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Anti-Inflammatory Potential of *Citrus aurantiifolia* Peel Decoction in LPS-induced RAW 264.7 via Inducible Nitric Oxide Synthase (iNOS) Expression Downregulation

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Abstract

Citrus aurantiifolia peel is a major industrial by-product that has potential to be developed into functional food due to its rich bioactive compounds. *C. aurantiifolia* peel contains bioactive compounds, including flavonoids, terpenoids, and limonoids that exhibited anti-inflammatory properties. This study aimed to evaluate anti-inflammatory properties of *C. aurantiifolia* peel aqueous extract (CPE). *C. aurantiifolia* peel were extracted using boiling water, then the extract anti-inflammatory properties were tested on LPS-induced RAW 264.7 cells. Molecular docking simulations of CPE compounds to inducible nitric oxide synthase (iNOS) (PDB ID: 3E6T) and TNF-α (PDB ID: 7KP8) were conducted to identify bioactive compounds responsible for anti-inflammatory properties. The results showed that CPE reduced NO production by 63.48 % at 3.125 µg/mL and iNOS expression by 71.67 % at 12.5 µg/mL, but TNF-α production was not inhibited. Molecular docking revealed that hesperidin exhibited the highest iNOS binding affinity with a docking score of -104.78 kcal/mol, while hesperetin exhibited the highest TNF-α binding affinity with a docking score of -87.3311 kcal/mol. In conclusion, this study demonstrates that CPE exhibits anti-inflammatory properties through the reduction of NO concentration and iNOS expression, showing promising potential for development into functional food products.

Keywords: Inflammation, hesperidin, lime peel, molecular docking

Introduction

Inflammation is a protective biological response to noxious stimuli caused by pathogen infection, damaged cells, irritants, and autoimmune reactions (1,2). The signs of inflammation include redness, swelling, heat, pain, and loss of function. While acute inflammation plays crucial role in eliminating pathogens and promoting tissue regeneration, chronic inflammation on the other hand is associated with various diseases, such as cancer, atherosclerosis, non-alcoholic fatty liver disease, lupus erythematosus, cardiovascular disorders, diabetes mellitus, and autoimmune disorders (3,4). Although non-steroidal anti-inflammatory drugs (NSAIDs) are widely prescribed as anti-inflammatory drugs, their use raises safety concerns due to adverse effects on the gastrointestinal tract, kidney, and cardiovascular system (5,6). Furthermore, over the counter NSAIDs availability and accessibility contribute to self-medication risks and account for nearly 11% of preventable drug-related hospital admissions (7,8).

The Citrus genus, which is a part of Rutaceae family, is a widely cultivated crop worldwide with 158 million tons of annual production in 2020. *C. aurantiifolia* (key lime) is one of the most widely used citrus species, with annual production up to 21.3 million tons (9). While industrial processing of *C. aurantiifolia* primarily focuses on extracting essential oil and juice, this process generates a substantial amount of discarded by-product, including fruit peel, seed, and pulp (10). *C. aurantiifolia* peel is the major , accounting for 11-12% of the fruit weight (9).

C. aurantiifolia peel contains bioactive compounds, such as flavonoids, terpenoids, essential oils, coumarins, and limonoids. It exhibits diverse functional properties such as, hypolipidemic, antioxidant, hepatoprotective, nephroprotective, and anti-inflammatory effects (12). Regarding its anti-inflammatory properties, administration of *C. aurantiifolia* peel ethanolic extract demonstrated anti-inflammatory activity in carrageenan-induced paw oedema and reduced inflammatory cell infiltration in renal tissue (13,14). Hesperidin, the predominant *C. aurantiifolia* flavonoid compound, exerts anti-inflammatory properties by reducing NO, PGE2, TNF-α, and IL-6 in LPS-induced RAW 264.7 cells (15). *C. aurantiifolia* fruit peel essential oils (CPEO) also demonstrated anti-inflammatory properties, reducing TNF-α by 87% and NF-κB by 36% on dystrophic mice muscle and reduced inflammation in formalin-induced mice at a dosage of 100 mg/kg (16,17).

