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EFFECT OF NANOPARTICLES CONTAINING ALPHA-HUMULENE AND CURCUMIN ON PRO-INFLAMMATORY CYTOKINES

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ABSTRACT

The study investigated the neuroprotective effects of curcumin- and alpha-humulene-containing nanoparticles on neuroinflammation induced by Amyloid Beta1-42 (Aβ1-42) toxin, with the aim of evaluating the modulation of inflammatory cytokines in an experimental model of Alzheimer's disease (AD). Neuroinflammation is a key factor in the progression of AD, and the compounds studied showed potential to mitigate the detrimental effects of this process. A total of 63 Wistar rats were used, divided into six groups, treated with curcumin, alpha-humulene or their respective formulations in nanoparticles. The nanoparticles were developed to optimize bioavailability and therapeutic efficacy, aiming to cross the blood-brain barrier. Flow cytometry analyses focused on the pro-inflammatory cytokines TNF-α and IL-1 and the anti-inflammatory IL-4. The results demonstrated that treatment with curcumin and alpha-humulene nanoparticles significantly reduced TNF-α levels and increased IL-4 levels, suggesting a more favorable neuroinflammatory response. However, there was no statistically significant variation in IL-1 levels between the treated groups. The findings suggest that these nanoparticles have a promising neuroprotective effect, offering a potential therapeutic alternative for AD.

Keywords: Microglial Activation. Neurofibrillation. Alzheimer's disease.

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INTRODUCTION

Neuroinflammation is a response that occurs in the Central Nervous System (CNS), mainly involving the brain and spinal cord, and is initiated by various stimuli, including infections, traumatic injuries, toxic substances, and autoimmune reactions (LENG; EDISON, 2021). The main mediators of neuroinflammation are CNS immune cells, particularly microglia and astrocytes, which are activated in response to these insults. This activation leads to the production of inflammatory mediators, such as cytokines, chemokines, and reactive oxygen species, which play critical roles in modulating the inflammatory response (WANG et al., 2023). Although neuroinflammation serves as a protective mechanism against further damage, its dysregulation can result in chronic inflammation, being characterized by sustained activation of glial cells, prolonged release of inflammatory mediators, and possible breakdown of the blood-brain barrier (BBB) (THAKUR et al., 2023). This inflammatory state is recognized as a contributing factor in the pathogenesis of several neurodegenerative diseases – such as Alzheimer's Disease (AD) – probably due to the deposition of amyloid beta peptides, as explained by the amyloid hypothesis (BREIJYEH; KARAMAN, 2020; VAISERMAN; KOLIADA; LUSHCHAK, 2020). Thus, understanding neuroinflammation, with both protective and harmful effects, requires an approach that takes into account the context, duration, and intensity of the inflammatory response (LENG; EDISON, 2021; WANG et al., 2023).

In addition, the interaction between pro-inflammatory and anti-inflammatory cytokines is fundamental for neuroinflammation, playing crucial roles in its regulation, influencing both the initiation and resolution of inflammatory responses (BECHER; SPATH; GOVERMAN, 2017). TNF-α is a key pro-inflammatory cytokine, acting as a mediator of neuroinflammation, facilitating the recruitment of immune cells to the sites of injury. Elevated levels of TNF-α have been associated with AD, contributing to neuronal damage and disease progression through mechanisms such as synaptic dysfunction and apoptosis (BORASCHI et al., 2023; WANG et al., 2015). IL-1 is another critical pro-inflammatory cytokine, and it plays a dual role in neuroinflammation, acting as both a mediator of inflammation and a neuroprotective factor in certain conditions. However, chronic overproduction of IL-1 can perpetuate inflammatory cycles and promote neurotoxic environments (BECHER; SPATH; GOVERMAN, 2017; KONSMAN, 2022). IL-4 is a cytokine that plays a protective role against neuroinflammation. It is primarily produced by type 2 helper T cells and is known to promote the differentiation of microglia into an anti-

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inflammatory phenotype. IL-4 exerts its effects by inhibiting the production of pro-inflammatory cytokines and increasing the expression of neuroprotective factors. This change in the cytokine environment may help resolve inflammation and promote tissue repair after CNS injury (DISABATO; QUAN; GODBOUT, 2016; GALEA; GRAEBER, 2023). Thus, elevated levels of TNF-α and IL-1 may indicate active neuroinflammation and may serve as biomarkers of disease progression or response to therapy, while increased levels of IL-4 may reflect an attempt to combat inflammatory processes and promote recovery (BORASCHI et al., 2023; DISABATO; QUAN; GODBOUT, 2016; WANG et al., 2015).

