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Tuna blood inhibits lipopolysaccharide-induced inflammatory mediators in RAW264.7 macrophages

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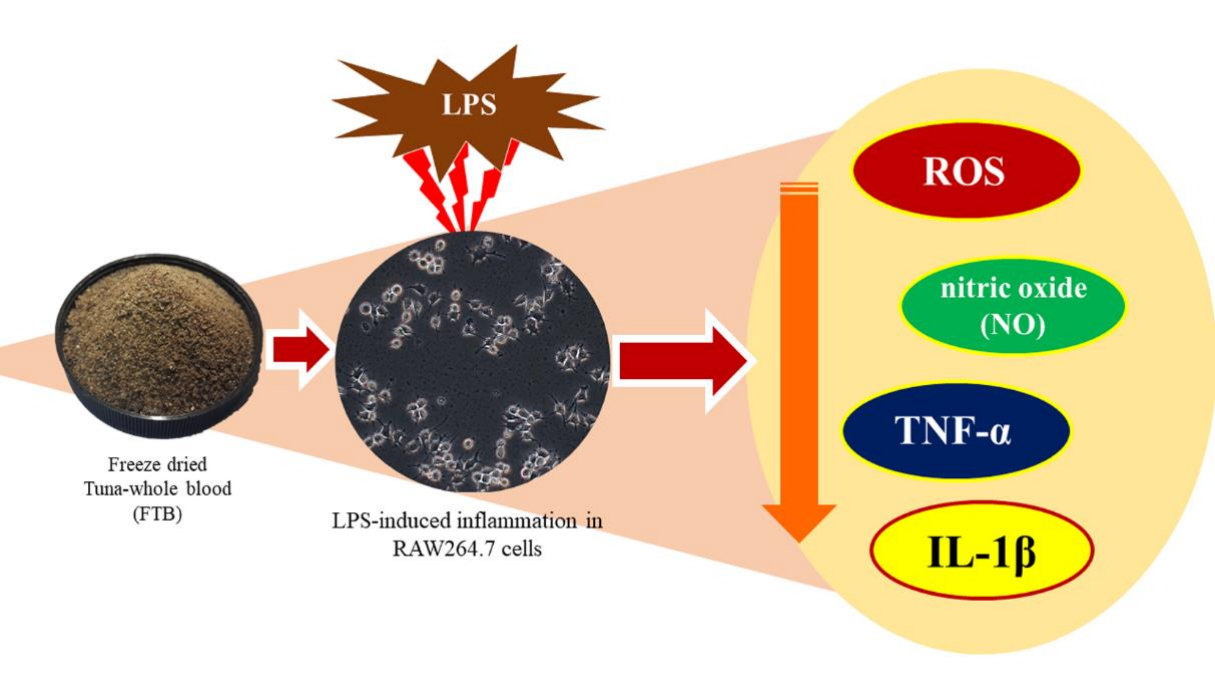
ABSTRACT

Background: This study aimed to evaluate the anti-inflammation effects of the freeze-dried tuna whole blood (FTB), and freeze-dried tuna blood cell (FTC) in LPS-induced RAW264.7 cells.

Methods: The RAW264.7 cells were pre-administered with FTB and FTC at different concentrations for 2 h and then stimulated with lipopolysaccharide (LPS) for 24 h. The production of reactive oxygen species (ROS), nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), and interleukin-1 beta (IL-1β) in RAW 264.7 cells were then determined.

Results: The results showed that only FTB remarkably abolished LPS-induced ROS in RAW264.7 cells. FTB and FTC significantly decreased LPS-induced NO which IC_{50} values of FTB and FTC after 24 h were 78.58 and 22.47 µg/mL, respectively. TNF-α and IL-1β secretion were abolished by FTB and FTC in LPS-stimulated macrophages which IC_{50} values of both FTB and FTC after 24 h were more than 25 µg/mL, respectively. However, the efficacy of FTC against inflammatory mediators was due to cytotoxic effects on RAW 264.7 cells.



Conclusion: Tuna whole blood potentially inhibits inflammation through modulating the synthesis of several mediators and cytokines associated with the development of inflammation. These findings suggest a role of tuna blood on anti-inflammatory activity.

