Investigating Cumulative Effect of Curcumin and Vitamin D as an Anti-Stress Therapy

By

Aafia Rasheed Warsi (280186)

Saleha Aziz (279491)

Umme Ammara (279681)

Zunaira Saadat (280673)

Thesis Supervisor: Dr. Saira Justin

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2022)

Investigating Cumulative Effect of Curcumin and Vitamin D as an Anti-Stress Therapy

By

Aafia Rasheed Warsi (280186)

Saleha Aziz (279491)

Umme Ammara (279681)

Zunaira Saadat (280673)

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

Bachelor of Sciences in

Applied Biosciences

Thesis Supervisor: Dr. Saira Justin

Atta-Ur-Rahman School of Applied Biosciences (ASAB)

National University of Sciences & Technology (NUST)

Islamabad, Pakistan

(2022)

THESIS ACCEPTANCE CERTIFICATE

We Certified that the final copy of BS FYP Thesis written by Ms. Aafia Rasheed Warsi (280186), Ms. Saleha Aziz (279491), Ms. Umme Ammara (279681), and Ms. Zunaira Saadat (280673) of Atta-Ur-Rahman School of Applied Biosciences (ASAB) had been vetted by undersigned, found complete in all respects as per NUST Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for the award of Bachelor's degree. It is further certified that necessary amendments as pointed out, during the final presentation of the scholar, have also been incorporated in the said thesis.

Signature: _____

Name of Supervisor: **Dr. Saira Justin**

Date: _____

Signature: _____

Name of Supervisor: **Dr. Sobia Manzoor**

Date: _____

Signature (Dean/Principal) _____

Name of Supervisor: **Professor Dr. Hussnain Janjua**

Date: _____

AUTHOR'S DECLARATION

We Aafia Rasheed Warsi, Saleha Aziz, Umme Ammara and Zunaira Saadat hereby state that our BS FYP thesis titled "*Investigating Cumulative Effect of Curcumin and Vitamin D as an Anti-Stress Therapy*" is our own work and has not been submitted previously by us for taking any degree from this university National University of Sciences & Technology (NUST) Islamabad, Pakistan or anywhere else in the country/world.

At any time if, our statement is found to be incorrect even after our graduation, the university has the right to withdraw our Bachelor's degree.

Signature: _____

Name of student: **Aafia Rasheed Warsi**

Date: 30th May, 2022

Signature: _____

Name of student: **Saleha Aziz**

Date: 30th May, 2022

Signature: _____

Name of student: **Umme Ammara**

Date: 30th May, 2022

Signature: _____

Name of student: **Zunaira Saadat**

Date: 30th May, 2022

CERTIFICATE FOR PLAGIARISM

It is certified that the BS FYP Thesis Titled "Investigating Cumulative Effect of Curcumin and Vitamin D as an Anti-Stress Therapy" by Aafia Rasheed Warsi, Saleha Aziz, Umme Ammara and Zunaira Saadat has been examined by me. I undertake the following:

a. Thesis has significant new work/knowledge as compared to already published or are under consideration to be published elsewhere. No sentence, equation, diagram, table, paragraph or section has been copied verbatim from previous work unless it is placed under quotation marks and duly referenced.

b. The work presented is original and the author's own work (there is no plagiarism). No ideas, processes, results or words of others have been presented as Author's own work.

c. There is no fabrication of data or results which have been complied/analyzed.

d. There is no falsification by manipulating research materials, equipment, or processes, or changing or omitting data or results such that the research is not accurately represented in the research record.

e. The thesis has been checked using TURNITIN (copy of originality report is attached) and found within limits as per HEC / NUST plagiarism Policy and instructions issued from time to time.

Date: _____ **Student Signature**

Date: _____ **Signature & Stamp of Supervisor**

"Whoever treads a path, seeking that path in knowledge, Allah will make easy for him the path to paradise."

Prophet Muhammad (ﷺ)

DEDICATION

"This thesis is dedicated to our parents, grandparents, siblings, and friends. Their combined love and support have been a constant source of comfort for all of us, and their presence in our lives has been the force that has led us forward. Finally, we would also like to dedicate our work to our teachers. Their brilliant minds and the confidence they put in us have made us see our true potential, and their excellence has helped us see the true beauty of our field."

-Aafia, Saleha, Umme Ammara and Zunaira

ACKNOWLEDGEMENTS

All praise and glory are for the Almighty, the most beneficent, the most merciful, who blessed us with the ability to think, learn and grow and picked us up when we fell and raised us to a place of peace and tranquility.

We feel the greatest honor to have the opportunity to express our indebtedness and joy to our supervisor, Dr. Saira Justin, for her never-ending support and her brilliance, dedication, perfection, and unparalleled knowledge. Her faith in us and our work made this project a success and a great learning curve.

We owe our most sincere gratitude to Dr. Hussnain Janjua, Principle, Atta-ur-Rahman School of Applied Biosciences (ASAB), for his endeavors in making research and learning with extreme quality possible. We would also love to extend our gratitude to Engr. Javed Mahmood Bukhari, rector National University of Sciences and Technology (NUST), to allow science and technology to flourish by ensuring the best facilities and opportunities.

We would also like to extend our heartiest gratitude to Mr. Fida Hussain, supervisor of lab animal house, NUST, for his time and cooperation throughout the project. Our heartiest gratitude to our seniors, Ms. Momina, Ms. Khushbakht, Ms. Sara Ishaq, Ms. Maryam Hamid, Ms. Aiman Tahir, Ms. Summayya, and Ms. Shumaila for their guidance and cooperation during the project. Special thanks to our teachers from the first semester to the last, who directed us towards the path of success through their experiences, suggestions, and wisdom, and all the staff members at ASAB for helping us during our research.

Above all, this paper would have been unthinkable without the adoration and persistence of our loved ones. They have been the backbone holding us together away from home, while our friends have been our stabilizers through thick and thin. Without the collective moral support of all of them, this thesis would not have been possible.

-Zunaira, Aafia, Saleha and Umme-Ammara

TABLE OF CONTENTS

DEDICATION ..... **vii**

ACKNOWLEDGEMENTS ..... **viii**

LIST OF FIGURES ..... **xiii**

LIST OF ABBREVIATIONS ..... **xv**

ABSTRACT ..... **xix**

Chapter 1 ..... **1**

INTRODUCTION **1**

1.1 RESEARCH OBJECTIVES 5

Chapter 2 ..... **6**

LITERATURE REVIEW **6**

2.1 STRESS 6

2.1.1 Stress Response 6

2.1.2 Types of Stress 6

2.1.3 Effects of Stress 7

2.1.3.1 Behavioral Effects 7

2.1.3.2 Cognitive Effects 7

2.1.3.3 Physical Effects 8

2.2 STRESS AND NERVOUS SYSTEM 8

2.2.1 Hippocampus 8

2.2.2 Prefrontal cortex (PFC) and its Functions 9

2.3 Brain-Derived Neurotrophic Factor (BDNF) GENE 9

2.3.1 Brain-Derived Neurotrophic Factor (BDNF) Synthesis 9

2.3.2 Brain-Derived Neurotrophic Factor (BDNF) Functions 11

2.3.3 Stress and Brain-Derived Neurotrophic Factor (BDNF) 12

2.4 STRESS HORMONE - CORTISOL 12
2.4.1 Functioning of Glucocorticoids 13
2.4.2 Release and Activation 13
2.4.3 Relation to stress 14
2.4.4 Reference Values 14
2.4.5 Importance of Cortisol 15
2.5 TURMERIC 15
2.5.1 Chemical Constituents of Turmeric Powder 16
2.5.1.1 Curcuminoid 17
2.5.1.1.1 Curcumin 17
2.5.1.1.1.1 Physiological Actions of Curcumin 18
2.5.1.1.1.2 Neuroprotective Actions of Curcumin 18
2.5.1.1.1.3 Pharmacokinetics Properties of Curcumin 18
2.6 VITAMIN D 18
2.6.1 Vitamin D Forms 19
2.6.2 Physiological Actions of Vitamin D in The Body 20
2.6.3 Neuroprotective Actions of Vitamin D 21
Chapter 3 22
METHODOLOGY 22
3.1 ETHICAL STATEMENT 22
3.2 ANIMAL MODELS 23
3.3 DRUGS– CURCUMIN AND VITAMIN D 23
3.4 TREATMENT OF EXPERIMENTAL MICE 23
3.4.1 Curcumin Feed 23
3.4.2 Vitamin D Feed 23
3.5 MATERIALS AND INSTRUMENTS 24

3.6 STUDY PLAN 26
3.7 STUDY DESIGN 26
3.8 RESTRAINED STRESS TO MICE 28
3.9 BEHAVIOR TESTING 29
3.9.1 SOCIAL PREFERENCE 29
3.9.1.1 Apparatus 29
3.9.1.2 Procedure 30
3.9.1.3 Evaluation 31
3.9.2 MARBLE BURYING 31
3.9.2.1 Apparatus 31
3.9.2.2 Procedure 32
3.9.2.3 Evaluation 32
3.9.3 EXIT CIRCLE 32
3.9.3.1 Apparatus 32
3.9.3.2 Procedure 33
3.9.3.3 Evaluation 33
3.10 SERUM ISOLATION 33
3.11 BRAIN EXTRACTION 33
3.12 RNA EXTRACTION 34
3.12.1 Sample Lysis and Phases Separation 34
3.13 RNA SOLUBILIZATION 35
3.14 RNA QUANTIFICATION 35
3.15 REVERSE TRANSCRIPTION – PCR (RT-PCR) 35
3.16 PCR-TO CHECK THE EXPRESSION OF ACTIN AND BDNF GENES 36
3.16.1 Gene Expression Study 37
3.17 STATISTICAL ANALYSIS 39

Chapter 4 ..... **40**

RESULTS **40**

4.1 EFFECT OF CURCUMIN AND VITAMIN D ON PHYSICAL PARAMETERS 40

4.1.1 Effect on Weight 40

4.1.2 Effect on Feed Consumption 41

4.2 EFFECT OF CURCUMIN AND VITAMIN D ON BEHAVIOR 42

4.2.1 Effect on Social Preference 42

4.2.2 Effect on Marble Burying 46

4.2.3 Effect on Exit Circle 47

Chapter 5 ..... **49**

DISCUSSION **49**

CONCLUSION **52**

FUTURE DIRECTIONS **53**

REFERENCES **54**

TURNITIN PLAIGARISM REPORT **63**

LIST OF FIGURES

Figure No. **Description** **Page No.**

Figure 2.1 The Conversion Process of precursor of brain-derived neurotrophic factor (proBDNF) to Mature BDNF(Thomas & Davies, 2005)..... 10
Figure 2.2 The precursor and mature forms of Brain-derived neurotrophic factor (BDNF) play different roles in our brain (Palasz et al., 2020)..... 11
Figure 2.3 Varying levels of Glucocorticoids according to changing internal and external situations. 13
Figure 2.4 Curcuma longa. a) Plant b) Rhizome 16
Figure 2.5 Chemical structure of curcumin (Sharifi-Rad et al., 2020). 17
Figure 2.6 Structural difference between two forms of vitamin D2 and Vitamin D3 (F. Bokhari & Albaik, 2020). 19
Figure 3.1 NUST-IRB Certificate..... 22
Figure 3.2 Timeline for the treatment of mice..... 26
Figure 3.3 Study Design. 27
Figure 3.4 Mouse restrained in a modified 50 ml Falcon tube with holes in the top and bottom (Mohamed et al., 2013)..... 28
Figure 3.5 Social Interaction Test Apparatus: The apparatus contains a three-chambered rectangular box with two metal cages to place stranger mice during the test and two boxes to block the entry to two chambers during habituation..... 30
Figure 3.6 Marble Burying Apparatus: (A) The side view of the marble burying setup. (B): The upper view of the marble burying setup containing a total of 20 marble divided in 4 rows of 5. 32
Figure 3.7 Brain harvested from mice after decapitation. 34
Figure 3.8 PCR Profile for gene expression.. 38
Figure 4.1 Percentage (%) weight variation 41
Figure 4.2 Feed Consumption..... 42
Figure 4.3 Session 1 Sociability. 43
Figure 4.4 Session 2 Social Novelty..... 44
Figure 4.5 Discrimination index for sociability..... 45
Figure 4.6 Discrimination index (DI) for social novelty 46
Figure 4.7 Innate Defensive Response..... 47
Figure 4.8 Intrinsic Inquisitiveness & Exploratory Behavior: Compared to the control group 48

LIST OF TABLES

Table No. **Description** **Page No.**

Table 2.1 Categories of Effects of Stress. 7
Table 2.2 Properties of Vitamin D2 (Ergocalciferol) and Vitamin D3 (Cholecalciferol). 19
Table 3.1 List of Chemicals and Reagents Used 24
Table 3.2 List of Kits Used 24
Table 3.3 List of Plastic Consumables and Miscellaneous 25
Table 3.4 List of Instruments Used 25
Table 3.5 List of software used 25
Table 3.6 Reverse Transcription PCR (RT-PCR) Recipe for Making 20 µL cDNA. 36
Table 3.7 PCR Reaction Recipe for a Single cDNA Sample. 37
Table 3.8 List of Primers along with their Annealing Temperatures and No. of Cycles. 38

LIST OF ABBREVIATIONS

%	Percentage
℃	Degree Celsius
AD	Alzheimer's Disease.
IUPAC	International Union of Pure and Applied Chemistry
mg	Milligram
IL-6	Interleukin-6
nm	Nanometer
UVB	Ultraviolet B
MARRS	Membrane Associated Rapid Response Steroid-Binding Receptor

CNS	Central Nervous System
GDNF	Glial Cell Line-Derived Neurotrophic Factor
SOCE	Store-Operated Calcium Entry
STIM	Stromal Interaction Molecule
GSH	Glutathione
Ca	Calcium
ROS	Reactive Oxygen Species
BDNF	Brain-Derived Neurotrophic Factor
ProBDNF	Precursor of Brain-Derived Neurotrophic Factor
mBDNF	Mature Brain-Derived Neurotrophic Factor

TGN	Trans Golgi Network
ROS	Reactive Oxygen Species
VDR	Vitamin D Receptor
HPA	Hypothalamic-Pituitary-Adrenal
GCs	Glucocorticoids
PFC	Prefrontal Cortex
SCN	Suprachiasmatic Core
PVN	Paraventricular Core
CRH	Corticotropin-Delivering Chemicals
AVP	Arginine Vasopressin

ACTH	Adrenocorticotropin Hormone
GRs	Glucocorticoid Receptors
MRs	Mineralocorticoid Receptors
p75NTR	p75 Neurotrophin Receptor
TrkB	Tropomyosin Receptor Kinase B

ABSTRACT

***Introduction*:** Chronic stress causes structural modifications in brain, ultimately impacting behavior, emotions and cognition. Therapeutic role of curcumin, principal curcuminoid of turmeric, is limited due to its *low* bioavailability. Similarly, vitamin D, a fat-soluble secosteroid, is reported to have neuroprotective properties.

***Objective*:** To study the cumulative effects of curcumin and vitamin D on social behavior in restrained stress mice model.

***Methods*:** Restrained stress was induced in BLAB/c mice for 30 days. Treatment with curcumin (30 mg/kg) and/or vitamin D (1500 IU/kg) was administered orally. Behavior tests were conducted to assess sociability, social novelty, inquisitiveness and innate defense response.

***Results*:** Social propensity was significantly reduced in stressed mice (51.0±11.5) compared to control (144.7±17.8). Improved sociability was seen following treatment with curcumin either alone (98.67±38.83) or in combination with vitamin D (120.7±4.3), with significant difference seen in combination therapy. Regarding social novelty, negligible decrease was seen in stress group (65.0±22.5) compared to control (74.67±25.33). Following treatment, an improved behavior was only seen in combination therapy (104.0±21.0), although the difference was insignificant. Marble burying was significantly reduced in stress group (3.0±0.9) compared to control (7.3±0.6). Stressed mice showed increased burying ability due to curcumin administration, either alone (5.3 ±1.2) or in combination with vitamin D (8.7±1.3), with a significant difference in the combination therapy. For exploratory behavior, compared to control group (16.67±3.33), time taken by stress group (36.0±10.50) to exit the circle was increased, showing impairment. Curcumin administration, either alone (24.67±0.33) or in combination with vitamin D (17.33±1.67) caused insignificant improvement in behavior.

***Conclusion*:** Restrained stress exhibited declined social behavior. Following curcumin administration either alone or in combination with vitamin D, an improvement in sociability, inquisitiveness & innate defense response was seen. Overall, the study showed no significant cumulative effect in the combination treatment. Further studies with focus on the molecular aspects are needed.

Chapter 1

INTRODUCTION

The human body's survival is largely dependent on maintaining its internal balance, also known as homeostasis. Stress poses threats to this homeostatic balance. In unbefitting situations, the distressing occurrence is known as the stressor, and the body's reaction to it is known as the stress response.

Stress is categorized into good stress, tolerable stress, and toxic stress (McEwen et al., 2015). It has widespread effects on the body and can be highly harmful in constant and prolonged exposures. The three categories that can be used to understand its effects are physical, cognitive, and behavioral effects. Physical effects include an upset stomach, insomnia, etc. Cognitive effects are worrying, forgetfulness, inability to focus, and poor judgment. Behavioral effects include changes in appetite, procrastination, alcohol/drug use, and nervousness.

Long-term exposure to stress harms multiple body systems, including cardiovascular (high blood pressure, heart attack), respiratory (asthma, emphysema), mental (memory impairment, depression), reproductive (infertility, menopause), gastrointestinal (heartburn, ulcers), and muscular (pain, muscle contractility) activities (Yaribeygi et al. 2017). Additionally, hormones like adrenaline, cortisol, and other neuropeptides are released as a part of the stress response and help regulate an individual's metabolism and other systems to withstand the stressor (Mariotti, 2015) successfully.

The effect of stress on the nervous system has shown that it leads to structural modifications in certain parts of the brain (Yaribeygi et al., 2017). These modifications range from loss of volume of certain structures to differences in neuronal plasticity because of atrophy and low spine density in regions like the prefrontal cortex (PFC) and the limbic system. Such alterations were found in patients suffering from mental disorders like depression, indicating that these modifications can be the root cause of such depressive disorders.

