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Research Article

Neuroprotective potential of optimized *Lactobacillus fermentum* extracts against Acetylcholinesterase activity in Zebrafish model and cytotoxicity studies in PC12 cell line

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Abstract
Alzheimer's disease is linked with reduced levels of acetylcholine due to elevated acetylcholinesterase (AChE) activity. Antioxidants and natural AChE inhibitors (AChEIs) are becoming more popular due to their potential neuroprotective benefits. The present work aimed to enhance the production of AChE inhibitors by *Lactobacillus fermentum* using phytogenic substrates and evaluate their neuroprotective effects in a zebrafish model. Media optimization was carried out using response surface methodology (RSM) with *Cinnamomum cassia* (0.5 mg/mL), *Syzygium aromaticum* (1 mg/mL), and *Phoenix dactylifera* seed (1 mg/mL) as substrates. AChE inhibition was predicted using a quadratic model (R^2 = 97.61%), and the maximal inhibition of 28.69% closely matched the expected value of 28.56% (Run no 11). The optimized *L. fermentum* chloroform extract (OLFCE) exhibited a strong antioxidant potential, with 75.09% DPPH scavenging activity. OLFCE exhibited minimal cytotoxicity on H_2O_2-induced PC12 cell line, maintaining 95–99% cell viability across all tested concentrations. Treatment of zebrafish with 2.5–10 mg/mL OLFCE significantly enhanced antioxidant enzyme activities (superoxide dismutase and catalase) and decreased AChE levels in brain, liver, and plasma. GC-MS analysis of OLFCE revealed high levels of potential AChEIs i.e. 3,4-dimethyl benzaldehyde (66.81%) and phenol, 2,4-bis(1,1-dimethylethyl) (16.84%). This study demonstrates, for the first time, that substrate-optimized *L. fermentum* can produce potent AChEIs and antioxidant compounds, as validated through *in vivo* zebrafish assays. The findings suggest that OLFCE has promising Acetylcholinesterase Inhibitor activity, which can be used for the treatment of Alzheimer's Disease in the future.

Keywords: Acetylcholinesterase, Antioxidant, *Lactobacillus fermentum*, Optimization

INTRODUCTION

Alzheimer's disease (AD) is a common neurodegenerative disorder that is the main cause of dementia and is characterized by a progressive decline in cognitive function accompanied by behavioral symptoms ("Alzheimer's Disease Facts and Figures," 2023).The pathogenesis of AD has been linked to several pathogenic variables, including the accumulation of amyloid-beta Aβ peptide and tau protein, excessive transition metals such as iron, zinc, and copper that increase oxidative stress, and decreased levels of acetylcholine (ACh) (A *et al.,* 2015).The pathophysiological mechanisms associated with the progression of AD include the degradation of neurons and synapses, primarily characterized by cholinergic dysfunction (Lombardo and Maskos, 2015). Due to this characteristic, only a few specialized drugs are currently available for the treatment of AD, and these drugs slightly relieve symptoms like cognitive and behavioral decline. Acetylcholinesterase inhibitors (AChEIs) are currently employed in pharmaceutical treatments for the mild to

moderate stages of Alzheimer's disease (AD) (Galimberti and Scarpini, 2016). The current FDA-approved AChEIs for mild to moderate symptoms of AD include Galantamine, Donepezil, Tacrine, and Rivastigmine (Agatonovic-Kustrin *et al.,* 2018). Although these drugs frequently cause adverse effects, such as hepatotoxicity, dizziness, and seizures etc. (Bezerra da Silva *et al.,* 2016). The use of AChE combined with other fermented phenolic compounds and antioxidant medicines is another strategy to enhance AChEI activity that has been researched (Dizdar *et al.,* 2018).

AChEIs can be synthesized chemically or naturally extracted from plants and microorganisms. The advantages of microbial-producing AChEIs over both phytochemical extraction and synthetic compounds include their ease of extraction, lower cost, and higher rate of bioactive production with low toxicity (Zaki *et al.,* 2020).

In the biochemical process of fermentation, the components of raw food materials are fermented by probiotic microorganisms, resulting in final products called postbiotics that have significantly different organoleptic properties, increased nutritional value, and improved bioactive compounds (Thorakkattu *et al.,* 2022). The consumption of fermented foods has a broad range of nutraceutical advantages, including antioxidant, anti-inflammatory, anticancer, antimicrobial, immunomodulatory, and hypocholesterolemic qualities, which are enhanced (Kumar *et al.,* 2022). Mutalib Abd *et al.* reported that Lactic acid bacteria (LAB) supplementation possesses neuroprotective qualities and exhibits improvements in cognitive and memory performance (Abd Mutalib *et al.,* 2023).

A unique variant of the *Lactobacillus* species, specifically *L. fermentum*, is considered a beneficial organism for the gut microbiota, exhibiting health-promoting qualities when consumed and possessing antimicrobial and physiologically efficient antioxidant activities. Recent research suggests that the development of Alzheimer's disease (AD) may also be influenced by changes in the gut microbiome composition. It was discovered that short-chain fatty acids, butyric acid, and propionic acid affected the activation of microglia and astrocytes and assisted in lowering inflammation and tau and Aβ protein aggregation in brain tissues (Di Benedetto *et al.,* 2022) (Garland *et al.,* 2022).

As a multifunctional medicinal herb, *C. cassia* (cinnamon) is widely utilized as a food ingredient, hot beverage, and condiment worldwide. Numerous reports indicate that cinnamon has anti-inflammatory, antimicrobial, antipyretic, anticancer, and antidiabetic properties (Darwesh *et al.,* 2023). During the fermentation of *C. cassia*, flavonoids and phenolic compounds increase, which is beneficial against oxidative stress and enhances the gut microbiome (Plamada and Vodnar, 2021). *S. aromaticum* (Cloves) contains various pharmaceutical compounds that have been used to promote wound healing, prevent ageing, and treat various digestive problems. It is also used in fragrances and flavors (Kang *et al.,* 2019). It has antioxidant, anticancer, antibacterial, anti-diabetic and anti-inflammatory properties (Lee *et al.,* 2018) (Haro-González *et al.,* 2021). *P. dactylifera* seed (Date seed) is not much used product but is rich in carotenoids, total dietary fiber (pectin, -glucan, and arabinoxylan), phenolic acids (allergic, epicatechin, catechol, chlorogenic), polyphenols (such as hesperidin, quercetin, and kaempferol), and a variety of other nutrients and functional elements are present (Moslemi *et al.,* 2022). Previous studies on the effects of date seeds on humans and animals have found that they are an effective, low-cost supplement for improving memory and learning impairments, oxidative stress parameters, inflammation, hyperglycemia, and hyperlipidemia (Dehghanian *et al.,* 2017) (Saryono *et al.,* 2020) (Djaoudene *et al*., 2019).

This study aimed to enhance the level of AChE Inhibition by using *L. fermentum*, optimizing the MRS medium supplemented with different compositions of cinnamon (*C. cassia*), cloves (*S. aromaticum*), and date seed (*P. dactylifera*).

MATERIALS AND METHODS

Materials

Cinnamon *C. cassia* (A), Cloves *S. aromaticum* (B) and Date seed *P. dactylifera* seed (C) were purchased from local markets in Coimbatore, India. Cinnamon, Date seed, and Cloves were pulverized into a fine powder (electric mill), sieved, and stored in a dry container for further study. *L. fermentum* (MTCC 8711) was procured from the Microbial Type Culture Collection (MTCC), India. DeMan, Rogosa, and Sharpe (MRS) broth was purchased from Hi Media, India. The PC-12 Cell line was obtained from the National Centre for Cell Sciences (NCCS), Pune. Cell Culture reagents such as Dulbecco's modified Eagle medium (DMEM), Horse serum, and Fetal Bovine Serum (FBS) were obtained from HiMedia, Mumbai. Analytical-grade chemicals and solvents, such as ethanol, chloroform, and H2O2, were purchased from Srl Chemicals Pvt Ltd, Mumbai, India. Acetylcholinesterase (AChE) enzyme (CAS 9000-81-1), 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB), Acetylcholine iodide (ATCI), Galantamine, Gallic acid, Folin-Coicalteau's (FC) reagent, Tris-HCl and 2,2-Diphenyl-1 -picrylhydrazyl (DPPH) were obtained from Sigma Aldrich. For *In vivo* studies, zebrafish (*Danio rerio)* were obtained from Sirago Aqua Agri Farm, Erode, India.

Subculturing of *Lactobacillus fermentum*

Lactobacillus fermentum strain was revived from cryopreserved stock and subjected to serial dilution ($1:10^{-5}$). Subsequent subculturing was performed in MRS broth, followed by incubation at 37 °C for 48 hours. The pure

culture was isolated from the inoculated MRS broth, and bacterial growth was monitored using a UV/Visible spectrophotometer (λ = 620 nm). The resultant culture was utilized for further optimization studies.

