Elucidating the Effect of Turmeric in Scopolamine-Induced Cognitive Impairment in Mouse

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2015

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A thesis submitted in partial fulfillment of the requirement for the
degree of Masters of Science
in
Healthcare Biotechnology

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2015

بِسْمِ اللّٰهِ الرَّحْمٰنِ الرَّحِيْمِ

Dedication

I express heartfelt dedication to my sweet and loving parents
"Musarat Masood" and "Dr. M Masood"
Whose affection, engorgement and prayers of day and night made me able to get such success and honor. Without them, I have no existence. I could never have done this without your faith, support, and constant engorgement. Thank you for teaching me to believe in ALLAH, myself and my dream

Acknowledgements

In the name of Allah, the Most Gracious and the Most Merciful

First and foremost, I thank Allah (subhana wa taala) All praises is to Allah and to Him alone. He endowing me with health, patience, strength, opportunity and knowledge to complete this dissertation finally, after all the challenges and difficulties. Words are insufficient to describe my gratefulness and appreciation to Him in the whole process of the preparation, compiling and writing of this thesis. In moments of distress, He guided me, showed me what to do, removed all obstacles from and lighted my path, inspired me, eased the tedious task of writing, and gave me surplus energy so that I may stay up night after night, putting down words on paper. Without Him I would not have been able to undertake this daunting task. He is my world, always there in every moment! What matters to me is that He accepts this contribution of mine that He inspired me to write; assisting me by sending me various messengers at every crucial turn. Thank You Allah!

I would like to express my greatest gratitude to my supervisor Dr. Touqeer Ahmed, Assistant Professor of Neurobiology, for his constant encouragement and support throughout the dissertation process, who make capable me to complete my study. Who have guide and helped me a lot. By the help of his inputs, attention, patience and great knowledge inspiration for research in the field of Neuroscience.

This appreciation is also dedicated to my co-supervisor Dr. Saadia Zahid, Assistant Professor of Neurobiology, I am deeply indebted and grateful. I also thank to my committee members Dr. Ria Khalid, for his constructive guidance, valuable advice and cooperation in all phases of this research.

I acknowledge, with deep gratitude and appreciation to Dr. Peter John, Assistant Professor, Dean and Principal, Atta-ur-Rahman School of Applied Biosciences (ASAB), for his determination to keep the environment cordial for research and study. I would like to special thanks to Engr. Muhammad Asghar, Rector National University of Science and Technology (NUST), for endorsing his vision of science and technology by creating research opportunities in NUST.

I acknowledge to my teachers Dr. Attiya Bhatti, Dr Shazina, Dr Amjad, Dr Saadia Andleeb, for their support and valuable suggestions. I would like to thank all staff members of ASAB for supporting me in any manner which eventually impinged upon the completion of this project.

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible and because of whom my graduate experience has been one that I will cherish forever. I am also thankful to Dr. Arshad Mumtaz and Dr. Husnain, NIH Islamabad for the assistance in the histological studies at NIH.

My sincere regards to my friends, Ufaq Tasneem, Sara Ahmed, Saleha Resham, Misbah Nazir, Haleema Tariq Bhatti, Syeda Qudsia, Nayab Nawaz, Syeda Ayesha Ali, Ataf Zahoor, Tahira Riasat, Maria Raheel for their support, care, and prayers and for being there for me in all difficult time. Special thanks to my seniors, all my class mates, and hostel mates for their support and care that helped me overwhelmed setbacks. Thanks for the sweet memories and the valuable friendship.

Finally the most important, none of this would have been possible without the love and patience of my family. My parents and my siblings Haleema, Amna, Danish, Anum Fatima, Hamid and Ayesha for their constant source of love, concern, moral support and strength all these years. I would like to express my heart-felt gratitude to my family.

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LIST OF ACRONYMS

CR	Conditioning Response
CS	Conditioned Stimulus
D	Donepezil
HPLC	High Pressure Liquid Chromatography
IRB	Internal Review Board
NaCl	Sodium Chloride
NIH	National Institute of Health
NOR	Novel Object Recognition
NS	Normal Saline
RT	Reverse Transcriptase
SC	Sub-cutaeous
Scop	Scopolamine
Tur	Turmeric
US	Unconditioned Stimulus

PAL	Paired Association Learning
mAChRs	Muscarinic Receptors
nAChRs	Nicotinic Receptor

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ABSTRACT

The cholinergic theory claims that the decline in cognitive functions in dementia is predominantly related to a decrease in cholinergic neurotransmission. This hypothesis has led to great interest in the putative involvement of the cholinergic neurotransmission in learning and memory processes. Turmeric, a potent anti-inflammatory compound, like donepezil has shown to have sound effect on cholinergic system improving memory and learning. BALB/c mice were administrated Scopolamine (1mg/Kg/day) through subcutaneous injections for a period of 26 days. On 11 day, Donepezil (4mg/Kg/day) and Turmeric (20mg/Kg/day) was given mixed in feed. Novel recognition test, fear contextual and fear conditioning test a memory tests were performed to evaluate memory consolidation and acquisition. RT-PCR was used to measure the mRNA expression of M1, M3, and M5 receptors in the cortex. Turmeric treatment significantly improved the expression of cholinergic muscarinic receptors M1 and M5 in the cortex as compared to Scopolamine-induced amnesia group. Moreover, turmeric significantly enhanced the expression of α7 in control and treated groups, the expression of α4 is reduced in turmeric treated and control group as compared to scopolamine-induced amnesia group and there is no-significant change in expression in β2 gene in groups. There were four proteins differentially expressed in turmeric treated group. The impairment in cortex dependent learning and memory was inverted in Scopolamine-induced amnesia group as evident from improvement in fear conditioning ($p<0.001$), contextual ($p< 0.001$), and fear extinction ($p<0.001$) in turmeric treated group as compared to Scopolamine-induced amnesia group. Recognition memory was also improved ($p<0.001$) following turmeric administration as compared to scopolamine-induced amnesia, suggesting the positive effect of turmeric. Furthermore, turmeric show significant improvement in low scoring PAL and RT test in human aged subject as compared to placebo group of human subject. Therefore in the light of these findings turmeric

may serve as a potential candidate in improving cognitive functions. It can also act as a therapeutic option for neurodegenerative disorders.

INTRODUCTION

Cholinergic system involved in cognitive pathway, is an excitatory system. Cholinergic system plays a primary role in learning and memory. In cholinergic system, there are two types of receptors, muscarinic and nicotinic acetylcholine receptors, receptors are involved in cognition. Alzheimer's, a neurodegenerative disease, cholinergic system involved in cognitive pathway, is an excitatory system. Cholinergic system plays a primary role in learning and memory. In cholinergic system, there are two types of receptors, muscarinic and nicotinic acetylcholine receptors, receptors are involved in cognition. Alzheimer's, a neurodegenerative disease. Different types of nicotinic receptors subtype are involved in diverse type of cognition for example alpha 4 receptor containing receptor seems decreased in Alzheimer's (Martin-Ruiz et al., 1999), while alpha 4beta2 and alpha7 receptors both seem to be decreased in schizophrenia (Papke et al., 2000, Furey et al., 2000).

Amnesia is a condition in which memory is loss that would be by a trauma, physical damage to brain or due to a disease like Alzheimer's. Amnesia is divided into two major classes: Retrograde and Anterograde amnesia. Another category involved, intentionally loss of memory by injecting an amnesia drug is known to be drug induced amnesia. Scopolamine, is an anticholinergic drug cause amnesia and it leads to severe cognition impairment with aging. It has been reported that high doses of scopolamine block nicotinic receptors (Schmeller et al., 1995). Studies on many components of turmeric, such as curcumin, demethoxycurcumin and bisdemethoxycurcumin have provided insights that support turmeric's medicinal use in AD. These three compounds are found in turmeric in a specific ratio (3–5% bisdemethoxycurcumin, 15–20% demethoxycurcumin, and

75–80% curcumin), and this combination is called curcuminoids or the curcuminoid mixture (Ahmed and Gilani, 2009).

Many herbal plants have been used as medicines and these are observed as natural and safe therapies as compared to those synthetic drugs. Several herbal products have been used traditionally for memory enhancers and provide auspicious effects to improve quality of life in terms of improving cognitive function associated with senescence. Turmeric is one of the herbal plant used since many centuries for many therapeutic treatments such as Turmeric has an abundant medicinal significance throughout the world. It is scientifically known as curcuma longa (Goel *et al*., 2008, Ahmed and Gilani, 2009). Curcumin has confirmed effectiveness in numerous models of learning and memory impairment, as well as defending effects against scopolamine-induced amnesia (Ali and Arafa, 2011) and it converses impaired cognition. Moreover, to the neuroprotective effects of curcumin on cognition, as early data have shown that consistent curcumin intake may be associated to improved cognitive function in the elderly (Ng *et al*., 2006). Because of turmeric have neuroprotective capacity, it can play in the therapy pf several central nervous system diseases (Chainani-Wu, 2003, Ghadami *et al*., 2012). Neuronal nicotinic acetylcholine receptors constitute a variety of receptor subtypes, which are important for a variety of neurobehavioral functions including cognitive function (Changeux and Edelstein, 1998). Nicotinic α4β2 receptors have been shown to be critically involved in cognitive function. Agonists of α4β2 nicotinic receptors produce significant long-lasting improvement in memory function (Levin and Christopher, 2002, Lippiello *et al*., 1996, Papke *et al*., 2000) and attentional performance in rats (Grottick et al., 2000). Both selective α7 and α4β2 nicotinic receptor agonists have been shown to play a role in attention, learning, and working memory (Buccafusco et al., 2009, Cincotta and Ziemann, 2008). Turmeric is highly safe even when used in large amounts,

according to the studies conducted in animal (Qureshi et al., 1992, Shankar *et al*., 1980) and human subjects (Lao *et al*., 2006) Cheng *et al*., in 2001 used turmeric in his study, with dose 500mg/day orally for 3 months for studying inflammation. In his study that oral curcumin is not toxic for humans even at very high dose of 8000mg/day (Cheng *et al*., 2001).

AD study purpose is comprising of cholinergic treatments is to inhibit further impairment moreover, regain the decline in memory and cognitive function that follows over the progression of the disorder. There are several drugs have been used to treat the cholinergic deficit in AD have the drugs those have efficacy of cholinergic inhibitors drugs such as Donepezil, Galantamine, Rivastigmine and Huperzine A these are derivative of tarcrine, typical cholinesterase inhibitor. The anticholinesterase drugs decrease circulating Aβ deposition in several dementia types, including AD (Ballard *et al*., 2007). Donepezil is an effective and drug of choice acetylcholinesterase (AChE) inhibitor (Ogura *et al.*, 2000a). Donepezil upregulates brain ACh contents (Kosasa *et al*., 1999) and it improves the cognitive function (Ogura *et al*., 2000b) Based on existing literature, cholinergic hypothesis of cognition and pharmacological properties of AZD3480 and donepezil, it was hypothesized that this drug will reverse the attentional impairment induced by Donepezil (0.01–1.0 mg/kg) also significantly ($p<0.005$) decreased the dizocilpine-induced attentional impairment.

1.1. Research objectives:

The objectives of the study

- To study pharmacological effect of turmeric on learning, memory and cognitive functions in scopolamine-induced mice by **behavior tests**.
- To determine the effect of turmeric and donepezil on cholinergic hypo function via expression of muscarinic (M1, M3, M5) and nicotinic receptor (α-7, α4, β2) genes in cortex.
- To check the effect of turmeric in Scopolamine-induced amnesia mouse on cellular density and expression of protein.
- To observe pharmacological effect of turmeric on learning, memory and cognitive functions in old aged individuals by **Memory tests (PAL and RT)**.

LITERATURE REVIEW

Amnesia is come from a Greek word "ἀμνησία" with ἀ denoting "without" and μνήμη denoting memory. Hence amnesia is well-defined as a insufficiency in memory initiated by a disease, brain damage, or psychological shock (Senior *et al*., 2006). It is lasting or temporary and can also be due to use of hypnotic drugs and various sedatives. Basically, a loss of memory is amnesia. Amnesia is broadly classified into two types: anterograde amnesia and retrograde amnesia. The incapability to memorize new knowledge and form long-term memory from the short-term memory is known as anterograde amnesia. In this type of amnesia, people lose the ability to remember things for long periods of time. The inability to retrieve memories that were formed before a particular time is known as retrograde amnesia (Winocur and Moscovitch, 1999). Depending on the extent of damage, the loss of memory may extend back to decades or a few months. However, the two types of amnesia are not mutually exclusive. They may occur simultaneously or separately.

2.1.1 Discovery of amnesia

A French psychologist Theodule-Armand Ribot was one of the first scientists who studied amnesia. He proposed a law known as "Ribot's Law" according to which "there is a time gradient in retrograde amnesia". Ribot's Law follows development of memory deficit caused by a disorder. The progression starts with losing most recent memories, then next in hierarchy comes subjective memories and ultimately the patient loses intellectual memories. Professor Ribot implied that the most recent memories were the first ones that were lost(Ribot, 1882). **Important case studies of amnesia**

Case studies have played a crucial role in the discovery of amnesia and areas of the brain that are affected in the disease. The studies provided important information into the physiology and pathophysiology of the disease. The studies provided scientists with a platform to gain insights about the disease and develop a cure or preventive measure. Three of the widely studied case reports were of patient Henry Molaison, G.D and R.B.

2.1.3. Henry Molaison

Henry Molaison suffered from severe epilepsy and dramatically reformed the people's perspective about memory. Unable to control his seizures with prescribed medicine, physicians performed brain surgery as a new approach. During the surgical procedure, his medial temporal lobe was removed bilaterally. As anticipated, his epileptic seizures did improve but as a consequence of the surgery, the patient lost his ability to form new long-term memories. Molaison suffered from anterograde amnesia however his ability to form short-term memory was not affected. When a list of words was given to him, he would forget the list of words in less than a minute. He would even forget that he was even given a list of words (Milner *et al*., 1968). This gave scientists insight that short-term and long-term memories are formed by two different procedures (Draaisma, 2013). Although apparently he was unable to form new memories, Molaison would learn things through implicit memory. The researchers would ask the patient to look at a piece of paper using a mirror and draw a figure on it. Afterwards, he would forget about the task but when asked to draw again, an improvement in the drawing was evident. This evidence showed that learning and remembering also occurs unconsciously (Rosenbaum *et al.*, 2012). 14 years after initial diagnosis, scientists conducted a follow up study on Molaison. To gain useful insight about his amnesia, researchers studied him for two weeks. Molaison was still unable to form new memories or recall information that happened since his surgery but he could still remember information prior to his surgery. It was

also seen that that the patient could answer about international or national events but his own personal memories were lost (Milner *et al*., 1968). Molaison donated his brain after death to science, and scientists were able to discover lesions in the areas of the brain which caused amnesia (Draaisma, 2013). Molaison's case gave useful insights into how anterograde amnesia works and areas of the brain involved.

2.1.4. Patient R.B. and role of Hippocampus in anterograde amnesia

Patient R.B. was presented with angina and underwent surgery twice for heart problems. He suffered from ischemic stroke (reduction of blood to the brain) during a bypass surgery of the heart. As a consequence of the stroke, patients suffered from anterograde amnesia but his retrograde memory was almost intact with a loss of few years of memory before the stroke. There was no sign of loss of any cognitive functions. After his death, researchers examined his brain and discovered that lesions were restricted to the CA1 region of the hippocampus. This study provided evidence about the role of hippocampus in formation of memory (Rempel-Clower *et al*., 1996).

2.1.5. Causes of amnesia

Amnesia can be caused by three main reasons; trauma (example: head injuries during a fall or an accident), psychological trauma (example: an event that is emotionally devastating), or physical deficiencies (example: atrophy of a part of the brain specially hippocampus). The majority of issues related to memory and amnesia result from the first two categories (Gazzaniga, 2009).

Trauma to the head is a broad category and includes any active action or injury that might cause loss of memory. Both kinds of amnesia may occur from a head trauma. Electroshock therapy is a form of bead trauma in which the patient loses both forms of memory (long term and short term) after receiving the shock. Traumatic events are more subjective. What is traumatic is dependent on what the person finds to be traumatic.

When a psychologically traumatic event; death of a close family member, war or accident; occurs in a person's life, the brain instead of dealing with the stress, selects to forget about it causing dissociative amnesia (Benbow, 2004).

2.1.6. Cholinergic System

It is demonstrated that both pre and postsynaptic neurochemical changes or modifications are existing in the brains of individuals suffering from range of neurodegenerative diseases (Enna et al., 1976b), (Enna *et al*., 1976a, Reisine *et al*., 1977, Reisine *et al*., 1978). Among the neurotransmitter structures influenced, the cholinergic system appears to be predominantly susceptible, with changes taking place in particular brain region in Alzheimer's, Huntington's and Parkinson's disorders. These diseases arise frequently in mid- to late life and are described by both mental and motor dysfunctions. Since comparable, however more unobtrusive, motor also, mental anomalies progress through aging, it appears probable that changes in the brain cholinergic system might additionally be a piece of the aging process. In fact, it has lately been proposed that age-linked anomalies in main cholinergic mechanisms play a principally vital part in the cognitive impairment that occur naturally with old age (Drachman and Leavitt, 1974, Bartus, 1978, Bartus and Uehara, 1979).

The concept that cognitive roles are vastly dependent on fundamental cholinergic neurotransmission (Bartus *et al*., 1985, Holttum and Gershon, 1992, Sahakian, 1988). Though other neurotransmitters were known to be convoluted in learning and memory performance, the functions of the cholinergic system in learning and memory were of major interest to learning and memory (Hagan and Morris, 1988). Aging and dementia research is possibly the keystone of the cholinergic assumption of learning and memory. Moreover, the importance of the finding that the

cognitive decline in aging and dementia is related to a decrease in cholinergic function can be assumed from the fact that the effects of cholinergic antagonists and lesions of cholinergic nuclei are often related to cognitive deficits similar to those observed in aging and dementia (Dawson *et al*., 1992, Drachman and Leavitt, 1974, Kopelman, 1986, McEntee and Crook, 1992).

Acetylcholine (ACh) is a chief neurotransmitter not merely within the somatic and autonomic motor neurons but also in the central nervous system (CNS). A population of local cholinergic interneurons can be found in the brainstem and forebrain, these projections are supposed to regulate cognitive functions such as attention, consciousness, learning, and memory (Baxter and Chiba, 1999); (Hasselmo, 1999); (Nobili and Sannita, 1997); (Perry *et al.*, 1999); (Sarter, 2000)). The cholinergic neurons in the basal forebrain region are distributed in the medial septum (Ch1 group), vertical and horizontal limb of the diagonal band (Ch2 and Ch3 groups) and the nucleus basalis of Mynert (Ch4 group), topographically innervate the entire cerebral cortex, including the hippocampus and amygdala. Acetylcholine (ACh) is consist of a fragment of choline and an acetyl functional group attached by an ester linkage. The synthesizing enzyme, choline acetyltransferase (ChAT), catalyzes the biosynthesis of ACh from acetyl coenzyme A and choline. The debasing enzyme, acetylcholinesterase (AChE), hydrolyzes ACh to acetate and choline. ACh does not freely cross lipid membranes because of its highly polar, positively charged ammonium group. Hence, ACh initiate its biologic effects within cells by triggering two different classes of cholinergic cell surface receptors, the nicotinic acetylcholine receptors (nAChRs) and the muscarinic acetylcholine receptors (mAChRs). The nAChRs are ligand-gated ion channel proteins which are inotropic receptors belongs to a large superfamily which formed by various combinations of transmembrane alpha, beta, gamma and E glycoprotein subunits (Galzi *et al*., 1991). In comparison, the mAChRs are single subunit transmembrane glycoproteins coupled to G-

proteins. There are several molecular subtypes of these "metabotropic" receptors, named from m1 to m5 mAChRs (Hulme *et al*., 1990).

