4. Methodology

4.1 Chemicals:

3-Nitroproponoic acid (CDH, India), Naringenin, Acetyl thiocholine iodide, DTNB reagent (5, 5'-dithio-bis-(2-nitrobenzoic acid), tris buffer, trichloro acetic acid, potassium phosphate buffer, O-dianisidine solution, potassium dihydrogen and dipotassium hydrogen phosphates, sodium phosphate buffer, xylene, hematoxylin, eosin stain, succinic acid, potassium ferricyanide, EDTA (Ethylene diamine tetra acetic acid).

4.2 Animals:

Healthy adult male albino Wistar rats of 3-4 months old, weighed (250-300g) were purchased from Mahaveer Enterprises, Hyderabad, India and were used for the experiment. The animals were acclimatized to standard laboratory condition with temperature (25±2ºC) and fed with standard animal pellet feed (Hindustan lever limited) and water ad-libitum. The protocol was approved by Institutional Animal Ethics Committee (IAEC) of ANU College of Pharmaceutical Sciences, constituted for the purpose of animal experimentation as per CPCSEA guidelines (Reg.No.1725/GO/Re/S/13/CPCSEA), with Approval no: (ANUCPS/IAEC/AH/P/11/2017) for the care and use of animals.

4.3 Drug treatment

The study was carried out for a period of 21 days. In this experimental design, rats were randomly divided into 5 groups, consists of 12 animals each and treatment schedule was followed by:

**Group - I** served as normal control with saline *i.p.*

**Group - II** was administered with 3-NP at a dose of 15 mg/kg, *i.p.*

**Group - III** was administered with only Naringenin at a dose of 75 mg/kg, *p.o.*

**Group - IV & V** were administered with Naringenin 25 & 75 mg/kg *p.o.* along with 3-NP at 15 mg/kg *i.p.* respectively.

All the treatments were continued for 21 days and Group - II, IV, and V groups were treated with 3-NP (15 mg/kg/day, *i.p.*) from 8th day to 21st day one hour after the regular treatment as above said. On 21st day after 3hr of treatment, the animals were assessed for various physical and behavioural parameters and on 22nd day animals were euthanized to isolate the brain samples and they were used for
biochemical estimations, measurement of lesions with TTC staining and for histopathological studies on different parts of the brain i.e in striatum, cortex and hippocampus.

4.4 Experimental design

4.5 Parameters estimated

- **Measurement of Body weights**
- **Motor co-ordination and behavioural assessments**
  - Motor co-ordination assessments
    - Neurological scoring
    - Locomotor activity
    - Narrow beam walking test
    - Hanging wire test
    - Rota rod test
    - Print length Analysis
  - Behavioural assessments
    - Elevated plus maze test
    - Y-Maze test
- Morris water maze test
- Forced swim test

- Oxidative stress parameters in striatum, cortex and hippocampus
  - Lipid peroxidation (MDA)
  - Reduced glutathione (GSH)
  - Super oxide dismutase (SOD)
  - Catalase activity (CAT)

- Enzyme estimations in striatum, cortex and hippocampus
  - Succinate dehydrogenase (SDH) (Complex-II)
  - Acetyl cholinesterase enzyme (AchE) activity

- Neurochemical estimations in striatum, cortex and hippocampus
  - Gamma-amino butyric acid (GABA)
  - Glutamate

- Brain lesion measurement
- Histopathological examination in striatum, cortex and hippocampus

4.6 Measurement of Body weights (BW):

Animals body weights were weighed on the first and last day of the study and percentage change in body weights were calculated with the help of the following formula.

\[
\frac{(\text{BW on 1}^{st} \text{ day} - \text{BW on 15}^{th} \text{ day})}{\text{BW on 1}^{st} \text{ day}} \times 100
\]

4.7 Motor co-ordination and behavioural assessments

- Motor co-ordination assessments:
  - Neurological scoring:

  Neurological scoring was assessed to measure the motor disturbance induced by 3-NP, based on their normal ambulatory movements. The scoring was done as per previous literature: 0 normal, 1 general slowness of displacement due to mild hind limb impairment, 2 co-ordination loss and significant abnormality in gait, 3
hind limbs paralysis, 4 inability to move due to impairment in both forelimbs and hind limbs, 5 recumbency (Jung-Eun Park. et al., 2013).

- **Locomotor activity:**

  On 7th, 14th and 21st day of the experimental protocol all the animals were observed for locomotor activity. Animals were acclimatized for 5 min in the Photoactometer after that each animal was allowed to move freely in the Photoactometer for 5 min (300 sec) which was accessed with light sensitive photocells to count the motor activity digitally. The count/5 min was measured for each animal for their locomotor activity (Kumar. et al., 2011).

