed under DM500 microscope. The digital image obtained from the microscope was captured using an ICC50 HD camera module (Leica). Histological changes of testicular tissue in each treatment group were observed and evaluated.

The histological testicular scoring system was evaluated according to the method by Dang-Cong and Nguyen-Thanh (2021) with minor modifications. A total of 20 seminiferous tubules for each testis of the mice were scored from 1 to 10 based on the description as shown in Table 3.5. The test was conducted in triplicate and the final score was calculated.

Table 3.5: Johnson's mean testicular biopsy score count (Dang-Cong and Nguyen-Thanh, 2021).

Score	Description
10	Complete spermatogenesis, organized germinal epithelium, open lumen.
9	Many present spermatozoa, a slight disorganized germinal epithelium, obliteration of lumen.
8	Few spermatozoa present in the tubular section.
7	No spermatozoa present but many spermatids in the tubular section.
6	No spermatozoa but few spermatids present in the tubular section.
5	No spermatozoa and spermatids but many spermatocytes present in the tubular section.
4	Only few spermatocytes present in the tubular section.
3	Only spermatogonia cells present in the tubular section.
2	Only Sertoli cells present in the tubular section.
1	No cells present in the tubular section.

3.3.8 Percentage of Mating Success and Post-Parturition Assessment

Prior to the start of the mating experiment, the estrous cycle of all female ICR-strained mice (8 weeks old, 30 ± 5 g) was monitored via vaginal inspection for at least two consecutive cycles to identify and synchronize their reproductive phases (Heykants and Mahabir, 2016). Only females mice in the proestrus or estrus phase, which indicates the peak of fertility, were selected for cohabitation to standardize mating receptivity and avoid cycle-related bias (Byes, et al., 2012).

Subsequently, 36 untreated female mice were randomly cohabited with the remaining 12 male mice from each group (1 male:3 females ratio) for a period of 14 days. Cohabitation occurred daily from 6:00 PM to 6:00 AM, in a quiet environment without external disturbance (Ananthasubramaniam and Meijer, 2020). The presence of vaginal plug of each female mouse was checked daily to indicate successful mating from their male partner before separating both gender mice (Stockley, et al., 2020). Any positive vaginal plug detected from female mice was immediately recorded for mating period assessment, later separated and observed until parturition. After 14 days of mating period, the mating success rate was calculated as per described by Martínez, et al. (2011):

$$\text{Mating success rate (\%)} = \frac{\text{Female mice with vaginal plug presence}}{\text{Total cohabitated female mice in a cage}} \times 100\%$$

(a) (b)

Figure 3.10: (a) Behaviour of natural mating between treated male and untreated female mice was caught right after cohabitation. (b) Newborn litters from the dam after 21 days gestation period from untreated female mice (postnatal day 1).

The number of pups was initially recorded on postnatal day 1 to determine the litter size at birth. After 14 days, the litter size and survival rate of the pups were re-evaluated and recorded. The pup survival rate was calculated using the following formula:

$$\text{Pups' survival rate (\%)} = \frac{\text{Number of Pups on Day 14}}{\text{Number of Pups on Day 1}} \times 100\%$$

3.4 Statistical Analysis

All triplicated collected data were expressed in mean ± standard error (SE). SAS® OnDemand for Academics was used to run the collected statistical data. All data was assessed for its normal distribution using Shapiro-Wilk test (Loh, et al., 2022). The normalized data was then conducted in one-way analysis of variance (ANOVA), followed by Fisher's post-hoc test (least significant difference (LSD)) while the non-normalized data was conducted in Kruskal–Wallis *H* (one-way ANOVA on ranks) test followed by Dwass, Steel, Critchlow-Fligner (DSCF) method (Steel, Torrie and Dickey, 1997; Hollander, Wolfe and Chicken, 2014). Statistical significance was evaluated using the threshold of p-value less than 0.05 ($p < 0.05$), indicating statistically significant differences in the study. Finally, Pearson's correlation was conducted to evaluate the relationships between sperm parameters, Johnson's mean testicular biopsy score count, serum testosterone, lipid peroxidation and mating period .

CHAPTER 4

RESULTS

4.1 Extraction yield, Total Phenolic Contents, Total Flavonoid Content and DPPH Scavenging Radical Activity Assessment of Extracts of *K. parviflora*

Table 4.1 recorded the yield of KPE of various solvent. Aqueous extraction of *K. parviflora* resulted in the highest yield (13.63 ± 1.49%), followed by the ethanolic extraction (5.58 ± 1.07%) and the hexane extraction (1.12 ± 0.13%), respectively ($p < 0.05$). However, a significantly higher phenolics content ($p < 0.05$) was observed in ethanolic extract of *K. parviflora* (60.28 ± 1.69 mg GAE/g dw), followed by hexane extract (24.86 ± 0.73 mg GAE/g dw) and aqueous extract (8.19 ± 1.23 mg GAE/g dw) respectively. Conversely, the hexane extract had the most significant ($p < 0.05$) flavonoid content (151.11 ± 5.88 mg QE/g dw), followed by the ethanolic extract with approximately half the flavonoid content (77.78 ± 5.88 mg QE/g dw) compared to hexane extract. The aqueous extract of the *K. parviflora* in this study contain the least flavonoid content (24.44 ± 8.01 mg QE/g dw). In terms of the assessment of antioxidant activity, the ethanolic extract of *K. parviflora* has the highest free radical scavenging activity which is implied by the significant lowest ($p < 0.05$) EC_{50} value (0.865 ± 0.004 mg/mL) for the DPPH assay, compared to hexane extract (4.559 ± 13.90 mg/mL) and aqueous extract (8.671 ± 0.050 mg/mL), approximately ten times greater than ethanolic extract of *K. parviflora*. Since the ethanolic extract of *K. parviflora* (KPE) showed the

highest antioxidative activity, thus, it was chosen for the subsequent phytochemical analysis and *in vivo* animal studies.

Table 4.1: Extraction yield, DPPH EC_{50} scavenging activities, total phenolic and flavonoid content of *K. parviflora* extracts.

Types of Solvent Extraction	Extracted yield (%)	TPC [(1)] (mg GAE / g dw)	TFC [(2)] (mg QE / g dw)	DPPH EC_{50} [(3)] (mg/mL)
Aqueous	13.63 ± 1.49^{c}	8.19 ± 1.23^{a}	24.44 ± 8.01^{a}	8.671 ± 0.050^{a}
Ethanol	5.58 ± 1.07^{b}	60.28 ± 1.69^{c}	77.78 ± 5.88^{b}	0.865 ± 0.004^{c}
Hexane	1.12 ± 0.13^{a}	24.86 ± 0.73^{b}	151.11 ± 5.88^{c}	4.559 ± 13.90^{b}

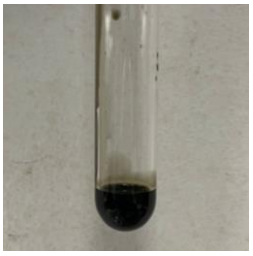
Data expressed as mean ± standard error (n = 3). [(1)]TPC represents total phenolic content in milligram gallic acid equivalents per gram of dry extract weight. [(2)]TFC represents total flavonoid content in milligram quercetin equivalents per gram of dry extract weight. [(3)]DPPH EC_{50} represents EC_{50} of DPPH scavenging activity values in microgram dry extract per millilitre. [abc]Means within the same column with different superscripts were significantly different ($p < 0.05$).

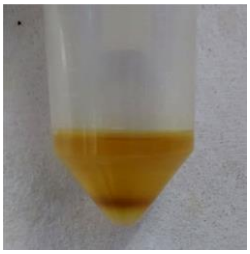
4.2 Determination of Bioactive Compounds in KPE

4.2.1 Physicochemical Properties of KPE

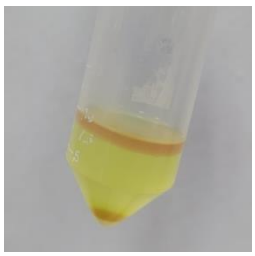
Table 4.2 shows the qualitative phytochemical screening results on KPE. The results indicates that the KPE contained phytochemical constituents such as tannins, saponins, alkaloids, cardiac glycosides, terpenes, volatile oil, phenolics and flavonoids, except anthraquinones, which was not detected in KPE.

Table 4.2: Observation of the qualitative phytochemical test for constituents' presence inside the KPE.

Constituents	Observation	Result
Tannin	 Yellow precipitate present.	+
Saponin	 Bubble foam was formed.	+
Alkaloid	 Reddish-brown precipitate present.	+
Anthraquinone	 Black coloured solution with no pink-red layer detected at ammonia lower layer.	-

Cardiac glycoside  +

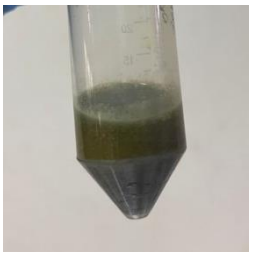
Brown ring interface was formed above the solution.

Terpene  +

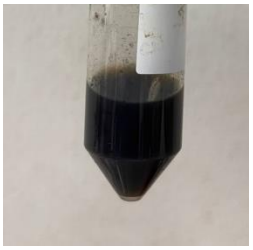
Reddish-brown layer formed on top of the yellow solution.

Volatile Oil  +

Yellow precipitate was formed after mixing the solution.

Phenolic  +

Solution turned from yellow to blue green.

Flavonoid		+
	Solution turned from colourless to dark magenta-red.	

+ denotes presence; - denotes absence.

4.2.2 GC-MS Assessment of KPE

The analysis via GC-MS detected a total of eight peaks in the ethanolic extract of *K. parviflora* (Table 4.3). Among the eight detected peaks, five compounds namely 5-hydroxy-3,7-dimethoxyflavone, 5-hydroxy-7,4'-dimethoxyflavone, 3,5,7,4'-tetramethoxyflavone, 5-hydroxy-3,7,3'4'-tetramethoxyflavone, 5,7,4'-trimethoxyflavone had been previously reported in other studies. While three compounds, 7-Hydroxy-3-methoxy-2-*p*-methoxyphenyl-4H-chromen-4-one, 2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one, 2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol) were not documented in any published research of KPE. Among the 8 compounds, 5-Hydroxy-3,7-dimethoxyflavone was the most abundant compound (29.94%), followed by 2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol) (22.39%) and 5,7,4'-Trimethoxyflavone (19.53%). In contrast, 5-Hydroxy-5,4'-dimethoxyflavone exhibited the lowest abundance, accounting for only 3.00% as shown in Table 4.3.

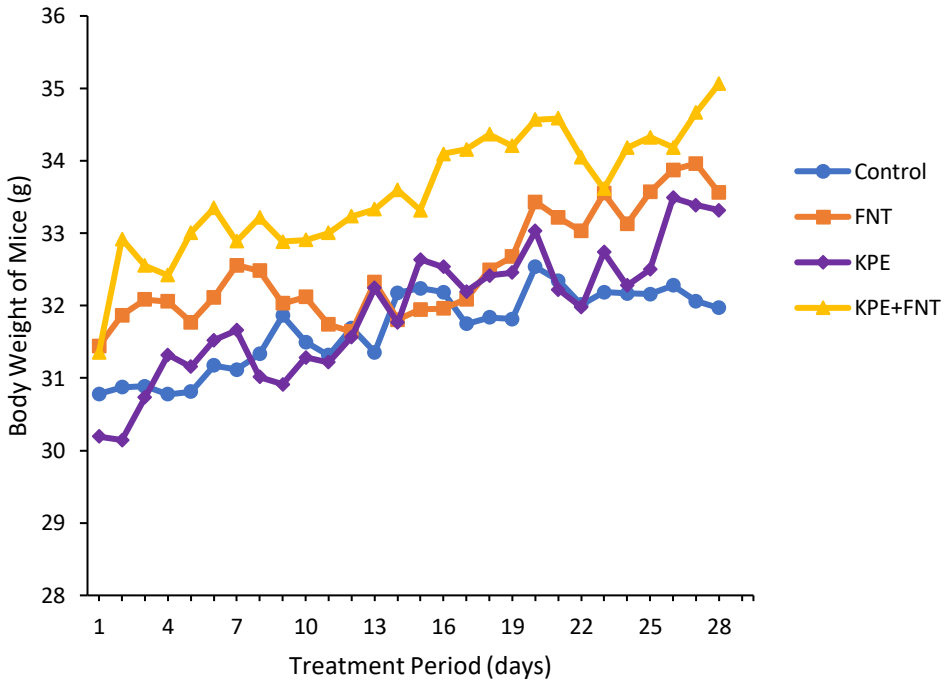
Table 4.3: **List of bioactive compounds present in KPE as detected by GC-MS.**

Retention time (min)	Compound detected	Peak Abundancy (%)
13.910	7-Hydroxy-3-methoxy-2-*p*-methoxyphenyl-4H-chromen-4-one*	5.48
14.945	5-hydroxy-3,7-dimethoxyflavone	29.94
16.257	2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one*	4.18
16.472	5-hydroxy-7,4'-dimethoxyflavone	3.00
18.242	3,5,7,4'-tetramethoxyflavone	12.30
18.298	5-hydroxy-3,7,3'4'-tetramethoxyflavone	3.18
18.430	5,7,4'-trimethoxyflavone	19.53
21.130	2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol)*	22.39

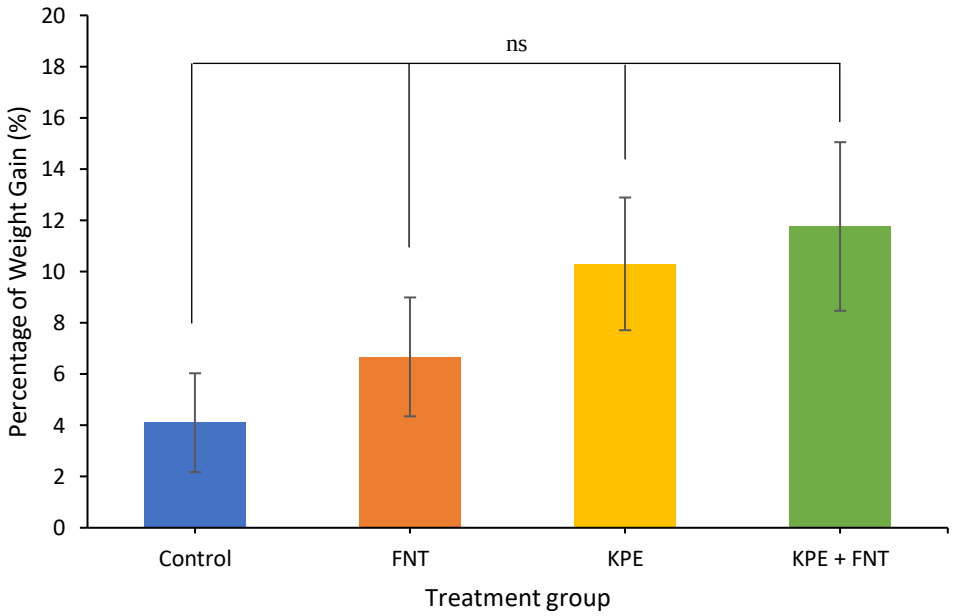
***Asterisk marked on the name of detected compound represents new compound which not found in other studies of KPE.**

4.3 Assessment of Mice Weight and Mortality Throughout 28 Days of Feeding Treatment

Weight changes in a direct indicator for the health status of the treated mice. Hence, bw was recorded daily for the observation of any signs of obesity. Figure 4.1(a) indicates a gradual increasing trend in weight gain across all treatment groups. The control group showed the smallest percentage of weight gain (4.10 ± 3.93 %), followed by FNT treatment group (6.67 ± 5.32 %), KPE treatment group (10.30 ± 2.59 %), and the co-administration of KPE and FNT group (11.76 ± 4.29%). However, the percentage of weight gain were not significantly different among the treatment groups ($p \geq 0.05$) as shown in Figure 4.1(b). No mortality or deaths were observed in any of the treatment groups throughout the 28-day study period.



(a)



(b)

Figure 4.1: Assessment of mice weights throughout 28 days of feeding treatment. (a) Trend of weight changes over 28 days (n = 6). (b) Percentage weight gain in each treatment group (n = 6). ^{ns}Means comparison between treatment group were not significantly different ($p \geq 0.05$).

4.4 Assessment of Relative Testis Weight After FNT or/and KPE Administration

The study of the testis is an important indicator of the male reproductive system. Hence, the relative weight of the testis was determined. FNT-administrated mice resulted in a 16% increase in relative testis weight (0.36 ± 0.030 %) compared to the control group (0.31 ± 0.014 %) (Figure 4.2). Conversely, KPE-administered group showed an approximately 6.5% decrease in relative testis weight (0.29 ± 0.002 %) as compared to control group. Interestingly, the co-administration of KPE and FNT have a slightly reduction in the relative weight of testis by approximately 11% as compared to the FNT

group. However, no significant differences were observed across all groups ($p \geq 0.05$).

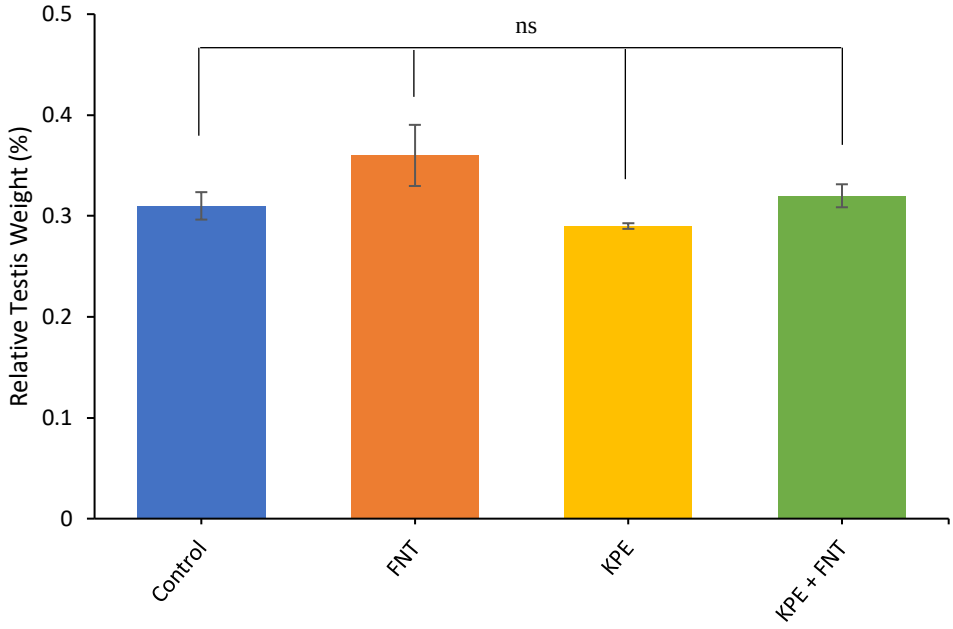


Figure 4.2: Relative testis weight of mice after 28 days treatment (n = 3). ^{ns}Means comparison between treatment group were not significantly different ($p \geq 0.05$).

4.5 Evaluation of Lipid Peroxidation Level in Male Mice After FNT or/and KPE Administration

The study of lipid peroxidation level is an important marker for oxidative stress determination. Therefore, serum malondialdehyde (MDA), a key indicator of lipid peroxidation was measured. In Figure 4.3, an approximately two-fold significant increase in serum MDA levels ($p < 0.05$) was observed in the FNT treatment group (41.30 ± 2.23 µmol/L) when compared to the control group (18.36 ± 2.28 µmol/L). Whereas the serum MDA levels in the KPE treatment group (23.96 ± 1.32 µmol/L) were about 30% lower than the control treatment group. Interestingly, an approximately 15% serum MDA levels reduction was observed in the co-administration of KPE and FNT (35.94 ± 1.45 µmol/L) as compared to FNT treatment alone, even though the serum MDA levels still significantly higher ($p < 0.05$) than control and KPE treatment groups.

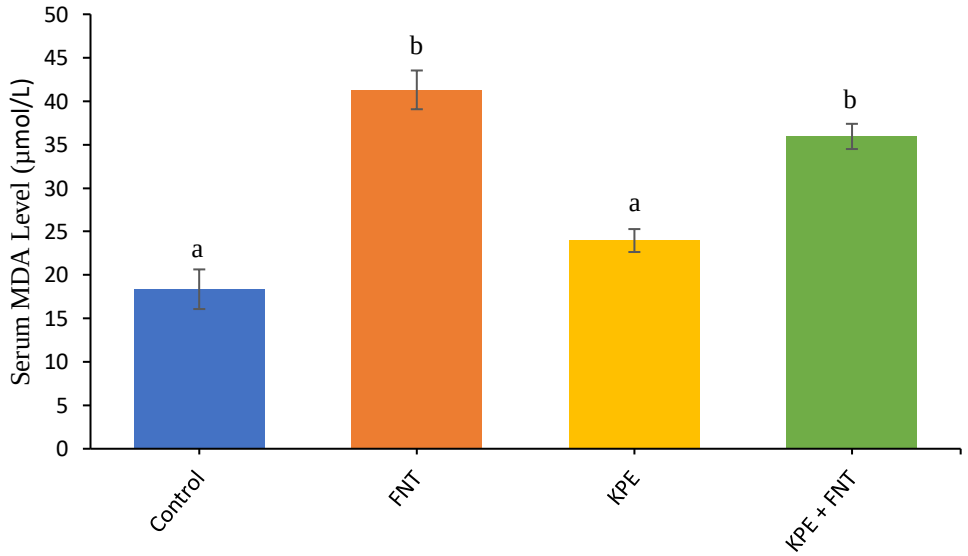


Figure 4.3: *In vivo* lipid peroxidation level in blood serum after 28 treatment (n = 3). [ab]Means with different superscript displayed on top of each bar were significantly different ($p < 0.05$) while means with same superscript displayed on top of each bar were not significantly different ($p \geq 0.05$).