Stressor initiates the sympathetic nervous system and hypothalamic-pituitary-adrenal (HPA) axis, resulting in physiological changes in the body (Kumar et al., 2013). In persistent stress, the HPA axis takes over through the peripheral systems. The hypothalamus delivers the corticotropin-releasing hormone to the pituitary, leading to the stress hormones glucocorticoids (GCs) through the adrenal cortex stimulation by pituitary-derived adrenocorticotropic

hormone. GCs reach the hippocampus and bind to the glucocorticoid receptors (GR), leading to their activation and termination of HPA axis response to intervene against the effects of stressors (Sheng et al., 2021; Osborne et al., 2015). Long-term exposure to stress has cognitive effects as the hippocampus is highly linked to memory strengthening and decision-making (Fogwe et al., 2021). Similarly, GCs contribute to memory adjustments and modification of physical behavior under stress (De Quervain et al., 2016).

PFC is one of the most important brain regions. It regulates our actions, emotions, and thoughts, playing a central role in cognitive control functions. The PFC performs these functions through extensive neuronal connections and the highly sensitive neurochemical environment. Therefore, slight changes in the environment and connections can drastically affect its functioning, e.g., cases of mild stress can lead to loss of the PFC abilities. Constant exposure to stress can lead to excessive modifications in the dendrites of the PFC (Arnsten, 2009).

Brain-derived neurotrophic factor (BDNF) is a widely expressed gene in numerous brain regions and plays a crucial role in the fortification and existence of neurons. In addition, it governs neurogenesis in particular brain areas counting the dentate gyrus (DG) and subventricular zone (SVZ) (Miao et al., 2020). Precursor of brain-derived neurotrophic factor (proBDNF) is firstly produced in the endoplasmic reticulum before its conversion into mature brain-derived neurotrophic factor (mBDNF) (Miao et al., 2020). BDNF has two receptors: p75 neurotrophin receptor (p75NTR) and tropomyosin receptor kinase B (TrkB). They both have a high affinity to proBDNF or mBDNF and work differently. BDNF can cross the blood-brain barrier (BBB) and is released by numerous cells, including neurons (Miao et al., 2020). mBDNF dimerizes and auto-phosphorylates the TrkB receptor after binding, activates various pathways like Ras, phosphatidylinositol 3-kinase (PI3K), phospholipase C-γ (PLC-γ), and mitogen-activated protein kinase (MAPK) pathways. These pathways regulate the development of synapses, growth of dendrites, learning- and memory-processes-dependent synaptic plasticity, survival, and apoptosis of neurons. (Palasz et al., 2020).

The reduction in BDNF mRNA and protein levels has been observed following acute and chronic restraint stress (CRS) in the hippocampus and the PFC. It is stated that the mBDNF levels are downregulated in the hippocampus in rats due to prenatal CRS. Conversely, the inhibition of the proBDNF to the mBDNF conversion process ultimately raises proBDNF levels (Miao et al., 2020).

Different treatments are employed to cope with stress. The traditional approaches include ecotherapy to boost self-esteem, complementary therapies like aromatherapy, exercises, medication, massage, and acupuncture. Conventional approach uses selective serotonin reuptake inhibitors (SSRIs) as the first line drug along with certain supplements (Lauren Ragland, 2021). The downside of conventional therapy is a long list of side effects associated with it. Because of this, researchers are now looking towards the bioactive compounds of human diet. Since diet is an integral part of our lifestyle therefore, a shift in the conventional approach with focus towards bioactive components of human nutrition sounds promising. This project focuses on two bioactive compounds, curcumin and vitamin D, to investigate their cumulative potential as an anti-stress therapy.

Turmeric, or *Curcuma longa*, belongs to the ginger family *Zingiberaceae* and is a popular compound for managing stressful conditions. It is a common spice that comes from the root of *Curcuma longa* and has a bitter, warm flavor (Meng et al., 2018). It is also considered an important herb in traditional Indian and Chinese medicinal systems to treat several ailments like cough, diabetic wounds, hepatic disorders, and cardiovascular diseases (Meng et al., 2018). There are various constituent parts of turmeric, among which curcumin is the main constituent.

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also called diferuloylmethane, is the main natural polyphenol found in the rhizome of *Curcuma spp* including *Curcuma longa* (Hewlings & Kalman, 2017). It is a potent anticancer agent which possesses anti-inflammatory, antioxidative, and anticancer activities. Moreover, curcumin has hepatoprotective, neuroprotective, cardioprotective, hypoglycemic, antirheumatic, and antidiabetic activities (Meng et al., 2018). Curcumin's neuroprotective potential has been used to prevent and treat age-related dementia arising from Alzheimer's Disease (AD), Parkinson's disease, and other neurological diseases of aging(Cole et al., 2007).

Similarly, vitamin D (also called "calciferol") is another popular compound to manage stress. It is naturally present in a few foods and is available as a dietary supplement. It is also produced endogenously when ultraviolet (UV) rays from sunlight strike the skin and trigger vitamin D synthesis in the body (Rockwell et al., 2018). It has two forms: Vitamin D2 (ergocalciferol) and Vitamin D3 (cholecalciferol).

Vitamin D performs many functions in the body. It promotes calcium absorption in the gut and maintains adequate serum calcium and phosphate concentrations to enable normal

mineralization of bone to prevent hypocalcemic tetany (involuntary contraction of muscles, leading to cramps and spasms). Vitamin D has other roles in the body, including reducing inflammation and modulation of cell growth, neuromuscular and immune function, and glucose metabolism (Calcium et al., 2011; International Life Sciences Institute., 2012; Ross et al., 2012). The involvement of vitamin D in the function of the central nervous system is supported by the presence of the enzyme 25(OH)D-1-hydroxylase, responsible for the formation of the active form of vitamin D, and the presence of vitamin D receptors (VDRs) in the brain, mainly in the hypothalamus and dopaminergic neurons of the substantia nigra. Vitamin D is also believed to play a similar role to neurosteroids.

Based on these observations, the current study was conducted to investigate the cumulative effect of curcumin and vitamin D as an anti-stress therapy.

1.1 RESEARCH OBJECTIVES

The research objectives of the study are as follows.

- To develop a physiological and physical stress model of mice
- To study effects of restrained stress on social wellbeing via behavior tests
- To study pharmacological effect of curcumin and vitamin D; alone and in combination with vitamin D, on the social aspects of health in stressed mice via behavior tests.

Chapter 2

LITERATURE REVIEW

2.1 STRESS

The survival of the human body is largely dependent on the proper functioning of homeostasis, the maintenance of the body's internal balance. Stress can be described as the effects of anything that threatens our homeostatic balance. In a stress stimulus, the stressful incident is called the stressor, and the body's response to it is known as the stress response. Even though central nervous system (CNS) initiates an integrative coping mechanism in response to stress, prolonged stress negatively impacts the survival of the organism through tissue disease and damage. (Schneiderman et al., 2005).

2.1.1 Stress Response

A stress response is generated as a coping mechanism to the stressor, the body tries to go back to the balance prior to the stressor to achieve equilibrium. Initially, the cortical centers in the brain can sense the stimulus and response through the activation of certain pathways. These pathways are supported by the limbic system for the stimulation of the renin-angiotensin system, sympathetic–adrenal–medullary axis and HPA axis. The response continues with a complex cascade flow of events that makes up the stress response. Hormones like adrenaline, cortisol and other neuropeptides are released as a part of the response and help in the regulation of the metabolism of an individual and other systems to successfully go through the stressor (Mariotti, 2015).

2.1.2 Types of Stress

Stress is categorized into good stress, tolerable stress, and toxic stress (McEwen et al., 2015). Good stress entails the kind of stress that allows the individual to rise to a challenge or take a risk that proves fruitful in the long run, however, if the results are not very beneficial the individual is able to overcome the feeling through his optimism and adaptivity. Secondly, tolerable stress implies that a person might suffer from distressing events, but they are able to cope with it through their own mental strength and support from their relatives and peers. Such incidents enable the individual to enhance their coping and adaptive strategies. Lastly, toxic

stress is the type of stress in which when an individual is faced with challenging situations, they are unable to cope with it due to lacking mental strength and support. Furthermore, the intensity and length of distress is much more (McEwen et al., 2015).

2.1.3 Effects of Stress

Stress has widespread effects on the body and can be very harmful if there is constant and prolonged exposure. The three categories that can be used to understand the effects are Physical, Cognitive, and Behavioral as shown in Table 2.1.

Table 2.1 Categories of Effects of Stress.

Physical Effects	Pain, Upset Stomach, Insomnia, etc.
Cognitive Effects	Worrying, Forgetfulness, Inability to Focus, Poor Judgement, etc.
Behavioral Effects	Changes in Appetite, Procrastinating, Alcohol or Drug Use, Nervousness, etc.

Constant exposure to stress can lead to various effects on the nervous system, including structural changes in the brain (Schneiderman et al., 2005). These changes further lead to differences in stress response, memory, and cognition.

2.1.3.1 Behavioral Effects

These include changes in appetite, procrastinating, alcohol or drug use, and nervousness. Long-term exposure to stress leads to a negative impact on multiple body systems, including cardiovascular (high blood pressure, heart attack), respiratory (asthma, emphysema), mental (memory impairment, depression), reproductive (infertility, menopause), gastrointestinal (heartburn, ulcers) and muscular (pain, muscle contractility).

2.1.3.2 Cognitive Effects

Stress can impact the cognitive ability of an individual and can lead to a short attention span, poor judgment and forgetfulness. Another effect that can manifest is unnecessary worrying and anxiety.

2.1.3.3 Physical Effects

Lastly, the physical effects of stress include sleeplessness, digestive problems and muscular pains. It can also lead to high blood pressure and consequently lead to cardiovascular complications.

2.2 STRESS AND NERVOUS SYSTEM

Studies that discuss the effect of stress on the nervous system have shown that stress leads to structural modifications of the brain (Yaribeygi et al., 2017). The modifications range from loss of volume of certain structures to differences in neuronal plasticity because of atrophy and low spine density in regions like the prefrontal cortex (PFC) and the limbic system. Such alterations were found in patients who were suffering from mental disorders like depression, indicating that these modifications can be the root cause of such depressive disorders. Furthermore, studies that discuss these modifications were aided by the presence of imaging technology that showed these modifications in patients with such disorders. These changes in the brain can also spread out into other connected parts and might lead to behavioral, cognitive, and emotional problems that are caused by stress (Mariotti, 2015).

2.2.1 Hippocampus

Hippocampus is highly linked to memory strengthening and decision making (Fogwe et al., 2021). Stress causes increased glucocorticoids (GC) and norepinephrine (NE) production. GCs reach the hippocampus and bind to the GR (Osborne et al., 2015). The activation of GRs leads to the termination of the HPA axis response in various brain regions, including the paraventricular nucleus, cortex, and hippocampus, which is necessary to intervene in the effects of stressors (Sheng et al., 2021). The hippocampus helps in stress regulation by activating GRs to avoid its effects on memory (Kim & Diamond, 2002).

Traumatic and prolonged stressors cause morphological changes in the hippocampus, e.g., reduced hippocampal volume, decreased number of dendritic spines, pyramidal neuronal branches, and reduced granule neuron production in the dentate gyrus regions of the hippocampus (Kim et al., 2015).

2.2.2 Prefrontal cortex (PFC) and its Functions

The PFC is one of the most important regions of the brain as it is involved in the regulation of our actions, emotions, and thoughts through massive connections with other regions of the brain. It also plays a central role in cognitive control functions, generates mental representations, and retains information without environmental stimuli. The PFC performs these functions through extensive neuronal connections and the highly sensitive neurochemical environment. Therefore, slight changes in the environment and connections can drastically affect the functioning of the PFC. For example, even minute amounts of stress can have a significant negative impact on the working of the PFC. Cases of mild stress can lead to loss of the PFC abilities, while constant exposure to stress can also lead to excessive modifications in the dendrites of the PFC (Arnsten, 2009).

Previously, the studies have shown rapid weakening of the PFC network and cognitive roles in rodents, primates, and humans in exposure to mild, acute, and uncontrollable psychological stress. Exposure to acute and uncontrollable stress causes the higher release of catecholamine levels in the PFC triggering the feedforward (FF) calcium-cAMP signaling pathways. In addition, it causes the opening of the adjacent potassium channels, which speedily weakens synaptic connectivity to decrease persistent firing. These signaling pathways also aggravate exposure to chronic stress causing loss of spines and subsequent marked cognitive impairment (Woo et al., 2021).

2.3 Brain-Derived Neurotrophic Factor (BDNF) GENE

Brain-derived neurotrophic factor (BDNF) plays a crucial role in the fortification and existence of neurons and is widely expressed in numerous brain regions. In addition, it governs neurogenesis in particular brain areas counting the DG and SVZ (Miao et al., 2020).

2.3.1 Brain-Derived Neurotrophic Factor (BDNF) Synthesis

The proBDNF is firstly produced in the endoplasmic reticulum before its conversion into mBDNF (Miao et al., 2020). The proBDNF passes through the Golgi apparatus to the trans-Golgi network (TGN) through constituted and regulated secretory pathways. First, BDNF binds to e lipid-raft-associated sorting receptor carboxypeptidase E (CPE) that sorts it into secretory vesicles of the regulated pathway, as shown in Figure 2.1. These vesicles are then transported to suitable places for activity-dependent secretion Figure 2.1 (Thomas & Davies, 2005).

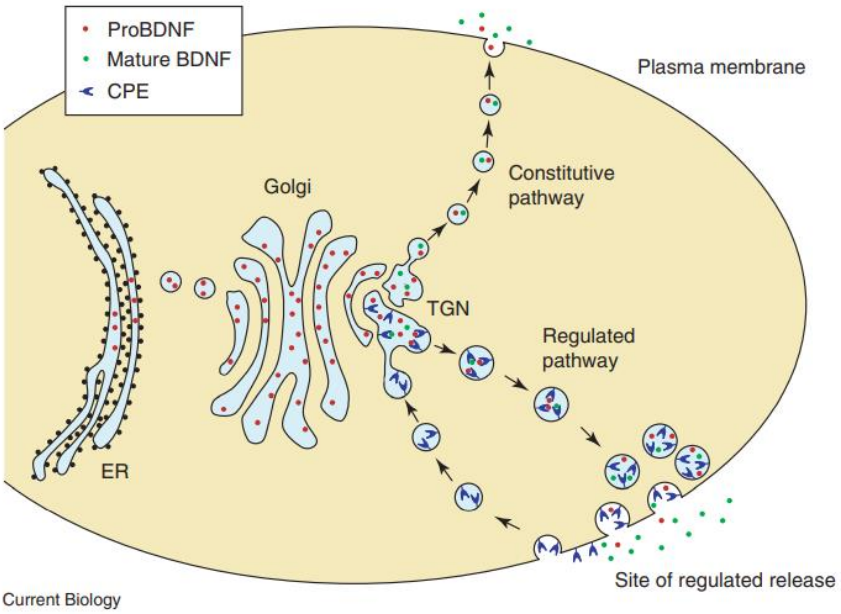

Figure 2.1 The conversion process of proBDNF to mBDNF: proBDNF is first produced in the endoplasmic reticulum and passes to the Golgi apparatus in vesicles. From where it moves toward the TGN and is passed towards regulated and constituted pathways. The vesicles contain convertases that convert proBDNF to mBDNF. Both pathways transport the mBDNF to specific sites depending on its activity. Precursor of Brain-Derived Neurotrophic Factor (proBDNF), Mature Brain-Derived Neurotrophic Factor (mBDNF), carboxypeptidase E *(CPE), trans-Golgi network (TGN), Endoplasmic Reticulum (ER) (Thomas & Davies, 2005).*

Postsynaptic spines and dendrites receive most of the BDNF from the regulated secretory pathway. BNDF also goes through activity-dependent transfer and anterograde axonal transport from pre- to postsynaptic sites. Regulated secretions allow the release of the vesicular contents through triggering signals. TGN and the secretory vesicles contain different protein convertases that cut proBDNF amino-terminal pro-domain and produce mBDNF (Thomas & Davies, 2005). Metal ions like Zn^{+2} and Cu^{+2} are involved in activating BDNF signaling and promoting proBDNF to mBDNF conversion, making them crucial elements in modulating cell activities (Miao et al., 2020).

BDNF has two receptors: the p75NTR and TrkB. They both have a high affinity for proBDNF or mBDNF but work differently. BDNF can cross the BBB and is released by numerous cells, including neurons (Miao et al., 2020).

2.3.2 Brain-Derived Neurotrophic Factor (BDNF) Functions

When proBDNF binds to p75 Neurotrophin Receptor (p75NTR), it activates c-Jun N-terminates (JNK), Ras homolog gene family member A (RhoA), and nuclear factor kappa B (NF-κB) signaling pathways activating neuronal apoptosis, growth, and survival Figure 2.2.

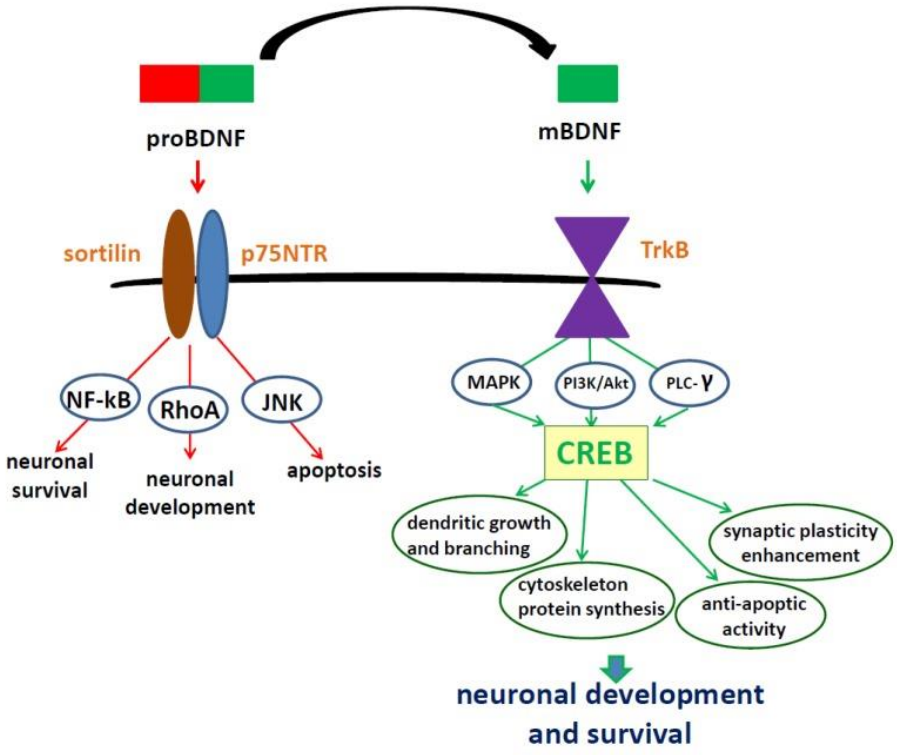

Figure 2.2 The precursor and mature forms of Brain-derived neurotrophic factor (BDNF) play different roles in our brain. The function depends on their interaction with two receptors, p75NTR and TrkB. The precursor form of Brain-derived neurotrophic factor (BDNF) binds to p75NTR and is crucial for apoptosis, neuronal survival, and development. In contrast, mature form Brain-derived neurotrophic factor (BDNF) binds to TrkB and is involved in neuronal survival and development, including dendritic growth, synaptic plasticity, and anti-apoptotic activity. Precursor of Brain-Derived Neurotrophic Factor (proBDNF), *Mature Brain-Derived Neurotrophic Factor* (mBDNF), *p75 Neurotrophin Receptor (p75NTR), tropomyosin receptor kinase B (TrkB, Nuclear factor kappa B (NF-κB), Ras homologous A (Rho A), c-Jun N-terminates (JNK), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and cAMP-response element binding protein (CREB)* (Palasz et al., 2020).

