Running Title: Anti-inflammatory activities of *Mesona procumbens in vivo*

Anti-inflammatory Activities of Aqueous Extract of *Mesona procumbens* in Experimental mice

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Abstract

BACKGROUND: The *Mesona procumbens* is consumed as an herbal drink and jelly-type dessert in Taiwan. This study aimed to determine the mechanism of anti-inflammatory activities of the aqueous extracts of *Mesona procumbens* (AMP) using model of λ-carrageenin (Carr)-induced paw edema in mouse model. In HPLC analysis, the fingerprint chromatogram of AMP was established. In order to investigate the anti-inflammatory mechanism of AMP, we have detected the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) and the levels of malondialdehyde (MDA) in the paw edema. Serum NO, tumor necrosis factor α (TNF-α), and (IL-1β) were evaluated.

RESULTS: Fingerprint chromatogram from HPLC indicated that AMP contains protocatechuic acid, chlorogenic acid, vanillic acid, and caffeic acid. In the anti-inflammatory test, AMP decreased the paw edema after Carr administration, increased the activities of CAT, SOD, and GPx and decreased the MDA level in the edema paw at the 5th hr after Carr injection. AMP affects the serum NO, TNF-α, and IL-1β levels at the 5th hr after Carr injection. Western blotting revealed that AMP decreased Carr-induced inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions.

CONCLUSION: *Mesona procumbens* have the potential to provide a therapeutic approach to inflammation-associated disorders.

Key words: *Mesona procumbens*; Anti-inflammation; MDA; NO; TNF-*α*

INTRODUCTION

The reactive oxygen molecules such as superoxide ($O_2^{\cdot -}$, $HOO^{\cdot -}$), hydroxyl ($OH^{\cdot}$) and peroxyl ($ROO^{\cdot}$) radicals play an important role in the degenerative or pathological processes of various serious diseases, such as aging, cancer, coronary heart disease, Alzheimer's disease, neurodegenerative disorders, atherosclerosis, and inflammation. [1] The use of traditional medicine is widespread and plants still represent a large source of natural antioxidants that might serve as leads for the development of novel drugs. Several anti-inflammatory, antinecrotic, neuroprotective, and hepatoprotective drugs have recently been shown to have an antioxidant and/or radical-scavenging mechanism as the basis of their activity [2].

The herb *Mesona procumbens* Hemsl., called Hsian-tsao in Taiwan, is consumed as a herbal drink and jelly-type dessert. It has been traditionally used for the treatment of heat-shock, hypertension, diabetes, liver disease, and muscle pains. In previous study, phenolic compounds extracted from Hsian-tsao significantly contributed to the antioxidant activity and free radical scavenging effects. [3] Yen et al. indicated that UV-C- and/or H_2O_2-induced DNA damage in human lymphocytes are/is significantly reduced by the aqueous extract of Hsian-tsao. [4] Recently, Hsian-tsao was also found to exhibit efficient protective action against *tert*-butylhydroperoxide-induced hepatic damage[5] and antihypertensive effect on blood pressure in rats. [6] However, no studies have been conducted to investigate the anti-inflammatory activity of Hsian-tsao *in vivo*.

Some researchers demonstrated that inflammatory effect induced by Carr could be associated with free radical. Free radical, prostaglandin and NO will be released when administrating with Carr for 1-6 h. [7] The edema effect was raised to maximum at the 3th

hr[8] and its MDA production was due to free radical attack on plasma membrane. [9] Thus, inflammatory effect would result in the accumulation of MDA. Therefore, in this paper we evaluated the anti-inflammatory effects of the aqueous extract of *Mesona procumbens* (AMP) on paw edema induced by Carr in mice. And we detected the levels of MDA, NO, and TNF-α in either paw edema or serum. Also, the activities of CAT, SOD, and GPx in paw edema at the 5th hr after Carr injection were measured to investigate the relationship between the anti-inflammatory mechanism of AMP and antioxidant enzymes.

