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

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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 PA/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 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 low 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.

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) (BPOM, 2016). 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 (BPOM, 2018). 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 (Suparmi et al., 2018) 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 (Abdullah et al., 2017), 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 (Bodi et al., 2014; Chen et al., 2017a; EFSA, 2017). The aim of the present study was to

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List of abbreviations

AAs	Aristolochic acids
ABs	Alkenylbenzenes
$BMDL_{10}$	Lower confidence limit of the benchmark dose resulting in a 10% extra cancer incidence
BPOM RI	Badan Pengawas Obat dan Makanan Republik Indonesia
CONTAM Panel	EFSA Panel on Contaminants in the Food Chain
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EMA	European Medicines Agency
GACP	Good Agricultural and Collection Practices
HMPC	Herbal Medicinal Products Committee
HVOD	Hepatic veno-occlusive disease
IARC	International Agency for Research on Cancer
iREP	interim Relative Potency
LOD	Limit of detection
LOQ	Limit of quantification
MMS	Matrix matched standards
MOE	Margin of Exposure
PoD	Point of departure
PAs	Pyrrolizidine alkaloids
PA-ILI	PA-Induced Liver Injury
PFS	Plant Food Supplements
SPE	Solid phase extraction
WHO-IPCS	World Health Organization, International Programme on Chemical Safety

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 (Mohabbat et al., 1976; Tandon et al., 1976; Wiedenfeld, 2011), whereas chronic exposure may lead to liver cirrhosis and pulmonary arterial hypertension (EFSA, 2011; Li et al., 2018). 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) (IARC, 2002).

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* (Bodi et al., 2014; EFSA, 2007; Fu et al., 2004; Liu et al., 2017; Qi et al., 2009; Wiedenfeld, 2011). 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 PA per day per person related to intake resulting from such contamination, for a 3 years period (HMPC, 2016). Recently HMPC (2019) 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 (EFSA, 2017).

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.

2. Materials and methods

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 Fig. 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

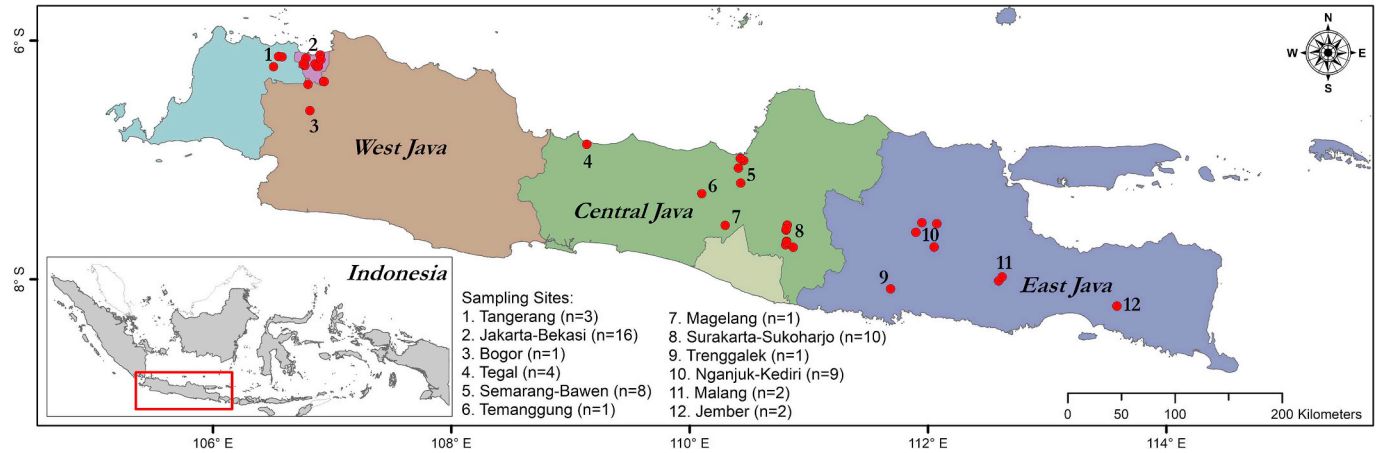

Fig. 1. Sampling locations of jamu in Banten, Jakarta, West Java, Central Java and East Java Provinces, Indonesia. The red dots represent the sampling locations of the products, including both PA- and non-PA-containing jamu and n is the number of collected samples in the respective city. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(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 (Suparmi et al., 2018), 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 Material 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.

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). A complete list of PA standards used in this study is presented in Supplementary Material 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.

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), 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 3500 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 3500 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.

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 0–1,000 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).

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 Material 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 Material 2. Data processing was conducted with MassLynx 4.1 software (Waters Corporation, Milford, MA, USA).

Table 1
The PA content detected in Indonesian jamu containing PA-producing botanicals, and the corresponding EDI without and with taking iREP factors into account.