In this sense, the use of herbal therapy constitutes an attractive approach to the treatment of various inflammatory disorders and, given the limitations of current treatments for AD, there is a growing interest in its therapeutic potential and bioactive compounds, especially curcumin and alpha-humulene, which can mitigate neuroinflammation and its consequences in AD, through modulation of the immune response and reduction of pro-inflammatory cytokines (BORDOLOI et al., 2024; WENDLER et al., 2024). Curcumin has attracted attention for its neuroprotective effects. It has potent anti-inflammatory, antioxidant, and anti-amyloidogenic properties, making it promising for the treatment of AD. Studies have shown that curcumin can inhibit the aggregation of Aβ peptides, reducing plaque formation and associated neuroinflammation. In addition, curcumin has been shown to modulate important signaling pathways, including the activation of brain-derived neurotrophic factor, which is crucial for neuronal survival and cognitive function (BORDOLOI et al., 2024; OLIVE TREE; PIENIZ, 2024). Alpha-humulene, a sesquiterpene found in hops, has also shown promise in the context of neuroinflammation and AD. Studies have highlighted its anti-inflammatory effects, particularly in models of Aβ-induced neuroinflammation (WENDLER et al., 2024). The safety profile and low incidence of side effects of these compounds increase their attractiveness as a complementary therapeutic strategy (BORDOLOI et al., 2024; NAGORI et al., 2023).

Also, for a therapy aimed at AD to be effective, the drugs must be able to cross the BBB. This acts as a first line of defense, preventing unwanted compounds from entering the brain (WU et al., 2023). In this context, nanocarriers emerge as a promising solution, as they protect drugs from degradation and preserve their therapeutic efficacy. These nanocarriers have the ability to recognize and bind to specific targets, providing precise action on target cells (AHLAWAT et al., 2020; ALOTAIBI et al., 2021). Nanomedicine also makes it possible to increase the intracellular levels of the drug by encapsulating it in

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various nanocarriers, such as polymeric nanoparticles (NPs) (AHLAWAT et al., 2020; PINHEIRO et al., 2021). NPs can be structured as nanocapsules or nanospheres. The nanocapsules have a vesicular structure, in which the drug is dissolved in a liquid nucleus surrounded by a polymeric capsule. Nanospheres, on the other hand, consist of a polymeric matrix where the drug is dispersed in the gaps or adsorbed on the surface of the sphere. Both types have advantages such as controlled release of drugs and the possibility of modifications on the surface to direct them to the brain, in addition to being safe, biodegradable and easily eliminated by the body. These characteristics have encouraged research on its use in the diagnosis and treatment of various neurodegenerative diseases (ABOZAID et al., 2022; AHLAWAT et al., 2020; ALOTAIBI et al., 2021; PINHEIRO et al., 2021; SASTRI et al., 2022; WANG et al., 2022; WENDLER et al., 2024).

Thus, the aim of this study was to evaluate the neuroprotective effects of nanoparticles containing alpha-humulene and curcumin in an experimental model of AD. Specifically, we sought to quantitatively analyze pro-inflammatory (TNF-α and IL-1) and anti-inflammatory (IL-4) cytokines after the induction of neuroinflammation by Amyloid Beta Toxin1-42 (Aβ1-42).

METHODOLOGY

SAMPLE CALCULATION

The sample size calculation was performed based on the central limit theorem, which holds that the mean of a random sample of a large population tends to approximate the mean of the total population. Considering a population of 2,000 individuals with Alzheimer's disease in the municipality of Guarapuava, a margin of error of 10%, a confidence level of 95% and a homogeneity of 99%, the calculated sample size was 63 animals. The formula used was: $n = p(1-p)Z^2/e^2$, where "n" represents the sample size, "p" is the expected proportion, "Z" is the value associated with the confidence level, and "e" is the margin of error.

SAMPLE

The sample consisted of 63 rats of the species Rattus norvegicus, Wistar lineage, weighing between 300 and 350 grams, from the Vivarium of the State University of Maringá (UEM), with approval from the ethics committee (protocol number 009/2021). The animals

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were housed in acrylic cages, each containing a maximum of four animals, kept on shelves under a 12-hour light cycle (from 7 am to 7 pm), with controlled temperature at 23 ± 1 ºC.

