JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

# Antigastritis effects of *Armillariella tabescens* (Scop.) Sing. and the identification of its anti-inflammatory metabolites

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**Keywords**
antigastritis; anti-inflammation; *Armillariella tabescens*; NF-kappa B; Tricholomataceae

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Received July 27, 2017
Accepted November 25, 2017

doi: 10.1111/jphp.12871

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## Abstract

**Objectives** This study demonstrates the biological and chemical analysis of the mushroom *Armillariella tabescens* (Scop.) Sing. (Tricholomataceae).

**Methods** Chemical structures of the isolates were determined by 1D and 2D NMR, and ESI-MS, as well as comparison with previously reported data. All isolates were tested for anti-inflammatory effects based on their ability to inhibit LPS-stimulated nitric oxide (NO) production in RAW264.7 cells.

**Key findings** We found that the MeOH extract of the fruiting bodies of *A. tabescens* showed antigastritis activity against ethanol-induced gastric damage in rats and notably reduced the gastric damage index compared to control in a concentration-dependent manner. Chemical investigation of the MeOH extract led to the isolation of four steroids (**1**–**4**), three alkaloids (**5**–**7**), two nucleic acids (**8**–**9**) and four fatty acids (**10**–**13**). This is the first study to report the identification of all isolates, except for compound **7**, from *A. tabescens*. Compounds **1**, **2**, **3**, **4** and **10** showed inhibition on LPS-stimulated NO production. Treatment with compound **10** inhibited expression of iNOS, COX-2, phospho-IKKα, IKKα, phospho-IκBα, IκBα and NF-kappa B in LPS-stimulated RAW264.7 cells.

**Conclusions** Compound **10** likely contributes to the health benefits of *A. tabescens* as an antigastritis agent through its anti-inflammatory effects.

## Introduction

Mushrooms are drawing attention as a potential new source of medicine, as they are of a wide source of natural traditional medicine.[1–4] There are reports on medicinal mushrooms that are used to treat diseases such as gastritis. *Phellinus rhabarbarinus*, a popular Chinese folk medicine, is reported to enhance immunity and treat diseases such as gastritis and cancer.[1] *Inonotus obliquus*, another popular medicinal mushroom, has been recognized as a remedy for gastritis, as well as cancer and ulcers.[2] Many mushrooms have been found to contribute to the prevention of gastritis to some degree, although the underlying pharmaceutical mechanism is yet to be fully understood.[3,4]

Our group has aimed to discover secondary metabolites from Korean wild mushrooms possessing biological activity,[5–10] and our endeavour in searching for bioactive secondary metabolites has led us to the collection of endemic mushroom species from the mountainous regions during the hot summer, to test in our bioactivity screening system. Among the collected mushrooms, we found that the MeOH extract of the fruiting bodies of *Armillariella tabescens* (Scop.) Sing. showed antigastritis activity against ethanol-induced gastric damage in rats.

*Armillariella tabescens* is a medicinal mushroom that belongs to the family Tricholomataceae and is known as 'Luminous Fungus' in China. As a traditional medicine, it has been used to treat cholecystitis, improve bile secretion and regulate the pressure of the bile duct to lessen inflammation as well as for proper function of liver.[11,12] In a previous research, armillarisin A, a chemical compound identified from *A. tabescens*, was reported to be the active


 

component for the treatment of infection of the biliary system, gastritis and hepatitis.[11,12] Armillarisin B, another component of *A. tabescens*, was reported to show a significant antifungal effect on mycelia growth of *Gibberella zeae*.[11] In addition, polysaccharides that have antitumour activity and immunomodulating activity were identified from this mushroom;[13–20] however, other significant chemical metabolites of *A. tabescens* have not yet been reported. To the best of our knowledge, our screening data demonstrate for the first time that the MeOH extract of *A. tabescens* showed antigastritis activity, suggesting its potential as a functional food with preventive and therapeutic benefits for gastritis. To identify chemical contributors to the antigastritis activity of the MeOH extract of *A. tabescens*, the extract was chemically analysed, and as a result, 13 compounds were isolated and identified as the main components of *A. tabescens*. Here, we describe the antigastritis effects of *A. tabescens* and the chemical analysis of the MeOH extract of its fruiting bodies as well as the bioactivity of the isolates with regard to anti-inflammatory activity.