Sample ID	Number of PAs detected	Total PAs level (μg/kg)[a]	Recommended daily use (g)	EDI (μg/kg bw/day)	EDI with iREP factors (μg riddelliine equivalents/kg bw/day)	% reduction in the EDI when taking iREP factors into account	Top three PAs and their concentration (μg/kg)[a]
TR-1	2	13.1	1.9	0.0005	0.0001	70.0	Monoester 7.90 (7.7), rinderine (5.3)
TR-2	3	135.3	14	0.035	0.034	3.2	Senkirkine (85.7), neosenkirkine (43.3), rinderine (6.3)
TR-3	–	<LOQ	200[b]	-[c]	-[c]	–	–
TR-4	3	31.8	3.4	0.002	0.001	70.0	Rinderine (16.8), echinatine (9.0), rinderine N-ox (5.9)
TR-5	37	35,066	1.1	0.691	0.682	1.3	Jacoline N-ox (16,762), jacobine N-ox (4016), senkirkine (3,042)
TR-6	26	17,435	5.6	1.808	1.805	0.2	Senkirkine (5,807), senecionine (4,918), neosenkirkine (4,153)
TR-7	7	73.4	2.9	0.004	0.001	61.8	Lycopsamine (19.0), echinatine (15.0), PA diester 11.45 (12.6)
TR-8	23	70,055	0.8	1.067	1.065	0.2	Neosenkirkine (44,446), senkirkine (15,674), integerrimine (2,981)
TR-9	31	74,837	2.9	3.951	3.874	2.0	Jacoline N-ox (30,507), onetine (16,468), jacoline (8,342)
TR-10	27	39,632	3	2.229	2.229	0.0	Senkirkine (15,505), otonecine ester 3.75 (9008), neosenkirkine (7,065)
TR-11	2	12.3	3.7	0.001	0.0005	43.9	Senkirkine (6.8), intermedine (5.5)
TR-12	40	105,099	2.4	4.761	4.711	1.1	Senkirkine (52,260), neosenkirkine (19,495), otonecine ester 3.75 (17,460)
TR-13	4	65.7	3	0.004	0.001	60.5	Rinderine (38.0), intermedine (13.3), senkirkine (8.0)
TR-14	2	131.1	45[b]	0.109	0.109	0.0	Senkirkine (123.0), neosenkirkine (8.2)
TR-15	27	21,516	0.5	0.202	0.200	1.3	Senecionine (6,634), senkirkine (6,588), neosenkirkine (4,743)
TR-16	4	33.2	1.1	0.001	0.000	74.3	Rinderine (12.0), echinatine N-ox (8.8), rinderine N-ox (7.6)
TR-17	31	235,376	3	13.256	13.256	0.0	Senecionine N-ox (114,071), senkirkine (66,713), integerrimine N-ox (23,784)
TR-18	4	29.5	30	0.016	0.004	77.9	Echinatine N-ox (8.5), lycopsamine N-ox (8.1), rinderine (6.9)
TR-19	8	453	2	0.017	0.004	77.6	Echinatine (187.4), rinderine (114.6), lycopsamine (58.0)
TR-20	9	276.6	2.9	0.015	0.002	89.1	Indicine N-ox (136.0), indicine (62.3), rinderine (22.8)
TR-21	8	270.2	6	0.030	0.002	94.3	PA diester 11.45 (91.2), lycopsamine N-ox (56.2), Lycopsamine (42.3)
TR-22	6	113.4	1.4	0.003	0.001	76.1	Echinatine (40.8), Rinderine (23.6), lycopsamine (16.1)
TR-23	23	51,425	1	0.933	0.932	0.1	Senkirkine (23,234), neosenkirkine (15,662), otonecine ester 3.75 (7749)
TR-24	37	63,877	0.9	1.065	1.048	1.6	Senkirkine (28,452), neosenkirkine (14,610), otonecine ester 3.75 (7870)
TR-25	13	1265	1.2	0.029	0.027	5.1	Neosenkirkine (699.3), senkirkine (189.7), Otonecine ester 3.75 (93.5)
TR-26	26	12,173	1.4	0.311	0.307	1.4	Senkirkine (4,679), neosenkirkine (2,539), senecionine (1,996)
TR-27	19	933.9	3.8	0.065	0.048	26.4	Senkirkine (237.2), senecionine N-ox (132.6), rinderine (127.1)
TR-28	34	65,763	0.7	0.813	0.803	1.2	Senkirkine (33,630), neosenkirkine (13,022), otonecine ester 3.75 (8894)

(*continued on next page*)

Table 1 (*continued*)

