AMELIORATIVE EFFECT OF THE BIOACTIVE METABOLITES DERIVED FROM *MURRAYA KOENIGII* (CURRY LEAF) ON CLOZAPINE INDUCED HYPERLIPIDEMIA IN WISTAR RATS

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY
in
Clinical Biochemistry

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DECLARATION

I, hereby declared that the presented work in the thesis entitled "Ameliorative effect of bioactive metabolites of *Murraya koenigii* (curry leaf) in Clozapine induced hyperlipidemia in Wistar rats" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Louis Cojandaraj, working as Associate professor, in the Medical Laboratory Sciences, School of Allied Medical Sciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Ameliorative effect of bioactive metabolites of *Murraya koenigii*(curry leaf) in Clozapine induced hyperlipidemia in Wistar rats" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Medical Laboratory Sciences, is a research work carried out by Pearl Pinto, 42100042, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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Name of supervisor: Dr. Louis Cojandaraj
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University: Lovely Professional university

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In honor of my late mother, Paulet Pinto, a dedicated teacher who took great pride in my accomplishments.

Pearl Pinto

Contents

ABBREVIATIONS

MK	Murraya Koenigii
AT	Antipsychotic
BD	Bipolar disorder
SGA	Second generation antipsychotics
SCZ	Schizophrenia
TRS	Treatment resistant schizophrenia
CNS	Central nervous system
CVD	Cardio vascular diseases
CLZ	Clozapine
HAL	Haloperidol
STAT	Statin
AEMK	Aqueous extract of Murraya koenigii
AQ	Aqueous
MEMK	Methanolic extract of Murraya koenigii
CEMK	Chloroformic extract of Murraya koenigii
T2DM	Type II Diabetes mellitus
CTAB	Cityltrimethylammonium bromide
PCR	Polymerase chain reaction
rBcl	Ribulose-bisphosphatecarboxylase
GAE	Gallic acid equivalent
ATS	Acute toxicity study
LD	Lethal dose
DMSO	Dimethylsulfoxide
DPPH	1,1-diphenyl-2-picryl hydrazyl
ABTS	2,2 –azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
FRAP	Ferric reducing antioxidant property
WAT	White adipose tissue
PANSS	Positive and Negative symptom score
TPTZ	2,4,6 –Tripyridyl-s-triazine
HMG CoA	hydroxymethylglutaryl-coenzyme A
Hmg-cr	3-hydroxy-3-methylglutaryl-CoA reductase
SREBP	Sterol regulatory element binding protein
Fasn	Fatty acid synthase
Pparg	Peroxisome proliferator-activated receptor
D	Dopamine
5HT2	Hydroxy tryptamine Serotonin receptor
H1	Histaminergic receptor
NO	Nitric oxide
H2O2	Hydrogen peroxide
Conc	Concentration
PI	Percent Inhibition
DNSA	Di nitro salicylic acid
OECD	Organization for Economic cooperation and Development
PUFA	Polyunsaturated fatty acids
BMW	Body mass weight
W/V	Weight per volume
V/V	Volume per volume

TBARS	Thiobarbituric reactive acid substances
BSA	Bovine serum albumin
p-NPB	para-Nitrophenylbutyrate
IC50	50% Inhibitory concentration
IAEC	Institutional Animal Ethics Committee
LPO	Lipid peroxidation
mM	milli molar
µL	micro Litre
HDL	High density lipid
TC	Total cholestrol
PE	Phosphatidyl ethanolamine
PC	Phoshatidyl choline
LDL	Low density lipoprotein
TG	Triglyceride
VLDL	Very Low density lipoprotein
TP	Total protein
ALP	Alkaline phosphatase
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvic transaminase
GGT	Gamma Glutamyl Transferase
Hb	Haemoglobin
FBS	Fasting Blood Sugar
PPBS	Post-prandial Blood Sugar
mg/ml	milligram per milliliter
b.w	Body weight
DTNB	5,5-dithio-bis-(2-nitrobenzoic acid)
NCBI	National center for biotechnology information
CYP7A1	Hepatic Cholestrol 7 alpha Hydroxylase
LFT	Liver function tests
RFT	Renal function test
CK	Creatine kinase
CKMB	Creatine kinase-heart fraction
TpI	Troponin I
GPx	Glutathione peroxidase
MDA	Malondialdehyde
GSH	Glutathione
CAT	Catalase
SOD	Superoxide Dismutase
OD	Optical density
CCl4	Carbon tetrachloride
i.p	intraperitoneal
ANOVA	Analysis of variance
DPX	Dibutylphthalate polystyrene xylene
N.S	Normal saline
S.D	Standard deviation
HPE	Histopathological examination
IU/L	International unit per liter
mmol/L	Millimol per liter
UV-VIS	Ultraviolet-visible spectrophotometer

nm	Nanometer
TPC	Total Phenolic content
RPM	Revolutions per minute
TFC	Total Flavanoid content
ROS	Reactive oxygen species
GOD POD	Glucose oxidase peroxidase

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ABSTRACT

Clozapine, an atypical antipsychotic, is a highly favored medication in the treatment of drug-resistant schizophrenia due to its unique pharmacological profile and efficacy in managing psychotic symptoms where other antipsychotic drugs often fail. It operates by inhibiting dopamine (D2), serotonin (5HT2c), and histamine (H1) receptors, a combination that allows Clozapine to exhibit moderate antagonism toward these pathways. This distinctive mode of action enhances its therapeutic impact against psychosis, making it invaluable for patients who do not respond to other treatments. However, Clozapine treatment is associated with significant metabolic side effects, including weight gain, dyslipidemia, and metabolic syndrome. These adverse effects pose a considerable challenge in the clinical management of schizophrenia, as they not only impact patient health but also reduce adherence to treatment due to the discomfort and risks posed by metabolic disorders. As such, while Clozapine's effectiveness in managing psychosis is well-recognized, the need to mitigate its adverse metabolic effects has become a prominent area of research.

The American Heart Association's 2013 guidelines recommend using lipid-lowering agents like statins to address abnormal lipid levels in patients, including those on antipsychotic medications such as Clozapine. Although statins can help regulate lipid levels, concerns about long-term reliance on pharmaceutical interventions and potential side effects have spurred interest in exploring alternative treatments, particularly herbal options with antihyperlipidemic properties. In this context, Murraya koenigii (commonly known as curry leaf) has gained attention due to its traditional use in herbal medicine and its broad range of medicinal properties. Curry leaves are rich in phytochemicals and bioactive compounds that may offer therapeutic benefits, including antioxidant and lipid-lowering effects. Thus, the current research seeks to investigate the potential of Murraya koenigii as a herbal alternative to help mitigate the adverse metabolic effects associated with Clozapine treatment.

This study focuses on the bioactive metabolites of Murraya koenigii to determine their potential in alleviating Clozapine-induced metabolic side effects, aiming to benefit both the psychiatric

patient community and clinicians managing schizophrenia. In August 2022, samples of Murraya koenigii were collected from the southern parts of Goa, India. These samples underwent morphological identification using standard taxonomic keys, confirming the sample as Murraya koenigii from the Rutaceae family. The investigation was designed to perform an in-depth analysis of the phytochemical composition, antioxidant potential, antihyperlipidemic activity, and in vivo effects of the chloroformic extract derived from Murraya koenigii. The study explored the phytochemical profile of Murraya koenigii using both qualitative and quantitative analysis methods, employing three distinct solvents: chloroform, water, and methanol. The aim was to understand how each solvent affects the extraction of phytochemicals, as different solvents have unique polarities that influence the solubility and extraction efficiency of bioactive compounds.

The results of the phytochemical analysis revealed a diverse range of compounds in Murraya koenigii, including saponins, tannins, terpenoids, steroids, anthraquinones, glycosides, flavonoids, coumarins, and phenols. Notably, all three extracts from the solvents—chloroform, water, and methanol—contained these compounds, though their concentrations varied depending on the solvent used. This variation in phytochemical composition suggests that different solvents can selectively extract certain compounds, potentially impacting the biological activities of each extract. Given the known antioxidant and antihyperlipidemic properties of many of these compounds, the presence of these phytochemicals in Murraya koenigii supports its potential role as a natural remedy for managing Clozapine-induced metabolic side effects.

The antioxidant activity of Murraya koenigii extracts was assessed using multiple assays, including DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging, ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation scavenging, Ferric Reducing Antioxidative Power (FRAP), and lipid peroxidation inhibition. These assays allowed for a comprehensive evaluation of the antioxidant potential of each extract. The chloroform and methanol extracts of Murraya koenigii demonstrated a concentration-dependent increase in DPPH radical inhibition, indicating significant antioxidant activity. Notably, the chloroformic extract exhibited the highest capacity for DPPH radical inhibition, suggesting it may be more effective than the other extracts in neutralizing free radicals and preventing oxidative stress. In the ABTS assay, the chloroformic extract also yielded the highest level of ABTS+ radical inhibition, measuring 91.18%. While the

methanolic extract showed slightly lower antioxidant activity in comparison, it still demonstrated substantial potential, reinforcing the efficacy of Murraya koenigii as a natural antioxidant source.

