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Therapeutic Effect of Fructus Hordei Germinatus Ethanol Extract on Hyperplasia of Mammary Gland in Rats

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Abstract: Fructus Hordei Germinatus (Poaceae, Hordeum) has been traditionally used for treatment of female galactorrhea or Hyperplasia of Mammary Gland (HMG) in China. The overall purpose of this study was to evaluate the Anti-Hyperplasia of Mammary Gland (HMG) effect of Fructus Hordei G Ethanol extract (FHG-E) in HMG rats. Forty virgin female Wistar rats were randomly divided into blank control, HMG Model, Rupisanjie Capsule (RPSJC, positive control) and FHG-E groups, 10 in each. Injection of estrogen and progestogen were given to prepare rat models of HMG, RPSJC and FHG-E were given at the same time. Changes of nipple heights were measured sex hormone levels (E2, P, PRL, FSH and LH) in serum were estimated uterus and ovory index were calculated. Pathologic changes of mammary gland in rats were also observed by microscope. FHG-E could decrease the increased nipple height and uterus index, reduce the numbers of mammary gland lobules and secretion in HMG rats. It could also decrease E2, PRL and FSH levels in serum and increase the serum levels of P and LH significantly compared with the model group. The histopathological observation revealed that FHG-E obviously alleviated the degree of HMG. The contents of total flavonoid total alkaloid and total phenolic contents in the extract were also determined. The results showed that the 50% ethanolic extract from FHE contained total flavonoids 3.95±0.06 mg Quercetin Equivalent (QE)/g extract total alkaloids 42.74±0.08 mg Hordenine Equivalent (HE)/g extract and total phenolics 52.46±0.12 mg Gallic Acid Equivalent (GAE)/g extract. These results suggest FHG-E has therapeutic effects on HMG rats induced by estrogen and progestogen.

Key words: Fructus hordei germinatus, anti-inflammatory, anti-hyperplasia of mammary gland, rats, China

INTRODUCTION

Hyperplasia of Mammary Gland (HMG) is a kind of pathologyical hyperplasia of lobules of mammary gland induced by the balance disorder of estrogen and progesterone (Bennett *et al*., 1990) which were controlled and secreted abnormally by hypothalamus-pituitary-gonadal axis. HMG is related to menstrual cycle, breast-feed, occupation, abuse of sex hormone drugs, diet and mental pressure (Xu, 2011; Jia, 2011). The number of patients with HMG is increasing in recent years and it has severe cancerous tendencies may have been mixed and cover with early breast cancer. Studies have proved that traditional Chinese medicine could improve the regulatory mechanism in the body to inhibit HMG (Qian *et al*., 2007).

Fructus Hordei Germinatus is a kind of herb germinating from barley widely used in China. In the ancient book, Zhonghuazi of Song Dynasty, it was used to treat female galactorrhea or breast pain effectively (Wang and Wu, 2010). It has the effect of regulating endocrine disorder and is popularly used for the treatment of HMG in clinical (Yang and Lin, 2012; Mou, 1996). Fructus Hordei G was reported to contain constituents such as flavonoids, alkaloids and phenolics (Hu, 1999). In the present study, the ethanol extract from Fructus Hordei G was used to be examined. The anti-inflammatory and anti-HMG effects of FHG-E in estrogen combined with progestogen induced HMG rats was evaluated. This is first study on anti-HMG effect of Fructus Hordei G *in vivo*.

MATERIALS AND METHODS

Preparation of Fructus Hordei G ethanol extract: Fructus Hordei G was collected in Bozhou, Anhui Province, China in August 2012 and a voucher specimen (FHG 2012006) was taxonomically identified by Professor Keli Chen, College of Pharmacy, Hubei University of Chinese Medicine, P.R. China.

The dried materials (2 kg) were powered and extracted twice with aqueous 75% EtOH under reflux for 3 h combined and concentrated the solvent to get residue FHG-E. The concentrated extract was then evaporated on a boiling water bath until a constant weight was obtained. The dried extract was weighed and the yield was calculated.