EXPERIMENTAL GROUPS

The animals were divided into six groups:

- Negative Control Group (NC): 13 animals treated only with water, in the same volume of treatments, with material collection and euthanasia with the other groups;
- Positive Control Group (PC): 10 animals treated with 5 mg of donepezil hydrochloride, 30 days after induction of neuroinflammation by amyloid beta1-42, by gavage, for 30 consecutive days, with material collection and euthanasia on the 120th day;
- Group treated with α-Humulene (HUM): 10 animals treated with 6.5 µg of α-Humulene, 30 days after induction of neuroinflammation by Amyloid Beta1-42, by gavage, for 30 consecutive days, with material collection and euthanasia on the 120th day;
- α-Humulene Nanoparticle (NHUM) Group: 10 animals treated in a similar way to the previous group, using α-Humulene encapsulated in nanoparticles;
- Curcumin-treated group (GCur): 10 animals treated with 6.5 µg of Curcumin, also 30 days after induction of neuroinflammation, by gavage, for 30 consecutive days, with euthanasia on the 120th day;
- α-Humulene and Curcumin Nanoparticles Treated Group (NHUM+Cur): 10 animals treated with nanoparticles containing 6.5 µg of Curcumin and 6.5 µg of α-Humulene, for 30 consecutive days, with euthanasia on the 120th day.

PREPARATION OF NANOPARTICLES

The nanoparticles were developed in the nanotechnology laboratory of the State University of the Midwest (UNICENTRO) using the anti-solvent precipitation method, following a methodology adapted from previous studies (ANTÔNIO et al., 2017; LIU et al., 2017; SUN; DAI; GAO, 2017). The technique involves coacervation between Zein and Chitosan nanoparticles, with both substances acting as stabilizers. The solutions of Zein and Chitosan were prepared, mixed in different proportions and subjected to controlled agitation and centrifugation processes, ensuring the formation of nanoparticles of adequate size.

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AVERAGE DIAMETER AND POLYDISPERSION INDEX

The average diameter and polydispersion index (PDI) of the nanoparticles will be measured using the dynamic light scattering (DLS) technique using the BIC 90 Plus equipment (Brookhaven Instruments Corp., Holtsville, NY). For the analyses, aliquots of nanoparticle suspensions will be collected before and after incubation with chitosan, allowing the comparison of sizes and PDI. The nanoparticles will be dispersed in ultrapure water (1:200 v/v) and placed in a cuvette for analysis. All measurements will be performed with a scattering angle of 90° to 25°C and with a laser wavelength of 659 nm.

ZETA POTENTIAL

The zeta potential will be determined from the electrophoretic mobility of the nanoparticles in suspension. The samples will be diluted (1:200 v/v) in a 1mM KCl solution and inserted into an electrophoretic cell at 25°C, under a potential of ±150 mV (ZetaSizer ZS, Malvern, UK). The zeta potential will be analyzed both before and after the incubation of the nanoparticles with chitosan. Measurements will be performed in triplicate and expressed as mean ± standard deviation.

DETERMINATION OF ENCAPSULATION EFFICIENCY

The encapsulation efficiency (EE) of α-Humulene in nanoparticles will be determined indirectly. An aliquot of the supernatant resulting from the ultracentrifugation of the nanoparticles will be diluted in the mobile phase, filtered on a 0.22 µm membrane and analyzed by high performance liquid chromatography (HPLC) in the Waters 2695-Alliance equipment (Milford, USA). The mobile phase will be composed of methanol, phosphate buffer (pH 6.8) and acetonitrile (63:30:7 v/v/v) with a flow rate of 0.9 mL/min, and the diode array detector (PDA) will be set to 306 nm. The percentage of EE will be calculated from at least three replications, using Equation 1. Results will be expressed as mean ± standard deviation.

Equation 1 %EE = [(initial amount of α-Humulene – amount recovered) / initial amount of α-Humulene] × 100

PRE-INDUCTION PERIOD

For 30 consecutive days, the animals were treated according to the experimental groups. After this period, neuroinflammation was induced via stereotactic, using the toxin

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Aβ1-42 for the formation of senile plaques. The animals remained under observation for 90 days before being euthanized.

