



UNIVERSITI PUTRA MALAYSIA

***RISK ASSESSMENT OF ARISTOLOCHIC ACIDS FROM
TRADITIONAL HERBAL MEDICINE MARKETED IN MALAYSIA
USING MARGIN OF EXPOSURE APPROACH***

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HERBAL MEDICINE MARKETED IN MALAYSIA USING MARGIN OF
EXPOSURE APPROACH**



BY

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Science in Environmental and Occupational Health with Honours from the
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ABSTRACT**RISK ASSESSMENT OF ARISTOLOCHIC ACIDS FROM TRADITIONAL HERBAL MEDICINE MARKETED IN MALAYSIA USING MARGIN OF EXPOSURE APPROACH****NUR ATIQAH BINTI ABDUL MANAF**

Introduction: Aristolochic acid (AA) is a natural chemical that is abundant in Aristolochia plants. Herbal remedies containing Aristolochia species are believed to heal snake bites, increase breastfeeding and urination, and reduce edema. Some of the herbal medicines like slimming pills containing AA may induce kidney tumor.

Objectives: The aim of this study was to assess the risk of AA from traditional herbal medicine marketed in Malaysia using the Margin of Exposure (MOE) approach. **Methodology:** A total of 31 samples of traditional herbal medicine suspected to contain AA were purchased via online or over the counter. Samples were extracted using the hot water extraction method and analyzed using Ultra Performance Liquid Chromatography. Then, an estimated daily intake (EDIs) value was calculated, and the risk was evaluated using the MOE approach. **Results and Discussion:** From 31 herbal medicine samples, two samples were found to contain aristolochic acids I ranging from 104.0 to 262.6 $\mu\text{g g}^{-1}$. The EDIs obtained were ranging from 3.468 to 8.755 $\mu\text{g kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$. The MOEs calculated from exposure to AA from these traditional herbal medicines were below 10,000. **Conclusion:** The calculated MOE from traditional herbal medicines containing AA marketed in Malaysia were below 10,000 indicating the necessity for risk management action and causing public concern.

Keywords: Risk assessment, Aristolochic acid, Margin of Exposure and Estimated Daily Intake

ABSTRAK

PENILAIAN RISIKO ASID ARISTOLOKIK DARIPADA PERUBATAN HERBA TRADISIONAL YANG DIPASARKAN DI MALAYSIA MENGGUNAKAN PENDEKATAN PENDEDAHAN TERHADAP MARGIN

NUR ATIQA BINTI ABDUL MANAF

Pengenalan: Asid Aristolokik (AA) adalah bahan kimia semulajadi yang banyak terdapat dalam tumbuhan Aristolochia. Ubatan herba yang mengandungi spesies Aristolochia dipercayai boleh menyembuhkan gigitan ular, meningkatkan penyusutan dan pembuangan air kecil, dan mengurangkan edema. Beberapa ubat herba seperti pil pelangsing yang boleh menyebabkan tumor buah pinggang. **Objektif:** Tujuan kajian ini adalah untuk menilai penilaian risiko AA daripada ubat herba tradisional yang dipasarkan di Malaysia menggunakan pendekatan pendedahan Margin. **Metodologi:** Sebanyak 31 sampel ubat herba tradisional yang disyaki mengandungi AA telah dibeli secara dalam talian atau di kaunter. Sampel diekstrak menggunakan kaedah pengekstrakan air panas dan dianalisis menggunakan Kromatografi Cecair Prestasi Ultra. Kemudian, anggaran nilai pengambilan harian (EDI) dikira, dan risiko dinilai menggunakan pendekatan pendedahan terhadap Margin (MOE). **Keputusan dan Perbincangan:** Daripada 31 sampel ubatan herba, dua sampel didapati mengandungi asid aristolokik I antara 104.0 hingga 262.6 $\mu\text{g g}^{-1}$. EDI yang diperoleh adalah antara 3.468 hingga 8.755 $\mu\text{g kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$. MOE yang dikira daripada pendedahan kepada AA daripada ubat-ubatan herba tradisional ini adalah di bawah 10,000. **Kesimpulan:** MOE yang dikira daripa ubatan herba tradisional yang mengandungi AA yang dipasarkan di Malaysia adalah di bawah 10,000 menunjukkan keperluan untuk tindakan pengurusan risiko dan menimbulkan kebimbangan orang ramai.

Kata kunci: Penilaian risiko, Asid aristolokik, pendekatan pendedahan terhadap margin, anggaran nilai pengambilan harian

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LIST OF ABBREVIATIONS

AAs	Aristolochic acids
EFSA	European Food Safety Authority
EDI	Estimated Daily Intake
MOE	Margin of Exposure
US EPA	The United States Environmental Protection Agency
BMD	Benchmark Dose Level
BMDL ₁₀	Benchmark Lower Dose Level of 10
FDA	Food and Drug Administration
EU	European Union
DI	daily intake
UPLC	Ultra-performance liquid chromatography
IARC	International Agency for Research on Cancer

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Nowadays, traditional medicines and herbal supplements have been used extensively due to its beneficial effects. This is a global concern especially on the ingredients of this medicine that might cause another negative effect if it is used for a long period of time. The traditional medicine and herbal supplement such as ginseng, teas, and turmeric are widely used without any indications of harmful effects to their health (Sellami et al., 2018). According to Sellami et al. (2018), athletes and non-athletes equally utilized the herbal product to boost endurance and strength of performance.

Herbal medicine and natural products are also called as dietary supplements where it can be in the form of pill, tablets, capsules, or liquid form (University of Michigan Health, 2020). According to University of Michigan Health (2020) people consume these herbal remedies as they found this can give many benefits like to treat constipation, pain, and used as relaxants and stimulants. Some herbs and plant products have been demonstrated to have some of the same benefits as conventional medicines, while others may have no effect or be hazardous (Alsanad et al., 2014).

However, some herbal remedies were found to be genotoxic carcinogens especially the use of Chinese herbs that contain Aristolochic acids (AA) with the effect of causing kidney damage (Abdullah et al., 2017; Cheung et al., 2006; Martena et al., 2007). Based on adequate evidence of carcinogenicity from human research and supporting facts on carcinogenesis pathways, AA are known to be human carcinogens (National Toxicology Programme, 2008).

AA are a set of acids found naturally in a variety of plants called Aristolochia (birthworts or pipevines) and Asarum (wild ginger) that grow all over the world (National Toxicology Program, 2008). Aristolochia species are a family of *Aristolochiaceae* that comprise of many species which are *Aristolochia fangchi*, *Aristolochia manshuriensis*, *Aristolochia contorta*, *Aristolochia debilis*, *Aristolochia contorta*, *Aristolochia debilis* and *Aristolochia debilis* (Yin et al., 2002). AA may contain two major constituents which are Aristolochic acids I (AAI) and Aristolochic acids II (AAII) (National Center for Biotechnology Information, 2002).

AA became a global concern when it was found that AA may induce cancer in humans (Yang et al., 2014). The first cases were detected in Belgium in 1991, where more than 100 female patients were diagnosed with end-stage renal disease (ESRD) as a result of AA poisoning (Martena et al., 2007).

