

# Anti-inflammatory Effects of Astaxanthin Extracted from Microalgae *Hematococcus pluvialis* and Combinations with Palm Tocotrienol Rich-Fraction in RAW 264.7 Macrophages

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## ABSTRACT

Astaxanthin and tocotrienols, known as antioxidants derived from natural compounds and shown to have anti-inflammatory properties. This study aims to investigate the effects of a combination of astaxanthin extracted from *Hematococcus pluvialis* microalga and palm tocotrienols rich-fraction (TRF) on inflammatory reaction in lipopolysaccharide (LPS)-stimulated mouse RAW 264.7 macrophages cells. MTT assay was used to test cell viability and nitrite oxide (NO) was determined using Griess assay. Isobologram confirmed that the combined treatment produced synergistic effect and measurement of inflammatory cytokines such as interleukin 6 (IL-6) and interleukin 12 (IL-12) through ELISA assay. Our results showed that the combination of astaxanthin and TRF exhibited inflammatory markers such as NO production. The concentration of astaxanthin ranging from 10 to 100 µg/mL and TRF at 4-25 µg/mL had no toxicity and achieved higher cell viability. The combination treatments led to more potent inhibition of NO production compared to single treatments. Combination Index (CI) was achieved from the combination treatments at IC80, resulting in synergism at a CI value of 0.81. Furthermore, ELISA showed that the combined treatment significantly further reduced the expression levels of pro-inflammatory cytokines IL-6 and IL-12. Our findings suggest that the combination of astaxanthin and TRF enhanced anti-inflammatory and antioxidant activities in stimulated macrophages and may act synergistically to produce health effects reducing inflammation.

**Key Words:** Inflammation, Tocotrienol-Rich Fraction, Astaxanthin, Antioxidant, Combination, Macrophages.

## INTRODUCTION

Inflammation is the immune system's natural response to injury and illness —inflammatory chemicals in the bloodstream work to protect your body from foreign invaders like bacteria and viruses. When someone is injured, a localized inflammatory response plays a critical role in the healing process. Inflammation is the immediate, defensive reaction by the body's white blood cells to protect us from infection, injury and foreign substances such as bacteria and viruses. Symptoms of inflammation are addressed by pain, redness and swelling of tissue. However, if the inflammation reaction is over-expressed, it could lead to tissue and capillary damage. Chronic inflammation is one of the significant contributors to cancer, neurodegenerative disorders and cardiovascular diseases.

In general, treatment for inflammation is aimed at either inhibiting the activity of inflammatory cells or inhibiting the production of inflammatory mediators.<sup>1</sup> At present, most inflammatory diseases are treated with steroidal and nonsteroidal anti-inflammatory drugs, which suppress the levels of pro-inflammatory cytokines, inducible nitric oxide synthase (iNOS) cyclooxygenase-2, and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>).<sup>2</sup> However, prolonged use of these conventional drugs may produce adverse side effects<sup>3</sup> in addition to long-term steroid use that suppresses the immune system.<sup>4</sup> New anti-inflammatory agents from natural sources with

fewer adverse effects are alternates that could be developed for long-term administration.

Nowadays, many anti-inflammatory pharmacotherapies inhibit the production of inflammatory mediators and therapies incorporated with antioxidants. Antioxidants are the front-line defenders against free radicals within the cells that serve as the free-radical scavengers.

Microalgae is a natural source that could be a sustainable source of bioactive compounds, and the anti-inflammatory activity of microalgae has been reported widely.<sup>5-8</sup> Several studies have demonstrated that bioactive compounds isolated from microalgae have anti-inflammation and anti-oxidative properties. These compounds can inhibit the production of pro-inflammatory cytokines and reduce the expression of inflammatory genes. Microalgae have been widely recognized as a valuable source of natural, bioactive molecules that benefit human health. Commercial values synthesized by the microalgal metabolism have been proven to display anti-inflammatory activity, including the carotenoids lutein and astaxanthin, the fatty acids EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid), and sulphated polysaccharides.<sup>9,10</sup> These molecules can accumulate to a certain extent in a diversity of microalgae species. A production process could become commercially feasible if the productivity is high and the overall production process costs are minimized. The productivity of anti-inflammatory molecules

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depends on each algal species and the cultivation conditions. The productivity and biochemical composition of microalgae depend strongly on the mode of cultivation, medium composition, and nutrient profile.<sup>11,12</sup>

As photoautotrophs, their simple growth requirements make them attractive for bioprocesses to produce high added-value compounds in great demand by the pharmaceutical market. A few compounds synthesized by microalgae have proven to possess anti-inflammatory, antiviral, antimicrobial, and antitumoral features. Astaxanthin, an antioxidant produced by *Hematococcus pluvialis*, is an illustrative example with significant anti-inflammatory and anti-tumour roles.<sup>13-15</sup> Among the commercially essential microalgae, *Hematococcus pluvialis* is the richest source of natural astaxanthin, which is considered a "super antioxidant." Natural astaxanthin produced by *H. pluvialis* also has significantly greater antioxidants such as astaxanthin.<sup>16,17</sup> A study also reported the inhibition of pro-inflammatory mediators and cytokines by *Chlorella vulgaris* extracts.<sup>18</sup> It is now evident that astaxanthin can significantly reduce free radicals and oxidative stress and help the human body maintain a healthy state.<sup>16,19</sup> With extraordinary potency and an increase in demand, astaxanthin is one of the high-value microalgae products of the future.

