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Research Paper

The safety of *Osmanthus fragrans* ethanol extract treatment in BALB/c mice

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Abstract

Background: *Osmanthus fragrans* flower, recently certificated as a new natural antioxidant, has been used in various foods. We performed an acute and subacute toxicological test for evaluating the safety and toxicity of 75 % ethanol extraction of *Osmanthus fragrans* (OFEE).
Method: In the acute toxicity study, a single dose of 5 g/kg and 10 g/kg of the extract was administered orally to six mice. General behaviour, mortality and toxic symptoms were determined daily for 14 days. For the subacute toxicity, two groups of 12 mice received 0.9% normal saline (control) and 1 g/kg of the extract daily for 28 consecutive days by oral gavage. The animals were observed daily for abnormal clinical signs and death. Body weight, haematological and biochemical parameters of blood as well as kidney, liver, lung and spleen tissues histology were evaluated.
Results: The total phenolic contents in OFEE were 371.71 ± 12.35 mg GAE/g extracts and total flavonoid contents were 47.23 ± 5.36 mg QE/g extract. OFEE did not cause any mortality or morbidity. Maximum tolerated dose (MTD) was 10 g/kg body weight in our BALB/c mice, which can be regarded as virtually non-toxic. Administration of OFEE at dose 1 g/kg/day for 28-day did not cause changed in hematological, biochemical assay, and histopathological conditions change, suggesting a no-observed-adverse-effect level (NOVEL) of 1 g/kg/day.
Conclusion: We found no evidence of adverse effects in our laboratory test and pathological studies. 75 % ethanol extraction may safely be used in the research of *Osmanthus fragrans* in animal and probably human studies.

Keywords: *Acute toxicity test, antioxidant activity, osmanthus fragrans, subacute toxicity test, total phenolic and flavonoid content*.

1. Introduction

Osmanthus fragrans, also known as sweet osmanthus of sweet olive, is a species of Osmanthus, which is native to Asia and, found from the eastern Himalayas through southern China and as far as Taiwan and southern Japan (Huxley 1992, Cochinch 1996). The flower of *O. fragrans*, called Kwai-fah in China, has been used as a beverage and as an additive for tea and foods, condiments, and beverages. It is popular because of its delicate fruity/floral aroma. Traditional Chinese medicine has applied *O. fragrans* in the treatment of weakened vision, halitosis, panting, asthma, cough, toothache, stomachache, diarrhea, and hepatitis. Although research for the biomedical use of the extract of *O. fragrans* flowers is limited, there is some evidence showing that the *O. fragrans* may be a promising source of natural antioxidants (Kihuchi 1984, Lee *et al*. 2007, Wang *et al*. 2010, Hung *et al*. 2012a). Lee *et al*. (2007) found dried flowers of *O. fragrans* to have abundant level of phenols and flavonoids and to exhibit antioxidant activity in the metal reducing, FRAP assay, the DPPH, and hydroxyl anion scavenging ability (Lee *et al*. 2007). Our group has analyzed many antioxidant components of *O. fragrans* flowers and found their antioxidant activity to be slightly weaker than that of green tea (Hung *et al*. 2012a).

Although there are no *in vivo* studies into therapeutic activity of *O. fragrans*, some in vitro investigation have studied its bioactivity. It has been found to inhibit lipid peroxidation through ferrous chloride in the mitochondria in rat brain, liver, heart, and kidney (Lee *et al*. 2007). It may also exert neuroprotective actions through its upregulation of the AKT survival pathway, attenuating neurotoxicity (Lee *et al*. 2007). Several lignans isolated from the flowers of *O. fragrans* var. aurantiacus were found to inhibit nitric oxide production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages (Lee *et al.* 2011). In addition, our team found that the *O. fragrans* flower extract to have anti-depressive (Hung *et al*. 2012b), anti-allergic (Tu *et al.* 2013) and anti-pulmonary fibrosis capabilities (Hung & Ye, 2011). All of these studies showed a strong enhanced antioxidant status after the consumption of the extract from the *O. fragrans* flower.

Recently, our group reported that *O. fragrans* flower beverage supplementation upregulated antioxidant parameters, including ORAC, GSH, GSSG, and GSH/GSSG ratio, GPx and SOD, in adults. After seven days of daily consuming 250 mL of a beverage made by brewing 2 g of dry *O. fragrans* flower in 1L of water, subjects were found to have increased plasma ORAC activity, increased GSH and GPx content, and decreased GSSG, suggesting

that consumption of this beverage might increase the antioxidant defense of the healthy individuals (Li *et al*. 2013).

