INVESTIGATING CUMULATIVE EFFECT OF CURCUMIN AND VITAMIN D ON LEARNING AND MEMORY USING RESTRAINT STRESS MICE MODEL

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A thesis submitted to the National University of Sciences and Technology in the partial fulfillment of the requirements for the degree of

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Thesis Supervisor: Dr. Saira Justin

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DECLARATION

We certify that this research work titled **"Investigating Cumulative Effect of Curcumin and Vitamin D on Learning and Memory using Restraint Stress Mice Model"** is our own work. The work has not been presented elsewhere for assessment. The work herein was carried out while we were undergraduate students at Atta-ur-Rahman School of Applied Biosciences, NUST under the supervision of **Dr. Saira Justin.** The material that has been used from other sources has been properly acknowledged / referred.

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DEDICATION

"This research is dedicated to our beloved families; without their unconditional support and love we wouldn't be able to achieve our goal."

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AChase	Acetylcholinesterases
ACTH	Adrenocorticotrophic hormone
BBB	Blood-brain barrier
BDNF	Brain-derived neurotropic factor
cAMP	Cyclic adenine monophosphate
CA1	Cornu ammonis
CHRM3	Chromosome M3
CNS	Central nervous system
CRF	Corticotropin releasing factor
CRH	Corticotropin-releasing hormone
CUMS	Chronic unpredictable mild stress
DH	Dorsal hippocampus
ED50	Median effective dose
GH	Growth hormone
GnRH	Gonadotrophin-releasing hormone
HPA axis	Hypothalamus-pituitary-adrenal axis
IP	Intraperitoneal
mAChR	Muscarinic acetylcholine receptors
MAOI	Monoamine oxidase inhibitors
MWM	Morris water maze
NDRI	Norepinephrine-dopamine reuptake inhibitor
NGF	Nerve growth factor
NSRI	Norepinephrine-serotonin reuptake inhibitor
NTC	No template control

SSRI	Selective serotonin reuptake inhibitor
SEM	Standard Error Mean
RSC	Retrosplenial cortex
T3	Triiodothyronine
T4	Thyroxine
TCA	Tricyclic antidepressants
TSH	Thyroid stimulating hormone
VDR	Vitamin D receptor

ABSTRACT

Background: Chronic stress causes memory impairment. Curcumin, main secondary metabolite of *Curcuma longa*, has limited applications as a neuroprotectant due to its low bioavailability. Vitamin D, a fat-soluble secosteroid, has independently shown to improve cognitive functions.

***Objective*:** To examine the synergistic effect of curcumin and vitamin D on memory impairment caused by restrained stress.

***Methods*:** Restraint stress was induced in BALB/c mice. 30 mg/kg of curcumin and/or 1500 IU of vitamin D were administered orally. Learning and memory were evaluated by behavior tests; novel object recognition, and Morris water maze (MWM) test.

***Results*:** Percentage recognition index which is a measure of animal's ability to discriminate between a familiar object and novel object, was decreased in stress group (39.4±6.95) as compared to control (60.7±3.37). An improvement in exploratory behavior and recognition memory was seen in stressed mice following treatment with curcumin and/or vitamin D (stress + curcumin = stress + curcumin + vitamin D). For spatial memory, decreasing trend in escape latencies was observed for all, with stress group performing equally well, indicating that restrained stress might not cause a notable impairment of spatial memory. On the other hand, reference memory, assessed from probe trial was significantly impaired in stress group (10.33±1.33). Following treatment with curcumin alone (31.33±2.02) or in combination with vitamin D (29.33±5.2) a significant improvement was seen in stressed mice; however, a cumulative effect was not observed.

***Conclusion*:** Restraint stress model showed significant decline in cognitive functions. Treatment with curcumin alone and in combination with vitamin D counteracted stress and improved learning and memory, although a significant cumulative effect was not seen. Further studies with focus on the molecular aspects of learning and memory are needed.

1. INTRODUCTION

1.1. Stress

Stress has been a focus of study for many scientists due to its significant and notable impact on human behavior, mental health, and productivity. Studies have been conducted to further understand how stress impacts our health and how it can be alleviated.

Scientifically, stress can be described as "a state of threatened homeostasis, following exposure to extrinsic or intrinsic adverse forces (stressors) (1). Intrinsic stressors include social anxiety, and a physical, or psychological illness whereas extrinsic stressors may be academic or work deadlines, electric shocks, or extreme weather conditions.

Due to the common nature of stress, it's hard to put an exact number on how many people suffer from stress. However, there have been multiple studies conducted to evaluate stress in the general population, the workforce, students, etc. Since the start of the COVID-19 pandemic, stress has only worsened due to the loss of jobs, deaths due to the pandemic, fluctuating economic conditions, and uncertainty over the future. Mandatory lockdowns around the world further isolated people and caused the development of various symptoms of anxiety, insomnia, and stress (1).

Chronic stress (stress that persists for weeks or months) has been found to lead to long-term mental illnesses and impairment of cognitive functions. Elevated levels of glucocorticoids, the stress hormones, have a detrimental impact on learning and memory. These hormones have a profound effect on the regions of our brain responsible for memory, learning, and emotional regulation (2).

Symptoms of stress include but are not limited to headaches, exhaustion, and insomnia. Long-term effects of these symptoms include anxiety, depression, hypertension, and memory impairment (3,4).

The mechanism through which stress exerts an effect on the brain is known as the hypothalamus-pituitary-adrenal axis (HPA axis). Following are the steps through which stress causes the release of glucocorticoids (5);

1. Stress acts on the hypothalamic region of the brain and induces the production of corticotrophin-releasing hormone (CRH).
2. CRH acts on the pituitary gland to release adrenocorticotrophic hormone (ACTH).
3. ACTH acts on the adrenal glands, present on the kidneys, to prompt the release of cortisol, a glucocorticoid.
4. Cortisol further acts upon various organ systems such as the nervous, immune, cardiovascular, respiratory, reproductive, musculoskeletal, and integumentary systems. It can bind to the glucocorticoid receptor on these cells and affect gene transcription which leads to the development of stress symptoms.

1.2. Muscarinic Receptors

Learning can be defined as the attainment of new information/knowledge or processing the already known information, values or skills, and behavior or preferences (6). Information that is received, encoded and stored in the brain is known as memory which is a cognitive process (7). Learning is the acquisition of skill or knowledge, while memory is the expression of what has been acquired.

The part of the brain associated with learning, memory, and decision-making is the prefrontal cortex. It is the largest part of the cerebrum and occupies 29% of the cerebral cortex and takes a longer time to mature than other association cortices, and is responsible for, attention, perception, thought and awareness, cognition and consciousness, and language (8). Located at the front of the frontal lobe, the prefrontal cortex is thought to be an essential brain area for examining human intelligence and creativity (9).

Muscarinic receptors are expressed in the brain like other organs in the body. They are G-coupled protein receptors involved in the parasympathetic nervous system. Five distinct muscarinic receptor subtypes (M_1–M_5) are known to exist. In humans, they are encoded by chromosome M3 (CHRM3). They are named due to their sensitivity to muscarine, which is a compound found in

mushrooms (10). Acetylcholine activates the muscarinic receptors (11). They are widely distributed throughout the human body and mediate distinct physiological functions. For example, smooth muscles, endocrine, exocrine glands, lungs, pancreas, and the brain.

All five muscarinic receptor subtypes are expressed in the brain (12). The M_1, M_3, and M_5 subtypes are coupled to the G_q /G_{11} family of the G proteins, while M_2 and M_4 subtypes interact with the G_i / G_0 family (62). The M_2, M_3, and M_4 are present in the dorsal horn of the spinal cord (63). Other subtypes, M_1 receptors, for example, are most abundant in the neocortex, hippocampus, and M_5 receptors are localized to the projection neurons of the hippocampus. The M_2 receptors are mainly expressed by the smooth muscles in the gastrointestinal tract along with a small population of M_3 receptors. M_4 muscarinic receptors are involved in dopaminergic neurotransmission. Dopaminergic neurons in the prefrontal cortex regulate motor and cognitive function; hence M_4 muscarinic receptor along with M_3 is involved in working memory and cognitive functions. Consistent with this distribution, muscarinic M_1 receptors are implicated in learning and memory processes. Enhanced cholinergic receptor activation, either by the use of acetylcholinesterases (AChase) or muscarinic agonists, ameliorates cognitive decline (13). Central cholinergic signaling via muscarinic acetylene choline receptors (mAChR) has been implicated in learning and memory. However, the exact role of acetylcholine in these processes remains elusive (14). Central muscarinic receptors are involved in higher cognitive processes such as learning and memory. Recent studies have shown the relation of the M_3 receptor with learning and memory (15).

To date several bioactive compounds were checked for their ability to counter the decline in cognitive functions. Among such compounds, curcumin has been shown to be beneficial against age and stress related decline in cognitive functions (16)

1.3. Curcumin

Turmeric scientifically known as *Curcuma longa* belongs to a ginger family and is indigenous to South and Southeast Asia. Turmeric is attributed for its stimulant, aromatic and carminative properties in ancient Hindu manuscripts,

and it is considered as a household antidote for relieving pain, swellings and for treating sprains as a result of an injury (17). Turmeric is traditionally used in India and China in medicines and as a curry spice in foods is obtained from the rhizome of the plant (18). The majority of the medicinal properties of turmeric are due to the active compounds known as curcuminoids which further contain a mixture of curcumin (75–80%), demethoxycurcumin (15–20%), and bisdemethoxycurcumin (3–5%) (19).

Curcumin has been reported to have many beneficial properties including antioxidant, anti-inflammatory, chemotherapeutic and chemoprotective properties (18,20). In central nervous system (CNS) related disorders such as depression and aging-related disorders such as Alzheimer's and Parkinson's diseases, curcumin was found to show neuroprotective properties (21,16). Improved cognitive functions have also been reported to be associated with the consumption of curry in old age (22). Over the past years, the protective effects of curcumin have been explored against Subjective Memory Impairment (SMI) (20). SMI is commonly known by the terms like forgetfulness, difficulty in concentration and leaving out the objects, etc., which have been identified in adults (23) Curcumin was shown to ameliorate memory deterioration using possible mechanisms such as oxidative stress (24), acetylcholinesterase activity moderation, improvement of mitochondrial function (20,25), neurogenesis, neuroprotective effects, anti-depressive & anxiolytic effects along with the preservation and alteration of the plasticity of neural terminals in the brain (20).

Even though studies have confirmed that curcumin is well tolerated and safe for consumption and has low toxicity in humans (26). However, despite the promising pharmacological profile of curcumin, it has low bioavailability which is limiting its therapeutic potential (18). And that's where integrative medicine steps in. The lack of efficacy can be addressed with the help of complementary therapies.

1.4. Vitamin D

Calciferol (vitamin D) comprises a group of fat-soluble seco-sterols and are divided into major forms named Cholecalciferol (vitamin D3) and Ergocalcifero (vitamin D2). They are regarded as prohormones and have the

identical potency to cure vitamin D–deficiency (27). Both forms are considered biologically inactive until they undergo two enzymatic reactions to produce the final active hormone called calcitriol (1,25-dihydroxyvitamin D) (27). The major source of vitamin D is skin exposure to solar ultraviolet B radiation with the range of 290–315 nm. The dietary sources include food and dietary supplements such as fatty fish, liver and egg yolk (27,28,29).

Vitamin D has been reported to play an important role in different regions of the body. It helps the body absorb calcium for bone development, mineralization, and preservation preventing muscle disorders such as osteomalacia and rickets. It is important for regulating cell growth and can reduce the progression of cancer. It regulates the immune system by acting as an immunosuppressant and exhibits a vital role in fighting bacterial infections and viral infections. It lowers blood pressure through the regulation of the renin angiotensin system and has been reported to reduce the risk of type 2 diabetes (27,30).

Vitamin D has been reported to enhance cognitive performance, particularly memory and attention. Vitamin D, particularly vitamin D3, crosses the blood-brain barrier (BBB) and influences gene expression in different parts of the brain and neurotransmitters including dopamine, gamma butyric acid, acetylcholine and serotonin (31,32). Vitamin D also reduces age-related tau hyper phosphorylation, the formation of amyloid-beta oligomers and increases amyloid clearance in Alzheimer's. Vitamin D has been reported to stabilize the structure of myelin sheath along with enhancement of transcription factors contributing to the preservation of memory in the aging process (33).

