EFFECT OF TURMERIC ON SCOPOLAMINE INDUCED CHOLINERGIC HYPO-FUNCTION IN MICE

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A thesis submitted in partial fulfilment of the requirement for the degree of Masters of Science

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بِسْمِ ٱللَّهِ ٱلرَّحْمَٰنِ ٱلرَّحِيمِ

فَتَبَسَّمَ ضَاحِكًا مِّن قَوْلِهَا وَقَالَ رَبِّ أَوْزِعْنِيٓ أَنْ أَشْكُرَ نِعْمَتَكَ ٱلَّتِيٓ أَنْعَمْتَ عَلَيَّ وَعَلَىٰ وَٰلِدَيَّ وَأَنْ أَعْمَلَ صَٰلِحًا تَرْضَىٰهُ وَأَدْخِلْنِي بِرَحْمَتِكَ فِي عِبَادِكَ ٱلصَّٰلِحِينَ ﴿١٩﴾

There upon he smilingly laughed at her works, and said, "**O my Lord, grant me guidance that I may be grateful for Your favors which You have bestowed upon me and my parents and that I may do such good works as would please You and admit me out of Your Mercy among Your those bondmen who deserve Your proximity.**" **(Chapter Naml, Ayat no. 19)**

Dedication

ALL MY ACHIEVEMENTS ARE DEDICATED TO MY GRAND PARENTS, PARENTS AND SIBLINGS, FOR THEIR ENDLESS LOVE, SUPPORT AND ENCOURAGEMENT.

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TABLE OF CONTENTS

Title — Page No.
Acknowledgements — V
Table of contents — Vii
List of acronyms — X
List of tables — Xii
List of figures — Xvi
Abstract — 1

Chapter 1

Introduction — 2

1.1 Research objectives — 4

Chapter 2

Literature Review — 5

2.1 Cholinergic system and its function — 5
- 2.1.1 Synthesis of Ach — 7
- 2.1.2 Nicotinic receptors — 8
- 2.1.3 Muscarinic receptors — 10
- 2.1.4 Importance of Hippocampus in cognitions — 11

2.2 Cholinergic system dysfunctions in neurodegenerative diseases — 13
- 2.2.1 Cholinergic dysfunction and Alzheimer's disease — 14

2.3 Models of cognitive impairment — 15
- 2.3.1 Scopolamine — 16

2.4 Treatment of cholinergic dysfunction — 17
- 2.4.1 Potential of novel drug targets for cholinergic system — 17
- 2.4.2 Synthetic Therapeutic Agents — 17
- 2.4.3 Donepezil and Alzheimer's Diseases — 19

2.5 Potential Of Medicinal Plants 20

2.5.1 Therapeutic Applications of Turmeric 21

2.5.2 Bioavailability of Turmeric 22

2.5.3 Turmeric and Neurodegenerative diseases 23

Chapter 3

Material and Methods 24

3.1 Drugs and chemicals 24

3.2 Ethical Statement 24

3.4 Study Design 25

3.5 Animal groups for study 26

3.5.1 Scopolamine and Normal saline

3.5.2 Oral Administration of Turmeric and Donepezil 28

3.6 Behavioral testing 29

3.6.1 Morris water maze test 29

3.5.2 Social preference test 31

3.7 Mouse decapitation and brain harvesting 32

3.7.1 RNA. extraction 32

3.8 Quality and quantification of RNA 34

3.8.1 Quality of the RNA 34

3.8.2 Reverse transcription polymerase chain reaction 35

3.8.3 Quantification of mRNA levels by RT-PCR 35

3.8.4 DNA contamination check 36

3.8.5 Gene expression study mRNA levels 37

3.9 Protein sample preparation 39

3.9.1 Protein estimation 39

3.9.2 Protein separation by SDS-PAGE 39

3.9 .3 Statistical analysis 40

3.10 Histology of brain tissues 41

3.10.1 Perfusion Fixation 41

3.10.2 Brain Fixation and Paraffin Embedding 42

3.10.3 Tissue Sectioning 43

3.10.4 Nissl (cresylviolet) staining 43

3.11 Nuclear Magnetic Resonance (NMR) Spectroscopy

Chapter 4

Results 45

4.1 Effect of turmeric on physical parameters 45

4.1.1 Weight variation 45

4.2 Effect of turmeric on behaviors 47

4.2.1 Effect of Turmeric on memory and Cognitive Functions 47

4.2.2 Effect of Turmeric on Social Preference 55

4.3 Effect of turmeric on mRNA levels 60

4.3.1 Effect of Turmeric on nAChR genes in hippocampus 61

4.3.2 Effect of Turmeric on mAChR genes in hippocampus 64

4.4 Protein profiling 66

4.4.1 Protein Quantification 66

4.4.2 Total Differential Proteome Profile 67

4.4.3 Effect of Turmeric on Protein Expression 68

4.5 Histological Studies 70

4.5.1 Cresyl Violet Stain 70

Chapter 5

Discussion 75

Conclusion 81

Future Directions 82

Chapter 6

References 82

LIST OF ACRONYMS

%	Percentage
µL	Micro Litre
µm	Micrometer
ACh	Acetylcholine
AChE	Acetylcholine Esterase
AD `	Alzheimer's Disease
Aβ	Amyloid Beta
ANOVA	Analysis of Variances
APP	Amyloid Precursor Protein
APPS	secreted APP
BBB	Blood brain Barrier
ChAT	Choline Acetyl Transferase
cm	Centimeter
CSF	Cerebrospinal Fluid
DEPC	Diethylpyrocarbonate
DG	Dentate Gyrus
DI	Discrimination Index
DNA	Deoxyribonucleic Acid
Hrs	Hours
Kg	Kilo-Grams
mAChRs	Muscarinic Acetyl Choline Receptors
mg	Milligram
mL	Millilitre
mm	Millimetre
mM	Millimolar
MWM	Morris Water Maze
n	Number of Variables
nAChRs	Nicotinic Acetyl Choline Receptors

nbm	Nucleus Basalis of Meynert
NFT	Neurofibrillary Tangles
NIH	National Institute of Health
NMDA	N-Methyl D-Aspartate
NMDAR	N-Methyl D-Aspartate Receptor
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
Rcf	Relative Centrifugal Force
RNA	Ribonucleic Acid
rpm	Rotation per Minute
RT	Reverse Transcriptase
RT-PCR	Reverse Transcriptase- PCR
sec	seconds
SEM	Standard Error Mean
w/v	Weight by Volume

LIST OF FIGURES

Figure No	Title	Page No
Figure 2.1	Diagrammatic presentation of Cholinergic neurons in brain	7
Figure 2.2	Schematic representation of Cholinergic synapse	9
Figure 2.3	Structure & signaling pathways of mAChRs and nAChRs	12
Figure 2.4	Aberrant proteins production in the neurodegenerative diseases	15
Figure 2.5	Chemical Structure of Scopolamine	17
Figure 2.6	Chemical Structure of Donepezil	20
Figure 2.7	Chemical Structure of turmeric isolated compounds	23
Figure 3.1	Study plan for the development of cholinergic hypo-function model	26
Figure 3.2	Schematic presentation of Drug treated groups	28
Figure 3.3	Subcutaneous Injection	29
Figure 3.4	Morris Water Maze (MWM) Apparatus	32
Figure 3.5	Apparatus for Social Interaction Test	34
Figure 3.6	RNA samples on 2% agarose gel	36
Figure 3.7	PCR Profile for gene expression	38
Figure 3.8	ImageJ, NIH software, for the quantification of band intensity	40
Figure 3.9	Perfusion Fixation	44
Figure 4.1	Percent weight variation graph	47
Figure 4.2	Effect of turmeric and donepezil on learning and memory using Morris water maze (MWM) test	50

Figure 4.3	Comparison between groups on 3rd, 4th and 5th day of Morris water Maze Test	51
Figure 4.4	Effect of turmeric and donepezil on reference memory	52
Figure 4.5	Representative pathway effect of turmeric donepezil on reference memory (MWM Test, Probe Trial).	53
Figure 4.6	Cartoonish presentation social preference novelty test	55
Figure 4.7	Effect of turmeric and donepezil on sociability and social novelty	56
Figure 4.8	Discrimination Index (DI) % of sociability and social novelty	57
Figure 4.9 (a)	RNA Gel Electrophoresis Image	58
Figure 4.9 (b)	Gel electrophoresis image PCR products	59
Figure 4.10	Effect of turmeric and donepezil on expression of nAChRs genes in hippocampus	60
Figure 4.11	Effect of turmeric and donepezil on expression of mAChRs genes in hippocampus	62
Figure 4.12	Bradford standard curve	63
Figure 4.13	The SDS-PAGE image of hippocampus proteins	64
Figure 4.14	Effect of turmeric and donepezil on expression of proteins in hippocampus	65
Figure 4.15	The cortical image of the mouse brain	67
Figure 4.16	Effect of turmeric and donepezil on cell density in cortex at 20X magnification	67
Figure 4.17	Effect of turmeric on cell density in C3 area of cortex	68
Figure 4.18	Effect of turmeric and donepezil on neuronal cell density in cortex	69

LIST OF TABLES

Table No	Title	Page No
Table 2.1	Schematic representation of distribution and function of nAChRs and mAChRs	13
Table 3.1	Morris Water Maze table for direction of release	31
Table 3.2	List of primers along with their thermal cycling conditions	39

Abstract

Cholinergic system plays an important role in higher cognitive functions and its chief modulator is acetylcholine. These neurons form a widespread network in brain regions. With passage of time various factors deteriorates their functions, which results in cholinergic dysfunction and ultimately cause cognitive impairment. Cholinergic dysfunction is a major cause in several neurodegenerative diseases. Turmeric is a yellow spice, also used as herbal medicine. Due to its neuro protective action, it has profound pharmacological effect in various central nervous system disorders. Donepezil hydrochloride is an acetylcholinesterase inhibitor (AChEI), is currently used for Alzheimer's disease (AD). The conformation of turmeric and donepezil can potentially rescue cholinergic hypo function. Therefore, the aim of this study is to observe synergistic effect of Turmeric (20mg/Kg/day) and donepezil (0.5mg/Kg/day) treatment on cognitive function and gene expression in scopolamine (1mg/Kg) induced amnesic mouse model. Scopolamine was used to induce cholinergic hypo-function in BALB/c mice. The effect of turmeric and donepezil on memory was investigated by Morris water maze (MWM) and social interaction test. In 5th day of MWM, Scopolamine + Turmeric + Donepezil group (5.29 ± 0.73) showed significant ($p < 0.001$) decrease in escape latency relative to scopolamine (24.9 ± 2.82) and control (18.37 ± 2.71) groups. Similarly in social preference test, Scopolamine + Turmeric + Donepezil treatment group (210.75 ± 45.23) displayed significant ($p < 0.001$) increase in sociability and social novelty as compare to scopolamine group (63.25 ± 14.44). The study also includes evaluation of the effect of turmeric powder on the expression of Nicotinic Receptors (nAChRs) like (α-7, α-4, and β-2) and Muscarinic Receptors (mAChRs) (M1, M3, and M5) in hippocampus of mice brain. Turmeric significantly increases ($p < 0.05$) the expression of α-7, α-4, β-2, M1, M3, and M5. Whereas, in Scopolamine + Turmeric + Donepezil also increase the expression but the difference in expression values was not significant. In protein profiling, the Scopolamine + Turmeric + Donepezil group has shown significant ($p < 0.01$) increase in protein expression at 35KDa, 55KDa and 70KDa as compare to Scopolamine and control. In histological studies, Scopolamine + Donepezil showed significant ($p < 0.05$) increase in neuronal cell density in relative to scopolamine. Thus, in the light of these findings

turmeric may serve as a potential candidate in improving brain functions and cognitive properties. Turmeric with donepezil's synergistic capability can be a potential therapeutic and preventive strategy for cognitive deficits. It can also act as a therapeutic option for psychiatric disorders and neurodegeneration disease.

INTRODUCTION

Cholinergic is major excitatory systems of the central nervous system. It is main targets for causing cognitive impairments and cellular dysfunction. Acetylcholine is chief neurotransmitter of the cholinergic system, that is involved in number of brain functions like learning, memory, attention, cognition, circadian cycles, motor functions (Gold, 2003). The cholinergic system play an important role in processing of new memory Foster (1999), and in attention (Sarter and Bruno, 1997). Central cholinergic systems dysfunctions due to various factors like environmental and genetic. Neurodegeneration leads to wide number of neuropsychiatric disorders and diseases, such as Alzheimer´s disease (AD), Parkinson's disease, Huntington's disease or psychological trauma (Francis et al., 1999, McKeith 2004). Central cholinergic receptors can be block with synthetic agents to stimulate pattern of cognitive decline and neurodegeneration (Buccafusco, 2000). Scopolamine is a synthetic anticholinergic drug that causes 'Cholinergic dysfunction'(Hulme et al., 1978) and induces cognitive impairments in animals and human (Pandit and Dundee, 1970, Klinkenberg and Blokland, 2010). Previous studies have demonstrated that muscarinic and nicotinic receptor blockade impairs attention and memory (Chen et al., 2004, Rezvani et al.,2002; Turchi et al., 1995;). Scopolamine, anti-cholinergic agent is commonly used to study the cholinergic receptor subtypes in screening memory enhancing drugs (Blokland, 2005). Prior studies also shown that deficits in cholinergic neurotransmission is a chief factor in age-related memory impairments and many other neurological disorders (Bartus et al., 1982, Aubert et al., 1995, Araujo et al., 2005)). Cognitive functions like learning and memory are facilitated by muscarinic receptors functions (Quirion et al., 1995, Van der Zee and Luiten, 1999, Rowe et al., 2003). The blockade of the muscarinic receptors with scopolamine treatment leads to the memory impairment and decline in neurons activity (Aubert et al., 1995, Schon et al., 2005). 1mg/Kg dose of scopolamine is sufficient to produce sedative effects in learning and memory impairment (Kim et al., 2009, Choi et al., 2012, Falsafi et al., 2012). To enhance the cholinergic function and reverse the cognitive impairments, two strategy are used; one is to stimulate the cholinergic receptors directly by cholinergic agonists, while the other alternative is modulating Acetylcholinesterase (AChE) by its anta-agonist. Donepezil hydrochloride is an acetylcholinesterase inhibitor

(AChEI), presently used for Alzheimer's disease (AD). Donepezil that modulates nAChR activity and promote neurogenesis. It also prevented nuclear fragmentation and glutamate-induced apoptosis *via* α4β2 and α7 nAChRs (Takada, et al., 2003). Furthermore, many studies have shown that donepezil induced upregulation of nicotinic receptors (nAChRs) (Akaike et al., 2010; Wang et al., 2005). Previous studies shown that nAChRs plays crucial role in neuroprotection through the involvement of the phosphatidylinositol 3-kinase (PI3K) pathway, via modulating AChE-I (Akaike et al., 2010; Takada-Takatori et al., 2009). Therefore, neuroprotection exerted by nAChR agonists could be useful in the treatment of neurodegenerative disorders. (Shen et al., 2010). The effect of donepezil with antioxidant plants in the pathology of neurological diseases are not fully determined.

Various medicinal plants have neuroprotective capability to cure cognitive impairments (Kannappan et al., 2011). The herbs produce antioxidant effect, anti-inflammatory effect , by modifying or by inhibiting the esterase enzyme (Akhondzadeh and Abbasi, 2006a). Among a range of herbs, Turmeric *(Curcuma longa)* is one of the candidate used to inhibit neurodegeneration by its antioxidant activity (Sreejayan and Rao, 1997). (Joshi and Parle, 2005) (Joshi and Parle, 2005) (Joshi and Parle, 2005) Turmeric has high medicinal values in traditional medicine system of South Asia which includes: aiding in wound healing, inflammatory conditions, and blood purification (Aggarwal et al., 2007 Kapoor, 1990; Krishnaswamy, 2008; Nadkarni, 1976). These listed studies indicate that turmeric has potential in multiple pathological states. Furthermore, according to the studies conducted in animals (Qureshi et al., 1992; Shankar et al., 1980) and human subjects (Lao et al., 2006) turmeric is considered safe if used in large concentration. Due to its neuroprotective action, it plays an active role in the treatment of various central nervous system disorders (Chainani-Wu, 2003, Ghadami et al., 2012). Although, underlying pharmacological mechanisms of many cholinergic dysfunction disorders are not yet understood. There is no single therapeutic approach due to complexed pathophysiology of these disorders. A combination of drugs having antioxidant, anti-inflammatory and cholinergic properties is used for their symptomatic treatment (Stewart et al., 1997, Hager et al.,2001, Herholz et al., 2004). Many plants have neuroprotective role (Kannappan et al., 2011), but evidence that turmeric with AChEIs have synergistically reverse cholinergic impairment and promotes cell proliferation has not be confirm in many

studies. Therefore, we have taken the opportunity to investigate the effect of turmeric extract in combination with standard drug, donepezil on cognitive functions and memory in cholinergic depleted mouse model. We hypothesized that turmeric and donepezil will demonstrate synergistic effect on behavioral impairments of the cholinergic dysfunction mice model. The effect of turmeric powder and donepezil on nicotinic and muscarinic receptors was also studied to ensure its potential role in modulating the cholinergic receptors in neurodegeneration. As turmeric has an incredible medicinal value, before it is used as pharmacological drug; it functional characteristics underlying neuronal signaling mechanism must be elucidate experimentally. This study will significantly aid to determine turmeric's future as healthful therapeutic compound.

Research Objectives

The objectives of the study were

- To study the effect of Turmeric and donepezil on learning, memory and cognitive functions in scopolamine induced cholinergic hypo-function mice model via behavior tests.
- To study the expression of nicotinic ($\alpha 7$, α 4 and $\beta 2$) and muscarinic receptors (M1, M3 and M5) in hippocampus in turmeric and donepezil treated mice model.
- Histological studies of brain tissue to observe the neuronal cells density.
- To investigate the effect of Turmeric and donepezil on the differential protein expression in hippocampus.

Literature Review

2.1 Cholinergic System and Its Function

Acetylcholine (ACh) is the main neurotransmitter in the central (CNS) and peripheral (PNS) nervous system. ACh synthesizing neurons are known as cholinergic neurons (M Tata et al., 2014) . These neurons form a widespread network in ascending area, where the cell bodies are densely present, and lesser are projecting towards the descending area. They are found in forebrain, midbrain and brainstem. The cholinergic neuronal inputs in hippocampus arise from medial septum and diagonal band of Broca through transverse fiber of the fimbria-fornix pathway (Fig 2.1) (Dutar et al., 1995, Cobb et al., 1999). The projecting neurons in septo-hippocampal area play a vital role in hippocampus functions like mediating neuronal network activity and particularly theta-rhythm oscillations in memory processing functions(Stewart and Fox, 1990, Buzsáki, 2002).

In the nervous system, ACh is widely spread that play an important role in functions like brain's activity, development, regulating blood flow in the brain regions, and circadian cycle. It is also involved in maintaining cognitive functions and attention, learning and mnemonic processes (Lamirault et al., 2003). It has vital role in modulating the neuronal circuits by developing synaptic connections between the cells that are further involved in major cognitive functions. (Berger-Sweeney 2003). These neurons with time (or other factors) experienced the deterioration which results in cholinergic hypo-function and ultimately cause cognitive impairment (Schliebs et al., 2006, Bigl 1990, Härtig 2002). There are many factors responsible for cholinergic degenerations mechanism, each can coexist with one or more factors like (1) excitotoxic injury; (2) growth factor deprivation; (3) oxidative stress; (4) inflammation; (5) mitochondrial dysfunction; and (6) beta-amyloid toxicity (Amenta and Tayebati, 2008).

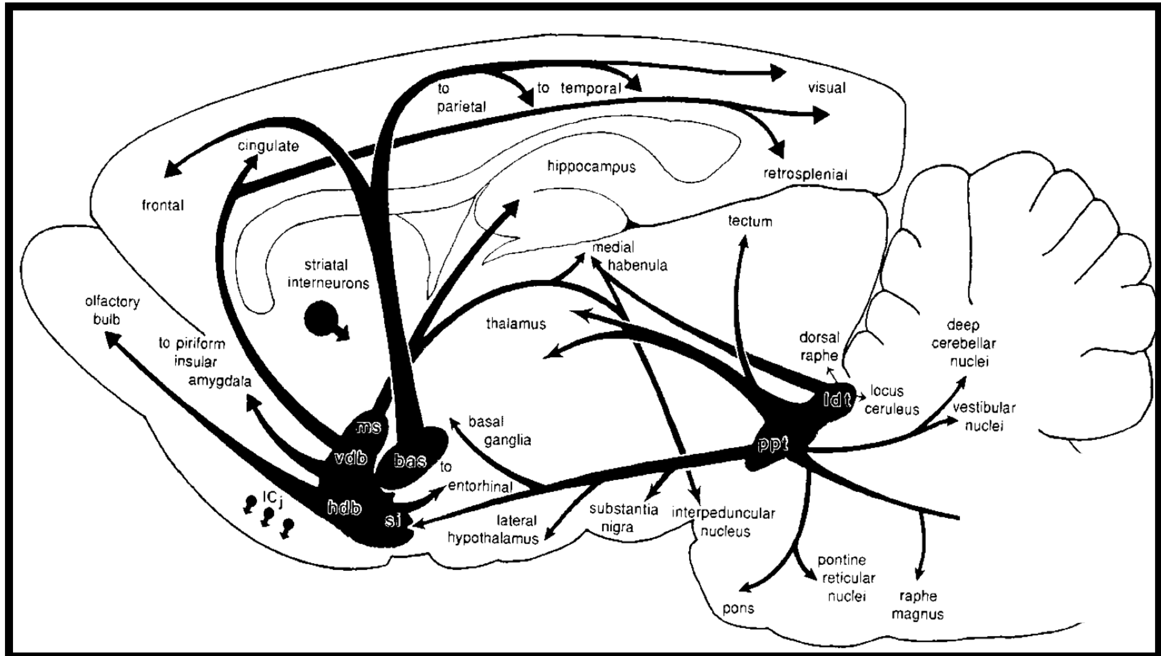


Figure. 2.1: The diagrammatical representation of cholinergic neurons in the major regions of the brain. Cholinergic neurons are projecting in the basal forebrain, including those in the medial septal nucleus (ms), vertical diagonal band nucleus (vdb), horizontal diagonal band nucleus (hdb), substantia innominata (si), and nucleus basalis (bas), project to the entire cerebral cortex, hippocampus, and amygdala. Adapted from (Woolf, 1997).

