UNIVERSITI PUTRA MALAYSIA

RISK ASSESSMENT OF GENOTOXIC AND CARCINOGENIC ESTRAGOLE IN PLANT FOOD SUPPLEMENTS (PFS) IN MALAYSIA

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RISK ASSESSMENT OF GENOTOXIC AND CARCINOGENIC ESTRAGOLE IN PLANT FOOD SUPPLEMENTS (PFS) IN MALAYSIA

By,

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ABSTRACT

RISK ASSESSMENT OF GENOTOXIC AND CARCINOGENIC ESTRAGOLE IN PLANT FOOD SUPPLEMENTS (PFS) IN MALAYSIA

NUR SYAHIRAH BINTI MOHD ESA

Introduction: Plant Food Supplements (PFS) are commonly used by the consumers without knowing the actual ingredients in the PFS as they thought all "natural" ingredients are "safe". Estragole is among of genotoxic and carcinogenic alkenylbenzenes derivatives that can naturally be found in plant such as basil, fennel and anise. **Objective**: This study was conducted to determine the risk from exposure to estragole via consumption of PFS using margin of exposure (MOE) approach. **Method**: Ten local PFS were purposively purchased and the level of estragole in the samples was quantified using Ultra High-Performance Liquid Chromatography (UHPLC). MOE approach was applied to calculate the risk for both genotoxic and carcinogenic substance. The MOE was calculated by dividing the Benchmark dose level ($BMDL_{10}$) to the estimated daily intake (EDI) of the PFS. **Results and Discussions:** Estragole was detected in all samples ranging between 371.8±275.5 µg/g to 1950.6±1575.4 µg/g. The EDI values calculated were from 0.021 mg/kg bw/day to 0.117 mg/kg bw/day. All samples were found to have MOE less than 10,000 with minimum value of 20-60 and maximum value of 150-310 indicating high priority for risk management action. **Conclusion**: It is concluded that Malaysian populations are exposed to estragole via consumption of PFS and risk management actions are needed in order to control the exposure to this natural genotoxic and carcinogenic chemical.

Keywords: estragole, plant food supplements (PFS), margin of exposure (MOE), risk assessment

ABSTRAK

PENILAIAN RISIKO TERHADAP GENOTOKSIK DAN KARSINOGENIK ESTRAGOL DALAM SUPLEMEN BERASASKAN TUMBUHAN (SBT) DI MALAYSIA

NUR SYAHIRAH BINTI MOHD ESA

Pendahuluan: Suplemen berasaskan tumbuhan (SBT) seringkali digunakan oleh pengguna tanpa mengetahui ramuan sebenar. Ini kerana, mereka berpendapat bahawa semua bahan "semulajadi" yang terdapat di dalam SBT adalah "selamat" untuk digunakan. Estragol merupakan salah satu sebatian '*alkenylbenzene*' yang merupakan bahan genotoksik dan karsinogenik yang boleh didapati secara semulajadi di dalam tumbuhan seperti daripada daun selasih, jintan manis dan bunga lawang. **Objektif:** Kajian ini dijalankan untuk menentukan risiko akibat pendedahan kepada estragol melalui penggunaan SBT dengan menggunakan kaedah '*Margin of Exposure (MOE)*'. **Metodologi:** Sepuluh SBT dibeli secara sengaja di mana tahap estragol di dalam sampel dianalisa dengan menggunakan '*Ultra High-Performance Liquid Chromatography (UHPLC)*'. Kaedah *'MOE'* digunakan dengan membahagikan *'Benchmark Dose Level (BMDL$_{10}$)*' kepada anggaran pengambilan harian SBT. **Hasil dan perbincangan:** Estragol telah dikesan di dalam semua sampel di dalam julat di antara 371.8±275.5 μg/g sehingga 1950.6±1575.4 μg/g. Nilai anggaran pengambilan harian SBT adalah di dalam julat 0.021 mg/kg bb/hari sehingga 0.117 mg/kg bb/hari. Kesemua sampel mempunyai nilai '*MOE*' kurang daripada 10,000 di mana nilai minima yang direkodkan adalah 20-60 manakala nilai maksima adalah 150-310. Ini menunjukkan keutamaan untuk tindakan pengurusan risiko yang tinggi. **Kesimpulan:** Secara kesimpulan, rakyat Malaysia terdedah kepada estragol melalui pengambilan SBT di mana tindakan pengurusan risiko diperlukan untuk mengawal pendedahan kepada bahan kimia semulajadi yang mempunyai ciri genotoksik dan karsinogenik.

Kata Kunci: estragol, suplemen berasaskan tumbuhan (SBT), *'Margin of Exposure (MOE)', penilaian risiko*

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LIST OF ABBREVIATIONS

ALARA	as Low as Reasonably Achievable
AUC	Area under Curve
BMDL	Benchmark Dose Level
IARC	International Agency for Research on Cancer
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
JECFA	Joint FAO/WHO Expert Committee on Food Additives
MOE	Margin of Exposure
NOAEL	No Observed Adverse Effect Level
PFS	Plant-based Food Supplements
USEPA	United States Environmental Protection Agency
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1. Background

People always equate "natural" with "safe", but it is not necessarily true. Natural substances are still chemicals; chemicals found in nature. According to the Royal Society of Chemists (2005), "*nature's poisons outrank those synthesised by chemists, both in number and in toxicity*". Thus, risk assessment of plant food supplements (PFS) containing different alkenylbenzenes was conducted based on the Malaysian market.

Alkenylenzenes have many derivatives that are naturally found in plant especially in tropical climate country such as cinnamon, fennel, basil or parsley that are common in Malaysia. Estragole is a known genotoxic carcinogen in rodents since 1976 where its mechanism of action seems to be similar in humans and can be commonly found in our herbs and spices such as tarragon, star anise and chervil (Superfoodly, 2017; Drinkwater et al., 1976).

1.1.1. **Herbal products in Estragole**

Herbal products formed an important component in Malaysian medicine system (Ramli et al., 2015; Sultana et al., 2014; Sooi & Keng, 2013; Jamal, 2006). Herbal products have been used since thousands of years ago and influenced by Indonesian, Chinese, Indian and Aboriginal's root (Sultana et al., 2014). It was believed that herbal medicines can act as remedies in promoting health maintenance (Yeoh, 2017).

In 2012, a survey conducted by the Forest Research and Institute Malaysia (FRIM) revealed that it was estimated that 73% of Malaysians consume herbal products, which was lower than estimated by World Health Organization (WHO) for developing countries with 80% (Ahmad, 2015). Meanwhile, the herbal industry in Malaysia was estimated to grow at a rate of 15% per annum, with the market value rising from RM7 billion in 2010, RM15 billion in 2014 and to some RM29 billion in 2020 where 91% of product buyers were locals (Rahman et al., 2017).

1.1.2. **Alkenylbenzenes**

Alkenylbenzenes such as estragole, methyleugenol, safrole and apioles (Figure 1.1) are natural compounds found in herbal plants such as fennel, basil, nutmeg, parsley and dill (Suparmi et al, 2018; Al-Malahmeh et al., 2017; EFSA, 2012; Van den Berg et al., 2012; Van Den Berg et al, 2011). These types of compounds can be found

commonly in PFS, herbal teas as well as flavouring agents (Martins et al., 2018; Rietjens et al., 2005).

Most of the alkenylbenzenes found in the PFS come in the form of pills, powders, liquid, tablet, herbal teas and even capsules (BiotechCorp, 2011). PFS are widely marketed in pharmacies, drug stores, health-food shops, supermarkets and via the internet including social media platform which are Instagram and Facebook page.

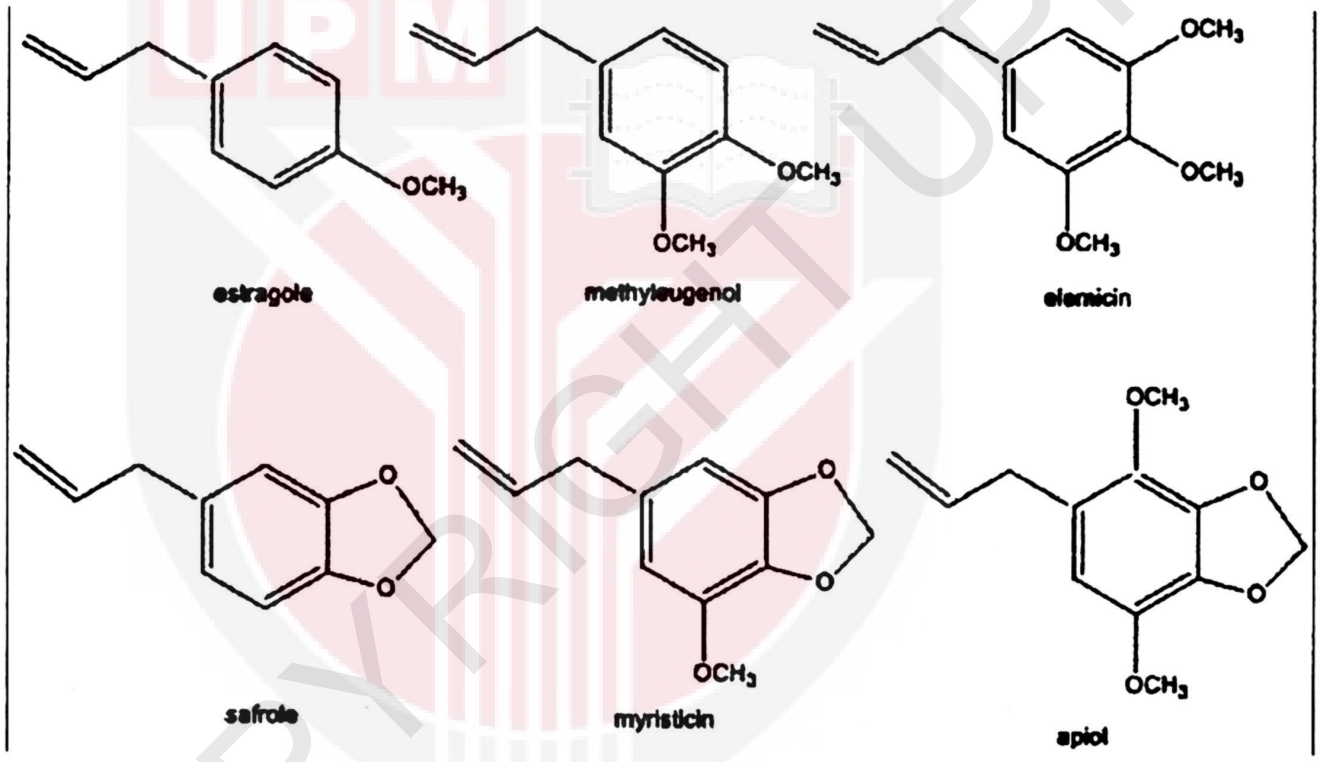


Figure 1.1: Structural formulas of alkenylbenzenes (Suparmi et al., 2018).

Alkenylbenzenes are known for their genotoxic and carcinogenic properties, due to metabolic conversion to the proximate carcinogens of 1'-hydroxymetabolites (Marabini et al., 2014). These compounds can be differentiated into two classes; allylbenzenes with 2,3-double bond and propenylbenzenes with 1,2-double bond (Ávila, Zougagh, Escarpa, & Ríos, 2009). The bioactivation of cytochromes P450 through genotoxic and carcinogenic activity of alkenylbenzenes,

leads to the formation of 10'-hydroxymetabolites (Figure 1.2), and the subsequent sulfonation of these 10'-hydroxymetabolites by sulfotransferases (SULTs), resulting in unstable DNA reactive 10'-sulfoxymetabolites (Van Den Berg et al., 2013).

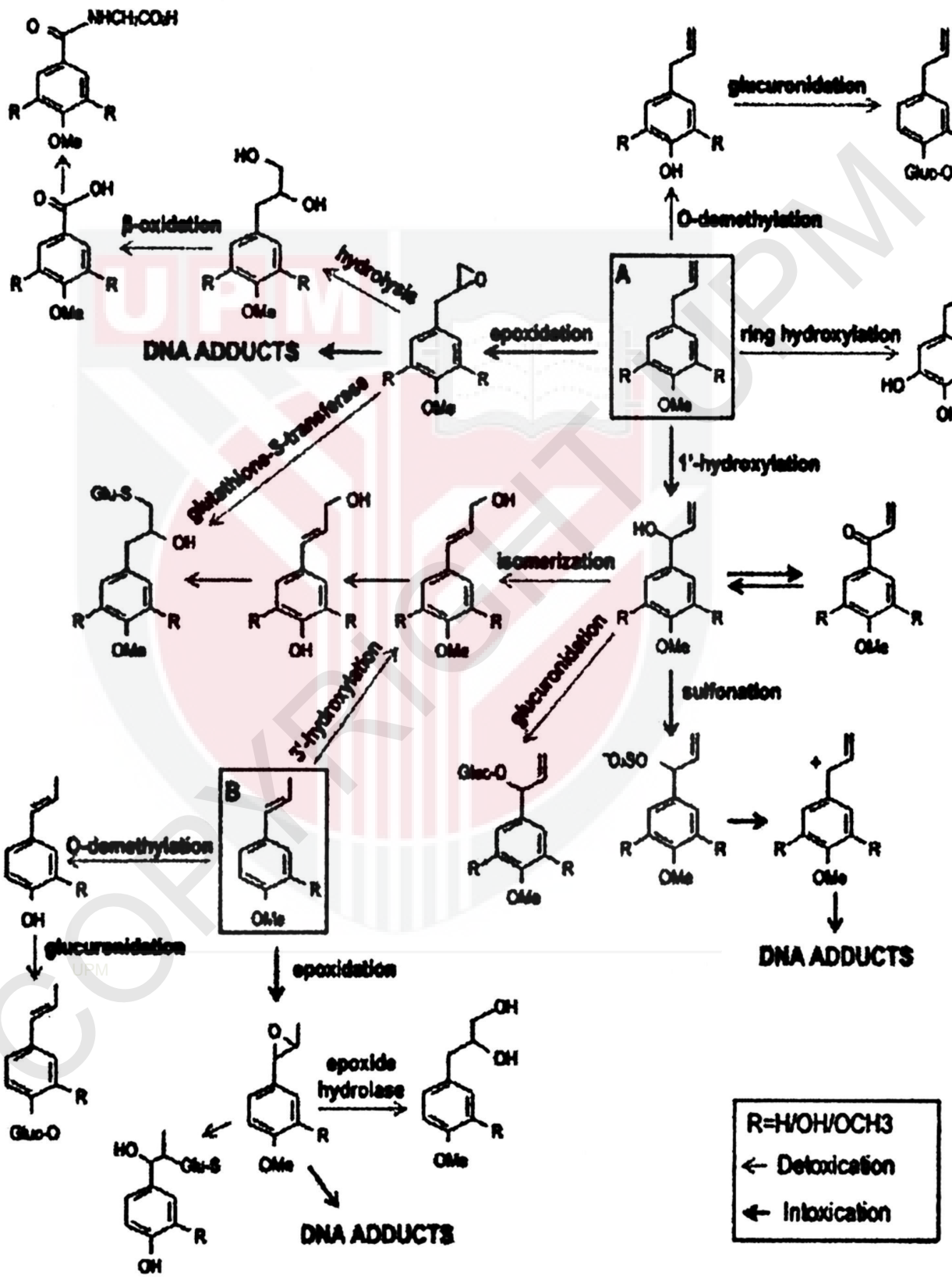


Figure 1.2: General Pathway of Alkenylbenzenes (Kobets et al., 2016).

