Neuroprotective and memory enhancing effect of Nose to Brain formulation of 6- Hydroxyflavone in icv-STZ-induced Alzheimer's disease mouse model

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Abstract

Alzheimer's disease characterized by memory and cognitive impairments as well as neurobehavioral abnormalities. Streptozotocin (STZ) can induce a condition analogous to alzheimer's disease by promoting the accumulation of plaques and neurofibrillary tangles, resulted in progressive cognitive decline. 6-hydroxyflavone (6HOF) is a flavone that occurs naturally in the foliage of *Barleria prionitis*, an Acanthaceae plant species native to India. The purpose of this study was to examine the effects of intranasal formulation of 6-Hydroxyflavon (6HOF-NTB) in mice that had been administered STZ (3mg/kg of icv) unilaterally. Neurodegeneration, and oxidative stress were used to evaluate the impact on working memory and neuroprotection. Memory impairment was developed by intracerebrovascular (icv) injections of streptozotocin (3mg/kg). Upon 21-day administration of 6HOF-NTB and Oral administrations of 6-HOF the behavioral parameter was studied using Morris water maze, novel object recognitions test, Y-maze, EPM etc. and the level of acetylcholinesterase and antioxidants was measured in brain homogenate.In a mouse model of a condition like Alzheimer's, demonstrated significant effect than the STZ-treated group. The 6HOF-NTB (1 mg/kg) increased transfer latency in the EPM, spontaneous alternation in the Y-maze, discrimination index, and novelty preference in the NOR. In addition, in the MWM, the number of platform crossings and time spent in the target quadrants increased, indicating an improvement in working spatial memory. In addition, the intranasal formulation inhibited oxidative stress and acetylcholinesterase levels in the brain, thereby reducing neurodegeneration. In the STZ induced memory impairment model in mice, the intranasal formulation of 6 HOF demonstrated desirable effects on cognitive functions and neuroprotection.

1. Introduction

Alzheimer's disease (AD) is the most common neurological disease that gets worsen over time. It is the leading cause of death in older people and is marked by problems with memory and thinking, as well as changes in behavior (Paladugu et al., 2021). It has a complicated pathophysiology that includes the buildup of amyloid beta (A) plaques, the accumulation of hyperphosphorylated tau proteins (pTau), which causes neurofibrillary tangles (NFTs), and neurodegeneration, which can lead to the loss of synapses and damage to neurons (Kowiański et al., 2018). this disorder is linked to inflammatory changes, mitochondrial failure, and oxidative stress in the cortical and hippocampus of the brain (Selkoe and Schenk, 2003). The prevalence of Alzheimer's disease increases with age, and it is estimated that about 5–8% of people over the age of 60 have Alzheimer's disease. By the age of 85, the prevalence of Alzheimer's disease is estimated to be around 30–40%. (Yamini et al., 2022a)

Most current therapies for Alzheimer’s disease and other CNS disorders use the peripheral routes of drug administration (parenteral and oral). This type of endeavour diminishes the efficacy and potency of the medication treatment. Nose-to-brain drug delivery allows the direct transport of therapeutic molecules by bypassing the BBB and increases drug concentration in the brain (Lochhead et al., 2015). In the current study for the purpose of bypassing BBB we formulate PLGA-6HOF nanoparticle emulsion in a polymer

termed poly (lactic-co-glycolic acid) using the emulsion diffusion technique (Fortuna et al., 2014, Pieper and Langer, 2017, Shah et al., 2021).

Intracerebroventricular streptozotocin (icv-STZ) is used to produce loss of brain function in similar way of Alzheimer's disease (Mehla et al., 2013, Salkovic-Petrisic et al., 2013). Streptozotocin (STZ) has a glucosamine-nitrosourea properties affects many parts of behavior, such as learning, memory, and cognitive functions. This impairment is caused by interfering with insulin signals in the brain, messing up the way energy was used, increased neuroinflammation, problems with the cholinergic system, oxidative stress in the brain, and problems with the way neurons work. (Grieb, 2016).

6-Hydroxyflavon (6-hydroxy-2-phenylchromen-4-one) is a naturally occurring flavone found in the leaves of *Barleria prionitis*, an Acanthaceae family plant native to India that is frequently used to treat neurological disorders such as paraplegia and sciatica(Banerjee et al., 2012, Gupta et al., 2016). 6-Hydroxyflavone is known to have several pharmacological effects, such as anti-anxiety(Ren et al., 2010), antioxidant (Stompor, 2016), anti-inflammatory(Wang et al., 2015), muscle relaxant, anti-asthmatic (Flores-Flores et al., 2019) and osteoblast differentiation (Lai et al., 2014)etc.

This study evaluate the effect of 6-hydroxyflavone through the nose, and alterations in behavior of swiss albino mice that had been given icv-STZ. This was determined by analyzing (a) learning and spatial memory in a working memory model of the Morris Water Maze task; (b) anxiety and exploratory behavior utilizing the Elevated Plus Maze; (c) novelty preference and exploratory behavior utilizing the Novel Object Recognition; and (d) spontaneous alternation and exploratory behavior utilizing the Y-Maze. e) oxidative stress as measured by oxidative parameters; f) Acetylcholine esterase levels in the brain as determined by Ellman's assay; g) histopathology.

2. Materials and Methods

2.1 Drug and chemicals:

6-Hydroxyflavone (Sigma-Aldrich), Donepezil (Donep-5, Alkem Science), Streptozotocin (A K Scientific), Ketamine (Themis Medicare limited, batch no. KMI 902) & Xylazine (xylo-B, brilliant biopharma batch no XLB1801), Poly (lactic-co-glycolic acid) (PLGA), Acetylthiocholine (Sigma-Aldrich), Hydrogen peroxide, Sodium hydroxide, di-sodium Hydrogen phosphate, acid, DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma-Aldrich) were purchased. Sodium monophosphate phosphate and potassium monophosphate phosphate were obtained from Rankem laboratories.

2.2 Nanoparticle PLGA-6HOF Formulation

In 2.5 ml ethyl acetate, 50 mg PLGA (Poly (lactic-co-glycolic acid)) was dissolved, and 200µl of a 6-hydroxyflavone solution (2.5 mg/ml) was added. This solution was then poured into 5 ml of 1% (m/V) PVA solution. This mixture was then homogenized for 5 minutes at 15,000 rpm. Then, 5 ml of PVA

solution was mixed with this pre-emulsion. The resultant nanoparticles were purified by centrifugation at 21,000 g for 15 minutes following overnight agitation. (Kumar et al., 2012).

2.3 Particle Size and Particle Size Distribution for Zeta Potential

The sample was added to the high concentration cell with a transparent electrode to test the nanoparticle's zeta potential using the Malvern Zeta sizer. The zeta potential represented the electrokinetic potential at the sample's sliding plane, as measured by electrophoretic mobility. (Mandial et al., 2018)

2.4 In Vitro Drug Release:

A dialysis bag containing nanoparticles loaded with 6-Hydroxyflavone at a concentration of 1 mg was utilized in this experiment. The 6-Hydroxyflavone was initially suspended in a buffered solution of 0.5 ml. The dialysis bag was then placed in a container filled with 4.5 ml of pH 4.5 buffered solution, resulting in a total volume of 5 ml (Arisoy et al., 2021). The container was subsequently subjected to incubation in an orbital agitator bath at a temperature of 37.5°C and a rotational speed of 100 rpm. To assess the release of the drug over time, 1 ml samples were collected at specific intervals of 30, 60, 120, and 240 minutes. After each collection, the withdrawn sample was replaced with 1 mL of fresh buffer solution at the corresponding time points of 30, 60, 120, and 240 minutes. The drug concentrations in the collected samples were then determined (Weng et al., 2020, Kim et al., 2021).

2.5 Experimental Animals:

Swiss albino mice weighing 25-30g (6-8week) were kept in the departmental animal house, all the animals were maintained in the standard polypropylene cages at standard temperature (22°C-26°C) with 12 h light/dark cycle and relative humidity of 40–55%. All the animals were provided standard pelleted feed and water. All the procedures were approved by the Institutional Animal Ethics Committee (approval number IAEC/RCPIPER/2022-23/15) of the R. C. Patel Institute of Pharmaceutical Education and Research in Shirpur, India, and 42 were approved for this study protocol.