Vitamin D deficiency can affect the brain through different mechanisms. Lack of vitamin D reduces the perineuronal nets in the hippocampus which act as a scaffolding for the brain. These scaffoldings stabilize important neurons and the connections between them are more easily degraded by enzymes when vitamin D levels drop. Emerging evidence also recommends that vitamin D deficiency may contribute to hippocampal volume loss and cognitive impairment leading to dementia (34,32). Lack of vitamin D has also been associated with depression.

Stress negatively impacts learning and memory and common treatment routes to alleviate stress incorporate allopathic medications (called “anxiolytics”) which pose risks of side effects such as sleepiness, fatigue, and impediment of mental functioning (35). To avoid these side effects, we are investigating the use of natural compounds such as curcumin and vitamin D in reducing stress and improving memory and learning. Our research aims to scrutinize the collective effect of curcumin and vitamin D to treat stress for ameliorating the negative effects of stress on learning and memory.

1.5. Research Objectives

Based on the reported literature, the objectives for the present work were set as follows:

1. To develop a physiological (stress) model of mice
2. To study stress-induced memory and learning impairment by behavior tests
3. To study the effect of curcumin and vitamin D; alone and in combination, on learning, memory and cognitive functions in stressed mice by behavior tests.

2. LITERATURE REVIEW

2.1. Stress

2.1.1. Definition

Stress is defined as "a state of threatened homeostasis, following exposure to extrinsic or intrinsic adverse forces (stressors) (36). Stressors can be divided into two categories: exteroceptive and interoceptive. Examples of the former include extreme weather conditions, noisy work environments, electric shocks, and socially stressful situations, etc. Examples of the latter include health problems like a painful illness or psychological problems such as phobias (3).

2.1.2. Prevalence

It's a prevalent condition across the globe, irrespective of socioeconomic status. According to a study conducted in 2020, 29.6% of the world's population was shown to suffer from stress (37). The same study showed that women were more vulnerable to stress and that stress was significantly more prevalent in 21 to 40 years old individuals. In another study conducted in 2021, 57.4% of individuals reported being stressed with 1.6% saying they were enduring "extremely severe stress" (38).

With the start of the COVID-19 pandemic, strict lockdowns were put in place to control its spread which led to an estimated people losing their source of employment (39). A study conducted during this period showed that confinement in quarantine can cause an individual to develop various symptoms of anxiety, insomnia, and stress, etc. (1). According to a meta-analysis conducted in 2020, the prevalence of stress due to the pandemic was 29.6% (40). A meta-analysis study conducted with participants from countries like China, Italy, Spain, etc., showed that the overall prevalence of stress varied from 8.1% to 81.9% (41). While in Pakistan, a study showed that 27% of individuals were suffering from stress (42) whereas another study reported that among 52 participants, 3.3% were dealing with mild to moderate stress (43).

2.1.3. Stress vs Anxiety vs Depression

Symptoms of stress can be confused with those of anxiety and depression, which are two different psychological conditions. According to the American Psychological Association, anxiety is "characterized by feelings of tension, worried thoughts, and physical changes like increased blood pressure" (44). Depression on the other hand is defined as "feelings of sadness and/or a loss of interest in activities (an individual) once enjoyed. It can lead to a variety of emotional and physical problems and can decrease (an individual's) ability to function at work and at home" (45).

Moreover, the difference lies in their nature and how they're presented in an individual. Stress occurs in response to one's perceived inability to cope with stressors. Anxiety involves the emotions of fear or panic as a response to stress. However, in anxiety, the fear/panic persists long after the stressor has ceased. Depression on the other hand is continual sadness that persists for a prolonged period of time (weeks or months) (46). In short, stress occurs only in the presence of a stressor, anxiety persists long after (or even before) the stressor has ceased to be, and depression occurs as a response to a traumatic event or hormonal imbalance. In this scenario, stress can lead to anxiety and anxiety can lead to the development of a depressive disorder.

2.1.4. Symptoms

Some common symptoms of stress include aches and muscle pain, headaches, digestive problems, chest pain, exhaustion, insomnia, nervousness, dry mouth, clenched jaw, and grinding teeth. Persistent stress can lead to long-term mental illnesses and impairment of cognitive functions (4).

2.1.5. Stress Hormones

Levels of various hormones change in response to stress (47).

1. Cortisol

Cortisol is classified as a glucocorticoid, and it's involved in regulating the body's stress response. It metabolizes amino acids, mobilizes free fatty acids, initiates gluconeogenesis, breaks down antibodies, impairs the immune system,

depletes beta cells, and decreases insulin production (48). Furthermore, it affects the sites in our brain responsible for memory, learning, and emotional regulation (2).

2. Catecholamines

These are the hormones produced by our adrenal glands (epinephrine and norepinephrine). Their effect on our sympathetic nervous system causes the release of adrenaline and noradrenaline (which induces our body to go into fight-or-flight mode) (61). Catecholamine production leads to the higher rate of cardiac output, skeletal muscle blood flow, sodium retention, and behavioral activation (47).

3. Vasopressin

Vasopressin is released from the hypothalamus' paraventricular nucleus along with corticotrophin-releasing hormone (CRH) under the conditions of acute stress. Adrenocorticotrophin-releasing hormone (ACTH)'s release from the pituitary gland occurs as a direct result of vasopressin's action on the vasopressin receptor (V1b receptor).

4. Gonadotropins

During stress, circulating gonadotropins and gonadal steroid hormones are suppressed which leads to disruption of the normal menstrual cycle. Continued exposure to such stress can cause impairment of reproductive function. Gonadotrophin releasing hormone (GnRH)'s transport to the pituitary gland is decreased as a result of increased endogenous CRH secretion.

5. Thyroid hormones

Stress causes the downregulation of triiodothyronine (T3) and thyroxine (T4) along with the inhibition of thyroid-stimulating hormone (TSH) due to glucocorticoids acting on the central nervous system.

6. Growth hormone

Acute physical stress can cause up to a two- to tenfold increase in growth hormone (GH) level. Since GH is antagonistic towards insulin, it may enhance metabolic activity. However, during psychological stress, GH responses are rare.

Instead, GH secretory defect may be noted along with prolonged psychosocial stress.

7. Insulin

Insulin decreases during stress. Antagonistic hormones' increase during stress leads to stress-induced hyperglycemia.

2.2. Hypothalamus-pituitary-adrenal axis (HPA axis)

Elevated levels of glucocorticosteroids due to chronic stress can have a detrimental impact on learning and memory and other cognitive functions and can lead to the development of various psychopathologies (5). The pathway utilized by these glucocorticosteroids to impact our body in stress conditions is called the hypothalamus-pituitary-adrenal axis (HPA axis) which is shown in the *Figure 1.*

Stress acts on the hypothalamic region of the brain to induce the production of CRH which subsequently acts on the pituitary gland and causes it to secrete ACTH. ACTH acts on the adrenal glands to prompt the production of cortisol which can act on the following organ systems due to the presence of glucocorticoid receptors; nervous, immune, cardiovascular, respiratory, reproductive, musculoskeletal, and integumentary. Cortisol acts as a primary messenger. Due to its hydrophilic properties, cortisol can cross the cytoplasmic membrane and enter the cell to bind to the glucocorticoid receptor. The cell's nucleus acts as the destination for this cortisol-receptor complex, thus affecting gene transcription. An example is the upregulation of the Period Circadian Regulator 1 (PER1) and Period Circadian Regulator 2 (PER2) genes, responsible for controlling the circadian rhythm (49). Cortisol induces neurobehavioral effects on attention, cognitive ability, memory, learning, impulsivity, and hyperactivity (50,51).

Glucocorticosteroid exerts its effect through rapid non-genomic or delayed genomic pathways. Non-genomic pathways which circumvent genetic transcription and so, ends up occurring at a quicker rate. Non-genomic pathways occur by trafficking nuclear receptors to the plasma membrane. Once there, these nuclear receptors can activate kinase pathways directly or indirectly.

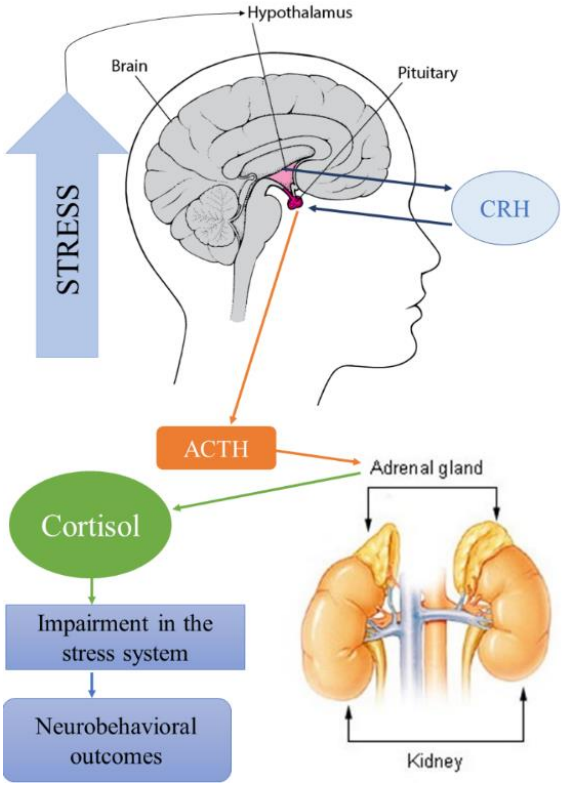

Figure 1: Hypothalamus-pituitary-adrenal (HPA) axis. The pathway begins with stress influencing the hypothalamus to release corticotrophin-releasing hormone (CRH) which acts on the pituitary gland to secrete adrenocorticotrophic hormone (ACTH), which consequently acts on the adrenal glands to produce cortisol. Cortisol is responsible for producing the stress response (5).

Non-genomic pathways possess the ability to regulate genomic pathways and vice versa. Early stress responses, including corticotropin-releasing factor (CRF), noradrenaline and rapid glucocorticoids' actions favor attentional processes and the encoding of relevant information. Delayed genomic glucocorticoid actions, however, would suppress neuronal activity and therefore reduce the processing of new information. This leads us to the assumption that stress enhances memory when it is experienced in the context and around the time of learning; stress out of the learning context would impair memory. To elaborate further on this, a study was conducted in which the participants learned words related or unrelated to the stressor shortly afterward psychosocial stress was induced. It was seen that stressed participants were able to recall more stressor-related words than their non-stressed peers. Other corroborating evidence is that rats' hippocampus' exhibit enhanced synaptic plasticity (important for memory formation) under the condition that repetitive stimulation

coincides with high corticosterone levels. Contrary to this, synaptic plasticity decreases when stimulation occurs before or after corticosterone administration (52).

2.2.1. Learning and Memory

Learning can be defined as the attainment of new information/knowledge or processing the already known information, values or skills, and behavior or preferences (6). It can be the result of a single event (e.g., having a near-drowning experience) but repeated experiences may result in the accumulation of much skill and knowledge. The changes resulting from learning are usually lifelong, but it's often difficult to discriminate apparently "lost" learned things from those which are not retrievable (2). Memory is the function of the brain, vital to experiences by which information is encoded, stored, and retrieves when required to influence future actions (54). It can be declarative (data storage and its retrieval from the conscious mind) or non-declarative i.e., data storage and its retrieval from the non-conscious mind (55,56). In short, learning is the acquisition of skill or knowledge, while memory is the expression of what has been acquired.

2.2.2. Pre-Frontal Cortex and its Role in Learning and Memory

The largest part of the cerebrum is the cerebral cortex in the mammalian brain, and it is most important in learning, memory, attention, perception, thought, awareness, cognition, consciousness, and language (56). The cerebral hemispheres contain the frontal lobe (precentral gyrus, prefrontal cortex, and orbitofrontal cortex). The prefrontal cortex is located at the front of the frontal lobe. The right prefrontal cortex is involved in spatial memory formation while the left prefrontal cortex is involved in verbal memory formation (57).

