Bioactive Flavonoids Improves Scopolamine-Induced Learning And Memory Impairments in Mice

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Abstract:

The increasing number of epidemiological studies have consistently shown a protective effect against many diseases of polyphenol-rich foods (fruit, wine, chocolate...). The evidence is confirmed by the findings of various studies carried out in animal models, with nutritionally realistic amounts of isolated flavonoids, and with flavonoid-rich foods in humans. There have been huge advances in research on flavonoids due to their wide variety of pharmacological properties and a great devotion towards such flavonoids has gained much interest in preclinical research to explore them at molecular level. Natural bioactive compounds have wide and broad therapeutic actions that can be used for management of chronic diseases like Parkinson, Alzheimer, in this propose investigation centralizing on two such most promising bioactive flavonoids like, Andrographolide and Hesperidin and with the goal of identifying the potential synergistic impact adjacent to Scopolamine administration (1mg/kg, i.p.) for 7 days. There was a serious impairment in the behavior pattern (memory and learning), increase acetylcholinesterase enzyme activity, decrease oxidative status. The findings of the current investigation indicate that scopolamine intraperitoneal administration in mice causes neurobehavioral and biochemical changes that resemble those seen in Alzheimer's disease. Treatment of mice with a combination of AG and HSD is likely to have substantial neuroprotection mediated by its antioxidant properties and offers a good rationale for improved clinical control of Alzheimer's disease.

Keywords: Alzheimer's, *Andrographolide*, Flavonoids, *Hesperidin*, learning, Memory.

Introduction:

Alzheimer's disease (AD) is a significant cause of cognitive deficits, memory loss, behavioral change and progressive healthcare obstacle. (Pena, *et al.*, 2019). Impairment in memory, judgment, decision making and language are the clinical hallmarks of Alzheimer's disease. (Nussbaum and Ellis, 2003). It also responsible for enlargement of brain ventricles along with the shrinkage of cerebral cortex and the medial temporal lobe (Matsuda, 2013). In AD, tau protein forms insoluble fibrils after abnormal hyperphosphorylation and deposits within the cell (Galimberti and Scarpini, 2010). This causes major structural and functional damage to the healthy brain. (Aisen, *et al.*, 20 l7). AD is characterized by basal forebrain loss of cholinergic neurons, deposition of extracellular senile plaques and brain-derived neurotrophic factor (BDNF) protein alterations, activity-regulated cytoskeleton-associated protein (Arc), glial fibrillary acidic protein (GFAP), immune dysregulation, neuroinflammation,

impairment in neuronal glial communication play contributing role in AD pathology (DeKosky, 2001). Neurofibrillary tangles in hippocampal and cortex region which ultimately leads to cognitive impairment (Masters CL *et al.*, 1985). Symptom includes hallucinations, depression, sleep disturbance, loss of appetite, uncontrollable twitches (Khairallah MI and Kassem LA 2011). The risk factors for AD are multifactorial and the biggest culprit in genetics is the e4 allele gene for apolipoprotein E (ApoE) and ApoE4 gene involved in transportation and metabolism of fats it is a principle cholesterol carrier in the brain mutation of which profound to be risk cause of AD (Van Cauwenberghe *et al.*,2016). Mutations in *PSEN2* are rare i.e., about 20% of familial cases. *PSENI* and *PSEN2* both encode for presenilin's which is a catalytic subunit of γ-secretase responsible in causing amyloid plaques (St George and Petit,2005).

Material and Method:

Animals: Male Swiss albino mice (4-weeks-old), 25-30 g in weight, were obtained from Krystal Biological Solutions pvt. ltd. (Pune, India). Maintained in a 12 h light/dark period under a steady temperature (23± l °C) and humidity (55±5 %) ad libitum was supplied with feed and water.

Reagents and Chemicals: Andrographolide (AG) and Hesperidin (HSD) were purchased from Phyto Life Sciences Pvt. Ltd. (Ahmadabad, India). Scopolamine Hydrochloride (SCP), was purchased from Sigma Aldrich (Bangalore, India). All other materials were obtained from normal commercial sources and were of the highest grade available.

Experimental protocol and treatment schedule:

The animals were divided randomly into twelve groups of ten animals each as shown in table.1 For a duration of 14 days, standard and test drugs were administered and scopolamine prepared freshly in normal saline solution used to induce Alzheimer's disease and was administered by intraperitoneal (i.p.) route for 7 days. AG and HSD was also freshly prepared in distilled water and administered 30 min before scopolamine administration (14 days) by orally using oral gavage needle.