These antioxidant properties are particularly relevant in the context of Clozapine treatment, as oxidative stress has been linked to weight gain, dyslipidemia, and other metabolic disturbances associated with antipsychotic medications. By countering oxidative stress, Murraya koenigii may help to reduce these adverse effects, making it a valuable natural adjunct in managing the metabolic side effects of Clozapine. Additionally, the study's findings on the antihyperlipidemic potential of Murraya koenigii suggest that the curry leaf could play a role in regulating lipid metabolism and reducing lipid accumulation in patients undergoing Clozapine treatment. This antihyperlipidemic effect, coupled with its antioxidant activity, highlights Murraya koenigii's potential as a dual-action herbal remedy for addressing the metabolic side effects commonly associated with antipsychotic medications.

Another promising outcome of this research is the potential application of Murraya koenigii for weight management. Weight gain is a significant issue for patients treated with Clozapine, often leading to increased health risks and reduced compliance with treatment. The results of this study indicate that the bioactive compounds in Murraya koenigii may support weight reduction by modulating lipid metabolism and reducing oxidative stress, thereby addressing one of the primary concerns in Clozapine treatment. As such, incorporating curry leaves into the diet of patients on Clozapine could provide a natural means of managing weight gain and enhancing overall metabolic health.

Overall, this research suggests that alterations in lipid metabolism induced by psychotropic medications like Clozapine may play a role in drug-related overeating and weight gain. The findings emphasize the need for further investigation into how Clozapine affects lipid metabolism in both the central nervous system and peripheral tissues. By understanding these metabolic pathways, researchers and clinicians can develop more targeted approaches to mitigate Clozapine's side effects, potentially improving patient outcomes. The study also opens the door for additional research into other natural compounds with similar properties, expanding the range of herbal alternatives available for managing metabolic syndrome in psychiatric patients.

In conclusion, the current investigation provides compelling evidence supporting the use of Murraya koenigii as a natural alternative to counteract Clozapine-induced metabolic disturbances. Its antioxidant and antihyperlipidemic properties make it a promising candidate for managing weight gain, dyslipidemia, and oxidative stress in patients undergoing Clozapine therapy. Future studies are needed to further explore the mechanisms by which Murraya koenigii exerts these effects and to evaluate its clinical efficacy in larger, more diverse patient populations. The insights gained from this research have the potential to significantly improve the quality of life for individuals with schizophrenia by offering a natural solution to some of the most challenging side effects associated with antipsychotic treatment.

Keywords: *Clozapine, Schizophrenia, Dyslipidemia, Murraya koenigii, Antioxidant, Agranulocytosis, Herbal alternative, Antihyperlipidemic ability.*

CHAPTER 1

INTRODUCTION

1.1 ANTIPSYCHOTICS

Antipsychotics have consistently served as fundamental treatments for mental illnesses, particularly in the context of schizophrenia. Schizophrenia is not solely a neurological disorder; it also disrupts the familial dynamics of those experiencing its symptoms. Schizophrenia is a debilitating mental disorder marked by psychosis, negative symptoms, and neurocognitive deficits (Kaddurah-Daouk, 2007). The life expectancy of these patients may decrease by approximately 20 years in comparison to the healthy population. Patients with schizophrenia typically exhibit positive symptoms, including delusions, hallucinations, and psychosis, as well as negative symptoms such as hypoactivity, neurocognitive deficits, difficulty initiating speech, and behavioral issues (Correia et al., 2021). Schizophrenia is getting more common. About 24 million people worldwide—roughly 0.32% of the population—are affected by schizophrenia. This is equivalent to roughly 1 person out of every 300. There were 23.6 million people with a diagnosis of schizophrenia in 2019, a 65.85% increase since 1990. Age-standardized disability adjusted life years (ASDR) were highest in the United States of America, Australia, and New Zealand (Xuanxuan et al.,2019). The overall incidence of schizophrenia spectrum disorders in India is estimated to be 1.41%, while the current prevalence is 0.42%. 72% of those diagnosed with schizophrenia do not receive the necessary care, indicating a significant treatment gap (Hegde et al.,2023).

Antipsychotics are classified into two categories: atypical antipsychotics and typical antipsychotics. A typical antipsychotic, also referred to as second-generation antipsychotics, include commonly prescribed medications such as Clozapine, Olanzapine, Risperidone, and Aripiprazole. While these drugs are considered safe, they may have adverse side effects. Antipsychotics typically function by modifying the actions of neurotransmitters such as dopamine, serotonin, acetylcholine, and histamine. The inhibition of these chemicals can mitigate the occurrence of hallucinations, delusions, and severe mood fluctuations. Research indicates that approximately 60% of individuals with schizophrenia do not exhibit the necessary response to standard treatments. Clozapine has been demonstrated to be more effective than other antipsychotics in treating drug-resistant schizophrenia (Howes et al., 2017).

Clozapine has been regarded as the most effective treatment for resistant schizophrenia since 1988,

despite the significant risk of agranulocytosis, its primary adverse effect (Kane, 1988). Other side effects include tremors and akathisia (Menaka et al., 2016). Clozapine is the preferred medication for patients exhibiting minimal to negligible responses to standard antipsychotic treatments.

All antipsychotics exert their effects by inhibiting dopamine D2 receptors in the mesolimbic and frontal brain regions (Fernø et al., 2005). Clozapine functions as an antagonist for dopamine receptors (D2 and D4), serotonin receptors (5HT2 and 5HT3), and additionally interacts with adrenergic, cholinergic, and histamine (H1) receptors. The moderate D2 antagonism enhances therapeutic efficacy against psychosis, as demonstrated by clozapine (Tauscher et al., 2004). Clozapine exhibits a greater affinity for H1 receptors (Reynolds & Kirk, 2010), indicating the role of these receptors in weight gain (Wirshing et al., 1998).

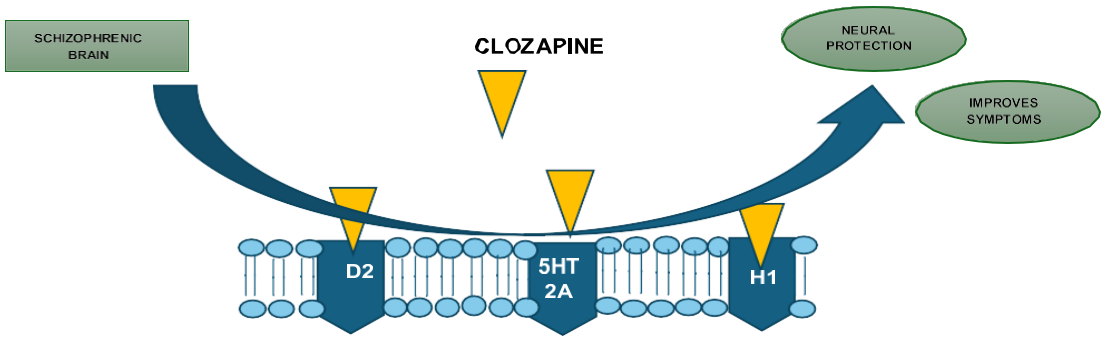

Figure 1 Antagonism of Dopaminergic, serotonergic and histamine receptors by Clozapine

Clozapine is associated with numerous adverse effects. Commonly observed are metabolic disturbances in glucose, insulin, lipids, and an increase in weight gain, collectively termed metabolic syndrome.

1.2 CLOZAPINE

Clozapine is an atypical antipsychotic medication primarily used for treatment-resistant schizophrenia. It is known for its efficacy in reducing the risk of recurrent suicidal behaviors in patients with schizophrenia or schizoaffective disorder.

This antipsychotic medication is primarily utilised for treatment-resistant schizophrenia and for reducing suicidal risk in individuals with schizophrenia. It is a tricyclic dibenzodiazepine that is soluble in Water and acetone exhibit high solubility in chloroform. The chemical formula is C18H19ClN4.

