In vivo Testing of Anticancerous and Antidepressant Agent Silymarin Against Glioblastoma Multiforme

BS Thesis

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National University of Sciences and Technology (NUST)

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

Bachelor in Applied Biosciences

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

(2023)

Thesis Acceptance Certificate

Certified that final copy of BS FYP Thesis written by **Aimen Akhtar** (Registration No. 00000323841), **Hadia Fatima** (Registration No. 00000324512), **Sania Sajjad** (Registration No. 00000323175), **Sara Sabir** (Registration No. 00000323395), of **Atta-ur-Rahman School of Applied Biosciences** has been vetted by undersigned, found complete in all respects as per NUST Regulations, is free of plagiarism, errors, and mistakes and is accepted as partial fulfillment for the award of BS degree. It is further certified that necessary amendments as pointed out, during final presentation of the scholar, have also been incorporated in the thesis.

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DEDICATION

"This thesis is dedicated to our supervisors at ASAB, and our parents for their persistent guidance and relentless support, enabled us to accomplish this project."

Declaration

We **Aimen Akhtar, Hadia Fatima, Sania Sajjad,** and **Sara Sabir,** declare that the work presented in this thesis titled **"*In vivo Testing of Anticancerous and Antidepressant Agent Silymarin against Glioblastoma Multiforme*"** is our own and has been generated as a result of our research. Where information has been derived from other sources, we confirm that this thesis has indicated this.

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ACKNOWLEDGEMENTS

Alhamdulilllah, most precious thanks to ALLAH and there is none worthy of worship but ALLAH and all praise to ALLAH, whom, with His will give us the opportunity to complete our thesis. All respects for His Holy Prophet Hazrat Muhammad (Peace Be Upon Him) who taught us the right path and enabled us to recognize the oneness of our creator.

First and foremost, we would like to express our deepest gratitude to our supervisor, **Prof. Dr. Aneela Javed,** for her unwavering support, indispensable guidance, and insightful feedback throughout this project. Their expertise, encouragement and dedication have been instrumental in the direction and quality of this research. We thankfully acknowledge the support and inspiration that we received from our supervisor.

We would also like to express our heartfelt appreciation to our teacher, **Dr. Saira Justin,** for her significant contribution, valuable insights, valuable comments, and their immense knowledge and advice to make this research possible.

We would also like to extend our sincere thanks to **Dr. Sadia Nazir** and our seniors **Momina Ijaz, Sabahat Ahmed** and **Iman Fatima** for their utmost help and support throughout this project. Their willingness to contribute has been essential to the completion of this research. We are all grateful to them for generously sharing their time and insights with us.

Lastly, we thank our friends and family, especially our parents for their constant care, support, and love for believing in us and keeping our spirits high during this process.

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List Of Abbreviations

Ach	Acetylcholine
BBB	Blood Brain Barrier
CDK4	Cyclin-Dependent Kinase 4
CDK6	Cyclin-Dependent Kinase 6
CNS	Central Nervous System
DMBA	Dimethylbenzanthracene
DMEM	Dulbecco's Modified Eagle Medium
EDTA	Ethylenediaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
EORTC	European Organization for Research and Treatment of Cancer
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
FLX	Fluoxetine
GBM	Glioblastoma Multiforme
GABA	Gamma-Aminobutyric Acid
H_2O_2	Hydrogen Peroxide
IL-6	Interleukin-6
IL-1β	Interleukin-1-Beta
IDH	Isocitrate Dehydrogenase
MAPK	Mitogen-Activated Protein Kinases
MDD	Major Depressive Disorder
MDM2	Murine Double Minute 2
MET	Mesenchymal-Epithelial Transition Factor (MET)
MTIC	5-(3-Methyl-1-Triazeno) Imidazole-4-Carboxamide

MYC	MYC Protooncogene
NCIC	National Cancer Institute of Canada Clinical Trial Group CE3
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PBS	Phosphate Buffered Saline
RT	Radiation Therapy
STAT3	Signal Transducer and Activator of Transcription 3
TTF	Tumor Treating Fields
TNF-A	Tumor Necrosis Factor Alpha
TPA	Tetradecannoylphorbol Acetate
U87-MG	U87-Malignant Cell Line

ABSTRACT

Glioblastoma multiforme (GBM) is a highly malignant grade IV astrocytoma comprising 54% of all glial cell tumors, with a median survival age of less than 12 months. It is characterized by cognitive decline, seizures, and development of major depressive disorder (MDD). Limited treatment options, lower efficacy of drugs to cross the blood brain barrier (BBB), emerging resistance against the standard treatment drug temozolomide (TMZ), and aggravation of MDD symptoms by TMZ present the need to develop novel treatment strategies. This study aimed to investigate the anticancer and antidepressant potential of silymarin (SIL) in an orthotopic GBM xenograft mouse model. The mouse model was constructed by injecting U-87 cells into the prefrontal cortex of the brain. Mice were divided into 8 groups (4 control and 4 GBM induced group), and were treated with normal saline, Temozolomide (TMZ), Fluoxetine (FLX) standard antidepressant and targeted compound Silymarin (SIL). Treatment doses were administered intraperitoneally. Six behavioral tests evaluating behavioral despair, anhedonia, anxiety, depression-like behavior, locomotion, and social interaction were conducted to evaluate the therapeutic effects of the drugs. TMZ elevated depression-like behavioral symptoms in both the control and GBM groups in all six experiments. Silymarin did not induce any depressive symptoms and proved to be effective in alleviating anxiety- and depression-like behaviors associated with GBM in all six experiments, showing greater efficacy than the standard antidepressant FLX. Moreover, Silymarin reduced inflammation in the GBM group. Thus, the efficacy of silymarin merits further molecular studies, and utilizing silymarin in combinatorial therapy could prove pivotal in the fight against GBM and other cancers.

KEYWORDS:

Anxiety, Astrocytoma, Fluoxetine, Glioblastoma Multiforme, Major Depressive Disorder, Radiation therapy, Seizures, Silymarin , Temozolomide.

Chapter 1

INTRODUCTION

The emperor of all maladies: "cancer," refers to more than 100 diseases that can occur if the cells in our normal healthy body evade cell cycle regulations and proliferate uncontrollably. The hallmarks of cancer are genetic mutations that transform proto-oncogenes into carcinogenic oncogenes, leading to proliferation of cancer cells and suppression of growth inhibitors. This leads to the formation of tumors that exhibit angiogenesis, metastasis, and resistance to apoptosis. Furthermore, tumors create a repertoire of ostensible normal cells in the tumor microenvironment that provide optimal conditions for cancer progression. (Weinberg, 1996)

Glioblastoma multiforme (GBM) is a grade IV malignant astrocytoma with a median survival rate of less than 12 months after diagnosis. (Krex, 2007) GBM accounts for 54% of all glial tumors. (Juweid., 2017 Sep 27.) The causative factors of GBM have not yet been identified; however, ionizing radiation is considered a risk factor. Molecular characteristic studies have identified EGFR, PDGFRA, MET, MDM4, MDM2, PIK3CA, MYC, CDK4, and CDK6 as recurrent targets for gene amplification along with loss of the tumor suppressor CDKN2A/B locus and somatic mutations. (Ignacio Veliz, 2015)The prognosis of GBM is characterized by headache, neurological impairment, and the development of major depressive disorder (MDD). (Rooney, 2013)

MDD is one of the greatest clinical implications of GBM. The inflammatory cascade, which is a hallmark of cancer, contributes to the development of MDD. The poor prognosis of MDD makes treatment of GBM more difficult. (Khan, 2022) According to a study by Xie et al. GBM and MDD possibly share three common signaling pathways in their pathogenies:

MAPK signaling pathway, cytokine-cytokine interaction, and chemokine signaling pathway. Salvadore et al. suggested that amino-acid neurotransmitter system malfunction plays a significant role in contributing to MDD pathophysiology, and the three significant neurotransmitters (GABA, Ach, and glutamic acid) of this system are also potential targets of GBM therapy. The bidirectional approach, suggesting that MDD can be a risk factor for developing GBM, is a complex phenomenon that requires further research. (Xie, 2018)

Although it has been reported that TMZ increases the median survival rate in patients by up to 26.5% in combination with radiotherapy, but its effectiveness is limited by several factors. (Liu, 2008) Acquired resistance to TMZ poses a major challenge in GBM treatment. DNA repair protein O^6-Methylguanine-DNA methyltransferase (MGMT) repairs the lesion produced by MTIC, rendering TMZ ineffective in inducing apoptosis. GBM, like other cancers, is also accompanied by inflammation. significant increase in interleukin-6 (IL-6), interleukin-1beta (IL-1β) and tumor necrosis factor- alpha (TNF-α) is observed in GBM cells. Chemotherapeutic drugs, such as TMZ, induce proinflammatory cytokines, leading to chronic inflammation, which is one of the causes of resistance to treatments. (Sowers, 2014) Furthermore, TMZ has been shown to cause nausea, myelosuppression, cognitive decline, and depression (Egeland et al., 2017). Anti-depressant Fluoxetine (FLX) is administered to counter the neurological side effects of TMZ. (Ma, 2016)

The efficacy of drugs targeting the intracranial region is greatly hampered by the blood–brain barrier (BBB). The BBB is a highly selective structural roadblock that restricts the entry of chemicals and microorganisms into the central nervous system (CNS). The BBB does not let large peptides and proteins pass. Large molecules due to their molecular weight and size are unable to cross the tight junctions formed by endothelial cells. (Abbott, 2013)

The efflux pumps present in the BBB also prevent pump toxins and drugs from the CNS. However, lipophilic molecules having molecular weight less than 500 Daltons can passively diffuse through the lipid bilayer. (Pardridge, 2012) TMZ can cross the BBB to a small extent, achieving a blood plasma concentration of only 20%-30%. (Lee, 2013)

Silymarin is a flavonolignan derived from milk thistle (Silybum marianum) plant. Silymarin exhibits inhibition of epidermal growth factor receptor (EGFR), and cyclin-dependent kinases (CDK), along with anti-inflammatory and anti-angiogenic properties. (Ramasamy, 2008)

Silymarin has been reported to alter the PI3K/Akt and Wnt/β-catenin pathways, which play an essential role in GBM invasion and cell proliferation. (Shahcheraghi, 2020) The potential of silymarin to treat GBM was tested in vitro. Silymarin is cytotoxic at a concentration of 0.01μM against U87 and U251 lines after 72 hours. It did not show cytotoxicity against normal cell lines.

Due to the limitations of TMZ, it is imperative to explore new therapeutics for GBM treatment. This study aimed to investigate the anticancer and antidepressant properties of Silymarin on U87 induced xenograft tumor mice model. And comparative analysis was conducted with TMZ and fluoxetine treatments

Chapter 2

LITERATURE REVIEW

2.1 Cancer and Tumor Introduction

Cancer is defined as the disease condition in which cells of the body divide abnormally or in uncontrolled manner (without following any check points just dividing without any brake). (Staff, 2022) **Tumor** is defined as cluster formation of abnormal cells in solid tissues of the body such as (organ, bone, and muscle). Tumor can be benign (non-cancerous/non-spreadable) or malignant (cancerous/spreadable). (Staff, 2022)

Benign Tumor	Malignant Tumor	Malignant Tumor Types	Primary Tumor	Secondary Tumor
Non-spreadable tumor and (not invade surrounding tissues and have slow	Spreadable tumor that (invades surrounding tissues and has high growth rate).	**Locally invasive tumor** that invades only surrounding tissues by sending	A tumor that originates from brain or the surrounding tissues of brain	A tumor that formed somewhere else in the body but due to spreading/metastatic nature reached brain and cause cancer called

growth rate). Having confined boundaries. Can be called as **low-grade tumor**. (Cancer, 2022)	Not confined boundaries. Life threatening and called **high grade tumor.**	cancerous projection to normal tissues. **Metastatic cancer** invades other tissues of body that are far from original tissues. (Cancer, 2022)	called **primary brain tumor**. (Group., 2022)	**secondary brain cancer.** (Group., 2022)

2.2 Brain and its Tumor Classification

The brain is a central and complex organ of the body that carries out many functions such as controlling thoughts, emotions, touch, feelings, temperature regulation, breathing pattern, memory, and every process that has a role in body regulation. Both brain and spinal that

extends together and they formed **the Central Nervous System (CNS)** of the body. (Brain Anatomy and How the Brain Works, 2022) The brain performs lots of functions by sending and receiving messages and tells our body how to respond in response to any signal. The brain has many parts, and each part has its function.

