In Vivo Anti-inflammatory and Analgesic Activities of a Purified Saponin Fraction Derived from the Root of Ilex pubescens

Jing-Rong Wang, Hua Zhou, Zhi-Hong Jiang, Yuen Fan Wong, and Liang Liu*

School of Chinese Medicine, Hong Kong Baptist University; Kowloon Tong, Kowloon, Hong Kong, People’s Republic of China. Received October 25, 2007; accepted December 14, 2007; published online January 23, 2008

The root of Ilex pubescens (Mao-Dong-Qing in Chinese, MDQ) has been commonly used for treatment of cardiovascular and inflammatory diseases in the Chinese medical system. The current studies aimed to investigate the anti-inflammatory and analgesic effects as well as the underlying mechanisms of a purified saponin fraction (PSF) derived from MDQ. PSF was found to significantly suppress the paw edema of rats induced by histamine given intraperitoneally at dosages ranging from 12.5—100 mg/kg. Meanwhile, PSF given orally at dosages of 200 and 100 mg/kg significantly inhibited acetic acid-induced abdominal writhing response of mice and prolonged the time required for mouse tail flick after exposure to a source of radiant heat. The mechanistic studies showed that cyclooxygenase-2 (COX-2) protein expression in carrageenan-injected paw tissues of rats was markedly attenuated by intraperitoneal injection of PSF at dosages of 12.5 to 100 mg/kg. PSF could also markedly inhibit production of proinflammatory cytokines, especially IL-1β, IL-6 and TNF-α, but enhance production of anti-inflammatory cytokines of IL-4 and IL-10 in the carrageenan-injected paw tissues in rats. These effects resulted in an overall attenuation of the ratio of proinflammatory/anti-inflammatory cytokines and, ultimately suppression of the paw edema. In conclusion, the current study has demonstrated the in vivo anti-inflammatory and analgesic effects of PSF, and suggested that the molecular mechanisms might be associated with inhibition of the elevated expression of COX-2 protein and the overproduction of the proinflammatory cytokines, as well as augmentation of the anti-inflammatory cytokines IL-4 and IL-10 in the carrageenan-injected paw tissues of rats.

Key words Ilex pubescens; saponin; anti-inflammatory activity; analgesic activity; cyclooxygenase; cytokine

Researches in the last decades have demonstrated that inflammation plays an essential role in the pathogenesis and progression of atherosclerosis.1—3 Anti-inflammatory agents have shown good effects in the prevention and treatment of atherosclerosis and coronary artery diseases.2—3

Mao-Dong-Qing (MDQ) in Chinese, the dried root of the plant Ilex pubescens Hook. et Arn. which is an evergreen bush with a wide distribution in Southern China, has been shown to contain triterpene saponins and phenolics.3—7 MDQ has been widely used in China for treatment of cardiorcerebral and cardiovascular diseases, such as stroke and coronary arterial disease, as well as peripheral vascular disease.8 It has been also often used for alleviating upper respiratory infections and other inflammatory conditions.9—11

Previous pharmacological studies have demonstrated that extracts of MDQ can enlarge blood vessels, improve microcirculation, lower blood pressure, inhibit platelet aggregation, prevent thrombus formation, reduce the excitation of the cardiac conduction system and enhance the ability of anoxia resistance of body.12,13

In our previous investigation on the active principles on MDQ, a purified saponin fraction (PSF) has been identified as a bioactive fraction possessing anti-inflammatory activity. PSF could inhibit carrageenan-induced paw edema significantly given intraperitoneally at dosages ranging from 12.5 to 100 mg/kg. Dominant constituents in PSF were triterpene saponins of ursane and oleanane types.14

In the present study, we aimed to further assess the anti-inflammatory and analgesic actions of PSF in rodent models and to investigate the underlying mechanisms focusing on the production of cyclooxygenases, proinflammatory and anti-inflammatory cytokines in the carrageenan-induced paw edema of rats.

MATERIALS AND METHODS

Plant Materials The raw materials of MDQ were purchased from Caizhiling Chinese Herbal Slice Co., Ltd., Guangzhou, China. These materials were authenticated by Prof. Lai Xiao Ping of Guangzhou University of Chinese Medicine. The authenticated voucher specimens were kept in the School of Chinese Medicine, the Hong Kong Baptist University.

