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EVALUATION OF LIMONIA ACIDISSIMA LEAF EXTRACT IN REDUCING OXIDATIVE STRESS AND NEURODEGENERATION INSCOPOLAMINE-INDUCED ANIMAL MODEL OF ALZHEIMERS DISEASE

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ABSTRACT

The present study was designed to evaluate limonia acidissima leaf extract in reducing oxidative stress and neurodegeneration in scopolamine-induced animal models of Alzheimer's disease. The extraction was carried out with an aqueous extract of Limonia acidissima leaves. The preliminary phytochemical screening showed the presence of glycosides flavonoids, tannins, phenolics, phytosterol, and saponins. This activity was evaluated by using the mice behavioral model i.e. T-maze performance and Elevated mazeperformance for 15 days, with an estimation of neurotransmitters in animals' brains. Anindividual animal was administered with 250mg/kg, 750mg/kg 2000mg/kg body weight doseone at a time orally. The animal was observed for 24 hours, the animal survived. Histology of hippocampal neurons in the case of control groups of Limonia acidissima and Donepezilshowed almost normal hippocampal cells when compared with their corresponding scopolamine treated group. The present study reveals that an aqueous preparation of Limoniaacidissima has a significant role in the management of cognitive impairment and oxidativestress.

KEYWORDS: Scopalamine, Limonia Acidissima, Leaf Extract, Alzheimer's, Dementia, Oxidative Stress, Neurodegeneration.

INTRODUCTION

According to a World Health Organization report,[1] plant-based medical systems are still in use in modern healthcare, with 80 percent of the human population relying on traditional medicine.. Starting with the isolation and commercialization of morphine (Papaver somniferum), notable leads identified from plants approved by the Food and Drug Administration for the treatment of various ailments[2] include digitoxin (Digitalispurpurea), quinine (Cinchona succirubra), pilocarpine (Pilocarpus jaborandi), and taxol (Taxus brevifolia). *Limonia acidissima* is a medium-sized deciduous tree found throughout India.[3] It is an aromatic, slow-growing tree that grows up to 9 meters tall and may be found throughout India.In indigenous medicine, all parts of Limonia are prescribed for the treatment of various diseases.Alkaloids, flavonoids, phenols, terpenoids, tannins, fat steroids, saponins, glycosides, gum, mucilage, and fixed oils were found in an early phytochemical investigation of *Limonia acidissima* plant parts.[4-7]

The term "neurodegenerative disorders" refers to a group of neurological conditions marked by a wide variety of clinical and pathological symptoms that cause particular parts of the brain to stop working normally.[8] Dementia is not a single disease; it manifests differently in different persons based on the brain regions involved and the sort of biochemical injury. There are several dementia classifications based on important clinical symptoms, anatomical sites, cell types affected by protein structure and metabolic abnormalities, and cellular and subcellular pathology in which pathogenic proteins are deposited in cell compartments.[9] Alzheimer's disease is one of the

most common forms of dementia, accounting for 60 to 80 percent of all cases. By the year 2050, it is anticipated that there would be 115 million cases of Alzheimer's disease. Though increasing age is a significant risk factor, Alzheimer's disease is not a natural component of aging. Patients' symptoms of Alzheimer's disease develop slowly and deteriorate with time, making it difficult to do daily tasks.[10] Long before any signs of the disease appear, the brain of an Alzheimer's patient undergoes multiple changes that linger for years and are referred to as pre-clinical AD. AD can be divided into three stages once symptoms begin to develop (National Institute of Aging, 2016). The possible risk factors for Alzheimer's disease include (National Institute of Aging, 2016). Aging, Family history Factors increasing vascular risk (diabetes, high cholesterol, and high blood pressure), Educational level Race and ethnicity, Head trauma Gender and Dietary factors The key pathological characteristics of AD,[11] have been recognized as alterations in the cholinergic system, deposition of -amyloid (A) plaque development of neurofibrillary tangles (NFT), glutamate excitotoxicity, inflammation, dystrophic neuritis, and neuropil threads. The major goal of this study was to see how effective Limonia acdissima leaf extract was in reducing oxidative stress and being neuroprotective in a scopolamine-induced Alzheimer's disease mice model. The objectives of this study is to provide evidence-based medication with a neuroprotective impact to investigate the plant's phytochemical contents and to investigate the histology of the brain

METHODS

1. Plant Collection and Extraction

Limonia acidissima leaves were obtained from the vicinity of our college in the Ranga Reddy district of Hyderabad. Botanical Survey of India, Hyderabad, has been certified and authenticated at the Department of Botany, Sri Venkateshwara University, Tirupati, Andhra Pradesh. For this experiment, fresh mature leaves were utilized. The leaves were ground in a mortar and pestle for a fresh preparation. For the preparation, four milliliters of water were added. It was taken for the experiment and given to the mice using a gastric feeding tube at a dose of 2500 mg per kilogram of body weight for 15 days.[12]

2. Qualitative Phytochemical Analysis[13]

The extract was subjected to qualitative phytochemical analysis for alkanoids, glycosides, naphthoquinones, brontager's test, Iridoidal glycosides, coumarin glycosides, cyanogenetic glycosides, sterols, triterpenoids, flavonoids, tannins, matchstick test, carotenoids, carbohydrates, proteins.

Pharmacological Investigation

1. Animals

In this investigation, 2 month old Mus musculus Swiss weighing between 25 and 30 g was employed. The mice were kept and grown at the Department of Pharmacology's animal house, with a 12 hour light/12-hour dark cycle. They were housed in standard cages (29 x 22x 14 cm) with three mice per cage and given rat pellets and tap water from stainless steel bottles. They were washed daily and fed ad libitum. In compliance with the criteria of the Committee for Control and Supervision of Animal Experiments (CPCSEA), Ministry of Social Justice and Empowerment, the experiments were conducted.

2. Oral Toxicity Study

The up and down approach was used to test the acute oral toxicity of *Limonia acidissima* leaf extracts (UDP). The main benefit of UDP is that it reduces the number of animals needed to calculate the acute oral toxicity of a chemical and the median fatal dosage. 6-10 animals of one sex can be used to determine the LD50. The animals are dosed one by one at 24-hour intervals. The dosage for the following animals is adjusted up or down depending on the outcome. If the animal lives, the dose is raised; if the animal dies, the dose is dropped. After reaching the reversal on the initial outcome, i.e., point where an increasing or decreasing dose pattern is reversed by giving a smaller or higher dose. Four additional animals have dosed thesame UDP. In absence of any information about the substance, the starting dose may be 200 or 500 mg/kg body weight. For further doses, a dose progression factor of 1:3 is used. The next dose was administered according to the mortality of the animal.[14]

Procedure

An oral dosage of 250 mg/kg body weight was given to each animal one at a time. The animal was kept under observation for 24 hours. The animal survived, and the 750mg/kg body weight dosage was given to the following animal one at a time orally. The animal was watched for another 24 hours. The animal survived, and the following animal received the 2000mg/kg body weight dosage orally one at a time. The animal was watched for another 24 hours. The animal lived, and four more animals were dosed with the same dose. There were no deaths, and three animals of the opposite sex were given the same dose. The test was ended if mortality was not recorded again.[15]

Design of Behavioural Experiments

1. T-Maze

The T-maze is a well-known tool for preference and spatial learning. Animals learn to switch arms based on their recall of previously visited arms or by selecting an arm based on the reward offered.[16]

A stem (35 X 12 cm), a choice area (15 X 12 cm), and two arms make up the wooden T-mazeequipment (35 X 12 cm). The apparatus's sidewall is 40 centimeters in height. The equipment was housed in a sound-proofed space. The animal was pushed to the back of the line. Animal feed was placed in one of the T-arms mazes in such a manner that the mice couldn't see it until it reached the end of the long arm. To get the meal, the

animal must walk ahead and turn left or right of the Amaze. The animal's movement toward the meal was seen as a good reaction. When it goes to the opposite end, it is seen as a negative response.

