Research work entitled as

"Potentiation of anti-Alzheimer activity of curcumin by probiotic *Lactobacillus rhamnosus* and diclofenac"

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Title: Potentiation of anti-Alzheimer activity of curcumin by probiotic *Lactobacillus rhamnosus* and diclofenac

1. Introduction

Alzheimer disease (AD) is an irrevocable brain disorder that degrades neurons and leads to dementia. Dementia is a syndrome that includes amnesia and deterioration in thinking, behaviour, and ability to carry out daily activities. 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]. In recent years, many techniques are used to increase solubility and permeability by reduction of particle size and creation of super saturation solution or encapsulation into nanoparticles. Although these tactics enhance 2-10-fold bioavailability, the effectiveness, safety, stability of this formulation remain the key concerns. Also these formulation only increasing the solubility or absorption. None of the approaches targets extensive metabolism. Targeting metabolism with specific potent and safe inhibitors, is one of the other and most important approaches for enhancing overall systemic bioavailability of polyphenol example the curcumin-piperine complex. [9-12]. Any substantial enhancement in bioavailability by which the dose is reduced as well as the 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]. 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. 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. Beta glucuronidase is generated form probiotics, *Lactobacillus rhamnosus* in the intestine and increase the absorption of the same [18-21]. Zebrafish model is used as it posses similar resemblance to that of humans. Zebrafish contains UGT conserved genes, additionally genes responsible for AD and bear antioxidant enzymes required for regulation of normal functions in brain. We have investigated the neuroprotective effect of curcumin in combination with L. rhamnosus and diclofenac (as bioenhancer) in Alzheimer’s disease using zebrafish and mice as animal model.

2. Objectives

1. Investigate the impact of diclofenac and probiotics on the bioavailability of curcumin.
2. Assess the potential of combining curcumin with diclofenac and probiotics in mitigating Alzheimer's disease symptoms using a zebrafish model induced with scopolamine.
3. Examine the synergistic effects of curcumin in conjunction with diclofenac and probiotics on scopolamine-induced Alzheimer's disease in Swiss albino mice.

3. Materials and Methods

3.1 Materials:

The strain of probiotic *Lactobacillus rhamnous* was procured from the Unique Biotech Limited, Hyderabad, India. Curcumin was 98% pure was procured from Loba Chemie Pvt.Ltd, Mumbai, India. All other chemicals and reagent was analytical grade.

3.2 The effect of diclofenac and probiotics on bioavailability of curcumin [17-19]:

Animals were divided into five groups, Curcumin (400mg/kg), Curcumin + Probiotics (10^6cfu), Curcumin + Diclofenac (2mg/kg), Curcumin + Diclofenac (4mg/kg), Curcumin + Probiotics + Diclofenac. Blood will be collected in tube. Then centrifuge the blood at 4000 rpm for 10 mins

under 4 º C. Serum will be collected and adjust pH 2 with 6N HCl. Then extracted two times with equal volumes of ethyl acetate/propanol (9:1, v/v). . The extraction recovery from plasma was approximately 95%. . The plasma samples were separated by centrifugation at 5000g for 10 min in a desktop centrifuge and filtered through 0.22 mM polyvinylidene difluoride membrane filters. Curcumin standard calibration graph was taken. Sample detection was achieved at 420 nm, and injection volumes were 20 microliter.

3.3 To determine neuroprotective effect of curcumin in combination with diclofenac and *Lactobacillus rhamnous in* scopolamine induced dementia in zebra fish.

3.3.1 Acute toxicity curcumin in combination with diclofenac in zebra fish*:*

According to OECD 203 guidelines toxicity study was performed in zebrafish. For limit test fishes were acclimatize and arbitrarily distributed into control and test group (n=7); test dose chosen was 100mg/L. To demonstrate whether the LC50 is greater than this concentration or not. For the main test group, healthy thirty-five zebrafish were randomly divided as control, group A, group B, group C and group D (n=7). Control receives only distil water and Group A to Group D received 0.88, 1.94, 4.26, 9.37mg/L of diclofenac respectively. The fish were exposed to compound for 96 hours and analyzes for their mortality rate. In every 24, 46, 72 and 96 hours, change in behavior movements like swimming, pigmentation and survival was observed[41].

3.3.2 Neuroprotective effect of curcumin in combination with diclofenac and *Lactobacillus rhamnous in* zebra fish*:*

3.3.2.1 Drug administration:

An additional set of forty-nine fishes was used in this study (n=7). The drug was added directly into the tank, daily for 10 consecutive days (each day water was changed and the drug was added/L). group I (CON) control group receiving distill water, group II (SCP) scopolamine receiving (200µM), group-III (DOZ) Standard treatment donepezil (0.75µg)+scopolamine (200µM), group IV(CUR) Curcumin(40mg/kg)+scopolamine (200µM),group V (CUR+LACTO) curcumin(40mg/kg)+ *Lactobacillus rhamnous*(10^6cfu) + scopolamine (200µM),group VI (CUR + DICL) curcumin(40mg/kg) + diclofenac (0.69mg/L) + scopolamine (200µM), group VII (COMBO) curcumin (40mg/kg) + diclofenac (0.69mg/L) + *Lactobacillus rhamnous*(10^6cfu) + scopolamine (200µM).

3.3.2.2 Behavioural test

T-maze test:

T maze is made up of acrylic glass with specific dimension 50cm x 10cm x 10 cm(stem), 20 cm x 10 cm x 10cm (2 arms), and 10cm x 10 cm x 10cm start box with a sliding door. The maze was filled with water 6cm height and the temperature was kept 26°C. Both the arms were covered with different color; one with the green and other with the red. Initial 6 days the fish was trained. During training the fish entering green arm was rewarded with the food; whereas one entering red arm was punished by swirling water with a glass rod. Fish was placed in the starting box for 1 min and the sliding door was opened once. For the duration of 4 mins fish was observed and the observation noted was whether it goes in green arm or red arm.. After treatment of 7 consecutive days; fish was given scopolamine dose and then was subjected to these tests where time no. of entries in the green arm was recorded[24-25].

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 the giving oral dose of scopolamine, each group was subjected to this test for 5 mins. Number of entries in the top zone and the bottom zone of the trapezoidal reservoir and distance travelled was assessed[24-25]

3.3.2.3 Neurochemical assay:

After the execution of behavioural parameters, zebrafish were euthanized by immersing in ice-cold water, for 2 min (till 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 mins. Supernant was taken for further neurochemical assays.

Assay of acetylcholinesterase (Ache) activity

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 mins. 0.1ml of freshly prepared acetylcholine chloride in the buffer (pH 8) was then added. Absorbance was recorded immediately with 1 min time intervals till 10 mins, at 412 nm. Acetylcholinesterase activity was expressed as Ache hydrolyzed/min/gram protein[28].

Assay of Lipid peroxidation (LPO)

Estimation of LPO was carried out using 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), the resulting solution was centrifuged at 3000 rpm for 10 mins. Collect 0.2 ml supernant and add 0.1 ml of thiobarbituric acid (0.375 %). Place this solution into a water bath at 80 °C for 40 min, cool at room temperature [35-36]. 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 by incubating 0.5ml 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[32].

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 (H2O2) each, for 10 mins at 37 °C. Then this reaction was stopped by adding 0.5ml of TCA solution. The resultant solution was then centrifuge at 3000rpm for 10 mins. 0.5ml supernant 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[29].

3.3.3 Neuroprotective effect of curcumin in combination with diclofenac and *Lactobacillus rhamnous* in scopolamine induced dementia in mice [25, 28, 31, 36].

