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New Phenomenon

Anti-inflammatory activity of total flavonoids from seeds of *Camellia oleifera* Abel

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Inflammation is the primary response to infection or injury that functions to clear the injurious material or agent and promote tissue repair. However, when inflammation persists, such as chronic inflammation, it can cause tissue damage and loss of function. Persistent inflammation is closely associated with many chronic diseases, such as cancer, arthritis, osteoporosis, asthma, Alzheimer's disease, obesity, diabetes, and cardiovascular disease [1]. Numerous molecules such as cytokines, prostaglandins, and nitric oxide (NO) are involved in the induction and maintenance of the inflammatory response. Inhibition and/or down-regulation of these pro-inflammatory molecules may exert anti-inflammatory effects.

In conventional therapy, steroidal anti-inflammatory drugs and non-steroidal anti-inflammatory drugs are used to treat acute inflammation. However, they fail to cure chronic inflammatory diseases, such as rheumatoid arthritis and osteoarthritis. Furthermore, these compounds have several undesired side effects. Recently, anti-inflammatory activity of natural bioactive compounds is attracting growing interest because these compounds may offer a safer and effective treatment for inflammation, especially for long-term use [2].

Camellia oleifera Abel. belongs to the *Camellia* genus in the Theaceae family, which is widely distributed in the central and southern China. Its seeds have been used as oil material in China for more than 1000 years. *Camellia* seed oil is not only used as cooking oil, but also traditionally applied as a medicine for stomach ache and burning injury in China. Pharmacological studies indicated that seeds of *C. oleifera* contain various bioactive substances including unsaturated fatty acids, flavonoids, saponins, polysaccharides, and proteins, and possess many bioactivities such as antioxidation, antibacterial, anticancer, hepatoprotection, and anti-inflammation [3].

Flavonoids are members of a class of natural compounds widely distributed in the plant kingdom, and possess many bioactivities including antioxidation, antibacterial, antiviral, and protective effects from many diseases such as cancer, cardiovascular, and inflammation [4]. Kaempferol and several kaempferol glycosides have been isolated from the seeds of *C. oleifera*, and kaemferol-3-O-[2-O-β-D-glucopyranosyl-6-O-L-rhamnopyranosyl]-β-D-glucopyranoside and kaemferol-3-O-[2-O-β-D-xylopyranosyl-6-O-α-L-rhamnopyranosyl]-β-D-glucopyranoside were identified as the two main flavonoids [5–10]. Various flavonoids such as genistein, quercetin, daidzein, flavone, isorhamnetin, naringenin, and pelargonidin from fruits, herbs, and spices have been found to possess important activity on the inflammatory process *in vitro* and *in vivo* [11]. However, little is known about the anti-inflammatory effects of flavonoids from the *C. oleifera* seeds.

In this study, total flavonoids were prepared from the 80% ethanol extract of *C. oleifera* seeds by semi-preparative HPLC (Waters, Milford, USA), and the composition was characterized by UPLC–UV–MS (Waters) analysis. The anti-inflammatory activity of total flavonoids was evaluated by NO inhibitory assay in RAW 264.7 cells. Moreover, the effects of total flavonoids on the mRNA and protein expression levels of pro-inflammatory enzymes and cytokines including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), and macrophage inflammatory protein-1α (MIP-1α) were determined by quantitative real-time-polymerase chain reaction (qRT-PCR) and western blot analysis, respectively.

Four compounds (1–4) were detected in total flavonoid fraction of *C. oleifera* seeds by UPLC–UV–MS, and numbered corresponding to their order of elution (**Supplementary Fig. S1**). Quasi-molecular ions $[M-H]^-$ for compounds 1–4 were observed at 755.19, 739.12, 725.19, and 593.17 in negative ion mode, respectively (**Supplementary Fig. S2**). The fragment ions at *m*/*z* 285 suggested that the aglycone was kaempferol. These results were consistent with the previous reports [5–8,10], and the four compounds were identified as kaemferol-3-O-[2-O-β-D-glucopyranosyl-6-O-L-rhamnopyranosyl]-β-D-glucopyranoside (compound 1), kaempferol 3-O-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranosyl-7-O-α-L-rhamnopyranoside (compound 2), kaemferol-3-O-[2-O-β-

