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ORIGINAL ARTICLE

Potentiation of anti-Alzheimer activity of curcumin by probiotic *Lactobacillus rhamnosus* UBLR-58 against scopolamine-induced memory impairment in mice

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Abstract

Curcumin, a major component of Indian saffron through clinical studies, revealed its neuroprotective effect in neurodegenerative diseases. However, it has not been utilized alone orally due to its low bioavailability. There are certain strategies to overcome the drawbacks such as poor absorption and low aqueous solubility. Many strategies are utilized to increase the systemic availability of curcumin. Among them, the steady intestinal and liver metabolism of curcumin by a curcumin adjuvant (enzyme inhibitor/inducer) is an important and less engrossed strategy for improving the overall systemic bioavailability of curcumin. Here, we assess the effect of probiotic *Lactobacillus rhamnosus* as a curcumin adjuvant (potentiate the effect of curcumin) in scopolamine-induced dementia in mice. To induce amnesia, scopolamine was used in a mouse model (1 mg/kg, daily for 10 days i.p.). After execution of behavioural tests (Morris water maze test), brains and liver were isolated for further neurochemical and histopathology examination. Our results showed a significant increase in antioxidant enzyme levels in curcumin with a probiotic group compared with curcumin alone. Besides, histopathology study results showed less neuronal damage of curcumin with probiotics as compared with the curcumin and scopolamine alone groups. Additionally, curcumin with probiotics improved memory and cognitive functions in the behavioural study with the significance of $p \leq 0.0001$. In conclusion, curcumin with probiotics has greater activity as compared with curcumin alone and reverses the hallmarks of Alzheimer's disease (AD).

Keywords Curcumin · Probiotic · *Lactobacillus rhamnosus* · Neurodegeneration · Beta-glucuronidase

Introduction

AD is an irrevocable brain disorder that degrades neurons and leads to dementia (Weller and Budson 2018). Dementia is a syndrome that includes amnesia and deterioration in thinking, behaviour, and ability to carry out daily activities (Bondi et al. 2017). Foremost, two histopathological features of AD reported the extracellular accumulation of amyloid-beta plaques (Kametani and Hasegawa 2018) and the formation of neurofibrillary webs (Chesser et al. 2013; Gong et al. 2018). According to the World Health Organization, currently worldwide 50 million individuals are suffering from dementia. In India, 4 million people suffered from Alzheimer's disease and the number was estimated to reach almost 7.5 million by the end of 2030 (Sathianathan and Kantipudi 2018). In 2019, the cost of this disease is $290 billion, including $195 billion to Medicare payments. However, USFDA permitted only a few drugs for the management of AD over the past years. Not merely that, these approved drugs include donepezil, galantamine, and rivastigmine or their combination (World Health Organization 2019), which most often provide short-term and inadequate symptomatic relief escorted by severe side effects at a low dose. These minimal effects were incapable of slowing down the development of AD. Therefore, there is

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an urge to develop new drugs to stop or reverse the development of AD. Numerous clinical studies indicated that curcumin (diferuloyl methane) is a major component (2–6%) of the perennial herb *Curcuma longa* Lin and has a protective role in AD by scavenging free radicals, hampering the development and disaggregation of amyloid-beta plaques, diminishing the hyperphosphorylation of tau and enhancing tau tangle clearance, impeding acetylcholinesterase and anti-inflammatory properties, and having metal chelation properties (Gupta et al. 2013; Li et al. 2008; Tang and Taghibiglou 2017). Till date, there are more than 6000 citations of curcumin for enhancing the bioavailability of curcumin and 215 clinical trials, out of which 95 studies are completed (Di Martino et al. 2017; Kunnumakkara et al. 2019). The poor bioavailability has inspired researchers to use countless ways to deal with incrementing the bioavailability of curcumin. In recent years, many strategies and techniques are used to increase solubility and permeability including modification of the solid state (amorphous solid dispersion), the reduction in particle size (micronization), and creation of a supersaturation solution (nanosuspension and nanoemulsion) or encapsulation into nanoparticles (Anand et al. 2007). However, the efficacy and safety and stability of this formulation are still the main concern, and these strategies increase bioavailability 2- to 10-fold (Heger et al. 2014; Prasad et al. 2014). Other and most significant methodologies for improving the overall systemic bioavailability of curcumin include slowdown or inhibition of intestinal and liver metabolism of curcumin by a curcumin adjuvant (enzyme inhibitor/inducer), i.e. curcumin-piperine complex. Several preclinical studies revealed that curcumin goes through rapid metabolism in its metabolite after oral administration. If enzyme β-glucuronidase, which hydrolyzes back curcumin, is raised then the problem of rapid metabolism will be overwhelmed and overall systemic bioavailability will be overwhelmed (Ozawa et al. 2017). The term *probiotics* is defined as "live microorganisms that when administered in adequate amounts, confer a health benefit on the host". *Lactobacillus rhamnosus* (*L. rhamnosus*) confers benefit by boosting the microbiota and possessing the counter aliment property (Westerik et al. 2018). *L. rhamnosus* strain UBLR-58 (MTTC 8712) was secluded by Unique Biotech Ltd., India. It is a gram-positive short heterofermentative anaerobe. In vitro studies in mice revealed that *L. rhamnosus* is safer for human consumption (LD_{50} is greater than 50 g/kg/day) (Zhou et al. 2000). Currently, clinical trials are being conducted on probiotic *L. rhamnosus* for dermatitis, diabetes mellitus, colorectal cancer, ulcerative colitis, Crohn's disease, constipation, diarrhoea irritable bowel diseases, candidiasis, rheumatoid arthritis, and polycystic ovary syndrome, and it shows potential as a probiotic (Athari et al. 2018; Biernat et al. 2019; Kajander et al. 2008; Sanders 2008; Westerik et al. 2018). As reported, *L. rhamnosus* synthesizes and generates beta-glucuronidase in the gut (Pham et al. 2000). We hypothesized that *L. rhamnosus* will potentiate the consequence of curcumin. Hence, to test this hypothesis we examined the curcumin combination with *L. rhamnosus* in scopolamine-induced dementia in mice.

Materials and methods

Probiotic

The probiotic strain used was *Lactobacillus rhamnosus* UBLR-58 (Unique Biotech Ltd., India). Acid tolerance test, bile tolerance test, and bile salt hydrolase (BSH) activity were evaluated by the reported method (Patel et al. 2020).

Animals and care

Thirty Swiss albino female mice weighing 25–30 g were purchased. The protocol was permitted by the Institutional Animal Ethics Committee (IAEC) and accompanied by the guidance of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Mice were housed in a temperature-controlled room (22 ± 2 °C, relative humidity 55 ± 3%) with a regimen of light darkness of 12 h × 12 h and had ad libitum access to food and water.

Induction of scopolamine-induced dementia and treatment

Animals were acclimatized for 1 week and then randomly assigned into five groups, each bearing six animals (Table 1):

- Group I (G1) Negative control—receives saline (1 ml/kg/day, 10 days, i.p.)
- Group II (G2) Scopolamine-induced—receives scopolamine (1 ml/kg/day, 10 days, i.p)
- Group-III (G3) Positive control—receives scopolamine (1 ml/kg/day, 10 days, i.p) + donepezil (2 mg/kg/day 10 days, i.p)
- Group IV (G4) Curcumin alone—receives scopolamine (1 ml/kg/day, 10 days, i.p) + curcumin (205 mg/kg/day, 10 days, p.o)
- Group V (G5) Curcumin combination with probiotics—receives scopolamine (1 ml/kg/day, 10 days, i.p) + curcumin (205 mg/kg/day, 10 days, p.o) + *Lactobacillus rhamnosus* (1 × 106 CFU/day, 10 days, p.o)

At the end of the study, behavioural parameters were evaluated. Instantly after performing behavioural tests, mice were sacrificed by a high dose of anaesthesia, and brains were isolated and further used for neurochemical assay and

Table 1 Neurochemical and antioxidant levels in the brain homogenate of mice

	Ache (min/g of tissue)	LPOx (μg/mg)	SOD (μg/mg)	GPx (μg/mg)	Catalase (μg/mg)
Control (G1)	2.61 ± 0.33	1.03 ± 0.54	74.88 ± 0.10	5.9 ± 0.93	19.16 ± 0.66
Scopolamine (G2)	$10.3 \pm 0.38^{****a}$	$5.15 \pm 0.64^{****a}$	$15.63 \pm 0.38^{****a}$	$1.83 \pm 0.34^{****a}$	$2.87 \pm 0.23^{****a}$
Donepezil (G3)	$6.1 \pm 0.79^{****b}$	$0.71 \pm 0.26^{****b}$	$46.45 \pm 0.65^{****b}$	$15.13 \pm 0.89^{****b}$	$18.60 \pm 0.23^{****b}$
Curcumin (G4)	11.4 ± 0.65^{ns}	4.35 ± 0.50^{ns}	23.16 ± 0.23^{ns}	2.37 ± 0.83^{ns}	3.61 ± 0.12^{ns}
Curcumin+ probiotics (G5)	$7.11 \pm 0.91^{****b}$	$0.53 \pm 0.19^{****b}$	$48.14 \pm 0.80^{****b}$	$15.39 \pm 0.21^{****b}$	$20.08 \pm 0.70^{****b}$

The values are the mean ± SD with $n = 6$ per group

Values of $p \geq 0.05$ = ns while p values ≤ 0.05, ≤ 0.01, ≤ 0.001 and ≤ 0.0001 were expressed as *, **, *** and ****, respectively

[a] The significance level between G1 and G2 groups

[b] The significance level between G2 in comparison with G3, G4, and G5 groups

histopathologic examination (the brains of two mice from each group). The experimental design is shown in Fig. 1.