However, mBDNF dimerizes and autophosphorylates the tropomyosin receptor kinase B (TrkB) receptor after binding and activates the Akt, and phospholipase C-γ (PLC-γ) dependent generation of inositol trisphosphate (IP3) and diacylglycerol (DAG) and Ras, phosphatidylinositol 3-kinase (PI3K), PLC-γ, and Ras stimulation of mitogen-activated protein kinase (MAPK) pathways. Figure 2.2 also shows the interaction of proBDNF and mBDNF with p75NTR and TrkB and the resulting actions. These pathways regulate the development of synapses, growth of dendrites, learning- and memory-processes-dependent synaptic plasticity,

survival, and apoptosis of neurons Figure 2.2. Thus, the proper production of BDNF is crucial in regulating stress (Palasz et al., 2020).

2.3.3 Stress and Brain-Derived Neurotrophic Factor (BDNF)

The stressors mainly impact brain functions through two different systems, including the reward-related system and the associated stress system covering the hippocampus and the HPA axis (Miao et al., 2020).

The reduction in BDNF mRNA and protein levels has been observed following acute and chronic restraint stress in the hippocampus and the PFC. However, BDNF expression was raised in the amygdala of the rodents. Further, a report states that the mBDNF levels are downregulated in the hippocampus in rats due to prenatal CRS. The inhibition of the proBDNF to the mBDNF conversion process ultimately raises proBDNF levels (Miao et al., 2020).

2.4 STRESS HORMONE - CORTISOL

Glucocorticoids (GCs) are hormones that act as markers for stress. At low levels, this hormone works to regulate energy in the body. The link with stress becomes significant at high levels (Busch & Hayward, 2009).

Several chemicals fall under the general term "Glucocorticoids"; Cortisol is used in humans, while in rodents, the chemical is called corticosterone. (Ramamoorthy & Cidlowski, 2016). Cortisol or corticosterone is produced by the adrenal glands and released in the cortex of these glands. Its levels are greatest immediately after waking up and minimum before going to sleep (Ramamoorthy & Cidlowski, 2016). It works as a metabolite, and its actions vary according to different levels in the blood as shown in Figure 2.3. Small amounts work well in basal regulation of salts and glucose, shown in black. Under normal external stimuli, the levels vary but stay in the modulated zone, indicating the body’s changes, as a result, shown in white. When stimulated physically or psychologically, the levels rise very high, entering the stress range shown in gray. It is here that the body carries out emergency responses. (Busch & Hayward, 2009).

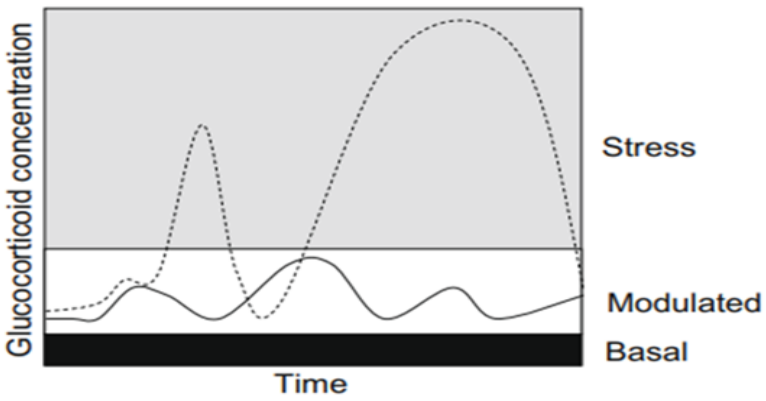

Figure 2.3 Varying levels of Glucocorticoids according to changing internal and external situations (Busch & Hayward, 2009).

2.4.1 Functioning of Glucocorticoids

Cortisol plays its role in cognition by interacting with two receptors, Mineralocorticoid Receptors (MRs) (Type I receptors), and Glucocorticoid Receptors (GRs) (Type II receptors). MRs have a greater affinity, around 6-10 times higher for Cortisol, than GRs (Ouanes & Popp, 2019). Different parts of the brain contain different quantities of these receptors. However, the hippocampus involved in episodic memory contains MRs and GRs, while the (PFC) in charge of the executive functions only contains GRs. MRs produce a positive/augmenting influence on cognitive functions, while GRs have inhibitory effects (Ouanes & Popp, 2019).

As the hippocampus contains both kinds of receptors, only high-affinity receptors, i.e., MRs, are activated during normal conditions when moderate Cortisol levels are produced and cause memory enhancement (Ouanes & Popp, 2019). When Cortisol levels are elevated, MRs are stimulated, and memory is highly enhanced. However, there comes the point when MRs are saturated, and at this point, a further rise in Cortisol levels will bind to the fewer affinity receptors, i.e., GRs. The high activation of GRs will cause damaging impacts on the memory. As the PFC only contains GRs, higher Cortisol levels will also deteriorate the executive functioning produced by PFC (Ouanes & Popp, 2019).

2.4.2 Release and Activation

Glucocorticoid discharged from the adrenal organs is managed by the HPA axis. Assistance from the suprachiasmatic core (SCN) invigorates the paraventricular core (PVN) of the nerve

center to deliver corticotropin-delivering chemicals (CRH) and arginine vasopressin (AVP). These chemicals follow up on the front pituitary, where they enact corticotrophin cells to release adrenocorticotropin chemicals (ACTH) into the overall flow. ACTH follows up on the adrenal cortex to invigorate the union and arrival of glucocorticoids. Once set free from the adrenal organs into the blood flow, glucocorticoids access target tissues to direct physiologic cycles, including digestion, immunity, skeletal development, cardiovascular functioning, comprehension, and reproduction.

Because of its lipophilic nature, glucocorticoids can't be premade and stored in adrenal organs; instead, they are quickly incorporated upon ACTH excitement. This feedforward instrument inside the HPA axis framework is adjusted by the negative feedback of glucocorticoids acting at both the anterior pituitary and inside the nerve center to restrain further arrival of ACTH and CRH.

2.4.3 Relation to stress

HPA axis is the focal point and the main response system for stress faced by humans. It consequently works to reverse stress faced to maintain homeostasis. In persistent stress, HPA axis takes over through the peripheral systems that are activated centrally. During this response, the hypothalamus delivers the corticotropin-releasing hormone to the pituitary, leading to GCs through the adrenal cortex stimulation by pituitary-derived adrenocorticotropic hormone. Numerous tissues and organs contain GC receptors that can respond to the GCs stress. Chronic stress results in situations where stress is overwhelming and equilibrium cannot be fully attained. The GC-dependent negative feedback fails to work in such situations leading to high concentrations of stress molecules that damage the immune system and numerous tissues and organs.

2.4.4 Reference Values

The levels of Cortisol in the blood vary according to the time of blood collection during the day. It is preferred to collect samples during the daytime for testing. In testing, blood collected during the daylight hours (6 am – 10 am), the range of Cortisol for humans is 5.0 – 25.0 mcg/dL (microgram per deciliter). During the night hours (6 pm – 10 pm), the range changes to 5.0 – 15.0 mcg/dL. This change can be due to the body’s metabolic activities and general exposure to hard work and stress during the day instead of during night hours. A higher-than-normal

level can indicate acute illnesses, Cushing's Syndrome, pituitary and adrenal gland tumors, thyroidism, and corticosteroid medications. Higher levels can also be due to stress or the intake of contraceptive pills. A lower-than-normal level in the serum indicates adrenal hyperplasia, hypopituitarism, hypothyroidism, Addison's disease, or possible strokes and head injuries (C. Chernecky & Berger, 2013; C. C. Chernecky & Berger, 2013; Vargatu, 2016).

2.4.5 Importance of Cortisol

Cortisol plays a crucial role in processing memories that trigger certain emotions, often resulting from stressful experiences. They improve the combination of fresh memories while impairing the ability to retrieve material locked in indelible memory. As most phobias and psychological disorders, including post-traumatic stress disorder, result from unforgettable memories locked away in the subconscious mind, the memory-adjusting properties of glucocorticoids have, as of late, impressive translational interest (De Quervain et al., 2016).

2.5 TURMERIC

Turmeric, *Curcuma longa*, is a flowering plant that, belongs to the ginger family, *Zingiberaceae*, the rhizomes of which are used in cooking Figure 2.4 (Priyadarsini, 2014). It is a common spice, has a bitter, warm flavor and is frequently used to flavor or color curry powders, mustards, butter, and cheese (Meng et al., 2018). It is indigenous to India and cultivated widely in the tropical and subtropical regions of South and Southeast Asia including countries China, Indonesia, along with some African areas with a wet and warm tropical climate. It is considered a very important herb in traditional Indian and Chinese medicinal systems for the treatment of several ailments like cough, diabetic wounds, hepatic disorders, and cardiovascular diseases (Meng et al., 2018).

Figure 2.4 Curcuma longa. a) Plant b) Rhizome.

Classification

Botanical name: *Curcuma longa*

Order: Zingiberales

Family: Zingiberaceae

Genus: *Curcuma*

Species: *longa*

2.5.1 Chemical Constituents of Turmeric Powder

There are many types of secondary metabolites including monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, curcuminoids, and the products conjugating curcuminoids with monoterpenes or sesquiterpenes, phenolic compounds, flavonoids (1 dihydroflavonol glucoside and 11 flavonol glycosides), saccharides, steroids, fatty acids, and alkaloids present in turmeric. The important anticancer, antioxidative, anti-inflammatory, antimicrobial, antidiabetic, lipid-decreasing, hepatoprotective, and neuroprotective activities of turmeric are due to these phytochemicals. The explanation of some of the chemical constituents that are present in turmeric are:

2.5.1.1 Curcuminoid

Curcuminoid is the main component of turmeric giving it an orange-yellow color. At present, 50 curcuminoids including three characteristic subtypes have been recognized:

1. Linear-curcuminoids
2. Cyclic-curcuminoids
3. Curcuminoids conjugated with monoterpenes or sesquiterpenes.

The most common curcuminoids found in turmeric are linear curcuminoids, among them the major ones are: 77% Curcumin, 17% demethoxycurcumin (DMC) and 3% bisdemethoxycurcumin (BDMC) (Sandur et al., 2007).

2.5.1.1.1 Curcumin

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), also called diferuloylmethane, is the main natural polyphenol found in the rhizome of *Curcuma longa* (turmeric) and in others *Curcuma spp* (Hewlings & Kalman, 2017).

IUPAC Name: (1E,6E)-1,7-bis (4-hydroxy- 3-methoxyphenyl) -1,6- heptadiene-3,5-dione

Chemical Formula: $C_{21}H_{20}O_6$

Molar mass: 368.38 g/mol

Appearance: Bright yellow-orange powder

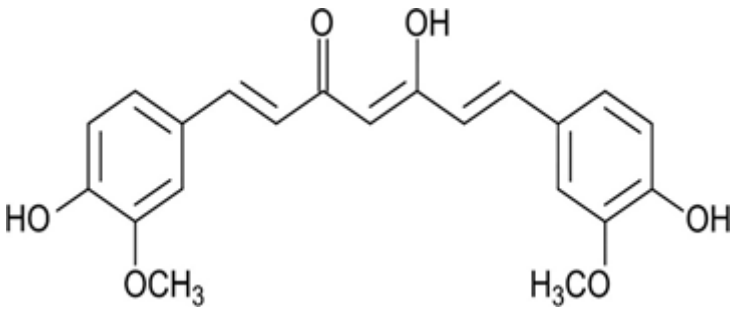

Figure 2.5 Chemical structure of curcumin (Sharifi-Rad et al., 2020).

2.5.1.1.1.1 Physiological Actions of Curcumin

Curcumin is a potent anticancer agent, which possesses anti-inflammatory and antioxidative activities. It is a highly pleiotropic molecule which can modulate several intracellular signaling pathways that maintain cell growth. Moreover, curcumin has hepatoprotective, neuroprotective, cardioprotective, hypoglycemic, antirheumatic, and antidiabetic activities (Meng et al., 2018). It also helps in the management of, metabolic syndrome, arthritis, anxiety, muscle soreness and hyperlipidemia. (Hewlings & Kalman, 2017).

2.5.1.1.1.2 Neuroprotective Actions of Curcumin

Curcumin has exhibited neuroprotective role in multiple models and has great potential for the prevention or treatment of age-related dementia arising from Alzheimer Disease (AD) or cardiovascular disease, Parkinson's disease and other diseases of aging(Cole et al., 2007).

One of curcumin’s lesser-known benefits is its effects on stress and anxiety (Martínez-rodríguez et al., 2022; Rajendran et al., 2022). Curcumin increases resilience to chronic social stress. Furthermore, curcumin supplementation has been found to regulate levels of corticosterone and to increase serotonin levels, a neurotransmitter that stabilizes our mood, feelings of well-being, and happiness (Kulkarni & Dhir, 2010; Aubry et al., 2019; Martínez-rodríguez et al., 2022 and Rajendran et al., 2022).

2.5.1.1.1.3 Pharmacokinetics Properties of Curcumin

Curcumin is poorly soluble in water, chemically unstable and has a low pharmacokinetic profile. Despite its safety and efficacy, the therapeutic potential of curcumin is indeed still debated due its low bioavailability in humans, even when administered at high dosage (12 g/day) (Anand et al., 2007). Generally, the oral bioavailability of curcumin is low due to a relatively low absorption by small intestine coupled to an extensive reductive and conjugative metabolism in the liver and an elimination through the gallbladder. The poor bioavailability is also exacerbated by the curcumin bindings to enterocyte proteins that can modify its structure (Heger et al., 2013).

2.6 VITAMIN D

Vitamin D (also referred to as "calciferol") is a fat-soluble vitamin. It is naturally present in a few foods, and available as a dietary supplement. It is also produced endogenously when

ultraviolet (UV) rays from sunlight strike the skin and trigger vitamin D synthesis in the body (Rockwell et al., 2018).

2.6.1 Vitamin D Forms

Vitamin D has two forms: Vitamin D2 (ergocalciferol) and Vitamin D3 (cholecalciferol)

Figure 2.6 and Table 2.2**.**

Chemical Structure Differences:

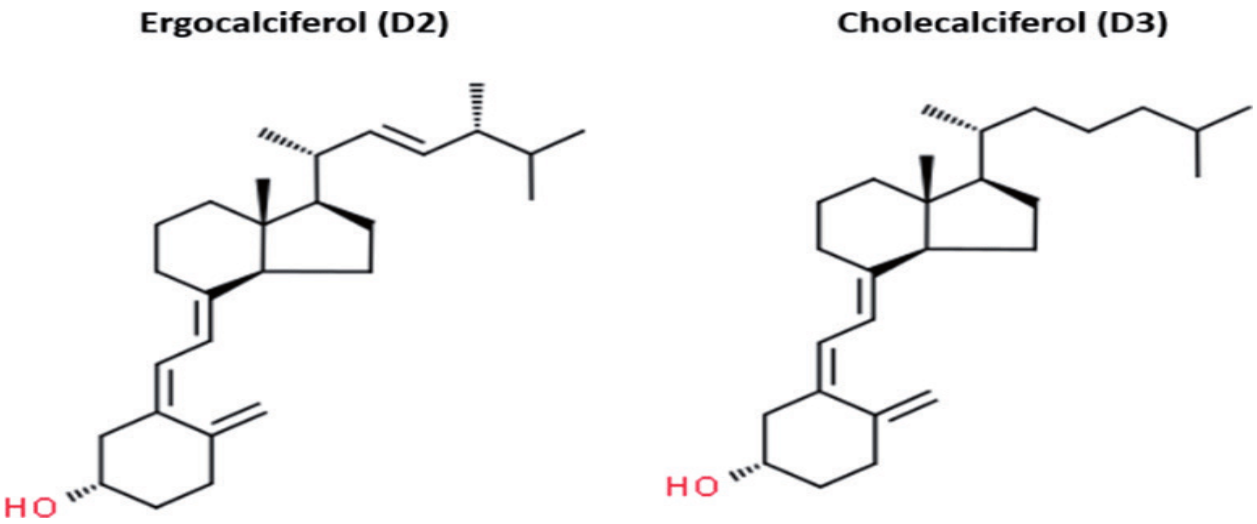

Figure 2.6 Structural difference between two forms of vitamin D2 and Vitamin D3 (F. Bokhari & Albaik, 2020).

Table 2.2 Properties of Vitamin D2 (Ergocalciferol) and Vitamin D3 (Cholecalciferol) ("Vitamin D3: Uses, Interactions, Mechanism of Action | DrugBank Online", 2022).