Preparation of PSF The dried plant materials (10 kg) were mechanically powdered and extracted with EtOH three times at room temperature. The EtOH extracts from the three extractions were combined and then concentrated under reduced pressure to yield a total of 451 g crude extracts (the yield rate is 4.51%). Then, the crude extracts were suspended in H2O and subjected to liquid–liquid partition by adding EtOAc and n-BuOH successively, yielding three fractions, i.e. EtOAc fraction, n-BuOH fraction and H2O fraction. The n-BuOH fraction was then chromatographed over Diaion HP 20 eluted with gradient MeOH in H2O. The 80—100% methanol elution was collected and further chromatographed on ODS to produce 20.6 g of the purified saponin fraction (PSF), and the yielding rate of PSF is 0.206% from the raw materials of MDQ.

Chemical Profiling Analysis of PSF Using HPLC Fingerprint Technology HPLC fingerprint technology was carried out on Agilent 1100 series HPLC system. Samples were separated on an Alltech Altima C18 column (250 mm× 4.6 mm, I.D.; particle size 5 μm) at room temperature. The mobile phase comprised water containing 0.4% formic acid (v/v, A) and acetonitrile containing 10% propan-2-ol (v/v, B). Elution was performed by using the following gradients: first, isocratic elution with composition of 65A/35B for...
Dalis yanhusuo (0.5% CMC-Na), an analgesic drug derived from the tuber of Siegmund Siegmund Siegmund, was orally administrated 0.5 h prior to a source of radiant heat, were evaluated according to the description of D’Amour and Smith. Briefly, each animal was placed in a plexiglas box that allowed its tail to be free, then the box was placed on the tail stimulator analgesia meter (IITC Inc., U.S.A.) with the tail occluding a slit over a photocell for radiant heat stimulation generated by a power lamp mounted in a reflector. The tail-flick response was elicited by applying radiant heat to the point 1/3 of length away from the tip of the tail. When the mouse felt pain and flicked its tail, the light of the lamp fell on the photocell such that the timer was automatically stopped. The intensity of the heat stimulus in the tail-flick test was adjusted so that the mouse flicked its tail within 3 to 6 s. A 12 s cut-off time was set in order to prevent tail tissues from being damaged. Before experiment, the heat stimulation latency of all animals were tested, and those with response time of <2 or >6 s to heat stimulation were excluded. The tail-flick responses were measured at 0.5, 1, 2, and 3 h after oral administration of PSF (200, 100, 50 mg/kg), vehicle (0.5% CMC-Na), or rotundine (50 mg/kg).

Western Blot Analysis for Determination of COX-1 and COX-2 Protein Expressions COX-1 and COX-2 protein expressions were investigated in carrageenan induced paw edema model in rats. The edema was induced according to Winter's method. In brief, each rat was injected with 0.1 ml of freshly prepared carrageenan (1% w/v) in physiological saline (0.9% w/v NaCl) into the subplantar tissues of the right hind paw. PSF (100, 50, 25, 12.5 mg/kg) or vehicle were intraperitoneally administrated 10 min in advance. The reference drug, indomethacin (10 mg/kg), was orally administrated 1 h prior to carrageenan injection. At 4 h after injection of carrageenan, the edema paw tissues from PSF, indomethacin, or vehicle treated animals were collected for COX-1 and COX-2 determination. The collected soft tissues were homogenized in RIPA buffer containing 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/ml aprotinin, 1 µM peptatin and 10 µM leupeptin. The homogenates were centrifuged at 12000 × g for 20 min, and then 50 µg of protein from the supernatants was separated on 8% sodium dodecylsulphate-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore, MA, U.S.A). Following the transferring, the membranes were blocked overnight at 4 °C with 5% skim milk in Tris-buffered saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5). The membranes were then incubated with rabbit polyclonal anti-COX-2 (3 µg/ml, Vision, Fremont CA, U.S.A), mice monoclonal anti-COX-1 (5 µg/ml, Cayman Ann Arbor, MI, U.S.A.) for 1 h at room temperature. The membranes were washed six times with Tris-buffered saline-Tween (TBST, 20 mM Tris, 500 mM NaCl, pH 7.5, 0.14% Tween 20). The membranes were then incubated with 1:10000 dilution anti-rabbit (Zymed, South San Francisco, CA, U.S.A.) or anti-mouse IgG secondary antibody (Zymed, South San Francisco, CA, U.S.A.), and then conjugated to horseradish peroxidase in 2% skim milk in TBST for 1 h at room temperature. The membranes were washed six times, and the immunoreactive proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Amersham International plc., Buckinghamshire, U.K.). The relative optical density of bands was quantified by densitometric scanning of the Western blots.

