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Original Research Article

Anti-inflammatory activity of *Cinnamomum cassia* bark on macrophage migration inhibitory factor (MIF) protein and expression levels of pro-inflammatory mRNAs and cytokines

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Abstract

Purpose: *To assess the inhibitory effect of extract of Cinnamomum cassia* (cinnamon) *bark on macrophage migration inhibitory factor (MIF) protein, expression levels of pro-inflammatory mRNAs and cytokines of macrophage RAW 264.7 cells.*
Methods: *Inhibition of MIF tautomerase activity following administration of cinnamon bark extract was measured and the IC_{50} value was determined. Expressions of pro-inflammatory TGF-β1, TNF-α and IL-8 mRNAs were determined using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Also, IL-1β and IFN-γ cytokines were evaluated using enzyme-linked immunosorbent assay (ELISA).*
Results: *Cinnamon bark extract displayed inhibitory activity on MIF protein with an IC_{50} value lower than 1 mg/L, and reduction of the expressed levels of TGF-β1, TNF-α and IL-8 mRNAs; and IL-1β, and IFN-γ cytokines of macrophage RAW 264.7 cells with varying levels of effectiveness depending on the specific pro-inflammatory mediators.*
Conclusion: *Extract of cinnamon bark decreases the expression of TGF-β1, TNF-α, and IL-8 pro-inflammatory mRNAs, as well as IL-1β and IFN-γ pro-inflammatory cytokines released by macrophage RAW 264.7 cells induced by LPS.*

Keywords: *Cinnamomum cassia, MIF, macrophage, Anti-inflammatory, Pro-inflammatory mRNAs, Cytokines, Chronic diseases*

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INTRODUCTION

Cinnamomum cassia (cinnamon) from the Lauraceae family has been used empirically to treat diseases such as cardiovascular, gastrointestinal, and other chronic diseases associated with inflammation. The plant parts usually used for medications are its branches and leaves. Cinnamon bark has a golden-brown colour and thickness of 1.5 cm. Cinnamon leaves are dark green in colour, oval in shape, and 17 – 20 cm in length.

The phytochemistry and pharmacology of cinnamon barks and leaves have been identified and reported in several studies which include antitumor and anti-inflammatory effect [1]. Terpenoid, phenylpropanoid, and cinnamaldehyde compounds are the most abundant components, with cinnamaldehyde being particularly representative of cinnamon quality [2]. The cinnamaldehyde compounds in cinnamon include cinnamaldehyde, cinnamate, and cinnamic acid. Cinnamon essential oil contains borneol, eugenol, α-copaene, coumarin, β-caryophyllene, α-terpineol, α-cubebene, cinnamyl acetate, trans-cinnamaldehyde, E-nerolidol, and caryophyllene oxide [3].

The eugenol compounds are found more abundantly in cinnamon leaves. Cinnamon barks comprise 1 – 2 % essential oil, the primary component containing 78 – 81 % cinnamaldehyde and a small amount of eugenol. Cinnamon barks are the most commonly used part of the plant. Extracting bioactive compounds from cinnamon depends on solubility of the compound in the solvent used, as it has low solubility in aqueous solvents, high solubility in ether and chloroform, and mix well with ethanol and oils [4]. Although cinnamon bark is known for various biological activities, the potential anti-inflammatory effect has not been fully investigated.

The anti-inflammatory evaluation of cinnamon bark extract in this study was conducted on macrophage migration inhibitory factor (MIF), a protein having an important role in inflammation by inhibiting random migration of macrophages [5], and on macrophage, the immune cells that play crucial roles in the inflammation progression [6–7]. Inhibition of MIF activity and reduction of expressed levels of pro-inflammatory mRNAs and cytokines of macrophage may contribute to its anti-inflammatory effects. Therefore, this study investigated the anti-inflammatory effect of extract of cinnamon bark in macrophage RAW 264.7 cells.

