The volatile constituents of rose, saffron and cardamom suppress the fibrillation of tau and not HEWL through the formation of non-toxic tau oligomers

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Abstract

The formation of fibrillar structures of tau is correlated with taupathies including Alzheimer's disease (AD). This study has aimed to find ways to prevent tau fibril formation. Here, we used dietary compounds including cinnamon (CN), damask rose (Rose), saffron (Saf) and green cardamom (Car), to evaluate the effects of their volatile constituents, on hen egg white lysozyme (HEWL), as a model protein (commonly used for fibrillation studies), as well as the brain-related tau protein. The study was done using different spectroscopic techniques as well as SDSPAGE, AFM and MTT assay. While the results suggested that the volatile constituents were unable to prevent HEWL fibril formation, most of the dietary compounds, in particular Saf, Rose and Car, were able to interfere with the mature fibril formation, by either maintaining the native form of tau or resulting in the entrapment of non-toxic oligomeric forms of tau. Moreover, the neurotoxicity analysis of tau samples on neuroblastoma SHSY5Y cells indicated that tau treated with Saf, Rose and Car were the least toxic. Overall, the findings indicate that the potential therapeutic impacts of the volatile constituents of Rose, Car and in particular Saf, may demonstrate significant efficacy in addressing neurodegenerative diseases such as AD.

Key words: Natural dietary compounds, Volatile constituents, Tau protein, Fibrillation, Alzheimer's disease, HEWL

1. Introduction

The study discusses the role of protein misfolding in neurodegenerative diseases like Alzheimer's disease (AD) and other tauopathies. These conditions are characterized by the accumulation of misfolded tau proteins, which form amyloid-like aggregates that contribute to neuronal dysfunction and death. Tau, primarily located in the central nervous system, regulates microtubule dynamics and axonal transport. The accumulation of abnormally phosphorylated tau leads to its fibrillation, a process that results in toxic intermediates and aggregates, making the inhibition of tau aggregation a potential therapeutic approach for reducing neurotoxicity in tauopathies.

Recent research has explored plant-derived volatile compounds as potential therapeutic agents for cognitive disorders like AD. These volatile compounds, found in aromatic plants, possess bioactive properties such as antioxidant, anti-inflammatory, and neuroprotective effects. Some studies have suggested that inhaling plant-derived volatile compounds can improve cognitive performance, alleviate agitation, and reduce psychotic symptoms in patients with brain disorders, including AD. These compounds are thought to exert their beneficial effects by suppressing apoptosis, reducing inflammation, providing antioxidant protection, and promoting neuroprotection.

However, limited research has focused on how these volatile compounds might influence protein fibrillation, particularly tau fibrillation. To explore this, the authors of the study investigated the effects of three volatile compounds—cinnamaldehyde (Cin), phenyl ethyl alcohol (PEA), and N,N,N',N'-tetramethylethylenediamine (TEMED)—on the fibrillation of hen egg white lysozyme (HEWL) and tau protein. Their previous findings suggested that Cin and PEA could prevent the formation of mature fibrils by disrupting intermediate species or preventing the formation of protofibrils. Building on this, the current study extended the investigation to volatile compounds derived from several dietary plant extracts—cinnamon (CN), damask rose (Rose), saffron (Saf), and green cardamom (Car). These extracts were chosen due to their known protective effects against AD. Cinnamon, for instance, has demonstrated neuroprotective activity by inhibiting the formation of amyloid beta plaques and neurofibrillary tangles, as well as reducing acetylcholinesterase activity. Similarly, rose, saffron, and cardamom have shown potential in improving memory and cognitive function, inhibiting AChE activity, and preventing the formation of neurotoxic aggregates in AD models. The study also identified the key volatile constituents in each plant extract. For example, cinnamon's active volatile compounds include cinnamaldehyde, eugenol, and linalool, while rose contains phenylethyl alcohol (PEA), citronellol, and geraniol. Saffron's key volatiles include safranal, and cardamom's main volatile is 1,8-cineol. These compounds have been linked to therapeutic effects via various mechanisms, such as anti-inflammatory and antioxidant properties. Since one of the challenges in treating AD is the difficulty in delivering therapeutic agents to the brain, the nasal route for drug delivery has been explored as a promising alternative. The small size of volatile compounds allows them to easily pass through the nasal cavity and reach the brain, making them ideal candidates for such treatment.

In conclusion, this study aims to further explore how volatile compounds from common dietary plants can influence the fibrillation process of tau proteins and contribute to potential treatments for neurodegenerative diseases such as Alzheimer's. By focusing on natural compounds with

known neuroprotective properties, this research opens the door for new therapeutic strategies targeting protein misfolding and aggregation in the brain.

