CHAPTER 6

IN VIVO NEUROPROTETIVE ACTIVITY OF POLYHERBAL FORMULATION ON AGED RATS.

6.1 INTRODUCTION

Senile dementia of Alzheimer's type is an age related progressive neurodegenerative disorder primarily affecting the elderly population [339, 340]. Researchers have hypothized various factors that are associated with pathogenesis of AD like oxidative stress, abnormal phosphorylation of tau protein, altered homeostasis of calcium, age, inflammatory markers, β-amyloid peptide accumulation etc. Studies have reported the existences of lipid preoxidation products such as 4-hydronoel HNE in the brain of AD patients [341]. AD is characterized by loss and degeneration of cholinergic neurons in the cerebral system [342, 343] which plays prime role in learning and memory [344]. Deficits in cholinergic transmission in the hippocampus and cortex due to loss of cholinergic neurons are strongly correlated with cognitive dysfunction in AD patients [345].

Plants have been used since ancient days as medicine, food and as a source for variety of daily needs [346]. Among the extensive floral biodiversity in India, only about 7000-7500 species are utilized in daily routine as medicine by traditional communities [347]. Ayurveda is one of the ancient systems of medicine in India; in ayurveda rasayana class of drugs is used to strengthen the biological system. The drugs that act on brain and cognition are called "medhyarasayana". The current study was designed to evaluate the neuroprotective activity of polyherbal formulation against ageing brain and scopolamine induced neurodegenerative model. This study may add further significance to its traditional use.

6.2 Methodology

6.2.1 Experimental animals

Adult sprauge dawley rats were obtained from the Central Animal House, IMS, BHU, Varanasi, India. Rats were housed in metallic cages (47 cm×34 cm×20 cm) lined with husk, replaced every alternate day. Rats were caged under controlled temperature and humidity at 22 ± 2°C and relative humidity 30 – 70% and 12:12 hrs light/dark cycle. The rats were fed with standard laboratory diet and RO water *ad libitum.* The experiments were carried out according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and approved by the Institutional Animal Ethics Committee.

6.2.2 Experimental model

The rats were randomly assigned to 5 groups of 6 animals each the groupings are as follows:

Group 1- Young controls (2 – 3 months) - orally given artificial cerebrospinal fluid (ACSF).
Group II- Aged (20-22 months).
Group III- Aged (20-22 months) + Polyherbal formulation 100mg/kg body wt.
Group IV- Aged (20-22 months) + Polyherbal formulation 200mg/kg body wt.
Group V- Aged (20-22 months) + Polyherbal formulation 300mg/kg body wt.
The sample was orally administered once in a day at 0.5 ml constant for 28 days after which the animals were tested for level of neurotransmitters and active avoidance learning.

6.2.3 Estimation of neurotransmitters content

6.2.3.1 Acetylcholine

Acetylcholine was detected by the fluorimetric method as described by Fellman [348].The method is based on the precipitation of acetylcholine-iodine complex, followed by the absorption of quaternary ester in cation exchange resin column, and the separation of fluorescent material, acetylhydrazylsalicylhydrazone and salicylaldehyde, which are detected in spectrophotofluorimeter. Brain cortex was homogenized in 10% TCA and 10 mL of the homogenate was added to 0.1mL of tetramethylammonium bromide (10 μM/mL)

followed by 0.4 mL of saturated potassium iodide solution. This reaction mixture was kept for incubation on ice for 20 min and then centrifuged at 4°C at 1000g at 15 min. After discarding the supernatant 5 mL of anhydrous ether was added to the precipitate and stirred to dissolve. 5 mL of water was added and mixed to remove ether layer containing iodine. This solution was extracted three times with 5 mL ether and the ether layer was discarded after every centrifugation. The remaining 5 mL of the sample was kept in water bath at 100°C for 10 min to remove excess ether residue and cooled. This was transferred on to 7 cm x 0.7 cm Bio Rex 70 (50 – 100 mesh) resin column. 20 mL of water was then added to rinse the column without disturbing the top of the resin. 0.1 mL of the hydrazine solution (2M) was added to top of column and incubated for 2 min. The column was eluted with 2.5 mL of HCl (0.07N).The eluate was placed in water bath at 37°C for 30 min. To this 0.1 mL of potassium borohydride solution (0.2M) was added and kept for 8 min, followed by the addition of 0.1 mL NaOH (2N). The reaction mixture was shaked and read immediately at an activation and emission wavelength of 370nm and 475 nm respectively. A standard acetylcholine as well as a reagent blank is run with each group of determinations.