Sample ID	Number of PAs detected	Total PAs level (μg/kg)[a]	Recommended daily use (g)	EDI (μg/kg bw/day)	EDI with iREP factors (μg riddelliine equivalents/kg bw/day)	% reduction in the EDI when taking iREP factors into account	Top three PAs and their concentration (μg/kg)[a]
TR-29	7	205.9	1.8	0.007	0.002	70.4	Echinatine (103.5), lycopsamine (37.2), rinderine (21.4)
TR-30	4	42.5	1.5	0.001	0.0002	79.8	Echinatine (15.9), rinderine (12.1), lycopsamine (8.2)
TR-31	36	104,842	3.4	6.556	6.497	0.9	Senkirkine (48,296), neosenkirkine (16,787), otonecine ester 3.75 (16,257)
TR-32	24	146,977	0.5	1.255	0.228	81.8	Echinatine (33,713), echinatine N-ox (33,563), lycopsamine (26,217)
TR-33	11	357.2	2.3	0.015	0.008	45.8	Rinderine N-ox (66.8), senkirkine (65.9), neosenkirkine (44.0)
TR-34	39	106,712	4.2	8.346	8.212	1.6	Senkirkine (49,156), neosenkirkine (18,997), otonecine ester 3.75 (16,757)
TR-35	2	15.1	30[b]	0.008	0.008	0.0	Neosenkirkine (8.4), senkirkine (6.6)

[a] Liquid samples are expressed in μg/L.
[b] In mL.
[c] No EDI was calculated because no PAs were detected above the LOQ.

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.

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 (1).

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

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 1) based on the information provided on the label (See Supplementary Material 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 (FAO, 2017). The factor 1,000 is added to convert W in g to kg or mL to L.

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 (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). A daily intake of PAs of 10 μg/kg bw/day established by WHO-IPCS (World Health Organization, International Programme on Chemical Safety) & WHO Task Group on Pyrrolizidine Alkaloids (1988) 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 (EFSA, 2005). The MOE was calculated as described in Equation (2).

$$MOE = \frac{BMDL_{10}}{EDI} \quad \text{(Equation 2)}$$

where the MOE is dimensionless, the $BMDL_{10}$ value used was 237 μg/kg bw/day established by EFSA (2017) for riddelliine and used as PoD for evaluating the risks of PA exposure, and EDI values (μg/kg bw/day) were calculated by Equation (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) 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

Table 2
The PA content detected in Indonesian jamu containing non-PA-producing botanicals and the corresponding EDI without and with taking iREP factors into account.

Sample ID	Number of PAs detected	Total PAs level (μg/kg)[a]	Recommended daily use (g)	EDI (μg/kg bw/day)	EDI with iREP factors (μg riddelliine equivalents/kg bw/day)	% reduction in the EDI when taking iREP factors into account	Top three PAs and their concentration (μg/kg)[a]
TR-36	–	<LOQ	14	-[c]	-[c]	–	–
TR-37	10	127.8	14	0.033	0.007	80.3	Indicine N-ox (36.5), heliotrine N-ox (19.9), echinatine N-ox (12.6)
TR-38	–	<LOQ	14	-[c]	0.000	–	–
TR-39	14	313.7	14	0.081	0.018	78.2	Indicine N-ox (91.9), heliotrine N-ox (66.2), europine N-ox (30.1)
TR-40	–	<LOQ	14	-[c]	-[c]	–	–
TR-41	–	<LOQ	14	-[c]	-[c]	–	–
TR-42	5	40.6	2.1	0.002	0.0003	83.9	Rinderine (14.7), intermedine (8.0), rinderine N-ox (6.5)
TR-43	10	144.8	14	0.038	0.006	85.1	Lycopsamine (36.2), rinderine N-ox (23.2), echimidine (20.6)
TR-44	–	<LOQ	3	-[c]	-[c]	–	–
TR-45	8	253.2	14	0.066	0.019	70.8	Echinatine N-ox (70.8), rinderine (43.5), echinatine (41.9)
TR-46	3	60.2	7	0.008	0.002	74.8	Rinderine (33.3), rinderine N-ox (16.8), intermedine (10.0)
TR-47	7	149.8	14	0.039	0.005	86.4	Indicine N-ox (48.9), echinatine N-ox (40.8), lycopsamine N-ox (15.6)
TR-48	11	436.8	10	0.081	0.008	90.7	Indicine N-ox (135.5), indicine (86.8), PA diester 11.45 (47.3)
TR-49	6	102.8	10	0.019	0.003	81.8	Rinderine (42.6), intermedine (16.9), lycopsamine (14.4)
TR-50	3	35.7	14	0.009	0.002	78.7	Rinderine (18.8), intermedine (10.8), echinatine (6.2)
TR-51	2	26.2	12	0.006	0.005	22.6	Monocrotaline (17.7), heliotrine N-ox (8.4), europine N-ox (4.7)
TR-52	3	49.5	1.7	0.002	0.0002	87.1	Rinderine (20.4), lycopsamine (15.7), intermedine (13.4)
TR-53	6	125.2	2.1	0.005	0.001	87.1	Indicine N-ox (33.3), rinderine N-ox (28.4), indicine (24.0)
TR-54	4	86.7	10	0.016	0.004	75.9	Echinatine (43.9), echinatine N-ox (19.8), lycopsamine (17.8)
TR-55	1	5.9	14	0.002	0.0005	70.0	Rinderine (5.9)
TR-56	7	70.1	14	0.018	0.004	79.7	Rinderine (17.7), echinatine N-ox (13.0), echinatine (9.1)
TR-57	–	<LOQ	5[b]	-[c]	-[c]	–	–
TR-58	4	3,421	3.6	0.228	0.228	0.0	Senkirkine (3,221), otonecine ester 3.75 (121.5), senecionine (52.3)

