Analgesic and anti-inflammatory effects of hydroalcoholic extract isolated from Semen vaccariae

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Abstract: Semen vaccariae, the seeds of *Vaccaria segetalis* (Neck.) Garcke, is usually used as an important medication for female mammary gland diseases; it has also been used to promote lactation for centuries in China. The purpose of this work was to evaluate the analgesic and anti-inflammatory effects of hydroalcoholic extract from semen vaccariae (HESV) with oral doses of 50, 100 and 200mg/kg·bw in mice and rats. We observed that the HESV could effectively inhibit acetic acid-induced abdominal contraction and could elevate the latency time to thermal stimuli in the hot-plate test in mice. In the xylene-induced ear-swelling test in mice, HESV could suppress the ear swelling. Additionally, HESV could significantly decrease the peritoneal capillary permeability and leukocyte infiltration in mice induced by the intraperitoneal injection of acetic acid. HESV also significantly reduced paw thickness 2-4 hours after the injection of carrageenan in the carrageenan-induced rat paw edema test. This study was the first to demonstrate that the oral administration of HESV might play an important role in the process of analgesia and anti-inflammation, supporting its use for female mammary gland diseases in traditional medicine.

Keywords: *Semen vaccariae*; Hydroalcoholic extract; analgesic activity; anti-inflammatory activity.

INTRODUCTION

Semen vaccariae, the ripe seeds of *Vaccaria segetalis* (Neck.) Garcke, is a traditional herb described in Compendium of Materia Medica and is considered as an important medication for treating female mammary gland diseases and for promoting lactation. Semen vaccariae is usually used to activate blood circulation, regulate menstrual disturbances, dispel edema, and promote lactation for centuries in China (Li and Liang, 2007; Sang *et al*., 2003a).

Recent studies have shown that semen vaccariae is rich in flavonoids, triterpene saponins, alkaloids, cyclic peptide, phenolic acid and steroids cyclopeptides (Sang *et al*., 2003b); it also possesses various bioactivities. Zhang *et al*. (2013) have demonstrated that polysaccharide isolated from semen vaccariae has an inhibitory effect on the benign prostatic hyperplasia in mice induced by Pule'an. Segetalin A and segetalin E from semen vaccariae strongly inhibit the proliferation of human microvascular endothelial cells (Hua *et al*., 2009), and segetalin A and segetalin B have an estrogen-like activity (Itokawa *et al*., 1995). Triterpenoid saponin, isolated from semen vaccariae, has an inhibitory effect on luteal cells (Sang *et al*., 2000). Tong *et al*. (2012) have demonstrated that semen vaccariae positively impacts mammary gland development by promoting the proliferation and secretion functions of bovine mammary epithelial cells and modulating the expression of E-cadherin and β-catenin via the Wnt signaling pathway *in vitro*, supporting the traditional use of semen vaccariae for promoting lactation. The aim of this study were to evaluate the analgesic and anti-inflammatory effects of hydroalcoholic extract from semen vaccariae (HESV) in mice and rats and to present evidence for the traditional use of semen vaccariae to treat female mammary gland diseases, such as mastitis.

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MATERIALS AND METHODS

Reagents and drugs

Carrageenan, indomethacin, Evans blue and crystal violet were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Acetic acid was purchased from Tianjin Damao Chemical Reagent Factory (Tianjin, China). Xylene was purchased from Tianjin Fuchen Chemical Reagent Factory (Tianjin, China). The control medicinal material of semen vaccariae and vaccarin reference substance were purchased from the National Institutes for Food and Drug Control (Beijing, China).

HESV preparation

Semen vaccariae was purchased from the local market in Lanzhou city, China. Dongan Cui, Ph.D., of the Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences, China performed botanical identification and thin layer chromatography analysis according to the protocols described by the Chinese pharmacopoeia (2010). In particular, heavy metals, residual pesticides and microbial contamination of semen vaccariae were also detected, and the results met the safety standards of traditional Chinese medicine in China.

