

Expression of AQP2, AQP4 and AQP 8 in mouse intestine induced by unprocessed and processed *Euphorbia lathyris*

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Abstract: The present research was designed to study expression of AQP2, AQP4 and AQP8 in mouse intestines induced by unprocessed and processed *Euphorbia lathyris*. KM mice were given by different dose lavage of unprocessed and processed *Euphorbia lathyris*, Euphorbia factor L₁, Euphorbia factor L₂, Euphorbia factor L₃. Samples of mouse intestine were collected for protein levels of AQP2, AQP 4 and AQP 8 which were assessed by immunohistochemical staining and mRNA expression of AQP2, AQP 4 and AQP 8 which were quantified by Real Time-PCR. Comparing to the normal control group, the protein levels of AQP2, AQP 4 and AQP 8 were significantly decreased ($P<0.05$) by Semen Euphorbiae group and Semen Euphorbiae Pulveratum group (unprocessed and processed *Euphorbia lathyris*) induced. Protein expression of AQP2, AQP 4 and AQP 8 in the Euphorbia factor L₁, Euphorbia factor L₂ and Euphorbia factor L₃ group were not significantly lower than normal control group. There had no differences on the levels of AQP2 and AQP 8 mRNA expressions between the high-dose group of semen Euphorbiae group, semen Euphorbiae Pulveratum group and positive control group, while significantly lower than normal control group ($P<0.05$). Expression of AQP4 mRNA in the Semen Euphorbiae group and Semen Euphorbiae Pulveratum group has not significantly decreased. But levels of AQP2, AQP 4 and AQP 8 mRNA in the Euphorbia factor L₁ group had no significant differences in normal control group and positive control group. These findings suggest that semen Euphorbiae could regulate expression of AQP2, AQP 4 and AQP 8 protein and mRNA, which may be the possible one reason of semen Euphorbiae induces diarrhea. The semen Euphorbiae group has more significant effects on the levels of AQP2, AQP 4 and AQP 8 protein and mRNA than semen Euphorbiae Pulveratum group, which may be one of the mechanisms of processing attenuation.

Keywords: Semen Euphorbiae, Semen Euphorbiae Pulveratum,; Euphorbia factor L₁, AQPs.

INTRODUCTION

The seed of *Euphorbia lathyris*, Semen Euphorbiae is a traditional Chinese medicine which has been used for the treatment of hydropsy, ascites, anuresis and constipation, amenorrhea, scabies. (Pharmacopoeia Commission of the People's Republic of China, 2015). Processing of herb is one of the characteristics of traditional medicine Chinese. Semen Euphorbiae was the representative herb of frostlike powder processing. Raw Semen Euphorbiae, as a poisonous traditional Chinese medicine, needs to be attenuated after processing. There were reports that toxicity and efficacy of *Euphorbia lathyris* L. may be induced by the same substance or similar substance (Zhang *et al.*, 2010). Modern research shows that Semen Euphorbiae has double physiological activities, not only could treat-tumor and leukemia, but also has a strong gastrointestinal mucosa irritation (Duan *et al.*, 2013; Zhu *et al.*, 2013; Duan *et al.*, 2014; Gallardo *et al.*, 2001). Toxicological studies demonstrated that Semen Euphorbiae induced strong gastrointestinal mucosa irritation. The gastrointestinal mucosa irritation mainly manifested as serious diarrhea. After processing, the

toxicity and the capacity of diarrhea is decreased obviously (Wang *et al.*, 2015). However, at present the attenuation of processing mechanism is still unclear.