However, not all alkenylbenzene compounds are genotoxic and carcinogenic. According to International Agency for Research on Cancer, estragole is classified as 'genotoxic and carcinogenic to rodents' by European Food Safety Authority (EFSA). It is found that estragole is genotoxic and carcinogenic to experimental rodents at high dose level (The Scientific Comittee on Food (SCF), 2001). As for the rest of the alkenylbenzene derivatives such as apiol, elemicin and myristicin, there are no scientific data on its toxicity.

Alkenylbenzenes are of concern due to the ability to form DNA adducts that contribute to the formation of hepatomas in rodent bioassays upon exposure at high dose levels (Alajlouni et al., 2016; Van den Berg et al., 2012). Alkenylbenzenes are rapidly absorbed after ingestion and transferred to the liver where the metabolic activations of these compounds lead to formation of a reactive 1'-sulfoxy metabolite that can form adducts with cellular macromolecules including proteins and DNA, thus contributing to the mode of action underlying the carcinogenicity (Alajlouni et al., 2017).

Due to their genotoxic and carcinogenic properties, the European Commission (EC) has prohibited the use of estragole, methyleugenol and safrole as pure compound in foods based on Regulation No 1334/2008 of the European Parliament and of the Council (European Commission, 2008).

1.1.3. **Estragole**

In recent years, estragole (Figure 1.3) has become an interest to many researchers because it is a major compound found in common plants such as fennel and nutmeg. Furthermore, this volatile compound could possess potential carcinogenic properties (Rodríguez-Solana et al., 2011).

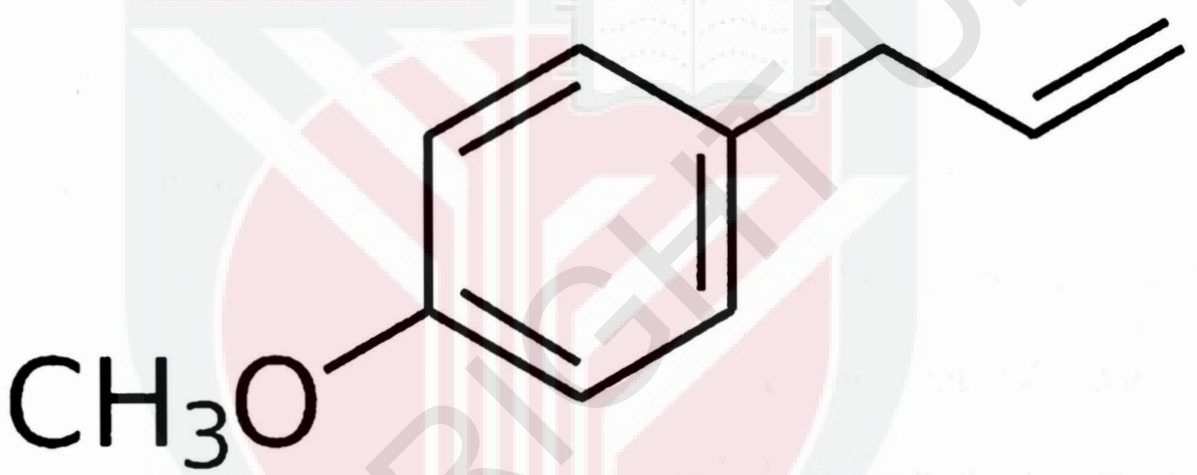


Figure 1.3: Chemical structure of estragole

Estragole (1-methoxy-4-(2-propenylbenzene) is a natural constituent of a number of aromatic plants essential oils such as basil, tarragon, anise, and fennel (Ismaiel, 2016; Martins et al., 2012; Zeller & Rychlik, 2009; De Vincenzi, 2000). Estragole (1-methoxy-4-(2-propenylbenzene); CAS 140-67-0) is a phenylpropene and an isomer of anethole that shares its anis-like flavour (Cabral et al., 2014).

Estragole naturally occurs in a variety of traditional foods, mainly in spices and is used as flavouring agent in seasonings, non-alcoholic beverages and condiments (Kfoury et. al, 2015; Zeller, Horst & Rychlik, 2009). This is due to their insecticidal, antiviral, antibacterial and acaricide activities (Kfoury et al., 2015; Hu et al., 2012; Chang, Cho, & Li, 2009). Besides, estragole exhibits characteristic of myorelaxant, anticonvulsant and anaesthetic, bradycardic, vasoactive and antioxidative and antimicrobial properties (Ponte et al., 2012).

For this reason, a legal limit for estragole of 10 mg/kg in non-alcoholic beverages was discussed in the European Union (EU) (The European Parliament and The Council of the European Union, 2006; Zeller & Rychlik, 2009). The U.S. Department of Food and Agriculture recorded the status of estragole along numerous essential oils containing estragole (e.g. extracts of bay leaves, basil, fennel, tarragon and anise) as "generally recognized as safe" (GRAS) for food purpose (McDonald, 2011).

1.2. Problem Statement

Herbal industry is one of the most promising industries in the future, as Malaysian government acknowledged that this industry is interconnected with the development of agriculture, pharmaceutical, life sciences, health care and food in Malaysia. Thus, it can be easily accessible in Malaysian market via online market, pharmacies, night markets and health boutique. This also includes social media platform such as Instagram and Facebook.

'Natural' does not necessarily mean 'safe' (Ning et al., 2018; Kristanc & Kreft, 2016; Van den Berg et al., 2011a; Van den Berg et al., 2011b; Ávila et al., 2009; Jeurissen, 2007; Rietjens et al., 2005). For instance, a large study of ginkgo that enrolled more than 3,000 older adults; found that it does not help to prevent, slow down dementia, or cognitive decline (Snitz et al., 2009). In addition, public can even have serious safety concerns for instance due to consumption of kava; a native plant in South Pacific that may be associated with severe liver damage (National Center for Complementary and Integrated Health (NIH), 2017).

Recent studies showed that alkenylbenzenes; which are the component in PFS are carcinogenic and genotoxic that can affect human health (AlMalahmeh et al., 2017; EFSA, 2017; Kobets et al., 2016; Paini et al., 2012; Auerbach et al., 2010).

Alkenylbenzene derivatives such as estragole, methyleugenol and safrole are listed in the International Agency of Research on Cancer (IARC) list as 2B which is possible carcinogenic on human; causing liver cancer, hepatotoxicity and breakdown of central nervous system (CNS). Besides, they are known to be natural genotoxic and carcinogenic to human health. Thus, this study presents an overview of the risk possess by PFS containing alkenylbenzenes in Malaysian populations.

1.3. Study Justification

This study is important to determine the presence of estragole and to quantify its level through the consumption of the PFS. It also acts a preliminary study to determine the risk from exposure to estragole via consumption of PFS obtained from Malaysian market. The Malaysian consumer were unaware of the presence of toxic ingredients in the PFS that they consumed may lead to adverse health effect.

Malaysian perceived herbal medicines as safe and effective because they are 'natural' and do not contain any dangerous chemicals. This is not exactly true because many drugs are based on substances from nature, from paracetamol to modern forms of chemotherapy. On the flip side, many poisonous items, such as cyanide and estragole, can be found in nature.

Besides, there are no specific guidelines or data for the manufacturer to comply on the control of the alkenylbenzenes' content in the PFS in Malaysia. This study could provide a baseline or reference that will help in developing the guidelines in the future. Consequently, this study could provide new information on the risk management plan of alkenylbenzenes exposure in PFS.

Since the alkenylbenzenes group especially estragole are known to be genotoxic and carcinogenic, this study can create public awareness among the consumers in purchasing natural products. The consumers can choose a variety of other natural ingredients that are much safer and healthier for their own health.

1.4 Objective

1.4.1. General Objectives

To determine the risk from exposure to estragole in PFS in Malaysia.

1.4.1. Specific Objectives

i. To determine the mean differences of estragole level between different samples.

ii. To determine the risk priority of estragole exposure from the intake of PFS.

1.5 Research Question

i. What is the level of estragole in PFS?

ii. What is the risk priority for risk management action in PFS containing estragole?

1.6 Hypotheses

i. There are significant mean differences of estragole level between samples.

ii. There is risk associated with estragole present in PFS in Malaysia.

1.7 Conceptual Framework

Figure 1.4 shows the conceptual framework of the present study.

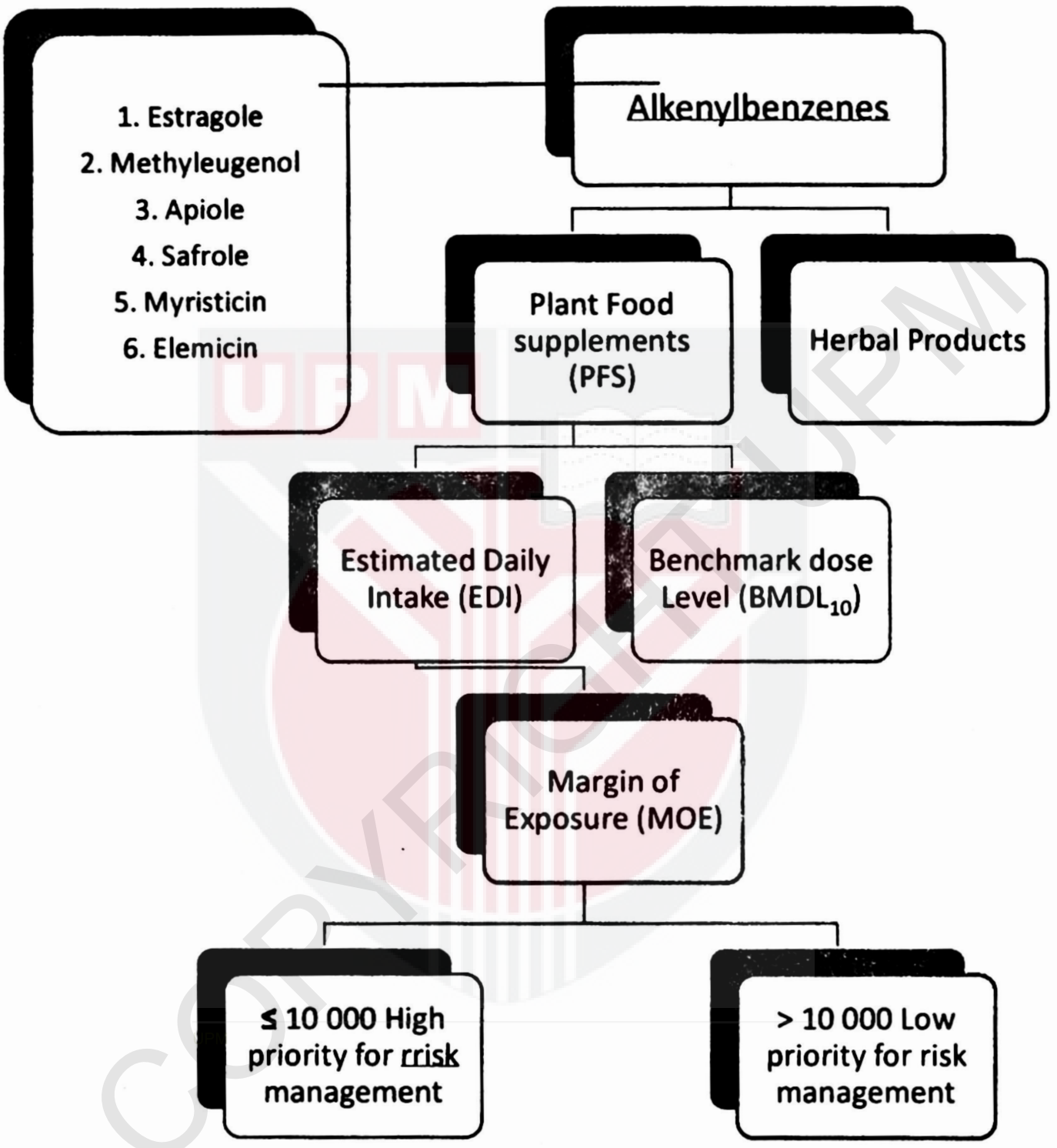


Figure 1.4: Conceptual Framework

1.8 Definition of Terms

1.8.1. Conceptual Definition

1.8.1.1. Plant Food Supplements

PFS are "foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of botanical preparations that have nutritional or physiological effect, alone or in combination with vitamins, minerals and other substances which are not plant-based. PFS are marketed such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities" (EFSA, 2005; Raats et al., 2014).

1.8.1.2. Margin of exposure (MOE)

MOE is "The ratio of the point of departure (POD), typically the Benchmark Dose – Lower Confidence Limit ($BMDL_{10}$) for a tumorigenic response in experimental animals, to the estimated human exposure for a genotoxic carcinogen (Boobis et al., 2013).

1.8.2. Operational Definition

1.8.2.1. Plant Food Supplements (PFS)

The PFS containing one or more plant species selected and listed as in literature review. The PFS selected are Malaysian products. The PFS are in capsules and paste forms.

1.8.2.2. Margin of Exposure (MOE)

Ratio of the $BMDL_{10}$ and estimated daily intake (EDI) in humans. $BMDL_{10}$ values were taken from the range of the $BMDL_{10}$ values from the tests using Benchmark dose software version 2.7 downloaded from USEPA. The EDI of the plant food supplements selected were calculated based on the daily intake recommended as stated by the suppliers.

$$MOE = \frac{BMDL_{10}}{EDI}$$ ---- Equation 1.1

Where;

MOE= Margin of Exposure

$BMDL_{10}$ = Lower confidence level of the benchmark dose giving 10% additional cancer occurrence.

