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RISK ASSESSMENT OF ESTRAGOLE IN PLANT FOOD SUPPLEMENTS USING MARGIN OF EXPOSURE APPROACH

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ABSTRACT

RISK ASSESSMENT OF ESTRAGOLE IN PLANT FOOD SUPPLEMENTS USING MARGIN OF EXPOSURE APPROACH

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Plant food supplements (PFS) are commonly used by the consumers without knowing the actual ingredients in the PFS and they thought that all "natural" ingredients are "safe". Estragole is one of alkenylbenzenes constituent that is a genotoxic and carcinogenic compound that is naturally exist 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 as suggested by European Food Safety Authority. 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 113.5±63.3 to 378.7±221 mg/g. The EDI values were calculated ranging from 1.11×10^{-4} to 3.36×10^{-3} mg/kg bw/day. Five samples were found to have MOE less than 10,000 (60% of recovery) while only three samples were found to have MOE less than 10,000 (100% of recovery) indicating the priority for risk management action. All of these samples are in paste forms and the EDI of the estragole in paste forms are higher compared to capsule form. The calculated MOE reflected a priority for risk management action, however it does not provide a quantitative estimate of risk. **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, margin of exposure, estimated daily intake

ABSTRAK

PENILAIAN RISIKO ESTRAGOL DALAM SUPLEMEN BERASASKAN TUMBUHAN MENGGUNAKAN KAEDAH MARGIN PENDEDAHAN

NUR 'AMIRAH BINTI A RAZAK

Makanan tambahan berasaskan tumbuhan lazimnya digunakan oleh pengguna tanpa mengetahui ramuan 'sebenar' yang terdapat dalam suplemen berasaskan tumbuhan (SBT) dan mereka berpendapat bahawa semua bahan "semulajadi" adalah "selamat". '*Estragole*' adalah salah satu unsur '*alkenylbenzene*' yang merupakan sebatian genotoksik dan karsinogenik yang secara semulajadi wujud dalam tumbuhan seperti basil, adas dan '*anise*'. **Objektif**: Kajian ini dijalankan untuk menentukan risiko daripada pendedahan kepada '*estragole*' melalui penggunaan makanan tambahan berasaskan tumbuhan menggunakan kaedah '*Margin of Exposure (MOE)*'. **Kaedah**: Sepuluh SBT tempatan secara telah dibeli dan tahap '*estragol*e' dalam sampel dikira menggunakan '*Ultra High Performance Chromatography Liquid*' (UHPLC). Pendekatan MOE digunakan untuk mengira risiko bahan genotoksik dan karsinogenik seperti yang dicadangkan oleh Pihak Berkuasa Keselamatan Makanan Eropah. MOE dikira dengan membahagikan paras dos '*Benchmark*' ($BMDL_{10}$) kepada anggaran pengambilan harian makanan tambahan berasaskan tumbuhan. **Keputusan dan Perbincangan**: '*Estragole*' telah dikesan dalam semua sampel dalam julat diantara 113.5 ± 63.3 - 378.7 ± 221 mg/g. Nilai anggaran pengambilan harian adalah dalam julat diantara 1.11×10^{-4} - 3.36×10^{-3} mg/kg bw/hari. Lima sampel didapati mempunyai MOE kurang daripada 10,000 (60% pemulihan) manakala hanya tiga sampel yang didapati mempunyai MOE kurang daripada 10,000 (100% pemulihan) yang menunjukkan keutamaan untuk tindakan pengurusan risiko. Semua sampel ini dalam bentuk pes dan anggaran pengambilan harian dari '*estragole*' dalam bentuk pes adalah lebih tinggi berbanding dalam bentuk kapsul. Pendekatan MOE dapat mengenal pasti keutamaan untuk tindakan pengurusan risiko, namun ia tidak memberikan anggaran kuantitatif risiko. **Kesimpulan:** Kesimpulannaya, populasi Malaysia terdedah kepada '*estragole*' melalui penggunaan SBT dan tindakan pengurusan risiko diperlukan untuk mengawal pendedahan kepada kimia semulajadi yang mempunyai ciri genotoksik dan karsinogenik.

Kata kunci: '*Estragole*', Makanan tambahan berasaskan tumbuhan, '*Margin of Exposure (MOE)*', anggaran pengambilan harian

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LIST OF ABREVATIONS

PFS	Plant food supplements
MOE	Margin of exposure
EDI	Estimated daily intake
SULT	Sulfotransferase
GRAS	Generally Recognize as Safe
IARC	International Agency for Research on Cancer
PBBK	Physiologically Based Biokinetic
DCA	Drug Control Authority
WHO	World Health Organization
EFSA	European Food Safety Authority
USEPA	United States Environmental Protection Agency
SD	Standard Deviation
BMR	Benchmark Dose Response
POD	Points of Departure

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CHAPTER 1

INTRODUCTION

1.1 Background

1.1.1 Alkenylbenzene

Alkenylbenzenes is a group of compounds that are naturally occurring in plants such as basil, nutmeg, fennel and clove flower (van den Berg et al., 2011a). These types of botanicals are commonly used in plant food supplements (PFS), herbal teas as well as flavoring agent. Alkenylbenzenes metabolites including estragole, myristicine, safrole and eugenol have been reported genotoxic and carcinogenic in rodents (Avila et al., 2009).

Avila et al., (2009) mentioned that chemically, alkenylbenzenes can be differentiate into two classes which are allylbenzenes (with a 2,3-double bond) and propenylbenzenes (with a 1,2-double bond). Allylbenzenes such as estragole, safrole, elemicin, myristicin, apiol and methyleugenol (Figure 1) are genotoxic and carcinogenic in rodents while propenylbenzenes such as isoeugenol or anethole are non-genotoxic. The alkenylbenzenes are converted to genotoxic and carcinogenic 1'-

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sulfooxymetabolites by cytochrome P450 and sulfotransferase (SULT) mediated. 1'-sulfooxymetabolites will bind to DNA and this will cause liver cancer (van den Berg et al., 2011b).

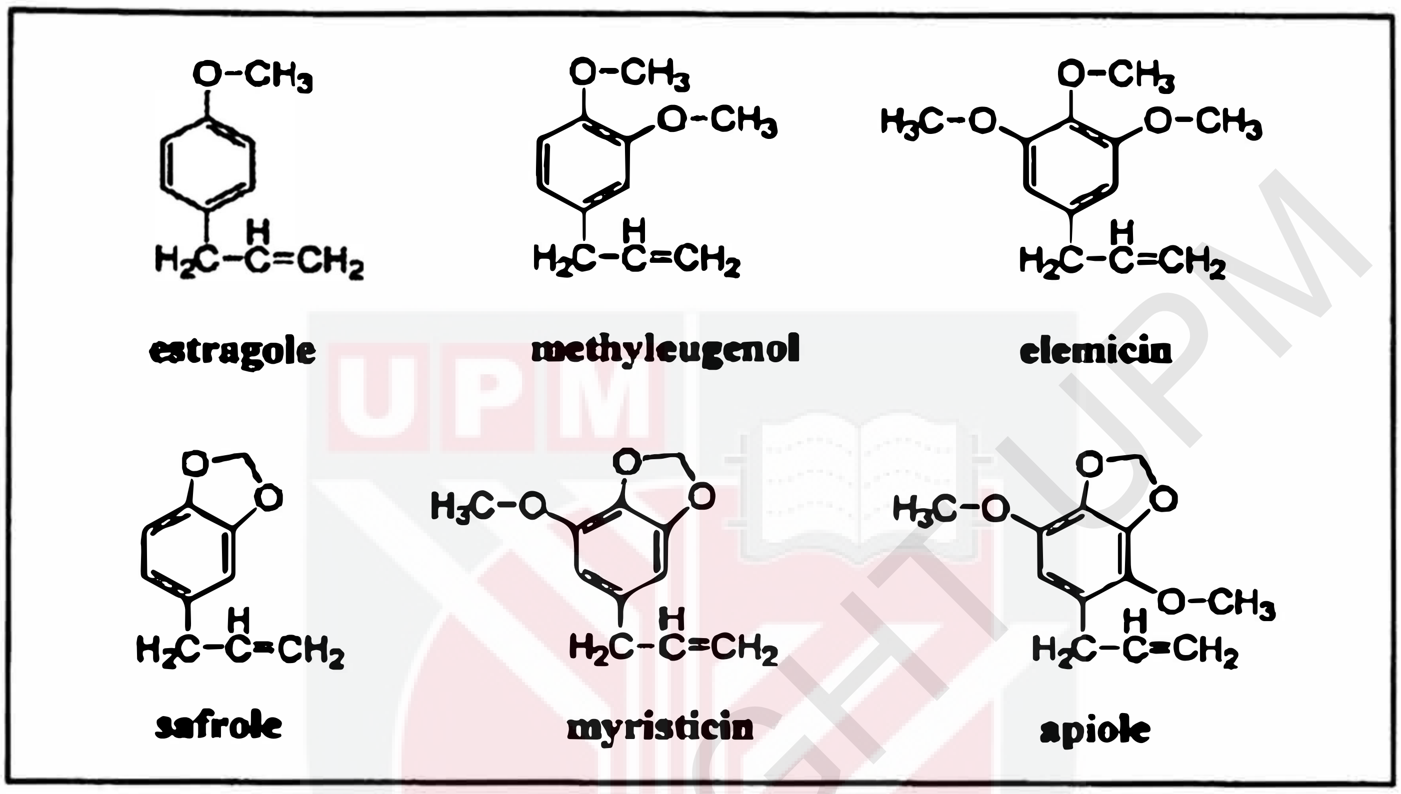

Figure 1.1: The structural formula of alkenylbenzene compounds (Rietjens et al., 2008)

There are studies conducted that aimed to evaluate the alkenylbenzenes toxicity on experimental animals (Phillips et al., 1981; Randerath et al., 1984) and risk assessment of alkenylbenzene (van den Berg et al., 2014). The research on experimental animals is important for the risk assessment. The toxicological data is needed in determination and extrapolation of reference point or point of departure. The selection of the available toxicological data are based on the quality and extent of the data to develop the strength of the reference point or point of departure (Barlow et al., 2006). Miller et al. (1983) found that the male mice that treated with safrole or estragole developed a hepatic tumor. This study was coincides with

Phillips et al. (1984) and Randerath et al. (1984) that safrole, estragole and methyleugenol resulted in significant increase of incidence of hepatic carcinomas.

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1.1.2 Estragole

Estragole (1-allyl-4-methoxybenzene; CAS 140-67-0) is one of the alkenylkbenzenes derivatives which is natural constituents in plants such as basil, tarragon, anise and fennel (Martins et al., 2012). 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, 1999).

The bioactivation of estragole to 1'-hydroxyestragole and sulfonation of 1'-hydroxyestragole will produce 1'-sulfooxyestragole, which is able to form DNA adducts in liver and eventually lead to cancer formation (Punt et al., 2009). Estragole shows toxicity to liver in male rats (Ding et al., 2015) but estragole was not mutagenic in any strains of *Salmonella typhimurium* (Drinkwater et al., 1976). Randerath et al. (1984) also discovered that alkenylbenzene compounds such as safrole, estragole and methyleugenol showed the most active metabolite that bind to DNA in the liver of mouse (1 adduct in 10,000-15,000 DNA nucleotides).

Margin of exposure (MOE) is a method that are recommended by European Food Safety Authority (EFSA) for risk assessment of substances that are both genotoxic and carcinogenic (EFSA, 2005). The MOE value of 10,000 or higher is consider low priority for risk management action and if the MOE value less than 10,000, there is priority for risk management action (Barlow et al., 2006). Estragole

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was found in basil and fennel PFS which have MOE value of 1-1,000, 000 and 3-20,000 respectively (van den Berg et al., 2011a)

In Malaysia, the management of the genotoxic and carcinogenic compounds in PFS is not harmonize. There is no specific regulation on the usage and risk management plan of the PFS containing alkenylbenzenes. In addition, alkenylbenzenes are not listed in the Drug Registration Guidance Document by Ministry of Health Malaysia. However, one of the genotoxic and carcinogenic compound which is aristolochic acids is already ban for its use in any PFS (MOH, 2011). Aristolochic acids have been classified in group 1 by International Agency for Research on Cancer (IARC) and have been demonstrated to cause genotoxicity, carcinogenicity and nephrotoxicity (Abdullah et al., 2017). Currently, one of the main challenge is on the assessment of possible risks for human health from the exposure to low levels of natural occurring food-borne compounds that are genotoxic and carcinogenic. In the present study, exposure level of estragole in PFS is quantified and MOE approach is used to assess the possible risk to human health and to determine the priority for risk management actions.