Table 1: Protocol for Alzheimer's disease in mice:

GROUP	TREATMENT	No. of ANIMALS
1	Normal saline (1 ml/kg body weight, *i.p.*)	10
2	SCP in normal saline (1 mg/kg body weight, *i.p.)*	10
3	Donepezil (1mg/kg *i.p.*)	10
4	SCP (1 mg/kg *i.p.*) + HSD (100 mg/kg *p.o.*)	10
5	SCP (1 mg/kg *i.p.*) + HSD (200 mg/kg *p.o.*)	10
6	SCP (1 mg/kg *i.p.*) + HSD (300 mg/kg *p.o.*)	10
7	SCP (1 mg/kg *i.p.*) + AG (30 mg/kg *p.o.*)	10
8	SCP (1 mg/kg *i.p.*) + AG (60 mg/kg *p.o.*)	10
9	SCP (1 mg/kg *i.p.*) + AG (120 mg/kg *p.o.*)	10
10	SCP (1 mg/kg *i.p.*) + HSD (50 mg/kg *p.o.*) + AG (15 mg/kg *p.o.*)	10
11	SCP (1 mg/kg *i.p.*) + HSD (100 mg/kg *p.o.*) + AG (30 mg/kg *p.o.*)	10
12	SCP (1 mg/kg *i.p.*) + HSD (150 mg/kg *p.o.*) + AG (60 mg/kg *p.o.*)	10

Behavioral Parameter

Morris Water Maze:

The apparatus is established in order to evaluate the spatial memory and learning which is related to the hippocampal damage. (Vorhees and Williams, 2006). Placing the mouse in the pool close to the bottom, facing the wall of the tank in one of the four quadrants, begins a trial. (Sharma, 2009). The

cumulative time each mouse took to locate the hidden platform in the pool of water was determined and used for the analysis. The animals were allowed to swim for maximum time of 60 second after which they were removed.

Biochemical Estimation:

Acetylcholinesterase Assay:

Reaction mixture contained 145 µl phosphate buffer of pH 7.2, 13.1 µl AchE preparation,5 µl of different concentration of HSD and AG (1- 1000 nm) which is incubated at room temperature for 10 minutes which is followed by addition of 5 µl of 75 mM acetylthiocholine iodide and 10 Mm ellman's reagent in 168 µl of total reaction mixture. Change in absorbance were determined by spectrophotometrically, a UV -spectrophotometer (Schirnadzu 1800) at 412 nm for 2 min at 30second of interval for the determination of the IC50 value, a fixed substrate concentration and varying inhibitor concentrations (1-1000 nm) were used at the point where 50 % inhibition of the enzymes catalytic activity occurred. Separate controls were also carried out in which the preparation of the enzyme is substituted by substrate at the concentration used in the assay. (Ellman GL *et al.*, 196l). % Inhibition= (sample — blank / blank) x 100

Lipid Peroxidation Assay:

This assay is based on the formation of a red adduct (maximum 532 nm absorption) between TBA and malondialdehyde (MDA), a colorless lipid peroxide decomposition end product. (Sandhu, 2013). 8.1 % SDS, 1.5ml of 20 % acetic acid solution modified to pH 3.5 with NAOH and 1.5ml of 20 % acetic acid solution were applied to the reaction mixture containing 0.2 ml tissue homogenate. 1.5 ml of 0.8% of aqueous TBA solution. The final mixture volume was balanced with distilled water to 4.0 ml, and then heated in a water bath at 95'C for 60 minutes. In the above reaction mixture, 1 ml of distilled water and 5 ml of the n-butanol and pyridine mixture (15:1, v/v) were applied to the mixture after cooling and shaken vigorously. The organic layer absorbance was measured at 532 nm using a UV spectrophotometer after centrifugation at 4000 rpm for 10 min. (Shalavadi *et al.*,2012). The extent of lipid peroxidation was to be expressed in terms of n moles of MDA / mg of proteins.

Statistical Analysis:

By using One Way ANOVA, followed by Sidak's multiple comparison test, all values are expressed as mean SEM, n= 10. 1. Negative is compared with normal, standard and all treated groups. 2. Combination is compared with single drugs of AG and HSD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, NS: Non-Significant. 3. Standard is compared with combination of both drugs. #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001, NS: Non- Significant.

