RESEARCH PAPER

Development and Evaluation of Formononetin Nanoformulation for Neuroprotection in Aluminium Chloride-Induced Alzheimer's Disease

Ms. Divya G. Thite[1*], Dr. Neelam L. Dashputre[2]

[1*]PhD Research Scholar, Department of Pharmacology, MET's Institute of Pharmacy, Bhujbal Knowledge City, Adgaon, Nashik, India

Email: thitedivya81@gmail.com

[2]Associate Professor, Department of Pharmacology, MET's Institute of Pharmacy, Bhujbal Knowledge City, Adgaon, Nashik, India

Abstract

Introduction:

Alzheimer's disease is a progressive neurodegenerative disorder characterized by memory loss, cognitive impairment, oxidative stress, and neuronal degeneration. Natural compounds with antioxidant and anti-inflammatory properties are being explored for neuroprotection. Formononetin, a naturally occurring isoflavone, has shown promising neuroprotective potential. However, its therapeutic effectiveness is limited due to poor aqueous solubility and low bioavailability. The present study aimed to develop a nanosuspension of formononetin to enhance its bioavailability and evaluate its neuroprotective effect in Aluminium chloride-induced experimental Alzheimer's disease in rats.

Materials and Methods:

Formononetin nanosuspension was prepared by the precipitation method using poly-lactic-co-glycolic acid (PLGA) as a polymer and polyvinyl alcohol as a stabilizer. The optimized formulation was evaluated for particle size, polydispersity index, and entrapment efficiency. Forty-eight male Wistar rats were randomly divided into six groups. Alzheimer's disease was induced using aluminium chloride (1 mg/kg). Treatment groups received formononetin nanosuspension (10, 20, and 40 mg/kg) intranasally for 21 days, while the standard group received donepezil (1 mg/kg). Behavioral tests including Morris water maze, Y-maze, novel object recognition, and passive avoidance tests were performed. Biochemical parameters such as superoxide dismutase (SOD), acetylcholinesterase (AChE), inflammatory cytokines, and histopathological examination of brain tissues were also evaluated.

Results:

The optimized nanosuspension showed particle size in the nanometer range with high entrapment efficiency (80–95%). Aluminium chloride significantly impaired learning and memory in rats. Treatment with formononetin nanosuspension significantly improved cognitive performance, increased antioxidant enzyme levels, reduced acetylcholinesterase activity, and decreased inflammatory markers. Histopathological analysis revealed reduced neuronal degeneration and improved hippocampal structure, particularly at the 40 mg/kg dose, showing effects comparable to donepezil.

Conclusion:

Formononetin nanosuspension exhibited significant neuroprotective effects against aluminium chloride-induced Alzheimer's disease by improving cognitive function and reducing oxidative stress and neuroinflammation, suggesting its potential as a promising therapeutic strategy for Alzheimer's disease.

Keywords: Formononetin, Nanosuspension, Alzheimer's disease, Neuroprotection, Oxidative stress, Intranasal delivery, PLGA.

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Introduction

Alzheimer's disease is a progressive neurodegenerative disorder characterized by gradual memory loss, cognitive decline, and behavioral impairment. It is one of the most common causes of dementia among the elderly population worldwide. The pathological features of Alzheimer's disease include the accumulation of β-amyloid plaques,

neurofibrillary tangles, oxidative stress, neuroinflammation, and degeneration of cholinergic neurons in the brain. These pathological changes ultimately lead to synaptic dysfunction and neuronal death, particularly in the hippocampus and cerebral cortex, which are regions associated with learning and memory.

Currently available drugs such as acetylcholinesterase inhibitors and NMDA receptor antagonists provide only symptomatic relief and do not effectively halt disease progression. Therefore, there is an increasing need to explore novel therapeutic strategies that can provide neuroprotection and target multiple pathological pathways involved in Alzheimer's disease. Natural phytoconstituents have attracted considerable interest due to their antioxidant, anti-inflammatory, and neuroprotective properties.

Formononetin is a naturally occurring isoflavone found in several medicinal plants. It has been reported to possess various pharmacological activities including antioxidant, anti-inflammatory, and neuroprotective effects. However, the clinical application of formononetin is limited due to its poor aqueous solubility and low bioavailability. Nanotechnology-based drug delivery systems such as nanosuspensions have emerged as promising approaches to enhance drug solubility, stability, and therapeutic efficacy.

In experimental research, Aluminium chloride is widely used to induce Alzheimer-like neurotoxicity in animal models. Aluminium exposure is known to promote oxidative stress, cholinergic dysfunction, and neuronal degeneration, thereby mimicking several pathological features of Alzheimer's disease.

Therefore, the present study aimed to develop a nanosuspension of formononetin and evaluate its neuroprotective potential against aluminium chloride-induced experimental Alzheimer's disease in rats through behavioral, biochemical, and histopathological assessments.

Materials and Methods:

1. Drug Authentication using analytical techniques:

1.1. Drug Procurement:

Drug (Formononetin) was procured from Yucca Enterprises, Mumbai. Yucca Enterprises provided Certificate of Analysis for authentication of purity of Formononetin.

1.2. Pre-formulation Studies:

Pre-formulation Studies of received Formononetin sample were conducted according to Indian Pharmacopoeia, 2014 to check the quality, efficacy and stability of the drug.

1.3. Organoleptic Properties:

2.Formulation of Nanoparticles:

2.2. Development of Nanosuspension:

The nanosuspension is prepared by precipitation method. The formononetin and poly-lactic co-glycolic acid (PLGA) dissolved in ethyl acetate. It is the organic phase of the formulation. The organic phase sonicated at room temperature for 15-20 min. After that 3% Polyvinyl Alcohol (aqueous phase) was added dropwise into organic phase on magnetic stirrer. Then the whole mixture was sonicated again in same conditions. Further to achieve more smaller size, prepared emulsion was subjected to high pressure homogenizer at 15 bar pressure. Particle size of each batch was determined. Further the prepared nanosuspensions stored in refrigerator (Ding *et al.,* 2018)

Table 2: Ingredients and quantity of nanosuspension batches

Ingredients	Low (10mg/kg)	Medium (20mg/kg)	High (40 mg/kg)	Use
Ethyl Acetate	84 ml	84 ml	84 ml	Solvent
Poly-Vinyl Alcohol	84 ml	84 ml	84 ml	Aqueous solvent
Formononetin	336 mg	672 mg	1344 mg	Drug
Poly-lactic co-glycolic acid (PLGA)	336 mg	672 mg	1344 mg	Polymer