The T-maze test was done on the first, eighth, and fifteenth days of the research. A total of ten trials were given every set, with a five-minute interval between them. The trials were carried out indefinitely until a set of 90 percent favorable responses was obtained. The percentage of positive response.[17] was calculated by adding the total number of positive replies across all sets and dividing it by the total number of trials delivered across all sets. The entire experiment took place after 9.00 p.m. in a dark room with weak red light. The animals were starved for 24 hours and given just water to drink before this experiment. During the trial, this makes the animal more active in its search for food.

The percentages of affirmative responses received in different animal groups were statistically analyzed for significance using the posthoc test (LSD – Least Significant Difference) function in the Statistical Package for Social Sciences (SPSS) version.[18]

2. Elevated Plus-Maze

With prior exposure to the open and closed arms, the animal's transfer latency (time it takes to go from an open arm to a closed arm) is lowered, which has been related to memory. Inflection ratio increases without tropic action. In addition, several studies on the effects of various nootropics and amnesic medications on EPM have validated its use in researching learning and memory processes in rodents198,[19] Day 1 transfer delay is considered acquisition (learning), whereas day 24 memory retention is measured.

The EPM gadget has four equal-sized arms: two open (50 x 10 cm) crossed with two closed (40 cm) arms. A central square (10 x 10 cm) connects the arms, giving the maze a plus sign look. This labyrinth is 50 cm high. The steps were the same as mentioned by.[20] The behaviour test was conducted between 9:00 a.m. and 6:00 p.m. Memory was tested twice, each 24 hours apart, using EPM. During training, the mice were placed near the open arm's end, distant from the centre platform. The rat's transfer latency (TL1) was timed by moving inside either of the enclosing arms with all four legs. If the rat did not enter one of the two enclosed arms within 90 seconds, it was gently pushed into one. After 10 seconds, the rat was returned to its cage. The maze was cleaned with 70% ethanol between runs to remove odour trails. A decrease in time latency (TL2) throughout the test session was viewed as an indicative of memory improvement. In both the training and test rounds, each rat had 90 seconds to explore the maze.

Pharmacological Drug Administration

Scopolamine butyl bromide manufactured by Kemwell biopharma Pvt. Ltd. and Done-10 tablets manufactured by Alkem Laboratories Ltd was used in the present study. Scopolamine was administered to the mice as an intraperitoneal injection at a dosage of 1 mg/kg body weight.[18] for 15 days 30 minutes before the start of behavioral experiments. Each Done-10 tablet contained Donepezil hydrochloride 10 mg and excipients and the tablets were powdered and mixed with sterile 0.9 % W/V normal saline. It was administered to the mice with an orogastric feeding tube at a dosage of 3 mg/kg body weight for 15 days and two hours before the start of the behavioral experiments.

Collection of Brain Sample

After the behavioral test, the animals were killed by cervical dislocation, and the brains were quickly removed and cleaned with 0.9 percent ice-cold saline. The tissues were weighed and homogenized with 10 times (W/V) ice-cold 0.1 M phosphate buffer (pH 7.4) in a pestle and mortar, then centrifuged at 5400 rpm for 20 minutes at 4oC to separate the supernatant usinga Sigma Aldrich cooling centrifuge. The supernatant was tested for acetylcholinesterase, SOD, and GST. A portion of the supernatant was combined with 10% TCA, well mixed, thencentrifuged at 4000 rpm for 10 minutes at 4oC, with the precipitate recovered and re-dissolved in 0.1NNaOH. An aliquot was used to compute soluble protein.

Biochemical Estimation

1. Protein Estimation

The protein content of diverse samples was calculated using the Lowry et al., 1951.[20] technique.

2. Principle

The approach is based on the Folin phenol reagent's color interaction with amino acids like tryptophan and tyrosine. These amino acids react with phosphomolybdic acid and phosphotungstic acid in Folin's reagent to produce a blue color, which was measured spectro photometrically. The color is caused by the reduction of phosphomolybdic acid and phosphotungstic acid in an alkaline media, as well as the Biuret reaction of protein with Cu2+.

The reagents include percent sodium carbonate (w/v) in 0.1 M NaOH, percent (w/v) copper sulfate ,Sodium potassium tartrate (w/v): 2% sodium potassium tartrate, 1 mg/ml Bovine Serum Albumin (BSA) and 0.1N Folin phenol reagent

Procedure

20 l of the sample, a BSA standard (10-200 g), and a blank were placed in separate tubes for the protein assay, and the final volume was reduced to 0.5 ml with 0.1 N NaOH. Then five milliliters of reagent C (made by combining 48 milliliters of 2% sodium carbonate, 1 milliliter of 1% copper sulfate, and 1 milliliter of 2% sodium potassium tartrate) were added. It was completely combined and set aside at room temperature for 10 minutes. A little amount of 1N FolinCiocalteau's reagent was added and mixed right away. The

ingredients were thoroughly The results were represented in milligrams of protein per milliliter or milligrams of protein per gram of tissue weight.

➢ **Estimation of Acetylcholinesterase Activity**

The technique of Ellmanet al., 1961723, was used to determine acetylcholinesterase activity. The principle behind this is using a spectrophotometer, the rise in yellow color generated by thiocholine when it interacts with di-this-nitrobenzoate (DTNB) with an absorbance of 412 nm was used to determine acetylcholinesterase activity.

Procedure

A 0.4 ml aliquot of the homogenate is put to a cuvette containing 2.6 ml phosphate buffer (0.1 ml pH 8) and 100 l of DTNB for AChE measurement. By bubbling air, the contents of the cuvette were well mixed, and the absorbance was measured in a spectrophotometer at 412nm. The baseline reading was taken when the absorbance reached a steady value. Then 20 l of the substrate (acetyl thiocholine iodide) was added, and the change in absorbance was monitored for 10 minutes at 2-minute intervals. As a result, the change in absorbance per minute is calculated.

➢ **Estimation of Lipid Peroxidation**

The production of lipid peroxide was measured using the Niehaus (1968)124 technique.

1. Principle

Malondialdehyde (MDA) is a breakdown product of peroxidized lipids that is measured using the Trichloroacetic acid- Thiobarbituric acid- Hydrochloric acid (TCA-TBA HCl) reagent (absorption maximum at 535 nm).

2. Procedure

In 0.1 M Tris HCl buffer, pH 7.5, the tissue homogenate was produced. About 1 mL of the homogenate was mixed well with 2 mL of the TCA-TBA-HCl reagent. In a boiling water bath, the solution was heated for 15 minutes. Centrifugation at 3000 rpm for 10 minutes was used to remove the flocculent precipitate after it had cooled. The sample's absorbance was measured at 535 nm against a blank that did not include any tissue homogenate. 1.56 X 105 M–1 cm-1 is the extinction coefficient of MDA.

➢ **Antioxidant defense status**

1. Superoxide dismutase

Superoxide dismutase is a metal-containing antioxidant enzyme that converts damaging oxygen free radicals to oxygen and hydrogen peroxide during normal metabolic cell processes. The technique of Kono (1978)[21] was used to calculate superoxide dismutase levels.

