Polyphenols Extracted from *Hibiscus sabdariffa* L. Inhibited Lipopolysaccharide-Induced Inflammation by Improving Antioxidative Conditions and Regulating Cyclooxygenase-2 Expression

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Oxidative stress and inflammation are related to several chronic diseases including cancer and atherosclerosis. *Hibiscus sabdariffa* Linnaeus has been found to possess antioxidant effects. In this study, polyphenols extracted from *Hibiscus sabdariffa* L. (HPE) were used to detect anti-inflammatory effects on nitrite and prostaglandin E2 (PGE2) in lipopolysaccharide (LPS) treated RAW264.7 cells. Sequentially, an animal model examination was performed to confirm the effects of HPE on LPS-induced hepatic inflammation. The results showed that HPE reduced 94.6% of xanthine oxidase activity in vitro, and decreased nitrite and PGE2 secretions in LPS-induced cells. In LPS-treated rats, HPE significantly decreased the serum levels of alanine and aspartate aminotransferase. Furthermore, in liver, lipid peroxidation and liver lesions decreased, and catalase and aspartate aminotransferase activity increased. The study also revealed that down-regulation of cyclooxygenase-2 (COX-2), p-c-Jun N-terminal kinase (p-JNK) and p-P38 might have been involved. In sum, this study found an anti-inflammatory potency of HPE both in vitro and in vivo.

Key words: anti-inflammatory; *Hibiscus sabdariffa* L.; lipopolysaccharide; macrophage; Malvaceae

COX-2 is expressed at very low levels but is strongly induced by pro-inflammatory stimulation, such as LPS, and by several activated oncogenes. The significance of COX-2 in prostaglandin synthesis and inflammation is highlighted by the observation that COX-2 inhibitors block the synthesis of PGE2, and as a result they inhibit inflammation and confer analgesia. Moreover, homozygous deletion of the COX-2 gene in mice leads to alleviation of the hepatocellular toxicity caused by LPS administration, suggesting the important physiological role of this enzyme in LPS-induced pathology.

NO produced by nitric oxide synthase (NOS) has been identified as an important molecule in inflammation and sepsis. Inducible nitric oxide synthase (iNOS) is not expressed under normal conditions. After exposure to endogenous and exogenous stimulators, it can be induced quantitatively in various cells, such as macrophages, smooth muscle cells, and hepatocytes, to trigger several disadvantageous cellular responses, as well as causing diseases, including inflammation, sepsis, and stroke. Hence, the level of iNOS might reflect the degree of inflammation, and can be used to evaluate the effects of drugs on the inflammatory process. In the liver, LPS activates iNOS in Kupffer cells, endothelial cells, and hepatocytes to promote NO production. Recently it was found that iNOS inhibitors might protect the liver from LPS-induced toxicity, and that natural antioxidants such as curcumin, resveratrol, and tea polyphenols exhibit an inhibition effect on LPS-induced iNOS and hepatic damage.

Many phenolic substances, particularly those present in edible and medicinal plants, have been reported to possess substantial anticarcinogenic and antiinflammatory activities. The majority of naturally occurring phenolics possessing antioxidative and anti-inflammatory properties contribute to their chemopreventive or chemoprotective activity. The flower of *Hibiscus sabdariffa*

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Abbreviations: ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; COX-2, cyclooxygenase-2; HPE, polyphenols extract from *Hibiscus sabdariffa* L.; JNK, c-Jun N-terminal kinase; NO, nitric oxide; iNOS, inducible nitric oxide synthase
Linnaeus calyx (family Malvaceae, local name Karkaday) is commonly used in cold and hot beverages and as a supplement because of its perceived potential health benefits. The flower extract has been reported to decrease blood pressure and to have anti-tumor characteristics as well as immune-modulating and anti-leukemic effects.\(^{13}\) It contains polyphenolic acids, flavonoids, protocatechueic acid (PCA), and anthocyanins. In our previous studies, the aqueous extract was found to inhibit the development of atherosclerosis in cholesterol-fed rabbits.\(^{14}\) Moreover, we have found that *Hibiscus* anthocyanin-rich extract (HAxs, about 2.5% of aqueous extracted *Hibiscus sabdariffa* L. flowers) inhibit LDL oxidation and ox-LDL-mediated macrophage apoptosis.\(^{15}\) In this study, a model of LPS-induced inflammation was conducted to determine the anti-inflammation of polyphenol extract from *Hibiscus sabdariffa* L. (HPE) *in vitro* and *in vivo*, and further to clarify the possible molecular mechanisms.