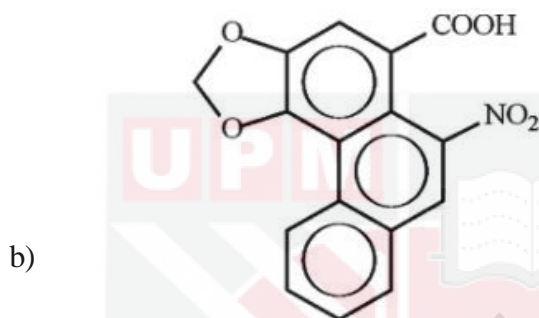
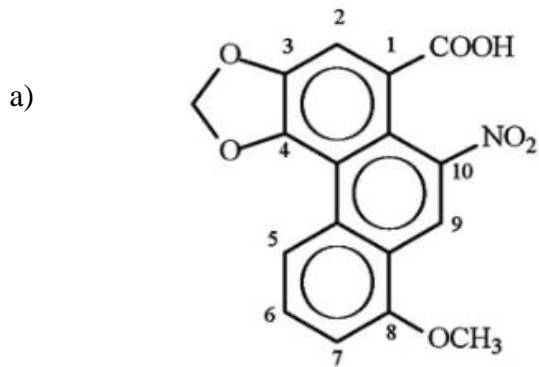


Figure 1: Structure of two major congeners of AA namely AAI (a) and AAII (b)

Figure 1 shows the structure of major congeners of AA namely AAI and AAII that differ in the structure. The main target organ of AA is the kidney (Mengs, 1988; Mengs & Stotzem, 1993). According to National Cancer Institute (2019), individuals who experienced kidney impairment caused by the ingestion of herbal items containing AA have been known to develop cancers of the upper urinary tract (renal pelvis and ureter) and bladder. Most of the country has banned the use of AA, however, there are still herbal products containing aristolochic acids that can be purchased via the online market.

1.2 Problem statement

Nowadays, traditional medicine and herbal supplements has been used worldwide. It is believed that 80 percent of the world's population relies on traditional herbal medicine for primary health care (Woo et al., 2012). The global market for herbal treatments was approximately USD 83 billion in 2008, and it is currently approximately USD 100 billion (Zhang et al., 2019). Despite the increasing use of herbal medicine used, the concern on its safety has been issued due to prolong use of the medicine. For instance, *Caulis Akebiae*, has been replaced by *Caulis Aristolochiae Manshuriensis*, and *Stephania tetrandra* has been replaced by *Aristolochia fangchi*, resulting in the major problem of "aristolochic acid nephropathy (AAN)" (Cheung et al., 2006; Martena et al., 2007). This showed that the incorrect usage of herbal remedies may lead to the adverse health effect.

The previous study shows that many Chinese herbs contain AA, including Ma Dou Ling (*Aristolochia debilis*), Tian Xian Teng (*Aristolochia contorta*), Qing Mu Xiang (*Aristolochia cucurbitifolia*), Guang Fang Ji (*Aristolochia fangchi*), Guan Mu Tong (*Aristolochia manshuriensis*), and Xixin (*Aristolochia manshuriensis*) Radix et Rhizoma Asari (Yang et al., 2014). AA are a natural compound that present in a plant called Asarum. It is highly genotoxic and they have been reported to be carcinogenic and nephrotoxic, and have been linked to kidney illnesses such as (AAN), which can lead to renal failure, various health concerns, and even death (Okhale, 2020). According to Duke Nus Medical School (2017), herbal medicine that contains AA can be purchased easily via online and occasionally mislabelled, making it difficult for suppliers and customers to know the real ingredient in mixture

of herbs. There is possibility that public purchased this herb online as there is no label on the product containing aristolochic acids.

1.3 Study justification

Based on the literature review, there were many studies regarding the toxicity of AA in herbal medicine. This study can be helpful to assess the risk of AA by using a margin of exposure (MOE) derived from Benchmark Model (BMD) from animal toxicological data. The study by Priestap et al. (2012) states that tumors were developed in numerous places when AAs were administered to rats, either orally or by injection, including the forestomach, stomach, kidney, lung, and uterine, but urothelial cancer is the most common in humans who have been exposed to AAs.

The consumption of herbal medicine is a global concern especially on the long terms effect of the usage of herbal remedies containing AA. Although there were many studies conducted worldwide, the study on exposure of AA among Malaysian population is still lacking. These studies might be helpful to prevent the increased use of herbal remedies and can promote the awareness on the present of the natural genotoxic and carcinogenic chemical in the traditional medicine. Next, the finding of the study can support the evidence of the previous study conducted by researchers and can promote more studies regarding AA in Malaysia.

Lastly, this study helps to summarize the data on AA level in different types of herbal medicine purchased in Malaysia.

1.4 Research question

This research was conducted based on the following research questions:

- a) What is the level of (AA) from traditional herbal medicine purchased in Malaysian market?
- b) What is the benchmark dose lower level of 10 (BMDL₁₀) of AA from in vivo toxicological data?
- c) What is the estimated daily intake (EDI) of aristolochic acids?
- d) How to assess the risk of aristolochic acids using a margin of exposure (MOE) approach?

1.5 Research objectives

1.5.1 General objective

To evaluate the risk assessment of Aristolochic Acids from traditional herbal medicine marketed in Malaysia using Margin of Exposure approach.

1.5.2 Specific objective

- a) To determine the level of aristolochic acids (AA) from traditional herbal medicine
- b) To establish the benchmark dose lower level of 10 (BMDL₁₀) of AA from in vivo toxicological data
- c) To calculate the estimated daily intake (EDI) of AA
- d) To assess the risk of AA using Margin of exposure (MOE) approach

1.6 Hypothesis

The MOE of AA from traditional herbal medicine marketed in Malaysia is below 10,000.

1.7 Research variables

Dependent variable: Risk assessment of aristolochic acids (AA)

Independent variable: Level of aristolochic acids, Estimated daily intake of aristolochic acids.

1.8 Conceptual definition

1.8.1 Traditional herbal medicine

It is the knowledge, skill, and practises supported theories, beliefs, and experiences indigenous to varied cultures, whether explicable or not, utilized in the upkeep of health also because the prevention, diagnosis, improvement, or treatment of physical and mental disease, whether explicable or not that used some herbs or herbal products from the derivative of plant (World Health Organization, 2022).

1.8.2 Risk assessment

A multi-step method that, when combined with an exposure assessment and other components, yields estimate of the risk of an unfavourable health outcome as a result of consuming, inhaling, or absorbing one or more dangerous substances.

1.8.3 Margin of exposure (MOE)

A tool used by risk assessors to analyze potential safety problems arising from the presence of genotoxic (DNA-damaging) and carcinogenic chemicals in food and feed (EFSA, 2009).

1.8.4 Aristolochic acids (AAs)

AA is a carcinogenic alkaloid derived from phenanthrene and carboxylic acid that is found predominantly in plants of the genus *Aristolochia*.

1.9 Operational definition

1.9.1 Traditional herbal medicine

Herbal medications are mostly used for health promotion and treatment of chronic, instead of life-threatening diseases. However, when mainstream medicine proves ineffectual in treating disease, such as in advanced cancer or in the face of emerging infectious diseases, the use of traditional treatments rises. Furthermore, traditional remedies are often regarded as being natural and risk-free.

1.9.2 Risk assessment

The MOE method was used to estimate the risk of AAs from the use of PFS and herbal items (Abdullah et al., 2017) and the calculations of MOE were based on animal data. EDI value of aristolochic acids can be obtained by using simple dose addition in combined exposure from a previous study (Abdullah et al., 2017).

1.9.3 Margin of exposure (MOE)

MOE is the value that shows priority management needed if the value is less than 10,000.

1.9.4 Aristolochic acids (AAs)

Aristolochic acids are a class of acids occurring naturally in various Aristolochia (birthworts or pipevines) and Asarum (wild ginger) plants that grow all over the world (National Cancer Institute) and it is usually presence in Chinese herb. Exposure can occur by the deliberate or unintentional use of aristolochic acid-containing herbal or culinary items (National Cancer Institute, 2019).

1.10 Conceptual framework

Figure below shows the conceptual framework of this study.

