UNIVERSITI PUTRA MALAYSIA

HEALTH RISK ASSESSMENT OF ALKENYLBENZENES IN PLANT FOOD SUPPLEMENTS USING MARGIN OF EXPOSURE APPROACH

AKMAL ARIF BIN MAT RAMLE

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BY

AKMAL ARIF BIN MAT RAMLE

Thesis submitted in fulfilment of the requirement for the degree of Bachelor of Science in Environmental and Occupational Health with Honours from the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia

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ABSTRACT

HEALTH RISK ASSESSMENT OF ALKENYLBENZENES IN PLANT FOOD SUPPLEMENTS USING MARGIN OF EXPOSURE APPROACH

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Introduction: For a long time, plants have been used as medicines to cure a variety of illnesses since it provides a wide range of functions, including beverage uses, as well as food and dietary supplementation. Plant food supplements (PFS) are dietary supplements made from plant extracts and botanical extracts. PFS are frequently sold in capsules or tablet form and are designed to supplement a person's regular diet. PFS derived from botanicals and botanical preparations are becoming increasingly popular around the world because consumers believe they are safe. However, regardless of their supposed health benefits, these supplements may contain potentially harmful ingredients such as alkenylbenzenes, which is estragole. Alkenylbenzenes are a major class of carcinogenic and genotoxic substances found in PFS. **Objectives:** The objective of this study is to evaluate the health risk of alkenylbenzenes in PFS using margin of exposure approach (MOE). **Methodology:** A total of 30 PFS samples have been collected and purchased via Malaysian market and online merchants. Each sample was extracted with methanol before being analysed using Ultra Performance Liquid Chromatography. To estimate consumer exposure to estragole in PFS, the estimated daily intake (EDI) was calculated using quantified estragole concentrations in PFS samples. MOE was used to determine the risk of estragole in the PFS sample. **Results and Discussion:** Estragole, an alkenylbenzene of interest, was found in 9 of 30 PFS samples. Estragole concentrations in PFS ranged from 55.03 to 418.02 μg/g. The EDI values ranged from 0.99-9.44 μg/kg bw per day. Based on the result obtained, all positive samples have MOE values less than 10 000. This emphasises the importance of risk management, especially for long-term consumption. MOE value of less than 10,000 is a high priority for risk management, whereas the MOE of more than 10,000 is a low priority. **Conclusion:** In conclusion, the presence of alkenylbenzenes in PFS still raises concern if it is consumed daily over a longer period.

Keywords: plant food supplements, health risk assessment, alkenylbenzenes, margin of exposure, estragole

ABSTRAK

PENILAIAN RISIKO KESIHATAN ALKENILBENZENA DALAM MAKANAN TAMBAHAN DENGAN MENGGUNAKAN PENDEKATAN PENDEDAHAN MARGIN

AKMAL ARIF BIN MAT RAMLE

Pengenalan: Untuk sekian lama, tumbuhan telah digunakan sebagai ubat untuk menyembuhkan pelbagai penyakit kerana ia menyediakan pelbagai fungsi, termasuk kegunaan minuman, serta makanan dan makanan tambahan. Makanan tambahan tumbuhan (PFS) adalah makanan tambahan yang diperbuat daripada ekstrak tumbuhan dan ekstrak botani. PFS sering dijual dalam bentuk kapsul atau tablet dan direka bentuk untuk menambah diet biasa seseorang. Penggunaan bahan botani dan persediaan botani yang semakin meningkat sebagai PFS di seluruh dunia adalah berdasarkan andaian bahawa produk semulajadi secara amnya selamat. Walau bagaimanapun, tanpa mengira manfaat kesihatan yang sepatutnya, suplemen ini mungkin mengandungi bahan-bahan yang berpotensi berbahaya seperti alkenilbenzena, iaitu estragole. Alkenilbenzena adalah kelas utama bahan karsinogenik dan genotoksik yang terdapat dalam PFS. **Objektif:** Kajian ini bertujuan untuk menilai risiko kesihatan alkenilbenzena dalam PFS menggunakan pendekatan margin pendedahan (MOE). **Metodologi:** Sebanyak 30 sampel PFS telah dikumpul dan dibeli melalui pasaran Malaysia dan peniaga dalam talian. Setiap sampel PFS akan diekstrak dengan metanol sebelum dianalisis menggunakan UPLC. Untuk menganggarkan pendedahan pengguna kepada estragole dalam PFS, anggaran pengambilan harian (EDI) dikira menggunakan kepekatan estragole terkuantiti dalam PFS sampel. Margin pendedahan, kaedah penilaian risiko, digunakan untuk menentukan risiko estragole dalam sampel PFS. **Keputusan dan Perbincangan:** 9 daripada 30 sampel PFS mengandungi alkenilbenzena yang dicari iaitu estragole. Nilai estragole dalam PFS adalah antara 55.03 hingga 418.02 μg/g. Nilai EDI adalah antara 0.99-9.44 μg/kg bw sehari. Berdasarkan keputusan yang diperolehi, kesemua sampel positif mempunyai nilai MOE kurang daripada 10 000. Ini menunjukkan bahawa terdapat keutamaan untuk pengurusan risiko terutamanya untuk penggunaan jangka panjang. Nilai MOE yang kurang daripada 10,000 adalah keutamaan yang tinggi untuk pengurusan risiko, manakala MOE yang melebihi 10,000 adalah keutamaan yang rendah. **Kesimpulan:** Kesimpulannya, kehadiran estragole dalam PFS masih menimbulkan kebimbangan jika ia digunakan setiap hari dalam jangka masa yang lebih lama.

Kata kunci: makanan tambahan tumbuhan, penilaian risiko kesihatan, alkenilbenzena, margin pendedahan, estragole

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LIST OF ABBREVATIONS

BMD	Benchmark Dose
$BMDL_{10}$	Benchmark Dose lower level of 10
BMR	Benchmark Response
CAGR	Compound Annual Growth Rate
DNA	Deoxyribonucleic acid
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
HRA	Health risk assessment
IARC	International Agency for Research on Cancer
ILSI	International Life Sciences Institute
IPCS	Inter-Organization Programme for the Sound Management of Chemicals
MOE	Margin of exposure
NTP	National Toxicology Program
PFS	Plant food supplements
REP	Relative potency factor
TD	Threshold dose
TEQ	Toxic equivalence
UDC	Uveodermatologic syndrome
UPLC	Ultra-performance liquid chromatography
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

The purpose of this introduction chapter is to provide the background of the study, the issues raised on the themes, and why this study is required. The objectives of the study and the predicted outcome were also outlined in the introduction.

1.1 Study Background

For a long time, plants have been used as medicines to cure a variety of illnesses (Veeresham, 2012). Plants provide a wide range of functions, including beverage uses, as well as food and dietary supplementation. Despite the fact that a large number of people in developed countries use nutritional supplements and that the number is growing, numerous studies have been unable or unwilling to establish their benefits, active ingredients, or adverse effects (Prinsloo et al., 2019). Herbal products, particularly plant food supplements (PFS), are becoming increasingly popular globally, especially among certain demographics such as children, women, and cancer patients. PFS are in high demand these days due to their ability to improve health and reduce disease risk (Van Den Berg et al., 2011). PFS are dietary supplements made from plant extracts and botanical extracts. PFS are frequently sold in capsules or tablet form and are designed to supplement a person's regular diet (Alajlouni et al., 2017).

PFS are widely available in Malaysia due to their low cost. It can be purchased without a prescription at supermarkets, health food stores, pharmacies, and even online

markets. Customers often accept PFS because they believe that "natural" implies "safe." However, this argument should be used with caution as it has been discovered that some plants are dangerous, even carcinogenic, and genotoxic (Rietjens et al., 2008). Alkenylbenzenes are a major class of carcinogenic and genotoxic substances found in plant food supplements. They include estragole, safrole, eugenol, myristicin, elemicin, trans-anethole, and β-asarone (BA), methyleugenol and apiol (Van Den Berg et al., 2011). These compounds can be found in a variety of botanicals, including fennel, parsley, tarragon, anise star, nutmeg, basil, coriander, lemongrass, and ginger. High dosages of estragole, methyleugenol and safrole were found to be hepatocarcinogenic in rats, with identical modes of action and tumour formation (Al-Malahmeh et al., 2017). Due to their mutagenic or carcinogenic effects on rodents, the European Commission has banned foods containing pure compounds of methyleugenol, estragole and safrole since September 2008. Hence, the objective of this study is to evaluate the health risk of alkenylbenzenes found in plant food supplements using the margin of exposure approach. The findings from this study can be utilised to inform risk management, allowing regulatory efforts to mitigate possible dangers associated with PFS intake to be prioritised.

1.2 Problem Statement

According to Lim (2021), the Malaysian dietary supplement market has grown from RM2.07 billion (488 million USD) in 2014 to RM3.1 billion (730 million USD) in 2019. A total of 50% of Malaysians are used health supplements. Herbal and traditional treatments account for roughly one-third of Malaysia's health supplement market. One in every four Malaysians takes supplements on a regular basis, with the

remaining two in ten using them on alternate days or once a week (Vodus, 2021). According to a Rakuten Insight survey conducted in July 2022, more than half of Malaysians use dietary supplements to help strengthen and improve their immune systems (Statista, 2022). In Malaysia, married Malay women frequently utilise herbal treatments to treat both general and gender-specific health issues. Postpartum care concerns, menstrual abnormalities, and infertility are just a few instances. Furthermore, Malay women enjoy herbal medicine because they believe that products obtained naturally are safer to consume (Mohamad et al., 2019). On the other hand, unauthorised sales of unregistered health supplements are a major concern in Malaysia. In August 2021, the Health Ministry seized RM9.7 million in unlawful health and beauty products. They were all sold through drop-shippers and e-commerce companies (John, 2021). Also, it has been reported that many consumers have a high level of trust and, as a result, have become a victim to dishonest sellers that engage in heavy marketing based only on testimonials or even unproven scientific evidence. Despite the fact that these dietary supplements are consumed for purported health benefits, they may include potentially dangerous compounds such as alkenylbenzenes, which are genotoxic and carcinogenic (Prinsloo et al., 2019). In fact, a lot of research is required to determine whether the dietary supplements on the market are safe and effective. This is because improper use of these supplements has resulted in adverse effects to human health.

