

RESEARCH ARTICLE

Anti-inflammatory and Anticancer Activities of Ethanol Extract of *Pendulous Monkshood Root in vitro*

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Abstract

Aim: *Pendulous monkshood root* is traditionally used for the treatment of several inflammatory pathologies such as rheumatisms, wounds, pain and tumors in China. In this study, the anti-inflammatory and anticancer activities and the mechanism of crude ethanol extract of *pendulous monkshood root* (EPMR) were evaluated and investigated *in vitro*. **Materials and Methods:** The cytotoxic effects of EPMR on different tumor cell lines were determined by the MTT method. Cell apoptosis and cell nucleus morphology were assessed by Hoechst 33258 staining. Moreover, nitric oxide (NO) levels and intracellular oxidative stress in peritoneal macrophages were determined to further elucidate mechanisms of action. **Results:** The data showed that EPMR could produce significant dose-dependent toxicity on three kinds of tumor cells. Furthermore, EPMR displayed obvious anti-inflammatory effects on LPS-induced mouse peritoneal macrophages at the dosage of 4 - 200 $\mu\text{g/mL}$. The results demonstrated the therapeutic potential of *Pendulous Monkshood Root* on cancer and inflammatory diseases. **Conclusion:** Our results indicate that EPMR has anti-inflammatory and anticancer properties, suggesting that *pendulous monkshood root* may be a useful anti-tumor and anti-inflammatory reagent in the clinic.

Keywords: *Pendulous monkshood root* - anti-inflammatory - anticancer - mouse peritoneal macrophages

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Introduction

Pendulous monkshood root (the dried roots of *pendulous monkshood*), belonging to the genus of *Aconitum* (Family *Ranunculaceae*), is well known for its anti-rheumatic and analgesic properties. It mainly distributes in Tibet, Yunnan and Sichuan province in China (Sato et al., 1979; Hikino et al., 1980). In the early Tibetan medica “*Jingzhu Bencao*” (Dimaer Danzeng Pengzhe, 1743), it has been documented as a remedy for infectious damp heat, vermination, leprosis and vesania, etc. It is also generally used by Qiang and Hui people in China to treat several inflammatory pathologies such as rheumatisms, wounds, pain and tumor. Moreover, recent investigations reveal that *Aconitum* herbs possess anticancer activity. The aconitic alkaloids as well as Chinese compound formula contained with *Aconitum* herbs were reported to be used as anti-cancer agents (Yang et al., 2005; Rao and Peng, 2010; Liu et al., 2004). It could be supposed that *Aconitum* herbs present *in vitro* cytotoxicity possible interest in cancer chemotherapy. (Chodoeva et al., 2005; Singhuber et al., 2009; Wang et al., 2012). However, few studies were carried out to support their ethnopharmacological use. Therefore, the present study was undertaken to investigate the anti-inflammatory and anticancer activities of ethanol extract of *Pendulous Monkshood Root* (EPMR) and to further discuss the mechanism. The evaluation will serve as the basis for further research on the isolation and pharmacological mechanisms of active constituents.

Materials and Methods

Extract preparation

The dried roots of *Pendulous Monkshood Root* were purchased commercially from Bozhou city, Anhui province in China in September 2010. The plant was identified by Dr. Liu Xinqiao, an associate Professor in Pharmacognosy at School of Pharmacy, South-central University for Nationalities. The voucher specimen (no. Huang X.J. 20120315) was deposited at the Herbarium of South-central University for Nationalities.

The dried roots of *Pendulous Monkshood Root* (1 Kg) were ground into powder and submerged in 95% ethanol (8 L \times 3, each 3 days) and left to macerate for three times. The combined solution was filtered and evaporated to complete dryness using a standard Buchi rotary-evaporator. Finally, 259 \pm 60 g (w/w) extract was obtained. The extract (solid sample) was stored in 4 C from where it was used when required.

The EPMR was freshly prepared with dimethyl sulfoxide (DMSO) and diluted by D-hanks at the desired concentrations just before use.