Properties	Vitamin D_2 (Ergocalciferol)	Vitamin D_3 (cholecalciferol)
IUPAC Name	(1S,3E)-3-[(2Z)-2-[(1R,3aR,7aR)-1-[(E,2S,5R)-5,6-dimethylhept-3-en-2-yl]-7a-methyl-2,3,3a,5,6,7-hexahydro-1H-inden-4-ylidene]ethylidene]-4-methylidenecyclohexan-1-ol	(1S,3Z)-3-[(2E)-2-[(1R,3aS,7aR)-7a-methyl-1-[(2R)-6-methylheptan-2-yl]-2,3,3a,5,6,7-hexahydro-1H-inden-4-ylidene]ethylidene]-4-methylidenecyclohexan-1-ol.
Molecular Formula	$C_{28}H_{44}O$	$C_{27}H_{44}O$
Physical Description	White crystals.	Solid and emulsifiable cream-colored powder.
Solubility	Soluble in alcohol, chloroform, ether, and fatty oils.	Insoluble in water.
Color/Form	White/Colorless crystals.	Colorless crystals.
Odor	Odorless.	Odorless.
Sources	Mushrooms (grown in UV light) Fortified foods Dietary supplements Since vitamin D_2 is cheaper to produce, it's the most common form in fortified foods.	Oily fish and fish oil Liver Egg yolk Butter Dietary supplements

2.6.2 Physiological Actions of Vitamin D in The Body

Vitamin D performs many functions in the body such as promoting calcium absorption in the gut and maintaining adequate serum calcium and phosphate concentrations to enable normal mineralization of bone (involuntary contraction of muscles, leading to cramps and spasms). It is needed for bone growth and bone remodeling by osteoblasts and osteoclasts and helps prevent hypocalcemic tetany, osteomalacia and osteoporosis (Calcium et al., 2011; International Life Sciences Institute., 2012; Ross et al., 2012).

Other roles of vitamin D includes reduction of inflammation as along with the modulation of cell growth, neuromuscular and immune function, and glucose metabolism (Calcium et al., 2011; International Life Sciences Institute., 2012; Ross et al., 2012). Many genes encoding

proteins that regulate cell proliferation, differentiation, and apoptosis are modulated in part by vitamin D.

2.6.3 Neuroprotective Actions of Vitamin D

The involvement of vitamin D in the function of the central nervous system is supported by the presence of the enzyme 25(OH)D-1-hydroxylase, responsible for the formation of the active form of vitamin D, as well as the presence of VDRs in the brain, mainly in the hypothalamus and dopaminergic neurons of the substantia nigra.

Vitamin D is believed to play a similar role to that of neurosteroids. Due to its interaction with the membrane associated rapid response steroid-binding receptor (MARRS) receptors, the hormonal form of vitamin D affects various intracellular metabolic pathways. Moreover, the enzyme 1-hydroxylase and the nuclear VDRs are also present in the microglia, i.e., nonneuronal cells of the CNS. This suggests both autocrine and paracrine effects for calcitriol on nerve cells. The influence of the active form of vitamin D on the nervous system is associated with modifying the production and release of neurotrophic factors such as nerve growth factor (NGF), which is essential for neuron differentiation, as well as increasing the levels of glial cell line-derived neurotrophic factor (GDNF) (Wrzosek et al., 2013).

The neuroprotective role of vitamin D involves the synthesis of proteins binding calcium (Ca) ions (e.g., parvoalbumin) and thus maintaining cellular calcium homeostasis, which is very important for brain cell function. Vitamin D also stimulates the influx of Ca ions through store-operated calcium entry (SOCE) channels, located in cells such as skeletal muscle cells or lymphocytes. This involves stromal interaction molecule (STIM) proteins that play the role of calcium level sensors in these cells and regulate the SOCE process (Wrzosek et al., 2013).

Neuron culture studies showed that 1,25- (OH)D increases glutathione levels in these cells. The reduced form of glutathione (GSH), supplied into nerve cells by astrocytes, is a fundamental antioxidant protecting cells against reactive oxygen species (ROS) and apoptosis caused by oxidation. This suggests an important neuroprotective effect for the active form of vitamin D, by counteracting oxidative damage to the CNS (Harms et al., 2011; Shinpo et al., 2000).

Chapter 3

METHODOLOGY

3.1 ETHICAL STATEMENT

Protocol approval was obtained from the Internal Review board (IRB), Atta-Ur- Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). The Laboratory Animal House of ASAB, NUST, was used for keeping all the animals under sustained conditions. All the experiments including the treatment of animals were done in accordance with the guidelines and rules of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Animals, 2011; Council,2011).

NUST-IRB Certificate

Research Project Title: **Investigating cumulative effect of curcumin & vitamin D as an antistress therapy**

Name of PI:	Dr. Saira Justin
Duration:	12 months
Name of Institution / Department	Healthcare Biotechnology, ASAB, NUST
IRB No.	12-2021-ASAB-02/02

- The project proposal entitled above has been reviewed by the NUST Institutional Review Board Meeting held on Dec 17, 2021.
- The Board approves project proposal on scale and criteria given below to be implemented before/during project execution.

a. Safety Measures:

b. Workspace Requirement:

c. Protection from potential hazards & Risks:

d. Confidentiality Requirements:

Note: The Ethical Review Committee reserves the rights to re-review the project during the project execution to address the suggested guidelines.

Signature & Seal of the Chairman NUST-IRB
Pro-Rector (RIC)
National University of Sciences and Technology
(Dr. Rizwan Riaz)

For official use only

Approved:	☐
Not Approved: (Comments)	☐
Comments: Proposal proforma has been reviewed at ASAB IRB committee meeting. Documents required has been fulfilled. Forwarded to RIC for necessary action and Approval	

Figure 3.1 NUST-IRB Certificate

3.2 ANIMAL MODELS

Our study used 21 BALB/c mice of age 4-6 weeks and weight of 20-45 g. Both male and female mice were used and grouped into 7 groups of 3 mice per group. The mice were provided with 10 g of feed and 300-500 mL of tap water. The treatment was done for 30 days in which behavior tests started from the 24^{th} day. The housing of these mice was done in a controlled environment, at a constant temperature of 25 ± 2°C with a natural 14:10 hours light-dark cycle.

3.3 DRUGS– CURCUMIN AND VITAMIN D

Curcumin (C1386-25G) was obtained from SIGMA and Vitamin D drops (Caltig-D3), manufactured by Dolphin Laboratories were acquired for the treatment.

3.4 TREATMENT OF EXPERIMENTAL MICE

Experimental mice received treatment in feed. Curcumin and vitamin D were added separately and together for the respective groups.

3.4.1 Curcumin Feed

The calculated dose of curcumin used in the study was 30 mg/kg. 0.02 g curcumin powder was added to 100 g finely crushed standard feed. Water was added to make medium-sized pellets that were air-dried and fed to the animals.

3.4.2 Vitamin D Feed

The calculated dose for vitamin D was 1500 IU per kg (Groves et al., 2016). Therefore, one drop of vitamin D containing 400 IU was added to 270 g crushed feed. The pellets were made similarly to curcumin, air-dried, and given to the animals.

3.5 MATERIALS AND INSTRUMENTS

Table 3.1 List of Chemicals and Reagents Used

Chemicals and Reagents	Manufacturer
Curcumin (C1386-25G)	SIGMA
Vitamin D	Caltig-D3, Dolphin Laboratories
Chloroform	SIGMA: UN1888, 24216-2.5L-R
Phosphate Buffer Saline (PBS)	SIGMA-ALDRICH Tablets: P4417, Ident-Nr. 10-100-94
TRIzol™ Reagent	Invitrogen
Isopropanol	SIGMA-ALDRICH: LOT no. STBJ7393
Ethanol (80%)	SIGMA-ALDRICH: 32221-M-2.5L
Ambion Nuclease Free Water	Thermo Scientific
Agarose Powder	BioWORLD Agarose I, Biotechnology Grade
Polymerase Chain Reaction (PCR) Water	Thermo Fisher Scientific
10mM DNTP's	Thermo Fisher Scientific
5mM oligo dT	Thermo Fisher Scientific
RNAse out	Thermo Fisher Scientific
Reverse Transcriptase (RT) Buffer (5X)	Thermo Fisher Scientific
Revert Aid Transcriptase Water	Thermo Fisher Scientific
Actin	Thermo Fisher Scientific
10X Taq Buffer	Thermo Fisher Scientific
10 mM dNTPS mix	Thermo Fisher Scientific
25 mM $MgCl_2$	Thermo Fisher Scientific
Forward Primer	Thermo Fisher Scientific
Reverse Primer	Thermo Fisher Scientific
Taq Polymerase	Thermo Fisher Scientific
Ladder 100bp	Thermo Fisher Scientific
Loading Dye	Thermo Fisher Scientific

Table 3.2 List of Kits Used

Kits	Manufacturers
cDNA Synthesis Kit	Thermofisher

Table 3.3 List of Plastic Consumables and Miscellaneous

Plastic Consumables	**Manufacturer**
Falcon Tubes (50ml)	Accumax
Polymerase Chain Reaction Tubes (RNase-Free, Thin-walled, Frosted Lid 0.2ml PCR Tubes)	Ambion, P/N: AM12225
Micro Pipette	0.1-2ul, 2-20ul, 10-100ul, 100-1000ul Nichipet EXII NICHIRYO
Pipette Tips	10ul, Tarsons: Cat no. 521000 200ul. PORLAB EstaSET pipette tips : Ref no. PTO2-0017
Disposable Syringes	UNISA(PVT) Limited
Latex Powdered Examination Gloves	SRITRANG

Table 3.4 List of Instruments Used

Instruments	**Manufacturer**
Weighing Balance	SF-400 Electronic Digital Scale
Centrifuge (Spectrafuge 24D)	Labnet
Refrigerated Centrifuge	HERMLE Z216MK
Minispin	WEALTEC
Vortex	VELP SCIENTIFICA
Ultrasonic Sonicate	Heilscher UP400S
Refrigerator	Haier
Nanodrop	BERTHOLD: LB915 Colibri
UV Transilluminator	WEALTEC
Thermocycler	Veriti
Airtight Sensitive Weight Machine	SHIMADZU

Table 3.5 List of software used

Software	**Manufacturer**
GraphPad Prism 9.3.1	GraphPad Software, Inc.

3.6 STUDY PLAN

The study plan was to utilize physical means of uneasiness to develop stress in mice. With this increase in stress, we gave treatment using combinations of Curcumin and Vitamin D via oral methods to check their individual and combined effects.

The total duration of testing was 30 days. The behavior tests lasted six days, starting on the 24th day. On the 30th day, the animal was sacrificed, its brain harvested, and RNA was extracted. Expression analysis was done using RT-PCR; our gene of interest expression was measured using our desired primers.

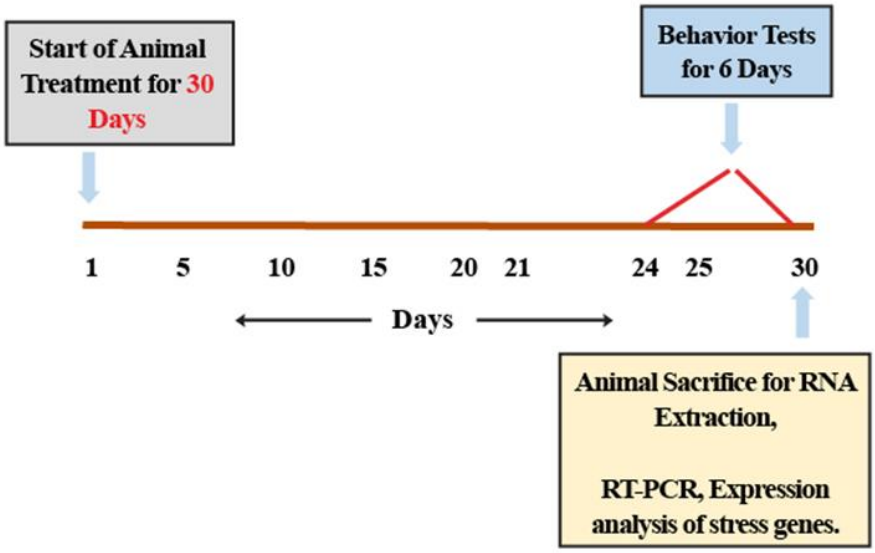

Figure 3.2 Timeline for the treatment of mice.

.

3.7 STUDY DESIGN

The design included seven groups. The mice were 4-6 weeks old, with the male and female mice separated into groups. Details of all the groups are mentioned below:

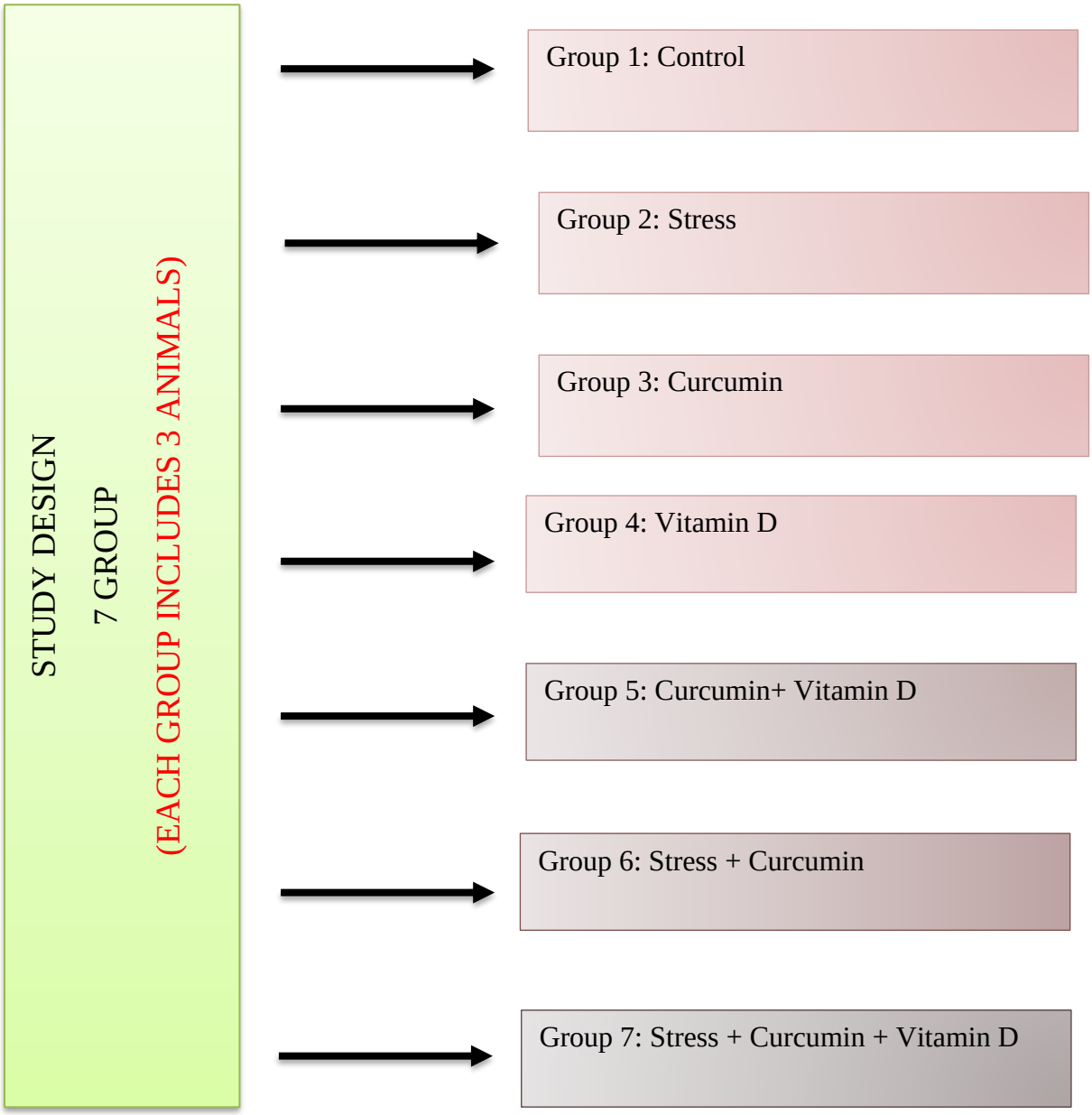

Figure 3.3 Study Design.

1. **Control Group:** Normal mice feed and tap water.
2. **Stress Group:** Normal feed and tap water. However, the restrained stress of 4 hours was given to each mouse daily.
3. **Curcumin Group:** Specialized treatment containing Curcumin (30 mg/kg) alone was given along with tap water. No stress was given throughout the treatment.
4. **Vitamin D:** Specialized feed containing Vitamin D (1500 IU/kg) was given throughout the treatment and tap water.
5. **Curcumin + Vitamin D Group:** Specialized treatment of Curcumin (30 mg/kg) + Vitamin D (1500 IU/kg) was given, along with tap water.
6. **Stress + Curcumin Group:** Specialized feed containing Curcumin (30 mg/kg) was given throughout, along with tap water. Additionally, 4 hours of stress was also given to each mouse daily.
7. **Stress + Curcumin + Vitamin D Group:** Specialized feed of Curcumin (30 mg/kg) + Vitamin D (1500 IU/kg) was given throughout the treatment and tap water. Additionally, the stress of 4 hours was given to each mouse daily.

3.8 RESTRAINED STRESS TO MICE

Body fit restrainers were made from plastic Falcon tubes of 50 mL. Holes were made in the tube at two ends: one to allow the mouse to breathe and the other on the cap to allow the tail to pass. The total stress duration was 4 hours daily. Once behavior tests were conducted, the stress duration was reduced to 3 hours a day (Shoji & Miyakawa, 2020).

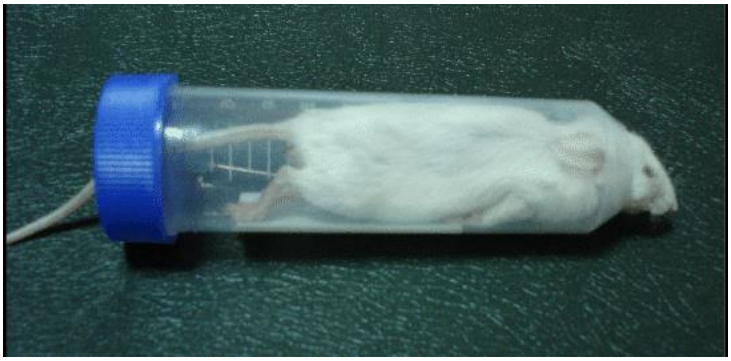

Figure 3.4 Mouse restrained in a modified 50 ml Falcon tube with holes in the top and bottom (Mohamed et al., 2013).

3.9 BEHAVIOR TESTING

Behavior tests began on the 24th day of the treatment and ended on the 29th day. The mice were transferred to the behavior room for 30 minutes before starting the tests to acquaint them with the room's environmental conditions. The behavior test room was aptly illumined, and its temperature was sustained at 25 ±2 ºC. Behavior tests were recorded using a video camera by fixing it on a tripod stand. Absence of human interference and disturbances were ensured during the test procedures.

