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Investigation of the Mechanism of Anti-Inflammatory Action and Cytotoxicity of a Semipurified Fraction and Isolated Compounds From the Leaf of *Peltophorum africanum* (Fabaceae)

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Abstract

Peltophorum africanum extracts have been shown to possess many important medicinal benefits, including anti-inflammatory and antiviral activities. However, the mechanism of action is poorly understood. The mechanism of anti-inflammatory action was determined by measuring the synthesis of cytokines in lipopolysaccharide (LPS) stimulated RAW 264.7 macrophage cells in vitro. Compound 1 (CP1), compound 2 (CP2), and fraction F3.3.0 (F3.3.0) significantly reduced the synthesis of interleukin 1β (IL-1β) from RAW 264.7 cells (1.18, 1.32, and 0.92 ng/mL), respectively. Similarly, CP1, CP2, and F3.3.0 inhibited the production of IL-2 and tumor necrosis factor–α (TNF-α) by RAW 264.7 cells (0.41, 0.60, 0.74 and 0.11, 0.27, 0.24 ng/mL, respectively. In addition, CP1 and CP2 had lower cytotoxicity toward RAW 264.7 cells, with CP2 indicating the lowest cytotoxicity (LD_{50} = 207.88 μg/mL). The mechanism of action was found to be via the inhibition of pro-inflammation cytokines (IL-1 β and TNF-α). This observation may support the use of *P africanum* to treat pain-related conditions.

Keywords

IL-1β, IL-2, TNF-α, anti-inflammatory, RAW 264.7, *Peltophorum africanum*

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In chronic inflammation, the release of pro-inflammatory cytokines by activated T-lymphocytes and macrophages is largely responsible for perpetuating the inflammatory process, often leading to irreversible tissue damage and permanent functional impairment. However, this complex process involves the coordinated activity of transcriptional factors and gene expressions in activated immune cells. Although, the complete process of the signaling pathways and gene expressions are not fully understood, recent work has attempted to explain the molecular basis of the inflammatory response.[1] Activities of transcription factors, kinases, and pro-inflammatory cytokines have been implicated in the pathogenesis of chronic inflammatory conditions. Nuclear factor kappa B (NF-κB) is involved in the regulation and synthesis of pro-inflammatory cytokines such as tumor necrosis factor–α (TNF-α), interleukin-1β (IL-1β), and IL-6.[2-4] Although, the predominant cells responsible for the synthesis and release of these cytokines are activated T-helper 1 and 2 (Th_1 and Th_2) subset of T-lymphocytes,[3] macrophages and endothelial cells are also actively involved.[4]

Extracts of medicinal plants such as *Peltophorum africanum* are widely used in traditional medicine in southern Africa to treat painful symptoms associated with chronic inflammatory conditions like rheumatoid arthritis. Preliminary evaluation of the crude acetone extracts of the leaf of *P africanum* indicated good activity against 15-lipoxygenase and inducible nitric oxide synthase,[5] which are potent mediators of inflammation. This observation led to a bioassay guided extraction of

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semipurified fractions F3.3 and F3.3.0 that contained among others, 2 isolated compounds, CP1 and CP2.

The primary objective of this study was to investigate the mechanism(s) of anti-inflammatory action of subfractions F3.3, F3.3.0 and isolated compounds CP1 and CP2. This was done by assessing the inhibitory activity of subfractions and isolated compounds on the synthesis of pro-inflammatory cytokines by lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cell line.

Understanding the molecular basis of action of plant-derived compounds could assist in the identification of a specific target candidate for the development of potent and safer anti-inflammatory drugs.