The mice were divided into six groups, with each group comprising six (n = 6) animals. Group 1: The vehicle control received saline at 1 mL/kg p.o; Group II: The SHAM group received CMC at 1 mL/kg p.o; Group III: STZ group 3 mg/kg in citrate buffer by ICV; Group IV: 6-Hydroxyflavone(6-HOF) 25 mg/kg p.o; Group V: PLGA-6-Hydroxyflavone nanosuspension intranasal formulation(6HOF-NTB)1 mg/kg daily; Group VI: The standard group received Donepezil(DON), 1 mg/kg p.o respectively through the oral route.

Figure 1: **Experimental Protocol for evaluating efficacy of nose to brain formulations of 6-hydroxyflavone**

2.6 Surgical procedures: Intracerebroventricular streptozotocin (STZ-ICV) injection.

The mice were anesthetized by using Ketamine (80 mg/kg, i.p.) and xylazine (6 mg/kg, i.p.). The cranium was exposed after the head was positioned in a stereotaxic apparatus (Harvard Apparatus, India) and a middle sagittal incision was made in the scalp. Bregma was identified according to the stereotaxic atlas(Franklin and Paxinos, 2019). One hole was drilled through the skull for unilateral placement of the microinjector (Stainless-steel cannula prepared in-house by 24G needle (Kokare et al., 2011) into the lateral cerebral ventricles at: 0.46 mm posterior, + 1 mm lateral to the midline, and 2.3 mm ventral with respect to the bregma. (Nakhate et al., 2018). On day 1 and 3, STZ (3 mg/kg) was injected unilaterally in two divided volumes using a Hamilton syringe. (Nakhate et al., 2018, Kaur et al., 2020).

3. Behavioral Evaluation

3.1 Elevated Plus Maze:

The exteroceptive behavior of mice was evaluated using the Elevated Plus Maze (EPM). If the animal has previously entered an open arm, transfer latency could be reduced (Parle and Dhingra, 2003, Hussain et al., 2022). The apparatus consists of two open arms (16 cm and 5 cm) and two enclosed arms (16 cm, 5 cm, and 12 cm). The apparatus was raised by 25 centimeters. Each mouse was placed at the corner of an open arm and the movement and time taken to enter from an open arm into close arm

(Transfer latency) was measured by using Any Maze video tracking system. The cut of time was 90 seconds. (Bohra and Kale, 2018, Retinasamy et al., 2020). Transfer Latency is expressed as inflexion ratio and was calculated by using following formula.

$$\text{Inflexion ratio} = \frac{[L0\text{-}L1]}{[L0]}$$

Where: L0- Initial escape latency, L1- Final escape latency

3.2 Y Maze

The Y-Maze test is based on the rodents' natural inclination to explore unfamiliar environment. The Y-maze apparatus consists of 3-arm maze, shaped like a capital ‘Y’ with each arm spaced at an angle of 120 degrees. The arm lengths range between 30 to 40 cm and are usually symmetrical. The pathway widths are approximately 7 cm. The maze was enclosed approximately by 20 cm high walls. There are 3 doors at all the 3 arms which can be opened and closed as required which was placed at 5 cm from the center of the maze. The spontaneous alternation task takes advantage of the subject's natural exploratory inclination and allows it to choose which limb to examine first. All the animal was observed for two minutes, and the percentage of spontaneous alternation was calculated (Kraeuter et al., 2019, Prieur and Jadavji, 2019)

$$\% \text{ Spontaneous Alternation} = \frac{\text{(No. of correct arm entries)}}{\text{(Total no. of entries} - 2)} * 100$$

3.3 Novel Object Recognition:

The new object recognition (NOR) paradigm is used to assess cognition, specifically recognition memory in mouse. This test is based on the mouse's natural inclination to spend more time investigating unfamiliar objects than familiar ones(Antunes and Biala, 2012). NOR test was carried in an open field arena (40 cm x 40 cm x 40 cm). Each animal was trained for 5 min durations in presence of the familiar objects. To evaluate the novelty preference and discrimination index, one of the familiar objects was replaced with unfamiliar object (novel object) and allow the mouse to explore for 5 min. The movement of mouse was recorded by using Anymaze 6.1 video-tracking system. (Lourenco et al., 2019).

$$\text{Discrimination index} = \frac{\text{(TB} - \text{TA)}}{\text{(TA+TB)}}$$

$$\text{Novelty preference} = \frac{\text{(TB)}}{\text{(TA +TB)}} * 100$$

TA = time spent exploring the familiar object; TB = time spent exploring the novel object

3.4 Morris Water Maze:

The Morris water maze consist of circular tank full of water (23 ± 2◦C) with a diameter of around 100 cm and height 50 cm. The water tank was divided into 4 quadrants by 4 initial points as: north, south, east, and west (NW, NE, SE, and SW) (Auti and Kulkarni, 2019). Each trial involved sequentially five days training to individual mouse to spend 60 seconds for search a platform hidden 1cm below the water's surface, followed by 20 seconds on the platform. The mice were gently trained to locate the platform within 60 seconds, or to land there for 20 seconds if they were unable to do so (acquisition phase). On the fifth day, the platform was removed, and a 60-second spatial probe trial was conducted (retention phase). (Tian et al., 2019, e Silva et al., 2020). ANY-maze video tracking software (USA) monitored the time spent in the target quadrant (expressed as percent of the time spent in the water pool), the distance swum in the target quadrant (expressed as percent of the distance swum in the water pool), and the number of escape trials from the border of the water pool were calculated.(Shalaby et al., 2019).

3.5 Preparation of homogenate of the brain

The brain of mice was isolated immediately after sacrificed by Urethane (1g/kg, ip). 10% (w/v) homogenate was prepared in phosphate-buffered saline pH 7.4 (PBS) and 1.15% (w/v) potassium chloride (KCL). After 10 minutes of centrifugation at 10,000 rpm and 4°C, the supernatant was collected and stored as 4°C. The activity of Acetylcholinesterase, Superoxide dismutase, Catalase, and Glutathione were measured in the PBS supernatant. The KCl supernatant was used to determine the level of lipid peroxidation.

3.6 Acetylcholinesterase (AChE) activity in Brain Tissue

Ellman's assay was used to estimate **Acetylcholinesterase activity** brain. homogenate. The assay reaction mixture was initiated by adding sodium phosphate buffer (140 l, 0.1 M), 20 µl of DMSO sample, and 20 µl of 0.25 IU/ml AChE solution(Yadang et al., 2020). The resultant mixtures were kept aside for 15 minutes before being combined with DTNB (10 µl, 2.5 mM) and ATCI (10 µl, 2.0 mM) at room temperature for 10 minutes. The absorbance of each sample mixture was measured at 412 nm using 96 well microplate reader. (Deshmukh et al., Pohanka et al., 2011).

3.7 Estimations of Antioxidants:

Glutathione Reduction (GSH):

Glutathione reductase (GR) aides in the maintenance of adequate intracellular glutathione (GSH) levels, thereby preventing oxidative damage within the cell. In conjunction with the enzyme glutathione peroxidase (GP), GSH is the acting reductant responsible for lowering hazardous hydrogen peroxide levels in cells. (Sharma et al., 2006). The GSH was estimated by previous reported method (Kumar et al., 2016), briefly, 20 µl of tissue homogenate was mixed with 180 µl of DTNB. Read the developed yellow color at 412 nm at 37oC. GSH concentrations were measured in µg/mg of protein.

Lipid Peroxidation (LPO):

Lipid Peroxidation (LPO) in cerebral tissue estimation MDA is a byproduct of lipid peroxidation, but it can also be produced in cells during prostaglandin formation. MDA reacts with amino groups on proteins and other biomolecules to form a variety of conjugates, including mutagenic and potentially carcinogenic adducts with DNA bases. As measured by MDA, increased levels of lipid peroxidation products. Lipid peroxidation in the Brain tissue was determined by measuring MDA content as described by (Goyal et al., 2016), briefly, 100 µl of the tissue homogenate was mixed with 600 µl of 1% w/v phosphoric acid and 200 µl of 0.6% thiobarbituric acid w/v. The reaction mixture was heated to 85°C for 45 minutes before cooling in an ice bath. After cooling, 4 mL of n-butanol was added to it, vortex, and centrifuged at 5000 rpm for 10 min, the organic layer separated. The absorbance of the generated pink color in the organic layer was measured at 532 nm. The levels were expressed as nmol/mg of protein (Ohkawa et al., 1979).