2.1.1 *Synthesis of ACh*

ACh is synthesized from choline and acetyl CoA by the choline acetyltransferase (ChAT) enzyme and stored in presynaptic vesicles as shown in fig 2.2. The synthesized ACh is packed in presynaptic vesicles by vesicular acetylcholine transporter (VAChT) and then released into synaptic cleft. ACh acts on two types of receptors: Nicotinic acetyl-choline receptors (nAChR) and muscarinic acetylcholine receptors (mAChRs). After its action on receptors, the Ach hydrolyzed into choline and acetate by acetylcholinesterase (AChE), this reaction is important for immediate and quick action of neurotransmitter. Half of the choline is derived from hydrolyzed ACh which was recovered by a presynaptic high-affinity choline transporter (CHT) where it aids in ACh release and production (Amenta and Tayebati, 2008). The acetylcholinesterase (AChE) enzyme specifically degrades ACh secreted from the pre-synaptic terminals and its action is necessary for a rapid and short lasting action of the cholinergic neurotransmitter. (M Tata et al., 2014)

In the mammalian brain, as well as in other target tissues, ACh binds to two classes of receptors: the metabotropic muscarinic ACh receptors (mAChRs) and the ionotropic nicotinic ACh receptors (nAChRs).

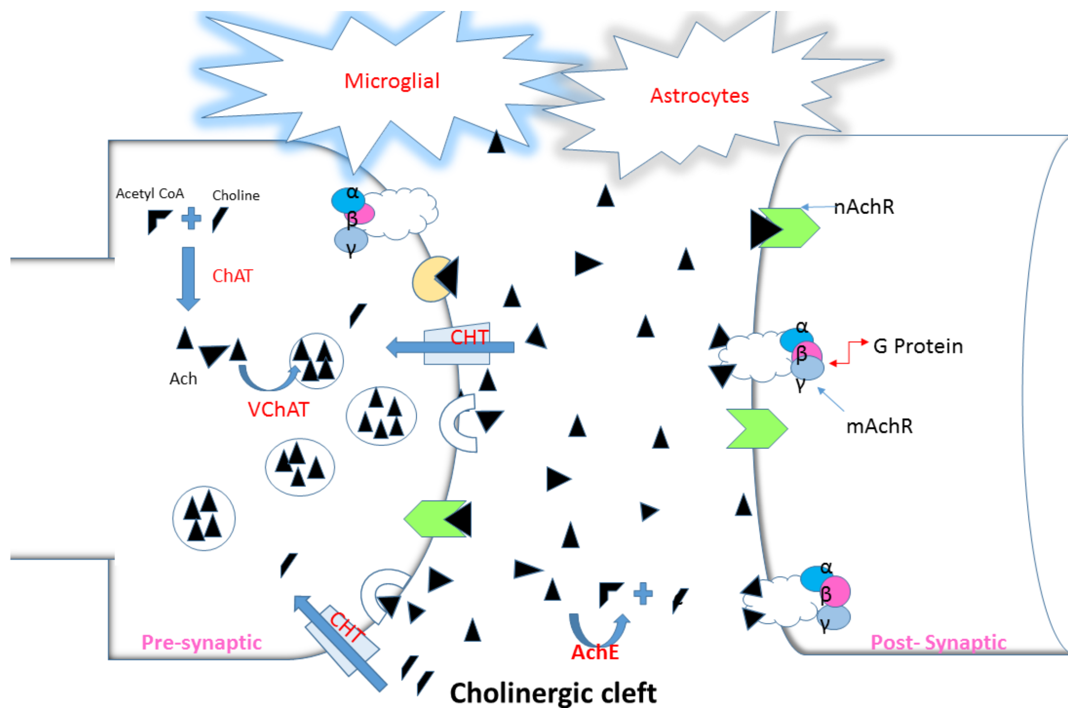


Figure 2.2: Schematic representation of a cholinergic synapse summarizing mechanisms of neurotransmitter synthesis, storage, and release. ACh: acetylcholine; AChE: acetylcholinesterase; Acetyl-CoA: acetyl coenzyme A; Ch: choline; ChAT: choline acetyltransferase; CHT: choline high affinity transporter; VAChT: vesicular acetylcholine transporter.

2.1.2 *Nicotinic receptors*

Throughout the CNS the nicotinic receptors are located in pre synaptic, post synaptic membranes and extra synaptic area. They are ionotropic receptors, which have 5 subunits (pentamers) positioned around a central hole, forming an ion channel. These subunits are about 280 KDa molecular mass and consist of two α subunits and one β, γ and δ subunit (Toews and Morell, 1999). nAChR are further divided into two different classes on the basis of Ca^{2+} permeability that is important for pharmacological, functional and structural properties, these classes are as follow:

(1) **Homopentameric nAChRs** containing subunits (α 7– α 9) that exhibit the highest measured Ca2+ permeability values.

(2) **Heteropentameric nAChRs**, always comprising at least one α (out of α 2– α 6) and one β (out of β 2– β 4) subunits, with lower measured Ca2+ permeability (Dajas-Bailador

and Wonnacott, 2004, Fucile, 2004). These combinations results in unique structural and functional properties (Fenster et al., 1997, Morris et al., 2003, Nai et al., 2003) In brain the α4β2 are the most commonly present (Cooper et al., 1991, Seguela et al., 1993, Zarei et al., 1999) whereas α7 is prevalent in the hippocampus, present at multiple loci (glutamatergic and GABAergic) presynaptic and postsynaptic nerve terminals. α7 showed high level of Ca^{2+} ion permeability which is important for neurotransmitter release and neuronal plasticity. (Wonnacott, 1997).

2.1.2.1 *Distribution*

In nervous system, the nAChRs are present in 3 different areas of neuronal plasma membrane:

1. Presynaptic terminal receptors, where they mediate rapid signal transmission and neurotransmitter release that influence on brain mechanism.
2. Preterminal axon receptors, where they rise the current's frequency.
3. Post synaptic receptors, where they modulate neuronal depolarization (Wonnacott (1997), (Hefft et al., 1999).

2.1.2.2 *Functions*

Animal model studies demonstrate that nAChRs are widely distributed in presynaptic neuron where it modulate the neurotransmitter release and facilitates higher cognitive functions (Gotti and Clementi, 2004).

Despite the scant innervation, cholinergic activity of nicotinic receptors drives a wide variety of functions and behaviors. It play a vital roles during neuronal development and plasticity, release of all major neurotransmitter from presynaptic terminal (Role and Berg, 1996, Broide and Leslie, 1999) increase arousal, heighten attention, learning and memory influence rapid eye movement sleep, produce states of euphoria and reward, decline in fatigue and anxiety, processing of pain, and involved in a number of cognitive functions (Rose and Levin, 1991, Everitt and Robbins, 1997, Adler et al., 1999, Marubio et al., 1999). Biological alteration in nicotinic mechanism leads to cognitive dysfunction which is associated with neurodegeneration diseases hence effecting the quality of life.

2.1.3 *Muscarinic receptors*

In CNS the mAChR are extensively expressed. They are also known as family of transmembrane receptors coupled to G-proteins or metabotropic receptors. There are Five receptor subtypes (M1-M5) which are further catergorized into two groups based on their signal transduction modulations(Caulfield and Birdsall, 1998).

1. The M1, M3 and M5 receptors are all combined through Gq/11 proteins to phospholipase C and result in intracellular Ca^{2+} mobilization and involved in signal transduction.
2. whereas the M2 and M4 receptors interact to adenylate cyclase via Gi/o family proteins and reduce cAMP levels (Lanzafame et al., 2003).

2.1.3.1 *Distribution*

In general the mAChRs are widely expressed in pre- and post-synaptic terminals of brain regions, M1 receptors are highly expressed subtype in brain region like cortex, hippocampus, striatum, amygdala and thalamus(Levey et al., 1991). M2 and M4 receptors are present with moderate concentration in cortical regions (Levey et al., 1991), and least expressions levels of M3 and M5 exist in CNS(Rouse et al., 1997, Van der Zee and Luiten, 1999) mostly in hippocampus, substantia nigra (Levey, 1996) and ventral tegmental area (Vilaró et al., 1990, Yasuda et al., 1993).

2.1.3.2 *Functional role*

The mAChRs subtypes play a key role in cognitive and molecular functions (Jafari-Sabet, 2011). M1 receptors activation is involved in ameliorate the cognitions (Fisher, 2000, Anagnostaras et al., 2003, Fisher, 2012). Through M2 and M4 receptors in the hippocampus, regulate the neurotransmitter (ACh) release and cognitive functions, whereas M2 specifically mediate neuronal plasticity and working memory (Tzavara et al., 2003). Motor functions, behavior, learning and mnemonic like higher cognitive functions are modulated by M3 receptors (Levey et al., 1994, Poulin et al., 2010). M5 receptors are involved in regulating the cerebrovascular mechanisms(Araya et al., 2006).

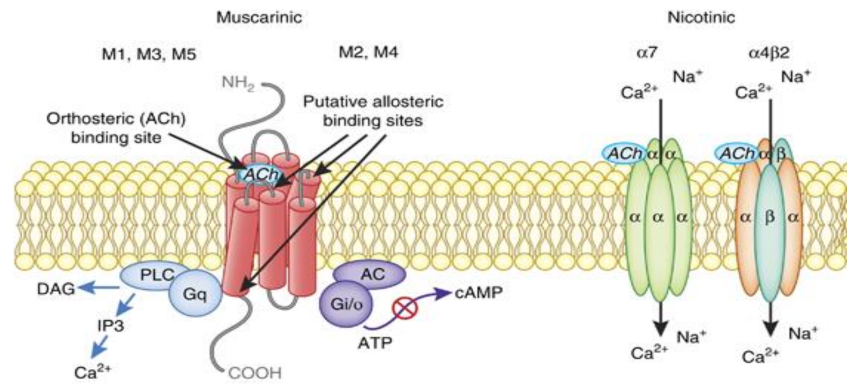


Figure 2.3: Modified diagram of the structure and signaling pathways of mAChRs and nAChRs. After the binding of Ach with receptor, it activates the secondary messenger which start the cascade of reactions and mechanisms, that play a vital role in learning, memory, higher cognitive functions and also involved in neurotransmitter release and different pathways (Jones et al., 2012).

2.1.4 Importance of Hippocampus in cognitions:

Hippocampus play a chief role in learning and memory, in the transmission and plasticity (Boyd et al., 2000, Egorov et al., 2002). The subtypes of cholinergic receptors at interneurons, pre-synaptic, and post-synaptic cleft demonstrate various properties and functions through downstream signaling mechanisms.(Lanzafame et al., 2003, Miyazawa et al., 2003). In the different regions of hippocampus like CA1or CA3, the ACh facilitate or cease the neuronal transmission, enhance the long term potentiate, neuronal plasticity and continuously proliferate the neuronal stem cells present in the dentate gyrus (Eriksson et al., 1998). Furthermore, the cholinergic inputs from medial septum are important in building the relation between cholinergic firing and theta rhythm (an oscillatory pattern in electroencephalography(EEG) signals) of hippocampus; as these play a vital role in higher cognitions like learning, memory and attention(O'Keefe and Recce, 1993, Buzsáki, 2002). The dorsal hippocampal area is crucial (Moser et al., 1993) for the memory formation and retrieval processes during the MWM (Morris Water Maze) task (Hollup et al., 2001) and is essential for long-term storage of mnemonic process(Riedel et al., 1999). For learning and memory processes, the hippocampal muscarinic receptors play a chief role (Bartus et al., 1982, Quirion et al., 1995, Sarter and Bruno, 1997, Van der Zee and Luiten, 1999) Following are nAChR and mAChR distribution and function in the hippocampus (Table. 1)(Drever et al., 2011).

Table. 2.1: Schematic representation of distribution and function of nAChRs and mAChRs in the nerves cells of hippocampus.

Cholinergic Receptor	Hippocampal Distribution	Downstream Mechanism
α7	Large fraction levels, interneuron, presynaptic & postsynaptic pyramidal neuronal cells	Ca^{2+} permeability s high, Na^{+} & K^{+} are also permeable
α4β2	Medium fraction levels, found on pre-synaptic inhibitory interneurons and post-synaptic on pyramidal nerves cell	Ca^{+2}, K^{+} & Na^{+} are permeable
α3β4	Low fraction levels, mostly on pre-synaptic excitatory nerves cells	Ca^{+2}, K^{+} & Na^{+} are permeable
M1	Large fraction, pyramidal neuronal cells and scantly present on terminals & axon. Predominantly on postsynaptic cells	Receptor Coupled with G-protein, G_q, low K^{+} conductance
M2, M4	Medium fraction- heteroreceptor and autoreceptor on non-cholinergic hippocampus presynaptic cells. Present on post synaptic, interneurons	Receptor Coupled with G-protein, G_i, high K^{+} conductance, low conductance of Ca^{+2}
M3, M5	Low fraction levels, mostly postsynaptic on pyramidal nerves cells	Receptor Coupled with G-protein, G_q, low conductance of K^{+}

2.2 Cholinergic System Dysfunctions in Neurodegenerative Diseases

As the central ACh neurotransmitter has important regulatory functions in learning and memory behavior. The maintenance of the normal functioning of this regulatory function is essential to ensure normal learning and memory (Lamirault et al., 2003)Natural causes of brain damage are aging, diseases or psychological trauma (Gazzaniga et al.). Degeneration of basal forebrain cholinergic cells has been observed in a number of other dementing disorders (fig:2.4), such as Parkinsons's disease (Arendt et al., 1983)(Jellinger, 1991, 2000), Down-syndrome, progressive supranuclear palsy, Jakob- Creutzfeld disease (Arendt et al., 1983)), Korsakoff's syndrome (Bohnen et al., 2003; Terry and Buccafusco, 2003) as well as after chronic ethanol intake (Arendt, 1994), and traumatic brain injury (Salmond et al., 2005). In these diseases is also associated with loss of synapses and neurons, synaptic dysfunctions, mitochondrial abnormalities and inflammatory stress (Selkoe, 2001, Reddy and Beal, 2005). Synaptic damage contributes to cognitive decline including changes in executive functions, memory and learning, deficit in language and visuospatial skills (Flicker et al., 1991, Rubin et al., 1998). The non-cognitive symptoms includes depression, anxiety, delusion, hallucination and behavior problems (Greene et al., 1982, Reisberg et al., 1987). These changes are due to progressive death of nerve cells that are responsible for the processing and storage of information (Mattson, 2004). These diseases has massive implications for society, in terms of both economic burden and human sufferings (Casserly and Topol, 2004).

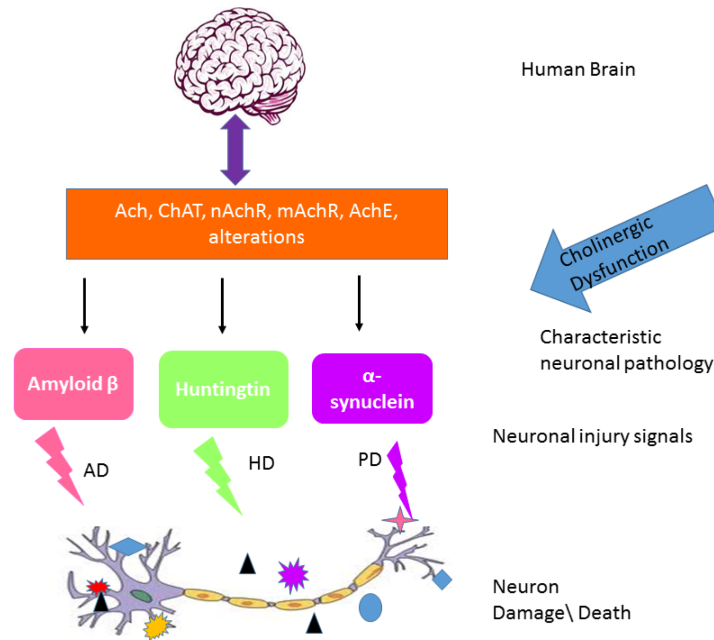


Figure 2.4: Aberrant proteins production in the neurodegenerative diseases, it contributes to neuronal death causing the deposition and aggregation of neurotoxic proteins.

2.2.1 *Cholinergic dysfunction and Alzheimer's disease*

In comparison with other neurodegenerative disorders, in Alzheimer's disease the cholinergic dysfunctions are accompanied by the occurrence of two major histopathological hallmarks such as β-amyloid protein plaques deposited extracellularly in neurons of cortex and hippocampus regions and neurofibrillary tangles that are present in cytoplasm of cortical neurons. AD pathogenesis is also contributed with loss of synapses and neurons, synaptic abnormalities, mitochondrial dysfunctions and inflammatory stress (Selkoe, 2001, Reddy and Beal, 2005). Synaptic dysfunction leads to decline in cognitive functions like variations in executive functions, memory and learning, deficit in language and visuospatial skills (Flicker et al., 1991, Rubin et al., 1998). The non-cognitive behavior symptoms are depression, anxiety, delusion, and hallucination (Greene et al., 1982, Reisberg et al., 1987). These alterations lead to neuronal death of cells that are responsible for the storing and processing the information (Mattson, 2004).

In cortex and hippocampus, oxidative stress produced Aβ-induced which cause in synaptic dysfunction (Forero et al., 2006 Reddy and Beal, 2008) Synaptic terminals consuming acetylcholine, glutamate and serotonin like neurotransmitters are generally

affected (Coyle et al., 1983, Levey, 1996). ACh deficiency appears to be in cholinergic hypo-function, which has been considered an important feature of AD (Fisher, 2012), as it is involved in proper functioning of cognition and memory (Elvander et al., 2004, Hasselmo, 2006, Woolf, 2006).

Progressively, at the synaptic level ACh level efflux by cholinergic neurons became smaller and unable to transmit the electric impulse. This presynaptic cholinergic deficit lead to cognitive dysfunction and the assembling of neuritic plaques and tangles. As the '**cholinergic deficit hypothesis'** proposes learning difficulties in dementia, could be due to lack of ACh. In AD mortems brains, postsynaptic muscarinic (M1, M3) receptors are conserved, whilst a reduction in the number of nicotinic and muscarinic (M2) receptors, located on presynaptic terminals are observed. Furthermore, the early onset of AD pathology are due to low ChAT activity, the abnormal disturbance of muscarinic M1 receptors and G-proteins coupling and in second messenger systems. The amyloid protein and cholinergic function interaction was assist by the Aβ metabolism which was activated either by mACh or nACh receptors, and in many cases the cholinergic dysfunction appeared to be a secondary effect of Aβ toxicity (Mori et al., 1995, Inestrosa et al., 1996, Auld et al., 1998, Pákáski and Kálmán, 2008).

Consequently, the reduction in cholinergic activity, diminished stimulation of protein kinase C may contribute to a higher tau hyper-phosphorylation. Thus, the imbalance in the neurotransmission may lead to increase amyloid β, tau phosphorylation and neurodegeneration (Mori et al., 1995, Inestrosa et al., 1996, Auld et al., 1998, Pákáski and Kálmán, 2008).

2.3 Models of Cognitive Impairment

As mentioned earlier, natural causes of amnesia are brain damage due to disease or psychological trauma (Gazzaniga et al. 1998). We can induced cognitive impairment by using sedatives and hypnotic drugs, which can cause temporary memory loss as they are absorbed in blood serum increases (Veselis et al., 2001, Lerner and Lerner, 2004). These drugs are also known as 'premedicants' because they are used to forget surgery or medical procedures (Walsh et al., 2011). Physostigmine, Lorazepam, Clozapine etc, are types of

sedative drugs administrated systematically in animals to create cognitively impaired models for research purposes (Mineau et al., 1994, Thiel et al., 2002, Li et al., 2007).

The administration of MK-801 are NMDA receptor blockers results in, dysfunction of the mnemonic processes (de Lima et al., 2005). Other strong muscarinic blockers are Scopolamine, Atropine and Propofol (Thiel et al., 2002, Miner and Burton, 2007). Scopolamine is commonly used in screening memory drugs due to its memory impairment properties (Blokland, 2005).

2.3.1 *Scopolamine*

Scopolamine, an anticholinergic agent (antimuscarinic), is used as a standard drug in both humans and animals for cognitive dysfunction (Klinkenberg and Blokland, 2010). Scopolamine is a type of tropane alkaloid drug that causes 'Cholinergic hypofunction' by binding non-selectively with muscarinic receptors (Hulme et al., 1978). Blocking central muscarinic receptors, produces the symptoms of cognitive decline like in AD patients (Graham and Buccafusco, 2000).

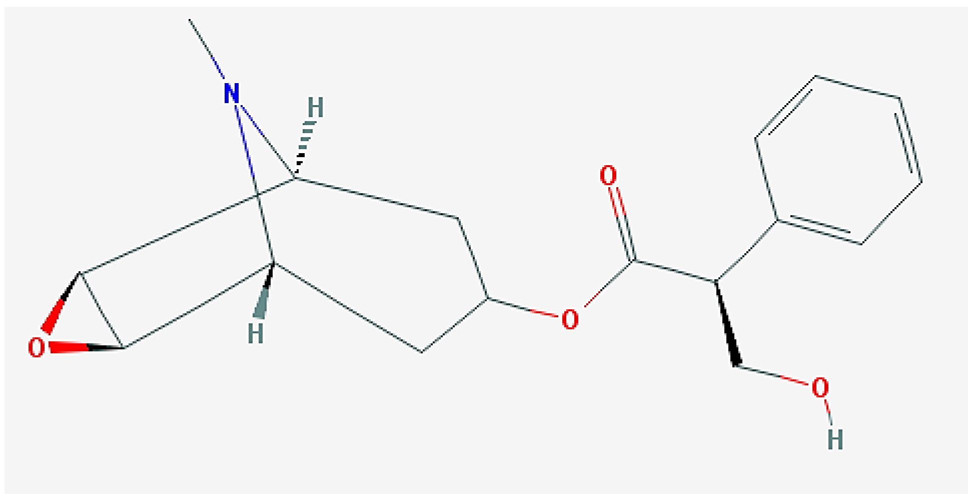


Figure 2.5: The structure of Scopolamine Hydro Bromide.

For this research study scopolamine HBr is selected as its well-characterized to cause cholinergic dysfunction. This anti-muscarinic antagonist has the capability to cross BBB more efficiently than other non-specific cholinergic antagonists like atropine (Consolo et al., 1972, Hughes, 1982).

Scopolamine HBr has property to induce effects on central cognitive impairment by easily cross BBB, so it is extensively used for experimental purposes (Hoffman and Lefkowitz, 1990, Anagnostaras et al., 1999). Scopolamine induced cognitive impairment in healthy volunteers has similar effects as found in demented and senile patients. When these individuals were clinically trialed they showed memory loss of recent events (Wiener and Messer, 1973). A number of previous studies have shown that the Scopolamine and Atropine when administered before training of behavioral tasks induces impairment learning and memory in rats (Ribot, 1882; Corkin et al., 1968).

