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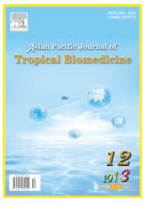

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Study on the antiulcer effects of *Veronicastrum axillare* on gastric ulcer in rats induced by ethanol based on tumor necrosis factor-α (TNF-α) and endothelin-1 (ET-1)

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PEER REVIEW

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Comments

This is a good study in which the authors evaluated the effect of water extract of *V. axillare* that are effective against gastric ulcer caused by ethanol. The results are interesting and suggest that *V. axillare* may be used as alternative therapy for gastric ulcer.
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ABSTRACT

Objective: To assess whether *Veronicastrum axillare* (*V. axillare*) can ameliorate ethanol-induced gastric mucosal lesions in rats, reduce the production of pro-inflammatory cytokines, suppress apoptosis and improve local microcirculation disturbances.

Methods: Totally 48 male Sprague-Dawley rats were randomly divided into six groups, eight rats in each group. Rats in the normal group and the model group were administered with 0.9% normal saline respectively. Rats in the positive group and ranitidine group were administered with 0.18% ranitidine suspension by intragastric administration respectively. Those in the high dose *V. axillare* group, the medium dose *V. axillare* group and the low dose *V. axillare* group were administrated with *V. axillare* at the daily dose of 2.8 g/kg, 1.4 g/kg and 0.7 g/kg by intragastric administration. Gastric mucosal lesions were produced by intragastric administration of absolute ethanol. Water extract of *V. axillare* was successively injected for 14 d and last day was injected 1 h before ethanol administration. Gastric mucosal ulcer index and ulcer inhibitory rate were counted by improved Guth methods. The tissue sections were made for pathological histology analysis. Also, we measured the concentrations of tumor necrosis factor-α (TNF-α) and endothelin-1 (ET-1) in gastric mucosal, as an index of the pro-inflammatory cytokines, apoptosis and local microcirculation. Besides, the mRNA contents of TNF-α and ET-1 were measured to verify effects on gene expression by real-time fluorescent quantitative PCR.

Results: Water extract of *V. axillare* significantly ameliorated the gastric mucosal lesions induced by ethanol administration ($P<0.01$). Pro-inflammatory cytokines, TNF-α and ET-1 were increased after ethanol administration and significantly reduced by water extract of *V. axillare*. The expressions of TNF-α and ET-1 mRNA were also be inhibited by water extract of *V. axillare*.

Conclusion: Current evidences show water extract of *V. axillare* is effective for defending against ethanol-induced gastric mucosal lesions, significantly inhibiting the production of pro-inflammatory cytokines and the expressions of TNF-α and ET-1 mRNA, which may be useful for inhibiting apoptosis and improving local microcirculation.

KEYWORDS

Veronicastrum axillare (Sieb. et Zucc) Yamazaki, Gastric mucosal lesions, Pro-inflammatory cytokines, TNF-α, ET-1

1. Introduction

Veronicastrum axillare (Sieb. et Zucc) Yamazaki (*V. axillare*), one of scrophulariaceous plants, is rich in resources in Zhejiang Province of China[1]. The chemical compositions of *V. axillare* are mainly sterol, mannitol, tannin, resin, *etc*[2]. Traditionally, the plant is externally used to treat injuries, mumps, boils, burn, snake bites, *etc.* and take orally for the treatment of pleural effusion in Jiangsu and Zhejiang folk of China[3]. Although *V. axillare* is

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widely used in folk, therapeutic effects of treating diseases and mechanism of action still remain unknown. Our recent studies are the first to reveal that water extract of *V. axillare* can significantly inhibit gastric ulcer induced by ethanol[4].