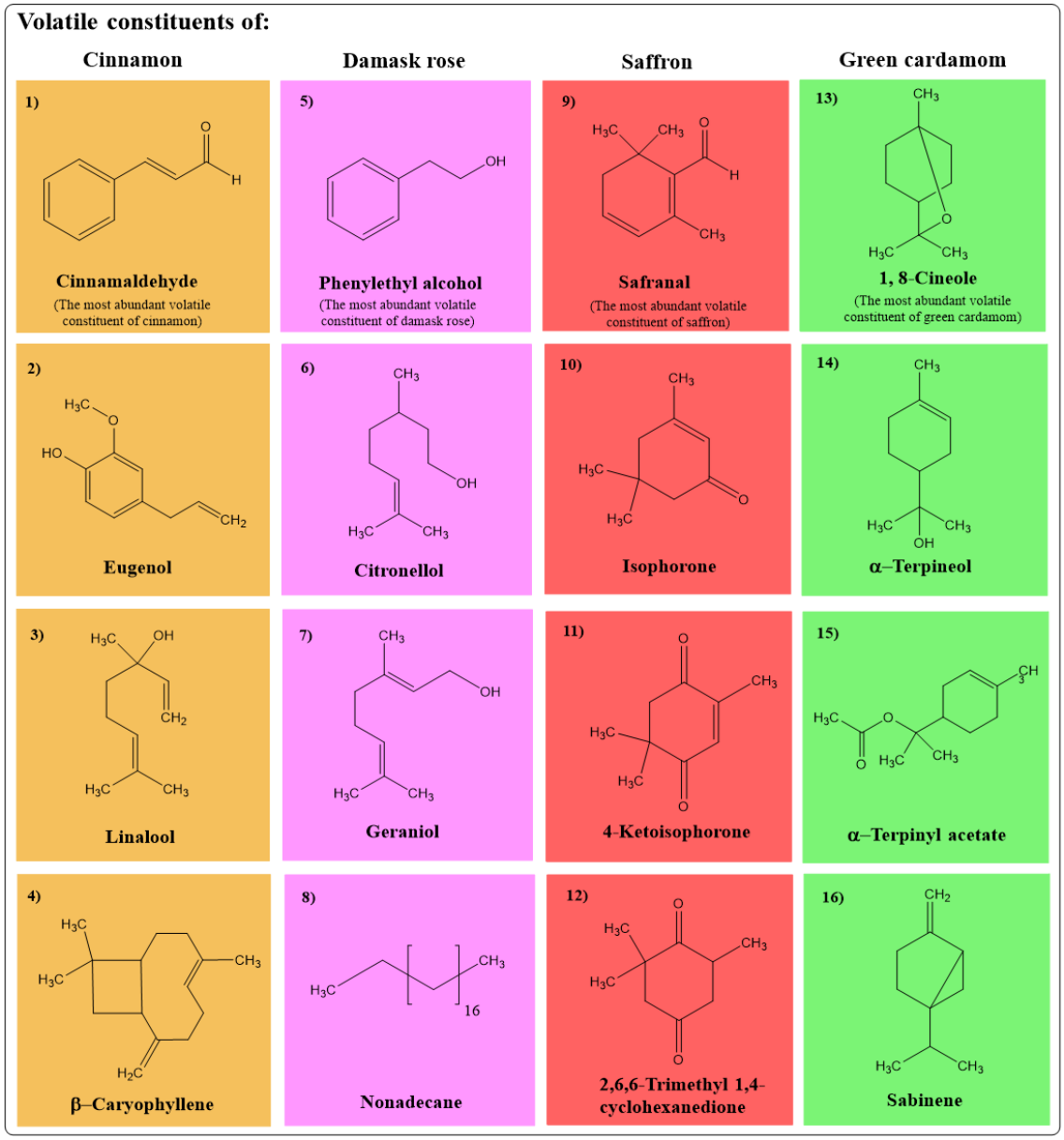

Figure 1. Structure of the main volatile constituents of CN, Rose, Saf and Car. 1-4) The main volatile constituents of cinnamon. 5-8) The main volatile constituents of damask rose. 9-12) The main volatile constituents of saffron. 13-16) The main volatile constituents of green cardamom. ChemDraw software (version 18.2) was used to draw the structures.

1. Results

2.1. Gel mobility shift analysis of HEWL and tau protein samples under fibrillation conditions and in the presence of volatile constituents of dietary compounds

Heparin is commonly used to induce tau protein fibrillation in vitro via the nucleation-elongation pathway. Tau, particularly the 4-repeat (4R) isoform, contains cysteine residues involved in disulfide bond formation, which is associated with the formation of aggregates that hinder mature fibril assembly. To prevent disulfide bond formation during heparin-induced tau fibrillation, dithiothreitol (DTT) was used to maintain the cysteines in their reduced form. This study explored the effect of volatile dietary compounds (CN, Rose, Saf, and Car) on the fibrillation of both hen egg white lysozyme (HEWL) and tau proteins.

The pH of protein samples was measured before and after incubation with the volatile compounds, showing no significant changes for most compounds, though CN caused a noticeable reduction in pH. SDS-PAGE analysis of HEWL and tau samples revealed the effects of volatile compounds on protein aggregation. In the case of HEWL, the not-treated sample exhibited significant aggregation and high molecular weight (HMW) species formation, but the volatile compounds did not significantly alter this pattern. For tau, the presence of heparin led to the formation of HMW species and aggregates. Volatile compounds from CN led to complete disappearance of the tau monomeric band, indicating severe aggregation, while Rose and Car treatments resulted in aggregates or protofibrils. Interestingly, the intensity of the monomeric tau band was higher for Rose and Car than for CN, suggesting these compounds were more effective in maintaining the native tau protein form. Quantification of monomeric protein band intensities showed that volatile compounds like Saf were particularly effective in preserving the native form of tau, with the highest monomeric band intensity (53.52%), compared to Rose (18.11%), Car (17.25%), and CN (no detectable monomeric band). Similarly, for HEWL, Saf was the most effective in reducing aggregation, followed by Car, Rose, and CN.

