Optimization of Anti-Inflammatory Potential of Astaxanthin and Tocotrienols Cocktails in Lipopolysaccharide (LPS) Stimulating RAW 264.7 Macrophages

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Abstract. The rise in inflammatory diseases necessitates effective anti-inflammatory strategies. Astaxanthin and tocotrienols exhibit anti-inflammatory potential, with possible synergistic benefits. Combining multiple anti-inflammatory properties can provide a broad spectrum of protection. However, the effectiveness of such combinations varies depending on the synergistic effect and their proportions. This study established an optimal inflammation model using RAW264.7 cell-line, determining 15,000 cells/well and 10 ng/mL LPS as ideal conditions. Both compounds, individually and in combination, proved non-toxic and effectively reduced Nitrite Oxide (NO), a marker of inflammation. To enhance cost-effectiveness and maintain NO regulation, lower concentrations than previously reported (200 µg/mL and 100 µg/mL) are recommended for astaxanthin-tocotrienols formulations.

Keywords: astaxanthin; tocotrienols; anti-inflammatory; RAW264.7 macrophages.

1 Introduction

Inflammation plays a crucial role in various pathological conditions, including Alzheimer's disease, cancer, and allergies. It is a response to disturbances in tissue homeostasis caused by factors like tissue injury, contaminants, or pathogens [1]. While inflammation is essential for survival in cases of physical injuries and infections, chronic inflammation can lead to several diseases [2]. Chronic inflammatory diseases account for 50% of worldwide deaths and include conditions like stroke, chronic kidney diseases, diabetes mellitus, ischemic heart disease, autoimmune and neurodegenerative conditions, and non-alcoholic fatty liver disease [1-2].

To develop therapeutic strategies for mitigating the adverse effects of inflammation, it is important to understand the cellular and molecular mechanisms underlying it. Key

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pathways involved in inflammation include nuclear factor kappa-B (NF-κB), mitogen-activated protein kinase (MAPK), and Janus kinase-signal transducer and activator of transcription (JAK-STAT). Dysregulation of these pathways can contribute to inflammation-associated diseases [3-4].

When studying inflammation and potential therapeutic compounds, established cell lines are commonly used for in vivo and in vitro analyses [5]. Macrophages, as key players in the innate immune system, serve as valuable models for studying inflammation. They can be activated by various signaling molecules and differentiate into different functional types. M1 macrophages are used to study inflammation, bactericidal activities, and immunostimulant, while M2 macrophages are utilized for tissue repair, immunosuppression, matrix remodeling, and angiogenesis stimulation [6].

In this study, RAW 264.7 macrophages derived from murine monocyte/macrophage lineage are employed due to their ability to mimic the immune response and inflammatory pathways [7]. However, the optimal cell seeding density for inflammatory experimentation using RAW 264.7 macrophages vary across studies. For instance, the osteoclast differentiation experiment showed that a seeding density of 1.8×10^4 yielded more osteoclasts over multinucleated cells, while similar studies indicated that the optimal cell density was 6.25×103 cells/cm^2 [8-9]. It is crucial to determine the best cell density for accurate and consistent results.

Lipopolysaccharide (LPS), a component of Gram-negative bacteria's outer membrane, is commonly used to stimulate macrophages and induce an inflammatory response [10]. The concentration of LPS used in previous studies varies. For instance, in a study inducing inflammation in THP-1 cells, a human leukaemia monocytic cell line used 5, 10, and 20 ng/ml of LPS for 24 hours. These three concentrations of LPS induced the inflammation in the THP-1 Cells to mimic bacterial infection [11]. Nevertheless, the optimal concentration is not being investigated in that work. In the other work, researchers have stimulated RAW 264.7 murine macrophages with a fixed concentration of LPS, which is 1 g/ml at varying times. The study showed that LPS managed to induce inflammation in the cells and treated the inflamed cells using amber extracts [12]. It is important to determine the optimal concentration for generating a physiologically relevant inflammatory response in macrophages.

Natural compounds like astaxanthin and tocotrienols have shown potential anti-inflammatory properties. However, their combined effects on inflammation have been less explored. Previous studies have focused on their combined antioxidative properties and memory improvement. The optimal combined concentrations of these compounds are also less reported in the literature [13-14]. Our recent studies investigate the individual concentrations of astaxanthin and tocotrienols in reducing inflammation where 10 to 100 μg/mL and tocotrienols at 4-25 μg/mL were utilized for individual treatments and astaxanthin-tocotrienols formulations were tested randomly for the combined treatments. Our initial study indicated that the combined compounds postulated anti-inflammatory activities and were able to produce health effects in reducing inflammation [15] Hence, the present work aims to optimize the concentration of astaxanthin and tocotrienols to address inflammation using a Box-Behnken Design.

All in all, this research seeks to develop and optimize a formulation of astaxanthin and tocotrienols that can effectively modulate inflammation in RAW 264.7 macrophages stimulated with LPS. By assessing the potential combination of these compounds, the study aims to identify a novel therapeutic approach for inflammation management. The outcomes of this research will contribute valuable insights into establishing a robust macrophage-based inflammation model and developing novel anti-inflammatory formulations. Ultimately, this study aims to advance our understanding of inflammation biology and facilitate the development of targeted interventions to mitigate the detrimental effects of chronic inflammation on human health.