First, washing, drying, pulverizing and screening with a 3-mm mesh, treated semen vaccariae; then, the powder (200g) was refluxed with 70% ethanol (2000mL) for 4h at 7^{o}C according to an established extraction possess (results not published). The HESV (20.21g) was obtained by freeze-drying with a lyophilizer (Beijing Songyuan Huaxing Technology Develop CO. Ltd., Beijing, China) after extraction with hydroalcoholic liquid evaporation under reduced pressure at 45°C and the yield ratio was 10.1%. The marker compound of semen vaccariae was vaccarin. The content of vaccarin was 60.19mg/g determined with high-performance liquid chromatography (HPLC) according to the method described by the Chinese pharmacopoeia (2010). Karl Fischer analysis was used to determine the water content, which was between 3.50 and 4.11%.

Animal care and handling

Balb/C mice (18~22g) and Sprague-Dawley (SD) rats (160~200g) were purchased from the experimental animal center of Lanzhou University and were housed in plastic cages with free access to food and water at a temperature of 22±1°C and a relative humidity of 50±10%. This study was approved by the Institutional Animal Care and Use Committee of Lanzhou Institute of Husbandry and Pharmaceutical Sciences of the Chinese Academy of Agricultural Sciences (SCXK20008-0003). The animal protocols were in compliance with the ethical guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermanm, 1983).

Acetic acid-induced abdominal constriction in mice

The acetic acid-induced abdominal constriction test in mice was conducted according to a described protocol (Cho *et al*., 2013; Young *et al*., 2005). The mice were randomly divided into one of five groups (n=10) and were given intraperitoneal injections of 0.7% acetic acid at a dose of 10mL/kg·bw 1h after oral administration of HESV (50, 100 and 200mg/kg·bw) in the treatment groups, oral administration of indomethacin (3mg/kg·bw) in the reference group, or oral administration of normal saline (10mL/kg·bw) in the control group. The number of writhing movements, abdominal musculature contraction followed by the extension of hind limbs, was registered within 30 min after the injection of acetic acid. The inhibition ratio was calculated using the following equation:

$$\text{Inhibition ratio} = \frac{\text{Nc - Nt}}{\text{Nc}} \times 100\%$$

Nc represents the number of writhing movements in the control group, and Nt represents the number of writhing movements in the treatment group.

Hot-plate test in mice

The hot-plate test was performed according to a previously described protocol (Shinde *et al*., 1999). Groups of 10 female Balb/C mice were treated orally with HESV (50, 100 and 200mg/kg·bw) or indomethacin (3 mg/kg·bw) as a reference; normal saline (10mL/kg·bw) was used as a control. The latency times to thermal stimuli were recorded before (0min) and 30, 60 and 90 min following treatment with a cut-off time of 60sec to avoid paw lesions.

Xylene-induced ear swelling in mice

The xylene-induced ear-swelling test was performed according to a previously described method (Kou *et al*., 2005). Male mice were randomly divided into five groups (n=10 in one group), and ear swelling was induced by smearing 30μL of xylene on the anterior and posterior surfaces of the right ear 1h following treatment with HESV (50, 100 and 200mg/kg·bw, p.o.), indomethacin (3 mg/kg·bw, p.o.) or normal saline (10mL/kg·bw, p.o.). The left ear was as control on the same mouse. One hour later, all mice were sacrificed by cervical dislocation. Circular sections of both ears were collected with an 8 mm diameter punch and were immediately weighed. The swelling degree and inhibition ratio were calculated using the following equations:

$$\text{Swelling degree (SD)} = \frac{\text{Wr - Wl}}{\text{Wl}} \times 100\%$$

$$\text{Inhibition ratio} = \frac{\text{SDc - SDt}}{\text{SDc}} \times 100\%$$

Wr represents weight of the right ear, and Wl represents weight of the left ear of the same mouse; SDc represents the swelling degree in the control group, and SDt represents the swelling degree in the treatment group.