Catalase (CAT):

Catalase activity was determined using the method by Aebi (Aebi, 1984), to a 50 µl of tissue supernatant add 1.0 ml of 50 mM phosphate buffer (PH 7) and 0.1 mL of 30 mM hydrogen peroxide. The optical

density was determined by the drop in absorbance, which was determined spectrophotometrically every five seconds for 30 seconds at 240 nm. (Dhawan et al., 1999, Weydert and Cullen, 2010).

Superoxide dismutase (SOD):

Enzyme photoreduction of NBT dye by the hippocampus of the brain served as the premise for estimating SOD activity (Hritcu et al., 2015). SOD activity was estimated by previously reported method (Marklund, 1984), in a nutshell assay mixture (100 µl of 500 mM Na2CO3, 100 µl of 1 mM EDTA, 100 µl of 240 M/ NBT, 640 µl of distilled water, 10 µl of 0.3 percent Triton x 100, and 25 µl of 10 mM hydroxylamine) was added to 25 µl of tissue supernatant. At 560 nm, the values were taken spectrophotometrically in kinetic mode at intervals of 1 to 3 minutes. The enzyme activity was measured in units per milligram of protein.

3.8 Histopathological study:

Immediately after the mice were sacrificed, brain's hippocampal region was separated histological examination. Each group's samples were fixed in 10% phosphate-buffered formalin, dehydrated in variable alcohol concentrations, and then embedded in paraffin sections. As a counterstain, hematoxylin and eosin were applied to glass transparencies, which were then mounted with this portion. The specimens were examined under a 40x magnification light microscope to determine the presence of tau tangle formation and pyknotic neurons in the hippocampus.

3.9 Statistical analysis:

The behavioral activities of the animals in the Elevated Plus Maze (EPM), Novel Object Recognition (NOR), Y-maze, and Morris Water Maze (MWM), as well as the biochemical parameter assay results, were statistically analyzed using one-way and two-way analysis of variance (ANOVA). As a post hoc analysis, Dunnett's multiple comparison test was conducted, and a $P < 0.05$ was considered statistically significant. The statistical analysis was conducted using GraphPad Prism version 6.00 for Windows, created by GraphPad Software in La Jolla, California, United States. All results were presented as the mean standard error of the mean.

4. Results

4.1 Formulation Parameter:

4.1.1: Particle size of PLGA-6HOF formulation:

particle size of PLGA-6HOF formulation was measured by using a Malvern zeta sizer (Model no: MAL1051945). The nanoparticle size distribution was 164.1 nm **(**Fig. 2**).** The formulation's sharp peak and PDI of 0.269 imply a tight and well-defined particle size distribution.

4.1.2 Zeta Potential of PLGA-6HOF formulation:

A Malvern zeta sizer (Model no: MAL1051945) measured the PLGA-6HOF formulation Zeta Potential. As depicted in Fig. 3, Nanoparticles showing their surface charge as Zeta Potential of -18.2 mV. The formulation had a 4.84 mV zeta deviance, indicating a steady and consistent Zeta Potential. These results and the sharp peak show good Zeta Potential and particle stability for intranasal formulation.

4.1.3: In Vitro Drug Release:

Drug released (percent control drug release (%CDR) from 6-hydroxy flavone-loaded PLGA nanoparticles was determined by using dialysis membrane, as indicated in Fig. 4 the %CDR at 60 min was 2.26% and at 300 min it was 5.5%

4.2Neurobehavioral Parameter:

4.2.1: Elevated plus maze test:

In the exteroceptive behavior study in elevated plus maze administration of 6HOF-NTB intranasal formulation 1mg/kg ,6-HOF (p.o) 25mg/kg and Donepezil (p.o) 1mg/kg significantly ($P < 0.001$) increased inflexion ratio as depicted in Fig. 5.This result showed that significant decrease ($P < 0.001$) the Transfer latency (expressed as Inflexion ratio) in STZ (3 mg/kg icv) treated group which indicated that the spatial long-term working memory of the mice was improved in nose to brain formulation treated group.

4.2.2: Y-Maze:

In the assessment of spatial learning memory in Y-maze Treatment with 6HOF-NTB intranasal formulation 1mg/kg (**$P < 0.01$), 6-HOF (p.o) 25mg/kg (*$P < 0.05$) and Donepezil (1 mg/kg p.o) significantly increased % spontaneous alternation as compared to STZ (3 mg/kg icv) treated group as shown in Fig. 6. Decrease in spontaneous alterations in STZ (3 mg/kg icv) treated group as compared to control group indicates that natural tendency of rodents to explore new environments was increased in nose to brain formulation treated group

4.2.3: Novel Object Recognition (NOR) Test:

Discrimination index and novelty preference indicated that to what extent animal can distinguish in between familiar object and novel object. As shown in Fig. 7 treatment with 6HOF-NTB intranasal formulation 1mg/kg (**$P < 0.01$), 6-HOF (p.o) 25mg/kg (*$P < 0.05$) and Donepezil (1 mg/kg p.o) significantly increased discrimination index and novelty preference as compared to STZ (3 mg/kg icv) treated group. Reductions in novelty preference and discrimination index in STZ (3 mg/kg icv) treated group as compared to control group (##$P < 0.01$), indicates that animal fails to distinguish between two objects thus animals' ability to distinguish two different objects indicates improvement in cognitive memory.

4.2.4: Morrise water maze test:

In the assessment of spatial learning (reference and working memory), Fig. 8 represents result of intranasal administration of PLGA-6HOF(1mg/kg) and oral administration of 6-HOF (25mg/kg) and donepezil (1mg/kg) was found to be significantly decrease (**P < 0.01) the latency to target quadrant and significantly increased (*P < 0.05, **P < 0.01) in the number of platform crossings and time spend in target quadrant on 20th day as compared to STZ (3 mg/kg icv) treated group. This indicates mouse was successfully escape and correctly locate the position of platform in a stressful situation in a large pool of water.

4.2.5: Effect of PLGA-6HOF Nanoparticles Intranasal Formulation on Acetylcholine esterase activity

Figure 9 indicated that, STZ (3mg/kg icv) treated group was showed significant increase ($^{####}p < 0.0001$) in brain Acetylcholinesterase level as compared to control group. After treatment with Donepezil (1mg/kg p.o.) and 6HOF 25mg/kg (p.o) and intranasal formulation of 6HOF-NTB (1mg/kg) we observed that there was significant decrease (****p < 0.0001) in acetylcholinesterase level as compared with STZ treated group. This indicate that the nose to brain administrations of intranasal formulations of 6-hydroxyflavon (6HOF-NTB) significantly lowers acetylcholinesterase level and hence improve memory.

4.2.6: Effect of PLGA-6HOF Nanoparticles Intranasal Formulation in antioxidant parameters:

In our study STZ (3mg/kg icv) induces significant deterioration in the level of catalase, reduced glutathione and superoxide dismutase and increase level of malondialdehyde ###(P < 0.001), Fig. 10 represent the result of treatment of intranasal administration of PLGA-6HOF(1mg/kg) and oral administration of 6-HOF (25mg/kg) and donepezil (1mg/kg) was significantly increased (*P < 0.05, **P < 0.01) in the level of CAT, SOD and GSH in brain homogenate. We also noted that significant decrease in level of MDA in drug treated group. Although oral administrations of 6HOF was not significantly change SOD level.

4.2.7: Histopathological examination:

In Fig. 11, histopathological study revealed the microscopic appearance of hippocampus cells of CA1, CA2, and CA3 region with pyramidal cell nucleus, no pyknotic neuron cells, No appearance of neurodegenerative changes in the hippocampus in control and SHAM group. In STZ (3mg/kg) treated group there was significant microscopic changes was observed with most of the vesicular nuclei and the formation of tau tangles with many pyknotic neurons. This indicates the significant neurodegenerative changes was takes place in the hippocampus. In a drug treated group some of the vesicular nuclei few pyknotic neurons are visible and slight appearance of reduced neurodegenerative changes in the hippocampus was observed.