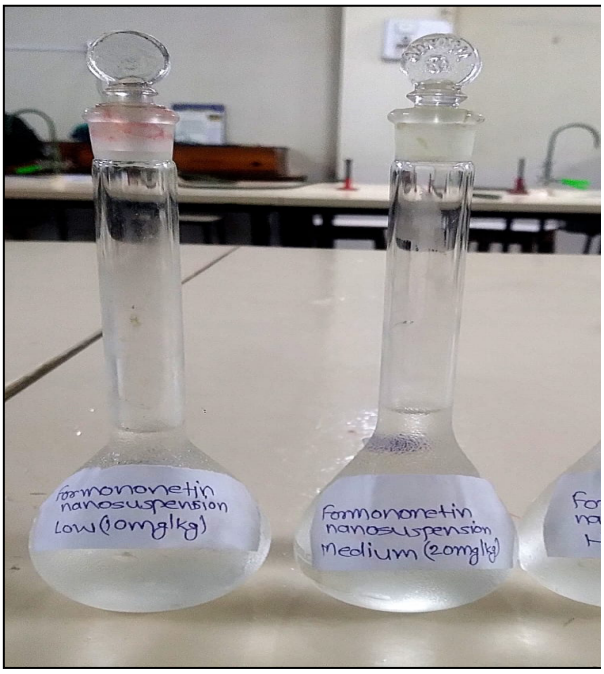

Fig : Final 3 batches of nanosuspension

Sr. No.	Groups	Dose	Route of administration	No. of Animals
1	Control group	Distilled water	Per oral	1
2	Test group	Formononetin nanosuspension (5 mg/kg)	Intranasal	1
3	Test group	Formononetin nanosuspension (10 mg/kg)	Intranasal	1
4	Test group	Formononetin nanosuspension (15 mg/kg)	Intranasal	1
5	Test group	Formononetin nanosuspension (50 mg/kg)	Intranasal	1
6	Test group	Formononetin nanosuspension (75 mg/kg)	Intranasal	1

3.1.Experimental design:

Main Study: Forty- eight male wistar rats of 180-220gm of weight and having age 6-8 weeks randomly selected and acclimatized for 7 days. The rats have the free access to diet and water.

Study design:

Forty- eight animals were randomly divided into the different six group of animals

Group I: The rats included in the normal group have free access to dist. water throughout the study for 21 days.

Group II: The Alzheimer's disease is induced to rats present in the disease control group with the help of $AlCl_3$ (1mg/kg) dissolved in saline solution by the oral route with the help of intubation canula (Okesina *et al.*, 2020).

Group III: Low treatment group were given $AlCl_3$ (1mg/kg) for induction of disease. after 72 hrs, Formononetin NS (10mg/kg)(Xu *et al.*, 2019) were given by Intranasal route for 21 days.

Group IV: Medium treatment group were given $AlCl_3$ (1mg/kg) for induction of disease. after 72 hrs, Formononetin NS (20mg/kg)(Xu *et al.*, 2019) were given by Intranasal route for 21 days.

Group V: High treatment group were given $AlCl_3$ (1mg/kg) for induction of disease. after 72 hrs, Formononetin NS (40mg/kg)(Xu *et al.*, 2019) were given by Intranasal route for 21 days.

Group VI: Standard treatment group were given $AlCl_3$ (1mg/kg) for induction of disease. after 72 hrs, Donepezil hydrochloride (1mg/kg) were given by Intranasal route for 21 days.

Table No. 4 : study design of main experimental animal study

Sr. No.	Group	Dose	Route of administration	No. of Animals
1.	**Group I** Control	Normal	Per Oral	8
2.	**Group II** Disease control	Disease Control ($AlCl_3$ mg/kg)	Intraperitoneal	8

3.	**Group III** Treatment	$AlCl_3$ + Formononetin Nanosuspension (10mg/kg)	Intraperitoneal Intranasal	8
4.	**Group IV** Treatment	$AlCl_3$ + Formononetin Nanosuspension (20mg/kg)	Intraperitoneal Intranasal	8
5.	**Group V** Treatment	$AlCl_3$ + Formononetin Nanosuspension (40mg/kg)	Intraperitoneal Intranasal	8
6.	**Group VI** Standard	$AlCl_3$ + Standard (Donepezil 1 mg/kg)	Intraperitoneal Per oral	8

3.2.Behavioural study:

1. Morris water maze test:

The Morris water maze is a black circular pool (80 cm in diameter and 35 cm in height) with a plain inner surface. The circular pool was loaded with water and a non-toxic, water-soluble black dye (20 ± 1°C). The pool is separated into four quadrants with equal areas. A transparent platform (4 cm in diameter and 18 cm in height) was centered in one of the pool's four quadrants and buried 1 cm below the water surface, rendering it undetectable at water level. Rats were given 120-second training sessions three times per day for three days. During each acquisition experiment, animals were free to locate the platform in the specified quadrant. Once the rat found the platform, it was allowed to stay on it for 10 seconds; if the animal did not reach the platform within 120 seconds, it was placed on it for 30 seconds. Escape latency for each rat was calculated (Lam *et al.*, 2016)

Fig No. 1 : Morris water maze test

2. Y maze test:

The Y-maze is a three-arm maze with walls that are 10 cm high and arms that are all 35 cm long and 5 cm wide, all at equivalent angles. The rat was first housed in a single arm, and throughout the duration of eight minutes, the order and quantity of arm entrances were noted. The rat was considered as entering an arm when all four paws were inside the arm's limit. Between tasks, the maze arms were properly cleaned to get rid of any lingering smells. The term "alternation" was defined as consecutive entries into the ABC, CBA, and BCA triplet set's three arms. The formula for calculating the alternation score (%) for each mouse was as follows: % Alternation = [(Number of alternations)/ (Total arm entries – 2)] × 100; this represents the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus two) multiplied by 100 (Ghofrani *et al.*, 2015).

