Investigating Accumulative Effect of Fluoxetine and *Fagonia indica* as an Anti-Stress Therapy

Submitted by:

Aamnah Shakeel (323055)

Leena Sajid (323017)

Murtaz Aziz Ahmad (325540) (Late)

Sameera Zafar (322578)

BS (Applied Biosciences)

Supervisor:

Dr. Saira Justin

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

National University of Sciences and Technology (NUST)

Islamabad, Pakistan.

(2023)

Investigating Accumulative Effect of Fluoxetine and *Fagonia indica* as an Anti-Stress Therapy

Submitted by:

Aamnah Shakeel (323055)

Leena Sajid (323017)

Murtaz Aziz Ahmad (325540) (Late)

Sameera Zafar (322578)

BS (Applied Biosciences)

This thesis is 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

Supervisor:

Dr. Saira Justin

Atta-Ur-Rahman School of Applied Biosciences (ASAB)
National University of Sciences and Technology (NUST)
Islamabad, Pakistan.

(2023)

THESIS ACCEPTANCE CERTIFICATE

It is certified that the final copy of BS FYP Thesis written by **Ms. Aamnah Shakeel** (Reg No. 323055), **Ms. Leena Sajid** (Reg No. 323017), **Mr. Murtaz Aziz Ahmad** (Reg No. 325540) (Late), and **Ms. Sameera Zafar** (Reg No. 322578) of **Atta-Ur-Rahman School of Applied Biosciences (ASAB)** has been vetted by undersigned, found complete in all respects as per National University of Sciences and Technology (NUST) Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for award the degree of Bachelor of Sciences in Applied Biosciences. It is further certified that necessary amendments as pointed out, during final presentation of the scholar, have also been incorporated in the said thesis.

Signature: _____

Dr. Saira Justin
Assistant Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

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

Date: 18-05-2023

Signature (HOD): _____

Dr. Sobia Manzoor
Tenured Professor
Head of Department (HoD)
Dept. of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad
21-6-23

HOD Healthcare Biotechnology: **Prof. Dr. Sobia Manzoor**

Date: 18-05-2023

Signature (Dean/Principal): _____

Dr. Hussnain A. Janjua
Principal
Atta-ur-Rahman School of Applied Biosciences (ASAB)
NUST, Islamabad

Principal ASAB: **Prof. Dr. Husnain Ahmed Janjua**

Date: 18-05-2023

iii

DECLARATION

We certify that this research work titled **"Investigating Accumulative Effect of Fluoxetine and *Fagonia indica* as an Anti-Stress Therapy"** is our own work. The work has not been presented elsewhere for assessment. The work here in was carried out while we were an undergraduate student at Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST) under the supervision of **Dr. Saira Justin.** The material that has been used from other sources has been properly acknowledged / referred.

Signature of Student _____

Aamnah Shakeel

Reg No. 323055

Signature of Student _____

Leena Sajid

Reg No. 323017

Signature of Student _____

Sameera Zafar

Reg No. 322578

Murtaz Aziz Ahmad (Late)

Reg No. 325540

iv

CERTIFICATE FOR PLAGIARISM

It is certified that the BS Thesis Titled **"Investigating Accumulative Effect of Fluoxetine and *Fagonia indica* as an Anti-Stress Therapy"** by Ms. Aamnah Shakeel (Reg No. 323055), Ms. Leena Sajid (Reg No. 323017), Mr. Murtaz Aziz Ahmad (Reg No. 325540) (Late) and Ms. Sameera Zafar (Reg No. 322578), has been examined by me. I undertake that:

a. Thesis has significant new work/knowledge as compared to already published or is 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 own work of the authors i.e., there is no plagiarism

c. There is no fabrication of data or results that the research is not accurately represented in the records. The thesis has been checked using TURNITIN (a copy of the originality report attached) and found within limits as per HEC plagiarism Policy and instructions issued from time to time.

Dr. Saira Justin
Assistant Professor
Deptt of Healthcare Biotechnology
Atta-ur-Rahman School of Applied Biosciences (ASAB), NUST Islamabad

Signature of Supervisor

Dr. Saira Justin

Assistant Professor

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

National University of Sciences and Technology (NUST)

Islamabad, Pakistan

v

"And those who strive in Our (cause), - We will certainly guide them to our Paths: For verily Allah is with those who do right."

Quran 29: 69

DEDICATION

"This work is dedicated to our beloved parents and teachers. Their continuous love and support have been our motivation to strive and move forward. We would like to dedicate our research to Mr. Murtaz Aziz Ahmad (Late), who left us too soon. His love, kindness and cheerful smile will remain in our hearts forever. Further, this study is dedicated to the 54 mice who sacrificed their lives for its successful completion."

ACKNOWLEDGEMENTS

All praises and glory are for the Highest God, the most beneficent, the most merciful who blessed us with the sight to observe, the mind to think, the courage to work more and the light when we were in dark.

We feel great pleasure to have the opportunity to express our earnest regards and gratitude to our supervisor **Dr. Saira Justin**, for her constant and immense support, kindness, guidance, motivation, dedication, deep interest, professionalism and tremendous knowledge. Her faith in us has made this project a success.

We would like to appreciate Prof. Dr. Husnain Janjua, Principal, Atta-ur- Rahman School of Applied Biosciences (ASAB), for his efforts to maintain the institution's mission for research and study. Special thanks to Engr. Javed Mahmood Bukhari, Rector, National University of Sciences and Technology (NUST), for encouraging science and technology by creating research opportunities in NUST.

We owe our deep gratitude to our teachers, Prof. Dr. Muhammad Qasim Hayat, Prof. Dr. Touqeer Ahmad, and Dr. Miriam K. Gomez for their support and valued recommendations. We would like to thank all staff members of ASAB for helping us in any manner which ultimately contributed towards the completion of our research. A very special thanks to all our friends, Ms. Maheen Imdad Chaudhry, Mr. Samiullah, Mr. Abdul Rafay, Mr. Huzaifa Ali Ahfaz, Ms. Haniya Javed, Mr. Syed Tauheed Ahmad and Ms. Ifrah Taqdees for always being a source of comfort and ease for us on our difficult days. Heartfelt thanks to our seniors, Mr. Muhammad Irfan, Ms. Maryam Hamid, Ms. Saleha Aziz, Ms. Zunaira, Ms. Tooba, and Ms. Atiqa, for their guidance and support.

We would also like to thank Ms. Maria Arif and Mr. Shamsul Haq for assisting us in the lab animal house and for the provision of animals. Special thanks to Ms. Fouzia and Mr. Izzat for their help with diagnostic lab. This project wouldn't have been completed without their support.

Above all, our work would not have been possible without the love, support and patience of our families. Without their encouragement and faith in us, this work could not have been possible.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentage
℃	Degree Celsius
≤	Less than equals to
AD	Alzheimer's Disease.
ACTH	Adrenocorticotropin Hormone
ADH	Antidiuretic Hormone
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
BDNF	Brain Derived Neurotropic Factor
cm	Centimeter
CNS	Central Nervous System
CRH	Corticotropin-Releasing Hormone
CYP2D6	Cytochrome P450 Enzyme
DI	Discrimination Index
dl	Deciliter
GC	Glucocorticoid
GC-MS	Gas Chromatography- Mass Spectrometry

HDL	High Density Lipoprotein
HPA	Hypothalamic–Pituitary–Adrenal
K^+	Potassium Ion
Kg	Kilogram
LDL	Low Density Lipoprotein
LFT	Liver Function Test
mg	Milligram
ml	Milliliter
ng	Nanogram
OCD	Obsessive Compulsive Disorder
PFC	Prefrontal Cortex
PTSD	Post Traumatic Stress Disorder
RFT	Renal Function Test
RI	Recognition Index
ROS	Reactive Oxygen Species
SSRI	Selective Serotonin Reuptake Inhibitor
U/L	Units/Liter

ABSTRACT

Introduction: Chronic stress causes structural modifications in brain, ultimately impacting behavior, emotions and cognition. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), acts as the first line of defense against stress, but unfortunately is associated with a long list of side effects. *Fagonia indica* has been found to have antioxidant, anti-inflammatory and anti-cancerous properties.

Objective: To study the cumulative effects of Fluoxetine and *Fagonia indica* on social behavior in restrained stress mice model.

Methods: The study was conducted on 48 female BALB/c mice, randomly divided into groups. Restrained stress was induced for 4 hours for a duration of 30 days. Treatment with Fluoxetine (18 mg/kg/day) and/or *Fagonia indica* plant extract (400 mg/kg/day) was administered orally. Behavior tests were conducted to assess anxiety, sociability, social novelty, intrinsic inquisitiveness, recognition memory and motor coordination. Biochemical tests were performed to check the effects of stress and drug treatments on critical organs, such as liver, kidneys and heart.

Results: Marble burying test revealed increased anxiety levels of stress mice (12.25 ± 3.4) compared to the control group (4.5 ± 1.6). Both of the monotherapies showed significant improvement, with Fluoxetine monotherapy (4.8 ± 1.1), *Fagonia indica* monotherapy (4.5 ± 0.6) and the combination therapy (2.75 ± 0.25), respectively. Insignificantly decreased social propensity was seen in stressed mice (0.58 ± 0.05) compared to the control (0.61 ± 0.03). Improved sociability was seen following treatments, with significant difference observed only in the combination therapy (0.66 ± 0.08). Interestingly, percentage exit circle test and recognition memory index results, revealed no drastic changes among the groups. Beam balance tests revealed impaired motor coordination in stressed mice (2.25 ± 0.75) compared to the control group (0.75 ± 0.25). Significant improvement was seen the *Fagonia indica* monotherapy group (0.75 ± 0.25) and the combination therapy group (0.5 ± 0.3).

Regarding cortisol levels, no significant changes were observed among the groups. Stressed group showed increased ALT (43.3 ± 4.3) and ALP levels (129.3 ± 22.2), compared to the control group, ALT (14.0 ± 6.7) and ALP (64.3 ± 17.1). All three treatments managed to bring ALT levels within

the normal reference range (22-32 U/L). Fluoxetine monotherapy group resulted in high ALP (189.3 ± 22.1) and urea levels (4.6 ± 0.3) which were counteracted by *Fagonia indica* in the combination therapy; ALP (133.0 ± 4.7) and urea (1.6 ± 0.5) respectively. Lipid profile revealed normal total cholesterol (81-208 mg/dl) and LDL: HDL among the groups except for *Fagonia indica* group where high levels were seen. Interestingly Fluoxetine group was found to counteract this in the combination therapy.

Conclusion: Restrained stress exhibited declined social behavior. Following Fluoxetine administration either alone or in combination with *Fagonia indica*, an improvement in anxiety, sociability, intrinsic inquisitiveness and motor coordination was seen. Regarding biochemical tests, overall *Fagonia indica* was able to counteract the adverse effects of Fluoxetine in the combination therapy. Although an improvement was seen in the combination therapy compared to the monotherapies, unfortunately it was not significant. Furthermore, high serum cholesterol and LDL: HDL levels in response to *Fagonia indica* treatment suggest that it might not be advisable for cardiac dysfunction patients. A detailed GCMS analysis of *Fagonia indica* plant extract and further studies with focus on the molecular aspects are needed.

Key Words: Stress, Fluoxetine, *Fagonia indica*, Integrative medicine, Mice model, Behavior tests, Biochemical tests.

INTRODUCTION

Any circumstance that tries to upset the balance between a living organism and its environment is referred to as "stress." Stress can be any internal or external stimulus that triggers a body's biological response (Yaribeygi et al., 2017).

When the body is under stress, multiple pathways are affected, and the normal state of the body is changed to help it cope with it. While this response is appropriate for dealing with the stressor in the short term, it can become harmful if it lasts too long. Depending upon the time, and the intensity of the stress, it can adversely affect the brain size and functioning. Numerous neurological conditions such as Alzheimer's disease (AD), depression, and post-traumatic stress disorder (PTSD) are triggered or aggravated because of chronic stress (Marin et al., 2011).

In reaction to stress, the adrenal glands release a hormone called corticosterone that regulates physiological processes, including glucose metabolism, immunological response, and cardiovascular function. Chronic stress exposure can cause the hypothalamic–pituitary–adrenal (HPA) axis to be dysregulated, which raises glucocorticoid levels. As a result, there may be detrimental impacts on one's health, such as a reduced capacity to handle stress, cognitive impairment, and a higher chance of contracting stress-related diseases including melancholy and anxiety (Pitman et al., 1988).

Antidepressant therapy seeks to relieve depressed symptoms while lowering the risks and difficulties of relapse. Antidepressants have been shown to be beneficial, but it takes a few weeks for them to reach their full potential, and they are also more likely to have unpleasant effects, which can lead to low compliance. One of the most commonly used anti-depressants are the Selective Serotonin Reuptake Inhibiters (SSRIs) (D J David, 2016).

By raising serotonin levels in the brain, SSRIs alleviate depression. One of the chemical messengers, or neurotransmitters, that communicate between brain nerve cells is serotonin (neurons). Serotonin reabsorption (reuptake) into neurons is inhibited by SSRIs. As a result, there is more serotonin accessible, which enhances neural communication. Despite their use for the treatment of depression, SSRIs also have side effects that vary from person to person. These include anxiousness and agitation, indigestion, constipation or diarrhea, blurring of vision, dryness

of mouth, weight loss due to reduced appetite, insomnia, headaches, reduced sex drive and erectile dysfunction. Because of the long list of side effects associated with conventional treatments, in recent years, there has been a shift towards integrative medicine. (Dulawa et al., 2004; Holick et al., 2008).

Fagonia indica contains a high number of flavonoids, saponins, polyphenols and alkaloids all of which confer antioxidative capabilities to the plant (A Atiq-ur-Rehman et al., 2021). Due to this the plant has proven neuroprotective and anti-inflammatory properties, which assist in alleviation of the symptoms and delay the onset of neurodegenerative disorders and neurological problems. *Fagonia indica* may possess antioxidant, anti-inflammatory, immunomodulatory, antibacterial, and anticancer effects, according to certain studies (Rawal et al., 2004a; Ali et al., 2019; S et al., 2021). Furthermore, studies have indicated that *Fagonia indica* may have positive benefits on the cardiovascular system, such as lowering blood pressure, enhancing lipid profiles, and lowering the risk of heart disease.

1.1 Research Objectives

Our research objectives have been demonstrated, keeping in view the extensive literature. These include:

- 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 effects of Fluoxetine and *Fagonia indica* extract; either alone and/or in combination, on cognitive functioning in stressed mice via behavior tests.
- To measure levels of serum cortisol and other biochemical compounds to see effects on metabolism on critical organs and systems.

LITERATURE REVIEW

2.1 Stress

2.1.1 Definition

Any intrinsic or extrinsic stimulus that induces a biological response is known as stress. Stress may be either external with environmental source, or caused by internal perceptions of the individual. The latter form, in turn can produce anxiety and negative emotions and feelings such as pressure, pain, sadness, etc., that result in serious psychological disorders such as post-traumatic stress disorder (PTSD) (Tse et al., 2007).

2.1.2 Stress Response

A compensatory response against any type of stress is referred as a stress response. A stress response serves as an adaptive mechanism to cope with stressors, aiming to restore the body's equilibrium. When a stressor is detected, specific pathways are activated in the cortical centers of the brain. These pathways are supported by the limbic system, which stimulates the renin-angiotensin system, sympathetic-adrenal-medullary axis, and the HPA axis. This initiation of the stress response sets off a complex cascade of hormones such as adrenaline, cortisol, and various neuropeptides to regulate the individual's metabolism and other systems, facilitating the successful adaptation to the stressor (Yaribeygi et al., 2017; Black, 2002; Mariotti, 2015).

2.1.3 Stress vs Anxiety

Experiencing stress, particularly during early life, has been found to have a substantial impact on the probability of developing anxiety and other mental disorders, such as depression. Anxiety disorders are prevalent psychiatric conditions that are frequently accompanied by depression and substance abuse (Bartlett et al., 2017).

The distinction between stress, anxiety, and how they manifest in an individual depends on their nature, severity and presentation. Stress arises when an individual perceives an inability to cope with stressors. On the other hand, anxiety involves experiencing emotions of fear or panic as a response to stress. However, in the case of anxiety, the fear or panic continues even after the stressor has been removed or resolved. Therefore, certain subtypes of anxiety disorders are categorized as "stress-related" due to their association with stressful experiences (Gholami et al., 2017).

2.1.4 Stress and Hormones

When an individual perceives a stressful situation, it initiates the activation of the HPA axis. Within this axis, a vital brain structure often referred to as the "master gland," releases corticotropin-releasing hormone (CRH). The pituitary gland responds by secreting adrenocorticotropic hormone (ACTH), which enters the bloodstream and reaches the adrenal glands, stimulating the release of stress hormones (Figure 2.1) (Lupien et al., 2008). To help the body react to a perceived threat, these hormones cause a series of physiological reactions, such as an increase in heart rate, blood pressure, and alertness (Godoy et al., 2018).

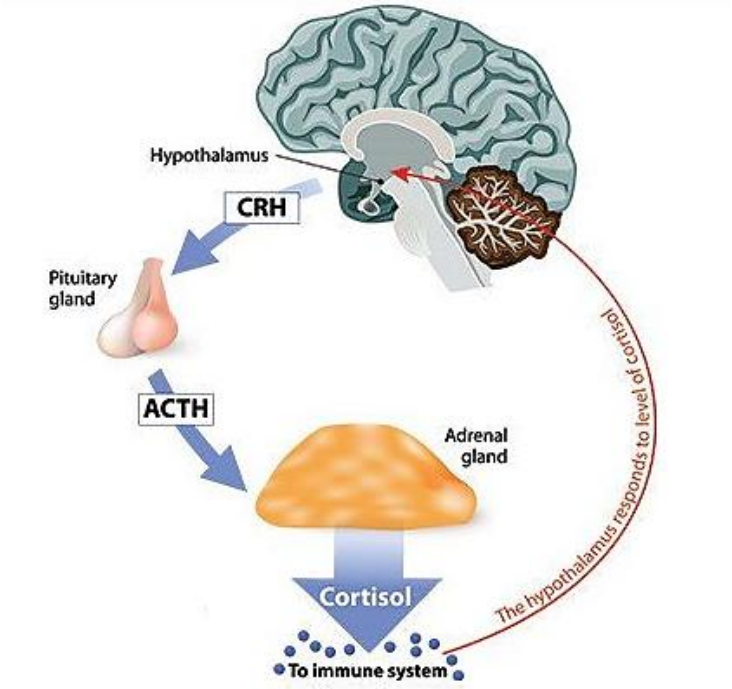


Figure 2.1: Effect of stress on the endocrine system (Cabej, 2018).

2.1.5 Hormonal Imbalance

Under stress, the hormonal balance in the body undergoes alterations. The stress response is linked to increased secretion of multiple hormones, such as glucocorticoids, catecholamines, growth hormone, and prolactin. These hormonal changes serve to enhance the mobilization of energy reserves and adapt the individual to its new circumstance (Ranabir & Keisam, 2011).

2.2 Treatments for Stress

Selective Serotonin Reuptake Inhibitors (SSRIs) are antidepressant drugs that help raise serotonin levels in the brain. They are of assistance in the treatment of depression and other associated disorders. Patient responses to antidepressant drugs can vary, and the full therapeutic benefit may

take several weeks to manifest. Certain precautions apply to SSRIs. They should not be stopped abruptly because this might cause discontinuation syndrome, which is characterized by flu-like symptoms, disorientation, irritability, and mood fluctuations. SSRIs may raise the risk of suicide thoughts or behaviors in some people, particularly children, adolescents, and young adults (James M. Ferguson, 2001).

2.3 Fluoxetine

Fluoxetine, commercially available as Prozac, is a widely used anti-depressant that is used to treat a wide variety of neurological disorders including depression, obsessive compulsive disorder (OCD), post-traumatic stress disorder (PTSD), and panic disorders etc. Fluoxetine falls under the category of SSRIs. This class of anti-depressants inhibit the reuptake of norepinephrine and serotonin, and results in an increase in the concentration of serotonin that improves mood, and energy levels. It is metabolized in the liver by the parent compound, and it has a several-day elimination half-life, allowing a steady state plasma concentration during long-term treatment.

2.3.1 Pharmacokinetics

When Fluoxetine is administered orally, the overall availability of the drug reduces because of its metabolism in the liver. Fluoxetine is extensively metabolized such that only ~2.5% of the administered dose is excreted unchanged in urine. Various oxidative metabolic pathways and conjugation results in its elimination. In the liver, and hepatic enzyme known as cytochrome P450 enzyme (CYP2D6) is involved in the elimination of various SSRIs including Fluoxetine. Fluoxetine works by moderating inhibiting this enzyme. CYP2D6 demonstrates genetic polymorphism. The lack of this enzyme will make it harder for people to metabolize Fluoxetine.