The cholinergic neurotransmitter system cells organize and initiate predominantly in the basal forebrain. These cells widely projected all over the cortex and, therefore, by project have extensive effect on information processing. Cholinergic neuromodulation is well known to effects several cognitive process, comprising memory and attention (Klinkenberg *et al*., 2011, Graef *et al*., 2011). Blockade of the cholinergic muscarinic and nicotinic receptors by the antagonist scopolamine results in impairment in learning and memory performances. Blockade of the cholinergic receptors by the antagonist scopolamine results in impaired performance on learning and memory tasks, (Sitaram *et al*., 1978), (Rusted and Warburton, 1988), (Barak and Weiner, 2010), (Blake *et al*., 2011), (Klinkenberg *et al*., 2011) and the performance of attention, (Klinkenberg *et al.*, 2010), (Wesnes and Warburton, 1984), although improving cholinergic function recovers memory and attention (Terry *et al*., 2011). Drugs that enhance cholinergic neuromodulation improve performance on short-term memory tasks both in animals and in humans (Furey *et al*., 2008), (Bartus *et al*., 1981),(Furey *et al*., 2000) and can reverse the memory deficits created by nBM lesions (Murray and Fibiger, 1985). Historically, the literature has identified acetylcholine as related to cognition and cognitive processing. A closer look at the literature, however, argues that cholinergic effects on cognitive functions, such as working memory and attention, occur specifically as a result of direct effects on stimulus processing mechanisms.

Cholinergic neurotransmission is determined by two sets of receptors, the G-protein coupled muscarinic family and the ligand-gated ion channel nicotinic family.

2.1.7. Nicotinic Receptor (nAChRs)

Nicotinic acetylcholine receptors (nAChRs) participate to sensory cognitive functions, as shown in several behavioral studies (Warburton 1992 ; Turchi *et al*. 1995; Rezvani *et al*. 2002). The involvement probably contributes nAChRs placed within sensory systems, containing sensory cortex, that assist the cholinergic role in some cognitive functions and in attention (Sarter *et al*. 2001). significant evidence exists about the molecular biology, cellular physiology, and general scattering in the brain of nAChR subtypes (Jones *et al*. 1999; Dani 2001; Leonard and Bertrand 2001), subsequent information on how nAChRs function within specific cortical circuits is needed to understand nicotinic functions in sensory cortex or, for that matter, anywhere in the CNS (Alkondon and Albuquerque, 2003; Clarke, 2003; Metherate and Hsieh, 2003). Numerous studies have observed nicotinic actions in prefrontal cortex, in part because of the known role of nAChRs in cognitive or attentional processes facilitated by prefrontal cortex (Granon *et al*., 1995).

The nAChR in neurons are a pentamer that can comprise a grouping of α and β subunits as a heteromeric receptor, or five α subunits as a homomeric receptor (Dani, 2001; Leonard and Bertrand, 2001). Though there are genes for a great number of subunits (α2–10, β2–4), utmost nAChRs in cortex are thought to exist as heteromers with α4 and β2 subunits or as α7 homomers. According to the functional point of view, meaningful the receptor configuration is significant because subunits pay different functional characteristics, as presented by studying different subunit combinations in expression systems (Role and Berg, 1996; Fenster *et al*., 1997).

In the studies of moving bars; Parkinson *et al*., 1988 and Liang and Metherate 2003 associate cortical nAChRs in the regulation of sensory responses. Significantly, because decline of induced responses occurred during transfer of antagonist alone (i.e., in the absence of exogenous

nicotine), the conclusions also suggest that stimulant release of endogenous ACh acts at nAChRs to regulate sensory responses. α4β2.

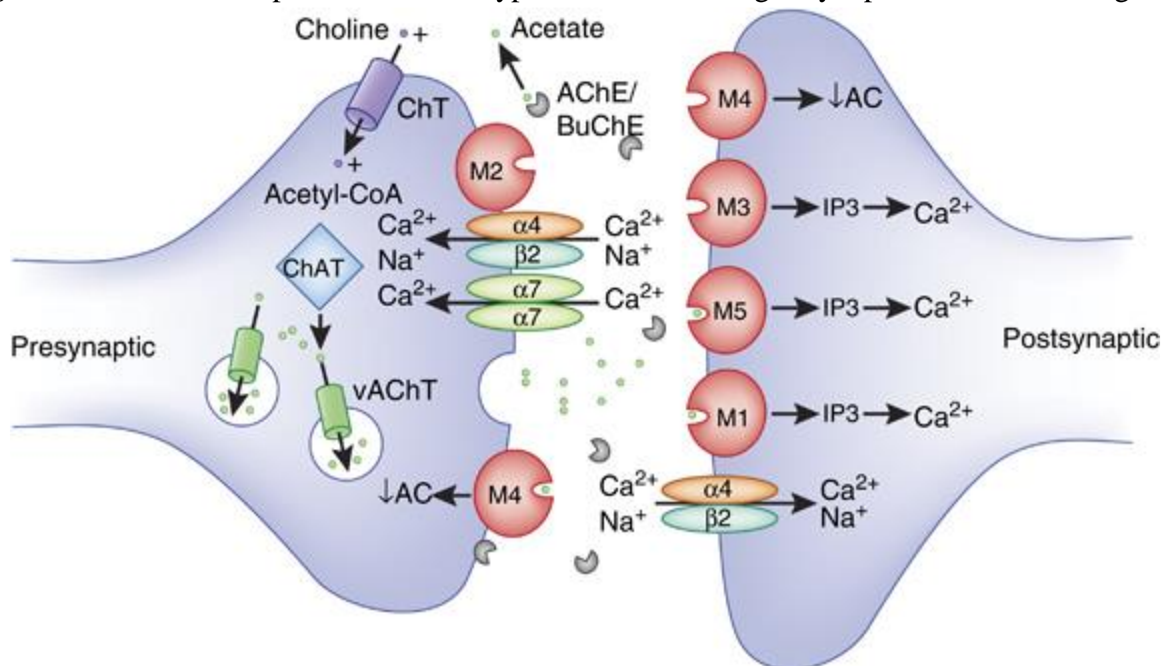
Notably, the author's annotation that subsequent to the earlier studies, it had been validated that high-affinity nicotine-binding sites reflect nAChRs containing α4 and β2 subunits. Thus, a contribution to the nicotine-induced increase in sensory cortex responsiveness may depend on presynaptic, α4 β2 containing nAChRs that regulate thalamocortical transmission in prefrontal cortex, as in mature sensory cortex.

2.1.8. Muscarinic Receptors (mAChRs)

Many studies have emphasize on mAChR subtypes since this family has more well-known roles in central cholinergic transmission and functions such as learning and memory (Coyle, 1983; Bartus, 1982). Numerous studies give proof that associate impaired cholinergic neurotransmission at mAChR as paying role in the dementia signs in AD, including: (a) constant reduction of choline acetyltransferase in neocortex and hippocampus in patients (Coyle, 1983; Perry, 1978), comprising equally initial and late onset types of AD (Etienne, 1986); (b) basal forebrain neurons, which present the majority of cholinergic innervation of neocortex and hippocampus, are reduced in number in AD (Whitehouse, 1981; Arendt, 1983); (c) association of choline acetyltransferase levels (Coyle, 1983 ; Perry, 1978) and numbers of basal forebrain neurons (Doucette, 1986, Lehericy, 1993) with the severity of dementia; and (d) lesions of basal forebrain neurons and pharmacological blockade of mAChR impair cognition in animals (Dunnett, 1985; Nilsson, 1992) . That ACh plays a necessary role in learning and memory and that it is sufficient to restore these functions in lesioned animals has been recently demonstrated using cholinergic specific lesioning methods and genetically modified grafts (Winkler, 1995).

There are five subtypes of mAChRs such as M1-M5 and these receptors are dispersed all over the brain (Levey *et al.,* 1991 b, Volpicelli and Levey, 2004). Subtypes individually play various roles in cognitive functions and their roles have been already well-known (Jafari-Sabet, 2011, Bubser *et al.,* 2012). M1-receptor is contributed in development of cognition (Fisher *et al.,* 2000, Anagnostaras *et al.,* 2003, Fisher, 2012) whereas acetylcholine efflux and cognitive processes are maintained through M2 and M4 receptors in the hippocampus (Tzavara *et al.,* 2003). M3 receptors are contributed in controlling motor functions, behavior, learning and memory (Levey *et al.,* 1994, Poulin *et al.,* 2010). M5 plays an important role in cerebrovascular function mediated by acetylcholine (Araya *et al.,* 2006). The distribution of M1 receptors is throughout the brain, with the maximum concentrations in cortical regions, including the neocortex, hippocampus, striatum, thalamus and amygdala (Levey *et al.,* 1991b).

Figure 1: Schematic representation of hypothetical cholinergic synapse demonstration general



localization of cholinergic receptors (mAChRs and nAChRs).

2.1.9. Cholinergic Antagonist

The effect of cholinergic antagonist and cholinomimetic on learning and memory. There are many examples as cause the loss of cognitive abilities are benzodiazepines like Midazolam or Flunitrazepam, Lorazepam (Page *et al*., 2002; Riss *et al*., 2008)and other robust antagonist are

Atropine Propofol and Scopolamine (Thiel *et al*., 2002, Miner and Burton, 2007). Scopolamine induced amnesia results in loss of cognitive abilities appeared to be comparable to that observed in old untreated subjects.

2.2. Donepezil

The most consist alteration in Alzheimer's disease (AD) is a deficit in central cholinergic neurotransmission. Donepezil hydrochloride has been demonstrated to be well novel group of cholinesterase (ChE) inhibitor, that it prevent degradation of acetylcholine (ACh) and stimulates central cholinergic system (Ogura *et al*., 2000a). Moreover it improves cognition and cholinergic function in AD patients (Burns, 1999).

Donecept (Donepezil hydrochloride) is known as chemically E2020; (±) -2,3-dihydro-5,6-dimethoxy-2-[[1-(phenylmethyl)-4- piperidinyl]methyl]-1H-inden-1-one hydrochloride (Aricept) is a piperidine-based agent that is chemically distinctive from other cholinesterase inhibitors (Cardozo (Cardozo *et al*., 1992), Sugimoto *et al*., 1990, Sugimito *et al*., 1992).

Donepezil has been presented potential to enhance performance on memory and learning tasks in preclinical studies it show that donepezil has better specificity for brain tissue and is more selective for AChE than either physostigmine or tacrine hydrochloride. Furthermore, donepezil also has a lengthier duration of inhibitory action than either of these agents (Roger *et al*., 1991).

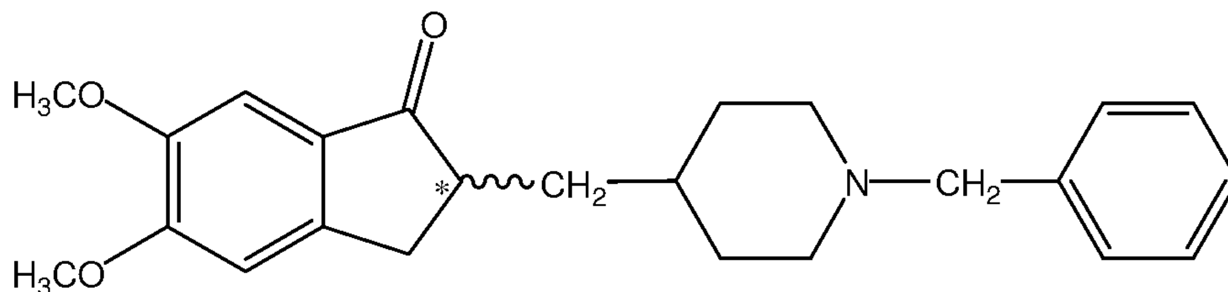


Figure 2: Illustration of the chemical structure of Donepezil Hydrochloride.

2.2.1. Turmeric

Turmeric is belong to family of Zingiberaceae known as curcuma longa and recognized culinary plant specifically in South Asia, well-known as fundamental element in the cooking items also used in coloring and food items (Ammon and Wahl, 1991; Govindarajan, 1980). Turmeric has a great medicinal importance in the conventional medicine technique of South Asia, which comprises helping in wound healing, inflammatory disorders, and purification of blood (Kapoor,

1990; Krishnaswamy, 2008; Nadkarni, 1976). Turmeric is a well-reported herb, and several studies have proved its number of medicinal benefits, which include presence of anti-inflammatory property (Ammon and Wahl, 1991; Brouet and Ohshima, 1995).

Moreover, most captivating property of turmeric is that it is very safe even while used in large quantity, conferring to the studies directed in animal (Qureshi *et al*., 1992; Shankar *et al*., 1980) and human subjects (Lao *et al*., 2006). It was studied that old individuals regularly eat turmeric have improved cognitive performance, but more studies are needed to support the scientific proof for this invention (Ng *et al*., 2006). Turmeric and curcuminoids are comparatively safe in human beings, even when administered in high doses (Lao *et al*., 2006).

There are many compounds that have been listed and isolated from turmeric in several studies. These contain curcumin, bisdemethoxycurcumin, demethoxycurcumin, eugenol, dihydrocurcumin, azulene, borneol, D-camphene, caprylic acid, cineol, turmerone, and zingiberine (Duke, 1992). Many studies have been in several components of turmeric, likewise curcumin, bisdemethoxycurcumin, and demethoxycurcumin have presented abilities that sustain turmeric's medicinal use in treatment of AD. These three components are present in turmeric in a particular proportion (3–5% bisdemethoxycurcumin, 15–20% demethoxycurcumin, and 75–80% curcumin), and this mixture is termed as curcuminoids or the curcuminoid combination (Ahmed and Gilani, 2009).

Numerous in vivo studies approved associating indication and stressed therapeutic potential of curcumin to an AD mouse model caused in a reduced serum Aβ level also decreased Aβ load in the brain, and this effect was significantly presented in the neocortex and hippocampus of the AD mouse model (Wang *et a*l., 2009). Curcumin have ability to cross the blood–brain barrier and inhibits Aβ fibril formation and is proficient of prompting disaggregation of preformed Aβ

fibrils and their extension (Garcia-Alloza *et al*., 2007; Ono *et al*., 2004; Yang *et al*., 2005). Curcumin repressed Aβ fibril development more proficiently than naproxen or ibuprofen (Yang *et al*., 2005). Curcumin therapy rescued the distorted neuritic morphology present near plaques, thereby providing a useful therapeutic effect in AD (Garcia-Alloza *et al*., 2007).

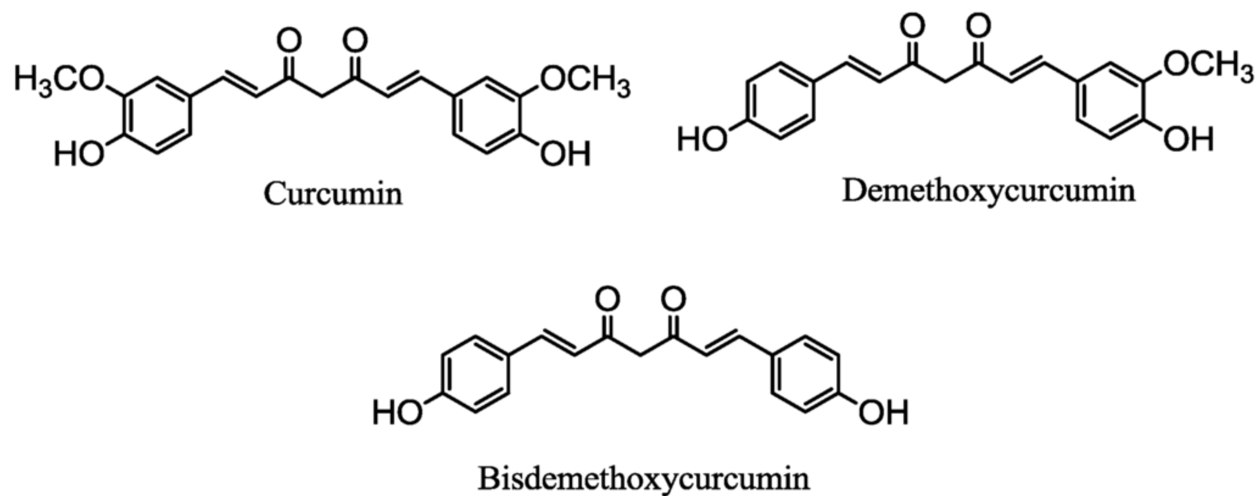


Figure 3: Illustration of the chemical structure of Curcumin, Demethoxycurcumin and Bisdemethoxycurcumin.

MATERIAL AND METHODS

3.1. Ethical statement

All experiments performed were in accordance with rules and regulations of 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: Eighth Edition, 2011). All protocols were obtained after taking a consent from the Internal Review board (IRB), Atta-Ur- Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST).

3.2. Drugs and chemicals

Scopolamine hydrobromide trihydrate (product code no. S-1101) was purchased from Sigma Aldrich USA. 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. Rhizomes of turmeric were ground into a fine powder and used in the experiments. All chemicals used for electrophoresis experiments were procured from Sigma. Distilled water was used throughout the study to evade any contamination and for the preparation of reagents and buffers used for SDS-PAGE. All the solutions were prepared fresh on the day of experiment

3.3. Animal Subject

BALB/c male mice were used in the study. The age of all animals was within of 3-6 months with average weight of 30-45g. The mice were kept under controlled conditions, room temperature of 22 ± 2°C under a natural 14 hour light and 10 hour dark cycle. Approximately 3-4 mice were kept in a single cage of 40cm x 25cm x 15cm dimensions with wood shavings as bedding. Animals were fed with food and water *ad libitum* consisting of crude protein (30%), crude

fiber (4%), crude fat (9%) and moisture (10%). 56 mice were used in the study which were divide in seven groups randomly, were treated for a period of 26 days. A total of 12 mice were used in each group and 2 mice were kept in one cage (40cm x 20.5cm x 2.5cm). Each cage and animal was given a different labelling and tail marks were given for differentiation. One week after acclimatization into the new environment, the experiments were commenced. 84 mice were divided into seven groups.

3.4. Study design

A study of 26 days was designed to determine the pharmacological effect of Turmeric on cholinergic systems in Scopolamine-induced amnesia. On 22nd day of the treatment and onwards, behavior tests were performed followed by sacrifice of micron 26th day. RNA and Protein was extracted from cortex and expression studies were performed. Mice were divided into seven groups and given treatments according to group.

3.4.1. Group I: Control group

Inject normal saline subcutaneously, Fed normal feed and distilled water for 26 days.

3.4.2. Group II: Scopolamine group

Inject scopolamine 1mg/Kg subcutaneously, Fed normal feed and distilled water for 26 days.

3.4.3. Group III: Scopolamine + Turmeric

Inject scopolamine 1mg/Kg subcutaneously for 26 days, on day 11 Fed turmeric 20mg/Kg in feed.

3.4.4. Group IV: Scopolamine + Donepezil

Inject scopolamine 1mg/Kg subcutaneously for 26 days, on day 11 Fed normal feed and 4mg/Kg Donepezil HCL in drinking water.

3.4.5. Group V: Scopolamine + Turmeric + Donepezil

Inject scopolamine 1mg/Kg subcutaneously for 26 days, on day 11 Fed turmeric 20mg/Kg in feed and 4mg/Kg Donepezil HCL in drinking water.

3.4.6. Group VI: Turmeric

Fed turmeric 20mg/Kg in feed and distilled water for 16 days.

3.4.7. Group VII: Donepezil

Fed normal feed and 4mg/ Kg Donepezil in drinking water for 16 days.

