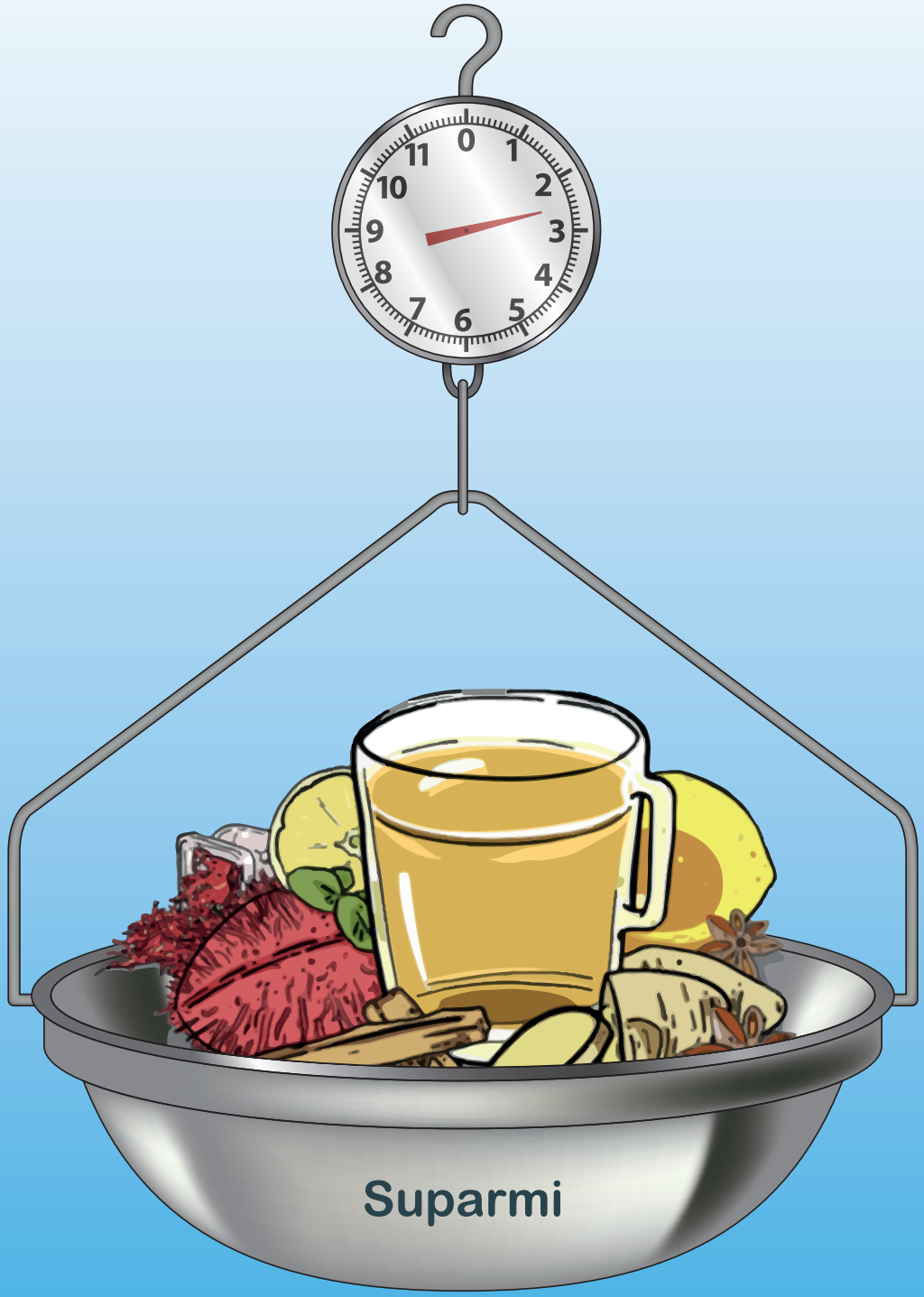
Risk and Benefit

Analysis of Herbal Products from Indonesia



Suparmi

Propositions

1. The type and level of genotoxic carcinogens in Indonesian herbal products are a risk for human health.
   (this thesis)
2. Combining in vitro toxicity with physiologically based kinetic modeling fills data gaps in the toxicological database of pyrrolizidine alkaloids.
   (this thesis)
3. The adverse effects of noise pollution in the marine ecosystem are underestimated.
4. The health benefits of regular fasting are underestimated.
5. Children are good advisors for parents with PhD-related stress.
6. A cultural shock supports success.

Propositions belonging to the PhD thesis entitled:
Risk and benefit analysis of herbal products from Indonesia

Suparmi
Wageningen, 21 April 2020

Risk and Benefit Analysis of Herbal Products from Indonesia

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This research was conducted under the auspices of the Graduate School VLAG (Advanced Studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences)

Risk and Benefit Analysis of Herbal Products from Indonesia

Suparmi

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 21 April 2020
at 04.00 p.m. in the Aula

Suparmi
Risk and Benefit Analysis of Herbal Products from Indonesia
226 pages

PhD thesis, Wageningen University, Wageningen, The Netherlands (2020)
With references, with summary in English
ISBN 978-94-6395-290-3
DOI: https://doi.org/10.18174/512309

Contents


BENEFIT
RISK

Chapter 1

General introduction

1.1. The aim of the thesis

The aim of the present thesis was to perform an assessment of potential risks and also some benefits of herbal products available on the Indonesian market. The model compounds chosen included naturally occurring genotoxic and carcinogenic botanical constituents including alkenylbenzenes and pyrrolizidine alkaloids. Beneficial effects focussed on potential PPARγ activation by the carotenoids bixin and crocetin. Existing but also novel testing strategies were used to evaluate the relevance of effects at estimated human intake levels. The botanicals and botanical preparations used for the studies were collected on the Indonesian market and consisted of herbal products including especially jamu and herbal beverages.

1.2. Herbal products in Indonesia

The healthcare system in Indonesia uses medicinal botanicals in many types of herbal products. Providing cultivation possibilities for 30,000 out of 40,000 medicinal botanicals available in the world potentially serves as an enormous supply to develop herbal products in Indonesia. Badan Pengawas Obat dan Makanan, Republik Indonesia (BPOM RI) (The National Agency for Drug and Food Control, Republic of Indonesia) has divided the herbal products in Indonesia into 3 categories based on preparation methods of the products and the level of proof required for their efficacy; i.e jamu, standardized herbal medicines, and fitofarmaka (phytomedicines) which are all regulated under regulation HK. 00.05.4.2411.[1]

Jamu is recognized as Indonesian traditional medicine for which the therapeutic effects are mainly based on empirical data, inherited across generations.[2] The efficacy of standardized herbal medicines has to be proven in preclinical studies and the standardisation of the level of their active ingredients is required, while the efficacy of fitofarmaka has to be proven in clinical studies.[1]

Herbal products have been industrialised for various kind of applications and in a variety of preparations. Use of herbal products may relate to for example medicine, health care, or beauty-care (cosmetics).[2] Since ancient times, the use of jamu has been mainly directed at maintaining well-being and the prevention of ailments, or as medicines used to cure a disease or relieve its symptoms. In the past, people consumed the freshly picked leaves or other parts of the plants, drank the decoction of jamu materials prepared in boiled water or rubbed their body with the preparation for topical treatment. Nowadays, people tend to consume herbal products rather in forms similar to other modern medicines such as in the form of pills, tablets, powders, pastilles, capsules, extracts, cream or ointments.[2] Furthermore, exposure to functional herbal products may originate from use of herbal teas, spices, coffees and/or natural food colorants.[3]

1.3. Consumption and regulatory trends of herbal products at the current state-of-the art in Indonesia

Nowadays herbal products and dietary supplements are widespread in the global market, being easily accessible to consumers via supermarkets, drugstores, natural health/food stores, herbal shops, and gyms while there are also ample possibilities to purchase these products via the internet. The notorious growth in the consumption of herbal products has been associated with the increasing demand for alternative therapies, in part due to the mistrust in conventional medicine and pharmaceutical drugs, and building on the perception that herbal product are "safe" and "natural" and thus "healthy", while at the same time facilitating the tendency for self-medication aiming for increased control over one's own health.[4-6]

In Indonesia, the market demand for jamu and other herbal products keeps growing, and as a result jamu increasingly provides economic and perceived clinical benefits. The developments already mentioned above, including easy accessibility of the products without a need for prescription and the trend of consumers to favour natural products also stimulate the production of herbal based jamu. As a result many jamu manufacturers keep developing the products and their potential health claims communicating the products and their potential benefits to consumers.[7] Nowadays, the Indonesian government established a task force to promote jamu to become fitofarmaka by improving the quality of raw materials, the manufacturing process, the standardization of the technology, the preclinical and clinical trials required to prove efficacy, traditional health service development, and the introduction and promotion of these traditional products in international markets.[8] In addition substantial research efforts focus on the exploration of Indonesia's indigenous botanicals and their potential beneficial effects providing a rationale for their use as sources of new therapeutic agents. In contrast, however, the research on potential adverse health effect of these herbal products that could be caused by toxic botanical constituents, remains limited.

As a result, the increasing use of herbal products has raised concerns among scientific and regulatory communities especially given the case studies related to cases of intoxication from misuse, misidentification of the botanical species or contamination with extraneous plants.[9, 10] Consumers sometimes only focus on the beneficial effects of the products while they may not be aware of the potential adverse health effects that can occur due to the presence of toxic botanical constituents. An effective system of risk assessment and control by competent authorities is needed in order to prevent the presence of potentially toxic constituents, possible adulterations, and/or to implement quality control in order to enforce measures to safeguard public health.[11] In this regard it is of interest to note

that BPOM RI reported that in 2018 there were some unscrupulous manufacturers and distributors who deliberately adulterated jamu through the addition of pharmaceutical drugs or analogue substances in order to increase product effectiveness.[8]

1.4. Existing regulatory status of herbal products in Indonesia and other countries

The safety, efficacy and standardization of herbal products should be controlled strictly to reach safer and more effective products to better guarantee consumer safety.[5, 7, 12] Within this framework it is interesting to note that the legislation and the risk assessment criteria for botanical supplements vary among different countries.[13]

The European Food Safety Authority (EFSA) published a compendium of botanicals that are reported to contain toxic, addictive, psychotropic, or other substances of concern[14] which was updated in 2012[15]. In the United States, the Food and Drug Administration (FDA) under the Dietary Supplement Health and Education Act of 1994 (DSHEA) recognized plant food supplements (PSF) as a separate category of foods and established its own requirements for safety and labeling.[16]

BPOM RI supervises the quality and safety of herbal products in Indonesia.[17] In case of jamu, criteria for safety and efficacy assessment of these products to assure the protection of consumers still need to be established.[12] Under regulation of the Ministry of Health (No. 659/MENKES/SK/X/1991) and the renewed regulation No. HK.00.05.4.1380 the producers and small-scale industries of herbal products have to refer to good manufacturing practice guidelines for traditional medicine, called CPOTB (*Cara Pembuatan Obat Tradisional yang Baik*) which provide guidelines for raw materials, the production process, quality control, factory building, workers, management, instrumentation, and sanitation.[18]

1.5. Risk assessment of genotoxic and carcinogenic compounds in herbal products using Margin of Exposure (MOE) approach

Although most people and even some medical practitioners assume that herbal products have fewer side effects than conventional drugs, the health risks associated with the use of herbal products cannot be neglected. The adverse health effect of consuming herbal products is influenced by many factors including natural occurrence of toxins in the plants, the dose of consumption and also consumer-related factors such as age, genetics, concomitant diseases and co-medication (herb–herb and herb–drug interactions).[10] Botanicals may contain a wide variety of toxic constituents (see EFSA compendium[15]) and this may include even compounds that

are both genotoxic and carcinogenic. Genotoxic and carcinogenic compounds that may naturally occur in botanicals include especially alkenylbenzenes (ABs), pyrrolizidine alkaloids (PAs) and aristolochic acid (AAs)[19] and previous risk assessment studies on botanicals and botanical preparations are available that report these type of constituents to pose a concern for human health indicating a priority for risk management action of the corresponding herbal products[19-24].

This risk assessment of the botanical constituents that are genotoxic and carcinogenic may be performed following the so-called Margin of Exposure (MOE) approach. The MOE approach is recommended by the European Food Safety Authority (EFSA), the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the International Life Sciences Institute (ILSI) for evaluating the risk of compounds that are both genotoxic and carcinogenic.[25-28] The result of the MOE approach can be used to determine priorities for risk management[26] taking relevant human exposure scenario's into account[29]. The MOEs are calculated by dividing a relevant reference point, for instance the $BMDL_{10}$, which is the lower confidence limit of the benchmark dose giving 10% extra tumour incidence above background levels, by the Estimated Daily Intake (EDI). The $BMDL_{10}$, is considered the most appropriate point of departure (POD) for calculating the MOE. An outcome of the MOE below 10,000 is considered a priority for risk management when the MOE is based on a $BMDL_{10}$ for carcinogenicity and an EDI for daily lifetime exposure. Exposure to the chemical would be considered a low concern for human health and a low priority for risk management when the MOE is higher than 10,000.[27] This MOE cut off value of 10,000 takes into account four uncertainty factors including a factor 10 for species differences in kinetics and dynamics, a factor 10 for human variability in kinetics and dynamics, a factor 10 for the variability in cell cycle control and DNA repair within humans, and a factor of 10 because the $BMDL_{10}$ is not a no observed adverse effect level (NOAEL).[27]

1.6. The genotoxic and carcinogenic herbal constituents evaluated in this thesis

1.6.1. Alkenylbenzenes (ABs)

ABs, including elemicin (3,4,5-trimethoxyallylbenzene), methyleugenol (1-allyl-3,4-dimethoxybenzene), myristicin (1-allyl-4,5-methylenedioxy-3-methoxybenzene), safrole (1-allyl-3,4- methylene dioxy benzene), apiol (1-allyl-3,4-dimethoxybenzene), and estragole (4-allyl-1-methoxybenzene) (Figure 1.1) occur naturally in botanicals used for the production of herbal products. Botanicals such as nutmeg, cinnamon, anise, black pepper, and basil are reported to contain safrole. Methyleugenol occurs mostly in nutmeg, lemongrass, star anise, ginger and fennel. Estragole mainly originates from tarragon, basil, fennel, and star anise. Whereas,

apiol and myristicin are mostly found in parsley, dill, and nutmeg.[5, 30-32] Humans can be exposed when consuming herbal products containing AB-producing-botanicals. In addition, exposure can result from food products formulated with the essential oils derived from these AB-producing-botanicals. This may include for example food products such as candy, ice cream, soft drinks, alcoholic beverages, sauces and spreads.[30-34]

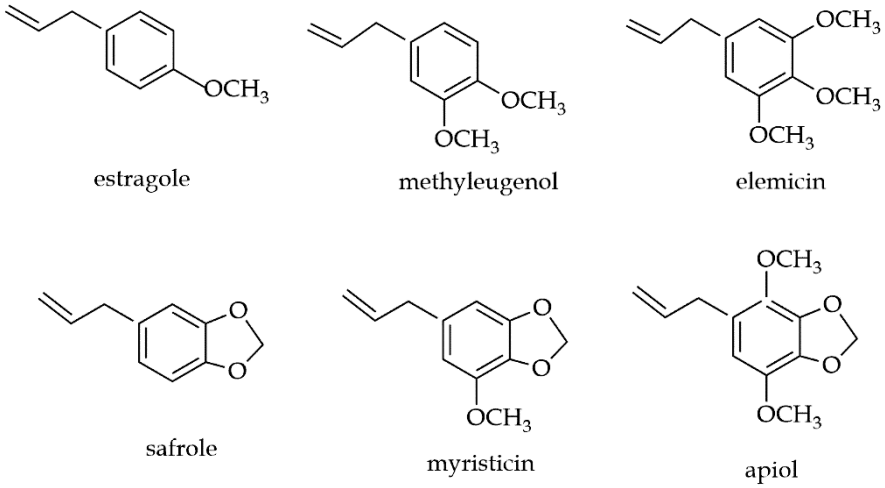


Figure 1.1. Chemical structures of the six alkenylbenzenes of interest.

The genotoxicity and carcinogenicity of the ABs require metabolic activation occurring mainly in the liver.[35] Figure 1.2 explains the metabolic pathways of safrole which are also relevant for methyleugenol, estragole and the other ABs. The bioactivation pathway starts with the formation of 1'-hydroxysafrole in a reaction catalysed by cytochromes P450 (CYP450s).[36, 37] The bioactivation process is continued by the sulfonation of 1'-hydroxysafrole resulting in the formation the ultimate carcinogenic metabolite identified as 1'-sulfoxysafrole in a reaction catalysed by sulfotransferases (SULTs).[38, 39] The unstable 1'-sulfoxysafrole metabolite deteriorates in the aqueous environment to generate a reactive carbocation that covalently binds to various nucleophilic molecules such a proteins, glutathione, RNA, and DNA. Consequently, DNA adducts are formed due to the conjugation of the carbocation at the 1'- position of the AB and the DNA adducts thus formed are considered to play a role in the subsequent tumor induction.[5, 35, 40] Figure 1.2 also present the various alternative metabolic pathways that can be considered to represent detoxification pathways.

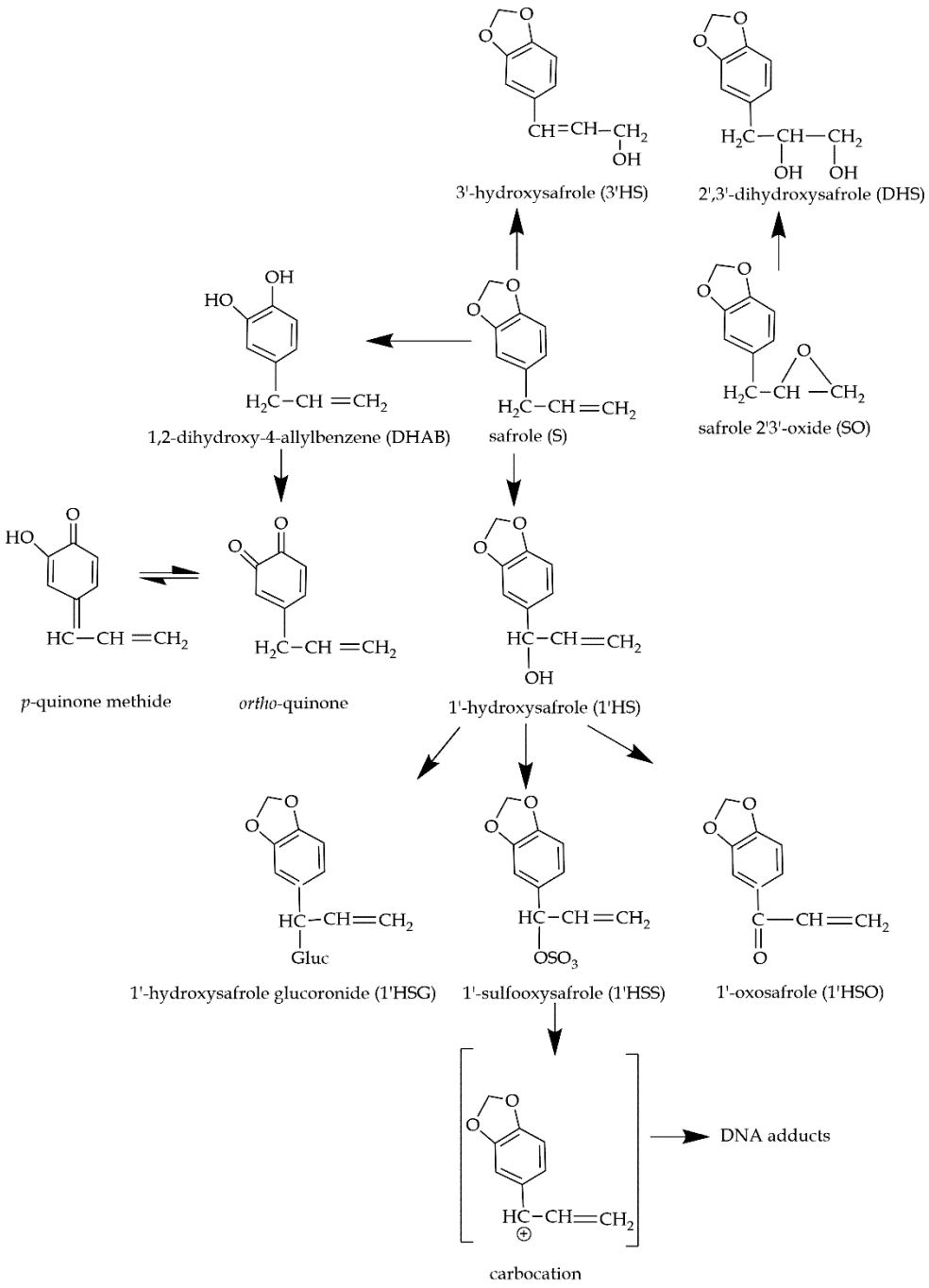


Figure 1.2. Bioactivation pathways of safrole to the proximate and ultimate carcinogen and additional detoxification pathways.

Given the presence of ABs in botanicals and botanical preparations, recent studies reported on their occurrence, actual levels detected and accompanying risk assessment in plant supplements and other foods.[19, 41-45] In these studies the MOE approach was used to assess the risk of AB-containing plant food supplements containing basil, cinnamon, sassafras, nutmeg, fennel or calamus[19, 41], dry fennel preparations and related infusions[42], parsley and dill based teas[46], nutmeg-based plant food supplements[44], basil-based pesto sauces[47] and plant food supplements and traditional Chinese medicines (TCM) and herbal teas[45]. Overall these studies showed that the consumption of AB-containing botanicals via these botanical preparations daily during a lifetime period would result in MOE values below 10,000 indicating a potential priority for risk management.

These MOE values are based on daily chronic lifetime exposure, although a shorter period of consumption of the herbal products should be taken into account to better reflect the real-life exposure scenario in which people do not consume these preparations every day during their lifetime. Given that these herbal preparations are often especially consumed during periods of illness it seems essential to also consider shorter than life time exposure scenario's. Although there is no officially established method to evaluate shorter than lifetime exposure to a genotoxic carcinogen, it has been suggested to use Haber's rule to estimate the effects for different exposure duration.[48, 49] Haber's rule states that the dose times the effect is constant, (C1×T1= constant = C2×T2) which implies that one could correct for shorter time of exposure in a linear way. Following Haber's rule and assuming consumption only for 2 weeks every year during periods of illness, the EDI values will be 52 weeks per year/2 weeks = 26 times lower and thus the MOE values 26 times higher than for lifetime exposure scenario's. In addition, Haber's rule can also be used to calculate the number of weeks of daily consumption of the different herbal products that would result in an MOE value above 10,000, the threshold for risk management concern.[27]

1.6.2. Pyrrolizidine alkaloids (PAs)

PAs consist of a group of more than 660 naturally occurring plant metabolites derived from an estimated over 600 different species, which are for the most part from the families Asteraceae (Compositae), Boraginaceae and Fabaceae (Leguminosae).[50, 51] PAs also may occur in botanical food products due to the contamination of the products with PA-producing botanicals like *Symphytum, Petasites, Tussilago,* coharvested as weeds.[21, 24, 52]

PAs share a common structure based on a 1-hydroxymethylpyrrolizidine (necine base) and are divided into four different structure types, i.e retronecine,

heliotridine, otonecine, and platynecine type PAs (Figure 1.3A). A necine base is coupled with 1 or 2 necine acids via ester linkages, which based on their esterification patterns result in PAs being categorized as monoesters, cyclic diesters (including monocrotaline and riddeliine) and open diester, like lasiocarpine (Figure 1.3B). In the present thesis, the occurrence of PAs in jamu was studied. Furthermore, the toxicity of monocrotaline a cyclic retronecine-type PA was studied in some more detail. Monocrotaline is categorized as being possibly carcinogenic in humans (category 2B) a classification also applicable for the related PAs lasiocarpine and riddeliine (Figure 1.3B).[53] Recently EFSA (2017)[24] listed monocrotaline as one of the 17 PAs to be monitored for their presence in food and feed due to the possible concern for human health to the exposure to PAs via consumption of tea and herbal infusions.

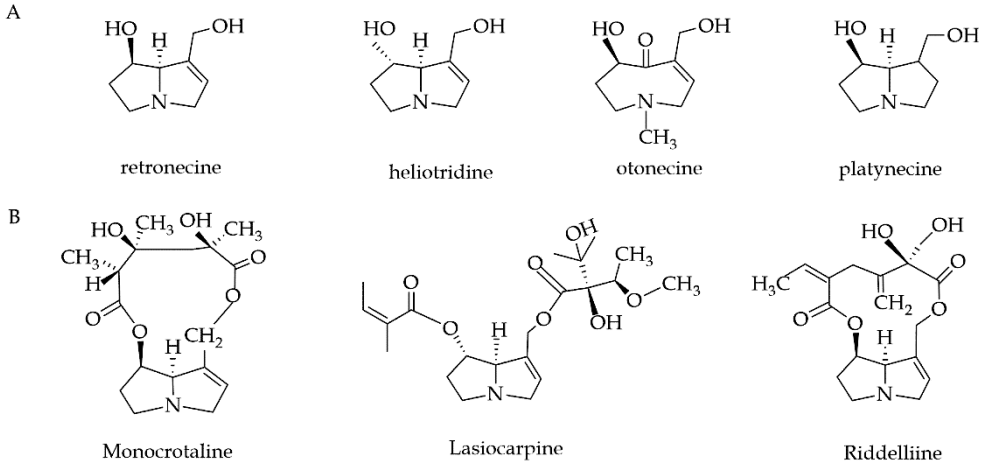


Figure 1.3. Overview of the 4 types of PAs based on necine bases (A) and the chemical structure of monocrotaline, lasiocarpine and riddeliine (B).

The double bond in 1,2-position of the necine base is required for toxicity of the PAs and at least one hydroxyl group at the ring system has to be esterified for exerting toxic effects.[54] Like all 1,2-unsaturated PAs monocrotaline itself is a pro-toxin (unreactive compound) requiring metabolic activation by cytochromes P450 to exert hepatic and pulmonary toxicity.[54] CYP3A4 and the CYP2B6 were found to be the major P450 enzymes in metabolic activation of monocrotaline in rat liver.[55, 56] According to another study Ruan et al. (2004)[57], the metabolism of PAs is generally occurring via three pathways, namely hydrolysis, N-oxidation, and dehydrogenation (Figure 1.4).

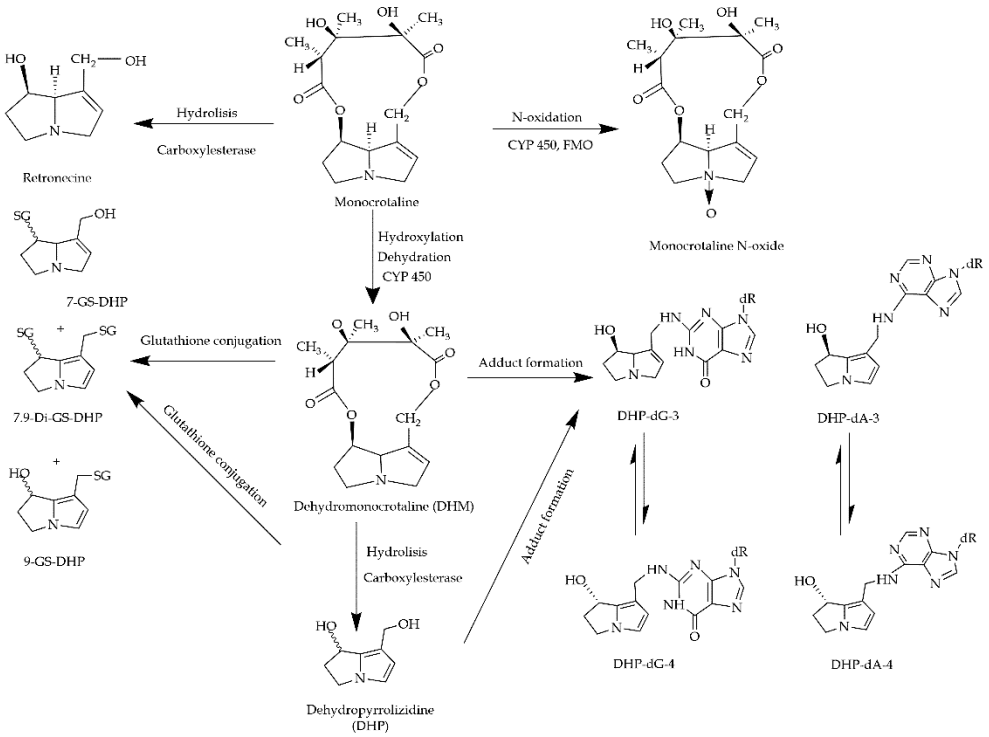


Figure 1.4. Schematic diagram of the metabolic pathways of monocrotaline and DNA adduct formation by monocrotaline metabolites relevant for rat and human.[55, 58, 59] FMO = flavin-containing monooxygenase, CYP 450 = cytochromes P450.

Upon dehydrogenation of monocrotaline, an unstable and highly reactive intermediate, named dehydromonocrotaline (DHM) is formed. Eventually, DHM can react with macromolecules including proteins and DNA to form protein- and DNA-adducts, which are considered to be responsible for the toxicity including the genotoxicity and carcinogenicity of monocrotaline.[60-62] Upon hydrolysis of DHM 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP) is formed that is also able to react with cellular macromolecules including glutathione (GSH) the latter resulting in formation of GSH-DHP and di-GSH-DHP. These conjugates are considered less toxic and more stable,[57, 63] although they may still also react with proteins and DNA to form the same DNA adducts formed by DHM and DHP themselves.[64]

For only a few PAs actual tumor data that enable definition of a $BMDL_{10}$ value for risk management using the MOE approach are available. Initially EFSA (2011) established a $BMDL_{10}$ value of 0.07 mg/kg bw/day for lasiocarpine derived from data obtained in a 2-year carcinogenicity study on induction of liver haemangiosarcomas in male rats.[65] A risk assessment based on the MOE approach

was performed using this $BMDL_{10}$ value and mean PA levels reported in literature for herbal teas and PFS. The results obtained indicated that consumption of one cup of tea a day during a whole lifetime would result in MOE values lower than 10,000 for several types of herbal teas, indicating a priority for risk management for these products. A refined risk assessment using interim relative potency (REP) factors for different PAs showed that there was a concern for 7 (54%) of 13 types of herbal teas and 1 (14%) of 7 types of PFS included in the study. A concern for risk management action was also raised for the preparations containing non-PA-producing botanicals, which may contain PAs as a result of co-harvesting of PA containing weeds.

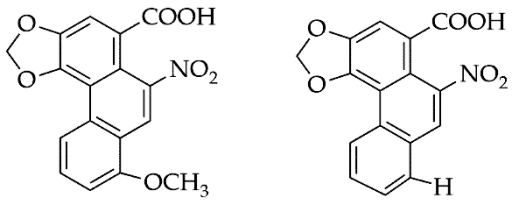
Recently, the EFSA Panel on Contaminants in the Food Chain (CONTAM) established a $BMDL_{10}$ of 237 µg/kg body weight per day as POD for calculating the MOE for chronic exposure to PAs, based on tumor data for riddelliine as the reference PA.[24] Using this value of $BMDL_{10}$ Chen et al. (2019) performed a risk assessment for Chinese herbal products.[66] Total PA concentrations that ranged from 404 to 7,883 µg/kg sample in 4 out of 8 samples of Chinese herbal medicines containing PA-producing botanicals, which resulted EDI values of 0.02-0.45 µg/kg bw. MOE values were between 110 and 530 (< 10,000) when assuming lifetime daily consumption indicating that chronic use of these herbal medicines may pose a potential risk for human health. An amount of 4.0-4.2 µg/kg PAs detected in 2 out of 4 samples containing non-PAs-producing botanicals resulting in EDI values of 0.0003-0.001 µg/kg bw also indicated MOE values <10,000 when daily consumption for a whole lifetime would be considered, indicating a concern for human health and a priority for risk management action.

The current existing risk assessment on PAs is mainly based on the assumption of equal potency of all PAs detected in the samples and comparison to the $BMDL_{10}$ of riddelliine, without taking into account differences in relative potency of the PAs present in the samples. Merz and Schrenk (2016)[67] defined interim Relative Potency (REP) factors for the toxic and genotoxic potency of 1,2-unsaturated PAs based on the available data on the genotoxic potency in *Drosophila melanogaster*, the cytotoxic potency in vitro in chicken hepatocellular carcinoma (CLR-2118) cells and their acute toxicity in adult rodents. More recently Louisse et al. (2019)[68] proposed REP factors based on results obtained in the γH2AX assay in HepaRG human liver cells for 37 PAs showing that open diester PAs (including lasiocarpine) and cyclic diester PAs (including riddelliine) display the highest potency. However due to the limited data on liver toxicity and carcinogenicity upon oral exposure to PAs, available for only lasiocarpine and riddelliine[69, 70], an alternative testing strategy instead of animal studies for estimating the relative toxicity of different PAs would be of use. Furthermore, a translation of the result of

alternative testing methods to the in vivo situation is required converting in vitro concentration-response curves to in vivo dose-response curves. This can be achieved by using physiologically based kinetic (PBK) modeling-based reverse dosimetry which is explained further in the Section 1.8.

1.6.3. Aristolochic acids

Aristolochic acids (AAs) are a group of nephrotoxic and carcinogenic compounds of which the most important congeners are 8-methoxy-6-nitrophenanthro-(3,4-d)-1,3-dioxolo-5-carboxylic acid (AA-I) and its 8-demethoxylated form (AA-II)[71] (Figure 1.5). *Aristolochia debilis*, *Aristolochia clematitis*, *Clematis armandii*, *C. montana*, *C. chinensis, Akebia quinata, A. trifolatia*, *Aucklandii lappa*, *Saussurea lappa* and *Asarum* species are reported as AA-containing botanicals.[72] Herbal medicines containing AA-producing botanicals are reported to be carcinogenic in humans, and as a result IARC [73] classified these preparations as group 1 carcinogens. In addition, AAs are also listed among the most potent 2% of known carcinogens[53, 74].



Aristolochic Acid I Aristolochic Acid II

Figure 1.5. Structural formulas of aristolochic acid I (AAI) and aristolochic acid II (AA-II).

Aristolochic Acid Nephropathy (AAN) is potentially a crucial problem in the Asian area since a lot of people in this region still are convinced that traditional Chinese herbal medicines, which frequently contain AA producing plants, are safer than chemically produced 'Western' drugs.[75] AAN was reported in Belgium in 1991, where over 100 young women suffered from end-stage renal disease and in several cases cancer in the kidneys and the upper urinary tract due to the confusing nomenclature, resulting in a replacement of *Stephania tetrandra* ('Han Fang Ji') by *Aristolochia fangchi* ('Guang Fang Ji') in a Chinese herb-based weight-loss preparation.[76] Similar to incidences of AAN, Balkan Endemic Nephropathy (BEN) occurring in Balkan regions in the 1950's, was ascribed to flour contaminated with *Aristolochia clematitis*.[77, 78] More cases of AAN were reported in other countries including Spain, Japan, France, Belgium, UK, Taiwan, USA, Germany, China, Korea, Hong Kong, Australia and Bangladesh. The use of AA containing botanicals in food

was banned worldwide.[79, 80] BPOM RI based on regulation No. 7, 2018, is banning use of the AA-containing botanicals, *Aristolochia spp, Stephania tetrandra* S.Moore, *Magnolia officinalis* Rehder & E.H.Wilson, and their preparations, as ingredients in foods in Indonesia.[81]

In spite of the ban installed in 2001 in The Netherlands, Martena et al. (2007)[82] found that from the 190 Chinese herbal medicines that were collected on the Dutch market between 2002 and 2006 using a targeted approach, 25 still contained AA-I and 13 of these 25 also contained AA-II. Furthermore, in a another study a risk assessment on AA-containing herbal supplements showed that 3 out of 18 samples contained AAs at levels that would result in intakes and corresponding MOE values that point at a priority for risk management.[20]

1.7. The carotenoids analysed in the thesis for their beneficial effects

In addition to potential hazards and risks of selected botanical constituents, the thesis also included a study on potential beneficial effects of some selected botanical ingredients. This included the carotenoids bixin and crocetin (Figure 1.6). Carotenoids are red, yellow, and orange pigments that are part of the human diet due to their presence in a variety of fruits, vegetables and other food products. There are two types of carotenoids; the carotenes, which consist of a long-chain conjugated polyene structure without oxygen and the xanthophylls, that possess one or more oxygen containing substituents at particular sites on the terminal rings.[83] The majority of the 600 carotenoids found in nature belong to the carotene group, and include compounds such as lycopene, α-carotene, β-carotene, γ-carotene, and ζ-carotene, which contain a long-chain conjugated polyene structure, no oxygen atoms, and are usually orange and red in colour. The xanthophyll group contains constituents like lutein, zeaxanthin, which possess one or more oxygen containing functional groups at particular sites on the terminal rings Figure 1.6 shows the chemicals structures of some carotenoids, including bixin, norbixin and crocetin which are the model carotenoids of the studies in the present thesis. The beneficial effects of carotenoids are considered to be influenced by their number of conjugated double bonds.[83, 84] Kiokias and Gordon (2003)[85] reported that polarity also influenced their beneficial effect, with the less polar carotenoids (α-carotenes, β-carotenes, lycopene and lutein) being more potent than the more polar carotenoids (bixin, capsanthin and capsorubin).

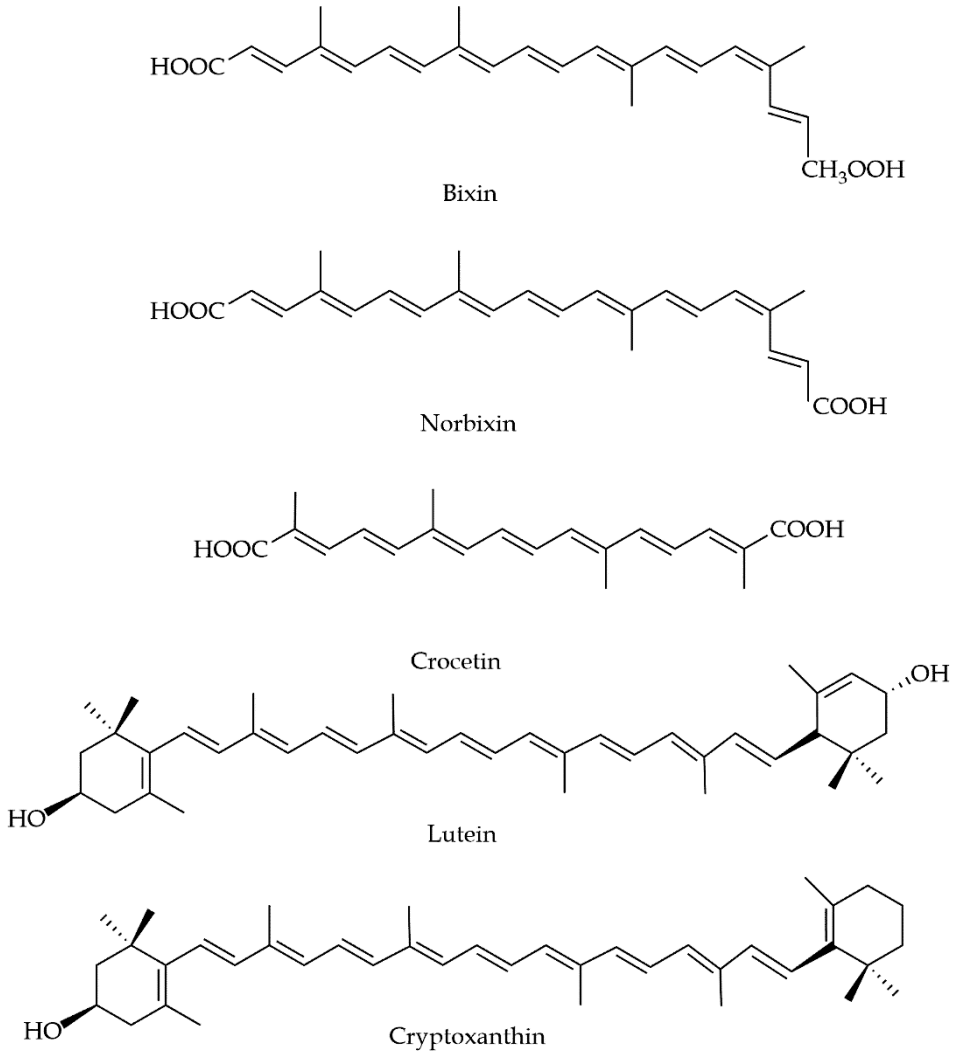


Figure 1.6. Chemical structures of some carotenoids including β-carotene, lycopene, bixin, norbixin and crocetin (carotene group) and lutein and cryptoxanthin (xanthophyll group).

Carotenoid and also bixin and crocetin containing botanical preparations are consumed in many countries, such as India, China, Japan, as a herbal medicine to treat type 2 diabetes mellitus (T2DM).[86, 87] The effects of intake of herbal preparations containing the aforementioned carotenoids on insulin sensitivity is suggested to be due to activation of peroxisome proliferator-activated receptor γ (PPARγ), a ligand-activated transcription factor and the receptor of the T2DM thiazolidinediones drugs.[88] The activation of PPARγ starts by ligand binding, leading to a conformational change in the ligand binding domain. Subsequently PPARγ forms a complex with retinoid X receptor (RXR) (Figure 1.7) upon which the

complex binds to peroxisome proliferator response elements (PPRE) to regulate gene transcription.

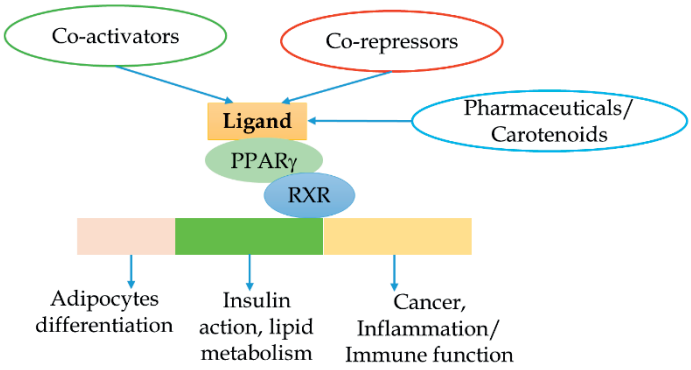


Figure 1.7. Mechanism of gene transcription by PPAR-γ as modified from Houseknecht et al. (2002).[89]

Upon activation of PPARγ, insulin-stimulated glucose uptake increases, free fatty acid levels partly decrease and lipid storage in adipose tissue increases.[90] Carotenoids and retinoids inhibited the adipocyte differentiation in 3T3-L1 adipose cells via RAR up-regulation and PPARγ2 suppression.[91] The direct interaction of the carotenoid molecules or their derivatives with ligand-activated nuclear receptors, or indirect modification of transcriptional activity of non-liganded transcription factors changes the expression of multiple proteins as the starting event of carotenoid mediated effects on gene transcription.[92] Sharoni and colleagues (2004)[92] reviewed that lycopene, phytoene, phytofluene, and *β*-carotene activated peroxisome proliferator response elements (PPREs) in cells co-transfected with PPAR-*γ*, although the activation by these compounds was lower than that of well-known PPAR ligands, like 15-deoxy-D12,14-prostaglandin J2 and ciglitazone. The exploration of carotenoids as alternative drugs to activate PPARγ is also of interest given that insulin-sensitizing medications such as synthetic ligands like rosiglitazone, were found to induce adverse side effects, for example weight gain, heart failure and edema, leading to limitations of their use in diabetic patients.[93]

1.7.1. Bixin

Bixin (methyl hydrogen 9′-*cis*-6,6′-diapocarotene-6,6′-dioate, $C_{25}H_{30}O_4$) belongs to the carotene group. A bixin containing extract prepared from the seed coat of the achiote tree (*Bixa orellana* L) is used as an approved food colorant (E160B) in many products such as butter, cheese, bakery products, oils, ice creams, sausages, and cereals.[94, 95] Also JECFA evaluated bixin containing annatto extracts as food colorant (E160b) and concluded that when all the pigment ingested would be bixin,

the estimated dietary exposure of 1.5 mg/day would result in an intake of bixin of 26 µg/kg bw per day, which would be below the established acceptable daily intake (ADI) of 6 mg/kg bw.[96] According to Code of Federal Regulation Tittle 21 (21CFR73.30) of FDA annatto extracts are categorized as colour additives exempt from batch certification, and there is "no maximum level" of annatto usage indicated as long as the use is consistent with good manufacturing practice (GMP).[97] EFSA also evaluated bixin containing annatto extracts (E160b) for use as food additive and concluded the safety of the currently authorised solvent-extracted bixin could not be assessed due to the lack of data, both in terms of identification and toxicological studies. EFSA also indicated that the exposure to bixin of the European population for all age groups was estimated to be below the established ADI of 6 mg/kg bw per day.[98, 99] BPOM RI approved annatto extracts (bixin based) as natural food colorant with Colour Index (CI) Number 75120 and an ADI of 0-12 mg/kg bw. The regulation also determined maximum permitted levels (MPLs) for bixin in many food products in mg bixin/kg food product also defining specifications limiting the amount of norbixin, the hydrolysed form of bixin (Figure 1.6) to 28% of the bixin level.[100]

Bixin served as an agonist for PPARγ in vitro, thus it can be considered a potential functional food-derived compound to regulate adipocyte functions for T2DM treatment.[101-104] Bixin at 70 µM significantly activated PPARγ 8.2 fold of solvent control in a chimera protein of PPARγ model, at a level that was 2.5-fold in the another luciferase assay using a full-length PPARγ protein and PPRE-luciferase reporter plasmid.[102] Studies in experimental animals revealed that bixin shows hypoglycemic activity, while norbixin appeared ineffective to protect against the hyperglycemia and dyslipidemia in streptozotocin-induced diabetic rats.[104] Studies in humans showed that plasma bixin level of volunteers in Quito, Equador reached a maximum, 11.6 µg/L (range 0-18) (0-0.046 µM) at 2 h and returned to zero at 8 h after ingestion of 1 ml of a commercial Annatto Food Color containing 16 mg of bixin[105] showing the systemic bioavailability of the compound upon oral intake.

1.7.2. Crocetin

Another carotenoid that is frequently consumed due to its use as food colorant is crocetin (8,8′-diapocarotene-8,8′-dioic acid) (Figure 1.6). Crocetin occurs naturally in the fruits of gardenia (*Gardenia jasminoides* Ellis) and in saffron (*Crocus sativus* L.).[106-109] JECFA recognized saffron, CI. (1975) No 75100, as a food ingredient rather than a food additive, so an ADI was not established for this compound.[110] FDA in 21CFR73.500 exempted saffron (E164), which is known to contain crocetin, from food additives certification.[111] The amount of saffron added to the food is considered safe as long as the procedures follow GMP. In the European Union (EU)

saffron is not authorized as food additive but is generally considered as a food colorant without E number.[112]

Crocetin was reported to enhance insulin sensitivity in insulin resistant rats,[113-116] suggesting its potential preventive and/or therapeutic roles in T2DM. The pharmacokinetic profile of crocetin was described in 10 healthy Filipino volunteers (5 men and 5 women) who consumed a capsule containing crocetin extracted from dried gardenia fruits at three single doses (7.5, 15 and 22.5 mg) with a one-week interval. Maximum plasma concentrations ranged from 100.9 to 279.7 ng/mL (0.31 - 0.85 μM) at 4.0 to 4.8 h after administration, and the compound was eliminated from human plasma with a mean elimination half-life ($T_{1/2}$) of 6.1 to 7.5 h.[117]

Given information on the beneficial effects and the pharmacokinetic data of bixin and crocetin in human mentioned above, in the thesis a novel strategy called physiologically based kinetic (PBK) modeling facilitated reverse dosimetry[118] was used to investigate whether at realistic human intake levels actual PPARγ mediated health effects of these compounds can be expected.

1.8. PBK modeling-based reverse dosimetry as a novel method for the prediction of in vivo toxicity and beneficial effects

A physiologically based kinetic (PBK) model is a set of mathematical equations which describe the absorption, distribution, metabolism and excretion (ADME) characteristics of a compound within an organism based on three types of parameters. These type of parameters include: i) physiological and anatomical parameters (e.g. cardiac output, tissue volumes and tissue blood flows), (ii) physico-chemical parameters (e.g. blood/tissue partition coefficients) and (iii) kinetic parameters (e.g. kinetic constants for metabolic reactions and/or metabolic clearance).[118] The translation of in vitro effective concentrations into in vivo effective dose levels can be achieved using PBK modeling with a reverse dosimetry approach. In this approach in vitro effect concentrations are considered as blood or tissue concentrations that would cause a relevant biological effect in the in vivo situation, upon which the PBK model can calculate the doses which are required to reach these blood or tissue concentrations.[118, 119] In this way concentration response curves obtained in vitro can be translated to in vivo dose response curves needed to judge the risks or benefits upon in vivo exposure of the human population.

So far, this approach has been used to translate in vitro toxicity data to in vivo dose response curves for a range of different endpoints, including for example liver toxicity, kidney toxicity, developmental toxicity, genistein induced estrogenicity, and hesperitin induced effects on inhibition of protein kinase A activity.[120-127] The

approach can be considered a novel non-animal-based testing strategy, contributing to the replacement, reduction and refinement (3Rs) of animal experiments. Recently, Chen et al. (2018)[128] and Ning et al. (2019)[129] developed PBK models and applied PBK model based reverse dosimetry to predict the *in vivo* liver toxicity of the PAs lasiocarpine and riddeliine in rat and human (Figure 1.8). In this thesis, a similar study was performed for monocrotaline as a model 1,2-unsaturated PAs in order to further investigate if this approach can assist in filling existing gaps in the toxicological data base for PAs.[24]

PBK modeling-facilitated reverse dosimetry approach applied in the present thesis includes the establishment of in vitro concentration-response curves for liver toxicity using rat hepatocytes, development of a PBK model using kinetic parameters based on in vitro assays, translation of the in vitro concentration-response curve into an in vivo dose-response curve for liver toxicity using PBK modeling-facilitated reverse dosimetry. Subsequently BMD analysis is applied to the dose-response data in order to obtain a potential POD for risk assessment. In a final step the predicted PBK model and obtained POD are evaluated against available literature data.[128, 129] This last step is also why monocrotaline was selected as the model PA given that in vivo data on its kinetics and liver toxicity are available[130-134], enabling evaluation of the PBK model and of predictions made.

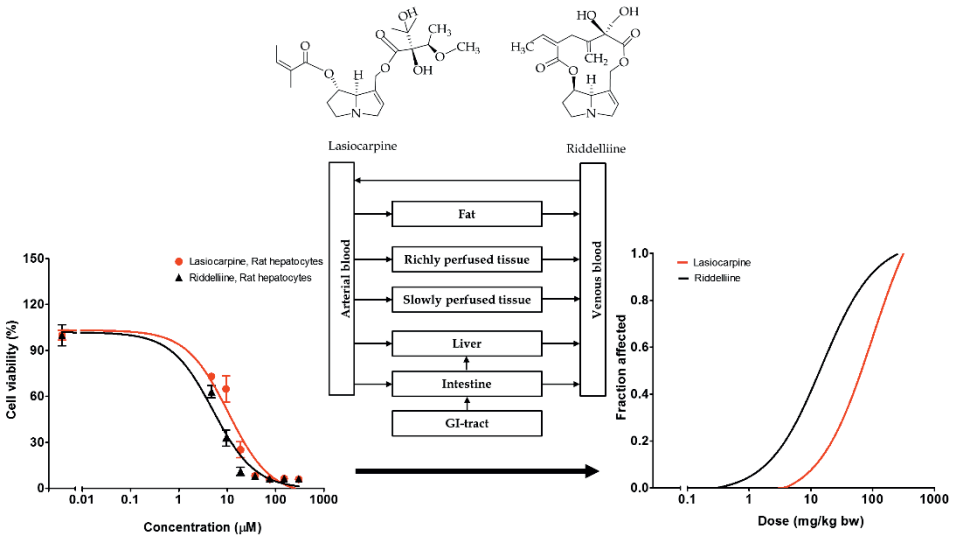


Figure 1.8. Schematic presentation of the PBK model based reverse dosimetry approach to predict the *in vivo* liver toxicity of lasiocarpine and riddeliine in rats based on an *in vitro* toxicity curve in rat hepatocytes and a rat PBK model. For further details see literature.[128]

Apart from its use in novel methods for the assessment of adverse health effects in the process of risk assessment, the PBK modeling-facilitated reverse dosimetry approach can also be used for benefit analysis. In the present study the approach was used to investigate whether PPARγ activating characteristics of bixin and crocetin may be expected at realistic human daily intake levels without the need to perform a human intervention study.

1.9. Outline of the thesis

As already explained, the aim of the present thesis was to perform an assessment of potential risks and also some benefits of herbal products available on the Indonesian market. In order to meet this aim, the thesis consists of 7 chapters, the content of which is schematically summarised in Figure 1.9.

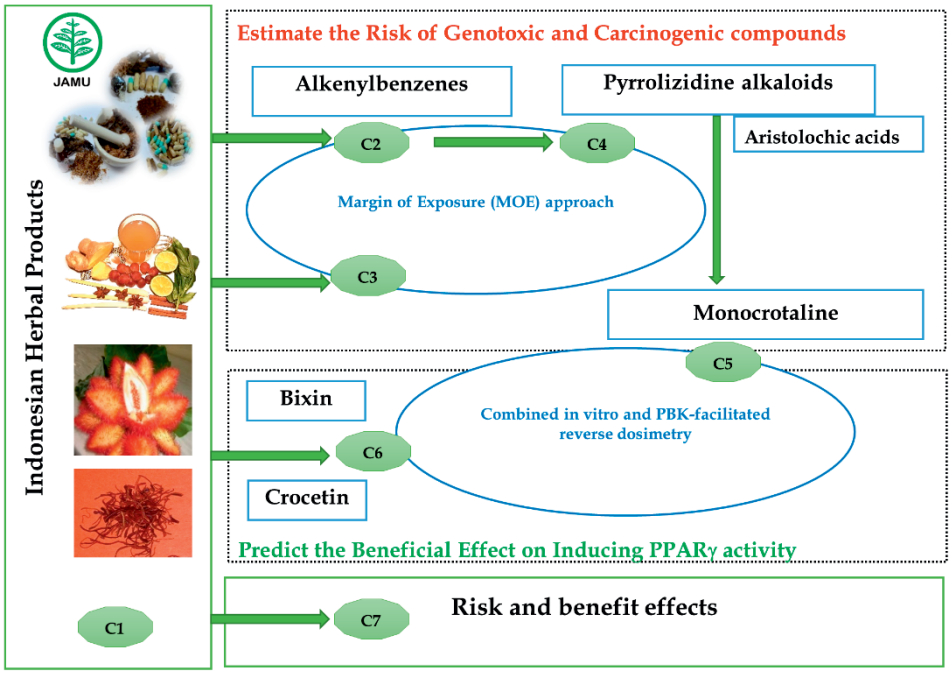


Figure 1.9. Flowchart illustrating the key content of each chapter related to aim of this thesis (C = Chapter).