3.3.3.1 Animals and care:

Thirty Swiss albino female mice weighing 25-30g were purchased. The protocol was approved by Institutional Animal Ethics Committee SSR/IAEC/2018/08 and was conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). Mice had *ad libitum* access to water and food. They were housed in a temperature-controlled room (22±2 °C, relative humidity 55±3%) with an automatic regimen of light darkness of 12 h ×12 h.

3.3.3.2 Experimental design:

All the mice were subjected to training for 6 days prior to any treatment. The mice were divided into

- Group I:- Control group receiving distill water.
- Group II: Scopolamine (i.p 1 mg/kg once daily for 10 days)
- Group III: Standard treatment donepezil (2 mg/kg once daily for 10 days) and scopolamine (i.p 1 mg/kg once daily for 10 days)
- Group IV: Curcumin(205 mg/kg once daily for 10 days)+ scopolamine (i.p 1 mg/kg once daily for 10 days)
- Group V: Curcumin(205 mg/kg once daily for 10 days) + *Lactobacillus rhamnous*(10^6cfu) + scopolamine (i.p 1 mg/kg once daily for 10 days)
- Group VI: Curcumin(205 mg/kg once daily for 10 days) + diclofenac (5 mg/kg) + scopolamine (i.p 1 mg/kg once daily for 10 days)
- Group VII: Curcumin(205 mg/kg once daily for 10 days) + diclofenac(10 mg/kg) + probiotic(10^6cfu)+scopolamine (i.p 1 mg/kg once daily for 10 days)

- Treatment was given for 10 days, on 10^{th} day, physical behavior was assessed.
- The mice were euthanized by high dose of anesthesia; brain was removed and then neurochemical parameters was performed.

3.3.3.3 Behavioural parameters:

Determining spatial memory using Morris water maze test [27-28]:

Morris water test is used to determine the spatial memory and reference. In this test mice navigates form the start location and finds a hidden platform which is submerged in to water. Mark the directions in the maze, then animals is place in the desired position into the maze and released into the water (not dropped). A timer is set at the moment when animals is released into the water. Stop the time when animal reaches or touch the platform. Trail limit of 120 secs is kept as a limit to reach to the platform. Leave the animal in to the platform for 15 secs; this will improved the learning memory in mice this is due to hypothermia induced performance in mice. Again place the animal in to the maze repeat this step till achieve a desire memory. Maximum trails given can be 4. Trail is repeated after the treatment is began for 0,1,3,5,7,9 days respectively. On 10^{th} day platform was removed and animal were placed in to the maze their behavior was recorded for 120s after removing the platform, as a probe trail they were allowed to swim for 120 secs to search platform which was placed during the training sessions. The time spent in the target quadrant was measured as an indicator of learning memory.

3.3.3.4 Neurochemical parameters [25]:

Once the training session is completed the mice are euthanized with high dose of anaesthesia then brain is isolated, and neurochemicals was performed.

Assay of acetylcholinesterase activity: same as in above section

Assay of lipid peroxidation: same as in above section

Assay of superoxide dismutase: same as in above section

Assay of glutathione peroxidase: same as in above section

Assay of catalase [26]: Brain homogenate 0.5 ml was mixed with phosphate buffer (0.1Mm,pH 7.4) and H2O2 3 ml which was measured at 240nm.The activity of the enzyme was calculated using the molar extinction coefficient 43.6 M/cm.

Assay of protein carbonyl [28-30]: Brain was homogenized in phosphate buffer (50 mM, pH 7.4) and centrifuged at 11,000×*g* for 15 min. The subsequent supernatant was used for reaction with DNPH. The difference in absorbance between the DNPH treated and the HCl treated samples was determined on spectrophotometer at 375 nm and the amount of carbonyl content (C) was calculated using a molar extinction coefficient (ε) of 22.0 m/M/cm for aliphatic hydrazones.

3.3.3.5 Histology sample preparation:

Brain was isolated from the mice, it was stored in 10% formalin solution, labelled and sent histology to Sakshi laboratories, Vadodara.

3.4 Statistical Analysis

One way ANOVA was used for the data analysis and present as mean + standard error of the mean using Graph pad prism software version 7.1.

4. Results

4.1 The effect of diclofenac and probiotics on bioavailability of curcumin

Time required to reach maximum concentration of curcumin and concentration is shown in table below**.** Curcumin level in blood was measured t max was 15 mins and Cmax was 0.2521 µg/ml. Curcumin level in blood with probiotics was measured t max was 45 mins and Cmax

was 1.201 µg/ml. Curcumin level in blood with diclofenac 1 combination was measured t max was 60 mins and Cmax was 0.6687 µg/ml. Curcumin level in blood with diclofenac 2 combination was measured t max was 45 mins and Cmax was 0.2362 µg/ml as shown in Figure 1.

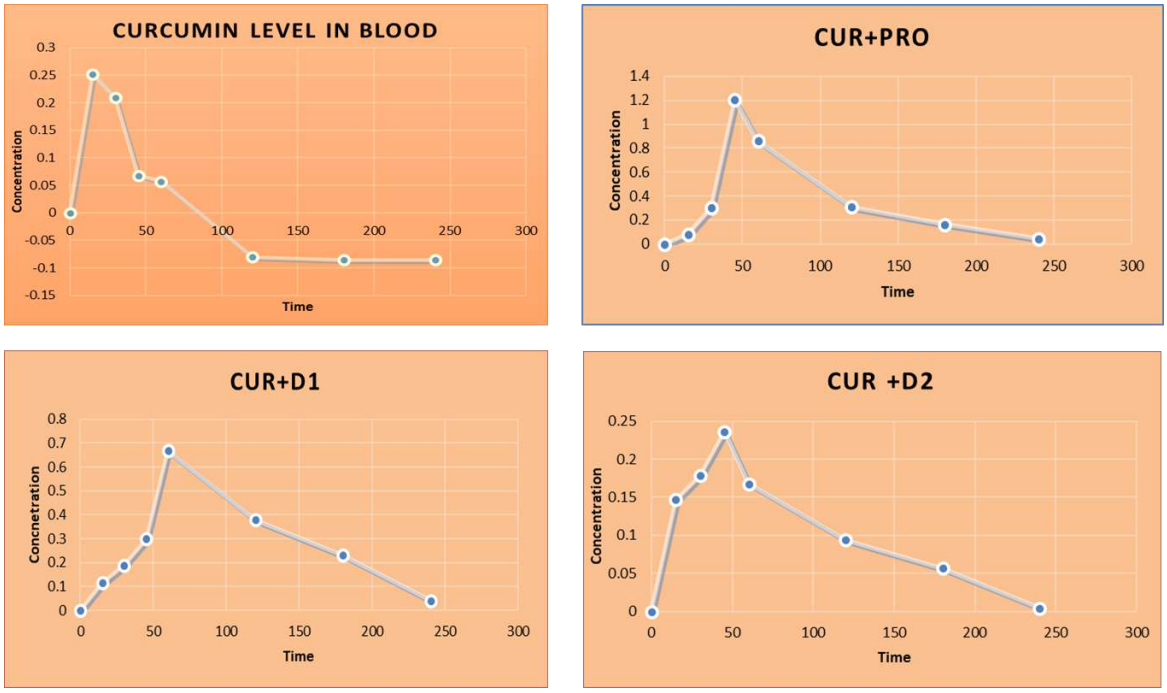

Fig. 1 Pharmacokinetic level of curcumin, curcumin + probiotic, curcumin + diclofenac 1 and curcumin + diclofenac 2 group.

4.2 Neuroprotective effect of curcumin in combination with diclofenac and *Lactobacillus rhamnous* in scopolamine induced dementia in zebra fish.