6.2.3.2 Biogenic amines (Nor epinephrine, Dopamine, Serotonin)

The levels of NE, DA and 5–HT were estimated using High Performance Liquid Chromatography with fluorimetric detection (HPLC - FD) as developed by Lakshamana and Raju [349] and adopted with some modifications [350, 351]. Brain cortex was removed and homogenized in 1 mL of ice cold 0.1M perchloric acid. This was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was filtered through 0.45µm filter membrane and 100 mL of the filtrate was injected into HPLC pump. The mobile phase employed was of sodium acetate (0.02M), methanol (16%), heptane sulphonic acid (0.1375%), EDTA (0.2mM) and dibutylamine (0.01%). After adjusting the pHT to 3.92 ± 0.01 with O-phosphoric acid the mixture was filtered through 0.45µm membrane filter and degassed. The flow rate was set to 1mL per minute. The column was washed with quartz distilled water and then with 80% methanol and then equilibrated with sodium acetate buffer. After separation, NE, DA and 5–HT were detected at the excitation/emission wavelength of 280 nm/315 nm, while keeping the slit width 10/10. The separations of biogenic amines were done in isocratic condition at room temperature. The biogenic amines peaks were identified by comparing the retention

period of the peaks with that of standards. Stock solutions of standard (1mg/mL) were prepared in 0.1N HCl and stored at – 20°C and used within two weeks of preparation. The working standard solution was prepared freshly in 0.1M perchloric acid for each experiment. The amount of standard was 3 ng in each injection for each NE, DA and 5 – HT.

6.2.3.3 Acetylcholinesterase (AChE) Inhibition Activity

The acetylcholinesterase (AChE) enzyme inhibition activity was measured following the method of Ellman et al. [352]. Brain cortex was homogenized in phosphate buffer (0.1M) to obtain 10% w/v homogenate. To 0.2 mL of homogenate was taken and 3 mL of phosphate buffer and 0.1 mL of dithionitrobenzoic acid (0.01M) was added to it and kept for incubation for 5 min at room temperature. To this reaction mixture 20 µL of acetylthiocholineiodide (25mM) was added and absorbance was taken at 412 nm at an interval of 30 sec for 3 min. Blank tubes were used which consisted of buffer and dithionitrobenzoic acid while control tubes without acetylthiocholineiodide were also run simultaneously.

6.2.3.4 Estimation of lipofuscin content

Lipofuscin content was detected spectrofluorimetrically [353, 354]. Brain cortex was homogenized in 10 mL of chloroform: methanol (2:1 v/v). The homogenate was centrifuged for 20 min at 4000 rpm, the supernatant was removed and deionized water was added to it after thorough mixing the supernatant was centrifuged for 20 min at 6000 rpm. The methanol–water layer was pipetted out and deionized water was added to the lower chloroform layer. The sample was then centrifuged at 6000rpm for 20 min. The lower clear chloroform portion was collected using a syringe and processed for recording excitation (350 nm) and emission (445 nm) spectra in a spectrofluorimeter. Standard curve was obtained by running quinine sulphate in the range of 2–100 µg/mL.