MATERIAL AND METHODS

Chemicals

λ-Carrageenan (Carr), indomethacin (Indo), and other chemical reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA). TNF-α and IL-1β were purchased from Biosource International Inc., (Camarillo, CA, USA). Plant materials were collected from Taichung country in Taiwan. They were identified and authenticated by Dr. Yuan-Shiun Chang, Professor, School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources, College of Pharmacy, China Medical University.

Fingerprint chromatogram of AMP extracts by HPLC

The chromatographic system consisted of a Qaternary Gradient Pump SFD 2100, a SFD 5200 autosampler, a Merck LiChrospher 100 RP-18e column (5 μm, 4.0 I.D.×250 mm) and a S-3210 photodiode-array detector (PDA) (Schambeck SFD GmbH, Bad Honnef, Germany). Peak area was calculated using a Schambeck HPLC-GPC-Software.

1 HPLC separation was accomplished on a Schambeck SFD GmbH LC model instrument.
2 For analysis a isocratic elution separations was applied, and elution was carried out with
3 solvent A (acetic acid/water (2:98 *v/v*)) and solvent B (acetic acid/acetonitrile/water
4 (2:50:48 *v/v*)) as mobile phase at a flow rate of 0.8 ml min^{-1}. The samples were analyzed
5 by HPLC on a Lichrospher 100 RP-18e column and detected at 280 nm. All phenolic
6 acids were identified by matching the retention time and absorption spectra
7 characteristics against those of standards.

8

9 **Mice**

10 This study was conducted in conformity with the policies and procedure details in
11 the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86–23
12 1985) and was approved by the ethics committee of the Institutional Animal Care and
13 Use Committee (IACUC) of China Medical University, Taichung, Taiwan. ICR strain
14 male mice (6−8 weeks old) were obtained from BioLASCO Taiwan Co., Ltd., Taipei,
15 Taiwan. The mice were housed in an environmentally controlled room (temperature
16 22 ± 1 ℃; relative humidity 55 ± 5%; 12 h dark–light cycle). They were given food and
17 water *ad libitum*.

18 After a 2-week adaptation period, male ICR mice (18-25 g) were randomly assigned
19 to five groups (n=6) of the animals. In the Carr-induced edema experiment, there were
20 randomly assigned to six groups (n=6) of the animals in the study. The control group
21 receives normal saline (i.p.). The other five groups include a Carr-treated, a positive
22 control (Carr + Indo) and AMP administered groups (Carr + AMP: 125, 250, and 500 mg

kg^{-1}).

Determination of carrageenan (Carr) induced edema

Carr-induced hind paw edema model was used for determination of anti-inflammatory activity.[10] After a 2-week adaptation period, male ICR mice (18 to 25 g) were randomly assigned to five groups (n = 6) including Carr, positive Indo control and three AMP-treated groups. Carr group received 1% Carr (50 μL). AMP at doses of 125, 250, and 500 mg kg^{-1} were orally administered 2 hrs before the injection with 1% Carr (50 μL) in the plantar side of right hind paws of the mice. And Indo (10 mg kg^{-1}) was intraperitoneally administered 90 min before the injection with 1% Carr (50 μL) in the plantar side of right hind paws of the mice. Paw volume was measured after Carr injection at 1, 2, 3, 4, and 5 hr intervals using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree of swelling induced was evaluated by *a* minus *b*, where *a* was the volume of the right hind paw after Carr treatment and *b* was the volume of the right hind paw before Carr treatment. Indo was used as a positive control.

In the later experiment, the right hind paw tissue was taken at the 5th hr. The right hind paw tissue was rinsed in ice-cold normal saline, and immediately placed in cold normal saline four times their volume and homogenized at 4 ℃. Then the homogenate was centrifuged at 12,000×*g* for 5 min. The supernatant was obtained and stored at −20 ℃ refrigerator for MDA and the antioxidant enzymes (CAT, SOD, and GPx) activities assays.