Vitamin E consists of two groups: tocopherols and tocotrienols, each with four distinct isomers ( $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ ).<sup>20</sup> The significant difference between these two groups lies in the unsaturated hydrophobic tridecyl side chain of tocotrienols absent in tocopherols. The four tocotrienols differ in the number and location of the methyl group on the chromanol ring.<sup>21</sup> Tocotrienols are reported to possess more superior antioxidant properties than  $\alpha$ -tocopherol due to the presence of the unsaturated side chain, which allows more efficient incorporation into tissues with saturated fatty layers such as the brain and liver.<sup>22</sup> Beyond the antioxidant capacity, tocotrienols are also shown to modulate inflammatory responses via regulating gene expression of pro-inflammatory cytokines.<sup>23</sup>

Tocotrienols are naturally found in edible oils such as palm oil, annatto oil, rice bran oil, and coconut oil.<sup>22</sup> Recent studies have shown that tocotrienols are superior to tocopherols in their antioxidant and anti-inflammatory activities.<sup>20,22</sup>

One study demonstrated that  $\alpha$ -tocotrienol has 40~60 times higher antioxidant activity than  $\alpha$ -tocopherol in preventing oxidative stress in rat liver microsomal membranes.<sup>21</sup> Tocotrienols are more evenly distributed in the phospholipid bilayer and more efficiently re-cycled, allowing for more effective interaction with free radicals. Tocotrienols are also attributed to their preferential cellular uptake<sup>21,24</sup> and more effective penetration into fatty tissues, where they can exhibit their preventive effects.<sup>22,25</sup> Since the chronic phase of inflammation constitutes the production of proteases and oxygen radicals from phagocytes, the usage of tocotrienols and tocopherols may prevent extensive tissue damage.

This study aims to investigate the anti-inflammatory effects of combination treatment between astaxanthin extracted from *Hematococcus pluvialis* microalgae and palm tocotrienol-rich fraction on the lipopolysaccharide (LPS) stimulated RAW 264.7 macrophages.

## MATERIALS AND METHODS

### Cell culture

RAW 264.7 cell lines were purchased from ATCC. RAW 264.7 were cultured in a fresh growth medium of RPMI (Roswell Park Memorial Institute) with an additional 25mM HEPES and L-glutamine, 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin. All cells were cultured at 37°C and 5% CO<sub>2</sub> in a humidified incubator.

### Methodology for astaxanthin extraction from *Hematococcus pluvialis* microalgae

*Hematococcus pluvialis* UTEX 2505 strain (University of Texas, USA), used in this research, are from the stock culture maintained in the Microalgae Research Laboratory (MRL), Faculty of Applied Sciences, UiTM Shah Alam, Selangor. MRL Modified Bold Basal Medium (MRLMBBM) comprised of 2.94 mM NaNO<sub>3</sub>, 0.17 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.3 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.43 mM K<sub>2</sub>HPO<sub>4</sub>, 1.29 mM KH<sub>2</sub>PO<sub>4</sub>, 0.43 mM NaCl, 0.179 mM FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.185 mM H<sub>3</sub>BO<sub>3</sub>, 8.82 ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.144 mM MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.57 mM CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.049 mM Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.071 mM ZnMoO<sub>4</sub>, 0.0025 mM biotin and 0.0015 vitamin B12 was used as the cultivation medium for the cultivation of strain UTEX2505.

In the green stage, the seed cells were inoculated into a 1 L flask with 1% CO<sub>2</sub> and continuous illumination at 50  $\mu$ mol photons m<sup>-2</sup> s<sup>-1</sup>. After 15 days of cultivation, the settling green cells were transferred into another 1 L flask supplied with 0.2% NaCl to induce the astaxanthin formation (red stage) for 7 days. The red cells were harvested via centrifugation (500 g, 10 min, 25 °C) and autoclaved (121°C, 15 minutes, 100 kPa). The resultant cell paste was then mixed with 5 g of glass beads and vortexed for 10 minutes. Subsequently, the cell paste was mixed with distilled water and palm olein at a 1:3 ratio before being vortexed for 10 minutes. The astaxanthin was allowed to be separated overnight based on the polarity of oil and water. The astaxanthin extracted in the oil layer was next quantified using a spectrophotometer at 480 nm before being used in the subsequent experiment.

### Test compound and drug preparation

Astaxanthin extracted from *Hematococcus pluvialis* and Tocotrienol Rich Fraction (TRF) (Sime Darby, Malaysia) were prepared in ethyl alcohol and stored at -20°C. Upon experiment, the test compounds were diluted in cultured media at various concentrations.

### Cell viability assay by MTT proliferation reagent

In triplicate wells, RAW 264.7 were plated at 1 × 10<sup>4</sup> cells per well (final volume of 200  $\mu$ L) in flat-bottomed 96-well plates for 24 hours of incubation. Astaxanthin, TRF and combination between astaxanthin and TRF were added onto the cells in the presence of LPS at 10ng/mL and incubated for 24, 48 and 72 hours. At each time point, cell viability was evaluated using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) assay (Sigma, USA) according to the supplier's instructions. MTT reagent was added to each well, and optical density (OD) absorbance values were measured at 560 nm by Synergy H1 Hybrid Multi-Mode Reader plate reader (BioTek Instruments). The percentage of proliferating cells was determined relative to the 100 per cent viability of control untreated cells.

### Griess assay

The Griess reagent assayed the nitrite oxide (NO) produced by macrophages. Briefly, 50  $\mu$ L of cell supernatant from each well were collected and distributed in 96-well plates and 50  $\mu$ L of Griess reagent (1:1, 0.1% N-(1-Naphthyl) ethylenediamine dihydrochloride (Sigma-Aldrich, United States) in distilled water and 1% sulfanilamide (Sigma-Aldrich, United States) in 5% phosphoric acid (Sigma-Aldrich, United States) were added. Plates were incubated at room temperature, and nitrite was read in a plate reader (BioTek, United States) at 540 nm. The nitrite value was calculated from a calibrated standard curve using sodium nitrite ranging from 0 to 100  $\mu$ M. The result was plotted in a bar graph.

### Synergistic effect analysis

The anti-inflammation effects of the interaction between astaxanthin and TRF were performed by measuring the combination-index (CI), a

quantitative representation of the pharmacological interaction between two drugs. The NO inhibition data was collected after administering astaxanthin and TRF individually and combined with a 1:1 ratio of doses correspondence of each drug doses, respectively. The CI values and isobologram curve between astaxanthin and TRF were performed using the median effect analysis described by Chou and Talalay, 1984 method.<sup>26</sup>  $CI < 1$ ,  $CI = 1$  and  $CI > 1$  indicate synergistic, additive, and antagonistic effects, respectively. The formula calculation is based on  $CI: (IC_c/IC_{Ax}) + (D_c/D_{TRF})$  where  $IC_c$  is the inhibition concentration of combination treatment,  $IC_{Ax}$  is the inhibition concentration from astaxanthin at the dosage used, and  $IC_{TRF}$  is the inhibition concentration of TRF at the dosage used.