[a] Liquid samples are expressed in μg/L.
[b] In mL.
[c] No EDI was calculated because no PAs were detected above the LOQ.

outcome, C is the concentration (or dose) of the toxic chemical and T is the duration of exposure) (Doull and Rozman, 2000; Felter et al., 2011; Gaylor, 2000). Using Haber's rule, the EDI of PAs can be expressed as follows:

$$EDI\ \ (2\ wk/yr\ during\ a\ lifetime) = \frac{EDI}{26} \qquad \text{(Equation 3)}$$

where the EDI for 2 weeks every year during a lifetime is the EDI for daily lifetime exposure obtained by Equation (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)) of daily consumption of the different samples that would result in an MOE value of 10,000:

$$Number\ of\ weeks = \frac{MOE \times 69 \times 52}{10{,}000} \qquad \text{(Equation 4)}$$

where the MOE is the value for lifetime exposure calculated by Equation (2), 69 represents the life expectancy of Indonesian people in years (WB, 2017), 52 is the number of weeks within a year, and 10,000, the threshold for health concern (EFSA, 2005).

To take the differences in relative potency between the PAs detected in the jamu samples into account, calculations of EDI and MOE values were performed using the interim Relative Potency (iREP) factors as reported by Merz and Schrenk (2016), to express the PA levels and EDI values in riddelliine equivalents (Supplementary Material 4). Using the EDI values thus obtained, MOE values were calculated for lifetime exposure and for exposure during 2 weeks every year during a lifetime and the number of weeks during which the jamu could be used to result in a MOE value of 10,000.

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 (Suparmi et al., 2018). The MOE values were calculated using the $BMDL_{10}$ of 15.3 mg/kg bw for the major alkenylbenzene in the mixture, methyleugenol (van den Berg et al., 2011), and the EDI resulting from summing up the EDIs of the individual alkenylbenzenes assuming equal potency (See Supplementary Material 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 (Abdullah et al., 2017) 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 (Mengs et al., 1982). The EDI values were calculated based on the AA levels determined using the UPLC method for quantification of AAs described previously (Abdullah et al., 2017).

3. Results

3.1. Levels of PAs in Indonesian jamu

As shown in Table 1 (for a full set of results see Supplementary Material 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).

PAs were also found in 17 out of 23 jamu samples containing non-PA-producing botanicals with levels ranging from 5.9 to 3,421 μg/kg (Table 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

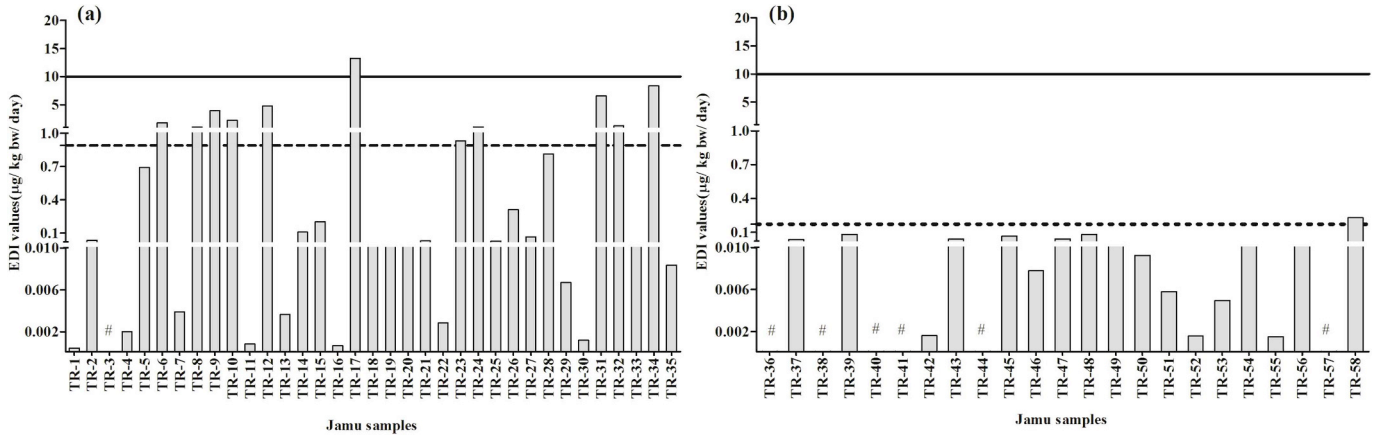

Fig. 2. EDI values for the consumption of PA detected in (a) 35 jamu samples containing PA-producing botanicals and (b) 23 jamu samples containing non-PA-producing botanicals. EDI values were calculated as explained in the Materials and Methods section (equation (1)). The dashed line (- - -) in figure (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 (EFSA, 2017). 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 (EFSA, 2017). 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 (WHO-IPCS, 1988). # indicates that the EDI value is not quantifiable due to a PA content < LOQ.