EDI= Estimated Daily intake

CHAPTER 2

LITERATURE REVIEW

2.1. Estragole

2.1.1. Characteristics of Estragole

Estragole is one of the alkenylbenzenes derivatives. Apart from estragole, there are many other derivatives of alkenylbenzenes such as apiol, safrole, methyleugenol and myristicin. Studies shows that alkenylbenzenes can cause liver tumours in animals especially in high dose (Alajlouni et al., 2016; Kobets et al., 2016; Alhusainy et al., 2012; Martins et al., 2012; Chichioco-Hernandez et al., 2011; Wiseman et al, 1987; Davles, Phillips & Reddy, 1984; Phillips, Reddy, & Randerath, 1984; Miller et al., 1983). Estragole is considered as 'genotoxic and carcinogenic in rodents' according to the EFSA compendium (2012) and it is found to be genotoxic and carcinogenic in experimental rodents at high dose level (Sharif et al., 2017; Alajlouni et al., 2016; Van Den Berg et al., 2013b; Alhusainy et al., 2012).

Estragole consist of one benzene ring substitute with a methoxy group and a propenyl group. Table 2.1 shows the general information of estragole. Genotoxic substance has the possibility to directly alter the genetic material (DNA) in an organism and cause cancer. There might be a risk related with the consumption of estragole even in small quantity, particularly if consumed on a routine basis (EFSA, 2005).

Table 2.1: General information of estragole

Name	Estragole
Chemical classification	Alkenylbenzenes
Molecular formula	$C_{10}H_{12}O$
CAS No	140-67-0
Molecular weight	148.2 g/mol
Synonym	1-Allyl-4-methoxybenzene; 1-Methoxy-4-(2-propenyl) benzene; 3-(p-methoxyphenyl) propene; Estragol; Estragon; 4-allylanisole; 4-allyl-1-methoxybenzene; chavicol methyl ether; esdragol; p-Allyl anisole; Chavicyl methyl ether; Isoanethole; Methyl chavicol; *p*-methoxyallylbenzene; 4-methoxy-2'propenylbenzene; *p*-allyl-methyl chavicol; tarragon

Estragole can be found naturally in variety of botanical species such as *Artemisia dranunculus L.* (tarragon) with 60-75% of essential oil, *Ocimum basilicum L.* (sweet basil) with 20-43% of essential oil, *Foeniculum vulgare* Mill. (sweet fennel) with 5-20% of essential oil, *Pimpinella anisum L.* (anis vert) with 1% of essential oil and *Illicium verum* Hook f. (anis star) with 5-6% of essential oil (Van den Berg et al., 2014; The Scientific Comittee on Food (SCF), 2001; Vincenzi et al., 2000). Estragole was identified in three samples of basil containing pesto sauce (Al-Malahmehet al., 2017), fennel-based teas (Van den Berg et al., 2014; Gori et al., 2012) and basil-containing plant food supplements (PFS) (Van Den Berg et al., 2013). Estragole is listed on the U.S. Environmental Protection Agency High Production Volume Chemicals list with an

estimated annual production volume of 2.8 to 3.8 million pounds (1,300 to 1,700 metric tons) (Masten & Tice, 1999).

2.1.2. **Metabolism of Estragole**

Figure 2.1 shows the bioactivation pathway of estragole in the body. The genotoxic and carcinogenic activity of estragole in the body started with the bioactivation of estragole by the enzyme cytochrome P450, prompting development of 1'-hydroxyestragole, and the following sulfonation of these 1'-hydroxyestragole by sulfotransferases produce unstable DNA reactive 1'-sulfoxyestragole. Then, the carbocation of this 1'-sulfoxyestragole will bind to the DNA and forming DNA adducts in the liver and eventually leads to tumour formation (Van Den Berg et al., 2013b; Rietjens et al., 2008). Researchers use physiologically based biokinetic (PBBK) modelling respectively to study the bioactivation and detoxification of estragole and it is found that the organ that mostly contribute to the development of 1'-hydroxyestragole is liver (Alhusainy et al., 2010; Ning et al., 2018; Paini et al., 2012; Punt et al., 2009).

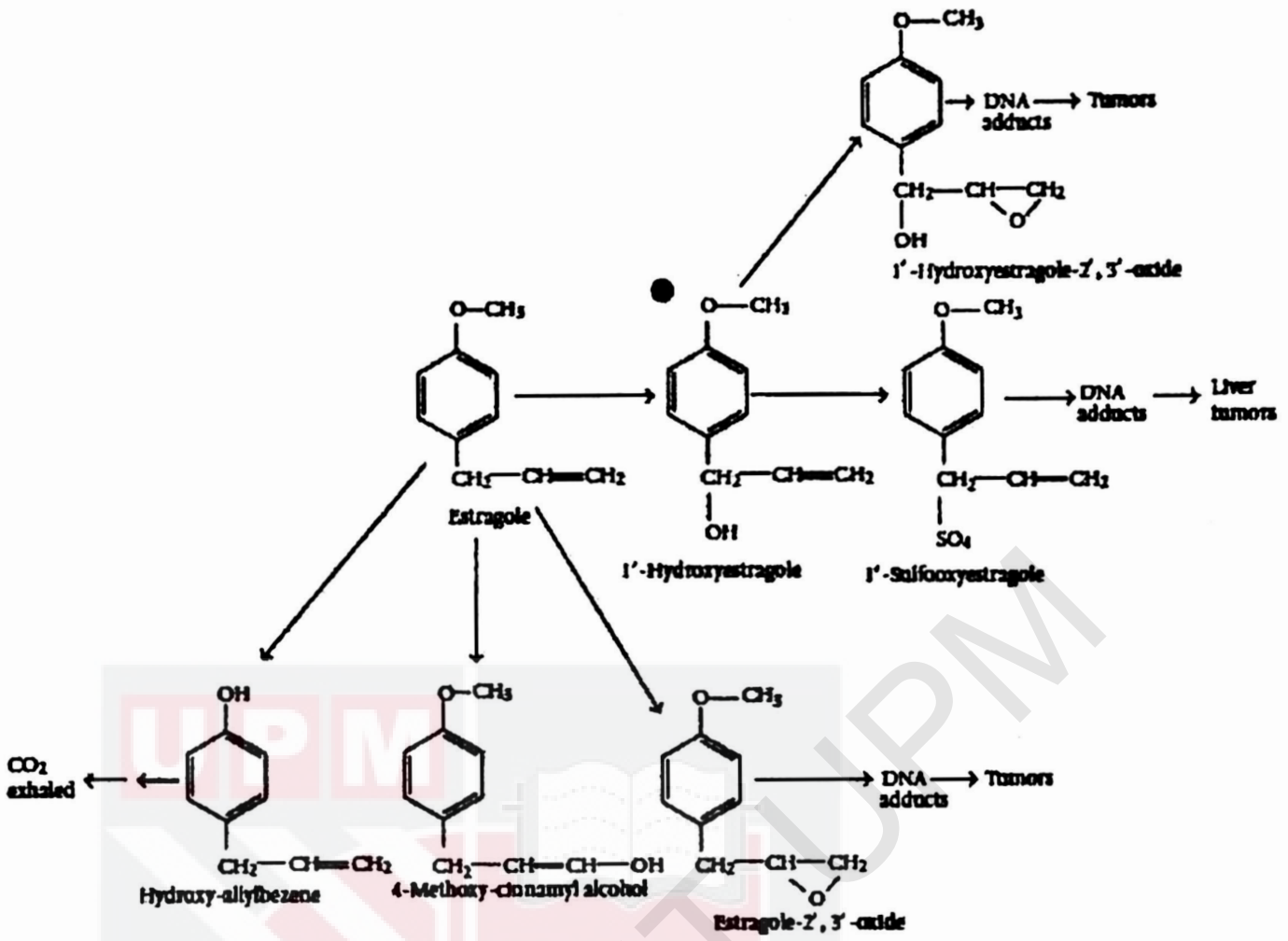


Figure 2.1: Bioactivation pathway of estragole (Gori et al., 2012).

There are several studies conducted to study the metabolism and bioactivation of estragole in animals. In rodents, the sulphate conjugate of 1'-hydroxyestragole is belief to be an extreme hepatotoxic and hepatocarcinogenic agent (Alhusainy et al., 2012). Upon oral exposure of estragole in human, it is metabolised in gastrointestinal tract, transported to the liver, and it is bio-activated to 1'-sulfooxyestragole (Alhusainy et al., 2012; Zeller et al., 2009). Formation of 1'- oxo estragole, which represents a minor metabolic route for 1'-hydroxyestragole in rat, is the main detoxification pathway of 1'-hydroxyestragole in humans (Punt et al., 2009).

In vitro research conducted by Suzuki (2012) and Paini (2012b) observed a moderate liver lymphocytic, which become severe over the

experiment time. Meanwhile, in the rat liver that treated with estragole, DNA adducts were detected, showing the induction of genotoxic activity.

2.1.3. **Genotoxicity and Carcinogenicity of Estragole**

The reported oral LD_{50} values of estragole are 1.2 and 1.8g/kg for rats and 1.25g/kg for mice (Bristol, 1991). Liver staining, mottling and blunting of lobe edges showed minor liver damage in rats exposed to four daily oral doses of estragole (Bristol, 2011). Several studies have been conducted to evaluate the mechanism of estragole carcinogenesis by examining DNA binding and characterising DNA adducts formed by estragole in mouse models (Paini et al., 2012; Suzuki et al., 2012; Liehr, 2000).

In a study conducted by Martins et al. (2012), they found that the adduct level was increase consistently after 2 hours of incubation of estragole in V79 cell. The study from Miller et al. (1983) showed that 61- to 73% of the male mice treated with safrole and estragole developed hepatic tumours and for which the average multiplicities of tumours per liver were 1.7 to 3.5 in about one year. Paini et al. (2012) also found that DNA-adduct formation was significantly higher in liver compared to the occurrence in metabolically active tissue such as lung and kidney in rat.

2.1.4. Plant Species Containing Estragole

The list of plant species containing estragole were present in Table 2.2 based on respective references. The references consist of journals, the statements from international agency and compendium from EFSA.

Table 2.2: Information on plant species containing estragole

Plant species	Remarks	Reference
Agastache foeniculum (Anise hyssop)	Estragole content: 555-12.160 ppm (plant)	European Medicines Agency, 2014 European Food Safety Authority, 2012
Agastache rugosa		European Food Safety Authority, 2012
Anthriscus cerefolium L. (Garden) chervil		European Medicines Agency, 2014 European Food Safety Authority, 2012
Artemisia dranunculus L. (Tarragon)		European Medicines Agency, 2014
Artemisia vallesiaca		European Food Safety Authority, 2012
Boswellia serrata **Roxb.**		
Cuminum cyminum L.		

Table 2.2 continue.

***Foeniculum vulgare* Mill.** (Sweet fennel)	Indicated in the EFSA compendium as 'genotoxic and carcinogenic in rodents.	European Medicines Agency, 2014 European Food Safety Authority, 2012 Van den Berg et al., 2011a
Foeniculum vulgare* Mill. *ssp. Vulgare var. vulgare (Sweet fennel)	3.5-12% estragole in essential oil	
***Foeniculum vulgare* Mill. *ssp. vulgare var. dulce* (Mill.) Batt. & Trab.** (Sweet fennel)	Seed essentail oil: estragole 3.4-8.1% Seeds approx. 0.3% estragole.	
***Illicium verum* Hook f.,** (Star anise)	Indicated in the EFSA compendium as 'genotoxic and carcinogenic in rodents' Estragole content: 280-6500 ppm (fruit), 0.6-6% (essential oil) Star anise oil: estragole 0.34-5.04%	European Medicines Agency, 2014 European Food Safety Authority, 2012 Van den Berg et al., 2011a
Melissa officinalis L. (Lemon balm)		European Medicines Agency, 2014
Myrrhis odorata (Sweet chervil)	Estragole (up to 75% in essential oil) Fruit essential oil: estragole 1.2-1.7%	European Medicines Agency, 2014
Myrtus communis L.	Essential oil estragole content: 58-88ppm	European Food Safety Authority, 2012

Table 2.2 continue.

***Ocimum basilicum* L.** (Sweet basil)	Estragole content: 238-8780 ppm (plant), 5-85% (essential oil) Presence of high amounts of estragole, genotoxic and carcinogenic in rodents It contains also camphor estragole (0.4% in the herb) Leaves and flowering tops essential oil: estragole 20-50% Indicated in the EFSA compendium as 'genotoxic and carcinogenic in rodents'	European Medicines Agency, 2014 European Food Safety Authority, 2012 Van den Berg et al., 2011a
***Ocimum canum* Sims.**	Essential oil estragole content:98%	European Food Safety Authority, 2012
***Ocimum nudicaule* Benth.**		
***Ocimum selloi* Benth.**	Estragole content: 51.1% (essential oil), 94.95% (essential oil from the leaves), 92.54% (essential oil of flower)	
Ocimum tenuiflorum L.	Estragole content: 39.950 ppm (leaves)	
Origanum majorana L.	Estragole content: 96-550 ppm	
***Persea americana* Mill *(Persea drymifolia* Schltdl. & Cham)**	Essential oil from leaves: estragole content: 3-85%	European Food Safety Authority, 2012
***Pimenta racemosa* (Mill.)**	Estragole content: 30-10.745 ppm	

Table 2.2 continue.

***Pimpinella anisum* L.** (Anise, Sweet cumin)	Estragole content: 400-1050ppm Essential oil estragole content: 400-1050 ppm	European Medicines Agency, 2014 European Food Safety Authority, 2012
Piper betle L. (Long pepper)	Estragole content: 1.02-4.0%	European Food Safety Authority, 2012
Salvia sclarea L.	Herb essential oil estragole content: 49%	
***Tagetes filifolia* Lag.**	Essential oil estragole content: 61.2%	
***Tagetes lucida* Cav.**	Essential oil estragole content: 45%	
***Vanillosmopsis arborea* (Gardner) Baker *(Eremanthus arboreus* (Gardner) MacLeish**	Essential oil (wood bark) estragole content: 36% Leaf essential oil estragole content: 3.6%	

2.2. **Plant Food Supplements (PFS)**

According to The European Union (EU) Directive on Food Supplements (2002/46/EC), food supplements are foodstuffs containing the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities (EFSA, 2005; Raats et al., 2014). Meanwhile, PFS are concentrated sources of dietary ingredients derived from various plants and herbal extracts (Al-Malahmeh et al., 2017).

PFS are well-known products and widely distributed. However, information regarding it risk is still limited (Lüde et al., 2016). The availability of botanicals or PFS in food supplements has far surpassed the availability of scientific information on their benefits, adverse effects and drug interactions. Information on the benefits of the PFS were covered partly by the 'tradition of use', but it is difficult to evaluate possible adverse clinical effects due to plant properties, plant misidentification or their interaction with pharmaceutical drugs and nutrients (Di Lorenzo et al., 2015).