Methods

Plant Materials

The collection and preparation of plant materials for bioassays was as described previously.[5,6]

Cell Culture

The RAW 264.7 macrophage cell line (American Type Culture Collection, ATTC TIB-71, Rockville, MD, USA) was used to determine the molecular mechanism of action of the test fractions or compounds. The cells were cultured in plastic culture flasks in Dulbecco's modified Eagle medium (DMEM) containing L-glutamine supplemented with 10% fetal calf serum (FCS) and 1% PSF (penicillin/streptomycin/fungizone) solution under 5% CO_2 at 37°C. The growing cells were split twice a week. The cells were subsequently seeded into a 96-well microtiter plate at 2×10^6 cells/well (200 µL) prior to the commencement of each bioassay. The cytokine induction from RAW 264.7 cell lines was performed using an adapted method of Abrham et al.[7]

Interleukin-1β Assays

For the evaluation of the inhibitory activity on IL-1β production, RAW 264.7 cells were incubated with the test compounds or subfraction (20 µL) for 30 minutes under 5% CO_2 at 37°C. The negative control contained no inhibitor and was supplemented with 20 µL growth media. After the incubation period, 5 ng/mL LPS was added to the appropriate wells and the microtiter plate was further incubated in 5% CO_2 at 37°C for 24 hours. At the end of the incubation period, 100 µL of the supernatant of each culture medium was carefully removed into labeled Eppendorf tubes and stored at −80°C until assayed for IL-1β using an IL-1β enzyme-linked immunosorbent assay (ELISA) kit (BioLegend, London, UK). Each sample was diluted 10-fold prior at the commencement of the assay. The amount of IL-1β present in each culture supernatant was then determined as described by the manufactures. Following the addition of stop solution to each well, the absorbance was read at 450 and 570 nm. The absorbance was corrected by removing the absorbance at 570 nm from that of 450 nm. The concentration of IL-1β in each sample was extrapolated from the standard curve generated by the standards included in the test kit.

Evaluation of the Inhibitory Activity on Production of Other Cytokines

The Bio-Plex suspension array system (BioRad, Hercules, CA, USA) uses Luminex xMAP technology, which allows for simultaneous detection of up to 100 cytokines in a single sample/control. For the evaluation of the effect of the test compounds on cytokine production, a Bio-Plex Pro-human cytokine Th_1/Th_2 assay kit (BioRad) for the detection of IL-2, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α was used. The RAW 264.7 macrophage cell line was treated as described above and the supernatants collected for cytokine determinations were stored at −80°C until use. The experimental procedure was then followed as described by the manufacturer. The final concentrations of the assayed interleukins in the culture supernatant were obtained using the Bio-Plex Manager 4.0 software (BioRad).

Cytotoxicity of Compounds on RAW 264.7 Macrophage Cell Line

In order to ascertain that the results obtained from the bioassays were not due to cytotoxicity of subfraction/compounds on RAW 264.7 macrophage cell lines, the samples were also evaluated for potential cytotoxicity using the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetra-zoliumbromide (MTT) assay. The RAW 264.7 cell lines were seeded at 2×10^6 cells/well (200 µL) in a 96-well microtiter plate. The microtiter plate was place in the incubator in 5% CO_2 at 37°C for 1 hour. After the incubation period, 50 µL of culture supernatant was carefully removed from each appropriate well and replaced with 50 µL test compound or 1 mM hydrogen peroxide (H_2O_2) solution as positive control. The cells were incubated in 5% CO_2 at 37°C for 48 hours. At the end of the incubation period, 10 µL MTT (5 mg/mL in DMEM) was added to each well and the microtiter plate was further incubated in 5% CO_2 at 37°C for 2 hours. After incubation, 20 µL isopropanol (with 0.04 M hydrochloric acid) was added to each well to dissolve the formazan precipitate that had formed. The absorbance of the solution on the microtiter plate was read at 570 nm. The lethal dose (LD_{50}) for each test compound or control was calculated as the concentration that resulted in 50% viability of the cell line in the well. The experiment was done at 3 different times in triplicate.

Statistical Analysis

All results are presented as the mean ± standard error of the means (SEM) of triplicate experiments. Differences between test extract in these experiments was assessed for significance using analysis of variance (ANOVA) and Student's t test, where probability $P \leq .05$ was considered significant.