### RNA extraction

Raw 264.7 cells were seeded in T75 flasks at a density of 500,000 cells/flask. The cells were treated with the respective test agents (astaxanthin, TRF, combination between astaxanthin and TRF) at their respective concentrations for 24 hours at 37°C in humidified 5% CO<sub>2</sub> incubator. The treated cells were pelleted by centrifugation (1,000 rpm for 5 minutes). Once the supernatant was discarded entirely, 350 µl of Buffer LBP was added to each sample. The homogenized cell lysate was pipetted into a NucleoSpin gDNA Removal Column and centrifuged (11,000 g for 30 seconds). The column is discarded, and the flow-through is collected. Next, 100 µl of Binding Solution BS is added to the flow-through and is mixed well by moderate vortexing or by pipetting up and down several times. The whole lysate is then transferred to the NucleoSpin RNA Plus Column that is placed in a Collection Tube and centrifuged (11,000 g for 15 seconds). Following this, 200 µl of Buffer WB1 is added to the NucleoSpin RNA Plus Column and centrifuged (11,000 g for 15 seconds). The flow-through is then discarded, and the column is placed into a new 2 mL Collection Tube. After that, 600 µl of Buffer WB2 to the NucleoSpin RNA Plus Column and centrifuged (11,000 g for 15 seconds). Flow-through is discarded, and 250 µl of Buffer WB2 is added to the column and centrifuged for 11,000 g for 2 minutes to completely dry the membrane. The column is then placed into a nuclease-free collection tube. Following this, 30 µl of RNase-free H<sub>2</sub>O is added and is centrifuged at 11,000 g for 1 minute. After that, 30 µl of RNase-free H<sub>2</sub>O can be added to another collection tube and is centrifuged at 11,000 g for 1 minute. Elution can be performed with 1 X 60 µl, but the yield can be slightly reduced compared to elution by 2 X 30 µl. Purified RNA was aliquoted into PCR tubes and stored at -80°C.

### RT-PCR method

RT-PCR analysis was conducted to detect mRNA expression of IFN-gamma in RAW 264.7 macrophages. Synthesis of cDNA from total RNA was carried out by RT reaction with superscript (SensiFAST™ cDNA Synthesis Kit). DNase-treated RNA samples were used for Real-Time RT-PCR to measure IFN-gamma expressions. For each 25 µl RT-PCR reaction mix, 5 µl of cDNA corresponding to 20 ng of concentration, 0.5 µl of both sense and anti-sense primers (100 µM), 0.5 µl of enzyme mix, 15 µl of 2x reaction (including SYBR Green dye), and 4.25 µl of PCR-grade water were mixed. After denaturation for 2 min at 94°C. RT-PCR assay was carried out for 40 cycles with denaturation of 1 min at 94°C, annealing for 30s at 60°C, and extension for 30s at 72°C. The specified mode of reaction was controlled with the melting curve. Real-Time RT-PCR analyses were performed with the BioRad CFX 96 real-time RT-PCR detection system. The primers used in the real-time PCR were as follows: mouse TNF-α sense 5'-GCCTCTTCTCATTCCCTGCTTG-3', anti-sense 5'-CTGATGAGAGGGAGGCCATT-3'; and mouse β-actin sense 5'-CGTGCGTGACATCAAAGAGAA-3', anti-sense 5'-TGGATGCCACAGGATTCCAT-3' [27].

### ELISA

The cell supernatant was measured by ELISA to analyze cytokine production of IL-6 and IL-12. 100 µl of capture antibody was added into

a 96-well plate and incubated overnight at 4°C. The 96-well plate was washed 3 times with wash buffer (1x PBS, 0.05% Tween 20), and 100 µl of blocking buffer was added into each well. The plate was incubated for an hour and washed again with wash buffer before samples, standards and blank were added into the wells. The final volume in each well was 100 µl. The plate was then incubated for 2 hours. After that, the plate was washed with wash buffer before detection antibody/enzyme was added into assay diluent and 100 µl dispensed per well. The plate was incubated for an hour at room temperature. 100 µl of substrate (50% substrate A and 50% substrate B) solution was added into each well after rinsing with wash buffer. 50 µl of stop solution was added into wells after the yellow colour developed. The plate was auto mixed for 5 seconds before being measured at the wavelength of 450 nm (BioTek, United States).

### Statistical analysis

The statistical analysis was done using SPSS version 20. Most of the data represented the means + SD of triplicate measurements, One Way ANOVA, Post HOC test with  $P < 0.05$ . The qRT-PCR data represented the means value with + SEM of triplicate measurements.

