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TEL: (+84-24) 38247058 FAX: (+84-24) 39348740
Email: tapchiduoclieu@nimm.org.vn; tapchiduoclieu@gmail.com

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PROGESTERONE-LIKE EFFECTS OF *HAPLOPTERIS FLEXUOSA* (FÉE) E.H. CRANE ON VAGINAL BLEEDING IN AN EXPERIMENTAL MURINE MODEL

Dinh Thi Minh[1], Vu Minh Chau[2], Dang Ngoc Tuan Anh[2], Dinh Thi Huyen Trang[3], Pham Thi Nguyet Hang[1,4,*]

[1]National Institute of Medicinal Materials (NIMM), Hanoi, 11022, Vietnam;
[2]Hanoi University of Pharmacy, Vietnam;
[3]Vinh University, Vietnam; [4]VNU University of Medicine and Pharmacy, Hanoi, Vietnam
*Corresponding author: nguyethangpt@nimm.org.vn
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Summary

The study evaluated the progesterone-like effects of the ethanol extract of *Haplopteris flexuosa* (Fée) E.H. Crane in the treatment of vaginal bleeding, with potential application in managing menorrhagia, using murine models that mimic human menstruation. The results showed that vaginal bleeding mice treated with the ethanol extract of *H. flexuosa* (at doses of 0.8 g/kg and 1.6 g/kg) and Utrogestan (progesterone) (at a dose of 0.05 g/kg) exhibited significant improvement in pathological symptoms, including an increase in progesterone levels, a subsequent reduction in the number of cornified cells in the vaginal lavage fluid, increased endometrial thickness, and reduced uterine-ovarian weight with a recovery trend. Furthermore, histology of the uterus showed an increased number of glands, with only mild shedding of the endometrial lining. In conclusion, *H. flexuosa* exhibits progesterone-like effects in a murine model of vaginal bleeding and may potentially be applied in the treatment of menorrhagia. These results provide valuable insights into the mechanisms and applications of *H. flexuosa* in progesterone-related therapies for women's health issues.

Keywords: *Haplopteris flexuosa*; *Vaginal bleeding; Menorrhagia; Heavy menstrual bleeding; Progesterone; Mice.*

1. Introduction

Vaginal bleeding due to abnormal uterine bleeding (AUB), including its prevalent subgroup (heavy menstrual bleeding - HMB), is a common problem among women of reproductive age [1]. The estimated prevalence of HMB in women of reproductive age is approximately 30% [2]. HMB significantly impacts patients' physical and mental well-being, including increasing the risk of anemia, fatigue, and depression [3]. Current treatments for HMB include combined oral contraceptives, progestogens, nonsteroidal anti-inflammatory drugs (NSAIDs), tranexamic acid, and levonorgestrel-releasing intrauterine system (LNG-IUS) [4]. Surgical intervention is only recommended when pharmacological therapy fails, or when there is severe anemia or additional uterine abnormalities [5]. However, due to the adverse drug reactions of modern medicines, an increasing number of women are turning to treatments of herbal medicine [6]. In recent years, the clinical use of traditional herbal medicines in the management of HMB has attracted more attention due to their strong efficacy and fewer side effects [7]. In this study, we evaluated the effects of *H. flexuosa* on vaginal bleeding. According to local knowledge in Ha Tinh, Vietnam, patients with menorrhagia and hormone-related abnormal uterine bleeding were commonly treated with *H. flexuosa* before receiving hormonal therapy, in order to minimize the side effects of hormonal medications. This approach yielded positive outcomes. *H. flexuosa* is a relatively new medicinal plant in Vietnam and has not yet been given a Vietnamese common name, so before conducting the study, the plant was identified by the Center for Medicinal Material Resources - National Institute of Medicinal Materials (NIMM). *H. flexuosa* is a fern species in the family Pteridaceae, this species was first scientifically described by E.H. Crane in 1997 [8]. Up until now, studies have only focused on botanical characteristics and structure, with very few examining its medicinal properties. Consequently, we explored its properties, to enrich Vietnamese herbal medicinal resources. Due to its traditional uses, we evaluated the effects of *H. flexuosa* in the treatment of vaginal bleeding or menorrhagia by using murine models mimicking menstruation. We focused on the progesterone-like effects of the plant by evaluating several criteria, including the number of cornified cells in vaginal lavage fluid, serum progesterone concentration in mice, uterine and ovarian weight, histology, and endometrial thickness.