In recent years, studies showed aquaporins (AQPs) are widely distributed in the stomach, intestine, brain, lung and other organs and play an important role in the water absorption or secretion (Ricanek *et al.*, 2015; Fang-qin, 2014). AQPs could be elucidated the pharmacology and toxicology of traditional Chinese medicine about Junxiazhuohui, Lishuishenshi, zaoshi and so on. They may become the new target that treat the diseases about water metabolism disorders such as ascites, edema. For further exploring the regulation role of AQPs, AQP2, AQP 4 and AQP 8 were the dominant three proteins, which study the relationship between traditional Chinese medicine and water metabolism disorders. The present studies reported that a reduction in the expression of AQP2, AQP 4 and AQP 8 appears to be correlated with increased disease activity with diarrhea. Based on the previous work, it is necessary to study relationship between diarrheas that Semen Euphorbiae caused and AQPs. Furthermore, whether the regulating effect of Semen Euphorbiae on expression of AQPs is associated with diarrhea has not yet reported. The major AQPs

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expressed in the colon are AQP2, AQP4, and AQP8 (Gallardo *et al.*, 2001; Wang *et al.*, 2015; Ricanek *et al.*, 2015; Fang-qin, 2014; Zhu *et al.*, 2014). Our study detected the variation of expression of AQP2, AQP4, and AQP8 in colon to investigate the relationship between regulating effect of Semen *Euphorbiae* on expression of AQP2, AQP4, and AQP8. Moreover, we also try to discuss the pathway which causes toxicity through processed Semen *Euphorbiae* in dose in order to reduce its adverse reactions.

MATERIALS AND METHODS

Reagents and instruments

Animals

KM mice (SPF grade, 18-22 g) were purchased from Sibeifu Co., Ltd (Beijing, China). All experiments were approved by the Animal Care Committee. Mice were kept at room temperature (23±1°C) and 55±5% humidity. All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animal, as adopted by the Committee on Animal Research at Beijing University of Chinese Medicine.

Chemicals and reagents

Standard compounds of *Euphorbia* factor L₁, *Euphorbia* factor L₂, and *Euphorbia* factor L₃ were provided by the laboratory of Chinese medicine processing technology from Shandong University of Traditional Chinese Medicine.

Non-immune goat serum, goat anti-rabbit IgG, HRP antibody, diaminobenzidine (DAB), and antibody dilution were purchased from Beijing Ding Guo Chang Sheng Biotechnology Co., Ltd (Beijing, China).

AQP2, AQP4, and AQP8 antibody (Rabbit polyclonal) were purchased from Bioss Co., Ltd. (Beijing, China). Total RNA extraction reagent (Trizol) was purchased from Tian Gen Biotech Co., Ltd. (Beijing, China). Prime Script™ RT reagent Kit with gDNA Eraser, SYBR® Premix Ex Taq™ II (TliR NaseH Plus) ROX plus, DL2000 DNA Marker were purchased from TAKARA.

Plants, extraction and isolation

Pieces of *Euphorbiae* Semen (Batch Number, 1203070692; origin, Jiangxi Province, China) were purchased from Anhui Bozhou Hu Qiao Chinese Herbal Pieces plant. Petroleum ether extract of Semen *Euphorbiae*, petroleum ether extract of Semen *Euphorbiae* Pulveratum was provided by Shandong University of Traditional Chinese Medicine. The extraction and isolation methods of Semen *Euphorbiae* had been published in these articles (Zhu *et al.*, 2013; Zhu *et al.*, 2014).

Instruments

Heated Incubators SKP-02 (Huang Shi Hengfeng medical instrument Co., Ltd), Pathological paraffin-embedded

machine LS-100 (Shenyang Long Shou Electronic Equipment Co., Ltd), Paraffin slicer Finesse325 (Beijing Hong TaiJia Ye Technology Development Co., Ltd), Microscope BA400 (Mike Dior Industrial Corporation), Imaging software system Motic Images Advanced 3.2 (Mike Dior Industrial Corporation), Centrifuge 5415D (Eppendorf), Spectrophotometer NANODROP 2000 (Thermo Scientific), Quantitative PCR instrument ABI7500 (Applied Biosystems).