SURGICAL PROCEDURE

To induce AD with Toxin Aβ1-42, the animals were anesthetized with a solution in the proportion of 80mg/kg of ketamine hydrochloride (ketamine, 10ml vial) to 15 mg/kg of xylazine hydrochloride (dopaser, 10ml vial) intraperitoneally, then taken to a stereotactic device (David Kopf, USA) where their heads were fixed by the temporal rock and maxillary incisors, under specific coordinates for the hippocampal area (AP=-3.0mm, ML=1.6mm, 1.6mm and DV= 3.0mm), taking bregma as a reference, receiving 4µl of Aβ1-42 Toxin through a Hamilton syringe in the hypocampic region of CA1 for the process of senile plaques (MIRI et al., 2018). After inducing the neuroinflammation process, the animals rested for a period of 30 days for the inflammatory processes of the hippocampal neurons to occur. One animal per group was euthanized to verify the presence of plaques in the hippocampus.

POST-SURGICAL ANALGESIA

Tramadol hydrochloride was administered at a dose of 10 mg/kg, orally, every 12 hours, for seven consecutive days, to control postoperative pain.

TREATMENT

The animals received 6.5 µg of α-Humulene, Curcumin or their nanoparticles orally (gavage) for 30 consecutive days.

FLOW CYTOMETRY

Two mL of blood were collected from each animal and then centrifuged at 1500 rpm for 10 min at room temperature. After centrifugation, the supernatant (serum) was pipetted and separated for analysis. The BD Cytometric Bead Array Mouse Inflammation Cytokine Kit (Becton Dickinson, USA) was used™. The cytokines analyzed were TNF-α; IL-1 and IL-4. According to the manufacturer's instructions and analyzed in the BD™ Accuri C6 Flow Cytometer (Becton Dickinson, USA), 10µL of each reagent was added to each sample. After this procedure, 50µL of the cytokine beads, 50µL of the sample (serum) and 50µL of the detection reagent were placed in a 1.5 mL eppendorf for each sample. The tubes were

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placed in the dark for two hours at room temperature. After two hours, 1 mL of the wash buffer was added to each eppendorf and centrifuged at 200g at room temperature for 5 minutes. After centrifugation, the supernatant was carefully removed and discarded from each sample, and then 300 µL of the wash buffer was added to each tube to resuspend the samples. The cytometer reading was performed manually by acquiring 10,000 events from each sample. Flow cytometry data were analyzed using the FCap 3.0 Array software (Becton Dickinson, USA) and the results were plotted on graphs of means and standard deviations.

EUTHANASIA

The animals were anesthetized with 80 mg/kg of ketamine and 15 mg/kg of xylazine. After verifying the anesthetic status, they received 175 mg/kg of lethal dose of pentobarbital intraperitoneally.

STATISTICAL ANALYSIS

The data were analyzed using the Prisma 9.3.1 software. The Shapiro-Wilk test was used to verify the normality of the data, and the One-Way ANOVA test with Tukey's post-test was applied for non-parametric samples.

RESULTS

The Zein nanoparticles showed different characteristics according to the encapsulated compound, as shown in Table 1. For the curcumin-only nanoparticles, the mean diameter was 243.9 nm, with a polydispersion index (PDI) of 0.347 and a zeta potential of -32.7 mV. The encapsulation efficiency (EE) was 95%, with a concentration of 617.5 µg/mL of curcumin. The nanoparticles containing α-humulene had an average diameter of 313.1 nm, PDI of 0.221 and zeta potential of -36.2 mV. The encapsulation efficiency of these nanoparticles was 91%, with a concentration of 591.5 µg/mL of α-humulene. Finally, nanoparticles containing both curcumin and α-humulene had the largest average diameter, at 351.1 nm, and a PDI of 0.482. The zeta potential of these nanoparticles was considerably lower, at -40 mV. Encapsulation efficiencies were maintained at 95% for curcumin (617.5 µg/mL) and 91% for α-humulene (591.5 µg/mL).

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Table 1 – Physicochemical Characteristics and Encapsulation Efficiency of Nanoparticles

Structure/Qualification	Average diameter (nm)	Polydispersion index	Zeta Potential (mV)	Encapsulation efficiency
Zein nanoparticle containing curcumin	243,9	0,347	-32,7	95% (617.5 µg/ml)
Zein nanoparticle containing alpha-humulene	313,1	0,221	-36,2	91% (591.5 µg/ml)
Zein nanoparticle containing curcumin and alpha-humulene	351,1	0,482	-40	95% Curcumin and 91% Alpha-Humulene (617.5 µg/ml Curcumin and 591.5 µg/ml Alpha-Humulene)

Source: Author (2025)

The data obtained from the analysis of pro-inflammatory (TNF-α and IL-1) and anti-inflammatory (IL-4) cytokines were presented in Figures 1, 2 and 3, highlighting the means and standard deviations of the different experimental groups.

