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Risk assessment of intake of pyrrolizidine alkaloids from herbal teas and medicines following realistic exposure scenarios

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A B S T R A C T

In this study five types of herbal teas were used to quantify the effect of comminution of the leaves on resulting PA exposure. Results show that PA levels extracted from intact leaves were consistently lower than from comminuted tea leaves. The Margin of Exposure (MOE) approach was applied to evaluate the consequences of this difference for the associated risks in the scenario of lifetime exposure. Furthermore, we considered medicinal use of these teas for shorter-than-lifetime exposure scenarios, and also analysed the risks of shorter-than-lifetime use of eight herbal medicines and 19 previously analysed plant food supplements. This analysis revealed that shorter-than-lifetime use resulted in MOE values $<$ 10,000 upon use for 40–3450 weeks during a lifetime, with for only a limited number of herbal teas and medicines use of two weeks a year (150 weeks during a 75 year lifetime) would still raise a concern. It is concluded that taking more realistic conditions into account markedly reduces the concerns raised for these herbal preparations. These results also illustrate the need for development of a generally accepted method for taking short term exposure into account in risk assessment of compounds that are genotoxic and carcinogenic.

1. Introduction

Botanicals and botanical preparations, such as herbal teas and herbal medicines, have been marketed for several decades. Due to (misleading) advertisement and/or overstatement of the benefits arising from consumption of these products and also because many customers equate 'natural' with 'safe', botanical preparations are widely used around the world (Rietjens et al., 2008). In addition to the fact that benefits of these preparations are often not scientifically proven and/or due to illegal adulteration with pharmaceutical ingredients (Ancuceanu et al., 2013; Carvalho et al., 2012; Reeuwijk et al., 2014), botanical preparations may even contain ingredients of concern. For example, herbal teas and plant food supplements (PFS) have been shown to frequently contain toxic pyrrolizidine alkaloids (PAs) (Bodi et al., 2014; EFSA, 2016; Mulder et al., 2018; Mulder et al., 2015). Especially 1,2-unsaturated PAs have been shown to be hepatotoxic, genotoxic and carcinogenic in rats and other experimental rodents (Hirono et al., 1979; Hirono et al., 1976; Kuhara et al., 1980; NTP, 1978, 2003; Schoental, 1970; Shumaker et al., 1976). These results also raise a concern for human health when levels of intake would be too high. A number of cases of intoxication and even death caused by PAs through consumption of herbal teas and herbal medicines have been reported (Lin et al., 2011; Margalith et al., 1985; Mohabbat et al., 1976; Ruan et al., 2015; Tandon et al., 1976; Weston et al., 1987). Intake of herbal teas and medicines has proven to be a major route for human exposure to PA-containing plants (Bodi et al., 2014; Edgar et al., 1992; Roeder, 2000).

In light of the health relevance of PAs derived from herbal teas, recently a number of studies have performed a risk assessment for PAs in herbal teas (BfR, 2013; Chen et al., 2017; EFSA, 2016). Given that 1,2-unsaturated PAs are genotoxic and carcinogenic, this risk assessment was based on the Margin of Exposure (MOE) approach. An MOE cut-off value of 10,000 is generally applied, which incorporates factors including possible inter-species and intra-species differences in toxicokinetics and toxicodynamics, inter-individual human variability in cell cycle control and DNA repair, and the fact that the $BMDL_{10}$ (the lower confidence limit of the dose level that results in 10% extra cancer incidence above background level), is not a no effect level (EFSA, 2005). MOE values below 10,000 indicate that there might be a potential concern for human health (EFSA, 2005). The risk assessments

Abbreviations: PAs, Pyrrolizidine Alkaloids; MOE, Margin of Exposure; $BMDL_{10}$, The lower confidence limit of the benchmark dose resulting in 10% extra cancer incidence; EDI, Estimated daily intake; PFS, Plant food supplements

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suggested that long-term consumption of certain herbal teas may pose a potential risk for human health, especially when considering lifetime exposure (BfR, 2013; Chen et al., 2017; EFSA, 2016). These studies evaluated the MOE values for lifelong daily use of herbal teas based on occurrence data obtained from extraction of finely ground, comminuted leaves. Considering the fact that consumption of teas usually occurs by hot water extraction of partially intact or coarsely ground leaves, extraction of comminuted leaves may represent a worst case, and not reflect a real life scenario. This is especially of importance given that previous studies demonstrated that hot water extraction of alkenylbenzenes from comminuted fennel fruits is more efficient than that from the whole fruits (Raffo et al., 2011; van den Berg et al., 2014). It is conceivable that also for PAs use of comminuted leaves may facilitate their extraction from the teas. This may influence the actual exposure and corresponding risk assessment. Therefore, the aim of the present study was to compare the extraction of PAs from comminuted leaves and intact leaves of herbal teas, and quantify the consequences of possible differences for the MOE based risk assessment. Furthermore, when considering realistic exposure scenario's it should also be taken into account that herbal teas are frequently used for medicinal purposes, a practice being quite popular in many developing and developed countries (Fu et al., 2002; Prakash et al., 1999; Roeder, 2000; Stegelmeier et al., 1999). Thus, people might consume herbal teas as a medicine only during certain periods of for example illness. This shorter-than-lifetime use also holds true for herbal medicinal supplements, while evaluation by the MOE approach generally assumes lifelong everyday use. According to a survey of usage of herbal preparations by European adults, the majority of all respondents indicated to take these botanicals only during a specified time period or when they had a worsened condition (Garcia-Alvarez et al., 2014). In addition shorter-than-lifetime use of these herbal preparations is in line with the EMA (European Medicines Agency) who indicates for example that bitter fennel preparations should not be taken for periods exceeding two weeks (EMA, 2008). The limit of two weeks was established by EMA considering the lack of available safety data on long term exposure and the traditional short term use of such herbal products in self-medication (EMA, 2007). The German and Netherlands regulations indicate that the use of PA containing preparations should be limited to short time (defined as 6 weeks) use at dose levels of 1 μg/day (Bundesgesundheitsamt, 1992; WKB, 2001). It is thus of significance to analyse the presence of PAs in extracts of comminuted and whole leaf teas and herbal medicines and perform a risk assessment taking shorter exposure duration into consideration. In our previous study, we used the data from Bodi et al. (2014), Mulder et al. (2015) and EFSA (2016) to perform a risk assessment for herbal teas and PFS based on daily consumption of one cup of tea or 200 mg of PFS per day during a lifetime, mainly because lifetime exposure is the default assumption in the MOE approach (Chen et al., 2017). This evaluation confirmed that consumption of certain herbal teas and PFS may raise a health concern when consumed regularly during a lifespan (Chen et al., 2017). The aim of the present study was to assess the risk of exposure to PAs from herbal teas, herbal medicines and PFS using the MOE approach considering shorter and more realistic exposure scenarios, also taking the potentially lower extraction efficiency from whole herbal tea leaves versus comminuted samples into account.

2. Materials and methods

2.1. Standards and reagents

Formic acid (analytical grade) and ammonium carbonate (analytical grade) were obtained from Sigma-Aldrich, Zwijndrecht, the Netherlands). Acetonitrile (LC-MS grade) and methanol (LC-MS grade) were obtained from Actu-all, Oss, the Netherlands). Fifty-four 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, trichodesmine N-oxide were in-house synthesized by the method of (Chou et al., 2003). See Supplementary Data 1 for a full list of PA standards used in this study.

Stock solutions of the 54 available PAs were prepared in methanol (100 μg/mL). A mixed solution (1 μg/mL in methanol) containing all PA standards was prepared from the stock solutions. This mixed standard solution was used to spike the herbal teas and medicine samples as described below.

