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IC_{50}- Based Comparison of Astaxanthin and Tocotrienols Across Raw 264.7, A549, and Calu-3 Cells

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Abstract

One of the most significant mediators of inflammation within immune and epithelial cells is nitric oxide (NO). It could be noted that NO production in excess may contribute to oxidative stress and tissue injury, thus, the measurement of this NO was considered as a beneficial endpoint in the search of nutraceutical compounds that possess anti-inflammatory effects. In the current work, the potential of astaxanthin and tocotrienols in inhibiting NO production across three cells, namely murine macrophages (RAW 264.7) and human lung epithelial cells (A549 and CALU-3) were compared. These cells were first induced with lipopolysaccharide (LPS, RAW 264.7) or interleukin-1β (IL-1β, A549 and CALU-3) and treated with increasing concentrations of astaxanthin or tocotrienols (1–25 µg/mL) for 24, 48, and 72 hours. For each time points, NO levels were measured by using the Griess assay while half-maximal inhibitory concentrations (IC_{50}) were derived from dose–response curves. The present work found that both natural compounds were able to inhibit the production of NO in a concentration-dependent manner among all studied cell lines. IC_{50} values ranged from 4.97 ± 0.29 to 9.85 ± 1.28 µg/mL for astaxanthin and 6.97 ± 1.57 to 13.50 ± 1.58 µg/mL for tocotrienols across all three cell lines and time points. Astaxanthin was more likely to exhibit lower IC_{50} values than tocotrienols in the studied cells. Nevertheless, statistically significant differences were only found in RAW 264.7 cells at 48 h and in CALU-3 cells at 48 h and 72 h, but not in A549 cells at any of the tested time points. The relatively narrow IC_{50} range across time points further supports the reproducibility of the inhibitory responses of both compounds. To

sum up, both studied compounds exhibited a comparable inhibitory activity against NO production. The results give a reliable comparative reference of the anti-inflammatory property of both compounds and can be a starting point of future mechanistic and in vivo research.

1. Introduction

Numerous chronic diseases like metabolic disorders, cardiovascular conditions and respiratory illnesses were derived from uncontrollable inflammation. One of the central mediators for inflammatory injury is the nitric oxide (NO), where this reactive nitrogen species was produced mostly by inducible nitric oxide synthase (iNOS) within activated epithelial cells and activated macrophages (Suriyaprom et al. 2023). NO levels play a significant role in signalling or host defense; however, excessive or continued production may usually lead to oxidative stress, tissue damage plus the progression of inflammatory pathologies. In studying the inflammation levels *in vitro* through the identification of the NO, Griess assay remains a widely used method to quantify the nitrite accumulation, especially in RAW 264.7 macrophages as well as lung epithelial cell lines (Sun et al. 2023). The cell models were recognized as established screening options for nutraceuticals that have anti-inflammatory activity potential.

A potent antioxidant and anti-inflammatory compound that has obtained a significant attention is astaxanthin, a naturally occurring xanthophyll carotenoid. Various studies have elucidated its potential in reducing pro-inflammatory mediators such as NO, TNF-α, IL-6, and COX-2, in LPS-stimulated macrophages and in various epithelial systems (Hwang et al. 2020; Al-Tarifi et al. 2022; Cheng and Eroglu 2021). The anti-inflammatory potential was associated with its lipid-soluble structure that facilitates membrane incorporation, protecting the cells against oxidative stress while also modulating the NF-κB transcription factors. Present literature has also illustrated its capacity in protecting the lung epithelial cells away from toxin-induced and pollutant- oxidative damage, showing their therapeutic potential within airway inflammation (Okudan Altındaş and Güner 2025). Not only that but astaxanthin had also been proposed as an adjunctive nutraceutical in modulating the cytokine storm responses in the event of COVID-19, thus illustrating its significance to inflammatory situations (Talukdar et al. 2020).

Tocotrienols, a potent antioxidant of the vitamin E family, have been studied for their bioactive components beyond that. It has been reported that this compound exerts pleiotropic effects that allow the modulation of NF-κB signaling, iNOS expression suppression while also works in downregulating the inflammatory cytokines (Qureshi 2022; Ranasinghe, Mathai, and Zulli 2022). Prior research has investigated the benefits of this compound in acting as epithelial barrier models, especially when they are exposed to environmental stressors. They were reported to alleviate inflammation while also maintaining the structural integrity (Neo et al. 2022). While both compounds had been reported on their anti-inflammatory potential, a direct comparative evaluation of their anti-inflammatory potency across different cell lines remains limited.

