Determination of benchmark doses for linear furanocoumarin consumption associated with inhibition of cytochrome P450 1A2 isoenzyme activity in healthy human adults

Zeyad Alehaideb [a,b,]*, Sabine Matou-Nasri [a,b,]*

[a] King Abdullah International Medical Research Center, Ministry of National Guard-Health Affairs, Riyadh, Saudi Arabia
[b] King Saud Bin Abdulaziz University for Health Sciences, Riyadh, Saudi Arabia

A B S T R A C T

Millions of individuals globally consume traditional herbal medicines (THMs), which contain abundant amounts of linear furanocoumarins. Linear furanocoumarins (*i.e.,* 8-methoxypsoralen, 5-methoxypsoralen, and iso-pimpinellin) are inhibitors of cytochrome P450 (CYP) isoenzymes including 1A2, a major enzyme involved in drug metabolism and carcinogen bioactivation. Despite the high consumption of furanocoumarin-containing THMs, no studies have measured the furanocoumarin consumption level that triggers an inhibition to CYP1A2 activity in humans. The first objective was to verify if the potencies of the three furanocoumarins are additive towards the inhibition of CYP1A2 activity *in vitro* using concentration-addition and whole-mixture chemical-mixture-assessment models. A second objective was to determine the benchmark dose (BMD) with the mixtures of furanocoumarin oral doses, expressed as 8-MOP equivalents, and to assess the *in vivo* CYP1A2 activity, expressed as inhibition percentages. The *in vitro* results indicated that the three furanocoumarin inhibitory potencies were additive in the THM extracts, validating the use of the concentration-addition model in total furanocoumarin dose-equivalent calculations. Using the USEPA BMD software, the BMD was 18.9 μg 8-MOP equivalent/kg body weight. This information is crucial for furanocoumarin-related health-assessment studies and the regulation of THMs. Further studies should be performed for the remaining major metabolic enzymes to complete the safety profile of furanocoumarin-containing THMs and to provide accurate warning labelling.

1. Introduction

Humans are exposed to natural pharmacoactive ingredients through the daily consumption of plant-based foods and beverages that may affect certain biological systems, including the metabolism. The consumption of such ingredients or phytochemicals can result in inhibitory interaction effects within the metabolic pathways of pharmacoactive drugs [1]. The outcome of such effects is referred to as herb-drug and food-drug interactions. One of the important phytochemical groups is the linear furanocoumarins, occurring in various frequently consumed plant families such as *Apiaceae, Rutaceae, Moraceae,* and *Leguminosae*

[2]. Several herb-drug and food-drug interactions are due to the prior consumption of linear furanocoumarins, including the known "grape-fruit juice effect" on intestinal cytochrome P450 (CYP) 3A4 substrates [3]. Linear furanocoumarins are classified as derivatives of the simplest furanocoumarin form of psoralen, as depicted in the Fig. 1.

Linear furanocoumarins, including 8-methoxypsoralen (8-MOP), 5-methoxypsoralen (5-MOP), and isopimpinellin (ISOP) have gained importance due to their abundance in several plant families and have potent inhibitory effects on xenobiotic metabolic processes when consumed. Human studies have shown that 8-MOP and 5-MOP treatments significantly reduced caffeine clearance in psoriasis patients,
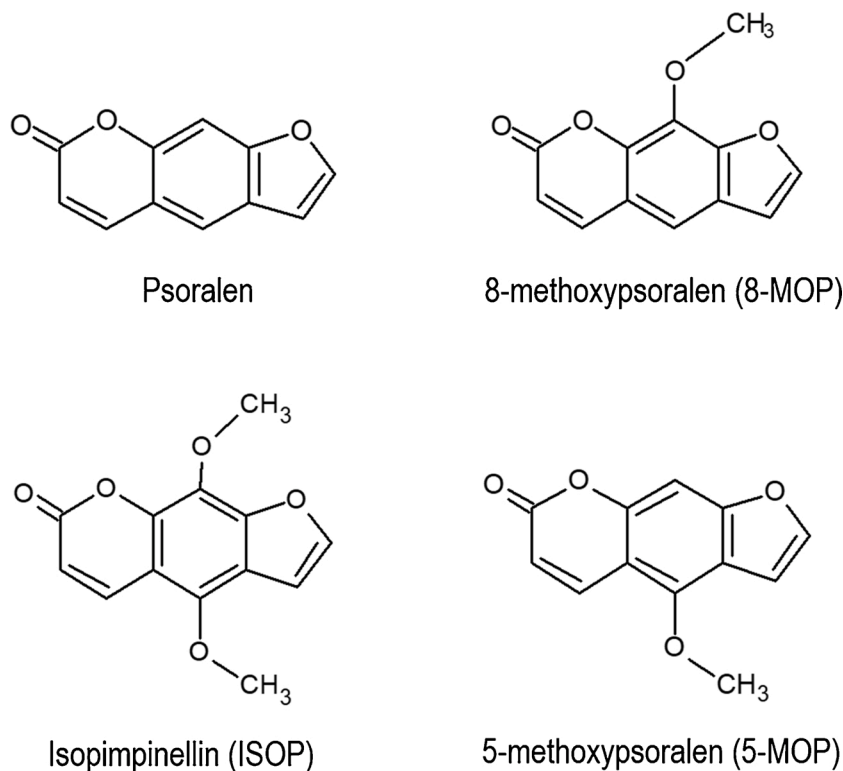
Fig. 1. Structures of linear furanocoumarins of psoralen and derivatives.

suggesting an *in vivo* inactivation of the CYP1A2 isoenzyme [4,5]. The three linear furanocoumarins are irreversible inhibitors of numerous rat and human CYP enzymes. For example, ISOP is characterized as an inactivator of CYP1A2 using microsomes from the yeast expressing human CYP1A2 isoenzyme [6]. Psoralen and angelicin are inactivators of the CYP1A2 isoenzyme using rat liver microsomes and human liver microsomes (HLM), respectively [7]. Based on our recent study, we characterized 8-MOP, 5-MOP, and ISOP as potent irreversible time-dependent inhibitors of the CYP1A2 isoenzyme using HLM [8].

In conventional dose-response or dose-effect assessments, the threshold levels of no-observed-adverse-effect level (NOAEL) and lowest-observed-adverse-effect level (LOAEL) are frequently used to determine the point-of-departure (POD) for chemicals and the associated effects, risks, or endpoints [9]. In recent years, the benchmark dose (BMD) and the BMD lower bound (BMDL) levels have been introduced as an alternative approach, and are extensively used in cancer and non-cancer risk assessment of environmental and food contaminants [10]. In principle, the BMD approach can be applied to any dose-effect relationship [11,12]. The BMD approach has gained increased importance in non-cancer risk assessment studies involving foods, herbs, and nutrition supplements, including the dose-effect and dose-benefit assessments [13–16].