2.4 Treatment of Cholinergic Dysfunction

2.4.1 *Potential of Novel Drug Targets for cholinergic system*

The anti-oxidative and anti-inflammatory stress are major approaches used to prevent cholinergic impairment in neurodegenerative diseases (McGeer and McGeer, 2007). Decreasing the inflammatory stress while using the drugs to examine the potential such as NSAIDs, vitamin C (antioxidant) and metal chelating agents (Singh et al., 2012). Animal models of AD demonstrate that GSK-3β enzymes is proved to be beneficial for lowering the tau phosphorylation (Terwel et al., 2008). Another effective strategy is active and passive immunization that declines and removes the Aβ and hence improves mnemonic processes (Christopher et al., 2000, Weiner et al., 2000, Brody and Holtzman, 2008). Previous studies reported that in-vivo immunization of Aβ and anti Aβ antibodies lowers the toxicity such as neuritic dystrophy (Oddo et al., 2004, Brendza et al., 2005).

2.4.2 Synthetic Therapeutic Agents

Many drugs are developed to improve the cognitive functions; based on understanding cholinergic dysfunction in pathophysiology of many neurodegenerative diseases. The drugs are used for symptomatic treatment and to halt the neuronal degeneration.

Previous studies demonstrate that cholinergic antagonist's administration results in intense memory impairment (Roldán et al., 1997, Fornari et al., 2000, Richetti et al., 2011)

so it depicts the importance of cholinergic receptors in learning and memory. Prior studies reported that reduction ChAT levels in the brain regions of AD patients, is one of the reason progression of disease (Mufson et al., 2008). Therefore, the impairment in cholinergic neurons in different brain regions lead to cognitive deficits.

There are two ways to enhance the cholinergic function; one is to stimulate the cholinergic receptors directly by cholinergic agonists, while the other option is to inhibit the activity of Acetylcholinesterase (AChE).

1. **Acetylcholinesterase inhibitors:** ACh is an essential neurotransmitter, which is hydrolyzed by AChE (endogenous enzyme) present in the synaptic cleft. Reduced level of ACh is major cause of disease progression in many nervous system disorders. Current available drugs for the treatment of the AD include AChE inhibitors(Wimo et al., 1999, Ballard, 2002). Federal Drug Administration (FDA) has approved the following drugs for treating cholinergic dysfunction in various neuronal disease. These are selective or non-selective acetylcholinesterase inhibitor (AChEI), Tacrine (Cognex®), Rivastigmine (Reminyl®), and Donepezil (Aricept®). Due to adverse effects of Tacrine, its usage is limited (Allain et al., 2003).
2. **Muscarinic receptor agonists**: M1 agonist, selective muscarinic receptor is one of the effective candidate for therapy(Fisher, 2000, Nitsch et al., 2000, Jiang et al., 2014) as in the hippocampus and cortex M1 receptor can be found densely (Caccamo et al., 2006). The muscarinic receptor stimulation modifies APP (Amyloid Precursor Protein) processing and inhibits Aβ production (Caccamo et al., 2006).Currently, there is limited availability of M1 agonists which have limitation either due to side effects, poor availability or penetration into blood brain barrier thus requiring larger doses(Fisher et al., 2003).
3. **Memantine:** it is NMDA receptor antagonist, it increases glutamate levels without interfering with physiological regulation of NMDA receptor (Reisberg et al., 2003, Wilcock, 2003). Clinical studies proves memantine to be neuroprotective as it demonstrates an effective for both mild and severe cognitive impairment treatments (Winblad et al., 2007).

4. **Antioxidants**: Aβ fragments produce oxidative radical's stress that may retard and slow down the disease onset (Behal et al., 1994: Butterfield 1994). In vivo studies as well as cell culture system have been demonstrate that vitamin E attenuates the Aβ- induced neurotoxicity (Yatin et al., 2000, Isaac et al., 2008). Vitamin E and C are the natural antioxidants.
5. The protective role of various drugs for curing cholinergic deficits like anti-inflammatory drugs (McGeer and McGeer, 2007), antioxidants (Rinaldi et al., 2003), calcium channel blockers (Yasar et al., 2005)and cholesterol lowering drugs (Refolo et al., 2001) are accepted.

To complement these pharmaceutical treatments, nutraceuticals or herbal supplements are used as supportive agents for memory enhancement or antioxidant effects.

2.4.3 Donepezil and Alzheimer's Diseases

Donepezil (Fig. 4) is a selective, reversible acetylcholinesterase inhibitors (AChE-Is). It is a chief candidate in the treatment of mild to moderate AD, some clinical studies demonstrate that it improves cognitive impairment in severe AD patients. The international pharmaceutical companies Eisai and Pfizer produces donepezil under the commercial name Aricept. It is administered via an orally route, it can easily cross the blood-brain barrier and excrete slowly as its bioavailability is 100%. The drug is available in 5 and 10 mg dose strengths and half-life is 70 hours.

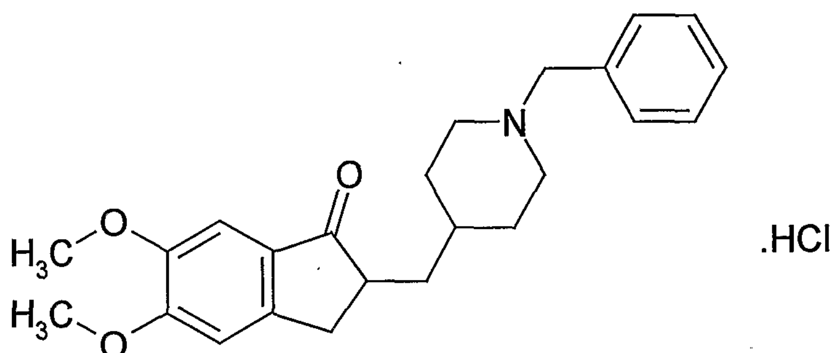


Figure 2.6: The chemical structure of Donepezil HCl.

In the neuronal diseases pathology, the loss of basal forebrain (BF) cholinergic neurons and reduction of ACh synthesis and its release significantly contribute to the cognitive impairment (Bartus et al., 1982; Bartus et al., 2000; Sarter et al., 2004). Donepezil prevent the hydrolysis of the residual ACh in the brain's synaptic cleft and currently used to improve or maintain central cholinergic function (D'Amelio et al., 2012, Pepeu et al., 2009). Many studies provide evidence that, AChEIs produce effective neuroprotection (Pepeu et al., 2009); against glutamate excitotoxicity, Aβ neurotoxicity and neuronal damage. Furthermore, they also upregulation the expression of nAChRs (Akaike et al., 2010; Wang et al., 2005). Essentially, α7 and α4 nAChRs play a critical role in neuroprotection, mainly participating through the phosphatidylinositol 3-kinase (PI3K) pathway, mediated by AChE-I (Akaike et al., 2010; Takada-Takatori et al., 2009). Moreover, isoflurane induced cholinergic and spatial memory impairment in aged mice are significantly prevented by donepezil (D'Amelio et al., 2012). It also diminished okadaic acid–induced cognitive impairment, apoptotic cell death, mitochondrial dysfunction (Pepeu et al., 2009), reduces oxidative stress, and caspase-3 activity through the specific stimulation of nAChRs (Meunier et al., 2006). Thus in our study donepezil (during behavioral task) is used with turmeric to evaluate its potential on the scopolamine induced cholinergic dysfunction in mice model.

2.6 Potential Of Medicinal Plants

To explore efficient, safe and cheap agents for treatment of chronic diseases has led to the herbal medicine for therapeutics effects. Due to nescience of chemically active agents and their mode of action, we are unable to explore their full potential. Presently 3500 medicinal plant species are used worldwide for therapeutic purpose (Maclennan et al., 2002). In comparison to synthetic, fewer adverse effects are reported with medicinal plants extract. In recent years the medicinal plants for dementia have become more accepted. Scientist have shown interest to cure and prevent neurodegeneration with medicinal plants (Anekonda and Reddy, 2005). The medicinal plants are effective by producing anti-apoptotic effect, antioxidant effect, enhancing synaptic functions anti-amyloid effect, inhibiting cholinesterase and NMDA receptors (Akhondzadeh and Abbasi, 2006b).

Several plants have potential therapeutic role in neurodegenerative disease such as *Curcuma longa* (tumeric) (Butterfield et al., 2002), *Zingiber officinale* (ginger) (Ha et al., 2012), *Huperzia serrata* (toothed clubmoss) (Wang et al., 2006), , *Ginkgo bilob* (DeKosky et al., 2008), *Glycyrrhiza glabra* (The Licorice Root) (Dhingra et al., 2004), *Panax ginseng* (Lee et al., 2008) *Salvia officinalis* (Wake et al., 2000). *Ungernia* sp. (daffodil bulbs) (Kihara et al., 2004). Herbal drugs have dominance over synthetic drug because therapeutically they demonstrate less toxicity, easily cross blood brain barrier and shows synergistic effects (Anekonda and Reddy, 2005). Thus medicinal plants may be used as treatment option in neurodegenerative disease, as the improve cholinergic system and cognitive functions (Anekonda and Reddy, 2005).

2.5.1 *Therapeutic Applications of Turmeric*

In South Asia, turmeric powder is (Curcuma longa), (fig, 2.7) commonly used spice in traditional cooking and medicine system. Apparently it is similar with ginger family, and slightly spicy in flavor.(Hong et al., 2004). It is most commonly used in Sub-continent and has a high medicinal importance especially in treating wound healing, inflammatory conditions and blood purification (Aggarwal et al., 2007). In 1910 for first time it was chemically determined, it is composed of almost 20 bioactive molecules, out of this 3% are active ingredients of curcumin (Pari et al., 2008). The fraction of turmeric contains chief agents are, curcumin (77%) demethoxycurcumin (17%), bisdemethoxycurcumin (3%), and the recently identified cyclocurcumin (TsUDA, 1993) as shown in fig: 2.7. Its active compound curcumin is widely prescribed as a potent remedy for numerous disorders such as, arthritis, diabetic wounds, runny nose, cough, and sinusitis, sprains and swelling. It is also used for the diseases associated with abdominal pain, liver, allergy, asthma, rheumatism, heart disease, diabetes, atherosclerosis and cancer (Cole et al., 2007, Kim et al., 2009). Turmeric has also been used for treating asthma, epilepsy, gall stones, cramps, congestion and anorexia. It is also used to balance cholesterol levels and for treating AD (Duke, 2002, Gilani et al., 2005, Ahmed and Gilani, 2014). Extensive research within the last two decades has revealed that curcumin exhibits antioxidant, anti-inflammatory, anti-proliferative, anti-invasive and antiangiogenic activity.

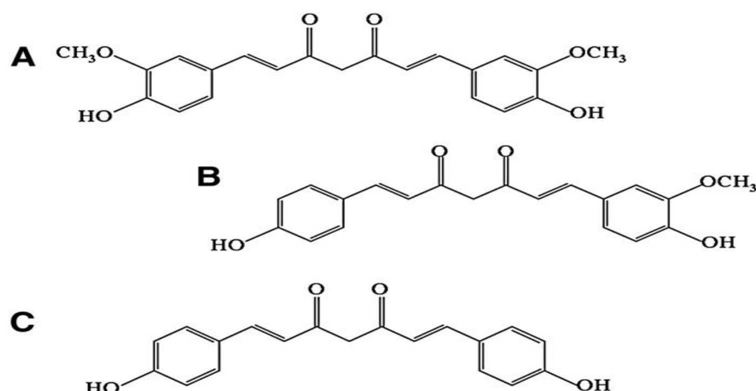


Figure 2.7: The chemical structures of turmeric's isolated compounds (a) curcumin (b) demethoxycurcumin (c), bisdemethoxycurcumin.

2.5.2 Bioavailability of Turmeric

Turmeric has phenolic ring structures that are chained with hydrocarbons (fig: 2.7), these rings make it little difficult to dissolve in plasma (Belkov et al., 2011) and it is also responsible for turmeric's yellow brown color. The turmeric structure also contain small fraction of water soluble hydroxyl and ether groups (Tayyem et al., 2006). Phenolic structure are responsible for low turmeric bioavailability tests, specifically in elderly population (Jäger et al., 2014). Whereas recent studies has demonstrate that these phenolic compounds play a vital role in preventing and curing oxidative stress, inflammation, and cancer(Mishra and Palanivelu, 2008). Researchers reported that turmeric accompanied with probiotics augment their absorption from the stomach (Pianpumepong et al., 2012). Previous study demonstrate that freshly grounded turmeric supplemented with lactic acid bacteria boost the metabolism and digestion in the body as compared to turmeric without lactic acid bacteria (Potter, 2013). Turmeric has shown greater bioavailability when ingested with food, as this lead to production of bile salts that can aid in curcumin-water insoluble emulsification and hence increased solubility in plasma (Welak et al., 2014).

2.5.3 Turmeric and Neurodegenerative diseases

Oxidative stress (Beal, 1995, Lu et al., 2004) results in the enhance production of the nitric acid, which mediate glutamate excitotoxicity and leads to neurodegenerative diseases (Beal, 1995). Previous study has shown the neuroprotective role of turmeric reverts the oxidative stress induced by acrylonitrile in the brain (Guangwei et al., 2010). The other study demonstrate that curcumin combine with free radicals and reactive oxygen spice and also augment the activity of cholinergic system in streptozotocin induced dementia in rats model. (Agrawal et al., 2010), and in acute traumatic injury(Sharma et al., 2009).

As turmeric has anti-inflammatory and antioxidant properties; it has been found to inhibit formation of Aβ fibrils, and causes destabilization of already formed Aβ plaques in AD related animals model. Curcumin component of turmeric spice also acts against abnormal growth and proliferation of neuroglial (gliosis) cells. For these reasons curcumin "could be a key molecule for the development of therapeutics for Alzheimer's disease" (Ambegaokar et al., 2003, Ono et al., 2004). Turmeric's biphenolic structure has the ability to scavenge with Aβ plaques thus decreasing Aβ-induced toxicity (Yang et al., 2005, Begum et al., 2008). Curcumin has ability to cross the blood-brain barrier, where it binds to and induces rapid dissolution of plaques. This anti-amyloid activity was verified using multi-photon microscopy in vivo(Garcia-Alloza et al., 2007).

The curcumin effects the disease pathology through various mode of actions like, growth factors receptors, transcription factors, cytokines receptors, neurotransmitter receptor, many protein kinase and inflammatory modulator(Gupta et al., 2012). Mechanisms of protection against inflammation, oxidative damage and cognitive decline make turmeric a promising natural agent in fighting aging and degenerative diseases. These finding suggest that turmeric treatment at any stage of neurodegeneration can attenuates the clinical symptoms.

MATERIAL AND METHODS

3.1. **Drugs and chemicals**

Scopolamine hydrobromide trihydrate (product code no. S-1101) was purchased from Sigma Aldrich, Germany. 0.9% solution of sodium chloride (NaCl) by Scharlau (product code no. SO0225) was prepared as Normal Saline (NS). Reverse transcriptase (RT), Taq polymerase, dNTPs and Tri-Reagent were purchased from Fermentas®, and Invitrogen respectively. Cresyl violet stain (229630250) used in histology of brain tissues were obtained from Scharlau. 2% Xylocaine (lignocaine HCl + Adrenaline), Paraformaldehyde (PA0095100), Ethanol (100983), sodium chloride (SO0225), sodium phosphate monobasic (567549), sodium phosphate dibasic (567550) and xylene (108684) were obtained from Merck. Rhizomes of turmeric were ground into a fine powder and used in the experiments. The turmeric powder was used as whole and was given to mice in their feed. Dose of turmeric was 20mg/Kg per day. All the solutions were prepared fresh on the day of experiment.

3.2. Ethical statement & Animals

All the protocols approval was attained from the Internal Review Board (IRB), the ethics committee of Atta- Ur- Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST). All the experiments and treatment of animals were maintained in accordance with the guidelines for ethical conduct developed by the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of laboratory Animals: 8th Edition, 2011).

60 male BALB/c mice (about 3-6 months of age and average weight of 30-45g) were used in experiment. The animals were housed in standard laboratory conditions, humidity and temperature is maintained to 25±2ºC under natural light and dark cycle (10 hours light and 14 hours dark). These mice were given water and a standard diet ad libitum. In each cage dimension of 40cm x 20.5cm x 2.5cm, 4 mice were kept with distinct individuality for differentiation. All the times mice were handled with special care to avoid disturbance and sudden noise.

3.3 STUDY DESIGN

Scopolamine is used to build cholinergic hypo-function model for 26 days to determine the effects of Turmeric powder and donepezil in mouse. From 21st day Behavior tests Morris Water Maze (for 5 days) and social preference were performed to observe the outcome on spatial memory and learning respectively. On 26th day, animals were sacrificed for study of mRNA expression analysis of hippocampus and thalamus, and some animals were perfused for histological study of brain tissues. Mice were divided into 5 groups and given treatments according to the group (table 3.1).

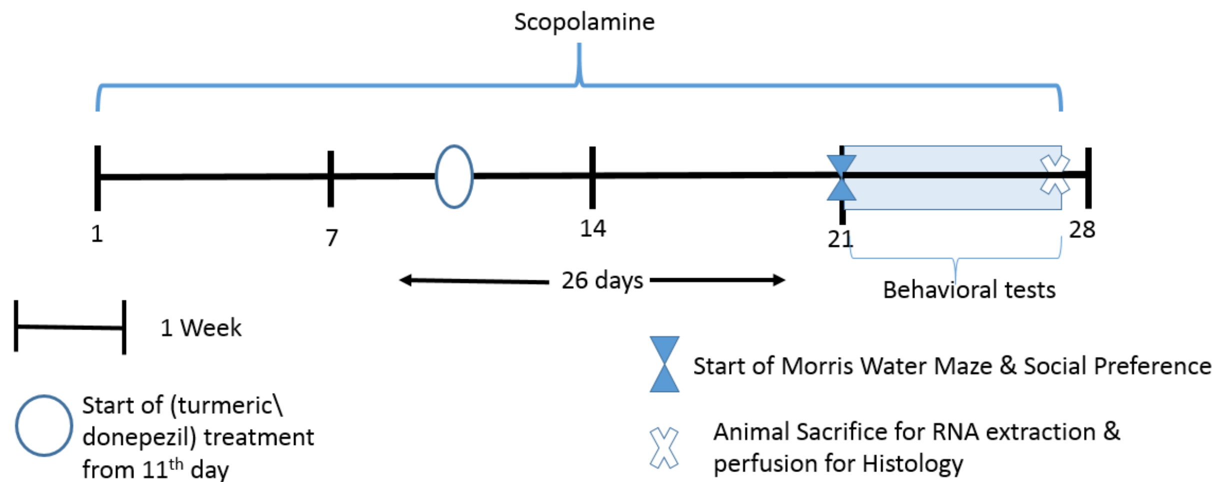


Figure 3.1: (a) Study plan for the development of cholinergic hypo-function model and to check the activity of raw turmeric and donepezil afterwards. Behavior Tests were performed from 21st to 25th day including five days Morris Water Maze Test and Social Preference Test. On 26th day, animals were decapitated for RNA extraction or subjected to perfusion for histological study.

3.5 ANIMAL GROUPS FOR STUDY

Male healthy BALB\c mice age of 3-6 months were used in the experiment. They were divided into 5 groups, each group contain 12 mice. Following are the details of all groups separately (Fig: 3.2).

a) Control: 0.9% w/v Normal Saline was given subcutaneously for 26 days. (Con)

b) Scopolamine: Scopolamine 1mg/Kg was injected subcutaneously each day for 26 days to produce cholinergic hypo-function model. (Scop)

c) Turmeric alone: 20mg/Kg of turmeric powder was mixed in their feed given orally to the mice for 16 days. (Turm)

d) Donepezil Alone: 0.5mg/Kg of donepezil powder was mixed in their water given orally to the mice for 16 days. (Donp)

e) Scopolamine + Turmeric: 1mg/Kg of Scopolamine was injected subcutaneously for 26 days. 20mg/Kg turmeric powder was mixed in the mice feed during last 16 days of treatment along with the regular injections of scopolamine (Scop+ Turm)

f) Scopolamine + Donepezil: for last 16 days 0.5mg/Kg of donepezil was given in water and 1mg/Kg of Scopolamine was injected subcutaneously for 26 days. (Scop+ Donp)

g) Scopolamine + Turmeric +Donepezil: 0.5 mg/Kg of donepezil (in water) and 20mg/Kg raw turmeric powder (in feed) was orally given for last 16 days along with regular injections of scopolamine. (Scop+ Turm+ Donp)

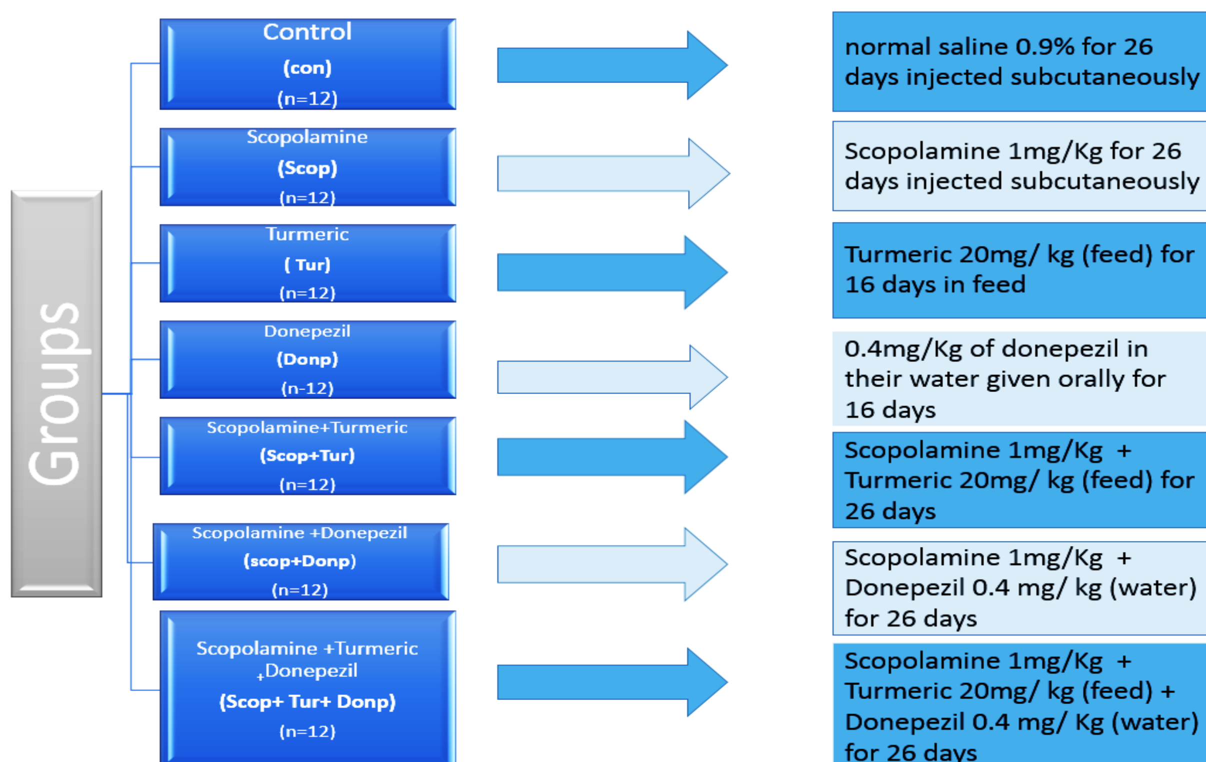


Figure: 3.2 (b) Schematic presentation of drug treated groups, animals divided into seven groups with their specifications.