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1.2 Problem Statement

Plant Food Supplements (PFS) are widely used in Malaysian diet. The PFS are easily accessible at the pharmacies, shops and at the night markets. The PFS also can be purchased easily via online market. Prevention is considered as better than cure because it is easier to stop the problem from happening than to solve the problem afterwards. Therefore, food and nutritional supplements are considered as a way to maintain human health. Food and nutritional supplements include minerals, sport nutrition products, vitamins, natural food supplements and other products that increase the diet nutritional content (International Trade Administration, 2017).

PFS contain natural ingredients for example herbs, plants and fruits. Generally, consumers would prefer to take PFS more because of the 'natural' ingredients containing in the supplements. The consumers usually consider that 'natural' equals 'safe'. The truth is not all 'natural' is actually 'safe'. This is a false concept that needed to be corrected by providing evidence so that public could choose PFS to be consumed wisely (van den Berg et al., 2011b). Some of the product manufacturers show misleading claims and testimonies to the consumer to convince them to buy their products without knowing the actual adverse effects of the PFS to the consumer's health. Some of the supplements do not have sufficient information on the package labelling. For example, the plant species that contain in the supplements are not listed properly.

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1.3 Study Justification

This study is the preliminary study to determine the risk from exposure to estragole via consumption of PFS in Malaysian diet. Due to the easily accessible to PFS, consumers are unaware of the toxic ingredients that may contain in the PFS that could lead to adverse effects to their health. There was no study been conducted yet in Malaysia on the risk assessment of alkenylbenzene compound in the supplements. This study is important to provide scientific evidence on the risk from the estragole exposed through the PFS consumption.

Human exposure to estragole is common as its use as flavoring agent, additive and fragrance in variety of foods and cosmetic products as well as through the consumption of herbs for instance basil and tarragon (Bristol, 2011). Other countries such as Netherland, USA and German had conducted many researches on genotoxicity, carcinogenicity and risk assessment of estragole in PFS. In this study, we are focusing on estragole exposure and its risk via consumption of PFS in Malaysia.

There are no specific guidelines on the control of the alkenylbenzenes content in the PFS for the manufacturer to comply. This study could provide a baseline or reference to develop the guidelines in the future. Therefore, this study could provide a new information on the risk management plan of estragole exposure in PFS.

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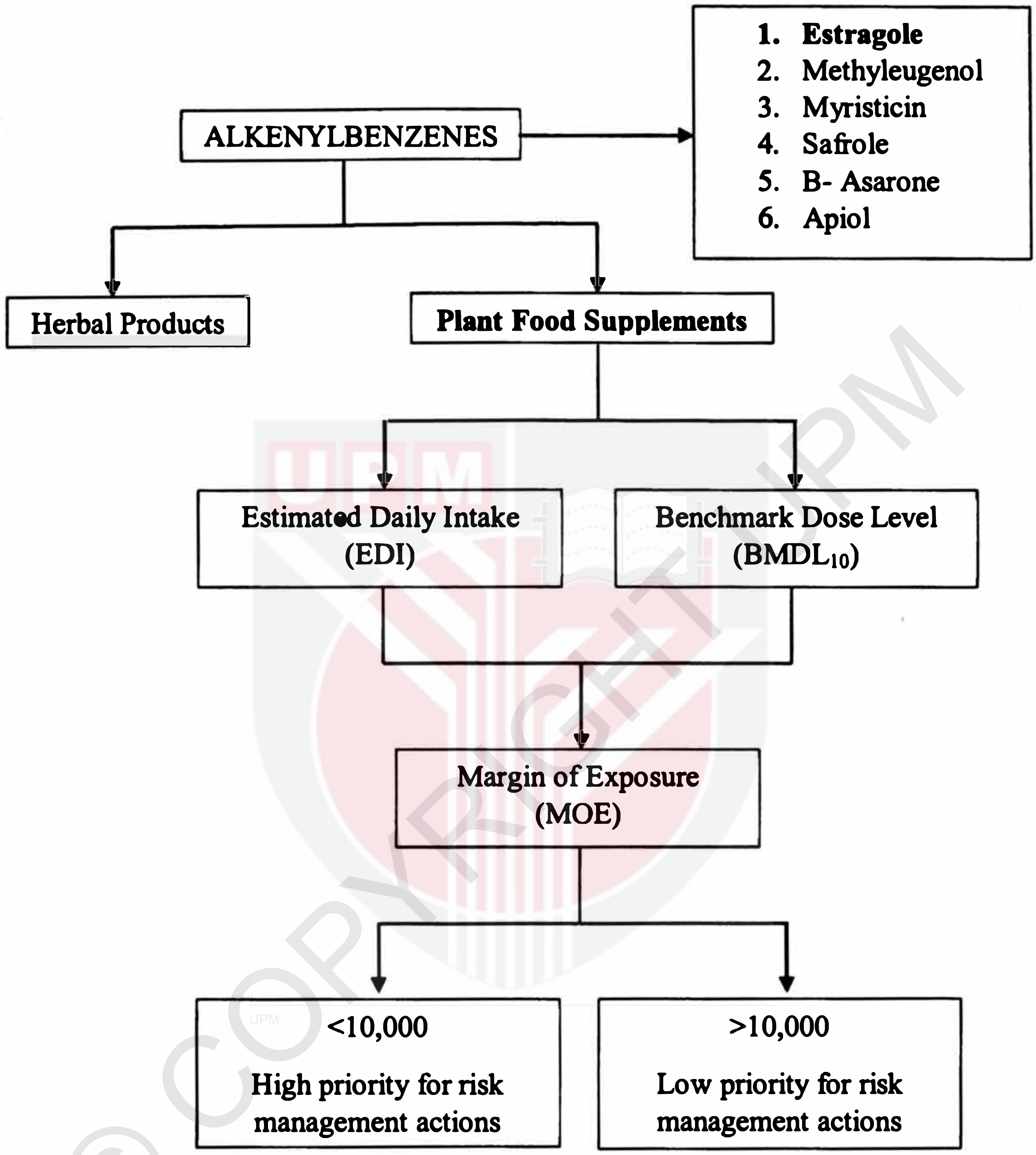
1.4 Conceptual Framework



1.5 Research Questions

i. What is the plant species containing estragole compound?

ii. What is the level of estragole in plant food supplements?

iii. What is the estimated daily intake of estragole in plant food supplement?

iv. What is the value of margin of exposure of estragole in plant food supplements?

v. What is the risk priority for risk management actions of estragole in plant food supplements?

1.6 Hypothesis

i. The margin of exposure value of estragole in plant food supplements is less than 10,000.

ii. There is a significant mean difference of estragole level between the samples.

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1.7 Objectives

1.7.1 General Objective

i. To determine the risk from exposure to estragole via consumption of plant food supplements using margin of exposure approach.

1.7.2 Specific Objectives

To identify the plant species that contain estragole compound.

ii. To measure the level of estragole in plant food supplements.

iii. To determine the mean differences of estragole level between the samples.

iv. To determine the estimated daily intake (EDI) of estragole from plant food supplement.

v. To calculate the margin of exposure of estragole in plant food supplements.

vi. To determine the risk priority of estragole exposure from the intake of plant food supplements.

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1.8 Definition of Terms

1.8.1 Conceptual Definition

1.8.1.1 Plant Food Supplement (PFS)

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 in dose form, 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" (Garcia-Alvarez et al., 2014).

1.8.1.2 Margin of Exposure

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

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1.8.2 Operational Definition

1.8.2.1 Plant Food Supplement

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

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.

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

Where;

MOE= Margin of exposure

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

EDI= Estimated Daily intake

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CHAPTER 2

LITERATURE REVIEW

2.1 Estragole

2.1.1 Characteristics of estragole

Estragole (Figure 2.1) is one of alkenylbenzenes derivatives. Estragole is considered as 'genotoxic and carcinogenic in rodents' according to the European Food Safety Authority (EFSA) compendium and it is found to be genotoxic and carcinogenic in experimental rodents at high dose level (EFSA, 2012). Figure 2.1 shows the structural formula of estragole that consist of one benzene ring substituted with a methoxy group and a propenyl group. Table 2.1 shows the general information of estragole. Substance that is genotoxic 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 the substance in even in small quantity, particularly if consumed on a routine basis (EFSA, 2005).

Estragole could be found in variety of botanical species such as *Illicium verum Hook.f.* (Clove flower), *Foeniculum vulgare Mill.* (Fennel) and *Ocimum basilicum L.* (Basil). (Van den Berg et al., 2011a). Estragole was identified in two

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samples of basil containing pesto sauce (Al-Malahmeh et al., 2017) and fennel based teas (Van den Berg et al., 2014). National Institute of Health of United States (2008) reported that about 19,980 pounds of estragole were produced in United States and 17,370 pounds were imported.

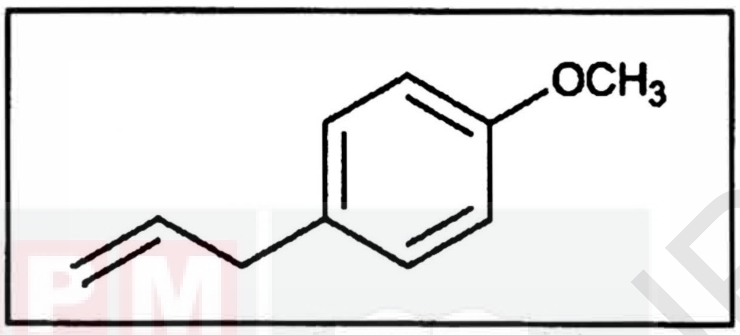

Figure 2.1: Structural formula of estragole

Table 2.1: General information of estragole

Chemical classification	Alkenylbenzenes
CAS registry number	140-67-0
IUPAC name	1-methoxy-4-prop-2-enylbenzene
Molecular formula	$C_{10}H_{12}O$
Molecular Weight	148.2g/mol
Synonyms	1-methoxy-4-(2-propenyl)-benzene (9CI); 1-allyl-4-methoxybenzene; 3-(p-methoxyphenyl)propene; 4-allylanisole; 4-allyl-1-methoxybenzene; chavicol methyl ether; esdragol; 1-methoxy-4-(2-propenyl)-benzene; *p*-methoxyallylbenzene; 4-methoxy-2'propenylbenzene; *p*-allyl-methyl chavicol; tarragon Methyl chavicol, 4-allylanisole,

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2.1.2 Metabolism of estragole

Figure 2.2 shows the metabolic pathway of estragole in the body. The genotoxic and carcinogenic activity of estragole in the body started with the bioactivation of estragole by 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 tumor formation (Rietjens et al., 2008). Punt et al. (2008) and Ning et al. (2017) used physiologically based biokinetic (PBBK) and kinetic (PBK) modelling respectively to study the bioactivation and detoxification of estragole and it was found that liver is the organ that mostly contribute to the development of 1'-hydroxyestragole.

There are several studies conducted to study the metabolism and bioactivation of estragole in experimental animals. In rodents, the sulfate conjugate of 1'-hydroxyestragole is belief to be an extreme hepatotoxic and hepatocarcinogenic agent (Alhusainy et al., 2012). According to Ding et al (2015), estragole undergoes the first metabolism in the gastrointestinal tract. Following absorption in the gastrointestinal tract, the estragole is transported to the liver and it is bio activated 1'-sulfooxyestragole. Safrole, estragole and methyleugenol induced a significant occurrence of hepatic carcinoma when a series of nine alkenylbenzene compounds were administered to newborn male mice (Phillips, Reddy & Randerath, 1984). Swanson, Miller & Miller (1981) stated that 1'-hydroxymetabolites are stronger hepatocarcinogens as compared to their parent compounds. This finding showed that

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estragole is carcinogen if it is consumed and bioactivated to 1'-hydroxyestragole in the body.

In vitro research conducted by Suzuki et al., (2012) 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.