## RESULTS

### The effects of astaxanthin and palm tocotrienol on cell viability LPS stimulated RAW 264.7

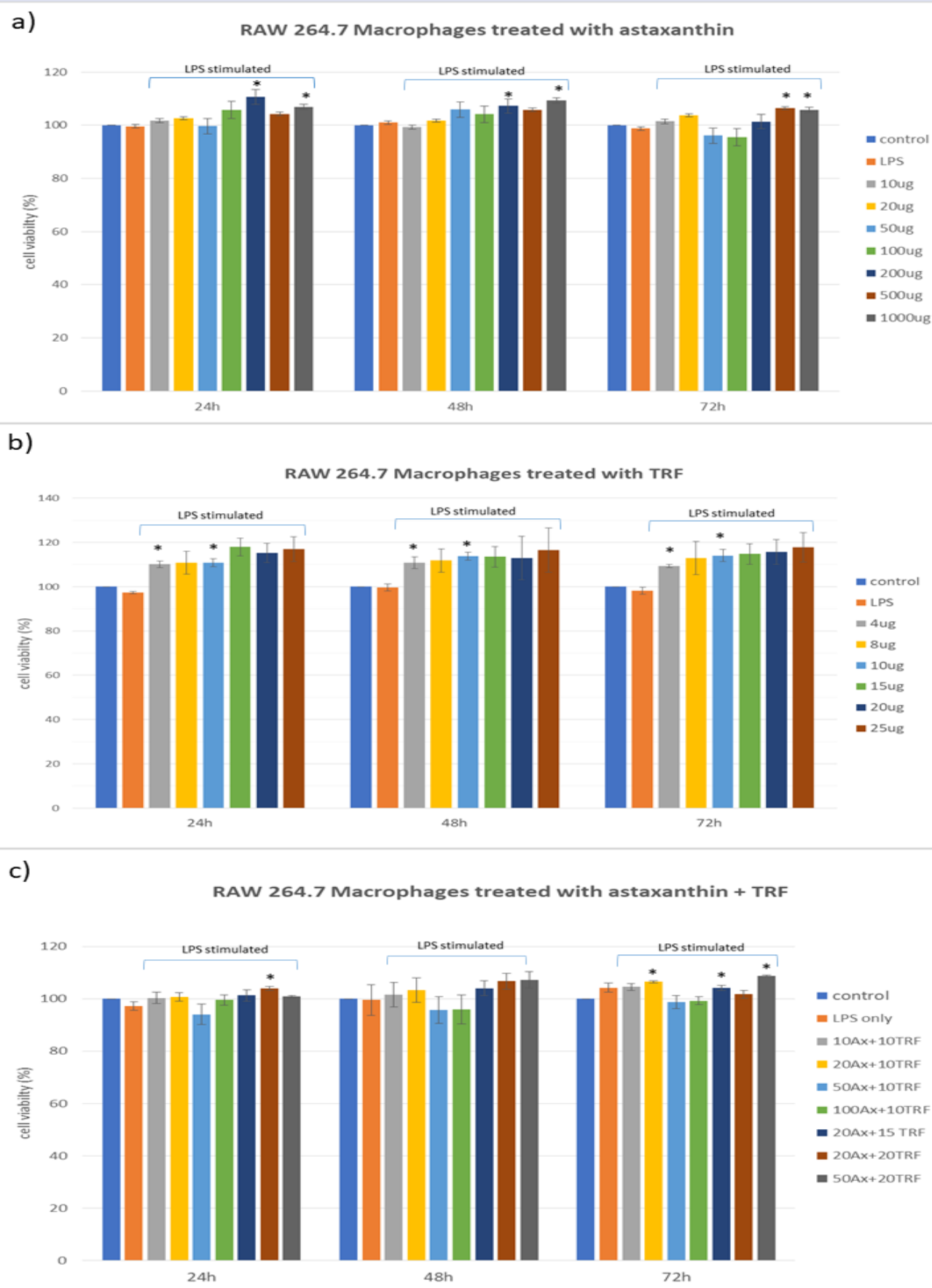
The cell viability of RAW 264.7 macrophages cells was tested on single compounds at various concentrations using MTT assay. Figure 1a showed the viability of cells in different concentrations of astaxanthin ranging from 10, 20, 50 and 100 µg/mL and treated for 24, 48 and 72 hours. It demonstrated that at 10 ng/mL of LPS did not cause any changes to the cell viability, and no cytotoxicity was observed in all treatments up to 100 µg/mL after 24, 48 and 72-hour incubation. A similar result was monitored in RAW 264.7 treated with TRF ranging from 4-25 µg/mL (Figure 1b). The combination treatment with astaxanthin and TRF showed no effects on the cell viability and no cytotoxicity effects (Figure 1c). Overall on cell viability, none of the treatments caused any noticeable cytotoxicity on LPS-treated macrophages with cell viability greater than 95% compared to the control cells.

### NO production decreased in single, and combination treatment in LPS stimulated RAW 264.7 macrophages

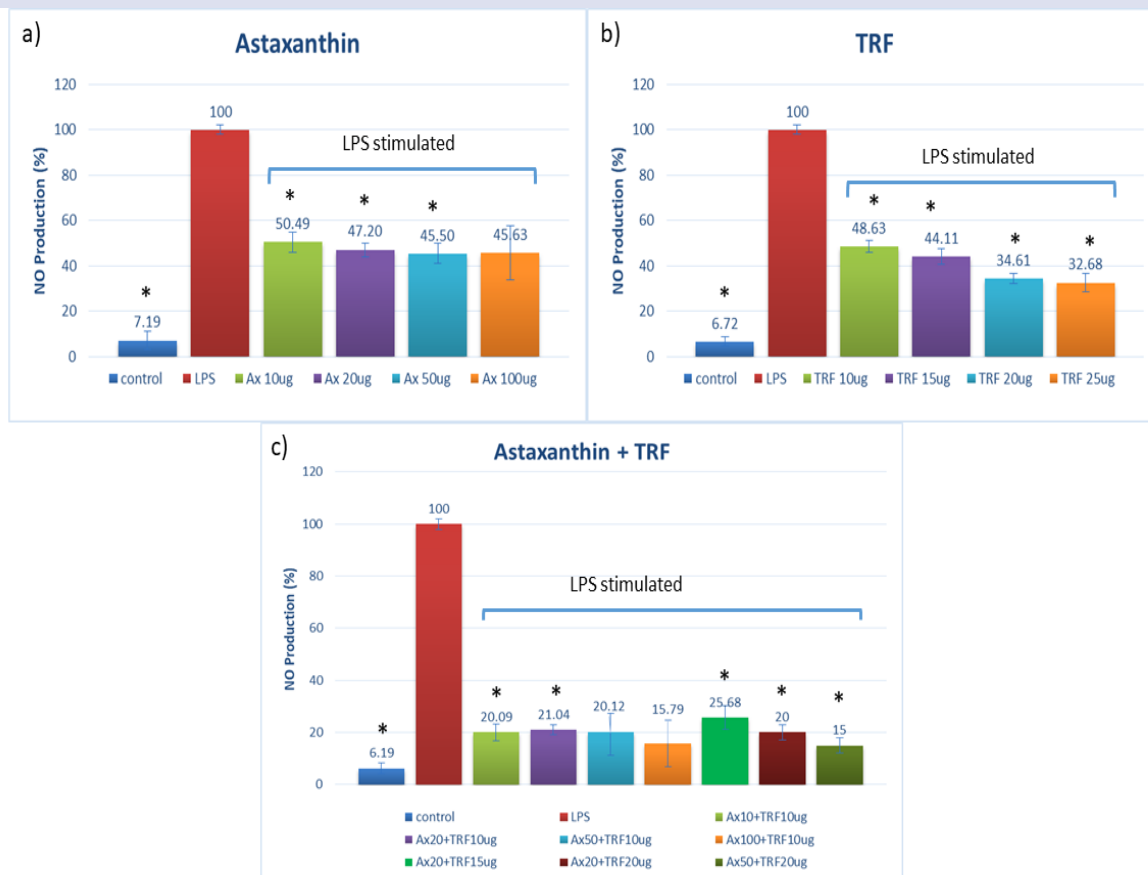
RAW 264.7 macrophages were treated with different concentrations of astaxanthin and TRF for 24 hours in the presence of 10 ng/mL of LPS with single treatments and in combination to determine the anti-inflammatory effects. Figure 2 showed that all treatments with astaxanthin and TRF inhibited the production of NO. Figure 2a showed that astaxanthin at concentrations 10, 20 and 50 µg/mL significantly inhibited the NO production at 50.5%, 47.2% and 45.5%, respectively. For TRF, stimulated LPS ranging from 10-25 µg/mL significantly inhibited NO production at 48.6% -32.7%, as shown in Figure 2b. With the combination treatments at different concentrations between astaxanthin and TRF (Figure 2c), NO production further decreased to 20% - 15%. It was observed that the combination treatments showed more potent inhibitory effects compared to single treatments either in astaxanthin or in TRF, respectively.