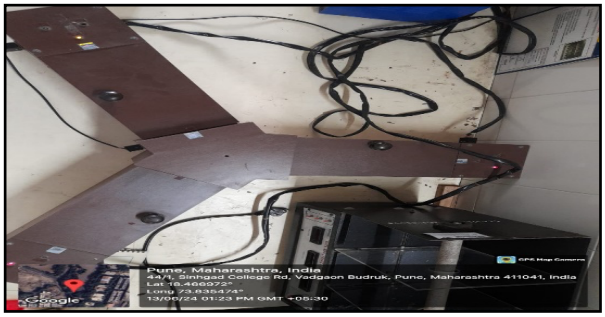

Fig No. 2 : Y maze test instrument

3. Novel object recognition test:

A popular behavioural test for examining several facets of rat learning and memory is the Novel Object Recognition Test (NORT). Three days are designated to the relatively easy NORT: training, testing, and habituation days. The rat is permitted to investigate two identical objects throughout training. One of the practice objects gets exchanged out for a new one of same texture and different size, shape on test day. Rats have an inbuilt desire for novelty, so if they see something they are familiar with, they will go toward the new item and spend most of their time there. This natural predilection eliminates the requirement for lengthy training regimens or positive or negative reinforcement (Lueptow *et al.,* 2017).

Fig No. 3 : Novel object recognition apparatus

1. Passive avoidance test:

The test is often used to assess avoidance memory retention in rats. The passive avoidance approach involves placing the rat in a shuttle box. The device is made up of two chambers separated by door. On the first day of the test, rat was placed in the apparatus for 10 minutes of habituation with the door and gate open. On the second day (acquisition trial), the rat was placed in a lighted container. After 30 seconds of acclimatization, the door was raised, and when the rat walked from the lighted area to the dark region, it received a 10-second electrical shock (0.5 Ma). On the third day (retention trial), the same methods as in the acquisition trial were followed, but no electric shock was administered. In all the acquisition and retention trials, the transfer latency time (sec) for each rat was measured (Ghofrani *et al.*, 2015).

Fig No. 4 : Passive avoidance instrument

1. Tissue Homogenate preparation:

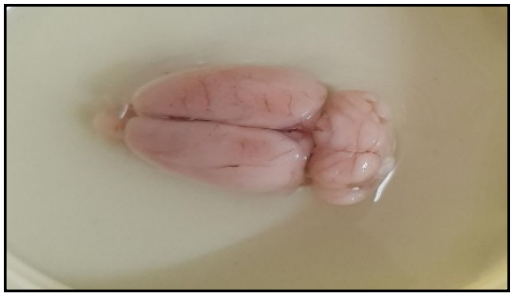

Fig No. 5 : Isolation of brain

For immunohistochemical investigation, brains were removed following 4% paraformaldehyde perfusion. Following an additional washing in ice-cold isotonic saline for biochemical estimates, the brains that were extracted were homogenized to yield 10%w/v homogenates using ice-cold 0.1 M phosphate buffer saline (Ph 7.4). Following another centrifugation for 15 minutes at 10,000 rpm and -4°C, these homogenates were used to estimate biochemical parameters.

Biochemical Parameters: -

7. Oxidative stress parameters: -

7.1. Superoxide dismutase (SOD) activity in brain:

SOD, which is found in numerous intracellular regions such as the cytosol, mitochondria, and blood plasma, can remove superoxide radicals, protecting the cell against oxidative stress. SOD shields the cell from oxidative stress by converting superoxide to oxygen and hydrogen peroxide (Rao *et al.*, 2021).

Procedure: Combine a portions amount of distilled water and brain tissue homogenate (supernatant), then add 0.25 Ml of ice-cold ethanol and 0.15 Ml of ice-cold chloroform. The liquid was well mixed with a cyclomixer before being centrifuged at 600 x g for 15 minutes at 4°C. This was combined with 0.5 ml of EDTA solution. To initiate the reaction, 0.4 Ml of epinephrine was introduced. The optical density change per minute at 480 nm was measured compared to a control sample (Nanaware *et al.*, 2017).

7.2. Glutathione peroxidase (GSH) activity in brain:

GSH is a significant endogenous antioxidant that is catalyzed by enzymes and is essential for detoxifying reactive oxygen species (ROS) and controlling the intracellular redox state. It is found in the brain in high amounts of 1-2Mm.

Procedure: TCA (20%) along with brain tissue homogenate (supernatant) were mixed in equal proportions. After centrifugation at 600 x g for 15 minutes at 4 °C, the precipitated fraction was mixed with 0.25 Ml of supernatant and 2 Ml of DTNB reagent. With phosphate buffer, the final volume was makeup upto 3.0Ml. At 412 nm, the color produced was compared to a reagent blank (Nanaware *et al.*, 2017).

7.3. Catalase (CAT) levels in brain:

Catalase is essential for the breakdown of H2O2 into nontoxic water and oxygen, which helps to manage illnesses associated with oxidative stress. As a crucial regulator, CAT influences H2O2 metabolism and, consequently, H2O2 levels, which are necessary for

signaling. The relationship between Aβ accumulation and the emergence of oxidative stress conditions in AD may be linked by the interaction between CAT and Aβ, which may greatly raise H2O2 levels inside the cells (Kamaljeet *et al.,* 2024).

Procedure: 1960 Ml of substrate, which consisted of 1800 Ml of 10 Mm H2O2, 100 Ml of Tris-HCl buffer, and 60 Ml of D/W, was added to 40 Ml of the supernatant of the brain tissue homogenate. Activity was measured using the exponential disappearance of H2O2 at 240 nm at 25 °C (Nanaware *et al.*, 2017).

7.4. Malondialdehyde (MDA) levels in brain

Procedure: Filling 100 µl of homogenate, or 0.9% NaCl, 250 µl of trichloroacetic acid (TCA), 20%, and 400 µl of TBA, 0.67%, into the glass screw-top tubes (blank and test) and sealing them. The mixture was placed in a water bath that was heated to 100 °C for 15 minutes, and it was then cooled for 30 minutes in a cold-water bath. The tubes were left open to allow the gasses generated during the reaction to escape. The supernatant's absorbance at 532 nm was evaluated in relation to a control sample following five minutes of centrifugation at 1500 rpm. In nmol/mg, the MDA concentration was stated (Nanaware *et al.*, 2017).

7.5. Nitric oxide (NO) levels in brain:

An example of an endogenously produced free radical is nitric oxide (NO)It is believed that the upregulation of NO production by Inos plays a significant role in the neurodegeneration linked to oxidative stress (Asiimwe *et al.*, 2016).

Procedure: Brain tissue homogenate sample solutions were produced in 100 Ml volumetric flasks with varying concentrations. Here, 0.1489 g of sodium nitroprusside was added, with a final concentration of 5 Mm, and allowed to incubate.5.6 Ml was taken at various intervals, to which 0.2 Ml of Griess reagent A was added and incubated for ten minutes at 30°C. The absorbance was measured at 542 nm against a blank after incubation, then 0.2 Ml of Griess reagent B was added and incubated for 10 minutes at 30°C (Nanaware *et al.*, 2017).