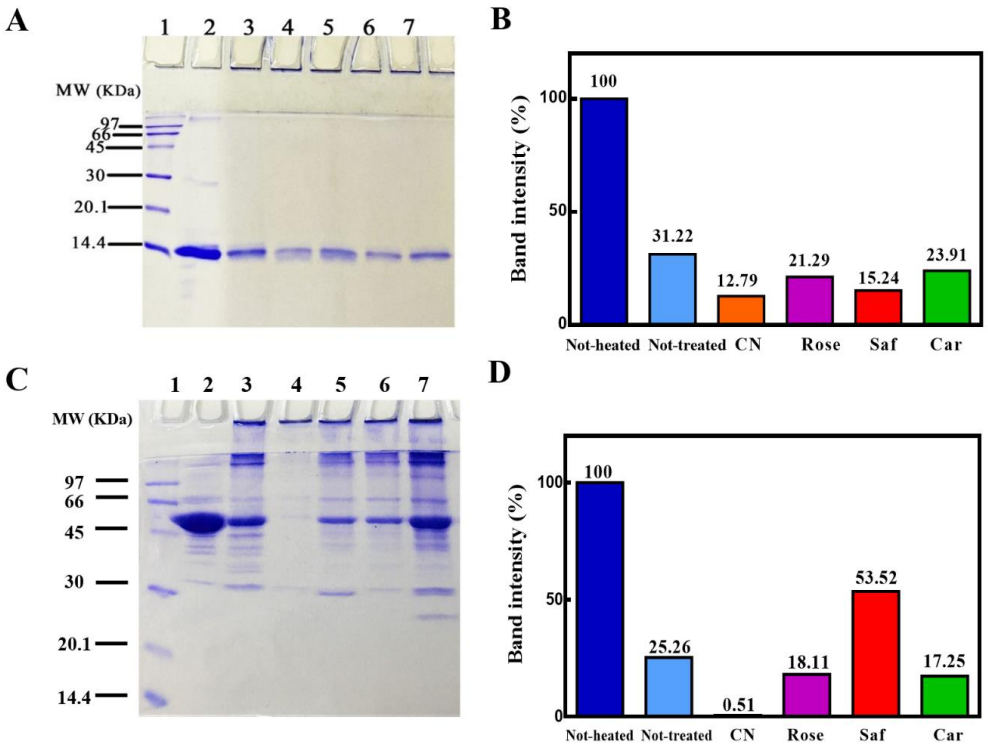

Figure 2. SDS-PAGE analysis of HEWL and tau protein samples, before and after incubation studies, in the presence or absence of volatile constituents. A) Gel electrophoresis of HEWL samples, which were shaken at 500 rpm for 24 h at 54^{o}C. Lane description is as follows: 1-Protein marker, 2- not-heated HEWL sample, 3- not-treated HEWL sample, 4-7: HEWL sample treated with CN, Rose, Saf and Car, respectively. B) Quantitative measurement of the monomeric band intensity of HEWL samples compared to the not-heated HEWL sample. C) Gel electrophoresis of samples obtained by incubating tau protein with heparin, DTT and volatile constituents. Lane description is as follows: 1- Protein marker, 2- not-heated tau sample, 3- not-treated tau sample, 4-7: tau sample treated with CN, Rose, Car, and Saf, respectively. D) Quantitative measurement of the monomeric band intensity of tau protein samples compared to the not-heated tau sample. All the tau samples except the not-heated sample were shaken at 500 rpm at 37 ^{o}C for 96 h. The visualization of the protein bands was done by using an appropriate CBB staining method.

2.2. ThT- fluorescence analysis of HEWL and tau protein samples under fibrillation conditions and in the presence of volatile constituents of dietary compounds

Thioflavin-T (ThT) fluorescence, a marker for amyloid fibril formation, was used to detect the effects of the volatile compounds on fibril formation. HEWL samples incubated with CN, Rose, Saf, and Car all showed an increase in ThT fluorescence, indicating the promotion of amyloid fibril formation. Tau samples also exhibited enhanced ThT fluorescence, suggesting fibril formation in the absence of volatile compounds, though some compounds, particularly Rose and Car, showed lower fluorescence, potentially due to decreased β-sheet content. Saf, however,

exhibited a significant increase in ThT fluorescence, possibly due to phenolic compounds that interact with protein hydrophobic regions, thus enhancing fluorescence

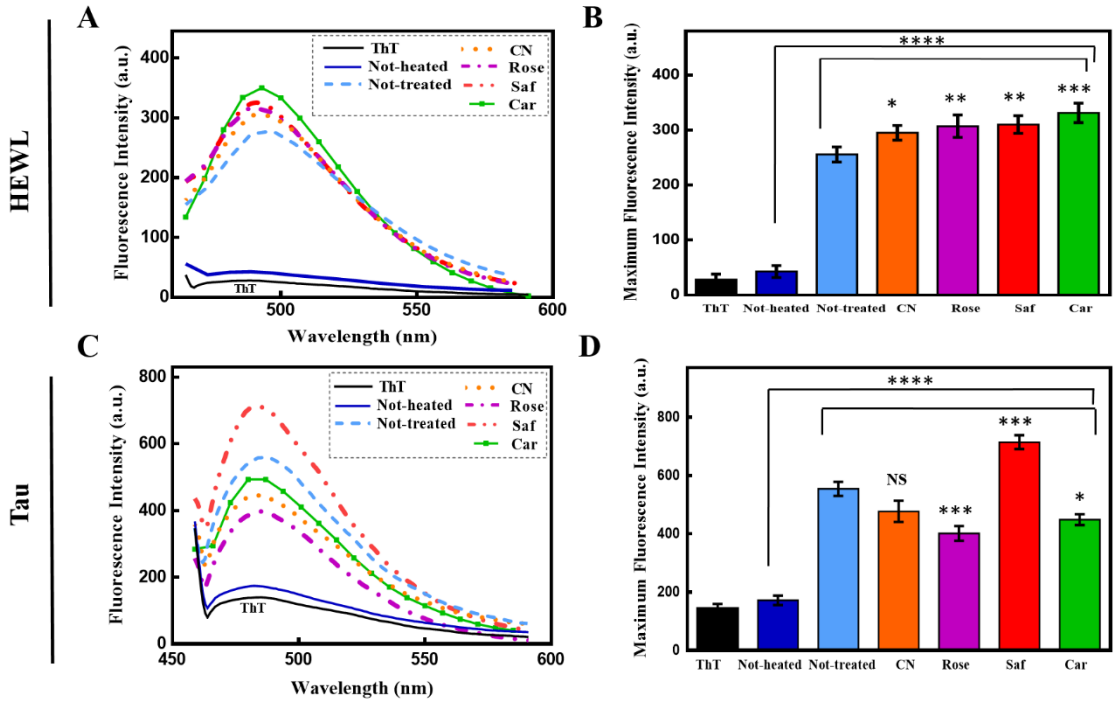

Figure 3. ThT fluorescence of HEWL and tau samples, before and after incubation studies, in the presence or absence of volatile constituents. The fluorescence intensity assessments of HEWL and tau protein samples were performed using 0.20 μM ThT. The excitation wavelength was fixed at 440 nm and the emission spectra were measured at 450-600 nm. A, C) Emission spectra of HEWL and tau protein samples treated with CN, Rose, Saf and Car. B, D) The maximum emission intensity of all the samples at 485 nm.

2.3 Visualization of the inhibitory effects of volatile constituents of dietary compounds on tau and HEWL fibrillation

Fluorescence microscopy further confirmed these findings, showing that HEWL samples treated with volatile compounds did not prevent fibril formation, while tau samples exposed to heparin and DTT alone formed mature fibrils. However, when tau was treated with volatile compounds, smaller intermediate species, rather than mature fibrils, were observed, particularly for Rose, Saf, and Car. Among these, CN had the least effect in preventing tau fibrillation, while Rose, Saf, and Car effectively inhibited fibril formation and maintained tau in a more native or intermediate state. In conclusion, the volatile constituents of dietary compounds demonstrated varying degrees of effectiveness in modulating tau fibrillation. Saf showed the strongest protective effect, followed by Rose and Car, while CN had the least impact on reducing tau aggregation. These findings suggest that certain volatile compounds can potentially be used to modulate protein aggregation and may have therapeutic implications for amyloid-related diseases.