3.5.1 Scopolamine and Normal saline

A total of seven groups of animals were used in this study and the duration of treatment was 26 days. In this study total five groups of animals were used for 26 day of treatment. The scopolamine and normal saline was administered through sub-cutaneous injections (SC). 10x Scopolamine stock solution was prepared and stored at -20°C, from this 1x solution was prepared freshly daily. Every day mice were weighed and 0.9% normal saline or scopolamine dose of 1mg/Kg body weight was given to them through insulin syringes (30guage x 0.3mm x 8mm needle). These injections were administered 60 minutes before the behavioral tests during day time. To avoid disturbance the cage lid was carefully removed. The mouse was restrained by one handed method. It was placed on lid of the cage, with 3rd and 4th finger gently tuck the tail, as this made the mouse to grasp the surface with all four paws and pull forward. Then with forefinger and thumb grasp the dorsal rump near the base of the head to make the scruff. Now with other hand insert the injection at the angle of 30-45° into the tented skin (fig. 3.2), a small swelling marked as successful SC

injection. Injections were given at alternate sites to minimize pain as the animal was administered injections every day (Machholz et al., 2011).

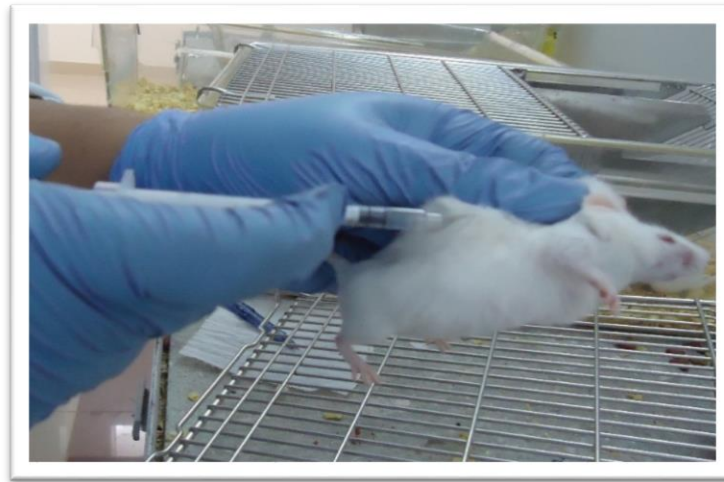


Figure 3.3: Subcutaneous Injection. Animal is restrained by holding its tail. Tent of the scruff is made and needle is injected just parallel to the skin. After the injection, bulge in the skin is formed indicating successful injection.

3.5.2 Oral Administration of Turmeric and Donepezil

Turmeric and Donepezil were routed orally in the form of mixed feed and in distilled water respectively. Turmeric powder was given to animals orally. 20mg/Kg/day dose was mixed in mice feed. 0.016g of turmeric powder was added in 100g of feed. Initially turmeric was homogenized in 5g of starch powder and then added to the calculated amount of finely crushed feed. Thorough mixing was ensured. Sufficient amount of water was added to make medium size pellets of feed. Pellets were then air-dried. Daily weighed amount of pellets were given to the animals as feed.

Administration of Donepezil

Donepezil HCl tablet "Donecept" brand was purchased from the market and it was finely grounded with pistil and motel. 0.5mg/Kg/day of donepezil is mixed in 100ml distilled water and finally poured in opaque drinking bottle as it is photo sensitive. The solution is mixed thoroughly to ensure that donepezil was well saturated in it.

3.6 ANIMALS BEHAVIOR TESTS

On 21st to 26th day all experiments were carried out between 09:00 and 16:00 to avoid possible variability because of the circadian rhythms. Before commencing the experiment 30 minutes habituation of the mice was done in the respective room. The behavior test room maintained with standard laboratory conditions i.e. room was properly lit, humidity and temperature (22 ± 2°C) with minimum level of disturbance. The experimenter was always sat at the same position. Video recording were done by fixing the camera on tripod stand (Lee et al., 2012).

3.6.1 Morris Water Maze

This behavior task was originally adapted from Morris (Morris, 1984)**,** a cognitive test to determine the spatial and reference memory. The test procedure was same as described previously (Bromley-Brits et al., 2011), with slight modifications. This test was conducted in a circular tank with 120cm diameter,60cm height and about 34cm depth, pool was filled with blue opaque water (26 ± 1 C). It was virtually divided into 4 quadrants. A submerged escape platform was placed in the North-West quadrant, 1cm below the water surface, was only escape from the water. On every trial this platform was situated in the same quadrant and were left unchanged throughout the test. Spatial cues of different shapes were placed in the wall of pool's four quadrants, to navigate the mice to find the hidden platform (fig: 3.4).

On the 21st day of treatment, five escape trails were given to all mice for five consecutive days During training days the starting points for mice was set to differ with each trial (Table: 3.1). A trial began by placing the mouse in the pool facing towards the wall of the starting points. If mouse was failed to found hidden platform within 90 s, it was gently placed on the platform and allowed to stay there for 20s. If mouse found the platform before 90s, were also allowed to stand there for 5s. 5–10 min was given as the inter-trial interval. The training would then end, and escape latency were recorded. The average of escape latency were calculated for each day.

On the 26th day of the treatment, the platform was removed in the probe trial. It was only trial, the mouse was released from west and allowed to swim for 90s. Reference

memory was checked by calculating the time spent in the platform quadrant as compare to other three quadrants in 90s.

Table: 3.1 Morris Water Maze table for direction of release

DIRECTIONS OF RELEASE					
No. of Days	**Trial 1**	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**
DAY 1	West	South	North	East	South
DAY 2	North	West	East	West	South
DAY 3	North	East	West	South	North
DAY 4	East	South	West	East	North
DAY 5	West	South	North	East	West
DAY 6	SINGLE TRIAL WITHOUT PLATFORM. RELEASE DIRECTION, **WEST*.**				

* On 6th day, Probe trial was performed with the platform removed and the time spent on Previously learned platform quadrant was noted.

Figure 3.4: Morris Water Maze (MWM) Apparatus. Pool filled with opaque (blue) water and divided into four quadrants. Distal cues are placed on the pool wall and a hidden platform is located in the North-West quadrant.

3.6.2 SOCIAL PREFERENCE TEST

This behavior task is performed to determine the sociability and preference social novelty of an animal established methods as previously explained by (Moy et al., 2004) with few modifications. The apparatus consisted of an opaque square box made up of iron alloy, with dimensions of 40cm x 40cm x 40cm. Two small wire gauze cages, each with 10cm in height and 8cm in diameter, were placed in the apparatus. In these cages the stranger mice of same gender were confined for social interaction. There were placed diagonally to each other and equal distant from the corner of the apparatus, as shown in fig 3.5. Before testing, the apparatus and cages were cleaned with 70% ethanol. This behavior test consist of 3 sessions: habituation, sociability and Preference Social Novelty Test (PSNT).

In habituation Session, each test mouse were allowed to freely move in the apparatus with two empty small iron cages for 5 minutes. During sociability (session 1), an unfamiliar male mice (stranger 1) was placed in the small cage and other cage was kept empty. The experimental mice was allowed to freely explore the apparatus and contact the small cages for 10 minutes. In this first session, sociability was checked by measuring the interaction time of the test animal with the stranger 1 and the empty cage respectively.

After the inter-session interval of 20 minutes PSNT (session 2) was performed, another unfamiliar mouse (stranger 2) was placed inside the empty cage while stranger 1 remained at its place where it was in the session 1. The experimental mouse was freely allowed to contact the wire cages housing the strangers for 10 minutes. In this session, PSNT was checked by measuring the interaction time of the test animal with the stranger 1 and the Stranger 2 respectively.

To ensure that tested mouse paid attention and showed interaction, the interaction less than 2s was not taken. Mouse's behavior was recorded by video camera mounted on tripod stand. Discrimination Index (DI), based on differences in the duration of interactions of previously encountered cage Stranger 1 and Stranger 2 and/or empty) by test mouse was calculated as an index of discrimination ability and memory.

DI = Time Spend with Stranger Mouse ÷ Total Time of Interaction

Figure 3.5: Apparatus for Social Interaction Test, showing a large iron box having two small hollow wire cages. 5 minutes habituation is carried out, having both the cages empty. In the first session, test for sociability is carried out, in which cage S1 is introduced with an unfamiliar Stranger 1 mouse and the cage marked S2 is kept empty. Second session is test of preference of social novelty in which Stranger 2 is placed in the previously empty cage whereas Stranger 1 remains as it is.

3.7 MOUSE DECAPITATION AND BRAIN HARVESTING

On the last day of behavior test (26th day) the total RNA was isolated from the Mouse brain. For this chloroform was used to anesthetized, skull was decapitated and brain was carefully harvested. In chilled Phosphate-Buffered Saline (PBS) on the petri-dish, brain was placed and hippocampus and thalamus tissues were extracted/ isolated (Hagihara et al., 2008), fig: 3.6.

3.7.1 RiboNucleic Acid (RNA) EXTRACTION

The isolated tissues were subjected to the RNA extraction by using TRI Reagent manufacturer's guide. The hippocampus and thalamus was placed into glass homogenizer containing 1ml and 0.5ml of Tri-reagent respectively, different specifications in Table. For complete dissociation of the nucleoprotein complex, homogenates were allowed to incubate at room temperature for 5 minutes. Then chloroform was added 200µl/1 mL of TRI Reagent and samples were vigorously mixed until it became milky. Incubating these samples for 10 minutes, after wards the first centrifugation was carried out at 12,000 rpm for 15 minutes at 4°C. This centrifugation formed 3 phases: a transparent phase (RNA) at the top, a whitish interphase (DNA), and pinkish organic phase (protein) at the bottom.

To a Fresh Eppendorf chilled isopropanol with 500µl/1mL of TRIzol, the aqueous phase was very carefully added to avoid genomic DNA contamination with the other phases. Solution was gently mixed and allowed to stand at room temperature for 10 minutes. Then second centrifugation was performed at 12,000 rpm at 4°C for 10 minutes, this precipitated RNA at the wall of tube in the form of whitish pellet. For third and last centrifugation (at 7,500 rcf at 4°C for 5 minutes), the supernatant was discarded and pellet was washed with 75% ethanol in Diethylpyrocarbonate (DEPC) water, 1mL/1mL of TRIzol. Samples were stored at -80°C until further use (Rio et al., 2010)

Ingredients	Hippocampus
TRIzol	1 ml
Chloroform	0.2 ml
	1st centrifugation
Aqueous phase+ isopropanol	0.5 ml
	2nd centrifugation
75% ethanol in DEPC	1 ml
	3rd centrifugation

3.7.1 RNA Solubilization

For RNA solubilization Reverse Transcription (RT) PCR, the RNA samples were removed from -80°C, thawed and centrifuged to get whole pellet. 75% ethanol was carefully discarded and pellet was allowed to dry (not completely dried) and re-suspended in 30µl of PCR water by pipetting up and down. To uncoil RNA, samples were subjected to slight heat shock on hot plate at 55°C for 5 minutes.

3.8 QUALITY AND QUANTIFICATION OF RNA

3.8.1 Quality of the RNA

The quality of isolated RNA was further quantified through gel electrophoresis and Nano Drop. All samples were run on 2% agarose gel at 90mV for 20-25 minutes with following specifications:

1.	Sample RNA	3µl
2.	Bromophenol blue loading dye	2µl
3.	PCR water	7µl

If the RNA samples showed sharp and clear bands of 18S and 28S RNA under UV light of Gel Documentation System (gel doc), then it was acceptable quality for further process. Nano Drop was performed to get the concentration of RNA in ng/µl. 3 readings were taken for a sample then the average was calculated for 1µg of RNA in 30µl.

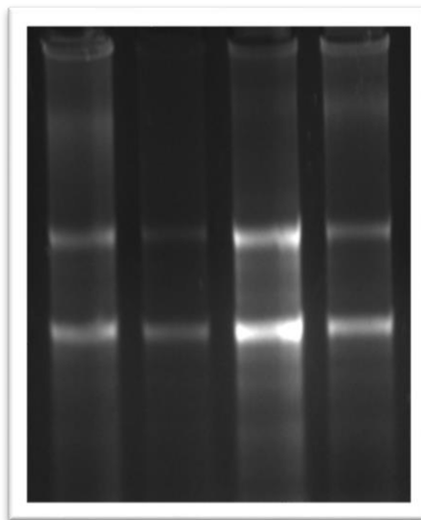


Figure: 3.6 RNA of samples run on 2% agarose gel

3.8.2 Reverse transcription polymerase chain reaction (RT-PCR)

For cDNA synthesis reverse transcription was performed, 1 µg of RNA from each samples mixed with 3 µl of 10mM dNTP's, 5 mM oligo dT then subjected to heat shock for 5 min at 55°C and followed by addition of 8 µl of 5x RT buffer, 4 µl of DTT and 2 µl of RT enzyme. Total volume of the mixture was made up to 40 µl by the addition of PCR water. This complete procedure was performed on ice. For RT-PCR 37°C for 10 minutes, 42°C for 60 minutes, 95°C for 10 minutes and 4c for 5 minutes conditions were maintained.

3.8.2 QUANTIFICATION OF mRNA LEVELS BY RT-PCR

Synthetic cDNA was used to create the polymerase chain reaction for the expression of genes. Recipe mentioned in table 3.3 and conditions shown in fig 3.8. All PCR ingredients like cDNA of sample, 10x buffer, 10 mM dNTP's, MgCl2, Taq enzyme, both forward and reverse primer for specific genes added and the finally addition of 22 µl of PCR water used for volume makeup.

The PCR mixture was amplified by setting profile as in fig with initial denaturation at 95°C (5 mins), then denaturation at 94° C (30 sec), annealing temperature (30 sec) and no. of cycles for specific genes in table, followed by extension at 72° C (30 sec). At the end final extension at 72° C (10 minutes) and cooling was done at 4°C for 5 minutes.

S.No.	Ingredient	Amount
1.	Taq Buffer (10X)	2µl
2.	2 mM DNTPs	2.5µl
3.	$MgCl_2$	2µl
4.	Forward Primer	1µl
5.	Reverse Primer	1µl
6.	Taq Polymerase	0.5µl
7.	cDNA of sample	3 µl
8.	PCR water	15 µl

3.8.4 DNA Contamination Check

To confirm that there is no genomic DNA contamination, PCR reaction was performed with no cDNA as negative control. This reaction's product was run on gel.

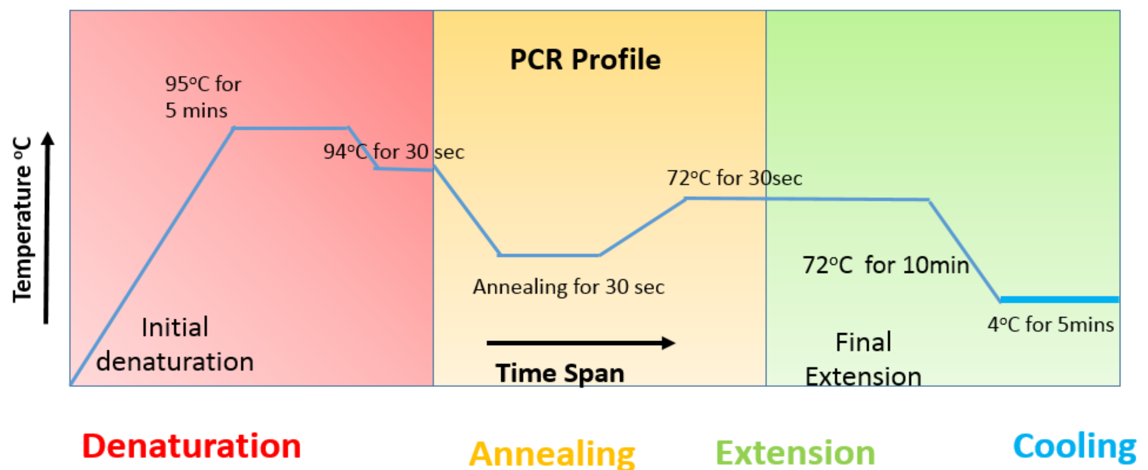


Fig: 3.7 PCR Profile for gene expression. Initial denaturation is at 95° for 5 minutes. This is followed by denaturation at 94°C for 30 sec, primer specific annealing temperature for 30 sec, and extension at 72°C for 30 sec.

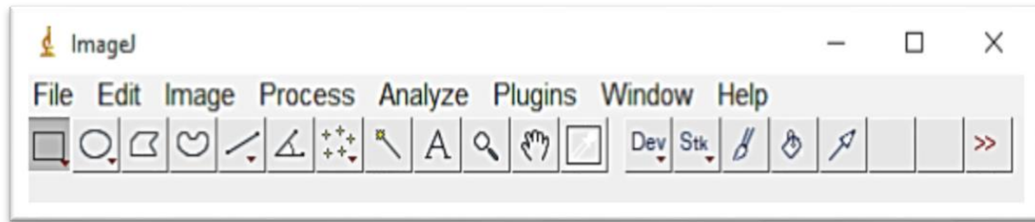
3.8.5 Gene Expression Study mRNA levels

The amplified PCR products of specific genes were normalized with Actin (housekeeping gene) in respective groups. These products were detected by 2% agarose gel containing ethidium bromide. The gel images were taken and its background was adjusted by using NIH software as shown in figure: 3.8. Each band intensities were quantified for densitometry, minimum and maximum values of band calculated by using Image J software and normalized with actin. Results were saved and plotted in Excel sheet.

Table 3.2: List of primers along with their thermal cycling conditions.

	Gene	Primer sequence (5’→3’)	Annealing temp °C	No. of cycles
1.	Actin	F= GCCTTCCTTCTTGGGTATGG R= CAGCTCAGTAACAGTCCGC	55	32
2.	M1	F= GTCCCATGGAAACCCTGAATCC R= GTCACTGACTTAGTCGCCCG	56	35
3.	M3	F= TCTTGAAGTGCTGCGTTCTGA R= GTTGGGAAACAAAGGCGAGG	56	35
4.	M5	F= AGCACCTCAACAACGGGAAA R= GGGGATCCAGGCCTTTTGTT	55	32
5.	α7	F=TGCAAAGAGCCATACCCAGA R=TGATCCTGGTCCACTTAGGC	66	35
6.	α4	F=GTCTAGAGCCCGTTCTGTGA R=TAGTCATGCCACTCCTGCTT	63	35
7.	β2	F=GATGACCAGAGTGTGAGGGA R=CCCCCACCGTTAACACTACT	63	35

(a)



(b)

. **(c)**

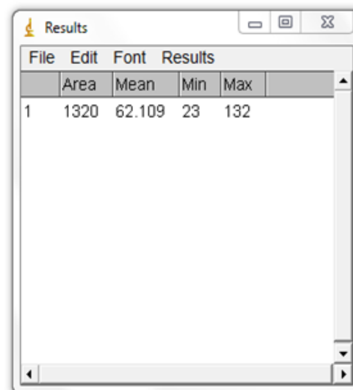


Figure 3.8: Interface of the NIH software used for the quantification of band intensity (a) software with open gel picture. (b) Band was selected and (c) its density was displayed in result box.

3.9 PROTEIN SAMPLE PREPARATION

The whole tissue lysates were prepared by suspension in 100µl of ice cold lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 10mM Phenyl methyl sulfonyl fluoride (PMSF), 1% Dithiothreitol (DTT), followed by sonication using a UP400S Ultrasonic Processor (Hielscher Ultrasound Technology). To increase dissolubility, the homogenates were stored at room temperature for 1 hour and centrifuged at 14000 rpm at 4℃ for 10 mins. Then supernatant was transferred to new tube and stored at -20 °C. In order to maximize the yield, 50µl lysis buffer was added to the pellet and treatment (procedure) was repeated. The two supernatant were then pooled and centrifuged at 14000 rpm for 90 mins. The final supernatant was stored at -80℃ until further used.

3.9.1 Protein Estimation (Bradford's assay)

The serial dilutions of bovine serum albumin (1mg/ml) were prepared in duplicate with ddH_2O. The sample was diluted with ddH_2O (1: 20) in duplicates. The final volume of each standard /sample was 20 µl and 1ml of Bradford reagent was added, followed by gentle vortexing. The samples were then incubated for 10 mins at room temperature. The absorbance of each sample was measured at 595 nm reagent blank using OPTIMA 300 spectrophotometer. A standard curve was derived by plotting standard absorbance against its concentration. This curve was used to estimate the protein concentration against the observed absorbance.

3.9.2 Protein separation by Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulphate- Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was used to separate the proteins based on their molecular weight. Resolving gel 12.5% (distilled water; monomer solution; 1.5M Tris-HCl pH 8.8; 10% SDS; 10% ammonium persulphate, TEMED) was prepared and poured immediately between the glass plates. Isopropanol was added to the top, the gel was left to polymerize for 45 mins to allow polymerization. After polymerization, the combs were removed and the glass plates were shifted to the electrophoresis tank which was filled with 1X electrode tank buffer. Samples were prepared by addition of sample diluting buffer (0.125M Tris-HCl pH 6.8; 20%

glycerol; 10% 2-Mercaptoethanol) in 1:1 ratio. The samples were heated at 100℃ for 3mins and given a short spin at 12000rpm for 3min. the samples were then loaded in the wells and the electrophoretic separation process was carried out at 100 Volts for 90mins. After the run, the gel was destained using 10% destaining solution (75ml glacial acetic acid; distilled water; 25ml of 100% ethanol) until a cleared background was attained.

3.9.3 Image and Statical Analysis

Quantity One® (BioRad) image analysis software was employed for gel, quantification and molecular weight calculation of protein bands. The differential expression of the proteins was calculated on the basis of relative quantity of each band. The data was statically analyzed by Student's t-test. A value of $p < 0.05$ was considered to be statically significant.

3.10 HISTOLOGY OF BRAIN TISSUES

3.10.1 ***Perfusion Fixation***

All mice were sacrificed after performing behavioral tasks. Three of the mice's brain were fixed through heart perfusion method as previously described by Gage et al., 2012. The animal was anesthetize via intra peritoneal injection (i.p) of ketamine (300µl/50g). Pinch-response method was used to ensure that mouse was deeply anesthetize. The mouse was ventrally placed on the operating table, appendages were fixed by piercing needles. An incision was made on the abdomen to exhibit the diaphragm. To expose the heart, the connective tissues of diaphragm and ribcage was carefully incised. After opening the thoracic cavity, with the aid of forceps pumping heart was hold at a fixed position and needle was inserted into left ventricle about 5mm in depth (fig. 3.8). To release the perfusion drip valve, the cold 0.9% normal saline was steadily allowed (5ml/min). As the heart was swelled, simultaneously the right atrium was cut with the sharp scissor to allowed free flow of 80ml solution through the vascular system. As the blood cleared from the body, normal saline was replaced with 4% paraformaldehyde (PFA) solution. After running 100ml of PFA the mouse liver decolorized and tail became stiff, now perfusion was stopped and brain was harvested. The brain was stored in 4% PFA for 24-48 hours at 4 ˚C and it was then processed for dehydration and tissue embedding.

