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Synergistic Neuroprotective Drug Combinations Targeting Amyloid-Beta Toxicity in *Caenorhabditis elegans*: A Multitarget Approach for Alzheimer's Disease

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Abstract

A progressive neurodegenerative disease, Alzheimer's disease (AD) is characterized by a multifactorial pathology that includes neuronal loss, oxidative stress, amyloid-beta (Aβ) accumulation, and mitochondrial dysfunction. Since currently available drugs like donepezil simply reduce symptoms, there is an urgent need for more potent, disease-modifying therapy. Using *Caenorhabditis elegans* (*C. elegans*) models of AD, we examined the treatment effectiveness of three novel antioxidant-based medication combinations: N-acetylcysteine + Curcumin (NAC+CUR), Mitoquinone + Butylated Hydroxyanisole (MitoQ+BHA), and Carnosine + Kynurenic Acid (CAR+KYNA). Phenotypic investigation was conducted using the wild-type N2 and transgenic CL4176 strains, the latter of which expresses human Aβ1–42 in muscle tissue. The Chou–Talalay approach was used for evaluating drug interactions in order to identify synergistic combinations. Lifespan, healthspan, Aβ-induced paralysis, stress tolerance, chemotaxis index, and fluorescence microscopy-measured amyloid burden were important outcomes. These results show that, in comparing with untreated and donepezil-M treated controls, all three drug combinations show synergistic interactions and significantly enhance physiological outcomes.

Keywords: Alzheimer's disease, Amyloid-beta, *C. elegans*, Combination therapy, Neuroprotection

Introduction

Over 55 million people worldwide suffer with Alzheimer's disease (AD), a chronic, progressive neurological illness that is the primary cause of dementia (28). Clinically, AD presents as behavioral abnormalities, cognitive dysfunction, and progressive memory loss. Extracellular amyloid-beta (Aβ) plaque buildup, intracellular neurofibrillary tangles made of hyperphosphorylated tau, extensive synaptic loss, oxidative damage, mitochondrial dysfunction, and persistent neuroinflammation are the neuropathological hallmarks of the condition (20, 22-23).

Current pharmacological treatments, such as cholinesterase inhibitors like donepezil and NMDA receptor antagonists like memantine, provide only symptomatic relief without stopping or correcting the underlying neurodegeneration, despite decades of dedicated research (29,11). The intricacy of AD pathophysiology, which includes several interrelated molecular cascades such oxidative stress, impaired proteostasis, mitochondrial dysfunction, calcium dyshomeostasis, and excitotoxicity, is highlighted by the limited effectiveness of monotherapies (5,11). As a result, there is a growing

understanding that effective disease-modifying therapies require a multitargeted therapeutic approach (15,11).

Combination therapy, in which two or more pharmacologically active drugs are provided concurrently to target various pathogenic pathways, has emerged as a viable option for complicated multifactorial disorders such as Alzheimer's disease (21). The efficacy and interaction dynamics of such drug combinations must be evaluated using accurate quantitative frameworks. The Chou-Talalay method, based on the median-effect principle, is well-known for its capacity to evaluate synergistic, additive, or antagonistic drug interactions through the calculation of a combination index (CI). CI < 1 indicates synergy, CI = 1 indicates an additive effect, and CI > 1 indicates antagonism (9-10).

In order to examine the therapeutic effectiveness of multitarget drug combinations for AD, we used the nematode *Caenorhabditis elegans* as a genetically feasible and physiologically relevant in vivo model. *C. elegans* has a short lifespan, a transparent body for live imaging, conserved stress-response and proteostasis pathways, and well-characterized transgenic strains (17,1). We employed two strains: the wild-type N2 for baseline assessments, and the transgenic CL4176, which expresses human $A\beta_{1-42}$ in body wall muscle cells upon temperature induction, leading to progressive paralysis—a phenotype that recapitulates key aspects of Aβ-induced proteotoxicity (18).

Based on their complementing redox-modulatory and neuroprotective actions, three novel pharmacological combinations - carnosine + kynurenic acid (CAR+KYNA), mitoquinone + butylated hydroxyanisole (MitoQ+BHA), and N-acetylcysteine + curcumin (NAC+CUR) were evaluated. In earlier research, each of these substances showed anti-inflammatory, antioxidant, mitochondrial-targeting, and anti-excitotoxic qualities (3,24,26,2). These combinations effectiveness was compared to that of the therapeutically prescribed cholinesterase inhibitor Donepezil-M.

A wide range of physiological and behavioral tests were used, including as chemotaxis for neurobehavioral evaluation, paralysis assay, oxidative and thermal stress tolerance, lifespan and healthspan analysis, and fluorescence-based Aβ aggregate detection. Chou–Talalay was used to quantitatively evaluate drug synergism (12). Finding synergistic neuroprotective combinations with possible translational significance is made possible by this integrative approach. These findings demonstrate the significance of *C. elegans* as a rapid and inexpensive model for preclinical drug discovery and seek to support the logical development of multitargeted treatment regimens for AD.

Materials and Methods

Synergism analysis

Drug synergism was evaluated using the Chou–Talalay method with CompuSyn software, as previously described. Briefly, dose–response data obtained from individual drugs and their combinations were analyzed to calculate the Combination Index (CI). CI values were interpreted as follows: CI < 1 indicates synergism, CI = 1 indicates an additive effect and CI > 1 indicates antagonism. Representative CI values from our previously reported synergism analysis were used to support the selection of drug combinations for subsequent evaluation in the *C. elegans* Alzheimer's disease model (12).

Combination drug preparation

N Acetylcysteine + Curcumin (2:1)

NAC- Water (100 mg /ml)

Curcumin – EtOH (10 mg/ml)

NAC	Curcumin	Total (2:1) uM
2	1	3
20	10	30
30	15	45
40	20	60

Butylated hydroxyanisole + Mito Q (5:0.5)

BHA- EtOH (50 mg/ml)

Mito Q – DMSO (30 mg/ml)

BHA	Mito Q	Total (5:0.5) uM
25	2.5	27.5
30	3	33
35	3.5	38.5
40	4	44

Carnosine + Kynurenic acid (1:1)

Carnosine – Water (20 mg/ml)

Kynurenic acid – 1M NaOH (50 mg/ml)

Carnosine	Kynurenic acid	Total (1:1) uM
5	5	10
10	10	20
15	15	30
20	20	40

Donepezil + Memantine (Market based drug for Comparison)

Donep-M – Water (10 mg/ml)

Donepezil	Memantine	Total (1:1) uM
5	5	10
10	10	20
15	15	30
20	20	40

Figure 1. Drugs from Sigma Aldrich (Merk), Market based drug Donep M

Source of C. elegans

The P.D. Patel Institute of Applied Sciences at Charotar University of Science and Technology (CHARUSAT Campus, Nadiad Petlad Highway, Anand) provided the N2 wild type worms. The CL4176 mutant worms were provided by the Central Drug Research Institute (Division of Neuroscience & Ageing Biology), Lucknow.

C. elegans strains and cultivation

N2 Bristol (wild-type) and CL4176 (dvIs27 [pAF29 (myo-3/Ab 1-42/let UTR) + pRF4 (rol-6(su1006)]) *C. elegans* strains were employed in the present study. On nematode growth medium (NGM) agar plates seeded with the Escherichia coli strain OP50 (4), all strains were cultivated and kept at 16 °C. By administering the drugs with NGM agar at varying concentrations, the effects of several combination drugs were investigated (25). After autoclaving and before pouring, combination drugs were added to NGM to create the drug-containing plates.

Preparation of Nematode growth medium [100 ml]

NaCl	0.3 Gram
Peptone	0.25 Gram
Agar	2 Gram
$MgSO_4$ (1 M)	100 µl
$CaCl_2$ (1 M)	100 µl
Cholesterol (5 mg/ml)	100 µl
KPO_4 buffer (Combination of KH_2PO_4 and K_2HPO_4) pH 6 (1.5 M)	1.625 ml

In a 100 ml conical flask, combine 0.2 g peptone, 2 g agar, and 0.3 g NaCl. Add Mili-Q water (97.5 ml). Wrap aluminum foil around the flask's mouth. 50 minutes of autoclaving.For 15 minutes, cool the flask in a water bath at 55°C.Add 1.625 ml of 1.5 M KPO4 buffer, 100µl of 1 M MgSO4, 100µl of 5 mg/ml cholesterol in ethanol, and 100µl of 1 M CaCl2. Mix thoroughly by swirling.Dispense the NGM solution into petri dishes using sterile techniques. Plates should be 2/3 full of agar.To identify impurities and allow excess moisture to evaporate, leave plates at room temperature for two to three days before to use (4).

Preparation of bacterial food source

E. coli strain OP50 use as a food source (Brenner, 1974). *E. coli* OP50 is a uracil auxotroph whose growth is limited on NGM plates.

Preparation of LB Broth for growth of OP50

Its primary function was the mainte-

nance and culture of recombination strains *(E. coli OP50*). distilled water with 20 grams of complete makeup. For 15 minutes, autoclave at 15 lbs (121^0 C) to sterilize. Add 50 microliters of *E. Coli OP50* to 5 milliliters of Luria broth. The Luria Broth should be kept in the incubator overnight at 37°C. NGM plate seeding can then proceed with the E. Coli OP50 solution (4).

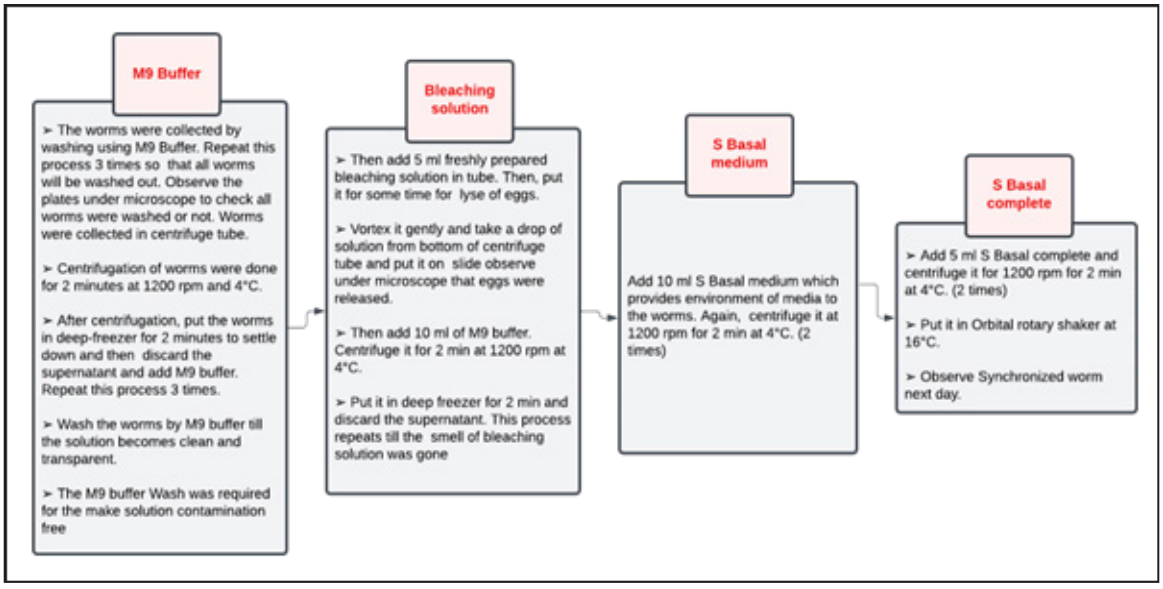
Sr. No.	Ingredients	Grams/litre
i	Tryptone	10
ii	Yeast Extract	5
iii	Sodium chloride	5
iv	Final PH (at 25°C)	7

Synchronization of worms

All worms are arrested in one stage of their life cycle during synchronization. This procedure is crucial for each assay or experimental observation. There were two synchronization techniques. 1. Bleaching 2. Laying eggs. Worms were synchronized through bleaching. The dissolution of their outer cuticle and the release of eggs from their guts caused by a bleaching solution killed hermaphrodites.

Lifespan assay

After being moved to new drug-containing NGM plates that also included 150 uM fluorodeoxyuridine (FUDR), the synchronized



L4 worms were seeded with E. coli OP50. The FUDR was introduced to keep the worm population synchronized since it prevents reproduction. Each experiment was carried out three times. Five alternate days were used to count the dead worms. When the worm did not react to repeated contact, it was marked as dead. The experiment came to an end when every worm was determined to be dead (25).

Health span assays

The physiological effects of monitoring pharyngeal pumping and body bending in drug-treated CL4176 worms and N2 Wild type worms over a duration of 1 minute and 20 seconds were conducted since Aβ accumulation can lead to muscle degeneration (7).