The graphical abstract presented in Figure 1.9 summarises the following content of the different chapters of the thesis.

Chapter 1 of the thesis (this chapter) starts with the aim of the thesis followed by an overview about herbal products in Indonesia, consumption trends of herbal products, and the existing regulatory status of herbal products in Indonesia and

other countries. This is followed by a description of the model compounds selected and used in the studies of the thesis and of the methods used for their evaluation, including the MOE approach for risk assessment of the genotoxic and carcinogenic botanical constituents and the PBK model based reverse dosimetry approach to translate in vitro data to in vivo data. This quantitative in vitro to in vivo extrapolation (QIVIVE) enables comparison of realistic exposure scenarios with estimated human intake levels to evaluate if beneficial or adverse effects could be expected upon intake of relevant preparations.

Chapter 2 of the thesis evaluates the consumer risks of jamu, Indonesian traditional herbal medicines. The levels of ABs in 25 samples collected by a targeted sampling strategy, collecting samples with AB-containing botanical ingredients, were quantified and the amount of ABs that would be consumed through consumption of the respective preparations was quantified. Three different approaches were used for estimating the EDI and subsequent calculation of the MOE values including: considering the individual ABs using their $BMDL_{10}$ values, combined exposure assuming equal potency of all ABs using the $BMDL_{10}$ value of the major AB in the mixture, and combined exposure using a toxic equivalency (TEQ) approach.

Chapter 3 evaluates whether the level of methyleugenol and related ABs in instant herbal beverages obtained on the Indonesian market would be safe for human consumption. Analysis of the methyleugenol and related AB levels and resulting EDI and MOE values was performed for 114 samples collected on the Indonesian market by a targeted sampling strategy. Model averaging as an update on BMD modeling for toxicological risk assessment was applied to calculate an updated $BMDL_{10}$ for methyleugenol. An overview was made of the current product registration type indicated on the label of the various herbal beverages to evaluate whether there is a need for establishment of maximum permitted levels (MPLs) in Indonesia also for methyleugenol, given MPLs for the related ABs estragole and safrole are already defined.

Chapter 4 investigates the occurrence of PAs in 58 Indonesian jamu products containing various mixed medicinal plants, including 35 samples containing PA-producing botanicals and 23 samples containing non-PA producing botanicals. Based on the levels of PAs present and directions for use given by the producers, an exposure and safety assessment of consumption of these jamu was performed. In addition, the risk assessment based on PA levels in the samples containing non-PA-producing botanicals was compared to the risk assessment of these samples based on their levels of ABs (detected in Chapter 2) and the AAs detected in 2 of the samples containing AA-producing botanicals.

Chapter 5 investigates whether an alternative testing strategy including quantitative in vitro to in vivo extrapolation (QIVIVE) can adequately predict the liver toxicity for monocrotaline. Monocrotaline was selected as the model compound because this PA appeared to be one of the few other PAs for which in vivo data on kinetics and liver toxicity are available thus enabling evaluations of the PBK model and of predictions made. PBK modeling facilitated reverse dosimetry was used for conversion of in vitro data for toxicity in primary hepatocytes to quantitatively predict in vivo acute liver toxicity for rat.

Chapter 6 investigates whether the same approach as applied in Chapter 5 would also adequately predict whether a potential therapeutic effect of bixin and crocetin via their supposed PPARγ activating activity may be expected at realistic human daily intake levels. To this end concentration response curves for bixin and crocetin induced activation of PPARγ mediated gene expression in U2OS PPARγ2 reporter gene cells were converted to expected in vivo dose response curves using PBK modeling based reverse dosimetry.

Chapter 7 summarizes the main outcomes obtained in the thesis and compiles the overall discussion. This chapter provides insight regarding the potential of risk assessments to support risk management in formulating regulatory actions to minimize the potential health risk for herbal products for consumers in Indonesia due to the occurrence of toxic ABs, PAs and AAs in these products. Furthermore, this chapter discusses the potential for the application of the PBK model based QIVIVE approach for predicting beneficial as well as adverse effects, without the need for animal experiments and/or human intervention studies. The chapter also present some future perspectives for research on the risks and benefits of botanicals and botanical preparations.

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BENEFIT
RISK

Chapter 2

Natural occurrence of genotoxic and carcinogenic alkenylbenzenes in Indonesian jamu and evaluation of consumer risks

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Published in: Food and Chemical Toxicology, 118 (2018): 53-67
DOI: 10.1016/j.fct.2018.04.059

Abstract

The consumer risks of jamu, Indonesian traditional herbal medicines, was assessed focussing on the presence of alkenylbenzene containing botanical ingredients. Twenty-three out of 25 samples contained alkenylbenzenes at levels ranging from 3.8-440 µg/kg, with methyleugenol being the most frequently encountered alkenylbenzene. The estimated daily intake (EDI) resulting from jamu consumption was estimated to amount to 0.2-171 µg/kg bw/day for individual alkenylbenzenes, to 0.9–203 µg/kg bw/day when adding up all alkenylbenzenes detected, and to 0.9-551 µg/kg bw/day when expressed in methyleugenol equivalents using interim relative potency (REP) factors. The margin of exposure (MOE) values obtained were generally <10,000 indicating a priority for risk management when assuming daily consumption. Using Haber's rule it was estimated that two weeks consumption of these jamu only once would not raise a concern (MOE >10,000). However, when considering use for two weeks every year during a lifetime, 5 samples still raise a concern. It is concluded that the consumption of alkenylbenzene containing jamu can be of concern especially when consumed on a daily basis for longer periods of time on a regular basis.

2.1. Introduction

Indonesian jamu represents one of the traditional herbal medicines, for which the efficacy claims are mainly based on user experiences for many decades or even hundreds of years.[1] Generally, the formula of jamu consist of selected and mixed medicinal plants to get the desired efficacy, usually from whole, fragmented or cut plants, and parts of plants.[2] In Indonesia, jamu is commercially available in many forms, including powder, liquid and/or plant simplicia, which are readily consumed by adding hot water and drinking the resulting preparation. The manufactures of jamu generally recommend daily uses, regularly 1–3 times a day for treatment of one or versatile diseases. Currently, jamu represents a major traditional medicine system in the world, being applied especially in Malaysia, Singapore, Hong Kong, Australia, and the Netherlands, where Indonesian communities are large. In order to support the development of jamu, most of the available scientific studies focused on their efficacy,[3-6] while the safety and risks of frequent and prolonged consumption of jamu have not yet been evaluated.

It has been recognised that the safety, efficacy, quality and standardization of botanical ingredients in general and/or of jamu should be controlled to reach safer and more effective products.[7-9] The existing law in Indonesia has not explicitly regulated the maximum limit of natural compounds present in jamu. The National Agency for Drug and Food Control, Republic of Indonesia (NADFC RI) reported that there were 48 and 16 electronic reports related to the adverse effects of consumption of traditional herbal medicines and health supplements in 2015 and 2016, respectively.[10, 11] An example is the detection of carcinogenic aflatoxin B1, B2 and G2 in 14 jamu preparations.[12] In addition to these mycotoxin contaminants, jamu may also contain botanical ingredients that may raise a concern because they are genotoxic and carcinogenic. This includes for example the presence of botanicals that contain alkenylbenzenes, including compounds like estragole, methyleugenol, elemicin, safrole, myristicin, and apiol (Figure 2.1) which have previously been shown to be naturally occurring in plant food supplement (PFS) and herbal teas and a possible priority for risk management,[13-18] Alkenylbenzenes can occur in one or more of the medicinal herbs and plants used in jamu. Cinnamon (*Cinnamomum burmannii* Blume.) as one of the well-known spices in Indonesia and present in almost all jamu products may contain safrole,[19] myristicin,[20] and estragole.[16] Nutmeg (*Myristica fragnan* Houtt.) contains myristicin, estragole, safrole, elemicin, and methyeugenol.[16, 21] *Thymus vulgaris* L. folium contains estragole and methyleugenol.[22] And estragole, myristicin and apiol are occurring in semen of *Nigella sativa* L.[23] and fennel (*Foeniculum vulgare* Mill.).[24]

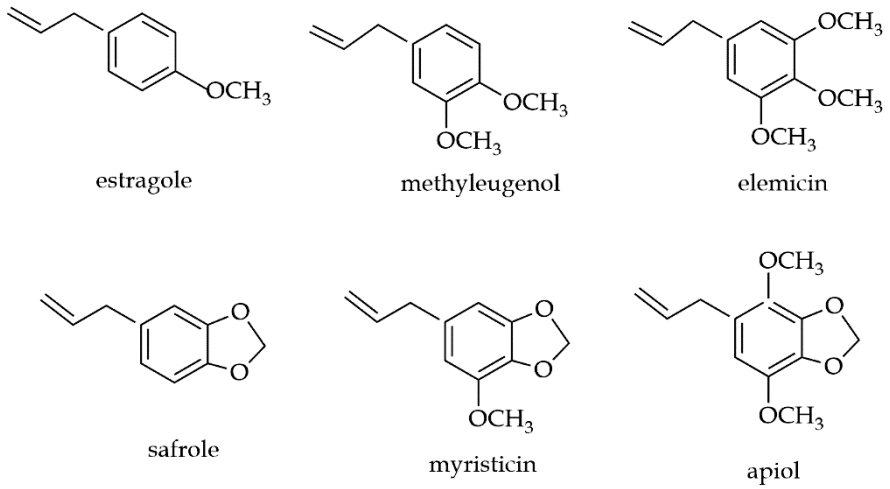

Figure 2.1. Structural formulas of alkenylbenzenes that are genotoxic and carcinogenic.

The DNA reactive properties of alkenylbenzenes have been well documented in in vitro and in vivo studies and even in humans.[22, 25] In human liver samples N^2-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine adducts were detected.[26-28] Safrole is categorized in IARC group 2B, probably carcinogenic to humans based on its carcinogenicity in rodent bioassays at high dose levels.[29-31] Estragole and methyleugenol are considered to be genotoxic and carcinogenic.[16, 32, 33]

The aim of the present study was to analyse the natural occurrence of alkenylbenzenes in 25 Indonesian jamu containing various mixed medicinal plants, and to evaluate the consumer risk of use of these jamu using the margin of exposure (MOE) approach. This MOE approach is recommended by expert groups of the European Food Safety Authority (EFSA), the Joint FAO/WHO expert committee on Food Additives (JECFA) and the International Life Sciences Institute (ILSI) for risk assessment of exposure to compounds that are both genotoxic and carcinogenic.[34-37] The results can be used to inform risk management to prioritize regulatory actions to reduce potential risks connected to jamu consumption and to define a safe jamu policy in Indonesia.

2.2. Materials and methods

2.2.1. Preparation of samples

A total of 25 jamu were collected from the traditional markets or jamu stores focussing on jamu samples with the name of possible alkenylbenzene containing botanicals on the label. The botanical names used in this targeted sampling were fennel (*Foeniculum vulgare* Mill.), bettel pepper (*Piper betle* L.), cinnamon (*Cinnamomum burmannii* Blume.), nutmeg (*Myristica fragrans* Houtt.), and ginger

(*Zingiber officinale* Rosc.). Details on the 25 jamu samples are presented in Table 2.1. The health claims written on the label are also presented in Table 2.1, while the class of disease listed in Table 2.1 was based on the International Classification of Diseases ver. 10.[38] The 25 jamu samples were purchased from jamu stores in Tegal (one store, 6 samples), Semarang (3 stores, each providing 2-3 samples), Surakarta (one store, 4 samples), and Nganjuk (3 stores, each providing 2-5 samples), as depicted in Figure 2.2.

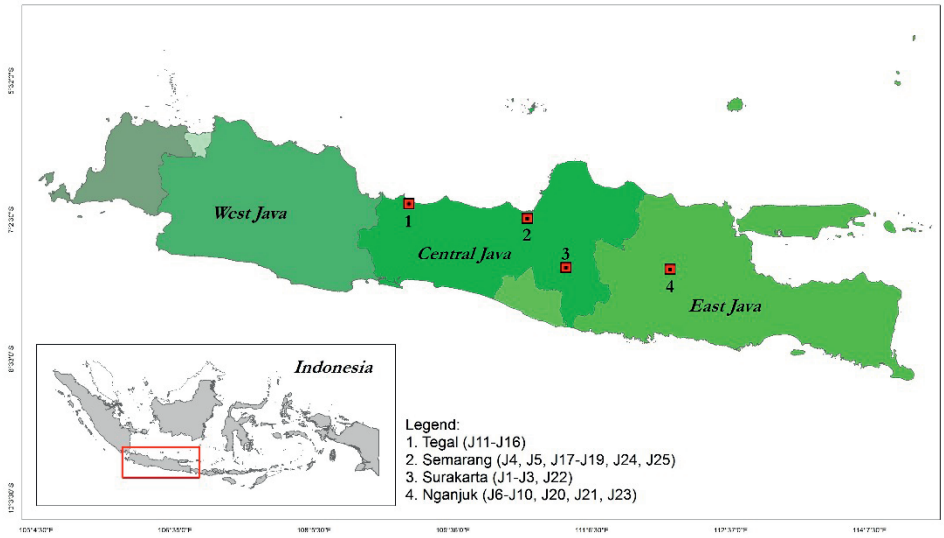

Figure 2.2. Sampling locations of jamu in East Java and Central Java Provinces, Indonesia. J1 until J25 represent the sample IDs used in the present study.

All of these samples were in powder form, packaged in sealed and labelled plastic sachet with net weights ranging between 5-7 g each (Table 2.1). The homogeneity of each sample was ensured by mixing manually in a ziplock plastic packet before taking samples for analysis.

Table 2.1. Jamu products from Indonesia used in the present study, their major characteristics and health claims. Plant ingredients possibly containing alkenylbenzenes are printed in bold.

Sample ID	Characteristics			Health claims (class of disease)
	Ingredients: plant species (part of plant)[a]	Weight per package (g)	Recommended daily use[b]	
J1	***Foeniculum vulgare* Mill.** (fruit) 0.25 g, ***Piper betle* L.** (leaf)1 g, *Merremiae mammosa* (tuber) 1 g, *Kaempferiae galanga* L. (rhizome) 1 g, *Piper cubeba* (fruit) 0.25 g, *Amomum cardamomum* (fruit) 0.5 g, ***Thymus vulgaris* L.** (leaf) 1 g	5	2 × a day (10 g)	Cure cough (respiratory diseases)
J2	*Phyllanthus urinaria* L. (herb) 500 mg, *Andrographis paniculata* Ness (herb) 500 mg, *Elaeocarpus grandiflorus* (leaf) 375 mg, *Azadirachtae indica* (leaf) 375 mg, *Leucaenae glauca* benth 250 mg, *Syzygium cumini (*L.) Skeels (seed) 250 mg, *Tinospora tuberculata* (bark) 250 mg, *Rauvolvia serpentina* Benth (root) 500 mg, *Alstonia macrophylla* Wall. Ex G. Don. (bark) 250 mg, *Strychnos ligustrina* (wood) 500 mg, *Alyxia reinwardtii* Bl. (bark) 250 mg, ***Foeniculum vulgare* Mill.** (fruit) 250 mg, *Curcuma longa* L. (rhizome) 375 mg	5	2-3 × a week (2.14 g)	Cure diabetes (nutritional and metabolic diseases)
J3	*Talinum paniculatum* Gaertner (rhizome) 10%, ***Cinnamomum burmannii* Blume** (bark) 10%, ***Foeniculum vulgare* Mill.** (fruit) 10%, *Baeckea frutescens* L. (leaf) 10%, and other ingredients until 100%	5	2 × a day (10 g)	To maintain health, cure headache, loss of appetite and spirit (musculoskeletal and connective tissue disorders)
J4	*Elephantopus scaber* L. (leaf) 0.70 g, ***Piper betle* L.** (leaf) 0.70 g, *Piper cubeba* (fruit) 0.70 g, ***Cinnamomum burmannii* Blume** (bark) 1.05 g, ***Foeniculum vulgare* Mill.** (fruit) 1.75 g, *Kaempferia galanga* L. (rhizome) 2.10 g	7	2-3 × a day (21 g)	Cure fever, influenza with headache, cough (respiratory diseases)
J5	*Melaleuca leucadendra* L. (fruit) 0.49 g, *Mesua ferrea* L. (flos) 0.56 g, *Merremia mammosa* CHOIS (tuber) 0.70 g, *Amomum cardamomum* (fruit) 0.70 g, *Paederia scandens* (Lour.) Merr. (leaf) 0.70 g, *Curcumae domesticae* (rhizome) 1.75 g, ***Foeniculum vulgare* Mill.** (fruit) 2.10 g	7	2-3 × a day (21 g)	Cure cough with phlegm (respiratory diseases)
J6	*Glycyrrhiza glabra* L. (root) 900 mg, *Boesenbergia pandurata* (Roxb.) (rhizome) 600 mg, *Kaempferia galanga* L. (rhizome) 300 mg, ***Foeniculum vulgare* Mill.** (fruit) 300 mg, *Curcumae domesticae* (rhizome) 600 mg, Gallae 600 mg, *Baeckea frutescens*	6	2 × a week (1.71 g)	Useful for the mothers after giving birth to reproduce, and purify milk, so the baby will grow up healthy, fresh and plump (female-specific diseases)

Sample ID	Characteristics			Health claims (class of disease)
	Ingredients: plant species (part of plant)[a]	Weight per package (g)	Recommended daily use[b]	
	L (leaf) 900 mg, *Terminalia arbareae* (fruit) 600 mg, *Rheum palmatum* L (root) 600 mg, *Coriandrum sativum* L (fruit) 600 mg			
J7	*Glycyrrhiza uralensis* Risch. (root) 900 mg, *Cinnamomum sintok* Bl. (bark) 1200 mg, *Alyxia reinwardtii* Bl. (bark) 600 mg, *Helicteres isora* L. (fruit) 900 mg, ***Myristica fragrans* Houtt**. (seed) 900 mg, *Coriandrum sativum* L (fruit) 900 mg, *Strychnos ligustrina* Bl (wood) 600 mg	6	2 × a day (12 g)	Useful for mothers who are nursing babies. Drink this herb seizures if the child sick, cranky, often surprised and diarrhea (gastrointestinal disorders)
J8	***Foeniculum vulgare* Mill.** (fruit) 14%, ***Nigella sativa* L.** (seed) 20%, *Amomum cardamomum* Willd (fruit) 20%, *Piper cubeba* L (fruit) 10%, *Centella asiatica* (leaf)10%, and other ingredients until 100%	7	2 × day (14 g)	Cure of cough, hoarse, shortness of breath (respiratory diseases)
J9	*Kaempferia galanga* L. (rhizome) 6%, ***Foeniculum vulgare* Mill.** (fruit) 9%, ***Zingiber officinale* Rosc** (rhizome) 8%, *Curcuma domestica* Val (rhizome) 8%, *Parkia roxburghii* G.Don (seed) 8%, and other ingredients up to 100%	7	2 × a day (14 g)	Cure of stomachache, relieve abdominal pain, diarrhea, bloating, heartburn, normalize digestive function and strengthen the intestinal lining, loss appetite (gastrointestinal disorders)
J10	***Foeniculum vulgare* Mill.** (fruit) 1000 mg, *Amomum cardamomum* Willd (fruit)1000 mg, *Alyxia reinwardtii* Bl. (bark) 500 mg, Ritae Herb 500 mg, *Curcuma domestica* Val (rhizome)1500 mg, *Kaempferia galanga* L. (rhizome) 750 mg, ***Zingiber officinale* Rosc** (rhizome) 1000 mg**, *Cinnamomum burmannii* Blume** (bark) 750 mg	7	1 × a day (7 g)	Treat and prevent diseases caused by seizures in infants and small children such as boils, itch, flatulence (gastrointestinal disorders)
J11	*Kaempferia galanga* L. (rhizome) 0.76 g, *Strychnos ligustrina* Bl (wood) 1.27 g, *Glycyrrhiza glabra* L. (root) 1.27 g, ***Foeniculum vulgare* Mill.** (fruit) 1.65 g, *Costus speciosus* Smith. (rhizome) 2.05 g	7	2 × a day (14 g)	Cure cough with phlegm and sore throat (respiratory diseases)
J12	*Carum copticum* (L.) Benth (fruit) 0.64 g, *Plantago major* L (leaf) 1.27 g, Piper cubeba L.f. (fruit) 1.27 g, ***Foeniculum vulgare* Mill.** (fruit) 1.91 g, *Curcuma domestica* Val (rhizome) 1.91 g	7	2 × a day (14 g)	Treat frequent stools/ diarrhea (gastrointestinal disorders)
J13	*Piper nigrum* L (fruit) 1.03 g, ***Foeniculum vulgare* Mill** (fruit) 1.27 g, *Piper retrofractum* Vahl (fruit) 1.27 g, *Kaempferia galanga* L (rhizome)1.27 g, *Zingiber zerumbet* SM (rhizome) 2.16 g	7	2 × a day (14 g)	Effective to relieves muscles and joints pain (musculoskeletal system and connective tissue disorders)

Sample ID	Characteristics			Health claims (class of disease)
	Ingredients: plant species (part of plant)[a]	Weight per package (g)	Recommended daily use[b]	
J14	*Cinnamomum sintok* Bl. (bark) 0.28 g, *Sindora sumatrana* (fruit) 0.35 g, ***Myristica fragrans* Houtt.** (seed) 0.7 g, *Parameria laevigata* (Juss.) Moldenke (bark) 1.12 g, *Curcuma domestica* Val (rhizome) 4.55 g	7	2-3 × a week (3 g)	Useful for maintaining health and add strength to the weak body, less blood and loss of appetite (musculoskeletal and connective tissue disorders) and reduce body odour
J15	***Nigella sativa* L** (seed) 0.63 g, ***Myristica fragrans* Houtt** (pericarpium) 0.77 *g, Piperis nigrum* L (fruit) 1.12 g, *Eurycoma longifolia* Jack. (root) 2.03 g, *Curcumae domesticae* (rhizome) 2.45 g.	7	2-3 × a week (3 g)	To maintain health and protect the body from disease for men who are always keen to work (musculoskeletal and connective tissue disorders)
J16	*Piper retrofractum* Vahl. (fruit) 2 g, ***Myristica fragrans* Houtt** (seed) 1 g, ***Foeniculum vulgare* Mill.** (fruit) 1 g, ***Piper betle* L.** (leaf)1 g	5	3 × a week (2.14 g)	Treatment of leukorrhoea/vaginalis, dysmenorrhoea (female specific diseases)
J17	*Piper retrofractum* Vahl. (fruit) 0.56 g, ***Eucalyptus alba* Reinw** (fruit) 0.84 g, *Zingiber aromaticum* Val (rhizome) 0.84 g, ***Zingiber officinale* Rosc** (rhizome) 0.84 g, *Curcuma xanthorrhiza* Roxb (rhizome) 0.56 g, other ingredients 3.36 g consist of: ***Foeniculum vulgare* Mill.** (fruit), *Alyxia reinwardtii* Bl. (bark), *Languas galanga* (L.) Stuntz (rhizome), *Smilax zeylanica* L. (rhizome), *Parkia roxburghii* G.Don (seed), *Orthosiphon spicatus* B.B.S. (leaf), ***Cinnamomum burmannii* Blume (bark)**, *Carum copticum* (L.) Benth (fruit), *Piper nigrum* L. (fruit), *Phyllanthus niruri* L (herb)	7	2 × a day (14 g)	Eliminate fatigue, aches and back muscular pain (musculoskeletal and connective tissue disorders)
J18	*Curcuma xanthorrhiza* Roxb (rhizome) 0.4 g, *Zingiber aromaticum* Val (rhizome) 0.5 g, ***Myristica fragrans* Houtt.** (seed) 0.05 g, ***Cinnamomum burmani* Bl (**bark**)** 0.05 g, other ingredients 7.3 g consist of: *Curcuma domestica* Val (rhizome), ***Zingiber officinale* Rosc (**rhizome), *Curcuma aeruginosa* Roxb (rhizome), *Kaempferia galanga* L. (rhizome), ***Cymbopogon nardus* L. Rendle** (leaf), *Pandanus amaryllifolius* Roxb (leaf), *Mangifera indica* L. (fructus), *Saccharum officinarum* L (in extract form)	7	2-3 × a day (21 g)	Cure of lose appetite, reduce symptom of trichinosis (gastrointestinal disorders)
J19	*Glycyrrhiza glabra* L. (root) 0.84 g, ***Eucalyptus alba* Reinw** (fruit) 0.84 g, *Caryophyllus aromaticus* L. (leaf) 0.56 g, *Amomum*	7	2 × a day (14 g)	Relieve breathing caused by smoking (respiratory diseases)

Sample ID	Characteristics			Health claims (class of disease)
	Ingredients: plant species (part of plant)[a]	Weight per package (g)	Recommended daily use[b]	
	cardamomum Willd (fruit) 0.56 g, *Curcuma xanthorrhiza* Roxb (rhizome) 0.56 g, and other ingredients until 7 g consist of ***Foeniculum vulgare* Mill.** (fruit), *Parkia roxburghii* G.Don (seed), ***Zingiber officinale* Rosc (**rhizome), *Piper cubeba* L.f. (fruit), ***Cinnamomum burmannii* Blume** (bark), *Carum copticum* (L.) Benth (fruit), *Stachytarpheta mutabilis* Vahl. (leaf), *Centella asiatica* Urb (herb), *Alyxia reinwardtii* Bl. (bark), *Plantago major* L (leaf)			
J20	*Equisetum debile* Roxb. (herb) 0.49 g, ***Zingiber officinale* Rosc** (rhizome) 0.70 g, *Blumea balsamifera* DC (leaf) 0.28 g, ***Myristica fragrans* Houtt** (seed) 0.63 g, *Curcuma xanthorrhiza* Roxb (rhizome) 1.50 g, *Curcuma domestica* Val. (rhizome) 1.50 g, *Kaempferia galanga* L. (rhizome) 1.00 g, *Orthosiphon spicatus* B.B.S. (leaf) 0.50 g, *Piper nigrum* L (fruit) 0.40 g	7	2 × a day (14 g)	Effective to relieves muscles and joints pain, refresh the body (musculoskeletal and connective tissue disorders)
J21	***Zingiber officinale* Rosc** (rhizome) 0.56 g, ***Foeniculum vulgare* Mill.** (fruit) 0.42 g, *Piper retrofractum* Vahl. (fruit) 0.14 g, *Pluchea indica* Less (leaf) 0.35 g, *Piper cubeba* L.f (fruit) 0.07 g, *Kaempferia galanga* L. (rhizome) 2 g, *Curcuma domestica* L. (rhizome) 1.8 g, *Centella asiatica* (herb) 0.86 g, *Melaleuca cajuputi* Powell (leaf) 0.8 g	7	2 × a day (14 g)	Cure cough and cold (respiratory diseases)
J22	*Maschosma polystachyum* Benth (leaf) 24%, *Euphorbia hirta* L. (herb) 23%, ***Myristica fragrans* Houtt**. (seed) 4%, ***Foeniculum vulgare*** (fruit) 4%, and other ingredients until 100%	5	2 × a day (10 g)	To strengthen heart (heart and blood vessels)
J23	*Melaleuca leucadendra* L. (fruit) 0.56 g, *Woodfordia floribunda* Salisb (fruit) 0.35 g, *Curcuma xanthorrhiza* Roxb (rhizome) 1.4 g, *Curcuma domestica* L. (rhizome) 1.05 g, *Piper retrofractum* Vahl.(fruit) 0.35 g, and other ingredients 3.2 g consist of *Amomum cardamomum* Willd (fruit), ***Piper betle* L.** (leaf), ***Foeniculum vulgare* Mill.** (fruit)	7	2 × a day (14 g)	Cure cough with phlegm and asthma (respiratory diseases)
J24	***Zingiber officinale* Rosc (**rhizome) 0.7 g, *Curcuma xanthorrhiza* Roxb (rhizome) 0.7 g, *Zingiber aromaticum* Val (rhizome) 0.7 g, ***Foeniculum vulgare* Mill.** (fruit) 0.7 g, Caryophylli Leaf 0.7 g,	7	2 × a day (14 g)	Cure fever, headache, nasal congestion (ear, nose and throat)

Sample ID	Characteristics			Health claims (class of disease)
	Ingredients: plant species (part of plant)[a]	Weight per package (g)	Recommended daily use[b]	
	Piper retrofractum Vahl (fruit) 0.35 g, *Languas galanga* (L.) Stuntz (rhizome) 0.35 g, *Kaempferia galanga* L. (rhizome) 0.35 g, Usnea *misaminensis* (Vain) Not. (herb) 0.35 g, *Piper nigrum* L (fruit) 0.35 g, ***Myristica fragrans* Houtt.** (seed) 0.35 g, *Parkia roxburghii* G.Don (seed) 0.35 g, *Zingiber purpureum* Roxb (rhizome) 0.35 g, ***Nigella sativa* L.** (seed) 0.35 g, *Blumea balsamifera* DC. (leaf) 0.35 g			
J25	***Foeniculum vulgare* Mill.** (fruit) 1.05 g, ***Zingiber officinale* Rosc** (rhizome) 0.7 g, *Abrus precatorius* L (leaf) 0.7 g, *Thymus vulgaris* L (herb) 0.7 g, *Ruta graveolens* L. (herb) 0.7 g, *Caryophyllus aromaticus* L. (leaf) 0.35 g, *Piper cubeba* L.f. (fruit) 0.28 g, ***Myristica fragrans* Houtt.** (pericarpium) 0.35 g, *Amomum cardamomum* Willd (fruit) 0.35 g, *Curcuma longa* L. (rhizome) 0.28 g, Usnea *misaminensis* (Vain) Not. (herb) 0.35 g, *Mentha arvensis* L (herb) 0.35 g, *Alyxia reinwardtii* Bl. (bark) 0.28 g, *Centella asiatica* Urb (herb) 0.21 g, *Cuminum cyminum* L (fruit) 0.21 g, *Stevia rebaudiana* (leaf) 0.14 g.	7	2 × a day (14 g)	Cure cough with phlegm (respiratory diseases)

[a] Plant species and its used part as mentioned on the label of the samples, if the label only mentioned the common name, the plant species was searched for on KNApSAcK Family Databases (http://kanaya.naist.jp/KNApSAcK_Family/) from Afendi et al. (2012).[4]

[b] The value presented in the blacket is weight of recommended daily use of jamu in g.

2.2.2. Chemicals and reagents

Estragole (purity 98% w/w), methyleugenol (purity 99% w/w), safrole (purity >97% w/w), and myristicin (purity >97% w/w) were purchased from Sigma-Aldrich (Zwijdrecht, The Netherlands). Apiol (purity >99%) was obtained from Extrasynthese (Genay Cedex, France). Elemicin was obtained from Synchem OHG (Felsberg, Germany). Methanol (HPLC supra gradient) was acquired from Sigma-Aldrich. Acetonitrile (ACN) (ULC/MS gradient), trifluoroacetic acid (TFA), and dimethyl sulfoxide (DMSO) was purchased from Merck. Nanopure water was obtained from an Arium pro UF/VF water purification system (Sartorius Weighting Technology GmbH, Goettingen, Germany).

2.2.3. Methanol extraction

Methanolic extraction was applied to optimally extract and quantify the total amount of different alkenylbenzenes present in the samples. Extraction was performed based on the method described by Gursale at al. (2010)[39] with minor modifications. Briefly, 10 mL of methanol was added to 1 g of jamu followed by sonication for 15 min. Upon sonication the extract was filtered using a 0.45 µm syringe filter and the filtrate was directly analysed using ultraperformance liquid chromatography (UPLC) analysis. Samples were extracted and analysed on UPLC in three independent experiments.

The accuracy of the developed method was assessed using a recovery study to account for possible losses during the extraction process. For recovery studies, 1 g of sample J18 was spiked with mixtures of 6 pure standards of alkenylbenzenes each in a final concentration of 10 µM. The spiked sample was analyzed by the same procedure as described above. The recoveries of elemicin, methyleugenol, myristicin, safrole, and estragole are 108.12±1.77; 103.83±0.13, 96.11±1.25, 105.59±2.98, 99.73±3.41%, respectively. These results of sample recovery were used to correct the levels of the alkenylbenzenes detected in the different jamu samples. To show the linearity of the extraction procedure J18 was analysed in a range of weight per volume of methanol ratio at 2.5%, 5%, 10%, 20% and 40% w/v.

2.2.4. UPLC analysis

The methanol extract was analyzed in undiluted form, and 3.5 µl aliquot of each sample was subjected to UPLC analysis as described before.[17] The sample analysis was carried out using a UPLC-DAD system consisting of a Waters (Milford, MA) Acquity binary solvent manager, sample manager, and photodiode array detector, equipped with a Waters Acquity UPLC BEH RP 18 column (1.7 µm, 2.1 × 50 mm). The column and sample temperature were set at 22°C and 10°C

respectively. The mobile phase used in UPLC consisted of nanopure water containing 0.1% (v/v) TFA (as eluent A) and ACN (as eluent B) with a flow rate of 0.6 mL/min. After equilibrating the column at the starting conditions of 30.5% ACN, the ACN was kept at this level for 15 min and then increased to 80% over 1 min and kept at 80% for 0.5 min, then decreased to 0% over 1.5 min and kept at 0% for 1 min after which the ACN was returned to the starting conditions. The levels of the alkenylbenzenes of interest were quantified based on the peak areas of the UV absorption at 206 nm for elemicin, 202 nm for methyleugenol and safrole, 210 nm for myristicin and apiol, 225 nm for estragole and comparison to the calibration curves of these compounds derived from commercially available standards.

The UPLC method was characterised by the limit of detection (LOD) and the limit of quantification (LOQ) according to the International Conference on Harmonization Guidance for the Validation of Analytic Methods.[40] The LOD and LOQ were determined for each alkenylbenzene using the standard of the response method, using the following equations:

$LOD = (3.3\sigma)/S$ (Equation 2.1)

$LOQ = (10\sigma)/S$ (Equation 2.2)

where σ is the standard deviation of the peak areas and S the slope of the calibration curve. Table 2.2 presents the LOD and LOQ obtained for analysis of the 6 alkenylbenzenes elemicin, methyleugenol, myristicin, apiol, safrole and estragole. Values varied somewhat among different alkenylbenzenes, but followed a similar trend when comparing the LOD and LOQ values.

Table 2.2. Limits of Detection (LOD, µV*Sec) and Quantification (LOQ, µV*Sec) for analysis of the 6 alkenylbenzes by UPLC.

Type of alkenylbenzene	LOD of area (µV*Sec)	LOQ of area (µV*Sec)
Elemicin	129.34	373.76
Methyleugenol	124.05	375.92
Myristicin	125.52	380.36
Safrole	125.89	381.48
Apiol	126.51	383.37
Estragole	128.03	387.96

2.2.5. Calculation of interim relative potency (REP) factors

Interim relative potency (REP) factors were determined to estimate the combined exposure of different alkenylbenzenes in the same sample using a toxic equivalency (TEQ) approach. Methyleugenol was chosen as the reference compound with a REP value of 1.00 because methyleugenol appeared to be the main alkenylbenzene detected in the samples (see Results section). The interim REP values (Table 2.3) were calculated as the average of interim REP values obtained from (1) DNA adduct formation by the different alkenylbenzenes in CD-1 mice[41],

(ii) physiologically based kinetic (PBK) modelling predicted relative formation of the proximate hepatocarcinogenic 1′-sulfoxy metabolite representing the relative importance of the bioactivation route at a dose level of 0.01 mg/kg bw[42-46], and (iii) the lower confidence limit of the benchmark dose giving 10% extra tumor incidence ($BMDL_{10}$) values derived from the carcinogenicity data[29, 47] and read-accross from safrole for myristicin and apiol[42, 44] and from estragole and methyleugenol for elemicin.[48] These average interim REP values are considered a suitable first estimate to take into account the differences in potency of the alkenylbenzenes. The accuracy of these interim REP values can obviously be further refined. However, given that the actual outcomes for the risk assessment when using just adding up the alkenylbenzene levels or using a TEQ based approach did not differ substantially we feel further refinement of the interim REP values is not required or indicated at this stage.

Table 2.3. Interim REP values as defined based on different available data set and the average interim REP values used in this study.

Compound	Interim REP derived from DNA adduct information	Interim REP derived from human PBK model based prediction of 1′-sulfoxy formation	Interim REP derived from the $BMDL_{10}$ for liver tumor formation	Average Interim REP value
Safrole	1.05	1.08	8.05	3.39
Myristicin	0.24	0.8	8.05	3.03
Apiol	0.07	0.53	2.68	1.09
Estragole	1.55	4.62	4.64	3.60
Methyleugenol	1.00	1.00	1.00	1.00
Elemicin	0.09	0.39	0.50	0.33

2.2.6. Estimation of daily intakes of alkenylbenzenes resulting from the consumption of jamu

In order to evaluate consumer risks, the estimated daily intake (EDI) was calculated based on the individual alkenylbenzene levels present in the samples, the recommended daily use as written on the labels (Table 2.1) and a mean Indonesian body weight (bw) of 54 kg/person.[49] For calculation of EDI values it was assumed that 1 sachet jamu would be added to one cup of hot water (±100 mL) followed by drinking all of the preparation. When the label lists use of 2-3 times a day, 3 times consumption was chosen to calculate the EDI. In the present study three different exposure scenario's were considered for calculating lifetime exposure. These included (i) calculation of the EDI for each individual alkenylbenzene for all the samples, expressed in µg/kg bw/day using the following equation:

$$EDI=\frac{\text{recommended daily use (g)} \times \text{level of alkenylbenzene (µg/g)}}{\text{bw (54 kg)}} \quad \text{(Equation 2.3)}$$

Since several samples were found to contain more than one alkenylbenzenes, the EDI values were also estimated taking combined exposure into account. (ii) In

this second approach, the EDI for combined exposure was calculated by assuming equal potency of the different alkenylbenzenes, adding up the EDIs of the individual alkenylbenzene.[13, 15] (iii) In a third approach, the combined EDI was calculated by a toxic equivalency (TEQ) approach using the average interim REP values presented in Table 2.3 taking methyleugenol as the reference compound. These EDI values were expressed in µg methyleugenol equivalents/kg bw/day, and calculated using the following equation:

EDI (µg methyleugenol equivalents/kg bw/day) $= \sum(EDI \times REP)i$ (Equation 2.4)

In addition, also 3 scenarios were included to take into account durations of exposure shorter than lifetime exposure. For these scenario's only the combined total alkenylbenzene exposures were taken into account. The 3 scenarios compared for differences in duration of exposure included: i) chronic lifetime exposure, which is the exposure generally considered in the MOE approach and described also above as the second scenario with the combined exposure based on equal potency, ii) short term exposure for only two weeks, correcting for the shorter than lifetime exposure by applying Haber's rule, and iii) regular short term exposure to mimic the situation where people use jamu as a remedy during periods of illness and thus likely will use it for short period of time but on a regular basis. For this last exposure scenario, it was assumed that people would use the preparations two weeks, once every year during a lifetime.

Although there is no formally accepted procedure for correcting the EDI and thus the MOE approach for shorter than lifetime exposure, previously it was suggested to apply, as a first approximation, Haber's rule.[50-52] Based on this rule the toxic outcome will be similar for situations where the product of the exposure time and the dose will be constant, ($k = C \times T$; $C1 \times T1 = C2 \times T2$, where k is the toxic outcome, C is the concentration (or dose) of the toxic chemical and T is the time of exposure). Using Haber's rule and a lifetime expectancy of Indonesian people of 69 years[53], the EDI of the total level of alkenylbenzenes could be corrected as follows:

EDI for n week exposure corrected to lifetime exposure $=$
$\frac{\text{recommended daily use (g)} \times \text{level of alkenylbenzene (µg/g)}}{bw\ (54\ kg)} : (\frac{69\ years \times 52\ weeks}{n\ week})$ (Equation 2.5)

2.2.7. Calculation of the Margin of Exposure

The MOE approach was used to perform a risk assessment of jamu. First, MOEs for individual alkenylbenzenes were calculated by dividing the $BMDL_{10}$ values for the individual alkenylbenzenes, 15.3 mg/kg bw for methyleugenol,[17] 1.9 mg/kg bw for safrole[54] and myristicin[42], and 3.3 mg/kg bw for estragole[55] by the EDI values of the individual alkenylbenzenes, using the following equation:

$$MOE = \frac{BMDL_{10}}{EDI}$$ (Equation 2.6)

Second, the MOE values were calculated using a combined exposure assessment assuming equal potency of all alkenylbenzenes. For these MOE calculation Equation 2.6 was applied using the $BMDL_{10}$ of 15.3 mg/kg bw for the major alkenylbenzene in the mixture, methyleugenol, and the EDI resulting from summing up the EDIs of the individual alkenylbenzenes assuming equal potency. Third, the MOE values were calculated based on a TEQ approach calculating the combined exposure in methyleugenol equivalents (Equation 2.4) and using the $BMDL_{10}$ of methyleugenol of 15.3 mg/kg bw.

For calculating MOE values for shorter than lifetime exposure the same equation was applied only using the corrected EDI values (Equation 2.5) assuming equal potency and the $BMDL_{10}$ of methyleugenol of 15.3 mg/kg bw. To further illustrate how short term exposure would affect the MOE values, the number of weeks of daily consumption of jamu samples analysed in this study that would result in a MOE of 10,000 indicating a low priority for risk management was also calculated using Equations 2.5 and 2.6.

2.3. Results

2.3.1. Levels of alkenylbenzenes in Indonesian jamu

As shown in Table 2.4, four different alkenylbenzenes, methyleugenol, myristicin, safrole, and estragole were detected in 23 out of 25 samples of Indonesian jamu, at levels ranging from 3.8 – 440 µg/g. Methyleugenol was the major alkenylbenzene detected in most (91.3%) of the positives samples (Table 2.5). Apiol and elemicin were not detected in the samples. Two positive samples contained 3 different alkenylbenzenes; methyleugenol, safrole and estragole in J7, and methyleugenol, safrole and myristicin in J24. Only 2 (J14 and 18) out of 25 samples did not contain detectable levels of any alkenylbenzene.

Table 2.4. The level and EDI of different alkenylbenzenes found in Indonesian jamu.

Sample ID	Alkenylbenzenes detected	Alkenylbenzene level (µg/g)[a]	% content of alkenylbenzene	Recommended daily use (g)	EDI of the individual alkenylbenzene (µg/kg bw/day)	Combined EDI based on assuming equal potency (µg/kg bw/day)	Combined EDI based on TEQ approach (µg methyleugenol equivalents per/kg bw/day)
J1	Safrole	18.8±3.2	100.0	10	3.5	3.5	11.8
J2	Methyleugenol	18.2±1.6	75.1	2.14	0.7	0.9	1.5
	Safrole	6.0±1.1	24.9	2.14	0.2		
J3	Methyleugenol	23.9±1.6	31.8	10	4.4	13.9	33.1
	Myristicin	51.1±24.4	68.2	10	9.5		
J4	Methyleugenol	82.3±6.5	15.8	21	32.0	203.2	550.6
	Myristicin	440.1±24.8	84.2	21	171.2		
J5	Methyleugenol	48.9±12.1	36.5	21	19.0	52.1	119.2
	Myristicin	85.0±8.9	63.5	21	33.1		
J6	Methyleugenol	27.1±3.5	100.0	1.71	0.9	0.9	0.9
J7	Methyleugenol	38.8±2.2	58.3	12	8.6	14.8	30.6
	Safrole	3.8±0.5	5.7	12	0.8		
	Estragole	23.9±6.3	36.0	12	5.3		
J8	Methyleugenol	13.8±3.4	100.0	14	3.6	3.6	3.6
J9	Methyleugenol	4.8±1.6	100.0	14	1.2	1.2	1.2
J10	Methyleugenol	19.4±2.9	23.6	7	2.5	10.7	27.2
	Myristicin	62.9±4.9	76.4	7	8.2		
J11	Myristicin	66.6±0.2	100.0	14	17.3	17.3	52.4
J12	Methyleugenol	31.2±2.9	100.0	14	8.1	8.1	8.1
J13	Methyleugenol	111.0±22.5	76.6	14	28.8	37.6	55.4
	Myristicin	33. 9±7.2	23.4	14	8.8		
J14	nd[b]	nd	nd	3	nd	nd	
J15	Methyleugenol	128.6±0.9	100.0	3	7.1	7.1	7.1
J16	Methyleugenol	51.3±4.2	38.4	2.14	2.0	5.3	11.9
	Myristicin	82.4±17.2	61.6	2.14	3.3		
J17	Methyleugenol	80.3±0.9	100.0	14	20.8	20.8	20.8
J18	nd	nd	nd	21	nd	nd	
J19	Methyleugenol	84.4±9.2	100.0	14	21.9	21.9	21.9
J20	Methyleugenol	84.9±5.1	35.3	14	22.0	62.2	143.9
	Myristicin	155.2±25.5	64.7	14	40.2		

Sample ID	Alkenylbenzenes detected	Alkenylbenzene level (µg/g)[a]	% content of alkenylbenzene	Recommended daily use (g)	EDI of the individual alkenylbenzene (µg/kg bw/day)	Combined EDI based on assuming equal potency (µg/kg bw/day)	Combined EDI based on TEQ approach (µg methyleugenol equivalents per/kg bw/day)
J21	Methyleugenol	63.2±6.8	29.6	14	16.4	55.4	134.6
	Myristicin	150.5±19.9	70.4	14	39.0		
J22	Methyleugenol	15.5±2.4	20.0	10	2.9	14.3	38.9
	Myristicin	48.4±12.5	62.7	10	9.0		
	Estragole	13.3±1.3	17.2	10	2.5		
J23	Methyleugenol	38.6±2.5	29.7	14	10.0	33.6	81.6
	Myristicin	91.2±3.0	70.3	14	23.6		
J24	Methyleugenol	96.3±5.3	42.7	14	25.0	58.5	128.6
	Safrole	22.2±5.9	9.9	14	5.8		
	Myristicin	107.0±1.9	47.4	14	27.7		
J25	Methyleugenol	99.1±2.3	66.6	14	25.7	38.6	64.8
	Myristicin	49.7±3.1	33.4	14	12.9		

[a]Mean of 3 independent analyses. Levels were corrected for the recovery result (see materials and methods); [b]Not detected

Table 2.5. Overview of the occurrence of the different alkenylbenzenes in the positive jamu samples analysed.

Detected compound	Number of jamu samples containing the compound	% of positive jamu samples containing the compound
Methyleugenol	21	91.3
Myristicin	13	56.5
Safrole	4	17.4
Estragole	2	8.7
Elemicin	0	0.0
Apiol	0	0.0

2.3.2. EDI of individual and combined alkenylbenzenes

Table 2.4 presents the EDI values obtained for the individual alkenylbenzene, amounting for the positive samples from 0.2 to 171 µg/kg bw/day. The EDI values estimated taking combined exposure into account by assuming similar potency amounted to 0.9 to 203 µg/kg bw/day. Using the TEQ approach EDI values expressed in methyleugenol equivalents/kg bw resulted in EDI values ranging from 0.9 to 551 µg methyleugenol equivalents/kg bw/day.

2.3.3. Risk assessment of jamu using the MOE

The MOE values for the individual alkenylbenzene occurring in the jamu samples are depicted in the Figure 2.3A. The MOE values obtained when considering combined exposure assuming equal potency of the alkenylbenzene and using the $BMDL_{10}$ of the major alkenylbenzene present in the samples, methyleugenol, are shown in Figure 2.3B. Only 3 samples (J2, J6 and J9) out of 23 positives samples were shown to give rise to MOE values higher than 10,000. For J2 the safrole level resulted in an MOE of less than 10,000. The observation that the MOE of most of the jamu samples (87%) were lower than 10,000 indicates a priority for risk management when these jamu would be consumed for long periods of time.

Figure 2.3C reveals the MOE values that resulted from dividing the $BMDL_{10}$ of 15.3 mg/kg bw/day for methyleugenol by the EDI for the respective jamu samples expressed in methyleugenol equivalents. In line with the MOE results from the 2 other approaches, also the combined risk assessment based on the TEQ approach, resulted in MOE values that were lower than 10,000 for a large number (87%) (20/23) of samples with the values for 65% (15/23) of the samples being even below 1,000. The results obtained using the combined risk assesment based on equal potency and the TEQ approach were comparable, with the MOE being lower than 10,000 for all samples except for samples J2, J6 and J9 for which the MOE values were above 10,000 for both methods. MOE values resulting from the TEQ approach were generally somewhat lower than the MOE values obtained when adding up the levels of the different alkenylbenzenes, mainly because the alkenylbenzenes being present in addition to methyleugenol have interim REP values higher than 1.00. Together the results depicted in Figure 2.3 indicate that the MOE values resulting from regular daily consumption of the majority of Indonesian jamu preparations indicate a priority for risk management.

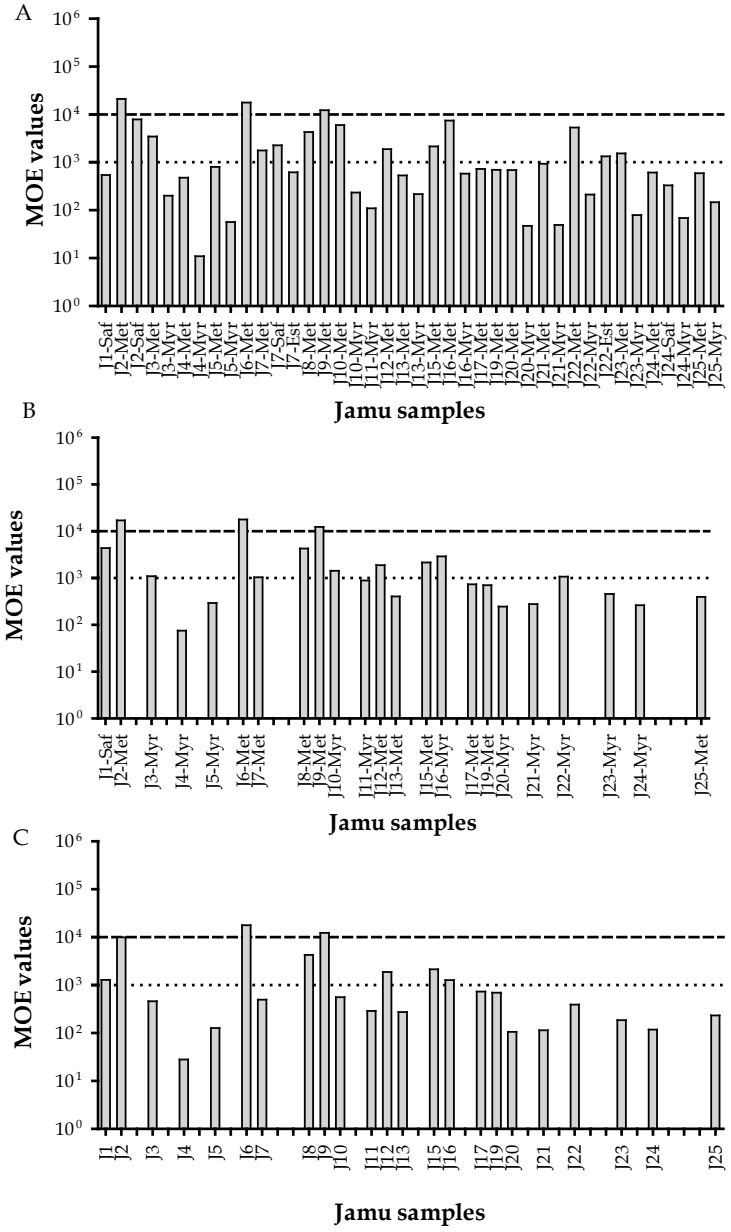

Figure 2.3. MOE values obtained for use of the jamu preparations on a daily basis during a lifetime based on the three approaches: A) considering the individual alkenylbenzenes using their $BMDL_{10}$ values, B) the combined exposure assuming equal potency of all alkenylbenzenes and using the $BMDL_{10}$ value of the major alkenylbenzene in the mixture, and C) the combined exposure using the TEQ approach based on methyleugenol equivalents and using the $BMDL_{10}$ value of methyleugenol. EDI and MOE values were calculated as explained in the Materials and Methods section (Equations 2.3, 2.4 and 2.6). The horizontal dashed and dotted lines represent MOE values of 10,000 (----) and 1,000 (.....), while Saf, Met, Myr, and Est, represent the alkenylbenzenes safrole, methyleugenol, myristicin and estragole, respectively.

The range of daily recommended consumption of jamu samples of 1.7 g to 21 g (Table 2.1) will be of low priority of risk management (MOE > 10 000) when the total alkenylbenzene level in the jamu sample would be less than 48 to 4 μg/g, respectively. This could be calculated using Equations 2.3 and 2.6 and considering lifetime exposure, a $BMDL_{10}$ of methyleugenol of 15.3 mg/kg bw and a 54 kg body weight.[49] When instead of lifetime use, two weeks of use of the jamu samples is considered, and Haber's rule is used for linear correction of the correponding intake estimates, the EDI values will be 69 year (=lifetime) × 52 weeks per year/2 weeks = 1,794 times lower (Equation 2.5) and thus, alkenylbenzene levels in the jamu samples can be 1,794 times higher before an MOE of 10,000 is reached (Equation 2.6). Thus, with a $BMDL_{10}$ of 15.3 mg/kg bw/day, two weeks intake of 1.7 or 21 g jamu (Table 2.1) would not raise a concern (MOE of more than 10,000) if the total alkenylbenzene level would be lower than 86 or 7 mg/g, respectively. Taken together the results indicate that at the current levels of alkenylbenzenes in Indonesian jamu, their consumption would be a concern especially for people who consume these jamu for long periods of time.