The development and progression of cancer in the cells of the brain and nervous system are termed **Brain Cancer or Central Nervous System cancer**. And brain cancer was first discovered by Russian scientist ***Gupta Longati*** in 1873 when he was observing the brain of a person who died because of a benign brain tumor but during his observation, he noticed that the person died because of a malignant nature brain tumor. (GuptaLongati And Brain Cancer, 2022) Globally the incidence rate of brain cancer and other central nervous system cancer is **25,050** that accounting (**for 14,170** in men and **10,710** in women) and the global death rate due to brain and central nervous system cancer is **18,280** cases out of which (**10,710** in men and **7,570** in women). (American Cancer Society | Cancer Facts & Statistics, 2022)

Tumors are classified based on their diagnosis and appearance under microscopic examination. Tumors grading is done by examining the cancer cells under a microscope and observing the size of tumor, rate of invasion and vascularity. Tumors are divided into 4 grades: Grade I, II, III, and IV. (A Primerof BrainTumors, 2015)

Grade I Tumor grows slowly and looks more like normal cells. The survival rate is high because of their less malignant nature.

Grade II Tumor grows relatively faster as compared to Grade I and the cells look comparatively abnormal as compared to first-grade tumor. This grade of tumor cells can invade surrounding tissue.

Grade III tumors grow actively and look more abnormal as compared to earlier grade tumors. This grade tumor is considered a high-grade tumor and can infiltrate into surrounding tissues.

Grade IV grows very fast and looks highly abnormal as compared to all previous grades' tumors. The cell at the center appears dead and they also can invade nearby tissues.

2.2.1 Brain Cancer and Glioblastoma

Brain cancer is classified as benign and malignant brain tumors and primary and secondary (metastatic) brain tumors like other cancers. But of all the brain and CNS tumors the most common tumor that occurs is Glioblastoma (GBM) which accounts for 14.6% of all primary brain and CNS tumors, 48.3% of malignant tumors, and 57.3% of all gliomas. GBM prevalence is more common in males as compared to females and its five-year survival rate is the lowest (6.8%) from all malignant brain and CNS tumors. (Quinn T Ostrom, 2019)

Gliomas are known as tumors that originate from different types of glial and precursor cells that includes **Astrocytoma:** is a common type of glioma tumor that originates from the star-shaped cells called **astrocytes** and represents 12% of all primary tumors and is classified into different categories based on their grading. (A Primerof BrainTumors, 2015) (Shah, 2018) **Grade IV Astrocytoma** is called **Glioblastomas** and the other name for this tumor is **Glioblastoma-multiforme,** and this is a highly fast-growing tumor that appears in different lobes of the brain and the lobes that are highly targeted are frontal, and temporal and the preferred age group for this tumor is 50-70 years old adults. (A Primerof BrainTumors, 2015) (Shah, 2018) Supra-tentorium (frontal, temporal, parietal, and occipital lobes) are the regions where gliomas are mostly present and account for 61.3% and very less chances of their presence in other areas of the CNS. (Quinn T Ostrom, 2019) 90% of

the GBM cases show wildtype IDH (isocitrate dehydrogenase) that predominates in the patient population aged above 55 years while IDH mutant accounts for 10% of GBM patients and is referred to as secondary glioblastoma. (Daniele Armocida, 2019)

The survival rate of GBM patients is less and most of the patients die within 2 years although there is advancement in microsurgery technique but still radiation therapy and chemotherapy are not enough to increase the life span of GBM patients. The therapies and treatments that are most used for GBM are maximal surgical resection and followed by radiation therapy (RT). And sometimes few chemotherapeutic agents are administered along with Radiation Therapy because few met analysis results showed that their concomitant administration increases the survival rates in GBM patients. (Takahiro Oike, 2013)

2.3 GBM Standard Drug (TMZ)

Temozolomide (TMZ, sold under the brand name Temodar) is a medication that is used for the treatment of patients having brain tumors. TMZ is an alkylating agent that acts as a second-line treatment option for astrocytoma and a first-line treatment option for glioblastoma. In the European Union and the United States, TMZ is indicated as a drug that can be administered concomitantly with radiation therapy or as monotherapy to newly diagnosed adults having glioblastoma multiforme. (TEMODAR- temozolomide capsule TEMODAR- temozolomide injection, powder, lyophilized, for solution, 2022)

The European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada Clinical Trial Group CE3 (NCIC) conducted a phase III trial in the year 2005 that trials included 573 glioblastoma multiforme patients after that trial TMZ is established as a standard chemotherapeutic agent for GBM treatment. In that

study first, they did surgical resection of the tumor as much as possible and after that, they administered TMZ **(75 mg/m2/day×7 days/week for 6 weeks**) along with RT **(total dose of 60 Gy delivered by a schedule of 2 Gy/day×5 days/week for 6 weeks).** After this study, they observed that the mean survival improved from 12.1 to 14.6 months duration and 10.4% to 26.5% improved for a 5-year survival rate. These results are further confirmed by a study trial that was published in 2009 demonstrating that for the patients with combined therapy (TMZ and RT) and those who received RT alone the 5-year survival rate is 9.8% and 1.9% respectively. (Takahiro Oike, 2013) (Dr Roger Stupp MD a, 2009) In addition to TMZ some other drugs are also available and approved by FDA that are used for the treatment of GBM and these drugs include nitrosourea reagents (lomustine, carmustine, and carmustine wafer implants), bevacizumab and tumor treatment fields (TTF).

Although TMZ is one of the standard chemotherapeutic drugs that is used for GBM patients, it has been observed even after effective treatment of tumors few cancer cells are still able to enter a new stage of differentiation and proliferation and then they play roles in the recurrence of tumor after some time. When this phenomenon is observed then it is estimated that GBM cells developed resistance against TMZ that's how they survived through the chemotherapy process. Then there is a study showing that stem cells of GBM express the high level of CD133 marker that displayed strong resistance phenomenon against TMZ by high expressing the MGMT expression. (Wei Yu, 2019)

MGMT known as O6-methylguanine-DNA methyltransferase is a repair enzyme that functions by transferring the methyl at the O6 site of guanine to cysteine residues and preventing the process of gene mutation, cell death, and tumorigenesis caused by different alkylating agents. So, TMZ efficiently kills all those tumor cells that do not have a high

expression of MGMT but cannot work efficiently for those that have a higher level of this repair enzyme. Then in other studies different blocking agents were used to block MGMT and decrease the resistance rate against TMZ. (Wei Yu, 2019)

2.4 GBM and Inflammation

Robust immune system response is one of the phenomena that also play roles in treating GBM because a complex array of genetic and epigenetic changes appeared in different gliomas that result in disruption of different signaling pathways that have roles in cell's survival, proliferation, and infiltration to surrounding tissues. Inflammation is a kind of natural immune response that occurs in response to any kind of threat that occurs on the body like entry of any pathogens, tissue injury, or any malfunction observed. In response to any threat, our resident macrophages get activated and blood flow increased to the affected area an increased release of proinflammatory cytokines and increased leucocyte number is observed at the site of infection or injury and when a situation comes under control then anti-inflammatory molecules number increased and pro-inflammatory molecules decreased. Although inflammation plays a great role in the inhibition of tumor development by increasing immune response, sometimes the inflammation roles are exchanged, and it starts promoting tumorigenesis. (Hui, 2013)

The process of inflammation promotes tumorigenesis by genetic alterations that happen because of different reactive oxygen and nitrogen species released from the surrounding macrophages that act as mutagenic enzymes. The inflammatory response is generated because cancer growth can be started due to many reasons like the entry of some pathogen, exposure to some ionizing radiation, or some environmental factors. When an immune response is generated because of any of these there are possibilities of the release of such

cytokines that starts promoting tumor growth by inhibiting the cell death process that is started because of the body's natural response or the chemotherapeutic drug that is given to a patient for tumor cells death. That inflammatory cytokine can trigger different pathways like STAT3 or NFκB that result in an increase in proliferation and a decrease in cell death. (Hui, 2013)

2.5 GBM and Depression

Depression is one of the complications that is linked with cerebral gliomas although the signs and symptoms of depression are difficult to differentiate either they are because of tumors or treatment. But the studies show that among all cancer patients, those who have gliomas show more psychiatric complications. Depression affects not only the quality of life but is an important factor that has a role in increasing the mortality of patients having cancer of the Central Nervous System. (Alasdair G. Rooney, 2011) Because the patient with gliomas are not only fighting against the oncological disease but also trying to figure out their changes in social functioning, cognition, and roles that's why these patients require physiological and supportive care to improve their life quality.

Glioblastoma itself is a complex disease that affects many local physiological processes and ability to affect surrounding tissues and cells, cause angiogenesis, and disruption of different neuronal signaling pathways that pathways disruption has a role in the development of depression. Depression is not only linked with the disruption in cognition and emotional and social interaction, but it is also linked with other organs like the cardiovascular and immune system. (Nazir S. , 2022) In a GBM patients' number of pathways that are associated with depression and different markers appeared dysregulated. Some studies

showed that depression and anxiety dysregulate the levels of cytokines, inflammatory makers, and some neurotransmitters and they are also observed during the heterogeneity phenomenon that appeared in GBM. Although some observational studies are showing that it could be possible that GBM and depression occur concomitantly but there is no extensive research on this topic.

2.6 Silymarin

Silymarin is a class of natural products that have been used to treat other diseases but not cancer, but this compound has antitumoral properties that were recognized after a long time and now it is used in certain circumstances to treat cancer. But still, there is a need to investigate this compound because this compound has the potential to treat cancer effectively. The Silymarin compound is derived from the milk thistle plant and scientifically it is known as *Silybum marianum* L which is a member of the Carduus marianum family. For centuries until now this compound has been used because of its medicinal properties and is widely used to treat not only liver diseases but also to treat snake and mushroom poisons effect. Silymarin plants are all thorny including flowers that are reddish-purple and part of the plant that has medicinal properties are seeds or fruits. (Tomas Koltai, 2022)

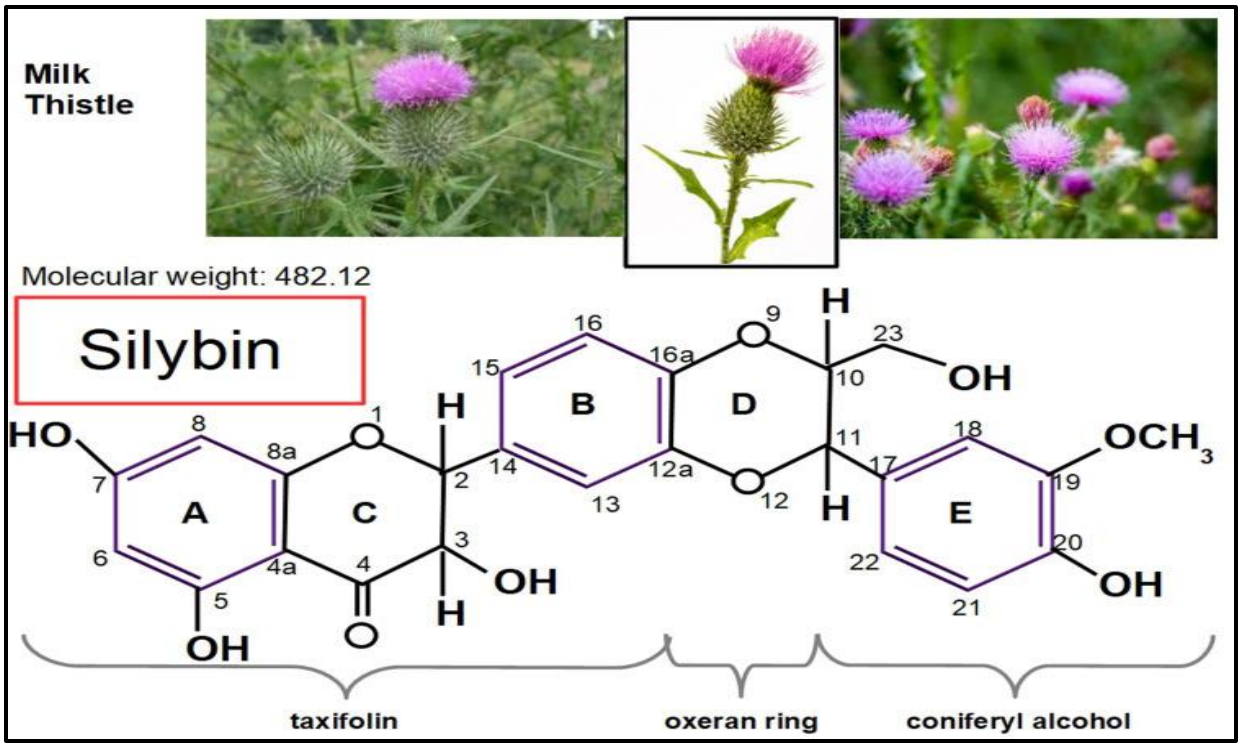

Figure 1: *Milk thistle plant, Molecular weight, and chemical structure of Silybin (C25 H22 O10) with its propriety numbering. (Tomas Koltai, 2022)*

The active component of this herb is Silymarin which contains many other sub-components like silybin A and B, Isosilybin A and B, and some other flavonolignans. Silymarin possesses many pharmacological properties like anticancerous, and anti-inflammatory, and can modulate the immune system by increasing the number of inflammatory cytokines like IFN-γ, IL-4, and IL-10. This compound also has anti-neoplastic effects and can induce apoptosis of endothelial cells through interacting with different pathways like p53 dependent pathway involving the release of cytochrome C or activation of caspase 3. (Tomas Koltai, 2022)

2.6.1 Silymarin and Cancer

Mehta and Moon in 1991 first time published that silymarin can provide possible benefits when used to treat cancer. In the study, they showed that when mouse mammary glands were treated with DMBA (dimethylbenzanthracene) and TPA (tetradecannoylphorbol

acetate) after that when Silymarin was used it acted as a preventive of cancer. In 1994 another study showed that this compound showed protective effects when used on skin that is treated with TPA. Silymarin also showed effective results when it is administered to Adenocarcinoma cells found in the colon and small intestine. All these studies show anti-tumor and protective properties of silymarin come to light. (Tomas Koltai, 2022).