Principle

Under aerobic circumstances, the reduction of Nitro Blue Tetrazolium (NBT) to blue formazan mediated by hydroxylamine hydrochloride was measured. The amount of inhibition of NBT reduction by superoxide anions, which are formed by photo-oxidation of hydroxylamine hydrochloride, was used as a measure of enzyme activity when superoxide dismutase was added.

Procedure

1.3ml of 50 mM sodium carbonate solution with 0.1 mM EDTA (pH 10.0), 0.5 ml of 96 MofNBT, and 0.1 ml of 0.6 percent triton-X-100 made up the reaction mixture. The reaction was started by adding 0.1 ml of 20 mM hydroxylamine hydrochloride (pH 6.0) to the reaction mixture, and the rate of NBT reduction was measured for around 30 seconds in the absence of the enzyme source. Small aliquots of supernatant were then introduced to the test and reference cuvettes, which were both devoid of hydroxylamine hydrochloride.

Finally, the percentage inhibition in the rate of NBT reduction was calculated, and one unit ofthe enzyme was defined as the inverse of the quantity of protein (mg) necessary to inhibit the rate of NBT reduction by 50%.

2. Glutathione-S-Transferase

Glutathione-S-transferase is a metabolic enzyme best known for catalyzing the conjugation ofthe reduced form of glutathione (GSH) to xenobiotic substrates for detoxification purposes. The technique of Habig et al., 1974126, was used to determine glutathione-S-transferase. As a substrate, 1-chloro-2,4-dinitrobenzene (CDNB) is utilized.

Principle

GST catalyzes the production of a glutathione-chloro-2,4 dinitrobenzene conjugate, which was detected at 340 nm (E 340 = 9.6Mm-1 cm-1).

Procedure

0.1ml of 1 m MCDNB (freshly produced) was added to 2.75 ml of 0.1 M phosphate buffer (pH 6.5). The volume was then increased to 3 ml by adding 0.1 ml of 1 mM GSH (freshly produced) and 50 l samples. CDNB's glutathione conjugate absorbs the most light at 340 nm. The extinction coefficient of 9.6 Mm-1 cm-1 was achieved using the Jasco V double beam spectrophotometer. The absorbance was measured every minute for a total of five minutes. The blanks represented a non-enzymatic interaction of GSH with the relevant substrate in allcircumstances.

The CDNB solution was diluted in ethanol with care taken to ensure that the alcohol content in the assay mixture did not exceed 5%, and the reference cuvette should contain 0.1 M phosphate buffer (pH 6.5).

➢ **Statistical Analysis**

The data were analyzed using SPSS version 16 and the post-hoc LSD program.

➢ **Histology**

Seven columns of alcohol were made, with dilutions of 50 percent, 60 percent, 70 percent, 80percent, 90 percent, 100 percent, and 100 percent. To dehydrate the tissue, the test samples were serially put through an increasing concentration of alcohol and subsequently into xylene. It was then placed in a wax melter overnight at 60 degrees Celsius, containing liquid paraffin wax, for wax penetration into the tissue. Following that, the tissues were embedded in paraffin wax blocks. The embedded blocks were cut into pyramids and mounted on a hardwood block before being placed on a microtome. Two-millimeter thick sections were collected. To disperse the pieces, the ribbon of sections was placed in a water bath at 40 degrees Celsius. The portions were mounted on glass slides with egg albumin adhesive and baked for one hour at 60 degrees Celsius. After that, the slides were cleaned with xylene to remove the wax. Then it went through an alcohol rehydration process.

➢ **Rehydration**

1 The slides are run through a sequence of increasing concentrations of alcohol.2-3 minutes xylene
2 2-3 minutes of absolute alcohol 95 percent alcohol: 2-3 minutes
3 70 percent alcohol: 2-3 minutes
4 60 percent alcohol: 2-3 minutes 3-5 minutes under running water

Gore's Ehrlisch Hematoxylin Eosin Staining

1. Hematoxylin Staining

In 100 mL ethanol, 2 grams of hematoxylin were dissolved and put into a flask filled with cotton. After filtering, the flask was left for a few days with periodic shaking. In 100 mL distilled water, three grams of ammonium alum were dissolved and well mixed. The filtrate collected before was added to this combination. Glycerine (100 mL) was added after the mixture was thoroughly mixed.

2. Eosin stain

About 2 grams of eosin were dissolved in 70% alcohol and 1000 mL of 0.9 percent saline was prepared. This stock solution was diluted with an equivalent amount of 70% alcohol and 2-3 drops of acetic acid.

3. Mayer's Egg Albumin

An equal amount of egg white and glycerine were combined well. A pinch of thymol crystals, which function as a preservative, was put to it.

Procedure

- The cervical dislocation was used to kill the Mice.
- The mice brains were dissected out and post-fixed with 10% formalin for 24 hours after being transcardially perfused with 0.9 percent physiological saline and then infused with 10% formalin.
- The tissue was dehydrated with an alcohol series of increasing concentrations before being cleaned with xylene, a clearing agent.
- After that, the tissue was encased in paraffin wax and cut into blocks.
- There were two micron-thick pieces created.
- Sections were placed on a glass slide with Mayer's egg albumin adhesive andincubated for one hour at 60 degrees Celsius.
- The pieces were then rehydrated by passing them through a graded alcohol series ranging from higher to lower concentrations for 3 minutes.
- After washing the rehydrated sections under running water, the sections were dipped in hematoxylin stain and the staining intensity was evaluated after 1 minute.
- After washing the parts under running water for 2-3 minutes, they were placed incounterstain eosin for 3 minutes.
- The slides were dehydrated by soaking them in a succession of alcohol solutionsranging from low to high concentrations before being put in xylene.
- The stained sections were then mounted in a DPX mountant with a cover slip for microscopic analysis.

Analysis

All of the groups' histology preparations were photographed and compared.

RESULTS

1. Extract and extractive value

The yield obtained was about 10%. i.e. from 500, gm *Limonia acidissima leaves* 50 gms of the extract was obtained. It is blackish-green in color.

2. Phytochemical Analysis

Table 1 shows the preliminary phytochemical analysis of methanol extract of *Limonia acidissima* leaves.

Table 1: Preliminary Phytochemical Analysis.

Sl. No.	Phytoconstituents	Aqueous
1	Alkaloid	-
2	Glycosides	+
2	Flavonoid	+
3	Triterpenoid	-
4	Phytosterol	+
5	Phenolic compound &Tannin	+
6	Saponin	+
7	Free anthraquinone	-
8	Coumarin	+
9	Carbohydrate	+
10	Protein/Amino acid	+
11	Lipid &Fat	+

(+) means present and (-) means absent.

3. Acute Toxicity and Dose Determination

The OECD guideline No. 423 was used to conduct the acute oral toxicity test. Before medication treatment, Wistar mice have fasted overnight. Three animals were

employed, each receiving a single oral dosage of methanol extracts of *Limonia acidissima* leaves in the amount of 2000 mg/kg body weight. The animals were monitored for changes in behavior, hypersensitive responses, and other factors over 24 hours. Over two weeks, any mortality was determined. As a result, the dose chosen was 250 mg/kg of body weight.

4. Behavioural studies

T-maze performance

Table 2: effect of *limonia acidissima* on t-maze performance ondementia-induced *mice* (N=6).

Sl. No		T-maze performance		
		1st day	8th day	15th day
1	Group I (Normal control)	72.13± 4.91	74.61 ± 2.92	76.40± 3.50
2	Group II (Scopolamine control)	36.41± 4.96**	27.01± 4.12	22.73± 4.54
3	Group III (*Limonia acidissima* control)	73.55± 3.36*	76.81± 3.34*	81.55± 2.88*
4	Group IV (*Limonia acidissima*+ Scopolamine)	70.42± 5.16**	72.05± 3.11**	74.55± 4.06**
5	Group V (Donepezil control)	76.24± 1.70	78.84± 3.76	84.67 ± 3.15
6	Group VI (Donepezil + Scopolamine)	70.72± 2.43*	73.10 ± 3.41*	76.73± 3.54*

The result is given as (mean sd, n=6). * Significant at a 5% level of significance; ** Significant at a 1% level of significance.