Bio-Plex Assay for Cytokines Determination of IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ, TNF-α in Rat

Animals Male ICR mice of 18—24 g and male SD rats weighing 150—210 g were purchased from the Laboratory Animal Services Center, Chinese University of Hong Kong, Hong Kong. All animals were acclimated for ≥1 week under 12 h light and 12 h dark cycle at room temperature of 22±1 °C. Chow diet and water were provided ad libitum. Animal care and treatment procedures conformed to the Institutional Guidelines and Animal Ordinance Hong Kong Special Administrative Region. Mice and rats were fasted for 16 h and 48 h, respectively, before experiment.

**Induction of Acute Inflammation in Rat Hind Paws by Histamine** Acute inflammation in the hind paws was induced by the subcutaneous injection of 0.05 ml of the prepared solutions of histamine (1%) into the right hind paws of the rats. The left hind paws without injection were used as control. The volumes (ml) of both hind paws of rat were measured using a plethysmometer (Plethysmometer 7150, UGO Basile, Italy) at 1 h before induction and 0.5, 1, 2, 3, 4, 6 h after induction of the inflammation. The increased volumes (paw edema) of the right hind paws of rats were calculated by the following equation: the increased rate (%), = (B−A)/A × 100, where A and B represent the paw volumes before induction of inflammation and at different time points after the induction, respectively. PSF (100, 50, 25, 12.5 mg/kg) or vehicle were intraperitoneally administrated 10 min prior to histamine injection. The reference drug, indomethacin (10 mg/kg), was orally administrated 1 h prior to histamine injection.

**Visceral Nociceptive Model Induced by Acetic Acid Stimulation in Mice** The abdominal writhing test induced by acetic acid stimulation in mice as originally described by Siegmund et al. Briefly, PSF (200, 100, 50 mg/kg) or vehicle (0.5% CMC-Na) were orally administrated 0.5 h prior to acetic acid injection. The reference drug rotundine (50 mg kg), an analgesic drug derived from the tuber of Corydalis yanhusuo W. T. Wang in China, was orally administrated 2 h before acetic acid injection. Immediately after intraperitoneal injection of 0.2 ml acetic acid (0.8% w/v) in physiological saline (0.9% w/v NaCl), animals were isolated for observation. The numbers of abdominal writhing episodes, which consisted of contraction of the abdominal area with extension of hind legs, were recorded during a 15 min period after injection of acetic acid in each animal.

**Central Nociceptive Model Induced by Radiant Heat Stimulation in Mice** The antinociceptive effect of PSF and the reference drug, represented by the time required for mice tail flick after exposure to a source of radiant heat, were evaluated according to the description of D’Amour and Smith. Briefly, each animal was placed in a plexiglas box that allowed its tail to be free, then the box was placed on the tail stimulator analgesia meter (IITC Inc., U.S.A.) with the tail occluding a slit over a photocell for radiant heat stimulation generated by a power lamp mounted in a reflector. The tail-flick response was elicited by applying radiant heat to the point 1/3 of length away from the tip of the tail. When the mouse felt pain and flicked its tail, the light of the lamp fell on the photocell such that the timer was automatically stopped. The intensity of the heat stimulus in the tail-flick test was adjusted so that the mouse flicked its tail within 3 to 6 s. A 12 s cut-off time was set in order to prevent tail tissues from being damaged. Before experiment, the heat stimulation latency of all animals were tested, and those with response time of <2 or >6 s to heat stimulation were excluded. The tail-flick responses were measured at 0.5, 1, 2, and 3 h after oral administration of PSF (200, 100, 50 mg/kg), vehicle (0.5% CMC-Na), or rotundine (50 mg/kg).
Paw Tissues To obtain paw exudates, rats were killed by Et₂O at 4 h after intraplantar carrageenan injection. Each hind paw was amputated at the level of the calcaneus bone and a cross incision was made in the surface of paws, and then centrifuged at 400 \( g \) for 15 min at 4 °C to collect the exudates (edema fluid). The levels of interleukin (IL)-1\( \beta \), IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-\( \gamma \) and TNF-\( \alpha \) in paw exudates at 4 h after carrageenan injection were measured by using Bio-Plex rat cytokine assay. Briefly, the premixed standards were reconstituted in 0.5 ml of culture medium, generating a stock concentration of 50000 pg/ml for each cytokine. The standard stock was serially diluted in the same culture medium to generate 8 points for the standard curve. The assay was then performed in a 96-well filtration plate supplied with the assay kit. Premixed beads (50 µl) coated with target capture antibodies were transferred to each well of the filter plate and washed twice with Bio-Plex wash buffer. Premixed standards or samples (50 µl each) were added to each well containing washed bead. The plate was shaken for 30 s and then incubated at room temperature for 30 min with low-speed shaking. After incubation and washing, premixed detection antibodies (50 µl) were added to each well. The incubation was terminated after shaking for 10 min at room temperature. After washing three times, the beads were resuspended in 125 µl of Bio-Plex assay buffer and then were read on the Bio-Plex suspension array system. Data were analyzed using Bio-Plex Manager software with 5PL curve fitting.

