ORIGINAL ARTICLE

Analgesic and Anti-inflammatory Activities of *Phellinus merrillii*

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Purpose. To investigate the analgesic and anti-inflammatory effects of ethanol extract of Phellinus merrillii (EPM) in ICR (Institute for Cancer Research) mice.

Methods. The authentic ingredient hispolon and the hispolon content of EPM were analyzed by high-performance liquid chromatography (HPLC). The analgesic effects of EPM were investigated by measuring the acetic acid-induced writhing response and the licking time of hind paws following formalin injection. λ-carrageenin (CARR)-induced paw edema was studied to explore the anti-inflammatory effect of EPM.

Results. HPLC analysis revealed that hispolon and EPM had similar peaks at a retention time of 6 min. This implied that EPM contained the active ingredient hispolon. Treatment of male ICR mice with EPM (2 g/kg) significantly inhibited the numbers of writhing response ($p < 0.001$). This inhibition by EPM (2 g/kg) was similar to that produced by a positive control indomethacin (10 mg/kg) ($p < 0.001$). EPM (1 and 2 g/kg) significantly inhibited ($p < 0.001$) the formalin-induced pain in the late phase. EPM (1 and 2 g/kg) also inhibited the development of paw edema induced by CARR ($p < 0.05$).

Conclusion. EPM may have analgesic and ant-inflammatory activities. **(Mid Taiwan J Med 2007;12:76-82)**

Key words

anti-inflammation, formalin, high-performance liquid chromatography, *Phellinus merrillii*, writhing response, λ-carrageenin

INTRODUCTION

In Asia, macrofungi are commonly given as a nutritional supplement to patients with a variety of diseases [1,2]. Several different species of *Phellinus* are believed to have anticancer and also antioxidant properties. For example, *Phellinus linteus* has been demonstrated to exhibit anti-tumor activity in several studies [3-7]. The methanolic extract of the basidiocarps of *Phellinus linteus* exhibits antioxidative effect [8, 9]. Studies have indicated that *Phellinus linteus* could protect primary cultured rat hepatocytes against hepatotoxins [10] and *Phellinus rimosus* (Berk) Pilat exhibited antioxidant and antihepatotoxic activities [11]. There is also a report indicating that n-BuOH subfraction of

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Phellinus linteus exhibited anti-inflammatory activity [12]. Hispolon, an active ingredient, was isolated from the fungus *Phellinus igniarius* [13]. Hispolon, a yellow pigment was first found in *Inonotus hispidus* in 1996 [14]. Hispolon had been reported to exhibit apoptotic effect on human epidermoid KB cells [15] and to exhibit antivirus activities [16]. Hispolon has also been shown to inhibit the chemiluminescence response of human mononuclear cells and suppress mitogen-induced proliferation of spleen lymphocytes in mice [17].

In our previous study (unpublished), we found that EPM displayed antioxidant activities in comparison with controls in models such as total antioxidant activity, DPPH (1, 1-diphenyl-2-picrylhydrazyl) staining and reducing power method in a series of in vitro tests. EPM also showed hepatoprotective and antioxidant effects in mice with carbon tetrachloride-induced liver damage.

However, little information is availabile on the analgesic and anti-inflammatory effects of *Phellinus merrillii*. Therefore, we examined the analgesic effects of *Phellinus merrillii* on nociception induced by acetic acid, and formalin. We also evaluated the anti-inflammatory effects of *Phellinus merrillii* on paw edema induced by λ-carrageenin in mice.

MATERIALS AND METHODS

Material

Fresh bodies of Phellinus merrillii were purchased from Ji-Pin mushroom store (Nantou , Taiwan). The origin of Phellinus merrillii was confirmed by Dr. Yu-Cheng Dai from the Institute of Applied Ecology, Chinese Academy of Science, China and Dr. Sheng-Hua Wu from the Department of Botany, National Museum of Natural Science,Taiwan.