Figure 2.4 depicts the MOE values that would be obtained for the jamu samples of the present study assuming two weeks consumption. From this result it follows that when consumption of jamu is limited to two weeks the MOE values of all samples are higher than 10,000 indicating there would no priority for risk management. However, in real life people tend to consume jamu for short periods but on a rather regular basis especially when considering its use as medicine. To take this regular short term use into account we finally estimated the MOE values assuming use for two weeks once every year during a whole lifetime. This lowers all MOE values obtained for 2 weeks exposure by a factor of 69, assuming 69 years to represent the average Indonesian lifetime.[53] The results thus obtained are depicted in Figure 2.5. Together the results depicted in Figure 2.5B indicate that the MOE values resulting from regular consumption during two weeks every year during a whole lifetime suggest a priority for risk management, for 5 out of 23 (21.7%) of the positives samples.

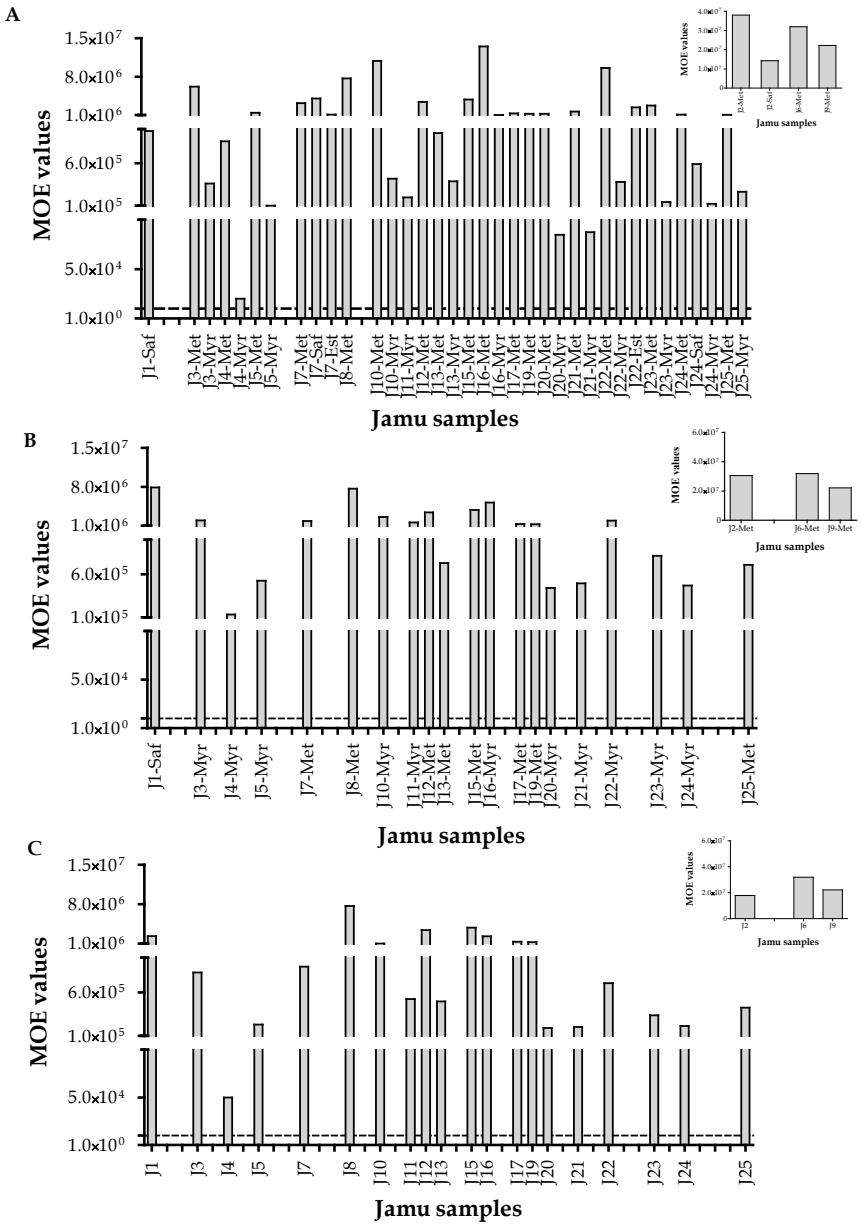

Figure 2.4. MOE values assuming 2 weeks consumption based on the three approaches: A) the individual alkenylbenzenes using their $BMDL_{10}$ values, B) the combined exposure assuming equal potency of all alkenylbenzenes and using the $BMDL_{10}$ value of the major alkenylbenzene in the mixture, and C) the combined exposure using the TEQ approach based on methyleugenol equivalents and using the $BMDL_{10}$ value of methyleugenol. EDI and MOE values were calculated as explained in the Materials and Methods section (Equations 2.5 and 2.6). The Y axis was dirrupted in 3 segments at 1-10^5, 10^5-10^6 and 10^6-1.5×10^7 for bottom, center and top segment, respectively. The inserted graph shows the MOE values for J2, J6 and J9. The horizontal dashed line (----) represents MOE values of 10,000, while Saf, Met, Myr, and Est, represent the alkenylbenzenes safrole, methyleugenol, myristicin and estragole, respectively.

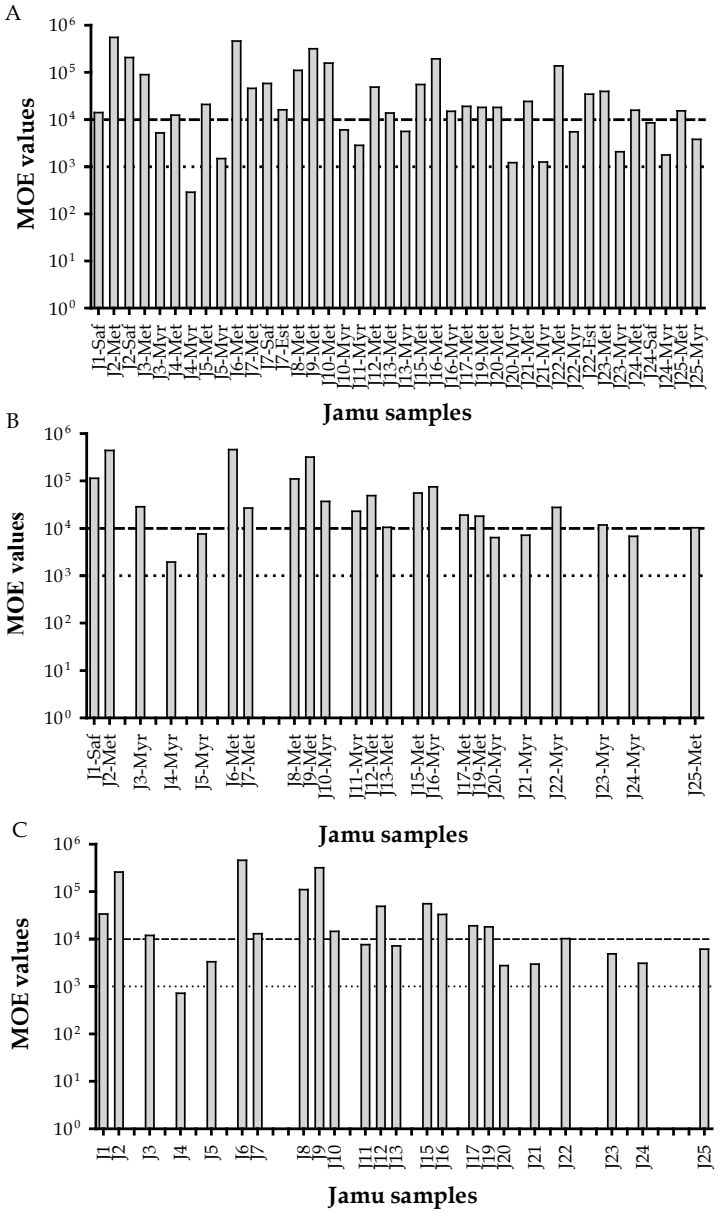

Figure 2.5. MOE values assuming jamu consumption 2 weeks every year for a whole lifetime (69 years)[53] based on the three approaches: A) the individual alkenylbenzenes using their $BMDL_{10}$ values, B) the combined exposure assuming equal potency of all alkenylbenzenes and using the $BMDL_{10}$ value of the major alkenylbenzene in the mixture, and C) the combined exposure using the TEQ approach based on methyleugenol equivalents and using the $BMDL_{10}$ value of methyleugenol. EDI and MOE values were calculated as explained in the Materials and Methods section (Equations 2.5 and 2.6). The horizontal dashed and dotted lines (----) represent MOE values of 10,000 and 1000 (.....), while Saf, Met, Myr, and Est, represent the alkenylbenzenes safrole, methyleugenol, myristicin and estragole, respectively.

Figure 2.6 shows the number of weeks of daily consumption of jamu samples analysed in this study that would result in a MOE value of 10,000 as calculated using Haber's rule (Equations 2.4 and 2.5) and assuming the equal potency approach. From the results obtained it can be seen that the shortest time of daily combined exposure to make the risk of low priority was for J4 (the sample with the highest EDI; 203.1 µg/kg/bw/day) amounting to 27 weeks. This implies that use of this sample is considered of low concern when during a whole lifetime it would be consumed for less than 27 weeks. When considering consumption for 2 weeks every year during a lifetime, there are 5 out of 23 positive samples that would raise a concern and be a priority for risk management.

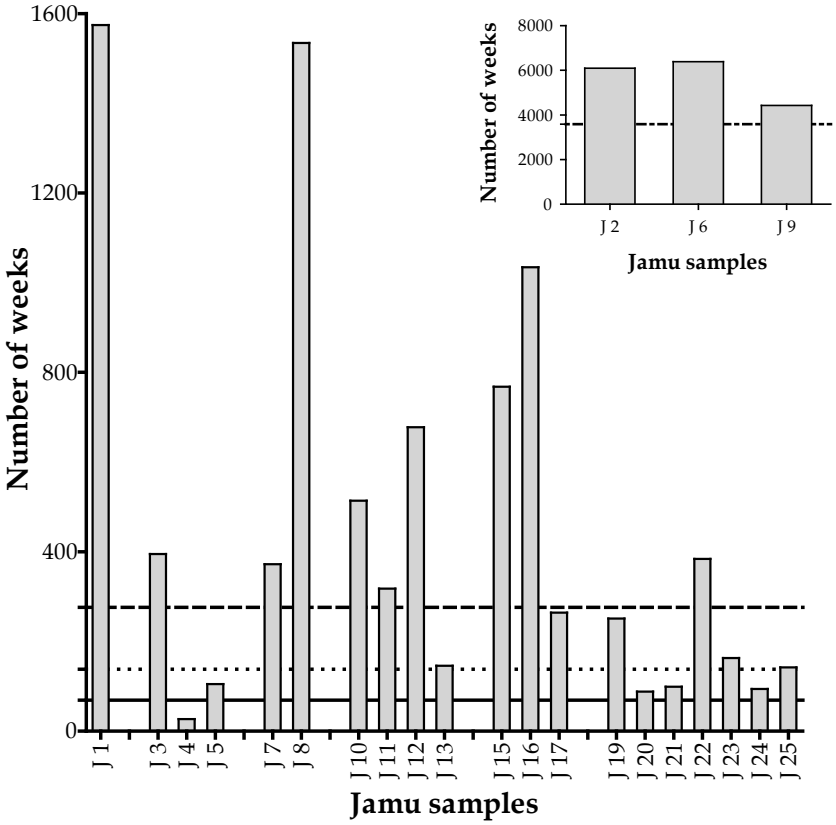

Figure 2.6. The number of weeks of use that would result in a MOE of 10,000 upon daily consumption of jamu samples analysed in the present study. The calculations assumed combined exposure and equal potency of the different alkenylbenzenes using the $BMDL_{10}$ of methyleugenol. The horizontal line (——), dotted line (.....), and dashed line (-----) represent one week, 2 weeks, and 4 weeks intake during lifetime. The inserted graph shows the number of weeks for J2, J6 and J9, dashed dotted line (-·-·-) represents the number of weeks when use these jamu for 3,588 weeks = 69 years so a whole lifetime.

2.4. Discussion

This study was conducted to obtain insight in possible consumer risks of jamu due to the natural occurance of alkenylbenzenes. This assessment is of interest considering the high number of jamu consumers, and the fact that alkenylbenzenes are genotoxic and carcinogenic compound that that can be naturally present in jamu. The Health Minister (MoH) of Indonesia in (2013) reported that about 49.0% out of 294,962 Indonesian households are actually consuming concoction that include jamu preparations for many reasons including; to maintain health and physical fitness (52.7%), more efficacious (18.4%), as a tradition and because the consumers belief that natural is always good for health (12.3%), low cost (6.8%), trial and error (2.8%), and as an option when medicinal treatment did not work (1.8%).[56]

Jamu preparations analysed in the present paper are generally consumed by mixing the powders with hot water. This may extract some of the active ingredients, while others remain bound to the matrix, which is however consumed as such with the beverage prepared. Thus, as a worst case assumption for risk assessment, it was assumed that all alkenylbenzenes present in a powder would become available in the gastrointestinal tract for uptake. For this reason, the powders were extracted with methanol in order to optimise the extraction. Methanol can break down the cell wall thereby improving release of the cellular substances,[14] moreover, the ultrasonification used in the extraction process provides an additional destructive effect on the cell wall and increases the permeability of the methanol to the cell and the extraction efficiency.[57] The extraction efficiency is also affected by the powder size, since previous studies with alkenylbenzene containing teas showed that extraction from course material is lower than from fine cut material.[14, 18]

The level and type of alkenybenzenes detected varies with the nature of the targeted plants in the samples. Fruit of fennel (*Foeniculum vulgare* Mill) is the main targeted plant, being present as an ingredients in most (80%) of the jamu samples at a variable amount or percentage. The remainder 20% (J7, J14, J15, J18 and J20) of the samples contained seed of nutmeg (*Myristica fragrans* Houtt). These two medicinal plants are known to naturally contain methyleugenol (4-allyl-1,2-dimethoxybenzene),[58] and this is in line with the observation that methyleugenol was detected in 91% (21/23) of the samples. The highest level of myristicin of 440 ±25 µg/g was detected in J4 which contains 3 myristicin-based plants, i.e: 25% w/w of fennel fruit, 14.71% w/w of cinnamon (*Cinnamomum burmannii* Blume) bark and 10% w/w of betel pepper (*Piper betle* L.) leaf. Safrole was detected as a minor alkenylbenzene in 4 samples, either as the only alkenylbenzene present (J1) or present in combination with other alkenylbenzenes including methyleugenol, myristicin and estragole. None of the sample appreared to contain dill or parsley as

an ingredient and this explains why apiol was not detected in the samples. The variability in the actual alkenylbenzene levels detected in the samples can also in part be due to the ecological factors at the different plant locations,[59] plant maturity at harvest, harvesting techniques, storage conditions, processing (e.g. drying), and method of measurement.[60, 61]

The MOE is a dimensionless ratio resulting from comparing the $BMDL_{10}$ with the EDI in humans.[62] This method has been used before to assess the risk of alkenylbenzene containing supplements,[17] fennel based tea,[18] parsley and dill based teas,[14] basil-based pesto sauces,[13] and nutmeg-based plant food supplements.[63] The same method was applied in the present study.

It is of interest to compare the three different approaches used for estimating the EDI and MOE values including: 1) considering the individual alkenylbenzenes using their $BMDL_{10}$ values, 2) combined exposure assuming equal potency of all alkenylbenzenes and using the $BMDL_{10}$ value of the major alkenylbenzene in the mixture, and 3) combined exposure using a TEQ approach.

Evaluating the risks associated with exposure to the individual alkenylbenzenes already indicated that several of the jamu preparations pose a concern with methyleugenol, the major alkenylbenzene present, giving rise to MOE values that range from 11 to 21,191. However, since several samples appeared to contain more than one alkenylbenzenes also a combined exposure and risk assessment was considered. Given the fact that alkenylbenzenes act by a similar mode of action and on a similar target organ,[64-67] it is at the current state of knowledge most logic to assume that combined exposure could best be modelled by dose addition, summing up the doses of the components considering equal potency. Thus, in the second approach the EDIs of the alkenylbenzenes were added up and combined exposure was used to calculate the MOE. For these MOE calculations the $BMDL_{10}$ of the major alkenylbenzene detected in the mixture, methyleugenol, was used. This approach may somewhat underestimate the risk when alkenylbenzenes like estragole, safrole and/or myristicin would be present in the mixture which are known to have higher relative potency than methyleugenol. Thus, in a third approach added exposure was calculated using a TEQ approach correcting for differences in relative potency between the different alkenylbenzenes using REP values and methyleugenol as the reference compound.

Definition of the interim REP values in Table 2.3 thus includes some surrogate endpoints for tumor formation. Such an approach to define interim REP values based on surrogate endpoints was used previously for pyrrolizidine alkaloids (PA)[68] calculating the average of data on the genotoxic potency in *Drosophila melanogaster*, the cytotoxic potency in vitro in chicken hepatocellular carcinoma

(CLR-2118) cells and their acute toxicity in adult rodents. In the present study interim REP values were calculated using methyleugenol as the reference compound (REP value = 1.00) since this appeared to be the major alkenylbenzene detected in 91.3% of the positives samples. Given that the interim REP values for other alkenylbenzenes detected in the jamu samples (estragole, safrole and myristicin) were higher than 1.00, the MOE values obtained when calculating the EDI values using the TEQ approach were lower than those obtained assuming equal potency albeit not to a large extent.

In addition to the three approaches for combined exposure and risk assessment also three approaches for the duration of exposure were evaluated. These include i) lifetime exposure as used for the three methods for (combined) exposure discussed above, but also two additional scenario's including different exposure durations to better reflect real life exposure and including ii) exposure for only two weeks and iii) exposure for two weeks on a regular (yearly) basis during a lifetime. The latter scenario better reflects the possible use of jamu preparations for short periods during periods of illness. For these approaches only combined exposure based on the sum of the total alkenylbenzenes was considered. This was possible because, as outlined above the different scenario's for calculating combined exposure did not vary substantially. To assess the risk for short term exposure Haber's rule was used (Equation 2.5).

It is important to note that in real life, Indonesian people do not consume jamu for their whole lifetime, but that the jamu preparations are generally consumed only during periods of illness. Using Haber's rule, the MOE values were corrected to take into account this shorter exposure. Applying this linear correction method results in MOE values for for example two weeks consumption that would be 1,794 times (69 years × 52 weeks/year/2 weeks) higher than MOE values based on daily consumption of jamu over a whole lifetime (69 years)[53], indicating far lower priority of risk management. However, given that in real life situations jamu is used for medical puposes Indonesian people tend to use the preparations for short intervals but on a regular basis for example two weeks once every year during a lifetime. Using this asumption, the MOE values for 5 out of 23 (21.7%) of positives samples analysed would indicate a priority for risk management. Gaylor (2000)[52] reported that Haber's rule can be applied for extrapolation from lifetime cancer incidences to different exposure periods when dose rate is not the determining factor and only total dose dictates the biological effect. The application of the rule is based on the assumption that there is a linear relationship between tumor incidence and low doses of genotoxic carcinogens or carcinogens that enhance background

carcinogenic processes.[69] Applying this rule to an MOE based risk assessment implies that shorter exposure will result in lower EDI and higher MOE values.[51]

It is important to note that at the present state of the art evidence supporting such linear behaviour of the adverse effects of alkenlbenzenes is limited to studies on bioactivation to the ultimate carcinogenic 1'-sulfoxymetabolites and linearity in DNA adducts formation from dose levels causing significant tumor formation down to realistic dietary exposure levels.[43, 46, 54, 70] To what extent this also holds for the tumor formation remains to be demonstrated. Nevertheless, these observations provide some support for the application of Haber's rule for the current risk assessment. It is however acknowledged that at the present state-of-the-art there is no formally adopted method to take short term exposure into account in an MOE based risk assessment. Nevertheless, applying Haber's rule provides a reasonable first approach to evaluate the risks connected to shorter than lifetime exposure.

Previously DNA adduct formation induced by dietary intake of methyleugenol was detected in human liver samples.[27] Given the use of jamu contaning alkenylbenzenes it would be of interest to study whether such DNA adducts can also be detected in Indonesian human liver samples although that remains a topic of interest for future research. In addition, further studies on repair of DNA adduct resulting from alkenylbenzene exposure might help to clarify the risks of consumption alkenylbenzene-containing jamu.

It is concluded that the consumption of alkenylbenzene containing jamu can be of concern especially when consumed on a daily basis for longer periods of time on a regular basis.

Conflict of Interest

The authors state no conflict of interest regarding this manuscript.

Acknowledgements

This research was supported by a grant from Beasiswa Pendidikan Indonesia/ Lembaga Pengelola Dana Pendidikan (the Indonesian Endowment Fund for Education, Ministry of Finance of Republic of Indonesia) [contract number: PRJ-365/LPDP/2016] for Suparmi.

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BENEFIT
RISK

Chapter 3

Levels of methyleugenol and eugenol in instant herbal beverages available on the Indonesian market and related risk assessment

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Published in: Food and Chemical Toxicology, 125 (2019):467-478
DOI: 10.1016/j.fct.2019.02.001

Abstract

The presence and accompanying risks of methyleugenol and eugenol in herbal beverages available on the Indonesian market were evaluated. Methyleugenol was detected in 49 out of 114 samples, at levels amounting to 2.6-443.7 µg/g, while 4 samples contained eugenol at 21.4-101.2 µg/g. The EDI resulting from drinking these preparations amounted to 0.1-51.2 µg/kg bw/day and 1.1-3.3 µg/kg bw/day, respectively for samples targeted at adults and children. A $BMDL_{10}$ value of 22.2 mg/kg bw/day for methyleugenol was defined using literature data and model averaging. MOE values were below 10,000 for 46 samples (40.4%), indicating a priority for risk management when assuming daily lifelong consumption, while the EDI for 4 samples containing eugenol did not exceed the ADI of 2.5 mg/kg bw thus did not raise a concern for human health. Using Haber's rule to correct for less than lifetime exposure, consumption of methyleugenol via these beverages would be of low concern when consumed for less than 2 weeks/year during a lifetime. This conclusion holds for herbal beverages collected by targeted sampling, not for all herbal beverages on the Indonesian market. The study provides data that can support establishment of a maximum permitted level (MPL) for methyleugenol in herbal beverages in Indonesia.

3.1. Introduction

Herbal beverages can be enriched in herbs such as ginger (*Zingiber officinale* Rosc), cloves (*Syzygium aromaticum* L.), cinnamon (*Cinnamomum burmannii* Blume), fennel (*Foeniculum vulgare* Mill.), betel pepper (*Piper betle* L.), star anise (*Illicium verum*), nutmeg (*Myristica fragnan*) or other herbs. Generally, such enrichment of herbal beverages with selected or mixed herbs is aiming at possible beneficial health effects.[1] Ginger containing beverages including preparations like *wedang jahe*, *bandrek*, *ronde*, *bajigur*, and *sekoteng*, are among the most popular herbal beverages consumed in Indonesia, because ginger has a long history as a thermogenic and antiemetic drink.[2] Traditionally, Indonesian people need to prepare the herbal beverages by using mixed fresh herbs, but nowadays more people tend to choose instant herbal beverages which are commercially available. These products are usually in powder form, packaged in sealed and labelled plastic sachet, and readily consumed upon adding hot water and then drinking the resulting preparation.

Nowadays, consuming instant herbal beverages in Indonesia shows an increasing trend due to the easy online access and the fact that websites provide information about all kind of possible beneficial health effects of these products including: maintaining and increasing health, prevention of diseases, counteracting cold, increasing vitality after work, and slimming and cosmetic effects especially for women.[3] In addition, the majority of people consider these herbal beverages to be safe, even during pregnancy, with less side effects than conventional medicines.[4] The limitation of knowledge related to possible adverse health effects of herbal beverages may put consumers at risk that may arise from (over)consumption of herbal beverages, and therefore the quality and safety of these products should be assessed and monitored.[5]

The safety regulation of herbal products varies internationally among jurisdictions.[6] Herbal products are classified by the US Food and Drug Administration (FDA) as dietary supplements or foods and are marketed pursuant to the Dietary Supplement Health and Education Act (DSHEA) of 1994. This implies that a dietary supplement cannot carry any health claim or medical advice on the label.[7] Similarly, in the European Union botanical preparations are considered food and these preparations should comply with broader requirements as defined for safe food while any health claim used needs scientific evaluation and approval by the European Food Safety Authority (EFSA).[8] Australia formed the Advisory Committee on Complementary Medicines (ACCM) in 2010 to address regulatory issues regarding the safety, efficacy and manufacturing quality of herbal remedies.[9] In Indonesia, botanical preparations can be registered as food[10] but also as traditional medicine.[11]

According to the Government Regulation 28/2004 on Food Safety, Quality and Nutrition, in Indonesia the food producer will receive a register approval number and get the marketing authorization number after passing the standard of safety and quality.[12] The product registration type is based on two parameters including: (1) the safety regulatory body in Indonesia where the product is registered, being either BPOM RI (*Badan Pengawas Obat dan Makanan Republik Indonesia*/National Agency for Drug and Food Control Republic of Indonesia, NADFC RI,) or Depkes RI (*Departemen Kesehatan Republik Indonesia*/Ministry of Health of Republic Indonesia, MoH RI) and (2) the category of the product being: MD (*Pangan Olahan Dalam Negeri*/Domestic Processed Food), ML (*Pangan Olahan Luar Negeri*/Foreign Processed Food), SD (*Suplemen Dalam Negeri*/Domestic Supplement), SL (*Suplemen Luar Negeri*/Foreign Supplement), TR (*Obat Tradisional Produksi Dalam Negeri*/ Indonesian Traditional Medicine), TI (*Obat Tradisional Impor*/Imported Traditional Medicine), TL (*Obat Tradisional Lisensi*/Licensed Traditional Medicine), and P-IRT (*Pangan Industri Rumah Tangga*/Food Household Industry). So far, all products with the label MD, ML, SD, SL, TR, TI and TL were registered by BPOM RI, therefore they are labelled BPOM RI MD, BPOM RI ML, BPOM RI SD, BPOM RI SL and BPOM RI TR, BPOM TI, BPOM TL respectively, while the household food product were labelled by Depkes RI P-IRT.[10]

The awareness and knowledge of herbal beverage producers in Indonesia related to the food safety and registration procedure is still limited.[13, 14] Therefore, the risks of consumption of the herbal beverages produced may not be adequately evaluated and/or regulated or guaranteed. The Indonesia Risk Assessment Center (INARAC), a body under the NADFC RI, after its initiation in November 2014 so far presented a Microbiology Risk Assessment (MRA) of chicken Salmonella and a risk assessment on Aflatoxin B1 (AFB1) levels in peanuts and their processed products in 2016,[3] while the safety and risks of frequent and prolonged consumption of herbal beverages have not yet been assessed.

Methyleugenol is a genotoxic and carcinogenic herbal ingredient that can be detected in basil (*Ocimum basilicum* L.) leaf,[15] star anise (*Illicium anisatum* L.) fruit, bay Laurel (*Laurus nobilis* L.) leaf, and ginger (*Zingiber officinale* Rosc) rhizome.[16] Recently, Suparmi et al. (2018)[17] reported that methyleugenol was the major alkenylbenzene detected in most (91.3%) of the samples testing positive for the presence of alkenylbenzenes in Indonesian jamu. The consumer risk based on the Margin of Exposure (MOE) approach showed that the consumption of jamu for two weeks once every year during a whole lifetime of Indonesian people presents a priority for risk management for 5 out of 23 (21.7%) of the positively tested samples. Herrmann et al. (2013)[18] reported that the exposure to methyleugenol leads to

substantial levels of hepatic DNA adducts in the liver of human subjects. Twenty-nine out of 30 human liver samples were reported to contain the N^2-(*trans*-methylisoeugenol-3′-yl)-2′-deoxyguanosine adduct. And based on experimental animal studies the induction of liver carcinogenesis upon exposure to high dose levels of methyleugenol is well established.[19]

Eugenol is another alkenylbenzene present in the herbs mentioned above and in the essential oils of botanicals frequently used in the herbal beverages including clove (*Syzygium aromaticum* L.), cinnamon (*Cinnamomum burmannii* Blume), and fennel (*Foeniculum vulgare* Mill.).[20, 21] In studies with eugenol in rats no carcinogenicity was observed while in a 2-year bioassay in mice the incidences of hepatocellular neoplasms were considered not significant and not dose-related.[22-25] Also, eugenol was considered not genotoxic at concentrations that did not result in cytotoxicity.[26-28] This lack of genotoxicity and carcinogencity of eugenol resulted in establishment of an acceptable daily intake (ADI) of 0-2.5 mg/kg bw/day by JECFA (1982)[26] and of 1.0 mg/kg bw/day by EFSA (2012),[28] while also a risk assessment by the Research Institute for Fragrance Materials (RIFM) Expert Panel concluded that eugenol does not present a concern for genetic toxicity in human health.[20] In line with this eugenol is listed as a permitted flavouring agent in the USA, EU, Australia and Indonesia.[29-32]

Based on these results it was anticipated that methyleugenol and eugenol may also be present and pose a risk in Indonesian instant herbal beverages. Therefore, the objective of the present study was to analyse methyleugenol and eugenol in a large number (114 samples) of instant herbal beverages containing various mixed herbs collected on the Indonesian market by a targeted sampling strategy, and to perform an associated human risk assessment using the MOE approach for methyleugenol and the ADI for eugenol. Also, an overview was made of their current product registration type indicated on the label. The results of this study may give information relevant for risk management aiming at prioritizing regulatory actions to reduce potential risks connected to instant herbal beverage consumption in Indonesia.

3.2. Materials and Methods

3.2.1. Herbal beverage samples

A targeted sampling approach was applied, collecting herbal samples with the name of possible methyleugenol containing herbs on the label. These herbs included ginger (*Zingiber officinale* Rosc), cloves (*Syzygium aromaticum* L.), cinnamon (*Cinnamomum burmannii* Blume), fennel (*Foeniculum vulgare* Mill.), betel pepper (*Piper betle* L.), star anise (*Illicium verum*), nutmeg (*Myristica fragnan*), lemongrass

(*Cymbopogon nardus* L. Rendle), carrot (*Daucus carota* subsp. sativus), and galangal (*Kaempferia galanga*), all known to contain methyleugenol.[23] One-hundred-fourteen samples of herbal instant beverages from different brands were purchased from traditional markets or supermarkets as depicted in Figure 3.1, including sampling in Medan (1 store, n = 2), Semarang (11 stores, n = 54), Bawen (1 store, n = 9), Magelang (1 store, n = 6), Purworejo (1 store, n = 1), Yogyakarta (2 stores, n = 9), Surakarta (2 stores, n = 7), Nganjuk (4 stores, n = 19), Kediri (1 store, n = 4), and Mojokerto (1 store, n = 3).

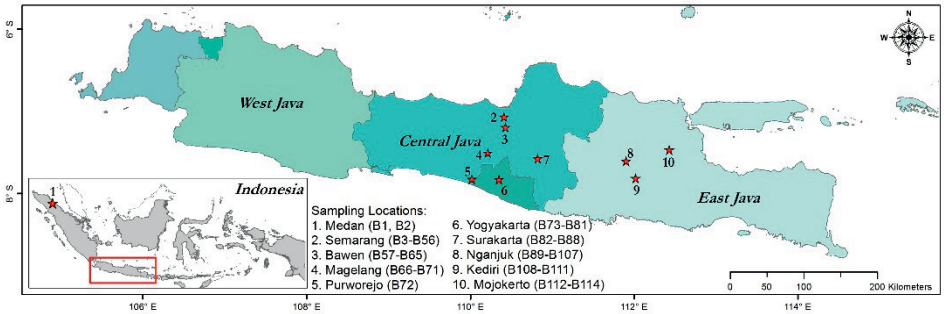

Figure 3.1. Sampling locations of the Indonesian instant herbal beverages used in this study. B1-B114 represent the sample IDs used in the present study.

Detailed information, including information on the respective herbs of concern present in the samples, the instructions for preparation and consumption of the beverages, beneficial effects claimed, and product registration according to the Indonesian system for labelling of these herbal samples outlined in the introduction, all as indicated on the label of each sample, are summarised in Supplementary materials (Table 1). All samples, denoted B1-B114 in the present study, were in powder form and packaged in sachets. Among the 114 samples 98 samples were targeted to be consumed by adults, and 16 samples were dedicated to use by children.

3.2.2. Chemicals and Reagents

Methyleugenol (purity 99%), estragole (purity 98%), safrole (purity 97%) and eugenol (purity 99%) were obtained from Sigma-Aldrich (Zwijdrecht, The Netherlands). Acetonitrile ULC/MS gradient, trifluoracetic acid (TFA) and dimethyl sulfoxide (DMSO) were purchased from Merck (Felsberg, Germany). Methanol ULC/MS gradient was purchased from Sigma-Aldrich, and nanopure water was obtained from an Arium pro UF/VF water purification system (Sartorius Weighing Technology GmbH, Gottingen, Germany).

3.2.3. Analysis of methyleugenol and eugenol levels

3.2.3.1. Methanol extract

The level of alkenylbenzenes present in the samples was determined by methanol extraction followed by UPLC analysis performed as described by Gursale et al. (2010)[33] with minor modifications. The homogeneity of each sample was ensured by mixing manually 100 g from 6-20 sachets of sample in a ziplock plastic packet before taking samples for extraction. For the extraction methanol (10 ml) was poured over 1 g of homogenized sample in a brown glass extraction bottle, and the sample was mixed and sonicated for 15 min at room temperature. Upon sonication, the sample solution was filtered using a 0.45 µm syringe filter (VWR international). The filtrate was placed into a 1 ml Ultra Performance Chromatography (UPLC) vial and subsequently injected into the chromatographic system. Every sample was prepared and analysed in triplicate.

The extraction efficiency was evaluated based on the Guidance for Industry Bioanalytical Method Validation[34] by spiking and mixing 1 g of sample B89, B114, with a methyleugenol standard dissolved in DMSO at a concentration that would give a final concentration of 10 µM upon addition of 10 ml methanol in the extraction procedure performed as described above. The average percentage of recovery was used to correct the levels of methyleugenol detected for the different instant herbal beverages samples. To confirm linearity of the method, samples were analysed in five different ratios of weight per volume of methanol in the range from 2.5 to 40 % (w/v). In a separate recovery experiment the samples B89, B114 were also spiked with a mixture of eugenol and methyleugenol to determine recoveries. Eugenol was included in the studies because some samples appeared to contain eugenol (see Results section). Since safrole and estragole were not detected in the respective samples their recovery was not further quantified. The limits of detection (LOD) amounted to 1.2, 3.0 and 5.5 µM for methyleugenol, safrole and estragole, respectively.

3.2.3.2. UPLC analysis

The methanol extracts were analysed in undiluted form. To this end 3.5 µl of each sample was subjected to UPLC analysis performed as described before.[35] The UPLC used was a UPLC-DAD system consisting of a Waters (Milford, MA) Acquity binary solvent manager, sample manager, and photodiode array detector, equipped with a Waters Acquity UPLC BEH RP 18 column (1.7 µm, 2.1 × 50 mm). The column was kept at 22°C, while the sample temperature was set at 10°C. UPLC analysis was performed using a mobile phase A of nanopure water containing 0.1% (v/v) TFA and mobile phase B consisting of acetonitrile and a gradient program with a flow

rate of 0.6 ml/min. The mobile phase composition started with 30.5% B, which was maintained for 15 min, followed by an increase to 80% B over 1 min and holding this for 0.5 min, followed by a decrease to 0% B in 1.5 min and keeping it at 0% for 1 min after which the eluents was returned to the initial conditions of 30.5% B for the next run in 20.5 min. Under the specified chromatographic conditions, the retention times for eugenol, methyleugenol, safrole and estragole were 2.2, 4.3, 8.1 and 9.0 min, respectively. Detection of safrole and estragole was done at 202 nm and used 225 nm respectively. For detection and quantitative analysis of methyleugenol, and eugenol, the wavelength was 202 nm. The peak intensity at this wavelength was compared to calibration curves of the compounds prepared using commercially available standards.

3.2.4. Exposure assessment resulting from the drinking of instants herbal beverages based on methyleugenol and eugenol levels detected

In order to assess the potential exposure to methyleugenol or eugenol resulting from drinking the herbal beverages, the estimated daily intake (EDI) was calculated. The EDI values were expressed in µg/kg bw/day using a body weight (bw) of 54 kg, the average bw for Indonesian male and female.[36] For the products targeted at children as their consumers, the EDI was calculated using a bw of 27.21 kg, the average bw of Indonesian boys and girls under the age of 1-17 years old.[36]

The EDI calculation was done using the levels detected in the various samples and the recommended daily consumption of these samples as presented on the product labels (Table 3.1). The weight of the recommended daily consumption (g) was based on the preparation method indicated on the label (see Table 1 at Supplementary materials), assuming that 1 teaspoon equals 6 g, and 1 tablespoon equals 10 g, representing the average of weighting the respective samples using a full tea spoon and tablespoon from Indonesia (3 replications). When there was no information on the label (unknown) the weight and frequency of consumption were assumed to be equal to 3 full teaspoons once a day. In line with the habits for use of the herbal beverages, it was assumed that consumption of the herbal beverage implies consumption of the whole preparation mixed with water. EDI values were calculated following Equation 3.1:

$$EDI\left(\frac{\mu g/kg\ bw/day}{day}\right) = \frac{recommended\ daily\ consumption\ (g/day) \times level\ of\ detected\ methyleugenol\ (\mu g/g)}{bw\ (kg)} \quad \text{(Equation 3.1)}$$

3.2.5. Benchmark Dose (BMD) modelling for methyleugenol

To define the lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence ($BMDL_{10}$) the quantal dose-response data for induction of hepatocellular carcinoma in male and female F344/N rat induced by methyleugenol and reported by the NTP (2000)[19] (see Supplementary materials at Table 2) were used for BMD modelling. In the 2-years study, 50 rats per group for both sexes, were administered methyleugenol orally in 0.5% methylcellulose at doses of 0, 37, 75, or 150 mg/kg, 5 days per week for 105 weeks. For the modelling, these experimental dose levels were converted to the time-adjusted dose levels (mg/kg bw/day) by multiplying the actual dose by 5/7 (to correct for the 5 instead of 7 days per week dosing regimen) in line with what was reported before by van den Berg et al. (2011)[35] and Benford et al. (2010)[37].

Previously $BMDL_{10}$ values for methyleugenol were obtained using EPA BMDS software version 2.6.0.1 using different models, including the Gamma, Logistic, Log-logistic, Probit, Log-probit, Multistage, Weibull and Quantal linear model.[35] This resulted in values between 15.3 - 34.0 mg/kg bw/day resulting from male and female rat data. In the present study the data were analyzed using model averaging, as recommended by the EFSA Scientific Committee (2017)[38], to derive the final $BMDL_{10}$ from multiple fitted benchmark dose models. To this end the online EFSA's web-tool for BMD analysis (https://shiny-efsa.openanalytics.eu/app/bmd), which implements statistical methods for R-package PROAST, version 65.7 Proast[39] was used. The $BMDL_{10}$ model averaging was performed using the default settings.

3.2.6. Safety assessment using the Margin of Exposure (MOE) approach for methyleugenol

The MOE approach was applied to assess the risk posed by the use of the methyleugenol containing herbal beverages, in line with the recommendations of EFSA for risk assessment of compounds that are both genotoxic and carcinogenic.[40] The final $BMDL_{10}$ resulting from model averaging using male rat data (providing a lower value than obtained from the female data, see Results section) and EDI values were used to calculate the MOE values according to Equation 3.2. MOE values were rounded to a single significant figure.

$$MOE = \frac{BMDL_{10}}{EDI} \qquad \text{(Equation 3.2)}$$

The MOE values are based on chronic lifetime exposure, although realistic use of the herbal drinks may be for shorter periods of time. Although there is no officially established method to evaluate shorter than lifetime exposure to a genotoxic carcinogen, it has been suggested to use Haber's rule to estimate the effects for

different exposure duration.[41, 42] Haber's rule states that the dose times the effect is constant, (C1×T1= constant = C2×T2) which implies that one could correct for shorter time of exposure in a linear way. Using this approach, the MOE values were also calculated for regular short term exposure, i.e. 2 weeks, once every year during a lifetime. This exposure scenario was chosen to mimic the situation where people consume a herbal beverage as a supplement to counteract cold or during periods of illness. In addition, Haber's rule was also used to calculate the number of weeks of daily consumption of the different herbal beverage samples that would result in an MOE value of 10,000, the threshold for risk management concern.[40]

3.2.7. Safety assessment of eugenol containing herbal beverages

The risk of consuming herbal beverages which contained eugenol was assessed using the Acceptable Daily Intake (ADI) of 0 - 2.5 mg/kg bw established by JECFA (2006)[27] and the ADI of 1 mg/kg bw/day established by EFSA (2012)[28], in line with the recommendations of EFSA for risk assessment of compounds that are non-genotoxic[43].

3.2.8. Evaluation of the product registration established in Indonesia based on the safety assessment results and product registration type

To evaluate the product registration as applied in Indonesia during the product registration, this product registration was reviewed based on the results of the safety assessment in the present study. The product registration on the label of 114 samples indicated BPOM RI MD (domestic processed food, 31 samples), BPOM RI ML (foreign processed food, 1 sample), BPOM RI SD (domestic supplement, 9 samples), BPOM RI TR traditional medicine (traditional medicine, 30 samples) and Depkes RI P-IRT samples sold as regular home industry food product (food household industry, 43 samples).

3.3. Results

3.3.1. Methyleugenol and eugenol levels in Indonesian instant herbal beverages

Table 3.1 presents the level of the alkenylbenzenes, methyleugenol and eugenol, as detected and quantified in the herbal beverage samples. Estragole and safrole were not detected. Methyleugenol was detected in 49 out of 114 samples, at levels ranging from 2.6 to 443.7 µg/g. Sample B91, sold as a household industry food product (labelled as P-IRT), appears to contain the highest methyleugenol level at 443.7 µg/g. In 65 of the 114 samples 65 levels of all three alkenylbenzenes were below their respective limit of detection (LOD) of 1.2, 3.0 and 5.5 µM for methyleugenol, safrole and estragole, respectively. Interestingly, 4 out of 114

samples, B5, B19, B83 and B109 appeared to contain eugenol at a level of 21.4 - 101.2 µg/g.

Table 3.1. The level of methyleugenol and eugenol detected in the instant herbal beverages and the resulting estimated daily intake (EDI) calculated using the recommendations for daily consumption on the label and assuming the level to equal the LOD of 1.2 mM (1.9 µg/g) for samples where methyleugenol was below the LOD to provide an upper bound EDI.

Sample ID	Detected compound	Level (µg/g)[a]	Recommended daily consumption (g) of the sample	Specified consumer	EDI (µg/kg bw/day)
B1	Methyleugenol	14.0±1.6	20	Adult	5.2
B2	Methyleugenol	94.2±1.4	18	Adult	31.4
B3	nd[b]		11	Adult	0.40 (upper bound)[c]
B4	nd		14	Adult	0.51 (upper bound)[c]
B5	Eugenol	101.2±20.6	25	Adult	46.9
B6	nd		8	Adult	0.3 (upper bound)[c]
B7	nd		8	Adult	0.3 (upper bound)[c]
B8	nd		8	Adult	0.3 (upper bound)[c]
B9	nd		25	Adult	0.9 (upper bound)[c]
B10	nd		11	Adult	0.4 (upper bound)[c]
B11	nd		5	Adult	0.2 (upper bound)[c]
B12	nd		6.3	Adult	0.2 (upper bound)[c]
B13	nd		21	Adult	0.8 (upper bound)[c]
B14	nd		4	Adult	0.1 (upper bound)[c]
B15	nd		7	Adult	0.3 (upper bound)[c]
B16	nd		10	Adult	0.4(upper bound)[c]
B17	nd		13.5	Adult	0.5 (upper bound)[c]
B18	nd		12	Adult	0.4 (upper bound)[c]
B19	Eugenol	21.4±3.3	12.6	Adult	5.0
B20	nd		12.6	Adult	0.5 (upper bound)[c]
B21	nd		30	Adult	1.1 (upper bound)[c]
B22	nd		25	Adult	0.9 (upper bound)[c]
B23	nd		8	Adult	0.3 (upper bound)[c]
B24	Methyleugenol	18.2±4.8	60	Adult	20.3
B25	Methyleugenol	34.3±1.6	20	Adult	12.7
B26	nd		15	Children	1.1 (upper bound)[c]
B27	nd		15	Children	1.1 (upper bound)[c]
B28	nd		21	Children	1.5 (upper bound)[c]
B29	nd		21	Children	1.5 (upper bound)[c]
B30	nd		21	Children	1.5 (upper bound)[c]
B31	nd		21	Children	1.5 (upper bound)[c]
B32	Methyleugenol	110.6±0.5	25	Adult	51.2
B33	nd		15	Children	1.1 (upper bound)[c]
B34	nd		15	Children	1.1 (upper bound)[c]
B35	nd		15	Children	1.1 (upper bound)[c]
B36	nd		15	Children	1.1 (upper bound)[c]
B37	nd		15	Children	1.1 (upper bound)[c]
B38	nd		15	Children	1.1 (upper bound)[c]
B39	nd		15	Children	1.1(upper bound)[c]
B40	nd		21	Adult	0.8 (upper bound)[c]
B41	nd		8	Adult	0.3 (upper bound)[c]
B42	Methyleugenol	65.06±48.05	23	Adult	27.7
B43	nd		28	Adult	1.0 (upper bound)[c]
B44	nd		25	Adult	0.9 (upper bound)[c]

Sample ID	Detected compound	Level (μg/g)[a]	Recommended daily consumption (g) of the sample	Specified consumer	EDI (μg/kg bw/day)
B45	nd		12.6	Adult	0.5 (upper bound)[c]
B46	nd		25	Adult	0.9 (upper bound)[c]
B47	nd		25	Adult	0.9 (upper bound)[c]
B48	Methyleugenol	16.1±2.5	25	Adult	7.5
B49	Methyleugenol	17.5±6.8	26	Adult	8.4
B50	nd		21	Children	1.5 (upper bound)[c]
B51	nd		25	Adult	0.9 (upper bound)[c]
B52	nd		8	Adult	0.3 (upper bound)[c]
B53	nd		8	Adult	0.3 (upper bound)[c]
B54	nd		8	Adult	0.3 (upper bound)[c]
B55	Methyleugenol	9.6±0.2	22.5	Adult	4.0
B56	Methyleugenol	31.2±0.3	30	Adult	17.3
B57	Methyleugenol	7.1±0.4	25	Adult	3.3
B58	nd		25	Adult	0.9 (upper bound)[c]
B59	Methyleugenol	4.2±0.3	25	Adult	1.9
B60	nd		21	Children	1.5 (upper bound)[c]
B61	nd		21	Adult	0.8 (upper bound)[c]
B62	Methyleugenol	4.2±1.7	21	Children	3.3
B63	nd		30	Adult	1.1 (upper bound)[c]
B64	Methyleugenol	11.9±2.0	25	Adult	5.5
B65	nd		27	Adult	1.0 (upper bound)[c]
B66	nd		25	Adult	0.9 (upper bound)[c]
B67	Methyleugenol	35.9±15.5	15	Adult	10.0
B68	Methyleugenol	28.4±17.5	15	Adult	7.9
B69	nd		15	Adult	0.5 (upper bound)[c]
B70	nd		15	Adult	0.5 (upper bound)[c]
B71	nd		15	Adult	0.5 (upper bound)[c]
B72	nd		10	Adult	0.4 (upper bound)[c]
B73	Methyleugenol	13.0±0.1	25	Adult	6.0
B74	Methyleugenol	16.1±1.3	10	Adult	3.0
B75	Methyleugenol	51.6±1.5	10	Adult	9.6
B76	Methyleugenol	19.8±1.6	25	Adult	9.2
B77	Methyleugenol	12.7±2.2	30	Adult	7.0
B78	Methyleugenol	11.4±9.1	20	Adult	4.2
B79	Methyleugenol	14.2±2.3	20	Adult	5.3
B80	Methyleugenol	11.4±1.3	18	Adult	3.8
B81	nd		18	Adult	0.7 (upper bound)[c]
B82	Methyleugenol	2.7±1.2	23	Adult	1.1
B83	Eugenol	37.8±6.9	25	Adult	17.5
B84	Methyleugenol	14.2±0.8	10	Adult	2.6
B85	Methyleugenol	42.6±6.6	33	Adult	26.1
B86	Methyleugenol	66.9±2.9	25	Adult	31.0
B87	Methyleugenol	24.0±3.7	40	Adult	17.8
B88	Methyleugenol	28.7±1.7	22	Adult	11.7
B89	nd		28	Adult	1.0 (upper bound)[c]
B90	nd		75	Adult	2.7 (upper bound)[c]
B91	Methyleugenol	443.7±31.5	6	Adult	49.3
B92	Methyleugenol	10.1±0.4	30	Adult	5.6
B93	Methyleugenol	9.3±0.4	15	Adult	2.6
B94	Methyleugenol	28.9±5.7	15	Adult	8.0
B95	Methyleugenol	36.9±1.2	15	Adult	10.2
B96	Methyleugenol	23.2±1.0	22	Adult	9.5
B97	Methyleugenol	36.3±3.0	23	Adult	15.5

Sample ID	Detected compound	Level (µg/g)[a]	Recommended daily consumption (g) of the sample	Specified consumer	EDI (µg/kg bw/day)
B98	Methyleugenol	24.1±1.3	20	Adult	8.9
B99	Methyleugenol	21.2±1.0	27.5	Adult	10.8
B100	Methyleugenol	2.6±0.3	25	Adult	1.2
B101	Methyleugenol	2.6±1.1	22.5	Adult	1.
B102	Methyleugenol	50.8±3.3	25	Adult	23.5
B103	Methyleugenol	85.0±6.5	20	Adult	31.5
B104	Methyleugenol	109.3±9.5	25	Adult	50.6
B105	Methyleugenol	33.1±0.3	25	Adult	15.3
B106	nd		25	Adult	0.9 (upper bound)[c]
B107	nd		24	Adult	0.9 (upper bound)[c]
B108	Methyleugenol	15.8±4.3	20	Adult	5.9 (upper bound)[c]
B109	Eugenol	26.2±5.7	30	Adult	14.6
B110	Methyleugenol	17.0±1.4	25	Adult	7.9
B111	Methyleugenol	31.5±1.5	10	Adult	5.8
B112	Methyleugenol	58.4±1.1	20	Adult	21.6
B113	nd		20	Adult	0.7 (upper bound)[c]
B114	nd		25	Adult	0.9 (upper bound)[c]

[a]Mean of 3 independent analyses. Levels were corrected for the recovery result (see materials and methods)
[b]Not detected
[c]The EDI values of samples in which levels were below the LOD, were calculated using the LOD of methyleugenol of 1.2 µM corresponding to 1.9 µg/g sample thus representing an upper bound.

3.3.2. EDIs of methyleugenol and eugenol resulting from daily consumption of the herbal beverages

Using the quantified levels of methyleugenol and eugenol in the various samples and the direction for their use as indicated on the labels (Supplementary materials Table 1), EDI values were calculated using Equation 3.1. EDI values thus obtained range from 1.1 to 51.2 µg methyleugenol/kg bw/day for the 49 positive samples containing methyleugenol (see Table 3.1). Sample B32, sold as a traditional medicine (labelled BPOM RI TR), appears to result in the highest EDI, followed by B104 (labelled BPOM RI MD) and B91 (labelled Depkes RI P-IRT) with EDI values amounting to respectively 50.6 and 49.3 µg/kg bw/day. For samples in which levels of methyleugenol were below the LOD, EDI values were calculated by a so-called upper bound approach, assuming levels to be at the LOD of 1.9 µg/g sample. The EDI values thus obtained for these 65 samples in which methyleugenol was below the LOD ranged from 0.1 to 2.7 µg/kg bw/day. This approach to use the LOD to substitute the results below the LOD to define an upper bound for the exposure was recommended by the WHO GEMS/Food EURO workshop.[44] Fifteen out of the 16 samples (94%) specified to be consumed by children appeared to contain no methyleugenol resulting, using the same upper bound approach, in upper bound EDI values of 1.1 to 1.5 µg/kg bw/day. The EDI of eugenol calculated for the 4 eugenol containing samples (out of 114) ranged from 5.0 to 46.9 µg/kg bw/day.

3.3.3. $BMDL_{10}$ value for methyleugenol obtained by model averaging

Given that $BMDL_{10}$ values for methyleugenol available in the literature were derived from single model fits in some cases selecting the lowest value, and that at the present state-of-the-art model averaging is considered the preferred method, the $BMDL_{10}$ value for methyleugenol was recalculated using model averaging. Tumor data analyzed were the data from the NTP study reporting dose-dependent incidences of hepatocellular carcinoma in rats exposed to methyleugenol via gavage for 2 years[19] (Supplementary Table 2). The time-adjusted dose levels (mg/kg bw/day) calculated by multiplying the actual dose by 5/7 were used to correct for the 5 instead of 7 days per week dosing regimen.[35, 37] The final $BMDL_{10}$ values resulting from model averaging were 22.2 mg/kg bw/day for male rats (Table 3.2) and 66.5 mg/kg bw/day for female rats (Table 3 of Supplementary materials). Figures representing the model fits are also presented in the Supplementary materials (Figure 1 and 2).

Table 3.2. Results from the BMD model averaging to derive a $BMDL_{10}$ for methyleugenol using online EFSA statistical models and the data from the NTP study on hepatocellular carcinoma incidences in methyleugenol-treated male rats exposed via gavage for 2 years[19] (Supplementary Table 2). Figure 3.1 (Supplementary materials) presents the corresponding figures.