Table 2 compares the percentage performance of different groups in the T- labyrinth on the first, eighth, and fifteenth days. The *Limonia acidissima* control group demonstrated a gain in performance throughout the trial period, as shown in Figure 1, and the difference in their averages was statistically significant at the 5% level (p0.05). The scopolamine-treated group demonstrated a deterioration in memory function from the first to the fifteenth day, which is a common symptom of dementia. The normal medicine donepezil +scopolamine group and the Limonia acidissima+scopolamine group both demonstrated a substantial performance improvement, with the difference in their means statistically significant at the 5% level (p0.05). When compared to the *Limonia acidissima* treated group, the regular medicine donepezil produced a superior effect.

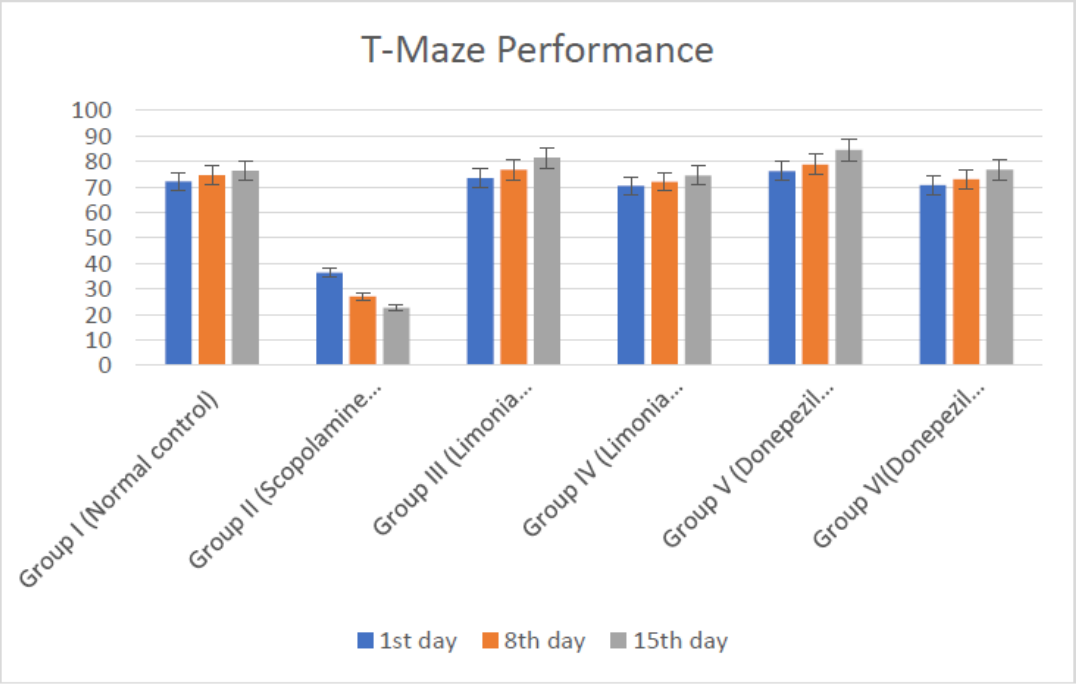


Figure 1: Effect of *limonia acidissima* on t-maze performance ondementia induced *mice* in different groups.

Elevated maze performance

The impact of the test, standard, and scopolamine treated groups were evaluated at the top of the 14th day. TL was recorded. It was seen that TL for all the treated groups was less on the 15th day as compared to the 14th day. Decrease IR indicates the induction of a state of mind, and exaggerated IR indicates an improvement in psychological features and memory impairment. Scopolamine-treated group animals were considerably shrunken compared with all the groups that indicated that state of mind is evoked. When compared to the scopolamine- treated group, the extract-only group exhibited a substantial increase.

Table 3: Effect Of Effect Of Methanolic Leaves Extract Of *Limonia Acidissima* On Ir And Tl.

Groups	IR	TL Day: 14	TL Day: 15
Group I (Normal control)	0.30	69.33±0.76	53±0.73
Group II (Scopolamine control)	0.28	104±2.42**	80.83±0.79**
Group III (*Limonia acidissima* control)	0.48	62±0.93**	41.83±0.60**
Group IV (*Limonia acidissima*+Scopolamine)	0.92	66.2 ± 6.2*	34.4 ± 5.4*
Group V (Donepezil control)	0.86	60.6 ± 3.8*	32.6 ± 4.6*
Group VI (Donepezil + Scopolamine)	0.60	61.6 ± 6.3*	38.4 ± 3.9*

The mean and standard error of the mean of n=7 mice/treatment is used to calculate thevalues. *p<0.05, **p≤0.01. TL: IR: Inflexion ratio, transfer delay.

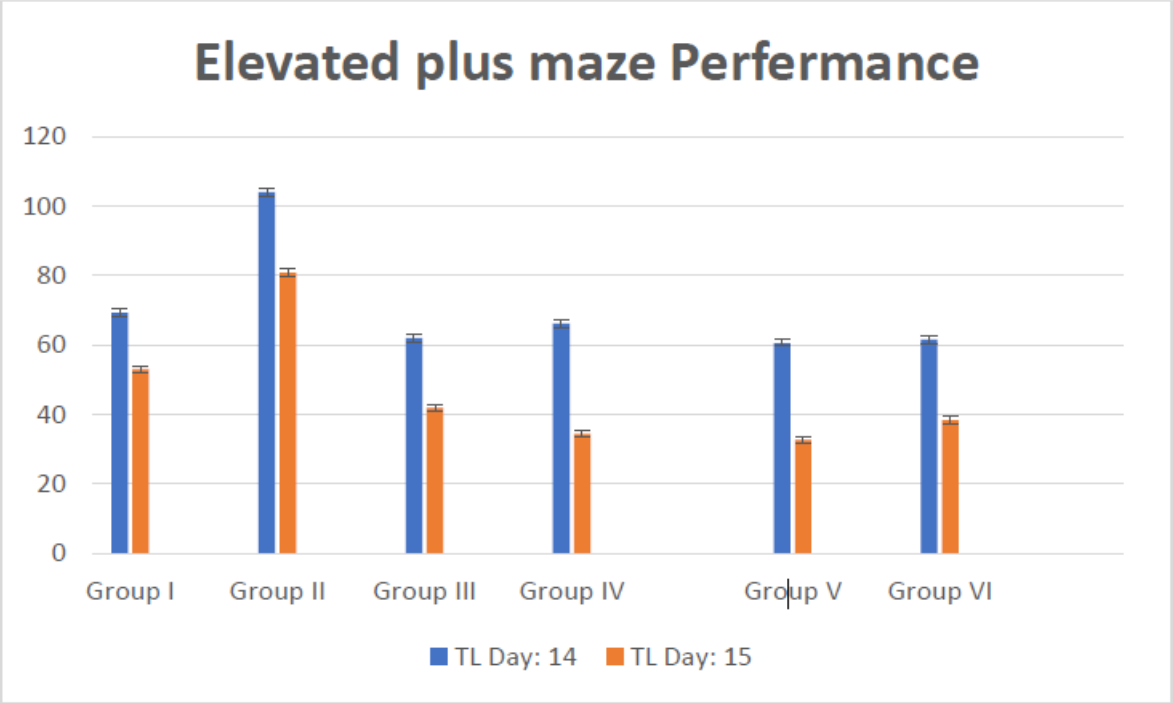


Figure 2: Effect Of Effect Of Methanolic Leaves Extract of *Limonia Acidissima* on And Tl.

