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Corilagin potential in inhibiting oxidative and inflammatory stress in LPS-induced murine macrophage cell lines (RAW 264.7)

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ABSTRACT

Objective(s): Inflammation is thought to be the common pathophysiological basis for several disorders. Corilagin is one of the major active compounds which showed broad-spectrum biological and therapeutic activities, such as antitumor, hepatoprotective, anti-oxidant, and anti-inflammatory. This study aimed to evaluate the anti-oxidant and anti-inflammatory activities of corilagin in LPS-induced RAW264.7 cells.

Materials and Methods: Anti-oxidant activities were examined by free radical scavenging of H_2O_2, NO, and *OH. The safe concentrations of corilagin on RAW264.7 were determined by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay on RAW264.7 cell lines. The inflammation cells model was induced with LPS. The anti-inflammatory activities measured IL-6, TNF-α, NO, IL-1β, PGE-2, iNOS, and COX-2 levels using ELISA assay.

Results: The results showed that corilagin had a significant inhibition activity dose-dependently in scavenging activities toward H_2O_2, *OH, and NO with IC_{50} values 76.85 µg/ml, 26.68 µg/ml, and 66.64 µg/ml, respectively. The anti-inflammatory activity of corilagin also showed a significant decrease toward IL-6, TNF-α, NO, IL-1β, PGE-2, iNOS, and COX-2 levels at the highest concentration (75 µM) compared with others concentration (50 and 25 µM) with the highest inhibition activities being 48.09%, 42.37%, 65.69%, 26.47%, 46.88%, 56.22%, 59.99%, respectively ($P<0.05$).

Conclusion: Corilagin has potential as anti-oxidant and anti-inflammatory in LPS-induced RAW 264.7 cell lines by its ability to scavenge free radical NO, *OH, and H_2O_2 and also suppress the production of proinflammatory mediators including COX-2, IL-6, IL-1β, and TNF-α in RAW 264.7 murine macrophage cell lines.

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Introduction

Over the past two years, inflammation has become a medical concern for researchers, especially those related to COVID-19. Inflammation is a complex mechanical process involved various compounds. Inflammation is a response to irritation and infection caused by pathogens, injury, and chemicals (1, 2). Reactive oxygen species (ROS) such as anion superoxide (O_2^{*-}), hydroxyl radical (*OH), peroxide radicals (ROO), and nitric acid radical (NO) are key signaling molecules that contribute to the progression of inflammatory diseases (3, 4).

Anti-oxidants are the components to neutralize the effect of free radicals, but the effect will be limited to specific anti-oxidants (5). The anti-oxidant agent plays an important role in setting up this intricate balance in the cells (6). The natural bioactive compounds in various plants have anti-oxidant activity. They may help in preventing several inflammatory diseases through free-radical scavenging activity (7). Corilagin was isolated from *Caesalpinia coriaria* (Jacq.) Willd. (dividivi) by Schmidt in 1951 for the first time (8). It was reported to exhibit anti-oxidant (9), anti-cancer (7), anti-inflammatory, and hepatoprotective activities (10). Previous reports have reported that corilagin can inhibit Tumor Necrosis Factor-α (TNF-α) expression and radiation-stimulated microglia activation by controlling the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (11). Corilagin also has the potential to remedy inflammation and oxidation-related diseases through the capability toward hepatic protection, by blocking the NF-κB pathway, has anti-oxidative effects, and can act as a hepatoprotective agent (10).

Based on the potential of corilagin, this study evaluates the anti-oxidant and anti-inflammatory activities of corilagin through scavenger ability on hydrogen peroxide (H_2O_2), hydroxyl (*OH), nitric oxide (NO) free radicals, and also inhibitory activity of pro-inflammatory mediators including induceble Nitrogen Synthase (iNOS), cyclooxigenase-2 (COX-2), Interleukin (IL)-6, IL-1β, and TNF-α in Lipopolysaccharide (LPS)-induced macrophage cells.

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Materials and Methods

H_2O_2 scavenging activity assay

Ferrous ammonium sulfate (1 mM, 12 µl), samples at various concentrations, and H_2O_2 (5 mM, 3 µl) were transferred into a 96-well plate and incubated at room temperature for 5 min. Then, 75 µl of 1,10-phenanthroline was added, and the plate was incubated again for 10 min in the same condition (12, 13). The mixture absorbance was read using a microplate reader at a wavelength of 510 nm and expressed as a percentage of H_2O_2 scavenging activity, which was computed using the equation below:

$$\% \ H_2O_2 \text{ Scavenging Activity} = (1-(As/Ac)) \times 100$$

Ac: Absorbance of negative control
As: Sample absorbance

OH scavenging activity assay

The reaction mixture contained 30 µl of different concentrations of sample, 10 µl of FeCl3-EDTA (3 should be written in subscript) (Merck, 1.039430250;), 5 µl of 20 mM H_2O_2 (Merck, 822287), 5 µl of 1 mM L-Ascorbic acid (Sigma-Aldrich, K3125), 10 µl of 28 Mm Deoxyribose (Sigma-Aldrich, 121649), and 70 µl phosphate buffer. The mixture was incubated at 37 °C for 30 min and then 25 µl of 5% TCA (Merck, 1008070250), and 1% TBA (Sigma-Aldrich, T5500) were added to be further incubated at 80–90 °C for 30 min. The absorbance was measured at 532 nm wavelength using a spectrophotometer (Multiskan GO Microplate Spectrophotometer,Thermo Fisher Scientific) (14).