2.2. Herbal teas and PA extraction

Five types of herbal teas derived from PA-producing plants were used to investigate the difference of PAs extraction efficiency between intact leaves and comminuted leaves, including coltsfoot (*Tussilago farfara*), comfrey (*Symphytum officinale*), borage (*Borago officinalis*), climbing groundsel (*Senecio scandens*) and sunn hemp (*Crotalaria juncea*) teas. These herbal teas were selected, because they are available on the market as intact leaves. Table 1 presents an overview of the herbal teas used in the present study, their country and year of origin, and also the presumptive health effects of the respective teas as derived from the literature.

The intact leaves of each tea were randomly selected and ground to produce the comminuted leaves using a grinder (HR2056, Philips, the Netherlands). The intact leaves as well as the comminuted leaves of these teas were used for hot water extraction and subsequent PA analysis. For PA extraction, 2 g of the sample were positioned in a 250 mL glass beaker and 150 mL of boiling water was poured onto the tea. The infusion was stirred 3 times in 10 min. This procedure was selected as a worst case scenario for extraction of bioactive ingredients upon hot water extraction based on literature (Molan et al., 2009; McKAY et al., 1995; Raffo et al., 2011). Then, the infusion passed through a paper filter. The extraction was performed in triplicate.

From each of the filtered infusions four aliquots were taken and transferred to autosampler vials. Of tea 1 (coltsfoot) aliquots of 400 μL; of tea 3 (borage) aliquots of 20 μL; of tea 2 (comfrey) and tea 4 (climbing groundsel) aliquots of 100 μL and of tea 5 (sunn hemp) aliquots of 200 μL were taken. For each infusion one of the aliquots was spiked with 25 ng/mL (25 μl of 1 μg/mL PA mix) and one was spiked with 100 ng/mL (100 μL of 1 μg/mL PA mix). The total volumes were made up to 1 mL with water.

2.3. Herbal medicines and PA extraction

A total of eight herbal medicines were purchased from the Chinese market. The products were in the form of capsules, pills or tablets, four of them containing PA-producing plants and four containing non-PA-producing plants. Table 2 presents an overview of the collected herbal medicine samples and their characteristics.

For PA analysis from each sample three test portions of 1.0 g were extracted with 20 mL of 1.0% formic acid solution by agitation for 30 min. Before extraction one of the test portions was fortified with the mixed PA standard solution at 500 μg/kg (500 μL of 1 μg/mL PA mix). After centrifugation 5 mL of supernatant was transferred to a new tube and brought to pH 6–7 with 1 M ammonium carbonate solution, pH 9. The extracts were further purified by SPE over a StrataX 200 mg, 6 cc cartridge. Cartridges were conditioned with 6 mL methanol, followed by 6 mL water. After the extract was passed through the cartridge, this was washed with 6 mL 1% formic acid, followed by 6 mL water and dried under vacuum using a vacuum manifold for 10 min. PAs were eluted with 6 mL of methanol and the eluates were dried under a stream of nitrogen at 50 °C using a TurboVap. The extracts were reconstituted

Table 1
The five types of herbal teas used and their characteristics.

Herbal tea sample	Type of herbal tea (Pin Yin name)	Latin name	Health claim from studies	Reference	Origin	Year
Tea 1	Coltsfoot (Kuan Dong)	*Tussilago farfara*	Eliminates irritation and inflammation of the respiratory system and persistent coughs	Lapenna et al. (2015)	Netherlands	2015
Tea 2	Comfrey (Ju He Cao)	*Symphytum officinale*	Treatment of rheumatoid arthritis, bronchitis, various allergies and for diarrhoea	Stickel and Seitz (2000)	China	2016
Tea 3	Borage (Liu Li Ju)	*Borago officinalis*	Suppression of inflammation, treatment of diabetic neuropathy	Oomah and Mazza (1999)	Spain	2015
Tea 4	Climbing groundsel (Qian Li Guang)	*Senecio scandens*	Induces diuresis, anthelmintic	Ling (2008)	China	2016
Tea 5	Sunn hemp (Tai Yang Ma)	*Crotalaria juncea*	Antiarthritic	Ashok et al. (2006)	China	2016

Table 2
Herbal medicines used in the study and their characteristics. PA containing botanicals are printed in bold.

Herbal medicine sample	Pin Yin name	Ingredients	Recommend daily intake	Health Claim indicated on the label
M1	Fufang Sanqi jiaonang	Panax notoginseng, eupolyphaga sinensis, chuanxiong, angelica, safflower, frankincense, myrrh, angelica	250 mg per capsule 6 capsules each time, 2 times per day (total: 3000 mg)	Improves blood circulation, detoxification
M2	Sanqi Pian	Panax notoginseng	600 mg per tablet 6 tablets each time, 3 times per day (total: 10,800 mg)	Improves blood circulation, reduces swelling and pain
M3	Sanxi Shangyao Pian	Panax notoginseng, kusnezoff monkshood root, shortstalk monkshood root, borneol, drynaria, safflower, elderberry, red peony	500 mg per tablet 3 tablet each time, 3 times per day (total: 4500 mg)	Relaxes the muscles, stimulates the blood circulation
M4	Feire Keli	Ephedra, bitter almond, gypsum, licorice, honeysuckle, forsythia, anemarrhena, scutellaria, indigowoad root, ophiopogon japonicus, houttuynia cordata	4 g per bag 2 bags each time, 3 times per day (total: 24,000 mg)	Heat-clearing, dispels cold
M5	Runfei Zhisou Wan	Asparagus, rehmannia, snakegourd root, pericarpium trichosanthis, perilla seed (fried), honey-made mulberry bark, bitter almond, aster tataricus, **coltsfoot**, campanulaceae, fritillaria, vinegar schisandra, radix peucedani, tangerine peel	6 g per pill 2 pills each time, 2 times per day (total: 24,000 mg)	Moistens the lung, reduces phlegm
M6	Qianbai Biyan Pian	**Senecio scandens,** selaginella, notopterygium, cassia, ephedra, chuanxiong, angelica	440 mg per tablet 4 tablets each time, 3 times per day (total: 5280 mg)	Heat-clearing and detoxification, activates blood circulation
M7	Qianxi Pian	Andrographis paniculata, **Senecio scandens**	310 mg per tablet 3 tablets each time, 4 times per day (total: 3720 mg)	Heat-clearing and detoxification
M8	Juhong Keli	Fructus trichosanthis, poria, licorice, campanulaceae, bitter almond, perilla seed (fried), aster tataricus, **coltsfoot**, pericarpium trichosanthis, fritillaria, rehmannia, ophiopogon, gypsum	11 g per bag 2 times per day (total: 22,000 mg)	Lung heat-clearing

in 500 µL of 10% methanol and filtered using 0.45 µm PTFE filtervials (UniPrep, Whatman, Maidstone, UK).

Medicines 5 and 8 contained senkirkine at concentrations exceeding the spiked concentration of 500 µg/kg. Senkirkine was quantified in these samples by spiking aliquots (1 mL) of the herbal extracts with senkirkine at 50 µg/mL (5 µL of a 10 µg/mL solution of senkirkine in methanol and at 250 ng/mL (25 µL of a 10 µg/mL solution of senkirkine). The fortifications correspond to 1000 and 5000 µg/kg in the herbal medicine, respectively.

2.4. LOD, LOQ, recovery, and precision data

In-house validated methods for 54 PAs in herbal tea infusions and in PFS were used. For PAs in herbal infusions the LOQs obtained were at 0.05 µg/L and in herbal supplements at 4–5 µg/kg. LODs were estimated at 0.01–0.02 µg/L in herbal infusions and at 1–2 µg/kg in PFS. For PFS recoveries (level: 100 µg/kg) varied from 73 to 107%. Repeatability (n = 5) ranged between 8.1 and 24% at 10 µg/kg, between 3.1 and 8.5% at 100 µg/kg and between 3.1 and 10.9% at 250 µg/kg. Linearity of the LC-MS/MS system was checked by analysis of 8-point calibration curves prepared in blank tea extract and in blank PFS extract over the range of 0–250 ng/mL.