Chapter 3

MATERIALS AND METHODS

Based on the objectives and aims of the research project in vitro study was conducted to evaluate the therapeutic potential of SIL in comparison with FLX in behavioural outcome. In addition to this behavioural changes and stress response developed due to the induction of GBM was also evaluated.

3.1 Chemicals

Valium (catalogue number. D0899-100MG), Ketamine hydrochloride (catalogue number. K2753-1G), Absolute chloroform (catalogue number. 288306-100ML), Eye ointment (Puralube Ophthalmic Ointment) (catalogue number.17033-211-38), DMEM High Glucose with L- Glutamine (catalogue number. 11965), Bone wax (catalogue number. Z046) Intraperitoneal injections of Dextrose, TMZ (catalogue number. PHR1437), FLX (catalogue number. F-132), Silymarin (SIL).

3.2 Ethical Approval Statement

All protocols and experiments conducted were approved by the Institutional Review Board (IRB) Atta-ur-Rahman School of Applied Biosciences (ASAB), National Institute of Sciences and Technology (NUST), Pakistan in accordance with principles set forth by Institute if Laboratory Animal Research, Division on Earth and Life Sciences, NIH, USA (Guide for the care and use of laboratory animals: Eight Edition, 2011). The number of mice and their suffering was tried to be minimised throughout all experiments.

3.3 Animals

Eight groups of BALB/c male mice of 10-12 weeks were used for the in Vivo testing of the therapeutic drugs. All animals housed at ASAB animal house facility, NUST, 4-5 mice were

kept per cage under constant conditions of: 12-12 hrs natural light-dark cycle and constant temperature conditions of (25±2◦C). Animals were initially purchased from National Institute of Health (NIH), Pakistan as well as from ASAB Animal House.

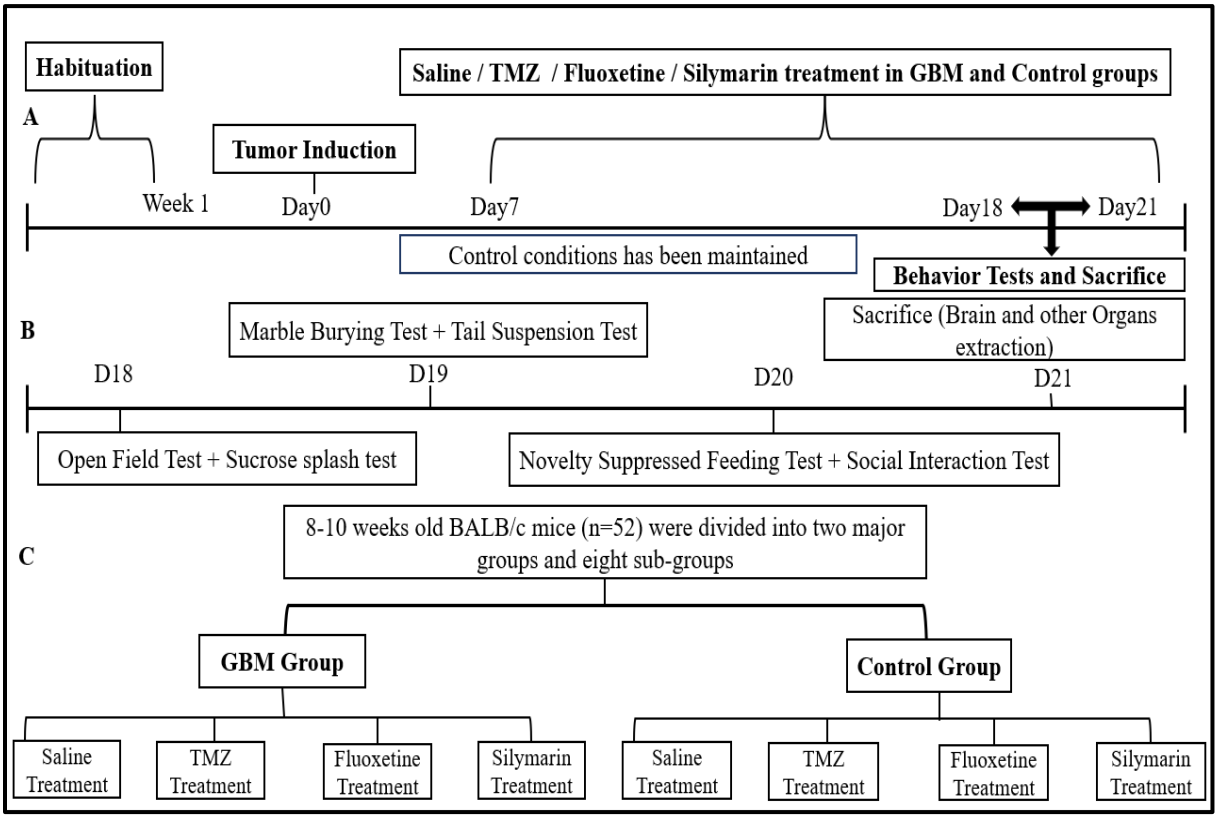

Figure 2: *Experimental Design. Tumor induction and treatment schedule. B. Behavior tests. to analyze the effect of treatment drugs. C. Grouping. experimental grouping contains two main groups GBM and Control with four subgroups in each.*

3.4 In-Vivo Experiments

3.4.1 Control Group Experimental Design

Twenty mice were randomly divided into four groups, namely Saline, TMZ, FLX and SIL (number of mice per group= 5); treated and tested in the same order. Administration of therapeutic drug was done intraperitoneal. Drugs were injected to animals in their home cages in the following concentrations: **Saline** (10 ml/kg body weight), **TMZ** (60 mg/kg body weight in normal saline 10 ml/ kg body weight), **FLX** (20 mg/kg body weight in

normal saline 10 ml/ kg body weight), **SIL** (50 mg/kg body weight in normal saline 10 ml/ kg body weight). Animal subjects were shifted to another room specifically reserved for behavioural analysis for habituation of thirty minutes before the test. After the completion of fourteen days treatment and behaviour tests the animals were euthanized.

3.5 Development of Orthotopic Xenograft GBM Mouse Model

3.5.1 Cell Preparation for Tumour Induction

The U87-MG cells were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) at 37 °C and with 5% CO_2 in the humidified incubator and split when sub-confluent. Trypsin-EDTA was used for the detachment of cells for passage. Orthotropic xenograft mouse model was developed. Briefly, Sub-confluent Monolayers of U87-MG cells were harvested by trypsinization, centrifuged at 1000 rpm for 10 minutes at 4°C, and the cell pellet was dissolved in PBS.

3.5.2 Tumour Cell Implantation in Brain

Valium and Ketamine (V: 10 mg/kg; K: 80-100 mg/kg) were injected intraperitoneal for anaesthesia. Eye ointment was applied, and the hair between the eye area was cut using sterile razor. Skin was then cleaned with alcohol swab and a fine incision was made 0.5 cm over the parieto- occipital lobe. 3% H_2O_2 was used to clean the exposed cranium. Once bregma became visible then a minute puncture was made on the skull for tumour cell injection. The puncture is made near Bregma (1mm anterior to coronal structure and 2mm right of Bregma) by the help of a scale.

Cell suspension was homogenised before injection. A concentration of 1×10^8 cells in 3µl of Serum-free DMEM were loaded into the Hamilton syringe. Before injection, to avoid contamination the needle tip was cleaned to prevent the formation of any extra cranial

tumours. The syringe was taken 3mm deep into the skull and at a placement of 2mm depth, cells were deliberately and slowly injected into the hole, it was made sure that no cell comes out. The Hamilton syringe was place at an angle of 90 degrees throughout the procedure. The syringe was kept in place for 2 minutes after cell dispensing. Hamilton syringe was slowly removed to, and skin and skull surface was wiped with 3% H_2O_2 to remove any cells present out of the injection site. The hole was sealed with mildly heated bone wax. The incision was then stitched with sutures. Treated mice were administered with 300µl/kg dextrose solution. Animals were monitored for 30 minutes until they recovered from anaesthesia and retained normal walk. (Ozawa, 2010)

3.6 GBM Mouse Model Experimental Design

Thirty- two BALB/c male mice weighting between 30-42 grams were randomly divided into 4 groups (no of mice in each group=8 mice). Each mouse in the four groups was injected with U-87 tumour cells to induce GBM. After GBM induction each group was treated with a different therapeutic drug regime. All the drugs were administered via intraperitoneal route. Group one was subjected to normal **Saline** (10 ml/kg body weight). Group two was given **TMZ** (60 mg/kg body weight in normal saline 10 ml/ kg body weigh), **FLX** (20 mg/kg body weight in normal saline 10 ml/ kg body weight), **SIL** (50mg/kg body weight in normal saline 10 ml/ kg body weigh). The treatment was started day 7 after tumour induction. And continued from 7-21 days. The behavioural tests were performed on day18,19 and 20. After the behavioural tests the mice were sacrificed.

3.7 Dissection and Brain Harvest

Mice were anesthetized with absolute chloroform. The neck was dislocated to sacrifice the neck. Cranium was broken for the complete removal of brain organ. The tumour tissues kept in liquid nitrogen and stored at -80°C.