Immunohistochemical staining

Animals grouping and drug administration

The mice were randomly assigned to six groups (n=3), normal control group, Semen *Euphorbiae* group, Semen *Euphorbiae* Pulveratum group, *Euphorbia* factor L₁ group, *Euphorbia* factor L₂ group, *Euphorbia* factor L₃ group. The crude drug dose of extract of Semen *Euphorbiae* and Semen *Euphorbiae* Pulveratum was 145.25g/kg. The three compounds were separately suspended in 1% carboxymethyl cellulose sodium, and then were given to mice orally at 1g/kg. The normal control group was administered with 0.9% physiological saline.

Sample preparation

Six hours later the animals were killed by breaking cervical. The colons were removed, washing the colons (3-5cm) with cold saline immediately for 3 times. One half of the colon was fixed in 10% neutral formalin. The other half of the colon was snap-frozen in liquid nitrogen, and then stored at -80°C.

Intestinal histological analysis

The colons fixed in 10% formalin, dehydrated in graded ethanol and embedded in paraffin. Thereafter, 5µm sections of tissue were cut to make paraffin section, stained with hematoxylin and eosin and assessed microscopically. The morphological structure of the colon were observed by microscopic examination.

Immunohistochemical staining

According to immunohistochemistry kit operation, the sections were dewaxed, hydration, microwave antigen retrieval. Thereafter, the sections were incubated in primary antibody AQP2, AQP4, and AQP8 antibody (1:50) in the condition at 4°C for 12h. When they had been rinsed in 0.01M PBS (pH 7.4, ×3, 5min), the sections were incubated in secondary antibody (goat anti-rabbit IgG conjugated to peroxidase, 1: 500) at 37°C for 40min, then rinsed in 0.01M PBS (pH7.4, ×3, 5min). Then diaminobenzidine (DAB) was used as chromogen. The immunohistochemical staining sections were observed under a microscope.