2.3.2 Pharmacodynamics

R- and S-Fluoxetine, the two enantiomers of Fluoxetine that make up the racemic combination, have different pharmacological properties. Both enantiomers of Fluoxetine undergo metabolism to create the active metabolite, norfluoxetine that has an extended half-life than Fluoxetine itself. R-Fluoxetine is an even more potent inhibitor of the reuptake of serotonin than S-fluoxetine. The recommended dosage of fluoxetine for initial treatment of individuals suffering from depression is 20mg/day, if clinical improvement is not seen after several weeks of treatment, the dosage may be increased up to 80mg/day. Patients with hepatic impairment should have their dosage decreased, and dosage reduction should also be considered for the aged and patients with concurrent disorders.

2.3.3 Side Effects

Side effects of Fluoxetine do not differ significantly from those experienced by other SSRIs. Studies show that patients receiving Fluoxetine most frequently experience nausea, sleeplessness, headaches, dry mouth, nervousness and sexual dysfunction etc. (Stahl et al., 2003; Cipriani et al., 2009).

Despite being the first line of defense, the downside of conventional therapy is a long list of side effects associated with it. And that's where integrative medicine steps in. A shift in the conventional approach with focus towards bioactive phytochemicals sounds promising for improved stress management.

2.4 *Fagonia indica*

Fagonia is a flowering plant that belongs to the family Zygophyllaceae which is represented by 35 different *Fagonia* species, including, *Fagonia arabica, Fagonia brugie, Fagonia cretica, Fagonia paulayana, Fagonia mycorrhizal, Fagonia olivieri* and *Fagonia indica*. These are found in dry, semi-arid and hilly regions in most parts of the world including Africa, Middle East, Central Europe, South Asia, and United States of America (USA). All the species have close similarity in their morphology and phytochemistry.

In Pakistan, *Fagonia spp* are found in all dry parts across the country. Molecular basis indicate that that the specie most commonly found here is *Fagonia indica* (Ali et al., 2019; Lam et al., 2014). It is locally known as Dhamasa and Suchi Booti (Kanwal et al., 2021).

2.4.1 Botanical Classification

Table 2.1: Botanical classification of Fagonia indica (Anil et al., 2012).

Kingdom	Plantae
Class	Magnoliopsida
Order	Zygophyllales
Family	Zygophyllaceae
Genus	*Fagonia*
Specie	*indica*

2.4.2 Morphology

Fagonia indica is annual to perennial, spiky shrub or herb with a height of 60 cm to 100 cm and a width of about one meter. It has pink and purple flowers with pointed spines. It has a woody stem with internodes 2.5 cm to 5 cm long at its erect cylindrical branches. This plant has a capsular fruit with locules. The petioles are 3 mm to 30 mm long and the leaves are oppositely arranged with 1-3 foliates. It consists of 2 sets of stipules having sharp thorns (Figure 2.2, Figure 2.3) (Puri & Bhandari, 2014; Farheen et al., 2017; Kanwal et al., 2021).

Figure 2.2: Morphology of Fagonia indica (S et al., 2021).

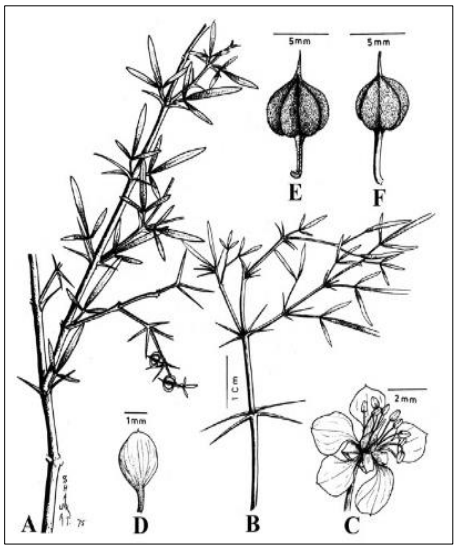


Figure 2.3: Illustration of Fagonia indica taken from eFlora of Pakistan; A, B Fruiting twigs; C Flower; D Petal; E, F Capsular fruit (S et al., 2021).

2.4.3 Traditional Uses

This plant has been found to be used in folklore. In Kharan, Jhalawan, Sindh and Afghanistan, it is used as a treatment for fever. It is even used as a prophylactic against smallpox in Peshawar. Its leaves are boiled and rubbed over skin to cure skin ailments and also used to over neck swelling as it is found to have cooling properties. In the Labella region, its powdered leaves are mixed with milk and stored for three days to be used a remedy for itch. The leaves and twigs are also used against snake bite. Moreover, it is traditionally used to cure toothache, hemorrhoids, stomach issues, asthma, urinary discharge and kidney diseases (Puri & Bhandari, 2014; Farheen et al., 2017; Ali et al., 2019). It is also used by women to help them with the regulation of their menstrual cycle (Anil et al., 2012).

2.4.4 Phytoconstituents

Fagonia indica is composed of many nutrients. The following tables contains in a list of these nutrients present in different parts of the plant:

Table 2.2: Nutritional composition of Fagonia indica (Dastagir et al. in 2014).

Plant Parts	**Moisture (%)**	**Ash (%)**	**Protein (%)**	**Fat (%)**	**Fiber (%)**	**Carbohydrate (%)**	**Gross Energy (Kcal/g)**
Roots	7.4	6.7	9.0	3.7	62.3	10.9	428.9
Stems	9.1	11.9	8.6	4.9	54.3	11.2	400.8
Leaves	8.4	16.5	10.0	8.9	41.3	14.9	399.5
Fruits	7.4	10.7	10.2	6.2	55.2	10.3	423.1
Mean	**8.07**	**11.45**	**9.45**	**5.9**	**53.27**	**11.8**	**413.0**

The plant is known to have many phytochemicals including alkaloids, flavonoids, saponins, phenols, coumarins, terpenoids, sterols and tannins. The following table consists the names of the different phytochemicals present in this plant:

Table 2.3: List of phytochemicals in Fagonia indica (Anil et al., 2012; S et al., 2021; A Atiq-ur-Rehman et al., 2021).

Name of Compound	**Chemical Nature**
Piperine	Alkaloid
Quercetin	Flavonoid
Isorhamnetin-α-3-o-rhamnoside	
Ursolic acid	Terpenoid saponins
Indicasaponin A	
Indicasaponin B	
Nahagenin	
β-amyrin	
Betulinic acid	
Hederagenin	
Oleanolic acid	
Fagonicin	
β-sitosterol	Sterol
Stigmasterol-3-o-β-d- glucoside	
17-(1,5-dimethylhexyl)- 10,13-dimethyl-4-vinylhexadecahydrocyclopenta[a] phenanthren-3-ol	Steroid

2.4.5 Therapeutic potential:

The unique phytochemical composition of the plant, attributes towards the different biological activities, like antioxidant, antifungal, antimicrobial, antitumor, and hepatoprotection (S et al., 2021). The plant extract of *Fagonia indica* has shown to exhibit anti-cancerous activities by inducing cell cycle arrest and apoptosis (Lam et al., 2012).

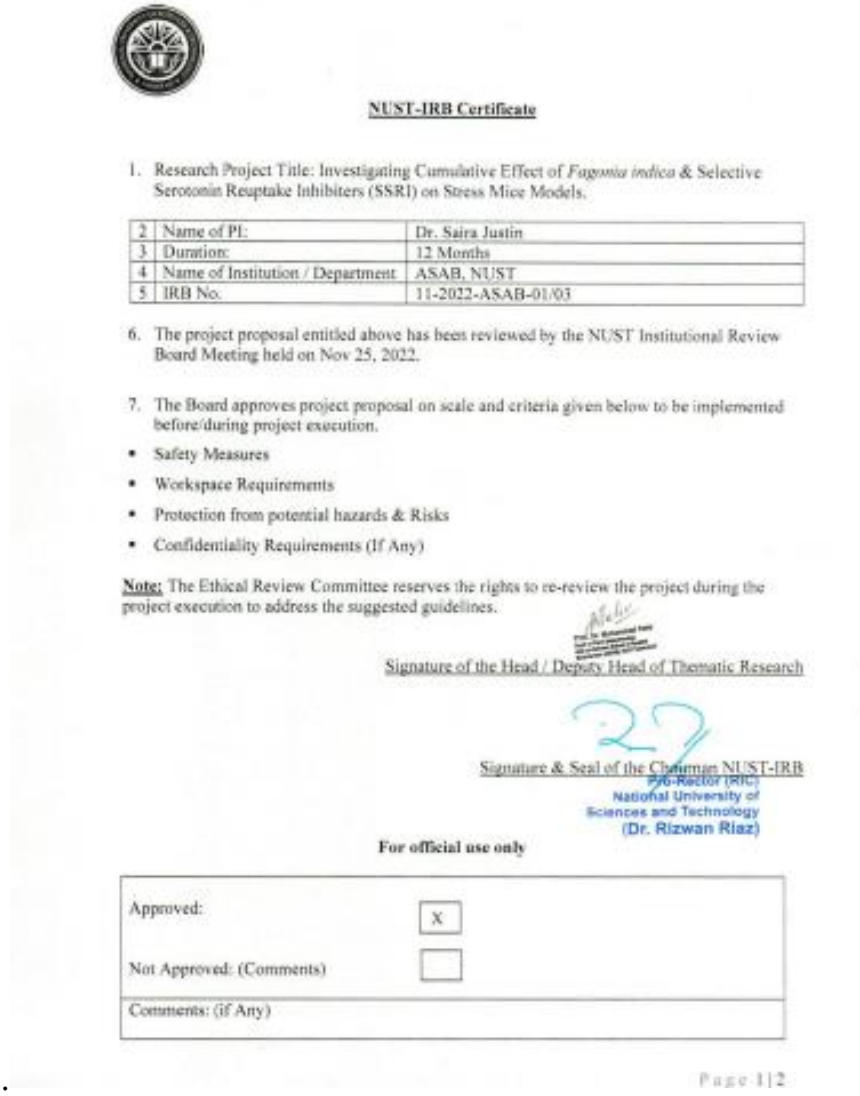
Furthermore, ethanolic extract of *Fagonia indica*, owing to it antioxidant properties, showed improved hormonal imbalance in polycystic ovarian syndrome (PCOS) rat models and caused weight reduction, comparable to the conventional drug, metformin. (Younas et al., 2022).

Species closely related to *Fagonia indica* have showed potential as antidepressants (Umbreen Rashid et al., 2022) as well, but neuroprotective effects of *Fagonia indica* are yet to be explored. Therefore, the aim of the present study is to evaluate accumulative effect of Fluoxetine and *Fagonia indica* as an anti-stress therapy.

METHODOLOGY

3.1 Ethical Statement

All the protocols performed including mouse care and use were approved by the Institutional Review Board (IRB), Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST) (Figure 3.1). All the animals were treated and experimented upon in accordance with the declarations of the Laboratory Animal Research Institute, Earth and Life Sciences Division, National Institute of Health (NIH), USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011).



NUST-IRB Certificate

1. Research Project Title: Investigating Cumulative Effect of *Fagonia indica* & Selective Serotonin Reuptake Inhibiters (SSRI) on Stress Mice Models.

2	Name of PI:	Dr. Saira Justin
3	Duration:	12 Months
4	Name of Institution / Department	ASAB, NUST
5	IRB No.	11-2022-ASAB-01/03

6. The project proposal entitled above has been reviewed by the NUST Institutional Review Board Meeting held on Nov 25, 2022.

7. The Board approves project proposal on scale and criteria given below to be implemented before/during project execution.

- Safety Measures
- Workspace Requirements
- Protection from potential hazards & Risks
- Confidentiality Requirements (If Any)

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

Signature of the Head / Deputy Head of Thematic Research

Signature & Seal of the Chairman NUST-IRB

Pro-Rector (RIC)
National University of
Sciences and Technology
(Dr. Rizwan Riaz)

For official use only

Approved:	X
Not Approved: (Comments)	
Comments: (if Any)	

Page 1|2

Figure 3.1: Institutional review board (IRB) certificate.

3.2 Materials and Instruments

Table 3.2.1: List of chemicals and reagents used

Chemicals and Reagents	**Manufacturers**
Prozac (Fluoxetine)	Eli-Lilly Pakistan (Pvt) Ltd
Methanol	SIGMA-ALDRICH: 24229-M-2.5L
Ethanol (80%)	SIGMA-ALDRICH: 32221-M-2.5L
Phosphate Buffer Saline (PBS)	SIGMA-ALDRICH Tablets: P4417, Ident-Nr. 10-100-94

Table 3.2.2: List of kits used

Kits	**Manufacturers**
ALP	LabKit Ref: 30133
ALT	LabKit Ref: 30253
Creatinine	LabKit
Urea	LabKit
Total Cholesterol	LabKit Ref: 30183
HDL	LabKit
LDL	LabKit

Table 3.2.3: Plastic consumables and miscellaneous

Plastic Consumables	**Manufacturers**
Falcon Tubes (50ml)	Accumax
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
Latex Powdered Examination Gloves	SRITRANG

Table 3.2.4: List of instruments

Instruments	**Manufacturer**
Weighing Balance	SF-400 Electronic Digital Scale
Centrifuge (Spectrafuge 24D)	Labnet
Refrigerator	Haier

Table 3.2.5: List of software used

Software	**Manufacturer**
GraphPad Prism (version 9)	GraphPad Software, Inc.

3.3 Plant Extract Preparation

In order to investigate the potential therapeutic properties of *Fagonia indica*, plant extract was prepared using maceration technique from dried plant material of *Fagonia indica*. The whole dried plant was grounded for 60 seconds in a grinder machine to obtain a fine powder. For maceration, 10 g of grinded plant was placed in labelled dark glass bottles along with 200 ml (1:20) of methanol as solvent (Figure 3.2a). The bottles were airtight locked and placed inside a cabinet to be kept protected from sunlight for approximately 10 days and were frequently agitated until most of the plant material had dissolved. After 10 days the extract was filtered using Whatman filter paper no. 42 and the pure extract was collected in a 50 ml falcon tube (Figure 3.2b). The plant extract was then poured in petri plates and placed for overnight incubation at 37°C for solvent evaporation (Figure 3.2c). Lastly, after 24hrs the dried extract was scraped off and stored in powdered form at 4°C (Figure 3.2d)

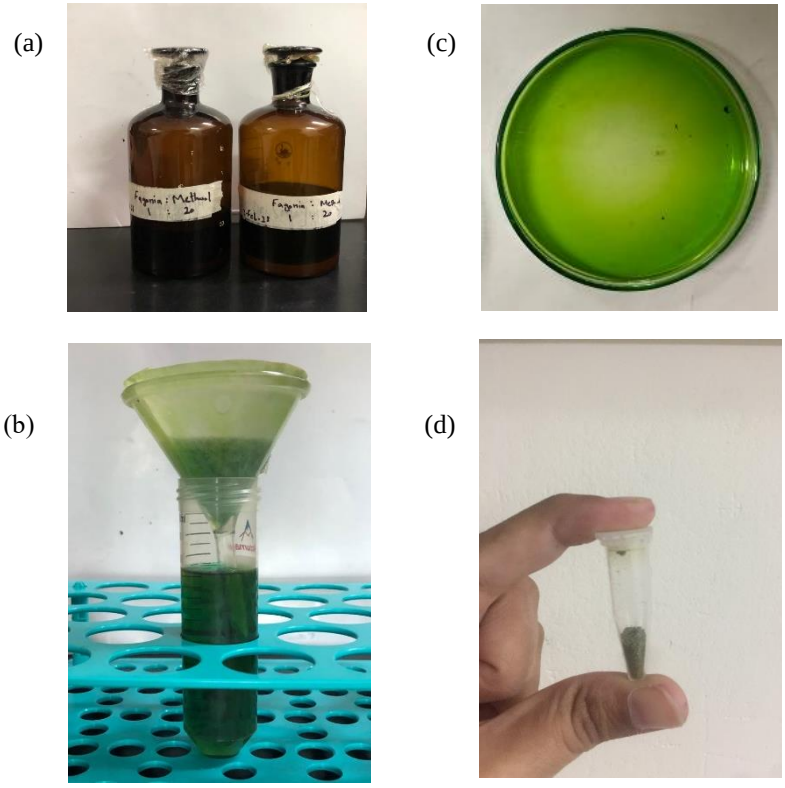


Figure 3.2: Plant extraction: (a) Maceration, (b) Filtration of extract, (c) Filtered extract poured in petri plates to be dried overnight at 37°C in incubator, (d) Dried extract to be stored at 4°C.

3.4 GC-MS of *Fagonia indica*

For the Identification of bioactive components, the extract was subjected to GC-MS analysis. GC-MS analysis was carried out on a GC-MS - SH-Rxi-5Sil MS present in advanced energy materials and systems lab at U.S.-Pakistan Center for Advanced Studies in Energy (USPCASE), NUST. It comprises of an auto sampler and a gas chromatograph interfaced to a mass spectrometer (GC-MS) instrument, employing the following conditions: Low-polarity phase: Crossbond™ silarylene phase 1,4-bis (dimethylsiloxy) phenylene dimethyl polysiloxane, low-bleed GCMS column, inertness for active compounds, similar phases: DB-5ms UI, DB-5ms, VF-5ms, SLB5ms column (30×0.25 mm, 0.25 µl thickness). Following were the requirements for the methanolic extract of our plant (Atiq-ur-Rehman et al., 2021):

Table 3.3: Requirements for GC-MS of methanolic extract of Fagonia indica (A Atiq-ur-Rehman et al., 2021).

Operating in electron impact mode	70eV.
Carrier gas	Helium (99.999%)
Constant flow	0.7 ml/min
Injection volume	1µl
Split ratio	Splitless
Injector temperature	250℃
Ion-source temperature	200℃.
Column temperature	60℃ with an increase of 10℃/min to 310℃
Mass spectra	70eV
Scan range	50-650 m/z
Detector temperature	250℃

3.5 Animal Model

The research was conducted on 48 female BALB/c mice, provided by ASAB Animal Lab House, NUST. All the mice were of 8-12 weeks old and had an average weight of 30-40 g. The lab room in which the mice were kept had an artificially controlled temperature of 22 ± 2°C. A natural 14:10 hours light and dark cycle were implemented. All the mice were kept in plastic cages in a group of 6. Cages of dimensions 40cm x 25cm x 15cm were used and 4-5 mice were kept in one cage,

with wood shavings as bedding. Standard housing conditions were maintained for the animals and were fed with food and water *ad libitum.*

3.6 Drugs – Fluoxetine and *Fagonia indica*

3.6.1 Fluoxetine Feed

Prozac (Fluoxetine) 20mg capsules were obtained from Eli-Lilly Pakistan (Pvt) Ltd. The calculated dose of Fluoxetine used was 18mg/Kg/day (Dulawa et al., 2004a; Holick et al., 2008) . Experimental mice received treatment orally mixed with standard feed. 8.3 mg Fluoxetine was added to 100 g finely crushed, powdered standard feed. Water was added to make medium-sized pellets that were air-dried and fed to the animals.

3.6.2 *Fagonia indica* Feed

Fagonia indica was acquired locally. A taxonomist was requested to identify the plant specie before starting the treatment. The calculated dose of *Fagonia indica* used in the study was 400mg/Kg/day (Abbas et al., 2014). Experimental mice received treatment orally mixed with standard feed.142.8 mg of *Fagonia indica* was added to 100 g finely crushed, powdered standard feed. Water was added to make medium-sized pellets that were air-dried and fed to the animals.

3.7 Study Plan

The total duration of testing was 30 days. The behavior tests lasted five days, starting on the 25th day. On the 30th day, mice were decapitated and their blood was collected for serum protein analysis and biochemical testing.