5. Discussion

Dementia is characterized by a decline in thinking, memory, and independence in daily work. Dementia is a cardinal sign of Alzheimer's disease (AD) causes neurodegeneration due to oxidative stress and neuroinflammation in the brain that are responsible for the production of amyloid beta protein and tau tangles (Yang et al., 2020, Jeremic et al., 2021). The prevalence of Alzheimer's disease increases with age, and it is estimated that about 5–8% of people over the age of 60 have Alzheimer's disease. By the age of 85, the prevalence of Alzheimer's disease is estimated to be around 30–40% (McDade, 2022).

The mouse model of Alzheimer's disease was developed by administering streptozotocin intracerebroventricularly (ICV) with the aid of stereotaxic surgery (Yamini et al., 2022b). STZ induces impairments in various behavioral aspects such as leaning, memory, and cognitive functions through the interference with cerebral insulin signaling, disruption of energy metabolism, and mechanisms including increased neuroinflammation, imbalances in the cholinergic system, oxidative stress in the brain, and dysfunction of existing neurons (Grieb, 2016). STZ was administered twice, with a dose of 1.5 mg/kg per day given after the surgery and a separate dose of 3 mg/kg given on a different day (Nakhate et al., 2018).

Most current therapies for Alzheimer's disease and other CNS disorders utilize peripheral routes of drug administration, such as parenteral and oral administration. However, these approaches often result in diminished efficacy and potency of the medication treatment. Nose-to-brain drug delivery, on the other hand, offers a promising solution by enabling the direct transport of therapeutic molecules without the need to pass through the blood-brain barrier (BBB). This approach significantly enhances the drug concentration in the brain, thereby improving the effectiveness of treatment (Lochhead et al., 2015). Generally, the nasal cavity is an ideal route for drug administration due to its high permeability and effective absorption of both small molecule medications and biopharmaceuticals through the nasal mucosa. Additionally, the proximity of the nasal cavity's roof to the brain, which houses nerves projecting to the brain, further enhances the potential for drug delivery. By bypassing the blood-brain barrier and blood cerebrospinal fluid barrier, nasal-to-brain drug administration offers a promising approach. The delivery of drugs from the nose to the brain is minimally invasive, exhibits good patient compliance, and provides opportunities for self-medication. It is noteworthy that nasal-to-brain delivery is not limited to small molecule medications alone; studies have shown that peptides, proteins, stem cells, viruses, and nucleotides can also traverse from the nose to the brain. Overall, nasal-to-brain drug delivery holds great potential as a non-invasive and effective means of delivering a wide range of therapeutic agents to the brain. (Gänger and Schindowski, 2018).

In the current study, a PLGA-6HOF intra nasal nanoparticle formulation was developed with the objectives of bypassing the blood-brain barrier (BBB). The doses given to the animals were adjusted based on their weight, and the nose-to-brain dosing was conducted using a micropipette with a volume of 5µl, delivered into one nostril(Hong et al., 2019).

In this study we administered freshly prepared intranasal formulation in PLGA for 21 days to experimental animals. The characterization of the formulation was carried out by zeta sizer and we

observed that sharp peak, indicating a well-defined particle size distribution of 164.1 nm (Fig. 2), and zeta potential of -18.2 mV (Fig. 3) which lies ins good category of formulation parameter reported in previous studies. (Weng et al., 2020). Also a good %CDR was observed at 60 min (2.26%) and at 300 min (5.5%) (Fig. 4).

The short-term, long-term, and spatial memory processing that was impacted by Alzheimer's disease (AD) will be evaluated by using The Morris Water Maze, Elevated Plus Maze, Novel Object Recognition, and Y-maze, which are four well-known hippocampus-dependent spatial memory tasks that have been used in the past to evaluate cognitive function (Gamberini et al., 2015, Kraeuter et al., 2019, Tian et al., 2019).

The findings of our study provide compelling evidence that the administration of 6-Hydroxyflavone through an intranasal formulation effectively promotes the development of spatial memory in a mouse model with Alzheimer's disease induced by STZ.

Mice were put through memory tests in the EPM, a paradigm for exteroceptive behavior (Gamberini et al., 2015). Similar changes were seen in our study, where drug therapy reduced the elevated inflexion ratio in the treatment groups. The inflexion ratio (Transfer latency) in the EPM was increased in STZ treated mice. When an intranasal formulation of 6-Hydroxyflavone was used the higher inflexion ratio in comparison to mice treated with STZ made this improvement clear, as seen in Fig. 5. These results imply that the intranasal formulation of 6-Hydroxyflavone (1mg/kg) used in this investigation had a more favorable effect than oral treatment of 6-Hydroxyflavone on the short-term memory inflexion ratio of STZ-treated mice within the EPM. According to Retinasamy et al., 2020 (Retinasamy et al., 2020), the inflexion ratio decreased in the STZ group as compared to the control group. However, treatment caused the inflexion ratio to rise, showing that the disease had improved.

In the Y-Maze, which is widely used to assess spatial learning and memory, spontaneous alternation was employed as a measure of working memory to evaluate the exploratory behavior of mice (Kraeuter et al., 2019). The administration of an intranasal formulation of 6-Hydroxyflavone (1mg/kg) to mice resulted in a significant enhancement of spatial working memory as seen in Fig. 6. This improvement was observed through an increased percentage of spontaneous alternation compared to STZ-treated mice. These findings indicate that the use of 6-Hydroxyflavone in this study exhibited a beneficial effect on short-term memory in STZ-treated mice within the Y-Maze.

To measure recognition memory, novel object recognition was one of a crucial neurobehavioral metrics that takes advantage of mice's innate propensity to look at unfamiliar items rather than familiar ones. The novelty preference reveals how much the mouse prefer the novel object, whereas the discrimination index reveals how much the mouse preferred the familiar object over the novel one (Pi et al., 2020). When mice were administered an intranasal formulation of 6-Hydroxyflavone, they exhibited a significantly longer exploration time of the novel object during the test phase. This increased exploration time suggests a higher preference for novelty and indicates an improvement in cognitive function. Additionally, both the intranasal formulation of 6-Hydroxyflavone and donepezil-treated mice demonstrated a higher discrimination index compared to the STZ-treated group as seen in Fig. 7. This indicates their ability to

distinguish between the familiar and novel objects (Sefati et al., (2023) and Akhtar et al (2021),previously reported in their study that after STZ induction, the discrimination index and inflexion ratio significantly decreased.

The Morris water maze test shows learning ability by assessing the latency to reach the platform during training. When the platform was taken down on the fifth day, the period of time spent there, the latency to the target quadrant, and the quantity of platform crossings were all examined (Ma et al., 2014). As shown in Fig. 8 we observed that intranasal formulation of 6-Hydroxyflavone sustain the long-term memory, as 6HOF-NTB and 6HOF p.o treated mice showed decreased in latency to target quadrant (Fig. 8a) and increase in number of platforms crossing(Fig. 8b)and time spent in target quadrant(Fig. 8c) as compared to STZ treated mice.

The cholinergic system plays a vital role in memory and cognition, and its deterioration is one of the major hallmarks of AD. Thus, treatments acting as AChE inhibitors may confer improvement in cholinergic function and hence cause memory restoration(Pohanka et al., 2011, Worek et al., 2012).

Most of the drugs used to treat Alzheimer's patients are acetylcholinesterase inhibitors, which have been demonstrated to improve cognition and hence act as symptomatic treatment. Hence, measuring AChE activity becomes a crucial parameter in proving that our treatment can also work as planned. The acetylcholinesterase assay was performed using the modified Ellman's method (Worek et al., 2012).

Figure 9 showed that administration of STZ significantly increased Acetylcholinesterase levels in brain. However, this increased AChE levels were significantly reduced by the administration of intranasal formulation of 6-Hydroxyflavone. These findings suggest that 6-Hydroxyflavone has the potential to modulate AChE activity, which may have implications for the treatment of AD.

Oxidative stress is a state of imbalance between the production of reactive oxygen species (ROS) and the ability of cells to neutralize or repair the damage caused by ROS. Neurodegeneration refers to the progressive loss of structure or function of neurons in the brain, which can lead to Alzheimer's disease(DeTure and Dickson, 2019). While due to STZ administration increase in oxidative stress was seen in the current study (Bathina et al., 2017).