Chemicals

Acetic acid and formalin were purchased from Merck (Darmstadt, Germany). λ-Carrageenin and indomethacin were obtained from Sigma (St. Louis, MO, USA). Hispolon was purchased from BJYM PHARM. & CHEM. CO. LTD (Beijing, China).

Compositional analysis of hispolon and EPM by HPLC

The standard (hispolon) and EPM were analyzed by HPLC according to Chen et al [15]. The hispolon purity was more than 95% based on reversed phase HPLC analysis (Instrument: Waters 2695; Column: Waters Cosmosil 5C18-AR-II, 4.6 × 150 mm, 5 μ; Mobile phase: AcCN: H_2O (50:50); Flow rate: 0.5 mL/min; PDA: 200 to 350 nm).

Extraction

Phellinus merrillii (1.5 kg) was soaked in 70% ethanol (each 10 L) at room temperature for 72 h. The samples were filtered with filter paper (Advantec No.1, Japan) and the residue was further extracted three times under the same conditions. The filtrates collected from these separate extratcions were combined and evaporated to dryness under vacuum at 50˚C. The yield obtained was 4% (60 g) from the ethanol extract of *Phellinus merrillii*.

Animals

Male *ICR* mice (18 to 25 g) were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ± 1˚C, relative humidity 55 ± 5% with 12 h dark-light cycle for at least 2 week before the experiment. They were given food and water *ad libitum*. All experimental procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals. The placebo groups were given intraperitoneally with 0.1 mL/10 g saline. A bent blunted 27-gauge needle connected to a 1 mL syringe was used to administer the placebo. All tests were conducted under the guidelines of the International Association for the Study of Pain [18].

Acetic acid-induced writhing response

After a 2-week adaptation period, male ICR mice (18 to 25 g) were randomly assigned to four groups (n = 8). These included a normal and a positive control, and 2 EPM-administered groups. Control mice received normal saline [19,20]. Positive control animals were pretreated with indomethacin (10 mg/kg, i.p.) 20 min

before acetic acid (0.1 mL/10 g). Each EPM-administered group was pretreated with either 1 g/kg EPM or 2 g/kg EPM p.o. 60 min before acetic acid (0.1 mL/10 g). Five minutes after the i.p. injection of acetic acid, the number of writhings during the following 10 min was counted.

Formalin test

The antinociceptive activity of the drugs was determined using the formalin test described by Dubuisson and Dennis [21]. Male ICR mice (18 to 25 g) were randomly assigned to four groups (n = 8). These included a normal and a positive control group, and 2 EPM-administered groups. The normal control group received only drugless vehicle (0.1 mL/10 g). The EPM (1 g/kg, 2 g/kg, p.o.) and indomethacin (10 mg/kg, i.p.) were suspended in tween-80 plus 0.9% (w/v) saline solution and administered i.p. in a volume of (0.1 mL/10 g). One hour before testing, the animal was placed in a standard cage (30 cm $\times$ 12 cm $\times$ 13 cm) that served as an observation chamber. EPM (1 g/kg, 2 g/kg, p.o.) was administered 60 min before formalin injection. Indomethacin (10 mg/kg, i.p.) was administered 30 min before formalin injection. The control group received the same volume of saline by oral administration. Twenty microlitres of 1.0% formalin was injected into the dorsal surface of the right hind-paw. The mice were observed for 40 min after the injection of formalin, and the amount of time spent licking the injected hind-paw was recorded. The first 5 min post formalin injection is referred to as the early phase and the period between 15 min and 40 min as the late phase. The total time spent licking or biting the injured paw (pain behavior) was measured with a stop watch. The activity was recorded in 5 min intervals.