**Materials and Methods**

**Chemicals.** LPS (lipo-polysaccharide; endotoxin from *Escherichia coli*, serotype 0127:88), kits for ALT (alanine aminotransferase) and AST (aspartate aminotransferase) and other chemicals were purchased from Sigma Chemical. (St. Louis, MO). Prostaglandin E2 immunoassay kit (R&D Systems, Boston, MA), anti-iNOS, COX-2, phospho-JNK, phosphor-P38, and anti-β-tubulin antibody (Transduction Laboratories, Lexington, KY), and a protein assay kit (Bio-Rad, Watford, UK) were obtained as indicated.

**Preparation and assay of HPE.** *Hibiscus sabdariffa* L. was obtained from Taitung Hsin Farmers’ Association, Taiwan, and identified by Professor Yi-Ching Li of Chung Shan Medical University. A voucher specimen has been kept for future reference at the Department of Pharmacology, Chung Shan Medical University. Extraction of HPE was carried out as previously described.\(^{16}\) Briefly, dried flower was extracted with methanol, and then the samples were filtered and concentrated with a vacuum rotary evaporator. The residue was extracted first with hexane to remove pigments, and then with ethyl acetate. It was redissolved in water and lyophilized to obtain HPE. The components of HPE, including protocatechuic acid (8.83%), catechin (9.97%), EGCG (10.23%), EGCG (20.20%), and caffeic acid (18.10%), were determined by HPLC analysis.\(^{16}\)

**Activity of xanthine oxidase assay.** Xanthine oxidase activity was evaluated to determine the formation of uric acid produced from xanthine. Xanthine (0.2 mM, 0.88 ml) in phosphate buffer (0.1 M, pH 7.8) with xanthine oxidase (0.12 U/ml) was incubated for 15 min at room temperature and read at 295 nm against a blank sample without the enzyme. The components of HPE, including protocatechueic acid (8.83%), catechin (9.97%), EGCG (10.23%), EGCG (20.20%), and caffeic acid (18.10%), were determined by HPLC analysis.\(^{16}\)

**Cell culture.** Rat macrophage RAW 264.7 cells were cultured in a humidified atmosphere of 95% air-5% carbon dioxide at 37 °C and with RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 20 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

**Cell viability assay.** RAW 264.7 cells (2 × 10⁵) were plated to perform the viability assay. The cells were treated with HPE (0-0.5 mg/ml) for 24 h, and then the medium was replaced with fresh medium containing 5 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and incubated for 4 h. The medium was then removed, and 1 ml of isopropanol was added to dissolve the crystals. The optical density of each sample was read at 563 nm against a blank prepared from cell-free wells.

**Nitrite oxide measurement.** Total nitrates in the culture medium were measured using the Griess reagent, as described previously.\(^{17}\) Cells incubated with LPS and HPE (0-0.5 mg/ml) at 37 °C for 12 h, and 100 μl of each culture medium were centrifuged and then mixed with equal volumes of Griess reagent (1 g/l sulfanilamide and 0.1 g/l N-1-naphthyl-ethylenediamine in 2.5% phosphoric acid solution). After incubation at room temperature for 10 min, the absorbance at 540 nm was read and compared with standard solutions of NaNO₂.

**Production of PGE₂.** The cells were plated at 1 × 10⁶ cells per well. After adherence, the previous medium was replaced with phenol red-free RPMI1640. After the cell were incubated with LPS and HPE (0-0.5 mg/ml) at 37 °C for 12 h, the production of prostaglandin E₂ (PGE₂) in the medium was determined with a commercial competitive PGE₂ ELISA kit.