### Synergistic effect analysis

The synergistic effects from combination treatment were determined using isobologram and CI values based on the NO production data. Figure 3a showed the isobologram analysis for the combination treatment of astaxanthin and TRF, which achieved 50% inhibition, IC<sub>50</sub> (Fa= 0.5, red line). All concentration pairs of astaxanthin and TRF in combination treatments produces the combination indexes (CI)



**Figure 1:** Percentage of cell viability of LPS stimulated RAW 264.7 macrophages treated with astaxanthin, TRF and combination at 24, 48 and 72 hours. The data represented as means of  $\pm$  SD triplicate wells. \* $P < 0.05$  compared with control, test with One-Way ANOVA.



**Figure 2:** NO production in LPS stimulated RAW 264.7 at different treatment and concentrations; a) astaxanthin, b) TRF and c) combination treatment between astaxanthin and TRF. The data represented as mean  $\pm$  SD of triplicate samples, \*P < 0.05 compared with LPS.

between 0.67 to 1.29, indicating the interaction between astaxanthin and TRF in inhibiting the NO production in LPS stimulated RAW 264.7 macrophages. CI value is a quantitative measure of drug combination divided by synergism (CI < 1), additive (CI = 1) and antagonism (CI > 1). The smaller CI value means a higher synergistic effect. Few doses selected for the combination treatments were plotted (Figure 3a and Figure 3b). The results showed that four (4 CIs: 0.67, 0.81, 0.86 and 0.88) out of six (6) combination treatments tested were found to be synergistic as an anti-inflammatory effect, and the remaining three (3) shows antagonistic effects (1.02, 1.26 and 1.29) as the CI values are more than one (CI > 1, antagonistic effect).

The combination of astaxanthin (10 $\mu$ g, 20 $\mu$ g, 50 $\mu$ g and 100 $\mu$ g) and TRF (10 $\mu$ g) had lower CI values at 0.81, 0.88, 0.86 and 0.67 respectively. The combination treatments of astaxanthin and TRF (astaxanthin/TRF: 10 $\mu$ g/mL/10g/mL and 20 $\mu$ g/mL/10 $\mu$ g/mL) were selected for further studies in-vitro treatments. This is because the fraction affected (fa) showed a similar number of 0.8 for both combination treatments (Figure 3b) and lower dose for working concentrations in producing the good synergistic effect.

### Combination treatments with astaxanthin and TRF down-regulated the expression of TNF-alpha in LPS stimulated RAW 264.7 macrophages

The effects of 24 hours treatments with astaxanthin, TRF and combination in LPS stimulated RAW 264.7 significantly down-regulated the expression of TNF-alpha specific mRNA genes in all tested compounds such as astaxanthin (10 $\mu$ g/mL and 20 $\mu$ g/mL) and 10 $\mu$ g/mL of TRF (Figure 4). From the results, significantly further