2 Methodology

2.1 Cell Culture

Raw 264.7 murine cell lines were acquired from the American Type Culture Collection (ATCC). The acquired cells were cultured in a fresh growth medium of DMEM (Dulbecco's Modified Eagle Medium), by which the medium was supplemented with 25 mM Hepes and L-glutamine, 10% fetal bovine serum (FBS) and 100 U of penicillin and streptomycin. The cell culture conditions were maintained at 37°C and 5% CO_2 in a humidified incubator. The cells were passaged twice before being used in the experiments.

2.2 Determination of Optimal Cell Density and LPS Concentration in Establishing RAW 264.7 Inflammatory Models

Cells maintained from the previous procedures have been plated in ACEA iCELLigence 8-well plates to determine the optimal cell density and LPS concentration to establish RAW 264.7 inflammatory models. In finding the optimal cell density, RAW 264.7 Macrophage cells were seeded at varied concentrations ranging from 5,000 to 40,000 cells per well in Agilent E-Plate. The cell index was monitored every 30 minutes via the RTCA SP instrument. In optimizing the LPS concentration, RAW 264.7 cells were stimulated with different concentrations of LPS throughout 72 hours in an 8-well plate. The concentration tested ranged from 10-1000 ng/ml of LPS. The data was tabulated in real time to determine the optimal cell density and LPS concentration for the RAW 264.7 macrophage cell line.

2.3 Astaxanthin and Tocotrienols Rich Faction (TRF)

Astaxanthin was obtained from the Microalgae Research Laboratory (MRL), Faculty of Applied Sciences, UiTM Shah Alam, Selangor, while TRF was acquired from Sime Darby, Malaysia. Astaxanthin and TRF were prepared in soyabean oil, which acts as a basal medium and stored at -20°C. Upon experimenting, the test compounds were diluted at various concentrations. Single compound treatment for astaxanthin was

diluted from 25-200 µg/ml, while for TRF, the concentration used for single treatment was 25-100 µg/ml.

2.4 Astaxanthin-Tocotrienols Cocktails Formulation using Box-Behnkn Design (BBD)

Astaxanthin concentration, tocotrienol concentration and the basal compound were the three independent variable factors used in the study. The basal compound utilized in the current work is pure soyabean oil. The factor levels for this BBD Design were divided into -1, 0 and +1. The levels represent the low, medium, and high, respectively. Reducing the number of experiments is considered one of the advantages of using this BBD Design, as this will reduce the cost of experimenting compared with the traditional univariate procedure [16-17]. Previous literature was used to indicate the highest level for Box-Behnkn Design, and its experimental design is postulated in Table 1 below.

Table 1. Box-Behnkn Design for Astaxanthin-Tocotrienol Cocktails

Independent Variables	Factor Level			Dependent Variable	Goal
	-1	0	+1		
Astaxanthin Concentration	50	100	200	Optimized Cocktails Formulation (ug/ml)	Maximized
Tocotrienols Concentration	25	50	100		
Basal Compound	5	10	20		

Using Minitab Statistical Software Version 21.1.0, the Box-Behnkn Design proposed has resulted into 15 formulations, as shown in Table 2 below.

Table 2. Formulated Astaxanthin-Tocotrienols Cocktails

Sample Number	Formulations (µg/ml)		
	Astaxanthin	Tocotrienols	Basal Compound
1	1 [200]	1 [100]	0 [10]
2	0 [100]	-1 [25]	-1 [5]
3	-1 [50]	0 [50]	1 [20]
4	1 [200]	0 [50]	-1 [5]
5	0 [100]	1 [100]	1 [20]
6	-1 [50]	1 [100]	0 [10]

7	1 [200]	0 [50]	1 [20]
8	-1 [50]	0 [50]	-1 [5]
9	0 [100]	0 [50]	0 [10]
10	0 [100]	0 [50]	0 [10]
11	1 [200]	-1 [25]	0 [10]
12	-1 [50]	-1 [25]	0 [10]
13	0 [100]	0 [50]	0 [10]
14	0 [100]	-1 [25]	1 [20]
15	0 [100]	1 [100]	-1 [5]

2.5 Cell Viability Assays by MTT Proliferation Reagent

Raw 264.7 macrophage cells were plated at 15,000 cells per well (final volume of 200 µL) in flat-bottomed 96-well plates for 24 hours of incubation in triplicate wells. Astaxanthin, TRF and combinations of astaxanthin and TRF were put into the cells with LPS at 10ng/ mL. The cells were incubated for 24, 48 and 72 hours, and cell viability was assessed using MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide, a tetrazole) assay (Sigma, USA) according to the supplier's protocols, at each time point. Optical density (OD) absorbance values after adding MTT were measured at 560 nm via Synergy H1 Hybrid Multi-Mode Reader plate reader (BioTek Instruments). The percentage of proliferating cells was compared relative to the 100 per cent viability of control untreated cells.