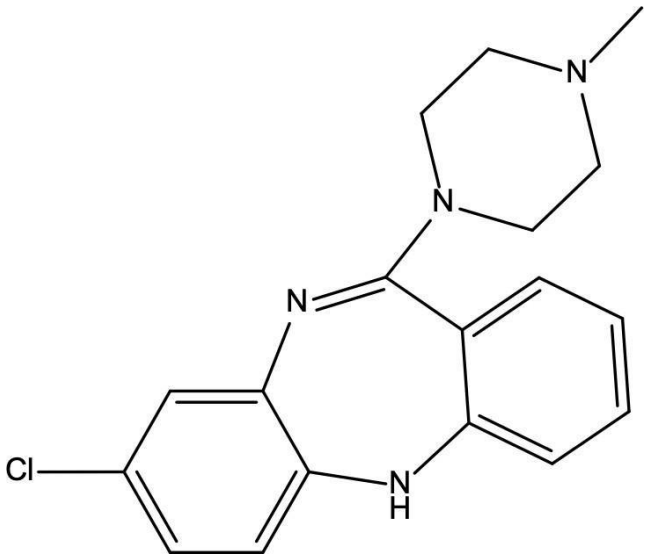

Figure 2 Structure of Clozapine

Clozapine is not only effective in reducing the primary symptoms of schizophrenia, such as hallucinations and delusions, but it is also valuable in managing a broader range of psychotic symptoms often present in other mental illnesses. This includes negative symptoms such as apathy, emotional withdrawal, and confusion (Kane, 1988). Unlike conventional antipsychotics, which

commonly cause catalepsy, Clozapine's unique pharmacology allows it to avoid this side effect, leading to a reduced risk of motor abnormalities. This distinction is primarily due to Clozapine's relatively loose and transient binding to dopamine D2 receptors. In contrast, other antipsychotics like Haloperidol tightly bind to D2 receptors, which contributes to a higher incidence of movement-related side effects. Although Clozapine also binds to other dopamine receptors (D1, D3, D4, and D5), the implications of these interactions are not yet fully understood (Miller, 2009).

Metabolized in the liver via the cytochrome P450 system, Clozapine is well absorbed and excreted through urine and stool. Due to the initial steps of its metabolic pathway, only about 60 -70% of the administered dose remains bioavailable, with food intake having minimal impact on its absorption. Clozapine has a half-life of approximately 8-14 hours, though this can vary between individuals (Jann et al., 1993).

1.2.1 ADVERSE METABOLIC EFFECTS OF CLOZAPINE

Among antipsychotic medications, Clozapine and Olanzapine are particularly associated with a higher risk of hyperlipidemia. Patients with chronic schizophrenia show a notable prevalence of metabolic syndrome, which poses long-term health risks. However, only some antipsychotics are linked to substantial weight gain, as observed through clinical experience (Panariello et al., 2011). The potential causes of lipid dysregulation in patients on antipsychotics include factors like weight gain, changes in diet, glucose intolerance, medication effects, and a sedentary lifestyle (Howes et al., 2017). Although weight gain associated with Clozapine is generally not considered life-threatening if managed properly, it remains a concern in long-term treatment (Henderson et al., 2000). A combination of genetic and environmental factors further increases the risk of cardiovascular disease (CVD) among individuals with schizophrenia (Ratna et al., 2019).

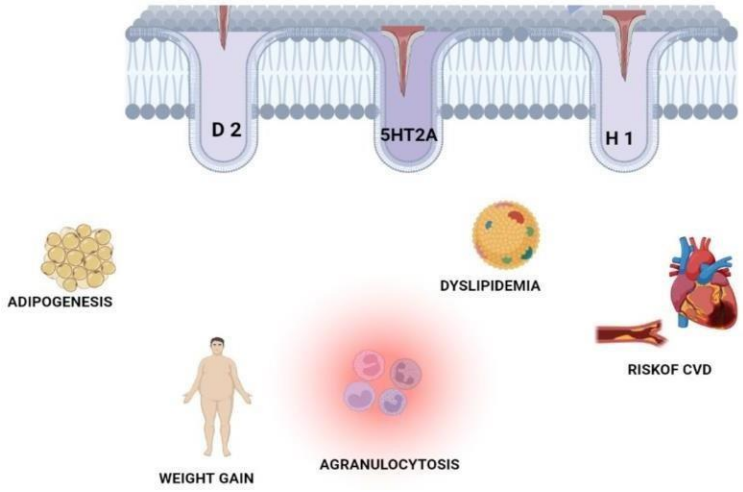

Figure 3 Adverse side effects of Clozapine treatment in Schizophrenia

⇒ **Weight Gain:** Among atypical antipsychotics, clozapine and olanzapine are particularly associated with significant weight gain. Studies indicate that the prevalence of obesity is 64% in adults on clozapine and 56% in those on other atypical drugs, compared to only 28% in non-medicated adults (Gonçalves et al., 2015). Antipsychotics, particularly in adult patients, tend to have an orexigenic effect, promoting increased appetite (Mata et al., 1967). The exact molecular mechanism behind clozapine-induced weight gain remains unclear, though inhibition of histamine receptors in the central nervous system (CNS) appears to play a significant role. Histamine H1 receptor blockade is believed to contribute to hyperphagia, leading to overeating (Coccurello & Moles, 2010). Additionally, the concurrent blockade of dopamine and serotonin receptors may further contribute to weight gain, though the primary factor is often attributed to H1 receptor inhibition (Yuen et al., 2021). Antipsychotics interact with various receptors, triggering signal transduction pathways that may underlie this weight gain effect (Tkachev et al., 2021). The hypothalamus, which regulates food intake and satiety through neurotransmitters and neuropeptides, is particularly affected by this multifaceted receptor action, leading to obesity in some patients (Keshavan et al., 2008). Proposed mechanisms of weight gain also include 5-HT2c receptor antagonism, hyperprolactinemia, elevated serum leptin levels (leading

to leptin resistance), and H1 receptor antagonism (Monteleone et al., 2002; Herrán et al., 2001).

⇒ **Increased Lipids:** Antipsychotics, particularly clozapine, may promote lipid metabolism disturbances through gene upregulation involved in lipogenesis. Clozapine is known for its superior therapeutic efficacy in treating refractory schizophrenia compared to other typical antipsychotics like haloperidol and chlorpromazine (Chakos et al., 2001). This drug also impacts several biochemical pathways, particularly those involving the sterol regulatory element-binding proteins (SREBPs), which are crucial in lipid regulation. Clozapine has been found to upregulate SREBP-1 and SREBP-2 pathways, leading to increased lipid and cholesterol synthesis (Lauressergues et al., 2010). This effect is accompanied by a significant rise in free fatty acids (Fernø et al., 2009) and lipid accumulation in the liver (Jassim et al., 2012). Additionally, clozapine-induced glucose intolerance has been linked to an increased risk of developing new-onset type 2 diabetes mellitus (T2DM).

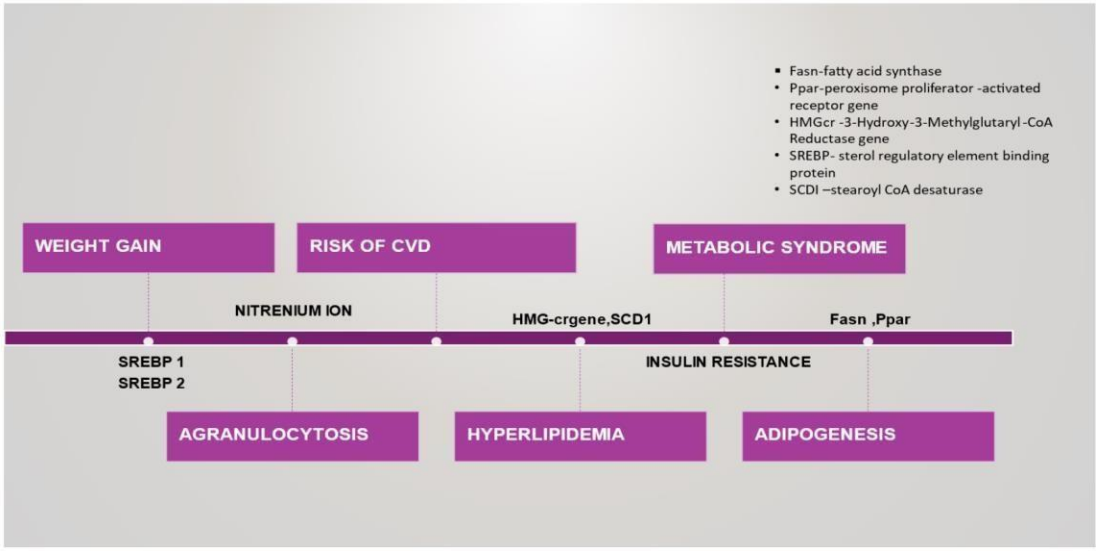

Figure 4 Mechanisms involving Gene alterations that induces metabolic effects in Clozapine treated patients

⇒ **Phospholipid Involvement:** Clozapine treatment leads to a significant reduction in polyunsaturated fatty acids (PUFAs), especially omega-3 and omega-6 fatty acids, as

well as in phospholipid levels. This reduction also includes decreased levels of choline and glycerophospholipids (Correia et al., 2021). PUFAs, such as omega-3 and omega-6, and phospholipids play a crucial role in cellular membrane integrity, and they can be incorporated into glycerophospholipids, which are essential for maintaining cell structure (Correia et al., 2021).