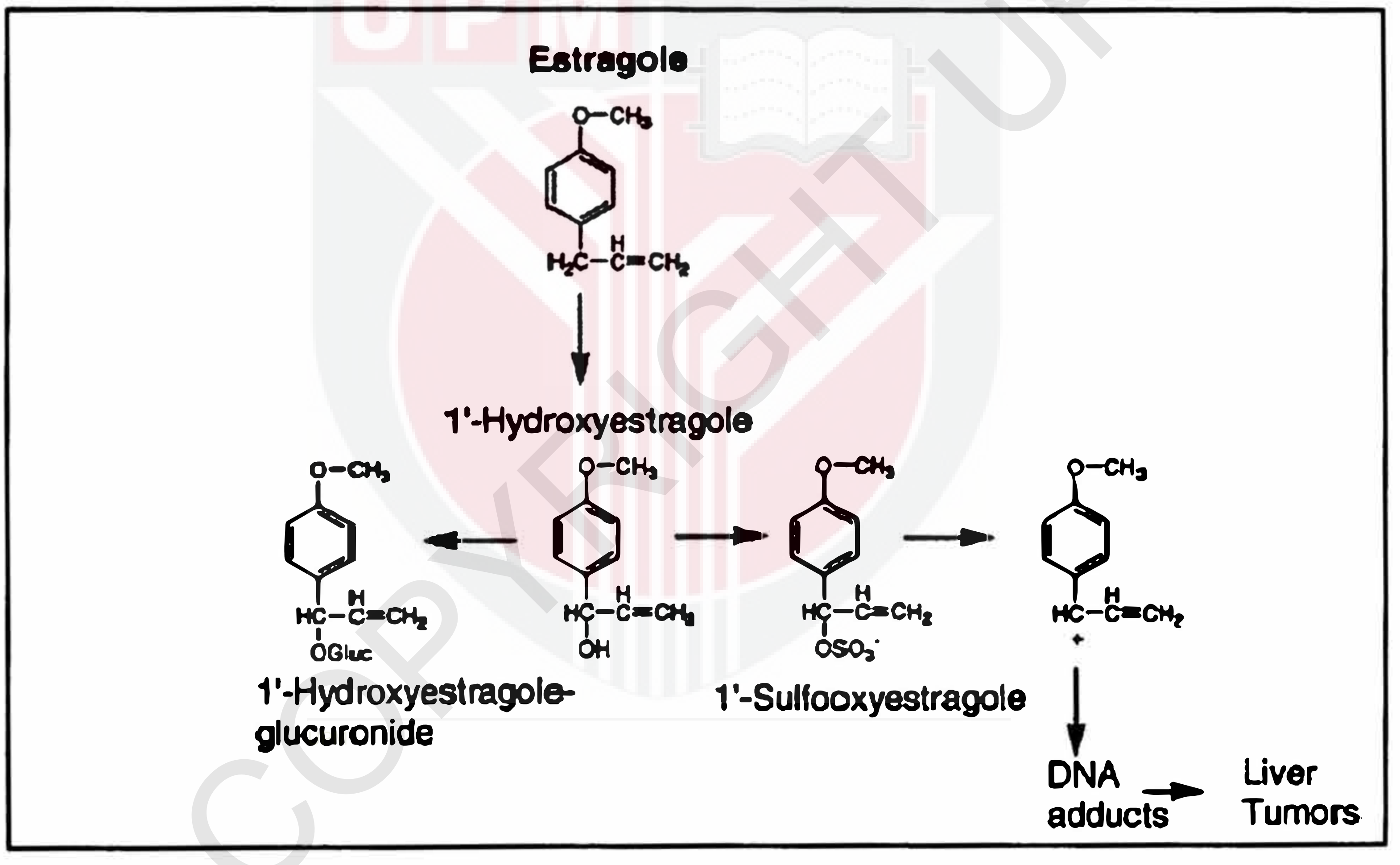

Figure 2.2: The metabolic pathway of estragole.

2.1.3 Genotoxic and carcinogenicity

Estragole is lightly toxic following acute exposure. The oral LD_{50} values reported are 1.2 and 1.8g/kg for rats and 1.25g/kg for mice. Liver staining, mottling and blunting of lobe edges showed minor liver damage in rats exposed to four daily oral doses of estragole (Bristol, 2011).

Many studies have been conducted on the toxicity of estragole by *in vivo* and *in vitro*. In a study conducted by Martins et al. (2012), they found that the adducts level was increase consistently after 2 hours of incubation of estragole in V79 cell. The toxicology data from Miller et al. (1983) was used to fit in the Benchmark dose modeling. The study showed that 61 to 73% of the male mice treated with safrole and estragole developed hepatic tumors and for which the average multiplicities of tumors 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.

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2.2 Plant Species Containing Estragole

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

Table 2.2: The list of plant species containing estragole

References	Plant species	Remarks
Van den Berg et al., 2011a	*Foeniculum vulgare* Mill., *Illicium verum* Hook f., *Ocimum basilicum* L	Indicated in the EFSA compendium as 'genotoxic and carcinogenic in rodents'.
European Medicines Agency, 2014	*Agastache foeniculum*, *Anthriscus cerefolium* L., *Artemisia dranunculus* L., *Foeniculum vulgare* Mill., *Illicium verum* Hook f., *Melissa officinalis* L., *Myrrhis odorata* L., *Ocimum basilicum* L., *Pimpinella anisum L.*	-
European Food Safety Authority, 2012	*Agastache foeniculum* (Pursh) Kuntze	Estragole content: 555-12.160 ppm (plant)
	Agastache rugosa Kuntze	Estragole content: 90% (essential oil)
	Artemisia vallesiaca All.	Small amount of estragole
	Boswellia serrata Roxb.	Essential oil: may contain up to 11% of methylchavicol (=estragole)
	Cuminum cyminum L.	Estragole content: 30ppm (fruit)

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Foeniculum vulgare Mill.	Herb essential oil: estragole 2.3-4.9%. Seed essential oil (not fully mature): estragole 11.88-29-65%; Seed essential oil (unripe seed): Estragole 56.1%; Seed essential oil (ripe seed): estragole 61.8 Estragole content: fruit (70-4.018 ppm), essential oil (0.8->80%)
Foeniculum vulgare Mill. *ssp. vulgare var. vulgare*	3.5-12% estragole in essential oil
Foeniculum vulgare Mill. *ssp. vulgare var. dulce* (Mill.) Batt. & Trab.	Seed essentail oil: estragole 3.4-8.1% Seeds approx. 0.3% estragole.
Hyssopus officinalis L.	Estragole content: 1-260ppm (bud) Herb and leaves essential oil: estragole 4.8%
Illicium verum Hook.f.	Estragole content: 280-6500 ppm (fruit), 0.6-6% (essential oil) Star anise oil: estragole 0.34-5.04%
Myrrhis odorata (L.) Scop.	Estragole (up to 75% in essential oil) Fruit essential oil: estragole 1.2-1.7%

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References	Plant species	Remarks
European Food Safety Authority, 2012	*Myrtus communis* L.	Essential oil estragole content: 58-88ppm
	Ocimum basilicum L.	Estragole content: 238-8780 ppm (plant), 5-85% (essential oil) Presence of high amounts of estragole, genotoxic and carcinogenic in rodents It contain also camphor estragole (0.4% in the herb) Leaves and flowering tops essential oil: estragole 20-50%
	Ocimum canum Sims.	Estragole content: 52%
	Ocimum nudicaule Benth.	Essential oil estragole content:98%
	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%
	Pimenta racemosa (Mill.)	Estragole content: 30-10.745 ppm
	Pimpinella anisum L.	Estragole content: 400-1050ppm Essential oil estragole content: 400-1050 ppm

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	Piper betle L.	Estragole content: 1.02-4.0%
	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%

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2.3 Plant Food Supplement (PFS)

Plant food supplements (PFS) can be described as a food supplements that contain natural botanical or botanical–derived ingredients. PFS are widely marketed such as at supermarkets, pharmacies and via internet that are easily accessible to the consumer. Public keep consuming the PFS because of their perception on what is natural is considered safe. But, the truth is not all natural is safe. Public know that the plant-based food supplements give benefits to their health, but some of the plants that are used as ingredients in the PFS are known to contain toxic compounds. Alkenylbenzenes are one of the examples of the compounds that are naturally present in plants (Van den Berg et al., 2013).

In Malaysia, the food and nutritional supplements consumption is primarily imported. This comes either in finished products or crude materials for local used. Intermittently, local importers cooperate with foreign manufacturers to obtain the private named products. Progressively, they produce their own supplements formulation (International Trade Administration, 2017). Under Drug Control Authority (DCA), health supplements products are managed as pharmaceutical products in Malaysia. A health supplements product is subjected to pre-advertising endorsement process. It can be classified as dietary supplement or customary medication, contingent upon the type and dosages of the formulation ingredients. Being directed under the pharmaceutical class in Malaysia, dietary supplements need to comply with the general wellbeing and quality requirements of a pharmaceutical products. Notwithstanding, organizations are not required to present a full drug enlistment dossier such as controlled drugs. Fundamental administrative and

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technical records, for example, product formula, GMP accreditation, analysis certification and stability study reports are compulsory (Poon, 2010).

2.4 Estimated Daily Intake (EDI)

The estimated daily intake of the PFS selected is based on the daily intake recommended as stated at the packaging label on the supplements by the suppliers. EDIs are evaluated using body weight of 62.65 kg (Azmi et al., 2009). The body weight of 62.65kg are the average body weight of 6,775 men and 3,441 women Malaysian aged 18 – 59 years. In previous study, the EDI was estimated using the content of alkenylbenzene in the food and a body weight of 70 kg, the default value for adult weight as recommended by EFSA (Abdullah et al., 2017) and the EDI was determined for specific compounds for all of the samples. But, there were other alkenylbenzene compounds that were founded in the samples. So, the subsequent risk assessment and combined exposure assessment were also conducted on the samples (Al-Malahmeh et al., 2017).

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2.5 Benchmark Dose

Mathematical modelling is utilized to characterize the benchmark dose (BMD) giving 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). The use of BenchMark Dose (BMD) modelling is a chosen approach to identify the reference point from the dose-response graph. The BMD approach makes utilization of various numerical models for the test of the observed carcinogenicity data. With this approach, a dose that causes predefined cancer reaction can be estimated, recognized as BenchMark Response (BMR). The BMD assessed the dosage that resulted a minimum but measurable response (BMR) from experimental animal data. Typically, a dose point that gives 1%, 5% or 10% extra cancer occurrence above the control. But, it is shown that the utilization of a measurements giving 10% additional cancer risk over background level ($BMDL_{10}$) is guided with the minimum uncertainties and is therefore favored.

The use of the BMDL instead of BMD will guarantee with 95% certainty that the value of the BMR will not go over predefined value (van den Berg et al., 2011a). $BMDL_{10}$ is the lower confidence level of the benchmark dose giving 10% additional cancer occurrence (Abdullah et al., 2017). In this study, the carcinogenicity data from Miller et al., 1983 was used to be analyze 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.

2.6 Margin of Exposure

Margin of exposure (MOE) approach is the preferred approach of risk assessment for both genotoxic and carcinogenic substance as suggested by World Health Organization (WHO) and European Food Safety Authority (EFSA) (Barlow and Schlatter, 2010). The MOE is a tool utilized by risk assessor to consider conceivable safety concerns rising from both genotoxic and carcinogenic substances present in the food (EFSA, 2012). According to van den Berg et al. (2011b), MOE is a ratio between reference points retrieve from dose-response data on experimental or epidemiologic statistics on tumor incidence in animals and the predicted daily intake in humans. 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).

There are many advantages of MOE approach including it set a base to set the priority of the botanical ingredient besides other priority in the area of genotoxic and carcinogenic compounds in food. 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). An

MOE value less than 10,000 express as high priority for risk management actions and exert high concern from a public health opinion. The value of 10,000 is interpreted based on recognizing many factors that can cause ambiguity in the MOE including;

1) A factor of 100 for species diversity and human variability in toxico-dynamics and toxico-kinetics.
2) A factor of 10 for inter-individual human variability in cell cycle management and DNA replacement.
3) 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 (van den Berg et al., 2011a)

However, the MOE does not provide the quantitative value of the estimated risk. It only present the ratio. The abstract description of a ratio may have issue on the people understanding (Barlow et al., 2006). The calculation of MOE generally using equation 2.1 below;

Equation 2.1

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

Where;
MOE= Margin of exposure
$BMDL_{10}$= Benchmark dose
EDI= Estimated Daily intake

CHAPTER 3

METHODOLOGY

3.1 Research Design

In this study, cross sectional study was carried out. The level of estragole in plant food supplements (PFS) were measured using Ultra-High Performance Liquid Chromatography (UHPLC) Flexar FX-15 UHPLC system.