Chemicals and reagents

2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA), Hoechst 33258, and LPS derived from *Escherichia coli* and *Salmonella typhosa* were obtained from Sigma (St. Louis, MO, USA). DMSO was obtained from Amresco (USA). The Dulbecco's modified Eagle's

medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin used in this study were obtained from Hyclone (Logan, Utah, USA). fetal calf serum was obtained from sijiqing biological engineering and materials Co. Ltd. (Hangzhou, PR China). RPMI-1640 medium was obtained from Invitrogen (Invitrogen Corporation, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were obtained from Gibco-BRH (Gibco, Grand Island, NY, USA). All chemicals were of the highest purity commercially available.

Cell culture of tumor cells and drug treatment

HepG2 (Human hepatocellular liver carcinoma cell line) and Hela (human cervix carcinoma) were obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. sP 2/0 (Mouse Myeloma Cell Line) was a kind gift from National Reference Laboratory of Veterinary Drug Residues (HZAU) / MOA Key Laboratory of Food Safety Evaluation, Huazhong Agricultural University, Wuhan, P. R. China. The cells were seeded at an appropriate density according to each experimental scale and cultured with DMEM, containing 10% FBS. All medium was included with penicillin (100 U/mL) and streptomycin (100 U/mL). Cultures were propagated at 37 °C in a humidified atmosphere of 5% CO₂.

All experiments were carried out 12 h after cells were seeded and the culture medium was refreshed with a new medium. The cells were exposed to various concentrations of EP MR (0 - 400 µg/mL) for 24 h. Control cells were treated with vehicle alone (final DMSO concentration not more than 0.5 %).

Data were obtained from different cell preparations. With each preparation, there were six replicates per treatment.

Cell culture of mouse peritoneal macrophages

Amidulin (Guangcheng, Tianjin, China)-elicited macrophages were harvested 3 days after intraperitoneal injection of 1.0 mL sterile amidulin into KM mice. The animals were sacrificed and sterilized by 75% ethanol. They were exsanguinated and their peritoneal cavity was washed with 5 mL of sterile RPMI-1640 medium, pH 7.4. Peritoneal cells were washed once (1000 rpm, 5 min, 4 °C) and peritoneal cell cultures (1 × 10⁶ cells/mL) were seeded in RPMI-1640 medium containing 10% fetal calf serum in culture flask. After 3 h of incubation, non-adherent and non-viable cells were removed by vigorous pipetting in order to enrich peritoneal macrophages. Adherent cells were then plated at a density of 2 × 10⁴ cells in a 96-well microplate. These cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

The cells were exposed to 10 µg/mL of LPS or EP MR (0 - 100 µg/mL) for indicated time. Control cells were treated with vehicle alone (final DMSO concentration not more than 0.5 %). Cell survival was observed with phase-contrast microscope (OLYMPUS, Japan).

Analysis of cell viability

Cell survival was observed with phase-contrast microscope (OLYMPUS, Japan) and evaluated by MTT assay. Briefly, cells (1 × 10⁵ cells/mL) were treated with

10 µg/mL LPS under the presence and absence of EP MR 24 h at 37 °C. After 3 h incubation with MTT (0.5 mg/mL), cells were lysed in DMSO and the amount of MTT formazan was qualified by determining the absorbance at 570 nm using a microplate reader (TECAN A-5082, meglan, Austria). Cell viability was expressed as a percent of the control culture value.

Morphological assessment of cell apoptosis by hoechst 33258 staining

The cells were exposed to different concentrations of EP MR for 12 h before staining. Cell apoptosis and cell nucleus morphology were detected using the method of hoechst 33258 staining (Araki et al., 1987; Yao et al., 2006). Briefly, the cells were stained by Hoechst 33258 (1 µg/mL) at room temperature in dark for 15 min. The cells were then washed twice with D-hanks, examined and immediately photographed under a fluorescence microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan). Apoptotic cells were defined on the basis of nucleus morphology changes, such as chromatin condensation and fragmentation.

Measurement of nitric oxide (NO) level

Peritoneal macrophages were pretreated with EP MR for 1 h and then exposed to LPS for 24 h. Cell-free supernatants were collected and NO release was measured using the Griess reaction.