![Figure 4 Actophotometer](image)

- **Narrow beam walking test:**

  Motor coordination of the rats was assessed by beam walking test on weekly intervals. In this test a wooden beam with (2.3 cm × 120 cm) measurements, elevated at a height of 50 cm from the ground. While doing experiment to prevent injury to the animals by falling from the beam on the ground, the ground was padded with foam cushion. During the test animals were allowed to move from home cage point to the end of the beam within 2 min of time. If the animals were unable to cross the beam within the time or fallen from the beam then the trail was ended. The animals crossed the beam within the time then the time of latency in crossing the beam was measured (Tinh. et al., 2011).
Hanging wire test:

In this test the grip strength was measured indirectly on weekly intervals by using hanging wire test. In this test, animals were allowed to hang with their forelimbs on a steel wire with a diameter of 2mm and length of 80 cm elevated to a height of 50 cm from the ground padded with a cushion to prevent injury to the animal. The latency in fall of time from the hanging wire on the ground was measured; the cut off time was kept for 90 sec (Hunter AJ. et al., 2000).

Rota rod test:

The skeletal muscle relaxation together with taming or calming effect, these agents reduce anxiety and tension. The loss of muscle-grip is an indication of muscle relaxation. This effect can be easily studied in animals using inclined plane or rotating rods. The difference in fall off time from the rotating rod between the normal and treated animal is taken as an index of muscle relaxation. The angle of the slope of the inclined plane, or the rate of rotation of the rod should be adjusted such that a normal rat can stay on the plane or on the rod for an appreciable time (3-
5 min) of time. Turn on the Rota-rod by selecting an appropriate speed (20-25 rpm). Place the animal one by one into several compartments. Note down the ‘fall off time’ when the rat falls from the rotating rod. A normal group of rats generally falls off within 3-5 minutes. Later the treated groups are followed by noting the fall off time (Sundara Veena et al., 2015).

![Figure 7 Rota rod](image)

- **Print length analysis:**

  In this test the animal hind limb paws were coloured with picric acid and animals were allowed to fall on a white paper from a height of 30 cm. The distance between the print of two hind legs were measured with the scale. This procedure was repeated 3 times for each rat to obtain an average value (Denny Joseph K.M, Muralidhara, 2014).

- **Behavioural assessments**

  - **Elevated plus maze test:**

    Elevated plus-maze is the simplest apparatus to study anxiety response and the effect of almost all type of anti-anxiety agents. Exposure of the animals to novel maze alley evokes on approach avoidance conflict which is stronger in open arm as compared to enclosed arm. Rodents have aversion for high and open space and prefer enclosed arm and therefore, spend greater amount of time in enclosed arm. When animals enter open arm, they freeze, become immobile, defecate and show fear-like movements. The plasma cortisol level is also reported to be increased, as a true reflection of anxiety. The elevated plus maze consisted of two opposite black open arms (50 cm × 10 cm), crossed with two closed walls of the same dimensions with 40 cm high walls. The arms were connected with a central square of...
dimensions 10 cm × 10 cm the entire maze was placed 50 cm high above the ground. Rats were placed individually at one end of the open arm facing away from the central square. The time taken by the animal to move from the open arm to the closed arm was recorded as transfer latency (TL) on 21st days (Puneet Kumar et al., 2010).

Figure 8 Elevated plus maze test

Y-maze test:

The Y-maze test was a horizontal maze (40 cm long and 14 cm wide, with walls 22 cm high) made of polyvinyl chloride (PVC) material with three arms (labelled A, B, and C) disposed at 120 to each other. Each rat was placed at the centre of the apparatus and allowed to move freely through the maze for 5 min. The number of alternations (i.e., consecutive entry sequences of ABC, CAB or BCA, but not BAB) and the numbers of arm entries were recorded. Maze arms were thoroughly cleaned between tests with water spray to remove residual odours. The percentage alternation was calculated according to the following equation: (Song X. et al., 2016).

\[
\text{Percentage alternation (\%) = \left(\frac{\text{number of alternations}}{\text{total arm entries} - 2}\right) \times 100.}
\]
Morris water maze test:

Morris water maze is an experimental method which is commonly used to evaluate spatial learning and memory in animal models. In this test, the rodents try to find the platform hidden beneath the water using the cues which is located on the around space. The water maze was a circular pool 136 cm diameter, 60 cm high and 30 cm deep. The pool was filled with water (24–25 °C), and a circular Plexiglas platform (10 cm diameter, 28 cm high) was placed inside it, 2 cm below the water surface in the central part of the southwest quadrant. Various visual signs were placed around the water maze pool, and a camera was mounted above the centre of the pool with which the rats motion was recorded. During the experiment the animal was allowed to swim freely, find the platform, and remain on the platform for 20 seconds. The animal was then taken out of the water. 20 seconds later, it was dropped into the water at another point. The time it took the animal to find the platform represented the amount of learning and memory and was recorded by camera. The animals were then dried and returned to the cage (Charles V Vorhees, Michael T Williams. 2006).
Forced swim test:

On 21st day of the experiment protocol, animals were placed individually in Plexiglas cylinders (40 cm in height and 18 cm in diameter) containing 25 cm water, maintained at 23–25°C. The animals were removed from the water cylinder after 5 min the total duration of immobility was measured. A rat was judged to be immobile when it remained floating passively in the water (Thangarajan. et al., 2016).

Figure.11 Forced swim test

4.8 Preparation of brain homogenate for biochemical estimations:

On the 21st day of the study protocol, animals were sacrificed with ether anaesthesia. Brain was removed and washed with ice-cold isotonic saline solution by removing the cerebellum part brain tissue was homogenized in ice-cold extraction buffer solution (10 mm Tris-Hcl, pH 7.4, 0.44 M sucrose, 10 mm EDTA and 0.1% BSA). The homogenate was centrifuged to at 5000 rpm with 4°C for 30 min of time to get a supernatant. The supernatant once again was centrifuged at 5000 rpm with 4°C for 45 min. The mitochondrial pellet was collected, washed

Figure.12 Isolation of rat brain
with extraction buffer and centrifuged the solution at 5000 rpm with 4°C for 45 min. the pellet was re-suspended in suspension buffer (0.44 M sucrose in 10 mM Tris-Hcl, pH 7.4). This suspension was used for the estimation of mitochondrial enzymes. Before re-centrifugation some part of the supernatant used for the assessment of biochemical parameters as described in following pages (Sandhir R. et al., 2010).

4.9 Oxidative stress parameters

➢ Lipid peroxidation (MDA)

The amount of lipid peroxidation products present in the serum samples/pleural fluid was estimated by the thiobarbituric acid reactive substances (TBARS) method which measures the malondialdehyde (MDA), reactive products by using UV-Visible spectroscopy.

Principle:

The reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), a secondary product of lipid peroxidation has been widely adopted as a sensitive assay method for measurement of lipid peroxidation in biological fluids. It is widely used as an index of the extent to which lipid peroxidation has progressed. Since the assay procedure estimates the amount of TBA reactive substances e.g. MDA, it is also referred to as TBARS (Thiobarbituric acid reactive substance) test.

Procedure:

To 0.5 ml of brain homogenate 0.5 ml of 30 % trichloro acetic acid (TCA) was added to precipitate the proteins and vortexed for 30 sec. Clear supernatant was taken after centrifuging at 3000 rpm for 10 min. To the supernatant, 500 µL of 1 % TBA solution and 500 µL of water was added and this solution was heated for 1hr at 98° C. Cool the solutions to room temperature and kept them in ice for 5 minutes. Then read the pink colour at 532 nm using spectrophotometer. Standard graph was plotted using TEP (1, 1, 3, 3-tetra ethoxy propane). The absorbance was observed at 532 nm. The values were expressed as nmol MDA/ mg protein and the calculation was done by using extinction coefficient of 1.56×10^5 M\(^{-1}\) cm\(^{-1}\) (Ohkawa. et al., 1979).
**Reduced glutathione (GSH)**

Glutathione forms a coloured complex with DTNB, which is measured spectrophotometrically.

**Reagents**

**Sodium phosphate buffer (0.3 M) pH 8:** Sodium dihydrogen phosphate (NaH$_2$PO$_4$) of 0.3 M of 4.68 g/100ml, were prepared in double distilled water. To 0.3 M disodium hydrogen phosphate solution, sufficient amount of 0.3 M sodium dihydrogen phosphate solution was added to get pH 8 phosphate buffer solution using pH meter.

**5-5’ (Dithiobis-2-Nitro benzoic acid) DTNB reagent:** DTNB (39.6 mg) was dissolved in 100 ml of 1% sodium citrate solution to give a concentration of 1 mm; sodium citrate has been selected for convenience, since its pH was appropriate both for solubility and stability of the reagent. The DTNB was stable in this medium for 13 weeks in refrigerator.