Box Behnken Design

A Box Behnken Design (BBD) (Ferreira *et al.,* 2007) was used to investigate the effects of multiple media composition on AChE inhibition activity. Response surface methodology (RSM) was employed to analyze the data using the Stat-Ease program (Design-Expert software, version 12). The independent variables consisted of three (levels) concentrations (0, 0.5, and 1 mg/mL) of Cinnamon (A), Clove (B), and Date Seeds (C) (Table 1). AChE activity was designated as the dependent variable.

Optimization of *Lactobaillus fermentum* medium

BBD set for 15 runs with triplicate center points (Table 2) was employed. Corresponding fermentations were prepared by combining varying concentrations (0, 0.5, 1 mg/mL) of Cinnamon, clove, and Date seed with MRS broth (Table 2) and were added into each conical flask, respectively. The mixtures were autoclaved at 121 °C for 20 minutes, cooled, and inoculated with 1 mL of *L. fermentum* culture. Fermentation proceeded in a shaking incubator at 120 rpm and 37 °C for 72 hours. The fermented broth was sonicated for 30 mins at room temperature to release the intracellular contents of the bacterial cells (Choi *et al.,* 2022). The supernatant obtained from centrifugation (5000 rpm for 15 mins) was subsequently subjected to liquid-liquid extraction with chloroform solvent for the isolation of secondary metabolites. Shake vigorously for a few minutes. Solvents were evaporated using a rotary evaporator, yielding dry powder. Subsequently, the dry-extracted powder was preserved in an air-tight container for further studies.

Acetylcholinesterase inhibitory activity of *Lactobacillus fermentum* chloroform extracts

A modified version of the procedure outlined by Ellman *et al.* (1961) was used to assess AChE inhibitory activity in a 96-well plate. The following mixture of reagents was added to the well: 40 µL of 1 mM DTNB, 5 µL of enzyme solution (AChE, 2 U/mL), and 125 µL of diluted sample solution. After 5-minutes of pre-incubation at 25 °C, 7.5 mM Acetylcholine iodide was added to initiate the reaction. After 15 minutes at room temperature (25° C), absorbance was measured at 412 nm. The control was phosphate buffer (200 mM, pH 7.7). The following formula was used to determine the AChE inhibition (%).

$$\% \text{ Inhibition} = \left(1 - \frac{\text{At}}{\text{A0}}\right) \times 100 \quad (1)$$

A0 is the control absorbance, At is the sample absorbance.

Antioxidant activity of optimized *Lactobacillus fermentum* chloroform extract (OLFCE)

DPPH free radical scavenging assay of OLFCE

The antioxidant activity of OLFCE was analyzed using the DPPH free radical scavenging assay, as described by Blois (1958). 0.1mM DPPH solution was prepared using methanol. Microbial extracts at concentrations of 20, 40, 60, 80, and 100 µg/mL were mixed with 1 mL of DPPH solution (1:1 ratio). After 30 minutes of incubation in darkness, the absorbance was measured at 517nm. The standard reference was taken as ascorbic acid (ASA), while the blank was taken as a solution of methanol and DPPH. From the following formula, the DPPH radical scavenging capacity was calculated.

$$\% \text{ DPPH Radical Scavenging} = \left(1 - \frac{\text{At}}{\text{A0}}\right) \times 100 \quad (2)$$

where A0 is the control absorbance, At is the sample absorbance. The results were expressed as *the* IC_{50} *concentration in* µg/mL.

Total antioxidant capacity (phosphomolybdate assay) of OLFCE

The total antioxidant capacity (TAC) of OLFCE was estimated following the protocol described by Prieto *et al.,* (1999). The phosphomolybdate reagent (0.6 M sulfuric acid, 4 mM ammonium molybdate, 28 mM sodium phosphate) was mixed with 300 µL of the sample solution (0.3 mg/mL in methanol). After being wrapped in silver foil, the test tube was placed in a boiling water bath set at 95 °C for 90 minutes before being allowed to cool to room temperature. Using a Spectrophotometer, absorbance was measured at 765 nm against a blank. The standard reference was ascorbic acid. Results were presented as milligrams of ascorbic acid equivalents per gram of extract (mg of AAE/g).

Total phenolic content (TPC) of OLFCE

The TPC of the OLFCE was determined using FC reagent method by Singleton *et al.,* (1999). To prepare the reaction mixture, 0.1 mg of OLFCE in 0.5 mL of methanol was mixed with 2 mL of 10% FC reagent and 3 mL of 7% Na_2CO_3. The samples were incubated at room temperature for 2 hrs and optical density (OD) was taken with a UV-spectrophotometer at 760nm. Gallic Acid was taken as standard. The Concentration of TPC was represented in milligrams of gallic acid equivalent (GAE) per gram of extract (mg GAE/g of extract)

***In vitro* cell culture analysis**

Culturing PC12 cell line

PC12 Neuronal cell line was used to analyze the neuroprotective effect of OLFCE. The PC12 cell line was acquired from NCCS, Pune. PC12 cells were cultivated in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10% horse se-

rum, 100 Units/mL of penicillin, and 100 µg/mL of streptomycin. Cell lines were grown at 37°C in a humidified CO_2 incubator with 5% CO_2. After reaching 80% confluency, the cells were taken for further assays (L. Wang *et al*., 2023).

Cell viability analysis of OLFCE

Cell Viability was analyzed using the MTT colorimetric assay (Ansari *et al.,* 2011). PC12 cells (1×10^5 cells/mL) were seeded in 96 well pates (100µL of cells/well) and exposed to different concentrations of OLFCE (20, 40, 60, 80, 100µg/mL). Galantamine served as the positive control, while untreated cells acted as the control. For inducing oxidative stress, H_2O_2 was added and the mixture was incubated for 24 hours. Then, MTT (20 µL) was added and incubated for 4hrs and the dark-blue formazan was dissolved in DMSO (150 µL). Absorbance was measured at 535nm using an ELISA plate reader. Experiments were performed in triplicate, and MTT reduction was calculated as a percentage of the absorbance of the control cells.

***In vivo* analysis**

Zebrafish Maintenance

Adult zebrafish (*Danio rerio*), aged three to six months, weighing 0.3-0.5 g, and measuring 3.1-0.4 cm, were purchased. Prior to experimentation, the zebrafish underwent a 2-week acclimation period to the laboratory environment. Zebrafish were maintained in dechlorinated tap water under controlled physio-chemical parameters: temperature (25.0 ± 0.5 °C), pH (7.2 ± 0.05), total hardness (17.4 ± 0.5 mg/L) and a 12:12 light-dark cycle. Zebrafish were fed commercial pellets twice daily, tanks were cleaned (removing metabolic debris and uneaten food), and the water was renewed daily. Habituated zebrafish served as test subjects for toxicity assessments.

Animal ethical approval

All procedures adhered to guidelines set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and were approved by the Institutional Animal Ethics Committee (IAEC/KITS/BT/23/08).

***In vivo* experimental setup**

For this study, zebrafish were distributed among six 5L tanks (24 cm length × 20 cm height × 25 cm width). The tanks were designated as the control and treatment groups. For treatment tanks, 1 mL of OLFCE (mg/mL of water) was added at four different concentrations: 2.5, 5, 7.5, and 10 mg/mL in four separate tanks. Six fish were added to each group. To induce oxidative stress, 0.1% ethanol (EtOH, v/v) was added to the water (Song *et al.,* 2022). Control fish were handled in the same manner without EtOH exposure and treatment at the same time. All the fish were exposed for 72hrs.

Analysis of antioxidant enzymes

On day 3, zebrafish were randomly selected from each concentration (n = 3) and then quickly euthanised in ice water (Ni *et al.,* 2019). For biochemical enzyme assay, liver, brain, and plasma samples were dissected. Samples were homogenised in ice-cold 100 mM Tris-HCl buffer (pH 7.4) and centrifuged at 12000 rpm for 15 minutes. The supernatants were taken for further studies (Umamaheswari *et al.,* 2021). Plasma was isolated from blood according to the procedure described by Babaei et al., (2013).

SOD activity was analyzed using the pyrogallol autoxidation method with slight modifications adapted from Marklund and Marklund, (1974), and Ramesh *et al.,* (2020)., 0.5 mL from liver, brain, and plasma supernatant were mixed with a reaction solution (50 mM Tris-HCl buffer, pH 8.4 with 1 mM EDTA and 2.64 mM Pyrogallol). The rate of pyrogallol autooxidation was measured at 420 nm using a 96-well plate photometer, and the activity was expressed as Units per milligram of protein.

CAT activity was analyzed by the following method of Sinha, (1972) with modification adopted from Hema *et al.,* (2023) The 0.1 mL supernatant of liver, brain, and plasma was mixed with 3 ml of reaction solution (containing 5% potassium dichromate and glacial acetic acid (1 : 3) and 10 mM phosphate buffer (pH 7.0). Then, the mixture was placed in a boiling water bath for 20 minutes and cooled to observe the colour change. The absorption of the solution was measured at 570 nm using a 96-microwell ELISA microplate reader, and activity was expressed as µmol of H2O2 utilised per minute per milligram of protein.