Alkenylbenzenes are a type of compound found in herbs such as fennel, nutmeg, basil, dill, and parsley. They include estragole, methyleugenol, safrole, and analogues (Ning et al., 2018). These compounds may pose a risk in plant food supplements. According to Ning et al (2018), alkenylbenzenes were found in 7 out of 10 PFS samples, and 4 out of 7 samples that tested positive contained more than one

alkenylbenzenes. In addition, animal studies have shown that these alkenylbenzenes generate deoxyribonucleic acid (DNA) adducts and liver tumours by producing reactive 10-sulfoxy metabolites (Rietjens et al. 2014). According to Herrmann et al. (2013), 29 percent of 30 human liver samples contained a high number of hepatic DNA adducts after exposure to methyleugenol. Since September 2008, the European Commission has banned foods containing pure compounds of methyleugenol, estragole and safrole because of their genotoxic and carcinogenic substances. However, aside from the general food law regulation, there is no specific regulation addressing the use of supplements containing alkenylbenzenes. The European Union standard for safrole in foods and beverages is 1 mg/kg. The European Union is unable to determine exposure limits for methyleugenol and estragole in food products. Clearly, further research is needed to determine exposure limits that will influence legal controls of these phytochemicals. Before natural products can be sold in Malaysia, they must be registered with the Drug Control Authority (DCA). Alkenylbenzenes may be found in any supplements approved by Malaysia's National Pharmaceutical Council, or NPRA since it is not listed as prohibited active ingredients. As a result, consumers may be exposed to genotoxic and carcinogenic alkenylbenzenes by consuming these registered PFS.

1.3 Study Justification

This study was able to determine the potential risks of alkenylbenzene-containing plant food supplements marketed in Malaysia. This is because there is currently no data on alkenylbenzenes exposure in Malaysia, even though a study found that long-term use of PFS with safrole, methyleugenol, and estragole may pose a risk

to human health. There has already been research on alkenylbenzene exposure in Indonesia. For example, Suparmi et al. (2018) studied the occurrence of alkenylbenzenes in 25 samples of Indonesian jamu. The study discovered that alkenylbenzenes were present in 23 of 25 jamu samples from Indonesia. This demonstrates the importance of this study by demonstrating that PFS consumption in Malaysia can be concerning, particularly when consumed daily for extended periods of time. If a study on alkenylbenzenes in PFS in Malaysia is conducted, we will be able to assess whether additional risk management action is required.

Furthermore, this study can help assess the risk of alkenylbenzenes by using a margin of exposure. MOE is the most widely used risk assessment method for determining the safety of genotoxic and carcinogenic substances. Once the safety evaluations are completed, risk management procedures can be implemented. It is believed that this research will assist us in determining when risk management measures are required, as well as which PFS do not require concerns, even if they contain low quantities of substances that may be carcinogenic and genotoxic.

Finally, this study raises awareness of the presence of alkenylbenzenes in plant food supplements. This research is required to change consumer perceptions, as many consumers believe that "natural" equals "safe" when consuming PFS. Also, it has been found that many people are easy to convince and fall for people who aggressively market products based only on testimonials or even unproven scientific evidence, which ends up being their own downfall (Ismail et al., 2020). Botanical ingredients and preparations, such as plant-based supplements and natural food ingredients, should be avoided because they may contain compounds that are harmful to human health, such as alkenylbenzenes. These alkenylbenzenes have been linked to DNA adducts and liver tumours in animal studies. Thus far, it is importance to analyze the natural

occurrence of alkenylbenzenes in plant food supplement in order to conduct a possible for a priority risk assessment.

1.4 Conceptual Framework

Figure 1.1 represents a conceptual framework for this research, which illustrates the risk assessment of plant food supplements when consumed over a lifetime, allowing this study to determine the presence of natural plant toxins such as alkenylbenzenes, which are genotoxic and carcinogenic because the compounds can cause DNA adducts and liver tumours.

Plant-derived food products are foods derived predominantly from plants. This includes herbs and spices as well as fruits and vegetables. Plants have been used for a lot of purposes, including PFS. PFS is commonly consumed to improve one's health, such as for strengthening health, constipation, menstruation and fever. Some people use PFS for a short period of time, while others have used it for their entire life. This is especially concerning because PFS may contain a potentially genotoxic and carcinogenic botanical constituent, and consumers are unaware of the substances used.

This highlights the significance of this study, which aims to analyse the possible risk of genotoxic carcinogens found in PFS, such as alkenylbenzenes. Alkenylbenzenes, which include compounds like estragole, are found in many plants, including fennel and parsley. These plants could have been used to make the supplements. Concerns have been raised regarding alkenylbenzenes because there is evidence linking alkenylbenzenes to genotoxic and carcinogenic effects, such as DNA adduct formation and the development of liver tumours.

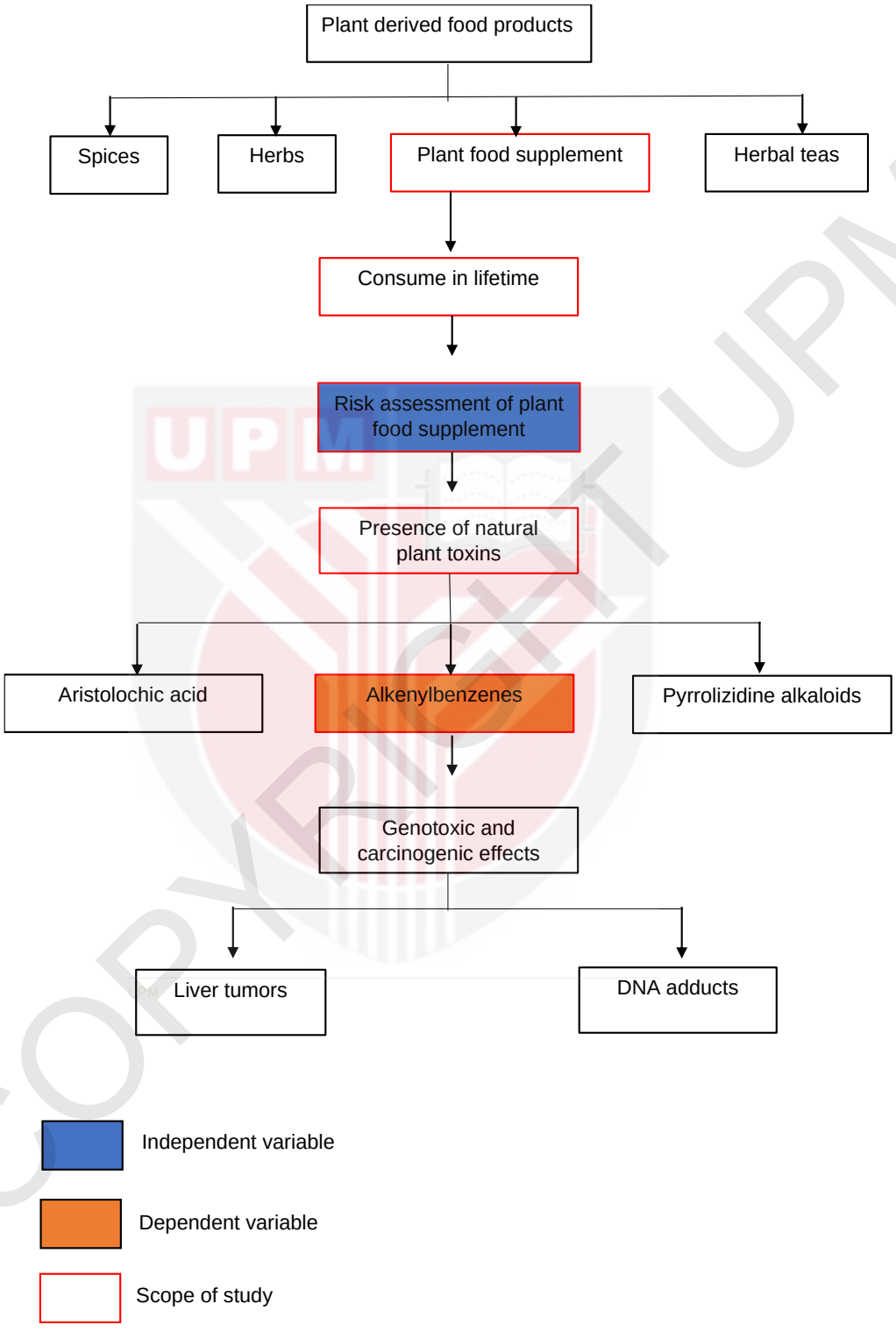

Figure 1.1: Conceptual framework

1.5 Definition of Terms

1.5.1 Conceptual Definition

i. Health Risk Assessment

A human health risk assessment evaluates the type and likelihood of chemicals in polluted environmental media causing harmful health effects in humans (USEPA, 2022).

ii. Alkenylbenzenes

Alkenylbenzenes are natural plant metabolites found in a wide range of herbs and spices, including basil and fennel seeds, in the plant families *Myristicaceae*, *Lauraceae*, *Labiatae*, *Acoraceae*, *Compositeae* and *Umbelliferae*, Alkenylbenzenes found in PFS include safrole, methyleugenol, and estragole (Ning et al., 2018; Eisenreich et al., 2021).

iii. Margin of Exposure Approach (MOE)

Risk assessors use the "margin of exposure" to consider the potential dangers of genotoxic and carcinogenic substances in food and ingredients. The MOE considers both the population as a whole and the amount of exposure to the substance under consideration when determining the severity of an adverse effect (ESFA, 2005).

1.5.2 Operational Definition

i. Health Risk Assessment

To assess the risk of consuming plant food supplements, the probability of developing cancer over the course of a lifetime is calculated. This can be accomplished by determining whether the range of values is greater than or less than 10,000 using the MOE method.

ii. Alkenylbenzenes

A literature review will be conducted to identify a list of plants that commonly contain alkenylbenzenes. Alkenylbenzenes will be extracted from plant food supplements using methanol, and the analysis will be completed with ultra-performance liquid chromatography quantification.

iii. Margin of Exposure Approach (MOE)

The MOE will be calculated by dividing $BMDL_{10}$, a reference point on tumour incidence acquired from epidemiological or experimental data, by the estimated daily intake in persons.

1.6 Research Objectives

1.6.1 General Objectives

Generally, this study aimed to evaluate the health risk of alkenylbenzenes in plant food supplements using margin of exposure approach.

1.6.2 Specific Objectives

1) To determine the level of alkenylbenzenes from the plant food supplements.
2) To calculate the estimated daily intake (EDI) of alkenylbenzenes.

3) To assess the health risk of alkenylbenzenes in the plant food supplement using margin of exposure approach.