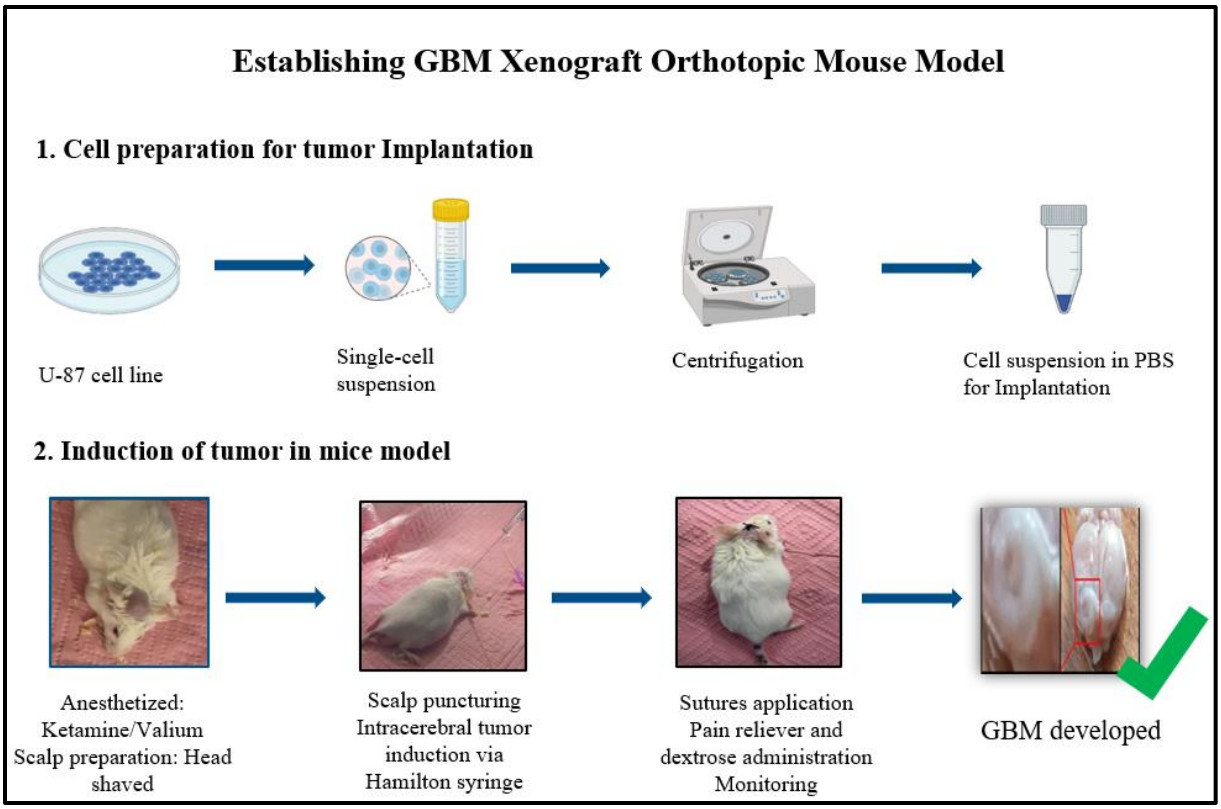

Figure 3: *Establishment of GBM Xenograft Orthotopic Mouse Model.1. Cell preparation for tumor implantation: Single cell suspension of U-87 cell lines are centrifuged and then suspended in PBS solution. 2. Induction of tumor mice model: The mice are anesthetized Ketamine and Valium. The head shaved, and a puncture is made in the skull followed by injection of tumor cells via Hamilton syringe. After recovery from anaesthesia pain reliever and dextrose are administered. Formation of lesions in cerebral region confirm development of GBM.*

3.8 Behaviors Test Protocols

3.9 Novelty Suppressed Feeding Test

One day prior to the test, all the mice were weighed and marked for easy and quick identification. New water bottles were then added. Mice were deprived of food.

The novelty suppressed feeding test was initiated approximately 24 h after food deprivation. The animals were transferred to the testing room for habituation 1 h before the test. The apparatus was placed in a standard mouse cage. New bedding was placed at the bottom, with a petri plate platform at the center. Weighed food pellets were placed on top of the platform and rendered immobile by helping threads and tape. The lightning in the room

increased. The subject mouse was placed in the corner of the cage, and the stopwatch was started immediately. The test was performed for 10 minutes. The timer stopped if the mice ate the pellet; otherwise, the test was continued for 10 minutes. After 10 min, the mouse and the pellet were removed. (Samuels, 2011)

3.9.1 Behavioral Analysis

The novelty suppressed feeding test, in addition to anxiety-like behavior, was used to assess anhedonia behavior. Anhedonia is the hallmark of depression. An increased latency time to approach the food pellet signifies anhedonia behavior in which the mouse has lost the ability to indulge in activities that are normally considered pleasurable by it.

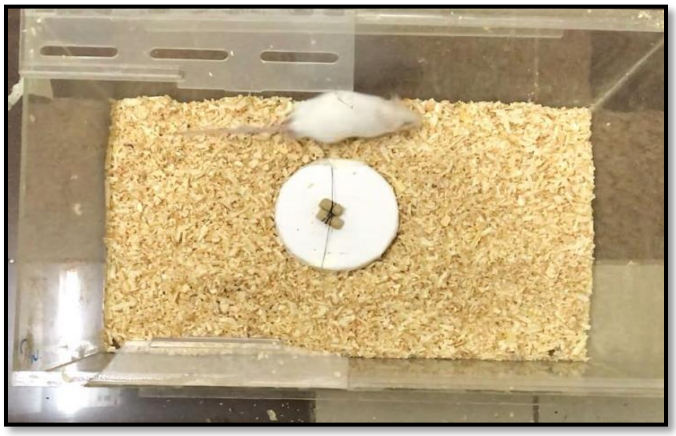

Figure 4: *Novelty Suppressed Feeding Test Apparatus*

Animals were likely first approach the food pellet and sniff without biting. This was not counted as eating. When the animal entered the center, grasped a food pellet with forepaws, and bit, then the time on stopwatch was paused marking the end of the test. (Samuels, 2011)

3.10 Sucrose Splash Test

The splash test involves spraying a 10% sucrose solution over a mouse's dorsal coat while it is still within its cage. The viscosity of the sucrose solution causes mouse hair to become

dirty, which prompts animals to begin grooming. Following the application of the sucrose solution, 5 min of grooming time was tracked as a measure of motivated and self-care behavior. The UCMS reduces grooming conduct, a type of motivated behavior thought to match some signs of depression, including apathy, according to a pharmacologically validated splash test. (Rosado, 2023)

3.10.1 Behavioral Analysis

A syntactic chain is composed of four phases: phase 1, which is characterized by a series of bilateral elliptical paw strokes made close to the nose (paw and nose grooming); phase 2, which is characterized by a series of unilateral strokes (each made by one paw); phase 3, which is characterized by a series of bilateral strokes made backwards and upwards by both paws simultaneously (head grooming); and phase 4, which is characterized by body licking. Any of these movements observed is considered grooming behavior. High grooming time behavior is an indication of a healthy mouse. Depressed mice lose the motivation to perform basic tasks hence depicting lower grooming time. (Rosado, 2023)

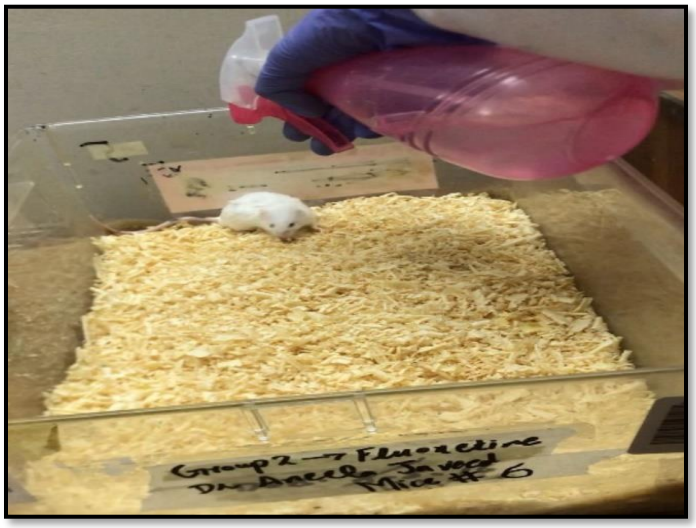

Figure 5: *Sucrose Splash Test Apparatus*

3.11 Open Field Test

An open-field maze with dimensions of 40 cm (length) × 40 cm (width) × 38 cm (height) was cleaned with 40% alcohol prior to the test. After alcohol evaporation, grids were drawn in the maze in the following manner: Apparatus was divided into central and peripheral zones shown below:

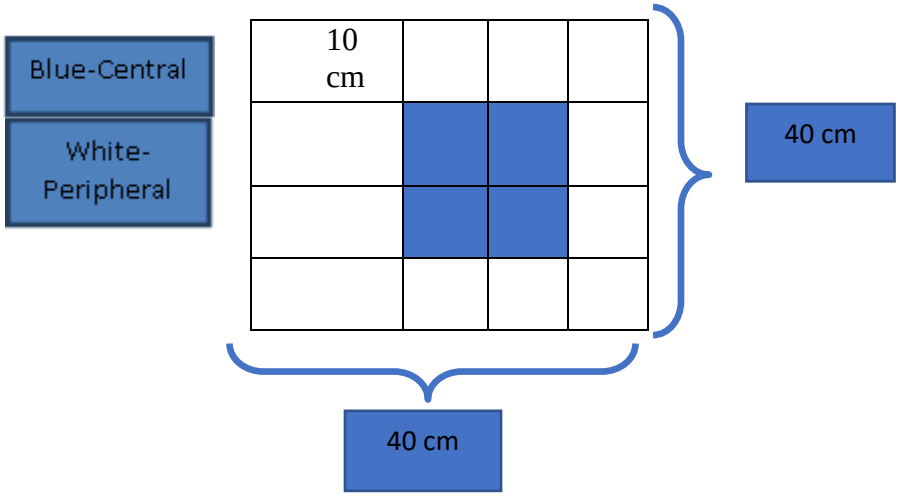

Figure 6: *Open Field Apparatus (Division of central and peripheral zones)*

The camera was suspended above the field and the mouse was placed in the central zone. The maze was left undisturbed for 30 min. After the conclusion of the test, fecal boli were manually counted and the mouse was returned to the home cage.

3.11.1 Behavioral Analysis

The open field test analyzes locomotor activity and anxiety-like behavior in mice. An anxious mouse is likely to spend more time in the peripheral zone near the walls than in the center.

Scoring was performed by analyzing the video recordings and calculating the number of entries and time spent in the central zone. Higher period spent in the central zone depicts are less depressed mouse.

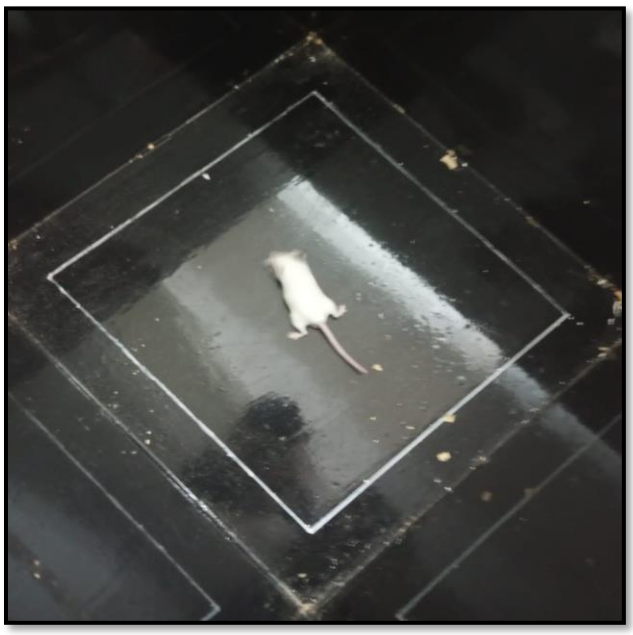

Figure 7: *Open Field Test Apparatus*

3.12 Tail Suspension Test

The camera was placed in a position to obtain the highest possible resolution of animals. Tape fragments were cut and marked before the session.

Before suspending the animal, the white noise generated started to cancel out any external noises. The animals were brought to the testing room 30 minutes – 1 hour before the test for habituation purposes. Marked end if the tape was adhered to tail in such a manner that 2-3 mm of tail remained outside the tape fragment. Once the tape was applied the recording device was started. The animals were suspended using the free end of the suspension bar or shelf. After six minutes (duration of one session) the animals were returned to their home cages and the tape was removed carefully.

