Food Chemistry 118 (2010) 315–322

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Anti-inflammatory effects of *Punica granatum* Linne *in vitro* and *in vivo*

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ARTICLE INFO

Article history:
Received 1 July 2008
Received in revised form 22 April 2009
Accepted 29 April 2009

Keywords:
Punica granatum L.
Granatin B
Ellagictannin
Anti-inflammation
Nitric oxide
Inducible nitric oxide synthase
Cyclooxygenase-2

ABSTRACT

Inflammation can cause various physical dysfunctions. *Punica granatum* Linne (pomegranate), a high phenolic content fruit, is widely used as an antipyretic analgesic in Chinese culture. Pomegranate has shown potential nitric oxide (NO) inhibition in LPS-induced RAW 264.7 macrophage cells. Moreover, pomegranate (100 mg/kg) significantly decreased carrageenan-induced mice paw edema for 1, 3, 4, and 5 h. Therefore, column chromatography combined with *in vitro* bioassay-guided fractionation was used to isolate the active anti-inflammatory components from the pomegranate. Punicalagin (**1**), punicalin (**2**), strictinin A (**3**), and granatin B (**4**) were obtained with yields of 0.093%, 0.015%, 0.003%, and 0.013%, respectively. All these hydrolysable tannins inhibited NO production and iNOS expression in RAW 264.7 cells. Among them, **4** showed the strongest iNOS and COX-2 inhibitory effects, and exhibited these effects in the inhibition of paw swelling and the PGE_2 level in carrageenan-induced mice. Taken together, we suggest that **4** could be used as a standard marker for the anti-inflammatory effect of pomegranate.

1. Introduction

Punica granatum Linne, pomegranate (Punicaceae), a common fruit in the Mediterranean and Iran, is widely used for therapeutic formulae, cosmetics, and food seasoning. Pomegranate, also easily acquired from traditional medicine markets, was usually used as an astringent agent (Alper & Acar, 2004), for eliminating parasites (Mudzhiri, 1954; Raj, 1975) and as an antipyretic. The pharmacological functions of pomegranate include antioxidation (Lansky & Newman, 2007), anti-tumour (Khan, Afaq, Kweon, Kim, & Mukhtar, 2007; Lansky & Newman, 2007), anti-hepatotoxicity (Kaur, Jabbar, Athar, & Alam, 2006), anti-lipoperoxidation (Reddy, Gupta, Jacob, Khan, & Ferreira, 2007) and anti-bacteria properties (Menezes, Cordeiro, & Viana, 2006). In hematology, pomegranate could reduce the common carotid intima-medium thickness, thus lowering blood pressure and decreasing low-density lipoprotein (LDL) oxidation and the incidence of heart disease (Aviram et al., 2002, 2004). In our previous studies, we found that extract from the dried peel of the pomegranate could significantly inhibit NO production. Hence, we suggested pomegranate contains the anti-inflammatory activity components.

In the last few years, many important functions of fresh fruits and vegetables have been reported, and they are now recognised as being good sources of natural antioxidants (Joseph, Shukitt-Hale, & Lau, 2007), such as grapes, apples, and guavas. The antioxidants can prevent lipid peroxidation, and DNA and protein damage. Polyphenols have been acknowledged to have health-beneficial effects, owing to derived products such as flavonoids, tannins, coumarins, and lignans. According to recent reports, the pomegranate is rich in polyphenols, including mainly ellagitannins, gallotannins (punicalin, punicalagin, pedunculagin, punigluconin, granatin B, and tellimagrandin I) (Satomi et al., 1993) and anthocyanins (delphinidin, cyanidine and pelargonidin) (Noda, Kaneyuki, Mori, & Packer, 2002). However, the correlation between the phytochemicals and the anti-inflammatory properties of the dried peel of the pomegranate has not been investigated. Therefore, this study aimed to clarify the anti-inflammatory activities of pomegranate and its active components.

Inflammation, the first physiological defense system in the human body, can protect against injuries caused by physical wounds, poisons, etc. This defense system, also called short-term inflammation, can destroy infectious microorganisms, eliminate irritants, and maintain normal physiological functions. However, long-term over-inflammation might cause dysfunctions of the regular physiology, i.e., asthma and rheumatic arthritis.

We used *in vitro* and *in vivo* models to confirm the anti-inflammatory activity of pomegranate. Lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophages were used in the *in vitro* study, and carrageenan-induced paw edema in mice served as the *in vivo* study. LPS can induce several cytokines, such as prostaglandins and nitric oxide (NO), which are involved in pro-inflammatory processes. NO can kill bacteria and viruses and is also an important

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doi:10.1016/j.foodchem.2009.04.123