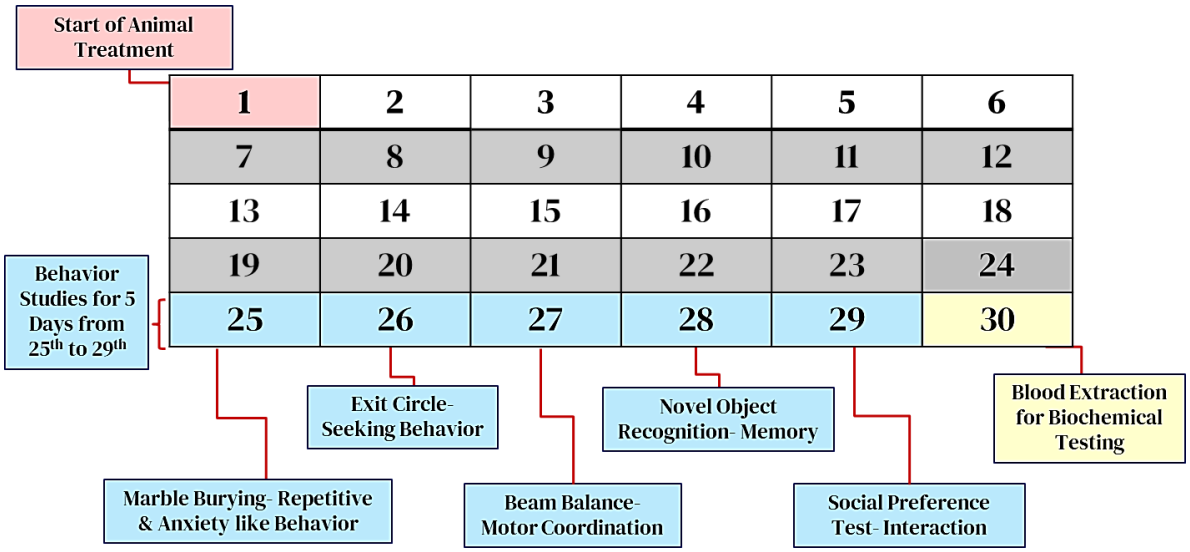


Figure 3.3: Study plan: The duration of the treatment was 30 days. During the last 5 days, behaviors were performed. On the last day, all animals were sacrificed and their blood collected for biochemical testing.

3.8 Animal Grouping

Animals were divided in to eight groups with six healthy mice placed in each group. Details of all the groups are mentioned below:

1) **Control group:** Normal balanced feed and tap water.
2) **Stress group:** The restrained stress of 4 hours was given to each mouse daily for 30 days. Normal feed and tap water.
3) **Fluoxetine group:** 18 mg/Kg daily oral dose of Fluoxetine mixed with normal feed and tap water. No stress was given throughout the treatment.
4) ***Fagonia indica* group:** 400 mg/Kg daily oral dose of *Fagonia indica* mixed in their feed along with tap water. No stress was given throughout the treatment.
5) **Fluoxetine + *Fagonia indica* group:** 18 mg/Kg daily dose of Fluoxetine and 400 mg/kg daily dose of *Fagonia indica* mixed in feed and tap water.
6) **Stress + Fluoxetine:** 4 hours of restraint stress was given to each mouse daily. Additionally, specialized feed containing18 mg/Kg daily oral dose of Fluoxetine was given throughout, along with tap water.

7) **Stress + *Fagonia indica* group:** 4 hours of restraint stress was given to each mouse daily. Specialized feed containing of 400 mg/Kg daily oral dose of *Fagonia indica* was given throughout, along with tap water.
8) **Stress + Fluoxetine + *Fagonia indica* group:** Restraint stress of 4 hours was also given to each mouse daily. Specialized feed of Fluoxetine (18 mg/Kg/day) + *Fagonia indica* (400mg/Kg/day) was given throughout the treatment, along with tap water.

3.8.1 Restraint Stress to Mice

Body fit restrainers were made from plastic Falcon tubes of 50 ml (Figure 3.4). 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 (Khalid et al., 2017; Shoji & Miyakawa, 2020).

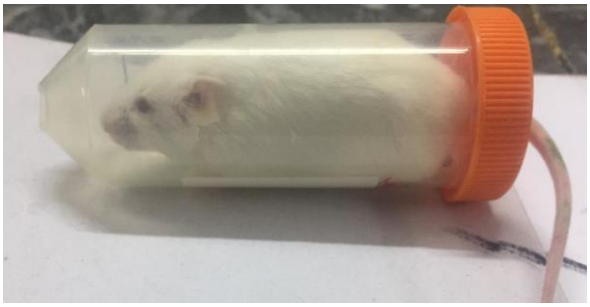


Figure 3.4: Mouse restrained in a 50 ml Falcon tube with holes at both ends for 4 hours.

3.9 Behavior Studies

The behavior tests began on the 25th day of the treatment and lasted till the 29th day. To familiarize the mice with the environment, they were shifted to the behavior room 30 minutes prior to the beginning of the tests. The room was properly illuminated and maintained at a temperature of 25±2˚C. Behavior tests were recorded using a video camera, without any disturbance or human interference. All the behaviors were performed in a sequence; from those that caused least stress to the animal to the most stressful ones.

3.9.1 Marble Burying Test

Mice exhibit various species-typical behaviors such as digging and burrowing. This behavior is sensitive to strain differences and drugs (Deacon et al., 2006). Marble burying test is normally used to identify obsessive compulsive disorder (OCD), anxiety-like, or repetitive behavior (Angoa-

Pérez et al., 2013). In this study, the test was performed to identify the digging behavior of mice and whether stress has impacted this behavior.

3.9.1.1 Apparatus

Standard mice cages with fitted filter-top covers cleaned with 70% ethanol were used for the marble-burying test. Fresh and unscented bedding was added 5cm deep to each cage. The bedding surface was leveled 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 5 rows of 4 marbles (Figure 3.5). The marbles were cleaned with 70% ethanol before and after the test was performed (Angoa-Pérez et al., 2013).

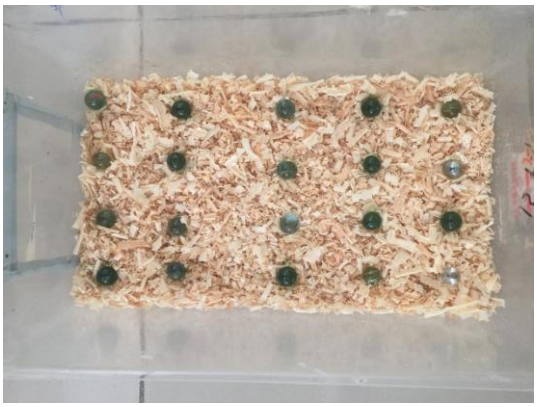


Figure 3.5: Apparatus for marble burying containing 20 marbles divided in 4 rows of 5.

3.9.1.2 Procedure

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

3.9.1.3 Evaluation

The blind scorers were asked to count the number of marbles that were buried about 2/3 in the cage bedding or were either displaced from original position. Each covered and displaced marble was scored as 1 point. The average of the total points was taken for each group (Angoa-Pérez et al., 2013).

3.9.2 Social Preference Test

Social interaction is essential for all animals. Similar to humans, mice also show social behavior to other mice. However, stress can affect this behavior for which the three-chamber social preference test is often used to assess social deficits in mouse models (Benjamin Rein et al., 2021). 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 refers to a mouse's ability to interact with a stranger mouse, while preference for social novelty represent the probability of spending time with a stranger mouse than with a familiar mouse. Generally, the mouse tends to interact more with unfamiliar mouse but stressed mouse doesn't interact much or interact more with familiar mouse (Kaidanovich-Beilin et al., 2011).

3.9.2.1 Apparatus

The apparatus used in this behavior test was a three-chambered, black rectangular box. The box has an open middle section with two circular openings providing access to each chamber. Two small wired cages were used to hold the stranger mice during the test (Figure 3.7) (Kaidanovich-Beilin et al., 2011).

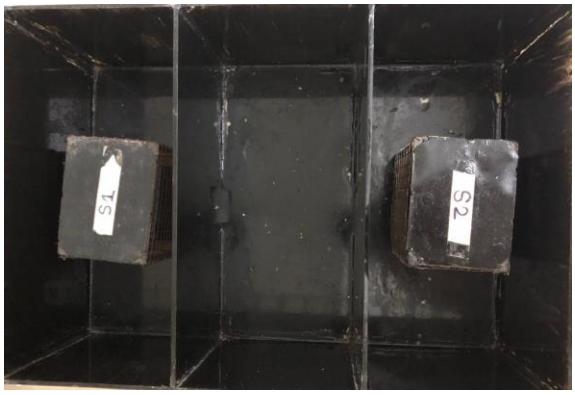


Figure 3.7: Apparatus for social preference test.

3.9.2.2 Procedure

Before starting the social preference test, mice were checked for their stage of estrous cycle. Only those mice were tested who were on their diestrus stage of the estrous cycle or were sexually non-receptive (Chari et al., 2020). The stage was identified using the visual method defined by (Byers et al., 2012).

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

Habituation: Firstly, the mouse was placed in the center compartment of a rectangular three chambered box. During the habituation phase of 05 minutes, the doorways into the two sides of the compartment were blocked with a piece of cardboard.

Session I: During the first session, an unfamiliar mouse of the same gender, female mouse in our case, as the test mouse was placed in a stranger 1 chamber while the stranger 2 chamber was kept empty. The stranger mouse was also habituated for 30 minutes in the behavior room before testing and it was made sure that it had no previous interaction with the test mouse. 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: Another unfamiliar mouse was added in the second chamber, which was previously empty. Each mouse was given 10 minutes to measure the social preference for the new stranger. The test mouse now had the choice between two mice, a previously explored mouse (Stranger 1) and a new, unfamiliar mouse (Stranger 2) (Moy et al., 2004).

Sessions I and II were recorded using video cameras. No disturbance or any kind of human interference was ensured during the test.

3.9.2.3 Evaluation

After the test, videos recorded were analyzed to see the duration of interaction of the test mouse with the empty cage and stranger 1 in first session and both the strangers in the second session, respectively. The time was recorded using a stopwatch as soon as the mouse showed signs of interaction (nose touch and sniffing). If the mouse climbed the cage and its face pointed upwards, it was not considered as an interaction.

The discrimination index (DI) was calculated to check its sociability, social preference and memory, by using the time spent with the empty cage, stranger 1 and/or stranger 2. The formula for DI is as follows:

DI = Time spent with stranger mouse (sec) ÷ Total interaction time (sec)

3.9.3 Exit Circle Test

Mice exhibit intrinsic inquisitiveness and exploratory behavior when placed in a new environment. The exit circle test was performed to identify if stress and the respective treatments has impacted their inquisitiveness and exploratory behavior.

3.9.3.1 Apparatus

A long steel cylinder with a 30 cm diameter and a small square opening of 5 cm x 5 cm was used (Figure 3.6).

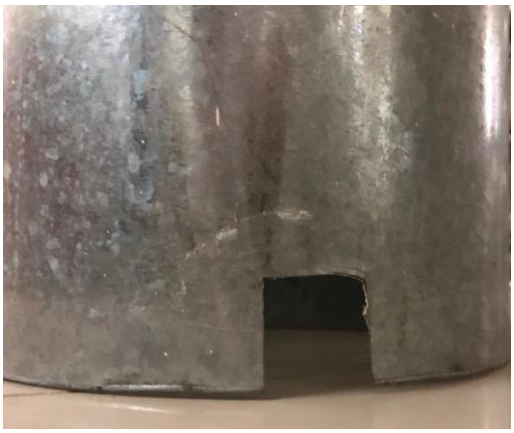


Figure 3.6: Apparatus for exit circle showing the small exit door

3.9.3.2 Procedure

The test mouse was placed in the steel cylinder, facing towards the wall. It was given 30 seconds to exit the circle from the small opening. A healthy mouse will find its way out of the circle within the given time while exhibiting spontaneous motor activity and exploratory behavior. The time was recorded using a stopwatch.

3.9.3.3 Evaluation

The scoring method was modified from (Fréchou et al., 2019) and is as follows:

Task	Description	Points
Exit Circle	Exit the circle within 10 sec.	3
	Exit the circle within 20 sec.	2
	Exit the circle within 30 sec.	1
Exploration	Exhibits no spontaneous motor activity.	0
	Moves but remains in the smallest circle.	1

3.9.4 Novel Object Recognition

Novel object recognition test was performed for investigating different phases of learning and recognition memory in mice (Lindsay M. Lueptow et al., 2017). Mice have a natural inclination towards novelty.

3.9.4.1 Apparatus

The apparatus consisted of a square box painted black, in which 2 similar objects for session 1 and one novel object for session 2 were placed diagonally for the mice to explore (Figure 3.8).

(a) (b)

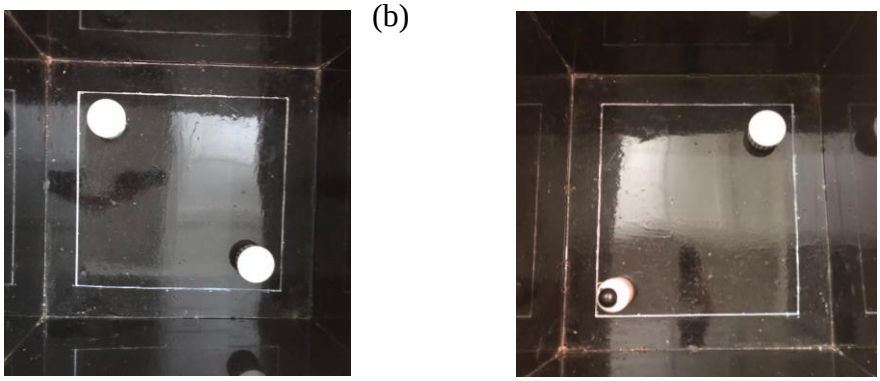


Figure 3.8: Apparatus for novel object recognition test; (a) Session I, (b) Session II.

3.9.4.2 Procedure

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

Habituation: During habituation, the mice were familiarized with the field in which they were to explore the objects. For this purpose, each mouse was put into the empty box for 5 minutes.

Training phase: In the second phase, i.e. the training phase, 2 similar objects were placed diagonally on either end of the box and the mouse was put into it to explore them for 10 minutes. The training session was followed by a 20 minutes gap period.

Testing Phase: In the third phase, i.e. the testing phase, one of the objects was replaced with a novel object and the mouse was put into the box again for 10 minutes to explore it.

3.9.4.3 Evaluation

After the test, videos recorded were analyzed to see the duration of interaction of the test mouse with the familiar object from session 1 and novel object in session 2. The time was recorded using a stopwatch as soon as the mouse showed signs of interaction (nose touch and sniffing). If the

mouse climbed the any of the objects and its face pointed upwards, it was not considered as an interaction.

The recognition index (RI) was calculated to check its recognition memory, by using the time spent with the familiar and the novel object. The formula for DI is as follows:

RI= Time spent with novel object (sec) ÷ Total interaction time

3.9.5 Beam Balance

The test is used in diseases where the motor ability of the animal is reduced. It helps to assess the fine motor coordination by observing if the mice are able to maintain its balance on a thin beam of 7 mm (Szczygielski et al., 2016).

3.9.5.1 Apparatus

The size of the beam was 100 cm long and 7 mm wide with an elevation of 30 cm (Figure 3.9).

Figure 3.9: Apparatus for beam balance test.

3.9.5.2 Procedure

The mouse to be tested was placed in the middle of the beam so it could balance itself on it for 60 seconds. The time was noted as soon as the mouse was released or it falls.

3.9.5.3 Evaluation

The scoring system was modified from (Szczygielski et al., 2016) and is as follows:

Task	Description	Points
Beam Balance for 60 seconds	Does not attempt to balance on the beam.	4
	Hangs on the beam with 2 paws but falls.	3
	Hugs the beam with all 4 paws.	2
	Balances with unsteady or shaky movements	1
	Balances with steady posture or walks	0

3.10 Serum Isolation

On the 30th day animals were sacrificed to collect blood for serum isolation. 1- 2 ml of chloroform along with carbon dioxide (CO_2) asphyxiation was used to anesthetize the mice. The mice were decapitated using mouse surgical kit. Blood was collected 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 4ºC for further procedures.

3.11 Biochemical Tests

Biochemical tests and serum protein analysis was performed to inspect how stress and drug treatments have affected the levels of enzymes and glucocorticoid hormone i.e. cortisol in mice. List of the tests performed is given below;

- Serum Cortisol Test
- Renal Function Tests
- Liver Function Tests
- Lipid Profile Tests

3.11.1 Serum Protein Analysis (Cortisol)

Cortisol is a steroid hormone released under stress. The blood from mice models was collected during decapitation of the mice from each group. It was ensured that the time for blood collection is same for each group (Dulawa et al., 2004a; Holick et al., 2008). The blood was collected between 12:00am to 14:00 pm. The serum sample was outsourced to Bio Care Laboratories, to perform serum cortisol test. The test was performed on Roche cobas e411 analyzer, a fully automated analyzer that works on the principle of electro-chemi-luminescence (ECL) for immunoassay analysis.

3.11.2 Liver Function Tests

The serum ALT and ALP levels were measured to check the extent of hepatocellular injury using commercially available UV-kinetic diagnostic of LabKit Ref: 30253 and Ref: 30133, respectively. The tests were performed according to manufacturer's instructions.

3.11.3 Renal Function Tests

Serum urea and creatinine were measured to evaluate the kidney injury using commercially available UV-kinetic diagnostic of LabKit. The tests were performed according to manufacturer's instructions.

3.11.4 Lipid Profile Tests

Lipid profile test was performed to measure the levels of various lipids (fats) and cholesterol in blood of mice. The test provides valuable information regarding the risk for developing cardiovascular conditions. Serum total cholesterol, HDL and LDL were measured using commercially available diagnostic kit, LabKit. The tests were performed according to manufacturer's instructions.

3.12 Statistical Analysis

All the results obtained were subjected to statistical analysis using GraphPad Prism (version 9). Two-way ANOVA followed by Tukey's multiple comparison test was used for percentage weight variation, percentage feed variation and both sessions of social preference test and novel object recognition test. Other than these, all behavior tests and biochemical tests were analyzed using one-way ANOVA followed by Tukey's multiple comparison test. A $p \leq 0.05$ was considered significant. The error bars represented ± standard error mean (SEM).

RESULTS

4.1 Effect of Fluoxetine and *Fagonia indica* on Physical Parameters

To evaluate the impact of 4 hours of daily restraint stress and the anti-stress treatments, 18mg/Kg/day of Fluoxetine and 400mg/Kg/day of *Fagonia indica* on the physical parameters, the weight and feed consumption was recorded in grams throughout the 30 days of the experiment.

4.1.1 Effect on Weight

The weight of the mice was recorded throughout the 30-day treatment to check for any significant changes. The graph below shows the weight measured on Day 1, Day 15 and Day 30 for all the groups. On Day 30, there was a significant decrease seen in weight between the control group (113 ± 2.2) and all other groups, with the stress group (106.8 ± 1.1), the Fluoxetine group (106.2 ± 1.0), the *Fagonia indica* group (107.5 ± 1.8), the Fluoxetine + *Fagonia indica* group (107 ± 1.3), the Fluoxetine monotherapy group (108.5 ± 1.2), the *Fagonia indica* monotherapy group (104 ± 1.5) and the combination therapy group (103.8 ± 0.9), respectively. Overall, the *Fagonia indica* monotherapy group and the combination therapy group had the least weight of all on Day 30 (Figure 4.1).

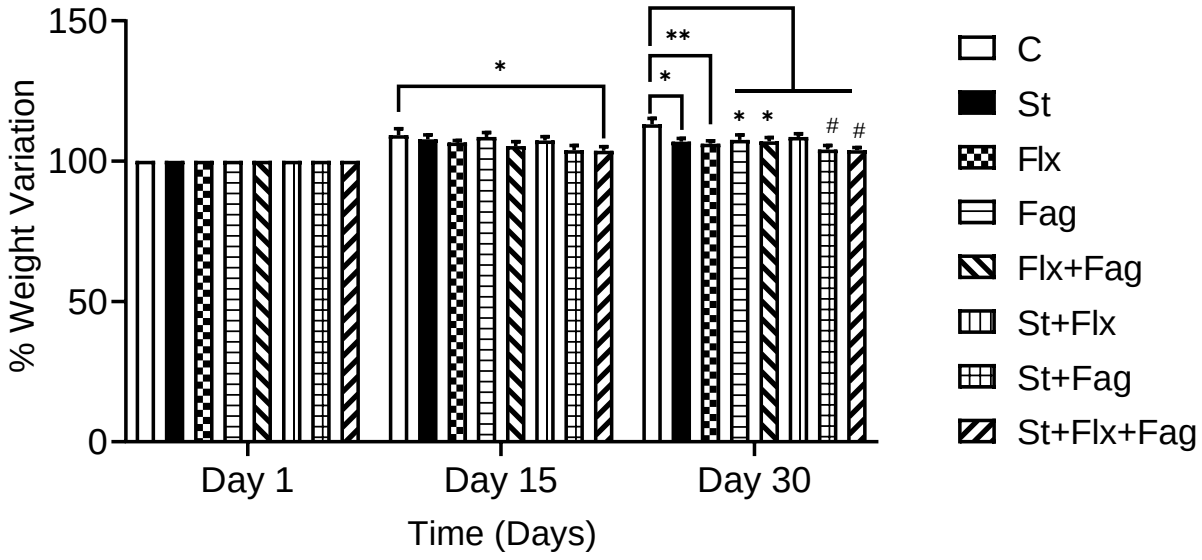


*Figure 4.1: Percentage weight variation: Weight variation was recorded after every 15 days. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=6. Two-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05; **p≤ 0.01; #p= ≤ 0.0001.*

4.1.2 Effect on Feed Consumption

To evaluate the impact of stress and treatment on feeding pattern, variation in feed consumption was observed throughout the 30-day treatment. The graph plotted here is for Day 1, Day 15 and Day 30. Overall, all the groups showed no significant change in feed consumption during the course of this experiment (Figure 4.2).