2.5. LC-MS/MS analysis

Analysis of PAs was performed 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). At least two MRM transitions were measured per analyte. Besides the 54 PAs for which an analytical standard was available, the samples were screened for another 35 1,2-unsaturated PAs for which no standards were available. These PAs could be included in the analytical method because mass spectrometric data were available from the analysis of extracts of authentic *Borago*, *Symphytum*, *Crotalaria*, *Senecio*, *Petasites* and *Tussilago* plant samples. See Supplementary Data 1 for an overview of the MS/MS transitions used for the complete set of PAs. Chromatographic separation was obtained on a 150 × 2.1 mm, 1.7 µm particle size, UPLC BEH C18 analytical column (Waters, Milford, MA, USA). Eluent A consisted of water containing 10 mM ammonium carbonate pH 9 and acetonitrile was used as eluent B. 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, 12.1–15.0 min 100% A/0% B. The column was kept at 50 °C and a flow rate of 400 µL/min was applied; 2 µL sample extract was injected. For some PA isomers, e.g. lycopsamine and intermedine and their N-oxide and 7-acetyl analogues no or only partial separation could be obtained under the chromatographic conditions used. For verification of the identity of the isomers, tea samples 2 (Comfrey) and 3 (Borage) were reanalysed using acidic chromatography, which allows the separation of lycopsamine and intermedine isomers (Bodi et al., 2014). Comfrey tea contained intermedine and lycosamine isomers in equal amounts, while borage tea contained only the lycopsamine isomer (data not shown). Representative LC-MS/MS MRM chromatograms of the herbal tea and medicine samples that contained PAs can be found in Supplementary Fig. 1.

2.6. Quantification of PAs in herbal tea infusions and in PFS

Quantification of the PAs in the herbal infusions was based on one-point standard addition of PAs to the infusion. Depending on the concentration found in the extract, either the standard addition at 25 ng/mL was used or the one at 100 ng/mL.

The PAs in the herbal medicines were quantified based on one-point standard addition of 500 µg/kg to the herbal product. Medicines 5 and 8 contained senkirkine at higher concentrations and these were quantified by spiking the herbal extracts with senkirkine at 50 and 250 ng/mL (corresponding to 1000 and 5000 µg/kg in the herbal medicine, respectively).

PAs for which no PA reference standard was available were semi-quantified by taking a structurally related PA reference standard, as indicated in the Supplementary Data 1.

2.7. Estimation of daily intakes of PAs resulting from the consumption of herbal teas and herbal medicines

To perform a risk assessment, the daily PA intake resulting from use of the herbal teas was estimated assuming daily consumption of the amount of PAs extracted by hot water extraction from 2 g tea, corresponding to one cup of tea, as described before (BfR, 2013). For estimation of the PA exposure resulting from herbal medicines, the concentration of the PAs quantified in the extracts was multiplied by the daily use of the herbal medicines as recommended by the supplier (Table 2). The estimated daily intake (EDI) values were calculated using a default body weight of 70 kg for an adult as proposed by EFSA (EFSA, 2012).

$$EDI = \frac{total\ PA\ concentration * daily\ use}{bw\ (70\ kg)}$$

2.8. Calculation of the Margin of Exposure (MOE)

Risk assessment was performed using the MOE approach. EFSA has recently set a reference point of 237 µg/kg bw/day for riddelliine as the point of departure (PoD) to assess the carcinogenicity risk of PAs (EFSA, 2017), based on an updated benchmark dose (BMD) modelling approach. This was done because it was considered that the $BMDL_{10}$ of 70 µg/kg/day obtained previously for lasiocarpine (EFSA, 2011) was affected by a high degree of uncertainty. In this study the MOE values were calculated by dividing the $BMDL_{10}$ of 237 µg/kg bw/day for riddelliine by the EDIs.

2.9. Real life exposure scenario

MOE values for the chronic lifetime exposure to herbal teas and herbal medicines were calculated based on daily exposure during a lifetime. We applied Haber's rule to correct the EDI values for this short term exposure (Doull and Rozman, 2000). According to Haber's rule, the toxic effect varies linearly with the time of exposure and the concentration or dose (i.e. C × T = k, where C is concentration or dose, T is time of exposure, and k is a constant toxic response for the specific substance) (Doull and Rozman, 2000). Using Haber's rule and a lifetime expectancy of 75 years (Felter et al., 2011; van den Berg et al., 2014), the EDI values for two weeks yearly exposure during a lifetime will be 52 weeks per year/2 weeks = 26 times lower than for daily lifelong exposure. EDI values for 6 weeks exposure a year, defined by the German and Netherlands regulations as short term exposure, would be 8.67 times lower. Another consideration to take into account when considering real life exposure scenario's for the use of herbal teas, is that these teas may not be ground before making the hot water extract. Using comminuted leaves is expected to facilitate diffusion of PAs into the hot water, and thus may result in a higher concentration of PAs in tea infusion then when using intact leaves. This will further influence the EDI and the MOE values. In the present study, five types of herbal teas were employed to compare the PA levels between comminuted leaves and intact leaves, as well as their resulting MOE values.

The possibility was considered that herbal teas or herbal medicines may be used for longer periods than 2 weeks or 6 weeks a year during a lifetime. For those herbal products, assuming a 75-year lifetime, the maximum number of weeks was calculated during which the product could be consumed to result in an MOE value of 10,000 given a $BMDL_{10}$ of 237 µg/kg bw per day:

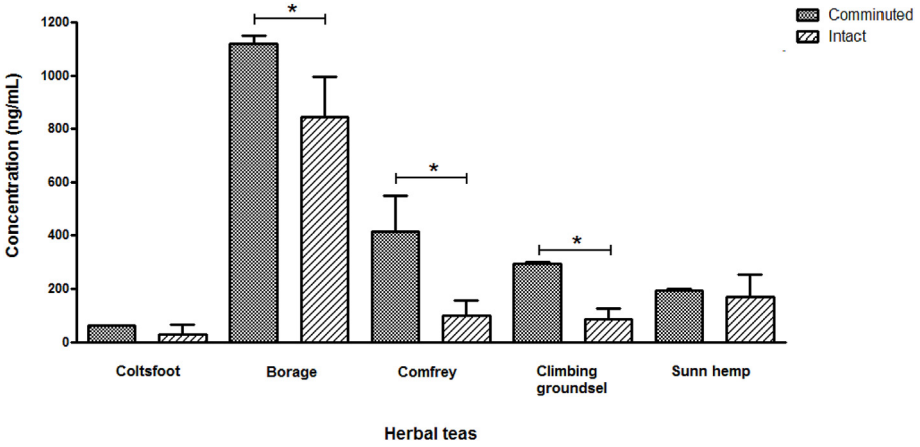


Fig. 1. The total PA concentrations of five herbal teas extracted either from intact leaves (bars filled with black dots) or from the corresponding comminuted leaves (bars filled with black lines). Average of three extractions. Data are expressed as mean ± SD and analysed using *t*-test. *p < 0.05.

$$EDI = \frac{\text{BMDL10}}{10{,}000} = \frac{total\ PA\ concentration * daily\ use}{bw\ (70\ kg)} : \left(\frac{75\ years * 52\ weeks}{n\ weeks}\right)$$

This calculation was also applied for the 34 types of herbal teas and 19 PFS, based on the data that have been previously reported by Bodi et al. (2014), EFSA (2016) and Mulder et al. (2015). In all calculations it has been assumed that the concentrations reported are representative for the specific tea or PFS and that exposure to PAs is only due to that tea or PFS.