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Chemicals: Rupisanjie Capsule (RPSJC) was used as the reference drug (positive control) it is a product of Shanxi Bailu Pharmaceutical Co., Ltd. RPSJC prescription comprises 11 Chinese medicines and the formula was used in China for many years which has been proven to be very effective in treating HMG. Estrogen injection was purchased from Tianjin Jinyao Amino acids Co., Ltd. progestogen injection was obtained from Zhejiang Xianju Pharmacetical Co., Ltd. urethane injection was got from Hubei Xing Galaxy Chemical Co., Ltd. Enzyme-Linked Immunosorbent Assay (ELISA) kits were purchased from Xin Bo Sheng Biological Technology Co., Ltd. (China) all other chemicals and reagents were of analytical grade and purchased from commercial sources.

Determination of total flavonoids content: Total flavonoids were analyzed using aluminum chloride colorimetric method. Sample (600 μg mL^{-1}) of 600 μL was mixed with 600 μL of 2% aluminum chloride solution. The mixture was allowed to stand at room temperature (27±2°C) for 10 min with intermittent shaking. The absorbance of the mixture was measured at 415 nm against a blank sample (methanol) without aluminum chloride using a UV-vis spectrophotometer (SHIMADZU, Japan). The total flavonoids content was determined using a standard curve of quercetin (0.4-15.8 μg mL^{-1}). The content was calculated as mean±SD (n = 3) and expressed as milligrams of Quercetin Equivalents (QE) in 1 g of the extract and dried powder.

Determination of total alkaloids content: Total alkaloids were determined using acid dye colorimetry method. The sample (600 μg mL^{-1}) or standard hordenine ethanol solution (10-80 μg mL^{-1}), 1.0 mL was mixed with 2.0 mL of phosphate buffer solution (pH = 7) 1.0 mL of bromothymol blue solution (1% w/v) and 3.0 mL of chloroform. The mixture was allowed to stand at room temperature (27±2°C) for 1 min with shaking then static for 60 min. Get the chloroform layer. The absorbance of the mixture was measured at 284 nm using a UV-vis spectrophotometer (SHIMADZU, Japan). The content of total alkaloid compounds was calculated as mean±SD (n = 3) and expressed as milligrams of Hordenine Equivalent (HE) in 1 g of the extract and dried powder.

Determination of total phenolics content: Total phenolics were determined using Folin-Ciocalteu procedure. The sample (300 μg mL^{-1}) or standard gallic acid solution (10-120 μg mL^{-1}), 0.4 mL was mixed with 1.0 mL of the Folin-Ciocalteu reagent (diluted 1:10 with deionized water) and 1.6 mL of sodium bicarbonate solution (8.0% w/v). The mixture was allowed to stand at room temperature (27±2°C) for 30 min with intermittent shaking. The absorbance of the mixture was measured at 765 nm using a UV-vis spectrophotometer (SHIMADZU, Japan). The content of total phenolic compounds was calculated as mean±SD (n = 3) and expressed as milligrams of Gallic Acid Equivalent (GAE) in 1 g of the extract and dried powder.

Animal model and drug treatments: Virgin female Wistar rats weighing 200-240 g and Kunming female mice (18-22 g) were obtained from Hubei Center for Disease Control and Prevention, Wuhan, Hubei. The animals had free access to food and water and were allowed to acclimatise for at least 1 week before use. All experiments conformed to the guidelines of the Principles of Laboratory Animal Care (NIH publication No. 80-23, revised 1996) and the legislation of the People's Republic of China for the use and care of laboratory animals.

Rats were randomly divided into 4 groups (n = 10), rats in the control group were administered with normal saline intramuscularly, rats in other groups were treated with estrogen (0. 5 mg kg^{-1}) intramuscularly for 25 days and followed with pro-gestogen (5 mg kg^{-1}) for another 5 days to induce HMG Model. From the 31st day, the rats in the normal control group and model group were received distilled water by gavage, the rats in RPSJC group were treated with RPSJC (800 mg kg^{-1}) and the rats in the FHG-E group were respectively administered with FHG-E (800 mg kg^{-1}) for 30 days. The nipple height, levels of sex hormone, uterus index and ovary index were measured. After the rats were sacrificed, blood was collected from the abdominal aorta and mammary glands were immediately removed. Blood samples were centrifuged at 3500 rpm for 10 min at 4°C to obtain blood serum. The mammary gland samples were fixed in formalin for pathological observation.