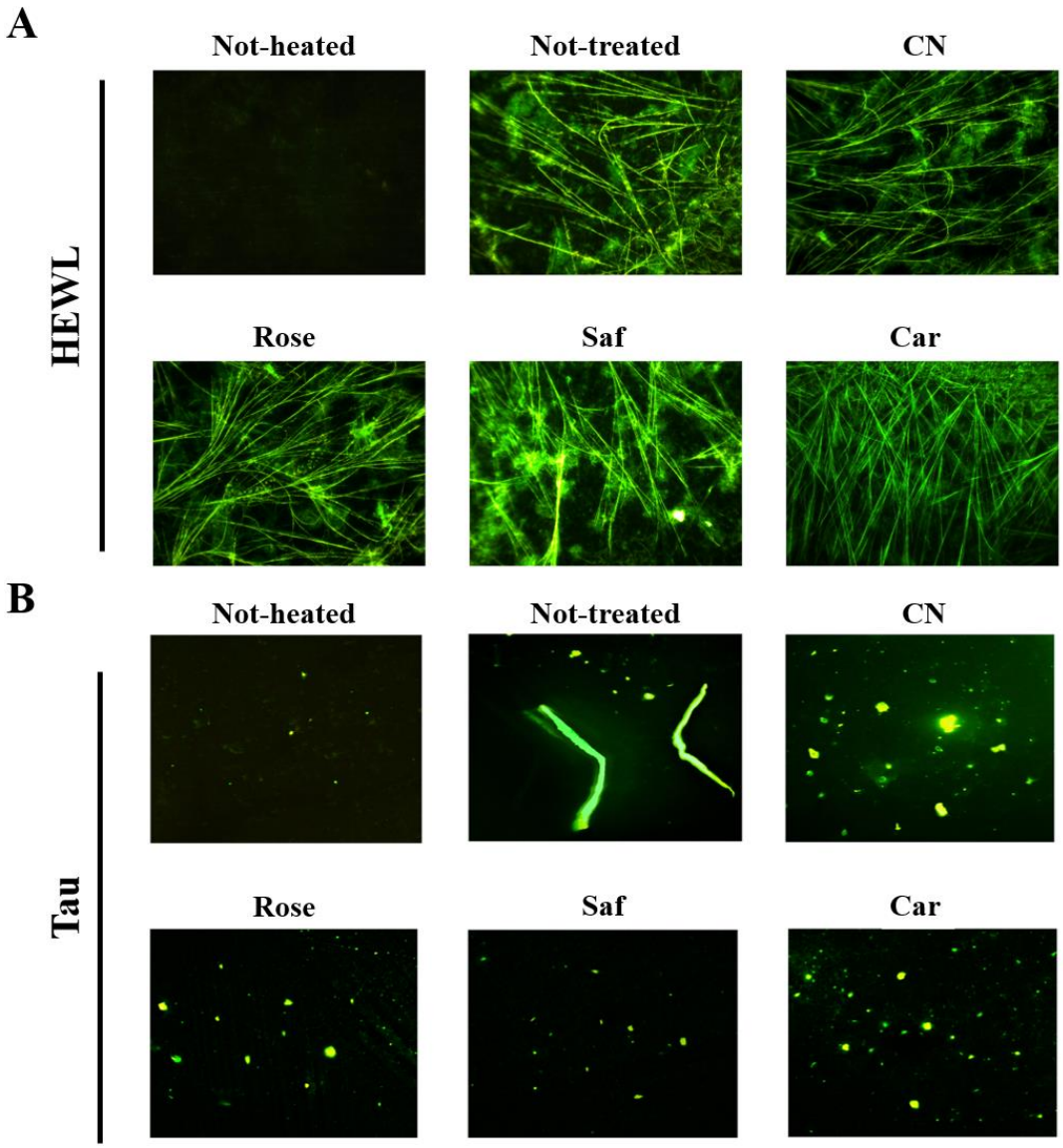

Figure 4. Fluorescence microscopy analysis of HEWL and tau samples, before and after incubation studies, in the presence or absence of volatile constituents. A) HEWL samples. B) Tau protein samples. Images were captured using a zeiss lens with 40X magnification and a 450-500 blue-fluorescence filter.

In the search for therapeutics to prevent tau fibrillation in Alzheimer's disease (AD) and other tauopathies, this study used atomic force microscopy (AFM) and fluorescence spectroscopy to evaluate the inhibitory effects of volatile compounds from dietary sources on tau fibrillation. AFM imaging showed that untreated tau formed mature fibrils, while tau samples treated with volatile compounds (CN, Rose, Saf, and Car) exhibited anti-fibrillation properties by forming different types of protofibrils and aggregates. Specifically, CN-treated tau formed spherical annular protofibrils with granular oligomers, whereas tau treated with Rose, Saf, and Car formed mostly

linear protofibrils and oligomeric aggregates, suggesting these compounds can prevent mature fibril formation by promoting intermediate species.