λ-carrageenin (CARR)-induced edema

The anti-inflammatory activity of EPM was determined by the CARR-induced edema test in the hind paws of mice. Male ICR mice (eight per group), 18 to 25 g, were fasted for 24 h before the experiment with free access to water. Fifty microlitres of a 1% suspension of CARR in saline was prepared 30 min before each experiment and was injected into the plantar side of right hindpaws of the mice. The EPM and indomethacin were suspended in tween-80 plus 0.9% (w/v) saline solution. The final concentration of tween-80 did not exceed 5% and did not cause any detectable inflammation. After 2 h, EPM at the doses of 1 and 2 g/kg were administered orally, and after 90 min, indomethacin was administered intra-peritoneally at a dose of 10 mg/kg before the CARR treatment. Paw volume was measured immediately after CARR injection and at 1, 2, 3, 4, 5 and 6 h intervals after the administration of the edematogenic agent using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by the ratio a/b, where a is the volume of the right hind paw after CARR treatment, and b is the the volume of the right hind paw before CARR treatment. Indomethacin was used as a positive control [22].

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range test). Statistical significance is expressed as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

RESULTS

We analyzed hispolon and the composition of EPM by HPLC. The HPLC finger print of EPM is shown in Fig. 1. Both the standard (hispolon) and EPM showed similar peaks at the retention time of 6 min. The chromatogram indicated that EPM contained the active ingredient hispolon and the HPLC finger print of EPM can provide the chemical basis for future repetitive trials.

The cumulative amount of abdominal stretching correlated with the level of acetic acid-induced pain (Fig. 2) EPM treatment (2 g/kg) significantly inhibited the number of writhings in comparision with the normal controls ($p < 0.001$). The inhibition by EPM (2 g/kg) was similar to

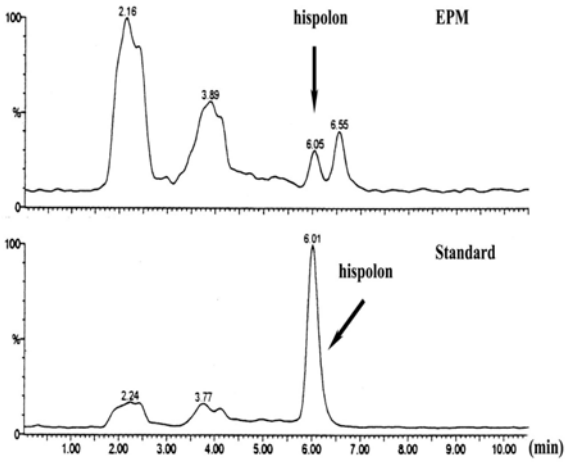

Fig. 1. HPLC chromatograms of EPM and hispolon (standard). Instrument: waters 2695; Column: cosmosil 5C_{18}-AR-II waters, 4.6 × 150 mm, 5 μ; Mobile phase: AcCN:H_2O (50:50); Flow rate: 0.5 mL/min; PDA: 200 to 350 nm. Retention time: 6.01. Sample (10 mg/10 mL MeOH); hispolon (2 mg/10 mL MeOH); Inj. vol. 10 μL.

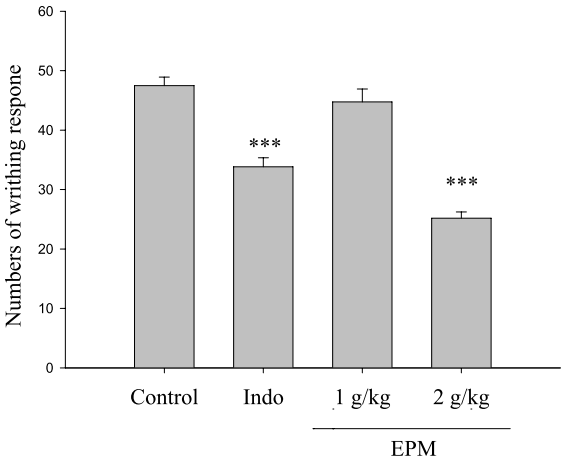

Fig. 2. The effect of EPM on 1% acetic acid-induced writhing response in mice. EPM (1 g/kg, 2 g/kg, p.o.) for 60 min or indomethacin (10 mg/kg, i.p.) for 20 min. Data represented as mean ± SEM (n = 8). ***$p < 0.001$ as compared with the control group. (Oneway ANOVA followed by Scheffe's test).