BMD modelling requires two sets of data, namely the exposure and the response or effect data. Examples of effect data are organ weight, body weight, and enzyme activity. Changes in enzymatic and hormonal activity have been used as biomarkers, or endpoints, in risk assessment studies [17–20]. The main advantage of using the BMDL/BMD thresholds, in comparison to the NOAEL/LOAEL, is the use of multiple dose levels with fewer animals or human data, with the consideration of the dose-effect curve shape, and non-dependency on dose-level spacing [9]. Many authors have compared the use of BMDL/BMD as an alternative to the conventional NOAEL/LOAEL and reported comparable values for the same historic studies [21].

In our previous studies, we detected three linear furanocoumarins namely the 8-MOP, 5-MOP and ISOP, in several plant products

belonging to the *Apiaceae* and *Rutaceae* families and concluded the furanocoumarins as the most prevalent in traditional herbal medicines (THMs) and foods [22]. We also conducted human pharmacokinetic interaction studies to investigate the *in vivo* pharmacokinetic effects of consuming furanocoumarin-containing THMs and found significant *in vivo* interactions with caffeine metabolism [8]. The three linear furanocoumarins were characterized as potent time-dependent inhibitors to CYP1A2 isoenzyme activity using HLM, as mentioned earlier. The study had three main objectives. Firstly, to validate the *in vitro* use of the concentration-addition (CA) model, in comparison to the whole-mixture (WM) model, using the inhibitory measurements of the linear furanocoumarin mixtures on the CYP1A2 isoenzyme activity in *Ammi majus* L. seeds (*A. majus*) and *Angelica archnangelica* L. roots (*A. archangelica*) aqueous extracts. Secondly, to establish the dose-effect curve based on the oral consumption of the combined three linear furanocoumarins (8-MOP, 5-MOP, and ISOP) on *in vivo* CYP1A2-mediated caffeine metabolism inhibition in healthy subjects. Thirdly, to determine the BMD concentrations for the established dose-effect curve using different built-in BMD software (BMDS) models developed by the United States Environmental Protection Agency (USEPA).

2. Materials and methods

2.1. Sources of plant-based products

The THMs were commercially obtained in Canada and the United States. *A. majus* was purchased from EverWilde (Fallbrook, CA). *A. archangelica*, *Apium graveolens* L. seeds (*A. graveolens* S), *Pimpinella anisum* L. seeds (*P. anisum*), and the *Ruta graveolens* L. leaves (*R. graveolens*) were provided by Mountain Rose (Eugene, OR). *Apium graveolens* L. flakes (*A. graveolens* F) and *Petroselinum crispum* (Mill.) Fuss leaves (*P. crispum*) were procured from A1SpiceWorld (Glen Head, NY). *Angelica pubescens* Maxim. roots (*A. pubescens*) and *Cnidium monnieri* (L.) Cusson (*C. monnieri*) were purchased from Spring Wind (San Francisco, CA) and Wellness House (Duncan, BC), respectively. The THMs were

authenticated by the suppliers and reported to be free of pesticides and preservatives. The THMs were further authenticated chromatographically in our previous study [22]. Voucher samples were kept in our laboratory for future reference and certificates of authenticity and analysis are available upon request.

2.2. Source of chemicals

Caffeine (≥99.0 %), 8-MOP (≥98.0 %), 5-MOP (99.0 %), ISOP (≥95.0 %) and β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH) (≥97.0 %) were purchased from Sigma-Aldrich (St. Louis, MO) and ChromaDex (Irvine, CA). Trichloroacetic acid (≥99.0), dipotassium phosphate (≥60.0), and monopotassium phosphate (≥60.0) were provided by Anachemia (Rouses Point, NY). Nitrogen gas (≥99.9 %) was procured from Praxair (Danbury, CT). Dimethyl sulfoxide (DMSO) (spectral grade) was provided by Caledon (Georgetown, ON). Radiolabeled caffeine [3-methyl-^{14}C] with specific activity of 50−60 mCi/mmol was purchased from American Radiolabeled Chemicals (St. Louis, MO). The scintillation cocktail fluids were obtained from PerkinElmer (Waltham, MA) and Amersham Biosciences (Piscataway, NJ). Ultrapure water was produced using a Millipore system (Billerica, MA) with a minimum resistivity of 16.0 MΩ cm at 25 °C.

2.3. Integrated dose/concentration determination for the mixtures of linear furanocoumarins

The integrated dose of a mixture of linear furanocoumarins was determined by using two different chemical mixture assessment models namely the CA model approach and the WM model approach [23–25]. The models were used to calculate the dose/concentration equivalent of 8-MOP for *A. majus* and *A. archangelica* extracts as these THMs contained significant and diverse amounts of linear furanocoumarins. The calculation of the integrated dose/concentration requires the determination of the inhibitory potencies (*i.e.,* IC$_{50}$) for the pure furanocoumarins (*i.e.,* 8-MOP, 5-MOP, and ISOP) and the two selected THM aqueous extracts (*i.e.,* *A. majus* and *A. archangelica*).

2.3.1. IC$_{50}$ measurement for the furanocoumarin inhibitory potencies on the CYP1A2

The IC$_{50}$ measurement experimental conditions and data analyses were similar to those described in our recent study [8]. Briefly, the dilution series of the pure furanocoumarins with the final concentrations were as follows: 1.74–1779.76 nM for 8-MOP, 4.44–1135.92 nM for 5-MOP, and 1.17–38448.70 nM for ISOP. Each concentration was incubated separately with Xenotech (Kansas City, KS) 50-donor pooled HLM (catalog number 1210267) (0.2 mg), non-labeled caffeine (82.0 μM), ^{14}C-labeled caffeine (0.2 μCi), NADPH (1.34 mM), and a potassium phosphate buffer (50.0 mM, pH 7.4) in a final volume of 200.0 μL with 1% DMSO. The incubation was conducted at 37 °C in a metabolic incubator with a 60 cycles/min shaking rate. At the end of the 10-min incubation, the reaction was terminated by the addition of ice-cold 10 % trichloroacetic acid solution (50.0 μL). The incubation mixture was centrifuged at 4000 ×*g*. An aliquot (300.0 μL) of the supernatant was applied to a pre-conditioned 3.0 mL Sigma-Aldrich Superclean ENVI-Carbon solid-phase extraction tube (0.25 *g*, 80–100 mesh). The demethylated metabolites of caffeine (*i.e.,* ^{14}C-formaldehyde and ^{14}C-formic acid) were eluted from the solid-phase extraction tube with 2.0 mL of water under gravity flow. The eluent was collected into a scintillation vial. After the addition of 15.0 mL scintillation cocktail, the radioactivity in the vial was counted in a liquid scintillation counter. The results were expressed as residual counts of the control incubation. The IC$_{50}$ values of the pure furanocoumarin were determined by plotting the common log (log$_{10}$) concentrations *versus* the percentage inhibitions of CYP1A2 isoenzyme activity using the GraphPad Prism software (San Diego, CA) built-in dose-response inhibition, log (inhibitor) *vs.* response-variable slope (four-parameter).

