1 Running Title: Antioxidant and anti-inflammatory properties of *Dichondra repens*

2

3 # Antioxidant and Anti-inflammatory properties of *Dichondra repens*
4 # Forst. and its reference compounds

5

6 Ming-Jyh Sheu[a], Ming-Hsing Huang[b], Chieh-Hsi Wu[a], Shyh-Shyun Huang[c],

7 Guan-Jhong Huang[c,*]

8 [a]*School of Pharmacy, College of Pharmacy, China Medical University, Taichung 404, ,*
9 *Taiwan*

10 [b]*Department of Cosmetic Science, Chia-Nan University of Pharmacy and Science,*
11 *Tainan 717, Taiwan*

12 [c]*School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources,*
13 *College of Pharmacy, China Medical University, Taichung 404, Taiwan;*

14

15 [*] **Corresponding author**

16 Dr. Guan-Jhong Huang

17 *School of Chinese Pharmaceutical Sciences and Chinese Medicine Resources,*

18 *College of Pharmacy, China Medical University, Taichung 404, Taiwan;*

19 Tel.: +886 4 2205 3366 ext 5508

20 Fax: +886 4 2208 3362.

21 E-mail address: gjhuang@mail.cmu.edu.tw

22

23 ABSTRACT

24 *Dichondra repens* (DR) is the main constituents in herbal beverage and
25 consumed daily as a nutrition supplement for the liver in Taiwan. The aims of this
26 study investigated the antioxidant in *in vitro* and anti-inflammatory effects of ethanol
27 extract of *Dichondra repens* Forst. (EDR) in *ex vivo* and *in vivo*. Fingerprint
28 chromatogram from HPLC indicated that EDR contains vanillin, umbelliferone and
29 scopoletin. The effects of EDR was evaluated for the antioxidant and LPS-induced
30 NO production in RAW264.7 cells. EDR decreased the LPS-induced NO production
31 and expressions of iNOS and COX-2 in RAW264.7 cells. *In vivo* anti-inflammatory
32 activities of EDR were assessed in mouse paw edema induced by λ-carrageenan
33 (Carr). We investigate the anti-inflammatory mechanism of EDR via studies of the
34 activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase
35 (GPx) in the liver and the levels of malondialdehyde (MDA) and nitrite oxide (NO) in
36 the edematous paw. Serum NO and TNF-α were also measured. EDR exerts
37 anti-inflammatory effects by suppressing TNF-α, NO, and might be related to the
38 decrement of the level of MDA in the edema paw via increasing the activities of CAT,
39 SOD, and GPx in the liver. The results showed that EDR might be a natural
40 antioxidant and anti-inflammatory agent.

41 *Keywords*: *Dichondra repens*; Antioxidant; Anti-inflammation; NO; TNF-α; MDA;

42

43 **1. Introduction**

44 Recent data have shown that inflammation is a critical component of tumor
45 progression. Many cancers arise from sites of infection, chronic irritation and
46 inflammation. The inflammatory process involves multiple physiological systems
47 with the immune system playing a central role (Coussens & Werb, 2002). Chronic
48 inflammation results in up-regulation of many enzymes and signaling proteins in
49 affected areas. These proinflammatory enzymes include the inducible forms of nitric
50 oxide synthase (iNOS) and cyclooxygenase-2 (COX-2). iNOS and COX-2 are
51 responsible for elevated levels of nitric oxide (NO) and prostaglandins (PGs),
52 respectively (Heiss, Herhaus, Klimo, Bartsch, & Gerhauser, 2001). Excessive
53 production of NO by iNOS or interaction of NO with other oxidants (*e.g.*,
54 peroxynitrite formation with superoxide anion) leads to its pro-inflammatory activities.
55 Study indicates that the chronic inflammation correlates with an increase in iNOS
56 activity (Lerouet, Beray-Berthat, Palmier, Plotkine, & Margaill, 2002). The most
57 significant evidence for NO as a mediator of tissue injury has been obtained from
58 studies on an animal arthritis model, human osteoarthritis, and rheumatoid arthritis

59 (Cochran, Selph, & Sherman, 1996). It has been demonstrated that the metabolism of
60 arachidonic acid by either cyclooxygenase or lipoxygenase pathway, generates
61 eicosanoids including PGs, thromboxanes and leukotrienes (LTs). Notably, PGs
62 and LTs are involved in angiogenesis, apoptosis, hyperproliferation,
63 immunosuppression and invasiveness (Jones, Adel-Alvarez, Alvarez, Broaddus, &
64 Das, 2003). COX has been found in two isoforms and COX-2 is an inducible form
65 responsible for the production of large amounts of pro-inflammatory PGs at the
66 inflammatory site (Weisz, Cicatiello, & Esumi, 1996). Tumor necrosis factor
67 (TNF)-α plays an important role in immunity and inflammation. TNF-α activates
68 vascular endothelium and increases vascular permeability, which leads to increased
69 entry of IgG, complement, and cells to tissues and increased fluid drainage to lymph
70 nodes as local effects. Numerous studies have indicated that NO and PGs participate
71 in inflammatory and nociceptive events. Inhibition of NO and PGs production via the
72 inhibition of iNOS and COX-2 expression is beneficial for treating inflammatory
73 diseases (Bogdan, 2001).