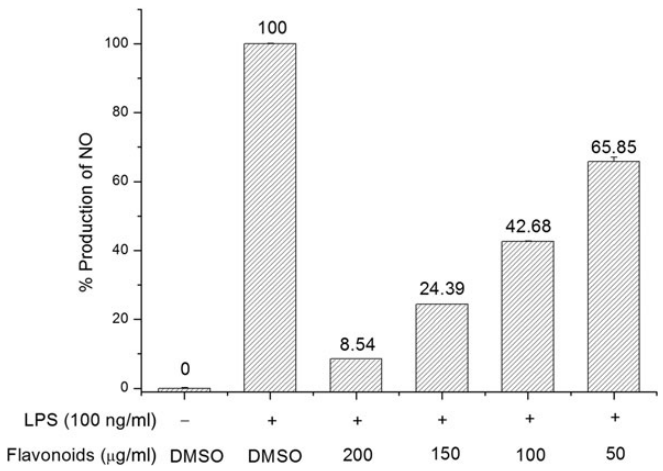

Figure 1. Total flavonoids of *C. oleifera* seeds reduce the NO production in LPS-stimulated RAW 264.7 cells Cells were incubated with different concentrations of the total flavonoids (0–200 μg/ml) for 2 h, and then co-treated with LPS (100 ng/ml) for 24 h. Cells treated without LPS and with vehicle (dimethyl sulfoxide, DMSO) were used as negative controls, and cells incubated with LPS and DMSO were used as positive controls. Data were represented as the mean ± SD from three independent experiments.

D-xylopyranosyl-6-O-α-L-rhamnopyranosyl]-β-D-glucopyranoside (compound 3), and kaemferol-3-O-α-L-rhamnopyranosyl (1→6)-β-D-glucopyranoside (compound 4). The structures of the four compounds are shown in **Supplementary Fig. S3**. The UPLC chromatogram showed that the percent composition of compounds 1–4 was 37.84%, 3.13%, 53.43%, and 4.19%, respectively, and the total percentage was 98.59%. This result indicated that the four kaempferol glycosides were the major flavonoids in *C. oleifera* seeds. Although several studies on the isolation and identification of flavonoids from the seed cake or seeds of *C. oleifera* have been previously carried out [5–10], this work for the first time reported a quick method for the UPLC–UV–MS analysis of flavonoids in *C. oleifera* seeds, which took only 10 min.

The anti-inflammatory activity of total flavonoids was determined in mouse macrophage-like cell line RAW 264.7. As shown in **Fig. 1**, lipopolysaccharide (LPS) treatment (100 ng/ml) obviously increased the amount of NO in RAW 264.7 cells. Total flavonoids significantly inhibited NO production in a concentration-dependent manner ($P < 0.05$). The