In Malaysia, most of the food supplements are imported, where it comes either in finished products or crude materials for local used. Local importers will cooperate with foreign manufacturers to obtain the private formulation and in the

end, making their own 'secret recipe' (Poon, 2010). Under Drug Control Authority (DCA), health supplements products are managed by the National Pharmaceutical Regulatory Agency (NPRA). This is in accordance with Regulation 7(1) (a), Control of Drugs and Cosmetics Regulations 1984 that requires all products to be registered with the DCA prior to being imported, manufactured, sold or supplied, unless the product is exempted under specific provisions of these regulations. The vitamins and dietary supplements led the market with a market share of around 49% during 2014 and is expected to lead the market until the end of 2019. The awareness of benefits from these supplements and the increase in the elder population are the key drivers for the growth of this segment.

2.3. **Benchmark Dose Level ($BMDL_{10}$)**

Bench Mark Dose (BMD) modelling is a preferred approach in selecting an appropriate reference point from the dose-response-curve (Barlow et al., 2006; EFSA, 2005). The BMD approach integrate different mathematical models for the analysis of the observed carcinogenicity data. Making use of this approach, a dose can be estimated that causes a pre- defined cancer response, known as the Bench Mark Response (BMR). BMR is typically produced in experimental animal studies (Benford, Leblanc, & Setzer, 2010).

The $BMDL_{10}$ (the 95% lower confidence bound of the BMD_{10}) is frequently used as a reference point to calculate the MOE (Edler et al., 2014; Van den Berg et al., 2011). The $BMDL_{10}$ is a standardized reference point derived by mathematical modelling, from the animal data within the observed range of experimental data (Al-Malahmeh et al., 2017). The use of the BMDL instead of

the BMD will assure with 95% confidence that the value of the BMR will not go beyond the predefined value of, for example, 10% (EFSA, 2005). $BMDL_{10}$ is the lowest confidence level of the benchmark dose giving 10% additional cancer occurrence (Abdullah et al., 2017). Generally, a dose that gives 1%, 5% or 10% extra tumour incidence compared to the background level is chosen. However, it is indicated that the use of a dose giving 10% extra cancer risk above the background level ($BMDL_{10}$) is attended with the least uncertainties and is therefore preferred (Barlow et al., 2006).

The BMD approach can be used to implement the recommendations in USEPA's Guidelines for Carcinogen Risk Assessment (2005) regarding modelling tumour data and other responses thought to be important precursor events in the carcinogenic process (USEPA, 2012). This mathematical modelling characterizes the BMD in regards of the benchmark response. The use of the lower limit of confidence interval on the BMD (the BMDL) was suggested that thus reflected uncertainties and statistical errors in the accessible dose-response data of cancer (Barlow et al., 2006).

2.4. Estimated Daily Intake (EDI)

The estimated daily intake (EDI) of the PFS selected is based on the daily intake recommended by the suppliers as stated at the packaging label on the supplements. EDIs are evaluated using body weight of 62.65 kg. The body weight of 62.65 kg is the average body weight of 6,775 men and 3,441 women Malaysian aged 18 – 59 years (Azmi et al., 2009). In previous study, the EDI was estimated using the content of alkenylbenzene in the food and a body weight of 60 kg, the

default value for adult weight as recommended by EFSA and the EDI was determined for specific compounds for all of the samples (EFSA, 2017; Van den Berg et al., 2014; Van Den Berg et al., 2013). However, there were other alkenylbenzene compounds that were founded in the samples. Therefore, the subsequent risk assessment and combined exposure assessment were also conducted on the samples (Al-Malahmeh et al., 2017).

2.5. Margin of Exposure (MOE)

MOE approach is recommended by expert groups of the European Food Safety Authority (EFSA), the Joint FAO/WHO expert committee on Food Additives (JECFA) and the International Life Sciences Institute (ILSI) for risk assessment of exposure to compounds that are both genotoxic and carcinogenic (Van Den Berg et al., 2011; Barlow et al., 2006; EFSA, 2005). The MOE is defined as the ratio between this reference point, the $BMDL_{10}$, and the estimated dietary intake (EDI) in humans (Rietjens et al., 2008). It is also expressed as the ratio between an appropriate Point of Departure (PoD) on the dose–response curve for a tumour response and a relevant estimate of human exposure (Edler et al., 2014). MOE is a tool used by risk assessor to consider possible safety concerns due to both genotoxic and carcinogenic substances present in the food (EFSA, 2012). EFSA concluded that the magnitude of an MOE could be used by risk managers for priority setting and was more informative than advising them that exposures should be reduced to ALARA (EFSA, 2005).

MOE is a dimensionless ratio based on a reference point obtained from epidemiologic or experimental data on tumour incidence, which is divided by the

estimated daily intake in humans (Abdullah et al., 2017). The MOE compares the exposure levels causing malignant tumours in experimental animals with dietary intake estimates (EDI) in humans, considering differences in consumption patterns (Al-Malahmeh et al., 2017). According to WHO, MOE is defined as the ratio of the no observed adverse effect level (NOAEL) or benchmark dose lower confidence limit (BMDL) for the critical effect to the theoretical, predicted, or estimated exposure dose or concentration (WHO, 2008). Thus, the MOE approach compares toxic effect levels with human exposure levels where it is considered a useful and pragmatic option for risk assessment of substances that may be both genotoxic and carcinogenic (Barlow et al., 2006). It allows comparison between compounds and prioritization of risk management actions, especially if the calculation of the MOE is accompanied by an appropriate narrative explaining inherent uncertainties (Van den Berg et al., 2011). The MOE approach was created to be used in situations where there is a requirement for an evaluation and guidance on the risks to the individuals who are, or have been, accidentally exposed, through food or substances that are both genotoxic and carcinogenic (Rietjens et al., 2008).

An MOE value more than 10,000 express as low priority for risk management actions and does not have any concern from a public health opinion (Van den Berg et al., 2011). The EFSA Scientific Committee considered that an MOE of 10,000 or more, based on animal cancer bioassay data, 'would be of low concern from a public health point of view and might reasonably be considered as a low priority for risk management actions" (EFSA, 2005). The value of 10,000 is interpreted based on recognizing many factors that can cause ambiguity in the MOE including (Van den Berg et al., 2011);

a) A factor of 100 for species diversity and human variability in toxicodynamic and toxicokinetic.
b) A factor of 10 for inter-individual human variability in cell cycle management and DNA replacement.
c) A factor of 10 because of the $BMDL_{10}$ is not equal to a no observe adverse effect level (NOAEL) when it is use as reference point.

However, MOE does not give a numerical risk estimate that may be regarded as quantification of the actual risk. It only displays the ratio that the public may have difficulties in understanding it (Barlow et al., 2006). The calculation of MOE generally using equation below;

$$MOE = \frac{BMDL_{10}}{EDI}$$

Where;

MOE = Margin of Exposure

$BMDL_{10}$ = Benchmark Dose

EDI = Estimated Daily intake

CHAPTER 3

METHODOLOGY

3.1. Research Design

In this study, a cross sectional and experimental research was carried out. The level of estragole in PFS were measured by using Ultra High-Performance Liquid Chromatography (UHPLC) Flexar FX-15 UHPLC system (BetaTech Scientific Sdn. Bhd.).

3.2. Sampling Technique

The purposive sampling was applied in this research. Based on the literature, the PFS samples were selected based on plant species that may contain estragole compound. Ten (10) samples of PFS containing one or more plant species containing estragole (Table 3.1) were bought over the counter.

Table 3.1: Product description of the PFS analysed in present study

Sample No.	Product	Product presentation	Dosage	Plant species
S1	Jamu Tradisi Wanita	Capsules	2 capsules, 2 times per day	*Coriandrum sativum*
S2	Kacip Fatimah Manjakani Plus	Capsules	2 capsules, 2 times per day	*Coriandrum sativum, Nigella sativa, Pimpinella anisum, Piper nigrum*
S3	Kapsul Tongkat Ali Hitam Plus	Capsules	2 capsules, 2 times per day	*Foeniculum vulgare, Nigella sativa, Piper nigum*
S4	Kapsul Tupai Dua Keluarga	Capsules	2 capsules, 2 times per day	*Coriandrum sativum, Piper longum Piper nigrum*
S5	OK Tongkat Ali	Capsules	2 capsules per day	*Coriandrum sativum, Pimpinella anisum, Piper nigrum,*
S6	Ma'jun Gamat Mengkudu Plus	Tablet	1 tablet per day	*Coriandrum sativum, Nigella sativa, Pimpinella anisum, Piper nigrum*
S7	Ma'ajun Kacip Fatimah Manjakani Plus	Tablet	1 tablet per day	*Coriandrum sativum, Nigella sativa, Pimpinella anisum, Piper nigrum*
S8	Ma'ajun Petani	Paste	Thumb-size, twice a day	*Coriandrum sativum, Nigella sativa, Pimpinella anisum, Piper longum*
S9	Warisan Maajun Maka	Paste	Thumb-size, once a day	*Coriandrum sativum, Pimpinella anisum, Piper nigrum*
S10	Arjuna	Tablet	1 tablet, 2 times per day	*Foeniculum vulgare, Nigella sativum, Piper nigrum*

3.3. Instrumentation

3.3.1. Materials and Chemicals

A total of 10 sample were collected from local herb shops or traditional market with the name of possible alkenylbenzene containing botanicals on the label. The botanical names used in this targeted sampling were fennel (*Foeniculum vulgare* Mill.), black pepper (*Piper nigrum* L.), coriander (*Coriandrum sativum* L.), fennel (*Nigella sativa* L.), anise (*Pimpinella anisum* L.) and long pepper (*Piper longum* L.). Estragole (purity 98% w/w) and was purchased from Sigma-Aldrich, United States. The methanol and acetonitrile (HPLC supra gradient) were purchased from Systerm Chemicals, Malaysia and Merck, Malaysia respectively. A 0.22µm nylon membrane syringe filters were obtained from Techub Allied Services, Malaysia. Ultrapure water was obtained from Mili-Q purification water system.

3.3.2. Methanol Extraction

Methanolic extraction was applied to optimally extract and quantify a total amount of different alkenylbenzenes present in the samples (Suparmi et al., 2018). The methanol extraction was conducted based on the method that was previously described in literature with some modifications (Suparmi et al., 2018; European Medicine Agency (EMA), 2014; Van den Berg et al., 2014; Van Den Berg et al., 2013b). In general, 25ml methanol was added to 1g of PFS followed by sonication for 15 minutes at room temperature. Upon sonication, the extract solution was

centrifuged at 4,000 rpm for 15 minutes. It was then filtered using a 0.22µm syringe filter and the filtrate was directly analysed using Ultra High-Performance Liquid Chromatography (UHPLC) analysis. Samples were extracted and analysed on UPLC in three independent experiments (n = 3).

3.3.3. Ultra High-Performance Liquid Chromatography (UHPLC)

The UHPLC analysis was conducted based on the method previously described by Al-Malahmeh et al. (2017) with minor modification. Flexar FX-15UHPLC system model was used for the analysis. 10µl of each sample was injected to UHPLC analysis. The chromatographic separation was achieved by using Ascentis C185µm column, 25cm × 4.6mm. The column was thermostated at 30°C. The acetonitrile and ultrapure water were filtered using 0.22µm nylon membrane filter. Then, both solvents were sonicated for 15 minutes at room temperature.

The separation was made from the isocratic gradient using a mixture of acetonitrile and ultrapure water. A gradient of 40:60 of acetonitrile and ultrapure water were mixed from 0 – 10 minutes with 0.6ml/min flow rate. The mobile phase gradient was set at 40% acetonitrile for 10 minutes. The flow rate was 0.6ml/min during the whole run. UV detector with the wavelength of 201nm was used to identify estragole in the samples. The retention time of estragole was identified by injecting the 98% pure estragole standard.

The calibration curve was constructed using six different concentrations of the standard which were 0μM, 50μM, 100μM, 150μM, 200μM and 250μM. All the concentrations were injected from low to high concentration using the same method as above. Then, concentration vs area under curve (AUC) was constructed and used to calculate the concentration of the estragole in the samples.

3.4. **Benchmark Modelling**

The carcinogenicity data from Miller et al., (1983) was used to be analyse using different mathematical model of Benchmark Dose Software version 2.7 (downloaded from United States Environmental Protection Agency (USEPA) website). The models used are: Gamma, Logistic, Log-Logistic, Probit, Log-Probit, Multistage, Weibull and Quantallinear model.

3.5. **Margin of Exposure (MOE) Calculation**

The risk assessment of estragole in PFS was performed using the MOE approach by dividing the $BMDL_{10}$ values with estimated daily intake (EDI). The value of $BMDL_{10}$ was obtained from the BMDL analysis. The calculation of the EDI for the PFS selected was based on the daily intake recommended by the manufacturers as stated at the packaging label on the supplements. EDIs were evaluated by using average body weight of 6,775 Malaysian men and 3,441 Malaysian women aged 18 -59 years old; which is 62.65 kg (Azmi et al., 2009).

$$MOE = \frac{BMDL_{10}}{EDI}$$

Where;

MOE = Margin of exposure

$BMDL_{10}$ = Lower confidence level of the benchmark dose giving 10% additional cancer occurrence

EDI = Estimated Daily intake

3.6. Quality Control

There are several steps taken for quality control and assurance in this study. Firstly, the extraction of the PFS and the estragole level quantification were repeated three times. The average value was then taken as data used in this study. Secondly, the extraction of the PFS and the quantification analysis was done on the same day. This is to avoid any estragole lost during analysis due its characteristics of high evaporability. Thirdly, the UHPLC were purged before the analysis and the solvent used were prepared fresh.

3.7. Data Analysis

The mean difference of the samples was analyse using IBM SPSS statistics Version 24. The normality of data was tested and One-way ANOVA analysis was used to analyse mean difference of estragole level between the samples.

CHAPTER 4

RESULTS

4.1 Levels of Estragole in Plant Food Supplements (PFS)

4.1.1. Calibration Curve

Six different concentrations of pure standard of estragole were prepared and injected into UHPLC. A calibration curve was constructed to calculate the concentration of estragole in PFS. The calibration curve was plotted as concentration vs the AUC (Figure 4.1). The equation, y= 9477.8x + 3282.4 was used to calculate the concentration of estragole in the PFS. The R^2 value, 0.9334 shows a strong correlation between the concentration and AUC.