Behavioral parameter

Determination of spatial memory using the Morris water maze (MWM) test

In the MWM test, a spherical maze of 122-cm diameter and 51-cm height with a circular acrylic platform of 10-cm diameter and 35-cm height was filled with water. Below the surface of 1 cm in the water, a platform was placed of about 10-cm diameter. In the training sessions, the mice were permitted to navigate the submerged platform. The maximum cutoff time given to mice for searching the submerged platform is 2 min and allowed to halt on the platform for about 15 s. Escape latency (time to reach the platform) was recorded in each trial, and the animals that failed to navigate to the platform within 120 s were placed on the platform. Animals were given trials of four sessions daily. The test session was performed during the treatment period on 0, 1, 3, 5, 7, and 9 days, respectively (Nunez 2008; Vorhees and Williams 2006).

Neurochemical assay

Assay of acetylcholinesterase (Ache) activity

Estimation of acetylcholinesterase activity was carried out by Ellman's method (Ellman et al. 1961), where 0.5 ml of brain homogenate was incubated with 0.1 ml of Ellman's reagent and 2.7 ml of phosphate buffer for 5 min, then 0.1 ml freshly prepared acetylcholine chloride was subsequently added in the buffer (pH 8), and absorbance was recorded immediately with 1-min time intervals till 10 min, at 412 nm. Acetylcholinesterase activity was expressed as Ache hydrolyzed per minute per gram of protein.

Lipid peroxidation (LPO) assay

Estimation of LPO was carried out using TBARS assay, where 0.5 ml of brain homogenate was mixed with 0.5 ml of distilled water and 1 ml of 10% trichloroacetic acid (TCA); the resulting solution was centrifuged at 3000 rpm for 10 min. The supernatant (0.2 ml) was collected, and 0.1 ml of thiobarbituric acid (0.375 %) was added (Ohkawa et al. 1979). The

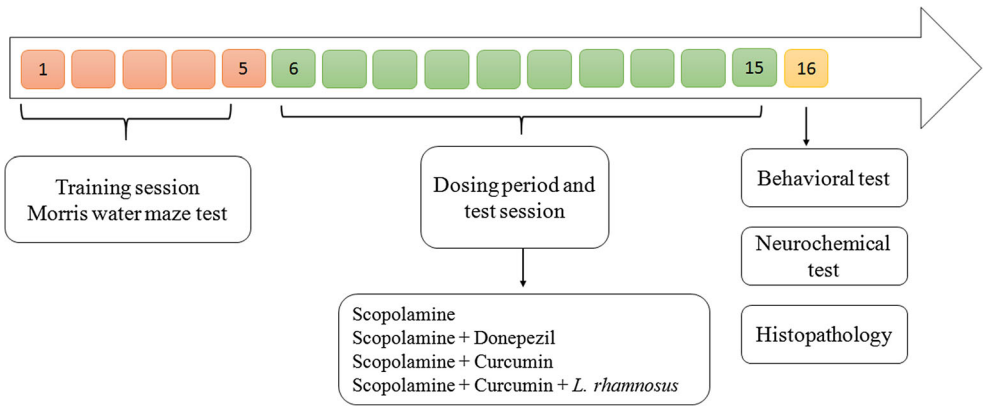

Fig. 1 Experimental design

solution was placed into a water bath at 80 °C for 40 min and cooled to room temperature. The amount of malondialdehyde (MDA) formed after reacting with TBARs was measured at 532 nm. The molar extinction coefficient used for calculation is 1.56×10^{-5}.

Assay of superoxide dismutase (SOD)

SOD activity was measured spectrophotometrically by incubating 0.5 ml of brain homogenate with 0.1 ml of NADH for 90 s. In the same solution, 0.5 ml, 10% acetic acid, and 4 ml butanol were added. The colour change was measured at 520 nm by collecting the upper layer of butanol. SOD activity was expressed in micrograms per milligram of tissue (Rinwa and Kumar 2012; Rukmini et al. 2004).

Assay of glutathione peroxidase (GPx)

Estimation of GPx was carried out by incubating 0.2 ml brain homogenate with 0.4 ml of sodium azide, disodium EDTA solution, and hydrogen peroxide (H_2O_2) each, for 10 min at 37 °C. Then, this reaction was stopped by adding 0.5 ml of TCA solution. The resulting solution was centrifuged at 3000 rpm for 10 min. The supernatant (0.5 ml) was collected, and 0.5 ml of DTNB along with 4 ml of disodium hydrogen phosphate was added. Absorbance was measured at 420 nm. GPx activity was expressed in micrograms per milligram of tissue (Weydert and Cullen 2010).

Assay of catalase (CAT)

CAT activity was assessed using a method of Luck and Bergmeyer (1971). Here hydrogen peroxide breakdown was assessed. In 0.2 ml brain homogenate add 3 ml of H_2O_2 phosphate buffer, absorbance was immediately recorded at 240 nm. CAT activity was expressed in micrograms per milligram of tissue (Goverdhan et al. 2012).

Statistical analysis

All the values were expressed as mean ± standard deviations ($n = 6$). Statistical analysis was performed using GraphPad Prism version 7.1, by one-way ANOVA followed by multiple comparisons.

Results

Acid and bile salt tolerance and BSH activity

The *L. rhamnosus* strain showed 94.23%, 84.25%, and 82.39% of survivability after 60 min, 120 min, and 180 min of incubation (pH 3), respectively. The *L. rhamnosus* strain showed 98.92%, 94.25%, and 89.21% survivability with 0.1, 0.3, and 0.5% bile salt for 3 h, respectively. Besides, it also showed high BSH ability (> 12 mm precipitation zones). This showed that *L. rhamnosus* strains can survive in gastric and intestinal fluids.

Behavioural parameter

During the test session, the control mice easily reached the platform, whereas mice of donepezil and curcumin in combination with the *L. rhamnosus* group initially on days 0 and 1 possess difficulty in finding the hidden platform, then afterwards showed improved memory to catch the hidden platform ($p \leq 0.0001$). In the scopolamine-only-administered mice, there was an increase in time to catch the platform which depicts that there is memory impairment in mice, as shown in Fig. 2.

Neurochemical assay

Effect on acetylcholinesterase activity

Mice, when exposed to scopolamine, possess a significant increase in acetylcholinesterase activity (10.3 ± 0.38, $p < 0.0001$) compared with negative control mice (2.61 ± 0.33). Positive control and curcumin combination with the *L. rhamnosus* group revealed significant decline in acetylcholinesterase activity (6.1 ± 0.79, 7.11 ± 0.91, $p < 0.0001$) when compared with G2 group mice.

Effect on lipid peroxidation activity

Revelation to mice with scopolamine showed an increase in the level of MDA in the brain tissue (5.15 ± 0.64, $p < 0.0001$) as compared with G1 mice (1.03 ± 0.54). Concurrent treatment of donepezil and curcumin combination with *L. rhamnosus* exhibited a significant reduction in MDA level (0.71 ± 0.26, 0.53 ± 0.19, $p < 0.0001$) as compared with those treated with scopolamine alone. Curcumin compared with the scopolamine-induced group showed no significance in lipid peroxidation (4.35 ± 0.50, ns)

Effect on superoxide dismutase activity

In the scopolamine-induced group, mice showed less SOD activity in the brain (15.63 ± 0.38, $p < 0.0001$) as compared with the G1 group (74.88 ± 0.10). There is no significant change in the enzyme activity in curcumin-alone-treated mice compared with G2 mice (23.16 ± 0.23, ns), whereas in positive control and curcumin combination with *L. rhamnosus* groups, there is a substantial increase in enzyme activity compared with the scopolamine group (46.45 ± 0.65, 48.14 ± 0.80, $p \leq 0.0001$).

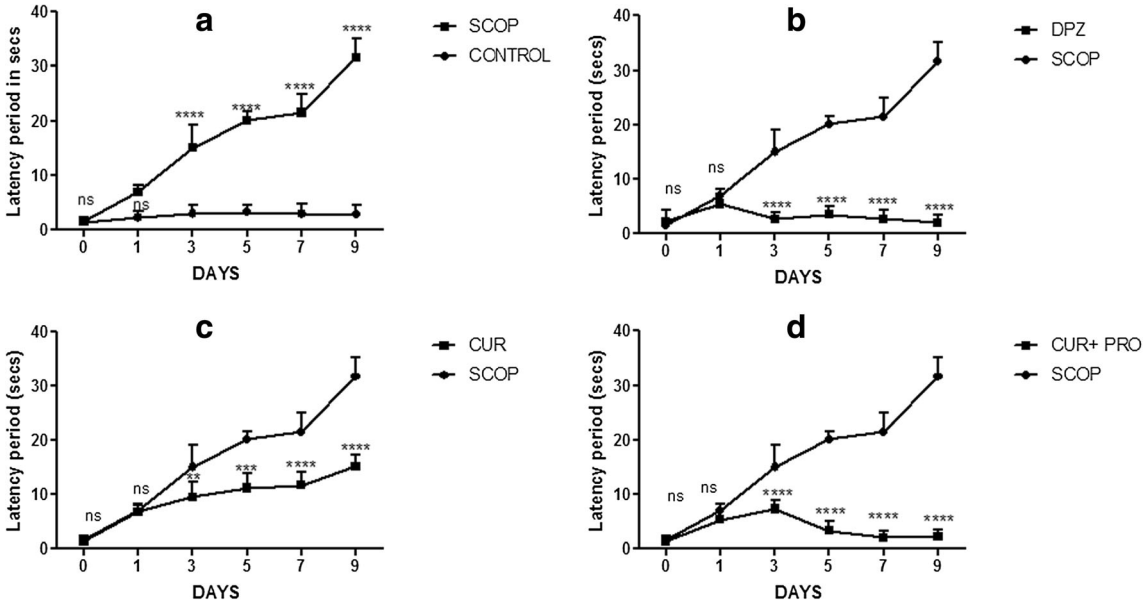

Fig. 2 Graph of the escape latency in Morris water test: between control and scopolamine, scopolamine and donepezil, scopolamine and curcumin, and scopolamine and curcumin with *L. rhamnosus*. The data are expressed as mean ± SD ($n = 6$). Value of $p \geq 0.05$ was shown by marking "ns" while p values ≤ 0.05, ≤ 0.01, ≤ 0.001 and ≤ 0.0001 were expressed as *, **, *** and ****, respectively