Incorporating *C. aurantiifolia* peel into functional food would enhance its economic value and reduce waste burden. It also complies to Sustainable Development Goals (SDGs), specifically SDG 3 (good health and well-being), SDG 7 (affordable and clean energy), and SDG 12 (responsible consumption and production). Additionally, traditional medicinal plants decoction was prepared by sorting, crushing, mixing, and boiling the plant materials in Indonesia (18). The decoction method in this study was employed to mimic the traditional practice and ensured that the biological activity remains relevant. Therefore, this study aims to evaluate anti-inflammatory properties of *C. aurantiifolia* peel using RAW 264.7 murine macrophages. Additionally, molecular docking simulations of reported *C. aurantiifolia* compounds to inducible nitric oxide synthase iNOS and TNF-α were conducted to identify active compounds responsible for anti-inflammatory properties.

Materials and Methods

Plant material and extraction

C. aurantiifolia fruit was collected from Cijeruk, Bogor, Indonesia (6.6815° S, 106.7971° E). The sample was identified and deposited at the Department of Biology, University of Indonesia with voucher specimen number J123-P-126. One hundred grams of dried *C. aurantiifolia* peel was extracted separately using 1000 mL of boiling water for 30 minutes. The extract was filtered and evaporated in a food dehydrator at 40°C to obtain concentrated *C. aurantiifolia* peel extract (CPE). To ensure reproducibility, the obtained extract was standardized in accordance with the Indonesian Herbal Pharmacopeia in a prior study (19).

CPE in-vitro anti-inflammatory properties
Cell culture

RAW 264.7 macrophage cells (ECACC 91062702) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-Amphotericin B solution (Gibco, USA). Cells were incubated until they reached 70-80% confluency.

Determination of cell viability

Cell viability was determined using an MTT reagent (CSH Protoc, USA). In a 96-well microplate, RAW 264.7 cells were seeded at a density of 1×10^5 cells per well in 100 µL of medium and incubated at 37°C with 5% CO_2 for 24 hours. Following the incubation, the cells were treated with 100 µL of CPE (1.56 – 200 µg/mL), then incubated under similar conditions. After treatment, 20 µL of MTT solutions was added to

100 µL of samples and cell mixture, then re-incubated under the same condition for 4 hours. The mixture was added with 150 µL of DMSO to stop the reaction and dissolve the formazan crystal, then the absorbance was measured using a microplate reader at 540 nm (20). The cell viability above 80% was considered non-toxic; 80%–60% viability was considered weak toxicity; 60%–40% was considered moderate toxicity, and less than 40% was considered strong cytotoxicity (21). Three highest nontoxic concentrations (viability > 80%) in RAW 264.7 cells were selected based on viability assays and further evaluated for antiinflammatory pathway analysis.

Determination of NO production

NO production assay determined using the Griess method was conducted in accordance with manufacturer's protocol (E-BC-K035-M, Elabscience, China). RAW 264.7 cells were treated with CPE (3.125 – 12.5 µg/mL) and incubated for 1 hour prior to LPS (1 µg/mL) induction, then incubated for 20 hours. Following the incubation, 100 µL of cell culture supernatant was mixed with 100 µL of Griess reagent, then the solution was incubated for 10 minutes and the absorbance was measured using a microplate reader at 540 nm.

Determination of iNOS production

iNOS production assay was conducted in accordance with manufacturer's protocol (E-EL-M0696, Elabscience, China) following the manufacturer's instructions. In brief, 100 µL of standard solution and CPE (3.125 – 12.5 µg/mL) were pipetted into a 96-well microplate followed by 90 minutes incubation at 37°C. Thereafter, wells were aspirated and added 100 µL of biotinylated detection Ab working solution, and further incubated for 30 minutes at 37°C. The wells were then washed three times with 350 µL of DPBS (1 minute each), added with 100 µL of HRP conjugate and incubated at 37°C for 30 minutes, and washed five times with 350 µL of DPBS (1 minute each). Subsequently, 90 µL of substrate reagent was pipetted into each well and incubated for 15 minutes at 37°C. The reaction was terminated by adding 50 µL of stop solution, and the absorbance was measured using a microplate reader at 450 nm.

