Running Title: antioxidant and anti-inflammatory properties of *Cardiospermum halicacabum*

Antioxidant and anti-inflammatory properties of *Cardiospermum halicacabum* and its reference compounds *ex vivo* and *in vivo*

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# Equal contribution to this work.

Abstract

Aims of the study: *Cardiospermum halicacabum* (CH) has been used in Chinese medicine for a long time. However, its fingerprint chromatogram, antioxidant, anti-inflammatory effects and mechanism are still needed to be explored. Therefore, the aims of this study investigated the antioxidant and anti-inflammatory effects of CH extracts and its reference compounds *ex vivo* and *in vivo*.

Materials and methods: In HPLC analysis, the fingerprint chromatogram of ethanolic extract of CH (ECH) was established. The effects of ACH (aqueous extract of CH) and ECH extracts were assessed for the antioxidant and LPS-induced NO production in RAW264.7 cells. *In vivo* anti-inflammatory activities of ECH were evaluated in mouse paw edema induced by λ-carrageenan (Carr). We investigate the anti-inflammatory mechanism of ECH via studies of the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) in the liver and the levels of malondialdehyde (MDA) and nitrite oxide (NO) in the edema paw. Serum NO and TNF-α were also measured.

Results: ECH had better antioxidant activity than that of ACH. In the anti-inflammatory test, ECH inhibited the development of paw edema induced by Carr and increased the activities of CAT, SOD and GPx in the liver tissue. ECH also decreased the level of NO in edematous paw tissue and in serum level, and diminished the level of serum TNF-α at the fifth hour after Carr injection.

Conclusions: ECH exerts anti-inflammatory effects by suppressing TNF-α and NO. The anti-inflammatory mechanism of ECH might be related to the decrement of the level of MDA in the edema paw *via* increasing the activities of CAT, SOD and GPx in the liver. The results showed that ECH might serve as a natural antioxidant and anti-inflammatory agent.

Keywords: *Cardiospermum halicacabum*; Chinese medicine; Antioxidant; Anti-inflammation; NO; TNF-α;

1. Introduction

Cardiospermum halicacabum L. (CH) has been used in Chinese medicine for a long time in the treatment of rheumatism, lumbago, nervous diseases, as a demulcent in orchitis and in dropsy (Sadique et al., 1987; Chandra and Sadique, 1989; Ra et al., 2006). Various pharmacological actions of CH have been investigated in animal models. The anti-inflammatory activity of ethanolic extract against inhibits LPS induced COX-2, TNF-α and iNOS expression in RAW264.7 cells (Sheeba and Asha, 2009). Experimental pharmacological studies have shown the analgesic and vasodepressant activities (Gopalakrishnan et al., 1976), antipyretic activity against yeast-induced pyrexia in rats (Asha and Pushpangadan, 1999), antimalarial (Waako et al., 2005), antioxidant activity (Kumar and Karunakaran, 2006), suppressing the production of TNF-α and nitric oxide in human peripheral blood mononuclear cells (Venkatesh Babu, 2006; Thabrew et al., 2004) and anti-ulcer activity against ethanol induced gastric ulcer in rats (Sheeba and Asha, 2006).

A number of compounds have been isolated and identified in CH, such as arachidic acid, apigenin, apigenin-7-O-glucuronide, chrysoeriol-7-O-glucuronide and luteolin-7-O-glucuronide (Khan et al., 1990; Subramanyam et al., 2007). Many studies have indicated that the reactive oxygen species scavenging inhibition and anti-inflammatory activities seen in herbs may be attributed to the various natural

84 phenolic components with antioxidant effects and anti-inflammatory present in it,
85 such as apigenin (Sharififar et al., 2009), apigenin-7-glucoside and
86 luteolin-7-glucoside (Miceli et al., 2005). Thus, it was necessary to explore the
87 phenolic compounds present in CH.