EXPERIMENTAL

Materials

Cinnamomum cassia barks and leaves were obtained from the organic garden of HRL International Company, in Mondoluku Village, Gresik Regency, East Java Province, Indonesia and confirmed by the Department of Information and Development of Traditional Medicine Center, Faculty of Pharmacy, University of Surabaya. The samples were dried in an oven-dryer at 40 °C for 3 days, pulverized and sieved through an 80-mesh sieve to obtain fine powder. The powdered samples were stored in plastic bags and tightly sealed in closed containers at room temperature (25 °C).

Extraction of cinnamon samples

Powdered cinnamon sample (10 g) was macerated in 100 mL 96 % ethanol (1:10) at 30 °C for 3 days with agitation at 175 rpm in a shaker incubator. Thereafter, it was filtered using filter paper and the filtrate was evaporated using a rotary evaporator. The dried extract was weighed and stored at 4 °C in the refrigerator till further use [8].

Evaluation with MIF protein

The MIF protein was produced from *E. coli* BL21(DE3). Inhibition of MIF tautomerase activity by the cinnamon extract was evaluated based on the absorbance of the borate complex with the enol form of 4-HPP as a product of the tautomerase reaction. The reaction mixture for positive control measurement was MIF protein in borate buffer pH 6.2, dimethyl sulfoxide (DMSO) solvent, and 1 mM 4-HPP substrate. Extract inhibition included all reaction components of the positive control measurement with extract solution in DMSO. For the positive inhibition control, Cu^{2+} solution (50 µM) was used instead of the extract solution. The negative control reaction mixture included all components of the positive control but borate buffer pH 6.2 without MIF protein [9].

Evaluation with macrophage RAW 264.7 cell

Cell viability assay was conducted on macrophage RAW 264.7 cells using MTS technique, which involves the conversion of the yellow salt of tetrazolium to a purple product of formazan following extract administration. Cells were seeded in a 96-well plate containing 5×10^5 cells in 180 µL of medium per individual well. The cells were allowed to grow at 37 °C for 24 h with 5 % CO_2 until confluent. Lipopolysaccharide (LPS) at 1 µg/mL was introduced to individual well and incubated for 18 h. Thereafter, different concentrations (ranging from 3.1 - 12.5 µg/mL) of cinnamon bark extract were added to the wells, while control wells received either only medium (negative control) or medium with LPS (positive control). After 24 h incubation, 20 µL per well of Cell Proliferation Assay Kit (Abcam ab197010) was introduced and absorbance read at 490 nm after 3 h [10].

Expression of inflammatory indices

The cells were plated in a similar manner and exposed to LPS for 18 h. Thereafter, extract of cinnamon (3.1 - 12.5 µg/mL) was administered for cell viability optimization. After 24 h, the cells were harvested, and centrifuged. Expressed levels of TGF-β1, TNF-α, and IL-8 mRNAs were evaluated using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), and enzyme-linked immunosorbent assay (ELISA) was done to quantify expression of IL-1β and IFN-γ cytokines. Expressed levels of pro-inflammatory mRNAs and cytokines were evaluated under inflammatory conditions by induction with LPS (positive controls) and after treatment with extract of cinnamon bark.

Expression of mRNAs

The total mRNA of the macrophage RAW 264.7 cell pellet was isolated using an RNA isolation kit (Zymo, R2073). Thereafter, 200 ng total mRNA was used for cDNA synthesis with Reverse Transcription Supermix (Bio-Rad 170-8841). The TGF-β1, TNF-α, and IL-8 gene copy numbers were determined using qRT-PCR with primers from Origene. Expressed level (i.e. the relative expression) of mRNAs was obtained by normalization to the β-actin reference gene.

Expression of cytokines

Individual well of the 96-well plate received 100 µL of cinnamon bark extract and incubated for 90 min at 37 °C. Any unbound component was eliminated, and phosphate-buffered saline (PBS) was used to wash the wells. Thereafter, 100 µL of biotinylated antibody was introduced to individual well and incubated for 60 min at 37 °C. The wells were washed with PBS, and 100 µL avidin-horseradish peroxidase was introduced to individual well and incubated for 30 min at 37 °C. The addition of 50 µL H_2SO_4 solution to each well was used to stop the reaction, and absorbance was read at 450 nm representing the expressed levels of IL-1β and IFN-γ cytokines.