Acetic acid-induced leukocyte infiltration and capillary permeability in mice

The test was performed according to a previously described method (Lucena *et al*., 2007). The mice were randomly divided into five groups (n=10 in one group), and 0.5% Evans blue solution (10mL/kg·bw) was intravenously injected to the tail veins of mice 1h following treatment with HESV (50, 100 and 200 mg/kg·bw, p.o.), indomethacin (3mg/kg·bw, p.o.) or normal saline (10mL/kg·bw, p.o.); 10 min later, leukocyte infiltration and capillary permeability were induced by the intraperitoneal injection of 0.7% acetic acid at a dose of 10mL/kg·bw. Twenty minutes after the intraperitoneal injection of 0.7% acetic acid, all mice were sacrificed by cervical dislocation, and the peritoneal cavity was immediately opened; the peritoneal fluid was then collected after washing with 5mL sterile saline. Finally, the total leukocyte counts were recorded in the peritoneal fluid (50μL of peritoneal fluid was taken and diluted in 450μL of Türk's solution) according to microscopic analysis. The remaining peritoneal fluid was centrifuged at 3000 rpm for 15 min and the supernatant was collected. The absorbance of the supernatant was determined at 606 nm using an Evolution 300 UV-VIS spectrophotometer (Thermo Scientific, USA). The concentration of Evans blue in peritoneal cavity indicated the peritoneal capillary

permeability induced by acetic acid (Lucena *et al*., 2007). The inhibition ratios of leukocyte infiltration (IRLI) and capillary permeability (IRCP) in the treatment groups were calculated using the following equations:

$$\text{IRLI} = \frac{\text{Lc - Lt}}{\text{Lc}} \times 100\%$$

$$\text{IRCP} = \frac{\text{Ec - Et}}{\text{Ec}} \times 100\%$$

Lc represents the number of leukocytes in the control group, and Lt represents the number of leukocytes in the treatment group; Ec represents the concentration of Evans blue in the control group and Et represents the concentration of Evans blue in the treatment group.

Carrageenan-induced paw edema in rats

The carrageenan-induced paw edema test was conducted in rats according to a previously described method (Mandegary *et al*., 2012; Posadas *et al*., 2004). The rats were randomly divided into five groups (n=10 in one group), and edema of the right hind paw was induced by hypodermic injection with 100μL of 1% carrageenan suspension in normal saline 1h after treatment with HESV (50, 100 and 200mg/kg·bw, p.o.), indomethacin (3 mg/kg·bw, p.o.) or normal saline (10mL/kg·bw, p.o.). The right hind paw thickness was determined using a vernier caliper before and 1, 2, 3 and 4h after the injection of carrageenan. The swelling degree and inhibition ratio were calculated with the following equations:

$$\text{Swelling degree(SD)} = \frac{\text{PTt - PT0}}{\text{PT0}} \times 100\%$$

$$\text{Inhibition ratio} = \frac{\text{SDc - SDt}}{\text{SDc}} \times 100\%$$

PT0 represents the right hind paw thickness before carrageenan injection, and PTt represents the right hind paw thickness 1, 2, 3, or 4h after carrageenan injection; SDc represents the swelling degree in the control group, and SDt represents the swelling degree in the treatment group.

STATISTICAL ANALYSIS

The data are expressed as means ± SE. Data were analyzed with SPSS (version 17.0, IBM SPSS Statistics; USA) using a one-way analysis of variance (ANOVA) followed by least significant difference (LSD) as the post hoc test. The results were considered statistically significant at $P<0.05$.

