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RESEARCH ARTICLE

Acute and developmental toxicity assessment of erincine A-enriched *Hericium erinaceus* mycelia in Sprague–Dawley rats

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ABSTRACT

This study aimed to establish an *in vitro* model to confirm the efficacy of erinacine A-enriched *Hericium erinaceus* (EAHE) mycelia and investigate its potential adverse effects in a preclinical experimental setting, including an assessment on the oral administration of EAHE mycelia in acute and prenatal developmental toxicity tests. At a single dose of 5000 mg/kg body weight, EAHE mycelia elicited no death or treatment-related signs of toxicity in ten Sprague–Dawley rats of both sexes during the 14 days of the experimental period. After considering the recommended dose range of EAHE mycelia from the acute toxicity test as well as the therapeutic doses, EAHE mycelia was administered to 66 pregnant rats in the low, medium, and high-dose groups by gavage at 875, 1750, and 2625 mg/kg body weight, respectively. All dams were subjected to a Caesarean section on the 20th day of pregnancy, and the fetuses were examined for any morphological abnormalities. Results indicated that weight of uterus, fetal body weight, number of corpora lutea, implantation sites, pre-implantation loss, and post-implantation loss of the treatment groups and the control group exhibited no statistical difference. In addition, no significant differences were observed in the fetal external, organ, and skeletal examinations. Taken together, it can be concluded that EAHE mycelia is considered safe and practically nontoxic for consumption within the appropriate doses and investigation period in this study.

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Introduction

Hericium erinaceus, also known as Lion's Mane or yamabushitake, is a medicinal and culinary mushroom that has been a part of traditional Chinese medicine and as a food in Asia for centuries. Habitually, this mushroom has been used for promoting digestive health, including improving liver health (Zhang *et al*. 2012), healing peptic and duodenal ulcers (Abdulla *et al*. 2008), and curing chronic gastric inflammation (Wong *et al*. 2013). Until recently, scientists have paid attention to the therapeutic potential of *H. erinaceus* and used it as a neuroprotective agent for the treatment and prevention of dementia (Phan *et al*. 2017).

In the adult brain, nerve growth factor (NGF) plays a key role in the survival and maintenance of basal cholinergic neurons in the forebrain (Lad *et al*. 2003). The forebrain is the largest section as it is involved in sensory integration, voluntary movement control, and high intellectual functions. Studies have shown that the bioactive compounds in *H. erinaceus*, known as erinacines, can stimulate the production of NGF in nerve cells (Ma *et al*. 2010), and thus help to maintain and protect the brain's cholinergic system.

Research has demonstrated specifically that erinacine A can increase NGF content in the central nervous system of rats (Yamada *et al*. 1997). Further research has shown erinacine A-enriched *H. erinaceus* (EAHE) mycelia are capable of delaying neuronal cell death in rats with neurodegenerative diseases, such as stroke (Lee *et al*. 2014), Parkinson's disease (PD) (Kuo *et al*. 2016), and Alzheimer's disease (AD) (Tsai-Teng *et al*. 2016). As there is evidence that EAHE mycelia can play a role in disease prevention, the consideration of the safe use of these ingredients is important.

Previous studies of genotoxicity (Li *et al*. 2014a) and systemic short-term toxicity (Li *et al*. 2014b) supported the use of EAHE mycelia in humans. However, there is inadequate acute toxicity and teratogenicity research on EAHE mycelia. To date, this study is the first attempt to investigate the impacts of EAHE mycelia exposure on acute toxicity as well as prenatal development of both the embryo and fetus.

Methods

Preparation of EAHE mycelia and analyses

HE mycelia were grown under optimal conditions to enhance the production of erinacine A, which was quantified by HPLC to be 5 mg/g (Li *et al*. 2014a, 2014b).