Statistical Analysis Values of the data were expressed as the means±S.E.M. Statistical significance of the differences was assessed by ANOVA and, if significance found, by multiple comparison with SNK method. \( p \) Values less than 0.05 were considered statistically significant.

RESULTS

Chemical Profiles of PSF Characterized by HPLC Chromatographic Fingerprints and Identification of the Representative Single Saponin Compounds in PSF Figure 1 shows the chemical profiles of PSF characterized by HPLC fingerprints with twenty peaks detected. Peaks 2 (10.23%), 9 (6.45%), 10 (12.27%), 12 (27.47%), 13 (8.76%) and 15 (5.09%) considered as the characteristic and representative saponin constituents in the fraction were identified as ilexsaponin B\(_3\), pubescenoside C, ilexsaponin B\(_2\), chikusetusaponin I\(_{Va}\), ilexsaponin A\(_{1}\), and ilexsaponin B\(_{1}\), respectively, by comparing the retention time of the peaks with the relevant standard chemical substances. Those six identified compounds accounted for 70.27% of the total peak area of PSF with twenty peaks in the HPLC chromatographic analysis (Fig. 1).

Inhibitory Effect of PSF on Histamine-Induced Paw Edema of Rats In histamine-induced rat paw edema, measurements were conducted at 0.5, 1, 2, 3, 4 and 6 h after subcutaneous injection of histamine. As shown in Fig. 2, the hind paw edema of rats peaked at 0.5 h and then rapidly decreased from 1 h onward after the injection. The left hind paws that were used as control showed no increase of paw

Fig. 1. HPLC Chromatographic Fingerprint of PSF with a Total Twenty Peaks

Peaks 2 (10.23%), 9 (6.45%), 10 (12.27%), 12 (27.47%), 13 (8.76%) and 15 (5.09%) considered as the characteristic and representative saponin constituents in the fraction were identified as ilexsaponin B\(_3\), pubescenoside C, ilexsaponin B\(_2\), chikusetusaponin I\(_{Va}\), ilexsaponin A\(_{1}\), and ilexsaponin B\(_{1}\), respectively. The areas of the identified six compounds in the HPLC chromatogram accounted for 70.27% in the total areas of twenty peaks of the chromatogram.
volume during the entire experiment (data not shown), suggesting that the right hind paw edema was successfully induced by intraplantar injection of histamine. Administration of PSF by i.p. injection with dosages of 100, 50, 25 and 12.5 mg/kg showed significant inhibition of the edema at 0.5, 1 and 2 h after the injection; This inhibitory effect could last for 4 h in the animals treated with PSF at 100, 50 and 25 mg/kg. These results suggested that the anti-inflammatory effect of PSF was time-dependent. In the early stage (0.5 to 1 h after histamine injection), PSF showed strong and dose-dependent inhibition on the paw edema with dosages of 25 to 100 mg/kg. However, in the late phase (2 h after histamine injection), PSF affect the paw edema to a less intensity without dose-dependence. Indomethacin showed also significant inhibition of paw edema for at least 4 h after histamine injection.