The prefrontal cortex is mainly responsible for complex cognition functions and the basic activity of this area is thought to be the orchestration of thoughts and actions according to internal goals (58, 60). The prefrontal cortex participates in a variety of higher cognitive functions, such as thinking, reasoning, planning, and decision-making. Therefore, it is thought to be an essential brain area for examining human intelligence and creativity. In humans,

the prefrontal cortex occupies 29% of the cerebral cortex and takes a longer time to mature than other association cortices, suggesting that it does not participate in basic sensory or motor information processing, but rather participates in more complex and highly integrated functions (59).

2.3. Muscarinic Receptors

Muscarinic receptors are G-coupled protein receptors involved in the parasympathetic nervous system. Evidence from molecular cloning indicates that there is separate intron-less human genes that encode five muscarinic receptor glycoproteins. Muscarinic receptor sequences have significant homologies with other members of this large super-family and the genes are very similar across mammalian species (60). In humans, they are encoded by chromosome M3 (CHRM3). They are named due to their sensitivity to muscarine, which is a compound found in mushrooms (10). Acetylcholine is responsible for the activation of the muscarinic receptors (11). Acetylcholine exerts its physiological actions via the activation of muscarinic acetylcholine receptors (mAChRs) (60).

2.3.1. Types of Muscarinic Receptors

The five subtypes of muscarinic receptors (M_1-M_5) share a high degree of sequence homology, but they also show pronounced differences in G-coupling preferences and physiological responses they mediate (61). Muscarinic receptors are widely distributed throughout the human body and mediate distinct physiological functions according to location and receptor subtype. For example, smooth muscles, endocrine, exocrine glands, lungs, pancreas, and the brain. Five distinct muscarinic receptor subtypes (M_1–M_5) are known to exist, although the exact location and functional role of all these subtypes have to date not been fully elucidated. In addition to the agonist-binding sites, muscarinic receptors possess allosteric sites at which compounds can modulate agonist activation (62). The nature of these allosteric sites differs both from the agonist-binding site and between subtypes, potentially allowing for the design of subtype-selective modulators (62).

2.3.2. Muscarinic Receptors in Brain

All five muscarinic receptor subtypes are expressed in the brain (12). M_1 receptors, for example, are most abundant in the neocortex, hippocampus, and neostriatum, whereas M_2 receptors are located throughout the brain. M_3 receptors (G_q) have been found in smooth muscles throughout the body specifically they are abundant in bronchi, gastrointestinal track, blood vessels and pupils. They are also found to be involved in vasodilation of blood vessels, constriction of smooth muscles, gallbladder, gastrointestinal track, bronchial constriction and pupil constriction. M_3 receptors use acetylcholine for the activation of M_3 receptors and are under sympathetic control (63). Studies have shown that the reduced expression of M_3 mAChR is responsible for weight loss, reduced adipose tissues, and reduced feed consumption in the mice at the start of their third postnatal week (64). In the CNS, muscarinic M_4 receptors are distributed in the corpus striatum, being co-localized with dopamine receptors on striatal projecting neurons (65,66). Levels of M_3 receptors are low, and localized in the hippocampus, whereas M_4 receptors are abundant in the neostriatum, and M_5 receptors have been localized to the projection neurons of substantia nigra, pars compacta, ventral tegmental area, and the hippocampus. Activation of spinal muscarinic receptors leads to potent anti-nociception (67,68) although the precise nature of the receptor subtype(s) mediating the response is unclear.

2.3.3. Role of Muscarinic Receptors in Learning and Memory

Muscarinic acetylcholine receptors play a significant role in the mediation of cognitive processes and previous studies have shown that the normal aging process and Alzheimer's disease are related to impaired muscarinic cholinergic neurotransmission (69). It has been found that mAChR is required for the retrieval and encoding of contextual memory in the retrosplenial cortex (RSC) and dorsal hippocampus (DH). Moreover, the cooperative activity of RSC M_1 and M_3 mAChR and hippocampal M_3 is required for the memory formation and the inactivation of multiple M_1-M_4 mAChR in DH or RSC is associated with the impairment of the memory retrieval than the inactivation of individual subtypes of muscarinic receptor (70). Researchers have shown that the various forms of learning and memory are mediated by M_3- mAChRs. There is evidence

for the involvement of both M_1- and M_3 subtypes in the mediation of learning and memory but whether they act synergistically or not is still unclear (71). M_3 receptors in particular are more abundant in the forebrain region and have distinct cellular localization, which suggests their various functions in the cholinergic circuits. They are involved in the modulation of memory-related synapses in the basal forebrain and hippocampus. The basal forebrain contains a group of cholinergic neurons, which provide innervation to the hippocampus. Previous studies on Alzheimer's disease have shown that synapse loss and dysfunction of cholinergic basal neurons cause memory impairment. Since acetylcholine activates all the muscarinic receptor subtypes including M_3, the depletion of acetylcholine transferase fails to activate these receptors in the hippocampus, leading to memory loss. Muscarinic M_1 receptors are also implicated in learning and memory processes. Enhanced cholinergic receptor activation, either by the use of acetylcholinesterases (AChase) or muscarinic agonists, ameliorates cognitive decline (13). Central cholinergic signaling via muscarinic acetylene choline receptors (mAChR) has been implicated in learning and memory. However, the exact role of acetylcholine in these processes remains elusive (14). The muscarinic M_5 receptor is the only muscarinic subtype expressed by the dopamine-containing neurons of the substantia nigra pars compacta, a structure that provides the principal dopamine innervation to the striatum. Activation of muscarinic M_5 receptors thus facilitates striatal dopamine release – although other muscarinic receptors, including the M_4 receptor, are involved (72). Central muscarinic receptors are involved in higher cognitive processes such as learning and memory. Recent studies have shown the relation of the M_3 receptor with learning and memory (15).

2.3.4. Available Antidepressant Treatments

Currently, several antidepressant drugs that are commonly used such as monoamine oxidase inhibitors (MAOI), norepinephrine-serotonin reuptake inhibitors (NSRI), tricyclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRI) and norepinephrine-dopamine reuptake inhibitor (NDRI) in clinical applications. However, currently used drugs have low remission rate that is 20-40% and provide inadequate treatment for depression (73). Additionally, currently used antidepressant drugs are associated with a number of side effects

such as dry mouth, constipation, sleeping trouble, neurotoxicity, cardiotoxicity, constipation, orthostatic hypotension and sexual dysfunction (73). Several new chemical agents derived from natural compounds have been tested both alone and along with the currently used antidepressants to augment their therapeutic efficacy to counter the inadequate efficacy of antidepressants, producing varied results (74). Due to less adverse effects along with their neuroprotective effects, herbal medicines are becoming an efficient pharmacological tool for the treatment of neurological disorders. Recently, curcumin's antidepressant effect has been recognized due to its efficacy in preventing the progression of depression-like behaviors in different animal models of depression (75). However, curcumin's neuroprotection activity is limited due to its poor bioavailability thus its combination with other bioactive compounds seems promising. Moreover, previous studies have shown that the combination of curcumin along with other compounds at lower and higher doses improved learning and memory (76). Hence for this study, a combined effect of curcumin and vitamin D was checked.

2.4. Curcumin

2.4.1. History

Turmeric has been used in the Vedic culture of India nearly 4000 years ago, it was conventionally used as a culinary spice and has religious importance. The use of turmeric became popular in China by 700 AD, in East Africa by 800 AD, in west Africa by 1200 AD, and in Jamaica by the eighteenth century (77). In Ayurveda, turmeric has been used to enhance memory when taken with honey and to treat snakebites when mixed with ghee (melted butter) (78). Turmeric is mainly cultivated in Pakistan, Peru, China and India and is a great source of bioactive compounds such as polyphenols, antioxidants and flavonoids and can be used as a substitute of antibiotics in food products and in food as well (79).

2.4.2. Classification

Turmeric scientifically known as *Curcuma longa* belongs to the herbaceous plant of the Ginger family (Zingiberaceae*) Table 1* is a perennial plant having underground rhizomes and is fairly tall (79,77,74). Roots and

rhizomes of the *Curcuma longa* are the primary sources of curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione) (17). The main rhizome with narrow and pointed ends is of 2.5-7.0 cm in length and 2.5 cm in diameter along with smaller tubers branching off. Upon drying the rhizome, pale yellow powder having a slightly acrid, bitter yet sweet taste can be obtained by grounding it (80).

Kingdom	*Plantae*
Subkingdom	Tracheobionts
Super division	Spermatophyta
Division	Mangoliophyta
Order	Zingiberales
Family	Zingiberaceae
Genus	Curcuma
Species	longa
Scientific name	*Curcuma longa*

Table 1: Scientific classification of Curcuma longa (79).

2.4.3. Constituents

Turmeric possesses both volatile and nonvolatile constituents, Turmerone zingiberene, ar-turmerone and curlone are the volatile constituents however curcuminoids are included in the nonvolatile constituents (79). The particular aroma of turmeric rhizome is due to aromatic volatile oil like turmerone (25%), curdione (11.58%) and ar-turmerone (8.5%) whereas phenolic compound like curcumin imparts a specific yellow color to the rhizomes of *Curcuma longa* (79). Phenolic compounds are powerful antioxidants that defend the human body against free radicals. The antioxidant activity of turmeric is attributed to the abundance of phenolic compounds such as curcuminoids (79). Other bioactive constituents such as desmethoxycurcumin, bisdemethoxycurcumin, and cyclocurcumin in addition to curcumin are also present in turmeric whereas at lower concentrations as shown in *Table 2*.

Altogether, these bioactive compounds known as curcuminoids constitute 2-4% of the turmeric powder (81).

Curcuminoids	***Concentration***
Bisdemethoxycurcumin	5%
Demethoxycurcumin	15%
Curcumin	80%

Table 2: Naturally occurring ratios of curcuminoids (81).

2.4.4. Chemical Properties

Curcumin, a beta-diketone with two of the hydrogens substituted with feruloyl groups is known as one of the major biologically active polyphenolic components present in turmeric (81,82) as shown in *Figure 2*.

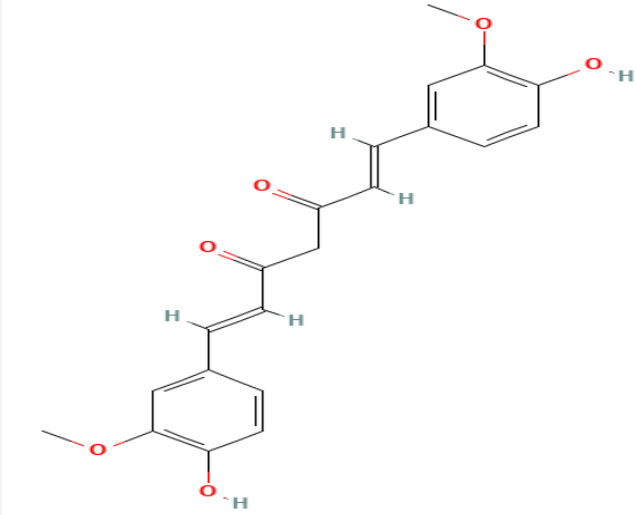

Figure 2: Chemical structure of curcumin (82).

The specific yellow color of curcumin is derived from the roots of the plant due to its bright yellow color it is often known as “Indian saffron”, and it is being widely used in Asia for a number of useful purposes such as; as a culinary spice, dye and as a conventional natural therapeutic compound (77,80). In 1815, curcumin was first isolated from the drug, however, its structure was elucidated in 1913. It is known to be soluble in acetone and ethanol but not in water (83).

2.4.5. Bioavailability

The dose of curcumin required to produce a therapeutic effect is 400-600 mg three times a day. In clinical trials of cancer patients, a dose of 4-8 g/day has been administered safely without any harmful effects (84). However, the oral doses needed to exert a significant anti-inflammatory response are significantly higher than the doses for intraperitoneal (i.p.) administration for producing a similar effect. A study has shown that for mice and rats the oral ED50 was 100.2 mg/kg and 48.0 mg/kg respectively (85).

Three major curcuminoids such as curcumin, demethoxycurcumin and bis-demethoxycurcumin present in the curcumin extract are responsible for producing desired pharmacological effects (74). Curcumin was shown to be beneficial for a number of diseases with chronic inflammation. Despite the high absorption of curcumin through lipid membranes due to its lipophilic nature, it has a low bioavailability after getting metabolized and accumulating in the liver, spleen and intestine without reaching to other organs. However, the main route of curcumin administration is oral (77). Absorption of curcumin is poor and despite getting absorbed into the body, it is excreted in feces after getting metabolized. Several drug delivery systems have been produced to counter the low bioavailability of curcumin which includes chitosan nanoparticles, polymer nanoparticles, colloidal nanoparticles, nano emulsion and ligand-targeted liposomes (86).