6.2.3.5 Oxidative stress markers

6.2.3.5.1 Lipid Peroxidation (LPO)

The levels of lipid peroxidation were estimated by the method of Nichans and Samuelson [355]. To 0.1 ml of brain tissue sample, 1.5 ml of TCA (1.22M) was added and allowed to

stand at room temperature for 15 min. The tubes were centrifuged and the supernatant was removed to which 1.5 mL of thiobarbituric acid solution was added and kept in boiling water bath for 15 minutes. On cooling, 3.0 mL of chloroform was added and the tubes were mixed thoroughly by vigorous shaking and then centrifuged at 2000 g for 10 minutes. The chromopore formed was measured at 530 nm. A series of standard solution in the range 2-10 nM/mL were treated in a similar manner and the values were expressed as nM of TBARS/mg protein.

6.2.3.5.2 Total glutathione content (GSH)

Total glutathione content was estimated following the method of Ellman [356]. 0.1 ml of the sample was mixed thoroughly with 1.8 ml of precipitating reagent that contains metaphosphoric acid, EDTA and NaCl, this was allowed to stand for 10 minutes and centrifuged at 2000g for 15 min. 1.0 ml of the supernatant was removed and 4.0 ml of phosphate buffer (0.2M) along with 0.5 ml of dithionitrobenzoic acid (0.6mM) were added to it. Standard glutathione in the range of 20 – 100 μg was also processed similarly. The color developed was read at 412 nm and the total glutathione level was expressed as mg/100 mg of wet tissue.

6.2.3.5.3 Superoxide dismutase (SOD)

Superoxide dismutase activity was estimated spectrophotometrically following the method of Misra and Fridovich [357]. To 0.1 mL of the sample, 0.8 mL of ethanol and 0.1 mL of ice chilled chloroform were added and centrifuged. To 0.5 ml of the supernatant 0.5 mL of EDTA (0.6mM) and 1 mL of bicarbonate buffer (0.1M) were added and absorbance was taken at 480 nm. Further, 0.5 mL of epinephrine (1.8 mM) was added to this mixture and the readings were taken at an interval of 30 sec for 2-3 min at 480 nm. The values were expressed as IU/mg protein.

6.2.3.5.4 Catalase

The activity of catalase was determined by the method of Sinha [358]. To 0.1 ml of tissue homogenate 3.0 mL of phosphate buffer (0.01M) and 0.2 ml hydrogen peroxide (0.2M) was added and mixed well. The reaction was stopped at the interval of 30 and 60

seconds by the addition of 1 ml dichromate-acetic acid reagent. The tubes were kept in boiling water bath for 10 minutes on the colour development the tubes were read at 620 nm. H_2O_2 standards (2-10 μM) were taken and treated similar to the test with a blank containing reagent alone. The enzyme activity was expressed as μM of H2O2 consumed/minute/mg of protein.

6.2.3.6 Active avoidance learning

Rats were trained for an active avoidance task before subjecting them to stress. During training, the rat was placed in the right electrified compartment of a shuttle box (Techno) and allowed to acclimatize for 5 min. Thereafter, the animal was subjected to 15 sec of a buzzer stimulus (CS) which was followed by electric shock (1 mA, 50 Hz) given through the grid floor (UCS). The rats were given at least 10 trails, with an inter-trial interval of 60 min, until they reached the criterion of 100% avoidance response of jumping to the unelectrified left chamber of the shuttle box during CS. The test was repeated on day 14^{th} and 28^{th} day in order to assess the retention of the active avoidance learning [360].

6.2.4 Statistical Analysis

All results were presented as mean ± SD. The intergroup variation was measured by one way analysis of variance (ANOVA) followed by Dunnett's and LSD *Posthoc* analysis through SPSS software. The statistical significance was considered at $P<0.05$.

6.3 Results

6.3.1 Effect of Polyherbal formulation on brain neurotransmitters

Neurotransmitters are required for proper brain memory functioning. In the present study reduced levels of neurotransmitters such as acetylcholine, serotonin, dopamine, and nor-adrenaline were found in aged rats. On treatment with polyherbal formulation the aged rats exhibited betterment in neurotransmitters levels which was dose dependent. However the increase was nearer to that of the normal control rats. Results are shown in Figure 6.1 the level of significance were $P<0.05$ and $P<0.01$ in treated groups compared to that of disease control group.