Measurement of intracellular Reactive oxygen species (ROS)

Determination of intracellular oxidative stress in peritoneal macrophages was based on the oxidation of DCFH-DA by intracellular ROS resulting in the formation of the fluorescent compound 2', 7'-dichlorodihydrofluorescein (DCF) (Huang et al., 2008). 200 µL cells were seeded in 96-well plates at density of 2 × 10⁶ / well. ROS prober dye 2', 7'-DCFH-DA (final concentration 10 µM) was added to each well. The plate was shielded from the light and stored for 30 min at 37 °C. After being washed with D-hanks, cells were exposed to different concentrations of EP MR in D-hanks. Then, the fluorescence was examined and immediately photographed under a fluorescence microscope (Nikon Corporation, Chiyoda-ku, Tokyo, Japan).

Statistical analysis

Values were expressed as the means ± S.E.M.. Analysis of variance (ANOVA) was used to assess the statistical significant difference of the means, with significance established at *P* < 0.05.

Results

The inhibitive effect of EP MR on cell proliferation of tumour cells

The anticancer activity of EP MR was evaluated with sP 2/0 cells, HepG2 cells and Hela cells. As shown in Figure 1, EP MR significantly inhibited the proliferations of sP 2/0 cells, HepG 2 cells and Hela cells in a dose-dependent manner. The cell viability of sP 2/0 cells decreased to

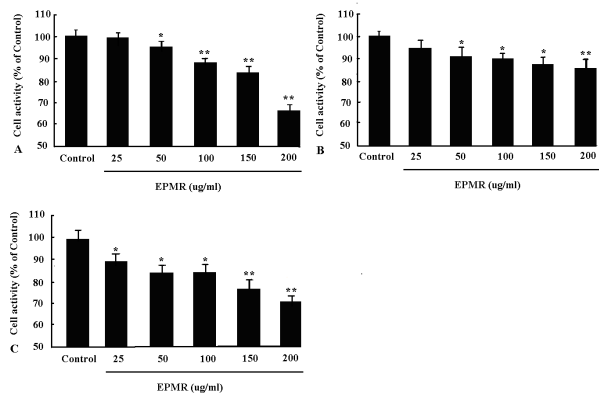


Figure 1. The Cytotoxicity of EPMR on (A) sP 2/0 cells, (B) HepG2 cells and (C) Hela cells (n = 6). Results were expressed as the means \pm S.E.M. (* P < 0.05 and ** P < 0.01 vs. control group)

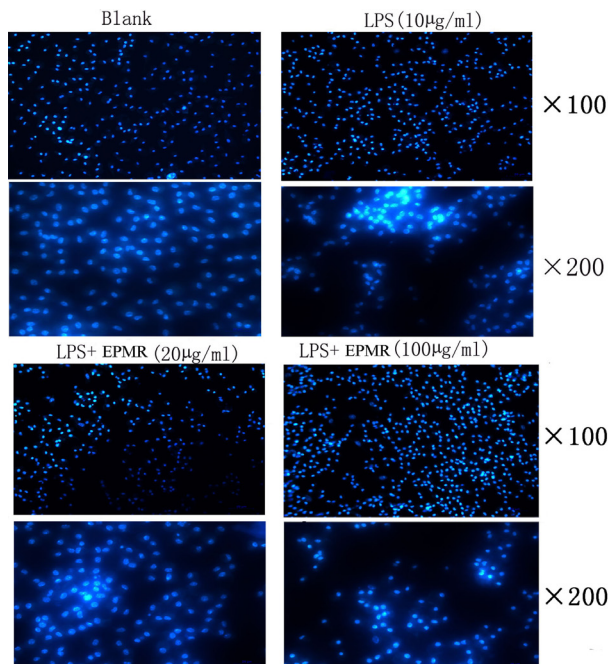


Figure 3. Effect of EPMR on the Apoptosis of Mouse Peritoneal Macrophages

(63.96 \pm 5.21)% of control after incubating with EPMR at the concentration of 400 μ g/mL. Comparatively, EPMR (400 μ g/mL) showed weaker inhibitive effects on HepG2 cells and Hela cells (cell viabilities decreased to 86.98 \pm 5.17 % and 70.12 \pm 4.35 % of control, respectively).