Pharyngeal pumping rate

The feeding behavior of worms treated with varying drugs concentrations and combinations is determined by the pharyngeal pumping rate. It was calculated by counting the pharyngeal rhythmic contractions over a one-minute period while keeping an eye on a single worm. Per minute, the mean pharyngeal pumping rate

was stated.

Body bending rate

The experiment was conducted using synchronized L4 worms. The ability of worms to move is connected to their body bending. A change in the reciprocating action of bending at the mid-body was called a body bend. N2 wild type, CL4176 control (untreated), and treated worms were used in the assay.

Stress tolerance assays

For the stress tolerance assay, the N2 wild-type and CL4176 worms were exposed to oxidative stress and changes in temperature. Age-synchronized young adult worms were moved to control and drug-containing NGM plates for both tests. After being cultivated until the fourth day (post-adulthood), they were exposed to oxidative stress and temperature changes. Worms, both treated and untreated, were subjected to a 12-hour temperature shift from 20 to 32 °C. Up to twenty-five hours, the dead worms were counted at regular intervals of five hours.

The worms were placed in a 10 mM hydrogen peroxide solution for two hours after being washed three or four times with M9 buffer to test for oxidative damage. The worms were moved to new NGM plates and incubated for 16 hours at 20 °C to recover from oxidative stress. Live animals were scored for both treated and untreated conditions after 16 hours (7).

Paralysis assay

` The paralysis experiment, which uses heat-inductive transgene of human Aβ1-42 in muscle cells, was performed using CL4176 transgenic worms. N2 wild is utilized as a control as well.In order to measure the expression of amyloid-beta (Aβ), which aggregates in muscle cells and causes paralysis, the synchronized L4 worms were moved to drug-containing and untreated plates and incubated at 16 °C (permissive temperature) for 48 hours before being moved to 25 °C (non-permissive temperature) for 20 hours.Following a 20-hour incubation period at 25°C, the number of paralyzed worms was counted every two hours until all of the worms had become paralyzed. Worms were considered paralyzed if they did not react to mechanical stimulation (13).

Gustatory plasticity assay

To accomplish pre-exposure to NaCl, the techniques outlined by (Hukema et al., 2006) were used. The well-fed young adult worms were gathered into 1.5 mL tubes and given three rounds of washing in wash buffer after being cultivated on either combination drug (+) or combination drug (−) plates. The worms were then pre-exposed to wash buffer containing 100 mM NaCl (conditioned) or without (mock-conditioned). Without food, 1.5 mL tubes were left at 20 °C for 15 minutes. It should be noted that drug was not added to the buffer during this conditioning phase. Following pre-exposure, worms were collected by centrifugation at around 450 × g for 60 s to remove NaCl from body surfaces, and they were then washed once with wash buffer devoid of NaCl.

Chemotaxis tests toward NaCl were used to assess gustatory plasticity. Tissue culture dishes (9 cm in diameter) with 5 mM KH-2PO4 (pH 6.0), 1 mM CaCl2, 1 mM MgSO4, and 17 g/L agar were utilized in these chemotaxis assays. Seven microliters of a 100 mM NaCl solution were spotted on the assay plate surfaces three and eighteen hours before to the test's commencement in order to create a gradient of NaCl concentration. To anesthetize the nematodes, 1 µL aliquates of 0.5 M sodium azide were then spotted onto the same places just prior to the chemotaxis assay. A control area was identified with 1 µL of 0.5 M sodium azide.

The worms were then separated by gently touching them with a Kimwipe after around 30 conditioned or mock-conditioned worms were put at original locations that were 2.8 cm apart from NaCl and control areas. The wash buffer that remained at the original location was then absorbed. For ninety minutes, the worms were free to roam around on the assay plate. A dissecting microscope was used to count the number of worms at the NaCl and control loca-

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tions (a circle with a radius of 1 cm) every 10 minutes. Chemotaxis indices were then calculated using the following formula: chemotaxis index= (number of worms at the NaCl location− number of worms at the control location)/(total number of worms on the plate). Chemotaxis indices were employed in this study at 90 minutes since they rose during the first 60 minutes and then stabilized during the 60–90 minute interval. All measurements were made at least three times, and assays were carried out during the day in a room that was kept at about 20 °C (27).

In vivo amyloid β staining by thioflavin-T on C. elegans (CL4176)

CL4176 worms (a model of Alzheimer's disease) were used to analyze Aβ in vivo. Through thioflavin-t staining, the efficacy of combination drugs on harmful Aβ accumulation produced in CL4176 worms was evaluated. To get the highest number of bacteria-free (E. coli OP50) worms for the thioflavin t staining assay, the treated and untreated worms were washed in S-basal media prior to staining, following a methodology (Link et al., 2003). 0.25% thioflavin was employed for staining at room temperature in a dark environment for half an hour after washing with S-basal media. After that, worms exposed to thioflavin were destaining with 50% ethanol until all stains were removed from the solution. Lastly, the stained worms were examined using a fluorescent microscope (Nikon Eclipse Ti 2), and the intensity of the fluorescence produced by the staining of thioflavin t when it reacted with Aβ aggregation on the body wall of treated and untreated worms was determined using ImageJ software (7).

Statistical analysis

Every experiment was carried out in triplicate, and the mean ± standard error of the mean (SEM) was used to express the findings. For graphical data, Graphpad Prisam 10 software was utilized, and Ordinary one-way ANOVA was employed to obtain $P < 0.0001$, which indicates statistically significant data.

Results and Discussion

Synergism analysis

Representative Combination Index (CI) values obtained from the Chou–Talalay analysis confirmed synergistic interactions among the selected drug combinations. NAC + Curcumin exhibited a CI value of 0.96, MitoQ + BHA exhibited a CI value of 0.76 and Carnosine + Kynurenic acid exhibited CI values ranging from 0.70 to 0.99 at different effect levels. Since CI values below 1 indicate synergism, these results confirmed the synergistic nature of the selected combinations and supported their further evaluation against amyloid-beta toxicity in the *C. elegans* Alzheimer's disease model(Divyata Desai and Mishra Kundan Kumar 2025).

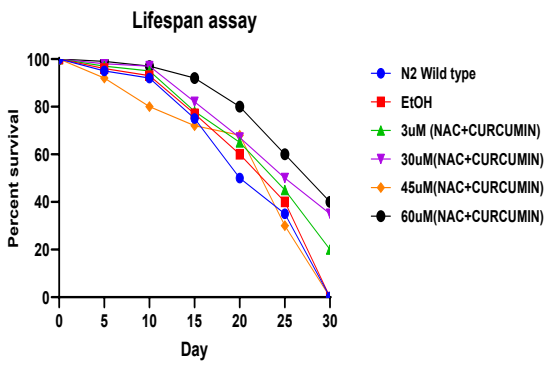
Drug Combination	Representative CI Value	Interaction
NAC + Curcumin	0.96	Synergistic
MitoQ + BHA	0.76	Synergistic
Carnosine + Kynurenic Acid	0.70	Synergistic
Donepezil + Memantine	0.81	Synergistic

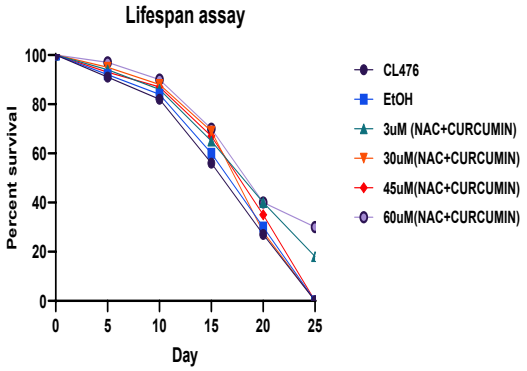
Lifespan assay

Both N2 wild-type and CL4176 transgenic *C. elegans* strains were used in a lifespan study to evaluate the effect of neuroprotective combination therapy on organismal longevity. Upon temperature stimulation, CL4176 mimics the proteotoxicity linked to Alzheimer's disease by expressing human amyloid-β_{1-42}.Four drugs combinations—N-acetylcysteine + Curcumin (NAC+CUR), Butylated Hydroxyanisole + Mitoquinone (BHA+MitQ), Carnosine + Kynurenic Acid (CAR+KYNA), and Donepezil + Memantine (D+M; clinical comparator)—were administered to worms in escalating quantities. Four increasing dosages of each drug were given, and survival was tracked on a regular basis. N2 Wild-Type Strain: When compared to untreated controls, all four combination therapies produced dose-dependent lifetime extensions ($p < 0.001$, one-way ANOVA). The strongest results were seen with BHA+MitQ and NAC+CUR: The

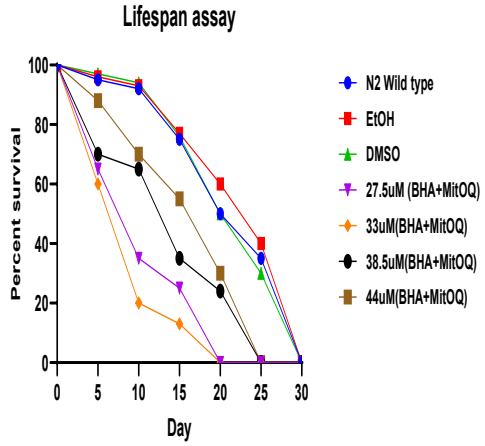
median lifespan of NAC+CUR (60 µM total) was almost 32% longer than that of controls. BHA+MitQ (44 µM total): ~28% increase in median longevity. CAR+KYNA (40 µM total): About 21% longer lifetime. Compared to untreated, D+M (40 µM total) showed a slight improvement (~19%). A concentration-dependent effect on longevity was confirmed by the graded effect of lower dosages of each combination. CL4176 Transgenic Strain: All combination therapy considerably increased survival in the CL4176 model, where Aβ expression often reduces lifespan, in contrast to the untreated Aβ-expressing controls: NAC+CUR (60 µM total): Significantly delayed early mortality and ~36% longer lifespan. BHA+MitQ: Increased median lifetime by around 30% (44 µM total). Lifespan increased by about 23% with CAR+KYNA (40 µM total), however it was marginally less robust than the other two combinations. D+M (40 µM total): Increased lifetime by about 25%. According to these results, the drug combinations not only prevent Aβ-induced toxicity but also decrease overall aging-related decline. Transgenic worms showed a stronger effect, suggesting their potential in the setting of neurodegenerative diseases.

