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Ameliorative Effect of Halo-Substituted Chromones on Scopolamine-Induced Memory Impairment in Mice

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Abstract

Alzheimer's disease is a neurodegenerative disorder caused by the progressive, recurrent, and irreversible loss of neuronal cells from certain areas of the brain. Flavonoid contents have differential efficacy against age-related toxic pathways within neurodegenerative disorders. Nine different halo-substituted chromones were synthesized from chalcones and these, 2-chloroflavone (C1-structurally 2-(2-chlorophenyl)-4H-benzopyran-4-one) were selected as more effective according to *in vitro* acetylcholinesterase activity. Our study also found that 2000mg/kg b.w of 2-chloroflavone was the maximum tolerated dose for mice by acute oral toxicity study. 2-chloroflavone produced the most promising learning and memory activity among scopolamine-treated amnesic mice when compared with the toxic control group. According to histopathological analysis, 2-chloroflavone protected the hippocampus from neuronal degeneration and the development of neuritic plaques in a dose-dependent way. The current study also demonstrated that 2chloroflavone at 200 and 400 mg/kg reduced oxidative stress by restoring catalase, superoxide dismutase, reduced glutathione, malondialdehyde, and glutathione peroxidase levels in the brain of mice treated with scopolamine. These results suggest that halo-substituted chromones offered protection against neuronal damage induced by scopolamine and have the potential to be developed into new drugs for the treatment of Alzheimer's disease.

Keywords: - Alzheimer's disease, Flavanoids, Halo-substituted chromone, 2-chloroflavone, Scopolamine induced memory impairment

INTRODUCTION

Dr. Alois Alzheimer, a German neurologist, and psychiatrist wrote about the case of Auguste D, a 51-year-old woman, in 1906. Normal physiological functions were changed due to progressive variations in the neuronal molecular environment and neurodegeneration [1]. The repeated and irreversible loss of neurons from particular brain regions is what causes the neurodegenerative disorder to progress[2]. It affects mainly mental performance, particularly memory processing. The main symptom of AD is dementia, which can be defined as an organic brain disease characterized by the depletion of intellectual abilities that obstruct social or occupational functioning [3, 4].

The majority of the elderly population is affected by AD, a chronic, progressive neurodegenerative disorder that is characterized by the disruption of numerous cortical functions, including loss of memory and cognition. [5-7]. Uncertainty surrounds AD's main cause. The main pathogenesis of AD is thought to involve beta-amyloid and tau protein aggregation, glutamatergic and calcium homeostasis, inflammatory hypothesis, metal ion hypothesis, oxidative stress, and reduced acetylcholine biosynthesis. Recent studies state that the inflammatory lesions and free radicals produced by oxidative stress are the other important aspects of the causes of AD [8].

Flavonoids are polyphenolic plant secondary metabolites from aromatic amino acids phenylalanine or tyrosine or malonate [9]. They are in charge of the primary organoleptic properties of plant-derived foods and beverages, particularly color and taste, as well as the nutritional value of fruits and vegetables. They are also involved in the pigmentation of flowers. About 8000 varieties of flavonoids are identified and have low toxicity [10- 12]. Flavonoids have been linked to a wide range of health-promoting effects, and they are an essential component in a wide range of nutraceutical, pharmaceutical, medicinal, and cosmetic applications. [13, 14]. Flavonoids are incorporated into the diet through fruits and vegetables, as well as herbal teas, due to their abundance in nature and safety. Flavonoids were used as main components in the preparation of herbal cosmetics, nutraceutical products, and medicinal and other toiletry preparations, owing to their preventive potential. There is sufficient evidence for flavonoids, which include flavones, flavonols, flavanones, isoflavones, and anthocyanins, to be effective at cellular levels for neuronal protection, inflammation inhibition, antioxidant properties, and inhibition of tumor-promoting enzymes. [15]. As a result, the current study focused on the ability of halo-substituted chromone to prevent scopolamine-induced AD in experimental mice as well as to demonstrate anticholinesterase activity in vitro. (Figure 1)

MATERIALS AND METHODS

Reagents and Chemicals:

2-chloro benzaldehyde, 3-chloro benzaldehyde, 4-chloro benzaldehyde, 2fluro benzaldehyde, 3-Fluro benzaldehyde, 4-Fluro benzaldehyde, 2-Bromo benzaldehyde, 3-Bromo benzaldehyde, 4-Bromo benzaldehyde, 2 hydroxy acetophenone, tris-hydrochloric acid buffer, folin phenol reagent, Acetylcholinesterase, 5,5'-dithiobis-2-nitro benzoic acid, ketamine, xylazine, and acetylthiocholine iodide were purchased from Sigma Aldrich, USA. Carboxymethyl cellulose, Sodium thiosulphate, Sodium hydroxide, hydrochloric acid, dimethylsulfoxide (DMSO), sodium potassium tartrate sodium carbonate, copper sulfate hydrogen peroxide, potassium dichromate, iodine crystals, glacial acetic acid, sodium citrate, phenol, cholesterol oxidase, cholesterol esterase, peroxidase, 4-amino antipyrine, glucose oxidase, anhydrous glucose, phosphate buffer, sodium azide, ethylene diamine tetra acetic acid (EDTA), hydrogen

peroxide, reduced glutathione, glutathione reductase enzyme, dithiothreitol, glutathione peroxidase, β-nicotinamide adenine dinucleotide phosphate, reduced nicotinamide adenine dinucleotide phosphate (NADPH), sodium dihydrogen phosphate and sodium monohydrogen phosphate were purchased from HiMedia Laboratories, Mumbai.

Normal saline was purchased from Baxter (India) Pvt. Limited, Tamil Nadu.

Scopolamine was obtained from Omega Laboratories Ltd. Coimbatore. Donepezil was obtained from Tanmed Pharmaceuticals, Chennai, India as a complimentary sample.

All other substances, including solvents, were of AR grade.

Animal Procurement and Ethical Clearance

The entire study protocol was approved by the Institutional Animal Ethics Committee (GCP/IAEC/21(1) P2) which was certified by the Committee for Control and Supervision of Experiments on Animals (CPCSEA). Sixty Swiss albino mice (54 male and 6 female) (25-30 g) were procured from the College of Veterinary and Animal Sciences, Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala, India.

Housing and feeding conditions

The animals were kept in clean and dry polycarbonate cages in a well-ventilated animal house with a 12-hour light and 12-hour dark cycle. The animals were acclimatized to the laboratory conditions for at least 5 days before the test and they are randomized and assigned to treatment groups. Studies were performed in a controlled environment under ambient temperature (22±3ºC) with relative humidity between 40-60% and they were made available with a normal diet and water *ad libitum*.