Determination of tissue lipid peroxidation

MDA was evaluated by the thiobarbituric acid reacting substances (TBARS) method.[11] Briefly, MDA reacted with thiobarbituric acid in the acidic medium at high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

Determination of nitric oxide (NO)

The production of NO was assessed indirectly by measuring the nitrite levels in plasma determined by a calorimetric method based on the Griess reaction.[12] Plasma samples were diluted four times with distilled water and deproteinized by adding 1/20 volume of zinc sulfate (300 g L^{-1}) to a final concentration of 15 g L^{-1}. After centrifugation at 10,000×*g* for 5 min at room temperature, 100 μL supernatant was applied to a microtiter plate well, followed by 100 μL of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylenediamine dihydrochloride in 2.5% polyphosphoric acid). After 10 min of color development at room temperature, the absorbance was measured at 540 nm with a MicroReader (Hyperion, Inc., FL, USA). Nitrite was quantified by using sodium nitrate as a standard.

Measurement of serum TNF-α and IL-1β by ELISA

Serum levels of TNF-αdetermined using a commercially available enzyme linked immunosorbent assay (ELISA) kit according to the manufacturer's instruction. TNF-α and IL-1β were determined from a standard curve for the calculation of these cytokines. The concentrations were expressed as pg mL^{-1}.

1 **Determination of antioxidant enzyme activity in paw tissue**

2 The following biochemical parameters were analyzed to check the protective
3 activity of AMP by the methods given below. Total SOD activity was determined by the
4 inhibition of cytochrome *c* reduction.[13] The reduction of cytochrome *c* was mediated by
5 superoxide anions generated by the xanthine/xanthine oxidase system and monitored at
6 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the
7 rate of cytochrome *c* reduction by 50%. Total CAT activity estimation was based on the
8 previously reported.[14] In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate
9 buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was
10 calculated by using a molar absorption coefficient, and the enzyme activities were
11 defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute.
12 Total GPx activity in cytosol was determined as previously reported.[15] The enzyme
13 solution was added to a mixture containing hydrogen peroxide and glutathione in 0.1 mM
14 Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated
15 from a calibration curve, and the enzyme activities were defined as nanomoles of
16 NADPH oxidized per milligram protein per minute. The protein concentration of the
17 tissue was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).
18

19 **Histological examination**

20 For histological examination, biopsies of paws were taken 5th h following the
21 interplanetary injection of Carr. The tissue slices were fixed in a solution (1.85%
22 formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded
23 ethanol and embedded in Paraffin (Sherwood Medical). Sections (thickness 5 μm) were

1 deparaffinized with xylene and stained with H & E stain. Neutrophils
2 (polymorphonuclear neutrophil; PMNs) were identified by positive staining and
3 morphology and were counted by using a BH2 Olympus microscopy. Only neutrophils
4 present within dermal region into the tissue were counted. Every 3~5 tissue slices were
5 randomly chosen from Carr, Indo, and AMP treated (500 mg kg^{-1}) groups. The number of
6 the neutrophils was counted in each scope (400 x) and thereafter we obtained their
7 average count from 5 scopes of every tissue slice. [16, 17]
8
9 **Protein Lysate Prepration and Western blot Analysis of iNOS and COX-2 in Paw**
10 Total protein was extracted with a RIPA solution (radioimmuno-precipitation assay
11 buffer) at -20℃ overnight. We used BSA (bovine serum albumin) as a protein standard
12 to calculate equal total cellular protein amounts. Protein samples (30 μg) were resolved
13 by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)
14 using standard methods, and then were transferred to PVDF membranes by
15 electroblotting and blocking with 1% BSA. The membranes were probed with the
16 primary antibodies (iNOS, COX-2, and β-actin) at 4℃ overnight, washed three times
17 with PBST, and incubated for 1 hr at 37 ℃ with horseradish peroxidase conjugated
18 secondary antibodies. The membranes were washed three times and the immunoreactive
19 proteins were detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL
20 reagent (Amersham International plc., Buckinghamshire, U.K.). The results of Western
21 blot analysis were quantified by measuring the relative intensity compared to the control
22 using Kodak Molecular Imaging Software and represented in the relative intensities.
23

Statistical analysis

Data are expressed as mean ± S.E. Statistical evaluation was carried out by one-way analysis of variance (ANOVA followed by Scheffe's multiple range test). Statistical significance is expressed as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

RESULTS

Fingerprint chromatogram of HPLC.