AChE activity was quantified according to the method described by Ellman et al. (1961). A reaction solution 0.075mM of acetylcholine solution (0.2 mL) and 10mM of DTNB solution (1 mL) were mixed with the supernatants of liver, brain, and plasma (50 µL). The mixture was then mixed with 30 mL of 0.1 M phosphate buffer and incubated for 10 minutes at 37°C. Using a microplate reader, the optical density of the samples was assessed at 412 nm. The AChE activity was expressed as nanomoles (nmol) of substrate hydrolysed per mi-

Table 1. Level of Independent variables in the Box Behnken Experimental Design

Independent variables	Level of variables		
	LOW	MID	HIGH
A	-1	0	1
B	-1	0	1
C	-1	0	1

A - Cinnamon (*Cinnamomum cassia*), B - Clove (*Syzygium aromaticum*), C - Date seed (*Phoenix dactylifera*)

Table 2. Optimization of physical variables for enhanced AChE Inhibitor production by *Lactobacillus fermentum*

Run order	A	B	C	AChE Inhibition %		Residual
				Experimental value	Predicted value (y)	
1	-1	0	1	8.772	10.219	-1.448
2	0	1	-1	0.376	0.309	0.066
3	0	-1	1	10.902	10.963	-0.061
4	1	-1	0	11.278	12.609	-1.331
5	1	1	0	14.787	16.211	-1.451
6	-1	0	-1	1.128	2.591	-1.464
7	0	0	0	8.008	8.700	-0.693
8	0	0	0	9.654	8.700	0.954
9	-1	1	0	7.393	5.981	1.412
10	1	0	-1	7.644	6.191	1.453
11	0	1	1	28.697	28.561	0.135
12	-1	-1	0	14.536	13.091	1.445
13	1	0	1	17.920	16.439	1.48
14	0	-1	-1	21.303	21.341	-.0.038
15	0	0	0	9.165	8.700	0.465

nute per milligram protein.

GCMS metabolite profiling of OLFCE

The gas chromatography-mass spectrometry (GC-MS) analysis was carried out using an Agilent GC 7890A coupled with an MSD 5977B (Agilent Technologies, USA). The chromatographic conditions included a helium carrier gas with a flow rate of 1 mL/min, an Agilent HP-5MS column (30 m × 250 µm × 0.25 µm), and a splitless injector at 20:1 with an injector temperature of 250°C. The quad temperature was set at 150°C, and the MS source temperature was set at 230°C. The oven's temperature was set at 60°C and kept maintained for one minute. After that, the oven temperature was increased to 300°C at 15°C per minute (maintained for 1 minute). The injection volume was 1 µL, and the scanning range was 50–800 mass ranges at an electron energy of 70 eV and with a solvent delay of 3 minutes. Compound identification was achieved by comparing the mass spectra with those in the NIST 2008 and PubChem databases. The analysis time per sample was 29 minutes.

Statistics

All the experiment was conducted in triplicate, and Microsoft Office Excel was used to evaluate the results. The results were displayed as mean ± Standard Deviation (SD), and SD shown by the error bar. One-way analysis of variance (ANOVA) was used for statistical analysis. Distinct letters ($p < 0.05$) indicate significant differences. To quantify the relationship between independent and dependent variables, quadratic regression equations were derived. Using the Stat-Ease software (Design-Expert software, version 12, Stat-Ease, Inc., Minneapolis, MN, USA), a three-dimensional response surface diagram was plotted to predict the ideal state.

RESULTS AND DISCUSSION

Optimization of microbial medium using Box Behnken Design and AChE Inhibitory activity

Inhibiting cholinesterase activity is a promising method for treating Alzheimer's disease. The cholinesterase enzyme (AChE) breaks down neurotransmitters, such as acetylcholine, which can disrupt brain function. This breakdown can lead to an increase in Aβ peptides, which form a harmful complex in the brain and contribute to neuronal disorders. Increasing acetylcholine levels and blocking the enzyme that breaks it down can help to prevent the formation of pathogenic Aβ peptides. Jabir *et al*. (2018) suggested that polyphenols can inhibit ACHE activity and Lactic acid fermentation increases phenolic content (Li *et al.,* 2024). Thus, following BBD, fermentation by *L. fermentum* was conducted using 15 distinct media compositions.

In this study, three independent variables (*C. cassia* (A), *S. aromaticum* (B), *and P. dactylifera* (C) were used to maximize the AChE inhibition potency by analyzing using RSM. The experimental results revealed that the maximum AChE inhibition was achieved at run 11 with the combination of 0.5 mg/mL of *C. cassia* (A) and 1 mg/mL of both *S. aromaticum* (B) and *P. dactylifera* (C), demonstrating a close relationship between the experimental and predicted optimized inhibition percentages.

Table 2 presents AChE inhibitory activities of single substrates: *S. aromaticum* (Run 6) exhibited low AChE inhibitory activity (1.18%), *P. dactylifera* (Run 12)

Table 3. ANOVA results for AChEI production by optimizing *Lactobacillus fermentum* in the quadratic model

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	9	726.461	80.718	22.65	0.002*
A	1	48.216	48.216	13.53	0.014*
B	1	5.900	5.900	1.66	0.255
C	1	159.758	159.758	44.84	0.001*
A*A (A^2)	1	9.320	9.320	2.62	0.167
B*B (B^2)	1	86.629	86.629	24.31	0.004*
C*C (C^2)	1	11.292	11.292	3.17	0.135
A*B	1	28.302	28.302	7.94	0.037*
A*C	1	1.716	1.716	0.48	0.519
B*C	1	373.069	373.069	104.71	<0.001*
Error	5	17.815	3.563		
Lack-of-Fit	3	17.075	5.692	15.38	0.062
Pure Error	2	0.740	0.370		
R^2 = 97.61					

DF- Degrees of freedom, Adj SS - Adjusted sum of Squares, Adj MS - Adjusted mean squares, * - Significant values at 95% confidence interval

showed moderate AChE inhibitory activity (14.5%), and *C. cassia* (Run 7), displayed higher AChEI inhibitory activity (21.3%) which is second highest AChEI activity out of 15 runs next to Run 11. These results indicated that *C. cassia* possessed superior AChE inhibitory activity compared to *S. aromaticum* and *P. dactylifera*. The positive control, Galantamine, demonstrated 34.2% inhibition.

The coefficients of the models were evaluated, and the significance was assessed through regression analysis, as shown in Table 3. The quadratic model with interaction between the independent variables was selected to describe the AChE inhibitory activity. An F-value of 22.65 further indicated that the model was statistically significant, while values less than 0.05 meant that some factors (A, C, B^2, A*B and B*C) were significant at a certain level. The lack-of-fit test had an F-value of 15.38 (p = 0.062), indicating non-significance and suggesting that the model fit within the defined parameter ranges. The mean and standard deviation of the present model were 11.44 and 1.94, respectively.

Multiple regression analysis was performed using a quadratic polynomial model to examine AChE inhibition and the relationship between influencing factors and the predicted responses.

$$Y = 8.70 + 2.455\ A - 0.859\ B + 4.469\ C - 1.589\ A*A + 4.844\ B*B + 1.749\ C*C + 2.660\ A*B + 0.655\ A*C + 9.658\ B*C \quad (3)$$

In this equation, the coefficients of *C. cassia* (A) and *P. dactylifera* (C) were positive, while S. aromaticum (B) was a negative independent variable. This indicated that increasing the content of *C. cassia* (A) and *P. dactylifera* (C), while decreasing *S. aromaticum* (B), enhanced the AChE inhibitory activity.

Equation (3) is visualized as a 3D response surface and is shown in Fig. 1. Figure 1(a) shows that the AChE inhibitory activity increased with the increase in *C. cassia* (A) content; however, *S. aromaticum* (B) decreased, which is counterintuitive, as it increases with AChEI activity. It can be understood that *S. aromaticum* suppresses AChE inhibitory activity. Figure 1(b) shows that increasing *P. dactylifera* (C) content enhances the AChE inhibition, while rising *C. cassia* (A) content AChE inhibitory activity slightly reduced. Figure 1(c) shows that when the concentration of both *P. dactylifera* (C) and *S. aromaticum* (B) decreased AChE inhibitory activity increased gradually. Quadratic multiple regression analysis of the three-factor system revealed that *Syzygium aromaticum* (B) decreased AChE inhibitory activity, while *Cinnamomum cassia* (A) and *Phoenix dactylifera* (C) positively contributed to the enhancement of AChE inhibition.