Preparation of EAHE extract

According to a previous study, ethanol was the most favorable condition for the extraction of erinacine A (Kawagishi

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et al. 1994). For the ethanol extract preparation, 1000 ml of absolute alcohol was added to 40 g of erinacine A powder, filtered through Whatman filter paper No. 4, and concentrated through a rotary evaporator (R-220; Büchi Labortechnik AG, Flawil, Switzerland). Dried ethanolic extract was then further reconstituted in DMSO at different concentrations for exposure to the cells.

Cell culture

Primary cultures of neonatal cortical neuronal cells were prepared according to a previous study (Wang *et al*. 2001) with minor modifications. Briefly, the cortex isolated from postnatal day 1 Sprague–Dawley rat pups by decapitation was digested in 0.5 mg/ml papain (P5306; Sigma-Aldrich, MO, USA) at 37 °C for 15 min and then was dissociated in Hibernate A medium (containing B27 supplement; Thermo Scientific, USA) by aspiring trituration. After the cell clumps were allowed to settle for 5 minutes, the supernatant was discarded, and 0.9 M sucrose was then added. The cells were triturated again before being passed through a sterile nylon mesh, pelleted at 750 g for 10 minutes, and resuspended in 10 ml neurobasal medium containing B27 supplement. Cells were plated onto poly-L-lysine-coated plates for four days, exposed to 5 μM cytosine-β-D arabinofuranoside (C1768; Sigma-Aldrich, USA) for one day to eliminate the proliferation of non-neuronal cells, and treated with different concentrations of EAHE extracts on the eighth day.

Immunofluorescence and imaging

Treated neuronal cells were fixed using 3.7% paraformaldehyde (158127; Sigma, USA) in PBS buffer at room temperature for 30 min. Following rinses in PBS, cells were blocked and permeabilized with 1% BSA/0.5% Triton X-100 (T8787; Sigma-Aldrich, MO, USA) in PBS at room temperature for 60 min to reduce nonspecific binding. Neuronal cells were then incubated with rabbit anti-microtubule associated protein 2 (MAP2) antibody (sc-20172; Santa Cruz Biotechnology, TX, USA) and mouse anti-tau antibody (sc-390476; Santa Cruz Biotechnology, USA) for overnight at 4 °C and then followed by appropriate fluorescent secondary antibodies Alexa 488 donkey anti-rabbit IgG (green, ab150073; Abcam, Cambridge, UK) and Alexa 568 donkey anti-mouse IgG (red, ab175472; Abcam, Cambridge, UK) for 1 h at 37 °C. Nuclei were labeled with Hoechst 33342 (H3570; Thermo Fisher Scientific, USA) for 30 min at room temperature. Images were acquired using a conventional inverted confocal microscope with a digital camera (LSM800; Carl Zeiss, Germany).

Experimental animals

All animal testing was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Five-week old male rats (weighing 264–298 g), female rats (weighing 182–207 g), and pregnant rats (weighing 211–296 g) were procured from BioLASCO Taiwan Co., Ltd. The animals were kept in pairs within polyethylene cages under standard laboratory conditions (22 ± 3 °C temperature, 55 ± 5% relative humidity, 12-h light/dark cycles, and 10–15 air changes/h) and had free access to commercial pellet diet (MGF; Oriental Yeast Co., Tokyo, Japan) and water.

Acute oral toxicity assay

This study was conducted as per Organization for Economic Co-operation and Development (OECD) test guidelines (OECD 2008) and approved by the Institutional Animal Care and Use Committee (IACUC#101-1b). Twenty rats, half male and half female weighing 264–298 g and 182–207 g respectively, were randomly assigned to one treatment group and the control group (five rats per sex per group). Following the period of fasting, animals were weighed and given EAHE once orally at a dose of 5 g/kg body weight while the control animals received distilled water. A constant volume of 10 mL/kg body vehicle control or EAHE was administrated to rats by adjusting the concentration to minimize variability in the test volume. Animals were observed individually for signs of toxicity, mortality, morbidity, and body weight changes during the study period. At the end of 14 days, all surviving mice were sacrificed, and the blood and organs were collected for hematology, clinical biochemistry, and pathological examination.