74 Dietary, vitamin supplements and other biological products from traditional Chinese
75 herbs are becoming increasingly popular in many medical situations, particularly among
76 patients with cancer. These nutrition supplements and biological products are mostly
77 used to reduce side effects, organ toxicity, protect and stimulate immunity, or to prevent

78 further cancer recurrences (Von Gruenigen, White, Kirven, Showalter, Hopkin, &

79 Jenison, 2001). *Dichondra repens* (DR) was widely used as an astringent,

80 anti-inflammation, diabetes and hypertension (Hsieh, 2004) in Taiwan. DR is frequently

81 added to herbal tea beverage and consumed daily by the local people as a tonic nutrition

82 for the liver. However, its antioxidant and anti-inflammatory effects has rarely been

83 investigated. In this study, the antioxidant and anti-inflammatory effects of ethanol

84 extract of *Dichondra repens* (EDR) were examined *ex vivo*. We also evaluated the

85 anti-inflammatory effects of EDR on paw edema induced by Carr in mice and

86 investigated the underlying mechanisms *in vivo*.

87 **2. Materials and methods**

88 *2.1. Materials*

89 Glutathione (GSH), 1, 1-diphenyl-2-picrylhydrazyl radicals (DPPH),

90 lipopolysaccharide (LPS; endotoxin from *Escherichia coli*, serotype 0127:B8), 2,

91 2'-azinobis-(3-ethylbenzothiazoline)-6- sulphonic acid (ABTS), butylated

92 hydroxytoluene *(*BHT*)*, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide

93 (MTT), λ-Carrageenan (Carr) and indomethacin (Indo) were obtained from

94 Sigma-Aldrich (St. Louis, MO, USA). Deionized water from a Milli-Q system

95 (Millipore, Bedford, MA, USA) was used to prepare all buffers and sample solutions.

96 *Dichondra repens* was collected from Taichung (Taiwan), identified and
97 authenticated by Dr. Shyh-Shyun Huang of the Institute of Chinese Pharmaceutical
98 Science, China Medical University, Taichung, Taiwan. Voucher specimens were
99 deposited at Institute of Chinese Pharmaceutical Science, China Medical University,
100 Tiachung, Taiwan. The purity of three marker standards was judged by a photodiode
101 array detector (Hitachi L-7455).

102 *2.2. Preparation of* ethanol extracts of *Dichondra repens*

103 Dried whole herb (100g each) was macerated with 1L 95% ethanol for 24 h at
104 room temperature. Filtration and collection of the extract were done three times. Then
105 the ethanol extract was evaporated to 10 mL and dried in vacuum at 40℃. The dried
106 extract was weighted and dissolved in 95% ethanol and stored in -20°C for further use.
107 The yield of the aqueous (ADR) and ethanol extracts (EDR) of *Dichondra repens*
108 obtained were 3.19% (W/W) and 5.03% (W/W), respectively.

109 *2.3. High performance liquid chromatography (HPLC) analysis*

110 HPLC was performed with a Hitachi Liquid Chromatograph (Hitachi Ltd.,
111 Tokyo, Japan), consisting of two model L-7100 pumps, and one model L-7455
112 photodiode array detector (254 nm). Samples of EDR (50 mg/mL) was filtered
113 through a 0.45 μm filter and injected into the HPLC column. The injection volume

114 was 20 μL and the flow rate was 0.8 ml/min. The separation temperature was 25 ℃.

115 The column was a Mightysil RP-18 GP (5 μm, 250×4.6 mm I.D.; Kanto Corporation,

116 Portland, OR, USA). The method involved the use of a binary gradient with mobile

117 phases containing: (A) phosphoric acid in water (0.1%, v/v) and (B)

118 $H_2O/CH_3CN/CH_3OH$ (2:4:4, v/v). The solvent gradient elution program was as

119 follows: 0-10 min, 100-75% A, 0-25% B; 10-15 min, 75-80% A, 25-20% B; 15-25

120 min, 80% A, 20% B; 25-40 min, 80-50% A, 20-50% B; 40-50 min, 50-30% A,

121 50-70% B; 50-60 min, 30-0% A, 0-100% B; and finally 60-70 min, 0% A, 100% B.

122 *2.4. Animals*

123 ICR mice (6–8 weeks male) were obtained from the BioLASCO Taiwan Co., Ltd.

124 The animals were kept in plexiglass cages at a constant temperature of 22 ± 1 ℃,

125 relative humidity 55 ± 5% with 12 h dark-light cycle for at least 2 weeks before the

126 experiment. They were given food and water *ad libitum*. All experimental procedures

127 were performed according to the NIH Guide for the Care and Use of Laboratory

128 Animals. This study was approved by the ethics committee of the Institutional Animal

129 Care and Use Committee (IACUC) of China Medical University. The control groups

130 were given 0.1 mL/10 g saline intraperitoneally using a bent blunted 27-gauge needle

131 connected to a 1 mL syringe.

132 *2.5. Cell culture and cell viability assay*

133 The murine macrophage RAW264.7 (1.0×10^6 cells/mL) cells were purchased
134 from Food Industry Research and Development Institute (Hsinchu, Taiwan) and
135 cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum,
136 streptomycin (100 μg/mL), and penicillin (100 U/mL) at 37℃ in a 5% CO_2
137 atmosphere. Cell viability was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,
138 5-diphenyltetrazolium bromide (MTT) assay. RAW264.7 cells (1.0×10^4 cells/mL)
139 were cultured in 96-well plates for 18 h, followed by treatment with LPS (100 ng/mL)
140 in the presence of various concentrations (0, 125, 250, 500, and 1,000 μg/mL) of EDR.
141 After 24 h incubation, MTT stock solution (50 μL; 2 mg/mL in PBS) was added to
142 the medium, and the medium was incubated for 4 hours. Then, the supernatant was
143 removed, and the obtained formazan crystals were dissolved in 200 μL of
144 dimethylsulfoxide (DMSO). Absorbance was measured at 540 nm. Percent of cells
145 showing cytotoxicity was determined relative to the control group.