3. Results

3.1. PA concentrations in herbal teas and the effect of hot water extraction of comminuted and intact leaves

Five types of herbal teas were used to compare the total amount of PAs that were extracted either from the intact or the comminuted leaves. The amounts of PAs extracted from the intact leaves of each tea were consistently lower compared to the levels extracted from the comminuted leaves (Fig. 1). The total PA concentrations varied from 30.7 to 845.4 μg/L for the intact leaves and from 61.3 to 1120 μg/L for the comminuted leaves. Overall, the PA levels extracted from intact leaves were 1.1- to 4.1-fold lower than from the corresponding comminuted leaves. The PA levels were significantly different between intact and comminuted leaves of borage, comfrey and climbing groundsel teas. The highest PA concentration was found in borage tea and lycopsamine N-oxide was the PA found in the highest concentration. In general, the same PAs and also similar profiles were found in intact and comminuted leaves of the teas (Table 3 and Supplementary Data 2). The tested herbal teas contained between 3 and 11 different PAs (Table 3 and Supplementary Data 2), the lowest number of PAs was detected in coltsfoot tea, the highest number in comfrey and sunn hemp tea. Interestingly, in sunn hemp tea trichodesmine N-oxide was the most abundant PA extracted from the intact leaves, whereas monocrotaline N-oxide was highest when extracted from comminuted leaves. This suggests that the size of the leaves may also have an impact on the relative extraction efficiency of PAs in the leaves.

We were interested whether the PAs present in the teas could be correlated with the botanical plant name listed on the label. Coltsfoot (*T. farfara*) is known to contain senkirkine as the dominant PA and varying but smaller amounts of senecionine (Roeder, 1995). In our sample senkirkine was indeed present but senecionine was not detected. Borago (*B. officinalis*) typically contains the monoesters lycopsamine and intermedine as well as their 7-acetyl derivatives (El-Shazly and Wink, 2014; Roeder, 1995). In the sample tested only lycopsamine and its 7-acetyl derivative were found. Comfrey (S. *officinale*) can contain a range of mono and diester compounds, including lycopsamine, intermedine, echinatine, echimidine, and heliosupine (El-Shazly and Wink, 2014). Lycopsamine, intermedine, echimidine and heliosupine were present in the tested sample, as well as leptanthine (a hydroxy analogue of lycopsamine) and an acetyl derivative of echimidine. Climbing groundsel (*Senecio scandens*) is reported to contain senecionine, seneciphylline (Roeder, 2000), although in another report adonifoline was identified as the main PA (Xiong et al., 2012). None of these PAs were detected in the tested sample. Based on the PA profile present in the extracts primarily senkirkine and lower levels of petasitenine and dehydrosenkirkine it is more likely to be a *Petasitis* (butterbur) species, e.g. *P. japonicus* (Hartmann and Witte, 1995). Sunn hemp (*C. juncea*) reportedly can contain trichodesmine, junceine, senecionine, integerrimine and seneciphylline (Roeder and Wiedenfeld, 2013). In the tested sample trichodesmine and integerrimine were indeed found, but senecionine, seneciphylline and junceine were not. Instead, monocrotaline and low levels of incanine and fulvine were detected. It is therefore possible that another or a mixture of *Crotalaria* species was used (Roeder and Wiedenfeld, 2013).

In several preparations trace levels PAs were found that could not be directly attributed to the botanical species reported on the label. These PAs can come from impurities present in the herbal teas, due to co-harvesting or processing of unrelated PA-containing plants.

3.2. Risk assessment for the herbal teas based on lifetime and shorter duration exposure

The MOE values based on the total PA levels that were extracted from either comminuted leaves or intact leaves of the five types of herbal teas assuming daily use during a whole lifetime are depicted in Fig. 2A. It is assumed that the PA concentration is representative for the specific tea. For the comminuted leaves, the MOE values ranged from 100 to 1,800, and from 130 to 3600 for the intact leaves. The MOE values were, regardless of the state and size of the leaves, all below 10,000 for these five types of herbal teas, the lowest MOE value was found for the borage tea.

Fig. 2B shows the MOE values for herbal teas in the form of comminuted leaves and intact leaves assuming consumption for two or six weeks/year during a lifetime. In particular consumption of borage tea still resulted in MOE values below 10,000, independent of the form of the leaves. Short term two weeks exposure to the extracts of comminuted leaves of comfrey and climbing groundsel teas resulted in MOE values just below 10,000, but for the intact leaves above 10,000. For coltsfoot and sunn hemp teas MOE values above 10,000 were obtained,

Table 3
Total number of PAs, total PA concentration and the top three PAs in the tested herbal teas. See Supplementary Data 2 for a complete data overview.

Herbal tea	Form	Number of PAs > LOD	Total PA concentration (μg/L)	EDI (μg/kg bw/day)	Top three PAs and their concentration (μg/L)
Coltsfoot	Comminuted	5	61.3	0.1	Senkirkine (58.2); Neosenkirkine (2.6); Echinatine N-oxide (0.27)
	Intact	3	30.7	0.07	Senkirkine (29.7); Neosenkirkine (0.94); Echinatine N-oxide (0.02)
Borage	Comminuted	4	1120	2.4	Lycopsamine N-oxide (889.3); Lycopsamine (209.5); 7-Acetyllycopsamine N-oxide (15.7)
	Intact	4	845.4	1.8	Lycopsamine N-oxide (679.7); Lycopsamine (149.2); 7-Acetyllycopsamine N-oxide (13.0)
Comfrey	Comminuted	10	415.0	0.9	Echimidine N-oxide (321.5); Echimidine (29.1); Leptanthine (22.7)
	Intact	11	101.1	0.2	Echimidine N-oxide (75.9); Echimidine (16.2); Leptanthine N-oxide (4.0)
Climbing groundsel	Comminuted	7	293.1	0.6	Senkirkine (248.2); Dehydrosenkirkine (22.8); Petasitenine (19.7)
	Intact	6	85.2	0.2	Senkirkine (74.3); Petasitenine (5.0); Dehydrosenkirkine (4.6)
Sunn hemp	Comminuted	11	192.8	0.4	Monocrotaline N-oxide (82.0); Monocrotaline (60.1); Integerrimine N-oxide (29.2)
	Intact	9	170.0	0.4	Trichodesmine N-oxide (61.8); Monocrotaline N-oxide (50.0); Integerrimine N-oxide (18.7)

irrespective of the form of the leaves. Assuming six weeks exposure/year during a lifetime, to mimic the definition of short term exposure in existing regulations, reduces the MOE values 3-fold resulting in a value < 10,000 for borage, comfrey and sunn hemp teas, independent of the form of the leaves. These results show that the size of the leaves and the duration of the short term exposure may influence the corresponding risk assessment.

3.3. PA concentrations in herbal medicines

Table 4 and Supplementary data 3 show the total PA concentrations found in eight herbal medicines, including four products (M5-8) that contain PA-producing plants such as coltsfoot or climbing groundsel as one of the ingredients. The other four herbal medicines (M1-4) were supposed to contain no PA-producing plants. In M1 and M2 the total PA levels were indeed below LOQ. A small amount of senkirkine (4.2 μg/kg) was found in M3 and traces of lycopsamine/intemedine (4.0 μg/kg) were detected in M4. In the medicines that contain a PA-producing plant ingredient, the measured total PA concentration ranged from 404 to 7883 μg/kg. Medicine 6 contained the highest total PA concentration as well as the highest number of different PAs. In this sample adonifoline was the dominant PA, accounting for approximately 98% of the total PA concentration. M5 and M8 contained coltsfoot (*T. farfara*) as one of the ingredients and in accordance with this, senkirkine, its isomer neosenkirkine, senecionine and its isomer integerrimine, were found as the main PAs (Roeder, 1995, 2000). The results for M6 and M7, both containing climbing groundsel (*S. scandens*) as an ingredient, were more diverse. The total PA concentration in M7 was only 5% of that of M6, what could be due to different inclusion levels of climbing groundsel in the products. However, also the PA profile in both medicines was quite different. M6 contained, besides the high concentration of adonifoline mentioned above, also lower levels of senecionine, seneciphylline and the monoesters lycopsamine, intermedine, echinatine and rinderine. The first three compounds have been reported for climbing groundsel (Roeder, 2000; Xiong et al., 2012). The monoesters are probably due to a contamination of the product (or an undeclared ingredient) with an *Eupatorium* or Boraginaceae species. The same monoesters were also found in M7, indicating a similar contamination, but for the rest this sample contained only senkirkine and lower levels of adonifoline.