Intragastric administration of ethanol to rats rapidly induces gastric mucosal lesions, which are commonly used to study both the pathogenesis and therapy of human ulcerative disease[5]. Absolute ethanol rapidly promotes the formation of hyperemic blisters in the stomach mucosa, which is essentially an acute inflammatory reaction[6]. Alcohol may contribute to gastric injury through a variety of mechanisms such as oxidative stress, lipid peroxidation, and glutathione depletion in gastric mucosa[7]. These mechanisms have already received attention. Tumor necrosis factor-α (TNF-α) is a major mediator of the acute inflammatory response that is generated during many disease states, including infection and inflammation[8]. Recently, enhanced apoptosis in the gastric epithelium has been demonstrated to be of pathophysiological importance in various kinds of gastric lesions like ethanol-induced ulcers[9-11]. Inflammatory cytokines, including TNF-α, have been postulated to play a role in gastric mucosal apoptosis[11]. Gastric mucosal apoptosis is known to be associated with the loss of mucosal integrity under many critical conditions such as stress, hemorrhage, microvascular leakage and may play an important role in ulcer development[12-14]. Endothelin, a 21 amino-acid peptide synthesized mainly by endothelial cells, exists in at least three isoforms: ET-1, ET-2, ET-3[15,16]. ET-1 is produced from the inactive big ET-1 precursor by endothelin-convertingenzyme-1, a membrane-bound metalloprotease which is characterized by its sensitivity to phosphoramidon[17,18]. Masuda *et al.* demonstrated that changes in ET-1 release induced by ethanol play a critical role in the pathogenesis of ethanol-induced gastric mucosal injury in rats[19]. ET-1 has strong effect in shrinking blood vessel and increasing blood pressure, further lead to gastric mucosa circulatory disturbance and significantly decline of gastric mucosal blood flow, further more lead to gastric mucosal injury[20,21].

On the basis of this evidence, we hypothesized that water extract of *V. axillare* would reduce ethanol-induced gastric mucosal injury by regulation the expression of TNF-α and ET-1. We report here that intragastric administration of *V. axillare* prior to ethanol substantially inhibits mucosal lesions, pro-inflammatory cytokines production, and may play a critical role in inhibiting apoptosis and improving local microcirculation.

2. Materials and methods

2.1. Animals

Totally 48 male Sprague-Dawley (SD) rats weighting (200 ±20) g were fed on a standard laboratory diet and water *ad libitum*, and housed in a temperature and humidity-controlled room with a 12-h dark-light cycle before and during the experiment. They were randomly divided into six groups, *i.e.* the normal group, the model group, the ranitidine group, the high dose *V. axillare* group, the medium dose *V. axillare* group and the low dose *V. axillare* group, eight rats in each group. The experimental protocol was approved by the Zhejiang Chinese medicine University Animal Care Committee.

2.2. Drugs and chemicals

Water extract of *V. axillare* was prepared by concentrating the decoction which was decocted with gentle heat for 1.5 h, successively for three times after *V. axillare* powder soaked 0.5 h with eight times volume water. The decoction were decocted into the concentration of 0.140, 0.070, 0.035 g/mL decoction with distilled water respectively. Positive control group, ranitidine, was dissolved in distilled water with the concentration of 0.0018 g/mL. The others were all analytical reagents.

2.3. Effect of V. axillare on ethanol-induced gastric mucosal lesions

The rats in the high dose *V. axillare* group, the medium dose *V. axillare* group and the low dose *V. axillare* group were administrated with *V. axillare* at the daily dose of 2.8, 1.4, 0.7 g/kg respectively by intragastric administration, once daily for 14-consecutive day[4]. Rats in the normal group and the model group were administered with 0.9% normal saline respectively. Rats in the ranitidine group were administered with 0.18% ranitidine suspension (at the daily dose of 0.027 g/kg) by intragastric administration. Prior to administration of absolute ethanol, animals were denied any food for 24 h but had free access to water. The gastric ulcer model was established using absolute ethanol in a volume of 1 mL 1 h later after the last intragastric administration. After 1 h, the animals were injected of 10% chloral hydrate at doses of 3 mL/kg. Then the stomach was collected and washed in normal saline. Subsequently, the stomach was incised along the greater curvature. The gastric mucosal lesions (ulcer index) were measured with the improved standard made by Guth using a magnifier and dividing ruler to examine the gastric mucosal lesions by two independent observers blinded to the treatments[4]. The ulcer inhibitory rate was calculated according to the formula:

$$\text{Ulcer inhibitory (\%)} = \frac{A_c - A_e}{A_e} \times 100$$

Where A_c is average ulcer index of the model group; A_e is average ulcer index of the experiment group.

For pathology morphological analysis of stomach tissue[22], the gastric tissue were fixed in 10% buffered formalin for 12 h, dehydrated, and embedded in paraffin wax. Sections (4 μm) were collected on coded slides and stained with hematoxylin and eosin.

2.4. Enzyme-linked immuno sorbent assay (ELISA) analysis

SD rats gastric tissues levels of TNF-α and ET-1 were quantified by using ELISAs according to the manufacturers' instructions. The ELISA kit of TNF-α and ET-1 were purchased from Shanghai Yuan Ye Bio-Technique Co. Ltd. Gastric mucosa tissue homogenate was prepared with nine times volume distilled water by homogenizer.