Effect on GPx activity

In the scopolamine group, there is a reduction in the enzyme activity of GPx (1.83 ± 0.34, $p < 0.0001$) compared with the G1 group (5.9 ± 0.93). In the positive control and curcumin combination with the probiotics group, results showed a significant rise in enzyme activity (15.13 ± 0.89, 15.39 ± 0.21, $p < 0.001$), whereas the curcumin-alone group showed insignificant change in enzyme level (2.37 ± 0.83, ns) when compared with the scopolamine group.

Effect on the activity of CAT

CAT is an enzyme that collapses hydrogen peroxide into water and a singlet oxygen molecule. An acquaintance of mice to scopolamine possesses a decline in the activity of CAT (2.87 ± 0.23, $p < 0.0001$) as compared with the negative control (19.16 ± 0.66) which depicts that there is oxidative damage in the brain. Additionally, treating mice with donepezil and curcumin combination with probiotics showed an elevated level of CAT enzyme activity (18.60 ± 0.23, 20.08 ± 0.70, $p < 0.0001$) as compared with those treated with scopolamine alone.

Histology study

In histopathology examination, as shown in Fig. 3, the G1 group possesses normal neuronal cells, whereas the G2 group showed neuronal damage with dark basophilic cytoplasm and dark condensed nuclei, less neuronal cells with pyknosis, and deposition of amyloid plaques (arrow). In the G3 group, the hippocampus possesses normal histology and mild neuronal damage. However, in the G4 group the hippocampus shows neuronal damage and presence of dark neurons with shrunken cytoplasm and pyknosis and reduced neuronal cells. The G5 group possesses mild neuronal damage, and cell integrity is not lost.

Discussion

AD is the sixth foremost reason for death in the USA. The predictable lifetime risk of AD at the age of 45–65 years is 10.6% and 19.5% in men and women, respectively (Bondi et al. 2017; Weller and Budson 2018). The affected region of the brain possesses neuron atrophy, astrogliosis, progressive loss of structure, and function of neurons (Glenner and Wong 1984; Karlinsky 1986; Spencer and Lal 1983). This leads to the degeneration of cholinergic neurons which are responsible for memory loss (Chen et al. 2004; Tsukada et al. 2004). Scopolamine a non-selective muscarinic blocker that blocks cholinergic transmission and causes impaired memory loss in mice (Konar et al. 2019). Scopolamine models used are considered most consistent and devour a value in experiments where an AD-like condition is observed (San Tang 2019). Therefore, in this study, probiotic *L. rhamnosus* with curcumin against scopolamine induced destruction of neurons with memory loss in animals. In the existing study, memory functions were appraised by the MWM test in scopolamine-induced mice and curcumin treatment in combination with *L. rhamnosus* significantly and dose-dependently improved memory loss in the MWM test. The cholinergic system, which is present in the hippocampus region, plays a

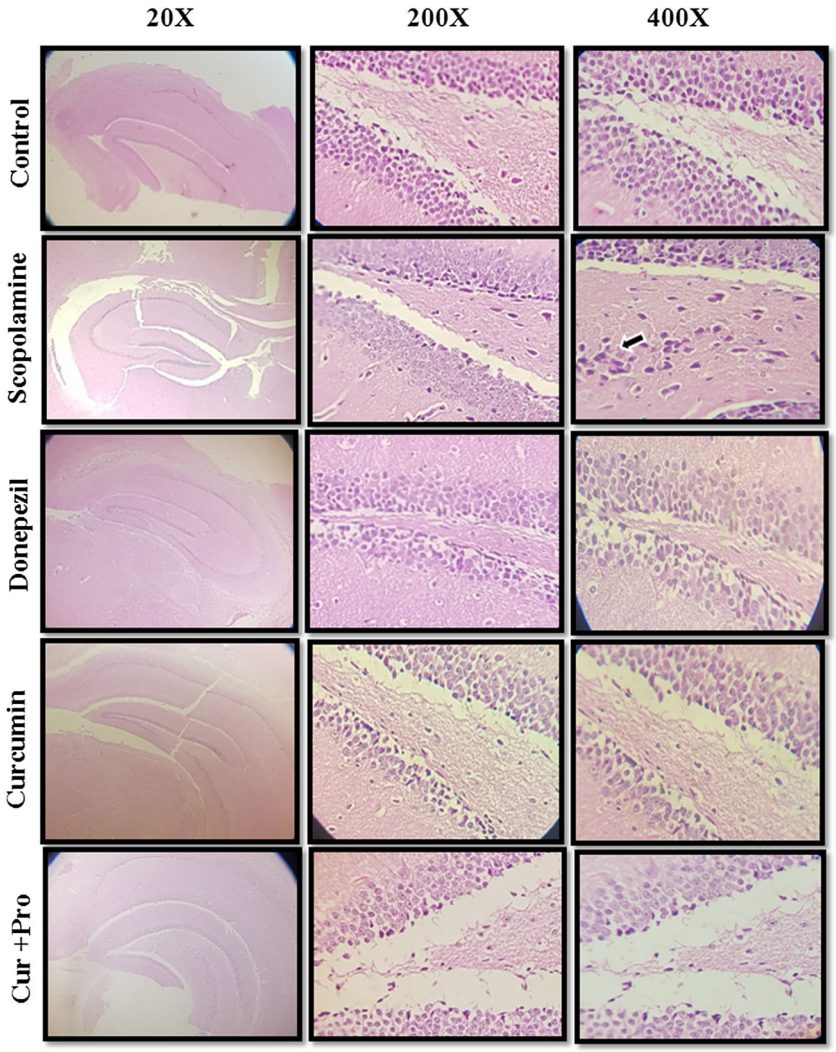

Fig. 3 Representative histology study of the mouse brain hippocampus region (×20, ×200, ×400). Control group: normal neuronal cells; scopolamine group: neuronal damage with dark basophilic cytoplasm and dark condensed nuclei, less neuronal cells with pyknosis, deposition of amyloid plaques (arrow); scopolamine + donepezil group: hippocampus showing normal histology, less neuronal damage; scopolamine + curcumin group: hippocampus showing neuronal damage and presence of dark neurons with shrunken cytoplasm and pyknosis and reduced neuronal cells; scopolamine + curcumin combination with probiotics: neuronal damage is less, cell integrity is not lost

chief role, as it possesses neurotransmitter acetylcholine which is associated with memory and learning ability (Maurer and Williams 2017). The degradation of this enzyme by acetylcholinesterase diminishes its pharmacological action. The AD level of acetylcholine is lowered due to the elevation of acetylcholinesterase (Decker and Duncan 2020). In the current investigation, scopolamine caused a substantial decline in acetylcholinesterase activity. Treatment of curcumin in combination with *L. rhamnosus* significantly restored acetylcholinesterase activity resulting in recovery and retaining memory processes. In addition to behavioural abnormalities, scopolamine promotes oxidative stress and demolishes the antioxidant defence system of the brain and results in neurodegeneration. Curcumin + *L. rhamnosus*-treated animals exhibited a significant rise in the antioxidant enzyme system compared with the scopolamine-induced mice. Despite its well-documented therapeutic activity, the development of curcumin has been limited by its poor oral bioavailability. Studies conveyed that curcumin gets metabolized in the liver and intestine. Thus, the concentration of curcumin after oral ingestion cannot be attained and maintained in the blood. In the existing study, oral bioavailability of curcumin co-administered with *L. rhamnosus* is enhanced. *L. rhamnosus* bacteria can synthesize and release a β-d-glucuronidase enzyme which reverts curcumin into the active form (Biernat et al. 2019; Kajander et al. 2008; Sanders 2008; Westerik et al. 2018). From this study, it was observed that the memory-enhancing effect and level of the antioxidant enzyme were elevated in the curcumin combination with *L. rhamnosus* compared with curcumin alone. The potentiation in the effects of curcumin indicates that *L. rhamnosus* might have raised the absorption of curcumin probably via the increasing free form of curcumin by induction of β-glucuronidase enzyme at an intestinal site.

Conclusion

The present study remarkably demonstrates that curcumin in combination with *L. rhamnosus* improves memory, learning,

and antioxidant system in scopolamine-induced mice. Besides, these outcomes offer a scientific justification of *L. rhamnosus* and curcumin co-administration, which might slow the progression of AD. Clinical pharmacokinetic studies are needed to address the probiotic *L. rhamnosus* as a bioavailability enhancer of curcumin.