In the aforementioned study, five phenolic compounds, including tyrosyl acetate (1), (+)-phillygenin (2), (8E)-ligustroside (3), rutin (4), and verbascoside (5), were isolated from the $CHCl_3$ layer of *O. fragrans*. Evaluation of the antioxidative properties of the isolated compounds (2), (4), and (5) revealed strong 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging activity and H_2O_2-scavenging ability. A higher percentage of rutin and verbascoside was found in an ethanol than a methanol extract of *O. fragrans* (data not shown). Considering that these findings suggest that further research in vivo studies, we decided to evaluate the safety of the 75 % ethanol extract of *O. fragrans* (OFEE). To do this, we conducted an acute and subacute toxicity test, following the safety assessment guidelines developed by the Department of Health of Taiwan (DOH 1999, Mehta *et al*. 2009). In both, female BALB/c mice were randomized to control and study groups which received an oral administration of OFEE. In the acute toxicity test, 5 g/kg and 10 g/kg of OFEE were administered (Cheng *et al.* 2010) and in the subacute toxicity study 1 g/kg was administered daily for 28 consecutive days.

2. Materials and methods

2.1. Animals

Healthy 6-week-old female mice (BALB/c, body weight, 16-18 g), obtained from Biolasco Taiwan Co., Ltd. (I-Lan, Taiwan), received a general physical examination when we received them and were acclimatized for 7 days. The animals were housed in cages (6 per cage) and provided with food (Lab Diet 5001 Rodent diet; Purina Mills LLC, St. Louis, MO, USA) and water ad libitum. The stainless steel cages were kept at 21 ± 2 ℃ with 50-70 % humidity under a 12-h light/12-h dark cycle. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of Chung Hwa University of Medical Technology (IACUC: A93-11).

2.2. Plant material

The dried flowers of *O. fragrans* were purchased from a traditional market at Guilin, Guangxi Province, China, in 2009. The samples were authenticated by Dr. Mo-shin Tang, Department of Pharmaceutical Sciences and Technology, Chung Hwa University of Medical Technology. Voucher specimen of *O. fragrans* (HCY091001) has been deposited at the herbarium of the Department of Food Nutrition, Chung Hwa University of Medical Technology, and Tainan, Taiwan.

2.3. Preparation

The dried flowers of *O. fragrans* were ground into a fine powder using a mill (RT-08, Rong Tsong, Taiwan), collected, sealed in a polyethylene plastic bag, and stored at 0–4°C for further use. For extraction, *O. fragrans* flowers (200 g) were soaked (72 h) 75% ethanol (3 L) twice and filtered through Whatman No. 1 filter paper. The combined extracts were concentrated under reduced pressure and freeze-dried to obtain dark syrup, which was stored at -20 °C for further use. The extraction yield of 75 % ethanol extract was presented as % weight (gram of 75 % ethanol extract for each 100 g of dry herbs) and the results are shown in Table 1.

2.4. Determination of total phenolic content

As described by Yen & Hung (2000), we mixed the sample solution in methanol (0.1 mL, 1 mg/mL) with 2 % Na_2CO_3 (2 mL). After 3 min, 50% Folin-Ciocalteaure agent (0.1 mL) was added. The mixture was allowed to stand at room temperature for 30 min with intermittent mixing. The absorbance at 750 nm was recorded. A standard curve using gallic acid was prepared. The total phenolic content was expressed as gallic acid equivalents (mg of GAE per g extract).

2.5. Determination of total flavonoid content

Following the methods described by Woisky & Salatino (1998) and also by Chang *et al.* (2002), 0.5 mL of sample solution was mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M KOAc, and 2.8 mL of distilled water. The mixture was allowed to stand at room temperature for 30 min, and absorbance was measured at 415 nm. The amount of sample solution was substituted by the same amount of a quercetin solution (0–200 μg/mL) as a standard. The amount of 10% $AlCl_3$ was substituted by the same amount of distilled water as a blank. Total flavonoid content was calculated from the plot of absorbance against quercetin concentration using linear regression analysis and expressed as quercetin equivalents (μg of QE per g extract).