4.2.1 Acute toxicity curcumin in combination with diclofenac and Lactobacillus rhamnous in zebra fish

The limit test was performed according to OECD guidelines at 100mg/L of diclofenac to determine that the LD50 might be greater than 100mg. All 7 fishes have gone under mortality in 3 hrs from this there is a proof that LC50 of diclofenac in zebrafish is less than 100mg/L. For the main study dose was selected as 0.88mg/L, 1.94 mg/L and 4.26mg/L. In the control group, swimming behaviour, respiration was normal, there was no pigmentation, and they survived until the end of the study. Zebrafish treated with test compound at the dose of 0.88mg/L, 1.94 mg/L and 4.26mg/L there was no change in swimming, pigmentation, respiration and survival. There were changes in swimming behavior, respiration, and pigmentation of zebrafish treated with test compound at the dose of 9.37 mg/L were observed.

From the result, the LD50 was found to be 6.9 mg for diclofenac. Hence, the dose of 1.874 mg/L (1/5) was selected for safety purposes.

4.2.2 Behavioural parameters

Zebrafish were evaluated for learning and memory using physical test T maze test and inhibitory avoidance test. In T-maze test, when fish were treated with scopolamine, group spent less time in green arm and less number of entries in the green arm than the normal group(<0.0001)) this indicates that there is memory impartment in zebrafish. Standard treatment donepezil and curcumin combination with probiotic and diclofenac attenuates the effect of the scopolamine in physical parameters. The time spent in green arm donepezil treated group was more and number of entries in the green arm was significantly higher($p<0.0001$) than scopolamine group. In curcumin combination with probiotics there was increased ($p<0.0001$) in the time spent and the number of entries compared to scopolamine group. In curcumin combination with diclofenac there was greater significance ($p<0.0001$) compared to scopolamine group. Curcumin alone also showed significance ($p<0.0001$)in the entries and time spent in that green arm. Results are showed in Fig no.2. Latency period was evaluated from the inhibitory avoidance test. The scopolamine treated group exhibited shorter latency period compared to normal group. In donepezil there was increased in the latency period than alone scopolamine group, in curcumin alone latency period was significant compared to scopolamine and combination also posses a greater significance than the induced group.

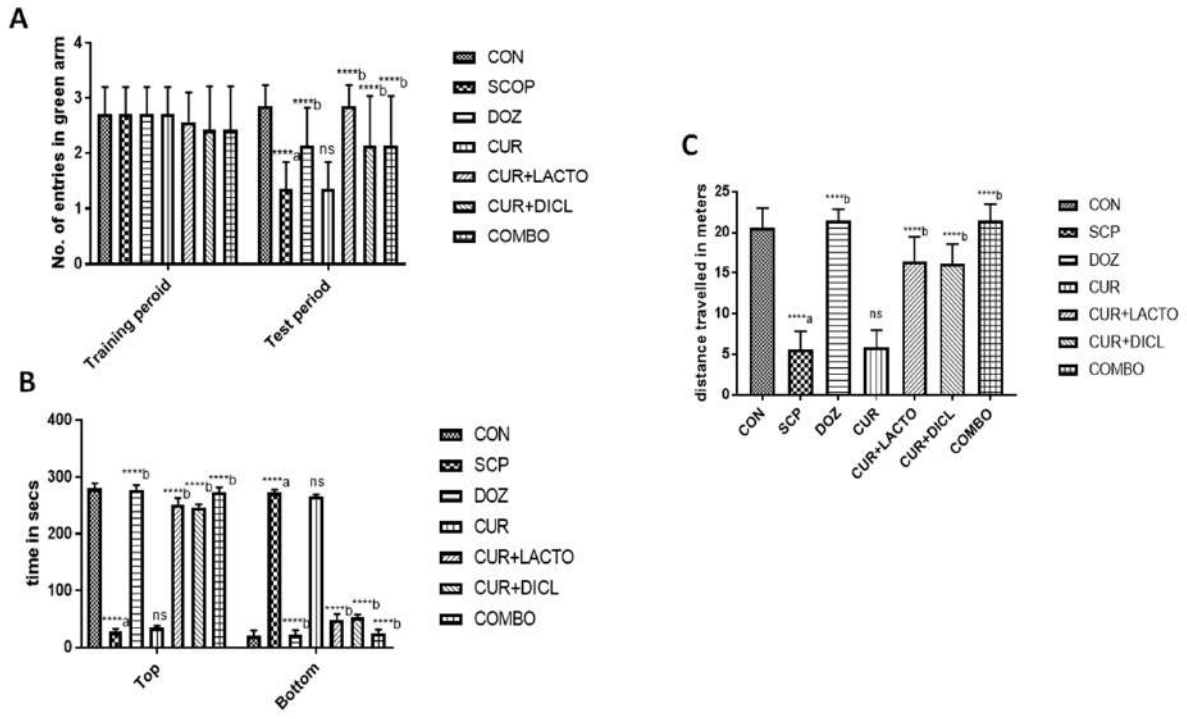

Fig. 2 A- Graph shows no. of entries in green arm, B- Graph shows time spent in top and bottom of tank, C- Graph shows distance travelled. The data are expressed as mean ± SD (n=7), a Control group significant compared to SCOP group whereas b DPZ group , Cur group and CUR+PRO group, CUR+DICL and CUR+COMBO group compared to SCOP 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.

4.2.2. Neurochemical parameters

4.2.2.1 Effect on acetylcholinesterase (Ache) activity

Zebrafish, when exposed to scopolamine, possess a substantial rise in the level of AchE in SCP group(10.73±0.10, p<0.0001) as compared to CON (6.62±0.28). In the CUR treatment group, there is no significant change in enzyme activity (9.45±0.54, ns). In DOZ and COMBO group their is petty the level of enzyme and shows higher significance as 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+COMBO also lowered the enzyme activity (7.96± 0.26, p<0.0001).

4.2.2.2 Effect on lipid peroxidation activity:

The substantial rise in the MDA levels in the SCP group (1.41 ±0.17, p≤0.0001) compared to the CON group (0.70 ±0.20); while the MDA level in DOZ and COMBO groups were expressively attenuated (0.54±0.23and 0.52±0.16, p≤0.0001) compared to the SCP group. Additionally, CUR+LACTO and CUR+COMBO groups have significant results (0.62±0.57 and 0.69±0.15, p≤0.0001). In CUR group there is a higher level of MDA which shows non-significant results (1.4 ±0.25, ns).

4.2.2.3 Effect on Superoxide dismutase activity:

Superoxide dismutase enzyme was low in the SCP group (2.90±0.10, p≤0.0001) as linked to the CON group (13.03±0.12). In the DOZ and COMBO group there is a noteworthy rise in the enzyme activity compared to the SCP group (12.59±0.08and 14.93±0.60, ≤0.001). Moreover, CUR+LACTO and CUR+COMBO have significant results (12.83 ±0.30 and 11.18 ±0.68, ≤0.001) In CUR group, there is no significance in the enzyme level in comparison to SCOP group (3.16 ±0.14, ns).

4.2.2.4 Effect on glutathione peroxidase activity:

In the standard group, DOZ, and COMBO group there was a substantial rise in the enzyme activity comparison to SCP group (13.24 ±0.45 and 13.67 ±0.36, p≤0.0001). Besides CUR+LACTO and CUR+ COMBO have significant results (11.79 ±0.38 and 10.8 ±0.36, p≤0.0001). Whereas the SCP group there is diminish in the enzyme activity (5.9±0.27, p<0.0001) glutathione peroxidase when compared to CON (14.32±0.11). CUR group posses insignificance result (6.9 ±0.30, ns) in comparsion with SCP group.