Thus, the work investigated and compared the half-maximal inhibitory concentration (IC_{50}) values of astaxanthin and tocotrienols in LPS-stimulated RAW 264.7 macrophages and IL-1β-stimulated A549 and CALU-3 lung epithelial cells. To our knowledge, no prior study has directly compared astaxanthin and tocotrienols under standardized NO-inhibition conditions in macrophage and epithelial models. By standardizing assay conditions and focusing on NO inhibition, this work provides a concise comparative reference for the anti-inflammatory potential of two nutraceuticals across distinct cellular contexts.

2. Methodology

2.1 Cell Culture and Induction

RAW 264.7 murine macrophages were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. A549 human lung adenocarcinoma cells were maintained in F-12K medium supplemented with 10% FBS, while CALU-3 human bronchial epithelial cells were grown in Minimum Essential Medium (MEM) containing 10% FBS and 1% penicillin-streptomycin. The studied cell lines were incubated for 24 hours at 37 °C in a humidified atmosphere of 5% CO_2 before being stimulated using the relevant stimulators.

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To induce nitric oxide (NO) production, RAW 264.7 cells were stimulated with lipopolysaccharide (LPS, 10 ng/mL), while A549 and CALU-3 cells were stimulated with interleukin-1β (IL-1β, 10 ng/mL). The stimulated cells were incubated for another 24 hours before being treated.

2.2 Treatment Conditions

Both astaxanthin and tocotrienols were prepared in corn oil as a basal vehicle and diluted in complete culture medium to obtain working concentrations. Cells were treated with a concentration range of 1–25 µg/mL for 24, 48, and 72 hours. Vehicle-only controls (corn oil without compounds) and induced controls (LPS or IL-1β without treatment) were included in each experiment.

2.3 Cell Viability Assay

MTT assay was used to measure the cell viability, according to manufacturer's instruction. All cells were seeded in 96-well plates and treated with a concentration range of 1–25 µg/mL for 24, 48, and 72 hours. Following each treatment, 10 µL of MTT solution was added to each well and incubated for 4 hours at 37. Absorbance was then measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to untreated control cells.

2.4 Nitric Oxide Measurement

Nitric oxide (NO) production was quantified using the Griess assay (Sun et al. 2003). Briefly, 100 µL of culture supernatant was mixed with 100 µL of Griess reagent, and absorbance was measured at 540 nm using a microplate reader. Nitrite concentrations were determined from a sodium nitrite standard curve. Percent inhibition was calculated relative to induced controls.

2.5 IC_{50} determination

IC_{50} is defined as the concentration of a compound needed to achieve 50% inhibition of nitric oxide production relative to the induced control. Nitric oxide production was assessed indirectly by measuring nitrite (NO_2^-) concentration using the Griess assay. Absorbance values were converted into NO_2^- concentrations based on the standard curve using the following equation:

$$NO_2\text{-}(\mu M) = \frac{Absorbance - 0.00075}{0.0208} \quad (1)$$

The calculated NO_2^- concentrations for the induced control, treated sample, and blank were then used to determine the percentage of nitric oxide inhibition according to the following equation:

$$\%\ inhibition = \frac{NO\ level\ in\ induced\ control - NO\ level\ in\ treated\ sample}{NO\ level\ in\ induced\ control - NO\ level\ in\ blank}\ x\ 100 \quad (2)$$

The resulting percentage inhibition data were fitted using nonlinear regression with a three-parameter logistic model (GraphPad Prism 9.0), and half-maximal inhibitory concentrations (IC_{50}) were derived from the best-fit curves (Srinivasan and Lloyd 2024). Data are presented as mean ± standard deviation (SD) from three independent experiments (n = 3). Paired-samples t-tests were performed to compare the IC_{50} values between astaxanthin and tocotrienols at each time point within the same cell line using SPSS Version 30.0. P values of less than 0.5 were considered as statically significant.

3. Results and Discussion

To ascertain whether nitric oxide inhibition was linked to cytotoxic effects, cell viability was determined through the MTT assay of all the tested concentrations and time in RAW 264.7, A549 and CALU-3 cells. The summary of cell viability ranges is presented in Table 1.