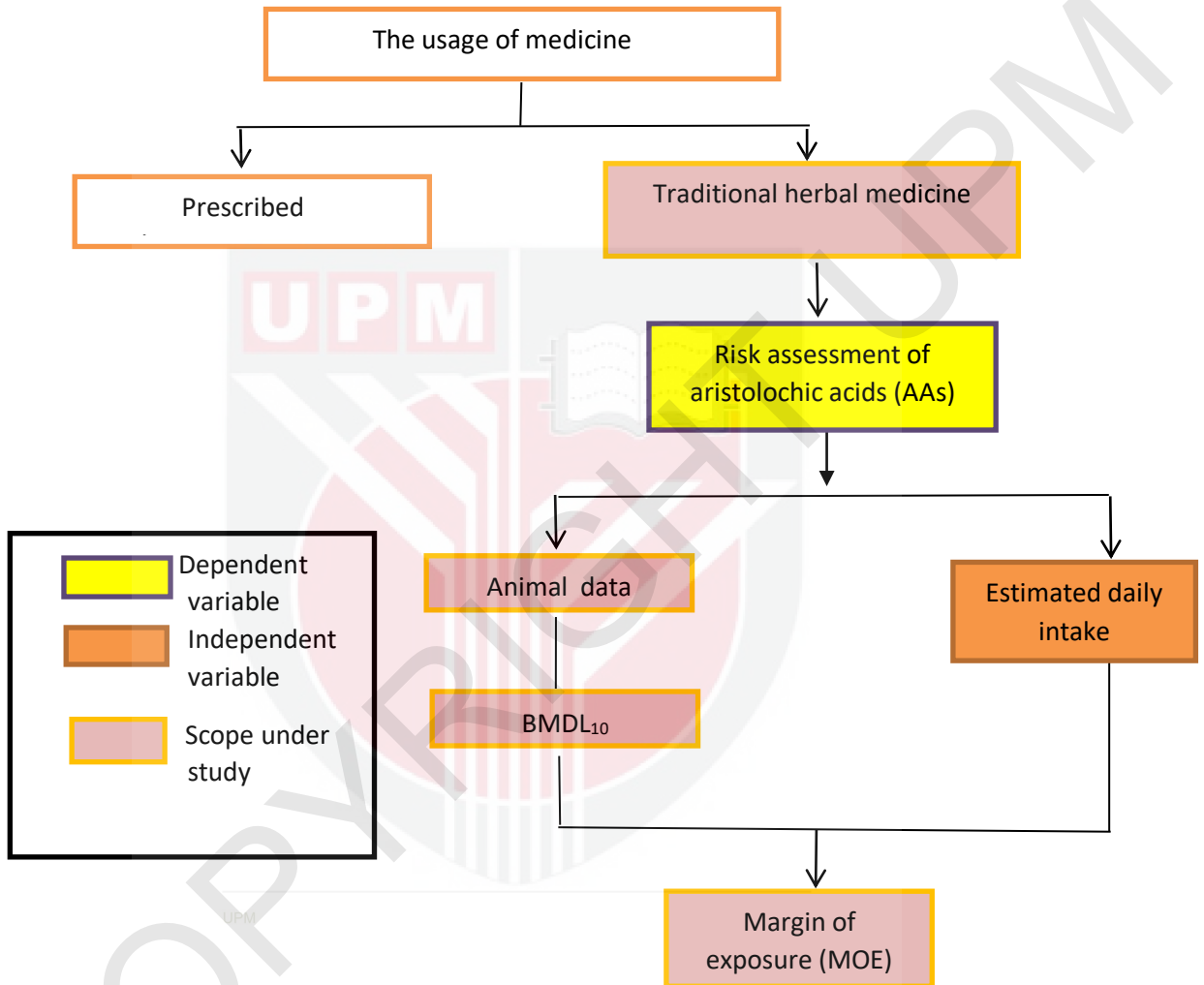


Figure 2: Conceptual Framework of this study

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Nowadays, traditional and herbal medicine is widely used in order to reduce or treat the disease. This includes the food supplement that has been consumed by some people to improve health. However, some studies found that herbal medicine has become a concern of human safety. According to Davey (2017), herbal remedies might cause kidney failure and liver damage in human because it might contain hazardous compounds such as heavy metals or interact negatively with other medications.

Traditional herbal medicine is usually taken directly from the plant, or it is extracted from the plants. Athletes and non-athletes equally utilize the herbal product to boost endurance and strength performance (Sellami et al., 2018). According to the World Health Organization (2022), approximately 4 billion people, or 80% of the world's population, use herbal medicine in certain medical care. There are many types of herbal products that have been consumed by most people which are echinacea where it has been used to prevent cold, ginseng (boost energy), ginkgo Biloba able to treat numerous illness, elder belly can treat cold and flu symptoms, St. John's Wort can treat depression, turmeric can treat arthritis, ginger can reduce nausea, valerian act as anti-anxiety aids and camomile able to treat a broad range of ailments (Hill, 2020). Herbal medicine is assumed to be safe to consume as it is

derived from natural products. However, the usage of herbal medicine for a long time may be toxic to humans.

2.2 Presence of Aristolochic acids in traditional herbal medicine

AAs are comprised of Aristolochic acids I and II are usually the compounds from *Aristolochia* species in plants. According to Martena et al. (2007), *Aristolochia* species such as *Aristolochia fangchi* and *Aristolochia manshuriensis* are used in traditional Chinese Medicine (TCM) to heal snake and beetle bites, increase breastfeeding and urination, and reduce edema. Indeed, this study also found that AA do not found in fruits (*Aristolochia manshuriensis* and *Aristolochia contorta*) namely *ma dou ling* but it is found in the roots of *A. contorta* namely *qing Mu Xiang* and *tian Xiang Teng* (herbs of *A. contorta*).The existence of Aristolochic acids hazardous chemicals in *Aristolochia bracteolata* L. methanol extract was also found in the study by Abdelgadir et al. (2011) by using a multi-component approach that included Column Chromatography (CC), Thin Layer Chromatography (TLC), and Preparative Thin Layer Chromatography (PTLC). In the previous study, out of six banned Chinese herbs that use in this study, four of them were found to have Aristolochic acids (Cheung et al., 2006). Table 1 shows the presence of AA in herbal medicine and herbal products from the literature.

Table 1 : List of Chinese Herbs containing Aristolochic acids (Yang et al., 2014)

Chinese name	herbal name	Botanical name	Chinese herbal formula containing this herb
Guang Fangchi (Fangchi)	Fang Ji	<i>Aristolochia fangchi</i>	Shu Jing Huo Xue Tang Shang Zhong Xia Tong Yong Tong Feng Fang Ji Huang Qi Tang Xiao Xu Ming Tang Jie Geng Tang Mu Fang Ji Tang
Xixin		<i>Radix et Rhizoma Asari</i>	Chuan Qiong Cha Diao San Xiao Qing Long Tang Du Huo Ji Sheng Tang
Guan Mu Tong		<i>Aristolochia manshuriensis</i>	Long Dan Xie Gan Tang Xin Yi San Ba Zheng San Gan Lou Xiao Du Dan Dao Chi San Dang Gui Si Ni Tang Mu Tong Guo Qi Yin Xiao Ji Yin Zi Ju He Wan

		Zheng Gu Zi Jin Dan
Qing Mu Xiang	<i>Aristolochia cucurbitifolia</i>	Xiang Sha Liu Jun Zi Tang
		Gui Pi Tang
		Zheng Gu Zi Jin Dan
Ma Dou Ling	<i>Aristolochia debilis</i>	
Tian Xiang Teng	<i>Aristolochia contorta</i>	



2.3 Exposure of aristolochic acids to human

The previous study showed that AA are from a Chinese herb that are usually used to treat the symptoms of certain diseases. However, the usage of this for a long time can give toxicity to human life. The risk of AA exposed to human is urothelial carcinoma where it first identified in Belgium. Aristolochic acid was shown to be the cause of serious renal failure in Belgian women who had taken weight-loss products containing *Aristolochia fangchi* in the 1990s (Wu & Wang, 2013) (Zhang et al., 2019). Zhang et al. (2019) state that rapidly progressing renal interstitial fibrosis led to chronic renal failure in these women, and around 5% of them had the end-stage renal disease (ESRD). This shows that aristolochic acids as a carcinogenic that is a concern to human safety. According to Zhang et al. (2019), some factors contribute to the toxicity of aristolochic acids to humans which are the high dose, duration of AA dose, and also activity of enzymes that catalyze the biotransformation of AA.