2.6. DPPH free radical scavenging assay

DPPH is a stable free radical that has a purple color which is reduced to a colorless compound by antioxidants. We used DPPH in an assay method modified by Shimada *et al*. (2002). Methanol (3.8 mL), sample solution in methanol (0.2 mL, 1 mg/mL) and 1 mM DPPH solution (1.0 mL) were mixed well and left to stand in the dark at room tempertrue for 30 min. The final concentration of the sample was 40 μg/mL. The absorbance at 517 nm was measured. The sample in methanol was used as a blank, and the DPPH radical in methanol solution was used as a control. The DPPH radical scavenging activity was calculated using the equation:

% of DPPH radical scavenging activity = $[1 - (A_{sample} - A_{blank})/A_{control}] \times 100$, where A is the absorbance at 517 nm.

The concentration providing 50% inhibition (IC_{50}) DPPH radical scavenging activity was calculated from the plot of inhibition percentage against sample concentration by linear regression.

2.7. Oxygen radical absorbance capacity (ORAC) assay

The total antioxidant activity of the organ samples was measured by using the oxygen radical absorbance capacity (ORAC) assay according to Chung *et al.* (2005). This assay was carried out in black-walled, 96-well plates at 37°C. All solutions were prepared in 75 mM phosphate buffer (Na_2HPO_4:NaH_2PO_4, pH 7.0) and pre-incubated at 37°C for 30 min before use. 15 μL of organ homogenate (diluted 100 times) and 100 μL of 0.1 μM β-PE (β-phycoerythrin) were transferred directly into the well to incubate for 10 min using the FLUOstar OPTIMA microplate reader system (Galaxy BMG LABTECH Inc., Cary, NC). 85 μL of 75 mM 2, 2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was added rapidly and the fluorescence measured immediately using fluorescence filters with an excitation wavelength of 480 nm and an emission wavelength of 520 nm. The fluorescence was recorded at 5 min intervals for 120 min until the final value was less than 5% of the initial value. ORAC values from samples were calculated by using the following equation and expressed as Trolox equivalents: ORAC value (mM) = $20 \times k \times (S_{sample} - S_{blank})/ (S_{Trolox} - S_{blank})$, where k is the sample dilution factor. The area under the curve (S) was calculated by the following equation:

$S = (0.5 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + f_{20}/f_0 + f_{25}/f_0 + \bullet\bullet\bullet\bullet\bullet + f_{120}/f_0) \times 5$

Where f_0 was initial fluorescence reading at 0 min and fn represented measurement at time n.

2.8. Single-dose acute study assessing the toxic effects of OFEE treatment

Female BALB/c mice were randomly assigned to a control group and two study groups treated with OFEE. The two study groups were treated with a single dose of 5 g/kg and 10 g/kg of OFEE, respectively. The control animals were gavaged with 0.9 % nor-

mal saline. The administration volume of 0.9 % normal saline was 10 mL/kg body weight. The animals were observed daily for signs of intoxication, including behavioral changes and changes body weight. Body weight was measured weekly for 14 days. On the next day, animals were anesthetized with pentobarbital and blood withdrawn via the abdominal aorta.

2.9. 28-day toxicity subacute study assessing the toxic effects of OFEE treatment

Female BALB/c mice were gavaged with OFEE at the dose of 1 g/kg orally for the 28 consecutive days. The control group was treated with 0.9 % normal saline (10 mL/kg). During the treatment period, weekly body weight and daily food consumption were recorded. On day 28, all animals were fasted overnight. On the next day, animals were anesthetized with pentobarbital and then blood withdrawal for blood biochemical analysis.

2.10. Histopathologic studies

During autopsy, select vital organs (spleen, lungs, liver and kidneys) were excised, blotted and weighed. All organs were observed by microscope of 400X. Tissues were fixed in 10 % buffered neutral formalin. H& E staining was also conducted.

2.11. Hematological analysis

Blood samples were analyzed for red blood cells (RBC) count, haemoglobin (Hb), haematocrit (HCT), mean corpuscular haemoglobin (MCH), and mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), and white blood cells count (WBC). Haematological analyses were performed using a fully automated haematological analyser (Abbott Cell-Dyn 3500, Abbott Laboratories, IL, and USA).