2.4.6. Therapeutic Applications

Curcumin possesses several beneficial properties such as antioxidant, anti-inflammatory, antineoplastic, neuroprotective and even anti-aging properties (77,87). The unique property of curcumin in rapidly emptying the gallbladder has been successfully used for the treatment of patients having subacute, recurrent or chronic cholecystitis (74). Moreover, curcumin has been used extensively in many preclinical trials against a range of human pathologies such as uveitis, rheumatoid arthritis, osteoarthritis, dyspepsia, several precancerous anomalies, inflammatory bowel disease, familial adenomatous polyposis, and pancreatic cancer (74).

2.4.7. Neuroprotective Role

Curcumin exerts its neuroprotective properties through the regulation of the signaling pathways of cognitive processes via inducing cyclic adenosine monophosphate (cAMP) response element-binding protein and then subsequent activation of brain-derived neurotropic factor (BDNF). The role of dietary curcumin in improving cognitive function and reducing the prevalence of dementia has also been investigated in epidemiological studies. Additionally, it has been reported that curcumin can enhance cognitive function in Alzheimer's disease by reducing amyloid deposition (87). Curcumin's ability in decreasing the secretion of corticosterone in the memory restoration process and in the reduction of oxidative stress in chronic and unpredictable stress seems to be its most expected impact (88).

2.5. Curcumin and Stress

In depression, neurotransmitters such as serotonin also known as 5-hydroxytryptamine (5-HT), dopamine, noradrenaline and glutamate have been observed to show disturbance in their activity. However, animal trial findings show that the activity of the neurotransmitters can be altered after curcumin administration; for instance, curcumin showed its stimulatory effect on the 5-HT1A receptor and reduced depression-like behavior in mice (89).

2.5.1. Curcumin's Potential Antidepressant Potential

Curcumin's ability to influence levels of serotonin, dopamine and noradrenaline in the central nervous system has been confirmed in both animals and in vitro studies. For instance, curcumin lowered serum corticosterone levels and reduced chronic unpredictable mild stress (CUMS) induced serotonin reductions in CUMS model with rats. However, curcumin's antidepressant potential is limited potential due to its poor absorption in the body which can be countered using the integrative medicine approach. Hence, to counter its poor bioavailability, vitamin D with neuroprotection ability seems a promising candidate to produce a synergistic effect for ameliorating the decline in cognitive functions (90).

2.6. Vitamin D

Calciferol (vitamin D) comprises a group of fat-soluble seco-sterols. Cholecalciferol (vitamin D3) and Ergocalcifero (vitamin D2) are the two main forms that are different only through their side-chain structure. They are regarded as prohormones and display the same responses in the body. They also have the identical potency to cure vitamin D–deficiency. However pre-clinical studies have displayed vitamin D2 as less toxic when compared to vitamin D3, but this has not been shown at the clinical level (27).

2.6.1. Activation

Either form is considered biologically inactive until it undergoes two enzymatic reactions. The first takes place in the liver where 25-hydroxylase produces 25-hydroxyvitamin D. The second reaction takes place in the kidney, where 1α-hydroxylase catalyzes the final reaction to produce an active hormone called calcitriol (1,25-dihydroxyvitamin D) (27).

2.6.2. Sources

The major source of vitamin D is skin exposure to solar ultraviolet B radiation with the range of 290–315 nm. The dietary sources include food and dietary supplements such as fatty fish, liver and egg yolk. Vitamin D is present in animal foods as vitamin D3. Vitamin D2 has been found in some wild mushrooms where a provitamin called ergosterol is converted into Vitamin D2 (27),(28),(29).

2.6.3. Functions of Vitamin D

Vitamin D has been reported to contribute to different regions of the body:

1. It stimulates the intestinal absorption of calcium and maintains blood levels of calcium and phosphorous along with bone development, mineralization, and preservation.
2. Its deficiency has been linked with muscle disorders such as osteomalacia and rickets.
3. It is involved in cell cycle regulation thus linked with cancer reduction.

4. It regulates the immune system by acting as an immunosuppressant and decreases the severity of autoimmune diseases.
5. It exhibits a vital role in fighting bacterial infections including tuberculosis, and viral infections, such as influenza.
6. It has cardiovascular protective effects based on lowering blood pressure through regulation of renin-angiotensin system.
7. It has been reported to reduce the risk of type 2 diabetes according to multiple observational studies by playing a role in insulin release.
8. An inverse relationship between depression and vitamin D deficiency has been also reported (27,91,30).

2.7. Role of Vitamin D in Neuroprotection

Vitamin D has been reported to enhance cognitive performance, particularly memory and attention (34,31). Vitamin D particularly vitamin D3 crosses the blood-brain barrier (BBB) and binds to the vitamin D receptor (VDR) in different parts of the brain including glial cells of the hippocampus, orbitofrontal-cortex, thalamus, cingulate and amygdala to influence gene expression. It promotes brain health through its neuroprotective, anti-inflammatory, and antioxidant effects on neurons. Vitamin D enhances the survival of both hippocampal and cortical neurons by producing neurotrophic factors like nerve growth factor (NGF). Vitamin D has also been reported to regulate neurotransmitters gene expression including dopamine, gamma butyric acid, acetylcholine and serotonin. Vitamin D reduces age-related tau hyper phosphorylation, the formation of amyloid-beta oligomers, increases amyloid clearance, and prevents neuronal death through activation of macrophages. Vitamin D has also shown its neuroprotective activity by upregulating the genetic expression of proteins that are needed for the formation of new synapses leading to the promotion of neurogenesis particularly in the hippocampus (32,31). The hippocampus plays a key role in spatial and temporal memory information and is associated with cognitive functioning. Emerging evidence recommends that vitamin D deficiency may contribute to hippocampal volume loss and cognitive impairment leading to dementia (34,32). Another study reports Vitamin D stabilizes the structure of myelin sheath along with

enhancement of transcription factors which facilitate cognition contributing to the preservation of memory in the aging process (33). Thus, vitamin D seems promising for improving the learning and memory of stressed mice in combination with curcumin

3. MATERIALS AND METHODS

3.1. Ethical Statement

All the protocols performed were approved by the Internal Review Board (IRB), Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology. All the animals were treated and experimented upon according to the guidelines and rules of the Institute of Laboratory Animal Research, Division of Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011).

3.2. Animals

21 male/female BALB/c mice (National Institute of Health, Islamabad) were used in the study and divided randomly into groups. All the animals fall in the range of 4-6 weeks of age and average weight of 34 g.

Animals were kept in a constant environment in the Lab Animal House of Atta-ur-Rahman School of Applied Biosciences, National University of Sciences and Technology. The lab room in which the mice were kept had a controlled temperature of 22 ± 2°C under a natural 14:10 hours light and dark cycle. Around 3-4 mice were kept in a single cage of 40 cm x 25 cm x 15 cm dimensions. The cages were bedded with wood shavings. Animals were fed with food and water (500 ml) ad libitum.

3.3. Drugs and Chemicals

Curcumin (C1386-25G) was obtained from SIGMA and the mice were administered a dose of 30mg/kg. Vitamin D3 (cholecalciferol) was obtained from “Caltig-D oral drops” by Dolphin Laboratories and was provided at a dose of 1500 IU/kg.

3.3.1. List of Chemicals & Reagents

1. Ambion™ Nuclease-free water (LOT: 2005355)
2. Invitrogen® TRIzol reagent
3. ThermoFisher Scientific® cDNA synthesis kit

4. Sigma Aldrich® ethanol
5. Sigma Aldrich® 2-propanol
6. Sigma Aldrich® chloroform

3.3.2. List of Plastic-and Glassware

1. Sritang® latex powdered examination gloves
2. Ambion™ RNase-free, thin-walled frosted lid 0.2 mL PCR tubes (Lot number: 1209113)
3. Porlab® EstaSET pipette tips for Gilson micropipettes (yellow) (Lot number: A04020-204)
4. Tarsons® Microtips graduated (Lot number: JT-210817)
5. Normax® 500 mL reagent bottle
6. Simax® 250 mL reagent bottle
7. NEST® 45 mL falcon tubes
8. ImuMed® blood collection tubes (Lot number: 20170710)
9. Kartell® 100 mL graduated cylinder
10. Borosil® 50 mL beaker
11. Gocl® 500 mL glass flask

3.3.3. List of Equipment

1. Shimadzu® weighing machine
2. Berthold Technologies® microvolume spectrometer
3. Veriti® 96-well thermal cycler
4. VELP® scientific vortex
5. Wealtec® e-centrifuge
6. Wealtec® UV transilluminator
7. Labnet® power station
8. Kentax® microwave oven
9. Cleaver scientific limited gel doc
10. Nichiryo® Nichipet EXII micropipettes (2 µL, 20 µL, 100 µL, 200 µL, and 1000 µL)
11. Hielscher® ultra-sonicator
12. Hermle® Refrigerated centrifuge

3.3.4. List of Software

1. Graph Pad Prism 9

3.4. Study Design

Our 30-day study design included stress induction and curcumin and vitamin D administration to check its effect on stressed mice with 6 days of behavior to assess mice's performance in learning and memory tests. After the completion of these tests, the mice were sacrificed to extract RNA from their cerebral cortex. After cDNA synthesis, gene expression studies are intended to be carried out as shown in *Figure 3.*

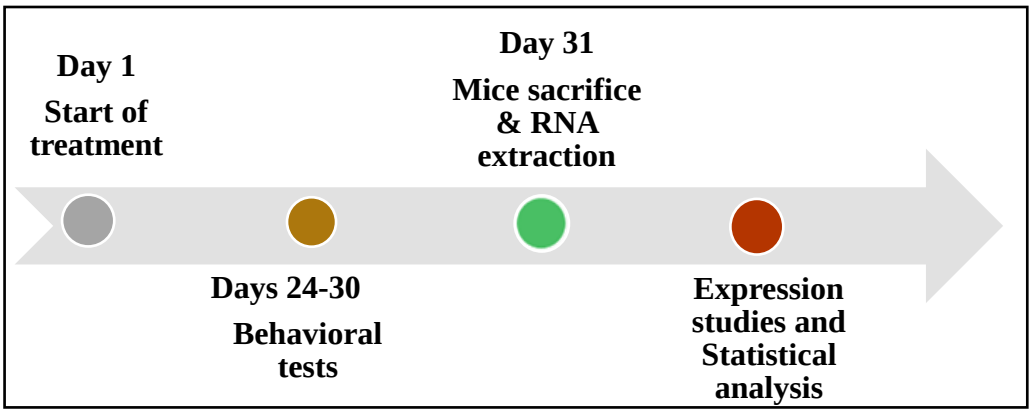

Figure 3: Diagrammatic representation of study design. Treatment was carried out for 30 days, with 6 days of behavior tests. Sacrifice was carried out on the 31st day.

For the study, animals were divided into seven groups comprising three mice each. All animals were healthy and aged between 4-6 weeks. The details of the groups are as follows.

i. **Control group:** Normal feed was given to the control group. The feed amount was approximately 10 g per animal per day.

ii. **Stress group:** Normal feed was given to the stress group i.e., 10 g per animal per day. The animals were restrained using a ventilated 50 ml falcon tube each day to induce stress.

iii. **Curcumin group:** The feed of this group included 30 mg/kg of curcumin mixed with the normal feed.

iv. **Vitamin D group**: 1500 IU/kg dose of vitamin D was used for preparing the feed of this experimental group.

v. **Curcumin + vitamin D group:** 30 mg/kg of curcumin and 1500 IU/kg of vitamin D dose were given to this experimental group.

vi. **Stress + curcumin group:** The animal feed consisted of curcumin mixed with normal feed. 30 mg/kg of curcumin was given to animals along with the restraint stress.

vii. **Stress + curcumin + vitamin D group:** For this experimental group 30 mg/kg of curcumin and 1500 IU/kg of vitamin D dose was given along with the restraint stress.