2.3.2. Integrated dose calculation using the whole-mixture (WM) model approach

The WM approach is a common method of evaluating a chemical mixture as a single entity without prior knowledge of the individual chemical concentrations in such a mixture, as seen in Eq. 1. A stock solution of *A. majus* (or *A. archangelica*) was prepared by precisely weighing 6.0 g of *A. majus* (or 9.0 g *A. archangelica*) powder and mixed with 600.0 mL of filtered water. The mixtures were boiled separately for approximately 3−4 h on a hot plate with a high temperature. When half of the volume evaporated, the THM preparation was cooled down to room temperature and filtered. Exactly 20.0 mL aliquot of the filtrate was removed, put into a glass tube, and evaporated to dryness in a vacuum concentrator. The remaining residues were redissolved in 2.0 mL DMSO. Serial dilutions were prepared to yield 6 serial concentrations (100−1.56%) relative to the full strength of each THM extract. The remaining incubation conditions and data analyses were similar to those described in subsection 2.3.1. for the pure furanocoumarins. The total dose/concentration for each furanocoumarin mixture was calculated using the IC$_{50}$ values of 8-MOP and each aqueous THM extracts as seen in Eq. 1:

Equation 1: The whole-mixture (WM) model approach

$$\text{Dose} \Big/ \text{Concentration equivalent} \ = \ \frac{\text{CM IC}_{50}}{\text{Extract IC}_{50}} \times \frac{\text{VAM}}{\text{VET}} \times \frac{\text{VHE}}{\text{DWH}} \qquad (1)$$

where "CM IC$_{50}$" is the concentration of the chemical marker (*i.e.,* 8-MOP) to elicit 50 % of the maximal inhibition (mg/mL), "extract IC$_{50}$" is the dilution factor of the prepared THM extract required to elicit 50 % of the maximal inhibition (unitless); VAM is the volume of the assay medium (mL); VET is the volume of extract tested (μL); VHE is the volume of THM stock extract (μL); and DWH is the dry weight of THM used to prepare the stock extract (g).

2.3.3. Concentration-addition (CA) model approach

The CA approach is considered as the most common and default chemical mixture assessment model [26]. The CA approach is a model-based method of relating individual chemical concentrations to a specific biological activity with the assumption that the individual chemical congeners exert similar mechanism of biological effect and are additive in nature. The CA approach is defined as the sum of individual furanocoumarin relative potency factor (RPF) multiplied with the measured individual furanocoumarin mass concentration (Eq. 2).

Equation 2: The concentration-addition (CA) model approach

$$\text{Dose} \Big/ \text{Concentration equivalent} = \sum \text{CI} \times \text{RPF} \qquad (2)$$

where CI is the mass concentration of the individual furanocoumarin in the chemical mixture (mg/g) and RPF is the relative potency of each chemical in comparison to the most potent chemical marker (*i.e.,* 8-MOP).

The individual furanocoumarin concentrations in the aqueous extracts of *A. majus* and *A. archangelica* were determined chromatographically in our previous study [22]. Briefly, the linear furanocoumarins were separated and measured using a Phenomenex (Torrance, CA) kinetex pentafluorophenyl column (250 × 4.6 mm, 5 μm particle size) and a gradient high-performance liquid chromatography (HPLC) method using water and acetonitrile as solvents with the ultra-violet detector set at 310 nm. The measured concentrations for 8-MOP, 5-MOP, and ISOP were 3.26 (±0.20), 0.72 (±0.01), and 7.97 (±1.49) mg/g dry weight for *A. majus* seeds and 0.72 (±0.28), 0.54 (±0.22), and 1.00 (±0.53) mg/g dry weight for *A. archangelica* roots. The RPF value for each furanocoumarin was determined by dividing the mean IC$_{50}$ of 8-MOP with each individual furanocoumarin (*i.e.,* 8-MOP, 5-MOP, and ISOP) mean IC$_{50}$ value.

Table 1
The measured individual linear furanocoumarin doses for participated healthy volunteers in this study.

Plant name	Plant part	Dose (g)	n[a]	Body weight (kg)	Human furanocoumarin oral dose (μg/kg body weight)[b]		
					8-MOP	5-MOP	ISOP
				Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
P. aniseum	seeds	10.0	3	74.7 ± 10.1	2.1 ± 0.3	n.d. [c]	n.d.[c]
A. pubescens	roots	12.0	3	78.7 ± 4.6	3.9 ± 0.2	5.0 ± 0.3	n.d.[c]
A. graveolens	seeds	10.0	3	82.0 ± 5.3	2.6 ± 0.2	2.1 ± 0.2	29.3 ± 2.6
A. archangelica	roots	4.5	5	73.2 ± 17.6	42.2 ± 11.2	25.5 ± 6.8	39.3 ± 10.4
R. graveolens	leaves	3.0	4	75.0 ± 8.2	56.3 ± 5.7	22.4 ± 2.3	12.3 ± 1.3
C. monnieri	fruits	3.0	5	75.8 ± 7.4	28.2 ± 3.0	71.3 ± 7.5	18.6 ± 2.0
A. archangelica [d]	roots	9.0	3	74.0 ± 15.6	38.7 ± 9.3	92.2 ± 22.1	224.4 ± 53.8
A. majus	seeds	6.0	5	72.4 ± 18.7	282.9 ± 80.3	63.1 ± 17.9	663.5 ± 188.4
A. majus [d]	seeds	12.0	3	74.0 ± 15.6	481.8 ± 115.6	86.9 ± 20.8	1065.4 ± 255.7

[a] Number of volunteers for each plant product.
[b] Based on 3–5 separate extractions.
[c] Not detected in plant aqueous extract.
[d] Double-dose levels of *A. archangelica* and *A. majus* plant products.

Table 2
The exposure and effect data sets used for BMD determination using the USEPA BMDS.

Plant name	Mean Integrated Dose[a]	Percentage Inhibition of In vivo CYP1A2 Enzyme Activity[b]
	(μg 8-MOP equivalent/kg body weight)	Mean ± SD
P. aniseum	2.1	11.2 ± 9.4
A. pubescens	8.3	32.7 ± 10.8
A. graveolens	12.3	28.5 ± 25.6
A. archangelica	75.3	53.4 ± 18.8
R. graveolens	79.4	35.6 ± 20.9
C. monnieri	96.3	54.2 ± 19.1
A. archangelica	180.7	60.4 ± 14.9
A. majus	517.3	77.0 ± 4.9
A. majus	845.5	83.2 ± 5.3

[a] Based on the sum of average 8-MOP, 5-MOP, and ISOP oral doses multiplied with their relative potencies (see subsection 2.3.2. for more information).
[b] Calculated from caffeine clearance data (see subsection 2.4. for more information).