4.6 Sperm Characterization and Testosterone Level Assessment of Male Mice After Treatment

Sperm concentration, motility, viability, and testosterone levels are key determinants in male fertility studies. Thus, these parameters were assessed. The assessment results of sperm and testosterone level of the treated male mice are shown in Table 4.4. Comparison on the sperm concentration between FNT treatment group (30.67 ± 0.15 $\times 10^6$ cells/mL) and control (40.16 ± 0.59 $\times 10^6$ cells/mL) showed a significant decrease ($p < 0.05$) of 23%. Meanwhile, both KPE treatment group (33.91 ± 0.67 $\times 10^6$ cells/mL) showed significant decrease ($p < 0.05$) of 15% of sperm count respectively when compared to control group. Furthermore, the co-administration of KPE and FNT mice group showed significantly higher ($p < 0.05$) sperm count (32.89 ± 0.74 $\times 10^6$ cells/mL) than the male mice treated with FNT only with a 7.2% difference indicating that the KPE might exert protective effect against FNT.

In this study, sperm progressive motility and sperm viability were recorded. FNT treatment group has significantly lower ($p < 0.05$) sperm progressive motility (33.00 ± 6.08 %) and sperm viability (60.38 ± 3.26 %) compared to control group (progressive motility: 79.00 ± 1.15 %; viability: 86.08 ± 0.81 %) respectively. In the meantime, there is no significant difference in sperm progressive motility (71.33 ± 2.19 %) and sperm viability (76.48 ± 1.57 %) observed in KPE treatment group when compared to control group. In addition, KPE with FNT treatment group has shown a significant improvement

($p < 0.05$) in sperm progressive motility by 95% (64.33 ± 2.19 %) and sperm viability by 21% (73.44 ± 2.12 %) compared to FNT treatment group (Table 4.4).

As for testosterone level, FNT-treated mice have a significantly decreased serum testosterone level ($p < 0.05$) by approximately 80% (4.50 ± 1.04 ng/mL) when compared to the control group (22.69 ± 1.58 ng/mL). Furthermore, KPE treatment group (44.05 ± 2.96 ng/mL) showed a nearly two-fold significant increase ($p < 0.05$) in serum testosterone levels compared to control. Interestingly, the co-administration of KPE and FNT resulted in about six-fold significantly elevated serum testosterone ($p < 0.05$) when compared with FNT alone (28.32 ± 2.20 ng/mL).

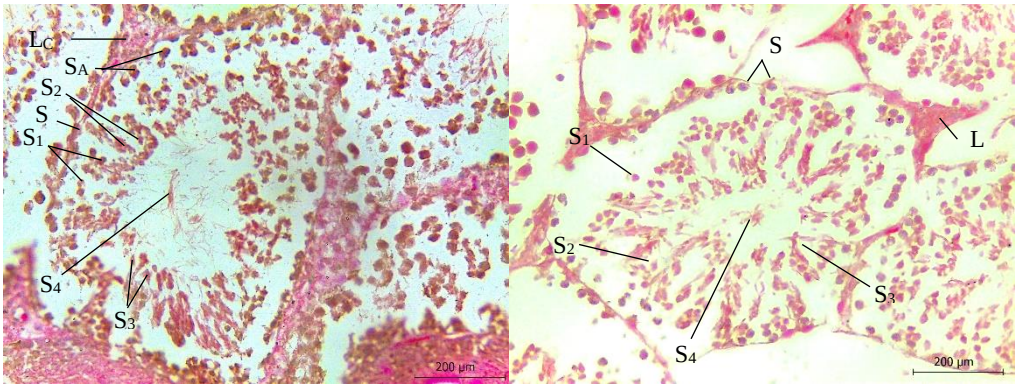
Table 4.4: Comparison of sperm concentration, progressive motility, viability and serum testosterone level of all treatment mice group.

Parameters	Control	FNT	KPE	KPE + FNT
Sperm concentration ($\times 10^6$ cells/mL^{-1})	40.16 ± 0.56^{c}	30.67 ± 0.15^{a}	33.91 ± 0.67^{b}	32.89 ± 0.74^{b}
Progressive motility (%)[(1)]	79.00 ± 1.15^{c}	33.00 ± 6.08^{a}	71.33 ± 2.19^{bc}	64.33 ± 2.19^{b}
Viability (%)	86.08 ± 0.81^{c}	60.38 ± 3.26^{a}	76.48 ± 1.57^{bc}	73.44 ± 2.12^{b}
Serum testosterone level (ng/mL)	22.69 ± 1.58^{b}	4.50 ± 1.04^{a}	44.05 ± 2.96^{c}	28.32 ± 2.20^{b}

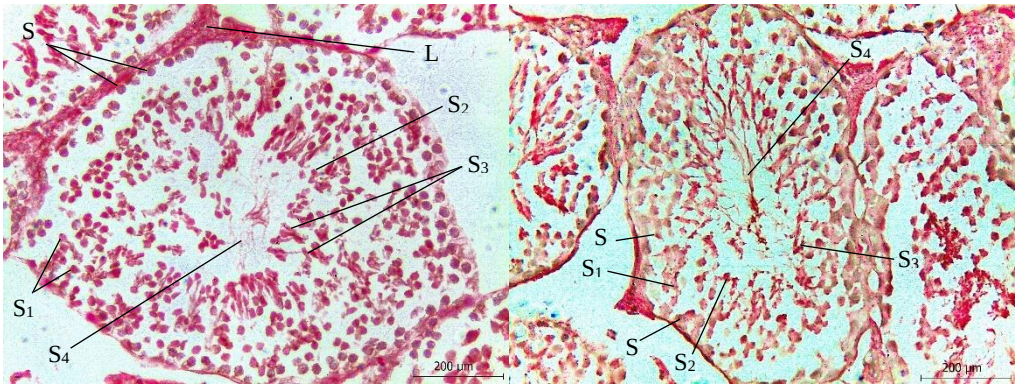
Data expressed as mean ± standard error (n = 3). [(1)]Percentage of sperm progressive motility which includes the sum of both grade A and grade B sperm motility. [abc]Means with different superscripts in a row within a group were significant different ($p < 0.05$).

4.7 Histopathology, Morphology of Testis and Johnson's Mean Testicular Biopsy Score Count After Treatment

Histological analysis of the testicular tissue allows the assessment of any present tissue damage. Hence, histology of testicular tissue was conducted in all treatment groups. From Figure 4.4, FNT treatment group had more disorganized spermatogonia, primary spermatocytes and spermatids with lower visibility of spermatozoa in the lumen of the testicular tissue than as of control group. In the meantime, KPE treatment group has maintained a testicular structure similar to the control group, with well-preserved seminiferous tubules and active spermatogenesis. However, more condensed testicular cells within the seminiferous tubules were observed in KPE treatment group when compared to the control group. Interestingly, KPE + FNT treatment group showed slightly improved testicular structure with relatively higher presence of spermatogonia, primary spermatocytes, spermatids and spermatozoa within the seminiferous tubules when compared to FNT treatment group.



(a) (b)



(c) (d)

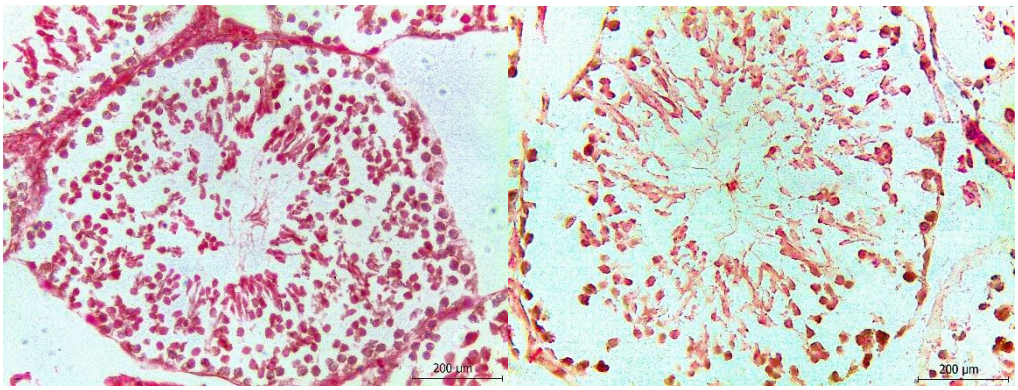
Figure 4.4: Histopathological images on seminiferous tubules of testis and spermatogenesis evaluation after treatments under light microscope at 400x magnification. (a) Control (b) FNT (c) KPE (d) KPE + FNT; L_C: Leydig cells, S_C: Sertoli cells, S_A: spermatogonia, S_1: primary spermatocytes, S_2: round spermatids, S_3: elongating spermatids, S_4: spermatozoa.

Notably, it was reflected in significantly lower Johnson's mean testicular biopsy score count ($p < 0.05$) for the FNT treatment group (7.15 ± 0.08) compared to the control (9.23 ± 0.04) group. Meanwhile, no significant was observed in KPE treatment (9.30 ± 0.08) group when compared to control group. Whereas the KPE + FNT treatment group showed significantly higher ($p < 0.05$) Johnson's mean testicular biopsy score count when compared to FNT treatment group as shown in Table 4.5. Individual Johnson's mean score assessments were corroborated with the corresponding histological images, as shown in Figure 4.5.

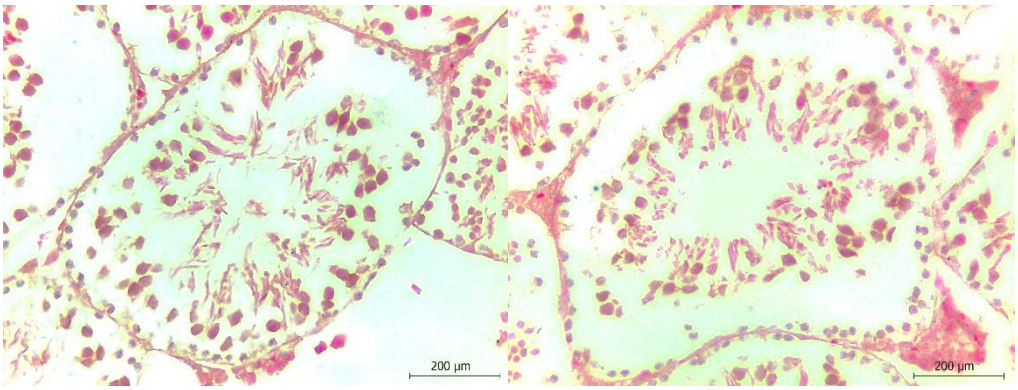
Table 4.5: Johnson's mean testicular biopsy score count of all treatment group.

Treatment group	Control	FNT	KPE	KPE + FNT
Johnson's mean score[(1)]	9.23 ± 0.04[c]	7.15 ± 0.08[a]	9.30 ± 0.08[c]	8.77 ± 0.09[b]

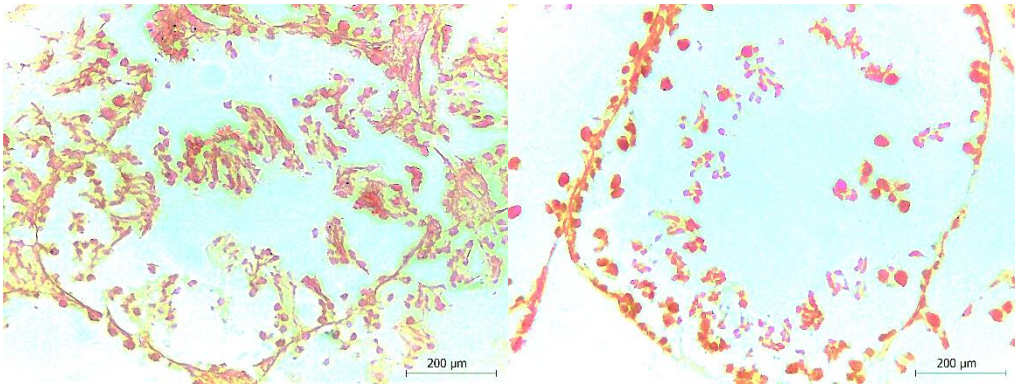
Data expressed as mean ± standard error (n = 3). [(1)]Johnson's mean testicular score count (score count out of 10). [abc]Means with different superscripts within a row were significant different ($p < 0.05$).



(a) (b)



(c) (d)



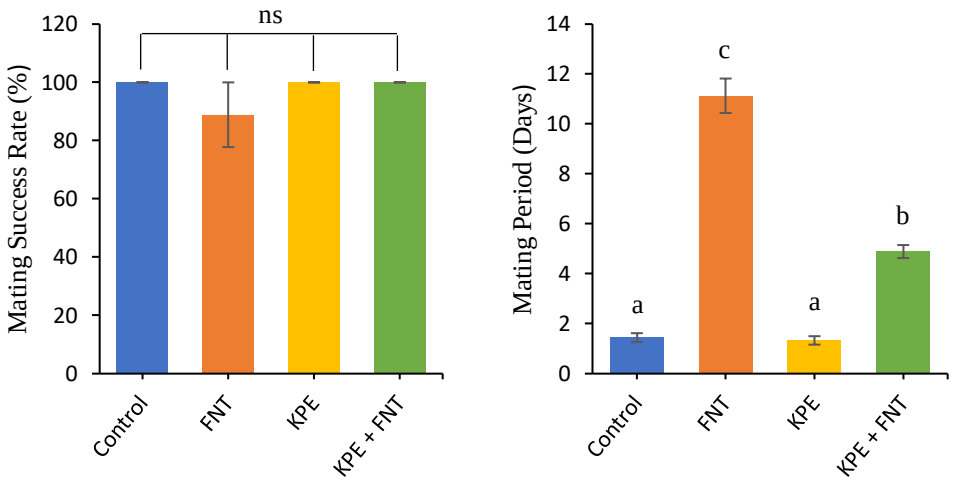
(e) (f)

Figure 4.5: Histopathological images on seminiferous tubules of testis of all treatment groups that matched according to the description of Johnson's mean testicular biopsy score count. (a) Score 10 (b) Score 9 (c) Score 8 (d) Score 7 (e) Score 6 (f) Score 5.

4.8 Mating and Post-Parturition Assessments After Treatment of KPE and/or FNT

Mating parameters are important for assessing fertility, while post-parturition assessments are crucial for evaluating offspring development. Therefore, mating and post-parturition assessments were done in all treatment groups. The mating success rate of FNT treatment group has decreased from 100% to 88.89 ± 11.11% when compared with the rest of the treatment groups. However, it was shown in Figure 4.6 (a) that the differences in mating success rate among the treatment groups were not statistically significant ($p \geq 0.05$). Notably in Figure 4.6 (b), FNT treatment group has shown delayed mating period of about 10% [(11.13 ± 0.69) days, $p < 0.05$] when compared to control group [(1.44 ± 0.18) days]. There is no significant difference ($p \geq 0.05$) observed in KPE treatment group [(1.33 ± 0.17) days] when compared to control group. Interestingly, a significantly lower mating period ($p < 0.05$) was observed in

KPE + FNT treatment group [(11.13 ± 0.69) days] when compared to FNT treatment group. All female mice with confirmed vaginal plugs successfully conceived and completed gestation within 20 to 21 days. No complications were observed during the gestation or parturition period.



(a) (b)

Figure 4.6: Mating assessments of all treatment groups were conducted over a 14-day observation period. (a) Mating success rate (n = 3). (b) Mating period (n = 3). ^{ns}Means comparison between treatment group were not significantly different ($p \geq 0.05$). ^{abc}Means with different superscript displayed on top of each bar were significantly different ($p < 0.05$) while means with same superscript displayed on top of each bar were not significantly different ($p \geq 0.05$).

Throughout the pregnancy period, the gestation length of all 36 female mice across the treatment groups was consistently recorded as 20 to 21 days, with no signs of early or delayed parturition observed. Notably in Table 4.6, the administration of FNT or KPE groups have exhibited relatively lower in the number of pups per dam 11.0 ± 0.38 and 11.1 ± 0.26 respectively as compared to control group (12.2 ± 0.36). Whereas test treatment group (KPE + FNT) has

an approximately 8% increase in the number of pups per dam (11.9 ± 0.26) relative to FNT treatment alone. In the meantime, 6% reduced chance of survival in the offspring was observed in FNT treatment group (89.90 ± 1.97%) when compared to control group (94.50 ± 1.84%). Surprisingly, KPE treatment group was recorded higher offspring survival of 95.3 ± 1.48%, a 0.8% higher as compared to control group. Furthermore, the co-administration of KPE and FNT group has displayed relative higher survival rate of pups by approximately 4.5% (94.40 ± 1.40%) when compared to FNT treatment group alone. Nevertheless, no significant difference was observed in both litter size per dam and pups' survival rates assessment among the treatment groups ($p \geqslant 0.05$).

Table 4.6: **Comparison of litter size per dam and pups' survival rate of all treatment mice groups.**

Parameters	Control	FNT	KPE	KPE + FNT
Litter size per dam, n	12.2 ± 0.36[a]	11.0 ± 0.38[a]	11.1 ± 0.26[a]	11.9 ± 0.26[a]
Pups' survival rate (%)	94.50 ± 1.84[a]	89.90 ± 1.97[a]	95.30 ± 1.48[a]	94.40 ± 1.40[a]

Data expressed as mean ± standard error (n = 9). [a]Means with same superscripts in a row within a group were not significantly different ($p \geq 0.05$).

4.9 Pearson Correlation Analysis Between Serum Testosterone Level, Serum MDA level, Sperm Concentration, Progressive Motility, Sperm Viability, Johnson's Mean Testicular Biopsy Score Count and Mating Period

Pearson correlation analysis was conducted to determine the significance of each variable. Figure 4.7 illustrates that all examined relationships between variables exhibited statistically significant negative correlations ($R^2 < 0$, $p < 0.05$). A moderate negative correlation was found between serum MDA and testosterone levels (R^2 = -0.5025). Stronger negative correlations were observed for serum MDA with sperm concentration (R^2 = -0.6558), progressive motility (R^2 = -0.6990) and viability (R^2 = -0.6873). Additionally, strong negative correlations existed between Johnson's testicular score and serum MDA (R^2 = -0.7520) and between serum testosterone and mating period (R^2 = -0.7585).

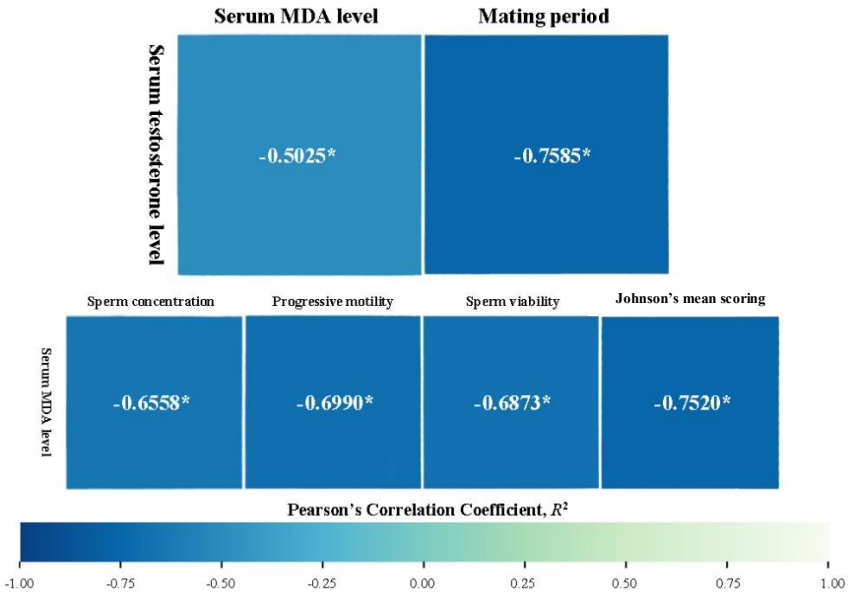


Figure 4.7: Inter-sample of *in vivo* study correlation heat map. R^2 or R squared represents the coefficient of determination. *Significant correlation between two variables at $p < 0.05$.