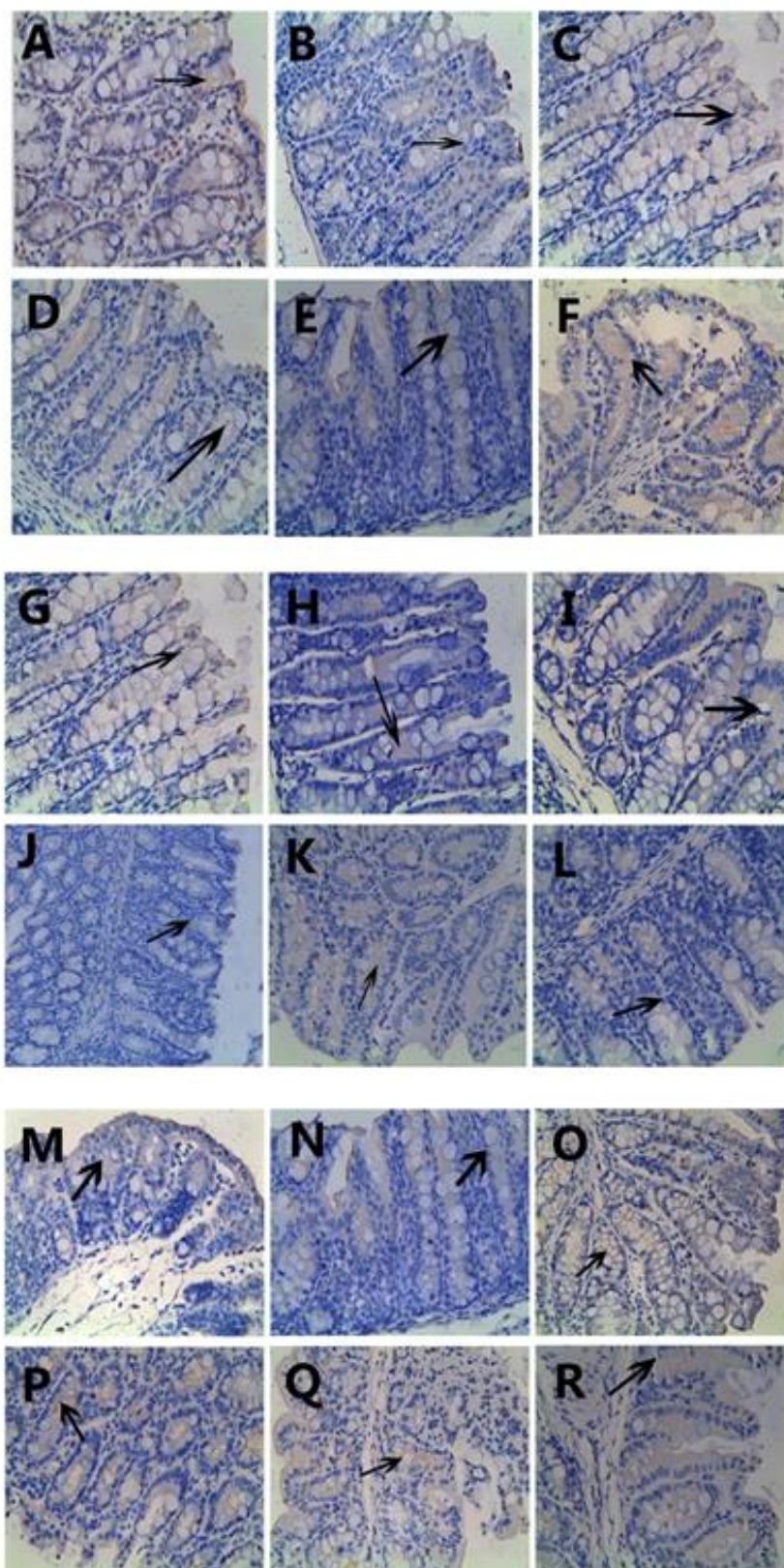


Fig. 1: Expression of AQP2, AQP4 and AQP 8 immunohistochemical results in mice
 Note: AQP2: A~F: The normal group; Semen Euphorbiae; Semen Euphorbiae Pulveratum; Euphorbia factor L₁; Euphorbia factor L₂; Euphorbia factor L₃.

Table 1: Primer Sequences of Mice mRNA

Gene		Primer sequences	Ampliconsize (bp)
GAPDH	Forward	5'- CTCATGACCACAGTCCATGC -3'	201
	Reverse	5'- CACATTGGGGGTAGGAACAC -3'	
AQP2	Forward	5'-TGTGCTCCAGATTGCCGT-3'	394
	Reverse	5'-GCCCAGGGTAACAGAGAAA-3'	
AQP4	Forward	5'-ATCAATCCCGCTGTGACT-3'	234
	Reverse	5'-AGTGAACACCAACTGGAAAG-3'	
AQP8	Forward	5'- ACATCAGCGGTGGACACTTCA-3'	239
	Reverse	5'-CAGAATGATCTCTATCCCCAGGG-3'	

Table 2: Expression of AQP2, AQP4 and AQP 8 immunohistochemical results ($\bar{x} \pm s$, n=3)

Group	Dose (g·kg ⁻¹)	AQP2	AQP4	AQP8
The normal group	0	0.198±0.03	0.192±0.03	0.212±0.03
Semen Euphorbiae	145.25	0.097±0.14**	0.094±0.10**	0.089±0.08**
Semen Euphorbiae Pulveratum	145.25	0.151±0.05**	0.143±0.05**	0.148±0.03**
Euphorbia factor L ₁	1	0.115±0.02	0.142±0.03	0.112±0.18
Euphorbia factor L ₂	1	0.201±0.05	0.210±0.04	0.220±0.03
Euphorbia factor L ₃	1	0.150±0.06	0.220±0.05	0.145±0.04

Note: *P<0.05, **P<0.01 compared with normal group. F1: Normal control group, 2: Semen Euphorbiae group, 3: Semen Euphorbiae Pulveratum group, 4: Euphorbia factor L₁ group, 5: Euphorbia factor L₂ group, 6: Euphorbia factor L₃ group

Table 3: Groups of mice colon tissue AQP2 mRNA, AQP4 mRNA, AQP8 mRNA expression ($\bar{x} \pm s$, n=3)