Prenatal developmental toxicity

The study was performed according to the OECD 414 guidelines (OECD 2001) with the IACUC's approval (#103–9 s). About 88 pregnant Sprague–Dawley rats were randomly divided into four test groups, including three dosages test groups and one negative control group. During the gestation period of 6–15 days, the three test groups received oral gavage of 10 mL of EAHE at 875, 1750, and 2625 mg/kg, respectively, while the negative control group received distilled water. Detailed clinical observation, individual body weight measurement, and food intake were monitored daily during the study period. On the 20th day, the female rats were euthanized via inhalation of CO_2, and subject uterus and fetuses were delivered immediately by cesarean sections. The number of implantations, corpora lutea, live fetuses, stillbirths, and absorbed fetus were counted. The percentages of pre-implantation and post-implantation loss were calculated as follows:

$$\text{Pre}-\text{implantation loss (\%)} = \frac{\text{number of corpora lutea} - \text{number of implantation sites}}{\text{number of corpora lutea}} \times 100$$

$$\text{Post}-\text{implantation loss (\%)} = \frac{\text{number of immplantation sites} - \text{number of livefetuses}}{\text{number of immplantation sites}} \times 100$$

The live fetuses were individually weighed, gender determined, and evaluated for external abnormalities. Afterwards, half of the live fetuses of each pregnant rat were fixed with

10% neutral buffered formalin for visceral examination while the remaining half were subjected to skeletal examination after 1% KOH Alizarin staining.

Statistical analysis

The dam body weight, feed intake, uterus weight, fetal weight, corpora lutea, implantations, pre-implantation loss, and post-implantation loss were conducted using one-way ANOVA followed by the Duncan's multiple range test using the SPSS statistical software. The gender ratio as well as the external, visceral, and skeletal examination data were analyzed by the Chi-squared test. All results were presented as a mean ± standard deviation and were considered to have statistically significant differences at $p < 0.05$.

Results

EAHE ethanolic extract induces neuritogenesis in postnatal cortical neurons

Primary postnatal cortical neurons were exposed to different concentrations of EAHE ethanolic extract and cultured for 48 h. The immunofluorescence staining data show that EAHE ethanolic extract enhances neurite growth of primary cortical neurons in a dose-dependent manner (Figure 1).

Acute toxicity study

All rats treated with EAHE mycelia showed no treatment-related toxicity or mortality, indicating that EAHE mycelia have a negligible toxicity on the growth of the animal. In addition, no body weight loss was detected (Figure 2), and all visceral organs examined during necropsy were free from pathological changes seen with the naked eye (Figure 3). Therefore, a lethal dose of orally administered EAHE mycelia is considered to be more than 5000 mg/kg in male and female rats.

Prenatal developmental study

Based on the recommended dose range of EAHE mycelia from the acute toxicity test and the therapeutic doses from a previous study (Kuo *et al.* 2016), dose levels of 875, 1750, and 2625 mg/kg body weight (50, 100, and 150 times the recommended daily intake, respectively) were chosen to be tested in the prenatal developmental toxicity study. EAHE mycelia administration during pregnancy resulted in no significant changes in the maternal body weight (Figure 4). Moreover, the mean uterus weight, mean corpora lutea, and number of implantation sites did not differ among groups at any time during the study (Table 1). Regarding fetal development, no significant difference was observed in any of the end-points, such as the litter number and the number of fetuses between the control and the treatment groups ($p > 0.05$, one-way ANOVA followed by Duncan's test) (Table 1). No between-group differences were observed as the mean weight of the fetuses was 4.2 ± 0.4 for the control group and 4.2 ± 0.4, 4.2 ± 0.4, and 4.2 ± 0.4 for the 875, 1750, and 2625 mg/kg EAHE groups, respectively. Similarly, there were no significant differences in the fetus gender ratio, the number of live fetuses, the number of stillbirths, the number of resorption fetuses, the pre-implantation loss, and the post-implantation loss among the four groups studied ($p > 0.05$, one-way ANOVA followed by Duncan's test) (Table 1).