Keywords: Anti-inflammatory activity, RAW 264.7 cells, red blood cell, tuna blood, waste utilization

INTRODUCTION

Canned tuna products have made Thailand one of the most important exporters in the global market. Waste or by-products included heads, bones, skin, viscera, and gills is accounted for 50-55% of the body weight in tuna production. Most of the by-products have been utilized for more valuable products as fish oil from tuna head [1], calcium from bone [2], or collagen from skin, scale, and bone [3]. However, tuna blood is the only remaining waste that has not been utilized. Normally, the blood is disposed into drainpipes causing a high cost of wastewater treatment for the industry. Recently, the recovery of the tuna blood to create the more valuable products is of great importance for the industry. To date, there has been a growing interest in the investigation of the biological properties of *Crocodylus siamensis* blood associated with the immune function, anti-bacterial [4], anti-inflammatory, and antioxidant activity [5-7].

The low-grade inflammation and immune stimulation are related with age-associated morbidity and mortality [8]. It has been concerned that the defense mechanism against external or internal stimuli in response to injury may lead to inflammation [9]. Appropriate control of the inflammatory response is crucial to restore the injury and prevent stress and cellular inflammation [10]. The gathering of

intracellular free radicals causes the secretion of many chemokines and cytokines, for example, interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and nitric oxide (NO) negotiate signal transduction pathways including nuclear-kappa β factor (NF-κβ) as member of the inflammatory reaction [11-13]. Phagocytes, for example, macrophages are the predominant donors to generate oxidative stress and their associated tissue injury [14-15]. Oxidation-inflammation has been reported to be correlated with the development of cellular damage in tissue and organ dysfunction, contributing to an increase in morbidity, mortality, and is associated in the aging process [8]. So, the reduction of oxidation-inflammation may result in the decrease of cellular dysfunction and organ damage, leading to improve health quality and longevity.

Interestingly, the anti-inflammatory effect of tuna blood on macrophages remains undetermined. Therefore, the present study aimed to examine the anti-inflammatory effects of freeze-dried tuna whole blood (FTB) and also freeze-dried tuna blood cell (FTC) against LPS-induced inflammation in murine macrophage RAW264.7 cells.

METHODS

Materials: Tuna blood was collected from a canned–tuna processing plant in Samut-Sakhon province, Thailand. The blood was packed in polyethylene bags and frozen overnight. The frozen tuna blood was transported to the laboratory within 2 hours, and it was immediately stored at -20 ^{o}C until use.

***Preparation of tuna blood*:** The frozen tuna blood was thawed at 3 - 6 °C for 24 h prior to the pasteurization process at 63 °C for 30 min then it was cooled at 0 – 4 °C before used.

***Preparation of tuna blood cell*:** Frozen tuna blood was thawed at 3 – 6 °C for 24 h then it was evaporated using an evaporator (50 °C, pressure at 30 mbar) to remove some excess water. After evaporation, the concentrated blood was centrifuged using a refrigerated centrifuge at 2,500 rpm for 30 min to obtain the sediment (blood cell). The tuna blood cell was further pasteurized at 63 ˚C for 30 min then it was cooled at 0 – 4 °C before used.

Preparation of dried tuna blood and dried tuna blood cell: The tuna blood and tuna blood cell were placed in aluminum trays and frozen at -20 °C for 24 h before being dried using a freeze dryer (Labogene, Scanvac coolSafe model, Denmark) for 24 h. The freeze-dried tuna whole blood (FTB), and freeze-dried tuna blood cell (FTC) were obtained after drying.

***Determination of mineral contents in FTB and FTC*:** The FTB and FTC were determined for iron (Fe), zinc (Zn), phosphorus (P), and magnesium (Mg) according to Poitevin [16].

***Cell culture*:** The murine macrophage RAW264.7 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). The complete DMEM media containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine (Gibco, USA) were used to maintain the RAW264.7 cells in an incubator with 5% CO_2 at 37 °C. RAW264.7 cells were subcultured every 3-4 days.

***Cytotoxicity assay*:** RAW264.7 macrophages were maintained with the complete DMEM medium in a 96-well plate. After cell detachment, FTB, and FTC at the concentration of 0, 1, 5, 10, 25, 50, 75, 100, and 250 µg/mL were then treated to the cells for 24 h. Colorimetric MTT assay was used to measure the viability of the RAW264.7 cells after treatment with FTB and FTC. A microplate reader (Bio-Tex Instruments, Inc., VT, USA) was used to detect the optical density (OD) of each well at 570 nm.

***Reactive oxygen species (ROS) level assay*:** The 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma, St. Louis, MO, USA) oxidation was used for evaluating the intracellular ROS generation in RAW264.7 cells. Briefly, FTB and FTC at the concentration of 5, 10, and 25 µg/mL were pre-treated for 2 h and subsequently the 1 µg/mL of lipopolysaccharide (LPS) from *Escherichia coli* 055: B5 (Sigma, St. Louis, MO, USA) was applied to RAW264.7 cells (5 x 10^4 cells/well) for 24 h. Finally, the DCFH-DA (50 µM) was added into each well and incubated in the dark for 1 h. An excitation wavelength of 485 nm and an emission wavelength of 530 nm was used to determine DCF fluorescence intensity by a fluorescence microplate reader (Bio-Tex Instruments, Inc., VT, USA).