2.4. Human exposure and effect data sets for the BMD determination

Based on our recent human pharmacokinetic studies (Alehaideb, 2021), we dosed healthy male volunteers between 20 and 35 years of age, with eight levels of furanocoumarin-containing THM extracts with concentrations ranging from 2.1–1634.1 μg total furanocoumarin dose per kg body weight as indicated in the Table 1. The studies were approved by Simon Fraser University (Office of Research Ethics) with approval number 2012s0565 and registration number ISRCTN83028296. Based on previous THM analysis studies, not all furanocoumarins of interest are present in each THM dose, and the ratio of the furanocoumarin concentrations is not similar at each dose level [22]. In an effort to address the variation in the furanocoumarin content in a consistent approach, we calculated the integrated external oral dose of the total furanocoumarins in each THM dose using the CA model. The calculated 8-MOP equivalent represents the total dose based on the concentration and the potency of each individual furanocoumarin, providing a more accurate numerical description of total furanocoumarin dose/concentration for human participants (Table 2).

The pharmacokinetic parameter of the plasma caffeine clearance was used to calculate the *in vivo* inhibition of the CYP1A2 enzymatic activity, expressed as the percentage inhibition as seen in Table 2. The full details of the human pharmacokinetic studies are described in our published studies [8,27]. Briefly, the volunteers were orally dosed with 200.0 mg of caffeine in the form of tablets. After dosage, saliva samples were collected at time points ranging between 0.5 and 48.0 h in addition to the pre-dose sample. The saliva samples were extracted once with ethyl acetate, evaporated with nitrogen gas, and reconstituted with the HPLC mobile-phase. The separation and measurement of the caffeine and internal standard were performed chromatographically, using an isocratic HPLC and ultra-violet detector method using a mobile-phase consisting of acetonitrile, water, and acetic acid. The study was performed twice for each volunteer to calculate the plasma caffeine clearance with and without prior THM pre-treatment. The inhibition percentages were determined using Eq. 3.

Equation 3: The calculation of the percentage inhibition of in vivo CYP1A2 isoenzyme activity

Percentage inhibition *of in vivo* CYP1A2 activity

$$= 100 - ((TCL/UCL) \times 100) \tag{3}$$

where TCL is the measured plasma caffeine clearance with prior THM pre-treatment and UCL is the measured plasma caffeine clearance without prior THM pre-treatment.

2.5. Benchmark dose (BMD) modelling

Based on USEPA technical guide recommendations, the establishment of a dose-effect relationship initially requires the selection of exposure and effect data sets [28]. Once the dose-effect curve is established, the exposure levels of BMD values are calculated statistically using the (BMDS) models.

2.5.1. The benchmark dose software (BMDS)

The dose-effect curve and thresholds of the BMD and the BMD lower (BMDL) and upper (BMDU) confidence limits were determined using the USEPA BMDS version 2.7.0.4 (Washington, D.C.) [29]. The BMDS provides a stepwise approach that includes the selection of the benchmark response (BMR), data set type, model selection, and data/statistical analysis. The software was downloaded from USEPA website (http://www.epa.gov/ncea/bmds/). We followed the guidelines, methodologies, and recommendations according to USEPA technical guidelines draft [28].

2.5.2. The benchmark response (BMR) selection

The BMD is a dose or concentration that produces a predetermined change in the response rate of an effect. This predetermined change in response is termed the benchmark response (BMR). Due to the absence of a specific endpoint for CYP1A2 baseline activity inhibition, we selected the BMR option of one standard deviation (SD) which was recommended as part of the USEPA set of recommendations for data reporting in the absence of a well-specified end-point and to determine the POD [28].
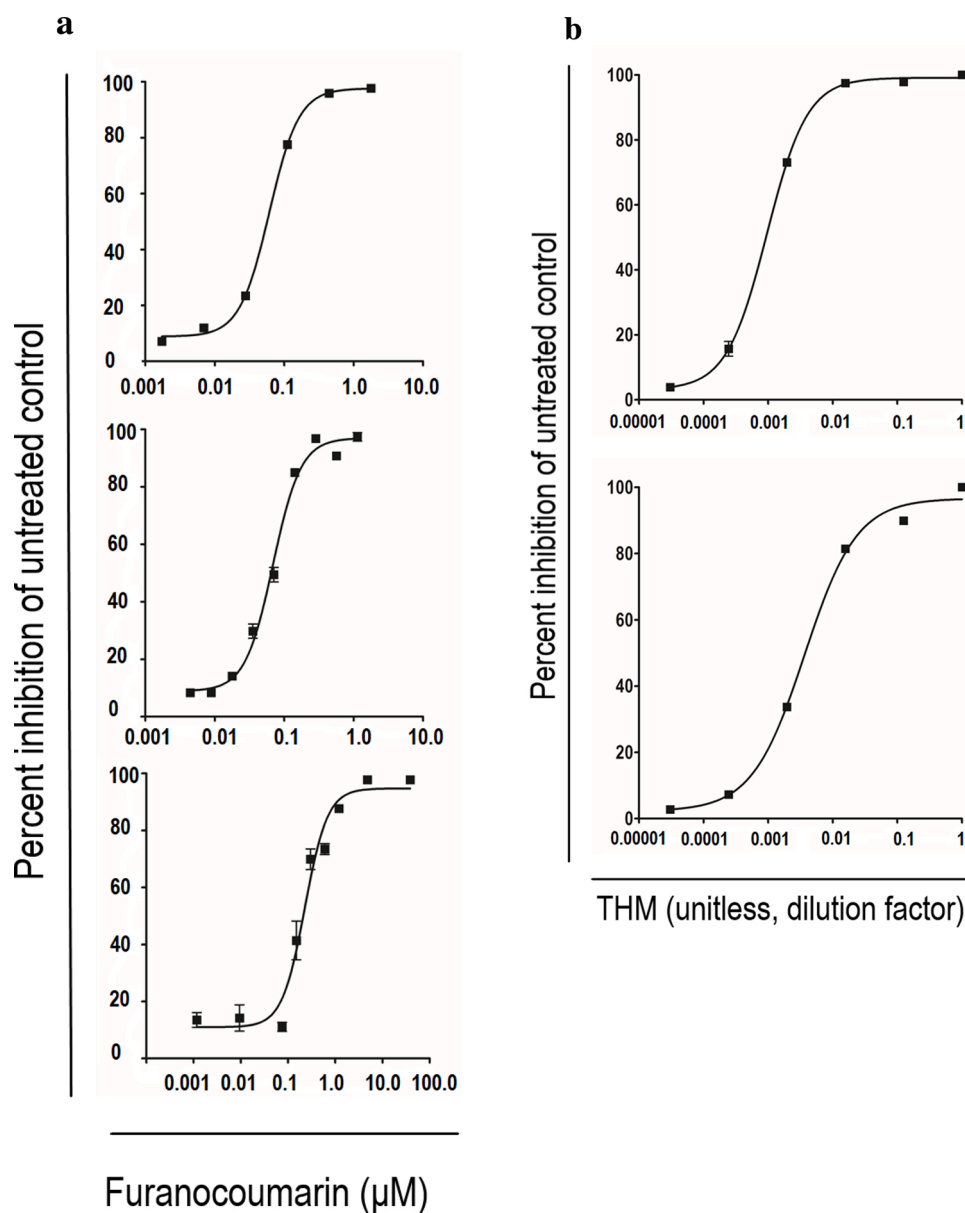
Fig. 2. (a) Concentration-inhibition plots for pure furanocoumarins of 8-MOP (top), 5-MOP (middle), and ISOP (bottom) on CYP1A2 isoenzyme activity *in vitro*. (b) Concentration-inhibition curves for *A. majus* seed (top) and *A. archangelica* root (bottom) aqueous extracts on CYP1A2 isoenzyme activity *in vitro*.