Antioxidant and total phenolic content activity of optimized lactobacillus fermentum chloroform extract

The antioxidant activity of OLFCE was analyzed using DPPH assay. The results were represented in Table 3. A maximum scavenging activity of 75.09% was observed at a concentration of 100 µg/mL, while the mini-

Table 4. Antioxidant activity and TPC of OLFCE

	DPPH IC_{50} µg/mL	TAC EC_{50} mg/mL (AAE)	TPC (mg GAE/mL)
OLFCE[1]	65.46	77.6±0.023	91.2±2.53

1- optimized *L. fermentum* chloroform extract (Cinnamon - 0.5mg, clove -1mg, Date seed - 1mg);Values are expressed as mean±S.D (n=3)

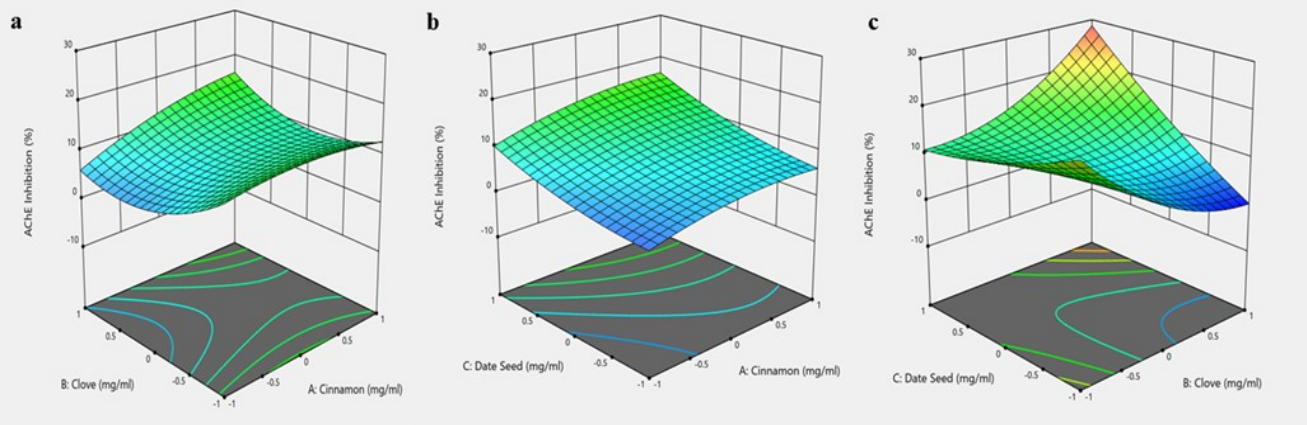

Fig. 1. *Response surface plots showing Interaction among physical variables for enhanced Acetylcholinesterase Inhibitor production from L. fermentum chloroform extract. Fig. 1a. % of AChE Inhibition and Concentration of Clove and Cinnamon (mg/mL). Fig. 1b. % of AChE Inhibition and Concentration of Date seed and Cinnamon (mg/mL). Fig. 1c. % of AChE Inhibition and Concentration of Date seed and Clove (mg/mL)*

mum activity of 15.68% was recorded at 20 µg/mL, indicating a dose-dependent increase. The IC_{50} value was observed to be 65.46 µg/mL. The above results was in accordance with the findings of Choi *et al*. (2022), who reported that, 86.5 ± 0.89% DPPH scavenging activity in *Leuconostoc mesenteroides* fermented with *Artemisia capillaris* at 300 µL. However, the results differ from those of John & Chandrapragasam, (2020), who reported a DPPH activity IC_{50} of 23.59 µg/mL in *Lactobacillus plantarum* fermented with cinnamon. They observed that the DPPH activity of OLFCE was enhanced due to the presence of Cinnamon, Date seed, and clove. Previous studies on *L. plantarum* have shown increased antioxidant activity due to the presence of bioactive compounds such as gallic acid, catechin, and chlorogenic acid in cinnamon (Eweys *et al.,* 2022a). The TAC of OLFCE, analyzed using a phosphomolybdate assay, displayed a remarkable EC_{50} activity of 77.6 mg/mL ascorbic acid equivalents (AAE). This suggested that OLFCE has a broad range of antioxidant compounds that can scavenge free radicals. The TPC of the OLFCE was estimated using the FC reagent, and the results were expressed in milligrams of gallic acid equivalents (GAE). The Total phenolic yield obtained in the microbial extract in optimized media is 91.2±2.53 mg GAE/g. This indicated that fermentation of *L. fermentum* was conducive to the release of phenolic acids. Similar results were found, where fermentation by *L. fermentum* of oat bran increased the TPC value from 1.05 ± 0.04 mg GAE/g to 2.33 ± 0.23 mg GAE/g (Li *et al.,* 2024). Additionally, it was demonstrated that fermentation by *L. plantarum* significantly increases the TPC content from 8.15 to 11.40 mg/g in lyophilised cinnamon extract (Eweys *et al.,* 2022b). Therefore, it has been observed that the chloroform extract of *L. fermentum* for 72hrs with the combination of Cinnamon (0.5mg/ml), clove (1mg/mL), Date seed (1mg/mL) in MRS media showed higher phenolic content (91.2±2.53 mg GAE/g), this result was very higher when compared to the results obtained by Li et al., (2024). They used oat bran as the substrate. From our findings, it was observed that cinnamon, Date seed, and Clove can be used as a substrate for media optimisation of *L. fermentum*, which resulted in increased production of phenolic contents and enhanced antioxidant activity (Table 4).

PC12 cell viablity of OLFCE

The cytotoxic effects of OLFCE on H_2O_2 induced PC-12 cells were investigated using an MTT assay. The OLFCE affected a minimal change of 95% in 20 µg/mL concentration on H_2O_2 induced PC12 cell viability compared to the control. No significant difference was observed in cell viability among all concentrations of OLFCE-treated groups. Figure 2 shows that 95 to 99% of cell viability was observed in H_2O_2-induced PC12 cells treated with different concentrations of OLFCE. The cell viability range, from 97 to 99%, occurred in galantamine (standard drug)- treated cells (Fig. 2). (Wang et al., 2023) reported 70.3% cell viability with *Brassica rapa* extract under similar oxidative stress conditions, indicating superior neuroprotective efficacy of OLFCE. Both OLFCE and galantamine demonstrated dose-dependent protective effects against H_2O_2-

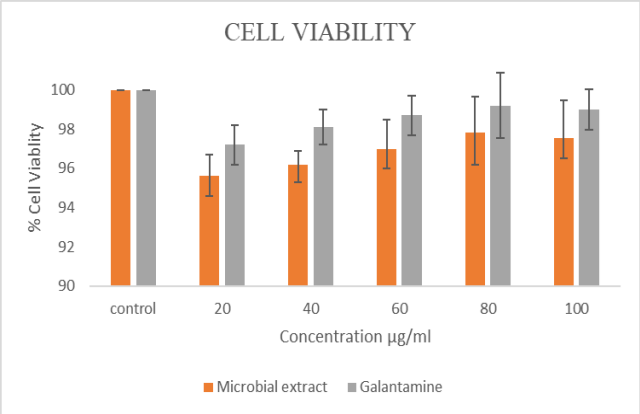

Fig. 2. *Effect of OLFCE in H_2O_2 induced PC12 rat pheochromocytoma cells. Cell survival was measured by the MTT assay; Results are presented as mean±SD (n=3)*

induced neuronal damage. Beniaich *et al*., (2023); Djaoudene, *et al*., (2019); Saxena, (2024) reported that bioactive compounds such as eugenol (clove), cinnamaldehyde (cinnamon), and phenolic acids (date seed) which exhibit capabilities to reduce oxidative stress, modulate inflammatory responses, inhibit cholinesterase activity, and prevent neuronal apoptosis. These findings were closely aligned with OLFCE, which contains a diverse array of neuroprotective compounds capable of enhancing cellular resilience to oxidative stress.

***In vivo* analysis**

In all the experimental groups, no mortality was recorded. All the water parameters were measured in triplicate, and they remained consistent among all the groups.

Antioxidant enzyme analysis of OLFCE treated fish

Superoxide dismutase (SOD) is a prime antioxidant enzyme present in nearly all organisms, from prokaryotes to eukaryotes, and plays a crucial role in cellular defence by neutralising superoxide anions, one of the most common reactive oxygen species (Zemlan *et al.,* 1989). Administration of ethanol (EtOH) significantly decreased the SOD-specific activity compared to the control group. The result (Fig. 3a) illustrates that the SOD activity in the brain, liver, and plasma was significantly increased ($p < 0.001$) in all OLFCE-treated groups (5, 7.5, 10 mg/mL) compared to the EtOH group. These result indicates that EtOH-treated fish showed promising antioxidant potential. Similar findings were reported, indicating that *L. fermentum* significantly increases ($p < 0.01$) the SOD activity in pig serum