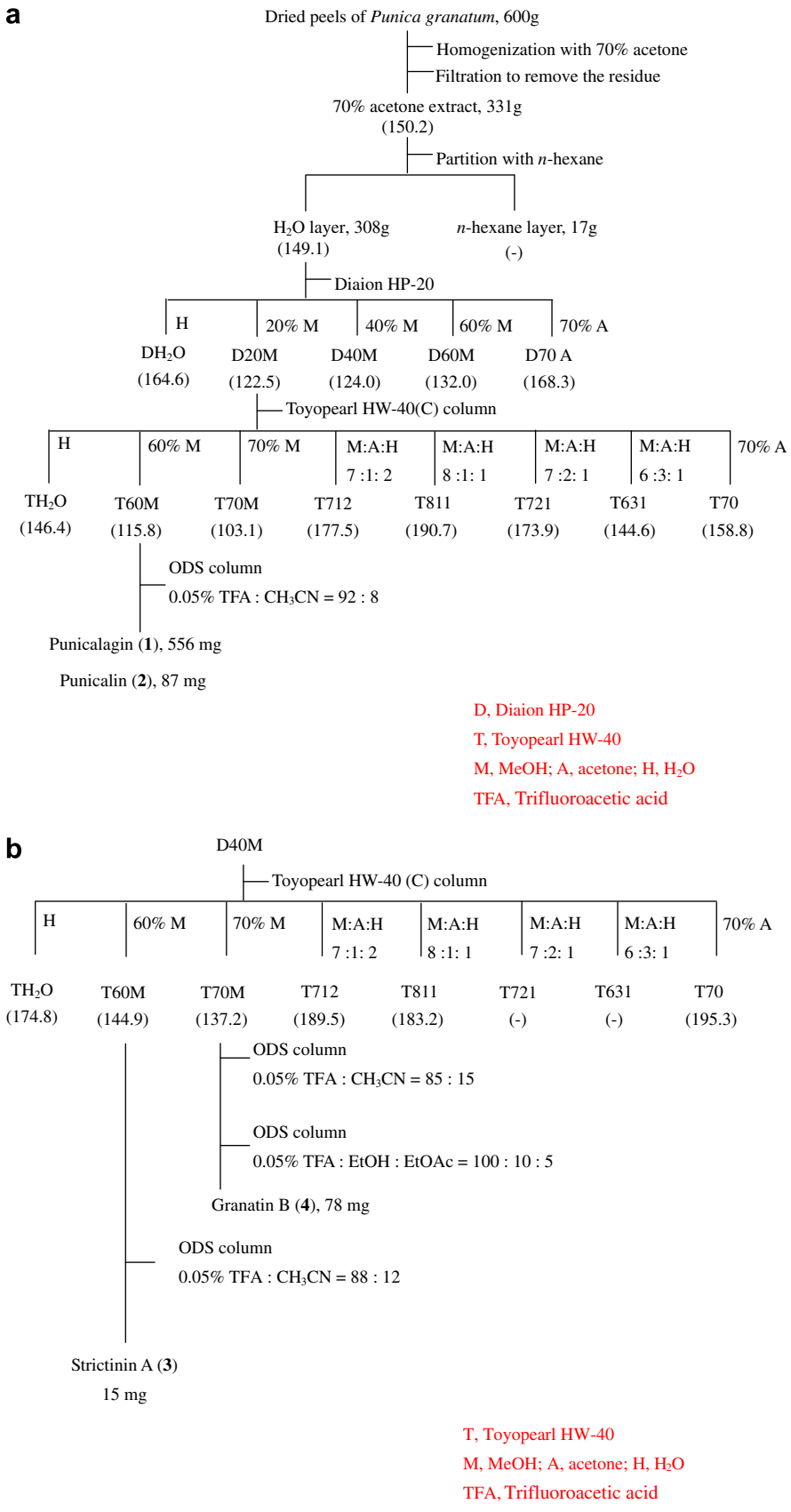

Fig. 1. Isolation flowchart of *Punica granatum* L. using anti-inflammation bioassay-guided fractionation. Numbers in the parentheses were the IC_{50} values (μg/ml) of NO inhibition. All test fractions displayed less than 10% cytotoxicity at 200 μg/ml, except D20M, D40M, D20M –TH_2O, and D20M – T60M. The IC_{50} values of the test sample exceeded 200 μg/ml.

mediator of vasodilatation; but too much NO might cause hypotension or septicemia (Moncada & Higgs, 2006). Prostaglandins have many physiological activities, such as inducing inflammation. Many tissues acutely or chronically generate excess NO and prostaglandin E_2 (PGE_2) by the overexpression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in the presence of various inflammatory stimulators. RAW 264.7 cells induced by LPS can produce the overexpression of NO and PGE_2, and their regulatory proteins, iNOS and COX-2. Therefore, we used this strategy, combined with chromatography, to isolate the active components, and we used carrageenan-induced paw edema in mice to confirm the *in vivo* anti-inflammatory effects of pomegranate.

2. Materials and methods

2.1. Chemicals

Dimethyl sulphoxide (DMSO), lipopolysaccharide (LPS), 3-(4.5-dimethylthiazol -2-yl) 2.5-diphenyltetrazolium bromide (MTT), indomethacin, *N*-nitro-L-arginine methyl ester (L-NAME), and the other chemicals were purchased from Sigma Industries (St. Louis, MO, USA). Dubecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics, and glutamine were purchased from GIBCO BRL (Grand Island, NY, USA). Diaion HP-20 gels were bought from Mitsubishi Chemical Industry (Tokyo, Japan). Western blotting was performed using an antibody specific to mouse. iNOS (sc-650), anti-COX-2 (sc-1745), and anti-GAPDH (sc-32233) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Sample preparation

Test solutions of pomegranate were prepared by dissolving pomegranate in 10% DMSO, which was then stored at 4 °C and used within 1 month. Serial dilutions of test solutions with culture medium were prepared before the *in vitro* assays.