## Materials and Methods

### Fungus material, extraction and isolation of compounds

The fresh fruiting bodies of *A. tabescens* were collected from Hwasung, Gyeonggi-do, Korea, in September 2014. The chopped fungus material (350 g) was extracted with 80% aqueous MeOH three times (each 3 l × 24 h) at room temperature. The resulting extracts were filtered, and the filtrate was evaporated under reduced pressure using a rotavapor. A crude MeOH extract (31.8 g) was acquired and was suspended in distilled water (700 ml) and MeOH (30 ml) and successively solvent-partitioned using hexane, dichloromethane ($CH_2Cl_2$), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH), which yielded a hexane-soluble layer (1.0 g), a $CH_2Cl_2$-soluble layer (1.1 g), an EtOAc-soluble layer (ESL) (0.4 g) and an *n*-BuOH-soluble layer (BSL) (1.9 g). The hexane-soluble and $CH_2Cl_2$-soluble layers were consolidated to a hexane- and $CH_2Cl_2$-soluble layer (HCSL) after confirmation with TLC analysis that they appeared to have similar major spots.

The HCSL (2.1 g) was subjected to silica gel column chromatography (CC) (hexane/EtOAc, from 30 : 1 to 1 : 1) to give eight fractions (Fr. A1–A8). Fr. A1 (480.0 g) was purified by semi-preparative HPLC (91% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min) to yield compound **10** (18.2 mg). Fr. A3 (237.9 mg) was separated with preparative HPLC (83% MeOH) using an Agilent Eclipse C18 column (21.2 × 250 mm i.d., flow rate: 5 ml/min) to give seven fractions (Fr. A31–A37). Fr. A37 (102.5 mg) was further purified by semi-preparative HPLC (88% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min) to yield compound **3** (2.1 mg). Fr. A4 (202.1 mg) was fractionated with preparative HPLC (85–100% MeOH) using an Agilent Eclipse C18 column (21.2 × 250 mm i.d., flow rate: 5 ml/min) to obtain five fractions (Fr. A41–A45). Fr. A45 (105.0 mg) was further purified using preparative HPLC (84% MeOH) with the same column to give five subfractions (Fr. A451–A455). Fr. A455 (68.5 mg) was separated by semi-preparative HPLC (87% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min), which gave compounds **12** (10.4 mg) and **13** (3.2 mg). Fr. A7 (28.6 mg) was directly subjected to semi-preparative HPLC (90% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min) to give compounds **1** (1.1 mg) and **2** (1.8 mg). Fr. A8 (400.8 mg) was separated on a Sephadex LH-20 column using a solvent system of $CH_2Cl_2$/MeOH (2 : 8), and five fractions were obtained (Fr. A81–A85). Fr. A83 (130.2 mg) was separated with preparative HPLC (70–100% MeOH) using an Agilent Eclipse C18 column (21.2 × 250 mm i.d., flow rate: 5 ml/min) to give five subfractions (Fr. A831–A835). Fr. A834 (17.7 mg) was further purified by semi-preparative HPLC (76% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min) to afford compound **11** (0.9 mg), and Fr. A835 (29.1 mg) was also purified by semi-preparative HPLC (85% MeOH) using the same column to give compound **4** (1.1 mg).

The ESL (0.4 g) was separated by preparative HPLC (30–80% MeOH) using an Agilent Eclipse C18 column (21.2 × 250 mm i.d., flow rate: 5 ml/min) to give eight fractions (Fr. B1–B8). Fr. B1 (68.0 mg) was separated by semi-preparative HPLC (5–10% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min), which gave six subfractions (Fr. B11–B16). Fr. B11 (31.0 mg) was further purified by semi-preparative HPLC (2% MeOH) using the same column, and compound **8** (2.4 mg) was obtained. Fr. B2 (15.4 mg) was purified by semi-preparative HPLC (5–20% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min), and the resulting subfraction (4.6 mg) was further purified using semi-preparative HPLC (2% MeCN) with the same method to yield compound **5** (9.2 mg). Fr. B4 (20.5 mg) was purified with semi-preparative HPLC (5–10% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min), and compounds **6** (0.8 mg) and **7** (0.9 mg) were obtained.