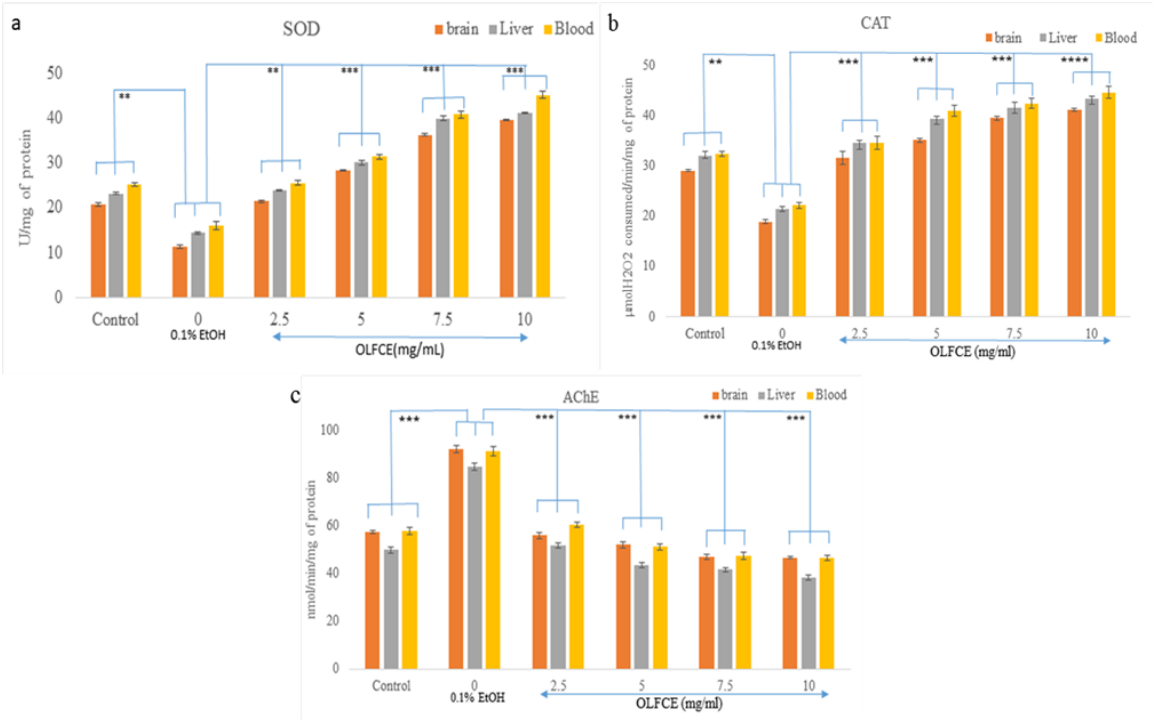

Fig. 3. *Optimized L. fermentum chloroform extract (OLFCE) (2.5, 5, 7.5, 10 mg/mL) exhibited anti AChE level and enhanced Zebrafish brain antioxidant activity. The enzyme's specific activities: (a) SOD; (b) CAT; (c) AchE; Values are represented as mean ± S.D. (n = 3). For Tukey's post hoc analyses: (a) Control vs. EtOH (0.1%): ** $p < 0.01$, EtOH vs. OLFCE (2.5 mg/L): ** $p < 0.01$, EtOH vs. OLFCE (5 mg/L): *** $p < 0.001$, and EtOH vs. OLFCE (7.5 mg/L): *** $p < 0.001$; EtOH vs. OLFCE (10mg/mL): *** $p < 0.001$. (b) Control vs. EtOH (0.1%): ** $p < 0.01$, EtOH vs. OLFCE (2.5 mg/L): *** $p < 0.001$, EtOH vs. OLFCE (5 mg/L): *** $p < 0.001$, and EtOH vs. OLFCE (7.5 mg/L): *** $p < 0.001$; EtOH vs. OLFCE (10mg/mL): **** $p < 0.0001$. (c) Control vs. EtOH (0.1%): *** $p < 0.001$, EtOH vs. OLFCE (2.5 mg/L): *** $p < 0.001$, EtOH vs. OLFCE (5 mg/L): *** $p < 0.001$, and EtOH vs. OLFCE (7.5 mg/L): *** $p < 0.001$; EtOH vs. OLFCE (10mg/mL): *** $p < 0.001$*

(Wang *et al.,* 2009). The catalase enzyme that protects the cells from oxidative damage by degrading hydrogen peroxide into water and oxygen (Nandi *et al.,* 2019). Figure 3b represented that CAT activity significantly decreased ($p < 0.01$) in EtOH-exposed zebrafish as compared to the control group. Treatment with OLFCE at concentrations of 2.5, 5, 7.5, and 10 mg/mL led to a significant increase in CAT activity compared to the ethanol-exposed group ($p < 0.001$). Notably, the 10 mg/mL dose showed the highest improvement in CAT activity, suggesting that OLFCE enhances the body's antioxidant defense in a dose-dependent manner. Lee *et al*., (2004) and Lee et al. (2003) proved that a phenolic substance called dietary cinnamate, present in cinnamon bark, increases CAT and Gpx activity in rats given a high-cholesterol diet and decreases lipid peroxidation. Similar findings were observed in *an in vivo* study of male albino rats, which reported that cinnamon oil plays a major role, exhibiting high potent antioxidant and anti-inflammatory effects against an overdose of acetaminophen that induced oxidative stress and inflammation in brain tissue. Cinnamon oil significantly upregulated the antioxidant enzyme and suppresses the inflammatory cytokines (Ashafaq *et al*., 2022). Acetylcholinesterase (AChE) is an enzyme involved in Alzheimer's disease, commonly characterised by β-amyloid plaques and neurofibrillary tangles (NFT), which alleviates disease symptoms associated with persistent cholinergic dysfunction (García-Ayllón *et al.,* 2011). Therefore, the main treatment for Alzheimer's disease involves drugs that increase the levels of acetylcholine through the inhibition of the acetylcholinesterase (AChE) enzyme, which is responsible for the breakdown of acetylcholine in the neural synapse (McGleenon *et al.,* 1999). Figure 3C illustrates that ethanol-treated zebrafish exhibited significantly elevated acetylcholinesterase (AChE) activity in the brain compared to the control group ($p < 0.001$). Treatment with OLFCE at concentrations of 2.5, 5, 7.5, and 10 mg/mL resulted in a significant, dose-dependent reduction in AChE activity ($p < 0.001$) compared to the Et-OH group. Notably, a significant decrease in AChE activity was observed even at the lowest tested concentration of 2.5 mg/mL. A similar study shows that 0.1% EtOH significantly increases AChE activity in the zebrafish brain; cotreatment with taurine at 400mg/L prevents the enhancement of AChE (Rosemberg *et al.,* 2010). The present results are in par with the literature, suggesting that the OLFCE has the ability to suppress oxidative damage by restoring antioxidant defense enzymes.

Gas Chromatography-Mass Spectrometry metabolite profile

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the OLFCE chemical composition, as illustrated in Table 5 and Figure 4, revealed the presence of several major bioactive compounds. The most abundant compound identified was 3,4-dimethylbenzaldehyde (66.81%), followed by phenol, 2,4-bis(1,1-dimethylethyl) (16.84%). Additionally, various other compounds containing functional groups, such as aldehydes, hydroxyls, carboxylic acids, esters, amines, and alkoxy groups, were detected. Each compound potentially contributes to the extract's diverse biological activities. Several bioactive compounds identified in OLFCE have been previously reported to exhib-

Table 5. Gas Chromatography-Mass Spectrometry analysis of optimized *Lactobacillus fermentum* chloroform extract

S.No	Compound	Molecular weight (g/mol)	Molecular formula	Retention time (min)	Area %
1	Benzaldehyde 3,4-dimethyl-	134.17	$C_9H_{10}O$	7.131	66.81
2	Phenol, 2,4-bis(1,1-dimethylethyl)	278.5050	$C_{17}H_{30}OSi$	9.586	16.84
3	Benzenepropanoic acid, 3,5-bis(1,1 -dimethylethyl)-4-hydroxy-, methyl ester	236.31	$C_{14}H_{20}O_3$	12.753	3.48
4	Cyclotrisiloxane, hexamethyl-	222.4618	$C_6H_{18}O_3Si_3$	16.308	1.80
5	1,2-Benzenediol, 3,5-bis(1,1-dimethylethyl)-	222.3233	$C_{14}H_{22}O_2$	17.196	1.76
6	Benzo[h]quinoline, 2,4-dimethyl-	207.2704	$C_{15}H_{13}N$	19.529	1.85
7	1,1,1,3,5,5,5-Heptamethyltrisiloxane	222.5049	$C_7H_{22}O_2Si_3$	19.618	2.10
8	Silane, trimethyl[5-methyl-2-(1-methylethyl)phenoxy]-	222.3987	$C_{13}H_{22}OSi$	20.452	2.27
9	Tetrasiloxane, decamethyl-	310.6854	$C_{10}H_{30}O_3Si_4$	20.951	1.67
10	Cyclotrisiloxane, hexamethyl-	222.4618	$C_6H_{18}O_3Si_3$	21.585	1.80
11	1,2-Benzisothiazol-3-amine tbdms	264.46	$C_{13}H_{20}N_2$ SSi	21.585	1.80

