JPP
Journal of
Pharmacy and Pharmacology

JPP 2010, 62: 381–388

Received August 29, 2009
Accepted December 17, 2009
DOI 10.1211/jpp/62.03.0014
ISSN 0022-3573

Research Paper

Gastroprotective activity of atractylenolide III from *Atractylodes ovata* on ethanol-induced gastric ulcer *in vitro* and *in vivo*

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Abstract

Objectives The rhizome of *Atractylodes ovata* De Candolle is popularly used in traditional Chinese medicine to treat gastrointestinal diseases. However, the major gastroprotective compounds of *A. ovata* have not been identified. This study reports on the principal gastroprotective component of *A. ovata*.

Methods Five sesquiterpenoids (atractylon, atractylenolides I, II, III and biatractylolide) were isolated from the extracts of *A. ovata* rhizome via silica gel column chromatography. The gastroprotective effects of these five sesquiterpenoids were measured in in-vitro ethanol-induced primary culture rat gastric mucosal (PRGM) cell damage and in-vivo ethanol-induced acute rat gastric ulcer models.

Key findings Atractylon, atractylenolide I and biatractylolide were strongly toxic in PRGM cells, whilst atractylenolides II and III were not. Atractylenolide II did not show cytoprotective effects, but oral administration of atractylenolide III dose-dependently prevented ethanol-induced PRGM cell death and cell membrane damage. The EC50 values were 0.27 and 0.34 mM, respectively. In the in-vivo assay, atractylenolide III 10 mg/kg significantly reduced 70% ethanol-induced Wistar rat gastric ulcer. Atractylenolide III could inhibit matrix metalloproteinase (MMP)-2 and MMP-9 expression through upregulation of tissue inhibitors of metalloproteinase from the gastric ulcerated tissues.

Conclusions Atractylenolide III was the major gastroprotective component of *A. ovata* in ethanol-induced acute gastric ulcer. It is suggested that the gastroprotective mechanism of atractylenolide III was via inhibition of the MMP-2 and MMP-9 pathway.

Keywords atractylenolide III; *Atractylodes ovata*; matrix metalloproteinases; primary culture rat gastric mucosal cells; tissue inhibitors of metalloproteinase

Introduction

Atractylodes ovata De Candolle (Bai Zhu), which is of the Compositae family, is classified as a tonic herb and is widely used in traditional Chinese medicine herbal prescriptions. Traditional properties and actions of *A. ovata* have been described as warm, bitter and sweet and entering the heart, spleen and stomach meridians. According to Ben-Cao-Gang-Mu, the traditional classical Chinese pharmacopoeia, *A. ovata* strengthened and nourished the stomach and could cure patients with splenic asthenia and oedema. It has been the principal drug in many Chinese medicinal prescriptions.[1] It has been used to treat symptoms of the gastrointestinal tract, such as anorexia, for two thousand years.[2] Sesquiterpenoid-type compounds are the major components of *A. ovata* and contributed to most of the pharmacological functions i.e. regulation of gastric emptying time, inhibition of tyrosinase and Na^+–K^+-ATPase activity, antihepatotoxicity and anticancer properties.[3–7] Evidence has shown that *A. ovata* maintained cell membrane potentials, controlled uterine contraction and decreased xylene-induced mouse ear oedema.[8,9] However, there have been few reports on the gastroprotective effects of *A. ovata* and the principal components of *A. ovata* have not been studied thoroughly.

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Gastric ulcers are a common gastrointestinal disorder worldwide. The integrity of the gastric mucosa is maintained by a balance of aggressive and defensive factors.[10] Although *Helicobacter pylori* is a recognized aggressive factor, other aggressive factors are involved in the pathogenesis of gastric ulcers, such as endogenous factors, like pepsin and gastric acid.[11] Besides, many unresolved issues play important roles in ulcer formation, such as the use of aspirin and non-steroidal anti-inflammatory drug (NSAID) treatment in elderly patients and the complication of patients' alcohol abuse.[12] Alcohol is one of the most generally abused chemicals. In the United States, the consumption of pure ethanol per year was approximately 10 l and this phenomenon may be even higher in Spain and France.[13] In the development of gastrointestinal tumours, alcohol consumption might cause haemorrhagic gastritis, thus increasing the risk of gastric ulcer occurrence.[14] Higher concentrations of ethanol (over 40% v/v) would cause the denaturation and dehydration of the gastroprotective mucus layer, leading to the direct damage of gastric mucosal cells.[15,16] Alcohol consumption also caused free radical chain reaction, activating matrix metalloproteinase (MMP) expression, resulting in the pathogenesis of acute gastric ulcers.[17] MMPs consist of a zinc-dependent endopeptidase family and act as the aggressive factor in damaging the extracellular matrix (ECM) in gastric mucosa. For example, MMP-2 and MMP-9 are gelatinase-type enzymes and digest basement membrane type IV collagen, resulting in the morbidity of gastric ulcer.[18] Swarnakar *et al*.[19] showed that secreted MMP-2 and MMP-9 activity of ulcerated mucosa increased in parallel with incremental doses of ethanol. Hence, acute gastric ulcer induced by ethanol had a high correlation to the expression of MMP-2 and MMP-9.