8. Immunohistochemistry analysis:

8.1. Estimation of brain-derived neurotrophic factor (BDNF).

Added 100ul Standard Diluent, prepared Standards and Samples to respective wells. Covered the plate and incubated for 120 minutes at 37°C. washed plate 4 times with diluted Wash Buffer (1X) and bloted residual buffer. Pipetted 100ul Biotinylated BDNF antibody Working Solution to all wells. Covered the plate and incubate for 60 minutes at 37°C. washed again. Pipette 100ul Streptavidin: HRP Conjugate Working Solution to all wells. Covered and incubated for 30 minutes at 37°C. Again washed. Pipette 100ul TMB Substrate in all the wells. Incubated plate at 37°C for 10 minutes. Positive wells should turn bluish in color. Add 100ul of Stop Solution to all wells. The wells should turn from blue to yellow in color. Read the absorbance at 450 nm with a microplate within 10-15 minutes after addition of Stop solution(Dashputre *et al.*, 2023)

8.2. Estimation of Tau protein:

Added 100ul Standard Diluent, prepared Standards and Samples to respective wells. Covered the plate and incubated for 120 minutes at 37°C. washed plate 4 times with diluted Wash Buffer and blot residual buffer. Add 100ul Biotinylated Tau-protein antibody Working Solution to all wells. Covered plate and incubated for 60 minutes at 37°C. washed. Added 100ul Streptavidin: HRP Conjugate Working Solution to all wells. Covered and incubated for 30 minutes at 37°C. washed. Added 100ul TMB Substrate in all the wells. Incubated the plate at 37°C for 10 minutes. Positive wells should turn bluish in color. Introduced 100ul of Stop Solution to all wells. The wells should turn blue to yellow in color. Read the absorbance at 450 nm with a micro plate within 10-15 minutes after addition of Stop solution (Dashputre *et al.*, 2023).

8.3. Estimation of β-amyloid protein.

Added 100ul Standard Diluent, prepared Standards and Samples to respective wells. Covered the plate and incubated for 120 minutes at 37°C. washed plate 4 times with diluted Wash Buffer and blot residual buffer. Added 100ul Biotinylated Beta Amyloid Protein Antibody Working Solution to all wells. Covered the plate and incubated for 60 minutes at 37°C. washed. Added 100ul Streptavidin: HRP Conjugate Working Solution to all wells. Covered the plate and incubated for 30 minutes at 37°C. washed. Added 100ul TMB Substrate in all the wells. Incubated the plate at 37°C for 10 minutes. Positive wells should turn bluish in color. Added 100ul of Stop Solution to all wells. The wells should turn from blue to yellow in color. Read the absorbance at 450 nm with a micro plate within 10-15 minutes after addition of Stop solution (Dashputre *et al.*, 2023)

8.4. Estimation of Acetylcholine Esterase:

Ellman's method is use for analysis of acetylcholine esterase. The brain homogenate (50 Ml), 3 Ml of 0.1 M phosphate buffer (Ph 8), 0.1 Ml of 14 Mm acetylcholine iodide, and add 0.1 Ml of 10 Mm 5,5-dithiobis(2-nitrobenzoate). The mixture incubates for 5 min. The increase in the absorbance record for 2 min at a 30 s interval at 412 nm using a spectrophotometer

(UV). The activity of AchE will expresse in enzyme unit/ mg of the protein (Pohanka *et al.,* 2015) .

9. Inflammatory Markers:

9.1. Tumour Necrosis Factor α:

Added 100ul Standard Diluent, prepared Standards and Samples to respective wells. Covered the plate and incubate for 120 minutes at 37°C. washed plate 4 times with diluted Wash Buffer (1X) and blot residual buffer. Pipette100ul Biotinylated TNF-alpha antibody Working Solution to all wells. Covered the plate and incubated for 60 minutes at 37°C. washed out. Pipette 100ul Streptavidin: HRP Conjugate Working Solution to all wells. Covered the plate and incubated for 30 minutes at 37°C and washed. Added 100ul TMB Substrate in all the wells. Incubated the plate at 37°C for 10 minutes. Positive wells should turn bluish in color. 100ul of Stop Solution to all wells. The wells turn from blue to yellow in color. Read the absorbance at 450 nm with a micro plate within 10-15 minutes after addition of Stop solution.

9.2. Interleukin-1β

For the estimation of pro inflammatory cytokines IL-1β in brain tissues, 10% tissue homogenate will prepare with PBS containing 1% protease inhibitor cocktail. Then, the homogenates will centrifuge at 12,000 g for 15 min and the supernatant obtained are used for the estimation of cytokines using respective rat ELISA kits.

10. Histopathology:

The brain of the rat was removed out and preserved in formalin buffered with a 10% neutral Ph. The tissue was processed using increasing alcohol concentrations for dehydration, xylene for cleaning, and paraffin wax for embedding. Slices of tissue blocks with embedded paraffin wax were cut at a thickness of 3–4 µm using a Rotary Microtome. Every single brain slide was stained using hematoxylin and eosin (H&E) stain. The light microscope has been employed to view and take pictures of the stained sections of the rat brain in order to analyze the morphological alterations.

A. Reagents –

1. Neutral buffered formalin: 4g of sodium dihydrogen phosphate, 6.5g of disodium hydrogen phosphate anhydrous, 100 ml of 40 % formalin, and 900 ml of distilled water were combined, and the Ph was then raised to 7.4.

2. Harris's haematoxylin: A solution containing 20 gm of potassium alum that had previously been dissolved in hot distilled water was combined with 1 gm of haematoxylin stain that had been dissolved in 10 ml of pure alcohol. 0.5 gm of mercuric oxide was added after bringing this mixture to a boil. This combination quickly cooled down in the ice water. After filtering, this was placed in a bottle of amber colour for storage.

3. Eosin stain (0.5%): 100 ml of distilled water were used to dissolve 500 mg of eosin yellow powder before being filtered and stored (Sivaraman *et al.,* 2015).