1.7 Research Hypothesis

The MOE of alkenylbenzenes in plant food supplements marketed in Malaysia is less than 10,000.

CHAPTER 2

LITERATURE REVIEW

This chapter aims to summarise current knowledge about the presence of estragole in PFS. This review also explored the mechanism of estragole and how it is distributed within the human body, as well as the negative consequences of estragole that are of concern to consumers' health. Furthermore, the methodologies for analysing estragole levels in PFS will be highlighted in this literature review.

2.1 Plant Food Supplement (PFS)

Secondary plant metabolites are particularly intriguing because they have medicinal properties that humans can use. Secondary metabolism produces an estimated 200,000 specialised compounds that are required for the plant to survive in its environment but do not aid in plant growth and development.

Plants are our primary source of energy and nourishment when it comes to sustaining human life. Plant based products were used as a source of vitamins and minerals, in addition to providing essential nutrients such as carbohydrates, proteins and fats as well as vitamin and minerals. There is no doubt that the addition of secondary substances that were previously considered flavourings and colorings, antinutritional, or toxic, increases the nutritional value of food significantly. Phytochemicals have grown in importance since the 1980s, despite the fact that people still do not fully understand how they work in the human body. This research has

resulted in the "rediscovery" of many well-known plants and substances, as well as the development of dietary supplements (Franz et al., 2011).

A "food supplement" is defined by the EU Directive on Food Supplements as "...supplemental foods that are concentrated sources of nutrients or other substances that have a nutritional or physiological effect, alone or in combination, marketed in dose form, such as forms such as capsules, pastilles, tablets and other similar shapes, powder sachets and ampoules of liquids, drop dispensing bottles, and other liquid and powder forms designed to be taken in measured small unit quantities." Food supplements can include a wide range of nutrients and other substances. Nutritional supplements may contain essential fatty acids such as omega-3 fatty acids, fibre, and various plant and herbal extracts in addition to vitamins and minerals (Directive, 2002). Plant-based food supplements are dietary supplements made from various plants and herbal extracts. A food supplement is defined in Malaysia as any substance that can be added to a person's diet to sustain, improve, or maintain the health of the human body (Team, 2022). It is available in the form of capsules, tablets, powders, and liquids, but not as sterile preparations such as injectables and eyedrops.

PFS is commonly sold as a powder or capsule and should be included in a healthy diet. Product availability, consumer awareness, and product acceptance all have an impact on the global market for herbal supplements. The global food supplement market is expected to grow at an 8.2 percent CAGR (Compound Annual Growth Rate) between 2020 and 2027, reaching a total value of 230.73 billion US dollar (Markets, 2020). In contrast, the global plant extracts market is anticipated to reach 61.30 billion US dollar by 2025, expanding at a CAGR of 6%. PFS's increased online marketing efforts are expected to increase online sales. There must be stricter

controls and safety tests on products made by PFS to protect consumers from potentially harmful side effects (Alajlouni et al., 2017).

The growing use of botanicals and botanical preparations as PFS around the world (Figure 2.1) is based on the assumption that natural products are generally safe. However, regardless of their supposed health benefits, these supplements may contain potentially harmful ingredients such as genotoxic and carcinogenic compounds (Prinsloo et al., 2019). PFS contains a variety of phytochemicals in varying concentrations. Alkenylbenzenes are a type of phytochemical found in aromatic oils, flavouring, detergents, perfumes, as well as vegetables and fruits such as fennel, basil, bananas, star anise, and oranges (Martins et al., 2018).

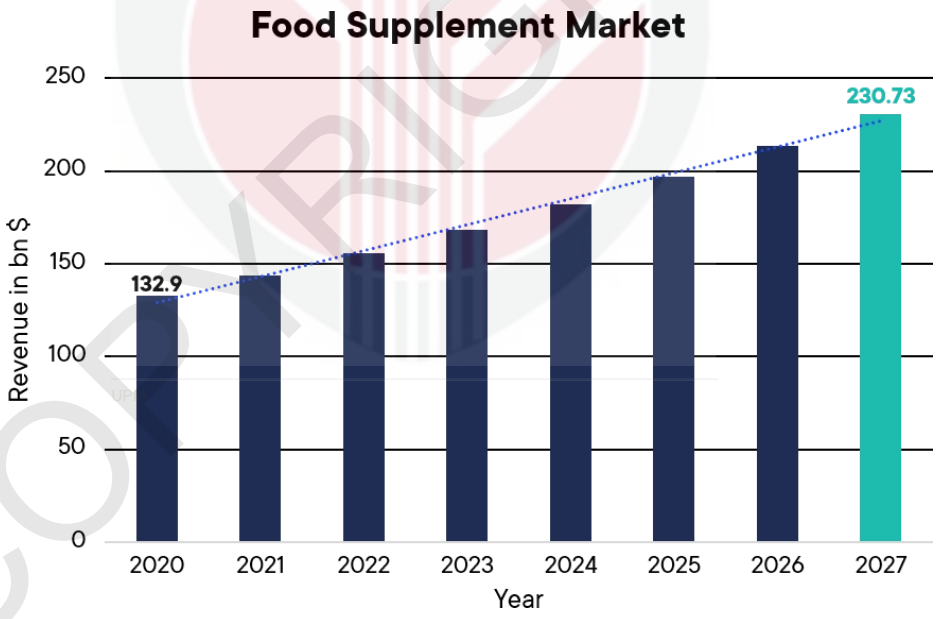

Figure 2.1: Global food supplement market revenue in USD per year (Markets, 2020)

2.2 Estragole

Estragole (1-Methoxy-4-(prop-2-en-1-yl)benzene) (Figure 2.2) is a volatile phenylpropanoid related to safrole, eugenol, methyleugenol, isoeugenol, anethole, isosafrole, apiol, elemicin and myristicin in the alkenylbenzene class. It has the chemical formula $C_{10}H_{12}O$ and a molecular weight of 148.20 g/mol.

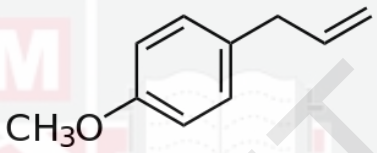

Figure 2.2: Molecular structure of estragole

Estragole, a naturally occurring compound, can be found in a variety of herbs and spices. Estragole is primarily derived from tarragon, sweet basil, sweet fennel, and their essential oils (Scientific Committee on Food, 2001). Estragole can also be found in small amounts in oranges, bananas, and grapefruit juice. Table 2.1 shows a list of plants that suspected to contain estragole.

Table 2.1: List of plant species containing estragole that is genotoxic and/or carcinogenic

Plant species	**Botanical family**	**Common name**
Foeniculum vulgare	*Apiaceae*	Fennel
Artemisia dracunculus	*Asteraceae*	Tarragon
Illicium verum	*Magnoliaceae*	Star anise
Melissa officinalis	*Lamiaceae*	Lemon balm

Ocimum basilicum	*Lamiaceae*	Sweet basil
Pimpinella anisum	*Apiaceae*	Anise, sweet cumin
Piper betle	*Piperaceae*	Betel/*Sireh*

Fennel, a primary source of estragole, has been used in traditional medicine for a long time. Fennel also have been used as a home remedy for a variety of ailments, particularly those related to the digestive system. Volatile compounds, phenolic compounds, and flavonoids in fennel have been linked to protection against oxidative stress-induced diseases like cardiovascular disease, cancer, and inflammation (Martati et al., 2018). It has long been used as an anti-inflammatory, diuretic, analgesic, carminative, laxative, stimulant, stomachic, expectorant, antioxidant, and integrative medicine. High doses of the estragole found in fennel essential oil were found to be carcinogenic and genotoxic in rats (SCF, 2001). The concentration of this substance is 5.45%.

According to its regulatory status, there are currently no restrictions regarding the use of estragole in medicinal products. The Council of Europe's Committee of Experts on Flavouring Substances recommended a maximum ingestion of 0.05 mg/kg for estragole in 2000. However, their statement does not specify whether the proposed limitations are based on the quantity or frequency of herbal medication use. Because estragole is genotoxic and carcinogenic, it is advised to minimize and reduce exposure (SCF, 2001).

In 2002, an expert panel decided that human exposure to estragole through diet from spices was not carcinogenic. This is due to the fact that just a few studies demonstrated that high-exposure metabolic, activation, and covalent binding patterns varied depending on the quantity of dosage received, but considerably decreased at

lower exposure levels (Smith et al., 2002). The JECFA has evaluated estragole and a group of additional allyls alkoxy benzenes present in foods and essential oils. The Committee assessed that a number of the six alkoxy-substituted allylbenzenes were hazardous and carcinogenic to rodents when supplied at high levels. A thorough scientific knowledge of these processes and their consequences for human risk will have a major impact on the determination of the health dangers posed by alkoxy-substituted allylbenzenes at food levels. At low exposure levels, alkoxy-substituted allylbenzenes, which are utilised as flavouring ingredients in foods and essential oils, may be detrimental to human health, while further research is needed to determine the severity of this problem.

2.3 Toxicokinetic of Estragole

Consumers consumed PFS orally. PFS contains alkenylbenzenes, such as estragole, which are quickly absorbed from the gastrointestinal tract after ingestion. Estragole has been discovered to go through various metabolic pathways, resulting in either bioactivation (making them more toxic) or detoxification (make them less toxic). The supplement species and dosage determine the length of each pathway (Martins et al., 2018). Figure 2.3 depicts the metabolic pathway of estragole, an example of an alkenylbenzene.