A previous study also found the diagnostic of aristolochic acids nephropathy. They were three criteria that can induce the development of AAN which were renal histology displaying interstitial fibrosis with a corticomedullary gradient, a history of consumption of herbal products which demonstrated the presence of AA, and therefore the presence of AA-DNA adducts (or the precise A: T → T: A transversion in p53 gene) during a kidney tissue sample or of a urothelial tumor (Jadot et al., 2017). The table 3 below shows the cases reported when ingesting the aristolochic acids contained in Chinese herbs or plant food supplements in certain countries.

Table 2: Summary of reported cases of AA intoxication (Jadot et al., 2017)

Country	Number of cases	Purpose of AA Ingestion <i>Suspected Aristolochia Species</i>	References
Belgium	9	Slimming pills containing Chinese herbs. <i>Aristolochia fangchi</i>	Vanherweghem et al. (1993)
Spain	1	An infusion made with a mixture of herbs. <i>Aristolochia pistolochia</i>	Peña et al. (1996)
Japan	1	Health food for atopic dermatitis.	Tanaka et al. (1997)
France	2	Slimming pills containing Chinese herbs.	Stengel and Jones (1998)
Belgium	100	Slimming pills containing Chinese herbs. <i>Aristolochia fangchi</i>	Vanherweghem et al. (1998)
UK	2	Herbal preparation for treatment of eczema. <i>Aristolochia manshuriensis</i>	Lord et al. (1999)
Taiwan	1	Chinese herbal medicine for peripheral extremities	Lee et al. (1999)

		weakness and numbness.	
USA	1	Chinese herbal medicine for pain relief.	Mayor et al. (2000)
Japan	2	Not described. <i>Aristolochia manshuriensis</i>	Tanaka et al. (2000)
Taiwan	12	Chinese herbal medicine for weight control, nutritional supplements, treatment of arthralgia, hypertension, or hepatitis.	Yang et al. (2000)
Germany	1	Chinese herbal medicine for hyperuricemia.	Krumme et al. (2001)
China	58	Not described.	Chen et al. (2001)
China	1	Chinese herbal medicine as a “tonic herbal remedy”.	Lo et al. (2004)
Korea	1	Chinese herbs mixture for slimming purposes.	Lee et al. (2004)
Japan	1	Chinese herbal medicine for sterility.	Kazama et al. (2004)
Taiwan	1	Chinese herbal medicines for “health improvement”.	Hong et al. (2006)

China	8	Chinese herb	Yang et al. (2007)
		“Guanmutong” <i>Aristolochia manshuriensis</i> .	
Australia	1	Chinese herbal medicine to treat psoriasis.	Chau et al. (2011)
China	300	Not described.	Yang et al. (2012)
Bangladesh		Snakebite remedies, sexual difficulties, stomach troubles, and a "tonic cure." <i>Aristolochia Indica</i>	Michi et al. (2013)

2.4 Metabolism of Aristolochic acids and their carcinogenicity

Aristolochic acids are plant toxins that comprise of Aristolochic Acid I (AAI) and II (AAII) (Yin et al., 2002). The most important component within the mixture of AAs within the plants is AAI or 1,3-dioxole-5-carboxylic acid (also referred to as 3,4-methylenedioxy-8-methoxy-10-nitro-1-phenanthrenecarboxylic acid or 3,4-methylenedioxy-8-methoxy-10-nitro-1-phenanthrenecarboxylic acid) while Aristolochic acid II (AA-II) (6-nitro-phenanthro[3,4-d]-1,3-dioxole-5-carboxylic acid or 3,4-methylenedioxy-10-nitro-1-phenanthrenecarboxylic acid) is generally present in small concentrations (Priestap et al., 2012).

AA are known to cause cancer in humans which was urothelial cancer. Aristolochic acid nephropathy and Balkan endemic nephropathy were rare renal disorders associated with upper urothelial carcinoma caused by the plant extract AA (Dedlková et al., 2020). In the previous study, the renal proximal tubule has been demonstrated in rats to be a major target of AA toxicity, which can lead to renal failure (Martena et al., 2007).

Aristolactam Ia, the main metabolite of AAI, was created by two metabolic routes, one via aristolactam I and the other via AA Ia (Arlt et al., 2002). Anaerobic incubation of AAI and AAII with rat liver S9 mix also produces aristolactam I and aristolactam II in vitro (Schmeiser et al., 1986). AA Ia is the predominant metabolite

generated by AAI under aerobic incubation conditions, while AAII is unaffected (Arlt et al., 2002). Although the phase II metabolism of both AAs has not been widely researched, considerable quantities of AA metabolites were found in conjugated form in the urine and faeces of rats, and were speculated to be either glucuronides or sulphate esters (Krumbiegel et al., 1987).

2.5 Animal toxicological study

According to American Physiological Society (2019), It is unethical to purposefully expose humans to health hazards to track the progression of a disease. Previous studies have found that AA was tested to increase the miRNA biomarkers on rat liver tissue that induces kidney tumors (Meng et al., 2014). Wistar rat that exposed to AA experimental study showed renal failure occurs at a dose of 25 mg/kg bw of aristolochic acids (Yin et al., 2002).

They also found that the toxic effect of AA in rat was observed at dose 5.0mg/kg bw given daily for four weeks (Yin et al., 2002). A previous study by Mengs & Stotzem (1993) reviewed by (Yin et al., 2002) also states that female Wistar rats are given single doses of 10, 50, or 100 mg/kg bw via intragastrical exposure developed renal lesions within three days. Table 2 shows mutations in oncogenes found in rodents treated in vivo with Aristolochic acids.

Table 3: Mutations in oncogenes found in rodents treated in vivo with aristolochic acids (AAs) (Yin et al., 2002)

Species	Treatment	Incidence and type of tumors	Method of analysis	No. of mutated genes/no. of tumors analyzed	References
Male Wistar rats	For three months, 8-week-old rats were administered 10 mg/kg bw AAI via gavage five times a week. After the treatment, rats were killed over 15 weeks.	15/40 forestomach tumors (SCC), 7/40 ear duct tumors (SCC), 23/40 adenocarcinomas or sarcomas of small intestine, 2/40 metastases of SCC in lung and pancreas	DNA isolated from excised forestomach tumors were transfected into NIH 3T3 cells which induced tumours in nude mice. c-Ha-ras fragments were amplified by PCR of DNA from nude mouse tumors and analyzed by sequencing. DNA extracted from rat tumors was amplified by PCR for regions of c-Ha-ras, c-Ki-ras, and c-N-ras gene and analyzed by selective oligonucleotide	5 7/7 ear duct tumors 14/14 forestomach tumors 1/8 tumors of the small intestine 1/1 metastasis in the pancreas 1/1 metastasis in the lung	Schmeiser et al. (1990)

				hybridization with probes carrying different ras base-pair substitution		
Female NMRI mice	5 mg/kg bw AA mixture, by gavage, daily for 3 weeks (80% AAI, 20% AAI).	SCC of the forestomach, adenocarcinoma of the lung	DNA extracted from histologically normal and neoplastic tissue in paraffin sections, c-Ha-ras fragments around codon 61 amplified by PCR and analyzed by oligo-nucleotide hybridization with probes carrying different c-Ha-ras base-pair substitution	1/1 forestomach tumor 1/3 lung tumors	Schmeiser et al. (1991)	
Male Wistar rats	10 mg/kg bw AAI given daily by gavage to 8-week-old rats, 5 times a week for 3 months. Rats were killed over 15 weeks	SCC of the forestomach, and pancreas	DNA extracted from histologically normal and neoplastic tissue in paraffin sections, c-Ha-ras fragments around codon 61 amplified by PCR and analyzed by oligonucleotide hybridization with	2/2 forestomach tumors 0/1 pancreas tumor	Schmeiser et al. (1991)	

after treatment

probes carrying different

c-Ha-ras base-pair

substitutions

2.6 Risk assessment approach

The MOE is a technique used by risk assessors to characterize the risk of genotoxic and carcinogenic chemicals present in food or feed (EFSA, 2005). According to EFSA (2005), MOE cannot be used to determine the safety of controlled chemicals that are being used in the food supply but they suggest that it might be beneficial in determining the safety of any genotoxic or carcinogenic contaminants present in such compounds at extremely low levels. This approach is helpful to keep the levels of substances as lower as possible. MOE is a dimensionless ratio that compares the BMDL10 (lower confidence bound of the benchmark dose that causes a 10% increase in cancer incidence) to the EDI whereas BMDL is a reference value generated from the Benchmark dose (BMD) that improves the characterization and quantification of possible hazards by extending the use of dose-response data from experimental animals or observational epidemiological research (EFSA, 2005). The MOE 10000 or more than that is considered as a low concern if it is based on the BMDL by referring to animal data.