Effect of EPMR on the activity of mouse peritoneal macrophages

As shown in Figure 2, 10 μ g/mL of LPS treatment for 24 or 72 h could both decrease the cell activity of mouse peritoneal macrophages. However, co-treatment of EPMR (4 - 100 μ g/mL) significantly suppressed the decrease of cell activity.

Effect of EPMR on the apoptosis of mouse peritoneal macrophages

As shown in Figure 3, an increased rate of apoptosis induced by LPS was determined by hoechst 33258 staining. EPMR (20 and 100 μ g/mL) treatment could

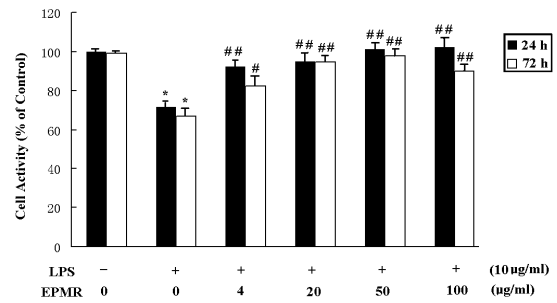


Figure 2. Effect of EPMR on the Activity of Mouse Peritoneal Macrophages. The mouse peritoneal macrophages were stimulated with 10 μ g/mL LPS with (+) or without (-) EPMR for 24 or 72 h (n = 6). Results were expressed as the means \pm S.E.M. (* P < 0.05 and ** P < 0.01 vs. control group; # P < 0.05 and ## P < 0.01 vs. the LPS-treated cells)

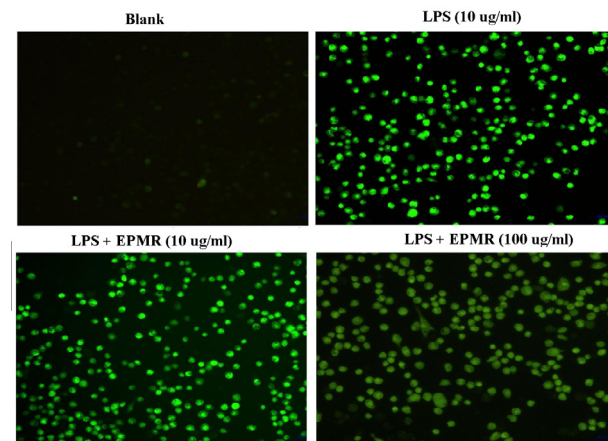


Figure 4. Inhibitive Effect of EPMR on the NO Production in LPS-treated Mouse Peritoneal Macrophages. The mouse peritoneal macrophages were stimulated with 10 μ g/mL LPS with (+) or without (-) EPMR for 24, 48 or 72 h (n = 6). Results were expressed as the means \pm S.E.M. (* P < 0.05 and ** P < 0.01 vs. control group; # P < 0.05 and ## P < 0.01 vs. the LPS-treated cells)

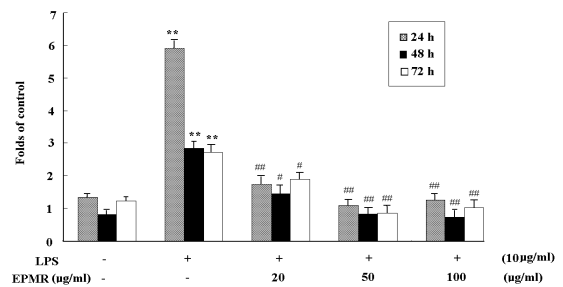


Figure 5. Effect of EPMR on Intracellular ROS in LPS-treated Mouse Peritoneal Macrophages

significantly decrease LPS-induced apoptosis of mouse peritoneal macrophages.