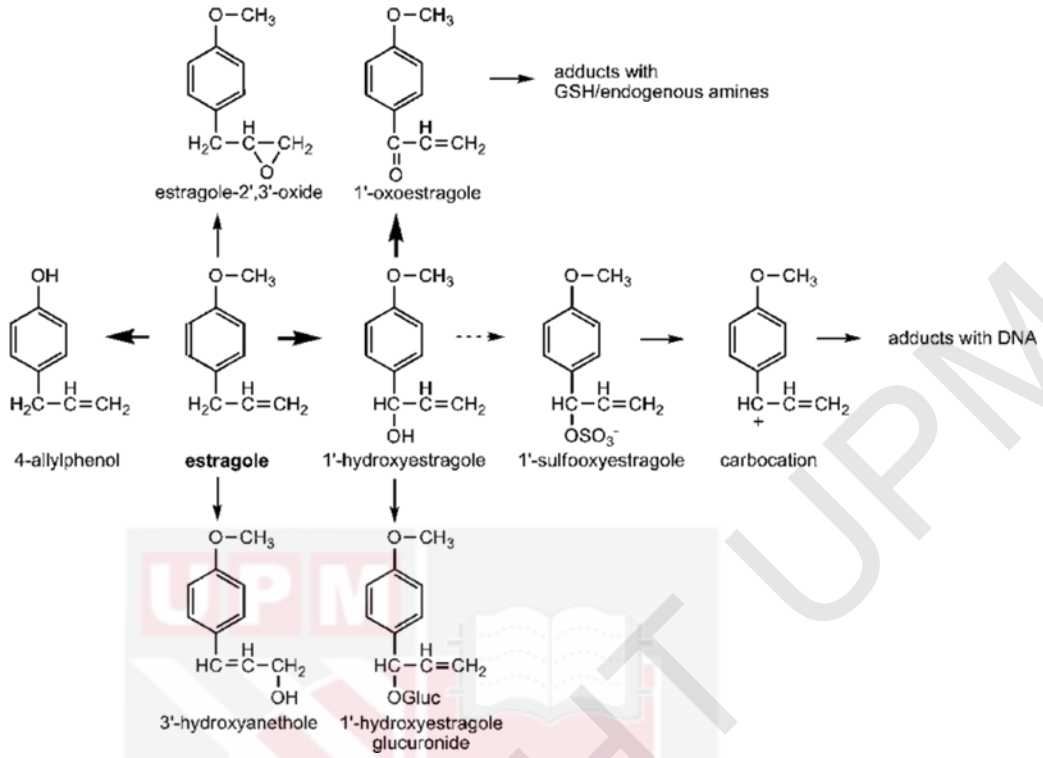

Figure 2.3: Estragole metabolic pathways (Punt et al., 2010)

The three principal metabolic processes found are o-demethylation of the aromatic ring's alkoxy substituents, 10-hydroxylation of the allylic side chain, and epoxidation at the allylic side chain's double bond. O-demethylation produces 4-allylphenol and other compounds, which are then subjected to a series of additional reactions (and the ultimate formation of CO2). The urine may contain glucuronic acid or sulphate as a byproduct of this process. Detoxification is accomplished through the process of O-demethylation. During the initial phase of sulfoconjugation, the active metabolite 1'-hydroxylation produces 1'-sulfooxyestragole, which can bind to DNA and protein. There is also 1'-hydroxyestragole glucuronidation and 1'-oxoestragole oxidation. Estragole conjugates, such as 1'-hydroxyestragole, are excreted in the urine of rats and humans. Notably, in a short period of time, epoxide hydrolase and

glutathione transferase convert estragole-2',3'-epoxide into detoxified metabolites (Guenthner et al., 2001). Furthermore, this route is thought to be detoxifying.

Cytochrome P450 metabolises estragole, producing a carcinogenic 1′-hydroxy metabolite. Sulfotransferases (SULTs) are required for further bioactivation of the 1′-hydroxy metabolite and for the liver to produce the carcinogenic 1′-sulfooxy metabolite. The covalent bonding of this unstable metabolite with the genetic material and proteins of the liver may contribute to the development of tumours (Ning et al., 2018). This mechanism raises concerns about alkenylbenzene consumption in PFS.

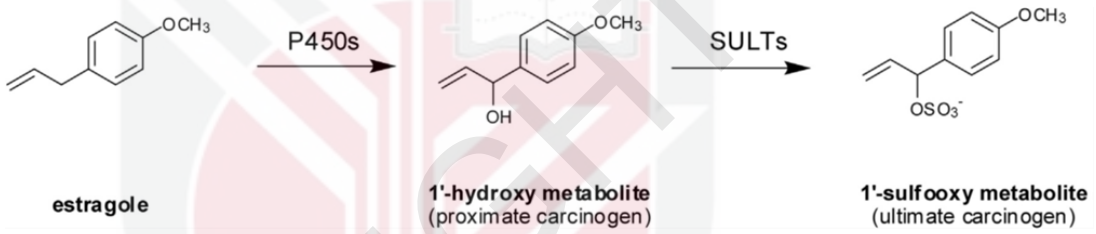

Figure 2.4: Simple mechanism of estragole

2.4 Genotoxic and Carcinogenic Effects of Estragole

Although long-term exposure to estragole in mice was found to increase the risk of cancer, similar studies in humans have not been reported. Malignant liver tumours developed after estragole or its metabolites were injected into adult mice or given orally to newborn mice of different strains. According Miller et al., (1983), after receiving estragole for a year, female CD-1 mice had significantly higher rates of liver cancer than control mice. Male CD-1 mice developed liver tumours after receiving ten oral doses of estragole, whereas female CD-1 mice were unaffected. High rates of hepatocellular carcinoma were produced when estragole was injected into the

peritoneum, skin, or abdomen of male newborn CD-1 mice or male B6C3F1 mice (Miller et al., 1983). Furthermore, in male B6C3F1 newborn mice, a single intraperitoneal dose of estragole increased the risk of liver cancer. The most toxic estragole metabolite, 1'-hydroxyestragole, was given to newborn male CD-1 mice subcutaneously, intraperitoneally, or orally (Miller et al., 1983). Several other estragole metabolites and synthetic derivatives, including estragole-2',3'-oxide, 1'-hydroxyestragole-2',3'-oxide, 1'-acetoxyestragole, and 1'-hydroxy-2',3'-dehydroestragole, have been shown in mice to be highly carcinogenic (Miller et al., 1983). Despite a lack of information on the subject, a study in which male rats were given estragole derivatives through the skin found no increase in tumour growth.

Estragole and its metabolites are toxic because DNA damage is what kills bacterial, yeast, and mammalian cells. Estragole was not effective against *Salmonella typhimurium*. This is likely the result of the protracted bioactivation process in living things. PAPS, a sulfation cofactor, increased strain TA1533's ability to produce estragole (To et al., 1982). One of toxic estragole metabolites, such as 1'-hydroxyestragole and estragole epoxides, were typically detected in mutagenicity assays with or without exogenous activation (McDonald, 1999). Estragole caused dose-dependent DNA damage in *Bacillus subtilis*, according to DNA repair assays. A separate study involving *B. subtilis* and *E. coli* came to the exact opposite conclusion (Sekizawa and Shibamoto, 1982). Numerous studies in rats, as well as in the livers of rats given estragole orally, have shown that estragole and its metabolites cause uveodermatologic syndrome (UDS). When toxic levels of estragole and its metabolite 1'-hydroxyestragole are present, DNA binds quickly to mice. As a result, a number of DNA adducts were discovered (Phillips, 1994). These adducts have the same binding properties as the carcinogen safrole.

Substances with structural similarities to estragole, such as safrole and methyleugenol, have been shown in rodents to be carcinogenic, particularly in the liver and other organs. Safrole causes hepatocellular carcinomas in rats and mice via the same genotoxic mechanism, and the evidence linking the two is compelling (Miller et al., 1983; Phillips, 1994). During a two-year feeding study, methyleugenol caused multiple site tumours in rats and mice, as well as newborn mouse hepatocellular carcinomas (Miller et al., 1983). All of these compounds, which have structural similarities to estragole or its metabolites, were given to rats. The formation of DNA adducts was strongly correlated with the number of liver tumours that later developed in neonatal mice exposed to estragole and related compounds (Phillips et al., 1984). While no "official" evaluation or determination has been reached by International Agency for Research on Cancer (IARC) or National Toxicology Program (NTP), estragole can be included in risk assessments similar to those undertaken for other recognised carcinogens because it is "reasonably believed to be a human carcinogen" (IARC class 2B). It should not be assumed that compounds that have not been classified do not cause cancer or are safe in general.

2.5 Analysis of Estragole Levels in PFS

2.5.1 Methanolic extraction

Methanolic extraction was one of the methods utilised to extract and determine the presence of estragole in the PFS samples. Methanol is an effective solvent for botanical extraction, yielding a high extract yield. The extractions were carried out using methanolic extracts, as explained by Gursale et al., (2009) with slight

modifications. 1 g of PFS samples were added to 25 ml methanol and ultrasonicated at room temperature for 15 minutes. The sample was then filtered through a 0.45-μm syringe filter, and the filtrate was kept at -20 °C until it was analysed using ultra performance liquid chromatography (UPLC). PFS samples were analysed three times using UPLC. The precision of the method was determined using a recovery test. PFS samples were spiked with pure estragole compounds to compensate for any loss during the extraction process. The same procedure was used to extract the samples as it was to extract the spiked samples.

2.5.2 Ultra-Performance Liquid Chromatography (UPLC) analysis

UPLC is the best method for separating nonvolatile chemical and biological compounds. Nonvolatile compounds include pharmaceuticals, salts, proteins, organic chemicals, and natural products such as plant extracts and herbal medicines. Liquid chromatography is the process of separating compounds from a liquid sample of interest. The ability of liquid chromatography to separate each and every compound while operating in ultra-mode is referred to as ultra-performance. A small volume of liquid sample is injected into a tube containing tiny particles known as the stationary phase (less than 2.5 to 5 microns in diameter) during the UPLC separation process. Liquid sample will go to stationary phase. A pump applies a high amount of pressure to a stationary column, causing a liquid (mobile phase) to flow through the column and down the packed tube (column). These components are separated by chemical and/or physical interactions between their molecules and the packing particles that occur during column packing. A flow-through device (detector) determines the concentration of these separated components at the exit of this tube (column). A liquid

chromatogram is the output of this detector. Each separated compound will be detected using a technique known as liquid chromatography.