88 In the present study, the antioxidant and anti-inflammatory effects of ACH and
89 ECH extracts were examined *ex vivo*. We also evaluated the anti-inflammatory effects
90 of ECH extract on paw edema induced by Carr in mice and investigated the
91 underlying mechanisms *in vivo*.

92

93 **2. Materials and methods**

94 **2.1. Materials**

95 Glutathione (GSH), 1, 1-diphenyl-2-picrylhydrazyl radicals (DPPH),
96 lipopolysaccharide (LPS; endotoxin from *Escherichia coli*, serotype 0127:B8), 2,
97 2'-azinobis-(3-ethylbenzothiazoline)-6- sulphonic acid (ABTS), butylated
98 hydroxytoluene (BHT), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
99 (MTT), apigenin-7-glucoside, apigenin, luteolin-7-glucoside, λ-Carrageenan(Carr),
100 indomethacin (Indo) and other chemicals were purchased from Sigma Chemical Co.
101 (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent was purchased from Merck

Co. (Santa Ana, CA, USA). Plant materials were collected from Taichung country in Taiwan. Also, they were identified and authenticated by Dr. Chao-Lin Kuo, Associate professor and Chairman, Department of Chinese Medicine Recourses, China Medical University, Taichung, Taiwan.

2.2. Preparation of the extracts of plant materials

A 100 g sample of CH was extracted with water (1 L) at 100 ℃ for 60 min and then centrifuged at 10,000 × *g* for 20 min. The extraction was repeated three times. The extracts were then combined and filtered through a No. 1 filer-paper. The filtrates were collected, concentrated with a vacuum evaporator until the volume was below 10 mL and then freeze-dried. The yield obtained was 6.3% (w/w). Dried sample of CH (100 g) was macerated with 1L ethanol for 24 h at room temperature. Filtration and collection of the extract was done three times. The filtrates were collected, concentrated with a vacuum evaporator until the volume was below 10 mL and then freeze-dried. The yield obtained was 8.2% (w/w).

2.3. Fingerprint chromatogram of CH extracts by HPLC

HPLC was performed with a Hitachi Liquid Chromatograph (Hitachi Ltd., Tokyo, Japan), consisting of two model L-7100 pumps, and one model L-7455 photodiode array detector (254 nm). Samples (10 mg/mL) were filtered through a 0.45 μm PVDF-filter and injected into the HPLC column. The injection volume was 10 μL and the separation temperature was 25 ℃. The column was a Mightysil RP-18 GP (5 μm, 250 mm × 4.6 mm I.D.). The method involved the use of a binary gradient with mobile phases containing: (A) phosphoric acid in water (0.1%, *v/v*) and (B) $H_2O/CH_3OH/CH_3CN$：20/40/40 (*v/v*). The solvent gradient elution program was as follows: 0–10 min, 100–75% A, 0–25% B; 10–15 min, 75–80% A, 25–20% B; 15–25 min, 80–80% A, 20–20% B; 25–40 min, 80–50% A, 20–50% B; 40–50 min, 50–30% A, 50–70% B; and finally 50–60 min, 30–0% A, 70–100% B. The flow-rate was kept constant at 0.8 mL/min. A precolumn of μ-Bondapak™ C_{18} (Millipore, Milford, MA, USA) was attached to protect the analytical column.

2.4. *In vitro* antioxidant activities of the crude extracts

2.4.1. Determination of antioxidant activity by DPPH radical scavenging ability

The effects of crude extracts and positive controls (GSH and BHT) on DPPH radicals were estimated according to the method of Huang et al., (2006). Aliquot (20 μL) of crude extracts at various concentrations were each mixed with 100 mM

Tris-HCl buffer (80 μL, pH 7.4) and then with 100 μL of DPPH in ethanol to a final concentration of 250 μM. The mixture was shaken vigorously and left to stand at room temperature for 20 min in the dark. The absorbance of the reaction solution was measured spectrophotometrically at 517 nm. The percentages of DPPH decolorization of the samples were calculated according to the equation: % decolorization = [1-(ABS_{sample} /$ABS_{control}$)] ×100. EC_{50} value was the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