Determination of TNF-α production

TNF-α production assay was conducted in accordance with manufacturer's protocol (E-UNEL-M0103, Elabscience, China) following the manufacturer's instructions. In brief, 100 µL of antibody working solution was added to each well and incubated overnight at 2-8°C. The antibody working solution was discarded, added 200 µL of blocking buffer, and incubated for 60 minutes at 37°C. The blocking buffer was discarded, then 100 µL of standard solution and CPE (3.125 – 12.5 µg/mL) were pipetted into a 96-well microplate followed by 90 minutes incubation at 37°C. Thereafter, wells were aspirated and added 100 µL of biotinylated detection Ab working solution. The wells were then washed three times with 350 µL of DPBS (1 minute each), added with 100 µL of HRP conjugate and incubated at 37°C for 30 minutes, and washed five times with 350 µL of DPBS (1 minute each). Subsequently, 90 µL of substrate reagent was pipetted into each well and incubated for 15 minutes at 37°C. The reaction was terminated by adding 50 µL of stop solution, and the absorbance was measured using a microplate reader at 450 nm.

Molecular docking

CPE compounds from a previous study were evaluated by molecular docking against iNOS (PDB ID: 3E6T) and TNF-α (PDB ID: 7KP8) from murine to align with the *in-vitro* anti-inflammatory assays (19). Molecular docking was performed using Molegro Virtual Docker V.5.0. Ligand preparation involved energy minimization in Chem3D. Protein preparation involved removal of water molecules and cofactors, and subsequently, internal validation was conducted to achieve RMSD value below 2 Å. The iNOS receptor (PDB: 3E6T) was analyzed using the Moldock score (scoring function) with iterated simplex (algorithm), with docking coor-

dinate of x,y,z = 123.29, 114.53, 35.94 and docking radius of 10 Å. The TNF-α receptor (PDB: 7KP8) was analyzed using Moldock score grid (scoring function) and iterated simplex (algorithm), with docking coordinate of x,y,z = -24.81, 18.59, -0.32 and docking radius of 9 Å.

Statistical analysis

The data analysis was carried out using SPSS software and analysed using ANOVA followed by Tukey post-hoc test with significant values $P < 0.05$. All data are presented as mean ± SD.

Results and Discussion

Determination of cell viability

Cell viability of CPE was determined using the MTT method, which measured the reduction of tetrazolium salt to formazan crystals by viable cells (22). The assay identified the non-cytotoxic concentration of for RAW 264.7 cells. As shown in Figure 1, CPE exhibited more than 80% cell viability up to 12.5 µg/mL, indicating the maximum non-toxic concentration for anti-inflammatory pathway analysis (21).

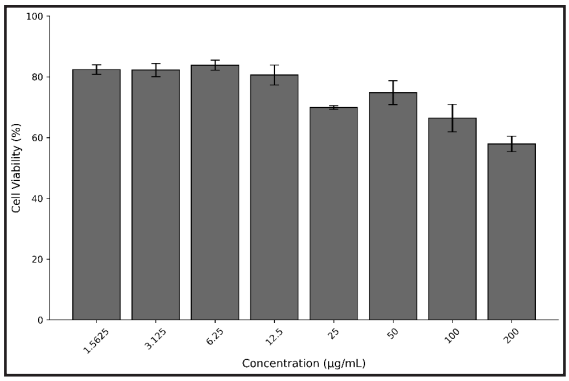

Figure 1. Cell viability assay of *C. aurantiifolia* peel extract (CPE) to RAW 264.7 cells. The results were presented as mean ± SD.

CPE anti-inflammatory effects in LPS-induced RAW 264.7 cells

CPE *in-vitro* anti-inflammatory effects were assessed using LPS-induced RAW 264.7 cells, and the levels of NO, iNOS, and TNF-α levels were quantified. As shown in Figure 2a-c, LPS significantly increased the concentrations of inflammatory mediator and pro-inflammatory cytokines. This inflammatory response is induced when LPS binds to Toll-like receptor 4, which subsequently triggers the NF-κB signaling pathway (23). Activation of NF-κB upregulates iNOS/NO pathway and enhances the secretion of proinflammatory cytokines, including IL-1, IL-6, IFN-I, and TNF-α (24). Figure 2a shows that CPE reduced NO production by 40.91 to 63.48 % in inverse concentration manner. Figure 2b shows that CPE reduced iNOS production by 25.63 – 71.67 %. However, as shown in Figure 2c, CPE did not reduce TNF-α production.