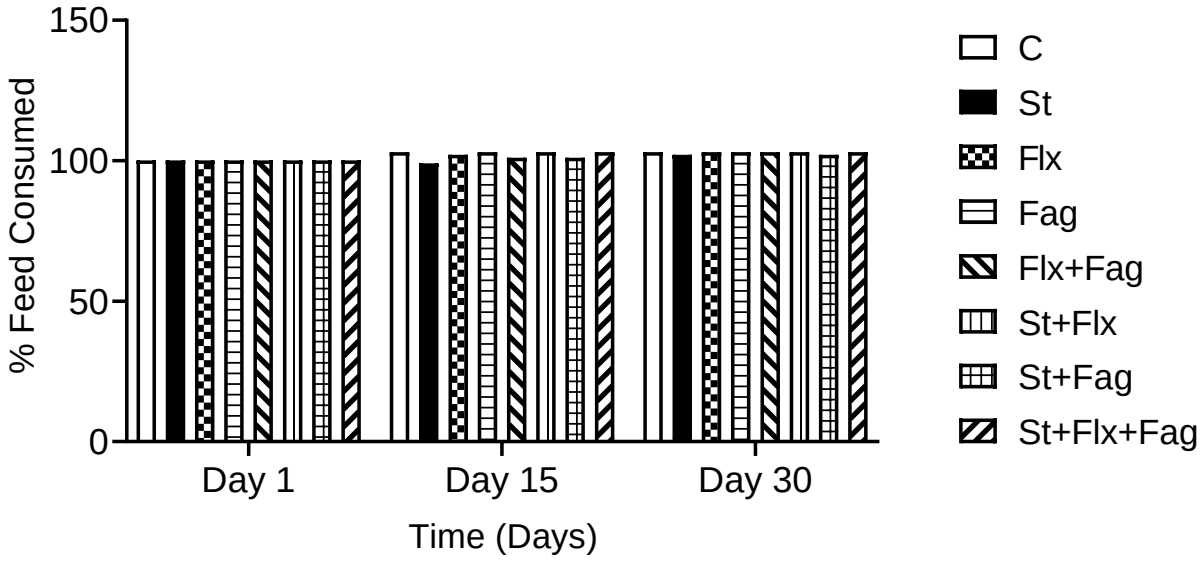


*Figure 4.2: Feed consumption***:** *The consumption of feed was recorded in grams after every 15 days. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=6. Two-way ANOVA followed by Tukey's multiple comparisons test was used.*

4.2 Effect of Fluoxetine and *Fagonia indica* on Behavior Tests

To evaluate the influence of anti-stress therapies, 18mg/Kg/day of Fluoxetine and 400mg/Kg/day of *Fagonia indica* in alleviating stress and the impairment linked to it, following behavior tests based on anxiety, social preference, learning and memory, motor coordination and inquisitiveness were performed.

4.2.1 Marble Burying

Mice have the intrinsic characteristic of burying objects. This test is used to measure repetitive and anxiety related behavior in mice. The number of marbles that the mouse buries under the wood shavings, gives an insight to its aggressive, repetitive and anxiety like behavior.

Compared to the control group (4.5 ± 1.5), the stress group (12.25 ± 3.4) demonstrated a significant ($p \leq 0.05$) increase in marble burying activity, indicating high levels of anxiety and aggressiveness. Both of the monotherapy groups and the combination therapy group showed a significant improvement in anxiety and repetitive behavior with, the Fluoxetine monotherapy group (4.8 ± 1.1), the *Fagonia indica* monotherapy group (4.5 ± 0.6) and the combination therapy group (2.75 ± 0.25), respectively. Although insignificant, the combination therapy group had a cumulative effect; (St + Flx+ Fag > St + Fag >St + Flx) (Figure 4.3).

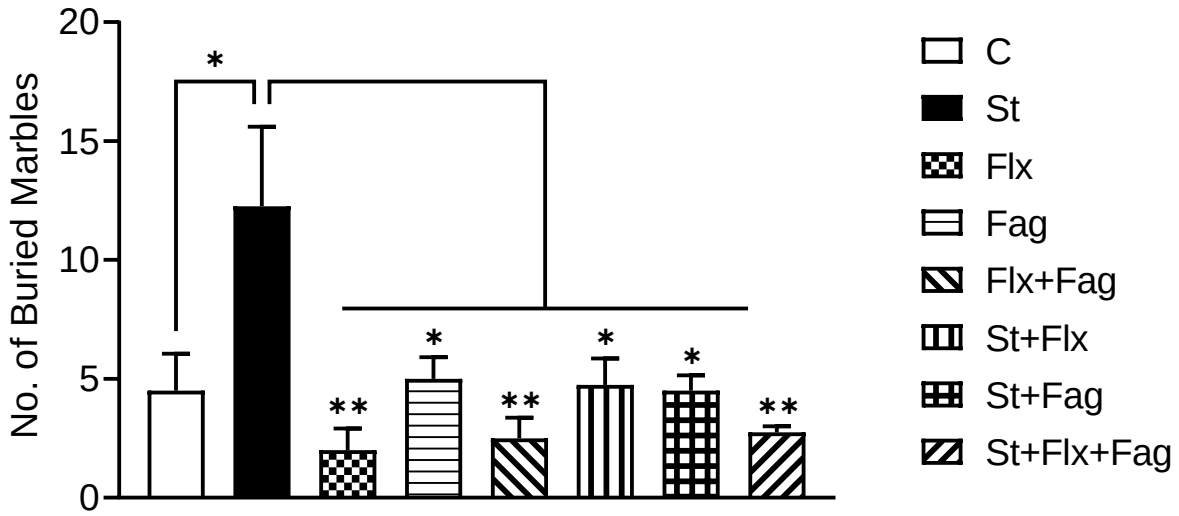


*Figure 4.3: Repetitive and anxiety-like behavior: Greater number of marbles buried indicate anxiety and aggressiveness. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. One-way ANOVA followed by Tukey's multiple comparisons test was used. *$p \leq 0.05$; **$p \leq 0.01$.*

4.2.2 Social Preference and Novelty

Mice display social behavior towards other mice. Stress and its related disorder can impair this ability. The social preference and novelty test was done to check the mouse's sociability and novelty towards another mouse. The test was performed in same sex pairs. The female mice that were on their diestrus stage of their estrous cycle were selected so they are sexually non-receptive and show normal social behavior towards another female mouse.

Social affiliation was checked in the first session and time was recorded in seconds. As compared to the control group (129.5 ± 29.9), the time spent by the stress group (84.3 ± 35.5) with Stranger 1 was quite less. Although both of the monotherapy groups and the combination therapy group showed improvement in behavior, significant difference was observed only in the Fluoxetine

monotherapy group (191.3 ± 36.7). Unfortunately, no additive effect in the combination therapy group (149.8 ± 20.9) was observed; (St + Flx > St + Fag > St + Flx + Fag). Overall, the Fluoxetine monotherapy group demonstrated the best social propensity (Figure 4.4).

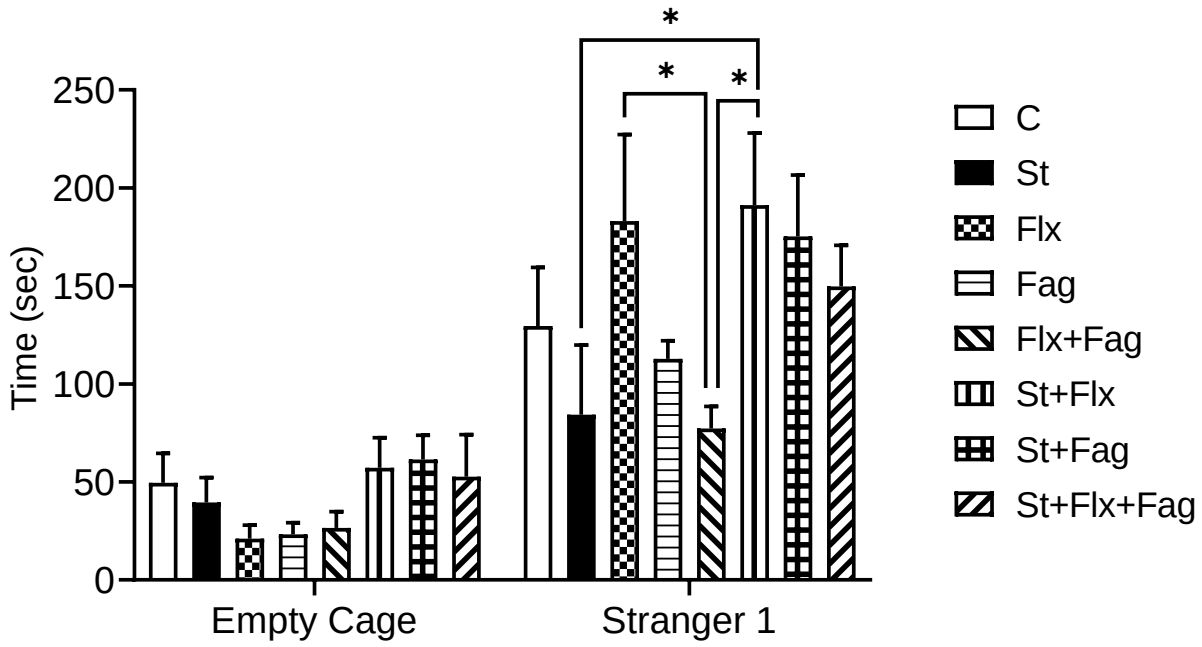


*Figure 4.4: Sociability: Social affiliation was assessed via time spent with Empty Cage and Stranger 1. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. Two-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05.*

In the second session of the test, the mouse's social preference was observed for a novel mouse (Stranger 2) in comparison to the acquainted mouse (Stranger 1). A non-significant, decrease was seen in the stress group (71.8 ± 27.7) as compared to the control group (102.0 ± 13.1), indicating reduced preference for social novelty. Both of the monotherapy groups and the combination therapy group showed improvement in behavior with the Fluoxetine monotherapy group (191.3 ± 6.7), the *Fagonia indica* monotherapy group (165.8 ± 42.3) and the combination therapy group (149.8 ± 20.9), respectively. Interestingly, the monotherapy groups showed better effect in comparison to the combination therapy group; (St + Fag > St + Flx > St + Flx + Fag) (Figure 4.5).

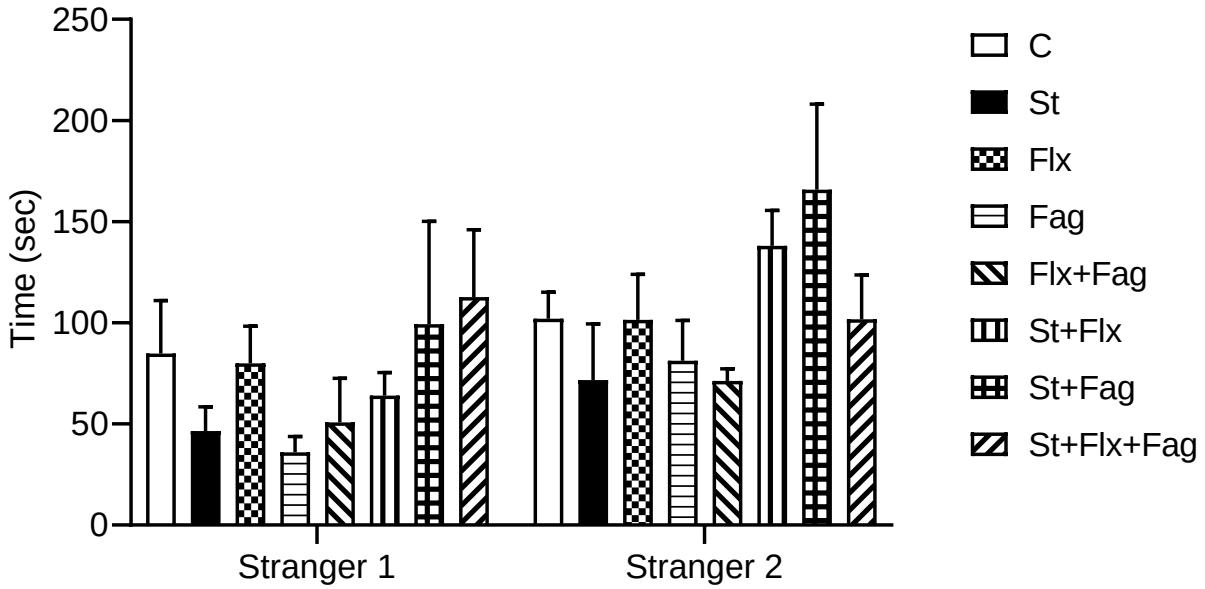


Figure 4.5: Social novelty: Preference between a familiar mouse (Stranger 1) vs a novel mouse (Stranger 2) was checked. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. Two-way ANOVA followed by Tukey's multiple comparisons test was used.

The discrimination index (DI) was calculated which is a measure of animal's memory and sociability. A high value of discrimination index (DI) indicates better social interaction. For session 1, test mouse's social interaction behavior was calculated. Compared to the control group (0.78 ± 0.02), a non-significant decrease was observed in the stress group (0.60 ± 0.10). Both of the monotherapy groups and the combination therapy group showed similar level of improvement in behavior with the Fluoxetine monotherapy (0.82 ± 0.04), the *Fagonia indica* monotherapy (0.80 ± 0.05) and the combination therapy (0.83 ± 0.04), respectively. A negligible additive effect of the combined treatment was seen as compared to the monotherapies; (St + Flx = St + Fag = St + Flx + Fag) (Figure 4.6).

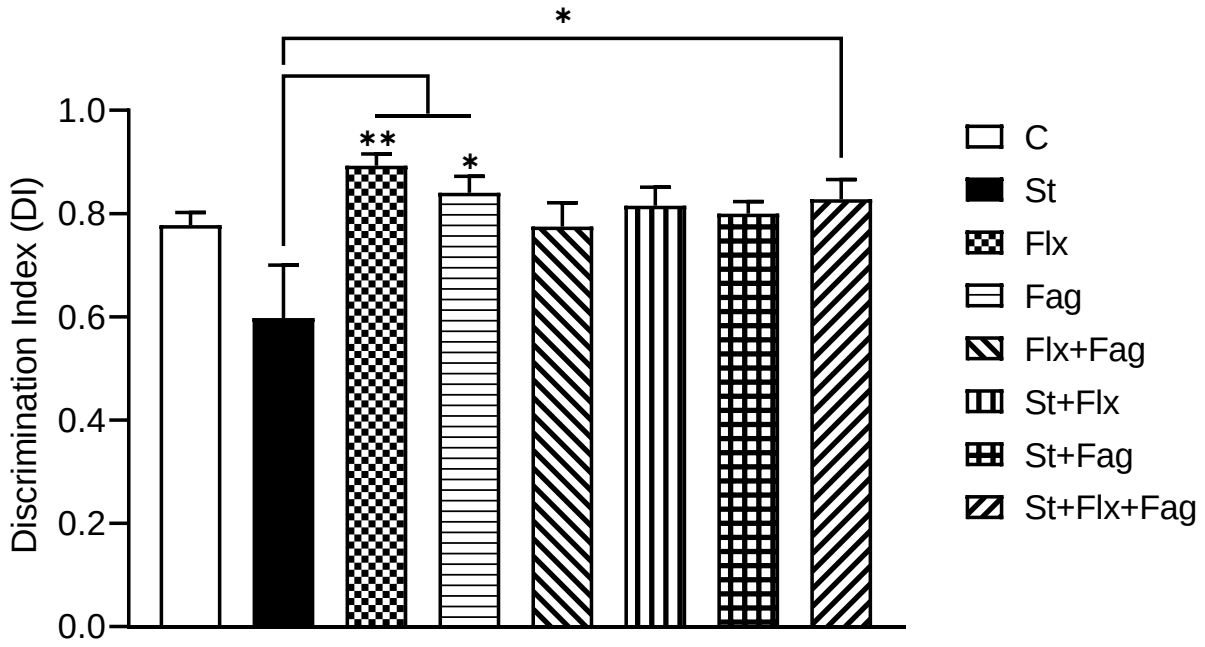


*Figure 4.6: Discrimination index (DI) for sociability: Preference for another mouse over an empty cage was checked. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05; **p≤ 0.01.*

For session 2, the DI was calculated as a measure of the mouse's ability to discriminate between the familiar and novel mouse on basis of its memory and social preference. No drastic changes were seen among the groups, with the control group (0.61 ± 0.03), the stress group (0.58 ± 0.05), the Fluoxetine monotherapy group (0.73 ± 0.02), the *Fagonia indica* monotherapy group (0.75 ± 0.07) and the combined therapy group (0.66 ± 0.08), respectively. Unfortunately, no additive effect was observed in the combination therapy group. DI for social novelty had a similar result among the treatments; (St + Flx = St + Fag = St + Flx + Fag) (Figure 4.7).

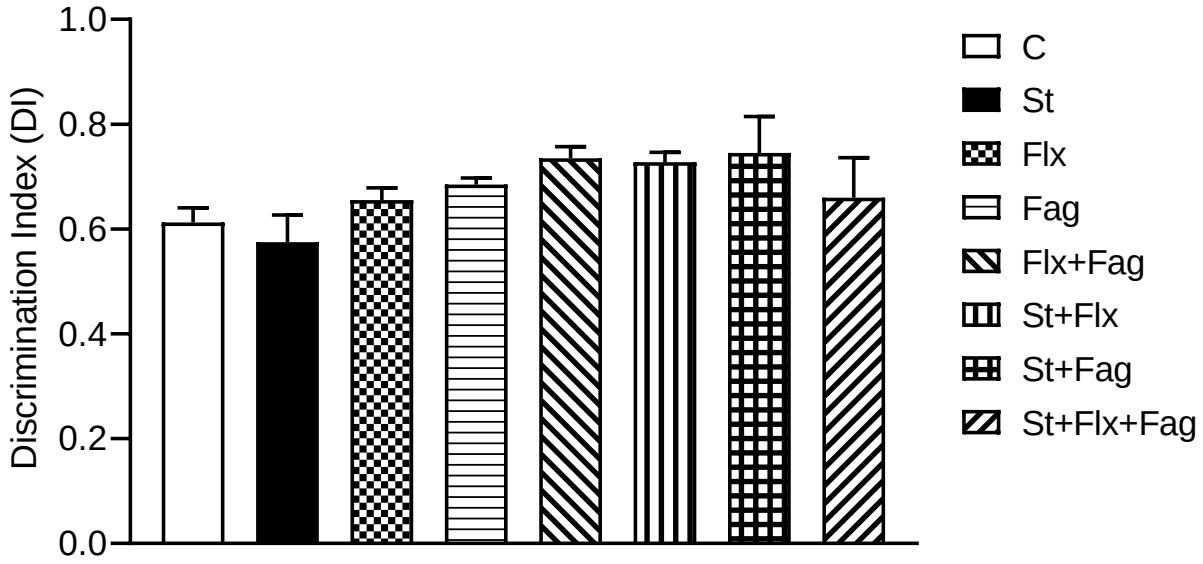


*Figure 4.7: Discrimination index (DI) for social novelty**:** The ability to differentiate between the familiar and novel mouse was checked. Control (C), Stress (St), 18mg/Kg/day Fluoxetine (Flx), 400mg/Kg/day Fagonia indica (Fag). Error bars represent ± standard error mean (SEM); n=4. One-way ANOVA followed by Tukey's multiple comparisons test was used.*

4.2.3 Exit Circle

This test evaluates the exploratory activity as well as the rapidity of the mice to exit a solid cylinder. The lower score indicates deficit in such a behavior.