CHAPTER 5

DISCUSSION

5.1 Extraction Yield, Antioxidant Activity, Total Phenolic and Flavonoid Contents of *K. parviflora* Extracts

Table 4.1 shows that the aqueous extract of *K. parviflora* has the highest extraction yield, followed by ethanolic extract and hexane extract respectively ($p < 0.05$). The methoxyflavone derivatives, which are abundantly present in the rhizomes of *Kaempferia parviflora*, are characterized by their branched methoxy groups, which contribute to their lower polarity (Chen, et al., 2018; Truong, et al., 2019). Due to their lipophilic nature, these compounds exhibit greater solubility in solvents with moderate to low polarity, such as ethanol or hexane, rather than highly polar solvents like water (Patanasethanont, et al., 2007a). This explains why ethanolic and hexane extractions tend to yield higher concentrations of methoxylated flavones, which are associated with the bioactive properties of *K. parviflora* (Chen, et al., 2018; Sitthichai, et al., 2022). Consistent with findings from other studies on *K. parviflora* extraction, lower extraction yields are typically observed when solvents with lower polarity are utilized (Rahman, et al., 2018; Krongrawa, et al., 2022; Sitthichai, et al., 2022). The present study confirmed these trends above, highlighting the significance of solvent selection based on the polarity of targeted compounds in phytochemical extraction (Chen, et al., 2018; Truong, et al., 2019). Even though aqueous yielded highest amount of extract in the present findings, the selection

of suitable extraction solvent remains unclear. These extracted phytochemicals require the assessment of antioxidant (DPPH scavenging activity), antimicrobial or other relevant therapeutic activities to evaluate on the overall their overall efficacy and potential applications (Baliyan, et al., 2022). The ideal solvent will be the one that maximizes the extraction of bioactive compounds, even if it does not produce the highest overall extract yield (Lefebvre, Destandau and Lesellier, 2021). This approach will ensure that the extracts obtained are not only abundant but also rich in the compounds responsible for *K. parviflora*'s therapeutic benefits. Thus, investigating the effects of different extraction methods and solvent systems on the chemical composition and bioactivity of *K. parviflora* extracts could provide valuable insights.

Meanwhile, the present study has shown that ethanolic extraction method of *K. parviflora* has the lowest EC_{50} value in the DPPH scavenging activity assay, followed by hexane and aqueous extracts, respectively. This indicates that ethanolic extract of *K. parviflora* contributes to the highest antioxidative activity. However, the EC_{50} DPPH value of ethanolic extract obtained in the present study were relatively higher than other studies. This could be due to the short maceration period of protocol which was used in this present study (Sudwan, et al., 2006; Choi, Kim and Yook, 2018; Rahman, et al., 2018). Chaisuwan, Dajanta, and Srikaeo (2022) highlighted that extending the maceration period could potentially enhance the extraction efficiency. Interestingly, Patanasethanont, et al. (2007b) discovered no significant variation

in antioxidant properties when the rhizome of *K. parviflora* were extracted with different ethanol concentrations ranging from 20% to 80% volume per volume.

It was also observed that ethanolic extract of *K. parviflora* contributes significantly higher phenolic content ($p < 0.05$), followed by hexane and aqueous extracts, respectively. The present findings above suggested that the ethanolic extract possesses the highest extraction yield of phenolic compounds from the rhizome of *K. parviflora*. This can be attributed to ethanol's effectiveness in extracting a broad spectrum of phytochemicals, including phenolic derivatives, due to its intermediate polarity (Singh, et al., 2017; Kozhantayeva, et al., 2024). In such, ethanol possesses unique ability to dissolve both polar and semi-polar compounds, enabling the extraction of diverse bioactive compounds like flavonoids, tannins, and phenolics more efficiently compared to solvents with narrower polarity ranges (Dai and Mumper, 2010; Hassan, et al., 2017; Lefebvre, Destandau and Lesellier, 2021).

Interestingly, the current study reported that hexane extract of *K. parviflora* yielded the highest total flavonoid content (TFC), almost twice flavonoid content observed in the ethanolic extract of *K. parviflora* while at least six times of total flavonoid content yielded in the aqueous extract of *K. parviflora*. This discrepancy may be due to the tendency of non-polar or moderately polar solvents to extract higher concentrations of methoxylated flavone derivatives during the extraction process (Chen, et al., 2018; Rahman,

et al., 2018). However, the overall TFC results obtained in the present study do not directly correlate with the TPC results. This is because TFC specifically quantifies flavone compounds, whereas TPC evaluates the overall phenolic compounds, which include flavones, phenolic acids, tannins, and other polyphenols (Siddiqui, et al., 2017; Shraim, et al., 2021). The broader scope of TPC explains why an extract may have a high TPC value despite a moderate TFC value, as observed in the ethanolic extract of *K. parviflora*. The present findings above align with previous research on the phytochemical composition of *K. parviflora* rhizomes, which consistently report that semi-polar solvents, such as methanol and ethanol, effectively extract higher concentrations of phenolic compounds while non-polar solvents like *n*-hexane are more efficient in extracting non-polar compounds, such as methoxylated flavonoids, leading to higher flavonoid content in these extracts (Julianti, Bakar and Wikantyasing, 2022; Sitthichai, et al., 2022).

In relation to DPPH radical scavenging activity, antioxidant properties were attributed to both phenolic and flavone compounds extracted from the rhizome of *K. parviflora* (Chaipech, et al., 2012; Chen, et al., 2018). However, despite the high flavonoid content observed in the hexane extract, it did not exhibit the lowest EC_{50} value in the DPPH assay. This suggests that the flavones extracted via hexane do not significantly contribute to the overall antioxidant activity. These findings indicate that other phenolic compounds, such as tannins and phenolic acids, may play a more substantial role in the antioxidative potential of *K. parviflora* (Chen, et al., 2018; Rahman, et al., 2018). Summary

to previous studies which demonstrates that phenolic acids and tannins exhibit strong free radical scavenging abilities (Chaisuwan, Dajanta, and Srikaeo, 2022). Furthermore, the lower EC_{50} value obtained for the ethanolic extract confirms its higher antioxidant efficacy, positioning it as the most potent extract among the three solvents tested.

Oxidative stress is a major mechanism by which FNT impairs spermatogenesis, damages sperm DNA, and disrupts overall testicular integrity and function, primarily through excessive ROS generation (Yusoff, et al., 2020; 2021). The flavonoid and phenolic compounds identified in *K. parviflora* extracts are known for their potent ROS-scavenging properties, as reflected in the DPPH radical scavenging activity observed in this study (Chen, et al., 2018; Mishra and Sharma, 2021).

Although the aqueous extract of *K. parviflora* yielded the highest extraction percentage, it exhibited relatively low levels of total phenolic and flavonoid contents, and correspondingly weaker antioxidant activity (Chow, Kim and Yook, 2018). This suggests that the aqueous solvent primarily extracted highly polar compounds, likely belonging to other phytochemical classes with limited antioxidant potential, making it less suitable for *in vivo* applications.

In contrast, while the hexane extract showed a lower overall yield than the ethanolic extract, it contained a high concentration of flavonoids, specifically methoxyflavones which reported as main constituent in the rhizome of *K. parviflora* (Okabe, et al., 2014; Chen, et al, 2018). However, its antioxidant activity was still inferior to that of the ethanolic extract, likely due to a lower phenolic content, which also plays a significant role in free radical scavenging (Chaipech, et al., 2012). Further research can be done to isolate and characterize individual methoxyflavone compounds from the hexane extract, and evaluate their specific bioactivities.

Given the balanced presence of both phenolic and flavonoid compounds, along with better antioxidant activity reflected in DPPH scavenging activity, the ethanolic extract of *K. parviflora* was selected for subsequent phytochemical profiling, GC-MS analysis and *in vivo* administration studies. From this point forward, the ethanolic extract will be referred to as *K. parvifl*ora extract (KPE).

5.2 Physicochemical Properties and GC-MS Assessment of KPE

Physicochemical analysis in the present study showed the presence of saponins, tannins, alkaloids, terpenes, cardiac glycosides, volatile oil, phenolics and flavonoids in KPE, apart from anthraquinones (Table 4.2). These findings are similar with the qualitative phytochemical study by Mishra and Sharma (2021). However, Julianti, Bakar and Wikantyasing (2022) reported that

saponins, terpenes, and glycosides were absent in KPE in their phytochemical study. This discrepancy may be due to the differences in extraction protocols, particularly the low concentration of powdered *K. parviflora* used during maceration in their study. A lower plant-to-solvent ratio or insufficient extraction duration could result in non-detectable levels of certain phytochemical constituents, leading to variations in reported findings (De Silva, Abeysundara and Aponso, 2017). In addition, other factors such as repetitive maceration of plant residues (percolation) would significantly improve the solubility and recovery of phytochemical by allowing prolonged solvent contact with the plant material (Chaisuwan, Dajanta, and Srikaeo, 2022). Meanwhile, Mishra and Sharma (2021) discovered that ethanolic extract has exhibited the highest presence of phytochemical constituents as compared to extraction using chloroform, ethyl acetate and aqueous. The findings once again highlight on the effectiveness of ethanol in extracting a broad range of bioactive compounds with different polarity from the rhizome of *K. parviflora* (Dai and Mumper, 2010; Truong, et al., 2019). This could also explain the widespread use of ethanolic extracts in the study of *K. parviflora* for investigating the potential bioactivities from the rhizome of *K. parviflora* in various studies (Sudwan, et al., 2006, Chen, et al., 2018; Al-Rawaf, Gabr and Alghadir, 2021). Since these phytochemical constituents are not the major components present in the rhizome of *K. parviflora*, thus they have received relatively limited attention in the context of their bioactive properties, particularly regarding their potential to ameliorate environmental toxicant-induced male reproductive toxicity, such as that caused by FNT in the study. Therefore, future studies can focus into deeper

investigation of the specific bioactivities of these minor compounds and their potential therapeutic roles.

Additionally, a total of eight distinct peaks were reported in KPE via GC-MS analysis (Appendix 6). Among these, there were five methoxyflavone compounds that have been previously reported as the main phytochemical compositions found in *K. parviflora* rhizomes (Toda, et al., 2016; Chen, et al., 2018). Notably, TMF, reported in present study, was confirmed to be amongst the top constituents that can be found in the ethanolic extract, consistent with earlier studies (Sutthanut, et al., 2007a; Pitakpawasutthi, Palanuvej and Ruangrungsi, 2018). However, DMF, commonly reported as a major constituent in *K. parviflora*, was not detected in the present study (Sutthanut, et al., 2007b). This discrepancy may be attributed to differences in extraction methods, solvent compositions, or variations in analytical techniques used to identify and quantify the compounds (Pitakpawasutthi, Palanuvej and Ruangrungsi, 2018; Wongpia, et al., 2021; Chaisuwan, Dajanta and Srikaeo, 2022). Solvent concentration, extraction time and solvent-to-solid ratio are crucial factors that influence the yield and composition of methoxyflavones, including DMF (Elshamy, et al., 2019; Krongrawa, et al., 2022).

Furthermore, another factor that may contribute to the discrepancy observed is the growing conditions of the plants. As a member of the *Kaempferia* species, the phytochemical composition of *K. parviflora* can vary

depending on the maturity of the rhizomes (Rahman, et al., 2018; Phurailatpam, et al., 2024). Several environmental factors, including presence of sunlight, temperature, soil composition and humidity, influence the biosynthesis and accumulation of bioactive compounds in the plant (Toscano, et al., 2019; Begum, et al., 2022). Rahman, et al. (2018) reported that the highest phenolic and flavonoid content was found in 8-month-old rhizomes of *K. parviflora*, suggesting that maturity plays a crucial role in determining its phytochemical profile. As the rhizomes mature, specific metabolic pathways become more active, potentially leading to increased methoxylation of flavonoids. The biosynthesis pathway of 4-coumaric acid (Figure 5.1) demonstrates that TMF can be synthesized from flavanone and flavone precursors (Cui, et al., 2019). This suggests that DMF could undergo hydroxylation followed by methylation at the 4'-position, leading to the formation of TMF or 3,5,7,4'-tetramethoxyflavone as the plant ages. Such modifications are likely catalyzed by flavonoid hydroxylases and *O*-methyltransferases (OMTs), enzymes that are influenced by developmental and environmental factors (Lam, et al., 2007; Seitz, Ameres and Forkmann, 2007). These findings indicate that the age of the rhizomes may affect the conversion of DMF into TMF, leading to variations in the detected phytochemical profile of KPE. Further studies should focus on tracking flavonoid biosynthesis at different growth stages to better understand these transformations.

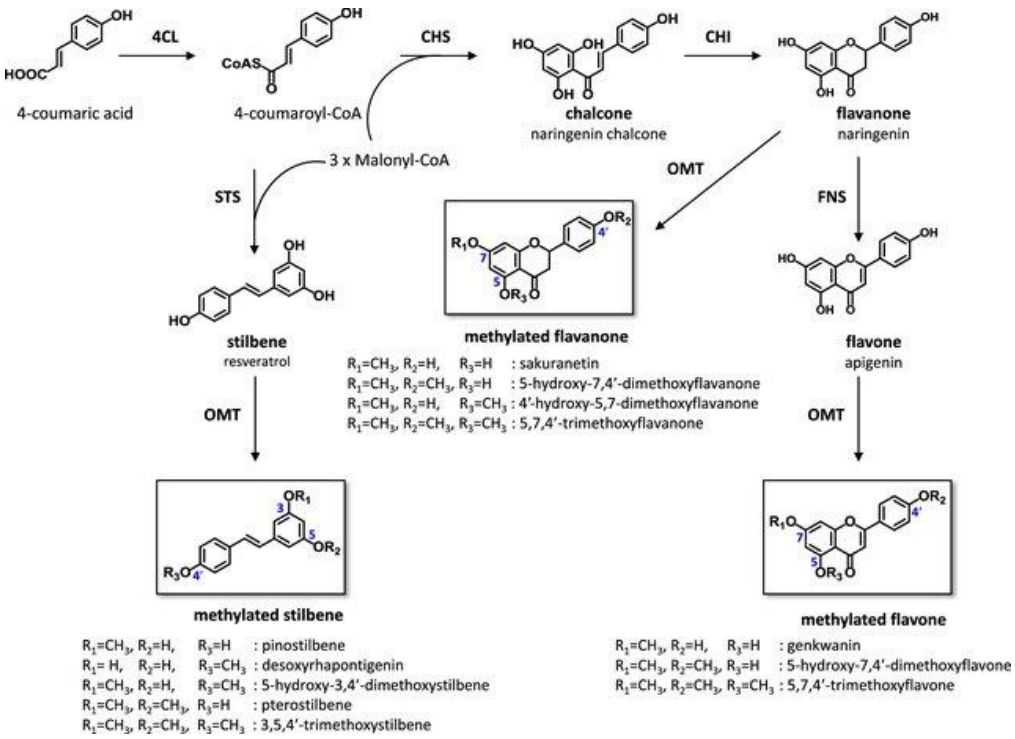


Figure 5.1: Biosynthesis pathway of 4-coumaric acid (Adopted from Cui, et al., 2019).

Interestingly, 5-hydroxy-3,7-dimethoxyflavone emerged as highest abundancy constituent in the present finding. This compound has garnered attention for its biological activity, particularly its ability to inhibit glycogen synthase kinase-3 beta (GSK-3β), enzyme which is highly involved in glycogen metabolism that enhance glycogen synthesis (Suanmali, 2016; Chen, et al., 2018). Such biological activity underlines the potential of 5-hydroxy-3,7-dimethoxyflavone as a therapeutic agent for metabolic disorders. Additionally, these compounds are known for their antioxidant, anti-inflammatory, and androgenic-modulating properties, which are highly relevant in protecting male reproductive toxicity, induced by environmental toxicants such as FNT (Park, et al., 2014; Potikanond, et al., 2017; Truong, et al., 2019). TMF and DMF have been reported to enhance testosterone levels by inhibiting phosphodiesterase

activity, thereby increasing cyclic AMP (cAMP) accumulation and promoting downstream protein signaling involved in steroidogenesis (Horigome, et al., 2016; Chen, et al., 2018). These compounds also exhibit free radical scavenging activities, which may be beneficial in counteracting FNT-induced oxidative stress in male mice (Vichitphan, Vichitphan, and Sirikhansaeng, 2007).

Among the novel findings, three compounds detected in this study had not been previously reported in KPE. The compound are 7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one (RT: 3.910 min), 2 (3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one (RT: 16.257 min), and 2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol) (RT: 21.130 min). The first novel compound, 7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one, was likely classified under methoxyisoflavone group due to its methoxyphenylchromen-4-one skeleton (Yoo and Koh, 2021). Such structural skeleton arrangement may possess potential anti-cancer properties like other reported methoxyisoflavone derivatives due to the methoxylation at its 4' position (Hyun, et al., 2012; Preedy and Society, 2013). Another identified novel compound in the present study, 2 (3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one, belongs to the class of methoxyflavone, also known as 3'-hydroxy-3,7,4'-trimethoxyflavone (Chen, et al., 2018). Up till date, this identified compound has not been associated nor reported with any specific bioactivity. However, based on structural characteristics, it is predicted to exhibit anti-allergic activity and AChE inhibitory effects due to the presence of methoxy groups at positions

4' and 7 (Tewtrakul, Subhadhirasakul and Kummee, 2008; Sawasdee, et al., 2009). In addition, this methoxyflavones may exert androgen-like effects or modulate the HPG axis, thereby improving testosterone regulation and spermatogenic function. The third novel compound, 2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol) also one of the major compounds reported in the present study. The compound belongs to the diarylmethane group and there is no reported studies or exploration conducted for its bioactivity. In general, these significant presence of the novel peaks highlights the need for future studies to isolate this compound from the rhizome of *K. parviflora* and investigate on its biological potential. Collectively, these findings underscore the complexity of *K. parviflora*'s phytochemical profile and the importance of continued research to fully understand its therapeutic applications.

5.3 Effect of FNT and KPE on Body Weight and Mortality

The observed changes in bw during the 28-day treatment period reflect on the potential effects of KPE and FNT in food intake and metabolic regulation. Weight changes in experimental animals are often indicative of metabolic alterations such as insulin resistance and physiological shifts like sudden increases or reductions in food consumption (Bachmanov, et al., 2002a; Wang and Liao, 2012). These fluctuations can provide valuable insights into how these treatments influence the overall energy balance and metabolic health (Bachmanov, et al., 2002b). In the present study, all treatment groups showed weight gain over the study period shown in Figure 4.1(a). This could be due to

the growing phase of the mice, as the animals were four weeks old at the start of the experiment (Nishikawa, et al., 2007). During this stage, natural growth, including increased muscle mass, may overshadow the subtle effects of treatment (Bradford, 1971; Castelhano-Carlos, et al., 2010). Furthermore, the relatively small sample size used in the present study may have limit the detection of potential significant differences between groups, underscoring the importance of larger cohorts in future studies to validate these findings (Bar-Hillel, 1979; Land, 1981). However, the differences in weight gain across the groups suggests that both KPE and FNT administration could possibly influence the metabolic activity, leading to different rates of growth.

As shown in Figure 4.2(b), FNT treatment group mice exhibited a higher percentage of weight gain compared to the control group mice, which may be attributed to the FNT-induced endocrine disruption. Previous studies have reported that organophosphate pesticides such as FNT can disrupt endocrine mechanisms, potentially accelerating or reducing weight gain through hormonal imbalances (Ngoula, et al., 2007; Noppakun and Juntarawijit, 2021). Sohoni, et al. (2001) deduced that FNT may acts as agonist of androgenic response on alpha kinase 1:antiperinuclear factor (Alpk:Apf) Sprague-Dawley (SD) male rats, but there is still not possible evaluation of the leading causes of the slow rate of weight gain in FNT-induced rats compared to control group. It is also possible that weight gain in the FNT group could involve fluid retention, a symptom associated with certain forms of endocrine disruption or cell-induced oxidative damages, which may transiently inflate bw without reflecting true

increases in lean or fat mass (Straub, 2014; Mottelson, Lundsgaard and Møller, 2020). This aspect should be further explored through analysis of body composition to determine the specific nature of the weight changes.

Meanwhile, KPE treatment group has also shown relatively higher weight gain when compared to control group. This suggests that KPE may enhance metabolism and energy utilization, potentially contributing to increased muscle mass or nutrient absorption. Previous studies have demonstrated that KPE supplementation influences metabolic pathways, particularly through its androgenic effects and flavonoid-induced modulation of lipid metabolism (Trisomboon, et al., 2009; Chen, et al., 2018). Study demonstrated that KPE supplementation increases the ratio of skeletal muscle weight to bw in adolescent subjects, indicating its potential role in muscle growth and development (Sripanidkulchai, et al., 2022). The presence of bioactive methoxyflavones in KPE has been linked to enhanced mitochondrial biogenesis, which could contribute to increased energy expenditure and weight gain (Okabe, et al., 2014). Kim, et al. (2018) reported that KPE treatment increases the expression of PPARGamma coactivator 1 alpha, which primary regulates mitochondrial biogenesis and energy. The upregulation of PGC-1α enhances mitochondrial transcription, leading to improved oxidative capacity and ATP production, which then promotes muscular endurance and performance (Olesen, Kiilerich and Pilegaard, 2010).