***Nitric oxide (NO) level assay*:** FTB and FTC at the concentration of 5, 10, and 25 µg/mL and dexamethasone (1 µg/mL), as a positive drug were pre-treated for 2 h and subsequently the 1 µg/mL of LPS was applied into RAW264.7 cells (5 x 10^4 cells/well) for 24 h. Then, one-hundred microliter of media was mixed with one-hundred microliters of Griess reagent. The optical densities of the nitrite-containing samples were detected by a microplate reader (Bio-Tex Instruments, Inc., VT, USA) at 540 nm. A standard curve of sodium nitrite was used to estimate nitrate concentrations in the medium.

***TNF-α and IL-1β level assay*:** FTB and FTC at the concentration of 5, 10, and 25 µg/mL and dexamethasone (1 µg/mL), as a positive drug were pre-treated for 2 h and subsequently the 1 µg/mL of LPS was applied to RAW264.7 cells (1 x 10^5 cells/well) for 24 h. After completion of incubation, enzyme-linked immunosorbent assay (ELISA) (Merck, Darmstadt, Germany) method was used to measure the secretion of TNF-α and IL-1β from RAW264.7 cells cultured medium.

***Statistical analysis*:** Values were expressed as mean ± standard error of results. For testing the differences of the mean values, ANOVA and Tukey post hoc test were utilized to compare between groups, and statistically significant was accepted at $P < 0.05$.

RESULTS AND DISCUSSION

***Mineral contents of FTB and FTC*:** Table 1 shows the mineral contents found in FTB and FTC. The results indicated that FTC had higher iron, zinc, and magnesium content than that found in FTB. It could be explained that the blood cell containing red blood cells normally contain haemoglobin, which is a rich source of heme iron and zinc [17] whereas the whole blood is composed of albumin, globulin, and fibrinogen and also haemoglobin [18]. Zinc plays a critical role in haemoglobin synthesis and erythropoiesis whereas iron is essential for oxygen transport and storage and many

other metabolic functions related to immunity, growth, bone strength, muscular activity, and the nervous system. [19]. However, phosphorus content found in FTB was similar to the amount found in FTC.

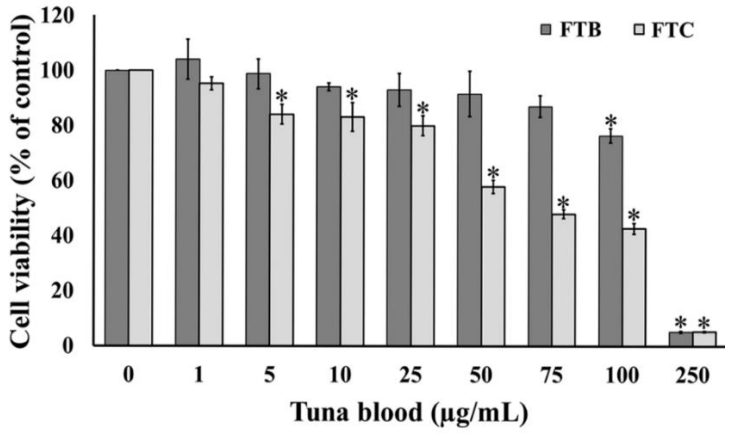
Table 1. The mineral content of freeze-dried tuna whole blood (FTB) and freeze-dried tuna blood cell (FTC)

Mineral	Content (mg/100 g)	
	FTB	FTC
Iron (Fe)	102.10	252.20
Zinc (Zn)	16.00	31.30
Phosphorus (P)	106.00	107.00
Magnesium	328.70	419.60