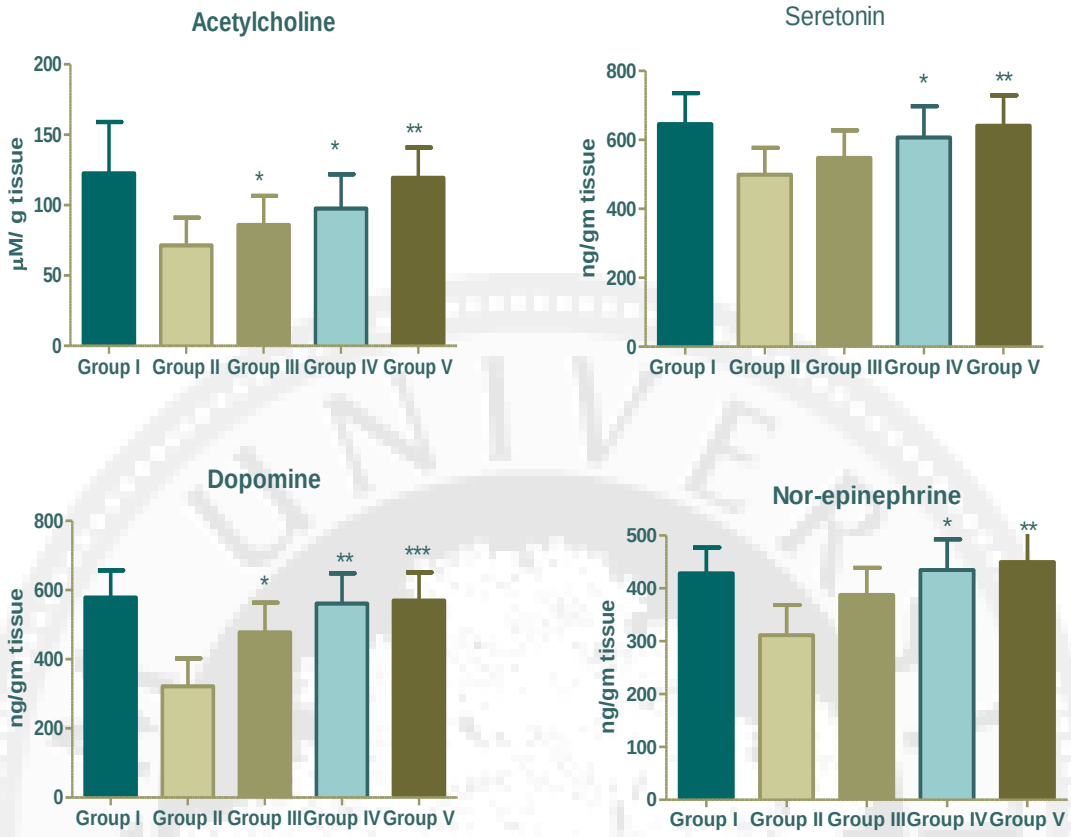

Figure 6.1 Concentration of neurotransmitters content in the cortex of young and aged treated or untreated rats. Values were expressed as mean ± SD of six animals in each group. Level of significance as calculated by one way ANOVA followed by *Posthoc* LSD.*$P < 0.05$,** $P < 0.01$ and***$P < 0.001$ for aged vs aged treated group.

6.3.2 Effect of Polyherbal formulation on antioxidant levels

Antioxidant enzymes like SOD, catalase, GSH are required for intracellular protection against oxidative stress. GSH is also one of the prime antioxidant enzymes in cell defense system. The levels of SOD, GSH and catalase along with TBARS to find the antioxidant index inside the brain tissues of aged rats. The results are shown in Figure 6.2. Aged rats showed 2-3 fold increase in TBARS level compared to control and treated group. The antioxidants were significantly (P<0.05 & P<0.01) higher in treated groups compared to aged induced rats.