2.7 Guidelines on the usage of herbal medicines

In certain countries, they usually developed guidelines for the safe use of herbal medicine to protect the consumers. World Health Organization (WHO) plays a significant role to develop certain guidelines involving herbal medicine or herbal product. WHO has published some guidelines regarding herbal medicine which are WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems (2004) and WHO guidelines for assessing the quality of herbal medicines about contaminants and residues (2007). According to European Medicines Agency (2005), there is a guideline regarding the *Aristolochia* species which is a public statement on the risk associated with the use of herbal products containing *Aristolochia* species.

AAs have been banned in some country including Malaysia. This has been stated under Guidelines on Registration of Natural Products that aristolochic acids has been banned due to reported adverse effect in appendix 7 (National Pharmaceutical Regulatory Division Ministry of Health Malaysia, 2021). Since 2000, the sale of AA-containing plants has been severely limited or prohibited in the United Kingdom, the United States, Canada, Australia, and New Zealand, and since 2001, the use of Mu Tong, Fang Ji, Ma Dou Ling, or Qing Mu Xiang in the United Kingdom has been prohibited (Martena et al.,2007). Martena et al. (2017) also states that the selling of *Magnolia Officinalis* and *S. tetrandra* was formerly prohibited under the Dutch Commodities Act "Herbal preparations" even though it has low risk.

Although many guidelines have been established in the use of herbal medicines, there is still a toxic substance that is widely used with a high risk to the consumer. For example, the use of Aristolochic acids is widely used nowadays and this herb was still present in the online and offline market (Abdullah et al., 2017; Cheung et al., 2006; Martena et al., 2007).



CHAPTER 3

METHODOLOGY

3.1 Study design

This study is an experimental study. Traditional herbal medicines were analyzed to determine the presence of AA. The secondary data was used in this study too. The secondary data were used to calculate benchmark dose lower level of 10 (BMDL₁₀) while EDI values were calculated based on the recommendation from the supplier or literature. The Margin of Exposure (MOE) values were calculated from the EDI and BMDL₁₀ data.

3.2 Collection of samples

A total of 31 samples of herbal medicine were purchased from online and over the counter from different sources. The samples were chosen based on the ingredient containing suspected botanical of concern like *Aristolochia* spp. and *Asarum* spp. Table 5 shows the product information and its botanical of concern. All dried herbs were homogenized to be in the form of powder by using a blender. Then, 1g of each sample was weighed using analytical balance before being extracted using the hot water extraction method (Van den Berg et al., 2014).

3.3 Chemicals

A standard compound of AAI was purchased from Sigma Aldrich from Merck (Darmstadt, Germany) whereas methanol (HPLC Grade) was purchased from R & M Chemicals (UK).

3.4 Hot water extraction

A total of 25mL freshly boil water were added to 1g of sample by using a measuring cylinder. The mixture of hot water and sample was put on the hot plate and then, incubated for 7 min and stirred three times to make sure the mixture mixed well. After that, the extracts were left at room temperature for about 30 minutes. Then the extract was centrifuged using a maximum speed of 40000rpm for 10 min (Tabletop Centrifuge, Kubota, Japan). After centrifugation, this extract was filtered using a syringe filter nylon 25mm 0.45 μ m into a UPLC via UPLC analysis.

3.5 Recovery analysis

Sample number 4 was used in the recovery analysis. During the extraction process, 100 µm of AAI was spiked to the sample. The percentage of the AAI that recovered was calculated using equation 3.1.

Equation 3. 1

$$\% \text{ recovery} = \frac{\text{conc of spike} - \text{conc of unspike}}{\text{known spike added concentration}} \times 100\%$$

3.6 UPLC analysis

UPLC analysis was used to identify the presence of AAI via chromatogram. A 3.5µl undiluted sample was injected into UPLC (Waters Acquity UPLC BEH C18 with 1.7µm 2.1x50mm) column. To summarize, a gradient was produced utilizing ultra-pure water as solvent A and 0.1 percent (v/v) TFA as solvent B. The flow rate was set at 0.6 ml min⁻¹. The gradient started at 80:20 (A:B), then changed to 75:25 from 1 to 3 min, then to 20:80 from 3 to 5 min, and stayed there for 1 min. To equilibrate the column, the gradient was altered from 6 to 7.3 min to 0:100 and held for another 0.2 min before the starting condition was reset from 7.5 to 8 min and kept for another 1 min. The chromatograms were examined at 240 nm using a photodiode array detector (Waters, Milford, MA, USA).

3.7 Quality Assurance and quality control (QAQC)

When doing the experiment, there are some QAQC that need to be considered to ensure that the experiment was acceptable. Firstly, gloves and lab coat has been worn before starting the experiment to avoid spillage of the chemical. Next, the triplicate of the sample was done to increase the validity and accuracy of the results. Other than that, negative sample was spike to calculate the percentage recovery of the compound. Lastly, after finished the experiment, all the apparatus that have been used in the laboratory like beaker and spatula has been washed by using decon and distilled water.

3.8 Estimated Daily Intake

To calculate the exposure to AA, an EDI calculation was used. Bodyweight of 60 kg for Malaysian users and 2.0 g daily consumption to make a cup of fennel tea were used to calculate EDIs as mentioned by Van den Berg et al. (2014). The EDIs value was calculated by using equation 3.2.

Equation 3. 2

$$\text{EDIs} = \frac{\text{level of AAI} \times \text{daily intake}}{\text{body weight}}$$

3.9 Risk assessment

The margin of exposure (MOE) approach was used to evaluate the risk of genotoxic and carcinogenic chemicals (EFSA, 2005). The MOE is a dimensionless ratio determined by comparing the lower confidence limit of the benchmark dose to the lower confidence limit of cumulative 10% tumor incidence (BMDL₁₀) derived from tumor data and EDIs calculations by using equation 3.3.

Equation 3. 3

$$\text{MOE} = \frac{\text{BMDL}_{10}}{\text{EDI}}$$

Where,

BMDL₁₀= Benchmark dose lower level of 10

EDI= Estimated daily intake

3.10 Study flowchart

Figure 3 shows the study flowchart of the present study.