Results and Discussion

Inflammation is mediated by the activities of cytokines released from cells of the immune systems in response to infections, external injuries, or in autoimmune conditions. The anti-inflammatory potential of *P africanum* fractions and isolated compounds was monitored in vitro by incubating RAW 264.7 macrophage cell lines with bacterial LPS, a major structural component of the outer wall of gram-negative bacteria. The amount of cytokines released into the cell cultured medium

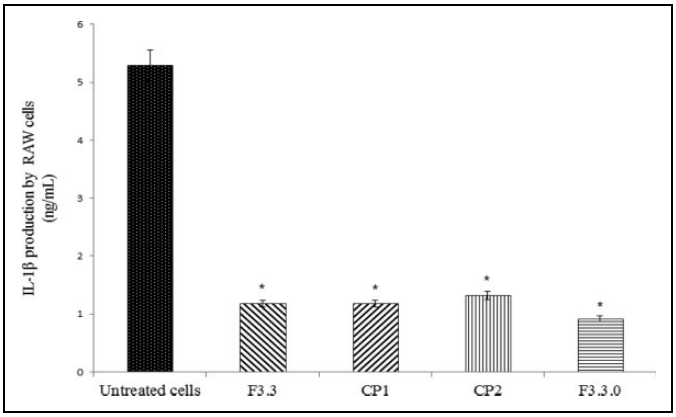

Figure 1. The inhibitory effects of F3.3, CP1, CP, and F3.3.0 (pooled rest of fractions) on interleukin-1β (IL-1β) synthesis (ng/mL) in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage cells. Untreated cells are activated RAW 264.7 macrophage cells without treatment with plant derivatives (*$P < .05$).

was quantified using ELISA (IL-1β) and Bio-Plex suspension array kits (IL-2, IL-5, GM-CSF, TNF-α) respectively.

Interleukin-1β Assay

IL-1β is a pro-inflammatory cytokine produced by endothelial cells, B cells, and fibroblast cells.[4] The synthesis and release of IL-1β is associated with acute and chronic inflammatory responses, edema, and promotes the synthesis of prostaglandins, as well as the induction of the acute phase reaction. All the samples tested (F3.3, CP1, CP2, and F3.3.0) reduced the synthesis of IL-1β by RAW 264.7 cells compared with untreated controls. Subfraction F3.3.0 had the highest inhibitory activity on IL-1β synthesis by RAW 264.7 cells (0.92 ng/mL) compared with untreated controls (5.29 ng/mL) (Figure 1). Neutralization of IL-1β synthesis is desirable in inflammatory conditions such as osteoarthritis, as it leads to rapid and sustained reduction in the stimulation and upregulation of other pro-inflammatory mediators.[8]

Interleukin-2 Assay

IL-2 is secreted by stimulated Th cells ($CD4^+$), cytotoxic T cells ($CD8^+$), and large granular lymphocytes and plays a central role in inflammation and immune signaling. It promotes the proliferation (colonal expansion) and differentiation of additional $CD4^+$ cells, B cells, and activates macrophages and oligodendrocytes (pro-inflammatory activity). The primary function of IL-2 is thought to be the regulation of regulatory T (T_{reg}) cells[9] and the generation of $CD8^+$ cytotoxic T cells, which are important for antiviral responses.[10] The results from this study indicate that CP1, CP2 and F3.3.0 also reduced the synthesis of IL-2 (0.41, 0.6, and 0.74 ng/mL, respectively), whereas F3.3 did not significantly affect IL-2 synthesis (1.87 ng/mL) when compared with untreated cells (1.9 ng/mL). These results suggest that the compounds had better inhibitory activity against IL-2 synthesis when in isolation than the sub-fraction (F3.3) from which they were isolated (Figure 2). The results are similar to the findings of Abrham et al.[7] Interleukin-2 is a pro-inflammatory cytokine that is capable of exacerbating inflammation.[9]

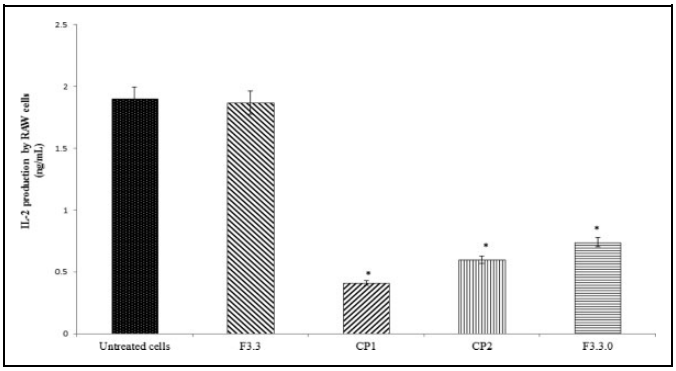

Figure 2. The inhibitory effects of F3.3, CP1, CP2, and F3.3.0 on interleukin-2 (IL-2) synthesis in lipopolysaccharide (LPS)-activated RAW 264.7 macrophage cell lines (*$P < .05$).

