Isolation of a Wild *Morchella* spp. Strain and the Effects of its Extract on Ethanol-Induced Gastric Mucosal Lesions in Rats

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A *Morchella* spp. strain was isolated from a wild morel mushroom, and the effects of its mycelia extract on the ethanol-induced gastric mucosal lesions of rats were investigated *in vivo*. Sequence analysis of internal transcribed spacer suggested that this *Morchella* spp. strain (strain No. M1) was clustered together with *M. conica* in the phylogenetic tree. The superoxide dismutase (SOD) activity increased significantly compared to the control. However, the malondialdehyde (MDA) level and myeloperoxidase (MPO) activity decreased significantly compared to the control. These results indicated that M1 is one member of *M. conica* and the protective effects of M1 extract against the ethanol-induced gastric lesions may be related to the increased SOD activity and decreased MDA level and MPO activity in rats.

Key words: *Morchella* sp., Ethanol-Induced Gastric Lesion, Malondialdehyde, Superoxide Dismutase

Introduction

The number of mushrooms on earth is estimated to be more than 140,000, although only 10% of them have been named. Recently, the extract or bioactive constituents of mushrooms have captured the attention of investigators because they exhibit hypolipidemic, hypoglycemic, immunomodulatory, and antitumour activities. They are widely used as nutritional foods and food-flavouring materials in many regions since centuries (Negi, 2006). Despite the widespread appreciation of these prized edibles, many aspects of their molecular biology are poorly understood. Traditionally, identification and characterization of macrofungi species have been based on morphological characters, such as conidial and appressorium shape and size, as well as pathogenicity tests (Gunnell and Gubler, 1992). Recently, molecular biological techniques have been applied successfully to identify fungi precisely and rapidly (Las Heras-Vazquez *et al*., 2003). The internal transcribed spacer (ITS) region has been generally considered as extensively sequenced molecular marker and an effective target for the molecular identification of fungi. The ITS region is a rapidly evolving region of the nuclear rDNA nested in the rDNA repeat between the highly conserved sequences of 18 S, 5.8 S, and 28 S subunits. It is commonly believed that ITS regions are variable between morphologically distinct species or even isolates of the same species (Green *et al*., 2004).

Members of the *Morchella* genus, commonly known as morels, are edible mushrooms most highly priced for their high gastronomic quality. In China, morels have been used in traditional Chinese medicines for their healthy properties for thousands of years. It has been previously reported that some extracts from *Morchella* spp. have demonstrated many pharmaceutical effects, such as fatigue-resisting, hepatoprotective, and antioxidant effects (Sun *et al*., 2001; Mau *et al.*, 2004; Zhou *et al*., 2006). However, prior to this study little was known on the molecular identification of *Morchella* strains and the effects of their extracts on ethanol-induced gastric mucosal lesions *in vivo*. Recent studies showed that acute ethanol challenge may induce oxidative stress, such as decreased superoxide dismutase (SOD) activity and increased malondialdehyde (MDA) level in gastric mucosal cells (Nermina *et al.*, 2007). Ethanol could activate neutrophil infiltra-

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tion that produces oxygen radicals and injures tissues, and myeloperoxidase (MPO) activity as an enzyme marker of leukocytes may be induced. The objectives of the present study are to identify *Morchella* strains based on ITS sequence analysis and to investigate the effects of the water extract of *Morchella* strains on the MDA level, SOD and MPO activity in rats.

Material and Methods

Animals

Male Wistar rats (180–200 g) were obtained from the Animal Facility of the Institute of Chinese Traditional Medicine, Sichuan, China. The protocols of feeding were formed in accordance with the Guidelines of Institute of Chinese Traditional Medicine Animals Research Committee. The rats, 5 per cage, were housed in a SPF level laboratory at (20 ± 2) °C with a 12-h light/dark cycle. They were fed with a standard rat chaw, and drinking water was available *ad libitum*.