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 to 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 1).

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

Table 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 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.

When taking the iREP factors proposed by Merz and Schrenk (2016) into account the EDI values of 17 out of 35 jamu samples containing PA producing botanicals decreased by less than 5%, while for 11 samples the values decreased by 70–90% (Table 1). For all but two jamu containing non-PA-producing botanicals with detectable levels of PAs, the EDI values decreased by 70–91% when taking the iREP factors into account (Table 2). This substantial decrease in many of the jamu samples is due to the fact that these samples contain relatively large amounts of mono and open-chain diester PAs with iREP factors of 0.01, 0.1 and 0.3. On the other hand, the samples with little or no reduction contain almost entirely macrocyclic PAs with iREP factors of 1.

3.3. Risk assessment of jamu based on PA levels

3.3.1. Acute exposure scenario

The EDI values for PAs resulting from the consumption of all jamu samples containing PA-producing botanicals (Fig. 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 (EFSA, 2017). This result indicates that jamu consumers are

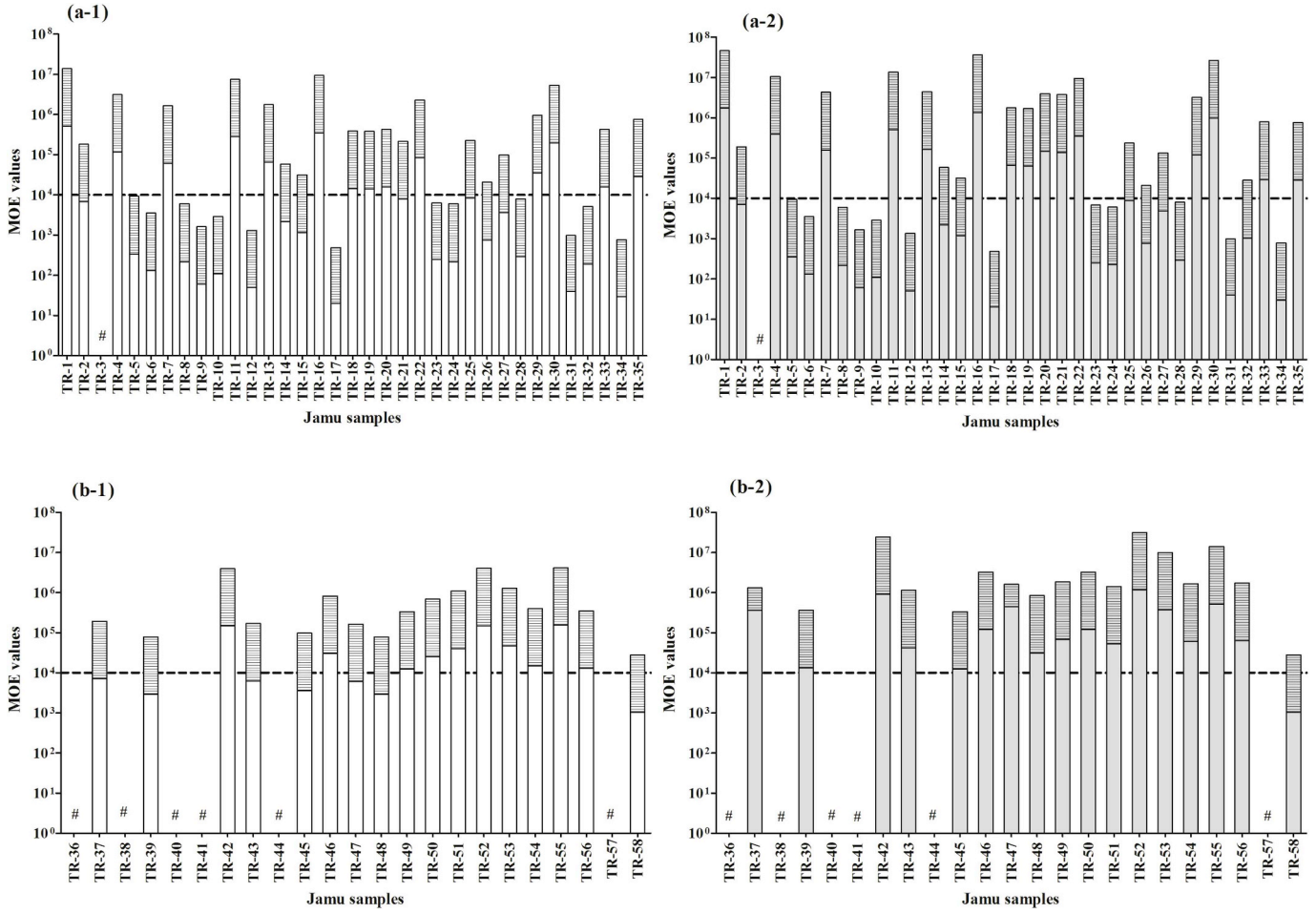

Fig. 3. MOE values obtained for the consumption of jamu samples containing (a) PA-producing botanicals and (b) non-PA-producing botanicals. (1) represents the MOE values calculated without taking iREP factors into account, based on daily lifetime exposure (white bars) and based on exposure for 2 weeks every year during a lifetime exposure (patterned bars), while (2) represents the MOE values calculated taking the iREP factors into account (Merz and Schrenk, 2016) based on daily lifetime exposure (grey bars) and based on exposure for 2 weeks every year during a lifetime exposure (grey patterned bars). MOE values were calculated as explained in the Materials and Methods section (equation (2)), using the $BMDL_{10}$ of 237 μg/kg bw/day for riddelliine (EFSA, 2017). The dashed line (- - -) represents the MOE value of 10,000 as a threshold for risk management action (EFSA, 2005). # indicates that no MOE value is calculated due to a PA content < LOQ.

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 (WHO-IPCS, 1988). 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), for consumption of an infusion of borage (*Borago officinalis*), a PA-producing plant consumed by a part of the European population (Fig. 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 (EFSA, 2016). Moreover, as can be seen in Fig. 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 (EFSA, 2017).