2.3. Determination of total phenols

The total phenol content was determined by the Folin-Ciocalteu method. Pomegranate extract was dissolved in distilled water and mixed with the Folin-Ciocalteu reagent and 7.5% aqueous Na_2CO_3 solution. After standing for 5 min at 50 °C, the absorbance was measured at 600 nm in an ELISA reader. The amount of total phenol was expressed as gallic acid equivalents (GAE, μg gallic acid/mg sample) through the calibration curve of gallic acid. The calibration curve ranged from 7.8 to 250 μg/ml (R^2 = 1) (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009).

2.4. Determination of the total flavones content

The total flavanol content was determined by the vanillin assay. Vanillin was dissolved in 80% H_2SO_4 to prepare the vanillin reagent. Pomegranate extract was dissolved in the distilled water and mixed with the vanillin reagent. After standing for 15 min at room temperature, the absorbance was measured at 530 nm in an ELISA reader. The amount of total flavanol was expressed as catechin equivalents (CE, μg catechin/mg sample) through the calibration curve of catechin. The calibration curve ranged from 7.8 to 250 μg/ml (R^2 = 1) (He, Liu, & Liu, 2008).

2.5. Determination of NO produced from LPS-induced RAW 264.7 cells

RAW 264.7 cells, a murine macrophage cell line, was obtained from American Type Cell Culture (ATCC no. TIB-71; Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin, and maintained at 37 °C and 5% CO_2. RAW 264.7 cells (4.0×10^5 cells/ml) were seeded in 96-well plates and then co-treated with LPS (500 ng/ml) and the test samples. After 18 h, the NO production was determined by mixing the culture supernatant with Griess reagent, and the absorption was detected in an ELISA reader at 530 nm. Anti-inflammatory activity was presented in terms of the NO production inhibition percentage. The viability of RAW 264.7 cells was detected by the MTT assay (Tseng, Lee, Chen, Wu, & Wang, 2006).

2.6. Isolation of polyphenols from P. granatum

Dried pomegranate peels (600 g) were purchased from a traditional Chinese medicine store in Taipei, pulverised and filtered through a 20# mesh. Pulverised peels were then homogenised with 70% aqueous acetone (3 L × 4) and the homogenate was then filtered. The filtrate was concentrated by evaporation under reduced pressure (*ca.* 40 °C) and further freeze-dried to yield the 70% acetone extract (330 g, yield 55%). The 70% acetone extract was dissolved in water and then partitioned with *n*-hexane to remove the non-polar compounds. The aqueous layer was chromatographed over a Diaion HP-20 gel column (9.5 × 40 cm) with stepwise aqueous MeOH ($H_2O \rightarrow 20\%$ MeOH → 40% MeOH → 60% MeOH → 70% acetone). Column chromatography was combined with an NO inhibition assay to clarify the bioactive fractions. We found that the 20% MeOH (D20M) and 40% MeOH (D40M) fractions of the Diaion column exhibited greater NO inhibition. Furthermore, the D20M fraction was chromatographed on a Toyopearl HW-40 (C) (Tosoh Bioscience, Montgomeryville, PA, USA) gel column (2.5 × 40 cm) and eluted stepwise with $H_2O \rightarrow 70\%$ MeOH → MeOH–acetone–H_2O (8:1:1, v/v/v) → MeOH–acetone–H_2O (7:2:1, v/v/v) → MeOH–acetone–H_2O (6:3:1, v/v/v) → 70% acetone. The 70% MeOH (D20M –T70M) eluate was applied to an ODS column (0.05% trifluoroacetic acid:CH_3CN, 92:8) and HLB® extraction cartridges (Waters, Milford, MA, USA) to yield punicalagin (556 mg, **1**) and punicalin (87 mg, **2**). The D40M fraction was chromatographed on a Toyopearl HW-40 (C) gel column (2.5 × 40 cm) and eluted stepwise with $H_2O \rightarrow 70\%$ MeOH → MeOH–acetone–H_2O (8:1:1, v/v/v) → MeOH–acetone–H_2O (7:2:1, v/v/v) → MeOH–acetone–H_2O (6:3:1, v/v/v) → 70% acetone. The 60% MeOH eluate (D40M –T60M) was purified on an ODS column with 0.05% trifluoroacetic acid–CH_3CN (88:12) to obtained 15 mg of strictinin A (**3**). The

Table 1
NO inhibitory effects of four hydrolysable tannins.

Compound	LPS-induced RAW 264.7 macrophage cells			Free radical-scavenging activity
	NO inhibition[a]	iNOS activity[b,c]		$ONOO^-$ scavenging[c]
	IC_{50} values (mM)	NO inhibition (%)	Cytotoxicity (%)	SNP clearance (%)
1	69.8	15.7 ± 0.4	35.5 ± 0.7	24.7 ± 6.4
2	78.6	46.7 ± 7.0	67.6 ± 1.4	30.7 ± 1.0
3	63.1	25.4 ± 2.4	45.8 ± 1.1	32.3 ± 0.0
4	33.6	14.8 ± 1.0	41.7 ± 0.7	29.5 ± 0.7
L-NAME[d]	–	46.4 ± 2.9	7.5 ± 1.6	–

Results are expressed as the mean ± SD of three experiments.
[a] Co-treatment of LPS and each test compound for 18 h and no cytotoxicites were found in these IC_{50} values.
[b] RAW 264.7 cells were stimulated with 1 μg/ml LPS and incubated overnight.
[c] Each test compound was 100 μM.
[d] L-NAME was used as positive control and its concentration was 400 μM.