Microorganism and culture

The morel strain (designated M1) was isolated from the fruit body of a wild *Morchella* sp. from a forest in the north region, Sichuan, China. The strain was maintained on synthetic potato dextrose agar (PDA) plates at 4 °C. Before the experiment, the slant was transferred from the active slant and maintained on a newly prepared synthetic PDA medium in a Petri dish at 25 °C for 7 d. Then it was transferred into the seed culture by punching out mycelia mat (ca. 1 cm^2) from the Petri dish and incubated on a rotary shaker incubator under agitation at 150 rev/min for 7 d at 25 °C. The seeds were grown in 250-ml Erlenmeyer flasks containing 100 ml liquid culture medium. The submerged fermentation experiment was carried out in 500-ml flasks containing 200 ml of liquid culture medium after inoculating with 10% (v/v) of the seed culture on a rotary shaker incubator under agitation at 150 rev/min for 6 d at 27 °C. The liquid culture medium was composed of 200 g/l potato, 20 g/l glucose, 2 g/l peptone, 1.5 g/l KH_2PO_4, 5 g/l $MgSO_4$. Mycelia were separated by centrifugation at 3,000 × *g* for 15 min. The precipitate was washed three times with a large amount of distilled water, freeze-dried, and stored at 4 °C for further use.

Morphology characterization

Morphology studies were carried out by plating 20 ml PDA medium in a 10-cm (i.d.) Petri dish and inoculating with 0.5 cm^2 mycelial disc. The mycelia were observed after culturing on a microscope slide with PDA medium at 25 °C for 3 d, and then colouring by fungus staining solution. The solution consisted of 20.0 g phenol, 20.0 ml lactic acid, 40.0 ml glycerin, 20.0 ml distilled water, and 0.5 g fuchsin acid.

DNA extraction

Total genomic DNA was extracted from about 50 mg mycelia following the modified cetyltrimethylammonium bromide (CTAB) method (Zheng *el al.*, 2007). The DNA was estimated spectrophotometrically (Bio-Rad Laboratories, Hercules, CA, USA), and quality was checked by agarose gel electrophoresis.

PCR amplification, sequencing, and analysis

PCR primers were universal sense primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and antisense primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') for fungi. Primers were synthesized by Shanghai Generay Biotech Co., Ltd, China. PCR reactions were performed in 50 μl total volume, containing about 100 ng of DNA, 5 μl of PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer, and 1.5 U of *Taq* DNA polymerase (Promega, Madison, WI, USA). The amplification was incubated in a Mycycler thermal cycler (Bio-Rad Laboratories). The PCR reaction mixture was denatured at 94 °C for 5 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 56 °C, 1 min at 72 °C, and a final extension step of 10 min at 72 °C. The amplified products were visualized in 1% (w/v) agarose gel in Tris-Borate-EDTA (TBE), and then were purified and sequenced by Invitrogen Biotechnology Co., Ltd (Shanghai, China).

DNA sequence similarity searching was performed using the BLAST standard nucleotide-nucleotide basicloc alignment search tool. A total of 20 *Morchella* spp. sequences and 2 outgroup sequences (*Verpa bohemica*, acession No. AM269502, and *Disciotis venosa*, acession No. DQ491503) were obtained from GenBank for phylogenetic analysis. Sequences were aligned using the multiple alignment program CLUSTAL X

1.83. Their phylogenetic analysis was performed using maximum parsimony as implemented by the PAUP* (Phylogenetic Analysis Using Parsimony, Version 4.0b) computer program. A heuristic search analysis was run with tree bisection-reconnection branch swapping to infer branch lengths with MULTREES option on, with ADDSEQ set at random and 1,000 randomized replicates. All characters were weighted equally. Bootstrap values from 1,000 replicates were calculated using the same settings as for heuristic searches (Yu *et al.*, 2008).

Preparation of M1 extract

After fermentation, the fermentation broth was collected and centrifuged for 10 min at 1,157 × *g*. The resulting precipitate was washed repeatedly with distilled water, and the mycelial pellets were dried at 65 °C. The dried mycelia of M1 were ground to 40 mesh, and then a 50-g sample was extracted by stirring with 1,000 ml of distilled water at 100 °C for 60 min and filtering through Whatman No. 4 filter paper. The residue was extracted twice by adding 500 ml of distilled water each time, as described above. The extracts were combined and dried for further experiments.