Synthesis of Flavonoids

Step I: Synthesis of chalcones

Chalcones were synthesized by base-catalyzed Claisen–Schmidt condensation reaction of substituted acetophenones and substituted benzaldehyde. A mixture of equimolar quantities of substituted benzaldehyde (2-chloro, 3-chloro, 4-chloro, 2-fluro, 3-fluro, 4-fluro, 2-Bromo, 3-Bromo, and 4-Bromo) and substituted acetophenone (2-hydroxy acetophenone) were dissolved in 10 ml rectified spirit in a 250 ml round bottom flask equipped with a mechanical stirrer and then 10 ml of sodium hydroxide (1 g in 10 ml water) was added dropwise to the reaction mixture. On vigorous stirring for 30 minutes the solution became turbid. The reaction temperature was maintained between 20-25°C with a cold water bath after 2-3 hours of vigorous stirring, 0.2 N HCl was added to the reaction mixture to neutralize it and encourage precipitation. Filtering the final product allowed the crude chalcones to dry in the air and be recrystallized using rectified spirit. [16].

Step II: Synthesis of flavonoids

Synthesized chalcones (0.01mol) were suspended in DMSO (10 ml) and crystals of iodine were added to it. After being refluxed for 30 to 40 minutes, the mixture was cooled to room temperature. Ice-cold water was used to dilute the reaction mixture, and the resulting precipitate was filtered and washed with 20% sodium thiosulfate. The crude product obtained was recrystallized by using ethanol [17-19].

***In Vivo* Study**

Acute oral toxicity study

By performing *in vitro* acetylcholinesterase inhibitory activity for all nine synthesized products, 2-chloroflavone (C1) (structurally 2-(2-chlorophenyl)-4H-benzopyran-4-one) was selected as a more potent active molecule and it was selected for further studies. An acute oral toxicity study was performed following the acute toxic class method employing the OECD guideline No. 423 (OECD 2001). It is a step-wise procedure. Three healthy young adult female Swiss albino mice were used for each step of the toxicity study. Females were nulliparous and non-pregnant, 8–12 weeks old, and weighed within the mean ± 20% range. The animals fasted for 3–4 hours before the test but had free access to water throughout the period. Following the fasting period, the animals were given the test sample orally at a dose of 2000 mg/kg body weight. Because the majority of the test sample has an LD50 value greater than 2000 mg/kg body weight, this was used as the starting dose.

Animals were observed immediately after dosing at least once during the first 30 minutes, and then every 24 hours for the next 14 days, with special attention paid during the first 2 to 6 hours of treatment and daily thereafter. Observations were including changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic, and central nervous systems, and somatomotor activity and behavior patterns. Attention will be directed to observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma. The study was repeated with the same dose to confirm the findings because there was no death as required by the guidelines.

Scopolamine-induced learning impairment

Learning impairment induction with scopolamine administration and CNS activity testing was carried out following the procedure explained by Hemant D Une *et.al*., (2014) Touqeer Ahmed, and Anwarul-Hassan Gilani., (2009) with slight modifications. In this study, thirty Swiss albino mice were chosen at random and grouped into five, each containing six animals. The training sessions were used to train the animals in the normal group. Learning impairment was induced among the second group of animals with single dose i.p. administration of scopolamine (0.4 mg/kg). These animals were also trained after 45 min of scopolamine administration. Animals in the standard group were given Donepezil (5 mg/kg) for seven days in a row, then a single dose of scopolamine was given i.p. on the seventh day, and learning and memory activities were performed 45 minutes later. Similarly, animals in the fourth and fifth groups were treated with low and high doses of test sample C1 orally for seven days in a row, and on the seventh day, learning and memory activity was performed after 45 minutes of the single dose i.p. injection of scopolamine.

Learning and Memory Activity Study

Step-down method

The Step-down apparatus consist of a box (27 cm x 27cm x 27cm) having three walls of wood and one wall of

plexiglass, featuring a grid floor (made up of 3 mm stainless steel rods set at 8 mm apart), was connected to a shock device which delivers scrambled foot shock, with the wooden platform (10cm x 7cm x 1.7cm) in the center of the grid floor. The box is illuminated with a 15W bulb during the experimental period. Electric shock (20V, AC) was delivered to the grid floor [23].

Elevated plus maze

The Elevated plus maze apparatus consists of two open arms (16cm x 5cm) and two closed arms (16cm x 5cm x 12cm). The arms extended from the central platform (5 cm x 5 cm), and the maze was raised to a height of 25cm off the ground. Each mouse was placed at the end of the open arm, facing away from a central platform, on the first day. All animals' transfer latency (TL) was measured on the first day. The mouse was given another 2 minutes to explore the maze before being returned to its home cage (i.e., on the seventh day). This learned task's retention was tested 24 hours after the first test (i.e., on the eighth day) [23].

CNS Toxicity Study

Locomotor activity

Most CNS medications affect movement. An actophotometer, which operates on photoelectric cells connected in a circuit with a counter, can easily measure a drug's locomotor activity. When the animal cut off the light beam falling on the photocell, a count was recorded. Individual animals were placed in the activity cage for 10 minutes and their activity was recorded. All of the animals' basal activity levels were recorded. The test was done 30 min before and after the drug administration on every alternative day. The photocell count was recorded and variation in locomotor activity was calculated [25].

Dissection, Collection of Blood and Brain Samples

Animals were euthanized with a xylazine and ketamine combination on the 9th day, and The animals were slaughtered. The blood was drawn. Each animal's brain was carefully removed from the skull and placed in ice-cold saline. The blood was centrifuged at 3000 rpm for 15 minutes to separate the serum for total cholesterol and glucose levels estimation. One-half of each brain was fixed in formalin buffer (10%) for histological investigation and the second half was weighed and transferred to a glass homogenizer and homogenized in an ice bath after adding 10 volumes of 0.9% sodium chloride solution. The homogenate was centrifuged at 3000rpm for 10 minutes and the resultant cloudy supernatant liquid was used for estimation of protein, brain acetylcholinesterase (AchE) activity, malondialdehyde (MDA), reduced glutathione (GSH), Super Oxide Dismutase (SOD), glutathione peroxidase and Catalase levels [26].

Biochemical Estimations

Estimation of Total Cholesterol Level

P J Lumb and B M Slavin's CHOD-PAP method was used to calculating serum total cholesterol., (1993). Using a micropipette, the blank, standard, and test samples were pipetted into the respective reaction vessels. 20µl of distilled water and 1000µl of working reagent were combined to make the blank sample. The standard sample contained 20µl of standard cholesterol and 1000µl of working reagent, while the test sample contained 20µl of serum and 1000µl of working reagent. These mixtures were incubated at 37°C for 10 minutes. Using an Auto analyzer, absorbance was measured at 510nm and 630nm (Filter 1 and Filter 2) against a blank sample (Erba Mannheim Chem-5 plusV2) [27].