Biochemical estimation

1. Effect of *limonia acidissima* on brain ache, sod, gst, andmda levels

Table 4: effect of *limonia acidissima* on brain ache, sod, gst, andmda levels in dementia-induced *mice* in different groups (N=6).

Sl. No	Treatment	Biochemical parameter			
		AChE	SOD	GST	MDA
1	Group I (Normal control)	124± 41.59	101± 10.23	15.59 ± 4.24	12.3836 ±0.061
2	Group II (Scopolaminecontrol)	194±44.12**	87.7± 13.4**	12.51±3.94**	10.4474 ±0.068**
3	Group III (*Limonia acidissima* control)	123±22.18**	136±25.96**	21.64 ±2.16**	12.3515 ±0.039**
4	Group IV (*Limonia acidissima*+ Scopolamine)	141±26.78**	111±24.83**	19.89±1.75**	12.3718±0.0522**
5	Group V (Donepezil control)	86.4 ± 18.24	159± 5.008	23.16 ± 1.32	11.2591 ±0.026
6	Group VI (Donepezil + Scopolamine)	127±41.97**	120± 26.53	21.94±3.78**	12.3502±0.0708**

The result is expressed in (mean ±SD, n=6). **Significant at 1 % level.

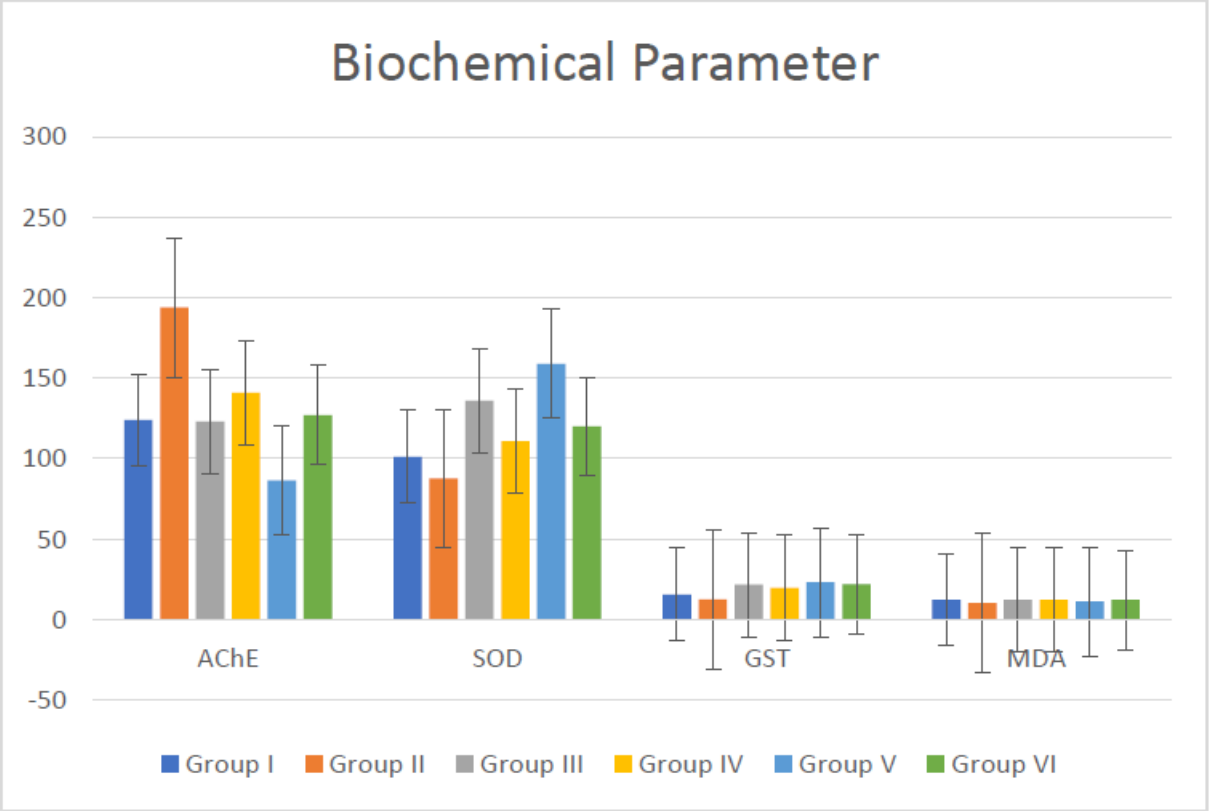


Figure 3: *Limonia acidissima* affects the levels of AChE, SOD, GST, and MDA in thebrain.

The levels of AChE, SOD, GST, and MDA in the brain are compared in Table 3. When scopolamine therapy was compared to a normal control group, AChE activity in the total brain was shown to be significantly higher (p0.01) following scopolamine treatment. The conventional medicine donepezil and the *Limonia acidissima* control groups both reduced brain AChE activity. The Limonia acidissima+ scopolamine group and the normal medicine donepezil + scopolamine group both reduced brain AChE activity, with the difference being statistically significant at the 1% level (p0.01). It is used as a marker for the suppression of AChE activity in the rat brain following 15 days of *Limonia acidissima* therapy. When compared to the *Limonia* -treated group, the usual medicine donepezil produced a superior effect.

The MDA activity of the total brain was significantly elevated (p0.01) following scopolamine therapy as compared to the normal control group, as shown in figure 3. The conventional medicine donepezil and the *Limonia acidissima* control groups both reduced MDA activity in the brain. The Limonia acidissima+ scopolamine group and the usual medicine donepezil + scopolamine group both had a 1 percent reduction in brain MDA activity (p0.01). When compared to the *Limonia acidissima* treated group, the regular medicine donepezil produceda superior effect.

Figure 10 shows that the antioxidant activity enzyme SOD levels were lower in scopolamine-treated groups as compared to the normal control group, and this difference was significant atthe 1% level (p0.01). When compared to the control scopolamine-treated group, *Limonia acidissima* reduced the loss of activity of this antioxidant enzyme, and the difference was significant at the 1% level (p0.01). When compared to the Limonia acidissima+ scopolamine group, the normal medicine donepezil + scopolamine group had a superior outcome, which was significant at the 1% level (p0.01).

In figure 5.2, the antioxidant activity of enzyme GST levels in scopolamine-treated groups was found to be lower than in the normal control group, and this difference was significant at the 1% level (p0.01). When compared to the scopolamine control group, *Limonia acidissima* reduced the loss of activity of this antioxidant enzyme, and the difference was significant at the 1% level (p0.01). When comparing the donepezil + scopolamine treated group to the Limonia acidissima+ scopolamine treated group, the difference was determined to be significant at the 1% level (p0.01).

Histology

Hippocampus neuron histology revealed that the normal control group had a row of normal hippocampal cells (LA1, LA2 andLA3). However, there were visible white patches or vacuolation around the neuronal cells in the scopolamine-induced dementia group. When Limonia acidissima and Donepezil control groups were compared to their scopolamine- treated counterparts, they exhibited virtually normal hippocampus cells (Figure 13-16).