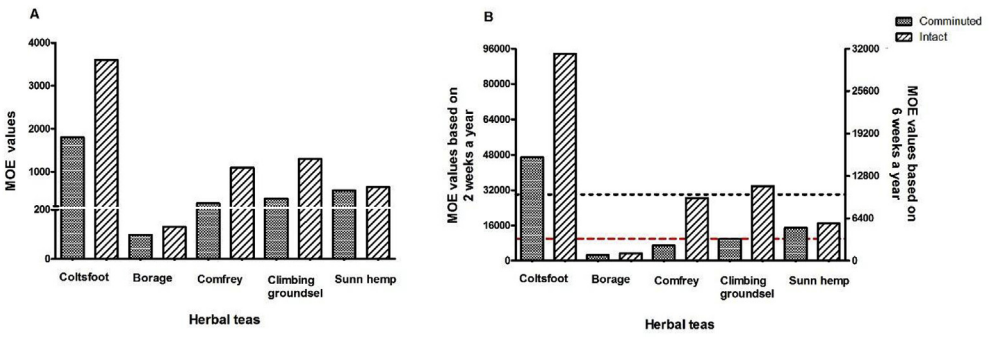


Fig. 2. The MOE values of five types of herbal teas obtained when assuming daily consumption of one cup of tea per day for a lifetime (A) and for 2 weeks (left Y axis) and 6 weeks (right Y axis) every year during a lifetime (B) using the total PA levels extracted from either comminuted leaves (bars filled with black dots) or intact leaves (bars filled with black lines). The red dashed line (———) and black dotted line (·····) represent the MOE values of 10,000 for 2 weeks and 6 weeks a year, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 4
Total number of PAs, total PA concentration and the top three PAs in the tested herbal medicines. See Supplementary Data 3 for a complete data overview.

Herbal medicine	Number of PAs detected	Total PA concentration (μg/kg)	EDI (μg/kg bw/day)	Top three PAs and their concentration (μg/kg)
M1	0	< LOQ	-	-
M2	0	< LOQ	-	-
M3	1	4.2	0.0003	Senkirkine (4.2)
M4	1	4.0	0.001	Lycopsamine/intermedine (4.0)
M5	6	6344	2.18	Senkirkine (5369); Neosenkirkine (868.9); Senecionine (79.7)
M6	8	7883	0.59	Adonifoline (7734); Seneciphylline (67.4); Senecionine (39.9)
M7	7	403.9	0.02	Senkirkine (292.1); Adonifoline (49.7); Echinatine (41.4)
M8	4	1431	0.45	Senkirkine (1215); Neosenkirkine (199.2); Senecionine (10.8)

-: Cannot be calculated because PA levels were < LOQ.

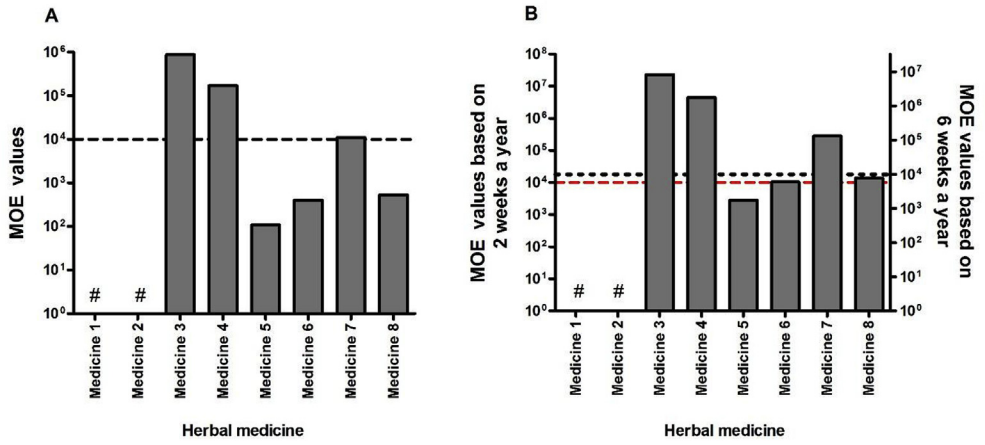


Fig. 3. The MOE values of eight different types of herbal medicines assuming lifelong daily consumption (A), and for 2 weeks (left Y axis) and 6 weeks (right Y axis) every year during a lifetime (B). The red dashed line (———) and black dotted line (·····) represents the MOE values of 10,000 for 2 weeks and 6 weeks a year, respectively. # implies PA content < LOQ, no MOE values obtained. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. Risk assessment for the tested herbal medicines based on chronic exposure and short term exposure scenarios

The MOE values for the eight types of medicines were evaluated according to three exposure scenario's including consumption at the recommended daily intake of that medicine daily throughout the whole lifespan (Fig. 3A), or shorter-than-lifetime during two or six weeks/year for 75 years, assuming a representative PA concentration and assuming exclusive exposure (Fig. 3B). Since M3 and M4 each only contained one PA at low concentrations, use of these two herbal medicines resulted in MOE values far above 10,000 irrespective of the duration of the exposure. However, of the four PA-producing plant containing herbal medicines, three samples resulted in low MOE values of between 110 and 530 when assuming lifelong daily consumption. For medicine 5 even short term consumption of two weeks/year resulted in an MOE value of 2800, well below 10,000, indicating that this medicine may pose a potential risk for human health. Considering shorter-than-lifetime exposure for 6 weeks/year resulted in MOE values < 10,000 for three medicines containing PA plant material.

3.5. Risk assessment for the herbal teas, herbal medicine and previously analysed teas and PFS based on shorter-than-lifetime use

In addition to the herbal teas and herbal medicines analysed in the present study also a risk assessment for shorter-than-lifetime exposure was made for the 34 types of (herbal) teas and 19 PFS for which PA levels were previously reported by Bodi et al. (2014), EFSA (2016) and Mulder et al. (2015). Given that the number of weeks a year selected for this shorter-than-life time exposure influences the MOE values and final conclusion, in this analysis the number of weeks during a lifetime that would result in an MOE of 10,000 was evaluated. The five types of herbal teas and eight types of herbal medicines analysed in the present study were also included in this evaluation.

Fig. 4 presents the maximum number of weeks during a 75-year lifetime that a herbal tea from the present study (Fig. 4A), a herbal medicine from the present study (Fig. 4B), a herbal tea analysed in previous studies (Fig. 4C) or a PFS sample analysed before (Fig. 4D) could be consumed to result in an MOE value of 10,000. From these data it follows for example that consumption of coltsfoot tea prepared from comminuted leaves, containing the PA concentration as determined in this study, for up to 700 weeks during a lifetime (corresponding to 9.3 weeks/year during 75 years) would be of little concern, whereas use of intact leaves to prepare the tea would increase this to 1400 weeks during a lifetime (18 weeks/year) (Fig. 4A). Similarly, use of intact borage, comfrey, climbing groundsel and sunn hemp tea leaves would raise no concern for, respectively, 50, 425, 500 and 250 weeks during a life time (corresponding to 0.6, 5.6, 6.7 and 3.3 weeks/year, respectively). However, use of comminuted borage, comfrey, climbing groundsel and sunn hemp tea, would result in an acceptable exposure for, respectively, 40, 100, 150 and 225 weeks during a lifetime (corresponding to 0.5, 1.3, 2.0 and 3.0 weeks/year) (Fig. 4A).