In comparison with the control group (4.0 ± 0), the stress group (3.5 ± 0.28) showed a negligible decrease. Upon treatment, the stressed mice showed improvement in the exploratory behavior with no significant changes among the treatment groups; (St + Flx + Fag > St + Flx >St + Fag) (Figure 4.8).

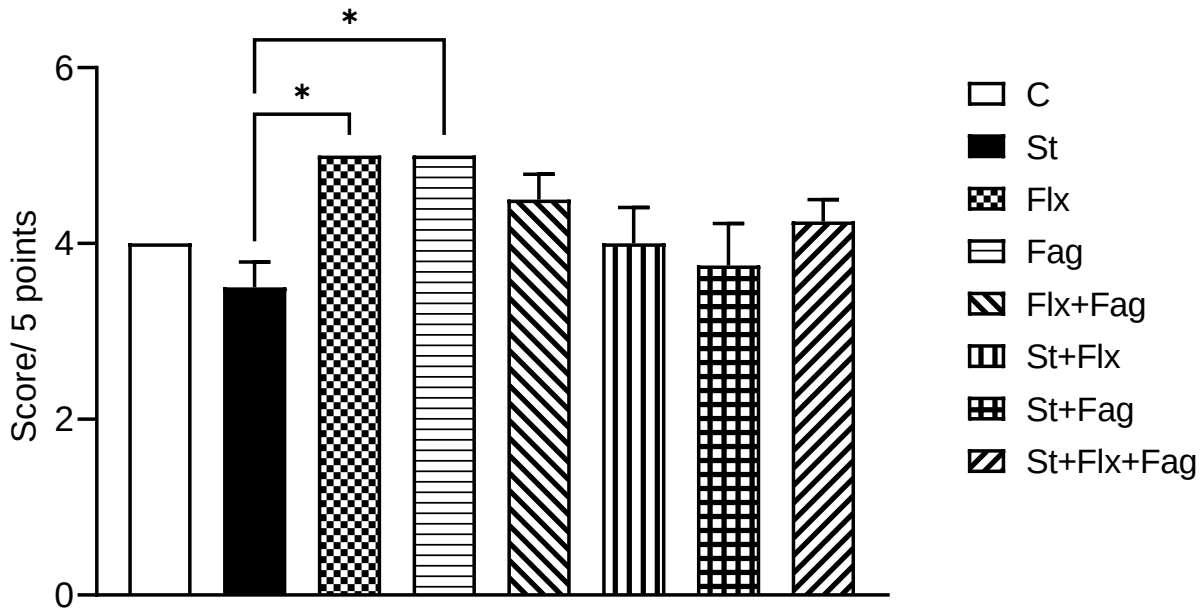


*Figure 4.8: Intrinsic inquisitiveness and exploratory behavior: The exploratory and rapidity of the mouse was checked. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05.*

4.2.4 Novel Object Recognition

To check the mouse's exploratory behavior and memory, novel object recognition test was done. The tendency to explore novel objects is an indication of the animal's use of learning and recognition memory.

During the familiarization session (training trial), mice were allowed to interact with two similar objects (Object 1 and Object 2) in an open box. The time spent in seconds with the objects is an indication of the exploratory behavior. For familiarization session, the interaction time between Object 1 and Object 2 for each group was almost the same with respect to each group. Compared to the control group (50.3 ± 13.5) (46.3 ± 7.9), the stress group (36.3 ± 7.8) (36.8 ± 4.1) spent slightly less time exploring the objects. The Fluoxetine monotherapy group (32.0 ± 7.7) (32.5 ± 3.9), the *Fagonia indica* monotherapy group (68.0 ± 51.8) (51.8 ± 7.8) and the combination therapy group (87.0 ± 24.2) (76.0 ± 25.6) showed increased exploratory behavior, but unfortunately a significant additive effect was not seen; (St + Flx + Fag > St + Fag > St + Flx) (Figure 4.9).

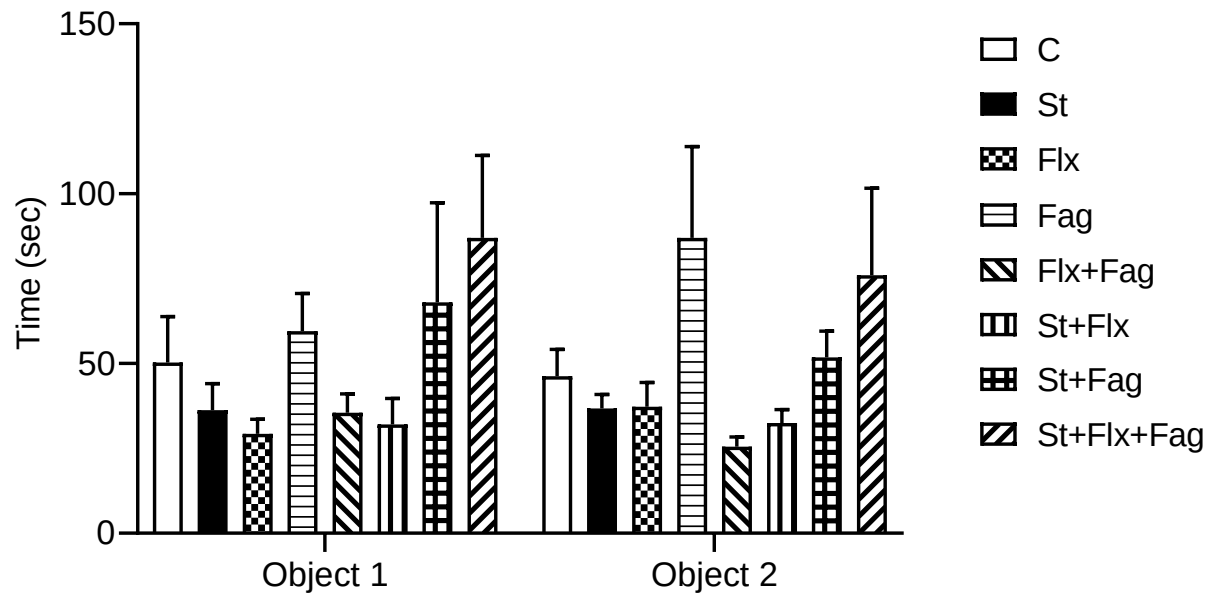


*Figure 4.9: Familiarization session (training trial)**:** Interaction between two similar objects (Object 1 and Object 2) was checked. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. Two-way ANOVA followed by Tukey's multiple comparisons test was used.*

During the second session (testing trial), Object 1 from training trial served as the familiar object and Object 2 was replaced with another object which served as the novel object to test for the learning and recognition memory. An insignificant decrease in the time spent with the novel object was observed in the stress group (34.0 ± 6.8), compared to the control group (38.3 ± 8.6). The combination therapy group (73.8 ± 21.9), showed improved behavior as compared to those administered with both of the monotherapies; the Fluoxetine monotherapy group (40.5 ± 8.7) and the *Fagonia indica* monotherapy group (45.6 ± 11.6), respectively. Even though an additive effect was seen in the combination therapy group, unfortunately it was not significant (St + Flx + Fag > St + Fag > St + Flx) (Figure 4.10).

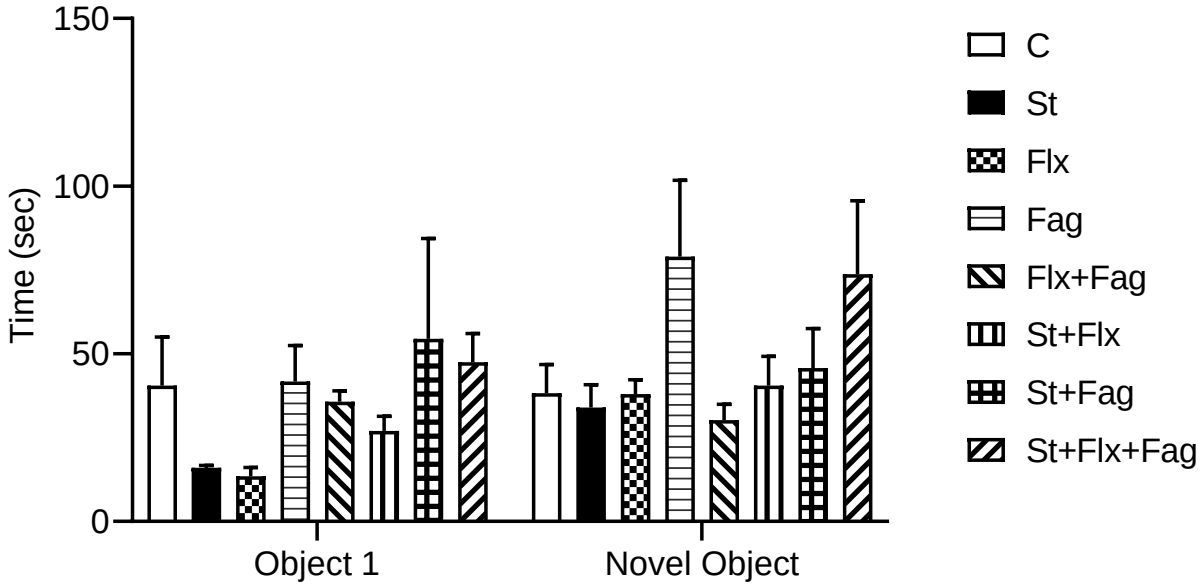


Figure 4.10: Testing trial: Learning and recognition memory was checked by observing the interaction with a novel object. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. Two-way ANOVA followed by Tukey's multiple comparisons test was used.

The recognition index (RI) for the second session is a measure of the recognition memory of the mice. A ratio of less than 0.5 means impaired memory and learning behavior. Overall, no drastic change was observed among all groups (Figure 4.11).

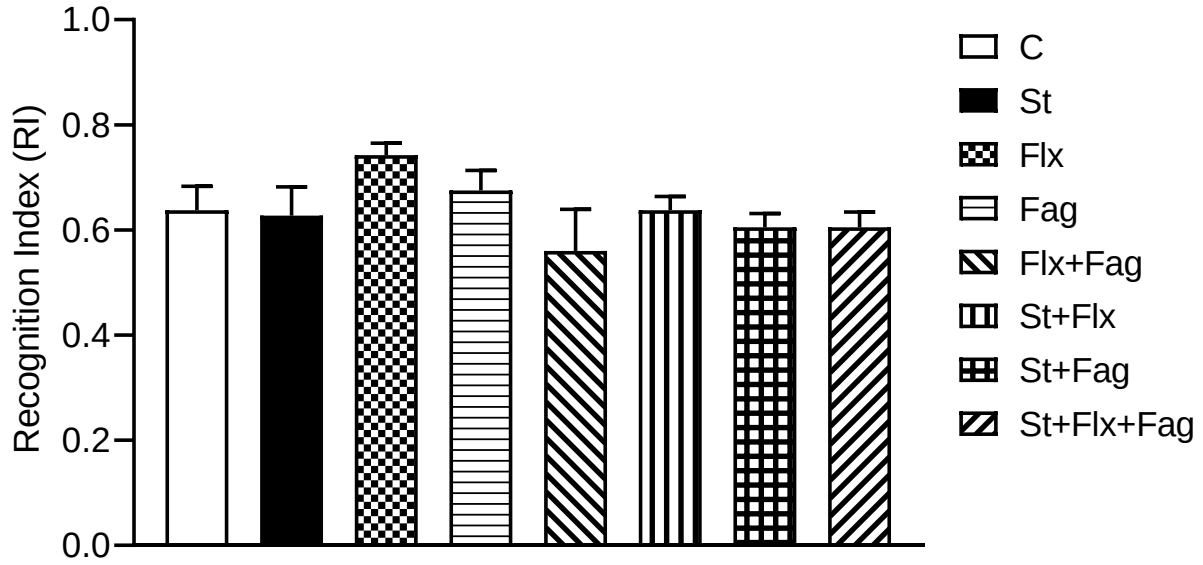


Figure 4.11: Recognition index (RI) for testing trial: The ability to recognize the familiar object and explore the novel object was checked. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia

indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. One-way ANOVA followed by Tukey's multiple comparisons test was used.

4.2.5 Beam Balance

This test is used to assess the motor coordination and reflexes of the mice when balanced on a beam for 60 seconds. The high impairment score means reduced motor function.

In comparison with the control group (0.75 ± 0.25), the stressed group (2.25 ± 0.75) had significantly ($p \leq 0.05$) high impairment score, indicating deficiency in motor abilities. Both of the monotherapy groups and the combination therapy group were able to improve the motor functions of the stressed mice, with significant difference seen in the *Fagonia indica* monotherapy group (0.75 ± 0.25) and the combination therapy group (0.5 ± 0.3), respectively. Even though an additive effect was seen in the combination therapy group, unfortunately it was not significant; (St + Flx + Fag > St + Fag > St + Flx) (Figure 4.12).

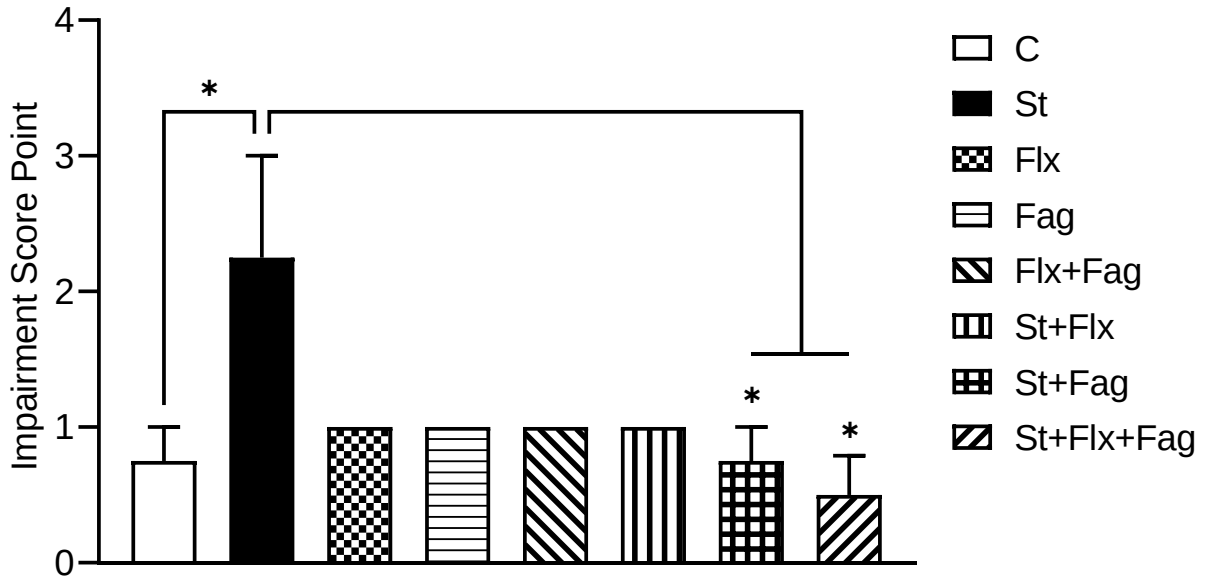


*Figure 4.12: Motor coordination: Motor coordination and the ability to balance on a 7mm beam for 60 seconds was checked. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=4. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05.*

4.3 Effect of Fluoxetine and *Fagonia indica* on Biochemical Tests

To evaluate the impact of stress, 18 mg/kg/day of Fluoxetine and 400 mg/Kg/day of *Fagonia indica* on the body's metabolism, following biochemical tests were performed:

4.3.1 Serum Cortisol Levels

Exposure to any type of stressor can have a significant effect on cortisol levels of mice, measuring which can be a useful tool for investigating the negative effects of stress on their behavior and physiology. Mice were sacrificed between 12:00 hours to 14:00 hours to collect serum for the hormonal assay. Compared to the control group (20.6 ± 6.7) a non-significant rise in cortisol levels was observed in the stress group (27.3 ± 7.7). Moreover, both of the monotherapy groups showed a non-significant decrease in cortisol levels; the Fluoxetine monotherapy group (15.7 ± 5.9) and the *Fagonia indica* monotherapy (15.0 ± 6.0) which lies in the normal reference range (8.3–17.06 ng/ml) (Manika kala et al., 2015) Surprisingly, an increase in the cortisol levels of the combination therapy (28.0 ± 5.6) was seen compared to the monotherapies (Figure 4.13).

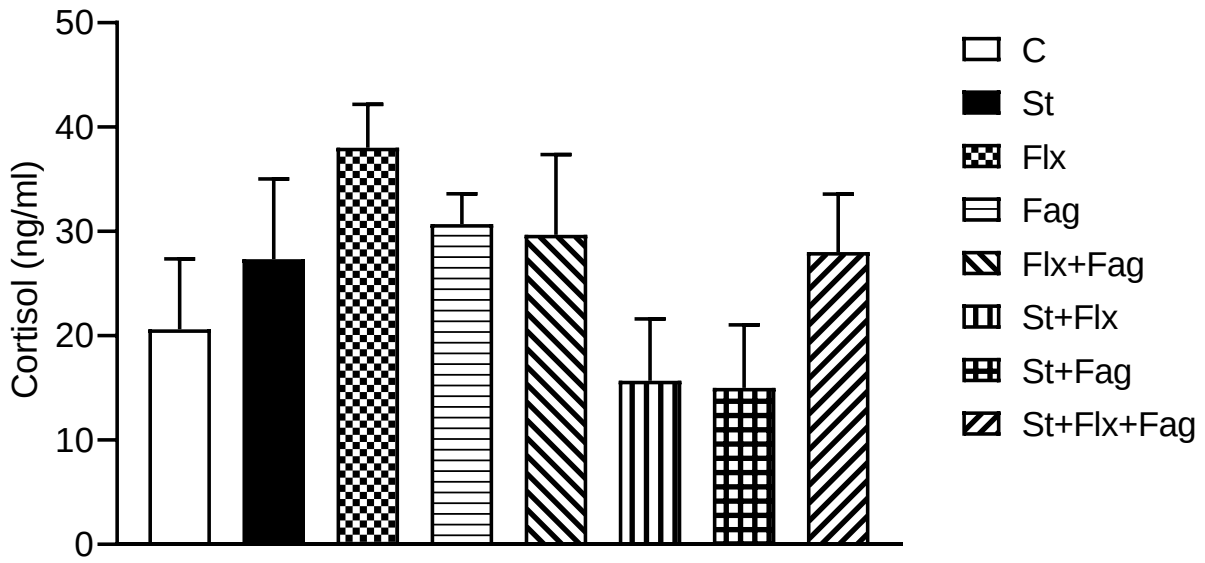


Figure 4.13: Serum cortisol levels: Cortisol being a biomarker for stress was evaluated. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used.

4.3.2 Liver Function Test

To assess the extent of hepatocellular injury, serum levels of alanine aminotransferase (ALT) and alkaline phosphatase (ALP) enzymes were measured.

4.3.2.1 Alanine Aminotransferase (ALT)

ALT is found in high levels in the liver and thus is a valuable biomarker for identifying liver dysfunction (Liu et al., 2014). The normal reference range for ALT 22-32 U/L (Gordon P Otto, 2016). Stress group showed significantly increased levels of ALT (43.3 ± 4.3) in comparison to the control group (14.0 ± 6.7). Both of the monotherapy groups and the combination therapy group were able to improve ALT levels of the stressed mice in a similar manner, though a significant difference was not seen; the Fluoxetine monotherapy group (24.0 ± 5.2), the *Fagonia indica* monotherapy group (30.3 ± 1.5) and the combination therapy group (29.7 ± 5.0), respectively (Figure 4.14).