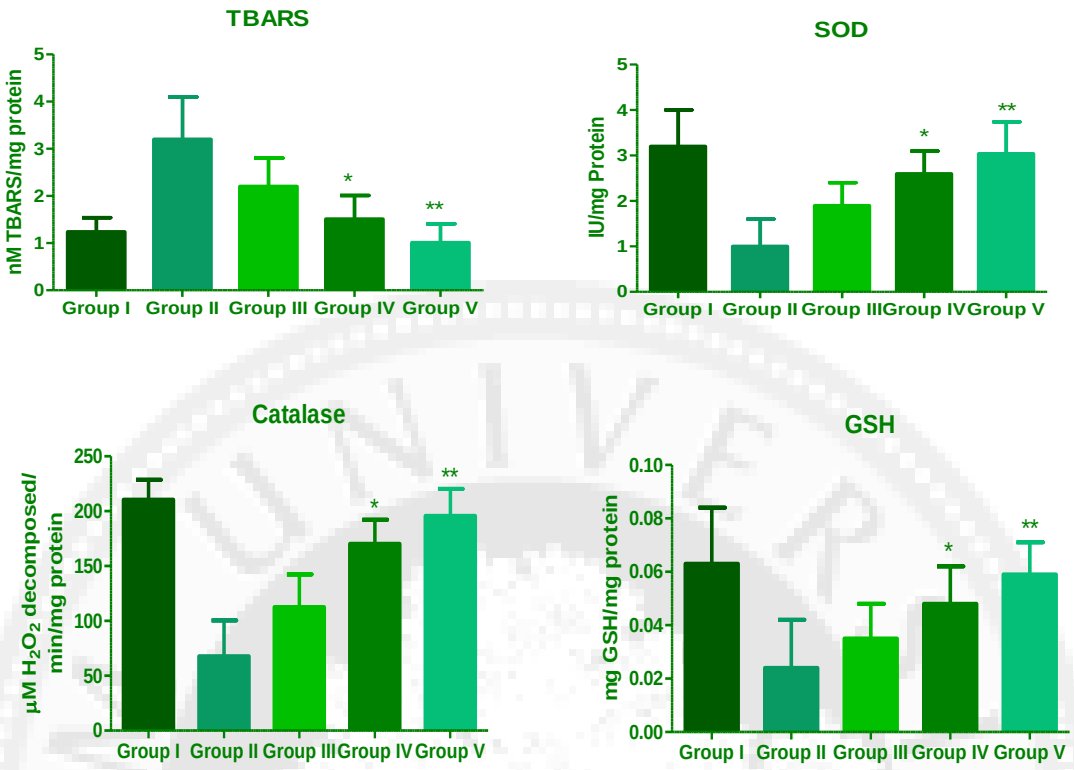

Figure 6.2 Concentration of antioxidant enzyme levels in the cortex of young and aged treated or untreated rats. Values were expressed as mean ± SD of six animals in each group. Level of significance as calculated by one way ANOVA followed by *Posthoc* LSD *$P < 0.05$,** $P < 0.01$ and***$P < 0.001$ for aged vs aged treated group.

6.3.3 Effect of Polyherbal formulation on cognitive function

Cognitive dysfunction was prominent in aged rats which significantly reduced the conditioned avoidance response (CAR) by 44.13% and increased unconditioned avoidance response (UAR) 3-4 folds compared to the normal group. Treatment with polyherbal formulation significantly increased CAR decreased UAR. The increase was dose dependent and showing highest CAR of 88.6% for 300mg.kg body wt of polyherbal formulation the results are given in Table 6.1.

Table 6.1 Effect of polyherbal formulation on Cognitive function.

Treatment groups	CAR%	UAR%
Group I	91.7±1.8##	9.12±1.24##
Group II	49.6±4.7	44.13±2.85
Group III	70.9±2.8*	22.12±3.11*
Group IV	81.7±3.4**	13.42±2.02**
Group V	88.6±2.1**	10.12±1.35**

Conditioned avoidance test was performed 30 min after treatment on the 28th day in trained rats. Value is represented as mean ± SD. One-Way ANOVA followed by Dunnett's multiple comparisons test.* P<0.05 aged vs. aged treated.