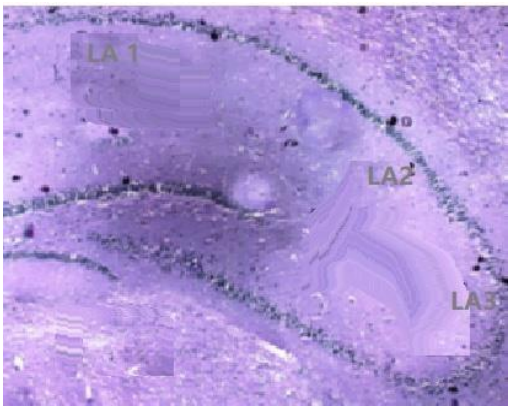
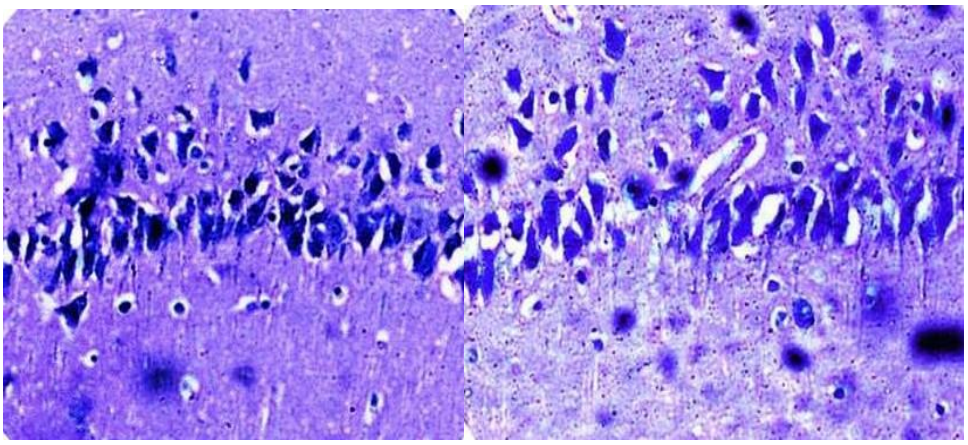
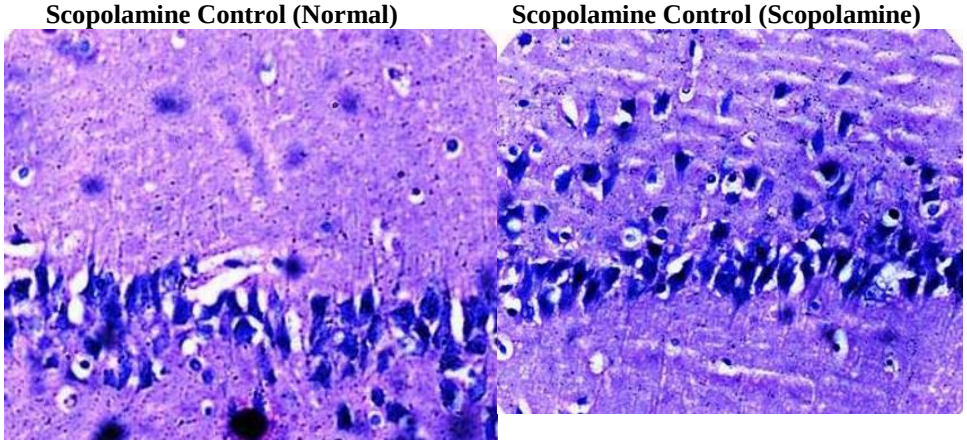


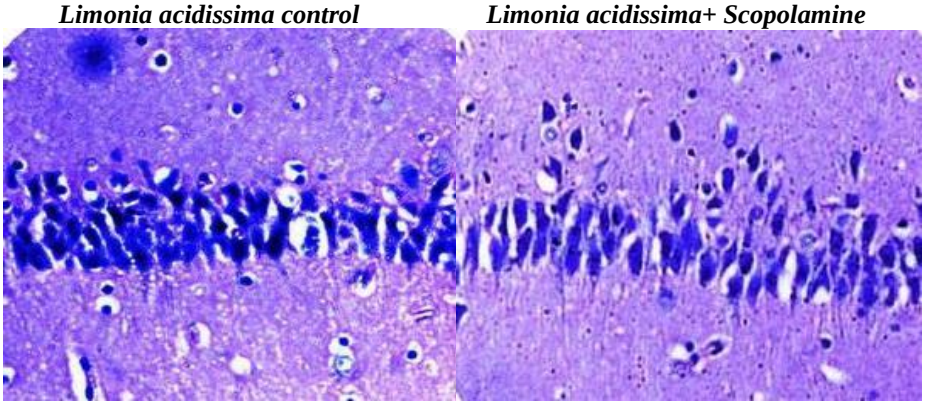
Figure 4: Photomicrograph of rat hippocampus cross slice with hematoxylin and eosin staining revealing la1, la2, and la3areas (4x).



Scopolamine Control (Normal) **Scopolamine Control (Scopolamine)**

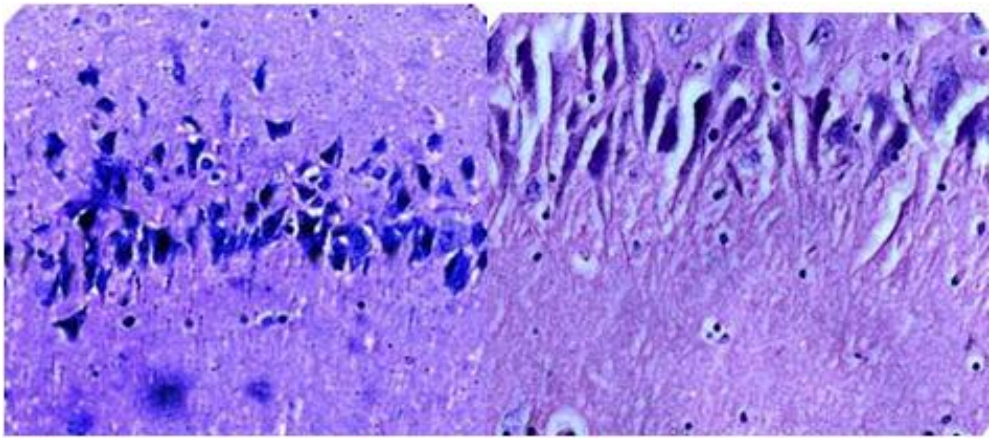


Limonia acidissima control ***Limonia acidissima+ Scopolamine***

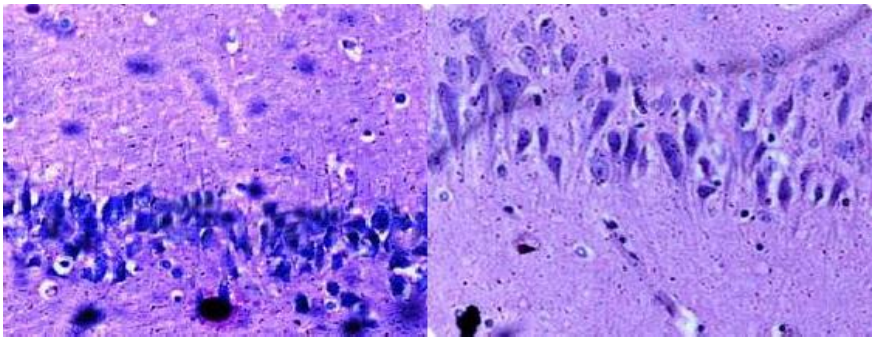


Donepezil control **Donepezil + Scopolamine**

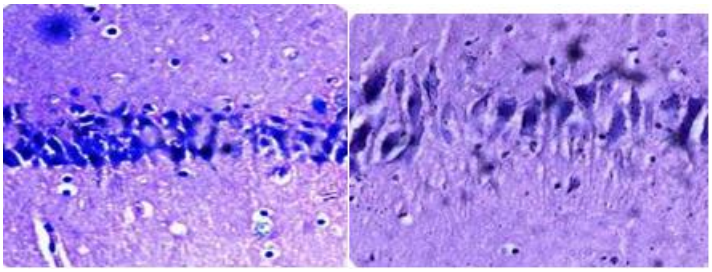
Figure 5: photomicrograph of the cross-section of differentgroups –la1 region of the hippocampus (20x).