3.6. Restraint Stress Model

Stress was induced in animals using ventilated 50 ml falcon tubes. Each day, the animals in the stress group i.e. (stress, stress + curcumin, and stress + curcumin + vitamin D) were restrained in the falcon tubes for four hours.

3.7. Treatment of Experimental Mice

The animals were treated and handled carefully. A quiet environment was ensured to prevent anxiety. The traditional tail handling method was used for mice. Gloves were worn every time the animals were handled.

3.8. Behavior Tests

Behavior tests were performed during the day between 9 am and 6 pm in order not to disturb the normal circadian cycle of the mice. Mice were habituated in the behavior room before conducting the tests. The habituation period was 30 minutes, after which the experiments were started. The camera was fixed on the tripod stand for the recording of the behavior tests. The temperature of the behavior room was between 24-25℃. During habituation and conduct of the experiments, human interference was kept to a minimum level.

3.8.1. Novel Object Recognition Test

Novel object recognition test was performed to assess retention memory in mice. This behavior test is a commonly used behavior assay to analyze various aspects of learning and memory in mice. Mice have a natural proclivity to explore novelty. They have an innate preference for novelty and hence, a mouse

that remembers a familiar object that it has explored earlier will spend more time exploring the novel object.

A novel object recognition test was started on the 26th day of the treatment. The apparatus consisted of a square box, in which 2 objects were taped for the mice to explore. This test was completed in 3 phases i.e., habituation, training and testing. A gap of 20 minutes was given among the last two phases. The duration of the habituation phase was 5 minutes while the rest of the phases were of 10 minutes duration. During habituation, the mice were familiarized with the field in which they were to explore the objects. For this purpose, each mouse was put into the empty box for 5 minutes. In the second phase, i.e. the training phase or first trial, 2 objects were placed on either end of the box at the diagonals and the mouse was put into it to explore them. In the test trial, one of the objects was replaced with a novel object and the mouse was put into the box again after 10 minutes to explore it. The retention memory of the mice was evaluated by the extent to which they remembered the familiar object during the second trial. If the mouse spent more time exploring the novel object, it meant that it had retained the memory of the object it explored earlier. In the other case, in which the mouse spent the same amount of time exploring both objects during the second trial, it meant that the mouse did not retain the memory of the familiar object.

3.8.2. Morris Water Maze Test

Morris water maze test was performed to observe the spatial learning and memory of the mice. The test is based on observing the ability of the mice to rely on cues and navigate to an escape platform submerged in a swimming arena from any start location shown in *Table 3*. Repeated trials help assess the spatial memory, while reference memory is determined during the probe trials by the preference of the animal for the platform area, when the platform is absent.

The apparatus consisted of a round steel tank with 122 cm diameter and 76 cm height. The tank was filled with water with a depth of 38 cm and divided into 4 hypothetical quadrants. The water was made opaque by using blue dye. The escape platform was submerged 2 cm below the water and had a 12 cm diameter and 36 cm height. It was placed in the northwest quadrant for the mice

to escape from the water. The walls of the tank had spatial cues for the mice to navigate their way out of the water onto the escape platform from the release location.

The behavior test was started on the 25th day of the treatment. A total of 5 trials were performed each day for 5 days for each mouse. The maximum limit of the trial was set at 90 seconds and mice were given rest for 10 minutes after each trial. The mice which found the platform within 90 seconds were allowed to stay on it for 5 seconds before placing them back in their cages. While those which did not find the platform in the stipulated cut-off limit were guided to the platform and allowed to stay there for 20 seconds. Escape latency was recorded for each trial and the average of the 5 trials was taken. The position of the platform was not altered throughout the trials. For each trial, the starting location was different. There was no repetition in start locations in any two consecutive trials, and the order of the locations in trials was also different. The lighting and water temperature were kept the same throughout the 6-day trials. On day 6, a probe trial was performed with the platform removed. The start location was kept the same for all mice. In the probe trials, the reference memory of the mice was determined by calculating the time they spent in the platform quadrant.

Days	***Direction of Release***				
	Trial 1	**Trial 2**	**Trial 3**	**Trial 4**	**Trial 5**
Day 1	West	South	North	East	South
Day 2	North	West	East	West	South
Day 3	North	East	West	South	North
Day 4	East	South	West	East	North
Day 5	West	South	North	East	South
Day 6*	Single Trial without Platform, Direction of Release: **WEST**				

Table 3: Directions of release for Morris Water Maze Test (MWMT) probe trial performed on 6th day with the platform removed. Time spent in the platform quadrant (previously learned) was calculated.

3.9. Mouse Dissection

Following were the step used for mice dissection and excision of brain tissues:

1. Three Eppendorf tubes along with a blood collection tube were labeled with the particular group and tag for a mouse.

2. Two falcon tubes containing chilled Phosphate Buffer Saline (PBS) solution and chloroform each along with a petri dish were placed in a filled ice bucket for steps following the dissection.

3. The bench surface along with instruments from the dissection kit was cleansed with 70% ethanol prior to dissection and allowed to dry on tissue papers.

4. Cling wrap was used to cover the dissection tray whereas ice packet was covered using aluminum foil and a biohazard waste bag was secured near the work bench.

5. Cotton was soaked with chloroform using thumb forceps and kept in a beaker to anesthetize the mouse.

6. This was followed by putting a mouse in the beaker containing cotton soaked with chloroform for 20 to 30 seconds. The beaker was covered with a lid until the mouse fainted out through asphyxiation by CO_2.

7. The mouse was then laid down ventrally on a dissection tray and a grip was maintained over the tail using the right hand. Plain forceps were used to hold the mouse skin making an incision with straight scissors. The curved scissors were further used to cut the skin in the middle position until it reached the rib cage. This was followed by cutting the underlying muscular layer beneath the skin in the middle position to expose the organs as shown in *Figure 4.*

8. The curved scissors were then used to cut the ribcage from its edges to expose the heart.

9. Then a 20 ml syringe was used at 45° to take the blood out of the heart. The blood was immediately put into a collection tube followed by its storage in ice box until the completion of dissection.

10. Afterward, a smooth end of plain scissors was placed behind the skull which was held down firmly with the right hand. The tail was rapidly pulled out with the left hand until the mouse skull was dislocated from the spinal cord.

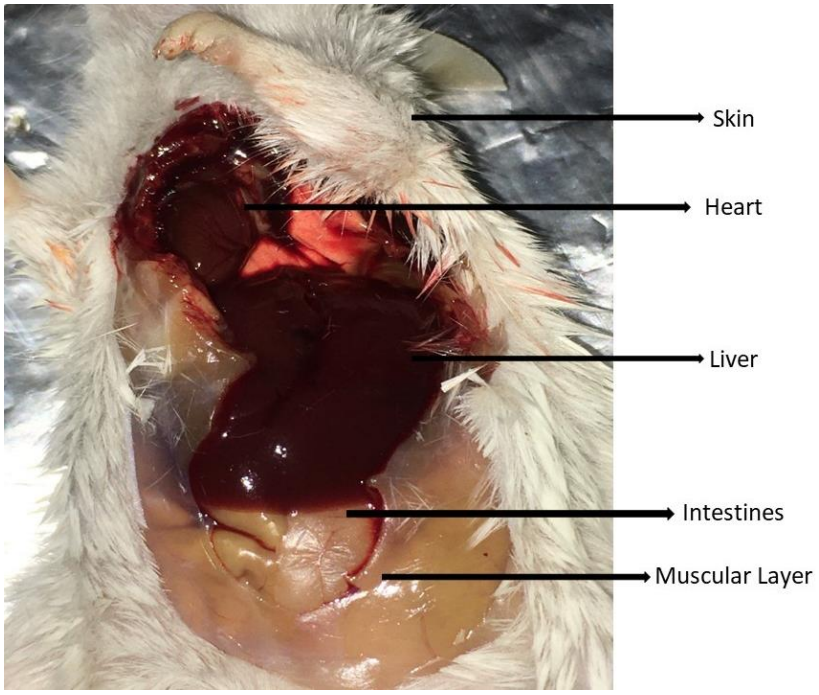

Figure 4: Organs of the mouse exposed after a precise cut in the muscular layer beneath the skin.

11. After cervical dislocation, decapitation was performed with the plain scissors just behind the skull to excise the brain.

12. This was followed by cutting the scalp skin from between the rodent's eyes down the midline using a razor blade while pulling the scalp to the lateral sides gently.

13. Afterward, the skull was cut laterally by placing one tip of the fine scissors into the foramen magnum. This was repeated for the other side. Then a gentle cut was made from the same cavity up the midline towards the nose making sure to keep the ends of the scissors as superficial as possible so as not to perturb the brain.

14. Then small cuts were made from the midline incision near the eyes laterally. This was followed by making small gentle cuts on the left and right sides of the cavity up the line towards the ear lobes.

15. Tooth forceps were used to apply gentle tangential, lateral pressure to either of the newly formed skull flaps. This was repeated for the

remaining side. Skull was fully removed when force was applied properly exposing the brain. However, when a piece of skull broke off before the respective hemisphere was exposed, it was discarded, and forceps were again used to coerce the remaining flap to the side.

16. A spatula was then used to gently transfer the brain to the petri dish containing 2 ml of chilled PBS solution to cleanse the brain tissue.

17. The tissue was later transferred to an ice packet covered with aluminum foil using a spoon and clean razor blade was used to remove and discard the cerebellum. The dorsal part was hemisected to separate the prefrontal cortex into the right and left parts followed by its transfer to respected labeled Eppendorf tubes. This was followed by the removal of Hippocampus (92).

18. Samples were then stored in ice box for RNA extraction.

3.10. RNA Extraction

RNA was extracted using right cortical portion of the mouse brain, 50 mg of the brain tissue was taken and added in 1 ml Invitrogen™ TRIzol™ Reagent according to the manufacturer's instructions. TRIzol Reagent maintains the integrity of the RNA through effective inhibition of RNase activity while disrupting cells and dissolving cell components during tissue homogenization.

Brain tissues were sonicated twice using a Hielscher, UP400S sonicator at 100% amplitude and 0.9 cycles for at least 30 seconds and samples were allowed to cool down for 10 seconds in between the sonication cycles. Sonicated tissue samples were then incubated on ice for 10 minutes to allow complete dissociation of the nucleoprotein complex. After incubation, 200 µl chloroform was added per 1 ml of TRI Reagent. Samples were vortexed for 10 seconds to ensure thorough mixing of the reagents then they were incubated on ice for 20 minutes. Samples were then centrifuged at 12,000 rpm for 20 minutes at 4°C. Following the centrifugation, the reaction mixture was separated into 3 phases: a pink organic phase (protein), milky interphase (DNA), and a transparent aqueous phase (RNA). The aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and the aqueous solution was pipetted out while the lower pink phase was discarded. Then, 500 µl of chilled

isopropanol was added to the transparent aqueous layer and incubated on ice for 15 minutes. Samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C. After centrifugation, the pellet can be seen attached to the bottom of the Eppendorf tube and the supernatant was discarded. 1ml of 80% ethanol in Diethylpyrocarbonate (DEPC) water was added and the RNA pellet was washed by carrying out centrifugation at 7500 rpm for 5 minutes. Ethanol washing was carried out twice to ensure the complete removal of the impurities. Extracted RNA was stored at -80°C for further downstream applications.

3.10.1. RNA Solubilization

RNA solubilization was carried out by removing the 80% ethanol from the Eppendorf tube containing the RNA pellet, and the pellet was air-dried. Pellet was not completely dried in order to prevent the decrease in solubility. Then, 30 µl of nuclease-free water was added. A slight heat shock at 55°C for 5 minutes was given to uncoil the RNA and to fully dissolve it in the nuclease-free water.

3.10.2. Quality and Quantification of RNA

The quality of RNA was checked through nanodrop and gel electrophoresis before carrying out the downstream processing.

Samples were run on 1% agarose gel. Samples loaded in the well had the following composition 3 µL of RNA, 2 µL of bromophenol blue loading dye, and 5 µL of PCR water. The gel was then run at 90 V for 40 minutes. The gel was then visualized using UV light of Gel Documentation System (gel doc). The presence of both 18S and 28S rRNA r as sharp bands after electrophoresis of total eukaryotic RNA showed that the RNA was intact. The 28S rRNA band should be approximately twice as intense as the 18S rRNA. The smearing of rRNA bands is an indication of degraded mRNA. A new sample of total RNA should be prepared if smearing occurs.