The BSL (1.9 g) was separated by preparative HPLC (10–80% MeOH) using an Agilent Eclipse C18 column (21.2 × 250 mm i.d., flow rate: 5 ml/min) to give five fractions (Fr. C1–C5). Fr. C3 (42.6 mg) was purified with semi-preparative HPLC (5–10% MeOH) using a Phenomenex Luna phenyl-hexyl column (250 × 10 mm i.d., flow rate: 2 ml/min) to yield compound **9** (8.2 mg).

### Ethanol-induced gastric mucosal injury in rats

All procedures involving the use of live animals described in this study were approved in November 2015 by the Institutional Animal Care and Use Committee of the Gachon University (approval number: GIACUC-R2015011), and the NIH guidelines were strictly followed for humane treatment of animals. Male Wistar rats weighing 200–220 g were used to evaluate the protective effect of *A. tabescens* MeOH extract against ethanol-induced mucosal injury in rats. The rats were deprived of food but had free access to water for 24 h before ulcer induction. Gastric mucosal lesions were induced by oral administration of 1 ml of a 60% ethanolic solution containing 0.15 M HCl. Rats were divided into four groups based on body weight and treated with either ethanol or ethanol + *A. tabescens*:

Group 1: Normal controls ($n = 3$), received water only.
Group 2: Ethanol ($n = 3$), received ethanol only.
Group 3: Ethanol + ranitidine 100 ($n = 3$), orally treated with ethanol and ranitidine (100 mg/kg) in aqueous solution.
Group 4: Ethanol + ranitidine 200 ($n = 3$), orally treated with ethanol and ranitidine (200 mg/kg) in aqueous solution.
Group 5: Ethanol + *A. tabescens* 100 ($n = 3$), orally treated with ethanol and *A. tabescens* extract (100 mg/kg) in aqueous solution.
Group 6: Ethanol + *A. tabescens* 200 ($n = 3$), orally treated with ethanol and *A. tabescens* extract (200 mg/kg) in aqueous solution.

All animals were pretreated with water or the pomegranate extract orally 1 h before the ethanol treatment. After 6 h, the rats were killed, and their stomachs were immediately removed.

### Determination of mucosal lesion level

The rat stomachs were removed and opened along the greater curvature to determine the extent of lesion damage. To perform quantitative analysis of the mucosal lesions, pictures of stomach tissue were captured and analysed using the image analysis software Leica Application Suite v3.8 (Leica, Seoul, Korea).

### Measurement of nitric oxide production

*Escherichia coli* LPS (strain 055:B5) was obtained from Sigma-Aldrich (Saint Louis, MO, USA). NO production was determined using the Griess reaction (in Appendix S1), which measures the accumulation of nitrite in the culture medium.

### Western blotting analysis

RAW264.7 cells seeded in 6-well plates were treated with compound **10** (25 and 50 μM).

Western blotting was performed by the method in Appendix S1. Specific proteins were analysed by staining with epitope-specific primary antibodies to iNOS, COX-2, inhibitor of κB-alpha (IκBα), IκB kinase (IKK), nuclear factor-kappa B (NF-κB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and horseradish peroxidase (HRP)-conjugated antirabbit secondary antibodies (Cell Signaling, Boston, MA).

### Statistical analysis

All data were presented as the average value and standard deviation (SD). Multiple comparison tests for the different dose groups were conducted. The Kruskal–Wallis test was used for the statistical analysis of each variable. SPSS statistical package was used for all analyses (IBM SPSS statistics version 21, Boston, MA). Statistical significance was considered at a *P*-value lower than 0.05.

## Results and Discussion

The fruiting bodies of *A. tabescens* were extracted with 80% aqueous MeOH at room temperature, and a crude MeOH extract was obtained after evaporating the resultant extracts under reduced pressure. The MeOH extract was tested for antigastritis activity against ethanol-induced gastric damage in rats, as ethanol is regarded as the major contributor to mucosal lesion formation.[21] The severity of gastric mucosal injury in rats that are induced by ethanol is shown in Figure 1a. The model group showed macroscopic morphological changes including glandular area hyperaemia and mucosal oedema accompanied by dot and linear haemorrhage necrosis. Figure 1b shows that when the gastric lesions were treated with the MeOH extract of *A. tabescens*, the ulcer formation is significantly reduced at doses of 100 and 200 mg/kg, in a dose-dependent manner. On the basis of this result, we have confirmed that *A. tabescens* was effective for the mitigation of ethanol-induced mucosal injury.