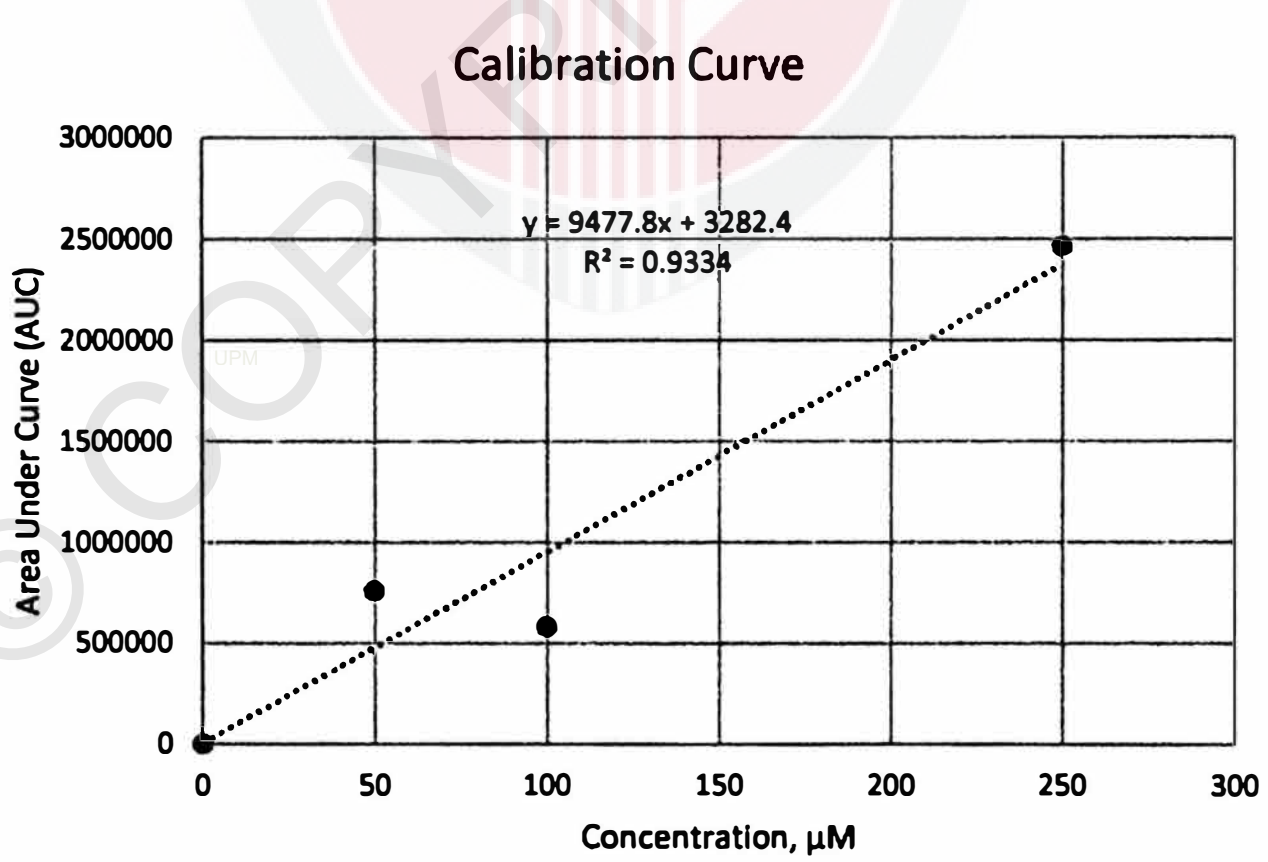


Figure 4.1: Calibration curve of Estragole

4.1.2. Level of Estragole in PFS

Figure 4.2 shows the ten samples of PFS that were analysed in the present study. The chromatogram (Figure 4.3) shows an example of the presence of estragole in sample number 8. It was shown that estragole was eluted at minutes 9.1 while, the peak at minutes 3.5 that is marked with asterisk was an unidentified peak.

Sample 1

Sample 2

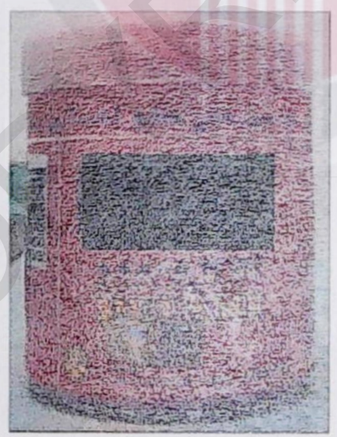
Sample 3

Sample 4

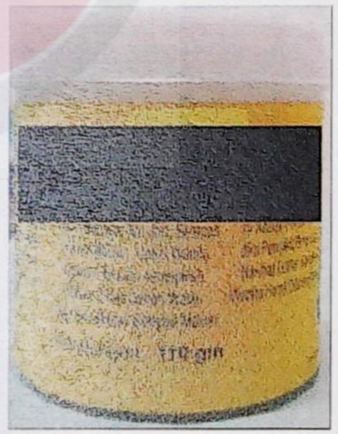
Sample 5

Sample 6

Sample 7



Sample 8



Sample 9

Sample 10

Figure 4.2: The PFS sample (Sample 1 – 5 is in capsules form while sample 6 – 10 is in paste or tablet form).

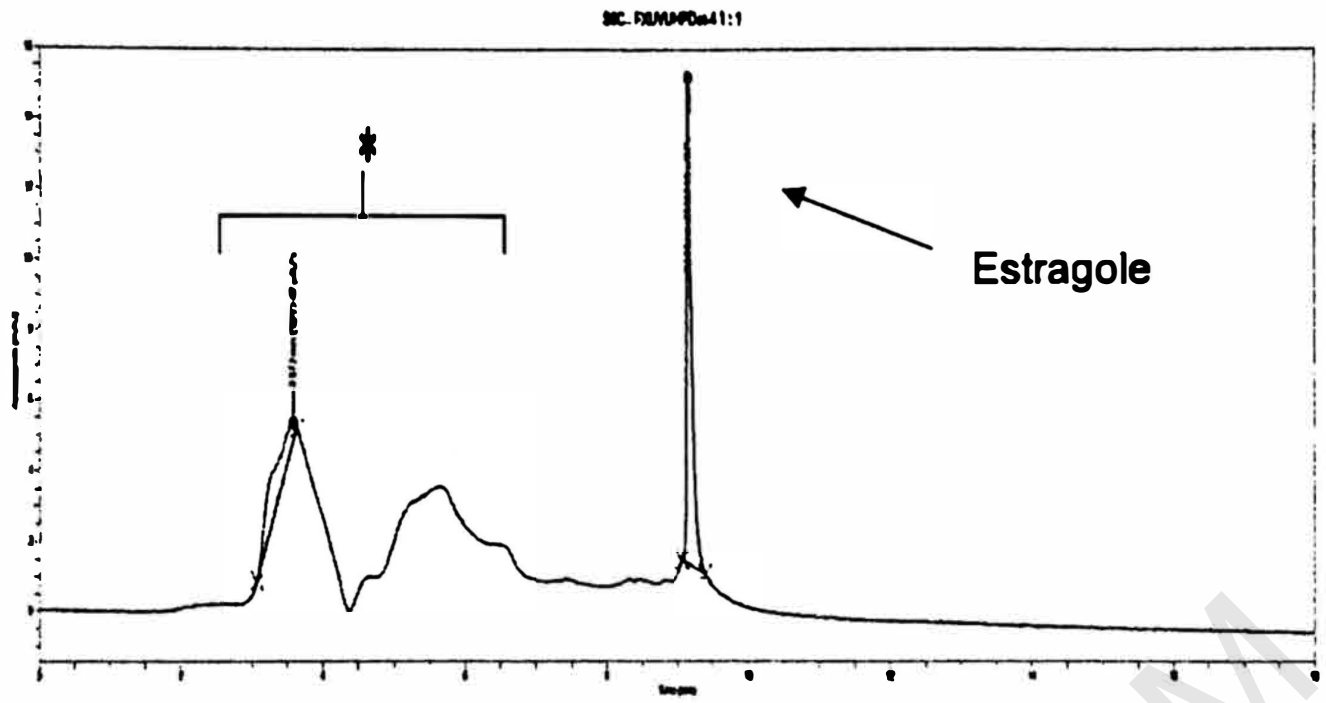


Figure 4.3: UHPLC chromatogram from sample 8. Peaks marked with asterisk (*) were not identified. The chromatogram was obtained at a wavelength of 201nm.

Figure 4.4 shows the bar graph of the mean (±standard deviation) of the level of estragole in the samples while Table 4.1 presents the results for the level of estragole in all the PFS. One Way ANOVA analysis was carried out to determine the significant difference of mean of estragole in between samples. Based on the analysis, estragole was found in all samples. For results, the highest estragole level was detected in sample 3 with 1950.6 µg/g (±1575.4 µg/g) while the lowest estragole level was found in sample 10 with 371.8 µg/g (±275.5 µg/g). The data of estragole level was normally distributed with p value more than 0.05 (Shapiro-Wilk). Based on the One-Way ANOVA analysis, there was no significant difference of the estragole level between the samples, where $F[(9,20) = 0.754, p = 0.658]$, $p>0.05$.

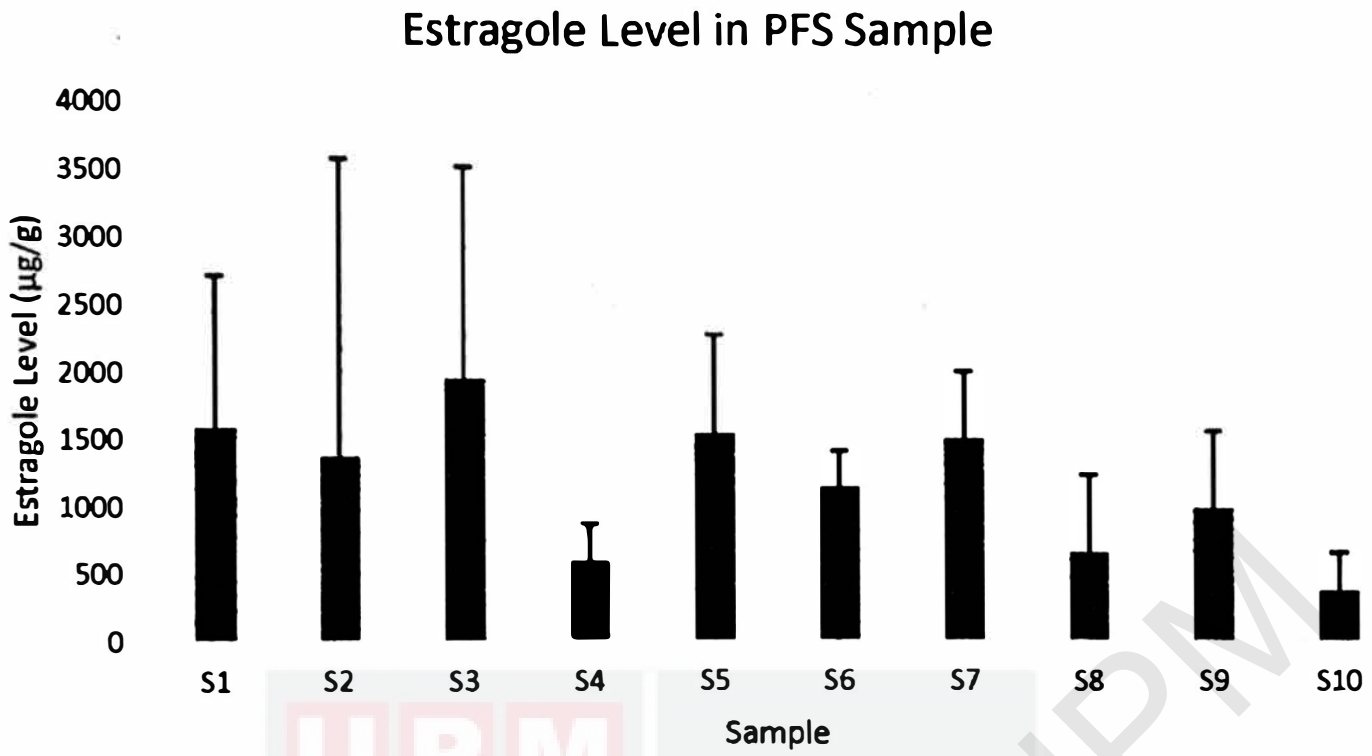


Figure 4.4: The mean and standard deviation of estragole level in PFS sample

Table 4.1: The level of estragole in PFS as determined by methanol extraction

Sample Number	Estragole Level, µg/g Mean (±SD)	F-statistics (df)	P-value*
1	1585.0 (1131.7)		
2	1374.5 (2210)		
3	1950.6 (1575.4)		
4	596.7 (276.9)		
5	1534.0 (736.5)		
6	1142.1 (264.5)	0.754	0.658
7	1499.2 (489.7)		
8	657.5 (566.3)		
9	979.3 (570.1)		
10	371.8 (275.5)		

*One-Way ANOVA
*p-value is significant at 0.05 level.
*Samples 1-5 is in capsules form while samples 6-10 is in paste/tablet forms

4.2 Malignant Carcinogenicity Data and $BMDL_{10}$

Table 4.2 shows the carcinogenicity data represent the incidence of hepacellular carcinomas in rodents that have been exposed to estragole from Miller et al. (1983). This data was used determine the $BMDL_{10}$ value using the mathematical model of Benchmark Dose Software version 2.7 (downloaded from United States Environmental Protection Agency (USEPA) website). Table 4.3 presents the results of BMD analysis consists of seven model including Gamma, logistic, Log logistic, Probit, Log Probit, Multistage, Weibull and Quantal-linear models. The $BMDL_{10}$ was ranged from 3.29 to 6.48 mg/kg bw/day. This is the same $BMDL_{10}$ values used in van den Berg et al. (2011) study.

***Table 4.2: Carcinogenicity data on the induction of hepatocellular in rodents** (Miller et al., 1983)*

Rodent's gender	Time-adjusted dose (mg/kg diet)	Number of animals	Cancer incidence
Female mice	0	50	0
	54	48	27
	107	49	35

Table 4.3: Results of BMD analysis based on data from table 4.2

Model	No. of parameters	Log Likelihood	p-value	Accepted*	BMD_{10} (mg/kg bw .day)	$BMDL_{10}$ (mg/kg bw .day)	Ratio between BMD_{10} and $BMDL_{10}$
Full	3	-62.21					
Null	1	-100.09					
Gamma	1	-62.21	0.61	Yes	8.03	6.48	1.24
Logistic	2	-70.84	0.00	No	23.71		
LogLogistic	1	-62.21	0.99	Yes	4.71	3.29	1.43
Probit	2	-70.14	0.00	No	22.95		
LogProbit	2	-62.21	1.00	Yes	4.86	ND**	-
Multistage	1	-62.71	0.61	Yes	8.03	6.48	1.24
Weibull	1	-62.71	0.61	Yes	8.03	6.48	1.24
Quantal-linear	1	-62.71	0.61	Yes	8.03	6.48	1.24

*Criteria for acceptance included $p>0.05$ and the ratio between BMD_{10} and $BMDL_{10}$
**ND-not determined, benchmark dose computation failed. Lower limit includes zero

4.3 Estimated Daily Intake (EDI)

The estimated daily intake (EDI) of the PFS selected was calculated based on the daily intake recommended as stated at the label on supplements packaging by the suppliers, Malaysian average body weight, 62.65kg (Azmi et al., 2009) and 74.8 of Malaysian life expectancy (Department of Statistics Malaysia, 2018). The total dose was calculate using the estragole level detected in the PFS. The EDI was calculated from the results of estragole level in PFS. As shown in Table 4.4, the EDI ranged from 0.021 to 0.117 mg/kg bw/day. Sample five recorded the lowest EDI with 0.021 mg/kg bw/day while the highest EDI is from sample seven with 0.117 mg/kg bw/day.