	Fitted Models								Weights for Model Averaging
Model	Number of parameter	Log-likelihood	AIC	Accepted	BMDL	BMDU	BMD	conv	
Null	1	-105.38	212.8		NA	NA	NA	NA	
Full	4	-84.05	176.1		NA	NA	NA	NA	
Two stage	3	-85.06	176.1	Yes	19.3	47.9	35.3	yes	0.08
Log-logistic	3	-84.70	175.4	Yes	21.9	49.8	35.0	yes	0.12
Weibull	3	-84.91	175.8	Yes	20.3	50.4	34.3	yes	0.10
Log-probit	3	-84.50	175	Yes	23.3	49.6	35.7	yes	0.15
Gamma	3	-84.77	175.5	Yes	21.1	50.4	35.2	yes	0.11
Logistic	2	-85.67	175.3	Yes	34.0	48.6	40.8	yes	0.12
Probit	2	-85.38	174.8	Yes	31.6	45.1	37.8	yes	0.07
LVM: Exponential model 3	3	-85.22	176.4	Yes	17.8	50.9	32.6	yes	0.07
LVM: Hill model 3	3	-85.10	176.2	Yes	18.8	50.5	33.2	yes	0.08
Final BMDL/D value					22.2	53.7			

AIC (Akaike information criterion); BMD (Benchmark dose); BMDL (lower bound of the BMD confidence interval); BMDU (upper bound of the BMD confidence interval), conv (convergence), NA (not available)

3.3.4. MOE based risk assessment for methyleugenol

Considering the target consumer for the herbal beverages samples, the MOE based risk assessment in this study separated into adults and children (Table 3.1). Figure 3.2A presents the MOE values calculated using the $BMDL_{10}$ of 22.2 mg/kg bw/day and the EDIs resulting from regular daily consumption of the 114 samples targeted at adults and children. The results presented in Figure 3.2A reveal that for 45 out of the 98 samples (45.9%) of Indonesian instant herbal beverages targeted at adults and 1 out of 16 samples (6.3%) targeted at children the MOE values based on the upper bound EDI were <10,000 indicating there is a priority for risk management when these herbal beverages would be used every day during a lifetime. For sample B90, which showed analytical results for methyleugenol below the LOD, use of the upper bound level and corresponding EDI resulted in MOE values that amounted to 8,200. For the samples targeted at children only 1, B62 in which methyleugenol could be detected and quantified indicated a priority for risk management (MOE<10,000). Most of the herbal beverage samples which were sold as traditional medicine (labelled BPOM RI TR) to cure trichinosis for children are a low priority for risk management (MOE > 10,000). Of the 49 samples in which methyleugenol could be quantified only 4 samples, B59, B82, B100 and B101, resulted in MOE values > 10,000 indicating a low concern for human health and a low priority for risk management when they would be consumed every day during a lifetime.

Considering that a scenario in which people consume the herbal beverage every day of their life for a whole lifetime seems unrealistic, a risk assessment for other, more realistic exposure scenario's was performed as well. Although there is no formal method to apply the MOE approach for less than lifetime exposures, previous studies have used Haber's rule to correct for shorter than lifetime exposure in a linear way.[41] In a more realistic scenario people might consume herbal beverages regularly for short periods of time, for example 2 weeks, every year during a lifetime. Following Haber's rule and thus a linear correction of the intake estimates, the EDI values will be 52 weeks per year/2 weeks = 26 times lower and thus the MOE values 26 times higher than what was presented in Figure 3.2A adult (98 samples) and children (16 samples) for lifetime exposure scenario's. This resulted in the MOE values depicted in Figure 3.2B in which all MOE values are > 10,000 indicating no priority for risk management for all 114 herbal beverages.

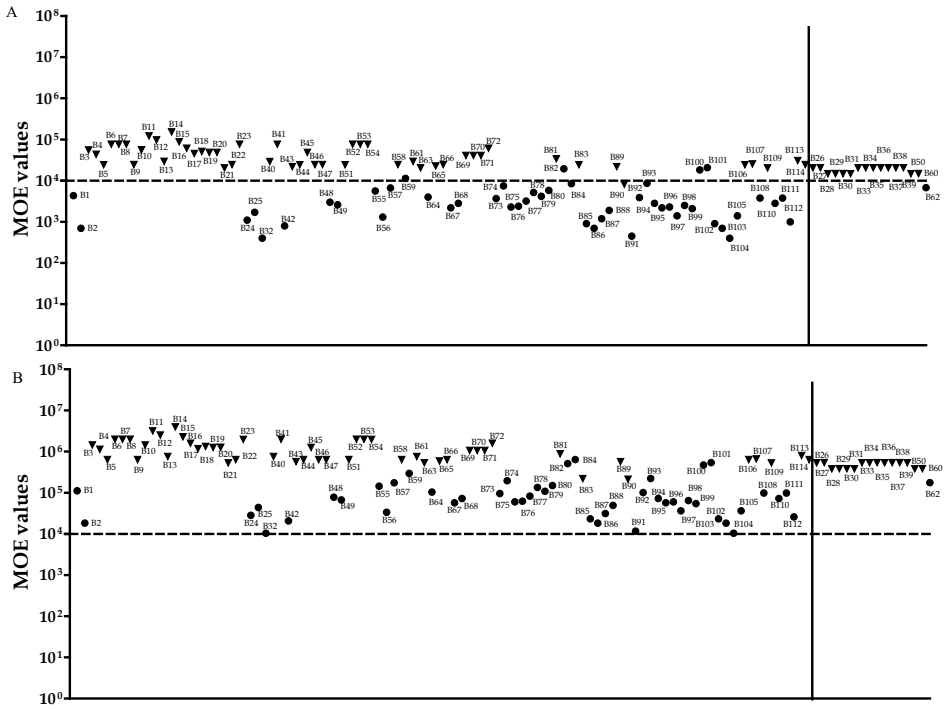

Figure 3.2. MOE values obtained for the evaluation of drinking the 114 herbal beverage samples targeted at adults (left of vertical black bar) and children (right side of the black vertical line) based on: A. daily lifetime exposure, B. 2 weeks every year exposure. MOE values were calculated as explained in the Materials and Methods section (Equation 3.2), using the $BMDL_{10}$ of 22.2 mg/kg bw/day resulting from model averaging (Table 3.2). The triangles show the MOE values calculated for the samples in which methyleugenol levels were below the LOD and EDI values were calculated as the upper bound (LOD) setting the levels equal to the LOD. The circles represent samples which contained methyleugenol above the LOD. The horizontal dashed line represents the MOE value of 10,000 (----) as a threshold for risk management action.[40]

In a final assessment it was calculated for the 46 herbal beverage samples that showed MOE values below 10,000 in the initial assessment (Figure 3.2A), how many weeks of exposure would result in an MOE value that is 10,000 or higher and thus would not raise a concern. Figure 3.3 depicts the results obtained and reveals that the shortest time of daily exposure without raising a concern was for B32 and B104 (the samples with the highest EDIs of 51.2 µg/kg/bw/day and 50.6 µg/kg/bw/day respectively) amounting to 144 weeks equal to about 2 weeks per year during a lifetime. Thus, it can be concluded that overall, if the herbal beverages would be consumed for less than 144 weeks (about 2 weeks a year) during a lifetime their level of methyleugenol would not raise a concern.

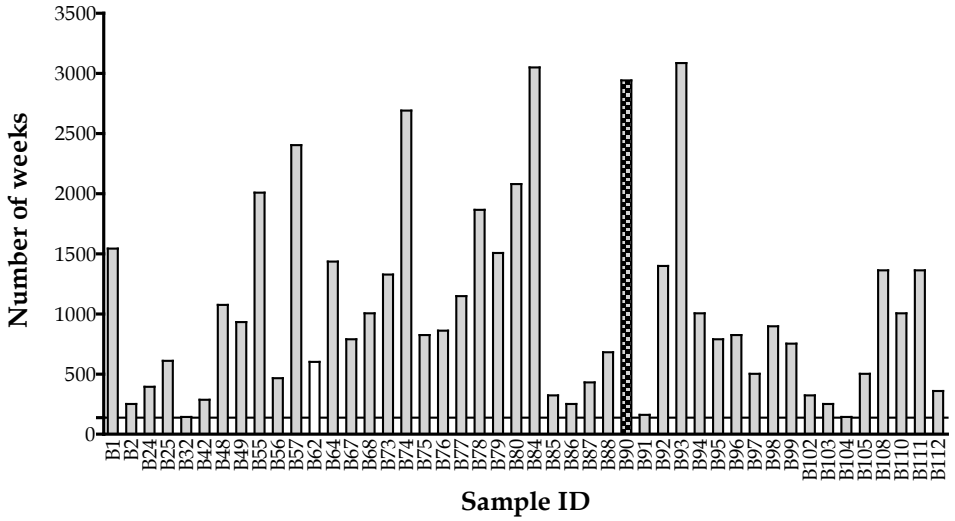

Figure 3.3. The number of weeks of exposure that would result in an MOE of 10,000 upon daily consumption of 46 Indonesian herbal beverage samples which showed concern for risk management. White bar represents a sample for children, while the grey patterned bar shows a sample in which methyleugenol levels were below the LOD. The horizontal black line represents 138 weeks, (2 weeks intake a year) during a lifetime.

3.3.5. Eugenol safety assessment

In 4 of the 114 samples eugenol was detected at a level that could be quantified. Given that eugenol is known to be non-genotoxic its risk assessment can be based on an ADI of 0 - 2.5 mg/kg bw/day established by JECFA (2006)[27] or the ADI of 1.0 g/kg bw/day established by EFSA (2012)[28]. All of the eugenol containing samples are specified to be consumed by adults. The EDI of eugenol calculated for the 4 eugenol containing samples ranged from 5.0 to 46.9 µg/kg bw/day, far lower than both ADI values, indicating there is no concern.

3.3.6. Evaluation of the safety assessment result based on the product registration type

Considering the product registration category, it appears that 31 out of the 43 samples (72.1%) produced and labelled as household herbal beverages (labelled Depkes RI P-IRT) appeared to result in MOE values < 10,000 and thus present a possible priority for risk management action upon lifetime consumption (Table 3.3). Seven out of 31 samples (22.6%) of the herbal beverages categorized as domestic processed food resulted in methyleugenol intakes that did raise a concern for human health. For traditional medicines this number amounted to 7 out of 30 (23.3%). This

analysis indicates that product registration may need to include a more detailed safety and/or risk-benefit assessment.

Table 3.3. Overview of the risk characterization using the MOE approach for the different Indonesian instant herbal beverages.

Registration code per target consumer	Category of product	Total samples	Concern for risk management	
			Yes	No
Children				
BPOM RI TR	Traditional medicine	16	1	15
Adult				
Depkes RI P-IRT	Food household industry	43	31	12
BPOM RI MD	Domestic processed food	31	7	24
BPOM RI TR	Traditional medicine	14	6	8
BPOM RI SD	Domestic supplement	9	0	9
BPOM RI ML	Foreign processed food	1	1	0
	Total	**114**	**46**	**68**

3.4. Discussion

The aim of the present study was to evaluate whether the level of methyleugenol in instant herbal beverages obtained by a targeted sampling strategy on the Indonesian market would be safe for human consumption. In this targeted sampling strategy samples were collected on the Indonesian market focussing on samples that listed methyleugenol-containing botanicals on their label followed by analysis of their methyleugenol content and resulting EDI and MOE values. Use of the MOE is in line with what is proposed by EFSA and others for risk assessment of compounds in food that are both genotoxic and carcinogenic.[37, 40, 45] Since in addition to methyleugenol in some samples also eugenol was detected a risk assessment for eugenol using the ADI of of 2.5 mg/kg/day established by JECFA (2006)[27] and the ADI of 1.0 mg/kg bw/day established by EFSA (2012)[28] was also performed.

Methyleugenol appeared to be the only alkenylbenzenes detected in 49 out of 114 samples of instant herbal beverages. The highest levels were measured in B91 a sample containing robusta coffee and ginger as ingredients. The high occurrence of methyleugenol in the herbal beverages is in line with the fact that most samples contain ginger as a major ingredient, since it was confirmed earlier that ginger (*Zingiber officinale* Rosc) naturally contains this alkenylbenzene.[46, 47] Furthermore Sigh et al. (2008)[48] reported that methyleugenol was identified at levels amounting to 0.5% of the oleoresin derived from *Z. officinale*. Methyleugenol naturally occurs in other herbs, like nutmeg, cloves, lemongrass, betel pepper and basil[47] and the presence of these botanical ingredients in the herbal beverage samples B2, B32 and B104, therefore also likely contributed to the methyleugenol levels detected.

It is of interest to note that the difference in the methyleugenol levels between the different beverages might be more than 100-fold. This relates to 1) the actual level

of the methyleugenol containing herb in the sample, and 2) the level of methyleugenol in this botanical. This latter level is known to vary with the part of the plant used, the geographic variants, the growth conditions, physiological variations, evolution and genetic factors, growth stages of the plant,[49] maturity of the plant at harvesting, the harvesting techniques, circumstance of storage, processing technologies, and measurement methods[50]. On the other hand, the methyleugenol level of 65 out of 114 was below the LOD. Although the results indicate that eugenol was detected in 4 out of 114 samples, the EDI resulting from use of these samples as herbal preparation appeared to be below the ADI of eugenol of 2.5 mg/kg bw/day[27] indicating this does not raise a concern.

The level of eugenol detected in 4 out of the 114 samples analysed also varied more than 4-fold. The highest level of eugenol was detected in sample B5, sold as a traditional medicine (labelled BPOM RI TR). The relatively high level of eugenol in this sample may be due to the fact that extract of tamarind (*Tamarindus indica* L.) pulp contributed 14% to the ingredients, in addition to extract of betel (*Piper betle* L.) leaf (5.6%) and honey (2.8%). These 3 ingredients have been reported to contain eugenol.[20] Cinnamon and clove, also well known to contain eugenol,[20] likely contributed the eugenol levels in samples B83 and B109, sold as household herbal beverages (both labelled Depkes RI P-IRT). The highest EDI for eugenol registered in the present study of 46.9 µg/kg bw/day (2.5 mg/day for a 54 kg person) resulting from intake of eugenol via herbal beverage sample B5 was lower than the maximum estimated intake in the EU from all sources previously reported to amount to 3 mg/day.[30] Spices and essential oil are the major contribution to the intake of eugenol in the EU. Given that even this highest intake was below the ADI of 2.5 mg/kg bw/day[27] and also below the ADI of 1.0 mg/kg bw/day established by EFSA[28] also exposure to eugenol via herbal drinks does not raise a concern.

The present study revealed an up to about 48 times difference in the EDI for methyleugenol resulting from consuming the different methyleugenol-containing herbal beverages, a difference that is caused by differences in their methyleugenol levels, but also by differences in the recommended daily use of the samples as indicated on the label, varying from 6 to 60 g per day. The highest EDI of 2,765.6 µg methyleugenol/person/day as calculated for B32 based on the EDI of 51.2 µg/kg bw/day and 54 kg bw for Indonesian people,[36] appears to be 34.4 fold higher than the estimated per capita intake of methyleugenol of 80.5 µg/person/day from spices and oil for the USA population and 288.1 fold higher than the 9.6 µg/person/day originating from nutmeg, mace and corresponding essential oils estimated for the EU population.[51]

Given that exposure to methyleugenol may occur also from other food sources it is of interest to also compare the EDI from herbal beverages estimated in the present study to the EDI for methyleugenol from all sources, estimated to amount to 190 μg/kg bw/day[52] or to 1-10 μg/kg bw/day[53]. This comparison reveals that intake from herbal drinks may contribute substantially to the dietary intake of methyleugenol. This is especially the case when it is considered that current intake of methyleugenol from the regular diet is likely substantially lower than what was estimated before, because at present addition of methyleugenol as a pure compound to flavour food is no longer allowed.[54]

Methyleugenol has been associated with carcinogenicity and genotoxicity, in animal studies, although epidemiological data to show the relevance of these effects for the human population are absent. In the absence of human data, risk assessment is based on animal data for tumor formation. In the present study the $BMDL_{10}$ derived from available data on dose-dependent induction of hepatocellular carcinoma in a 2 year rat study[19] was used as a reference point to calculate the Margin of Exposure (MOE). Model averaging as an update on BMD modelling for toxicologically based risk assessment was applied to calculate the $BMDL_{10}$ used. Model averaging is preferred over selecting the lowest $BMDL_{10}$ from results of fitting separate models.[38] Model averaging estimates the $BMDL_{10}$ as a weighted average of the outcomes of individual models in which the weight factor is determined by the Akaike Information Criterion (AIC). The AIC represents the goodness of fit of different mathematical models to a dose-response data set.[38] The $BMDL_{10}$ value obtained for methyleugenol based on the male rat data by model averaging amounted to 22.2 mg/kg bw/day and appeared to be lower than the value derived from the data for female rats that amounted to 66.5 mg/kg bw/day, reflecting the higher sensitivity of male rats. This value of 22.2 mg/kg bw/day was used to calculate the MOE values. The value falls within the range of $BMDL_{10}$ values of 15.3 - 34.0 mg/kg bw/day reported before when analysing the rat male data by individual models,[35] and is somewhat higher than the lowest $BMDL_{10}$ of 15.3 mg/kg bw used before in risk assessment of methyleugenol containing food supplements or samples of pesto.[35, 55]

Given that an MOE value below 10,000 indicates a possible concern from a public health point of view and points at a priority for risk management actions,[40] the results of the present study indicate that for 46 out of 114 herbal beverages there is a priority for risk management. This risk assessment is however based on lifetime daily consumption of the herbal beverages and one may question whether this is a realistic exposure scenario.

In the absence of a generally established method to take less than lifetime exposure scenario's into account in risk assessment for genotoxic carcinogens by the MOE approach, an estimate of the risk associated to short term use of the herbal beverages was obtained using Haber's rule. Such a shorter period of consumption of the herbal beverages should be taken into account to better reflect the real life exposure scenario in which Indonesian people do not consume these preparations every day during their lifetime. Applying Haber's rule and assuming use for only 2 weeks every year of a lifetime the MOE values were 26 times higher than the MOE values for lifetime exposure, and were all >10,000 indicating a low priority of risk management. It is of importance to note that Haber's rule can be applied provided there is a linear relationship between tumour incidence and the dose of the carcinogen.[56] This linearity is assumed and used more often in risk assessment of genotoxic carcinogens. It is used for example when correcting the dosing regimen in 2 year rodent carcinogenicity studies from 5 to 7 days exposure as done in the present and other studies[35, 37] for the data used for BMD modelling of the NTP carcinogenicity data for methyleugenol. However such linearity in the dose- and time- dependent response behaviour for tumour induction by methyleugenol is not available, although the existing evidence does support linearity in the dose- and time-dependent bioactivation of methyleugenol to its ultimate carcinogenic 1'-sulfooxy metabolite, and for the DNA adduct formation in both cell models or experimental animals exposed to methyleugenol.[57, 58]

As depicted in Table 3.3, most of the herbal beverage samples, namely 31 out of the 43 samples included in the study (72.1%), categorized as household food (labelled Depkes RI P-IRT), raise a concern when people would consume them every day during a lifetime. Also, a substantial number of herbal beverage samples registered as domestic processed food, or traditional medicine indicated a concern for human health. The limitation of producers' knowledge related to food safety and low-quality control processes for these products may cause the high content of methyleugenol containing herbs in these products. Putri (2018)[14] reported that the house-hold industry in Indonesia can easily get a license to build independent businesses for their economy improvement, while at the same time the limitation of monitoring from NADFC causes their food products to fail to comply with the quality and safety standard. Regulation NADFC 12/2016 Article 6 on Criteria of Prossessed Food stipulates that to be registered every processed food should meet 3 criteria's: (1) safety parameters namely the maximum limit of microbial, physical and chemical contamination, (2) quality parameters, including fulfilment of quality requirements in accordance with the existed standards and requirements; and (3) nutritional parameters according to the established requirements. Beside these 3

criteria's the processed food should comply the label requirement, good manufacturing practices and good distribution practices.[10] The results of the present study suggest that it would be of use that the process and regulation of manufacturing homemade herbal beverages is monitored more closely and may need to be updated in order to reduce the level of methyleugenol.

Currently, the National Agency for Drug and Food Control of Republic of Indonesia (BPOM RI) regulated estragole and safrole as natural food flavouring, with maximum permitted levels (MPLs) of 10 mg/kg for estragole and 0.1 mg/kg for safrole both in ready to drink beverages. The lower MPL for safrole than for estragole is due to consideration of the natural occurrence and use of estragole as a food flavouring.[10] However, MPLs for methyleugenol in herbal beverages in Indonesia have not yet been established. The levels of methyleugenol of 2.6-443.7 µg/g now encountered are higher than 0.1 mg/kg and for several samples even higher than 10 mg/kg, so they would not be in compliance with the MPLs set in Indonesia for related alkenylbenzenes like safrole and estragole. It is of interest to note that, based on the risk assessment provided in the present study, an MPL value of 10 mg/kg would not be low enough to support safe daily consumption of these beverages during a lifetime, when recommended daily use would amount to the highest recommended daily consumption of 75 gram, resulting in an MOE of about 1,600. At an MPL of 0.1 mg/kg or 1 mg/kg the MOE would amount to 160,000 or 16,000, and use of the herbal preparation would not be of concern. An MPL of 1 mg/g would be in line with what is established by the European Union (EU), EC Regulation 1334/2008.[54] An MPL of 10 mg/kg would however support safe consumption when use would be limited to 2 weeks a year during a lifetime. On the other hand, reducing the recommended daily consumption of herbal beverages to 0.3 g per day during a lifetime will be safe to meet the MOE value of 10,000 using the highest methyleugenol level of 443.7 µg/g. Obviously the approach to be taken is subject to a risk management decision.

Further evaluations of the present study revealed that with the levels of methyleugenol detected in the samples, their use would not raise a safety concern or priority for risk management if it would be limited to less than 144 weeks (about 2 weeks per year) during a lifetime. This result indicates that risk management of these herbal beverages may focus on providing information on the label for limitation of the consumption duration. Hermanu (2016)[59] reported that the implementation of food safety aspects for many home industry food items in Indonesia is still limited, and the results of the present study provide an examples of how this could be improved.

In conclusion, consumption of methyleugenol-containing herbal beverages can be considered safe when consumed for less than about 2 weeks a year during a lifetime. This conclusion holds for herbal beverages collected by targeted sampling on the Indonesian market. The study does support the establishment of an MPL for methyleugenol in foods and beverages in Indonesia, in line with what has been done for the related alkenylbenzenes estragole and safrole.

Conflict of Interest

The authors declare that there are no conflict of interest regarding this manuscript.

Acknowledgements

Financial support was received from the Indonesian Endowment Fund for Education, Ministry of Finance, Republic of Indonesia through a Beasiswa Pendidikan Indonesia Lembaga Pengelola Dana Pendidikan (BPI LPDP) doctoral scholarship for the first author [contract number: PRJ-365/LPDP/2016].

Supplementary materials

Supplementary materials of this article can be downloaded from https://doi.org/10.1016/j.fct.2019.02.001

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BENEFIT
RISK

Chapter 4

Detection of pyrrolizidine alkaloids in jamu available on the Indonesian market and accompanying safety assessment for human consumption

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Published in: Food and Chemical Toxicology 138 (2020): 111230
DOI: 10.1016/j.fct.2020.111230

Abstract

The occurrence and accompanying risks of pyrrolizidine alkaloids (PAs) in Indonesian jamu were evaluated. PAs were detected in 34 out of 35 jamu containing PA-producing botanicals, in the range of 12.3-235,376 µg/kg. A total PA level of 5.9-3,421 µg/kg was found in 17 out of 23 jamu made of non-PA-producing botanicals pointing to contamination with PA-producing plants. Short-time consumption of jamu is unlikely to result in acute toxic effects, although one sample would exceed an intake of 10 µg PAs/kg bw/day which may cause hepatic veno-occlusive disease (HVOD) in humans. The risk assessment for the genotoxic and carcinogenic potential of PAs, revealed Margin of Exposure (MOE) values below 10,000 for 27 out of all samples analysed (46.6%), indicating a potential priority for risk management when assuming daily lifelong consumption. Assuming consumption for two weeks every year during a lifetime, and using Haber's rule, 13 out of 35 jamu samples containing PA-producing botanicals (37%) still pose a priority, while the jamu consisting of non-PA-producing botanicals would be of no priority (MOE>10,000). This study provides data that can support risk management actions in Indonesia to minimize the potential health risk for jamu consumers due to the occurrence of toxic PAs in these products.

4.1. Introduction

Indonesian jamu represents one of the traditional herbal medicine practices in Indonesia. Jamu products are available in the market mainly with BPOM RI TR labelling, referring to BPOM RI, the *Badan Pengawas Obat dan Makanan Republik Indonesia* being the regulatory body where the product is registered, while TR refers to the product category being *obat tradisional produksi dalam negeri* (Indonesian traditional medicine).[1] Jamu is available in many forms, including powder, tablet, pill, caplet, capsule, liquid or simplicia (dried/fresh raw jamu botanicals). The jamu in powder form and simplicia are readily consumed by adding hot water and drinking the resulting preparation, while the other forms can be consumed directly as supplement. Considering the increasing demand for jamu in both local and international markets, BPOM RI is tightly monitoring the quality, safety and efficacy of the products. However, knowledge gaps regarding the possible adverse health effects of hazardous drugs and/or toxic constituents in the jamu currently hamper its monitoring activity.[2] This issue may put consumers at risk especially when they are regular jamu users.

Botanical constituents of special concern are compounds known to be genotoxic and carcinogenic, which may be naturally occurring in the botanical ingredients of jamu and thus may pose a safety issue. In our previous work for example[3] the alkenylbenzene (AB) methyleugenol, appeared to be a major ingredient, detected in 91.3% of the jamu samples testing positive for ABs. Quantification of methyleugenol levels and exposure resulting from use of the respective jamu products resulted in Margin of Exposure (MOE) values generally <10,000, indicating a priority for risk management when assuming daily consumption during a lifetime. Another group of genotoxic compounds are the aristolochic acids (AAs) that can occur in plant food supplements (PFS) and herbal products at levels that raise a health concern for their consumers. A review of the literature showed that the levels of AA-1 and AA-II reported in selected PFS resulted in MOEs below 10,000 for 206 out of 573 (35.9%) of the samples analysed[4], clearly indicating that herbal products containing AA1 and AAII were a priority for risk management. Recent data on PFS revealed that pyrrolizidine alkaloids (PAs) may represent a third category of botanical ingredients of concern.[5-7] The aim of the present study was to investigate the potential presence of PAs in jamu and to perform a risk assessment. In humans, acute exposure to PAs can cause hepatic veno-occlusive disease (HVOD) with severe liver damage, in some cases with fatal outcome,[8-10] whereas chronic exposure may lead to liver cirrhosis and pulmonary arterial hypertension[11, 12]. Furthermore, 1,2-unsaturated PAs, including lasiocarpine, monocrotaline and riddelliine, are considered genotoxic carcinogens

due to their potency to be metabolized into reactive pyrroles. Therefore, the International Agency for Research on Cancer (IARC) classified these compounds as being possibly carcinogenic to humans (category 2B).[13]

PAs are naturally occurring heterocyclic phytotoxins that are widely distributed and present in more than 6,000 flowering plant species, particularly from the genera *Senecio*, *Crotalaria*, *Heliotropium, Echium*, *Trichodesma*, *Symphytum*, *Petasites*, *Tussilago*, *Eupatorium* and *Gynura*.[6, 10, 14-17] Moreover in some botanical products including herbal teas, herbal medicines and food supplements, the detected PAs appeared to result from contamination of the non-PA-containing plant material, used to prepare the products, with PA-containing weeds during the cultivation or collection of these botanicals. In response, risk management actions were formulated by the European Medicines Agency (EMA) to reduce this level of contamination. In 2016 the Herbal Medicinal Products Committee (HMPC) of EMA has established a transitional limit of intake of 1.0 µg PAs per day per person related to intake resulting from such contamination, for a 3 years period.[18] Recently HMPC (2019)[19] announced a consensus to extend the transitional period for a further 2 years.

In their assessment of the potential cancer risks resulting from chronic PA exposure, the EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) established a lower confidence limit of the benchmark dose resulting in a 10% extra cancer risk ($BMDL_{10}$) of 237 µg/kg body weight per day, derived from tumour data on riddelliine, as point of departure (POD) for calculating the MOE.[5]

The purpose of this current work is to investigate the occurrence of PAs in 58 Indonesian jamu products containing various mixed medicinal botanicals, including 35 samples containing PA-producing botanicals and 23 samples containing non-PA-producing botanicals. Based on the levels of PAs present and directions for use given by the producers, an exposure and safety assessment of consumption of these jamu was performed. The results of the study can support risk management in formulating regulatory actions to minimize the exposure to PAs via use of jamu.

4.2. Materials and methods

4.2.1. Collection and preparation of samples

A targeted sampling approach was applied to collect 58 samples of jamu from different brands. The samples were purchased from traditional markets or jamu stores in Indonesia as depicted in Figure 4.1, including sampling in Tangerang (4 stores, n = 4), Jakarta-Bekasi (12 stores, n = 16), Bogor (1 store, n = 1), Tegal (1 store, n = 4), Semarang-Bawen (3 stores, n = 7), Temanggung (1 store, n = 1), Magelang (1 store, n = 1), Surakarta-Sukoharjo (7 stores, n = 10), Trenggalek (1 store, n = 1),

Nganjuk-Kediri (4 stores, *n* = 9), Malang (2 stores, *n* = 2), and Jember (1 store, *n* = 2). A total of 35 jamu samples (TR-1 – TR-35) were collected with the name of possible PA-containing botanicals on the label, including lithospermi radix (*Lithospermum orientale* (L.) L.), *Gynura pseudochina* (L.) DC., *Gynura procumbens* (Lour.) Merr., *Gynura segetum* (Lour.) Merr., *Gynura divaricata* (L.) DC., bandotan (*Adenostemma lavenia* (L.) Kuntze), *Ageratum conyzoides* (L.) L., flos farfarae (*Tussilago farfara*) and comfrey (*Symphytum officinale* L.). To monitor the possible contamination of jamu with PA-producing botanicals, a set of 23 samples that, according to the label, did not contain PA-producing botanicals, were included in the study (TR-36 – TR-58). Of these 23 samples, 21 were previously collected and analysed for ABs[3], while 2 samples, collected during the targeted sampling exercise, were included because their label indicated the presence of aristolochic acid (AA) producing botanicals. Detailed information, including an overview of the respective botanicals of concern present in the samples, the health claims and recommended daily use written on the label, is summarised in Supplementary materials 1.

The 58 samples included in the study were marketed in different forms including caplet (*n*= 1), capsule (*n* = 27), liquid (*n* = 4), pill (*n* = 2) and powder (*n* = 24). The homogeneity of each sample (except the liquid sample) was ensured by mixing the content from 10 packages manually in a ziplock plastic bag before taking samples for analysis. The powder samples were weighted and put into the plastic bag directly, the capsule samples were opened first and only the weighted content inside the capsule was put into the bag. The pill and caplet samples were weighted and ground with a mortar and the resulting powder was collected in the plastic bag.

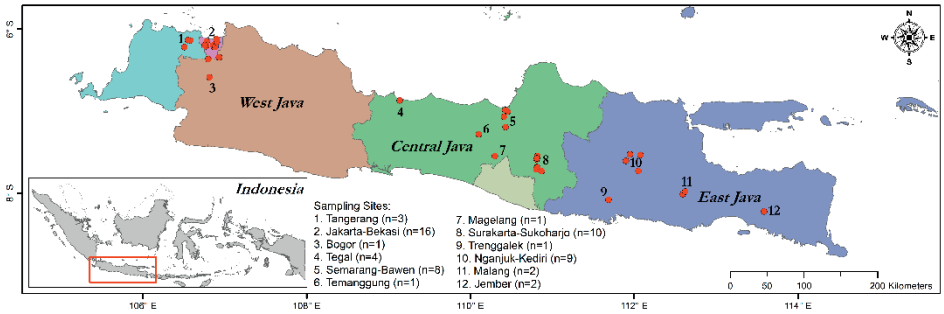

Figure 4.1. Sampling locations of jamu in Banten, Jakarta, West Java, Central Java, and East Java Provinces, Indonesia. The red dots represent the sampling locations of the products, including both PA- and non-PA-containing jamu and n is the number of collected samples in the respective city.

4.2.2. Chemicals and reagents

Water used was deionised MilliQ with a minimal resistance of 18.2 M. Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were obtained from Actu-

all (Oss, the Netherlands). Formic acid (analytical grade, 99-100%) and ammonium carbonate (analytical grade) were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Fifty-nine PA analytical standards were sourced from Phytoplan (Heidelberg, Germany), except for: heliotrine and trichodesmine from Latoxan (Valence, France); usaramine from BOC Sciences (Shirley, NY, USA), florosenine from PRISNA (Leiden, the Netherlands), echimidine, indicine, indicine N-oxide, intermedine, intermedine N-oxide, lycopsamine, lycopsamine N-oxide, monocrotaline, monocrotaline N-oxide and otosenine from Phytolab (Vestenbergsgreuth, Germany). Usaramine N-oxide, spartioidine N-oxide and trichodesmine N-oxide were in-house synthesized by the method of Chou et al. (2003)[20]. A complete list of PA standards used in this study is presented in Supplementary materials 2.

Stock solutions (100 µg/mL) of the individual PA standards were prepared in methanol, from these stock solutions a mixed solution (1 µg/mL in methanol) containing all PA standards was prepared. This mixed standard solution was used to spike the jamu samples as described below.

4.2.3. Extraction and purification

The extraction procedure was based on an in-house validated method and performed as described by Chen et al. (2019)[21], for the analysis of herbal teas and herbal medicines. Briefly, 20 mL of 0.2% formic acid solution was added to 1 g of jamu (1 mL for liquid samples) followed by agitation in a rotary tumbler for 30 min. Before extraction one of the test portions was fortified with the mixed PA standard solution at 250 µg/kg (250 µL of 1 µg/mL PA mix). Upon agitation the extract was centrifuged for 15 min at 3,500 g. After centrifugation, 5 mL of supernatant was transferred to a new tube and subsequently the supernatant was neutralized to pH 6–8 using approximately 350 µL of 1 M ammonium carbonate solution and the supernatant was centrifuged for another 15 min at 3,500 g.

The extracts were purified by solid phase extraction (SPE) using Strata-X Polymeric reversed phase 200 mg/6 ml cartridges (Phenomenex, Palo Alto, CA, USA). Cartridges were conditioned with 6 mL methanol, followed by 6 mL water. The extract was passed through the cartridge, which was then washed with 6 mL 1% formic acid, followed by 6 mL water. The cartridges were dried for 10 min under reduced pressure using an SPE vacuum manifold. PAs were eluted with 6 mL of methanol and the eluates were dried under a stream of nitrogen at 50 °C using a TurboVap (Biotage, Uppsala, Sweden). The residues were reconstituted in 500 µL 10% methanol in water and filtered using 0.45 µm PTFE filtervials (UniPrep,

Whatman, Maidstone, UK). The vials were closed with help of a compressor. The purified extracts were stored at -20°C until analysis.

4.2.4. LC-MS/MS analysis

The sample analysis was carried out in positive electrospray mode on an LC-MS/MS system consisting of a Waters Acquity UPLC coupled to a Xevo TQ-S tandem mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation was obtained on a 150 × 2.1 mm, 1.7 µm particle size, UPLC BEH C18 analytical column (Waters, Milford, MA, USA). The column and sample temperature were set at 50°C and 10°C, respectively. The mobile phase used in LC-MS/MS analysis consisted of water containing 10 mM ammonium carbonate pH 9 (as eluent A) and acetonitrile (as eluent B) with a flow rate of 0.4 mL/min. A gradient elution was performed as follows: 0.0 min 100% A/0% B, 0.1 min 95% A/5% B, 3.0 min 90% A/10% B, 7.0 min 76% A/24% B, 9.0 min 70% A/30% B, 12.0 min 30% A/70% B, 12.1–14.2 min 100% A/0% B. Of each sample extract, 2 µL was injected.

Matrix matched standards (MMS) were used to assess the linearity of the LC-MS/MS system and to confirm that the sample pre-treatment was done correctly. For MMS, 8 subsamples of 1 g of a blank plant food supplement, in which no PAs had been detected in a previous analysis (<LOD), were spiked with a mixture of the 59 PAs standards in a concentration range of 0-1000 µg/kg. After waiting for 10 min, the MMS samples were processed and analysed by the same procedure as described above. LOQs obtained were 5 µg/kg for individual PAs in dried plant material and 5 µg/L in liquids. Recovery and repeatability data were presented in Chen et al. (2019).[21]

Detection of PAs was done based on at least two MRM transitions measured per analyte. For detection and confirmation of PAs in the samples, retention times and ion ratios were compared to those of the calibration curves of the compounds prepared using the MMS. Besides the 59 PAs for which an analytical standard was available, the samples were screened for another 32 1,2-unsaturated PAs for which no standards were available. These PAs were included in the analytical method based on mass spectrometric data obtained from the analysis of selected extracts by running the LC-MS/MS in parent ion scanning mode. Fragment ions typically present in the fragmentation spectra of PAs were selected: ions with *m/z* 94; 118; 120 and 138 for retronecine-type PAs and ions with *m/z* 122; 150 and 168 for otonecine-type PAs. When two or more fragment ions were produced from the same parent ion (the protonated molecular ion), the latter was marked as a potential PA and the corresponding transitions were included in the MRM method. See Supplementary

materials 2 for an overview of the MS/MS transitions used for the complete set of PAs.

Quantification was performed by single level standard addition (250 µg/kg) to each sample. For those compounds for which no reference standard was available, a semi-quantitative concentration was obtained by comparison of the peak areas with that of the most closely related analogue (e.g. an isomer). For metabolites with tentative or unknown structures, no close related standard could be identified. In such cases the concentration was estimated by taking the sum of the two most intense MRM transitions and comparing this with the sum area of a selected reference standard, as indicated in Supplementary materials 2. Data processing was conducted with MassLynx 4.1 software (Waters Corporation, Milford, MA, USA).

Samples that contained PAs in a concentration exceeding 250 µg/kg were reanalysed. This was the case for 16 samples. Depending on the (range of) PA levels present in the samples, various dilutions of the purified extracts were made in triplicate. One of the 3 replicates was spiked with a mixed PA standard solution to obtain a concentration in the diluted extract of 50 ng/mL, one was spiked at 200 ng/mL and one extract was left unspiked. Samples TR-5, TR-6, TR-8, TR-15 and TR-37 were diluted 40-fold (25 µL), TR-9, TR-10, TR-23, TR-26 and TR-32 were diluted 100-fold (10 µL), TR-24, TR-28 and TR-31 were diluted 40-fold (25 µL) as well as 200-fold (5 µL), TR-12, TR-17 and TR-34 were diluted 40-fold (25 µL) as well as 400-fold (2.5 µL). The final volume after dilution with water in all cases was 1 mL.

4.2.5. Exposure assessment resulting from the drinking of jamu based on PA levels detected

In order to assess the potential exposure to PAs resulting from consuming the jamu, the estimated daily intake (EDI) was calculated according to Equation 4.1.

$$EDI = \frac{W \times total\ PAs}{BW \times 1{,}000} \qquad \text{(Equation 4.1)}$$

where the EDI values are expressed in µg/kg bw/day. W is the weight, expressed in g or mL, of recommended daily use of these samples (Table 4.1) based on the information provided on the label (see Supplementary materials 1). For the liquid samples and when there was no information on the label regarding the weight of recommended daily use, this was estimated from the average weight of 3 replicate samples. Total PAs is the total amount of PAs detected in the sample by LC-MS/MS, expressed in µg/kg for solid samples and in µg/L for liquid samples. BW is body weight of 54 kg, the average body weight for Indonesian male and female.[22] The factor 1,000 is added to convert W in g to kg or mL to L.

4.2.6. Safety assessment based on PA levels detected in the jamu

To assess the acute risks for consumers of jamu containing PAs, the EDI values calculated by Equation 4.1 were compared to the dose range of 1-3 mg PA/kg bw/day at which acute/short-term adverse effects in humans were reported upon consumption for 4 days up to 2 weeks periods, as described by EFSA (2017)[5]. A daily intake of PAs of 10 µg/kg bw/day established by WHO-IPCS (1998)[23] which may cause HVOD in humans, was used to evaluate the acute toxicity resulting from PAs intake via jamu consumption.

The MOE approach was applied to assess the chronic risk posed by the use of the PA-containing jamu, in line with the recommendations of EFSA for risk assessment of compounds that are both genotoxic and carcinogenic[24]. The MOE was calculated as described in Equation 4.2.

$$MOE = \frac{BMDL_{10}}{EDI} \quad \text{(Equation 4.2)}$$

where the MOE is dimensionless, the $BMDL_{10}$ value used was 237 µg/kg bw/day established by EFSA (2017)[5] for riddelliine and used as POD for evaluating the risks of PA exposure, and EDI values (µg/kg bw/day) were calculated by Equation 4.1. MOE values were rounded to one significant figure.

The MOE values are based on chronic lifetime exposure, although realistic use of the jamu may be for shorter periods of time. As previously suggested Doull and Rozman (2000)[25] Haber's rule was applied to correct the EDI and thus the MOE approach for shorter than lifetime exposure. Based on this rule the toxic outcome will be similar for situations where the product of the exposure time and the dose will be constant, ($k = C \times T$; $C1 \times T1 = C2 \times T2$, where k is the toxic outcome, C is the concentration (or dose) of the toxic chemical and T is the duration of exposure).[25-27] Using Haber's rule, the EDI of PAs can be expressed as follows:

$$EDI\ (2\ wk/yr\ during\ a\ lifetime) = \frac{EDI}{26} \quad \text{(Equation 4.3)}$$

where the EDI for 2 weeks every year during a lifetime is the EDI for daily lifetime exposure obtained by Equation 4.1 adapted to only 2 weeks yearly during a whole lifetime. To further illustrate how short term exposure would affect the MOE values, Haber's rule was also used to calculate the number of weeks (Equation 4.4) of daily consumption of the different samples that would result in an MOE value of 10,000:

$$Number\ of\ weeks\ = \frac{MOE \times 69 \times 52}{10{,}000} \quad \text{(Equation 4.4)}$$

where the MOE is the value for lifetime exposure calculated by Equation 4.2, 69 represents the life expectancy of Indonesian people in years,[28] 52 is the number of weeks within a year, and 10,000, the threshold for health concern[24].

4.2.7. Safety assessment based on PA levels compared to the AB and AA levels detected in the jamu containing non-PA producing botanicals

We compared for samples TR-36 to TR-56 the safety assessment on PA levels to the safety assessment of ABs. The MOE values for PA intake calculated from samples TR-36 to TR-56 were compared to previously reported MOE values for intake of ABs resulting from the AB-producing botanicals in these jamu products.[3] The MOE values were calculated using the $BMDL_{10}$ of 15.3 mg/kg bw for the major alkenylbenzene in the mixture, methyleugenol,[29] and the EDI resulting from summing up the EDIs of the individual alkenylbenzenes assuming equal potency (See Supplementary materials 1). Samples TR-57 and TR-58 contained AA-producing botanicals, and for these samples the MOE values determined for PAs were compared to the MOE values calculated for the AA intake from these 2 jamu samples using the $BMDL_{10}$ of 10 µg/kg bw/day[4] estimated from reported data on kidney tumour formation by a mixture of AAs (71% of AAI and 21% of AAII) upon oral exposure in rats[30]. The EDI values were calculated based on the AA levels determined using the UPLC method for quantification of AAs described previously.[4]

4.3. Results

4.3.1. Levels of PAs in Indonesian jamu

As shown in Table 4.1 (for a full set of results see Supplementary materials 3), PAs were detected in 34 out of 35 jamu samples containing PA-producing botanicals. The number of different PAs detected ranged from 2 to 40, and levels ranged from 12.3 to 235,376 µg/kg. The highest PA level was found in sample TR-17 in which senecionine N-oxide was present at the highest concentration, amounting to 114,071 µg/kg. Rinderine, senkirkine, and neosenkirkine were the top three most frequently found PAs, in 28, 26 and 23 samples, respectively, out of the 34 positive tested samples containing PA-producing botanicals. In one sample, TR-3, the levels of all PAs were below the LOQ.

According to the labelling, 29 of the 35 samples consisted of a *Gynura* species (mostly *G. procumbens* or *G. segetum*) or contained it as one of the ingredients. The 6 other samples contained *Sympytum officinale* (2 samples), and single samples of *Adenostemma lavenia, Ageratum conyzoides, Lithospermum orientale* and *Tussilago farfara.* Interestingly, 14 samples containing *Gynura* had high levels of PAs (> 12,000 µg/kg), while in 15 samples containing *Gynura* only moderate or even low levels (between <LOQ and 1270 µg/kg) of PAs were found. Most strikingly are jamu samples TR-7, TR-13, TR-16 and TR-11, that according to the label consisted solely

of *Gynura* leaf or an extract prepared from *Gynura,* but analysis revealed only very low levels of PAs (between 12 and 73 µg/kg).

Table 4.1. The level and EDI of PAs detected in Indonesian jamu containing PA-producing botanicals.

Sample ID	Number of PAs detected	Total PAs level (µg/kg) [a]	Recommended daily use (g)	EDI (µg/kg bw/day)	Top three PAs and their concentration (µg/kg)[a]
TR-1	2	13.1	1.9	0.0005	Monoester 7.90 (7.7), rinderine (5.3)
TR-2	3	135.3	14	0.035	Senkirkine (85.7), neosenkirkine (43.3), rinderine (6.3)
TR-3	-	<LOQ	200 [b]	- [c]	-
TR-4	3	31.8	3.4	0.002	Rinderine (16.8), echinatine (9.0), rinderine N-ox (5.9)
TR-5	37	35,066	1.1	0.691	Jacoline N-ox (16,762), jacobine N-ox (4016), senkirkine (3,042)
TR-6	26	17,435	5.6	1.808	Senkirkine (5,807), senecionine (4,918), neosenkirkine (4,153)
TR-7	7	73.4	2.9	0.004	Lycopsamine (19.0), echinatine (15.0), PA diester 11.45 (12.6)
TR-8	23	70,055	0.8	1.067	Neosenkirkine (44,446), senkirkine (15,674), integerrimine (2,981)
TR-9	31	74,837	2.9	3.951	Jacoline N-ox (30,507), onetine (16,468), jacoline (8,342)
TR-10	27	39,632	3	2.229	Senkirkine (15505), otonecine ester 3.75 (9,008), neosenkirkine (7,065)
TR-11	2	12.3	3.7	0.001	senkirkine (6.8), intermedine (5.5)
TR-12	40	105,099	2.4	4.761	Senkirkine (52,260), neosenkirkine (19,495), otonecine ester 3.75 (17,460)
TR-13	4	65.7	3	0.004	Rinderine (38.0), intermedine (13.3), senkirkine (8.0)
TR-14	2	131.1	45[b]	0.109	Senkirkine (123.0), neosenkirkine (8.2)
TR-15	27	21,516	0.5	0.202	Senecionine (6,634), senkirkine (6,588), neosenkirkine (4,743)
TR-16	4	33.2	1.1	0.001	Rinderine (12.0), echinatine N-ox (8.8), rinderine N-ox (7.6)
TR-17	31	235,376	3	13.256	Senecionine N-ox (114,071), senkirkine (66,713), integerrimine N-ox (23,784)
TR-18	4	29.5	30	0.016	Echinatine N-ox (8.5), lycopsamine N-ox (8.1), rinderine (6.9)
TR-19	8	453	2	0.017	Echinatine (187.4), rinderine (114.6), lycopsamine (58.0)
TR-20	9	276.6	2.9	0.015	Indicine N-ox (136.0), indicine (62.3), rinderine (22.8)
TR-21	8	270.2	6	0.030	PA diester 11.45 (91.2), lycopsamine N-ox (56.2), Lycopsamine (42.3)
TR-22	6	113.4	1.4	0.003	Echinatine (40.8), Rinderine (23.6), lycopsamine (16.1)
TR-23	23	51,425	1	0.933	Senkirkine (23,234), neosenkirkine (15,662), otonecine ester 3.75 (7,749)
TR-24	37	63,877	0.9	1.065	Senkirkine (28,452), neosenkirkine (14,610), otonecine ester 3.75 (7,870)
TR-25	13	1,265	1.2	0.029	Neosenkirkine (699.3), senkirkine (189.7), Otonecine ester 3.75 (93.5)
TR-26	26	12,173	1.4	0.311	Senkirkine (4,679), neosenkirkine (2,539), senecionine (1,996)

Sample ID	Number of PAs detected	Total PAs level (µg/kg) [a]	Recommended daily use (g)	EDI (µg/kg bw/day)	Top three PAs and their concentration (µg/kg)[a]
TR-27	19	933.9	3.8	0.065	Senkirkine (237.2), senecionine N-ox (132.6), rinderine (127.1)
TR-28	34	65,763	0.7	0.813	Senkirkine (33,630), neosenkirkine (13,022), otonecine ester 3.75 (8,894)
TR-29	7	205.9	1.8	0.007	Echinatine (103.5), lycopsamine (37.2), rinderine (21.4)
TR-30	4	42.5	1.5	0.001	Echinatine (15.9), rinderine (12.1), lycopsamine (8.2)
TR-31	36	104,842	3.4	6.556	Senkirkine (48,296), neosenkirkine (16,787), otonecine ester 3.75 (16,257)
TR-32	24	146,977	0.5	1.255	Echinatine (33,713), echinatine N-ox (33,563), lycopsamine (26,217)
TR-33	11	357.2	2.3	0.015	Rinderine N-ox (66.8), senkirkine (65.9), neosenkirkine (44.0)
TR-34	39	106,712	4.2	8.346	Senkirkine (49,156), neosenkirkine (18,997), otonecine ester 3.75 (16,757)
TR-35	2	15.1	30[b]	0.008	Neosenkirkine (8.4), senkirkine (6.6)

[a] Liquid samples are expressed in µg/L. [b] In mL. [c] The EDI cannot be calculated because no PAs were detected above the LOQ.

PAs were also found in 17 out of 23 jamu samples containing non-PA-producing botanicals with levels ranging from 5.9 – 3,421 µg/kg (Table 4.2), indicating there is a contamination with PA-producing plants. Senkirkine was the PA present at the highest level (3,221 µg/kg) in sample TR-58. The jamu made from non-PA-producing botanicals that tested positive for PAs contained between 1 and 14 different PAs, with rinderine being the PA most often found (14 out of 17 positive samples); albeit at relatively low levels (the highest concentration amounting to 43.5 µg/kg). Also its isomers intermedine, lycopsamine, echinatine and indicine, as well as the corresponding N-oxides were often present, in levels ranging from 5.2 – 135.5 µg/kg. It should be noted that the levels of PAs present in jamu made from non-PA-producing botanicals were much lower than the PA levels found in many of the jamu made from PA-producing botanicals. The total PA level in sample TR-58 was approximately 70 times lower than the level in TR-17, the sample with the highest PA content (Table 4.1).

Table 4.2. The level of PAs detected in Indonesian jamu containing non-PA-producing botanicals and the corresponding EDI

Sample ID	Number of PAs detected	Total PAs level (µg/kg) [a]	Recommended daily use (g)	EDI (µg/kg bw/day)	Top three PAs and their concentration (µg/kg)[a]
TR-36	-	<LOQ	14	- [c]	-
TR-37	10	127.8	14	0.033	Indicine N-ox (36.5), heliotrine N-ox (19.9), echinatine N-ox (12.6)
TR-38	-	<LOQ	14	- [c]	-
TR-39	14	313.7	14	0.081	Indicine N-ox (91.9), heliotrine N-ox (66.2), europine N-ox (30.1)
TR-40	-	<LOQ	14	- [c]	-
TR-41	-	<LOQ	14	- [c]	-

Sample ID	Number of PAs detected	Total PAs level (µg/kg) [a]	Recommended daily use (g)	EDI (µg/kg bw/day)	Top three PAs and their concentration (µg/kg)[a]
TR-42	5	40.6	2.1	0.002	Rinderine (14.7), intermedine (8.0), rinderine N-ox (6.5)
TR-43	10	144.8	14	0.038	Lycopsamine (36.2), rinderine N-ox (23.2), echimidine (20.6)
TR-44	-	<LOQ	3	- [c]	-
TR-45	8	253.2	14	0.066	Echinatine N-ox (70.8), rinderine (43.5), echinatine (41.9)
TR-46	3	60.2	7	0.008	Rinderine (33.3), rinderine N-ox (16.8), intermedine (10.0)
TR-47	7	149.8	14	0.039	Indicine N-ox (48.9), echinatine N-ox (40.8), lycopsamine N-ox (15.6)
TR-48	11	436.8	10	0.081	Indicine N-ox (135.5), indicine (86.8), PA diester 11.45 (47.3)
TR-49	6	102.8	10	0.019	Rinderine (42.6), intermedine (16.9), lycopsamine (14.4)
TR-50	3	35.7	14	0.009	Rinderine (18.8), intermedine (10.8), echinatine (6.2)
TR-51	2	26.2	12	0.006	Monocrotaline (17.7), heliotrine N-ox (8.4), europine N-ox (4.7)
TR-52	3	49.5	1.7	0.002	Rinderine (20.4), lycopsamine (15.7), intermedine (13.4)
TR-53	6	125.2	2.1	0.005	Indicine N-ox (33.3), rinderine N-ox (28.4), indicine (24.0)
TR-54	4	86.7	10	0.016	Echinatine (43.9), echinatine N-ox (19.8), lycopsamine (17.8)
TR-55	1	5.9	14	0.002	Rinderine (5.9)
TR-56	7	70.1	14	0.018	Rinderine (17.7), echinatine N-ox (13.0), echinatine (9.1)
TR-57	-	<LOQ	5[b]	- [c]	-
TR-58	4	3,421	3.6	0.228	Senkirkine (3,221), otonecine ester 3.75 (121.5), senecionine (52.3)

[a] Liquid samples are expressed in µg/L. [b] In mL. [c] The EDI cannot be calculated because no PAs were detected above the LOQ.

4.3.2. The estimated daily intake (EDI) of PAs resulting from consumption of jamu

Table 4.1 presents the EDI values of total PAs calculated for the consumption of positive samples of jamu containing PA-producing botanicals. The values range from 0.0005 to 13.3 µg/kg bw/day. The highest EDI of 13.3 µg/kg bw/day was calculated for consumption of jamu TR-17, which of all samples also had the highest level of PAs. As depicted in Table 4.2, the EDI values for intake of total PAs from jamu samples containing non-PA-producing botanicals ranged from 0.002 to 0.228 µg/kg bw/day. The highest EDI for this group of jamu products, calculated for TR-58, was still almost 60 times lower compared to the highest EDI (TR-17) resulting from consumption of jamu containing PA-producing botanicals.