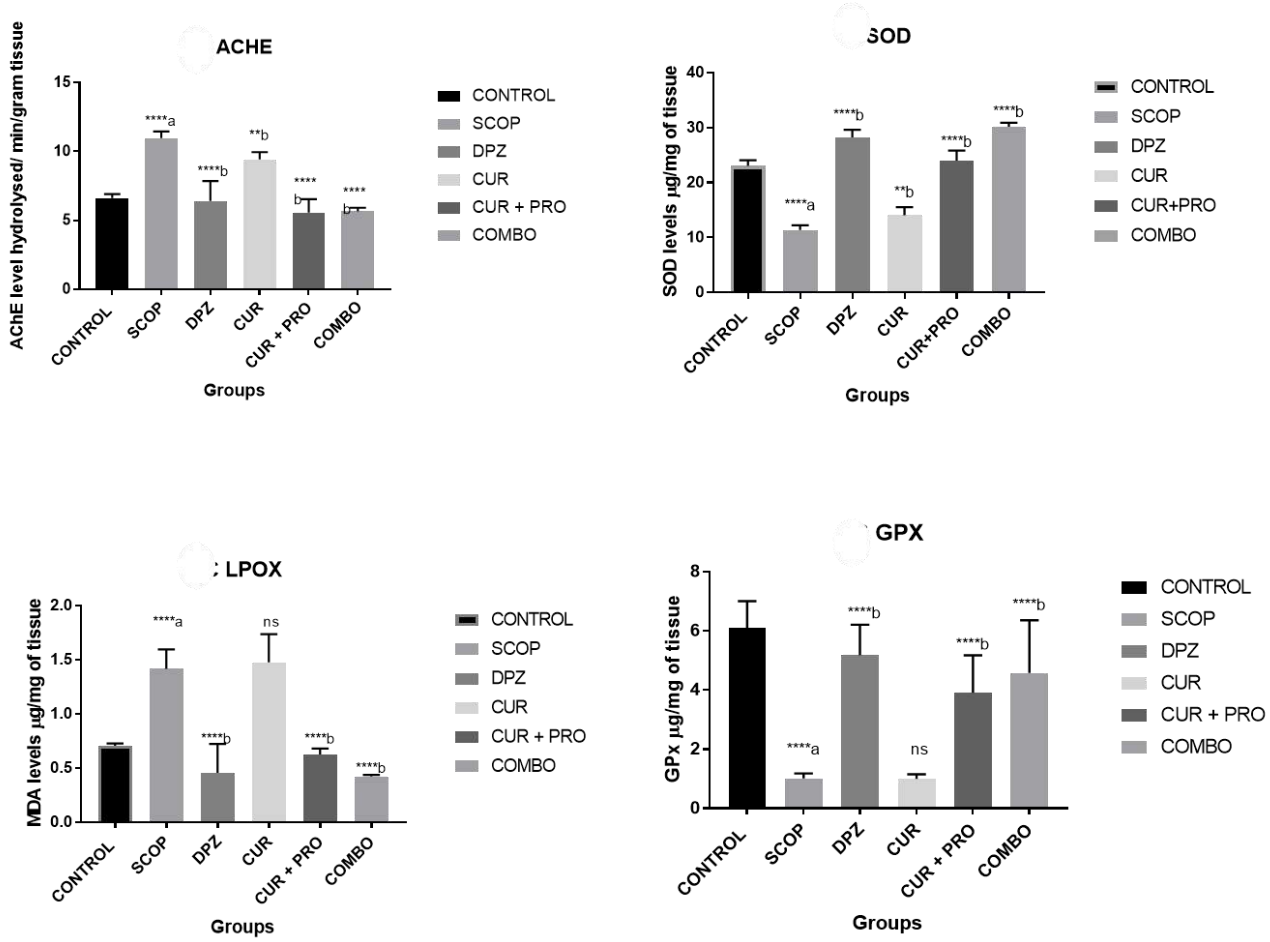

Fig. 3 Graph of Neurochemical parameters in zebrafish The values are the mean ± SD with n=7 per group; a represents the significance level between control and SCOP groups; b represents the significance level between DPZ group , Cur group, and CUR + PRO group CUR+ *COMBO* group compared to SCOP group. Value of $P \geq 0.05$ was shown by marking "ns" while *P*-values ≤0.05, ≤0.01, and ≤0.001 were expressed as *, **, and ***, respectively.

4.3 Neuroprotective effect of curcumin in combination with diclofenac and *Lactobacillus rhamnous* in scopolamine induced dementia in mice.

During the test session, the control mice readily reached the platform. However, the mice treated with a combination of donepezil and curcumin along with the L. rhamnosus group initially faced challenges in locating the hidden platform on days 0 and 1. Subsequently, they exhibited improved memory and successfully located the hidden platform ($p \leq 0.0001$). In contrast, the mice administered with only scopolamine displayed an increased time in reaching the platform, indicating memory impairment, as illustrated in Fig. 4.

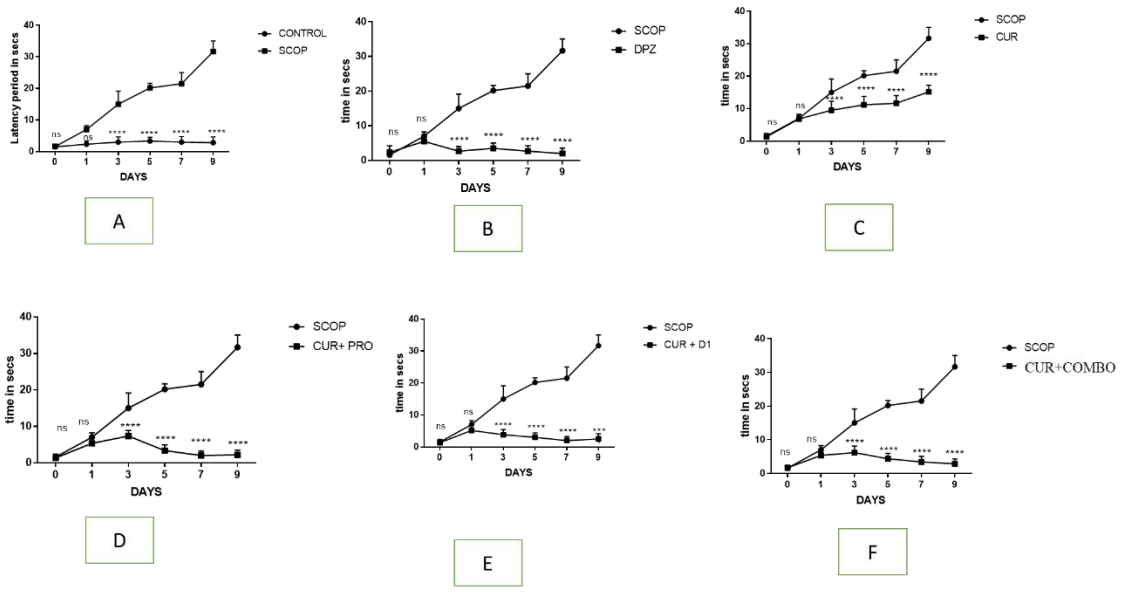

Fig. 4 Graph shows the escape latency in Morris water test between A-control group and scopolamine treated group (1 mg/kg), B- scopolamine treated group (1 mg/kg) and donepezil treated group (2mg/kg), C- Graph shows the escape latency in Morris water test between scopolamine treated group (1 mg/kg) and curcumin treated group (205mg/kg), D- scopolamine treated group (1 mg/kg) and curcumin + probiotic group (205 mg/kg + 10^6 cfu), E- scopolamine treated group (1 mg/kg) and curcumin + Combo treated group (205 mg/kg+ 10^6 cfu +10mg/kg), F- scopolamine treated group (1 mg/kg) and curcumin + diclofenac 1 treated group (205 mg/kg+5mg/kg). 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.