RESULTS

Analgesic activity of HESV

The results of the analgesic effects of HESV are presented in tables 1 and 2. In the acetic acid-induced abdominal constriction test, HESV caused 37.54%, 53.50% and 57.83% inhibition of constrictions at oral doses of 50, 100, and 200mg/kg·bw. There was a significant and dose-

Table 1: Effect of HESV on acetic acid-induced abdominal constriction in mice

Groups	Dose (mg/kg·bw)	Number of writhing	Inhibition (%)
Control	-	41.10±5.69 ^{A}	-
Indomethacin	3	16.20±3.94^{B}	60.58
HESV	50	25.67±4.97^{B}	37.54
	100	19.11±3.86 ^{B}	53.50
	200	17.33±5.89 ^{B}	57.83

Table 2: Effect of HESV on the hot-plate test in mice

Groups	Dose (mg/kg·bw)	Latency period (s)			
		0min	30min	60min	90min
Control	-	22.23±4.53	18.59±3.87^{a}	18.16±4.98^{A}	17.14±5.85 ^{A}
Indomethacin	3	21.89±3.44	23.32±8.47	26.84±6.14^{B}	33.41±7.77 ^{B}
HESV	50	21.91±4.13	20.54±3.36	22.31±3.47	24.35±5.06 ^{B}
	100	20.18±6.35	20.03±5.99	23.86±4.18 ^{B}	27.25±5.29 ^{B}
	200	21.85±3.98	24.44±5.81^{b}	25.69±4.66 ^{B}	28.16±4.58 ^{B}

Table 3: Effect of HESV on xylene-induced ear-swelling in mice

Groups	Dose (mg/kg·bw)	Swelling (%)	Inhibition (%)
Control	-	64.16±5.20 ^{A}	-
Indomethacin	3	30.76±4.29 ^{B}	52.06
HESV	50	53.68±3.70 ^{B}	16.33
	100	40.80±4.62 ^{B}	36.41
	200	25.89±3.27 ^{B}	59.65

Values within the same row that are marked with different superscript letters differ significantly: a, b = $P<0.05$; A, B = $P<0.01$.

Table 4: Effect of HESV on acetic acid-induced leukocyte infiltration in mice

Groups	Dose (mg/kg·bw)	Total leukocytes ($\times 10^6$)	Inhibition (%)
Control	-	5.13±0.72 ^{A}	-
Indomethacin	3	3.27±0.36^{B}	36.26
HESV	50	4.07±0.62 ^{B}	20.66
	100	3.49±0.43 ^{B}	31.97
	200	3.14±0.45 ^{B}	38.79

Table 5: Effect of HESV on the acetic acid-induced Evans blue leakage test in mice

Groups	Dose (mg/kg·bw)	Evans blue (μg/mL)	Inhibition (%)
Control	-	7.13±0.99 ^{A}	-
Indomethacin	3	4.68±0.20 ^{B}	34.36
HESV	50	5.32±0.40 ^{B}	25.39
	100	4.81±0.32 ^{B}	32.54
	200	4.61±0.16 ^{B}	35.34

Table 6: Anti-inflammatory effect of HESV on carrageenan-induced paw edema in rats

Groups	Dose (mg/kg·bw)	1h		2h		3h		4 h	
		Swelling (%)	Inhibition (%)	Swelling (%)	Inhibition (%)	Swelling (%)	Inhibition (%)	Swelling (%)	Inhibition (%)
Control	-	32.94 ±5.75^{a}	-	58.59 ±3.75^{A}	-	71.61± 9.92^{A,a}	-	62.61± 4.66 ^{A}	-
Indomethacin	3	30.94 ±3.53	6.07	44.13 ±4.80^{B}	24.68	45.12± 3.43^{B}	36.99	26.95± 7.29^{B}	56.96
HESV	50	32.86 ±2.11	0.24	54.64 ±5.07	6.74	65.43± 2.61^{b}	11.42	41.67± 4.48 ^{B}	33.45
	100	32.10 ±2.09	2.55	44.40 ±4.73 ^{B}	24.22	52.84± 3.63 ^{B}	26.21	31.55± 5.72 ^{B}	49.61
	200	28.76 ±3.92^{b}	12.69	39.01± 3.40 ^{B}	33.42	42.29± 5.89 ^{B}	40.94	25.10± 4.64 ^{B}	59.91

Values within the same row that are marked with different superscript letters differ significantly: a, b = $P<0.05$; A, B =$P<0.01$.

dependent analgesic effect of HESV at 50~200mg/kg·bw compared to the control. In the hot-plate test, there was no significant effects were observed at 60 min after treatment at an oral dose of 50mg/kg·bw. However, there was a significant increase in the latency to thermal stimuli at 100mg/kg·bw at 60 min after drug administration and the effect of HESV (200mg/kg·bw) was significantly higher. In both cases, HESV increased the latency time, at 90 min after drug administration, at doses of 50, 100 and 200 mg/kg·bw.