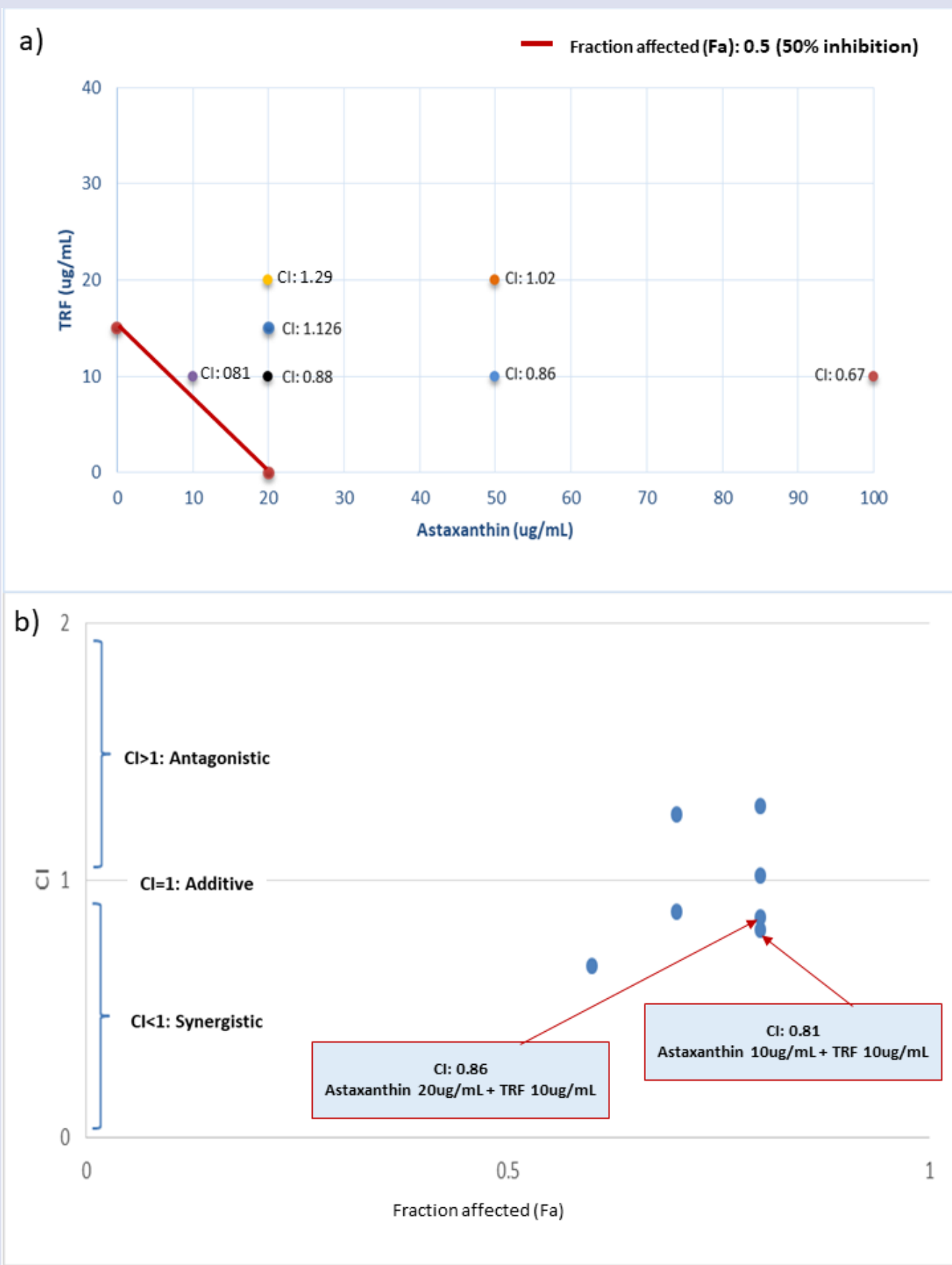
down-regulated of TNF-alpha showed in combination treatments (10 $\mu$ g/mL astaxanthin+10 $\mu$ g/mL TRF and 20 $\mu$ g/mL astaxanthin+10 $\mu$ g/mL TRF) when compared with single treatments and LPS group as represented in Figure 4.

### The expression of IL-6 and IL-12p70 in LPS stimulated RAW 264.7 down-regulated the inflammatory cytokines

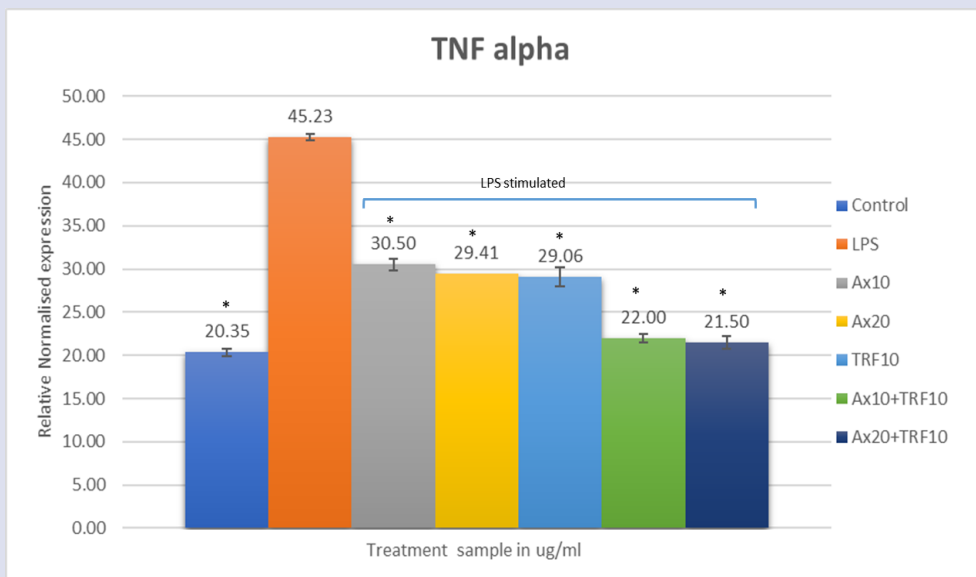
The production of IL-6 and IL-12p70 were measured in the cultures supernatants using ELISA kits. Treatment of RAW 264.7 cells with LPS alone resulted in a significant increase in cytokines productions in IL-6 and IL-12p70 compared with the control group (Figure 5a and 5b). However, in IL-6 productions (Figure 4a) showed treatment with astaxanthin at 10 and 20  $\mu$ g/mL and TRF at 10  $\mu$ g/mL significantly decreased the level of IL-6 to 132.55pg/mL, 136.10pg/mL and 95.87pg/ml respectively. We can observe that the combination treatments (10 $\mu$ g/mL astaxanthin+10 $\mu$ g/mL TRF and 20 $\mu$ g/mL astaxanthin+10 $\mu$ g/mL TRF) further inhibited the production of IL-6 to 67.01pg/mL and 68.02pg/mL. A similar inhibition pattern was monitored in IL-12p70 production with LPS stimulated RAW 264.7 for all treatments tested with further inhibition in combination treatments (Figure 5b). We can conclude that the combination treatments with astaxanthin and TRF considerably inhibited LPS induction of IL-6 and TNF-a in a dose-dependent manner.