3.12.1 Behavioral Analysis

The consistent recognition of movements recorded as legitimate mobility is the most significant component of the TST behavioral analysis. Mice exhibited clear escape-related behaviors, particularly early in the session. These included vigorous body shaking, limb

movements resembling running, and attempts to reach the suspension bar and apparatus walls. Mobility was clearly demonstrated by these actions. These actions then stop or become subdued. Small movements that are restricted to the front legs in our lab and do not include the hind legs are not accounted for in mobility. Additionally, oscillations and swings resembling pendulums that result from the momentum build-up during earlier mobility bouts are not included in the definition of mobility. (Can, 2011)

The prevailing behavior during each 5-s phase of the 360-s test was recorded using a time-sampling approach. These behaviors were also evaluated. (3) Curling: A mouse was judged to be curling when it actively twisted its entire body while actively moving its paws in the vertical position. (1) Immobility: A mouse was judged to be immobile when it hung by its tail without engaging in any active behavior. (2) Swinging: A mouse was judged to be swinging when it continuously moved its paws in the vertical position while keeping its body straight. (3) Curling: a mouse was judged to be curling when it engaged in active twisting movements of the entire body. Behavioral scoring was performed by a single experienced observer blinded to the treatments. The test sessions were then scored a second time by the observer to determine test–retest reliability. (Can, 2011)

Figure 8: *Tail Suspension Test Apparatus*

3.13 Marble Burying Test

Standard mouse cages with fitted-filter top covers were used for testing. Fresh, unscented bedding material was placed in each cage at a depth of 5 cm. The bedding was then levelled using another cage of the same size. Standard glass marble toys were cleaned with ethanol and placed on the bedding surface in an assortment of four marbles in five rows. Next, the stopwatch timer was set to 30 min (the duration of each test). Mice were brought from their cages to the testing room and allowed to habituate for 30 min. At one time, one mouse was placed in the testing cage. The mice were placed in the corner of the marble-containing cage; after placing the filter top, the cage was not disturbed for 30 min. Upon completion of the test, mice were removed from their home cages. The marbles were counted to estimate the anxiety and exploratory behavior of the mice. Then the bedding was disposed, and marbles retrieved. (Angoa-Pérez, 2013)

3.13.1 Behavioral Analysis

The marble burying test is used as a parameter to observe anxiety and exploratory behavior in mice. The greater the number of mice buried the more anxious the mice will be. Scores were taken by counting the number of marbles buried and displaced from their place. A marble was scored as buried if two-thirds of its surface area was covered by bedding. Then the average scores for the number of marbles buried for each mouse were calculated. (Angoa-Pérez, 2013)

Figure 9: *Marble Burying Test Apparatus*

3.14 Social Interaction Test

The test mouse was initially placed in the middle compartment and allowed to investigate. During this habituation period, plastic boxes were placed in front of the doors, leading to two side chambers. An unfamiliar C57BL/6J male (stranger 1), who had never interacted with the subject mice, was placed in one of the side chambers after the habituation phase. Between trials, the position of stranger 1 in the left- and right-side chambers was consistently switched. The unfamiliar mouse was housed in a tiny, circular wire cage that permitted nose-to-nose contact between the bars but discouraged fighting. The bottom diameter was 10.5 cm, the height of the cage was 11 cm, and the distance between the bars was 1 cm. Male BALB/c mice that had grown accustomed to being kept in tiny cages served as strangers. The individual was then given ten minutes to explore the full social test box after the doors to the side chambers had been opened. A human observer situated five feet away from the machine measured the length of time spent in each chamber and the number of entries into each room. (Nazir S. F., 2022)

3.14.1 Behavioral Analysis

The numbers of entries and time spent in each chamber were recorded. A higher amount of time spent in central zone depicts that the mouse was depressed and had lesser capability of

social interaction with the intruder mouse. A graph was plotted denoting the time spent in central zone to assess the social interaction depression like behavior of mice. (Nazir S. F., 2022)

Figure 10: *Social Interaction Test Apparatus*

Chapter 4

RESULTS

4.1 Novelty Suppressed Feeding Test

A novelty suppressed feeding test was performed to analyze anhedonia and anxiety-like behavior in the mice model. Latency to feeding was plotted on the y-axis, higher latency towards the feeding indicates increased stress levels in mice models. Following the tumor induction, the GBM mice showed a significantly high latency time (389.4 seconds) to feeding as compared to the control mice treated with saline (205.6 seconds). This finding suggests that tumor induction leads to the development of depression-like disorder in the GBM group. To assess the antidepressant potential of our proposed compound SIL under these circumstances, when it was injected into the GBM group the latency towards the food significantly decreases (196.2 seconds) which indicated SIL alleviated the symptoms of depression in the GBM group. Similarly, when the standard antidepressant FLX was administered the mice show a decrease in latency time towards feeding (205 seconds), however in this case the antidepressant effect of our proposed compound was comparable to that of the standard antidepressant FLX. In conclusion, the results of novelty suppressed feeding test demonstrate that tumor induction in the GBM group leads to an increase in latency time to feeding, indicating the presence of depression-like symptoms. Treatment with our proposed compound significantly reduced the latency time, suggesting its potential as an effective antidepressant against the GBM group. Furthermore, SIL exhibits a similar antidepressant effect to that of FLX.

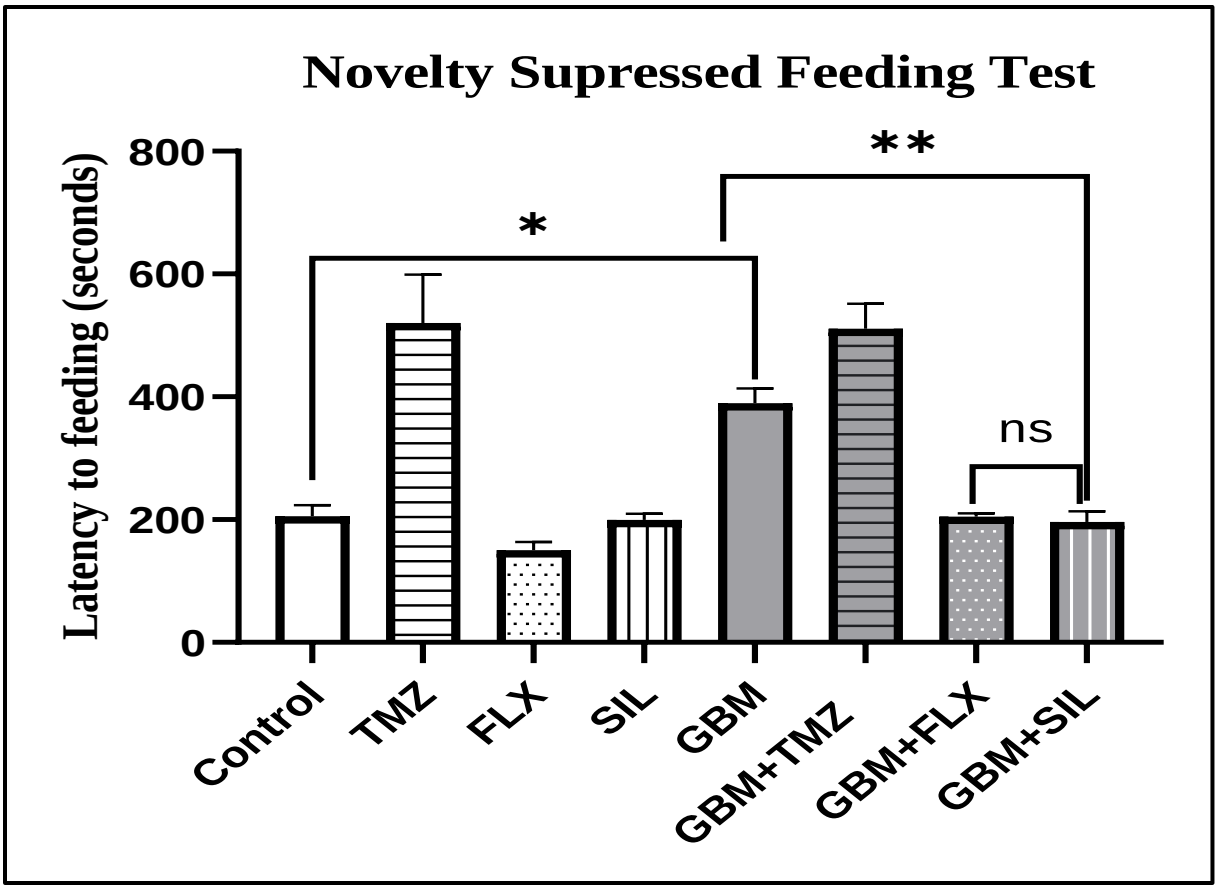

Figure 11: *Novelty Suppressed Feeding Test. Comparison of latency to feeding for 10 minutes following 24 hours of food deprivation. *: p=0.0119 (Control vs GBM), **: p=0.0071 (GBM vs GBM+SIL), ns: non-significant; p=>0.999 (GBM+SIL vs GBM+FLX).*

4.2 Sucrose Splash Test

The sucrose splash test was performed to analyze the grooming behavior in rodents. Time spent in grooming was plotted on the Y-axis, and lesser time spent in grooming was linked to a higher depression level in an animal. Mice induced with GBM showed significantly reduced grooming time (91 seconds) compared to those treated with saline (156.6 seconds) this indicates that the tumor induction leads to depression-like behavior in the GBM group. To access the antidepressant effect of our proposed compound SIL, it was administered to the GBM group. Notably, the grooming time of GBM group mice increased (146.6 seconds) this indicated that our proposed compound produced antidepressant effects in the GBM group. Similarly, When the standard antidepressant FLX was administered to the GBM

group, an increase in grooming behavior was observed (122.6 seconds), however, SIL has a significantly high effect on grooming behavior as compared to FLX, the standard antidepressant. In conclusion, the sucrose splash test results demonstrate that tumor induction in the GBM group leads to reduced grooming behavior, indicative of depression-like behavior. Treatment with our proposed compound SIL significantly increased the grooming time in the GBM group confirming it has potential as a potent antidepressant, much more effective than FLX.

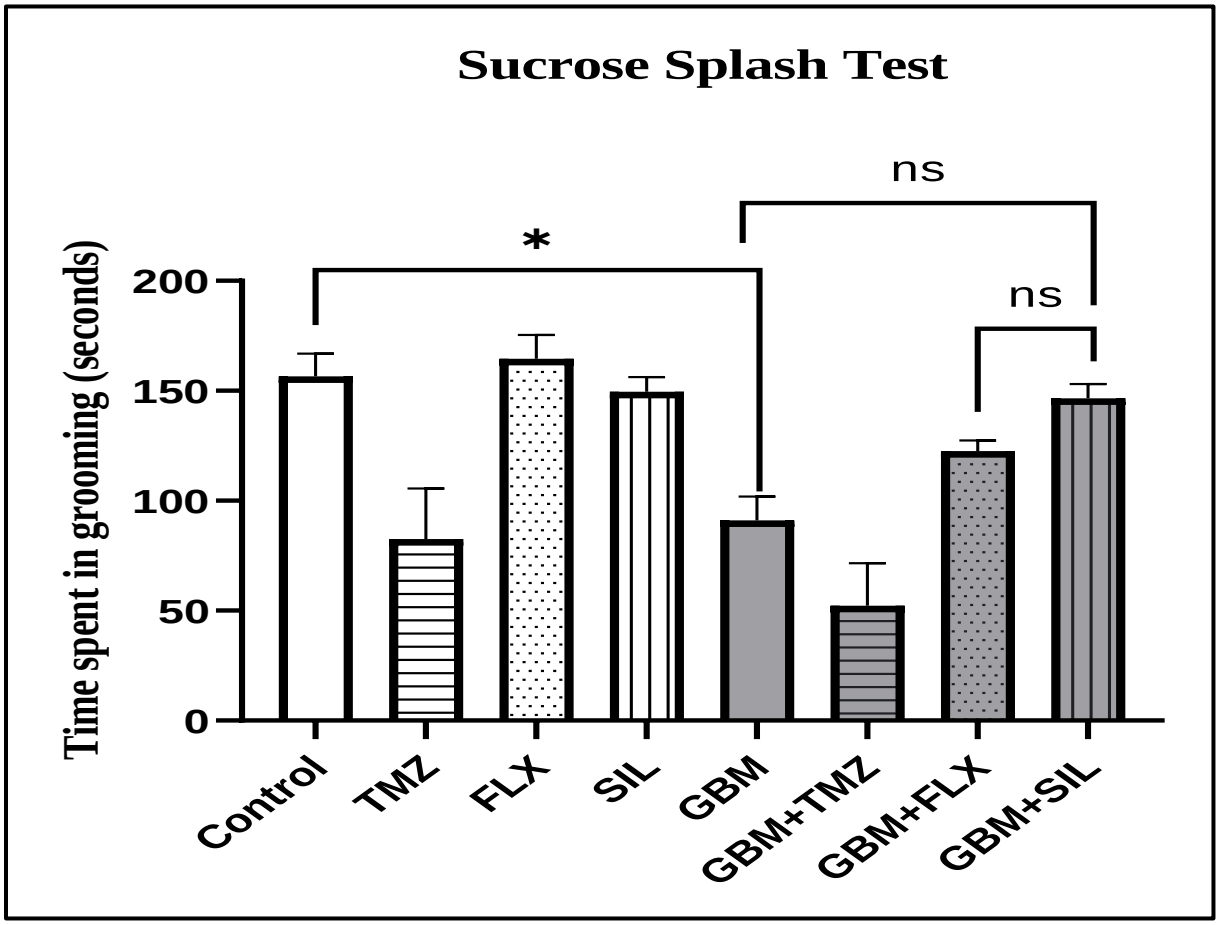

Figure 12: *Sucrose Splash Test. Comparison of time spent in grooming (seconds) after applying 10% sucrose solution on mice fur following drug treatment. *: p=0.0229 (Control vs GBM), ns: non-significant; p=0.0818 (GBM vs GBM+SIL), ns: non-significant; p= 0.8906 (GBM+SIL vs GBM+FLX).*

4.3 Open Field Test

Open Field Test is used to assess anxiety related behaviors in the laboratory animal models and this test was done by analyzing different activity of model animals like activity to approach different sides or zones of a testing apparatus because these all locomotory activities play roles in better understanding of depression and anxiety.