When mice were exposed to scopolamine alone, there was a significant increase in the level of AchE, an enzyme responsible for acetylcholine degradation ($p<0.0001$), compared to the control group. Furthermore, treatment with a combination of donepezil and curcumin along with probiotics exhibited a significant inhibition of brain enzyme activity ($p<0.0001$) when contrasted with those treated with scopolamine alone. In the group treated solely with curcumin, there was no noteworthy change in enzyme activity (ns). The scopolamine-treated group displayed reduced activity of the enzyme glutathione peroxidase compared to the control group ($p<0.0001$).

In the standard group treated with donepezil, curcumin combined with probiotics, and diclofenac, there was a noteworthy increase in enzyme activity ($p<0.001$ for all three groups). However, the administration of curcumin alone did not induce any significant changes, as depicted in Fig. 4. The scopolamine-treated group exhibited reduced activity of the enzyme superoxide dismutase compared to the control group ($p\leq0.0001$).

Conversely, the group treated with a combination of donepezil and curcumin along with probiotics displayed a significant increase in enzyme activity compared to the scopolamine group. In the curcumin alone group, there were no significant changes in enzyme activity compared to the scopolamine-treated group (ns). MDA (malondialdehyde) levels significantly increased in the scopolamine-induced group in comparison to the control group ($p<0.0001$). Conversely, the levels of MDA in groups treated with a combination of donepezil and curcumin along with probiotics and diclofenac were significantly attenuated ($p<0.0001$ for all three groups). In the curcumin alone group, a significant change ($p<0.001$) was observed compared to the scopolamine group, as shown in Fig 5.

Exposure of mice to scopolamine led to a substantial increase in protein carbonyl levels in the brain ($p<0.0001$) compared to the control group. However, concurrent treatment with a combination of donepezil and curcumin along with probiotics and diclofenac resulted in a significant reduction in brain protein carbonyl levels ($p<0.0001$) compared to curcumin

treatment.

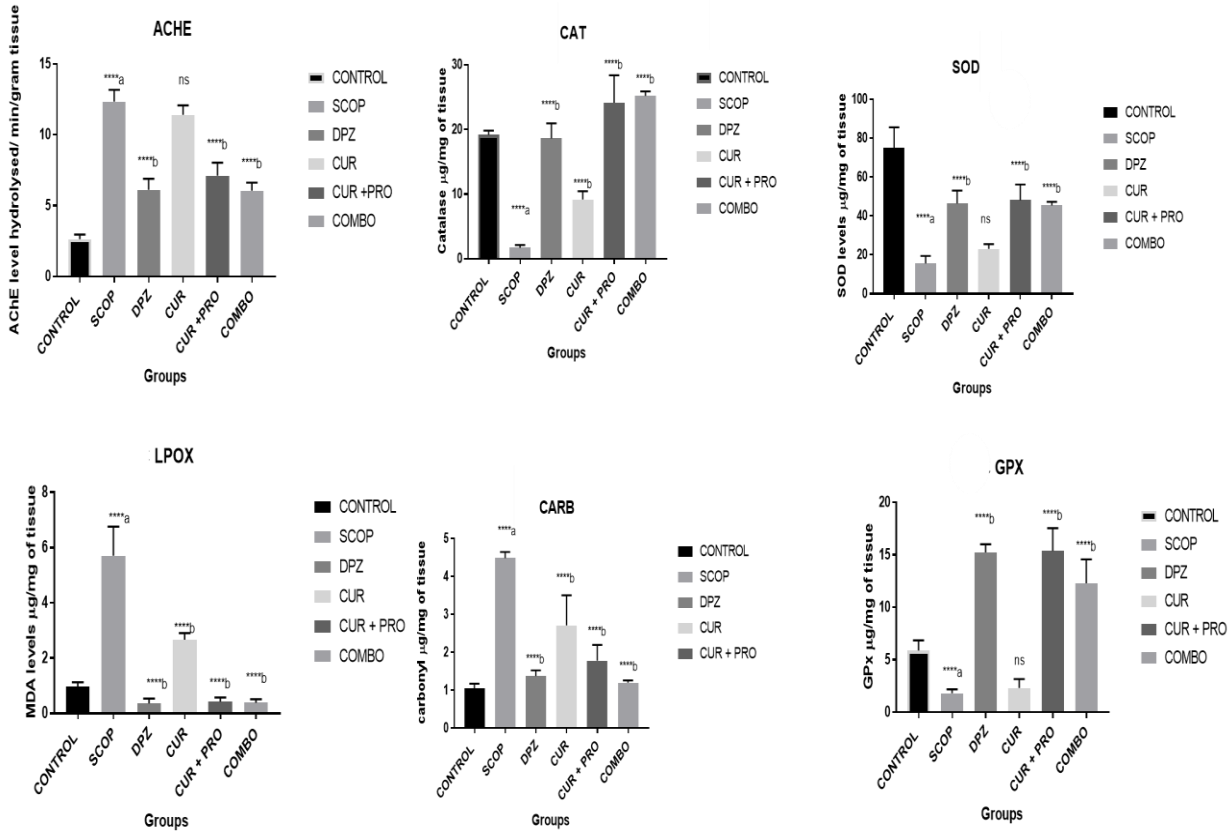

Fig. 5 Graph of Neurochemical parameters in mice. The values are the mean ± SD with n=6 per group; a represents the significance level between CON and STZ groups; b r The values are the mean ± SD with n=7 per group; a represents the significance level between control and SCOP groups; b represents the significance level between DPZ group , Cur group, and CUR + PRO group and CUR+COMBO compared to SCOP group. Value of $P \geq 0.05$ was shown by marking "ns" while *P*-values ≤0.05, ≤0.01, and ≤0.001 were expressed as ∗, ∗∗, and ∗∗∗, respectively.

4.4 Histology:

In the histopathology examination, as illustrated in Fig. 8, the control group exhibited normal neuronal cells. In contrast, the SCOP group displayed neuronal damage characterized by dark basophilic cytoplasm and condensed nuclei. This group also exhibited fewer neuronal cells with pyknosis, and there was a notable deposition of amyloid plaques (indicated by arrows).

In the DPZ group, the hippocampus displayed a histologically normal appearance with only mild neuronal damage as shown in fig 6. In comparison, the CUR group exhibited hippocampal neuronal damage, along with the presence of dark neurons showing shrunken cytoplasm and pyknosis. This was further associated with a reduction in neuronal cell count.

The CUR+PRO and CUR+COMBO groups exhibited mild neuronal damage, with no significant loss of cell integrity.