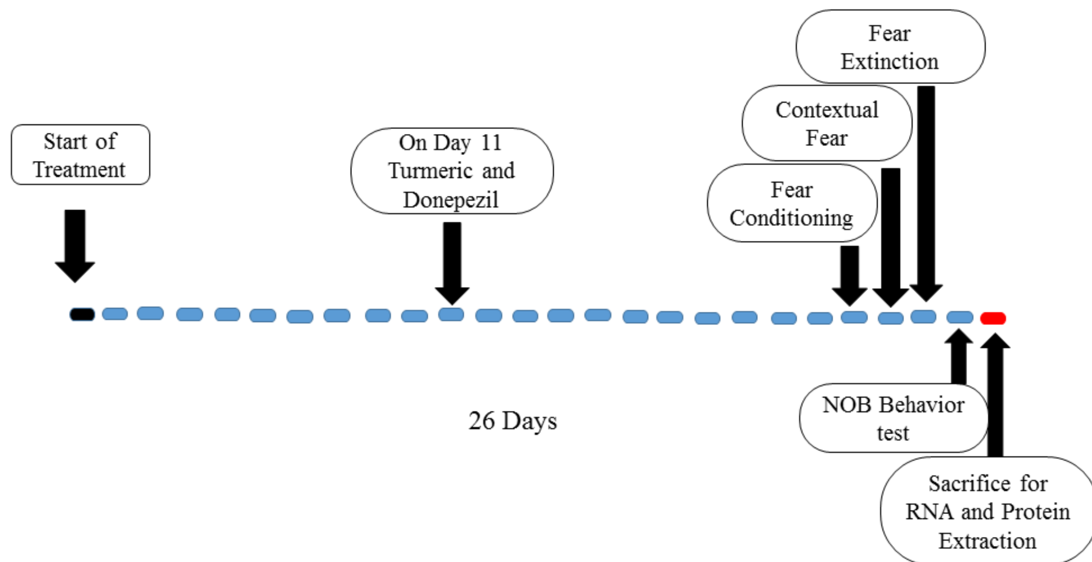


Figure 4. Study design to evaluate the pharmacological effect of Turmeric on cholinergic systems in Scopolamine-induced amnesia. On 22nd day fear conditioning was performed, followed by contextual fear after 24 hours and fear extinction 24 hours after contextual fear. Novel object recognition (NOB) test were performed on 25th day and mice were sacrificed on 26th day of the treatment.

3.4.9 Behavioral tests

Behavior tests were performed during the light cycle of the day. In order to allow the subject animal to habituate to the testing room conditions, the animal was placed in the behavior testing room 30 minutes prior to the beginning of the experiment. The temperature and light conditions of the behavior room were kept in consistence with the conditions of the mice facility room (25$_o$C±2 temperature). All the behaviors were recorded with the aid of a video camera and it was ensured that no excessive disturbance or human presence was in the room during testing procedures.

3.5. Fear conditioning

Fear conditioning is a form of associative learning in which an organism learns to predict aversive events. It is a long lasting form of learning and is acquired in a short period of time. It is considered a standard model of cognition and memory (Wood and Anagnostaras, 2011). In the classical Pavlovian fear conditioning employs pairing of a conditioned stimulus (CS) which can be a specific tone, smell or light (explicit cue) to an unconditioned stimulus (US) which is in most cases, an electric foot shock (0.3 mA for 1 second). The animal learns to associate the contextual cues (background, environment, surroundings) with the aversive unconditioned stimulus (foot shock). The freezing response, also known as the conditioning response (CR) is used to monitor expression of fear and is considered as a reliable index to measure fear in rodents (Blanchard and Blanchard, 1969). The protocol for fear conditioning was adopted from Dineley *et al.,* 2010 with slight modifications according to the requirements of our experiment. Context A was used in fear conditioning memory test. Context A was a rectangular chamber with a shock grid floor made of stainless steel bars. The shock grid was connected to a shocker scrambler unit to deliver shock of defined intensity and time duration. The walls of the chamber were made of clear plastic with a

sliding door in the front. The top of the chamber was open and a video recording device was connected on top of the chamber. The whole setup was placed in a square compartment connected to a computer fig. Any Maze software was used to design and implement the protocol for tone and shock duration and intensity. The inside of the box was cleaned with 70% ethanol before and after the experiment to prevent bias based on olfactory cues. The testing procedure started with habituation of the subject animal in the empty chamber for 5 minutes to allow the animal to explore the new environment and to avoid any freezing response due to anxiety of the new space during the experimental procedure. Following habituation, 7 tones (70dB each at 3000 Hz) were given with each tone lasting for 30 seconds with inter-tone interval of 2 minutes. On the 29th second of each tone, a foot shock of 0.3 mA was administered for 1 second. Freezing (Conditioning Response) was calculated by the software (Any Maze) and subsequent data was plotted fig.

3.5.1. Contextual fear conditioning

The protocol for Contextual fear was adopted from Wood and Anagnostaras, (2011) with some modifications. 24 hours after the initial exposure in the Context A during fear conditioning, the subject animal was placed back in the chamber (Context A) for a period of 5 minutes without any Conditioned (CS) or Unconditioned stimulus (US). Subject animal tried to recognize the context in which an aversive stimulus was given and subsequently showed freezing (Conditioning Response, CR). The CR was recorded and data was plotted.

3.5.1. Fear extinction

Fear extinction is classically conditioned behavioral paradigm. When the conditioned stimulus is presented alone without association of an unconditioned stimulus, it no longer predicts the forthcoming of an aversive stimulus (foot shock in this case) and hence conditioned freezing response gradually declines. For fear extinction, the subject animal was placed in context B in

which the walls and the floors of the chamber were replaced so that the animal could only associate and recall memory formed with the Conditioned Stimulus (CS). In order to avoid bias in behavior based on the new environment, the subject was placed in context B and allowed to habituate and explore for 7 minutes followed by 20 tones (70 dB and 3000 Hz each) with inter tone interval of 30 seconds (fig. 3.6). The behavior was recorded and subsequent data was plotted as percentage freezing according to the formula:

$$\% \text{ Freezing} = \frac{\text{Freezing observations}}{\text{Tone interval (30 sec)}} \times 100$$

3.5.2. Novel object recognition test

Novel Object Recognition (NOR) test is used to evaluate recognition memory and cognition in rodents. The protocol for NOR, previously described by (Ennaceur *et al., 2005*) was adopted with slight modifications as per requirement of our experiment. It is based on differential exploration of already familiar and novel objects and spontaneous inclination to explore and spend more time with novel object in comparison with familiar object. The tendency to explore novel object is an indicative of the use of learning and recognition memory (Ennaceur *et al., 2005*). The test was conducted in an open arena (1.5 x 1.5 x 1.5 ft.). Before the test, the subject was allowed to habituate in the chamber for 5 minutes to avoid bias based on intrinsic anxiety in new spaces. After habituation, two objects of approximately consistent height and volume but different shapes were placed equidistant to each other were placed in the arena and the subject was allowed to explore for 10 minutes. 20 minutes after session I, the subject was placed in the same space but one of the objects of Session I was replaced with a new object of different height and volume. The subject was allowed to explore the familiar object and the novel object for 10 minutes. The entire

apparatus was cleaned with 70% ethanol before and after the test to prevent bias based on olfactory cues.

3.5.3. Perfusion for whole tissue fixation

For the fixation of whole brain tissue, heart perfusion method was performed as previously described (Gage *et al.,* 2012). Ketamine (300µl/50g) was used to anesthetize mice via intra peritoneal injection. An incision with sharp scissors was made on abdomen to expose the diaphragm. The diaphragm was cautiously cut to approach the ribcage and heart was exposed by cutting ribs through midline. Holding the heart at a fixed position with the aid of forceps, the needle was inserted directly in to left ventricle about 5mm in depth. After insertion of the needle, a steady and slow flow rate of 5 ml/minute for normal saline was established. The heart swelled upon and a cut in right atrium was made instantly with a pointed sharp scissor. When approximately 80 ml of normal saline was injected, the normal saline was replaced with 4 percent paraformaldehyde solution. After 100ml of paraformaldehyde was injected, and the tail, heart and liver showed hardening of tissues, perfusion was stopped and animal brain was isolated. The isolated brain tissue was placed in 4% paraformaldehyde for 24-48 hours at 4˚C. Afterwards it was processed for the fixation.

3.5.4. Brain Fixation and paraffin embedding

After 24 hours of incubation in 4% paraformaldehyde, different concentrations of organic solvent (ethanol) were used to dehydrate the brain tissue followed by paraffin infiltration. The subsequent order of concentrations was used:

70% ethanol for 1 hour

95% ethanol for 1 hour

100% ethanol for 1 hour

To further harden the brain, ethanol was replaced with xylene. It was done by placing the brain in xylene for 4 hours. After the dehydration step, paraffin embedding was performed. For this purpose, the dehydrated brain tissue was kept in molten paraffin in incubator for 4 hours at 60°C.

Brain became embedded in mould with paraffin in the form of a block for microtome sectioning at room temperature. The paraffin was solidified at 4 °C.

3.5.5. Sectioning and Cresyl Violet Staining

The paraffin fixed brain block was processed for sectioning of 4 µm thickness by SLEE mainz (CUT6062) microtome. The 4 µm thick slices were transmitted to the glass slide. The slides were placed on hot plate at 65°C for 20 minutes to bind the tissue to the slide. To remove paraffin, the ready slides were kept in xylene for 10 minutes.

Rehydration was performed in organic solvent as following:

95 percent ethanol for 5 minutes

70 percent ethanol for 1 minute

50 percent ethanol for 1 minute

After rehydration of the tissue sections, staining was performed as following: Cresyl violet stain for 4 minutes A few dipping in distilled water to remove excess stain on slide

50 percent ethanol for 1 minute

70 percent * acid alcohol for 2 minutes. (*2 ml glacial acetic acid in 200 ml of 70 %ethanol)

Acid was removed by dipping the slides in 70 % ethanol

95 percent ethanol for 2 minutes

The slide was then dried for 2 hours after placing cover slip

Finally slide was examined under light microscope

3.5.6. RNA extraction for RT-PCR

RNA extraction was done using Tri-reagent according to the directions provided by the manufacturer. Animals were anesthetized using Chloroform and sacrificed. Cortex were isolated shortly after decapitation. The isolated tissue was homogenized in microfuge tube containing 1 ml of Tri-reagent. To confirm complete separation of the nucleoprotein complex, samples were allowed to stand at room temperature for 5 minutes. Following the stand, 0.2 ml of chloroform per ml of Tri-reagent was added to sample. Samples were rigorously shaken for 15 seconds until it turned milky and allowed to stand at room temperature for 10 minutes. The mixture was centrifuged at 4_{o}C and 12,000 rpm for 15 minutes. After centrifugation, 3 phases of the mixture were formed: a red organic phase at the bottom of the tube containing proteins, a whitish interphase containing DNA, and a colorless aqueous phase on the top containing RNA. Aqueous phase was removed carefully with a pipette ensuring no contamination with the other phases eliminating the chance of genomic DNA contamination. The separated aqueous phase was transferred to a new labeled tube and 0.5ml of isopropanol was added followed by a stand at room temperature for 10 minutes. After the stand, sample was centrifuged at 4_{o} C and 12,000 rpm for 10 minutes. After the second centrifugation, the RNA was precipitated out forming a pellet on side of the tube. The supernatant was removed carefully without dislodging the pellet. The RNA pellet was washed by adding 1 ml of 75% ethanol in DEPC treated water per 1 ml of Tri-reagent used in sample preparation. The RNA pellet in 75% ethanol was then centrifuged at 4_{o} C and 7500 rcf for 5 minutes. RNA sample was stored at -80_{o} C until use. For Reverse Transcription (RT), RNA stored at -80_{o} C was taken and thawed. It was centrifuged at 4_{o} C and 12000 rpm for 2 minutes. 75% ethanol was removed and pellet of total RNA was allowed to dry followed by re-suspension in 30µl PCR water.

3.5.7. Quality of the RNA

In order to ensure that the RNA isolated was of good quality, RNA of all samples was run on 2% agarose gel. It also ensured that the isolated RNA was not degraded. The quality of RNA was important for the reproducibility and accuracy of the results. If the RNA samples exhibited sharp and clear bands of 18S and 28S RNA, it was of acceptable quality.

3.5.8. RT-PCR for quantification of mRNA levels

Quantification of RNA was performed using Biophotometer and 1 µg of RNA in 30 µl was used to reverse transcribe (RT) it into cDNA. 3 µl of 10mM dNTP's, 5 mM oligoN dT were added followed by heat shock for 5 min at 55_o C to allow complete dissociation of secondary structures. Afterwards 8 µl of 5x RT buffer, 4 µl of DTT and 2 µl of RT enzyme was added. Total volume of the reaction mixture was made up to 40 µl by the addition of PCR water accordingly. For PCR reactions the synthesized cDNA was used with the recipe of each primer containing, 2.5 µl of 10x buffer, 0.5 µl of 10 mM dNTP's, 1.2 µl of $MgCl_2$, 1 µl of both forward and reverse primer specific for particular genes, 0.25 µl of Taq enzyme and then the final volume makeup of 22 µl was done by adding PCR water. At the final step 3 µl of cDNA was added. PCR profile was set up with the initial denaturation at 95_o C for 5 minutes, followed by denaturation at 94° C for 30 seconds, annealing for 30 seconds (at temperature given in table 1), then extension at 72° C for 30 seconds for number of cycles given for each gene in table. This was followed by final extension at 72° C for 10 minutes. Number of cycles and annealing temperature was optimized initially using gradient PCR to have good quality bands on agarose gel. PCR products were separated on 2 % agarose gel, visualized by ethidium bromide (addition of 10µl of ethidium bromide solution for each 100 ml of gel) staining. Actin was used as a housekeeping gene and used to normalize PCR products.

Table 1: List of primers used in the study along with their respective Annealing Temperatures

Sr. no:	Genes	Primer Sequence (5'-3')	Annealing Temperatures	No. of cycles
1.	Actin	F= GCCTTCCTTCTTGGGTATGG R= CAGCTCAGTAACAGTCCGC	55	35
2	Alpha 4	F= GTCTAGAGCCCGTTCTGTGA R= TAGTCATGCCACTCCTGCTT	63	35
3	Alpha 7	F= TGCAAAGAGCCATACCCAGA R= TGATCCTGGTCCACTTAGGC	66	32
4	Beta 2	F= GATGACCAGAGTGTGAGGGA R= CCCCCACCGTTAACACTACT	63	30
5	M1	F=GTCCCATGGAAACCCTGAATCC R= GTCACTGACTTAGTCGCCCG	56	35
6	M3	F= TCTTGAAGTGCTGCGTTCTGA R= GTTGGGAAACAAAGGCGAGG	56	35
7	M5	F= AGCACCTCAACAACGGGAAA R= GGGGATCCAGGCCTTTTGTT	55	35

3.5.9. Analysis of PCR Products by Gel Electrophoresis

To analyze the PCR products 3% agarose gel was prepared in 1X Tris acetate EDTA (TAE) buffer and was run in the same buffer composition. For gel preparation, working solution of 10X TAE (pH 8.3) was prepared by diluting the stock solution by 1:10 with distilled water. Agarose (2.1 g) was dissolved in 70 ml of 1X TAE and heated in microwave oven to dissolve agarose. The gel mixture was cooled to ~60 °C and upon cooling, 7 µl of ethidium bromide was added. PCR product (5 µl) along with 1X loading dye was run on the gel at a constant current of 90 V for 30-40 minutes. After the complete electrophoretic run the gel was visualized under ultra-violet light and photographed by gel documentation system (Wealtech Sparks, USA).

3.6. DNA contamination check

Negative control was used to check DNA contamination. Every sample was used in PCR to check for DNA contamination, using primers that can amplify DNA fragments with no cDNA.

3.6.1. Expression studies

On the 26th day of the experiments, animals were sacrificed. Cortex were isolated and RNA was extracted using Tri-Reagent. RNA samples were then quantified and processed for Cdna formation through Reverse transcription and PCR for expression studies (McKee *et al.,* 2008).

3.6.2. Quantification of band intensities

Quantification of band intensities was done through Image J software. A same background level was adjusted for every gel through NIH software. The minima and maxima of band intensity were calculated and value of band intensity was derived by dividing the minimum score from the maximum score. Then every band density was normalized with respective actin band and

expression was measured dividing desired gene density by actin and results were presented in graphs. The area of the band was kept constant (fig. 3.9).

3.6.3. Statistical analysis

Results were analyzed using Graph pad Prism version 2.0. One-Way ANOVA was used for data analysis followed by Bonferroni post-hoc test. Unpaired t-test was applied to determine the statistical significance of the data. Data was represented as mean ± Standard Error of the Mean (SEM) up to two significant figures with a confidence interval of 95%. Results were considered significant only if the *p* value was less than or equal to 0.05.

Figure 5: User interface of NIH software ImageJ used for quantification of band intensities. Gel image with a selected band and subsequent value of band intensity is shown in the results window.

3.6.4. Protein Sample Preparation

Tissue homogenates were prepared by suspending the cortex in urea/thiourea buffer (7M Urea, 2M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 10M Phenyl methyl sulfonyl fluoride (PMSF), Dithiothreitol (DTT) separately. Briefly 100 microliter of ice cold lysis buffer was added and homogenized using sonicator. For increased dissolubility the homogenates were incubated at room temperature for 1 hour and centrifuge at 14000 rpm at 4C for 10 minutes. The supernatant was transferred and stored at -20C in a new eppendropf tube. In order to maximum the yield, 50 microliter lysis buffer was added to the pellet and treated similarly as before. The two supernatants were pooled and centrifuged at 14000 rpm for 90 minutes. The final supernatant was stored at -80 C until use.

3.6.5. Protein Quantification by Bradford's Assay

Bradford's Assay was employed to measure the concentration of protein extracted from the cortex tissue. Different dilutions of bovine serum albumin (1mg/1ml) were prepared in duplicate with distilled water. The sample were diluted with distilled water (1:20) in duplicates. The final volume of each standard/sample was kept 20 microliter, 1ml Bradford reagent was added to each standard/sample and mix by gentle vortexing, followed by incubation at room temperature for 10 minutes, Absorbance of each standard/sample was measured at 595 nm reagent blank using OPTIMA SP-300 spectrophotometer. Standard curve was derived by plotting the standard absorbance against its concentration.

3.6.6. Protein profiling by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulaphate PolyAcrylamide 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 min. This was followed by the preparation of the 5% stacking gel (distilled water; monomer solution; 1.5 M Tris-HCl pH 6.8; 10%SDS; 10%APS; TEMED). Stacking gel was poured on top of the polymerized resolving gel, followed by the immediate insertion of the comb in to the gel. The gel was left for another 45 min 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 100ºC for 3 min and given a short spin at 12000 rpm for 3 min. The samples were then loaded in the wells and the electrophoretic separation process was carried out at 100 volts for 90 min. After the run, the gel was placed in coomassie brilliant blue staining solution (0.025%), overnight. The gel was destained, using 10% destaining solution (75ml glacial ac acetic acid; distilled water; 25 ml of 100% ethanol) until a clear background was attained.

3.6.7. Image and statistical analysis

3.6.8. Protein Quantification

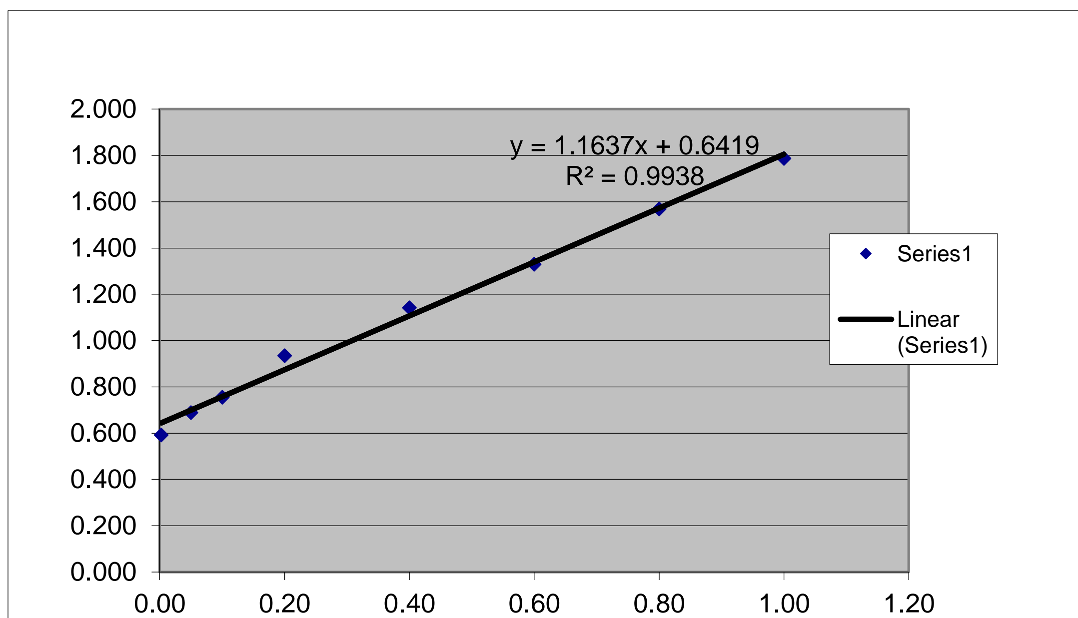


Figure 6: Bradford standard curve. Bradford standard curve plotted for eight standard values. Concentration was plotted on the x-axis (independent variable). Absorbance measured at 595 nm was plotted on the y-axis (dependent variable). This graph represents linear regression for the eight standard points. The obtained linear regression value was 0.9938 ($R2=0.993$).