146 *2.6. In vitro antioxidant activity of EDR*

147 *2.6.1. ABTS radical scavenging test*

148 ABTS was dissolved in distilled water to a final concentration of 7 mM,
149 containing 2.45 mM potassium persulphate. This solution was stored at room
150 temperature in the dark for 16 hours to become stable free radical green-blue $ABTS^{\bullet+}$.

151 Then it was diluted with ethanol under 734 nm, until the absorbance of $ABTS^{\bullet+}$

152 solution was at 0.75 ± 0.05. Different concentration standards of (trolox) 2 μL were

153 added into 20 μL ethanol, followed by 180 μL $ABTS^{\bullet+}$ solution. After the above

154 procedure, the absorbance was measured within one min at 734 nm. The sample

155 solution was detected by the same method. Trolox was used as a reference standard,

156 and results were expressed as TEAC values (mM). These values were obtained from

157 at least three different concentrations of each extract tested in the assay giving a linear

158 response between 20 and 80% of the blank absorbance. Moreover, all analyses were

159 done in triplicate (Huang et al., 2010).

160 *2.6.2. DPPH radical scavenging test*

161 An aliquot (20 μL) of EDR at various concentrations were mixed with 80 μL

162 Tris-HCl buffer (100 mM, pH 7.4) and then with 100 μL of the DPPH in ethanol to a

163 final concentration of 250 μL. The mixture was shaken vigorously and stood at room

164 temperature for 20 min in the dark. The absorbance at 517 nm of the reaction solution

165 was measured spectrophotometrically. The percentage of DPPH decolorization in the

166 samples was calculated according to the equation: % decolorization =

167 [1–(ABSsample/ABScontrol)] × 100. EC_{50} value was the effective concentration at

168 which DPPH radicals were scavenged by 50% and was obtained by interpolation from

169 linear regression analysis (Huang et al., 2010).

170 *2.6.3. Ferric reducing antioxidant property (FRAP)*

171 FRAP assay was done according to the protocol (Xu, Zhang, Cao, & Lu, 2010)
172 with some modifications. The stock solutions were 300 mM acetate buffer (pH 3.6),
173 TPTZ solution (10 mM TPTZ in 40 mM HCl) and 20 mM $FeCl_3 \cdot 6H_2O$ solution.
174 Working FRAP solution was prepared freshly by mixing 25 mL of acetate buffer,
175 2.5 mL TPTZ solution and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution, and then warmed to 37 ℃
176 before use. 150 μL of individual extract solutions (containing 25, 50, 100 and 200 μg
177 of extracts, respectively) was allowed to react with 2.85 mL of FRAP solution for
178 30 min in dark. Absorbance was read at 593 nm. Percentage Fe^{3+} reduction (to Fe^{2+})
179 were calculated by a $FeSO_4$ standard calibration curve. Percentage scavenging was
180 also evaluated in ascorbic acid equivalence (AAE) (in μg).

181 *2.6.4. Determination of antioxidant activity by lipid peroxidation assay*

182 Four mL linoleic acid emulsion was mixed with 20 μL sample extract of
183 different concentrations at 40°C in the dark. This mixture was added to 100 μL FTC
184 method solution, 200 μL of 25% TCA and 200 μL of 1% TBA. The solution was
185 maintained at 100°C for 10 min, and then centrifuged at 3,000 rpm for 20 min. The
186 absorbance was recorded at 532 nm (Huang et al., 2010).

187

188 *2.6.5. Determination of total polyphenol content*

189 Twenty μL of ADR and EDR (125 μg/mL) was added to 200 μL distilled water
190 and 40 μL of Folin-Ciocalteu reagent. The mixture was allowed at room
191 temperature for 5 min and then 40 μL of 20% sodium carbonate was added to the
192 mixture. The resulting blue complex was then measured at 680 nm. Catechin was used
193 as a standard for the calibration curve. The polyphenol content was calibrated using
194 the linear equation based on the calibration curve. Total polyphenol content was
195 expressed as mg catechin equivalent (CE)/g dry weight (Xu, Zhang, Cao, & Lu,
196 2010).

197 *2.7. Effects of EDR on lipopolysaccharide (LPS)-induced cell viability of RAW 264.7*
198 *cell lines*

199 Cytotoxic effects of compounds were evaluated by the MTT assay. Briefly, the
200 cells were stimulated with 100 ng/mL of LPS in the absence or presence of EDR (125,
201 250, 500 and 1,000 μg/mL) for 24 h. EDR was added 1 h. before incubation with
202 LPS.

203 *2.8. Inhibition of iNOS and COX-2 protein expressions by EDR in LPS-induced RAW*
204 *264.7 cells*

205 Cells were incubated with LPS (100 ng/mL) and various concentrations of EDR
206 (125, 250, 500, and 1,000 μg/mL). EDR was added 1 h before incubation with LPS.
207 The cells were collected, then lysed in a lysis solution followed by incubation at 95°C
208 for 5 min. Total proteins were separated using SDS-PAGE before being transferred to
209 PVDF membranes, blocked with 5% (v/v) nonfat dry milk in PBS-Tween 20 and
210 probed with the desired antibody iNOS and COX-2 (Santa Cruz, USA) overnight at
211 4°C. The blots were then incubated with horseradish peroxidase-linked secondary
212 antibody for 1 h followed by development with the electrochemoluminsence (ECL)
213 reagent and exposure to Hyperfilm (Amersham, Arlington Height, IL, USA). The data
214 were analyzed by Gel-Logic 200 Imaging Systems, Molecular Imaging Software.