2.5. Real-time fluorescent quantitative PCR analysis

Total RNA of SD rats gastric tissues were extracted using Trizol® Reagent according to the manufacturer's directions (invitrogen™). For each sample, first-strand cDNA was synthesized using 5 μL total RNA with iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions and the concentration of total RNA. Real-time PCR amplification of cDNA was performed in an automated thermal cycler (Minioption™ Real-time PCR System; BIO-RAD) in a final volume of 25 μL containing 2 μL cDNA solution,12.5 μL SYBR® Premix Ex Taq II (2×) , 1 μL of each primer(10 μmol/L), and 8.5 μL dH_2O. The cycling reactions were 95 °C for 10 seconds, plate read followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 45 seconds. Primers specific for murine TNF-α, ET-1 and β-actin were designed and purchased from Shanghai Shengong Info-Technology Corp, Shanghai, China. The primer sequences are listed in Table 1. Expression levels were determined by the comparative CT method (Opticon Monitor 3 System, BIO-RAD). TNF-α and ET-1 mRNA expressions were normalized by the expression of β-actin described in Table 1.

Table 1
Primers used for reverse transcriptase-PCR.

Primers	Sense 5′-3′	Anti-sense 5′-3′	Product size (bp)
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC	150
TNF-α	GTCGTAGCAAACCACCAAG	GGTATGAAGTGGCAAATCG	214
ET-1	GTGTGTCTACTTCTGCCACCTGGAC	GGGCTCGGAGTTCTTTGTCTGC	212

TNF: tumor necrosis factor; ET: endothelin.

2.6. Statistical analysis

All results were recorded as mean±SE of the mean. Differences among groups were evaluated by One-way analysis of variance (ANOVA) and LSD (L) test of SPSS19.0. Values of $P<0.05$ were regarded as statistically significant.

3. Results

3.1. Anti-ulcer effect of V. axillare on ethanol-induced gastric lesions

As showed in Figure 1, severe mucosal lesions were observed in model group rats. They consisted of linear and dotted erosions in the stomach. In contrast, the ulcer formation was clearly inhibited by pretreatment with *V. axillare*, whereas no abnormalities or lesions were found in the normal group treated with normal saline alone.

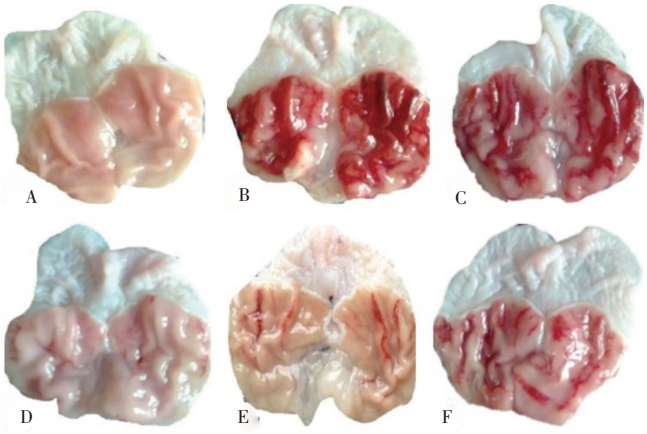

Figure 1. Macroscopic findings of ethanol-induced gastric mucosal lesions (n=8).
A, normal; B, model; C, ranitidine; D, 2.8g/kg·d; E, 1.4 g/kg·d; F, 0.7 g/kg·d (D, E, F represent the dose of V. axillare,respectively).

Macroscopic gastric mucosal damage after alcohol was significantly attenuated by pretreatment with *V. axillare*. The gastric mucosal lesions (ulcer index) in model group rats were (130.1±21.6) scores (Figure 2). The ulcer index in rats pretreated with *V. axillare* which were set at dose of 0.7 g/(kg•d),1.4 g/(kg•d) and 2.8 g/(kg•d) was significantly suppressed, respectively to (13.1±4.5), (37.3±10.3), (67.9±13.1) scores (Figure 2). The positive group, ranitidine, was up to (90.6±11.8) scores. Ulcer inhibitory rates of *V. axillare* setting at high, medium, low concentration and positive groups were up to 89.9%, 71.4%, 47.9%, 30.3%, respectively.