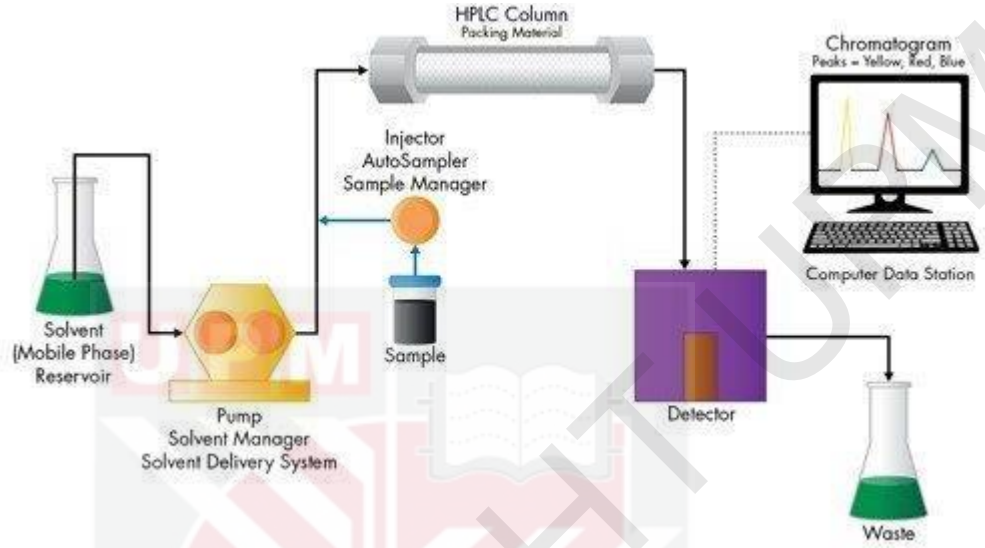

Figure 2.5: Representation of an UPLC system

2.5.3 Margin of Exposure Approach (MOE)

There are several methods for determining the possibility of exposure to genotoxic and carcinogenic substances. The International Life Sciences Institute (ILSI) Europe Expert Group was formed with the goal of investigating and evaluating existing risk assessment methods, as well as the scientific basis of genotoxic carcinogen mode of action and dose-response correlations. According to Barlow et al. (2006), both the World Health Organization (WHO) and the European Food Safety Authority (ESFA) recommend the margin of exposure technique as the preferable risk assessment tool for carcinogenic and genotoxic substances. The margin of exposure is

the ratio of estimated human exposure to the reference point based on dose-response data from experiments or epidemiological studies.

The European Food Safety Authority (EFSA) considered a number of potential approaches in order to establish a baseline for dose-response data. These included calculating a carcinogenic potency index, for instance the threshold dose (TD), TD_{25} or TD_{50}, as well as a benchmark dose (BMD), which was largely based on animal study data. The BMD is the smallest dose at which an effect can be measured. In most cases, the BMR which is benchmark response is set to be 5-10% higher than the control level (EPA, 1995). The BMD method, originally proposed by Crump (1984) to model dose-response, was improved by the US EPA (EPA, 1995). The ESFA and WHO have both concluded that BMD is the most effective method of establishing a standard. Data from animal bioassays and, less frequently, human cancer research are used to inform a variety of mathematical models that are used to simulate the disease. There are criteria for determining whether or not the models are a good match (Barlow et al., 2006). The BMD is calculated using each carefully simulated dose-response curve. The lower of these two values obtained from an acceptable modelled dose response curve is typically chosen because it is the most reasonable estimate of the BMD. The lower limit of a 95% confidence interval with only one side on the BMD is known as the benchmark dose lower limit (BMDL). Given the inherent uncertainty and statistical errors in cancer bioassay data, using this lower bound ensures (with 95% confidence) that the selected BMR is not exceeded. The EFSA recommends the $BMDL_{10}$, which is generated with a 10% BMR, as the optimal reference point. This is because a 10% rise in incidence is the smallest statistically significant increase that can be observed in the great majority of studies, requiring little or no extrapolation beyond the experimental data. Inter-Organization Programme for the Sound Management of

Chemicals (IPCS) provided recommendations for dose-response modelling using the BMD method. Furthermore, the EPA has software that makes it simple to model BMD.

Different MOEs for each substance can be determined whenever there is more than one carcinogenic target and different exposure estimates for different sectors of the human population. These MOEs were derived from multiple tumour data sets. A genotoxic and carcinogenic substance is evaluated in the same way as any other pollutant. According to the $BMDL_{10}$ data obtained from animal research and the overall ambiguity of the interpretation, the EFSA Scientific Committee determined that an MOE of 10,000 or higher would be of low concern from a public health perspective and may be considered a low priority for risk management measures (EFSA, 2005). The Committee determined that an MOE of 10,000 or higher would be negligible due to differences in toxicokinetics and toxicodynamics between species and humans, differences in how humans regulate the cell cycle and how well they can fix DNA, and the fact that the reference point is an effect level. Despite the fact that the Committee recognised that an MOE of this size or larger does not rule out the use of risk management strategies to restrict people's exposure, it also stated that this is a decision for risk managers to make. There is no agreement on whether a specific value or range of values should signify a given level of worry when it comes to the importance of MOE size (Barlow et al., 2006). Nonetheless, most individuals agree that the higher the level of concern, the lower the MOE.

CHAPTER 3

METHODOLOGY

3.1 Study Design

This is a quantitative study. This study employs both a general research study design and an experimental research design. The information was gathered and analysed in a laboratory setting. In this study, the independent variable was alkenylbenzenes in plant food supplements. The health risk assessment was the dependent variable. The objective of this research was to evaluate the health risks of alkenylbenzenes found in plant food supplements. The risk was evaluated in conjunction with the EDI, as well as using margin of exposure approach.

3.2 Study Location

The research was conducted at the Environmental and Occupational Health Laboratories at Universiti Putra Malaysia (UPM) and the Malaysian Agricultural Research and Development Institute (MARDI) in Serdang. Plant food supplements (PFS) were extracted in UPM laboratories, and the samples were analysed in MARDI.

3.3 Study Instruments

3.3.1 Chemicals

4-Allylanisole (purity >98%) were purchased from Sigma-Aldrich (USA). Acetonitrile and methanol (UPLC/MS grade) were obtained from Biosolve (Valkenswaard, the Netherlands).

3.3.2 Sample collection

A literature review was performed to identify a list of plants that frequently contain alkenylbenzenes. Alkenylbenzenes were screened using their plant family names, and the plant names were listed using their Latin names, which are based on the genus and species nomenclature systems. Estragole is the alkenylbenzenes of interest in this study because it has been shown to cause DNA damage and to cause liver tumours. Based on previous research, estragole is the most detectable compound in five out of ten PFS samples, followed by apiol and myristicin (Ning et al., 2018). Estragole occurs naturally in the plant groups *Apiaceae, Asteraceae, Magnoliaceae, Lamiaceae,* and *Piperaceae.* By researching the scientific names of the suspected botanicals on the ingredient list, 30 PFS samples were selected for collection from products purchased from a Malaysian market and online merchants. Thirty samples were chosen because for nonhuman samples, 30 samples are sufficient to do statistical analysis. All PFS samples purchased were in capsule and tablet form and were packed in their original package. The botanicals chosen in this case were *Foeniculum vulgare, Illicium verum, Pimpinella anisum, Piper betle, Melissa officinalis* and *Ocimum basilicum* as shown in Table 3.1. The plant was chosen because it is suspected to

contain estragole as shown in Table 2.1. Table 3.1 also shows manufacturer-recommended dosages used to calculate EDI.

Table 3.1: Characteristics of the PFS samples utilised in this study

PFS sample	Ingredients	Recommendation for daily intake	Content per unit	Health claims
S1	***Foeniculum Vulgare*** (100mg), *Azadiratna Indica* (50mg), *Curcuma Longa* (50mg), *Culminum Cyminum* (50mg)	1 capsule, 2-3 daily after meal	250mg	Traditionally used for health and body strengthening
S2	*Eucommia Ulmoides* (50mg), *Corni Officinalis* (10mg), *Rubus Idaeus* (10mg), *Rehmania Glutinosa* (20mg), *Poria Cocos* (12.5mg), *Morinda Officinalis* (20mg), *Broussonetia* (10mg), *Schisandra Chinensis* (10mg), *Cuscuta Chinensis* (12.5mg), *Acorus Gramineus* (5mg), *Rosa Laevigata* (7.5mg), *Adenophora Stricta* (10mg), *Cistanche Deserticola* (25mg), *Polygala Tenuifolia* (5mg), *Glycyrrhiza Glabra* (12.5mg), *Diosocrea Batatas* (10mg), ***Foeniculum vulgare*** (10mg), *Cordyceps Sinensis* (10mg)	1-2 capsules each time after food. 2 times daily	250mg	Traditionally used for health and body strengthening
S3	***Foeniculum vulgare*** (285mg), *Tetrapanax papyriferum* (180mg), *Poria cocos* (30mg)	Chew 2 tablets each time, 3 times daily after meal	500mg	Traditionally used for general health
S4	***Foeniculum vulgare*** (50mg), *Mentha piperita* (50mg), *Anethum graveolens* (40mg), *Carum carvi* (40mg), *Cuminum cyminum* (40mg), ***Ocimum basilicum*** (30mg), *Piper nigrum* (30mg), *Zingiber officinale*	2 capsules, twice a day	350mg	Traditionally used for dyspepsia, flatulence and indigestion

	(30mg), *Coriandum sativum* (20mg), *Elettaria cardamomum* (20mg)			
S5	*Euphrasia officinalis* (50mg), ***Foeniculum vulgare*** (20mg), *Daucus carota* (50mg), *Spirulina platensis* (100mg), *Chlorella pyrenoidosa* (100mg)	1 tablet, once daily	600.57mg	Traditionally used to support eye health
S6	*Cassia angustifolia* (50mg), *Zingiber minus* (50mg), ***Illicium verum*** (75mg), *Eugenia caryophyllata* (75mg), *Carum copticum* (75mg), *Piper nigrum* (75mg), *Cinnamomum zeylanicum* (100mg)	1 capsule after meal (morning and evening)	500mg	Traditionally used to relieve muscular pain, waist pain and joints pain, mild constipation
S7	*Aloe vera* (50mg), *Zingiber minus* (25mg), ***Illicium verum*** (150mg), *Eugenia caryophyllata* (75mg), *Carum copticum* (100mg), *Piper nigrum* (100mg)	1 capsule after meal (morning and evening)	500mg	Traditionally used to regulate menstruation, relieve menstrual pain and vaginal discharge, constipation, joints pain
S8	*Aloe vera* (50mg), *Zingiber minus* (25mg), ***Illicium verum*** (50mg), *Eugenia caryophyllata* (50mg), *Carum copticum* (75mg), *Piper nigrum* (50mg), *Astragalus membranaceus* (100mg), *Angelica sinensis* (100mg)	1 capsule after meal (morning and evening)	500mg	Traditionally used for women's health to regulate menstruation, relieve menstrual pain and vaginal discharge, constipation, joints pain
S9	*Glycyrrhiza glabra* (100mg), ***Pimpinella anisum*** (2.5mg), *Fritillaria cirrhosa* (2mg), Maltitol (564.6mg), Isomalt (256.4mg), Talc powder (30mg), Peppermint oil (7mg), Citric acid (5mg), Menthol (1.5mg), *Stevia rebaudiana* (1mg)	1-2 tablets, 3 times per day	1g	Traditionally used to treat phlegm, coughs, and sore throats
S10	*Eurycoma longifolia* (354mg), ***Pimpinella anisum*** (10mg), *Cuminum cyminum* (22mg), *Zingiber officinale*	1 capsule, 2 times per day	500mg	Traditionally used to relieve weak limb, smoothing blood