Cotton pellet induced granuloma (Marroquin-Segura *et al.*, 2009): Sterile cotton pellets (10 mg) were implanted subcutaneously in groin of anesthetized mice(18-22 g). Ten animals were used for every treatment. The animals received 200, 400 and 800 mg kg^{-1} of FHG-E, RPSJC (800 mg kg^{-1}) or saline (10 mL kg^{-1}) orally, once a day through an oral cannula over 7 consecutive days. On the 8th day, the mice were sacrificed and the cotton pellet removed, dried overnight at 60°C and weighed. The increase in weight of cotton pellet was determined and used for further calculateion.

Measurements of biochemical assay, uterus and ovory: The contents of sex hormones E2, P, PRL, FSH and LH were determined using ELISA kits. Uterus index were

calculated as uterus weight divided by body weight and ovory index were calculated as ovory weight divided by body weight.

Histopathological observations: Tissues of mammary gland from each group rats were fixed in 10% buffered formalin, embedded in paraffin, sectioned into 4 μm thickness, stained with Hematoxylin-Eosin (H-E) and Masson-Trichrome (M-T) and examined using optical microscopy. The severity of mammary gland hyperplasia was based on four parameters (hyperplasia of gland alveolus, lobule, shape and thickness of vessel and secretion).

Statistical analysis: Values are expressed as means±SEM. Multiple group comparisons were performed using one-way Analysis of Variance (ANOVA) followed by Dunnett's test to detect intergroup differences. The $p<0.05$ was considered significant in all cases.

RESULTS AND DISCUSSION

Total flavonoids total alkaloids and total phenolics contents of FHE: Yield, total flavonoids total alkaloids and total phenolics contents in the 50% ethanolic extract of Fructus Hordei G are shown in Table 1.

Cotton pellet induced granuloma: Results revealed that FHG-E had a significant and dose-related inhibition of the dried weight of the cotton pellet granuloma. The inhibitory values for 200, 400 and 800 mg kg^{-1} of FHG-E were 17.69, 21.82 and 27.38%, respectively. RPSJC inhibited granuloma tissue formation with a value of 16.8%, a lower value than that observed with the 200 mg kg^{-1} dose of FHG-E. FHG-E suppressed granulomatous tissue formation during chronic inflammation effectively (Table 2).

Effect of FHG-E on nipple height in rats: The heights of nipples (right 2 and 3) of rats were significantly decreased by RPSJC (800 mg kg^{-1}) and FHG-E (800 mg kg^{-1}) treatment as compared with HMG Model group (Table 3).

Effect of FHG-E on serum sex hormone levels in rats: After injecton of estrogen and progestogen, E2, PRL and FSH contents were significantly increased while P and LH levels was decreased in HMG Model rats. E2, PRL and FSH were obviously decreased by FHG-E (800 mg kg^{-1}) treatment as compared with model group ($p<0.01$), P and LH contents was remarkably increased compared with HMG Model group ($p<0.01$) (Table 4).

Table 1: Yield, total flavonoids total alkaloids and total phenolics contents of 50% ethanolic extract from Fructus Hordei G

Yield of crude extract (dry weight %)	Total flavonoids content (mg QE g^{-1})	Total alkaloids content (mg HE g^{-1})	Total phenolics content (mg GAE g^{-1})
36.15±0.03	3.95±0.06	42.74±0.08	52.46±0.12

Table 2: Effect of FHG-E in mice cotton pellet induced inflammation

Groups	Dosages (g kg^{-1})	Animals	Granuloma weight (mg)	Inhibition rate (%)	p-values
Control	-	10	73.92±9.450	-	-
RPSJC	0.8	10	61.50±11.75	16.80	>0.05
FHG-lowdose	0.2	10	60.84±18.48	17.69	>0.05
FHG-middle dose	0.4	10	57.79±13.24	21.82	<0.05
FHG-high dose	0.8	10	53.68±12.94	27.38	<0.05