2.5.3. Model selection and statistics

The final step is the selection of the mathematical model to best describe the dose-effect relationship using the available human exposure and effect data sets. We used the BMDS built-in models for continuous data to calculate the BMD thresholds at the pre-specified BMR level as mentioned. The BMDS provides numerous model options, including hill, exponential M2-M5, linear, polynomial, and power models. The best-fit model was selected based on USEPA recommendations, which include goodness-of-fit (*i.e.*, *t*-test), Akaike's information criterion (AIC) value, and visual inspection of the fitted dose-effect curve. The uncertainty of BMD determination was reflected as 95 % confidence intervals (*i.e.*, BMDL and BMDU), which was computed by the USEPA BMDS based on the profile likelihood method as mentioned in USEPA BMDS guidelines. Due to different volunteers giving different initial response values for each furanocoumarin-equivalent dose level, the response value for the no-treatment control group was set to zero percentage as default.

3. Results and discussion

In this study, we report the *in vivo* inhibition of the CYP1A2 isoenzyme activity, following the exposure to different doses of linear furanocoumarin-containing mixtures in the form of THMs. We attempted to illustrate the inhibition in a human dose-effect relationship fitted by the USEPA BMDS, and using the data sets of exposure doses expressed as 8-MOP equivalents using the CA model for chemical-mixture-assessment model and effect-based measurements expressed as percentage inhibition of *in vivo* CYP1A2 activity.

In the presence of different CYP1A2 drug probes, caffeine was chosen in this study to calculate the CYP1A2 activity due to its well-known pharmacokinetic profile and it is relatively safe to consume at levels not exceeding 400.0 mg per day [30]. From *in vitro* and *in vivo* studies, the CYP1A2 has been reported to be the main isoenzyme to metabolize caffeine. For instance, *in vitro* studies involving specific CYP enzymes demonstrated that the 1A2 isoenzyme specifically catalyses caffeine oxidation at 3-*N* and 1-*N* to form paraxanthine and theobromine, respectively, with a minor 1A2 role in the remaining caffeine metabolic

Table 3
Comparative outcomes for BMD determination using different USEPA BMDS built-in models.

Model[a]	BMD[b]	BMDL[c]	BMDU[d]	p value[e]	AIC[f]
	µg 8-MOP equivalent/ kg body weight				
Hill	18.86	8.58	42.00	0.190	246.22
Polynomial	94.36	66.29	159.99	0.003	257.75
Power	275.46	205.08	416.98	0.000	264.61
Linear	275.46	205.08	416.98	0.000	264.61
Exponential Model 2	416.46	336.07	576.56	< 0.0001	268.51
Exponential Model 3	416.46	336.07	576.56	< 0.0001	268.51
Exponential Model 4	28.01	17.35	51.57	0.119	247.72
Exponential Model 5	28.01	17.35	51.94	0.119	247.72

[a] Using default USEPA BMDS models and settings.
[b] Calculated with BMR set at 1.0 standard deviation.
[c] Determined at 0.95 lower confidence limit.
[d] Determined at 0.95 upper confidence limit.
[e] Based on likelihood ratio test performed by USEPA BMDS (acceptable model-fit if p value is > 0.1).
[f] Akaike's information criterion performed by USEPA BMDS.

pathways [31]. In addition, the same research group indicated that CYP1A2 is the main isoenzyme involved in caffeine metabolism, accounting for more than 70 % of 100 µM caffeine substrate concentration [32]. *In vivo* studies revealed that CYP1A2 is the main isoenzyme involved in caffeine metabolism in humans. For example, Miners and Birkett [33] concluded that CYP1A2 is the main isoenzyme to catalyze all three *N*-demethylations for caffeine, which accounts for more than 90 % of caffeine metabolism *in vivo*. Perera et al. [34] concluded that caffeine is the preferred probe for evaluating CYP1A2 isoenzyme activity *in vivo* in comparison to alternative probe drugs. Carrillo et al. [35] concluded that caffeine is a preferred probe substrate for both *in vitro* and *in vivo* studies related to CYP1A2 enzyme activity evaluation.

In this present study, we measured the inhibitory potencies of the pure furanocoumarin chemical on the CYP1A2 isoenzyme activity with IC_{50} values, determined as 61.6 (± 2.41), 68.8 (± 0.21), and 277.0 (± 10.4) nM for 8-MOP, 5-MOP, and ISOP, respectively (Fig. 2a). The inhibitory parameters were obtained using a multi-donor pooled HLM to mimic the true *in vivo* metabolic medium, to provide more accurate measurements. The calculated RPF values for 8-MOP, 5-MOP, and ISOP were 1.00, 0.88, and 0.27, respectively. The IC_{50} values were selected for the RPF calculation to obtain an accurate comparison of the inhibitory potencies in the three linear furanocoumarins, compared to the inhibitory values near the extremes of the dose-effect curve slope (*i.e.,* IC_{20} or IC_{80}). The dose-response curves of the three linear furanocoumarins were relatively parallel to each other with slopes of 2.036, 2.165, and 1.798 for 8-MOP, 5-MOP, and ISOP, respectively (Fig. 2a). The conditions for applying the CA model were met using 8-MOP as the chemical marker, as per Eq. 2.

Fig. 2b presents the inhibition curves for *A. majus* and *A. archangelica* aqueous extracts on CYP1A2-mediated caffeine 3-*N* demethylation activity using HLMs. The IC_{50} values were calculated at a 0.000959 (± 0.000133) and 0.003564 (± 0.000154) dilution factor for *A. majus* and *A. archangelica* extracts, respectively. The calculated integrated concentrations using the WM model were 6.97 (± 0.94) and 1.24 (± 0.05) mg 8-MOP equivalent/g dry weight for *A. majus* and *A. archangelica* extracts, respectively. However, the calculated integrative concentrations using the CA model were 6.04 (± 0.35) and 1.46 (± 0.08) mg 8-MOP equivalent/g dry weight for *A. majus* and *A. archangelica* extracts, respectively. The proximity of the WM and CA derived integrated concentration/dose results indicate that 8-MOP, 5-MOP, and ISOP are the main inhibitors of the CYP1A2 isoenzyme activity *in vitro* and most likely *in vivo*.