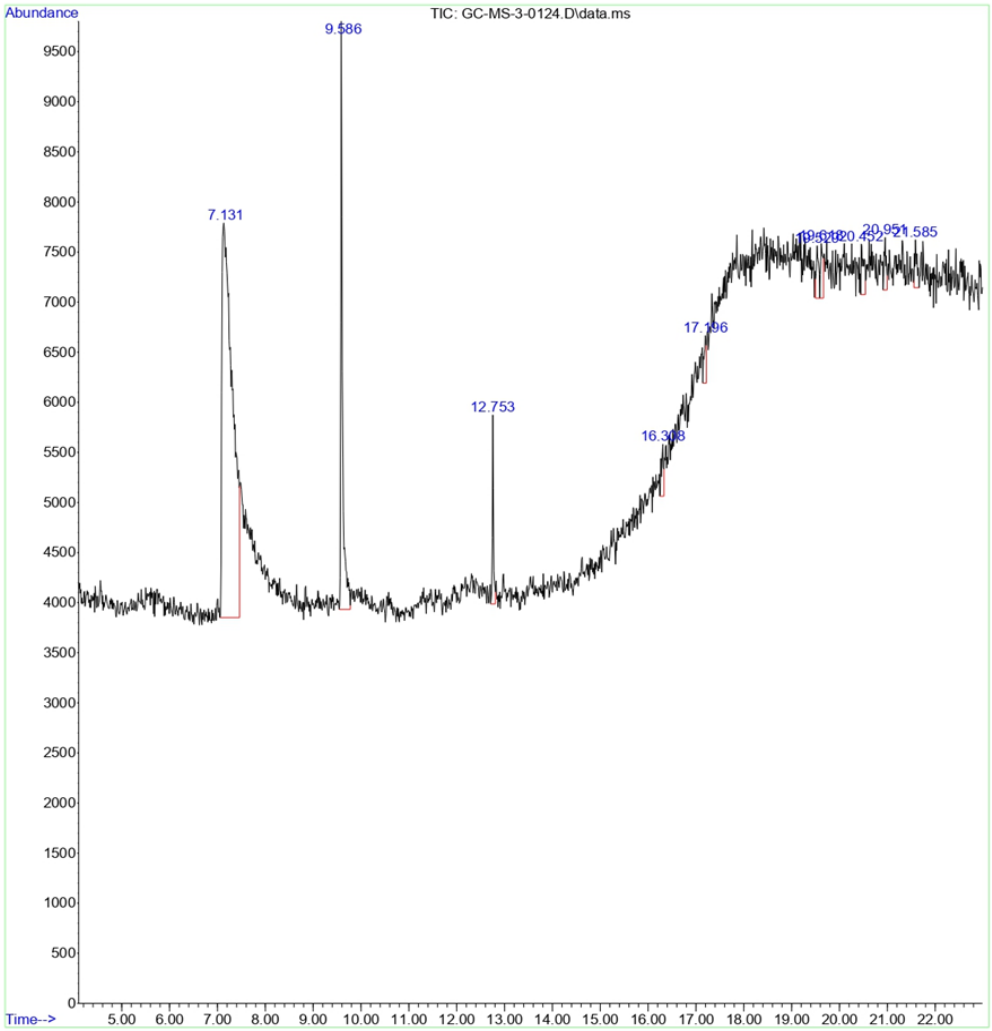

Fig. 4. *Gas Chromatography-Mass Spectrometry analysis of optimized Lactobacillus fermentum chloroform extract*

it significant pharmacological properties. The major constituent of OLFCE 3,4-Dimethylbenzaldehyde has been reported in various pharmacological studies to possess antioxidant, anti-inflammatory, and antimicrobial properties *(*Api *et al.*, 2020; Cheng *et al.*, 2025). Maria *et al*. (2014) reported that 2,4-bis(1,1-dimethylethyl) phenol is a phenolic compound with potent antioxidant properties that can protect against reactive oxygen species. Alqahtani *et al.* (2020) proved that Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester has antimicrobial and antioxidant properties. (2019) demonstrated that Cyclotrisiloxane, hexamethyl-, has antioxidant and cytotoxic properties. Piruthiviraj *et al.* (2024) reported that Silane, trimethyl[5-methyl-2-(1-methylethyl)phenoxy], possesses high antibacterial and antifungal activities. Naik *et al.* (2009) showed that 2,4-Dimethylbenzo [h]quinoline has highly antioxidant, wound-healing, and anti-inflammatory properties. Several studies have reported that Ethyl 4-t-butylbenzoate possesses high gastroprotective properties, increasing gastric wall mucus, enhancing antioxidant capacity, upregulating Hsp70 proteins, and suppressing the expression of BAX (Halabi *et al.,* 2014). 1,2-Benzisothiazol-3-amine TBDMS derivatives exhibit antimicrobial, antiproliferative, antioxidant, and anti-inflammatory activities. Notably, they can bind and activate the AMPK receptor, which is beneficial for antidiabetic and mitochondrial biogenesis (Devi, 2023; Murugan *et al.*, 2025). From the following GCMS analysis, it was observed that OLFCE revealed a diverse range of bioactive compounds with documented antioxidant, anti-inflammatory, antimicrobial, and cytoprotective properties, supporting its potential neuroprotective efficacy.

Conclusion

The present study concluded that OLFCE demonstrated superior antioxidant and acetylcholinesterase inhibitory activities. *In vitro* studies using H_2O_2-induced PC12 cell line showed high cell viability percentages. Furthermore, *in vivo* zebrafish studies revealed significantly high antioxidant enzyme and AChE inhibition activities at a minimal concentration of 2.5 mg/mL. From the GCMS results, 3,4-dimethylbenzaldehyde and phenol, 2,4-bis(1,1-dimethylethyl), were the compounds pre-

sent at the highest levels in OLFCE and can be used as inhibitors for AcHE. These findings suggest that OLFCE possesses potential antioxidant and antidementia properties, making it a promising study for probiotic formulation. To the best of our knowledge, this is the first study to report the involvement of *L. fermentum* in the modulation of AChE activity. The observed inhibitory effects indicate that the fermentation process not only increases the antioxidant potential of these botanicals but also increases their neuroprotective efficacy. These results suggests that OLFCE could help protect the brain from oxidative stress and support its potential use in managing conditions like Alzheimer's disease. However, further study on animal model research is necessary to validate its efficacy and safety as a therapeutic potential.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. Alzheimer's disease facts and figures. (2023). *Alzheimer's & Dementia*, *19*(4), 1598–1695. https://doi.org/10.1002/alz.13016
2. A, K., A, S., & None, E. (2015). A review on Alzheimer's disease pathophysiology and its management: An update. *Pharmacological Reports : PR*, *67*(2). https://doi.org/10.1016/j.pharep.2014.09.004
3. Abd Mutalib, N., Syed Mohamad, S. A., Jusril, N. A., Hasbullah, N. I., Mohd Amin, M. C. I., & Ismail, N. H. (2023). Lactic Acid Bacteria (LAB) and Neuroprotection, What Is New? An Up-To-Date Systematic Review. *Pharmaceuticals*, *16*(5), Article 5. https://doi.org/10.3390/ph16050712
4. Agatonovic-Kustrin, S., Kettle, C., & Morton, D. W. (2018). A molecular approach in drug development for Alzheimer's disease. *Biomedicine & Pharmacotherapy*, *106*, 553–565. https://doi.org/10.1016/j.biopha.2018.06.147
5. Alqahtani, S. S., Makeen, H. A., Menachery, S. J., & Moni, S. S. (2020). Documentation of bioactive principles of the flower from *Caralluma retrospiciens (Ehrenb)* and *in vitro* antibacterial activity – Part B. *Arabian Journal of Chemistry*, *13*(10), 7370–7377. https://doi.org/10.1016/j.arabjc.2020.07.023
6. Ansari, K. A., Vavia, P. R., Trotta, F., & Cavalli, R. (2011). Cyclodextrin-Based Nanosponges for Delivery of Resveratrol: In Vitro Characterisation, Stability, Cytotoxicity and Permeation Study. *AAPS PharmSciTech*, *12*(1), 279–286. https://doi.org/10.1208/s12249-011-9584-3
7. Api, A. M., Belsito, D., Biserta, S., Botelho, D., Bruze, M., Burton, G. A., Buschmann, J., Cancellieri, M. A., Dagli, M. L., Date, M., Dekant, W., Deodhar, C., Fryer, A. D., Gadhia, S., Jones, L., Joshi, K., Lapczynski, A., Lavelle, M., Liebler, D. C., … Tsang, S. (2020). RIFM fragrance ingredient safety assessment, 4-ethylbenzaldehyde, CAS Registry Number 4748-78-1. *Food and Chemical Toxicology*, *146*, 111700. https://doi.org/10.1016/j.fct.2020.111700
8. Ashafaq, M., Hussain ,Sohail, Alshahrani ,Saeed, Madkhali ,Osama, Siddiqui ,Rahimullah, Khuwaja, Gulrana, Alam ,M. Intakhab, & and Islam, F. (2022). Role of cinnamon oil against acetaminophen overdose induced neurological aberrations through brain stress and cytokine upregulation in rat brain. *Drug and Chemical Toxicology*, *45*(2), 633–640. https://doi.org/10.1080/01480545.2020.1747484
9. Babaei, F., Ramalingam, R., Tavendale, A., Liang, Y., Yan, L. S. K., Ajuh, P., Cheng, S. H., & Lam, Y. W. (2013). Novel Blood Collection Method Allows Plasma Proteome Analysis from Single Zebrafish. *Journal of Proteome Research*, *12*(4), 1580–1590. https://doi.org/10.1021/pr3009226
10. Beniaich, G., Zouirech, O., Allali, A., Bouslamti, M., Maliki, I., El Moussaoui, A., Chebaibi, M., Nafidi, H.-A., Bin Jardan, Y. A., Bourhia, M., & Taleb, M. (2023). Chemical Characterization, Antioxidant, Insecticidal and Anti-Cholinesterase Activity of Essential Oils Extracted from Cinnamomum verum L. *Separations*, *10*(6), Article 6. https://doi.org/10.3390/separations10060348
11. Bezerra da Silva, C., Pott, A., Elifio-Esposito, S., Dalarmi, L., Fialho do Nascimento, K., Moura Burci, L., de Oliveira, M., de Fátima Gaspari Dias, J., Maria Warumby Zanin, S., Gomes Miguel, O., & Dallarmi Miguel, M. (2016). Effect of Donepezil, Tacrine, Galantamine and Rivastigmine on Acetylcholinesterase Inhibition in Dugesia tigrina. *Molecules*, *21*(1), 53. https://doi.org/10.3390/molecules21010053
12. Cheng, Y., Zhang, L., Jiang, D., Wang, M., Du, X., Zong, H., Lu, X., & Zhuge, B. (2025). Fermentation of highly toxic reed hydrolysate using *Candida glycerinogenes* to produce platform compound glycerol. *International Biodeterioration & Biodegradation*, *201*, 106069. https://doi.org/10.1016/j.ibiod.2025.106069
13. Choi, J., Yoon, J., & Kim, M. (2022). Optimization of Fermentation Conditions of Artemisia capillaris for Enhanced Acetylcholinesterase and Butyrylcholinesterase. *Foods*, *11*(15), Article 15. https://doi.org/10.3390/foods11152268
14. Darwesh, O. M., Eweys, A. S., Zhao, Y.-S., & Matter, I. A. (2023). Application of environmental-safe fermentation with Saccharomyces cerevisiae for increasing the cinnamon biological activities. *Bioresources and Bioprocessing*, *10*(1), 12. https://doi.org/10.1186/s40643-023-00632-9
15. Dehghanian, F., Kalantaripour, T. P., Esmaeilpour, K., Elyasi, L., Oloumi, H., Pour, F. M., & Asadi-Shekaari, M. (2017). Date seed extract ameliorates β-amyloid-induced impairments in hippocampus of male rats. *Biomedicine & Pharmacotherapy*, *89*, 221–226. https://doi.org/10.1016/j.biopha.2017.02.037
16. Devi, V. S. M. and V. N. M. (2023). Molecular Docking Study For Adenosine Monophosphate-Activated Protein Kinase Agonist From Syzygium Cumini For Treatment Of Diabetes Mellitus-Type 2. *Journal of Pharmaceutical Negative Results*, 1078–1085. https://doi.org/10.47750/pnr.2023.14.S02.132
17. Di Benedetto, G., Burgaletto, C., Bellanca, C. M., Munafò, A., Bernardini, R., & Cantarella, G. (2022). Role of Microglia and Astrocytes in Alzheimer's Disease: From Neuroinflammation to Ca2+ Homeostasis Dysregulation. *Cells*, *11*(17), 2728. https://doi.org/10.3390/cells11172728
18. Dizdar, M., Vidic, D., Požgan, F., Štefane, B., & Maksimović, M. (2018). Acetylcholinesterase Inhibition and Anti-