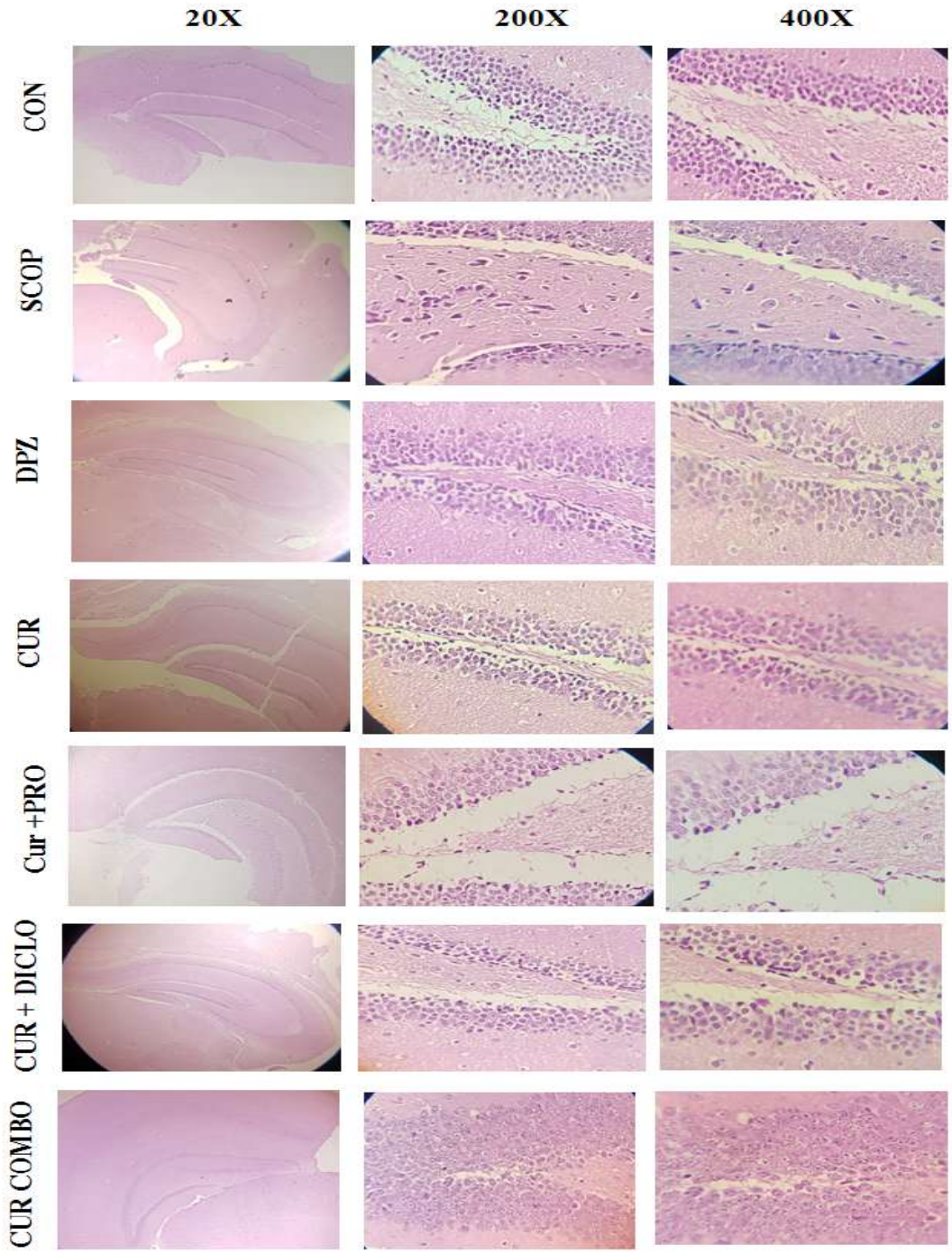

Fig. 6 Histology of mice brain section

Liver histology was done in order to check any effect on liver due to consumption of diclofenac as shown in Fig 7

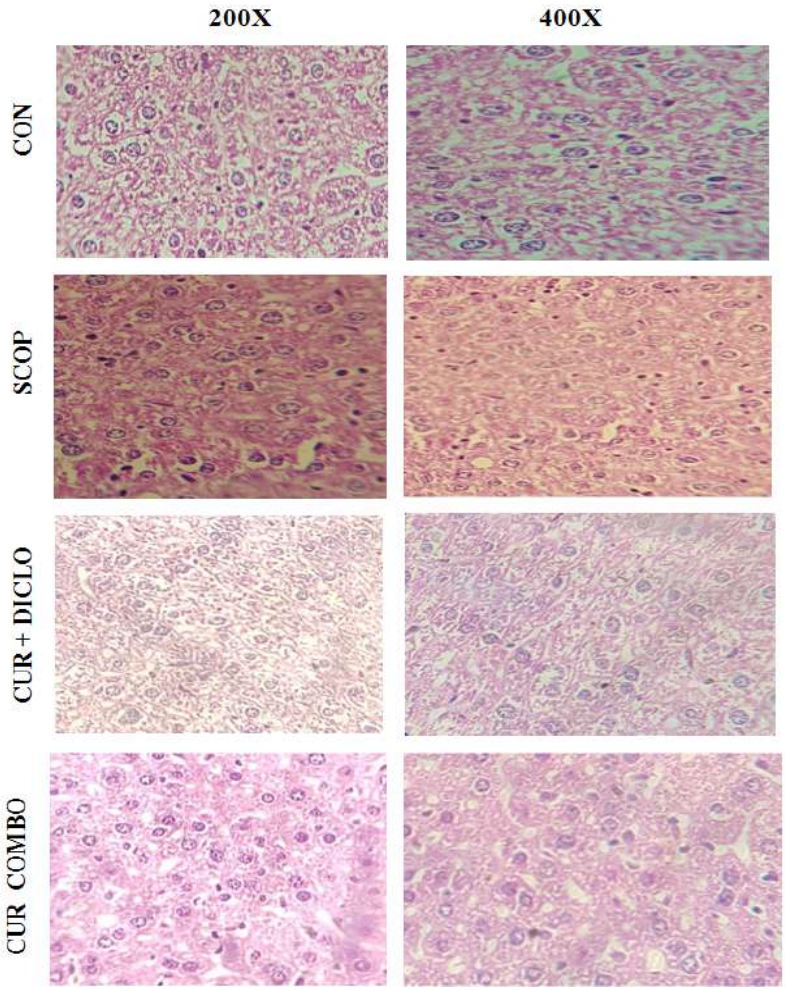

Fig. 7 Histology of mice liver section

In the liver histopathology study, the control group, as well as the scopolamine-treated group, displayed normal cellular morphology with intact cell integrity. Kupffer cells appeared normal, and sinusoids were present in the sections examined. However, in the curcumin combination with diclofenac group 2 and the curcumin combination group, there was evidence of kupffer cell hyperplasia.

5. Discussion

Alzheimer's diseases are the sixth leading cause of death in United States. Estimated lifetime risk of AD Gender wise at age of 45-65 year is; at age 45 risk of AD in male is 10.6% and

women are 19.5%. At age of 65 males are at risk of 11.6% whereas women 21.1%. Form this it can be concluded that women are more prone to this disease compared to men [1-3]. The affected region of brain possesses neuron atrophy, astrogliosis, and progressive loss of structure and function of neurons by the deposition of two abnormal structures one is senile plaques and other is neurofibrillary tangles (NFTs). Also multifactorial mechanism of AD, which has been proposed and states that mechanism involved in the AD pathogenesis also comprises of oxidative stress, inflammation, metal ion deregulation and cell cycle regulatory sequence [10]. This leads to loss of neurons, particularly cholinergic neurons which are responsible for the memory [15-16]. Here we have selected scopolamine a non-selective muscarinic blocker which blocks the cholinergic transmission and cause impaired memory loss in zebrafish and mice. Also, scopolamine leads to increase in the oxidative stress and reduce the level of the anti-oxidant system in the brain. As reported scopolamine if given longer duration will cause amyloid beta formation. In study we also found a significant increase in the Ache level in the brain of mice [31-33].

In this study, we have found the neuroprotective effect of curcumin with probiotics and diclofenac in scopolamine induced dementia in zebrafish and mice. Curcumin itself have numerous role in the AD conditions by scavenging free radicals, hinders the formation and promotes the disaggregation of amyloid beta plaques, diminishes the hyper phosphorylation of tau and enhances its clearance, inhibits acetylcholinesterase, anti-inflammatory, have metal chelation property and also possess other beneficial effects but due to its low bioavailability it is not used alone.