2.6 Griess Assays

In general, an enormous amount of nitrite oxide (NO) is being produced at the sites of inflammation, and the Griess reagent assays the NO released by macrophages. In the current work, 50 µl of cell supernatant from each well was pooled and distributed in 96-well plates. Then, 50 µl 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 put on. The 96-well plates were incubated at room temperature, and nitrite was read in a plate reader (BioTek, United States) at 540 nm. The nitrite value was determined based on a calibrated standard curve using sodium nitrite ranging from 0 to 100 µM. The result was presented in a bar graph.

2.7 Statistical Analysis

Microsoft Excel 2019 has been deployed to record and calculate the data obtained in the current work. Analysis of variance (ANOVA), Response Surface Analysis and

Response Optimizer have been utilized in this study to observe the significance and optimal astaxanthin and tocotrienol cocktail formulations. A value of $P < 0.05$ will be considered statistically significant. Minitab 21.1.0 has been used to design the experiment and analyze the data collected from all experiments. Most of the data represented the means + SD of triplicate measurement.

3 Results

3.1 Optimization of RAW 264.7 cell density using ACEA iCELLigence e-plate

Figure 1 indicates the cell density measured using ACEA iCELLigence e-plate in RAW 264.7 macrophage cell line.

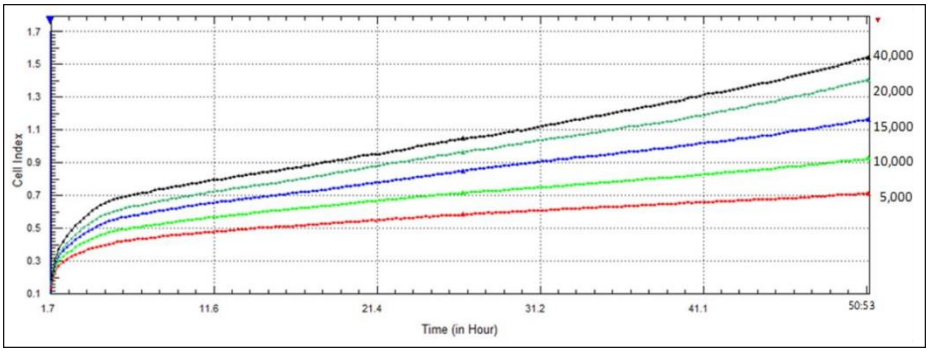

Fig. 1. Optimization of RAW 264.7 Cell Density using ACEA iCELLigence e-plate

The cell density based on varied cell numbers was plated in triplicate into a 96-well plate. The cell numbers tested in the current work are 5000, 10000, 15000, 20000 and 40000 cells/well. The cell index was measured to determine the optimal number for RAW 264.7 macrophage cells. Based on the data obtained in the current work, it could be noted that 15,000 cells per well was considered the best cell density to establish inflammatory models in RAW 264.7 macrophages because the cells are proliferating at a more stable rate compared to other concentrations.

3.2 Optimization of LPS concentration for RAW 264.7 Macrophages cell line

In establishing the inflammatory models for the RAW 264.7 macrophage cell line, the LPS concentration used in inducing the inflammation shall be considered. Figure 2 postulates the different LPS concentrations in inducing inflammation in macrophage cells.

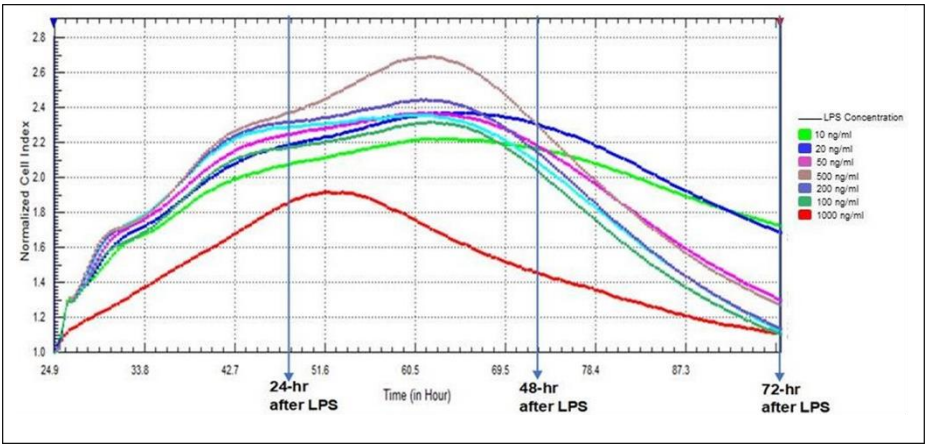

Fig. 2. Optimization of LPS Concentration in Establishing RAW264.7 Inflammatory Models

In the current work, RAW 264.7 macrophages have been infected with LPS at 10-1000 ng/ml concentrations. The result suggested that 10ng/mL of LPS is sufficient to induce inflammation in RAW 264.7 Macrophage cell lines models as it does not affect cell proliferation after 72 hours.