This Study showed that there was a notable rise in oxidative stress levels in STZ treated mice which assessed by measuring the activity of antioxidant enzymes such as superoxide dismutase (SOD), glutathione (GSH), and catalase. Additionally, the levels of malondialdehyde, a marker of lipid peroxidation and oxidative damage were measured (Sodhi and Singh, 2013). The results in Fig. 10 demonstrated that after administration of 6HOF -NTB, significant increase in the activity of antioxidant enzymes, including CAT (Fig. 10a), SOD (Fig. 10b), and GSH (Fig. 10c), indicating an enhanced antioxidant defense system, moreover, there was a decrease in the levels of malondialdehyde(MDA) (Fig. 10d), suggesting reduction in oxidative damage in the brain.

Furthermore, the administration of 6-Hydroxyflavone demonstrated not only a reduction in brain histopathological alterations induced by intracerebroventricular STZ treatment but also a decrease in neutrophil infiltration and the presence of pyknotic cells in brain tissue slices stained with hematoxylin and eosin (H&E) **(**Fig. 11**).** These beneficial effects were comparable to those observed with the administration of donepezil.

Conclusion

The present study has explored the potential of nanocarriers for transporting drugs to the brain through the intranasal route of administration. Polymeric nanoparticles prepared using PLGA and solid lipid nanoparticles were evaluated for their efficacy in delivering 6HOF to the brain for treating STZ-induced Alzheimer 's-like conditions. Overall pharmacokinetic behavior of 6HOF-NTB was improved through nanoparticles compared to solution or suspension, and the intranasal route was proved to be advantageous over the oral route of administration. Among nanoparticles, the 6HOF-NTB group showed comparatively better efficacy in delivering drugs to the brain, which was evident by neurobehavioral and oxidative parameters that show certainly significant results in comparison to oral 6HOF. Therefore, it may be concluded that 6-HOF has shown efficacy in dementia of AD type in mice as a neuroprotective agent. It can be explored as a novel therapeutic medication for the management of memory impairment and neuropathological changes linked with dementia or Alzheimer’s disease. Nevertheless, further studies are required to confirm the mechanism of action.

Declarations

Acknowledge

The authors would like to acknowledge the valuable support of Management and Principal of R. C. Patel Institute of Pharmaceutical Educations and Research, Shirpur for providing laboratory facility to carry out this research work

Ethical Statements

The experimental protocol was approved by Institutional Animal Ethics Committee of R.C. Patel Institute of Pharmaceutical Education and Research Shirpur (Protocol approval number: IAEC/RCPIPER/2015-16/10) and was carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) New Delhi**.**

Competing interest: The authors declare no conflicts of interest.

Author contributions

Manish Gagarani: Methodology, Material preparations, Pharmacological Experimentation, and writing.

Prakash Patil: Conceptualization, Monitoring and Data interpretation

Funding: No funding source

Availability of data and materials: Data available on request to corresponding author

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Figures

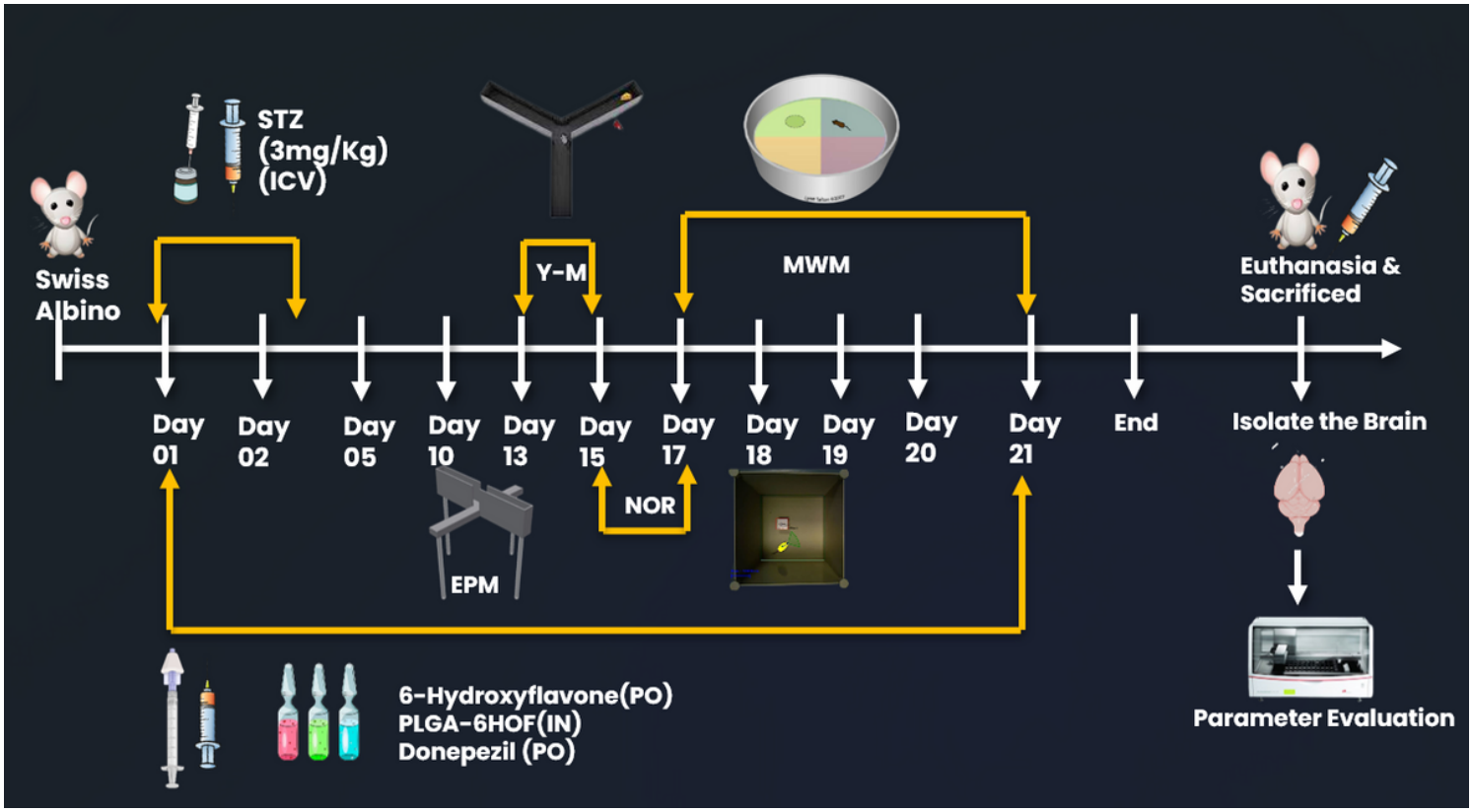


Figure 1

Size Distribution Report by Intensity

v2.1

Malvern

Sample Details

Sample Name:	6 Hydroxy Flavone 1
SOP Name:	mansettings.nano
General Notes:	

File Name:	Zeta sizer-2022.dts	**Dispersant Name:**	Water
Record Number:	120	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.01	**Measurement Date and Time:**	Monday, December 19, 2022 ...