Fetal external and organ analyzes recorded among the groups were statistically equivalent ($p > 0.05$, one-way ANOVA followed by Duncan's test) (Table 2). Although no significant difference was apparent in the skeletal examination between the groups, there was, however, a significant decrease in the number of unossified 5th sternebrae in the

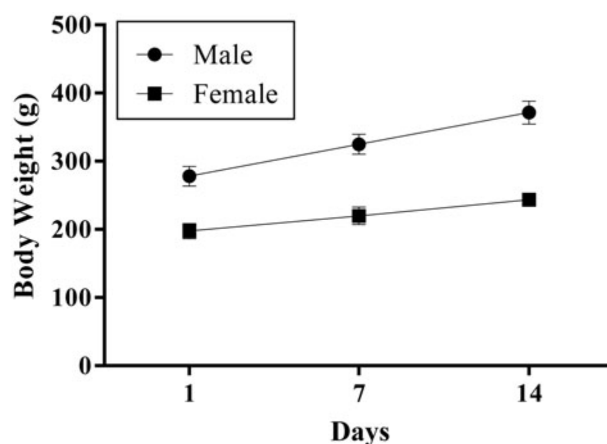

Figure 2. Body weight changes of male and female rats exposed to a single dose of EAHE mycelia at a dose level of 5000 mg/kg body weight. Data are expressed as mean ± SD.

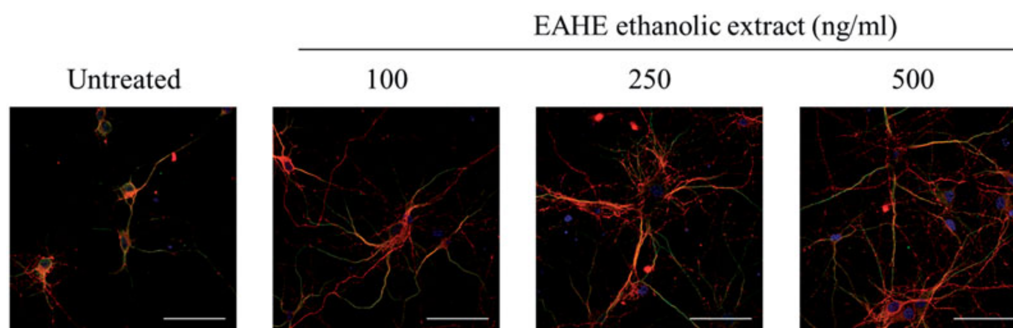

Figure 1. EAHE ethanolic extract induced neuronal growth and neurite elongation in postnatal cortical neurons in a dose-dependent manner. Cells were treated with or without EAHE ethanolic extract for 2 d, fixed, and labeled with DAPI (nucleus; blue), tau-1 antibody (axonal marker; red), and anti-MAP2 (somatodendritic marker; green). Scale bar, 50 µm.