Time spent in central zone is plotted on y-axis and lesser time spent in central zone is indication of depression. The results were analyzed, and a graph was taken showing the difference between depressed and not depressed mice. The more depressed mice spent less time in the central zone and remained for a longer time in the peripheral zone.

This graph shows that the TMZ, GBM and GBM+TMZ spend less time in the central zone and that indicated that these groups of mice are more depressed as compared to FLX and GBM+FLX groups because Fluoxetine is a standard antidepressant drug and therefore these groups mice were less depressed. Then the results of our tested compound Silymarin results, depicted under SIL and GBM+SIL bars, in the above graphs, and mice of these two groups are also less depressed, as compared to GBM and GBM+FLX. From these results it is indicated that GBM induction induced depression in mice but when FLX is administered to mice GBM+FLX then reduction in depression is observed a depression also decreases when SIL administered to GBM mice.

Similarly, in the control group FLX and SIL group, mice spent more time in the central zone and showed that these mice were not depressed but TMZ group mice were depressed, which also showed that administration of TMZ to mice induced depression in them.

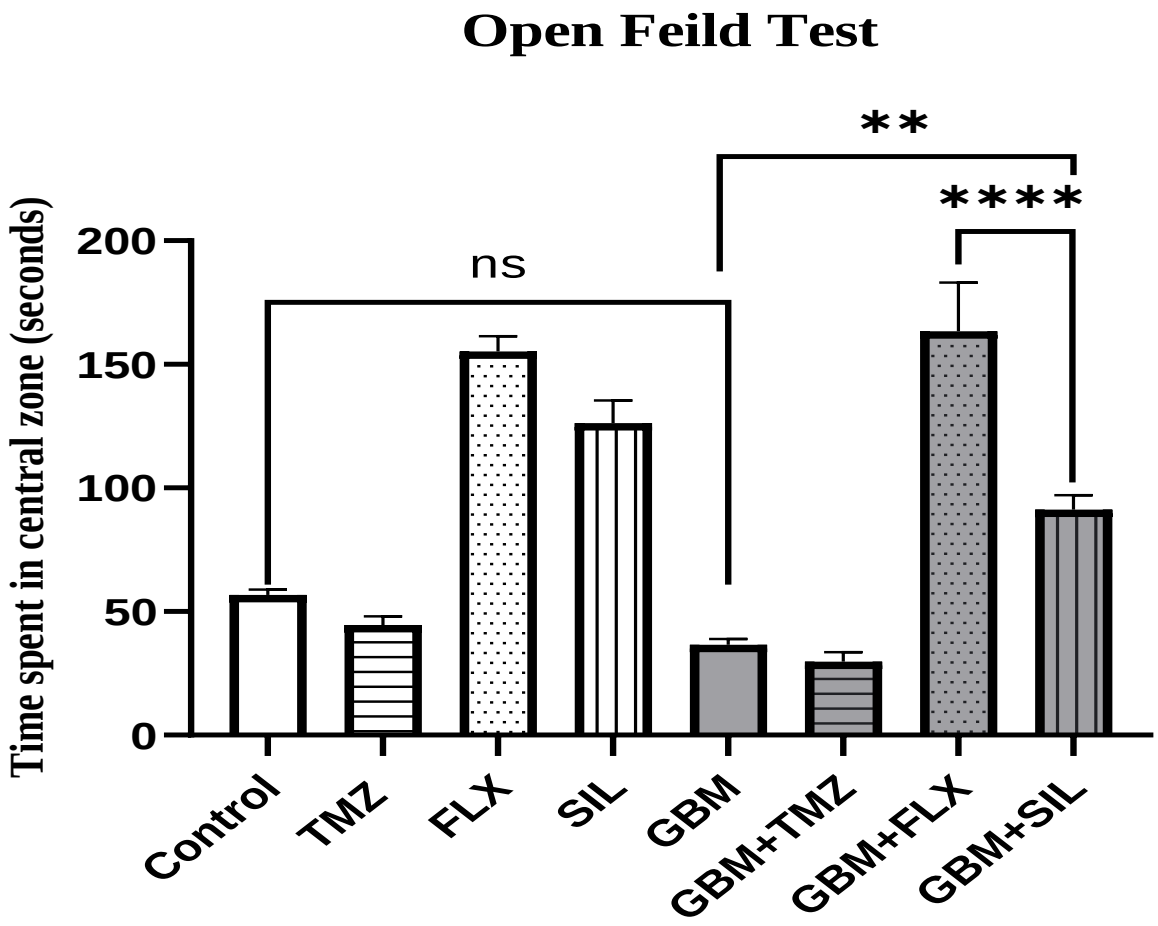

Figure 13: *Open Field Test result graphs showing the comparison of time spent by each test group mice in the central zone. ns: non-significant; p=0.7038 (Control vs GBM), **: p=0.0018 (GBM vs GBM+SIL), ****: p=0.0001 (GBM+SIL vs GBM+FLX).*

Similarly, in the control group FLX and SIL group, mice spent more time in the central zone and showed that these mice were not depressed but TMZ group mice were depressed, which also showed that administration of TMZ to mice induced depression in them.

4.4 Tail Suspension Test

The tail suspension test was conducted to observe behavioral despair or learned helplessness. The evaluation of behavioral despair is depicted by the immobility time of

mice in 6 min of tail suspension from the bar. The greater the immobility time, the more depressed the mouse and the less motivated it is to escape.

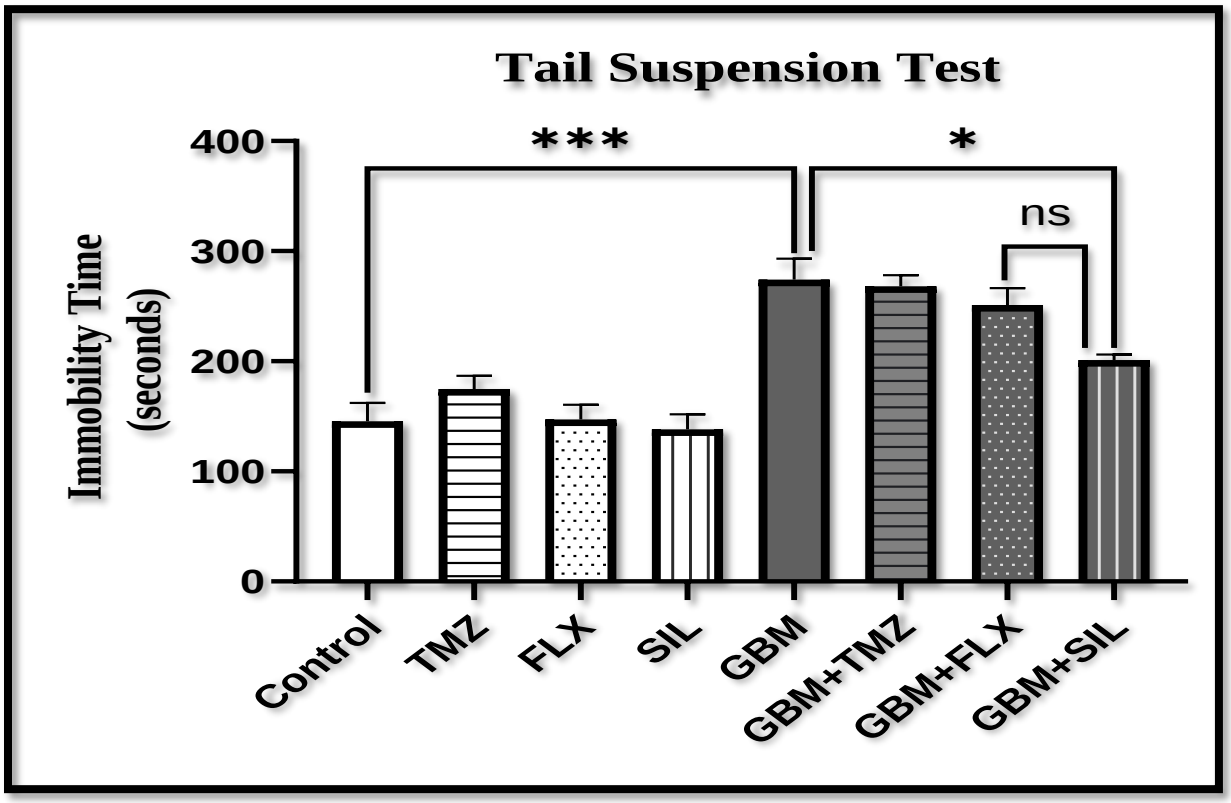

Figure 14: *Tail Suspension Test. Comparison of immobility time displaced in 6 minutes hanging time. ***: p=<0.0001 (Control vs GBM), *: p=0.0155 (GBM vs GBM+SIL), ns: non-significant; p=0.2125 (GBM+SIL vs GBM+FLX).*

The immobility time in seconds is plotted against the y-axis. GBM induced group when compared with the saline group shows a significant increase in the immobility time (control: 145 seconds, GBM: 274 seconds). An analysis of the GBM group with GBM + SIL showed a significant decrease in immobility time (GBM+ SIL:201 seconds). This means that the GBM group treated with SIL had reduced immobility time, contributing to a reduction in behavioral despair behavior. However, when the immobility time of GBM + FLX was compared with the immobility time of GBM + SIL, there was a non-significant decrease.

4.5 Marble Burying Test

Marble burying test was utilized to analyses anxiety and exploratory behavior. A total of 20 marbles were placed at the start of the test. At the conclusion of the test, the number of marbles buried were counted. 20 being the highest number of marbles buried while 0 being the lowest.

An increase in the number of marbles is associated with anxiety like behavior.

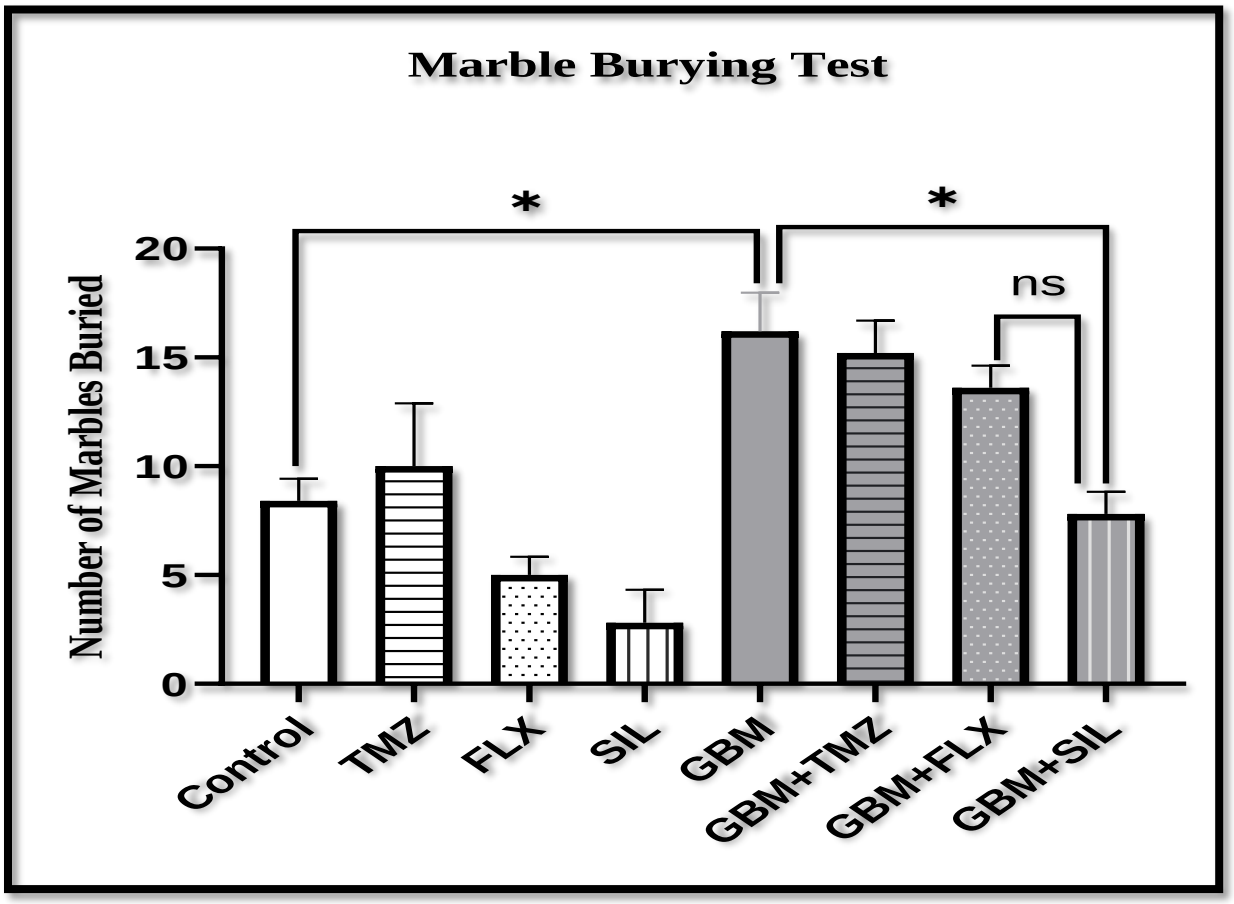

Figure 15: *Marble Burying Test. Comparison of number of marbles buried after 30 minutes. *: p=0.0275 (Control vs GBM), *: p=0.0140 (GBM vs GBM+SIL), ns: non-significant; p=0.1947 (GBM+SIL vs GBM+FLX).*