## DISCUSSION

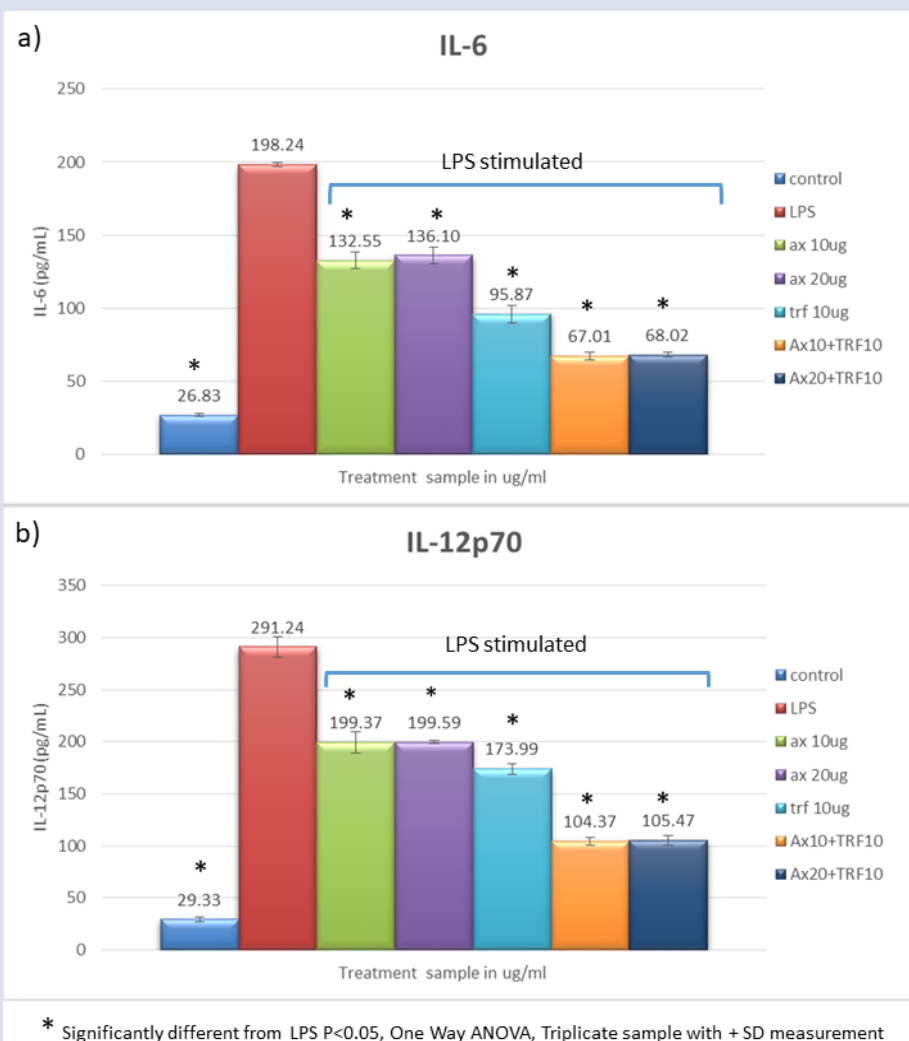
Antioxidants play a critical role in immunity and inflammation. Macrophages are essential in inflammation, immune response<sup>28</sup> and famous innate immune system players, which produce various inflammatory mediators such as nitric oxide (NO), cytokines, and



**Figure 3:** a) Isobologram and b) Combination index (CI) analysis for the inhibition pattern of LPS stimulated RAW 264.7 that induced NO treated with single compound and combination of astaxanthin and TRF.



**Figure 4:** IFN-gamma production in LPS stimulated RAW 264.7 macrophages with different concentrations of astaxanthin and TRF and the combination treatments. The data represented  $\pm$ SEM for triplicate measurement with  $P < 0.05$  as compared with the LPS group ( $*P < 0.05$ ).



**Figure 5:** The productions of a) IL-6 and b) IL-12p70 in LPS stimulated RAW 264.7 macrophages with different concentrations of astaxanthin and TRF and combination treatments. The data represented as  $\pm$  SD of triplicate data measurement, Post HOC test with  $P < 0.05$  ( $*P < 0.05$ ).

prostaglandins against various infectious pathogens such as bacteria, viruses, fungus, and parasites.<sup>28</sup> The combination of compounds in treating inflammation diseases has received attention due to the possibility of producing enhanced efficacy while reducing the dosage to avoid the risk of potential side effects and reduce the development of any treatment resistance.<sup>29</sup> This study aims to evaluate the potential synergistic anti-inflammatory effects from the combination treatments of astaxanthin and TRF.

In this study, we used LPS stimulated RAW 264.7 macrophages as a model of inflammation. No cytotoxicity effects were observed in all treatments, either in single or combination treatments. The cells are still viable even at higher concentrations of astaxanthin and TRF. The previous study by Yam et al. (2009)<sup>30</sup> showed that tocotrienol rich-fraction has non-toxic effects on the viability of cells at a concentration ranging from 1 µg/mL to 15 µg/mL. RAW 264.7 cells were pre-treated with various concentrations of AST for 24 hours and subsequently stimulated with 10 ng/mL of LPS for 24 hours did not change cell viability even at a concentration of 25 µM of astaxanthin.<sup>31</sup> Our study showed that both single and combination treatment of astaxanthin and TRF has non-cytotoxic effects from the treatments.

Nitrite oxide (NO) plays an essential role in mediating many aspects of inflammatory responses and excessive NO will be produced during various inflammatory diseases such as rheumatic diseases, including systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), vasculitis, rheumatoid arthritis (RA), and osteoarthritis (OA).<sup>32</sup> NO is one of the most critical cellular mediators secreted at inflammatory sites, which involves physiological and pathophysiological diversion mechanisms in cardiovascular, nervous and immunological systems.<sup>33,34</sup> Inhibition of NO level is considered as an improvement of tissues in the inflammatory sites. Our study showed that the combination of astaxanthin and TRF in LPS stimulated RAW 264.7 further inhibited NO production compared to a single treatment either in astaxanthin or TRF itself. The IC<sub>50</sub> was observed in 10, 20, and 50 µg/mL of astaxanthin and 10, 15 and 20 µg/mL of TRF treatments. Combination treatments in this study significantly further decreased the NO productions to IC<sub>70</sub> (inhibition at 70%) and IC<sub>80</sub> (inhibition at 80%) in tested concentrations.

In order to find the best combination treatment in this study, the isobologram analysis and CI values were conducted based on the NO production in LPS stimulated samples. The isobologram analysis showed that astaxanthin and TRF achieved 50% inhibition in one to one ratio (Fa:0.5) as shown in Figure 3a and four (4) out of seven (7) combination treatments tested achieved synergism (CI: 0.67, 0.81, 0.86 & 0.88) which indicated the CI number lower than one (CI<1). The results showed that there was a synergistic anti-inflammatory effect between astaxanthin and TRF. The results yielded two combination treatments (Astaxanthin/TRF = 10 µg/mL/10 µg/mL and 20 µg/mL/10 µg/mL) were chosen based on the fraction affected (Fa), CI values and lower dosage of treatment as this would be cost-effective, prevent adverse effects from high dose administration and reducing the development of drug resistance.