Estimation of Blood Glucose Level

As done by Sharma et al., (2017) Using an auto analyzer, the GOD-POD method was used to estimate blood glucose levels. Using a micropipette, the blank, standard, and test samples were pipetted into the respective reaction vessels. 10µl of distilled water and 1000µl of working reagent were combined to make the blank sample. The standard sample contained 10µl of standard glucose and 1000µl of working reagent, while the test sample contained 10µl of serum and 1000µl of working reagent. These mixtures were incubated at 37°C for 15 minutes. Using an Auto-analyzer, the absorbance was measured at 510nm and 630nm (Filter 1 and Filter 2) against a blank sample (Erba Mannheim Chem-5V2) [28].

Estimation of brain acetylcholinesterase level

Ellman's method was used to determine acetylcholinesterase levels. A cuvette containing 2.6 ml of 0.1M phosphate buffer and 100 µl DTNB was filled with 0.4 ml of brain homogenate. The contents were thoroughly mixed, and the absorbance at 412nm was measured with a spectrophotometer. The basal reading was taken when the absorbance reached a stable value. The absorbance was measured for 10 minutes at 2-minute intervals after 100 µl of acetylthiocholine iodide was added. The change in absorbance was calculated [29].

Enzymatic activity was calculated using the formula:

$$\mathbf{R = 5.74(10^{-4})\ \Delta A/Co}$$

R = Rate in moles substrate hydrolyzed/min/gm of tissue

ΔA = Change in absorbance/min

Co = Original concentration of tissue (mg/ml)

Anti-oxidant activity of C1 on mice brain

Estimation of catalase (CAT)

Catalase activity was assayed by the method of Luck (1971). Through this method, the breakdown of hydrogen peroxides (H_2O_2) was measured at 240 nm. The assay mixture consisted of 3.0 ml of H_2O_2 phosphate buffer and 0.05 ml of supernatant of tissue homogenate (10%), and a change in absorbance was recorded at 240 nm with a UV spectrophotometer. The results were expressed as micromole H_2O_2 decomposed/mg protein /min [30].

Estimation of superoxide dismutase (SOD)

SOD was assessed by the method of *Kakkar* and *Vishwanathan* in1984. A single unit of the enzyme was expressed as 50% inhibition of nitro blue tetrazolium chloride (NBT) reduction/min/mg protein by superoxide and was measured at 560nm. In brief, a mixture (1.5 ml) containing 1.0 ml of 0.01M phosphate buffer, pH 7.0, 0.2 ml of tissue homogenate, and 0.4 ml of 2M H_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in a 1:3 ratio). The concentration of SOD was expressed as units/mg of protein [31].

Estimation of Brain-Reduced Glutathione (GSH)

Reduced glutathione was measured by the method of Ellman, in 1959. About 1 ml of supernatant was treated

with 0.5 ml of Ellman's reagent (19.8 mg of DTNB in 100 ml of 0.1% sodium citrate solution) and 3.0 ml of 0.2M phosphate buffer of pH 8.0. Absorbance was measured at 412nm. The activity of GSH was expressed as nmoles/min/mg protein [29].

Estimation of Brain Malondialdehyde (MDA)

Malondialdehyde is an indicator of lipid peroxidation and was determined as described by Ohkawa et al, with a small change. The reaction mixture contained 0.2ml of 8.1 % sodium lauryl sulfate, 1.5ml 0f 20% of acetic acid (pH-3.5), and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid was added to the 0.2 ml of processed brain homogenate. The mixture was made up to 4.0 ml with distilled water and heated at 95^0C for 60 minutes. After cooling with tap water, 5 ml of n-butanol and pyridine (15:1 v/v) and 1 ml of distilled water were added and centrifuged. The organic layer was separated and its absorbance was measured at 532 nm using a UV-Visible spectrophotometer and MDA content was expressed as nmole/mg of protein [32, 33].

Estimation of glutathione peroxidase

By monitoring the change in absorbance at 460 nm caused by O-dianisidine oxidation in the presence of H_2O_2 and enzyme, peroxidase activity will be assessed spectrophotometrically. The reaction mixture will be incubated at 37°C for 15 minutes with 0.2 ml of a 15 mM potassium phosphate buffer (pH 5.0) and 0.2 ml of hydrogen peroxide will be added to start the reaction, and after about 3–5 minutes at 37°C, the absorbance at 460 nm will be measured against a blank spectrophotometrically. The µmoles O-dianisidine oxidized at 37°C will be used as the unit of enzyme activity. [32, 33].

Histopathological Investigations

After 24 hours of brain tissue fixation with formalin, washing was done in tap water, and then serial dilutions of alcohol were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56^0C in a hot air oven for 24 hours. Paraffin beeswax tissue blocks were prepared for sectioning at 4µ thick by sludge microtome. The obtained tissue sections were collected on glass slides, de-paraffinized, and stained by hematoxylin and eosin (H&E) stain for histopathological investigation.

Statistical analysis

The results were statistically analyzed by one way-ANOVA followed by

Dunnett's test using GraphPad Prism software. All *in vitro* determinations were done in triplicate and *in vivo* studies (n=6) and the values are expressed as mean ± SEM. $P < 0.05$ was considered to be significant when contrasted with the placebo and the standard medication.

RESULT AND DISCUSSION

Synthesis of Flavonoids

The synthesis of chalcones was carried out by using the Claisen-Schmidt condensation method and various halo-substituted flavonoids were synthesized from chalcones. The yield percentage of all synthesized compounds ranged from 69 to 84%. MR-VIS visual melting range apparatus was used to determine the melting point. The R_f values of synthesized flavonoids ranged from 0.64 to 0.88 respectively by using solvent chloroform: water (9:1).

***In Vitro* Acetylcholinesterase Inhibitory Assay**

All of the synthesized halo-substituted flavonoid compounds (C1 to C9) and donepezil (standard drug) had their percentage inhibition determined. For the common medication donepezil, a dose-dependent rise in percentage inhibition was seen for compounds C1 to C9. Among the nine-halo substituted flavonoid compounds, C1 was highly inhibitory with a greater inhibition percentage. The order of potency of flavonoid compound in acetylcholinesterase inhibitory assay was C1 >C2 >C9 > C5 >C7 > C4 > C8 > C3 > C6. ***In Vivo* Studies**

Acute toxicity study of the test sample (C1)

An acute toxicity study was done with test sample C1 by using the Acute Toxic Class method of OECD (Guideline No: 423). After oral administration of test sample C1 to the experimental animals, they were observed for changes in skin, eyes, mucous membranes, and also respiratory, circulatory, autonomic, and central nervous systems, and somatomotor activity, behavior pattern mortality. Even at 2000 mg/Kg, there was no mortality seen in the test sample. There were no behavioral changes until the end of the observation period, and all the animals were found to be healthy. These findings showed that the test sample was safe up to a dose of 2000 mg/kg of body weight, considered the maximum tolerated dose (MTD). Thus, 1/10th and 1/5th of MTD were chosen from this, and the effective doses were set at 200 and 400 mg/kg for additional pharmacological research.