70% MeOH eluate (D40M – T70M) was purified on an ODS column with 0.05% trifluoroacetic acid–CH_3CN (85:15) and 0.05% trifluoroacetic acid–EtOH–EtOAc (100:10:5, v/v/v) to obtain 78 mg of granatin B (**4**) (Fig. 1). The structures of these four compounds were identified by nuclear magnetic resonance and the purity of each compound (>99.0%) was confirmed by high-performance liquid chromatography. The formulae and molecular weights of punicalagin (**1**), punicalin (**2**), strictinin A (**3**), and granatin B (**4**) are $C_{43}H_{28}O_{30}$ (MW 1048.7), $C_{34}H_{22}O_{22}$ (MW 782.53), $C_{43}H_{28}O_{30}$ (MW 634.46), and $C_{41}H_{28}O_{27}$ (MW 952.66), respectively.

2.7. Determination of NO radical-scavenging activity

Sodium nitroprusside (SNP) is a stable NO donor. We used SNP as an NO donor to evaluate the direct NO radical clearance of samples. The SNP solution was prepared with H_2O. Test solution was added to the sample volume of SNP solution (50 mM). The mixture was incubated at 37 °C, for 5 h. Then, we took the supernatant (1, mixed it with the Griess reagent, and read the absorption on an ELISA reader at 530 nm (Bor, Chen, & Yen, 2006).

2.8. iNOS activity assay in LPS-induced RAW 264.7 cells

Initially, RAW 264.7 cells were pre-stimulated by 1 μg/ml LPS for 24 h to activate the iNOS. Then, cells were collected and washed twice with PBS to remove the excessive LPS, and the activated RAW 264.7 cells (4.0×10^5 cell/ml) were seeded in 96-well plates. Crude extract and active components were immediately added to the cells. The 96-well plate was incubated at 37 °C overnight. The NO detecting method was the same as previously described. We used *N*-nitrol-arginine methyl ester (L-NAME, 400 μM), a specific inhibitor of NO synthase enzyme, and as the positive control.

2.9. iNOS and COX-2 expression assay in LPS-induced RAW 264.7 cells

Total cellular protein was extracted with a RIPA solution (radioimmuno-precipitation assay buffer) at −20 °C overnight. We used BSA (bovine serum albumin) as a protein standard to calculate equal total cellular protein amounts. Protein samples (15 μg) were resolved by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using standard methods, and then were transferred to nitrocellulose (Hybond-PVDF) membranes by electroblotting and blocking with 1% BSA. The membranes were probed with the primary antibodies (iNOS, COX-2 and GAPDH) at 4 °C overnight, washed three times with PBST, and incubated for 1 h at 37 °C with alkaline phosphatase-conjugated secondary antibodies. Then, we used an NBT/BCIP commercial kit (Gibco) as the visualising agent. The intensity of the bands was quantified by computer-assisted image analysis using AlphaInnotech Digital Imaging Systems. Western blotting results are representative of three independent experiments for every data point.

2.10. Model of 1% carrageenan-induced paw edema in ICR mice

Male ICR mice weighing 25 ± 2 g were bought from the National Science Council, Taipei, Taiwan, and maintained at 21 ± 2 °C with food and water *ad libitum*. They were kept on a 12-h light/12-h dark cycle. All mice used in this experiment were cared for according to