As an endeavour to search for the chemical contributors to the antigastritis effects of the MeOH extract of

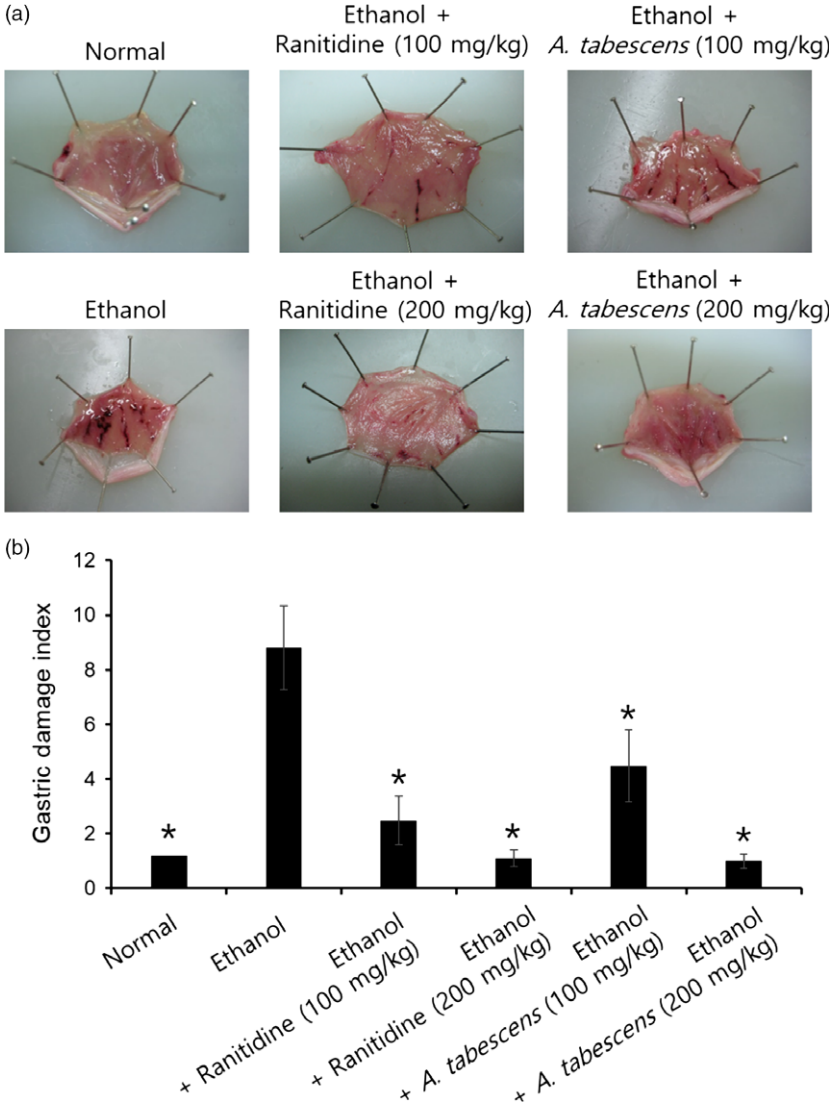


**Figure 1** Inhibitory effect of *A. tabescens* on ethanol-induced mucosal lesions in rats. (a) Representative pictures of a rat stomach. (b) The intensity of the lesioned area analysed by image analysis software. Ranitidine is a positive control for ethanol-induced mucosal lesions in rats. *$P < 0.05$ compared with ethanol-treated group. [Colour figure can be viewed at wileyonlinelibrary.com]