2. Materials and methods

2.1. Materials

❖ *Sample*

Whole plants of *Haplopteris flexuosa* (Fée) E.H. Crane were harvested in Ha Tinh (March 2022) and identified by the Center for Medicinal Materials Resources, NIMM. To prepare a 70% ethanol extract of *H. flexuosa* (abbreviated as HE), 1000 g of *H. flexuosa* was extracted with 10 liters of 70% ethanol for 2.5 hours at the solvent's reflux temperature, then the mixture was filtered to obtain the extract. The residue was extracted two more times with 70% ethanol (8 liters each time) under reflux for 2 hours and 1.5 hours, respectively. The filtrates from all three extractions were combined, and ethanol was recovered under reduced pressure. The remaining water was evaporated using a water bath, and the final product was dried in a vacuum oven, yielding 205.92 g of extract with a moisture content of 3.71%, corresponding to an extraction yield of 20.59%.

The dose used in mice is adjusted to the dose commonly used in humans in traditional medicine (16-32 g of crude plant material per person per day), corresponding to ethanol extract of *H. flexuosa* at doses of 800 mg/kg and 1600 mg/kg.

❖ *Animals*

Healthy *Swiss albino* mice of both sexes, aged 8-10 weeks and weighing 35-40 g, were obtained from the National Institute of Hygiene and Epidemiology (NIHE). All animals met experimental standards. The animals were housed under standard laboratory conditions: temperature at 25 ± 1°C, relative humidity at 65 ± 5%, and a 12/12-hour light/dark cycle (light period from 7:00 to 19:00). Food and water were available *ad libitum*. They were acclimatized for 5 days prior to the start of the experiment.

❖ *Chemicals*

Utrogestan 100 mg (Besins, France) containing progesterone used as a positive control at a dose of 0.05 g/kg (dispersed in Span 20 and diluted in CMC 0.5%); Progesterone assay kit (Thermo Fisher Scientific, USA); Sesame oil for intrauterine injection (Sigma-Aldrich, USA); Hematoxylin (Merck, Germany) and Eosin (Sigma-Aldrich, USA) for staining.

2.2. Methods

We used a murine model of menstruation as described by Marion Rudolph in 2012 [9]. The experimental design is summarized in the following diagram:

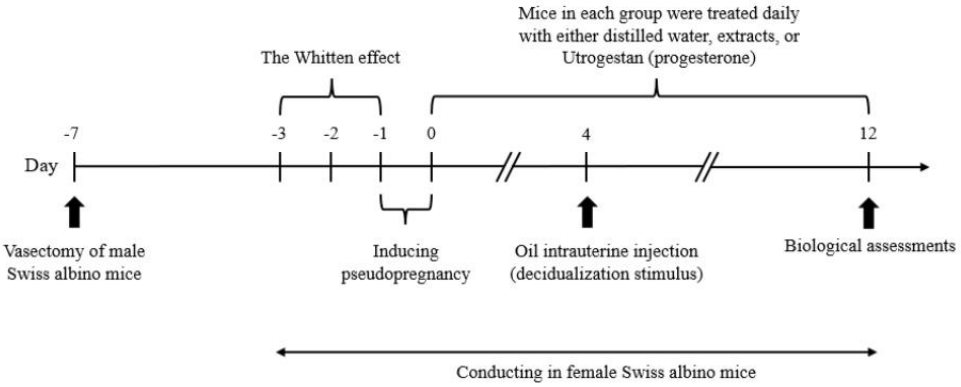

Fig. 1. Experimental design diagram.