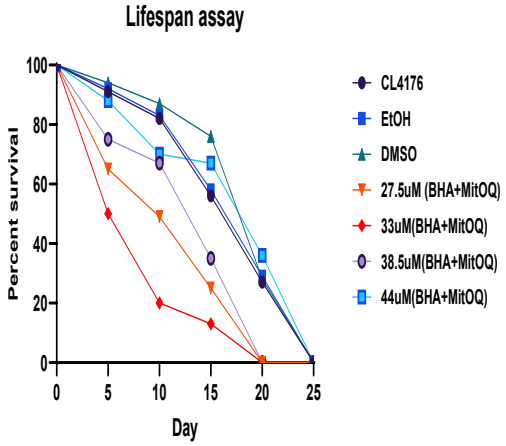
N- acetylcysteine + Curcumin



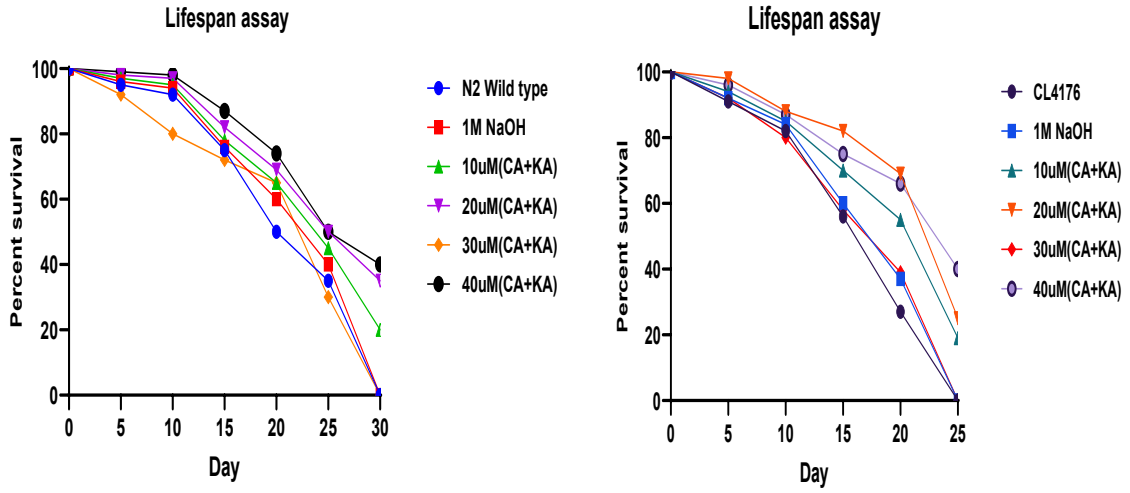


Butylated hydroxyanisole + Mitoquionone





Carnosine+ Kynurenic acid



Donepezil + Memantine

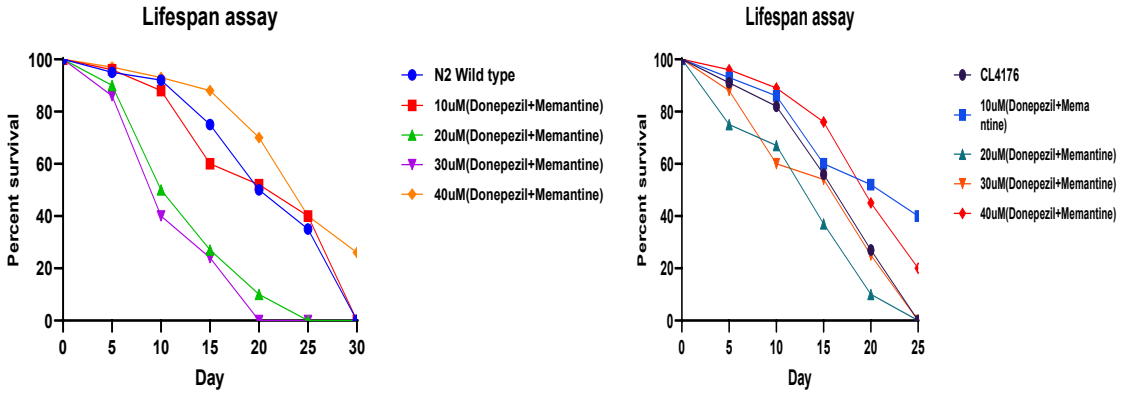


Figure 2. Lifespan analysis of *C. elegans* N2 wild-type and CL4176 transgenic strains following treatment with various combination drugs. (A) Survival curves of N2 wild-type worms treated with increasing concentrations of NAC+Curcumin, BHA+MitQ, CA+KA, and Donepezil+Memantine. (B) Survival curves of CL4176 worms treated under the same drug regimens. Control groups included EtOH, DMSO, and 1M NaOH as appropriate. Treatments were administered at multiple concentrations, and survival was monitored daily. Each combination exhibited a dose-dependent effect on lifespan, with specific concentrations showing significant extension or reduction in survival compared to controls.

Health span Assay

To assess the neuromuscular function and general healthspan of *C. elegans* following treatment with neuroprotective drug combinations, two behavioral parameters were analyzed: pharyngeal pumping rate (feeding activity) and body bending rate (locomotor function). Both N2 wild-type and CL4176 transgenic worms were used in these assays.

Pharyngeal Pumping Rate

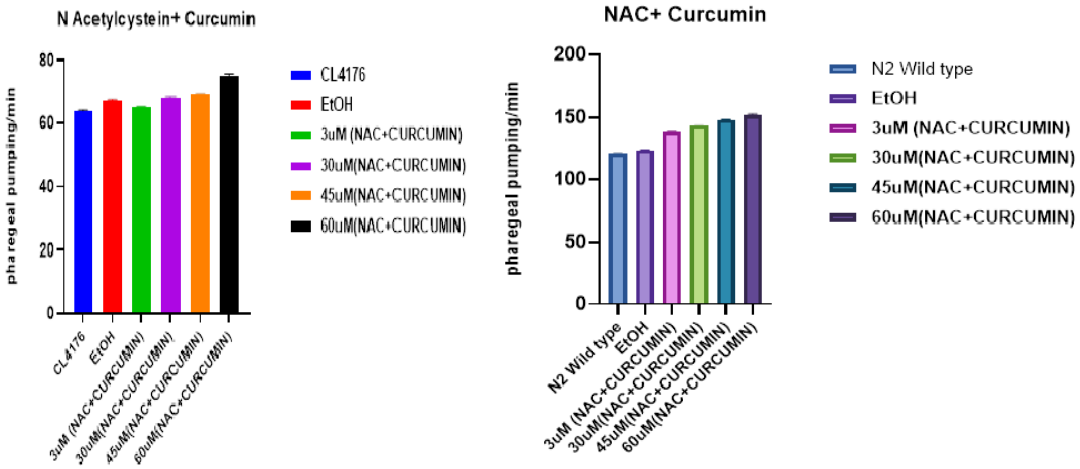
Treatment with all four drug combinations led to a measurable increase in the pharyngeal pumping rate compared to untreated worms. This suggests a preservation of neuromuscular integrity and feeding efficiency. Treatment with all four drug combinations led to a measurable increase in the pharyngeal pumping rate compared to untreated worms. This suggests a preservation of neuromuscular integrity and feeding efficiency. NAC+Curcumin significantly increased the pumping rate in CL4176 worms from 64 ± 0.26 to 75 ± 0.54, and in N2 worms from 120 ± 0.56 to 152 ± 0.67 Pump / min. BHA+MitQ treatment also enhanced the pumping rate (CL4176: 64 ± 0.26 to 67 ± 0.69, N2: 120 ± 0.56 to 127 ± 0.42). CAR+KYNA showed moderate improvement (CL4176: 64 ± 0.26 to 69 ± 0.64, N2: 120 ± 0.56 to 127 ± 0.48). Donepezil+Memantine increased the rate from 59 ± 0.38 to 69 ± 0.25 in CL4176, and from 120 ± 0.56 to 125 ± 0.87 in N2 worms.

Body Bending Rate

Body bending rate, an indicator of locomotor activity and neuromuscular coordination, was also positively influenced by all drug treatments in both worm strains. NAC+Curcumin showed the strongest effect, increasing bending in CL4176 from 20 ± 0.67 to 26 ± 0.48, and in N2 from 34 ± 0.64 to 38 ± 0.40 bends/min. BHA+MitQ enhanced movement (CL4176: 20 ± 0.67 to 24 ± 0.67, N2: 34 ± 0.64 to 36 ± 0.96). CAR+KYNA led to a moderate increase (CL4176: 20 ± 0.67 to 23 ± 0.64, N2: 34 ± 0.64 to 36 ± 0.84). Donepezil+Memantine increased rates to 24 ± 0.58 in CL4176 and 38 ± 0.87 in N2.

Collectively, these data demonstrate that the tested combination therapies not only mitigate neurodegeneration-associated pathology but also promote physiological functionality in C. elegans. NAC+Curcumin consistently emerged as the most effective combination, supporting its potential utility in preserving neuromuscular health in Alzheimer's disease contexts.

Pharyngeal Pumping Rate



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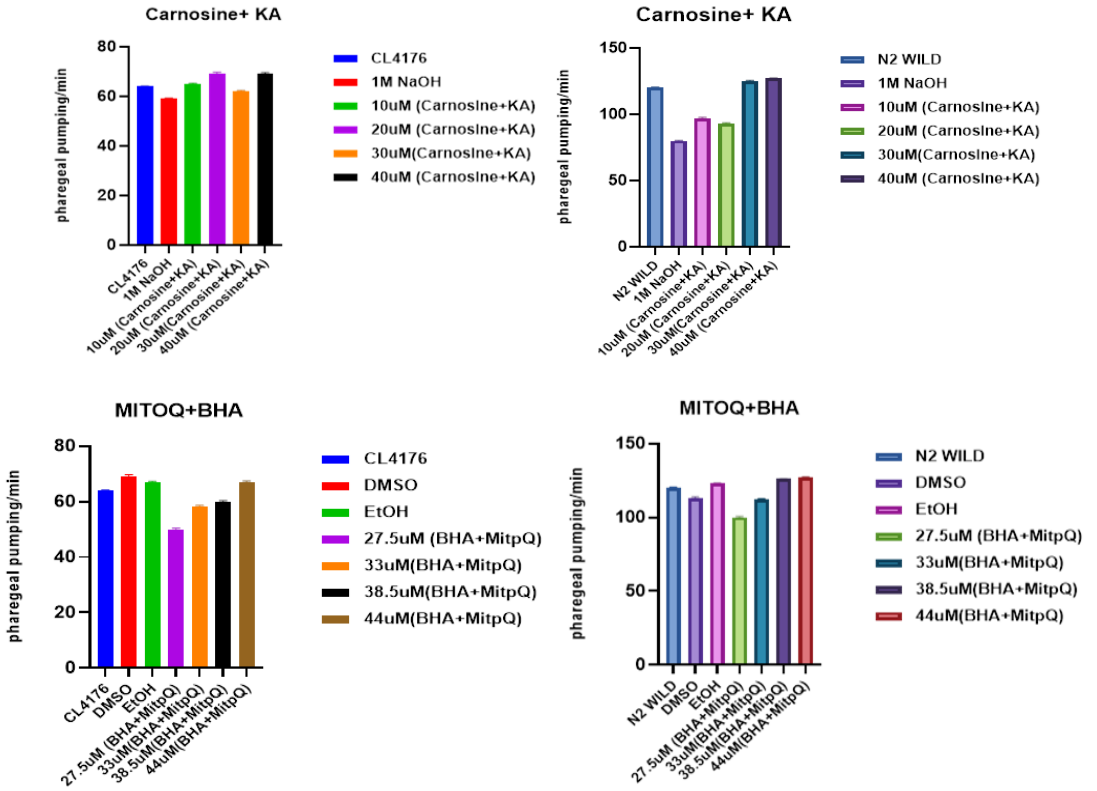
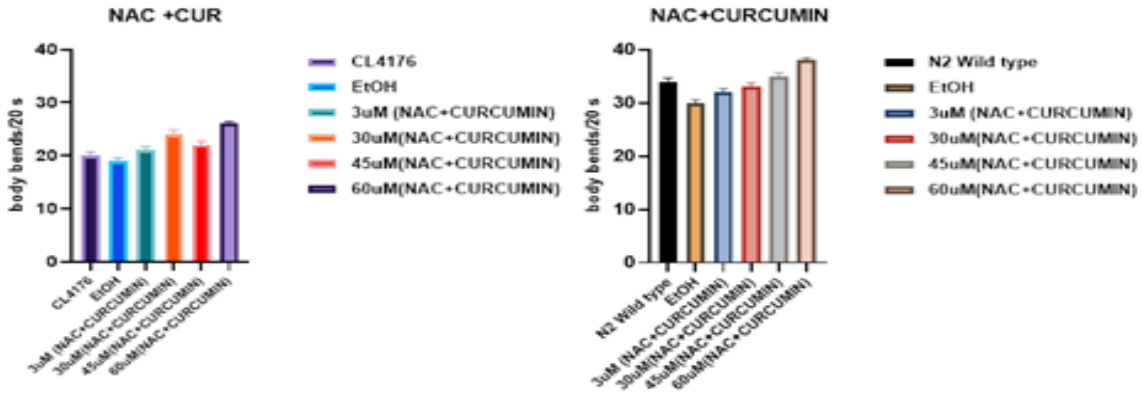


Figure 3. Effect of combination drug treatments on pharyngeal pumping rate in *C. elegans.* The pharyngeal pumping rate was quantified as an indicator of feeding activity and neuromuscular function in N2 wild-type and CL4176 transgenic worms after treatment with NAC+Curcumin, BHA+Mitoquinone, Carnosine+Kynurenic Acid, and Donepezil+Memantine. All drug combinations showed an increase in pumping rate relative to untreated controls, with the most significant enhancement observed in the NAC+Curcumin group. Data represent the mean ± SEM (n = 3)

Body bending Rate



Synergistic neuroprotective drug combinations targeting amyloid-beta toxicity in *Caenorhabditis elegans:* a multitarget approach for alzheimer's disease