3.2 Sampling Technique

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

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Table 3.1: Product description of the PFS analyzed in the present study.

Sample No.	Product	Product presentation	Dosage	Plant species
S1	Jamu Tradisi Wanita	Capsule	2 capsules, 2 times per day	*Coriandrum sativum*
S2	Kapsul Kacip Fatimah	Capsule	2 capsules, 2 times per day	*Pimpinella anisum, piper betle, piper nigrum*
S3	Kapsul Tongkat Ali Hitam Plus	Cupsule	2 capsules, 2 times per day	*Foeniculum vulgare*
S4	Kacip Fatimah Manjakani	Capsule	2 capsules, 2 times per day	*Piper nigrum, pimpinella anisum*
S5	Jamu Rapat Plus	Capsule	2 capsules, 2 times per day	*Piper nigrum*
S6	Makjun Pak Tani Tongkat Ali Plus	Paste	1-2 pack, 2 times per day	*Piper nigrum, Foeniculum vulgare*
S7	Makjun Pak Tani Paste	Paste	1 pack, 2 times per day	*Piper nigrum, Foeniculum vulgare*
S8	Makjun Petani	Paste	2 pack, 2 times per day	*Pimpinella anisum*
S9	Makjun Ubijaga	Paste	1 pack, once per day	Acorus calamus, piper nigrum
S10	Makjun Kacip Fatimah	Paste	1 pack, once a day	*Pimpinella anisum, piper nigrum*

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3.3 Study Instrument

3.3.1 Material and chemicals

The PFS were bought from the local herbs shop and supermarket. All the PFS bought were local products. Estragole (purity 98%) were purchased from Acros Organics, (Geel, German). The HPLC grade methanol and acetonitrile were purchased from Systerm Chemicals (Selangor, Malaysia) and Merck (Selangor, Malaysia) respectively. A 0.22µm nylon membrane syringe filters were obtain from KSFE (Selangor, Malaysia). Ultrapure water was obtain from Mili-Q purification water system.

3.3.2 Methanol Extraction

The samples were extracted using methanol extraction for quantification of the total estragole in the plant food supplements. The methanol extraction were conducted based on the method that was previously described by van den Berg et al. (2011a) with modification. In general, 1g of the PFS was added into 25ml of methanol followed by sonication for 15 minutes at room temperature. Then, the extract solution was centrifuged at 4,000rpm for 15 minutes. The extract were filtered using 0.22 µm nylon membrane syringe filter and 10µL of each sample was subjected to UHPLC analysis (n=3)

3.3.3 Ultra-high Performace Liquid Chromatography

The UHPLC analysis was conducted based on the method previously described by Al-Malahmeh et al. (2017). Flexar FX-15UHPLC system model (United States) was used for the analysis. For each sample, 10µL of was injected to UHPLC analysis. The chromatographic separation was achieve by using Ascentis C185µm column, 25cm × 4.6mm (Pennsylvania, United States). The column was thermostated at 30°C. The separation was made from the isocratic gradient using a mixture of acetonitrile and ultrapure water (Table 3.2). The mobile phase gradient was set at 40% acetonitrile for 10 minutes. The flow rate was 0.6 ml/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 identify by injecting the 98% pure estragole standard.

The acetonitrile and ultrapure water were filtered using 0.22 µm nylon membrane filter. Then, both solvents were sonicate for 15 minutes at room temperature.

Table 3.2: Isocratic solvent gradient for the analysis

Time (minutes)	Acetonitrile gradient	Ultra-pure water gradient	Flowrate (ml/min)
0	40	60	0.6
10	40	60	

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The calibration curve was constructed using six different concentration of estragole which were 50µM, 100µM, 500µM, 1mM, 10mM and 50mM. All the concentrations were injected from low to high concentration using the same method as above. Then, concentration vs area under the curve calibration curve was constructed and identify the y=mx+c equation of the calibration curve. This equation was used to calculate the concentration of the estragole in the samples by substitute the area under curve values.

3.4 Accuracy

A recovery study was conducted to determine the percentage of estragole loss during the experiment. The recovery study was conducted by spiking three different concentrations, 10 mM, 50mM and 100mM into sample 9 to determine the percentage recovery. Sample 9 was choose randomly as one of the sample to conduct the recovery analysis. Sample 9 also had a stable trend of chromatogram. So, it is easier to determine the estragole peak, calculate and compare with the spiked one. The area under the curve was compared and calculated to get the percentage. The estragole level in all samples were recalculated using this recovery. The results were compare between the absolute and relative value. Absolute value is the results without recovery (100%) and relative results is the results calculated with the percentage recovery. The extraction and analysis of each samples were repeated three times and the average were calculated and used as data. The extraction was done before the estragole quantification using UHPLC on the same day. This was important to avoid the estragole from degraded and to get the maximum and reliable data of estragole concentration in the PFS samples.

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3.5 Benchmark Modelling

The carcinogenicity data from Miller et al. (1983) was used to analyze different mathematical model of Benchmark Dose Software version 2.7 (downloaded from United States Environmental Protection Agency (USEPA) website) to obtain the $BMDL_{10}$ data. The models used are: Gamma, Logistic, Log-Logistic, Probit, Log-Probit, Multistage, Weibull and Quantallinear model.

3.6 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 from the BMDL analysis. The calculation of the EDI of the PFS selected was based on the daily intake recommended as stated at the packaging label on the supplements by the suppliers. EDIs were evaluated using body weight of 62.65 kg (Azmi et al., 2009). The body weight of 62.65kg are the average body weight of 6,775 men and 3,441 women Malaysian aged 18 – 59 years. The MOE was calculated using absolute (100% recovery) and relative results (recovery percentage from the analysis). Then, the MOE from these two types of results then compared directly.

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3.7 Data Analysis

The estragole level presented as mean± SD (standard deviation). The mean difference of the samples was analyze using IBM SPSS statistics Version 22. The normality of data was tested and One Way ANOVA analysis was used to analyze the mean difference of estragole level between the samples.

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CHAPTER 4

RESULTS

4.1 Levels of Estragole in Plant Food Supplements (PFS)

4.1.1 Calibration Curve

Six different concentration of pure standard of estragole and methanol was prepared and injected into UHPLC. Estragole was eluted at 3 minute as shown in Figure 4.1. A calibration curve was constructed to calculate the concentration of estragole in PFS. The calibration curve was plotted as concentration vs the area under curve (Figure 4.2). The equation, y= 520532x – 726.398 was used to calculate the concentration of estragole in the PFS. The R^2 value, 0.9848 shows a strong correlation between the concentration and area under the curve. The level of estragole were calculated without and with recovery value to obtain absolute and relative results of the estragole level in PFS respectively. The relative results was calculated using the percentage recovery which is 60%, means that 40% of estragole loss during the experiment.

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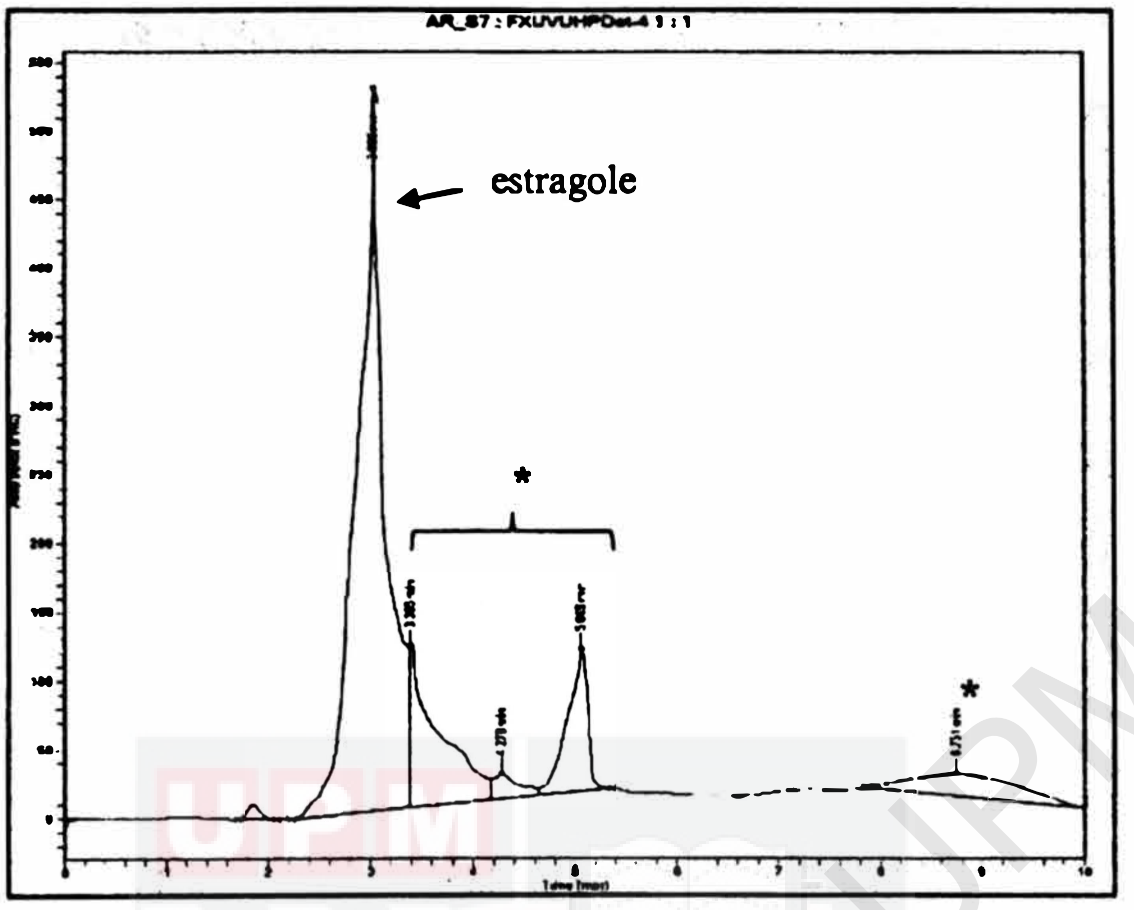

Figure 4.1: UHPLC chromatogram from sample 7. Peaks marked with asterisk (*) were not identified. The chromatogram was obtained at a wavelength of 201nm.