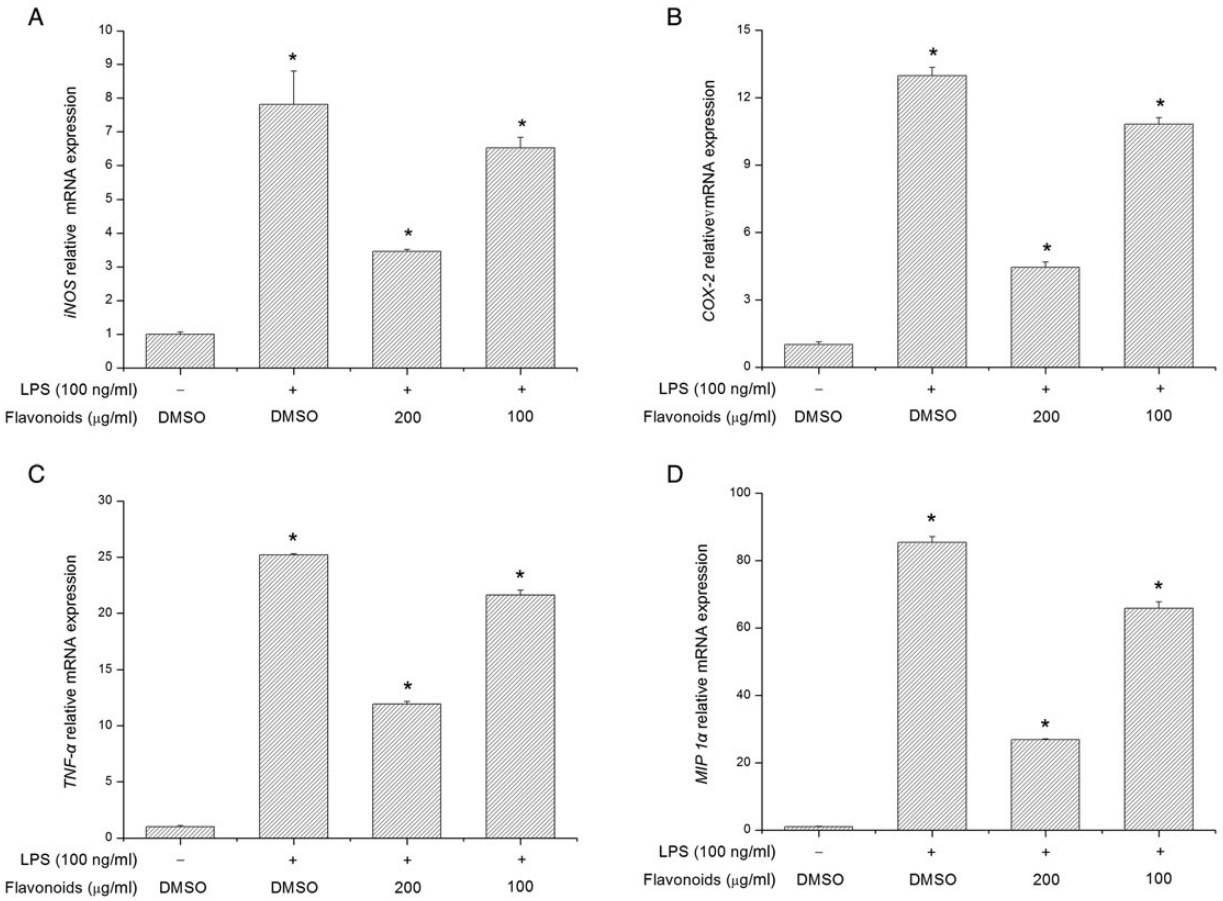

Figure 2. Total flavonoids of *C. oleifera* seeds reduce the mRNA over-expression of *iNOS*, *COX-2*, T*NF-α*, and *MIP-1α* in LPS-stimulated RAW264.7 cells determined by qRT-PCR (A) *iNOS*; (B) *COX-2*; (C) *TNF-α*; (D) *MIP-1α*. Cells were incubated with different concentrations of total flavonoids (0–200 μg/ml) for 2 h, and then co-treated with LPS (100 ng/ml) for 24 h. Cells treated without LPS and with vehicle (DMSO) were used as negative controls, and cells incubated with LPS and DMSO were used as positive controls. *GAPDH* mRNA levels were quantified in each sample and were used as a normalization control. Relative mRNA expression was calculated by the mean value with the comparative Ct method (ΔΔCt). Data were represented as the mean ± SD from three independent experiments. *$P < 0.05$ vs. control.

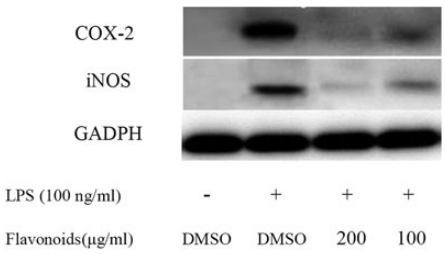

Figure 3. Total flavonoids of *C. oleifera* seeds reduce the protein over-expression of iNOS and COX-2 in LPS-stimulated RAW264.7 cells determined by western blot analysis Cells were incubated with different concentrations of total flavonoids (0–200 μg/ml) for 2 h, and then co-treated with LPS (100 ng/ml) for 24 h. Cells treated without LPS and with vehicle (DMSO) were used as negative controls, and cells incubated with LPS and DMSO were used as positive controls. GAPDH was used as an internal control.

NO production with the sample treatment (50–200 μg/ml) ranged from 65.85% to 8.54% compared with the control group. The IC_{50} value of the tested sample for NO production was estimated to be 87 μg/ml. MTT assay demonstrated that the total flavonoids have no significant inhibitory effect on RAW 264.7 cell survival up to 200 μg/ml (**Supplementary Fig. S4**), indicating that NO inhibitory activity was not due to the cytotoxic effect of samples.

The mRNA expression levels of *iNOS*, *COX-2*, *TNF-α*, and *MIP-1α* were determined by qRT-PCR using primers shown in **Supplementary Table S1**. As shown in **Fig. 2**, LPS significantly increased the mRNA levels of these genes, and total flavonoids remarkably reversed their overexpression in a dose-dependent manner ($P < 0.05$). This result indicated that total flavonoids of *C. oleifera* exerted anti-inflammatory effect by down-regulating the expression of *iNOS*, *COX-2*, *TNF-α*, and *MIP-1α*.