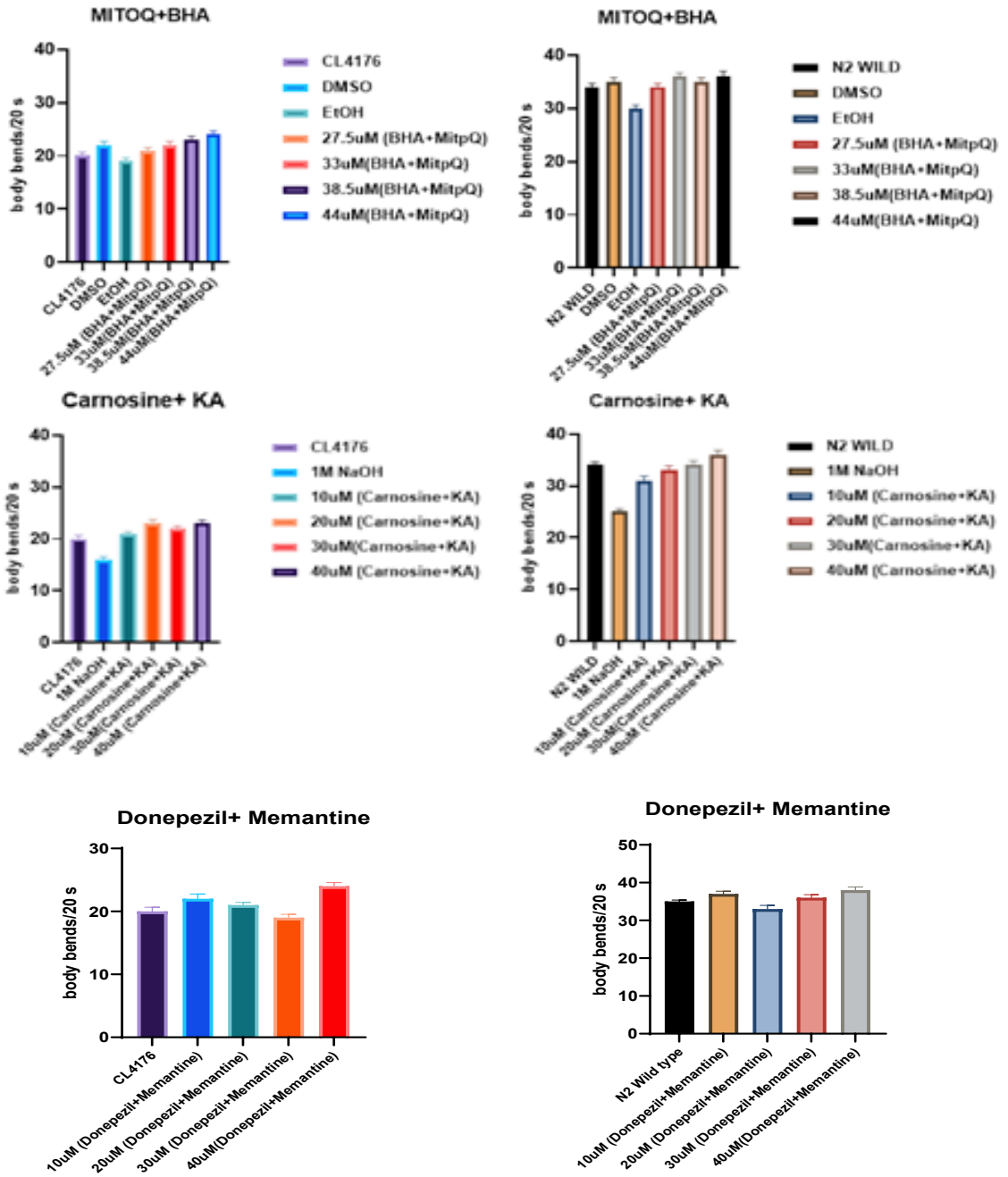
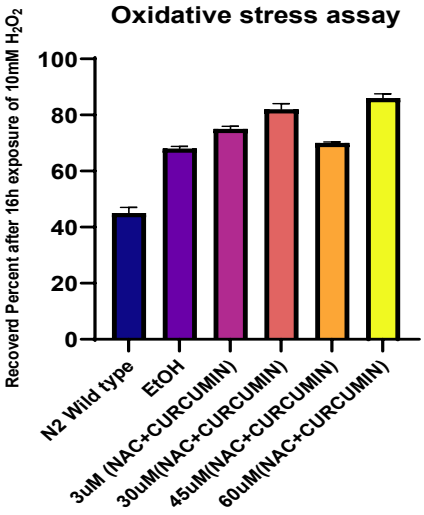


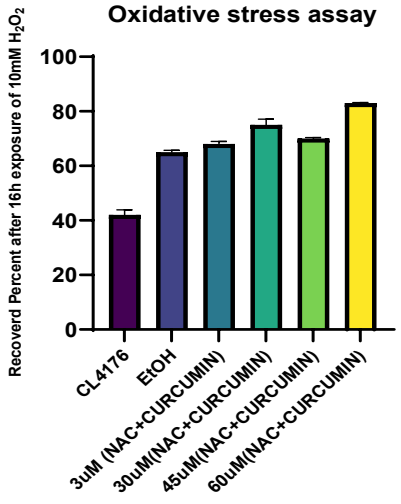
Figure 4. Effect of combination drug treatments on body bending rate in *C. elegans.* Body bending frequency was measured as a proxy for locomotor activity and neuromuscular health. Both N2 and CL4176 worms exhibited increased bending rates after treatment with all four drug combinations, with the greatest improvements again noted for NAC+Curcumin. Results are expressed as mean ± SEM (n = 3).

Oxidative stress Assay

To evaluate the neuroprotective potential of the drug combinations under oxidative stress conditions, both N2 wild-type and CL4176 transgenic *C. elegans* were pre-treated with four different combination therapies and then exposed to 10 mM hydrogen peroxide (H_2O_2) for 16 hours. Post-exposure recovery rates were recorded to assess survival under acute oxidative damage.The results demonstrated that all four drug combinations improved oxidative stress resistance compared to untreated controls in a dose-dependent manner, with notable differences in efficacy: NAC + Curcumin emerged as the most effective treatment, showing the highest survival rate at 60 µM in both N2 and CL4176 strains. This is likely due to NAC's ability to replenish intracellular glutathione and Curcumin's potent antioxidant and anti-inflammatory properties, which together enhance redox homeostasis. BHA + Mitoquinone also conferred robust protection, particularly at 44 µM. Mitoquinone, a mitochondria-targeted antioxidant, combined with the lipid-peroxidation-inhibiting properties of BHA, appears to strongly support mitochondrial integrity and oxidative defense mechanisms. Carnosine + Kynurenic Acid showed moderate protective effects. Both compounds are known for their neuroprotective and anti-glycation actions, and their combined efficacy in CL4176 worms suggests specific benefit in mitigating amyloid-β-induced oxidative burden. Donepezil + Memantine, though primarily designed to modulate cholinergic and glutamatergic neurotransmission, also improved oxidative stress resilience, especially in CL4176 worms. This could indicate a secondary antioxidant or neuroprotective role under AD-mimicking stress conditions.Importantly, CL4176 worms—prone to Aβ-induced oxidative damage—showed more pronounced benefit from combination therapy, underscoring the potential of these treatments to target amyloid-related oxidative pathology. The statistically significant survival improvements ($p < 0.05$ to $p < 0.001$) validate the therapeutic relevance of these combinations. Together, these findings highlight that targeting oxidative stress using multi-compound strategies can meaningfully enhance cellular resilience and may be critical in delaying or mitigating Alzheimer's disease progression.

N- acetylcysteine + Curcumin





Synergistic neuroprotective drug combinations targeting amyloid-beta toxicity in *Caenorhabditis elegans:* a multitarget approach for alzheimer's disease

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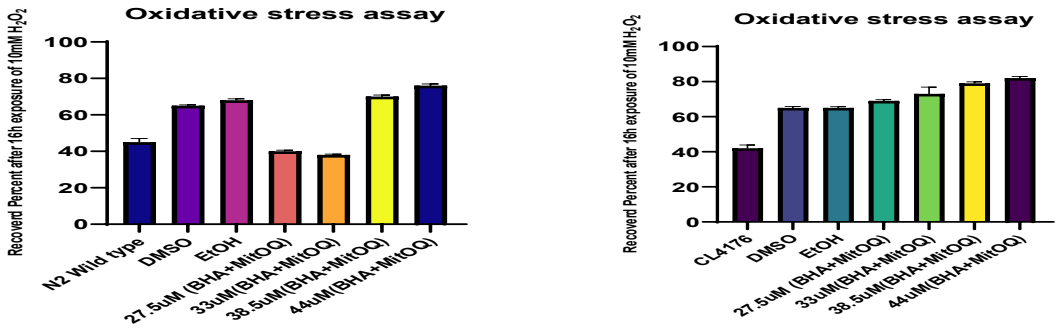
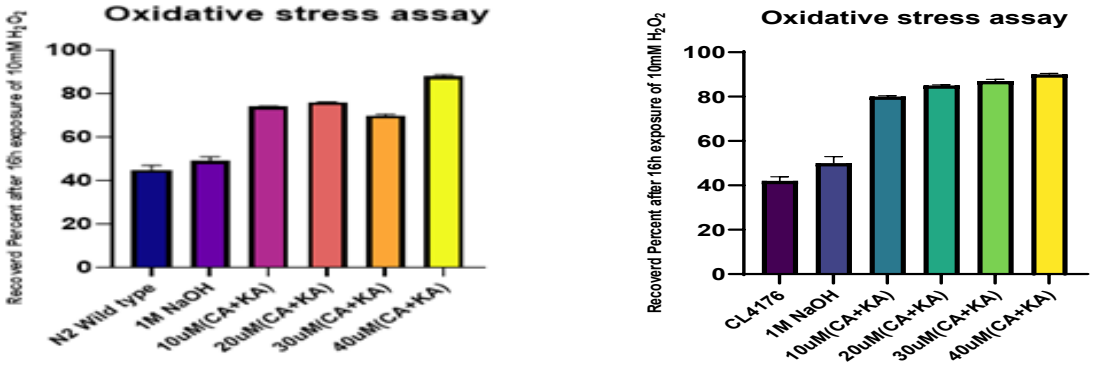
Butylated hydroxyanisole+ Mitoquionone



Carnosine+ Kynurenic acid



Donepezil + Memantine

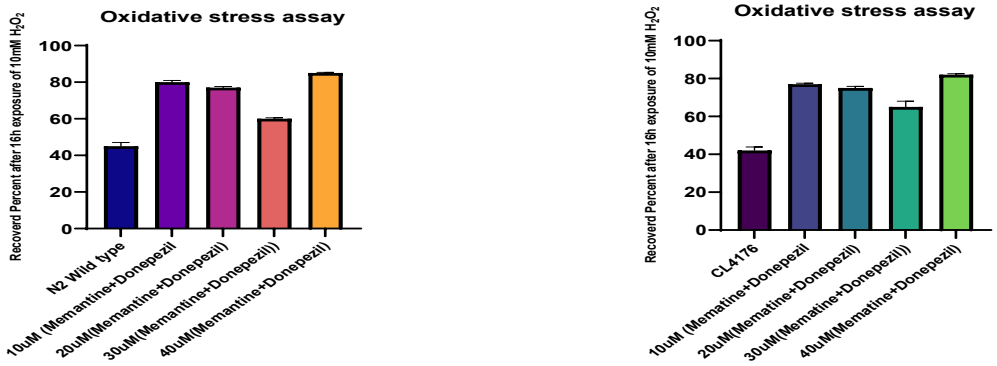


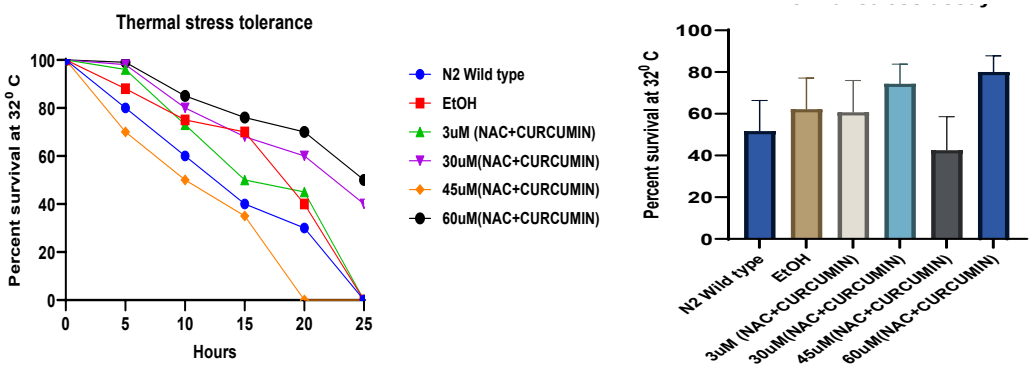
Figure 5. Combination drug treatments enhance oxidative stress resistance in C. elegans N2 wild-type and CL4176 strains. Worms were pre-treated with different concentrations of four drug combinations—N-acetylcysteine (NAC) + Curcumin, Butylated hydroxyanisole (BHA) + Mitoquinone (MitQ), Carnosine (CA) + Kynurenic acid (KA), and Donepezil + Memantine—followed by exposure to 10 mM hydrogen peroxide (H_2O_2) for 16 hours to induce oxidative stress. Post-exposure survival (recovery rate) was recorded for both N2 wild-type and CL4176 transgenic strains.

Across all treatments, drug combinations showed dose-dependent effects in enhancing oxidative stress resistance. Notably, NAC+Curcumin and BHA+MitQ significantly improved post-stress survival in both strains, with the highest recovery observed at 60 µM and 44 µM respectively. CA+KA and Donepezil+Memantine also conferred protective effects, particularly in CL4176, suggesting potential mitigation of amyloid-β-associated oxidative damage. Data represent the mean recovery percentages, with statistical significance evaluated using one-way ANOVA followed by post hoc analysis ($p < 0.05$ to $p < 0.001$), indicating meaningful enhancement of oxidative resilience through combination therapies.