RAW264.7 cell culture

The RAW 264.7 (ATCC®TIB-71™) murine macrophage cell line was obtained from the Biomolecular and Biomedical Research Center, Aretha Medika Utama. RAW 264.7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Biowest, L0104) supplemented with 10% fetal bovine serum (FBS) (Biowest, S1810) and 1% antibiotic–antimycotic (Gibco, Massachusetts, USA, 15240062). The cells were incubated at 37 ºC and 5% CO_2 in relative humidity (95-98%) until confluent (80%–90%). Trypsin-EDTA 0.25% (Gibco, 25200072) was used to harvest the cells which were then seeded on plates for the assays (2, 8, 15–18).

Viability assay

The cytotoxicity of corilagin was determined by the viability of RAW 264.7 cells using MTS assay (Promega, Madison, G3580). This method determines the safe and nontoxic concentrations of sample for the next assay. Briefly, 100 µl cells (5×10^3 cells per well) in medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) were plated in a 96-well plate and incubated for 24 hr at 37 °C in a humidified atmosphere incubator with 5% CO_2. The medium was then washed and 99 µl new medium and 1 µl of corilagin were added in different concentrations and DMSO in triplicate, then the plate was incubated for 24 hr. Untreated cells served as the control. Briefly, 20 µl MTS was added to each well. The plate was incubated in 5% CO_2 at 37 °C incubator for 4 hr. The absorbance was measured at 490 nm on a microplate reader (2,8,15–19).

Pro-inflammatory activation of RAW264.7 cell lines

The cells were seeded in a 6-well plate at the density of 5×10^5 cells per well and incubated for 24 hr at 37 °C in a humidified atmosphere and 5% CO_2. The medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) was then washed and supplemented with 1,600 µl growth medium and 200 µl corilagin (75, 50, 25 µM). Around 1–2 hr later, 200 µl lipopolysaccharide from *Escherichia coli* (LPS) (1 µg/ml) (Sigma-Aldrich, L2630) was added to the medium and incubated for 24 hr at 37 °C in a humidified atmosphere and 5% CO_2. After incubation of RAW 264.7 cells with LPS for 24 hr, the quantity of IL-6, TNF-α, NO, IL-1β, PGE-2, iNOS, and COX-2 was accumulated in the cell-free supernatant. The cell-free supernatant then was taken for the next assay by centrifugation at 2,000 g for 10 min. The supernatant was stored at -79 °C for measuring IL-6, TNF-α, NO, IL-1β, PGE-2, iNOS, and COX-2 levels (2, 15, 17–19).

Quantification of IL-1β, TNF-α, COX-2, IL-6, PGE-2, and iNOS levels in LPS-induced RAW 264.7 cells

The IL-1β, TNF-α, COX-2, IL-6, PGE-2, iNOS levels were assayed using ElabScience Elisa Kit IL-1β (E-EL-M0037), TNF-α (E-EL-M0049), COX-2 (E-EL-M0959) IL-6 (E-EL-M0044), PGE-2 (E-EL-0034), and iNOS (E-EL-M0696), based on manufacture protocols (2, 8, 15–20).

Quantification of NO level in LPS-induced RAW 264.7 cells

The nitrite associated with NO production was measured using NO Colorimetric Assay (ElabScience, E-BC-K035-M) which was performed based on manufacturing protocols. The sodium nitrite standard curve was determined to measure the nitrite quantity (17).

Total protein assay

Bovine standard albumin (BSA) standard solution was made from dilution series of BSA stock. The stock was obtained by dissolving 2 mg of BSA (Sigma Aldrich, A9576) in 1000 µl ddH_2O; briefly, 20 µl of standard solutions and 200 µl Quick Start Dye Reagen 1X (Biorad, 5000205) was added corilagin into each well plate. After 5 min of incubation at room temperature, absorbance was measured by a microplate reader at 595 nm (19, 20).

Statistical analysis

All data were obtained after performing in triplicate. The data were presented as mean ± standard deviation. The data were analyzed using ANOVA and *post hoc* test using Tukey HSD with $P<0.05$ using SPSS software (version 20.0).

Results

Effect of various concentrations of corilagin on H_2O_2 scavenging activity

Effect of corilagin treatments in H_2O_2 scavenging activity has been shown in Figure 1. Based on Figure 1, The corilagin treatment in concentration 500 µg/ml showed a significant increase in the inhibitory activity (94.86 ± 4.90%) compared with other treatment concentrations in H_2O_2 scavenging activity ($P<0.05$). Corilagin also has an IC_{50} value of H_2O_2 scavenging

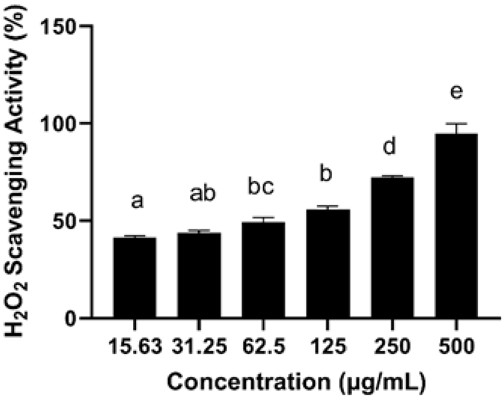

Figure 1. Effect of corilagin on H_2O_2 scavenging activity
*The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, ab, bc, d, e) showed significant differences among treatments at *P*<0.05 (Tukey HSD *post hoc* test)

activity with a value of 76.85 µg/ml (Table 1). This data indicates corilagin has anti-oxidant activity due to H_2O_2 scavenging activity.