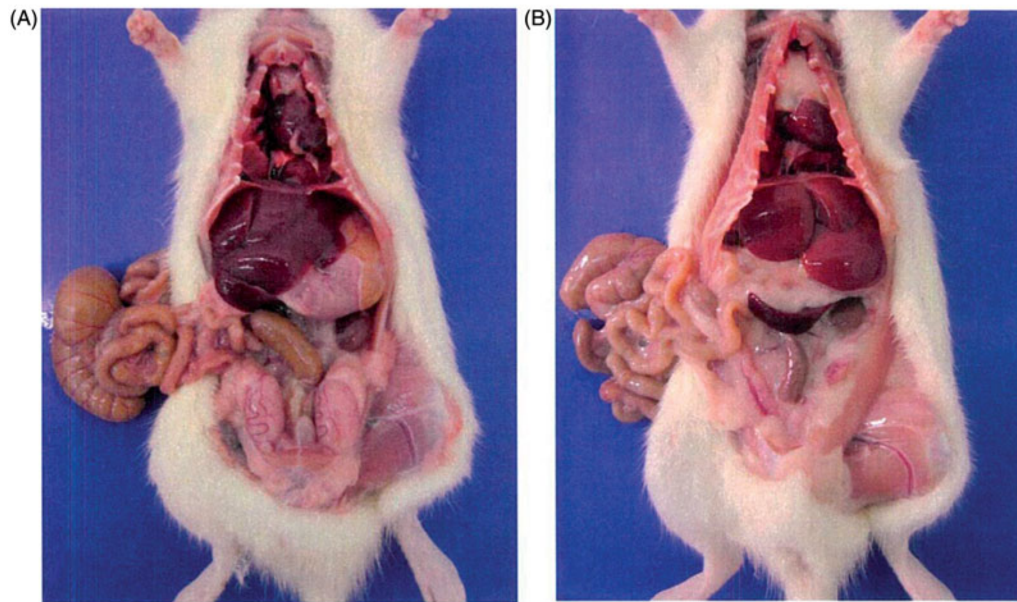

Figure 3. Representative photos of major organs in (A) male and (B) female rats at the end of the experiment.

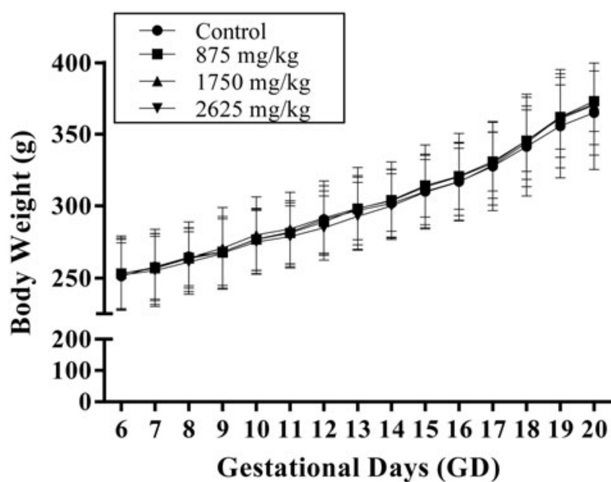

Figure 4. Changes in maternal body weights of rats exposed to EAHE on gestational day 6 until parturition. Data are expressed as mean ± SD.

low dose group (8/136) when compared to the control group (25/116) ($p < 0.05$, one-way ANOVA followed by Duncan's test) (Table 3).

Discussion

Following maternal exposure to EAHE, nearly all therapeutic agents cross into the placenta and enter the fetus (Wallach *et al*. 1991). However, there is an estimation of 15–50% of expectant mothers that were unaware of their pregnancy when taking pharmaceutical agents during the sensitive phase of organogenesis (Zaki and Albarraq 2014). The safe use of these agents providing medical health benefits during gestation, therefore, has become an increasingly contentious issue as medicines have a tendency to produce congenital malformations in the offspring at birth until proven otherwise.

Hericium erinaceus is an ideal culinary-medicinal mushroom and has recently attracted considerable attention for its positive effects on brain and nerve health by inducing NGF from its bioactive ingredient, erinacine A. This natural inducer of NGF synthesis with a low-molecular weight not only demonstrated its efficacy in several mouse models of age-associated neurological disorders but also achieved such effectiveness via oral delivery (Lee *et al*. 2014, Kuo *et al*. 2016, Tsai-Teng *et al*. 2016). Moreover, EAHE mycelia products have successfully launched onto the market, so there is a high likelihood that these may be used by pregnant women intentionally or unintentionally. Hence, there is an urgent need to acquire more data regarding the safety of their use during pregnancy.