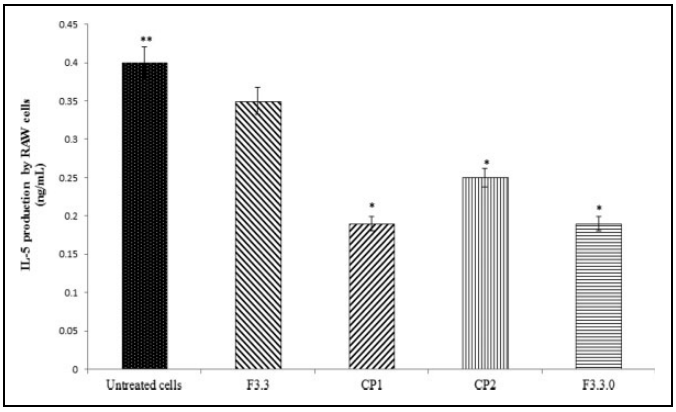

Figure 3. The inhibitory effects of F3.3, CP1, CP2, and F3.3.0 on interleukin-5 (IL-5) synthesis in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cell lines. Compounds 1, 2, and F3.3.0 significantly reduced the synthesis of IL-5 (*P vs **P, $P = .014$) compared with untreated positive controls.

Interleukin-5 Assay

IL-5 forms part of the Th_2 type cytokine response that includes IL-4, -5, and -13.[11] IL-5 induces terminal differentiation of activated B cells into antibody-forming cells and enhances proliferation and differentiation of eosinophil precursors into mature eosinophils.[9] Overexpression of IL-5 significantly increases eosinophil and $CD4^+$ T-cell numbers and antibody levels in vivo, hence it has pro-inflammatory activity.[12] The results in this study indicate that the compounds tested inhibit IL-5 synthesis from RAW 264.7 cells in a similar manner to that observed for IL-2 synthesis (Figure 3). CP1, CP2, and F3.3.0 significantly ($P = .014$) inhibited IL-5 synthesis (0.19, 0.25, and 0.19 ng/mL, respectively). On the other hand, F3.3 only slightly decreased its levels (0.35 ng/mL) compared with untreated cells (0.4 ng/mL).

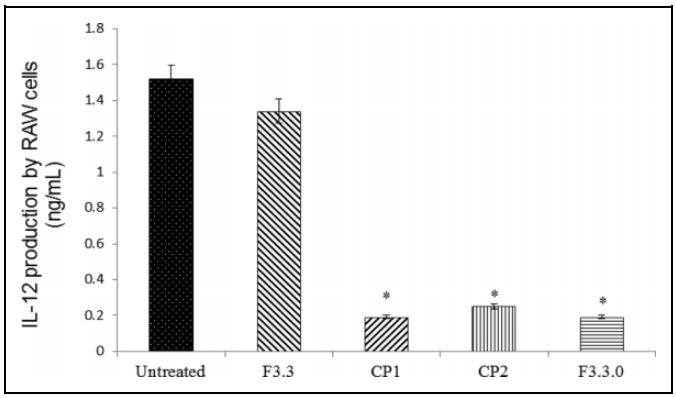

Figure 4. The inhibitory effects of F3.3, CP1, CP2, and F3.3.0 on interleukin-12 (IL-12) synthesis in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cell lines (*$P < .05$).