Learning and Memory Activity Study

Step-down Method

SDL of the seventh and eighth days of drug treatment showed an improvement in both learning and memory. On the seventh and eighth days, mice treated with C1 showed a dose-dependent increase in SDL, indicating significant improvement in both learning and memory (Table 1 and Figure 2).

Elevated plus maze method

C1 treatment resulted in a dose-dependent decrease in TL on the seventh and eighth days, indicating significant improvement in both learning and memory. (Table 2 and Figure 3).

CNS Toxicity Study

Locomotor activity

On the seventh and eighth days, mice treated with C1 showed a dose-dependent increase in locomotor activity, indicating a significant improvement in CNS activity. (Table 3 and Figure 4)

Biochemical Estimation

Estimation of Total Cholesterol Level

The experimental animals given C1 (200 mg/kg and 400 mg/kg) and donepezil hydrochloride (5mg/kg) orally for 7 days showed a significant reduction in total cholesterol levels when compared to the toxic control group, which had higher cholesterol levels than the normal control group. As a result, the test sample has a promising cholesterol-lowering property. (Table 4 and Figure 5)

Estimation of blood glucose level

The toxic control group showed a higher blood glucose level than the normal group. The animals that received the test drug, C1 (200mg/kg and 400mg/kg), and standard

drug, donepezil hydrochloride (5mg/kg) orally for 7 days showed a significant reduction in blood glucose level compared to the toxic control group (Table 4 and Figure 6).

Estimation of brain acetylcholinesterase level

C1-treated mice showed greater acetylcholinesterase levels in the brain.(Table 5 and Figure 7)

Anti-oxidant activity of C1 on mice brain

Detoxifying enzymes like glutathione peroxidase, superoxide dismutase, and catalase with antioxidant mechanisms are involved in the defense system against free radical injury. Glutathione can protect synapses from damage by peroxynitrite [36]. An imbalance between the formation of oxygen free radicals and the protective mechanisms has been proposed as a major factor for aging and Alzheimer's disease [37]. The present research depicts that C1 (200 mg/kg and 400 mg/kg) decreased oxidative stress by restoring catalase, SOD, GPx, and GSH levels in scopolamine-treated (Table 6).

Estimation of catalase (CAT):-

A significant (P<0.01) Catalase levels dropped in the toxic control group given scopolamine treatment(1.91±0.50 µmoles/min/mg protein) compared to the control group (4.22±0.051 µmoles/min/mg protein) which did not receive any treatment with test drugs. Administration of C1 (200 mg/kg) increased significantly (P<0.05) the catalase level to 2.89±1.04 µmoles/min/mg protein and with 400 mg/kg the level significantly (P<0.01) increased to 3.20±0.039 µmoles/min/mg protein in comparison to toxic control. Donepezil (5 mg/kg) treatment led to a significantly (P<0.01) elevated catalase level in the brain (3.12±0.072 moles/min/mg protein). Compared to the standard drug-treated group, the higher dose group treated animals had significantly higher levels of catalase; however, the increase was less pronounced compared to normal control mice(Table 6 & Figure 8).

Estimation of superoxide dismutase (SOD)

An enzyme antioxidant called superoxide dismutase converts the superoxide anion to hydrogen peroxide, reducing the toxicity of this radical. The reduction in the activity of this enzyme may result in deleterious effects due to the accumulation of superoxide radicals [34]. The SOD level was significantly (P<0.01) reduced to 1.47±0.093 units/mg tissue protein in the scopolamine-administered group compared to the control group (3.31±0.09 units/mg tissue protein). Administration of C1 (200 mg/kg and 400 mg/kg, p.o.) significantly (P<0.01) reversed (2.15±0.05 and 2.51±0.67 units/mg tissue protein) the scopolamine-induced reduction of SOD. Treatment with donepezil (5 mg/kg, p.o.) has shown a significant (P< 0.01) increase in the level of SOD (3.14±1.28 units/mg tissue protein) in brain homogenate of mice (Table 6 & Figure 9).

Estimation of Brain-Reduced Glutathione (GSH)

A vital tripeptide known as glutathione can neutralize free radicals like H2O2, O2, and alkoxy radicals while maintaining the membrane protein thiols. It also serves as a substrate for two enzymes: glutathione peroxidase and glutathione transferase. [35]. Test drug C1 (200 mg/kg and 400 mg/kg, p.o.) showed a significant (P<0.01) increase (2.33±0.023 and 3.10±0.25 nmoles/min/mg tissue protein) decreased glutathione level in the brain as compared to the scopolamine administered group (1.75±0.013 nmoles/min/mg tissue proteinDonepezil (5mg/kg, p.o.) significantly (P<0.01) raised the level of reduced glutathione in the brain (3.22±0.065 nmoles/min/mg tissue protein). (Table 6 & Figure 10).

Estimation of Brain Malondialdehyde (MDA)

An increase in the level of lipid peroxides in the brain reflects neuronal damage. The depletion of antioxidant defenses and raise in free radical production deteriorates the pro-oxidant antioxidant balance, leading to oxidative stress and cell death. Scopolamine-induced oxidative stress has been associated with an increased amount of lipid peroxidation. Cell death is frequently brought on by the autocatalytic process known as lipid peroxidation. Free radicals, in particular, as well as lipid peroxidative tissue damage caused by reactive oxygen species, have been linked to the pathogenesis of numerous diseases. Test drug C1 (200 mg/kg and 400 mg/kg, p.o.) showed significantly (P<0.01) decreased (0.93±0.46 and0.73±0.038 nmoles/min/mg tissue protein) malondialdehyde level in brain as compared to the scopolamine administered group (4.66±0.96 nmoles/min/mg tissue protein). Malondialdehyde levels in the brain were significantly (P< 0.01) reduced(0.65±1.31 nmoles/min/mg tissue protein) by donepezil (5 mg/kg, p.o.), and this suggests that C1 flavonoid may have antioxidant properties to lessen oxidative stress-induced membrane lipid peroxidation. (Table 6 & Figure 11).

Estimation of glutathione peroxidase

Our study revealed that oxidative stress is induced by the administration of scopolamine for 7 days and further administration of it has resulted in depletion of the glutathione peroxidase in mice. In the present study, it reveals that treatment with C1 flavonoid compound at both doses 200 and 400 mg/kg could restore the activity of the glutathione peroxidase antioxidant enzyme to 0.94±0.050 and 1.11±0.60 respectively, and possibly could reduce the generation of free radicals and neuronal damage. The GPx level was noticeably reduced (P<0.05) in the scopolamine-treated group to 0.56±0.096 when compared to the normal mice (1.23±0.01) and it was found to be significant P<0.01) in comparison with the control. There is a significant (P<0.01) restoration of the enzymatic activity of GPx in donepezil treated group (1.10±0.057) and this suggests that the drug can restore the level of GPx and reduce the formation of free radicals and associated oxidative stress (Table 6 & Figure 12).