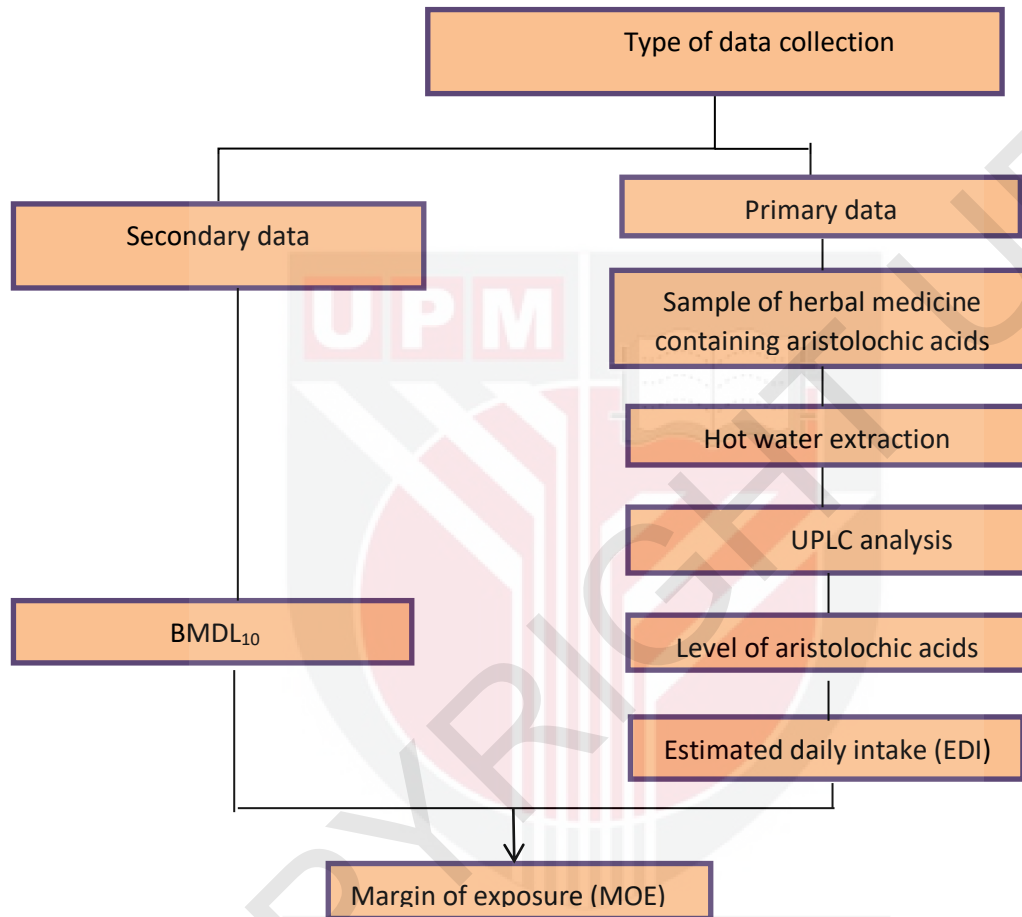


Figure 3: Study Flowchart

CHAPTER 4

RESULTS

4.1 Collection of herbal medicine samples for analysis

Table 4 shows the description of the traditional herbal medicine under investigation in this study. Traditional herbal medicines were bought from online and offline market. All the samples were originated from China and marketed in Malaysia.

Table 4: The description of the traditional herbal medicine under investigation in this study

Sample	Product name	Product presentation	Dosage	Suspected botanical ingredient	Country of origin
S1	Aristolochia	Fruit (dried)	2000 mg per day	<i>Aristolochia contorta</i> Bunge; Ma Dou Ling	China
S2	Guan Mu Tong	Plant stem (dried)	2000 mg per day	<i>Aristolochia manshuriensis</i> caulis; Guan Mu Tong	China
S3	Min Shan Chuan Xiong Cha Tiao San	Tablet (small pills)	8 pills, 3 times per day	<i>Aristolochia sp.</i>	China
S4	Ta Huo Lo Tan	Tablet (pills)	1 pill, 2 times per day	<i>Aristolochia sp.</i>	China
S5	Ren Shen Feng Shi Wan	Capsules	2-4 capsules, 3 times per day	<i>Aristolochia sp.</i>	China
S6	Wild ginger	Plant (dried)	Stem 2000 mg per day	<i>Asarum canadense</i> Linn; Wild Ginger	China
S7	Wild ginger	Plant (dried)	Stem 2000 mg per day	<i>Asarum canadense</i> Linn; Wild Ginger	China
S8	Coltsfoot Flower (Jingfangji)	Flower (dried)	2 mg per day	Syn. <i>Asarum canadense</i> var.reflexum; False coltsfoot	China
S9	Akebia (Huai Tong)	Stem Mu (dried)	Plant Stem 2000 mg per day	<i>Akebia spp</i> : Mu Tong	China
S10	Fruit of Fiveleaf Akebia	Fruit (dried)	2000 mg per day	<i>Akebia quinata</i> (Houtt) Decne; Fiveleaf akebia; Chocolate vine; Mu Tong	China

S11	Chinese Wild Ginger	Plant (dried)	Root	2000 mg per day	<i>Asarum canadense</i> Linn; Wild Ginger	China
S12	Chinese Wild Ginger	Whole (dried)	plant	2000 mg per day	<i>Asarum canadense</i> Linn; Wild Ginger	China
S13	Chinese Wild Ginger	Powder		2000 mg per day	<i>Asarum canadense</i> Linn; Wild Ginger	China
S14	Xi Xin (Wild Ginger)	Plant (dried)	Root	2000 mg per day	<i>Asarum heterotropoides</i> or <i>Asarum sieboldii</i>	China
S15	Zhu Po Chuanbei Hou Zao San	Pills		1 to 6 years old: 1/2 teaspoon (0.4g) 3 times a day 7 to 12 years old: 1 teaspoon (0.8g) 3 times a day. Adults: 2 teaspoons (1.6g) 3 times a day, (Teaspoon is provided, 1 teaspoon=0.	<i>Aristolochia sp.</i>	China
S16	Chuan Qiong Cha Tiao Wan	Capsules		8 pills, 3 times per day	<i>Aristolochia sp.</i>	China
S17	Soo Hup Pills	Tablets (small pills)	(small pills)	Adults: 1-2 pills to be taken with water or ginger soup. Children: 1 pill divided into 3 portions (take with water /ginger soup).	<i>Aristolochia sp.</i>	China
S18	Teck Aun Chi Kit Pills	Tablets (small pills)	(small pills)	Adults 1 packet, 4 times daily Children 3 years and above 1/2 packet, 4 times daily to be taken with warm water Children 2 – 3 years 10 pills, 4 times daily Children 1 – 2 years 5 pills. 4 times daily to be taken with warm water	<i>Aristolochia sp.</i>	China
S19	Chinese Clematis Root	Root (dried)		2000 mg per day	<i>Clematis Chinensis</i> ; <i>Akebia sp.</i>	China
S20	Hua Chuliang Chu Shizi	Powder (extract granule)		2000 mg per day	<i>Aristolochia sp.</i>	China
S21	Wumeiwei Lingxian Mulberry Teabag	Root (dried)		2000 mg per day	<i>Aristolochia sp.</i>	China
S22	Wild Weiling Xian	Powder		2000 mg per day	<i>Clematis manshurica</i>	China
S23	Chuan Mu	Plant	stem	2000 mg per day	<i>Aristolochia</i>	China

	Tong	(dried)			<i>manshuriensis</i> ; Chuan Mu Tong	
S24	Cocculus Indica	Liquid		Dosage should be directed by the physician	<i>Cocculus spp.</i>	China
	Dilution 30 CH					
S25	Wild Costustoot Radix Aucklandiae	Plant (dried)	stem	2000 mg per day	saussurea lappa, saussurea costus, Mu Xiang, costustoot	China
S26	Fangji	Plant (dried)	stem	2000 mg per day	<i>Aristolochia fangchi</i> root;guang fang ji	China
S27	Fangha Mufangji	Plant (dried)	stem	2000 mg per day	<i>Clematis spp.</i>	China
S28	Stephania Tetrandra	Plant (dried)	stem	Cook with 3 bowls of water to 1 bowl, and take it	Hang fang Ji	China
S29	Stephania Tetrandra	Plant (dried)	stem	2000 mg per day	Hang fang Ji	China
S30	Huang Qi Spot Fangji Fuling Decoction Teabag	Plant (dried)	stem	2000 mg per day	<i>Stephania Tetrandra</i> (Han fang ji)	China
S31	Wild Ginger	Whole (dried)	plant	2000 mg per day	<i>Asarum canadense</i> Linn; Wild ginger	China

4.2 UPLC Analysis

The UPLC study was performed to determine whether AAs were present in herbal medicine. It was found that AAI was eluted at 4.8 minutes. The chromatogram was obtained at a wavelength of 240nm. The AA levels were computed using the obtained calibration curves (Figure 4) for AA-I ($R^2=0.9856$) and adjusted using the percentage of recovery for the various sample types. Calculation of the recovery of AAs from spiked samples was used to assess the method's accuracy. Out of 31 samples analyzed in UPLC, two (2) samples were found containing Aristolochic acids 1 which were sample 1 (Figure 5) and sample 9 (Figure 6).