4.3.3. Risk assessment of jamu based on PA levels

4.3.3.1. Acute exposure scenario

The EDI values for PAs resulting from the consumption of all jamu samples containing PA-producing botanicals (Figure 4.2a) and non-PA-producing botanicals are far below the dose range of 1-3 mg PA/kg bw/day at which acute/short-term adverse effects in humans have been reported by (EFSA, 2017)[5]. This result indicates that jamu consumers are not at risk for acute toxicity of PAs when consuming those preparations for short periods of 4 days up to 2 weeks. However, in one jamu sample, TR-17, the EDI value is higher than 10 µg/kg bw/day, which has been linked to the prevalence of HVOD in humans.[23] There are 11 (31%) jamu samples containing PA-producing botanicals that would give rise to EDI values higher than 890 ng/kg bw per day, which is the highest estimated acute/short-term exposure level reported by EFSA (2016)[31], for consumption of an infusion of borage (*Borago officinalis*), a PA-producing plant consumed by a part of the European population (Figure 4.2a). On the other hand, the EDI of 13.3 µg/kg bw/day, resulting from the consumption of 2 capsules of TR-17 three times a day, was in the same range as the estimated acute exposure of 11.55 or 25.82 µg/kg bw/day from consumption of one tablet/capsule of the PA-producing plants boneset (*Eupatorium perfolatum*) or hemp agrimony (*Eupatorium cannabinum*), respectively.[31] Moreover, as can be seen in Figure 4.2b, out of 17 jamu samples tested positive for PAs while not containing PA-producing botanicals, the EDI of only one sample exceeded 170 ng/kg bw/day. This value estimated by EFSA represents for mean adult consumers the upper end of the acute exposure range based on the reported contamination levels in the different food commodities combined.[5]

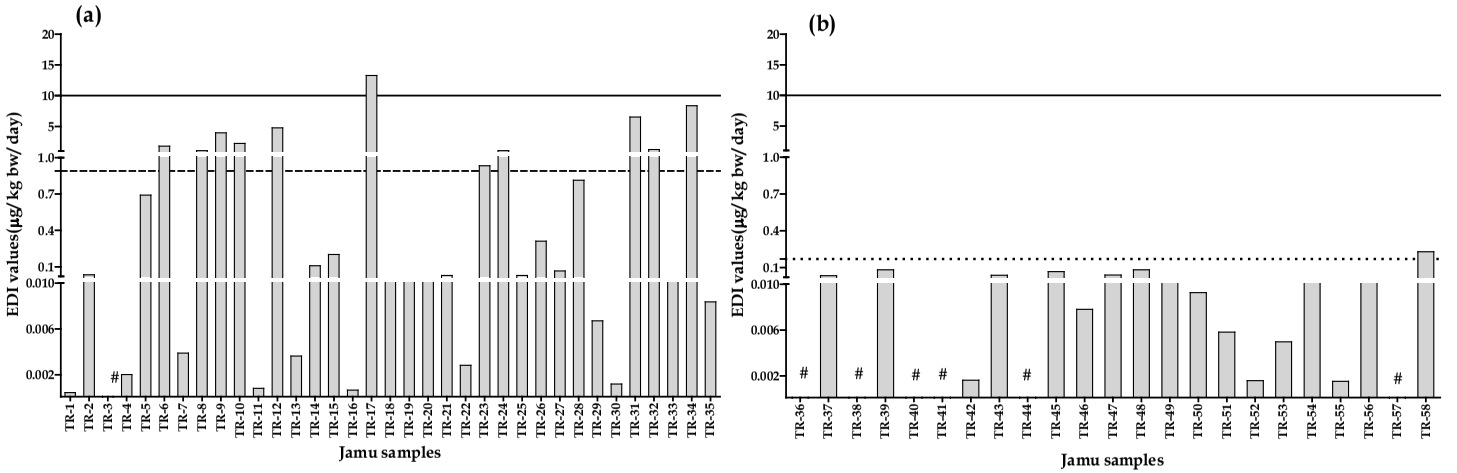

Figure 4.2. EDI values for the consumption of PA detected in (a) 35 jamu samples containing PA-producing botanicals and (b) 23 jamu samples containing non-PA-producing botanicals. EDI values were calculated as explained in the Materials and Methods section (Equation 4.1). The dashed line (- - -) in figure (a) represents the highest EDI of 890 ng/kg bw per day, estimated by EFSA for the acute/short term exposure resulting from consumption of infusions based on PA-producing plants in the European population.[5] The dotted line (.....) in figure (b) represent the EDI of 170 ng/kg bw/day, which is the high end of the acute exposure range for mean adult consumers estimated by EFSA, based on the contamination levels in the different food commodities combined.[5] The horizontal line in both figures represent the EDI value of 10 µg/kg bw/day, which is linked to the prevalence of HVOD in humans.[23] # indicates that the EDI value is not quantifiable due to a PA content < LOQ.

4.3.3.2. Chronic exposure scenario

The MOE values calculated for the jamu samples containing PA- and non-PA-producing botanicals, assuming daily lifetime consumption and 2 weeks of daily use every year during a lifetime, are depicted in Figure 4.3. The MOE values were calculated assuming equal potency for all PAs and using the $BMDL_{10}$ of riddelliine of 237 µg/kg bw/day as POD[5]. For 20 out of 35 (57%) jamu containing PA-producing botanicals the MOE values were below 10,000, indicating there is a potential health concern (Figure 4.3a). Consumption of jamu TR-17 and TR-34 resulted in MOE values of only 20 and 30, pointing at intake levels that are approaching the dose levels that caused liver tumours in rodent studies. Correcting for shorter-than-life-time exposure resulted in MOE values below 10,000 for 13 out of 35 (37%) jamu samples containing PA-producing botanicals (Figure 4.3a). MOE calculations for jamu samples containing non-PA-producing botanicals showed that 7 samples out of 23 would result in MOE values lower than 10,000 when assuming lifetime daily use, while there is no health concern (MOE >10,000) when these jamu would be consumed for a period of 2 weeks yearly during a lifetime (Figure 4.3b).

Figure 4.4 indicates the maximum number of weeks over a 69-year lifetime during which the jamu could be consumed based on the PA levels detected in the samples. From Figure 4.4a it follows for example that jamu TR-1 could be consumed without raising a concern for up to 184,480 weeks, corresponding to far more than a lifetime, and thus would be of little concern. On the other hand consumption of jamu TR-17 and TR-34 would be of no concern only when consumed for 6-10 weeks during a lifetime, what corresponds to one day or less per year. For jamu containing non-PA-producing botanicals, the maximum number of weeks of use that would result in an acceptable exposure during a lifetime exceeded 2 weeks every year (Figure 4.4b).

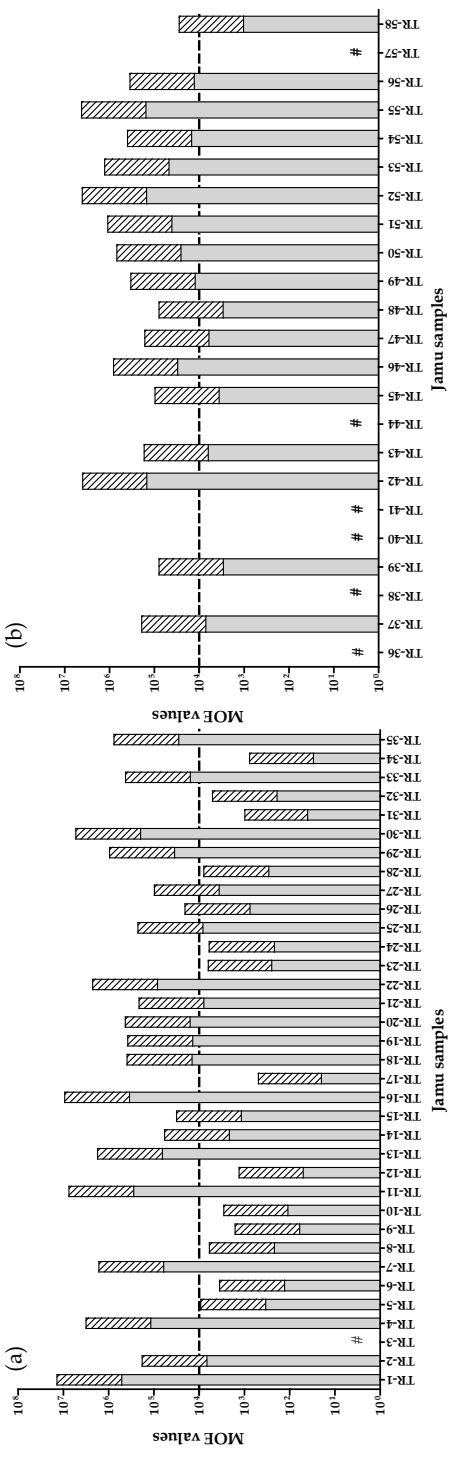

Figure 4.3. MOE values obtained for the consumption of jamu samples containing (a) PA-producing botanicals and (b) non-PA-producing botanicals based on daily lifetime exposure (grey bars) and based on exposure for 2 weeks every year during a lifetime exposure (patterned bars). MOE values were calculated as explained in the Materials and Methods section (Equation 4.2), using the $BMDL_{10}$ of 237 µg/kg bw/day for riddelliine.[5] The dashed line (- - -) represents the MOE value of 10,000 as a threshold for risk management action[24]. # indicates that no MOE value is calculated due to a PA content < LOQ.

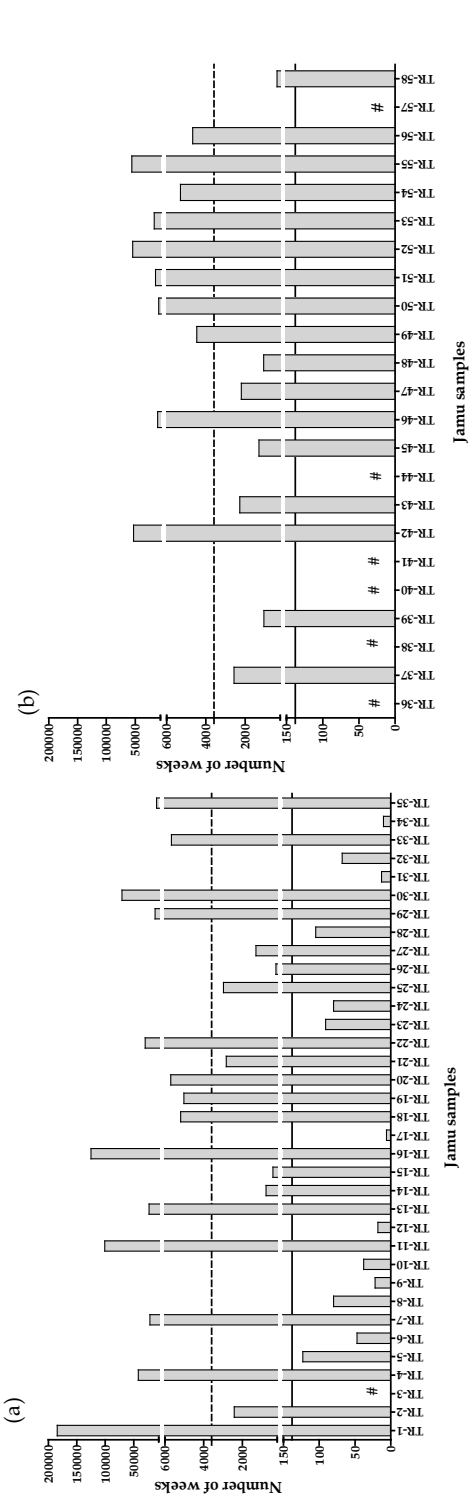

Figure 4.4. The number of weeks of use that would result in an MOE of 10,000 upon daily consumption of (a) jamu containing PA-producing botanicals and (b) jamu containing non-PA-producing botanicals. The calculations assumed equal potency of the different PAs using the $BMDL_{10}$ of 237 µg/kg bw/day for riddelliine.[5] The dashed (- - -) and horizontal line (—) represent the number of weeks equal to a lifetime (69 years = 3,588 weeks) and 2 weeks intake a year during a lifetime (138 weeks), respectively. # indicates that the EDI value is not quantifiable due to a PA content < LOQ.

4.3.4. Risk assessment of jamu containing non-PA-producing botanicals based on PA, AB and AA levels

Figure 4.5 presents the MOE values obtained for the exposure to PAs combined with the MOE results of a risk assessment on ABs detected in samples TR-36 to TR-56 and on AA levels detected in samples TR-57 and TR-58. It can be seen in Figure 4.5a that in 5 samples, TR-36, TR-38, TR-40, TR-41, TR-44, no PAs were detected (thus no MOE could be calculated), but that two of these samples (TR-36 and TR-38) with respect to their AB concentrations could be considered a priority for risk management, even when consumption is 2 weeks every year during a lifetime (Figure 4.5b). Overall the data presented in Figure 4.5 reveal that for samples TR36-TR56 collected in a targeted sampling approach for jamu containing AB-producing botanicals, the health risk due to exposure to ABs is substantially higher than the risk emerging from exposure to PAs from co-harvested PA-containing weeds.

Since the level of PAs in sample TR-57 was below the LOQ, it does not present a risk. However, since the list of botanical ingredients included Saussureae Radix and Magnoliae cortex, which are known to contain aristolochic acids, this sample was also analysed for the presence of AAs. The AAII level detected in this sample amounted to 10,500 ± 1,900 µg/kg, which resulted in MOE values for lifetime exposure and for 2 weeks exposure per year during a lifetime of 10.3 and 267.4, respectively, being both lower than 10,000 (insert in Figure 4.5), indicating a health concern. Jamu TR-58 consisted of *Aristolochia debile* as an AA producing botanical, and it contained AAI at 21,600 ± 6,000 and AAII at 9,600 ± 1,400 µg/kg. With a recommended use of 3.6 g per day this results in a MOE value for lifetime use of 4.8, and when consumed 2 weeks every year of 125, which are far below 10,000, indicating a health risk. This indicates that in TR-58 AAs present a larger concern than the PAs.

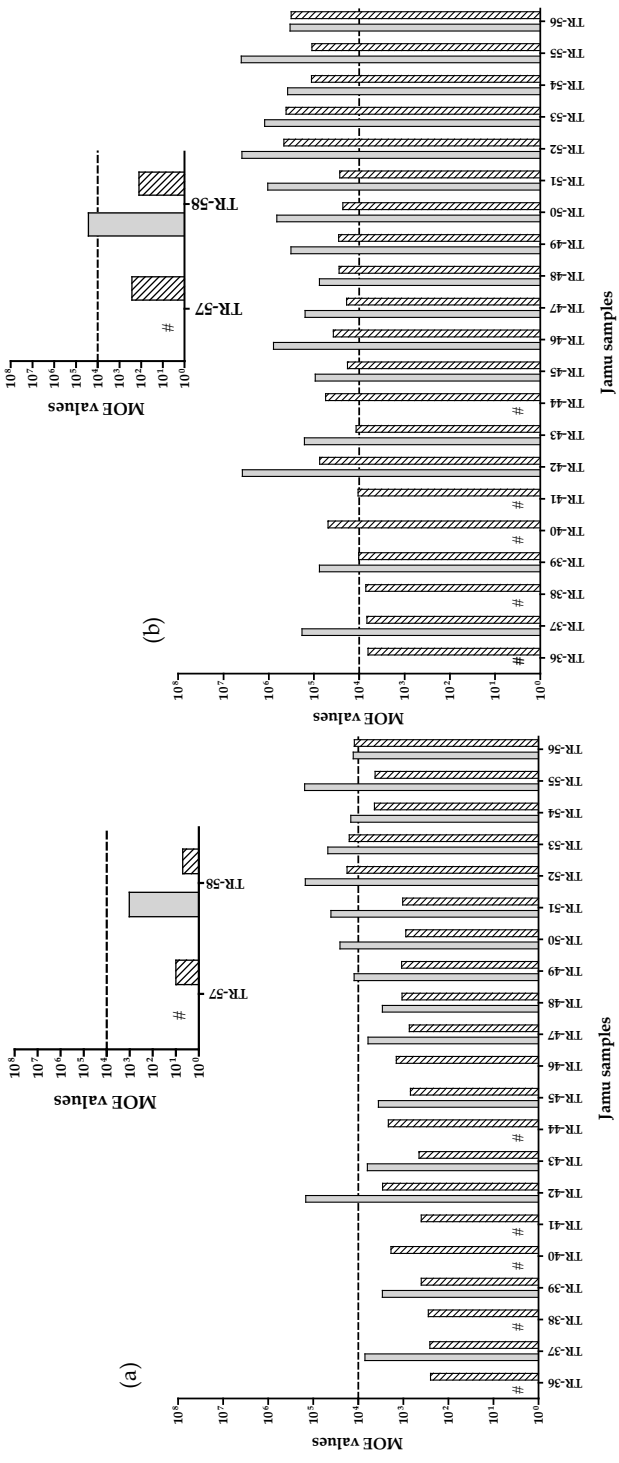

Figure 4.5. MOE values obtained for the consumption of 23 jamu samples containing non-PA-producing botanicals, based on (a) daily lifetime exposure and b) exposure for 2 weeks every year during a lifetime. MOE values based on PA levels (grey bars) were calculated as explained in the Materials and Methods section (Equation 4.2), using the $BMDL_{10}$ of 237 µg/kg bw/day for riddelliine.[5] The patterned bars are the MOE values based on the AB level previously detected in samples TR-36 to TR-56[3] using the $BMDL_{10}$ of 15.3 mg/kg bw for the major alkenylbenzene in the mixture, methyleugenol.[29] The inserted graph shows the MOE values calculated for the intake of PA levels (grey bars) compared to that for AAs (patterned bars) detected in jamu TR-57 and TR-58, using the $BMDL_{10}$ of 10 µg/kg bw/day [4]. The dashed line (- - -) represents the MOE value of 10,000 as a threshold for risk management action [24]. # indicates that no MOE value is calculated due to a PA content < LOQ.

4.4. Discussion

This study investigated the presence of pyrrolizidine alkaloids in jamu containing PA-producing botanicals and in jamu containing non-PA-producing botanicals, with the aim to assess whether there is a potential health risk for consumers of these preparations. This assessment is of interest considering the increasing number of jamu consumers, and the fact that botanical ingredients in jamu may contain PAs which, due to their hepatotoxic, genotoxic, and carcinogenic properties, can pose a potential risk for consumers.

The analysis of jamu revealed high total PA levels of up to 235,376 µg/kg in the samples containing PA-producing botanicals. Twenty-nine of the 35 PA-plant containing jamu products contained a *Gynura* species. The genus *Gynura* belongs to the tribe Senecioneae of the Asteraceae family, and the genus contains PAs typical for this well-known and broad family of plant species.[32] Interestingly, about half of these *Gynura* products contained high levels of PAs, while the other half contained relatively low levels. Many of the jamu samples made from *Gynura* that contain high PA levels show a specific profile in which otonecine-type PAs such as senkirkine, neosenkirkine, dehydrosenkirkine and various other otonecine-type analogues dominate. Senecionine and integerrimine are important PAs in these samples as well. This profile is very similar to that reported in a Chinese study for two closely related species, *G. bicolor* and *G. divaricata*.[33] Senkirkine, senecionine, integerrimine, seneciphylline, spartioidine and retrorsine, together with several unidentified otonecine and cyclic ester analogues were reported for these two species. Senecionine and senkirkine were reported as important constituents of *G. pseudo-china*.[34] Senecionine, integerrimine, retrorsine, usaramine, spartioidine, seneciphylline and seneciphyllinine (acetylseneciphylline) have been reported as characteristic PAs for *G. segetum* (syn. *G. japonica*).[17, 35, 36] These PAs are indeed present in the *Gynura* jamu samples high in PAs. However, 2 jamu samples, TR-5 (extract of *G. segetum*) and TR-9 (extract of *G. procumbens*), contain a rather different PA profile. Both contain high levels of jacobine, jacoline and jaconine and relatively low levels of the PAs mentioned above. Jacobine, jacoline and jaconine are (almost) absent in the other jamu samples containing *Gynura*.

Chen et al. (2017)[33] reported for 8 herbal samples of *G. bicolor* and *G. divaricata* a total PA content of 1,400-39,690 µg/kg. These levels are somewhat lower than present in the group of 14 jamu samples with a high PA content (12,173-235,376 µg/kg). In contrast, Ji et al. (2019)[37] reported very low PA levels in 12 herbal samples of *G. procumbens* (15.6-848 µg/kg), which would be in line with the results for the group of 15 samples of *Gynura*-containing jamu in which only low levels of PAs were found. The authors also investigated 8 commercial herbal products

containing *G. procumbens* and found 7 samples to contain low levels (9.9-160.5 µg/kg) as well. However, one commercial sample contained a high amount of PAs (33,900 µg/kg), what is in the range of the levels found in the high PA-group. Aizhen et al. (2019)[35] reported very high PA concentrations in batches of *G. japonica* (*segetum*) collected in China: in leaves the levels ranged from 460-2,860 mg/kg and in roots from 1,750-7,420 mg/kg.

Jamu TR-32 consisted of *Ageratum conyzoides*, a plant of the Boraginaceae family. This sample contained a high amount of PAs, 146,977 µg/kg, mainly composed of the monoesters echinatine, lycopsamine, intermedine, rinderine and their respective N-oxides. The composition is in general agreement with literature[38, 39] that report lycopsamine and echinatine as main constituents (together with acetyllycopsamine and dihydro analogues).

In 17 out of 23 jamu samples that had no PA-producing plants listed on their label, PAs were detected with the highest level amounting to 3,421 µg/kg. This points at contamination with PA-producing plants that may be caused by the co-harvesting of PA-containing weeds during cultivation or harvesting of the materials. Contamination with PA-producing plants has been reported for herbal teas[6, 40, 41] and Chinese herbal medicines[21]. In the jamu samples mono-esters such as rinderine, indicine, lycopsamine and echinatine as well as their respective N-oxides were the most frequently present. These mono-esters are typically found in species of the Boraginaceae family.[42]

The present study revealed a very wide variation in the EDI of PAs resulting from consuming the different jamu containing PA-producing herbs. This is due to the fact that there was a difference in their total PA levels, but also a wide range in the recommended daily use of the samples as indicated on the label, varying from 0.4 to 30 g per day for certain powders and up to 200 ml per day for liquids. The highest EDI of 715.8 µg/person/day was calculated for TR-17 based on the EDI of 13.3 µg/kg bw/day and a body weight of 54 kg for Indonesian people.[22] This EDI exceeded the transitional limit of intake of 1.0 µg PAs per day per person, set by HMPC (2016)[18] for herbal traditional medicinal products more than 700-fold. Considering the high level of PAs detected in a large proportion of *Gynura*-based jamu, strict monitoring and quality control of these products may be necessary to reduce the related health risk for consumers.

The average EDI of 0.038 µg/kg bw/day resulting from use of jamu containing non-PA-producing botanicals was 37 times lower than the average EDI of 1.4 µg/kg bw/day from use of the jamu containing PA-producing botanicals. Notwithstanding the much lower levels, exposure to PAs resulting from contamination of jamu products may contribute to the total dietary intake of PAs.

Edgar et al. (2011)[43] reported that exposure to PAs via contamination of some widely consumed foods (e.g. grains, milk, meat, eggs, honey, pollen) can exceed the maximum tolerable daily intakes and/or maximum levels determined by a number of independent risk assessment authorities. The results of the present study underlines the importance of vigilance and the establishment of good manufacturing practises with respect to the harvesting and handling of plant materials used in jamu in Indonesia to reduce the contamination of jamu with PAs-producing weeds in order to protect their consumers.

The risk assessment based on acute exposure showed that use of jamu for short-term periods of, for example, 4 days up to 2 weeks does not raise a health concern for acute adverse effects in humans because the EDI based on PAs levels detected in all jamu samples were far below the value of 1-3 mg/kg bw/day as reported by EFSA to result in acute human toxicity based on available case studies.[5] However, the EDI value of TR-17 indicate that there is a concern for the prevalence of HVOD in humans because this EDI may exceed the daily intake associated with HVOD of 10 µg/kg bw/day and 15 µg/kg bw/day reported by WHO-IPCS (1988)[23] and Ridker et al. (1985)[44], respectively. Consumption of PA-containing *G. segetum* in the form of Chinese herbal products for 5 days up to 2 years reportedly caused PA-induced liver injury (PA-ILI) in 15 patients in China.[45] The herbs ingested by the patients contained seneciphylline, senecionine, and their N-oxides as predominant PAs at levels ranging from 274 to 13,645 mg/kg. Wang et al. (2018)[46] in a retrospective study reported that *Gynura segetum*-induced HVOD patients show 5-year surrvival rates of 57%, underlining the importance to prevent the potent toxicity of *G. segetum*. The mode of action behind the PA-ILI and *Gynura segetum*-induced HVOD is linked to pyrole-protein adduct formation resulting upon bioactivation of the PA to reactive pyrrole metabolites by cytochrome P450 enzymes.[45, 47, 48] Although in most cases the PA levels in the Indonesian jamu are lower than the concentrations reported for *Gynura* in the Chinese studies, the risk of PA-ILI and HVOD due to exposure to PAs via *Gynura*-based jamu cannot be neglected, particularly for regular consumers. It clearly indicates that, in addition to concerns over the genotoxic carcinogenicity, some jamu also raise a concern with respect to PA-ILI and HVOD, further supporting the need for risk management actions.

When considering the chronic exposure, the MOE values for the PAs occurring in the jamu samples show that for 20 out of 35 (57%) jamu products containing PA-producing botanicals this MOE was lower than 10,000 indicating there would be a concern for human health upon daily lifetime exposure. However, in real life jamu is likely to be used for medical purposes, so that Indonesian people

tend to use the preparations for short intervals albeit on a regular basis. Therefore, an estimate of the risks accompanying this shorter-than-lifetime exposure (2 weeks every year during a lifetime) was made applying Haber's rule and resulted in MOE values that were 26 times higher than the MOE values for lifetime daily exposure. For this shorter-than-life-time exposure scenario MOE values < 10,000 indicated that there is still a health concern for 13 out of the 35 (37%) jamu samples containing PAs-producing botanicals. MOE values <10 000 were also obtained for daily lifetime consumption of 7 out of the 17 positive jamu samples containing non-PA-producing herbs but found to be contaminated with PA-producing weeds. Their consumption for only 2 weeks a year during lifetime, however, did not raise a concern for human health.

It is important to note that the present risk assessment is based on the assumption of equal potency of all PAs detected in the samples and comparison to the $BMDL_{10}$ of riddelliine, without taking into account differences in relative potency of the PAs present in the samples. Merz and Schrenk (2016)[49] defined interim Relative Potency (REP) factors for the toxic and genotoxic potency of 1,2-unsaturated PAs based on the available data on the genotoxic potency in *Drosophila melanogaster*, the cytotoxic potency in vitro in chicken hepatocellular carcinoma (CLR-2118) cells and their acute toxicity in adult rodents. Most recently Louisse et al. (2019)[50] proposed REP factors based on results obtained in the γH2AX assay in HepaRG human liver cells for 37 PAs showing that open diester PAs (including lasiocarpine) and cyclic diester PAs (including riddelliine) display the highest potency. Taking into account the REP values in the evaluation of jamu can be useful to refine the risk assessment of these products and to facilitate a proper management action of these traditional medicines. However, given that the REP values for most of the major PAs detected in the jamu samples would be 0.3 or 1.0 it is expected that taking the REP values into account would not substantially change the outcomes of the risk assessment. This would be in line with the results from a previous risk assessment for PA-containing herbal teas and food supplements.[33]

Further evaluation of the results obtained in the present study revealed that for samples containing non-PA-producing botanicals, but collected in a targeted sampling for AB-containing botanicals, the risk assessment based on PA, AB and AA levels reveals that the presence of co-harvested PAs is in general of a lower concern than the levels of ABs and AAs present in these samples. This result indicates that risk management should focus on providing information to jamu producers regarding the genotoxic carcinogenic compounds that can naturally occur in specific botanicals, to minimize exposure to these compounds via consumption of jamu. In

addition, regulations that control the use of *Gynura* plants in jamu need to be established.

In conclusion, consumption of Indonesian jamu that consist of PA-producing botanicals can be considered safe only when consumed for less than about 6 weeks during a lifetime. In addition, the results of the risk assessment highlight the need for monitoring actions and to update the process and regulation of manufacturing jamu, with the aim to reduce the level of PAs that occur in these products either naturally or via contamination. Applying Good Agricultural and Collection Practices (GACP) and the establishment of control measures may help to reduce potential PA contamination in jamu.

Conflict of Interest

The authors state no conflict of interest regarding this manuscript.

Acknowledgements

Financial support was received from the Indonesian Endowment Fund for Education, Ministry of Finance, Republic of Indonesia through a Beasiswa Pendidikan Indonesia Lembaga Pengelola Dana Pendidikan (BPI LPDP) doctoral scholarship for Suparmi [contract number: PRJ-365/LPDP/2016].

The authors want to thank Elena de Vries for her assistance in the analysis of the jamu samples.

Supplementary materials

Supplementary materials of this article can be downloaded from https://doi.org/10.1016/j.fct.2020.111230

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BENEFIT
RISK

Chapter 5

Monocrotaline-induced liver toxicity in rat predicted by a combined in vitro-physiologically based kinetic modeling approach

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Submitted to: Archives of Toxicology (2019): *Under review*

Abstract

The aim of the present study was to use an in vitro-in silico approach to predict the in vivo acute liver toxicity of monocrotaline and to characterize the influence of its metabolism on its relative toxic potency compared to lasiocarpine and riddelliine. In the absence of data on acute liver toxicity of monocrotaline upon oral exposure, the predicted dose-response curve for acute liver toxicity in rats and the resulting benchmark dose lower and upper confidence limits for 10% effect ($BMDL_{10}$ and $BMDU_{10}$) were compared to data obtained in studies with intraperitoneal or subcutaneous dosing regimens. This indicated that the predicted $BMDL_{10}$ value to be in line with No-observed-adverse-effect-levels (NOAELs) derived from availabe in vivo studies. The predicted $BMDL_{10}$-$BMDU_{10}$ of 1.7-6.3 mg/kg bw/day also matched the oral dose range of 1-3 mg PA/kg bw/day at which adverse effects in human are reported. A comparison to the oral toxicity of the related pyrrolizidine alkaloids (PAs) lasiocarpine and riddelline revealed that, although in the rat liver hepatocytes study monocrotaline was less toxic than lasocarpine and riddelliine, due to its relatively inefficient clearance its in vivo acute liver toxicity was predicted to be comparable. It is concluded that the combined in vitro-PBK modeling approach can provide insight in monocrotaline-induced acute liver toxicity in rats thereby filling existing gaps in the database on PA toxicity. Furthermore, the results reveal that the kinetic and metabolic properties of PAs can vary substantially and should be taken into account when considering differences in relative potency between different PAs.

5.1. Introduction

Monocrotaline (Figure 5.1) is a secondary metabolite that belongs to a group of cyclic di-ester 1,2-unsaturated pyrrolizidine alkaloids (PAs). It is naturally present in Crotalaria species including *Crotalaria spectabilis*, *C. sagittalis* L., *C. retusa* L., and *C. aegyptiaca* Beth.[1, 2] High acute toxicity of monocrotaline towards animals and humans has been reported.[3-6] Recently EFSA (2017)[7] listed monocrotaline as one of the 17 PAs to be monitored for their presence in food and feed because of possible concern for human health related to exposure to these PAs via food including consumption of tea and herbal infusions. PAs including monocrotaline are of concern because of their hepatotoxicity and the fact that they are genotoxic carcinogens.[7] Monocrotaline is categorized as being possibly carcinogenic in humans (category 2B).[8]

Like all 1,2-unsaturated PAs monocrotaline is a pro-toxin (unreactive compound) requiring hepatic metabolic activation by cytochromes P450 to exert hepatic toxicity and carcinogenicity.[9, 10] CYP2A6 and CYP2E1 were found to be the major P450s active in metabolic activation of monocrotaline in rat liver.[11, 12] Metabolism of PAs is generally occurring via three pathways, namely hydrolysis, N-oxidation, and hydroxylation followed by dehydrogenation (Figure 5.1).[13] Upon this dehydrogenation an unstable and highly reactive intermediate, named dehydromonocrotaline is formed. Dehydromonocrotaline can react with cellular macromolecules including proteins and DNA to form protein- and DNA-adducts, which are considered to be responsible for the toxicity including the genotoxicity of monocrotaline.[14-16] Alternatively, dehydromonocrotaline can be detoxified through hydrolysis resulting in 6,7-dihydro-7-hydroxy-1-hydroxymethyl-5*H*-pyrrolizine (DHP) and via glutathione (GSH) conjugation resulting in formation of GSH-DHP and di-GSH-DHP (Figure 5.1). These molecules are considered less toxic and more stable,[13, 17] although they may still also react with proteins and DNA to form the same DNA adducts formed by dehydromonocrotaline and DHP.[18]

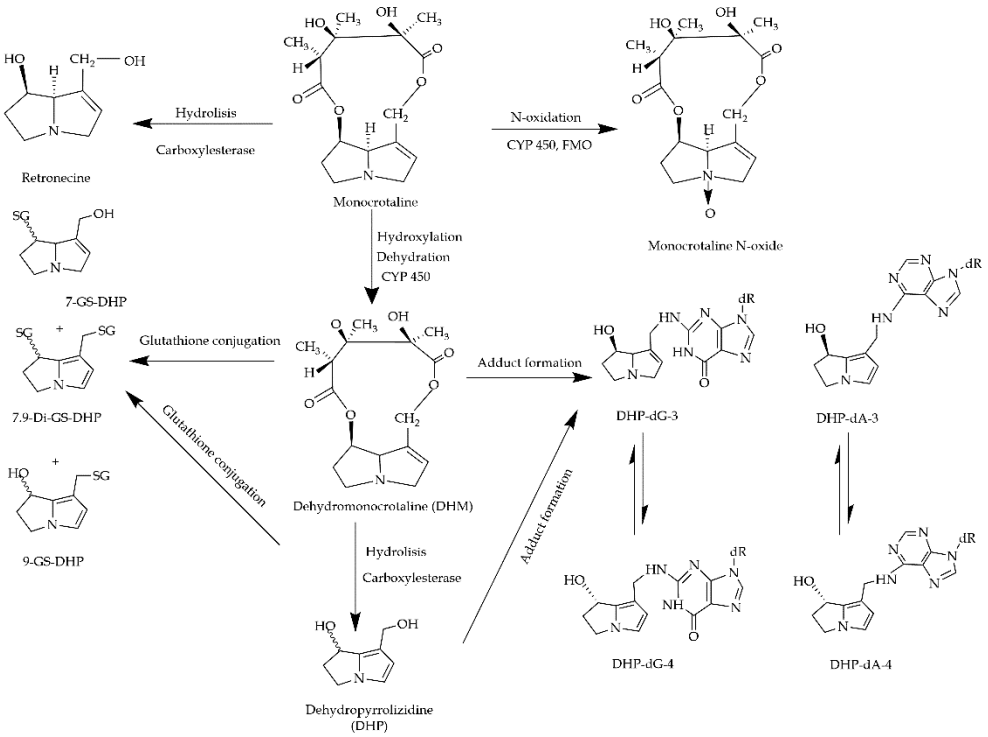

Figure 5.1. Schematic diagram of the metabolic pathways of monocrotaline and DNA adduct formation by monocrotaline metabolites relevant for rat and human.[11, 18-20] FMO = flavin-containing monooxygenase, CYP 450 = cytochromes P450.

Upon bioactivation, monocrotaline causes a variety of toxic insults including pulmonary endothelial apoptosis, acute lung injury, pulmonary fibrosis, necrotizing pulmonary arteritis, myocarditis, hepatic veno-occlusive disease (HVOD), pulmonary hypertension, and right ventricular hypertrophy,[9, 21-25] in addition to an increased risk of developing liver carcinomas.[26] In human, acute exposure to PAs can cause HVOD with severe liver damage with in some cases fatal outcomes,[27, 28] whereas chronic exposure is considered to increase the risk of developing cancer.[7]

However, only for a limited number of 1,2-unsaturated PAs in vivo toxicity data are available, and this implies that alternative testing strategies including read-across and in vitro to in vivo extrapolation (IVIVE) become important. In previous studies we reported the development and evaluation of physiologically-based kinetic (PBK) models for the PAs lasiocarpine and riddelliine for rat and human, and their use for conversion of in vitro data for toxicity in primary hepatocytes to quantitatively predict in vivo acute liver toxicity for both rat and human.[29, 30] Marked differences in toxicokinetics were observed between these two PAs influencing the predicted in vivo toxicity. This importance of toxicokinetics in the

relative differences in toxic potency between different PAs was also noted in a recent study that characterized the intrinsic relative potency of a series of PAs showing a role for the rate and extent of their metabolism.[31] The aim of the present study was to use the in vitro-PBK model facilitated reverse dosimetry approach to predict the in vivo acute liver toxicity of monocrotaline and to characterize the influence of its metabolism on its relative toxic potency compared to lasiocarpine and riddelliine. Monocrotaline was selected as the model compound because this is one of the few PAs in addition to lasiocarpne and riddelliine for which in vivo data on kinetics and liver toxicty are available thus enabling evaluations of the PBK model and predictions made.

5.2. Material and Methods

5.2.1. Chemicals and biological materials

Monocrotaline (>98%) was purchased from MedChemExpress (Huissen, The Netherlands). The plateable cryopreserved male rat (Sprague-Dawley) hepatocytes (RTCP10™), the thawing and plating supplement (serum-containing, CM 3000) pack, the cell maintenance supplement pack (serum free, CM4000), and Williams E Medium without phenol red (WEM, A1217601) were purchased from ThermoFisher (Naarden, The Netherlands). Pooled liver and intestinal microsomes from male Sprague-Dawley rats were purchased from Xenotech (Lenexa, USA). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium). Acetonitrile (UPLC/MS grade) was obtained from Biosolve (Valkenswaard, The Netherlands). Potassium hydrogen phosphate (K_2HPO_4) and trifluoroacetic acid (TFA) were purchased from Merck (Darmstadt, Germany). Fetal calf serum (FCS) and the reduced form of β-nicotinamide adenine dinucleotidephosphate sodium salt hydrate (NADPH) were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) solution was purchased from Roche (Woerden, The Netherlands). Rapid equilibrium dialysis (RED) devices were purchased from Thermo Fisher Scientific (Bleiswijk, The Netherlands). Phosphate-buffered saline (PBS) was obtained from Invitrogen (Breda, The Netherlands).

5.2.2. Outline of the PBK modeling-facilitated reverse dosimetry approach

The prediction of in vivo monocrotaline induced liver toxicity in rat using a combined in vitro-PBK modeling approach consisted of the following steps: (1) establishment of in vitro concentration-response curves for the toxicity of monocrotaline in primary rat hepatocytes, (2) development of a PBK model describing in vivo kinetics of monocrotaline, using kinetic parameters defined based

on in vitro assays using rat liver and intestinal samples, (3) evaluation of the PBK model predictions against available literature data on dose dependent blood levels of monocrotaline, (4) translation of the in vitro concentration-response curves for acute liver toxicity into in vivo dose-response curves for acute liver toxicity in rat using the PBK model taking into acount differences in protein binding of monocrotaline in the in vitro and in vivo situation, (5) benchmark dose (BMD) analysis on the predicted in vivo dose-response data to obtain a point of departure (POD), and (6) evaluation of the predicted POD for liver toxicity against available literature data.

5.2.3. In vitro liver toxicity assay with primary rat hepatocytes

The monocrotaline-induced liver toxicity was tested in vitro using the WST-1 assay which measures the formazan formation by the metabolically active cells from WST-1. Pooled cryopreserved plateable male rat (Sprague-Dawley) hepatocytes (RTCP10™) were thawed and seeded in accordance with the manufacturer's protocol. Briefly, cells were seeded in 96-well plates (Greiner bio-one, Alphen aan den Rijn, The Netherlands) at a concentration of 5×10^5 cells/ml to give 1.25×10^4 cells/well and incubated at 37 °C, 5% CO_2 in a humidified atmosphere for 6 h to allow cell adherence. After incubation, medium was aspirated and then replaced by 100 µl/well of exposure medium (serum free) containing the required concentration of monocrotaline. The cells were incubated for 24 h at increasing concentrations (0 - 600 µM) of monocrotaline in exposure medium added from 200 times concentrated stock solutions in DMSO. The solvent DMSO (0.5% (v/v) final concentration in exposure medium) was used as a negative control and triton X (final concentration 1% (v/v) in exposure medium) served as a positive control in all cytotoxicity assays. After exposure for 24 h, 5 µl (1:20 dilution) WST-1 reagent were added to each well and plates were incubated for an additional 1 h. Then, the plate was shaken at 1000 rpm for 1 min, and absorbance was measured at 440 nm (background absorbance at 620 nm was subtracted) using a SpectraMax M2 (Molecular Devices, Sunnyvale, USA).

Data are presented as mean values ± SE from three independent experiments with 3 different batches of rat hepatocytes. The cell viability was expressed as percentage of the solvent control, with the solvent control set at 100%. The obtained concentration–response curves for hepatoxicity were fitted with a symmetrical sigmoidal model (Hill slope) which was further used to derive IC_{50} values using log (inhibitor) vs. normalized response using GraphPad Prism software (version 5.00 for Windows, GraphPad software, San Diego, USA).

5.2.4. In vitro incubations of monocrotaline with rat liver and intestinal microsomes to derive the kinetic parameters for the PBK model

The kinetic parameters for the PBK model of monocrotaline in rats were estimated by a substrate depletion approach using the protocol for microsomal incubations reported by Wang et al. (2009)[19] with little modifications. The liver microsomal incubations were carried out in a total volume of 100 µl containing 0.1 M K_2HPO_4 (pH 7.4), 0.5 mg protein/ml of pooled rat liver/intestinal microsomes, and monocrotaline at final concentrations ranging from 0 to 500 µM (added from 100 times concentrated stock solutions in 0.1 M HCl, the latter in line with the protocol of Wang et al. (2009)[19], and shown to have no effect on the incubation pH). After 5 min of pre-incubation in a shaking water bath at 37 °C, the reactions were started by the addition of 1 mM NADPH. The reactions were carried out for 1 h and 2 h for liver and intestinal microsomes, respectively. For each incubation, a corresponding control incubated in the absence of NADPH was included by adding buffer instead of NADPH. To stop the metabolic conversion, 100 µl of ice-cold methanol were added and the sample was put on ice, then centrifuged at 5,000 × g for 20 min at 4°C using a microcentrifuge (CT15RE, VWR, Leuven, Belgium). Supernatants were diluted 200 times in 90% (v/v) acetonitrile and transferred to LC-MS vials. LC-MS analysis was performed using a Shimadzu Nexera XR LC-20AD SR UPLC system in tandem with a Shimadzu LCMS-8040 mass spectrometer (Shimadzu, Kyoto, Japan). From each incubation, 1 µl of supernatant was loaded onto a Luna Omega polar C18 100A LC column (1.6 µm 100 × 2.1 mm, Phenomenex) fitted with a FP polar precolumn (Phenomenex), using a flow rate of 0.3 ml/min. The temperature was set at 40 °C and 5 °C for column and sample, respectively. The mobile phase consisted of ultrapure water (solvent A) and acetonitrile (solvent B) both containing 0.1% (v/v) formic acid. The gradient began with 100% solvent A (0% B) for 1 min to wash away unwanted salts, followed by a linear gradient from 0 to 5% B till 8 min and a further increase to 100% B in 2 min, keeping the elution at 100% B for 0.5 min, then the column was set back to the starting conditions and equlibrated for 3.4 min before the next injection. The concentration of monocrotaline in the samples was quantified using a calibration curve prepared using a commercially available standard. For all incubations, three independent replicates were performed.

The time dependent decrease in the concentration of monocrotaline detected in NADPH containing reaction mixtures corrected for the time dependent decrease in the concentration of monocrotaline in the corresponding controls without the cofactor NADPH was used to determine the rate of monocrotaline depletion. The data for the monocrotaline concentration dependent rate of monocrotaline depletion

thus obtained were fitted to the standard Michaelis–Menten equation (Equation 5.1) using GraphPad Prism, 5.0 software (San Diego, CA, USA).

$$V = \frac{V_{max} \times [S]}{(K_m + [S])} \quad \text{(Equation 5.1)}$$

with [S] representing the monocrotaline concentration, V_{max} being the apparent maximum velocity (nmol/min/mg microsomal protein), and Km being the apparent Michaelis-Menten constant (µM). The ratio of V_{max} and K_m was calculated as the in vitro catalytic efficiency (k_{cat}) expressed in nmol/min/g tissue. The rat microsomal protein yield of 35 mg microsomal protein/g tissue and 20.6 mg microsomal protein/g tissue for liver and small intestine, respectively[32, 33] were used to scale V_{max} and k_{cat} values obtained from the in vitro microsomal incubations to in vivo V_{max} and k_{cat} values expressed in nmol/min/g tissue and ml/min/g tissue, respectively. The rat liver weight of 8.5 g and small intestine weight of 3.5 g (see Table 5.1)[34] were used to scaled the in vivo k_{cat} values to values expressed in ml/min/tissue.

5.2.5. Determination of fraction unbound (f_{ub}) of monocrotaline in rat serum and correction for protein binding

The monocrotaline-induced liver toxicity is assumed to be dependent on the concentration of unbound monocrotaline available for bioactivation. To correct for the difference in protein binding in the in vitro incubations and the in vivo situation the fraction unbound (f_{ub}) of monocrotaline in the in vitro and in vivo situations was determined. Since the in vitro toxicity was determined in serum-free assay medium the concentrations of monocrotaline tested were considered to be equal to the unbound concentration in the assay ($f_{ub,in\ vitro} = 1.0$). The $f_{ub,\ in\ vivo}$ was determined by rapid equilibrium dialysis (RED).[35] Briefly, 200 µl of spiked rat serum containing 150 µM monocrotaline (final concentration, 0.5% v/v DMSO) were added to the serum chambers of the RED device insert, while 350 µl dialysis buffer (PBS) were added to the buffer chamber. The device was sealed with tape and incubated at 37 °C on a shaker at 250 rpm. After incubation for 5 h when the system reached equilibrium[36], 50 µl of post-dialysis samples were collected from the serum and buffer chambers into separate eppendorf tubes. Subsequently, 50 µl of rat serum was added to the buffer samples and 50 µl of PBS was added to the serum samples. To precipitate the protein, 300 µl of ice-cold acetonitrile (90% v/v) was added to both tubes. After putting the mixtures on ice for 30 min, the mixtures were centrifuged at 15,000 g for 30 min at 4 °C, and the supernatants were diluted 5 times in 90% (v/v) acetonitrile and analyzed by LC-MS as described above. The measurements were performed in triplicate.

The concentration of monocrotaline detected in each chamber was used to calculate $f_{ub,\ in\ vivo}$ using Equation 5.2.[35, 36] The value of $f_{ub,\ in\ vivo}$ was used in the PBK modelling-based reverse dosimetry to calculate the total concentration of monocrotaline in rat liver blood according to the Equation 5.3.

$$f_{ub,in\ vivo} = \frac{C_b}{C_s} \quad \text{(Equation 5.2)}$$

$$C_{monocrotaline,rat\ blood} = \frac{C_{ub,in\ vitro}}{f_{ub,in\ vivo}} \quad \text{(Equation 5.3)}$$

where $f_{ub,\ in\ vivo}$ represents the fraction unbound of monocrotaline in rat serum, C_b is the concentration of monocrotaline in the buffer chamber (μM), C_s is the concentration of monocrotaline detected in the serum chamber (μM), $C_{monocrotaline,\ rat\ blood}$ is the total concentration of monocrotaline in rat blood (μM), $C_{ub,\ in\ vitro}$ is the unbound concentration of monocrotaline in the in vitro culture medium which in the present study equals the concentration tested because $f_{ub,in\ vitro}$ equals 1.0.

5.2.6. Development and evaluation of a PBK model for monocrotaline in rat

A PBK model for monocrotaline in rat was developed based on the models for lasiocarpine and riddelliine in rats (Chen et al., 2018)[30]. Figure 5.2 depicts the conceptual PBK model, which consists of 7 separate compartments connected via the blood circulation. The physiological and anatomical parameters for rats were obtained from literature[34], while the blood/tissue partition coefficients for monocrotaline were estimated using the formula reported by DeJongh et al. (1997)[37] based on the water/octanol partition coefficient (log Kow) of monocrotaline of -0.65 predicted by ChemDraw 18.1 (Perkin-Elmer, USA) as presented in Table 5.1.

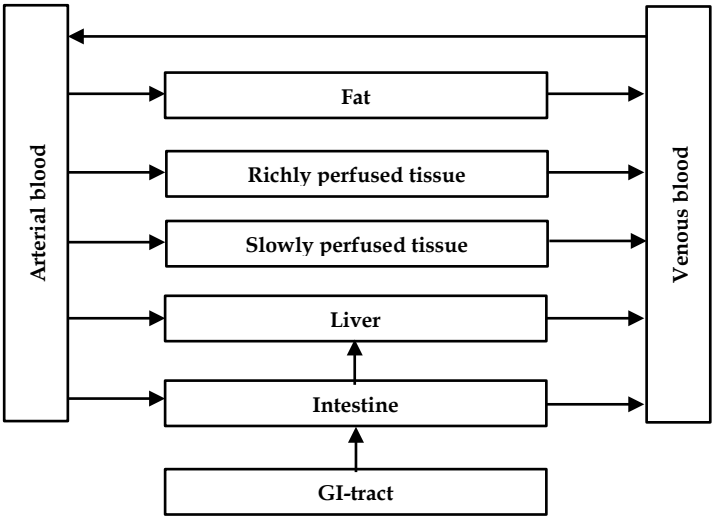

Figure 5.2. Schematic diagram of the PBK model for monocrotaline in rat, based on the model previously developed for lasiocarpine and riddelliine.[30]

Table 5.1. Physiological and physicochemical parameters for rats applied in the PBK model for monocrotaline.

Parameters	Symbol	Value
Physiological parameters[34]		
Body weight (kg)	BW	0.25
Tissue volume (fraction of body weight)		
Fat	VFc	0.07
Liver	VLc	0.034
Small intestine	VSic	0.014
Blood	VBc	0.074
Richly perfused tissue	VRc	0.042
Slowly perfused tissue	VSc	0.75
Cardiac output ($L/h/kg^{0.74}$)	QC	15
Blood flow to tissue (fraction of cardiac output)		
Fat	QFc	0.07
Liver	QLc	0.132
Small intestine	QSic	0.118
Richly perfused tissue	QRc	0.51
Slowly perfused tissue	QSc	0.17
Physicochemical parameters[37]		
Tissue/blood partition coefficients		
Fat	PF	0.46
Liver	PL	0.77
Small intestine	PI	0.77
Richly perfused tissue	PR	0.77
Slowly perfused tissue	PS	0.42

The absorption rate constants (K_a) for the uptake of monocrotaline from the intestinal compartment into the liver was estimated based on the reported K_a for adonifoline, using the correlation of Caco-2 permeation and molecular properties described in literature[38], as follows:

$$\log P_{app} = -5.469 + 0.236 \log P \quad \text{(Equation 5.4)}$$

$$\frac{\log P_{app\ monocrotaline}}{Ka_{\ monocrotaline}} = \frac{\log P_{app\ \text{adonifoline}}}{Ka_{\ \text{adonifoline}}} \quad \text{(Equation 5.5)}$$

where log P_{app} is the log value of the permeability coefficient (P_{app}), logP is the water/octanol partition coefficient predicted by ChemDraw 18.1 (Perkin-Elmer, USA) being -0.65 and -1.49 for monocrotaline and adonifoline, respectively. The $logP_{app\ monocrotaline}$ and $logP_{app\ adonifoline}$ calculated by Equation 5.4 are -5.62 for monocrotaline and -5.82 for adonifoline. Using the the reported K_a for adonifoline of 0.6/h, the value of Ka monocrotaline derived from Equation 5.5 is 0.58, which was used in the PBK modeling. This Ka value was assumed to reflect efficient uptake of monocrotaline and thus to also include the potential contribution of the organic cation transporter 1 (OCT1) reported by Tu et al. (2013)[39] to play a role in active transport of monocrotaline into the liver.

The model code in Berkeley Madonna (version 9.1.14, UC Berkeley, CA, USA) using Rosenbrock's algorithms for stiff systems for the developed PBK models of monocrotaline in rats is presented in Supplementary materials 1. In the PBK model

the excretion of monocrotaline into urine was not included due to the fact that the excretion of monocrotaline as a parent compound in urine is negligible.[40]

5.2.7. Evaluation of the PBK model

To evaluate the PBK model performance, predicted monocrotaline concentrations in blood were compared to reported concentrations of monocrotaline equivalents in rat blood upon intravenous (iv) injection of 60 mg/kg bw (10 µCi/kg) of [^{14}C] monocrotaline.[41] To this end the predicted time dependent monocrotaline concentration in blood was compared to the time dependent monocrotaline equivalent concentration curve reported by Estep et al. (1991)[41] which was derived from the published curve of monocrotaline equivalents (in nmol/g) against time (in h) using webPlotDigitizer (https://automeris.io/WebPlotDigitizer/) under the assumption that the weight of blood plasma (g) is equal to the volume of blood (mL). The final concentration of monocrotaline equivalents (µM) in whole blood was obtained by added up the concentration values in plasma and in red blood cells (Supplementary materials 2, Figure S1 and Table S1).[41]

In addition a sensitivity analysis was performed to identify the key parameters which contribute most to the predicted maximum concentrations in liver blood at an oral dose of 1 and 3 mg/kg bw which representing the lowest and highest dose in the range for the estimated daily human intake of PAs reported by EFSA (2017)[7] that might result in adverse health effects if consumed for 4 days up to a 2 weeks periods.