Note: the values obtained from 2 replication of measurement

***Effects of FTB and FTC on cell viability in RAW264.7 cells*:** It is essential to analyze the cytotoxicity of the FTB and FTC on the macrophages to confirm the safety of each sample before using it as a functional food product for human consumption. RAW264.7 murine macrophages were used to study the response of each compound to the immune system, which is comparative to the human body defense system [13, 20]. MTT cell viability assay was first performed for the cytotoxicity evaluation of FTB and FTC on macrophages. RAW264.7 cells incubated with FTB and FTC (0-250 µg/mL) for 24 h. As shown in Figure 1A, a concentration of 5-250 µg/mL FTC significantly decreased ($p < 0.001$) the percentage of cell viability of RAW264.7 cells after 24 h treatment. However, the percentage of cell viability was higher than 80% after treatment with FTC at the concentration range of 5-25 µg/mL. In contrast to FTC, a concentration range of 1-75 µg/mL FTB did not affect the viability of the RAW264.7 cells while 100-250 µg/mL of FTB significantly decreased ($p < 0.001$) the percentage of cell viability of RAW264.7 cells after 24 h treatment (Figure 1A). The half-maximal inhibitory concentration (IC_{50}) values of FTC and FTB after 24 h were 71.05, and 124.83 µg/mL, respectively (Figure 1B). Based on our results, FTC showed higher toxicity to the macrophage than FTB with the lower IC_{50}. The presence of higher level of iron in the FTC might contribute to the reduction of the RAW264.7 murine macrophage viability. Normally, the small level of iron is necessary for the formation of the blood cells component and other normal physiologic functions [21]. However, a high concentration of iron can interact with hydrogen peroxide to generate the hydroxyl radical, which can lead to injury to the biomolecules such as lipids, proteins, and DNA [22-23]. Besides, overproduction of iron contributes to the development of cardiovascular disease, for example, ischemic heart disease or atherosclerosis, and also inhibits the role of macrophages against the development of cancer [24-25].

(A)



(B)

Tuna Blood	IC_{50} (µg/mL)
FTB	124.83
FTC	71.05

Figure 1. (A) Effects of FTB and FTC at different concentrations on cell viability. (B) IC_{50} of FTB and FTC. Data are presented as mean ± S.E.M. for four independent experiments (n = 4). *p < 0.001 compared to the 0 µg/mL.

***Effect of FTB and FTC on intracellular reactive oxygen species (ROS) level in RAW264.7 cells*:** It is well known that oxidative stress is associated with inflammation. Oxidative stress, an increase of intracellular ROS levels, was identified as a powerful inflammatory mediator [26]. It has been known that ROS acts as a valuable mediator for host defense mechanisms and is produced by the phagocytic cells, for example, macrophages in response to microbial or inflammatory stimuli [27]. ROS overproduction causes the provocation of pro-inflammatory cytokines secretion [28-29]. Moreover, our previous study found that ROS is also a secondary messenger enhanced in LPS-induced inflammatory response [11,13]. As shown in Figure 2, LPS caused an increase in the production of intracellular ROS in RAW264.7 macrophages when compared to the untreated control group ($p < 0.001$). The concentration of 10 and 25 µg/mL of FTB significantly diminished ($p < 0.01$) the LPS-induced ROS production in RAW264.7 macrophages (Figure 2). However, the concentration of 5, 10, and 25 µg/mL of FTC insignificantly downregulated the ROS production in LPS-induced RAW264.7 macrophages (Figure 2) compared to the untreated group. Our results are consistent with those found by Phosri et al. [7], describing the antioxidant activity of blood from the crocodile in fibroblasts MRC-5 cells and RAW264.7 macrophages.

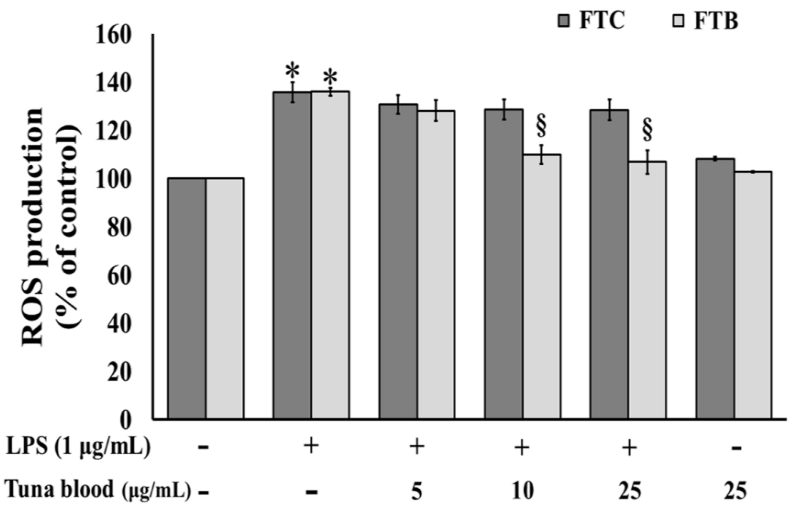


Figure 2. Effect of FTB and FTC on intracellular reactive oxygen species (ROS) level in RAW264.7 cells. Data are presented as mean ± S.E.M. for four independent experiments (n = 4). *p < 0.001 compared to the normal control group. §p < 0.01 compared to the LPS group.