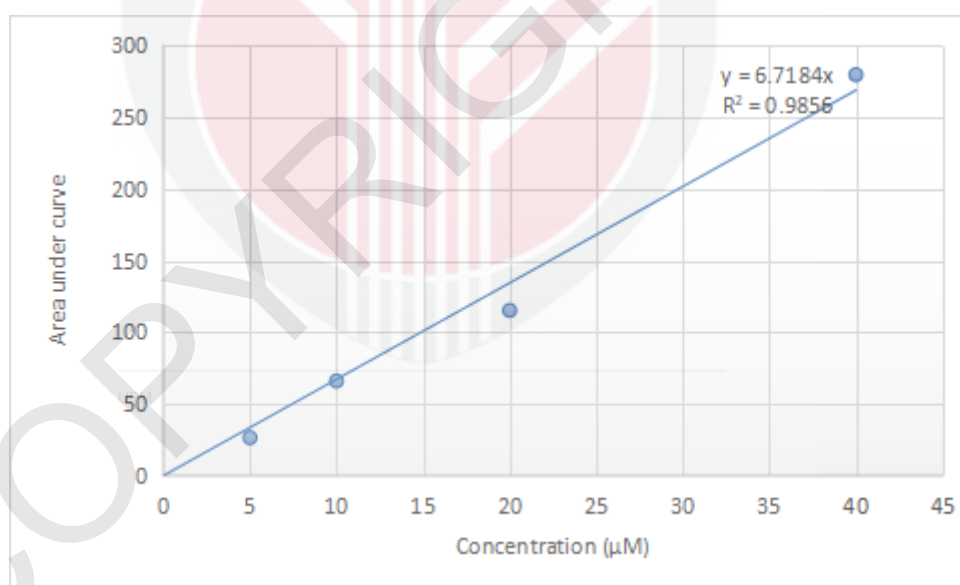


Figure 4: Calibration curve of AAI from UPLC analysis

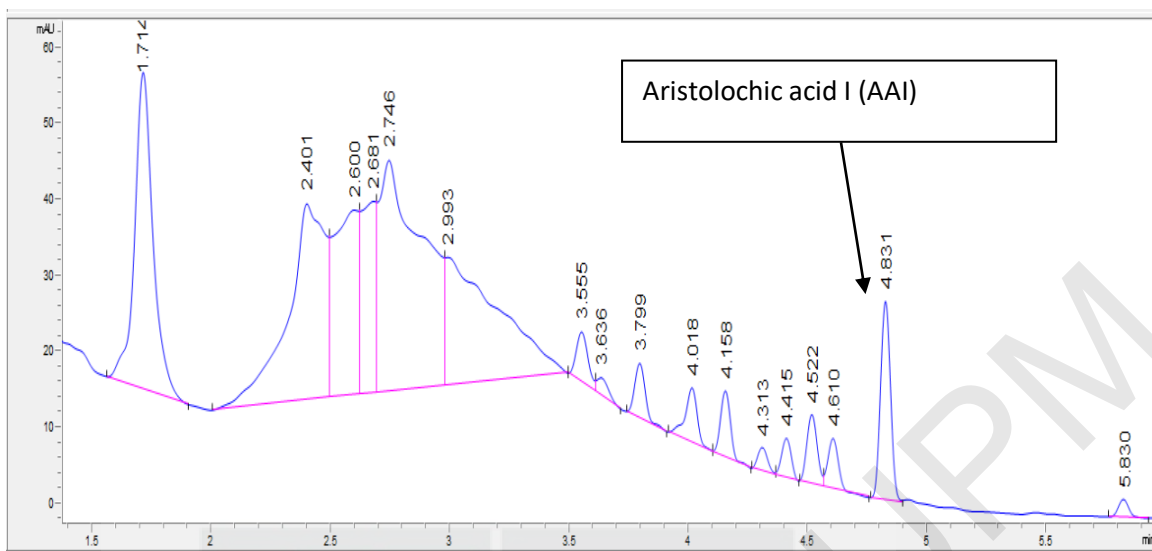


Figure 5: UPLC chromatogram of hot water extract of sample 1 (declared to contain *Aristolochia Contorta Bunge*; Ma Dou Ling). The chromatogram was obtained at a wavelength of 240nm.

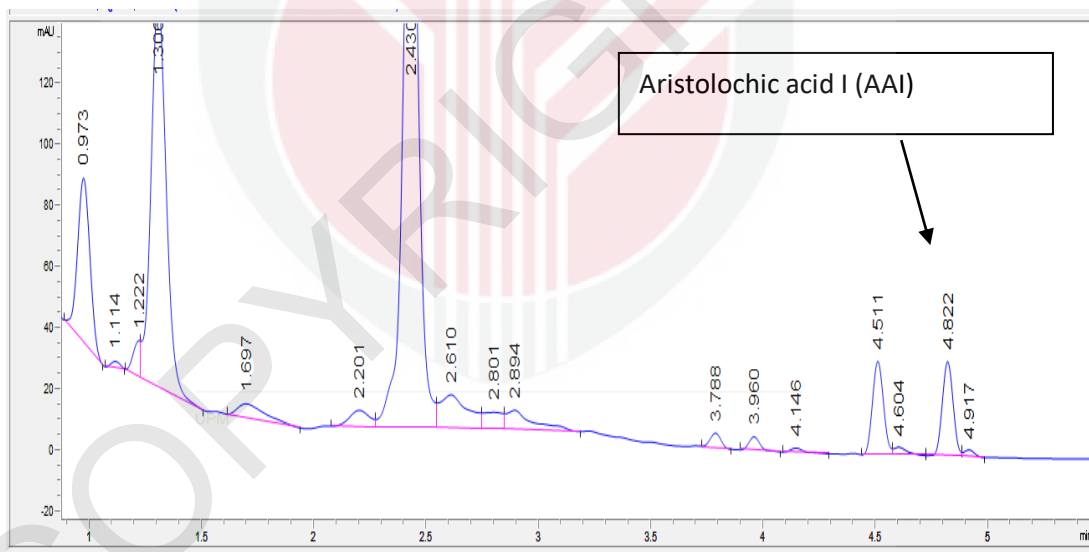


Figure 6: UPLC chromatogram of hot water extract of sample 9 (declared to contain *Akebia sp*; Mu Tong). The chromatogram was obtained at a wavelength of 240nm

Figure 4 shows the calibration curve for concentration 5 μ M, 10 μ M, 20 μ M and 40 μ M. This calibration curve was obtained from analysing at Ultra-Performance Liquid Chromatography by adding methanol and different concentration of aristolochic acids. The level of AAs were adjusted by using the percentage of recovery for the various sample type. Only 5% of the spiked sample's AAI was recovered, according to sample 4. However, due to time constraint, the analysis was not conclusive enough. Thus, the percentage recovery from a previous study by Mabuza et al. (2021) which was 74%, used for further investigation. Out of 31 samples that has been analyzed through UPLC using hot water extraction, two samples were found positive (containing AAs) which were sample 1 and sample 9 (6.47%). These two samples contain AAI that ranged from 104.0 to 262.6 μ g g⁻¹ respectively (Table 5).

Table 5: Levels of AAI in respective samples

Sample number	Aristolochic acids (μg g⁻¹)
S1	262.6 \pm 186.8
S9	104.0 \pm 86.9

In this study, only AAI can be detected due to lower concentration using the hot water extraction method. Thus, AAI that consider to have been lower concentration than AAI was unable to detect. S1 in the form of dried fruit showed the highest AA levels of the two samples containing AAI, followed by S9 in the form of the dried plant stem. Aristolochia contorta Bunge, also known as Ma Dou Ling, and Akebia spp., also known as Mu Tong, were found in each sample that tested positive for AAI.