Table 3: Effects of FHG-E on nipple height in rats

Groups	Dosages (g kg^{-1})	Animals	Nipple height (mm) Right 2	Nipple height (mm) Right 3
Control	-	10	1.56±0.15***	1.62±0.21***
Model	-	10	2.98±0.27	2.58±0.29
RPSJC	0.8	10	2.06±0.24***	2.09±0.09***
FHG-E	0.8	10	1.72±0.08	2.06±0.08***

*p<0.05 compared with model group; **p<0.01 compared with model group; ***p<0.001 compared with model group

Effects of FHG-E on uterus and ovary index in rats: Uterus index in HMG Model rats were significantly increased compared with normal control group ($p<0.01$). Uterus index were significantly decreased by RPSJC (800 mg kg^{-1}) ($p<0.01$) and FHG-E (800 mg kg^{-1}) ($p<0.05$) treatment as compared with HMG Model group. Ovary index was not shown distinct difference compared with control group (Table 5).

Effects of FHG-E on breast pathological morphology: Histopathologic examination showed no histological abnormalities, no proliferative lesions, less acinars, no mammary ducts secretion, no mammary ducts in the mammary gland of normal control group rats (Fig. 1a and b). Intense hyperplasia of gland alveolus and lobule much secretion, vessel arborization, lymphoplasia and plasma cells were observed in mammary gland of model group rats (Fig. 1c and d). In RPSJC-treated group at week 4, the hyperplasia of lobules and gland alveolus of mammary gland lessened, secretion and lymphocyte decreased in intracavitary (Fig. 1e and f). FHG-E (800 mg kg^{-1}) treatment markedly alleviated the degree of HMG. There was few hyperplasia in gland alveolus and lobules of mammary gland and no secretion and lymphocyte was seen (Fig. 1g and h).

There is evidence that chronic inflammation brought about by persistent chemical, bacterial or viral agents is a risk factor for cancer (Hussain and Harris, 2007; Mantovani *et al*., 2008; Mantovani, 2010; Lin and Karin, 2007). Inflammation by innate immunity which is required to fight microbial infections, heal wounds and maintain

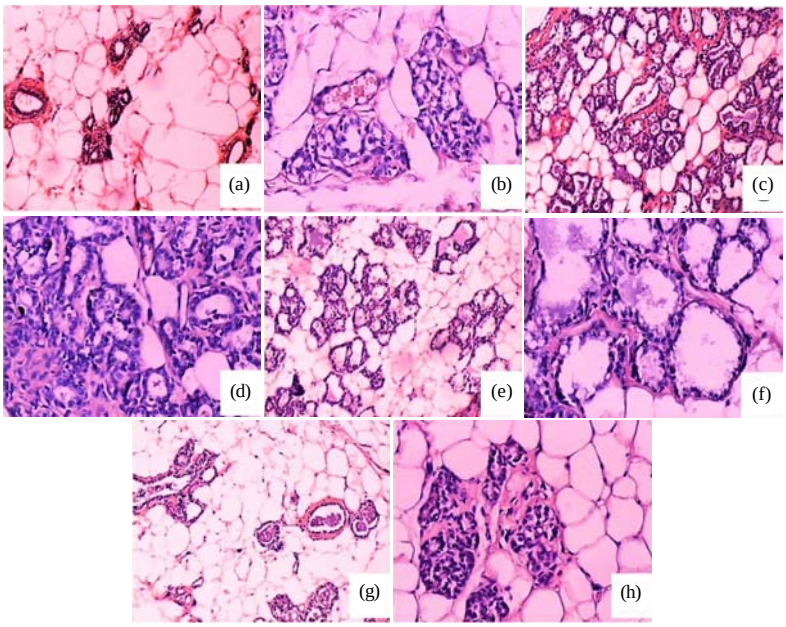

Fig. 1: Histological image of mammary gland tissues by microscope (100 or 200 magnification); the normal mammary gland in control rat ((a) x100 and (b) x200); the mammary gland of the model rat ((c) x100 and (d) x200); RPSJC treatment rat ((e) x100 and (f) x200); FHG-E (800 mg kg^{-1}) treatment rat ((g) x100 and (h) x200)