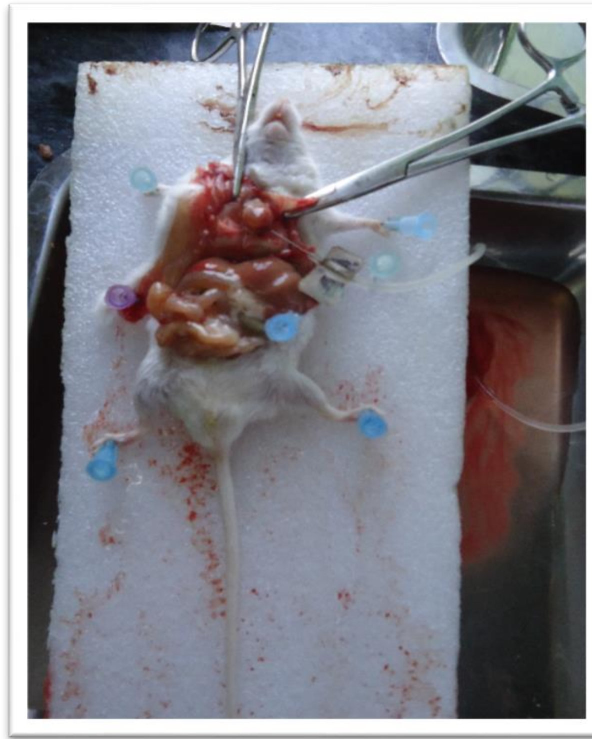


Figure 3.9: Perfusion Fixation. Test mouse under anesthesia, placed on the operating table with its back downwards. Appendages held by needles through them. Rib cage is accessed by cutting through the connective tissues at the bottom of diaphragm. The heart is fixed at a position with forceps, and needle is inserted into left ventricle protrusion about 5mm deep. Opened thoracic cavity with pumping heart is shown. Liver is gaining whitish color indicating successful insertion of needle in heart.

3.10.2 Brain Fixation and Paraffin Embedding

After incubating mouse's brain in PFA, it was further processed for paraffin infiltration. For this brain was first dehydrated with different concentration of ethanol (organic solvent) which are as follows:

1.	70% Ethanol	For 60 mins
2.	95% Ethanol	For 60 mins
3.	100% Ethanol	For 60 mins

To further hardened tissue, alcohol was replaced by xylene for 4 hours. Then paraffin embedding was performed by incubating the dehydrated brains in molten paraffin for 4hrs at 60°C. To made blocks for microtome sectioning, brain was embedded in mold

with molten paraffin. At the end, paraffin was solidified at 4°C and were ready for sectioning at room temperature. Or store until use in histological studies.

3.10.3 Tissue Sectioning

Embedded brains were sectioned on SLEE mainz (CUT6062) microtome. The 3-4μm thick sliced of tissue were transferred to a clean glass slide. Brain tissues were coronally sectioned at a thickness of 40 μm through the hippocampus and thalamus. These slides were placed on hot plate (at 60°C) for 20 mins to melt the paraffin and bound the tissue to the slide. To deparaffinized, the slide were kept in xylene for 10mins which was followed by rehydration

	Rehydration in Organic solvent	Duration
1	95 % ethanol	for 5 minutes
2	70 % ethanol	for 1 minutes
3	50 % ethanol	for 1 minutes

3.10.4 Nissl (cresylviolet) staining

Microtome sections were stained with cresyl violet as previously described by (Swarnkar et al., 2011). Sections were rehydrated in 70% isopropanol for 10 minutes and washed under running water. Slide was stained in Cresyl Violet dye for 4 minutes and again washed in water. Then sections were dipped in 70% acid alcohol* for 2mins (*2ml glacial acetic acid in 200ml of 70% ethanol), then twice dips were gave in xylene and allowed them to dry. Finally, the mounting was done by placing Canada Balsam adhesive on cover slip and gently placed over slide to avoid air bubbles. Final immersed in xylene for few seconds and allowed it to dry. Stained sections were captured at100x magnification under light microscope.

Results

4.1 Effect of turmeric and donepezil on physical parameters

4.1.1 Effect on Weight

Weight was measured to check any significant change in weight of mice after scopolamine, turmeric and donepezil treatment. Throughout the 26 days protocol treatment weight was recorded after every 7 days. There was no significant difference in Scopolamine group (39.15 ± 0.94) after subcutaneous injections of 1mg/Kg dose as compare to control (39.98 ± 0.28). No remarkable effect on weight of Scopolamine + Turmeric treated group was observed during the whole treatment. A significant ($p < 0.05$) difference was observed in case of turmeric alone group (43.18 ± 0.44) as compared to Donepezil (38 ± 0.86) Scopolamine + Turmeric (38.93 ± 0.912) Scopolamine + Donepezil (38.84 ± 0.67) Scopolamine + Turmeric + Donepezil (39.9 ± 0.90) at last days of treatment (Fig: 4.1). This can be because of turmeric's properties to balance cholesterol level, it can lower plasma and tissue cholesterol (Soudamini et al., 1992, Chainani-Wu, 2003).

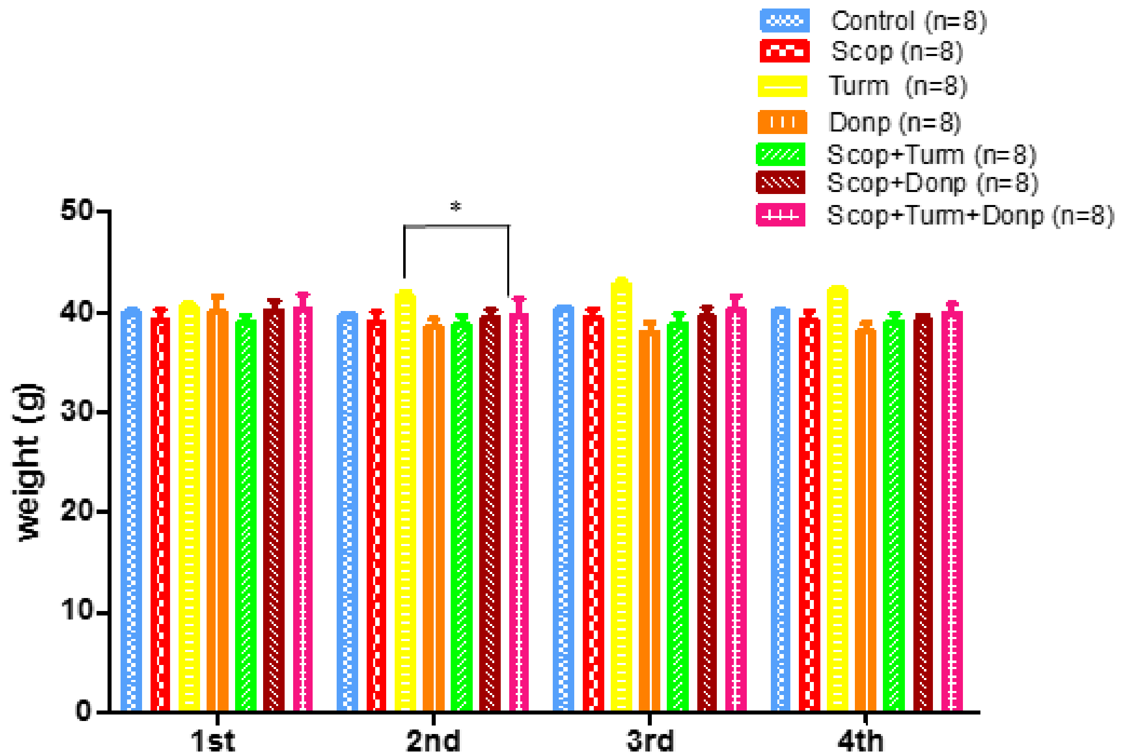


Figure 4.1: Percent weight variation after every 7 days during the treatment plan of all groups that are control group, scopolamine group (Scop), Turmeric alone (Turm), Donepezil (Donp) Scopolamine + Turmeric (Scop + Turm) and Scopolamine + Turmeric + Donepezil (Scop + Turm + Donp) groups. Error bar represent Mean ± SEM, n=8. Last bar for turmeric group is for day 15 % weight variation, as the total period of treatment for turmeric alone group was 16 days.

4.2 EFFECT OF TURMERIC ON BEHAVIORS

4.2.1 Effect of Turmeric on memory and Cognitive Functions (Morris water Maze)

Effect of turmeric and donepezil on spatial learning and reference memory was checked by Morris water maze test. Repeated training results on the acquisition of an escape response to the hidden platform. Average escape latency of mice to reach hidden platform is a direct reflection of effect of turmeric and donepezil on spatial memory. It was expressed as the average latency of mice to reach the platform as shown in fig 4.4. During acquisition all the groups showed improvement, with Control (con), Turmeric (Turm) and Donepezil (Donp) groups consistently finding the platform at almost 15sec at day 4th and 5th whereas, and Scopolamine + Donepezil (Scop + Donp), Scopolamine + Turmeric + Donepezil (Scop + Turm + Donp) finds the platform within 10 sec . Cholinergic impaired model of Scopolamine (Scop) less spatial memory than control mice with escape latency at 30 sec at day 4th and almost 25 sec at day 5th. Scopolamine + Turmeric (Scop + Turm) group show improved spatial information with latency time at almost 15 sec at day 4th and 7 sec at 5th (Fig: 4.2).

For 3rd, 4th and 5th day, Escape latency are shown in fig 4.3 (a), (b) (c) respectively. At 3rd day, Scopolamine group (44.9 ± 9.61) showed impaired memory as latency time to find the platform was high as compared to Control (29.5 ± 4.47), Turmeric alone (26.97 ± 7.38) and donepezil alone (14.98 ± 2.08) groups. Scopolamine + Turmeric group (20.96 ± 4.94) showed decrease in latency time, Scopolamine + Donepezil (17.2 ± 5.00) and turmeric alone (26.97 ± 7.38) groups showed improved spatial memory ($p < 0.05$) whereas Scopolamine +Turmeric +Donepezil (12.81 ± 2.34) showed significant difference ($p < 0.01$) relative to scopolamine group.

At 4th day, Scopolamine + Turmeric (14.3 ± 4.24) showed non-significant improved spatial memory relative to control (15.87 ± 1.83). Decrease in escape latency ($p < 0.05$) was observed in Turmeric (12.15 ± 5.07) and Scopolamine + Donepezil (12.07 ± 3.17) showed whereas significant ($p < 0.01$) improvement in spatial memory of Donepezil (5.55 ± 0.53) and Scopolamine + Turmeric+ Donepezil (7.02 ± 0.77) compare to Scopolamine (30.09 ± 7.16) group.

Similar results were also seen at day 5th, Turmeric alone (5.3 ± 1.096), Donepezil (4.78 ± 0.39), Scopolamine + Turmeric group (7.6 ± 1.81), Scopolamine + Donepezil (4.32

± 0.61) and Scopolamine + Turmeric + Donepezil (5.29 ± 0.73) groups showed decrease in latency time ($p < 0.05$) compare to Scopolamine (24.9 ± 2.82) and Control (18.37 ± 2.71) groups. This indicates that turmeric powder and donepezil improved spatial learning.

On day 6th probe trial was performed for the assessment of reference memory. Time spent in the goal quadrant was recorded to check the exploration time for the previously placed hidden platform (Fig: 4.4 (a)). The scopolamine group (32.75 ± 2.38) showed significant ($p < 0.01$) decline in reference memory as they spent less time in the target quadrant as compared to control (52.37 ± 2.82) and donepezil (49.75 ± 3.01). On the other hand, the time spent by the turmeric alone (62.25 ± 3.79), Scopolamine + Turmeric group (61.5 ± 4.25), Scopolamine + Donepezil (53.25 ± 2.62), Scopolamine + Turmeric + Donepezil (55.37 ± 2.85) in the goal quadrant was significantly ($p < 0.001$) increased relative to scopolamine group. Indicating that turmeric and donepezil alone and in combination synergistically overcame the mnemonic impairment effect of scopolamine and developed reference memory.

Same results for probe trial were also depicted by the number of crossing the goal area that is the position where platform was previously positioned as shown in fig 4.4 (b). Scopolamine group (1.37 ± 0.32) showed significant ($p < 0.01$) decline indicating memory relative to control group (3.75 ± 0.67). Scopolamine + Turmeric + Donepezil (4.5 ± 0.68) had the highest ($p < 0.001$) number of crossing in contrast to Scopolamine group (1.37 ± 0.32) showed significant ($p < 0.001$) decline indicating memory. The crossing number and reference memory significantly ($p < 0.01$) improved in the Turmeric group (5 ± 1.16), Donepezil (4.62 ± 0.77), Scopolamine + Turmeric (4.5 ± 0.86) and Scopolamine + Donepezil (4.25 ± 0.81) as compare scopolamine group. Representative mouse pathways for the probe trial are shown in fig 4.4 (c).

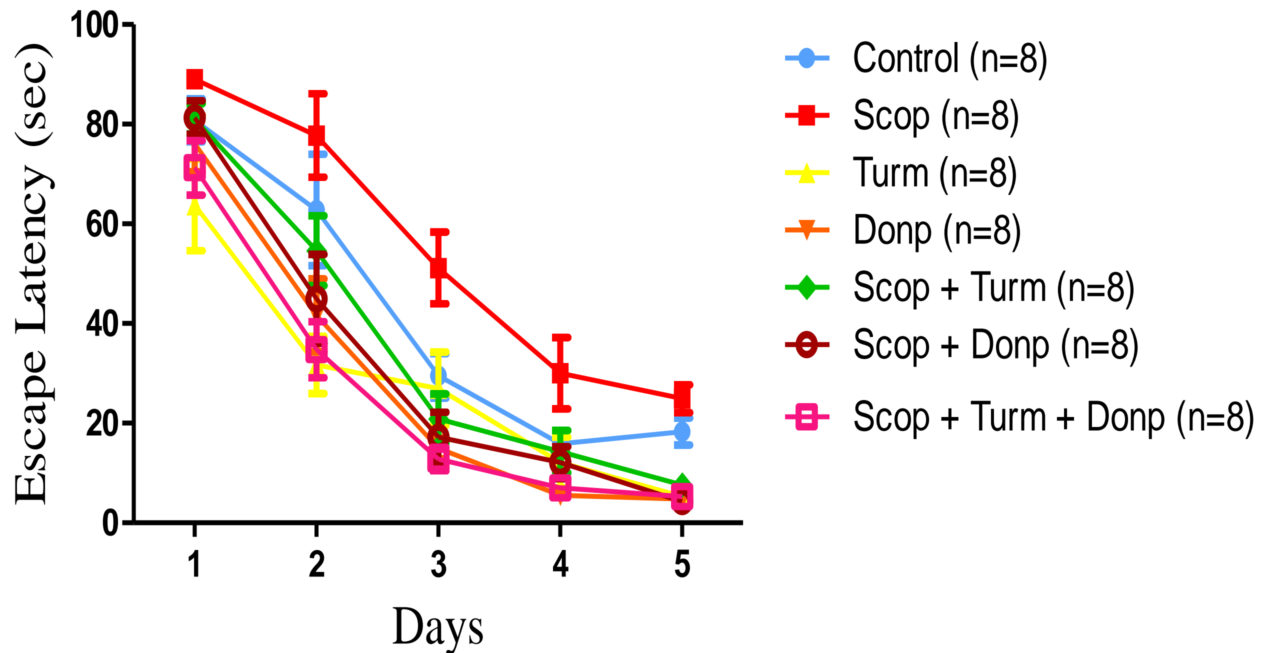


Figure 4.2: Effect of turmeric powder and donepezil on learning and memory using Morris water maze (MWM) test: Graph depicts escape latency (sec) of spatial memory between Control, Scopolamine (Scop), Turmeric alone (Turm), Donepezil (Donp) Scopolamine + Turmeric (Scop + Turm), Scopolamine + Donepezil (Scop+ Donp) and Scopolamine + Turmeric +Donepezil (Scop+ Turm+ Donp) groups. Scopolamine treated mice learn and retain spatial memory less quickly than control mice. The data indicate that the Scopolamine + Turmeric + Donepezil group find the location much faster than the cognitive impaired scopolamine group. Error bars represent mean ± SEM; n= 08.

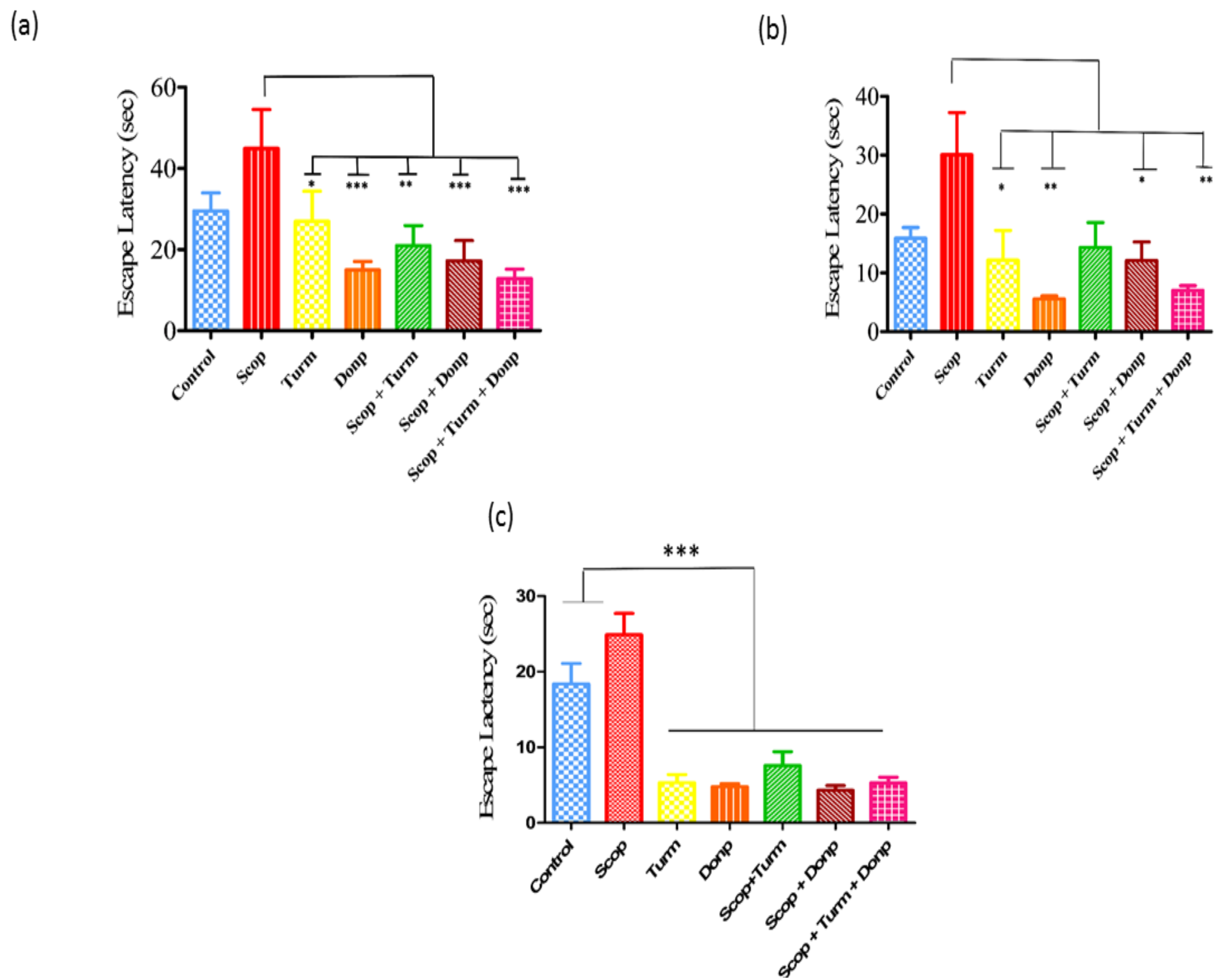
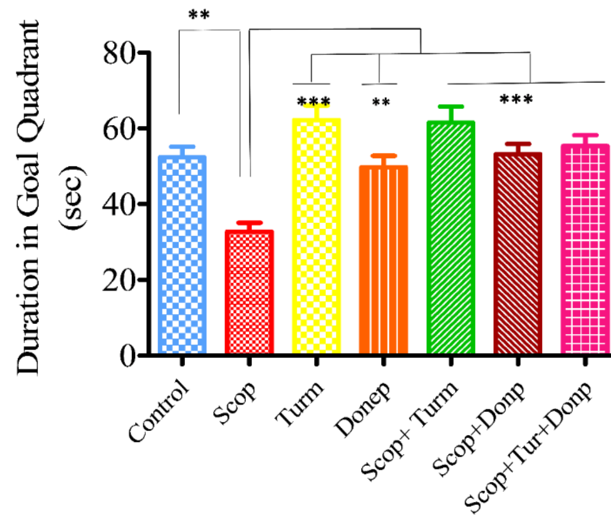


Figure 4.3: Comparison between seven groups on day 3rd **(a),** 4th **(b)** and day 5th **(c)**: Significant difference ($p < 0.01$), between Scopolamine (Scop) and Scopolamine + Turmeric + Donepezil (Scop + Turm + Donp) on 3rd and 4th days, whereas significantly improvement ($p < 0.001$) was shown on 5th day. This depicted that Scop + Turm +Donp was much faster than the cognitively dysfunction group due to the synergistically enhancing effect of turmeric and donepezil in learning and memory. *=$p < 0.05$, **=$p < 0.01$ and **=$p < 0.001$. Error bars represent mean ± SEM; n= 08. One way ANOVA followed by Bonferroni Multiple Comparison Test.