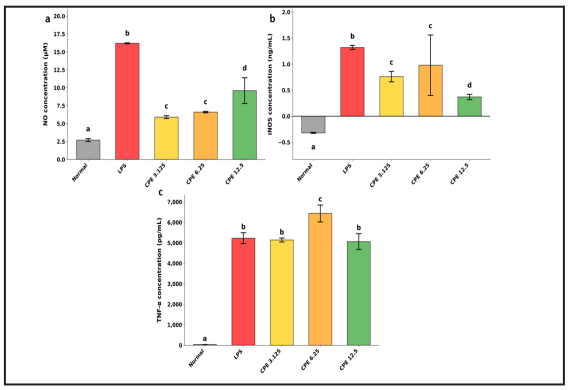

Figure 2. In vitro anti-inflammatory properties of *C. aurantiifolia* peel extract (CPE) in LPS-induced RAW 264.7 cells. (a) NO levels, (b) iNOS levels, and (c) TNF-α levels. The results were presented as mean ± SD. Different letters show $p < 0.05$.

Molecular docking

Molecular docking simulations were performed to support the *in-vitro* iNOS and TNF-α inhibition results. As shown in Table 1, hesperidin exhibited the highest iNOS inhibition with a docking score of -104.78 kcal/mol, while hesperetin exhibited the highest TNF-α inhibition with a docking score of -87.3311 kcal/mol. Hesperidin formed hydrogen bonds with seven amino acid residues to iNOS, in which four hydrogen bonds with the glycoside moiety (Tyr A:341, Asp A:346, Trp A:366, and Pro A:344),

three hydrogen bonds occurred to flavonoid skeleton (Arg A:382, Gln A:257, and Arg A:260), and two hydrophobic interactions with Val A:346 and Arg A:375 (Figure 3a). Hesperetin interactions to TNF-α dominantly due to hydrophobic interactions of C ring and methoxy group at C4' to Tyr C:59, Leu A:57, Val A:122, and Leu A:156 with one additional hydrogen bond at the C5 hydroxyl group (Figure 3b).

Table 1. Docking score of CPE compounds to iNOS (PDB ID: 3E6T) and TNF-α (PDB ID: 7KP8)

No	Compounds	Docking score (kcal/mol)	
		iNOS (PDB ID: 3E6T)	TNF-α (PDB ID: 7KP8)
1	Hesperidin	-104.7800	34.219
2	Hesperetin	-71.1009	-87.3311
3	Naringin	-101.7450	20.7599
4	Narigenin	-64.2070	-79.3106
5	Limonin	-77.9147	1.8350
6	Bergaptol	-58.3752	-69.7889
7	Citric Acid	-65.3851	-55.7935
8	Quercetin	-76.5731	-71.3107
9	Rutin	-91.9005	26.3548
10	Scoparone	-62.3862	-68.1584

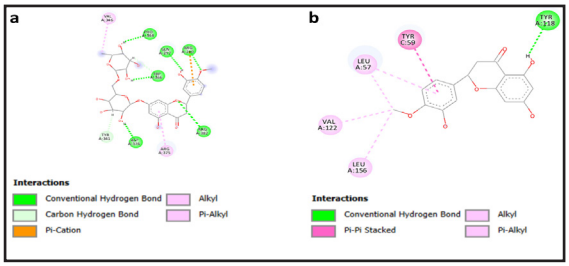

Figure 3. Docking visualization of a) Hesperidin with iNOS and b) Hesperetin with TNF-α

Discussion

Several studies have demonstrated *C. aurantiifolia* peel in-vivo anti-inflammatory properties. Previous study showed that *C. aurantiifolia* peel ethanol extract reduced oedema in carrageenan-induced paw oedema within 3 hours at a dose of 250 mg/kg and within 2 hours at 500 mg/kg. Additionally, it significantly reduced inflammatory cell infiltration in kidney tissue at a dose of 100 mg/kg and ameliorated kidney inflammation at a dose of 400 mg/kg in doxorubicin-induced kidney damage (14).

CPEO has been shown to reduce pro-inflammatory cytokines and inflammatory mediators. CPEO reduced TNF-α levels by 87% and NF-κB by 36% on dystrophic muscle (17). CPEO also showed anti-inflammatory effects in formalin-induced mice at a dose of 100 mg/kg. Further investigation showed that it reduced leukocyte migration, TNF-α, and IFN-γ in carrageenan-induced inflammation at a dose of 30 mg/kg and 100 mg/kg, while reducing protein extravasation, IL-1β, and NO concentration at dose of 100 mg/kg (16). The pronounced CPEO anti-inflammatory properties are suggested to be due to the abundance of D-limonene content, measured at 29.69% (25). D-limonene has exhibited anti-inflammatory properties (16,26). However, CPEO administration demonstrated myelotoxicity due to its high citral concentration and sub-chronic toxicity (16,27). On the other hand, *C. aurantiifolia* extract showed no toxicity up to 2000 mg/kg, making it a safer option for functional food application (28).