Nanodrop was done first to see the purity and the concentration of the RNA samples. For each sample, 3 readings were taken, and the average was calculated. The nanodrop reading of a sample is shown in *Figure 5*.

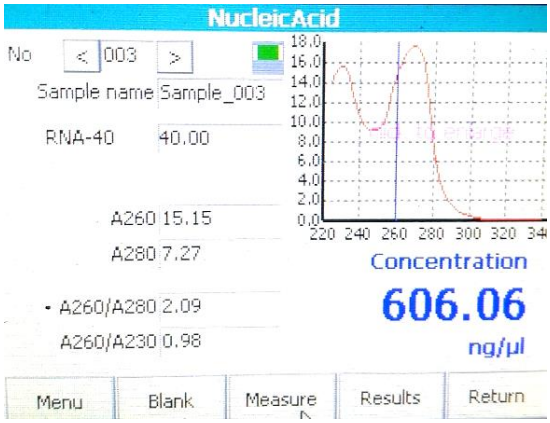

Figure 5: Nanodrop readings of RNA sample obtained using TRI Reagent.

3.11. Reverse Transcription PCR for cDNA Synthesis

cDNA was synthesized using Thermo Scientific K1621 RevertAid First Strand cDNA Synthesis Kit through reverse transcriptase PCR followed by the quantification of products through conventional PCR.

The reaction mixture was prepared on ice. The First 5µl RNA template, 1 µl oligo dT Primer, and 6 µl nuclease-free water was added in a sterile nuclease-free tube making the total volume up to 12 µl. The reaction mixture was incubated at 65°C for 5 minutes. After that 5X reaction buffer, ribonuclease inhibitor, 10mM DNTP mix, and RevertAid M-MuLV RT were added in the reaction mixture. The reaction mixture of 20 µl was briefly vortexed.

Sr. No	***Ingredients***	***Amount***
1.	Template RNA	5 µl Approx.
2.	Oligo(dT_{18}) Primer	1 µl
3.	Water, nuclease-free	6 µl
4.	5X Reaction Buffer	4 µl
5.	RiboLock RNase Inhibitor	1 µl
6.	10 mM dNTP Mix	2 µl
7.	RevertAid M-MuLV RT	1 µl
	Total volume	20 µl

Table 4: Recipe for Reverse Transcription PCR (RT-PCR) for cDNA synthesis

Then, it was incubated at 42°C for 60 minutes and the reaction was terminated at 70°C by heating for 5 minutes. cDNA synthesized was *stored at -20*°C until further usage. The recipe used for cDNA synthesis is mentioned in *Table 4.*

3.11.1 Gene Expression Study

For gene expression study, mRNA levels were checked for Beta Actin, the housekeeping gene, and for *Mus musculus* brain-derived Muscarinic 3 receptor gene expressions studies will be carried out. A list of primers for the Beta Actin and Muscarinic 3 genes along with their annealing temperatures and a number of cycles are mentioned in *Table 5.*

Sr.No	***Gene***	***Primer Sequence (5'-3')***	***AT****	***No of Cycles***
1.	Actin F	GCCTTCCTTCTTGGGTATGG	60 ℃	35
2.	Actin R	CAGCTCAGTAACAGTCCGC	55 ℃	35
3.	Muscarinic F	TCTTGAAGTGCTGCGTTCTGA	57 ℃	35
4.	Muscarinic R	GTTGGGAAACAAAGGCGAGG	57 ℃	35

Table 5: List of primers along with their annealing temperatures.

3.11.2 DNA Contamination Check

To check out the genomic DNA contamination, no template control (NTC) was used, in which PCR water was added to the reaction mixture instead of the cDNA template. The PCR profile is shown in *Figure 6*.

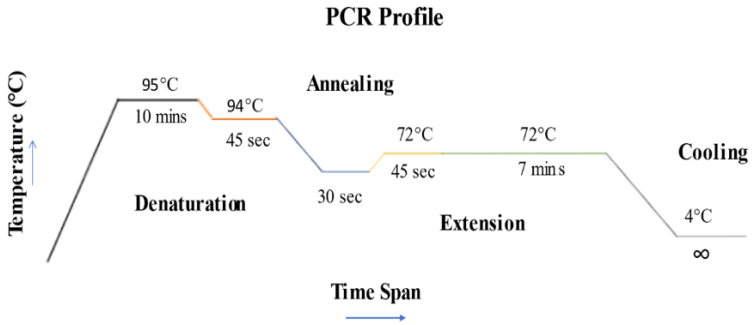

Figure 6: PCR Profile for gene expression. Initial denaturation is at 95° for 10 minutes. This is followed by denaturation at 94°C for 45 seconds, primer specific annealing temperature for 30 seconds, and extension at 72°C for 45 seconds.

3.11.3 Gel Imaging of PCR Products

Actin gene's amplified PCR products were run on 2% agarose gel using a 100bp ladder. Gel images of amplified PCR products were taken using WEALTEC UV Transilluminator, save and then analyzed.

3.12. Statistical Analysis

GraphPad Prism software (version 9) was used for statistical analysis. Paired T-test was applied to the data for analysis. P-value less than 0.05 was considered significant. The data was represented in line or bar graphs as mean ± Standard Error of the Mean (SEM).

4. RESULTS

4.1 Effect of Curcumin and Vitamin D on Physical Parameters

4.1.1 Feed Consumption

Each mouse was given 10g/day pellet feed for a period of 30 days after observing their feeding habits for the first five days. A graph of animal feed consumption is plotted for three different days' i.e. day 1st, day 15th and day 29th of the experiment.

A standardized pattern for feed consumption was shown by control (≈8g/mouse/day), curcumin alone (≈ 8g/mouse/day) and vitamin D alone (≈ 7g/mouse/day) groups. Whereas a decrease was observed in the feed consumption for the stress group (9g/mouse on the 1st day to 5g/mouse on the 29th day). For the rest of the groups; curcumin + vitamin D, stress + curcumin and stress + curcumin + vitamin D; variation in feed consumption was observed throughout the treatment as shown in *Figure 7.*

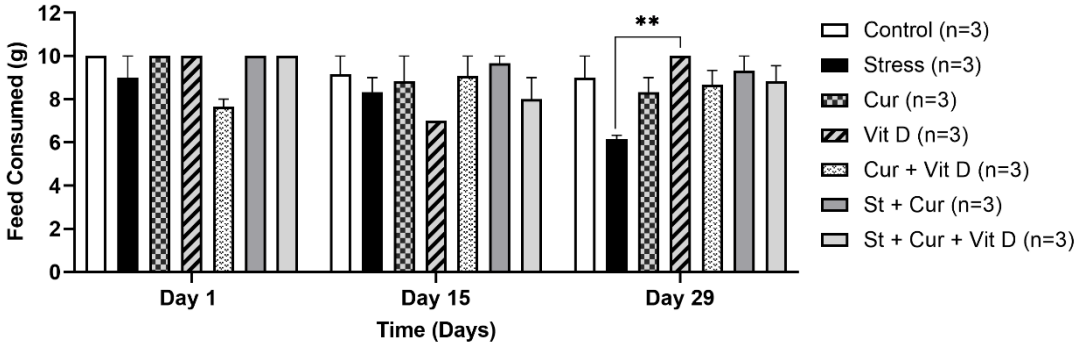

*Figure 7: Feed consumed Bar graph depicting feed consumption (in grams) for three different days i.e. day 1st, day 15th and day 29th of the experiment. Error bars represent mean ± SEM. n = 3. Paired t-test was used. St (Stress), Cur (Curcumin), Vit D (Vitamin D). **P = 0.0076. Two-way ANOVA was used.*

4.2 Effect of Curcumin and Vitamin D on Behaviors

To evaluate the impact of stress-induced memory and learning impairment and the cumulative effect of curcumin and vitamin D in alleviating that impairment, two behavior tests were performed: Novel object recognition and the Morris water maze test.

4.2.1. Effect of Curcumin and Vitamin D on Cognitive Functions

1. Recognition Memory and Exploratory Behavior

Exploratory behavior and recognition memory were checked using a novel object recognition test. The tendency to explore novel objects is indicative of the use of learning and recognition memory.

During the familiarization session (training trial), mice were allowed to interact with two objects (object 1 and object 2) in an open box. Time spent with each object was recorded in seconds respectively, as shown in *Figure 8*. For the familiarization session, the interaction time between object 1 and object 2 for each group was almost the same with respect to each group. An interesting finding was that all the vitamin D treatment groups (vitamin D (48.33±13.51), curcumin + vitamin D (57.16±8.09), and stress + curcumin + vitamin D (49.67±8.57)) showed increased exploratory behavior in comparison to the control (17.833±5.04) and other groups though the difference was not significant.

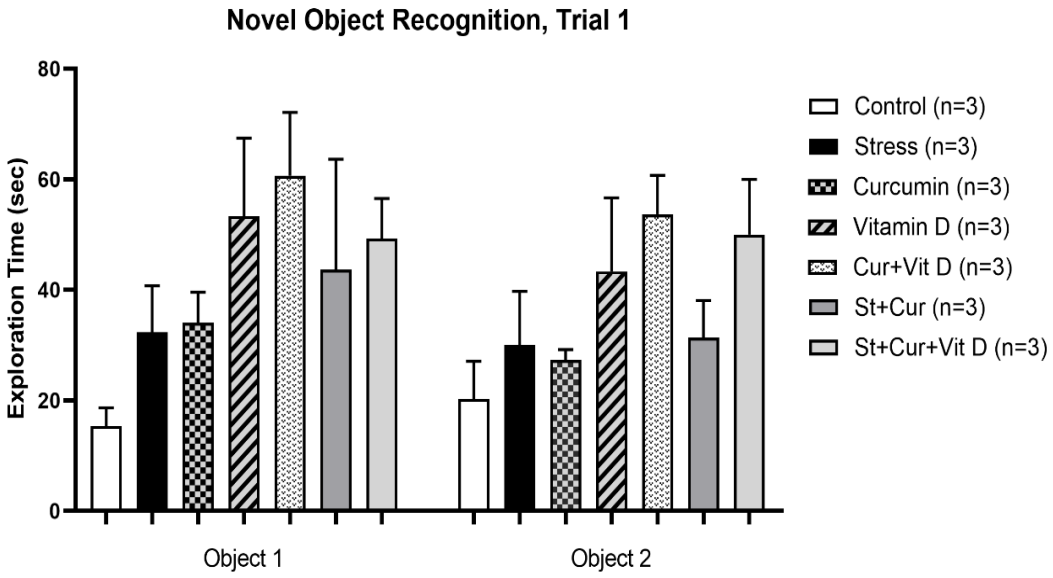

Figure 8: Effect on exploratory behavior (Novel Object Recognition Test): Bar graph depicts time spent (seconds) exploring objects 1 and 2 during the familiarization trial. Interaction time between object 1 and object 2 for each group was almost the same with no significant difference. St (Stress), Cur (Curcumin), Vit D (Vitamin D). Error bars represent mean ± SEM. n = 3.

During the second session (testing trial), object 1 from the training trial served as the familiar object l. Object 2 was replaced with a new object which served as the novel object. Once again, time spent with both objects is recorded in seconds and a bar graph was created as shown in *Figure 9*. If more time is spent with a novel object as compared to the familiar object, the mouse is said to have a preference for novelty thus indicating improved learning and memory whereas if more time is spent with the familiar object as compared to the novel object, it indicates impaired learning and memory.

The recognition memory was found to be impaired in the stress group (23.33±10.92) showing a decrease in the exploration of the novel object compared to the control (54.33±26.39) though the difference was not significant. Stressed mice showed improved recognition memory as a result of curcumin administration, either alone (38±6.11) or in combination with vitamin D (45±2.64), but the difference was not significant. No significant increase was observed in the combination therapy in comparison to the curcumin monotherapy in stressed mice though a slight improved behavior was seen.