215 *2.9. λ-Carrageenan (Carr)-induced edema*

216 Carr-induced hind paw edema model was used for determination of
217 anti-inflammatory activity (Winter, Risley, & Nuss, 1962). After a 2-week adaptation
218 period, male ICR mice (18–25 g) were randomly assigned to six groups ($n = 8$)
219 including Carr, positive Indo control and three EDR-treated groups. Carr group
220 received 1% Carr (50 μL). EDR at doses of 0.1, 0.5, and 1.0 g/kg were orally
221 administered 2 h before the injection with 1% Carr (50 μL) in the plantar side of right
222 hind paws of the mice. And Indo (10 mg/kg) was orally administered 90 min before

223 the injection with 1% Carr (50 μL) in the plantar side of right hind paws of the mice.
224 Paw volume was measured immediately after Carr injection at 1, 2, 3, 4, and 5 h
225 intervals using a plethysmometer (model 7159, Ugo Basile, Varese, Italy). The degree
226 of swelling induced was evaluated by the ratio a/b, where a was the volume of the
227 right hind paw after Carr treatment, and b was the volume of the right hind paw before
228 Carr treatment. Indo was used as a positive control.

229 Therefore, the right hind paw tissue and liver tissue were dissected at the 5 h.
230 The right hind paw tissue was rinsed in ice-cold normal saline, and immediately
231 placed in cold normal saline four times their volume and homogenized at 4 ℃. Then
232 the homogenate was centrifuged at 12,000×g for 5 min. The supernatant was obtained
233 and stored at −20 ℃ refrigerator for MDA assays. The whole liver tissue was rinsed
234 in ice-cold normal saline, and immediately placed in cold normal saline one time their
235 volume and homogenized at 4 ℃. Then the homogenate was centrifuged at 12,000g
236 for 5 min. The supernatant was obtained and stored in the refrigerator at −20 ℃ for
237 the antioxidant enzymes (CAT, SOD, and GPx) activity assays. Also, blood was
238 withdrawn and kept at −80 °C for NO and TNF-α assay. The protein concentration of
239 the sample was determined by the Bradford dye-binding assay (Bio-Rad, Hercules,
240 CA).

241 *2.9.1. Determination of nitric oxide (NO)*

242 Nitrite, a stable endproduct of NO, was then measured using the Griess reaction.
243 100 μL aliquots of sample mixed with 100 μL of Griess reagent (0.1% N-(1-naphthyl)
244 ethylenediamide dihydrochloride, 1% sulfanilamide in 5% phosphoric acid), followed
245 by spectrophotometric measurement at 550 nm. Nitrite concentrations in the
246 supernatants were determined by comparison with a sodium nitrite standard curve.

247 *2.9.2. Measurement of serum TNF-α by ELISA*

248 Serum levels of TNF-α were determined using a commercially available enzyme
249 linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.
250 TNF-α was determined from a standard curve for the cytokine. The concentrations
251 were expressed as pg/mL.

252 *2.9.3. MDA assay*

253 MDA was evaluated by the thiobarbituric acid reacting substances (TBARS)
254 method (Ohishi, Ohkawa, Miike, Tatano, & Yagi, 1985). Briefly, MDA reacted with
255 thiobarbituric acid in the acidic high temperature and formed a red-complex TBARS.
256 The absorbance of TBARS was determined at 532 nm.

257 *2.9.4. Antioxidant enzymes activity measurements*

258 The following biochemical parameters were analyzed to check the
259 hepatoprotective activity of EDR by the methods given below.

260 Total SOD activity was determined by the inhibition of cytochrome *c* reduction.
261 The reduction of cytochrome *c* was mediated by superoxide anions generated by the
262 xanthine/xanthine oxidase system and monitored at 550 nm. One unit of SOD was
263 defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction
264 by 50%. In brief, the reduction of 10 mM H_2O_2 in 20 mM of phosphate buffer (pH 7)
265 was monitored by measuring the absorbance at 240 nm. The activity was calculated
266 using a molar absorption coefficient, and the enzyme activity was defined as
267 nanomoles of dissipating hydrogen peroxide per milligram protein per minute. Total
268 GPx activity in cytosol was determined according to previously paper (Paglia &
269 Valentine, 1967). The enzyme solution was added to a mixture containing hydrogen
270 peroxide and glutathione in 0.1 mM Tris buffer (pH 7.2) and the absorbance at 340
271 nm was measured. Activity was evaluated from a calibration curve, and the enzyme
272 activity was defined as nanomoles of NADPH oxidized per milligram protein per
273 minute.

274 *2.10. Statistical analysis*

275 Data are expressed as mean ± S.E.M. Statistical evaluation was carried out using
276 one-way analysis of variance (ANOVA followed by Scheffe's multiple range tests).
277 The criterion for statistical significance is $P < 0.05$.