2.4.2. Determination of antioxidant activity by $ABTS^{\cdot+}$ scavenging ability

The $ABTS^{\cdot+}$ scavenging ability was determined according to the method of Huang et al., (2006). Aqueous solution of ABTS (7 mM) was oxidized with potassium peroxodisulfate (2.45 mM) for 16 hours in the dark at room temperature. The $ABTS^{\cdot+}$ solution was diluted with 95% ethanol to an absorbance of 0.75 ± 0.05 at 734 nm (Beckman UV-Vis spectrophotometer, Model DU640B). An aliquot (20 μL) of each sample (125 μg/mL) was mixed with 180 μL $ABTS^{\cdot+}$ solution and the absorbance was read at 734 nm after 1 min. Trolox was used as a reference standard.

2.5. Determination of total polyphenol content

The total polyphenol contents of the crude extracts were determined according to the method of Huang (2008). 20 μL of each extract (125 μg/mL) was added to 200 μL distilled water and 40 μL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min, and then 40 μL of 20 % sodium carbonate was added to the mixture. The resulting blue complex was then measured at 680 nm. Catechin was used as a standard for the calibration curve. The polyphenol content was calibrated using the linear equation based on the calibration curve. The total polyphenol content was expressed as mg catechin equivalence (CE)/g dry weight.

2.6.1. Cell culture.

A murine macrophage cell line RAW 264.7 (BCRC No. 60001) was purchased from the Bioresources Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were cultivated in plastic dishes containing Dulbecco's Modified Eagle Medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA) in a CO_2 incubator (5% CO_2 in air) at 37℃ and subcultured every 3 day at a dilution of 1:5 using 0.05% trypsin–0.02% EDTA in Ca^{2+}-, Mg^{2+}- free phosphate-buffered saline (DPBS).

2.6.2. Cell viability.

Cells (2 x 10^5) were cultivatedd in 96-well plates containing DMEM supplemented with 10% FBS for 1 day to become nearly confluent. Then cells were cultivated with samples in the presence of 100 ng/mL LPS (lipopolysaccharide) for 24 h. After that, the cells were washed twice with DPBS and incubated with 100 μL of 0.5 mg/mL MTT for 2 h at 37℃ testing for cell viability. The medium was then discarded and 100 μL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

2.6.3. Measurement of Nitric oxide/Nitrite.

Nitrite levels in the cultured media and serum, which reflect intracellular nitric oxide synthase activity, were determined by Griess reaction. The cells were incubated with samples in the presence of LPS (100 ng/mL) at 37℃ for 24 h. And then cells were dispensed into 96-well plates, and 100 μL of each supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% phosphoric acid) and incubated at room temperature for 10 min. Using sodium nitrite to generate a standard curve, the concentration of nitrite was measured form absorbance at 540 nm Huang et al., (2007).

2.7. Animals.

Imprinting control region (ICR; 6-8 weeks male) mice were obtained from the BioLASCO Taiwan Co., Ltd. The animals were kept in plexiglass cages at a constant temperature of 22 ±1℃, and relative humidity of 55 ± 5 % with 12 h dark-light cycle

for at least 2 weeks before the experiment. They were given food and water *ad libitum*. All experimental procedures were performed according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. In addition, all tests were conducted under the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

After a 2-week adaptation period, male ICR mice (18-25 g) were randomly assigned to six groups (n=6) of the animals in the study. The control group received normal saline (intraperitoneal; i.p.). The other five groups include a Carr-treated, a positive control (Carr + Indo) and ECH administered groups (Carr + ECH: 100, 200 and 400 mg/kg).