4.4. Margin of Exposure (MOE)

The MOE was calculated where it shows all of the samples have MOE ranged less than 10,000 which indicate high priority for risk management action. This is regardless of the nature of the PFS; either in paste or capsule form. Sample seven recorded the lowest MOE with 20 - 60 while the highest MOE was 150 - 310 obtained from sample five. All of the sample have less than 10,000 value of MOE which ranged from 20 till 310 as in Table 4.4.

Table 4.4: EDI and the respective MOE of the PFS samples

Sample number	Recommended daily intake (g) of the PFS	EDI* (mg /kg bw/ day)	MOE**
1	2.367	0.060	50 - 110
2	1.427	0.031	100 - 200
3	2.407	0.075	40 – 90
4	2.367	0.023	140 - 280
5	0.873	0.021	150 - 310
6	4.957	0.090	30 - 80
7	4.893	0.117	20 - 60
8	3.393	0.036	90 - 190
9	4.027	0.063	50- 110
10	8.693	0.052	60 – 130

*EDI were calculated using average body weight of 62.65 (Azmi et al., 2009)

**MOE= $BMDL_{10}$ (mg/kg bw/day)/EDI (mg/kg bw/day)

CHAPTER 5

DISCUSSION

According to EFSA, there are many type of plants that contain estragole being widely used in PFS (EFSA, 2012). This includes the fruits of fennel, star anise and sweet cumin, basil, shoot of tarragon, parsley and root of licorice (European Medicine Agency (EMA), 2014; Van den Berg et al., 2014; Godfrey, McNaughton, & Evans, 2013; EFSA, 2012; Gori et al., 2012; Bristol, 2011; The Scientific Comittee on Food (SCF), 2001; Masten & Tice, 1999; Douglas W. Bristol, 1991).

In Malaysia specifically, herbs and spices like cinnamon, nutmeg parsley and lemongrass are commonly used in our delicacies and also as natural remedies. In Asia, a number of botanical products that contain genotoxic and carcinogenic alkenylbenzenes have been found in Chinese market with herbs like anise, fennel and basil (Ning et al., 2018). Meanwhile, Suparmi et al. (2018) reported that cinnamon, betel pepper and ginger that are being used in Indonesian jamu is proved to contain genotoxic and carcinogenic alkenylbenzenes such as estragole, apiol and myristicin.

In this present study, ten (10) PFS containing fennel (*Foeniculum vulgare* Mill.), black pepper (*Piper nigrum* L.), coriander (*Coriandrum sativum* L.), fennel (Nig*ella sativa* L.), anise (*Pimpinella anisum* L.) and long pepper (*Piper longum* L.) were selected from local market. Since there are many types of PFS being sold in the market, the sample only limited to PFS that are in capsules form and also

paste or tablet form. The PFS were then purchased and the estragole level in the PFS were quantified and the MOE value were then calculated.

Methanol extraction was used to allow total quantification of the estragole from the PFS since the PFS itself is taken as a whole pill or capsule. Methanol may extract some of the active ingredients but, others will remain bound to the matrix (Suparmi et al., 2018). Therefore, it is safe to assume that the estragole present in the PFS would be available in the gastrointestinal tract for further uptake. Due to this reason, the PFS were extracted with methanol to optimise the extraction. Methanol act as an ingredient in breaking down the cell wall thus releasing the cellular substance (Alajlouni et al., 2016). In addition, the ultrasonification used in the extraction process further destruct the cell wall and improve extraction efficiency (Zayas, 1986). The extraction efficiency is also affected by powder size, which is why the PFS were since previous studies from Alajlouni et al.(2016) and Van den Berg (2014) showed that extraction from fine cut material is higher than course material.

The results show that estragole was present in all samples with the range of 371.8±271.5 µg/g to 1950.6±1575.4 µg/g. The extraction of the PFS was done fresh before the UHPLC analysis to reduce the estragole degradation in the samples. A total of five (5) samples were in paste form while the rest are in capsules or pills form. Sample one till five were in capsules form while sample six to ten were in paste or tablet form. The daily intake of the PFS were once or twice daily, depending on the instruction. It is found that the consumers are exposed to higher level of estragole via consumption of PFS in capsules form as compared topaste or tablet form.

According to Van den Berg et al. (2011), the level of estragole ranging between 0.07±0.005 to 241.56±62.02 mg/g showing that the current results are within the range found from the literature. In 2018, study from Ning et al. found estragole in 30 samples out of 71 samples from Chinese market meanwhile, Suparmi et al. recorded 23 out of 25 samples to contain alkenylbenzenes derivatives including estragole. These samples from the studies were obtained from the botanical ingredients in the PFS and Indonesian jamu, respectively. Apart from that, studies from Ismaiel et al. (2016) also found the level of estragole to be between 0.0074 mg to 16.74 mg in Fennel, Chinese and Japanese Star Anise. Van den Berg et al. (2011) also reported 0.07 to 241.56 mg/g PFS containing estragole.

Sample ten contain the lowest level of estragole with 371.8 µg/g while sample three have the highest estragole level with 1950.6 µg/g; where both are in paste and capsule form, respectively. The p-value of the estragole level in PFS is 0.658 using One-way ANOVA analysis which indicates that there was no significant difference of the estragole level between the samples. The recommended daily intake of both sample ten and three are 8.693g and 2.407g. This indicates that sample ten have the highest daily intake recommendation while sample five recorded the lowest with 0.873g.

The EDI of estragole was calculated by using the recommended daily intake of the PFS as suggested by the manufacturer. This can be found at the back of the PFS bottle itself. Azmi et al. (2009) reported 62.65kg as Malaysian average body weight where this was used to calculate the EDI. This data has been used since the first study conducted on the risk assessment of estragole in Malaysia.

Meanwhile, EFSA (2012) suggested to use 70kg as default value for the European adult.

The EDI of estragole from the consumption of PFS ranging from 0.021 mg/kg bw/day to 0.117 mg/kg bw/day. The minimum value for EDI are from sample five which is in capsules form while the maximum value of EDI recorded from sample 7 that are in paste form. From the data, it can be concluded that the paste form of PFS contributes to higher EDI value than the PFS in capsules form. This is because the PFS in paste form usually have higher daily intake as compared to PFS in capsules form.

Apart from that, study from Ning et al. (2018) recorded EDI values ranging from 0.02 to 278.0 μg estragole equivalents/kg bw/day. Van den Berg et al. (2011) on the other hand stated 0.001 to 4.78 mg/g PFS containing estragole in her research study.

MOE is the ratio of the no-observed-adverse-effect level (NOAEL) or benchmark dose lower confidence limit (BMDL) for the critical effect to the theoretical, predicted, or estimated exposure dose or concentration (WHO, 2009). According to JECFA (2005), MOE is the preferred option in calculating risk management especially for genotoxic and carcinogenic substances since the NOAEL cannot be ruled out. Similarly, the EFSA concluded that the magnitude of an MOE could be used by risk managers for priority setting and was more informative than advising that exposures should be reduced to ALARA (EFSA, 2005).

There are many advantages of MOE approach. One of it is that it sets a base in identifying priority of the botanical ingredient besides other priority in the area of genotoxic and carcinogenic compounds in food (Van den Berg et al., 2011). The MOE is the most scientifically reliable way to deal with the detailing of guidance since it includes the intake or exposure and the accessible data on the dose-response relationship are utilized without extrapolation or the generation of conceivably unverifiable risks estimates (Barlow et al., 2006).

The MOE value for the PFS were calculated by using computed $BMDL_{10}$ and the calculated EDI value. From the results, all PFS have MOE value less than 10,000 indicate that there is priority for risk management action. Interestingly, all of these samples recorded MOE value ranging from 20 to 150 for 3.29 mg/kg bw/day of $BMDL_{10}$ and 60 to 310 for $BMDL_{10}$ with 6.48 mg/kg bw/day showing high priority of risk management. This is proven through sample seven that recorded highest EDI, therefore contribute to lowest MOE value that indicates highest priority of risk management.

This is in accordance with studies from Suparmi et al. (2018), where the MOE value of alkenylbenzenes in Indonesian jamu ranging from 11 to 21,191. Besides, Van Den Berg et al. (2011) reported MOE values range between 3 to 20,000 for estragole in fennel containing PFS and 1 to 2000 for estragole in basil containing PFS. Meanwhile, studies from Van den Berg (2013) shows MOE values ranging between 200 to 1000 and 1 to 40 for PFS consisting powdered basil material or its oil essence.

In contrast, MOE identify the priority for risk management action but it does not provide a quantitative estimate of risk. Therefore, it might be misinterpreted as giving measure of a risk (Barlow et al., 2006) where further explanation should be included. MOE provide guidance on priority setting for the risk management actions (Barlow et al., 2006) and it is a method that can be used by risk assessors to prioritize the risk from the exposure estragole via consumption of PFS. The effects of estragole are likely to be less when estragole is consume in a PFS compared to pure estragole as it goes through many chemical process that might lessen its reactivity. The carciniogenicity data for the $BMDL_{10}$ analysis (Miller et al., 1983) was administered pure estragole to the rodents. This may result in overestimation of the risk management priority (Van Den Berg et al., 2013)

The result of MOE for all PFS involved shows that there is high priority for risk management action. This is because the MOE value for the samples does not only lower than 10,000 value but also less than 1,000. Prompt action should be plan and taken in order to minimize the risk of genetic mutation and also cancer among the PFS consumer due to consumption of these samples.

CHAPTER 6

CONCLUSION, STUDY LIMITATION AND RECOMMENDATION

6.1 Conclusion

In conclusion, there is a high priority for risk management actions due to exposure of estragole via consumption of PFS. Consumers are exposed to 0.021 mg/kg bw/day to 0.117 mg/kg bw/day estragole from consumption of the PFS. This is considered high as all of the PFS have MOE value less than 10,000 regardless of the nature of the PFS; either in capsule or paste form. All parties including the consumer, manufacturer, related agencies or authorities and government are held responsible in creating safe environment and products or all. Awareness is among of the key factor in risk management action of estragole in PFS. This can be done to health campaign, talk or advertisements through mass media to communicate the risk of consumption of PFS containing estragole. Risk management plan can be conducted by the government and also related authorities or agencies in dealing with this issue while the consumers should be able to have the knowledge on the presence of genotoxic and carcinogenic chemical by reading the PFS label thus avoid consuming any PFS that contain estragole.

6.2 Study Limitation and Recommendation

There are a few limitation that could have been improved in this study. First, limited number of samples being used in this study that covers only a small percent of the PFS available in Malaysian market. This includes only PFS that comes in capsules or pills form and also paste form while there are vast of other form of PFS sold in Malaysia such as in tea bag and sachets as well as in liquid form.

Apart from that, the accuracy and percentage of recovery of the estragole experiment was not satisfied due to limited research time. This is needed for the correction of the estragole amount that lost during the extraction procedure. This will determine a more validated result in regards with the chemical properties of the estragole that are easily evaporated.

Based on the findings, these are some recommendations for future study. Extensive range of PFS should be selected which includes both local and imported PFS. Next, one should consider to sample a variety of plant species containing estragole before choosing the sample. The experiments should also be repeated or replicated for more accurate data. This includes the analysis for the recovery and calibration curve of the model compound, as it is a critical steps to determine the level of estragole in the PFS. In addition, it is recommended to use food frequency questionnaire for the consumers to identify the most popular PFS used among Malaysian and also their real intake of PFS.

The result from this study confirms that high priority for risk management action should be taken against the level of estragole in PFS. Thus, responsible parties such as Ministry of Health Malaysia could develop a proper risk management plan in order to educate the consumers regarding this issue as well as to control the amount of natural products that may contain genotoxic and carcinogenic estragole in PFS.