Hence, we have used technique to overcome the poor bioavailability by targeting the metabolism step by diclofenac and increasing the absorption of curcumin by probiotics. In metabolism of curcumin there is an enzyme UGTs which metabolized curcumin into its metabolite. This enzyme will be inhibited by diclofenac; also, there is an enzyme beta glucuronidase which is generated from probiotics *Lactobacillus rhamnous* in intestine hydrolyzed back the curcumin metabolite into the curcumin by this there is increase the activity of curcumin for its therapeutic effect. Level of curcumin if given orally in the brain was less than 2% or not detected in the brain. Models used is zebra fish and mice model.

Zebrafish possess a high degree of similarity between the brain anatomic compared to humans. The *psen1* and *psen2* gene of zebrafish has some resemblance to that of humans PSEN1 and PSEN2. While BACE1 and BACE2 orthologues are also similar to that of humans. The study of learning and cognition in zebrafish is likely to be a rich and productive endeavor.

Additionally, they contains UGTs enzymes. UGT1 and UGT2 are present in the zebrafish. Mice they possess UGTs present in humans.

From this study it was found that curcumin if given alone showed less effect in all the parameters, also some neurochemical parameter such as AchE, GPx and SOD showed no significant change compared to the scopolamine group. Donepezil and curcumin combination with probiotics and diclofenac showed significant change with p value ≤0.001 when compared with scopolamine.

Zebrafish were evaluated for learning and memory using physical test T maze test and results depicts that when fishes treated with scopolamine spent less time in green arm and less number of entries in the green arm than the normal group(<0.0001)) this indicates that there is memory impartment in zebrafish. Standard treatment donepezil and curcumin combination with probiotic and diclofenac attenuate the effect of the scopolamine in physical parameters. The time spent in green arm donepezil treated group was more and number of entries in the green arm was significantly higher (p<0.0001) than scopolamine group. In Morris water maze test, the physical behavior was evaluated against scopolamine group. Parameters were performed on 0,1,3,5,7 and 9 th day. All group on 0 and 1 st day did not showed any significant change. From 3rd day there was significant change in the parameters with p value ≤0.001, from which it is concluded that there was a progressive improvement in memory to find out the hidden platform in the water. Whereas scopolamine induced group took more time to find the hidden platform which submerse that memory was impaired in that group.

In pathological conditions there is elevated lipid and protein oxidation due to oxidative stress. The level of antioxidant enzyme in the synaptic neurons are lowered. Curcumin upregulates the expression of genes that encodes the antioxidants such as heme oxygenase, catalase superoxide dismutase [34-37]. Curcumin overwhelms the microglia activation and attenuates the release of cytokines and acts as anti-inflammatory. Also reported in literature that Curcumin lead to inhibition of amyloid beta plaques by downregulating the BACE1 gene. Additionally, the two hydroxyl groups present in the structure of curcumin interacts with the polar pockets of the A-beta peptide which then hinders the stabilization of the sheet. It involves in the tau tangle clearance by upregulating the BAG 2 gene. AChE activity was reversed by curcumin. Curcumin possess metal chelation property curcumin chelated the copper and inhibited the fibril formation in the brain. **S**terol regulatory element is the transcription factors which synthesis the cholesterol. Curcumin inhibits this SREB and leads to less neurotoxicity [38-40].

From present study it was concluded that curcumin combination with probiotics and diclofenac possess much greater effect compared to alone curcumin. This is because of the production of the curcumin from its metabolite and remaining for more time in the body and inhibition of UGTs, it was clearly observed the level of antioxidant enzyme was elevated in the curcumin combination with the probiotic and diclofenac compared to the scopolamine and curcumin alone. Also physical behavior examination possesses a significant change in the combination compare to curcumin alone. The level of Ache, lipid peroxidation and protein carbonyl was less in the combination group; which gives us a more confirmatory evidence that the combination leads to more synergistic effect as compared to alone. Histology also showed that there is an amyloid plaques formation in scopolamine group as compared to control, donepezil, curcumin combination with probiotics and diclofenac group showed less accumulation of plaques and less neuronal damage which depicts the neuroprotective effect.

6. Impact of the research in the advancement of knowledge or benefit to mankind

Polyphenolic compounds sourced from natural origins are widely acknowledged for their safety, particularly among elderly patients. However, the challenge lies in their limited oral bioavailability, which can hinder their effectiveness in reaching the brain and producing the desired therapeutic effects. This becomes particularly crucial as these plant-based compounds tend to exit the body rather swiftly.

To address this issue and advance the field, various methods have been explored to enhance the systemic bioavailability of curcumin. However, many of these methods are associated with high costs, reduced stability, and patient inconvenience. Therefore, a significant approach emerges—targeting metabolism through the use of specific, potent, and safe inhibitors. One illustrative example is the curcumin-piperine complex, which has demonstrated a notable increase in bioavailability.

Understanding that curcumin undergoes metabolism by intestinal enzymes, some of which eliminate them rapidly while others convert them back to their original form, opens up a path to improving oral bioavailability of polyphenols. By strategically inhibiting these enzymes, the potential to optimize the therapeutic effects of polyphenols is promising, particularly in addressing and possibly even curing Alzheimer's disease.

In conclusion, this research not only contributes to the expansion of scientific knowledge but also holds the potential to significantly benefit humanity. The ability to enhance the oral

bioavailability of polyphenols, specifically curcumin, can lead to ground-breaking advancements in the treatment of Alzheimer's disease, improving the quality of life for numerous individuals and potentially offering new avenues for medical intervention.

7. Literature reference

1) Weller J, Budson A (2018) Current understanding of Alzheimer's disease diagnosis and treatment. F1000 Res 7. https://dx.doi.org/10.12688%2Ff1000research.14506.1

2) Bondi MW, Edmonds EC, Salmon DP (2017) Alzheimer's disease: past, present, and future. J Int Neuropsych Soc 23(9-10):818-31. https://dx.doi.org/10.1017%2FS135561771700100X

3) Kametani F, Hasegawa M (2018) Reconsideration of amyloid hypothesis and tau hypothesis in Alzheimer's disease. Front Neurosci 12:25. https://doi.org/10.3389/fnins.2018.00025

4) Chesser A, Pritchard S, Johnson GV (2013) Tau clearance mechanisms and their possible role in the pathogenesis of Alzheimer disease. Front Neurosci 4:122. https://doi.org/10.3389/fneur.2013.00122

5) Gong CX, Liu F, Iqbal K (2018) Multifactorial hypothesis and multi-targets for Alzheimer's disease. J Alzheimers Dis 64(s1): S107-17. https://doi.org/10.3233/JAD-179921

6) Sathianathan R, Kantipudi SJ (2018) The dementia epidemic: Impact, prevention, and challenges for India. Indian J Psychiatry 60(2):165. https://dx.doi.org/10.4103%2Fpsychiatry.IndianJPsychiatry_261_18

7) Alzheimer's association report (2019) FDA-approved treatments for Alzheimer's. https://www.alz.org/media/documents/fda-approved-treatments-alzheimers-ts.pdfalz.org Accessed 16 November 2019

8) Gupta SC, Kismali G, Aggarwal BB (2013) Curcumin, a component of turmeric: from farm to pharmacy. Biofactors 39(1):2-13. https://doi.org/10.1002/biof.1079

9) Tang M, Taghibiglou C (2017) The mechanisms of action of curcumin in Alzheimer's disease. J Alzheimers Dis 58(4):1003-16. https://doi.org/10.3233/JAD-170188

10) Li HL, Liu C, De CoutoG, Ouzounian M, Sun M, Wang AB et al (2008) Curcumin prevents and reverses murine cardiac hypertrophy. J ClinInvest 118(3):879-93. https://doi.org/10.1172/JCI32865.