Result and Discussion:

Present practice illustrated the neuroprotective effect as well as anti-alzheimer's potential of AG, HSD and combination of both on scopolamine induced mice model of alzheimer's disease. Pre-treatment, treatment of AG, HSD and its combination demonstrated complex effects like reduction in the level of AchE, lipid peroxidation. Scopolamine, a tropane alkaloid and a non-selective muscarinic receptor antagonist is a widely used gold standard drug for the induction of amnesia in rodents and humans (Kwon *et al.*, 2010) after administration it produces spatial memory deficits due to elevation in the level of AchE, increased oxidative stress markers and lipid peroxidation, deposition of amyloid plaques and neurofibrillary tangles are vital patterns in pathogenesis of AD (Wang *et al.*,2013). Scopolamine is also responsible for induction of neuroinflammation leading to elevated level of oxidative stress (Ahmad *et al.*, 2014). It also interferes with BDNF, GFAP and Arc protein (Konar *et al.*,2015). Therefore, administration of

SCP for a period of 7 days was found to be beneficial in order to induce Alzheimer's disease and for evaluation of potential of both bioactive to reverse neuronal blemish induced by SCP.

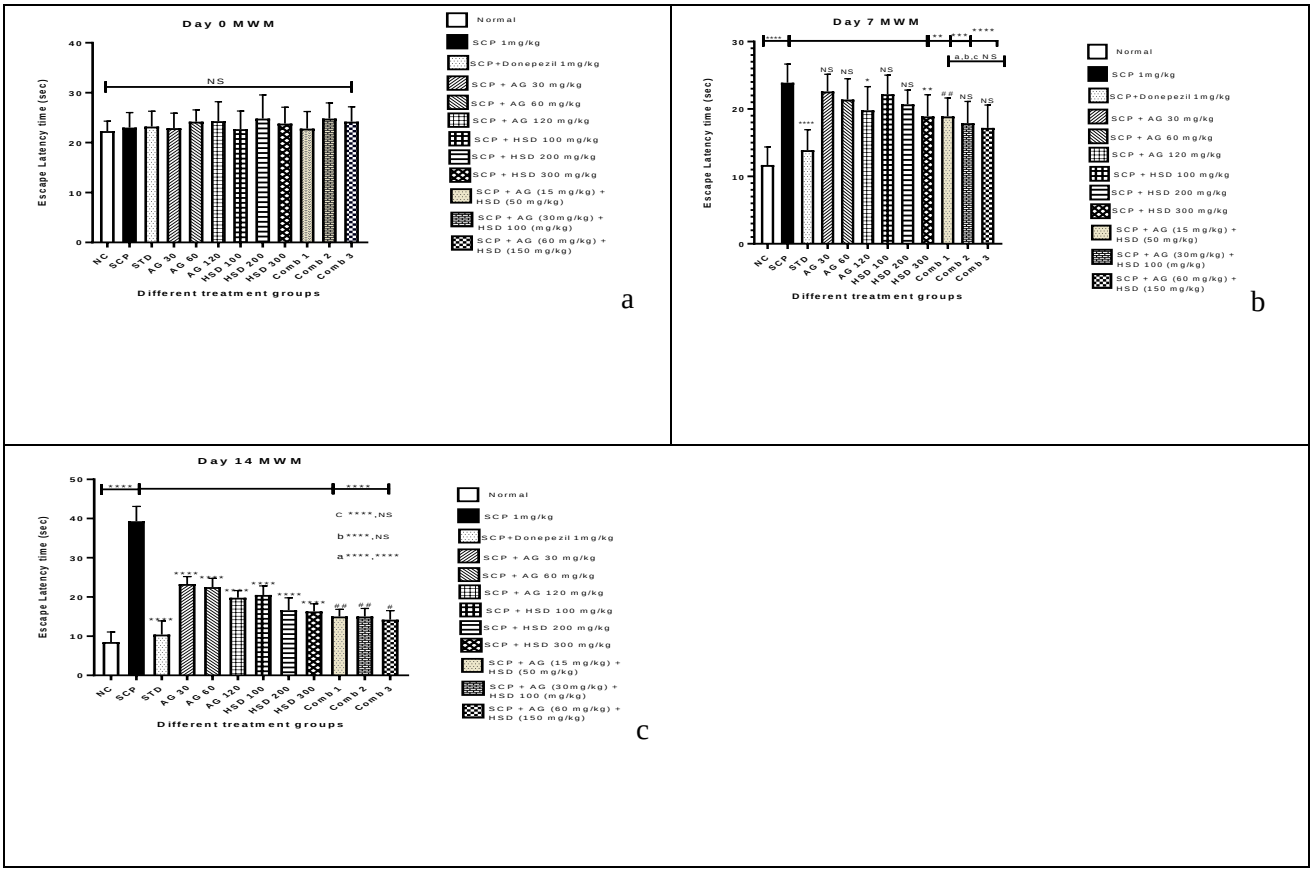

Figure 1: Escape latency following exposure to scopolamine, AG, HSD and combination of both in simultaneous treatment along with standard donepezil