TNF-α

The Negative Control Group (NC) had a mean of 0.6983 ± 0.05025 pg/ml, while the Positive Control Group (PC) had a mean of 0.6900 ± 0.03090 pg/ml. The group treated with Curcumin (GCur) showed a reduction, with an average of 0.5905 ± 0.12213 pg/ml, compared to the control groups. Treatment with α-Humulene (HUM) resulted in an average of 0.6713 ± 0.08390 pg/ml, while the group treated with α-Humulene nanoparticles (NHUM) had an average of 0.6293 ± 0.06013 pg/ml. The group treated with α-Humulene and Curcumin nanoparticles (NHUM+Cur) had an average of 0.6000 ± 0.03283 pg/ml. Statistically significant differences were observed between the CN/NHUM+Cur, CP/NHUM+Cur and GCur/NHUM+Cur groups ($p<0.05$), indicating that the treatment with the combined nanoparticles was more effective in reducing TNF-α levels.

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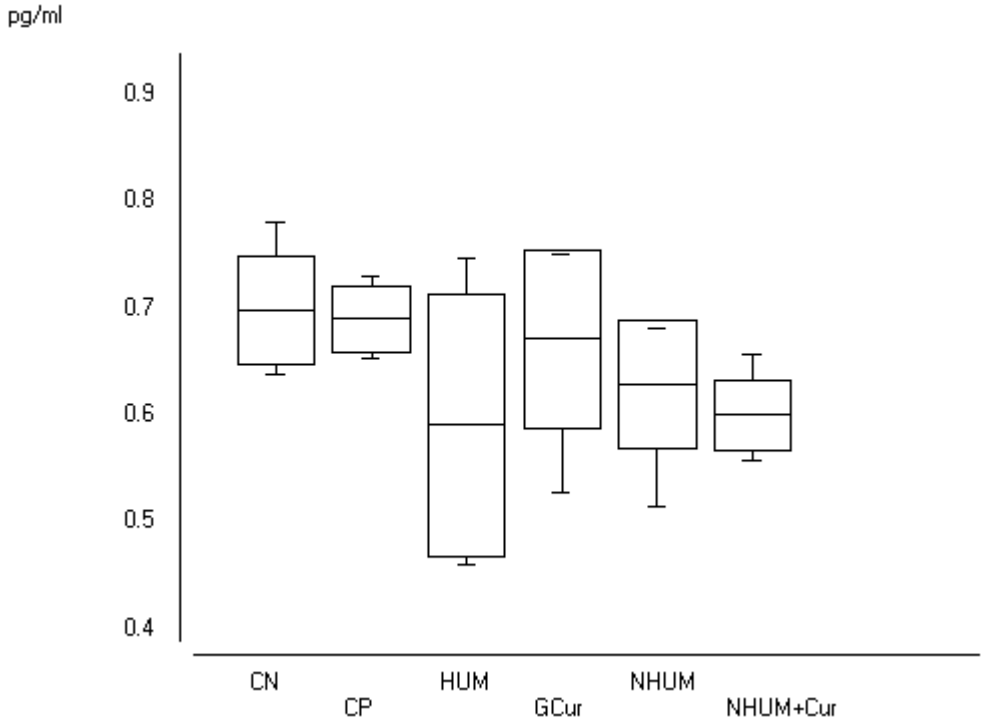
Figure 1 – Mean and Standard Deviation for TNF-Alpha.



Source: Author (2025)

A statistically significant difference was observed between the CN/NHUM+Cur, CP/NHUM+Cur and GCur/NHUM+Cur groups for $p<0.05$.

3.2 IL-1

The levels of IL-1 in the experimental groups varied less significantly. The Negative Control Group (NC) had a mean of 0.5150 ± 0.05782 pg/ml, while the Positive Control Group (PC) had a mean of 0.5788 ± 0.07736 pg/ml. The group treated with Curcumin (GCur) had a mean of 0.5000 ± 0.08211 pg/ml, followed by the HUM (0.5187 ± 0.13611 pg/ml), NHUM (0.5125 ± 0.05600 pg/ml) and NHUM+Cur (0.4775 ± 0.09968 pg/ml) groups. No statistically significant differences were observed between the groups after Dunn's test.