***Effect of FTB and FTC on nitric oxide (NO) level in RAW264.7 cells*:** The NO overproduction may contribute to cytotoxic effects through the formation of peroxynitrite and is linked to chronic inflammatory diseases and aging [30]. Subsequently, an inhibiting of NO production could decrease endotoxin-stimulated inflammation. As shown in Figures 3A and 3C, the nitrite level in RAW264.7 macrophages cultured medium was significantly enhanced by LPS compared to the untreated control group (p < 0.001). FTB at the concentration of 5, 10, and 25 µg/mL significantly decreased (p < 0.01) NO level in LPS-induced RAW264.7 macrophages (Figure 3A). The percentage of NO inhibition was upregulated in all concentrations of FTB with increasing FTB concentrations after 24 h with values of 40.28±9.32, 40.47±7.26, and 45.78±6.05% after treatment with 5, 10, and 25 µg/mL FTB, respectively (Figure 3B). In addition, the concentration of 5, 10, and 25 µg/mL FTC significantly downregulated (p < 0.01) NO level in LPS-induced RAW264.7 macrophages (Figure 3C) when compared to the untreated group. The percentage of NO inhibition was also significantly increased after 5, 10, and 25 µg/mL of FTC treatment with values of 35.40±4.91, 42.44±4.03, and 50.83±4.52%, respectively (Figure 3B). However, the NO inhibition of FTC might be due to chemically induced cytotoxicity at any dosages that were measured by MTT (Figure 3D). IC_{50} values of FTB and FTC after 24 h were 78.58 and 22.47 µg/mL, respectively. Our results are similar to those found by Phosri et al. [7] in fibroblasts cultures exposed to hydrogen peroxide (H_2O_2) and treated with the blood extract from crocodile blood. Additionally, a positive drug, dexamethasone (1 µg/mL) significantly suppressed the secretion of nitrite in the medium of LPS-induced RAW264.7 macrophages. So far, many researchers have targeted inhibition of the NO secretion, which can be employed for novel anti-inflammatory therapeutics agents [11,13,31-32].