2.8. 1. λ-Carrageenan (Carr)-induced edema

The Carr-induced hind paw edema model was used for determination of anti-inflammatory activity (Chang et al., 2009). Animals were i.p. treated with ECH (100, 200 and 400 mg/kg), Indo or normal saline, 30 min prior to injection of 1% Carr (50 µL) in the plantar side of right hind paws of the mice. The paw volume was measured immediately after Carr injection and at 1, 2, 3, 4 and 5 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 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. After 5 h, the animals were sacrificed and the Carr-induced edema feet were dissected and stored at -80 ℃. Also, blood were withdrawn and kept at -80 ℃.

Therefore, the right hind paw tissue and liver tissue were taken at the 5 h. 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 assays. The whole liver tissue was rinsed in ice-cold normal saline, and immediately placed in cold normal saline one time their volume and homogenized at 4 ℃. Then the homogenate was centrifuged at 12,000*g* for 5 min. The supernatant was obtained and stored in the refrigerator at −20 ℃ for the antioxidant enzymes (CAT, SOD and GPx) activity assays.

2.8.2. Total Protein Assay.

The protein concentrations of the sample were determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA).

2.8.3. MDA Assay.

MDA from Carr-induced edema foot was evaluated by the thiobarbituric acid reacting substances (TRARS) method (Chang et al., 2009). Briefly, MDA reacted with thiobarbituric acid in the acidic high temperature and formed a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

2.8.4. Antioxidant enzymes activity measurements.

The following biochemical parameters were analyzed to check the hepatoprotective activity of ECH by the methods given below. Total superoxide dismutase (SOD) activity was determined by the inhibition of cytochrome *c* reduction (Flohe and Otting 1984). The reduction of cytochrome *c* was mediated by superoxide anions generated by the xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%. Total catalase (CAT) activity estimation was based on that of Aebi (Aebi 1984). In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7) was monitored by measuring the absorbance at 240 nm. The activity was calculated by using a molar absorption coefficient, and the enzyme activity was defined as nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total GPx activity in cytosol was determined as previously reported (Paglia and Valentine, 1967). The enzyme solution was added to a mixture

containing hydrogen peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the absorbance at 340 nm was measured. Activity was evaluated from a calibration curve, and the enzyme activity was defined as nanomoles of NADPH oxidized per milligram protein per minute.

2.9. Histological Examination.

For histological examination, biopsies of paws were taken 5 h following the interplanetary injection of Carr. The tissue slices were fixed in (1.85% formaldehyde, 1% acetic acid) for 1 week at room temperature, dehydrated by graded ethanol and embedded in Paraffin (Sherwood Medical). Sections (thickness 5 μm) were deparaffinized with xylene and stained with hematoxylin and eosin (H&E) stain. All samples were observed and photographed with Nikon microscopy. Every 3~5 tissue slices were randomly chosen from Carr, Indo and ECH-treated (400 mg/kg) groups. Histological examination of these tissue slices revealed an excessive inflammatory response with massive infiltration of neutrophils [ploymorphonuclear leukocytes (PMNs)] by microscopy. The numbers of neutrophils were counted in each scope (400 x) and thereafter obtain their average count from 5 scopes of every tissue slice.

2.10. Statistical analysis.

Data are expressed as mean ± S.E.M. 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 ***$p < 0.001$.

3. Results

3.1. Antioxidant Assay.

Table 1 showed the DPPH radical scavenging activity of ACH and ECH. EC_{50} value of ECH and ACH were 198.26± 4.62 and 357.18± 2.58 μg/mL. This result suggested that ECH had better antioxidant activity in DPPH scavenging ability. ABTS assay was expressed as trolox equivalent antioxidant activity (TEAC) values. Higher TEAC value represented that the sample had a stronger antioxidant activity. TEAC value of the ECH and ACH were also shown in Table 1. It was observed that ECH (166.98± 2.07 μg/mg extract) had higher antioxidant potentials than ACH (126.52 ± 1.60 μg/mg extract). Since the total polyphenol contents of natural products are regular indices of their antioxidant activity, the catechin equivalence (CE) was thus determined for polyphenol. The results showed that the ACH and ECH contained polyphenol amounts equal to 108.05 ± 2.03 and 131.31 ± 0.78 mg CE/g, respectively.