*A. tabescens*, chemical analysis of the MeOH extract was carried out. The MeOH extract was solvent-partitioned using hexane, dichloromethane ($CH_2Cl_2$), ethyl acetate (EtOAc) and *n*-butanol (*n*-BuOH), yielding respective layers. The hexane-soluble and $CH_2Cl_2$-soluble layers were consolidated after TLC analysis, and the combined layer was subjected to repeated column chromatography and preparative and semi-preparative HPLC purification, which resulted in the isolation of eight compounds (**1**–**4** and **10**–**13**). Using the same method, four compounds (**5**–**8**) were isolated from the EtOAc-soluble layer, and compound **9** was obtained from the *n*-BuOH-soluble layer. With the aid of spectroscopic methods including 1D ($^1$H and $^{13}$C NMR), 2D NMR and LC/MS analysis, the structures of the isolated compounds (**1**–**13**) were elucidated (Figure 2). These compounds were identified as 9,11-dehydroergosterol peroxide (**1**),[22] ergosterol peroxide (**2**),[22] (17*R*)-17-methylincisterol (**3**),[23] (3β,5α,22*E*)-ergost-22-en-3-ol (**4**),[24] 3-(aminocarbonyl) pyridine (**5**),[25] 3-carboxyindole (**6**),[26] armillarisin B (**7**),[11] uridine (**8**),[27] adenosine (**9**),[28] ((*Z*,*Z*)-9,12-octadecadienoic acid (**10**),[29] (9*E*)-8-oxo-9-octadecenoic acid (**11**),[30] hexadecanoic acid (**12**)[31] and (9*Z*)-9-octadecenoic acid (**13**)[32] by comparing their obtained spectroscopic data with the reported values. To the best of our knowledge, this study reports the first identification

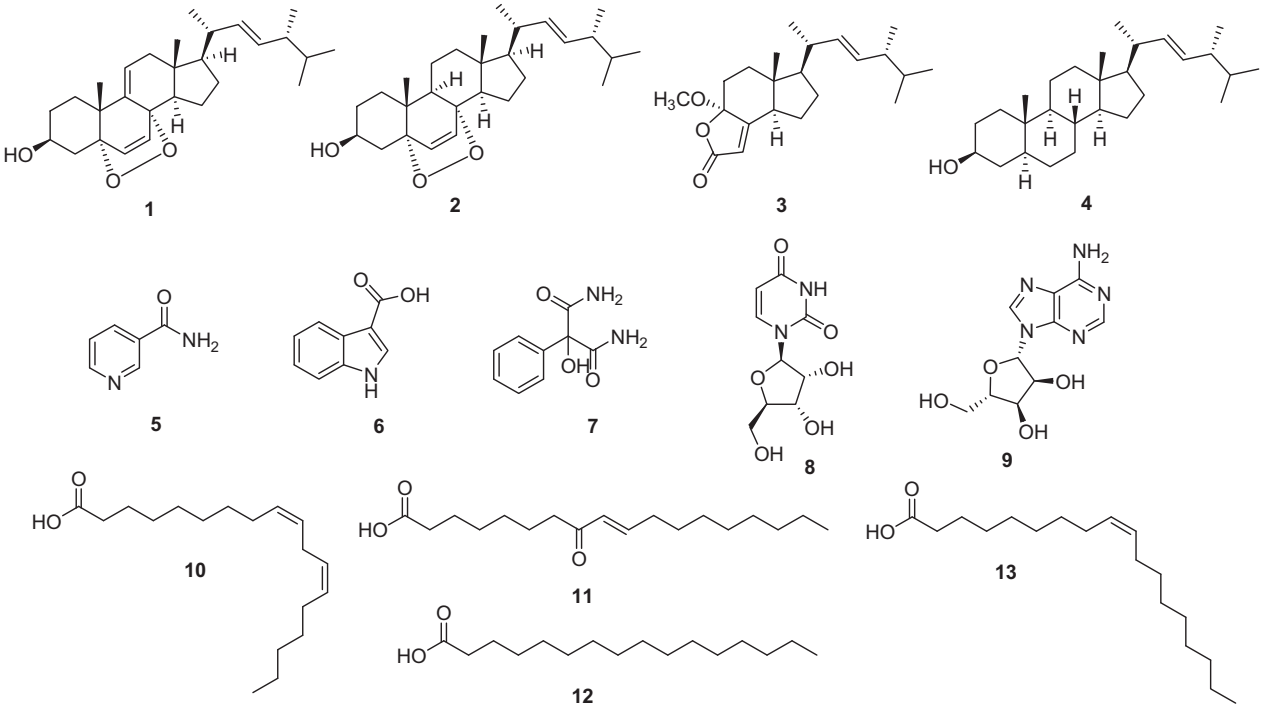


**Figure 2** Chemical structures of compounds **1**–**13**.

of these compounds, except for armillarisin B (**7**), from *A. tabescens*.