3.6.9. Total Proteome Profile

The total proteome profile of the cortex for all five groups was attained by 1D gel separation (SDS-PAGE). The gel image analysis using Quantity One® software revealed total of 20 and 15 protein bands in cortex and hippocampus respectively (Figure).

3.7. Human Subject

Two separate groups of normal, healthy, old 50 to 70 year of age, *n* = 40 divided. Volunteers took part in the drug study. All the subjects performed The paired associate learning (PAL) and Reaction time (RT) test which are part of the CANTAB battery (Downes *et al*., 1989) and None of the volunteers was receiving concurrent medication, nor had a history of liver kidney disease, Diabetes patients, gallbladder problem psychiatric, neurologic, or cardiovascular illnesses or other medical conditions that could interfere with central nervous system functions or analysis of the results. The studies were approved by the local ethical committee and national drug regulatory authority, and all the subjects provided a written informed consent. The number of test sessions was limited to two at the request of the local ethical committee and after taking a consent from the Internal Review board (IRB), Atta-Ur- Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST).

3.7.1. Dosage and Pharmacological Direction

One group *n* = 40 individual received 500/kg turmeric powder and other group also *n* = 40 individual received 500 mg/kg, for period of 30 days placebo PO in capsule form for period of 30 days, or the appropriate oral placebo, 90 min before starting the test session. The two test was performed on day first and on 31 day, Retest of battery memory test was take to evaluate the improvement in memory. The Reaction Time and Paired Association Learning test, a battery memory tests was performed to evaluate memory consolidation and acquisition. Subjects from each group appeared on two sessions with 30 days between sessions, and received the relevant pharmacological dosage on one time in a day daily for 30 days, and an appropriate placebo on the other in a counterweighed order for group, placebo-controlled (Hills and Armitage., 1979). Test sessions were started at the same time of each testing day for each individual subject. The tests

were given as a part of our study to investigating the effects of turmeric powder on attentional, and memory functions. The entire session lasted 60 to 90 min for all the subjects, and the testing began 90 min after ingestion of capsule for all the subjects.

3.7.2. Paired Associates Learning (PAL)

PAL is a test of optical pattern and visuospatial memory and learning, which contains characteristics of straight paired associate's procedure and a conditional learning test which are part of the CANTAB battery (Downes *et al*., 1989). The subjects were firstly presented with four white boxes arranged about the outside of the card, which were then "showed up" by the card to reveal four different colored patterns or images, one at a time. One of the four patterns was then displayed in the center of the card, and the subjects were instructed to touch the box that had "contained" that particular shape. Without giving any feedback as to the accuracy of the response, the card then presented another pattern and the subjects were asked to respond in the same way, until all six patterns had been placed. The test began at a very easy level with a single pattern in one of the boxes and then steadily become more challenging with two and three pattern sets before the test with ten items. If the subjects had made no errors with the four-box problem, then the procedure was repeated with eight boxes and patterns. If however, at least one error had been made, then all six patterns were presented to the subjects in the same location as before, and subjects were not informed which shapes had been incorrectly placed. This was repeated until the subjects had placed all six patterns correctly, within a maximum of 2 trials. All subjects were given 500mg/day orally turmeric orally increased the number of stages or responses to reach the criterion.

3.7.3. Reaction Time

The test provides assay for motor and mental response speed and can be analyze of movement time, reaction time, response accuracy and impulsivity, which consist aspects of responsive reaction time which are part of the CANTAB battery (Downes *et al*., 1989). The subject were showed with 10 boxes patterned around the outside of the card, which were then visible to subject with 10 different colors, the test trial was divided in to five stages which requires increasable chains of response. In each card, the subject must response as soon as a yellow dot appears at each with different location. Yellow dot appeared in one of 10 different locations, and the subject must respond. Measured the movement time, reaction time, response accuracy and impulsivity. All subjects were given 500mg/day orally turmeric orally increased the number of stages or responses to reach the criterion.

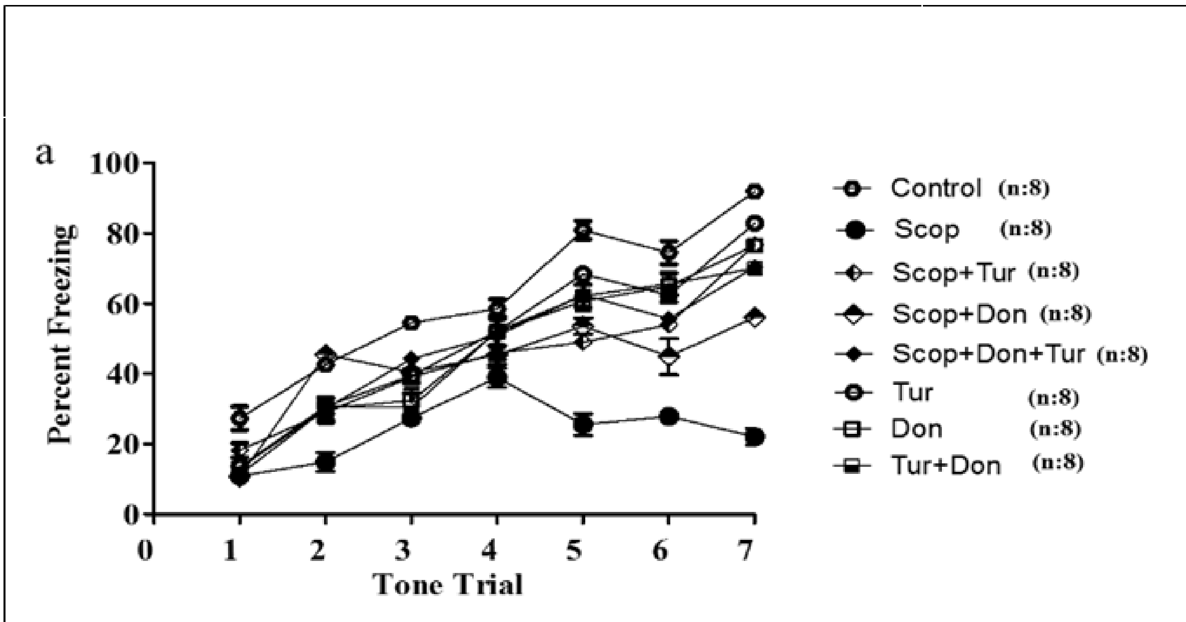
RESULTS

4.1. Behavioral tests

In order to evaluate the effect of Turmeric on learning and memory, fear conditioning, contextual fear, fear extinction and novel object recognition were accomplished.

4.1.1. Effect of Turmeric on fear conditioning

In order to measure cortex dependent associative learning, fear conditioning test was performed. Condition-stimulus (CS) and context-dependent memory was governed by measuring the freezing response (a). Learning of CS- and context-dependent fear memory was significantly reduced ($p < 0.001$) in Scopolamine-induced amnesia group as at the end two tone test, there was less freezing response in Scopolamine-induced amnesia group (10.91 ± 1.12) compared to control group (91.95 ± 3.32). Significantly high ($p < 0.001$) freezing was shown in Turmeric treated group (82.87 ± 1.49) as compared to Scopolamine-induced amnesia group (10.915 ± 1.04). Scopolamine + turmeric group (76.75 ± 0.81). Which indicated that deficiency in fear memory learning in amnesia was reversed by Turmeric. Moreover donepezil group (76.50 ± 1.23) as compared to Scopolamine-induced amnesia group again showed increase the recovery of fear memory learning in amnesia. On the other hand freezing response significant ($p < 0.01$) in Scopolamine + donepezil group (56.05 ± 2.04), and Scopolamine + turmeric + donepezil (69.37 ± 0.62). Whereas turmeric + donepezil group (62.16 + 3.28) did not show synergistic effect.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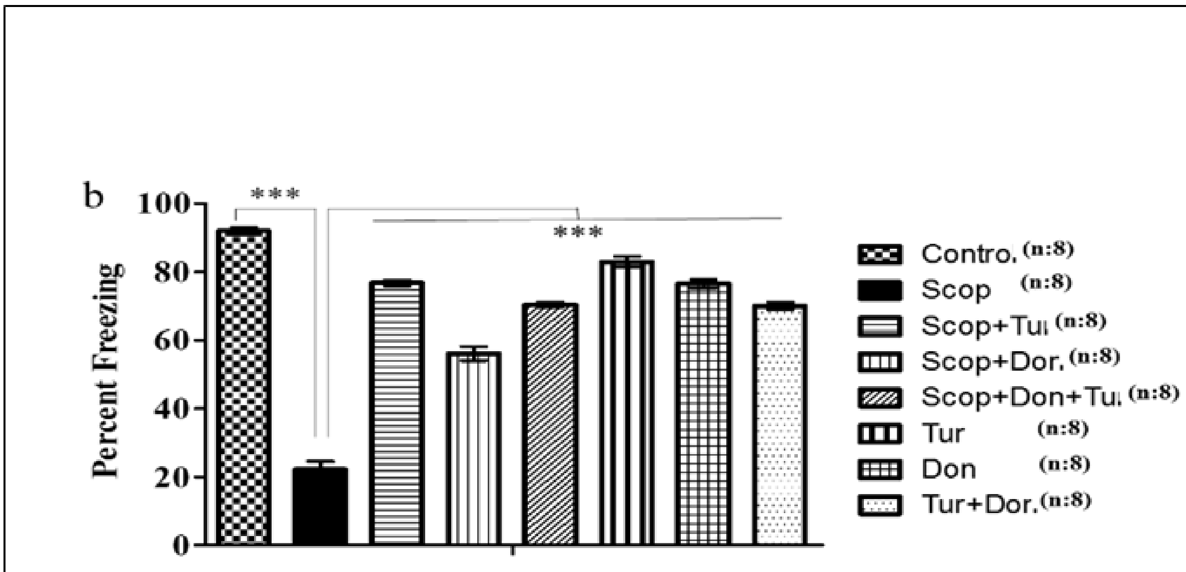
Don (n:8)
Tur+Don (n:8)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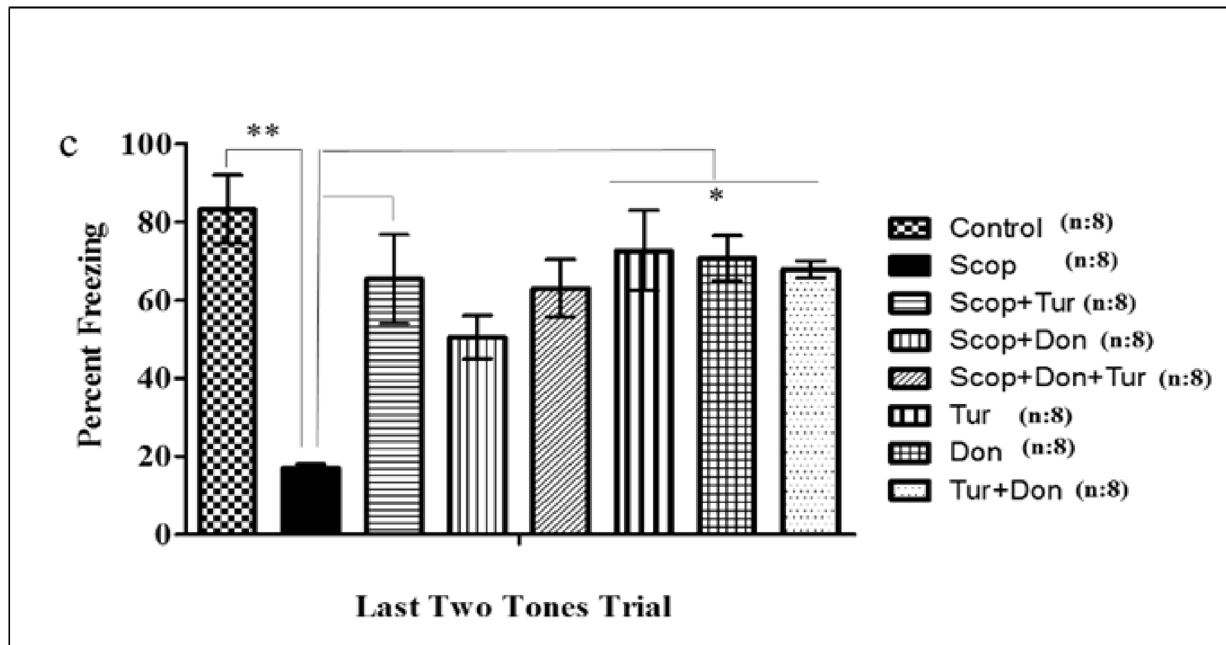


Figure 7: Effect of Turmeric on freezing response: (a) The graph shows the comparison of percentage freezing based on CS and US among control, Scopolamine-induced amnesia group, Turmeric treated group and Turmeric alone group. (b) The bar diagram represent the difference in fear conditioning between groups at last two trials. Error bars represents mean SEM ± n=8. ***p < 0.001. (c) Results of the last two tone trials in bar diagram illustrating the comparison of control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil, Donepezil alone and Turmeric only groups. Error bars show mean SEM ± n=8. **=p < 0.01.

4.1.2. Effect of Turmeric on context based memory

The freezing response of mice was assessed by placing them in the same context, where conditioning was done without conditioned stimulus. Scopolamine-induced amnesia group (33.50 ± 5.19) showed significant ($p < 0.001$) impairment in learning relative to control group (88.62 ± 1.55). A significant improvement ($p < 0.001$) in memory acquisition was seen in Turmeric treated group (68.50 ± 4.20) in comparison to Scopolamine-induced amnesia group (33.50 ± 5.19). On the other hand the significant ($p < 0.05$) in Scopolamine + turmeric group (49.0 ± 1.25) comparison with Scopolamine-induced amnesia group (33.50 ± 5.19).

4.1.3. Effect of Turmeric on fear extinction

Fear extinction memory was calculated by showing the animal to a new context (changed from where fear conditioning was done). It was seen that there is a robust relationship among CS and memory which affect the response (freezing) ultimately fig. At the final five tone test, there was a significant difference ($p < 0.001$) in fear extinction among control group and Scopolamine-induced amnesia group. Increased freezing was showed in Scopolamine-induced amnesia group (67.59 ± 6.77) as compared to control group (13.66 ± 3.24). It presented that even after 20 tone trials, amnesic mice were unable to learn new memory i-e; fear extinction. Freezing response was significantly decreased ($p < 0.01$) in Turmeric treated group (37.27 ± 6.64), and Scopolamine + turmeric group (35.89 ± 3.23) as compared to Scopolamine-induced amnesia group (67.59 ± 6.77) with the consecutive tones which indicated improvement of new learning by Turmeric. It shown that Turmeric enhanced fear extinction learning alike to control group that was reduced in Scopolamine-induced amnesia group. The group Scopolamine + turmeric + donepezil show highly significant ($p < 0.001$) difference in fear extinction (22.17 ± 2.44) with Scopolamine-induced amnesia group (67.59 ± 6.77). On the other hand, Turmeric + donepezil showed non-significant

data. Its mean the combination of both herbal and synthetic drug does not show any synergism effect.

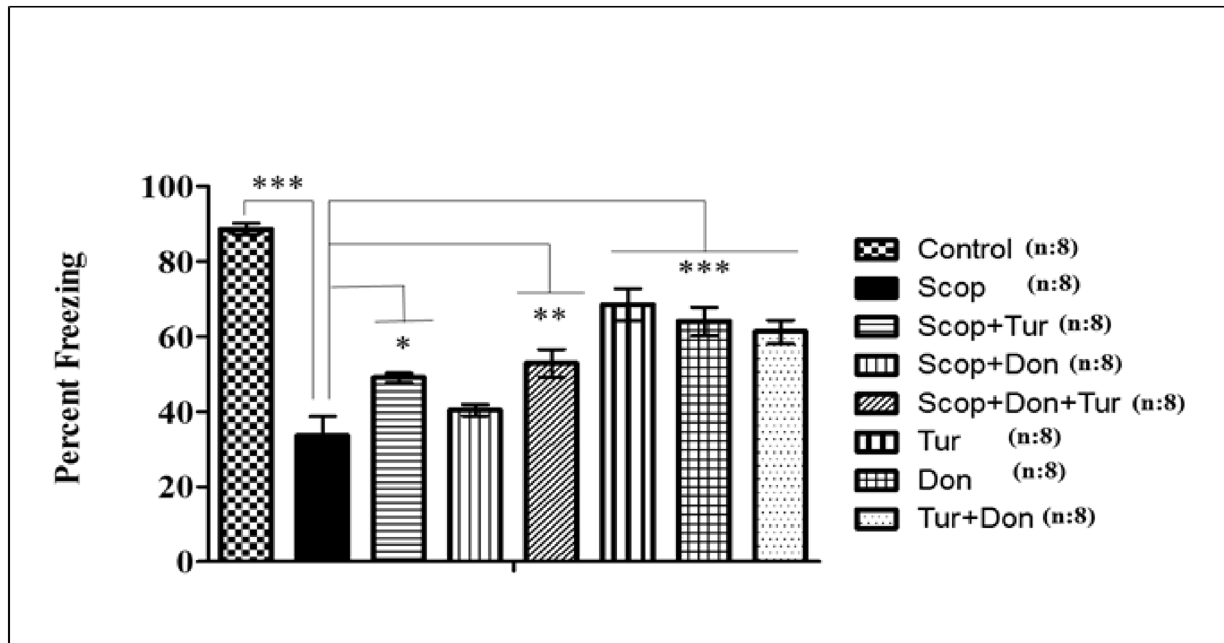


Figure 8: Effect of Turmeric on contextual memory: The bar diagram shows the comparison of context based memory after conditioning between control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil, Donepezil alone and Turmeric only groups. Error bars represents mean SEM ± n=8. ***=p < 0.001.