Groups	AQP2	AQP4	AQP8
Normal control group	0.778±0.21*	1.434±0.22*	2.087±0.08*
Positive control group	0.301±0.12*	0.273±0.07*	0.460±0.08*
High dose group of Semen Euphorbiae Pulveratum group	0.596±0.06*	0.642±0.24	0.600±0.03*
Medium dose group of Semen Euphorbiae Pulveratum group	0.697±0.21*	1.093±0.38	0.916±0.15*
Low dose group of Semen Euphorbiae Pulveratum group	0.793±0.34*	1.265±0.45	1.241±0.24*
High dose group of Semen Euphorbiae group	0.283±0.01*	0.644±0.28	0.556±0.21*
Medium dose group of Semen Euphorbiae group	0.425±0.09*	0.679±0.25	0.663±0.31*
Low dose group of Semen Euphorbiae group	0.453±0.17*	1.019±0.04	1.035±0.03*
Euphorbia factor L ₁ group	0.3366±0.15*	0.6793±0.19*	0.7542±0.22*

Note: * P <0.05 compared with the normal control group; * P <0.05 compared with the positive control group

RT-PCR

Animal grouping and drug administration

KM mice were randomly assigned to 9 groups (n=3), the normal control group (0.9% physiological saline), the positive control group (mannitol with dosage of 14g/kg). Diarrhea rate reached 35% as for the standard. There were stains in filter paper as for the standard of diarrhea. The drug groups were divided into low dose, middle dose and high dose Semen Euphorbiae group and Semen Euphorbiae Pulveratum group (crude drug dose of 48.42g/kg, 145.25g/kg, and 435.75g/kg respectively).

Mice were killed by breaking cervical after administered six hours later. The colons were removed and snap-frozen in liquid nitrogen and then stored at -80°C.

Extracting total RNA

AQP2, AQP4, AQP8 and GAPDH primers were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd,

as shown in table 1. After tissues were grinded in liquid nitrogen, total RNA was extracted by Trizol and quantitative research was made. Nanodrop2000 Ultraviolet spectrophotometer and 1% agarose electrophoresis were used to detect the purity and concentration of RNA. The cDNA retrovirus reaction was carried out in accordance with reverse transcription kit instructions. GAPDH was reference substance in fluorescence quantitative PCR assay. SYBR Green I dye method was used to PCR amplification reaction in accordance with Kit instructions.

STATISTICAL ANALYSIS

Results are presented as the mean ±standard deviation of the experiments. Statistical analyses were performed by SPSS17.0 software and One-way ANOVA was used to compare the mean of each group. Mean comparison in

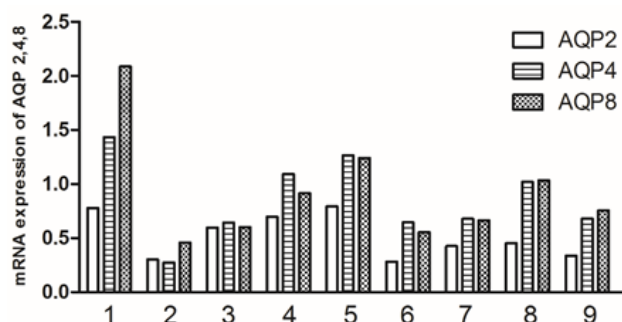
two groups was conducted with the least significant difference. $P < 0.05$ was considered statistically significant.

RESULTS

Immunohistochemical results

The comparison of vital sign changes after dosing 6 h.

No obvious change was observed in weight, back hair, response, activity, and eating behavior in normal control group. Both experimental mice appeared dim back hair, weary, burnout, lethargy, retardation, slow action. Experimental groups appeared obvious diarrhea after orally administration, both defecation quantity and the water content increased. Some mice even accompanied by loose stools, soft stool and watery stool. Diarrhea symptoms of Semen Euphorbiae group were more severe than other groups. However Euphorbia factor L₁ group, Euphorbia factor L₂ group and Euphorbia factor L₃ group had no significant difference.