System

Temperature (°C):	25.0	**Duration Used (s):**	180
Count Rate (kcps):	40.9	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	11

Results

			Size (d.nm):	**% Intensity**	**Width (d.nm):**
Z-Average (d.nm):	164.1	**Peak 1:**	147.1	100.0	31.30
PdI:	0.269	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.984	**Peak 3:**	0.000	0.0	0.000
Result quality :	Good				

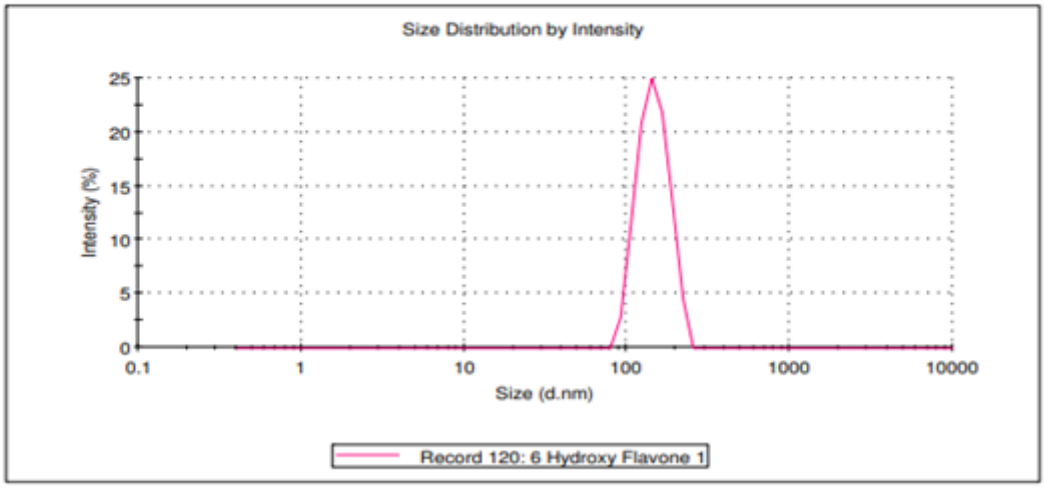


Figure 2

Particle size distribution of 6HOF-NTB nose to brain formulation

Zeta Potential Report

v2.2

Malvern

Sample Details

Sample Name: 6HF Zeta 1
SOP Name: mansettings.nano
General Notes:

File Name: Zeta sizer-2022.dts
Record Number: 121
Date and Time: Monday, December 19, 2022 3:38:26 PM
Dispersant Name: Water
Dispersant RI: 1.330
Viscosity (cP): 0.8872
Dispersant Dielectric Constant: 78.5

System

Temperature (°C): 25.0
Count Rate (kcps): 130.7
Cell Description: Clear disposable zeta cell
Zeta Runs: 15
Measurement Position (mm): 2.00
Attenuator: 9

Results

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -18.2	**Peak 1:**	-18.2	100.0	4.84
Zeta Deviation (mV): 4.84	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 0.205	**Peak 3:**	0.00	0.0	0.00

Result quality : Good

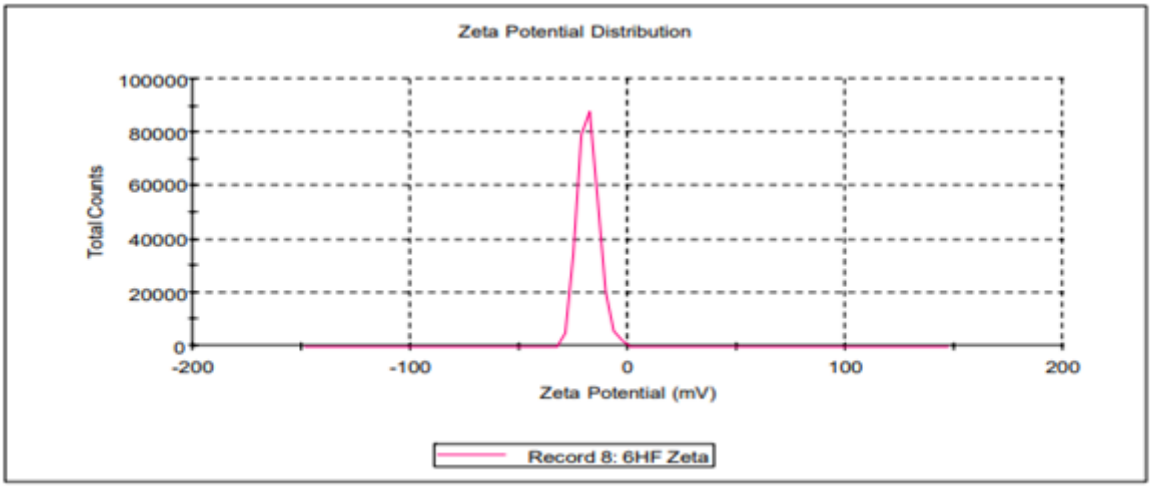


Figure 3

Zeta Potential of 6HOF-NTB nose to brain formulation

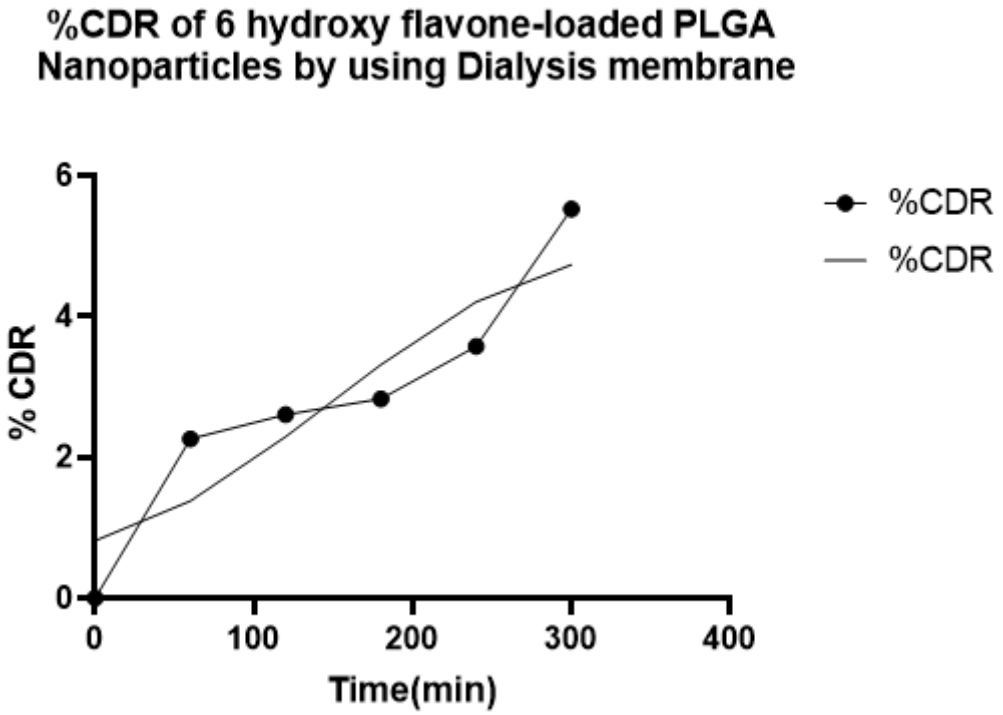
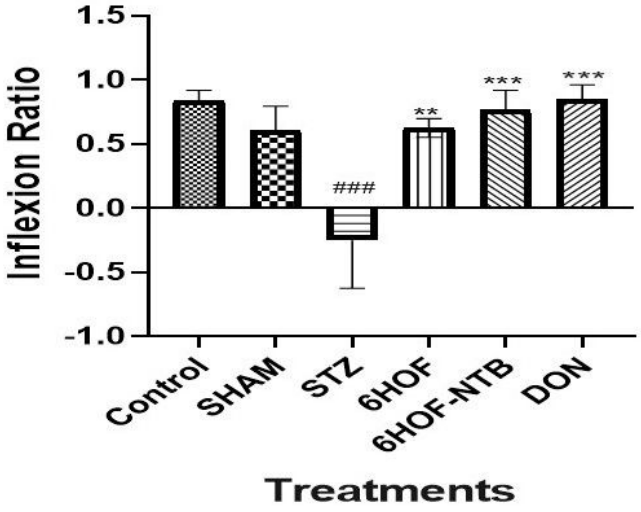


Figure 4

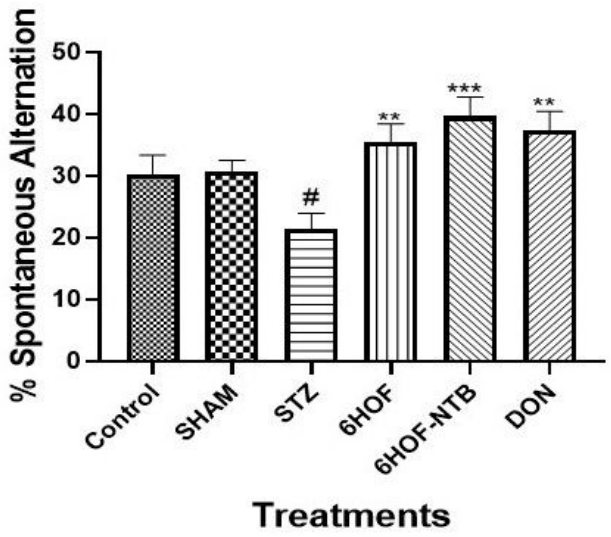
% control drug release of 6HOF-NTB formulation in dialysis membrane.