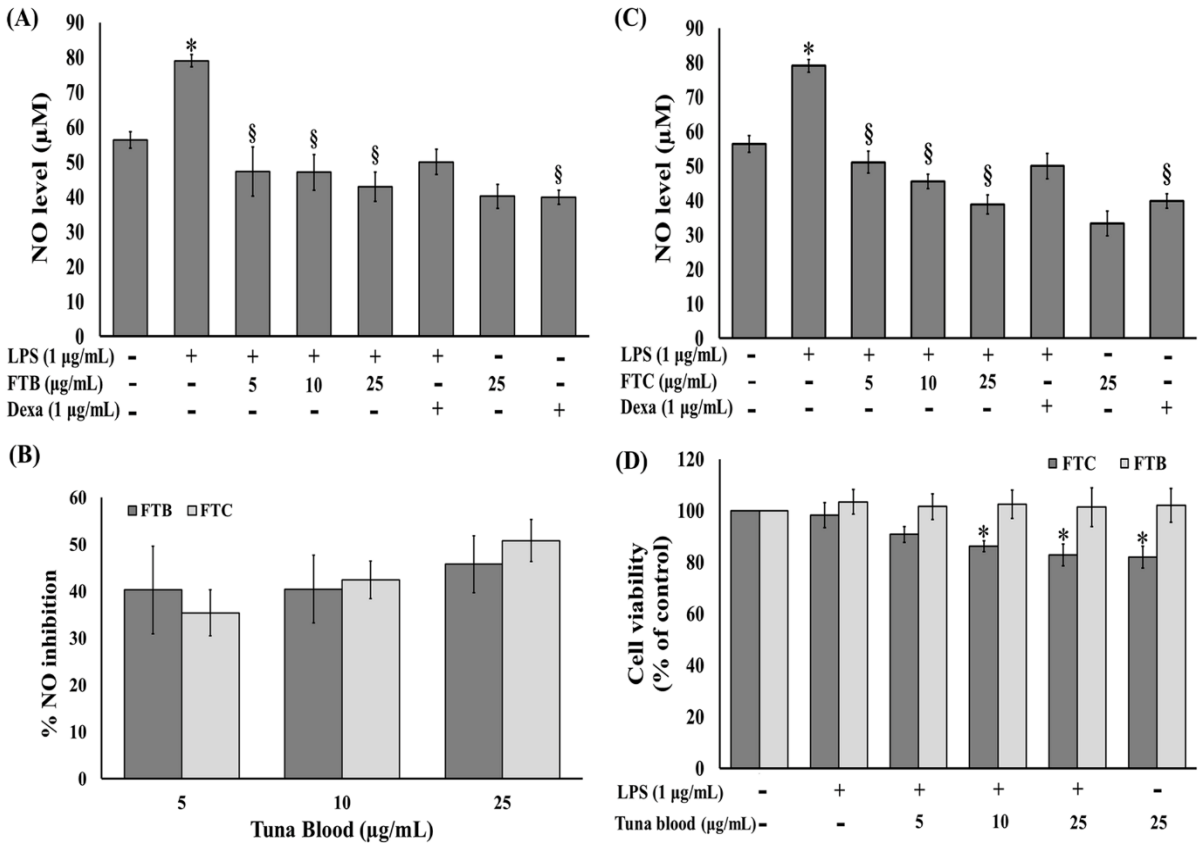


Figure 3. (A) Effect of FTB at different concentrations (5, 10, and 25 μg/mL) on nitric oxide (NO) level in RAW264.7 cells. (B) % Inhibitory effects of FTB and FTC cell on NO level in LPS-induced RAW264.7 cells. (C) Effect of FTC at different concentrations (5, 10, and 25 μg/mL) on NO level in RAW264.7 cells. (D) Effects of FTB and FTC on cell viability in LPS-induced RAW264.7 cells. Data are presented as mean ± S.E.M. for four independent experiments (n = 4). *$p < 0.001$ compared to the normal control group. $^{§}p < 0.01$ compared to the LPS group. $^{\#}p < 0.05$ compared to the LPS group.

***FTB and FTC inhibits the production of TNF-α and IL-1β level in RAW264.7 cells*:** Immune cells and macrophages control the inflammatory response and then produce numerous pro-inflammatory mediators such as ROS, NO, TNF-α, and IL-1β in reaction to injurious stimuli such as LPS [13,33]. The abundance of NO, TNF-α and IL-1β mediators induces the progression of inflammatory disorders [34]. Thus, suppression of NO, TNF-α, and IL-1β overproduction is generally used for seeking anti-inflammatory agents [13,33]. In this study, LPS significantly enhanced the secretion of TNF-α and IL-1β levels in cultured medium of RAW264.7 macrophages compared to the normal control group ($p < 0.001$; Figure 4 and Figure 5). The concentration of 10 and 25 μg/mL FTB significantly inhibited the release of TNF-α level in the cultured medium of LPS-induced RAW264.7 macrophages ($p < 0.05$ and $p < 0.01$; Figure 4A). The percentage of TNF-α inhibition was increased with enhancing FTB concentrations after 24 h with the values of 11.25±1.14, and 24.24±2.03% after treatment with 10 and 25 μg/mL FTB, respectively (Figure 4C). While FTC at the concentration of 10 and 25 μg/mL significantly diminished the TNF-α secretion in LPS-induced RAW264.7 macrophages cultured medium ($p < 0.01$; Figure 4B) compared with the untreated group. FTC at

the concentration of 10 and 25 µg/mL increased the percentage of TNF-α inhibition with the values of 10.79 ±2.18 and 21.67±3.48%, respectively (Figure 4C). IC_{50} values of both FTB and FTC after 24 h were more than 25 µg/mL. Additionally, the secretion of IL-1β into the medium of LPS-induced RAW264.7 macrophages significantly decreased after FTB at all concentrations of treatment ($p < 0.05$, and $p < 0.01$; Figure 5A). The percentage of IL-1β inhibition was upregulated in all concentration of FTB with increasing FTB concentrations after 24 h with the values of 26.88 ±2.59, 36.77±1.75, and 52.30±1.49% after treatment with 5, 10, and 25 µg/mL of FTB, respectively (Figure 5C). Even though the IL-1β level in the medium of LPS-induced RAW264.7 macrophages was significantly downregulated after treatment with 10 and 25 µg/mL of FTC ($p < 0.01$; Figure 5B) when compared to the untreated group. The percentage of IL-1β inhibition increased after 10 and 25 µg/mL FTC treatment with the values of 22.20±4.45, and 30.83±5.84%, respectively (Figure 5C). However, the TNF-α and IL-1β inhibition of FTC might be due to chemically induced cytotoxicity at any dosages that were measured by MTT (Figure 3D). Our results are consistent with the previous reports on fibroblasts cultures exposed to H_2O_2 and treated with the blood extract from crocodile [7] who reported the anti-inflammatory effects through downregulation of TNF-α and IL-1β production. Based on our results, tuna blood downregulates the production of the pro-inflammatory mediators (IL-1β and TNF-α) that will be helpful for the prevention and treatment of inflammatory-related disorders. However, it remains unclear on the exact pathway that regulates the anti-inflammatory activity of tuna blood in LPS-induced RAW264.7 cells.