Note: * $P < 0.05$ compared with normal group.

Fig. 2: groups of mice colon tissue AQP2 mRNA, AQP4 mRNA, AQP8 mRNA expression

Effects of experimental groups on colons morphology

Normal control has shaped-regularly colon, mucosal epithelium cells arranged in neat rows but occasionally there are Lymphocyte infiltration between epithelium. However, after diarrhea, proximal colonic mucosa epithelial showed edema, partial fusion, interstitial edema, congestion, oozy, inflammatory cells infiltration; the structure of cellular became irregularly, with unclear boundaries between epithelial cells, more lymphocyte infiltration, necrosis and shedding were also occurred in part of the epithelial; the stroma between with layers of intestinal mucosa has more inflammatory cells infiltration, but the tunica muscular and serosa are integrated.

The results and analysis of the immunohistochemical detection

Every colon tissue took three sections, each section counted three horizons. The subtypes of all the observed AQPs were discerned under high microscope ($\times 400$). Motic Images imaging software was developed for the determination of the optical density of each perspective calculated, compared the average. Performance of AQP2, 4, 8 were shown in table 2 and fig. 1.

Results as shown in fig. 1, positive cells of AQP 2, 4 and 8 expression were observed in colon, positive cells were colored in colon epithelial cell membrane, cytoplasm also have distribution, a tan, and no positive expression of nuclei. Compared with group 1, AQP 2, 4, 8 of group 2 and 3 expressed significantly lower ($P < 0.05$). What is more, compared with group 3, AQP 2, 4, 8 of group 2 expressed obviously lower ($P < 0.05$). When group 2 and 3 had the equal dosage, AQP 2, 4 and 8 of group 2 were lower than group 3. We could suspect that group 3 may have a weaker catharsis effect than group 2. AQP2, 4 and 8 of group 4 were decreased than group 5 and 6. That means diarrhea effect of group 4 was stronger than group 5 and 6. It was in accordance with previous animal acute toxicity experiment results.

Quantitative polymerase chain reaction results

RNA purity and concentration of the sample test results

The results showed that there is no degradation of RNA, and electrophoretic bands 28s, 18s is complete and clear. Photometrical quantification was performed (Nanodrop 2000) for the 260/280 ratio. Only samples which had 1.7-2.0 and presented good quality after RNA electrophoresis in agarose gel (1.0%) were used.

Real time PCR relative quantitative analysis results

According to Real Time PCR original test results, the $2^{-\Delta\Delta CT}$ method was be used.

Calculate expression of results such as table 3, as shown in fig. 2

The results showed AQP2, 8 mRNA of different doses of Semen Euphorbiae groups were significantly lower than normal control group ($P < 0.05$); compared with positive control group, there was no significant difference. Compared with the normal control group, AQP2 and AQP 8 mRNA of medium and low dose of Semen Euphorbiae Pulveratum groups had a significant difference ($P < 0.05$), while there was no significant difference compared with positive control group. Comparing the normal and high dose group, the expression of AQP2 mRNA in high dose of Semen Euphorbiae Pulveratum group had a significant difference with normal control ($P < 0.05$). Expression of AQP4 mRNA in Semen Euphorbiae and Semen Euphorbiae Pulveratum groups did not significant different, but the expression trend was the same to AQP2 and AQP 8 mRNA. The expression of AQP2, AQP4 and AQP8 mRNA in Euphorbia factor L₁ group were lower than the normal control group, but there was no significant differences. In conclusion, the findings showed that Semen Euphorbiae and Semen Euphorbiae Pulveratum could inhibit the expression of AQP2, AQP4 and AQP8 mRNA. And inhibitions of AQP2, AQP4 and AQP8 mRNA in Semen Euphorbiae Pulveratum groups were dose-dependent, however, Semen Euphorbiae groups were not dose-dependent. Then down-regulation of AQP2, AQP4 and AQP8 mRNA of Semen Euphorbiae Pulveratum was lower than Semen Euphorbiae's