⇒ **Adipogenesis:** Fat cells in adipose tissue are essential for glucose and lipid metabolism. The enzyme stearoyl CoA desaturase (SCD1) catalyzes the conversion of saturated fatty acids to monounsaturated fatty acids, playing a role in the synthesis of triglycerides, cholesterol esters, and phospholipids. SCD1, regulated by the SREBP system, has a direct impact on lipid homeostasis (Lauressergues et al., 2010). Clozapine may lead to weight gain and lipid accumulation in adipose tissue by reducing lipolysis, specifically the breakdown of triglycerides, contributing to higher adiposity levels (Parvathi et al., 2020).

⇒ **Agranulocytosis:** Agranulocytosis is a reduction in leukocytes, particularly neutrophils, which can be life-threatening if unmonitored. Though clozapine is generally safe, it can occasionally lead to agranulocytosis, necessitating regular hematological monitoring (Alvir, 1993; Nazir et al., 2021; Willcocks et al., 2021). The exact mechanism linking clozapine to neutropenia remains unclear, but it may involve the production of reactive nitrenium ions during clozapine metabolism. These ions could damage neutrophils by binding to their structural proteins, possibly as part of an immune response (Malech et al., 2019). This risk may intensify with infections (Leon et al., 2020; Willcocks et al., 2021).

Clozapine has also been shown to increase mitochondrial protein oxidation in both neuroblastoma cells and the lymphoblastoid cells of schizophrenia patients. This alteration affects energy metabolism, mitochondrial function, and the expression of critical proteins in the electron transport chain, such as cytochrome oxidase and succinate dehydrogenase (Streck et al., 2007). Clozapine intake may disrupt the electron transport chain in red blood cells (Walss-Bass, 2010) and elevate oxidative

stress across tissues, impacting cell structure and function (Reinke et al., 2004). Protein oxidation due to clozapine can lead to the generation of reactive oxygen species (Streck et al., 2007; Heart et al., 2013).

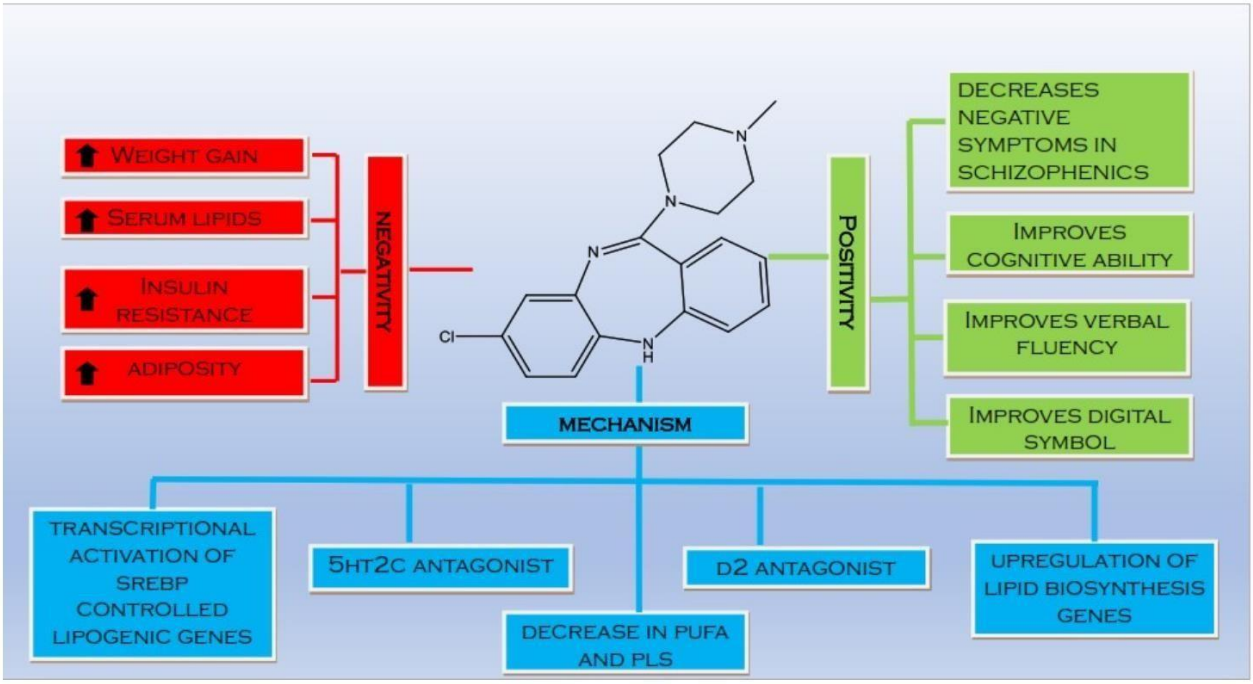

Figure 5 Pros and Cons of Clozapine treatment

1.3 DYSLIPIDEMIA

An increase in cholesterol, triglycerides, LDL cholesterol, and HDL cholesterol is a defining feature of dyslipidemia, a condition of impaired lipid metabolism (Chait1990.Pdf, n.d.). One risk factor for cardiovascular disease is dyslipidemia. It results in atherosclerosis, which can lead to stroke and coronary heart disease (CHD). There are many modifiable risk factors that might contribute to CVD: Physical activity, alcohol use, smoking, atherogenic diet, dyslipidemias, hypertension, obesity, diabetes, and metabolic syndrome (Halpern et al., 2010).

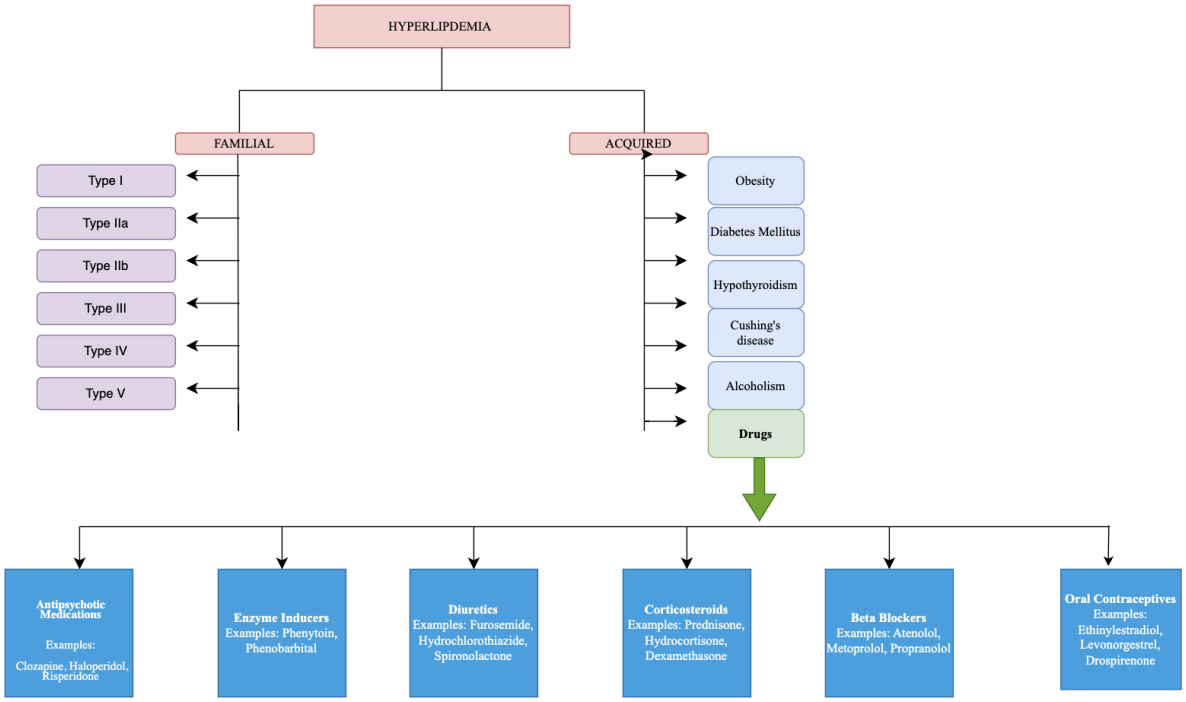

Figure 6 Classification of Familial and Acquired hyperlipidemias focusing on drug induced lipidemia (source: Fredrickson's classification of hyperlipidemias,1967., Acquired hyperlipidemias by Alan Chait (Chait1990.Pdf, n.d.)