Table 1 *Cell viability range (%) of astaxanthin- and tocotrienol-treated RAW 264.7, A549, and CALU-3 cells at 24, 48, and 72 hours*

Compound	Cell line	24 h	48 h	72 h
Astaxanthin	RAW 264.7	88.41–97.65	88.75–98.03	88.20–97.16
	A549	89.00–99.01	88.98–98.81	88.96–98.76
	CALU-3	89.37–98.85	89.24–98.80	89.18–98.77
Tocotrienols	RAW 264.7	105.15–110.91	102.19–107.50	101.73–107.14
	A549	98.19–107.85	98.72–108.62	98.63–108.01
	CALU-3	98.51–108.07	98.63–108.52	98.67–107.94

Note: Cell viability values are expressed relative to the induced control group. All values remained above 80%, indicating absence of cytotoxicity under the tested conditions.

In the case of astaxanthin, cell viability was over 80 percent across the treatment groups, with the range of 88.20% to 99.01% based on cell line and incubation time. Similarly, tocotrienols also maintained cell viability above 80%, with values ranging from 98.19% to 110.91%. These results suggest that the inhibitory action of the two compounds on nitric oxide was not due to cytotoxicity within the experimental conditions.

On the other hand, astaxanthin and tocotrienols inhibited nitric oxide (NO) production in a concentration-dependent manner across RAW 264.7 macrophages, A549, and CALU-3 epithelial cells. IC_{50} values derived from nonlinear regression are summarized in Table 2 and visualized in Figure 1.

Table 2 *Half-maximal inhibitory concentration (IC_{50}, μg/mL) values of astaxanthin and tocotrienols in RAW 264.7, A549, and CALU-3 cells at 24, 48, and 72 hours. Values are presented as mean ± SD with 95% confidence intervals (CI)*

Cell Line	Time (h)	Astaxanthin IC_{50} (μg/mL) [95% CI] ± SD	Tocotrienols IC_{50} (μg/mL) [95% CI] ± SD
RAW 264.7	24	9.67 [5.87–16.99] ± 0.31	12.15 [7.83–14.20] ± 1.58
RAW 264.7	48	9.06 [5.53–15.76] ± 0.31	13.50 [10.15–18.46] ± 1.58
RAW 264.7	72	9.49 [7.37–12.42] ± 0.31	12.53 [8.64–19.00] ± 1.58
A549	24	8.36 [5.75–12.53] ± 1.28	9.26 [5.63–12.85] ± 1.02
A549	48	7.31 [4.76–11.65] ± 1.28	9.07 [7.88–10.48] ± 1.02
A549	72	9.85 [8.24–11.86] ± 1.28	10.36 [9.83–10.94] ± 1.02
CALU-3	24	5.41 [2.84–10.94] ± 0.29	6.97 [3.81–7.45] ± 1.57
CALU-3	48	5.53 [3.70–8.45] ± 0.29	8.38 [5.26–14.10] ± 1.57
CALU-3	72	4.97 [3.35–7.52] ± 0.29	7.26 [6.02–8.82] ± 1.57

Note: IC_{50} values were calculated from nonlinear regression (three-parameter logistic fit). Each value represents the mean of three independent experiments (n = 3).

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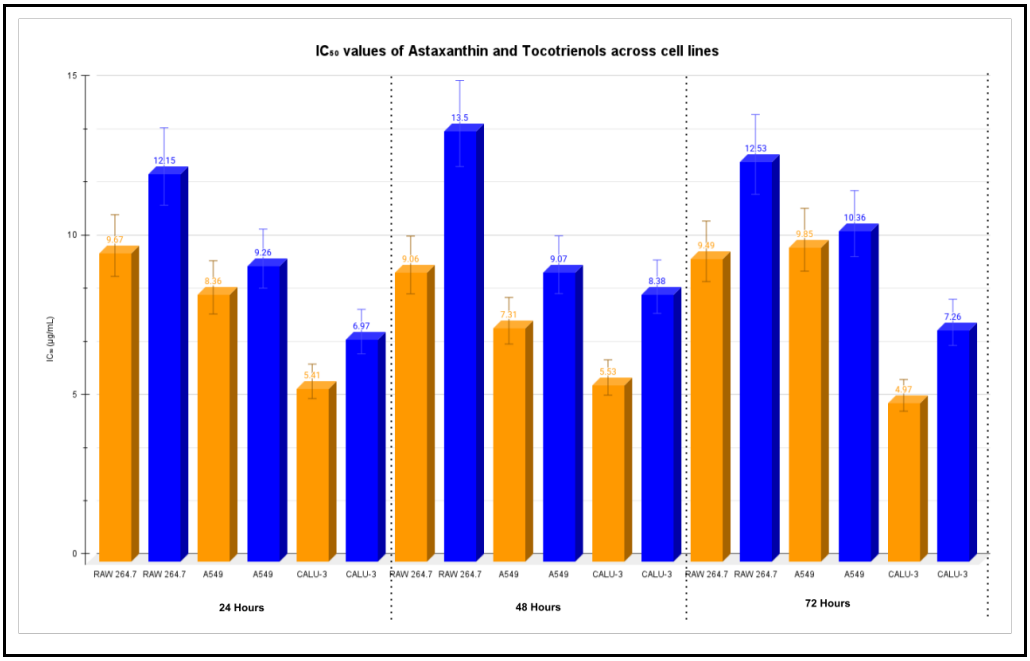