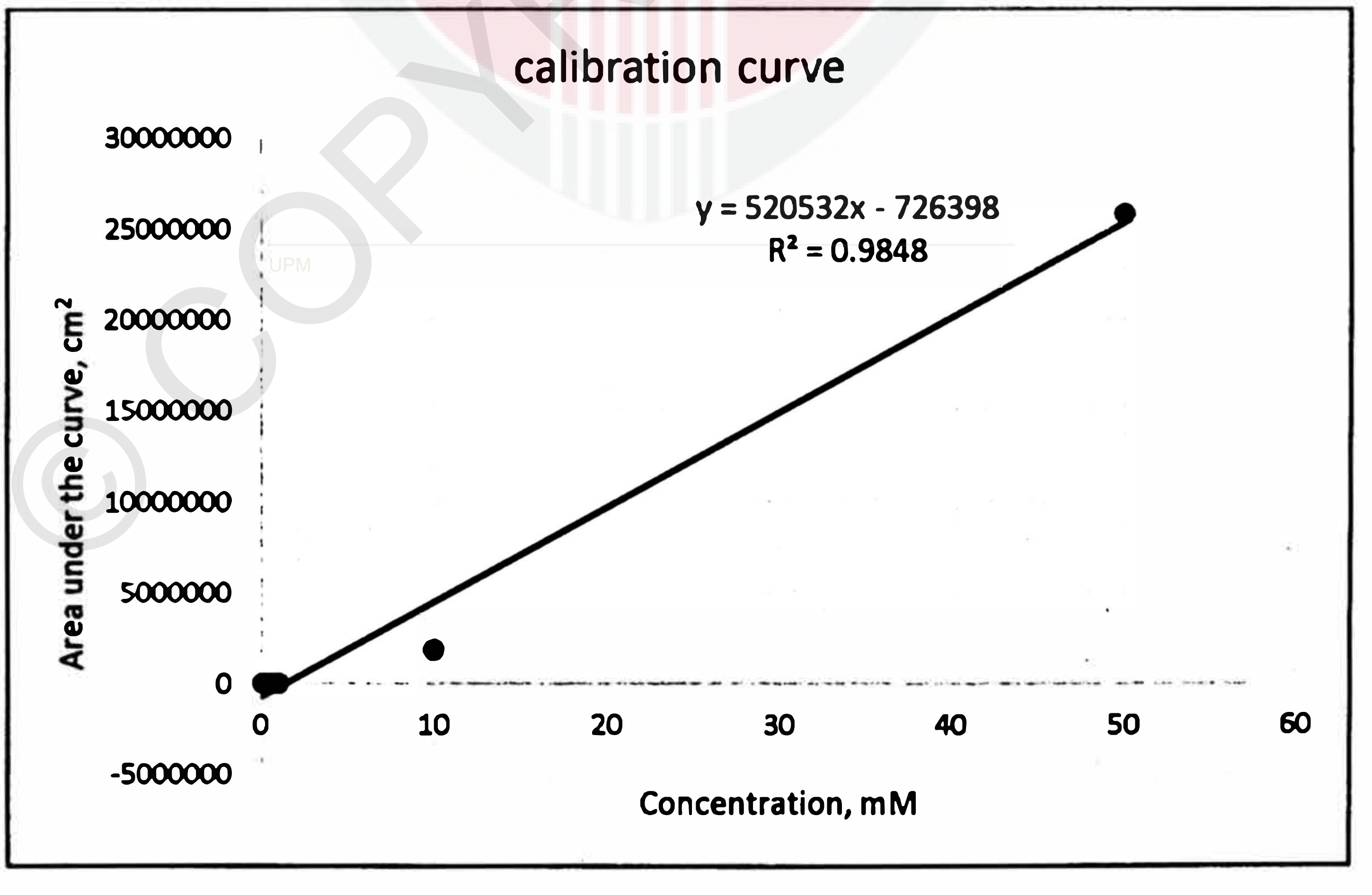

Figure 4.2: Calibration curve of estragole

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4.1.2 Level of estragole in PFS

Figure 4.3 shows the PFS samples used in this study. The recovery value of estragole from this study was 60%. The estragole level in PFS also recalculate using this recovery value for the relative results. Table 4.1 presents the absolute (100% recovery) and relative (60% recovery) results for the level of estragole in all the PFS and One Way ANOVA analysis. Figure 4.4 shows the bar graph of the mean and standard deviation of the level of estragole in the samples. Estragole was found in all samples. For absolute results, the highest estragole level 227.25mg/g (±132.6mg/g) and 68.1mg/g (±38mg/g) was the lowest found in sample four and seven respectively. For relative results, 378.7mg/g (±221mg/g) was the highest and 113.5mg/g (±63.3mg/g) the lowest estragole concentration, were found in sample four and seven respectively. 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 with p value is more than 0.05.

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Figure 4.3: The PFS samples

Table 4.1: The level of estragole in PFS as determined by methanol extraction (absolute and relative)

Sample number	Estragole level,mg/g mean(±SD)		*F*-statistic (df)	*p*-value*
	Absolute (100% recovery)	Relative (60% recovery)		
1	88.847 (±84.7)	148.1 (±141.1)	1.251 (9,20)	0.321
2	84.8 (±74)	141.3 (±123.3)		
3	110.3 (±67.7)	183.8 (±112.8)		
4	227.25 (±132.6)	378.7 (±221)		
5	84.2 (±9.4)	140.54 (±15.6)		
6	191.1(±162.9)	318.6 (±271.4)		
7	68.1 (±38)	113.5 (±63.3)		
8	128.7 (±61.5)	214.5 (±102.5)		
9	76.5 (±43.5)	127.6 (±72.5)		
10	84.5 (±12)	140.8 (±19.6)		

**p*-value is significant at 0.05 level.
Results were analyzed by One-way ANOVA

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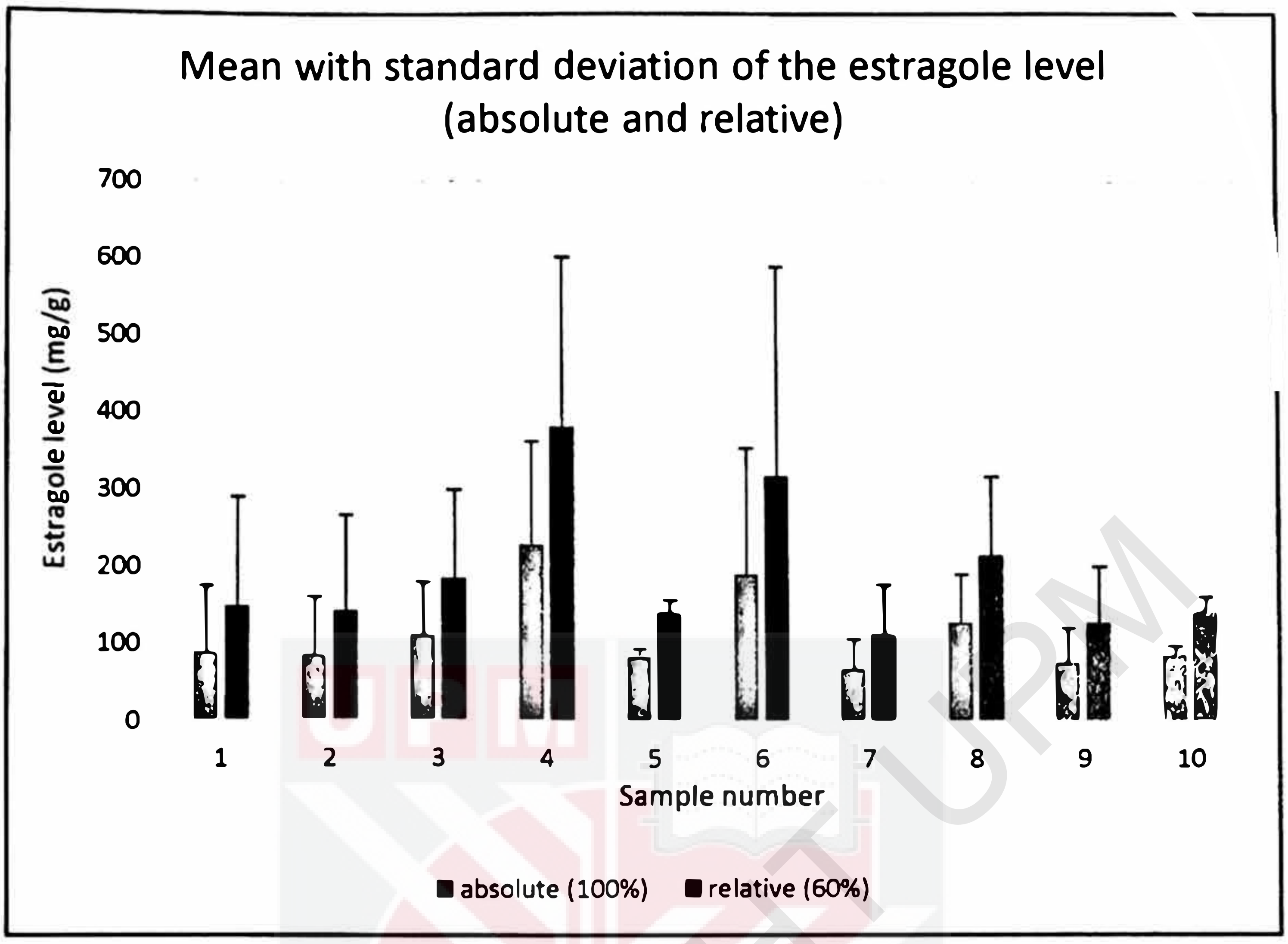

Figure 4.4: The mean and standard deviation of estragole level in the selected PFS for absolute and relative results.

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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 from the data in 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	18.82	1.26
LogLogistic	1	-62.21	0.99	Yes	4.71	**3.29**	1.43
Probit	2	-70.14	0.00	No	22.95	18.36	1.25
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

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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 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 using both absolute and relative results of estragole level in PFS. As shown in Table 4.4, the EDI ranged from 9.84×10^{-5} to 1.35×10^{-3} mg/kg bw/day for absolute results. Sample eight and two had the lowest and highest EDI respectively for absolute results. EDI ranged from 1.11×10^{-4} to 3.36×10^{-3} mg/kg bw/day for relative results. The lowest EDI is sample five while the sample six had the highest EDI for relative results.

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4.4 Margin of Exposure (MOE)

The MOE was calculated for absolute and relative results. The results shows three samples has MOE ranged less than 10,000 for absolute results and for the relative results, five samples has MOE ranged less than 10,000 indicate the priority for the risk management action.The five samples that showed the priority for risk management actions are from the PFS in paste forms. From the absolute results, the lowest MOE of 2,000 and the highest MOE was 97,000 were calculated from sample five and eight respectively. For the relative MOE results, sample six showed the lowest MOE with 900 and the highest MOE value, 58,000 was from sample five.

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Table 4.4: EDI and the respective MOE of the PFS samples (absolute and relative)

Sample number	Recommended daily intake (mg) of the PFS	[a]EDI ($mg\ kg^{-1}\ bw\ day^{-1}$) Absolute	Relative	[b]MOE using absolute results	[b]MOE using relative results
1	1828	9.5×10^{-5}	1.58×10^{-4}	34,000-68,000	20,000-40,000
2	1984	9.84×10^{-5}	1.64×10^{-4}	33,000-65,000	20,000-39,000
3	2140	1.38×10^{-4}	2.30×10^{-4}	23,000-46,000	14,000-28,000
4	1072	1.42×10^{-4}	2.37×10^{-4}	23,000-45,000	13,000-27,000
5	1348	6.64×10^{-5}	1.11×10^{-4}	49,000-97,000	29,000-58,000
6	18016	2.01×10^{-3}	3.36×10^{-3}	**[c]1,000-3,000**	**[c]900-1,000**
7	9676	3.85×10^{-4}	6.42×10^{-4}	**[c]8,000-16,000**	**[c]5,000-10,000**
8	17964	1.35×10^{-3}	2.25×10^{-3}	**[c]2,000-4,000**	**[c]1,000-2,000**
9	4476	2.0×10^{-4}	3.34×10^{-4}	16,000-32,000	**[c]9,000-19,000**
10	4291	2.12×10^{-4}	3.53×10^{-4}	15,000-30,000	**[c]9,000-18,000**

[a]EDI were calculated using life expectancy of 74.8years (Department of Statistics Malaysia, 2018) and average body weight of 62.65 (Azmi et al., 2009)
[b]MOE= $BMDL_{10}$ (mg/kg bw/day)/ EDI (mg/kg bw/day)
[c]The MOE value less than 10,000 indicating that priority for risk management action.

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CHAPTER 5

DISCUSSIONS

In this study, the risk from exposure to estragole via consumption of PFS was assessed using MOE approach. Ten PFS were purchased and the level of estragole in the PFS was quantified and the MOE value was calculated.

For absolute results, the highest estragole level 227.25mg/g (±132.6mg/g) and 68.1mg/g (±38mg/g) was the lowest found in sample four and seven respectively. For relative results, 378.7mg/g (±221mg/g) was the highest and 113.5mg/g (±63.3mg/g) the lowest estragole concentration, were found in sample four and seven respectively.