oxidant Activity of N-trans-Caffeoyldopamine and N-trans-Feruloyldopamine. *Scientia Pharmaceutica*, *86*(2), 11. https://doi.org/10.3390/scipharm86020011

19. Djaoudene, O., López, V., Cásedas, G., Les, F., Schisano, C., Bachir Bey, M., & Tenore, G. C. (2019). Phoenix dactylifera L. seeds: A by-product as a source of bioactive compounds with antioxidant and enzyme inhibitory properties. *Food & Function*, *10*(8), 4953–4965. https://doi.org/10.1039/c9fo01125k
20. Djaoudene, O., López, V., Cásedas, G., Les, F., Schisano, C., Bey, M. B., & Tenore, G. C. (2019). Phoenix dactylifera L. seeds: A by-product as a source of bioactive compounds with antioxidant and enzyme inhibitory properties. *Food & Function*, *10*(8), 4953–4965. https://doi.org/10.1039/C9FO01125K
21. Eweys, A. S., Zhao, Y.-S., & Darwesh, O. M. (2022a). Improving the antioxidant and anticancer potential of *Cinnamomum cassia* via fermentation with *Lactobacillus plantarum*. *Biotechnology Reports*, *36*, e00768. https://doi.org/10.1016/j.btre.2022.e00768
22. Eweys, A. S., Zhao, Y.-S., & Darwesh, O. M. (2022b). Improving the antioxidant and anticancer potential of *Cinnamomum cassia* via fermentation with *Lactobacillus plantarum*. *Biotechnology Reports*, *36*, e00768. https://doi.org/10.1016/j.btre.2022.e00768
23. Ferreira, S. L. C., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandão, G. C., da Silva, E. G. P., Portugal, L. A., dos Reis, P. S., Souza, A. S., & dos Santos, W. N. L. (2007). Box-Behnken design: An alternative for the optimization of analytical methods. *Analytica Chimica Acta*, *597*(2), 179–186. https://doi.org/10.1016/j.aca.2007.07.011
24. Galimberti, D., & Scarpini, E. (2016). Old and new acetylcholinesterase inhibitors for Alzheimer's disease. *Expert Opinion on Investigational Drugs*, *25*(10), 1181–1187. https://doi.org/10.1080/13543784.2016.1216972
25. García-Ayllón, M.-S., Small, D. H., Avila, J., & Saez-Valero, J. (2011). Revisiting the Role of Acetylcholinesterase in Alzheimer's Disease: Cross-Talk with P-tau and β-Amyloid. *Frontiers in Molecular Neuroscience*, *4*. https://doi.org/10.3389/fnmol.2011.00022
26. Garland, E. F., Hartnell, I. J., & Boche, D. (2022). Microglia and Astrocyte Function and Communication: What Do We Know in Humans? *Frontiers in Neuroscience*, *16*. https://www.frontiersin.org/articles/10.3389/fnins.2022.824888
27. Halabi, M. F., Shakir, R. M., Bardi, D. A., Al-Wajeeh, N. S., Ablat, A., Hassandarvish, P., Hajrezaie, M., Norazit, A., & Abdulla, M. A. (2014). Gastroprotective activity of ethyl-4-[(3,5-di-tert-butyl-2-hydroxybenzylidene) amino] benzoate against ethanol-induced gastric mucosal ulcer in rats. *PloS One*, *9*(5), e95908. https://doi.org/10.1371/journal.pone.0095908
28. Haro-González, J. N., Castillo-Herrera, G. A., Martínez-Velázquez, M., & Espinosa-Andrews, H. (2021). Clove Essential Oil (Syzygium aromaticum L. Myrtaceae): Extraction, Chemical Composition, Food Applications, and Essential Bioactivity for Human Health. *Molecules*, *26*(21), 6387. https://doi.org/10.3390/molecules26216387
29. Hema, T., Poopal, R.-K., Ramesh, M., Ren, Z., & Li, B. (2023). Developmental toxicity of the emerging contaminant cyclophosphamide and the integrated biomarker response (IBRv2) in zebrafish. *Environmental Science: Processes & Impacts*, *25*(8), 1391–1406. https://doi.org/10.1039/D3EM00186E
30. Jabir, N. R., Khan, F. R., & Tabrez, S. (2018). Cholinesterase targeting by polyphenols: A therapeutic approach for the treatment of Alzheimer's disease. *CNS Neuroscience & Therapeutics*, *24*(9), 753–762. https://doi.org/10.1111/cns.12971
31. John, S. K., & Chandrapragasam, V. (2020). In vitro antioxidant activity of Lactobacillus plantarum against hydrogen peroxide-induced neuronal damage on PC12 cells. *Journal of Applied Biology and Biotechnology*, *8*(5), 84–87. https://doi.org/10.7324/JABB.2020.80511
32. Kang, M., Park, J., & Yoo, S. (2019). Effect of clove powder on quality characteristics and shelf life of kimchi paste. *Food Science & Nutrition*, *7*(2), 537–546. https://doi.org/10.1002/fsn3.833
33. Kumar, M. R., Azizi, N. F., Yeap, S. K., Abdullah, J. O., Khalid, M., Omar, A. R., Osman, Mohd. A., Leow, A. T. C., Mortadza, S. A. S., & Alitheen, N. B. (2022). Clinical and Preclinical Studies of Fermented Foods and Their Effects on Alzheimer's Disease. *Antioxidants*, *11*(5), 883. https://doi.org/10.3390/antiox11050883
34. Lee, H.-S., Yang, E.-J., Lee, T., & Song, K.-S. (2018). Laccase Fermentation of Clove Extract Increases Content of Dehydrodieugenol, Which Has Neuroprotective Activity against Glutamate Toxicity in HT22 Cells. *Journal of Microbiology and Biotechnology*, *28*(2), 246–254. https://doi.org/10.4014/jmb.1709.09052
35. Lee, S., Han, J.-M., Kim, H., Kim, E., Jeong, T.-S., Lee, W. S., & Cho, K.-H. (2004). Synthesis of cinnamic acid derivatives and their inhibitory effects on LDL-oxidation, acyl-CoA:cholesterol acyltransferase-1 and -2 activity, and decrease of HDL-particle size. *Bioorganic & Medicinal Chemistry Letters*, *14*(18), 4677–4681. https://doi.org/10.1016/j.bmcl.2004.06.101
36. Li, Y., Zhang, Y., Dong, L., Li, Y., Liu, Y., Liu, Y., Liu, L., & Liu, L. (2024). Fermentation of *Lactobacillus fermentum* NB02 with feruloyl esterase production increases the phenolic compounds content and antioxidant properties of oat bran. *Food Chemistry*, *437*, 137834. https://doi.org/10.1016/j.foodchem.2023.137834
37. Lombardo, S., & Maskos, U. (2015). Role of the nicotinic acetylcholine receptor in Alzheimer's disease pathology and treatment. *Neuropharmacology*, *96*, 255–262. https://doi.org/10.1016/j.neuropharm.2014.11.018
38. Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, *47*(3), 469–474.
39. McGleenon, B. M., Dynan, K. B., & Passmore, A. P. (1999). Acetylcholinesterase inhibitors in Alzheimer's disease. *British Journal of Clinical Pharmacology*, *48*(4), 471–480. https://doi.org/10.1046/j.1365-2125.1999.00026.x
40. Moslemi, E., Dehghan, P., & Khani, M. (2022). The effect of date seed (Phoenix dactylifera) supplementation on inflammation, oxidative stress biomarkers, and performance in active people: A blinded randomized controlled trial protocol. *Contemporary Clinical Trials Communications*, *28*, 100951. https://doi.org/10.1016/j.conctc.2022.100951
41. Murugan, S., Natarajan, S., Soundararajan, S., Boobalan,