Thermal stress assay

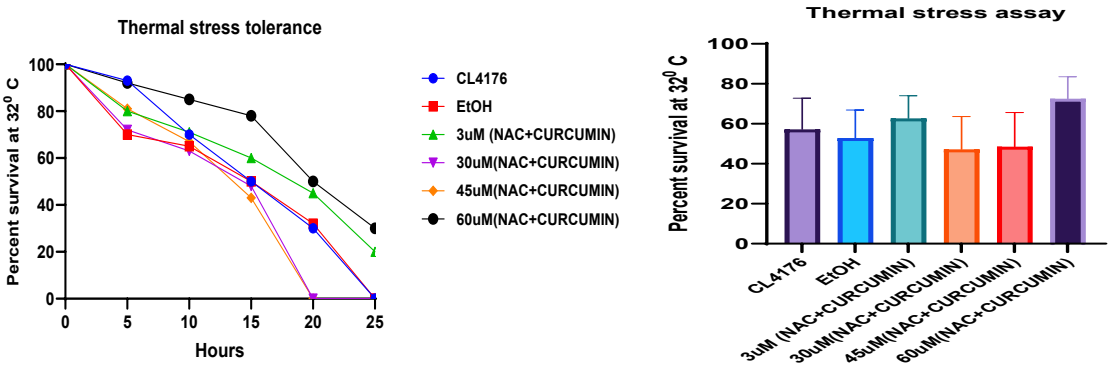
To determine whether antioxidant-based drug combinations can enhance resistance to heat-induced proteotoxicity, thermal stress assays were conducted on N2 wild-type and CL4176 transgenic C. elegans strains. Worms were treated with four neuroprotective drug combinations—NAC+Curcumin, BHA+MitQ, Carnosine+Kynurenic Acid, and Donepezil+Memantine—and then exposed to 32 °C for a defined period. Post-stress survival rates were calculated to assess thermotolerance. Across all treatment groups, a dose-dependent improvement in survival was observed compared to untreated controls: NAC + Curcumin treatment resulted in the highest survival rates in both strains. This combination was particularly effective in the CL4176 strain, likely due to NAC's glutathione-replenishing activity and Curcumin's antioxidant and anti-aggregative properties, which mitigate amyloid-induced stress. BHA + MitQ also significantly enhanced thermotolerance, with survival increasing at all tested concentrations. MitQ's mitochondrial targeting may play a key role in stabilizing cellular energy dynamics during thermal insult. Carnosine + Kynurenic Acid showed a moderate but consistent improvement in survival across both strains. Their known roles in anti-glycation and anti-excitotoxicity likely contributed to reduced protein misfolding and oxidative burden under stress. Donepezil + Memantine, though mechanistically different, conferred a notable increase in thermotolerance in CL4176 worms, indicating indirect protective effects possibly linked to reduced glutamate toxicity and improved synaptic stability under stress. CL4176 worms, which model Alzheimer's disease pathology, were significantly more sensitive to heat, yet they showed clear benefit from each drug combination—especially NAC+Curcumin and Donepezil+Memantine. These findings indicate the potential of such therapies in protecting against both environmental and pathological stress. Statistical analysis confirmed that survival differences were significant ($p < 0.05$ to $p < 0.001$), reinforcing the therapeutic relevance of multitarget antioxidant interventions for conditions involving thermal and proteotoxic stress.

N- acetylcysteine + Curcumin (N2 Wild type)

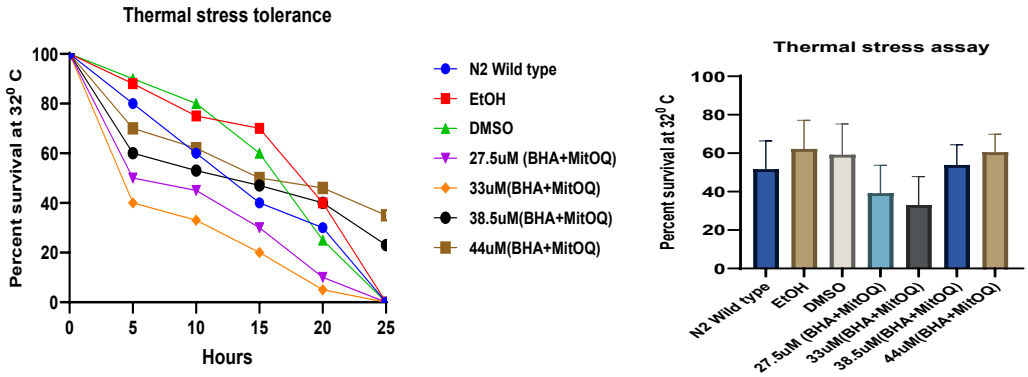


Synergistic neuroprotective drug combinations targeting amyloid-beta toxicity in *Caenorhabditis elegans:* a multitarget approach for alzheimer's disease

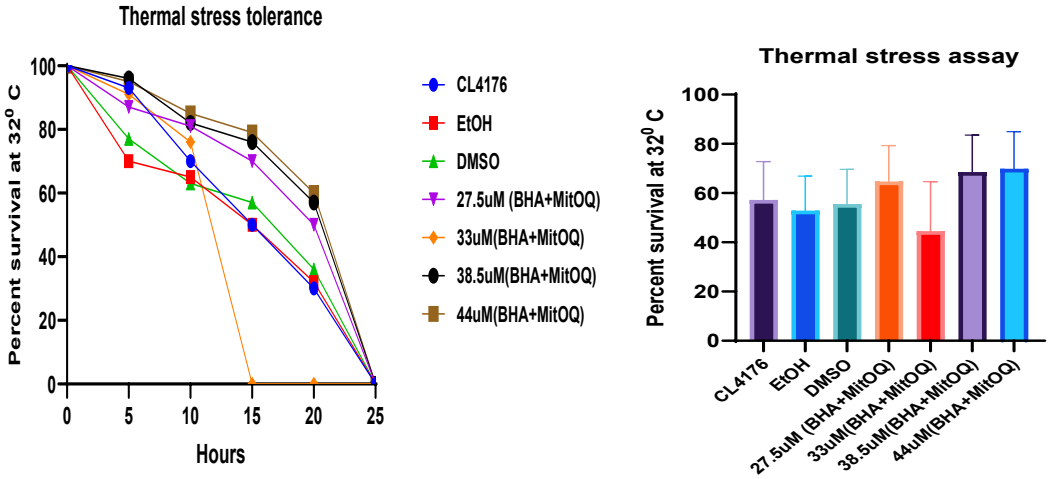
N- acetylcysteine + Curcumin (CL4176)

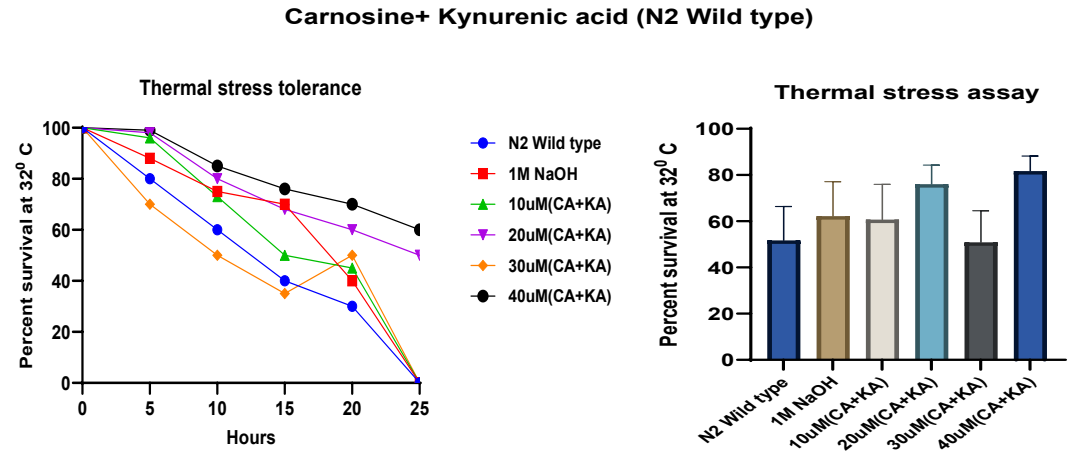


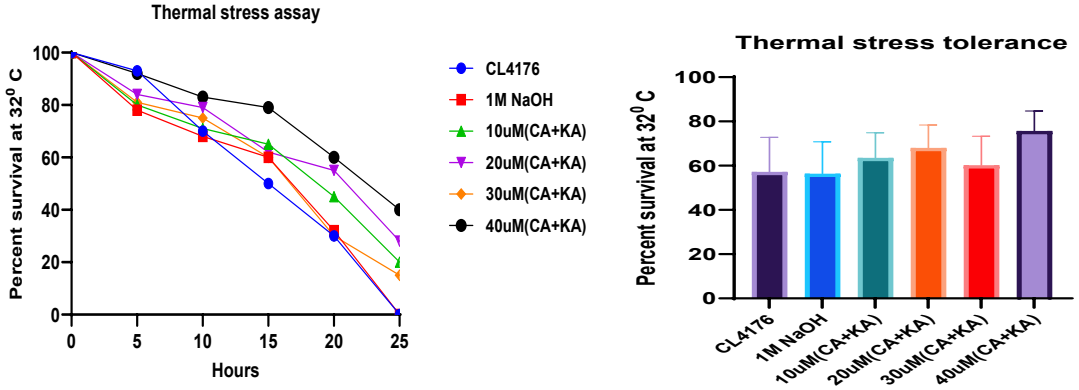
Butylated hydroxyanisole+ Mitoquionone (N2 Wild type)

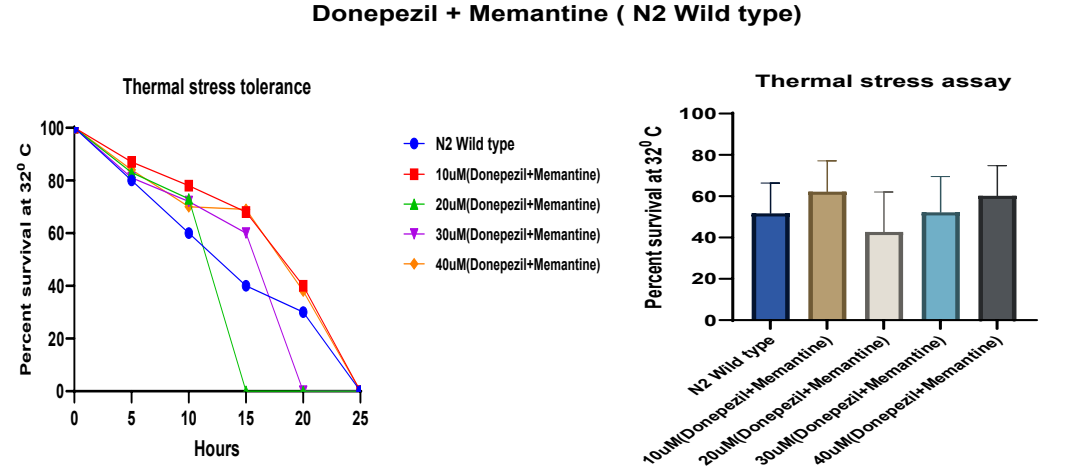


Butylated hydroxyanisole+ Mitoquionone (CL4176)




Carnosine+ Kynurenic acid (N2 Wild type)
Thermal stress tolerance
Percent survival at 32⁰ C
Hours
N2 Wild type
1M NaOH
10uM(CA+KA)
20uM(CA+KA)
30uM(CA+KA)
40uM(CA+KA)
Thermal stress assay
Percent survival at 32⁰ C
N2 Wild type
1M NaOH
10uM(CA+KA)
20uM(CA+KA)
30uM(CA+KA)
40uM(CA+KA)


Carnosine+ Kynurenic acid (CL4176)
Thermal stress assay
Percent survival at 32⁰ C
Hours
CL4176
1M NaOH
10uM(CA+KA)
20uM(CA+KA)
30uM(CA+KA)
40uM(CA+KA)
Thermal stress tolerance
Percent survival at 32⁰ C
CL4176
1M NaOH
10uM(CA+KA)
20uM(CA+KA)
30uM(CA+KA)
40uM(CA+KA)