3.2. Fingerprint chromatogram of HPLC.

To establish the fingerprint chromatogram for the quality control of ACH and ECH. Apigenin, apigenin-7-glucoside and luteolin-7-glucoside were used as markers.

297 An optimized HPLC-DAD technique was employed. Meanwhile, HPLC
298 chromatograms showed two marker components present in ACH and three marker
299 components present in ECH. As shown in Fig. 1A and 1B, these phenolic components
300 have been identified as luteolin-7-glucoside (retention time, 32.5 min),
301 apigenin-7-glucoside (34.5 min) and apigenin (54.6 min) by their retention time and
302 UV absorbance of purified standards. Butyl *p*-hydroxybenzoate was an internal
303 standard (IS). According to the plot of peak-area ratio (*y*) vs. concentration (*x*, μg/mL),
304 the regression equations of the three constituents and their correlation coefficients (*r*)
305 were determined as follows: luteolin-7-glucoside, $y = 0.0337x + 0.0239$ ($r^2 = 0.9964$);
306 apigenin-7-glucoside, $y = 0.0403x - 0.0623$ ($r^2 = 0.9976$); apigenin,
307 $y = 0.0594x - 0.1535$ ($r^2 = 0.9995$). The relative amounts of the three phenolic
308 compounds found in ECH and ACH were in the order of apigenin-7-glucoside (24.17
309 ± 0.52 mg/g) > luteolin-7-glucoside (8.22 ± 0.35 mg/g) > apigenin (0.64 ±
310 0.12 mg/g) and apigenin-7-glucoside (17.52 ± 0.14 mg/g) > luteolin-7-glucoside
311 (14.02 ± 0.38 mg/g) > apigenin (undetected), respectively.
312
313 **3.3. Inhibition of NO production.**
314 The anti-inflammatory activity of ACH, ECH and its reference compounds (luteolin
315 and apigenin) were studied *ex vivo*, analyzing their inhibitory effects on chemical

316 mediators released from macrophages. Once activated by inflammatory stimulation,
317 macrophages produce a large number of cytotoxic molecules. Treatment of RAW
318 264.7 macrophages with LPS (100 ng/mL) for 24 h induced NO production, as
319 assessed by measuring the accumulation of nitrite. As shown in Table 2, ACH, ECH,
320 luteolin and apigenin showed inhibition of LPS-induced NO production in RAW264.7
321 cells with an IC_{50} value of 407.82± 3.82, 104.06± 2.38, 38.47 ± 1.32 and 27.36 ± 1.58
322 μg/mL, respectively.
323
324 **3.4. λ-Carrageenan (Carr)-induced edema**
325 ECH (400 mg/kg) significantly inhibited the development of Carr-induced paw
326 edema after 5 h ($P < 0.001$) of treatment. Indo (10 mg/kg) significantly decreased
327 the Carr-induced paw edema after 3 h ($P < 0.01$), 4 h ($P < 0.001$) and 5 h of treatment
328 ($P < 0.001$) (Fig. 2).
329
330 **3.4.1. Effects of ECH on NO measurement**
331 ECH treatment (100, 200 and 400 mg/kg) significantly decreased the NO levels
332 in serum (Fig. 3A). Indo (10 mg/kg) significantly decreased the NO level in serum at
333 5 h post-Carr injection ($P < 0.001$). The NO levels were inhibited in ECH extract

group by 18.84, 38.81 and 53.85%, respectively, when compared to Carr group samples.

3.4.2. Effects of ECH on TNF-α level

ECH decreased the TNF-α level in serum at 5 h post-Carr injection (Fig. 3B). Indo (10 mg/kg) significantly decreased the TNF-α level in serum at 5 h post-Carr injection ($P < 0.001$). ECH treatment (100, 200 and 400 mg/kg) significantly inhibited the Carr-induced TNF-α level in comparison with the Carr group. The TNF-α levels were reduced to compare with the Carr group was 7.41%, 25.88%, and 42.69%, respectively.