The number of buried are plotted against the y axis. GBM group showed a greater number of marbles buried (16.2) compared with control saline group (8.4). When the number of marbles buried for GBM group are compared with the number of marbles buried by the

GBM group treated with the targeted compound SIL treated group a significant decrease in number of marbles buried was observed. SIL treated group buried 7.8 marbles. It depicts that SIL was effective in reducing anxiety like behavior induced in GBM mice. However, when the burials of GBM SIL treated group are assessed against GBM+FLX treated group, FLX being the standard antidepressant, a non- significant decrease is observed. GBM+FLX treated group buried more marbles (13.6 marbles).

4.6 Social Interaction Test

This assay is a commonly used method to assess anxiety like behavior in the laboratory mice and the main parameter of this test was time spent by the subject mouse in the chamber containing the conspecific stranger in a wire cage, versus the chamber containing an empty wire cage. This test has the advantage of letting the subject initiate the social approach to the enclosed stimulus and prevent aggressive contact.

This graph shows that the TMZ, GBM and GBM+TMZ spent less time in intruder chamber interacting with the mice, indicating that these groups of mice are more depressed and not able to socially interact as compared to FLX and GBM+FLX groups because Fluoxetine is a standard antidepressant drug and therefore these groups mice were not depressed. As the Glioblastoma Control (GBM) group increases the depression like behavior so when we compare the bar with GBM+SIL, we can clearly see that silymarin increases the interaction time with the intruder. Therefore, decreasing depressive like behavior in the GBM mice. From these results GBM induction induced depression in mice but when (F) is administered to GBM+FLX group then reduction in depression is observed a depression also decreases when SIL administered to GBM mice (GBM+SIL) also decreases in depression observed as compared to GBM mice.

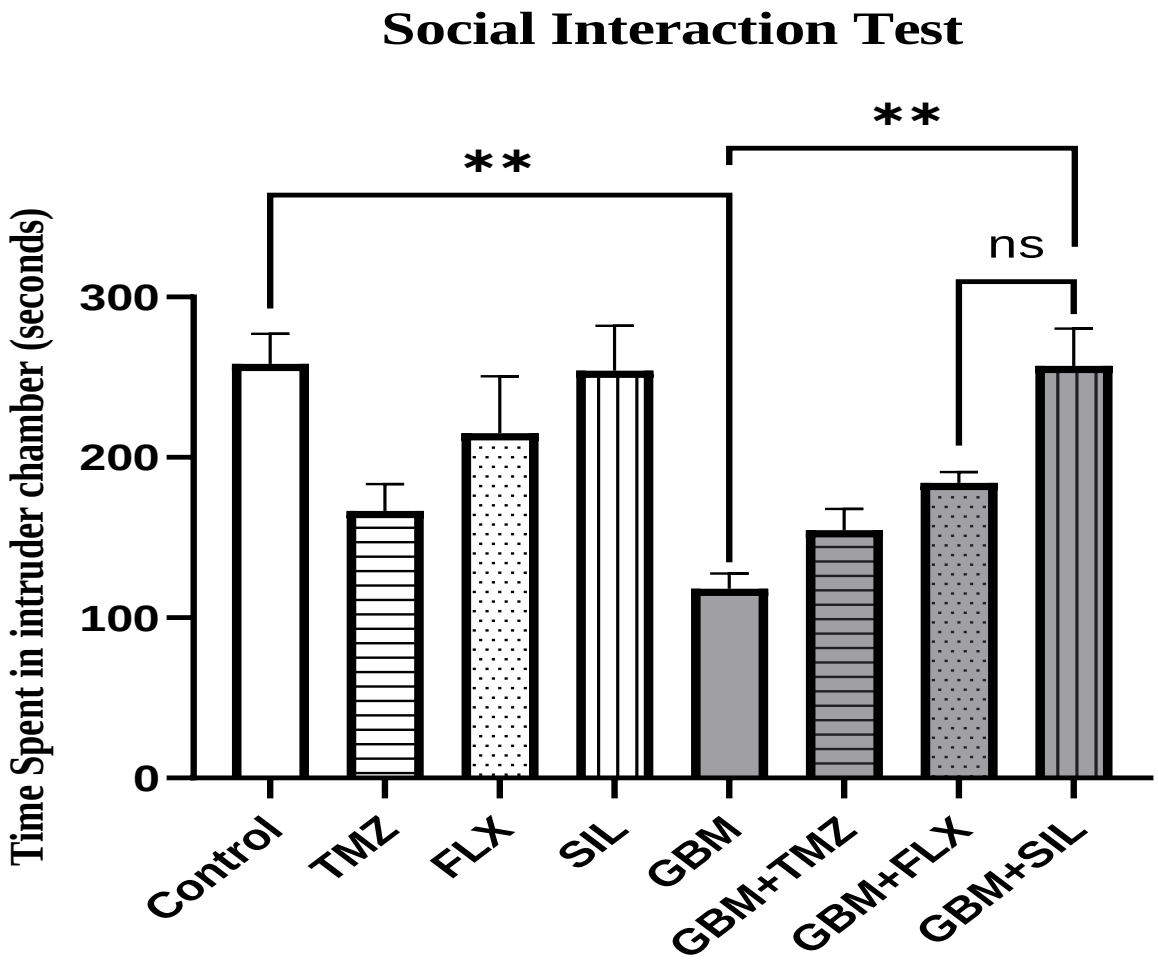

Figure 16: *Social Interaction Test result graphs showing the time spent in intruder chamber by each test mice. **: p=0.0099 (Control vs GBM), **: p=0.0015 (GBM vs GBM+SIL), ns: non-significant; p=0.009 (GBM+SIL vs GBM+FLX).*

Chapter 5

DISCUSSION

Glioblastoma multiforme (GBM) is a grade IV malignant astrocytoma with a median survival rate of less than 12 months after diagnosis and it accounts for 54% of all glial tumors. Molecular characteristic studies have identified EGFR, PDGFRA, MET, MDM4, MDM2, PIK3CA, MYC, CDK4, and CDK6 as recurrent targets for gene amplification along with loss of the tumor suppressor CDKN2A/B locus and somatic mutations. TMZ is established as a standard chemotherapeutic agent for GBM treatment but in addition to TMZ some other drugs are also available and approved by FDA that are used for the treatment of GBM. Glioblastoma itself is a complex disease that affects many local physiological processes and ability to affect surrounding tissues and cells, cause angiogenesis, and disruption of different neuronal signaling pathways that pathways disruption has a role in the development of depression. Some studies showed that depression and anxiety dysregulate the levels of cytokines, inflammatory makers, and some neurotransmitters and this type of dysregulation was also observed during the heterogeneity phenomenon that appeared in GBM.

5.1 Behavior Tests Analysis

Behavior tests were performed to analyze the antidepressant effect of our compound Silymarin and to compare this compound's results with the standard antidepressant drug Fluoxetine (FLX). This compound is also compared with TMZ, a standard FDA-approved chemotherapeutic drug used to treat GBM. Many studies are showing that glioma cells can develop resistance against TMZ, which increases the cases of tumor recurrence and the reason behind the resistance is the phenomenon of heterogeneity displayed by cells of

glioma. during the process of inflammation, some of the pro-inflammatory cytokines instead of killing cells and promoting cell death stands in favor of the anti-apoptotic process. One study shows that glioma cells show increased expression of MGMT that transfers the methyl from the O6 site of guanine to cysteine residues and preventing the process of gene mutation, cell death, and tumorigenesis caused by different alkylating agents and repairing the DNA damage and preventing the cells undergoing apoptosis.

Behavior tests like novelty suppressed feeding, sucrose splash, open field, marble burying, tail suspension, and social interaction tests were performed. These tests were performed to check how much the animal models were depressed after the administration of different drugs like TMZ, FLX, and SIL. All these tests were performed to measure depression levels through different ways and to perform these tests we had 8 test groups that were categorized as follows: Control Group and Experimental Group.

A control group is further divided into 4 sub-groups (Control-treated with Saline, TMZ-treated with the standard chemotherapeutic drug Temozolomide, FLX-treated with Fluoxetine, the standard antidepressant drug, and SIL-treated with the test compound Silymarin). The experimental group was also further treated with the same drugs but the main difference between the control and experimental was that GBM cells were induced into the experimental group and after tumor induction, this group was treated with these drugs to check their efficacy.

Behavior tests results were analyzed, and it was observed that the control group that were treated with saline were not depressed and performed all activities that clearly showed no depression symptoms, but when GBM was induced in the experimental group, the GBM control group that was not treated with any drug and only saline was administered to that

group showed depression when their behaviors were analyzed. The GBM mice group showed less grooming behavior, latency to approach food pellets placed at the center of brightly lit areas, spending less time in the central zone, increased immobility time, and more burying of marbles. These results confirmed that upon induction of GBM, depression also developed in those mice.

5.2 Drugs Results Analysis

From behavior test results, it was also observed that when FLX was given to mice, the mice were less depressed and performed all tests very well that confirmed that these mice were not depressed. But other results that were obtained from the behavior tests were that when TMZ is administered to control group mice they start showing depression and anxiety-like symptoms that were not first observed in the saline treated group. These results put another question mark on TMZ. Is it safe to give TMZ to GBM patients who already have depression and anxiety and after TMZ administration there is the possibility that their depression-like condition will become worse?

And the results of our test compound Silymarin showed that this compound has anti-depressant properties because in the control group after silymarin administration, mice did not show any depression symptoms and depression was also reduced when this drug was administered to the GBM mice groups. These results showed that Silymarin has antidepressant properties and can reduce depression when given to GBM-induced mice models. But testing of anti-cancerous properties of this compound is still under testing and in that part, work is still going on. But after behavior tests, it is confirmed that Silymarin can reduce depression in GBM models, while depression levels increase when TMZ is administered to control or GBM mice models

Chapter 6

CONCLUSION

6.1 Conclusion

Silymarin, a natural compound with antioxidant, anti-inflammatory, and anticancer properties, was evaluated in a xenograft orthotopic mouse model to determine its effects on GBM. This study demonstrated significant outcomes in reducing GBM growth and mitigating depressive symptoms associated with GBM, as supported by behavioral testing. TMZ was counter-effective, as it aggravated the MDD symptoms associated with GBM. Based on these findings, silymarin has demonstrated high efficacy in reducing GBM tumors and alleviating depression associated with GBM and thus merits further in vivo testing and molecular analysis.

6.2 Future Aspects

- Evaluate systemic toxicity of silymarin in various organs.
- Experiments to check dose dependent tumor reduction studies using in vivo models.
- Testing silymarin as combinatorial therapy against GBM using in vivo models.