In this study, CPE demonstrated anti-inflammatory properties by reducing NO and iNOS concentrations. NO is a physiological molecule synthesized from L-arginine by nitric oxide synthases (NOS), which exist in three isoforms: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Among these isoforms, iNOS is responsible for produces high levels of NO during pathogen infection. Although NO and iNOS play important functions against pathogen infection, excessive production of NO and iNOS in chronic inflammation generates more reactive nitrogen species (RNS) that led to tissue damage and autoimmune diseases (29). From molecular docking results (Table 1), hesperidin and naringin showed the highest iNOS inhibition. These findings are aligned with previous studies showing hesperidin significantly

reduced iNOS expression and NO concentration at 10 μM (30). Naringin reduced NO and TNF-α production in LPS-induced mice and reduced NO, iNOS, TNF-α, COX-2, and IL-6 in LPS-induced RAW 264.7 cells. Anti-inflammatory properties of naringin are suggested due to NF-κB inhibition (31).

TNF-α is an inflammatory cytokine produced by activated macrophages, natural killer cells, and T-lymphocytes. TNF-α is responsible in inflammatory response against pathogens, however overproduction of TNF-α may lead to rheumatoid arthritis, psoriasis, Crohn's disease, and other autoimmune disease (32,33). As shown in Figure 2c, CPE did not reduce TNF-α concentration. Molecular docking results supported the finding, revealing that major CPE compounds such as hesperidin, naringin, and limonin showed negligible inhibition (Table 1). The molecular docking results align with previous study that demonstrated hesperidin as the major flavonoid compound in CPE demonstrated inferior TNF-α production inhibition than hesperetin (15). However, other studies have demonstrated Citrus peel properties to inhibit TNF-α production. *C. unshiu* reduced TNF-α concentration and TNF-α mRNA expression at 2.5 μg/mL*, C. aurantium* reduced TNF-α mRNA expression at 10 μg/mL through NF-κB pathway*, and C. reticulata* reduced TNF-α production at 200 μg/mL (34–36).

The structure-activity relationship (SAR) of hesperidin also supports the iNOS inhibition rather than TNF-α production inhibition. The C2-C3 double bond, hydroxyl groups at C3' and C4', and the absence of a hydroxyl group at C3 favour both activities. The presence of hydroxyl groups at C5 and C7 is also important in TNF-α inhibition. While both activities show similar SAR patterns, sugar moiety enhances the iNOS inhibition, but is detrimental for TNF-α inhibition (37). The results presented in Table 1 align with the SAR of iNOS and TNF-α inhibition. Hesperidin, naringin, and rutin showed higher docking affinity to iNOS compared to the aglycone counterpart (hesperetin, naringenin, and quercetin). Conversely, for TNF-α inhibition, hesperetin, naringenin, and quercetin showed higher docking affinity than the glycosylated counterpart (hesperidin, naringin, and rutin).

Conclusion

Overall, this study demonstrated anti-inflammatory properties of CPE by reducing NO concentration and iNOS expression. The observed anti-inflammatory effects of CPE suggest its potential for further investigation into functional food development. However, this study was also limited to the modulation of NO, iNOS and TNF-α, and additional pathway studies are required to fully elucidate the anti-inflammatory mechanism. Furthermore, further *in-vivo* anti-inflammatory and toxicity evaluations need to be conducted to describe the effects in complex biological systems.

Authorship contribution

N.M.D.S.: Conceptualization, methodology, investigation, resources, writing - review & editing, supervision, visualization, project administration. Y.D.: Investigation, methodology, supervision, writing - review & editing. D.W.I.: Investigation, methodology, supervision, writing - review & editing. D.K.P.: Investigation, methodology, supervision, writing - review & editing. S.R.: Investigation, writing – original draft. F.X.: Investigation, writing – original draft.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

Not applicable

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Data availability

Data will be made available upon request

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