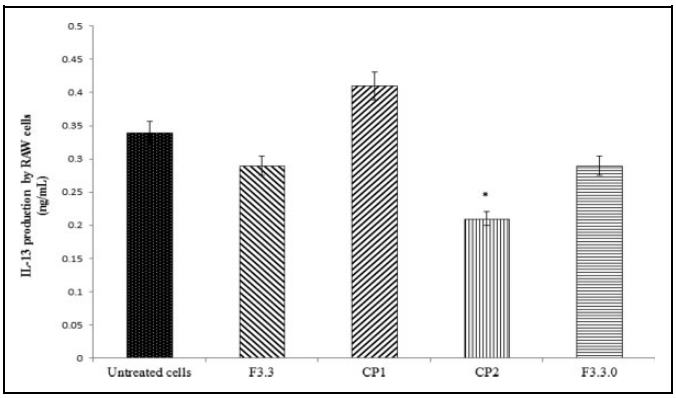

Figure 5. The effects of F3.3, CP1, CP2, and F3.3.0 on interleukin-13 (IL-13) synthesis in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cell line (*$P < .05$).

Interleukin-12 Assay

IL-12 is recognized as a master regulator of adaptive type 1, cell-mediated immunity, the critical pathway involved in protection against neoplasia and many viruses.[13] It is a pro-inflammatory cytokine that enhances Th_1 type responses in macrophage and natural killer (NK) cells by inducing the release of interferon-γ (IFN-γ). In addition to its noted effects in the priming of Th_1 cell responses and IFN-γ production by T- and NK cells, more recent studies support its critical role as a third signal for $CD8^+$ T-cell differentiation, and its ability to serve as an important factor in the reactivation and survival of memory $CD4^+$ T cells. This is particularly relevant in the repolarization of $CD4^+$ T cells from dysfunctional antitumor Th_2 into Th_1 cells in cancerous cells. However, cytokines such as IL-10 and transforming growth factor–β1 can negatively regulate IL-12 production.[13] The expression of IL-12 during infection regulates innate responses and determines the type of adaptive immune responses. Also, IL-12 induces IFN-γ production and triggers $CD4^+$ T cells to differentiate into Th_1 cells.[14] The results indicate that CP1 and F3.3.0 (0.19 ng/mL) significantly inhibited the synthesis of IL-12 ($P = .004$). CP2 and F3.3 (0.25 and 1.34 ng/mL, respectively) also inhibited the synthesis of IL-12, albeit to a lesser extent compared with untreated control cells (1.52 ng/mL) (Figure 4). The finding that plant extracts are able to inhibit IL-12 and IL-1β, has previously been demonstrated by Verma et al[4] who were able to show that extracts of *Grindelia robusta* and *Quassia amara* significantly inhibit IL-12 and IL-1β in LPS-induced murine macrophage cell lines by 50%.

Interleukin-13 Assay

IL-13 has both structural and functional similarities to IL-4 by promoting B-cell differentiation and the inhibition of the synthesis of pro-inflammatory cytokines from macrophages (anti-inflammatory activity).[11] Only CP2 appears to inhibit IL-13 synthesis (0.21 ng/mL) whereas F3.3, CP1, and F3.3.0 (0.29, 0.41, and 0.29 ng/mL, respectively) did not affect IL-13 synthesis when compared with untreated (0.34 ng/mL) RAW 264.7 cell lines (Figure 5). Interestingly, CP1 appears to enhance IL-13 synthesis and release, which is a beneficial process to limit inflammation and initiate the repair process. This observation further suggests that F3.3, CP1, and F3.3.0 have anti-inflammatory properties and promote the process of inflammatory resolution.

Granulocyte-Macrophage Colony Stimulating Factor

GM-CSF is an important hematopoietic growth factor and immune modulator.[15] This cytokine has profound pro-inflammatory effects on the functional activities of various circulating leucocytes. It is produced by a variety of cell types, including T cells, macrophages, endothelial cells, and fibroblasts on receiving immune stimuli.[16] Other substances such as bacterial LPS, IL-1β, IL-6, and TNF-α, are potent inducers of GM-CSF.