In the present study, an *in vitro* model was first established as it played a role in confirming the efficacy of EAHE mycelia. Once mass functional EAHE mycelia containing 5 mg/g erinacine A were produced using submerged culture, the dried mycelia were extracted with ethanol and screened on this *in vitro* platform to assess whether they have the potential to stimulate neurite outgrowth and neuronal differentiation. In the absence of NGF, the EAHE mycelia extract was able to induce neurite outgrowth in primary cultures of rat cortical neurons in a concentration-dependent manner. A functional maturation of a neurite into an axon and a dendrite was also observed by the immunostaining of MAP2 and tau, respectively, as these proteins are present only in differentiated neurons (Dehmelt and Halpain 2004). Moreover, quantitative real-time PCR analysis consistently revealed significantly higher NeuroD1, MAP2, and Tuj1 (mature neuronal markers) gene expression levels in the EAHE-treated primary cortical neurons compared to untreated and NGF-treated cells (data not shown). Since NGF is a large molecule and cannot cross the blood–brain barrier, along with being susceptible to degradation via enzymatic attacks, a safer therapy using EAHE that promote NGF synthesis inside the brain (Ma *et al*. 2010) as well as induce postnatal cortical neuronal growth with differentiation suggested its further potential in the treatment of neurodegenerative diseases.

Table 1. Effects of EAHE on uterus examination, ovaries examination, and fetal survival.

	Control 0 (mg/kg)	Low dose 875 (mg/kg)	Medium dose 1750 (mg/kg)	High dose 2625 (mg/kg)
Mean uterus weight (g)	80.2 ± 23.5	77.2 ± 26.5	82.4 ± 13.1	82.0 ± 17.4
Number of corpora lutea	12.7 ± 4.8	14.3 ± 1.7	13.7 ± 3.5	14.1 ± 2.8
Number of implantation sites	11.4 ± 5.1	13.0 ± 1.9	12.4 ± 3.5	13.0 ± 2.8
Litter number	20	22	21	22
Number of fetuses	252	286	273	285
Mean weight of fetuses (g)	4.2 ± 0.4	4.2 ± 0.4	4.2 ± 0.4	4.2 ± 0.4
Fetus gender ratio[a]	0.98	1	0.87	1.05
Number of live fetuses	252	286	273	285
Number of stillbirths	0	0	0	0
Number of resorption fetuses	16	18	12	11
Pre-implantation loss[b]	6.3 ± 19.1	4.5 ± 6.6	4.2 ± 6.0	5.6 ± 8.6
Post-implantation loss[c]	7.1 ± 10.3	6.0 ± 7.4	4.5 ± 7.2	4.1 ± 5.6

Data are expressed as mean ± SD.
[a]Fetus gender ratio = number of male rats ÷ number of female rats.
[b]Pre-implantation loss (%) = (number of corpora lutea − number of implantation sites) ÷ number of corpora lutea ×100%.
[c]Post-implantation loss (%) = (number of implantation sites − number of live fetuses) ÷ number of implantation sites ×100%.
*Significantly different from the negative control group ($p < 0.05$).

Table 2. Effects of EAHE on fetal external and visceral examinations.

	Control 0 (mg/kg)	Low dose 875 (mg/kg)	Medium dose 1750 (mg/kg)	High dose 2625 (mg/kg)
Number of litters for external examination (*n*)	20	22	21	22
Number of fetuses for external examination (*n*)	252	286	273	285
Number of fetuses exhibited abnormal findings				
Number/percentage	0/0	1/0.35	0/0	1/0.35
Number of litters for visceral examination (*n*)	20	22	21	22
Number of fetuses for visceral examination (*n*)	136	150	148	153
Type of visceral variations				
Ureter dilation				
Number of fetuses/percentage	2/1.5	2/1.3	3/2.0	2/1.3
Number of litters/percentage	2/10.0	2/9.1	3/14.3	2/9.1
Renal pelvis dilation				
Number of fetuses/percentage	2/1.5	1/0.7	1/0.7	1/0.7
Number of litters/percentage	2/10.0	1/4.5	1/4.8	1/4.5

Table 3. Effects of EAHE on fetal skeletal examination.