The sensitivity analysis was performed as described previously[42] calculating normalized sensitivity coefficients (SCs):

$$SC = \frac{(C'-C)}{P'-P} \times \left(\frac{P}{C}\right) \qquad \text{(Equation 5.6)}$$

where C is the initial value of the model output, C′ is the modified value of the model output resulting from an increase in parameter value, P is the initial parameter value and P′ is the modified parameter value. Each parameter was analyzed individually by changing one parameter at a time (5% increase) and keeping the other parameters the same.[42]

5.2.8. Translation of in vitro liver toxicity to in vivo liver toxicity

The in vitro concentration–response curve for monocrotaline-induced cytotoxicity in primary rat hepatocytes was translated into a predicted in vivo dose–response curve for acute liver toxicity using PBK modeling-facilitated reverse dosimetry. Within this translation a correction was made to take the difference in protein binding between the in vitro incubations ($f_{ub,\ in\ vitro}$=1.00) and the in vivo

situation ($f_{ub,\ in\ vivo}$ determined as described above) into account. This was done because it was assumed that only the free fraction of monocrotaline will be available to be bioactivated and exert the effects. Each concentration tested in the cytotoxicity assay, corrected by Equation 5.3 to calculate the corresponding total blood concentration, taking differences in in vitro and in vivo protein binding into acount, was set equal to the maximum concentration of monocrotaline in the liver blood and the developed PBK model was used to determine the coresponding oral dose. The dose response curve for monocrotaline-induced liver toxicity resulting from this translation was compared to the previous predicted dose-response curves for lasiocarpine and riddelliine.[30]

5.2.9. BMD analysis of in vitro concentration-response data and of predicted in vivo dose-response data

To define the benchmark dose resulting in a 10% increase in liver toxicity over the background level (BMD_{10}) the predicted in vivo dose-response data for monocrotaline-induced acute liver toxicity in rats were used for BMD modeling. To compare the toxic potency of monocrotaline with that of lasiocarpine and riddelliine, the predicted dose response curves reported previously for these PAs[30] was also used for BMD modeling. Dose-response modeling and BMD analysis were performed using the EFSA BMD modeling webtool (PROAST version 66.38, https://shiny-efsa.openanalytics.eu/app/bmd).[43] The lowest Akaike Information Criterion (AIC) value among the available models were used to judge the the goodness of fit application of the models.

5.2.10. Evaluation of the predicted PoD for liver toxicity against available literature data

The predicted $BMDL_{10}$-$BMDU_{10}$ values of monocrotaline in this study were compared to the PODs derived from in vivo rat acute liver toxicity data on monocrotaline reported in the literature.[3, 4, 6, 44] When the data from these in vivo studies were not suitable for BMD analysis due to the limited number of data points and/or insufficient distribution of the data points over the dose-response curves, the no observed adverse effect level (NOAEL) was used for the comparison. When only a lowest observed adverse effect level (LOAEL) was available, the NOAEL was calculated using the LOAEL divided by a factor of 10.[45]

5.3. Results

5.3.1. Monocrotaline-induced liver toxicity in vitro

Monocrotaline induced liver toxicity in primary rat hepatocytes with an IC_{50} value of 225 µM as shown in Figure 5.3. The highest concentration of 600 µM decreased cell viability by over 60% while limited solubility prevented testing of higher concentrations and reaching 100% cytotoxicity. The EC_{50} obtained for monocrotaline is 20.7- and 35.7-fold higher than the EC_{50} values previously obtained in the same model system for lasicarpine (EC_{50} 10.9 µM) and riddelliine (EC_{50} 6.3 µM) respectively.[30]

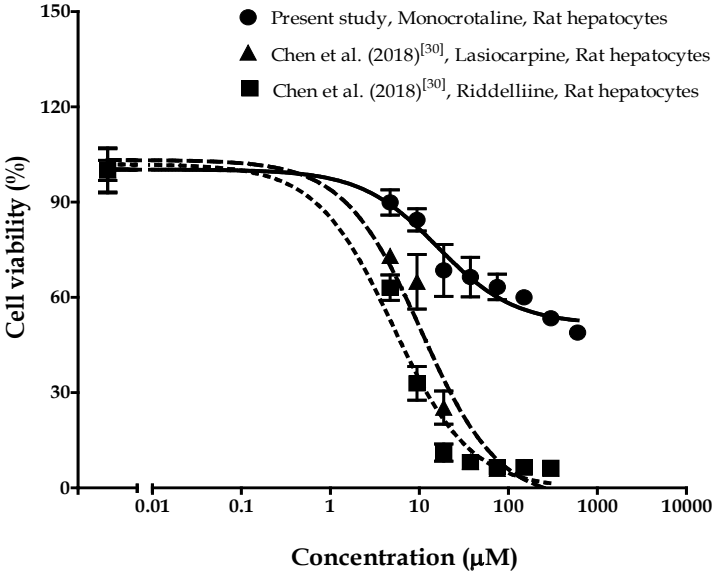

Figure 5.3. Concentration-response curves for effects of monocrotaline (circles with solid line) on cell viability of primary rat hepatocytes exposed for 24 h (means ± SE) and, for comparison, for effects of lasiocarpine (triangles with dashed line) and riddelliine (squares with dotted line) as reported by Chen et al. (2018)[30].

5.3.2. Metabolic clearance of monocrotaline by rat liver and intestine microsomes

Figure 5.4 shows the monocrotaline concentration dependent rate of conversion of the compound in incubations with rat intestinal and liver microsomes. Table 5.2 presents the V_{max} and K_m values derived from these curves and also the catalytic effciency (k_{cat}) for clearance of monocrotaline calculated as V_{max}/K_m. For comparison Table 5.2 also presents the kinetic parameters for depletion of lasiocarpine and riddelliine previously reported[30]. It appears that monocrotaline is converted by the liver microsomes with an in vivo scaled k_{cat} (ml/min tissue) that is 18 times higher than the conversion rate by intestinal microsomes (Figure 5.4 and Table 5.2). Lasiocarpine and riddeliine showed the same trend where the scaled

catalytic efficiency for conversion expressed per intestinal tissue was 15.4 and 253 times, respectively, lower than that for the liver indicating the intestinal contribution to PA clearance to be minor (Table 5.2). The scaled k_{cat} for conversion of monocrotaline in the liver was 41.8 and 4.3 times lower compared to the scaled liver k_{cat} of lasiocarpine and riddelliine, respectively, indicating that the metabolism of monocrotaline was the lowest among the three PAs. The total scaled in vivo k_{cat} sum of liver and intestine) for depletion of monocrotaline was 42.1 and 4.1 fold, respectively lower than that for lasiocarpine and riddelline.

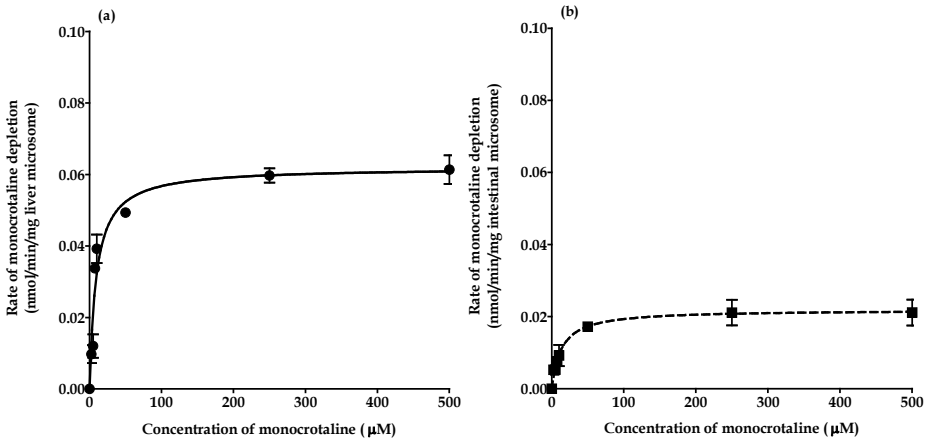

Figure 5.4. Concentration-dependent rate of monocrotaline depletion in incubations with: (a) rat liver microsomes and (b) intestinal microsomes. Values are presented as means ± SE derived from 3 independent experiments.

Table 5.2. Kinetic parameters for metabolic conversion of monocrotaline (present study), lasiocarpine and riddelliine[30] in incubations with pooled rat liver and instestine microsomes.

Compound Organ	V_{max} (nmol/min/mg microsomal protein)	Km (μM)	K_{cat} (ml/min/mg microsomal protein)	Scaled V_{max} (nmol/min/g tissue)[a]	Scaled K_{cat} (ml/min/g tissue)[a]	Scaled K_{cat} (ml/min/ tissue)[b]
Monocrotaline (present study)						
Liver	0.06	9.2	0.01	2.1	0.2	1.9
Intestine	0.02	13.4	0.001	0.4	0.03	0.1
Lasiocarpine [30]						
Liver	5.3	19.5	0.27	186	9.5	80.9
Intestine	1.7	23.4	0.07	35.0	1.50	5.2
Riddelliine [30]						
Liver	2.1	75.7	0.03	73.5	0.97	8.2
Intestine	0.1	221	0.0005	2.06	0.009	0.03

[a]Scaled V_{max} and k_{cat} calculated from the in vitro V_{max} and k_{cat} based on a microsome protein yield of 35 mg microsomal protein/(g liver) or 20.6 mg microsomal/(g small intestine)[32, 33].
[b]Scaled in vivo k_{cat} (mL/min/tissue) derived from the in vivo k_{cat} (ml/min/g tissue) based on the liver weight of 8.5 g or small intestine weight of 3.5 g[34]

5.3.3. PBK model predictions and evaluation

Due to unavailability of in vivo kinetic data for monocrotaline upon oral administration in rat, the blood concentration-time curves of monocrotaline as predicted by the developed PBK model upon iv administration were evaluated against the available concentration of monocrotaline equivalents in rat blood upon the iv administration of 60 mg/kg of [^{14}C] monocrotaline[41]. The predicted blood concentrations were on average 1.6- to 3.4-fold higher than the blood concentrations observed in vivo (see Table S1 Supplementary materials 2). Given this limited deviation it was concluded that the PBK model could be used for the in vitro to in vivo extrapolations.

5.3.4. Sensitivity analysis

The performance of the developed PBK model was further evaluated by a sensitivity analysis to determine the parameters which affect the prediction of the maximum concentration of monocrotaline in liver blood. The parameters that result in a normalized sensitivity coefficient higher than an absolute value of 0.1 are shown in Figure 5.5. At an oral dose level of of 1 and 3 mg/kg bw, representing the lowest and highest dose in the range for the estimated daily human intake of PAs that might result in adverse health effects if consumed for 4 days up to a 2 weeks periods[7], the predicted maximum concentration of monocrotaline in liver blood was affected by the fraction of liver volume (VL_C), the partition coefficient of monocrotaline into liver tissue (PL), the partition coefficient into slowly perfused tissue (PS), the absorption rate from the intestinal compartment into the liver (Ka), the liver microsomal protein yield, and the kinetic parameters (V_{max} and K_m) for monocrotaline depletion in the liver. The predicted monocrotaline concentration in liver blood was not sensitive to the kinetic parameters for monocrotaline depletion in the small intestine-related parameters in line with the earlier observation that monocrotaline metabolism is this organ is substantially less efficient (Figure 5.4).

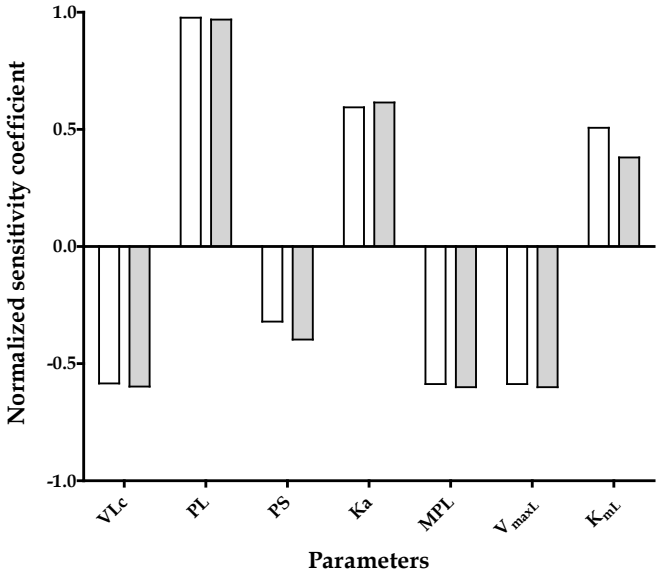

Figure 5.5. Normalized sensitivity coefficients for the parameters of the rat PBK model for monocrotaline of influence on the predicted maximum concentration in liver blood at a single oral dose of monocrotaline of 1 mg/kg bw (white bars), or 3 mg/kg bw/day (grey bars) PAs. VLc = fraction of liver volume, PL = liver/blood partition coeffient, PS = slowly perfused tissue/blood partition coefficient, Ka = uptake rate constant, MPL = liver microsomal protein yield, V_{maxL} and K_{mL} = the maximum rate of depletion and the Michaelis-Menten constant for depletion of monocrotaline in liver

5.3.5. Predicted hepatotoxicity of monocrotaline in rats and application of PROAST modeling on predicted dose-response data to derive PODs

The RED assay resulted in an $f_{ub,in\ vivo}$ of monocrotaline in rat serum of 0.53±0.12, a value used to correct for the differences in protein binding between the in vivo and in vitro situation. With this $f_{ub,in\ vivo}$ the concentrations tested in the cytotoxicity assay were converted to in vivo total blood concentrations by equation 5.3 and then converted to the corresponding dose levels using the PBK model. The dose levels thus obtained were used to create the corresponding dose response curve for acute liver toxicity.

The predicted in vivo dose-response curve thus obtained is shown in Figure 5.6. For comparison also the dose-response curves previously predicted for lasicarpine and riddelliine by the same approach[30] are included in the figure. From the results obtained it can be concluded that monocrotaline is predicted to be somewhat less toxic than riddelliine and somewhat more toxic than lasiocarpine. A BMD analysis was performed on the predicted dose-response data resulting in a predicted BMD_{10} and range of $BMDL_{10}$-$BMDU_{10}$ values for monocrotaline, lasiocarpine, and riddelliine as presented in Table 5.3. The predicted BMD_{10} for

monocrotaline appeared to be 1.5 fold higher than that obtained from the predicted dose-response curve for riddelliine[30], while the value was 8.6 fold lower than that predicted for lasiocarpine.

Comparison of these predicted differences in in vivo toxicity to the relative differences observed in vitro (Figure 5.3) shows that the differences in in vitro toxicity between monocrotaline, lasiocarpine and riddelliine were subtantially different from the differences observed in vitro where lasiocarpine and riddelliine were 35.7 and 20.7, respectively more toxic than monocrotaline. This shift towards relatively higher toxicity for monocrotaline in the in vivo situation is due to the differences in kinetics where monocrotaline appeared to be metabolised with a catalytic efficiency that was 4.1- and 42.1-fold lower than that for lasiocarpine and riddelliine, respectively. This implies that at similar dose levels the accompanying blood concentrations and thus toxicity will be relatively higher for monocrotaline. This result corroborates that differences in kinetics substantialy influence the relative in vivo potencies of PAs, and should not be ignored when defining relative potency factors.

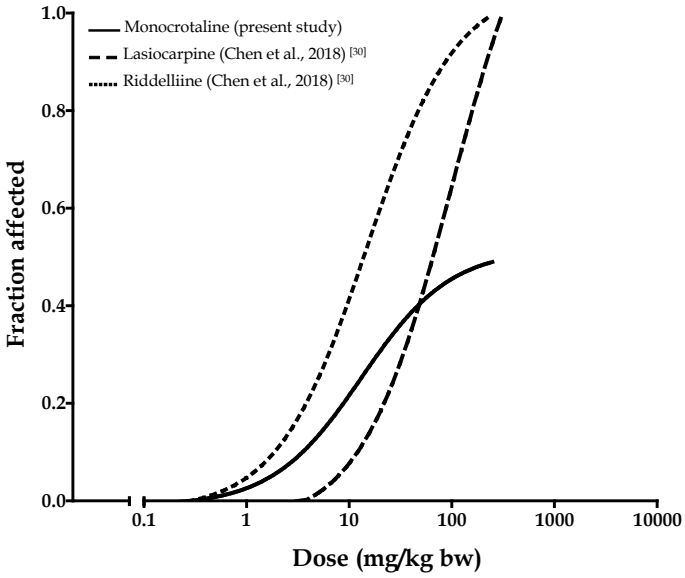

Figure 5.6. Predicted in vivo dose-response curves for acute liver toxicity in rats obtained by combining in vitro data in primary rat hepatocytes and PBK modeling-based reverse dosimetry for monocrotaline (black line). For comparison the predicted dose response curves for liver toxicity of lasiocarpine (dashed line) and riddelliine (dotted line) reported by Chen et al. (2018)[30] are also presented.

Table 5.3. Predicted $BMDL_{10}$-$BMDU_{10}$ values derived from the dose–response curves presented in Figure 5.6 predicted by PBK modeling-facilitated reverse dosimetry.

Compound	Predicted $BMDL_{10}$-$BMDU_{10}$ (mg/kg bw/day)	Predicted BMD_{10} (mg/kg bw/day)	Source of the predicted dose response-curve
Monocrotaline	1.7-6.3	3.8	Present study
Riddelline	1.3-3.7	2.6	Chen et al. (2018)[30]
Lasiocarpine	17.6-55.8	32.5	Chen et al. (2018)[30]

5.3.6. Comparison of the predicted PODs to PODs derived from the reported data for liver toxicity in rats

To further evaluate the in vitro-PBK modeling facilitated reverse dosimetry approach for prediction of monocrotaline-induced acute liver toxicity, the predicted $BMDL_{10}$ for monoctotaline induced liver toxicity was compared to the corresponding PODs (NOAEL values) derived from available in vivo studies for liver toxicity of monocrotaline in rats. Table 5.4 provides the overview of reported data on monocrotaline-induced acute liver toxicity in rats based on the endpoints of increased level of bound pyrrolic metabolites, increased alanine aminotransferase (ALT) activity, apoptosis of hepatic parencymal cells (HPC) and hepatic congestion [3, 4, 6, 44]. Since results from oral toxicity studies were not avalable, studies included in this comparison were studies with ip or sc dosing regimens. Given that the data of none of these studies enabled BMD modeling, the PODs from the available studies were based on the NOAEL or, when a NOAEL was not available, derived from the LOAEL value by assuming the NOAEL would amount to the LOAEL divided by 10[45] (Table 5.4).

Table 5.4. Monocrotaline-induced liver toxicity data reported for in vivo studies in rats.

Species	Exposure route	Dose (mg/kg bw/day)	Type of exposure	Effect	Type of POD	POD values (mg/kg bw)	Study
Male Sprague-Dawley (bw 200-250 g)	IP	0; 65	Single	increased level of bound pyrrolic metabolites levels 24 h after dosing	NOAEL (= LOAEL/10)	6.5	[44]
Male Sprague-Dawley (bw 100-130 g)	IP	0; 100; 200; 225; 300	Single	increased plasma ALT 12 h after dosing	NOAEL	100	[3]
Male Sprague-Dawley (bw 90-150 g)	IP	0; 300	Single	apoptosis of HPC 14-18 h after dosing	NOAEL (= LOAEL/10)	30	[4]
Male Sprague-Dawley (bw >200 g)	SC	0;60	Single	hepatic congestion 24 h after dosing	NOAEL (=LOAEL/10)	6	[6]

Figure 5.7 presents a comparison of the predicted $BMDL_{10}$-$BMDU_{10}$ value of monocrotaline to the PODs data of Table 5.4. This comparison reveals that the reported toxicity data upon ip exposure vary substantially, and that the predicted $BMDL_{10}$ value is in line with especially the NOAEL derived from the study with ip dosing reported by Yan and Huxtable (1996)[44] and sc dosing reported by Lachant et al. (2018)[6]. The $BMDL_{10}$-$BMDU_{10}$ of 1.7-6.3 mg/kg bw/day predicted by the in vitro-in silico approach of the present study is in line with this estimated toxic oral dose range of 1-3 mg PA/kg bw/day[7].

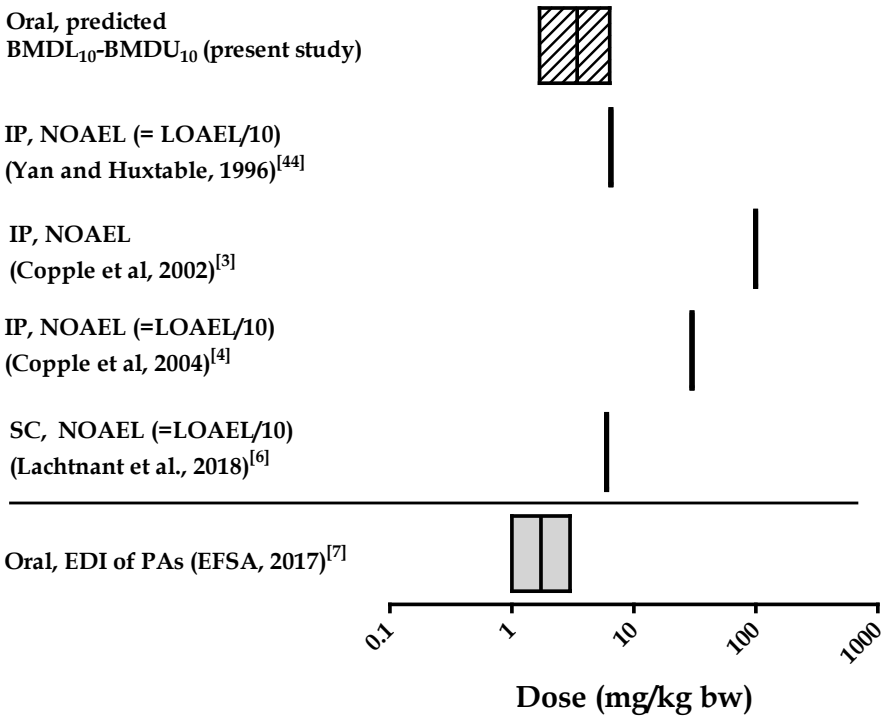

Figure 5.7. $BMDL_{10}$-$BMDU_{10}$ values for liver toxicity in rats predicted by the PBK modeling-facilitated reverse dosimetry approach using data for toxicity of monocrotaline in rat hepatocytes (patterned bar), compared to PODs derived from literature data on in vivo liver toxicity of monocrotaline in rats from studies with sc or ip dosing presented in Table 5.4 (vertical black bars). The grey bar below the line represents an oral dose range of 1-3 mg PA/kg bw/day at which acute/short-term adverse effect in humans may occur.[7]

5.4. Discussion

The aim of the present study was to use an in vitro-in silico approach to predict the in vivo acute liver toxicity of monocrotaline and to characterize the influence of its metabolism on its relative in vivo toxic potency compared to lasiocarpine and riddelliine. This in vitro-in silico approach was recently shown able to predict the acute liver toxicity of lasiocarpine and riddelliine.[30] The results now

obtained for monocrotaline further validate the approach as a possible method to fill existing gaps in the database on PAs relevant in food. Furthermore, comparison of the results to those previously obtained for lasiocarpine and riddelliine[30] corroborated the influence of metabolism on the relative toxic potency of these three PAs.

The in vitro concentration-response data for monocrotaline-induced toxicity were obtained using primary rat hepatocytes. Primary rat hepatocytes from pooled male Sprague-Dawley rats were used because male rats were previously reported to be more sensitive towards monocrotaline toxicity than female rats[46] and also because most in vivo data available for the liver toxicity of monocrotaline were obtained in male rats (Table 5.4). Comparison of the in vitro toxicity data to the in vitro study of Louisse et al. (2019)[47] which showed that monocrotaline did not exhibit cytotoxicity in HepaRG cells upon 24 h exposure, indicates that rat hepatocytes are more sensitive to the toxicity induced by monocrotaline. This result is in line with data from Ning et al. (2019)[29] who reported that rat hepatocytes are more sensitive towards lasiocarpine and riddelliine induced liver toxicity than HepaRG cells. Primary rat hepatocytes likely contain higher levels of the cytochrome P450 enzymes required for metabolism including the bioactivation of parent PAs.[11, 12] In the in vitro assay with rat primary hepatocytes the IC_{50} value of monocroaline was 20.7- and 35.7- fold higher than the IC_{50} values previoulsy reported in the same model system for lasiocarpine and riddelliine, respectively.[30] The lower toxicity of monocrotaline in in vitro liver model systems is also in line with what has been observed in other studies using HepG2 or HepaRG cells.[47, 48]

Since in the in vitro models used the liver toxicity of monocrotaline is quantified depending on the concentration of the parent compound, which is metabolised to its toxic metabolites within the cells of the in vitro model system, the PBK model developed in the present study describes the kinetics of monocrotaline and not of its metabolites and also the reverse dosimetry is based on concentrations of the parent compound. The subtrate depletion analysis indicated that monocrotaline was slowly metabolized in the incubations with rat liver and intestinal microsomes. The kinetic eficiency for monocrotaline conversion appeared to be 42.1- and 4.1- fold lower compared to that previously obtained for lasiocarpine and riddelliine, respectively using the same approach as Chen et al. (2018)[30]. This indicates that the metabolism of monocrotaline was the lowest among these three PAs. This result is line with the study performed by Lester et al. (2019)[31] who reported that monocrotaline is metabolically stable in the rat sandwich culture hepatocyte cell system. Marked differences in metabolic degradation among PAs was also reported recently by Geburek et al. (2019)[49] using in vitro incubations with

rat liver microsomes indicating as well that conversion of monocrotaline was lower than that of riddelliine. In the present study these kinetic differences were taken into account when translating the concentration-response curves for in vitro toxicity to the predicted dose-response curves for acute liver toxicity using PBK model-facilitated reverse dosimetry approach.

Evaluation of the developed PBK model for monocrotaline showed that the predicted concentrations of monocrotaline in blood were in line with the kinetic data available for monocrotaline in rats.[41] The PBK model used was also similar to that previously developed and evaluated for the PAs lasiocarpne and riddelliine.[30] Chen et al. (2018)[30] demonstrated that the developed PBK model could adequately predict blood concentrations of riddelliine and also adequately translate the in vitro liver toxicity induced by lasiocarpine to a predicted in vivo dose-reponse curve for liver toxicity. The results of the present study reveal that the same approach can quantitatively predict the reported in vivo acute liver toxicity of monocrotaline. The predicted $BMDL_{10}$ value appeared to be in line with the NOAELs derived from availabe in vivo studies, although the comparison also revealed that especially the NOAELs derived from the reported toxicity data upon ip exposure vary substantially, in part due to the fact that the NOAELs or LOAELs were the lowest dose levels tested, leaving room for the actual LOAEL and NOAEL being lower than what has now been derived from the data. The predicted $BMDL_{10}$ was in line with the NOAEL derived from the study with sc dosing reported by Lachant et al. (2018)[6]. The differences observed may in part also be ascribed to the difference in dosing regimen with the predicted values refering to oral exposure while the in vivo were from studies with ip or sc dosing. Due to the lack of data for monocrotaline induced acute toxicity via oral intake in rats, the predicted $BMDL_{10}$-$BMDU_{10}$ value was also compared to the oral dose range of 1-3 mg PA/kg bw/day at which acute/short-term adverse effects in human are reported to occur when consuming a combination of PAs via teas or herbal infusions[7]. The $BMDL_{10}$-$BMDU_{10}$ of 1.7-6.3 mg/kg bw/day predicted by the in vitro-in silico approach of the present study is in line with this estimated toxic oral dose level, indicating that the toxicity of monocrotaline would match the overall toxicity estimated for PAs.

The result of the present study also indicated that taking the kinetics into account the predicted in vivo differences in toxicity between monocrotaline and lasiocarpine and riddelliine appeared to be smaller than what would be predicted based on the vitro data obtained in primary hepatocytes. The predicted $BMDL_{10}$ value for acute liver toxicity of monocrotaline obtained in the present study supports the classification of monocrotaline as a toxic PA, with a potency for acute liver toxicty that seems comparable to that of lasiocarpine and riddelliine. To what extent

this conclusions also holds for the carcinogenicity of these PAs remains to be estabished. The conclusion of similar potency is in line with the provisional relative potency factors (pRPF) derived by Merz and Schrenk (2016)[50] indicating that monocrotaline, as well as riddelliine are categorized as the most potent congeners with a pRPF similar to that of lasiocarpine of 1.0. This result is in contrast to the ranking presented by Xia et al. (2013)[51] based on the formation of DNA adducts, who ranked monocrotaline as group II with moderate tumour formation. Louisse et al. (2019)[47] classified monocrotaline into group 3 with an pRPF of 0.06 based on its in vitro γH2AX induction potency in the human liver cell line HepaRG, while lasiocarpine and ridddelliine were categorized as group 1 with a pRPF of 1.08 and 1, respectively. However these in vitro studies are based on different endpoint and also do not take potential differences in in vivo toxicokinetics into account, while the result of the present study clearly indicate that this will hamper the translation of in vitro RPFs to the in vivo situation. The lower metabolic clearance of monocrotaline than of lasiocarpine and riddelliine observed in the present study is in line with the results from Lester et al. (2019)[31] and Geburek et al. (2019)[49], and will result in higher relative in vivo concentrations and potential toxicity than predicted based on in vitro concentration-response curves.

In conclusion, the results of the present study illustrate that a combined in vitro-in silico approach can be used to obtain insights in monocrotaline-induced acute liver toxicity in rats. Furthermore, the comparison of its relative toxic potency to lasiocarpine and riddelliine indicates that the kinetic and metabolic properties of these PAs should be taken into account when defining relative differences in in vivo toxic potency. This insight can be used to obtain a promising alternative testing strategy in risk and safety evaluation of PAs.

Conflict of interest

The authors state no conflicts of interest.

Acknowledgments

This research was supported by a grant from the Indonesian Endowment Fund for Education, Ministry of Finance, Republic of Indonesia through a Beasiswa Pendidikan Indonesia Lembaga Pengelola Dana Pendidikan (BPI LPDP) doctoral scholarship [grant number: PRJ-365/LPDP/2016] awarded to Suparmi.

Supplementary materials

Supplementary materials 1 and 2 can be downloaded from https://drive.google.com/open?id=1vXdSIdgp58Bs0Vr053HoZY86875wEPrK.

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BENEFIT
RISK

Chapter 6

Combining in vitro data and physiologically based kinetic modeling facilitated reverse dosimetry to define in vivo dose-response curves for bixin- and crocetin-induced activation of PPARγ in humans

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Published in: Molecular Nurition and Food Research (2019) 1900880:1-9
DOI: 10.1002/mnfr.20190088

Abstract

Scope: It is investigated whether at realistic dietary intake bixin and crocetin could induce peroxisome proliferator–activated receptor γ (PPARγ)–mediated gene expression in humans using a combined in vitro–in silico approach.

Methods and results: Concentration–response curves obtained from in vitro PPARγ-reporter gene assays are converted to in vivo dose–response curves using physiologically based kinetic modeling-facilitated reverse dosimetry, from which the benchmark dose levels resulting in a 50% effect above background level (BMD_{50}) are predicted and subsequently compared to dietary exposure levels. Bixin and crocetin activated PPARγ-mediated gene transcription in a concentration-dependent manner with similar potencies. Due to differences in kinetics, the predicted BMD_{50} values for in vivo PPARγ activation are about 30-fold different, amounting to 115 and 3,505 mg/ kg bw for crocetin and bixin, respectively. Human dietary and/or supplemental estimated daily intakes may reach these BMD_{50} values for crocetin but not for bixin, pointing at better possibilities for in vivo PPARγ activation by crocetin.

Conclusion: Based on a combined in vitro–in silico approach, it is estimated whether at realistic dietary intakes plasma concentrations of bixin and crocetin are likely to reach concentrations that activate PPARγ–mediated gene expression, without the need for a human intervention study.

6.1. Introduction

Bixin (methyl hydrogen 9′-cis-6,6′-diapocarotene-6,6′-dioate and crocetin (8,8′-diapocarotene-8,8′-dioic acid) (Figure 6.1) are food-borne carotenoids.[1, 2] Bixin is present in the extract prepared from the seed coat of annatto (*Bixa orellana* L). Annato extracts containing bixin are an approved food color additive (E160b), for which the European Food Safety Authority (EFSA) established an acceptable daily intake (ADI) of 6 mg/kg bw/day.[3-5] Crocetin occurs naturally in the fruits of gardenia (*Gardenia jasminoides* Ellis) and in the stigma of saffron (*Crocus sativus* L.) frequently consumed due to its use as food colorant and flavouring.[6] Saffron containing crocetin is recognized as food additive in the USA, while JECFA recognized saffron as a food ingredient rather than a food additive.[7] In addition to use as food additives, bixin and crocetin have been considered as potential functional food ingredients with beneficial effects in various diseases, including type 2 diabetes mellitus (T2DM).[8, 9]

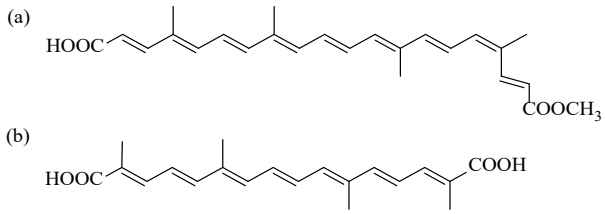

Figure 6.1. Chemical structures of bixin (a) and crocetin (b).

Studies in experimental animals revealed that bixin shows hypoglycemic activity in streptozotocin-induced diabetic rats,[10] and that crocetin enhances insulin sensitivity in insulin resistant rats,[11-13] suggesting their potential beneficial roles in T2DM. The interest to explore the carotenoids as potential functional food ingredients is increasing, due to the growing reports about side-effects associated with current T2DM medication. Thiazolidinediones (TZDs), which once were the most widely used drugs for treatment of T2DM,[14] have been reported to cause body weight gain and increased risks for myocardial infarction, peripheral edema and bone fracture.[15] TZDs are believed to exert their therapeutic effects via activation of peroxisome proliferator-activated receptor γ (PPARγ), which is also suggested as mode of action underlying the potential beneficial effects of bixin and crocetin.

PPARγ activation has been reported to increase insulin sensitivity,[16] decrease free fatty acid levels in plasma and increase lipid storage in adipose tissue.[17] Several carotenoids, including bixin and also norbixin, β-carotene, lutein, neoxanthin, phytoene, lycopene, β-carotene, astaxanthin, β-cryptoxanthin, zeaxanthin, γ-carotene, δ-carotene have been shown able to activate PPARγ-mediated gene expression in vitro.[18-21] It remains to be established however, whether the reported

PPARγ activating characteristics can also be expected at realistic human dietary intake levels.

Therefore the aim of the present study was to investigate whether the reported PPARγ activating characteristics of bixin and crocetin may be expected at realistic human daily intake levels. To this end, concentration-response curves for bixin- and crocetin-dependent activation of PPARγ-mediated gene expression in a stably transfected U2OS PPARγ reporter gene cell line were converted to predicted in vivo dose response curves using so-called physiologically based kinetic (PBK) modeling facilitated reverse dosimetry. This approach facilitates evaluation of whether PPARγ activating characteristics of bixin and crocetin may be expected at realistic human dietary intake levels without the need for a human intervention study.

A PBK model can predict the concentration of a compound and its relevant metabolites in any tissue at any point in time and for any dose level, within its applicability domain.[22] After the PBK model is validated with the available in vivo data, it can be used to convert in vitro concentrations, set equal to internal concentrations in blood or a tissue of choice, to corresponding in vivo dose levels, by so-called reverse dosimetry.[23, 24] In PBK modeling facilitated reverse dosimetry, the PBK model is used in the reverse order compared to the forward dosimetry that is generally applied in pharmacokinetics. Forward dosimetry is applied to calculate the internal concentration of a compound or its metabolite that can be expected in blood or a relevant tissue upon a given dose level. In the reverse dosimetry approach, in vitro concentrations are set equal to blood or tissue levels of the respective compound in the PBK model, following which the PBK model is used to calculate the corresponding in vivo dose level for any given route of administration. Subsequent benchmark dose (BMD) modeling can be applied on the predicted in vivo dose-response data, to determine effective exposure levels for humans, like a BMD value defining the dose levels inducing a limited but measurable response above background level and the BMDL values, the lower confidence limits of the BMD.[23]

6.2. Experimental Section

6.2.1. In vitro PPARγ CALUX assay of bixin and crocetin

Bixin (96.5% purity by HPLC) was purchased from International Laboratory (San Fransisco, USA). Norbixin was extracted from annatto seeds using extraction with 8% ethanol in dichloromethane (CH_2Cl_2). Norbixin was purified from this extract by preparative thin layer chromatography (TLC). Crocetin (98% purity by HPLC) was purchased from Carotenature (Lupsingen, Switzerland). The

cytotoxicity of bixin, norbixin and crocetin was tested in vitro as previously decribed using the cytotox CALUX cell line to ascertain that the test compounds did not affect the luciferase activity themselves under the conditions tested.[25] PPARγ-mediated gene expression was tested using the PPARγ2-reporter gene assay in PPAR-γ2 CALUX cells provided by BioDetection Systems BV (Amsterdam, The Netherlands).[26] To analyse the effects of bixin, norbixin and crocetin on PPARγ-mediated gene expression, the cells were incubated for 24 h at increasing concentrations (0.01–100 µM) of the compounds in culture medium added from 200 times concentrated stock solutions in THF. The final concentration of THF in exposure medium was 0.5% (v/v). 1 µM rosiglitazone, a well-known PPARγ agonist,[27] was included in every plate as positive control (added from a 200 times concentrated stock solution in DMSO). Luciferase activity of the lysate was quantified at room temperature using a luminometer (Glowmax Multi Detection System, Promega Madison USA).

Data are presented as mean values ± SD from three independent experiments with six replicates per plate. The PPARγ responses were expressed relative to the response of the rosiglitazone positive control set at 100%. The obtained concentration–response curves were fitted with a symmetrical sigmoidal model (Hill slope) using GraphPad Prism software (version 5.00 for Windows, GraphPad software, San Diego, USA) which was further used to derive EC_{50} values.

6.2.2. Determination of model parameter values for hepatic clearance

Pooled human cryopreserved hepatocytes (HEP10) for suspension were purchased from Life Technologies (Bleiswijk, The Netherlands). The cells were thawed and assessed for metabolic stability in accordance with the manufacturer's protocol (Supporting information 1). The intrinsic clearance (CL_{int}) values of bixin and crocetin were estimated by a substrate depletion approach using the protocol provided by the supplier for in vitro assessment of metabolic stability in suspensions with cryopreserved pooled mixed gender human hepatocytes (HEP10) with little modifications. The rate of disappearance of the parent compounds at a single, low substrate concentration (i.e. 3 µM) were scaled to in vivo clearance values to describe the hepatic clearance of the parent compounds in the PBK model. After incubation at time points 0, 7.5, 15, 30, and 60 min, the residual parent compounds were analysed using a Waters UPLC-DAD-System. For all incubations, three independent replicates were performed.

The slope of the linear curve for the time dependent percent residual parent compound from the HEP10 containing reaction mixtures corrected for the percent residual parent compound in the corresponding blancs without cells was used to

determine the in vitro $t_{1/2}$ (expressed in min) of the parent compound. Using the elimination rate constant k = $0.693/t_{1/2}$, $CL_{int,\ in\ vitro}$ expressed in µL/min/ 10^6 cells can be described as Equation 6.1.

$$CL_{int,in\ vitro} = \frac{0.693}{t_{1/2}} \times \frac{V}{N} \quad \text{(Equation 6.1)}$$

where V is the volume of the incubation (expressed in µL) and N is number of hepatocytes per well (expressed in 10^6 cells).[28] The human physiological parameters reported by Soars et al.[29] were used to scale the in vitro CL_{int} values to in vivo CL_{int} values which were applied in the PBK models (Equation 6.2).

$$CL_{int,in\ vivo} = WL \times bw \times Hep \times CL_{int,in\ vitro} \times 60 \times 10^{-6} \quad \text{(Equation 6.2)}$$

where $CL_{int,\ in\ vivo}$ is in vivo CL_{int} (L/h), WL is liver weight of 20 g kg/bw, bw is human body weight of 70 kg used in the PBK models, Hep is hepatocellularity of 120 × 10^6 cells/g liver, $CL_{int,\ in\ vitro}$ is in vitro CL_{int} (µL/min/ 10^6 cells), 60 is the value of 60 min within 1 h, 10^{-6} to convert from µL to L.

As norbixin, which is a likely metabolite of bixin, was unable to induce PPARγ-mediated gene transcription even at the highest concentration tested, and in line with literature[18], it was not considered in the clearance studies and subsequent PBK modeling.

6.2.3. Development and evaluation of a PBK model for bixin and crocetin

A PBK model is a set of mathematical equations which describe the absorption, distribution, metabolism and excretion (ADME) characteristics of a compound within an organism based on three types of parameters, i.e: i) physiological and anatomical (e.g. cardiac output, tissue volumes and tissue blood flows), (ii) physico-chemical (blood/tissue partition coefficients) and (iii) kinetic parameters (e.g. kinetic constants for metabolic reactions).[23] Figure 6.2 depicts the conceptual PBK model, which consists of separate compartments for the gastrointestinal (GI) tract, liver, slowly perfused tissues (e.g. skin, muscle, bone), rapidly perfused tissues (e.g. heart, lung, brain), fat and blood.

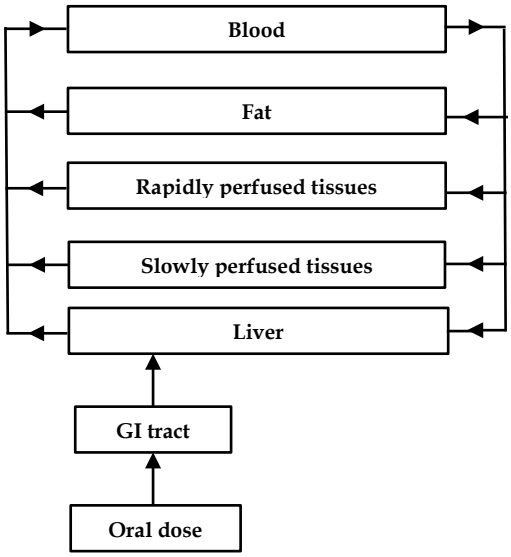

Figure 6.2. Schematic representation of the conceptual PBK model for bixin and crocetin in human.

The values of human physiological and anatomical parameters were obtained from literature,[30] while the blood/tissue partition coefficients were estimated using the formula using Log P values of olive oil, pKa and fraction unbound in serum as input,[31] and as shown in the supporting information 2 **(**Table S1 and Table S2). Log Kow values were estimated using ChemBio-Draw Ultra 14.0 (Cambridge-Soft, USA). Kinetic parameters for hepatic clearance of bixin and crocetin were determined using HEP10 incubations performed as described above. Berkeley Madonna 8.3.18 (Macey and Oster, UC Berkeley, CA) was used to code and numerically integrate the PBK models applying Rosenbrock's algorithm for stiff systems. Compared to other algorithms in Berkeley Madonna (BM), the Rosenbrock's algorithm serves better for stiff systems[32-34] and was shown to provide adequate results in previous studies providing proofs of principle for the PBK model based reverse dosimetry.[35-42]

The model code for the developed PBK models of bixin is presented in Supplementary information 3.

To evaluate the PBK model performance, predicted maximum bixin and crocetin concentrations in the blood were compared to reported maximum blood concentrations in humans as reported in the literature.[43, 44] Maximum concentrations of bixin and crocetin in blood were predicted by PBK modeling using a ka value of 1 h^{-1} for each compound assuming fast and complete uptake.[45]

In addition a sensitivity analysis was performed to identify the key parameters which contribute most to the predicted maximum blood concentrations

(C_{max}) at an oral dose of 0.23 mg/kg bw for bixin and 0.25 mg/kg bw for crocetin. This sensitivity analysis was performed as described previously[46] calculating normalized sensitivity coefficients (SCs) by Equation 6.3.

$$SC = \frac{(C'-C)}{P'-P} \times (\frac{P}{C}) \quad \text{(Equation 6.3)}$$

where C is the initial value of the model output, C′ is the modified value of the model output resulting from an increase in parameter value, P is the initial parameter value and P′ is the modified parameter value. Each parameter was analyzed individually by changing one parameter at a time (5% increase) and keeping the other parameters the same.

6.2.4. Translation of in vitro PPARγ concentration respon curves to in vivo PPARγ dose response curves

The in vitro concentration–response curves for bixin- and crocetin-induced activation of PPARγ mediated gene transcription were translated into predicted in vivo dose–response curves using PBK modeling-facilitated reverse dosimetry. This reverse dosimetry was based on the concentration of the parent compound, which was assumed to represent the form of the carotenoids activating PPARγ-mediated gene expression.

Furthermore, within this translation a correction was made to take the differences in albumin and lipid concentrations between in vitro and in vivo conditions into account. This was done because it was assumed that only the free fraction of the carotenoid will be available to exert the effects. Extracellular instead of intracellular concentrations were used because unbound concentrations in blood were considered to best match the in vitro model where cells were exposed to the carotenoids dissolved in the medium on top of the cell layer. The unbound fraction ($f_{ub,\ in\ vitro}$) was estimated to determine the fraction bound ($f_{b,\ in\ vitro}$) to lipid and protein in culture medium.[47] Each nominal concentration applied in the in vitro PPARγ-mediated gene expression assay ($EC_{in\ vitro}$) of bixin and crocetin was extrapolated to an in vivo effect concentration ($EC_{in\ vivo}$) according to the extrapolation rule of Gülden and Seibert (2003)[47] as described in supporting information 4. Each in vivo concentration ($EC_{in\ vivo}$), thus obtained was set equal to the blood C_{max} of bixin and crocetin in the PBK model. The PBK model was subsequently used to calculate the corresponding oral dose levels in humans to derive the in vivo dose-response curves.

To define the benchmark dose resulting in a 50% increase over the background level of PPARγ activation (BMD_{50}) the predicted in vivo dose-response data for bixin- and crocetin-induced PPARγ-mediated gene expression in human

were used for BMD modeling. Dose-response modeling and BMD analysis were performed using the EFSA BMD modeling webtool (PROAST version 66.38, https://shiny-efsa.openanalytics.eu/app/bmd).[48] Data were analysed using the exponential model for continuous data because this model appeared to provide the best (goodness of) fit with the lowest Akaike Information Criterion (AIC) value among the available models. In the visualization result of PROAST, a CES (critical effect size), CED (critical effect dose), CEDL (lower bound of the CED), CEDU (upper bound of the CED) correspond to the BMR, BMD_{50}, $BMDL_{50}$ (lower bound of the BMD_{50} 95%-confidence interval), and $BMDU_{50}$ (upper bound of the BMD_{50} 95%-confidence interval), respectively.

6.3. Results

6.3.1. Bixin- and crocetin-induced activation of PPARγ-mediated gene expresssion

Bixin and crocetin increased PPARγ-mediated gene expression in a concentration-dependent manner, while norbixin appeared unable to induce PPARγ-mediated gene expression up to the highest concentration tested of 100 µM (Figure 6.3). Bixin and crocetin were of similar potency and had an EC_{50} of 23.5 and 17.7 µM, respectively. Using the cytotox CALUX cell line it was confirmed that at the concentrations tested there was no cytotoxicity and the test compounds did not affect the luciferase activity (data not shown).

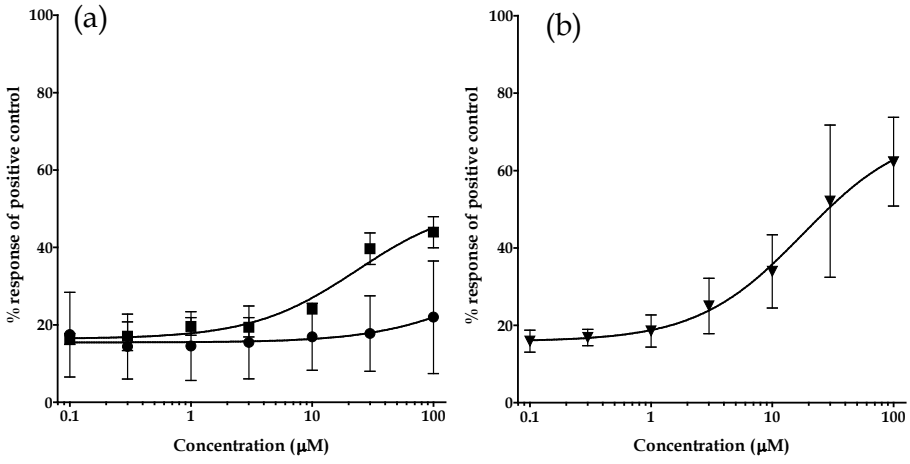

Figure 6.3. Concentration-dependent induction of PPARγ-mediated gene expression by (a) bixin (squares) and norbixin (circles), and (b) crocetin (triangles) expressed as percentage of the response induced by the positive control 1 µM rosiglitazone set at 100%. The induction by roziglitazone was between 7- and 8-fold. Values are presented as means ± standard deviations derived from 3 independent experiments.

6.3.2. Hepatic clearance of bixin and crocetin

The hepatic clearance of bixin and crocetin was determined for subsequent PBK modeling using incubations with primary human hepatocytes. Figure 6.4 shows that bixin concentrations decreased during the incubation, resulting in an in vitro clearance ($Cl_{int\ in\ vitro}$) of 36.13 µL/min/ 10^6 cells, and a scaled in vivo clearance ($CL_{int\ in\ vivo}$) of 364.16 L/h. Crocetin concentrations were not clearly affected along the 60 minutes incubation with human hepatocytes and therefore, for subsequent PBK modeling, hepatic clearance was assumed to be negligible ($CL_{int\ in\ vivo} = 0$).

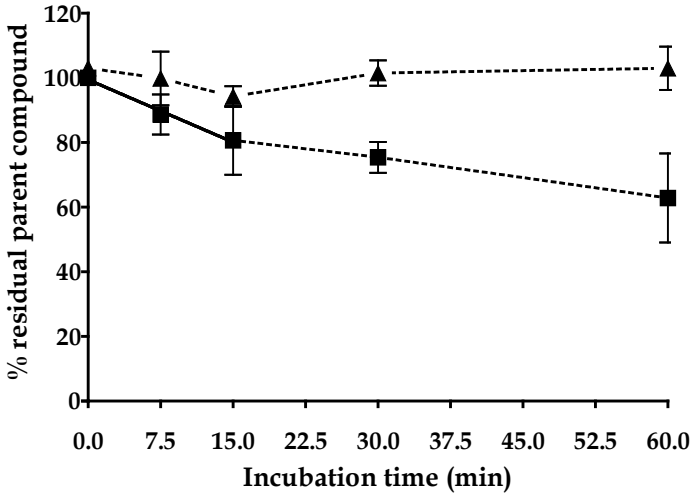

Figure 6.4. Hepatic clearance of bixin (square) and crocetin (triangle) during the incubations with primary human hepatocytes for 60 min. The slope for linear regression until 15 min (straight line) was used to determine the in vitro half-life ($t_{1/2}$) of bixin.

6.3.3. Evaluation of the PBK models for bixin and crocetin

To evaluate the PBK models, the dose-dependent blood concentrations of bixin and crocetin in humans were predicted and compared to blood concentrations resulting from oral intake of bixin and crocetin reported in literature. For bixin, the one study available reported a maximum blood concentration (C_{max}) of 0.029 µM after an oral dose of 0.23 mg/kg bw.[43] This predicted C_{max} value accurately matched the PBK model based predicted C_{max} value of 0.027 µM. For crocetin, also a single human study was available reporting C_{max} values after oral intake at three different dose levels of 0.125, 0.25, and 0.374 mg/kg bw.[44] The PBK model based predicted C_{max} values at these dose levels amounted to 0.12, 0.25, and 0.37 µM which were 2.5-, 2.5-, and 2.3-fold lower than the reported values of 0.31, 0.61 and 0.85 µM, respectively. Thus, comparison of the predicted and reported blood levels of bixin and crocetin reveals that the PBK models adequately predicted the C_{max} values.

Furthermore, comparison of the C_{max} values of bixin and crocetin reveals that the C_{max} values for bixin are about 5-14 times lower than those of crocetin.

The performance of the developed models was further evaluated by a sensitivity analysis to assess the parameters that affect the prediction of the C_{max} of bixin and crocetin in blood to the largest extent. The sensitivity analysis was performed at an oral dose of 0.23 mg/kg bw for bixin and 0.25 mg/kg bw for crocetin, which are dose levels applied in the available in vivo kinetic studies. Only the parameters that resulted in a normalized sensitivity coefficient higher than 0.1 (in absolute value) are shown in Figure 6.5. The results obtained reveal that the prediction of C_{max} in the PBK model is most sensitive to the parameters related to the liver including the hepatic clearance (CL_{int}), the absorption rate constant for uptake from the GI tract into the liver (ka) and hepatocellularity (Hep).

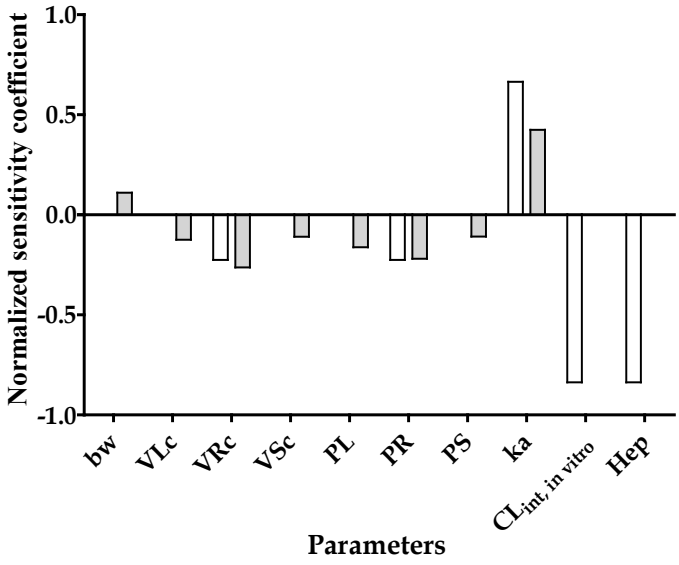

Figure 6.5. Normalized sensitivity coefficients for the parameters of the PBK model for bixin and crocetin on predicted C_{max} in blood at a single oral dose of 0.23 mg/kg for bixin (white bars) and 0.25 mg/kg (grey bars) for crocetin. bw = body weight, VLc = fraction of liver volume, VRc = fraction of rapidly perfused tissues volume, VSc= fraction of slowly perfused tissues volume, PL = liver/blood partition coefficient, PS = slowly perfused tissue/blood partition coefficient, PR = rapidly perfused tissue/blood partition coefficient, ka = uptake rate constant, $C_{int, in\ vitro}$ = in vitro intrinsic clearance of bixin/crocetin, Hep = hepatocellularity.