3.3 Cell Viability of Single and Combined Compounds against LPS Stimulated RAW 264.7 Macrophages

In finding the optimal concentration of astaxanthin and tocotrienols for treating inflamed RAW 264.7 macrophage cells, a thorough literature study was conducted to find the usual or most concentration used in the previous work for these potent anti-inflammatory compounds. It is noticeable that the highest concentration for astaxanthin used in the previous work was 200 µg/ml, while for tocotrienols, it was approximately 100 µg/ml. Based on the available data from the literature, the plated RAW 264.7 macrophage cells were tested with single compounds treatment and the cell viability of the treated cells was tested using MTT assays. Figure 3 indicates the viability of cells in different concentrations of astaxanthin ranging from 25 – 200 µg/ml, with an interval of 25 µg/ml. The treatment was applied to inflamed RAW 264.7 macrophage cells for 24, 48 and 72 hours. It was observed that 10 ng/mL of LPS applied to the cells did not cause any changes to the cell viability, and no cytotoxicity was noticed in all treatments up to 200 µg/mL after 24, 48 and 72-hour incubation. Figure 4 illustrates the cell viability of single tocotrienols treatment ranging from 25 – 100 µg/ml, with an interval of 25 µg/ml for 24, 48, and 72 hours, respectively. The results were like astaxanthin, where no cytotoxicity was observed in all treatments.

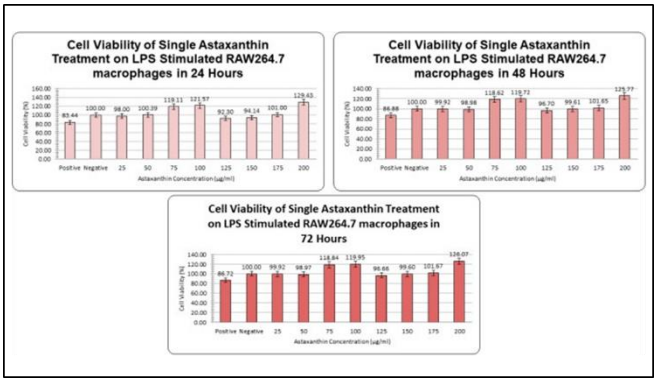

Fig. 3. Percentage of cell viability of LPS stimulated RAW 264.7 macrophages treated with astaxanthin at 24, 48 and 72 hours. The data represented as means of ± SD triplicate wells.

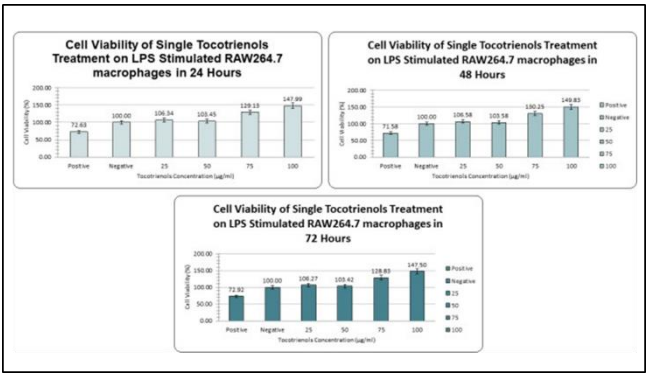

Fig. 4. Percentage of cell viability of LPS stimulated RAW 264.7 macrophages treated with tocotrienols at 24, 48 and 72 hours. The data represented as means of ± SD triplicate wells

Based on the information from previous literature, a Box-Behnken Design (BBD) was utilized to form cocktails formulation of astaxanthin-tocotrienols. Based on the previous literature, 200 µg/mL astaxanthin and 100 µg/ml tocotrienols were the best and highest concentrations for these single compounds to reduce inflammation, and hence, it has been set to be the highest level in BBD formulations. The highest level is +1, and 0 plus -1 concentration was divided into half. That would mean +1, 0 and -1 levels of astaxanthin were 200, 100 and 50 µg/ml, respectively. The same thing was applied to tocotrienols, and soyabean oil was set as the basal medium in the current work. Using Box-Behnken Design, 15 astaxanthin-tocotrienols formulations were formulated in the study. These 15 formulations were tested over the LPS-stimulated RAW 264.7 macrophages. Figure 5 postulated the viability of cells when 15 different formulations of astaxanthin-tocotrienols were applied. The combination treatment with astaxanthin and TRF did not affect cell viability, and no cytotoxicity effects were observed. Overall, in cell viability experiments, none of the treatments caused any

noticeable cytotoxicity on LPS-treated macrophages, with cell viability more than 95% compared to the control cells.