## Biological activity

Macrophages are known to produce various inflammatory cytokines such as lipopolysaccharide (LPS), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin-1β (IL-1β), IL-12 and IL-6, thereby being involved in the development of inflammatory diseases. Nitric oxide (NO), a gram-negative endotoxin in macrophage cell lines, is an important pro-inflammatory cytokine induced by LPS,[33–37] and thus, increase in the amount of LPS directly leads to the secretion of NO, provoking inflammatory responses.

As the MeOH extract of *A. tabescens* showed protective activity against gastritis defined by inflammation of the gastric mucosa, all of the isolated compounds **1–13** were tested for inhibition of NO production in LPS-activated RAW264.7 macrophages to investigate their anti-inflammatory properties. Among tested isolates, compounds **1**, **2**, **3**, **4** and **10** showed inhibition on LPS (1 μg/ml)-stimulated NO production at concentrations of 25 and 50 μM. As shown in Figure 3, low concentrations (3.125–12.5 μM) of these compounds did not inhibit NO production in RAW264.7 cells. Compounds **2**, **3** and **4** exhibited anti-inflammatory activity with $IC_{50}$ values of 38.12 ± 0.50 μM, 36.48 ± 0.42 μM and 40.75 ± 1.28 μM, respectively. Notably, compounds **1** and **10** showed outstanding inhibition on NO production with $IC_{50}$ values of 22.46 ± 1.59 μM and 24.71 ± 1.66 μM, respectively. In comparison, L-NAME, an inhibitor of NO synthase, showed inhibition on NO production with $IC_{50}$ value of 178.0 ± 0.2 μM. Although the active compounds **1** and **10** exhibited the similar extent of inhibitory effects, compound **10** was confirmed to be the major component with the most substantial proportion of the extract among the isolated compounds (the isolated amount of compound **10** was about 17 times higher than that of compound **1**), so the mechanism underlying the anti-inflammatory activity of compound **10** was further studied.

To further confirm the anti-inflammatory properties of compound **10**, we investigated its effect on the expression of iNOS, COX-2, phospho-IKKα, IKKα, phospho-IκBα, IκBα and NF-kappa B, which are involved in NO production.[38–41]

In inflammatory responses, macrophages activated by LPS induce secretion of pro-inflammatory mediators such as NO, iNOS, COX-2 and $PGE_2$, as well as pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β.[42,43] LPS binds to toll-like receptor 4 (TLR4) that subsequently activates nuclear factor-kappa B (NF-kappa B). This activation is regulated by IκB kinase (IKK), which is composed of subunits including IKKα, IKKβ, and IKKγ and phosphorylates IκBα.[44–46] When activated, NF-kappa B translocates