Various medications have been documented to influence lipid profiles. Cardiovascular medications, antipsychotics, anticonvulsants, hormones, and specific immunosuppressants are recognised for their potential to alter lipid levels (Zlatković et al., 2014). Antipsychotic medications, combined with an unhealthy lifestyle, contribute to elevated cholesterol and triglyceride levels (Kim et al., 2019). Polyunsaturated fatty acids, which are crucial components of cell membranes, are typically modified. It is proposed that defective lipids play a role in the pathophysiology of schizophrenia (Kaddurah-Daouk et al., 2007).Lower levels of fatty acids are observed in the cell membranes of schizophrenia patients, as evidenced by Orešič et al. (2012), in both the acute and chronic stages of the disease (Kim et al., 2019; Yuen et al., 2021). The involvement of membrane lipids, particularly polyunsaturated fatty acids (PUFA), in disease symptoms has been suggested, as indicated by the abnormal lipid levels observed during acute psychotic episodes in schizophrenia, which normalise post-episode. Dyslipidaemia may also arise from the effects of oxidative stress on serum lipid levels (Reinke et al., 2004). In schizophrenia,

serum and membrane lipid levels appear to be abnormal (S. Ghosh et al., 2017; Orešič et al., 2011; Tessier et al., 2016).

Phospholipids are critical components of brain cell membranes, and their metabolism may play a significant role in schizophrenia, as the structural integrity and functional properties of neurones are notably impacted in patients with this condition (S. Ghosh et al., 2017; Wood et al., 2015). Furthermore, inadequate uptake and biosynthesis, as well as excessive degradation of phospholipids from the brain membrane, have been hypothesised to be linked to schizophrenia and dysfunctional synapses (Tessier et al., 2016). Consequently, the elevated levels of phospholipids in the SCZ animal model receiving antipsychotics HAL or CLZ may suggest a potential de novo stimulated synthesis of phospholipids to compensate for their deficiency (Correia, Nani, Aladares Ricardo, et al., 2021).

Five primary classes of lipid-lowering agents exist: Statins, Fibrates, Bile acid binding agents, Cholesterol absorption inhibitors, and Nicotinic acid derivatives. Synthetic drugs for dyslipidaemia treatment primarily include HMG CoA reductase inhibitors (statins), which specifically reduce cholesterol levels, and fibrates, which address fatty acids and triglycerides (Pahan et al., 2006). Commonly prescribed statins include Atorvastatin, Simvastatin, Rosuvastatin, and Fluvastatin.

Weight gain, dyslipidaemia, and metabolic syndrome are significant concerns in the treatment of schizophrenia with antipsychotic medications. Additional risk management strategies should be incorporated during antipsychotic treatment, such as lifestyle interventions that involve engaging patients or their carers in self-management strategies to improve monitoring effectiveness. Lipid-lowering agents, such as statins, may be indicated based on an individual's cardiac risk group, as outlined in the 2013 American Heart Association guidelines, in the presence of abnormal lipid parameters. Side effects of statins, including hyperglycemia and peripheral neuropathy, may deter individuals from using these medications (Reynaldi et al., 2021). Consequently, there is a need for alternative treatments, such as herbal medicines, for managing dyslipidaemia.

1.4 NEED OF ALTERNATIVE MEDICATION -EASILY AVAILABLE MURRAYA KOENIGII

For centuries, traditional medicine has extensively utilised medicinal plants. Medicinal plants encompass a variety of biologically active compounds that contribute to enhancing quality of life. Murraya koenigii is recognised as a herb of medicinal significance. The various components of M. koenigii, including its leaves, root, bark, and fruit, are recognised for their ability to enhance a range of biological activities. These substances serve various purposes in Indian cuisine, including their roles as antihelminthics, analgesics, digestives, and appetisers. "The medicinal properties of M. koenigii are attributed to various chemical constituents. "

Various carbazole alkaloids, known for their oxidative properties, alongside other significant metabolites such as terpenoids, flavonoids, phenolics, carbohydrates, carotenoids, vitamins, and nicotinic acid, are derived from different parts of the M. koenigii plant. Plant-based diets have demonstrated effectiveness among the various alternative therapies available for lipid control.

Figure 7 Murraya koenigii -a sub-tropical tree

Murraya koenigii is a member of the Rutaceae family, often referred to as a sub-tropical tree, reaching heights of 4-6 meters (Fig 7) and featuring a medium-sized trunk (Mhaskar et al.). This tree originates from India, Sri Lanka, and various Southeast Asian nations. Numerous holistic treatments, such as Ayurveda and Unani, have utilised the beneficial properties of these leaves to address various gastrointestinal disorders, diabetes, and metabolic issues. Dried leaf decoction is utilised by individuals focused on health to assist in weight loss efforts. Murraya koenigii has been utilised in culinary practices across nearly all South Asian nations for an extensive period. These contain a wealth of vitamins, minerals, and various nutrients. (Zheng et al., 2012) Curry leaves are typically introduced to hot oil at the onset of cooking to effectively extract their flavour and aroma. The green leaves of M. koenigii serve as a direct flavouring agent for soups, curries, fish, and meat, and are also incorporated into various food preparations, including dal, sambar, and chutney. Dried leaf powder possesses additional properties such as antibacterial, antifungal, and antihelminthic effects, making it a potent agent against infections and skin-related disorders (Chaudhary, 2020). The leaves possess the capability to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals. Free radicals have been associated with the development of several chronic diseases, including diabetes, cardiovascular conditions, Parkinson's, and Alzheimer's diseases (Igara et al., 2016). Numerous metabolic irregularities have been documented in relation to the onset of glucose intolerance and diabetes. These abnormalities encompass a reduction in glucose transport and oxidation rate, as well as a decline in glycogen synthesis. Obesity is linked to insulin resistance and factors that increase the risk of cardiovascular diseases. The treatment with Murraya koenigii leaves demonstrated a significant enhancement in glucose intolerance (Sakarkar DM, Tembhume SV, 2017; Tembhurne & Sakarkar, 2010). Numerous animal studies clearly demonstrate that the leaf extract can significantly lower serum cholesterol and triglyceride levels.

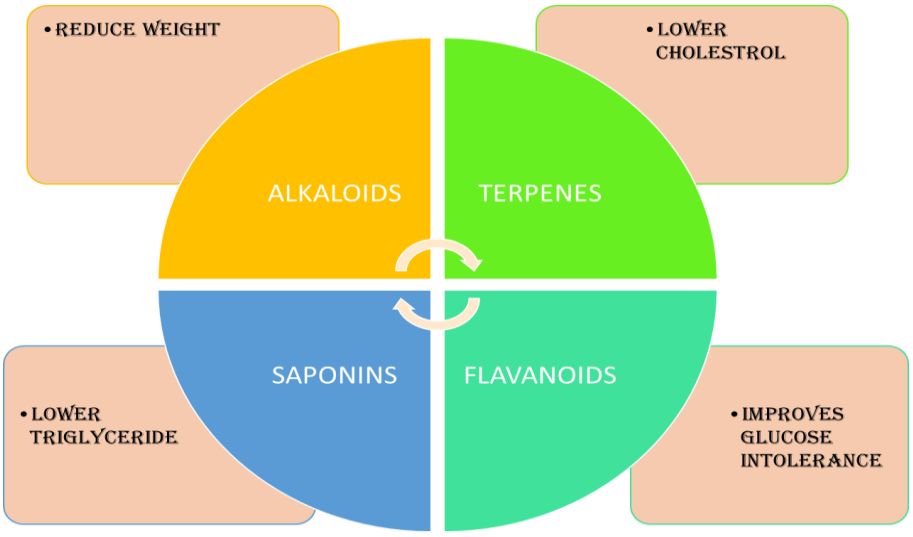

Figure 8 Importance of phytoconstituents of Murraya koenigii in improving health

1.5 SIGNIFICANCE OF *MURRAYA KOENIGII* AS AN ANTIHYPERLIPIDEMIC AGENT

There is substantial evidence that reducing cholesterol can help stabilize arterial plaques, ultimately lowering the risk of cardiovascular diseases. Three enzymes—acetoacetyl-CoA thiolase, HMG Co-A synthase, and HMG Co-A reductase—are promising targets for hypocholesterolemic agents, making them potential focal points for pharmaceutical interventions (X. Liu et al., 2015). Key constituents of *Murraya koenigii* highlight its potential as an alternative therapeutic option.