Effect of various concentrations of corilagin on OH scavenging activity

The effect of corilagin concentrations in the scavenging activity of OH can be seen in Figure 2. The inhibitory activity of corilagin shows the highest activity in concentration 26.67 µg/ml with a value of 49.30 ± 3.34% (*P*<0.05). Corilagin also has an IC_{50} value of H_2O_2 scavenging activity with a value of 26.68 µg/ml (Table 2), which indicated corilagin has anti-oxidant properties through scavenging OH free radicals.

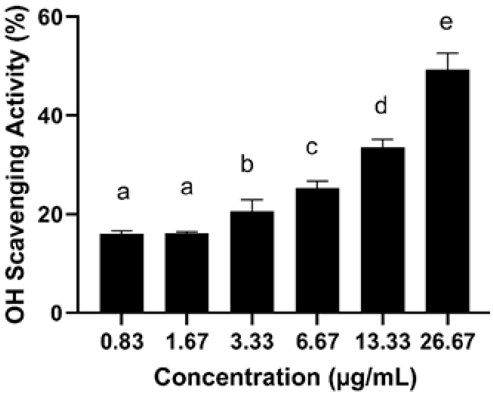

Figure 2. Effect of corilagin on OH scavenging activity
*The data were presented as mean ± standard deviation from 3 replications. Different superscripts (a, b, c, d, e) showed significant differences among treatments at *P*<0.05 (Tukey HSD *post hoc* test)

Effect of various concentrations of corilagin on NO scavenging activity

Effect of corilagin concentrations in NO scavenging activity were exhibited in Figure 3. The inhibitory activity of corilagin shows the highest activity in concentration

Table 1. IC_{50} value H_2O_2 scavenging activity of corilagin

Sample	Equation	R^2	IC_{50} (µg/ml)
Corilagin	y = 0.1101x + 41.539	0.99	76.85

Table 2. IC_{50} value OH scavenging activity of corilagin

Sample	Equation	R^2	IC_{50} (µg/ml)
Corilagin	y = 1.292x + 15.524	0.99	26.68

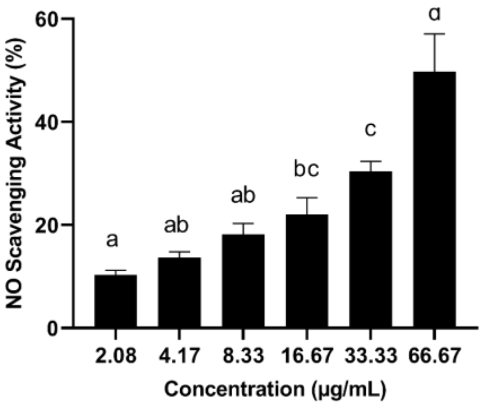

Figure 3. Effect of corilagin on NO scavenging activity
*The data were presented as mean ± standard deviation from 3 replications. Different superscripts (a, ab, bc, c, d) showed significant differences among treatments at *P*<0.05 (Tukey HSD *post hoc* test)

Table 3. IC_{50} value NO scavenging activity of corilagin

Sample	Equation	R^2	IC_{50} (µg/ml)
Corilagin	y = 0.5798x + 11.36	0.99	66.64

Table 4. Effect of various concentrations of corilagin toward the viability of RAW264.7 cell lines

Treatments	Cell Viability (%)
Control	100.00 ± 8.49^{bc}
Corilagin 100 µg/ml	78.96 ± 4.46^{a}
Corilagin 75 µg/ml	87.74 ± 2.07^{ab}
Corilagin 50 µg/ml	92.59 ± 4.21^{abc}
Corilagin 25 µg/ml	102.45 ± 5.14^{c}

*The data were presented as mean ± standard deviation from 3 replications. Different superscript letters in the same column (a, ab, bc, abc, c) showed significant differences among treatments at *P*<0.05 (Tukey HSD *post hoc* test)

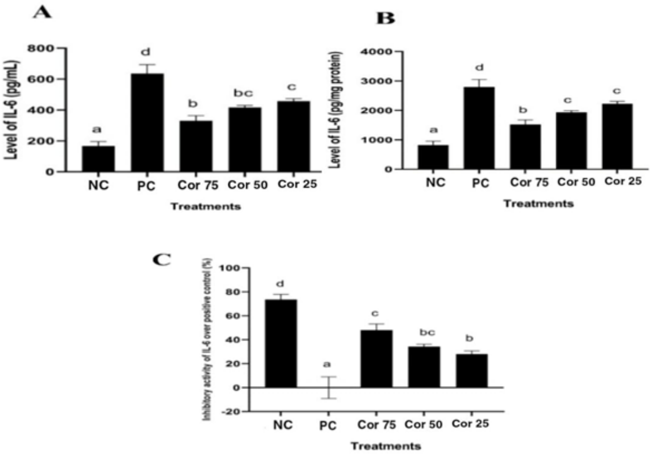

Figure 4. Effect of various concentrations of corilagin toward level and inhibition activity of IL-6 on LPS-induced RAW 264.7 cell lines
(A). IL-6 level (pg/ml). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, bc, d) for IL-6 level (pg/ml) for IL-6 inhibitory activity showed significant difference among treatments at $P<0.05$ (Tukey HSD *post hoc* test). (NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Positive Control + Corilagin 75 μM; Cor 50: Positive Control + Corilagin 50 μM; Cor 25: Positive Control + Corilagin 25 μM)
(B). IL-6 level (pg/mg protein). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, d) for IL-6 level (pg/mg protein) for IL-6 inhibitory activity showed significant difference among treatments at $P<0.05$ (Tukey HSD *post hoc* test). (NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Positive Control + Corilagin 75 μM; Cor 50: Positive Control + Corilagin 50 μM; Cor 25: Positive Control + Corilagin 25 μM)
(C). IL-6 inhibition activity (%). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, bc, c, d) for IL-6 inhibitory activity showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). (NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Positive Control + Corilagin 75 μM; Cor 50: Positive Control + Corilagin 50 μM; Cor 25: Positive Control + Corilagin 25 μM)