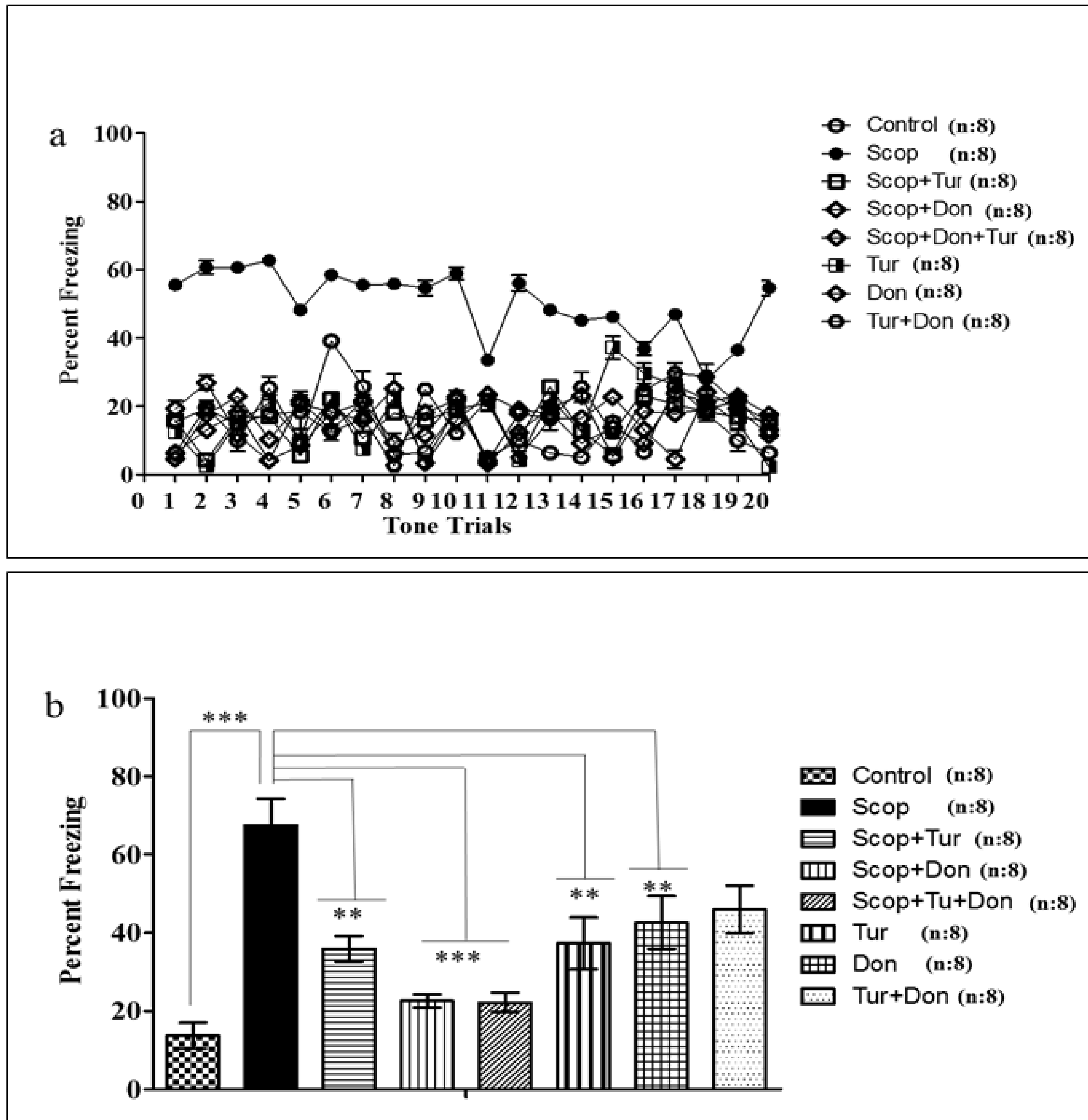


Figure 9: Effect of Turmeric on extinction memory: The bar diagram shows the comparison of context based memory after conditioning between control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil, Donepezil alone and Turmeric only groups. Error bars represents mean SEM ± n=6. ***=p < 0.001.

4.1.4. Effect of Turmeric on Recognition memory

Recognition memory and analytical behavior was evaluated by the means of novel object recognition test, by showing the trial animal to dissimilar objects i-e; habituation session (same objects) and test session (one known from session I and the other new). Recognition memory was impaired in Scopolamine-induced amnesia group (190.50 ± 16.65) and it showed a significant ($p < 0.001$) reduction in exploration of the novel object as compared to control (387.12 ± 23.09). This showed that exploratory behavior in Scopolamine-induced amnesia group was diminished. Turmeric treated group (357.37 ± 15.20) showed significant progression in exploratory behavior ($p < 0.001$) compared to Scopolamine-induced amnesia group (190.50 ± 16.65) in trial session. The results proposed the reestablishment of recognition memory by Turmeric in amnesia fig. On the other hand, group Scopolamine + turmeric + donepezil show highly significant ($p < 0.001$) difference in fear extinction (292.62 ± 15.36) with Scopolamine-induced amnesia group (67.59 ± 6.77). On the other hand, Turmeric + donepezil showed non-significant data. Its mean the combination of both herbal and synthetic drug does not show any synergism effect.

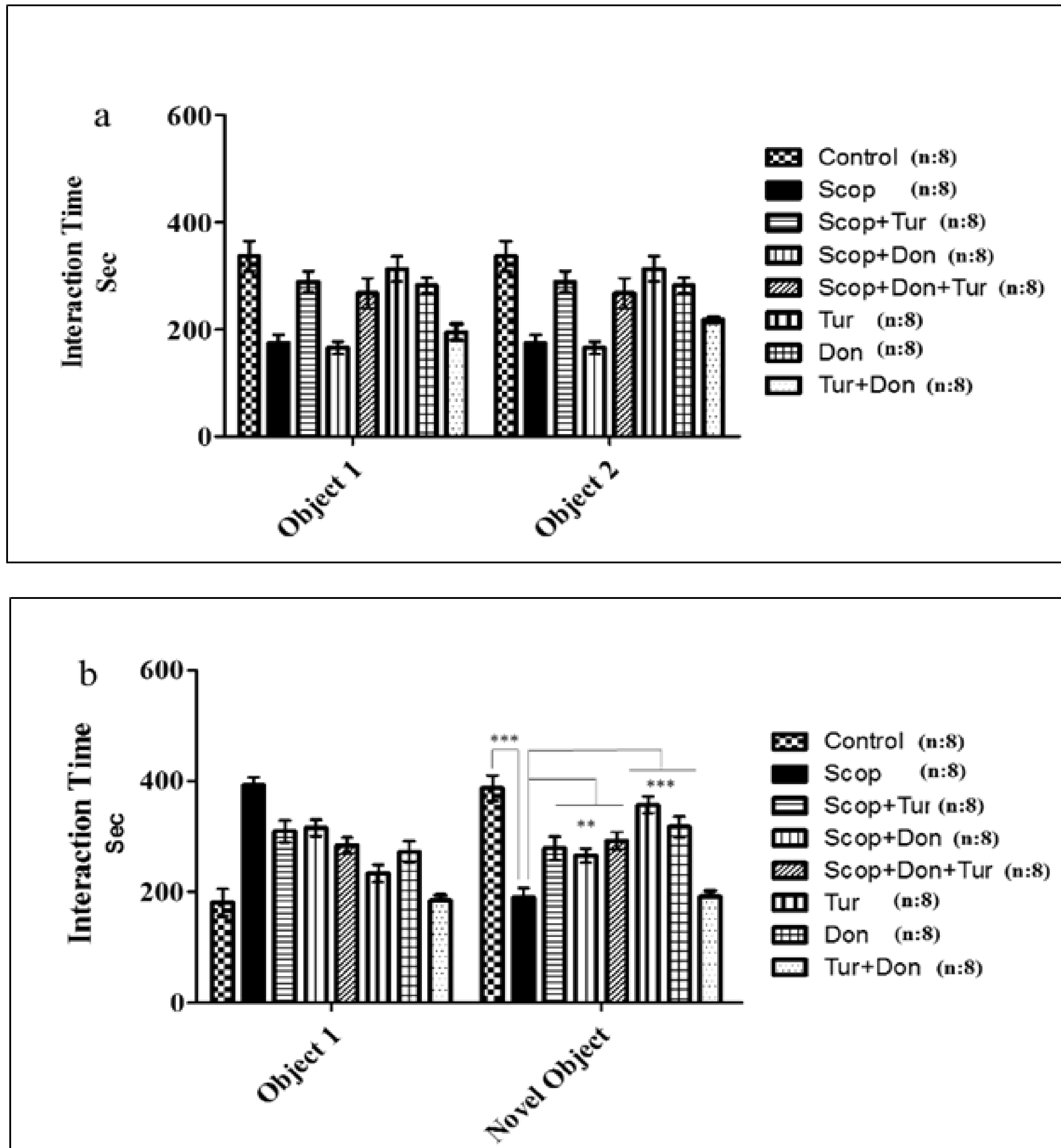


Figure 10: Novel object recognition test: Graphs represents the effect of turmeric (a) Comparison of exploratory and working memory in session 1 (Familiarization phase). Graph representing the time spend by all eight groups control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil,

Donepezil alone and Turmeric only groups. (b) Comparison of exploratory and novel preference in session II (Test phase). Test phase. Error bars represent mean SEM ± n=8. ***=p < 0.001

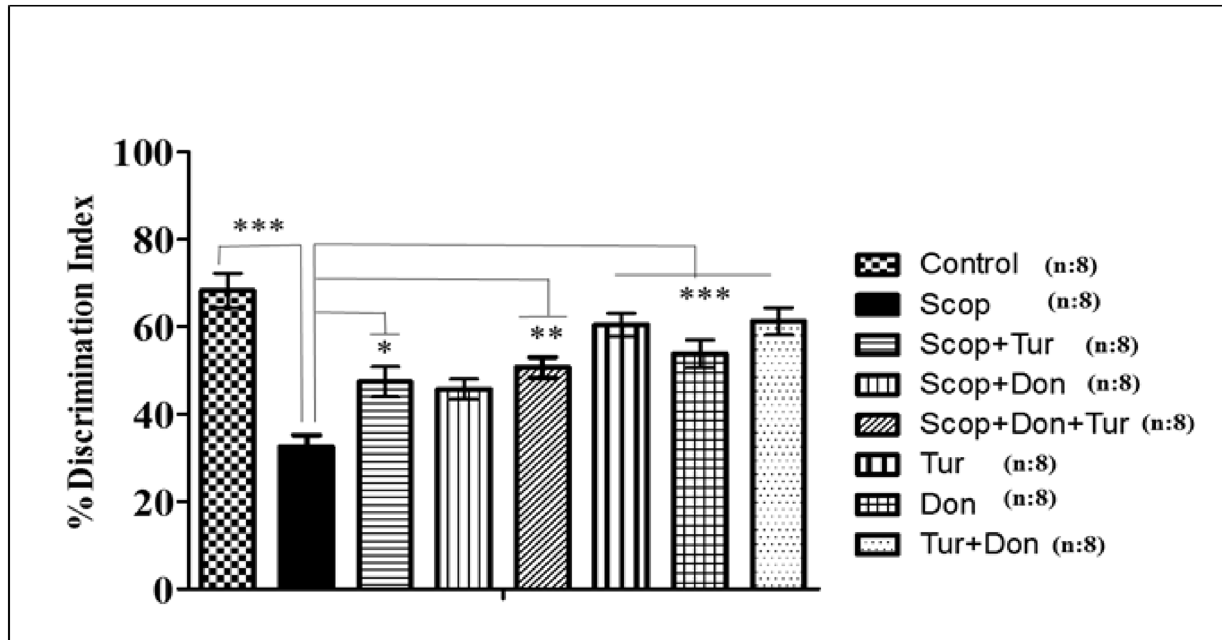


Figure 11: Effect of Turmeric on novelty test percent discrimination index: The bar diagram shows the comparison of context based memory after conditioning between control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil, Donepezil alone and Turmeric only groups. Error bars represents mean SEM ± n=8. ***=p < 0.001.

4.1.5. Effect of Turmeric on expression of genes

The expression of genes was evaluated by RT-PCR. The RNA extracted from brain tissues was measured and subjected to quality control check. To confirm quality, respectively sample of RNA was run on gel and gel electrophoresis was carried out using 2% agarose gel. The sharp ribosomal bands confirmed the quality of RNA (Fig). RNA quantification is essential since RNA is turned into proteins. The first step of protein production in the cell is the synthesis of RNA. RNA is then transcribed and subsequently translated to form a functional protein. These is complex steps, though RNA expression control the protein formation at bottom level. Therefore we studied RNA level in cortex of the brain, to demonstrate the pharmacological effects of Turmeric in amnesia.

4.1.6. Effect of Turmeric on nAChR genes in cortex

The effect of Turmeric was determined on the expression of nicotinic acetylcholine receptor (nAChR) genes (α4, α7 and β2) in the cortex of the Scopolamine-induced amnesia group. In cortex of Scopolamine-induced amnesia group, there was significantly reduced ($p < 0.001$) expression of α4 (23.60 ± 3.61, n=4x2), as contrasted to control group of α4, (150.86 ± 2.74, n=4x2). Following treatment with turmeric, there was a substantial increase ($p < 0.01$) in the expression of α4, (70.13 ± 4.79, n=4x2) in Turmeric treated group as compared to Scopolamine-induced amnesia group (23.60 ± 3.61, n=4x2).

The expression of nicotinic acetylcholine receptor (nAChR) genes α7 in the cortex of the Scopolamine-induced amnesia group. In cortex of Scopolamine-induced amnesia group, there was significantly increase ($p < 0.001$) expression of α4 (0.89 ± 0.01, n=4x2), as compared to control group of α4, (0.62 ± 0.004, n=4x2). Subsequent treatment with turmeric, there was a significant reduction ($p < 0.001$) in the expression of α4, (0.31 ± 0.009, n=4x2) in Turmeric treated group as compared to Scopolamine-induced amnesia group (0.89 ± 0.01, n=4x2).

Whereas the effect of Turmeric was determined on the expression of nicotinic acetylcholine receptor (nAChR) genes β2 in the cortex of the Scopolamine-induced amnesia group. There was no significant changes in expression of turmeric treated as compared to Scopolamine-induced amnesia group. On the other hand there was non-significant expression of α4, α7 and β2 in Scopolamine + Turmeric + Donepezil as compared to Scopolamine-induced amnesia group.

4.1.7. Effect of Turmeric on mAChR genes in cortex

The effect of Turmeric was determined on the expression of muscarinic acetylcholine receptor (mAChR) genes (M1, M3 and M5) in the cortex of the Scopolamine-induced amnesia group. In cortex of Scopolamine-induced amnesia group, there was significantly reduced ($p < 0.01$) expression of M1 (0.40 ± 0.02, n=4x2), M3 (0.16 ± 0.05, n=4x2) and M5 (0.10 ± 0.09, n=4x2) as compared to control group of M1 (0.92 ± 0.05, n=4x2), M3 (1.21 ± 0.10, n=4x2) and M5 (1.07 ± 0.08, n=4x2). Following treatment with Turmeric, there was a significant increase ($p < 0.01$) in the expression of M1 (0.80 ± 0.02, n=4x2), M3 (1.03 ± 0.05, n=4x2) and M5 (0.95 ± 0.13, n=4x2) in Turmeric treated group as compared to Scopolamine-induced amnesia group (0.40 ± 0.02, 0.16 ± 0.05 and 0.10 ± 0.09 respectively, n=4x2) (fig). On the other hand there was non-significant expression of M1, M3 and M5 in Scopolamine + Turmeric + Donepezil as compared to Scopolamine-induced amnesia group.

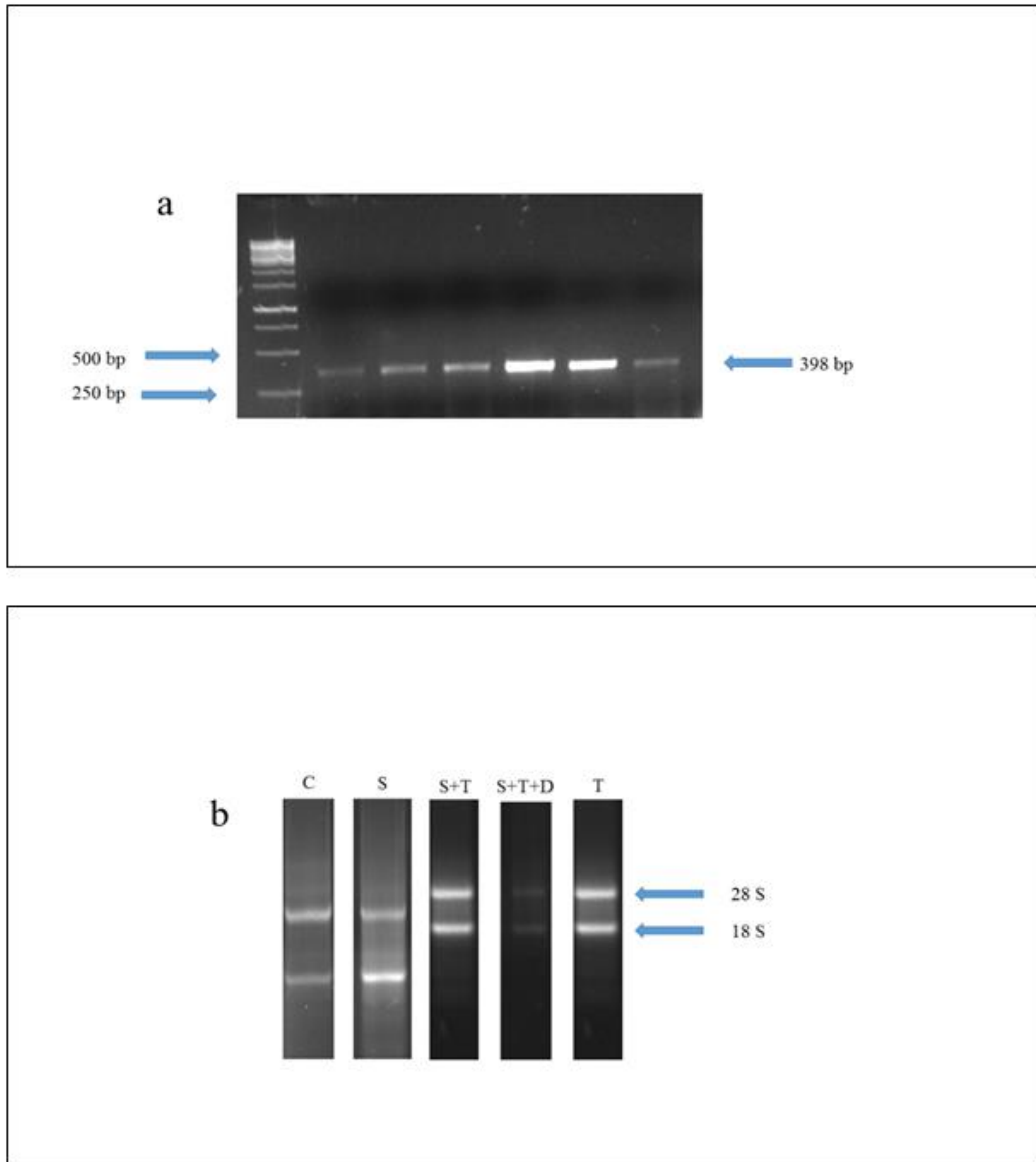


Figure 12: (a) RNA gel electrophoresis image with ladder for assuring correct band size and for reassurance of quality of whole RNA of the cell. In each well 2µg RNA of cortex was loaded, electrophoresed and seen by using ethidium bromide dye. **(b)** Two sharp bands in every sample show 18S and 28S which show that RNA is not degraded. C = control group, S = Scopolamine-treated group and S+T = Scopolamine + Turmeric-treated group, Scopolamine + Turmeric + Donepezil-treated group and T= Turmeric only group.