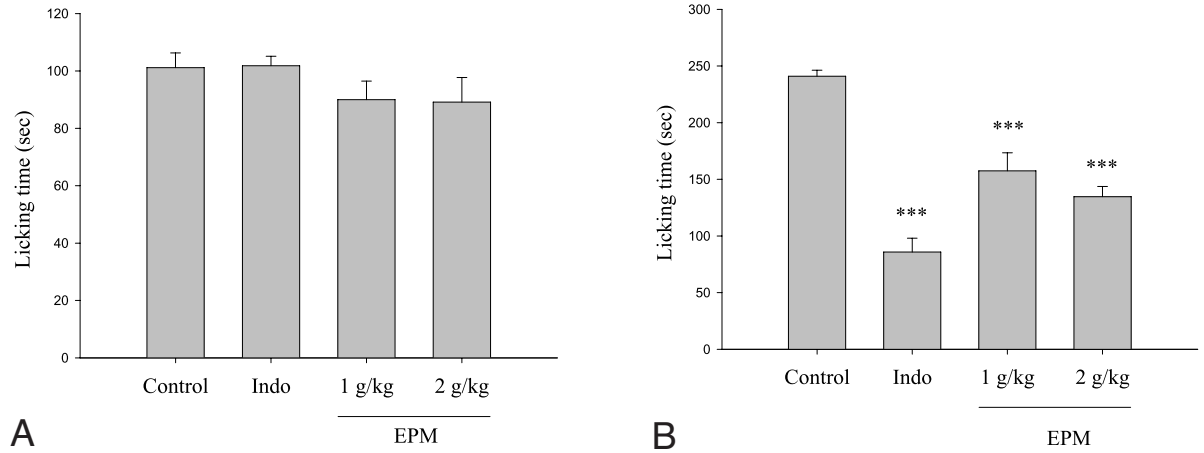

Fig. 3. The effects of EPM on the early phase A (0 to 5 min) and late phase B (20 to 30 min) on 1% formalin-induced inflammation in mice. Data represented as mean ± SEM (n = 8). ***$p < 0.001$ as compared with the control group. (One-way ANOVA followed by Scheffe's test).

that produced by indomethacin (10 mg/kg) ($p < 0.001$).

EPM (1 g/kg, 2 g/kg) significantly ($p < 0.001$) inhibited formalin-induced pain in the late phase (Fig. 3B), however, it did not show any inhibition in the early phase (Fig. 3A). The positive control indomethacin (10 mg/kg) also significantly ($p < 0.001$) inhibited the formalin-induced pain in the late phase.

As shown in Fig. 4, CARR induced paw edema. EPM (1 g/kg, 2 g/kg) significantly inhibited ($p < 0.05$) the development of paw edema induced by CARR after 3 h. of treatment; however, it only showed inhibition at earlier time. Indometacin (10 mg/kg) significantly decreased the CARR-induced paw edema after 3 ($p < 0.01$), 4, 5 and 6 h of treatment ($p < 0.001$).

DISCUSSION

The acetic writhing test is normally used to study the peripheral analgesic effects of drugs. Although this test is nonspecific (e.g., anti-

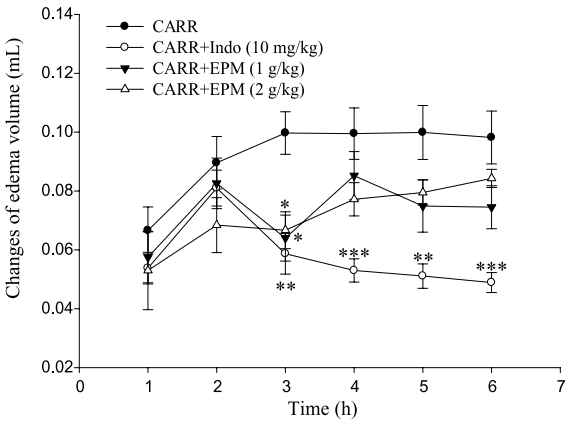

Fig. 4. The effects of EPM and indomethacin on mice hind paw edema induced by λ-carrageenin. Data represented as mean ± SEM (n = 8). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared with the control group. (Oneway ANOVA followed by Scheffe's test).