Fig. 1 *Comparative IC_{50} values of astaxanthin (orange) and tocotrienols (blue) across RAW 264.7, A549, and CALU-3 cells at 24, 48, and 72 hours. Bars represent mean IC_{50} values, and error bars indicate standard deviation (SD)*

On the findings above, the current work provides a direct comparison of the anti-inflammatory potential of astaxanthin and tocotrienols in macrophage and lung epithelial cell models with nitric oxide inhibition as the main endpoint. Both compounds exhibited concentration-dependent effects of NO inhibition in the RAW 264.7, A549, and CALU-3 cells, indicating that both compounds could reduce the production of inflammatory NO under the conditions tested. This general trend aligns with past literature demonstrating that astaxanthin can inhibit NO production and associate inflammatory mediators in activated cellular models, such as macrophage models, by regulating oxidative stress and inflammatory circuits like NF-κB and iNOS (Hwang et al., 2020; Al-Tarifi et al., 2022). Similarly, tocotrienols were also reported to have an anti-inflammatory effect by inhibiting the NF-κB signaling pathway, as well as other inflammatory markers, which confirms the usefulness of these bioactive nutraceutical compounds in models of inflammation (Qureshi, 2022; Ranasinghe et al., 2022; Ang et al., 2025).

Astaxanthin was found to have a low observed IC_{50} at each time point of 24 h, 48 h and 72 h in RAW 264.7 macrophages compared to tocotrienols. Statistical analysis, however, made it clear that this numerical difference was only significant at 48 h. No significant difference was observed at 24 h, $t(2) = 0.708$, $p = 0.552$, or at 72 h, $t(2) = -1.603$, $p = 0.250$, whereas a significant difference was detected at 48 h, $t(2) = -10.601$, $p = 0.009$, with tocotrienols exhibiting the higher mean IC_{50} value. This indicates that astaxanthin achieved half maximal inhibition at a significantly lower concentration than tocotrienols only at the intermediate exposure period in RAW 264.7 cells. The use of LPS in this macrophage model was appropriate because RAW 264.7 cells are a well-established inflammatory screening model in which LPS activates TLR4-dependent pathways, including NF-κB signaling, and strongly induces iNOS expression and NO production (Suriyaprom et al., 2023; Rajapakse et al., 2008).

A somewhat similar but statistically weaker trend was observed in A549 cells. Astaxanthin once again presented lower observed IC_{50} values as compared to tocotrienols after 24 h and 48 h, yet the two were closer to each other after 72 h. Nevertheless, paired-samples t-tests showed no significant differences between the two compounds at any tested time point (24 h, $t(2) = 0.592$, $p = 0.614$, 48 h, $t(2) = -1.406$, $p = 0.295$, and 72 h, $t(2) = -1.165$, $p = 0.364$). These findings indicate that whereas astaxanthin was generally lower in concentration to achieve the midpoint inhibitory response, the similarity between the two compounds in the inhibitory activity in A549 cells can be viewed as broadly similar. The rationale behind the use of IL-1β instead of LPS in A549 cells is that A549 is an epithelial model, and IL-1β is a biologically relevant pro-inflammatory

cytokine activating NF-κB and MAPK-linked inflammatory programs in airway and alveolar epithelial cells (Peng et al., 2018). In this context, IL-1β provides a more epithelial-relevant inflammatory trigger than the macrophage-centered endotoxin response elicited by LPS.