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 Fig. 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 (EFSA, 2017). For 20 out of 35 (57%) jamu containing PA-producing botanicals the MOE values were below 10,000, indicating a priority for risk management (Figs. 3a–1). 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 (Figs. 3a–1). 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 low priority for risk management (MOE >10,000) when these jamu would be consumed for a period of 2 weeks yearly during a lifetime (Figs. 3b–1).

At first sight the data presented in Fig. 3 do not reveal substantial differences by taking the iREP factors into account. Closer analysis, however, reveals some subtle differences. When taking iREP factors into account, the number of jamu containing PA-producing botanicals with an MOE <10,000 decreased from 13 to 12. For jamu sample TR-21 the PA levels and EDI values, when expressed in riddelliine equivalents, decreased by 94% and as a result, the MOE based on lifetime exposure increased from 7,900 to 140,000 (Figs. 3a–2). For the other jamu samples containing PA-producing plants the MOE value did not change to a level where it affected the outcome of the risk assessment. For 6 samples of jamu containing non-PA-producing botanicals, the MOE increased from <10,000 to >10,000 when the iREP factors were taken into account (Figs. 3b–2). Only one jamu sample (TR-58) remained with an MOE <10,000. The effect of iREP on the calculated MOE values for consumption of 2 weeks every year during a lifetime was limited: the total number of jamu samples with an MOE <10,000 decreased from 13 to 12 (Figs. 3a–2 and Figs. 3b–2).

Fig. 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 Fig. 4a it follows for example that jamu TR-1 would be of low concern when consumed for up to 184,480 weeks, corresponding to far more than a lifetime. 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 (Fig. 4b).

Fig. 4 also presents the number of weeks resulting in low concern (MOE >10,000) when taking the iREP factors into account. For 11 out of the 35 jamu samples containing PA-producing botanicals the number of weeks increased more than threefold with the largest increase (17.5-fold) for TR-21.

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

Fig. 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 Fig. 5a that in 5 samples, TR-36, TR-38, TR-