The results show that estragole was present in all samples with the range of 227.25mg/g (±132.6mg/g) to 68.1mg/g (±38mg/g) for absolute results and 113.5±63.3 to 378.7±221 mg/g for relative results. The extraction of the PFS was done fresh before the UHPLC analysis to reduce the estragole degradation in the samples. Sample six to ten were in paste form and the weight of one pack of the PFS was more than one capsule of the PFS. It shows that the consumers are exposed to estragole through consumption of PFS in paste form more than in capsule form. The samples consist of botanical species that may contain

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estragole as reported in the literature review such as *Foeniculum vulgare, Piper nigrum and Pimpinella anisum.*

The level of estragole from van den Berg et al. (2011a) study was ranging between 0.07±0.005 to 241.56±62.02 mg/g showing that the current results are within the range that have found from the literature. There were no estragole detected in PFS that have *Sassafras*, Nutmeg, Cinnamon and Calamus from van den Berg et al. (2011a). Recently, estragole was found in 30 samples out of 71 samples from Chinese market ranged from 7.2 to 341.1 μg/g (Ning et al., 2018). The samples from the study were based on the botanical ingredients in the PFS. 4 different alkenylbenzenes including estragole were detected in 23 out of 25 samples of Indonesian jamu, at levels ranging from 3.8 to 440 μg/g (Suparmi et al., 2018)

The estragole level in the PFS were calculated based on absolute and relative results. Absolute results were calculated based on the real data assuming that all estragole was extracted during the extraction process while relative results were calculated using recovery percentage from this study which was 60%. The MOE from both results was compared. The recovery percentage from van den Berg et al. (2011a) was 80% which was higher than recovery from this study. The lower recovery might be due to lack of quality control at some parts of the experimental procedure. This was also due to the unstable instrumentation, UHPLC. UHPLC needs to be purge first before injecting the sample. Purging was done to eliminate the air bubble produced in the UHPLC line. The bubble formation might be due to insufficient sonication of the solvent and the bottle of the solvent accidentally shaken during the transfer. The air bubble may cause the unidentified peak formation in the chromatogram. This will cause the unstable baseline in the chromatogram.

The benchmark dose (BMD) approach is applicable to all toxicological effects. It use all the data from dose-response to assess the overall dose-response relationship shape for certain endpoint (EFSA, 2017). In conventional method for risk assessment, the point of departure is derived from one dose in animal data that induce 1 cancer in million of animals that lead to high uncertainties. However, in this new method, all data from the experiment is used to derive the point of departure for safety assessment. Benchmark dose commonly makes no specific assumption about the biological basis of observed dose-response relationships other than the significance of response (relating to background response level) does not normally decrease with higher dose (USEPA, 2012). In this study, the carcinogenicity data was referred to a study conducted by Miller et al. (1983). This study used female CD-1 mice for the experiment. The mice were fed by pellets so the mice were exposed to estragole by ingestion. This was important that the human exposure to the estragole compound also through ingestion of plant food supplement. Both EFSA and WHO suggest the BMD approach to determine the point of departure (POD). EFSA recommended the $BMDL_{10}$ to be the preferred POD, calculated for a 10% Benhmark response (BMR) because the value is the lowest statistically significant increased incidence that can be measured in the most studies conducted, and would require less or no extrapolation outside the observed experimental data (Barlow & Schlatter, 2010). When a health-based guidance value is based on BMD analysis, it consider all the data points from the dose-response curve and it provides better method to identify the risk in situations where health-based guidance is exceeded, thus produce better risk communication (EFSA, 2017)

The estimated daily intake (EDI) of estragole were calculated using the recommended daily intake of the plant food supplements (PFS) as suggested by the manufacturer. Malaysian average body weight, 62.65kg (Azmi et al., 2009) and Malaysian life expectancy, 74.8 years (Department of Statistic Malaysia) were used to calculate the EDI. The EDI of estrgagole from the consumption of PFS ranged from 9.84×10^{-5} to 1.35×10^{-3} mg/kg bw/day for absolute results and 1.11×10^{-4} to 3.36×10^{-3} mg/kg bw/day for relative results. Since this is the first study conducted on the risk assessment of estragole in Malaysia, the average body weight and life expectancy used to calculate the EDI were data among Malaysian. EFSA (2012) suggested to use 70kg as default value for the European adult.

The MOE value for the PSF were calculated using computed $BMDL_{10}$ and the calculated EDI value. From the results, five PFS have MOE value less than 10,000 indicate that there is priority for risk management action. Interestingly, all of these samples are in paste forms and the EDI of the estragole in paste forms are higher compared to capsule form. MOE identify the priority for risk management action, however it does not provide a quantitative estimate of risk. Therefore, it might be misinterpreted as giving measure of a risk (Barlow et al., 2006). Further explanation should be presented with the MOE. 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. Three samples has MOE ranged less than 10,000 for absolute results and for the relative results, five samples has MOE ranged less than 10,000 indicate the priority for the risk management action. Several compound may contain in one PFS. . But, in this study, only estragole was determined and calculated for the MOE. Matrix effects can reduced the bioactivation of estragole (van den Berg et al., 2011b). Matrix effect is define as interference from matrix components that are unrelated to the analyte (Kelmac Medical, 2018). Nevadensin was demonstrated to be significantly inhibit the sulfotransferase (SULT)-mediated bioactivation and-DNA adduct formation of estragole *in vivo* (Alhusainy et al., 2013). The effects of estragole are likely to be less when estragole is consume in a PFS compared to pure estragole. The carciniogenicity data for the $BMDL_{10}$ analysis (Miller et al., 1983) was obtained by administered pure estragole to the rodents. Therefore, this may result in overestimation of the risk management priority (van den Berg et al., 2013)

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CHAPTER 6

CONCLUSION, STUDY LIMITATIONS AND RECOMMENDATIONS

6.1 CONCLUSION

In conclusion, there is priority for management actions of the risk from the consumption estragole via PFS consumption. Consumers are exposed to 1.11×10^{-4} mg/kg bw/day to 3.36×10^{-3} mg/kg bw/day estragole from consumption of the PFS. Five PFS showed priority for the risk management action and all positive samples were in paste forms. The responsible authorities should be aware of this issue and conduct risk management plan to control estragole in PFS. The consumers also should be aware of the presence of natural genotoxic and carcinogenic chemical by reading the label of the PFS and avoid from consuming the PFS containing estragole.

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6.2 STUDY LIMITATIONS AND RECOMMENDATIONS

This study had a few limitations including limited number of samples. The ten samples are in capsule (five smples) and in paste (five samples). The percentage of recovery of the estragole experiment was not satisfied due to limited time. The MOE calculated only for on type of alkenylbenzene only which is estragole. The MOE did not calculated for the combine exposure. Some of the PFS marketed did not have sufficient information at the package labelling such as the plant species contain as the ingredients.

Based on the findings, there are some recommendations for future study. Wide range of PFS should be selected including the local and imported PFS. study on several types of compound should be conducted to determine the combined effect of the compound. Next, when choosing the samples, one should consider to sample variety of plant species containing the estragole. It is also recommended to repeat or replicate the experiments for reliable data. For example, in the analysis for the recovery and calibration curve of the model compound. This two steps were critical steps to determine the level of estragole in the PFS. In addition, food frequency questionnaire could be distributed among PFS consumer to identify the real intake of the PFS among Malaysian.

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The results from this study indicate that the estragole in the PFS have the priority for risk management action. So, the responsible parties such as Ministry of Health Malaysia could develop management plan to control the estragole level in PFS and conduct activities such as campaign or talk to give knowledge and awareness to the consumers on this issue. The consumers also should be aware of the presence of natural genotoxic and carcinogenic compound in the supplements they consume.

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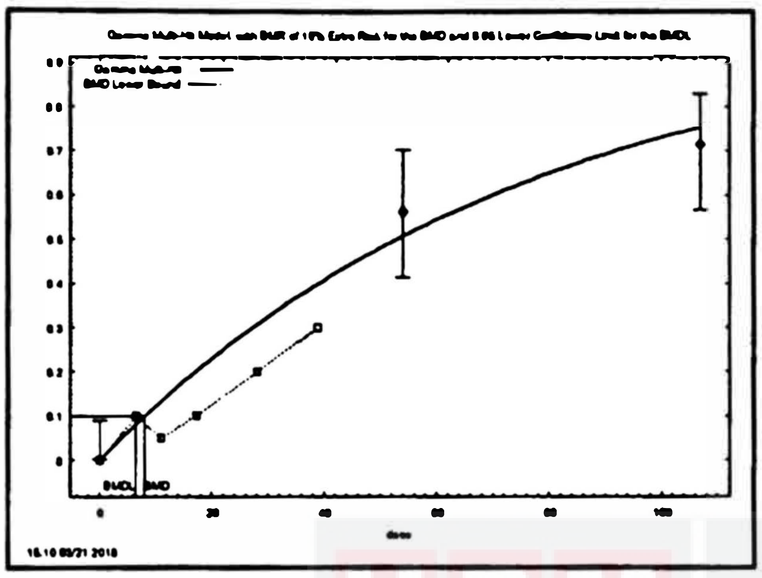
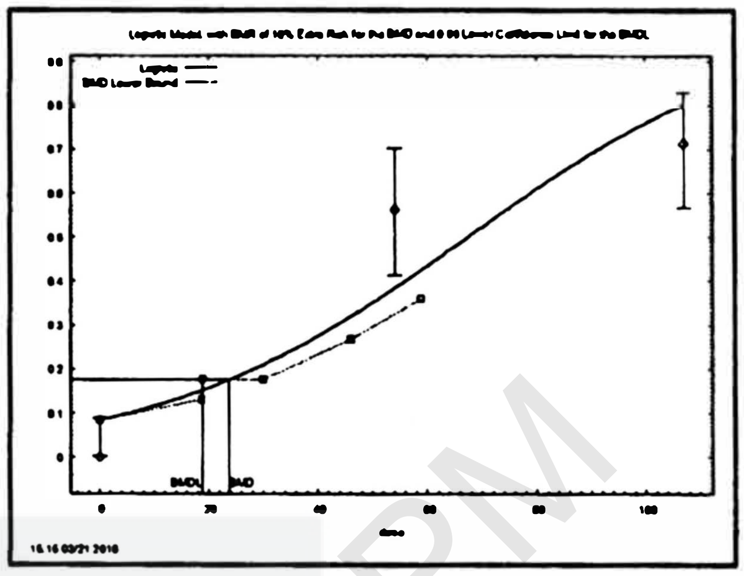
APPENDIX A: THE BMDL MODELLING ANALYSIS