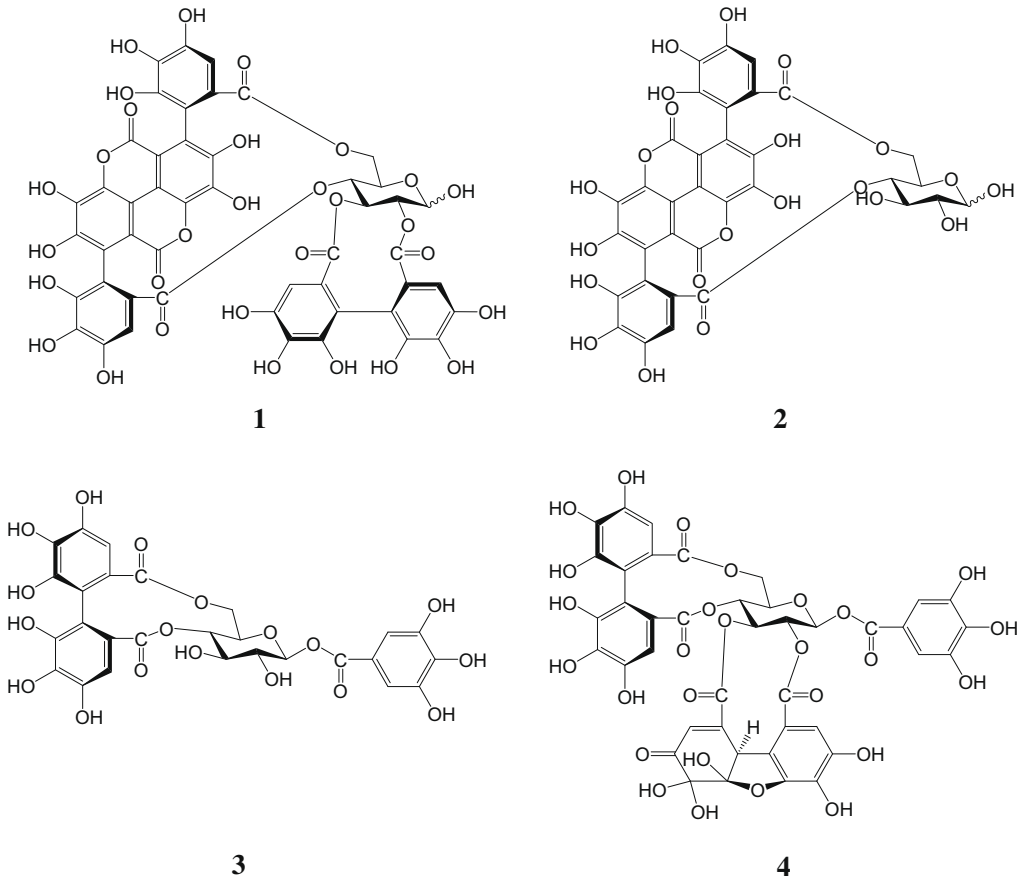

Fig. 2. Structures of the four hydrolysable tannins, punicalagin (**1**), punicalin (**2**), strictinin A (**3**), and granatin B (**4**), isolated from *Punica granatum* L.

the Ethical Regulations on Animal Research of our university. Edema in the left hind paw of the mice was induced by injecting 50 μl of 1% (w/v) carrageenan into the subplantar region. The perimeter of the paw was measured 1 h before the carrageenan injection and after 1–6 h, using calipers. One hour before the injection, the pomegranate (100 mg/kg), the active component (2.5 and 10 mg/kg), and indomethacin (10 mg/kg, as a positive control) were given orally, while the control group was given distilled water. The blank group was injected with normal saline and given distilled water. Each group consisted of five animals. After 6 h, mice were sacrificed and the serum collected for the PGE_2 measurement (Tseng et al., 2006).

2.11. Measurement of PGE_2 production

Serum and cell culture mediums were determined the PGE_2 concentrations by the PGE_2 ELISA kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.12. Statistical analysis

The data are presented as mean and standard deviation (SD). Significance was calculated using the Student's *t*-test. Differences were considered significant for $p < 0.05$.

3. Results

3.1. Polyphenols isolation from P. granatum L.

The phytochemical components of the 70% acetone extract of pomegranate peels were screened using the Folin-Ciocalteu method and vanillin assay. The screening found that pomegranate was rich in total phenol and flavanol (471.0 ± 32.0 μg gallic acid equivalent/mg in total phenol and 257.0 ± 19.6 μg catechin equivalent/mg in total flavanol).

The NO inhibition bioassay-guided fractionation flowchart of pomegranate is shown in Fig. 1. These four hydrolysable tannins, punicalagin (**1**), punicalin (**2**), strictinin A (**3**), and granatin B (**4**), were isolated from D20M and D40M of the pomegranate fractions, with stronger inhibition of NO production; the yields were 0.093%, 0.015%, 0.003% and 0.01%, respectively.

3.2. NO inhibitory effects of four hydrolysable tannins

The NO inhibitory effects of **1**–**4** were measured in LPS-induced RAW 264.7 cells for 18 h. We collected the culture medium to detect the NO levels using the Griess reaction. Compounds **1**–**4** showed less than 10% cytotoxicities in LPS-induced RAW 264.7 cells for 18 h. Compound **4** displayed a more potent NO inhibition