Analgesic Effect of PSF in Nociceptive Models of Mice

The abdominal writhing assay of writing induced by peritoneal injection of acetic acid and tail flicking evoked by radiant heat stimulation in mice were employed in the study. Figure 3 shows the numbers of abdominal writhing episodes evoked by i.p. injection of acetic acid in the PSF-treated and control animals. It can be seen that treatment with PSF at doses of 200 and 100 mg/kg could significantly reduce the number of writhing episodes of mice in comparison with that of the vehicle-treated animals. The reference drug rotundine which has been known to exert its analgesic activity by blocking dopamine receptor produced potent analgesic effect at dosage of 50 mg/kg.18)

In Fig. 4, it can be seen that the tail flick reaction time of
animals in the control group held constant at around 5 s for 0.5, 1 and 2 h after dosing with the vehicle; in contrast, treatment with PSF at a dosage of 200 mg/kg or with the reference drug rotundine at 50 mg/kg significantly prolonged tail flick reaction time at 0.5, 1 and 2 h after drug administration. In addition, an almost identical potency of analgesia was obtained in the animals treated with PSF at a dosage of 200 mg/kg or with rotundine at a dosage of 50 mg/kg. Administration of PSF with dosages of 100 mg/kg only showed significance at 1 h after administration, and 50 mg/kg showed only mild degrees of analgesia. These results suggested that PSF could exert the strongest analgesic effect at 1 h after oral administration, and at this time point, it showed in a dose-dependent manner.

**Inhibition of COX-2 Protein Expression in Rat Paw Tissues Induced by PSF** At 4 h after the induction of paw edema caused by intraplantar injection of carrageenan, protein expressions of both COX-1 and COX-2 enzymes in edema hind paw tissues of rats were examined. In Fig. 5, significant protein expressions of COX-1 and COX-2 can be

---

**Fig. 6. Influence of PSF on the Production of IL-1β (A), IL-6 (B), TNF-α (C), IL-2 (D), GM-CSF (E), IFN-γ (F), IL-4 (G) and IL-10 (H) in Intraplantar Carrageenan-Injected Paw Tissues of Rats**

PSF (100, 50, 25 mg/kg, i.p.) was administrated 10 min before carrageenan injection. At 4 h after the injection, the paws exudates were collected for cytokines measurement using Bio-Plex rat cytokine assay. The vehicle (30% propanediol, i.p.) treated rats served as the control group. Each column represents the mean ± S.E.M. (n=6—9). *p<0.05, **p<0.01, ***p<0.001, compared with the control group.
seen in the paw tissues of control animals. In the paw tissues of treated animals, PSF at all tested doses showed no inhibition on COX-1 protein expression, while the reference drug indomethacin demonstrated some degree of inhibition (the left side of Fig. 5). However, with regard to COX-2 protein expression, treatment with PSF at dosages of 12.5 to 100 mg/kg demonstrated significant suppression, as the positive drug indomethacin did (the right side of Fig. 5). These indicate that PSF possesses a selective inhibitory potency on COX-2 protein expression but not on COX-1, which is probably different from the mode of drug action produced by indomethacin.

**Effects of PSF on Levels of IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ and TNF-α in Paw Tissues of Rats**

Levels of IL-1β, IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN-γ and TNF-α were determined in the paw tissues of rats collected at 4 h after intraplantar injection of carrageenan were determined by using Bio-Plex assay, a recently developed suspension bead array technology that allows simultaneous measurement of multiple cytokine targets from a single sample in a microplate format. The Bio-Plex assay allows for the detection of multiple cytokines in a single sample, which is highly beneficial for the study of pathogenesis and pathology of the inflammatory and arthritic illnesses. In our study, PSF was shown to have anti-inflammatory activity, which is transient, the inflammation decreases very quickly after induction. Hence the early inflammatory response appears to be mediated mainly by histamine. In our study, PSF showed strong and dose-dependent inhibition in the paw edema in the early phase of the inflammation, implying that PSF exert the anti-inflammatory effect by acting on the early phase of the inflammation. In the late phase, PSF showed a weak anti-inflammatory effect which didn’t show dose-dependency, this might be due to the decrease in activity of PSF which might result from the elimination of PSF, as well as the possible synergistic or offsetting effects between individual components in PSF, which induced different patterns of the overall effect of PSF at different dosages.