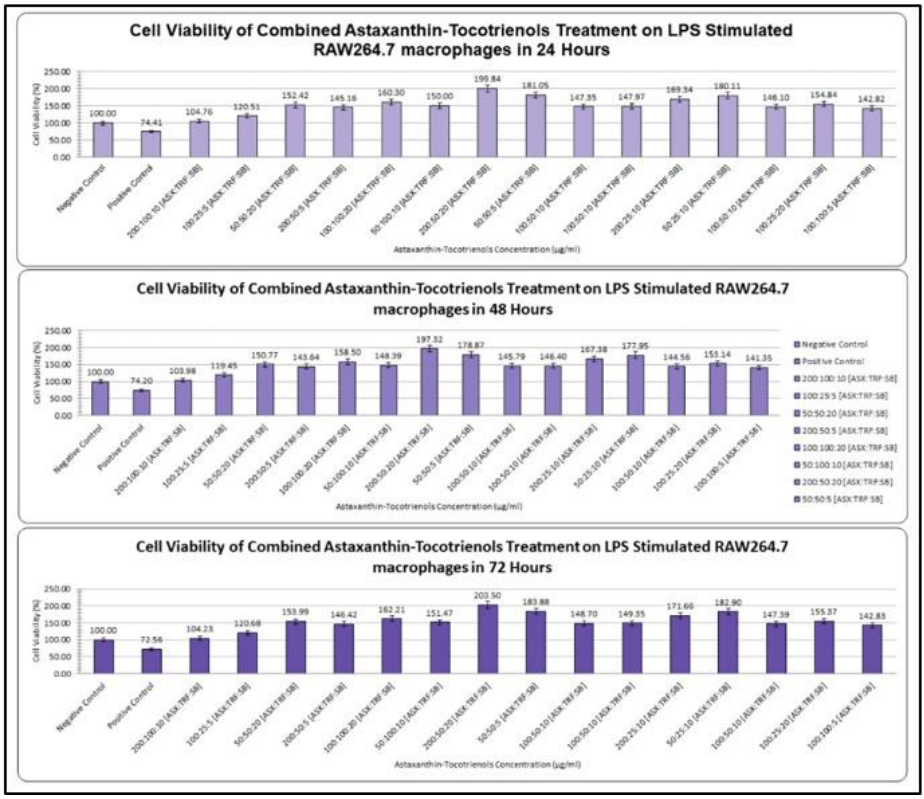

Fig. 5. Percentage of cell viability of LPS stimulated RAW 264.7 macrophages treated with astaxanthin-tocotrienols at 24, 48 and 72 hours. The data represented as means of ± SD triplicate wells, represented as means of ± SD triplicate wells

3.4 Nitrite Oxide (NO) Production Decreased in both Single and Combined Astaxanthin-Tocotrienols in LPS Stimulated RAW 264.7 macrophage cells

In determining the anti-inflammatory effects of single and combined treatments, RAW 264.7 macrophages were treated with different concentrations of astaxanthin and TRF for 24, 48, and 72 hours in the presence of 10 ng/mL of LPS. Figures 6 and 7 indicated that all treatments with astaxanthin and tocotrienols hindered NO production. Figure 6, for instance, showed that astaxanthin at concentrations 25 to 200 µg/mL significantly inhibited the NO production from 19.57% to 6.80% for 24 hours, 23.17% to 8.10% for 48 hours and from 22.37% to 7.30% for 72 hours, respectively. The percentage of inhibition was compared to the control group (untreated infected cells). On the other hand, when the infected cells were being treated with individual tocotrienols

compounds, it could be noted that tocotrienols at 25 to 100 µg/mL notably inhibited NO productions from 8.47% to 2.07%, 8.57% to 2.17% and 8.27% to 1.87% in 24, 48 and 72 hours, respectively. With the combination treatments at different concentrations between astaxanthin and TRF, as in Figure 8, the most NO production inhibition was seen in the formulation with 200:50:5 µg/mL [Astaxanthin: Tocotrienols: Soyabean Oil], with 16.12%, 15.08%, and 13.74% in 24, 48 and 72 hours, respectively. Nevertheless, it could be noted that the inhibition of NO production tends to be lower in a single treatment than in a combined treatment, suggesting that the higher concentration of formulation used in the previous studies may not be optimal in formulating the combination of these two compounds. All in all, for NO production, it could be noted that all treatments, whether single or combined, can reduce the NO production, showing that the compound possesses anti-inflammatory properties.

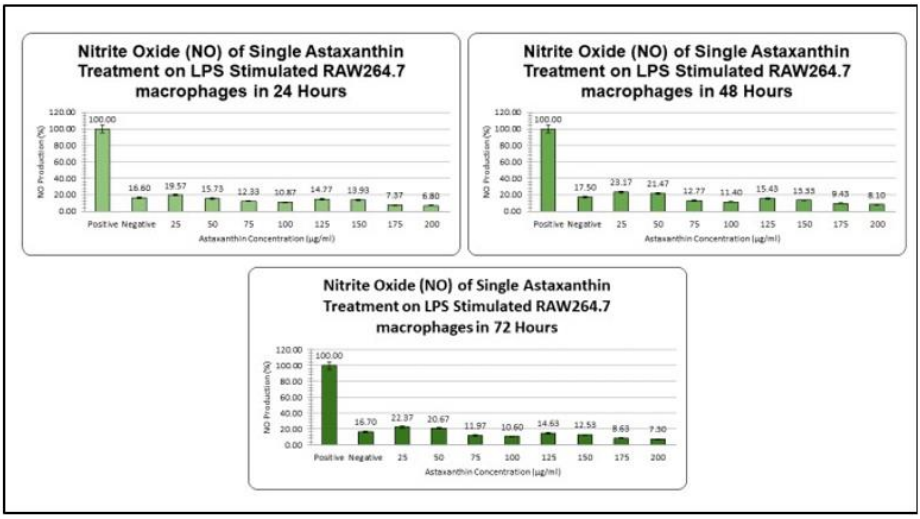

Fig. 6. Percentage of NO production of LPS stimulated RAW 264 macrophages treated with astaxanthin at 24, 48 and 72 hours. The d represented as means of ± SD triplicate wells