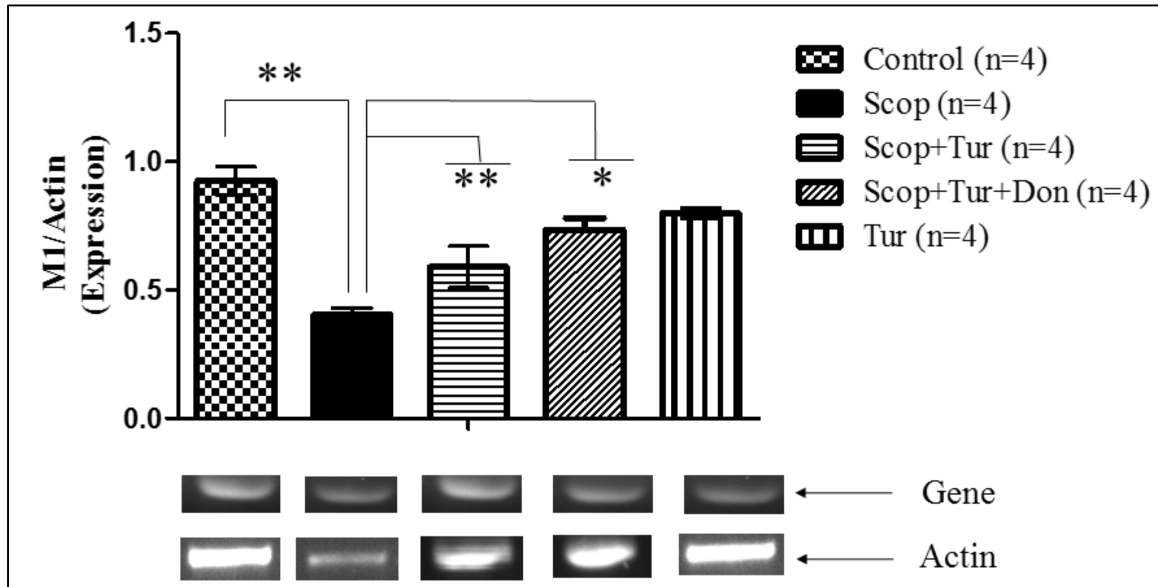


Figure 13: Graph representing expression of muscarinic receptor (M1) in cortex. Comparison among control, Scopolamine treated, Turmeric-treated (Scopolamine + Turmeric, Scopolamine + Turmeric + Donepezil) and Turmeric only group; **= $p < 0.01$, Data represent mean ± SEM; n = 4x2.

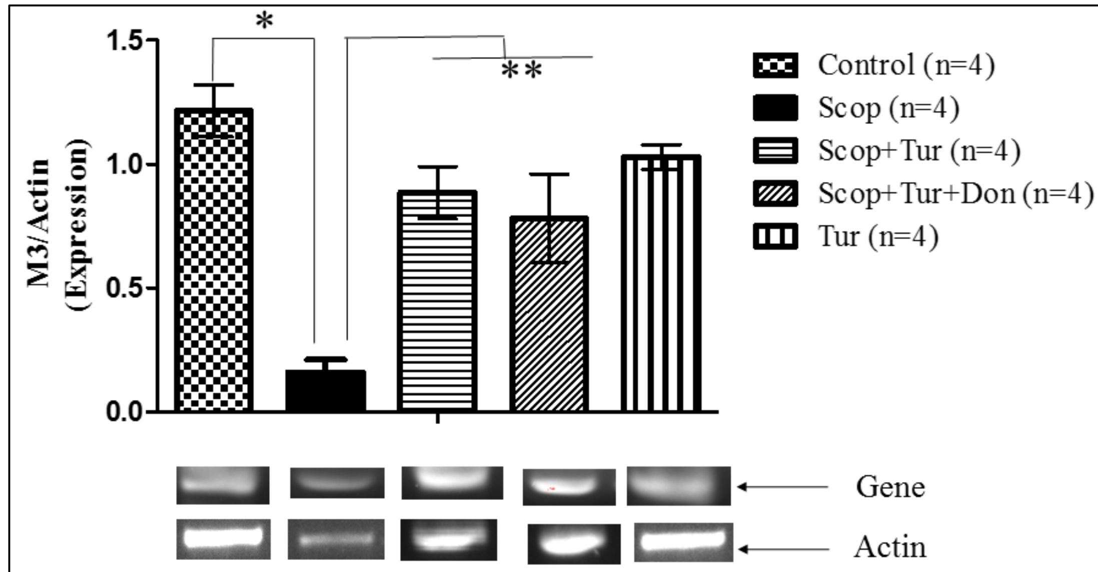


Figure 14: Graph representing expression of muscarinic receptor (M3) in cortex. Comparison among control, Scopolamine treated, Turmeric-treated (Scopolamine + Turmeric, Scopolamine + Turmeric + Donepezil) and Turmeric only group; **= $p < 0.0001$, Data represent mean ± SEM; n = 4x2.

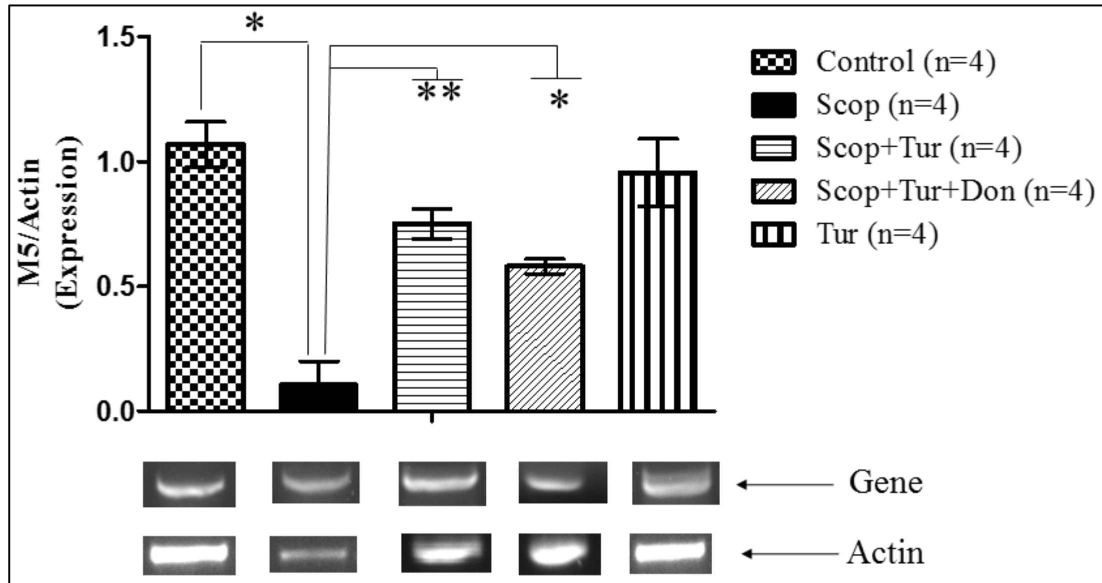


Figure 15: Graph representing expression of muscarinic receptor (M5) in cortex. Comparison among control, Scopolamine treated, Turmeric-treated (Scopolamine + Turmeric, Scopolamine + Turmeric + Donepezil) and Turmeric only group; **= $p < 0.0001$, Data represent mean ± SEM; n = 4x2.

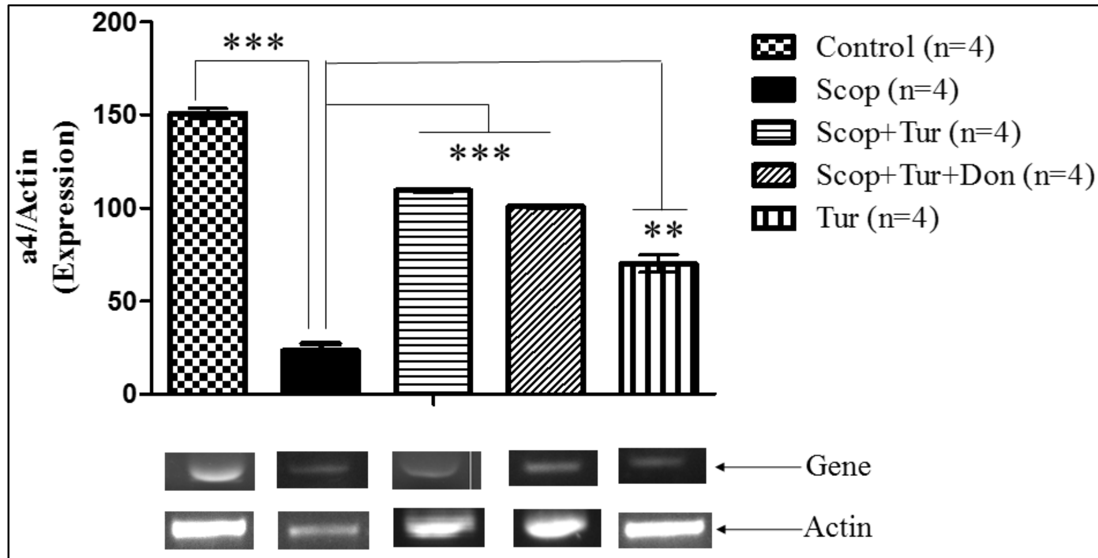


Figure 16: Graph representing expression of nicotinic receptor (alpha4) in cortex. Comparison among control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric, Scopolamine + Turmeric + Donepezil) and Turmeric only group; ***= $p < 0.0001$, Data represent mean ± SEM; n = 4x2.

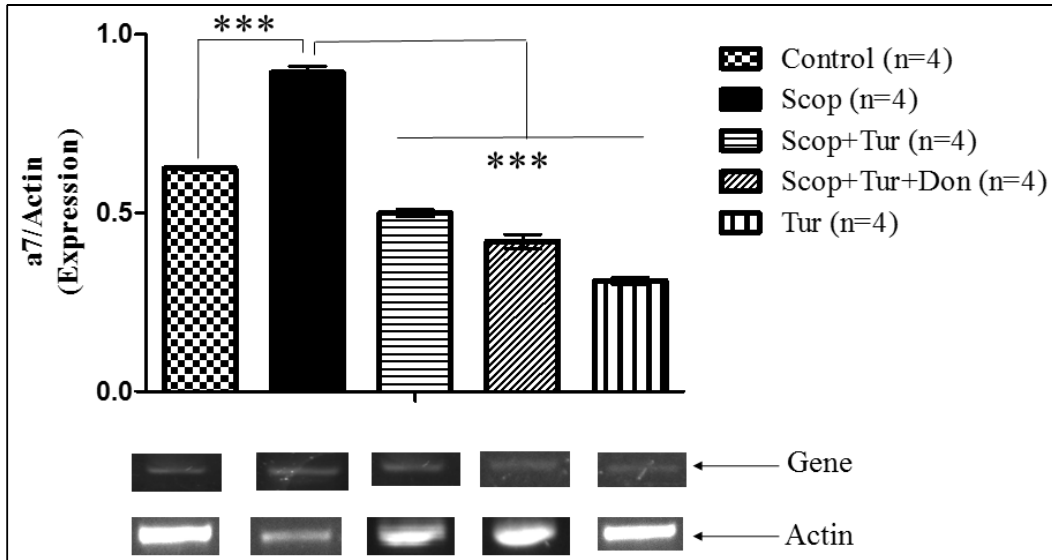


Figure 17: Graph representing expression of nicotinic receptor (alpha7) in cortex. Comparison among control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric, Scopolamine + Turmeric + Donepezil) and Turmeric only group; ***= $p < 0.0001$, Data represent mean ± SEM; n = 4x2.

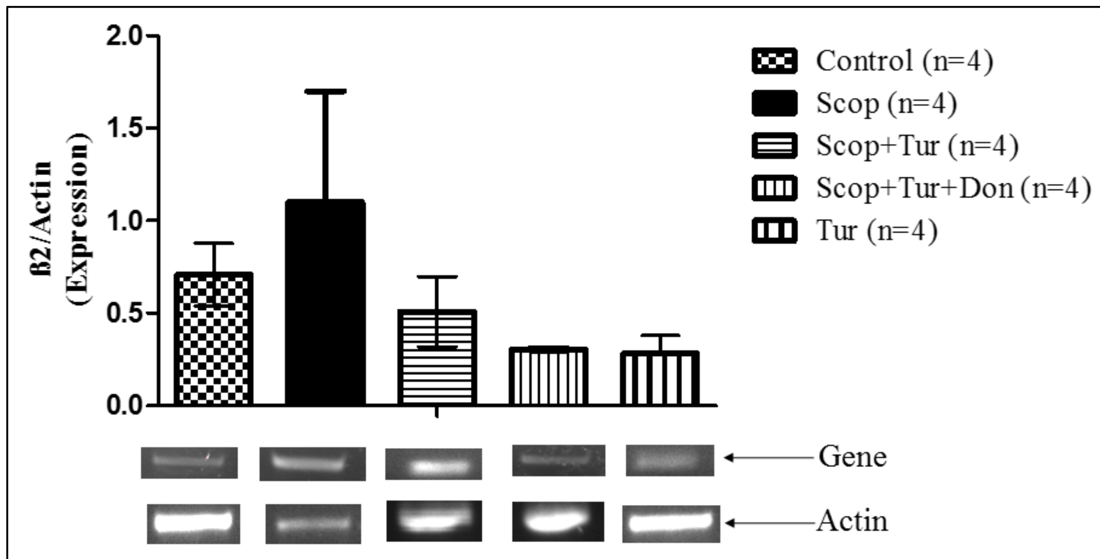


Figure 18: Graph representing expression of nicotinic receptor (beta2) in cortex. Comparison among control, Scopolamine treated, Turmeric-treated (Scopolamine + Turmeric, Scopolamine + Turmeric + Donepezil) and Turmeric only group; non-significant ± SEM; n = 4x2.

4.1.8. Effect of Turmeric on Expression profile of cortex proteins

The cortex 35 KDa protein revealed a significant increase in expression level ($p < 0.001$) in the Scopolamine-induced amnesia group (155.44 ± 7.02) as compared to the control (95.33 ± 3.28). Upon comparison between the Scopolamine-induced amnesia cortex (155.44 ± 7.02) and Scopolamine + turmeric, Scopolamine + turmeric + donepezil and turmeric (62.39 ± 9.27, 57.13 ± 2.50, and 71.50 ± 3.43, p value < 0.001 respectively, significantly reduction in protein quantity was observed, however the turmeric treatment did not restore normal protein expression completely.

The cortex 54 KDa protein revealed a significant increase in expression level ($p < 0.01$) in the Scopolamine-induced amnesia group (26.84 ± 0.41) as compared to the control (95.33 ± 3.28). Upon comparison between the Scopolamine-induced amnesia cortex (26.84 ± 0.41) and Scopolamine + turmeric, Scopolamine + turmeric + donepezil and turmeric (12.80 ± 0.83, 13.21 ± 1.63, and 11.50 ± 2.46, p value < 0.001 respectively, significantly reduction in protein quantity was observed, however the turmeric treatment did not restore normal protein expression completely.

The cortex 67 KDa protein revealed a significant decrease in expression level ($p < 0.001$) in the Scopolamine-induced amnesia group (27.35 ± 4.01) as compared to the control (100.57 ± 15.38). Upon comparison between the Scopolamine-induced amnesia cortex (27.35 ± 4.01) and Scopolamine + turmeric (86.85 ± 0.86, p value < 0.01), significantly increase in protein quantity was observed, however the turmeric treatment did not restore normal protein expression completely.

The cortex 47 KDa protein revealed a significant decrease in expression level ($p < 0.01$) in the Scopolamine-induced amnesia group (40.62 ± 0.71) as compared to the control (128.50 ± 8.86). Upon comparison between the Scopolamine-induced amnesia cortex (40.62 ± 0.71) and Scopolamine + turmeric + donepezil (120.73 ± 22.22, p value < 0.05), significantly increase in protein quantity was observed, however the turmeric treatment did not restore normal protein expression completely.

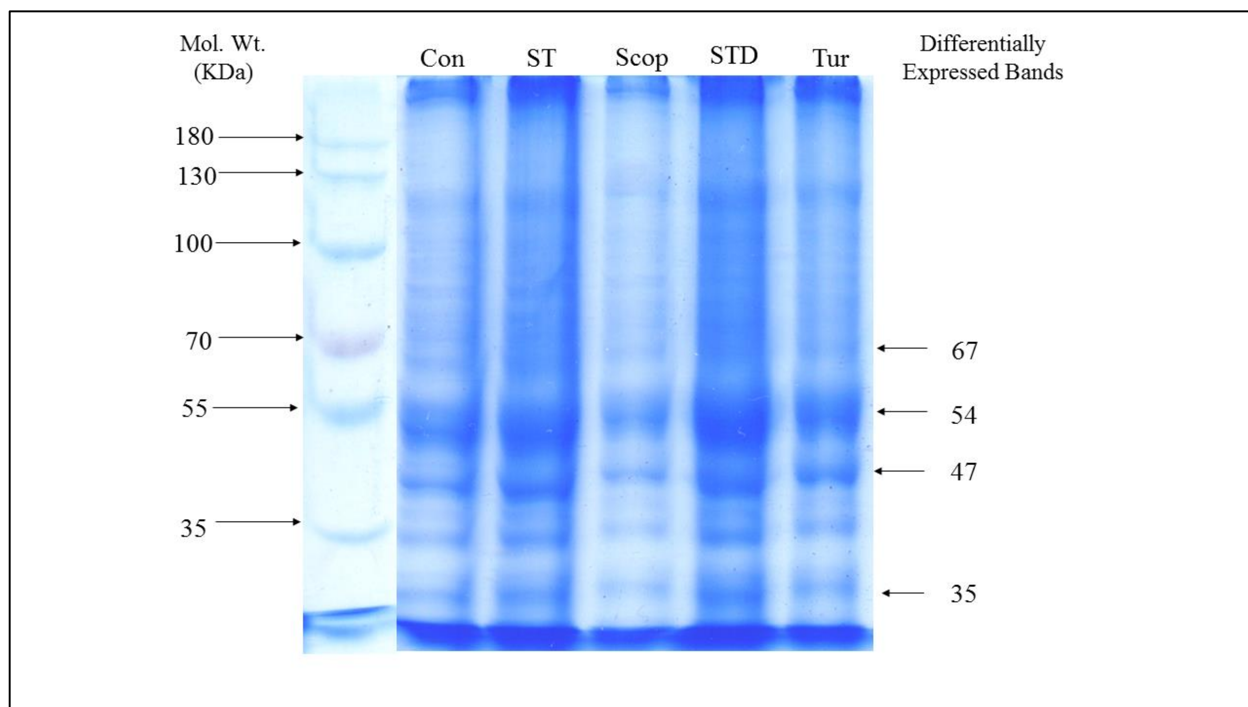
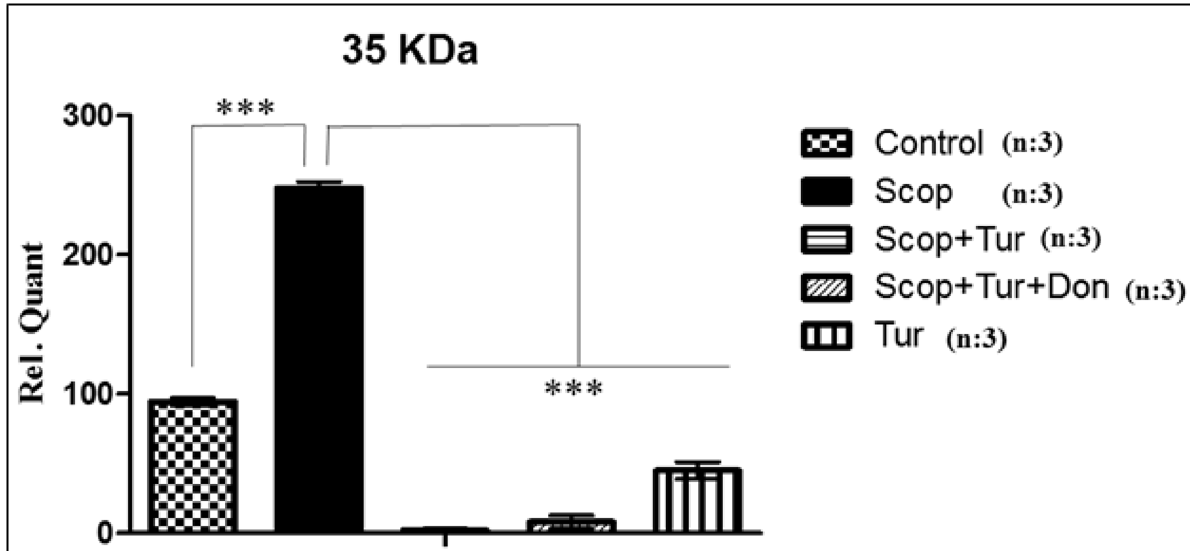