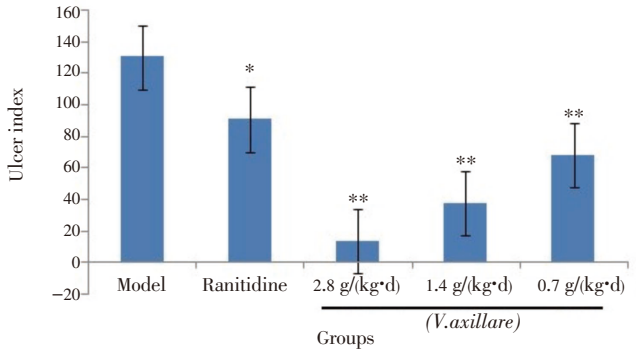

Figure 2. Effects of water extract of *V. axillare* on gastric lesions induced by absolute ethanol in rats.
Drugs and model groups were administered orally 1 h before absolute ethanol (1 mL/rat). Values are mean±SD, n=8. $^{*}P<0.05$, $^{**}P<0.01$ compared to the model group.

The protective effects of *V. axillare* were also confirmed histologically. Ethanol produced a large area of epithelial cells and mucous gland loss, the isolation of epithelium and matrix, erosions, mucosal bleeding. In contrast, pretreatment with water extract of *V. axillare* was

associated with fewer disruption of the gastric mucosal epithelium, smaller erosions and mucosal bleeding (Figure 3).

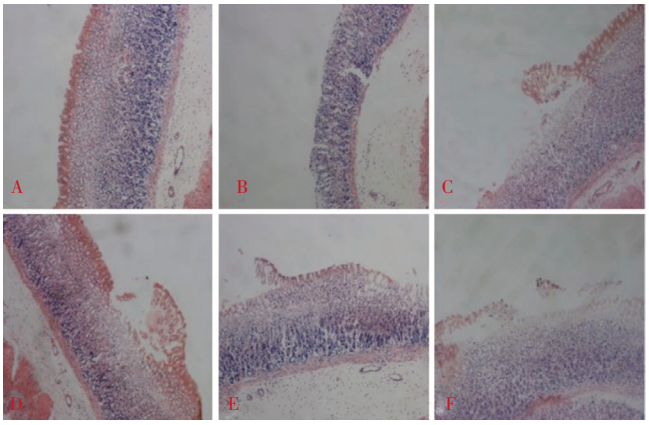

Figure 3. Pathological histology sections analysis of the stomach tissue in different groups (H.E.×100).
In model group, mucosal bleeding , erosion, and epithelial cells loss were observed. Following treatment with water extract of *V. axillare*, smaller erosions and few epithelial cells loss were demonstrated. A: normal; B: model; C: ranitidine; D: 2.8 g/(kg•d); E: 1.4 g/(kg•d); F: 0.7 g/(kg•d) (D, E, F represent the dose of *V. axillare*, respectively).

3.2. Effects of V. axillare on gastric mucosal concentration of TNF-α and ET-1 cytokines

The concentrations of TNF-α and ET-1 in gastric mucosal tissue were detected by ELISA analysis. As shown in Figures 4 and 5, the gastric mucosal concentration of TNF-α and ET-1 in the model group were significantly increased compared with that in the normal group (P<0.01). With the intervention of *V. axillare*, the expressions of TNF-α and ET-1were significantly decreased compared with the model group (P<0.01). And the positive group, ranitidine, could decrease the expressions of TNF-α and ET-1 to some extent. Besides, there was a significant correlation between gastric mucosal TNF-α, ET-1 concentrations and gastric mucosal damage in Figures 1 and 3.

3.3. Effects of V. axillare on gastric mucosal TNF-α and ET-1 mRNA expressions.

TNF-α and ET-1 mRNA expressions in gastric mucosal tissue were detected by real-time PCR. The expressions of TNF-α and ET-1 mRNA in the model groups increased significantly compared with that of the normal group (P<0.01). Differences between concentrations of *V. axillare* in TNF-α and ET-1 mRNA expressions are shown in Figures 6 and 7, showing the relatively high concentration of *V. axillare* that could significantly decrease the expressions of TNF-α and ET-1 mRNA compared with that of the model group (P<0.01).