	(10mg), *Stichopus variegatus* (10mg), *Piper nigrum* (10mg), *Coriandum sativum* (6mg), *Alpina galanga* (21mg), *Curcumae zadoaria* (35mg), Garlic oil macerate (22mg)			circulation and relieve mild pain in joints and muscle
S11	*Eurycoma longifolia* (50mg), *Zingiber officinale* (25mg), *Acorus calamus* (20mg), *Nigella sativa* (15mg), *Coriandum sativum* (10mg), *Piper longum* (35mg), *Trachyspermum ammi* (30mg), ***Pimpinella anisum*** (15mg), *Croton caudatum* (15mg), *Eugenia aromatica* (35mg)	2 capsules, 2 times per day	250mg	Traditionally used for men's health and energy, relieving waist ache, body weakness and joints pain, improving blood circulation, urination and bowel movement
S12	*Labisia pathoina* (60mg), *Parameriae laevigata* (45mg), Gallae (45mg), *Trachyspermum ammi* (10mg), *Zingiber officinale* (35mg), *Eugenia aromatica* (7.5mg), *Piper nigrum* (7.5mg), *Croton caudatum* (8.5mg), *Coriandum sativum* (9mg), *Nigella sativa* (15mg), ***Pimpinella anisum*** (15mg), *Usnea barbata* (24mg)	2 capsules, 2 times per day	281.5mg	Traditionally used to treat menstrual irregularities, headaches, increase blood circulation, and menstruation
S13	*Croton caudatum* (40mg), *Labisia pathoina* (30mg), *Trigonella foenum graecum* (5mg), *Allomorphia malacensis* (10mg), *Eugenia aromatica* (20mg), *Amomum kepulaga* (25mg), ***Pimpinella anisum*** (30mg), *Cuminum cyminum* (30mg), *Curcuma domestica* (35mg), *Zingiber officinale* (30mg), *Nigella sativa* (35mg), *Piper longum* (10mg)	2 capsules, 2 times per day	300mg	Traditionally used to relieve menstrual pain, mild itch, regulate menstruation

S14	*Labisia pathoina* (71.96mg), *Quercus infectoria* (70.37mg), *Allium sativum* (39.47mg), *Kaempferia galanga* (21.80mg), ***Pimpinella anisum*** (21.50mg), *Cuminum cyminum* (21.50mg), *Trigonella foenum graecum* (21.50mg), *Trachyspermum ammi* (21.50mg), *Curcuma domestica* (21.50mg), *Zingiber officinale* (21.50mg), *Piper longum* (21.50mg), ***Piper betle*** (21.50mg), *Anethum sowa* (21.50mg), *Coriandum sativum* (17.30mg), *Holothurian stichopus* (11.30mg), *Piper nigrum* (10.50mg), *Eugenia caryophyllus* (10.50mg), *Myristica fragrans* (10.50mg), *Cassia angustifolia* (10.50mg), *Cinnamomum zeylanicum* (10.50mg), *Ferula asafoetida* (10.50mg), *Helicteres isora* (10.30mg)	4 capsules per day	500mg	Traditionally used to promote general well-being and to strengthen the body following childbirth
S15	*Zingiber officinale* (100mg), *Nigella sativa* (100mg), ***Pimpinella anisum*** (100mg), *Cuminum cyminum* (100mg)	2 capsules, 2 times per day	400mg	Traditionally used for general health
S16	*Acorus calamus* (25.45mg), *Amomum kepulaga* (34.74mg), *Piper betle* (10.33mg), *Piper longum* (54.51mg), ***Pimpinella anisum*** (15.71mg), *Myristica fragrans* (6.74mg), *Languas galanga* (27.94mg), *Hippocratea indica* (67.84mg), *Croton caudatum* (6.74mg)	2 capsules, once per day	250mg	Traditionally used for menstruation, regulate blood circulation
S17	***Piper betle*** (175mg), *Azadirachta indica* (175mg)	2 capsules, 2 times per day	350mg	Traditionally used to regulate blood circulation, relief of cough, fever

S18	*Quercus infectoria* (150mg), *Labisia pumila* (150mg), *Parameria laevigata* (120mg), ***Piper betle*** (75mg), *Curcuma domestica* (75mg)	3 capsules once daily after meal	570mg	Traditionally used to enhance menstruation flow, relieve menstrual pain, vaginal discharge, and flatulence
S19	*Labisia pumila* (100mg), *Coriandum sativum* (50mg), *Usnea barbata* (60mg), *Piper nigrum* (60mg), *Zingiber minus* (60mg), *Nigella sativa* (60mg), ***Foeniculum vulgare*** (50mg), *Parkia roxburghii* (60mg)	2 capsules, 2 times per day	500mg	Traditionally used to improve menstrual flow for women after childbirth
S20	*Eurycoma longifolia* (120mg), *Nigella sativa* (120mg), *Globia pendula* (50mg), *Curcuma domestica* (50mg), *Piper nigrum* (10mg), *Languas galanga* (100mg), ***Foeniculum vulgare*** (50mg)	2 capsules, 2 times per day	500mg	Traditionally used for men's health and energy, relieving waist ache, body weakness and joints pain
S21	*Saraca indica* (82mg), *Abroma augusta* (82mg), *Symplocos racemos* (82mg), *Salmalia malabarica* (50mg), *Quercus infectoria* (25mg), *Emblica officinalis* (25mg), *Nymphae lotus* (25mg), *Woodfordia fruticosa* (25mg), *Cyperus rotundus* (12.5mg), ***Foeniculum vulgare*** (12.5mg), *Tinospora cordifolia* (12.5mg), Red ochre (25mg), Bronopol (0.7mg), Sodium benzoate (20mg)	1 capsule, 3 times daily after food	560mg	Traditionally used to enhance menstruation flow, relieve menstrual pain, vaginal discharge
S22	*Aloe vera* (137.5mg), *Elephantopus scaber* (110mg), *Phyllanthus niruri* (110mg), ***Foeniculum vulgare*** (82.5mg), *Curcuma domestica* (55mg), *Zingiber officinale* (55mg)	2 capsules, 2 times per day	550mg	Traditionally used to regulate menstruation

S23	*Guazumae ulmifola* (150mg), *Parazumeric laevigata* (150mg), ***Piper betle*** (100mg), *Coriandum sativum* (50mg), *Amoni compactum* (50mg)	1-2 capsules, 3 times per day	500mg	Traditionally used for women's health
S24	*Nigella sativa* (130mg), *Cinnamomum burmani* (65mg), *Zingiber officinale* (65mg), *Eugenia caryophyllata* (65mg), *Curcuma xantorrhiza* (65mg), *Curcuma domestica* (65mg), *Andrographis paniculata* (97.5mg), *Elaeocarpus grandiflora* (65mg), ***Foeniculum vulgare*** (32.5mg)	2 capsules, 2 times per day	650mg	Traditionally used to reduce pains and joins fatigue by excessive uric acid
S25	***Melissa officinalis*** (150mg), *Passiflora incarnata* (125.2mg), *Valeriana officinalis* (75mg), *Tilia cordata* (50mg)	1-2 capsule per day	400.2mg	Traditionally used for difficulty in sleep
S26	*Labisia pumila* (135mg), *Quercus infectoria* (135mg), *Parameria laevigata* (200mg), ***Piper betle*** (50mg), *Curcuma longa* (50mg)	1 capsule, 3 times per day	570mg	Traditionally used to reduce excessive mucus in the feminine area
S27	*Piper retrofractum* (120mg), *Amomi cardamomi* (48mg), *Alstonia scholaris* (40mg), ***Foeniculum vulgare*** (120mg), *Curcuma domestica* (48mg), *Nigella sativa* (24mg)	2 capsules, 2 times per day	500mg	Traditionally used to care the health of women
S28	*Nigella sativa* (60mg), ***Foeniculum vulgare*** (45mg), *Cuminum cyminum* (45mg), *Curcuma longa* (30mg), *Zingiber officinale* (30mg), *Trigonella foenum-graecum* (30mg), *Alpinia galanga* (15mg),	2 capsules per day	300mg	Traditionally used for health and strengthen the body

	Cinnamomum verum (15mg), *Eugenia aromatic* (15mg), ***Illicium verum*** (15mg)			
S29	*Imperata cylindrica* (70mg), ***Piper betle*** (55mg), *Andrographis paniculata* (50mg), *Centella asiatica* (40mg), *Gynura segetum* (35mg)	2 capsules, 3 times per day	250mg	Traditionally used for inner heat
S30	*Angelica dahurica* (170mg), *Agastache rugosa* (230mg), *Poria cocos* (170mg), *Mentha haplocalyx* (70mg), ***Foeniculum vulgare*** (210mg), *Eugenia caryphyllata* (210mg), *Glyeyrrhiza uralensis* (70mg), *Atractylodes lancea* (170mg), *Citri reticulata* (170mg), Honey (530mg)	Adult: 1 bottle (2g) each time Children: ½ bottle (1g) each time, 3 times daily	2g	Traditionally used for stomach ache, mild diarrhoea, flatulence and vomiting

3.3.3 Extraction of Estragole in PFS

Each PFS sample was extracted using a modified methanol extraction procedure from previous research (Gursale et al., 2010). Grinding was the first step in converting a solid PFS sample into fine particles or powder. For samples in capsule form, the capsules were opened and transferred to a tube. Next, 1 g of powdered sample must be weighed and transferred to another tube. Then, 1 g of PFS powder was dissolved in 10 ml of methyl alcohol, followed by 15 minutes of room temperature ultra-sonification. After ultra-sonification, the solution was centrifuged for 20 minutes at 20,000 rpm. This is an additional approach for separating particles suspended in a liquid that is being enhanced for this research. The solution was filtered through a 0.22µm syringe filter into a UPLC vial to remove any impurities before being kept at -80^{o}C and analysed with UPLC. The extraction and UPLC analysis were carried out in triplicates. The precision of the extraction method was determined using a recovery test. To compensate for any loss during the extraction process, S5 and S26 were spiked with pure estragole compounds.