We explored different BMD modelling options and restrictions. We concluded that using the default BMDS settings and restrictions provided the best modelling outcomes, as reported [21]. The goodness-of-fit

test results indicate adequate fit for the hill and exponential M4/M5 models with BMDS-calculated *p*-values more than 0.1 (Table 3). The 8-MOP equivalent doses and the CYP1A2-mediated caffeine metabolism inhibition percentages were best-fit using the BMDS hill model as it displayed the lowest AIC value (Table 3) (see supplementary 1 for more information). These results agree with a study concluding that the hill and exponential models fit for most toxicological dose-effect relationships [36]. Nevertheless, the human data in this study was fitted using all the available models provided by the BMDS, as per USEPA recommendations (see Supplementary 1 for more detailed information). Of note, we explored the use of the Netherland's National Institute for Public Health and the Environment (RIVM) software for dose-response modelling with benchmark dose analysis (PROAST), and we concluded that the USEPA BMDS provided the best-fit for the available data presented in this study.

As seen in the Table 3, the hill and exponential M4/M5 models provided good fits with the BMDS-calculated AIC values close to one another with less than 2.0 value difference. Moreover, the BMDL levels from the aforementioned models were within 3-fold difference, which are considered as non-divergent by BMD modellers [9,37]. Thus, an averaged BMDL value for the best-fit models (*i.e.,* hill and exponential M4/M5) could be considered as the POD as recommended [28]. In this current study, the averaged BMDL value was 14.4 (± 5.1) µg 8-MOP equivalent/kg body weight. It should be noted that this is not similar to the emerging approach of model-averaging that is advocated in recent years by modellers, which involves weighing adequately fitting models [38]. Worth mentioning, the determined BMD and BMDL values obtained in this current study were comparable to LOAEL and NOAEL levels reported in our previous study [8]. The NOAEL and LOAEL levels were also determined based on a series of human experiments involving various oral doses of THMs, with the total furanocoumarin content ranging from 2.1–845.5 µg 8-MOP equivalent/kg body weight. Based on Student's paired *t*-test, comparing the caffeine area-under-curve ratio with and without prior pre-treatment by furanocoumarin-containing THMs for the same dose group, the NOAEL and LOAEL levels were 13.1 and 71.9 µg 8-MOP equivalent/kg body weight, respectively. It is noteworthy that the LOAEL and NOAEL are depended on dose-level spacing, which gives advantage to the BMD/BMDL approach as mentioned earlier.

Despite the successful establishment of the dose-effect relationship for linear furanocoumarin mixture consumptions and the percentage inhibition of the CYP1A2-mediated caffeine metabolism determined in healthy volunteers, this study has limitations and assumptions, which should be addressed. The use of herb extracts instead of pure phytochemicals to determine the BMD and BMDL levels could be considered as a limitation in the current study due to the presence of other phytochemical groups, including flavonoids. These flavonoids, such as apigenin, quercetin, and naringenin, are known to exhibit inhibitory effects on CYP1A2 enzyme activity [39,40]. Nevertheless, the flavonoid contents in such herbs and foods are significantly reduced with prolonged boiling temperature treatment, as seen in traditional decoction preparations [41]. Based on our previous THM analysis study, osthole coumarin, detected in *C. monnieri* and *A. pubescens* extracts, was stable at boiling temperature (unpublished data). However, osthole coumarin has been characterized as a moderate inhibitor of CYP1A2 activity [42], which suggests osthole coumarin to play a minimal inhibitory role in the aforementioned herbs, especially with the abundant presence of potent irreversible inhibitors of linear furanocoumarins, which degrade the CYP1A2 isoenzyme.

However, the literature confirmed that the linear furanocoumarins, including 8-MOP, 5-MOP, and ISOP, are stable at boiling temperatures [22,43]. In the present study, the participants were dosed with THM extracts, treated at a boiling temperature, resembling a decoction preparation. As a result, confounding inhibitory effects were unlikely to occur. It should be also mentioned that the current study is not the first study to establish a dose-effect relationship and to determine BMD levels
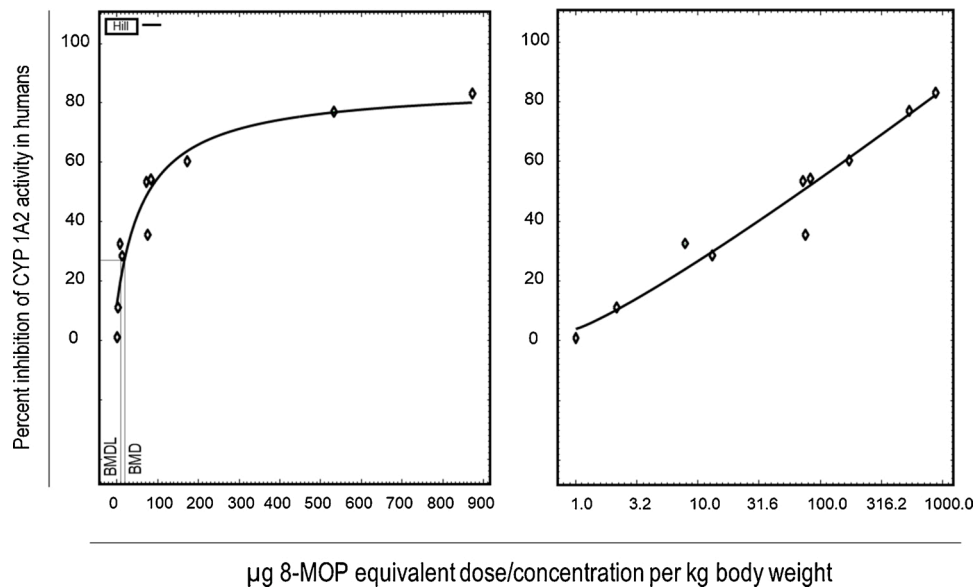
Fig. 3. Fitted BMDS hill-model curve for total furanocoumarin consumption expressed as 8-MOP equivalents (left), $\log_{10}$-transformed dose concentrations (right), and *in vivo* inhibition of CYP1A2-mediated caffeine metabolism expressed as percentage inhibition.

from exposure data using different natural products. Wu and Wang [44] established a dose-response relationship for end-stage renal disease with the consumption of aristolochic acid, present in different Chinese herbs, decoctions, and preparations. Our current results clearly show an ascending trend of both CYP1A2 isoenzyme activity inhibition and furanocoumarin-equivalent concentrations (Fig. 3).