Figure 6.6 presents the in vivo dose reponse curves obtained for bixin and crocetin when, upon correction for the differences in unbound fraction, the in vitro concentrations were converted to corresponding in vivo dose levels. BMD modeling of these data (for details see supporting information 5 Figure S1), resulted in the

BMD_{50}, $BMDL_{50}$ and $BMDU_{50}$ values presented in Table 6.1. From these data it follows that the BMD_{50} of bixin is about 30 times higher than that of crocetin.

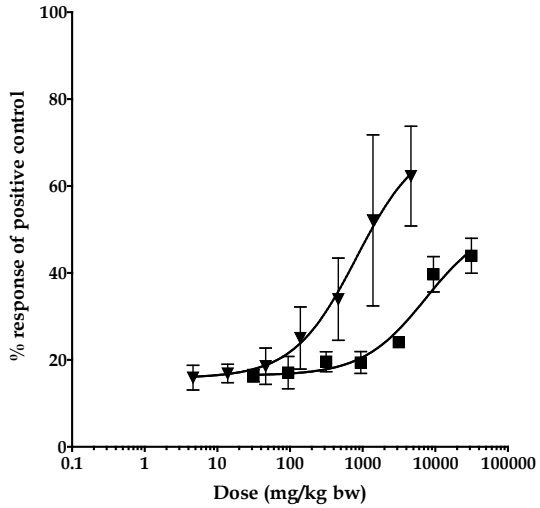

Figure 6.6. Predicted in vivo dose-response curves for PPARγ-mediated gene expression of bixin (square) and crocetin (triangle) in human. Predicted dose–response data were obtained using PBK modeling-facilitated reverse dosimetry for conversion of in vitro concentration–response data obtained in the PPARγ CALUX reporter gene assay (Figure 6.3).

Table 6.1. BMD_{50} and $BMDL_{50}$-$BMDU_{50}$ values derived from the dose-response curves predicted using PBK modeling-facilitated reverse dosimetry to convert the in vitro concentration-response curves as obtained in the present study to in vivo dose-response curves.

Compound	BMD_{50} (mg/kg bw)	Predicted $BMDL_{50}$ -$BMDU_{50}$ (mg/kg bw)
Bixin	3,505	1,710-5,220
Crocetin	115	0.32-374

6.3.4. Comparison to human dietary intake levels

The predicted BMD_{50} values including the $BMDL_{50}$ and $BMDU_{50}$ values thus obtained were compared to the reported dose levels of bixin and crocetin resulting from daily intake in humans as taken from the literature. Figure 6.7 shows a comparison of the predicted BMD_{50} values (presenting also the $BMDL_{50}$-$BMDU_{50}$ range) for bixin- and crocetin-mediated induction of PPARγ activity in vivo and the estimated dietary intake levels, resulting from use of the compounds as food additives and/or as functional food ingredients in food supplements.

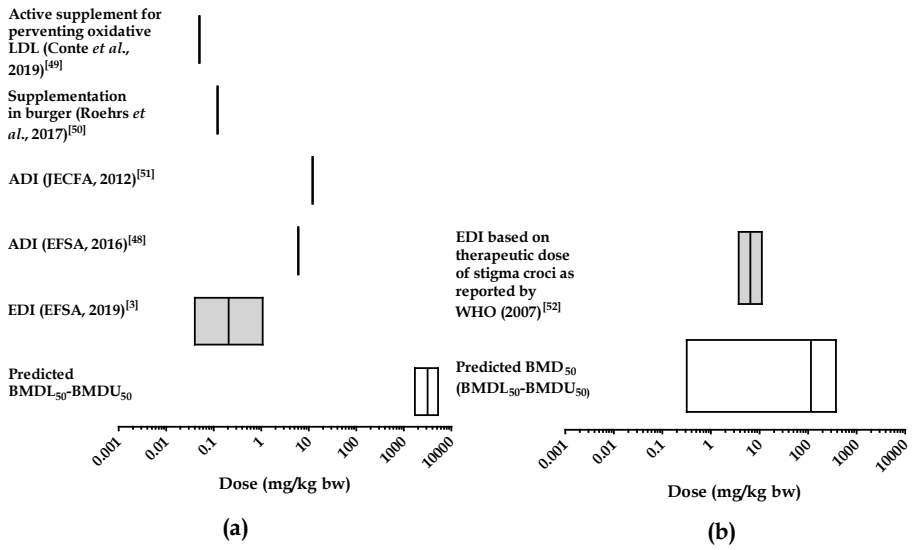

Figure 6.7. Comparison of the predicted in vivo BMD_{50}, $BMDL_{50}$ – $BMDU_{50}$ for PPARγ activation with available EDI values for a) bixin and b) crocetin in human. For comparison also the available ADI values are included.

The recent exposure assessment performed by EFSA[3] reported the estimated maximum level of dietary exposure to bixin-based annatto extracts (E 160b) from its use as food additive to amount to 0.04-1.07 mg/kg bw/day (95th percentile). This value is 3 to 5 orders of magnitude lower than the predicted BMD_{50} for PPARγ acivation, which reveals that normal dietary intake of bixin is expected to not result in activation of PPARγ-mediated gene expression. Also bixin supplementation at a level of 0.05 mg/kg bw in healty human subjects which was reported to be an active dose to prevent early oxidative modifications in LDL as key event of atherosclerosis[49] is several orders of magnitude below the predicted BMD_{50} for inducing the PPARγ-mediated gene expression. This result is in line with results reported before concluding that bixin supplementation amounting to 1.2 mg/kg bw (10% of the ADI) had no effect on the postprandial oxidative LDL levels and thus seemed inactive in preventing the risk of cardiovascular disease and insulin resistance.[50] Furthermore comparison of the predicted BMD_{50} values to the ADI values for bixin of 6 mg/kg bw/ day and 0-12 mg/kg bw/ day established by EFSA[3] and JECFA[51] reveals that these ADI values are also 2 to 3 orders of magnitude lower than the BMD_{50} indicating that they will prevent effective PPARγ-mediated gene expression.

For crocetin there are no existing values for the EDI resulting from its use as a food ingredient. However, the WHO (2007)[52] based on the Pharmacopoeia of the

People's Republic of China reported that the recommended therapeutic daily dose of stigma croci (saffron stigma) is 3-9 g. Considering the level of crocin of 25.95 mg/100 mg dry saffron[53] and the mass ratio of crocetin to crocin, this dose of stigma croci is estimated to be equivalent to an intake of crocetin of 3.74 – 11.2 mg/kg bw/day for a 70 kg person (see supporting information 6 for the detailed calculation). Comparison of this EDI to the predicted BMD_{50} and $BMDL_{50}$-$BMDU_{50}$ range for crocetin reveals that the recommended therapeutic dose as reported by the WHO (2007)[52] is predicted to represent a dose levels where PPARγ activation in human might be expected, although it must be noted that the confindence intervals in the predicted dose-response data for crocetin are large.

6.4. Discussion

PPARγ has been identified as a ligand-regulated nuclear receptor reported to increase insulin sensitivity in the treatment of T2DM. This made PPARγ a target for drug development and also resulted in reports on various natural dietary ingredients able to activate PPARγ-mediated gene expression. This includes reports on activation of PPARγ by various carotenoids as detected in in vitro reporter gene assays.[18-21] Some carotenoids, including the model compounds of the present study bixin and crocetin have also been proposed for use as functional food ingredients and/or are used in traditional medicine to treat T2DM-related symptoms.[54] For crocetin, the therapeutic use of crocetin-containing stigma croci has been proposed at dose levels amounts to 3-9 g per person, estimated in the present study to be equivalent to 3.74-11.2 mg crocetin/kg bw for a 70 kg person.[52] The aim of the present study was to investigate at what dose levels bixin and crocetin would be expected to induce PPARγ-mediated gene expression in humans in vivo by using a combined in vitro-in silico based testing strategy without the need for a human intervention study. Thus, the present study especially investigated whether dose-response curve for in vivo PPARγ activation in human by bixin and crocetin can be quantitatively predicted by PBK modeling-facilitated reverse dosimetry of PPARγ activation data obtained in an in vitro PPARγ reporter gene assay.

The results of the in vitro study indicate that both bixin and crocetin can activate PPARγ-mediated gene expression in U2OS PPARγ2 cells (Figure 6.3). This observation is in line with earlier reports on PPARγ activation by related carotenoids.[18-21] The results also match the results which reported that branched fatty compounds represent a group of natural PPARγ agonists able to enhance insulin sensitivity of adipocytes.[18] The EC_{50} values for bixin- and crocetin-dependent induction of PPARγ-mediated gene expression in the U2OS PPARγ2 cells

were similar indicating a similar intrinsic potency of the carotenoids to induce PPARγ activity. The absence of PPARγ induction by norbixin, the metabolite resulting from hydrolysis of bixin, as observed in the present study is in line with results previously reported by Takahashi et al. 2009[18] who reported that the activity of norbixin for PPARγ activation was substantially lower than that of bixin when tested in the luciferase assay using a chimera protein of PPARγ and the PPAR full-length system, respectively. Moreover Roehrs et al. (2014)[10] found the opposite effect of bixin and norbixin on potentially PPARγ related effects in vivo; where the highest dose of norbixin increased dyslipidaemia and oxidative stress in streptozotocin-induced diabetes rats, bixin showed an antihyperglycemic effect, improving lipid profiles, and protecting against damage induced by oxidative stress in the diabetic state.

To enable the translation of the in vitro concentration-response curves to in vivo dose-response curves for PPARγ activation by bixin and crocetin, PBK models for bixin and crocetin were developed. Characterisation of the model parameters for hepatic clearance revealed that hepatic clearance of crocetin was limited as compared to that observed for bixin. This result explains the observed differences in reported and also in the PBK modeling-based predicted C_{max} levels for crocetin and bixin in blood at comparable dose levels. The C_{max} values for crocetin were about 10-20 fold higher than those for bixin at comparable dose levels. Furthermore, comparison of the predicted C_{max} values to C_{max} values actually observed in available in vivo kinetic studies in human[43, 44] revealed that for both bixin and crocetin these differences were limited. The predicted C_{max} of bixin of 0.027 µM was similar to the reported value of 0.029 µM.[43] For crocetin there was only a 2-fold difference between the PBK model predictions and the reported C_{max} values[44], the predicted values being somewhat too low.

Upon evaluation of the PBK models the available in vitro concentration–response curves for bixin- and crocetin-mediated PPARγ activation were converted to in vivo dose–response curves using PBK modeling-facilitated reverse dosimetry. The BMD_{50} and $BMDL_{50}$-$BMDU_{50}$ values derived from the dose-response curves thus obtained were compared to estimated daily intakes for bixin and crocetin resulting from realistic exposure scenarios. These comparisons revealed that EDI values for bixin resulting from its use as a food additive[3] or as food supplement[49, 50] are unlikely to result in PPARγ-mediated gene expression in humans. In contrast, use of crocetin-containing stigma croci at dose levels amounting to 3-9 g per person, estimated to be equivalent to 3.74-11.2 mg crocetin/kg bw for a 70 kg person[52], were predicted to more likely result in substantial induction of PPARγ-mediated gene

expression in human. However, it must be noted that the confindence intervals in the predicted dose-response data for crocetin are large and that the BMD_{50} of the predicted dose-response data is about 10 times higher than the intake at therapeutic dose levels. On the other hand, since clearance of crocetin was measured to be negligible in our in vitro studies, crocetin clearance in vivo is expected to be limited as well so that internal concentrations may increase upon daily repeated crocetin intake, resulting in lower predicted effective dose levels.

It is of interest to note that in spite of the intrinsic similar potency of bixin and crocetin to induce PPARγ-mediated gene expression, as reflected by similar EC_{50} values in the PPARγ reporter gene assay, the predicted in vivo BMD_{50} values differed 30-fold with the value for crocetin being lower. This can be ascribed to the more efficient clearance of bixin than of crocetin, resulting in lower dose levels required to reach effective in vivo C_{max} levels for crocetin than for bixin. This difference in clearance was observed in the in vitro incubations with the primary hepatocytes used in the present study. The few articles reporting on the pharmacokinetics of crocetin in human confirm the inefficient, albeit not negligible, clearance of crocetin.[44, 55-57]

The present study used PBK modeling-based reverse dosimetry converting in vitro data to predicted in vivo dose-reponse curves enabling definition of effective in vivo dose levels. In previous studies this combined in vitro-in silico approach appeared already valid for other endpoints including for example genistein-induced estogenicity[36], hesperitin-induced effects on inhibition of protein kinase A activity[35], azole-[37], phenol-[38], retinoic acid-[39] and glycol ether-mediated developmental toxicity[40], and lasiocarpine- and riddelliine-induced liver toxicity[41, 42]. The results of the present study illustrate that this combined in vitro-in silico approach can also be used to obtain insights in human responses to potential functional food ingredients. This insight can be used to select the promising compounds for subsequent human intervention studies and can help in the selection of doses in such studies.

Conflict of interest

The authors state no conflicts of interest.

Acknowledgments

This research was supported by a grant from the Indonesian Endowment Fund for Education (LPDP), Ministry of Finance, Republic of Indonesia [grant number: PRJ-365/LPDP/2016] awarded to Suparmi.

Supporting information

Supporting information to this article can be downloaded from: https://doi.org/10.1002/mnfr.201900880.

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BENEFIT
RISK

Chapter 7

General discussion

7.1. Overview and general discussion of main findings

The high consumer demand for beneficial alternative medicines provokes a notorious production of herbal products in Indonesia. Based on the research on medicinal botanicals and jamu in 2017, Indonesia has 2,848 species of medicinal botanicals from which 32,014 efficacious formulae of herbal products have been developed.[1] The preference of Indonesian people for use of traditional medicine increased by 44.3% during 2010-2018.[2] While the consumers perceive that herbal products are "safe" and "natural" and thus "healthy", a risk and benefit assessment is crucial to support the safe and effective use of herbal products.[3-5]

The aim of this thesis was to analyze potential health risks due to the presence of constituents that are genotoxic and carcinogenic in botanicals and botanical products on the Indonesian market. The constituents of concern were alkenylbenzenes (ABs), pyrrolizidine alkaloids (PAs) and aristolochic acids (AAs). Their levels were determined in botanical preparations collected by a targeted sampling approach on the Indonesian market, followed by an exposure and risk assessment using the so-called Margin of Exposure (MOE) approach. In addition, a novel testing strategy for quantitative in vitro to in vivo extrapolation (QIVIVE) was applied to investigate if this novel testing strategy can help to fill data gaps in the currently available data base on the toxicity of PAs. The approach was used to predict acute liver toxicity in rats of the PA monocrotaline as the model compound. In addition, the same novel testing strategy was applied to provide a proof of principle to predict potential beneficial effects of botanical constituents in humans. To this end, the in vitro activity for activation of peroxisome proliferator receptor gamma (PPARγ) mediated gene expression by the model carotenoids bixin and crocetin was translated to an in vivo dose response curve for PPARγ activation in humans. This allowed comparison of supplemental dose levels of bixin and crocetin to predicted dose levels for in vivo activity to evaluate whether these supplemental dose levels would be potentially active in vivo, activating PPARγ and potentially contributing to improvement of insulin sensitivity. Figure 7.1 summarizes the main findings of the thesis, which are discussed in some more detail in this chapter.

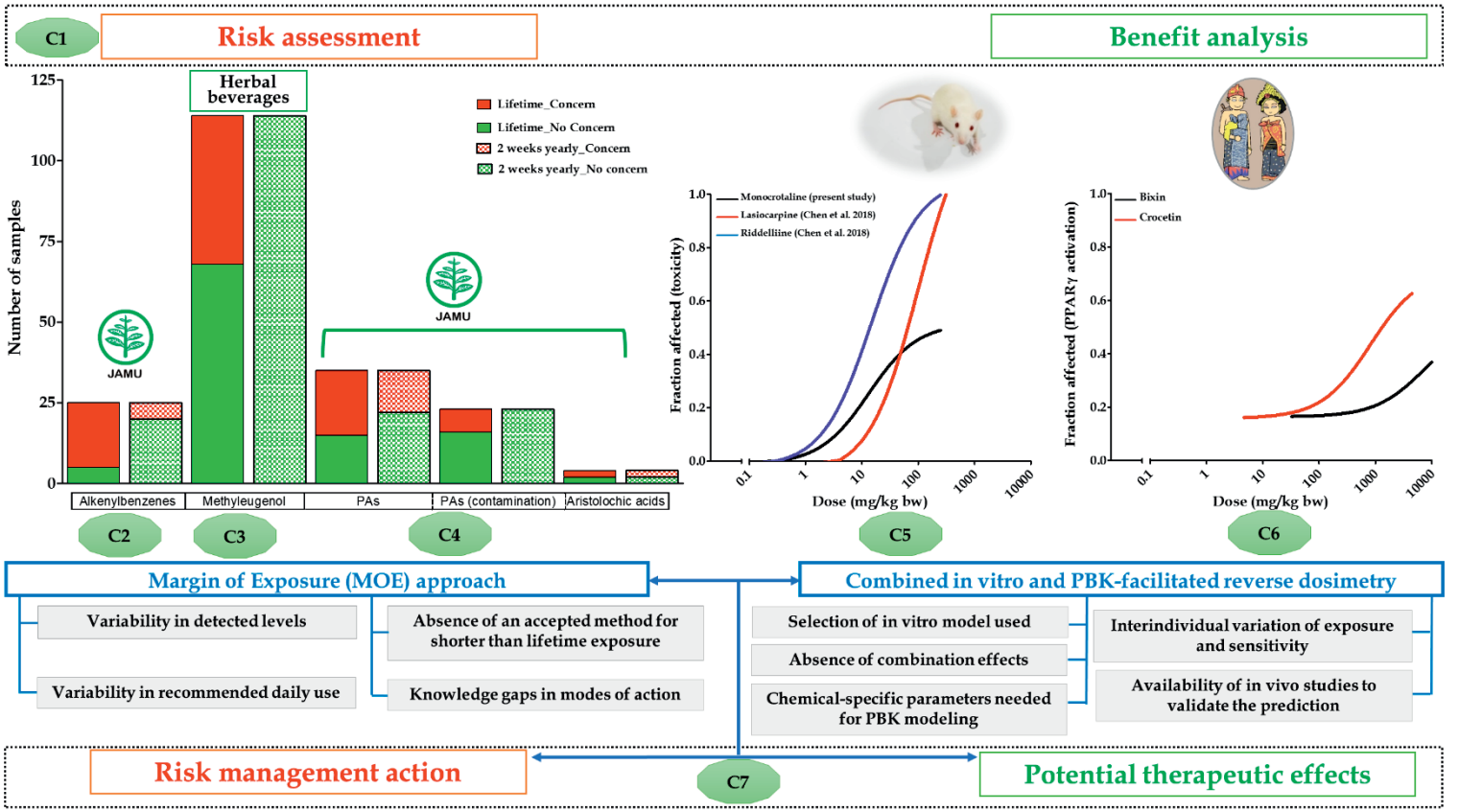

Figure 7.1. Flowchart illustrating the main finding of each chapter (C = Chapter). Grey boxes show the factors influencing the application of the MOE approach and combined in vitro-PBK modeling-facilitated reverse dosimetry in risk and benefit analysis of herbal products, also discussed in some more detail in this chapter.

Chapter 1 presented an overview of the importance of the herbal market in Indonesia and the available regulatory framework. In addition, the methods for risk and benefit assessment of herbal products used in the thesis, including the alternative testing strategy applied, were presented in some more detail, and the selected compounds ABs, PAs including monocrotaline, AAs, bixin and crocetin were introduced. In **Chapter 2, 3 and 4** series of botanical preparations collected by targeted sampling on the Indonesian market were analyzed for the presence of constituents of concern because they are genotoxic and carcinogenic. This enabled a risk assessment using the MOE approach. Overall, the MOE values obtained were generally <10,000, indicating a priority for risk management when assuming daily life-long consumption of AB-containing jamu (**Chapter 2**), methyleugenol-containing herbal beverages (**Chapter 3**), and jamu containing PA-producing botanicals and non-PA-producing botanicals (**Chapter 4**). It was also considered that risk assessment using the MOE is generally based on the assumption that estimated exposures are relevant every day during a whole lifetime.

For use of botanicals and botanical preparations, which are rather expected to be used during shorter periods of illness, this shorter-than-lifetime exposure was also considered. Although a formally accepted method to take this shorter-than-lifetime-exposure into account is currently not available, some studies suggest using Haber's rule to correct in a linear way for the duration of exposure. Using Haber's rule, it was estimated that exposure to ABs, including methyleugenol, and to PAs, via the majority of products analyzed, would be of low concern when the herbal preparations would be consumed for less than 2 weeks per year during a lifetime, although some samples still raised a concern.

Furthermore, combining in vitro bioassays with physiologically based kinetic (PBK) modeling-facilitated reverse dosimetry was shown to enable quantitative prediction of the acute liver toxicity of monocrotaline in rats (**Chapter 5**), while the method was also able to estimate whether at realistic dietary intake the carotenoids bixin and crocetin could be expected to induce PPARγ-mediated gene expression in humans (**Chapter 6**). Factors influencing the results presented in this thesis are discussed in this chapter (**Chapter 7**). The chapter also proposes some future perspectives related to the findings, indicating topics to consider in continued work in the field of risks and benefits of botanicals and botanical preparations.

In the risk assessment performed in **Chapter 2**, **3** and **4**, MOE values were calculated by dividing relevant $BMDL_{10}$ values (lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence) by the estimated daily intakes (EDI) of the targeted compounds. The EDI values were based on experiments in which the levels of the ABs, PAs and AAs were quantified in jamu and herbal

beverages obtained on the Indonesian market via targeted sampling. Using the results of these chemical analyses and the dose of the product recommended by the respective manufacturers, intake was estimated and compared with $BMDL_{10}$ values to calculate MOE values. When studying the risk assessments using the MOE approach, there are many factors that may need further consideration in future studies. These aspects include: **variability in detected levels of the targeted compounds, variability in recommended daily use mentioned on the label** that can influence the risk assessment. Other bottlenecks to be considered to a further extent are **absence of a generally accepted method to take shorter-than-lifetime exposure into account,** and **knowledge gaps in modes of action**. When applying a QIVIVE approach for the risk and benefit analysis as done in the present study, **selection of the in vitro model used,** the **absence of combination effects**, the **chemical-specific parameters needed for PBK modeling, interindividual variation of exposure and sensitivity** among Indonesian people, and the **availability of in vivo studies to validate the predictions** are issues for further consideration and future research. In the following sections, these additional considerations are discussed in more detail.

7.1.1. Variability in detected levels of the targeted compounds

Variety in occurrence of the targeted compounds is one of the challenges in risk assessment of herbal products. The profile and level of detected compounds are known to vary widely in different types of herbal products (**Chapter 2** and **3**) and may vary even within the product brand. Table 7.1 shows that using the same sampling strategy and analytical procedure the profile of detected ABs showed substantial variation among four types of herbal products, including jamu (**Chapter 2**), herbal beverages (**Chapter 3**), herbal teas (unpublished results) and botanical food spices (unpublished results). None of the samples contained apiol, because parsley (*Petroselinum crispum*) and dill (*Anethum graveolens*), main apiol-producing plants[6, 7] were not used as botanicals materials in the Indonesian herbal products investigated. Methyleugenol appeared to be a major AB detected in all the types of samples, while for this AB a maximum residue level has not been established in Indonesia.

Table 7.1. Overview of AB occurrence in the different types of samples analyzed in the present thesis.

Type of samples	n[a]	n positive (%)[b]	Number of samples containing each individual AB (% of positive samples containing the compound)						
			Methyl-eugenol	Myristicin	Safrole	Estragole	Elimicin	Apiol	Eugenol
Jamu	25	23 (92)	21 (91.2)	13 (56.5)	4 (17.4)	2 (8.7)	-[c]	-	-
Herbal beverages	114	49 (43)	49 (100)	-	-	-	-	-	4 (3.5)[d]
Herbal teas	9	8 (88.9)[e]	7 (87.5)	6 (75)	-	-	1 (11.1)	-	-
Botanical food spices	25	24 (96)	10 (41.7)	7 (29.2)	16 (66.7)	8 (33.3)	1 (4.2)	-	-

[a] Number of samples analyzed
[b] Number of samples that were found to contain ABs (level > LOD) out of the total number of samples detected
[c] Not detected
[d] Additional AB detected in the samples (% out of the total sample analyzed): eugenol is not a genotoxic and carcinogenic AB.
[e] ABs for these samples were detected in methanol extracts

This variety of occurrence for ABs but also for PAs and AAs (**Chapter 4**), relates to the raw materials, sample preparation, extraction and analytical methods applied to prepare the botanical preparations. Crews et al. (2010) reported that extracting, separating, identifying, and measuring a wide variety of PAs in very different matrices including plants, seeds, honey, pollen, body fluids, and insects is challenging for the analyst.[8] The variability of the AB, PA and AA levels may be due to differences in extraction efficiency[9-11], but also to ecological factors[12, 13], harvesting time, harvesting techniques, storage circumstances, processing technologies, and measurement methods[14-16]. Therefore, a standardized production procedure is essential to minimalize the variability in the levels of these important toxins in food and botanicals samples[8, 17], while better characterisation of batch to batch variability will also be of use for adequate future risk assessment and risk management.

Furthermore, the sample strategy chosen and thus how well the sample analysed represents the whole batch, plays an important role in the reliability of any conclusions drawn from the sample analysis. In the present thesis (**Chapter 2-4**) samples were collected in retail packages, ten consumer packages were selected from the same batch, then pooled by mixing the contents before analysis as recommended by WHO[17]. In addition, storage of the samples collected should be optimized to prevent the degradation of targeted compounds to ascertain that storage will not have a significant influence on the compound profiles.[18, 19]

The variability in composition of botanical materials affects the variability of detected levels of the compounds of interest. AB-producing botanicals appeared to be present in many herbal products sold by the sellers in the sampling locations.

Ginger (*Zingiber officinale* Rosc) rhizome, fennel (*Foeniculum vulgarei* Mill.) fruit, and cinnamon (*Cinnamomum burmannii* Blume) bark, lemongrass (*Cymbopogon nardus* L. Rendle) leaf, betel (*Piper betle* L.) leaf, nutmeg (*Myristica fragrans* Houtt.) seed, clove (*Syzygium aromaticum* (L.) flower, basil (*Ocimum basilicum*) leaves, all well-known AB-producing botanicals[20-22], were present in many of the collected samples. For example, ginger, fennel, and cinnamon were found in respectively 26, 23 and 22 of the 55 jamu samples (registered as BPOM RI-TR) analysed in **Chapter 2** and **3**. According to the label, the content of these AB-producing botanicals contributed around 0.1 to 90% to the weight of the sample. In contrast, occurrence of PA- and AA-producing botanicals appeared to be less common in jamu, in part because the use of (some) PA- and AA-producing botanicals has been restricted in Indonesia. For example comfrey (*Symphytum officinale*) as a PA-containing botanical has been listed as a banned ingredient in Indonesian traditional medicines based on regulation BPOM RI No 7:2018.[23] In **Chapter 3** comfrey was only found in 2 out of 35 jamu samples containing other PA-producing botanicals, including *Lithospermum orientale*, *Gynura pseudochina*, *Gynura* sp., *Gynura procumbens*, *Gynura segetum* and *Adenostemma lavenia*. Botanical *Aristolochia spp, Stephania tetrandra* S.Moore, *Magnolia officinalis* Rehder & E.H.Wilson, and their preparations, have been banned as ingredients in traditional medicines in Indonesia based on Regulation BPOM RI No. 7, 2018.[23] Only 1 out of 15 jamu collected in the targeted approach for AA containing plants appeared to contain *Aristolochia* spp. The other 14 samples targeted for AA containing plants contained *Saussurea lappa*, *Clematis chinensis*, *Stephania tetrandra*, *Asarum sieboldii*, and *Aucklandii lappa* as AA-producing botanicals.[24]

In case of PA occurrence due to the contamination of jamu samples by PA-producing botanicals (**Chapter 4**), Schulz et al. (2015)[25] reported that properties of the contaminating botanicals or of part of the botanicals in the samples caused the variation in the PA content within different samples of the same batch. To reduce the impact of unhomogenized composition during sample analysis, it might be useful to reduce the sample size, but increase the number of samples from a specific batch while keeping the same sample/extraction volume ratio. One could even consider use of statistical approaches to define optimal sampling strategies, as is current practice when for example sampling mycotoxins.[26, 27]

7.1.2. Variability in recommended daily use mentioned on the label

The potential exposure to carcinogenic genotoxic compounds resulting from consuming the herbal products analysed in the present thesis (**Chapter 2-4**) was estimated using the direction for their use as indicated on the labels. For some products the recommended daily use is clearly indicated on the label, but for others

there appeared to be no information on recommended use on the label. Based on Regulation BPOM RI No 31: 2018, the label of food should indicate product name, ingredients, net weight, name and address of producer/importer, halal information if relevant, date and production code, information on expiring date, marketing authorization number and source of certain ingredients.[28] The regulation does not prescribe the presence on the label of information on directions for use and use levels.

Based on the information available on the labels of the samples analysed in the present thesis, the recommended daily consumption appeared to vary substantially among the types of herbal products, and this obviously influenced the risk assessment (Table 7.2). Jamu, registered as BPOM RI TR, appeared to show a high variability of the weight of recommended daily use (0.5 - 200 g), with especially the high use levels resulting in a high number of samples (23.6% out of 246 samples) raising a health concern and indicating a priority for risk management action. The recommended daily intake also varied among herbal beverages produced by household industry (Depkes RI P-IRT) with also high use level preparations showing the highest number of samples which raise concern for human health. In contrast, the products registered as domestic processed food (BPOM RI MD) indicated the lowest variability in the range of recommended daily uses (1 - 30 g). The high variability of use and use level recommendations for the consumers on the labels of jamu and their variable quality in terms of absence of constituents of concern, may relate to the large number of jamu producers and to what extent each of them complies with the regulation on jamu production. Clearly harmonisation of use levels and quality control provide important issues for future risk management actions.

Table 7.2. Overview of recommended daily intake in the different types of samples analyzed in the present thesis and the percentage raising health concerns upon use over a lifetime.

Registration code of marketing authorization	Type of product	Total samples analysed	Recommended daily intake (g)		Total samples which raise concern (%)[a]
			Range	Average ± SD	
BPOM RI TR	Traditional medicine	131	0.15 - 200.0	12.2 ± 19.3	58 (23.6)
BPOM RI MD	Domestic Processed Food	54	1.0 - 30.0	12.2± 10.1	20 (8.1)
BPOM RI SD	Domestic supplement	9	4.0 - 21.0	12.2±6.0	0 (0.0)
BPOM RI ML	Foreign Processed Food	1	18.0	-	1 (0.4)
Depkes RI P-IRT	Food Household Industry	51	1.0 - 75.0	18.8±12.3	33 (24.6)
Total		**246**			**112**

[a] Percentage of total samples analysed

7.1.3. Absence of a generally accepted method to take shorter-than-lifetime exposure into account

The fact that the duration of herbal product consumption is unlikely to extend over a whole lifetime as assumed in the MOE approach for risk assessment is another factor that needs future attention. People tend to consume these preparations during periods of illness, and therefore in this thesis the MOE approach was combined with Haber's rule to take shorter-than-lifetime, i.e. more realistic, exposure scenarios into account. This approach was previously proposed by Felter et al. (2011)[29] as a framework for assessing the risk from shorter-than-lifetime exposures to potential human carcinogens. The application of Haber's rule, however, is not a generally accepted approach when using the MOE for risk assessment of exposure to genotoxic carcinogens. Under this rule, the toxic outcome is assumed to be similar for situations where the product of the exposure time and the dose will be constant, ($k=C\times T$; $C1\times T1=C2\times T2$, where k is the toxic outcome, C is the concentration (or dose) of the toxic chemical and T is the time of exposure). This implies a linear relationship between the response and the dose as well as the exposure time.[29]

The question of whether the rule also holds for nonlinear dose-response relationships has been debated by various authors.[30] The use of Haber's rule in risk assessment should be critically evaluated when the response is nonlinear, considering the mode of action (MOA) and toxicokinetics/toxicodynamics of the compound(s) of interest. Several approaches could be considered for use of Haber's rule for nonlinear responses,[29], including its use with the application of a dose-rate correction factor (DRCF) [31], and/or assigning "default" adjustment factors.[32]

Felter et al. (2011)[29] indicated that use of Haber's rule assumes that chemical-specific carcinogenicity data are available, and that the data support a linear dose-response relationship. To what extent such a linearity relationship holds for the induction of liver tumors by ABs, PAs, and AAs remains to be established and may depend on the MOA underlying the carcinogenicity. It is important to note that at the present state-of-the-art evidence supporting such linear behaviour of the adverse effects of ABs consists of PBK-based studies showing dose dependent linearity in the bioactivation to the ultimate carcinogenic 1'-sulfoxymetabolites and in DNA adduct formation, increasing in a linear way from realistic dietary exposure levels up to dose levels causing significant tumor formation.[33-36] However, the linearity of the subsequent mutagenic and carcinogenic effects of the 1'-sulfoxy AB DNA adducts, has not been quantified so far. For AAs and PAs such information is also presently not available. Thus, it can be concluded that providing such information on dose-dependent linearity of the adverse response, and definition of a generally accepted approach to deal with shorter than lifetime exposure in risk assessment of

genotoxic and carcinogenic botanical constituents is an important topic for the future risk assessment of botanicals and botanical preparations.

7.1.4. Knowledge gaps in the mode of action (MOA)

Other challenges remaining in the risk assessment of AB-, PA- and AA-containing botanicals and botanical preparations relate to the MOA underlying the carcinogenic and genotoxic effects. For ABs, for example, knowledge on the level of DNA adduct formation required to raise risks on tumor formation in humans above background levels remains to be elucidated. The 1'-sulfoxymetabolite and the subsequently formed DNA adducts play an important role in the AB-induced carcinogenicity, and knowledge on the levels of DNA adduct formation in human livers at relevant dietary intake levels would be of use to facilitate human risk assessment. This is especially of interest because previously, DNA adduct formation induced by dietary intake of methyleugenol was detected in 29 out of 30 human liver samples at levels that amounted for the maximal and median levels to 37 and 13 adducts per 10^8 nucleosides respectively.[37] Given the use of jamu containing the AB methyleugenol, it would be of interest to study whether such DNA adducts can also be detected in Indonesian human liver samples, and to develop methods that would allow estimation of the corresponding risks, in order to evaluate whether these risks would substantially increase background tumor incidences.

Such a method enabling estimation of the risks resulting from DNA adduct formation in human liver upon realistic daily exposure to ABs could be based on combining PBK models predicting dose dependent DNA adduct formation in the liver with data on DNA adduct formation at dose levels causing liver tumors in experimental animals. In this approach the PBK models will facilitate rat to human and high to low dose extrapolations. In addition, further studies on repair of DNA adducts resulting from AB, PA and AA exposure might help to clarify the risks of consumption of botanicals and botanical preparations containing these constituents at realistic low dose levels. Primary hepatocytes could likely be used to study possible repair and stability of the DNA adducts and would thus provide insight in whether formation of adducts, even during a short period, could have irreversible effects and thus be deleterious, or whether they are likely to be efficiently repaired, thereby decreasing the risk. For PAs, DNA adduct formation can be considered a surrogate measure of the reactive pyrrolic ester intermediate that forms intracellularly.[38] DNA adducts, DHP-dG-3, DHP-dG-4, DHP-dA-3, and DHP-dA-4, are a common biological biomarker of PA-induced liver tumor formation[39, 40], so to better link their formation to a MOA based risk assessment for PA exposure seems an important topic for future research.

7.1.5. Selection of the in vitro model used for QIVIVE

The quality of in the vitro studies used to define the concentration response curves that from the basis of the PBK model based QIVIVE, also need some further consideration. The in vitro assay, endpoints and readout parameters selected should cover the relevant MOAs of targeted compounds and are crucial for reliable predictions of the in vivo situation made by PBK-modeling facilitated reverse dosimetry.

For the analysis of beneficial effects of bixin and crocetin in PPARγ activation (**Chapter 6**), the PPAR-γ2 CALUX cells appeared to provide an adequate in vitro cell model to quantify PPARγ-mediated gene expression. Gijsbers et al. (2011)[41] demonstrated that the PPARγ1 CALUX and PPARγ2 CALUX cells, U2OS cells transfected by an expression vector for PPARγ1 or PPARγ2 and a pGL3–3xPPRE–tata-luc or pGL4–3xPPRE–tata-luc reporter, provide in vitro tools to test (mixtures of) chemicals, endogenous ligands, and (food) compounds for their ability to activate PPARγ1-mediated and PPARγ2-mediated gene expression. These cell lines express the complete human PPARγ receptor,[41] potentially allowing more faithful translation to effects in humans than the systems based on a chimeric PPARγ–GAL4 receptor[42, 43].

When studying the liver toxicity of PAs, as done in the present thesis, maintaining metabolic activity within the cell model used determines the quality of the in vitro studies. Lauschke et al. (2016)[44] reported that ideally, in vitro systems for studies on drug metabolism and toxicity, should accurately correspond to the phenotypes observed in vivo, including the expression of P450 and phase II enzymes, transporters, and nuclear receptors. Furthermore, viability, functionality, and phenotypes of cells should be stable for multiple weeks in culture to allow repeated dose toxicity studies. Primary hepatocytes in suspension or in monolayer culture are currently considered the most appropriate model for the evaluation of integrated drug metabolism, toxicity/metabolism correlations, mechanisms of hepatotoxicity, and the interactions (inhibition and induction) of xenobiotics with drug-metabolising enzymes.[45, 46] However, at the present state-of-the-art stability of this in vitro model is somewhat limited, hardly enabling studies beyond 24-72 hours duration.

Monolayer cultures of primary hepatocytes have been the gold standard for in vitro hepatotoxicity testing for acute effects.[47] In **Chapter 5**, the cryopreserved monolayer culture of primary rat hepatocytes was used to assess monocrotaline-induced acute liver toxicity. Although the cryopreservation was reported to slightly reduce cell viability compared to fresh isolated hepatocytes,[48, 49] the cell viability used in the present study was more than 90% after thawing. This indicates that

cryopreserved primary rat hepatocytes can be used as an adequate cell model to quantify in vitro liver toxicity of PAs.

Chapter 6 used pooled human cryopreserved hepatocytes (HEP10) in suspension to define the kinetic parameters for clearance in the PBK models for bixin and crocetin. The use of this cell model for clearance studies is supported by results reported by Griffin and Houston (2005)[50] showing that hepatocyte monolayer cultures offer the potential for extending measurements for predicting in vivo clearance to the lower end of the clearance range (below 0.1 μmol/min/10^6 cells). An important limitation of these primary cell models is the donor-to-donor variability in terms of the activity of phase I (CYP) and phase II (UGT and SULT) enzymes. To prevent the donor-to-donor variability, human induced-pluripotent stem cells (iPSCs) may provide a limitless supply of hepatocytes for high-throughput screening with minor batch-to-batch variability.[45]

Thus, selection of the appropriate cell model is essential to ascertain that the in vitro data reflect the relevant kinetics and toxicity. To further illustrate this argument, as an example, Figure 7.2 shows the in vitro concentration-response data for monocrotaline-induced toxicity in rat hepatocytes, HepG2 cells, and HepaRG cells, indicating that the rat hepatocytes are more sensitive to the toxicity induced by monocrotaline. This indicates that when biotransformation is an essential condition for toxicity, it is important to select an in vitro system that is metabolically competent. The fact that monocrotaline does not induce toxicity towards HepG2 and HepaRG cells reflects that the expression level of the relevant CYPs in HepaRG cells is generally lower than that in rat or human primary hepatocytes.[51] Finding ways to extend this model to a model that allows repeated dose toxicity studies would be of use to obtain insight in repeated dose toxicity and facilitate prediction of also chronic liver toxicity of PAs by the novel in vitro-PBK model based testing strategy. Currently, sandwich-cultured hepatocytes (SCH) may provide an interesting model to achieve this goal, since they are recognized as a powerful in vitro tool that can be utilized to study drug-drug interactions at the transport level, hepatotoxicity and drug-induced liver injury (DILI) for longer exposure time.[52, 53]

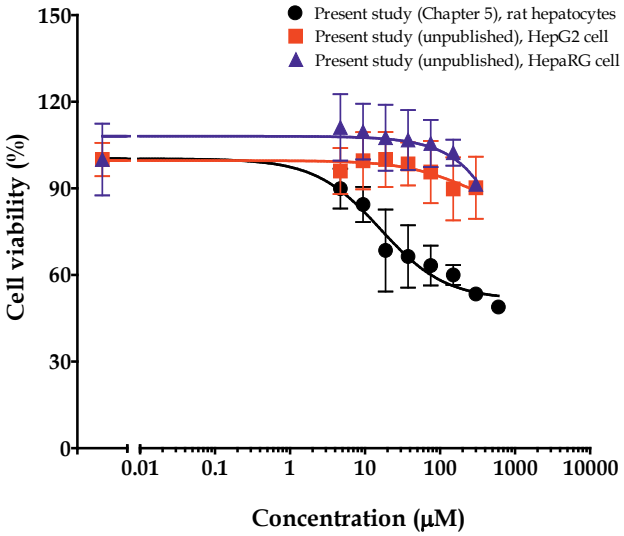

Figure 7.2. Concentration-response curves for effects of monocrotaline on cell viability of primary rat hepatocytes (black), HepG2 cells (red) and HepaRG cells (blue) exposed for 24 h (means ± SE).

7.1.6. Absence of combination effects

It should also be pointed out that the toxicity of monocrotaline (**Chapter 5**) has been mainly studied in this thesis using the parent compound monocrotaline in isolation. In the risk assessment (**Chapter 4)** combined exposure to PAs has been taken into account assuming similar potency of all PAs detected. In the real scenario, consuming a herbal product may result in simultaneous exposure to more than one PA give the occurrence of PAs in the botanical preparations, while these PAs may have different potency. In addition, some herbal products appeared to contain more than one class of genotoxic and carcinogenic ingredients, so to contain ABs and/or AAs in addition to PAs. Synergistic and antagonistic actions of the various ingredients in herbal product may fortify or weaken the toxicity of individual constituents when taken in the form of a herbal preparation. Chou and Fu (2006)[40] reported that toxic DNA adducts were not only detected in the liver DNA of rats treated with riddelliine but also following the administration of PA containing comfrey root extract, coltsfoot root extract, flos farfara extract, coltsfoot tussilage, and comfrey compound oil. An additive effect was demonstrated by Li et al. (2013)[54] for senecionine and seneciphylline in which the extract of *Gynura segetum*, a senecionine and seneciphylline-containing herb, exhibited cytotoxicity to HepG2 cells compared to the toxicity caused by the sum of these two PAs tested individually. Taking into consideration the profiles of PAs detected in samples of jamu in the present thesis (**Chapter 4**) in which samples contained from 2 up to 40

types of PAs, in vitro assays testing the potential combination toxicity of the PAs present in the herbal preparations in primary rat/human hepatocytes seems of interest for future studies. Also, the definition of relative potency (REP) factors to take different potency of the different PAs into account in the risk assessment appears to be an important topic for further research.[55, 56]

Merz and Schrenk (2016)[56] defined interim REP factors for the relative potency of 1,2-unsaturated PAs based on the available data on the genotoxic potency in *Drosophila melanogaster*, the cytotoxic potency in vitro in chicken hepatocellular carcinoma (CLR-2118) cells and their acute toxicity in adult rodents (LD_{50}). EFSA however indicated that the REP factors for PAs should be further refined before taking them into account in risk assessment.[55] It is of importance to note that due to the limitation of carcinogenicity data for PAs, the interim REP values did not (yet) take the relative potency of PAs for tumor formation into account.

In the present thesis REP factors were used in **Chapter 3** to estimate the combined exposure of different ABs in the same sample using a toxic equivalency (TEQ) approach in which methyleugenol was used as the reference compound (REP value = 1.00). Comparison of the outcomes obtained taking these REP factors into account to those obtained assuming equal potency of the ABs, did not substantially influence the outcomes, mainly because methyleugenol was the major AB detected and the REP values for the other ABs were not substantially different from that for methyleugenol. As discussed in **Chapter 4**, incorporating the REP in the evaluation of jamu will refine the risk assessment of these traditional medicines.

Human dietary exposure to ABs, PAs and AAs occurs in a complex of other herbal ingredients, where, interactions in a complex food matrix can occur that can affect the bioavailability and toxicity of these compounds.[4, 57] In case of for example estragole, Jeurissen et al. (2007)[58] reported that bioactivation and subsequent adverse effects of estragole might be lower in a matrix of other basil ingredients than what would be expected on the basis of experiments using estragole as a single compound. Furthermore, co-exposure to estragole and (mixtures of) different basil derived flavonoids, especially nevadensin, resulted in substantial inhibition of the SULT-mediated bioactivation of estragole and subsequent DNA adduct formation in liver cells both in vitro and in vivo.[59, 60] Such matrix effects should be taken into account in the risk and safety assessment of botanicals and botanical preparations on a case-by-case basis also considering the underlying mode of action.

7.1.7. Chemical-specific parameters needed for PBK modeling

The MOE approach needs carcinogenicity data to define the $BMDL_{10}$. However, not for all ABs, PAs, and AAs tumor data that would enable the definition

of a $BMDL_{10}$ for risk assessment are available. Therefore, novel strategies including PBK model based QIVIVE and read across could be applied to obtain the $BMDL_{10}$ of compounds of interest. Such an approach has already been applied to predict the $BMDL_{10}$ for tumor formation forelemicin by PBK model based read across from estragole and methyleugenol[61], or the $BMDL_{10}$ for tumor formation by myristicin and apiol via PBK model based read across from safrole[62, 63]. The approach could also be applied for read across from lasiocarpine and riddelliine to other PAs.[64, 65] Since many $BMDL_{10}$ values needed for risk assessment of botanical ingredients that are genotoxic and carcinogenic are unavailable, this QIVIVE approach seems a promising strategy for the future.

Given this conclusion it is also of interest to note that the development of PBK modeling based applications are time and effort consuming, because PBK models need chemical-specific parameters describing the absorption, distribution, metabolism and excretion (ADME) processes.[66] This indicates a need for further development of generic PBK models that can be based on parameters obtained by in silico and efficient in vitro methodologies. An efficient PBK modeling based read across would enable selection of the chemicals that could be prioritized for further risk assessment and/or in vivo testing facilitating an efficient and time-and-cost-saving risk assessment of genotoxic carcinogens for which rodent tumor data are not available.[67] The use of in silico modeling, such as quantitative structure activity relationship (QSAR) modeling should be considered for the prediction of model parameter values as was done also for several of the parameters required for the PBK models of the present thesis (**Chapter 5** and **6**) in which tissue:blood partition coefficients were predicted by the LogP value of the chemical.[68, 69] However, the potential of in silico modeling for estimating values for kinetic model parameters remains to be further developed and evaluated.

Another aspect that needs to be incorporated in future PBK models is the effect of repeated dosing on the in vivo kinetic characteristics. In **Chapters 5** and **6**, the prediction was made for single oral exposure, while in real situations rats/humans are exposed to chemicals repeatedly. The repeated exposure may influence expression levels of metabolizing enzymes. This should be incorporated in the PBK models used, preferably based on adequate in vitro models that must be further optimized and validated to make them suitable for making quantitative predictions on enzyme induction in the in vivo situation.

7.1.8. Interindividual variation of exposure and sensitivity

The intensity, frequency, route and duration of herbal product consumption varies among Indonesian population. For example, Kemenkes-RI (2010) reported

that 59.12% of the total Indonesian population above 15 years of age consume jamu as traditional medicines, with the frequency of consumption being every day (4.36%), seldom (once a week/month, 45.03%), and never (9.73%).[70] This consumption behavior may cause interindividual variation in the exposure to the ABs, PAs, and AAs via drinking the preparations. However, the risk and benefit analysis via consumption of herbal products was estimated in the present thesis for the average Indonesian population, without taking interindividual differences into account. In **Chapter 2**, **3**, **4** the exposure was assessed by an approach using the detected levels of ABs/PAs/AAs in the samples and recommended daily intake mentioned on the label. For the future it may be of interest to consider the use of biomonitoring strategies, enabling personal monitoring,[71, 72] since these strategies are expected to provide a more reliable insight into actual levels of exposure and will enable to take intraindividual aspects of variability in types and dose of toxic compounds consumed into account in the exposure and risk characterization.

Slob (2006)[73] reported that probabilistic dietary exposure assessments based on Monte Carlo sampling may facilitate this analysis of the interindividual variation in exposure. In addition, exposure from different consumer products can be considered simultaneously in assessing the exposure. In addition, a risk assessment may take into account interindividual differences in sensitivity by defining compound specific adjustment factors instead of default uncertainty factors as discussed later in this chapter (Section 7.2.6.).

7.1.9. Availability of in vivo studies to validate the prediction

An important aspect of novel QIVIVE approaches is the fact that in vivo data are still needed to evaluate the PBK model predictions on both kinetics and toxicity. Comparing model predictions with experimental data, such as area under the curve (AUC), peak plasma concentration (C_{max}), time to peak concentration (t_{max}), and plasma half-life is often used to evaluate the PBK models. Setting deviation thresholds of 0.5 to 2-fold, is often used to examine the performance of the model. Sensitivity analysis can be used to evaluate the impact of variations in model parameters on model outputs.[74] Ideally, the in vivo data used in an evaluation step should be representing the same situation as what has been targeted, including type of compound, administration route and species. However, due to the limitation in the amount, nature or even quality of reported in vivo data, the evaluation and validation of the predictions may sometimes be difficult, as discussed in **Chapter 5** and **6**.

Defining PBK models taking insights in the MOA into account will increase the model's capability to predict and extrapolate to the in vivo situation. To enhance

the acceptance of PBK models at an international level, currently a good modelingpractice (GMP) is established to guide the use of the in vitro and in silico methodologies in developing PBK models without the need of in vivo data. In the GMP, assuming there is no possibility of generating in vivo animal data for the model calibration, the model can be built when there are: (1) in vitro and in silico alternatives available to generate ADME parameters (including prediction of metabolism) of sufficient quality and (2) modeling platforms available and accessible.[75] The available in vitro and in silico methods can be obtained from in silico metabolic simulation tools for microbial and human metabolism[76] taking into account the guideline to choose QSAR models for ADME endpoints.[77] Several modelingplatforms, such as PK-Sim (www.systems-biology.com), GastroPlus (www.simulations-plus.com) and SimCyp (https://www.certara.com) are available and accessible to generate GMP.[75] Use of these available commercial platforms may prove a way forward that will facilitate use and acceptance of PBK model-based strategies by larger groups of scientists including regulators and risk managers.

7.2. Future perspectives

A collaboration between farmers, health professionals (physicians, pharmacists and nurses), consumers, academia, industry, and government is needed to promote the safety of Indonesian herbal products. Figure 7.3 shows the summary of alternative solution for factors which currently hamper the risk and benefit analysis discussed above, followed by some action plans for risk management action including all stakeholders involved.

Based on this scheme seven future actions are proposed in order to further improve safety and efficacy of herbal preparations on the Indonesian market. These actions include, (1) improvement of the quality of botanicals by applying Good Agricultural and Collection Practices (GACP) for the farmers, (2) increasing the safety of herbal products by applying good manufacturing practice (GMP) of herbal product and food safety training for the manufacturers/producers, (3) development of a toxicity database of medicinal botanicals used in Indonesia, (4) restriction of the exposure to genotoxic carcinogenic compounds by establishing MPLs and refining the label requirements for botanicals and botanical preparations, (5) use of human biomonitoring (HBM) and PBK modeling for a more refined exposure and risk and benefit analysis of Indonesian herbal products, (6) incorporation of Chemical Specific Adjustment Factors (CSAFs) for interspecies and interindividual variation in kinetics within the human population, and finally (7) exploring the beneficial effects of botanicals and botanical preparations.

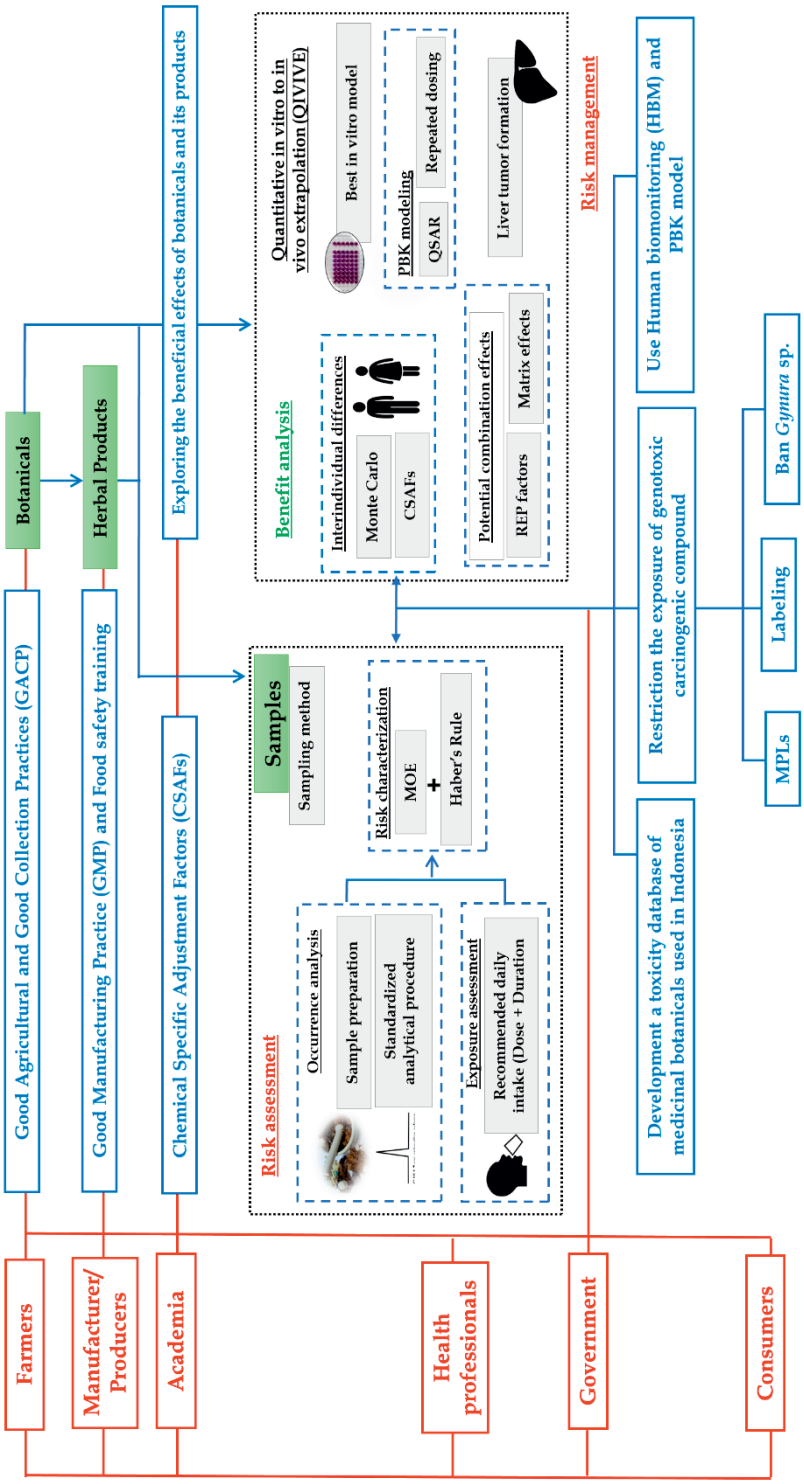

Figure 7.3. Overview of alternatives solutions for factors which hamper the risk and benefit analysis (grey boxes) and some action plans for risk management action (blue boxes) including all stakeholders involved (red boxes) being farmers, health professionals (physicians, pharmacists and nurses), consumers, academia, industry, and government.