3.3.4 Quantification using UPLC

The concentrations of estragole in PFS were determined using a modified version of the procedures described by Alajlouni et al. (2017). The presence of estragole in the extract was detected and quantified using UPLC with a Waters Acquity system equipped with a Waters BEH C18 column. An injection volume of 3.5 µL was made onto a Waters Acquity UPLC BEH C18 column (50 mm x 2.1 mm x 1.7 µm). The mobile phase was 0.1% (v/v) ultra-pure water in TFA (A) and 0.1% (v/v) ultra-

pure water in acetonitrile (B). The desired flow rate was set at 0.6 mL/min. Initially, a gradient of 69:31 (A:B) was applied and held for 5 minutes. After that, the concentration was raised to 20:80 and held for 4 minutes and the concentration was kept for 1 minute. After that, the concentration was lowered to 100:0 and held for 1.5 minute and kept 0% for 1 minute. Finally, after 13.5 minutes, the eluents were returned to their initial conditions of 69:31 for the next run. Estragole chromatograms were analysed using a photodiode array detector at 235 nm. To determine the concentrations of estragole in the samples, a calibration curve was created. A 10 mM stock solution of estragole in methanol was prepared and stored in a refrigerator at 4 °C. Calibration standards were created by diluting the stock solution in methanol to concentrations of 25, 50, 100, 125, and 250 µM. The calibration curve's accuracy is improved by measuring it in three independent experiments.

3.3.5 Calculation of Estimated Daily Intake (EDI)

The EDI was calculated using the quantified estragole concentrations in the samples to assess consumer exposure to estragole in PFS. The concentration of estragole in the samples was multiplied by the supplier's recommended daily intake and then divided by an adult's 70 kg body weight to calculate estimated daily intakes. When the label recommends a daily usage of 2-3 times, the EDI was calculated using 3 times consumptions. The following equation was used to calculate the EDI of potentially harmful constituents:

$$EDI = \frac{W\ X\ L}{BW}$$

Based on the equation, the “EDI” is given in µg/kg bw per day. “W” represents the recommended daily intake of PFS samples, in gram, based on product label

information. “L” is the amount of estragole detected in the samples in µg/g. “BW” is the body weight, which is set to 70 kg by default (ESFA, 2012).

3.3.6 Calculation of MOE

Since estragole are genotoxic and carcinogenic, it is advised that risk management measures be determined using a standard method known as the margin of exposure (MOE). MOE method is utilised to determine the safety of genotoxic and carcinogenic substances (EFSA, 2005). By dividing the $BMDL_{10}$ by the EDI, the MOE is calculated. $BMDL_{10}$ value for estragole was obtained from previous research (Van Den Berg et al., 2011). A $BMDL_{10}$ from an animal study with the MOE of less than 10,000 is a high priority for risk management, whereas the MOE of more than 10,000 is a low priority.

$$Margin\ of\ exposure = \frac{BMDL_{10}}{EDI}$$

Based on the equation, “$BMDL_{10}$” is the lower confidence limit for the benchmark dose that causes a 10% increase in tumour incidence in µg/kg bw/day. “EDI” is estimated daily intake in humans in µg/kg bw/day.

3.4 Quality control

To create calibration curves for each analyte, standard mixes with concentrations ranging from 25 to 250 µM were injected into the UPLC. The linearity of the calibration curve was tested using the coefficient of determination (R^2). Following that, each PFS sample was subjected to three independent experiments in order to provide a more precise estimation of the data. To evaluate the extraction

recovery, two PFS samples were spiked with a pure estragole standard. Recovery tests are performed using the same extraction process. Both S5 and S26 were extracted and spiked at the same time, with the sample divided into two groups: the first (S5a and S26a) was extracted using the standard extraction method, while the second (S5b and S26b) was spiked with estragole prior to UPLC analysis. Using the formula below, the percentage of recovery % was used to adjust the concentrations of estragole in detected PFS.

$$Recovery\ (\%)\ = \frac{C_{spike}\ + C_{unspiked}}{C_{added}}\ x\ 100$$

where C_{spiked} is the concentration of sample measured in a spiked sample, $C_{unspiked}$ is the concentration of sample measured in a unspiked sample, and C_{added} is the known concentration added to the sample.

Next, the instrument is calibrated prior to use in order to transfer solution for calibration curve. Pipette calibration is necessary to avoid inaccuracies and achieve accurate results, performance, and pipette longevity. Furthermore, PFS samples that have already been extracted must be stored at -80^{o}C prior to UPLC analysis to protect the samples from degradation. Lastly, the PFS sample must be placed in a clean tube to avoid contamination from other sources.

CHAPTER 4

RESULTS

4.1 Calibration curve

Figure 4.1 shows the calibration curve of estragole. The five range of calibration curve demonstrated good linearity across the concentration range of 25 to 250 µM, with correlation coefficients (R^2) values of 0.9923. Table 4.1 shows the calibration parameters for estragole that was used to calculate the concentration of estragole in positive samples. The following equation was used to calculate the concentration of estragole:

$$y = mx + c$$

$$y = 6.6536x + 35.286$$

where y is the area under curve of positive samples, m is the slope, c is the intercept and x is the concentration of estragole (µM).

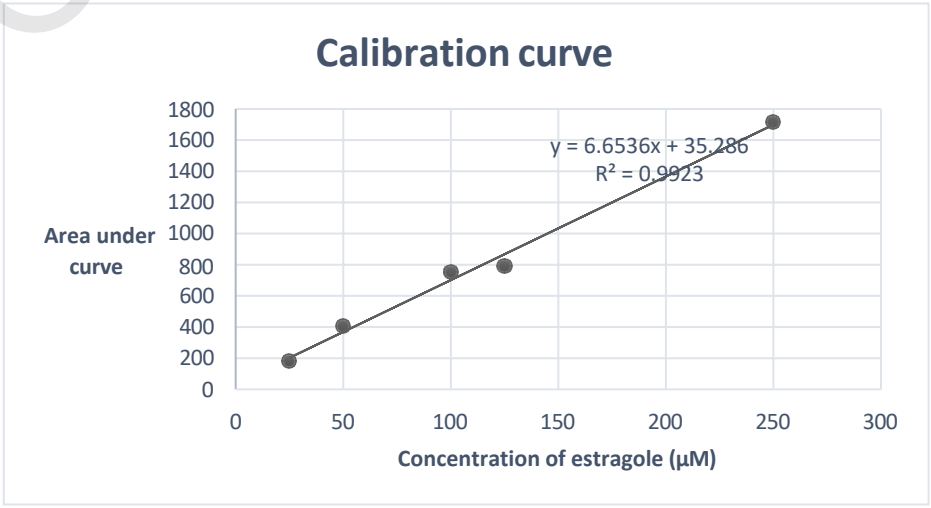

Figure 4.1: Calibration curve of estragole

Table 4.1: Calibration parameters for estragole

Parameters	Estragole
Linearity range (μM)	25-250
Slope	6.6536
Intercept	35.286
Correlation coefficients (R^2)	0.9923

4.2 Analysis of samples

A UPLC analysis was performed to determine the presence of estragole in PFS and whether a risk evaluation was required. Based on UPLC chromatogram, estragole elution time ranged between 6.305 - 6.327 minutes (Figure 4.2). Estragole concentrations were determined using the linear relationship between the concentration of estragole (R^2 = 0.9923) and the percentage of recovery from two different sample brands. Table 4.2 shows the estragole levels found in PFS obtained from the Malaysian market. It was discovered that 9 out of 30 PFS samples have the presence of alkenylbenzene of interest, estragole. In PFS, the concentrations of estragole ranged from 55.03 – 418.02 μg/g. Among the eleven positive samples, S16 had the highest concentration of estragole, followed by S18 and S23. All positive estragole samples contained three suspect ingredients which are *Foeniculum vulgare, Pimpinella anisum,* and *Piper betle*.

Table 4.2: Estragole levels in positive PFS samples

Sample number	Level of estragole in PFS (µg g–1)
S13	55.03 ± 26.22
S16	418.02 ± 272.09
S18	368.19 ± 186.62
S19	61.47 ± 31.62
S20	90.82 ± 34.59
S22	229.35 ± 116.53
S23	290.85 ± 143.58
S24	233.60 ± 145.80
S26	164.72 ± 28.12

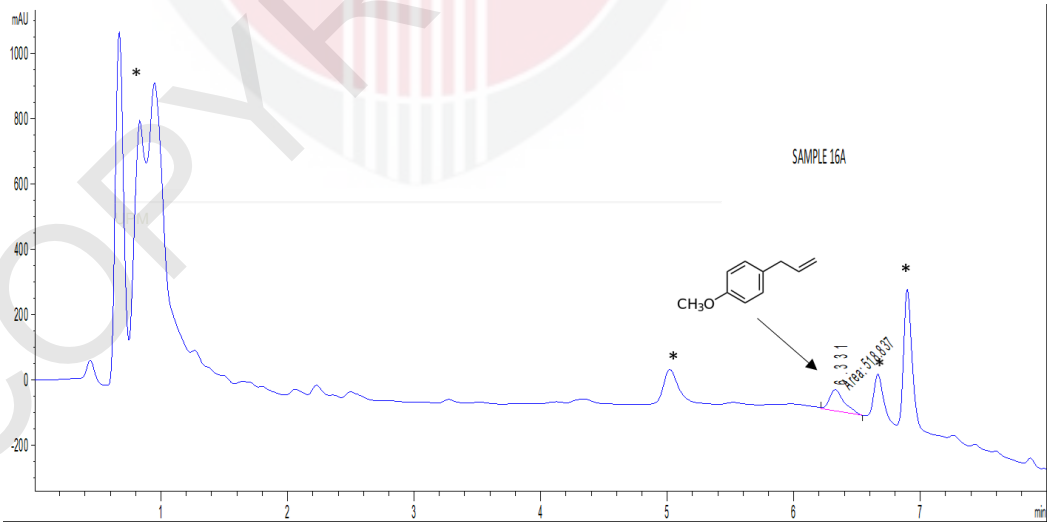

Figure 4.2: UPLC chromatogram of S16 with retention time 6.3 min (estragole).

Peaks marked with an asterisk (*) were unidentified.

4.3 EDI of estragole resulting from consumption of PFS

The EDI values were derived using the amount of estragole found in the PFS samples, the manufacturer's recommended daily dosage, and the weight of a 70-kilogram person. The recommended dosage for positive samples ranged from 500 mg to 2.6 g per day. Table 4.3 shows the EDI values of estragole in PFS. The EDI values obtained from positive PFS samples ranged from 0.99 - 9.44 μg/kg bw per day.