Table 4: Effects of FHG-E on serum sex hormone levels in rats

Groups	Dosage (g kg^{-1})	Animals	E2 (pmol L^{-1})	P (ng mL^{-1})	PRL (pg mL^{-1})	FSH (IU L^{-1})	LH (mIU mL^{-1})
Control	-	10	2.801±0.148***	1.413±0.169**	286.375±7.080**	0.124±0.021***	1.851±0.106**
Model	-	10	13.856±0.467	0.435±0.065	411.265±8.910	0.523±0.011	0.784±0.077
RPSJC	0.8	10	3.118±0.183***	1.120±0.204**	354.29±12.240*	0.288±0.270**	1.480±0.104**
FHG-E	0.8	10	2.670±0.168***	1.237±0.120**	278.936±8.420**	0.218±0.016**	1.776±0.096**

*p<0.05 compared with model group; **p<0.01 compared with model group; ***p<0.001 compared with model group

Table 5: Effects of FHG-E on uterus and ovary index in rats

Groups	Dosages (g kg^{-1})	Animals	Uterus index (mg g^{-1})	Ovary index (mg g^{-1})
Control	-	10	2.13±0.41**	0.68±0.07
Model	-	10	2.87±0.51	0.56±0.14
RPSJC	0.8	10	2.15±0.22**	0.48±0.07
FHG-E	0.8	10	2.28±0.46	0.49±0.08

*p<0.05 compared with model group; **p<0.01 compared with model group

tissue homeostasis can lead to the hallmarks of cancer (Trinchieri, 2011; Hanahan and Weinberg, 2011; Chang, 2010). Several recent studies suggested that inflammation has an important role in all phases of tumor development including tumor initiation, tumor promotion, invasion, metastatic dissemination and evading the immune system (Hanahan and Weinberg, 2011; Trinchieri, 2012). The development of many types of cancer including breast cancer is related with inflammation. Epidemiological studies have revealed that the use of non-steroidal anti-inflammatory drugs can decrease the risk of developing breast cancer (Howe *et al*., 2005; Reed *et al*., 2009). The study revealed that FHG-E had strong anti-inflammatory activities in chronic inflammation model mice. The inflammatory granuloma is a typical feature of a chronic inflammatory process. The dried weight of the pellets correlates with the amount of granulomatous tissue. Therefore, the cotton pellet granuloma method has been widely used to evaluate the proliferative components of chronic inflammation.

In the study, FHG-E inhibits chronic proliferative inflammation processes with a dose-dependent inhibition of granuloma formation in mice and FHG-E also has therapeutic effects on HMG rats induced by estrogen and progestogen. The heights of nipples of rats were significantly decreased by FHG-E (800 mg kg^{-1}) treatment as compared with HMG Model group. Results showed thatuterus index were remarkably decreased by FHG-E (800 mg kg^{-1}) treatment as compared with HMG Model group. Ovary index was not shown distinct difference compared with control group. After administrating of FHG-E (800 mg kg^{-1}) in HMG Model rats, it showed that E2, PRL and FSH were remarkably decreased and P and LH significantly increased ($p<0.01$) as compared with model group. Histopathologic observation showed that FHG-E (800 mg kg^{-1}) treatment significantly alleviated the degree of HMG, numbers of lobules and acinars, secretion

and lymphocyte decreased obviously. The mechanism of FHG-E in treating HMG might be by way of regulating endocrinal and immune functions to balance hormonal levels in the body to inhibit the pathological proliferation of mammary gland. There is no report about the pharmacological studies on anti-HMG effect of FHG-E at present. The study is the first report of establishing the anti-HMG activity of FHG-E.

CONCLUSION

In this study, the granulomatous tissue weight, nipple height, serum sex hormone levels, uterus and ovary index and breast pathological morphology revealed that the FHG-E could treat HMG effectively. Fructus Hordei G is a promising herb in treating HMG which should be studied and developed deeply.

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