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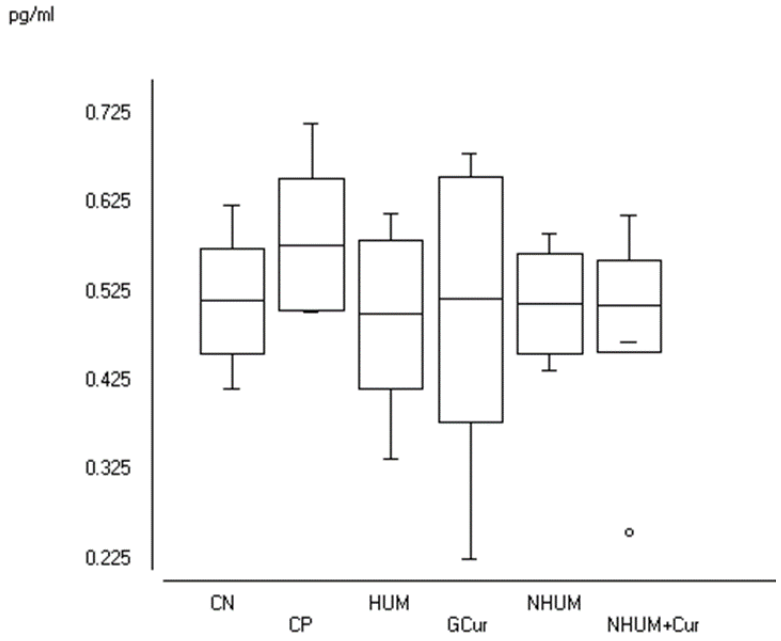
Figure 2 – Mean and Standard Deviation for IL-1.



Source: Author (2025)

No statistically significant difference was observed between the groups after Dunn's test.

IL-4

The NC group had a mean of 0.3813 ± 0.04673 pg/ml for IL-4, while the CP had a mean of 0.4413 ± 0.07936 pg/ml. The Curcumin-treated group (GCur) had an average of 0.3763 ± 0.04749 pg/ml. The groups treated with α-Humulene (HUM) and α-Humulene nanoparticles (NHUM) recorded averages of 0.4363 ± 0.14050 and 0.4113 ± 0.16111 pg/ml, respectively. The group treated with α-Humulene and Curcumin nanoparticles (NHUM+Cur) showed a significant increase, with an average of 0.5613 ± 0.08408 pg/ml. Statistically significant differences were observed between the CN/NHUM+Cur, CP/NHUM+Cur, and HUM/NHUM+Cur groups ($p<0.05$), suggesting that the combined treatment was effective in increasing IL-4 levels, indicating a more robust anti-inflammatory response.

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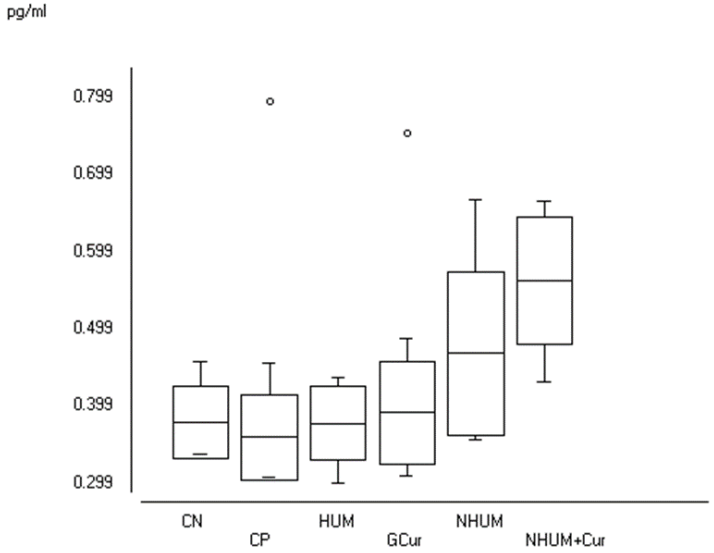
Figure 3 - Mean and Standard Deviation for IL-4.