3.9.1 SOCIAL PREFERENCE

Social interaction is necessary for all animals. Mice also show social behavior to other mice. However, stress and its associated illnesses can affect this behavior. The test was performed to check the sociability and social preference in mice and if the restrained stress has any impact on it (Kaidanovich-Beilin et al., 2011).

Sociability defines the capability of test mouse to interact with a stranger while preference for social novelty represent the probability of spending time with a stranger mouse than with a familiar mouse. Normally, the mice interact more with unfamiliar mice but stressed mice don’t interact much or interact more with familiar mice (Kaidanovich-Beilin et al., 2011).

3.9.1.1 Apparatus

The apparatus employed in this behavior test was a three-chambered rectangular box as shown in the Figure 3.5. Each chamber and the dividing walls were composed of acrylic and painted black. The box has an open middle section with two circular openings providing access to each chamber. Two small and round wired cages were used to hold the stranger mice during the test (Kaidanovich-Beilin et al., 2011).

Figure 3.5 Social Interaction Test Apparatus: The apparatus contains a three-chambered rectangular box with two metal cages to place stranger mice during the test and two boxes to block the entry to two chambers during habituation.

3.9.1.2 Procedure

The apparatus was cleaned using 70% ethanol. The test had three sessions.

Habituation Firstly, the test mouse was placed in the middle chamber to explore the box for 5 minutes. Plastic boxes blocked both chambers to hinder the entrance to other chambers.

Session I: During the first session, an unfamiliar mouse of the same gender as the test mouse was placed in a stranger 1 chamber while the stranger 2 chamber was kept empty. The stranger mouse had no previous interaction with the test mouse. Stranger mouse was put in a round wired glass, which permits only nose or paw contact between the mice. The stranger mice were also habituated for 30 minutes in the behavior room before testing. The openings of the middle section were opened to let the subject mouse explore the whole apparatus for 10 minutes (Satoh et al., 2011).

Session II: Each mouse was given 10 minutes to measure the social preference for the new stranger. Another unfamiliar mouse was added in the second chamber, which was previously empty. The test mouse now had the choice between the two mice, a previously explored mouse (Stranger 1) and a new, unfamiliar mouse (Stranger 2) (Moy et al., 2004).

The mice weren't disturbed during the test. Sessions I and II were recorded using video cameras.

3.9.1.3 Evaluation

The recorded videos were analyzed after the test. The social interaction of the test mice was evaluated by the time spent with stranger 1 and the empty cage in the first session and the time spent with each stranger in the second chamber. The interaction between the test and stranger mice of more than 2 seconds was considered “an interaction”.

Discrimination index (DI) established on the differences in the interaction time of formerly encountered cage (Empty and/or Stranger 1) and Stranger B by test mouse was evaluated as an index of memory and ability. The following formula is used to calculate the DI.

DI = Time Spend with Stranger Mouse ÷ Total Interaction Time

3.9.2 MARBLE BURYING

Mice exhibit digging behavior for finding or storing food, making secure nursery area for the young, and creating a shelter from predators. Similarly, laboratory mice actively dig wood shavings in deep bedding (Deacon, 2006).

Marble burying test is normally used to identify obsessive compulsive (OCD), anxiety-like, or repetitive behavior (Angoa-Pérez et al., 2013). However, some studies suggest that marble burying is also linked to the normal digging behavior of mice (Thomas et al., 2009) rather than anxiety-related behavior (Li et al., 2006; (Lazic, 2015)). So, the test was performed to identify the digging behavior of mice and whether stress has impacted this behavior.

3.9.2.1 Apparatus

Standard mice cages with appropriate top covers cleaned with 70% ethanol were used for the marble-burying test. Fresh and fragrance-free bedding was added 5cm deep to each cage. The bedding was flattened using another cage of a similar size. A total of 20 normal-sized glass marbles of equal dimensions and various colors were placed softly on the bedding surface in 4 rows of 5 marbles as shown in Figure 3.6. The marbles were cleaned with 70% ethanol before the test and washed with detergent after the test.(Angoa-Pérez et al., 2013).

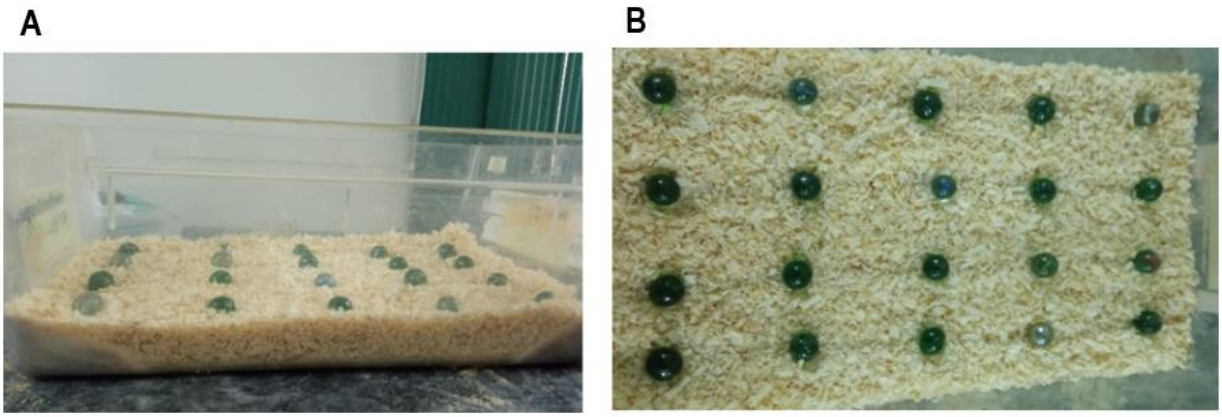

Figure 3.6 Marble Burying Apparatus: (A) The side view of the marble burying setup. (B): The upper view of the marble burying setup containing a total of 20 marble divided in 4 rows of 5.

3.9.2.2 Procedure

The test mouse was carefully placed in a cage, away from the marbles and facing the wall. Food and water weren't given during the test. The mouse remained uninterrupted for 30 minutes. After the test, the mouse was removed cautiously not to disturb the position of the marbles and replaced in its home cage after the test. Marbles were recollected after evaluation, and bedding was disposed off (Angoa-Pérez et al., 2013).

3.9.2.3 Evaluation

Three scorers counted the number of buried marbles. The average of these scores was taken for all test mice. The marbles are counted buried if the bedding shields 2/3rd of their surface area (Angoa-Pérez et al., 2013).

3.9.3 EXIT CIRCLE

Mice exhibit exploratory behavior when placed in a new environment. The exit circle test was performed to identify if stress has impacted their exploratory behavior. Healthy and active mice normally explore the environment. However, injured, or stressed mice don’t explore much rather they sit aside. The test mouse is placed in a steel circle and is given 3 minutes to exit it. Early exit shows increased exploratory behavior while late exit can be due to stress or other illness (Flierl et al., 2009).

3.9.3.1 Apparatus

A long circular steel container containing an exit door was used as apparatus for this test. The steel circle has 30 cm diameter and a small 5cm x 5cm square exit for escaping.

3.9.3.2 Procedure

The mouse was placed in the container facing the walls. It was given 3 minutes to exit the circle (Flierl et al., 2009).

3.9.3.3 Evaluation

Each mouse was given a score depending on the time it took to exit the circle. If the mouse exited the circles in less than 3 minutes, it received 0 point. In contrast, the mice who didn't exit the circle within 3 minutes got 1 point. The healthy mice normally exit within 3 minutes due to their seeking capability (Flierl et al., 2009).

3.10 SERUM ISOLATION

Animals were sacrificed on the 30^{th} day to harvest their brain. Chloroform was used to anesthetize the mice. Blood was taken from the heart during dissection and transferred to yellow blood collection tubes as it contains an anticoagulant Acid Citrate Dextrose (ACD) solution.

The blood was centrifuged at 13000 rpm for 10 minutes to isolate serum. After centrifugation, the yellowish aqueous part at the top was shifted to an eppendorf tube and labeled accordingly. Serum samples were stored at -20ºC for further procedures.

3.11 BRAIN EXTRACTION

The mouse was decapitated to harvest the whole brain as given in Figure 3.7. A few drops of ice-cold Phosphate Buffer Saline (PBS) were added onto the brain (Meyerhoff et al., 2021), and subsequently, the PFC was separated. The PFC was further treated to extract RNA.

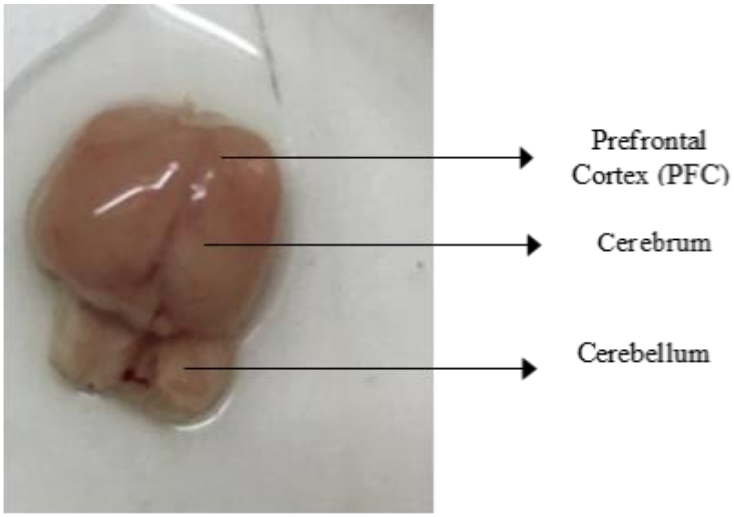

Figure 3.7 Brain harvested from mice after decapitation.

3.12 RNA EXTRACTION

The brain region PFC was used for RNA extraction. For this Invitrogen TRIzol™ Reagent was used according to the manufacturer's instructions.

3.12.1 Sample Lysis and Phases Separation

Samples were lysed and homogenized in Invitrogen TRIzol™ Reagent according to the starting material i.e., tissue. 1 mL of Invitrogen TRIzol™ Reagent per 50-100 mg of tissue was added to the sample. The samples were then subjected to sonication using a Hielscher UP400S Ultrasonic Sonicator. A frequency of 0.2 cycles/ 30 seconds was used twice with 30 seconds intervals. After sonication for further lysis of the sample 0.2 mL of chloroform per 1 mL of Invitrogen TRIzol™ Reagent was added and thoroughly mixed by vortexing for a few seconds. After 20 minutes of incubation, the sample was centrifuged for another 20 minutes at 12,000 rpm at 4°C. The mixture was separated into a lower red phenol-chloroform, interphase, and a colorless upper aqueous phase. Upper aqueous phase containing the RNA was transferred to a new tube by angling the tube at 45° and pipetting the solution out.

3.12.2 RNA Precipitation

To precipitate the RNA, 0.5 mL of isopropanol was added to the aqueous phase, per 1 mL of Invitrogen TRIzol™ Reagent and then incubated for 15 minutes at 4°C. The samples were centrifuged for 15 minutes at 12,000 rpm at 4°C, the total RNA was precipitated as a white gel-like pellet at the bottom of the tube and the supernatant was discarded using a micropipette.

3.12.2 RNA Washing

The pellet was resuspended in 1 mL of 80% ethanol per 1 mL of Invitrogen TRIzol™ Reagent followed by centrifugation for 5 minutes at 7500 rpm at 4°C. The supernatant was discarded using a micropipette and vacuum or air-dried the RNA pellet for 5–10 minutes.

3.13 RNA SOLUBILIZATION

For further processing of RNA, following steps were carried out:

Pellet was re-suspended in 30 µL of RNase-free water, 0.1 mM EDTA, or 0.5% SDS solution and mixed by pipetting up and down and incubated it in a water bath or heat block at 55–60°C for 10–15 minutes, then stored the RNA at –80°C or proceeded for the downstream applications such as nanodrop.

3.14 RNA QUANTIFICATION

The quantity of RNA was examined through Thermo Scientific Nano Drop. For a single sample, three readings were taken for each sample and then the average was calculated. The Thermo Scientific Nano Drop gave the concentration in ng/µL. From the given value, calculations were made for 1000 ng of RNA in 20 µL, for cDNA synthesis

3.15 REVERSE TRANSCRIPTION – PCR (RT-PCR)

After quantification of RNA, reverse transcription (RT-PCR) was performed to get the complementary DNA (cDNA). For this, the recipe for the reaction of 20 µL of PCR product is given in Table 3.6. The complete procedure of making the master mix was performed on ice. At first 1000 ng template RNA, 1 µL Oligo (dT) 18 primer, 6 µL nuclease free water were added and make the total volume up to 12 µL and subjected to heat shock at 65°C for 5 minutes. The rest of the ingredients including 4 µL 5X Reaction Buffer, 1 µL RiboLock RNase Inhibitor (20 U/µL), 2 µL 10 mM dNTP Mix, 1 µL RevertAid M-MuLV RT (200 U/µL) were added to make a total volume of 20 µL. Then Polymerase Chain Reaction (PCR) was performed. The conditions for RT-PCR were 65°C for 5 minutes, 42°C for 60 minutes, and 70°C for 5 minutes. The synthesized cDNA is stored at -20°C until further use.

Table 3.6 Reverse Transcription PCR (RT-PCR) Recipe for Making 20 µL cDNA.

S. No	Ingredient	Amount
1.	Template RNA	1000 ng
2.	Oligo (dT) 18 primer	1µL
3.	Nuclease free water	6 µL
4.	5X Reaction Buffer	4 µL
5.	RiboLock RNase Inhibitor (20 U/µL)	1 µL
6.	10 mM dNTP Mix	2 µL
7.	RevertAid M-MuLV RT (200 U/µL)	1 µL

3.16 PCR-TO CHECK THE EXPRESSION OF ACTIN AND BDNF GENES

To check the expression of the actin and BDNF genes, cDNA will be processed through PCR. The recipe is given in Table 3.7. All the ingredients including 2.5 µL 10x buffer, 2 µL $MgCl_2$, 0.5 µL 10mM dNTPs, and 1 µL both forward and reverse primer specific for genes, 0.5 µL of Taq enzyme per reaction will be added, and then the reaction volume will made up to 23 µL by adding 15.5 µL PCR water. Taq polymerase enzyme will added at the end. The last step involved adding 2 µL of cDNA and then PCR will be performed by giving the following conditions as shown in Figure 3.8. The number of cycles and the annealing temperature of primer will be optimized initially to find out the appropriate conditions where the band becomes sufficiently visible when run on 2 % agarose gel. The steps of denaturation, annealing and extension made one cycle. The numbers of cycles were specific for each primer just like the annealing temperature. In the end, the final extension was carried out at 72°C for 7 minutes and cooling was done at 4°C for infinity.

Table 3.7 PCR Reaction Recipe for a Single cDNA Sample.

S. No	Ingredient	Amount
1.	PCR water	15.5µL
2.	10X Buffer	2.5µL
3.	$MgCl_2$	2µL
4.	10mM dNTPs	0.5µL
5.	Forward Primer	1µL
6.	Reverse Primer	1µL
7.	Taq Polymerase	0.5µL

3.16.1 Gene Expression Study

mRNA levels will be checked for Beta Actin, the housekeeping gene, and for Mus musculus BDNF. List of primers for the BDNF genes along with their annealing temperatures and number of cycles are mentioned below in Table 3.8.

3.16.2 DNA Contamination Check

To check out the genomic DNA contamination, no template control was used, in which PCR water will be added in the reaction mixture instead of cDNA template.

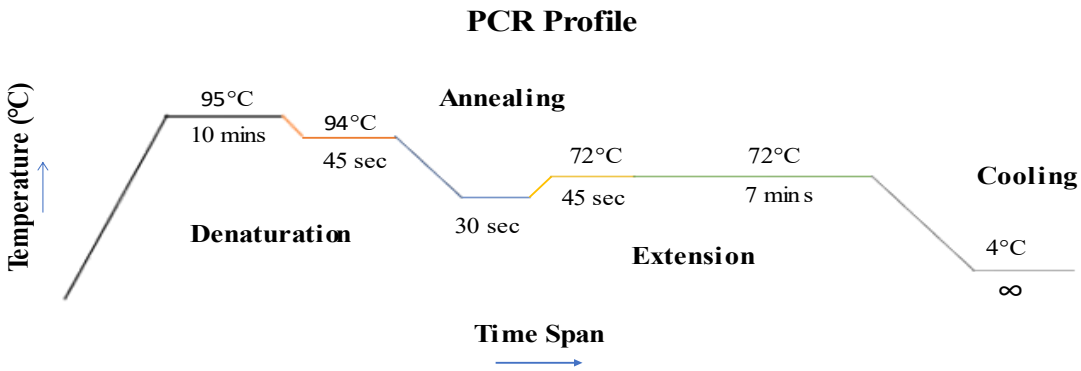

Figure 3.8 PCR Profile for gene expression. Denaturation at 94°C for 45 seconds, primer specific annealing temperature for 30 seconds, and extension at 72°C for 45 seconds.

Table 3.8 List of Primers along with their Annealing Temperatures and No. of Cycles.

S. No	Gene	Primer Sequence (5' to 3')	Annealing Temp (°C)	No. of Cycles
1.	Beta Actin (Mouse)-F	GCCTTCCTTCTTGGGTATGG	60	35
2.	Beta Actin (Mouse)-R	CAGCTCAGTAACAGTCCGC	60	35
3.	BDNF (Mouse)-F	CCCAAAGCTGCTAAAGCGGGAGGAAG	Will be optimized	Will be optimized
4.	BDNF (Mouse)-R	GAAGTGTACAAGTCCGCGTCCTTA	Will be optimized	Will be optimized

3.16.3 Gel Imaging of PCR Products

Actin and BDNF genes amplified PCR products will run on 2% agarose gel using 100bp ladder. Gel images of amplified PCR products will be taken using WEALTEC UV Transilluminator, save and then analyzed.

3.17 STATISTICAL ANALYSIS

GraphPad Prism Software (Version 9.3.1) was used for statistical analysis. Descriptive statistics was applied to the data for the analysis. P value less than 0.05 was considered significant. The data was shown as Mean ± Standard Error of Mean (SEM). 2-way ANOVA was applied for weight, session 1 and session 2 of social interaction test graphs and for feed, marble burying, exit circle, discrimination index (DI) graphs Paired t-Test was applied.