2.2.1. Vasectomy of male *Swiss albino* mice:

Bilateral vasectomy using 8-10-week-old male mice was conducted as follows:

Step 1: Anesthetize mice with pentobarbital at a dose of 0.05 g/kg using intraperitoneal injection.

Step 2: Place each mouse in a prone position, then expose and disinfect the shaved surgical area.

Step 3: Make a 1 cm incision on each side of the spine, approximately 0.5 cm lateral to the spine and 3 cm from the base of the tail. Cut through the skin, subcutaneous tissue, and muscle layer. Remove the surrounding adipose tissue to expose the vas deferens, which is then ligated.

Step 4: Suture the connective tissue layers and muscles, then suture the skin and disinfect the area with 10% Betadine solution.

Step 5: After surgery, mice are placed in a warm cage and monitored until full recovery from anesthesia.

2.2.2. The Whitten effect

The Whitten effect (Whitten WK (1968)) synchronizes the estrus cycle in female mice through volatile pheromones secreted in the urine of male mice [10]. The vasectomized male mice were placed in cages above female mice for 48 hours before mating. The purpose was to expose female mice to pheromones from male mouse urine through airflow, stimulating the sense of smell and inducing estrus according to the Whitten effect. After 2 days, the estrus status was determined by observing the vagina with a magnifying glass based on characteristic signs. In the proestrus stage, the vagina is open, the mucosal tissue is pink-red, moist, and has many folds running along the length of the 2 labia majora; and in the estrus stage, signs are similar to that of the proestrus stage but the tissues are brighter pink and less moist, the folds are also more obvious. Mice clearly defined in the estrus stage were selected for the next steps.

2.2.3. Establishment of murine models mimicking human menstruation and evaluation of the effects of HE in the treatment of vaginal bleeding

Female mice in estrus were mated overnight with vasectomized male mice. Pseudopregnant females were identified based on the presence of a vaginal plug (marking day 0 of the experiment). A sham group was prepared consisting of physiologically normal mice without inducing pseudopregnancy and vaginal bleeding. The animals were divided into 5 experimental groups, with 6-8 mice per group:

- Sham (1): physiologically normal mice without vaginal bleeding were orally received distilled water.
- Vaginal bleeding (VB) (2): Vaginal bleeding mice were orally received distilled water.
- VB + HE (3 and 4): Vaginal bleeding mice were orally administered the HE at doses of 0.8 g/kg and 1.6 g/kg, respectively.
- VB + Utrogestan (5): Vaginal bleeding mice were orally administered Utrogestan (progesterone, at a dose of 0.05 g/kg).

Mice in each group were treated as previously described once daily for 12 days at 10 mL/kg body weight. On day 4 of pseudopregnancy, 100 µL of sesame oil was injected intrauterinely into the female mice to induce decidualization. On day 12, criteria were assessed 1 hour after drug administration as follows:

❖ *Assessment of the extent of endometrial shedding through the number of cornified cells*

The extent of endometrial shedding was assessed by counting the number of cornified cells [11]. First, the vaginal canal of the mice was washed with 40 µL of phosphate-buffered saline (PBS). Then, 3 µL of the vaginal lavage fluid was placed on a glass slide, dried, and stained with H&E (hematoxylin and eosin). The number of cornified cells on each slide was counted at 5 random points, and the average value was calculated.

❖ *Determination of mouse serum progesterone by ELISA*

Tail blood was collected from mice to determine serum progesterone levels on day 12. The levels were determined according to the instructions of the Progesterone Kit. The progesterone level of each sample was calculated based on the standard curve.

❖ *Histological analysis*

The weights of the uterus and ovaries were determined using an analytical scale. Samples of uterine tissues were fixed in PBS-buffered 4% formaldehyde. After conducting paraffin infiltration of uterine tissue, paraffin-embedded sections (5 µm thick) were stained with hematoxylin and eosin (H&E staining). Histopathological evaluation was performed under a light microscope to assess endometrial thickness and the number of uterine glands. The thickness was determined by measuring the distance from the endometrial surface to the boundary with the myometrium. Measurements were taken at 3 to 5 different locations, and the average value was calculated.