278

279 **3. Results.**

280 *3.1. Fingerprint chromatogram of EDR extracts by HPLC*

281 The free radical scavenging activities of *Dichondra repens* were contributed to
282 various natural polyphenolics including vanillin, umbelliferone, and scopoletin (Liu,
283 Liang, Zhang, Wu, Xu, & Luo, 2002). Vanillin, umbelliferone, and scopoletin were
284 selected as three marker compounds for HPLC chromatographic fingerprint analysis
285 of *Dichondra repens*, and the chromatogram was shown as in Fig. 1. Based on the
286 plots of the peak-area (y) vs. concentration (x, μg/mL), the regression equations of the
287 three phenolic constituents and their correlation coefficients (r^2) were as follows:
288 vanillin, $y = 0.0157x - 0.0046$ ($r^2 = 0.996$); umbelliferone, $y = 0.0073x + 0.0031$ ($r^2 =$
289 0.999); and scopoletin, $y = 0.0218x + 0.0045$ ($r^2 = 0.989$). In a comparison of the
290 amounts of the three polyphenolic compounds present in the *Dichondra repens*,
291 umbelliferone (24.5 mg/g extract) >scopoletin (4.1 mg/g extract) > vanillin (0.3 mg/g
292 extract). Total flavonoids (49.6±0.79 mg/g extract), total flavonols (14.45±0.35 mg/g

293 extract) and total polyphenolics (186.58±7.37 mg/g extract) in the EDR were
294 determined.

295 *3.2. Antioxidant Assay.*

296 In DPPH radical scavenging activity assay, our results indicated that EC_{50}
297 values of ADR and EDR were 198.54 ± 0.79 and 188.53 ± 0.26 μg/mL, respectively.
298 This result suggested that EDR had better antioxidant activity in DPPH scavenging
299 ability (Table 1). ABTS assay was expressed as trolox equivalent antioxidant activity
300 (TEAC) values. Higher TEAC value represented that sample had a stronger
301 antioxidant activity. Our results showed that EDR (107.02 ± 2.61 μM/mg) had higher
302 antioxidant potentials than ADR (93.86 ± 0.96 μM/mg) (Table 1). In ferric reducing
303 antioxidant power assay (FRAP), it was observed that EDR (4.14 ± 0.02 μmol
304 Fe^{2+}/mg) had higher antioxidant potentials than ADR (3.23 ± 0.03 μmol Fe^{2+}/mg)
305 (Table 1). To further verify the antioxidant activity of EDR correlates with its three
306 polyphenolic constituents, vanillin, umbelliferone, and scopoletin were conducted on
307 ABTS, FRAP, and DPPH assays. Our results demonstrate that vanillin, umbelliferone,
308 and scopoletin showed significant radical scavenging activity (Table 1).

309 *3.3. Effects of EDR on LPS-induced cell viability, iNOS and COX-2 expressions*

310 To examine whether EDR has any toxicity to RAW264.7 cells, cell viability of

311 LPS-treated RAW264.7 cells at various concentrations of EDR was performed. EDR
312 at various doses (125, 250, 500, and 1,000 μg/mL) did not affect the LPS-induced
313 RAW264.7 macrophages cell viability (Fig. 2A). EDR at doses 250, 500 and 1,000
314 μg/mL decrease the NO levels (Fig. 2B) in RAW 264.7 macrophages. Also, EDR at
315 doses 500 and 1,000 μg/mL significantly inhibits LPS-induced iNOS and COX-2
316 protein expressions in RAW 264.7 macrophage cells (Fig. 2C,D). Further experiments
317 were conducted to determine the NO production from EDR, vanillin, umbelliferone,
318 and scopoletin. Our results demonstrate that EDR, vanillin, and scopoletin showed
319 significant inhibition of NO production. Inhibition of NO production (IC_{50}) in
320 LPS-induced RAW 264.7 macrophages by EDR and its reference compounds were
321 demonstrated as EDR (635.21 ± 3.15 μg/mL), vanillin (12.02 ± 0.25 μg/mL),
322 scopoletin (18.45 ± 0.11 μg/mL), umbelliferone (21.69 ± 0.21 μg/mL), and
323 indomethacin (Indo) (65.32 ± 0.12 μg/mL) (Table 1).

324 *3.4. λ-Carrageenan (Carr)-induced edema*

325 EDR (1.0 g/kg) significantly inhibited the development of carr-induced paw
326 edema after 5 h of treatment. Indo (10 mg/kg) significantly decreased the carr-induced
327 paw edema after 2 ($P < 0.01$), 3 ($P < 0.01$), 4 ($P < 0.001$), and 5 h of treatment
328 ($P < 0.001$) (Fig. 3A).

329 *3.4.1. Effects of EDR on NO measurement in serum*

330 EDR (0.5 and 1.0 g/kg) significantly decreased the NO levels in serum (Fig. 3B)
331 at the 5 h after Carr injection. Indo (10 mg/kg) significantly decreased the NO level in
332 serum at the 5 h after Carr injection ($P < 0.001$).

333 *3.4.2. Effects of EDR on TNF-α level*

334 EDR (0.1, 0.5, and 1.0 g/kg) decreased the TNF-α level in serum at the 5 h after
335 Carr injection (Fig. 3C). Indo (10 mg/kg) significantly decreased the TNF-α level in
336 serum at the 5 h after Carr injection ($P < 0.001$). The inhibition rates TNF-α levels
337 compared with the Carr group are 17.71%, 38.47%, and 41.71% at the 0.1, 0.5 and 1.0
338 g/kg of EDR, respectively.

339 *3.4.3. Effects of EDR on MDA level*

340 MDA level in the edematous paw induced by Carr was significantly increased.
341 However, MDA level was decreased significantly by treatment with EDR (0.5 and 1.0
342 g/kg), as well as 10 mg/kg Indo (Fig. 3D). The inhibition rates MDA levels compared
343 with the Carr group are 6.34%, 16.56%, and 28.16% at the 0.1, 0.5, and 1.0 g/kg of
344 EDR, respectively.