3.4.3. Effects of ECH on MDA level measurements.

MDA levels in the edema paw induced by Carr were significantly higher. However, MDA levels were lowered significantly upon treatment with ECH, as well as 10 mg/kg Indo (Fig. 4). ECH treatment (100, 200 and 400 mg/kg) significantly inhibited the Carr-induced MDA level in comparison with the Carr group. The MDA levels were reduced in the Carr group by 33.04, 43.05 and 54.29%, respectively.

3.4.4. Histological examination.

352 Paw biopsies of control animals showed marked cellular infiltration in the
353 connective tissue. The infiltrates accumulated between collagen fibers and into
354 intercellular spaces. Paw biopsies of animals treated with the ECH (400 mg/kg)
355 showed a reduction in Carr-induced inflammatory response. Actually inflammatory
356 cells were reduced in numbers and were confined to near the vascular areas.
357 Intercellular spaces did not show any cellular infiltrations. Collagen fibers were
358 regular in shape and showed a reduction of intercellular spaces. Moreover the
359 hypodermal connective tissue was not damaged (Fig. 5). Neutrophil levels were
360 significantly increased with Carr treatment ($P < 0.001$). Indo and ECH (400 mg/kg)
361 could decrease the neutrophils numbers as compared to the Carr-treated group ($P <$
362 0.001 or $P < 0.01$), significantly (Fig. 5E).
363
364 **3.4.5. Effects of ECH on the activities of antioxidant enzymes**
365 At the 5 h following the intrapaw injection of Carr, liver tissues were also
366 analyzed for the biochemical parameters such as CAT, SOD and GPx activities (Table
367 3). CAT activity of the livers was significantly higher in ECH (400 mg/ kg) treatment
368 group as well as in Indo (10 mg/kg) group. SOD activity in liver tissue was decreased
369 significantly by Carr administration. SOD activity was significantly higher in ECH
370 (200 and 400 mg/kg) treatment group as well as Indo (10 mg/kg) group. Carr

administration markedly lowered CAT activity in the livers. GPx activity in the liver tissue was decreased significantly by Carr administration. GPx activity of the livers was significantly higher in ECH (400 mg/ kg) treatment group as well as in Indo (10 mg/kg) group.

4. Discussion

The DPPH or ABTS have been popular radical scavenging tests for natural products. Free radicals could induce biological damage and pathological events, such as inflammation, aging, and carcinogenesis (Halliwell, 1999). In this study, ECH showed significant antioxidant activities. The HPLC chromatogram of ECH demonstrated three phenolic components identified as apigenin, apigenin-7-glucoside and luteolin-7-glucoside (Fig. 1). The higher radical scavenging activity of ECH than that of ACH seems to be closely correlated with its polyphenolic constituents though other active components in the ECH could also play important roles in its antioxidative effect. Apigenin, a flavonoid, is a potent antioxidant (Sharififar et al., 2009). Apigenin-7-glucoside and luteolin-7-glucoside were shown as main active compounds, implicated as anti-inflammatory and antioxidative (Miceli et al., 2005). Apparently, these marker compounds in ECH could contribute to its antioxidant effects. They could account for the high anti-inflammatory and antioxidative of ECH.

Phenolic compounds have been reported to be beneficial in the treatment of chronic inflammatory diseases associated with overproduction of NO (Jiang and Dusting, 2003). Anti-inflammatory activity was evaluated using LPS-stimulated