❖ *Statistical analysis*

Statistical analyses were performed using IBM SPSS Statistics version 20 and GraphPad Prism software. Data are presented as the mean ± standard error of the mean (S.E.M) and analyzed by unpaired Student's t-test for two groups or one-way ANOVA followed by the *Student-Newman-Keuls* test for multiple comparisons. P values < 0.05 were considered statistically significant.

3. Results

3.1. Evaluation of the effect of HE on endometrial shedding based on the number of cornified cells

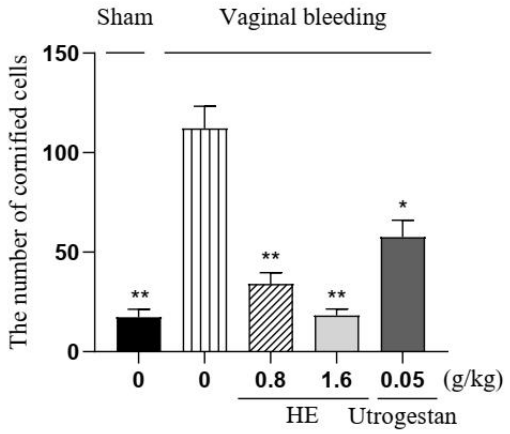

Fig. 2. Effect of HE on the improvement of endometrial damage in the vaginal bleeding (VB) mice.
*n = 6-8; significant values are shown as *p < 0.05; **p <0.01 versus the VB group.*

Fig. 2 shows that the number of cornified cells in the vaginal lavage fluid on day 12 in the VB group was higher than in the sham group, with $p < 0.01$. The number of cornified cells in HE groups (0.8 g/kg and 1.6 g/kg) was significantly lower than those of the VB group, with $p < 0.01$. Mice in the Utrogestan-treated group also had a lower number of cornified cells compared to the VB group ($p < 0.05$). Notably, the decrease in cornified cells in the higher dose was greater than that of the Utrogestan-treated group ($p < 0.05$).

Accordingly, it can be concluded that ethanol extract of *H. flexuosa* at doses of 0.8 and 1.6 g/kg has an effect in reducing the number of cornified cells in the vaginal lavage fluid, which is related to the reduction of endometrial shedding in the vaginal bleeding murine model.

3.2. Evaluation of the effect of HE on serum progesterone concentration in mice

Progesterone has been shown to be highly effective in the treatment of vaginal bleeding and menorrhagia [12],[13]. Therefore, measuring the serum progesterone concentration is an important criterion for evaluating the therapeutic efficacy of HE. The serum progesterone concentration was measured on day 12 using the ELISA technique.

Fig. 3 shows that on day 12, the progesterone concentration in the VB group was significantly lower than that in the sham group ($p < 0.01$). HE groups at doses of 0.8 and 1.6 g/kg showed a significant recovery in serum progesterone levels in the vaginal bleeding mice, with a statistically significant difference compared to the VB group ($p < 0.01$). The positive control drug, Utrogestan (0.05 g/kg), also significantly restored the progesterone reduction in vaginal bleeding mice ($p < 0.01$)

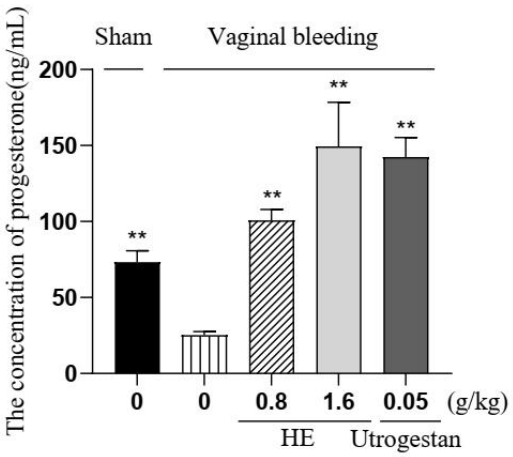

Fig. 3. Effect of HE on serum progesterone concentration in vaginal bleeding mice.
*n = 6-8; significant values are shown as **p <0.01 versus the VB group.*

3.3. Evaluation of the effect of HE on the weight of mouse uterus and ovaries

When serum progesterone levels decrease significantly, the uterus tends to enlarge considerably [9]. Therefore, uterine-ovarian weight serves as a criterion to evaluate the progesterone-enhancing effect of HE in the treatment of vaginal bleeding. On day 12, the mice were sacrificed to measure the uterine-ovarian weight.