Code availability Not applicable

Author contributions All data were generated in-house, and no paper mill was used. The experiments performed were planned and conceptualized by SP and CP. Experimental methodology was executed by SP. GraphPad Prism 7 software used to interpret results was done by SP and CP. Data curation was done by SP, SA, and CP. Investigation was done by SP and CP. CP and SP wrote and prepared the original draft. CP reviewed and edited the paper. SA supervised the experiment.

Data availability Not applicable

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethics approval IAEC (Institutional Animal Ethics Committee) approved the experimental protocol SSR/IAEC/2019/03.

Consent to participate Not applicable

Consent for publication Authors give consent for information to be published in the journal- Naunyn-Schmiedeberg's Archives of Pharmacology

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RESEARCH ARTICLE

Lactobacillus Rhamnosus UBLR-58 and Diclofenac Potentiate the Anti-Alzheimer Activity of Curcumin in Mice

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Abstract:

Background: Curcumin a providential spice has its role in protecting the brain from neurodegeneration. Despite its ubiquitous role, it is not exploited alone due to its hampered bioavailability. By restraining the intestinal and liver enzymatic metabolism, one can boost the bioavailability of curcumin and promotes reabsorption of the curcumin. Diclofenac inhibits uridine 5'-diphospho-glucuronosyltransferase enzymes specifically responsible for metabolism and elimination of curcumin. The *Lactobacillus rhamnous* able to synthesize and release β-d-glucuronidase enzyme which reverts curcumin into the active form.

Objective: In this research, we are combining curcumin with *Lactobacillus rhamnosus* and diclofenac as an adjuvant with curcumin to potentiate anti-Alzheimer effect in mice impaired with memory by scopolamine.

Methods: To induce amnesia, scopolamine was used in mice model (1mg/kg, daily for 10 days i.p.). After execution of behavioural tests (Morris Water Maze test), brains and liver were isolated for further neurochemical and histopathology examination.

Results: Our finding showed a marked rise in the level of antioxidant enzymes in curcumin with *L. rhamnosus* and diclofenac compared to curcumin alone. Additionally, in the behavioural study revealed that cognition in mice with curcumin adjuvant with *L. rhamnosus* and diclofenac showed a marked improvement. The histology study proves that curcumin alone possesses less and a non-significant neuroprotective effect as compared to curcumin with *L. rhamnosus* and diclofenac.

Conclusion: This entire outcome ratifies that curcumin with *L. rhamnosus* and diclofenac have higher activity as compared to curcumin alone, which reversed the cognition in the Alzheimer disease model.

Keywords: Alzheimer's disease; curcumin; Probiotics; diclofenac; *Lactobacillus rhamnosus*.

1. INTRODUCTION

Alzheimer's disease (AD) leads to dementia due to neurodegeneration. Histopathological manifestations of AD involve accretion of amyloid-beta plaques and hyperphosphorylated tau protein [1-3]. The Alzheimer association report clearly states 47 million individuals have dementia, which is to be dual in coming years [4], and yet, there are few USFDA approved drugs for AD. These approved drugs include cholinesterase inhibitors and N methyl-D-aspartate (NMDA) receptor antagonist that act by same mechanism, gives merely short-term relief and possess severe side effects. There is a need to develop a drug, which gives relief from AD. Curcumin is a golden drug, which produces effect in AD by numerous mechanisms some of them includes scavenges free radicals in the brain, hampers the creation and endorses the disaggregation of plaques, enhances neurofibrillary tangles clearance, inhibits acetylcholinesterase, anti-inflammatory, possess metal chelation property and also have other beneficial effects [5-7]. However, use of this molecule is hampered due to its low oral bioavailability with reasons mentioned as poor aqueous solubility, poor absorption and faster metabolism [8]. This is an imperative problem, which creates costlier treatments to overcome this problem such as liposomes, nanovesicles, nanoparticle, these works by increasing solubility and absorption [9-12]. Any substantial enhancement in bioavailability by which the dose is reduced as well as the

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cost of the therapy will be in greater demand. Here we are using strategy to increase bioavailability by targeting the metabolism step and increasing the absorption of curcumin so that the level of curcumin remains more time for its effect. UDP-glucuronosyl transferase (UGTs) is an enzyme, which metabolized curcumin. UGTs involved in curcumin metabolism are UGT1A1, UGT1A3, UGT1A8, UGT1A10, UGT1A9 and UGT2B7 [13]. Diclofenac is a known inhibitor of UGTs, which inhibits UGT1A1, UGT1A3, UGT1A9 and UGT2B7, hence diclofenac will reduce the metabolism of curcumin [14-16]. An additional enzyme, which hydrolyzes back curcumin metabolites into free form curcumin is, Beta-glucuronidase [17]. Beta glucuronidase is generated form probiotics, *Lactobacillus rhamnous* in the intestine and increase the absorption of the same [18-21]. We have investigated the neuroprotective effect of curcumin in combination with *L. rhamnosus* and diclofenac (as bioenhancer) in Alzheimer's disease.

2. MATERIALS AND METHOD

2.1 Chemical compound and probiotic

Curcumin 98% pure was procured from Loba Chemie Pvt. Ltd, Mumbai, India. The strain of probiotics *L. rahmnous* was procured from Unique Biotech Ltd, India. Diclofenac was procured from chemie.

2.2 Animals and care

Female Swiss albino mice 25-28 gm were used in the experiment. The protocol was permitted by IAEC (SSR/IAEC/2019/03) and was performed as per the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines. Mice were acclimatized for seven days' prior experiment. Were housed in a temperature-controlled room (22±2 °C, relative humidity 55±3%) with light darkness of 12 h × 12 h. with ad libitum access to water and food

2.3 Induction of scopolamine-induced dementia and treatment

Group A (CON) Negative control-receive saline (1ml/kg/day, i.p.);

Group B (SCOP) Scopolamine-induced- receives scopolamine (1mg/kg/day, i.p.);

Group-C (DPZ) Positive control- receives scopolamine (1mg/kg/day, i.p.) + Donepezil (2 mg/kg/day, i.p.)

Group D (CUR) Curcumin alone- receives scopolamine (1mg/kg/day, i.p.) + Curcumin (205 mg/kg/day, p.o.)

Group E (CUR + PRO) Curcumin combination with probiotics receives scopolamine (1mg/kg/day, i.p.) + Curcumin (205 mg/kg/day, p.o.) + *Lactobacillus rahmnous* (1 x 10^6 CFU/day, p.o)

Group F (CUR +DICLO) Curcumin combination with probiotics receives scopolamine (1mg/kg/day, i.p.) + Curcumin (205 mg/kg/day, p.o.) + Diclofenac sodium (2mg/kg, p.o.)

Group G (CUR + COMBO) Curcumin combination with probiotics receives scopolamine (1mg/kg/day, i.p.) + Curcumin (205 mg/kg/day, p.o.) + *Lactobacillus rahmnous* (1 x 106 CFU/day, p.o.) + Diclofenac sodium (2mg/kg, p.o.)

Induction and treatment was given simultaneously for 10 days. After completion of behavioral tests; mice were sacrificed by a high dose of anesthesia; brains were isolated for further neurochemical and histopathology examination.

2.3.1 Behavioral parameter

Determination of spatial memory through the MWM test

In MWM test, a spherical maze of 122 cm diameter and 51 cm height with a circular acrylic platform of 10 cm diameter and 35 cm height was filled with water. Below the surface of 1 cm in the water, a platform was placed of about 10 cm diameter. In the training sessions, the mice were permitted to navigate the submerged platform. The cut-off-time maximum given to mice for searching the submerged platform is 2 min and allowed to halt on the platform for about 15 s. Escape latency (time to grasp the platform) was noted and animals that failed to navigate to the stage in 120 s were placed on the stage. Daily animals were given trails of four sessions. The test session was performed during the treatment period on 0, 1, 3, 5, 7, 9 days respectively [22-23].

2.3.2 Neurochemical assay

Assay of acetylcholinesterase (Ache) activity

Estimation of acetylcholinesterase activity was carried out by Ellman's method [24]; where 0.5 ml of brain homogenate was incubated with 0.1 ml of Ellman's reagent and 2.7 ml of phosphate buffer for 5 min, then subsequently add 0.1 ml freshly prepared acetylcholine chloride in the buffer (pH 8), and absorbance was recorded with 1 min time intervals till 10 mins, at 412 nm. Acetylcholinesterase activity was expressed as Ache hydrolyzed/min/gram protein.

Lipid peroxidation (LPO) assay

Estimation of LPO was carried out using TBARS assay; where 0.5 ml of brain homogenate was mixed with 0.5 ml of distilled water and 1 ml of 10% trichloroacetic acid (TCA), the resulting solution was centrifuged at 3000 rpm for 10 mins. Collect 0.2 ml supernatant and add 0.1 ml of thiobarbituric acid (0.375 %) [25]. Place this solution into a water bath at 80 °C for 40 min, cool at room temperature. The amount of malondialdehyde (MDA) formed after reacting with TBARs was measured at 532 nm.

Assay of superoxide dismutase (SOD)

SOD activity was measured spectrophotometrically. 0.5 ml of brain homogenate incubated with 0.1 ml of NADH for 90 secs. In the same solution add 0.5 ml, 10% acetic acid and 4 ml butanol. The colour change was measured at 520 nm. SOD activity was expressed in µg/mg tissue [26].