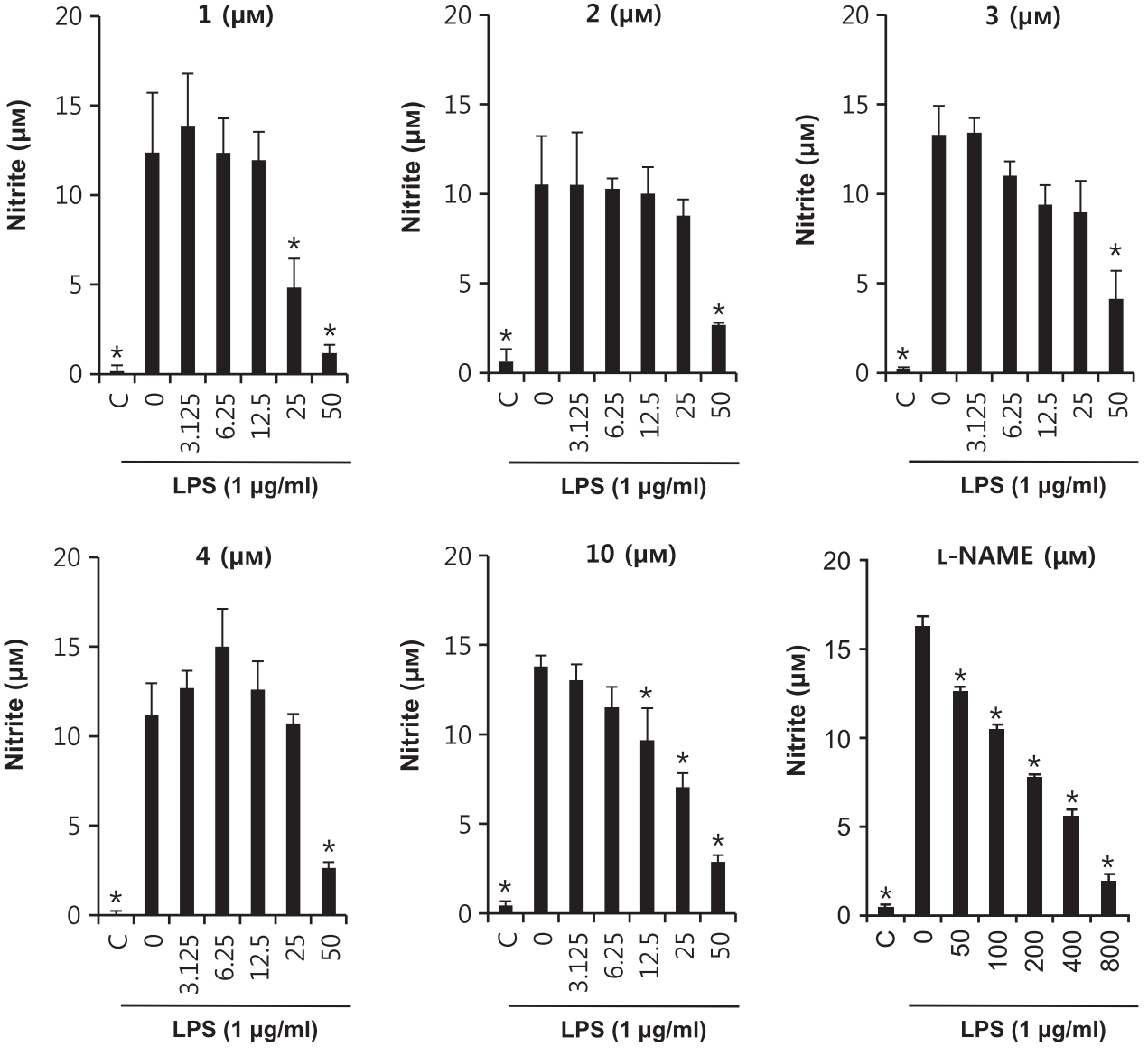


**Figure 3** Inhibitory effects of the isolated compounds **1**, **2**, **3**, **4**, **10** and L-NAME on NO production in LPS-activated RAW264.7 macrophages. L-NAME is a positive control for NO inhibition. NAME; *N*-nitroarginine methyl ester. *$P < 0.05$ compared with LPS-treated cells.

to the nucleus and binds to specific binding sites in the promoter regions of pro-inflammatory mediator genes such as iNOS and COX-2, involved in inflammatory responses.[43,47] iNOS and COX-2 generate NO and prostaglandins, respectively.[42]

Results from Western blotting analyses in the present study showed that compound **10** effectively suppressed protein levels of phospho-IKKα, IKKα, phospho-IκBα, IκBα, NF-kappa B, iNOS and COX-2 in a concentration-dependent manner (Figure 4). Comprehensively, the results suggest that compound **10** exhibits anti-inflammatory effects on the production of NO through downregulating expression of iNOS, COX-2, phospho-IκBα, IκBα, phospho-IKKα, IKKα and NF-kappa B in LPS-stimulated RAW264.7 cells.

## Conclusion

In the current study, we have discovered that the MeOH extract of the fruiting bodies of *A. tabescens* exhibits antigastritis activity against ethanol-induced gastric damage in rats. Chemical investigation of the MeOH extract revealed the identification of 13 compounds, where 12 compounds were identified for the first time from *A. tabescens*. Among the