To establish the fingerprint chromatogram for the quality control of AMP. Protocatechuic acid, chlorogenic acid, vanillic acid, and caffeic acid were used as markers. An optimized HPLC-PDA technique was employed.[18] Meanwhile, HPLC chromatograms showed four marker components present in AMP. As shown in Fig. 1A and 1B, these phenolic components have been identified as protocatechuic acid (retention time, 10.2 min), chlorogenic acid (17.8 min), vanillic acid (29.5 min), and caffeic acid (33.7 min) by their retention time and UV absorbance of purified standards. According to the plot of peak-area ratio (y) vs. concentration (x, μg/mL), the regression equations of the four constituents and their correlation coefficients (r) were determined as follows: protocatechuic acid, y =53316x+118394 (r^2=0.999); chlorogenic acid, $y = 75775x$ - 1E+06 ($r^2 = 0.9984$); vanillic acid, $y = 52766x - 899466$ ($r^2 = 0.9976$); and caffeic acid, $y = 111501x$ - 1E+06 ($r^2 = 0.9984$). The relative amounts of the four phenolic compounds found in AMP was in the order of chlorogenic acid (2.42 mg/g) > caffeic acid (1.33 mg/g) > vanillic acid (0.52 mg/g) > protocatechuic acid (0.47 mg/g), respectively.

AMP alleviated Carr-induced mouse paw edema

1 To determine whether anti-inflammatory effects of AMP occurred *in vivo*,
2 Carr-induced mouse hind paw edema test was conducted. As was expected, there was a
3 gradual increase in edema paw volume of mice in the Carr-treated group. However, in the
4 test groups, AMP (250 and 500 mg kg^{-1}) significantly inhibited Carr-induced mouse paw
5 edemas in a dose-dependent manner with a maximum attend (Fig. 2A). The dose-related
6 inhibition of hind paws edema between 3th to 5th h was observed. Indo as positive control
7 (10 mg kg^{-1}) produced a significant inhibitory effect compared to Carr-treated group.

8

9 **AMP reduced MDA, NO, TNF-α, and IL-1β production *in vivo***

10 MDA level increased significantly in the edema paw at the 5th h after Carr injection
11 ($p < 0.001$). As expected, administration of 10 mg kg^{-1} Indo significantly reduced the
12 MDA level in the edema paw. In this time, MDA level was also decreased
13 dose-dependently by treatment with AMP (500 mg kg^{-1}) ($p < 0.001$) (Fig. 2B).

14 In Fig. 2C, the NO level increased significantly in the edema serum after 5th h
15 carrageenan injection ($p < 0.001$). AMP (250 and 500 mg kg^{-1}) markedly decreased the
16 serum NO level ($p < 0.01$ or $p < 0.001$) in Carr-treated mice. The inhibitory potency was
17 similar to that of Indo (10 mg kg^{-1}) at 5th h after induction.

18 Data from ELISA assay showed that TNF-*α* and IL-1β levels increased significantly
19 in serum after 5th h Carr injection ($p < 0.001$). However, AMP (500 mg kg^{-1}) and Indo (10
20 mg kg^{-1}) decreased the TNF-α and IL-1β levels in serum at the 5th h after Carr injection
21 ($p < 0.001$) (Fig. 2D and 2E).

22

23 **Effects of AMP on activities of antioxidant enzymes**

At the 5th h following Carr injection, paw edema tissues were analyzed for the biochemical parameters such as CAT, SOD, and GPx activities (Table 1). CAT, SOD, and GPx activities in paw edema tissue were decreased significantly by Carr administration. CAT, SOD and GPx activity were increased significantly after treated with AMP and 10 mg kg^{-1} Indo (P<0.01) (Table 1).