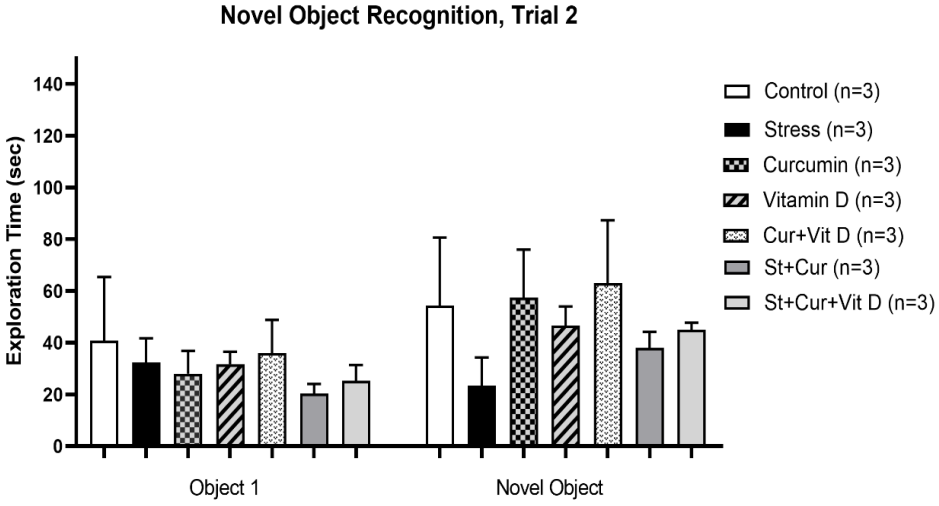

Figure 9: Effect on recognition memory (Novel Object Recognition Test): Bar graph depicts time spent (in seconds) exploring either object 1 (a familiar object from the previous trial) or a new object (novel object) in the test trial. Impaired recognition memory was in the stress group as shown by a decrease in exploration of the novel object in comparison to control. Stressed mice showed improved recognition memory as a result of curcumin administration either alone or in combination with vitamin D. All the observations were insignificant. St (Stress), Cur (Curcumin), Vit D (Vitamin D). Error bars represent mean ± SEM. n = 3.

An interesting finding was the overall improvement in recognition memory as a result of curcumin administration either alone or in comparison (curcumin + vitamin D > curcumin).

The overall picture of novel object recognition test results indicates that vitamin D might have more impact on the exploratory behavior whereas curcumin has the potential to complement the recognition memory of mice.

The percent recognition index, which is a measure of an animal's ability to discriminate between the known object and novel object, was also calculated. The following formula was used to calculate the novel recognition index;

N_t = Time spent with novel object

F_t = Time spent with familiar object

$T_t = N_t + F_t$ = Total time spent with both objects

$$Novel\ recognition\ index\ \% = \frac{\mathrm{Nt}}{\mathrm{Tt}} \times 100$$

If the novel recognition index >50%, a preference for a novel object is proved and the memory of the mouse is said to be improved. However, if the novel recognition index is <50%, memory is said to be impaired.

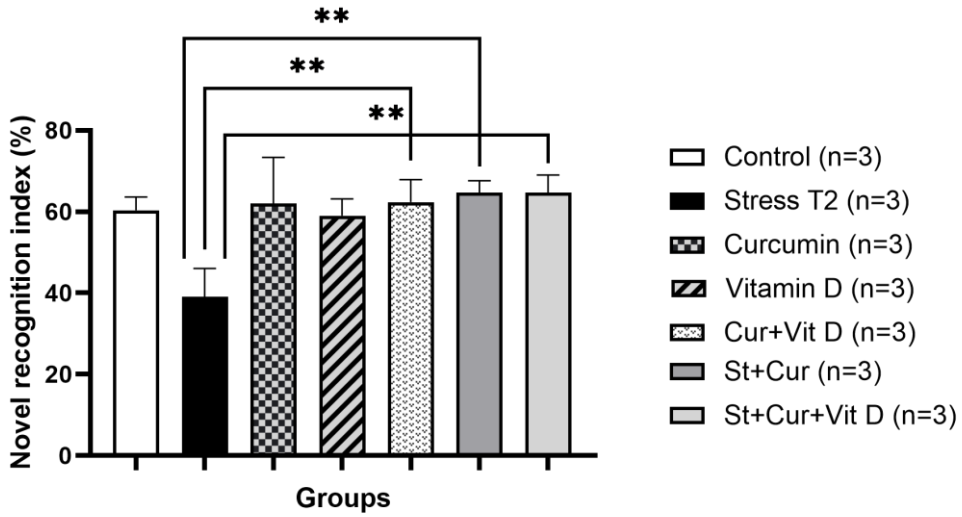

*Figure 10: Novel recognition index %: Measure of animal's ability to discriminate between the known object and novel object. The stress group showed impaired recognition memory. A significant improvement was seen in stress treated groups (St + Cur = St + Cur + Vit D). St (Stress), Cur (Curcumin), Vit D (Vitamin D). Error bars represent mean ± SEM. n = 3. Paired t-test was used. * = P < 0.05, ** = P < 0.01.*

The stress group showed a novel recognition index of less than 50% (39±6.95) showing impaired recognition memory in comparison to the control (60±3.37). A significant improvement in explorative behavior and recognition memory was seen in stressed mice as a result of curcumin treatment either alone (65.36±2.99) or in combination with vitamin D (64.89±4.23) shown in *Figure 10.*

2. Effect on Learning and Memory

For the assessment of spatial and reference memory, the Morris water maze test was performed in which repeated training results in the acquisition of an escape response to the hidden platform. The average time taken by the mice to locate the platform is a direct reflection of the effect of the treatment used.

During training days, improvement in the escape latency was seen for all of the experimental groups. On day 5th, all of the groups were able to locate the platform within 11 seconds. The stress group performed equally well when compared to the control and other treatment groups. Although escape latencies were improved for each group over a 5-day training trial, however, the difference in escape latencies between the groups wasn't significant. A line graph representing the escape latency over 5 training days is shown in *Figure 11.*

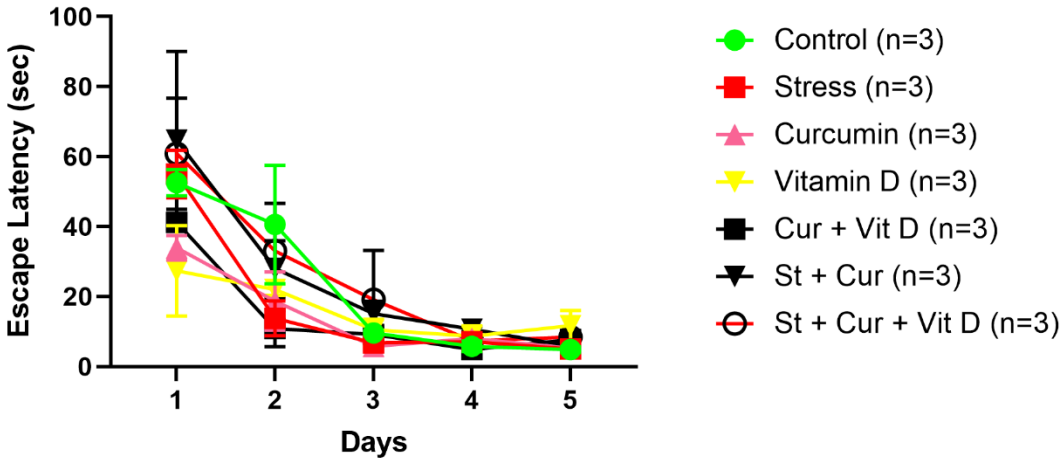

Figure 11: Effect on spatial memory (MWM Test): The graph shows the learning curve for the 5 days of training. A decreasing trend in the escape latency of all groups was observed, with the stress group performing equally well. St (Stress), Cur (Curcumin), Vit D (Vitamin D). Error bars represent mean ± SEM. n = 3. Paired t-test was used.

Probe trial was performed on day 6th for the assessment of the reference memory. Exploration time for the previously placed hidden platform was measured by the time spent in the goal quadrant. The stress group (10.33±1.33) showed a decline in the reference memory as less time was spent in the goal quadrant where the hidden platform was placed previously, in comparison to the control group (26.00±5.29), On the other hand, the stress + curcumin group (31.33±2.02) and stress + curcumin + vitamin D group (29.33±5.20) spent more time in the goal quadrant which indicates that treatment of curcumin alone and in combination with vitamin D improved the learning and memory of stressed mice as shown in *Figure 12.*

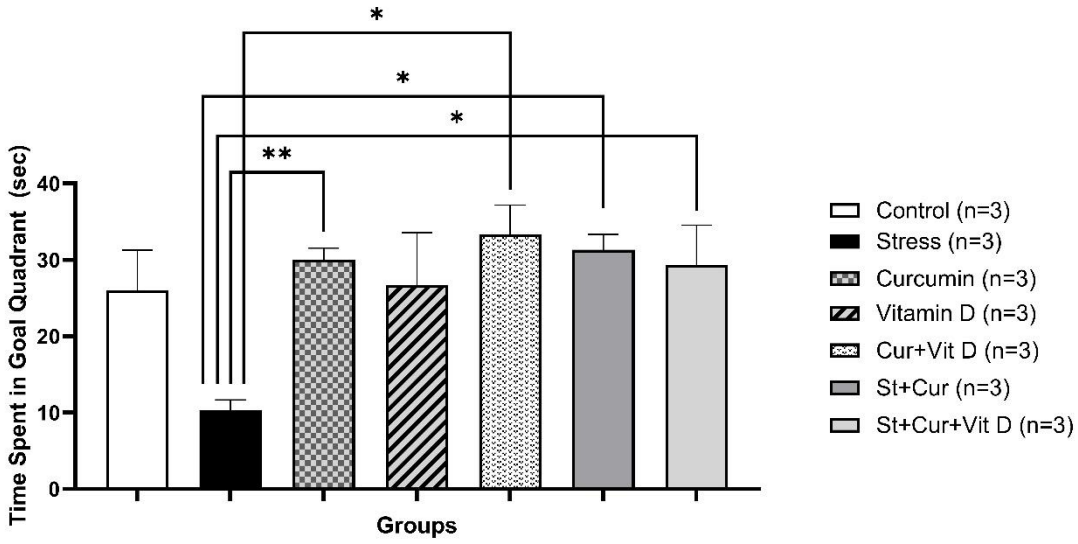

*Figure 12: Effect on reference memory: Time spent in goal quadrant (MWM Test, Probe Trial). Impaired reference memory of the stress group is depicted by the least amount of time spent in the goal quadrant. Concerning stress-treated groups, a significant improvement in the reference memory (St + Cur > St + Cur + Vit D was observed. St (Stress), Cur (Curcumin), Vit D (Vitamin D). Error bars represent mean ± SEM. n = 3. Paired t-test was used. * = P < 0.05, ** = P < 0.01.*

For the number of crossings in the goal quadrant, a similar trend was observed as shown in *Figure 13*. A non-significant decline was observed between the stress group (2.6±0.3) and the control group (4.5±0.5). Curcumin and vitamin D treatments either alone or in combination exhibited better reference memory compared to the stress group although a significant difference was only seen in comparison to the curcumin group. Stressed mice showed improved reference memory as a result of curcumin administration either alone

(5.0±1) and in combination with vitamin D (4.3±1.6); (stress + curcumin > stress + curcumin + vitamin D), though the difference was not significant Furthermore, an interesting finding was the overall improvement in reference memory as a result of curcumin administration either alone or in combination (curcumin > curcumin + vitamin D > stress + curcumin > stress + curcumin + vitamin D). An additive effect was not seen in the case of combination therapy.