**Procedure:**

To 0.5 ml of brain homogenate, 0.5 ml of 5% trichloroacetic acid (TCA) solution was added to precipitate the proteins and centrifuged at 3000 rpm for 20 minutes. To 0.1 ml of supernatant, 1ml of sodium phosphate buffer and 0.5 ml of DTNB reagent was added. The absorbance of the yellow colour developed was measured at 412 nm. The glutathione content was determined from standard graph by using pure glutathione. The values were expressed as nmol GSH/ mg protein and the calculation was done by using extinction coefficient of $1.36 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$ (Ellman G. L, 1959).

**Super oxide dismutase (SOD)**

**Procedure:** Assay of superoxide dismutase (SOD) was based on the ability of the enzyme to inhibit the autooxidation of pyrogallol. The assay was performed by using 1.5 ml tris buffer (0.05 M) and 0.5ml EDTA (1 mm) as blank. 1.5 ml tris buffer, 0.5 ml EDTA (1 mm) and 1 ml pyrogallol (0.2mm) as control and the test sample consisted of 1.5 ml tris buffer (0.05 M), 0.5 ml EDTA (1 mm), 0.05 ml serum and 1 ml pyrogallol (0.2 mm). Change in optical absorbance of sample per minute with reference to blank was recorded at a wavelength of 420 nm using SICO Spectrophotometer. The enzyme inhibition caused by the serum was calculated and
the enzyme activity was expressed as superoxide anion reduced/mg protein/min (Marklund, 1974).

- **Catalase activity (CAT)**
  Measurement of Catalase activity was done based on the ability of catalase to oxidize hydrogen peroxide (Beers, Sizer, 1952)
  The absorbance of hydrogen peroxide solution can be easily measured at 240 nm, on decomposition of hydrogen peroxide by catalase, the absorbance decreases with time. The enzyme activity could be arrived at from this decrease.

**Reagents:**
**Potassium phosphate buffer (65 mm, pH 7.8):** Potassium dihydrogen phosphate (KH₂PO₄) of 2 mg and 11.23 g of dipotassium hydrogen phosphate (K₂HPO₄) were dissolved in 250 ml and 1 litre distilled water respectively. The pH was adjusted to 7.8 with KH₂PO₄. Hydrogen peroxide solution (7.5 mm): 21 micro litres of commercial Hydrogen peroxide solution (30 %) was made up to 100 ml with distilled water.
**Procedure:** Phosphate buffer of 2.5 ml was added to 0.1 ml of brain homogenates and incubated at 25° C for 30 minutes. After transferring in to a cuvette the absorbance was measured at 240 nm, 650 μL of Hydrogen peroxide solution was added to initiate the reaction. The change in absorbance was measured for 3 minutes. The average change in absorbance per minute for each assay was calculated and the results were expressed in terms of IU/ml of tissue homogenate. One international unit of catalase is the enzyme that decomposes one μM of Hydrogen peroxide per minute at 25° C.

4.10 Enzyme estimations

- **Succinate dehydrogenase (SDH) (Complex-II)**
  The principle involved in the estimation of succinate dehydrogenase is by the conversion of succinic acid to fumaric acid in presence of an electron acceptor potassium ferricyanide. The mitochondrial suspension (0.05ml) was added to the reaction mixture of 1.5 ml of phosphate buffer (0.2M, pH 7.8), 0.2 ml of succinic acid (0.6 M, pH7.8), 0.3 ml (1% w/v) of BSA and 0.1 ml of potassium ferricyanide
(0.3M). To measure the succinate dehydrogenase the decrease in absorbance at 420 nm from 3 min using water as blank. Values were expressed as nmol succinate oxidized/ min/mg protein (Tsou E.King, 1967).

- **Acetyl cholinesterase (AchE) activity**

  Acetylcholinesterase (AchE) activity was assessed by using acetylthiocholine iodide and Dithiobisnitrobenzoic acid (DTNB). The assay is based upon the conversion of acetylthiocholine iodide into DTNB thiocholine adduct in the presence of AchE of the tissue sample by hydrolyzing the acetylthiocholine iodide. The hydrolyzed product thiocholine forms adduct with DTNB which is proportional to the AchE activity. The absorbance of the DTNB and thiocholine adduct is used as a measure of AchE activity in the tissue.

**Procedure:**

**Reagents:**

- 100 ml Phosphate buffer saline (0.2M, pH 8),
- 39.6mg DTNB reagent (39.6 mg of DTNB with 15mg NaHCO₃ is dissolved in 10 ml of 0.2M phosphate buffer pH 7.0),
- 21.67mg Acetyl thiocholine (21.67mg of acetyl thiocholine is dissolved in 1 ml of distilled water).