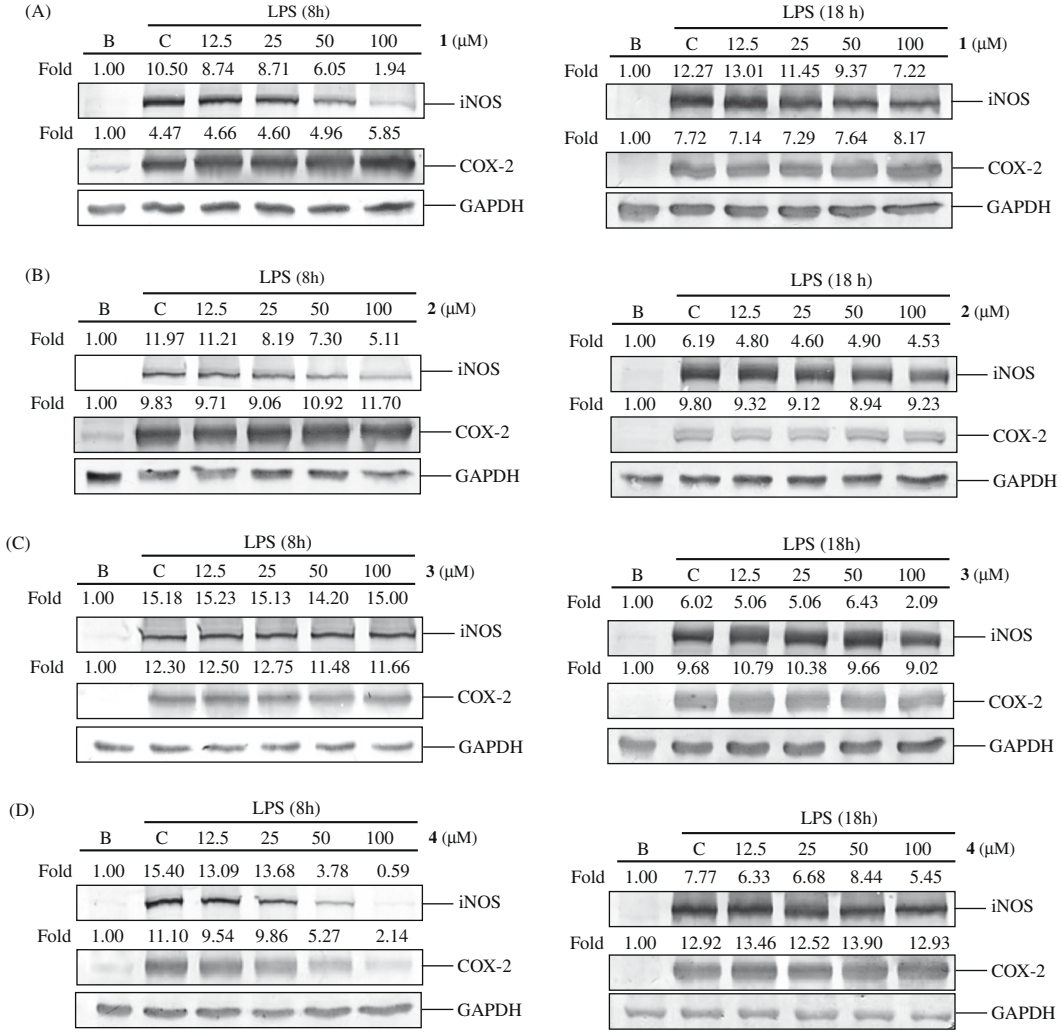

Fig. 3. Inducible NOS and COX-2 expression of punicalagin (A), punicalin (B), strictinin A (C) and granatin B (D) induced by LPS (500 ng/ml) in RAW 264.7 cells for 8 and 18 h. (B) Non-LPS-induced group. (C) Solvent control group (H_2O). GAPDH was used as an internal control to identify equal amounts of protein loading in each lane. The intensity of the iNOS and COX-2 protein was examined by densitometrus analysis, and expressed as the ratio of iNOS/GAPDH and COX-2/GAPDH. Data from three separate experiments were used, and the picture for one of which is shown.

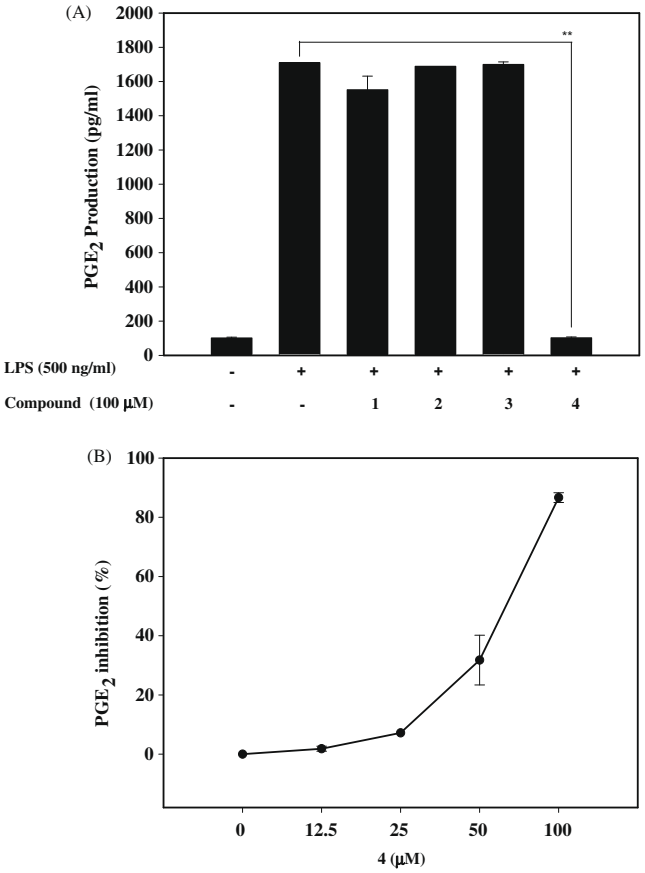

Fig. 4. Prostaglandin E_2 production of **1**–**4** (A) and a series dose–response curve of **4** (B) induced by LPS-induced RAW 264.7 cells for 8 h. $^{**}p < 0.001$. $n = 3$ All data were expressed as the mean ± SD.

in LPS-induced RAW 264.7 cells for 18 h than did the others, and its IC_{50} value was 33.6 μM (Table 1).

Nevertheless, we would like to clarify whether these hydrolysable tannins had NO radical-scavenging activity or not. SNP was used as an NO radical donor to evaluate the NO-scavenging activity of these four hydrolysable tannins. As shown in Table 1, the four hydrolysable tannins did not significantly exhibit SNP clearance at 100 μM.

3.3. iNOS expression and activities of four hydrolysable tannins in LPS-induced RAW 264.7 cells

Based on the above data, we suggest that the NO inhibition effects of the four hydrolysable tannins could be brought about through the influence of iNOS protein expression or activity. The iNOS protein expression in LPS-induced RAW 264.7 cells for 8 and 18 h were observed by Western blot assay. As shown in Fig. 3, the four hydrolysable tannins displayed dose-dependently inhibitory effects on the iNOS expression at 12.5–100 μM. In addition, the 8 h group expressed more significantly iNOS inhibitory effects than the 18 h group. Therefore, these four hydrolysable tannins could decrease the iNOS protein expressions from the early stage (8 h) to the late stage (18 h). Taken together, **1**–**4** significantly decreased the iNOS expressions in LPS-induced RAW 264.7 cells, and **4** was the strongest among them.