Consumption of herbal medicines that contain a PA-producing plant as ingredient, would not raise a concern for medicines 5, 6 and 8 when consumption is less than approximately 40, 150 and 200 weeks during a lifetime (0.5, 2.0 and 2.7 weeks/year). The other herbal medicines could be consumed on a daily basis, provided the medicine is the only source of PA exposure (Fig. 4B).

For the herbal teas for which PA levels were reported by Bodi et al. (2014), EFSA (2016) and Mulder et al. (2015), 29 out of in total of 34 types of herbal teas displayed a maximum number of weeks exceeding a lifetime of 3900 weeks, assuming a daily consumption of one cup of tea (Fig. 4C). Of the regular (herbal) tea types, only rooibos tea appeared to be contaminated with PA levels that would require them to be consumed shorter-than-life-time, for not more than 1750 weeks (23.3 weeks a year) to be of low concern. Regarding the 5 types of herbal teas, derived from PA-producing plants, the acceptable exposures ranged from 110 weeks (1.5 weeks a year) for borage tea to more than lifetime consumption for Eupatorium tea (Fig. 4C). The exposures calculated for

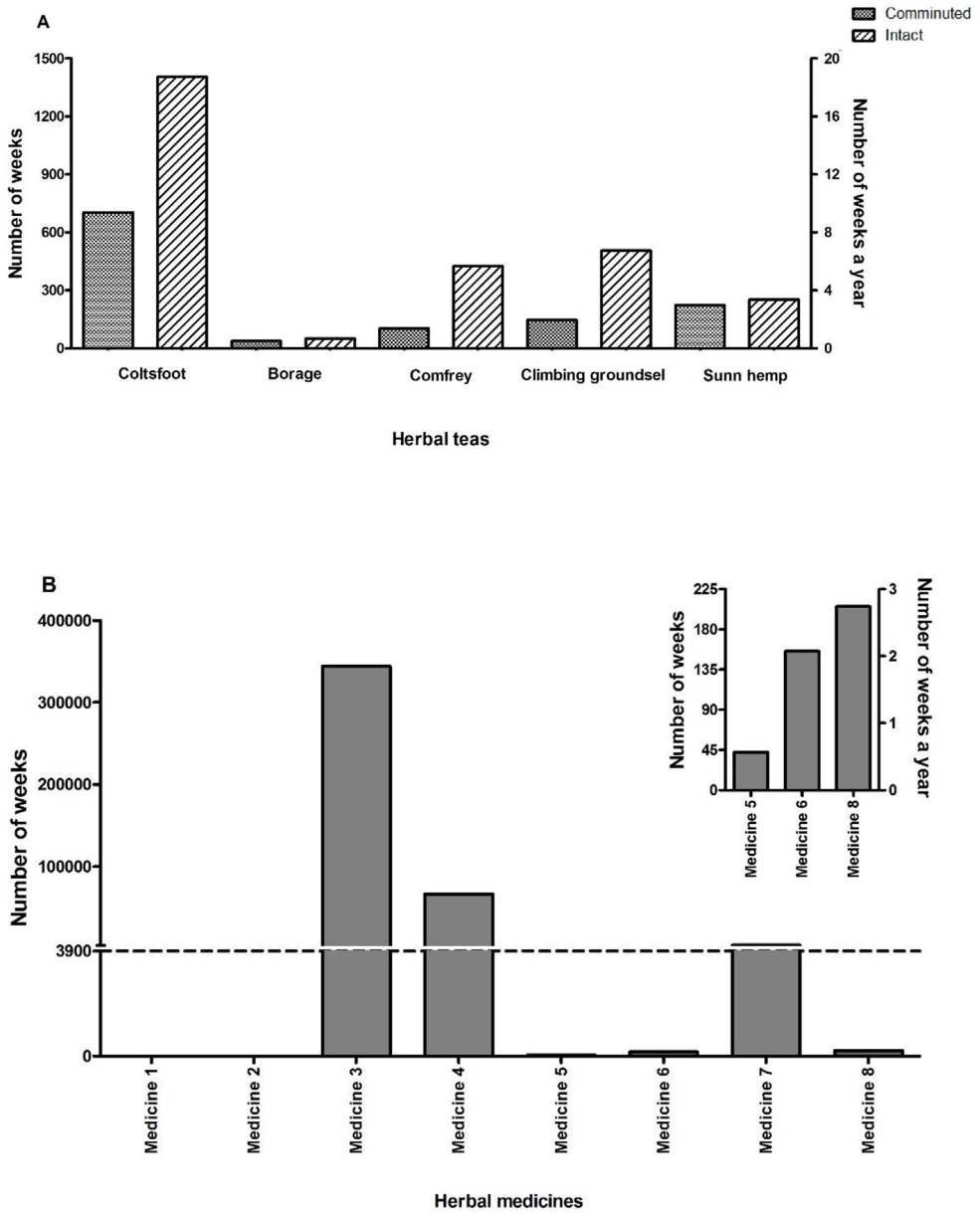


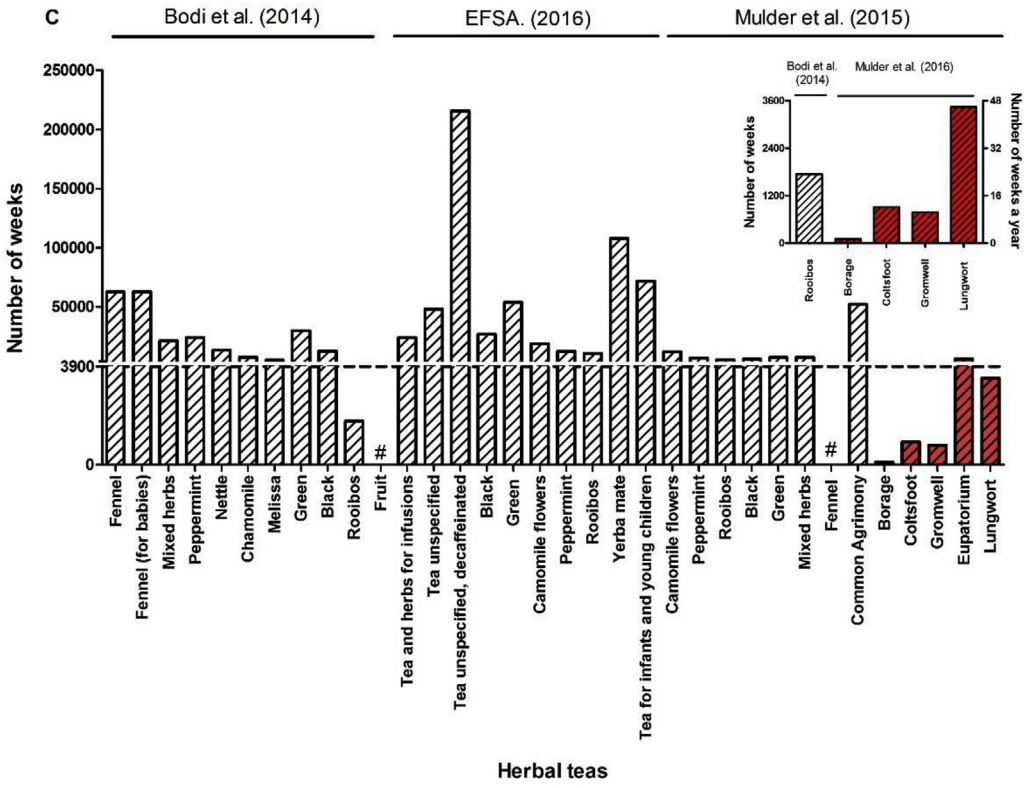
Fig. 4. The maximum number of weeks during a 75-year lifetime that a tea analysed in the present study (A), a medicine analysed in present study (B), a type of tea reported in the literature (C) or a type of PFS reported in the literature (D) could be consumed to result in an MOE of 10,000, assuming consumption of one cup of tea (150 mL) or 200 mg PFS. Bars filled with black dots represent the herbal teas prepared from comminuted leaves, bars filled with black slanted lines represent the herbal teas from intact leaves. The red bars represent the samples derived from PA-producing plants. The black dashed line represents daily intake during a 75 year lifetime (equal to 3900 weeks). # indicates that data are not quantifiable due to a PA content < LOQ. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