Source: Author (2025)

A statistically significant difference was observed after Dunn's test between the CN/NHUM+Cur, CP/NHUM+Cur and HUM/NHUM+Cur groups for $p<0.05$.

DISCUSSION

The results of the present study present solid evidence about the neuroprotective effects of alpha-humulene and curcumin nanoparticle formulations on the modulation of neuroinflammation associated with AD. The significant reductions in pro-inflammatory cytokines, especially TNF-α, along with increased levels of the anti-inflammatory cytokine IL-4, indicate that these treatments can effectively alter the neuroinflammatory response to a more favorable phenotype. This finding is consistent with previous research indicating that curcumin and alpha-humulene possess strong anti-inflammatory properties, demonstrating to mitigate neuroinflammation in various models of neurodegenerative diseases (DUAN et al., 2023; LEE et al., 2020).

The efficacy observed in the formulation of nanoparticles is in line with the growing literature advocating the use of nanotechnology in drug delivery systems to overcome the challenges posed by the BBB. Nanoparticles have been shown to increase the bioavailability of therapeutic agents and facilitate their targeted delivery to the CNS (ASIMAKIDOU et al., 2024; DUAN et al., 2023; OLIVE TREE; PIENIZ, 2024; WENDLER et al., 2024). The results show that the diameter of the nanoparticles varied according to the encapsulated compound. The nanoparticles containing both compounds, curcumin and α-

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humulene, had a larger diameter (351.1 nm), which can be explained by the greater complexity of the encapsulation matrix by accommodating two active ingredients simultaneously. This increase in size is also associated with a higher PDI (0.482), suggesting a more heterogeneous size distribution.

The zeta potential, an indicator of colloidal stability, varied significantly among the samples. Nanoparticles containing only α-humulene had the highest negative zeta potential (-36.2 mV), indicating good suspension stability. Nanoparticles containing both curcumin and α-humulene showed the lowest zeta potential (-40 mV), which may indicate lower stability due to the interaction between the two encapsulated compounds. Regarding encapsulation efficiency, the nanoparticles demonstrated high efficiency in all formulations, with values of 91% for α-humulene and 95% for curcumin, both in individual samples and in combined formulations. These results confirm the viability of zein as an encapsulation material for these compounds, while maintaining high levels of encapsulation regardless of the combination.

The data suggest that the combination of curcumin and α-humulene in the same nanoparticles influences the physical properties of the formulation, such as diameter and zeta potential, while the encapsulation efficiency remains high, indicating the possibility of effective applications in controlled-release systems. The findings of this study reinforce the idea that nanoparticle-based drug delivery systems (NDDS) can play a crucial role in the treatment of AD by improving the therapeutic efficacy of compounds such as curcumin and alpha-humulene.

However, this study has some limitations. The relatively small sample size may restrict the generalization of the findings. In addition, the duration of treatment and the observation period may not adequately reflect the long-term effects of the therapies. Future studies should include larger sample sizes and longer observation periods to better assess the durability of therapeutic effects. Furthermore, while cytokine analysis has provided valuable insights into the inflammatory response, a more comprehensive assessment, incorporating additional biomarkers of neuroinflammation, could offer a deeper understanding of the underlying mechanisms.

An unexpected finding was the absence of statistically significant variation in IL-1 levels between the treatment groups. This suggests the existence of complex regulatory mechanisms that control IL-1 expression and its role in chronic neuroinflammation.

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Additional research is needed to clarify the temporal dynamics of cytokine expression after treatment, particularly in the context of chronic neuroinflammation states.

Future research should also explore the potential synergistic effects of combining alpha-humulene and curcumin with other therapeutic agents. Studying the mechanisms by which these compounds exert their effects may provide valuable insights into their potential as multi-target therapies for AD.

CONCLUSION

In conclusion, this study demonstrates that alpha-humulene and curcumin nanoparticle formulations significantly modulate neuroinflammatory responses in an experimental model of Alzheimer's disease. The findings highlight the potential of these compounds as therapeutic agents, especially in the context of their ability to alter cytokine profiles associated with neuroinflammation. Given the limitations of current pharmacological treatments for AD, the results advocate further exploration of alternative therapies combined with nanotechnology as innovative strategies for neuroprotection. Ongoing research is essential to elucidate the underlying mechanisms of action and optimize treatment protocols, paving the way for new therapeutic interventions in the management of neurodegenerative diseases.

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