Donepezil + Memantine (N2 Wild type)
Thermal stress tolerance
Percent survival at 32⁰ C
Hours
N2 Wild type
10uM(Donepezil+Memantine)
20uM(Donepezil+Memantine)
30uM(Donepezil+Memantine)
40uM(Donepezil+Memantine)
Thermal stress assay
Percent survival at 32⁰ C
N2 Wild type
10uM(Donepezil+Memantine)
20uM(Donepezil+Memantine)
30uM(Donepezil+Memantine)
40uM(Donepezil+Memantine)

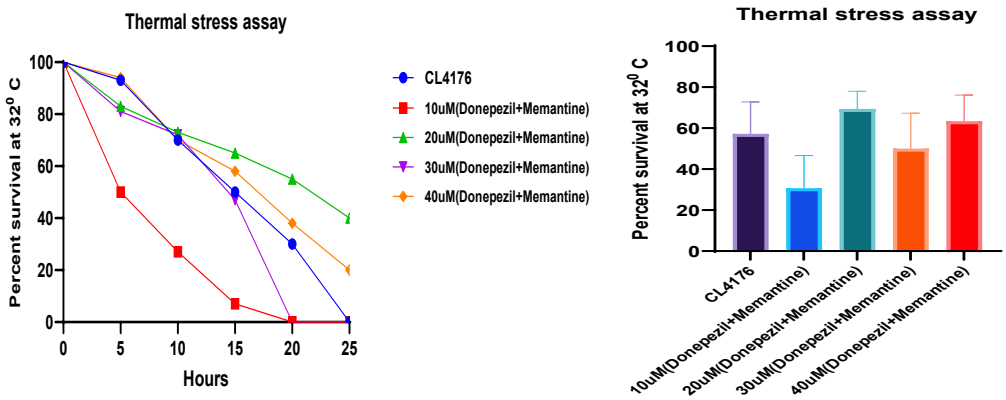


Figure 6. Effect of combination drug treatments on thermal stress resistance in *C. elegans N2* wild-type and CL4176 strains. Thermal stress assays were conducted to evaluate the protective efficacy of four neuroprotective and antioxidant drug combinations—N-acetylcysteine (NAC) + Curcumin, Butylated hydroxyanisole (BHA) + Mitoquinone (MitQ), Carnosine (CA) + Kynurenic acid (KA), and Donepezil + Memantine—on N2 wild-type and transgenic CL4176 worms.

Worms were pre-treated with varying concentrations of each drug combination and then exposed to elevated temperature (typically 32 °C) for a defined duration. Post-stress survival was recorded to assess thermotolerance. All four drug combinations demonstrated varying degrees of enhancement in thermal stress resistance, with NAC+Curcumin and BHA+MitQ showing the most pronounced protective effects in both strains. CL4176 worms, which express human amyloid-β and exhibit heat-sensitive neurodegenerative phenotypes, showed greater sensitivity to thermal stress but also responded positively to treatments—particularly with Donepezil+Memantine and CA+KA—indicating potential mitigation of Aβ-induced vulnerability. Data are expressed as percentage survival post-stress; statistical analysis using one-way ANOVA and post hoc comparisons confirmed significance of observed effects ($\mathbf{p} < 0.05$ to $\mathbf{p} < 0.001$).

Paralysis assay

The paralysis assay revealed that all four tested drug combinations significantly reduced amyloid-β-induced paralysis in the CL4176 strain compared to untreated controls. A one-way ANOVA test showed a statistically significant difference among the treatment groups ($p < 0.0001$), indicating that the observed effects were unlikely due to chance.Among the combinations, NAC+Curcumin exhibited the most pronounced protective effect, likely due to synergistic antioxidant and anti-inflammatory mechanisms. Donepezil+Memantine, a clinically used combination in Alzheimer's therapy, also showed strong efficacy, supporting its relevance in preserving neuronal function and delaying paralysis in the Aβ-expressing model. BHA+MitQ provided moderate protection, likely through mitochondrial antioxidant activity and free radical scavenging. The CA+KA group also demonstrated a significant reduction in paralysis, possibly due to modulation of excitotoxicity and support of mitochondrial homeostasis. These findings suggest that multi-targeted drug combinations can effectively counteract Aβ toxicity in vivo. The statistically robust outcome ($p < 0.0001$) underscores the therapeutic potential of these compounds for further preclinical development against Alzheimer's disease.

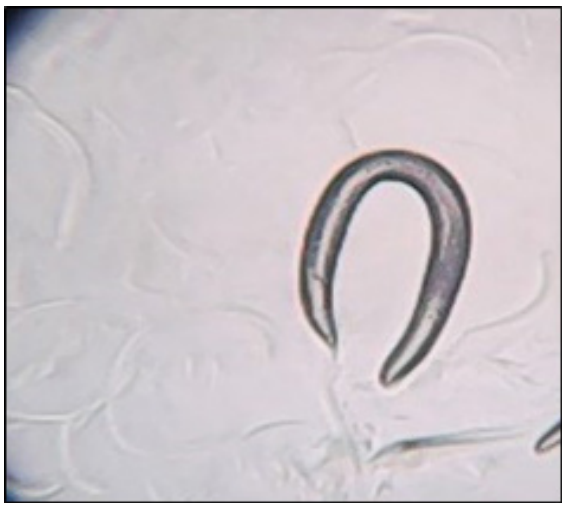
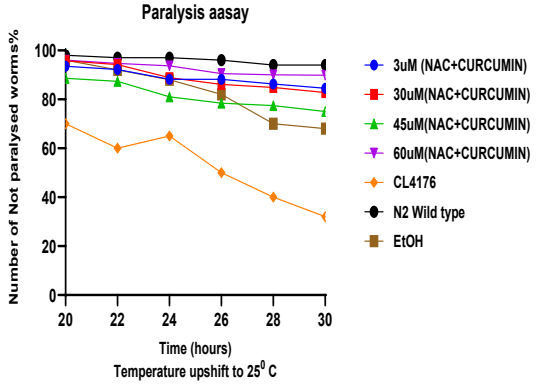
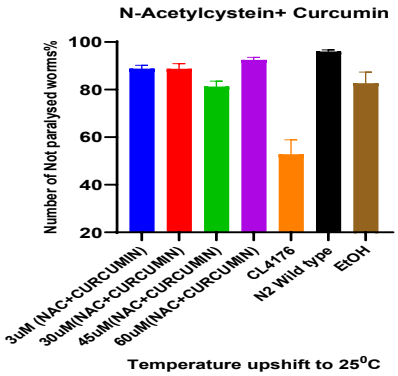


Figure 7 : Paralyzed worm CL4176

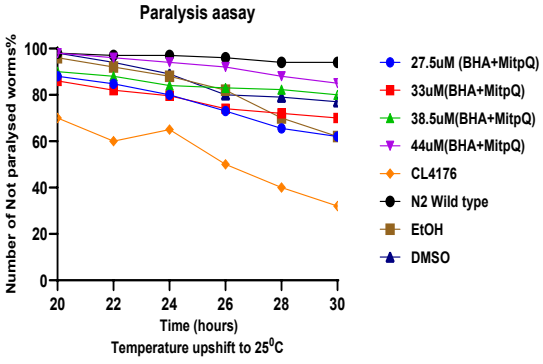
Figure 8: Non-Paralyzed worm CL4176

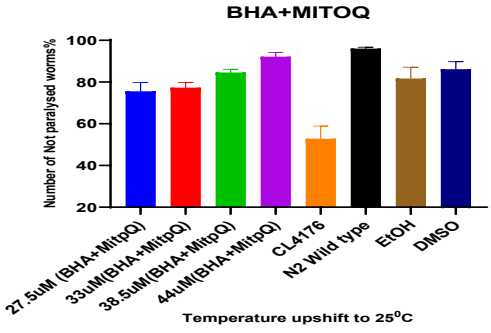
N- acetylcysteine + Curcumin





Butylated hydroxyanisole+ Mitoquionone





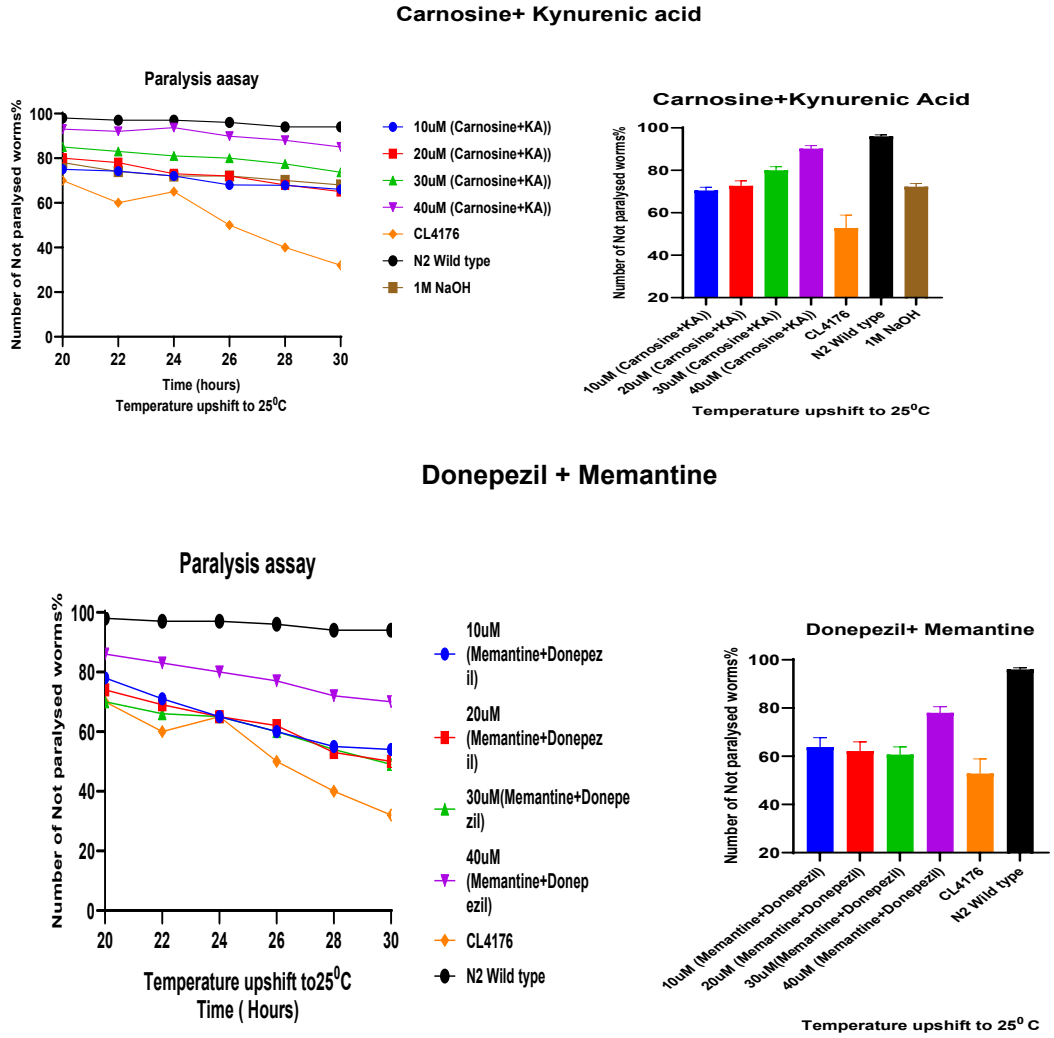


Figure 9. Effect of combination drug treatments on paralysis in the CL4176 *C. elegans* Alzheimer's disease model. The graph shows the percentage of paralyzed worms after treatment with N-acetylcysteine (NAC) + Curcumin, Butylated hydroxyanisole (BHA) + Mitoquinone (MitQ), Carnosine (CA) + Kynurenic Acid (KA), and Donepezil + Memantine. Drug treatments were administered prior to thermal induction of human Aβ1–42 expression. All combinations significantly reduced the rate and extent of paralysis compared to untreated controls. Statistical analysis using one-way ANOVA revealed a highly significant difference among treatment groups ($p < 0.0001$), confirming the efficacy of these neuroprotective combinations in attenuating amyloid-β-induced neuromuscular dysfunction.