Chapter 4

RESULTS

4.1 EFFECT OF CURCUMIN AND VITAMIN D ON PHYSICAL PARAMETERS

4.1.1 Effect on Weight

Weight is an important physical parameter and any change in the homeostasis and feed consumption can impact body weight. Therefore, weight variation was measured to find any substantial alteration in the weight of mice after getting curcumin and/or vitamin D treatment. Weight was recorded every day during the 30 days treatment to determine the influence of stress, curcumin, and/or vitamin D treatment. However, the mice weight graph is plotted on three different days, i.e., 1, 15, and 29.

As compared to the control group (98.28 ± 1.32), the continuous loss in weight was observed in stress (96.44 ± 1.81) group, that can be due to the disturbance in homeostasis. Stressed mice showed weight gain as a result of administration of curcumin alone (103.8 ± 2.01) whereas an insignificant weight loss when curcumin was given in combination with vitamin D (94.78 ± 2.69) Figure 4.1.

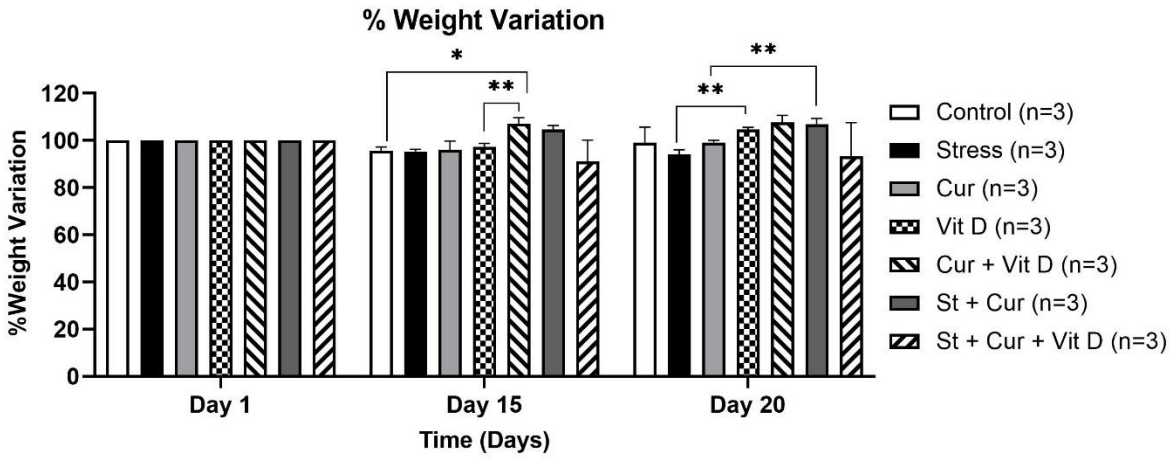

*Figure 4.1 Percentage (%) weight variation: Weight variation was accessed after every 15 days. Weight was reduced in stressed mice (96.44 ± 1.81) as compared to the control (98.28 ± 1.32). However, weight gain was observed following treatment with curcumin alone (103.8 ± 2.01). Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. * = $p \leq 0.05$, ** = $p \leq 0.01$. Ordinary 2-way ANOVA was applied.*

4.1.2 Effect on Feed Consumption

Stress also impacts feeding pattern. Thus, feed. consumption of each group was recorded to determine stress and treatment influence. Each mouse received 10 g of feed daily, and their feed consumption was recorded for 30 days. The graph has been plotted against three days, i.e., 1, 15, and 29 of the treatment plans.

A standardized pattern for feed consumption was shown by control throughout the period of 30 days. As compared to the control group (9.4 ± 0.31), decrease in feed consumption was observed in stress group (7.83 ± 0.85). Stressed mice showed increase feed consumption as a result of administration of either curcumin alone (9.70 ± 0.20) or in combination with vitamin D (8.94 ± 0.58). Furthermore, of all groups only vitamin D group (9.00 ± 1.00) showed the significant difference in comparison to the stress group Figure 4.2.

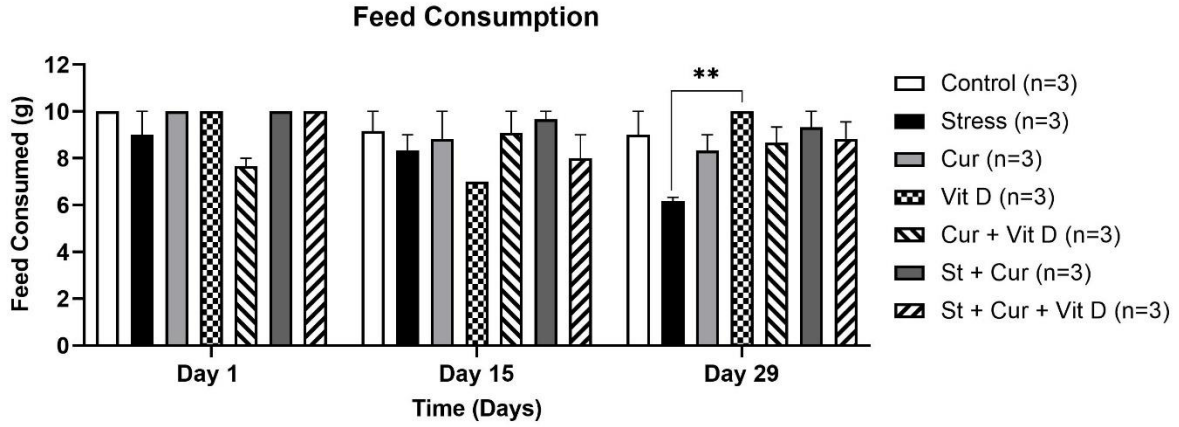

*Figure 4.2 Feed Consumption: Consumption of feed was measured in grams after every 15 days. Feed consumption was reduced in stressed mice (7.83 ± 0.85) as compared to the control (9.38 ± 0.31). However, feed consumption was increased following treatment with curcumin alone (9.67 ± 0.19) or in combination with vitamin D (8.94 ± 0.58) though the difference was not significant in comparison to stressed mice. Furthermore, of all groups only vitamin D group (9.00 ± 1.00) showed the significant difference in comparison to the stress group. Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. **p ≤ 0.01. Ordinary 2-way ANOVA was applied.*

4.2 EFFECT OF CURCUMIN AND VITAMIN D ON BEHAVIOR

4.2.1 Effect on Social Preference

Mice show social behavior to other mice. Stress and other related disorders can reduce this social behavior. Social interaction test was performed to check mice's sociability and social novelty preferences. The test was performed in same sex pairs.

The social affiliation aspect was checked in the first session. As compared to the control group (144.7 ± 17.80), the time spent by the stress group (51.0 ± 11.50) with Stranger 1 was significantly less ($p \leq 0.01$), showing that stressed animals lack innate social propensity. Stressed mice showed improve social behavior as a result of curcumin administration, either alone (98.67 ± 38.83) or in combination with vitamin D (120.7 ± 4.30). A significant difference was seen in the combination therapy (stress + curcumin + vitamin D > stress + curcumin) compared to the stress group. Although no significant cumulative effect of curcumin and vitamin D was observed Figure 4.3.

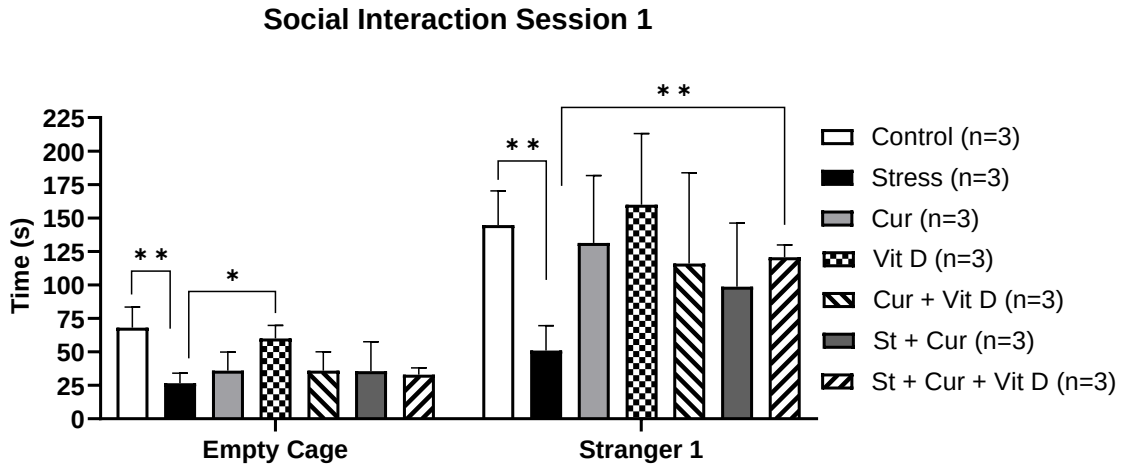

*Figure 4.3 Session 1 Sociability: Social affiliation was assessed via time spent with the empty cage & stranger 1. Social propensity was significantly reduced in stressed mice (51.0 ± 11.50) as compared to the control (144.7 ± 17.8). Improved sociability was observed following treatment with curcumin either alone (98.67 ± 38.83) or in combination with vitamin D (120.7 ± 4.30). A significant difference was seen in the combination therapy compared to stress (St + Cur + Vit D > St + Cur). Although no significant cumulative effect of curcumin & vitamin D was observed. Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. *p ≤ 0.05 and **p ≤ 0.01. Paired t-Test was used.*

In the second session animal's choice between a familiar mouse (Stranger 1) and a novel mouse (Stranger 2) was evaluated to check the animal's preference for social novelty. A non-significant decrease was observed in the stress group (65.0 ± 22.5) compared to the control (74.67 ± 25.33), indicating decreased preference for social novelty. An improved behavior was seen as a result of 30-day treatment (stress + curcumin + vitamin D > stress + curcumin), unfortunately the difference was insignificant in comparison to stressed mice. Furthermore, even though an additive effect was seen in the combination therapy, it was not significant Figure 4.4.

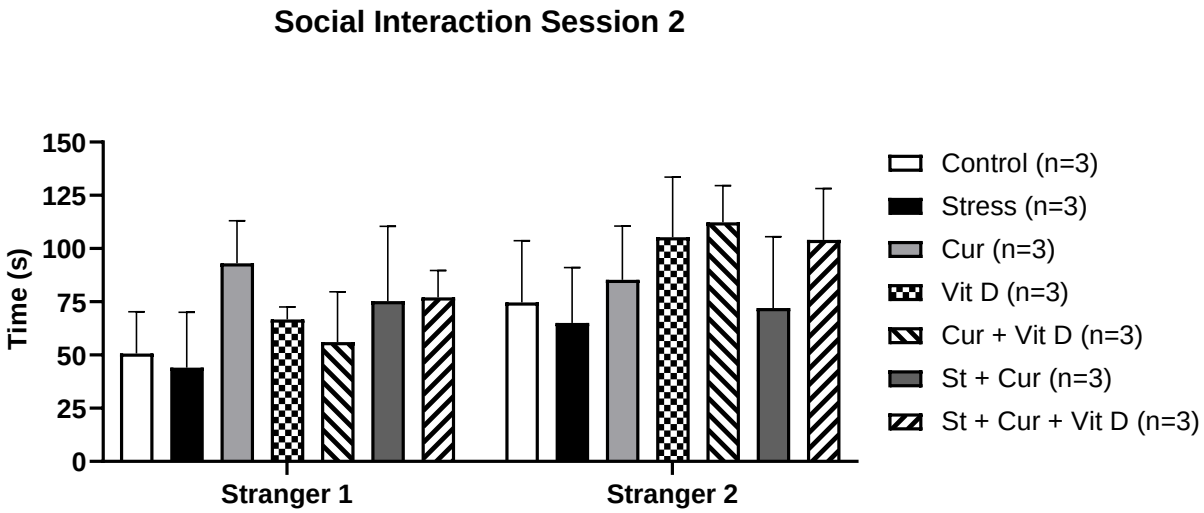

Figure 4.4 Session 2 Social Novelty: Preference between a familiar mouse (Stranger 1) vs a novel mouse (Stranger 2) was checked. A negligible decrease in social novelty was observed in the stress group (65.0 ± 22.5) compared to the control (74.67 ± 25.33). A minor improvement was seen in stressed mice due to curcumin (72.0 ± 28.0) administration. Whereas, an improved behavior was seen in combination therapy (104.0 ± 21.0), although the difference was insignificant. Furthermore, an insignificant additive effect was seen in the combination therapy (St + Cur + Vit D > St + Cur). Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. Paired t-Test was used.

Discrimination Index (DI) was calculated which is a measure of animal's memory and sociability. Interaction time with empty cage and the Stranger 1 was measured in the first session. A negligible decrease in the DI was seen in the stress group (0.64 ± 0.01) as compared to the control group (0.68 ± 0.01). In stressed mice, administration of curcumin either alone (0.76 ± 0.04) or in combination with vitamin D (0.79 ± 0.02) increased the DI (stress + curcumin + vitamin D ≥ stress + curcumin) though the difference was not significant in comparison to the stress group. Furthermore, of all the groups, curcumin group (0.79 ± 0.01) showed the most social aptitude Figure 4.5.

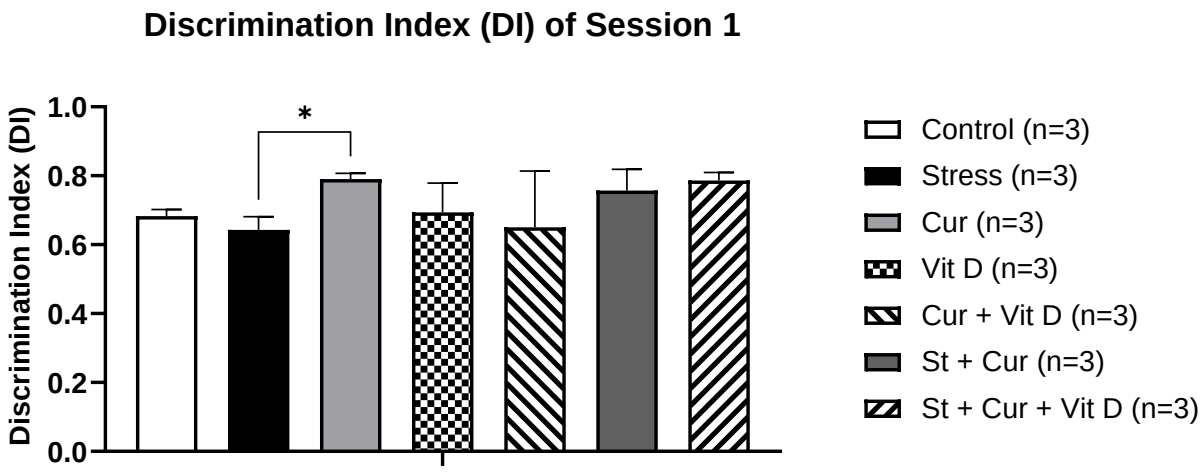

*Figure 4.5 Discrimination index for sociability: Social propensity was significantly reduced in stressed mice (0.64 ± 0.01) as compared to the control (0.68 ± 0.01). Improved sociability was observed following treatment with curcumin either alone (0.76 ± 0.04) or in combination with vitamin D (0.79 ± 0.02) increased the DI (St + Cur + Vit D ≥ St + Cur) though the difference was not significant in comparison to the stress group. Furthermore, of all the groups, Curcumin group (0.79 ± 0.01) showed the most social aptitude. Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. *=p ≤ 0.05. Paired t-Test was used.*

In the second session, DI is measured for social novelty between Stranger 1 and Stranger 2. The stress group (0.61 ± 0.15) exhibited almost the same DI as the control group (0.61 ± 0.02). An insignificant improvement in social novelty was observed following treatment with curcumin either alone (0.50 ± 0.08) or in combination with vitamin D (0.57 ± 0.01), unfortunately no additive effect was seen in the combination therapy Figure 4.6.

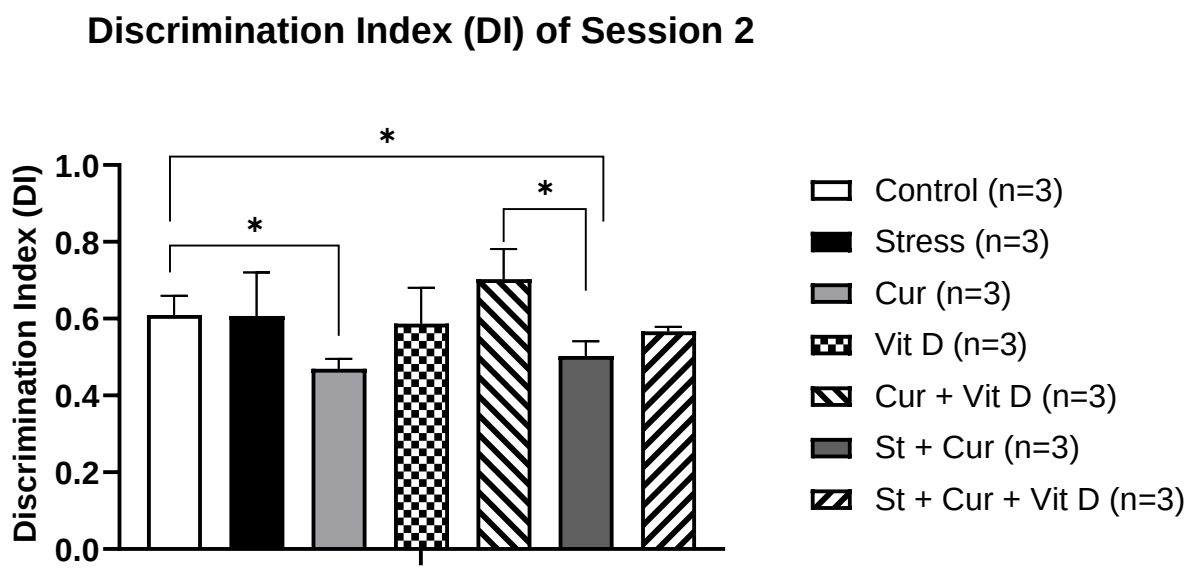

*Figure 4.6 Discrimination index (DI) for social novelty: The stress group (0.61 ± 0.15) exhibited the same DI as the control group (0.61 ± 0.02). No improvement in social novelty was observed following treatment with curcumin alone (0.50 ± 0.08), however improvement was seen in combination therapy with vitamin D (0.57 ± 0.01), (St + Cur + Vit D ≥ St + Cur) though the difference was not significant in comparison to the stress group. Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. *=p ≤ 0.05. Paired t-Test was used.*

Our results indicate that curcumin alone and combined with vitamin D (stress+ curcumin + vitamin D ≥ stress + curcumin) positively impacts sociability and social novelty in stress-induced groups. Unfortunately, no significant additive effect in the combination treatment was observed.