2.12. Serum biochemical analysis

Blood samples were kept at room temperature for 1 h, and then centrifuged at 1000 g for 10 min to obtain serum. The serum biochemical parameters, including glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), blood urea nitrogen (BUN), creatinine, albumin (ALB) and total protein (TP) were assayed using commercially available kits from Randox Laboratories Co. (Antrum, UK).

2.13. Statistics

The results are expressed as mean ± SD. Sample sizes are indicated by n. Variation analysis was performed using ANOVA software. Two-tailed unpaired student's t-test was used to compare the differences within a group or between groups. In all cases, a p value of < 0.05 was considered significant

3. Results

3.1. The total phenolic, flavonoid contents, DPPH scavenging effects and ORAC of OFEE

The total phenolic contents in OFEE were 371.71 ± 12.35 mg GAE/g extracts and total flavonoid contents were 47.23 ± 5.36 mg QE/g extract. As seen in Table 1, the ethanol extract of DPPH IC_{50} was 8.6 µg/mL, which was less than methanol extract (12.8 µg/mL), and trolox (4.9 µg/mL) (Hung *et al*. 2012). As previously reported, *O. fragrans* was a rich phenolic- and flavonoid-content and found to exhibit great antioxidative activity (Hung *et al*. 2012).

3.2. Single-dose acute toxicity of OFEE in BALB/c mice

During the 14-day post dosing observation period, all test mice appeared healthy and normal (data not shown). No abnormal signs or death was observed. The maximum tolerated dose (MTD) of 75 % ethanol extract of *O. fragrans* was more than 10.0 g/kg. Thus, the 75 % ethanol extract of *O. fragrans* at 5 g and 10 g/kg body weight had no acute toxic effect based on the criteria established for mice (Department of Health of Taiwan 1999).

3.3. Body weight of mice treated for 28-day with OFEE

Mice treated with *O. fragrans* extract for 28 consecutive days were weighted weekly. The mean group weekly body weights over time are presented in figure 1. Body weight was found to be slightly increased on day 7, 14, and 28. (Day 0: controls 16.37 ± 0.85 and OFEE treated groups 17.78 ± 0.75; Day 7: controls 19.27±1.18 and OFEE treated groups 18.73 ± 1.42; Day 14: controls 19.58 ±1.36 and treated groups: 19.40 ± 0.77; Day 28: controls 20.10 ± 1.38 and treated groups 19.38 ± 1.08) (n=6 in each group, all $p > 0.1$). We found no significant differences in body weight changes between control and study groups. All weight changes were within the normal range, and, therefore, attributed to normal biological variation.

Table 1: The total phenolic, flavonoid contents, DPPH scavenging effects and ORAC in OFEE.

	Extraction yield	Total phenolic contents	Total flavonoid contents	DPPH scavenging effects	ORAC
	(%)	(mg GAE/g extract)	(mg QE/g extract)	IC_{50}(µg/mL)	(mM trolox equivalent)
OFEE	6.4	371.71 ± 12.35	47.23 ± 5.36	8.6	0.38 ± 0.02

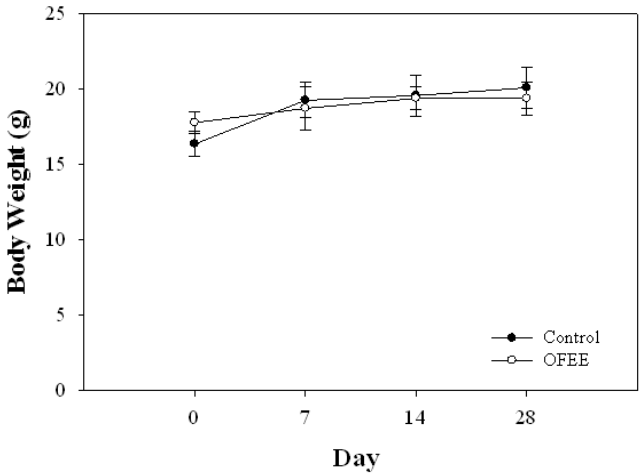

Fig. 1: Body weight changes of after oral administration with OFEE for 28-day. No significant difference was observed in the body weight of mice after daily gavage with OFEE or control group.