Gustatory plasticity assay

Gustatory plasticity assays were performed to assess associative learning and cognitive behavior in the CL4176 *C. elegans* Alzheimer's disease model following treatment with various neuroprotective combination drugs. In this assay, the chemotaxis index (CI)—a quantitative measure of the worm's ability to associate

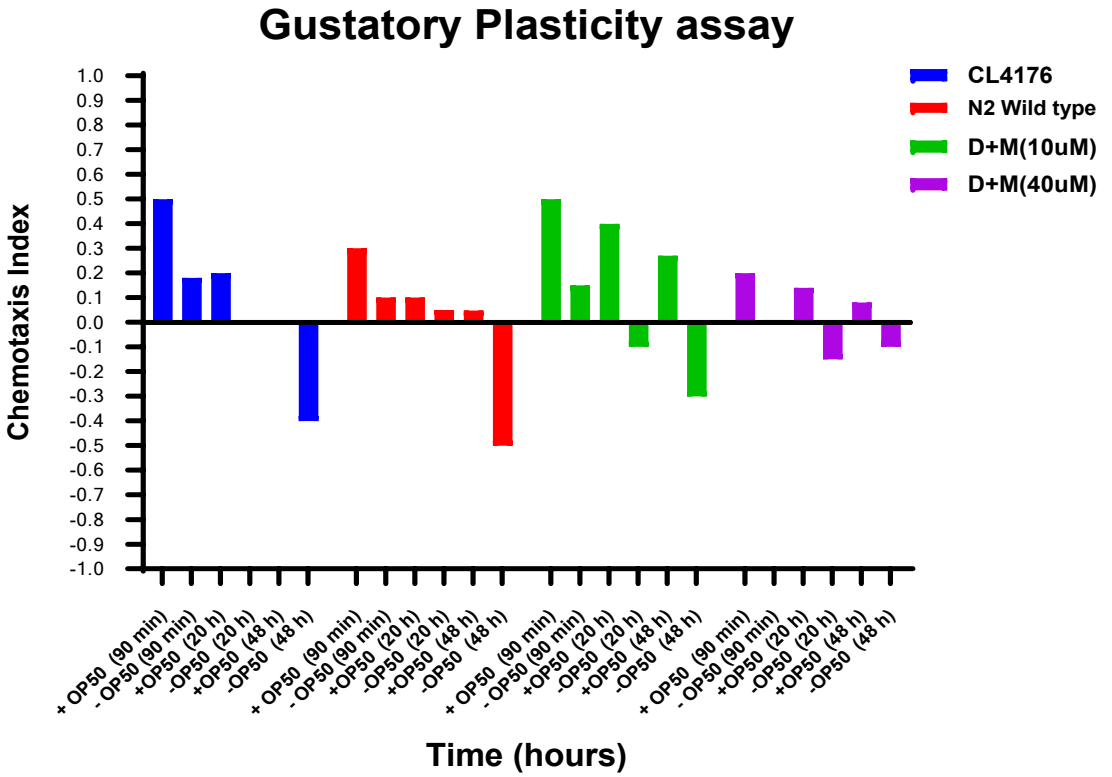
salt cues with food presence—was used to evaluate cognitive function. A CI value approaching 1.0 indicates normal or strong chemotactic behavior (intact neuronal function), whereas a value significantly less than 1.0 reflects impaired neuronal plasticity and cognitive decline due to amyloid-β (Aβ) expression.

Untreated CL4176 worms exhibited a markedly reduced chemotaxis index (CI < 0.3), consistent with Aβ-induced neurotoxicity and cognitive impairment. In contrast, all drug-treated groups demonstrated significantly improved chemotaxis indices.

The combination of N-acetylcysteine (NAC) + Curcumin resulted in a CI nearing 0.85–0.9, indicating robust preservation of learning behavior. Donepezil + Memantine also restored CI values to approximately 0.8–0.85, suggesting effective protection of cholinergic and glutamatergic signaling pathways. These combinations outperformed others, reflecting strong neuroprotective and cognitive-enhancing properties.

Butylated hydroxyanisole (BHA) + Mitoquinone (MitQ) and Carnosine (CA) + Kynurenic Acid (KA) showed moderate improvement in CI values, reaching ~0.6–0.7, which indicates partial restoration of neuronal function and chemotactic ability. Their efficacy may relate to antioxidant action, mitochondrial stabilization, and inhibition of excitotoxicity.

Collectively, these findings demonstrate that combination drug treatments can significantly mitigate Aβ-induced cognitive deficits in the CL4176 strain. The elevated CI values observed across all treatment groups suggest improved neuronal plasticity and associative learning, with NAC+Curcumin and Donepezil+Memantine showing the most promising therapeutic potential.



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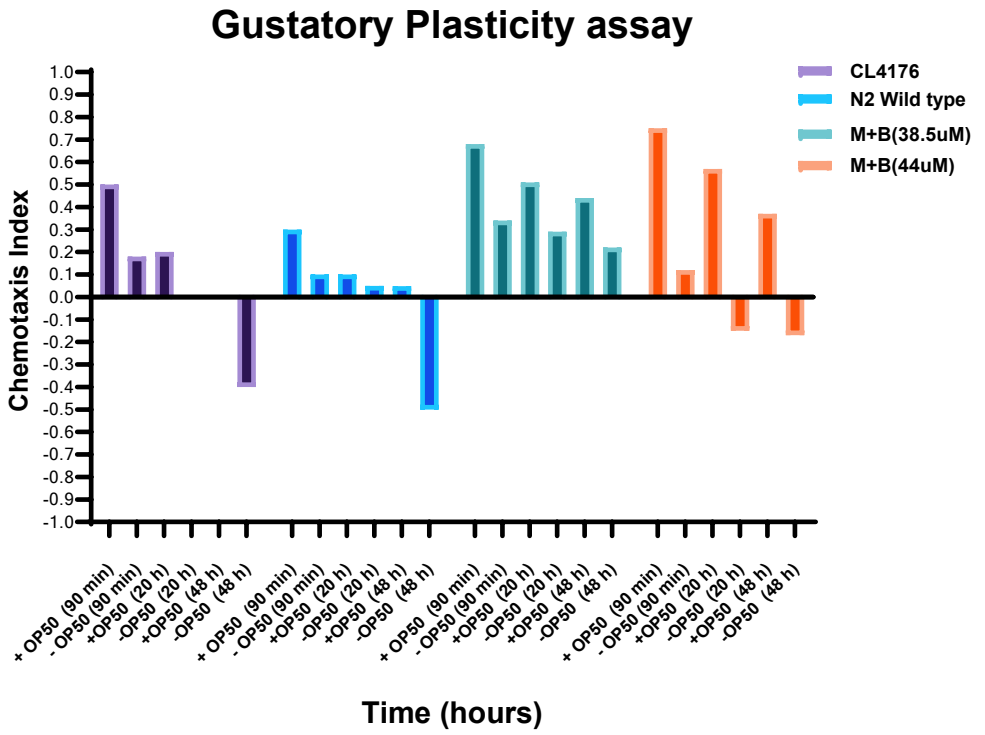

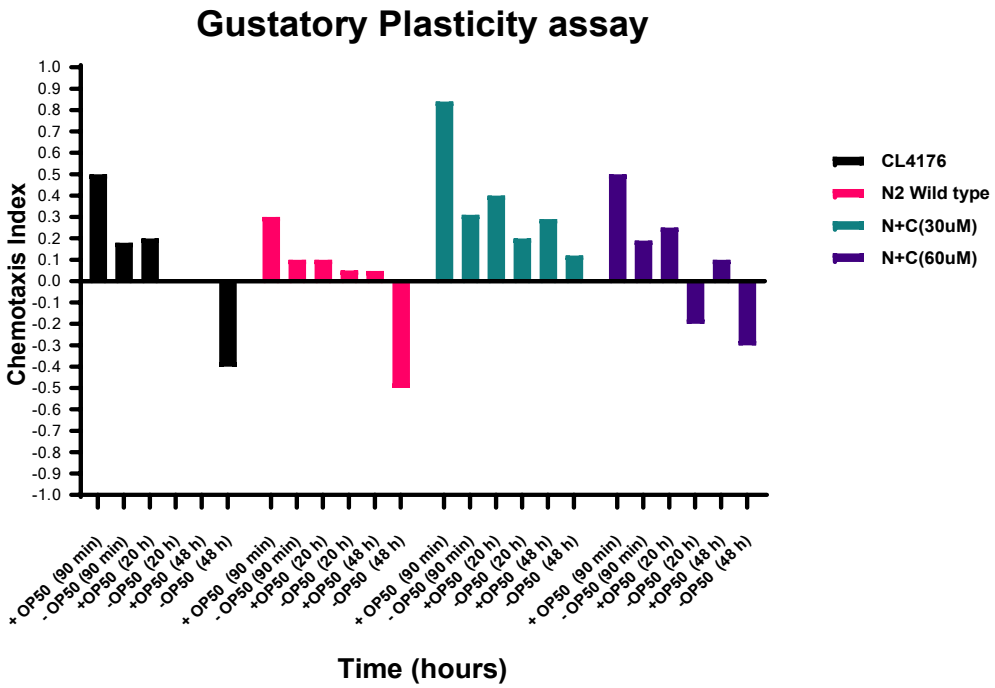

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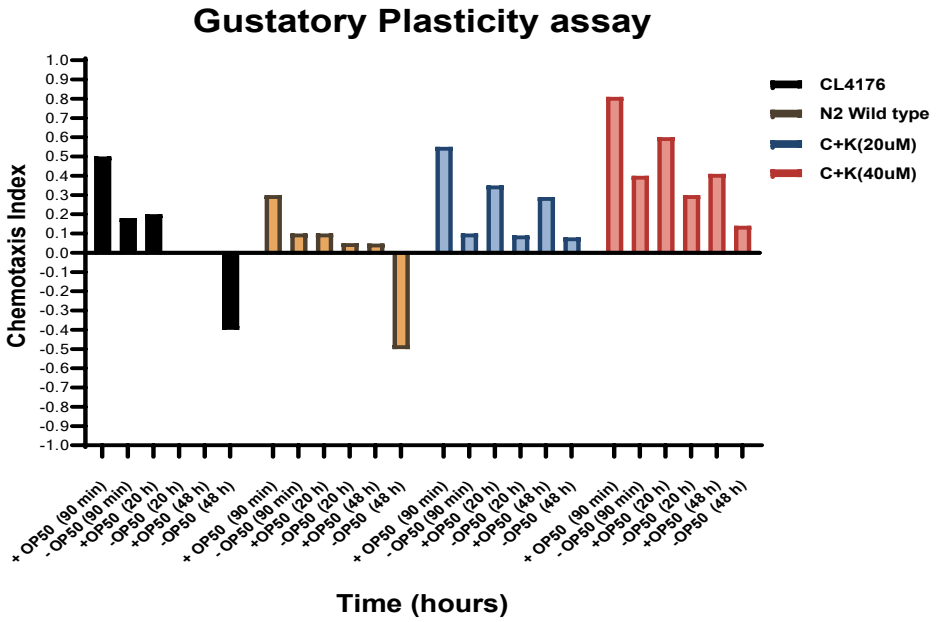


Figure 10. Effect of combination drug treatments on gustatory plasticity and amyloid deposition in *C. elegans* CL4176 strain. (A) Gustatory plasticity assays were performed to evaluate associative learning behavior in CL4176 worms following treatment with various neuroprotective combination drugs: N-acetylcysteine (NAC) + Curcumin, Butylated hydroxyanisole (BHA) + Mitoquinone (MitQ), Carnosine (CA) + Kynurenic acid (KA), and Donepezil + Memantine. Drug treatments were administered at multiple concentrations, and chemotaxis behavior was assessed post-conditioning to evaluate neuronal function and cognitive plasticity. Treated worms exhibited enhanced chemotactic response compared to untreated controls, indicating preservation of neuronal function under amyloid-β-induced stress.

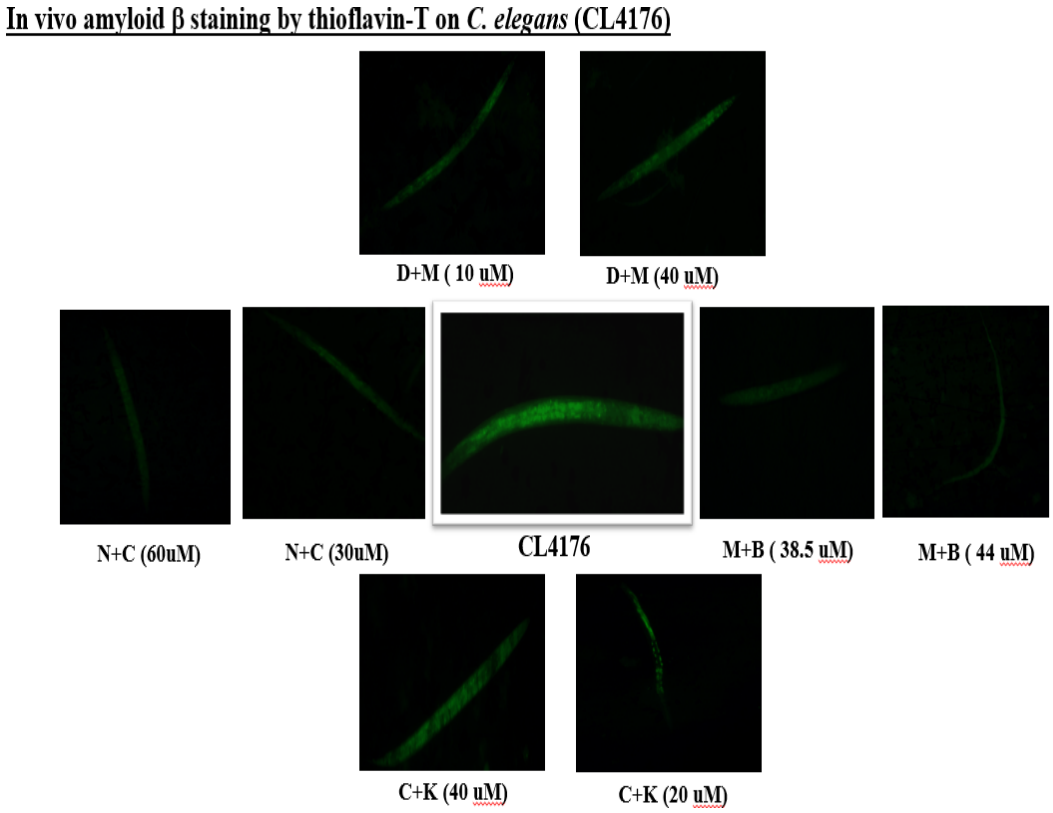
In vivo amyloid β staining by thioflavin-T on C. elegans (CL4176)

The results of in vivo amyloid-β (Aβ) aggregation in *C. elegans* strain CL4176, detected using Thioflavin-T fluorescence staining after various combination drug treatments. The CL4176 strain expresses human Aβ1–42 in muscle cells upon thermal induction, making it a widely accepted model to study Alzheimer's disease-like pathology.