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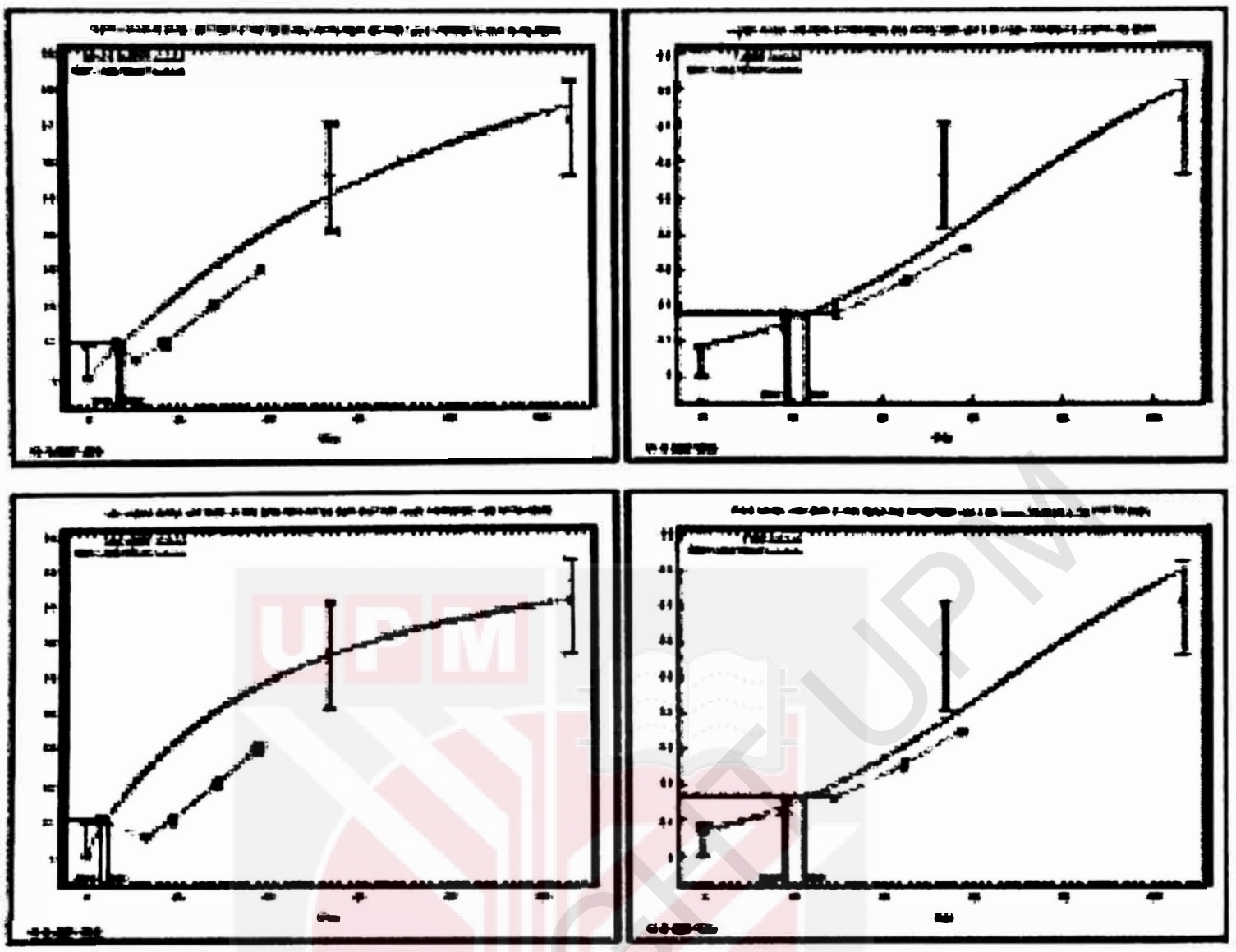
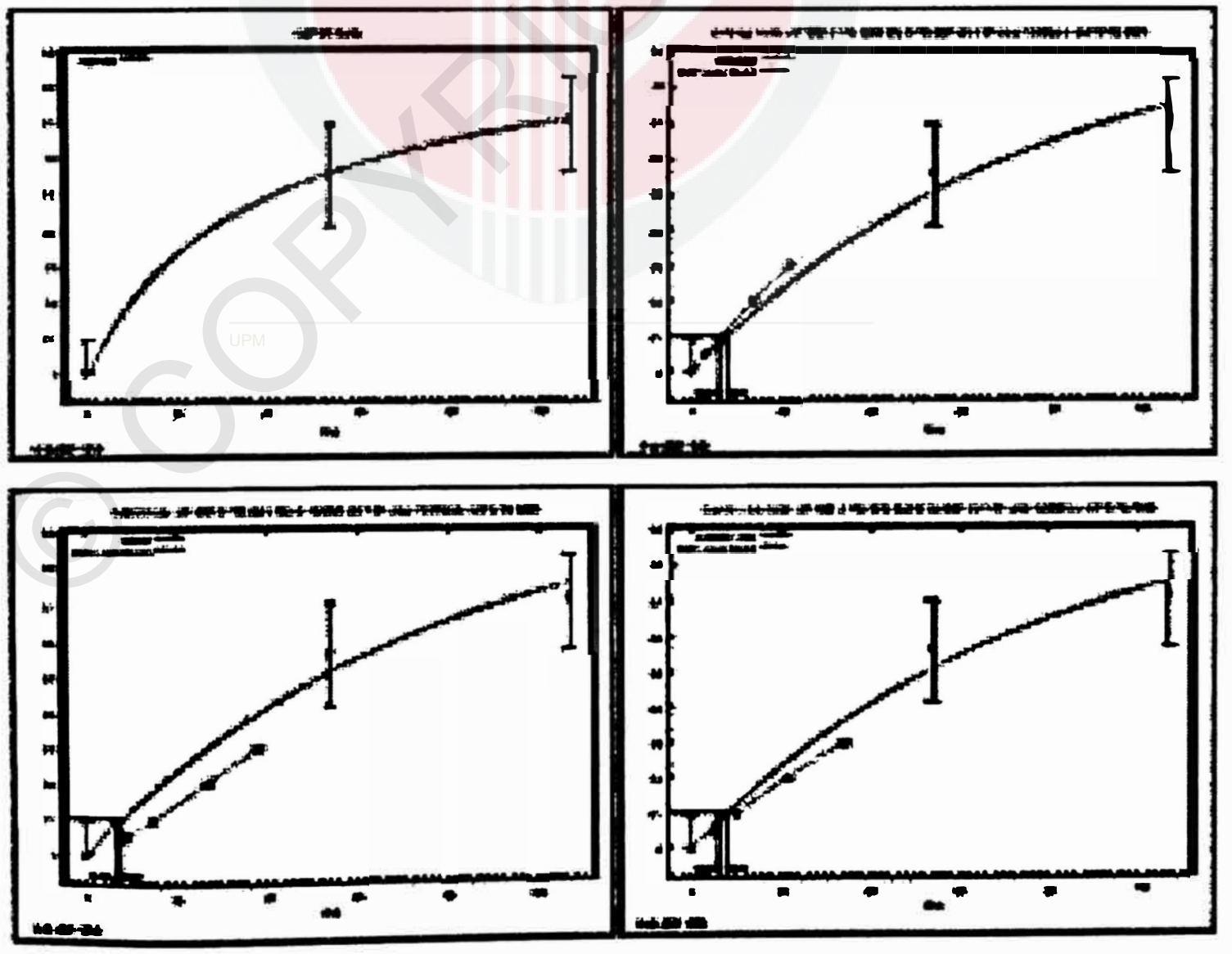
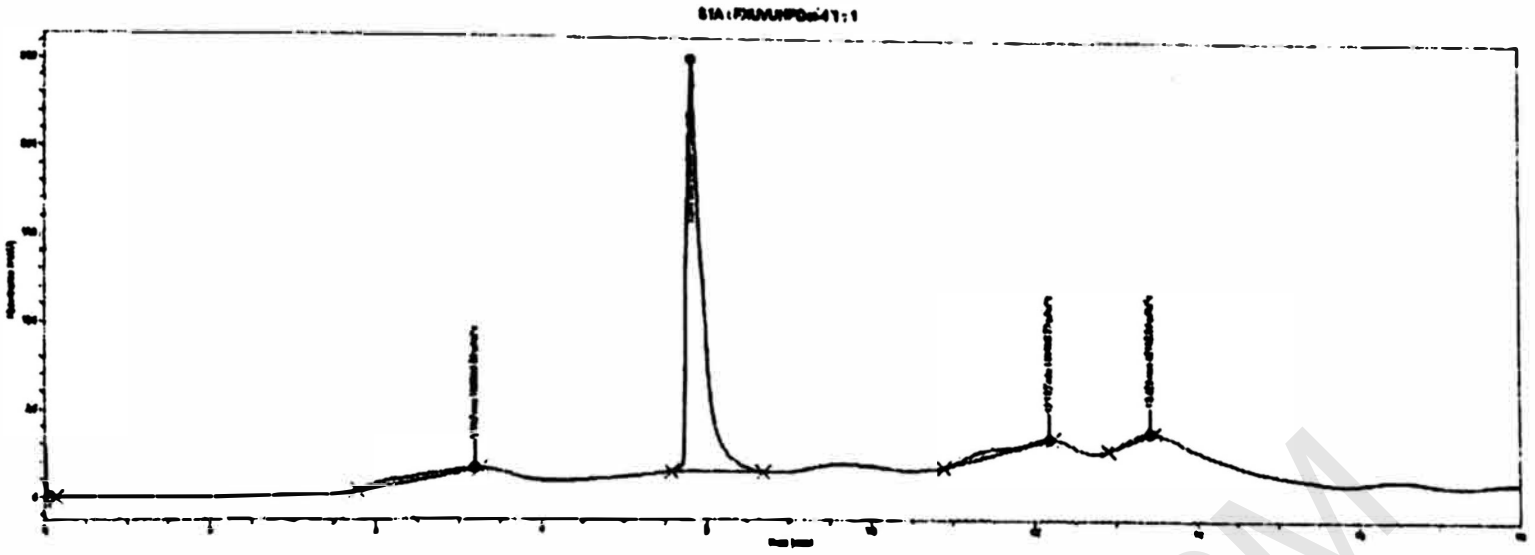
Appendix 1: The BMDL Modelling Analysis