Interestingly, the co-administration of KPE and FNT group showed the highest percentage of weight gain among the group (Figure 4.1(b)), suggesting a potential interplay between KPE and FNT. The observed weight gain in mice may indicate that KPE suppress the adverse metabolic effects induced by FNT, possibly by exerting its antioxidative and androgenic properties (Trisomboon, et al., 2009; Mishra and Sharma, 2021). While FNT may contribute to weight gain through endocrine disruption or fluid retention, KPE could counteract this by promoting weight regulation and metabolic stability (Huang, et al., 2022; Park, et al., 2023). In addition, KPE increases muscle mass by mitochondrial biogenesis (Olesen, Kiilerich and Pilegaard, 2010; Kim, et al., 2018). As a result, the overall increase in weight gain observed in KPE-treated mice may be attributed to elevated muscle mass rather than excessive fat accumulation (Toda, et al., 2016; Huang, et al., 2022). In addition, the presence of methoxyflavones in KPE may contribute to the prevention of oxidative damage, caused by FNT at cellular level, which could reduce further fluid accumulation in the body (Huang, et al., 2024). This protective effect may partly explain the higher weight gain observed in the KPE-treated group compared to other treatment groups. However, this hypothesis requires further investigation through food intake measurement, as well as, detailed metabolic assessments, including measures of glucose tolerance, insulin sensitivity, lipid profiles, fluid retention and genome profiling to better understand the co-administration effects of KPE and FNT on appetite, weight and metabolic health (Hidaka, et al., 2017; Ochiai, et al., 2019). Further studies should also explore on muscle mass and fat composition which will enable better understanding the actual condition of the co-administrative treatment of FNT and KPE on mice. These factors are closely linked to

metabolic health and could provide deeper evaluation by underlying the mechanisms of the observed effects of the co-administration of FNT and KPE (Nickelson, et al., 2012). In addition, by expanding these analyses with larger sample sizes and longer treatment durations will help clarify the impact of these treatments on obesity and other metabolic conditions (Falconer, 1953; Land, 1981). These efforts will enhance the understanding of the actual mechanism of natural compounds found in KPE in mitigating and protecting against the adverse effects of environmental toxins like FNT on weight and overall health.

Additionally, there was no signs of distress, illness, or mortality were observed in any of the treatment groups. This finding suggested that the dosages of FNT and/or KPE used in the study were well-tolerated and did not induce acute toxicity in male mice. This observation aligns with previous toxicological studies, where subchronic oral exposure to FNT at controlled doses did not lead to immediate lethality, but instead FNT induced biochemical and histological alterations primarily through oxidative stress pathways (El-Shenawy, 2010; Saber, Abd El-Aziz and Ali, 2016). Similarly, studies on KPE have consistently demonstrated a high safety margin, even at doses up to 2000 mg/kg body weight, without signs of systemic toxicity or mortality in rats (Chivapat et al., 2004; Yoshino et al., 2019). In general, these findings have reinforced the hypothesis that the administered doses in the current study fall within a non-toxic range and support further investigation into KPE's protective role against FNT-induced toxicity.

5.4 Effect of KPE and FNT Administration on Testis Weight

FNT treatment group in the present study exhibited a relatively higher testicular weight when compared to the control group as shown in Figure 4.2. The increase in testis weight could be due to the accumulation of fluid (hydrocele) at the site of inflammation in the seminiferous tubules (Wang, Naito and Nakajima, 2012a; Taib, et al., 2013). Hydrocele formation, commonly associated with inflammation-induced fluid retention, is a recognized pathological response to toxicant exposure and has been reported in organophosphate-induced testicular damage models (Wang, Naito and Nakajima, 2012b; Saber, Abd El-Aziz and Ali, 2016; Yusoff, et al., 2020). Such fluid retention can contribute to apparent testis enlargement without necessarily reflecting increased cellular mass or functional tissue.

In contrast, no significant difference was observed in relative testis weight between the KPE treatment group and the control group ($p \geq 0.05$). This suggests that KPE at the administered dosage is non-toxic and does not cause testicular inflammation or fluid accumulation. The hypothesis can be further associated with earlier findings by Chivapat, et al. (2004), who reported that KPE is safe at doses lower than 13.3 g/kg bw. Study by Chivapat, et al. (2010) reported no apparent toxicity symptoms in male mice administered with 2000 mg/kg bw KPE, emphasizing on the safety of KPE when used at appropriate dose. Additionally, Luangpirom and Komnont (2011) and Yoshino, et al. (2019)

also reported that appropriate doses of KPE do not induce adverse effects on testicular tissue.

Interestingly, the co-administration of KPE and FNT resulted in normalization of relative testis weight, bringing it closer to levels observed in the control group. This suggests a potential protective effect of KPE against FNT-induced testicular alterations. The high antioxidant content of KPE, particularly methoxyflavones and phenolic compounds, may help neutralize the ROS generated by FNT exposure, reducing inflammation and thereby limiting fluid retention or hydrocele formation in testicular tissues (Wang, Naito and Nakajima, 2012; Sripanidkulchai, et al., 2020). The protective effect of KPE may also involve in preventing further oxidative damage in the seminiferous tubules induced by FNT, thereby supporting testicular function and structural integrity, and maintaining tissue homeostasis (Dare, Oyeniyi and Olaniyan, 2013; Asadi, et al., 2017).

While the present findings have highlighted on the protective potential of KPE in counteracting FNT-induced testicular toxicity. However, genomic profiling and molecular pathway analysis are recommended to better elucidate the mechanisms through which FNT induces testicular toxicity and how KPE exerts its restorative effects.

5.5 Effect of KPE and FNT Administration on Lipid Peroxidation Level

In the present study, FNT-treated mice showed significantly higher serum malondialdehyde (MDA) levels than the control group (Figure 4.3). This elevated MDA level serves as a biomarker of oxidative stress and lipid peroxidation, reaffirming FNT's known mechanism of inducing ROS (El-Shenawy, 2010; Milošević, et al., 2018). Previous studies have reported that FNT exposure increases MDA levels, leading to oxidative stress and cellular damage in murine model (Taib, et al., 2013; 2014). In addition, several studies have also documented that organophosphate pesticides like FNT induce oxidative stress in spermatozoa, leading to spermatotoxicity and testicular dysfunction (Saber, Abd El-Aziz and Ali, 2016; Yusoff, et al., 2020). The mechanism involves ROS-induced damage to sperm cell membranes and epigenetic modifications in sperm DNA, ultimately impairing male fertility (Ito, et al., 2014; Mobasheri and Babatunde, 2019; Yusoff, et al., 2021). Furthermore, oxidative stress has also been implicated in promoting inflammatory responses and capillary permeability, which may lead to fluid retention in tissues, such as hydrocele formation in the testes, contributing to tissue swelling and weight gain (Wang, Naito, and Nakajima, 2012; Saber, Abd El-Aziz and Ali, 2016).

On the other hand, there are no significant differences observed between the KPE and the control groups on serum MDA levels ($p \geq 0.05$), indicating that KPE did not induce lipid peroxidation or oxidative stress at the administered dosage (Yoshino, et al., 2019). This supports previous findings suggesting that

KPE is safe for administration when used at appropriate doses (Chivapat, et al., 2010; Luangpirom and Komnont, 2011). While the present study reported that KPE did not induce oxidative stress at the tested dosage, further studies are still needed to evaluate the lipid peroxidation profile at higher doses of KPE administration. It is essential to investigate whether elevated doses may alter lipid peroxidation levels, particularly in prolonged administration.

Even though KPE + FNT treatment group showed no significant different in serum MDA level when compared to FNT treatment group, a reduction in serum MDA level were observed. It shows that KPE has the potential to overcome the oxidative stress induced by FNT. It was reported by other studies that the methoxyflavone derivatives found in KPE may play a critical role in scavenging ROS activity, which may also reduce lipid peroxidation activity by breaking the propagation of free radical chain reactions (Taib, et al., 2014; Sripanidkulchai, et al., 2020). These actions potentially prevent further oxidative damage to testicular tissues and support sperm cell integrity (Park, et al., 2014; Asadi, et al., 2017). Though a reduction in serum MDA levels was observed in the co-administration of KPE and FNT, it is suggested that a higher dosage of KPE may be required to achieve a more pronounced and significant reduction in lipid peroxidation levels, in which may further preventing fluid retention occurrence in the body due to high lipid peroxidation level and oxidative stress.

While the above findings highlight the therapeutic potential of KPE in counteracting FNT-induced oxidative stress, the exact mechanisms by which methoxyflavone derivatives exert their antioxidative effects remain unclear. Thus, further studies are required to elucidate the molecular pathways involved in ROS scavenging by FNT and the protective effects of KPE from oxidative damage. Additionally, future research should also include biomarker analysis of oxidative stress, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to comprehensively assess the oxidative balance in response to increasing KPE doses. These findings would contribute to establishing the optimal dosage range for the therapeutic use of KPE while ensuring its safety and efficacy.

5.6 Effect of KPE and FNT Administration on Sperm Characterization and Testosterone Level

Male fertility is commonly assessed through key parameters such as sperm count, viability, progressive motility and testosterone level (Iwamoto, Nozawa and Yoshiike, 2007; World Health Organisation, 2010). These indicators provide a comprehensive understanding of reproductive health, as they assess sperm quantity, sperm quality and hormonal balance which are necessary for spermatogenesis and reproductive function (Khandwala, et al., 2017). Thus, making it as a critical factor for successful fertilisation in clinical study on infertile male.

In present study, FNT treatment group has significantly impaired male fertility ($p < 0.05$), marked by the low sperm count, sperm progressive motility, sperm viability and testosterone level (Table 4.4). This could be the oxidative damaged to spermatozoa and Leydig cells (testosterone producer) upon the exposure of FNT (Saber, Abd El-Aziz and Ali, 2015; Yusoff, et al., 2020; 2021). Similar observation by previous studies, showing that ROS generated by FNT can disrupt testicular functions and impaired sperm quality (Taib, et al., 2013; 2014; Ito, et al., 2014). The generated ROS can lead to DNA damage and mutation in spermatozoa and Leydig cells, affecting their integrity and functions (Taib, et al., 2013; Yusoff, et al., 2021). Yusoff, et al. (2020) reported that FNT disrupts androgen receptor function indirectly by inducing oxidative stress and disrupting testosterone synthesis. This hormonal imbalance can further impair spermatogenesis, leading to reproductive toxicity (Dutta, et al., 2021; Karunyam, et al., 2023).

Additionally, a significantly higher serum testosterone level was observed in the KPE treatment group compared to the control group. This suggests that KPE may enhance androgenic activity by stimulating testosterone production. This aligns with previous studies indicating that methoxyflavones in KPE possess androgenic properties, which may promote Leydig cell function and steroidogenesis (Trisomboon, et al., 2009; Lert-Amornpat, Maketon, and Fungfuang, 2017). However, sperm progressive motility and viability were not showing significant different with control, indicating that KPE did not exert a direct effect on sperm quality or movement. This finding is consistent with

earlier research suggesting that KPE primarily influences testosterone levels rather than sperm parameters (Sudwan, et al., 2006; Chaturapanich, et al., 2008). Interestingly, a significantly lower sperm concentration was observed in the KPE treatment group compared to the control group. This could be due to the hormonal feedback suppression. Methoxyflavones in KPE may stimulate testosterone production via androgenic pathways, but chronic or high-dose androgenic stimulation can trigger negative feedback on HPG axis (Sripanidkulchai, et al., 2022). Abnormal testosterone production or androgenic stimulation may suppress LH and FSH, which are essential for spermatogenesis (Christian-Maitre and Young, 2022). In addition, the present finding above may also indicate a potential trade-off between enhanced androgenic activity and sperm production. A study reported that high level of testosterone may suppress gonadotropin and sperm production (Mastumoto, 1990; Ismael, AL-Anbari and Mossa, 2017). This suppression could slightly reduce sperm concentration, as observed in the KPE treatment group. Nevertheless, further studies are still needed to investigate the long-term effects of KPE on gonadotropin regulation and sperm production efficiency.

Interestingly, the co-administration of KPE and FNT has shown significantly restoration of all sperm concentration, progressive motility and viability as well as serum testosterone level when compared to FNT treatment group ($p < 0.05$, Table 4.4). The above findings could be attributed to antioxidative properties of KPE, that scavenges and protects sperm and Leydig cells from ROS and oxidative damages induced by FNT (Chen, et al., 2018;

Kowalczyk, 2022; Sitthichai, et al., 2022). Protective effects of antioxidant were also reported by Saber, Abd El-Aziz and Ali (2015) where quercetin, scavenges ROS and inhibits radical propagation. Thus, it can mitigate spermatotoxicity caused by FNT. Another study by Taib, et al. (2013; 2014) also demonstrated the TRF preserve the male reproductive function by inhibiting the propagation of free radical chain induced by FNT, therefore protecting the spermatozoa from oxidative damage. Thus, it is suggested that KPE possesses protective effect against FNT-induced spermatotoxicity and testosterone reduction. However, to further elucidate the molecular mechanisms underlying the protective effects of KPE, future studies should focus on genomic and transcriptomic profiling.

5.7 Effect of KPE and FNT Administration on Testicular Morphology

Testicular morphology is a crucial indicator of the male reproductive health, reflecting both structural and functional integrity (Bergmann, Behre and Nieschlag, 1994). Whereas the Johnson's mean testicular biopsy score count is a grading system used to evaluate spermatogenesis and testicular integrity (Mushtaq, Alam and Khan, 2013; Dang-Cong and Nguyen-Thanh, 2021). It provides valuable histological insights in infertility studies, particularly for evaluating testicular damage and recovery following exposure to harmful substances or protective treatments. In present study, there are some morphologies of the seminiferous tubules align with the descriptions provided by Johnson's mean testicular biopsy score count (Figure 4.5).

Figure 4.4 shows that FNT treatment group exhibited more disorganized testicular cells with lower visibility of spermatozoa in the lumen of the testicular tissue than control group. Additionally, Johnson's mean testicular biopsy score count revealed that the FNT treatment group had a significantly lower score compared to the control group. This indicates that FNT exposure adversely affected spermatogenesis, leading to structural disorganization and reduced sperm production (Saber, Abd El-Aziz and Ali, 2015). The observed disorganization of testicular cells suggests the degeneration of the germinal epithelium, which may be attributed to oxidative stress-induced damage caused by FNT-induced ROS (Taib, et al., 2013; 2014; Yusoff, et al., 2020). Meanwhile, other study also reported that prolonged FNT exposure subsequently caused inflammatory damage towards the testicular tissue (Abdel-Ghany, et al., 2016). The significant damaged can be observed by the reduction in spermatogenic cells, degenerated and disorganised epithelium structures, as reflected in the present study (Saber, Abd El-Aziz and Ali, 2015; Yusoff, et al, 2020). The adverse testicular histomorphology effects were also observed in FNT-treated rats over a longer treatment of 4 to 9 weeks (Taib, et al., 2013; Ito, et al., 2014; Saber, Abd El-Aziz and Ali, 2015). Additionally, the degenerative germ cells found in the seminiferous tubules is likely due necrotic or apoptotic events caused by FNT-induced oxidative damages (Taib, et al., 2013; Abdel-Ghany, et al., 2016). Supporting evidence from prior research suggested that FNT entered testicular tissue through bloodstream, impaired the process of germ cells maturation and differentiation, and lastly disrupting the overall spermatogenesis occurred in the seminiferous tubules (Ito, et al., 2014; Saber, Abd El-Aziz and Ali, 2015; Yusoff, et al., 2020).

Meanwhile, there was no adverse effect of germ cells nor abnormality of seminiferous tubules detected in mice administered with KPE when compared to control (Figure 4.4). This non-significant difference between KPE and control groups was also reflected in Johnson's mean testicular score count ($p \geq 0.05$), as shown in Table 4.5. The results suggested that KPE has no adverse effect or toxicity towards spermatogenesis and testicular tissue in mice. Chivapat, et al. (2010) reported on no visual of testicular atrophy or orchitis found in KPE-administered rats at doses from 5 to 500 mg/kg bw for 6 months treatment. Collectively, previous studies also strongly emphasized on the safe administered dose of KPE for testicular health in mice, supporting the observations from present histomorphological analysis (Chivapat, et al., 2004; Yoshino, et al., 2019).

Interestingly, similar protective and restorative properties on testicular tissue were observed in the co-administration of KPE and FNT group when compared to FNT treatment group (Figure 4.4). The significant improvement observations include the increase in the number of testicular germ cells, mild thickened layer of smooth muscle surrounds the seminiferous tubules and feeble congestion of testicular cells in seminiferous tubules. The present findings were consistent with previous findings by Saber, Abd El-Aziz and Ali (2015), which reported Specifically, rats treated with both quercetin and FNT showed improvement on testicular histology compared to those treated with FNT alone. The observations included a reduction in histopathological alterations such as degeneration of seminiferous tubules and interstitial edema. Based on the study

above, the observations from the present finding highlight on the potential antioxidative properties of KPE, which possess protective and restorative effects toward FNT-induced testicular damages (Chen, et al., 2018; Kowalczyk, 2022). It can be supported by Mekjaruskul, Jay and Sripanidkulchai (2012b) where methoxyflavones of KPE were found to gather in testes of mice after less than 2 hours of administration and they remained in the body for up to 6 hours before complete excretion. The rapid accumulation of the phytochemicals may protect the testicular from oxidative stress-induced injury in a time-dose-dependent way (Saber, Abd El-Aziz and Ali, 2015; Chen, et al., 2018; Sitthichai, et al., 2022). These findings collectively emphasize the potential of KPE as a natural therapeutic agent to counteract FNT-induced testicular toxicity while supporting male reproductive health.

5.8 Effect of KPE and FNT Administration on Mating and Post-Parturition Assessments

In the present findings, the FNT treatment group exhibited a lower mating success rate compared to the control group, as shown in Figure 4.6(a). Although the difference was not statistically significant ($p \geq 0.05$), the observed reduction may reflect the potential of FNT to act as an endocrine disruptor and oxidative stressor, thereby interfering with normal mating behavior and reproductive function in male mice. The short-term exposure of EDC such as FNT may exert insignificant impact on mating and post-parturition assessment, as shown in the present finding above. However, prolonged exposure of EDCs

can cause cumulative toxic effects, leading to reduced sperm concentration, motility and viability in males, thereby lowering mating success rates (Feroe, et al., 2017; Robaire, et al., 2022). Studies have reported that spermatozoa are more vulnerable to environmental damage (Soubry, et al., 2014; Wu, et al., 2015). Thus, chronic exposure to EDCs may affect epigenetic reprogramming of sperm during spermatogenesis, potentially leading to transgenerational toxic effects (Anway, et al., 2005; Donkin and Barrès, 2018; Mobasheri and Babatunde, 2019). A study by Yusoff, et al. (2020) investigated the effects of different doses of FNT on male sexual behaviour and reproductive performance in SD rats. They found that higher doses of FNT (20 mg/kg bw) had a more pronounced effect on reducing sexual behaviour and male fertility than the lower dose (10 mg/kg bw). Meanwhile, the co-administration of KPE and FNT group has shown higher mating success rate than FNT treatment group. This may be attributed to the protective effects of methoxyflavones found in KPE, which help prevent oxidative damage and enhance androgenic activity by promoting testosterone production in male mice. Studies have shown that methoxyflavones found in KPE possess antioxidant properties and promote androgenic activity in male rats (Sudwan, et al., 2008; Chen, et al., 2018). Sudwan, et al. (2006) reported that at high dose of KPE (240 mg/kg bw) may have adverse impacts on pre-copulatory behaviour of male rats, which may affect mating success rate as compared to low dose of KPE (60 and 120 mg/kg bw). However, the study also reported that lower doses of KPE did not significantly affect sexual behaviour of male rats. The findings suggest that moderate doses of KPE may not enhance or impair mating behaviour. Therefore,

future studies should explore the mechanism behind this effect and evaluate the long-term reproductive safety of KPE at different doses.

However, the overall mating success parameters in the present study did not fully support this evidence, as no statistically significant differences were observed in mating success rates across all treatment groups ($p \geq 0.05$). It could be attributed to several limitations such as small sample size, lack of dosage or treatment duration applied in this study. Such limitations may not provide a significant impact of FNT or KPE on mating success rate (Bar-Hillel, 1979; Land, 1981). The duration of treatment and the applied dosage in *in vivo* studies play a role in determining mating success rate and post-parturition outcomes. Both factors can significantly influence hormonal regulation, reproductive function, and fetal development, leading to varying reproductive success in animal models (Mahgoub and El-Medany, 2001; Najafi, et al., 2010). Future studies can be done with larger sample sizes, extended treatment durations, and a range of dosages to comprehensively assess the reproductive and developmental outcomes.

FNT treatment group showed significant lengthened mating period compared to control group. It is likely caused by testosterone suppression. Lower testosterone might lead to the diminished in sexual drive of the male mice which causes delayed mating behaviour (Wang, et al., 2012; Saber, Abd El-Aziz and Ali, 2015; Yusoff, et al., 2020). Remarkably, the co-administration

between KPE and FNT were recorded with significant restoration of testosterone level, thus showing a shorter mating period when compared to FNT treatment group (Chaturapanich, et al., 2012; Toda, et al., 2016; Chen, et al., 2018). Supporting evidence from previous studies indicates that KPE administration improves libido by enhancing androgen production, while FNT exposure reduces testosterone, leading to diminished sexual behaviour (Chen, et al., 2018; Yusoff, et al., 2020). These findings further suggest that KPE's potential to counteract the reduction in courtship drive caused FNT-induced reproductive impairments. Furthermore, a significantly strong inverse correlation between mating period and serum testosterone levels observed in the present study (Figure 4.6) further supports the critical role of testosterone in regulating mating behaviour and reproductive efficiency. The result suggests that higher testosterone levels are associated with shorter mating periods, indicating that testosterone enhances sexual drive and facilitates overall mating success.