- **Polyphenols:** Polyphenols, a diverse group of compounds, are broadly classified into flavonoids and non-flavonoids (Boccellino & Angelo, 2020). Studies suggest that flavonoids and phenolic compounds inhibit the porcine pancreatic lipase enzyme by binding to the enzyme-substrate complex, thereby reducing lipid absorption (Villa-Ruano et al., 2013). This inhibition provides antioxidant properties, making polyphenols useful in combating diseases caused by reactive oxygen species (Gajaria et al., 2015). Flavonoid compounds like quercetin, rutin, and catechin also exhibit anti-atherosclerotic effects and

have notable antilipid peroxidation and radical-scavenging activities (Chatuphonprasert et al., 2011).

- **Saponins:** Saponins, abundant in the leaves and stem of *Murraya koenigii*, are known to reduce lipid absorption, thus exhibiting anti-obesity properties. Studies show they help decrease adipose tissue, liver weight, and total body weight (Ji et al., 2019; X. Liu et al., 2015). By binding with cholesterol and forming non-dissolvable complexes, saponins prevent cholesterol absorption in the intestine. They also increase triglyceride excretion, demonstrating antihypertriglyceridemic activity (Reynaldi et al., 2021; Elekofehinti, 2014).
- **Tannins:** Tannins can inhibit fat absorption in the intestine by interacting with mucosal proteins and intestinal epithelial cells (Pinto & Cojandaraj, 2024). They form deposits on the small intestine's surface, reducing cholesterol absorption efficiency (Vinarova et al., 2015; Ekananda, 2015; Rahayu, 2005).
- **Alkaloids:** *Murraya koenigii* contains over fifty carbazole-type alkaloids with significant pharmacological properties. Key alkaloids, such as koenimbine, mahanimbine, and mahanine, exhibit cytotoxic, antimicrobial, antioxidant, antidiabetic, and antihyperlipidemic effects (Abeysinghe et al., 2021a; Balakrishnan et al., 2020; Mohan et al., 2013). Additionally, *M. koenigii* is effective in wound healing, protecting injury sites from infections and accelerating connective tissue formation (Meriga & Parim, 2014).

CHAPTER 2
REVIEW OF LITERATURE

2.1 DATA SOURCES AND SEARCH STRATEGY

We conducted a comprehensive search across multiple databases, including PubMed, Medline, Google Scholar, and the Google search engine. Our search spanned from the inception of these databases until May 2024. To identify pertinent articles, we employed a series of specific keywords related to our area of interest, such as '*Clozapine*,' '*Antipsychotics,*' '*Schizophrenia,*' '*Murraya koenigii,*' '*Agranulocytosis,*' and '*Hyperlipidemia*.' Additionally, we reviewed the references of the articles we located to uncover any additional studies that met our inclusion criteria.

2.2 CLOZAPINE

Clozapine was originally developed by Wander AG (Kane, 1988). In 1975, there were documented reports of 16 cases of agranulocytosis linked to clozapine, resulting in 8 fatalities, which prompted recommendations for stringent monitoring during the initial 18 weeks of treatment. The pivotal Clozaril Collaborative Study Group highlighted clozapine's significance in managing treatment-resistant schizophrenia, demonstrating considerable benefits associated with its use (Feldman, 1996; The Lancet, 1975). However, it took 14 years for the outcomes of the pivotal US Clozaril Study to be fully reported, eventually establishing clozapine's critical role in clinical practice (Kane, 1988). It wasn't until a 1990 study that the US Food and Drug Administration officially approved clozapine for use. While clozapine is effective, it carries the potential for severe, life-threatening side effects, including neutropenia, orthostatic hypotension, bradycardia, syncope, seizures, myocarditis, and increased mortality risk. Common adverse effects encompass constipation, sedation, hyperglycemia leading to diabetes mellitus, and weight gain. Impaired glucose metabolism and obesity are significant contributors to metabolic syndrome, which elevates the risk of cardiovascular disease. Evidence suggests that clozapine may induce more pronounced

metabolic side effects compared to other atypical antipsychotics (Keshavan et al., 2008). The advantages of clozapine extend beyond individual psychopathological dimensions, effectively addressing a wide range of psychotic symptoms, including negative manifestations such as blunted affect, emotional withdrawal, apathy, and confusion (Kane, 1988).

Ramachandra (2021) conducted a study to estimate the prevalence of metabolic syndrome among schizophrenia patients receiving both typical and atypical antipsychotic medications, assessing them at the start of therapy and three months later. The sample consisted of two groups of 50 patients each: Group A, which received typical antipsychotics, and Group B, which received atypical antipsychotics. A semi-structured, self-designed proforma was employed to collect socio-demographic and clinical data, with metabolic parameters evaluated before treatment initiation and three months afterward. The study found an overall metabolic syndrome prevalence of 30%, with rates of 25% in males and 35.42% in females (Lamba et al., 2021).

Tkachev et al. (2021) undertook a study analyzing the differences in concentrations of 322 blood plasma lipids at two distinct time points among 92 hospitalized schizophrenia patients. The investigation aimed to examine the relationship between lipid profile changes and symptom improvement. The results indicated that 20 specific triglyceride species were elevated in individuals with minimal improvement in their Positive and Negative Syndrome Scale (PANSS) scores, in contrast to those with significant reductions. These triglyceride species were notably distinct, exhibiting a lower number of carbon units in their fatty acid chains and being less prevalent than the primary triglyceride species found in plasma (Tkachev et al., 2021).

Panariello et al. (2011) identified excess body weight as a common health issue among individuals with schizophrenia, which increases the risk of various medical conditions, including type 2 diabetes mellitus, coronary heart disease, osteoarthritis, and hypertension. This contributes to a life expectancy that is, on average, 20% shorter than that of the general population. In patients with severe mental illness, obesity is often linked to an unhealthy lifestyle, genetic predispositions, and the effects of psychotropic medications, especially antipsychotics. While the introduction of newer ‘atypical’ antipsychotic medications marks significant progress compared to older ‘typical’ drugs, clinical observations indicate that not all newer medications result in substantial weight gain. Extensive research has been conducted on animal models related to antipsychotic-induced weight

gain, including transgenic models that alter the expression of antipsychotic receptor genes to assess changes in obesity-related gene expression and phenotypes. Moreover, pharmacogenomic studies have identified over 300 candidate genes potentially associated with weight gain due to antipsychotics. In his review, Panariello discusses contemporary perspectives on: (1) the influence of polymorphisms in various candidate genes, (2) the roles of different neurotransmitters and neuropeptides in this adverse effect, and (3) the current development status of animal models in this field (Panariello et al., 2011).

Although schizophrenia affects approximately 1% of the population, it places a disproportionate burden on society and the healthcare system (Nazir et al., 2021). A recent Canadian study estimated that, in 2004, the total economic burden associated with schizophrenia, encompassing both direct and indirect costs, amounted to 6.85 billion dollars (Goeree et al., 1999). Researchers worldwide have sought to enhance understanding of the various side effects of clozapine to improve its acceptability among clinicians and patients (Grover & Kathiravan, 2024; Id et al., 2020). A nationwide study revealed that clozapine constituted only 1.2% of all antipsychotic prescriptions among patients with psychotic disorders (Grover et al., 2016), reflecting low prescription rates given that 20-30% of schizophrenia patients are classified as having treatment-resistant schizophrenia (Smart et al., 2019). Recent updates to the criteria for treatment-resistant schizophrenia (TRS) and clozapine-resistant schizophrenia (CRS) aim to refine research focus and create a more homogeneous group of TRS patients to explore various aspects of the condition (Howes et al., 2017).

2.3 CLOZAPINE-INDUCED ADVERSE EFFECTS

2.3.1 CLOZAPINE AND WEIGHT GAIN

Clozapine, along with olanzapine, has been identified as a significant contributor to weight gain among atypical antipsychotics. Research indicates that the obesity prevalence is alarmingly high, with 64% of adults on clozapine exhibiting obesity, compared to 56% among those on other atypical antipsychotics and only 28% in a non-medicated adult group (Gonçalves et al., 2015). A study by Goran et al. (2012) highlighted a rapid increase in serum glucose, free fatty acids, and glucagon following acute administration of clozapine, as well as olanzapine, leading to lipid

accumulation in the liver, a phenomenon observed independently of food intake (Fernø et al., 2009) and weight gain (Keshavan et al., 2008). This suggests that weight gain may be a direct clinical reaction (Lindenmayer et al., 2003). Cai et al. (2015) also documented metabolic parameter elevations caused by atypical antipsychotic drugs (AAPD), which occurred even prior to visible weight gain, establishing significant links between antipsychotic use and metabolic disturbances. Moreover, some evidence implies that metabolic abnormalities associated with antipsychotics can manifest without concurrent weight gain (Keshavan et al., 2008). Intriguingly, variability in lipid concentrations among individuals may contribute to these discrepancies, with intrinsic (hormonal regulation), extrinsic (diet), and biological factors influencing lipid levels (Procyshyn et al., 2007). In murine studies, a single intraperitoneal injection of clozapine resulted in transcriptional changes in the liver mediated by genes regulating key transcription factors, including SREBP, liver X receptors, and peroxisome proliferator-activated receptors, leading to lipid deposition in hepatocytes and likely upregulation of lipogenesis. Notably, this lipid accumulation occurred independently of dietary influences, with triglycerides, cholesterol, and phospholipids all elevated within 48 hours post-injection (Birkenaes et al., 2008).