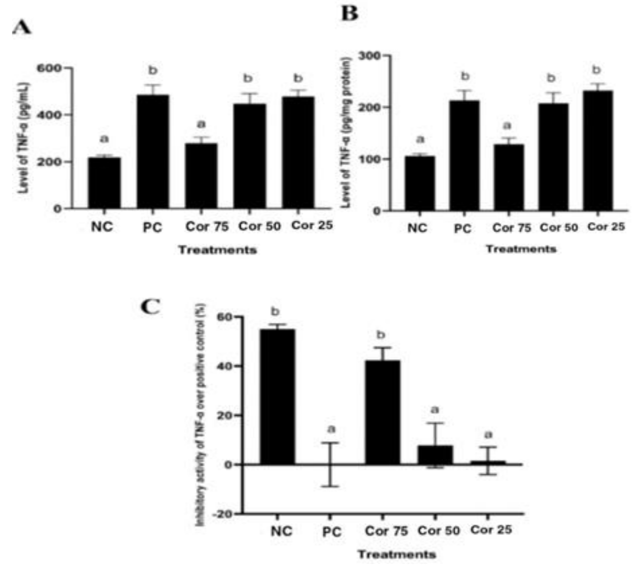

Figure 5. Effect of various concentrations of corilagin toward level and inhibition activity of TNF-α on LPS-induced RAW 264.7 cell lines
(A). TNF-α level (pg/ml). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(B). TNF-α level (pg/mg protein). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(C). TNF-α inhibition activity (%). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM

66.67 μg/ml with a value of 49.73 ± 7.38% ($P<0.05$). In Table 3, the IC_{50} value of corilagin as NO scavenger is 66.64 μg/ml. This data indicated corilagin has NO scavenging activity.

Effect of corilagin toward RAW264.7 cells viability

The viability of RAW264.7 cell lines is presented in Table 4. The increased concentration was correlated with increasing toxicity (<90% viability cells). Table 4 shows the cytotoxicity of corilagin concentration on RAW264.7 cell lines. The viability of cells was decreased in a dose-dependent manner. Concentration 100 μg/ml demonstrated the lowest viability of cells by corilagin with a value of 78.96 ± 4.46% ($P<0.05$).

Effect of corilagin toward IL-6 level in LPS-induced cells

Essential pro-inflammatory cytokine expression of IL-6 in LPS-induced cells was measured (Figure 4). As shown in Figure 4A, LPS (1 μg/ml) administration for 24 hr significantly increased this cytokine (635.95 ± 57.77 pg/ml) compared with the negative control group (167.95 ± 27.74 pg/ml). Corilagin treatment in 75 μM exhibited the decrease of IL-6 level (330.14 ± 33.03 pg/ml; 1523.36 ± 152.42 pg/mg protein) and also showed the highest increase in inhibitory activity in IL-6 level (48.09 ± 5.19%) compared with positive control and other treatments ($P<0.05$) (Figures 4A-C).

Effect of corilagin toward TNF-α level in LPS-induced cells

Cytokine expressions of TNF-α in LPS-induced cells were shown in Figure 5. Corilagin at concentration 75 μM resulted significant decrease TNF-α level (279.61 ± 24.95 pg/ml; 129.02 ± 11.51 pg/mg protein). The result was comparable with negative control (218.18 ± 9.41 pg/ml; 106.10 ± 4.58 pg/mg protein) which indicates the treatment has good anti-inflammatory capability (Figures 5A and 5B). The inhibitory activity of TNF-α also shows the highest presentation (42.37 ± 5.14%) compared with other treatments and positive control ($P<0.05$) (Figure 5C).

Effect of corilagin toward NO level in LPS-induced cells

The effect of corilagin treatment toward NO level in LPS-induced cells has been shown in Figure 6. Corilagin resulted from the lowest NO level at the highest concentration (75 μM) with a value of 11.82 ± 2.95 μM; 54.53 ± 13.60 μM/mg protein with inhibitory

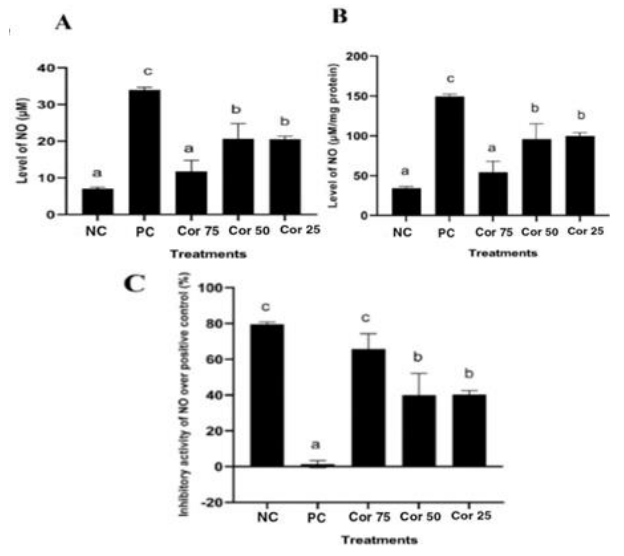

Figure 6. Effect of various concentrations of corilagin toward level and inhibition activity of NO on LPS-induced RAW 264.7 cell lines
(A). NO level (μM). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post ho*c test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(B). NO level (μM/mg protein). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post ho*c test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(C). NO inhibition activity (%). The data were presented as mean ± standard deviation from 3 replications. NO inhibitory activity showed significant differences among treatments at $P<0.05$ (Tukey HSD *post ho*c test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM

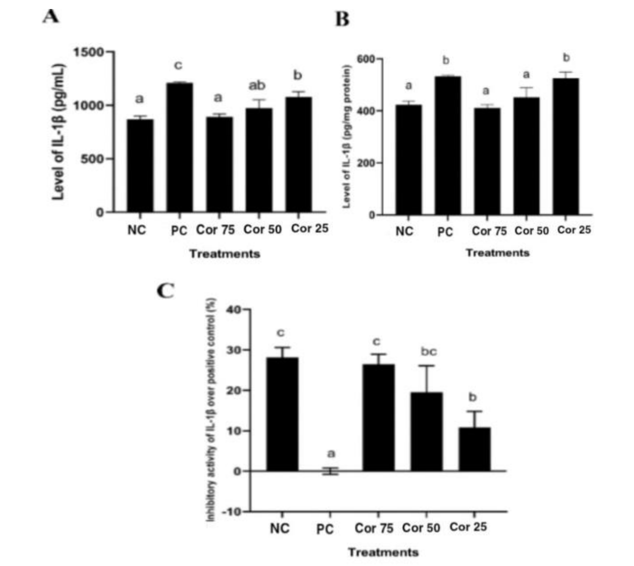

Figure 7. Effect of various concentrations of corilagin toward level and inhibition activity of IL-1β on LPS-induced RAW 264.7 cell lines
(A). IL-1β level (pg/ml). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, ab) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post ho*c test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(B). IL-1β level (pg/mg protein). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post ho*c test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(C). IL-1β inhibition activity (%). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, bc) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post ho*c test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM

activity of 65.69 ± 8.56% compared with control and other treatments ($P<0.05$) (Figures 6A-C). The results demonstrated that corilagin possessed anti-oxidant activity.

Effect of corilagin toward IL-1β level in LPS-induced cells

The effect of corilagin toward level and inhibitory activity of IL-1β has been shown in Figure 7. There was significant ($P<0.05$) decrease in level of IL-1β (890.00 ± 29.46 pg/ml; 410.67 ± 13.59 pg/mg protein) (Figures 7A and 7B) and increase in inhibitory activity of IL-1β (26.47 ± 2.43%) in the highest concentration of corilagin treated group compared with positive control (1210.31 ± 9.57 pg/ml; 0.00 ± 0.79%, respectively) (Figures 7A and 7C).

Effect of corilagin toward PGE-2 level in LPS-induced cells

In the LPS group (positive control) the PGE-2 level was significantly increased (903.15 ± 120.07 pg/ml) when compared with the negative control group (429.42 ± 36.87 pg/ml) ($P<0.05$). With corilagin intervention, PGE-2 level expression was significantly reduced in the highest concentration (479.76 ± 15.99 pg/ml; 2,213.74 ± 73.77 pg/mg protein) (Figures 8A and 8B) and inhibitory percentage of 46.88 ± 1.77% when compared with the LPS group ($P<0.05$) (Figure 8C).

Effect of corilagin toward iNOS level in LPS-induced cells

The effect of corilagin on the iNOS level has been shown in Figure 9. The level of iNOS in the positive control group significantly increased before being stimulated by LPS (negative control) with values 11.35 ± 0.45 and 3.16 ± 0.04 ng/ml, respectively. Corilagin treatment showed the lowest iNOS level in concentration 75 μM with value 4.97 ± 0.09 ng/ml; 2.42 ± 0.04 ng/mg protein ($P<0.05$) compared with another group (Figures 9A and 9B). Corilagin also had the highest inhibitory activity compared with positive control with a value of 56.22 ± 0.81% (Figure 9C).

Effect of corilagin toward COX-2 level in LPS-induced cells

The effect of corilagin on the COX-2 level has been shown in Figure 10. In the positive control group, the COX-2 level was significantly increased (9.94 ± 0.60 ng/ml) when compared with the negative control group (2.44 ± 0.03 ng/ml) ($P<0.05$) (Figure 10A). COX-2 level