Ethanol-induced gastric lesions in rats

Fifty male rats (180–200 g) were randomly divided into five groups, each consisting of ten rats. Animals were administered with 10 ml/kg M1 extract of different doses (300, 600, and 1,200 mg/kg) or 10 ml/kg of distilled water (control and normal group) for 10 consecutive days. They were deprived of food for 24 h but had free access to water before the experiments. After administration of the test substances for 30 min, the animals were given 1 ml absolute ethanol or 1 ml distilled water (normal group). After administration of ethanol for 60 min, the rats were sacrificed by cervical dislocation. Then, the stomachs were separated from the surrounding viscera and filled by injection of 10 ml 10% formalin to fix the inner. The inflated stomachs were immersed in a formalin bath for 2 h. After that, the stomachs were opened along the greater curvature. Gastric lesions were examined at 10 × magnification by 2 observers unaware of the treatments. The length (mm) and the width (mm) of the mucosal hemorrhagic ulceration were measured in each mucosal lesion. The total area of lesions was calculated and expressed in mm^2 (Ancha *et al.*, 2003).

Histological studies

For histological study, the stomach tissues of animals were examined by light microscopy. The stomach tissues were fixed in 10% neutral formaline, dehydrated in ethanol, and then embedded in paraffin. Sections from tissue blocks taken from ulcerated areas were stained with hematoxyline/eosin for routine histological examination (Ahmet *et al.*, 2003).

Lipid peroxidation assay

MDA is an important toxic byproduct of lipid peroxidation in animal and plant tissues. The measurement of the MDA content has been widely used as an index of lipid peroxidation. The MDA levels in rat stomachs were measured using the thiobarbituric acid test (Ohkawa *et al.*, 1979). Briefly, the rat stomachs were promptly excised and rinsed with cold saline. To minimize the possibility of interference of hemoglobin with free radicals, any blood adhering to the mucosa was carefully removed. The corpus mucosa was scraped, weighed, and homogenized in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added to a solution containing 0.2 ml of 80 g/l sodium lauryl sulfate, 1.5 ml of 200 g/l acetic acid, 1.5 ml of 8 g/l 2-thiobarbiturate, and 0.3 ml distilled water. The mixture was incubated at 98 °C for 1 h. Upon cooling, 5 ml of *n*-butanol/pyridine (15:l, v/v) were added. The mixture was vortexed for 1 min and centrifuged for 30 min at 2,075 × *g*. The absorbance of the supernatant was measured at 532 mn. A standard curve was generated using 1,1,3,3-tetramethoxypropane. Lipid peroxidation is expressed as MDA content in nanomoles MDA per gram tissue (nmol/g tissue).

Assay of MPO activity

The tissues of gastric mucosa were homogenized in 50 mM phosphate buffer (pH 7.2, 1/10, w/v). The homogenate was centrifuged at 15,294 × *g*. The pellet was again homogenized in an equal volume of 50 mM phosphate buffer, pH 7.2, containing 0.5% hexadecyltrimethyl ammonium bromide (HETAB) and 10 mM EDTA. The supernatant was used immediately or frozen and stored at –80 °C for assaying the enzyme activity

at a later date. The MPO activity was determined by adding 0.2 ml of *o*-dianisidine hydrochloride and 0.0005% hydrogen peroxide into 4 ml buffer containing 0.2 ml tissue sample using an MPO activity measurement kit (Jian-cheng Biological Engineering Institute, Nanjing, China). The change in absorbance at 460 nm for each sample was recorded on a spectrophotometer. MPO activity is expressed as units per gram tissue under assay conditions.

Assay of SOD activity

The stomach tissues (0.5 g) were homogenized in 150 mM saline solution (pH 7.0, 1/10, w/v). The homogenate was centrifuged at 15,294 × *g* for 10 min. The supernatant was used for assaying the SOD activity. SOD (E.C.1.15.1.1) activities were measured using a SOD activity measurement kit according to the method described by Jian-cheng Biological Engineering Institute. One unit of SOD was defined as the amount of enzyme that caused 50% inhibition of the photoreduction of nitrobluetetrazolium (NBT) under the assay conditions. SOD activity is expressed as units per milligram protein under assay conditions.