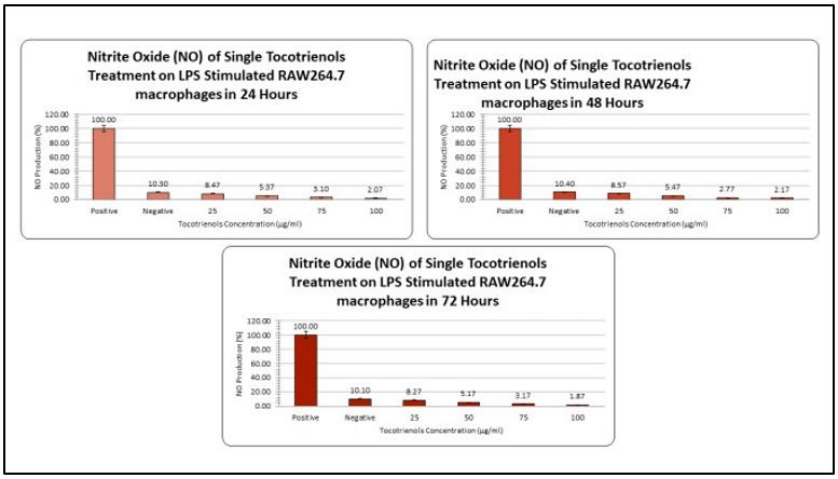

Fig. 7. Percentage of NO production of LPS stimulated RAW 264.7 macrophages treated with tocotrienols at 24, 48 and 72 hours. The data represented as means of ± SD triplicate wells.

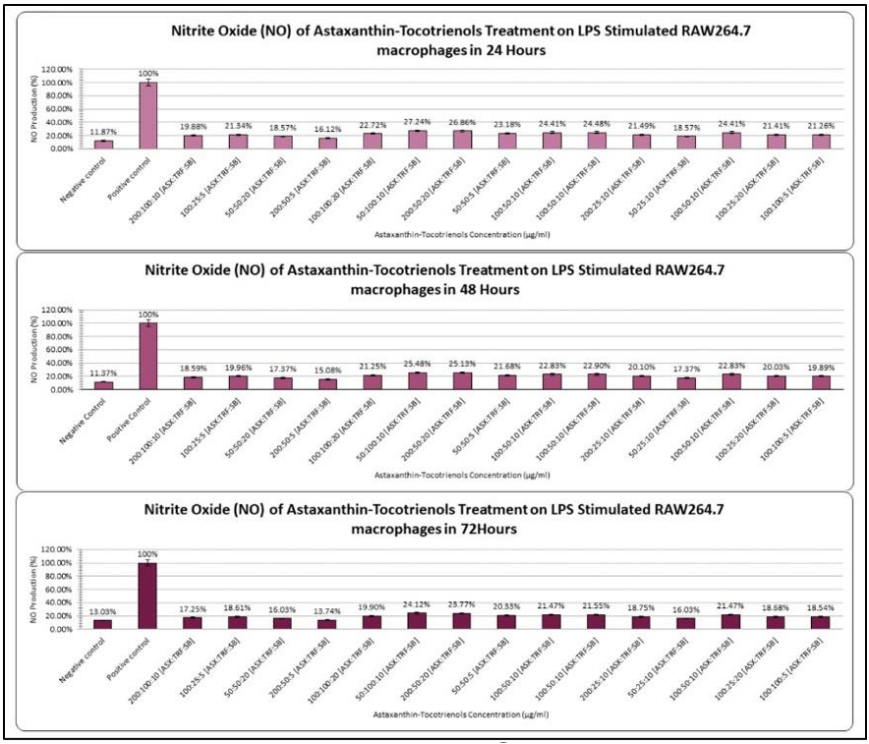

Fig. 8. Percentage of NO production of LPS stimulated RAW 264.7 macrophages treated with astaxanthin-tocotrienols at 24, 48 and 72 hours. The data represented as means of ± SD triplicate wells.

Note: ASX is for astaxanthin, TRF is for Tocotrienols, and SB is for Soyabean oil