4.3 Estimated Daily Intake

The average of the triplicate analysis was used to compute the EDI of AAI. Based on research by Van den Berg et al. (2014) for a 60 kg individual, the EDIs were calculated using the amount of AA found in traditional herbal medicine and assuming that the samples' consumption was similar to the recommended daily intake of 2.0 g to make a cup of fennel tea. The EDIs value calculated for sample 1 was $8.755 \mu\text{g kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$ while for sample 9 was $3.468 \mu\text{g kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$. This was shown in Table 6.

Table 6: EDIs values of respective samples

Sample number	Daily consumption (g) of the herbal medicine	EDI ($\mu\text{g kg}^{-1} \text{bw}^{-1} \text{day}^{-1}$)
S1	2.0	8.755
S9	2.0	3.468

4.4 Risk Assessment of Traditional Herbal Medicine using Margin of Exposure (MOE) Approach

The risk assessment for herbal medicine was done to see if consuming AAs through herbal medicine is a high priority for risk management. Furthermore, the lowest BMDL₁₀ was also obtained from the study by Abdullah et al (2017) based on the data for a kidney tumor. The MOEs were then determined using the EDIs and the lowest BMDL₁₀ of 10 g AAs kg⁻¹ bw⁻¹ day⁻¹. The MOEs calculation was 1.142 for sample 1 and 2.883 for sample 9 (Table 7) which was lower than 10,000 indicating the need for risk management thus raising high public concern.

Table 7: MOEs values of respective samples

Sample number	MOE
S1	1.142
S9	2.833

CHAPTER 5

DISCUSSION

This present study aimed to evaluate the presence and levels of AAI in traditional herbal medicine purchased from online and over the counter sources and carry out an associated risk assessment.

A total of 31 samples were bought to identify whether there was a presence of AAI in traditional herbal medicine. All these samples were analyzed in Ultra-Performance Liquid Chromatography (UPLC) by using the hot water extraction method. UPLC was used in this study to separate the compound. UPLC is better than HPLC because of its high sensitivity and ability to analyze small particles size in its column (Churchwell et al., 2005). Out of 31 samples, two samples (6.45%) were found positive containing AA-I which were sample 1 and sample 9. The level of AAI in these two samples was 262.2 and 104.0 $\mu\text{g g}^{-1}$. The results showed that AAs are still present in the market although it is already banned from being marketed in Malaysia. This conclusion was in line with the previous study that Aristolochic acids are still present in herbal medicine (Abdullah et al., 2017; Cheung et al., 2006; Martena et al., 2007). It's worth noting that AA-containing supplements might be bought from nations that haven't enacted any rules and delivered through the internet in countries that have.

The spike sample used in this study was sample 4 which was negative sample by using added concentration 100 μM . This was to determine the average recovery percentage to adjust the concentrations of Aristolochic acids I.

However, the percentage recovery of spike sample was not used in this study as we only got 5% recovery which means that only 5% AAs were found, and the other 95% lost during extraction. This shows that the result was not conclusive enough with our study and we were unable to repeat the experiment due to technical issue. Thus, we used percentage recovery found from previous study by Mabuza et al (2021) which was 74% recovery by using hot water extraction method.

Both samples that have been found containing AAI (Sample 1 and 9) were originally from China. Sample 1 was in the form of fruit (dried) while sample 9 was in the form of a plant stem (dried).

In sample 1, we detected the presence of *Aristolochia contorta* Bunge; Ma Dou Ling whereas in sample 9, we detected *Akebia* spp; Mu Tong as a suspected botanical ingredient. Both traditional herbal medicines can be used for medicinal purposes. *Aristolochia contorta* dried mature fruits are used to treat haemorrhoids, coughs, and asthma, epigastric discomfort, arthralgia, and oedema are treated with the dried stems or leaves (National Center for Biotechnology Information, 2002). *Akebia* was used to treat oedema, increase urination, calm severe urinary tract infections, and cure mouth and tongue ulcer (Mao, 2020). The *akebia*'s fruits have lately been employed in breast and digestive system tumour therapies (Mao, 2020).

However, in this study we used dried fruit as part of our experiment. The anti-tussive and anti-asthmatic herb *Aristolochia contorta* Bunge was commonly used to treat respiratory problems for dried fruit (In et al., 2005). It was also found that in Chinese medicine, *Aristolochia Contorta* can also be used to treat hemorrhoids,

cough, and other lung-related diseases (National Toxicology Program, 2008b) whereas the Japanese name for Akebia Caulis is 'mokutsu' and it was used as a diuretic and antiphlogistic for plant stems (Kawata et al., 2007).

In this study, daily doses were obtained from the study by Van den Berg (2014) which was 2.0 g while the bodyweight used was the default bodyweight of Malaysian people which was 60 kg. From this value, the EDIs value calculated was 8.755 for sample 1 and $3.468 \mu\text{g kg}^{-1} \text{bw day}^{-1}$ for sample 9. Indeed, daily consumption of more than 0.15 g of AAs obtained from Chinese herbal items was linked to an elevated risk of urinary tract cancer (Lai et al., 2010). This effect can occur through the consumption of herbal medicine regularly for a long period approximately around 7 to 8 years. A comparison of the intakes and the health risks linked with them suggests that ingestion of herbal remedies consisting of botanical of concern daily may be a health risk.

The results in this study shows that level of AAI much lower than the previous study by Abdullah et al (2017) as different extraction method was used. In this study, we used hot water extraction method which was lower concentration compared to methanol extraction method. Hot water extraction method corresponding to our daily life as we usually use to prepare a cup of fennel tea.

The "benchmark dosage level" is a research endpoint that is linked with a 10% increased chance of adverse impact in the exposed test animals. BMDL₁₀ was 10% of the total BMDL found from the previous literature review. The study by Abdullah et al (2017), reveal that BMDL₁₀ was $10 \mu\text{g kg}^{-1} \text{bw day}^{-1}$ from the analysis

data for kidney tumor formation using BMDS software version 2.5 to calculate the MOE values for the risk evaluation of herbal medicine obtain from the previous study based on the rat data from oral consumption. The MOEs value was 1.142 for sample 1 and 2.883 for sample 9 which was lower than 10,000 of the default value. For both of sample that contain Aristolochic acid I, it was indicate that MOE value lower than 10,000 shows that risk management is needed to prevent the development of kidney tumors due to exposure to AA from this traditional herbal medicine that increase public health concerns. In fact, the Scientific Committee of EFSA believes that a margin of exposure of lower than 10,000 if based on the BMDL10 from an animal study and taking into account overall uncertainties in the interpretation, would be of high public health concern and could be considered as a priority for risk management actions (EFSA, 2005).

CHAPTER 6

CONCLUSION

In conclusion, AAs was found in 2 out of 31 traditional herbal medicine samples (6.45%), which might include AA-containing herbs, suggests that the danger of unintended AA exposure remains high for people who use these traditional herbal medicines. This presence indicates that consumers are still in danger as they are exposed more to Aristolochic acids without knowing the risk that they will face. These findings point to the need for risk management measures to safeguard the population from AA exposure through herbal and traditional medicine. One of the strengths of this study is we used a primary data to determine the level of AA in a purchased sample. Analysis was done using UPLC. However, there are limitations and recommendations in this study. Firstly, this study was done in a short period of time due to the pandemic. Some analysis was not able to be repeated to increase the reliability of the data. It is recommended to repeat the experiment for recovery analysis to increase the validity and reliability of the outcomes. Other than that, the botanical product needs to be labelled so that public are aware of the ingredient of the traditional medicine especially to avoid the banned ingredients. Lastly, the government needs to take immediate action regarding the toxicity of the consumption of AA. To preserve public health, we believe that governments should enact more stringent rules requiring the elimination of all AA-containing botanicals of concern to avoid any harmful effects to humans. The government should provide financial assistance for development of educational programs and for technical support It is also recommended that future research can investigate ways for successful

detoxification or pharmaceuticals that can lower AA toxicity and limit the emergence and progression of AA-induced adverse effects.



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