393 RAW264.7 macrophages. Stimulation of RAW 264.7 macrophages by LPS induces
394 iNOS and overproduction of NO. NO is considered to play a key role in the
395 inflammatory response, based on its occurrence at inflammatory sites and its ability to
396 induce many of the hallmarks of the inflammatory response. The beneficial effects of
397 ACH and ECH on inhibition of the production of inflammatory mediators in
398 macrophages could be mediated through oxidative degradation of the products of
399 phagocytes, such as O^{2-} and HOCl. These results show that ECH possess better
400 anti-inflammatory activity than that of ACH (Table 2). The HPLC chromatogram for
401 ECH demonstrates three phenolic components identified as luteolin-7-glucoside,
402 apigenin-7-glucoside, and apigenin. Several articles have discussed the
403 anti-inflammatory activities of flavonoids, particularly that of apigenin and luteolin.
404 Few data are available for the glucopyranoside of apigenin-7-glucoside and
405 luteolin-7-glucoside; that showed significant activity on NO production inhibition.
406 Apigenin-7-glucoside showed an IC_{50} value of 25 μg/mL (Conforti et al., 2010), while
407 luteolin-7-glucoside showed an IC_{50} value of 20 μg/mL (Hu and kitts, 2004). These
408 phenolic components in the ECH could also play important roles in anti-inflammatory
409 activity.

410 *In vivo* anti-inflammatory activity, Carr-induced edema test, is highly sensitive to
411 nonsteroidal anti-inflammatory drugs, and has long been accepted as a useful
412 phlogistic tool for investigating new anti-inflammatory drugs (Just et al., 1998).

413 The L-arginine–NO pathway has been proposed to play an important role in the
414 Carr-induced inflammatory response (Salvemini et al., 1996). The expression of the
415 inducible isoform of NO synthase has been proposed as an important mediator of
416 inflammation (Cuzzocrea et al., 1997). In our study, ECH at 100, 200, and 400 mg/kg

417 significantly decreased the levels of NO in serum, indicating that ECH elicits an
418 anti-inflammatory response via the L-arginine–NO pathway (Fig. 3A). The
419 Carr-induced inflammatory response has been linked to neutrophil infiltration and the
420 production of neutrophil-derived free radicals, such as hydrogen peroxide, superoxide
421 and hydroxyl radicals, as well as the release of other neutrophil-derived mediators
422 (Dawson et al., 1991). Researchers demonstrated that inflammatory effect induced by
423 Carr is associated with free radical. Free radical, prostaglandin and NO will be
424 released when administrating with Carr for 1–6 h. The edema effect was raised to the
425 maximum at the third hour (Dudhgaonkar et al., 2006). Janero (1990) demonstrated
426 that MDA production is due to free radical attack plasma membrane (Janero et al.,
427 1990). Thus, inflammatory effect would result in the accumulation of MDA.
428 Enhances the level of glutathione conducive toward favor reduces MDA the
429 production. It was suggested that endogenous glutathione plays an important role
430 against Carr-induced local inflammation (Cuzzocrea et al., 1999). In this study, there
431 was a significant increase in CAT, SOD and GPx activities with ECH treatment.
432 Furthermore, there was a significant decrease in MDA level with ECH treatment. We
433 assume the suppression of MDA production is probably due to the increases of CAT,
434 SOD, and GPx activities.

435 TNF-α is a major mediator in inflammatory responses. It induces innate immune
436 responses by activating T cells and macrophages, and stimulates secretion of other
437 inflammatory cytokines (Veterichelvan et al., 2000; Yun et al., 2008). Also, TNF-α is
438 a mediator of carr-induced inflammatory incapacitation, and is able to induce the
439 further release of kinins and leukotrienes, which are suggested to play an important
440 role in the maintenance of long-lasting nociceptive response (Dawson, 1991; Kumar

et al., 2010). In this study, we found ECH lowered TNF-α level in serum after Carr injection (Fig. 3B).

. In summary, this study shows that ECH possesses antioxidant and anti-inflammatory activities, which supports its ethnopharmacological use. Moreover, ECH and its reference compounds (luteolin-7-glucoside, apigenin-7-glucoside, and apigenin) could contribute to the antioxidant and anti-inflammatory activities. We suggest that the mechanisms of ECH may be associated with the inhibition of inflammatory mediator overproduction, including NO and TNF-α. These findings suggest that ECH may be therapeutically useful for mitigating inflammatory pain.

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