Statistical analysis

Data were arranged in a completely randomized design with three replicates and expressed as means ± S.D. The total variation and difference between the means were analysed by one-way analysis of variance. Significance was calculated using Student's *t*-test. Values of *P* less than or equal to 0.05 were considered significant.

Results and Discussion

Mycelia grown in the centre of PDA agar media almost covered the whole Petri dish (90 mm) within 3 d at 25 °C. Colonies on PDA were white, flocculated, and orbicular after 5 d. They became brown with accumulation of microsclerotia near the centre after 7 d. After 14 d, larger sclerotia began to form in some distance from the centre, and colonies developed a darker brown. The hyphae were septate, ramose and no clamp connections were observed by a microscope (Fig. 1). In the present study, we observed the morphology of M1 in a PDA Petri dish at 25 °C. Mycelial colour change and sclerotia formation in the PDA medium were the morphological characteristics of the *Morchella* spp. strain. The present findings suggest that the morphological characters of *Morchella* spp. correspond with previous reports on its colonization characteristics (Perihan and Oğuz, 2000; Richard, 2006; Erbil and Fatih, 2008).

The mycelial growth of morels is strongly influenced by the medium components and environmental conditions, and it is difficult to form

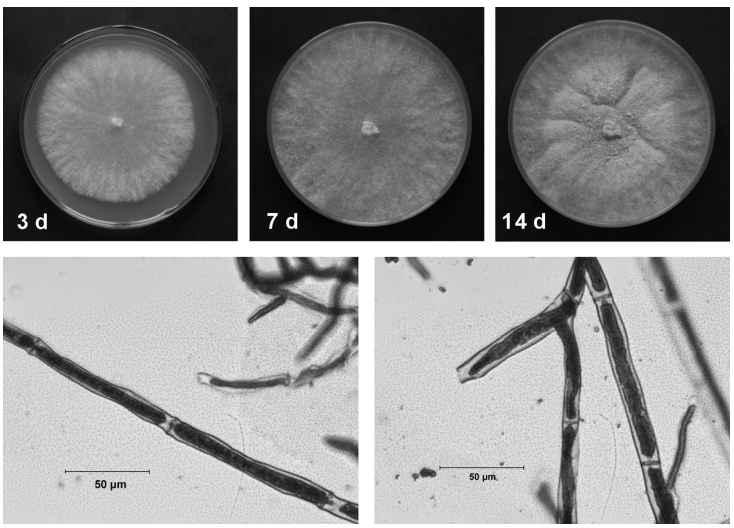

Fig. 1. The hyphal morphological characters of *Morchella* spp. (M1). The fungus was grown in PDA medium at 25 °C for 3, 7, and 14 d, respectively. The hyphae are coloured by fungus staining solution for light microscopy observations.