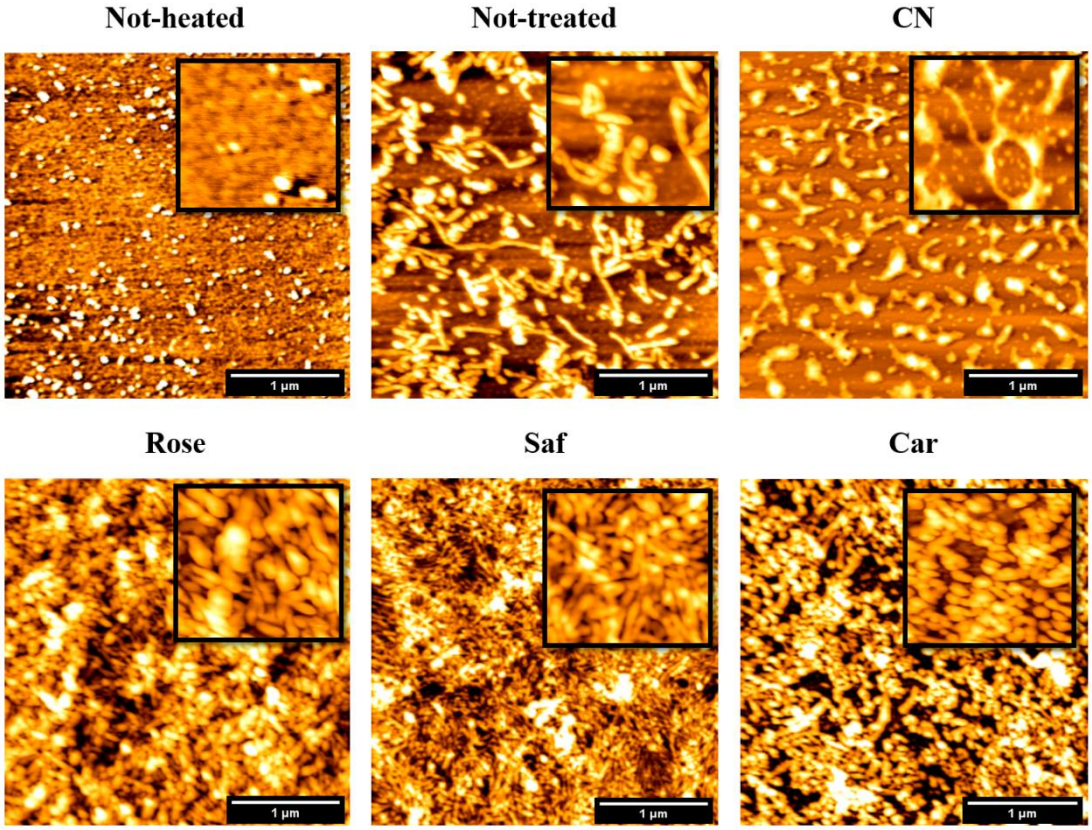

Figure 5. AFM analysis of tau protein samples, before and after incubation studies, in the presence or absence of volatile constituents. AFM images of different tau samples after 96 h of incubation. The scale bar in all images: 1 μm. The figure insets show magnified images.

2.4 Intrinsic fluorescence and circular dichroism analyses of HEWL and tau protein samples incubated with volatile constituents of dietary compounds

2.4.1. Intrinsic fluorescence assessment

To further assess the structural changes in proteins, Trp and Tyr fluorescence analyses were employed. For HEWL samples, both Trp and Tyr fluorescence intensities decreased in the not-treated sample, indicating structural changes. Volatile compounds like Rose, Saf, and Car did not significantly affect these fluorescence levels, suggesting they did not prevent the structural changes in HEWL. In contrast, CN caused a further reduction in both Trp and Tyr fluorescence, indicating that CN induced more profound structural changes in HEWL.

For tau protein, which lacks Trp residues, Tyr fluorescence spectroscopy was used. In the not-treated tau sample, Tyr fluorescence emission significantly decreased compared to the not-heated

sample. Tau samples treated with volatile compounds showed altered emission patterns, especially with CN and Saf. These treatments led to a shift in the Tyr fluorescence emission spectrum, with two peaks appearing in the spectrum and a reduction in intensity around 335 nm. This shift was attributed to interactions between volatile compounds and the proteins, which likely modified the positioning of charged amino acids near Tyr residues, altering the fluorescence emission.

Among the samples, tau treated with Saf showed the least decrease in Tyr fluorescence, suggesting that Saf was most effective in inhibiting tau fibrillation and maintaining tau's monomeric structure. In contrast, CN caused the greatest reduction in Tyr emission, promoting the formation of oligomeric and irregular tau structures. Rose and Car samples also altered tau's structure, but their effects were less pronounced compared to Saf and CN. Overall, the results suggest that volatile dietary compounds can modulate tau protein structure, with Saf being the most effective in preserving tau's native form and preventing fibrillation.