Data analysis

Data calculations and graph presentations were conducted either with GraphPad Prism version 8.0 software (GraphPad Software, USA) or with Microsoft Excel 365 software.

RESULTS

Inhibition of MIF activity

The bark and leaf extract were evaluated for anti-inflammatory effects by inhibiting MIF protein. The results indicated that the bark extract has a lower IC_{50} value compared to the leaf extract (Figure 1).

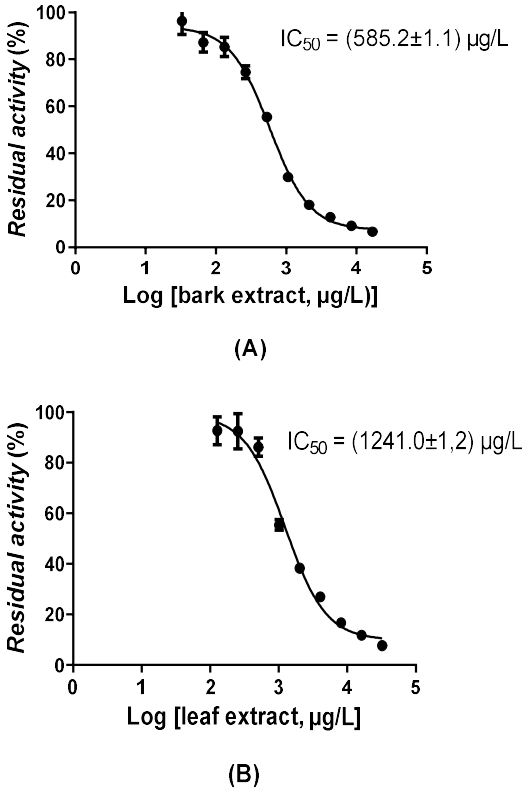

Figure 1: IC_{50} values of (A) cinnamon bark extract and (B) cinnamon leaf extract in inhibiting MIF tautomerase activity. Determinations were done in triplicates

Cell viability and anti-inflammatory activity

Results on cell viability using macrophage RAW 264.7 cells indicated that cinnamon bark extract at 3.1 to 12.5 µg/mL demonstrated higher cell viability percentage compared to LPS-induced inflammation (positive control; Figure 2).

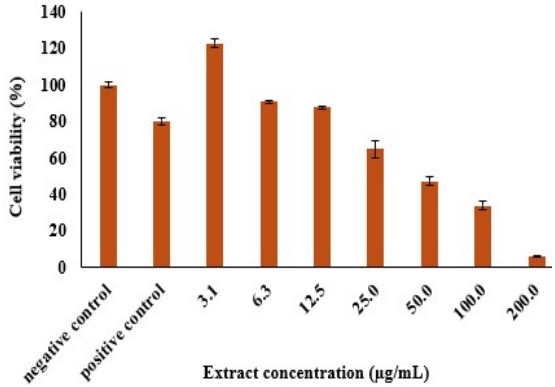

Figure 2: Viability of macrophage RAW 264.7 cells upon cinnamon bark extract treatment. The experiments were conducted in three replications

Anti-inflammatory activity on macrophage RAW 264.7 cells

Extract concentrations (3.1 to 12.5 µg/mL) demonstrated higher cell viability percentage compared to LPS-induced inflammation (positive control). Furthermore, cinnamon extract decreased expression levels of TGF-β1 (Figure 3 A), TNF-α (Figure 3 B), and IL-8 (Figure 3 C) pro-inflammatory mRNAs (Figure 3).