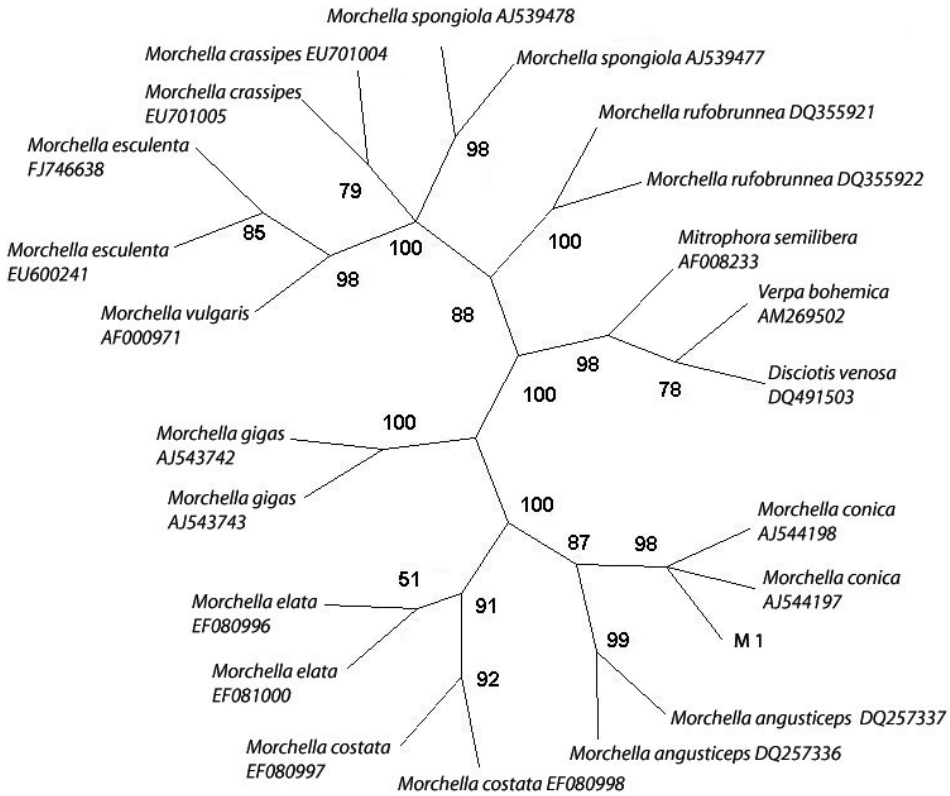

Fig. 2. Maximum parsimony bootstrap consensus tree. Bootstrap values of 1,000 replicates are labeled at the nodes.

the fruit body under artificial cultures (Richard, 2006). Thus, conventional methods are not accurate enough for identifying this fungus. Different molecular approaches have been used to characterize fungi species. They include isozyme comparisons, mitochondrial DNA restriction fragment length polymorphisms (RFLP), polymerase chain reaction (PCR), ribosomal DNA restriction analysis, and ribosomal DNA sequencing (Green *et al*., 2004). In addition, the ITS region has been used as an effective target for the molecular identification of fungi. In the present study, we compared and analysed the ITS sequence of M1 and other *Morchella* species. It is apparent that M1 and *M. conica* were combined in the same clade of the phylogenetic tree. Therefore, fungus M1 was primarily identified to be one of the *M. conica*. The ITS sequence of M1 was 692 bp when primers ITS1 and ITS4 were used. BLAST results indicated that the ITS sequence of M1 showed 99% similarity with *M. conica*. The phylogenetic tree was generated from 23 aligned sequences by the maximum parsimony method, and M1 was located in the same clade with *M. conica* (Fig. 2).

The gastric mucosal defense system consists of the pre-epithelial mucosal layer, the epithelial cell barrier, the mucosal microvasculature, the supply of the mucosa by enteric, extrinsic sensory and extrinsic autonomic neurons, and the mucosal immune system (Pai *et al.*, 1998). Ethanol-induced tissue damage in rodents is a useful model for evaluation of ethanol-induced pathologic changes in the gastric mucosa. The *in vivo* model of ethanol-induced gastric damage in animals has been widely used to evaluate the effects of drugs against this gastric damage (Arslan *et al.*, 2003). Histological changes in the stomach of rats exposed to M1 extract are shown in Fig. 3. Fig. 3A shows the normal gastric mucosa in rats that are smooth and complete. Intragastric administration of absolute ethanol induced linear hemorrhages, mucosal erythema, and edema, scattered petechiaes and erosions along the axis of the glandular stomach (Fig. 3B). Histological examination indi-