6.3.4 Effect of Polyherbal formulation on AchE level

The acetylcholinesterase activity was evaluated since acetylcholinesterase inhibitors antagonize drug induced amnesia. The effect of polyherbal formulation on AchE activity is given in Figure 6.3. Aged rats irregular brain AchE activity as compared with the normal control group. Administration of polyherbal formulation helped in normalizing the AchE activity in treated group respectively as compared to control group.

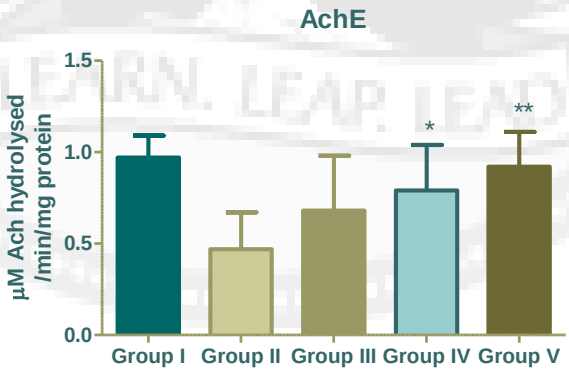

Figure 6.3 Concentration of AchE enzyme activity in the cortex of young and aged treated or untreated rats. Values were expressed as mean ± SD of six animals in each group. Level

of significance as calculated by one way ANOVA followed by *Posthoc* LSD.*$P < 0.05$,** $P < 0.01$ and***$P < 0.001$ for aged vs aged treated group.

6.4.5 Effect of Polyherbal formulation on Lipofuscin level

The effect of polyherbal formulation on lipofuscin content is given in Figure 6.4. Aged rats showed increased brain lipofuscin accumulation compared with the normal control group. Administration of polyherbal formulation decreased the lipofuscin accumulation by 27.24%, 46.40% and 59.24%, respectively as compared to control group.

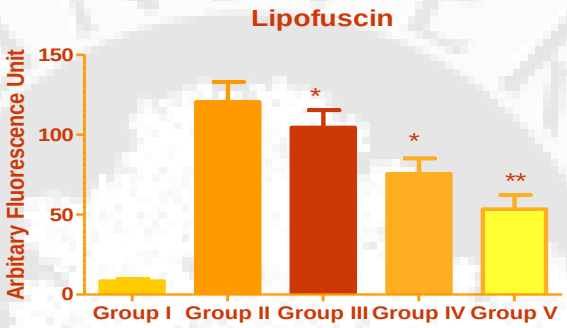

Figure 6.4 Concentration of lipofuscin content in the cortex of young and aged treated or untreated rats. Values were expressed as mean ± SD of six animals in each group. Level of significance as calculated by one way ANOVA followed by *Posthoc* LSD.*$P < 0.05$,** $P < 0.01$ and***$P < 0.001$ for aged vs aged treated group.

6.4 Discussion

The *In vivo* study demonstrated the neuroprotective mechanism of action of polyherbal formulation for promotion of healthy brain ageing and its implications in order to prevent the SDAT progression in aged sprauge dawley rats.

Age being prime risk factor for plethora of age related neurodegenerative disorders including Senile Dementia of Alzheimer‘s Type (SDAT) and SDAT progresses on accelerated ageing, which has been supported by many hypothesis [361]. Since no curative treatment options exist for age associated complications and neurodegenerative disorders like SDAT, long term neuroprotection has emerged as one of the essential therapeutic strategy to promote successful ageing and prevent SDAT.

The results of the present study demonstrated the anti–ageing property of the polyherbal formulation which was noted from the significant decrease in the levels of lipofuscin content in aged rat brain cortex. Lipofuscin is a yellow brown auto-fluorescent pigment, which accumulates progressively with age and this accumulation is hazardous to cellular functions. Lipofuscin accumulation has been found to increase the sensitivity of aged neurons to oxidative injury [362].