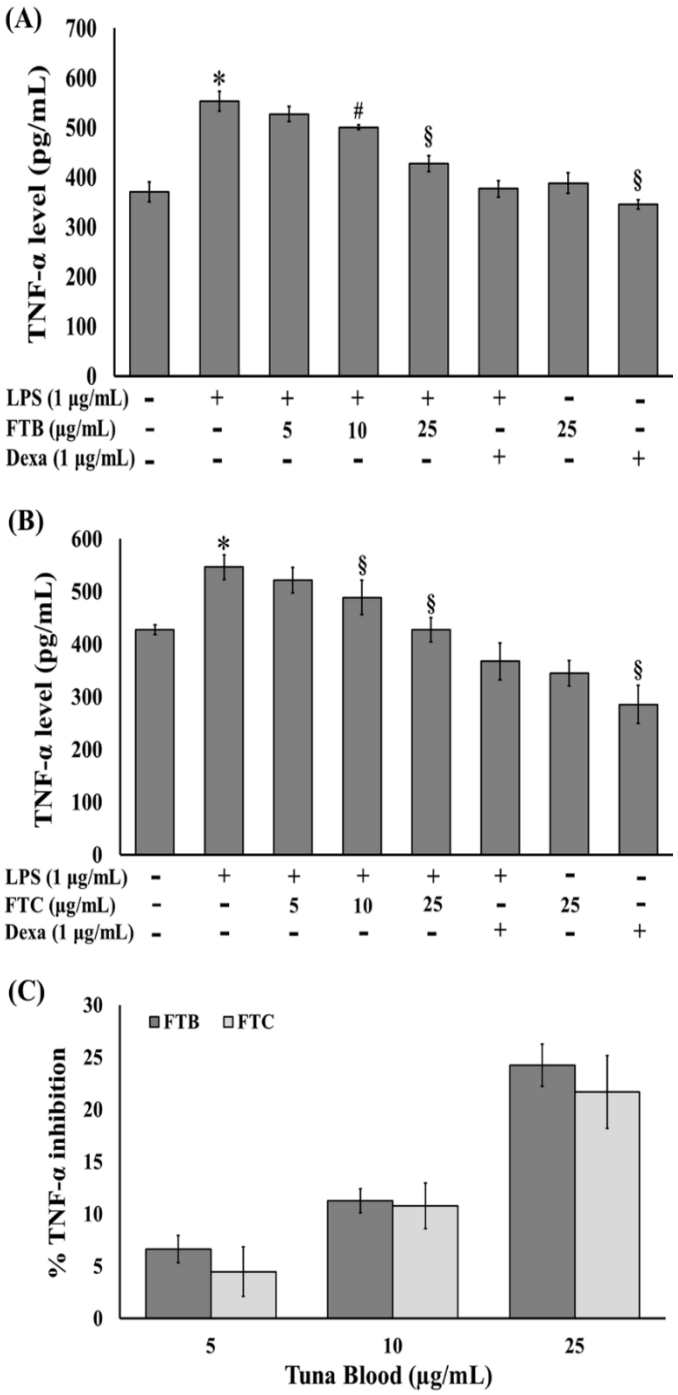


Figure 4. Effect of FTB (A) and FTC (B) at different concentrations (5, 10, and 25 µg/mL) on TNF-α level in RAW264.7 cells. (C) % inhibitory effects of FTB and FTC on TNF-α level in LPS-induced RAW264.7 cells. Data are presented as mean ± S.E.M. for four independent experiments (n = 4). *$p < 0.001$ compared to the normal control group. $^{§}p < 0.01$ compared to the LPS group. $^{#}p < 0.05$ compared to the LPS group.