In Table 4.6, FNT-treated group produced a slightly smaller litter size per dam and exhibited a lower pup survival rate compared to the control group. These findings may be attributed to reduced sperm quality caused by FNT-induced oxidative damage, which likely impaired fertilization success during mating. Furthermore, the potential transmission of sperm DNA damage or epigenetic alterations may have contributed to embryonic or postnatal anomalies, thereby reducing pup survival. A study by Yusoff, et al. (2021) reported that offspring anomalies, including short or absent tails and defective

feet, were observed following a four-week treatment of FNT in paternal SD rats. These findings suggest that FNT exposure may induce mutation or epigenetic modifications in sperm cells, leading to multigenerational impact (Anway, et al., 2005; Mobasheri and Babatunde, 2019). Additionally, the exposure of FNT may also reflect reduced pup survival due to potential congenital abnormalities, as oxidative stress and DNA mutations in sperm caused by FNT exposure could result in the transmission of defective genetic material, leading to organ malformations or developmental impairments in the offspring (Yusoff, et al., 2021). This aligns with previous findings where paternal exposure to organophosphates like FNT led to adverse reproductive outcomes, including reduced litter size, pup mortality, and even congenital anomalies (Okahashi, et al., 2005; Donkin and Barrès, 2018). Meantime, it is also shown in the present study that the co-administration of KPE and FNT group resulted in slight improvements in litter size and pup survival rates as compared to FNT treatment group. These improvements may be linked to the antioxidative properties of KPE, which could protect spermatozoa from FNT-induced oxidative damage, thus enhancing fertilization success and normal pup development (Kowalczyk, 2022; Sitthichai, et al., 2022). Additionally, several studies have reported that chronic exposure to KPE, even at increased doses, does not cause significant adverse effects in experimental models (Chivapat, et al., 2004; 2010; Yoshino, et al., 2019). These findings suggest that KPE is generally safe for oral administration in murine models and is unlikely to exert harmful effects on offspring. However, the absence of studies investigating the multigenerational impact of KPE exposure underscores the need for future research to evaluate potential long-term and transgenerational safety outcomes. Interestingly, no

gross anomalies were found in the offspring of all treatment groups. This finding can be further validated in future studies by increasing dosage and treatment duration, which can generate a more impactful observation in litter size per dam and pups' survival rate.

Nevertheless, there were no significant found in litter size and survival success rate of pups across all treatment groups ($p \geq 0.05$). The findings could be attributed to the limitations such as a small sample size, insufficient of treatment duration and dosage applied, which might not adequately capture the significant impact of FNT or KPE on these reproductive and post-parturition parameters (Bar-Hillel, 1979; Land, 1981). Meanwhile, the variability in experimental conditions, including dosage, exposure methods and the substances' interactions with the biological system, may also affects the significant of the results (Gumbo, Angulo-Barturen and Ferrer-Bazaga, 2015; Hicks, et al., 2020; Tyson, et al., 2020). Meanwhile, studies reported that the adverse effects of FNT can vary depending on the dosage applied in animal models, with higher doses generally leading to more severe toxicological outcomes (Ecobichon, Comeau, and Cameron, 1980; Trottier, et al., 1980). Thus, future studies should consider increasing the sample size, treatment duration, and dosages of both FNT and KPE to better evaluate their potential effects. This approach may help uncover significant impacts on reproductive outcomes and allow for more comprehensive assessment in multigenerational studies.

5.9 Summary Discussion of Research

The present study demonstrated that the choice of solvent significantly influences the extraction yield, phytochemical composition and antioxidant activity of *K. parviflora*. Aqueous extraction method yielded the highest extract quantity due to its efficiency in extracting polar phytochemicals such as saponins, tannins and glycosides. Whereas ethanolic extraction method resulted in the highest phenolic content and antioxidant activity, as indicated by the lowest EC_{50} value in the DPPH assay. In contrast, hexane extraction method produced the highest flavonoid content, likely due to its ability to extract non-polar methoxylated flavones from *K. parviflora*. However, the flavonoid-rich hexane extract did not exhibit the strongest antioxidant properties, suggesting that other phenolic compounds, such as tannins and phenolic acids, contribute more significantly to antioxidative potential. The discrepancy between total phenolic content (TPC) and total flavonoid content (TFC) further highlights the differences in solvent polarity and compound solubility. Notably, ethanol proved to be the most effective solvent, as it extracted a broad spectrum of bioactive compounds while maximizing antioxidant activity. Although the EC_{50} value of the ethanolic extract was relatively higher than in some previous studies, extending the maceration period may enhance extraction efficiency. Given its superior antioxidant activity and balanced phytochemical composition, the ethanolic extract of *K. parviflora* was selected for further phytochemical screening, GC-MS analysis and *in vivo* studies to explore its therapeutic potential.

Meanwhile, the subsequent present study highlights the importance of physicochemical assessment and GC-MS analysis in evaluating the solubility, extraction efficiency, and bioactive composition of KPE. Ethanolic extraction demonstrated the highest phytochemical diversity, efficiently extracting saponins, tannins, alkaloids, terpenes, cardiac glycosides, phenolics and flavonoids, while anthraquinones were absent. The GC-MS analysis identified eight major compounds, including five known methoxyflavones such as TMF, a major bioactive constituent of KPE. However, DMF, commonly reported in previous studies was not detected in the present findings. This can be due to the variations in plant maturity, environmental conditions and extraction protocols. It is suggested that DMF may undergo hydroxylation and methylation over time, thus converting into TMF as the rhizomes mature. Interestingly, 5-hydroxy-3,7-dimethoxyflavone was the most abundant compound, recognized for its potential glycogen metabolism regulatory properties. Furthermore, there were also three novel compounds identified in the present study, including 7-Hydroxy-3-methoxy-2-p-methoxyphenyl-4H-chromen-4-one, which may possess anti-cancer properties and 2-(3-Hydroxy-4-methoxyphenyl)-3,7-dimethoxy-4H-chromen-4-one, predicted to exhibit anti-allergic and AChE inhibitory activity. Whereas another major compound, 2,6-Bis-tert-butyl-2',6'-dimethoxy(4,4'-methylenebisphenol), was detected but has no reported biological activity, warranting further investigation. These findings reinforce the therapeutic potential of KPE and its diverse bioactive profile, emphasizing the need for further research to explore its pharmacological applications and potential health benefits.

In the subsequent *in vivo* analysis of the research, it provides an in-depth exploration of the toxicological effects of FNT on male reproductive health and the potential protective role of KPE in FNT-administered mice. The present findings have demonstrated that FNT, induces oxidative stress in the body of experimental model by the generation of ROS. The generation of ROS by FNT may contributes to lipid peroxidation, protein oxidation, and DNA damage. Above pathological events may result in apoptosis and degeneration within the seminiferous epithelium. The damages of testicular tissues may cause the significant spermatotoxicity and disrupted testosterone levels, as evidenced by the significant decreased sperm parameters and reduced serum testosterone as shown in the present findings. These effects align with the existing literature, highlighting the adverse effect of FNT which impaired germ cell development and Leydig cell function by the induced ROS in the testicular tissue. Above findings suggested that oxidative stress plays a pivotal role in the reproductive toxicity of FNT, as evidenced by the disrupted spermatogenesis and testicular tissue damage in the exposed animals. Furthermore, subsequent disrupted endocrine functions by FNT could potentially lead to the reduction in sexual drive significantly by elongating the mating period as observed in the present study. In short, the exposure of FNT may induce oxidative damages that disrupt spermatogenesis and testosterone level. Therefore, it leads to low sperm quality and reduces sexual drive. Collectively, the above findings highlight the critical role of FNT exposure which potentially leads to oxidative stress and causes male reproductive toxicity in murine model.

On the other hand, KPE demonstrates promising antioxidative and protective effects against FNT-induced oxidative damages. The present study showed no significant sperm nor testicular toxicity in mice treated solely with KPE, consistent with previous studies which indicate the safety administration of KPE at up to 2000 mg/kg bw. Moreover, the administration of KPE in FNT treatment mice enhanced the testicular structure and function as compared to FNT treatment group. It was shown by the improvements in sperm concentration, motility, viability and testosterone levels. These protective effects are likely attributed to the methoxyflavones extracted from the rhizome of *K. parviflora*, which possess antioxidant properties. These antioxidants can scavenge ROS and preventing oxidative damage of the testicular tissues. The ability of methoxyflavones to localize in testicular tissue within a short period further supports their role in preventing lipid peroxidation and cellular damages. The present study also highlighted the importance of dosage and duration in evaluating the efficacy of KPE. The observed improvements in sperm parameters and testosterone levels in the co-administration group suggest a dose-dependent protective mechanism. Whereas a higher dose of KPE is expected to further reduce serum MDA levels in the *in vivo* study. Similar effects have been noted with other antioxidant compounds, such as quercetin and tocotrienol-rich fractions, which ameliorate FNT-induced oxidative stress, restore testosterone levels and promote the improvement of reproductive health. The combination of KPE and FNT also showed minor histopathological changes on testicular tissue, such as thickened smooth muscle and slight congestion on the seminiferous tubules, which are comparable to findings in studies involving other antioxidant treatments. These structural modifications

may indicate a partial restoration of testicular tissue, further supporting the protective effects of KPE. Thus, KPE can be a potential therapeutic agent for improving male fertility by reducing oxidative damages and enhancing testosterone level.

Meanwhile, the present study raises questions about the long-term implications of FNT exposure and the potential multigenerational effects of KPE. The present study showed that only slight improvements in litter size and pups' survival were observed in the co-administration of KPE and FNT group as compared to FNT treatment group. The findings above proposed the potential role of KPE in ameliorating FNT-induced genetic changes on spermatozoa. The findings also associate with the existing evidence linking oxidative stress to mutation in sperm DNA, which have higher impact on male fertility and offspring development.

In short, the present findings have provided critical insights into the dual role of oxidative stress by FNT and antioxidants by KPE in affecting male reproductive health. While FNT has drawn the attention of inducing adverse effects like other environmental toxicants on male fertility, whereas KPE highlights the naturopathy potential of plant-derived antioxidants in protecting such induced-toxicities. Future study should explore into the molecular pathways such as genomic and metabolic profiling in underlying these effects,

focusing on the long-term impacts and multigenerational implications of KPE and FNT exposure in murine model.

5.10 Future Studies

Future research should consider a longer duration of the treatment (2 months or more) can be implemented on the research which can be used to assess on the chronic toxicity of FNT as well as the determination of the potential of KPE in lowering the cytotoxicity induced by FNT. By extending the duration of administration, it could provide further insights into progressive reproductive toxicity, oxidative stress accumulation and other hormonal imbalances, which may not have been fully observed within the 28-day treatment.

Additionally, it is observed that current KPE dose did not completely reduce serum MDA level in the co-administration of KPE and FNT group. Therefore, future studies should incorporate multiple dosage variations for both KPE (50 mg/kg bw and 100 mg/kg bw) and FNT (50 mg/kg bw and 100 mg/kg bw) to assess the dose-dependent efficacy of KPE in ameliorating FNT-induced reproductive toxicity. The present study found no significant difference on mating success rate at a low FNT dose (20 mg/kg bw), suggesting that a higher FNT concentrations may be necessary to induce more severe reproductive impairments, which could then be mitigated by different doses of KPE.

Parameters such as the number of implantation sites in females can provide additional insight into mating success and early embryonic development, complementing the evaluation of litter size per dam. This addition would strengthen your fertility assessment by distinguishing between successful fertilization and post-implantation loss.

Moreover, epigenetic studies should be conducted in future study to explore how FNT exposure and KPE intervention influence DNA methylation, histone modifications, and microRNA regulation in testicular cells. Since sperm undergo epigenetic reprogramming during development, FNT-induced oxidative stress may lead to epigenetic alterations that can affect sperm quality, fertility and offspring health. In contrast, KPE may modulate epigenetic markers and ameliorate transgenerational reproductive toxicity. To further assess the heritable effects of FNT and the protective potential of KPE, multigenerational studies should be considered. These studies can determine whether FNT-induced reproductive toxicity is passed on to offspring and if KPE treatment prevents or reverses these adverse effects in subsequent generations. By assessing fertility parameters, reproductive success and developmental outcomes in successive generation, they would provide valuable insights into the long-term consequences of pesticide exposure and KPE intervention.

Lastly, transcriptomic and metabolomic analyses should be performed to elucidate the molecular pathways involved in KPE-FNT interactions. These

advanced techniques can help identify gene expression changes, metabolic shifts and biomarker alterations. Thus, it offers a more comprehensive understanding of how KPE ameliorates FNT-induced toxicity at the cellular and systemic levels.

CHAPTER 6

CONCLUSION

In conclusion, ethanolic extract of *K. parviflora* was selected to proceed for animal study as it exhibited higher preservation of antioxidant properties as compared to hexane and aqueous extracts. The co-administration of KPE and FNT has shown an increase in mice weight over the course of 28 days of administration period. However, the observed weight gain is likely due to the normal growth of the mice, rather than a sign of obesity. This indicates the co-administration of FNT and KPE did not cause adverse effects on the overall health, nor induce obesity at the administered dosage. In addition, no mortality or deaths were observed in any of the treatment groups throughout the 28-day administration period, indicating that the dosages of KPE and/or FNT used in this study were well tolerated and did not elicit overt toxicity in male mice. Meanwhile, KPE administration was shown to reduce the lipid peroxidation and oxidative stress with the reduction of serum MDA as compared to FNT treatment group. Furthermore, KPE co-administration also restore Johnson's mean testicular score and mating period when compared to FNT treatment group. In addition, KPE was proven to possess protective effect towards FNT-induced male reproductive impairment. The protective effect of KPE in reducing oxidative stress and protecting testicular tissues from oxidative damage induced by FNT was likely contributed by the methoxyflavones, identified by GC-MS analysis, which has been identified as the major compound in the KPE.

Furthermore, the co-administration of KPE and FNT has shown a significantly shorter mating period as compared to FNT treatment alone. This signifies the protective effect of KPE on FNT-induced male reproductive toxicity in mice. However, there is no significant difference observed within all treatment groups in mating success rate, as well as post-parturition evaluation (litter size per dam, pups' survival rate). A future study with a larger number of mice per treatment could give a more significant finding for these parameters.

The overall summary of the current research has given an insight on the use of *K. parviflora* rhizome as a new and alternative naturopathic treatment in preventing oxidative damages and hormonal imbalances after chronically exposed to hazardous pesticides.

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APPENDICES

APPENDIX 1

List of Chemicals and Reagents

Chemicals and Reagents	Brand
2-Propanol	Merck
Absolute ethanol	Chemiz
Acetic acid	HmbG Chemicals
Acetic acid glacial	QR*ë*C
Acetic anhydribe	Acros Organics
Agarose D5	CONDA Pronadisa
Aluminium chloride	Merck
Ammonia	Merck
Ascorbic acid	Systerm
Chloroform	QR*ë*C
DPPH	Sigma-Aldrich
D.P.X. Mountant	R&M Chemicals
di-Potassium dihydrogen phosphate	Systerm
di-Sodium hydrogen phosphate dihydrate	Systerm
Eosin	Leica
Fenitrothion	DREHRENSTORFER
Folin-Ciocalteu's phenol reagent	Merck
Formaldehyde	VWR Chem
Gallic acid	R&M Chemicals

Haematoxylin	Leica
Hydrochloric acid	R&M Chemicals
Iodine	Fisher Scientific
Iron (III) chloride	Acros Organics
Nigrosine	Acros Organics
Phosphate buffer saline salt tablet	Takara
Potassium dihydrogen phosphate	Systerm
Potassium iodide	QR*ë*C
Quercetin	Acros Organics
Sodium carbonate	R&M Chemicals
Sodium dihydrogen phosphate	R&M Chemicals
Sodium hydroxide	Merck
Sodium nitrite	QR*ë*C
Sulphuric acid	ACI Labscan
Xylene	Bendosen
Zinc powder	Prochem

APPENDIX 2

List of Instruments and Equipments

Instrument and Equipment	Brand
Autoclave machine	Hirayama (HVE-50)
Electronic weighing balance	Sartorius
Freeze drier	CHRIST (Alpha 1-2 LSC)
Fume Hood	ESCO
Gas chromatography / mass spectrometry system	Agilent (5977C)
Horizontal Laminar Flow Cabinet	ESCO
HPLC System	Shimadzu (UFLC)
Inverted fluorescent microscope	Olympus (BX41)
Magnetic stirrer	IKA (C-MAG HS 7)
Microcentrifuge machine	Becjman Coulter
Micropipette	Eppendorf
Microplate reader	Fisher Scientific (51119700DP)
Microscope camera	Leica (ICC50 HD)
Orbital Shaking Incubator	HandyLAB System
pH meter	Mettler-Toledo Inc.
Refrigerator	Toshiba (GR-RT466WE-PMY (58))
Rotary evaporator	BUCHI
Rotary microtome	Histo-Line laboratories

Slide drier	Histo-Line laboratories (TEC 2602)
Sonicator	Powersonic (P2600D-45)
Tissue embedding cryo module	Tissue-Tek VIP 5 Jr.
Vacuum infiltration tissue processor	Tissue-Tek VIP 5 Jr.
Vortex mixer	Science Lab Asia
Water bath	Histo-Line laboratories (WB 2800)

APPENDIX 3

List of stock solution preparation

Stock and Reagent solution	Preparation method
DPPH (0.2 mM)	39.4 mg of DPPH 500 mL of absolute ethanol The solution was incubated in dark for 2 hours at room temperature before use.
Formalin (pH 7.0)	Four gram of NaH_2PO_4 6.5 g of Na_2HPO_4 100 mL of 36% formaldehyde 900 mL of ultra-pure distilled water
Phosphate buffered saline (pH 7.4, 1×)	One PBS tablet 100 mL ultra-pure distilled water
Potassium phosphate dibasic (30 mM, pH 8.0)	5.2254 g K_2HPO_4 1000 mL ultra-pure distilled water The pH of the solution was adjusted to pH 8.0 using 1 M H_3PO_4
TFC reagent	0.6 mL of 10 % $AlCl_3$ Eight millilitre of distilled water 0.6 mL of 5 % $NaNO_2$ Four millilitre of one molar NaOH
Wagner's Reagent	Two grams of iodine Six grams of potassium iodide 100 mL of distilled water

APPENDIX 4

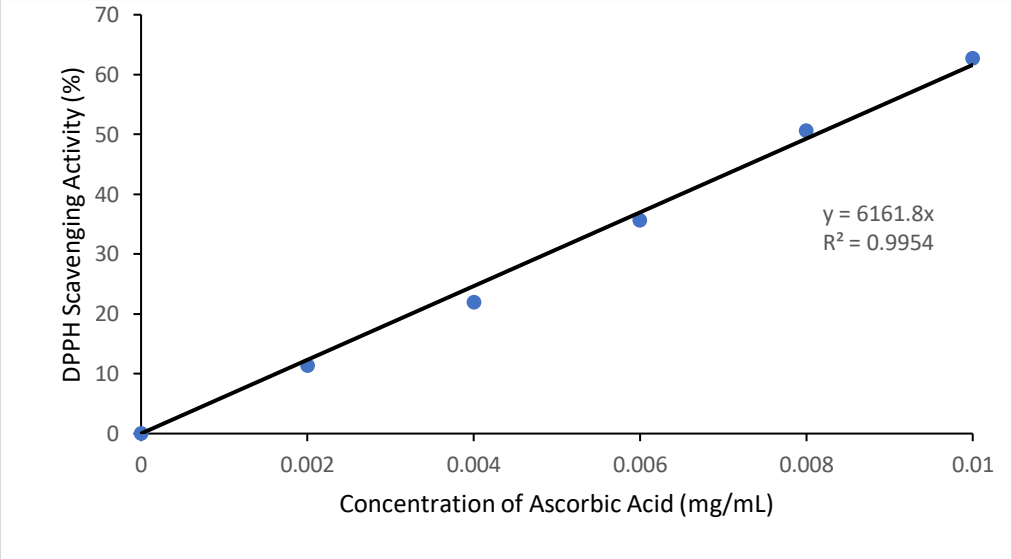
Composition of mouse feed pellet bought from Gold Coin Feedmills Pte. Ltd.