**Animal treatment.** Male Sprague-Dawley rats (260 ± 10 g), used in the studies, were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The animals were housed under laboratory conditions (18–23 °C, humidity 55–60%, 12 h light/dark cycle) for at least 1 week before each study. All animal studies were conducted according to the guidelines for care and use of laboratory animals approved by the Institute of Animal Care and Use Committee (IACUC) of Chung Shan Medical University. The rats were provided food and water *ad libitum*, and were divided to 5 groups (five rats per group). HPE (10, 20, and 40 mg/kg) was given daily to the animals for 5 consecutive d using a gavage. On the fifth d, h after HPE treatment, LPS (5 mg/kg) or distilled water as solvent control was injected (i.p.). The rats were decapitated 6 h later and the blood samples were collected for further detection. The livers were excised to detect lipid peroxidation levels and antioxidation enzyme activity, and the pathological histology was determined according to procedures described below. The body and liver weights were also recorded at the end of experimentation.

**Hepatotoxicity assessment.** Hepatic enzymes ALT, AST and ALKP were used as biochemical markers of early acute hepatic damage. The levels of uric acid were used to evaluate the activity of xanthine oxidase in the HPE-treated rats. The activities of enzymes and biochemical values were determined by enzymatic colorimetric methods using a standard Sigma commercial kit (Sigma, St. Louis, MO).

**Pathological histology of liver.** After removal from the animals, hepatic tissue was immediately fixed in 10% buffered formaldehyde and processed for histological examination by conventional methods by hematoxylin and eosin (H&E) stain. The liver lesions observed were classified according to morphology changes, such as neutrophil infiltration. Severity of liver damage was evaluated by examining the sections under 10 randomly selected high-power fields (×400). The number of fields that showed lesions and the areas of the lesions were recorded.

**Thiobarbituric acid-reacting substances (TBARS).** The lipid peroxidation was determined by measuring TBARS. Liver (0.5 g) tissue was homogenized with 5 ml of phosphate buffer and centrifuged (1,000 × g) for 30 min to obtain supernatant homogenate. Homogenate (0.3 ml) was supplemented with 0.3 ml of TBA (1% thiobarbituric acid in 0.3% NaOH) to react for 40 min at 95 °C in the dark. After the reaction, samples were analyzed in a Hitachi F2000 spectrophotometer with excitation at 532 nm and emission at 600 nm. The concentrations of TBARS were expressed as equivalents of malondialdehyde (MDA), which was used as standard.

**Catalase assay.** Catalase activity in the rat liver homogenate was assayed by a previous method.\(^{16}\) Briefly, 20 μl of homogenate was added to 980 μl of H₂O₂ solution (containing 30 μl of dlH₂O₂, 50 μl of Tris-HCl-EDTA, pH 8.0, and 900 μl of 10 nM H₂O₂). After 10 s at room temperature, the optical density of H₂O₂ was recorded at 240 nm for 1 min. One unit of catalase activity was defined of H₂O₂ consumed units/mg of protein.

**Determination of GSH content.** We determined the liver GSH content by the method of Hissin and Hilf.\(^{19}\) A stock solution of fluorescent probe o-phthalaldehyde (OPT) was freshly prepared in
Student's individual magnitudes. Statistical differences were analyzed by standard.

To perform immunoblotting, 50 μg of protein samples were separated in a 10% polyacrylamide gel and then blotted onto an nitrocellulose membrane (Sartorius, Goettingen, Germany) to react with primary antibodies (anti-p-JNK, p-P38, iNOS, COX-2 were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, and anti-α-tubulin, actin as internal control were from Sigma, St. Louis, MO) for 2 h and secondary antibody (peroxidase-conjugated goat antimouse antibody from Sigma) for 1 h. All incubations were performed at 37 ºC, and intensive PBS washing was carried out between the incubations. The reacted bands were revealed by enhanced chemiluminescence using an ECL commercial kit, and the relative photographic negatives by a gel documentation and analysis system (Alpha Imager 2000, Alpha Innotech, San Leandro, CA).

To investigate the effects of HPE on iNOS and COX-2 mediated by LPS, cells treated with 100 ng/ml of LPS and various concentrations of HPE (0.01–1.0 mg/ml) for 18 h. Total proteins (50 μg/lane) were prepared and subjected to immunoblot analysis as described above.

Statistical analysis. The results were reported as means ± S.D. from individual magnitudes. Statistical differences were analyzed by Student’s t-test, and differences were considered significant at *P < 0.05.

Results

**Effects of HPE on xanthine oxidase activity in vitro**

As shown in Fig. 1, HPE displayed the strongest inhibitory effect on xanthine oxidase activity, up to about 93% (EC50 = 0.742 mg/ml).