Assay of glutathione peroxidase (GPx)

0.2 ml brain homogenate incubated with 0.4 ml of sodium azide, disodium EDTA solution and hydrogen peroxide (H2O2) each, for 10 mins at 37 °C for the estimation of GPx. The reaction was stopped by adding 0.5 ml of TCA solution. Centrifuge the resultant solution at 3000 rpm for 10 mins. Collect 0.5 ml supernant and add 0.5 ml of DTNB along with 4 ml of disodium hydrogen phosphate. Measure absorbance at 420 nm. GPx activity was expressed in µg/mg tissue [27].

Assay of catalase (CAT)

CAT activity was assessed using a method of Luck, 1971. Here hydrogen peroxide breakdown was assessed. In 0.2ml brain homogenate add 3 ml of H_2O_2 phosphate buffer, absorbance was immediately recorded in 240nm. CAT activity was expressed in µg/mg tissue [28-29].

2.4 Statistical analysis

All the standards expressed as mean ± standard deviations (n=6). Statistical analysis achieved using graph pad prism version 7.1, one-way ANOVA followed by multiple comparisons.

3. RESULTS

3.1 Behavioral parameters

In the Morris water maze test, SCOP group find more difficulty in finding the hidden platform during the test session as compared to the CON group which indicates the memory impairment ($p \leq 0.0001$). Whereas DPZ and CUR COMBO showed a significant change during the test session as compared to the SCOP group by possessing less period to catch the dais as matched to scopolamine group ($p \leq 0.001$). CUR monotherapy group also find difficulty in finding the hidden platform which depicts there is still memory loss. While CUR + PRO and CUR + DICLO have results are shown in figure 1.

3.2 Neurochemical parameters:

The effect on the Acetylcholinesterase activity

Ache levels were significantly elevated in SCOP group(12.32±0.41, $p<0.0001$) as compared to CON (3.46±0.22). In DPZ and CUR COMBO group there is a reduced level of the enzyme and shows higher significance as compared to SCOP group (7.6±0.19 and6.26±0.29, $p<0.0001$).In the only CUR treatment group, there is no significant change in enzyme activity (12.96±0.65, ns). CUR+ PRO reduced the Ache activity (7.8±0.11, $p<0.0001$) and CUR+DICLO also lowered the enzyme activity (8.11±0.51, $p<0.0001$).

The effect on lipid peroxidation activity

The substantial rise in the MDA levels in the SCOP group (7.15±0.74,$p \leq 0.0001$) compared to the CON group (1.12±0.24); while the MDA level in DPZ and CUR COMBO was expressively attenuated (1.26±0.36 and 1.56±0.16, $p \leq 0.0001$) compared to the SCOP group. CUR group mice possess a higher level of MDA with non-significant results (6.95±0.51, ns). Additionally, CUR+ PRO and CUR+DICLO groups have significant results (2.25±0.76 and 3.12±0.32, $p \leq 0.0001$).

The effect on Superoxide dismutase activity

Superoxide dismutase enzyme was low in the SCOP group (19.53±0.28, $p \leq 0.0001$) as compared to the CON group (44.86±0.80). In DPZ and the CUR COMBO group there was a significant rise in the SOD activity compared to the SCOP group (39.15±0.25 and 38.93±0.60, ≤ 0.001). Moreover, CUR+ PRO and CUR+DICLO have significant results (38.22±0.50 and 37.81±0.30, ≤ 0.001) In the CUR group, there is no significance in the enzyme level in comparison to the SCOP group (18.16±0.63, ns).

The effect on the glutathione peroxidase activity

In standard group DPZ and CUR COMBO there was a significant increase in the enzyme activity (5.21±0.31 and 6.45±0.33, $p \leq 0.0001$) whereas the SCOP group there is diminished in glutathione peroxidase enzyme activity (1.94±0.24, $p<0.0001$) when compared to CON (7.4±0.12). Besides CUR+ PRO and CUR+DICLO have significant results (5.89±0.31 and 4.55±0.61,$p \leq 0.0001$).

The effect on the activity of catalase

An acquaintance of mice to SCOP caused a substantial decline in catalase activity (10.36±0.63, $p< 0.0001$) as compared to CON (21.16±0.66). Furthermore, treatment of DPZ and CUR COMBO in mice possess a significant rise in the enzyme level (19.64±0.63 and19.10±0.27, $p< 0.0001$) as compared to those treated with SCOP alone. CUR has no significant results when compared to SCOP group (9.56±0.18, ns). Besides CUR+ PRO and CUR+DICLO have significant results (15.64±0.70, 16.22±0.20, $p<0.0001$). Neurochemical and antioxidant levels in the brain homogenate of mice are shown in table 1.

3.3 Histolopathology study

In histopathology examination of the brain, as shown in Fig 2, Group-A possess normal neuronal cells. Whereas the Group-B exhibited neuronal damage with murky basophilic cytoplasm and dusky condensed nuclei, less neuronal cells with pyknosis, In Group-C, the hippocampus possesses normal histology, mild neuronal damage. While in the Group-D hippocampus abides neuronal damage and the

presence of dark neurons with shrunken cytoplasm and pyknosis and reduced neuronal cells. Group-E possess least neuronal damage, cell integrity is not lost. While in the Group-F hippocampus abides less neuronal damage and less shrunken cytoplasm. Group-G possess mild neuronal damage, cell integrity is not lost. Whereas histology study of liver in figure 3; all groups A, B, F, G, showed normal morphology the cells did not lose their integrity, kupffer cells are normal with no hyperplasia and absences of sinusoids.

4. DISCUSSION

From results, it is cleared that curcumin activity is increased when combined with *L. rhamnosus* and diclofenac. In this study, we checked the effect of curcumin with *L. rhamnosus* and diclofenac in scopolamine-induced dementia in mice model. Amnesia inducing agent used is scopolamine; (non-selective muscarinic blocker) that blocks the cholinergic transmission and leads to dementia in mice [30-31]. Scopolamine models used are considered most reliable and devour a value in experiments where AD-like condition is tempted [32-35]. In the MWM test, the physical behaviour was evaluated against scopolamine group on 0, 1, 3, 5, 7 and 9 the day. All groups showed a significant change in the parameters with p-value ≤0.0001, from which it is concluded that there was a progressive improvement in memory to catch the hidden platform. Where the scopolamine-induced group and curcumin alone group took additional time to catch the hidden platform which submerses that memory was impaired in that group. In neurochemical assays, the result showed that Ache level in scopolamine group is more compared to control. The cholinergic system, in the hippocampus region, has a dominant role as it possesses neurotransmitter acetylcholine and this neurotransmitter is involved in learning cycles [36]. Acetylcholinesterase degrades acetylcholine and the taper its pharmacological action. In AD level of acetylcholine is lowered due to the elevation of acetylcholinesterase [37-38]. Also, all treatment groups showed marked lower Ache activity. But, curcumin showed non-significant results in all neurochemical parameters. Level of MDA in scopolamine and curcumin group was more which shows the damage of neurons as oxidative stress do lipid peroxidation. In SOD, GPx and catalase assay the level of enzymes in scopolamine and curcumin alone group was lower and in the treatment group was elevated, which showed the effect, other groups. Histopathology study also showed the change in the deposition of amyloid-beta in the brain. Scopolamine has an abnormal structure of the brain neurons, whereas curcumin alone has little effect on the brain but the neurons are damages at most rate. Combination groups have a good effect on the brain where it showed a protective effect. In histology study of liver depicts that there is no damage in the liver due to diclofenac. Form all this physical and neurochemical test it was found that curcumin alone did not have good activity than a combination. The possible effect of the combination is by inhibiting the metabolism of curcumin and making reabsorb in the body for its therapeutic effect.

5. CONCLUSION

The existing study remarkably demonstrates that curcumin monotherapy possesses no effect as it gets excreted from the body very fast, whereas curcumin in combination with *L. rhamnosus* and diclofenac have its extraordinary activity by improving memory, learning and antioxidant system in scopolamine-induced mice. Besides, these outcomes offer a scientific justification of *L. rhamnosus* and diclofenac with curcumin as an adjuvant that might slow the progression of the AD. Clinical pharmacokinetic studies are needed to address the probiotic – *L. rhamnosus* and diclofenac as a bioavailability enhancer of curcumin.

LIST OF ABBREVIATIONS

NSAID	Non-steroidal anti-inflammatory drug
i.p.	Intraperitoneal
p.o.	Peroral
AD	Alzheimer's disease
USFDA	United State Food and Drug Administration
NMDA	N methyl-D-aspartate
UDP	Uridine 5'-diphospho
SCOP	Scopolamine
CON	Control
DPZ	Donepezil
CUR COMBO	Curcumin with *L. rhamnosus* and diclofenac
CUR	Curcumin
CUR+PRO	Curcumin with *L. rhamnosus*
CUR+DICLO	Curcumin with diclofenac
Ache	Acetylcholinesterase
MDA	Malondialdehyde
LPO	Lipid peroxidation
SOD	Super oxidase dismutase
GPx	Glutathione peroxidase
CAT	Catalase
IAEC	Institutional Animal Ethics Committee
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
MWM	Morris water maze
TBARS	Thiobarbituric acid
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid
EDTA	Edetate disodium
H202	Hydrogen peroxide

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

The protocol was permitted by IAEC (SSR/IAEC/2019/03) and was performed as per CPCSEA guidelines.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

None.

CONFLICT OF INTEREST

The authors have no conflict of interest.

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Figure legends:

Fig.1. The graph shows the escape latency in the MWM test. The statistics are expressed as mean ± SD (n=6). Value of P≥ 0.05 was shown by marking "ns" while P-values ≤0.05, ≤0.01, ≤0.001 and ≤0.0001 were expressed as *, **, *** and **** respectively.