11. Statistical analysis: All the observations were presented as Mean ± SEM. The whole data were expressed by the one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 8.4. #P<0.05, ##P<0.01, ###P<0.001 when compared with normal control, *P<0.05, **P<0.01, ***P<0.001 compared with disease control group

Result

1. Pre-formulation Studies of Formononetin

1.1. Organoleptic Properties

Formononetin was observed for organoleptic characters like colour, odour and appearance

Identification Test	Observed	Reported
Colour	White	White
Odour	Odourless	Odourless
Appearance	Amorphous	Amorphous

Table no:5

2. Formulation Study:

2.1. Optimization Data Analysis:

Table No. : Optimization of Central composite design

Number	PLGA	No of cycle	Partical size (nm)	PDI	% EE
1	10	6	987.1	0.109	80.5
2	10	8	756.9	0.123	85.4
3	10	10	587.45	0.134	87.5
4	20	6	472.56	0.15	87.8

				4	
5	20	8	453	0.164	89
6	**20**	**10**	**398.5**	**0.168**	**90.3**
7	40	6	289.34	0.290	91.5
8	40	8	184.23	0.300	93
9	40	10	137.45	0.302	95.2

Entrapment efficiency for the 9 batches was determined and it was observed that it was in the range 80 % to 95 %. HPH cycles had a positive effect on the reduction of particle size i.e. as HPH cycles increased, the reduction in the particle size was observed. Also, the Polymer had positive effect on entrapment efficiency and it was found to be 80 % to 95%.Particle size for the 9 batches was observed that it was in the range 137 nm to 987 nm. The particle size increases as the PLGA concentration increases and it decreases as the concentration of PLGA decreases. From this, we found 6 batch as an optimized batch.

5. Behavioral Parameters

5.1. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$ - induced Alzheimer's disease in Morris Water Maze Test:

Retention Test-

The time spent in quadrant in normal control group of animals was 30.36 ± 0.31 sec. In the TMT induced group-2, after 3 weeks of dosing there was significant ($p < 0.001$ each) decrease in retention time (7.50 ±0.51sec) compared to normal control group-1. Donepezil hydrochloride (1 mg/kg) and Formononetin nanosuspension (40 mg/kg) treatment respectively showed a significant ($p < 0.001$ each) increase in the retention time (27.87 ± 0.29 and 22.18 ± 0.95 sec). Formononetin nanosuspension (20 mg/kg) treatment also showed significant ($p < 0.01$) each increase in retention time (14.41 ± 0.56 sec) compared to $AlCl_3$ induced AD group-2. Treatment with Formononetin nanosuspension (10 mg/kg) show less significant increase in retention time (12.02 ± 0.63 sec) value.

Table No.17: Retention Time

Groups	Retention Time (sec) Mean ± SEM
Control	30.36 ± 0.31
Disease control ($AlCl_3$ 1 mg/kg)	7.50 ±0.51 ###
Formononetin NS (10 mg/kg)	12.02 ± 0.63 *
Formononetin NS (20 mg/kg)	14.41 ± 0.56 **
Formononetin NS (40 mg/kg)	22.18 ± 0.95 ***
Standard (Donepezil)	27.87 ± 0.29 ***

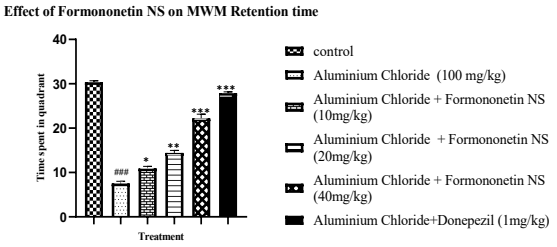

Fig : Values are expressed as mean ± SEM (n=6). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with $AlCl_3$ group and ### $p < 0.001$ compared to normal control group.

5.2. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$ - induced Alzheimer's disease in Y maze test:

Spontaneous Alterations-

The alteration percent in normal group of animals found to be 84.50 ± 0.76 %. In the $AlCl_3$ group-2, after 3 weeks there was significant ($p < 0.001$ each) decrease in alteration percentage (36.83 ± 0.60 percent) compared to normal control group. Donepezil hydrochloride (1 mg/kg) and Formononetin nanosuspension (40 mg/kg) treatment respectively showed a significant ($p < 0.001$ each) increase in the alteration percent (79.83 ± 0.47 and 73.5 ± 1.02 %). Formononetin nanosuspension (40 mg/kg) treatment showed significant ($p < 0.01$) increase in alteration percentage (50.16 ± 1.14 %), compared with $AlCl_3$ group. However, treatment with Formononetin nanosuspension (40 mg/kg) also showed significant

($p<0.05$) increase in alteration percentage (43.16 ± 0.70 %) in $AlCl_3$ induced AD in rats.

Table No.18: Spontaneous Alteration

Groups	Alteration percentage (%) Mean ± SEM
Control	84.50 ± 0.76
Disease control ($AlCl_3$ 1 mg/kg)	36.83 ± 0.60 ###
Formononetin NS (10 mg/kg)	43.16 ± 0.70 *
Formononetin NS (20 mg/kg)	50.16 ± 1.14 **
Formononetin NS (40 mg/kg)	73.5 ± 1.02 ***
Standard (Donepezil)	79.83 ± 0.47 ***

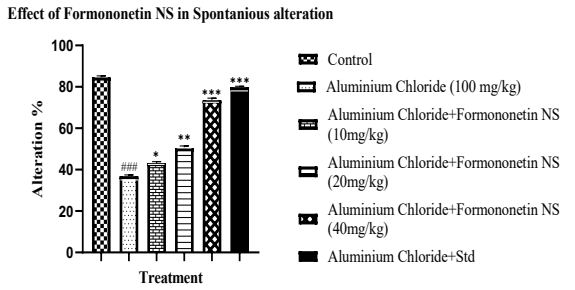

Fig : Values are expressed as mean ± SEM (n=6). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with $AlCl_3$ (1mg/kg) group and ### $p < 0.001$ compared to normal control group.

5.3. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$ (1mg/kg) -induced Alzheimer's disease in Passive Avoidance Test:

The passive avoidance (group-II) rats showed significant ($p < 0.001$) decrease in the step through latency. The step through latency in normal control group of animals was found to be 124 ± 1.65 sec. $AlCl_3$ (1mg/kg) group-II rats showed significant ($p < 0.001$) decrease in step through latency (44 ± 1.18 sec). Treatment with Donepezil hydrochloride (1 mg/kg) and Formononetin nanosuspension (40 mg/kg) significantly ($p < 0.001$) brought back those values toward near to normal (112 ± 1.57 and 94 ± 0.73 sec) respectively. Treatment with Formononetin nanosuspension (20 mg/kg) showed significant ($p < 0.01$) increase in step through latency (70.05 ± 1.97 sec) compared to $AlCl_3$ (1mg/kg) induced AD rats. However, Formononetin nanosuspension (10 mg/kg) show less significant ($p < 0.01$) increase in latency (54.05 ± 1.66 sec).