borage tea and coltsfoot tea (900 weeks or 12 weeks/year) were in the same range as calculated for the samples of the same type in this study.

With respect to the PFS, 17 of 19 PFS showed that a daily use of 200 mg during a lifetime would not raise a concern, and only for two samples, a plant extract formula reported by EFSA (2016) and a plant extract formula (PA-plant) reported by Mulder et al. (2015), the consumption should be substantially shorter than lifetime, 690 weeks (9.2 weeks/year), and 160 weeks (2.1 week/year), respectively (Fig. 4D).

4. Discussion

In the present study, we investigated the extraction of PAs in hot water from intact leaves and the corresponding finely ground leaves of five herbal PA containing teas and also examined the presence of PAs in eight samples of commercially available herbal medicines. For preparation of the infusion, the tea sample was steeped in the water for 10 min and the infusion was stirred 3 times. This procedure was selected as a worst case scenario for extraction of bioactive ingredients upon hot water extraction. For instance, Molan et al. (2009) investigated the effects of infusion time and stirring on the total phenolic levels extracted from green teas. The results showed that the total phenolic contents increased from 52.76 ± 2.19 to 102.83 ± 2.33 mg/g (95%) by increasing the infusion time of 3 min–10 min at 100 °C ($P < 0.0001$). The extraction was not further improved upon increasing the infusion time from 10 min to 30 min, at which time point the total phenolic levels was 106.8 ± 3.29 mg/g. In addition, infusion with stirring resulted in an increased total phenolic level of



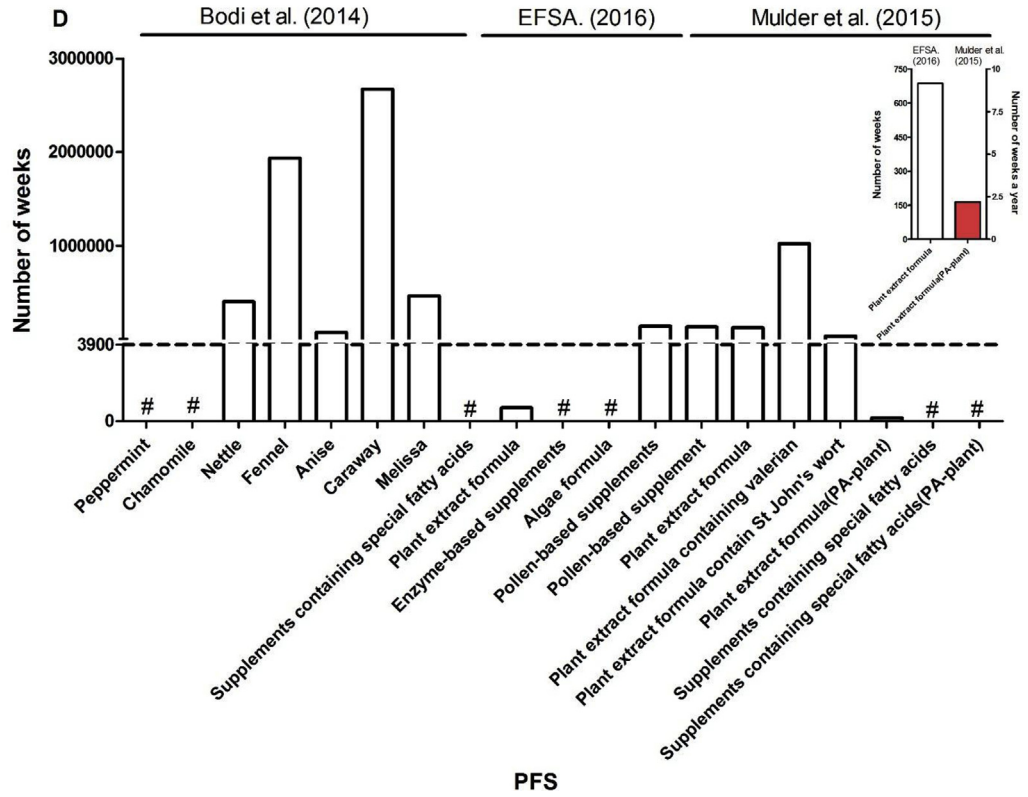


Fig. 4. (*continued*)

96.12 ± 2.67 mg/g, which is increased by 37% compared to a total phenolic level of 70.39 ± 0.27 mg/g resulted from infusion without stirring (P ≤ 0.0110–0.0001) (Molan et al., 2009). Similarly, McKAY et al. (1995) found that stirring during tea infusion resulted in a marked increase on oxalate concentrations in black teas (McKAY et al., 1995). EFSA also pointed out that the infusion time and stirring may have an influence on the extraction of PAs during consumer preparation (EFSA, 2016). Raffo et al. (2011) suggested that stirring 3 times could reflect best the scenario of preparing tea infusions by the consumer in general (Raffo et al., 2011).

In order to minimize the risk to miss relevant PAs, the samples were analysed for a comprehensive set of 89 1,2-unsaturated PAs. The previous studies on exposure and risk assessment for herbal teas and supplements were based on a set of 28 PAs (Bodi et al. (2014); EFSA (2016); Mulder et al. (2015)). The herbal teas and medicines investigated in this study, contained several PAs, that had not been included in previous studies, such as adonifoline, trichodesmine N-oxide, petasitenine, isomers of senkirkine and isomers of intermedine and lycopsamine. Herbal medicine M6 contained adonifoline in substantial amounts. When analysed with the set of 28 PAs, for this sample a relatively low amount of 131 μg/kg would have been found, instead of 7883 μg/kg with this method (Supplementary Data 3). In the other PA-containing medicines, the set of 28 PAs accounted for 74%–86% of the total content found using the comprehensive set. With respect to the five herbal teas, the difference in total PA concentration was relatively small, except for sunn hemp tea. For this tea the 28 PAs accounted for 42% (intact leaves) to 75% (comminuted leaves), while for the other preparations it ranged from 85% to 98% (Supplementary Data 2). It may be concluded that the set of 28 PAs is not always sufficient to get a reliable impression of the PAs present in these herbal teas and medicines.