In this study, we have isolated the sesquiterpenoids from *A. ovata* and explored the gastroprotective principal component against ethanol-induced acute gastric ulcer. We have discussed the gastroprotective mechanism of *A. ovata*.

Materials and Methods

Chemicals and reagents

HPLC-grade acetonitrile and absolute ethanol were purchased from Merck (Darmstadt, Germany). Penicillin–streptomycin, trypsin–EDTA, fetal bovine serum (FBS) and Hanks' balanced salt solution (HBSS) were obtained from Gibco (Gibco-BRL, Paisley, UK). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), collagenase type IV and hyaluronidase were purchased from Sigma–Aldrich (St Louis, MO, USA). Western blotting antibodies against anti-MMP-2 (clone 2C1) anti-TIMP-1 (tissue inhibitor 1 of metalloproteinase; clone R-18), anti-TIMP-2 (clone H-140) and anti-α-tubulin (clone TU-02) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-MMP-9 antibody was obtained from Sigma–Aldrich). Cell culture medium, Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 1 : 1 (DMEM/F12), was purchased from Gibco. A Millipore Milli-RO system (Milli-QRG, Billerica, MA, USA) was used to prepare the purified deionized water.

Isolation of sesquiterpenoids

A. ovata De Candolle was purchased from a traditional Chinese medicinal market in Taipei and identified by Dr Hsien-Chang Chang. A voucher specimen of *A. ovata* (AO-1013) was deposited in the Graduate Institute of Pharmacognosy Science, Taipei Medical University. The isolation procedure was modified from a previous study.[20,21] Evidence has shown that the major active components of *A. ovata* were sesquiterpenoids. Thus, we chose the nonpolar solvent *n*-hexane as the extraction solvent. However, few studies have reported the bioactivity of the polar components of *A. ovata,* e.g. polysaccharide, so the gastroprotective properties of the polar components of *A. ovata* have not been discussed in this study. *A. ovata* was immersed in *n*-hexane for two weeks and the *n*-hexane solvent was removed by a rotary evaporator to obtain the *n*-hexane extracts. The *n*-hexane extracts were subjected to silica gel column chromatography (45 × 9 cm i.d.) with an *n*-hexane–ethyl acetate gradient elution. Atractylon was obtained from recrystallization of the *n*-hexane fraction with cold 95% ethanol.[20] Atractylenolides I, II (AT-I, AT-II) and biatractylolide were obtained from rechromatography of the *n*-hexane–ethyl acetate (90 : 10 v/v) fraction with the mobile phase of *n*-hexane–ethyl acetate (90 : 10→85 : 15 v/v). Atractylenolide III (AT-III) was obtained from the recrystallization of the *n*-hexane–ethyl acetate (80 : 20 v/v) fraction with *n*-hexane. Structural estimation of these five sesquiterpenoids was compared with published papers.[22–27] The purity of each compound reached 99.0% by HPLC analysis. The UV spectrum of each compound was checked also (data not shown).

Animals

Male Wistar rats (approximately 250 ± 10 g) were purchased from the BioLASCO Taiwan Co., Ltd and kept on a 12 : 12-h day : night cycle. Animals were maintained in polycarbonate cages at 21 ± 2°C with food and water freely available. All experimental procedures involving animals followed the ethical regulations of Taipei Medical University.

Primary culture of rat gastric mucosal cells

A primary culture of rat gastric mucosal (PRGM) cells was isolated from Wistar rats. Before the operation, rats were starved for 24 h with free access to water. After the rats had been killed by CO_2 asphyxiation, the stomachs were removed. The fresh stomach was washed with HBSS and then cut into 0.3-mm^3 pieces. Gastric mucosal cells were isolated from the tissues by incubating them with a mixture of 0.05% type IV collagenase and 0.025% hyaluronidase for 90 min at 37°C with 5% CO_2, and then filtering through a 40-μm cell strainer (BD Biosciences, MA, USA). Cells were washed with HBSS, centrifuged for 10 min at 500*g* and harvested in DMEM/F12 containing 10% FBS, 1 μg/ml streptomycin and 1 IU/ml penicillin. After three days harvesting, the medium was changed to remove non-adhering cells. The PRGM cells were confirmed by periodic acid–Schiff (PAS) staining. After staining with PAS solution, mucopolysaccharides of PRGM cells reacted with the periodic acid and Schiff's reagent to form pararosaniline adducts which revealed a pink colour. Through staining of

PAS, it was confirmed that almost 95% of isolated cells were PRGM cells. The cells from passages three to five were used for the following experiments.