Fig.2. Histology examination of brain section: where 20x, 200x, 400x magnification have been used where CON group: possess a normal neuronal cell. SCOP group: showing neuronal damage with dark basophilic cytoplasm and dark condensed nuclei, less neuronal cells with pyknosis, deposition of amyloid plaques (arrow) DPZ group hippocampus showing normal histology, less neuronal damage. CUR group: hippocampus showing neuronal damage and presence of dark neurons with shrunken cytoplasm and pyknosis and reduced neuronal cells. CUR+ PRO: neuronal damage is less, cell integrity is not lost, CUR+DICLO: posses less neuronal damage and absence of pyknosis CUR COMBO: least neuronal damage, cells integrity is not lost.

Fig.3. Histology examination of the liver section: where, 200x, 400x magnification have been used. In liver histopathology study all groups; CON, SCOP, CUR+DICLO and CUR COMBO section showed normal morphology the cells did not lose their integrity, kupffer cells are normal with no hyperplasia and absences of sinusoids.

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RESEARCH ARTICLE

Effect of *Lactobacillus rhamnosus* and Diclofenac with Curcumin for Neuronal Restoration and Repair Against Scopolamine Induced Dementia in Zebrafish (Danio rerio)

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Abstract: ***Background*:** Clinical studies have already revealed the ubiquitous neuroprotective role of curcumin in neuronal deterioration, but it cannot be used alone due to its truncated bioavailability. Currently, many such approaches are functional, which overcome this issue either by increasing the solubility or absorption. These approaches carry a costlier treatment. One more tactic is present but less focused *i.e*., by limiting the intestine and liver enzymatic metabolism; by this approach, curcumin will be more available for its beneficial outcome.

***Objective*:** The goal of this study was to evaluate the impact of *Lactobacillus rhamnosus* and diclofenac on the neuroprotective effects of curcumin against scopolamine-induced dementia.

***Methods*:** Physical parameters involved a novel tank test, T maze test, whereas neurochemical parameters include brain oxidative stress and acetylcholinesterase (Ache) inhibition activity in a zebrafish dementia model.

***Results*:** Our results demonstrated that curcumin with *Lactobacillus rhamnosus* and diclofenac significantly (p<0.05) reduced anxiety, memory deficits, and brain oxidative stress compared to the alone curcumin-treated group.

***Conclusion*:** This result approves that curcumin with *L.rhamnosus* and diclofenac have superior activity compared to curcumin alone. However, further clinical studies are needed to validate these findings.

Keywords: Zebrafish, Alzheimer's disease, curcumin, diclofenac, probiotics, neurodegenerative

1. INTRODUCTION

Alzheimer's disease (AD) is an irretrievable brain disorder which leads to neuronal degradation and, ultimately, memory loss [1-3]. Worldwide, 47 million individuals are affected by dementia, and the ratio in the upcoming years will double in number [4], besides these; the United States Food and Drug Administration (USFDA) official drugs for AD are few in number and act by one mechanism. These drugs give short-term relief and possess severe side effects. Developing a drug that works by numerous mechanisms and provides long relief from AD is needed. Curcumin is a major component (2-6%) of Curcuma longa Lin.

Clinical studies revealed its beneficial effects in treating AD through the generation of free radicals in the brain, inhibiting the creation and endorsing the dissemblance of plaques, reducing the hyperphosphorylation of tau and enhancing its removal, inhibiting acetylcholinesterase, anti-inflammatory, and possessing metal chelation properties [5-7]. Curcumin cannot be used alone due to its low oral bioavailability. Certain reasons such as poor aqueous solubility, poor absorption, and quicker metabolism hinder its bioavailability. This problem is imperious and creates an expensive treatment [8-10]. Nanovesicles, nanoparticles, liposomes, nanoemulsions, and phytosomes are formulated to overcome this problem; it either increases solubility or absorption or increases 2-10-fold bioavailability [11-14]. Still, the effectiveness, safety, and stability of this formulation are the leading concerns [15-19]. One of the foremost approaches for improving systemic bioavailability occurs by hindering the intestinal and liver metabolism of curcumin by curcumin adjuvant [20]. Any substantial improvement in bioavailability by which the dose is reduced, as well as the cost of the therapy, will be in larger demand. Here we are using a strategy to increase bioavailability by targeting the metabolism step and increasing the absorption of curcumin so that the level of curcumin remains for more time for its effect. Curcumin is metabolized by the enzyme UDP-glucuronosyl transferase (UGTs) [21, 22]. Utmost for the metabolism of

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curcumin, predominant UGTs involved are UGT1A1, UGT1A3, UGT1A8, UGT1A10, UGT1A9, UGT2B7 [23]. Diclofenac is a known inhibitor of UGTs, which inhibits UGT1A1, UGT1A3, UGT1A9, UGT2B7, and thereby reduces the metabolism of curcumin [24]. There are other drugs that target UGTs, but the cost and safety are the major concerns [25-27]. We chose the medicine diclofenac because it is affordable, conveniently accessible, and USFDA-approved. On the contrary, beta-glucuronidase enzymes hydrolyze back curcumin metabolites in the initial form, making it simple to reabsorb curcumin. Beta-glucuronidase is generated by *Lactobacillus rhamnosus* [28-34]. The issue of fast metabolism will be solved and overall systemic bioavailability will be improved by mixing diclofenac and *L. rhamnosus* [35–36]. In a mouse model, we looked at the impact of curcumin, along with diclofenac and L. rhamnosus. Aquatic fauna is durable, petite, relatively inexpensive to maintain, and a key model for drug development, thus there is still a need to examine their significance [37]. In biomedical research, zebrafish is considered as a notable model as they lay hundreds of eggs at a time, to prove the repeatability and accuracy of results, one needs to conduct research many times so these animals can be chosen as offspring produced in large numbers [38-40]. Here we investigated the neuroprotective effect of curcumin in combination with *L.rhamnosus* and diclofenac in AD zebrafish model. Due to the presence of the Psen1, Psen2, BACE1, BACE2, APOE, and gamma-secretase complex genes, zebrafish is widely used to investigate AD [41-43]. These genes are essential for understanding AD pathogenies. Zebrafish learning and cognition are also probably a rich and fruitful effort. Zebrafish have larger quantities of antioxidant enzymes than other birds and animals normally do, and their anatomical brains are very comparable to those of humans [44]. Given that zebrafish contain UGT-conserved genes that are similar to those in the human gut, they can serve as ideal models for research involving gut microbes [45-47]. UGT1, UGT2, and UGT5 are the families of UGTs present in zebrafish and highly expressed, specifically UGT1A1, UGT1A2, UGT1A3, UGT1A5, UGT1B1, UGT1B7, UGT2A4, UGT5G2 [48]. Probiotics are used in zebrafish model as it modulates the gene expressed in the gut for producing a microbe. Zebrafish models employ probiotics because they regulate the genes that are expressed in the gut to produce microbes. They also express beta-glucuronidase enzymes, which makes them better candidates for the study. As a result, this model can be preferred since it resembles people in terms of neuroanatomical structure, behavior, pathology, and metabolism [49].

2. MATERIALS AND METHOD

2.1. Drugs and Reagents

Curcumin 98% pure was procured from Loba Chemie Pvt., Ltd, India. The strain of probiotics *L. Rhamnosus* was procured from Unique Biotech Ltd. Diclofenac was procured from Chemie. Other reagents as mentioned Ellman's reagent, acetylcholine chloride, trichloroacetic acid, thiobarbituric acid, NADH, butanol, acetic acid, sodium azide, disodium, EDTA, hydrogen peroxide, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid). Ellman's reagent, acetylcholine chloride, and DTNB were dissolved in phosphate buffer pH 8; whereas other reagents thiobarbituric acid, NADH, sodium azide, and disodium EDTA were prepared freshly in deionized water.

2.2. Animals

In total, ninety-eight adult zebrafish (Danio rerio) (male and female 2-3-month-old, 2-3cm in length, and weighing 0.5–0.12g) were procured from authenticated sources. They were acclimatized for one week in glass aquariums comprising of dechlorinated water, pH 7.0, and air pumps with submerged filters at 25°C (14:10 h light/dark circadian cycle). The fish were fed libitum before experiments. The protocol was permitted by Institutional Animal Ethics Committee SSR/IAEC/2019/03 and was performed in accordance with the Committee for Control and Supervision of Experiments on Animals (CPCSEA). For the culmination study, fish were euthanized in ice-cold water for 5 min until loss of movement, and the brain was isolated for neurochemical parameters.

2.3. Acute Toxicity

According to OECD 203 guidelines, a toxicity study was performed on zebrafish [50]. For the limit test, fish were acclimatized and arbitrarily distributed into the control and test groups (n=7); the test dose chosen was 100mg/L. To demonstrate whether the LC50 is greater than this concentration or not. For the main group, healthy thirty-five zebrafish were randomly divided as controls, group A, group B, group C, and group D (n=7). The control received only distilled water and Group A to Group D received 0.88, 1.94, 4.26, and 9.37mg/L of diclofenac, respectively. The fish were exposed to the compound for 96 hours and analyzed for their mortality rate. In every 24, 46, 72, and 96 hours, a change in behavior movements like swimming, pigmentation, and survival was observed.

2.4. Drug Administration

An additional set of forty-nine fish was used in this study (n=7). The drug was added directly into the tank daily for 10 consecutive days (each day the water was changed and the drug was added/L).