The AchE was assessed by taking, 0.4 ml of homogenate was added to cuvette containing 2.6 ml phosphate buffer (0.2M, pH 8) and 100 µl of DTNB was mixed well by bubbling air and absorbance measured at 412 nm in Spectrophotometer. When the absorbance was reached to a stable value, 20 µl of acetylthiocholine iodide was added and change in absorbance was measured with an interval of 1 min. (George L. Ellman. et al., 1961)

AchE activity was calculated by using the formula.

$$ R = 5.74 \times 10^{-4} \times \frac{A}{CO} $$

Where,

- \( R \) = Rate in moles of substrate hydrolyzed /minute /gm of brain tissue
- \( A \) = Change in absorbance / min.
- \( CO \) = Original concentration of the tissue (mg/ml)
4.11 Neurochemical estimations

- **GABA and Glutamate**

The level of Glutamate was estimated by multiple development paper chromatography technique.

**Reagents:**
2. Ninhydrin reagent: 0.25%
3. Copper sulphate solution: 0.005%
4. Standard glutamate: 2.942 mg of glutamate in 10 ml distilled water.

**Procedure**

Brain homogenate supernatant of 1.0 ml was evaporated to dryness at 70°C in an oven and the residue is reconstituted in 100 ml of distilled water. Standard solutions of glutamate and GABA at a concentration of 2mm along with the sample are spotted on Whatman No. 1 chromatography paper using a micropipette. It was placed on a chamber containing butanol: acetic acid: water (12: 3: 5 v/v) as solvent. When the solvent front reached the top of the paper, it was removed and dried. A second run is performed similarly, after which the papers are dried sprayed with ninhydrin reagent and placed in an oven at 100°C for 4 minutes. The portions which carry glutamate corresponding with the standard are cut and eluted with 0.005% CuSO₄ in 75% ethanol. Their absorbance is read against blank at 515 nm in spectrophotometer. (Raju. et al., 2004).

**Calculation:**
The levels of glutamate and GABA are calculated by using the following formula:

\[
A = \frac{\text{Unknown OD} \times \text{Standard in mg (3µg)} \times 1000}{\text{Standard OD} \times \text{Volume spotted (10µl)} \times W}
\]

Where
- \(A\) = Amino acid content in µmoles/gram wet weight tissue
- 1000 = Conversion factor for gram wet weight tissue
- \(W\) = Weight of the tissue in gram
4.12 Brain lesion measurement

![Figure 13 TTC staining](image)

For the measurement of infarct area we use immersion method of 2,3,5-triphenyltetrazolium chloride. Rats of each group were decapitated under deep anaesthesia by using ketamine hydrochloride (50 mg/kg, i.p), xylazine (10 mg/kg, i.m) and the brains were removed and kept in freezer at -4°C for 20 minutes to facilitate sectioning. Brain was sliced coronally from frontal pole into 2 mm-thick sections, sections were kept in a petri dish containing 2% of 2,3,5-triphenyltetrazolium chloride (TTC) in normal saline and glass coverslips wetted with the TTC solution were placed on top of each slice to facilitate even staining. The dishes were covered with aluminium foil to prevent exposure to light and incubated at 37°C for 30 minutes. TTC was replaced by 10% neutral-buffered formalin and kept for overnight. The images of the stained sections were photographed within 3rd day and brain infract area was measured.

4.13 Histopathological examination in striatum, cortex and hippocampus

After spatial memory evaluation, in each group (n=3) were anesthetized by ketamine hydrochloride (50 mg/kg, i.p). Then brains were isolated and fixed in 4% paraformaldehyde for 3-4 days. Then the brain tissue was sliced into thin blocks followed by placing in 95% ethanol later on carried out with subsequent dehydration and for clearing purpose it was subjected to 4°C in refrigerator. There after embedding in paraffin, and then the coronal sections of 5µm in thickness, including hippocampus, striatum and cortex were sectioned and used for pathological changes. For estimation of necrotic neurons which were characterized
by pyknotic or karyotic nuclei and cytoplasmic shrinkage. Staining was performed with hematoxylin and eosin (H&E).

Figure 14 Collection of brain samples for histopathology

4.14 Statistical Analysis:

All the data were expressed as Mean ± SEM and were analyzed by One-way ANOVA followed by Tukey’s multiple comparison tests. A ‘p’ value of ≤0.05 was considered as statistically significant. Data was analyzed by using Graph Pad prism software.
References:


- Puneet Kumar, Anil Kumar, “Protective Effect of Sesamol against 3-Nitropropionic Acid-Induced Cognitive Dysfunction and Altered Glutathione Redox Balance in Rats”. *Basic & Clinical Pharmacological & Toxicology*. 2010; 107(1): 577-582