2.3.2 CLOZAPINE'S ROLE IN LIPID DERANGEMENT

Fernø et al. (2005) identified several upregulated genes associated with cholesterol and fatty acid biosynthesis regulated by SREBP transcription factors. This examination, conducted on human glioma cells exposed to clozapine and haloperidol, utilized microarray technology to reveal gene expression profiles. The upregulation of these genes could elucidate the metabolic effects associated with antipsychotic treatments. Clozapine is notably more effective than typical antipsychotics like haloperidol and chlorpromazine in treating refractory schizophrenia (Chakos et al., 2001). In his study, the concentrations of haloperidol (10 μM) and clozapine (30 μM) that activated SREBP in glioma cultures were found to be approximately 400 and 5 -fold higher than their therapeutic levels, respectively. Moreover, the activation of genes related to lipid biosynthesis was observed at just 10 μM of clozapine, also fivefold above therapeutic levels. This suggests that clozapine can induce SREBP activation, promoting lipogenesis in vivo (Fernø et al., 2005). Clozapine significantly upregulates SREBP-1 and SREBP-2, further regulating these genes to enhance lipid and cholesterol synthesis (Lauressergues et al., 2010), as evidenced by an increase in free fatty acids following a single clozapine injection (Fernø et al., 2009) and subsequent lipid

accumulation in the liver (Jassim et al., 2012). A biphasic pattern was observed in white adipose tissues, where an initial upregulation was followed by downregulation of SREBP-controlled lipogenic genes correlating with decreasing drug concentrations (Jassim et al., 2012). Genes involved in fatty acid biosynthesis (e.g., Fasn), adipogenesis (e.g., Pparg), and cholesterol biosynthesis (HMGCR) exhibited upregulation in mesenteric white adipose tissues, similar effects also being noted in ovarian WAT (Jassim et al., 2012). One plausible mechanism suggested by Jassim et al. (2012) is that the rapid increase in serum free fatty acids is mediated by lipase activity, with triglyceride degradation occurring swiftly in white adipose tissue. This theory is supported by an observed increase in lipase gene expression (e.g., Hsl, Lpl) in both ovarian and mesenteric WAT. The enzyme stearoyl-CoA desaturase (SCD1), which catalyzes the conversion of saturated fatty acids to monounsaturated fatty acids and plays a critical role in synthesizing triglycerides, cholesterol esters, and phospholipids, is a target of the SREBP system and directly influences lipid homeostasis (Hulver et al., 2005). Numerous studies have linked antipsychotic-induced upregulation of SREBP-controlled lipogenic genes, primarily SCD1, to these metabolic disturbances (Fernø et al., 2005; Lauressergues et al., 2010). The rapid lipolytic activity in WAT, alongside increased free fatty acids in serum, may trigger stress responses, such as heightened sympathetic nervous activity and catecholamine release (Bartness et al., 2005), establishing a connection between clozapine and alpha-2 adrenoceptors, given that clozapine blocks antilipolytic alpha-2 adrenoceptors (Langin, 2006).

Atypical antipsychotics, particularly clozapine, are well-documented for inducing weight gain and lipid accumulation in adipose tissues by reducing lipolysis, specifically triglyceride hydrolysis. Compelling evidence suggests that these drugs are associated with significant adiposity among patients with schizophrenia (Parvathi et al., 2020). The upregulation of the SREBP1 system in the liver (Fernø et al., 2009) and adipose tissues of rats treated with atypical antipsychotics exhibiting dyslipidemia (Minet-Ringuet et al., 2007) indicates a potential mechanism for inducing dyslipidemia. Furthermore, clozapine may lead to adverse metabolic outcomes through mechanisms such as suppression of insulin release, insulin resistance, and impaired glucose utilization by cells (Wirshing et al., 1998).

2.3.3 CLOZAPINE AND THE ROLE OF PHOSPHOLIPIDS

Correia et al. (2021) examined lipid content alterations following a 30 -day clozapine treatment, revealing significant changes in lipid profiles that contribute to a deeper understanding of metabolic derangements in patients receiving antipsychotic therapy. Their findings demonstrated increases in cholesterol, omega-3 and omega-6 fatty acids, cardiolipins, phosphocholine, and sphingomyelins, which play crucial roles in cell membrane integrity (Correia et al., 2021). These results align with previous studies that noted downregulation of phosphocholine (Kaddurah-Daouk et al., 2007) as observed by Daouk et al. (2007). Accumulation of cholesterol within the nigrostriatal pathway has been implicated in dopaminergic neuronal degeneration in animal models, with significant cholesterol levels found in the blood of schizophrenia-afflicted animals, potentially contributing to cognitive impairments (Zhao et al., 2009). Moreover, the excessive utilization of phospholipids from brain membranes, coupled with insufficient biosynthesis, has been hypothesized to relate to the pathophysiology of schizophrenia and nonfunctional synapses (Ghosh et al., 2017). Thus, elevated phospholipid levels in clozapine-treated models may reflect an adaptive response to replenish deficiencies through de novo synthesis (Correia et al., 2021).

Ghosh et al. (2017) investigated lipid composition disturbances in frontal white matter among individuals with schizophrenia (SCZ) and bipolar disorder (BD) to establish molecular and cellular correlations. Using high-pressure liquid chromatography, they quantified phospholipids such as phosphatidylethanolamine (PE) and phosphatidylcholine (PC) in white matter adjacent to the dorsolateral prefrontal cortex of subjects with BD (n=34), SCZ (n=35), and non-psychiatric controls (n=35). Further quantification of individual fatty acid species and plasmalogens in PE and PC fractions was conducted via gas liquid chromatography. Results indicated a significant decrease in PC levels in the BD group compared to controls, while fatty acids such as PE22:0, PE24:1, and PE20:2n6 were elevated, and PC20:4n6, PE22:5n6, and PC22:5n6 were decreased in the BD group. In contrast, the SCZ group exhibited higher levels of PE22:1, while showing lower levels of PC20:3n6, PE22:5n6, and PC22:5n6 compared to controls. These findings provide compelling evidence of altered lipid compositions in white matter that may contribute to the dysfunction of frontal white matter circuits in SCZ and BD (S. Ghosh et al., 2017).

2.4 HERBAL MEDICINE

Historically, plants have been pivotal as a source of medicinal remedies. Observations of wild animals reveal their instinctive capacity to select certain flora to treat various health issues. In Asia, the legacy of herbal medicine runs deep, with extensive documentation highlighting the origins of numerous globally recognized medicinal plants from China and India. In Europe and North America, the adoption of herbal remedies is on the rise, particularly as a response to health imbalances stemming from modern diets and lifestyles. A growing number of individuals are integrating herbal products into their daily health regimens, not only for treating specific ailments but also for enhancing overall well-being. During the early development of modern medicine, bioactive compounds extracted from higher plants were crucial in formulating medications for pain relief and disease treatment. According to the British Pharmacopoeia (1932), over 70% of organic monographs were associated with plant-derived products. However, the advent of synthetic drugs and antibiotics resulted in a significant decline in the prominence of plant-based therapeutic agents, especially in economically developed nations (Dev, 1997).

This renewed enthusiasm for herbal remedies has been fueled by several key factors (WHO, 2002; WHO, 2005; Kong et al., 2003):

The last two centuries have seen significant exploration into the chemical and biological properties of plants, yielding compounds that have dramatically influenced the evolution of synthetic organic chemistry, paving the way for innovative and more effective therapeutic agents (Dash et al., 2005; Nair et al., 2007). Research by the World Health Organization highlights that up to 80% of the global population relies on plant-based sources for their health care (Farnsworth et al., 1985; WHO, 2002; Dash et al., 2005). The growing interest in phytopharmaceuticals stems from the perception that ‘green medicine’ is safer and more reliable than many expensive synthetic drugs, which often come with undesirable side effects (Parekh and Chanda, 2006). Researchers are increasingly focusing on linking the phytochemical makeup of plants to their pharmacological effects, correlating botanical attributes with therapeutic activities (Rawat et al., 1997).