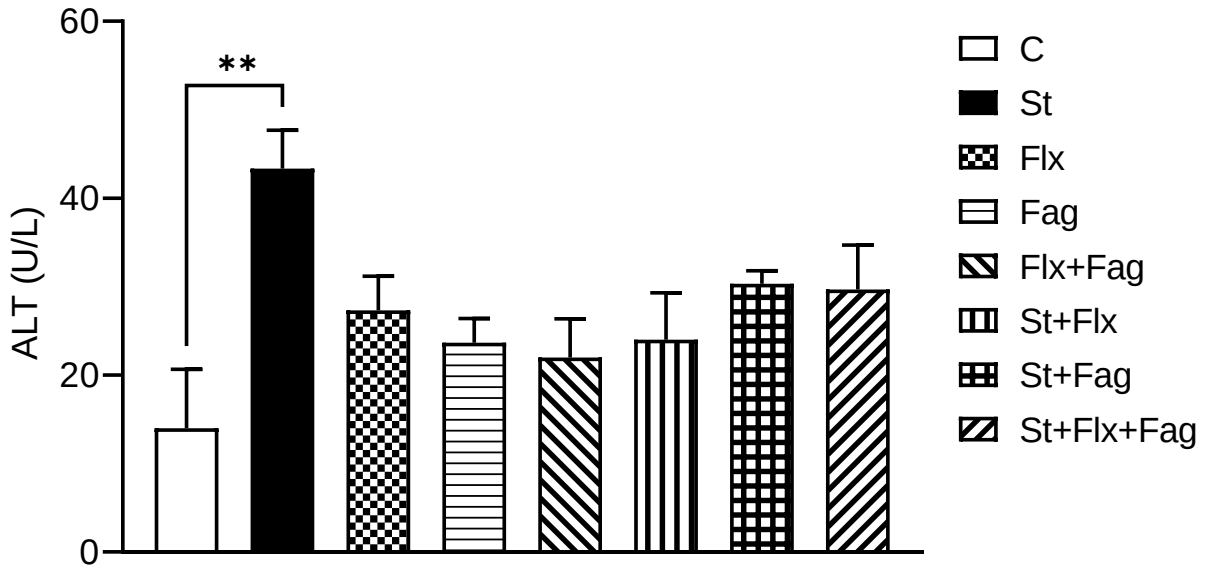


*Figure 4.14: Serum ALT levels: ALT being a biomarker of hepatocellular injury was evaluated. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used. **p≤ 0.01.*

4.3.2.2 Alkaline Phosphatase (ALP)

ALP is present in nearly all tissues, primarily bone and liver. Its serum levels were measured to evaluate of liver injury. The results showed a non-significant increase of ALP levels in the stress group (129.3 ± 22.1) in comparison to the control group (64.3 ± 17.3). Under treatment, no significant change in ALP levels was observed; the Fluoxetine monotherapy group (189.2 ± 22.1), the *Fagonia indica* monotherapy group (124.7 ± 6.8) and the combination therapy group (133.0 ± 4.7), respectively. The normal ALP level is 122-148 U/L (Gordon P Otto, 2016). Unfortunately, a significant additive was not seen in the combination therapy (Figure 4.15).

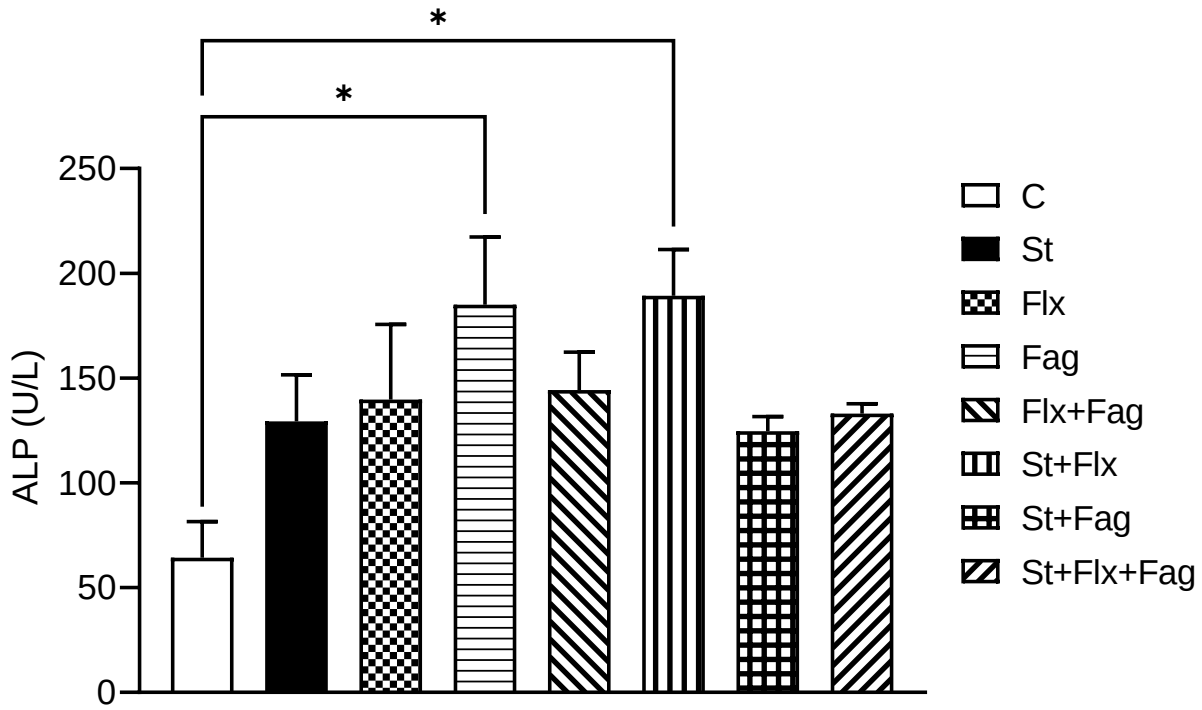


*Figure 4.15: Serum ALP levels: ALP used as a biomarker of hepatocellular injury was evaluated. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05.*

4.3.4 Renal Function Tests

To assess the extent of influence of stress and the treatments on kidney function, serum creatinine and serum urea tests were done.

4.3.4.1 Creatinine

Creatinine is entirely removed by the kidneys. Therefore, measuring serum creatinine levels is an important tool in assessing renal function and detecting potential kidney dysfunction. No significant change was seen in creatinine levels of the stress group (0.3 ± 0.03) as compared to the control group (0.3 ± 0.06). The Fluoxetine group (0.5 ± 0.01) showed the highest levels of creatinine among all the groups. The Fluoxetine monotherapy group (0.2 ± 0.07) was able to decrease creatinine levels of the stressed mice as compared to the *Fagonia indica* monotherapy group (0.4 ± 0.06) and the combination therapy group (0.24 ± 0.02); (St + Flx > St + Flx + Fag > St + Fag) (Figure 4.16).

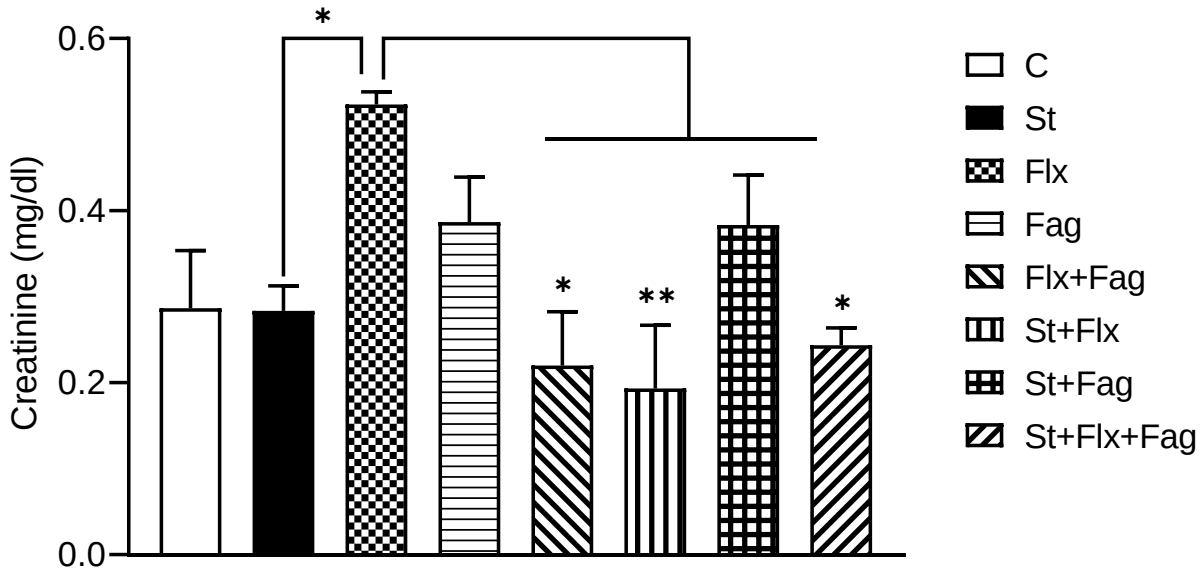


*Figure 4.16: Serum creatinine levels: Creatinine used to evaluate kidney function. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05; **p≤ 0.01.*

4.3.4.2 Urea

No significant change was seen in urea levels of the stress group (3.4 ± 0.3) as compared to the control group (0.3 ± 0.06). The Fluoxetine group (1.6 ± 0.3) showed significant decrease in urea levels compared to the control group. The Fluoxetine monotherapy group (4.6 ± 0.3) was able to cause non-significant increase in the urea levels of stressed mice. On the other hand, the *Fagonia indica* monotherapy group (2.7 ± 0.1) and the combination therapy group (1.6 ± 0.5), were seen to have decreased urea levels. Even though an additive effect was seen but it was not significant (Figure 4.17).

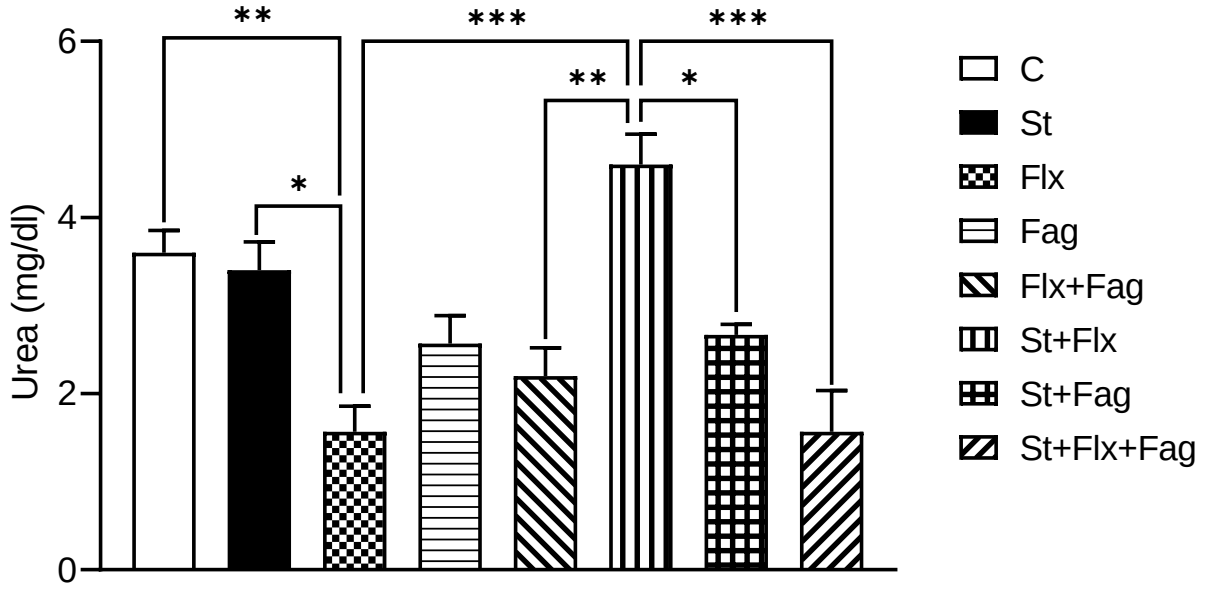


*Figure 4.17: Serum urea levels: Urea used to evaluate kidney function. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05, **p≤ 0.01, ***p= < 0.001.*

4.3.5 Lipid Profile

To assess the risk of cardiovascular dysfunction, levels of total cholesterol, HDL and LDL were measured.

4.3.5.1 Total cholesterol

Serum total cholesterol is a potential predictive biomarker for ascertaining cardiac dysfunction. The stress group (59.3 ± 3.3) showed a non-significant decrease in cholesterol levels as compared to the control group (95.7 ± 9.3). The Fluoxetine group (151.3 ± 37.4) and the *Fagonia indica* group (185.7 ± 49.6) showed increased levels of total cholesterol. Surprisingly, no drastic change was observed in the treatment groups and all groups were under the normal reference range (81-208 mg/dl) (Colonies, 2008) (Figure 4.18).

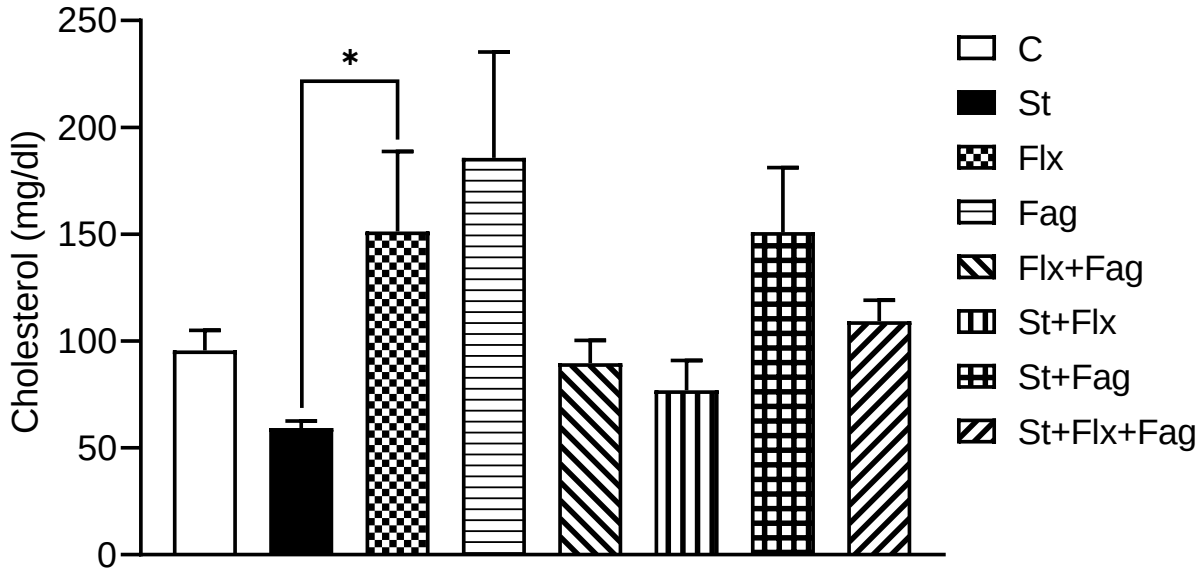


*Figure 4.18: Total serum cholesterol**:** Cholesterol used to evaluate cardiac function. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05.*

4.3.5.2 Low Density Lipoprotein (LDL)

The stress group (79.7 ± 14.3) showed a non-significant decrease in LDL levels as compared to the control group (121.0 ± 15.3). Increased LDL levels were seen in the Fluoxetine group (234.7 ± 21.4) and the *Fagonia indica* group (529.7 ± 35.1). Surprisingly, in comparison with these two groups, the levels were decreased in the Fluoxetine + *Fagonia indica* group (197.3 ± 6.8). All three treatments resulted in increased LDL levels, though the difference was not significant as compared to the stressed mice; the Fluoxetine monotherapy group (310.7 ± 80.3), the *Fagonia indica* monotherapy group (465.3 ± 87.8) and the combination therapy group (428.3 ± 83.3), respectively (Figure 4.19).

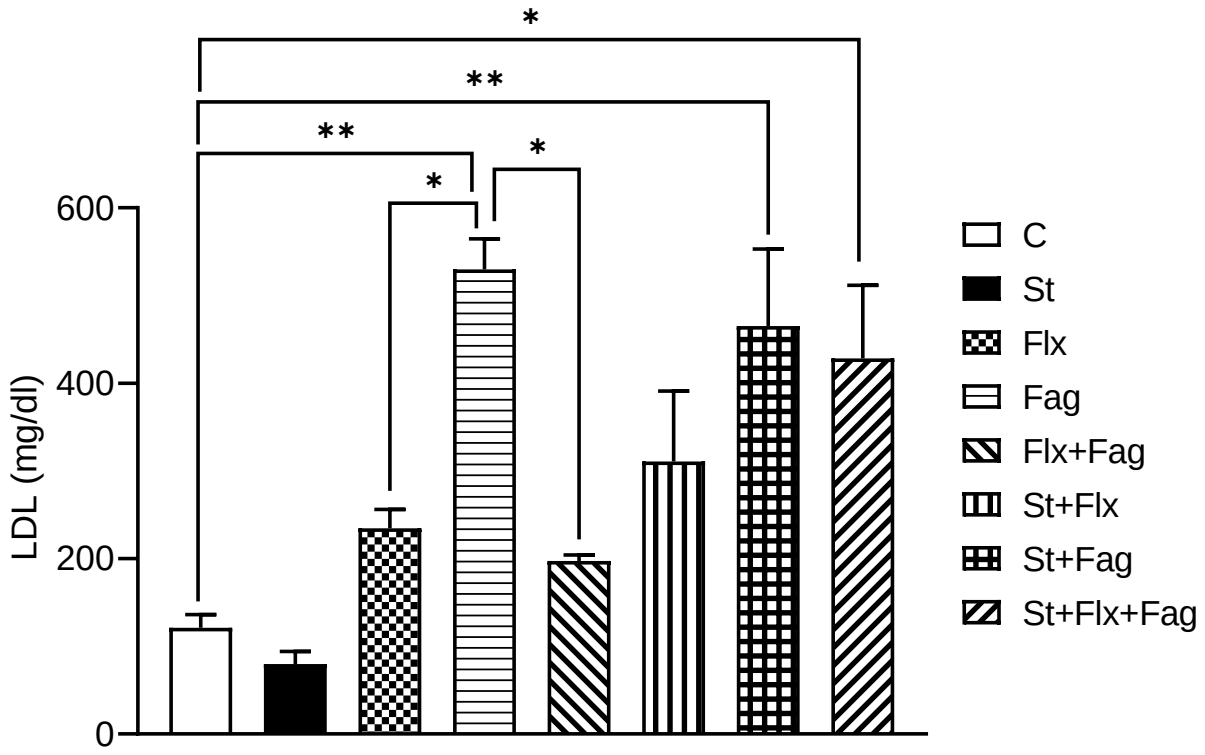


*Figure 4.19: Serum LDL: LDL used to evaluate cardiac function. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used. *p≤ 0.05; **p≤0.01.*

4.3.5.3 High Density Lipoprotein (HDL)

No drastic change was seen in HDL levels of the stress mice (196.7 ± 24.3) compared to the control group (167.7 ± 19.37). The Fluoxetine group (418.7 ± 197.3) resulted in insignificant increase in HDL levels, but on the other hand the *Fagonia indica* group (107.7 ± 33.84) was seen to have the lowest levels. Upon treatment of stressed mice, the Fluoxetine monotherapy group (532.0 ± 149.8) showed the highest levels of HDL. Unfortunately, the difference was insignificant. Interestingly, no significant changes were seen in the *Fagonia indica* monotherapy group (265.0 ± 59.4) and the combination therapy group (255.0 ± 52.6), respectively (Figure 4.20).

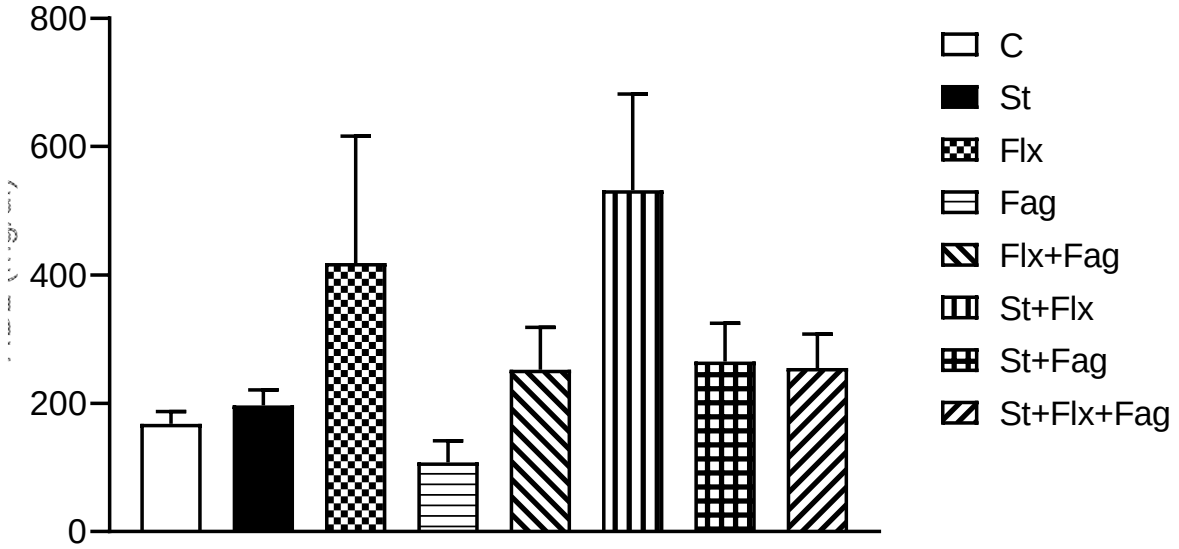


*Figure 4.20: Serum HDL**:** HDL used to evaluate cardiac function. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used.*

4.3.5.4 LDL: HDL

The LDL:HDL ratio aligns with the results of serum cholesterol. This ratio should normally be 1:3. All groups except for the *Fagonia indica* group showed normal range for this ratio. The lowest ratio is observed for the stress group (Figure 4.21).