3.4. Histopathologic assessment on the organs of mice treated for 28-day with OFEE

Histopathological assessment is required to reveal the intoxication phenomena in organisms. The livers, spleens, lungs, and kidneys of mice treated with OFEE for 28-day was subjected to such an assessment. On day 29, mice received a detailed post-mortem examination of the internal organs. No macroscopic differences were found in size, color or texture of the organs. Microscopic findings on H&E stained sections are shown in fig. 2. No cellular atrophy, necrosis or any cellular damage in organ tissues was found (controls vs. treatment group: liver A1 and A2; spleen B1 and B2; lung C1 and C2; and kidney D1 and D2). Under micro-

scope observation, no significant lesions were found in livers, kidney, spleen and lung of the treated mice. Morphological analysis of the kidney sections revealed no significant acute tubular injury, including vacuolization, loss of brush borders, sloughing of tubular cells into the lumen, or flattening of the tubular epithelium. The administration of OFEE did not affect liver tissue of mice, as evidence by a lack of morphological differences in the lining between the study mice and the controls. Hepatocellular cells were arranged in neat rows and showed, no signs of porridge necrosis or fibrosis, suggesting no portal chronic inflammation. The other vital organs (lung and spleen) appeared normal and did not show any alterations in structure. Analysis of tissues from the mice showed no feeding toxicity.

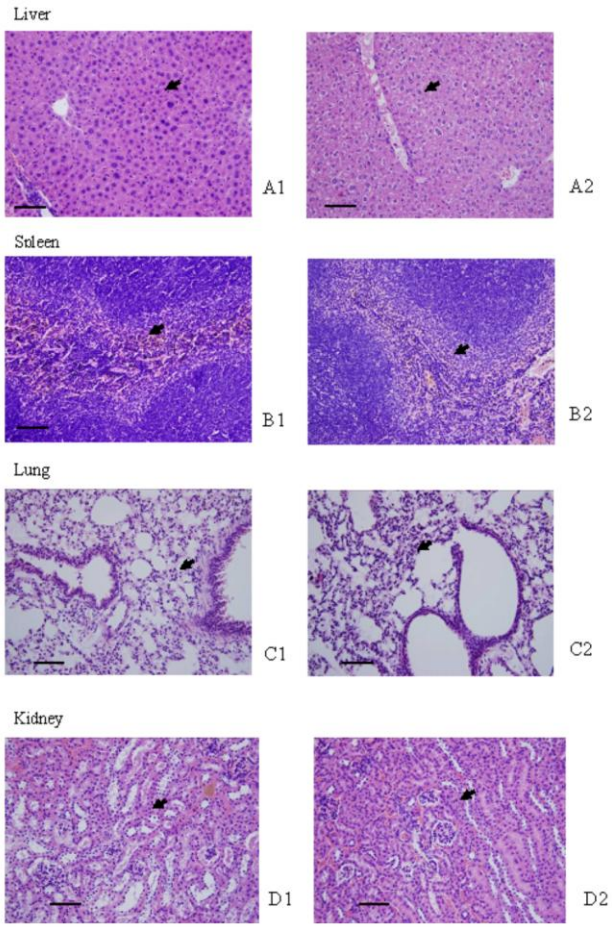

Fig. 2: Histopathologic of liver in all treated groups with OFEE for 28-day. A: Control; B: OFEE (Scale bar = 50μm) (400x).

2.5. Hematological and biochemical assays performed for mice treated with OFEE for 28-day

The results of hematological and biochemical assays are also crucial toxicological indices in organisms. We examined such indices, including white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), hematocrit (HCT), and mean cell volume (MCV) (Table 2). We also examined biochemical indices, including glutamate oxaloacetate transaminase (GOT), glutamic-pyruvic transaminase (GPT), blood urea nitrogen (BUN), creatinine, albumin and total protein (TP). The administration of OFEE did not lead to increased activity of serum toxicity marker enzymes (GOT, GPT), indicating normal liver function (Table 3).
No significant difference was found any of the hematologically examined items between controls and study groups. Furthermore, no significant differences were found in any of the biochemically examined items between control and treatment groups.

Table 2: Hematological parameters in BALB/c mice treated with OFEE or control.

Hematological	Unit	control	OFEE	P value
White blood cell	$\times 10^9$/l	7.47(0.17)	7.39(0.15)	0.11
Red blood cell	$\times 10^{12}$/l	8.06(0.12)	8.12(0.13)	0.10
Hemoglobin	g/dl	12.26(0.14)	12.31(0.12)	0.31
Hematocrit	%	44.83(2.95)	46.33(3.01)	0.08
MCV	fl	52.58(1.08)	53.16(1.20)	0.07
MCH	pg	15.39(0.20)	15.49(0.14)	0.08
MCHC	g/dl	29.29(0.10)	29.33(0.07)	0.13

Table 3: Biochemistry parameters in BALB/c mice treated with OFEE or control.