The human data sets were based on a subpopulation of healthy males from 20 to 35 years of age and exclude subpopulations of females and sensitive individuals, including children, elderly, and patients, which could be considered as another limitation in this study. However, the threshold levels of BMD and BMDL were based on observed human data, which account for the absorption, protein binding, distribution, metabolism, and elimination, providing more accurate measurements with minimal confounding uncertainties compared to results obtained from scaling animal toxicological data, which strengthened this study. A point to consider is the fact that linear furanocoumarins induce CYP isoenzymes in mice and rats, which may interfere with the conclusions of the present study. For instance, Letteron et al. [45] dosed 125 μmol/kg of 8-MOP or 5-MOP to rats for three days and reported that the monooxygenases activity remained high before the subsequent doses but were immediately lowered after the 8-MOP or 5-MOP dose. They concluded that 8-MOP and 5-MOP are both inactivators and inducers. Mays et al. [46] dosed rats with 50.0 mg/kg for three days, resulting in an increased caffeine clearance from 0.25 to 1.08 L/kg/hr. Apseloff et al. [47] dosed rats with 8-MOP at 0, 25.0, and 50.0 mg/kg for three days with an average theophylline clearance of 1.7, 2.4, and 9.5 mL/min/kg respectively. In addition, Tantcheva-Poór et al. [48] dosed human patients undergoing psoralen-ultraviolet A treatment with 0.6 mg 8-MOP/kg/-day with no observed increase in caffeine clearance levels after six to ten days of treatment suggesting no induction of CYP1A2 isoenzyme expression in humans by 8-MOP.

4. Conclusion

We successfully determined the threshold oral dose for linear furanocoumarins, expressed as 8-MOP equivalents, at which a significant inhibition of CYP1A2-mediated caffeine metabolism can occur. The results of this study can be used to predict herb-drug interactions involving linear furanocoumarin-containing THMs, as well as narrow-therapeutic-indexed drugs mainly metabolized by the CYP1A2 isoenzyme, such as theophylline and tizanidine. In addition, this study demonstrated the

successful application of the chemical-mixture-assessment models in a dose-effect relationship, involving phytochemicals. The study also supports advocates for the appropriate regulation of natural health products with significant potent pharmacoactive ingredients, which might pose a serious risk for consumer health.

Authors contribution

ZA: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Writing-Original Draft. SMN: Validation, Writing-Review & Editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2021.07.013.

References

[1] M. Hu, L. Fan, H.H. Zhou, B. Tomlinson, Theranostics meets traditional Chinese medicine: rational prediction of drug–herb interactions, Expert Rev. Mol. Diagn. 12 (8) (2012) 815–830.
[2] M.A. Pathak, F. Daniels Jr., T.B. Fitzpatrick, The presently known distribution of furocoumarins (psoralens) in plants, J. Invest. Dermatol. 39 (3) (1962) 225–239.
[3] D.G. Bailey, J.D. Spence, C. Munoz, J.M.O. Arnold, Interaction of citrus juices with felodipine and nifedipine, Lancet 337 (8736) (1991) 268–269.

[4] D.C. Mays, C. Camisa, P. Cheney, C.M. Pacula, S. Nawoot, N. Gerber, Methoxsalen is a potent inhibitor of the metabolism of caffeine in humans, Clin. Pharmacol. Ther. 42 (6) (1987) 621–626.

[5] E.K. Bendriss, Y. Bechtel, A. Bendriss, Ph. Humbert, G. Paintaud, J. Magnette, Pm Agache, P.R. Bechtel, Inhibition of caffeine metabolism by 5-methoxypsoralen in patients with psoriasis, Br. J. Clin. Pharmacol. 41 (5) (1996) 421–424.

[6] A.Y. Kang, L.R. Young, C. Dingfelder, S. Peterson, Effects of furanocoumarins from apiaceous vegetables on the catalytic activity of recombinant human cytochrome P-450 1A2, Protein J. 30 (7) (2011) 447–456.

[7] X.M. Zhuang, Y.H. Zhong, W.B. Xiao, H. Li, C. Lu, Identification and characterization of psoralen and isopsoralen as potent CYP1A2 reversible and time-dependent inhibitors in human and rat preclinical studies, Drug Metab. Dispos. 41 (11) (2013) 1914–1922.

[8] Z. Alehaideb, M. Sheriffdeen, F.C. Law, Inhibition of caffeine metabolism by Apiaceous and Rutaceae families of plant products in humans: in vivo and in vitro studies, Front. Pharmacol. 12 (2021), 641090.

[9] J.A. Davis, J.S. Gift, Q.J. Zhao, Introduction to benchmark dose methods and US EPA's benchmark dose software (BMDS) version 2.1. 1, Toxicol. Appl. Pharmacol. 254 (2) (2011) 181–191.

[10] E. Budtz-Jørgensen, Guidance of the Scientific Committee on the request from EFSA on the use of the benchmark dose approach in risk assessment, Efsa J. 1150 (2009) 1–72.

[11] S.D. Muri, J.R. Schlatter, B.J. Brüschweiler, The benchmark dose approach in food risk assessment: Is it applicable and worthwhile? Food Chem. Toxicol. 47 (12) (2009) 2906–2925.

[12] W. Slob, Benchmark dose and the three Rs. Part I. Getting more information from the same number of animals, Crit. Rev. Toxicol. 44 (7) (2014) 557–567.

[13] D.P.H. Hsieh, C.F. Chiang, P.H. Chiang, C.P. Wen, Toxicological analysis points to a lower tolerable daily intake of melamine in food, Regul. Toxicol. Pharmacol. 55 (1) (2009) 13–16.

[14] D.W. Lachenmeier, M. Uebelacker, Risk assessment of thujone in foods and medicines containing sage and wormwood–evidence for a need of regulatory changes? Regul. Toxicol. Pharmacol. 58 (3) (2010) 437–443.

[15] T.Ø. Fotland, J.E. Paulsen, T. Sanner, J. Alexander, T. Husøy, Risk assessment of coumarin using the benchmark dose (BMD) approach: children in Norway which regularly eat oatmeal porridge with cinnamon may exceed the TDI for coumarin with several folds, Food Chem. Toxicol. 50 (3–4) (2012) 903–912.

[16] D.W. Lachenmeier, C. Steffen, O. el-Atma, S. Maixner, S. Löbell-Behrends, M. Kohl-Himmelseher, What is a food and what is a medicinal product in the European Union? Use of the benchmark dose (BMD) methodology to define a threshold for "pharmacological action", Regul. Toxicol. Pharmacol. 64 (2) (2012) 286–295.

[17] IPCS, Environmental health criteria 155. Biomarkers and Risk Assessment: Concept and Principles, World Health Organization, Geneva, 1993.

[18] L.L. Carlock, W.L. Chen, E.B. Gordon, J.C. Killeen, A. Manley, L.S. Meyer, et al., Regulating and assessing risks of cholinesterase-inhibiting pesticides: divergent approaches and interpretations, J. Toxicol. Environ. Health B Crit. Rev. 2 (2) (1999) 105–160.

[19] T. Zhou, D.G. Ross, M.J. DeVito, K.M. Crofton, Effects of short-term in vivo exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats, Toxicol. Sci. 61 (1) (2001) 76–82.

[20] R.W. Setzer, C.A. Kimmel, Use of NOAEL, benchmark dose, and other models for human risk assessment of hormonally active substances, Pure Appl. Chem. 75 (11–12) (2003) 2151–2158.