The results in our study indicate that F3.3.0 (15.64 ng/mL) significantly ($P = .01$) suppressed the synthesis of GM-CSF by RAW 264.7 macrophage cells, better than F3.3, CP1, CP2 (46.51, 25.4, and 24.23 ng/mL, respectively) and untreated cells (40.31 ng/mL) (Figure 6). Over-expression of GM-CSF leads to severe inflammation.[17,18]

Tumor Necrosis Factor-α Assay

TNF-α is a pro-inflammatory cytokine produced by a variety of immunocompetent cells including macrophages, Th_1/Th_2 cells, dendritic cells, and neutrophils.[4] TNF, along with IL-1β, and IL-6 are potent, pro-inflammatory cytokines[18] capable of cell recruitment and initiation of inflammation. Increased concentrations of TNF-α are found in acute and chronic inflammatory conditions such as trauma, sepsis, infections and rheumatoid arthritis.[19]

The results obtained here indicate that CP1 (0.11 ng/mL) significantly ($P = .002$) reduced the secretion of TNF-α by RAW 264.7 cells compared with F3.3, CP2, F3.3.0 (0.3, 0.27, 0.24, ng/mL, respectively) and untreated cells

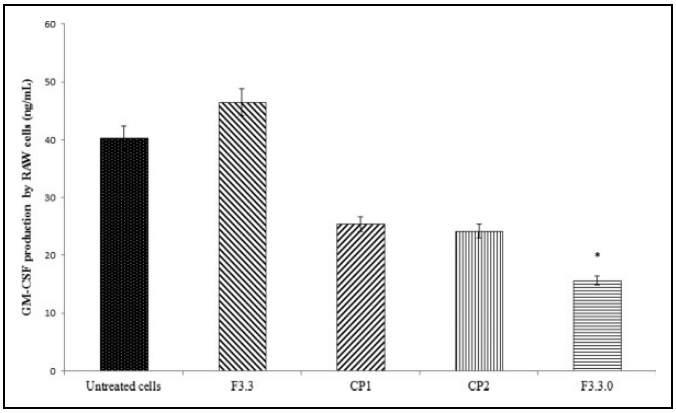

Figure 6. The effects of F3.3, CP1, CP2, and F3.3.0 on granulocyte-macrophage colony-stimulating factor (GM-CSF) synthesis in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cell lines (*$P = .01$).

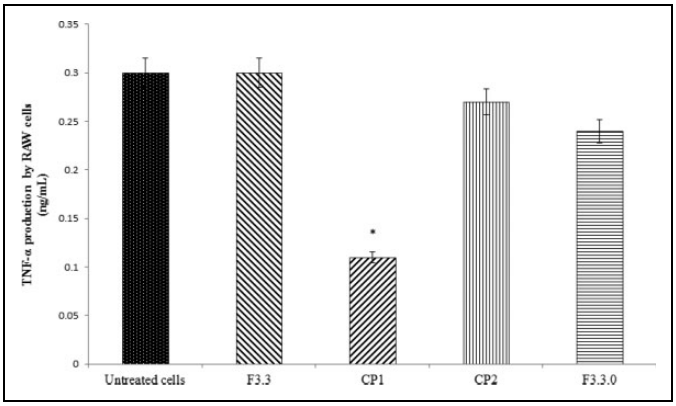

Figure 7. The inhibitory effects of F3.3, CP1, CP2, and F3.3.0 on the synthesis of tumor necrosis factor–α (TNF-α) in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cell lines (*$P = .002$).

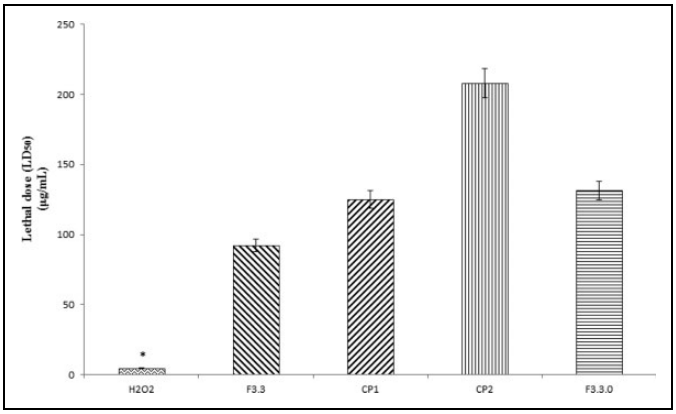

Figure 8. Cytotoxicity assessments of F3.3 (92.25 μg/mL) and CP1 (124.91 μg/mL), CP2 (207.88 μg/mL), F3.3.0 (131.41 μg/mL), respectively, compared with H_2O_2 (4.60 μg/mL) positive controls in RAW 264.7 macrophage cell line in vitro (*$P = .03$).