Conversely, the highest apparent sensitivity to both compounds was found in CALU-3 cells in which all the IC_{50} values were below 10 μg/mL. Astaxanthin recorded an IC_{50} of 5.41, 5.53 and 4.97 μg/mL at 24 h, 48 h and 72 h respectively, whereas tocotrienols registered slightly elevated values of 6.97, 8.38 and 7.26 μg/mL at the same time. The statistical analysis revealed that there was no significant difference at 24 h, $t(2) = 1.057$, $p = 0.401$, but significant differences were observed at 48 h, $t(2) = -5.058$, $p = 0.037$, and 72 h, $t(2) = -15.451$, $p = 0.004$. These results imply that as the duration of exposure increased, the difference of the two compounds increased in CALU-3 cells. One likely reason is that CALU-3 cells are bronchial epithelial cells and are distinct in terms of epithelial barrier properties, inflammatory responsiveness, and downstream signaling behaviors, which may impact compound uptake and sensitivity (Cormet-Boyaka et al., 2011; Foster et al., 2010). IL-1β was also an appropriate inducer in CALU-3 because cytokine-driven airway epithelial inflammation is a relevant biological context for this model, and CALU-3 cells are widely used to study respiratory epithelial inflammatory responses.

Combined, these results suggest that the inhibitory efficacy of astaxanthin and tocotrienols in comparison to each other varied based on the cell type and duration of exposure. Even though in many instances astaxanthin exhibited less observed IC_{50}, statistically significant differences were only observed in RAW 264.7 cells at 48 h and CALU-3 cells at 48 h and 72 h. This implies that the comparative inhibitory strength of the two compounds may not be consistent in all the systems tested. Rather, the data supports a more context-dependent interpretation in which cellular origin, inflammatory trigger, and exposure duration collectively influence the comparative response.

The comparatively small IC_{50} variations recorded over time points also corroborate the overall reproducibility of the inhibitory reactions, especially of astaxanthin in CALU-3 cells and of both compounds in a number of other systems. This consistency can be beneficial in the context of dietary bioactives as adjunctive anti-inflammatory interventions, as consistent activity over multiple exposure times can reflect a consistent response pattern and not a time-dependent effect. This observation is generally in line with the literature that characterizes both astaxanthin and tocotrienols as anti-inflammatory nutraceuticals whose effects are related to the control of oxidative stress and NF-κB-related inflammatory response (Cheng and Eroglu, 2021; Ranasinghe et al., 2022; Ang et al., 2025).

The present study lacked pharmacological positive control; thus, this should be considered a limitation. Inclusion of a reference anti-inflammatory compound would have given more pharmacological background to the interpretation of the absolute potency of the measured IC_{50} values. However, the primary goal of the present study was to compare directly astaxanthin and tocotrienols at the same assay conditions in the various cell models as opposed to a comparison to a therapeutic standard. In that respect, the present IC_{50} comparison still provides a useful internal comparative framework, while future studies should incorporate a standard anti-inflammatory reference compound to strengthen biological contextualization.

NO inhibition is another limitation to this study since it is the main endpoint. Despite the popularity of NO as an indicator of inflammatory activation, especially in macrophage screening systems, inflammation is a more holistic process that encompasses the release of cytokines, the control of COX-2, and various transcriptional pathways. Nonetheless, NO inhibition is a viable and informative initial marker of comparative nutraceutical screening, particularly when it is used in conjunction with viability data as in the current study. Subsequent studies should thus build on these results using mechanistic studies on iNOS, NF-κB, MAPK, cytokine profiling and in vivo validation to further characterize the relative anti-inflammatory effects of astaxanthin and tocotrienols.

4. Conclusion

All in all, both studied compounds demonstrated reproducible, dose-dependent inhibition of nitric oxide production in macrophage and lung epithelial cell models. IC_{50} values were consistently within the 5–14 μg/mL range. Although astaxanthin frequently showed lower observed IC_{50} values than tocotrienols, statistically significant differences were only identified in RAW 264.7 cells at 48 h and in CALU-3 cells at 48 h and 72 h, while no significant differences were observed in A549 cells. These findings provide a concise comparative reference for the anti-inflammatory activity of two nutraceuticals across distinct cellular contexts and establish a foundation for future mechanistic and in vivo investigations.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of the paper.

Author Contribution

Khairul Adzfa Radzun and Sitti Rahma Abd Hafid; ***conceptualized the research idea and study design****. Muhamad Helmi Husaini and Maliya Azilah;* ***conducted the experiments, collected the data, and were considered equal first authors for the current work****. Nabiha Iran, Faezah Pardi, Asmida Ismail, and Wan Razarinah Wan Abdul Razak;* ***statistical analysis and interpreted the results****. All authors critically reviewed and approved the final version of the manuscript.*

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