Normal Control **Scopolamine Control**

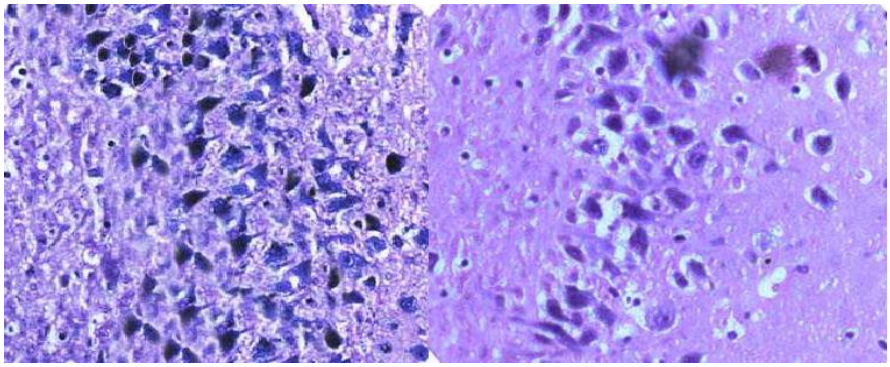


Limonia acidissima control ***Limonia acidissima+ Scopolamine***

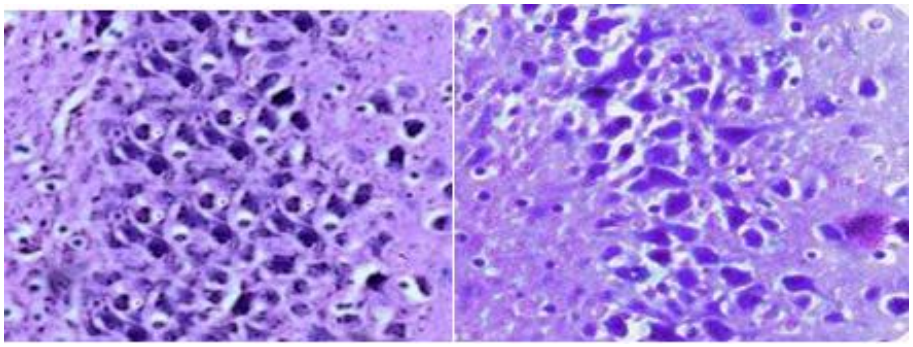


Donepezil control **Donepezil + Scopolamine**

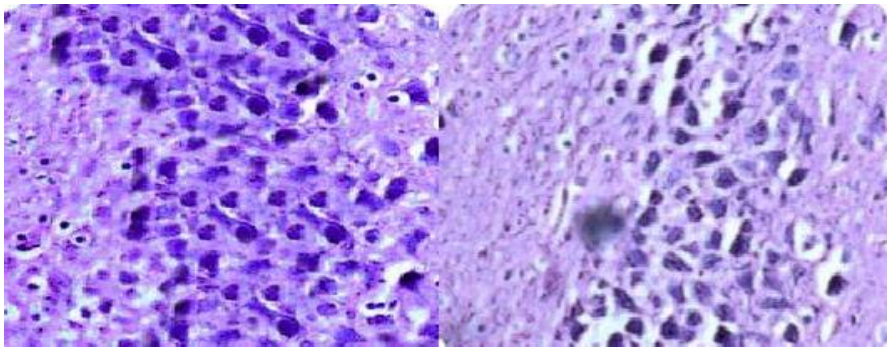
Figure 6: Photomicrograph of the cross-section of differentgroups – la2 region of the hippocampus (20x).



Normal Control **Scopolamine Control**



Limonia acidissima control *Limonia acidissima+ Scopolamine*



Donepezil control **Donepezil + Scopolamine**

Figure 7: Photomicrograph of the cross-section of different groups – LA3 regions of thehippocampus (20X).

LA1, LA2, and LA3 are three regions of the hippocampus named by the name of thetested plant.

DISCUSSION

Memory formation is a multi-step process that involves several neural circuits and chemicals. The cholinergic neural system is widely recognized for its function in memory in humans and animals[22-24]. Dementia is a neurological condition that begins slowly but eventually leads to memory loss[25-26]. Many attempts have been attempted to repair cognitive impairments by enhancing brain cholinergic activity with acetylcholinesterase inhibitors[27], based on the cholinergic theory. Despite the severity and widespread incidence of this disease, the allopathic medical establishment has failed to provide an acceptable antidote.

As a result, the current work focuses on investigating Limonia acidissima's memory- enhancing potential in a scopolamine-induced dementia paradigm in mice.

The scopolamine-induced dementia test in mice is a well-known animal model of memory impairment that is frequently used as a key screening test for anti-Alzheimer drugs.[28-32] Memory impairment in a scopolamine-induced animal model has been linked to an increase in oxidative stress in the brain.[33-35] When compared to young people, scopolamine produces cognitive impairment in healthy elderly subjects.[36]

Treatment with muscarinic receptor agonists and AChE inhibitors improves cognitive impairments in Alzheimer's disease through increasing cholinergic neuron transmission.[37]

1. T-Maze Test

Figure 1 depicts a percentage comparison of memory performance in the T-maze for several animal groups. The performance of the normal mice improved from the first to the fifteenth day of the trial. The performance of normal mice given Limoniaacidissima was superior to that of the normal control group throughout the trial. Scopolamine-induced memory impairment was seen in the groups. Scopolamine, according to review research, is capable of generating a variety of behavioral alterations in a variety of animal species,[38-42] indicating that it can impair performance in learning and memory tests.[43-44] Scopolamine-induced cognitive deterioration in mice has been linked to altered brain oxidative stress state, cholinergic dysfunction, and memory impairment.[45] There was a significant enhancement in memory in the scopolamine-induced group that was treated with Limonia acidissima. Dementia may be connected to a poor diet, which can be mitigated by eating a high- antioxidant diet. The current finding suggests that the Limonia acidissima has a nootropic effect and, as a result of its free radical scavenging activity, may protect against debilitating disorders such as dementia.

2. Elevated Maze Performance

Scopolamine significantly decreased the spontaneous iteration behavior compared with a control group. However, this decreased spontaneous alteration behavior induced by scopolamine was significantly inhibited by

the plant extract and standard drug.

3. Acetylcholinesterase

Scopolamine inhibition of muscarinic cholinergic receptors inhibits the encoding of new memories but not the recall of previously acquired memories, according to pharmacological investigations in human subjects.[46-47] Drugs that stimulate nicotinic receptors, on the other hand, increase the encoding of new information.[48-49] The cholinergic neural system is widely established for its role in cognitive impairments linked to dementia, aging, and neurodegenerative disorders.[50] Estimating acetylcholinesterase activity provides a quick and simple way to understand cholinergic function.[51]

The AChE activity of the total brain was found to be significantly elevated following scopolamine therapy, as shown in figure 3. Scopolamine is a muscarinic receptor antagonist that inhibits central cholinergic neuronal activity,[52,53] affects learning and short-term memory, and lowers the amount of acetylcholine in the hippocampum.[54] In the rat brain, it affects the expression of a wide range of genes involved in muscarinic receptor signaling pathways, apoptosis, cytoskeleton rebuilding, protein trafficking, and cell differentiation.There is a considerable change in the level of AChE in the *Limonia acidissima* control group. *Limonia acidissima* is said to have anti-inflammatory, antioxidant, and antipathogenic properties. Because the action of *Limonia acidissima* is on several target areas, it plays a critical function in memory retention. Scopolamine given in combination with *Limonia acidissima* for 15 days resulted in a considerable drop in brain acetylcholinesterase activity, presumably enabling cholinergic transmission and increasing the animals' memory.