Effects of AMP **on Carr-Induced iNOS and COX-2 Protein Expressions in Mice Paw Edema.**

To investigate whether the inhibition of NO production was due to a decreased iNOS and COX-2 protein level, the effect of AMP on iNOS and COX-2 proteins expression were studied by Western blot. The results showed that injection of AMP (500 mg kg^{-1}) on Carr-induced for 5th h inhibited iNOS and COX-2 proteins expression in mouse paw edema (Fig. 3A). The intensity of protein bands were analyzed by using Kodak Quantity software in these three independent experiments and the result showed an average of 67.4% and 56.4% down-regulation of iNOS and COX-2 protein, respectively, after treatment with AMP compared with the Carr-induced alone (Fig. 3B). In addition, the protein expression showed an average of 60.6% and 54.1% down-regulation of iNOS and COX-2 protein after treatment with Indo at 10.0 mg kg^{-1} compared with the Carr-induced alone.

Histological examination.

Paw biopsies of control animals showed marked cellular infiltration in the connective tissue. The infiltrates accumulated between collagen fibers and into intercellular spaces.

Paw biopsies of animals treated with AMP (500 mg kg^{-1}) showed a reduction in Carr -induced inflammatory response. Actually inflammatory cells were reduced in numbers and were confined to be near the vascular areas. Intercellular spaces did not show any cellular infiltrations. Collagen fibers were regular in shape and showed a reduction of intercellular spaces. Moreover, the hypodermal connective tissue was not damaged (Fig. 4A). The number of neutrophil was significantly increased upon Carr treatment ($P < 0.001$). However, Indo and AMP (500 mg kg^{-1}) could effectively decrease the neutrophil numbers as compared to the Carr-treated group ($P < 0.001$) (Fig. 4B).

Discussion

In the present study, we demonstrated anti-inflammatory activities of AMP in *in vivo* experimental systems, using a mouse model of topical inflammation. Dual inhibitory activities were against iNOS and COX-2 as shown in *in vivo* efficacy of AMP in mouse, Carr-induced, paw edema, comparable with a potent and well known COX inhibitor, Indo, suggested its potential therapeutic usage as a novel topical anti-inflammatory source of health food.

The Carr test is highly sensitive to non-steroidal anti-inflammatory drugs, and has long been accepted as a useful phlogistic tool for investigating new drug therapies. [19] The degree of swelling of the Carr-injected paws was maximal 4th h after injection. Statistical analysis revealed that AMP and Indo significantly inhibited the development of edema 4th h and 5th h after treatment ($p<0.01$) (Fig. 2A). It is well known that the 3th or 4th h of the Carr-induced edema, where the edema reaches its highest volume, characterized by the injection of Carr into the mice paw induces the liberation of bradykinin, which later

induces the biosynthesis of prostaglandin and other autacoids, which are responsible for the formation of the inflammatory exudates. [20]

Edema formation is the result of interaction among various inflammatory mediators that increase vascular permeability and/or blood flow. Carr-induced edema has been described as a biphasic event. The early phase, observed about 1 h (up to 2 h) after Carr injection, is related to the production of serotonin, histamine, leukotrienes bradykinin, and cyclooxygenase products in the inflamed tissue, while the late phase (2–6 h) is due to neutrophil infiltration, as well as to the continuing production of arachidonic acid metabolites. [2] In a number of pathophysiological conditions associated with inflammation or oxidant stress, these ROS have been proposed to mediate cell damage via a number of independent mechanisms including the initiation of lipid peroxidation, the inactivation of a variety of antioxidant enzymes.[21]

Excessive production of NO plays a critical role in the aggravation of circulatory shock and chronic inflammatory diseases, such as septic shock, inflammatory hepatic dysfunctions, inflammatory lung disease and colitis.[22] As many of these conditions exhibit rapid onset and development, often resulting in the failure of conventional anti-inflammatory therapies and extremely high mortality rates, a simultaneous suppression of NO production pathways, as shown by AMP, may satisfy the so far unmet need for control of the rapid progression of the inflammatory process. In the studies of the mechanism on the inflammation, L-arginine–NO pathway has been proposed to play an important role in the Carr-induced inflammatory response. [23] Our present results also confirm that Carr-induced paw edema model results in the production of NO. The expression of the inducible isoform of NO synthase has been proposed as an important

1 mediator of inflammation. In our study, the level of NO was decreased significantly by
2 treatment with 250 and 500 mg kg^{-1} AMP. We suggest the anti-inflammatory mechanism
3 of AMP may be through the L-arginine–NO pathway because AMP significantly inhibits
4 the NO production.