The herbal teas selected in this study are available for the consumer in the form of intact leaves. Comparing total PA levels in hot water extracts of the intact and comminuted leaves from five PA-plant containing herbal teas, we found that, overall, the PA concentrations extracted from the comminuted leaves were 1.1–4.1 times higher compared to concentrations extracted from the intact leaves. It is of interest that, not only higher levels of PAs were extracted from the comminuted leaves but sometimes also different PAs were found in the hot water extracts from the comminuted leaves. This indicates that PA extraction efficiency can be influenced by the particle size of the botanical sample. In line with this, previous studies have demonstrated that using comminuted fruits increased the extraction efficiency of alkenylbenzenes as compared to the whole fruits (Raffo et al., 2011; van den Berg et al., 2014). The results of the current study suggest that the use of comminuted tea materials to prepare hot water extracts, as routinely done in studies on PAs in herbal teas (Bodi et al., 2014; EFSA, 2016; Mulder et al., 2015) may overestimate the levels and thus, also the corresponding exposure and risk. It was noted that hot water extraction from intact leaves resulted in a larger variation in the PA levels than observed for comminuted leaves. This may be due to the fact that PA levels can vary between the individual intact leaves, which are likely to originate from different plants. The leaves may also originate from different batches of teas, from different locations, which were mixed during processing and packaging. Therefore, it is conceivable that the PA levels of intact leaves derived from a single bag of tea are variable. Grinding the leaves and homogenizing the ground product will strongly reduce differences in the samples.

It is of interest to note that the form of the leaves also may have an effect on the type of PAs extracted. For example trichodesmine N-oxide was the PA present at the highest concentrations in sunn hemp teas extracted from the intact leaves, whereas monocrotaline N-oxide was the most abundant PA in the extract from the comminuted leaves. In hot water extracts from intact and comminuted leaves the amount of PA N-oxides dominated over that of PA free bases, which is in line with other studies However, in the herbal medicines the PAs were present solely in the free-base form. In line with this, Griffin et al. (2014) reported that only 6 samples out of a total of 54 herbal medicines contained PA N-oxides (Griffin et al., 2014).

Using the total PA levels, we performed a risk assessment for these herbal products taking the use of intact tea leaves as well as shorter-than-lifetime exposure scenario's into account. In addition, we analysed the consequences of shorter-than-lifetime use for the risk assessment of herbal teas and PFS reported by Bodi et al. (2014), EFSA (2016), and Mulder et al. (2015).

We previously found that whole lifetime exposure to PA-plant containing herbal teas, including borage and coltsfoot teas with consumption of one cup of those teas per day would result in the MOE values below 10,000 (Chen et al., 2017). However, results of the present study indicate that when considering shorter-than-lifetime exposure the outcome of the risk assessment may be different. When use of the herbal teas analysed in the present study was assumed to be limited to 2 weeks a year during a lifetime, coltsfoot tea was shown to result in an MOE value higher than 10,000. Considering use during 2 (or 6) weeks a year for a lifetime increased the MOE values 26- (or 8.67-) fold, while using the $BMDL_{10}$ for riddelliine of 237 μg/kg bw per day instead of that for lasiocarpine of 70 μg/kg bw per day in our previous study, additionally increased the MOE values by a factor of 3.4 (EFSA, 2017).

We also estimated the number of weeks one could consume the different types of teas and PFS that have been analysed before by Bodi et al. (2014), EFSA (2016) and Mulder et al. (2015) during a 75-year lifetime. The results showed that the regular consumption of herbal teas derived from PA-producing plants except for eupatorium tea would not raise a concern when consumed for periods varying from 40 to 3450 weeks during a lifetime, which is equivalent to 0.5–46 weeks/year for 75 years. Obviously, the outcome strongly depends on the average PA content in these teas and the amount of tea consumed. Similarly, the weeks of regular consumption of PFS that would not raise a concern varied from 160 to 690 weeks during a life time, equivalent to 2.1–9.2 weeks a year during 75 years.

It is of interest to note that the values from 2.1 to 9.2 weeks a year obtained for safe consumption of PA containing PFS, are all covered by the limit of two weeks established by EMA for the short term use of such herbal products in self-medication (EMA, 2007), but not fully covered by the 6 weeks defined as short term exposure in the German and Netherlands regulation (Bundesgesundheitsamt, 1992; WKB, 2001). This regulation however also defines a maximum daily use of 1 μg/day. This means that a yearly 6-weeks intake of 1 μg/day by a 70 kg person would result in an MOE of about 144,000, and thus not raise a concern. In fact, an intake of 1 μg/day during a lifetime by a 70 kg person would result in an MOE of 16,600, which is still a sufficient safety margin. It is of interest to note that the PA content in the herbal products evaluated in the present study can result in intakes far beyond the levels specified in regulations for PAs in botanicals and botanical preparations. As outlined above this includes the regulations in Germany and the Netherlands, where the government has established maximum limits for daily intake of 1 μg/day (for short term use up to 6 weeks) and 0.1 μg/day (for long term use) and a maximum PA content for herbal supplements of 1 μg/kg or 1 μg/L (Bundesgesundheitsamt, 1992; WKB, 2001). In Austria and Belgium, this regulation is even more strict, with a “zero tolerance” approach towards PAs, (Bundesgesetzblatt, 1994; Koninklijk besluit, 1997). So far there are no maximum limits established in the EU for daily PA intake (other than what is specified for specific medicinal herbal products). The EU is currently considering maximum limits (ML) for PAs in teas and supplements in general. These maximum limits will likely be based on the evaluation made by EFSA regarding the occurrence of PAs in herbal teas and PFS, and the consumption of these products by different age and population groups (EFSA, 2016; EFSA, 2017). Depending on the level of safety that is pursued and the evaluation on what is reasonably achievable in lowering the contamination levels in these products, it is not unlikely that

the maximum limits will be set in the range of 100–500 μg/kg, with the possibility that different MLs may be set for different (types of) products. On the other hand, in other countries such as China, there are no specific regulations for the maximum limits of daily PA intake (CP Commission, 2010). This situation may raise a possible concern for human health especially when these preparations would be used for longer periods of time.

In the current study we focused primarily on the individual MOEs for a set of herbal teas and medicines, assuming that there is no additional exposure to PAs from any other source. One could argue that our approach for herbal teas and PFS could be prone to underestimate the risks in terms of the amounts of PAs consumed, since individuals may drink different types of herbal teas at the same time with addition of honey, a product which contains PAs (EFSA, 2011). Importantly, according to the EFSA risk assessment report, there are consumer groups that have a relatively high exposure to PAs due to consumption of contaminated teas and honey (EFSA, 2017). It is conceivable that these consumers may be at greater risk in case that they would also use the PA-containing herbal products such as PFS. A more complex scenario could be developed that considers short exposure to combinations of herbal teas, PFS, or other herbal products which are derived from PA-producing plants. More detailed information on consumption habits of herbal teas by the average population and by the 95th percentile population (heavy consumers) should be collected to further evaluate the influence of shorter-than-lifetime exposure scenario's. However, at the moment data regarding combined consumption of different types of herbal preparations are mostly lacking, which makes the related risk assessment difficult to perform. Such data await to be collected and will contribute to risk assessment for the current exposure to PAs.

Taken together the results of the present study illustrate the need for development of a generally accepted method for taking shorter-than-lifetime exposure into account when analysing the risks of botanicals and botanical preparations containing compounds that can be genotoxic and carcinogenic. Using Haber's rule to correct for shorter periods of use may prevent from an overestimation of the actual risk to human health. The application of Haber's rule is based on the assumption that the tumour incidence and carcinogenic processes induced by carcinogens have a linear relationship with the cumulative dose (Crump et al., 1976). At present there is only limited data available that supports such a linear dose-response relationship for tumour formation. Currently, there is no valid method on how to take a shorter-than-lifetime exposure into account in an MOE based risk assessment. Nevertheless using Haber's rule gives a reasonable first approach to assess the risks related to shorter-than-lifetime exposure. It is obvious that future developments in risk assessment should consider how to further advance this issue of taking shorter-than-lifetime exposure into account when applying the MOE approach in risk assessment of compounds that are both genotoxic and carcinogenic.

Note

The authors declare that they have no conflict of interest.

Declaration of interests

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.2019.05.024.

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