4.2.2 Effect on Marble Burying

The marble burying test for mice is designed to assess anxiety-like behavior, repetitive behavior and innate defensive response. Number of marbles buried depends largely on the intensity of the animal's digging behavior, and is not directed at the marbles themselves. Marble burying form the well-characterized innate response of mice to bury threatening objects i.e., defensive response

Compared to the control group (7.3 ± 0.6), stressed mice marble burying ability was significantly decreased (3.0 ± 0.9). Stressed mice showed increased marble burying due to curcumin administration, either alone (5.3 ± 1.2) or in combination with vitamin D (8.7 ± 1.3). A significant difference was seen in the combination therapy (stress + curcumin + vitamin D > stress + curcumin) in comparison to the stress group. Although no significant cumulative effect

of curcumin and vitamin D was observed. Interestingly, vitamin D group (10.7 ± 0.8) exhibited the most marble burying behavior compared to all the other groups Figure 4.7.

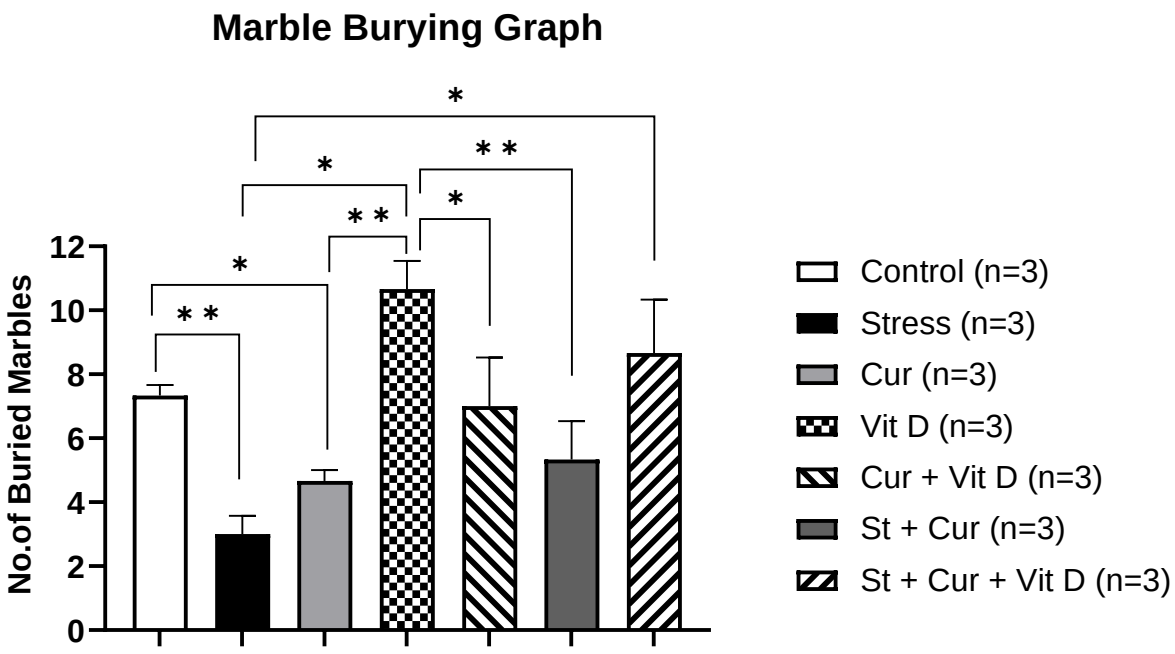

*Figure 4.7 Innate Defensive Response: Marble burying ability was significantly reduced in stress group (3.0 ± 0.9) compared to the control (7.3 ± 0.6). Stressed mice showed increased burying ability due to curcumin administration, either alone (5.3 ± 1.2) or in combination with vitamin D (8.7 ± 1.3), with a significant difference in the combination therapy. However, no significant cumulative effect of curcumin and vitamin D (St + Cur + Vit D > St + Cur) was observed. The average of three scores was taken for each mouse. Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. =p ≤ 0.05 and *=p ≤ 0.01. Paired t-Test was applied.*

4.2.3 Effect on Exit Circle

The exit circle test is a simple exploratory test that helps quantify the curiosity of mice. Owing to their intrinsic inquisitiveness, healthy mouse usually exits the circle within given time of 3 minutes (180 sec). The graphical representation of the results is shown in Figure 4.8.

The control group collectively took (16.67 ± 3.33) seconds to exit the apparatus. The stress group took the maximum time among the groups i.e. (36.0 ± 10.50) seconds exhibiting impaired behavior. Stressed mice showed improvement as a result of curcumin administration either alone (24.67 ± 0.33) or in combination with vitamin D (17.33 ± 1.67). However, all the observations were insignificant.

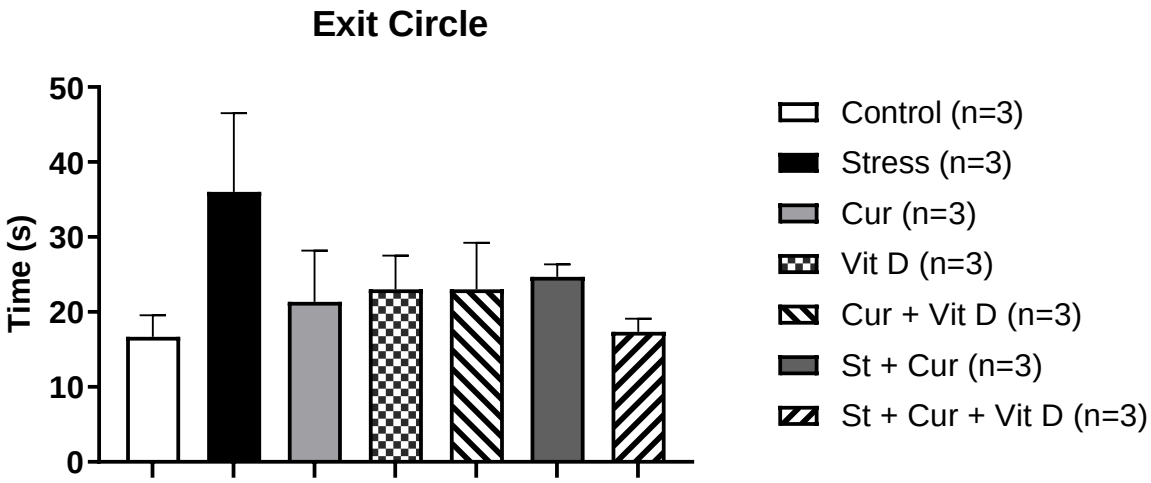

Figure 4.8 Intrinsic Inquisitiveness & Exploratory Behavior: Compared to the control group (16.67±3.33), time taken by stress group (36.0±10.50) to exit the circle was increased, showing impaired exploratory behavior. Stressed mice showed improvement as a result of curcumin administration, either alone (24.67±0.33) or in combination with vitamin D (17.33±1.67). All the observations were insignificant. Stress (St), curcumin (Cur), vitamin D (Vit D). Error bars represent mean ± SEM; n= 03. Paired t-Test was applied.

Chapter 5

DISCUSSION

Stress affects homeostasis, which maintains the internal balance of the body (Kumar et al., 2013). Hormones like adrenaline, cortisol, and other neuropeptides are released in response to stress and help regulate an individual's metabolism and other systems to successfully go through the stressful condition (Mariotti, 2015). Persistent stress, causes physiological changes by triggering the sympathetic nervous system and HPA axis (Kumar et al., 2013).

Restrained stress affects the nervous system, leading to the brain's structural modifications (Yaribeygi et al., 2017) making it one of the commonly used stress models. It is a modified form of immobilization stress in which inescapable physical and mental stress is induced (Das et al., 2000). This is a validated experimental stressor which induces both physical and psychological effects at the same time. In the present study chronic stress was induced by restraining animals for 4 hours for 30 days (Khalid et al., 2017).

Curcumin also called as diferuloylmethane, is the main natural polyphenol found in the rhizome of *Curcuma longa* and others *Curcuma spp.* (Hewlings & Kalman, 2017). Curcumin is neuroprotective and is a popular compound for managing stress conditions (Meng et al., 2018). However, its therapeutic potential is limited due to its poor absorption, rapid metabolism, chemical instability, and rapid systemic elimination (Lopresti, 2018). Therefore, scientists are exploring combination treatments of curcumin along with other compounds (Dai et al., 2020).

Another popular compound for managing stress is a steroid hormone, vitamin D (called "calciferol"), which is involved in the functioning of the central nervous system. It is supported by the presence of the enzyme 25(OH)D-1-hydroxylase, responsible for forming the active form of vitamin D and the presence of VDRs in the brain. Vitamin D is believed to play a similar role to that of neurosteroids (Gezen-Ak et al., 2014). Hence, the combination effect of curcumin with vitamin D as an anti-stress therapy was investigated in the present study. The dose of curcumin used for this study was 30 mg/kg, it is considered as a safe dose in mice with no harmful side effects (Cole et al., 2007; Gupta et al., 2013). For humans, dose-escalating studies have indicated the safety of curcumin at doses as high as 12 g/day over 3 months (Gupta et al., 2013). For the present study the dose used for vitamin D was 1500 IU/kg, it is considered as a safe dose with no such side effects (Groves et al., 2016).

As any change in the homeostasis can impact physical parameters like feed consumption and body weight. Stress is usually linked to either weight gain (Kivimäki et al., 2006) or weight loss (Ayanian et al., 2009) in different conditions. Therefore, these factors were observed and recorded throughout the 30 day treatment in the present study. Feed consumed revealed decreased consumption in the stress group (Heinrichs & Richard, 1999; Richard et al., 2002). But overall, there was no drastic change in the percentage weight variation for all the study groups respectively.

To access the impact of stress three behavior tests named as social interaction, marble burying and exit circle, were performed. In the social interaction test, sociability and social novelty were checked. In the first session sociability was accessed. It was seen that social propensity was significantly reduced in stressed mice (51.0 ± 11.50) as compared to the control (144.7 ± 17.80) mice. However, improved sociability was observed following treatment with curcumin either alone or in combination with vitamin D. A significant difference was seen in the combination therapy (stress + curcumin + vitamin D > stress + curcumin) compared to the stress group. In second session, social novelty was accessed. A negligible decrease in social novelty was observed in the stress group (65.0 ± 22.5) as compared to the control group (74.67 ± 25.33). A negligible improvement was seen in stressed mice due to the administration of curcumin. Whereas, an improved behavior was seen in combination therapy, although the difference was insignificant. Overall, stressed mice getting combination therapy performed better than the ones getting monotherapy (stress+ curcumin + vitamin D > stress + curcumin), but unfortunately there was no significant additive effect seen in the combination therapy. Previous studies have revealed improved social behavior of stressed mice following treatment with turmeric (García-Serna & Morales, 2019; Khalid et al., 2017).

In marble burying test innate defensive response was checked, in which the average of three scores was taken for each mouse. The burying ability was significantly reduced in stress group (3.0 ± 0.9) compared to the control group (7.3 ± 0.6). However, stressed mice showed increased burying ability due to curcumin administration either alone or in combination with vitamin D, with a significant difference in the combination therapy (stress + curcumin + vitamin D > stress + curcumin). However, no significant cumulative effect of curcumin and vitamin D was observed.

Moreover, intrinsic inquisitiveness & exploratory behavior were accessed through exit circle test. Stressed mice (36.0 ± 10.50) showed impaired behavior compared to the control group

(16.67 ± 3.33). An improvement was seen in stressed mice as a result of curcumin administration either alone or in combination with vitamin D (stress + curcumin > stress + curcumin + vitamin D). Although the observations were insignificant. Previous studies have revealed improved social behavior of stressed mice following treatment with turmeric (Becker et al., 2005; Khalid et al., 2017).

Our findings highlight the significance of curcumin and vitamin D for improving social preference, innate defensive response and exploratory behavior. This approach might be useful for stressed individuals to improve their isolated behavior.

CONCLUSION

Restrained stress exhibited declined social behavior. Following curcumin administration either alone or in combination with vitamin D, an improvement in sociability, intrinsic inquisitiveness & innate defense response was seen in stressed mice, however, a significant additive effect in the combination therapy was not seen. Further studies are required to investigate the impact of these compounds on the molecular mechanisms, define a beneficial dose for humans and aid in the treatment of other stress-related disorders.

FUTURE DIRECTIONS

The findings of this study indicate that curcumin powder and vitamin D alone and combination might be effect candidates to ameliorate stressful conditions. However, the underlying molecular mechanisms of these natural compounds in the stressed brain are needed to be inquired into further strengthen their therapeutic potential.

Molecular testing of the brain-derived neurotrophic factor (BDNF) gene should be conducted to further investigate its role in stress and neuroplasticity.

REFERENCES

Anand, P., Kunnumakkara, A. B., Newman, R. A., & Aggarwal, B. B. (2007). Bioavailability of curcumin: Problems and promises. *Molecular Pharmaceutics*, *4*(6), 807–818. https://doi.org/10.1021/mp700113r

Angoa-Pérez, M., Kane, M. J., Briggs, D. I., Francescutti, D. M., & Kuhn, D. M. (2013). Marble Burying and Nestlet Shredding as Tests of Repetitive, Compulsive-like Behaviors in Mice. *JoVE (Journal of Visualized Experiments)*, *82*, e50978. https://doi.org/10.3791/50978

Arnsten, A. (Yale). (2009). stress signalling pathways and PFC. *Molecular Biology*, *26*(2), 148. https://doi.org/10.1038/nrn2648.Stress

Aubry, A. V., Khandaker, H., Ravenelle, R., Grunfeld, I. S., Bonnefil, V., Chan, K. L., Cathomas, F., Liu, J., Schafe, G. E., & Burghardt, N. S. (2019). A diet enriched with curcumin promotes resilience to chronic social defeat stress. *Neuropsychopharmacology*, *44*(4), 733. https://doi.org/10.1038/S41386-018-0295-2

Ayanian, J. Z., Block, J. P., He, Y., Zaslavsky, A. M., & Ding, L. (2009). Psychosocial stress and change in weight among US adults. *American Journal of Epidemiology*, *170*(2), 181–192. https://doi.org/10.1093/aje/kwp104

Becker, A., Eyles, D. W., McGrath, J. J., & Grecksch, G. (2005). Transient prenatal vitamin D deficiency is associated with subtle alterations in learning and memory functions in adult rats. Behavioural Brain Research, 161(2), 306–312. https://doi.org/10.1016/J.BBR.2005.02.015

Busch, D. S., & Hayward, L. S. (2009). Stress in a conservation context: A discussion of glucocorticoid actions and how levels change with conservation-relevant variables. Biological Conservation, 142(12), 2844–2853. https://doi.org/10.1016/J.BIOCON.2009.08.013

Calcium, I. of M. (US) C. to R. D. R. I. for V. D. and, Ross, A. C., Taylor, C. L., Yaktine, A. L., & Valle, H. B. Del. (2011). Dietary Reference Intakes for Calcium and Vitamin D. *Dietary Reference Intakes for Calcium and Vitamin D*. https://doi.org/10.17226/13050

Cole, G. M., Teter, B., & Frautschy, S. A. (2007). NEUROPROTECTIVE EFFECTS OF CURCUMIN. *Advances in Experimental Medicine and Biology*, *595*, 197. https://doi.org/10.1007/978-0-387-46401-5_8

Chernecky, C., & Berger, B. (2013). Laboratory Tests and Diagnostic Procedures 6th Edition. *Elevier Saundres, St. Louis*, *1232*, 808–809.

Chernecky, C. C., & Berger, B. J. (2013). Blood gases, arterial (ABG) - blood. *Laboratory Tests and Diagnostic Procedures*, 208–213.