2.5. Behavioural Parameters

2.5.1. T-maze Test

T maze is made up of acrylic glass with specific dimensions 50cm x 10cm x 10 cm(stem), 20 cm x 10 cm x 10cm (2 arms), and a 10cm x 10 cm x 10cm start box with a sliding door. The maze was filled with water 6cm in height and the temperature was kept at 26°C. Both arms were covered with different colors; one with green and the other with red. For initial 6 days, the fish was trained. During training, the fish entering the green arm was rewarded with food; whereas the one entering the red arm was punished by swirling water

with a glass rod. The fish was placed in the starting box for 1 min and the sliding door was opened once. For the duration of 4 mins, the fish was observed and the observation noted was whether it goes in the green arm or red arm. After treatment of 7 consecutive days; the fish was given a scopolamine dose and then was subjected to these tests where the time and number of entries in the green arm were recorded [51].

2.5.2. Novel Tank Test

Novel tank test comprises of specific dimensions as 15.2 cm × 27.92 cm × 22.5 cm ×7.1 (height×top×bottom× width). This tank is divided equally into two parts (top and bottom) filled with 1.5L water. After giving an oral dose of scopolamine, each group was subjected to this test for 5 min. The number of entries in the top zone and the bottom zone of the trapezoidal reservoir and the distance traveled were assessed [52].

2.6. Neurochemical Assay

After the execution of behavioral parameters, zebrafish were euthanized by immersing in ice-cold water for 2 min (until loss of movement). Brains were isolated for neurochemical parameters. The brain was homogenized in phosphate buffer (pH 7.4). The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was taken for further neurochemical assays [53].

2.6.1. Assay of Acetylcholinesterase (Ache) Activity

The estimation of acetylcholinesterase activity was carried out by Ellman's method; 0.5 ml of brain homogenate was incubated with 0.1 ml of Ellman's reagent and 2.7 ml of phosphate buffer for 5 min. 0.1ml of freshly prepared acetylcholine chloride in buffer (pH 8) was then added. Absorbance was recorded immediately with a 1 min time interval until 10 min at 412 nm. Acetylcholinesterase activity was expressed as Ache hydrolyzed/min/gram protein [54].

2.6.2. Lipid Peroxidation (LPO) Assay

The estimation of LPO was carried out using the TBARS assay; 0.5 ml of brain homogenate was mixed with 0.5 ml of distilled water, and 1 ml of 10% trichloroacetic acid(TCA), and the resulting solution was centrifuged at 3000 rpm for 10 mins. 0.2 ml supernatant was collected and 0.1 ml of thiobarbituric acid (0.375%) was added. This solution was placed into a water bath at 80°C for 40 min, and cooled at room temperature. The amount of malondialdehyde (MDA) formed after reacting with TBARs was measured at 532 nm [55].

2.6.3. Assay of Superoxide Dismutase (SOD)

SOD activity was measured spectrophotometrically by incubating 0.5 ml of brain homogenate with 0.1 ml of NADH for 90 secs. In the same solution, add 0.5 ml 10% acetic acid and 4 ml butanol. The color change was measured at 520 nm by collecting the upper layer of butanol. SOD activity was expressed in µg/mg tissue [56].

2.6.4. Assay of Glutathione Peroxidase (GPx)

The estimation of GPx was carried out by incubating 0.2 ml brain homogenate with 0.4 ml of sodium azide, disodium EDTA solution, and hydrogen peroxide (H_2O_2) each, for 10 min at 37°C. Then this reaction was stopped by adding 0.5ml of TCA solution. The resultant solution was then centrifuged at 3000 rpm for 10 min. 0.5 ml supernatant was collected and 0.5 ml of DTNB was added along with 4ml of disodium hydrogen phosphate. Absorbance was measured at 420nm. GPx activity was expressed in µg/mg tissue [57, 58].

2.7. Statistical Analysis

All standards were expressed as mean ± standard deviation (n=6). Statistical analysis was achieved using Graph pad prism version 7.1, one-way ANOVA followed by multiple comparisons.

3. EXPERIMENTAL

Zebrafish were acclimatized for one week prior to starting the study. Each group contains seven fishes and the grouping is shown below

Group I (CON)- Control group received distilled water,

Group II (SCP)- Scopolamine received (200 µM),

Group III (DOZ)- Standard treatment donepezil (0.75µg) + scopolamine (200 µM)

Group-III (DOZ)- Standard treatment donepezil (0.75µg)+ scopolamine (200 µM),

Group IV (CUR)- Curcumin (40mg/kg) + scopolamine (200 µM),

Group V (CUR+LACTO)- Curcumin (40 mg/kg) + *L. rhamnosus* (106cfu) + scopolamine (200 µM),

Group VI (CUR + DICL), curcumin (40 mg/kg) + diclofenac (0.69 mg/L) + scopolamine (200 µM),

Group VII (COMBO) curcumin (40 mg/kg) + diclofenac (0.69 mg/L) + *L. rhamnosus* (106cfu) + scopolamine (200 µM).

The experimental design is shown in Fig. (**1**). Treatment was given for 10 days and on the 10th day scopolamine was given to each group after one hour of drug administration and behavioral parameters were assessed.

4. RESULTS

4.1. Behavioral Parameters

4.1.1. T-maze Test

In the T-maze test, fishes treated with scopolamine possess fewer entries in the green arm than the CON group (<0.0001), which indicates that there is memory impartment in zebrafish. Standard treatments DOZ, COMBO, CUR+LACTO, and CUR+DICL attenuate the effect of scopolamine on physical parameters; where the number of en-

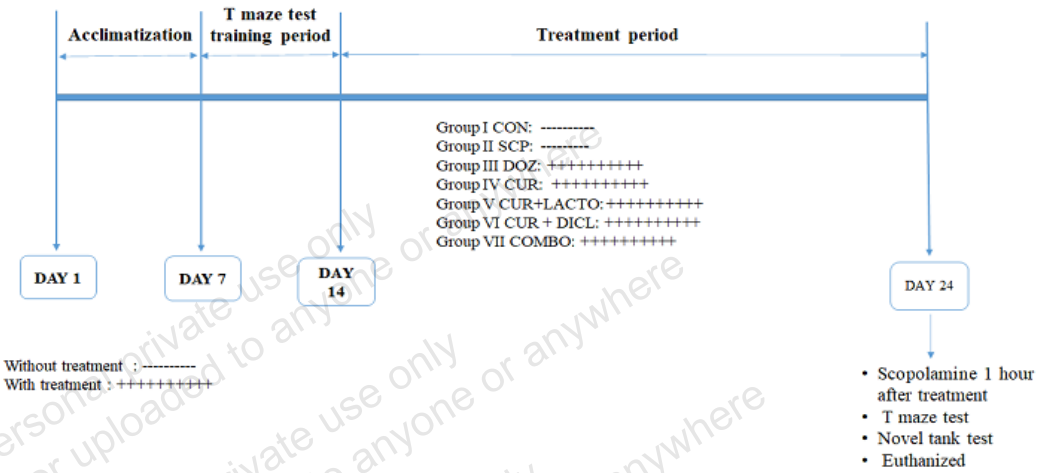

Fig. (1). Diagrammatic representation of experiment design.

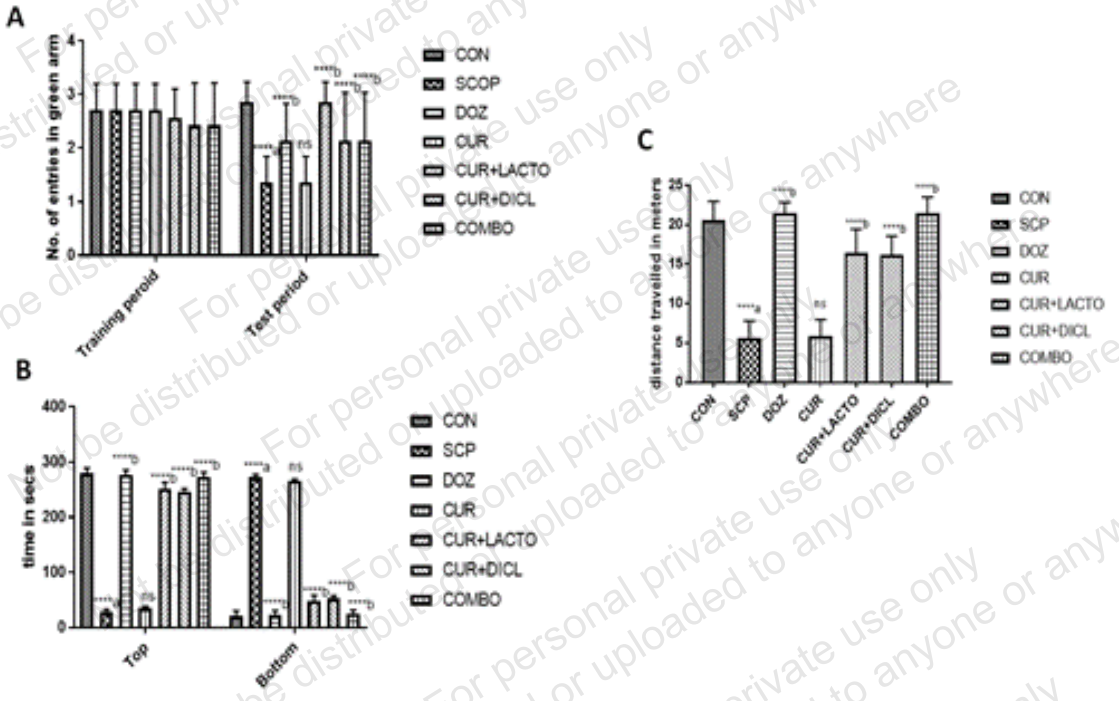

Fig. (2). Graph shows **A**) the number of entries in the green arm; **B**) Number of entries in the top zone and the bottom zone of the trapezoidal reservoir; **C**) the distance travelled in meters; The data are expressed as mean ± SD (n=7), a CON group significant compared to SCP group; whereas b DOZ group, CUR group, CUR+LACTO group, CUR+DICL and COMBO compared to SCP group. Value of P≥ 0.05 was shown by marking "ns" while *P*-values ≤0.05, ≤0.01, ≤0.001and ≤ 0.0001 were expressed as *, **, *** and **** respectively.

tries in the green arm was more. CUR group showed non-significant results compared to SCP, as shown in Fig. (**2A**).