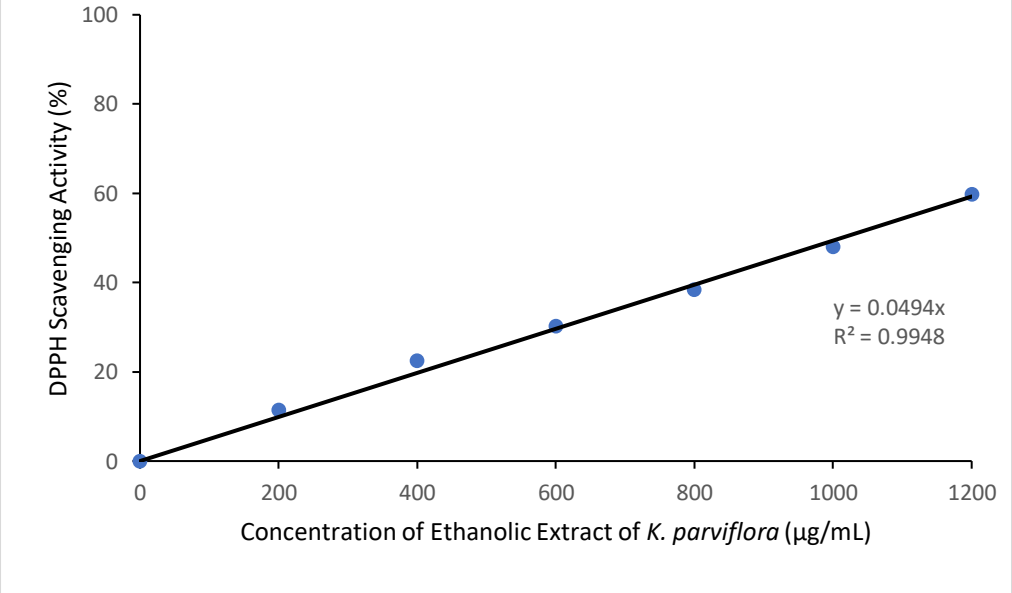
Composition specifications	Total amount (%)
Crude protein (minimum)	21.0
Crude fibre (maximum)	5.0
Crude fat (minimum)	3.0
Moisture (maximum)	13.0
Ash (maximum)	8.0
Calcium (minimum)	0.8
Phosphorus (minimum)	0.4

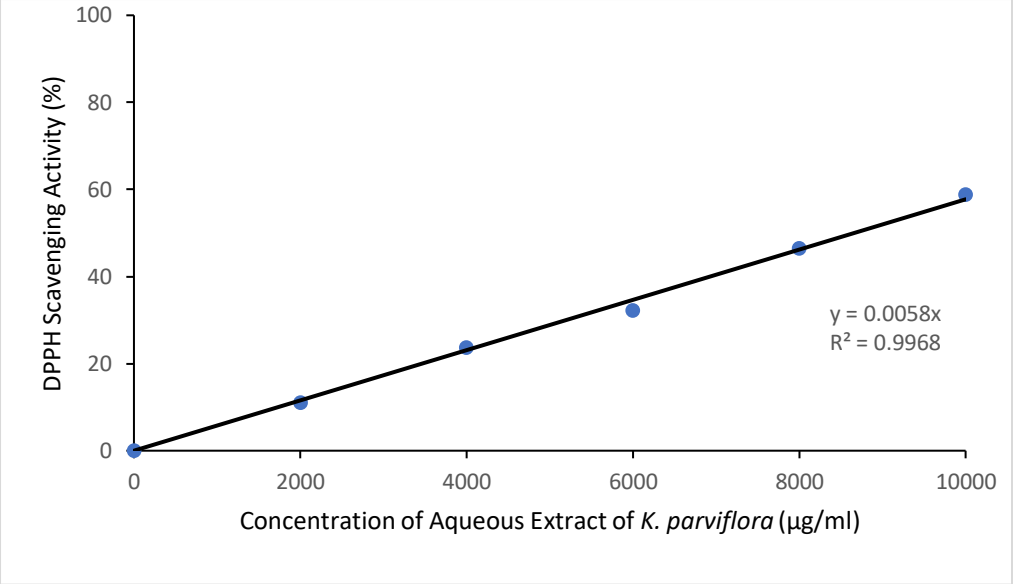
APPENDIX 5

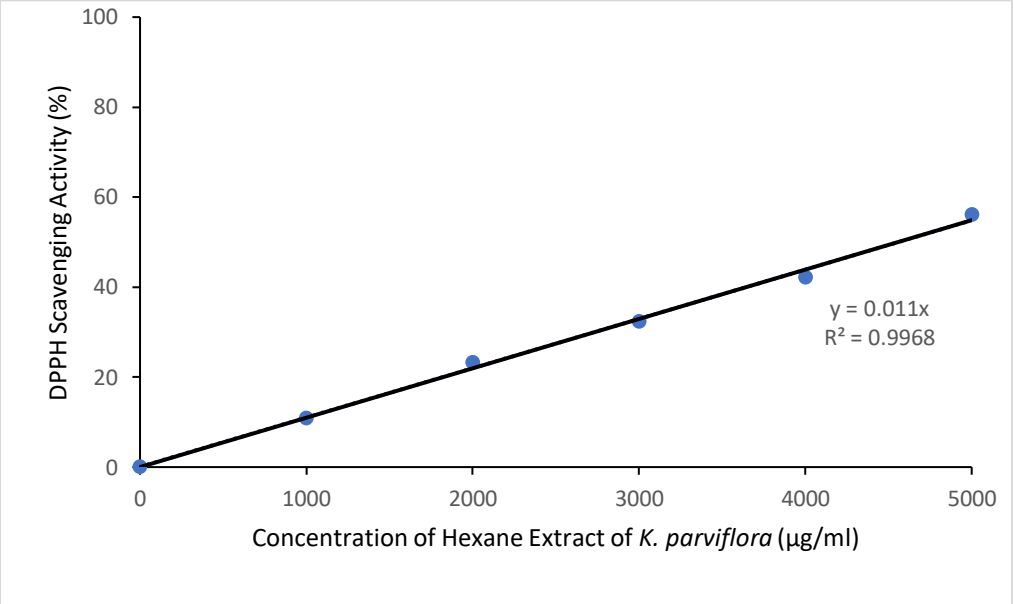
DPPH, TPC and TFC standard curve and sample calculation

5.1 DPPH Ascorbic Acid Standard curve and Sample graph



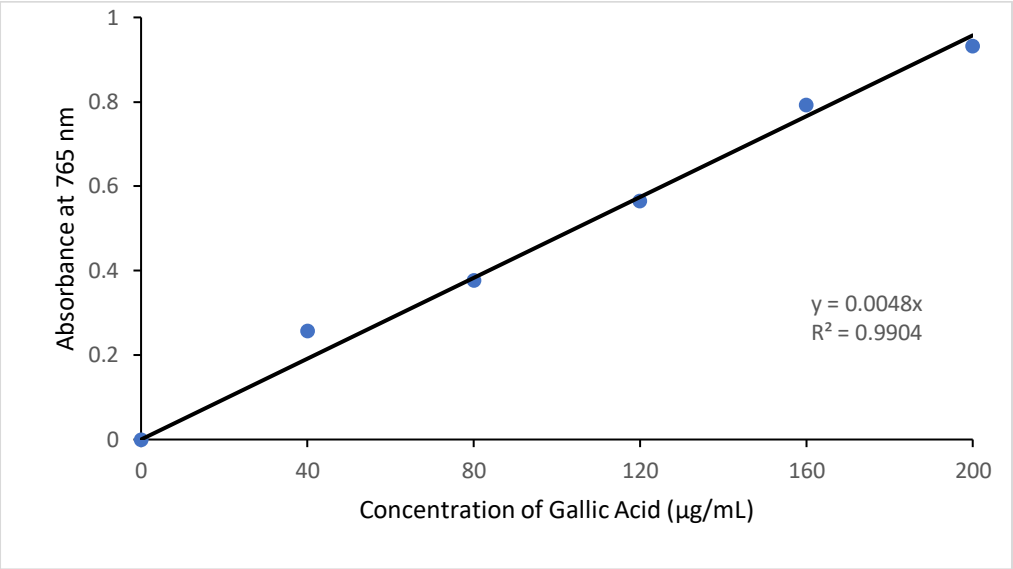






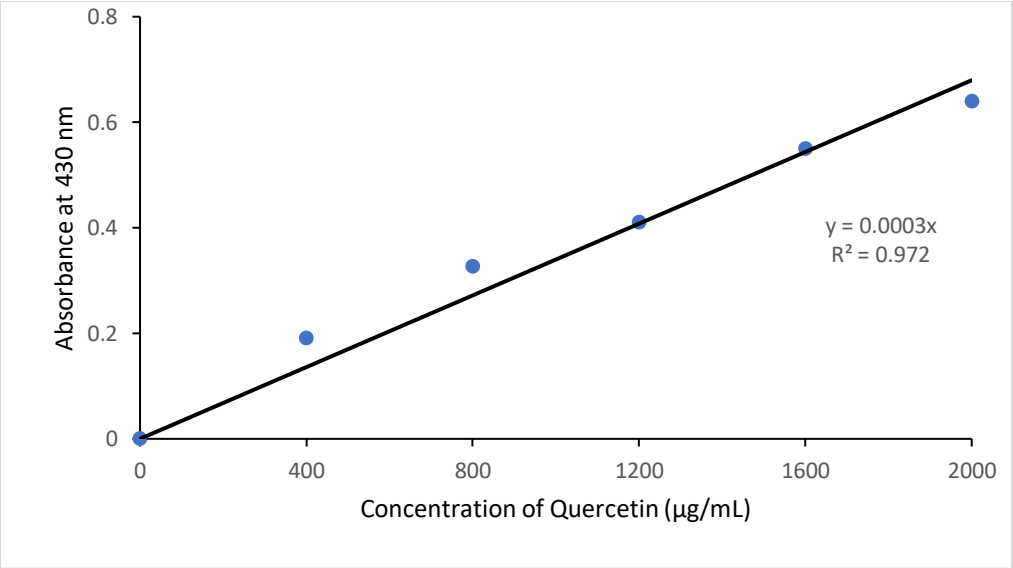
	Ascorbic Acid	KPE		
		EtOH	H_2O	Hexane
DPPH EC_{50} (µg/mL)	8.11 ± 1.48	865.05 ± 5.76	8671.10 ± 8.41	4559.36 ± 6.90

5.2 TPC Gallic acid standard curve and sample determination



KPE	Absorbance at 765 nm			Total phenolic content (mg GAE / g dw)		
	1	2	3	1	2	3
EtOH	0.152	0.138	0.144	63.33	57.50	60.00
H_2O	0.024	0.021	0.014	10.00	8.75	5.83
Hexane	0.057	0.063	0.059	23.75	26.25	24.58

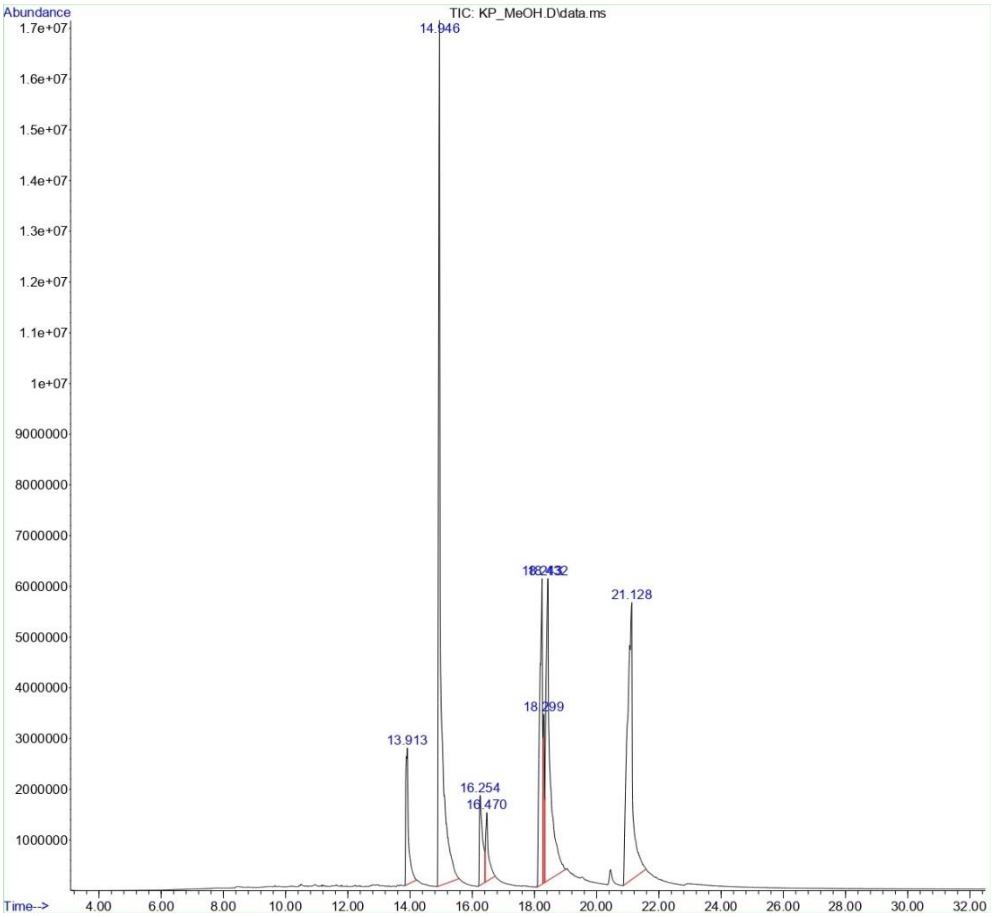
5.3 TFC Quercetin standard curve and sample determination



KPE	Absorbance at 430 nm			Total flavonoid content (mg QE / g dw)		
	1	2	3	1	2	3
EtOH	0.010	0.012	0.013	66.67	80.00	86.67
H_2O	0.003	0.002	0.006	20.00	13.33	40.00
Hexane	0.024	0.023	0.021	160.00	153.33	140.00

APPENDIX 6

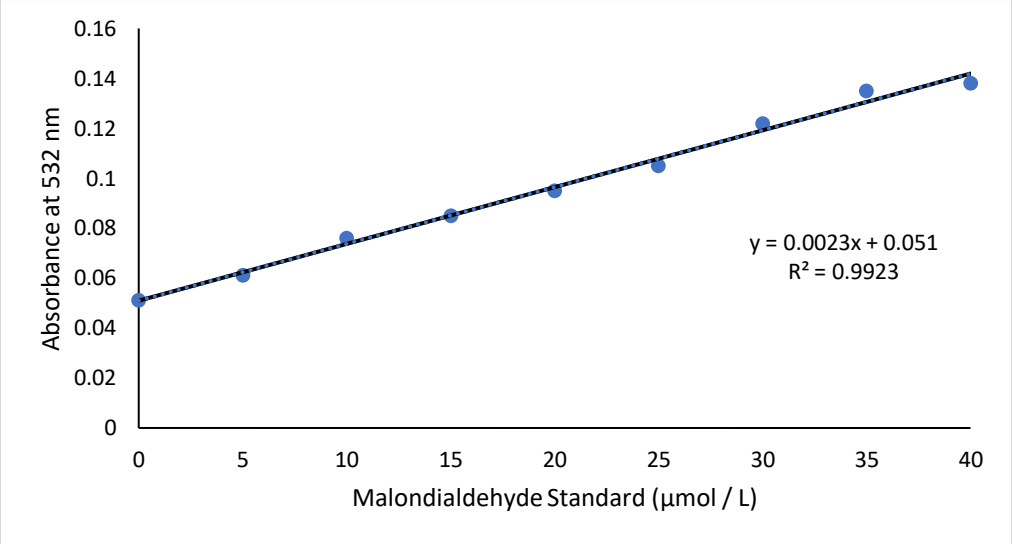
GC-MS Chromatogram of Ethanolic Extract of *K. parviflora*



APPENDIX 7

Lipid Peroxidation Assay via Serum MDA Determination Kit

7.1 Standard Calibration Graph of MDA Standard



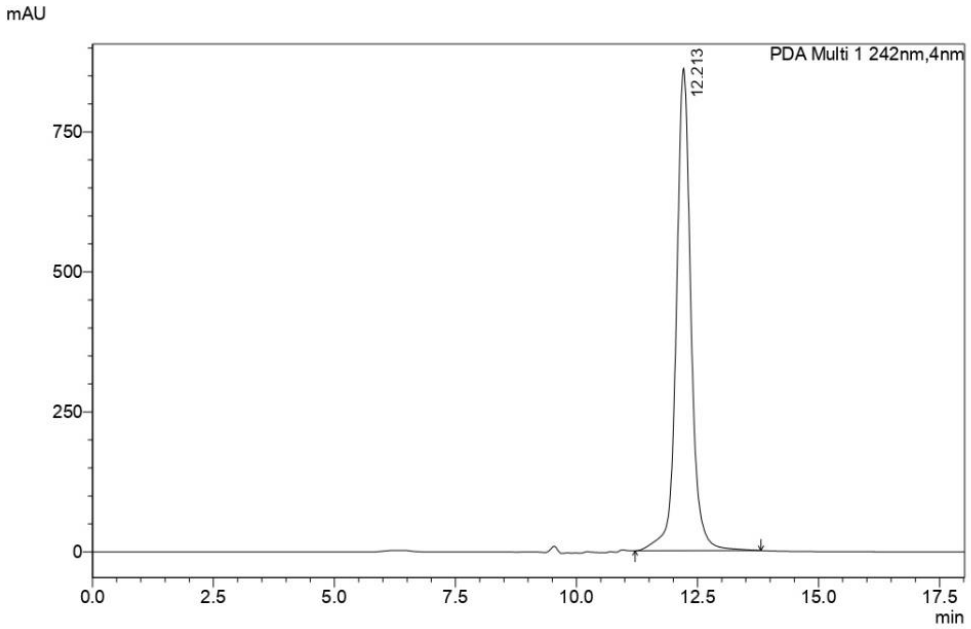
7.2 Absorbance Readings and MDA Level in All Study Groups

Mice study group	Absorbance at 532 nm			MDA (µmol/L)		
	1	2	3	1	2	3
Control	0.128	0.132	0.115	19.71	21.45	13.91
FNT	0.132	0.179	0.133	21.30	41.74	21.74
KPE	0.143	0.132	0.139	25.94	21.45	24.49
KPE + FNT	0.161	0.164	0.172	33.77	35.36	38.70

APPENDIX 8

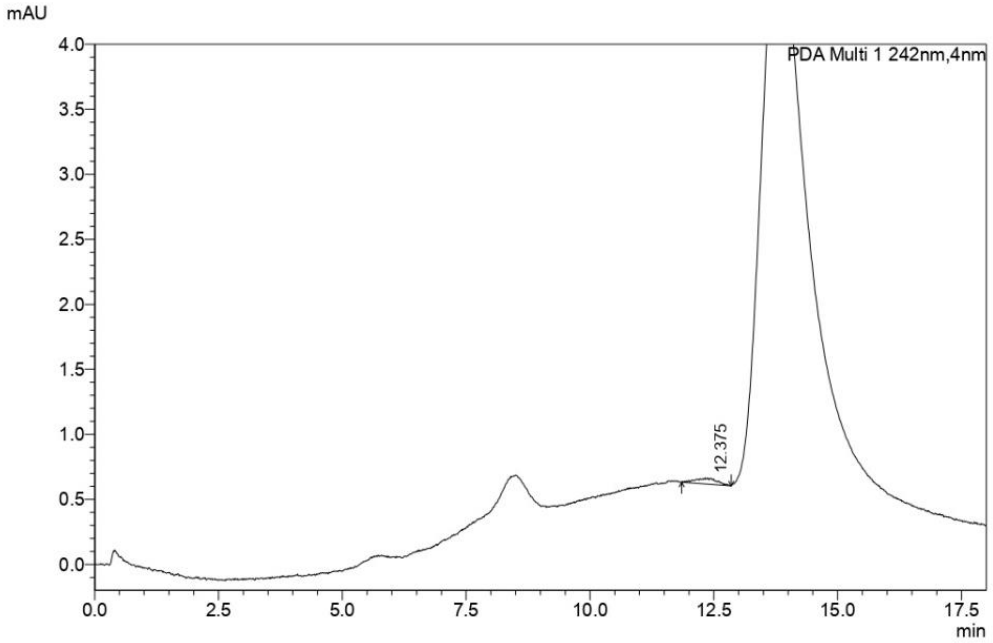
Serum Testosterone Level Analysis via HPLC

8.1 Standard Area Peak and All Specifications



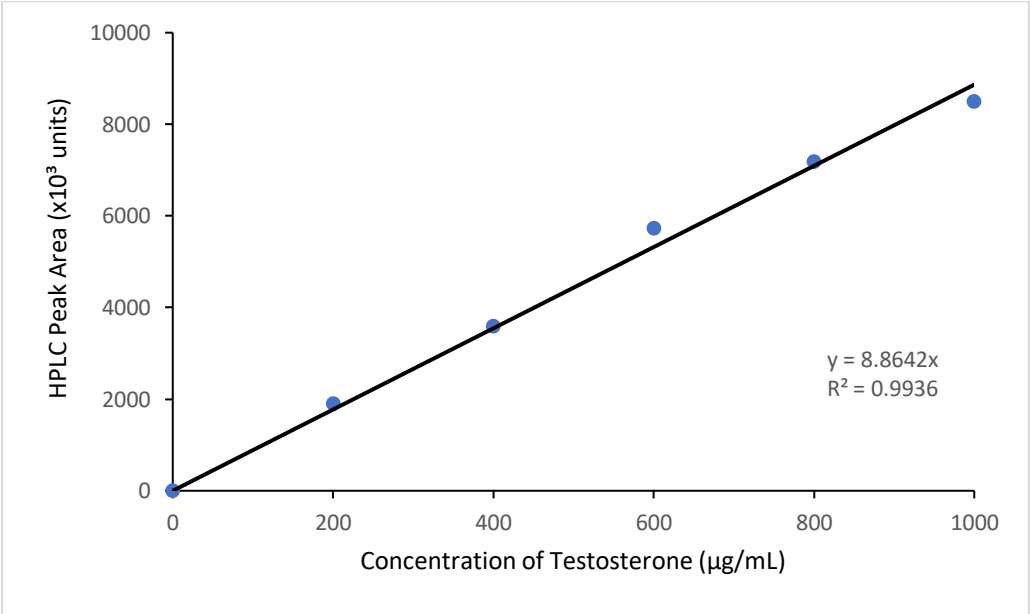
Peak Name	Ret. Time (min)	Area	Height	Theoretical Plates/meter	Tailing Factor
Testosterone	12.213	18108645	861355	58366	1.064