Cockrem, J. F. (2013). Individual variation in glucocorticoid stress responses in animals. *General and Comparative Endocrinology*, *181*(1), 45–58. https://doi.org/10.1016/j.ygcen.2012.11.025

Dai, C., Zhang, X., & Zhang, K. (2020). New Discovery of Curcumin Combination Therapy and Action Mechanism. Evidence-Based Complementary and Alternative Medicine : ECAM, 2020. https://doi.org/10.1155/2020/4793058

Das, A., Kapoor, K., Sayeepriyadarshini, A. T., Dikshit, M., Palit, G., & Nath, C. (2000). Immobilization stress-induced changes in brain acetylcholinesterase activity and cognitive function in mice. Pharmacological Research, 42(3), 213–217. https://doi.org/10.1006/PHRS.2000.0678

Deacon, R. M. J. (2006). Digging and marble burying in mice: simple methods for in vivo identification of biological impacts. *Nature Protocols*, *1*(1), 122–124. https://doi.org/10.1038/NPROT.2006.20

De Quervain, D., Schwabe, L., & Roozendaal, B. (2016). Stress, glucocorticoids and memory: Implications for treating fear-related disorders. *Nature Reviews Neuroscience*, *18*(1), 7–19. https://doi.org/10.1038/nrn.2016.155

F. Bokhari, F., & Albaik, M. (2020). Vitamin D and Its Deficiency in Saudi Arabia. *Vitamin D Deficiency*. https://doi.org/10.5772/INTECHOPEN.88745

Flierl, M. A., Stahel, P. F., Beauchamp, K. M., Morgan, S. J., Smith, W. R., & Shohami, E. (2009). Mouse closed head injury model induced by a weight-drop device. *Nature Protocols*, *4*(9), 1328–1337. https://doi.org/10.1038/nprot.2009.148

Fogwe, L. A., Reddy, V., & Mesfin, F. B. (2021). Neuroanatomy, Hippocampus. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK482171/

García-Serna, A. M., & Morales, E. (2019). Neurodevelopmental effects of prenatal vitamin D in humans: systematic review and meta-analysis. Molecular Psychiatry 2019 25:10, 25(10), 2468–2481. https://doi.org/10.1038/s41380-019-0357-9

Gezen-Ak, D., Dursun, E., & Yilmazer, S. (2014). The Effect of Vitamin D Treatment On Nerve Growth Factor (NGF) Release From Hippocampal Neurons. Noro Psikiyatri Arsivi, 51(2), 157–162. https://doi.org/10.4274/NPA.Y7076

Groves, N. J., Bradford, D., Sullivan, R. K. P., Conn, K. A., Aljelaify, R. F., McGrath, J. J., & Burne, T. H. J. (2016). Behavioural effects of adult Vitamin D deficiency in BALB/c mice are not associated with proliferation or survival of neurons in the adult hippocampus. *PLoS ONE*, *11*(4). https://doi.org/10.1371/journal.pone.0152328

Gupta, S. C., Patchva, S., & Aggarwal, B. B. (2013). Therapeutic Roles of Curcumin: Lessons Learned from Clinical Trials. The AAPS Journal, 15(1), 195. https://doi.org/10.1208/S12248-012-9432-8

Harms, L. R., Burne, T. H. J., Eyles, D. W., & McGrath, J. J. (2011). Vitamin D and the brain. *Best Practice & Research. Clinical Endocrinology & Metabolism*, *25*(4), 657–669. https://doi.org/10.1016/J.BEEM.2011.05.009

Heger, M., van Golen, R. F., Broekgaarden, M., & Michel, M. C. (2013). The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in Relation to cancer. *Pharmacological Reviews*, *66*(1), 222–307. https://doi.org/10.1124/PR.110.004044

Heinrichs, S. C., & Richard, D. (1999). The role of corticotropin-releasing factor and urocortin in the modulation of ingestive behavior. Neuropeptides, 33(5), 350–359. https://doi.org/10.1054/NPEP.1999.0047

Hewlings, S. J., & Kalman, D. S. (2017). Curcumin: A Review of Its Effects on Human Health. *Foods (Basel, Switzerland)*, *6*(10). https://doi.org/10.3390/FOODS6100092

International Life Sciences Institute. (2012). *Present knowledge in nutrition.*

Jagota, A., & Reddy, M. Y. (2007). The effect of curcumin on ethanol induced changes in suprachiasmatic nucleus (SCN) and pineal. *Cellular and Molecular Neurobiology*, *27*(8), 997–1006. https://doi.org/10.1007/S10571-007-9203-8

Kaidanovich-Beilin, O., Lipina, T., Vukobradovic, I., Roder, J., & Woodgett, J. R. (2011). Assessment of social interaction behaviors. In *Journal of Visualized Experiments* (Vol. e2473, Issue 48, pp. 1–6). Journal of Visualized Experiments. https://doi.org/10.3791/2473

Khalid, A., Shakeel, R., Justin, S., Iqbal, G., Shah, S. A. A., Zahid, S., & Ahmed, T. (2017). Pharmacological Effects of Turmeric on Learning, Memory and Expression of Muscarinic

Receptor Genes (M1, M3 and M5) in Stress-induced Mouse Model. *Current Drug Targets*, *18*(13). https://doi.org/10.2174/1389450118666170315120627

Kim, E. J., Pellman, B., & Kim, J. J. (2015). Stress effects on the hippocampus: a critical review. *Learning & Memory*, *22*(9), 411. https://doi.org/10.1101/LM.037291.114

Kim, J. J., & Diamond, D. M. (2002). The stressed hippocampus, synaptic plasticity and lost memories. *Nature Reviews Neuroscience 2002 3:6*, *3*(6), 453–462.

Kivimäki, M., Head, J., Ferrie, J. E., Shipley, M. J., Brunner, E., Vahtera, J., & Marmot, M. G. (2006). Work stress, weight gain and weight loss: evidence for bidirectional effects of job strain on body mass index in the Whitehall II study. International Journal of Obesity 2006 30:6, 30(6), 982–987. https://doi.org/10.1038/sj.ijo.0803229

Kulkarni, S. K., & Dhir, A. (2010). An overview of curcumin in neurological disorders. Indian Journal of Pharmaceutical Sciences, 72(2), 149. https://doi.org/10.4103/0250-474X.65012

Kumar, A., Rinwa, P., Kaur, G., & Machawal, L. (2013). Stress: Neurobiology, consequences and management. Journal of Pharmacy & Bioallied Sciences, 5(2), 91–97. https://doi.org/10.4103/0975-7406.111818

Kugler, J., Lange, K. W., & Kalveram, K. T. (1988). Influence of bleeding order on plasma corticosterone concentration in the mouse. *Experimental and Clinical Endocrinology*, *91*(2), 241–243. https://doi.org/10.1055/S-0029-1210754

Lazic, S. E. (2015). Analytical strategies for the marble burying test: Avoiding impossible predictions and invalid p-values. *BMC Research Notes*, *8*(1). https://doi.org/10.1186/s13104-015-1062-7

Leblanc, E. S., Rizzo, J. H., Pedula, K. L., Ensrud, K. E., Cauley, J., Hochberg, M., & Hillier, T. A. (2012). Associations Between 25-Hydroxyvitamin D and Weight Gain in Elderly Women. *Journal of Women's Health*, *21*(10), 1066. https://doi.org/10.1089/JWH.2012.3506

Leenaars, C. H. C., van der Mierden, S., Durst, M., Goerlich-Jansson, V. C., Ripoli, F. L., Keubler, L. M., Talbot, S. R., Boyle, E., Habedank, A., Jirkof, P., Lewejohann, L., Gass, P., Tolba, R., & Bleich, A. (2020). Measurement of corticosterone in mice: a protocol for a mapping review. *Laboratory Animals*, *54*(1), 26–32. https://doi.org/10.1177/0023677219868499

Li, X., Morrow, D., & Witkin, J. M. (2006). Decreases in nestlet shredding of mice by serotonin uptake inhibitors: Comparison with marble burying. *Life Sciences*, *78*(17), 1933–1939. https://doi.org/10.1016/j.lfs.2005.08.002

Lopresti, A. L. (2018). The Problem of Curcumin and Its Bioavailability: Could Its Gastrointestinal Influence Contribute to Its Overall Health-Enhancing Effects? Advances in Nutrition, 9(1), 41–50. https://doi.org/10.1093/ADVANCES/NMX011

Mariotti, A. (2015). The effects of chronic stress on health: new insights into the molecular mechanisms of brain-body communication. Future Science OA, 1(3), FSO23. https://doi.org/10.4155/fso.15.21

Martínez-rodríguez, A., Martínez-olcina, M., Mora, J., Navarro, P., Caturla, N., & Jones, J. (2022). Anxiolytic Effect and Improved Sleep Quality in Individuals Taking Lippia citriodora Extract. *Nutrients*, *14*(1). https://doi.org/10.3390/NU1401021

Maurya, V. K., & Aggarwal, M. (2017). Factors influencing the absorption of vitamin D in GIT: an overview. *Journal of Food Science and Technology*, *54*(12), 3753. https://doi.org/10.1007/S13197-017-2840-0

McEwen, B. S., Bowles, N. P., Gray, J. D., Hill, M. N., Hunter, R. G., Karatsoreos, I. N., & Nasca, C. (2015). Mechanisms of stress in the brain. Nature Neuroscience, 18(10), 1353–1363. https://doi.org/10.1038/nn.4086

Meng, F., Zhou, Y., Ren, D., & Wang, R. (2018). Turmeric: A Review of Its Chemical. In *Natural and Artificial Flavoring Agents and Food Dyes*. Elsevier Inc. https://doi.org/10.1016/B978-0-12-811518-3/00010-7

Meyerhoff, J., Muhie, S., Chakraborty, N., Naidu, L., Sowe, B., Hammamieh, R., Jett, M., & Gautam, A. (2021). Microdissection of Mouse Brain into Functionally and Anatomically Different Regions. *Journal of Visualized Experiments: JoVE*, *2021*(168), 1–14. https://doi.org/10.3791/61941

Miao, Z., Wang, Y., & Sun, Z. (2020). The relationships between stress, mental disorders, and epigenetic regulation of bdnf. In *International Journal of Molecular Sciences* (Vol. 21, Issue 4). MDPI AG. https://doi.org/10.3390/ijms21041375

Miranda, M., Morici, J. F., Zanoni, M. B., & Bekinschtein, P. (2019). Brain-Derived Neurotrophic Factor: A Key Molecule for Memory in the Healthy and the Pathological Brain.

In *Frontiers in Cellular Neuroscience* (Vol. 13). Frontiers Media S.A. https://doi.org/10.3389/fncel.2019.00363

Miyazawa, T., Nakagawa, K., Kim, S. H., Thomas, M. J., Paul, L., Zingg, J. M., Dolnikowski, G. G., Roberts, S. B., Kimura, F., Miyazawa, T., Azzi, A., & Meydani, M. (2018). Curcumin and piperine supplementation of obese mice under caloric restriction modulates body fat and interleukin-1β. *Nutrition & Metabolism*, *15*(1). https://doi.org/10.1186/S12986-018-0250-6

Moy, S. S., Nadler, J. J., Perez, A., Barbaro, R. P., Johns, J. M., Magnuson, T. R., Piven, J., & Crawley, J. N. (2004). Sociability and preference for social novelty in five inbred strains: an approach to assess autistic-like behavior in mice. *Genes, Brain and Behavior*, 287–302. https://doi.org/10.1111/j.1601-183X.2004.00076.x

Ouanes, S., & Popp, J. (2019). High cortisol and the risk of dementia and alzheimer's disease: A review of the literature. *Frontiers in Aging Neuroscience*, *11*, 43. https://doi.org/10.3389/FNAGI.2019.00043/BIBTEX

Osborne, D. M., Pearson-Leary, J., & McNay, E. C. (2015). The neuroenergetics of stress hormones in the hippocampus and implications for memory. *Frontiers in Neuroscience*, *9*(APR), 1–16. https://doi.org/10.3389/fnins.2015.00164

Palasz, E., Wysocka, A., Gasiorowska, A., Chalimoniuk, M., Niewiadomski, W., & Niewiadomska, G. (2020). BDNF as a Promising Therapeutic Agent in Parkinson's Disease. *International Journal of Molecular Sciences*, *21*(3). https://doi.org/10.3390/IJMS21031170

Parikh, S. J., Edelman, M., Uwaifo, G. I., Freedman, R. J., Semega-Janneh, M., Reynolds, J., & Yanovski, J. A. (2004). The relationship between obesity and serum 1,25-dihydroxy vitamin D concentrations in healthy adults. *The Journal of Clinical Endocrinology and Metabolism*, *89*(3), 1196–1199. https://doi.org/10.1210/JC.2003-031398

Priyadarsini, K. I. (2014). The Chemistry of Curcumin: From Extraction to Therapeutic Agent. *Molecules 2014, Vol. 19, Pages 20091-20112*, *19*(12), 20091–20112. https://doi.org/10.3390/MOLECULES191220091

Rajendran, K., Ramaswamy, R., L., S., Rajendran, R., Bharadwaja, R., K., L., & Deep, D. K. (2022). A randomized, double-blind, parallel, placebo-controlled study to evaluate efficacy and safety of a synergistic multi-herbal extract blend KaraHeartTM in supporting healthy

cholesterol levels. *International Journal of Basic & Clinical Pharmacology*. https://doi.org/10.18203/2319-2003.IJBCP20220737

Ramamoorthy, S., & Cidlowski, J. A. (2016). Corticosteroids. Mechanisms of Action in Health and Disease. Rheumatic Disease Clinics of North America, 42(1), 15–31. https://doi.org/10.1016/j.rdc.2015.08.002

Richard, D., Lin, Q., & Timofeeva, E. (2002). The corticotropin-releasing factor family of peptides and CRF receptors: their roles in the regulation of energy balance. European Journal of Pharmacology, 440(2–3), 189–197. https://doi.org/10.1016/S0014-2999(02)01428-

Rockwell, M., Kraak, V., Hulver, M., & Epling, J. (2018). Clinical management of low vitamin D: A scoping review of physicians' practices. *Nutrients*, *10*(4). https://doi.org/10.3390/NU10040493

Ross, A. C., Caballero, B., Cousins, R. J., Tucker, K. L., & Ziegler, T. R. (2012). Modern nutrition in health and disease: Eleventh edition. *Modern Nutrition in Health and Disease: Eleventh Edition*, 1–1616. https://doi.org/10.1097/01.ccm.0000236502.51400.9f

Sandur, S. K., Pandey, M. K., Sung, B., Ahn, K. S., Murakami, A., Sethi, G., Limtrakul, P., Badmaev, V., & Aggarwal, B. B. (2007). Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and anti-proliferative responses through a ROS-independent mechanism. *Carcinogenesis*, *28*(8), 1765–1773. https://doi.org/10.1093/CARCIN/BGM123

Satoh, Y., Endo, S., Nakata, T., Kobayashi, Y., Yamada, K., Ikeda, T., Takeuchi, A., Hiramoto, T., Watanabe, Y., & Kazama, T. (2011). ERK2 Contributes to the Control of Social Behaviors in Mice. *The Journal of Neuroscience*, *31*(33), 11953. https://doi.org/10.1523/JNEUROSCI.2349-11.2011

Sharifi-Rad, J., Rayess, Y. El, Rizk, A. A., Sadaka, C., Zgheib, R., Zam, W., Sestito, S., Rapposelli, S., Neffe-Skocińska, K., Zielińska, D., Salehi, B., Setzer, W. N., Dosoky, N. S., Taheri, Y., El Beyrouthy, M., Martorell, M., Ostrander, E. A., Suleria, H. A. R., Cho, W. C., … Martins, N. (2020). Turmeric and Its Major Compound Curcumin on Health: Bioactive Effects and Safety Profiles for Food, Pharmaceutical, Biotechnological and Medicinal Applications. *Frontiers in Pharmacology*, *11*, 1021. https://doi.org/10.3389/FPHAR.2020.01021/BIBTEX

Sheng, J. A., Bales, N. J., Myers, S. A., Bautista, A. I., Roueinfar, M., Hale, T. M., & Handa, R. J. (2021). The Hypothalamic-Pituitary-Adrenal Axis: Development, Programming Actions of Hormones, and Maternal-Fetal Interactions. *Frontiers in Behavioral Neuroscience*, *14*(January), 1–21. https://doi.org/10.3389/fnbeh.2020.601939

Shinpo, K., Kikuchi, S., Sasaki, H., Moriwaka, F., & Tashiro, K. (2000). Effect of 1,25-dihydroxyvitamin D3 on cultured mesencephalic dopaminergic neurons to the combined toxicity caused by L-buthionine sulfoximine and 1-methyl-4-phenylpyridine. *Journal of Neuroscience Research*, *62*(3), 374–382. https://doi.org/10.1002/1097-4547(20001101)62:3<374::AID-JNR7>3.0.CO;2-7

Schneiderman, N., Ironson, G., & Siegel, S. D. (2005). Stress and Health: Psychological, Behavioral, and Biological Determinants. Annual Review of Clinical Psychology, 1(1), 607–628. https://doi.org/10.1146/annurev.clinpsy.1.102803.144141

Shoji, H., & Miyakawa, T. (2020). Differential effects of stress exposure via two types of restraint apparatuses on behavior and plasma corticosterone level in inbred male BALB/cAJcl mice. Neuropsychopharmacology Reports, 40(1), 73–84. https://doi.org/10.1002/NPR2.12093

Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L. A., & Paylor, R. (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology*, *204*(2), 361–373. https://doi.org/10.1007/s00213-009-1466-y

Thomas, K., & Davies, A. (2005). Neurotrophins: A ticket to ride for BDNF. In *Current Biology* (Vol. 15, Issue 7). Cell Press. https://doi.org/10.1016/j.cub.2005.03.023

Vargatu, I. (2016). WILLIAMS TEXTBOOK OF ENDOCRINOLOGY. Acta Endocrinologica (Bucharest), 12(1), 113. https://doi.org/10.4183/AEB.2016.113

Vitamin D3: Uses, Interactions, Mechanism of Action | DrugBank Online. (2022). Retrieved 28 May 2022, from https://go.drugbank.com/drugs/DB00169

Woo, E., Sansing, L. H., Arnsten, A. F. T., & Datta, D. (2021). Chronic Stress Weakens Connectivity in the Prefrontal Cortex: Architectural and Molecular Changes. *Chronic Stress*, *5*. https://doi.org/10.1177/24705470211029254

Wrzosek, M., Lukaszkiewicz, J., Wrzosek, M., Jakubczyk, A., Matsumoto, H., Piatkiewicz, P., Radziwon-Zaleska, M., Wojnar, M., & Nowicka, G. (2013). Vitamin D and the central nervous

system. *Pharmacological Reports*, *65*(2), 271–278. https://doi.org/10.1016/S1734-1140(13)71003-X

Xu, Y., Ku, B., Cui, L., Li, X., Barish, P. A., Foster, T. C., & Ogle, W. O. (2007). Curcumin reverses impaired hippocampal neurogenesis and increases serotonin receptor 1A mRNA and brain-derived neurotrophic factor expression in chronically stressed rats. *Brain Research*, *1162*(1), 9–18. https://doi.org/10.1016/J.BRAINRES.2007.05.071

Yaribeygi, H., Panahi, Y., Sahraei, H., Johnston, T. P., & Sahebkar, A. (2017). The impact of stress on body function: A review. EXCLI Journal, 16, 1057–1072. https://doi.org/10.17179/excli2017-480

TURNITIN PLAIGARISM REPORT

Thesis

ORIGINALITY REPORT

11%	10%	12%	7%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	vitamindwiki.com Internet Source	3%
2	Fan-Cheng Meng, Yan-Qing Zhou, Dai Ren, Ruibing Wang, Chunming Wang, Li-Gen Lin, Xiao-Qi Zhang, Wen-Cai Ye, Qing-Wen Zhang. "Turmeric: A Review of Its Chemical Composition, Quality Control, Bioactivity, and Pharmaceutical Application", Elsevier BV, 2018 Publication	2%
3	www.cse.dmu.ac.uk Internet Source	1%
4	www.mdpi.com Internet Source	1%
5	pubmed.ncbi.nlm.nih.gov Internet Source	1%
6	healthjade.net Internet Source	1%
7	coek.info Internet Source	1%
8	www.ncbi.nlm.nih.gov Internet Source	1%
9	Submitted to Stephen F. Austin State University Student Paper	1%
10	Submitted to Southern Cross University Student Paper	1%
11	Handbook of Experimental Pharmacology, 2014. Publication	1%

Exclude quotes On
Exclude bibliography On
Exclude matches < 1%