(a)



(b)

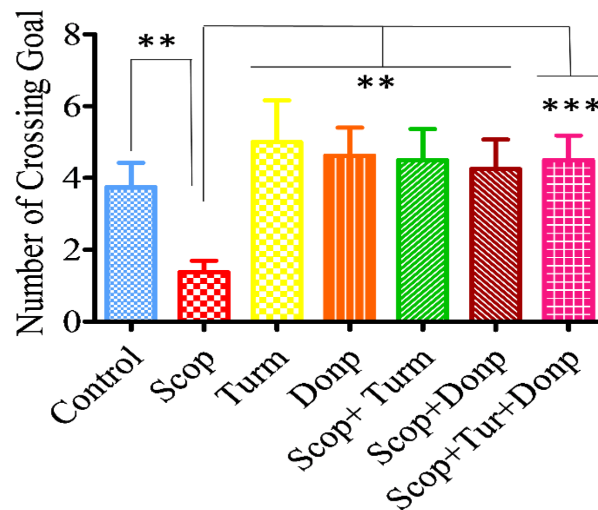


Figure 4.4: Effect of turmeric and donepezil on reference memory (MWM Test, Probe Trial): Time spent by test animals in goal quadrant, where platform was placed is shown in bar diagram **(a)**. Number of crossing the goal area i.e. where platform was previously positioned **(b)**. Significant difference ($p < 0.01$) is present between control and Scopolamine (Scop) group depicting impaired reference memory in cognitive impaired animals. Scop again shows significant difference ($p < 0.01$) with Turmeric, Donepezil, Scopolamine + Turmeric (Scop + Turm), and Scopolamine + Donepezil (Scop + Donp). Scopolamine + Turmeric+ Donepezil (Scop + Turm +Donp) Significantly ($p < 0.001$) spent more time in the target quadrant relative to as time spent by the Scop group, and so is the number of crossings; indicating that reference memory is restored by the synergistic effect of turmeric and donepezil. **=$p < 0.01$ and ***=$p < 0.001$. Error bars represent mean ± SEM; n= 08. One way ANOVA followed by Bonferroni Multiple Comparison Test was applied.

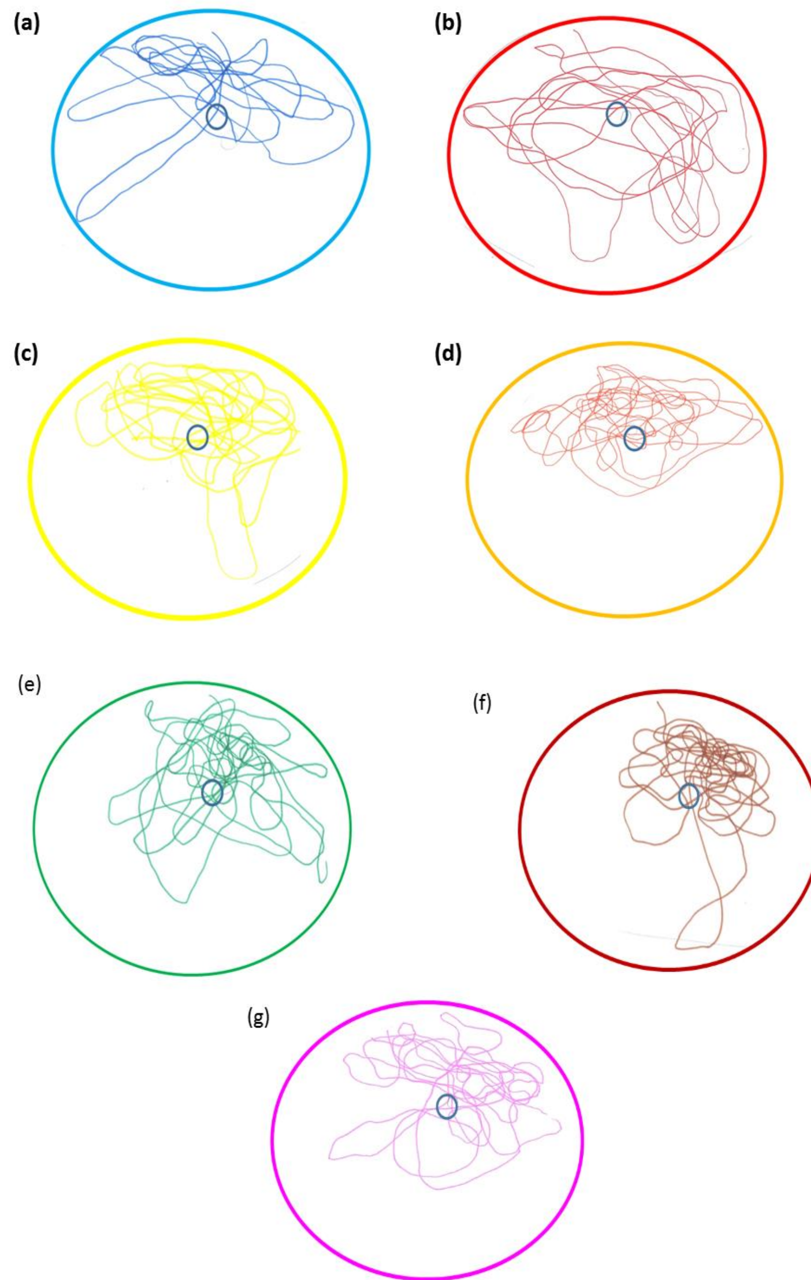


Figure 4.5: Effect of turmeric and donepezil on reference memory (Probe Trial, MWM Test): Representative pathways of mouse swimming in probe trial are shown Control mice (a) center their search location around the previous platform location, whereas scopolamine mice (b) are less systematic, spending more time along the perimeter. Turmeric mice (c) and donepezil mice (d) spent more time in the goal quadrant. Reference memory is improved by the intake of turmeric and donepezil in scopolamine + turmeric mice (e) scopolamine + donepezil mice (f) and scopolamine + turmeric + donepezil mice (g).

4.2.2 Effect of turmeric and donepezil on social preference

Mice being social species exhibit behavioral social interaction. Social preference test was carried out to check levels of sociability and preferences for social novelty in cognitive impaired animals and then to check turmeric and donepezil effect on it. In first session pair-bonding was checked to assess sociability. As compared to Control group (144 ± 16.12), time spent by Scopolamine group (85.62 ± 18.81) with Stranger 1 was quite less depicting lack innate social aptitude. This was also shown by the highest interaction with the empty cage by Scopolamine group relative to all other six groups. Moreover Turmeric alone group (192 ± 40.36) Donepezil (203 ± 18.26), Scopolamine + Turmeric (143.12 ± 14.82), Scopolamine + Donepezil (222 ± 38.40) and Scopolamine + Turmeric + Donepezil (233 ± 42.23) showed significant ($p < 0.05$) increase in sociability as compared to scopolamine indicating positive effect of turmeric and donepezil on social behavior (Fig: 4.5 (a)).

Second session was performed to check animal's choice between a familiar mouse (Stranger 1) or a novel mouse (Stranger 2) in order to evaluate animal's preference for social novelty. Donepezil alone group (240 ± 33.55) spent significantly ($p < 0.001$) more time with unfamiliar Stranger 2 mouse than already investigated Stranger 1 mouse, as compared to Scopolamine group (63.25 ± 14.44), showing that cognitively impaired animals have less preference for social novelty. In Scopolamine + Turmeric + Donepezil (210 ± 45.23) group the synergistic effect of turmeric and donepezil on social novelty was shown by the significant ($p < 0.01$) increase in interaction time with unfamiliar Stranger 2 mouse. Scopolamine + Donepezil (193 ± 30.23) showed significant improvement ($p < 0.05$) in social behavior. Control (76 ± 16.53), Turmeric alone (122 ± 21.96) groups and Scopolamine + Turmeric group (110 ± 16.17) has showed social preference but it was non-significant (Fig 4.5 (b)).

Discrimination Index (DI) was calculated as an index of discrimination ability and memory. In first session, time of interaction with Stranger 1 vs. empty cage is depicted. Control group (81 ± 1.700) spent significantly ($p < 0.05$) more time with the mouse than Scopolamine group (66 ± 5.53). Turmeric group (79.25 ±5.11), Donepezil (65.01 ± 5.08) Scopolamine + Turmeric (71.87 ±2.49), Scopolamine+ Donepezil (68.87 ± 3.865) Scopolamine + Turmeric + Donepezil (71.25 ± 4.43) group showed sociability in DI as

compared to scopolamine group but it was non-significant (Fig: 4.6 (a)). In second session, DI is between Stranger 1 vs. Stranger 2, and is a measure for social novelty. Scopolamine group (56.37 ± 3.48) showed a significant ($p < 0.05$) decrease in the interaction time as compared to turmeric alone (73.12 ± 4.04) and Scopolamine + Turmeric (68.5 ± 2.13) groups, indicating less preference of cognitive impaired animals for social novelty. Scopolamine+ Turmeric+ Donepezil (70.25 ± 4.22) has showed significant ($p < 0.01$) increase in interaction time with unfamiliar Stranger 2 mouse this depicting the synergistic effect of turmeric and donepezil.

Scopolamine+ Donepezil (67.5 ± 3.93) Donepezil (66.62 ± 4.15) has more interaction with novel mouse Stranger 2 as compared to scopolamine group control (61.37 ± 6.60) but the difference was non-significant (Fig: 4.6(b)). This Indicates that turmeric powder and donepezil has positive impact on sociability and social novelty and improves the deficits caused by scopolamine drug.

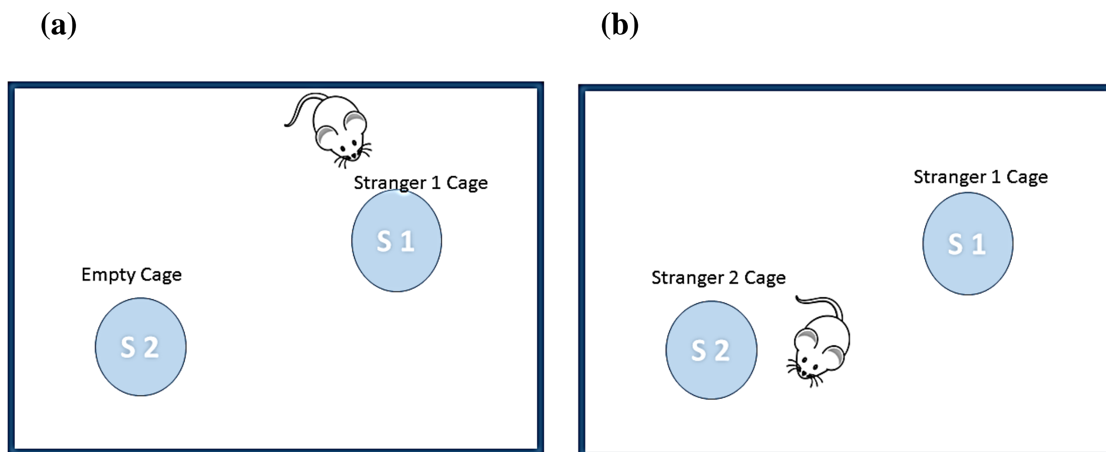
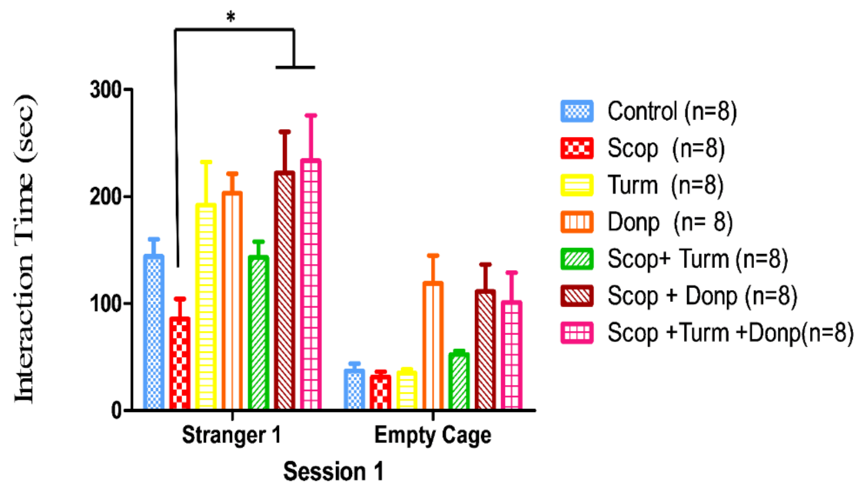


Figure 4.6: The cartoonish representation of Sociability (a) and Preference Social Novelty Test (PSNT) (b).

(a)



(b)

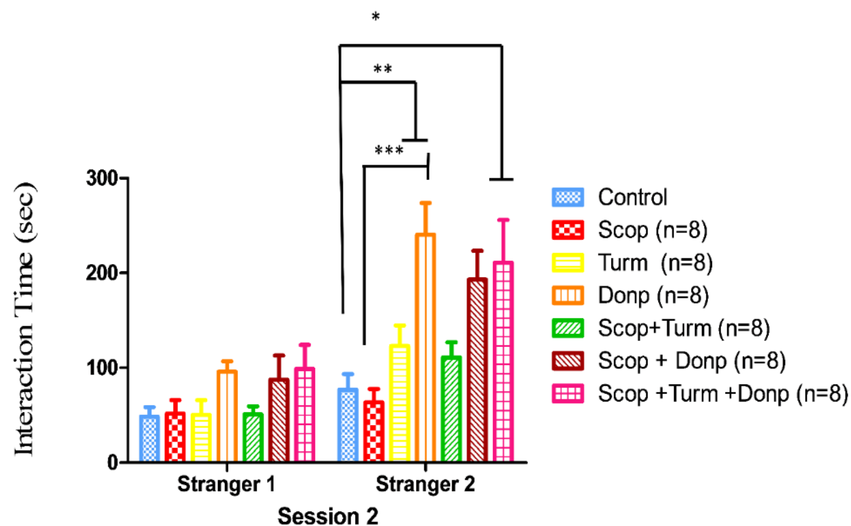
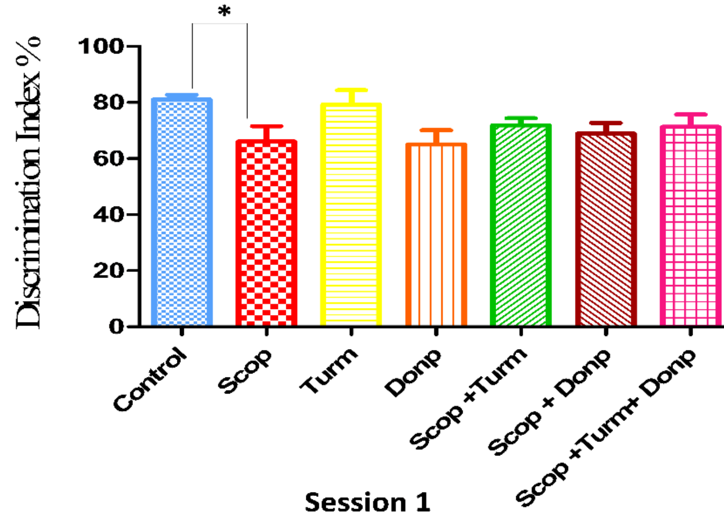


Figure 4.7: Effect of turmeric and donepezil on sociability and social novelty: The graph depicts comparison of Control, Scopolamine (Scop), Turmeric (Turm), Donepezil (Donp), Scopolamine + Turmeric (Scop + Turm), Scopolamine + Donepezil (Scop + Donp) and Scopolamine + Turmeric + Donepezil (Scop + Turm + Donp) groups. **(a)** First session for sociability, duration measures were taken i.e. preference for Stranger 1 mouse vs. an empty cage. **(b)** Second session for social novelty i.e. preference between a novel mouse (Stranger 2) vs. first familiar mouse (Stranger 1). Error bars represent mean ± SEM; n= 08. *=$p < 0.05$ and ***=$p < 0.001$. One way ANOVA followed by Bonferroni Multiple Comparison Test was applied, between group comparisons for Stranger 1 in first session and for Stranger 2 in second session.

(a)



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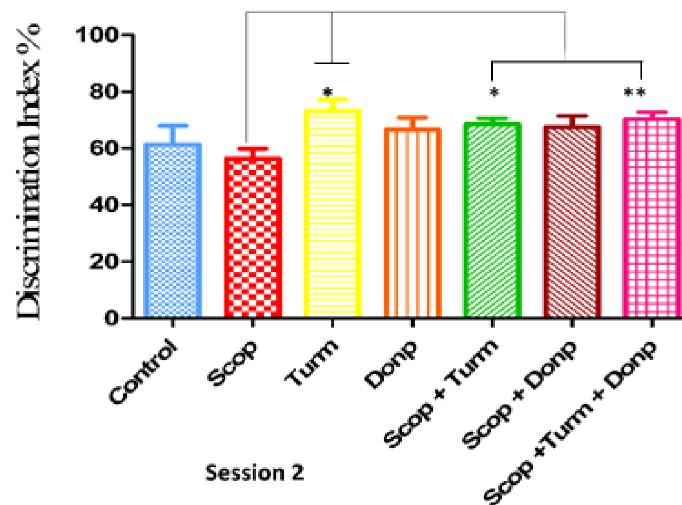

Figure 4.8: Effect of turmeric and donepezil of sociability and social novelty preference between the groups: Control, Scopolamine (Scop), Turmeric (Turm), Donepezil (Donp), Scopolamine + Turmeric (Scop + Turm), Scopolamine + Donepezil (Scop + Donp) and Scopolamine + Turmeric + Donepezil (Scop + Turm +Donp **(a)** Discrimination Index (DI) % of first session for sociability. **(b)** Second session DI % for the social novelty. Error bars represent mean ± SEM; n= 08. One way ANOVA followed by Bonferroni Multiple.

4.3 Effect of turmeric and donepezil on mRNA levels

RNA was extracted from hippocampus region of the mouse brain. Extracted RNA was quantified and then quality control check was carried out. Quality of RNA was checked through gel electrophoresis. RNA samples were run on 2% agarose gel at 90mV for 20-25 minutes. Good quality was indicated by sharp ribosomal bands (28S and 18S) of RNA (Fig: 4.7 (a)).

To check transcriptional basis of the effect of turmeric, mRNA levels was studied in hippocampus. For this RNA quantification was done through RT-PCR. Therefore gene expression of nicotinic (alpha-7, alpha-4 and beta-2) and muscarinic (M1, M3, and M5) were studied in hippocampus region of the mouse brain to show pharmacological effects of turmeric and donepezil on cognitive impairment caused by scopolamine.

To normalize respective genes, the housekeeping gene used was Actin. PCR products were separated on agarose gel 2% stained with ethidium bromide. Analysis of the PCR products was carried out using NIH software; Image J. Expression was measured by dividing desired gene density by Actin density. Results were plotted in graphical form

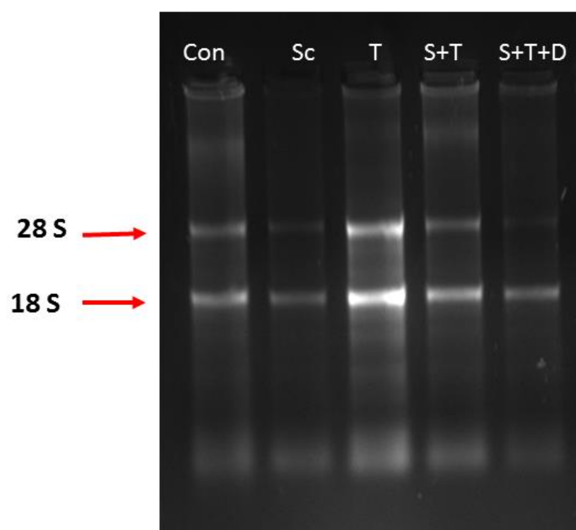

Figure 4.9 (a): RNA Gel Electrophoresis Image: 3μL of each RNA sample extracted from hippocampus was loaded in 2% agarose gel for quality check. Ethidium bromide staining was used and bands were visualized under UV light of Gel Documentation System (gel doc). Samples with sharp and clear bands for 28S and 18S RNA, shows that the RNA is of good quality (not degraded) and is acceptable for further downstream procedures.

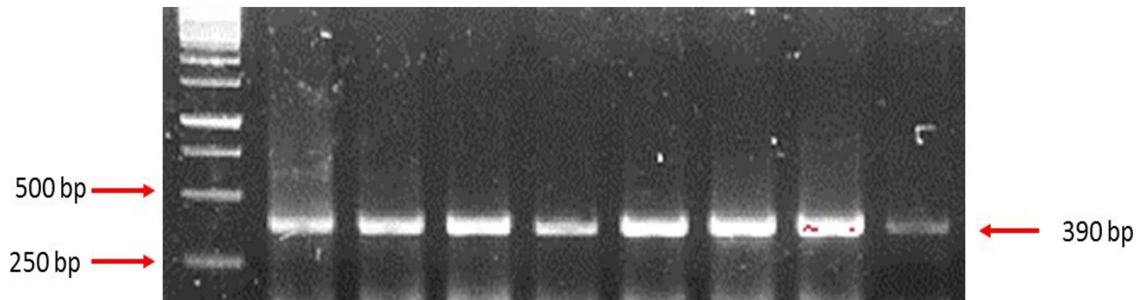

Figure 4.9 (b): Gel Electrophoresis Image: Amplified PCR products were run on agarose gel. 2% gel was used for Actin products used for nAchR (α-7, α-4 and β-2). The product size for α-7 is 364bp. α-4 has 316bp while β-2has 390bp respectively. Above gel picture is for nicotinic receptors PCR products.

4.3.1 Effect of turmeric and donepezil on nAChR genes in hippocampus

Gene expression studies were also carried for hippocampus region of the brain. In hippocampus alpha-7 expression was significantly high ($p < 0.01$) in Turmeric group (1.24 ± 0.09) as compared to scopolamine group (0.42 ± 0.07). Scopolamine + Turmeric + Donepezil (0.71 ± 0.09) Scopolamine + Turmeric group (0.77 ± 0.17), Control group (0.7852 ± 0.1512) had moderate expression. The difference was not significant.

In case of alpha-4, expression was significantly ($p < 0.01$) high in Turmeric group (0.58 ± 0.16) as compared to Scopolamine group (0.21 ± 0.08) and Control group (0.32 ± 0.005). A significant increase ($p < 0.05$) in Scopolamine + Turmeric group (0.45 ± 0.06) was observed in contrast to scopolamine group. Increase expression level was observed in case of Scopolamine + Turmeric + Donepezil group (0.41 ± 0.02) but the data was non-significant.

Significantly high expression ($p < 0.05$) of Beta-2 was depicted in turmeric group (1.52 ± 0.18) and Scopolamine + Turmeric group (1.21 ± 0.02) as compared to Scopolamine group (0.56 ± 0.09). Scopolamine showed low level of expression just by decimal points. A decrease in Scopolamine + Turmeric + Donepezil group (0.81 ± 0.02) was observed in contrast to Control group (1.21 ± 0.31) but there was no significance difference among the groups. Expression levels of alpha-7, alpha-4 and beta-2 in hippocampus are shown in fig: 4.11.