The effect of total flavonoids of *C. oleifera* seeds on the protein expression of iNOS and COX-2 was assessed by western blot analysis. As shown in **Fig. 3**, protein levels of iNOS and COX-2 were reduced in a dose-dependent manner, which confirmed the inhibitory effect of total flavonoids on the expression of iNOS and COX-2 in LPS-induced RAW 264.7 cells.

Excessive inflammation is considered to be a critical factor in many human diseases and conditions, including obesity, cardiovascular diseases, neurodegenerative diseases, diabetes, aging, and cancer. Numerous molecules are involved in the induction and maintenance of the inflammatory response [12]. Natural anti-inflammatory compounds have been used to mediate the inflammatory process and often have little side effect [13].

This study demonstrated that the four kaempferol glycosides were the major flavonoids in *C. oleifera* seeds. The total flavonoids of *C. oleifera* seeds exhibited excellent anti-inflammatory activity in suppressing NO production in LPS-stimulated macrophages. This activity might attribute to the regulations of pro-inflammatory enzymes and cytokines including iNOS, COX-2, TNF-α, and MIP-1α. Thus the flavonoids in *C. oleifera* seeds may have the potential to treat inflammation and related diseases. However, further studies are needed to purify each compound and investigate their effect on the inflammatory signaling pathways *in vitro* and *in vivo*.

Supplementary Data

Supplementary data are available at *ABBS* online.

Funding

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References

1. Lucas SM, Rothwell NJ and Gibson RM. The role of inflammation in CNS injury and disease. Br J Pharmacol 2006, 147: S232–S240.
2. Maroon JC, Bost JW and Maroon A. Natural anti-inflammatory agents for pain relief. Surg Neurol Int 2010, 1: 80.
3. Jin X. Bioactivities of water-soluble polysaccharides from fruit shell of *Camellia oleifera* Abel: antitumor and antioxidant activities. Carbohydr Polym 2012, 87: 2198–2201.
4. Havsteen BH. The biochemistry and medical significance of the flavonoids. Pharmacol Ther 2002, 96: 67–202.
5. Chen JH, Liau BC, Jong TT and Chang CMJ. Extraction and purification of flavanone glycosides and kaempferol glycosides from defatted *Camellia oleifera* seeds by salting-out using hydrophilic isopropanol. Sep Purif Technol 2009, 67: 31–37.
6. Du LC, Wu BL and Chen JM. Flavonoid triglycosides from the seeds of *Camellia oleifera* Abel. Chin Chem Lett 2008, 19: 1315–1318.
7. Gao DF, Xu M, Zhao P, Zhang XY, Wang YF, Yang CR and Zhang YJ. Kaempferol acetylated glycosides from the seed cake of *Camellia oleifera*. Food Chem 2011, 124: 432–436.
8. Li B and Luo Y. Studies on chemical constituents of *Camellia oleifera* Abel. Chem J Internet 2003, 5: 20–23.
9. Chen JH, Wu HY, Liau BC, Chang CMJ, Jong TT and Wu LC. Identification and evaluation of antioxidants defatted *Camellia oleifera* seeds by isopropanol salting-out pretreatment. Food Chem 2010, 121: 1246–1254.
10. Chen L. Extraction and separation, chemical structure characterization and biological Activity of flavonoid glycosides in camellia oleifera seed cake. Academic Dissertation. Nanchang University, 2011, 40–48.
11. Hämäläinen M, Nieminen R, Vuorela P, Heinonen M and Moilanen E. Anti-inflammatory effects of flavonoids: genistein, kaempferol, quercetin, and daidzein inhibit STAT-1 and NF-κ B activations, whereas flavone, isorhamnetin, naringenin, and pelargonidin inhibit only NF-κ B activation along with their inhibitory effect on iNOS expression and NO production in activated macrophages. Mediators Inflamm 2007, 2007: 45673.
12. Sharma J, Al-Omran A and Parvathy S. Role of nitric oxide in inflammatory diseases. Inflammopharmacology 2007, 15: 252–259.
13. Yuan G, Wahlqvist ML, He G, Yang M and Li D. Natural products and anti-inflammatory activity. Asia Pac J Clin Nutr 2006, 15: 143–152.