4 Discussion

The current work was conducted to establish the optimal cell number and lipopolysaccharide (LPS) concentration in an inflammation experimental model using RAW 264.7 macrophages. Previous studies have indicated that different cells may have different behavior; hence, it is crucial to investigate the optimal RAW 264.7 cells per well to be pursued in inflammation experimentation. In the recent work, it could be noted that the optimal cell seeding density observed for the RAW 264.7 cell line would be 15,000 cells per well. The optimization of cell density was done by using iCELLigence e-plates. This tool is widely used in monitoring cells in real time [18]. 15,000 cells per well was considered the optimal cells number for RAW 264.7 cell line compared to other cells because the cells are proliferating at a more stable rate compared to other concentrations. The cell index was monitored over time as this indicator measures cell adhesion, spreading, and cell behavior. A cell number is considered optimal when the cell index curve pleatues or reaches a stable phase [19]. Five thousand (5,000) cells per well should not be used for experiments using RAW 264.7 macrophages as the cells did not spread and proliferate much due to low cell index. A higher cell index would indicate that the cells attached and spread well [18-19]. Forty thousand (40,000) cells per well also should not be used as the cell index plateauing at a high value, indicating that the cells become too densely packed, leading to inaccurate measurements as the cells may stack on top of each other [19]. The optimization of cell density or cell numbers per well is significant for at least three reasons: allowing researchers to measure the inflammatory response in a precise and cost-effective while ensuring consistency and responsibility for the results. Having the correct number of cells is vital, as too few of them may be too weak to observe the inflammatory response and too many may be too saturated. It is cost-effective, as using too many cells may be wasteful and expensive. Maintaining a consistent cell number per well also ensures that the experiments are reproducible [20].

Apart from the cell density, it is also essential to determine the LPS concentration to be applied to the RAW 264.7 cell line in establishing the inflammatory models. The optimal LPS concentration would vary for one cell. For instance, immune cells such as the dendritic and macrophage cells are highly responsive; hence, they may need a lower concentration of LPS rather than another cell type [21]. The current work has tested several concentrations of LPS in RAW 264.7 macrophages cell line, and it could be noted that 10 ng/mL of LPS is a sufficient concentration to induce inflammation in RAW 264.7 macrophage cell line models, and it does not affect the cell proliferation. The data obtained in the current work contrasted with other experiments conducted using THP-1 human monocytic cells. In the work using THP-1 cells, it could be noted that 100 ng/mL is the optimal LPS amount to induce inflammation in that cell type, for instance [21,22]. In determining the optimal LPS concentration in the current work, the cell index was monitored 24, 48 and 72 hours after the cells were stimulated with different LPS concentrations. The cell index is indeed a measure of cell behaviour; hence, one can distinguish how it changes when exposed to different concentrations of LPS [19]. Based on the curve plotted, when 10 ng/mL of LPS was applied to RAW 264.7 macrophage cells, it could be noted that there was no sudden decrease in cell

index as compared with other concentrations. Cells induced with 10 ng/mL have shown the highest cell index amount after being infected with LPS for 72 hours. It could be noted that a sudden decrease in cell index may indicate acute cytotoxicity as a significant number of cells are dying or started to detach from the surface [23]. The main reason why 10 ng/mL of LPS is sufficient in inducing inflammation in RAW 264.7 macrophages may be because RAW 264.7 macrophage cells possessed Toll-like receptor 4 (TLR-4), the receptor for LPS. Hence, a low concentration of LPS can activate a robust inflammatory response in these macrophage cells [24].

Based on the facts mentioned above, including the data obtained over the study, it could be observed that in establishing an optimal, cost-efficient inflammatory model for RAW 264.7 macrophage cells, the cells must be seeded with a cell number of 15,000 cells per well and the LPS concentration that would need to be applied to these cells would be 10 ng/mL.

Apart from finding the optimal cell density and LPS concentration to establish inflammatory models of RAW 264.7 macrophage cells, the current work was also conducted to explore the optimized astaxanthin and tocotrienols cocktails formulation in reducing the inflammation in LPS stimulating macrophage cells. It is undeniable that both astaxanthin and tocotrienols are potent anti-inflammatory compounds, and the findings related to this have been reported widely in the literature. However, pure astaxanthin is a premium compound (Sigma: RM6817/mg), and information on the synergistic effects of astaxanthin with a less-premium antioxidant, tocotrienols (Sigma: RM70/mg), is minimal [25]. Few studies reported the potential of combining these compounds, but most aimed to investigate antioxidant properties and improve memories when human subjects were given astaxanthin and tocotrienols [13-14]. Our initial study in investigating the potential of combining these compounds showed a positive outcome; nevertheless, the investigation of optimal cell number and LPS concentration had not been conducted before, and the concentration of single and combined compounds tested was up to 100 μg/mL for astaxanthin and 25 μg/mL for tocotrienols [15]. Therefore, research on the synergistic anti- inflammatory effects and comparative anti-inflammatory efficiency analysis with the individual compounds is essential.

In finding the individual concentrations for both astaxanthin and tocotrienols, a thorough literature review was conducted, and it could be observed that the previous studies indicated that the highest and best concentrations for astaxanthin in reducing the inflammation was 200 μg/mL [26,27]. On the other hand, for tocotrienols, the maximum concentration utilized from the previous studies was 100 μg/mL [28]. Hence, in establishing the optimum concentration of astaxanthin and tocotrienols, the concentration for single and combined treatments of both compounds was set to be from 25-200 μg/mL for astaxanthin with the interval of 25 μg/mL and 25-100 μg/mL for tocotrienols, respectively. The relevance of choosing such concentrations for individual treatment was that previous work reported the highest and best concentration for these compounds to reduce inflammation.