Table No.19: Step through latency

Groups	Step through latency (sec) Mean ± SEM
Control	124 ± 1.65
Disease control ($AlCl_3$ 1mg/kg)	44 ± 1.18 ###
Formononetin NS (10 mg/kg)	54.05 ± 1.66 *
Formononetin NS (20 mg/kg)	70.05 ± 1.97 **
Formononetin NS (40 mg/kg)	94 ± 0.73 ***
Standard (Donepezil)	112 ± 1.57 ***

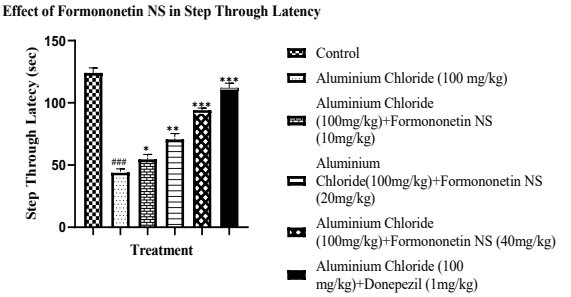

Fig : Values are expressed as mean ± SEM (n=6). Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared with $AlCl_3$ group and ### $p < 0.001$ compared to normal control group.

5.4. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$ - induced Alzheimer's disease in Novel Object Recognition Test:

The novel object recognition (group-2) rats showed significant ($p < 0.001$) decrease in the discrimination index. The discrimination index in normal control group of animals was found to be 85.50 ± 0.76 %. $AlCl_3$ 1mg/kg group-2 rats showed significant ($p < 0.001$) decrease in discrimination index (29.50 ± 0.76 %). Treatment with Donepezil hydrochloride (1 mg/kg) and Formononetin nanosuspension (40 mg/kg) significantly ($p < 0.001$) brought back those values toward near to normal (77.66 ± 1.28 and 57.16 ± 1.22 %) respectively. Treatment with Formononetin nanosuspension (20 mg/kg) showed significant ($p < 0.01$) increase in discrimination index (46.83 ± 0.60 %) compared to $AlCl_3$ induced AD rats. However, Formononetin nanosuspension (10 mg/kg) show less significant ($p < 0.05$) increase in discrimination index

(36.83 ± 0.60 %).

Table No.20: Discrimination Index

Groups	Discrimination Index (%) mean ± SEM
Control	85.50 ± 0.76
Disease control ($AlCl_3$ 1mg/kg)	29.50 ± 0.76 ###
Formononetin NS (10 mg/kg)	36.83 ± 0.60 *
Formononetin NS (20 mg/kg)	46.83 ± 0.60 **
Formononetin NS (40 mg/kg)	57.16 ± 1.22 ***
Standard (Donepezil)	77.66 ± 1.28 ***

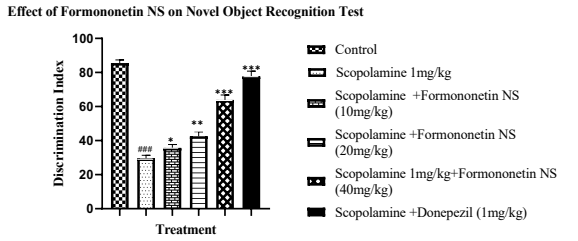

Fig : Values are expressed as mean ± SEM for n = 6 rats and statistical analysis was carried out by one-way ANOVA followed by post hoc Tukey's test;* $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with **$AlCl_3$** (group-2) and ###$p < 0.001$ compared to normal control group

6. Oxidative Stress Parameters

6.1. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$ - induced Alzheimer's disease in Superoxide dismutase (SOD):

The SOD level of brain tissues of control group was found to be 8.60 ± 0.15 nmole/mg, which was significantly ($p < 0.001$) decreased in $AlCl_3$ 1mg/kg control group and found to be 3.15 ± 0.17 nmole/mg. Treatment with Donepezil hydrochloride (1 mg/kg) and Formononetin nanosuspension (40 mg/kg) significantly ($p < 0.001$) increase in brain tissues SOD level (7.66 ± 0.14 and 6.64 ± 0.12 nmol/mg) respectively when compared to $AlCl_3$ induced group. Treatment with Formononetin nanosuspension (20 mg/kg) showed significant ($p < 0.01$) increase (5.69 ± 0.14 nmol/mg) in brain tissues SOD level, when compared to $AlCl_3$ control group. However, Formononetin nanosuspension (10 mg/kg) showed (3.97 ± 0.14 nmol/mg) less significant ($p < 0.05$) increase in brain tissues SOD level when compared to $AlCl_3$ control group.

Table No.21: Superoxide dismutase (SOD)

Groups	SOD (nmol/mg) Mean ± SEM
Control	8.60 ± 0.15
Disease control ($AlCl_3$ 1mg/kg)	3.15 ± 0.17 ###
Formononetin NS (10 mg/kg)	3.97 ± 0.14 *
Formononetin NS (20 mg/kg)	5.69 ± 0.14 **
Formononetin NS (40 mg/kg)	6.64 ± 0.12 ***
Standard (Donepezil)	7.66 ± 0.14 ***

Superoxide dismutase plays a crucial role in the antioxidant defense system of the brain by converting superoxide radicals into less harmful molecules such as hydrogen peroxide and oxygen. Adequate levels of SOD are essential for maintaining neuronal integrity and protecting brain tissues from oxidative stress. In neurodegenerative disorders such as Alzheimer's disease, oxidative stress is considered one of the major contributing factors leading to neuronal damage and cognitive impairment.

Administration of Aluminium chloride is known to induce oxidative stress in the brain by generating excessive reactive oxygen species (ROS) and impairing the endogenous antioxidant defense system. This results in a marked reduction in SOD activity, indicating increased oxidative damage to neuronal cells and disruption of normal brain function.