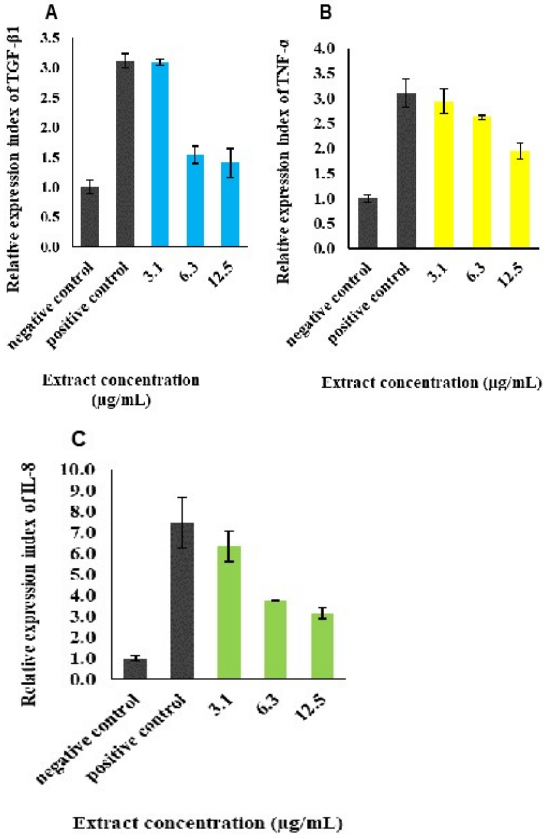

Figure 3: Reduction of relative expression of pro-inflammatory (A) TGF-β1, (B) TNF-α, (C) IL-8 mRNA of macrophage RAW 264.7 cells following treatment with cinnamon bark extract

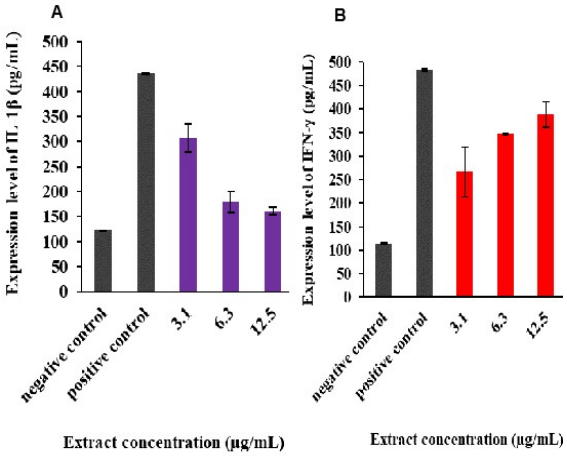

Figure 4: Reduction of expressed levels of (A) IL-1β, (B) IFN-γ cytokine of macrophage RAW 264.7 cells following administration of extract of cinnamon bark. Determinations were done in triplicates

Effect on inflammatory cytokines

The results indicated a significant decrease in the expression level of IL-1β ($p < 0.05$; Figure 4 A), and a dose-dependent increase in the expression of IFN-γ at lower concentrations compared to that at higher concentrations (Figure 4 B).

DISCUSSION

It has been reported that certain ligands of cinnamate analogues bind with Pro-1 of MIF protein in *in-silico* study. Similarly, some potent MIF inhibitors were found among cinnamic acid derivatives. Also, cinnamaldehyde at micromolar concentrations was reported to inhibit pro-inflammatory cytokines secretion from monocytes/macrophages by inhibiting intracellular signaling [11]. Furthermore, MIF protein is one of the mediators that stimulate production of pro-inflammatory cytokines suggesting that cinnamaldehyde inhibits MIF activity.

The findings of this study align with the previously reported outcomes. Extracts of cinnamon barks and leaves showed inhibitory activities on MIF protein. The IC_{50} of cinnamon bark extract was lower compared to leaf extract. A lower IC_{50} on MIF activity of the bark extract compared to leaf extract indicates greater capability to inhibit the activity of MIF protein and a higher potential not to inhibit the random migration of macrophages, hence it may alleviate inflammation. Based on this greater potential, cinnamon bark extract was used for the

evaluation of levels of expression of pro-inflammatory mRNAs and cytokines of macrophage RAW 264.7. Results of cell viability assay indicated that the bark extract at 12.5 µg/mL demonstrated higher cell viability exceeding 80 % surpassing that of the LPS-induced inflammation (positive control). This suggests that cinnamon bark extract at a concentration of up to 12.5 µg/mL is considered safe for cells.