11) Di Martino RM, Luppi B, Bisi A, Gobbi S, Belluti F. (2017) Recent progress on curcumin-based therapeutics: a patent review Part I: curcumin. Expert Opin Ther Pat 4;27(5):579-90. https://doi.org/10.1080/13543776.2017.1276566

12) Kunnumakkara AB, Harsha C, Banik K, Vikkurthi R, Sailo BL, Bordoloi D, et al (2019) Is curcumin bioavailability a problem in humans: lessons from clinical trials. Expert Opin Drug Metab Toxicol 15(9):705-33. https://doi.org/10.1080/17425255.2019.1650914

13) Anand P, Kunnumakkara AB, Newman RA, Aggarwal BB et al (2007) Bioavailability of curcumin: problems and promises. Mol Pharm 4(6):807-18. https://doi.org/10.1021/mp700113r

14) Prasad S, Tyagi AK, Aggarwal BB (2014) Recent developments in delivery, bioavailability, absorption and metabolism of curcumin: the golden pigment from golden spice. Cancer Res Treat 46(1):2. https://doi.org/10.4143/crt.2014.46.1.2

15) Heger M, van Golen RF, Broekgaarden M, Michel MC (2014) The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. Pharmacol Rev 66(1):222-307. https://doi.org/10.1124/pr.110.004044

16) Ozawa H, Imaizumi A, Sumi Y, Hashimoto T, Kanai M, Makino Y et al (2017) Curcumin β-D-glucuronide plays an important role to keep high levels of free-form curcumin in the blood. Biol Pharm Bull 40(9):1515-24. https://doi.org/10.1248/bpb.b17-00339

17) Westerik N, Kort R, Sybesma W, Reid G (2018) Lactobacillus rhamnosus probiotic food as a tool for empowerment across the value chain in Africa. Front Microbiol 10;9:1501 https://dx.doi.org/10.3389%2Ffmicb.2018.01501

18) Zhou JS, Shu Q, Rutherfurd KJ, Prasad J, Gopal PK, Gill HS (2000) Acute oral toxicity and bacterial translocation studies on potentially probiotic strains of lactic acid bacteria. Food Chem Toxicol 1;38(2-3):153-61 https://doi.org/10.1016/s0278-6915(99)00154-4

19) Kajander K, Myllyluoma E, Rajlic-Stojanovic M, Kyronpalo SS, Rasmussen M, Jarvenpaa et al (2008) Clinical trial: multispecies probiotic supplementation alleviates the symptoms of IBS and stabilises intestinal microbiota. Aliment Pharmacol Ther. 27(1):48-57 https://doi.org/10.1111/j.1365-2036.2007.03542.x

20) Sanders ME (2008) Probiotics: definition, sources, selection, and uses. Clin Infect Dis. 46(Supplement_2):S58-61. https://doi.org/10.1086/523341

21) Athari Nik Azm S, Djazayeri A, Safa M, Azami K, Ahmadvand B, Sabbaghziarani F, et al. (2018) Lactobacilli and bifidobacteria ameliorate memory and learning deficits and oxidative stress in β-amyloid(1–42) injected rats. Appl Physiol Nutr Metab 43(7):718-26. https://doi.org/10.1139/apnm-2017-0648

22) Pham PL, Dupont I, Roy D, Lapointe G, Cerning J (2000) Production of exopolysaccharide by Lactobacillus rhamnosus R and analysis of its enzymatic degradation during prolonged fermentation. Appl Environ Microbiol 66(6):2302-10. https://dx.doi.org/10.1128%2Faem.66.6.2302-2310.2000

23) Biernat KA, Pellock SJ, Bhatt AP, Bivins MM, Walton WG, Tran BN (2019) Structure, function, and inhibition of drug reactivating human gut microbial β-glucuronidases. Sci Rep 9(1):1-5. https://www.xmol.com/paperRedirect/979031

24) Todirascu-Ciornea E, El-Nashar HA, Mostafa NM, Eldahshan OA, Boiangiu RS, Dumitru G, et al. Schinusterebinthifolius Essential Oil Attenuates Scopolamine-Induced Memory Deficits via Cholinergic Modulation and Antioxidant Properties in a Zebrafish Model. Evid Based Complement Alternat Med 2019;2019.

25) Hamilton TJ, Morrill A, Lucas K, Gallup J, Harris M, Healey M, et al. Establishing zebrafish as a model to study the anxiolytic effects of scopolamine. Sci Rep 2017; 7(1):1-9.

26)Goverdhan P, Sravanthi A, MamathaT (2012) Neuroprotective effects of meloxicam and selegiline in scopolamineinduced cognitive impairment and oxidative stress. Int J Alzheimers Dis 2012. https://doi.org/10.1155/2012/974013

27)Rinwa P, Kumar A (2012) Piperine potentiates the protective effects of curcumin against chronic unpredictable stress-induced cognitive impairment and oxidative damage in mice. Brain Res 1488:38-50. https://doi.org/10.1016/j.brainres.2012.10.002

28)Vorhees CV, Williams MT (2006) Morris water maze: procedures for assessing spatial and related forms of learning and memory. Nat Protoc 1(2):848. https://doi.org/10.1038/nprot.2006.116

29)Nunez J (2008) Morris water maze experiment. J Vis Exp (19):897 https://dx.doi.org/10.3791%2F897

30)Ellman GL, Courtney KD, Andres Jr V, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7(2):88-95. https://doi.org/10.1016/0006-2952(61)90145-9

31)Ohkawa H, Ohishi N, Yagi K (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 95(2):351-8. https://doi.org/10.1016/0003-2697(79)90738-3

32)Rukmini MS, D'souza B, D'souza V (2004) Superoxide dismutase and catalase activities and their correlation with malondialdehyde in schizophrenic patients. Indian J Clin Biochem 19(2):114. https://dx.doi.org/10.1007%2FBF02894268

33)Weydert CJ, Cullen JJ (2010) Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat Protoc 5(1):51. https://doi.org/10.1038/nprot.2009.197.

34)H. Luck, H. U. Bergmeyer (1971) Catalase in Methods of Enzymatic Analysis. Academic Press, NewYork, USA.

35) Karlinsky H (1986) Alzheimer's disease in Down's syndrome: a review. J Am Geriatr Soc 34(10):728-34. https://doi.org/10.1111/j.1532-5415.1986.tb04304.x

36)Glenner GG, Wong CW (1984) Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. Biochem Biophys Res Commun 120(3):885-90. https://doi.org/10.1016/s0006-291x(84)80190-4

37)Spencer D, Lal H.(1983) Effects of anticholinergic drugs on learning and memory. Drug Dev Res 3(6):489-502. https://doi.org/10.1002/ddr.430030602

38)Chen KC, Baxter MG, Rodefer JS (2004) Central blockade of muscarinic cholinergic receptors disrupts affective and attentional set- shifting. Eur J Neurosci 20(4):1081-8. https://doi.org/10.1111/j.1460-9568.2004.03548.x

39)Tsukada H, Nishiyama S, Fukumoto D, Ohba H, Sato K, Kakiuchi T (2004) Effects of acute acetylcholinesterase inhibition on the cerebral cholinergic neuronal system and cognitive function: functional imaging of the conscious monkey brain using animal PET in combination with microdialysis. Synapse 52(1):1-0. https://doi.org/10.1002/syn.10310

40)Konar A, Gupta R, Shukla K, Maloney B, Khanna V, Wadhwa R, et al (2019) M1 muscarinic receptor is a key target of neuroprotection, neuroregeneration and memory recovery by i-Extract from Withania somnifera. Sci Rep 9, 13990 https://doi.org/10.1038/s41598-019-48238-6

41) OECD Guidelines for the Testing of Chemicals, Test system no 203, Fish, Acute Toxicity Test, available on https://www.oecd-ilibrary.org/environment/test-no-203-fish-acute-toxicity-test_9789264069961-en Accessed on 02 Nov 2019