An impaired cholinergic neurotransmission was found with ageing which was indicated by the reduction in acetylcholine content in aged rat's brain as observed in the present study. The reduction may be attributed either to the decline in muscarinic acetylcholinergic receptors activity or acetylcholine synthesizing enzyme cholineacetyltransferase activity due to dsyregulated choline biosynthesis [363]. Similarly, an age-related decrease in AchE activity was observed in the present study as explained by the previous research works on ageing brains [364], as the AchE content is linearly correlated with the acetylcholine content. Thus the present findings confirmed an age related cholinergic degeneration with ageing which contributes for the deficits in memory and learning among the aged [365].

Polyherbal formulation significantly prevented this age associated alterations in monoaminergic neurotransmission in aged rats. Previous research findings have demonstrated that bacosides play a significant role in the modulation of mono-amerigic amines 5-HT and DA content in middle aged and aged rats with least effect in the levels of NE [366].

In the present study it was well illustrated that disruptive antioxidant status in the aged brain which was noticed with increased lipid peroxidation and reduced activity of endogenous antioxidant enzymes. Several studies evidenced that progressive oxidative damage is a conserved, prime mechanism for functional decline in aged [367]. Excessive level of oxidative stress significantly contributes to the neuronal loss and thus alleviating the age related neurochemical and cognitive deficits as well as increased susceptibility to develop SDAT. In the present study, increased levels of lipid peroxidation products was found to be elevated in aged rat brain cortex, which was in line with the previous studies [368, 369]. Along with excessive lipid peroxidation, a considerable decline in the levels of

GSH levels in aged rats. In addition, a significant decrease in the levels of SOD, GPx and catalase were also noticed in the aged brain reports, which stand with the previous reports [370, 371].

The plants present in the polyherbal formulation are well known for its antioxidant activity and also earlier studies have reported *B. monnieri* extract to exhibit potent anti–lipofuscinogenic property in aluminium mediated neurotoxicity and in brains of aged rats respectively [372] and the present study results was in confirmation with these reports. Polyherbal formulation significantly prevented the depletion of acetylcholine content in aged rat brain cortex. Bhattacharya et al in his study has demonstrated that the acetylcholine enhancing activity of bacosides present in *B. monnieri* was attributed to its cholineacetyltranferase activity [373].

The cognitive enhancing effect of *B. monnieri* was due to its active component bacoside A, Bacosides induce membrane dephosphorylation, with a lateral increase in protein and RNA turnover in specific regions of the brain as studied by Singh et al, and it has also shown to enhance protein kinase activity in the hippocampus which could also bestow to its nootropic action. Thus the modulation of neurotransmission system on treatment with polyherbal formulation will also contribute in the delaying of ageing progression, as the reduction in lipofuscin content was inversely correlated with enhanced monoaminergic neurotransmission aged rats respectively

In the present study treatment with polyherbal formulation significantly prevented the age associated depletion in the levels of antioxidant enzyme in aged rats which was similar to the report as described by Anabarsi and his co-workers in 2006 stated that treatment with bacoside was found to increase antioxidant level with superoxide quenching activity [374]. The activity of GPx was found to be significantly elevated in aged rat brain, which was consistent with the previous reports by Bhattacharya et al. *H. rhamnoides* being rich in antioxidants and *D. bulbifera* possessing phytoestrogens act as excellent antioxidant and protect the neurons from oxidative stress induced damage and increase the antioxidant levels in the brain.

6.6 CONCLUSION

The outcome of the study proved that the anti-ageing, neuroprotective and cognitive enhancement activity of polyherbal formulation was the synergistic property of its active biomolecules. The formulation would prevent degeneration of neurons from oxidative stress and improve aged induced decrease in neurotransmitter levels which in turn improve the cognitive function. Hence, it may be advocated to treat age related neurodegenerative disorders.