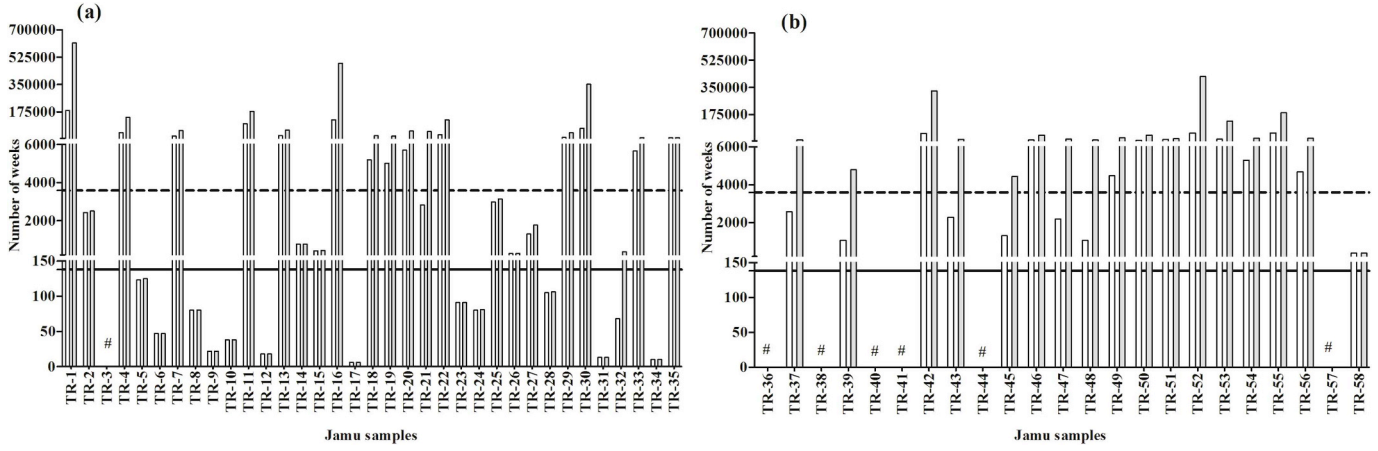

Fig. 4. The number of weeks of use that would result in an MOE of 10,000 upon daily consumption of (a) jamu containing PA-producing botanicals and (b) jamu containing non-PA-producing botanicals. White bars represent the calculations assuming equal potency of the different PAs using the $BMDL_{10}$ of 237 μg/kg bw/day for riddelliine (EFSA, 2017), while grey bars represent the number of weeks taking iREP factors into account (Merz and Schrenk, 2016). The dashed (- - -) and horizontal line (—) represent the number of weeks equal to a lifetime (69 years = 3588 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.

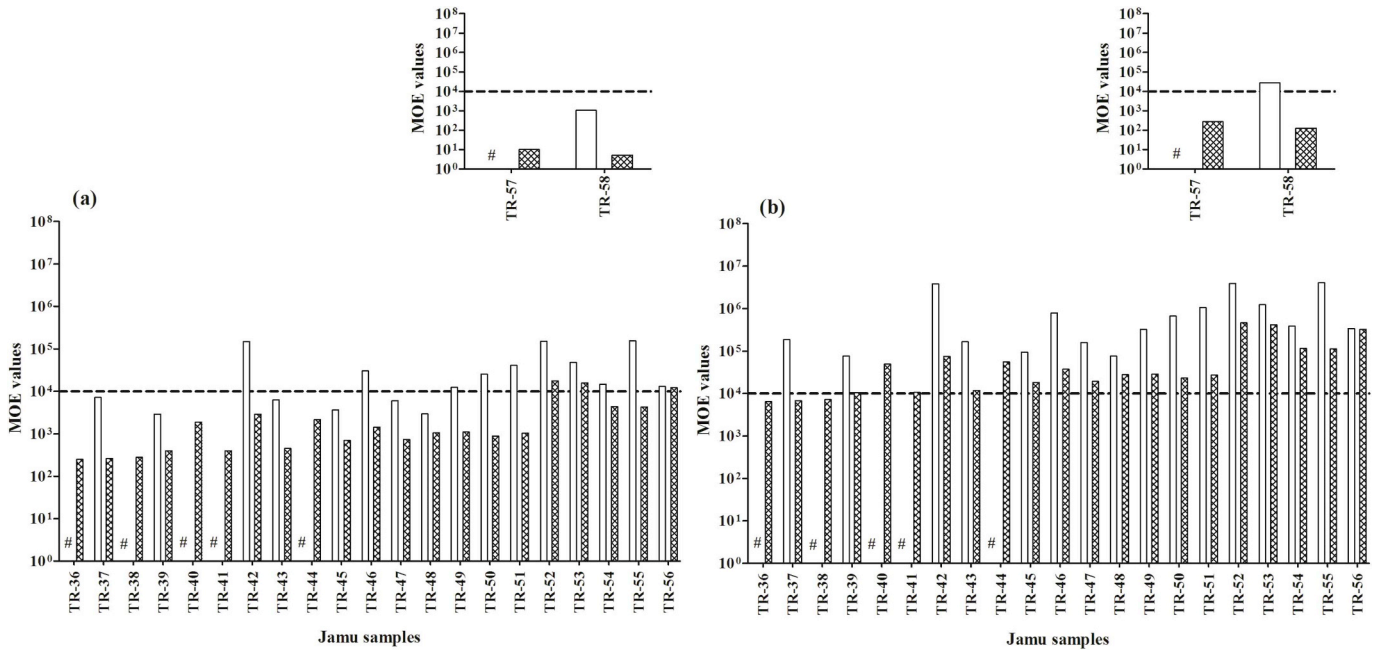

Fig. 5. MOE values obtained for the consumption of 23 jamu samples containing non-PA-producing botanicals, based on (a) daily lifetime exposure and b) exposure for 2 weeks every year during a lifetime. MOE values based on PA levels (white bars) were calculated as explained in the Materials and Methods section (equation (2)), using the $BMDL_{10}$ of 237 μg/kg bw/day for riddelliine (EFSA, 2017). The patterned bars are the MOE values based on the AB level previously detected in samples TR-36 to TR-56 (Suparmi et al., 2018) using the $BMDL_{10}$ of 15.3 mg/kg bw for the major alkenylbenzene in the mixture, methyleugenol (van den Berg et al., 2011). The inserted graph shows the MOE values calculated for the intake of PA levels (white 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 (Abdullah et al., 2017). The dashed line (- - -) represents the MOE value of 10,000 as a threshold for risk management action (EFSA, 2005). # indicates that no MOE value is calculated due to a PA content < LOQ.