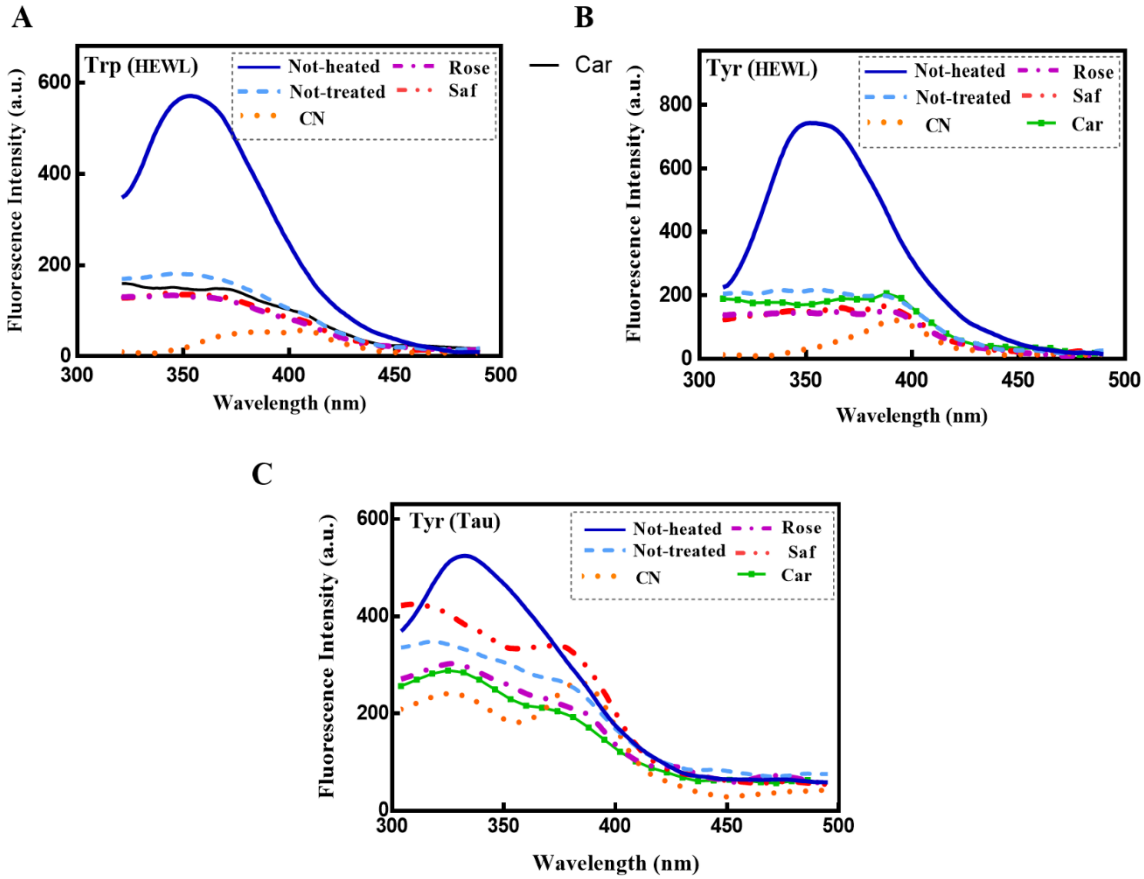

Figure 6. Intrinsic fluorescence emission spectroscopy analysis of HEWL and tau protein samples, before and after incubation studies, in the presence or absence of volatile constituents. The excitation wavelength of Trp (A) and Tyr (B and C) was fixed at 295 and 280 nm, respectively. The emission spectra were obtained in the range of 300-500 nm.

2.4.2. Circular dichroism spectroscopy

The secondary structural changes of HEWL and tau proteins treated with volatile constituents of dietary compounds were analyzed using Far-UV Circular Dichroism (CD) spectroscopy. For HEWL, the native (not-heated) sample showed typical alpha-helix features, with negative peaks at 208 and 222 nm. In contrast, the not-treated HEWL sample, which underwent structural changes, displayed a weak peak at 222 nm and a positive peak at 204 nm, indicative of non-parallel beta sheets, a key step in amyloid formation. HEWL treated with CN, Saf, and Car also showed a positive peak at 204 nm, though with reduced intensity. The Rose-treated sample did not exhibit significant changes.

For tau, the native (not-heated) sample displayed a negative peak at 204 nm, reflecting its random coil structure. The not-treated tau sample showed a shift to a negative peak at 217 nm, suggesting the formation of beta-sheet structures, a hallmark of amyloid fibrils. Tau treated with CN showed a negative peak at 220 nm, while samples treated with Rose, Car, and Saf showed peaks at 210-209 nm.

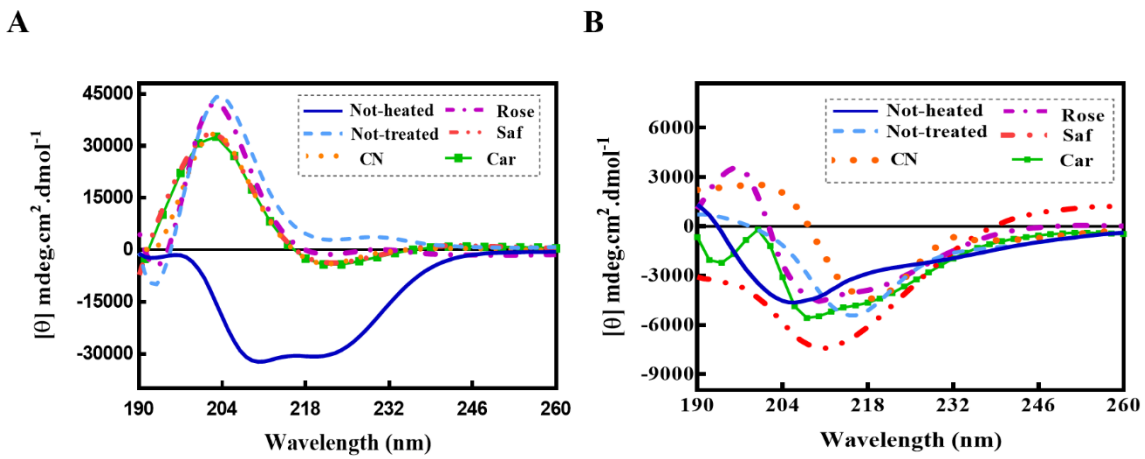

Figure 7. Evaluation of changes in secondary structure content in HEWL and tau protein samples, before and after incubation studies, in the presence or absence of volatile constituents. A and B) Far-UV CD spectra of HEWL and tau protein samples, respectively**.** The CD spectra of the protein samples are shown in the wavelength range of 190 to 260 nm.

Using the BeStSel online tool, secondary structure predictions revealed that HEWL under fibrillation conditions transitioned from alpha-helix to antiparallel beta-sheets, which was mitigated by volatile compounds, increasing random coil and beta-turn structures. For tau, the not-treated sample had a mix of random coil and beta-sheet structures. In contrast, treatments with CN, Rose, Saf, and Car reduced parallel beta-sheet content and promoted the formation of intermediate

species, indicating that these compounds likely prevented the formation of tau fibrils by promoting protofibrils and oligomers.

Table 2. The percentage of secondary structure content of HEWL and tau protein samples, before and after incubation studies, in the presence or absence of volatile constituents, using the BeStSel online web site.

HEWL						Secondary structure (%)	Tau					
NH	NT	CN	Rose	Saf	Car		NH	NT	CN	Rose	Saf	Car
40.4	0.0	0.0	1.0	0.0	1.5	**α helix**	2.7	0.0	0.0	10.5	15.0	6.1
23.8	60.1	56.6	55.0	51.3	52.1	**Antiparallel β**	31.2	34.9	39.2	29.8	27.4	23.2
0.0	0.0	0.0	0.0	0.0	0.0	**Parallel β**	0.0	9.8	0.0	0.3	3.3	6.0
8.3	15.1	14.7	14.2	13.3	13.8	**β turn**	15.8	12.8	9.6	15.4	13.8	14.9
27.5	25.2	30.1	31.5	34.4	32.6	**Random coil**	50.3	42.5	51.2	44.0	40.5	49.9

2.5 Studying the effects of volatile constituents of dietary compounds on thiol contents of HEWL and tau protein

In this study, the fluorescence dye Monobromobimane (mBBr) was used to detect free thiol groups in protein samples, with fluorescence emission at 490 nm indicating the amount of free thiol. For HEWL, the not-heated sample showed the lowest fluorescence intensity, indicating minimal free thiols, as the protein's cysteines form disulfide bridges in its native state. However, the not-treated HEWL sample and those treated with volatile compounds (CN, Rose, Saf, and Car) showed a significant increase in fluorescence, suggesting that the proteins lost their native fold and exposed thiol groups. The fluorescence intensity of these treated samples did not differ significantly from the not-treated sample.