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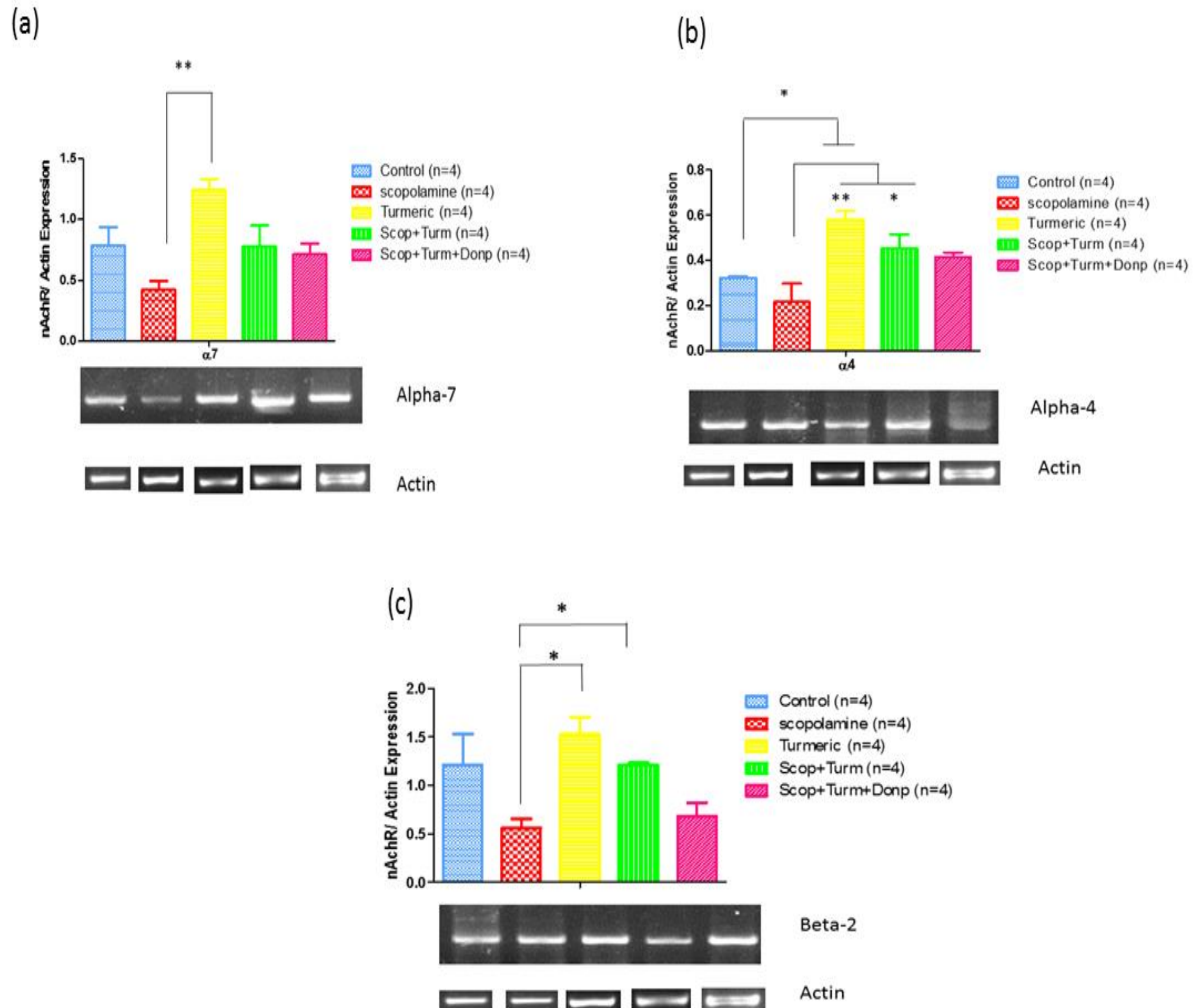


Figure 4.10: Effect of turmeric and donepezil on expression of genes in hippocampus: alpha-7, alpha-4 and beta-2 expression in hippocampus of Control group, Scopolamine (Scop) group, turmeric group, Scopolamine + Turmeric (Scop + Turm) treated group and Scopolamine + Turmeric + Donepezil (Scop + Turm + Donp) treated group. Evaluation of expression level was carried out through RT-PCR. Bar diagram shows the intensity of nAchR level normalized with Actin. Error bars represent mean ± SEM; n= 04. One way ANOVA followed by Bonferroni Multiple Comparison Test was applied, *=p < 0.05, and **=p < 0.01.

4.3.2 Effect of turmeric and donepezil on mAChR genes in hippocampus

Gene expression studies were also carried for hippocampus region of the brain. In hippocampus M1 expression was significantly decreased ($p < 0.001$) in Scopolamine group (0.63 ± 0.04) as compared to control group (1.15 ± 0.05). In contrast, Scopolamine + Turmeric group (1.97 ± 0.52) demonstrate significantly increase ($p < 0.01$) in expression as compared to scopolamine induced amnesic model. Scopolamine + Turmeric + Donepezil (0.62 ± 0.009) and Turmeric group (0.83 ± 0.17) had moderate expression. The difference was not significant.

In case of M3, expression was significantly ($p < 0.05$) lowered in Scopolamine group (0.25 ± 0.08) as compared to Control group (0.49 ± 0.08). A significant increase expression ($p < 0.001$) in Turmeric group (0.53 ± 0.02) and ($p < 0.01$) Scopolamine + Turmeric group (1.03 ± 0.17) was observed in contrast to Scopolamine group. Whereas, moderate expression level was observed in case of Scopolamine + Turmeric + Donepezil group (0.31 ± 0.09) but the data was non-significant.

Significantly decrease expression ($p < 0.01$) of M5 was depicted in Scopolamine group (0.17 ± 0.01) as compared to Control group (0.52 ± 0.09). Turmeric group (0.46 ± 0.12) significantly increase ($p < 0.05$) in expression as compared to amnesic model. Likewise Scopolamine + Turmeric group (0.90 ± 0.22) and Scopolamine + Turmeric + Donepezil group (0.69 ± 0.11) demonstrate significantly increase ($p < 0.01$) in gene expression as compared to Scopolamine treated group. Expression levels of M1, M3 and M5 in hippocampus are shown in fig: 4.11.

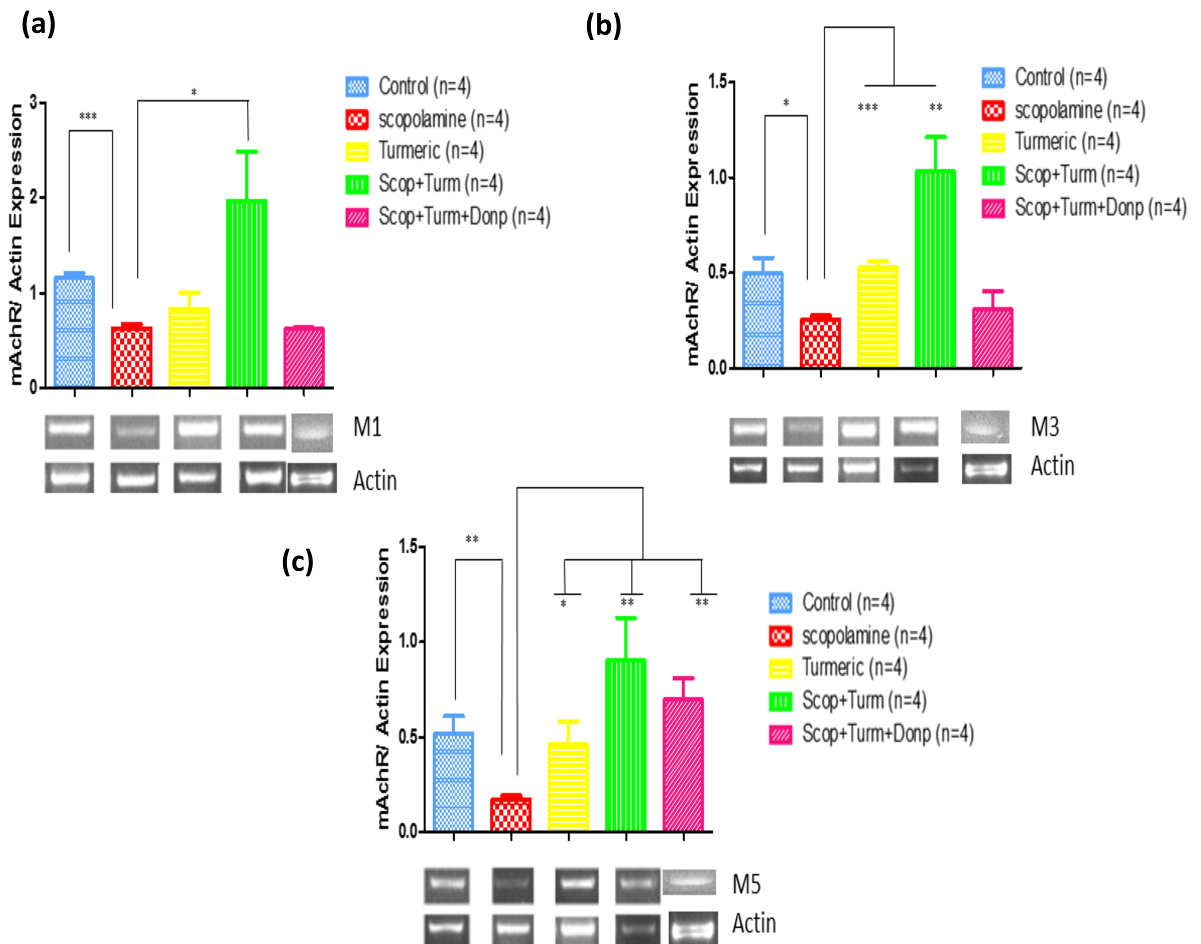
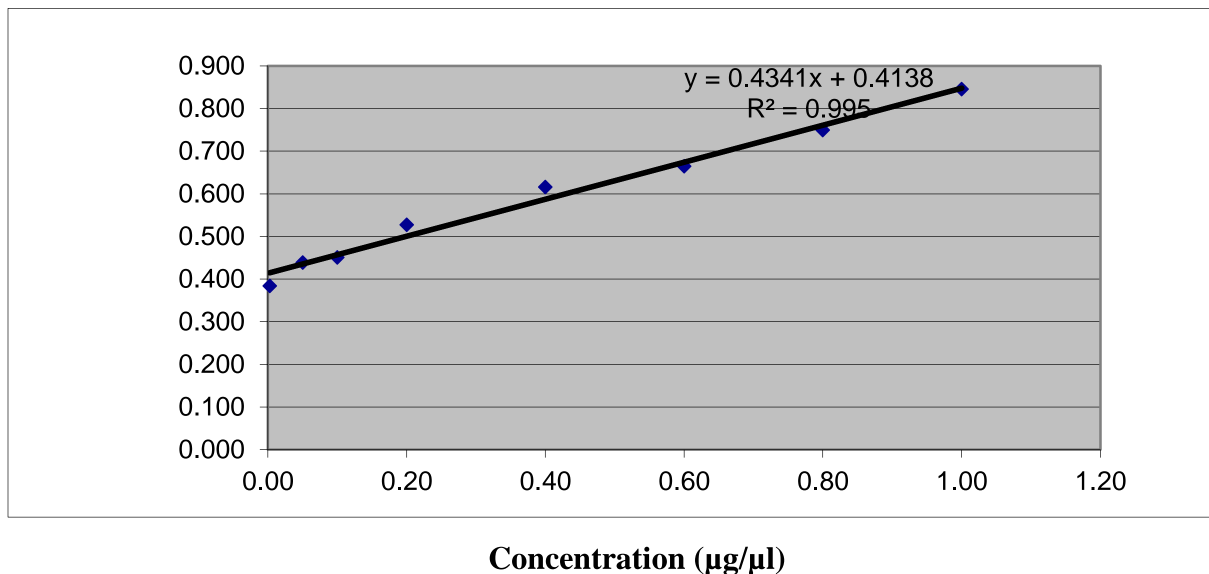


Figure 4.11: Effect of turmeric and donepezil on expression of genes in hippocampus: M1, M3 and M5 expression in hippocampus of Control group, Scopolamine (Scop) group, and Turmeric group, Scopolamine + Turmeric (Scop + Turm) treated group and Scopolamine + Turmeric + Donepezil (Scop + Turm + Donp) treated group. Evaluation of expression level was carried out through RT-PCR. Bar diagram shows the intensity of mAchR level normalized with Actin. Error bars represent mean ± SEM; n= 04. One way ANOVA followed by Bonferroni Multiple Comparison Test was applied, *=p < 0.05, **=p < 0.01 and ***=p < 0.001.

4.4 Effect of turmeric and donepezil on protein expression in hippocampus

4.4.1 Protein Quantification

Protein concentration of each sample was determined by plotting the absorbance value of the colored reaction product on the standard curve. The intensity of the colored product is directly proportional to the protein content of the sample.



Concentration (µg/µl)

Figure 4.12: Bradford standard curve plotted for eight standard values. Concentration was plotted on the x-axis (independent variable). Absorbance measured at 595 nm was plotted on the y-axis (dependent variable). This graph represents linear regression for the eight standard points. The obtained linear regression value was 0.995 (R^2=0.995).

4.4.2 Total differential protein expression

The total proteome profile of the hippocampus was attained by 1D gel separation (SDS-PAGE). Marked differences in the expression of one protein in hippocampus. The gel image analysis was done using Chemi Doc software.

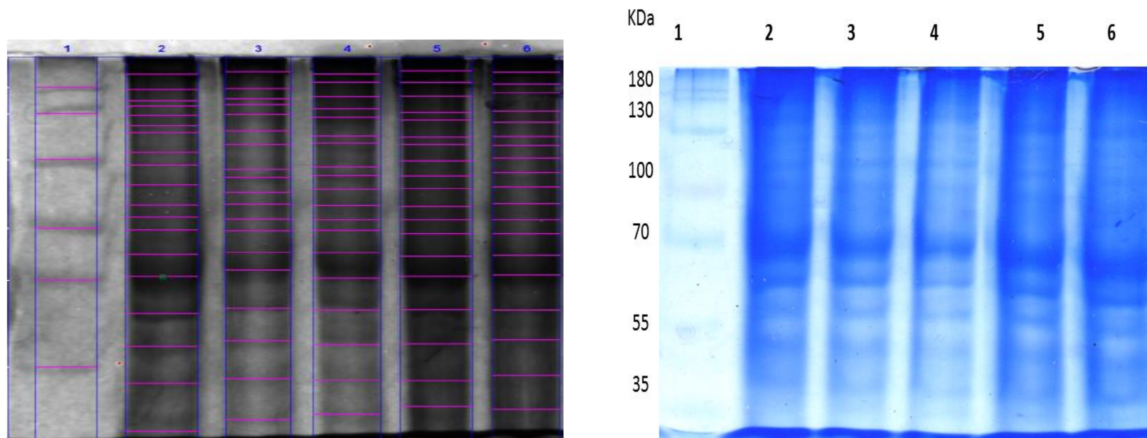


Figure 4.13: The SDS-PAGE image of hippocampus proteins. Extracted proteins were separated in 10% resolving gel and stained with coomassie Brilliant Blue. Lane 1: protein marker, lane 2: healthy control, lane 3: Scopolamine treated, Lane 4: Turmeric treated, lane 5: Scopolamine + Turmeric treated, Lane 6: Scopolamine+ Turmeric +Donepezil treated. The amount of hippocampus protein loaded in each well was 75µg.

4.4.3 Effect of turmeric and donepezil on differential expression of hippocampal protein

Protein expression studies were also carried for hippocampus region of the brain. In hippocampus 35K Da expression was significantly high ($p < 0.01$) in scopolamine group (31.03 ± 0.56) as compared to control group (8.57 ± 0.67) and Scopolamine + Turmeric + Donepezil group (9.72 ± 2.75). Scopolamine + Turmeric group (17.89 ± 3.34) and Turmeric alone group (19.90 ± 4.19) has was non-significant protein expression as compare with Scopolamine. (Fig 4.12)

In case of 55K Da, expression of protein was significantly ($p < 0.01$) expressed in Scopolamine +Turmeric + Donepezil group (89.47 ± 17.57) as compared to scopolamine group (16.27 ± 1.43) and control group (29.88 ± 6.75). Scopolamine + Turmeric group (59.65 ± 3.61) in and Turmeric group (39.70 ± 4.30) has shown improves in expression as compared to scopolamine induced mice model, whereas there is no significant difference.

In 70K Da the expression of protein was significantly ($p < 0.01$) high in Scopolamine + Turmeric + Donepezil group (101.7 ± 18.55) as compared to scopolamine group (31.01 ± 3.08). Scopolamine + Turmeric group (66.72 ± 13.14) and Turmeric group (48.85 ± 3.73) has shown increase in protein expression as compared to control group (40.18 ± 5.58) but the data was non-significant.

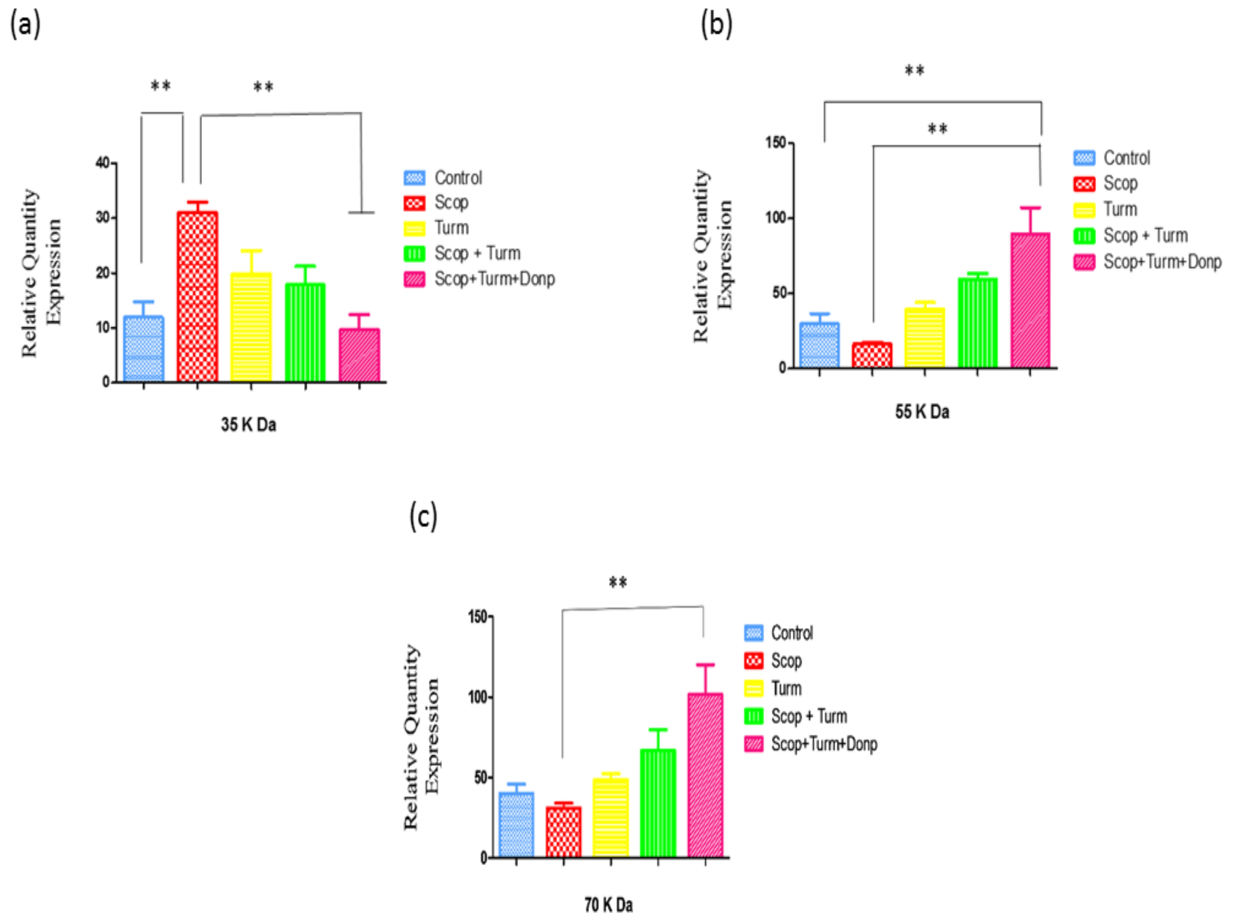


Figure 4.14: Effect of turmeric and donepezil on expression of proteins in hippocampus: 35 K Da (a), 55K Da (b), 70K Da (c), proteins expression in hippocampus of Control group, Scopolamine (Scop) group, Turmeric (Turm) group, scopolamine + turmeric (Scop + Turm) treated group and Scopolamine + Turmeric + Donepezil (Scop + Turm + Donp) treated group. Evaluation of expression level was carried out through SDS-PAGE. Bar diagram shows the intensity of respected protein in drug treated groups. In 35K Da proteins samples, the Scopolamine treated group showed significantly ($p < 0.01$) increase in expression as compare to Scopolamine + Turmeric + Donepezil and control group. In 55K Da (b) and 70K Da (c), Scopolamine + Turmeric + Donepezil treated group showed significantly ($p < 0.01$) increase in protein expression as compared to scopolamine treated group. Error bars represent mean ± SEM; n= 03. One way ANOVA followed by Bonferroni Multiple Comparison Test was applied, **=$p < 0.01$

4.5 HISTOLOGICAL STUDIES

Coronal sections of mouse brain were stained with Cresyl violet and Congo red. Cresyl Violet was used for neurodegeneration detection. Cortex region was examined under 10X magnifications.

4.5.1 Cresyl Violet Stain

Staining showed that there was neurodegeneration in scopolamine-induced cholinergic hypo-function model in mouse brain as compared to the control. Turmeric and donepezil significantly restored neurodegeneration in scopolamine-induced model showing neuroprotective effect of turmeric as shown in 10X and 20X magnification under the microscope. These findings are the qualitative results based on the morphology of neuronal cell bodies (Fig 4.13). For the cell quantification the C3 area of the cortices was selected. To observe the neurodegeneration in the brain region, the slides were identified into six cortical layers and each layer was further quantified at the area of 100 X 100 µm of the 4 µm thick of brain's slice (Fig 4.14).

4.5.1 *Effect of turmeric and donepezil on neuronal cell density*

In I cortical layer the scopolamine group (8.00 ± 0.57) has shown significant ($p < 0.05$) neuronal degeneration as compare to control (11.50 ± 0.28). The Scopolamine + Donepezil (11± 0.57), Scopolamine +Turmeric + Donepezil (11.33 ± 0.66) groups has shown significant increase ($p < 0.05$) in cell density as compared to scopolamine but the data was non-significant. Scopolamine + Turmeric (10 ± 0.57), Turmeric alone group (11.33 ± 1.20) and Donepezil alone group (12 ± 0.57) has moderate increase in cell count (Fig 4.17).

In II -IV cortical layer scopolamine + Donepezil (36.67 ± 1.45) has shown significant ($p < 0.05$) increase as compare to scopolamine (25.67 ± 2.60). Turmeric (33.00 ± 1.52), donepezil (36.00 ± 1.45), scopolamine +Turmeric (30.67 ± 0.57), Scopolamine +Turmeric + Donepezil (29.67 ± 2.60) has shown increase in cell count as compared to control (30.33 ± 0.33), but this data was non-significant

In V–VI cortical layer of the mouse, the scopolamine group (51.67 ± 1.85) has shown significantly ($p < 0.01$) decrease in cell density as compared to Scopolamine +

Donepezil (72.33 ± 2.18), control group (65.33 ± 6.36), whilst the turmeric alone group (66.33 ± 3.52), donepezil alone group (71.67 ± 1.45) Scopolamine +Turmeric (66.67 ± 5.78), Scopolamine + Turmeric + Donepezil (62.33 ± 1.20) demonstrate non-significantly increase in cell count.