	Control 0 (mg/kg)	Low dose 875 (mg/kg)	Medium dose 1750 (mg/kg)	High dose 2625 (mg/kg)
Number of litters for skeletal examination (*n*)	20	22	21	22
Number of fetuses for skeletal examination (*n*)	116	136	125	132
Type of skeletal variations				
Vertebrae, thoracic centrum, bifid				
Number of fetuses/percentage	5/4.3	6/4.4	5/4.0	10/7.6
Number of litters/percentage	5/25.0	5/22.7	5/23.8	7/31.8
Sternebrae 5th, unossified				
Number of fetuses/percentage	25/21.6	8/5.9*	10/8.0	18/13.6
Number of litters/percentage	10/50.0	5/22.7*	7/33.3	13/59.1
Sternebrae 6th, unossified				
Number of fetuses/percentage	17.14.7	15/11.0	11/8.8	9/6.8
Number of litters/percentage	10/50.0	10/45.5	9/42.9	9/40.9
Metacarpals 2nd or 5th digits, unossified				
Number of fetuses/percentage	3/2.6	1/0.7	2/1.6	1/0.8
Number of litters/percentage	2/10.0	1/4.5	2/9.5	1/4.5
Rib 14th lack				
Number of fetuses/percentage	0/0.0	0/0.0	0/0.0	1/0.8
Number of litters/percentage	0/0.0	0/0.0	0/0.0	1/4.5

*Significantly different from the negative control group ($p < 0.05$).

After confirming the effectiveness of EAHE mycelia, the safety of the ingredient should also be considered. While studies on acute toxicity have appeared in the literature (Li *et al.* 2014b), this study is the first to report a complete acute toxicity test of EAHE mycelia administered orally at a dose level of 5000 mg/kg body weight to both sexes of Sprague–Dawley rats. Since no remarkable changes were observed in the general symptoms, clinical signs, gross pathology, and mortality rates with up to 5000 mg/kg body weight of EAHE administered orally for 14 d, it can be inferred that EAHE is nontoxic. Acute toxicity evaluation is usually the initial step in assessing the toxic characteristics of

a substance and can be useful in providing information to predict potential human hazards (Chinedu *et al.* 2013). However, in order to achieve the applicability of animal experiments to the human condition, a prenatal developmental toxicity should also be conducted to develop a complete toxicological database.

As EAHE exposure could differentiate and express neuronal markers from our previous *in vitro* results, there is a possibility that it may affect the normal course of fetal development when the ectoderm differentiates to form the nervous system. Since the fetus is exceptionally vulnerable to unwanted environmental factors during several developmental processes that have been shown to be particularly susceptible to toxic exposures (Grandjean and Landrigan 2006), the effect of EAHE on the developing fetus cannot be ignored or overlooked. In this study, the administration of EAHE mycelia to pregnant rats throughout day 6–15 of gestation had no adverse effect on maternal toxicity and embryo-fetal development. The body weight and feed intake of dams from the three treatment groups were not significantly different from the control group, which indicated that EAHE mycelia had no adverse effects on the weight gain of dams. In addition, the uterus, ovaries, and fetal examinations were not significantly different between the treatment and control groups, suggesting that the EAHE treatment had no effect on the body weight and survival rate of the fetuses. Although the total numbers and the incidence rate of unossified 5th sternebrae were significantly reduced in the low-dose group in comparison with the control group, this change was not dose-dependent and can therefore be considered as an individual variation and not a toxic effect induced by EAHE mycelia.

Conclusions

Results indicated that weight of uterus, fetal body weight, number of corpora lutes, implantation sites, pre-implantation loss, and post-implantation loss of the EAHE mycelia treatment groups and the control group exhibited no statistical differences. Furthermore, no fetal deformations were observed from the fetal external, organ, and skeletal examinations, indicating that administering EAHE mycelia at even the highest dose level resulted in no detrimental changes on rat fetal development. In converting the mouse dose to human dose, the recommended daily allowance of EAHE mycelia is 1050 mg/60 kg body weight/day.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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