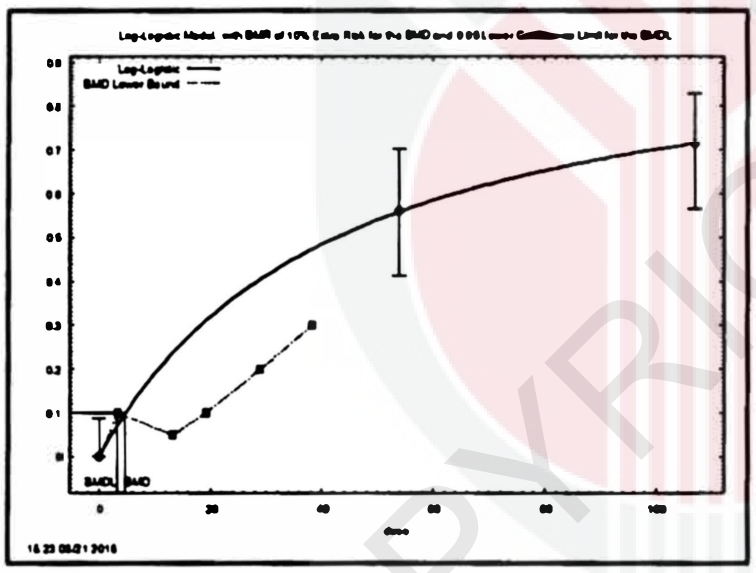
Gamma Model



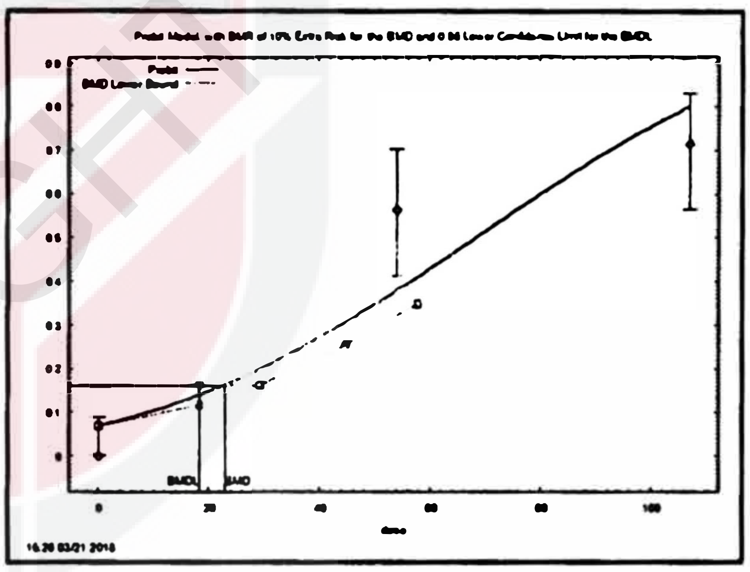
Logistic Model



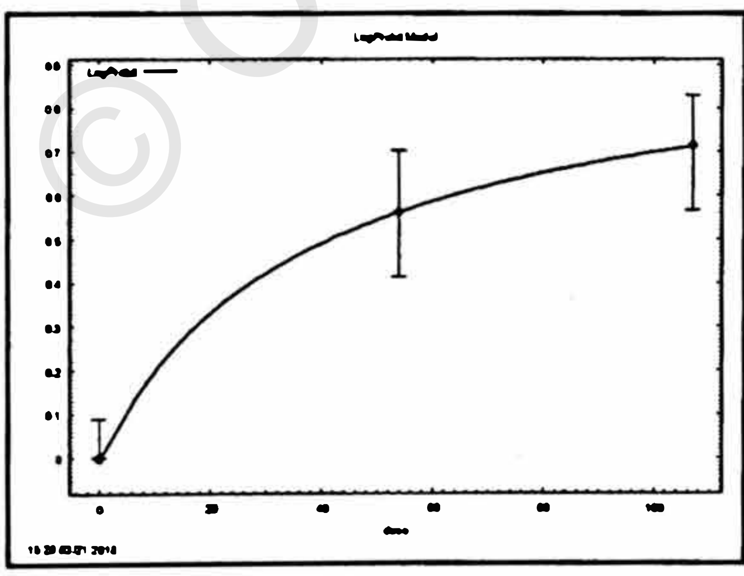
Log-Logistic Model



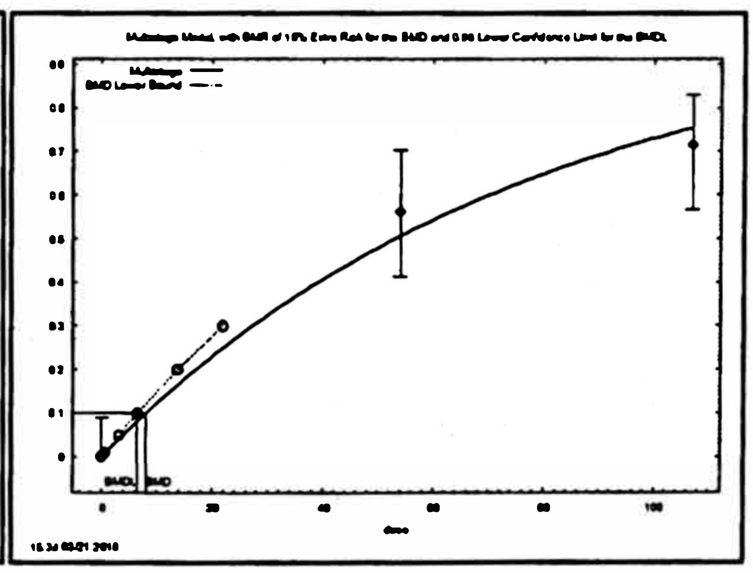
Probit Model



Log-Probit Model



Multistage Model



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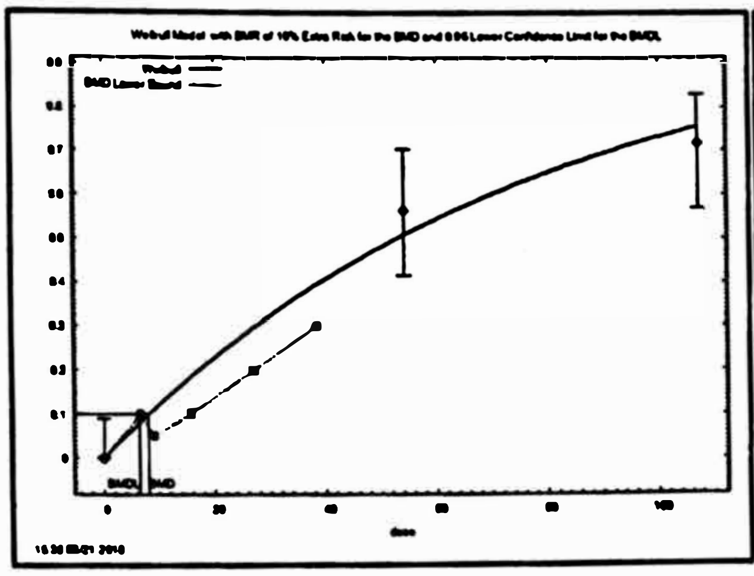
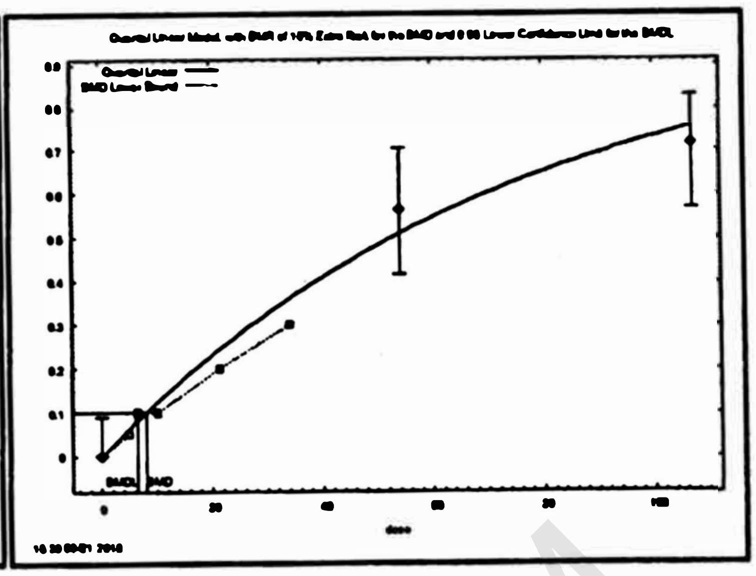
Weibull Model



Quantal Linear model



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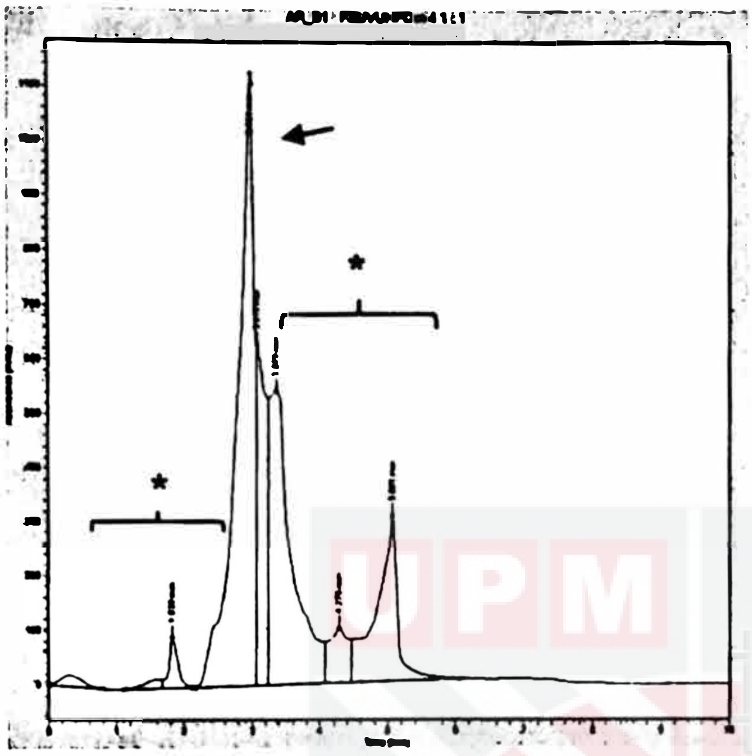
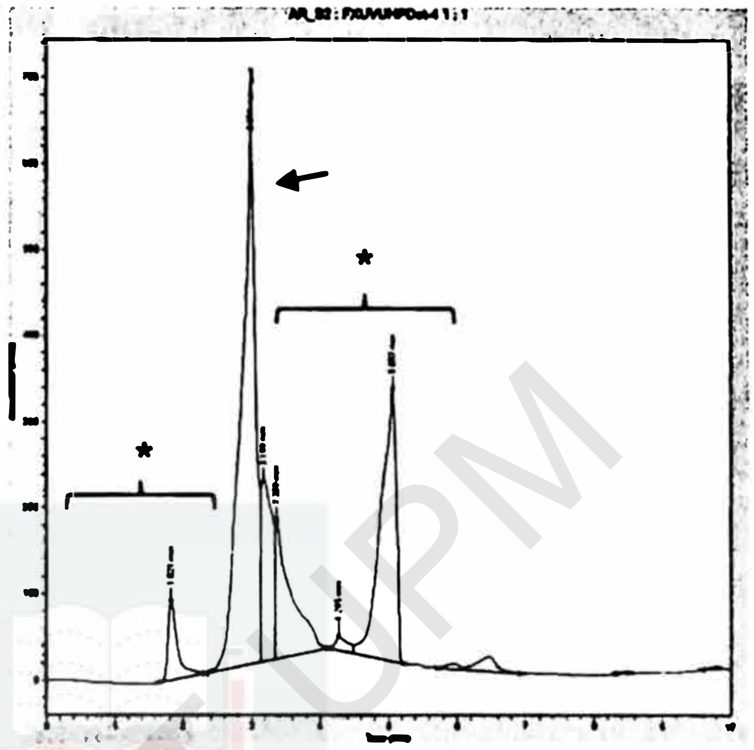
APPENDIX B: THE CHROMATOGRAMS OF THE SAMPLES