5 It was reported that phenolic phytochemicals show strong antioxidative and
6 anti-inflammatory activities.[24] In previous study, five phenolic compounds,
7 protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, and syringic acid,
8 were isolated from AMP extracts.[25] In the present study, the following components were
9 found in AMP HPLC analysis protocatechuic acid, chlorogenic acid, vanillic acid, and
10 caffeic acid. Those phenolic compounds played important antioxidative roles in AMP
11 extracts and that caffeic acid and chlorogenic acid had high yield and antioxidant
12 activities. Some paper evaluated that protocatechuic acid may inhibit the expression of
13 TNF-α, IL-β, and COX-2 in LPS-stimulated RAW264.7 cells.[26] Vanillic acid inhibits
14 inflammatory TNF-α and IL-6 by suppressing NF-κB in lipopolysaccharide-stimulated
15 mouse peritoneal macrophages.[27] Then caffeic acid and caffeic acid derivatives exert *in*
16 *vitro* and *in vivo* anti-inflammatory actions, being their actions mediated, at least by the
17 scavenging of NO and their ability to modulate iNOS expression and probably that of
18 other inflammatory mediators.[28] Chlorogenic acid inhibits lipopolysaccharide-induced
19 cyclooxygenase-2 expression in RAW264.7 cells through suppressing NF-kappaB and
20 JNK/AP-1 activation.[29] These phenolic components in the AMP could also play
21 important roles in anti-inflammatory activity. These results suggest that the antioxidative
22 and anti-inflammatory activities of AMP are related to their phenolic compounds.

TNF-α and IL-1β are the mediators of Carr-induced inflammatory incapacitation, and are able to induce the further release of kinins and leukotrienes, which are suggested to have an important role in the maintenance of long-lasting nociceptive response[30]. In this study, we found that **AMP** decreased the TNF-α and IL-1β levels in serum after Carr injection by treatment with 250 and 500 mg kg^{-1} **AMP**, significantly ($p<0.01$ or $p<0.001$).

The Carr-induced inflammatory response has been linked to neutrophils infiltration and the production of neutrophils-derived free radicals, such as hydrogen peroxide, superoxide and hydroxyl radicals, as well as the release of other neutrophils-derived mediators.[31] Some researchers demonstrated that inflammatory effect induced by Carr is associated with free radical. Free radical, prostaglandin and NO will be released when administrating with Carr for 1-6 h. The edema effect was raised to the maximum at 4 h. MDA production is due to free radical attack on plasma membrane. Thus, inflammatory effect would result in the accumulation of MDA. Glutathione (GSH) acts as a oxyradical scavenger by scavenging NO and other oxidants. The increased GSH level may favor to reduce MDA production. GSH plays an important role against Carr-induced local inflammation.[32] In this study, there is significantly increased in CAT, SOD, and GPx activities with **AMP** treatment. Furthermore, there are significantly decreases in MDA level with **AMP** treatment. We assume the suppression of MDA production is probably due to the increases of CAT, SOD, and GPx activities in the paw edema.

CONCLUSION

These results suggested that **AMP** possessed anti-inflammatory effects. The anti-inflammatory mechanism of **AMP** may be related to iNOS and it is associated with

1 the increase in the activities of antioxidant enzymes (CAT, SOD, and GPx). **AMP** may be
2 used as a pharmacological agent in the prevention or treatment of disease in which free
3 radical formation is a pathogenic factor.

4

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