Chapter 7

REFERENCES

A Primerof BrainTumors. (2015). Retrieved August 18, 2022, from American Brain Tumor Association: https://health.mo.gov/living/healthcondiseases/chronic/cancer/pdf/A_Primer_of_Brain_Tumors.pdf

Abbott, N. J. (2013). Blood–brain barrier structure and function and the challenges for CNS drug delivery. *Journal of Inherited Metabolic Disease, 36*(3), 437-449. doi:https://doi.org/10.1007/s10545-013-9608-0

Alasdair G. Rooney, A. C. (2011, January 5). Depression in Cerebral Glioma Patients: A Systematic Review of Observational Studies. *JNCI: Journal of the National Cancer Institute, 103*(1). doi: https://doi.org/10.1093/jnci/djq458

American Cancer Society | Cancer Facts & Statistics. (2022). Retrieved August 17, 2022, from American Cancer Society | Cancer Facts & Statistics: https://cancerstatisticscenter.cancer.org/#!/cancer-site/Brain%20and%20other%20nervous%20system

Angoa-Pérez, M. K. (2013). Marble burying and nestlet shredding as tests of repetitive, compulsive-like behaviors in mice. *Journal of Visualized Experiments, 82*. doi:https://doi.org/10.3791/50978

Brain Anatomy and How the Brain Works. (2022). Retrieved August 18, 2022, from Hopkinsmedicine.org: https://www.hopkinsmedicine.org/health/conditions-and-

diseases/anatomy-of-the-brain#:~:text=What%20is%20the%20brain%3F,central%20nervous%20system%2C%20or%20CNS.

Can, A. D. (2011). The Tail Suspension Test. *Journal of Visualized Experiment*. doi:https://doi.org/10.3791/3769

Cancer. (2022, August 25). Retrieved August 17, 2022, from Stanfordhealthcare.org: https://stanfordhealthcare.org/medical-conditions/cancer/cancer.html

Daniele Armocida, 1. A. (2019, November 30). Long Term Survival in Patients Suffering from Glioblastoma Multiforme: A Single-Center Observational Cohort Study. *Diagnostics (Basel)., 9*(4). doi: 10.3390/diagnostics9040209

Dr Roger Stupp MD a, M. E. (2009, May). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *The Lancet Oncology, 10*(5). doi:https://doi.org/10.1016/S1470-2045(09)70025-7

Group., J. M. (Ed.). (2022, August 28). *Understanding Brain Tumors*. Retrieved August 12, 2022, from Cancer.org.au: https://www.cancer.org.au/assets/pdf/understanding-brain-tumour-booklet

GuptaLongati And Brain Cancer. (2022). Retrieved August 18, 2022, from prezi.com: https://prezi.com/erleq_tkx5if/guptalongati-and-brain-cancer/

Hui, R. P. (2013, March 1). Inflammation and Gliomagenesis: Bi-Directional Communication at Early and Late Stages of Tumor Progression. *Curr Pathobiol Rep*. doi:10.1007/s40139-012-0006-3

Ignacio Veliz, 1. Y. (2015, January). Advances and challenges in the molecular biology and treatment of glioblastoma—is there any hope for the future? *Ann Transl Med*. doi:doi: 10.3978/j.issn.2305-5839.2014.10.06

Juweid., A. F. (2017 Sep 27.). *Epidemiology and Outcome of Glioblastoma.* (De Vleeschouwer S, Ed.) Codon Publications.

Khan, H. N. (2022). Fabrication and assessment of Diosgenin encapsulated stearic acid solid lipid nanoparticles for its anticancer and antidepressant effects using in vitro and in vivo models. *Frontiers in Neuroscience*. doi:https://doi.org/10.3389/fnins.2021.806713

Krex, D. K. (2007). Long-term survival with glioblastoma multiforme. *Case Reports in Oncology, 130*(10), 2596–2606.

Lee, E. Q. (2013). Treatment options in newly diagnosed glioblastoma. *Current Treatment Options in Neurology, 15*(3), 281-288. doi:https://doi.org/10.1007/s11940-013-0226-9

Liu, L. X. (2008). Bradykinin increases blood–tumor barrier permeability by down-regulating the expression levels of ZO-1, Occludin, and Claudin-5 and rearranging actin cytoskeleton. *Journal of Neuroscience Research, 86*(5). doi:https://doi.org/10.1002/jnr.21558

Ma, J. Y.-R.-H.-D.-L.-Z.-C. (2016). Fluoxetine synergizes with temozolomide to induce the chop-dependent endoplasmic reticulum stress-related apoptosis pathway in glioma cells. *Oncology Reports, 36*(2). doi:https://doi.org/10.3892/or.2016.4860

Nazir, S. (2022). *Evaluating Role of Inflammation in Glioblastoma Multiform Induced Depression and Major Depressive Disorder for Alternate Therapeutics.*

Nazir, S. F. (2022). Therapeutic effect of thymoquinone on behavioural response to UCMS and neuroinflammation in hippocampus and amygdala in BALB/C mice model. *Psychopharmacology, 239*(1), 47-58. doi:https://doi.org/10.1007/s00213-021-06038-9

Ozawa, T. &. (2010). Establishing intracranial brain tumor xenografts with subsequent analysis of tumor growth and response to therapy using bioluminescence imaging. *Journal of Visualized Experiments*. doi:https://doi.org/10.3791/1986-v

Pardridge, W. M. (2012). Drug transport across the blood–brain barrier. *Journal of Cerebral Blood Flow & Metabolism, 32*(11). doi:https://doi.org/10.1038/jcbfm.2012.126

Quinn T Ostrom, 1. G.-S. (2019, November). CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2012–2016. *Neuro Oncol., Volume 21(Suppl 5)*. doi:10.1093/neuonc/noz150

Ramasamy, K. &. (2008). Multitargeted therapy of cancer by Silymarin. *Cancer Letters, 269*(2), 352–362. doi:https://doi.org/10.1016/j.canlet.2008.03.053

Rooney, A. G. (2013). Depression in glioma: A Primer for clinicians and researchers. *Journal of Neurology, Neurosurgery & Psychiatry, 85*(2), 230–235. doi:https://doi.org/10.1136/jnnp-2013-306497

Rosado, A. F. (2023). Behavioral flexibility impacts on coping and emotional responses in male mice submitted to social defeat stress. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. doi:https://doi.org/10.1016/j.pnpbp.2022.110696

Samuels, B. A. (2011). Novelty-suppressed feeding in the mouse. *Mood and Anxiety Related Phenotypes in Mice,*, 107–121. doi:https://doi.org/10.1007/978-1-61779-313-4_7

Shah, V. (2018, January 17). Brain Cancer: Implication to Disease, Therapeutic Strategies and Tumor Targeted Drug Delivery Approaches. *Recent Patents on Anti-Cancer Drug Discovery, 13*(1). doi:10.2174/1574892812666171129142023

Shahcheraghi, S. H.-N. (2020). Wnt/beta-catenin and PI3K/AKT/mtor signaling pathways in glioblastoma: Two main targets for drug design. *Current Pharmaceutical Design, 26*(15). doi:https://doi.org/10.2174/1381612826666200131100630

Sowers, J. L. (2014). The role of inflammation in brain cancer. *Advances in Experimental Medicine and Biology*, 75–105. doi:https://doi.org/10.1007/978-3-0348-0837-8_4

Staff, D.-F. (2022). *Tumor Definition: What You Need to Know | Dana-Farber Cancer Institute*. Retrieved August 12, 2022, from Dana-Farber Cancer Institute: https://blog.dana-farber.org/insight/2018/05/difference-cancer-tumor/#:~:text=What%20is%20the%20difference%20between,organ%2C%20muscle%2C%20or%20bone.

Takahiro Oike, Y. S.-i. (2013, November 12). Radiotherapy plus Concomitant Adjuvant Temozolomide for Glioblastoma: Japanese Mono-Institutional Results. *PLoS ONE 8(11), 8(11)*. doi:https://doi.org/10.1371/journal.pone.0078943

TEMODAR- temozolomide capsule TEMODAR- temozolomide injection, powder, lyophilized, for solution. (2022, November 22). Retrieved May 20, 2023, from https://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=046a9011-3911-4d3f-a15f-fbb56d5aad56

Tomas Koltai, a. L. (2022, January 12). Role of Silymarin in Cancer Treatment: Facts, Hypotheses, and Questions. *J Evid Based Integr Med.* doi:10.1177/2515690X211068826

Wei Yu, 1. L. (2019). O6-Methylguanine-DNA Methyltransferase (MGMT): Challenges and New Opportunities in Glioma Chemotherapy. *Front Oncol*. doi:10.3389/fonc.2019.01547

Weinberg, R. A. (1996). How cancer arises. *Scientific American, 275*(3), 62-70. doi:https://doi.org/10.1038/scientificamerican0996-62

Xie, Y. W. (2018). Transcriptomics evidence for common pathways in human major depressive disorder and glioblastoma. *International Journal of Molecular Sciences, 19*(1). doi:https://doi.org/10.3390/ijms19010234

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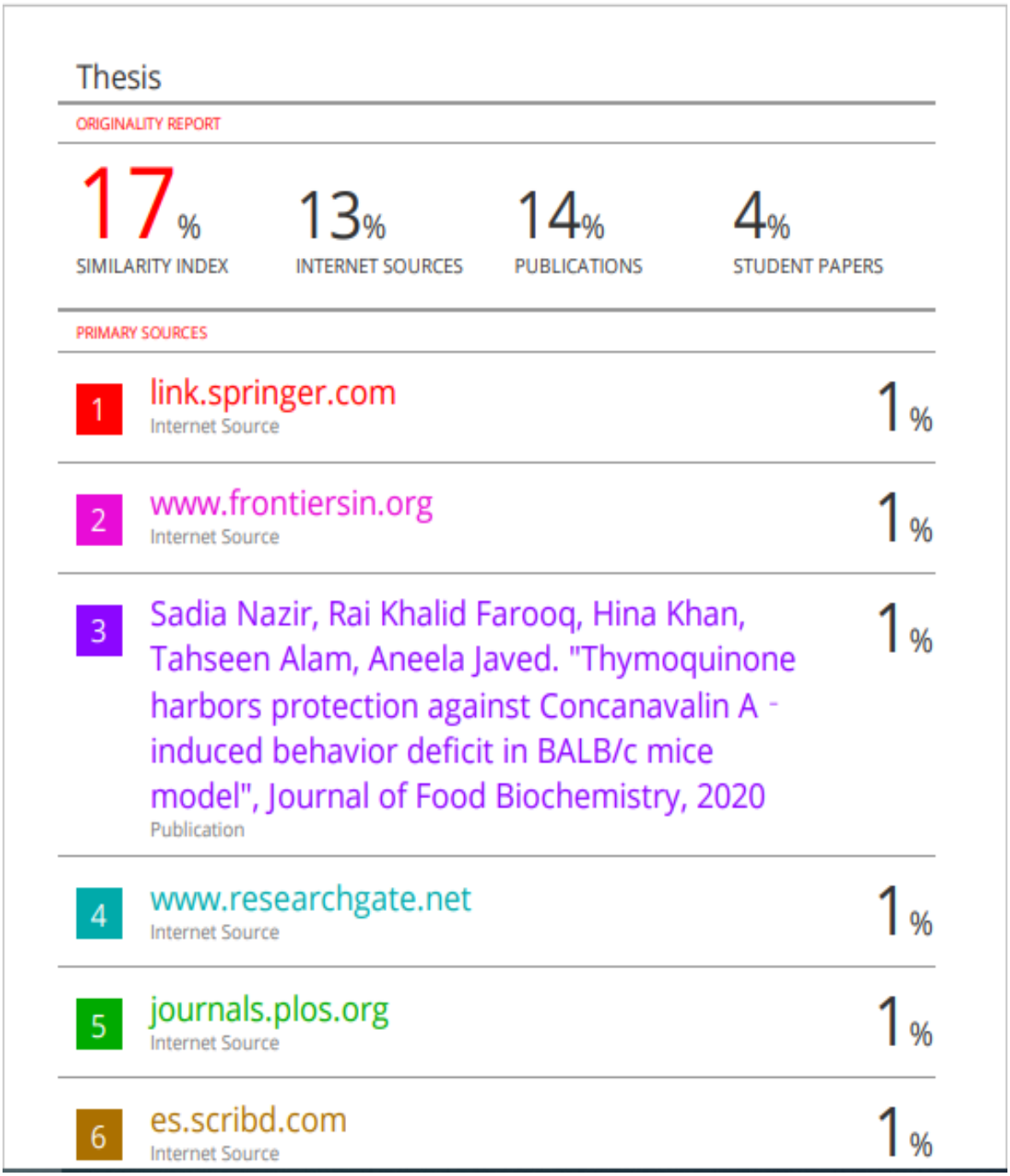

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