TNF alpha is a potent pro-inflammatory agent that regulates many aspects of macrophage function and is considered a master regulator of pro-inflammatory cytokine production.<sup>35</sup> TNF alpha plays a critical role in developing many chronic inflammatory diseases and is involved in inflammatory cell activation and recruitment.<sup>36</sup> Our finding showed that all treatments were able to down-regulate the expression of TNF alpha significantly in LPS stimulated RAW 264.7 macrophages and further down-regulated the expression in both of the combination treatments that have been tested. We also observed that the combination treatments selected showed almost similar expression, which is 22 fold in 10 µg/mL astaxanthin + 10 µg/mL TRF and 21.5 fold in 20 µg/mL astaxanthin + 10 µg/mL TRF. The same data reported by

Lee et al. (2003) also demonstrated a reduction of TNF alpha in a single treatment of astaxanthin in *in-vitro* and *in-vivo* in LPS stimulated RAW 264.7 macrophages.<sup>35</sup> Some studies<sup>30,37</sup> reported the inhibition of TNF alpha on tocotrienol treatments in LPS stimulated macrophages and in the mouse model.

Furthermore, we investigated the effects of combination treatments of astaxanthin and TRF in reducing the production of IL-6 and IL-12p70. Our experimental data showed that after 24 hours of single and combination treatments with LPS, both compounds significantly decreased the production of IL-6 and IL-12p70 in LPS-induced mouse macrophages at tested concentrations ( $P < 0.05$  vs LPS alone), as shown in Figure 5a and 5b. IL-6 is produced at the site of inflammation and plays a vital role in the acute phase response as defined by various clinical and biological features such as the production of acute-phase proteins.<sup>38</sup> The IL-12p70 is a heterodimer composed of p35 and p40 and is a pro-inflammatory cytokine that enhances Th1, cytotoxic CD8<sup>+</sup> T, and NK cell responses by increasing IFN- $\gamma$  production.<sup>39</sup> From the observation, combination treatments both in astaxanthin/TRF = 10 µg/mL/10 µg/mL and 20 µg/mL/10 µg/mL showed an almost similar pattern of inhibitions and further decreased the productions of IL-6 and IL-12p70 when compared with single treatment and LPS group ( $P < 0.05$ ).

## CONCLUSION

In summary, the combination of astaxanthin and TRF exerted anti-inflammatory and antioxidant activities in LPS stimulated RAW 264.7 macrophages cells, which inhibited the production of NO, productions of IL-6 and IL-12p70, and down-regulated the expression of TNF alpha. Since both combination treatments in astaxanthin/TRF = 10 µg/mL/10 µg/mL and 20 µg/mL/10 µg/mL tested and measured in TNF alpha, IL-6 and IL-12p70 expressed almost similar reductions; we selected the combination of treatment of lowest concentration which is astaxanthin at 10 µg/mL and 10 µg/mL of TRF to be used in our future treatment in our mouse model study.

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## CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

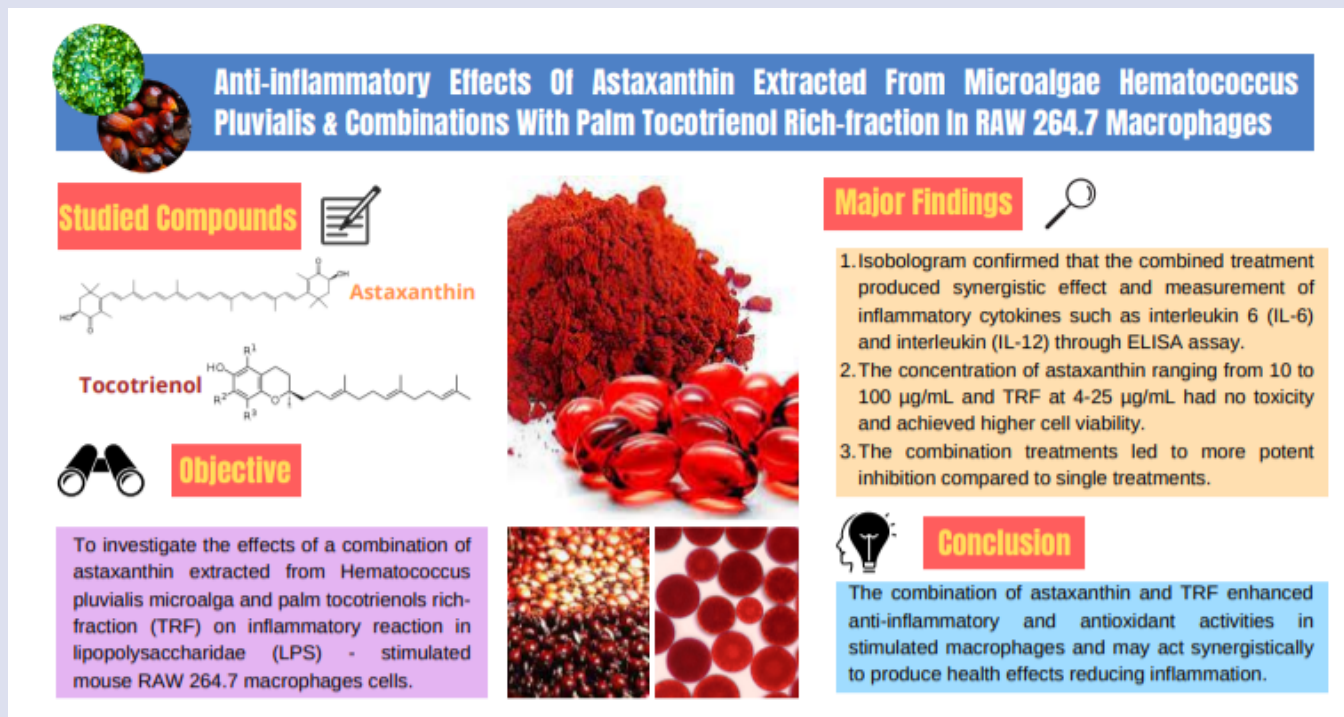
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## GRAPHICAL ABSTRACT



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