7.2.1. Improvement of the quality of botanicals by applying Good Agricultural and Collection Practices (GACP) for the farmers

Farmers have an important role as a supplier for the botanical materials of herbal products. The contamination of jamu products made from non-PA producing plants by especially PA-producing botanicals presents an issue raising concern for these products (**Chapter 4**). The contamination may occur during the cultivation, or the harvesting of the jamu materials, resulting in compounds of concern ending up in intermediate or finished products. Many PA-producing botanicals like *daun dewa* (*Gynura segetum*) are weeds that may accidentally contaminate medicinal botanicals. For this reason, applying Good Agricultural and Collection Practices (GACP)[78], may help to reduce potential PA contamination in jamu. In addition the quality of botanicals that are selected for herbal products can be controlled by an appropriate procedure in plant identification (plant part) and adequate control of factors influencing the presence of constituents of concern during plant production, harvesting and post-harvesting procedures.[79] It may also be reconsidered whether it is prudent to use PA-containing botanicals in herbal products at the levels currently in use.

7.2.2. Increase the safety of herbal products by applying Good Manufacturing Practice (GMP) of herbal product and food safety training for the manufacturers/producers

Table 7.3 shows the number of samples which appeared of concern for human health pointing at a need for risk management actions and reveals that this number varied among the registration code for their market. Herbal products registered as domestic processed food (BPOM RI MD) presented only 8% of samples of concern because of the presence of genotoxic and carcinogenic constituents. This relatively lower number of samples raising a concern may be related to the fact that the herbal products marketed as domestic processed food have to meet the requirements of Good Manufacturing Practice (GMP) and are considered to be of higher quality than herbal products marketed as jamu. This result of the present thesis indicates that risk management actions for Indonesian herbal products may be prioritized for jamu, one of the traditional medicines used by Indonesians. This priority is of interest considering the high number of jamu consumers and the fact that ABs, PAs and AAs were detected in the products (**Chapter 2-4**).

Table 7.3. Overview of the risk characterization using the MOE approach for the different Indonesian herbal products.

Registration code per chapter in this thesis	Targeted compound	Total samples	Concern for risk management	
			Yes	No
Chapter 2 (Jamu)	Alkenylbenzenes			
BPOM RI TR		25	20	5
Chapter 3 (Herbal beverages)	Methyleugenol			
Depkes RI P-IRT		43	31	12
BPOM RI MD		31	7	24
BPOM RI TR		30	7	23
BPOM RI SD		9	0	9
BPOM RI ML		1	1	0
Chapter 4 (Jamu)	Pyrrolizidine alkaloids			
BPOM RI TR		35	20	15
BPOM RI TR		23[a]	7	16
Unpublished result (Jamu)				
BPOM RI TR	Aristolochic acid	15	2	13
Unpublished result (Botanical food spices)	Alkenylbenzenes			
Depkes RI P-IRT		5	2	3
BPOM RI MD		20	13	7
Unpublished result (Herbal teas)[b]	Alkenylbenzenes			
Depkes RI P-IRT		3	0	3
BPOM RI MD		3	0	3
BPOM RI TR		3	2	1

[a] Samples containing non-PA-producing botanicals to assess the contamination of PAs
[b] MOE values based on the level of ABs extracted using hot water and 1 cup of tea a day

Based on regulation BPOM (246/MENKES/Per/V/90 and HK.00.05.41.1384, 2005), the production and distribution of traditional medicines should comply with the GMP of traditional medicines (*Cara Pembuatan Obat Tradisional yang Baik* = CPOTB). Until now, in Indonesia there are 1,247 herbal medicine manufacturers consisting of 129 traditional medicine industries (*industri obat tradisional* = IOT), with the remaining ones consisting of middle (*Usaha Menengah Obat Tradisional*=UMOT) and small business enterprises of traditional medicines (*Usaha Kecil Obat Tradisional* = UKOT). However, until 2018 only 111 facilities for the production of traditional medicines received the certificate of CPOTB[80], indicating that the risks of consumption of the respective herbal products produced may not be adequately evaluated and/or regulated or guaranteed.

Providing a training or workshops on food safety and registration procedures for producers of herbal products can be a first step in overcoming the limitations of producer awareness and knowledge. This can be achieved not only by supporting IOT and UKOT to get CPOTB certificates which has been done by BPOM-RI (2018)[80], but also through detailed training on the assessment of the adverse health effects potentially arising from incorrect formulations which use botanicals of concern because of constituents that are genotoxic and carcinogenic. As seen in Table 7.3 and discussed in **Chapter 3**, the high number of samples of herbal beverages

(Depkes RI-PIRT) which raise concern for human health indicating a priority for risk management may be due to the limited awareness and knowledge of the producers on the food safety and registration procedure.[81, 82] A study on jamu sellers in Semarang reported that the sellers formulate jamu based on their knowledge and experiences on the main types of compounds in botanical materials and health effects. The sellers use traditional equipment to produce the jamu.[83] Limyati and Juniar (1998)[84] reported the contamination of raw material and products of *jamu gendong* with bacteria, yeasts and molds, indicating the low application of food safety among sellers. Another example is the detection of carcinogenic aflatoxin B1, B2 and G2 in 14 jamu preparations.[85] Thus, it can be concluded that improving awareness among producers and implementing GMP within the field of jamu production will improve the safety of these products.

7.2.3. Development a toxicity database of medicinal botanicals used in Indonesia

The toxicity data and knowledge on the occurrence of adverse reactions as a result of use of Indonesian herbal products are still limited. The result of a monitoring program on adverse health effects resulting from the consumption of traditional medicine and food supplements showed that there were 19 and 37 electronic reports related to adverse effects of consumption of traditional herbal and health supplements in 2018.[80] From January - until September 2019, there were 149 reports on adverse health effects due to consumption of traditional medicines in Indonesia.[86] Although the report did not mention the compounds involved in causing the reported adverse effects, it seems likely that these reports relate to cases of acute toxicity, while the results of the MOE approach-based risk assessment in this thesis provide evidence that also chronic toxicity such as the toxicity resulting from the exposure to carcinogenic genotoxic compounds in the products, should be taken into consideration.

Analysing the side effects of herbal products is reported to be much more complex than analysing side effects of conventional pharmaceuticals, especially in the case of chronic toxicity, where causality can be very difficult to establish.[87] Only a few reports on adverse effects of jamu have been reported in the peer reviewed literature so far. Paul et al. (2005)[88] reported a case on agranulocytosis and citrobacterial infection in a 75-year-old woman with osteoarthritis after consuming jamu adulterated by phenylbutazone (to enhance the analgetic and anti-inflammatory effect). Besides adulteration, the jamu was also contaminated with *Klebsiella pneumoniae*, *Enterobacter sakazakii* and *Clostridium* species. Recently, a study on costs of illness due to consumption of drug-adulterated herbal medicines resulting in kidney failure showed that adulterated jamu contributed 0.02-2.69% to

the costs of kidney failure in Indonesia.[89] The examples provided by these 2 case studies, could form the basis for a toxicity database of toxic compounds in botanicals and botanical preparations specific for the Indonesian market.

Afendi et al. (2012)[90] already built the KNApSAcK Family database (http://www.knapsackfamily.com/jamu/top.jsp) that contains the formula names and botanical ingredients of 5,310 formulae out of more than 7,000 commercial jamu registered at BPOM RI. The database encompasses 550 medicinal plants and 12 morphological segments. However, a database of jamu toxicity and constituents of potential concern has not been established yet. Such information on adverse effects, toxicity or constituents of concern in jamu formulations could be included in the KNApSAcK Family database. However, it may also be of use if the toxicity database will be built in a different online platform to cover botanical constituents of concern for all Indonesian herbal products, and not be restricted to only jamu.

The databases should cover the toxic compound, toxicity data (in vitro and/or in vivo), as well as data on toxicokinetics, toxicogenomics, and mode of action. The EFSA Compendium of Botanicals (https://www.efsa.europa.eu/en/data/compendium-botanicals) can be used as an example of a database on botanicals that are reported to contain naturally occurring substances of possible concern for human health when present in food.[91] Such an online toxicity database may be a first step towards prioritization amongst issues related to the adverse reactions that have been associated with different herbal products. Using the database, toxicants can be identified easier and earlier and potentially be removed or modified during the production process,[92] for example by reducing the proposed use and use levels, and/or via removal of the constituent of concern for example by using other botanicals or varieties with lower levels of the respective constituent.[19]

Furthermore, the database can be used to counterbalance the perception of consumers, and sometimes even medical practitioners, that herbal products are harmless. Numerous examples of herb–drug, herb–herb interactions and herb-induced side effects with serious clinical consequences have been documented in other regions/countries[93-96], indicating the importance of similar research to further document what is clinically relevant to the situation in Indonesia. Users, prescribers and producers of jamu should be aware of this. More and continuous control is needed on traditional medicines such as jamu to guarantee safety for the consumer. This should preferably be regulated on a national level and implemented in the manufacturing process to ensure the safety in use of products on the market.[97]

7.2.4. Restriction the exposure to genotoxic carcinogenic compound by establishing MPLs, refining the labels and regulation for botanicals and botanical preparations

The exposure to ABs and PAs via consumption of herbal products can also be restricted by establishing maximum permitted levels (MPLs). BPOM RI (2016)[98] has stipulated an MPL of 10 mg/kg for estragole and of 0.1 mg/kg for safrole based on Regulation of Head BPOM RI No. 22, while MPLs for methyleugenol and PAs have not (yet) been established. As discussed in **Chapter 3**, an MPL value of 0.1-1 mg/kg can be considered to reduce the exposure to methyleugenol via consuming the herbal products to a level that would raise less concern.

In addition, to reducing the exposure to PAs via reducing the consumption of jamu an MPL for PAs also can be set based on the result of **Chapter 4**. Assuming an average recommended daily use of jamu containing PA-producing botanicals of 10.8 g and a $BMDL_{10}$ value of 237 µg/kg bw/day for riddelliine,[55] an MPL of 0.1 mg PAs/kg jamu will result in an MOE of 10,000 and thus would not be of concern for human health. Furthermore, the high level of PAs detected in a large proportion of especially the *Gynura*-based jamu, indicates that banning the use of *Gynura* sp. as botanical constituent in herbal products will increase consumer safety. The highest PA level of 114,071 µg/kg detected in sample TR-17 was 93.3-fold higher than the level of PAs in dried comfrey (*Symphytum officinale*) leaves of 2,523.1 µg/kg (analysed using the same method as described in **Chapter 4**), a constituent already banned from use in herbal preparations including jamu.[99] The level of *Gynura*-based jamu (TR-17) was 3 times higher than the level of PAs detected in dried farfara (*Tussilago farfara*) flos of 84,585.9 µg/kg, which was also relatively high. Based on these results, the regulation HK.00.05.23.3644[100] can be refined by adding *Gynura* sp. and *Tussilago farfara* to the list of banned ingredients in Indonesian traditional medicines. BPOM RI can consider warning or paying more attention to the inclusion of *Gynura* sp. and *Tussilago farfara* in herbal products marketed in Indonesia.

To reduce the exposure to ABs and PAs, also the labelling regulation can be refined by adding restrictions on the recommended daily dose, the duration of consumption, and the adverse effects upon prolonged consumption on the label of herbal products to further support consumer safety. The labelling is useful not only for consumers and risk assessors, but also for general practitioners, so they can prescribe the product in the appropriate dose for an appropriate duration. So far, on the label of Indonesian herbal products there is no information about the limitation of consumption duration or use levels.

Chapter 2-4 indicated that short-term consumption can reduce the risk of exposure to the carcinogenic genotoxic herbal constituents. This recommendation

would be comparable to Stevinson et al. (2002)[101] who reported that when taken as a short-term monotherapy at recommended doses, kava (*Piper methysticum*) extracts appear to be well tolerated by most consumers. However, long-term use can cause dermatological reactions, neurological complications and, of greatest concern, liver damage. Another example is ginger (*Zingiberis officinalle*), which is mostly used as an ingredient in Indonesian herbal products, which can cause heartburn and act as a gastric irritant in doses exceeding 6 g of dried ginger.[102] In **Chapter 4**, it is concluded that consumption of Indonesian jamu that contain PA-producing botanicals can be considered safe when consumed for less than about 6 weeks during a lifetime. This limitation of consumption duration is also recommended by European Medicine Agency (EMA)[103] for PA exposure via drinking bitter fennel-based herbal preparations. The same recommendation to consume the PA-containing herbal preparations for a short-time only (defined as 6 weeks) at dose levels not exceeding 1 µg PAs/day is also regulated by Germany and The Netherlands.[104, 105]

7.2.5. Use of human biomonitoring (HBM) and PBK modeling for improved exposure and risk and benefit analysis of Indonesian herbal products

The risks resulting from exposure to natural toxins via consumption of herbal products have not yet been assessed by the Indonesia Risk Assessment Center (INARAC), a body under the BPOM RI. So far INARAC finalized the Microbiology Risk Assessment (MRA) of chicken Salmonella and a risk assessment on Aflatoxin B1 (AFB1) levels in peanuts and their processed products.[106] Currently, the body is working on the risk assessment of 3-monochloro-propane-1,2-diol (3-MCPD) esters and glycidol esters (GE) present as contamination in palm oil, and acrylamide in coffee [107]. Based on the results of risk assessment of ABs, PAs, and AAs present in botanicals and botanical preparations on the Indonesian market reported in the present thesis, a further risk assessment, as well as a risk management action and related risk communication actions could be considered by the Indonesian authorities.

Further actions could also include a further study in Indonesia on the exposure to and effects of the compounds of concern due to frequent and prolonged consumption of herbal products. The study will be useful to inform the risk managers on the importance of actions to monitor the safety of herbal products. In case of the effect of exposure to ABs, PAs, and AAs via consuming herbal products on the incidence of liver cancer in Indonesia, epidemiological data would be needed and, if possible, further analysis of liver samples from the patients to quantify levels of relevant DNA adducts. Also, a more refined dietary exposure assessment of ABs,

PAs and AAs including both acute and chronic exposure should be conducted in the Indonesian population. The data on occurrence resulting from the present thesis can be used as a basis to perform an exposure assessment by combining them with the consumption data of Indonesian herbal products, as was done for the European population by EFSA (2016)[108]. Furthermore, a more refined exposure assessment for these compounds could be done by human bio-monitoring (HBM) for example by measuring specific metabolites and/or haemoglobin adducts as biomarkers of exposure.[109]

Biomarkers of exposure may involve measurements of the parent compound, metabolites or DNA- or protein adducts and reflect internal doses, the biologically effective doses or target organ doses.[109] Biomarkers in blood and urine are most commonly used as biomarkers of exposure and cells in blood may provide surrogate endpoints for effects in internal organs. Occurrence of persistent DNA-adducts would be ideal parameters for HBM to evaluate the mutagenicity of ABs, PAs or AAs and potentially related elevated cancer risks.[72] Furthermore, when liver samples from patients with liver cancer who consumed herbal products would become available, this HBM approach can be further refined for use in risk assessment.

HBM data can be of use in both forward and backward methods. Forward methods analyse the measured intake doses to predict body burden and related biomarker levels, while backward (reverse) analysis uses urinary/blood HBM data to reconstruct past exposure. For the reverse dosimetry methods, the fractional urinary excretion (FUE), defining the fraction of the dose that ends up as a defined biomarker in a relevant matrix is needed to convert the urinary level of a biomarker into an oral dose level [110]. When applying the reverse dosimetry methods PBK-modeling also will prove to be a way forward to translate the biomarker levels to oral dose levels.

Recently, PBK modeling has already been accepted as a tool for risk assessment or for use as supporting information in some of the chemical-specific dossiers evaluated by the Scientific Committee on Consumer Safety (SCCS), EFSA, the US Environmental Protection Agency (EPA), the US Food and Drug Administration (FDA) and the EMA[75], and this approach could also become of use to tackle the lack of epidemiological data in exposure and safety assessment of Indonesian herbal products. As reported by Paini et al. (2019)[75] application of new generation-PBK models would be extremely valuable in the generation of virtual population/patient libraries for exposure assessment, studies on the effect of enzyme polymorphisms and of drug-drug interactions and interindividual variability in relation to chemical exposures and toxicological outcomes.

7.2.6. Incorporation of Chemical Specific Adjustment Factors (CSAFs) for interspecies and interindividual variation in kinetics within the human population

In the MOE approach a factor 10 for interindividual variability is included in the default cutoff value of 10,000 to evaluate whether there is a concern or not. The factor 10 is consisting of a default value of 3.16 for kinetics and 3.16 for dynamics.[30] Integrating PBK modeling with Monte Carlo simulations using human in vitro data can be used as a strategy to quantify inter-individual variations in kinetics and take these into account in risk assessment in a chemical specific way defining so-called chemical specific adjustment factors (CSAFs) that can replace the default factor of 10 for interindividual differences. This approach has been used to predict inter-individual and inter-ethnic variation in bioactivation and liver toxicity of the PA lasiocarpine[111], the AB estragole[112], phenol[113], and *trans*-2-hexenal[114]. This approach can be extended to other ABs, PAs and AAs and to the individual Indonesian people. Quantifying inter-individual variations will enable a more refined risk assessment for the Indonesian population.

7.2.7. Exploring the beneficial effects of botanicals and its products

The health claims of Indonesian herbal products mentioned on the label or advertised via various media are provoking the increasing consumption trends of the products (Chapter 1). Figure 7.4 depicts an overview of the classes of diseases which are targeted and/or claimed to be cured by the 197 samples analysed in **Chapter 2**, **3** and **4**. The health claims mentioned on the label were used to classify the diseases based on the International Classification of Diseases (ICD)-10 ver. 2016.[115] Beneficial effects were not mentioned on the label of 24.4% (48/197) of the total samples (NA).

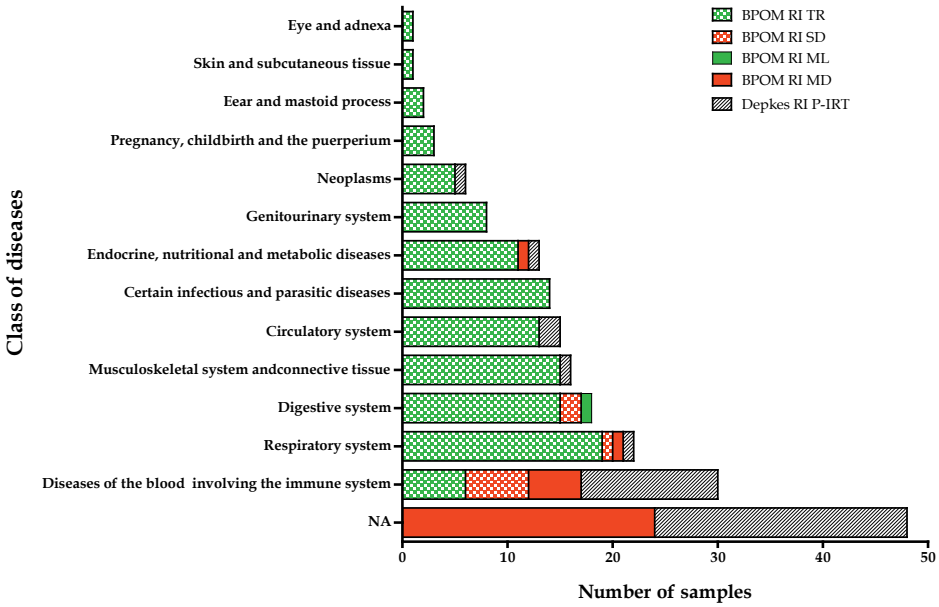

Figure 7.4. Overview of type of diseases which are targeted and/or claimed to be cured by the Indonesian herbal product samples analysed in Chapter 2, 3 and 4. The detail of actual health claims mentioned on the labels has been described in the respective chapters. NA refers to No available information on health claims mentioned on the label of the sample.

As depicted in the figure, health claims targeting the blood and blood-forming organs and certain disorders involving the immune system are linked to the highest number of samples (24.4% out of the total samples), while only a few samples target eye, ear, and skin-related diseases. Interestingly 6 samples claim that after consuming the products consumers can cure their neoplasm-related diseases. The respective labels indicate that the products help to maintain the health condition of tumour and cancer patients, and to prevent and treat cancer (cervix, breast, liver, brain, lung, leukaemia, and other diseases related with cancers, cysts and tumours). Diseases of the respiratory system such as cough, cough with phlegm, asthma, and sore throat are claimed to be treated after consuming 19 of the samples of jamu (registered as BPOM RI TR).

These claimed beneficial effects of the products are considered to be related to the biological activity of constituents of the botanical(s) inside the products. For example, in jamu claimed to have an effect on ailments related to the respiratory system, fennel (*Foeniculum vulgare* Mill.) bark is the active botanical ingredient in the preparations. The use of fennel as traditional medicine for a wide range of effects has been reviewed.[116, 117] Spasmolytic effects on contracted smooth muscles is reported as the mode of action underlying the anti-asthma effects of fennel.[118, 119]

Another example is ginger (*Zingiberis officinale*) of which the rhizome is present in many samples analysed in this thesis linked with beneficial effects on digestive system-related illness. Gingerols and shogaols and their activity on cholinergic M receptors and serotonergic 5-HT and 5-HT receptors are reported as the MOA of the effect of ginger to cure nausea and vomiting.[120] However, since the jamu ingredients consist of mixed medicinal plants to get the desired efficacy, the beneficial effects of the preparation can be explored further to support the efficacy of jamu.

As discussed in **Chapter 1**, the therapeutic effects of jamu are mainly based on empirical data, inherited across generations[121, 122], and thus generation of science based evidence for the beneficial effects of the Indonesian botanicals and their products remains to be performed. The efforts can support the program *saintifikasi jamu* established by the Indonesian government. This program is aiming to collect data on jamu efficacy based on a scientific basis understanding the clinical practice, context of usage, safety of usage, effectiveness, elucidation of active compounds related to the therapeutic effect, and elucidation of the underlying therapeutic mechanism.[123] QIVIVE using the combination of in vitro assay and PBK modeling-facilitated reverse dosimetry, as also applied in **Chapter 6** of the present thesis, can be a solution to prove the efficacy of jamu and other Indonesian herbal products. The method can be used to obtain insights in human responses to potential functional food ingredients. This insight can be used to select the promising botanicals for subsequent human intervention studies and can help in the selection of doses to be applied in such studies.

Conclusion

The research presented in this thesis supports risk management aimed at prioritizing regulatory actions to reduce potential risks connected to the exposure to genotoxic carcinogens, ABs, PAs an AAs via consumption of Indonesian herbal products. In addition, a novel testing strategy, combining in vitro and PBK modeling-facilitated reverse dosimetry, was found to facilitate risk and benefit assessment of botanical compounds without the need for animal experiments and/or human intervention studies. Many aspects, including variability in detected levels of the targeted compounds, variability in recommended daily use mentioned on the label, interindividual variation of exposure among Indonesian people, absence of a generally accepted method to take shorter-than-lifetime exposure into account, the knowledge gaps in modes of action, selection of the best in vitro model for QIVIVE, potential combination effects, the chemical-specific parameters needed for PBK modeling and availability of in vivo studies to validate the predictions

should be considered for future research. Seven actions including (1) applying Good Agricultural and Collection Practices (GACP) for farmers, (2) applying good manufacturing practice (GMP) of herbal product and food safety training for manufacturers and producers, (3) development of a toxicity database of medicinal botanicals used in Indonesia, (4) restriction of the exposure to genotoxic carcinogenic compounds by establishing MPLs and refining the label requirements for botanicals and botanical preparations, (5) use of human biomonitoring (HBM) and PBK modeling for a more refined exposure, risk and benefit analysis of Indonesian herbal products, (6) incorporation of Chemical Specific Adjustment Factors (CSAFs) for interspecies and interindividual variation in kinetics within the human population in the risk assessment, and (7) exploring the beneficial effects of botanicals and botanical preparations, were proposed to improve safety and efficacy of botanicals and botanical preparations on the Indonesian market.

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Chapter 8

Summary

Summary

In Indonesia, the market demand for herbal products keeps growing, and as a result, herbal products increasingly provide economic and perceived clinical benefits. A risk and benefit assessment are crucial to be performed to support the safe use of herbal products although the consumers perceive herbal product as "safe" and "natural" and thus "healthy" (**Chapter 1**). The aim of the present thesis was to perform an assessment of potential risks and some benefits of herbal products available in the Indonesian market. The model compounds chosen included especially naturally occurring genotoxic and carcinogenic botanical constituents including alkenylbenzenes (ABs) and pyrrolizidine alkaloids (PAs). Beneficial effects focussed on potential PPARγ activation by the carotenoids bixin and crocetin. Existing but also novel testing strategies were used to evaluate the relevance of effects at estimated human intake levels.

The consumer risks of jamu, Indonesian traditional herbal medicines, was assessed focussing on the presence of AB-containing botanical ingredients (**Chapter 2**). Methyleugenol, appeared to be a major AB present, being detected in 91.3% of the collected jamu samples. Quantification of AB levels and exposure resulting from use of the respective jamu products resulted in Margin of Exposure (MOE) values generally <10,000, indicating a priority for risk management when assuming daily consumption during a lifetime. Using Haber's rule, it was estimated that two weeks consumption of these jamu only once would not raise a concern (MOE >10,000). However, when considering use for two weeks every year during a lifetime, 5 samples still raised a concern. It is concluded that the consumption of AB-containing jamu can be of concern especially when consumed daily for longer periods of time.

Based on these results it was anticipated that methyleugenol may also be present and pose a risk in Indonesian instant herbal beverages. **Chapter 3** analysed methyleugenol in many instant herbal beverages containing various mixed herbs collected on the Indonesian market by a targeted sampling strategy. Interestingly, eugenol was detected in a few samples at a level that resulted in an estimated daily intake (EDI) lower than the acceptable daily intake (ADI) of 2.5 mg/kg bw thus not raising a concern for human health. The MOE for methyleugenol intake by targeted consumers, including both adults and children, revealed that several of the herbal products targeted at adults would require a risk management action, while only a few samples targeted at children pointed at a priority for risk management, when the respective herbal beverages would be used every day during a lifetime. However, when assuming the consumption for 2 weeks, every year during a lifetime and using Haber's rule then all MOE values were estimated to be > 10,000 indicating no priority for risk management. An overview of the current product registration

type of these samples indicated that herbal beverages registered as household food (labelled as 'Depkes RI P-IRT'), would raise a concern when people would consume them every day during a lifetime. The study provided data that can support establishment of a maximum permitted level (MPL) for methyleugenol in herbal beverages in Indonesia.

Another group of genotoxic compounds potentially present in botanicals and botanical preparations and raising a health concern are PAs. In **Chapter 4,** the occurrence and accompanying risks of PAs in Indonesian jamu were evaluated. PAs were detected in 97.1% of the jamu containing PA-producing botanicals and in 74% of the jamu samples that had no PA-producing botanicals listed on their label. This latter point shows contamination with PA-producing plants due to co-harvesting of PA-containing weeds during cultivation or harvesting of the materials. Short-time, 4 days up to 2 weeks, consumption of jamu, is unlikely to result in acute toxic effects, although one sample would exceed an intake of 10 µg PAs/kg bw/day which may cause hepatic veno-occlusive disease (HVOD) and PA-induced liver injury (PA-ILI) in humans. When evaluating the potential risk for genotoxicity and carcinogenicity via the MOE approach, MOE values below 10,000 were obtained for 46.6% of the samples, indicating a priority for risk management when assuming daily lifelong consumption. Assuming consumption for two weeks every year during a lifetime, and using Haber's rule, 37% of the jamu samples containing PA-producing botanicals still raised a concern, while the jamu consisting of non-PA-producing botanicals would be of low concern. This study provided data that can support risk management actions in Indonesia to minimize the potential health risk for jamu consumers due to the occurrence of toxic PAs in these products.

Exposure to these PAs, including monocrotaline via herbal product consumption is of concern because of their hepatotoxicity and the fact that they are genotoxic carcinogens. Considering that only for a limited number of 1,2-unsaturated PAs in vivo toxicity data are available, hampering risk assessment where differences in relative potency between different PAs are considered, alternative testing strategies including read-across and quantitative in vitro to in vivo extrapolation (QIVIVE) become important for risk analysis. In **Chapter 5**, a combination of in vitro-physiologically based kinetic (PBK) modeling-facilitated reverse dosimetry was used to predict the in vivo acute liver toxicity of the PA monocrotaline and to characterize the influence of its metabolism on its relative toxic potency compared to lasiocarpine and riddelliine. In the absence of data on acute liver toxicity of monocrotaline upon oral exposure, the predicted dose-response curve for acute liver toxicity in rats and the resulting benchmark dose lower and upper confidence limits for 10% effect ($BMDL_{10}$ and $BMDU_{10}$) were compared to

data obtained in studies with intraperitoneal or subcutaneous dosing regimens. This indicated the predicted $BMDL_{10}$ value to be in line with no-observed-adverse-effect-levels (NOAELs) derived from available in vivo studies. The predicted $BMDL_{10}$-$BMDU_{10}$ of 1.7-6.3 mg/kg bw/day also matched the oral dose range of 1-3 mg PA/kg bw/day at which adverse effects in human are reported. A comparison to the oral toxicity of the related pyrrolizidine alkaloids (PAs) lasiocarpine and riddelline revealed that, although in the rat liver hepatocytes study monocrotaline was less toxic than lasocarpine and riddelliine, due to its relatively inefficient clearance the in vivo acute liver toxicity was predicted to be comparable. It was concluded that the combined in vitro-PBK modeling approach can provide insight in monocrotaline-induced acute liver toxicity in rats thereby filling existing gaps in the database on PA toxicity. Furthermore, the results reveal that the kinetic and metabolic properties of PAs can vary substantially and should be considered when considering differences in relative potency between different PAs.

The combined in vitro-PBK modeling approach was also applied for benefit analysis of the therapeutic effect of bixin and crocetin in type 2 diabetes mellitus suggested to occur via PPARγ activation. **Chapter 6** investigated whether at realistic dietary intake from botanical preparations bixin and crocetin could induce PPARγ-mediated gene expression in humans. Concentration-response curves obtained from in vitro PPARγ-reporter gene assays were converted to in vivo dose-response curves using PBK modeling-facilitated reverse dosimetry, from which the benchmark dose resulting in a 50% effect above background level (BMD_{50}) values were predicted and subsequently compared to dietary exposure levels. In the PPARγ reporter gene assay bixin and crocetin activated PPARγ-mediated gene transcription in a concentration-dependent manner with similar potencies. Due to differences in kinetics, the predicted BMD_{50} values for in vivo PPARγ activation was about 30-fold different, amounting to 115 and 3,505 mg/kg bw for crocetin and bixin, respectively. Human dietary and/or supplemental estimated daily intakes may reach these BMD_{50} values for crocetin but not for bixin, pointing at better possibilities for in vivo PPARγ activation by crocetin. The results presented further show that based on a combined in vitro-in silico approach, it could be estimated whether at realistic dietary intakes plasma concentrations of bixin and crocetin are likely to reach concentrations that activate PPARγ-mediated gene expression, without the need for a human intervention study.

The results obtained in the present thesis support the conclusion that there is a need for risk management formulating regulatory actions to minimize the potential health risks for consumers in Indonesia due to the occurrence of toxic ABs, PAs and AAs in herbal products (**Chapter 7**). It is important to note that this

conclusion holds for herbal products collected by targeted sampling, and not for all herbal products on the Indonesian market. Several methodological considerations are formulated that need to be considered when performing the risk and benefit analysis to avoid over-/under-estimation of risks or benefits. Furthermore, the relevance of risk assessment to support risk management action is highlighted, and considerations regarding the potential for the application of PBK model based QIVIVE for predicting beneficial as well as adverse effects, without the need for animal experiments and/or human intervention studies are formulated.

Altogether, it can be concluded that the risk assessment using the MOE approach combined with Haber's rule can be used to prioritize risk management actions to prevent the adverse health effects of consuming Indonesian herbal products containing genotoxic carcinogens. In addition, a novel testing strategy, combining in vitro and PBK modeling-facilitated reverse dosimetry was found to facilitate risk and benefit assessment of botanical compounds without the need for animal experiments and/or human intervention studies.


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Appendices

Acknowledgements
About the author
List of publications
Overview of completed training activities

Acknowledgements

In this opportunity I would like to acknowledge my sincere gratitude to many people for helping me to finish my PhD as presented in this thesis.

First and foremost, I would like to thank my promotor Prof. Dr I.M.C.M. Rietjens who made the biggest difference in my life. Thanks for your Letter of Acceptance which brings me to this wonderful department with the new facilities in Helix. Since then, she has been there for last four years, motivating and inspiring every bit of me towards new possibilities in life. Not only in the research, I found her as an excellent supervisor and my role model to become a good teacher. Her intelligent ideas, thought provoking discussions and comprehensive understanding, when I was struggling to synthesize my research outcomes were much appreciated.

I would like to extend my gratitude to my previous daily supervisors Dr. Karsten Beekmann and Dr. Jochem Louisse for the involvement in my early stages of the PhD life. Although it was quite a challenge in my first year, without your valuable guidance, the bixin and crocetin paper would not have been possible to publish. Karsten, your critical scientific views have inspired me to thrive for excellence. Jochem, thanks for teaching me the PBK modeling.

I would like to acknowledge other co-authors of my publications. Dr. Sebas Wesseling who extended his supports and acted as my unofficial daily supervisor and provided technical help in LC-MS and UPLC. Special thanks to Laura for answering all technical questions regarding cell cultures and providing valuable suggestions for balancing between job and family. Bert, it was a great pleasure to work in UPLC labs with your joke and storytelling on raising children. My sincere thanks to Dr. Patrick Mulder from Wageningen Food Safety Research who provided valuable suggestions on my targeted sampling for PA-containing jamu and writing the PA paper. Furthermore, I would like to extend my gratitude to Elena, who helped and taught me during performing solid phase extraction in your lab.

I would like to thank the current and former staff members at Toxicology for the nice times and the comradery atmosphere. My special thanks to Lidy for the arrangement of administrative matters I needed even before arrived in Wageningen until finalized my thesis. I will miss your voice to sing *lang zal ze leven in de gloria* during my birthday. Letty, thank you for your help with administrative matters of PET course and the fun talking during the coffee breaks and lab trip. Carla and Gerda, thanks for always opening your door for any administrative help. Carla, thanks for arranging ticket and hotel for my opponents, I enjoy our discussion about Indonesia with you. Hans, thank you for always opening your door for me for any sort of technical help, especially when my technician buddies were absent. Wouter thanks for some nice talks during coffee break. My sincere thanks to Dr. Nico van den Brink and Dr. Hans Bouwmeester for the constructive comments during my research in progress presentations.

The friendship amongst the PhD-candidates is very crucial for my PhD journey. Room 4033 is my second home with a nice environment, many thanks for my former (Jia, Lu, Gogo) and current officemates (Ixchel, Nina, Jingxuan and Tessa) who allowed me to pray and even cry in our office, also to share the happiness. The former PhD students at TOX Jonathan, Nacho, Rozaini, Rung, Sunday, Myrtho, Marcia, Samantha, Amer, Abdul, Leny, Mebrahtom, Martha, and Aziza thank you for all the good time when doing activities together. Current *Intoxicated PhDs* Diana, Mengying, Annelies, Katja, Shengsheng, Dora, Mengying, Shuo, Felicia, Isaac, Danlei, Menno, Bohan, Myoying, Biyao, Weija, Jing, Pim, Chen, Artem and Asraf, thank you for sharing during the experiments in the lab, the discussion during the lunch or coffee break, and the good times outside of work especially during our PhD trip in Japan, PET course and lab trips. New *Intoxicated PhDs* Jin, Merel, Yi Ming, Veronique, Edith, Maartje, Akansha, Aafke, Frances, Ren and Orsi, thanks for nice conversations and friendship. Thanks to my PhD friends from my husband group, MAE: Chris (for the monthly Donald Duck magazine), Celia (for the aquarium), Park, Erik, Misha and Diede (thanks for nice friendship and the dinner).

I would also like to thank my former MSc students Imron, Yizhi, Diana, Alex, Siti, Chodijah, Charlotte, Wenting and Yogi for the efforts to contribute in my project.

I would also like to express my gratitude to the committee members Prof. Dr E.J.M. Feskens, Dr M. Lahaniati, Prof. Dr Taufiqurrachman Nasihun, M.Kes., Sp.And, and Dr D. Wahidin for the time and efforts to evaluate this thesis.

My paranymphs, Diego and Ixchel, thank you for being great friends during my PhD life. Diego, who always ask 'How are you?" with the slow motion and smooth voice admiring me and supporting me to -albeit slow- but for sure finishing my PhD. Ixchel, you are my sister in TOX, thank you for all the heart-talk sessions and for being a real friend that I can trust during our PhD journeys.

To my Indonesian friends in Wageningen, thanks for being a real family in Wageningen when we are far from our home country. To all of the parents and their children in Wageningen: OBS de Tarthorst's Ouders (Mbak Aulia, Mbak Andra, Mas Zukri, Teh Nia, Mbak Nurul, Mbak Najmi, Mbak Mutia, Mbak Hanum, Mas Anto, Mbak Fifi, Mas Anto (Satria), Mas Hardi, Mas Edo, Pak Darmanto, Pak Gede, Mbak Ningrum, Mbak Ika, Mbak Lia); OBS de Wereld Ouders (Mbak Hermi, Mbak Uli, Mbak Ita, Bundo Rovanty, Mbak Dian, Mbak Lia, Mbak Natal, Mbak Ayu, Mbak Fitri, Mbak Vinna), thank you for sharing experiences in parenting, cooking, playing together and keep watching my sons after school or during the weekend. I also would like to thank the PhD-Post Doct Indonesian Association for the sharing about PhD life in WUR, especially for Pak Eko, Pak Fariz, Pak Gede, Pak Ery, Pak Iman, Pak Dikky, Pak Dadan, Pak Fajar, Mas Ayusta, Mbak Ita, Mas Alim, Mas Gumi (and Mbak Gendis), Mas Hachi, Mbak Windi (and Mas Firin), Mbak Aviv, Mbak Atik, Mbak Nila, Vina, Mas Farid, Teh Novi, Kang Indra, Mbak Mega, Mbak Hikmah, Mas

Asrul, Mas Satria, Mbak Uun, Mbak Eva, Mas Emil, Ana, Mas Ludi and many others that I could not mention one by one. Thanks to Yasin Taklim Wageningen, Indonesian Students'Association in Wageningen (PPIW), *TPQ* Wageningen and PCINU Belanda for the gathering. The previous Indonesian MSc students at WUR: Mas Anan and Mbak Khusnul, thank for the friendship during your thesis at TOX; Mbak Lisa, Mbak Isna, Mbak Atina, Mas Rausan, Mas Ikrom, Mas Iqbal, Mas Yitno, Mbak Astin for the nice friendship and helping to take care my sons during my Hajj; Mbak Icha and Mas Arif thanks for being caregiver, friend, and chef of my sons after their school day during the 2nd and 3rd year of my PhD; Mas Adhiet and Mbak Adi thanks for carrying my samples from Indonesia to Wageningen; Hendy thanks for collecting my samples in Solo. Thanks to current MSc students: Mas Garry for designing the cover of this thesis; Mas Kukuh for connecting me with Mas Garry; Mas Ikbar, Mas Yanda, Mas Nuve thanks for caring my sons to Jumah prayer when my husband was absent.

Thanks to my Pakistanese friend, Aulia and her parents for the discussion on traditional use of saffron, sending me a nice picture of saffron which published in the graphical abstract of my crocetin paper, and for the delicious dinner. Hajj Family 2018: Ustadz Fatah and Yai Hasyim, thank you for being our spiritual mentors. Bu Yeni (Pak Wim), Bu Armis, Bu Berni, Bu Ita (Pak Akmal), Bu Efi (Mr. Daniel), Bu Maya (Pak Oscar), Bu Leni, Bu Teti, Bu Anita, Bu Sunu, Bu Nurhayati, Bu Riana, Bu Lilis, Bu Lilis (*geulis*), Mas Fikri, Pak Yuli, Mbak Vica and Mohammad thank you for the togetherness before, during and after the Hajj. My special thanks to Bu Iik who called me "Nok" for sharing your personal life stories, giving kitchen stuffs, and sewing my dress when I was a paranymph of my college.

I would like to thank my home university, Universitas Islam Sultan Agung (UNISSULA), especially Faculty of Medicine (FK) for keeping my employment. Pak Is (chair of Biology), Pak Iwang, Bu Puji (former Dean and Vice Dean) thank you for being supported me to achieve LPDP scholarship and to continue my PhD study in WUR; Pak Setyo, Bu Ijah dan Pak Hadi (current Dean and Vice Dean) thank for for allowing me to stay in Wageningen for another 1 year after I finished my PhD. Pak Anies (former Rector) thank you for visiting me during WISE 2017; Pak Prabowo, Bu Eni, Pak Bedjo (current rector and vice rectors) and Prof Imam who visiting during your meeting in Rotterdam. Colleagues in UNISSULA: my labmate Mbak Dina thanks for the heart-chat sessions and updating me the situation in UNISSULA; Bu Endang and Bu Minidian, thanks for the sharing about study in the Netherlands; Bu Dian, Bu Mila and Bu Ayu thanks for the chatting about batik, hajj, and Indonesian food; Bu Hesti thank for bringing *oleh-oleh*. I am indebted to Pak Harka who supported a part of my family allowance especially during the first year. Thanks to Mbak Aang, Mbak Okti, Mbak Eva, Mbak Fuah, Mas Andi, Mas Adin, Mas Didik Karno, Mas Hamdani, Mas Adji, Mbak Ekki, Pak Dwi, Mas Andika and

Mas Wisnu for the arrangement of administrative matters I needed to keep my employment in track.

I would like to grateful my lecturers from Diponegoro University who visiting when they came to WUR: Prof Ocky, Prof Jeki and Bu Tita for sharing experinces on research grant and research collaboration; Bu Riri and Bu Resti for cooking class and *oleh-oleh*. Mas Purwanto thanks for offering free luggage.

I would like to thank my dearest family, *Papa*, *Kakak* and *Adek*. My dear husband *Papa* Achmad Sahri, thank you very much for your love, your support, and for always making me calmer to finalize my thesis. We are doing our PhDs together and with you at my side, my PhD journey can safely finish on time. Thank you for taking care of our sons and taking over the household duty during my courses or conferences outside Wageningen or my prolonged work in lab and office. Thank you for the map of my sampling location in the 3 published papers, the beautiful pictures for the graphical abstracts in all my papers and the great cover design and layout of this thesis. I would like to extend my gratitude to my husband's promotor, Prof. Dr Tinka Murk who gives him a flexible time to come at his office and even allow him to work at home in the meanwhile accompanying our sons. My dear sons, Syifa Brillant and Fadli Habile, thank you for being my advisor of my PhD-related stress. *Kak* Brillant, you are special, your silent is a gold, and you can feel what I felt. *Dek* Habile, your enthusiastic and talkative characteristics make me wake up from the misfortune of my works.

My extended thanks to: my son's teachers at OBS de Tarthorst especially their class teachers since in Group 1/2 until 4/5 (Juf Ditje, Juf Ann, Juf Marloes, Juf Els, Juf Martine, Juf Fleur, Juf Manon, Juf Marjan) and Plus Group (Yuf Marianne, Mister Jorik); Sekolah Indonesia den Haag (teachers: Bu Siska and Bu Hesty, commitee: Pak Mulyono); Habile's korfbal trainer (Allin), Brillant's chess trainer (Hans). Thanks to KVW parents especially Janneke, Elise, and Juf Sandra.

The most important persons throughout my life, my mother, *Mboke* and my father, *Pake* (*almarhum*) thank you for all your constant supports and endless love. Without your *ridho* to me, I would not have been able to achieve this PhD. This degree is for you *Mboke*, also for you *Pake* (even we are in different realm). My grandfather, Pak Surat, thanks for your *doa* and endless love. Thanks also for my parents in law, *Mimih* and *Abah* (*almarhum*) for your *ridho* and *doa* to my little family. To all my sisters, Yu Yak (and Mas War), Sulis and Ayu; my niece Khoir; my nephew Imam; my aunts and uncles, my cousin's thanks for all your support and for always being there with *Mboke*. My sister Sulis, my cousin Ulfi and my brother in law Nana thank you for helping me to collect jamu samples.

Finally, I would like to thank my sponsor, Lembaga Pengelola Dana Pendidikan (LPDP) from The Ministry of Finance, The Republic of Indonesia for the financial support to my PhD journey.

About the Author

Suparmi was born on 26th June 1983 in Nganjuk, Indonesia. Before her PhD, she obtained her Bachelor's degree at Biology Department, Faculty of Science and Mathematics, Diponegoro University, Indonesia in 2005. Her BSc thesis on the effect of chitin supplementation in mice was published in a local journal. After one year of being a freelance teacher, she continued her education with a scholarship from *Beasiswa Unggulan*, Ministry of Education of Republic Indonesia to follow the MSc program in the field of natural pigments and its application for food and industry at Biology, Satya Wacana Christian University, Indonesia. She also was granted the research funding from the Beasiswa Peneliti, Penulis, Pencipta, Seniman, Wartawan, Olahragawan, dan Tokoh (P3SWOT) and published some research articles as first author at national journals and proceedings.

In October 2008, she was employed as a lecturer at Faculty of Medicine, Universitas Islam Sultan Agung, Semarang (UNISSULA) Indonesia. During these works, she received some grants from the Ministry of Research, Technology and Higher Education for research and research based-community services. Her research explored the beneficial effect of plant pigments, chlorophyll and carotenoids for food supplements with published research articles in local journals or magazines, accredited national journals and international journals. In November 2015, she was granted the StuNed Scholarship Programme Nuffic-Neso Indonesia for 2 weeks of Short Course: Right Based Approach to Food and Nutrition Security on 25th January – 5th February 2016 at CDI-Wageningen University and Research (WUR), The Netherlands.

In December 2016, she received a doctoral scholarship from the Indonesian Endowment Fund for Education, Ministry of Finance, Republic of Indonesia through a Beasiswa Pendidikan Indonesia Lembaga Pengelola Dana Pendidikan (BPI LPDP) for her PhD study at WUR. She started her PhD research described in this thesis at the Department of Toxicology WUR on 2nd May 2016. This PhD study brought practical opportunities for her to work to understand the food safety and risk assessment of herbal products from Indonesia. During her PhD, she followed a postgraduate education program and registered as toxicologist-in-training, to become a European Registered Toxicologist after graduation. After completing her doctoral study and one year additional live in Wageningen, she will return to serve as a lecturer at her home university, UNISSULA.

List of publications

Peer reviewed publications

S. Suparmi, D. Widiastuti, S. Wesseling, I.M.C.M. Rietjens. Natural occurrence of genotoxic and carcinogenic alkenylbenzenes in Indonesian jamu and evaluation of consumer risks. *Food and Chemical Toxicology*, 118 (2018), pp. 53-67, https://doi.org/10.1016/j.fct.2018.04.059

S. Suparmi, A.J. Ginting, S. Mariyam, S. Wesseling, I.M.C.M. Rietjens. Levels of methyleugenol and eugenol in instant herbal beverages available on the Indonesian market and related risk assessment. *Food and Chemical Toxicology*, 125 (2019), pp. 467-478, https://doi.org/10.1016/j.fct.2019.02.001

Suparmi, S., de, L., Spenkelink, A., Louisse, J., Beekmann, K., Rietjens, I. M. C. M., Combining in vitro data and physiologically based kinetic modeling facilitates reverse dosimetry to define in vivo dose–response curves for bixin- and crocetin-induced activation of PPARγ in humans. *Molecular Nutrition and Food Research* (2020), 64, 1900880, pp 1-9, https://doi.org/10.1002/mnfr.201900880.

S. Suparmi, Patrick P.J. Mulder, Ivonne M.C.M. Rietjens, Detection of pyrrolizidine alkaloids in jamu available on the Indonesian market and accompanying safety assessment for human consumption. *Food and Chemical Toxicology*, 138 (2020), 111230, pp. 1-13, https://doi.org/10.1016/j.fct.2020.111230

S. Suparmi, S. Wesseling, I.M.C.M. Rietjens. Monocrotaline-induced liver toxicity in rat predicted by a combined in vitro-physiologically based kinetic modeling approach. *Under review,* Archives of Toxicology (2019).

Abstracts/conference proceedings

S. Suparmi, Laura de Haan, Albertus Spenkelink, Jochem Louisse, Karsten Beekmann, Ivonne M.C.M. Rietjens, 2017. Activation of peroxisome proliferator-activated receptor γ (PPARγ)- mediated gene expression by bixin and norbixin from pericarp *Bixa orellana* seed, In: Wageningen Indonesia Scientific Exposure 2017.

S. Suparmi, D. Widiastuti, S. Wesseling, I.M.C.M. Rietjens. 2018. Consumer risk analysis for jamu in Indonesia based on natural occurrence of genotoxic and carcinogenic alkenylbenzenes. **In**: Wageningen Indonesia Scientific Exposure 2018 **and** NVT Annual Meeting and Young Scientist Day.

S. Suparmi, A.J. Ginting, S. Mariyam, S. Wesseling, I.M.C.M. Rietjens. 2019. Are the levels of methyleugenol in instant herbal beverages available on the Indonesian market safe for human consumption? **In:** 40th NVT Anniversary Annual Meeting

Suparmi, A.J. Ginting, S. Mariyam, S. Wesseling, I.M.C.M. Rietjens. 2019. The importance to establish a maximum permitted level (MPL) for methyleugenol in Indonesia: A risk assessment study based on methyleugenol levels. **In:** Wageningen Indonesia Scientific Exposure 2019.

S. Suparmi, Laura de Haan, Albertus Spenkelink, Jochem Louisse, Karsten Beekmann, Ivonne M.C.M. Rietjens. 2019. In vitro-in silico-based prediction of peroxisome proliferator-activated receptor γ (PPARγ) activation by bixin and crocetin in humans, **In**: Toxicology Letters 314S1 (2019) page S304

S. Suparmi, S. Wesseling, I.M.C.M. Rietjens. 2020. Prediction of in vivo monocrotaline-induced liver toxicity in rat using an in vitro-in silico approach. **In:** The Toxicologist: Late-Breaking Supplement, the Society of Toxicology 59th Annual Meeting (2020)

Overview of Completed Training Activities

Discipline specific courses		
Molecular Toxicology	PET	2016
Pathobiology	PET	2016
Risk Assessment	PET	2016
Organ Toxicology	PET	2017
Mutagenesis and Carcinogenesis	PET	2017
Toxicogenomics	PET	2017
Epidemiology	PET	2018
Cell Toxicology	PET	2019
Meetings and conferences		
Wageningen Indonesia Scientific Exposure (WISE)	WUR, The Netherlands	2017
39th NVT Annual Meeting and Young Scientist Day	Hilversum, The Netherlands	2018
Wageningen Indonesia Scientific Exposure (WISE)	Indonesian Institute of Sciences, Indonesia	2018
Wageningen Indonesia Scientific Exposure (WISE)	WUR, The Netherlands	2019
40th Anniversary Annual Meeting Netherlands Society of Toxicology (NVT)	Ede, The Netherlands	2019
55th Congress of the European Societies of Toxicology (EUROTOX 2019)	Helsinki, Finland	2019
General courses		
Information Literacy Including Endnote Introduction	WGS	2016
Reviewing a Scientific Paper	WGS	2016
VLAG PhD Week	VLAG	2016
The Essentials of Scientific Writing and Presenting	WGS	2016
Laboratory Animal Science: Design and Ethics in Animal Experimentation	WUR	2017
Orientation on Teaching for PhD Candidates	WGS	2017
Supervising BSc and MSc Thesis Students	WGS	2018
Philosophy and Ethics of Food Science and Technology	VLAG	2019
Reviewing a Scientific Paper	WGS	2019
Start to Teach	WGS	2019
Optional activities		
Thesis Proposal	TOX-WUR	2016
Attending scientific presentations	TOX-WUR	2016-2020
General Toxicology	WUR	2016
Environmental Toxicology	WUR	2019
PhD Trip to Japan	TOX-WUR	2018

Approved by Graduate School VLAG

The research described in this thesis was financially supported by the Indonesian Endowment Fund for Education (LPDP), Ministry of Finance, Republic of Indonesia through a Beasiswa Pendidikan Indonesia Lembaga Pengelola Dana Pendidikan (BPI LPDP) doctoral scholarship for Suparmi [contract number: PRJ-365/LPDP/2016].

Colophon
Front cover: schematic image of weighing process of herbals and the resulting herbal preparation
Back cover: schematic image of Mbok jamu, a woman who sells fresh jamu carrying the jamu using *gendong* (long wide shawl) on the back of her body.
Cover & layout design: M. Pramaditya Garry Hanantyo, Achmad Sahri & Suparmi
Printed by: Digiforce || Proefschriftmaken (www.proefschriftmaken.nl)

Financial support from LPDP and Toxicology Department, Wageningen University for printing the thesis is gratefully acknowledged.