8.2 Sample Area Peak and All Specifications



Peak Name	Ret. Time (min)	Area	Height	Rel. Ret. Time
Testosterone	12.375	516	48	0.987

8.3 Standard Calibration Graph of Concentration Of Testosterone Standard Against HPLC Peak Area



8.4 Sample Peak Area and Testosterone Level in All Study Groups

Mice study group	Peak Area			Testosterone (ng/mL)		
	1	2	3	1	2	3
Control	210	225	169	23.69	25.38	19.07
FNT	75	22	22	8.46	2.48	2.48
KPE	448	379	344	50.54	42.79	38.81
KPE + FNT	253	267	233	28.54	30.12	26.29

APPENDIX 9

Statistical Analysis

9.1 Statistical Analysis for *In vitro* Studies

9.1.1 ANOVA analysis of Extraction Yield in All Extracts

The ANOVA Procedure

Dependent Variable: yield

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	241.0571556	120.5285778	35.77	0.0005
Error	6	20.2148667	3.3691444		
Corrected Total	8	261.2720222			

R-Square	Coeff Var	Root MSE	yield Mean
0.922629	27.09037	1.835523	6.775556

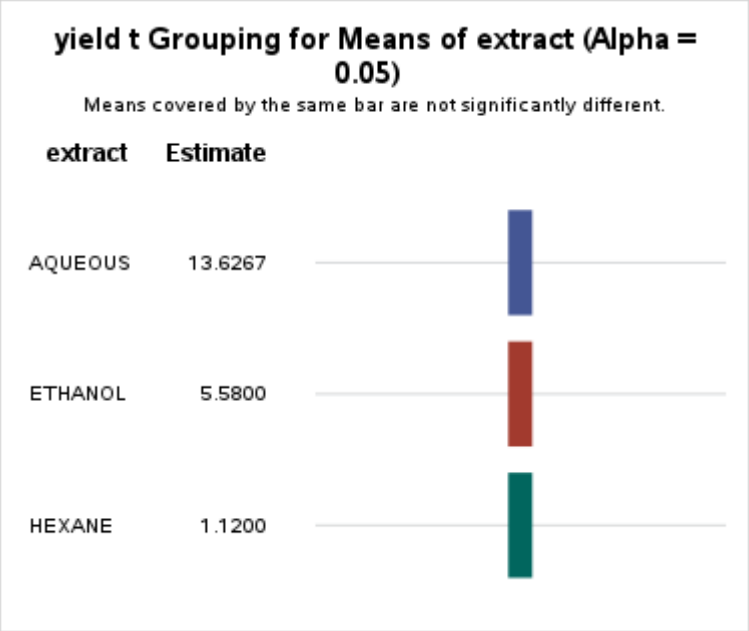
Source	DF	Anova SS	Mean Square	F Value	Pr > F
extract	2	241.0571556	120.5285778	35.77	0.0005

Levene's Test for Homogeneity of yield Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
extract	2	29.0716	14.5358	2.34	0.1771
Error	6	37.2381	6.2063		

t Tests (LSD) for yield

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	3.369144
Critical Value of t	2.44691
Least Significant Difference	3.6672



9.1.2 ANOVA analysis of EC_{50} DPPH Value in All Extracts

The ANOVA Procedure

Dependent Variable: EC50_DPPH

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	91488832.15	45744416.08	16640.4	<.0001
Error	6	16494.02	2749.00		
Corrected Total	8	91505326.18			

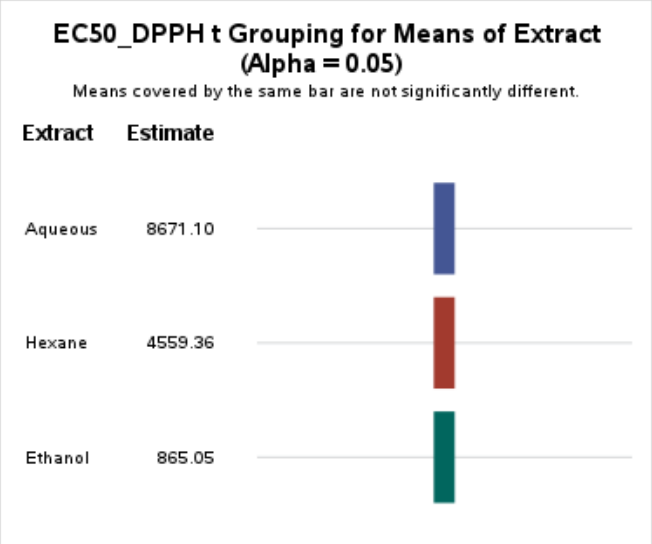
R-Square	Coeff Var	Root MSE	EC50_DPPH Mean
0.999820	1.115907	52.43094	4698.503

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Extract	2	91488832.15	45744416.08	16640.4	<.0001

Levene's Test for Homogeneity of EC50_DPPH Variance ANOVA of Squared Deviations from Group Means					
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Extract	2	47733288	23866644	3.67	0.0908
Error	6	38980687	6496781		

t Tests (LSD) for EC50_DPPH

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	2749.004
Critical Value of t	2.44691
Least Significant Difference	104.75



9.1.3 ANOVA analysis of Total Phenolic Content in All Extracts

The ANOVA Procedure

Dependent Variable: TPC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	88132216.54	44066108.27	15946.8	<.0001
Error	6	16579.90	2763.32		
Corrected Total	8	88148796.44			

R-Square	Coeff Var	Root MSE	TPC Mean
0.999812	1.107196	52.56726	4747.781

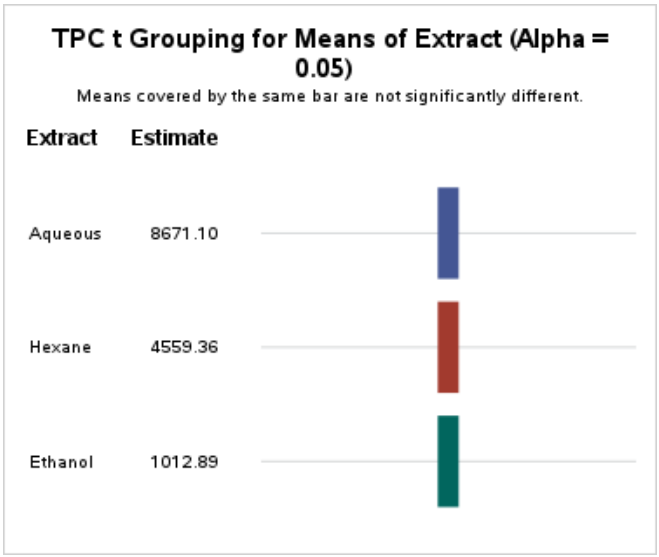
Source	DF	Anova SS	Mean Square	F Value	Pr > F
Extract	2	88132216.54	44066108.27	15946.8	<.0001

Levene's Test for Homogeneity of TPC Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Extract	2	47425068	23712534	3.65	0.0918
Error	6	38984368	6497395		

t Tests (LSD) for TPC

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	2763.316
Critical Value of t	2.44691
Least Significant Difference	105.02



9.1.4 ANOVA analysis of Total Flavonoid Content in All Extracts

The ANOVA Procedure

Dependent Variable: TFC

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	4244.791667	2122.395833	431.51	<.0001
Error	6	29.511133	4.918522		
Corrected Total	8	4274.302800			

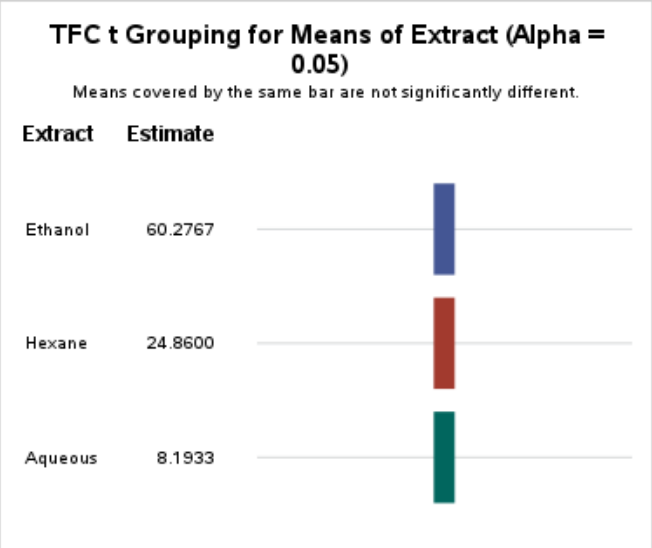
R-Square	Coeff Var	Root MSE	TFC Mean
0.993096	7.128814	2.217774	31.11000

Source	DF	Anova SS	Mean Square	F Value	Pr > F
Extract	2	4244.791667	2122.395833	431.51	<.0001

Levene's Test for Homogeneity of TFC Variance ANOVA of Squared Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Extract	2	32.2771	16.1385	1.50	0.2961
Error	6	64.5223	10.7537		

t Tests (LSD) for TFC

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	4.918522
Critical Value of t	2.44691
Least Significant Difference	4.4309



9.2 ANOVA analysis of Percentage of Weight Gain in All Treatment Group

The ANOVA Procedure

Dependent Variable: weight_gain

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	217.681771	72.560590	0.70	0.5613
Error	20	2064.416799	103.220840		
Corrected Total	23	2282.098571			

R-Square	Coeff Var	Root MSE	weight_gain Mean
0.095387	123.7762	10.15977	8.208175

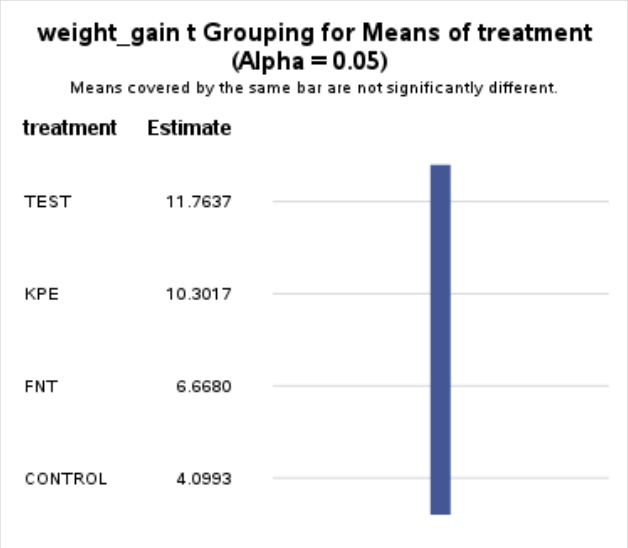
Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	3	217.6817715	72.5605905	0.70	0.5613

Levene's Test for Homogeneity of weight_gain Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment	3	35730.0	11910.0	1.10	0.3704
Error	20	215585	10779.2		

t Tests (LSD) for weight_gain

Alpha	0.05
Error Degrees of Freedom	20
Error Mean Square	103.2208
Critical Value of t	2.08596
Least Significant Difference	12.236



9.3 Statistical Analysis for Relative Testis Weight, Serum MDA Level, Serum Testosterone Level and Johnson's Mean Testicular Score Count

9.3.1 Normality test of Relative Testis Weight

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.370	3	.099	.786	3	.081
	FNT	.255	3	.200*	.963	3	.630
	KPE	.219	3	.200*	.987	3	.780
	KPE + FNT	.376	3	.092*	.772	3	.049**

*. This is a lower bound of the true significance. ** $p < 0.05$, data not normally distributed.

a. Lilliefors Significance Correction

9.3.2 Kruskal-Wallis analysis of Relative Testis Weight After Treatment

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable testis_index Classified by Variable treatment

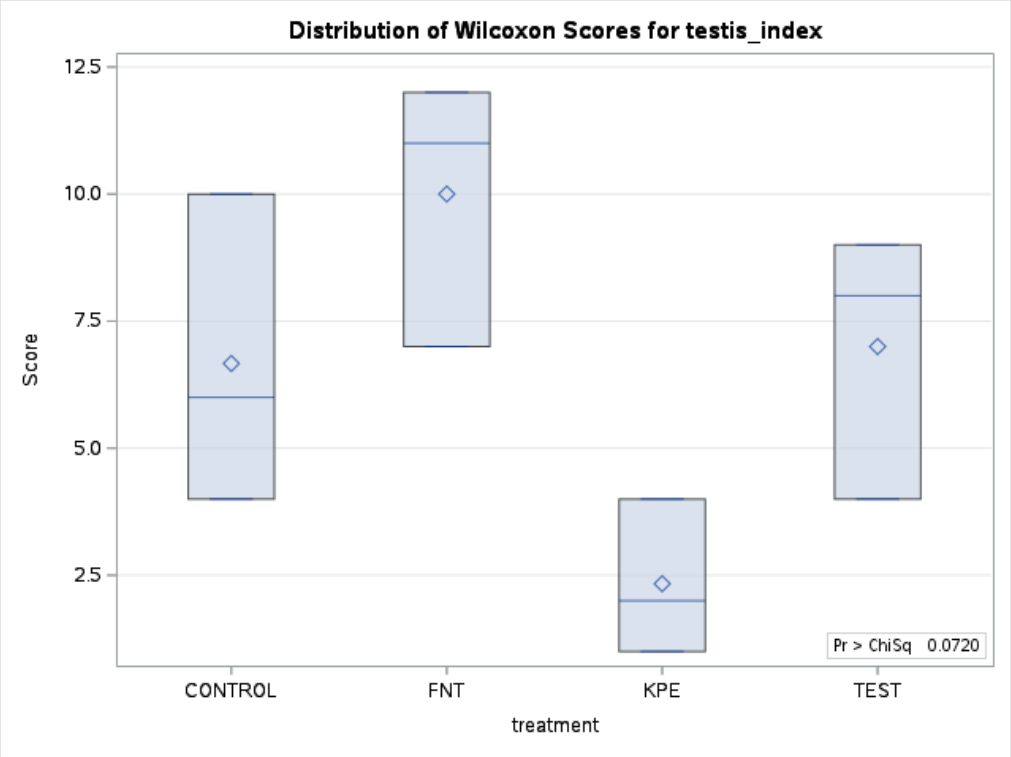
treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
CONTROL	3	20.0	19.50	5.370373	6.666667

Wilcoxon Scores (Rank Sums) for Variable testis_index Classified by Variable treatment					
treatment	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
FNT	3	30.0	19.50	5.370373	10.000000
KPE	3	7.0	19.50	5.370373	2.333333
TEST	3	21.0	19.50	5.370373	7.000000
Average scores were used for ties.					

Kruskal-Wallis Test		
Chi-Square	DF	Pr > ChiSq
6.9953	3	0.0720

The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: testis_index			
treatment	Wilcoxon Z	DSCF Value	Pr > DSCF
CONTROL vs. FNT	-1.5275	2.1602	0.4209
CONTROL vs. KPE	1.7712	2.5049	0.2873
CONTROL vs. TEST	0.0000	0.0000	1.0000
FNT vs. KPE	1.9640	2.7775	0.2017
FNT vs. TEST	1.0911	1.5430	0.6950
KPE vs. TEST	-1.7712	2.5049	0.2873



The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: testis_index			
treatment	Wilcoxon Z	DSCF Value	Pr > DSCF
CONTROL vs. FNT	-1.5275	2.1602	0.4209
CONTROL vs. KPE	1.7712	2.5049	0.2873
CONTROL vs. TEST	0.0000	0.0000	1.0000
FNT vs. KPE	1.9640	2.7775	0.2017
FNT vs. TEST	1.0911	1.5430	0.6950
KPE vs. TEST	-1.7712	2.5049	0.2873

9.3.3 Normality test of Serum MDA Level

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.259	3	.082	.805	3	.024**
	FNT	.203	3	.200*	.898	3	.240
	KPE	.202	3	.200*	.952	3	.711
	KPE + FNT	.157	3	.200*	.980	3	.962

*. This is a lower bound of the true significance, ** $p < 0.05$, data not normally distributed.

a. Lilliefors Significance Correction

9.3.4 Kruskal-Wallis analysis of Serum MDA Level After Treatment

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable MDA Classified by Variable group

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
CONTROL	3	9.0	19.50	5.408327	3.000000
FNT	3	32.0	19.50	5.408327	10.666667
KPE	3	12.0	19.50	5.408327	4.000000
TEST	3	25.0	19.50	5.408327	8.333333

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	DF	Pr > ChiSq
9.0513	3	0.0286

The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: MDA			
treatment	Wilcoxon Z	DSCF Value	Pr > DSCF
CONTROL vs. FNT	-3.4952	4.9429	0.0027
CONTROL vs. KPE	-1.6361	2.3138	0.3582
CONTROL vs. TEST	-2.8315	4.0044	0.0240
FNT vs. KPE	3.5781	5.0602	0.0020
FNT vs. TEST	2.5179	3.5609	0.0572
KPE vs. TEST	-3.5762	5.0576	0.0020

9.3.5 Normality test of Serum Testosterone Level

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.185	3	.200*	.978	3	.956
	FNT	.320	3	.010	.839	3	.057
	KPE	.188	3	.200*	.942	3	.608
	KPE + FNT	.137	3	.200*	.963	3	.831

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

9.3.6 ANOVA analysis of Serum Testosterone Level After Treatment

The ANOVA Procedure

Dependent Variable: testosterone

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	7194.076035	2398.025345	62.02	<.0001
Error	8	1237.371719	38.667866		
Corrected Total	11	8431.447754			

R-Square	Coeff Var	Root MSE	testosterone Mean
0.853243	24.98539	6.218349	24.88794

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	3	0.02176813	0.00725604	0.70	0.5614

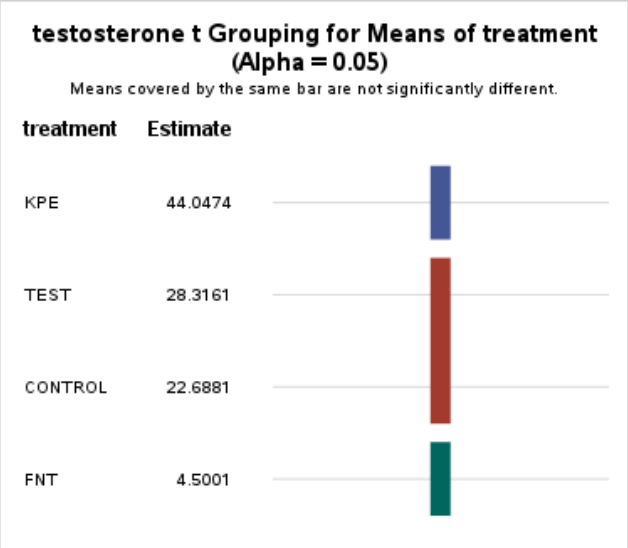
Levene's Test for Homogeneity of testosterone Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment	3	19470.1	6490.0	1.56	0.2171

Levene's Test for Homogeneity of testosterone Variance ANOVA of Squared Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Error	8	132748	4148.4		

t Tests (LSD) for testosterone

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	38.66787
Critical Value of t	2.03693
Least Significant Difference	5.971



9.4 Statistical Analysis for Sperm Parameters

9.4.1 Normality test of Sperm Concentration

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.237	3	.154	.840	3	.058
	FNT	.152	3	.200*	.928	3	.461
	KPE	.182	3	.200*	.911	3	.320
	KPE + FNT	.267	3	.064	.872	3	.131

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

9.4.2 ANOVA analysis of Sperm Concentration After Treatment

The ANOVA Procedure

Dependent Variable: conc

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	148.7467583	50.1424333	47.90	<.0001
Error	8	8.2803333	1.0350417		
Corrected Total	11	157.0270917			

R-Square	Coeff Var	Root MSE	conc Mean
0.947268	2.956683	1.017370	34.40917

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	3	148.7467583	49.5822528	47.90	<.0001

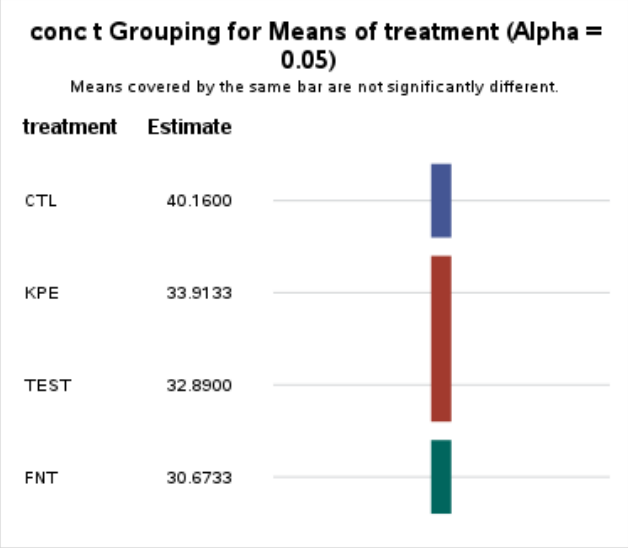
Levene's Test for Homogeneity of conc Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment	3	1.9172	0.6391	1.34	0.3281