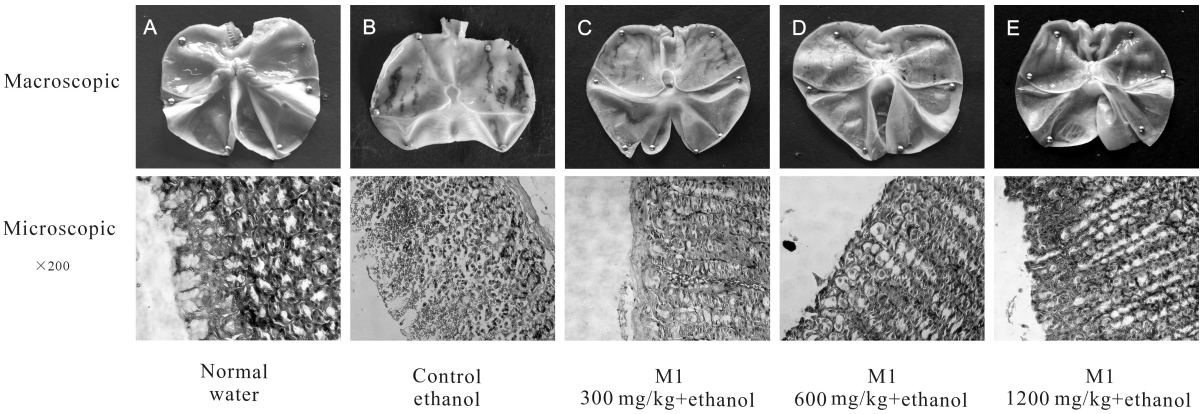

Fig. 3. Macroscopic and microscopic images of the rat stomach. (A) Normal group; (B) ethanol-treated group; (C) low-dose group (300 mg/kg); (D) middle-dose group (600 mg/kg); (E) high-dose group (1,200 mg/kg).

cated that absolute ethanol caused inflammatory tissue damage, such as diffuse coagulative cell necrosis, multiple superficial erosions, and marked vascular congestion (Fig. 3B). A high dose of M1 extract produced only a slight submucosal edema and ecstatic blood vessels, no extravasation of erythrocytes and no significant damages were observed in the infiltrateor mucosa (Fig. 3E). Changes in the gastric lesion area of stomachs are shown in Table I. The gastric lesion area increased significantly when treated with absolute ethanol compared to the control group. However, the gastric lesion area decreased significantly by pretreatment with 300, 600, and 1,200 mg/kg M1 extract compared to the control group, respectively. These results suggest that the M1 extract may inhibit significantly the formation of gastric lesions induced by ethanol.

Since lipid peroxidation is a well-established mechanism of cellular injury, we measured the changes of the MDA levels as an indicator of lipid peroxidation in the gastric mucosa. As shown in Table I, the MDA levels increased significantly in ethanol-induced gastric mucosa compared to the control group, and the increment represents 1.96 times that of the control. However, pretreatment with 300, 600, and 1,200 mg/kg M1 extract decreased the MDA contents significantly by 7.98%, 32.2%, and 40.1% compared to the control group, respectively. It was shown that the MDA level is significantly increased in gastric mucosa exposed to ethanol administration (Brzozowski *et al.*, 2005). Decreases in MDA contents in the gastric tissues of the M1 extract-treated group might be explained by the antioxidant and MPO activity (Table I). Thus, the present results suggest that the M1 extract has a potent protective activity on injury of ethanol-induced gastric mucosa in rats.

Many studies have indicated that oxygen-free radicals play an important role in the pathogen-

Table I. Effects of the M1 extract on ethanol-induced changes of lesion area, MDA contents, SOD and MPO activities.

Treatment	Lesion area [mm^2]	MDA content [nmol/g]	SOD activity [U/mg protein]	MPO activity [U/g]
Normal (water)	0	2.30 ± 0.98	693.6 ± 145.5	0.38 ± 0.07
Control (ethanol)	106.6 ± 4.31$^{\#\#}$	4.51 ± 1.4$^{\#}$	433.3 ± 118.3$^{\#}$	0.49 ± 0.12$^{\#}$
M1, 300 mg/kg + ethanol	98.7 ± 3.83	4.15 ± 1.73	485 ± 98. 6	0.48 ± 0.08
M1, 600 mg/kg + ethanol	50.34 ± 2.12*	3.06 ± 1.39*	595.1 ± 121.7	0.39 ± 0.23*
M1, 1,200 mg/kg + ethanol	16.13 ± 0.65**	2.70 ± 0.79*	644.2 ± 97.7*	0.36 ± 0.11*