As for the combined treatment concentrations, the current work has utilized the Box-Behnkn Design (BBD) to formulate the cocktails. Typically, a Box-Behnken Design involves three levels for each factor: low, medium, and high, denoted by -1, 0 and +1,

respectively. The +1 level for astaxanthin and tocotrienols were set to be 200 μg/mL and 100 μg/mL, respectively, and 0 plus -1 levels were reduced in half. Using such BBD has allowed the production of 15 combined formulations to be pursued in the experiment. Reducing the number of experiments is considered one of the advantages of using this BBD, as this will reduce the cost of experimenting compared with the traditional univariate procedure [16-17].

These combined and single treatments were tested in RAW 264.7 macrophage cells that utilized optimal cell number and LPS concentration for the inflammation experiment. MTT assays and Nitrite Oxide (NO) production were the indicators pursued in the current work in establishing the combined astaxanthin-tocotrienols compound. Due to its quantitative nature, sensitivity, usability, and versatility, the MTT test is a practical and frequently used tool for determining cell cytotoxicity and viability in various research applications [28]. The control and treated cells' absorbance readings using combined and single compounds were recorded after 24, 48, and 72 hours, respectively. The cell viability was calculated relative to the control group, which is untreated cells. The calculated cell viability percentage indicates the portion of viable cells in the samples compared to the control. Higher values indicate greater cell viability, while lower values suggest reduced viability, which may indicate cytotoxic effects or changes in cell behaviour due to treatments or conditions [29]. The current work's data suggested that all combined and single compound treatments possessed no cytotoxicity as Individuals and combination treatments did not affect RAW 264.7 macrophage cell viability. In a nutshell, for the 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.

Nitrite Oxide (NO) production was monitored in testing anti-inflammatory properties once the LPS-stimulated RAW 264.7 macrophage cells were subjected to the treatment. Briefly, immune cells generate NO as a part of their defense mechanisms during an inflammatory reaction. NO functions as a signaling molecule to control various elements of inflammation, such as the recruitment of immune cells, dilating blood vessels, and modulating inflammatory mediators. A compound or drug is considered to possess anti- inflammatory properties when the NO production shows reduction, indicating that the treatment has managed to suppress the pro- inflammatory aspects of the immune response [30]. In the current study, it could be observed that combined and single treatments managed to reduce the NO production compared to the control group (infected untreated cells). Astaxanthin at concentrations of 25 to 200 μg/mL significantly inhibited the NO production from 19.57% to 6.80% for 24 hours, 23.17% to 8.10% for 48 hours and from 22.37% to 7.30% for 72 hours, respectively. The same thing was observed in a single tocotrienols experiment where tocotrienols at the concentration of 25 to 100 μg/mL notably inhibited NO productions from 8.47% to 2.07%, 8.57% to 2.17% and 8.27% to 1.87% in 24, 48 and 72 hours, respectively.

Nevertheless, for the combined treatment, the highest NO reduction can only be observed when the LPS-stimulated RAW 264.7 macrophage cells were subjected to formulation with 200:50:5 μg/mL [Astaxanthin: Tocotrienols: Soyabean Oil], with NO reduction to 16.12%, 15.08%, and 13.74% in 24, 48 and 72 hours, respectively. Though most NO reduction was observed in 200 μg/mL astaxanthin and 100 μg/mL

tocotrienols, it could be observed that the combined compounds at such high concentrations were not able to reduce the NO percentage as much as the single treatment. Though the combined compounds could not reduce NO as much as single treatment in all 15 formulations, it is indeed good news as the aim is to utilize the most minor concentration of astaxanthin and tocotrienols in managing inflammation as both compounds were expensive. Based on the results obtained, it is suggested that for finding the optimal astaxanthin-tocotrienols compound, the +1 levels of astaxanthin and tocotrienols shall be reduced to 25 µg/mL instead as the single compound treatment showed that with 25 µg/mL, the NO production has been reduced to 16.60%, 17.50% and 16.70% in 24, 48 and 72 hours respectively for astaxanthin and 10.30%, 10.40% and 10.10% in 24, 48 and 72 hours respectively for tocotrienols. After all, the NO level should not be too low as it serves critical functions and regulatory effects in the human body, especially in the blood flow regulation, immune function, and wound healing [31].

5 Conclusion

In conclusion, this study successfully determined optimal conditions for conducting inflammation experiments using the RAW 264.7 macrophage cell line. A cell density of 15,000 cells per well and an LPS concentration of 10 ng/mL were identified as the most suitable parameters for reliable and cost-effective inflammation studies. Furthermore, the research explored the potential anti-inflammatory effects of combining astaxanthin and tocotrienols, revealing promising results for future investigations. It could be noted that the combined compounds significantly reduce the NO production as compared to the control group; nevertheless, the +1 level in the Box-Behnkn Design was too high for both astaxanthin and tocotrienols and shall be reduced to 25 µg/mL respectively. These findings provide valuable guidance for researchers working on inflammation-related studies and therapeutic interventions, ensuring robust and reproducible experimental models.

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Paper Contribution to Related Field of Study. The present work elucidates a comprehensive framework for advancing the development of anti-inflammatory strategies using astaxanthin and

tocotrienols. The establishment of an optimized in vitro model and the exploration of safe and effective concentration ranges for combination therapy pave the way for innovative approaches to combat inflammatory-related diseases.

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