Treatment with the standard drug **donepezil hydrochloride** significantly restores antioxidant defense by improving SOD activity and protecting neurons from oxidative stress. Similarly, treatment with Formononetin nanosuspension demonstrates a notable improvement in SOD levels in brain tissues. This effect may be attributed to the strong antioxidant properties of formononetin, which help neutralize free radicals and enhance the endogenous antioxidant defense system

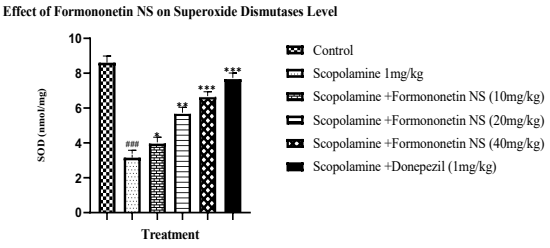

Fig: Values are expressed as mean ± SEM for n = 6 rats and statistical analysis was carried out by one-way ANOVA followed by post hoc Tukey's test;* $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with $AlCl_3$ (group-2) and ###$p < 0.001$ compared to normal control group.

6.6. Immunohistochemistry analysis:

6.6.2. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$-induced Alzheimer's disease in Estimation of brain-derived neurotrophic factor (BDNF):

The BDNF level of brain tissues of control group was found to be 1179.07 ± 11.51 Pg/mg, which was significantly ($p < 0.001$) decreased in $AlCl_3$ control group and found to be 539.45 ± 3.80 Pg/mg. Treatment with Donepezil hydrochloride (1 mg/kg) and Formononetin nanosuspension (40 mg/kg) significantly ($p < 0.001$) increase in brain tissues BDNF level (1055.87 ± 9.66 and 867.14 ± 7.32 Pg/mg) respectively when compared to $AlCl_3$ induced group. Treatment with Formononetin nanosuspension (20 mg/kg) showed significant ($p < 0.01$) increase (660.56 ± 9.28 Pg/mg) in brain tissues BDNF level, when compared to TMT control group. However, Formononetin nanosuspension (10 mg/kg) showed (620.42 ± 14.28 Pg/mg) less significant ($p < 0.05$) increase in brain tissues BDNF level when compared to $AlCl_3$ control group.

Table No: 26

Groups	BDNF (Pg/mg) Mean ± SEM
Control	1179.07 ± 11.51
Disease control ($AlCl_3$ 1mg/kg)	539.45 ± 3.80 ###
Formononetin NS (10 mg/kg)	620.42 ± 14.28 *
Formononetin NS (20 mg/kg)	660.56 ± 9.28 **
Formononetin NS (40 mg/kg)	867.14 ± 7.32 ***
Standard (Donepezil)	1055.87 ± 9.66 ***

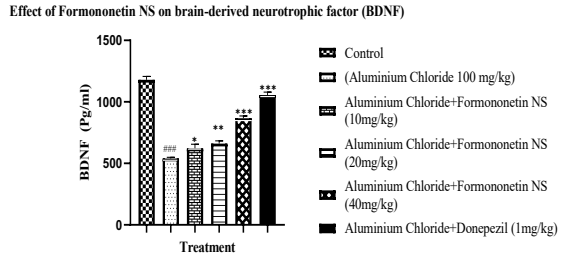

Fig : Values are expressed as mean ± SEM for n = 6 rats. Data are expressed as mean ± SEM and statistical analysis was carried out by one-way ANOVA followed by post hoc Tukey's test; * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with $AlCl_3$ (group-2) and ###$p < 0.001$ compared to normal control group.

6.6.4. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$-induced Alzheimer's disease in Estimation of Acetylcholine Esterase (AChE):

The AChE level of brain tissues of control group was found to be 12.66 ± 2.34 nmol/min/mg, which was significantly ($p < 0.001$) increased in $AlCl_3$ control group and found to be 41.5 ± 3.72 nmol/min/mg. Treatment with Donepezil hydrochloride (1 mg/kg) significantly ($p < 0.001$) decrease in brain tissues AChE level (14.33 ± 2.98 nmol/min/mg) when compared to $AlCl_3$ induced group. Treatment with Formononetin nanosuspension (40 mg/kg) showed significant ($p < 0.01$) decrease (19.16 ± 3.21 nmol/min/mg) in brain tissues AChE level, when compared to $AlCl_3$ control group. Treatment with Formononetin nanosuspension (20 mg/kg) showed significant ($p < 0.05$) decrease (25.16 ± 2.65 nmol/min/mg) in brain tissues AChE level, when compared to $AlCl_3$+ control group. However, Formononetin nanosuspension (10 mg/kg) showed (34.33 ± 3.32 nmol/min/mg) no significant in brain tissues AChE level.

Table No: 28

Groups	AchE (nmol/min/mg) Mean ± SEM
Control	12.66 ± 2.34
Disease control ($AlCl_3$ 1mg/kg)	41.5 ± 3.72 ###
Formononetin NS (10 mg/kg)	34.33 ± 3.32 ns
Formononetin NS (20 mg/kg)	25.16 ± 2.65 *
Formononetin NS (40 mg/kg)	19.16 ± 3.21 **
Standard (Donepezil)	14.33 ± 2.98 ***

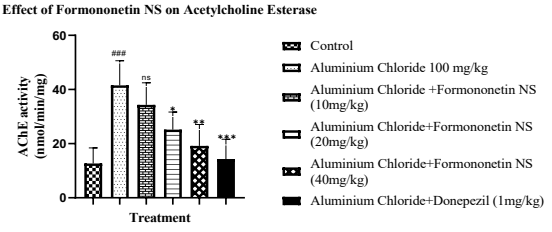

Fig : Values are expressed as mean ± SEM for n = 6 rats. Data are expressed as mean ± SEM and statistical analysis was carried out by one-way ANOVA followed by post hoc Tukey's test; * $p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with $AlCl_3$ (group-2) and ###$p < 0.001$ compared to normal control group.

6.6.6. Effect of Formononetin nanosuspension (10, 20 and 40 mg/kg) & Donepezil (1mg/kg) on $AlCl_3$ -

induced Alzheimer's disease in Estimation of Interlukin-1 (IL-1):

The IL-1 level of brain tissues of control group was found to be 49.5 ± 7.37 Pg/mg, which was significantly ($p < 0.001$) increased in $AlCl_3$ control group and found to be 186.5 ± 8.17 Pg/mg. Treatment with Donepezil hydrochloride (1 mg/kg) and Formononetin nanosuspension (40 mg/kg) significantly ($p < 0.001$) decrease in brain tissues IL-1 level (72.33 ± 6.39 and 90.83 ± 5.35 Pg/mg) respectively when compared to $AlCl_3$ induced group. Treatment with Formononetin nanosuspension (20 mg/kg) showed significant ($p < 0.01$) decrease (129.83 ± 3.02 Pg/mg) in brain tissues IL-1 level, when compared to $AlCl_3$ control group. However, Formononetin nanosuspension (10 mg/kg) showed (146.5 ± 8.16 Pg/mg) less significant ($p < 0.05$) decrease in brain tissues IL-1 level when compared to $AlCl_3$ control group.