Ethanol-induced PRGM cell death

PRGM cells (1×10^5 cells/ml) were seeded on a 24-well plate for one day. To determine a suitable concentration of ethanol, cells were treated with different concentrations of ethanol (0, 30 and 70%) for 4 h. Different doses of atractylon, AT-I, AT-II, AT-III and biatractylolide (100, 200 and 400 μM) were used to evaluate the cytotoxicity in PRGM cells. In addition, the nontoxic compounds were pretreated for 1 h and co-treated with ethanol for 4 h to evaluate the cytoprotective effects. The commercial gastroprotective drug, Gelfos gel (Laboratoires Biotherax, La Plaine Saint Denis, France) whose major component was colloidal aluminium phosphate, was used as the positive control.

Cytotoxicity assay

Cell viability was determined by examining the mitochondrial-dependent reduction of the tetrazolium compound MTT to formazan. Following treatment with different samples and ethanol, cells were washed with phosphate-buffered saline (PBS) and MTT was added to the medium for 4 h. The supernatant was removed, and formazan crystals were dissolved by 0.04 M HCl in isopropanol. The optical density was measured at 600 nm with a μQuant spectrophotometer ((BioTek, Winooski, VT, USA).

Cell membrane integrity assay

Release of lactate dehydrogenase (LDH) from the culture medium of damaged cells was determined using a Roche cytotoxicity detection kit. LDH is a stable cytoplasmic enzyme present in all cells and rapidly released into the cell culture supernatant upon damage of plasma membranes. The amount of enzyme activity detected in the culture supernatant is correlated to the proportion of lysed cells. The maximum amount of releasable LDH enzyme activity was determined by lysing cells with Triton X-100. In a 96-well flat-bottom microtitre plate, culture medium was added to the reaction mixture in each well, and incubated for up to 30 min at room temperature while being protected from light. The optical density was measured at 490 nm with a μQuant spectrophotometer (BioTek).

Ethanol-induced acute gastric ulcer in Wistar rats

AT-III (5 and 10 mg/kg) was used to test the gastroprotective functions. Wistar rats were starved for 24 h with free access to water. The gastroprotective effects of AT-III were determined by co-treating different samples with 70% ethanol (v/v) at a dose of 0.1 ml/10 g body weight per rat by oral administration for 4 h. The control group was treated with 70% ethanol only, while the sham group received distilled water but no ethanol. The rats were killed with CO_2 and the stomachs were isolated. A section of stomach was cut from the greater curve and washed with PBS. Pictures of different levels of lesions were taken with a proportional scale, and ulcerated areas were analysed with Scalar Digital Scale 1.0E software (Scalar Corporation, Tokyo, Japan). Calculation of the ulcer index was modified from Swarnakar *et al.*[19]: 0 = no lesion formation; 1 = less than 10 minor lesions; 2 = more than 10 minor lesions; 3 = less than five haemorrhagic bands; 4 = more than five haemorrhagic bands; 6 = complete lesions with haemorrhage. Tissue protein of the whole stomach was extracted with a modified method.[28]

Histopathological analysis

After scoring the ulcerated level, the fundic stomach was fixed in 10% paraformaldehyde, gradually dehydrated in ethanol, and embedded in paraffin. Sections (5 μm) were cut using a microtome, stained with haematoxylin and eosin (H&E) and observed under a light microscope.[29]

Gelatin zymography

Firstly, protein of the mucosal extracts (80 μg) was quantified with the Bradford method.[30] Non-reducing SDS-polyacrylamide gel with 0.1% gelatin was used to analyse MMP-2 and MMP-9 activity. After gel electrophoresing, gel was washed with 2.5% Triton X-100, incubated with the reaction solution (40 mM Tris-HCl, pH 8.0; 10 mM $CaCl_2$; 0.01% NaN_3) at 37°C for 12 h and stained with 0.25% Coomassie Blue R-250. The gelatinolytic results were represented as a clear band against a blue background by reacting with the destaining solution. MMP activity was determined by scanning the gelatinolytic zone and this was analysed with the AlphaImager Imaging System (Cell Biosciences, Inc., Santa Clara, CA, USA).