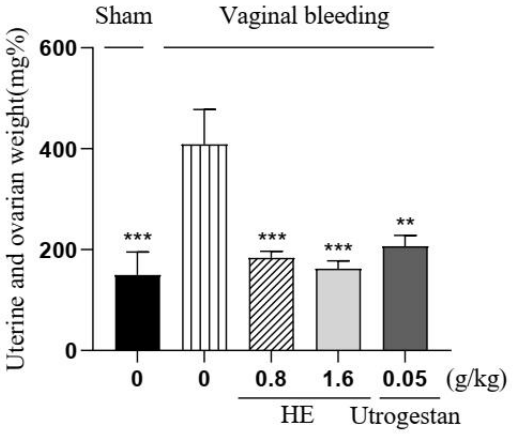

Fig. 4. Effect of HE on uterine-ovarian weight in vaginal bleeding mice.
*n = 6-8; significant values are shown as **p <0.01; *** p < 0.001 versus the VB group.*

The results show that on day 12, the uterine-ovarian weight in the VB group increased significantly compared to the sham group ($p < 0.001$). In HE groups (0.8 g/kg and 1.6 g/kg) and the Utrogestan group, the uterine-ovarian weight was substantially reduced compared to the VB group, with statistically significant differences ($p < 0.001$, $p < 0.001$, and $p < 0.01$, respectively).

3.4. Evaluation of the effect of HE on uterine histology in mice

During the menstrual cycle, a significant drop in progesterone levels causes the endometrial lining to become thinner, leading to its shedding and increased bleeding [14]. Therefore, to evaluate the effect of the treatment for vaginal bleeding, we conducted a histological assessment of the uterus on day 12.