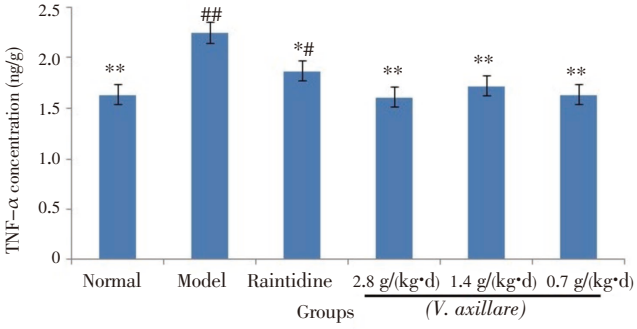

Figure 4. Effect of *V. axillare* on expression of TNF-α cytokine in the gastric mucosa lesions induced by absolute ethanol in rats.
Values are mean±SD, n=8. $^{*}P$<0.05, $^{**}P$<0.01 compared to model. $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared to normal.

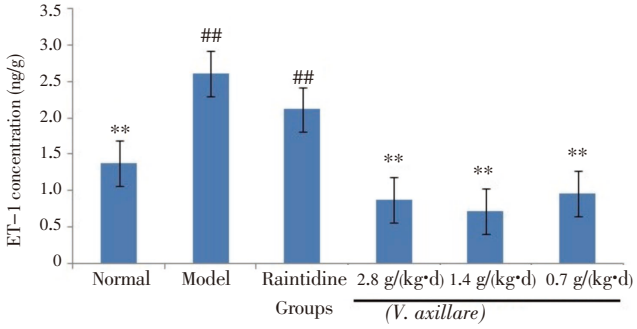

Figure 5. Effect of *V. axillare* on expression of ET-1 cytokine in the gastric mucosa lesions induced by absolute ethanol in rats.
Values are mean±SD, n=8. $^{*}P$<0.05, $^{**}P$<0.01 compared to model. $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared to normal.

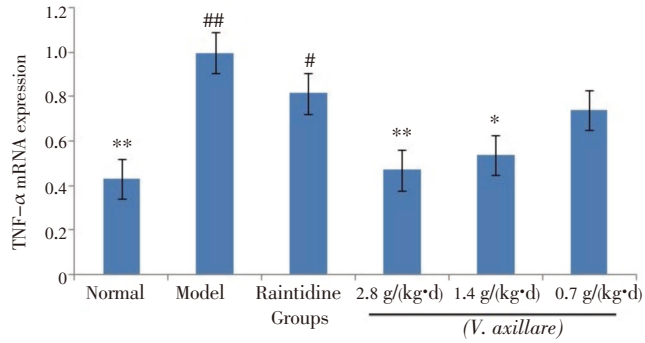

Figure 6. Effect of *V. axillare* on expression of TNF-α mRNA in the ethanol-induced gastric mucosa lesions in rats.
Values are mean±SD, n=8. $^{*}P$<0.05, $^{**}P$<0.01 compared to model. $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared to normal.

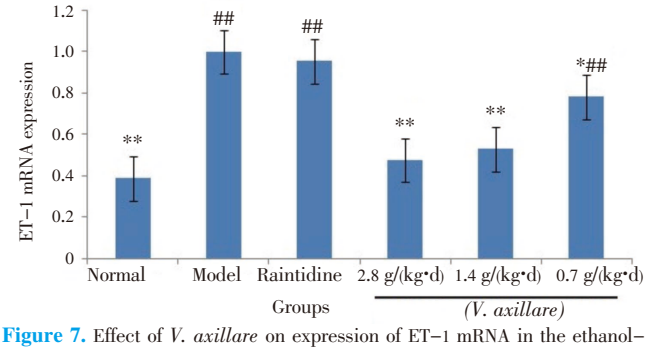

Figure 7. Effect of *V. axillare* on expression of ET-1 mRNA in the ethanol-induced gastric mucosa lesions in rats.
Values are mean±SD, n=8. $^{*}P$<0.05, $^{**}P$<0.01 compared to model. $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared to normal.