[21] H. Izadi, J.E. Grundy, R. Bose, Evaluation of the benchmark dose for point of departure determination for a variety of chemical classes in applied regulatory settings, Risk Anal. An Inter. J 32 (5) (2012) 830–835.

[22] Z. Alehaideb, M. Sheriffdeen, F.C. Law, Furanocoumarin bioactives in the *Apiceae* and *Rutaceae* families of plants, Can. J. Appl. Sci. 11 (2) (2017) 4157–4167.

[23] S.H. Safe, Hazard and risk assessment of chemical mixtures using the toxic equivalency factor approach, Environ. Health Perspect. 106 (suppl 4) (1998) 1051–1058.

[24] A. Lorenzen, J.G. Hendel, K.L. Conn, S. Bittman, A.B. Kwabiah, G. Lazarovits, D. Massé, T.A. McAllister, E. Topp, Survey of hormone activities in municipal biosolids and animal manures, Environ. Toxicol. 19 (3) (2004) 216–225.

[25] Z. Alehaideb, K.C. Chin, M.C. Yao, F.C. Law, Predicting the content of anthraquinone bioactive in Rhei rhizome (Rheum officinale Baill.) with the concentration addition model, J. Saudi Pharm. Soc. 27 (1) (2019) 25–32.

[26] J. Seed, R.P. Brown, S.S. Olin, J.A. Foran, Chemical mixtures: current risk assessment methodologies and future directions, Regul. Toxicol. Pharmacol. 22 (1) (1995) 76–94.

[27] M.M. Sheriffdeen, Z.I. Alehaideb, F.C. Law, Caffeine/Angelica dahurica and caffeine/Salvia miltiorrhiza metabolic inhibition in humans: in vitro and in vivo studies, Complement. Ther. Med. 46 (2019) 87–94.

[28] USEPA, Benchmark Dose Technical Guidance, US Environmental Protection Agency, 2012.

[29] USEPA, Benchmark Dose Software (BMDS), Downloaded June 19, 2015, EPA, 1999, http://www.epa.gov/ncea/bmds/.

[30] O. Commissioner, Of the Spilling the Beans: How Much Caffeine Is Too Much? [(Accessed on 1 April 2020)]; 2019. Available online FDA, 2019, https://www.fda.gov/consumers/consumer-updates/spilling-beans-how-much-caffeine-too-much.

[31] M. Kot, W.A. Daniel, The relative contribution of human cytochrome P450 isoforms to the four caffeine oxidation pathways: an in vitro comparative study with cDNA-expressed P450s including CYP2C isoforms, Biochem. Pharmacol. 76 (4) (2008) 543–551.

[32] M. Kot, W.A. Daniel, Caffeine as a marker substrate for testing cytochrome P450 activity in human and rat, Pharmacol. Rep. 60 (6) (2008) 789–797.

[33] J.O. Miners, D.J. Birkett, The use of caffeine as a metabolic probe for human drug metabolizing enzymes, Gen. Pharmacol. 2 (27) (1996) 245–249.

[34] V. Perera, A.S. Gross, A.J. McLachlan, Measurement of CYP1A2 activity: a focus on caffeine as a probe, Curr. Drug Metab. 13 (5) (2012) 667–678.

[35] J.A. Carrillo, J. Christensen, S.I. Ramos, C. Alm, M.L. Dahl, J. Benítez, L. Bertilsson, Evaluation of caffeine as an in vivo probe for CYP1A2 using measurements in plasma, saliva, and urine, Ther. Drug Monit. 22 (4) (2000) 409–417.

[36] W. Slob, R.W. Setzer, Shape and steepness of toxicological dose–response relationships of continuous endpoints, Crit. Rev. Toxicol. 44 (3) (2014) 270–297.

[37] L.T. Haber, M.L. Dourson, B.C. Allen, R.C. Hertzberg, A. Parker, M.J. Vincent, A. Maier, A.R. Boobis, Benchmark dose (BMD) modeling: current practice, issues, and challenges, Crit. Rev. Toxicol. 48 (5) (2018) 387–415.

[38] S.M. Jensen, F.M. Kluxen, C. Ritz, A review of recent advances in benchmark dose methodology, Risk Anal. 39 (10) (2019) 2295–2315.

[39] U. Fuhr, K. Klittich, A. Staib, Inhibitory effect of grapefruit juice and its bitter principal, naringenin, on CYP1A2 dependent metabolism of caffeine in man, Br. J. Clin. Pharmacol. 35 (4) (1993) 431–436.

[40] S. Peterson, J.W. Lampe, T.K. Bammler, K. Gross-Steinmeyer, D.L. Eaton, Apiaceous vegetable constituents inhibit human cytochrome P-450 1A2 (hCYP1A2) activity and hCYP1A2-mediated mutagenicity of aflatoxin B1, Food Chem. Toxicol. 44 (9) (2006) 1474–1484.

[41] K.H. Miean, S. Mohamed, Flavonoid (myricetin, quercetin, kaempferol, luteolin, and apigenin) content of edible tropical plants, J. Agric. Food Chem. 49 (6) (2001) 3106–3112.

[42] L.P. Yang, Z.W. Zhou, X.W. Chen, C.G. Li, K.B. Sneed, J. Liang, S.F. Zhou, Computational and in vitro studies on the inhibitory effects of herbal compounds on human cytochrome P450 1A2, Xenobiotica 42 (3) (2012) 238–255.

[43] G.W. Ivie, D.L. Holt, M.C. Ivey, Natural toxicants in human foods: psoralens in raw and cooked parsnip root, Science 213 (4510) (1981) 909–910.

[44] F. Wu, T. Wang, Risk assessment of upper tract urothelial carcinoma related to aristolochic acid, Cancer Epidemiol. Biomarkers. Prev. 22 (5) (2013) 812–820.

[45] P. Letteron, V. Descatoire, D. Larrey, M. Tinel, J. Geneve, D. Pessayre, Inactivation and induction of cytochrome P-450 by various psoralen derivatives in rats, J. Pharmacol. Exp. Ther. 238 (2) (1986) 685–692.

[46] D.C. Mays, S. Nawoot, J.B. Hilliard, C.M. Pacula, N. Gerber, Inhibition and induction of drug biotransformation in vivo by 8-methoxypsoralen: studies of caffeine, phenytoin and hexobarbital metabolism in the rat, J. Pharmacol. Exp. Ther. 243 (1) (1987) 227–233.

[47] G. Apseloff, D. Shepard, M. Chambers, S. Nawoot, D. Mays, N. Gerber, Inhibition and induction of theophylline metabolism by 8-methoxypsoralen. In vivo study in rats and humans, Drug Metab. Dispos. 18 (3) (1990) 298–303.

[48] I. Tantcheva-Poór, M. Servera-Llaneras, K. Scharffetter-Kochanek, U. Fuhr, Liver cytochrome P450 CYP1A2 is markedly inhibited by systemic but not by bath PUVA in dermatological patients, Br. J. Dermatol. 144 (6) (2001) 1127–1132.