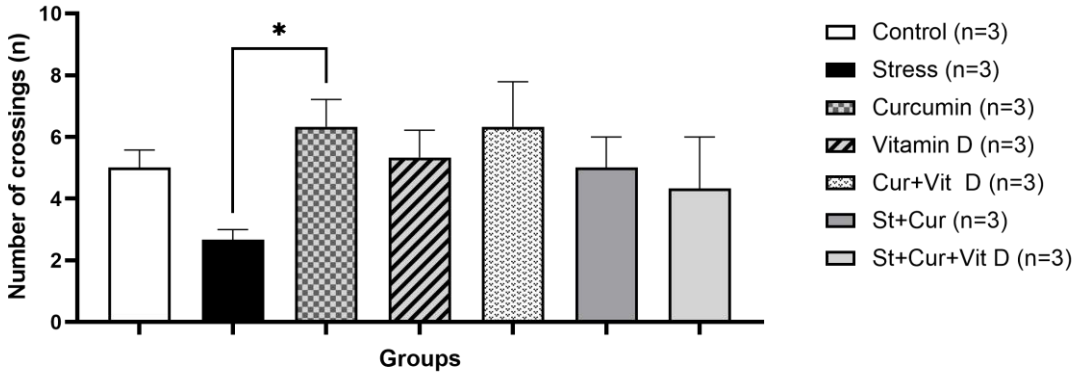

*Figure 13: Effect on reference memory: A number of crossings in the target area (MWM Test, Probe Trial). A non-significant decline was observed in the stress group in comparison to the control. Stressed mice showed improved reference memory as a result of curcumin administration either alone or in combination with vitamin D (St + Cur > St + Cur + Vit D), St (Stress), Cur (Curcumin), Vit D (Vitamin D). Error bars represent mean ± SEM. n = 3. Paired t-test was used. * = P < 0.05.*

Swimming patterns of the mice in the probe trial are also shown in *Figure 14.* This shows that groups treated with curcumin and vitamin D spent more time in the goal quadrant as compared to the stress group.

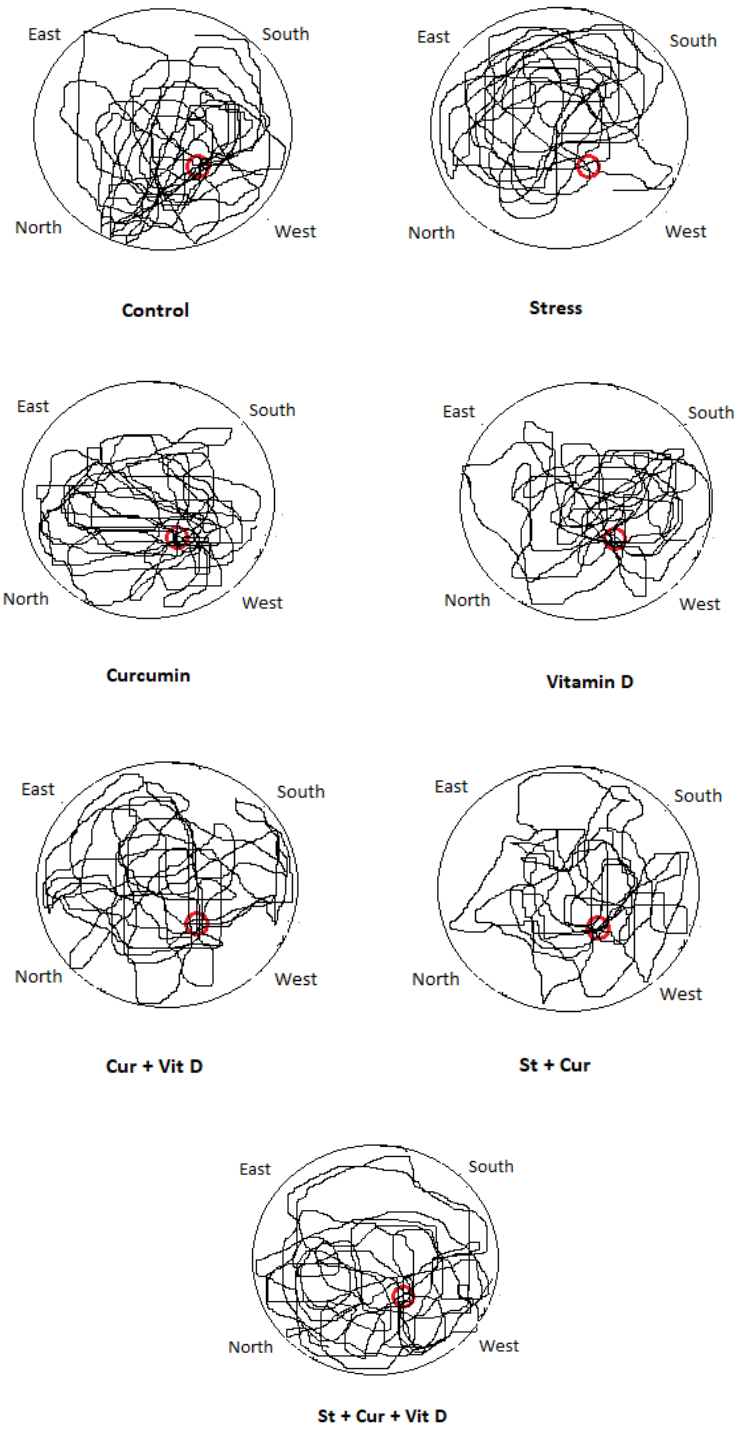

Figure 14: Effect on reference memory: Representative swimming patterns (MWM Test, Probe Trial), depicting the number of times mouse crossed the location of the platform and its motion in the target quadrant. The "red circle" shows the position where platform was previously placed. Control mice centered their search around the previous platform location, whereas stressed mice are less systematic, spending more time along the perimeter. Stressed mice showed improved reference memory as a result of curcumin administration, alone and in combination with vitamin D. St (Stress), Cur (Curcumin), Vit D (Vitamin D).

5. DISCUSSION

Stress is prevailing at a large scale across the globe and is regarded as a state of threatened homeostasis, following exposure to extrinsic or intrinsic adverse forces (stressors) (93). 29.6% of the world's population was shown to suffer from stress as per a study conducted in 2020 (94). Our study aims at evaluating the therapeutic role of curcumin and vitamin D on learning and memory at the pre-clinical stage using the restraint stress mice model.

Stress has been found to cause neuronal damage and impaired brain-cell communication in areas associated with learning and memory, with the frontal cortex and hippocampus being the most susceptible brain regions (95,96). The composition of circulating plasma is changed as a result of stress stimuli from the external and internal environment. These fluctuations are restricted from the central nervous system (CNS) through the blood-brain barrier (BBB) which particularly plays a significant part in supporting homeostasis. However, studies have shown that stress increases the permeability of BBB in rodents (97) along with humans (98) which is associated with disruptions in CNS homeostasis and neuronal death (99).

Restraint stress is one of the commonly used models and is a modified form of immobilization stress. Inescapable physical and mental stress is induced during this procedure by placing the animals in a plastic tube in order to restrict their movements (100). This is a validated experimental stressor that induces both physical and psychological effects at the same time (101). In the present study chronic stress was induced by restraining animals for 4 hours for 30 days (101).

Curcumin has been identified as a potential treatment to reduce memory and learning impairment, owing to its antioxidant, anti-inflammatory and neuroprotective role (102). However, poor absorption, rapid metabolism, chemical instability, and rapid systemic elimination of curcumin limit its therapeutic potential. Therefore, scientists are exploring combination treatments of curcumin along with other compounds at lower doses (103,104,105). Similarly, vitamin D has also been suggested to play a vital role in the regulation of various neurotransmitters along with playing a protective role against

neurological diseases (117). Moreover, vitamin D administration has been linked to better performance of memory-related tasks and cognitive functions. In one of the studies conducted earlier on aging rodents with human modulated vitamin D serum levels ranging from deficient to sufficient were tested to identify its effect on cognitive functions. It was found that aged rats with high doses of vitamin D were able to perform better in the complex memory-related tasks with blood levels in the optimal range as compared to the rats fed with low and vitamin D in the normal dietary range. This finding suggests that vitamin D improves cognitive functions. It also recommends that changes in hippocampal gene expression may be mediated by vitamin D and can improve the chances of successful aging of the brain (106). Previously, one study reported improvement in learning and memory after exposure to curcumin and vitamin D3 treatment in Alzheimer's disease animal model (107). But no such study was conducted using the combination treatment of curcumin and vitamin D against cognitive decline caused by stress. Hence, the combined effect of curcumin with vitamin D was explored for the first time against stress-induced learning and memory impairment in this study.

The dose of curcumin used for this study was 30mg/kg. Kumar et. Al administered 15-30mg/kg of curcumin to mice along with D-galactose and a dose-dependent improvement with curcumin was observed. In a study conducted by Dolatabadi et. al high dose of curcumin 100 mg/kg was used in rats which significantly improved memory and neuronal deficits (108).

Although the recommended doses of curcumin for mice is 100.2 mg/kg and for rats is 48.0 mg/kg (17), studies have been conducted on mice with chronic administration of higher curcumin doses (200 and 400 mg/kg) along with the induction of stress which showed dose-dependent effects on cognitive functions (109). Literature shows that curcumin doses required are much higher for the oral administration as compared to the intraperitoneal (i.p.) administration, to produce a similar effect (110). For humans, curcumin doses as high as 12 g/day over 3 months have been shown safe in the studies conducted (111). For the present study, the dose used for vitamin D was 1500IU/kg. Studies have shown treatment of mice for 16 weeks with a high dose of vitamin D that is 1000 IU/kg significantly improved the cognitive impairment caused by

diabetes (112). Moreover, doses as high as 5000 IU/kg have been administered in mice as anticancer therapy and are shown to be safe. Possible side effects such as an increase in the serum calcium levels resulting in hypercalcemia weren't observed with the 5000 IU/kg dose (113). Hence, we can say that the dose of 1500 IU/kg we used in our study is safe.

In this study, feed consumption was recorded which revealed decreased consumption in the stress group. In several studies, chronic exposure to restraint stress has been shown to negatively impact the intake of feed and weight of rodents (114,115). Joon Yee et. al reported a reduction in the body weight and feed intake of the mice due to the induction of chronic restraint stress in mice for 15 days (115). The previous study has shown that restraint stress and novelty stress-induced decreases food intake (116). Since variation in feed consumption was observed throughout the treatment and food intake slightly decreased upon curcumin supplementation for some of the groups; curcumin + vitamin D, stress + curcumin and stress + curcumin + vitamin D; it was most likely accounted to curcumin's bitter taste (117).

The present study showed a decrease in the percentage recognition index of the stress group indicating impaired memory. Wang et. al, has also reported reduced novelty preference and impaired recognition memory as a result of acute stress which negatively affected recognition memory (118). This could be because of increased corticosterone levels, as literature shows a negative effect on memory retrieval due to increased stress-induced plasma corticosterone levels or due to systemic corticosterone administration (119). The present study showed an improvement in explorative behavior and recognition memory in stressed mice as a result of curcumin treatment either alone or in combination with vitamin D (stress + curcumin = stress + curcumin + vitamin D). Sanei et. al, Zhang et. al, and Kamali et. al, have shown that curcumin improves memory impairment in novel object recognition tasks (120,121,122) Though the effects on short-term and long-term memory are dose-dependent; the higher the dose, the more significant is its effect on recognition memory (123). Similarly, the administration of oral curcumin improves learning and memory and protects against unpredictable chronic stress-induced oxidative damage and cognitive impairment in rats and mice (124).

Morris water maze is a test for rodents to assess their spatial learning memory that depends on the animals' learning and memory ability to navigate to the submerged platform in an open swimming pool with the help of distant cues. In this study, stressed mice showed shorter escape latency similar to the other non-stressed and treatment groups. It was further observed that the difference between the escape latencies wasn't significant. Gehring et. al reported that stressed animals tend to cover more distance with longer path lengths and move faster than non-stressed animals. Therefore, the reason for low escape latencies of the stress group could be hyperactivity in terms of locomotion due to the development of an anxious state (125). Hence, in this case, spatial memory solely on the basis of escape latency can't be assessed (120).

Reference memory was evaluated via probe trial in which exploration time for the previously placed hidden platform is measured. The stress group showed impairment in reference memory. A significant improvement was seen in stress treated groups (stress + curcumin > stress + curcumin + vitamin D).

Our results show that curcumin either alone or in combination with vitamin D provides neuroprotective effects against restraint stress and improves learning and memory. Unfortunately, a significant cumulative effect in the combination treatment was not observed.

CONCLUSION

Restraint stress affects exploratory behavior along with recognition and reference memory. Curcumin treatment alone and in combination with vitamin D counteracted the effects of restraint stress and resulted in improved behavior, learning and memory. Unfortunately, an additive effect of the combination treatment was not observed. In conclusion, the combination of curcumin and vitamin D holds potential for improving cognition, but further dose-dependent studies are required. Moreover, studies at the molecular level of histological studies are required for better understanding.

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