Spatial and learning memory enervation based on cholinergic dysregulation in the hippocampus of mice was evaluated in the MWM test on SCP administration. The latency time for escape (i.e., time required by mice to find out the hidden platform) were recorded on day 0, 7 and 14 as shown in figure 1. On day 7, the significant reduction in the escape latency time were observed in the groups receiving pre-treatment with standard donepezil ($p<0.0001$) and combination of both flavonoids showed significant reduction ($p<0.0001$) as shown in figure 1.b. On day 14, AG (30, 60 and 1 20 mg/kg) and HSD (100, 200 and 300 mg/kg) showed significant reduction ($p<0.000$ 1) respectively as shown in figure 1.c. Combination 1, 2 and 3 of both the flavonoids showed significant reduction ($p<0.0001$) in escape latency time as compared to scopolamine treated group. All three combinations were compared with standard donepezil. Combination 1 and 2 showed significant reduction ($p<0.01$ respectively) as shown figure 1. The reduced escape latency time revealed the increased spatial learning and memory in mice as compared with SCP treated group.

In-vitro study-acetylcholinesterase enzyme assay:

The in-vitro acetylcholineesterase inhibition activity of *Andrographolide* and *Hesperidin* alone and in combination was evaluated by using brain homogenate. The combination of both the drugs was found to be more effective as compared single drug doses. The inhibition of acetylcholinesterase by AG (IC_{50}-312.53 nM), HSD (IC_{50}-316.45 nM) and combination (IC_{50}-359.53 nM) were found to be less potent as compared with IC_{50} value of standard donepezil (IC_{50}- 53.6 nM) as shown figure 2.