Histopathological Studies

The histopathological assessment shows histological changes in mice's hippocampus region following scopolamine oral administration. By administration of scopolamine, histology showed signs of protein accumulation, development of plaques, degeneration of neurons, and dystrophic neuritis (B). The Standard treated group (Donepezil hydrochloride 5mg/kg) reduced the rate of degeneration of neurons, protein accumulation,

development of plaques, and dystrophic neuritis (C). Test drug- C1 treated group showed a dose-dependent decrease in protein accumulation, degeneration of neurons, and plaque formation.

Thus, the histopathological investigation showed that halo-substituted chromones prevent the hippocampus from degeneration and neuritic plaque formation in a dose-dependent manner.

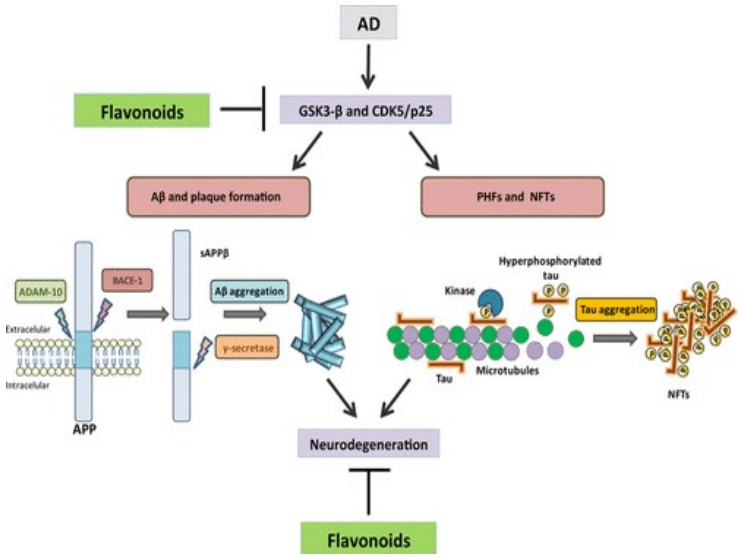

Figure 1:- Pathogenesis of Alzheimer's disease (AD) and effect of flavonoids on its prevention

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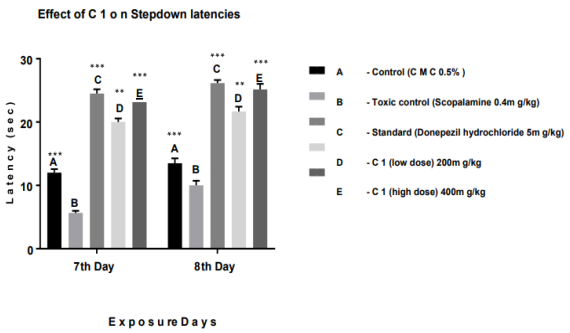