Western blotting

Equal amounts of protein from each group were prepared and separated on 8–12% non-reducing SDS-polyacrylamide gels. Gels were transferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA) and nonspecific binding was blocked with 1% bovine serum albumin at 37°C for 1 h. Blots were incubated overnight at 4°C with anti-MMP-2, anti-MMP-9, anti-TIMP-1, anti-TIMP-2, anti-GAPDH and anti-α-tubulin antibodies. Membranes were washed with PBS–Tween 20 (PBST) and incubated for 1 h at 37°C with alkaline phosphatase-conjugated secondary antibodies. Protein expression was visualized by staining with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate solution (BCIP/NBT). Protein expressions were analysed with the AlphaImager Imaging System.

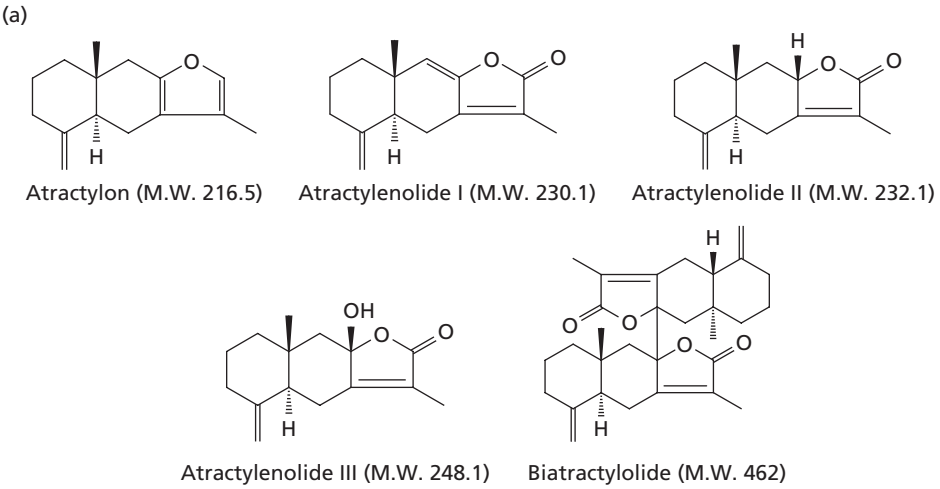
Statistical analysis

Data were presented as the mean and SD. Data were analysed with one-way analysis of variance by SPSS 12 software (SPSS Inc., Chicago, IL, USA) and the Student's *t*-test.

Results

Cytotoxicity of sesquiterpenoids in PRGM cells

Five sesquiterpenoids (atractylon, AT-I, II, III and biatractylolide) were isolated from *A. ovata*. Their structures and molecular weights are shown in Figure 1a. Of the five compounds, atractylon, AT-I and biatractylolide exerted a strong cytotoxic effect in PRGM cells at 400 μM for 24 h. AT-II and AT-III did not show any cytotoxicity at 400 μM in PRGM cells (Figure 1b).



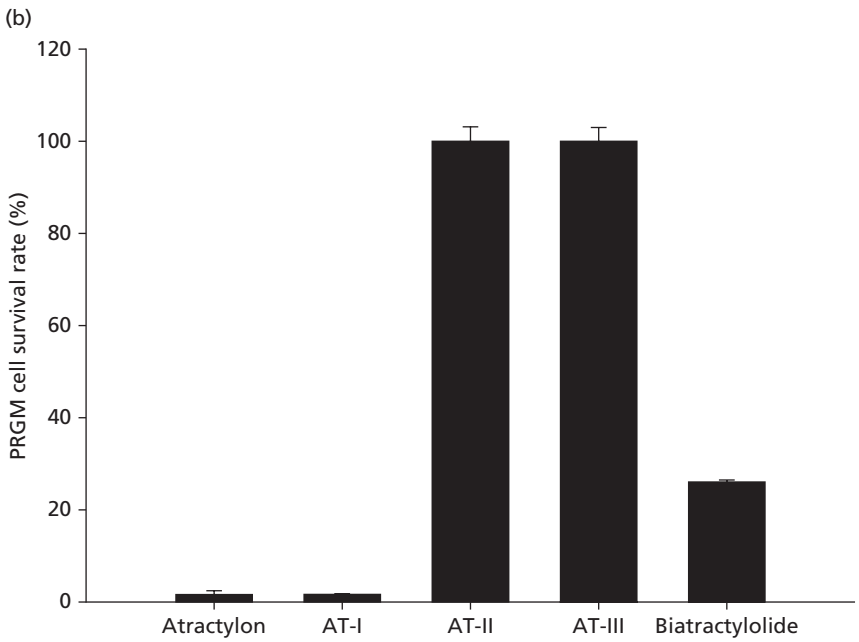


Figure 1 Five sesquiterpenoids isolated from extracts of *Atractylodes ovata.* (a) Chemical structures and molecular weights of the five sesquiterpenoids atractylon, atractylenolide I (AT-1), atractylenolide II (AT-II), atractylenolide III (AT-III) and biatractylolide. (b) Cytotoxicity effects of atractylon, AT-I, AT-II, AT-III, and biatractylolide at 400 μM for 24 h in PRGM cells