Anti-inflammatory activity of HESV

In this study, HESV significantly suppressed xylene-induced ear-swelling in mice in a dose-dependent manner (See table 3). Additionally, the inhibition ratio of HESV (200mg/kg·bw, p.o.) exceeded that of indomethacin at 3 mg/kg·bw. After the intraperitoneal injection of acetic acid, there were significant increases in total leukocyte numbers and the Evans blue content extruded into the mouse peritoneal cavity in the control group (tables 4 and 5). Additionally, compared to the control, HESV could effectively decrease the total leukocyte numbers and the Evans blue content in the peritoneal fluid. Furthermore, in the acetic acid-induced leukocyte infiltration test, HESV-mediated inhibition at a dose of 200mg/kg·bw exceeded that of indomethacin at a dose of 3mg/kg·bw. In the carrageenan-induced paw edema, the hind paws of rats exhibited marked swelling at 3h after stimulation by carrageenan in the control and treatment groups; the degrees of swelling in the indomethacin and HESV (50, 100, and 200mg/kg·bw, p.o.) treatment groups were significantly decreased in the endpoint 4h of observation compared to the control (table 6).

DISCUSSION

Semen vaccariae has been widely available for centuries in China as an important medication for female mammary gland diseases. The present results provided the first evidence of the analgesic and anti-inflammatory activities of HESV in mice and rats.

In the acetic acid-induced abdominal constriction test and the hot-plate test, HESV significantly inhibited the number of mouse writhing and increased the latency to thermal stimuli. Thus, HESV might possess a strong analgesic effect. It exerted analgesic activity through peripheral mechanisms and central mechanisms in part.

The inflammatory reaction is a combination of a number of overlapping reactions within the body. In the early stages of this process, many types of inflammatory mediators, such as prostaglandins, bradykinin and histamine are produced by the inflamed tissue during the vascular reaction stage. Under the effect of these mediators, the endothelial cells of blood vessels shrink and endothelial cell gaps form. Additionally, local vasopermeability was enhanced by leukocyte-mediated endothelial cell injury (Santos *et al*., 2012). Peritoneal capillary permeability and leukocyte infiltration in the abdominal cavity are aggravated by intraperitoneal injection of acetic acid in mice (Li *et al*., 2010). HESV significantly suppressed xylene-induced ear-swelling in mice and decreased the total leukocyte numbers and the Evans blue content in the peritoneal fluid induced by intraperitoneal injection of acetic acid. Given these results, it was suggested that HESV might exhibit anti-inflammatory effects through modulating the cascade reaction of inflammatory mediators and decreasing leukocyte-mediated endothelial cell injury. Carrageenan-induced paw edema, including edema and hyperalgesia, is a reliable and repeatable model for evaluating the anti-inflammatory activities of natural products (Huang *et al*., 2011). In this study, the degree of swelling stimulated by carrageenan were significantly decreased by HESV compared to the control. It is a well-established fact that nonsteroidal anti-inflammatory drugs exert their analgesic and anti-inflammatory activities by inhibiting cyclooxygenase activity to decrease the inflammatory reaction (Rosa *et al*., 1971; Vane, 1971). We tentatively hypothesized that HESV could decrease inflammatory reactions by inhibiting cyclooxygenase activity in rats.

CONCLUSION

The present work is the first report on the analgesic and anti-inflammatory activities of HESV. We demonstrated that the oral administration of HESV might play an important role in the process of analgesia and anti-inflammation, supporting its traditional use to treat female mammary gland diseases, such as mastitis. Future investigations will focus on the broader involvement of the chemical constituents and mechanism(s) responsible for the pharmacological activities.

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