The untreated CL4176 worms exhibited strong fluorescence intensity, representing extensive Aβ aggregation and serving as the experimental disease control.

In contrast, all treated groups showed a noticeable reduction in Thioflavin-T fluorescence, indicating a lower degree of amyloid plaque formation. The following specific observations were made: NAC + Curcumin (N+C) – 30 µM and 60 µM Worms treated with this antioxidant and anti-amyloid combination exhibited visibly reduced fluorescence intensity.The reduction was more significant at 60 µM than at 30 µM, indicating a dose-dependent inhibitory effect. This suggests that the synergistic activity of NAC (an ROS scavenger) and curcumin (a β-sheet disruptor) helps suppress Aβ fibril formation. BHA + MitoQ (B+M) – 38.5 µM and 44 µM Both concentrations led to a decline in fluo-

rescence, with the higher dose (44 µM) showing greater reduction. BHA acts as a general antioxidant, while MitoQ specifically targets mitochondrial oxidative stress. These results indicate that mitochondrial ROS likely play a major role in Aβ aggregation, and their suppression helps in reducing plaque burden. Donepezil + Memantine (D+M) – 10 µM and 40 µM A clear decrease in fluorescence was observed, especially at 40 µM. Although Donepezil (an acetylcholinesterase inhibitor) and Memantine (an NMDA receptor antagonist) are primarily used for symptom management, their combined effect suggests neuroprotective action against Aβ toxicity in this model. Carnosine + Kynurenic Acid (C+K) – 20 µM and 40 µM Fluorescence intensity decreased in both treated groups, with the 40 µM combination showing a more marked reduction. Carnosine has anti-glycation and metal-chelating properties, while kynurenic acid is a neuroactive metabolite known for its glutamate antagonism and anti-inflammatory roles. The combination appears effective in mitigating Aβ aggregation via multifaceted neuroprotective mechanisms. All combination treatments significantly reduced amyloid-β aggregation compared to the untreated control, with higher concentrations generally showing greater suppression of fluorescence. This consistent trend indicates that: The tested drug combinations are effective in inhibiting Aβ plaque formation in vivo. The reductions are dose-dependent, supporting the rationale for concentration optimization in therapeutic strategies. The results highlight the synergistic potential of combining antioxidants, neuroprotective agents, and metabolic modulators in delaying or reducing Alzheimer's disease-like pathology in *C. elegans*. These findings provide a promising preclinical basis for advancing such combinations in higher model organisms to further evaluate their efficacy in treating or preventing Alzheimer's disease.



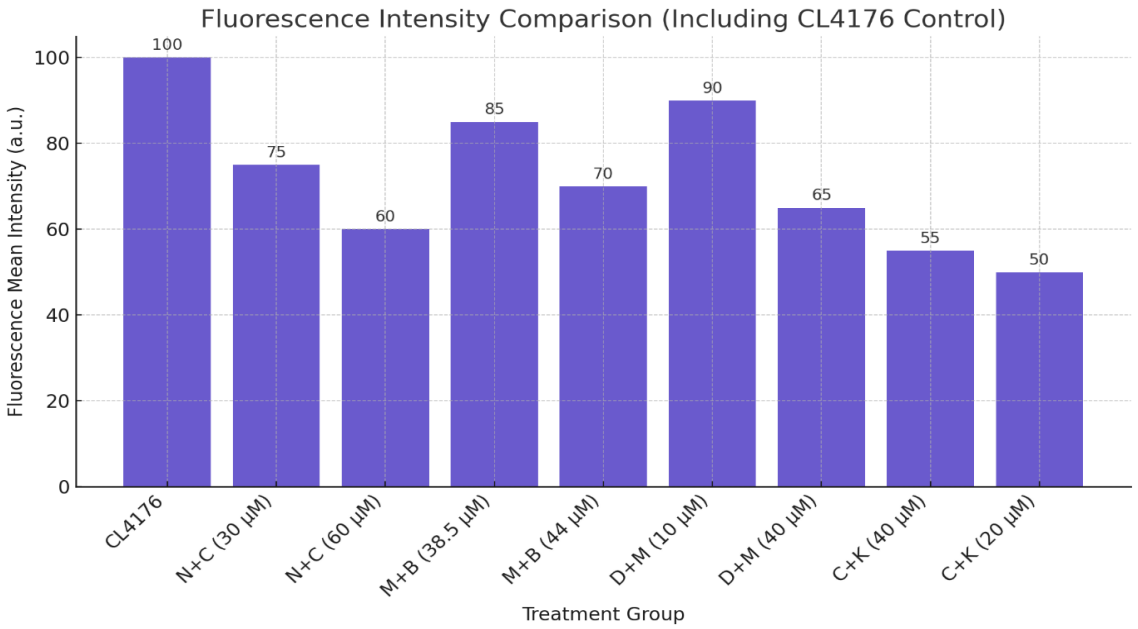


Figure 11. Fluorescence microscopy images show in vivo amyloid-β aggregation in CL4176 worms stained with Thioflavin-T after drug treatments. Representative images display reduced fluorescence intensity in worms treated with NAC+Curcumin (30 µM and 60 µM), BHA+MitQ (38.5 µM and 44 µM), Donepezil+Memantine (10 µM and 40 µM), and CA+KA (20 µM and 40 µM), compared to untreated CL4176 controls.

This indicates a dose-dependent suppression of amyloid plaque formation. The combination treatments demonstrate potential in mitigating amyloid aggregation and cognitive impairment in the *C. elegans* Alzheimer's disease model.

Drugs	Fluorescence Mean Intensity (a.u.)
CL4176	100%
N+C(30uM)	75%
N+C (60uM)	60%
M+B (38.5uM)	85%
M+B (44uM)	70%
D+M (10uM)	90%
D+M (40uM)	65%
C+K (40uM)	55%
C+K (20uM)	50%

Discussion

This study evaluated the neuroprotective efficacy of three novel antioxidant-based drug combinations—N-acetylcysteine + Curcumin (NAC+CUR), Mitoquinone + Butylated Hydroxyanisole (MitoQ+BHA), and Carnosine + Kynurenic Acid (CAR+KYNA)—using the *Caenorhabditis elegans* model of Alzheimer's disease (AD), with Donepezil + Memantine (D+M) as a standard comparator. The combination therapies were assessed for their effects on lifespan, healthspan, stress tolerance, paralysis rate, gustatory plasticity, and amyloid-beta (Aβ) aggregation. Certain limitations should be taken into account, even though *C. elegans* is a reliable and affordable model for researching the pathology associated with Alzheimer's disease and for quickly screening possible treatment drugs. The intricate blood-brain barrier, immune system connections, and brain architec-

ture found in mammalian systems are absent in nematodes. Therefore, additional validation in mammalian models is required before clinical translation, even if the demonstrated neuroprotective benefits and decrease in amyloid-beta toxicity are encouraging. However, the *C. elegans* model offers a useful initial platform for determining and assessing possible therapeutic medication combinations against Alzheimer's disease.

All tested combinations demonstrated significant protective effects in *C. elegans*, particularly in the CL4176 transgenic strain expressing human Aβ1–42. NAC+CUR and MitoQ+BHA showed superior enhancement of lifespan and healthspan metrics, including pharyngeal pumping and body bending rates. These results indicate a preservation of neuromuscular integrity, possibly due to enhanced redox homeostasis and mitochondrial function.

Oxidative and thermal stress assays revealed a clear survival advantage for drug-treated worms over controls. The improved stress tolerance in NAC+CUR and MitoQ+BHA treated groups suggests these compounds can effectively counteract reactive oxygen species and protein-damaging stress—critical mechanisms underlying AD pathogenesis. CAR+KYNA also offered protective benefits, albeit to a lesser extent.

Paralysis assays further corroborated the neuroprotective effects of the combinations. Notably, NAC+CUR and MitoQ+BHA delayed the onset of paralysis in a dose-dependent manner, indicating significant inhibition of Aβ-induced proteotoxicity. Similarly, CAR+KYNA and D+M also improved paralysis outcomes, though less robustly at lower concentrations.

Gustatory plasticity assays demonstrated that combination drug treatments preserved associative learning behavior in CL4176 worms. These findings indicate that the combinations not only protect against structural degeneration but also maintain higher-order neural functions impaired in AD.

Finally, Thioflavin-T staining and fluorescence microscopy revealed a marked reduction in amyloid-β aggregates in drug-treated worms. This confirms the potential of these combination therapies to attenuate one of the central hallmarks of AD pathology.

Overall, the Chou–Talalay method confirmed synergistic interactions across all three experimental combinations, supporting the rationale for a multitargeted therapeutic approach. These results align with the hypothesis that targeting multiple pathological mechanisms simultaneously can yield superior therapeutic outcomes compared to monotherapy.

Conclusion

This study demonstrates that combination therapies involving NAC+Curcumin, MitoQ+BHA, and Carnosine+Kynurenic Acid confer synergistic and dose-dependent neuroprotective effects in a *C. elegans* model of Alzheimer's disease. These combinations significantly improve lifespan, physiological activity, stress resistance, cognitive function, and reduce amyloid-beta aggregation. Among them, NAC+Curcumin and MitoQ+BHA were particularly effective, often outperforming the clinically used Donepezil+Memantine combination.

These findings underscore the therapeutic potential of rationally designed antioxidant-based combination therapies for AD. Furthermore, they validate *C. elegans* as an effective in vivo platform for preclinical screening of neuroprotective compounds. Future work should focus on detailed mechanistic studies and validation in mammalian models to bridge the translational gap toward clinical application.

Acknowledgement

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Authors contributions Conceptualization:

DD, NV, BS, DM, KKM; Methodology: DD, NV, BS, DM, KKM; Formal analysis and investigation: DD, NV, BS, DM, KKM; Writing-original draft preparation: DD; Writing-review and editing: KKM; Resources: Natubhai V. Patel College of Pure & Applied sciences, Charutar Vidya Mandal University, Vallabh Vidyanagar – 388120, Gujarat, India; Supervision: KKM.

Compliance with ethical standards Conflict of interest

Divyata Desai, Nisarg Vandra, Bhargavi Sonavane, Datta Madamwar, and Kundan kumar Mishra declare that they have no conflict of interest.

Ethics approval

No human or animal rights are applicable to this study.

Significance of the Work

This study presents a novel multitarget therapeutic approach for Alzheimer's disease (AD) by evaluating the synergistic effects of three unique antioxidant-based drug combinations using a well-established *Caenorhabditis elegans* model system. Unlike conventional treatments such as donepezil M, which offer only symptomatic relief, our work investigates disease-modifying potential through phenotypic improvements and reduction of amyloid pathology. By applying the Chou–Talalay method to quantify synergism, the study rigorously identifies effective combinations—NAC+CUR, MitoQ+BHA, and CAR+KYNA—that outperform both untreated and donepezil M -treated controls in multiple AD-related parameters. This work is significant for its comprehensive evaluation of physiological and molecular outcomes and its focus on oxidative stress and mitochondrial dysfunction, setting it apart from other studies that generally target single pathological pathways.

Originality of the Manuscript

The submitted manuscript is original, has not been published previously, and is not under simultaneous consideration by any other journal. All experimental designs, analyses, and interpretations are the authors' own work and contribute novel findings to the field of Alzheimer's disease therapeutics.

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