Effect of PLGA-6HOF intranasal formulation on inflection ratio in elevated plus maze test. Values are expressed as Mean ± SEM(n=6). Compared with control group ###P < 0.01, compared with STZ treated group *P < 0.05 ***P < 0.001.

Figure 5

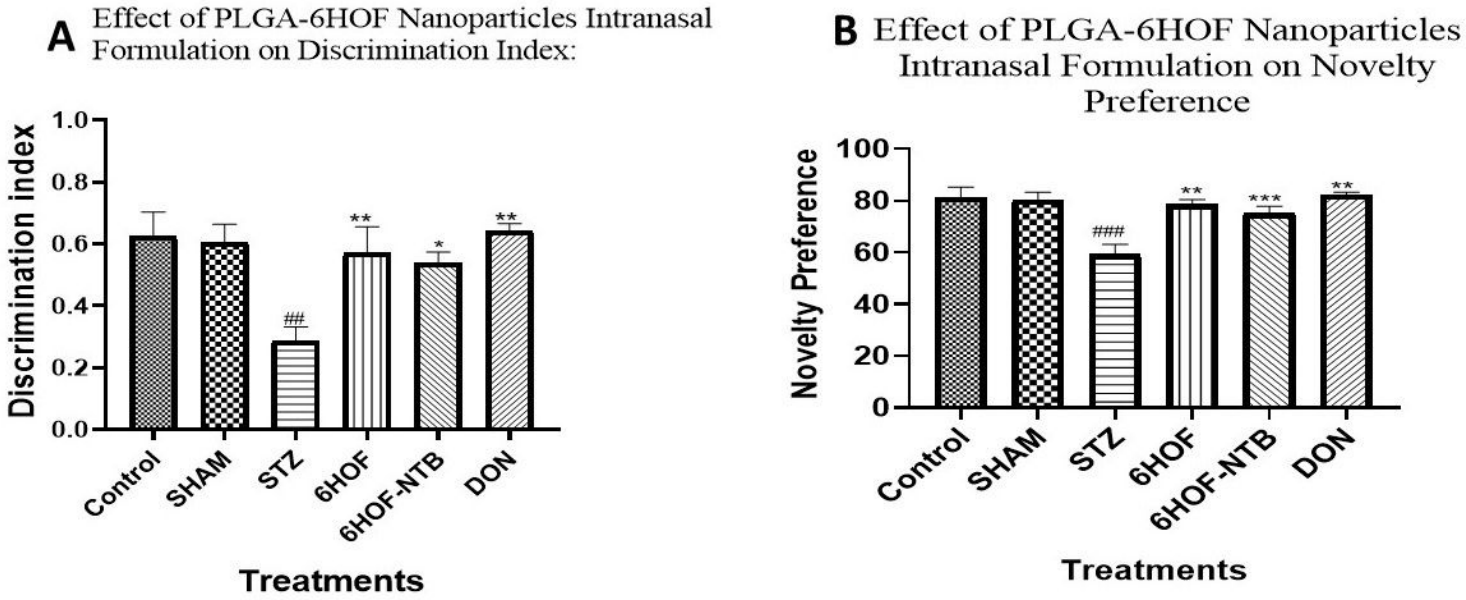
Effect of PLGA-6HOF formulation on inflection ratio in elevated plus maze test



Effect of PLGA-6HOF intranasal formulation on precent spontaneous alteration in Y-maze test, Values are represented as Mean ± SEM (n=6)., Compared with control group #P < 0.05, as compared to STZ treated group *P < 0.05, **P < 0.01 ***P < 0.001.

Figure 6

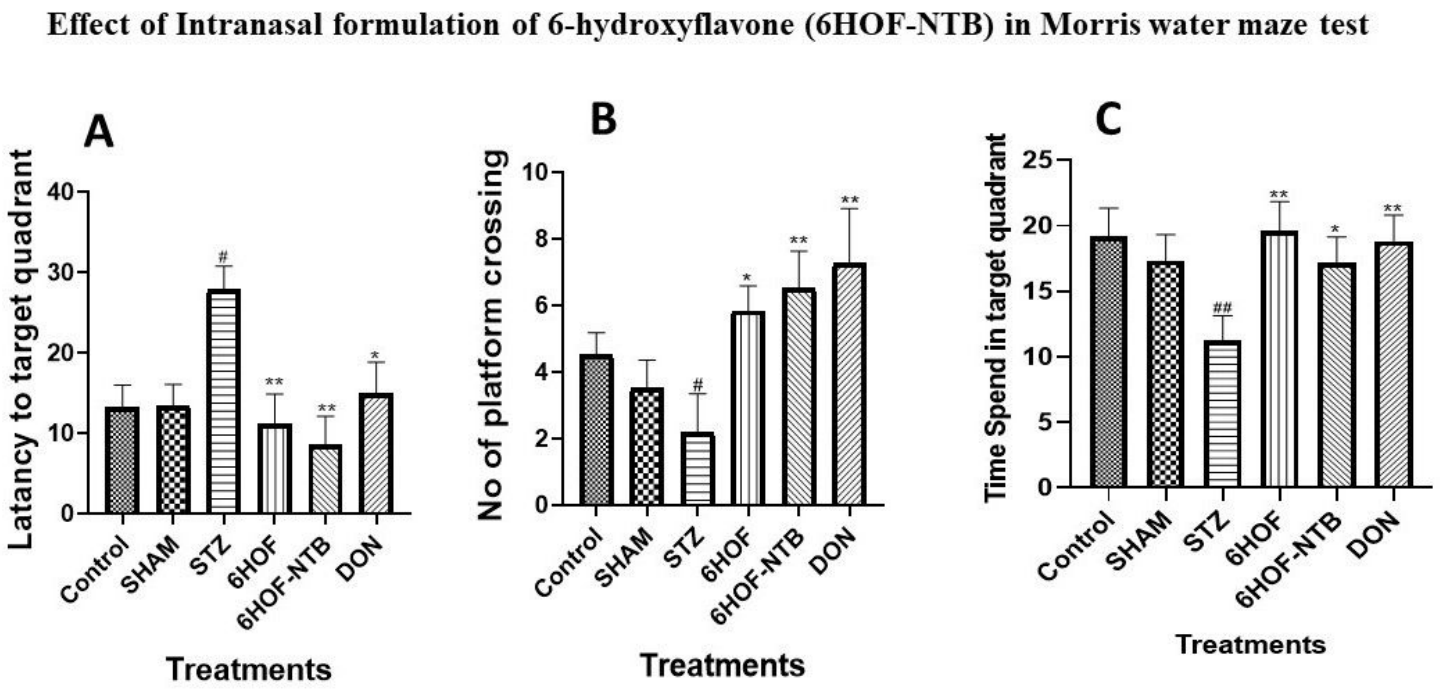
Effect of PLGA-6HOF formulation on % Spontaneous alterations in Y-maze test



Effect of PLGA-6HOF intranasal formulation on Discrimination index (A) and Novelty Preference (B). Values are expressed as Mean ± SEM(n=6). Compared with control group ##P < 0.05,###P < 0.01, compared with STZ treated group *P < 0.05 **P < 0.01.