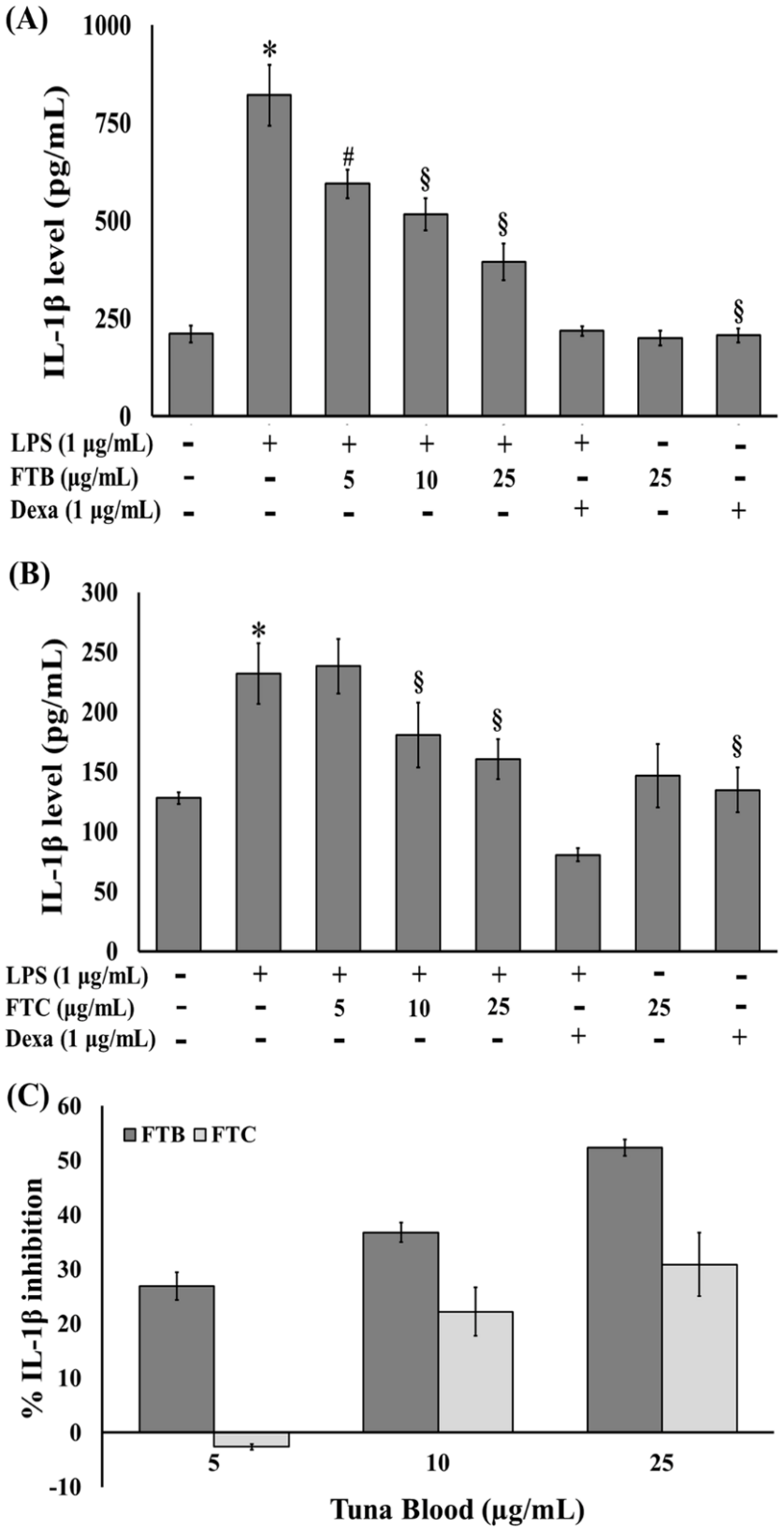


Figure 5. Effect of FTB (A) and FTC (B) at different concentrations (5, 10, and 25 μg/mL) on IL-1β in RAW 264.7 cells. (C) % inhibitory effects of FTB and FTC on IL-1β level in LPS-induced RAW264.7 cells. Data are presented as mean ± S.E.M. for four independent experiments (n = 4). *$p < 0.001$ compared to the normal control group. §$p < 0.01$ compared to the LPS group. #$p < 0.05$ compared to the LPS group.

CONCLUSION

Altogether, our data clearly acknowledges that tuna blood exhibits anti-inflammatory properties through its capability to control the generation of ROS, NO, TNF-α, and IL-1β in LPS-induced RAW264.7 cells. However, the mechanism of the anti-inflammatory effect of tuna blood in both in vitro and in vivo needs to further clarify.

List of abbreviations: FTB: freeze-dried tuna whole blood, FTC: freeze-dried tuna blood cell, LPS: lipopolysaccharide, ROS: reactive oxygen species, NO: nitric oxide, TNF-α: tumor necrosis factor-alpha, IL-1β: interleukin-1 beta

Authors' contributions: WK and WS designed the research, conducted the experiments, and prepared the manuscript; MH and NN analyzed the data; PC and ML prepared the manuscript.

Competing Interests: The authors declare no conflict of interest.

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