On the other hand, RAW 264.7 cells were pretreated with LPS for 24 h to observe the iNOS activity inhibition of these four hydrolysable tannins. As seen in Table 1, L-NAME acted as a positive control and displayed significant iNOS activity inhibition at 400 μM. However, these four hydrolysable tannins had no iNOS activity inhibitory effects and even exhibited cytotoxicity at 100 μM. Hence, these four hydrolysable tannins inhibited NO production through directly decreasing the iNOS protein expressions.

3.4. COX_2 and PGE_2 inhibitory effects of four hydrolysable tannins

The COX-2 and PGE_2 inhibitory effects of these four hydrolysable tannins were evaluated in LPS-induced RAW 264.7 cells for 8 and 18 h. As shown in Fig. 3, Compound **4** inhibited COX-2 protein expression in dose-dependent (Fig. 3) and PGE_2 productions (Fig. 4A) more significantly than the others after treatment with LPS for 8 h. Moreover, **4** inhibited PGE_2 productions in a dose-dependent manner, and the IC_{50} value was 66.22 ± 9.4 μM (Fig. 4D). However, COX-2 protein expression of LPS-induced RAW 264.7 cells was not inhibited after treatment with the four hydrolysable tannins for 18 h (Fig. 3). Hence, we suggested that **4** could inhibit COX-2 and PGE_2 production in the early stage (8 h) because there was less COX-2 expression at that stage, but in the late stage (18 h), COX-2 expression reached a stable state, so **4** had difficulty inhibiting its expression.

3.5. Inhibitory effect of pomegranate and 4 on carrageenan-induced paw edema in ICR mice

Carrageenan-induced paw edema in ICR mice was used to evaluate the *in vivo* anti-inflammatory model. We used indomethacin, a common non-steroidal anti-inflammatory drug (NSAID) as a positive control. Mice were treated with pomegranate (100 mg/kg) or indomethacin (10 mg/kg) 1 h before carrageenan induction, and had detected paw edema for 6 h. Results showed that carrageenan significantly induced paw edema during the entire experiment in the control group (Table 2). Pomegranate significantly reduced paw edema by more than 50% at 1–5 h after the carrageenan injection, and had even greater potential paw edema inhibitory effects than indomethacin.

Table 2
Effects of pomegranate and granatin B on the paw perimeter of carrageenan-induced mice paw edema.

Group	Time after injection of carrageenan (h)					
	1	2	3	4	5	6
Control	1.74 ± 0.30	1.41 ± 0.17	1.41 ± 0.23	1.21 ± 0.13	0.92 ± 0.16	0.88 ± 0.08
Indomethacin (10 mg/kg)	0.70 ± 0.35*	0.90 ± 0.34*	0.79 ± 0.33*	0.72 ± 0.15*	0.75 ± 0.09*	0.62 ± 0.19*
Pomegranate (100 mg/kg)	0.86 ± 0.10*	0.60 ± 0.07**	0.59 ± 0.28*	0.42 ± 0.30*	0.4 ± 0.36*	0.34 ± 0.50
*Granatin B (**4**)*						
2.5 mg/kg	1.08 ± 0.13*	0.84 ± 0.20*	0.77 ± 0.07**	0.75 ± 0.17*	0.63 ± 0.03*	0.59 ± 0.15*
10.0 mg/kg	0.98 ± 0.40*	0.89 ± 0.19*	0.68 ± 0.29*	0.50 ± 0.41*	0.23 ± 0.52*	0.48 ± 0.26*

Values were presented as the mean ± SD of the increasing mice paw edema (mm) of four animals for each group.
* $p < 0.05$.
** $p < 0.001$ were presented as significantly different from the control.

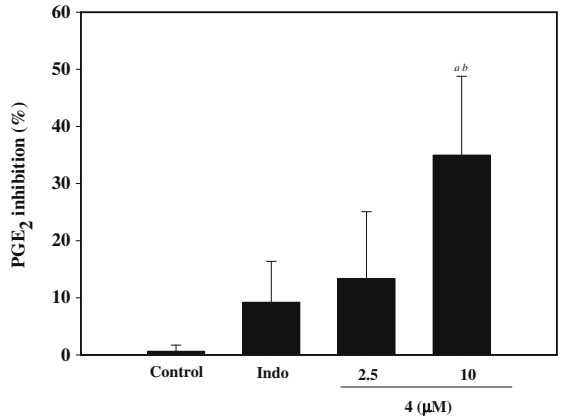

Fig. 5. Prostaglandin E_2 concentrations of **4** on carrageenan-induced mice paw edema for 6 h. Indomethacin served as a positive control and 10 mg/kg dosage used. [a]$p < 0.01$. The p value was calculated by comparison with the control group. [b]$p < 0.05$. The p value was calculated by comparison with the indomethacin group ($n = 3$). All data were expressed as mean ± SD.