Figure 2:- Effect of C1 on mice's step-down latencies

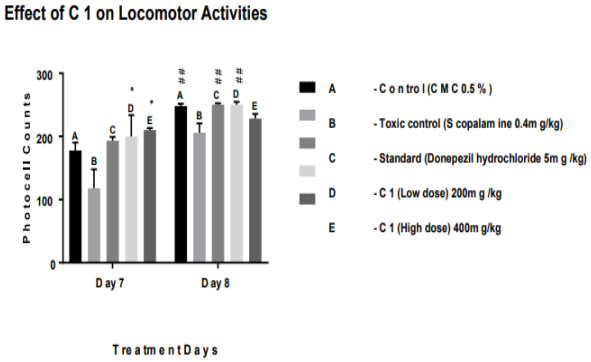

Figure 4:- Effect of C1 on Locomotor activity (actophotometer) in mice

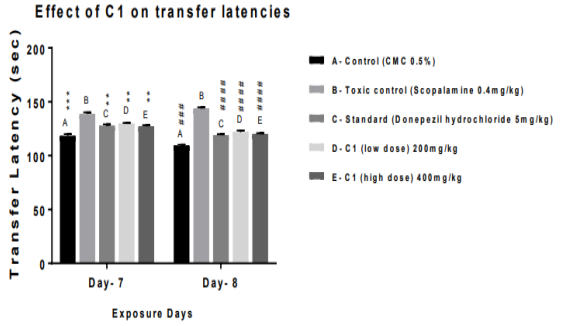

Figure 3:- Effect of C1 on Transfer latencies of mice

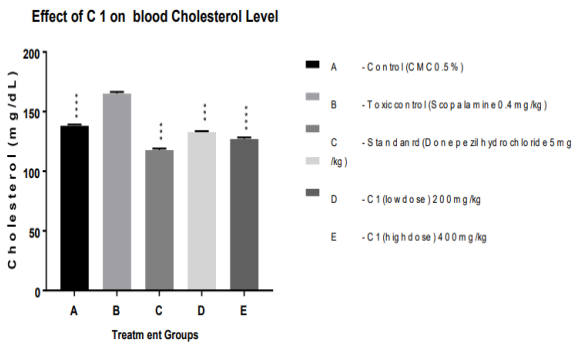

Figure 5:- Effect of C1 on Blood cholesterol level

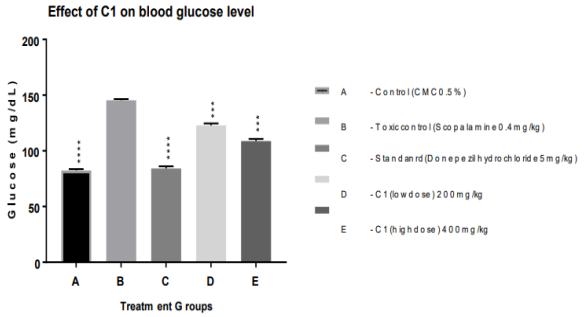

Figure 6:- Effect of C1 on Blood glucose level

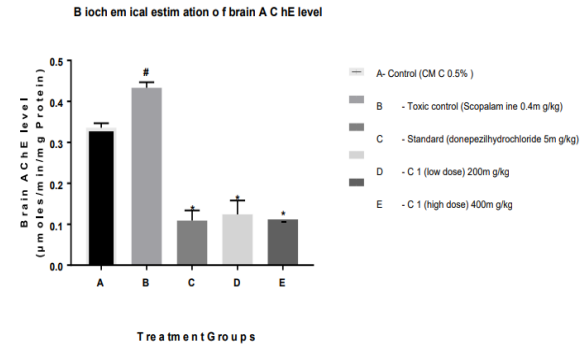

Figure 7:- Effect of C1 on brain acetylcholinesterase level

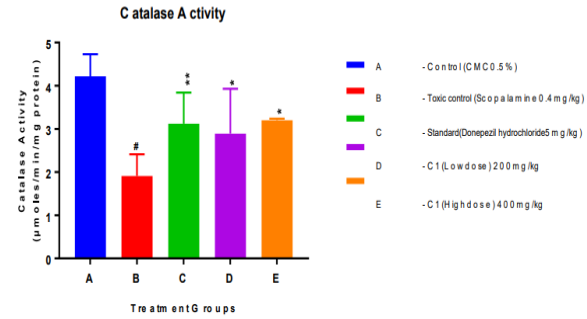

Figure 8:- Effect of C1 on brain catalase activity

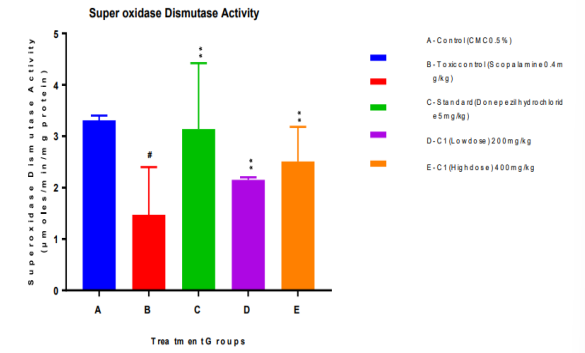

Figure 9:-Effect of C1 on brain Superoxide dismutase activity

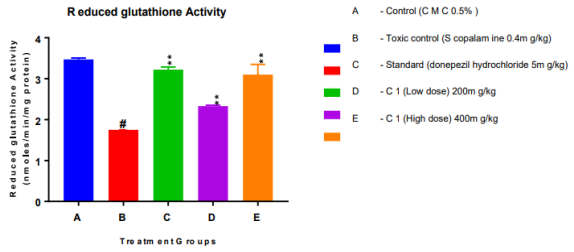

Figure 10:- Effect of C1 on brain reduced glutathione activity

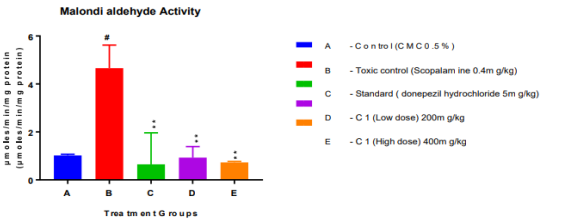

Figure 11:- Effect of C1 on brain malondialdehyde activity

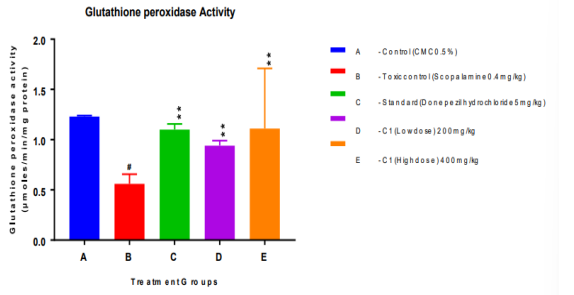

Figure 12:- Effect of C1 on brain glutathione peroxidase activity

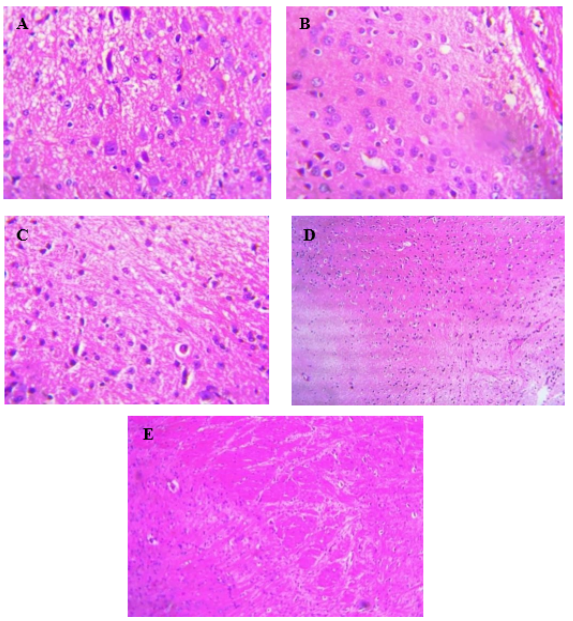

Figure 13:- Photomicrographs of the hippocampal region of the mice brain from each experimental group. The original magnification of all images is × 40. (A) Control group, (B) Toxic control (Scopolamine 0.4mg/kg) group, (C) Standard (Donepezil hydrochloride 5mg/kg) treated group, (D) Test Drug C1 Low dose treated group, (E) Test Drug C1 High dose treated group

Table 1:- Effect of C1 on step down latencies of mice

Group	Step down latency on 7th day (sec)	Step down latency on 8thday (sec)
Control (CMC 0.5%)	12 ± 0.5781***	13.5 ± 0.7638***
Toxic control (Scopolamine 0.4mg/kg)	5.66 ± 0.333	10 ± 0.7300
Standard drug (Donepezil hydrochloride 5mg/kg)	24.5 ± 0.6710***	26.16 ± 0.4770***
Test drug C1 (low dose) 200mg/kg	20 ± 0.5770**	21.66 ± 0.760**
Test drug C1 (high dose) 400mg/kg	23.16 ± 0.6010***	25.16 ± 0.872***

Values are expressed as mean±SEM, n=6 for each group; the values were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. ***P < 0.001, **P<0.01 when compared to toxic control.

Table 2:- Effect of C1 on Transfer latencies of mice

Group	Transfer latency on 7th day (sec)	Transfer latency on8thday(sec)
Control (CMC 0.5%)	118.33 ± 1.563***	109.33±0.7149###
Toxic control (Scopolamine 0.4mg/kg)	138.67 ± 1.453	143.67 ± 1.116
Standard drug (Donepezil hydrochloride 5mg/kg)	127.67 ± 1.229**	119.2±0.6540####
Test drug C1 (low dose) 200mg/kg	129.76 ± 0.5164**	121.7 ± 1.606####
Test drug C2 (high dose) 400mg/kg	127.33 ± 0.8028**	120.2 ± 0.6540####

Values were expressed as mean±SEM, n=6 for each group; the values were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. **** P<0.0001, ***P<0.001, **P<0.01 when compared to toxic control on 7th day.
P<0.0001, ###P<0.001 when compared to toxic control on 8th day.