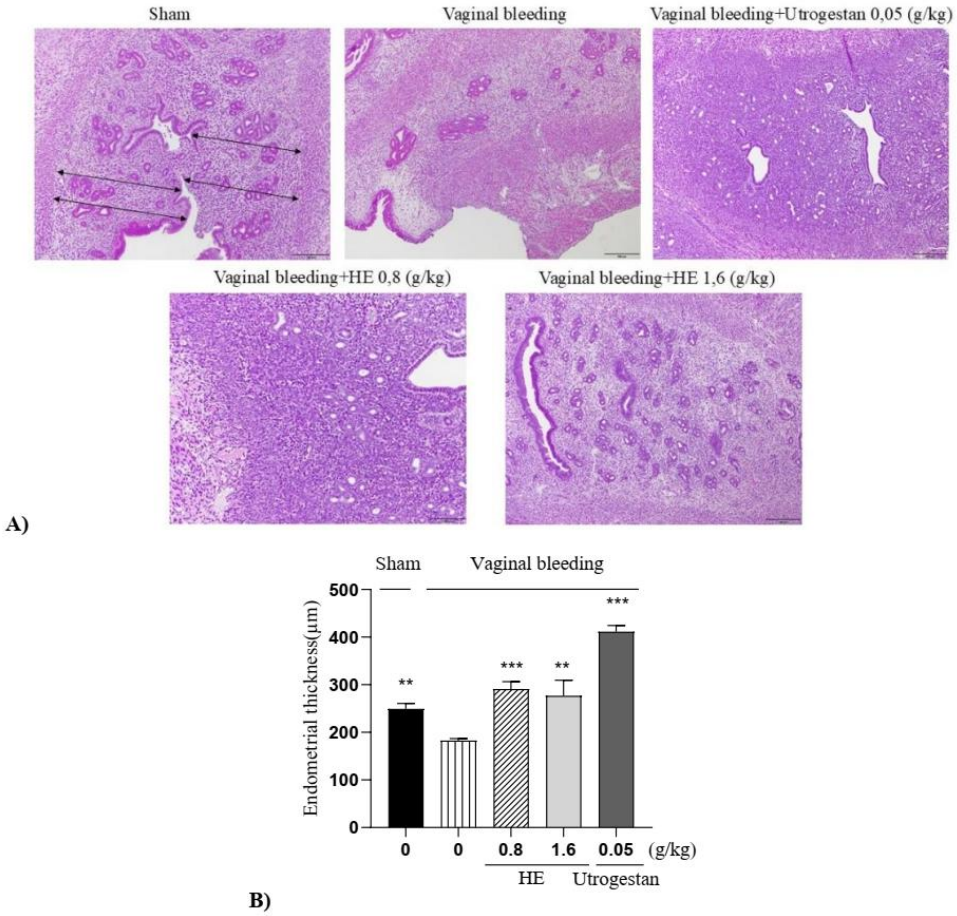

Fig. 5. Effect of HE on the uterus of mice with vaginal bleeding. On day 12, mice were sacrificed to collect the uterus and ovary. The uterus was subjected to histological analysis (A) and the endometrial thickness was assessed (B). *n = 6; significant values are shown as **p <0.01; *** p < 0.001 versus the VB group. Black arrow indicates the uterine thickness.*

Histological observation of the uterus on day 12 (Fig. 5A) showed that the VB group exhibited a reduction of gland number, as well as signs of endometrial shedding, congestion, and hemorrhage. However, in the HE-treated group at the dose of 0.8 g/kg, the number of glands increased, with mild congestion and no hemorrhage. In the HE-treated group at the dose of 1.6 g/kg and Utrogestan group, there was an increase in gland number, no signs of congestion or hemorrhage, and a relatively thick endometrium.

Fig. 5B indicates that endometrial thickness in the VB group was significantly lower than in the sham group ($p < 0.01$). Vaginal bleeding mice treated with HE (0.8 and 1.6 g/kg) showed a

significant increase in endometrial thickness compared to the VB group ($p < 0.001$ and < 0.01, respectively). However, the Fig. 5 in both HE-treated groups were notably lower than that of the Utrogestan group. In the Utrogestan group, endometrial thickness increased substantially compared to the VB group, with a statistically significant difference ($p < 0.001$).

4. Discussion

In adolescents and adult women, vaginal bleeding due to abnormal uterine bleeding (AUB), including its subgroup heavy menstrual bleeding (HMB), is a common condition. Since HMB is the prevalent subgroup of AUB, its causes are categorized using the PALM-COEIN classification system developed by FIGO (International Federation of Gynecology and Obstetrics) to classify the etiologies of AUB [1]. Among these categories, ovulatory dysfunction (AUB-O) is one of the most frequent categories. Ovulatory dysfunction results in a deficiency of progesterone and excessive estrogen (E2) production by ovarian follicles, leading to endometrial proliferation. This makes the endometrium more prone to irregular and excessive menstrual bleeding [14]. Progesterone treatment has been shown to significantly reduce menstrual blood loss, supporting its effectiveness in the management of heavy menstrual bleeding (HMB) [12],[13]. Therefore, investigating the progesterone-like effects of HE may serve as an appropriate therapeutic strategy for managing HMB and vaginal bleeding.