345 *3.4.4. Effects of EDR on histological appearance*

346 Histological appearance of the mouse hind footpad after a subcutaneous injection
347 with 0.9% saline (control group) or Carr stained with H&E stain. Control rats show
348 the normal appearance of dermis and subdermis without any significantly lesion (Fig.
349 4A). Hemorrhage with moderately extravascular red blood cell and large amount of
350 inflammatory leukocyte mainly neutrophils infiltration in the subdermis interstitial
351 tissue of mice following the subcutaneous injection of Carr only. Moreover, detail of
352 the subdermis layer show enlargement of the interstitial space caused by edema with
353 exudates fluid (Fig. 4B). Indo significantly reduced the level of hemorrhage, edema
354 and inflammatory cell infiltration compared to subcutaneous injection of Carr only
355 (Fig. 4C). EDR significantly show morphological alterations compared to
356 subcutaneous injection of carr only. The numbers of neutrophils were counted in
357 each scope and thereafter obtain their average count from 5 scopes of every
358 tissue slice (Fig. 4D & 4E).

359 *3.4.5. Effects of EDR on the activities of antioxidant enzymes*

360 In Table 2, data showed that at the 5 h following the intrapaw injection of Carr,
361 liver tissues were also analysed for the biochemical parameters such as CAT, SOD
362 and GPx activities (Table 2). CAT and SOD activity in liver tissue was decreased
363 significantly by Carr administration. CAT activity was increased significantly after

364 treated with 1.0 g/kg EDR ($P < 0.05$) and 10 mg/kg Indo ($P < 0.001$). SOD activity
365 was increased significantly after treated with 0.5 g/kg ($P < 0.05$) and 1.0 g/kg
366 ($P < 0.01$) EDR and 10 mg/kg Indo ($P < 0.001$). Carr administration markedly
367 decreased GPx activity in the livers. GPx activity of the livers was increased
368 significantly by EDR (0.5 and 1.0 g/kg), as well as Indo (10 mg/kg).

369

370 **4. Discussion**

371 HPLC was used to quantify the components of ethanol extract of *Dichondra*
372 *repens*. The fingerprint chromatograms demonstrate vanillin, umbelliferone, and
373 scopoletin as its ingredients. Other study indicates that the free radical scavenging
374 activities of EDR were contributed to various natural polyphenolics including vanillin,
375 umbelliferone, and scopoletin (Liu, Liang, Zhang, Wu, Xu, Luo, 2002). Vanillin
376 (2-hydroxy-3-methoxybenzaldehyde) inhibited LPS-stimulated nuclear factor kappa B
377 (NF-κB) activation and COX-2 gene expression in RAW264.7 murine macrophage
378 cell line (Murakami et al., 2007). Umbelliferone significantly demonstrated
379 anti-inflammatory and antioxidant effects (Singh, Singh, Singh, Kumar, Kumar, &
380 Arora, 2010). Scopoletin, the phenethyl alcohol derivatives showed COX-2/5-LOX
381 dual inhibitory activity and suppressed the production of pro-inflammatory cytokines

382 and exerted inhibitory activity on LPS-induced PGE_2 production through the
383 depression of COX-2 expression (Kim et al., 2006). Our results also demonstrated that
384 1 g of dry weight of EDR contains polyphenol amounts equal to 186.58±7.37 mg
385 CE/g, respectively. This result confirms that total polyphenol contents of natural
386 products are regular indices of their antioxidant activity. Therefore, we aimed to
387 investigate the effect of EDR on oxidative and inflammatory stress in RAW264.7
388 macrophages and ICR mice, and further investigate possible signals related with its
389 anti-inflammatory effects.

390 ABTS assay are often used in evaluating antioxidant power of single compound
391 and complex mixtures of various plants. In this assay, ABTS radical monocation was
392 generated directly in stable form from potassium peroxodisulfate. Generations of
393 radical before the antioxidants were added to prevent the interference of compounds,
394 which affected radical formation. This modification made the assay less susceptible to
395 artifacts and prevented overestimation of antioxidant power (Long, Kwee, &
396 Halliwell, 2000). ABTS assay was expressed as TEAC value. The TEAC values for
397 EDR and ADR indicated that EDR had higher antioxidant potencies than ADR (Table
398 1). Both FRAP and TEAC assay were used to estimate the total antioxidant power
399 because they were quick and simple to perform, and the reaction was reproducible and
400 linearly related to the molar concentration of the antioxidants (Xu, Zhang, Cao, & Lu,

401 2010). The FRAP values also indicated that EDR had higher antioxidant potencies
402 than ADR (Table 1). DPPH• is used to study free radical scavenging effects of natural
403 materials, such as polyphenolic compounds, anthocyanins and some extracts from
404 traditional chinese medicine. However, a decrease or an absence of absorbance was
405 observed after adding antioxidant reagents (Xu, Zhang, Cao, & Lu, 2010). The EC_{50}
406 values of five different concentrations from EDR and ADR were determined. It
407 suggested that EDR and ADR share similar antioxidant activity in DPPH scavenging
408 ability (Table 1). The experiment were also conducted to verify that three phenolic
409 constituents demonstrate antioxidant activities through ABTS, FRAP and DPPH
410 assays (Table 1).

411 Carr-induced edema test is highly sensitive to nonsteroidal anti-inflammatory
412 drugs, and has long been accepted as a useful phlogistic tool for investigating new
413 anti-inflammatory drugs (Just et al., 1998). The degree of swelling of the carr-injected
414 paws was maximal 3 h after injection and the mean increase in volume at that time
415 was about 100% in the control group. Statistical analysis revealed that EDR (1.0 g/kg)
416 significantly inhibited the development of carr-induced paw edema after 5 h of
417 treatment (Fig. 3A).