Table 3:- Effect of C1 on Locomotor activity (actophotometer) in mice

Group	Locomotor activity on 7th day	Locomotor activity on the 8th day
Control (CMC 0.5%)	177.66± 12.7	248.36± 3.52##
Toxic control (Scopolamine 0.4mg/kg)	118 ±29.87	206±14.57
Standard drug (Donepezil hydrochloride 5mg/kg)	193.25±5.96	250.50±1.96##
Test drug C1 (low dose) 200mg/kg	200±33.80*	228.15±4.33##
Test drug C2 (high dose) 400mg/kg	210.33±2.90*	250.35±7.38

For each group, values were expressed as mean±SEM, n=6; the values were analyzed by one-way ANOVA followed by Dunnett's multiple comparison test. **** P<0.0001, ***P<0.001, **P<0.01 when compared to toxic control on 7th day. ##P<0.01 when compared to toxic control on the 8th day

Table 4:- Effect of C1 on cholesterol and glucose levels of mice

Group	Glucose (mg/dL)	Cholesterol (mg/dL)
Control	82.33 ± 1.33****	138.167 ± 0.9458****
Toxic control	145.33± 1.116	165.167 ± 1.308
Donepezil hydrochloride 5mg/kg	84.167±1.922****	117.833 ± 1.249***
Test drug C1 (low dose 200mg/kg)	122.833±1.778***	132.833 ± 0.8724***
Test drug C1 (high dose 400mg/kg)	108.833±1.905***	127.00 ± 1.302****

Values for each group were expressed as mean±SEM, n=6, and were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. In comparison to the toxic control, **** P<0.0001, ***P<0.001.

Table 5:- Biochemical estimation of brain AChE level

Groups	Brain AChE level (µmoles/min/mg Protein)	AchE inhibition (%)
Control	0.3356±0.011	-
Toxic control	0.433±0.014#	28.37±0.445#
Donepezil hydrochloride 5mg/kg	0.109±0.025*	82.99±0.020*
Test drug C1 (low dose 200mg/kg)	0.124±0.034*	48.46±0.195*
Test drug C1 (high dose 400mg/kg)	0.112±0.006*	80.87±0.135*

The values were expressed as mean±SEM, with n=6 for each group; one-way ANOVA was used to analyze the data, followed by Dunnett's multiple comparison test. *P<0.01 significant when compared to negative control, #P<0.01 significant when compared to control.

Table 6:- Anti-oxidant activity of C1 on mice brain

Groups	CAT	SOD	GPx	GSH	MDA
Control Normal Saline (10 ml/kg b.w) (p.o.)	4.22 ± 0.51	3.31 ± 0.09	1.23 ± 0.01	3.47 ± 0.036	1.02 ± 0.05
Toxic control Scopolamine 0.4mg/kg b.w) (p.o.)	1.91 ± 0.50#	1.47 ± 0.93#	0.56 ± 0.096#	1.75 ± 0.013#	4.66 ± 0.96#
Standard Donepezil hydrochloride (5mg/kg b.w) (p.o.)	3.12 ± 0.72**	3.14 ± 1.28**	1.1 ± 0.057**	3.22 ± 0.065**	0.65 ± 1.31**
Test drug C1 (low dose 200mg/kg b.w) (p.o.)	2.89 ± 1.04*	2.15 ± 0.05**	0.94 ± 0.050**	2.33 ± 0.023**	0.93 ± 0.46**
Test drug C1 (high dose 400mg/kg b.w) (p.o.)	3.2 ± 0.039*	2.51 ± 0.67**	1.11 ± 0.60**	3.1 ± 0.25**	0.73 ± 0.038**

The values are presented as Mean± SEM, with n=6 in each group. #denotes P< 0.01 versus control; **denotes P< 0.01 versus toxic control; *denotes P< 0.05 versus negative control (one-way ANOVA followed by Dunnett's test). SOD (Superoxide dismutase): units/mg tissue protein; GPx (Glutathione peroxidase) = moles/min/mg protein; GSH (Reduced glutathione): nmoles/min/mg protein; CAT (Catalase): µmoles/min/mg protein. Malondialdehyde (MDA) = moles/min/mg protein.

CONCLUSION

Flavonoids were reported to possess various pharmacological activities such as neuroprotective activity, anti-diabetic activity anti-inflammatory activity, anti-arthritic activity, anti-platelet activity, vasorelaxant activity, and anti-cancer activity. Therefore, identifying and characterizing flavonoids like moieties is a promising approach to fighting an illness such as Alzheimer's disease. Rapid and simple synthesis or chemical modifications of flavonoids allow rapid production of a large number of novel compounds.

Halo-substituted flavonoids were selected and synthesized. Based on the electro-negativity, the electron-withdrawing groups (chloro, fluoro, and Bromo) produced good AChE inhibition activity. *In vivo* studies were performed on scopolamine-induced mice model only with C1 (structurally 2-(2-chlorophenyl)-4H-benzopyran-4-one) because, among nine synthesized halo-substituted flavonoids, C1 showed higher AChE inhibitory activity. So it was compared with donepezil hydrochloride *in vitro* and *in vivo* ways for its activity against neurodegeneration.

This study suggests that the protection of antioxidant enzyme activity along with the increased level of brain acetylcholinesterase inhibition may be potentially helpful in the treatment of Alzheimer's disease. A great deal of research has been done regarding AChEIs as a therapeutic drug for the treatment of AD. Challenges to drug development include potency, safety, side effects, and comparison to current AChEIs.

Results of this study indicated the presence of oxidative damage after treatment with scopolamine and further synthesized flavonoids produced free radical scavenging activity and offered protection by enhancing the learning and memory impairment in mice. Finally, the results suggest that the synthesized compound C1 (structurally 2-(2-chlorophenyl7 -4H-benzopyran-4-one) offered protection against neuronal damage induced by scopolamine and has the potential to be developed into new drugs for the treatment of Alzheimer's disease. However, a more thorough preclinical investigation into the involved receptor, as well as cellular and molecular targets, is required before these chemical moieties are used in clinical trials.