4.1.2. Novel Tank Test

The time consumed in the bottommost area of the tank is more and the top area is less by the SCP and CUR group compared to the CON group ($p < 0.0001$), indicating an anxiogenic effect. Whereas DOZ and COMBO groups have petty time in the bottom and prolonged time spent in the top areas, respectively, compared to SCP ($p < 0.0001$). In CUR+LACTO and CUR+DICL groups, the time spent in the top area and the bottom area is modest compared to SCP ($p <$ 0.0001). In the SCP and CUR group, the distance traveled is less compared to CON ($p < 0.0001$). All other groups traveled more distance ($p < 0.0001$) in meters indicating the reverse action of SCP as shown in Figs. (**2B** and **C**), respectively.

4.2. Neurochemical Assay

4.2.1. Effect on Acetylcholinesterase (Ache) Activity

Zebrafish, when exposed to scopolamine, possess a substantial rise in the level of ache in the SCP group (10.73 ± 0.10, $p<0.0001$) as compared to CON (6.62 ± 0.28). In the

Table 1. Neurochemical parameters in zebrafish.

Group	Ache (µg/mg)	LPO (µg/mg)	SOD (µg/mg)	GPX (µg/mg)
CON	6.62 ± 0.28	0.70 ± 0.20	13.03 ± 0.12	14.32 ± 0.11
SCP	10.73 ± 0.10 ****a	1.41 ± 0.17 ****a	2.90 ± 0.10 ****a	5.9 ± 0.27 ****a
DOZ	6.41 ± 0.14 ****b	0.54 ± 0.23 ****b	12.59 ± 0.08 ****b	13.24 ± 0.45 ****b
CUR	9.45 ± 0.54 ns	1.4 ± 0.25 ns	3.16 ± 0.14 ns	6.9 ± 0.30 ns
CUR+LACTO	7.58 ± 0.96 ****b	0.62 ± 0.57 ****b	12.83 ± 0.30 ****b	11.79 ± 0.38 ****b
CUR+DICL	7.96 ± 0.26 ****b	0.69 ± 0.15 ****b	11.18 ± 0.68 ****b	10.8 ± 0.36 ****b
COMBO	6.69± 0.18 ****b	0.52± 0.16 ****b	14.93± 0.60 ****b	13.67± 0.36 ****b

Note: All values are the mean ± SD with n=7 per group; a represents the significance between CON and SCP; b represents the significance level between SCP in comparison with DOZ, CUR, CUR+LACTO,CUR+DICL and COMBO groups. Value of $P \geq 0.05$ = ns while *P*-values ≤0.05, ≤0.01, ≤0.001 and ≤0.0001 were expressed as *, **, *** and **** respectively.

CUR treatment group, there was no significant change in enzyme activity (9.45 ± 0.54, ns). In DOZ and COMBO groups, there is a petty level of enzyme and shows higher significance compared to SCP group (6.41 ± 0.14and 6.69 ± 0.18, $p<0.0001$). CUR+LACTO reduced the ache activity (7.58 ± 0.11, $p<0.0001$) and CUR+DICL also lowered the enzyme activity (7.96 ± 0.26, $p<0.0001$).

4.2.2. Effect on Lipid Peroxidation Activity

The substantial rise in the MDA levels in the SCP group (1.41 ± 0.17, $p\leq0.0001$) compared to the CON group (0.70 ± 0.20); while the MDA levels in DOZ and COMB0 groups were expressively attenuated (0.54 ± 0.23 and 0.52 ± 0.16, $p\leq0.0001$) compared to the SCP group. Additionally, CUR+LACTO and CUR+DICL groups have significant results (0.62 ± 0.57 and 0.69 ± 0.15, $p\leq0.0001$). In the CUR group, there is a higher level of MDA which shows non-significant results (1.4 ± 0.25, ns).

4.2.3. Effect on Superoxide Dismutase Activity

The superoxide dismutase enzyme was low in the SCP group (2.90 ± 0.10, $p\leq0.0001$) as linked to the CON group (13.03 ± 0.12). In the DOZ and COMBO groups, there is a noteworthy rise in the enzyme activity compared to the SCP group (12.59 ± 0.08 and 14.93 ± 0.60, ≤0.001). Moreover, CUR+LACTO and CUR+DICL have significant results (12.83 ± 0.30 and 11.18 ± 0.68, ≤0.001) in the CUR group, there is no significance in the enzyme level in comparison to the SCP group (3.16 ± 0.14, ns).

4.2.4. Effect on Glutathione Peroxidase Activity

In the standard group, DOZ, and COMBO group, there was a substantial rise in the enzyme activity in comparison to SCP group (13.24 ± 0.45 and 13.67 ± 0.36, $p\leq0.0001$). Besides, CUR+LACTO and CUR+DICL have significant results (11.79 ± 0.38 and 10.8 ± 0.36, $p\leq0.0001$), whereas, in the SCP group, there was declination in enzyme activity (5.9 ± 0.27, $p<0.0001$) glutathione peroxidase when compared to CON (14.32 ± 0.11). CUR group possessed as an insignificance result (6.9 ± 0.30, ns) in comparison with the SCP group as shown in Table **1**.

5. DISCUSSION

In AD, the region of the brain, neuron atrophy, astrogliosis, progressive loss of structure, and function of neurons are observed [59, 60]. Due to this, there is memory loss, as it leads to the degradation of cholinergic neurons. Scopolamine is a non-selective muscarinic blocker that antagonizes cholinergic transmission and impairs memory loss in zebrafish [61, 62]. Scopolamine models are used, which are considered the most consistent and devoured value in experiments where AD-like condition is tempted [63, 64]. Zebrafish as a model due to its simplicity, accuracy, and similar genetic resemblance to the humans used as an AD model. Additionally, UGTs involved in the metabolism of curcumin are also present. Previously, we evaluated the effect of curcumin combination in mice as a model [65-67]. Still focused on aquatic abodes made as they can be used to explore drug discovery due to its similar genetic resemblance to Homo sapiens [68-71]. From this investigation, it is clear that curcumin activity is increased when combined with *L.rhamnosu*s and diclofenac against scopolamine-induced dementia in the zebrafish model. In the T maze test, the number of entries of the SCP group and CUR group was less, which shows that there is an interruption in cognition. All other groups showed a greater number of entries in the green arm, which depicts the protective role of each in AD. In neurochemical parameters, the results show that the ache level in the SCP group is more compared to CON. The cho-

linergic system in the hippocampus region has a dominant role as it possesses the neurotransmitter acetylcholine, and this neurotransmitter is involved in learning cycles [72-75]. Acetylcholinesterase degrades acetylcholine and tapers its pharmacological action. In AD, the level of acetylcholine is lowered due to the elevation of acetylcholinesterase [76-78]. CUR also showed non-significant results; whereas all other groups, DOZ, CUR+LACTO, CUR+DICL, and COMBO showed significant results. The level of MDA was more in SCP and CUR, which depict the damage of neurons due to oxidation. In SOD and GPx, the level of this protective enzyme is more in DOZ, CUR+LACTO, CUR+DICL, and COMBO and less in SCP and CUR. Combination groups have the most equivalent results compared to standard therapy. From all these behavioral and neurochemical tests, it was found that curcumin alone did not have good activity than a combination. The possible effect of the combination is by inhibiting the metabolism of curcumin and making a reabsorb in the body for its therapeutic effect.

CONCLUSION

This study concludes that curcumin with *L.rhamnosus* and diclofenac can be used together for improving the oral bioavailability of curcumin. As combination has greater activity compared to curcumin alone. Although, further clinical studies are required to validate these findings.

LIST OF ABBREVIATIONS

Ache	=	Acetylcholinesterase
AD	=	Alzheimer's Disease
USFDA	=	United States Food and Drug Administration
UGT	=	UDP-Glucuronosyl Transferase
DTNB	=	5,5'-dithiobis-(2- Nitrobenzoic Acid)
EDTA	=	Ethylenediamine Tetraacetic Acid
NADH	=	Nicotinamide Adenine Dinucleotide Hydrogen
CPCSEA	=	Committee for the Purpose of Control and Supervision of Experiments on Animals
OECD	=	Organisation for Economic Co-operation and Development
LPO	=	Lipid Peroxidation
SOD	=	Superoxide Dismutase
GPx	=	Glutathione Peroxidase

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The protocol was permitted by Institutional Animal Ethics Committee SSR/IAEC/2019/03 and was performed in accordance with the Committee for Control and Supervision of Experiments on Animals (CPCSEA).

HUMAN AND ANIMAL RIGHTS

No humans were used in this study. Animal research procedures were followed in accordance with the standard of the institutional animal ethics committee.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data and supportive information is available within the article.

FUNDING

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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