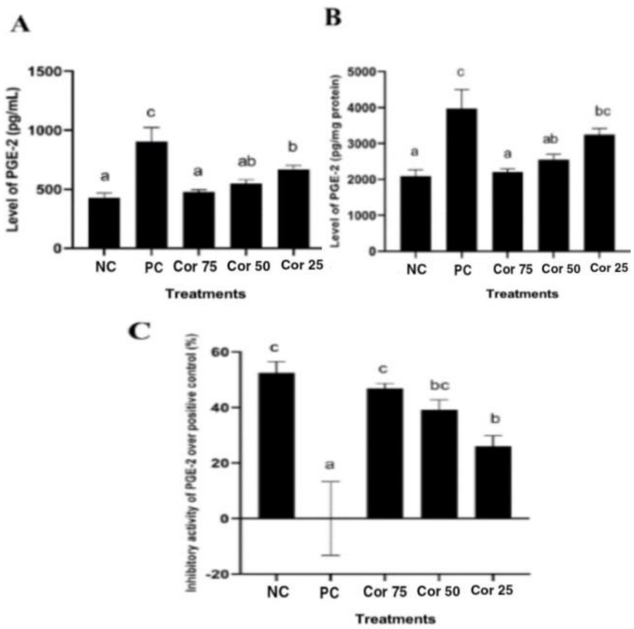

Figure 8. Effect of various concentrations of corilagin toward level and inhibition activity of PGE-2 on LPS-induced RAW 264.7 cell lines
(A). PGE-2 level (pg/ml). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, ab, c) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(B). PGE-2 level (pg/mg protein). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, ab, bc, c) showed significant differences among treatments at $P<0.05$ (Tukey HSD post hoc test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(C). PGE-2 inhibition activity (%). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, bc) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM

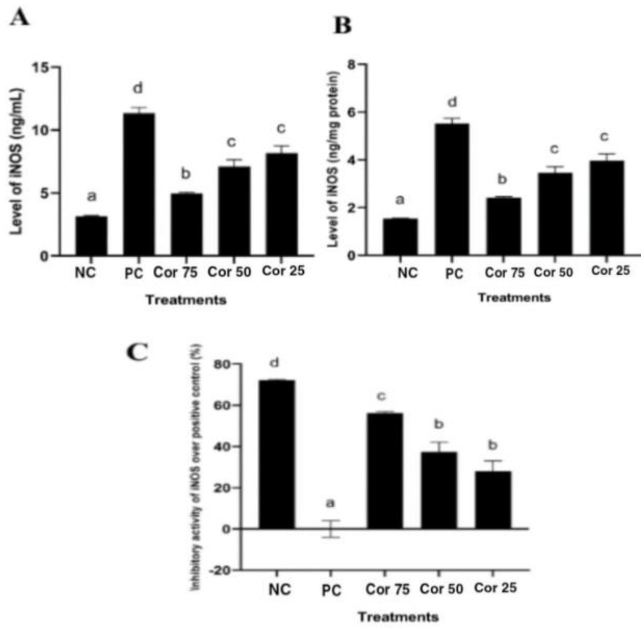

Figure 9. Effect of various concentrations of corilagin toward level and inhibition activity of iNOS on LPS-induced RAW 264.7 cell lines
(A). iNOS level (ng/ml). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, d) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(B). iNOS level (ng/mg protein). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, d) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(C). iNOS inhibition activity (%). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, d) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM

expression was significantly reduced in the highest concentration (75 μM) with a value of 3.98 ± 29.46 ng/ml; 1.93 ± 0.29 ng/mg protein compared with other treatment and also has inhibitory activity with value 59.99 ± 6.09% ($P<0.05$) (Figures 10A-C). Figure 11 shows the effect of various concentrations of corilagin toward cells morphology and density in LPS-induced RAW 264.7 cells line.

Discussion

Corilagin is a phenolic compound that has been reported to show promising pharmacological effects including anti-inflammatory and anti-oxidant (8, 21). In anti-oxidant activity, higher concentration of corilagin showed a significant increase in H_2O_2 scavenging activity (94.86 ± 4.90%) (Figure 1). A Previous study shows corilagin has slightly higher anti-oxidant activity than the standard (vitamin C) and depends on concentration (22).

Free radicals such as hydroxyl radicals (OH) and superoxide anion radicals (O_2^{*-}) can be generated by normal physiological reactions in living organisms (23). In recent studies, corilagin has *OH inhibitory activity in a dose-dependent manner with the highest value (49.30 ± 3.34%) (Figure 2). Another study has reported that corilagin isolated from longan pericarp extract possessed the highest *OH inhibitory rate of 75.90 ± 0.30% compared with other phenolic compounds, and also has DPPH radical scavenging rate of 71.80 ± 0.50% (24). Corilagin showed anti-oxidative activity due to scavenging of nitrite and blocking of nitrosamine synthesis (8). Corilagin significantly reduces oxidative stress by increasing the total anti-oxidant capacity and decreasing malondialdehyde (MDA) in the spinal tissue injured rat. Corilagin treatment also decreases the level of cytokines in the spinal tissue of spinal cord injured rats. Oxidative stress and inflammatory mediators trigger the activity of caspase cascade and result in increasing activity of caspase 3, Bax, and a decrease in Bcl-2 in injured spinal cord tissues (25).

The anti-oxidant effect of corilagin also was measured by its inhibitory potency on NO. In this study, corilagin shows the highest NO scavenging activity is 49.73 ± 7.38% in a concentration of 66.67 μg/ml ($P<0.05$)