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REFERENCES

1. Goodman and Gilman's, The pharmacological basis of therapeutics,13th edition, page no.172-173.
2. Ganguli R and Guha D (2008). Alteration of brain monoamines and EEG wave pattern in a rat model of Alzheimer's disease and protection by *Moringa oleifera*. Indian Journal of Medical Research 12: 744-751.
3. Nivsarkar M, Banerjee A, Deval S, Jaimie T, Manoj P, Bapu C and Harish P (2006). Reduction in aluminium induced oxidative stress by meloxicam in rat brain. Iranian Biomedical Journal 10(3): 151-155.
4. Thompson SB (2011). Alzheimer's disease: a comprehensive review of etiology, diagnosis, assessment recommendations and treatment. Web med Central Review Article 81: 1-42.
5. Parle M and Vasudevan M (2007). Memory enhancing activity of Abana: an Indian Ayurvedic poly-herbal formulation. Journal of Health Science 53(1): 43-52
6. Tripathi KD (2008). Essentials of medical pharmacology. 6th edition. Jaypee brother's medical publishers (P) LTD. New Delhi: 471-473.
7. Uma S, Kavimani S, and Ramanan, KV (2010). Effect of *Saraswatharishta* on learning and memory. International Journal of psychopharmacology 1(1): 15-19.
8. Yang MH, Yoon KD, Chin YW, Park JH and Kim SH (2009). Neuroprotective effects of *Dioscorea opposite* on scopolamine-induced memory impairment *in vivo* behavioral tests and in-vivo studies. Journal of Ethnopharmacology 121: 130-134.
9. Muller WE, Schaffer S, Weber CC, Eckert V and Schmitt S (2005). Flavonoids and the aging brain. Journal of Physiology and Pharmacology 16:23-36.Janicijevicet al, 2007
10. Tapas AR, Sakarkar DM, and Kakde RB (2008). Flavonoids as nutraceuticals: a review. Tropical Journal of Pharmaceutical Research 7 (3): 1089-1099Krishna et al, 2001;
11. Ducrot PH, Safi NE and Souhila G (2007). Flavonoids: chemosynthesis, reactivity, characterization, and free radical scavenging activity. Molecules 12: 2228-2258.
12. Agrawal AD (2011). The pharmacological activity of flavonoids: A review. International Journal of Pharmaceutical Science and Nanotechnology 4(2): 1394-1398.
13. Agrawal AD (2011). The pharmacological activity of flavonoids: A review. International Journal of Pharmaceutical Science and Nanotechnology 4(2): 1394-1398.
14. Karak,P.(2019). Biological activities of flavonoids: an overview. International Journal of Pharmaceutical Sciences and Researches, 10(4), 1567-1574.
15. Alka NC and Juyal V (2011). Synthesis of chalcones and their derivatives as antimicrobial agents. International Journal of Pharmacy and Pharmaceutical Sciences 3(3): 125-128.
16. Vidyasagar NC and Nanda RK (2012). Synthesis of some flavonoid derivatives and study of their antioxidant and in vivo antidiabetic activity. Contemporary Investigations and Observations in Pharmacy1(1): 9-18.
17. Sreedhar NY and Jayapal MR (2011). Synthesis of 2, 4-dihydroxy substituted chalcones using silica sulphuric acid reagent under solvent-free conditions. Asian Journal of Pharmaceutical and Clinical Research 4(1): 106-108.
18. Huang WH, Chien PY, Yang CH, Lee AR. Novel synthesis of flavonoids of *Scutellaria baicalensis* G EORGI. Chemical and pharmaceutical bulletin. 2003;51(3):339-40..
19. Ellman GL, Courtney DK, Andres V and Featherstone RM (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical pharmacology 7: 88-95.
20. Sacan O, Yanardag R. Antioxidant and anti-acetylcholinesterase activities of chard (*Beta vulgaris* L. var. cicla). Food and chemical toxicology. 2010 May 1;48(5):1275-80.
21. OECD 2002, Acute oral toxicity, Acute oral toxic class method guidelines423adopted23.03.1996, In Eleventh Addendum to the OECD guidelines for the testing of chemicals organization for economic cooperation and development, Paris, June-2000.
22. Une HD, Ejaj MA, Tarde VA. Nootropic activity of saponins obtained from *Tinospora cordifolia* stems in scopolamine-induced amnesia. Int J Pharma Res Rev. 2014 Feb;3(2):28-35.
23. Ahmed T, Gilani AH. The inhibitory effect of curcuminoids on acetylcholinesterase activity and attenuation of scopolamine-induced amnesia may explain the medicinal use of turmeric in Alzheimer's disease. Pharmacology Biochemistry and Behavior. 2009 Feb 1;91(4):554-9.
24. Vogel HG, Müller G, Sandow J, Schölkens BA. Drug discovery and evaluation: pharmacological assays. Vogel HG, Vogel WH, editors. Berlin: Springer; 1997 Jan.
25. Goverdhan P, Sravanthi A, Mamatha T. Neuroprotective effects of meloxicam and selegiline in scopolamine-induced cognitive impairment and oxidative stress. International Journal of Alzheimer's Disease. 2012 Mar 22;2012
26. Lumb PJ, Slavin BM. Determination of serum cholesterol concentration in the presence of ascorbate. Journal of clinical pathology. 1993 Mar 1;46(3):283-4..
27. Sharma SP, Anjankar AP, Kale A. Comparison of glucose levels using a glucometer and GOD-POD Method in diabetic patients. Int. J. Clin. Biochem. Res. 2017;4(1):6-10.
28. Nandhini S, Vadivu R, Jayshree N. Memory strengthening activity on seeds of Abelmoschus moschatus. International Journal of Research in Pharmacy and Chemistry. 2014;4(2):346-50.

29. Ramanathan M, Sivakumar S, Anand vijayakumar PR, Saravanababu C, Pandian PR. Neuroprotective evaluation of standardized extract of Centella asciatica in monosodium glutamate treated rats.
30. Singh S, Ramandeep S, Ajay SK and Gaurav G (2014). Neuroprotective role of antioxidant and pyranocarboxylic acid derivative against $AlCl_3$ induced Alzheimer's disease in rats. Journal of Coastal Life Medicine2(7): 571-578.
31. Aly HF, MetwallyFM and Ahmed HH (2011).Neuroprotective effects of dehydroepiandrosterone (DHEA) in a rat model of Alzheimer's disease. Acta Biochimica Polonica 58, (4) 513–520.
32. Yassin NAZ, Siham MA, Karam AM, Nadia AM, Goudad AHM, Razik HF and BassantMMI(2013). Effect of *Boswellia serrata* on Alzheimer's disease induced in rats. Journal of the Arab Society for Medical Research 8: 1–11
33. Chandan R, Ghosh T K and Debjani G (2008). Dose-dependent activity of Benincasahispida on colchicine induced experimental rat model of Alzheimer's disease. International Journal of Pharmacology 4: 237-244.
34. Murugesh KS, Yeligar VC, Maiti BC and Maity TK (2005). Hepatoprotective and antioxidant role of *Berberis tinctoria* Lesch leaves on paracetamol-induced hepatic damage in rats. Iranian Journal of Pharmacology and Therapeutics 4:64-69.
35. Raghavendran HR, Sathivel A and Devaki T (2004). Hepatoprotective nature of seaweed alcoholic extract on acetaminophen-induced hepatic oxidative stress. Journal of Health Science 50: 42-46.
36. Halliwell B and Gutteridge JM (1984). Oxygen toxicity, oxygen radicals, transition metals, and disease. Journal of Biological Chemistry 21:1–14.
37. Drake J, Kanski J, Varadarajan S, Tsoras M and Butterfield DA (2002). Elevation of brain glutathione by γ-glutamylcysteine ethyl ester protects against peroxynitrite-induced oxidative stress. Journal of Neuroscience Research 18: 776-784