Biochemistry	Unit	control	OFEE	P value
GOT	U/l	181.84(2.25)	180.52(1.39)*	0.038
GPT	U/l	53.51(2.17)	52.72(2.14)	0.22
BUN	mg/dl	24.04(0.29)	23.88(0.15)	0.065
Creatinine	mg/dl	0.37 (0.03)	0.36(0.04)	0.27
ALB	g/l	31.76(0.37)	31.57(0.31)	0.07
TP	g/l	58.82(0.39)	58.77(0.40)	0.65

*Significant difference between the control group and OFEE group at $p < 0.05$

4. Discussion

To the best of our knowledge, there are no published studies on the toxicological profile of OFEE following acute and subacute exposure. In our acute toxicity study, a high dose of OFEE at 10 g/kg was used expecting that it would induce some observable toxic effects in the BALB/c mice. However, this preliminary toxicological study found no such toxic effect up to 10 g/kg body weight of OFEE when administered orally. Our subacute toxicity study (28-day) indicated the absence of cumulative toxicity and a no-observed-adverse-effect level (NOAEL) of 1 g/kg of OFEE.
In the present studies, the results suggested that 75% ethanol can be safely used in the extraction of *O. fragrans*, as evidenced by no significant change in weight and no damage to the organ tissues in mice treated for 28-day consecutively. In addition, there were no significant differences in hematological and biochemical assay finding between the study group and controls. None of the mice subjected to our acute toxicity test died or showed any abnormalities. All tested mice appeared healthy and normal (data not shown).
The 28-day subacute toxicity study involved a dosage equivalent to human consumption of 937.5 g *O. fragrans* per 60 kg body weight per day. This is a 937.5 to 468.8-fold higher dose than the estimated average human dietary intake (1-2 g *O. fragrans*/ 250 mL one cup per day). A commonly reported average dose of *O. fragrans* is about 15.625 g dried herb, which is equivalent to 1 g of the OFEE (yield of 75 % ethanol extract = 6.4 % of the dried material) considering an average body weight of an adult of 60 Kg. This dose for a 60 kg human is equal to 60 g of OFEE /day, or 937.5 g *O. fragrans*/day.
As new drugs are developed, it is important to translate the drug dosage from one animal species to another, including humans. It has been evidenced that animal dose should not be extrapolated to a human equivalent dose (HED) by a simple conversion based on body weight. The evaluation of animal toxicity screening systems can be used as a tool to enable safe introduction of new drugs into humans, although the authors of those studies did not attempt to relate therapeutic doses in various species (Freireich *et al.* 1966, Schein *et al.* 1970). Reagan-Shaw *et al*. (2007) suggest using the body surface area (BSA) normalization method to obtain the most appropriate conversion of drug doses from animal studies to human studies. BSA correlates well across several mammalian species with several biological parameters, including oxygen utilization, caloric expenditure, basal metabolism, blood volume, circulating plasma proteins, and renal function. However, the Food and Drug Administration (2002) has suggested that the extrapolation of animal dose to human dose is correctly performed only through

normalization to B.S.A., which often is represented in mg/m2. Reagan-Shaw *et al.* (2007) advocate the use of BSA as a factor when converting a dose for translation from animals to humans. To convert the dose used in a mouse to a dose based on surface area for humans, multiply 1000 mg/kg (Baur's mouse dose) by the 3 Km factor for a mouse and then divide by the 37 Km factor for a human. This calculation results in a human equivalent dose for OFEE of 81.1 mg/kg, which equates to a 4864.9 mg dose of OFEE, or 76.01 g *O. fragrans* for a 60 kg person. Based on these models of calculation, *O. fragrans* extract and *O. fragrans* flower beverages are safe.

5. Conclusion

In conclusion, based on evidence by our acute and sub-acute toxicity studies in rats in our study, the consumption of *O. fragrans* extract is safe. It can be used in herbal formulas without any toxic effects and be used safely in the development of health foods.

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Conflicts of Interest

The authors declare no conflict of interest.

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