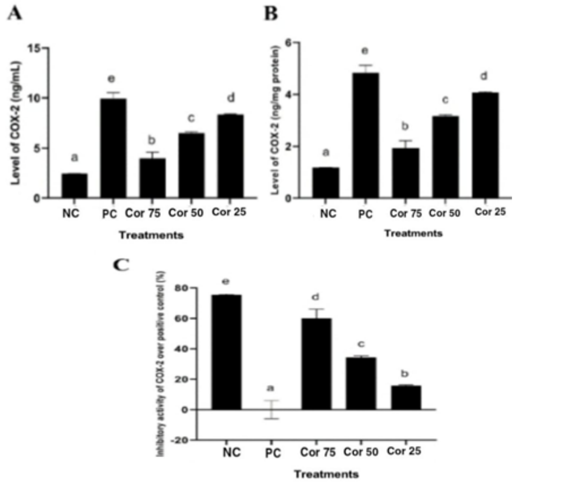

Figure 10. Effect of various concentrations of corilagin toward level and inhibition activity of COX-2 on LPS-induced RAW 264.7 cell lines.
(A). COX-2 level (ng/ml). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, d, e) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(B). COX-2 level (ng/mg protein). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, d, e) showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM
(C). COX-2 inhibition activity (%). The data were presented as mean ± standard deviation from 3 replications. Different superscript letters (a, b, c, d, e) for COX-2 inhibitory activity showed significant differences among treatments at $P<0.05$ (Tukey HSD *post hoc* test). NC: Negative Control (untreated cell); PC: Positive Control (LPS-induced cell); Cor 75: Corilagin 75 μM; Cor 50: Corilagin 50 μM; Cor 25: Corilagin 25 μM

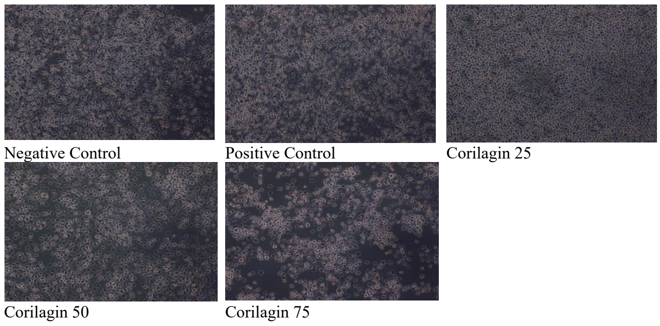

Figure 11. Effect of various concentrations of corilagin toward cells morphological, density in LPS-induced RAW 264.7 cells line

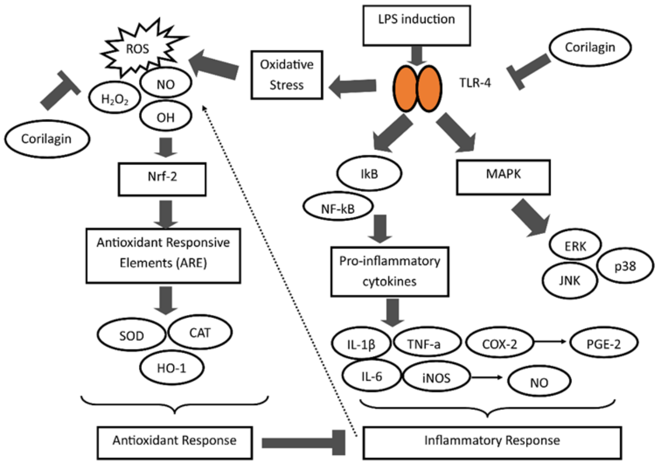

Figure 12. Proposed mechanism of corilagin effects as anti-oxidant and anti-inflammatory activity

(Figure 3). Corilagin was shown to exhibit NO scavenging activity with IC_{50} 7.2 μM (26).

In this study, the cytotoxic assay was performed on RAW 264.7 cells to rule out any adverse effects before using corilagin. Based on the results of cell viability) assays, corilagin treatment showed not toxic and safe in concentrations 75, 50, and 25 μM.

LPS-induced in macrophage cell lines (RAW 264.7) as pro-inflammatory activation caused the increase in inflammatory cytokines IL-6, TNF-α, NO, IL-1β, PGE-2, iNOS, and COX-2. In this study, the inflammation model using LPS-induced cells as a positive control indicated increasing of inflammatory cytokines such as IL-6, TNF-α, NO, IL-1β, PGE-2, iNOS, and COX-2 compared with the negative control. Macrophages become activated and produce pro-inflammatory mediators through the extracellular region of Toll-Like receptor 4 (TLR4), which will become activated through the MAPK (JNK, ERK1/2, p38) pathway (17, 27). The activated TLR4 will activate cascade events in the cytoplasm which leads to MAPK (JNK, ERK1/2, p38) pathway activation. The phosphorylation of MAPKs allows the inhibitory factor-kappa B (IkB) to be phosphorylated. Nuclear factor-kappa B (NF-kB) is activated and translocated to the nucleus as a result of this. NFκB is a widely distributed transcription factor that regulates the expression of genes involved in apoptosis, immune responses, and the cell cycle (17, 28).

IL-6 is a monocyte-derived cytokine, these have an important acute phase reaction medium in inducing the inflammation process (29). Corilagin treatment in a concentration of 75 μM showed the highest inhibitory activity in the IL-6 level (48.09 ± 5.19%) compared with positive control and other treatments (Figure 4C). Corilagin possessed anti-inflammatory capability, through intervention the expression of TLR4 was greatly decreased in the inflammatory phase. Corilagin intervention, IL-1β, and IL-6 expression were significantly reduced when compared with the LPS group ($P<0.01$) (30).

TNF-α is a highly inflammatory cytokine developed and secreted by mast cells and plays a key role in some pathogenesis (31, 8, 17). In the present study, we have demonstrated that corilagin 75 μM has anti-inflammatory activity due to suppressing of TNF-α level (42.37 ± 5.14%) compared with other treatments (Figure 5C). This result is in line with Gambari *et al.* (2012) study that showed corilagin can inhibit the expression of (TNF-α) and IL-6 protein (32).