Table 4.3: Estimated daily intakes (EDIs) of estragole

Sample number	Recommended daily intake (g) of the PFS	EDI (μg/kg bw per day)
S13	1.2	0.99
S16	0.5	3.13
S18	1.71	9.44
S19	2.0	1.84
S20	2.0	2.72
S22	2.2	7.56
S23	1.5	6.54
S24	2.6	9.11
S26	1.71	4.22

4.4 Risk assessment of PFS consumption using the MOE approach

Table 4.5 displays the MOE values for consuming plant food supplements depending on level of estragole, which was determined using the appropriate individual EDIs from Table 4.3 and the corresponding $BMDL_{10}$ values from published

research (Van Den Berg et al., 2011) as shown in Table 4.4. According to the EFSA-defined MOE approach, all MOE values are based on lifetime exposure which is 75 years. Based on the result obtained, all positive samples have MOE values less than 10,000 as shown in Figure 4.3. Among the nine samples with MOE values under 10,000, S18 had the lowest MOE values, followed by S24 and S22. The highest estragole concentration was found in S18. Given that all of the PFS had MOEs of less than 10,000, it is clear that minimising potential risks is necessary before taking these supplements over an extended period of time.

Table 4.4: $BMDL_{10}$ values for estragole derived from literature (Van Den Berg et al., 2011)

Compound	$BMDL_{10}$ (mg/kg bw/ day)	$BMDL_{10}$ (µg/kg bw/ day)
Estragole	3.3	3300

Table 4.5: Estragole MOE values based on PFS consumption

Sample number	MOE values
S13	3333
S16	1053
S18	350
S19	1790
S20	1212
S22	436
S23	505
S24	362
S26	781

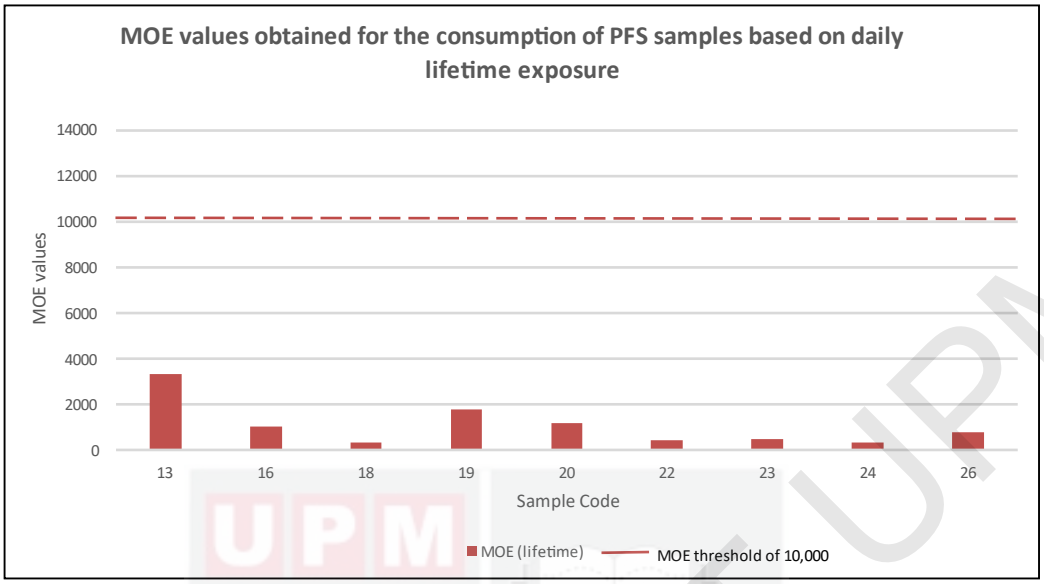

Figure 4.3: MOE values of PFS containing estragole

CHAPTER 5

DISCUSSION

The MOE approach was utilised in this study to analyse the risk of alkenylbenzenes caused by the use of PFS and herbal products. This approach has previously been used to evaluate the safety of alkenylbenzene-containing plant food supplements (Ning et al., 2018; Alajlouni et al., 2017; Van Den Berg et al., 2011), Indonesian jamu (Suparmi et al., 2018), nutmeg-based PFS (Alajlouni et al., 2017), basil-based pesto sauces (Al-Malahmeh et al., 2017), parsley and dill-based teas (Alajlouni et al., 2016), and fennel-based tea (van den Berg et al., 2014). Between 2011 and 2018, researchers evaluated the presence of alkenylbenzenes in 143 samples of PFS and other herbal products and discovered that 104 (72.7%) of those samples contained alkenylbenzenes. Thirty samples were obtained online in order to assess current alkenylbenzenes exposure through PFS consumption. Nine of these samples (30.0%) were found to contain estragole at levels as high as 418.02 μg/g. These results are consistent with prior research that looked at the occurrence of alkenylbenzenes in plant-based dietary supplements and other herbal supplements. When the findings of five previous investigations were combined, 43 out of 143 (30.1%) of the samples tested appeared to contain estragole at concentrations ranging from 1.4 – 1474.8 μg/g (Ning et al., 2018), 66.3 μg/g (Alajlouni et al., 2016), 17.17 μg/g (Alajlouni et al., 2017), 13.3 – 23.9 μg/g (Suparmi et al., 2018) and 120 – 1029 μg/l (Raffo et al., 2011). The amount of estragole found in samples varies depending on the plant species. In the majority of positive samples, 4 out of 9 PFS (44.4%) samples contain suspected

botanicals, *Foeniculum vulgare* which is known as fennel/*jintan hitam*. The other PFS sample contains suspected botanicals such as *Pimpinella anisum* and *Piper betle*. These two botanicals also known as anise/*jintan manis* and betel/*sirih*. Estragole is the most prevalent alkenylbenzene in fennel, which is consistent with the presence of fennel in 37 of the 71 samples from previous research (Van Den Berg et al., 2011). Estragole levels in botanical extracts ranged from 55.03 - 418.02 ug/g in this study. The difference between the level of estragole in the samples may be due to the botanical composition itself, but it could also be due to geographical impacts in growing, genetic factors, harvesting time, drying method and processing and manufacturing circumstances (Ning et al., 2018). The European Medicines Agency has published a public statement regarding the use of herbal products containing the active ingredient estragole (EMA, 2021). The EMA stated in this statement that estragole is a genotoxic carcinogen and suggested that exposure to estragole be maintained as low as practically possible. However, the EMA also said that short-term which is a maximum 14-day ingestion of 0.5mg per person per day is acceptable for herbal medicinal products.

Based on the results, it is evidently proven that estragole-containing PFS is available on the Malaysian market. These products are widely available on online shopping platforms such as Lazada and Shopee. According to a survey conducted by Mohamed et al. (2019), 22.4% of herbal products are purchased online by customers. This consumer group prefer to purchase herbal products via online because they are easily accessible and convenient. It is quite alarming that not all consumers check the ingredients and label before purchasing PFS. Consumers typically seek for herbal products that can improve their health and provide advantages, regardless of the PFS contents. Consumers will continue to buy the products as long as they are effective,

increasing their exposure to the suspected botanicals over time. The number of herbal plants planted in Malaysia, as well as the number of herbal supplements registered for use in general health, has increased dramatically during the last two decades. Over the last 20 years, Malaysia has seen a significant increase in the planting of medicinal plants and the licencing of herbal products for health and wellbeing. According to this survey, 71.89% of respondents had used herbal products in the previous year, which is consistent with recent research that evaluated the prevalence of use among university students in Klang Valley (Yan et al., 2021). This could lead to serious issues since long-term usage of herbal products containing suspected ingredients may result in serious complications, such as liver tumours. The government must enforce the law prohibiting the use of estragole-containing compounds in PFS since estragole has been shown to cause genotoxic and carcinogenic. Since MOE only calculates lifelong exposure to be 75 years, the consumer may take it for a short length of time. This is why short-term exposure need be considered. If only a short period of time is considered, MOE results in this study may be greater than 10,000, indicating a low priority for risk management.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

5.1 Conclusion

This study aimed to assess the health risk of alkenylbenzenes in plant food supplements using a margin of exposure technique. The findings from this study concluded that PFS sold in Malaysia contained estragole, a genotoxic and carcinogenic alkenylbenzene with MOE of less than 10,000. The findings identified that 9 out of 30 PFS samples (30.0%) contained estragole and all positive samples (100.0%) have MOE values less than 10,000 indicating the priority for risk management action. Because of the high MOEs for estragole exposure from various botanical preparations, risk management may need to be prioritised, especially for long-term exposure. We believe that there is a need to be concerned about the likelihood of estragole exposure from ingestion of some PFS obtained from the Malaysian market, especially over long periods of time. Even though the toxicological significance of these well-studied compounds remains debatable when consumed in small amounts, it appears very evident from a toxicological standpoint that excessive intake levels, such as those that may come from certain plant food supplements, should be avoided. Because MOE calculates a lifetime exposure of 75 years, short-term exposure must be considered. This is because even short-term exposure can result in MOE values lower than 10,000, indicating that risk management has to be addressed.

5.2 Limitation and Recommendation

The limitation in this study is that it only calculates lifetime exposure because the MOE approach is based on lifetime exposure. Short-term exposure must also be considered because MOE values may exceed 10,000, indicating a low priority for risk management. Several suggestions must be made based on the conclusions reached in order to improve this research in the future. First, researcher in Malaysia should conduct more study on alkenylbenzenes in PFS in the future in order to evaluate and prioritise risk management. Next, additional study on other alkenylbenzenes such as methyleugenol and safrole is required in Malaysia. This is due to the presence of other alkenylbenzenes, which have the same mechanism as estragole might be found in PFS. Furthermore, it is important to measure combined exposure of alkenylbenzenes in PFS sold in Malaysia, as many PFS samples may include multiple alkenylbenzenes. In this research, estragole is the only alkenylbenzene being evaluated in PFS, hence EDI and MOE values are determined based on individual alkenylbenzenes. The EDI and MOE can be calculated in the combined exposure technique by assuming equal potency of the few alkenylbenzenes, combining the EDIs of the individual alkenylbenzenes, and computing a toxic equivalence (TEQ) using the average interim relative potency factor (REP) values. Lastly, labeling requirements should be updated to include limitations on the recommended daily dose, duration of use, and information on the detrimental effects of prolonged use to further protect consumers from alkenylbenzenes. The labelling can also help general practitioners prescribe the optimal dosage and duration of consumption for the consumer.

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