Figure 7

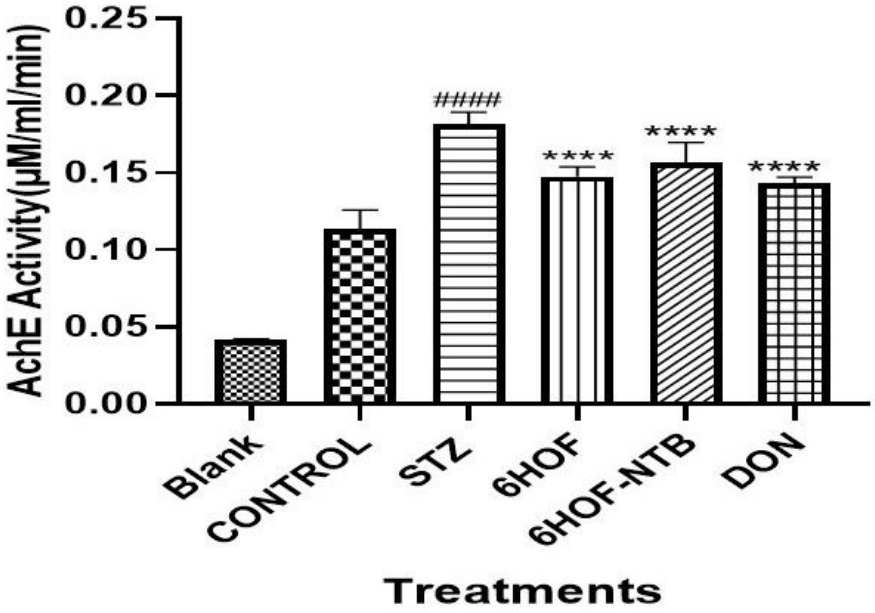
Effect of PLGA-6HOF formulation in Novel object recognition test



Effect of PLGA-6HOF intranasal formulation on Latency to target quadrant(A) number of platform crossings(B) and time spent in an target quadrant (C). Values are expressed as Mean ± SEM(n=6). Compared with control group #P < 0.05,##P < 0.01, compared to control group, *P < 0.05 **P < 0.01 as compared to STZ treated group

Figure 8

Effect of PLGA-6HOF formulation in Morrise water maze test

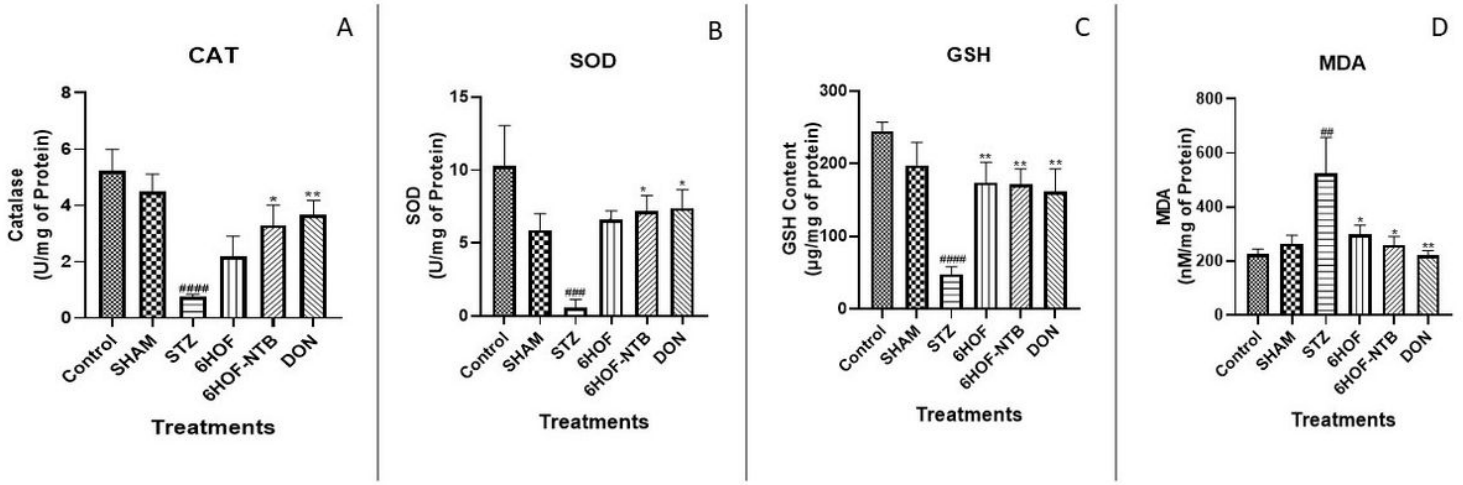


Effect of PLGA-6HOF intranasal formulation on acetylcholinesterase activity. Values are represented as Mean ± SEM (n=6)., Compared with control group ####p<0.0001 , as compared to STZ treated group *****p < 0.0001

Figure 9

Effect of PLGA-6HOF formulation on acetylcholinesterase activity.

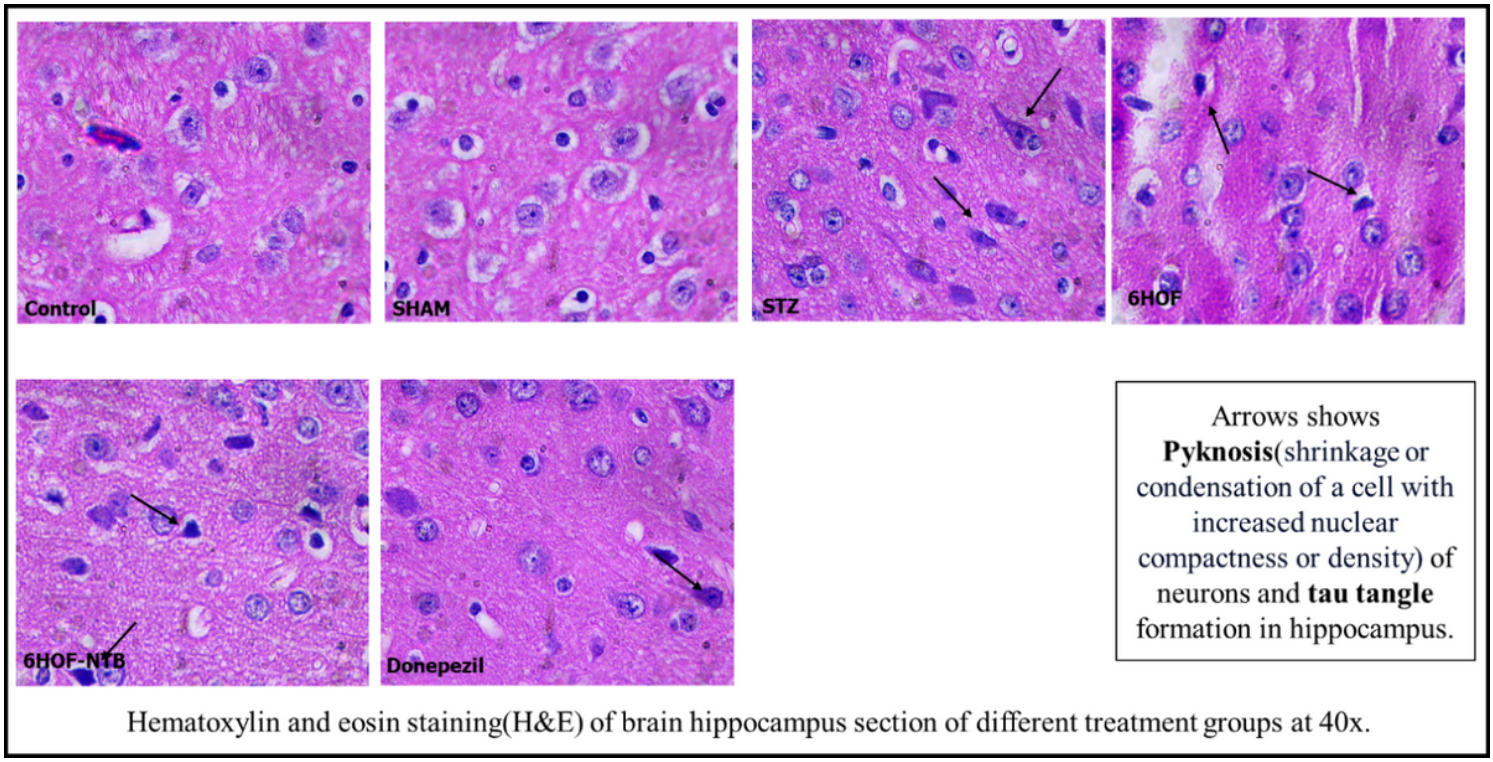
Effect of Intranasal formulation of 6-hydroxyflavone (6HOF-NTB) on antioxidants defense parameter



Effect of PLGA-6HOF intranasal formulation on Catalase (A), Superoxide dismutase(B), Reduced glutathione (C) and Malondialdehyde(D), Values are expressed in mean ± SEM (n=6) ,$^{###}P < 0.001$ as compared to control group. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to STZ treated group.

Figure 10

Effect of PLGA-6HOF formulation in antioxidant parameters.



Hematoxylin and eosin staining(H&E) of brain hippocampus section of different treatment groups at 40x.

Figure 11

Histopathological changes in different treatment groups