This study has also revealed that cinnamon bark extract at concentration of 3.1 µg/mL reduced the relative expression of pro-inflammatory mRNAs. Similarly, administering the extract at that particular concentration reduced expression levels of pro-inflammatory cytokines in ELISA assay. At extract concentration of 6.3 µg/mL, TGF-β1, TNF-α, and IL-8 mRNAs relative expression decreased further, suggesting that the anti-inflammatory potency of the extract at higher concentration is fundamentally greater compared to lower concentrations.

Initial stages of chronic diseases are usually marked by increased inflammation due to a rise in TGF-β1 expression. However, this cytokine may help reduce inflammation in the later stages of inflammatory diseases [12]. Hence TGF-β1 is an important cytokine for homeostasis of the immune system [13]. In this study, LPS-induced inflammation showed around 200 % rise in the relative expression of TGF-β1 mRNA (from 1.0 to 3.1), indicating initial stage of inflammation. Treatment with extract of cinnamon bark reduced the expression of TGF-β1 by about 65 % (from 3.1 to 1.5) at 6.3 µg/mL concentration, and also by about 65 % (from 3.1 to 1.4) at 12.5 µg/mL.

Primary cytokine produced by induced macrophages is TNF-α. Its elevated expression enhances inflammation response and is linked to the progression of some chronic diseases [12].
In this study, LPS-induced inflammation increased the relative expression of TNF-α mRNA by around 200 % (from 1.0 to 3.1), indicating inflammation. Cinnamon bark extract treatment lowered this level by 6.5 % (from 3.1 to 2.9) at 3.1 µg/mL, 16 % (from 3.1 to 2.6) at 6.3 µg/mL and 39 % (from 3.1 to 1.9) at 12.5 µg/mL.

The cytokine widely used for diagnosing and predicting outcomes of various inflammatory conditions is IL-8 [14]. It is produced mainly by macrophages and has a role in the progression of rheumatoid arthritis. Following LPS induction, the relative expression of IL-8 mRNA rose by around 650 % (from 1.0 - 7.5), indicating inflammation which was reversed following administration of extract of cinnamon bark.

Another cytokine for initiating and triggering expression of pro-inflammatory cytokines (such as IL-8) is IL-1β [15]. The expression level of IL-1β cytokine rose by 250 % (from 125 to 435 pg/mL) following LPS-induced inflammation which was reversed by extract of cinnamon bark. The higher the extract concentration, the greater the decrease of IL-1β expression level is.

Another important cytokine in the development of inflammatory disorder is IFN-γ. Following stimulation by IFN-γ, macrophage enhances TNF-α gene transcription [12]. Expression of IFN-γ cytokine in this study rose by 300 % following LPS-induced inflammation, and this was followed by a rise in the relative expression of TNF-α mRNA. Treatment with extract of cinnamon bark demonstrated that expression of IFN-γ cytokine is greater at relatively lower concentrations compared to higher concentrations.

Previous studies reported the MIF activity inhibition by lime peel extract (IC_{50} of 70 mg/L) [14,16] and probiotics extract (IC_{50} of 8 mg/L) [9]. This study revealed that extract of cinnamon bark inhibited MIF protein with an IC_{50} lower than 1 mg/L, reduced relative expression of TGF-β1, TNF-α, IL-8 mRNAs, IL-1β and IFN-γ cytokines.

CONCLUSION

The ethanol extract of cinnamon bark inhibits MIF tautomerase activity and reduces the expressions of TGF-β1, TNF-α, IL-8, IL-1β, and IFN-γ pro-inflammatory mediators of macrophage RAW 264.7 cells. Thus, *Cinnamomum cassia* bark has potentials as an anti-inflammatory agent in inflammation-associated chronic diseases.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

Tjie Kok designed the project, supervised the experiments, analyzed the data, and revised the manuscript; Marco Hadisurya Susilo conducted the experiments, analyzed the data and wrote the manuscript. All authors have read and agreed to the published version of manuscript.

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