4. Discussion

This study showed for the first time that water extract of *V. axillare*, 1 h prior to the administration of absolute ethanol, is remarkably effective at attenuating the extent of gastric mucosal lesions, as evidenced by both macroscopic and histologic findings. Intragastric administration absolute ethanol to 24 h fasted rats produced linear hemorrhagic lesions, extensive submucosal edema, mucosal friability, inflammatory cells, and epithelial cell loss in the stomach, which are typical characteristics of alcohol injury[23]. Ethanol also causes constriction of submucosal venules with subsequent stasis of blood flow in mucosal microcirculation as well as arteriolar dilatation and plasma leakage from the vascular bed, which shows that it could lead to the formation of bandlike blisters in the gastric mucosa[6,24]. Moreover, this protection is correlated with the inhibition of the pro-inflammatory cytokines production in gastric mucosal tissue. In order to study the protective mechanism, we evaluated the effect of *V. axillare* on pro-inflammatory cytokines TNF-α and ET-1. We examined gastric mucosal TNF-α , ET-1 cytokines concentrations and mRNA expressions using ELISA and real-time PCR analysis. The results obtained revealed that with the intervention of *V. axillare*, the concentration of TNF-α cytokine and expression of TNF-α mRNA both significantly decreased compared with the model group, and the high concentration group of *V. axillare* had better effects in decreasing the expressions of TNF-α cytokine and TNF-α mRNA. TNF-α is a major mediator of the acute inflammatory response that is generated during many disease states, including infection and inflammation[8], and TNF-α has been learned to play an important role in gastric mucosal apoptosis[11]. Therefore, we concluded that *V. axillare* may play an important role in inhibiting inflammation and gastric mucosal apoptosis.

In addition, we studied the effects of *V. axillare* on the concentration of ET-1 cytokine and expression of ET-1 mRNA. They are both significantly decreased by *V. axillare* compared with the model group. ET-1 is also a major mediator of the acute inflammatory response which relates to proinflammatory cytokines production such as TNF-α and IL-1β[25]. An increase in endogenous ET-1 release causes gastric mucosal microcirculatory disturbance in gastric mucosal injury by ethanol in rats[26] and significantly decline of gastric mucosal blood flow, further more leading to gastric mucosal injury. Therefore, we concluded that the effects of *V. axillare* against gastric mucosal lesions may depend on inhibiting inflammation and improving local microcirculation.

V. axillare, one of scrophulariaceous plants, is rich in resources in Zhejiang Province of China with complex components. The effective components against gastric mucosal lesions induced by ethanol in rats are still unknown. So it remains to be verified which components could account for the mitigation of ethanol-induced gastric mucosal injury through intragastric administration of water extract of *V. axillare*. Further studies to separate and purify the chemical compositions of *V. axillare* are necessary for understanding the effective components. So far, pathological mechanism of treating disease on *V. axillare* has not been reported yet. So it needs to be further studied on the pharmacological efficacies and mechanisms of *V. axillare*.

In conclusion, water extract of *V. axillare* significantly inhibits the acute gastric mucosal injury induced by ethanol in rats. This effect may be due to inhibition of pro-inflammatory cytokines production, a reduction of TNF-α and ET-1 mRNA expressions into the gastric mucosa, inhibiting apoptosis in gastric mucosa tissues and improving local microcirculation of gastric mucosa tissues. Our results demonstrate for the first time that water extract of *V. axillare* is useful for the therapy of gastric mucosal damage induced by ethanol. Further studies to elucidate the precise mechanism of action and effective components of *V. axillare* are important for understanding the protective effects against gastric mucosal damage induced by ethanol. Furthermore, *V. axillare* is rich in resources which offer very high potential for exploitation[1].

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Gastric ulcer has become a worldwide public healthy problem. As the rise of people living standard, the increase of social activities and social work stress are aggravating. Drinking alcohol has become a universal social problem between east and west. Heavy drinking hard liquor, in particular, can cause gastric mucosa injury, resulting in gastric ulcer. Research and development of new drugs against gastric ulcer caused by ethanol is essential.

Research frontiers

The study was conducted in order to verify the effect of *V. axillare* against gastric ulcer caused by ethanol. It focused on the effects of anti-inflammatory, inhibiting apoptosis, and improving local microcirculation.

Related reports

Guifang *et al*. (2012) reported antiulcer effects and action pathways of *V. axillare* on gastric ulcer in rat induced by ethanol. However, this study is the first time for report using real time fluorescent quantitative PCR and pathology morphological analysis on the water extract of *V. axillare* against gastric ulcer.

Innovations and breakthroughs

This study showed that water extract of *V. axillare* may have the effect of anti-inflammatory, inhibiting apoptosis, and improving local microcirculation which has potential for exploitation.

Applications

Due to the property against gastric ulcer caused by ethanol on water extract of *V. axillare*, it may be a good alternative for the treatment of gastric ulcer after completing necessary study.

Peer review

This is a good study in which the authors evaluated the effect of water extract of *V. axillare* that are effective against gastric ulcer caused by ethanol. The results are interesting and suggest that *V. axillare* may be used as alternative therapy for gastric ulcer.

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