418 The L-arginine–NO pathway has been proposed to play an important role in the
419 carr-induced inflammatory response (Salvemini, Wang, Bourdon, Stern, Currie, &

420 Manning, 1996). The expression of the inducible isoform of NO synthase has been
421 proposed as an important mediator of inflammation (Cuzzocrea, Costantino,
422 Zingarelli, Mazzon, Micali, & Caputi, 1999). In our study, EDR significantly decrease
423 the levels of NO in cells (Fig. 2B) and edematous serum (Fig. 3B). We also
424 demonstrate that EDR at 500 and 1,000 μg/mL significantly decrease the iNOS
425 protein expressions (Fig. 2C). Inhibition of NO production (IC_{50}) in LPS-induced
426 RAW 264.7 macrophages by EDR and its reference compounds were demonstrated as
427 EDR (753.31±5.12 μg/mL), vanillin (12.02±0.25 μg/mL), scopoletin (18.45 ± 0.11
428 μg/mL) and Indomethacin (65.32 ± 0.12 μg/mL). This could indicate that EDR elicits
429 an anti-inflammatory response via the L-arginine–NO pathway.

430 In addition, COX-2-mediated PGE_2 production from LPS-treated RAW 264.7
431 cells has been involved in the progression of inflammation. In RAW 264.7 cells, we
432 demonstrate that EDR inhibits COX-2 protein expression (Fig. 2C). This might
433 explain that EDR could have an anti-inflammatory effect the regulation of COX-2
434 expression.

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 (Beutler & Cerami, 1989). Also, TNF-α is a mediator of

438 carr-induced inflammatory incapacitation, and is able to induce the further release of

439 kinins and leukotrienes, which are suggested to play an important role in the

440 maintenance of long-lasting nociceptive response (Tonussi & Ferreira, 1999). In this

441 study, we found EDR decrease the TNF-α level in serum after Carr injection (Fig.

442 3C).

443 The Carr-induced inflammatory response has been linked to neutrophil

444 infiltration and the production of neutrophil-derived free radicals, such as hydrogen

445 peroxide, superoxide and hydroxyl radicals, as well as the release of other

446 neutrophil-derived mediators (Dawson, Sedgwick, Edwards, & Lees, 1991). Some

447 researches demonstrate that inflammatory effect induced by Carr is associated with

448 free radical. Free radical, prostaglandin and NO will be released when administrating

449 with Carr for 1–6 h (Dudhgaonkar, Tandan, Bhat, Jadhav, & Kumar, 2006). The

450 edema effect was raised to the maximum at the 3 h (Kirkova, Kassabova, & Russanov,

451 1992). It was demonstrated that MDA production is due to free radical attack plasma

452 membrane (Janero, 1990). Thus, inflammatory effect would result in the accumulation

453 of MDA. Glutathione is a known oxyradical scavenger. Enhances the level of

454 Glutathione conducive toward favor reduces MDA the production. Cuzzocrea

455 suggested that endogenous glutathione plays an important role against Carr-induced

456 local inflammation (Cuzzocrea, Costantino, Zingarelli, Mazzon, Micali, & Caputi,

1999). In this study, there is significantly increased in CAT, SOD, and GPx activities with EDR treatment (Table 2). Furthermore, there is a significant decrease in MDA level with EDR treatment (Fig. 3D). We assume the suppression of MDA production is probably due to the increases of CAT, SOD, and GPx activities.

We propose that EDR elicits an antioxidant and anti-inflammatory activity. We suggest that the mechanisms of EDR may be associated with the inhibition of inflammatory mediator overproduction, including NO and TNF-α. The anti-inflammatory mechanism of EDR 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.

Acknowledgements

The authors want to thank the financial supports from the National Science Council (NSC 97-2313-B-039-001-MY3) and China Medical University (CMU) (CMU96-113, CMU97-232 and CMU99-S-29). This study is supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004). The authors would like to thank Dr. Jeffrey Conrad for critically reading the manuscript.

475

476 **References**

477 Beutler, B., & Cerami, A. (1989). The biology of cachectin/TNF-α primary mediator

478 of the host response. *Annual Review of Immunology*, *7*, 625-655.

479 Bogdan, C. (2001). Nitric oxide and the immune response, *Nature Immunology*, *2*,

480 907-916.

481 Cochran, F. R., Selph, J., & Sherman, P. (1996). Insights into the role of nitric oxide

482 in inflammatory arthritis. *Medicinal Research Reviews, 16,* 547-563.

483 Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature, 420,* 860-867

484 Cuzzocrea, S., Costantino, G., Zingarelli, B., Mazzon, E., Micali, A. & Caputi, A. P.

485 (1999). The protective role of endogenous glutathione in carrageenan-induced

486 pleurisy in the rat. *European Journal of Pharmacology*, *372,* 187-197.

487 Dawson, J., Sedgwick, A. D., Edwards, J. C., & Lees, P. (1991). A comparative study

488 of the cellular, exudative and histological responses to carrageenan, dextran and

489 zymosan in the mouse. *International Journal of Tissue Reactions, 13,* 171-185.