cholinergic, antihistaminic and other agents also show activity in the test), it is widely used for analgesic screening [23]. Kim et al indicated that ethanol extract of *Phellinus linteus* exhibited antinociceptive effect [12]. In our study, we found that ethanol extract of *Phellinus merrilii* (2 g/kg) also exhibited antinociceptive effect in acetic acid-induced writhing response.

The formalin test is a valid and reliable model of nociception and is sensitive for various classes of analgesic drugs. The formalin test produces a distinct biphasic response and different analgesics may act differently in the early and late phases of this test. Therefore, the test can be used to clarify the possible mechanism of an antinociceptive effect of a proposed analgesic [24]. Centrally acting drugs such as opioids inhibit both phases equally [23], but peripherally acting drugs such as aspirin, indomethacin and dexamethasone only inhibit the late phase. The inhibitory effect of EPM on the nociceptive response in the late phase of formalin test suggested that the anti- nociceptive effect of the EPM could be due to its peripherial action.

The CARR test is highly sensitive to nonsteroidal antiinflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies [25]. The degree of swelling of the CARR-injected paws was maximal 3 h after injection. Statistical analysis revealed that EPM at doses of 1 and 2 g/kg significantly inhibited the development of edema 3 h after treatment ($p < 0.05$).

Phellinus merrillii contains hispolon. Hispolon is a phenolic compound. Recently, polyphenols were reported to exhibit anti-inflammatory and analgesic effects [26,27]. Therefore, it seems that analgesic and anti-inflammatory profile of EPM might be related to the hispolon present in the EPM.

In conclusion, this study demonstrated that EPM possessed analgesic and ant-inflammatory activities. Further studies are necessary to elucidate the mechanisms.

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梅里爾針層孔菌之鎮痛及抗發炎活性

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目的 本研究探討梅里爾針層孔菌的乙醇抽出物之鎮痛和抗發炎作用於ICR小鼠。

方法 利用標準品(hispolon)與梅里爾針層孔菌的乙醇抽出物之高效液相層析分析。研究梅里爾針層孔菌的乙醇抽出物，以醋酸誘導扭體試驗探討鎮痛作用和以福馬林注射足蹠之舔足時間試驗探討梅里爾針層孔菌的乙醇抽出物之鎮痛作用。最後λ-角叉菜膠誘導足蹠浮腫測試，可用於探討梅里爾針層孔菌乙醇抽出物之抗發炎作用。

結果 在高效液相層析方法中，梅里爾針層孔菌的乙醇抽出物的指紋圖譜被建立，hispolon與梅里爾針層孔菌的乙醇抽出物顯示出有相似的波峰與相同的6分鐘的滯留時間，這暗示梅里爾針層孔菌的乙醇抽出物含有活性成份hispolon。給予梅里爾針層孔菌的乙醇抽出物(2 mg/kg) 對小鼠醋酸扭體反應次數有顯著抑制作用($p < 0.001$)，正對照組indomethacin (10 mg/kg)也有明顯抑制的效果($p < 0.001$)。梅里爾針層孔菌的乙醇抽出物(1和2 mg/kg)對福馬林誘導的後期疼痛有明顯的抑制效果，正對照組indomethacin (10 mg/kg)也有明顯抑制的效果($p < 0.001$)。梅里爾針層孔菌的乙醇抽出物(1和2 mg/kg)也會抑制λ-角叉菜膠誘導的足部浮腫($p < 0.05$)。

結論 梅里爾針層孔菌的乙醇抽出物可能具有鎮痛和抗發炎活性。（**中台灣醫誌 2007;12: 76-82**）

關鍵詞

抗發炎，福馬林，高效液相層析法，梅里爾針層孔菌，扭體反應，λ-角叉菜膠

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