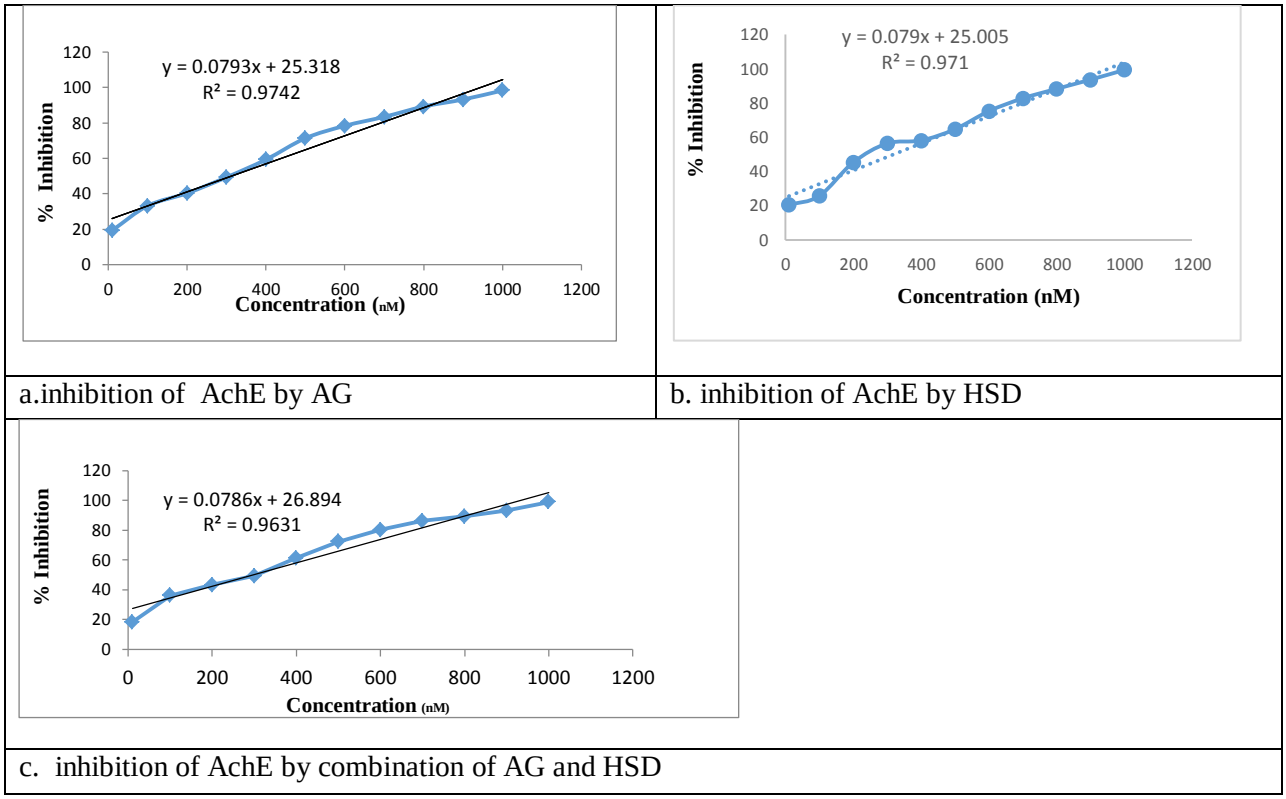

Figure 2: Concentration dependent and %inhibition of AchE.

Acetylcholinesterase is an enzyme playing role in degradation of neurotransmitter acetylcholine. Acetylcholine depletion result in memory loss. SCP administration significantly elevated the level of enzyme AchE as compared with normal treated group ($p<0.0001$). All the treatment groups of AG and HSD alone and in combination reversed the AchE level as compared to SCP treated group ($p<0.0001$) as shown figure in 3.a. On the basis of result findings, both the bioactive alone and in combination were found to be significant in reducing the level of AchE. Administration of SCP increased level of MDA leading to increased lipid peroxidation as compared to the normal control group ($p<0.0001$). Same time, treatment with highest dose of AG (120 mg/kg) and HSD (200and 300 mg/kg) reduce the level of lipid peroxidation ($p<0.05$) respectively as shown in figure 3.b. Combination 2 and 3 showed significant alteration ($p<0.0001$) while combination 1showed significant change ($p<0.01$) as compared with scopolamine treated group which indicating potential of phytoconstituent to prevent cells from neuronal degeneration.

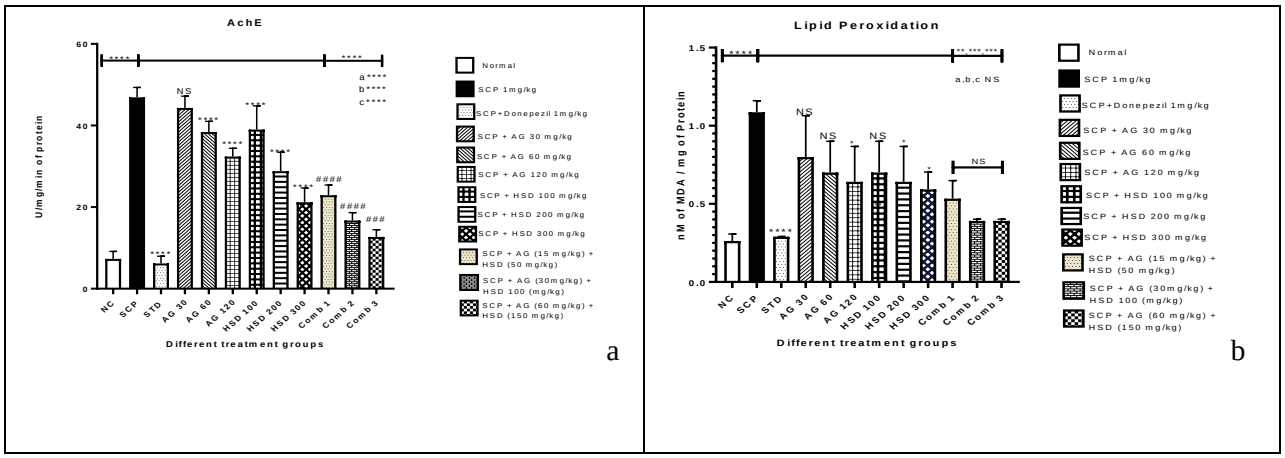

Figure 3: Biochemical estimations with respect to AchE and LPO

Histopathological Study:

All the preserved organs/tissues samples such as brain from twelve groups were processed routinely and embedded in paraffin. Microscopic examination of brain of normal control group did not showed any neuronal degeneration. Standard donepezil group showed normal neuronal cells. Whereas scopolamine treated group showed severe neuronal desecration in the CA_1, CA_2 and CA_3 regions of the hippocampus as shown in figure 4. Mice treated with AG (30 and 60 mg/kg) showed normal neuronal cells with mild neurodegeneration in the hippocampus. The AG 120 showed moderate to severe neuronal degeneration in hippocampus. Mice treated with HSD (100 and 200 mg/kg) showed significant effect. Whereas HSD 300 showed moderate neuronal degeneration in CA_1, CA_2 and CA_3 regions of the hippocampus. The combination of AG and HSD (Combination 1 and 2) showed similar effect as that of the standard donepezil group by showing normal neuronal cells. Combination 3 showed mild neuronal denegation in the CA_1, CA_2 and CA_3 regions of the hippocampus.

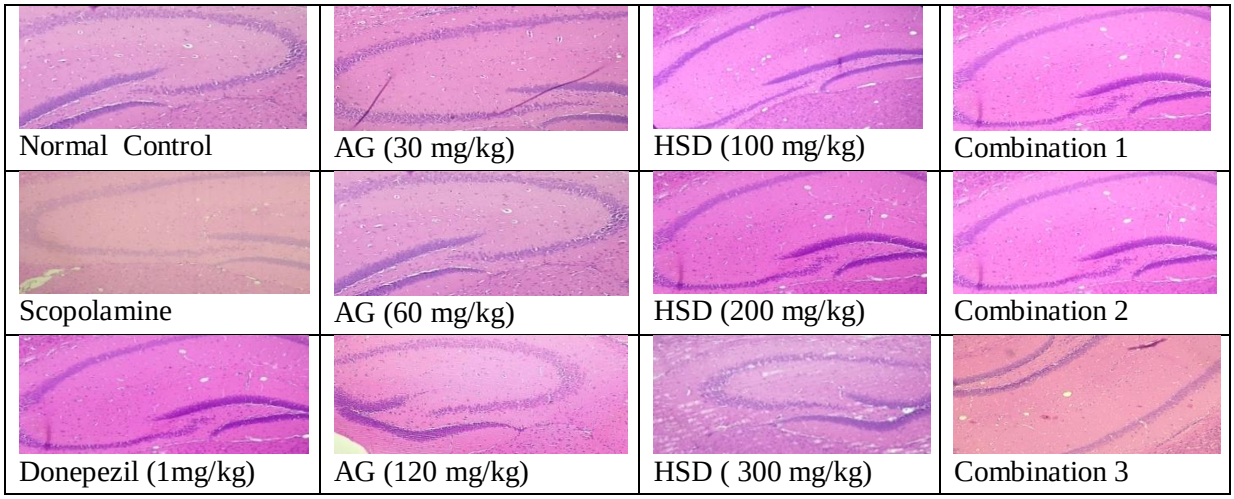

Figure 4: Histopathology of different treatment groups.

Conclusion:

Alzheimer's which is most common disorder marked by gradual loss of cholinergic neurons in forebrain with deposition of amyloid plaque deposition and tau accumulation associated with poor memory performance. Proposed hypothesis was to evaluate the neuroprotective effect of *Andrographolide* and *Hesperidin* for behavioral and biochemical alteration in scopolamine induced mice model for Alzheimer's disease. Study comes with scientific evidences as combinational therapy was found to be effective. It also provides justification that proposed study shows synergistic effect in combination of two bioactive. Proposed research also shows that combinational therapy is a natural alternative and/or complementary source of antioxidative and neuroprotective agents thus economical. Ultimately it will give a better therapeutic management against cognitive dysfunction and neurodegeneration. Present research work with different treatments of AG and HSD in combination refined behavioral performance in terms of improvement in memory and exploratory behavior with raised learning process. Combinational therapy also showed refinement in biochemical parameters by restoring antioxidant status and balancing mitochondrial respiratory chain. Thus, combinational therapy shows correlation to proposed hypothesis i.e., its improved antioxidant status, improved antiapoptotic: apoptotic ratio, reduction of peroxidation and AchE enzymes activity. Performed research work was found to be correlated with hypothesis and the effect may be attributed by improving cognitive performance. Thus, combinational therapy of *Andrographolide* and *Hesperidin* may be effective as neuroprotective in synergistic manner. This proposed research will further broaden the research

aspect on newly identified isolated flavonoids for their rational and scientific uses in suffering community for the therapeutic management of various chronic diseases.

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Conflicts of Interest

- The authors declare that they have no conflict of interest for this study.
- Funding Support
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