The current study indicates the possible mechanism by which Limonia acidissima(250mg/kg per os) lowered brain acetylcholinesterase activity by improving learning and memory performance. It was showing that these samples had stimulating effects on the cholinergic system. As a result, the anticholinesterase activity of compounds can be ascribed to their memory-enhancing impact.

4. Enzyme Status

Antioxidant enzymes aid in the improvement of alertness, memory, and general mental function.[55-56] By preventing the generation of free radicals and lipid peroxidation,[57] it protects brain cells from harm. Oxygen-free radicals are implicated in the age-related deterioration in cognitive function and may be to blame for the onset of dementia-likesymptoms in the elderly.[58-59] Because of its high oxygen consumption and high amounts of polyunsaturated fatty acids.[60] the brain is particularly vulnerable to oxidative stress. Oxidative stress is linked to damage to a broad spectrum of molecular species.[61-62] It is caused by an imbalance between free radical generation and antioxidant defenses.

5. Malondialdehyde

Many aldehyde products are produced by lipid peroxidation in living systems, with malondialdehyde being the most important166. Malondialdehyde is a useful biomarker of the degree of lipid peroxidation.[63] since it is one of the end-products of polyunsaturated fatty acid peroxidation. Increased oxidative stress has been linked to scopolamine-induced memory impairment in several studies. Malondialdehyde (MDA) is a biomarker for oxidative stress, and a higher MDA level causes more free radicals to be produced.[64-65] which may damage biomolecules. This research found that MDA levels rose dramatically in the brains of scopolamine-treated mice, but that the rise was moderated after pre-treatment with *Limonia acidissima* (figure 2). Scopolamine administration was linked to a change in brain antioxidant status.[66] but *Limonia acidissima* pre-treatment dramatically reduced this action. *Limonia acidissima* leaves are an excellent source of proteins, minerals, vitamin A, vitamin B complex, essential amino acids, and vitamin E.[67] These chemicals have anti-oxidant and memory-enhancing properties,[68] according to research. In the scopolamine-induced group, *Limonia acidissima* administration decreased MDA levels. *Limonia acidissima* leaves' antioxidant properties may contribute to MDA levels in the brain being reduced.

The *Limonia acidissima's* and donepezil-treated groups performed nearly as well as thedonepezil-treated groups , indicating *Limonia acidissima's* therapeutic effectiveness against oxidative stress.

6. Superoxide Dismutase

Superoxide dismutase is an enzyme that helps cells break down potentially damaging oxygen molecules, possibly preventing tissue damage.[69-70] SOD levels in the brains of *Limonia acidissima's* -treated mice rose considerably as compared to the control group, according to this research. SOD activity was considerably decreased when scopolamine was given alone, suggesting oxidative stress (figure 3). *Limonia acidissima* and scopolamine co-administration reduced the degree of oxidative stress in this group via increasing SOD activity. The superoxide toxicity was reduced and SOD levels were dramatically enhanced when *Limonia acidissima* was administered, suggesting that the plant interacts with reactive oxygen species or boosts antioxidant enzyme synthesis. SOD's catalytic elimination of reactive oxygen species is critical because it prevents a variety of lipid peroxidation by products, protein adduction, and organ dysfunction. *Limonia acidissima* extracts include a lot of phenolic compounds and flavonoids,[71-72] which are known to efficiently scavenge free radicals, according to their plant metabolite profiles. Its greater antioxidant activity may be responsiblefor these benefits.

Superoxide dismutase is a metalloprotein that is engaged in antioxidant defense by reducing the steady-state

oxygen level. SOD is a naturally occurring enzyme that converts superoxide radicals to H2O2,[73] and is extensively distributed to protect cells from the harmful effects of the superoxide anion. When compared to the *Limonia acidissima* -treated group, the conventional medicine donepezil produced a better outcome, which was also superior to the donepezil -treated group and the scopolamine-treated group.

7. Glutathione-S-Transferase

Limonia acidissima leaves contain several phytocompounds, vitamins, and carotenoids, which are primarily responsible for the antioxidant qualities and biological activities of the plant. The antioxidant capabilities of phenolic compounds from *Limonia acidissima* have been linked to the capacity to scavenge free radical formation.[74] Glutathione-S-transferase and other antioxidant enzymes may be produced in response to certain phenolic compound.[75]

When scopolamine-treated groups were compared to normal control groups, the antioxidant activity of GST was considerably decreased (figure 3). When compared to the scopolamine- treated group, *Limonia acidissima* reduced the loss of antioxidant enzyme activity. Oxidative stress.[76] is linked to a substantial decrease in GST levels in scopolamine-treated groups. Oxidative stress causes a rise in the generation of reactive oxygen species and lipid peroxidation, and it is more prevalent in those with cognitive impairment.[77]

Antioxidant enzymes and oxidative free radical scavenging enzymes like glutathione are vital in reducing oxidative stress in the brain. GST has been shown to have an important function in shielding cells from oxidant-mediated damage in many investigations.

Catalyzes the breakdown of lipid hydroperoxides produced by oxidative damage to cellular lipidmolecules.[77-79]

The increased quantity of GST protects cellular proteins from oxidative stress caused by scopolamine exposure, as shown by the results. The occurrence of oxidative stress in a range of dementia-related illnesses is supported by a wide body of experimental data.[80-88]

Glutathione S-transferase is an important part of the body's defense systems against oxidative damage. When compared to the normal control group, the scopolamine-treated group saw a significant decrease in GST levels. This drop-in GST might be attributed to scopolamine-induced oxidative stress in the brain during the study.

8. Histopathology

Normal control rats had a row of normal hippocampal neurons, but scopolamine-induced amnesiac mice had shrunken hippocampus neurons with a white patch in the LA1, LA2, and LA3 areas (Figure 5, 6&7). Scopolamine is a nonselective muscarinic receptor antagonist that may cause a variety of behavioral alterations in a variety of animals. It may also decrease the number of neurons in hippocampal formation subregions. The histological alterations in hippocampal morphology were linked to changes in animal behavior in the scopolamine- induced amnesia paradigm. In the scopolamine control group, shrunken hippocampus neuronal cells were noticeable, however, this was improved in *Limonia acidissima*-treated Mice.

CONCLUSION

In summary. The histological alterations in hippocampus morphology coincided with the change in behavior of mice in a scopolamine-induced amnesia scenario. Scopolamine therapyresulted in vacuolation surrounding the neurons, and the morphology of neurons in the LA1, LA2, and LA3 areas shrank, however, this improved with *Limonia acidissima* administration.

The current research shows that an aqueous preparation of *Limonia acidissima* may help mice with cognitive impairment and oxidative stress by preventing lipid peroxidation, increasing endogenous antioxidant enzymes, and lowering acetylcholinesterase activity. Given the above, it could be useful to investigate the plant's potential for treating cognitive impairment.

Further Investigations

Further investigations are to be continued to explore the actual compound responsible for this effect and possible involvement of other neurotransmitters such as glutamate, Gamma- aminobutyric acid(GABA), and catecholamines for the memory improving property of *Limonia acidissima* at a molecular level.

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