Data are expressed as means ± S.D., $n = 10$ in each group.
$^{\#}P < 0.05$ compared with the normal group. $^{*}P < 0.05$ compared with the control group. $^{\#\#}P < 0.01$ compared with the normal group. ** $P < 0.01$ compared with the control group.

esis of acute gastric damage induced by ethanol. Ethanol can induce vascular endothelium injury of gastric mucosa, disorder of microcirculation and ischemia as a result of increased production of oxygen-free radicals, which attack essential cell constituents and induce peroxidation of cytomembrane lipids producing MDA and other toxic compounds, which are leading to cell death. Oxygen-free radicals, generated from gastric mucosal injury, are scavenged by SOD or other antioxidant enzymes (Ahmet *et al.*, 2003; Gazzieri *et al.*, 2007). As shown in Table I, the SOD activity of controls that were ethanol-treated decreased significantly by 37.5% compared to the normal group. This decrease in SOD activity may be due to the inactivation of SOD by highly produced O_2-radicals ($O_2^{\cdot-}$). However, the SOD activity of groups pretreated with 300, 600, and 1,200 mg/kg M1 extract increased significantly by 11.9%, 27.2%, and 48.6% compared to the controls which were ethanol-treated, respectively. It is suggested that SOD is critical to eliminate $O_2^{\cdot-}$ and H_2O_2 when it is formed at the site of generation. Thus, the elimination of $O_2^{\cdot-}$ by SOD is an important factor in the protection process. It has been reported that $O_2^{\cdot-}$ is involved in the pathogenesis of gastric mucosal damage and that pretreatment with SOD reduces ethanol-induced gastric mucosal injuries (Gazzieri *et al.*, 2007). There is an increasing interest in medicinal plant extracts, the greatest value of which may be due to constituents that contribute to the modulation of the oxidative balance *in vivo*. Various plant-originated gastroprotectors have been used in clinical and folk medicine due to their beneficial effects on the gastric mucosa. Literature has centered primarily on their pharmacological action in experimental animals using different models of gastric lesions induction (Zayachkivska *et al.*, 2005; Olaleye and Farombi, 2006). Based on the above results, increased SOD activity has a specific role in protecting the gastric mucosa against the ethanol-induced gastric lesions.

MPO is an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMNs), and tissue MPO activity correlates significantly with the number of PMNs determined histochemically in inflamed tissues (Bradley *et al.*, 1982). Thus, tissue-associated MPO activity was utilized as an indication of accumulation of neutrophils. As shown in Table I, the MPO activity increased by 28.94% after ethanol administration compared to the control group. Some authors reported that the MPO activity in the gastric mucosa exposed to ethanol increased significantly (Martin *et al.*, 1998; La Casa *et al.*, 2000). These results suggest that there was a considerable neutrophil influx into the mucosa in response to ethanol injury in rats. However, the MPO activity decreased by 2%, 20.4%, and 26.5% at doses of 300, 600, and 1,200 mg/kg M1 extract compared to the control group, respectively. According to our results, the M1 extract, which had anti-inflammatory properties, prevented the increase in MPO activity and thus protected the gastric mucosa from the deleterious effects of activated neutrophil infiltration. The present results are in agreement with reports that explain the anti-inflammatory activity of medicinal plant extracts (Periera da Silva *et al.*, 2000). In addition, this is supported by the finding that M1 extract administration decreased MDA contents and increased the SOD activity. Thus, our results suggest that the M1 extract may inhibit the neutrophil infiltration to the tissues, and protect ethanol-induced gastric mucosa lesions.

In conclusion, a *Morchella* spp. strain has been isolated and identified using ITS sequence analysis. In addition, the present study reports that a morel M1 water extract has gastroprotective effects on the ethanol-induced gastric mucosal lesions of rats *in vivo* by activation of the SOD activity as well as inhibition of MDA contents and MPO activity. However, further research needs to be carried out to clarify the detailed gastroprotective mechanisms of active compounds of the M1 extract, as well as its chemical value.

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