S. K., & Subbiah, M. (2025). Comparative insights into ultraviolet-b radiation-induced biochemical modulations in senna auriculata: a gas chromatography-mass spectrometry profiling study. *Asian Journal of Pharmaceutical and Clinical Research*, 82–89. https://doi.org/10.22159/ajpcr.2025v18i2.53568

42. Nandi, A., Yan, L.-J., Jana, C. K., & Das, N. (2019). Role of Catalase in Oxidative Stress- and Age-Associated Degenerative Diseases. *Oxidative Medicine and Cellular Longevity*, *2019*, 9613090. https://doi.org/10.1155/2019/9613090
43. Ni, H., Peng, L., Gao, X., Ji, H., Ma, J., Li, Y., & Jiang, S. (2019). Effects of maduramicin on adult zebrafish (Danio rerio): Acute toxicity, tissue damage and oxidative stress. *Ecotoxicology and Environmental Safety*, *168*, 249–259. https://doi.org/10.1016/j.ecoenv.2018.10.040
44. Piruthiviraj, P., Swetha, B. R. M., Balasubramanian, C., Krishnamoorthy, R., Gatasheh, M. K., Ahmad, A., Parthasarathi, R., Pandurangan, P., Bhuvaneshwari, V. K., & Vijayakumar, N. (2024). Exploring the potential: Inhibiting quorum sensing through marine red seaweed extracts – A study on *Amphiroa fragilissima*. *Journal of King Saud University - Science*, *36*(4), 103118. https://doi.org/10.1016/j.jksus.2024.103118
45. Plamada, D., & Vodnar, D. C. (2021). Polyphenols—Gut Microbiota Interrelationship: A Transition to a New Generation of Prebiotics. *Nutrients*, *14*(1), 137. https://doi.org/10.3390/nu14010137
46. Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric Quantitation of Antioxidant Capacity through the Formation of a Phosphomolybdenum Complex: Specific Application to the Determination of Vitamin E. *Analytical Biochemistry*, *269*(2), 337–341. https://doi.org/10.1006/abio.1999.4019
47. Ramesh, M., Angitha, S., Haritha, S., Poopal, R.-K., Ren, Z., & Umamaheswari, S. (2020). Organophosphorus flame retardant induced hepatotoxicity and brain AChE inhibition on zebrafish (Danio rerio). *Neurotoxicology and Teratology*, *82*, 106919. https://doi.org/10.1016/j.ntt.2020.106919
48. Rosemberg, D. B., da Rocha, R. F., Rico, E. P., Zanotto-Filho, A., Dias, R. D., Bogo, M. R., Bonan, C. D., Moreira, J. C. F., Klamt, F., & Souza, D. O. (2010). Taurine prevents enhancement of acetylcholinesterase activity induced by acute ethanol exposure and decreases the level of markers of oxidative stress in zebrafish brain. *Neuroscience*, *171*(3), 683–692. https://doi.org/10.1016/j.neuroscience.2010.09.030
49. Saryono, Warsinah, Isworo, A., & Sarmoko. (2020). Anti-inflammatory activity of date palm seed by downregulating interleukin-1β, TGF-β, cyclooxygenase-1 and -2: A study among middle age women. *Saudi Pharmaceutical Journal*, *28*(8), 1014–1018. https://doi.org/10.1016/j.jsps.2020.06.024
50. Saxena, B. (2024). Eugenol as Neuro-Phytomedicine: Recent Trends Pertaining to the Treatment of Neurological Disorders. In *NeuroPhytomedicine*. CRC Press.
51. Singleton, V. L., Orthofer, R., & Lamuela-Raventós, R. M. (1999). [14] Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. In *Methods in Enzymology* (Vol. 299, pp. 152–178). Academic Press. https://doi.org/10.1016/S0076-6879(99)99017-1
52. Sinha, A. K. (1972). Colorimetric assay of catalase. *Analytical Biochemistry*, *47*(2), 389–394. https://doi.org/10.1016/0003-2697(72)90132-7
53. Song, L., Li, M., Feng, C., Sa, R., Hu, X., Wang, J., Yin, X., Qi, C., Dong, W., & Yang, J. (2022). Protective effect of curcumin on zebrafish liver under ethanol-induced oxidative stress. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *258*, 109360. https://doi.org/10.1016/j.cbpc.2022.109360
54. Thorakkattu, P., Khanashyam, A. C., Shah, K., Babu, K. S., Mundanat, A. S., Deliephan, A., Deokar, G. S., Santivarangkna, C., & Nirmal, N. P. (2022). Postbiotics: Current Trends in Food and Pharmaceutical Industry. *Foods*, *11* (19), 3094. https://doi.org/10.3390/foods11193094
55. Umamaheswari, S., Priyadarshinee, S., Bhattacharjee, M., Kadirvelu, K., & Ramesh, M. (2021). Exposure to polystyrene microplastics induced gene modulated biological responses in zebrafish (*Danio rerio*). *Chemosphere*, *281*, 128592. https://doi.org/10.1016/j.chemosphere.2020.128592
56. Wang, A. N., Yi, X. W., Yu, H. F., Dong, B., & Qiao, S. Y. (2009). Free radical scavenging activity of Lactobacillus fermentum in vitro and its antioxidative effect on growing–finishing pigs. *Journal of Applied Microbiology*, *107*(4), 1140–1148. https://doi.org/10.1111/j.1365-2672.2009.04294.x
57. Wang, J., Xiao, S., Cai, Q., Miao, J., & Li, J. (2023). Antioxidant Capacity and Protective Effects on H2O2-Induced Oxidative Damage in PC12 Cells of the Active Fraction of Brassica rapa L. *Foods*, *12*(10), Article 10. https://doi.org/10.3390/foods12102075
58. Wang, L., Li, K., Cui, Y., Peng, H., Hu, Y., & Zhu, Z. (2023). Preparation, structural characterization and neuroprotective effects to against H2O2-induced oxidative damage in PC12 cells of polysaccharides from *Pleurotus ostreatus*. *Food Research International*, *163*, 112146. https://doi.org/10.1016/j.foodres.2022.112146
59. Zaki, A. G., El-Sayed, E.-S. R., Abd Elkodous, M., & El-Sayyad, G. S. (2020). Microbial acetylcholinesterase inhibitors for Alzheimer's therapy: Recent trends on extraction, detection, irradiation-assisted production improvement and nano-structured drug delivery. *Applied Microbiology and Biotechnology*, *104*(11), 4717–4735. https://doi.org/10.1007/s00253-020-10560-9
60. Zemlan, F. P., Thienhaus, O. J., & Bosmann, H. B. (1989). Superoxide dismutase activity in Alzheimer's disease: Possible mechanism for paired helical filament formation. *Brain Research*, *476*(1), 160–162. https://doi.org/10.1016/0006-8993(89)91550-3