In addition, PSF exhibited potent analgesic effect in nociceptive models of mice. Mechanistic studies have shown that PSF treatment could selectively suppress COX-2 protein expression, decrease the levels of multiple proinflammatory cytokines, especially the levels of IL-1β, IL-6 and TNF-α, and increase the levels of anti-inflammatory cytokines of IL-4 and IL-10 in the hind paw edema paw tissues of rats evoked by intraplantar injection of carrageenan, in comparison with that of the vehicle-treated animals.

Inhibition of the protein expression of cyclooxygenases has been widely accepted as one of the important molecular targets of anti-inflammatory agents. Classic anti-inflammatory agents, non-steroidal anti-inflammatory drugs (NSAIDs), are used worldwide as anti-inflammatory, antipyretic and analgesic remedies through the inhibition of COX enzymes activities. Since the early 1990s, it has been commonly known that two COX enzymes, COX-1 and COX-2, are responsible for the production of prostaglandin (PG) H₂, the first step in prostanooids biosynthesis. COX-1 is responsible for the physiological production of prostanooids while COX-2 induces the elevated production of prostanooids that occurs at the sites of disease and inflammation. Clinically, although commercially available NSAIDs are often prescribed as anti-inflammatory agents, serious adverse side effects significantly limit their usage. A hypothesis has been accordingly suggested to explain the effects of NSAIDs that inhibition of COX-1 accounts for the side effects while inhibition of COX-2 accounts for the therapeutic benefits of NSAIDs. Thus, selective inhibition of the COX-2 enzyme appears to be the desirable target for anti-inflammatory/analgesic agents with improved gastrointestinal tolerability. In the past two decades, several COX-2 selective inhibitors such as Celecoxib, Valdecoxib, Parecoxib Sodium, Rofecoxib, Etoricoxib and Lumiracoxib have been developed. Inhibition of COX-2 protein expression has also become the most popular target for screening anti-inflammatory agents as well as for the study of pathogenesis and pathology of the inflammatory and
nociptive processes in animal models.\textsuperscript{24} In the current study, it was showed that PSF at dosages of 12.5 to 100 mg/kg could significantly suppress the protein expression of COX-2 but not COX-1 in the edema paw tissues of rats, while indomethacin could inhibit both COX-1 and COX-2 expression significantly. These results suggest that the PSF of MDQ could play a role in anti-inflammation in the model of carrageenan-induced paw edema of rats through inhibition of COX-2 protein expression at the sites of inflammation.

As cytokines are critical to the pathogenesis of inflammatory disorders, inhibition of their production and action provides therapeutic benefits in various inflammatory and arthritic diseases. Injection of the inflammatory agent, carrageenan, in the rat hind paws is a model frequently employed to study acute inflammation and evaluate the anti-inflammatory activity of various candidate compounds and agents. Previous studies have shown significant correlations among cytokines production, COX-2 protein expression and prostaglandins synthesis in the paw tissues of rats in which edema was invoked by intraplantar injection of carrageenan.\textsuperscript{24,25} In the current study, besides significant inhibition of COX-2 protein expression by pretreatment of PSF, the induction of COX-2, with a consequent reduction in the production of prostaglandins.\textsuperscript{32,33} Our current study showed that PSF at a relatively high dosage could significantly inhibit the production of IL-1\(\beta\), IL-6, and TNF-\(\alpha\) in hind paw tissues of rats was also decreased by PSF treatment. Thus, inhibition of the proinflammatory cytokines production is believed to be one of the pathways by which PSF exerts its anti-inflammatory effect in this model.