In humans, the reproductive cycle is referred to as the menstrual cycle, whereas in mice it is termed the estrous cycle. Both cycles involve the proliferation of endometrial stromal cells and ovulation, followed by corpus luteum formation in the ovary, which produces progesterone. The key difference is that in humans, stromal cells differentiate into decidual cells (decidualization). Decidualization occurs in response to rising progesterone levels during the luteal phase, even without embryo implantation. In mice, decidualization occurs only in response to implantation, typically on day 4 post-mating. The decidua provides a vascular network for gas and nutrient exchange to support embryonic development before the placenta is fully established. In both cycles, if implantation does not occur, the corpus luteum degenerates, leading to a drop in progesterone levels. This decline triggers endometrial tissue breakdown and shedding, resulting in menstruation (discharge of blood and endometrial tissue through the vagina) in humans, or reabsorption of the endometrium during the estrous cycle in mice [9]. Therefore, this study applied a murine model mimicking human menstruation by inducing pseudopregnancy and injecting sesame oil into the uterus on day 4 to evaluate the potential of the ethanol extract of *H. flexuosa* to increase progesterone levels and reduce menstrual-like bleeding.

According to the model by Marion Rudolph (2012), mice experience peak uterine bleeding on day 9, when progesterone levels are at their lowest, with spontaneous recovery observed by day 12 [9]. However, in our study, cornified cell counts from vaginal lavage fluid taken from day 6 to day 15 in normal mice group revealed that cornified cells peaked on day 12, accompanied by detectable vaginal bleeding, with spontaneous recovery by day 15. This discrepancy may be attributed to differences in mouse strains used across studies. As a result, we chose day 12 as the time point for biological assessments.

Progesterone plays a key role in regulating vaginal bleeding and is commonly used in clinical management of HMB and AUB. The efficacy of progesterone and related compounds in treating AUB and menstrual disorders is demonstrated [12]. Cyclic progesterone therapy administered for 14 days using oral norethindrone acetate or medroxyprogesterone acetate has been shown to reduce menstrual blood loss in 2%-30% of women. In contrast, continuous 21-day therapy (from day 5 to day 26 of the cycle) resulted in a reduction of blood loss in 63%-78% of women [15]. Based on these findings, we administered continuous therapy to mice with vaginal bleeding in this study (from day 0 to day 12).

The results showed that treatment with HE at doses of 0.8 g/kg and 1.6 g/kg significantly increased serum progesterone levels in mice with induced vaginal bleeding. According to Xu et al. (2007), epithelial cell degradation and detachment from the endometrium were observed when progesterone levels decreased [16]. These findings are consistent with the reduction in the number of cornified cells in vaginal bleeding mice treated with HE at doses of 0.8 g/kg and 1.6 g/kg. Moreover, when serum progesterone levels significantly declined, the size of the uterus increased and became considerably enlarged [9]. This also aligns with the results obtained in this study, which showed a reduction in uterine weight in the treatment groups. Furthermore,

progesterone has been shown to be closely related to the thickness of the endometrial lining. A sudden drop in progesterone leads to menstruation, and the endometrial lining becomes thinner, with only a few epithelial cells remaining at the base of the glands. The endometrial lining undergoes degeneration up to 65% of its thickness [14]. This was also demonstrated in the present study, with the effect of HE in increasing endometrial thickness and reducing endometrial shedding in vaginal bleeding mice. Therefore, it is necessary to develop phytochemical studies on *H. flexuosa*. To date, no studies have been reported on the chemical composition of either the species *H. flexuosa* or the genus *Haplopteris*; existing research has focused only on the family *Pteridaceae*. This family includes many species with notable phytochemical potential - 58 of which have been reported to possess therapeutic properties for various human ailments, including uterine disorders, wounds, hypertension, and a wide range of other diseases and health conditions [17].

5. Conclusion

The study demonstrated that HE has progesterone-like effects of *H. flexuosa*, to reduce vaginal bleeding, as indicated by reduced endometrial shedding, and decreased uterine and ovarian weights. These findings suggest that *H. flexuosa* may be a promising candidate for the treatment of heavy menstrual bleeding.

Acknowledgments: *This research is funded by the Institute of Medicinal Materials on the project "Establishment of murine models mimicking human menstruation and their application in researching the effects of Leonurus japonicus (*Houtt*) and Haplopteris flexuosa (*Fée*)* E.H. Crane *on vaginal bleeding in mice". We thank Prof. William R. Folk, University of Missouri for his suggestions and guidance for the manuscript.*

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