**Effects of HPE on LPS-induced NO and PGE2 production in macrophages**

The results summarized in Table 1 indicate that concentrations of HPE up to 0.5 mg/ml did not affect the viability of the cells (P > 0.05). After treatment with LPS (100 ng/ml) for 12 h, nitrate concentration in medium increased remarkably. When cells were treated with HPE accompanied by LPS, HPE significantly inhibited nitrite production. We also investigated the effects of HPE on LPS-induced PGE2 production in the macrophages. The results showed that HPE significantly suppressed the LPS-induced amount of PGE2 (Table 1).

**Effects of HPE on LPS-induced iNOS and COX-2 protein expression in macrophages**

As shown in Fig. 2, LPS induced the expression of iNOS protein in macrophages, and 0.5 mg/ml of HPE reduced iNOS protein to 20%. After co-treatment with HPE, the expression COX-2 protein was also inhibited significantly, in a dose-dependent manner.

**Effects of HPE on LPS-induced hepatic lesions in rats**

The liver sections of the control group had no inflammatory cells showing cellular damage (Fig. 3A). After LPS administration, liver lesions were found to exhibit infiltration by inflammatory neutrophils (Fig. 3B). In the 10 randomly selected high-power fields (× 400), lesions with an area extending over half of the field were observed. The liver pathological changes caused by LPS in the animals pretreated with HPE (10–40 mg/kg) were reduced (Fig. 3C, D and E). Only a few fields with lesions were observed, and the lesion areas in the HPE-treated groups were reduced as compared to the LPS-treated control group.

![Fig. 1. Inhibition of Xanthine Oxidase by HPE.](image)

![Fig. 2. Effects of HPE on LPS-Induced COX-2 and iNOS Protein Expression in Macrophages.](image)

![Table 1. Effects of HPE on LPS-Treated Macrophages](table)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cell viability (%)</th>
<th>Nitrite (μM)</th>
<th>Prostaglandin E2 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPE</td>
<td>LPS (100 ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>100.00 ± 8.16</td>
<td>4.81 ± 0.67</td>
<td>0.28 ± 0.00</td>
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<tr>
<td>0.05</td>
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</tr>
<tr>
<td>0.1</td>
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<td>50.26 ± 1.32</td>
<td>17.07 ± 0.04</td>
</tr>
<tr>
<td>0.5</td>
<td>110.19 ± 8.99</td>
<td>21.42 ± 1.03</td>
<td>16.77 ± 0.11</td>
</tr>
</tbody>
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Data are mean ± SD calculated for three independent examinations. Statistical significance analyzed by Student’s t-distribution.

*p < 0.005 compared with the normal group

*p < 0.05, **p < 0.001 compared with the group stimulated with LPS
peroxidation in the livers in contrast to the livers of rats.

This result was consistent with the increases were significantly diminished by 20–40 mg/kg administration of LPS significantly increased serum uric acid in acute hepatitic inflammation, the serum uric acid evaluate the role of xanthine oxidase in LPS-induced inflammation, also decreased after pretreatment with HPE (10–40 mg/kg). However, neither the liver nor the body weight was affected even by 40 mg/kg of HPE. To evaluate the role of xanthine oxidase in LPS-induced acute hepatic inflammation, the serum uric acid concentration was analyzed. The results indicated that administration of LPS significantly increased serum uric acid levels as compared to the control group. The increases were significantly diminished by 20–40 mg/kg of pretreated HPE. This result was consistent with the in vitro xanthine oxidase activity assay.

Effects of HPE on LPS-induced lipid peroxidation in rats

6 h of LPS administration caused marked lipid peroxidation in the livers in contrast to the livers of the control rats using MDA as standard. Under pretreatment with HPE (10–40 mg/kg) for 5 consecutive days using a gavage, MDA levels decreased significantly (Table 2).

Effects of HPE on hepatic antioxidative enzyme activity induced by LPS

After LPS (5 mg/ml) treatment, hepatic GSH levels and catalase activity decreased, and, gavaged with HPE for 5 d before LPS injection, the liver tissue lysate showed a dose-dependent increase in GSH levels and catalase activity (Table 2).