Fig 19: The SDS-PAGE pattern of cortex protein. Extracted proteins were separated in 12.5% resolving gel and stained with Coomassie Brilliant Blue. Cortex: Lane 1=protein marker, Lane 2= Control group, Lane 3= Scop+ Tur treated group, Lane 4= Scop group, Lane 5= Scop + tur + Don treated group and Lane 6= Turmeric treated group.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Scop+Tur+Don (n:3)
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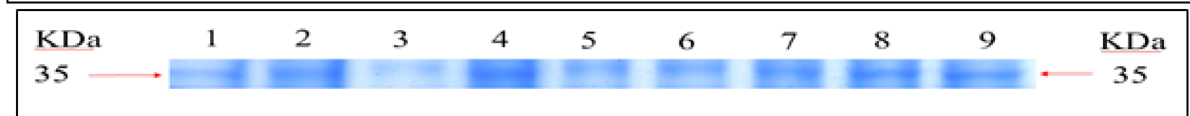
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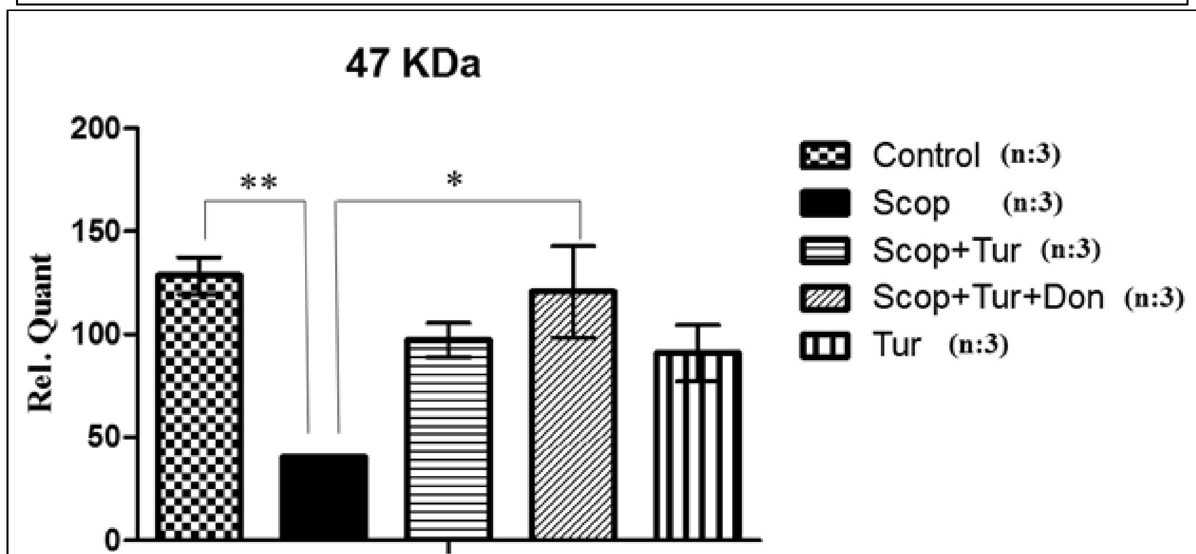
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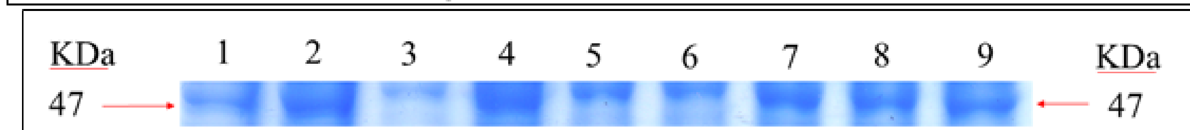
KDa
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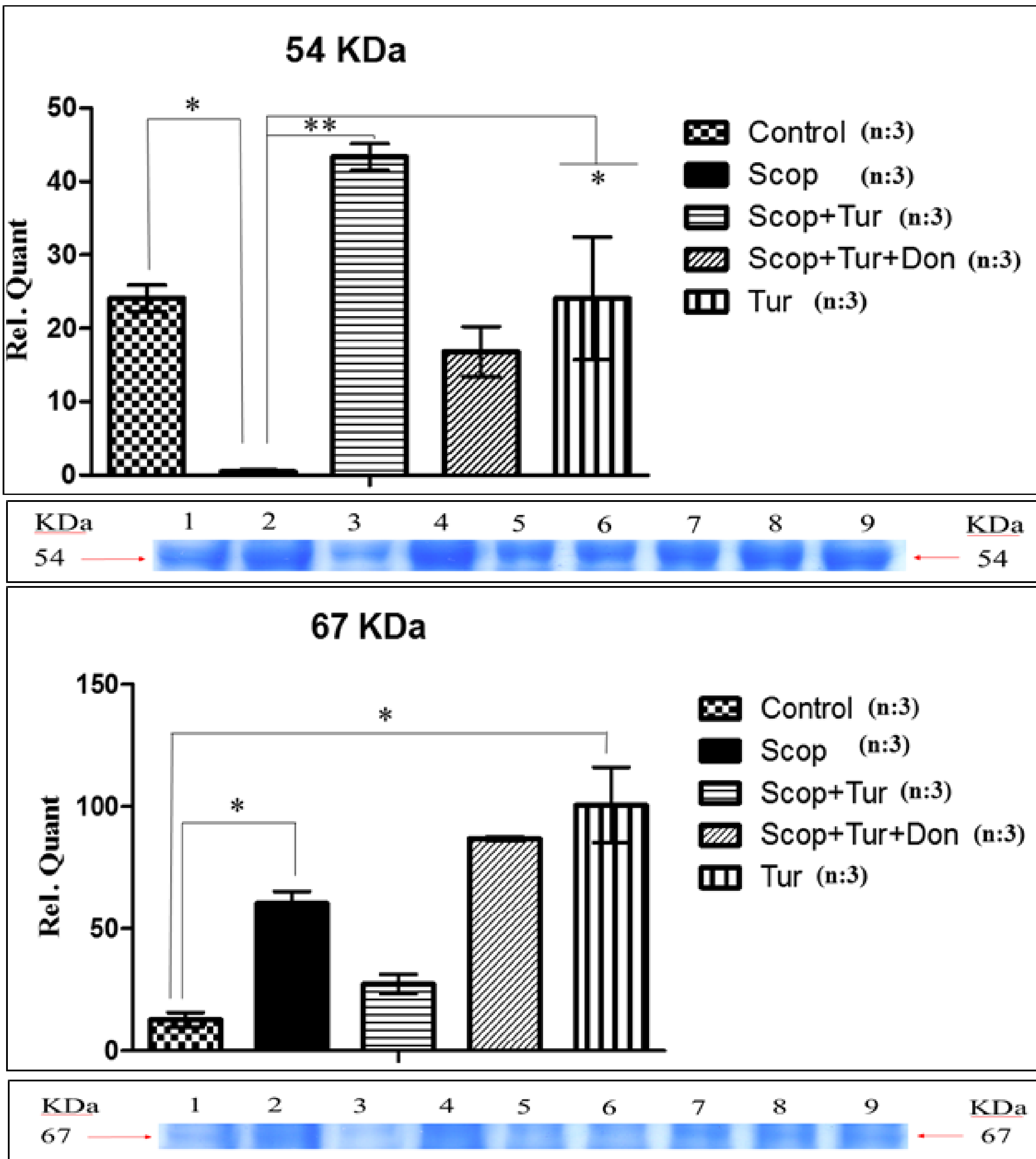
Figure 20: Expression levels of differentially expressed 35 KDa protein in the cortex. The extracted protein were separated on 10% SDS-PAGE and stained with coomassie brilliant blue. Image analysis was performed with Quantity One (Bio-Rad). Lane 1: Cortex Control, lane 2: scopolamine treated cortex, lane 3: Scopolamine + Turmeric treated cortex, lane 4: Scopolamine + Turmeric + Donepezil treated cortex, lane 5: Turmeric treated cortex and same sequence of group run in duplicate from lane 6 to 9 same sequence of groups. Expression levels of differentially expressed 47 KDa protein in the cortex. The extracted protein were separated on 10% SDS-PAGE and stained with coomassie brilliant blue. Image analysis was performed with Quantity One (Bio-Rad). Lane 1: Cortex Control, lane 2: scopolamine treated cortex, lane 3: Scopolamine + Turmeric treated cortex, lane 4: Scopolamine + Turmeric + Donepezil treated cortex, lane 5: Turmeric treated cortex and same sequence of group run in duplicate from lane 6 to 9 same sequence of group


54 KDa
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Control (n:3)
Scop (n:3)
Scop+Tur (n:3)
Scop+Tur+Don (n:3)
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KDa
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67 KDa
150
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50
67

Figure 21: Expression levels of differentially expressed 54 KDa protein in the cortex. The extracted protein were separated on 10% SDS-PAGE and stained with coomassie brilliant blue. Image analysis was performed with Quantity One (Bio-Rad). Lane 1: Cortex Control, lane 2: scopolamine treated cortex, lane 3: Scopolamine + Turmeric treated cortex, lane 4: Scopolamine + Turmeric + Donepezil treated cortex, lane 5: Turmeric treated cortex and same sequence of group run in duplicate from lane 6 to 9 same sequence of groups. Expression levels of differentially expressed 67 KDa protein in the cortex. The extracted protein were separated on 10% SDS-PAGE and stained with coomassie brilliant blue. Image analysis was performed with Quantity One (Bio-Rad). Lane 1: Cortex Control, lane 2: scopolamine treated cortex, lane 3: Scopolamine + Turmeric treated cortex, lane 4: Scopolamine + Turmeric + Donepezil treated cortex, lane 5: Turmeric treated cortex and same sequence of group run in duplicate from lane 6 to 9 same sequence of groups.

4.1.9. Light Microscopic Observations

Microscopic study of mouse brain region of cortex stained with Cresyl violet illustrated that there was neurodegeneration in Scopolamine-induced amnesia group as compared to the control. Turmeric significantly restored neurodegeneration in Scopolamine-induced amnesia group which showed that Turmeric and donepezil has neuroprotective effect. The quantitative analysis of slides showed the decrease in cell density of Scopolamine-induced amnesia group in comparison to the control group. After treatment with Turmeric and donepezil the cell density was significantly improved but the combination.

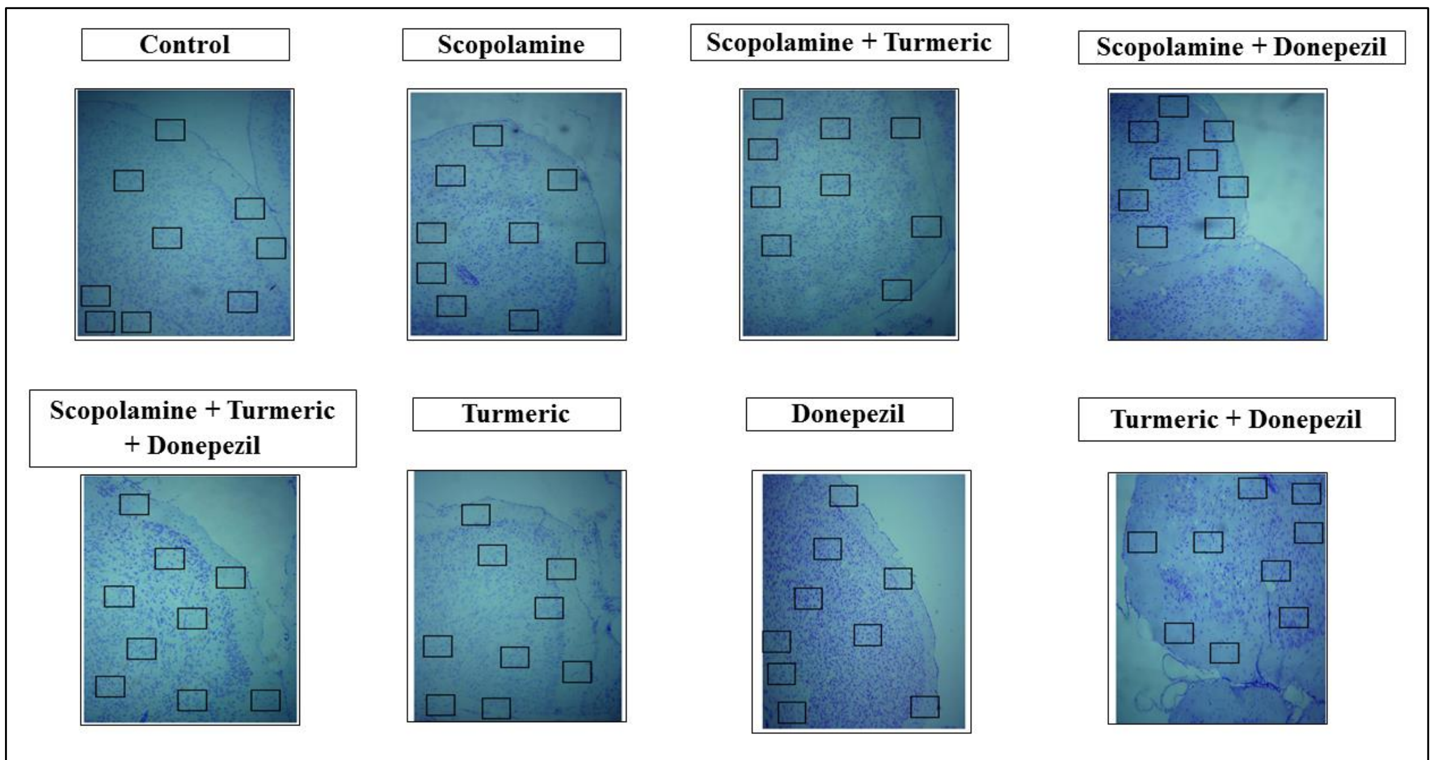

Figure 22: Microscopic examination of cortical sections of mouse brain stained with Cresyl violet.

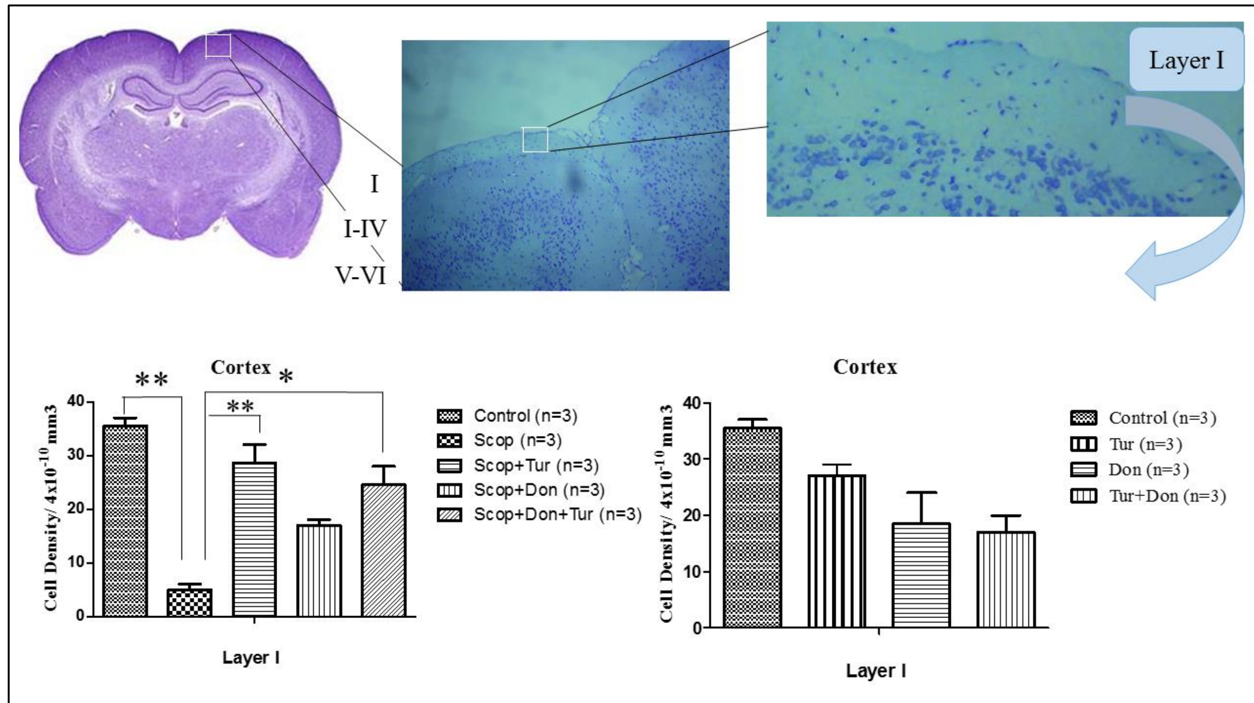

Figure 23: Microscopic examination of cortical sections of mouse brain stained with Cresyl violet. Graph representing the cell density in cortex area one in layer one. Comparison of total cell density in control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil, Donepezil alone and Turmeric only groups. Error bars represents mean SEM ± n=3. **=p < 0.01.

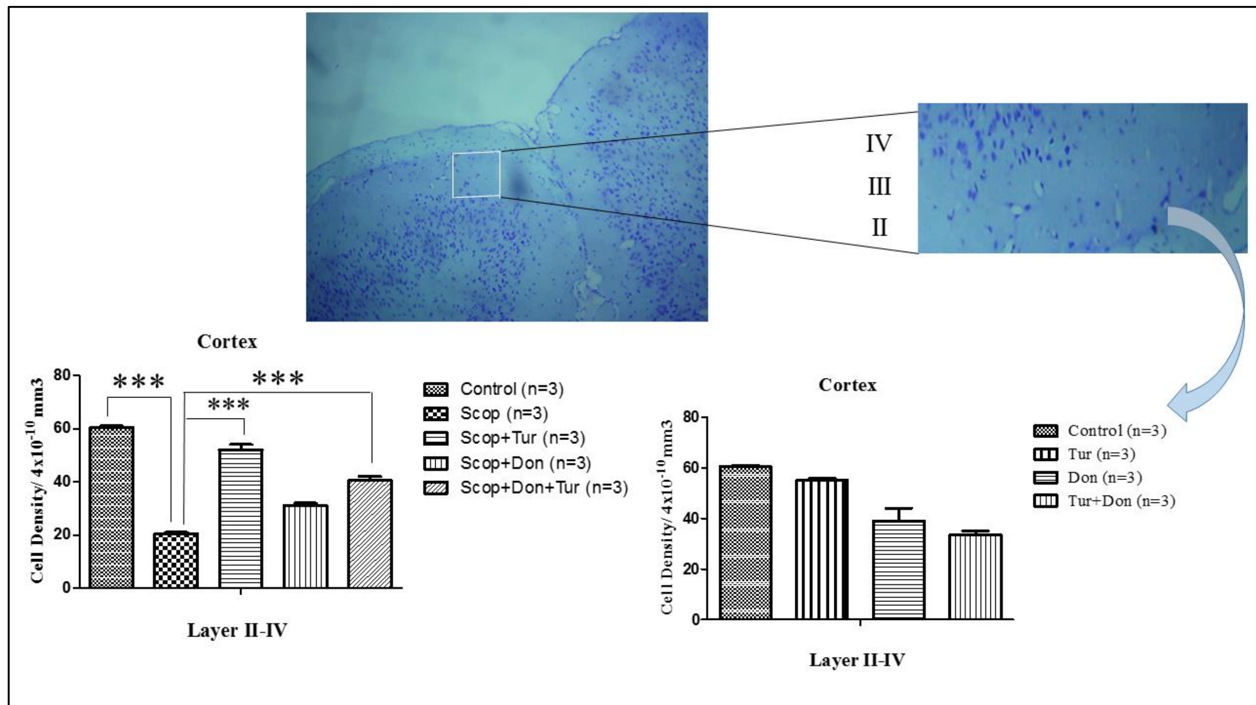

Figure 24: Microscopic examination of cortical sections of mouse brain stained with Cresyl violet. Graph representing the cell density in cortex area one in layer two to four. Comparison of total cell density in control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil, Donepezil alone and Turmeric only groups. Error bars represents mean SEM ± n=3. ***=$p < 0.001$.