In-vitro gastroprotective effects of AT-III

The in-vitro 6% ethanol-induced PRGM cell death model was established in this study. AT-II and AT-III did not show cytotoxicity in PRGM cells, and they were used to detect the gastroprotective effects in ethanol-induced PRGM cell death. As shown in Figure 2a, AT-III significantly protected PRGM cell death. Due to the good protective effects of AT-III at 400 μM, lower doses of AT-III were tested. As shown in Figure 2b, AT-III significantly prevented 6% ethanol-induced PRGM cell death and cell membrane damage in a dose-dependent manner. Gelfos, the commercial Western gastroprotective drug, also displayed good cytoprotective effects at 50 μg/ml.

In-vivo gastroprotective effects of AT-III

Acute gastric ulcer of Wistar rats was induced by 30% and 70% ethanol. Figure 3a shows that the increasing doses of ethanol significantly increased the ulcerated areas and ulcer index. In addition, protein expression and enzyme activity of MMP-2 and MMP-9 were significantly induced by 70% ethanol (Figure 3b and c). Taken together, 70% ethanol was used as the inducer in this gastric ulcer in-vivo model.

In pathological analysis, AT-III significantly and dose-dependently suppressed gastric ulcer formation, and reached maximal inhibitory effects at 10 mg/kg (Figure 4a). We investigated further the MMPs expression profiles after AT-III administration. The untreated group was presented as the sham

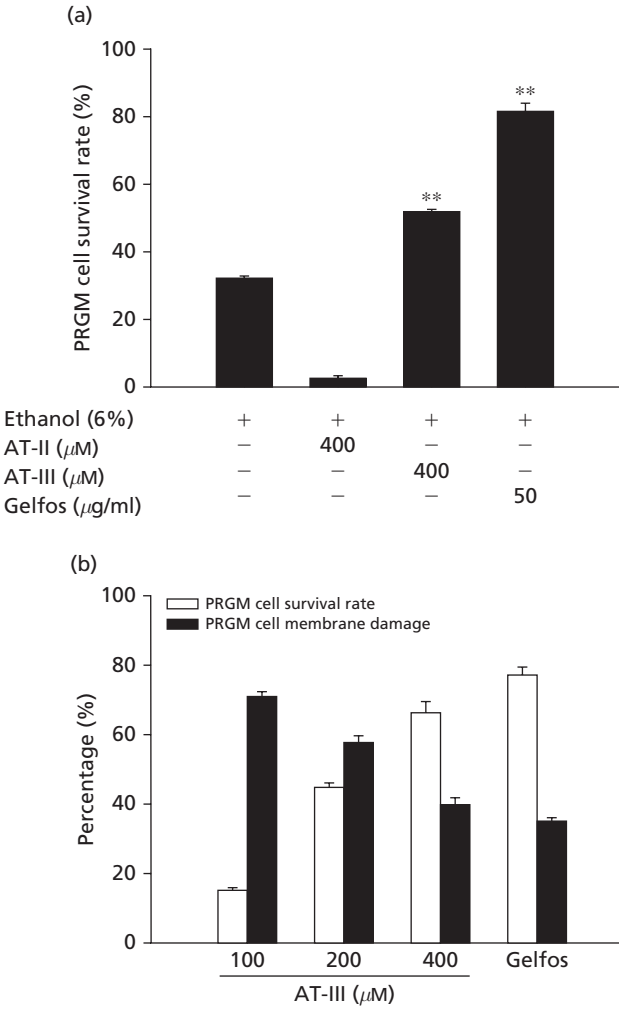


Figure 2 Cytoprotective effects of atractylenolide II and atractylenolide III extracted from *Atractylodes ovata.* (a) Cytoprotective effects against 6% ethanol-induced PRGM cell death and (b) cell membrane damage. Atractylenolide II, AT-II; atractylenolide III, AT-III. Gelfos (50 μg/ml) was used as the positive control. $^{**}P< 0.005$ compared with the ethanol only group. All data were expressed as the mean and SD, analysed through one-way analysis of variance by SPSS software. $n = 3$