Effect of EPMR on NO release in mouse peritoneal macrophages

It is known that NO is the major cause of macrophage cell death/apoptosis induced by LPS (Ramana et al., 2007). Hence, we investigated the effect of EPMR on LPS-induced NO levels in macrophages. Figure 4 showed that the NO production of mouse peritoneal macrophages increased to 3 - 6 folds in response to LPS treatment for 24, 48 or 72 h. EPMR significantly inhibited LPS-mediated

NO production in mouse peritoneal macrophages.

Effect of EPMR on intracellular ROS in mouse peritoneal macrophages

LPS treatment for 1 h caused a marked increase in ROS formation in mouse peritoneal macrophages as observed in Figure 5. Co-incubation with EPMR prevented the increase of ROS level in LPS-treated cells. These observations suggested that EPMR could ameliorate the oxidative stress induced by LPS in mouse peritoneal macrophages.

Discussion

In the presence of pathogens or irritants and associated molecules, the body mounts a strong immune response termed as “inflammation” aimed at preventing tissue injury and combating infection. Mononuclear phagocytes residing in tissues are the first to be activated during innate immune response. They recognize pathogens and molecules associated with tissue damage such as bacterial cell wall LPS and danger/damage associated molecular patterns (DAMPs) (Kipanyula et al., 2012).

Although individual etiologic factors follow distinct mechanisms of tumor development, local persistent tissue inflammation is commonly involved in carcinogenesis. Inflammatory reactions are ceased following the elimination of pathogens, but can persist in case of chronic infection or following chronic exposure to DAMPs (Duckworth et al., 2012), resulting in tissue fibrosis and carcinogenesis. Cancer arises via a heterogeneous disease process that underlies diverse etiologic factors, all of which contribute to abnormal intracellular signal transduction and genetic alterations (Kundu et al., 2012).

Extensive research over the past several decades has made substantial progress in unfolding the mechanistic links between chronic inflammation and cancer. The connection between inflammation and cancer, first perceived in the nineteenth century, is now accepted as enabling characteristic of cancer (Grivennikov et al., 2010; Sodir et al., 2011; Balkwill et al., 2012). Epidemiologic data have strongly indicated that chronic inflammation is associated with increased risk of cancer (Grote et al., 2012). Current estimates suggest that about 25% of cancers are associated with chronic inflammation sustained by infections (e.g. hepatitis) or inflammatory conditions of diverse origin (e.g. prostatitis) (Grivennikov et al., 2010). Moreover, tumors that are not epidemiologically related to inflammation are characterized by the presence of inflammatory cells and mediators (Grivennikov et al., 2010; Sodir et al., 2011; Balkwill et al., 2012).

In this study, the anti-inflammatory and anticancer effects of EPMR were evaluated with the purpose of better understanding the relationship between inflammation and cancer. Our present results showed that EPMR significantly reduced the production of pro-inflammatory factor NO and intracellular ROS in LPS-treated mouse peritoneal macrophages, demonstrating the good anti-inflammatory properties of EPMR. Inducible nitric oxide synthase (iNOS) is an enzyme catalyzing NO production induced by TNF- α , IL-1 β , and NF- κ B, among other

inflammatory factors (Hussain et al., 2004), which was found to be over expressed in chronic inflammatory diseases and various types of cancer (Kim et al., 2005). It has been reported that the production of NO in tissues contributes to the carcinogenesis process (Liu and Hotchkiss, 1995; Tamir and Tannenbaum, 1996), because overproduction of NO could lead to enhanced replication of genes and oxidative damage to DNA.

In the present study, EPMR not only significantly inhibited the production of NO, but also effectively exerted cytotoxicity on sP 2/0 cells, HepG 2 cells and Hela cells. The results indicated that there might be some relationship between inhibitory activities on productions of NO and intracellular ROS and cytotoxic effects against cancer cell lines. Further studies will be needed to investigate the precise mechanisms of extract, fractions and isolated triterpenes from Pendulous Monkshood Root on cytotoxic activities against cancer cell lines and anti-inflammatory activities. The detailed phytochemistry, pharmacological action and in vivo studies of the active compounds in the plant should be further clarified in the future study.

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