For tau, the not-treated sample showed a marked decrease in fluorescence compared to the not-heated sample, indicating reduced free thiol content due to disulfide bond formation during heparin-induced fibrillation. Tau samples treated with CN and Car also showed a significant

reduction in mBBr intensity, while other treatments had little effect on fluorescence compared to the not-treated sample.

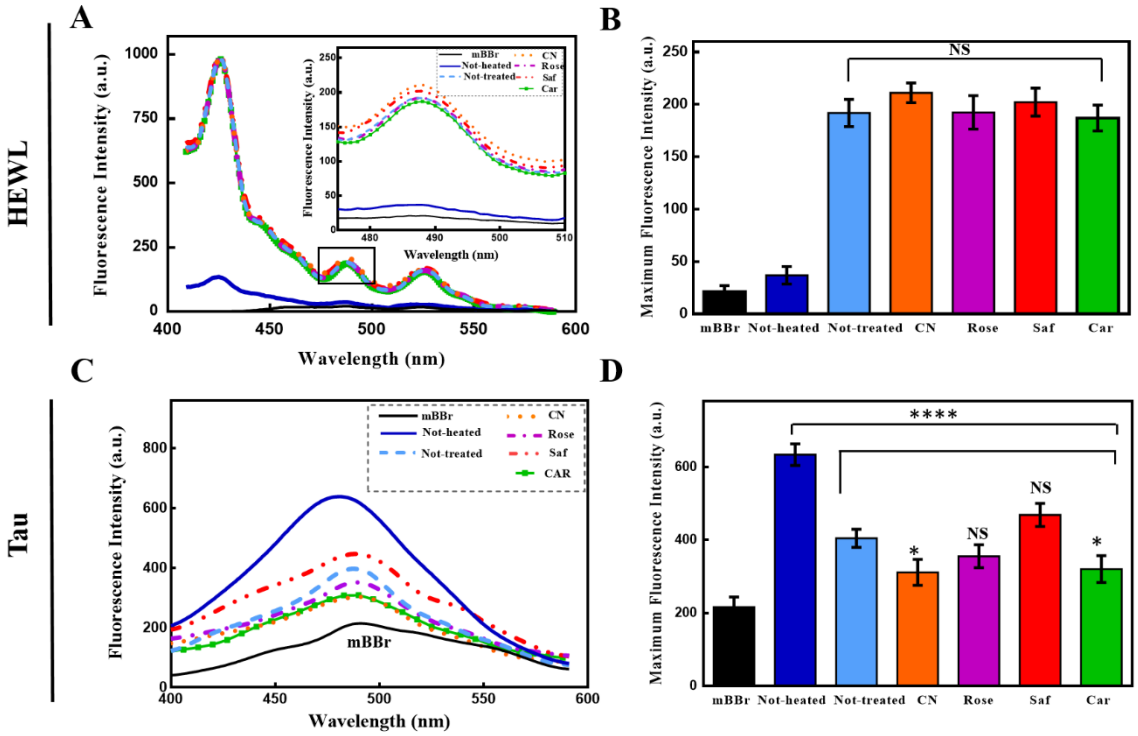

Figure 8. mBBr fluorescence spectra of HEWL and tau protein samples, before and after incubation studies, in the presence or absence of volatile constituents. A, C) mBBr fluorescence spectra of post-incubated HEWL and tau samples. B, D) Maximum fluorescence intensity of the samples at 490 nm. The excitation wavelength of mBBr was fixed at 380 nm.

2.6. Neuronal toxicity analysis of tau samples in the presence or absence of volatile constituents of dietary compounds

To evaluate the toxicity of tau samples, SH-SY5Y neuroblastoma cells were treated with 5 and 10 µM tau for 24 hours. All tau samples, including those treated with volatile dietary compounds, increased neuronal toxicity and cell death compared to the not-heated tau sample. However, tau samples treated with Saf, Rose, and Car showed reduced toxicity, as these compounds helped maintain the native monomeric form of tau and prevented fibrillation. In contrast, tau treated with CN caused more neuronal death, suggesting that oligomeric species formed by CN were more toxic. Additionally, higher tau concentrations (10 µM) led to increased neuronal toxicity compared to 5 µM.

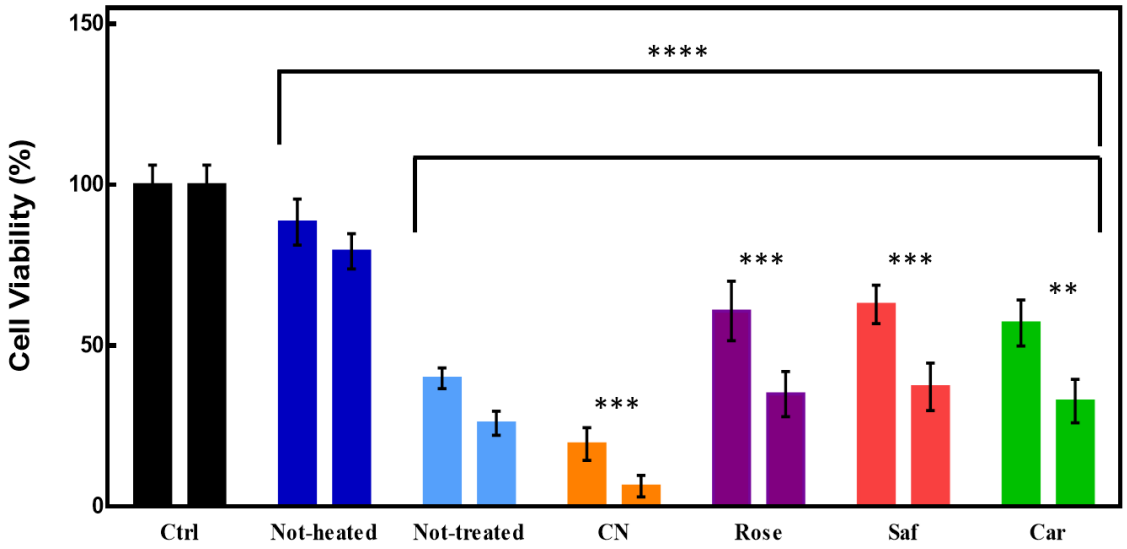

Figure 9. Cell toxicity assay of tau protein samples, before and after incubation studies, in the presence or absence of volatile constituents. The SH-SY5Y cells were treated with tau protein samples at a final concentration of 5 and 10 μM for 24 h. For each sample, the first and the second column represented the cell viability of 5 and 10 μM concentration of tau protein samples, respectively