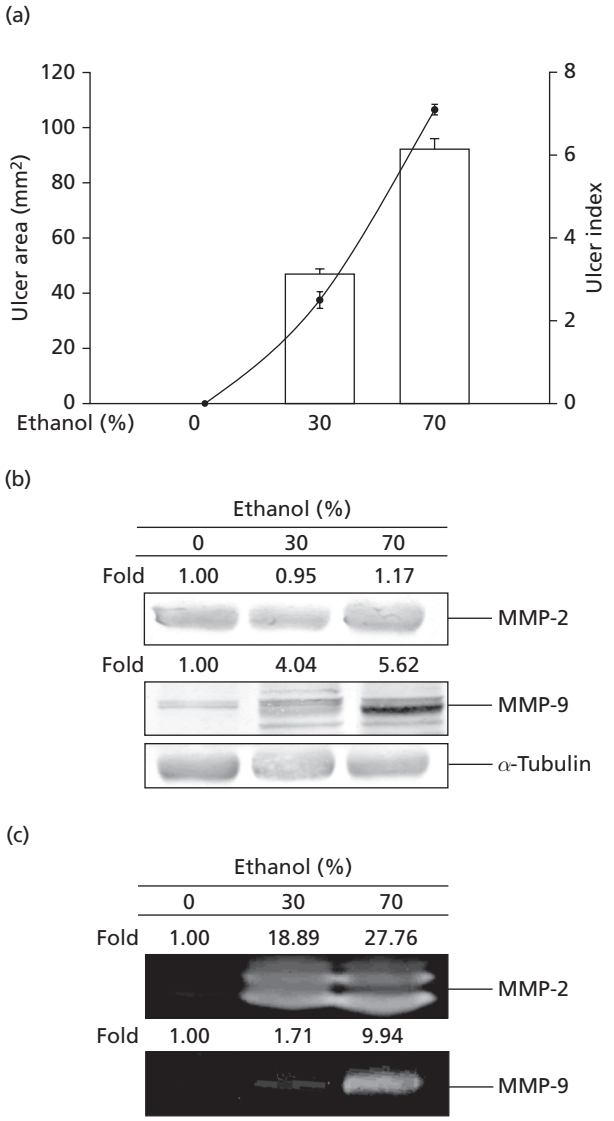


Figure 3 Effects of different concentrations of ethanol on gastric ulcer and correlation of ulcer areas and ulcer index with matrix metalloproteinase protein expression and enzyme activity. (a) Ulcerated areas and index were caused by different concentrations of ethanol. $n = 8$. Protein expression (b) and enzyme activity (c) of matrix metalloproteinase (MMP)-2 and MMP-9

group. As Figure 4b shows, the highly induced MMP-2 and MMP-9 protein expression was dose-dependently diminished by AT-III at 5 and 10 mg/kg. Expression of MMP-2 and MMP-9 was regulated by TIMP-2 and TIMP-1. The results suggested that AT-III dose-dependently downregulated the MMP-2 and MMP-9 expression through activating the TIMP-2 and TIMP-1 expressions. In addition, co-treatment with AT-III significantly decreased the enzyme activity of MMP-2 and MMP-9 by approximately 20–40% (Figure 4c). In the histological analysis, acute degeneration, necrotic erosion and deep ulceration of gastric tissues were found in the 70% ethanol-treated group. Moreover, infiltrations of inflammatory cells were found in the ulcerated tissues (Figure 5, control group). Histological inspection of the tissues indicated that co-treatment of AT-III reduced the necrotic erosion of gastric mucosa (Figure 5, AT-III group), suggesting the strongly gastroprotective effects of AT-III. Active proliferation was required for rejuvenation of the mucosal layer. In the AT-III-treated group, many proliferated mucosal cells were found.

Discussion

In this study, we modified the isolation procedure from our previous study and obtained an additional compound, biatractylolide. Biatractylolide was classified as a bisesquiterpene lactone, which has been reported as having blood pressure lowering effects via negative inotropic and chronotropic activities.[27] Biosynthetically, recent studies have reported that biatractylolide could originate from the dimerization of AT-II or AT-III, while atractylon was a possible intermediate