1: Normal control group, 2: Positive control group, 3: High dose group of Semen *Euphorbiae Pulveratum* group, 4: Medium dose group of Semen *Euphorbiae Pulveratum* group, 5: Low dose group of Semen *Euphorbiae Pulveratum* group, 6: High dose group of Semen *Euphorbiae* group, 7: Medium dose group of Semen *Euphorbiae* group, 8: Low dose group of Semen *Euphorbiae* group, 9: *Euphorbia* factor L₁ group

DISCUSSION

The aquaporins (AQPs) are a family of small, integral membrane proteins that facilitate water transport across the plasma membranes of cells in response to osmotic gradients (Verkman, A. S. *et al.*, 2014). They also widely expressed in the body, particularly in cell types that are involved in fluid transport, such as epithelial cells in several organs such as kidney, intestine and lung (Martins, A. 2014; Gallardo P *et al.*, 2001). There had studies shown that AQPs play an important role in the diarrhea of body. When expression of AQP2 and AQP4, AQP8 were down regulated, intestinal absorption function was damaged, resulting in changes in the secretion of intestinal liquid and diarrhea. That means expression of AQP2 and AQP4, AQP8 and body diarrhea are closely related (Zhao Kai-ke *et al.*, 2016; Li Zihui, *et al.*, 2011).

This work highlights the relationship between expression of AQP2, AQP4 and AQP8 and Semen *Euphorbiae Pulveratum* using immunohistochemical staining and RT-PCR. What is more, based on our work, we furtherly analyzed the relationship between *Euphorbia lathyris* after processed and toxic effects after administration. As described in previous literature (Cristia E *et al.*, 2007; Gallardo P *et al.*, 2001; King LS, *et al.*, 2004), the expression of AQP2, AQP4 and AQP8 may play a role in its pathogenesis. The findings mean that processing or not may lead to the differences on the toxicity extract which induce diarrhea. Immunohistochemistry and RT-PCR results told us that expression of AQP2, AQP4 and AQP8 incubated by the equal crude drug dose of extract of raw *Euphorbia lathyris* were lower than the *Euphorbia lathyris* processed, which consistent with the previous study on animals experiment. That is to say, *Euphorbia lathyris* processed may cause weaker diarrhea and lower toxicity. Our article also for the first time find that *Euphorbia* factor L₁, *Euphorbia* factor L₂, *Euphorbia* factor L₃ may down-regulate the expressions of AQP2, AQP4 and AQP8, but was obviously different with extract of Semen *Euphorbiae* and Semen *Euphorbiae Pulveratum*. It indicates that single component may not play a major role in the extracts, may be the multiple components that act on the target of AQP2, AQP4 and AQP8 has additive effect (Xu Feng, *et al.*, 2014; Qing, Wang Xuan, *et al.*, 2015).

Research reported that abnormal distribution and expression of AQP2, AQP4 and AQP8 mRNA and protein

were induced in response to inflammatory factors (Ito *et al.*, 2006; Lehmann *et al.*, 2008; ONG *et al.*, 2012). And then the decreased expression of AQP2, AQP4 and AQP8 caused the intestinal fluid disorder balance which leads to the decrease of intestinal mucosal defense function and aggravates the intestinal mucosal inflammation and injury.

CONCLUSION

Our work found that *Euphorbia lathyris* could regulate to inflammatory factors such as TNF- α . We found that regulations to TNF- α of Semen *Euphorbiae Pulveratum* were weaker than Semen *Euphorbiae* and regulations to AQP2, AQP4 and AQP8 of Semen *Euphorbiae Pulveratum* were also weaker than Semen *Euphorbiae*. Thus we had come to a conclusion that regulation to inflammatory factors and AQP2, AQP4 and AQP8 may be one of the key mechanisms for processing attenuated of *Euphorbia lathyris*. From these results, it could recommend scientific methods and ideas for *Euphorbiaceae* plants that have the same effect.

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