Effects of HPE on the expression of COX-2 and MAPK proteins in LPS-induced rat

Immunoblotting analysis showed that the protein expression of COX-2 increased following LPS stimulation, and that COX-2 protein gradually decreased with increasing concentrations of HPE (Fig. 4A). To investigate further whether the MAPK proteins (JNK and/or p38) are involved in the anti-inflammatory
mechanisms, the rat liver lysate was analyzed. As shown in Fig. 4B, pretreatment with HPE reduced LPS-induced protein expression of phosphorylated JNK and phosphorylated P38.

Discussion

Many phenolic substances present in dietary and medicinal plants possess antioxidative and anti-inflammatory properties that to some extent contribute to their cancer chemopreventive potential. The flower of Hibiscus sabdariffa Linnaeus calyx has been reported to possess antioxidation potential. In the present study, we confirmed that HPE possessed anti-oxidation activity in vitro, inhibited LPS-induced NO and PGE₂ production, and reduced COX-2/iNOS protein expression in LPS-treated macrophages. In an in vivo system, Sprague-Dawley rats were used to determine the effects of HPE in regulating LPS-induced hepatic damage. It was found that AST, ALT, and ALKP increased in the plasma after LPS injection. Nevertheless, HPE pretreatment significantly decreased these inflammatory markers induced by LPS. HPE was also found to have inhibitory potential on LPS-induced hepatic neutrophil infiltration. These findings suggest that HPE can be used to prevent inflammation. Decreased GSH levels and antioxidant enzymes such as catalase have been identified in the process of LPS treatment.

In our results, both GSH levels and catalase activity decreased in LPS-induced inflammatory liver. In the groups pretreated with HPE, the GSH levels and catalase activity improved significantly. Moreover, HPE also reduced the level of lipid peroxidation induced by LPS.

Mechanically, macrophage activation by bacterial lipopolysaccharide (LPS) promotes the secretion of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), and certain secondary mediators, such as leukotrienes and prostaglandins (PGs). These substances are important regulators of both innate and adaptive immunity. But, uncontrolled expression of them can cause acute and chronic inflammatory syndromes. An acute inflammatory syndrome induced by these mediators is the septic shock syndrome, characterized by fever, hypotension, disseminated intravascular coagulation, and multiple organ failure. Moreover, LPS induced iNOS and COX-2 gene expression in the rat liver, and the COX enzyme possesses both cyclooxygenase and peroxidase functions. Prostaglandins formed due to COX impair immune surveillance and modulate proliferation in a variety of cell types. The peroxidase function contributes to the activation of procarcinogens. During infection and inflammation, high production of NO has been found to cause DNA damage as well as mutations in vivo. It has also been found that overexpression of COX-2 or iNOS might be intimately involved in the pathogenesis of many diseases, such as colon cancer, multiple sclerosis, neurodegenerative diseases, and heart infarction. Our results indicate that HPE can decrease the levels of PGE₂, iNOS, and COX-2, but improve the antioxidative condition. Base on this, HPE can decelerate chronic diseases progression by regulating inflammatory or antioxidative molecules.

Activation of NFκB is necessary for LPS-induction of the iNOS and COX-2 promoter. NFκB is composed mainly of two proteins: p50 and p65. Exposed to proinflammatory stimuli, IκB becomes phosphorylated, ubiquitinated, and then degraded. Thus the liberated NFκB dimers are transllocated to the nucleus, where transcription of target gene is induced. In particular, JNK and p38 MAPK are known to play key roles in the LPS induced signal transduction pathway involving NFκB activation. In this study, we found that administration of LPS to rats brought about activation in the liver of JNK and p38 MAPK, which were then inhibited after pretreatment with HPE for 5 consecutive d. These results suggest that HPE, by inhibiting the activation of JNK and p38 MAPK, can suppress LPS-induced NFκB translocation and subsequently decrease the protein levels of COX-2 in in vivo experimentation. However, the direct relationship between NFκB and HPE remains to be further clarified.

Taken together, these results strongly suggest that HPE has the ability to prevent inflammation and that this extract, besides its anti-oxidative profile, is capable of impairing COX-2 induction by down-regulating JNK and p38 MAPK. It is therefore recommended that HPE be developed and applied as an anti-inflammatory agent.

Acknowledgment

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