Levene's Test for Homogeneity of conc Variance ANOVA of Squared Deviations from Group Means					
Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Error	8	3.8154	0.4769		

t Tests (LSD) for conc

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	1.035042
Critical Value of t	2.30600
Least Significant Difference	1.9155



9.4.3 Normality test of Sperm's Progressive Motility (grade A + grade B)

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.175	3	.200*	1.000	3	1.000
	FNT	.204	3	.200*	.993	3	.843
	KPE	.337	3	.200*	.855	3	.253
	KPE + FNT	.337	3	.200*	.855	3	.253

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

9.4.4 ANOVA analysis of Sperm Progressive Motility After Treatment

The ANOVA Procedure

Dependent Variable: progressive motility

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	3667.583333	1222.527778	34.04	<.0001
Error	8	287.333333	35.916667		
Corrected Total	11	3954.916667			

R-Square	Coeff Var	Root MSE	prog Mean
0.927348	9.679222	5.993052	61.91667

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	3	3667.583333	1222.527778	34.04	<.0001

Levene's Test for Homogeneity of progressive motility Variance ANOVA of Squared Deviations from Group Means

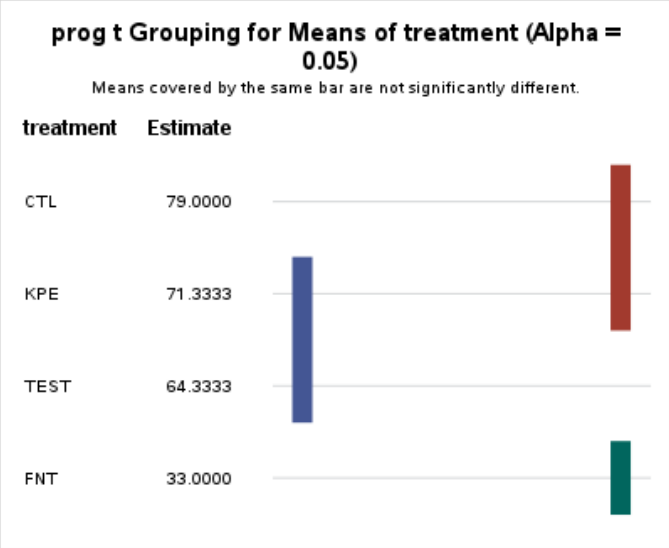
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment	3	10117.1	3372.4	3.17	0.0850
Error	8	8498.6	1062.3		

t Tests (LSD) for progressive motiliy

Note: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	35.91667

Critical Value of t	2.30600
Least Significant Difference	11.284



9.4.5 Normality test of Sperm's Viability

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.236	3	.200*	.932	3	.510
	FNT	.135	3	.200*	.962	3	.821
	KPE	.147	3	.200*	.962	3	.822
	KPE + FNT	.183	3	.200*	.931	3	.495

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

9.4.6 ANOVA analysis of Sperm Viability After Treatment

The ANOVA Procedure

Dependent Variable: viability

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	1127.001825	375.667275	9.88	0.0046

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Error	8	304.090667	38.011333		
Corrected Total	11	1431.092492			

R-Square	Coeff Var	Root MSE	viability Mean
0.787512	8.358735	6.165333	73.75917

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	3	1127.001825	375.667275	9.88	0.0046

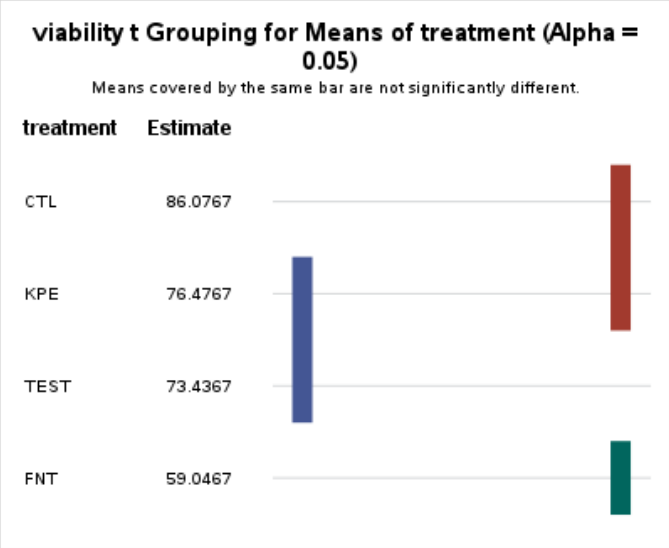
Levene's Test for Homogeneity of motility Variance
ANOVA of Squared Deviations from Group Means

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
treatment	3	9929.5	3309.8	3.00	0.0949
Error	8	8817.7	1102.2		

t Tests (LSD) for viability

Note: This test controls the Type I comparisonwise error rate, not the experiment wise error rate.

Alpha	0.05
Error Degrees of Freedom	8
Error Mean Square	38.01133
Critical Value of t	2.30600
Least Significant Difference	11.608



9.5 Statistical Analysis for Post-Parturition Studies

9.5.1 Kruskal-Wallis analysis of mating success rate in all treatment groups

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable mating Classified by Variable group

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
CONTROL	9	171.0	166.50	7.794229	19.0
FNT	9	153.0	166.50	7.794229	17.0
KPE	9	171.0	166.50	7.794229	19.0
TEST	9	171.0	166.50	7.794229	19.0

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	DF	Pr > ChiSq
3.0000	3	0.3916

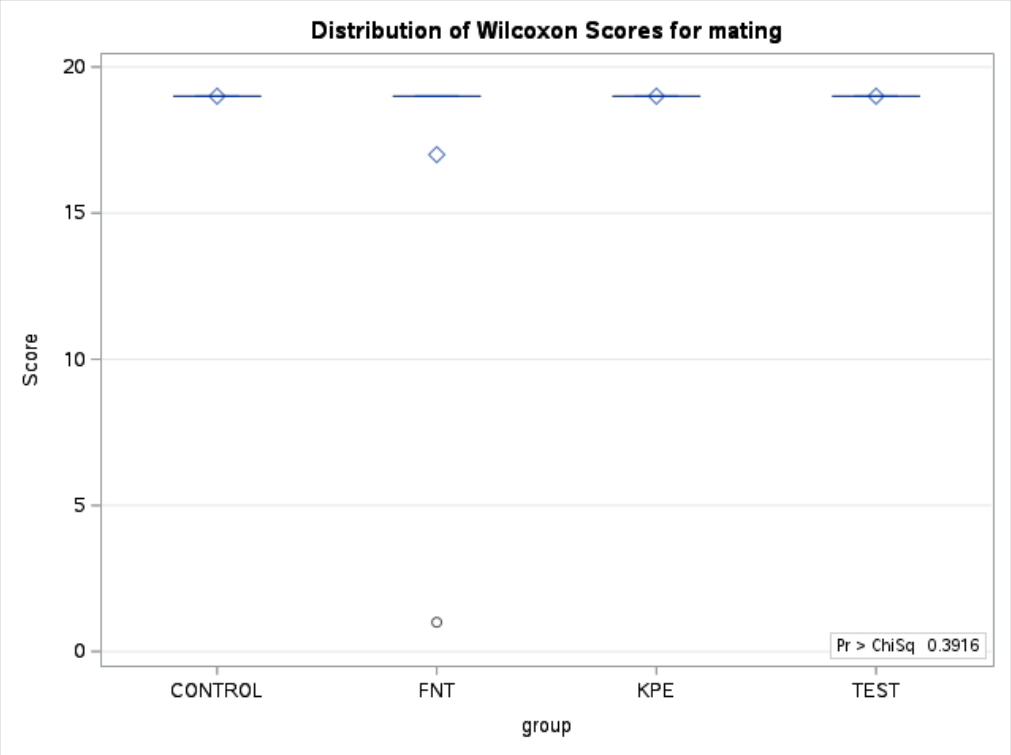
The NPAR1WAY Procedure

Pairwise Two-Sided Multiple Comparison Analysis

Dwass, Steel, Critchlow-Fligner Method

Variable: mating

group	Wilcoxon Z	DSCF Value	Pr > DSCF
CONTROL vs. FNT	1.0000	1.4142	0.7494
CONTROL vs. KPE	0.0000	0.0000	1.0000
CONTROL vs. TEST	0.0000	0.0000	1.0000
FNT vs. KPE	-1.0000	1.4142	0.7494
FNT vs. TEST	-1.0000	1.4142	0.7494
KPE vs. TEST	0.0000	0.0000	1.0000



9.5.2 Normality test of Mating Period

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.356	9	<.001	.655	9	.0004**
	FNT	.342	8	<.001	.801	8	.030**
	KPE	.414	9	<.001	.617	9	.0002**
	KPE + FNT	.223	9	.200*	.838	9	.055

*. This is a lower bound of the true significance, ** $p < 0.05$, data not normally distributed.

a. Lilliefors Significance Correction

9.5.3 Kruskal-Wallis analysis of Mating Period After Treatment

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable mating_period Classified by Variable GROUP

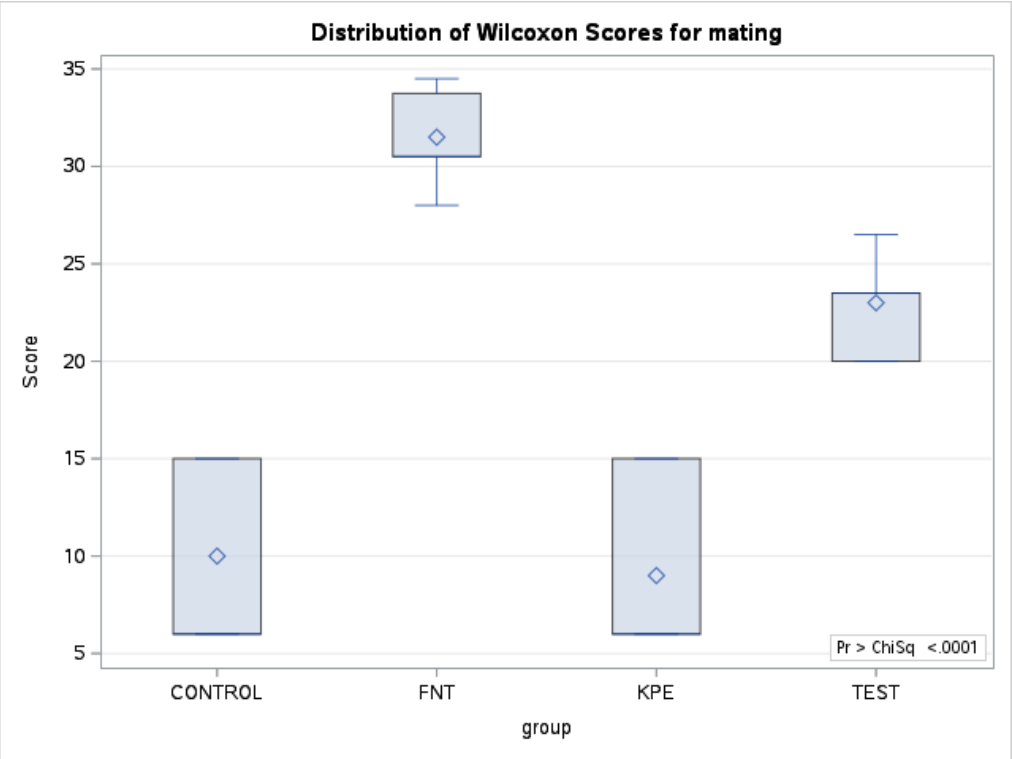
GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
CONTROL	9	90.0	162.0	25.928894	10.00
FNT	8	252.0	144.0	24.911676	31.50
KPE	9	81.0	162.0	25.928894	9.00
TEST	9	207.0	162.0	25.928894	23.00

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	DF	Pr > ChiSq
29.7140	3	<.0001

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: mating_period			
GROUP	**Wilcoxon Z**	**DSCF Value**	**Pr > DSCF**
CONTROL vs. FNT	-3.5546	5.0269	0.0021
CONTROL vs. KPE	0.4699	0.6645	0.9657
CONTROL vs. TEST	-3.6623	5.1793	0.0014
FNT vs. KPE	3.5754	5.0563	0.0020
FNT vs. TEST	3.5206	4.9789	0.0024
KPE vs. TEST	-3.6803	5.2047	0.0013



9.5.4 Normality Test of Survival Rate of Pups

Tests of Normality

		Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Group	Statistic	df	Sig.	Statistic	df	Sig.
Day 0	Control	.284	9	.035	.819	9	.034**
	FNT	.256	8	.233	.904	8	.316
	KPE	.308	9	.015	.722	9	.003**
	KPE + FNT	.358	9	.100*	.705	9	.002**

*. This is a lower bound of the true significance, ** $p < 0.05$, data not normally distributed.

a. Lilliefors Significance Correction

9.5.5 Kruskal-Wallis analysis of Survival Rate of Pups After Treatment

The NPAR1WAY Procedure

Wilcoxon Scores (Rank Sums) for Variable SURVIVAL Classified by Variable GROUP

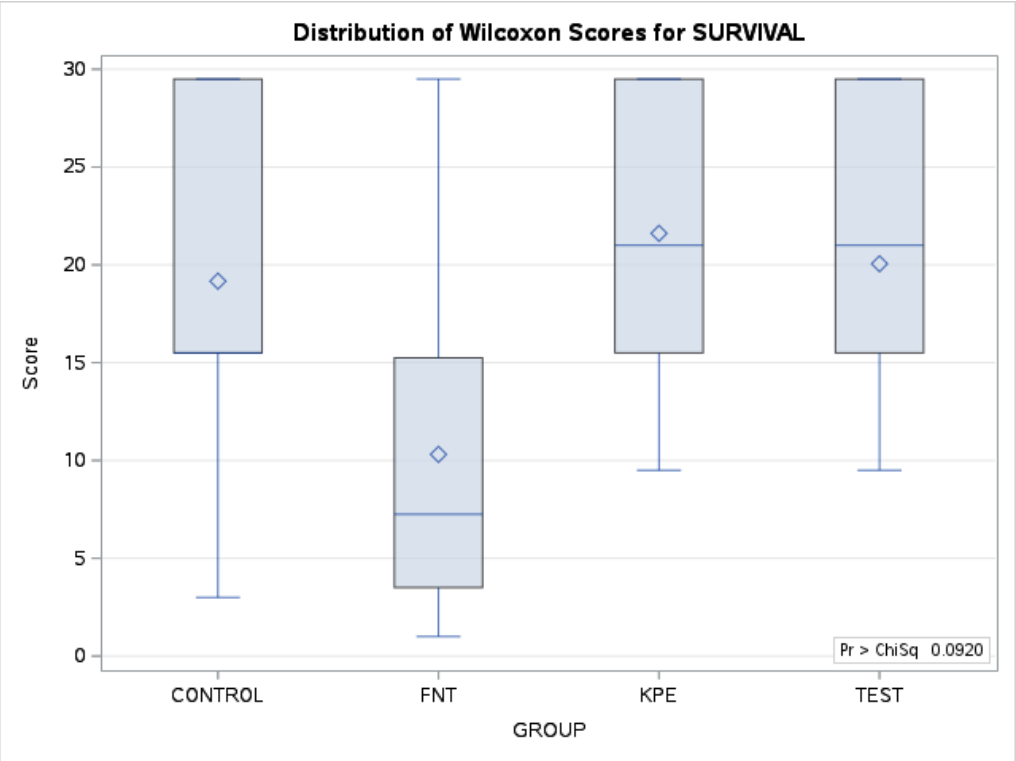
GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
CONTROL	9	172.50	162.0	25.780587	19.166667
FNT	8	82.50	144.0	24.769187	10.312500
KPE	9	194.50	162.0	25.780587	21.611111
TEST	9	180.50	162.0	25.780587	20.055556

Average scores were used for ties.

Kruskal-Wallis Test

Chi-Square	DF	Pr > ChiSq
6.4421	9	0.0920

Pairwise Two-Sided Multiple Comparison Analysis			
Dwass, Steel, Critchlow-Fligner Method			
Variable: SURVIVAL			
GROUP	**Wilcoxon Z**	**DSCF Value**	**Pr > DSCF**
CONTROL vs. FNT	1.6657	2.3556	0.3420
CONTROL vs. KPE	-0.4652	0.6578	0.9666
CONTROL vs. TEST	-0.1381	0.1953	0.9991
FNT vs. KPE	-2.2622	3.1993	0.1070
FNT vs. TEST	-2.1026	2.9735	0.1522
KPE vs. TEST	0.4149	0.5867	0.9759



APPENDIX 10

List of Publication

1. Ong, S.S.T.X, Kwong, P.J., Chan, M.Y. and Tan, G.C., 2025. Effect of Co-administration Treatment of *Kaempferia parviflora* Extract and Fenitrothion on Male Reproductive Parameters in Murine Model. *Asian Pacific Journal of Reproduction*. 14(2), pp.84–94.

APPENDIX 11

Confirmation of Plant Species Letter

INSTITUT BIOSAINS
INSTITUTE OF BIOSCIENCE
اينستيتوت بيوساءين س

Reference : UPM/IBS/UB/H22/25
Date : 28th April 2025

Shawn Samson Ong Tze Xian
Faculty Of Science
Universiti Tunku Abdul Rahman,
Kampar Campus, Bandar Barat
31900, Kampar, Perak

Mr.,

CONFIRMATION OF PLANT SPECIES

With all respect the above matter is referred,

Please be informed that the plant specimens submitted for identification by:

Student : **Shawn Samson Ong Tze Xian**

Supervisor : **Dr. Tan Gim Cheong, Dr. Kwong Phek Jin, Chan Mun Yee**

Voucher No.	Family Name	Scientific Name	Local Name
KM 0212/25	Zingiberaceae	*Kaempferia parviflora* Wall. ex Baker	Kunyit hitam

Herbarium : **Institute of Bioscience**
Person In-Charge : **Mohd Hafizi Adzmi Hanafi**
Determined : **Dr. Khairil Mahmud**

It should be noted that we are not involved with anything as a result of research conducted by the host.

Thank you.

"With Knowledge We Serve"

I who carry out the trust,

.....
DR. KHAIRIL MAHMUD
Biodiversity Unit
Institute Of Bioscience
Universiti Putra Malaysia

Institut Biosains, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
603-97692111 603-97692101 http://www.ibs.upm.edu.my

APPENDIX 12

Ethical Approval Letter

UNIVERSITI TUNKU ABDUL RAHMAN DU012(A)
Wholly owned by UTAR Education Foundation Co. No. 578227-M

Re: U/SERC/254/2022

29 November 2022

Dr Tan Gim Cheong
Department of Allied Health Sciences
Faculty of Science
Universiti Tunku Abdul Rahman
Jalan Universiti, Bandar Barat
31900 Kampar, Perak

Dear Dr Tan,

Ethical Approval For Research Project/Protocol

We refer to your application which was tabled for discussion at the UTAR Scientific and Ethical Review Committee (SERC) meeting held on 29 November 2022. We are pleased to inform you that your application for ethical approval for your research project involving the use of animals has been approved by SERC.

The details of the project are as follows:

Research Title	Potential of *Kaempferia Parviflora* Extract in Ameliorating Male Infertility Induced by Fenitrothion in Murine Model
Investigator(s)	Dr Tan Gim Cheong Dr Kwong Phek Jin Ms Chan Mun Yee Ms Lim Pooi Gaik (Sophea Fertility Centre) Shawn Samson Ong Tze Xian Lim Wei Shan Gan Jing Xian Go Juin Yin Benjamin Khoo Sheng Yuan
Research Area	Science
Research Location	UTAR, Kampar Campus
Animal Type/Number	80 Mus musculus (laboratory mouse/ICR strain)
Approval Validity	29 November 2022 - 28 November 2023

We note the following:

(1) The mice supply will be obtained from Animal House, UKM for breeding to support the experimental needs.

(2) The laboratory officer in Department of Agriculture and Food Science and Department of Allied Health Sciences will be responsible for the overall monitoring of the animals, while the postgraduate and undergraduate students involved will be responsible for the husbandry of animals, daily.

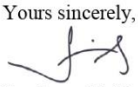
Kampar Campus : Jalan Universiti, Bandar Barat, 31900 Kampar, Perak Darul Ridzuan, Malaysia
Tel: (605) 468 8888 Fax: (605) 466 1313
Sungai Long Campus : Jalan Sungai Long, Bandar Sungai Long, Cheras, 43000 Kajang, Selangor Darul Ehsan, Malaysia
Tel: (603) 9086 0288 Fax: (603) 9019 8868
Website: www.utar.edu.my

Please note that the conduct of this project must be in compliance with procedures set out in related policies of UTAR such as the UTAR Research Ethics and Code of Conduct, Code of Practice for the Care and Use of Animal for Scientific Purposes and other related policies/guidelines.

The University wishes you all the best in your research.

Thank you.

Yours sincerely,



Professor Ts Dr Faidz bin Abd Rahman
Chairman
UTAR Scientific and Ethical Review Committee

c.c Dean, Faculty of Science
Director, Institute of Postgraduate Studies and Research

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