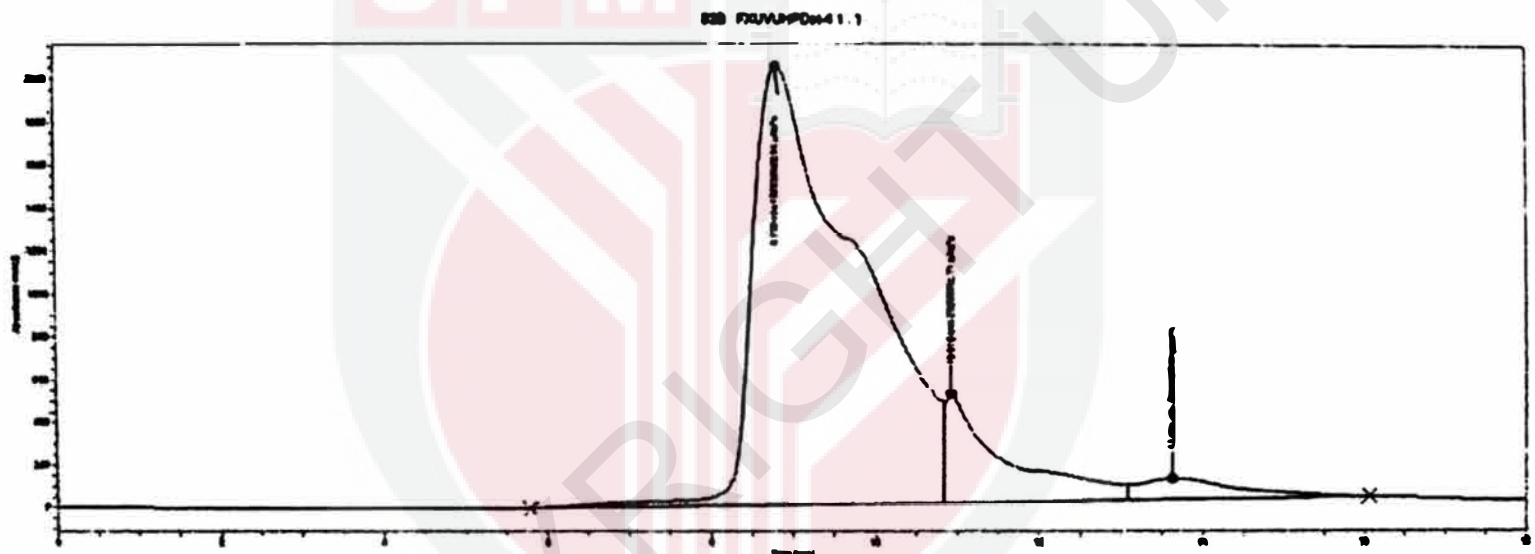


Appendix 2: The chromatograms of the samples

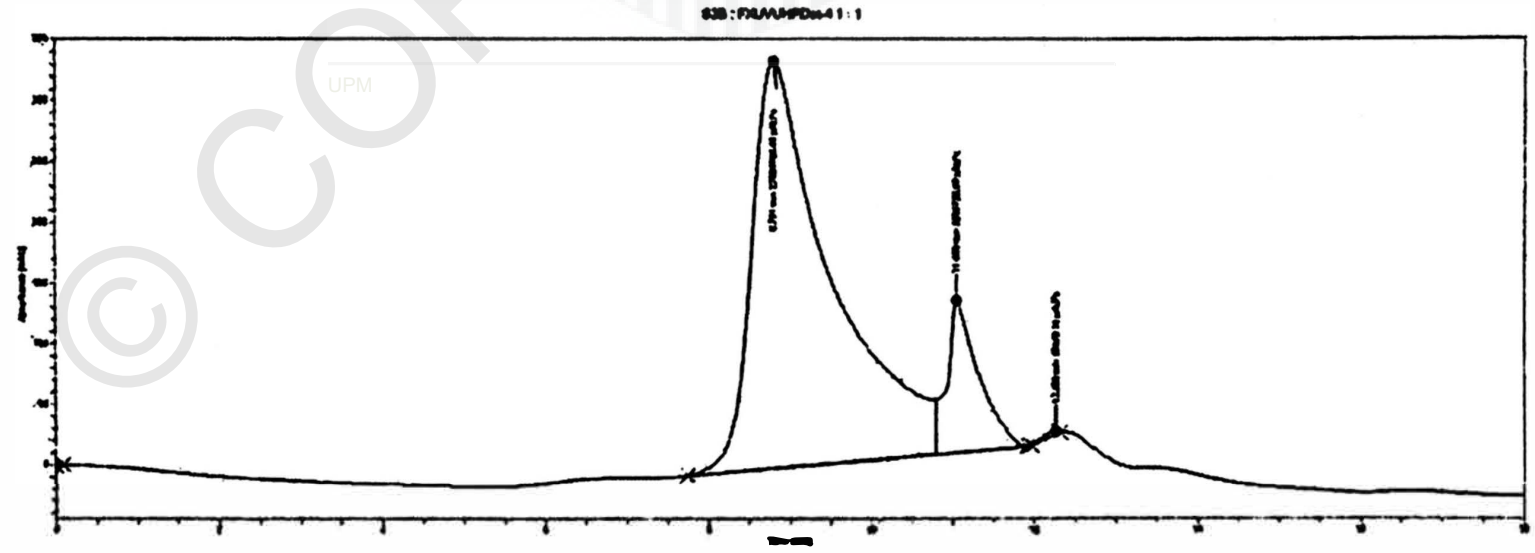
Sample 1



Sample 2



Sample 3



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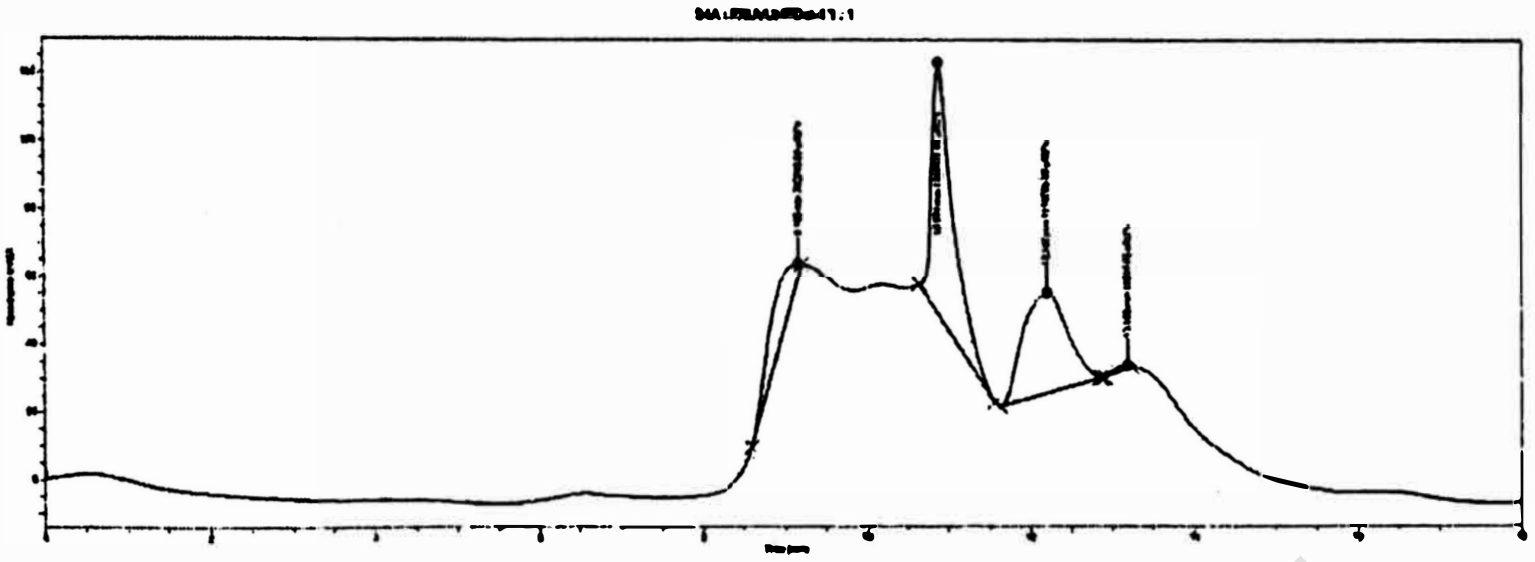
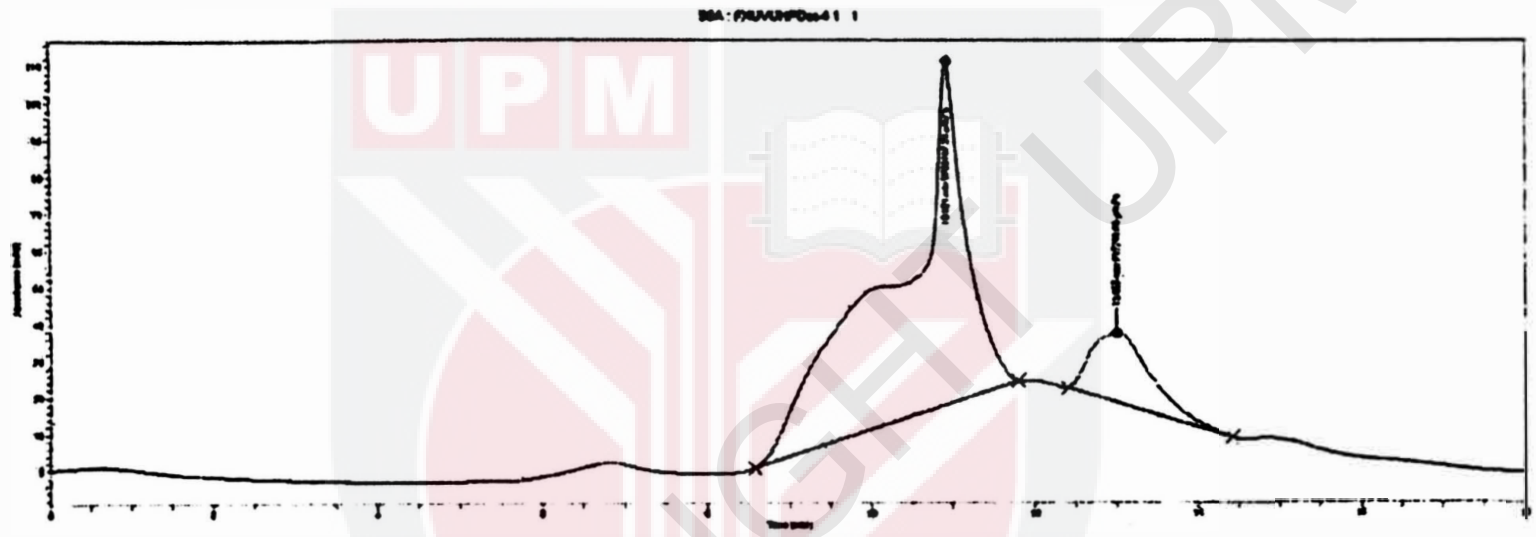


Sample 5



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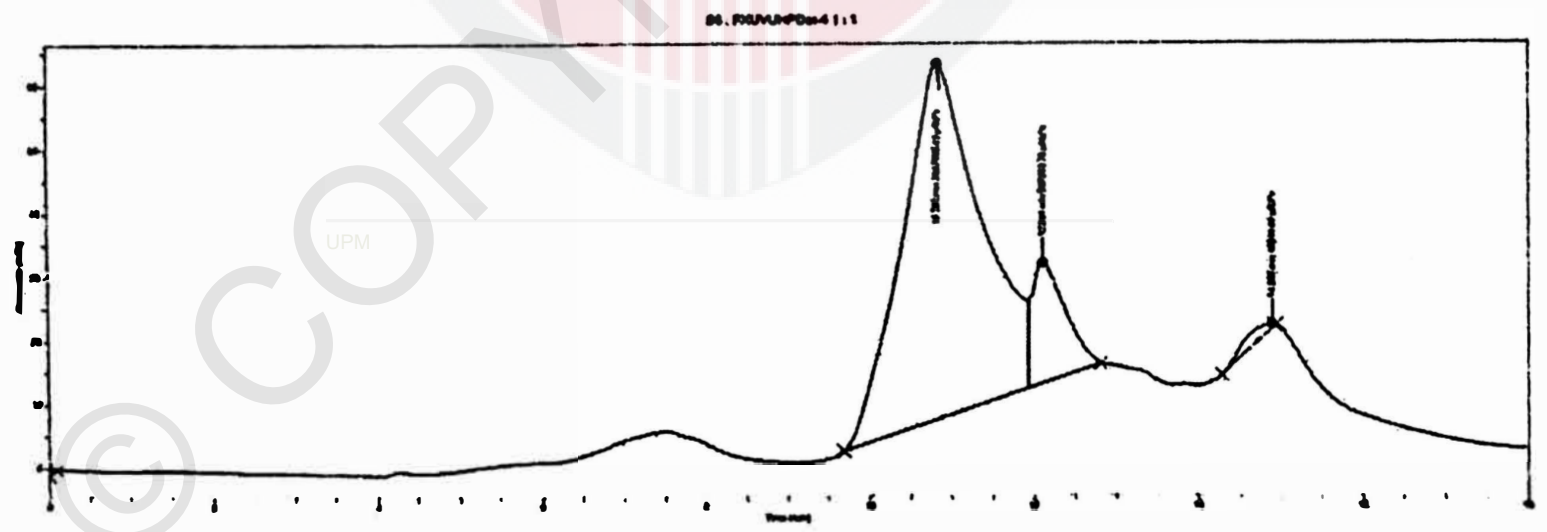
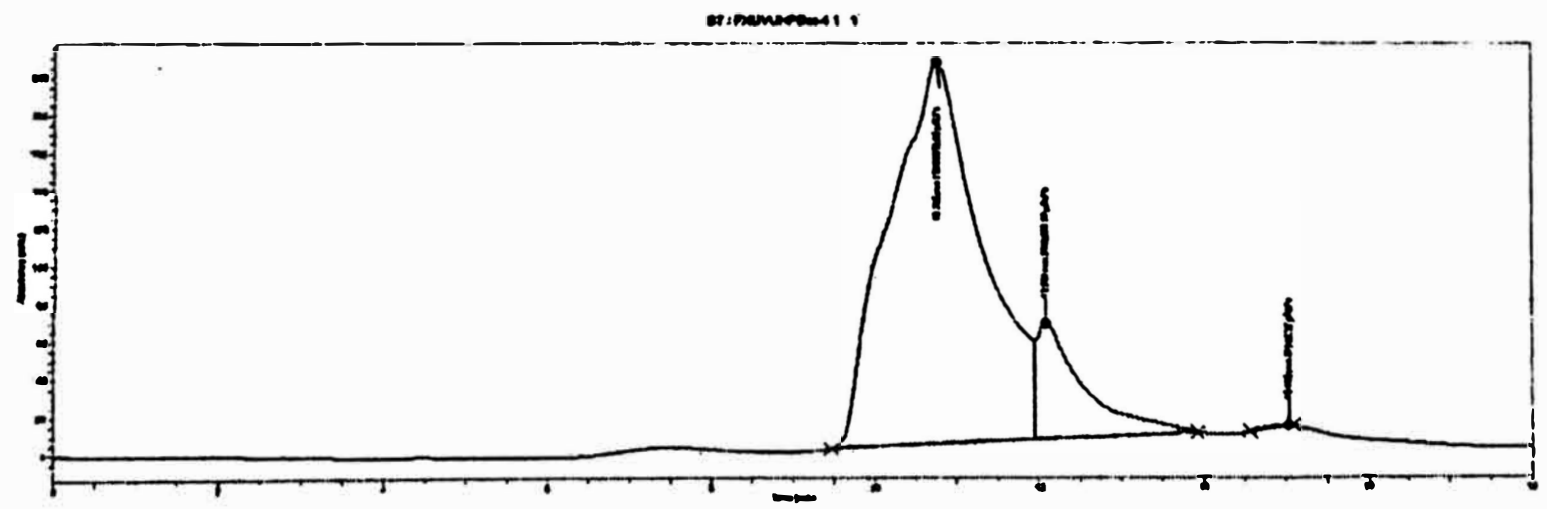
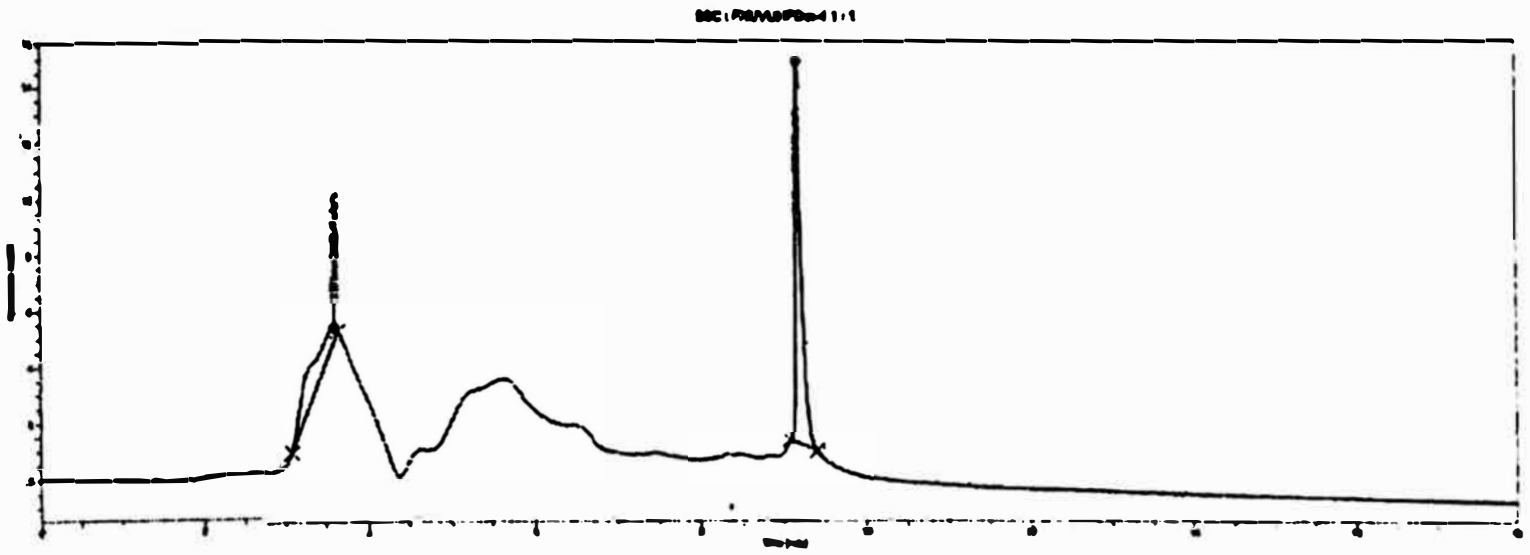


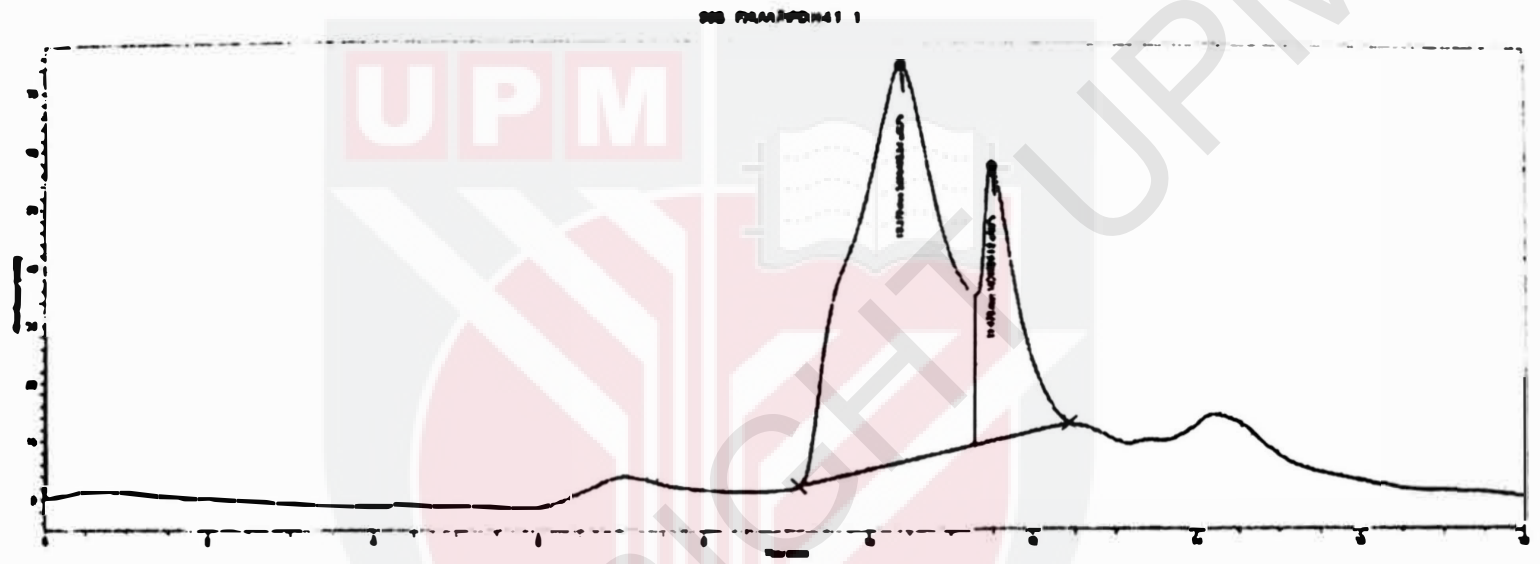
Sample 7



Sample 8



Sample 9



Sample 10