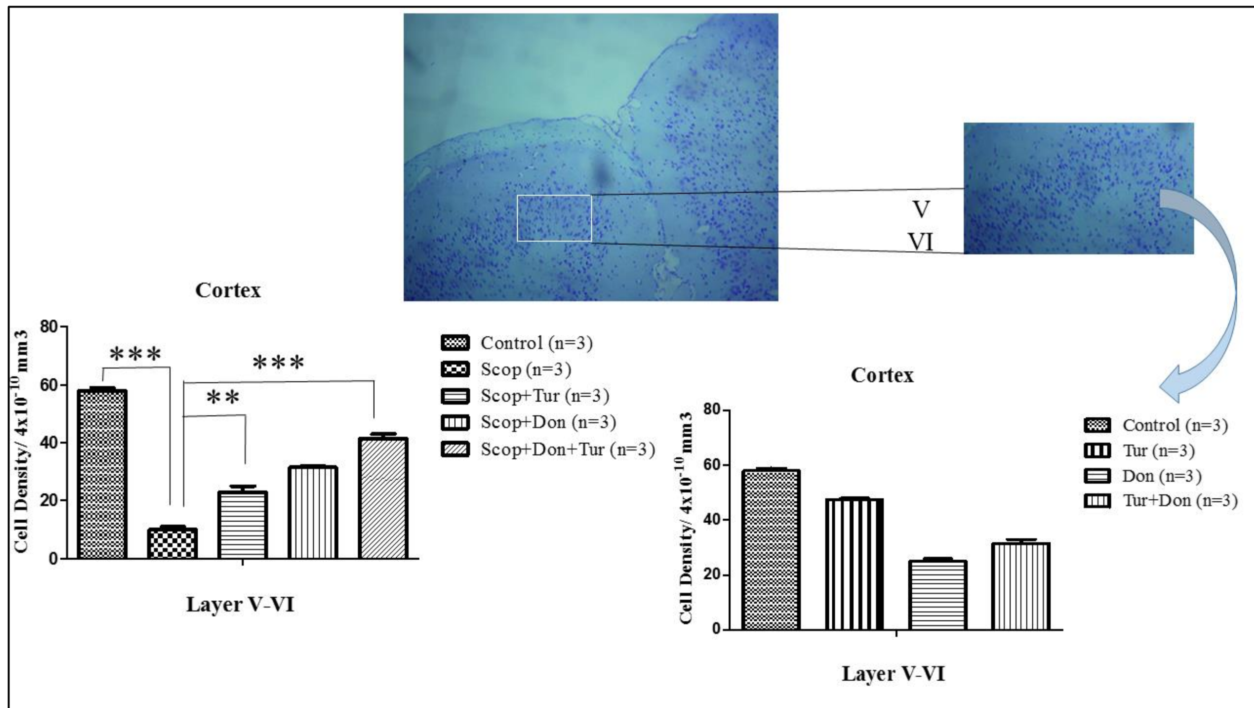

Figure 25: Microscopic examination of cortical sections of mouse brain stained with Cresyl violet. Graph representing the cell density in cortex area one in layer five and six. Comparison of total cell density in control, Scopolamine-treated, Turmeric-treated (Scopolamine + Turmeric), Donepezil-treated (Scopolamine + Donepezil,) Scopolamine + Turmeric + Donepezil, Donepezil alone and Turmeric only groups. Error bars represents mean SEM ± n=3. ***=p < 0.001.

4.2. Effect of Turmeric on memory consolidation and acquisition by Paired Association learning

Memory consolidation and acquisition behavior was assessed using paired association learning battery memory test, by test trial performed human subject with different patterns of figures on cards i.e; familiarization session (remember the patterns) and test session (recognize the position of pattern which pattern placed on which box) and PAL is a test of optical pattern and visuospatial memory and learning, which contains characteristics of straight paired associate's procedure and a conditional learning test. Consolidation memory was impaired in placebo group (4.50 ± 3.50) and it showed a significant ($p < 0.001$) reduction in association of patterns of paired association learning as compared to turmeric. Turmeric treated group (4.321 to 11.68) showed significant improvement in memory consolidation and acquisition memory battery test ($p < 0.001$) compared to placebo group (15.54 ± 1.50) in test session. The findings suggested the improvement in memory consolidation is improved by turmeric in aged people.

4.2.1. Reaction Time

The test provides assay for motor and mental response speed and can be analyze of movement time, reaction time, response accuracy and impulsivity, which consist aspects of responsive reaction time. Motor and mental response behavior was assessed using reaction time battery memory test, by test trial performed human subject with different patterns of figures on cards i.e; The subject were showed with 10 boxes patterned around the outside of the card, which were then visible to subject with 10 different colors, the test trial was divided in to five stages which requires increasable chains of response. Motor and mental response speed and can be analyze of movement time and the mental response was slow rate in placebo group (138.0 ± 62.0) and it showed a significant ($p < 0.001$) reduction in response to the identification of color in reaction time test as

compared to turmeric. Turmeric treated group (10.32 ± 1.68) showed significant improvement in mental and motor response in reaction time memory battery test ($p < 0.001$) compared to placebo group (138.0 ± 62.0) in test. The findings suggested the improvement in motor and mental response is improved by turmeric in aged people.

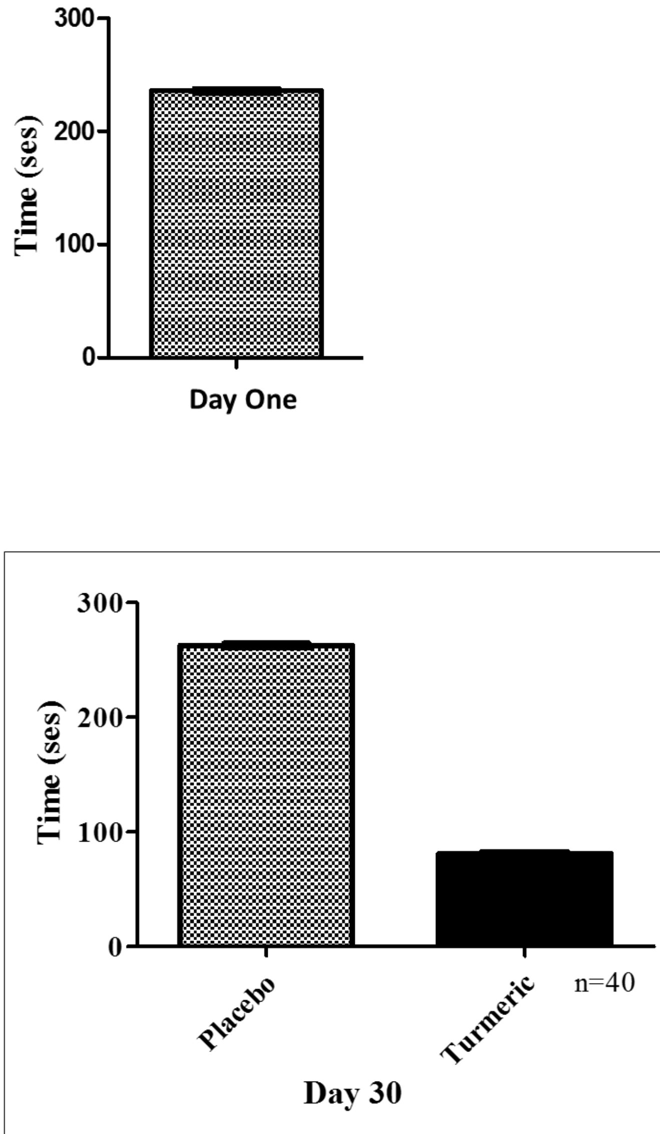

Figure 26: Effect of Turmeric on Reaction Time: The bar diagram shows the comparison of context based memory after conditioning between Placebo (Control), and Turmeric only groups. Error bars represents mean SEM ± n=40. ***=p < 0.001.

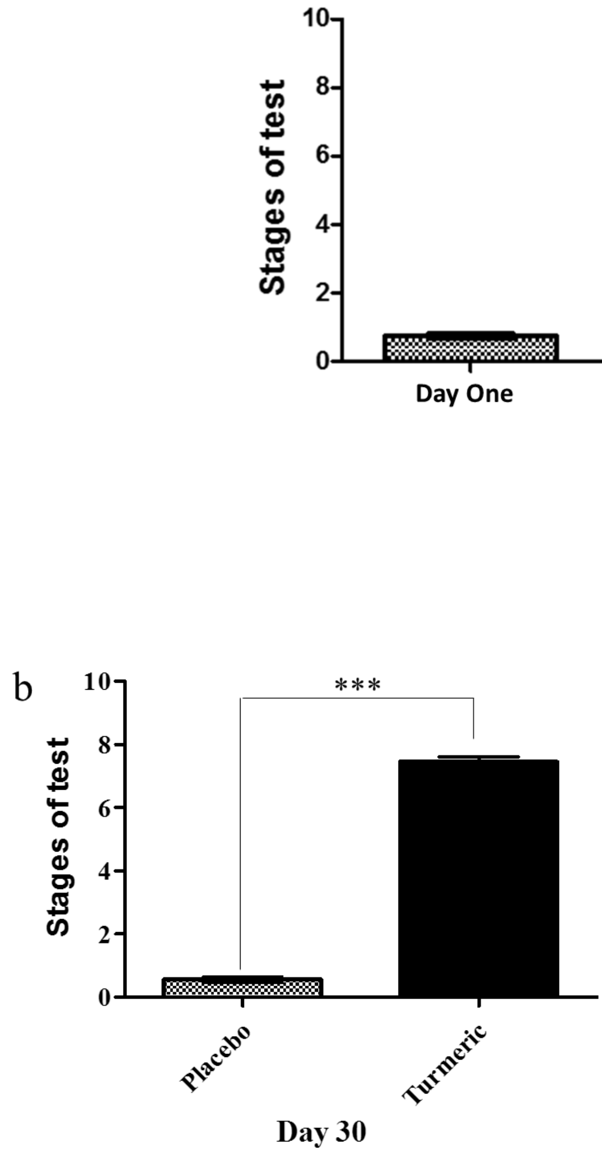

Figure 27: Effect of Turmeric PAL: The bar diagram shows the comparison of context based memory after conditioning between Placebo (Control), and Turmeric only groups. Error bars represents mean SEM ± n=40. ***=$p < 0.001$.

DISCUSSION

Since past two decades, evidence has gathered from both animal and human studies intending that central cholinergic systems are important mechanisms of the neural circuitry of learning, memory and cognition. Central cholinergic systems have been implicated in mediating learning and memory processes (Perry, 1986). Degeneration of basal forebrain (BF) cholinergic nuclei is associated with cognitive decline, and this effect is believed to be mediated by neuronal dysfunction in the denervated cortical area.

The cholinergic hypothesis suggested that a dysfunction of acetylcholine containing neurons in the brain provides substantially to the cognitive decline and it is observed in those with advanced age individuals and Alzheimer's disease (AD) patients (Terry and Buccafusco, 2003). Many studies, for example, have demonstrated that anti-muscarinic and anti-nicotinic agents such as atropine and scopolamine can disrupt both the acquisition and performance of a variety of learned behaviors (Aigner and Mishkin, 1986) (Hagan and Morris, 1988).

In this study, the scopolamine-induced amnesia model has been used as an experimental model to study the memory deficits observed in AD. AD is associated with degeneration and dysfunction of cholinergic neurons (Bartus et al., 1982). Since scopolamine-induced amnesia is due to blockage of the cholinergic neurotransmission, it provides a basis for the use of this drug to model the cognitive deficits that are observed in aging and dementia (Crow and Grove-White, 1973) (Drachman and Leavitt, 1974). In the present study, administration of scopolamine produced impairment of improvement and memory as reflected by the passive avoidance behavior of mice and in this study. Turmeric significantly improved CS-dependent fear conditioning, contextual and fear extinction memory deficits in Scopolamine-induced amnesia group. The change in behavior of animals in scopolamine-induced amnesia model associated with the

histological changes in cortex morphology and decreased cell density in cortex area one may depict brain antioxidant levels though other putative mechanisms may be involved. Treatment with turmeric with scopolamine induced mouse observed improvement in behavior as well as in cell density. In histological examination of cortex the cell density increase in turmeric treated group as compared to combination with donepezil. Loss of neuronal cells in the cortex area one region of the cortex which is seen in amnesia and was noted following scopolamine administration and it was improved in mice treated with turmeric and donepezil. The effects of scopolamine on brain antioxidant levels is in line with previous studies, which demonstrated that administration of scopolamine was associated with an alteration in the brain antioxidant status(Sharma et al., 2010) (El-Sherbiny et al., 2003).

Muscarinic receptors chiefly mediate the neuromodulatory functions of cholinergic system (Feng (Feng et al., 2004). Moreover, muscarinic receptors play significant role in memory circuits in diverse brain regions like cortex and hippocampus (Levey, 1996),(Jafari-Sabet, 2011). Previous studies have shown that muscarinic receptors M1, M3 and M5 play a key role in learning and memory (Seeger, 2004), (Araya et al., 2006), (Poulin et al., 2010), (Haley, 2011). Continuously, blockage of muscarinic receptors earlier to a learning event, for example using the unspecific antagonist scopolamine, constrain spatial memory. Turmeric also improved the expression of M1, M3 and M5 receptors in cortex. This result is consistent with the fact that muscarinic receptors play an important role in learning and development of new memories (Young et al., 1994) (Gale et al., 2001). Hence based on the potential therapeutic role of Turmeric in Scopolamine-induced amnesia, muscarinic receptors prove to be a valuable and novel target for Turmeric. The role of M1 receptor is essential in cortical memory formation (Anagnostaras et al., 2003).

Cortex help in learning and memory formation in Novel object recognition test (Daenen et al., 2002) (Balderas et al., 2008) (Antunes and Biala, 2012). Subsequent disturbance of learning and memory through muscarinic antagonists, Turmeric significantly enhanced recognition memory in mice. Improved expression of M1 in cortex through Turmeric additional supports the contribution of muscarinic receptors in cortex dependent learning and memory formation constant with the earlier reported studies (Daniel et al., 2005, Balderas et al., 2008), (Zheng et al., 2012), (Uslaner et al., 2013).

A consensus is developing on some probable functions of nAChRs in sensory neocortex. Cortical nAChRs expected act to enhance sensory responsiveness, which may contribute to improved behavioral performance in sensory–cognitive tasks. Nicotinic acetylcholine receptors (nAChRs) contribute to sensory– cognitive functions, as demonstrated in numerous behavioral studies (Warburton, 1992) (Turchi et al., 1995) (Rezvani et al., 2002). General, block of nicotinic receptors lead to spatial memory deficits

Impaired neurotransmission at muscarinic cholinergic synapses may contribute to the devastating loss of memory and other cognitive abilities in AD. Identification of a family of five mAChR genes encoding highly related receptor subtypes with markedly different cellular and synaptic distributions in brain, raises the exciting possibility that individual receptors may be targets for improved therapies. Presently used cholinergic compounds suffer from a lack of subtype-selectivity and potency, which favor negative peripheral side effects and may limit cognitive effects because of weak and your opposing actions in brain.

In general, the brain of rodents the expression of nAChRs is differently as α4 is dense, α7 is moderately expressed and β2 is scattered and dense in cortex. Most currently, selective nAChR subtype specific compounds have been applied. Because of their low bioavailability, direct

administration of antagonists against α4β2 heteromeric and α7 homomeric nACh receptors, correspondingly, into preselected brain regions was performed with the overall consequence of impaired spatial memory. These receptors have currently become the focus of interest as novel therapeutic targets in the treatment of neurodegenerative disorders with cognitive impairment. Stimulation of nAChRs by nicotine has been reported to improve cognition in both normal and dysfunctional animals, in healthy volunteers and patients with AD (Levin, 1992) (Newhouse et al., 1988) (Rusted et al., 1995).

According to previous studies, several separated chief compounds of Turmeric have exposed therapeutic potential in neurological diseases alike Alzheimer's disease that influence in learning and memory (Ahmed and Gilani, 2009), though, this study explicated the effect of raw Turmeric powder on learning and memory and cholinergic systems. Results have shown that Turmeric significantly improves the expression of muscarinic and nicotinic receptors M1, M3, M5 and β2, α4 and α7 that play an important role in memory formation. Furthermore, significant improvement in cortex dependent cognitive functions demonstrated that Turmeric has therapeutic potential and is an economical and instantly accessible therapeutic candidate for Alzheimer's disease and other disorders involving cholinergic disruptions affecting learning and memory.

In present study, it was observed that differential protein expression present in cortex region of mice brain. Four proteins were differentially expressed in the cortex (molecular weight: 35 KDa, 47 KDa, 54 KDa and 67 KDa). In the 35 KDa cortex protein, significant increase in the expression of scopolamine induced amnesia treated mouse cortex was observed as compared to the control. Treatment with Turmeric in the cortex had significant reduction in the expression of the 35 KDa protein as compared to scopolamine treated cortex, the reduction was significant as turmeric components combatted the effect of scopolamine, and however the expression of protein

was not restored completely when compared to the cortex control. A similar pattern of expression was observed in the 54 KDa protein in the cortex. Whereas In the 47 KDa cortex protein, significant decrease in the expression of scopolamine induced amnesia treated mouse cortex was observed as compared to the control. Treatment with Turmeric in the cortex had significant increase in the expression of the 47 KDa protein as compared to scopolamine treated cortex, the increase was significant as turmeric components combatted the effect of scopolamine, and however the expression of protein was not restored completely when compared to the cortex control. A similar pattern of expression was observed in the 67 KDa protein in the cortex. A possible reason for this could be the dose of turmeric used in this study which was 20mg/kg. Turmeric can be promising as it brought down expression of the 35 KDa and 54 KDa proteins as compared to the scopolamine treated to the control, turmeric can be potent enough to combat the damaging effect of scopolamine once the dose is optimized.

This study illustrated the differential expression of four cortical proteins. Moreover further characterization using advanced proteomic techniques such as Mass Spectrometry is essential to completely comprehend the role of these protein in causing cholinergic system and to enlighten the neuronal properties of turmeric in combating scopolamine induced amnesia. This study provides preliminary data which will be supportive in the identifying the biomarker and drug designing for therapeutic treatment of amnesia patients or other cholinergic deficit disorders such as Alzheimer’s disease.

The PAL test involves learning an relationship between visual stimuli and distinct spatial locations on a trial by trial basis, which has been demonstrated to decline with age in factor-analytic studies involving large samples (Rabbitt and Lowe, 2000) (Robbins et al., 1994). The turmeric used in the present study, qualitatively partly similar and partly distinct effects on

performance in tests measuring visual paired associates learning (PAL) and reaction time (RT). In the PAL test, Turmeric improved performance, because the subjects treated with 500 mg/daily for thirty days made fewer memory errors at the most difficult level of the test as compared to control group (placebo). While In the Reaction time test, turmeric treated, subject take less time as compared to placebo group of subject, because starch is used as placebo and it has been no effect on performance. Elderly subjects perform poorly on the tests of memory, as do subjects with history any trauma or more advanced aged individuals. Turmeric shows in this study improvement in the tests performance which indicate turmeric have therapeutic potential for the treatment of neurological disorder like AD.

CONCLUSION

One of the most major struggles of the last decade in research is to gain a better understanding on the mechanisms convoluted in cognitive function. The present investigation has illustrated that Turmeric has a significant therapeutic potential and has distinct effects on cortex correlated learning and memory. Furthermore substantial enhancement in M1, M3 and M5 receptor expression has indicated that Turmeric has the aptitude to control Acetylcholine neurotransmission. However lessen α7 and β2 receptor expression while improvement in α4 receptor expression has reflected that turmeric has the potential to control cholinergic both nAChRs and mAChRs expression and can be used in disorders with cholinergic hypo function and disturbance. . For years pharmacological research has been looking for novel routes and therapeutic approaches to address the problem of memory impairment. These modern findings aid the fact that herbal medicinal plants have pharmacological properties that need to be studied as potential lead compounds for drug designing.

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