3. Discussion

Despite significant efforts, diagnosing and treating amyloid-related disorders like Alzheimer's Disease (AD) remains challenging. Essential oils, known for their therapeutic potential, have recently gained attention for their role in treating brain disorders. This study explores the anti-fibrillary effects of volatile constituents of dietary compounds on tau protein, a key player in neurodegenerative diseases like AD.

The study used HEWL (hen egg white lysozyme) protein, a model for amyloid fibril formation, to assess the impact of volatile compounds from cinnamon (CN), rose, saffron (Saf), and carrot (Car) on protein aggregation. Previous studies showed that compounds like cinnamaldehyde (Cin) and PEA inhibited fibril formation in HEWL and tau. Here, the effects of these volatile compounds on tau fibrillation were analyzed through SDS-PAGE, ThT fluorescence, AFM, and CD spectroscopy. The results showed that Saf, Rose, and Car retained more of tau's native monomeric form compared to untreated tau, which formed higher-molecular-weight (HMW) aggregates. Among these, Saf was the most effective in preventing fibrillation. Fluorescence microscopy and AFM images indicated that the volatile compounds prevented tau fibril formation, maintaining intermediate structures such as protofibrils and oligomers.

Further analysis of the tau protein treated with CN revealed increased HMW aggregates, which correlated with higher toxicity levels. This suggests that CN may induce more toxic oligomeric species. In contrast, tau treated with Rose, Saf, and Car showed less toxicity in MTT assays, suggesting that these compounds prevent tau fibrillation while maintaining less toxic intermediate species. The study concludes that volatile compounds from Saf, Rose, and Car, particularly their

active components (safranal, 1,8-cineole, and PEA), could serve as potential therapeutic agents for AD, as they reduce tau fibrillation and exhibit lower toxicity compared to untreated tau.

4. Materials and Methods

4.1. Materials:

Materials:

Hen egg white lysozyme (HEWL) was purchased from Sigma-Aldrich. Nickel NTA agarose resin, IPTG, DTT, heparin sodium, and other chemicals were sourced from DNAbiotech and Iran Hormone Pharmaceutical Company. Tau protein was provided by Dr. Mohammad Ali Nasiri Khalili (University of Tehran). Thioflavin-T (ThT) and Monobromobimane (mBBr) were also obtained from Sigma-Aldrich.

4.2. Methods:

Expression and Purification of Recombinant Tau Protein:

Recombinant tau protein (1N4R isoform) was expressed in E. coli BL21 (DE3) cells. The protein was purified using affinity chromatography with Ni-NTA agarose resin. After transformation and culture, protein expression was induced with IPTG, and cells were lysed for protein extraction. Tau protein was then purified and confirmed by SDS-PAGE, dialyzed, concentrated, and stored at -70°C. Protein concentration was determined using UV absorbance at 280 nm.

Treatment of HEWL and Tau Protein with Volatile Compounds:

HEWL (30 µM) was treated with 1 mg of powdered cinnamon (CN), rose, saffron (Saf), and carrot (Car) dissolved in water. These volatile compounds were placed in small perforated tubes inside 20 mL bottles with HEWL samples, allowing exposure to their volatiles. Tau protein (20 µM) was treated with 5 µM heparin and 5 mM DTT, in the presence or absence of the same volatile compounds. The samples were incubated at 37°C for tau protein and 54°C for HEWL, shaking at 500 rpm. Control samples included proteins without volatile compounds, incubated at 4°C.

SDS-PAGE Analysis:

Protein samples were analyzed using SDS-PAGE with 12% gels for tau and 18% gels for HEWL. The samples were treated under reducing conditions with DTT and visualized using Coomassie Brilliant Blue dye.

Fluorescence Microscopy:

To visualize amyloid fibril formation, ThT fluorescence was used. ThT (20 µM) was added to post-incubated HEWL and tau samples and incubated for 10 minutes in the dark. The samples were then placed on glass slides and observed under a fluorescence microscope with a 40X objective, using a blue-fluorescence filter.

Atomic Force Microscopy (AFM):

AFM was used to observe the morphology of the tau protein samples. Post-incubation, 5 µL of each tau sample was fixed onto freshly prepared mica, dried, washed with deionized water, and dried again before imaging.

Fluorescence Spectroscopy:
Fluorescence spectra of tau and HEWL were measured using a Cary-Eclipse spectrofluorometer. ThT fluorescence was used to assess amyloid fibril formation. Additional measurements were performed for tryptophan and tyrosine fluorescence. mBBr fluorescence was used to quantify free thiol groups in the protein samples, indicating possible structural changes.

Circular Dichroism (CD) Spectroscopy:
CD spectra of the proteins were collected in the far-UV range (200-260 nm) to assess secondary structure alterations, such as changes in α-helix and β-sheet content. Data were analyzed using the BeStSel online tool.

Cell Viability Assays:
Human SH-SY5Y neuroblastoma cells were cultured and treated with tau protein samples (both untreated and treated with volatile compounds). Cell viability was assessed using the MTT assay, measuring the absorbance at 570 nm after incubation with MTT. Viability was compared to untreated control cells.

Data Analysis:
Data were analyzed using GraphPad Prism software. Statistical comparisons were made using One-Way and Two-Way ANOVA followed by Dunnett's multiple comparisons test and T-tests.

This experimental approach evaluates the effects of volatile compounds from dietary sources on the aggregation of HEWL and tau proteins, assessing their potential impact on amyloid fibrillation, structural changes, and neurotoxicity.

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