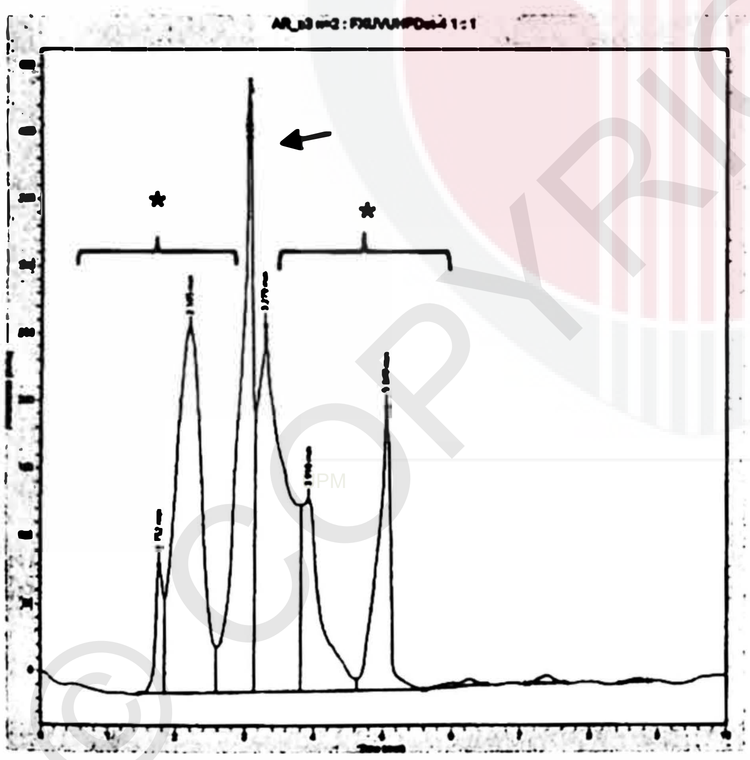
Sample 1



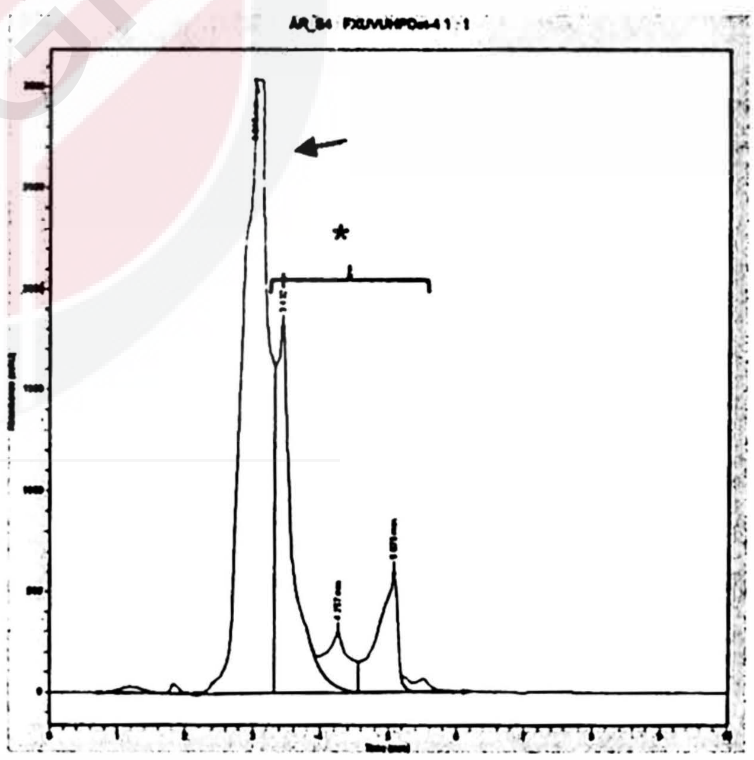
Sample 2



Sample 3



Sample 4



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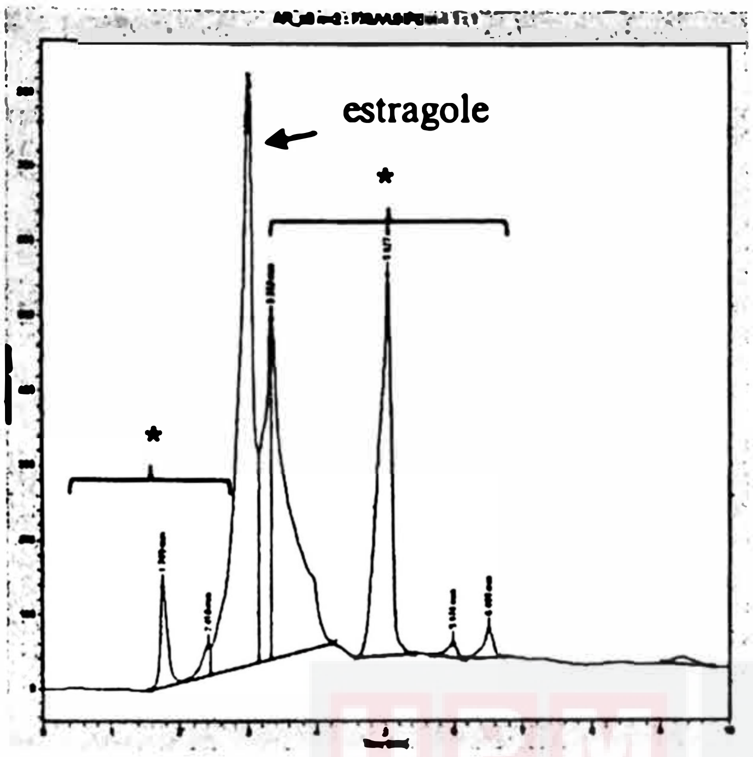
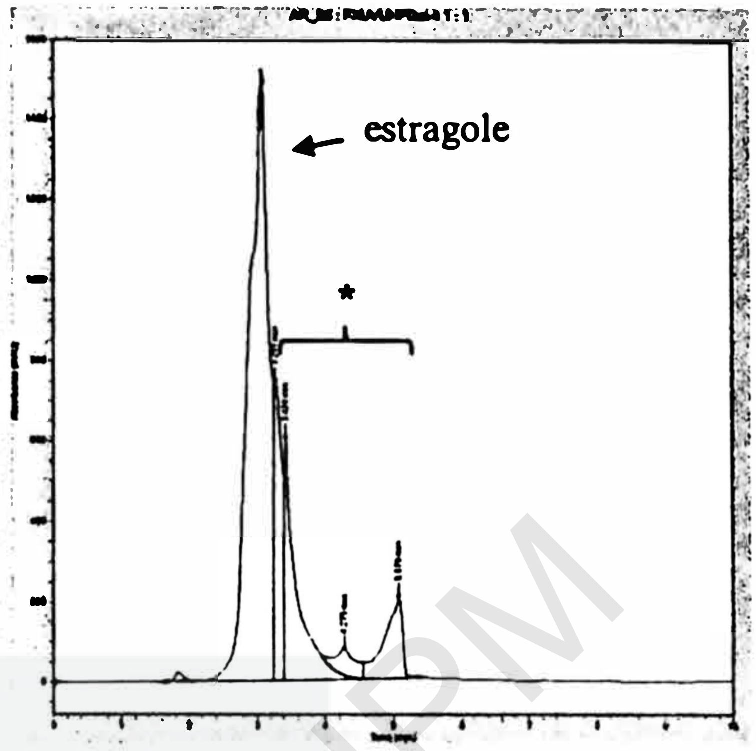
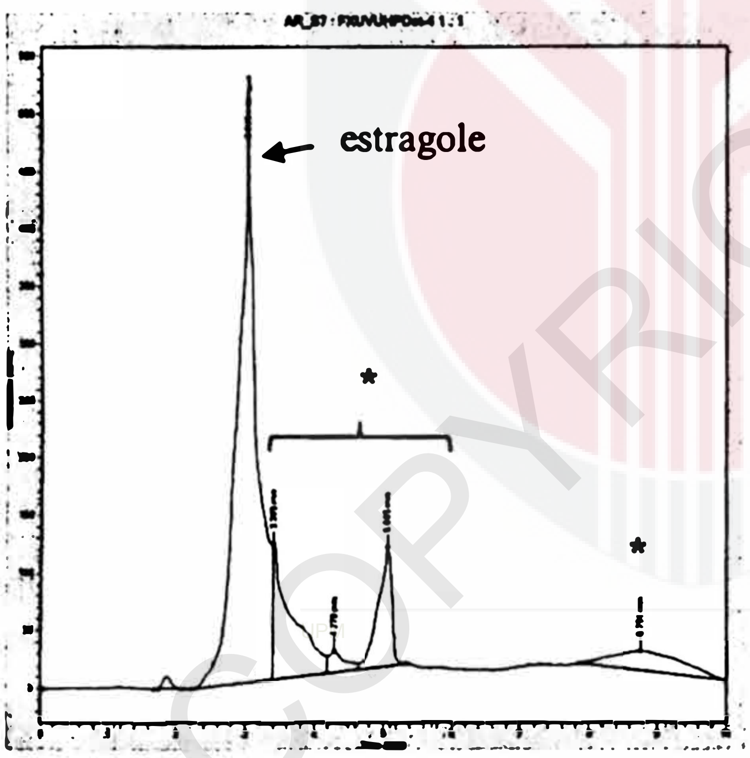
Sample 5



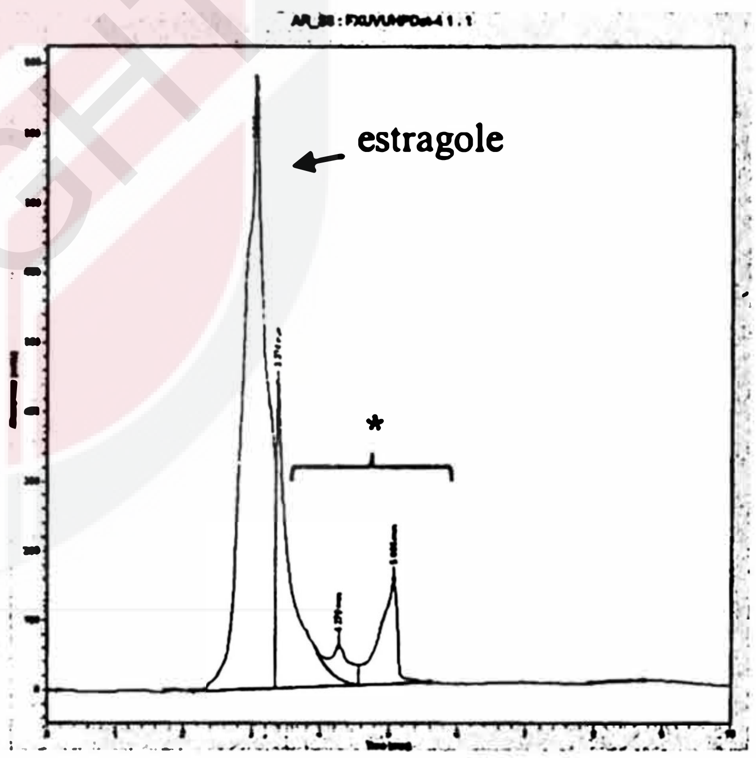
Sample 6



Sample 7



Sample 8



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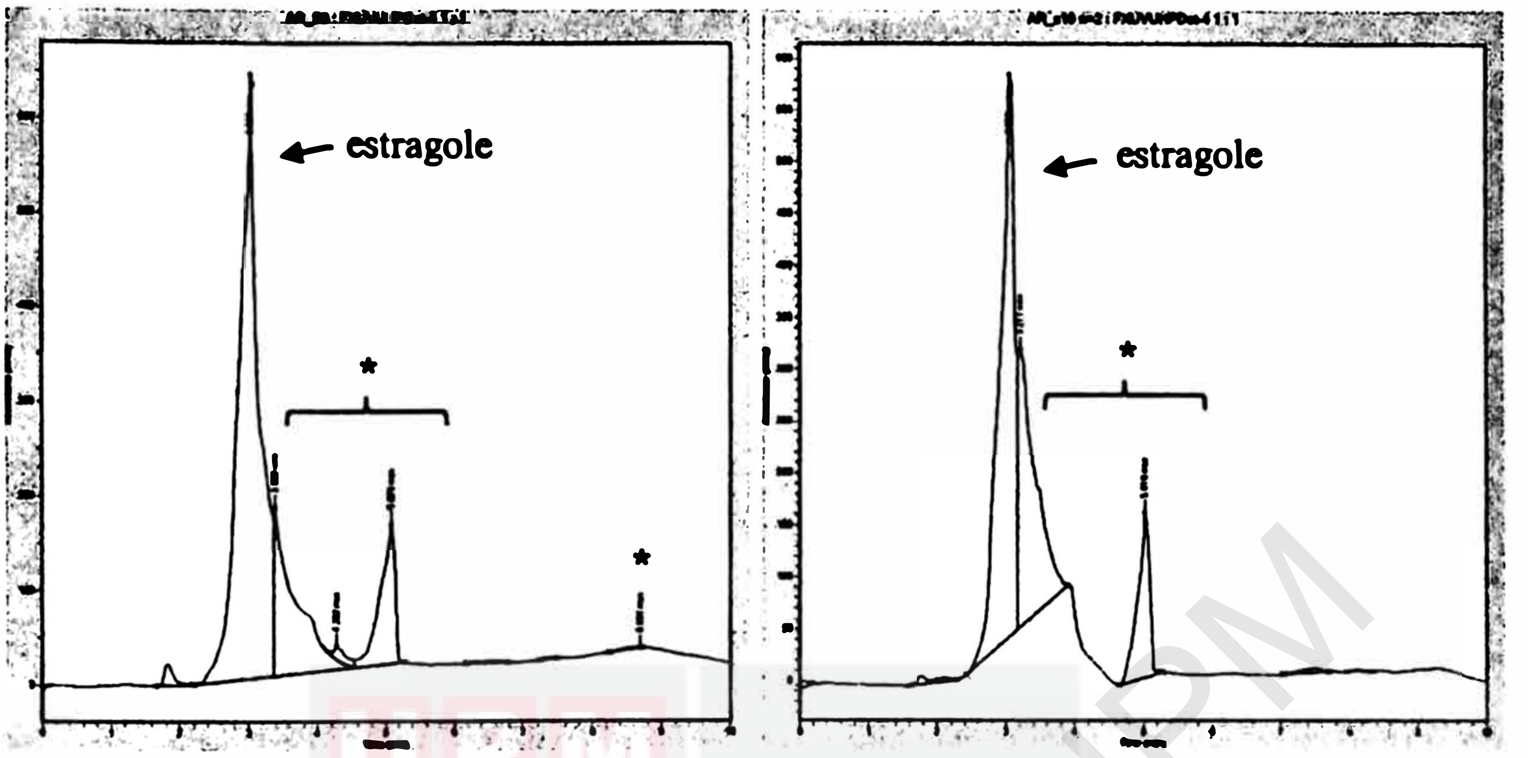
Sample 9 **Sample 10**



Peak mark with asterisk (*) were not identified. The chromatogram was obtained at a wavelength of 201nm.