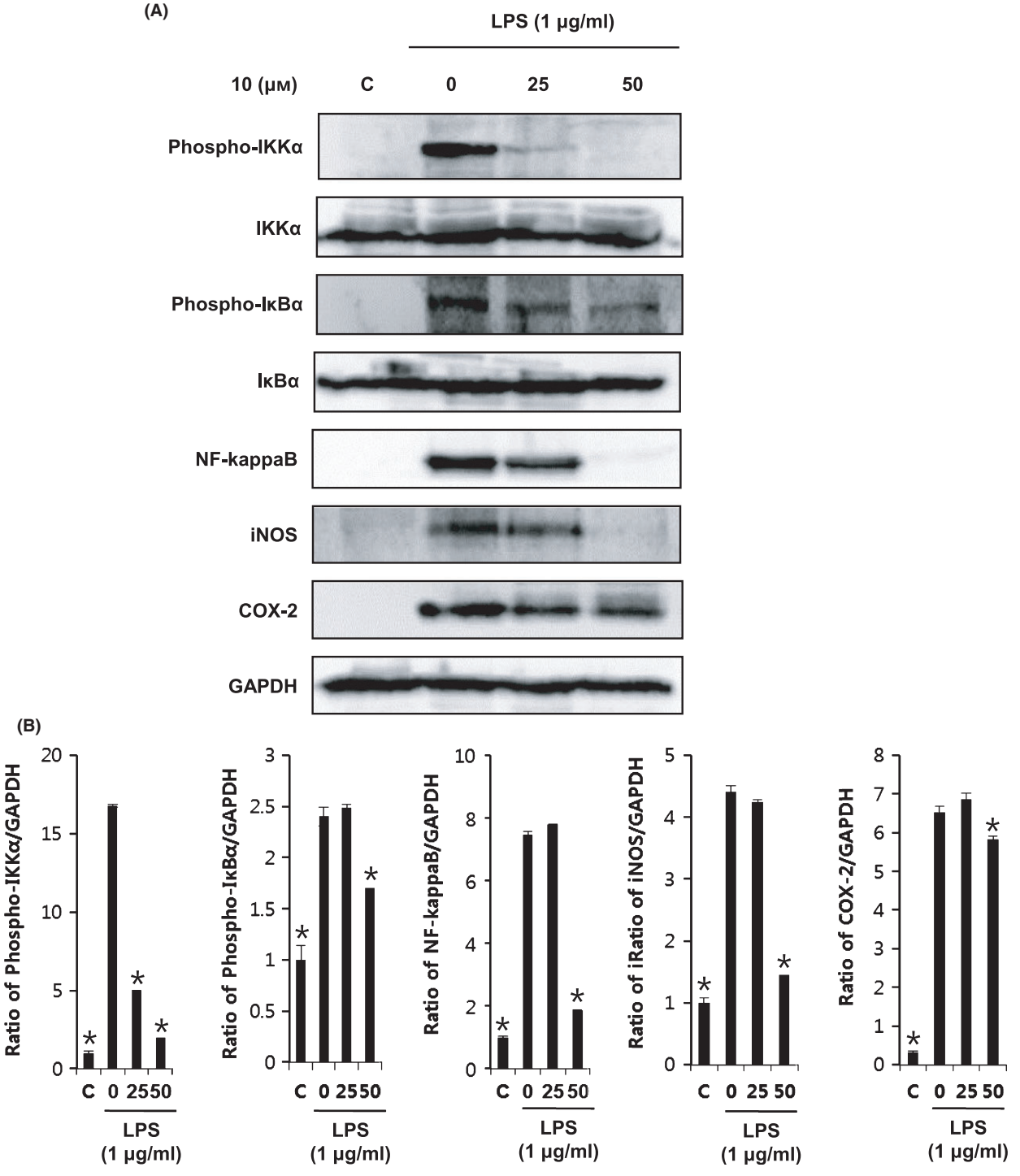


**Figure 4** Inhibitory effects of the isolated compound **10** on the NF-kappa B-dependent inflammation pathway in LPS-activated RAW264.7 macrophages. (a) Representative Western blotting result. (b) Quantitative graph for Western blots. *$P < 0.05$ compared with LPS-treated cells.

isolates, compounds **1**, **2**, **3**, **4** and **10** inhibited NO production in LPS-activated RAW264.7 macrophages. The major component of the extract, compound **10**, showed the most prominent inhibitory effect on NO production, by downregulating the protein expression of iNOS, COX-2, phospho-IκBα, IκBα, phospho-IKKα, IKKα and NF-kappa B. Thus, it can be concluded that compound **10**, as a main contributor to the health benefits of *A. tabescens* as an

  

antigastritis agent, might have remarkable potential in treating inflammation involved in the development of gastritis.

## Declarations

### Conflict of interest

The Authors declare that they have no conflict of interests to disclose.

### Acknowledgements

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2015R1C1A1A02037383). This work was also supported by the Basic Science Research Program for Young Researchers funded by the Richwood Pharmaceutical Company.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Appendix S1**. Materials and methods.