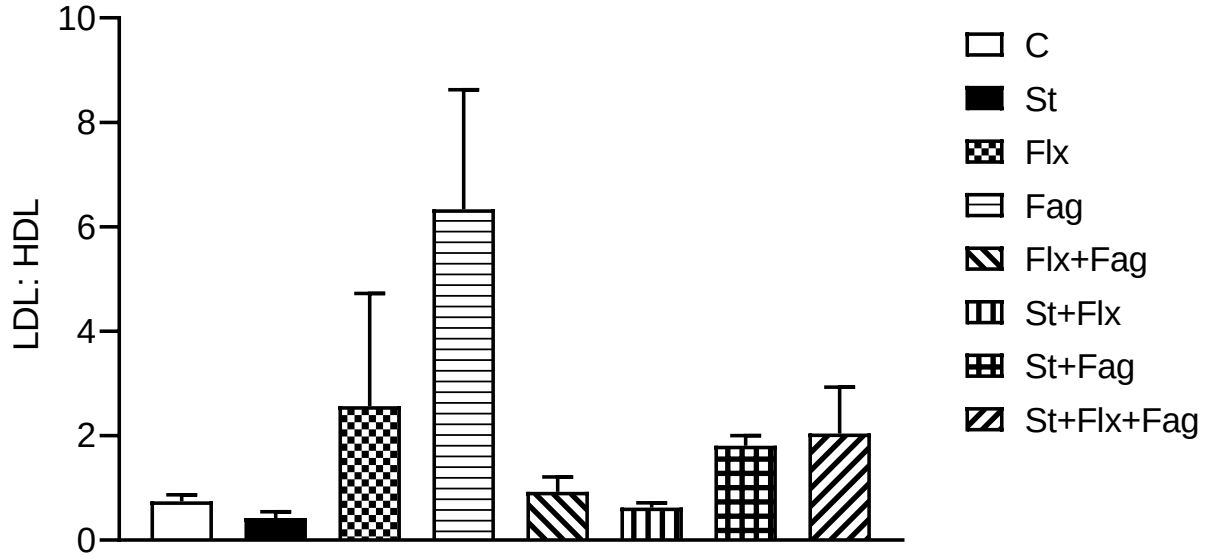


*Figure 4.21: Ratio of serum LDL to HDL**:** LDL: HDL used to evaluate cardiac function. Control (C), Stress (St), Fluoxetine 18mg/Kg/day (Flx), Fagonia indica 400mg/Kg/day (Fag). Error bars represent ± standard error mean (SEM); n=3. One-way ANOVA followed by Tukey's multiple comparisons test was used.*

DISCUSSION

This study was aimed to evaluate the antistress and neuroprotective properties of the medicinal plant, *Fagonia indicia*, either as a monotherapy or as an additive component to the conventionally used SSRI, Fluoxetine.

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).

Flouxetine is the most commonly used SSRI which is administered orally. The present study uses safe dosage of 18mg/Kg/day which previously has been shown to be the most effect for the treatment of depression (Holick et al., 2008). For this study the dosage was determined by keeping in consideration the average body weight of each mouse. Though effective, the conventional therapy is associated with a long list of side effects, and therefore, scientists are exploring integrative medicine for possible combination treatments with phytochemical compounds for improved stress management.

Fagonia indica has shown to exhibit antioxidant, anti-inflammatory, anticoagulatory and anti-cancerous capabilities (Anil et al., 2019). Still, no studies have been conducted to evaluate therapeutic potential of *Fagonia indica* against cognitive decline and social impairment caused by stress. Hence, the combined effect of Fluoxetine with *Fagonia indica* plant extract was explored for the first time against stress-induced social impairment in this study. For the present study, the dose used for *Fagonia indica* was set at 400/Kg/day, as this dose was used to check for anti-depressant properties of a closely related specie, *Fagonia olivieri* in rodents (Umbreen Rashid et al., 2022a).

Under stress conditions, the body’s homeostasis changes which can influence the physical parameters like weight and feed consumption. Hence, these two parameters were assessed during the 30-day experiment. Overall, no variation in the percentage feed consumption was observed. However, a slight decrease in weight of the stress group was observed. This is in line with the

previous studies that showed weight loss in rodents under restraint stress (Loprinzi & Frith, 2019; Ruth et al., 2002; Marti et al., 1994). Similarly, the Fluoxetine group (106.2 ± 1.0) had quite a significant ($p \leq 0.01$) decrease in weight as compared to the control group (113 ± 2.2). Previously-treatments with Fluoxetine has been linked to cause weight loss (Aggarwal et al., 2016; Lyons et al., 2011). In the present study, the groups treated with *Fagonia indica*, either alone or in combination demonstrated significant weight loss as well. *Fagonia indica* is known to produce hyperglycemic effects (Abbas et al., 2014) that can lower the body weight (Mahmood, 2016). Our findings were consistent with the study done on female rats with polycystic ovaries, 500mg/Kg/day dose of *Fagonia indica* caused decrease in their weight (Younas et al., 2022).

To assess the impact of stress various behavior tests named as marble burying, social interaction, novel object recognition, beam balance and exit circle, were performed. Marble burying is done to reveal repetitive and aggressive behavior. Mice take the marble as a novel stimulus that causes increased levels of anxiety and mice that are depressed have shown to bury more marbles. (Angoa-Pérez et al., 2013). In our experiment, the stress group (12.25 ± 3.4) buried the most marbles showing high levels of compulsive behavior. In the Fluoxetine monotherapy group, there was a significant reduction in the number of buried marbles which was in line with a previous studies showing reduction in anxiety with administration of Fluoxetine (Kobayashi et al., 2008; Dulawa et al., 2004). The *Fagonia indica* monotherapy group (4.5 ± 0.6) also demonstrated reduced aggressiveness which can be due to the presence of various alkaloids, saponins and flavonoids that have antioxidant effect (Seyidoglu & Aydin, 2015) ultimately leading to reduction in stress (Umbreen Rashid et al., 2022b; Ali et al., 2019). An additive effect of the combination therapy was seen, but it was not significant.

Social performance tests for mice are exploratory examines used to research their social conduct. These tests include estimating a mouse's inclination for social collaboration with different mice contrasted with different nonsocial stimuli. The female mice used were sexually non-receptive at the time of their performance, to rule out any biasness owing to their estrous cycle (Chari et al., 2020). Lack of sociability exhibited by the stressed mice was in alignment with the previous studies suggesting a decrease in ability to socialize due to alterations in the prefrontal cortex (PFC), a part of the brain involved in social behaviors, in the stressed mice (Dalla, 2012; Kokras, 2017). Interestingly, assessment of social novelty revealed no drastic changes among the groups.

To assess the inquisitiveness and exploratory behavior of mice exit circle test was performed. The animal models with neurological deficit have shown impairment in such behavior (Lopez-Rodriguez et al., 2015). Our study showed, a deficit behavior of stressed mice (3.5 ± 0.29) compared to the control (4 ± 0). All three treatment groups resulted in improved behavior, with the combination therapy group showing the most improvement (4.25 ± 0.25), owing to the neuroprotective properties of Fluoxetine (Rogóz & Skuza, 2011) and *Fagonia indica* (Rawal et al., 2004b).

Studies have shown that the hippocampal region and the perirhinal cortex are involved in memory-based learning. Any damage to these parts will result in impairment of this behavior (Antunes & Biala, 2012). Chronic stress can lead to a reduction in hippocampal neurogenesis due to a decrease in the expression of brain-derived neurotrophic factor (BDNF) gene which has been linked to memory impairment (Loprinzi & Frith, 2019; Patki et al., 2013). Moreover, under stressful conditions, a high level of circulating corticosterone in blood has also been linked to memory impairment (Roozendaal, 2002). Therefore, novel object recognition was done to test for the recognition memory and learning. Our study showed a negligible reduction in recognition index (RI) of the stress group (0.63 ± 0.05) as compared to the control group (0.64 ± 0.05). An improved recognition index (RI) in the Fluoxetine group (0.74 ± 0.02) and the Fluoxetine monotherapy group (0.64 ± 0.03) was seen. Such an improvement in recognition memory was also observed in rodents with cognitive impairment induced by chemotherapy that were given 10mg/Kg/day Fluoxetine as treatment (Lyons et al., 2011). This is due to the Fluoxetine's effect on promoting BDNF expression which in turn causes neurogenesis (Lyons et al., 2011; David et al., 2009). Unfortunately, no such effect in the *Fagonia indica* monotherapy group or the combination therapy group was observed.

Chronic stress has been shown to cause motor coordination dysfunction. A study where mice were given stress early in their life by separating them from their mothers for 3 hours daily during their lactation, impaired motor coordination was observed in their adulthood. This was linked to reduced volume of the cerebellum and neuroinflammation (Kokubo et al., 2018; Schimmel et al., 2017). Therefore, to evaluate the motor coordination, beam balance test was performed. The highest impairment score was seen in the stress group, indicating a deficit behavior (Szczygielski et al., 2016), whereas the most improvement in the impairment score was observed under the

combination therapy. The additive effect ($p \leq 0.05$) might be due to synergism of increased serotonin levels leading towards the development of cerebral regions involved in motor coordination (Nichols, 2011), and the anti-inflammatory effects of *Fagonia indica* (Umbreen Rashid et al., 2022a),

To assess the impact of stress on the hormonal levels of mice different biochemical test such as cortisol, liver function, renal function and lipid profile were performed. Cortisol, a typical glucocorticoid hormone, is released from the adrenal cortex of the kidney in response to stress stimuli (Ramamoorthy & Cidlowski, 2016). Although corticosterone is considered to be the major glucocorticoid hormone involved in regulation of stress response in rodents, several studies have reported increase in plasma and adrenal cortisol as well (Nakamura et al., 1990; Thurston & Hauhart, 1989; Won & Lin, 1995). Cortisol have been used as the index for stress activation in mice (Ayada et al., 2002; Chen et al., 2005; Zhang et al., 2011). A study by (Sentari et al., 2019) observed a significant increase in cortisol levels of mice induced with depression. In the present study, overall, there were no significant changes in the cortisol levels, among the groups. A non-significant increase of the cortisol levels than the normal reference range i.e., 8.3–17.06 ng/ml (Kala & Nivsarkar, 2015) as a result of 30-day restraint stressor could be an indication that the mice have adapted to the repeated restraint stress or the stressor may not be severe enough to provoke a significant cortisol response. (Shuai et al., 2015) reported that under stressful conditions glucocorticoid hormones increase to the highest level in early days of the repeated-restraint stress, but after that the concentration of cortisol do not show any significant change (Shuai et al., 2015). Among the groups, only the Fluoxetine monotherapy group (15.7 ± 5.9) and the *Fagonia indica* monotherapy (15.0 ± 6.0) were able to reduce the cortisol levels of the mice to within the normal reference range. Fluoxetine has been shown to competitively bind with the serum albumin protein against cortisol, decreasing its serum levels (Rezaei-Tavirani et al., 2012). The flavonoids present in *Fagonia indica* may have reduced the cortisol levels in the blood as it has been shown that antioxidant and anti-inflammatory activity of flavonoids inhibits the glucocorticoid response (Ruijters et al., 2016). Unfortunately, an additive effect was not seen, rather the combination therapy group had insignificantly increased cortisol level. Further studies, at molecular level are required to find out the under lying cause of increased cortisol levels under the combination therapy.

To assess the risk of hepatocellular damage liver function tests were performed. The results showed that the stress group had higher levels of ALT and ALP as compared to the control group. Previous studies have reported that stress have negative influences on the liver causing hepatotoxicity and liver injury (Zhu Qing & Gu, 2014). Both of the monotherapies and the combination therapy were able to bring ALT levels close to the normal range of 22-32 U/L (Gordon P Otto, 2016). On the other hand, the Fluoxetine monotherapy group resulted in an increase of ALP levels (122-148 U/L) (Gordon P Otto, 2016). Fluoxetine is extensively metabolized by the liver and can cause an increase in liver enzymes (Glenn et al., 1988). Increased ALT and ALP levels in our study, suggests possible liver damage. Although, non-significant the owing to the hepatoprotective effect of *Fagonia indica,* the combination therapy seemed to counteract the possible liver damage caused by the intake of Fluoxetine (Rashid et al., 2016).

In order to investigate renal responsiveness and proper functioning of kidneys, renal function tests were performed to assess the serum levels of creatinine and urea. Previous study done on mice, has shown that a restraint stress for 6 hours can cause nephrotoxicity by inducing oxidative stress and inflammation in the kidneys (Said et al., 2021). Furthermore, rodents exposed to restraint stress of 6 hours, show increased levels of creatinine and nitrogen urea (Arakawa et al., 1997). Surprisingly, our study found no significant change in creatinine and urea levels between the stress group and the control group, suggesting that 4-hour, 30-day restraint stress, might not be enough to cause acute kidney damage. Fluoxetine is excreted from the human body primarily through the urinary system, where approximately 10% is eliminated as the parent compound (Fluoxetine) and the remainder is eliminated as norfluoxetine (Hiemke & Härtter, 2000; Said et al., 2021). In our study, decreased levels of urea in the Fluoxetine group were observed, which could be due to inhibitory action of Fluoxetine on kidney epithelial K^+ channels (Vieira-Coelho & Martel, 2023) and/or could be due to possible liver damage as indicated by increased ALT and ALP levels. On the other hand, increased levels of urea in the Fluoxetine monotherapy group (4.6 ± 0.3) were observed. Studies have reported that Fluoxetine intake effects inappropriate secretion of antidiuretic hormone (ADH), causing increased water reabsorption by the kidneys, a leading cause towards renal insufficiency and nephrotoxicity, which ultimately is associated with acute increases in serum creatinine and urea levels (Ng et al., 2014; Schattner et al., 1996). On the other hand, the *Fagonia indica* monotherapy group (2.7 ± 0.1) and the combination therapy group (1.6 ± 0.5),

showed decreased urea levels. Various functional and bioactive compounds found in *Fagnoia indica* have been identified as having critical properties in preventing renal dysfunctions including flavonoids, sterols and alkaloids (Anil et al., 2012; Olaiya et al., 2015; Szliszka et al., 2009; Yakubu & Musa Fakai, 2012), that might have played role in counteracting the negative side effect of Fluoxetine by reducing urea levels in the combination therapy. Unfortunately, the additive effect observed was not significant.

To evaluate the risk of cardiovascular dysfunction levels of total cholesterol, HDL and LDL were measured. The reference range for cholesterol in mice is 81- 208 mg/dl (Colonies, 2008). In present study, the stress group showed a non-significant decrease in total cholesterol levels compared to the control group. This might be the cause of weight loss observed in stressed mice. Surprisingly, the LDL/HDL ratio of the stressed mice was within the normal reference range. Important finding was increased LDL/HDL ratio of the *Fagonia indica* group. According to the literature the GCMS analysis of *Fagonia indica* shows presence of fat content, due to the presence of sterols and fatty acids (Dastagir1 et al., 2014). Interestingly, Fluoxetine was able to counteract this when given in combination with *Fagonia indica*. Increased levels of total cholesterol, with increased LDL/HDL ratio caused by the intake of *Fagonia indica* implicates a potential risk for cardiac dysfunction. Therefore, further investigations are advised if *Fagonia indica* is safe to be administered to the patients with cardiac disorders.

Overall, our results show that the combination therapy has a positive effect on anxiety, social preference, recognition memory, inquisitiveness and motor coordination. Moreover, in the biochemical tests, an improved level of LFTs and RFTs were seen for the combination therapy suggesting protective role of *Fagonia indica* in counteracting the adverse effects caused by Fluoxetine administration.

CONCLUSION

Restrained stress exhibited declined social behavior. Following co-administration of Fluoxetine with *Fagonia indica*, an improvement in anxiety, sociability, motor coordination and intrinsic inquisitiveness was seen. Regarding biochemical tests, overall *Fagonia indica* was able to counteract the adverse effects of Fluoxetine in the combination therapy. Although an improvement was seen in the combination therapy compared to the monotherapies, unfortunately it was not significant and so, studies with dose variations are required. Furthermore, high cholesterol and LDL: HDL levels in response to *Fagonia indica* treatment suggest that it might not be advisable for cardiac dysfunction patients without further investigations.

FUTURE PROSPECTS

Our study indicates that the combination therapy of Fluoxetine and *Fagonia indica* might be effective in alleviating stress. However, further research is required to analyse the GCMS of *Fagonia indica*, along with the neuroprotective effect of the phytochemicals present at molecular and cellular levels. Moreover, additional investigations are advised to confirm if *Fagonia indica* is safe to be administered to the patients with cardiac disorders.

REFERENCES

A Atiq-ur-Rehman, A.-R., Talib, F., & Aftab, T. (2021). FTIR, HPLC, GC-MS Analysis and Investigation of Hypoglycemic Effects of Leaves Extracts of Fagonia indica. *Pharmacognosy Communications*, *11*(2), 109–118. https://doi.org/10.5530/pc.2021.2.21*ajpregu.2002.282.1.r77*. (n.d.).

Ali, A., Malik, A., & Ali, Q. (2019). *International Journal of Botany Studies Comparative in vitro anti-oxidant and anti-fungal potential profiles from methanol extract of Fagonia indica, Fagonia bruguieri and Fagonia... Non-coding RNAs in cancer diagnosis and therapy View project Phytochemicals activities View project*. www.botanyjournals.com

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. *Journal of Visualized Experiments : JoVE*, *82*, 50978. https://doi.org/10.3791/50978

Anil, P., Nikhil, B., Manoj, G., & Prakash, N. B. (2012.). PHYTOCHEMICALS AND BIOLOGICAL ACTIVITIES OF FAGONIA INDICA. In *IRJP* (Vol. 2012, Issue 6). www.irjponline.com

Antunes, M., & Biala, G. (2012). The novel object recognition memory: Neurobiology, test procedure, and its modifications. In *Cognitive Processing* (Vol. 13, Issue 2, pp. 93–110). https://doi.org/10.1007/s10339-011-0430-z

Arakawa, H., Kodama, H., Matsuoka, N., & Yamaguchi, I. (1997). Stress Increases Plasma Enzyme Activity in Rats: Differential Effects of Adrenergic and Cholinergic Blockades. *Journal of Pharmacology and Experimental Therapeutics*, *280*(3), 1296. http://jpet.aspetjournals.org/content/280/3/1296.abstract

Ayada, K., Tadano, T., & Endo, Y. (2002). Gnawing behavior of a mouse in a narrow cylinder. *Physiology & Behavior - PHYSIOL BEHAV*, *77*, 161–166. https://doi.org/10.1016/S0031-9384(02)00844-2

Bartlett, A. A., Singh, R., & Hunter, R. G. (2017). Anxiety and epigenetics. In *Advances in Experimental Medicine and Biology* (Vol. 978, pp. 145–166). Springer New York LLC. https://doi.org/10.1007/978-3-319-53889-1_8

Black, P. H. (2002). Stress and the inflammatory response: A review of neurogenic inflammation. In *Brain, Behavior, and Immunity* (Vol. 16, Issue 6, pp. 622–653). https://doi.org/10.1016/S0889-1591(02)00021-1

Brooks BW, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, La Point TW. (2003). Aquatic ecotoxicology of fluoxetine. Toxicol Lett; 142(3):169-83. doi: 10.1016/s0378-4274(03)00066-3. PMID: 12691711.

Byers, S. L., Wiles, M. V., Dunn, S. L., & Taft, R. A. (2012). Mouse estrous cycle identification tool and images. *PLoS ONE*, *7*(4). https://doi.org/10.1371/journal.pone.0035538

Cao YL, Lin JH, Hammes HP, Zhang C. (2022). Flavonoids in Treatment of Chronic Kidney Disease. Molecules; 27(7):2365. doi: 10.3390/molecules27072365. PMID: 35408760; PMCID: PMC9000519.