Table 1. The Selectivity Index (SI) of F3.3, CP1, CP2, and F3.3.0 as a Measure of Their Anti-Inflammatory Activity Relative to the Value of Their Cytotoxicity on RAW 264.7 Cell Lines.

Sample	LD_{50} (μg/mL) on RAW cells	Selectivity Index (SI = LD_{50} in μg/mL/Value of Bioactivity in μg/mL)						
		IL-1β	IL-2	IL-5	IL-12	IL-13	GM-CSF	TNF-α
F3.3	92.25	0.15	0.29	0.02	0.02	0.03	0.21	0.19
CP1	124.91	0.19	0.16	0.13	0.09	0.3	0.09	0.16
CP2	207.88	0.32	0.21	0.16	0.1	0.16	0.17	0.04
F3.3.0	131.41	0.20	0.1	0.14	0.1	0.04	0.16	0.05
H_2O_2	4.60	—	—	—	—	—	—	—

Abbreviations: GN-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; LD_{50}, median lethal dose; TNF-α, tumor necrosis factor–α.

(0.3 ng/mL) (Figure 7). Compounds that are capable of suppressing the synthesis and release of TNF-α are potential candidates for use as templates for development of potent anti-inflammatory drugs.

Cytotoxicity Assessments

Cytotoxicity assessment of plant extracts, fractions, and isolated compound is a measure of the safety profile of the samples when exposed to a particular cell line in culture. All the fractions/compounds tested demonstrated low cytotoxicity on the RAW 264.7 macrophage cell line compared with the H_2O_2 positive controls ($P = .03$). CP2 was the least cytotoxic fraction tested ($LD_{50} = 207.88$ μg/mL) compared with the H_2O_2 positive controls ($LD_{50} = 4.60$ μg/mL) (Figure 8). The results also indicate that the biological activities of the subfraction and test compounds were not due to cytotoxicity on the RAW 264.7 macrophage cell line. This was evaluated using the selectivity index (SI),[20] to ascertain that the observed anti-inflammatory effect was not due to a general metabolic toxic effect on the RAW 267.4 cell lines (Table 1). A higher SI value is indicative of selective anti-inflammatory (biological) activity while a low SI value indicates higher cellular toxicity. The SI for fraction X and isolated compounds ranged between 0.02 and 0.32.

Conclusions

The fractions/compounds isolated from the leaf of PA tested in this study significantly inhibited the synthesis of some pro-inflammatory cytokines; IL-1β, IL-2, and TNF-β. This was especially noted for CP1, which had good inhibitory activity on most of the pro-inflammatory cytokines, with no significant effect on IL-13 synthesis. These activities are probably orchestrated via the inhibition of NF-κB, which is responsible for stimulating the synthesis of these pro-inflammatory cytokines. The results indicate that CP1, CP2, and F3.3.0 are potential candidates that could be used for treating inflammation initiated by the actions of these pro-inflammatory cytokines. It was determined that the extracts have low cytotoxicity and, therefore, have the potential to be used as templates in the

development of new anti-inflammatory drugs. The structures of CP1 and CP2 are currently being ascertained.

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Author Contributions

The draft manuscript as well as biological assays, data entry, and analysis were done by SAA. HCS critically reviewed the manuscript and participated in the study design and choice of assay methods. LJS and JNE conceived the idea, reviewed the draft manuscript, and were instrumental in the study design, data analysis and interpretation. All authors read and approved of the final manuscript for submission.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

This study received ethics clearance from the Research and Ethics committee of the Faculty of Science at the Tshwane University of Technology, as human and animal subjects were not used (2012/209306254).

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