Since **4** showed the strongest COX-2 and iNOS inhibitory effects in the *in vitro* study, we examined the *in vivo* anti-inflammatory effect of **4**. We found that **4** (2.5 and 10 mg/kg) could significantly and dose-dependently reduce paw edema, and the inhibitory abilities of the higher dose (10 mg/kg) were the same as that of the indomethacin (Table 2). In addition, **4** had more significant PGE_2 inhibitory effects than the indomethacin at 6 h (Fig. 5).

4. Discussion

Pomegranate has been used for centuries as a therapeutic agent for the treatment of inflammatory diseases. According to current reports, pomegranate is a polyphenol-rich fruit, and showed potential as an anti-inflammatory, anti-oxidative and anti-cancer agent in several experimental models (Shukla, Gupta, Rasheed, Khan, & Haqqi, 2008). Chemical analyses have also shown that the phenol compounds of pomegranate contain significantly high levels of hydrolysable tannins, such as punicalin, punicalagin, pedunculagin, punigluconin (Dudonné et al., 2009). Therefore, we used an NO inhibition assay combined with column chromatography to determine which components in pomegranate would have effective anti-inflammatory activity. In this paper, four hydrolysable tannins, punicalagin (**1**), punicalin (**2**), strictinin A (**3**), and granatin B (**4**), were isolated from pomegranate by bioassay-guided fractionation. Each of them displayed a dose-dependently and significantly inhibitory effect on NO production in LPS-induced RAW 264.7 cells (Table 1). Furthermore, granatin B (**4**) more strongly inhibited PGE_2 production and COX-2 expression in LPS-induced RAW 264.7 cells than the others.

Structure–activity relationships (SAR) of natural products have been found to influence the various pharmacological functions, such as antioxidant and anti-inflammation activities. Up to the present, over 3000 kinds of tannins have been identified, chiefly as secondary metabolites in green plants. Tannin is involved in a large proportion of phenolic derivatives in plants and is divided into two types: hydrolysable and condensed tannins. Many possible permutations are offered by substitution and conjugation, and this could explain why so many tannin derivatives occur naturally (Fylaktakidou, Hadjipavlou-Litina, Litinas, & Nicolaides, 2004). Based on the structural similarities of these four hydrolysable tannins, we divided them into two groups. One was punicalagin (**1**) and punicalin (**2**) and the other was strictinin A (**3**) and granatin B (**4**) (Fig. 2). Among them, **1** and **2** were previously recognised as inhibitors of the pro-inflammatory for anti-edematogenic activity on carrageenan-induced paw edema in rats (10 mg/kg) (Lin, Hsu, & Lin, 1999). However, there is no scientific evidence that **3** and **4** have anti-inflammatory activities. In this study, we first found that both **3** and **4** had potential NO inhibitory effects in LPS-induced RAW264.7, and that **4** was the better one (Table 1). Moreover, **4** showed the strongest COX-2 and iNOS inhibitory effects in an *in vitro* assay, and significantly reduced paw edema and PGE_2 inhibitory effects in an *in vivo* assay.

Dehydrohexahydroxydiphenoyl (DHHDP), a substitution in C-2 and C-3 of **4**, was the difference between **4** and **3**, and probably played an important role in anti-inflammation. In related investigations, tannins with DHHDP units invariably had strong anti-inflammatory and anti-oxidant activity. (Feldman, 2005) Geraniin, a well-documented hydrolysable tannin, has been reported to have excellent NO radical-scavenging and iNOS inhibitory activities (Kumaran & Karunakaran, 2006). Mallotusinic acid and euphorbin E also had potent scavenging effects on DPPH free radicals. (Okuda, 2005) Hence, we concluded once the hydrolysable tannins had the DHHDP group, they would appear to have better anti-inflammatory activities.

Many studies have demonstrated that the massive production of NO and PGE_2 *via* the pro-inflammatory proteins iNOS and COX-2 played an important physiological role in inflammation. Evidence has shown that NO production was initiated by treating LPS for 8 h and reached a stable state for 18 h, and that COX-2 expression began at 6–8 h and reached the maximal level at 16–24 h. (Caughey, Cleland, Penglis, Gamble, & James, 2001; Reher, Harris, Whiteman, Haiand, & Meghji, 2002). Therefore, we used two treatment times (8 and 18 h) to measure the anti-inflammatory effects of these hydrolysable tannins, because of the different mechanism and production time of these inflammation mediators (COX-2, iNOS, etc.). On the basis of the above results, we found that **4** could significantly decrease NO and PGE_2 production through inhibiting iNOS and COX-2 expression. Little NO production was found in LPS-induced RAW 264.7 for 8 h. NO was released from the process of converting L-arginine to L-citruline by iNOS, while NO was initially produced at 8 h and reached the maximum level at 18 h; therefore, NO production from LPS-induced RAW 264.7 was not detected at 8 h. Unlike NO production, activated COX-2 could convert arachidonic acid to PGE_2 in only 30 min. Hence, the COX-2 and PGE_2 inhibitory effects of **4** from LPS-induced RAW 264.7 at 8 h were stronger than those at 18 h. In summary, we suggest that **4** is an effective anti-inflammatory compound and has dual roles in anti-inflammation, by decreasing PGE_2 production in the early stage and decreasing NO production in the late stage.

In conclusion, **4** not only displayed the best NO inhibitory abilities in LPS-induced RAW 264.7, but also had the strongest PGE_2 inhibitory effects in the *in vitro* and *in vivo* assays. Taken together, **4** could be used as a standard marker compound to determine the potential anti-inflammatory effect of pomegranate.

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