Chari, T., Griswold, S., Andrews, N. A., & Fagiolini, M. (2020). The Stage of the Estrus Cycle Is Critical for Interpretation of Female Mouse Social Interaction Behavior. *Frontiers in Behavioral Neuroscience*, *14*. https://doi.org/10.3389/fnbeh.2020.00113

Chen, Y., Kong, L.-D., Xia, X., Kung, H.-F., & Zhang, L. (2005). Behavioral and biochemical studies of total furocoumarins from seeds of Psoralea corylifolia in the forced swimming test in mice. *Journal of Ethnopharmacology*, *96*(3), 451–459. https://doi.org/https://doi.org/10.1016/j.jep.2004.09.033

Colonies, A. (2008). *BALB/C Mouse Biochemistry*.

Dastagir[1], G., Hussain[1], F., Khanzadi, A., & Khattak[2], F. (2014). NUTRITIONAL EVALUATION OF PLANTS OF FAMILY ZYGOPHYLLACEAE AND EUPHORBIACEAE. In *Pak. J. Bot* (Vol. 46, Issue 5).

David, D. J., Samuels, B. A., Rainer, Q., Wang, J. W., Marsteller, D., Mendez, I., Drew, M., Craig, D. A., Guiard, B. P., Guilloux, J. P., Artymyshyn, R. P., Gardier, A. M., Gerald, C., Antonijevic, I. A., Leonardo, E. D., & Hen, R. (2009). Neurogenesis-Dependent and -Independent Effects of Fluoxetine in an Animal Model of Anxiety/Depression. *Neuron*, *62*(4), 479–493. https://doi.org/10.1016/j.neuron.2009.04.017

Dulawa, S. C., Holick, K. A., Gundersen, B., & Hen, R. (2004a). Effects of chronic fluoxetine in animal models of anxiety and depression. *Neuropsychopharmacology*, *29*(7), 1321–1330. https://doi.org/10.1038/sj.npp.1300433

Gholami, N., Sabzvari, B., Razzaghi, A., & Salah, S. (2017). Effect of stress, anxiety and depression on unstimulated salivary flow rate and xerostomia. *Journal of Dental Research, Dental Clinics, Dental Prospects*, *11*, 247–252. https://doi.org/10.15171/joddd.2017.043

Godoy, L. D., Rossignoli, M. T., Delfino-Pereira, P., Garcia-Cairasco, N., & Umeoka, E. H. de L. (2018). A comprehensive overview on stress neurobiology: Basic concepts and clinical implications. In *Frontiers in Behavioral Neuroscience* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fnbeh.2018.00127

Gordon P Otto. (2016). Clinical Chemistry Reference Intervals for C57BL/6J, C57BL/6N, and C3HeB/FeJ Mice (Mus musculus). *Journal of the American Association for Laboratory Animal Science*.

Gerecke KM, Kolobova A, Allen S, Fawer JL. (2013). Exercise protects against chronic restraint stress-induced oxidative stress in the cortex and hippocampus. Brain Res; 1509:66-78. doi: 10.1016/j.brainres.2013.02.027. Epub 2013 Mar 13. PMID: 23499928.

Hiemke, C., & Härtter, S. (2000). Pharmacokinetics of selective serotonin reuptake inhibitors. *Pharmacology & Therapeutics*, *85*(1), 11–28. https://doi.org/https://doi.org/10.1016/S0163-7258(99)00048-0

Holick, K. A., Lee, D. C., Hen, R., & Dulawa, S. C. (2008). Behavioral effects of chronic fluoxetine in BALB/cJ mice do not require adult hippocampal neurogenesis or the serotonin 1A receptor. *Neuropsychopharmacology*, *33*(2), 406–417. https://doi.org/10.1038/sj.npp.1301399

James M. Ferguson, M. D. (2001). SSRI Antidepressant Medications: Adverse Effects and Tolerability. *Primary Care Companion to The Journal of Clinical Psychiatry*, *3*(1), 22–27.

Kala, M., & Nivsarkar, M. (2015). Role of cortisol and superoxide dismutase in psychological stress induced anovulation. *General and Comparative Endocrinology*, *225*. https://doi.org/10.1016/j.ygcen.2015.09.010

Khalid, A., Shakeel, R., Justin, S., Iqbal, G., Shah, S. 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*, 1545–1557. https://doi.org/10.2174/1389450118666170315120627

Kobayashi, T., Hayashi, E., Shimamura, M., Kinoshita, M., & Murphy, N. P. (2008). Neurochemical responses to antidepressants in the prefrontal cortex of mice and their efficacy in preclinical models of anxiety-like and depression-like behavior: A comparative and correlational study. *Psychopharmacology*, *197*(4), 567–580. https://doi.org/10.1007/s00213-008-1070-6

Kokubo, M., Toya, S., Amano, I., & Takatsuru, Y. (2018). Early-life stress induces motor coordination dysfunction in adult mice. *Journal of Physiological Sciences*, *68*(5), 663–669. https://doi.org/10.1007/s12576-017-0580-6

Liu, Z., Que, S., Xu, J., & Peng, T. (2014). Alanine aminotransferase-old biomarker and new concept: A review. In *International Journal of Medical Sciences* (Vol. 11, Issue 9, pp. 925–935). Ivyspring International Publisher. https://doi.org/10.7150/ijms.8951

Loprinzi, P. D., & Frith, E. (2019). Protective and therapeutic effects of exercise on stress-induced memory impairment. In *Journal of Physiological Sciences* (Vol. 69, Issue 1). Springer Tokyo. https://doi.org/10.1007/s12576-018-0638-0

Lupien, S., Maheu, F., Tu, M., Fiocco, A., & Schramek, T. (2008). The effects of stress and stress hormones on human cognition: Implications for the field of brain and cognition. *Brain and Cognition*, *65*, 209–237. https://doi.org/10.1016/j.bandc.2007.02.007

Lyons, L., Elbeltagy, M., Umka, J., Markwick, R., Startin, C., Bennett, G., & Wigmore, P. (2011). Fluoxetine reverses the memory impairment and reduction in proliferation and survival of hippocampal cells caused by methotrexate chemotherapy. *Psychopharmacology*, *215*(1), 105–115. https://doi.org/10.1007/s00213-010-2122-2

Marin, M.-F., Lord, C., Andrews, J., Juster, R.-P., Sindi, S., Arsenault-Lapierre, G., Fiocco, A. J., & Lupien, S. J. (2011). Chronic stress, cognitive functioning and mental health. *Neurobiology of Learning and Memory*, *96*(4), 583–595. https://doi.org/https://doi.org/10.1016/j.nlm.2011.02.016

Mariotti, A. (2015). The effects of chronic stress on health: New insights into the molecular mechanisms of brain-body communication. *Future Science OA*, *1*, 1–6. https://doi.org/10.4155/fso.15.21

Marti, O., Marti, J., & Armario, A. (1994). Effects of Chronic Stress on Food Intake in Rats: Influence of Stressor Intensity and Duration of Daily Exposure. In *Physiology & Behavior* (Vol. 55, Issue 4).

Nakamura, K., Aoike, A., Hosokawa, T., Rokutan, K., Koyama, K., Nishi, Y., Yoshida, A., & Kawai, K. (1990). Effect of food-restriction stress on immune response in mice. *Journal of Neuroimmunology*, *30*(1), 23–29. https://doi.org/https://doi.org/10.1016/0165-5728(90)90049-S

Ng TM, Cao DX, Patel KA, Wong YM, Prasad M, Lou M, Elkayam U. (2014). Association of hyponatremia to diuretic response and incidence of increased serum creatinine levels in hospitalized patients with acute decompensated heart failure. Cardiology; 128(4):333-42. doi: 10.1159/000360604. PMID: 24942293.

Nichols, R. A. (2011). Serotonin, presynaptic 5-HT 3 receptors and synaptic plasticity in the developing cerebellum. In *Journal of Physiology* (Vol. 589, Issue 21, pp. 5019–5020). https://doi.org/10.1113/jphysiol.2011.219782

Olaiya, C., Esan, A., & Alabi, T. (2015). Ameliorative effects of β-sitosterol on some biochemical indices of hypertension in wistar albino rats. *African Journal of Medicine and Medical Sciences*, *43*, 157–166.

Patki, G., Solanki, N., Atrooz, F., Allam, F., & Salim, S. (2013). Depression, anxiety-like behavior and memory impairment are associated with increased oxidative stress and inflammation in a rat model of social stress. *Brain Research*, *1539*, 73–86. https://doi.org/10.1016/j.brainres.2013.09.033

Pitman, D. L., Ottenweller, J. E., Natelson, B. H., Ottenweller, J. E., & Natelson, B. H. (1988). Plasma Corticosterone Levels During Repeated Presentation of Two Intensities of Restraint Stress: Chronic Stress and Habituation. In *Physiology & Behavior* (Vol. 43).

Podsevatkin VG, Kiriukhina SV, Podsevatkin DV, Podsevatkina SV, Blinov DS. (2008) Dynamics of the behavioral response and cortisole level caused by the combined action of mexidole,

diazepam, thymogen, and hyperbaric oxygenation in mice under immobilization stress conditions. Eksp Klin Farmakol; 71(1):22-5. Russian. PMID: 18365482.

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

Ranabir, S., & Keisam, R. (2011). Stress and hormones. *Indian Journal of Endocrinology and Metabolism*, *15*, 18–22. https://doi.org/10.4103/2230-8210.77573

Rashid, U., Khan, M. R., & Sajid, M. (2016). Hepatoprotective potential of Fagonia olivieri DC. against acetaminophen induced toxicity in rat. *BMC Complementary and Alternative Medicine*, *16*(1). https://doi.org/10.1186/s12906-016-1445-x

Rawal, A. K., Muddeshwar, M. G., & Biswas, S. K. (2004). Rubia cordifolia, Fagonia cretica linn and Tinospora cordifolia exert neuroprotection by modulating the antioxidant system in rat hippocampal slices subjected to oxygen glucose deprivation. *BMC Complementary and Alternative Medicine*, *4*. https://doi.org/10.1186/1472-6882-4-11

Rezaei-Tavirani, M., Tadayon, R., Mortazavi, A., Medhet, A., Kalantari, S., & Noshinfar, E. (2012). *Fluoxetine Competes with Cortisol for Binding to Human Serum Albumin*.

Roozendaal, B. (2002). Stress and memory: Opposing effects of glucocorticoids on memory consolidation and memory retrieval. In *Neurobiology of Learning and Memory* (Vol. 78, Issue 3, pp. 578–595). Academic Press Inc. https://doi.org/10.1006/nlme.2002.4080

Ruijters, E. J. B., Haenen, G. R. M. M., Willemsen, M., Weseler, A. R., & Bast, A. (2016). Food-derived bioactives can protect the anti-inflammatory activity of cortisol with antioxidant-dependent and -independent mechanisms. *International Journal of Molecular Sciences*, *17*(2). https://doi.org/10.3390/ijms17020239

S, M. P., Singh, V., & Kumar, Y. (2021). *THE CHEMISTRY AND PHARMACOLOGY OF FAGONIA GENUS: A REVIEW*. www.ijsdr.org

Said, O., Okafor, C., Haji, H., Babu, P., Nayak, V., Obianagha, N., & Galano, E. (2021). Effect of Short and Long Term Restraint Stress on the Histology of Liver, Kidney and Suprarenal Gland in

Albino Mice during Postweaning Period. *Journal of Pharmaceutical Research International*, *32*, 6–19. https://doi.org/10.9734/JPRI/2020/v32i4031029

Sentari, M., Harahap, U., Sapiie, T., & Ritarwan, K. (2019). Blood Cortisol Level and Blood Serotonin Level in Depression Mice with Basil Leaf Essential Oil Treatment. *Open Access Macedonian Journal of Medical Sciences*, *7*, 2652–2655. https://doi.org/10.3889/oamjms.2019.819

Seyidoglu, N., & Aydin, C. (1995). *Stress, Natural Antioxidants and Future Perspectives*. www.intechopen.com

Shuai, G., Miao, Y.-L., Jiao, G.-Z., Sun, M.-J., Li, H., Lin, J., Luo, M.-J., & Tan, J.-H. (2015). Dynamics and Correlation of Serum Cortisol and Corticosterone under Different Physiological or Stressful Conditions in Mice. *PLOS ONE*, *10*, e0117503. https://doi.org/10.1371/journal.pone.0117503

Szliszka, E., Czuba, Z. P., Domino, M., Mazur, B., Zydowicz, G., & Krol, W. (2009). Ethanolic Extract of Propolis (EEP) Enhances the Apoptosis- Inducing Potential of TRAIL in Cancer Cells. *Molecules*, *14*(2), 738–754. https://doi.org/10.3390/molecules

Thurston, J. H., & Hauhart, R. E. (1989). Effect of momentary stress on brain energy metabolism in weanling mice: Apparent use of lactate as cerebral metabolic fuel concomitant with a decrease in brain glucose utilization. *Metabolic Brain Disease*, *4*(3), 177–186. https://doi.org/10.1007/BF01000294

Tse, J., Flin, R., & Mearns, K. (2007). Facets of job effort in bus driver health: Deconstructing "effort" in the effort-reward imbalance model. *Journal of Occupational Health Psychology*, *12*, 48–62. https://doi.org/10.1037/1076-8998.12.1.48

Umbreen Rashid, Muhammad Rashid Khan, Jasia Bokhari, Shumaila Jan, Hammad Ismail, & Bushra Mirza. (2022a). In vivo assessment of pharmacological potentials of different fractions of Fagonia olivieri DC. *World Journal of Advanced Research and Reviews*, *16*(2), 761–770. https://doi.org/10.30574/wjarr.2022.16.2.1243

Vieira-Coelho, M. A., & Martel, F. (2023). Inhibition of kidney potassium channels by fluoxetine: In vivo and in vitro studies. *Fundamental & Clinical Pharmacology*, *37*(2), 226—234. https://doi.org/10.1111/fcp.12833

Won, S. J., & Lin, M. T. (1995). Thermal stresses reduce natural killer cell cytotoxicity. *Journal of Applied Physiology*, *79*(3), 732–737. https://doi.org/10.1152/jappl.1995.79.3.732

Yakubu, M., & Musa Fakai, I. (2012). Liver and Kidney Functional Indices of Pregnant Rats Following the Administration of the Crude Alkaloids from Senna alata (Linn. Roxb) Leaves. *Iranian Journal of Toxicology*, *6*.

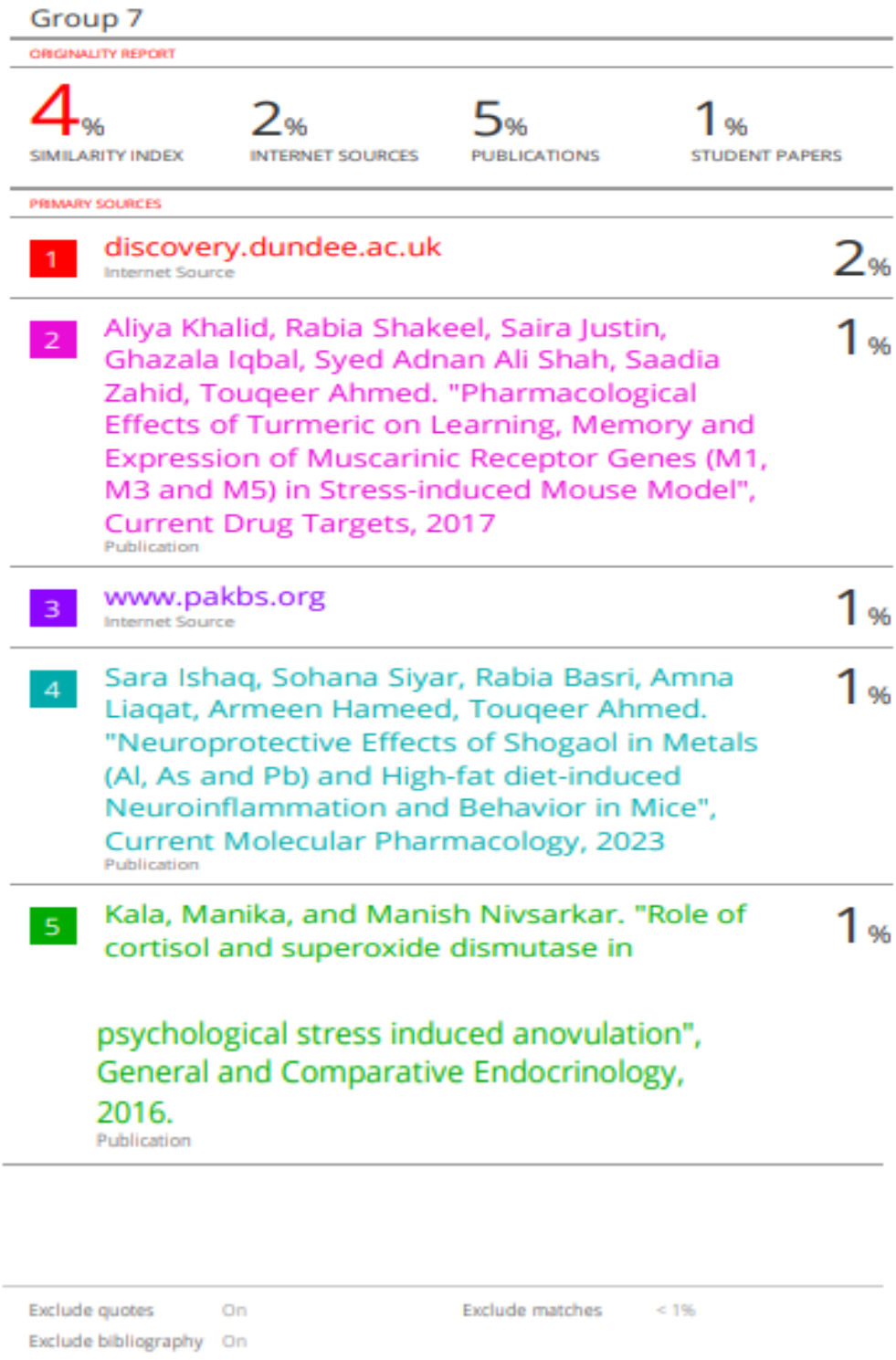
Yaribeygi, H., Panahi, Y., Sahraei, H., Johnston, T., & Sahebkar, A. (2017). The impact of stress on body function: A review. *EXCLI Journal*, *16*, 1057–1072. https://doi.org/10.17179/excli2017-480

Zhang, S.-Y., Wang, J.-Z., Li, J.-J., Wei, D.-L., Sui, H.-S., Zhang, Z.-H., Zhou, P., & Tan, J.-H. (2011). Maternal Restraint Stress Diminishes the Developmental Potential of Oocytes1. *Biology of Reproduction*, *84*(4), 672–681. https://doi.org/10.1095/biolreprod.110.087890

Zhou P, Lian HY, Cui W, Wei DL, Li Q, Liu YX, Liu XY, Tan JH. (2012). Maternal-restraint stress increases oocyte aneuploidy by impairing metaphase I spindle assembly and reducing spindle assembly checkpoint proteins in mice. Biol Reprod; 86(3):83. doi: 10.1095/biolreprod.111.095281. PMID: 22133696.

Zhu Qing AND Gu L, Wang Y, Jia L, Zhao Z, Peng S, Lei L. (2014). The Role of Alpha-1 and Alpha-2 Adrenoceptors in Restraint Stress-Induced Liver Injury in Mice. *PLOS ONE*, *9*(3), 1–9. https://doi.org/10.1371/journal.pone.0092125

TURNITIN PLAGIARISM REPORT



Group 7

ORIGINALITY REPORT

4%	2%	5%	1%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	discovery.dundee.ac.uk Internet Source	2%
2	Aliya Khalid, Rabia Shakeel, Saira Justin, Ghazala Iqbal, Syed Adnan Ali Shah, Saadia Zahid, Touqeer Ahmed. "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, 2017 Publication	1%
3	www.pakbs.org Internet Source	1%
4	Sara Ishaq, Sohana Siyar, Rabia Basri, Amna Liaqat, Armeen Hameed, Touqeer Ahmed. "Neuroprotective Effects of Shogaol in Metals (Al, As and Pb) and High-fat diet-induced Neuroinflammation and Behavior in Mice", Current Molecular Pharmacology, 2023 Publication	1%
5	Kala, Manika, and Manish Nivsarkar. "Role of cortisol and superoxide dismutase in psychological stress induced anovulation", General and Comparative Endocrinology, 2016. Publication	1%

Exclude quotes	On	Exclude matches	< 1%
Exclude bibliography	On		