Table No:30

Groups	NO (µmol/mg) Mean ± SEM
Control	49.5 ± 7.37
Disease control ($AlCl_3$1mg/kg)	186.5 ± 8.17 ###
Formononetin NS (10 mg/kg)	146.5 ± 8.16 *
Formononetin NS (20 mg/kg)	129.83 ± 3.02 **
Formononetin NS (40 mg/kg)	90.83 ± 5.35 ***
Standard (Donepezil)	72.33 ± 6.39 ***

Interleukin-1 beta is an important pro-inflammatory cytokine involved in neuroinflammation and neuronal damage associated with Alzheimer's disease. Increased levels of IL-1 in brain tissue are commonly linked with activation of microglial cells, oxidative stress, and progressive neuronal degeneration. These inflammatory processes contribute significantly to cognitive decline and the pathological progression of neurodegenerative disorders.

Exposure to Aluminium chloride is known to induce neurotoxicity and trigger inflammatory responses in the brain. Aluminium-induced toxicity stimulates the release of pro-inflammatory cytokines, including IL-1, which further promotes neuronal injury and disrupts normal brain function. This elevation in inflammatory mediators reflects the development of Alzheimer-like pathological changes in experimental models.

Treatment with the standard drug donepezil hydrochloride helps reduce neuroinflammation by improving cholinergic neurotransmission and protecting neuronal cells from inflammatory damage. Similarly, treatment with Formononetin nanosuspension demonstrates a significant reduction in IL-1 levels in brain tissues. This effect may be attributed to the antioxidant and anti-inflammatory properties of formononetin, which help suppress inflammatory signaling pathways and protect neurons from oxidative damage.

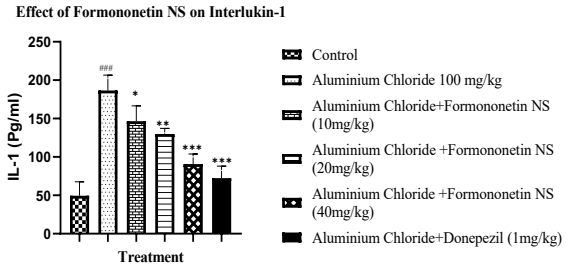

Fig : Values are expressed as mean ± SEM for n = 6 rats. Data are expressed as mean ± SEM and statistical analysis was carried out by one-way ANOVA followed by post hoc Tukey's test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with $AlCl_3$ (group-2) and ### $p < 0.001$ compared to normal control group.

6.9. Histopathological Examinations:

Hippocampus was observed by staining with Hematoxylin and Eosin stain to detect the degeneration of neurons. The disease control ($AlCl_3$) animals (fig. B) showed decreased cellular density with presence of degenerating dark neurons at hippocampus when compared with normal control animals (fig. A) showing normal appearance of brain cells and neurons. Treatment with formononetin nanosuspension (10 mg/kg) (fig. D) showed appearance of brain cells and neurons with mild formation of focal amorphous eosinophilc materials. At a dose formononetin nanosuspension (20 mg/kg) (fig. E) treatment showed normal appearance of brain cells and neurons, no fibrotic changes and neuritic threads and normal distribution of neurons when compared with disease control group. At a dose formononetin nanosuspension (40 mg/kg) (fig. F) treatment showed normal appearance of brain cells and neurons, no fibrotic changes and neuritic threads and normal distribution of neurons when compared with disease control group. Treatment with combination of formononetin nanosuspension (10 mg/kg) (fig. D) did not prevent the neuronal degeneration of neurons. However, treatment with formononetin nanosuspension (20 mg/kg) (fig. E) and formononetin nanosuspension (40 mg/kg) (fig. F) showed normal histology, normal layer of neuronal cell when compared with disease group. The results of

formononetin nanosuspension (40 mg/kg) treatment showed comparable results as that of donepezil (1 mg/kg) treated group (fig. C) with mild to less degeneration of neurons showing normal structure of hippocampus in the rat's brain.

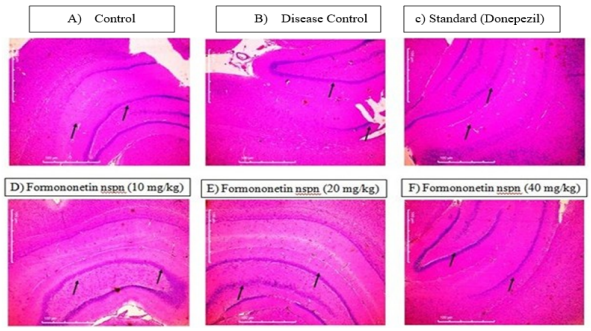

Table.31: Histopathological examinations of rat's brain.

Images ID	Description
A) Normal control	Normal appearance of brain cells and neurons, no fibrotic changes and neuritic threads. normal distribution of neurons. No abnormality detected.
B) Disease control ($AlCl_3$)	Degeneration and pyknosis in neurons with formation of focal amorphous eosinophilc materials. Abnormality detected.
C) Donepezil (1 mg/kg)	Normal appearance of brain cells and neurons, no fibrotic changes and neuritic threads. normal distribution of neurons. No abnormality detected.
D) formononetin nanosuspension (10 mg/kg)	Normal appearance of brain cells and neurons, mild formation of focal amorphous eosinophilc materials. Mild abnormality detected.
E) formononetin nanosuspension (20 mg/kg)	Normal appearance of brain cells and neurons, no fibrotic changes and neuritic threads. normal distribution of neurons. No abnormality detected.
F) formononetin nanosuspension (30 mg/kg)	Normal appearance of brain cells and neurons, no fibrotic changes and neuritic threads. normal distribution of neurons. No abnormality detected.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the research work presented in this report.

Consent for Publications

All authors have read and approved the final version of this manuscript for publication.

Availability of Data and Material

All data generated or analyzed during this study are included in the manuscript and are available within the document.

Authors' Contributions

Ms. Divya G. Thite designed and conducted the research work, performed data analysis, and prepared the manuscript.

Dr. Neelam L. Dashputre supervised the research design, guided the experimental work, and reviewed and approved the final version of the manuscript.

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