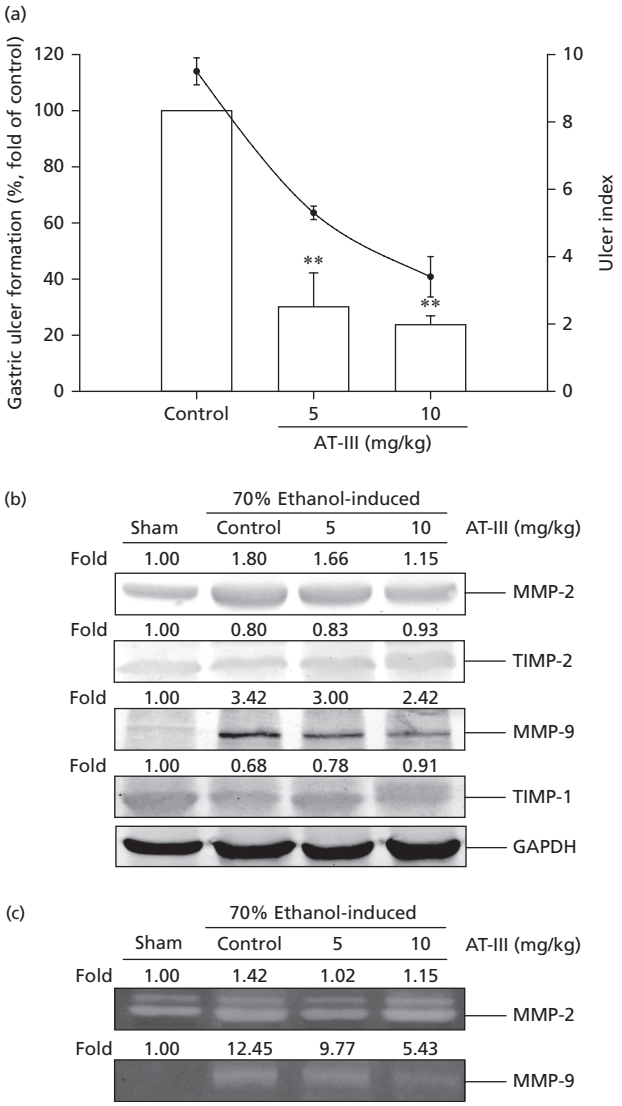


Figure 4 Effects of atractylenolide III against 70% ethanol-induced gastric ulcers and correlation with matrix metalloproteinase protein expression and enzyme activity. Atractylenolide III (AT-III) was extracted from *Atractylodes ovata*. (a) Gastroprotective effects were presented as the percentage of ulcer formation and ulcer index, $n = 8$. Protein expressions of matrix metalloproteinase (MMP)-2, MMP-9, tissue inhibitors of metalloproteinase (TIMP)-1 and TIMP-2 (b) and enzyme activity of MMP-2 and MMP-9 (c). $^{**}P < 0.005$. All data are expressed as the mean and SD

during the synthesis.[26,27] Evidence has shown antitumour effects of atractylon and AT-I on leukaemia cell lines, and these two compounds also displayed strong cytotoxicity in PRGM cells (Figure 1b).[20,31,32] However, biatractylolide also showed significant cytotoxicity effects in PRGM cells and so we chose the noncytotoxic compounds, AT-II and AT-III, for the gastroprotective assay. Due to the stronger PRGM cell protective effects of AT-III, we used AT-III to evaluate the in-vivo gastroprotective effects (Figure 2a, b).

The aetiology of gastric ulcer involves many aggressive factors, including ethanol, NSAIDs, stress and *H. pylori* infection. Ethanol is a well-known denaturant and will cause the denaturation of protein. In the ethanol-induced gastric ulcer formation, it would disrupt the gastric barrier, directly damaging the superficial mucosal cells and causing the breakdown of DNA strands.[33] Recent study has shown several mechanisms for the pathogenesis of ethanol-induced gastric ulcer, while MMPs play a crucial role in the ulcer formation.[34] Different types of MMPs selectively caused the degradations of extracellular matrix (ECM) i.e. collagen, gelatin, elastin and fibronectin, and could cause ECM remodelling. During the normal biological functions, balance of activation and inactivation of MMPs were regulated by tissue inhibitors of metalloproteinase (TIMP). The imbalance of MMPs would cause the pathogenesis of many diseases i.e. gastric ulcer. MMP-2 and MMP-9 are gelatinases-type enzymes and specifically regulate acute and chronic gastric ulcers.[34] Evidence has shown that ethanol would increase the expression of MMP-2 and MMP-9, resulting in acute gastric ulcer.[19,34,35] Analysing the expression of MMPs, AT-III downregulated MMP-2 and MMP-9 expression through upregulating TIMP-2 and TIMP-1 expressions during the prevention of ethanol-induced gastric ulcer (Figure 4b). In addition, the attenuation of MMP-2 and MMP-9 activity was correlated to the downregulation of protein expression (Figure 4c).