490 Dudhgaonkar, S. P., Tandan, S. K., Bhat, A. S., Jadhav, S. H. & Kumar, D. (2006).

491 Synergistic anti-inflammatory interaction between meloxicam and

492 aminoguanidine hydrochloride in carrageenan-induced acute inflammation in
493 rats. *Life Science*, *78*, 1044-1048.
494 Heiss, E., Herhaus, C., Klimo, K., Bartsch, H., & Gerhauser, C. (2001). Nuclear
495 factor kappa B is a molecular target for sulforaphane-mediated
496 anti-inflammatory mechanisms. *Journal of Biological Chemistry, 276*,
497 32008-32015.
498 Hsieh, W. C. (2004). Illustration of common medicinal plants in Taiwan. Taipei:
499 CCMP Press.
500 Huang, M. H., Huang, S. S., Wang, B. S., Wu, C. H., Sheu, M. J., Hou, W. C., Lin, S.
501 S., et al. (2010). Antioxidant and anti-inflammatory properties of
502 Cardiospermum halicacabum and its reference compounds ex vivo and in vivo.
503 Journal of Ethnopharmacol. 2010 Nov 9. (doi:10.1016/j.jep.2010.11.005)
504 Janero, D. R. (1990). Malondialdehyde and thiobarbituric acid-reactivity as
505 diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free*
506 *Radical Biology and Medicine, 9*, 515-540.
507 Jones, R., Adel-Alvarez, L. A., Alvarez, O.R., Broaddus, R., & Das, S. (2003).
508 Arachidonic acid and colorectal carcinogenesis. *Molecular and Cellular*
509 *biochemistry, 253,* 141-149.

510 Just, M. J., Recio, M. C., Giner, R. M., Cuellar, M. J., Manez, S., Bilia, A. R., et al.
511 (1998). Anti-inflammatory activity of unusual lupane saponins from *Bupleurum*
512 *fruticescens*. *Planta Medica, 64*, 404-407.

513 Kim, J. S., Kim, J. C., Shim, S.H., Lee, E. J., Jin, W., Bae, K., et al. (2006). Chemical
514 constituents of the root of Dystaenia takeshimana and their anti-inflammatory
515 activity. *Archives of Pharmacal Research, 29*, 617-623.

516 Kirkova, M., Kassabova, T., & Russanov, E. (1992). *In vivo* effects of
517 indomethacin--I. Activity of antioxidant enzymes and lipid peroxidation.
518 *General Pharmacology, 23*, 503-507.

519 Lerouet, D., Beray-Berthat, V., Palmier, B., Plotkine, M., & Margaill, I. (2002).
520 Changes in oxidative stress, iNOS activity and neutrophil infiltration in severe
521 transient focal cerebral ischemia in rats. *Brain Research, 958*, 66-75.

522 Liu, Y., Liang, G., Zhang, J., Wu, K., Xu, B., Luo, B. (2002). Studies on chemical
523 constituents of *Dichondra repens*. *Chinese Pharmaceutical Journal*, 37,
524 577-579.

525 Long, L. H., Kwee, D. C., & Halliwell, B. (2000). The antioxidant activities of
526 seasonings used in Asian cooking. Powerful antioxidant activity of dark soy
527 sauce revealed using the ABTS assay. *Free Radical Research, 32,* 181–186.

528 Murakami, Y., Hirata, A., Ito, S., Shoji, M., Tanaka, S., Yasui, T., et al. (2007).
529 Re-evaluation of cyclooxygenase-2-inhibiting activity of vanillin and guaiacol
530 in macrophages stimulated with lipopolysaccharide. *Anticancer Research,* 27,
531 801-807.
532 Ohishi, N., Ohkawa, H., Miike, A., Tatano, T., & Yagi, K. (1985). A new assay
533 method for lipid peroxides using a methylene blue derivative. *Biochemistry*
534 *International, 10*, 205-211.
535 Paglia, E. D., & Valentine, W. N. (1967). Studies on the quantitative and qualitative
536 characterization of erythrocytes glutathione peroxidase. *Journal of Laboratory*
537 *Clinical Medicine. 70*, 158-169.
538 Singh, R., Singh, B., Singh, S., Kumar, N., Kumar, S., & Arora, S. (2010).
539 Umbelliferone – An antioxidant isolated from *Acacia nilotica* (L.) Willd. Ex.
540 Del. *Food Chemistry 120*, 825–830
541 Salvemini, D., Wang, Z. Q., Bourdon, D. M., Stern, M. K., Currie, M. G., & Manning,
542 P.T. (1996). Evidence of peroxynitrite involvement in the carrageenan-induced
543 rat paw edema. *European Journal of Pharmacology, 303*, 217-220.
544 Tonussi, C. R., & Ferreira, S. H. (1999). Tumour necrosis factor-alpha mediates
545 carrageenin-induced knee-joint incapacitation and also triggers overt
546 nociception in previously inflamed rat knee-joints. *Pain, 82*, 81-87.

547 Von Gruenigen, V. E., White, L.J., Kirven, M.S., Showalter, A. L., Hopkin, M. P., &
548 Jenison, E. L. (2001). A comparison of complementary and alternative medicine
549 use by gynecology and gynecologic oncology patients. *International Journal of*
550 *Gynecological Cancer, 11,* 205-209.

551 Weisz A., Cicatiello I., & Esumi H. (1996). Regulation of the mouse inducible-type
552 nitric oxide synthase gene promoter by interferon-gamma, bacterial
553 lipopolysaccharide and NG-monomethyl-L-arginine. *Biochemical Journal*, *316*,
554 209-215.

555 Winter, C. A., Risley, E. A., & Nuss, G. W. (1962). Carrageenin-induced edema in
556 hind paw of the rat as an assay for antiiflammatory drugs. *Proceedings of the*
557 *Society for Experimental Biology and Medicine, 111*, 544-547.

558 Xu, C., Zhang, Y., Cao, L., & Lu, J. (2010). Phenolic compounds and antioxidant
559 properties of different grape cultivars grown in China. *Food Chemistry*, *119,*
560 *1557-1565.*