Several mediators, including NO, control leukocyte recruitment to the infection site. NO is a vascular tone regulator that also controls leukocyte adherence in blood micro-capillaries (33-35). Corilagin 75 μM can suppress NO level with a percentage of 65.69 ± 8.56 (Figure 6C). The previous reports demonstrated that corilagin suppresses the levels of NO and modulates oxidative stress to reduce liver damage induced by Hepatitis C Virus (HCV) proteins (36). Corilagin also elicited prominent anti-inflammatory activity in RAW264.7 macrophages via suppressing LPS-induced TLR4 and NOX2 activation, thereby repressing the corresponding ROS production, MAPKs activation, and NF-κB translocation from the cytoplasm to the nucleus, where it mediates the expression of pro-inflammatory mediators, such as TNF-α, IL-6, NO, and PAI-1 in activated macrophage cells (37). Moreover, corilagin lightened the herpes simplex virus (HSV)-1-induced inflammatory damage by inducing the apoptosis of infected microglial cells and inhibiting the production of TNF-α, NO, and IL-1β (38).

IL-1β production caused TLR activation and also stimulated up-regulation of NF-kB in inflammatory diseases (39). This study demonstrated that corilagin has IL-1β inhibitory activity (26.47 ± 2.43%) (Figure 7C). Corilagin in pomegranate fruit has been shown to inhibit iNOS expression and IL-1β-induced tissue destruction in periodontitis (40).

Prostaglandin E2 (PGE2) is a pro-inflammatory lipid mediator produced by COX-2 and microsomal PGE synthase-1 (mPGES-1) from arachidonic acid (41). The present study showed that corilagin can act as a PGE-2 inhibitor (46.88 ± 1.77%) (Figure 8C). Phenolic compounds like corilagin can reduce LPS-stimulated PGE-2 production, they exhibit anti-inflammatory activity by targeting different inflammation-related cytokines (42).

Inflammatory activity is accompanied by iNOS, which produces NO, which stimulates COX-2 catalytic activity by forming peroxynitrite anion. (43, 44). Stimuli that increase NOS-2 and NO formation also may induce COX-2 expression (44). In this study, corilagin 75 μM had anti-inflammatory toward iNOS level with inhibitory activity 56.22± 0.81% (Figure 9C), decreased COX-2 level 59.99 ± 6.09% (Figure 10C); all had the highest value compared with positive control and other concentration in LPS-induced cells. Similar results were obtained by Zhao *et al*. (2008), who found that corilagin repressed the discharge of the pro-inflammatory cytokines COX-2 and NO through inhibiting the NF-κB pathway in LPS-stimulated RAW 264.7 (45). Corilagin also can inhibit the production of pro-inflammatory cytokines and mediators including COX-2 through suppressing the DSBs (DNA double-strand breaks)-triggered NF-κB signaling pathway in the murine microglial cell line (BV-2) (46).

The morphological change in macrophage RAW 264.7 cells was induced by LPS and treated with various concentrations of corilagin (75, 50, 25 μM), furthermore incubated for 24 hr. (Figure 11). The negative control (normal cells, without LPS) was a round shape, the positive control became irregularly shaped, and increased spreading after LPS stimulation. The cotreatment with 25, 50, and 75 μM corilagin decreased the degree of cell spreading and irregular shape. Treatment of 75 μM corilagin was the most active to decrease the spreading and density of cells. There result data were in line with previous research showing that LPS stimulation changed the cells' round shape to irregular and increased the spreading of cells.

The anti-oxidant activities possibly contribute to the anti-inflammatory of corilagin (26). Oxidative stress has played a role in activating signal pathways such as NF-κB, an important transcription factor in the nucleus. The excessive oxidative stress release caused NF-κB activation, and then nuclear translocation and binding specific sites in the promoter regions of target genes. It up-regulates gene expressions of numerous pro-inflammatory cytokines and inflammatory mediators including TNF-α, IL-1β, and COX-2 (47-49). However, corilagin has protective effects against LPS-induced RAW264.7 cell lines by suppressing ERK/JNK MAPK and NF-κB signaling pathways. Moreover, the inflammatory mediators and ROS have been related to the actuation of mitogen-activated protein kinase (MAPK) that controls the intracellular signal transduction pathway in oxidative stress-induced cells (50). Its mechanism is attributed to anti-inflammatory and anti-oxidative properties (37). In summary, corilagin may play a critical part in the oxidative stress and inflammatory response (51). Based on these results, we proposed anti-oxidant and anti-inflammatory mechanism of corilagin in LPS-stimulated RAW264.7 cells (Figure 12).

Conclusion

Corilagin has the potential as an anti-oxidant as measured through H_2O_2, NO, and *OH scavenging activities. Corilagin also has anti-inflammatory properties through suppression of IL-6, TNF-α, NO, IL-1β, PGE-2, iNOS, and COX-2 levels. However, corilagin protects against LPS-stimulated RAW264.7 cells via inhibiting oxidative stress and inflammation. This study can be the basis for further research on the exploration of the bioactivity of corilagin and on the treatment of inflammatory diseases with natural compounds.

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Authors' Contributions

WW, SA, and HSWK Study conception and design; HS WK, CDW, MAM, and CRW Data analysis and draft manuscript preparation; WW, HSWK, and CRW Critical revision of the paper; EA and CDW Supervision of the research; WW, HSWK, EA, and RR Final approval of the version to be published.

Conflicts of Interest

The authors declare that no conflict of interest exists.

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