Dysregulation of gastric inflammatory response led to an inappropriate recruitment of macrophages into the gastric mucosa, causing gastric mucosal injury. Administration of high concentrations of ethanol led to the protein denaturation of gastric mucosa, neutrophil infiltration, acute gastric inflammation and mucosal barrier damage.[36] Tumour necrosis factor-α (TNF-α), which is secreted by active macrophages, is a pleiotropic inflammatory cytokine and can be induced by several pro-inflammatory agents e.g. lipopolysaccharide and ethanol. Previous studies have shown that ethanol enhanced and triggered TNF-α production, which in turn activated the MMP-2 and MMP-9 gene expression, resulting in gastric ulcer formation.[37,38] Besides the ECM damage by MMPs, release of TNF-α would inhibit microcirculation and cell proliferation of the ulcerated tissues and impair ulcer healing.[39] That *A. ovata* had significant downregulatory action on TNF-α production was supported in a recent study.[40] In Li *et al*.,[40] AT-III acted as the active component on the anti-inflammatory activity, and the possible mechanism was through inhibition of TNF-α production.[40] Taken together, we suggested that the gastroprotective mechanism of AT-III was through arresting expression of MMP-2 and MMP-9.

Conclusions

Of the five sesquiterpenoids of *A. ovata*, AT-III was the principal gastroprotective component in ethanol-induced gastric mucosal damage in in-vitro and in-vivo models. The gastroprotective mechanism of AT-III was via inhibition of MMP-2 and MMP-9 expression, decreasing the ECM damage and preventing gastric ulcer formation. In traditional Chinese medicine, *A. ovata* has been used to treat gastrointestinal tract symptoms. This study has been the first to explore the gastroprotective role of AT-III from *A. ovata* on

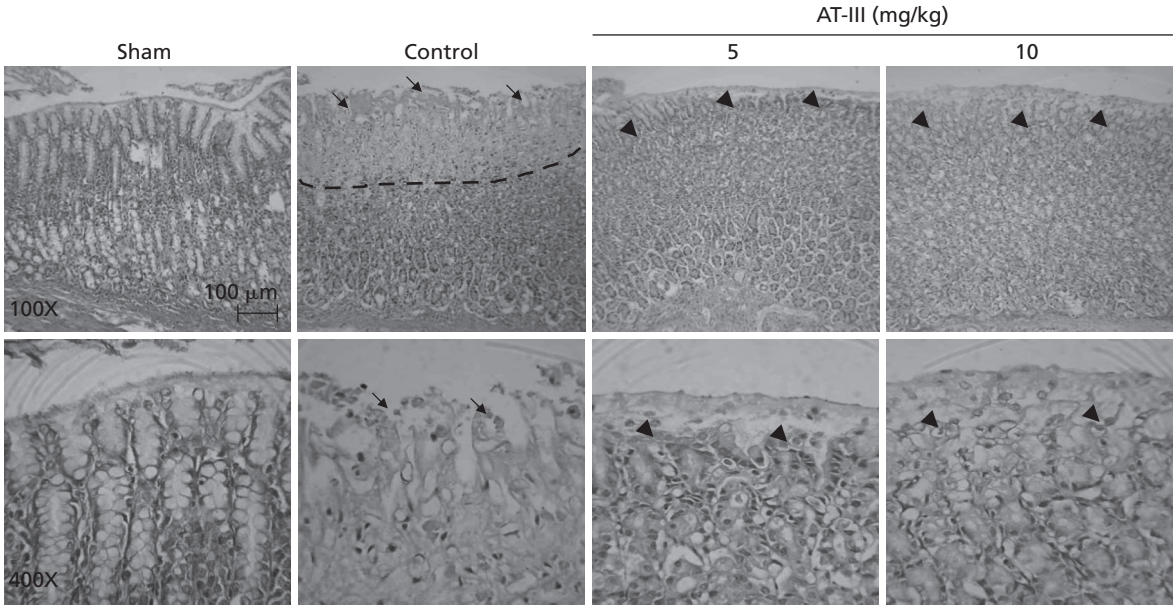


Figure 5 Histological analysis of the gastroprotective effects of atractylenolide III against ethanol-induced gastric ulcer in rats. Atractylenolide III (AT-III) was extracted from *Atractylodes ovata.* Gastric tissues were stained with haematoxylin and eosin at 100× and 400× magnifications. In the control group, gastric mucosa displayed acute degeneration, necrosis erosion and deep ulceration (above the dotted line region, black arrows). In the AT-III-treated group, many proliferated mucosal cells were found for the rejuvenation of the mucosal layer (solid black triangles)

ethanol-induced gastric ulcer. We found that AT-III was the principal gastroprotective component of *A. ovata* and this has improved our understanding of the gastroprotective mechanisms of *A. ovata*.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was supported by a grant from the Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, Taiwan (CCMP96-CP-009).

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