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 (Fig. 5b). Overall the data presented in Fig. 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 Fig. 5), indicating a priority for risk management. 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 a value of 125 when consumed 2 weeks every year, which are far below 10,000, indicating a priority for risk management. This indicates that in TR-58 AAs present a larger concern than the PAs.

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 (Langel et al., 2011). 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* (Chen et al., 2017b). 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* (Windono et al., 2012). Senecionine, integerrimine, retrorsine, usaramine, spartioidine, seneciphylline and seneciphyllinine (acetylseneciphylline) have been reported as characteristic PAs for *G. segetum* (syn. *G. japonica*) (Aizhen et al., 2019; Qi et al., 2009; Roeder, 2000). 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., 2017b 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)

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) reported very high PA concentrations in batches of *G. japonica* (*segetum*) collected in China: in leaves the levels ranged from 460 to 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 (Bosi et al., 2013; Wiedenfeld and Roder, 1991) 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 (Bodi et al., 2014; Mulder et al., 2018; Schulz et al., 2015) and Chinese herbal medicines (Chen et al., 2019). 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 (El-Shazly and Wink, 2014).

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 (FAO, 2017). This EDI exceeded the transitional limit of intake of 1.0 μg PA per day per person, set by HMPC (2016) 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) 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 (EFSA, 2017). 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) and Ridker et al. (1985), 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 (Ruan et al., 2015). 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) 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 (Lin et al., 2011; Ma et al., 2018; Ruan et al., 2015). 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 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 priority for risk management 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 priority for risk management 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, was of low concern (MOE$>$10,000).

It is important to note that the application of Haber's rule is not a generally accepted approach when using the MOE for risk assessment of short-term exposure to genotoxic carcinogens. Based on this rule the toxic outcome will be similar for situations where the product of the exposure time and the dose is 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) (Doull and Rozman, 2000; Felter et al., 2011; Gaylor, 2000). Haber's rule thus describes a linear relationship between the response and the dose and between the response and the exposure time (Felter et al., 2011). Felter et al. (2011) also indicated that the use of Haber's rule assumes that chemical-specific carcinogenicity data are available, and that the data support such a linear dose- or time-response relationship. To what extent such a linear relationship holds for the induction of liver tumours by PAs in the low dose ranges relevant for realistic human exposures, remains to be established and may depend on the mode of action (MOA) underlying the carcinogenicity. For risk assessment of the PAs such evidence is not (yet) available and the $BMDL_{10}$ of riddelliine of 237 μg/kg bw/day is taken as the point of departure for calculation of the MOE. This $BMDL_{10}$ was derived by EFSA using benchmark dose software and applying model averaging, fitting data at the high dose levels required to induce measurable tumor incidences in experimental rodents in a non-linear way (EFSA, 2017). Thus, experimental data supporting a linear response of tumor incidences at low dose or shorter than life time exposure levels

remain to be provided. However, this evidence cannot be easily obtained given that at low dose levels tumor incidences in rodent bioassays will be too low to be detected. Nevertheless, application of Haber's rule can be considered a first tier approach to estimate the consequences of shorter-than-life-time exposure.

It is important to note that the present risk assessment of PAs can be performed without or with taking into account differences in relative potency of the PAs present in the samples. Merz and Schrenk (2016) defined interim Relative Potency (iREP) 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) proposed iREP 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 iREP factors 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. Substantial reductions were estimated for jamu samples primarily containing PAs of the mono/open-chain diester types which were reported to have iREP factors ranging from 0.01 to 0.3 (Merz and Schrenk, 2016) (see Supplementary Material 4). However, for other samples little or no EDI reduction was calculated due to the fact that these samples almost exclusively contained macrocyclic PAs, for which the assigned iREP factor is 1. Importantly, most of the jamu samples with the highest PA levels contained only macrocyclic PAs. The risk assessment was different for only 7 out of the 58 jamu samples: indicating a low concern. Six of them were jamu containing non-PA producing botanicals. Overall, the impact of applying iREP factors on the calculated MOE values was limited. Therefore, also given the uncertainty in the current iREP factors (EFSA, 2017), a risk assessment on PAs present in jamu without taking the relative potency differences into account, can provide a useful first indication and serve to set priorities for risk management actions. This would be in line with the results from a previous risk assessment for PA-containing herbal teas and food supplements (Chen et al., 2017a).

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.

CRediT authorship contribution statement

Suparmi Suparmi: Conceptualization, Resources, Investigation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Patrick P.J. Mulder:** Methodology, Resources, Investigation, Validation, Formal analysis, Writing - review & editing. **Ivonne M.C.M. Rietjens:** Conceptualization, Resources, Writing - review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2020.111230.

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