In contrast to the proinflammatory cytokines, some ‘antagonist cytokines’, such as IL-10, IL-4 and IFN-\(\gamma\), have been reported to inhibit the production of proinflammatory cytokines.\textsuperscript{26,27} IL-10 could inhibit the production of IL-2, IL-1\(\beta\), TNF-\(\alpha\), GM-CSF.\textsuperscript{27} It can also induce the formation of IL-1 receptor antagonist, another anti-inflammatory cytokine.\textsuperscript{29} Injection of a monoclonal antibody to mouse IL-10 can potentiate carrageenan- and TNF-\(\alpha\)-evoked inflammatory pain.\textsuperscript{29} These results, taken together, suggest that the administration of IL-10 may have therapeutic potential in acute and chronic inflammatory diseases. In addition to IL-10, another inhibitory cytokine, IL-4 is known to limit production of the proinflammatory cytokines, IL-1\(\beta\), IL-6, IL-8 and TNF-\(\alpha\).\textsuperscript{30} Also, IL-4 can suppress delayed-type hypersensitivity responses in experimental animals and in man.\textsuperscript{31} Moreover, both IL-4 and IL-10 have been reported to inhibit the induction of COX-2, with a consequent reduction in the production of prostaglandins.\textsuperscript{32,33} Our current study showed that PSF at a relatively high dosage could significantly increase the production of anti-inflammatory cytokines of IL-10 and IL-4 in the paw tissues of rats, which is believed to be beneficial to the amelioration of the paw edema of animals. In contrast, indomethacin could not augment but, rather, significantly inhibit the production of IL-4 and IL-10. These results demonstrate the differences in drug properties between indomethacin and PSF of MDQ. Moreover, both PSF and indomethacin demonstrated inhibitory but not stimulatory effect to the production of IFN-\(\gamma\) in the paw tissues of rats.

Besides the inhibitory effect on several proinflammatory cytokines and enhancing effect on anti-inflammatory cytokines production, PSF also showed a kind of biphasic effect on the production of IL-2, GM-CSF, IFN-\(\gamma\) and IL-4. This biphasic effect might be due to complex constituents of PSF which might interact with each other to yield synergistic or offsetting interactions.

In addition, like the positive agent rotundine, PSF demonstrated potent analgesic effect both in the visceral and central nociceptive mouse models induced by i.p. injection of acetic acid and by radiant heat stimulation, respectively. Although the analgesic mechanisms of PSF were not investigated in the current study, previous studies showed that acute inflammatory pain is usually characterized by hypernociception due to the sensitization of primary sensory nociceptive neurons, also referred to as hyperalgesia or allodynia; while eicosanoids and sympathetic amines are the most important primary mediators ultimately responsible for the mechanical hypernociception in rodents. Moreover, even though the release of primary hypernociceptive mediators is not directly stimulated by inflammatory stimuli, it is preceded by the release of a cascade of proinflammatory cytokines.\textsuperscript{34} Therefore, there might be underlying correlations between the anti-inflammatory and analgesic actions of PSF.

Regarding chemical constituents of PSF, HPLC chromatographic analysis showed that it is a saponin fraction with twenty peaks in the chromatogram, from which six saponins have been identified, including two novel saponins obtained. Previous studies showed that saponins are the major chemical ingredients in medicinal herbs responsible for most of the observed biological activities.\textsuperscript{35,36} Recent reports further indicated that most saponins can suppress the expression of COX-2 and iNOS, thus resulting in a marked lowering of levels of prostaglandin E\(_2\) (PGE\(_2\)).\textsuperscript{37} Moreover, the soybean saponins were reported to significantly inhibit the release of TNF-\(\alpha\)\textsuperscript{38} while platycodon D (PD) and PD3 increase the secretion of TNF-\(\alpha\) as well as expression of TNF-\(\alpha\) mRNA from RAW 264.7 cells \textit{in vitro}.\textsuperscript{21} Taken together, the saponin fraction of MDQ is believed to be valuable of further investigation.

In conclusion, the present study demonstrated that PSF exhibits potent suppressive effect on histamine-induced acute paw edema in rats. The molecular mechanisms are considered to be closely related to the inhibition of COX-2 protein expression, proinflammatory cytokines production, and especially decreasing levels of IL-1\(\beta\), IL-6, and TNF-\(\alpha\) in the paw tissues of the PSF-treated animals. In addition, PSF can significantly elevate the tissue levels of anti-inflammatory cytokines of IL-4 and IL-10, which helps relieving inflammation. Besides, PSF also showed significant analgesic effect in both visceral and central nociceptive models. Although there might be some correlations and common molecular pathways, such as inhibition of proinflammatory cytokines production, between the effects of anti-inflammation and analgesia of PSF, the exact mechanisms by which PSF exerts its analgesic effect need to be further investigated.

Acknowledgements The authors wish to thank Dr. Martha Dahlen for her professional English editing for this paper. This research is funded by the Faculty Research Grants of Hong Kong Baptist University (FRG/04-05/II-41) and by the Innovation and Technology Commission of Hong Kong Special Administrative Region, China (GHP/054/05).
REFERENCES