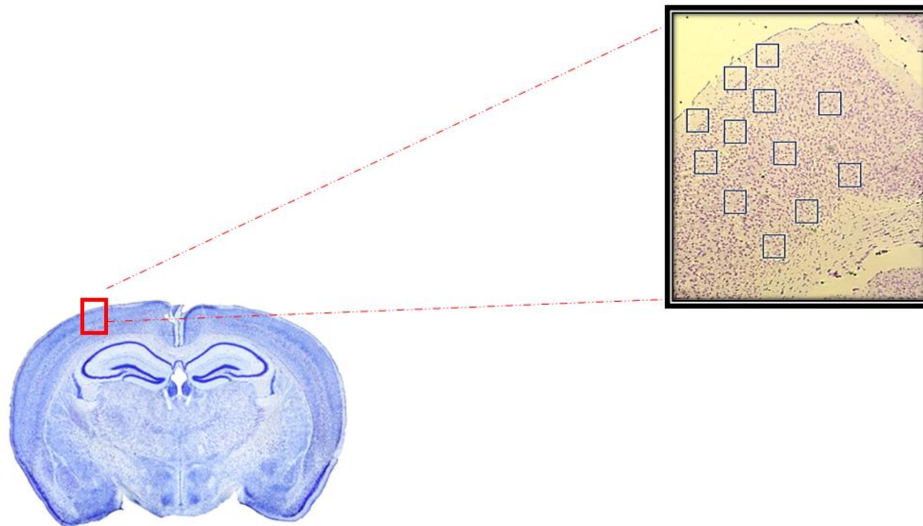


Figure 4.15: The cortical image of the mouse brain. The cell density was determined under light microscope, the cortical layers of C3 area was identified and quantified at the area of 100 X 100 µm per layer of brain's slice and SEM was calculated.

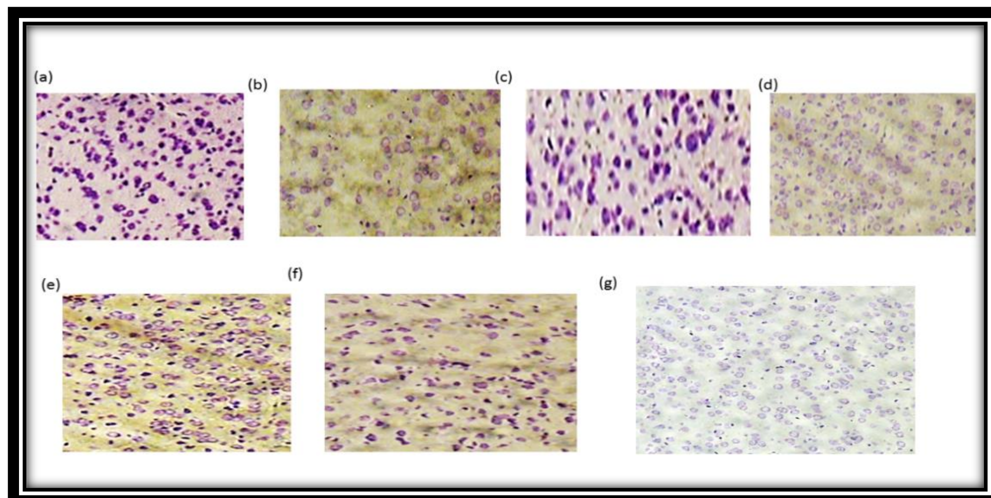


Figure 4.16: Effect of turmeric and donepezil on cell density in cortex. Cresyl violet staining of brain tissue cortex (4µm thickness), at and 20X magnification: Control (a), Scopolamine (b), Turmeric (c), Donepezil (d), Scopolamine+ Turmeric (e), Scopolamine+ Donepezil (f), Scopolamine+ Turmeric + Donepezil (g).

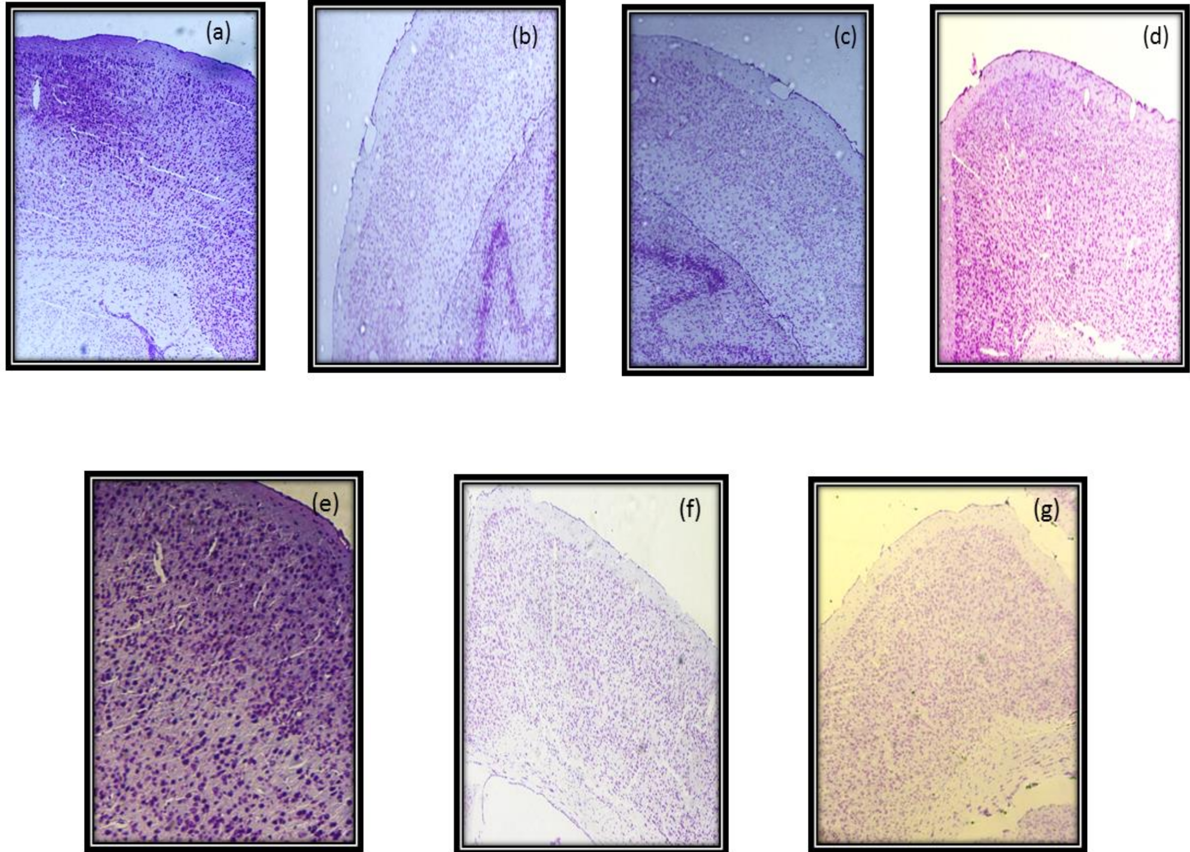
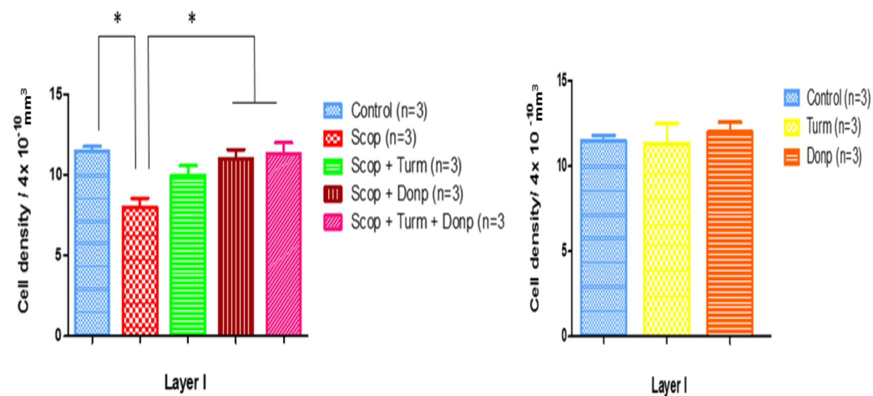
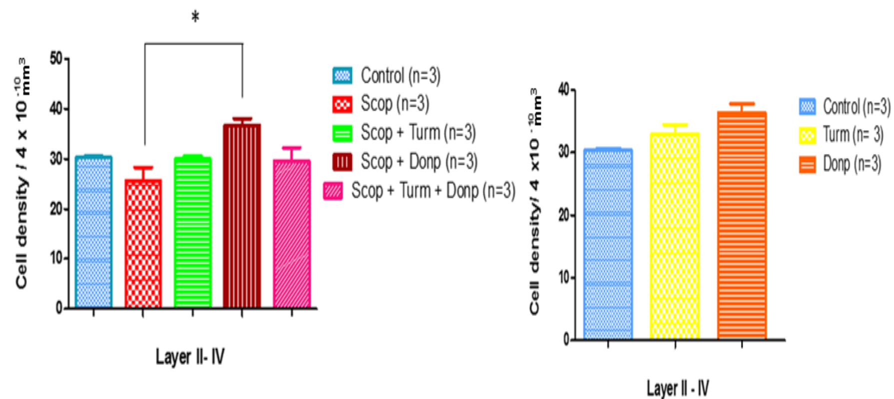


Figure 4.17: Effect of turmeric on cell density in C3 area of cortex: Cresyl violet staining of brain tissue cortex (4μm thickness), at and 10X magnification. Control (a), Scopolamine (b), Turmeric (c), Donepezil (d), Scopolamine+ Turmeric (e), Scopolamine+ Donepezil (f), Scopolamine + Turmeric + Donepezil (g).

(a)



(b)



(c)

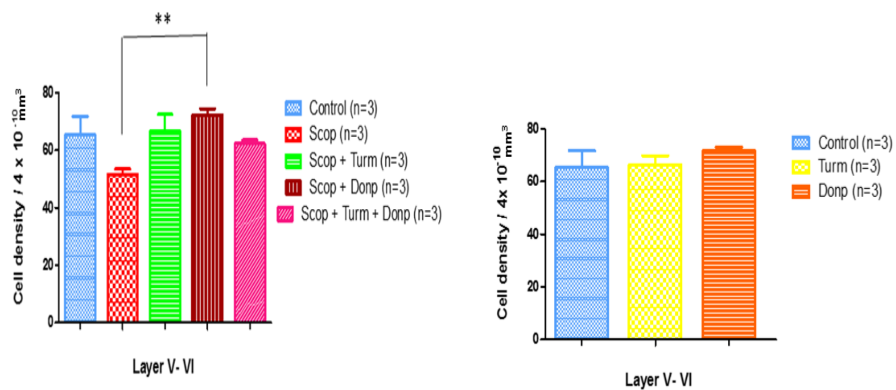


Figure 4.18: Effect of turmeric and donepezil on neuronal cell density in cortex: control group, scopolamine group, turmeric group, scopolamine + turmeric treated group and Scopolamine + Turmeric + Donepezil treated group. Evaluation of cell density was carried out through cresyl violet stain. Bar diagram shows the density of neuronal cells count in drug treated groups relative to control. The Scopolamine show significant decrease ($p < 0.05$) in cell count as compare to control. Error bars represent mean ± SEM; n= 03. One way ANOVA followed by Bonferroni Multiple Comparison Test was applied, *=$p < 0.05$

Discussion

In this comparative study we aimed to evaluate the neuroprotective properties of turmeric with standard drug donepezil along with scopolamine to induce cognitive dysfunction in mice model. Scopolamine model has proven to be a useful for investigating, understanding and developing therapeutic approaches for neurodegenerative diseases in humans (Bartus, 2000). Turmeric and donepezil have shown synergistic improvement on the cognitive behavior tasks.

Morris water maze test is best suited for evaluating spatial learning and memory to determine reference memory (Morris, 1981, Vorhees and Williams, 2006, Bromley-Brits et al., 2011). We found that scopolamine has impaired spatial memory as observed from the results of MWM test which was consistent with previous studies (Burešová et al., 1986, Blokland et al., 1992, Ghadami et al., 2012). Escape latencies observed on a day-to-day basis reflect long-term memory (Morris, 1981). In the present study, long-term memory impairment was observed in scopolamine-treated mice as it took too long to find the hidden platform as compare to control and drug treated mice. The oral intake of turmeric and donepezil (in Scopolamine+ Turmeric+ Donepezil group) along with scopolamine administration showed significantly shortened escape latencies during trial sessions. Whereas, turmeric and donepezil administered alone and along with scopolamine significantly improves the spatial reference memory as compare scopolamine treated group. This result shows that turmeric and donepezil synergistically suppress the effects of muscarinic antagonists. This behavior test demonstrates that test model spending more time; swim greater distances in the target quadrant during probe trials built spatial memories (Blokland et al., 2004, Kim et al., 2009). Similarly, during probe trial sessions, the number of crossing the goal area Scopolamine + Turmeric + Donepezil significantly improves swimming time within the target quadrant. As previous study suggested that donepezil has capability to attenuate scopolamine-induced cognitive impairments in healthy animals (Buccafusco et al. 2008; Cachard-Chastel et al. 2008). These results suggest that turmeric and donepezil synergistically improves the memory and decreased the latency time in Morris water maze test.

Deficits in social behavior are characteristic of various mental disorders. Along with having an important role in spatial and non-spatial memory formation, the cholinergic system also participates in social recognition and olfactory memory (Wang et al., 2002, von Linstow Roloff et al., 2007). In this study turmeric had positive effect on recognition and social interaction. The scopolamine group caused impairment in sociability and preference for social novelty due to its anti-cholinergic properties (Winslow and Camacho, 1995, Riedel et al., 2009). On the other hand turmeric showed significant potential for improving social behavior in scopolamine treated mice, exhibiting neuroprotective role. An important finding is the increase in sociability in turmeric alone group as compared to the control group, although the data was not significant. Whereas, in this study we also observed that donepezil alone and donepezil in combination with turmeric (Scop+ Turm+ Donp) significantly enhanced interaction time and novelty in social preferences session. This behavior can be due to increase in the synaptic transmission of neurons by the intake of turmeric and donepezil. This result demonstrate donepezil with turmeric synergistically increases sociability and preference for social novelty. The donepezil along with scopolamine drug significantly increases social preferences as compare to scopolamine treated group. Riedel et al. (2009) reported that donepezil aids in reversing the social memory impairment in scopolamine-induced deficits. Our result suggest that scopolamine act as muscarinic antagonist to induce behavioral disinhibition. Previous studies demonstrate that cognitive functions effected by scopolamine (Wiener and Messer, 1973), was recused by AChE-Is, like donepezil (Pepeu etal., 2009). Previous studies shows that donepezil administration improves cognition and behavior in AD patients (Rogers et al. 1998; Burns et al. 1999; Winblad et al. 2001), aged monkeys (Buccafusco and Terry 2004), and transgenic mouse models of AD (Dong et al. 2005; Van Dam et al. 2008). In healthy animal models, donepezil has also been used to reverse scopolamine-induced behavioral impairments (Buccafusco et al. 2008; Cachard-Chastel et al. 2008).

On molecular level, we studied both subtypes of cholinergic receptors that is mAChRs and nAChRs. Our current research reveals that in nAChRs turmeric treated groups showed significant up regulation of β2 receptor in contrast with scopolamine induced cholinergic depleted group. This is due to Curcumin (CU) that helps to decline the AChE expression produced by scopolamine as demonstrated by prior studies (Baum et al.,

2008). Neurotransmitters like Acetylcholine play an important role in learning and memory formation. Cessation of Ach and synaptic dysfunction of cholinergic systems has been implicated in the cognitive deficit that is associated with many neurodegenerative diseases (Terry and Buccafusco, 2003). In cholinergic dysfunction, the defects in synaptic transmission and in nAChR plays major role in the disease progression. Alteration in ACh and in cholinergic subtype receptors induce oxidative stress that causes synaptic dysfunction. This synaptic disturbances lead to degeneration in basal cholinergic neurons.(Forero et al., 2006, Reddy and Beal, 2008, Ondrejcak et al., 2010, Marcello et al., 2012).

We observed that turmeric treated mice showed significant increase in α7 receptor in contrast with scopolamine. Turmeric and its combination with scopolamine treated mice demonstrated significant increase in α-4 receptor expression. Similarly, Scopolamine+ Turmeric+ Donepezil group synergistically increases expression as compared to scopolamine treated group; whereas, the data was non-significant due to decimal difference with turmeric. This result illustrate that turmeric along with donepezil prevents neuron degeneration, and up regulates the expression of receptors, in the cholinergic depleted model. As indicated in prior studies, donepezil modulate neuroprotective effects by upregulation of nAChR (Akasofu et al., 2008), and activation of the AChR/PI3K pathway (anti-apoptotic mechanism) via secondary messenger (Takada-Takatori et al., 2009). Nicotinic acetylcholine receptor has high potential to be modulated by allosteric factors such as donepezil; that prolongs the presence of Ach before broken down in synapse. (Meunier et al., 2006b, Meunier et al., 2006a). Previous study showed that in neuronal cells culture, AChEIs (nAChRs allosteric factors) promotes BCL2 (anti-apoptotic protein) expression via activating PI3K pathway (Takada-Takatori et al., 2009). Nicotinic not only proliferates these cells but also phosphorylate apoptotic protein: the BAX (Bcl2-associated X), to inhibit its activity and decrease its movement from cytosol to mitochondria.(Xin and Deng, 2005) Martín et al., (2006) also demonstrated in *Drosophila melanogaster* neurons, that over-expressing PI3K enhances synaptic functions and synaptogenesis.(Martín-Peña et al., 2006) Therefore, it is possible that turmeric and donepezil synergistically act on nAChR and promote cells proliferation and stability.

Regarding the expression of muscarinic receptors our result suggests that turmeric augment the expression of muscarinic acetylcholine receptor like; M1, M3 and M5 in hippocampus. This lead to the fact that learning and formation of new memories are regulated by muscarinic receptors (Young et al., 1994, Gale et al., 2001). Therefore, the therapeutic potential of turmeric prove to be a valuable and novel target. The up regulation of muscarinic receptors helps in learning and building memory like cognitions, as M1 receptors have a chief role in cortico-hippocampal memory formation (Anagnostaras et al., 2003). Based on previous studies, various active components of Turmeric have shown therapeutic potential in neurodegeneration diseases that affect learning and memory (Ahmed and Gilani, 2009). However, this study for the first time reveals the effect of raw Turmeric powder and donepezil drug on cholinergic systems and cognitive behaviors. Results have shown that Turmeric significantly improves the expression of muscarinic receptors M1, M3 and M5 that play an important role in memory formation. The turmeric and donepezil along with scopolamine shows up-regulation but the data was non-significant. This proposes that turmeric and donepezil reverse the effect of scopolamine, a muscarinic antagonist. Moreover, significant improvement in hippocampus dependent cognitive functions proved that Turmeric is an inexpensive and readily available therapeutic candidate for neurological disease.

In current protein expression studies, we observe differential expression in 35KDa protein. This protein was highly expressed in scopolamine induced cholinergic depleted mice as compare to control and drug treated mice. At 55KDa and 70KDa the turmeric along with donepezil show significantly increase in protein expression as compare to scopolamine induced cholinergic hypo-function. These protein profiles are helpful in identifying and determining the function of differentially express hippocampal proteins in: memory storage (Maquet, 2001), formation and retrieval (Rutishauser et al., 2008), attention and learning processes, and higher cognitive functions. In most neurological disorders the alter protein leads to neuronal loss and cholinergic dysfunction, these changes are region specific like hippocampus and cortex in AD (Sultana et al., 2007). The significant increase in protein expression in the turmeric treated group suggest that may be turmeric has therapeutic potential to increase the protein expression in hippocampus. Although the turmeric and donepezil along with scopolamine treated group has

demonstrate the augmentation in expression. The research suggest that turmeric alone or along with donepezil has synergistic effects on protein expression, and it will aid us to elucidate the function of protein in specific region of brain and how their differential expression alters function at molecular and cellular level. Further advance techniques like two-dimensional gel electrophoresis (2DE) and Mass spectrometry would be applied for detail characterization of differentially expressed proteins; that could enable us to identify disease related biomarker in neurodegenerative disorders, and to underlying the therapeutic potentials of turmeric.

In histological studies we found that scopolamine treated group shows significant decrease in cell density in almost all cortical layers of cortex. This is a novel finding as scopolamine lowers the cell count. Hulme and Buccafusco demonstrated that scopolamine stimulates pattern of cognitive decline in AD patients by non-selective blockage of central muscarinic receptors (Hulme et al., 1978, Buccafusco, 2000). Thus blocking muscarinic receptors by scopolamine is found to impair cognition, and also inhibiting cholinergic neurotransmission that causes deficits in attention, perception, and memory (Bartus et al., 1982, Renner et al., 2005). Turmeric and donepezil in combination (Scop+ Turm+ Donp) enhances the neuronal cell count but this data is non-significant. This is due to turmeric and donepezil absorbed by nerve cells, and their synergistic effect augment their proliferation, if this treatment will prolong it will help the neurons in stability and synaptogenesis.

CONCLUSION

The results of this study show that turmeric powder with donepezil has the synergistic ability to improve cognitive functions especially attention, learning and memory. It has promising effects on learning and memory as well as the emotional behavior which are severely impaired in neurodegenerative and psychiatric disorders. It is concluded that turmeric can be an excellent therapeutic potential to increase synaptic transmission and neurogenesis.

Limitations & future directions

A major consideration in turmeric's applicability in treatment of neurodegeneration is its bioavailability since the compound, when taken orally, is subject to low absorption and limited tissue distribution because of its relatively poor solubility in water. Though we investigated this concern to some level but it does not determines all details of turmeric effectiveness. So, in future studies, some of these challenges may be overcome by co-administration of compounds such as piperine, nanoparticle encapsulation and bioconjugates which can improve not only the absorption of curcumin, but may also increasing its bioavailability and improving brain penetration.

This present research proposed that turmeric alone or/with donepezil has synergistic effects on protein expression, and it will aid us to elucidate the function of proteins in specific region of brain and how their differential expression alters function at molecular and cellular level. Further advance techniques like two-dimensional gel electrophoresis (2DE) and Mass spectrometry needed to be carried out for detail characterization of differentially expressed proteins; that could enable us to identify disease related biomarker in neurodegenerative disorders, and to underlying the therapeutic potentials of turmeric.

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