2.5 MURRAYA KOENIGII: AN ANTI-HYPERLIPIDEMIC AGENT

2.5.1 NATIONAL STATUS

Murraya koenigii, commonly known as curry leaves, has a rich history in traditional medicine, where it has been valued for its diverse therapeutic properties. This plant has been utilized as an antiemetic, anti-diarrheal, febrifuge, blood purifier, tonic, and stomachic, as well as a flavoring agent in various culinary applications, including curries and chutneys. Recent studies have shed light on the bioactive compounds present in Murraya koenigii, particularly through the work of Bhopal Chandra, who explored its potential through dichloromethane and ethyl acetate extracts. When administered orally at a dosage of 300 mg/kg/day to high-fat diet-induced obese rats over a two-week period, significant reductions in body weight gain, plasma total cholesterol, and triglyceride levels were observed (V et al., 2016).

Furthermore, Kesari et al. demonstrated the hypoglycemic effects of aqueous extracts from Murraya koenigii leaves in both normal and streptozotocin-induced diabetic rats, indicating the plant's potential to mitigate severe complications associated with diabetes (Kesari et al., 2007).

Comparative studies involving hydroalcoholic extracts of Murraya koenigii alongside other medicinal plants have revealed its potent inhibitory effects on pancreatic lipase and α-glucosidase enzymes. Notably, Rajan et al. reported that the hydroalcoholic extract of Murraya koenigii exhibited the most significant pancreatic lipase inhibition, highlighting its therapeutic potential (Rajan et al., 2014). Similarly, research by Phatak et al. (2019) suggested that the antihyperlipidemic activity of Murraya koenigii is linked to its bioactive compounds, as it led to a decrease in serum lipid parameters, including total cholesterol and LDL-C, thereby reducing atherogenic risk factors.

Reddy et al. (2020) investigated the anti-Parkinson's activity of aqueous extracts from Murraya koenigii leaves against paraquat-induced Parkinsonism in rats. Their study focused on various behavioral parameters, including catalepsy, muscle rigidity, and locomotor activity, alongside neurochemical assessments of malondialdehyde, catalase (CAT), glutathione (GSH) reductase, and GSH peroxidase. The results indicated that chronic paraquat administration resulted in significant motor dysfunction, elevated lipid peroxidation levels, and reduced GSH, CAT, and GSH levels. However, daily administration of Murraya koenigii extract significantly improved motor performance and attenuated oxidative damage, confirming its neuroprotective effects.

Sakarkar et al. further corroborated these findings by demonstrating the antidiabetic and hypolipidemic effects of Murraya koenigii in streptozotocin-induced diabetic rats. In their study, treatment with Murraya koenigii (200 mg/kg, p.o.) for seven days led to a significant reduction in total cholesterol and triglycerides compared to the control group, suggesting its lipid-lowering capabilities (Tembhurne & Sakarkar, 2012).

The hypolipidemic effects of both aqueous and methanol leaf extracts of Murraya koenigii were explored in male Sprague Dawley rats. The subjects were divided into three groups, with the control group receiving a vehicle, while the other two groups received daily oral doses of 600 mg/kg body weight of the aqueous extract and 200 mg/kg body weight of the methanol extract, respectively, over eight weeks. Significant decreases in plasma cholesterol, triglycerides, and phospholipid levels were observed in the treated groups compared to controls, attributed to the leaf constituents that stimulate insulin secretion (Vinuthan et al., 2007, 2015).

Birari et al. confirmed the hypolipidemic properties of ethyl acetate extracts from Murraya koenigii, noting significant reductions in body weight gain, plasma total cholesterol, and triglyceride levels in high-fat-diet-induced obese rats over a two-week period (Birari et al., 2010). These results were supported by Khan et al. (1996), who conducted extensive biochemical, hematological, and histological studies in rats supplemented with curry leaves and mustard.

Molly et al. (2016) conducted a groundbreaking human trial involving forty menopausal women from rural communities (ages 45-65) suffering from hyperlipidemia. Participants were administered curry leaves powder (5 g) once daily for 45 consecutive days. The study collected demographic data through questionnaires and assessed liver (serum transaminases) and renal (urea and creatinine) functions before and after the intervention. The findings revealed a statistically significant decrease in transaminases, urea, and creatinine, indicating that curry leaves pose no harmful effects on liver or kidney functions (Molly et al., 2017).

2.5.2 INTERNATIONAL STATUS

On an international scale, research by Yakuzo et al. (2011) emphasized the necessity of investigating Murraya koenigii's beneficial effects on diabetes-induced renal damage to substantiate its efficacy, as recognized by local traditional practitioners. Aqueous extracts were administered to both normal and streptozotocin-induced diabetic male rats over a 30-day period. The study showed a significant dose-dependent decrease in serum urea and creatinine levels, alongside marked increases in plasma antioxidant capacity in diabetic rats, with minimal variations

in normal controls. Histological evaluations indicated tissue regeneration in the kidneys of treated animals (Yakuzo et al., 2011).

Xie et al. (2006) reported that curry leaf extract significantly lowered blood cholesterol and glucose levels in diabetic ob/ob mice, with daily intraperitoneal injections demonstrating significant reductions in both metrics after treatment. This suggests the clinical importance of Murraya koenigii in managing high cholesterol levels and type 2 diabetes mellitus.

Zheng et al. (2012) further investigated the hypolipidemic effects of aqueous and methanol leaf extracts on male Sprague Dawley rats, finding significant reductions in plasma cholesterol, triglycerides, and phospholipids in treated groups. The study concluded that the extracts exhibited hypolipidemic activities due to constituents that stimulate insulin secretion.

Bajes et al. (2020) highlighted various natural substances derived from plants and algae with anti-obesity potential, emphasizing that certain medicinal plants from Jordan have demonstrated efficacy in reducing lipid metabolism and absorption.

A rigorous experimental study by Reynaldi et al. (2021) involved male Sprague-Dawley rats subjected to a high-fat diet and subsequently administered curry leaf extract at varying doses. The study found no significant differences in total cholesterol levels pre- and post-administration, although triglyceride levels did exhibit significant changes, suggesting that the duration of treatment may have been insufficient to effect substantial lipid level reductions.

2.6 EXTRACTION

The Soxhlet procedure is mainly utilised for extracting nonvolatile and semivolatile organic compounds from solid materials. This extraction method was first employed for extracting milk fats and has since become widely used for the extraction of fats and oils, as well as other mainly nonpolar solutes, from solid and semi-solid matrices. The efficiency of extraction exceeds that of numerous manual methods.(Kinghorn, 1992) The Soxhlet extraction method ensures a strong interaction between the sample matrix and the extraction solvent (3540c, n.d.).Extracting active compounds from plants necessitates the use of appropriate methods and techniques that produce extracts and fractions abundant in bioactive ingredients. This approach offers a distinct advantage over alternative methods by efficiently extracting large amounts of drug while utilising

considerably less solvent. This leads to significant reductions in time, energy, and, ultimately, financial resources (Longo & Rakesh, n.d.)

The main elements affecting the extraction efficiency of this method consist of the amount of solvent utilised, the temperature, the extraction time, the particle size of the drug powder, and the solvent-to-drug ratio (Peanparkdee & Iwamoto, 2019).Conventional approaches require a considerable amount of organic solvent, ranging from 50 to 200 ml for a 10-g sample (Ridgway et al., 2012). The solvent's flow rate requires meticulous regulation to guarantee sufficient time for penetration into plant cells and the effective extraction of the desired phytochemicals. On the other hand, an excessively slow solvent percolation rate could lead to higher solvent consumption in order to achieve full extraction. For each kilogramme of plant material, it is advisable to use a solvent flow rate of around 5 mL per minute. The choice of extraction solvent depends on the chemical characteristics of the secondary metabolites intended for extraction. A combination of water and alcohol is often utilised, as it promotes effective extraction; the water hydrates the plant cell walls, while the alcohol possesses chemical similarities to numerous active compounds present in the plant material (Bitwell et al., 2023).

Martin and Emilio investigated how different extraction methods influence the recovery of bioactive phenolic compounds obtained from food industry by-products. These phenolic compounds hold considerable importance owing to their abundance in plant residues and the increasing market interest in their functional attributes, including natural antioxidant activity, which hold significant importance in various applications, including nutraceutical, cosmetological, and biomedical fields. In the realm of ‘bench-to-bedside’ advancements, the extraction of samples holds significant importance, as the valorisation process depends on the desorption from the matrix and the solubilisation of the desired phytocompounds. Essential elements affecting the release and stability of these compounds encompass the composition and polarity of the extractant, the optimal particle size of the sample, the sample-to-solvent ratio, along with pH, pressure, and temperature. Moreover, modern principles of green chemistry require extraction techniques that reduce the use of hazardous materials and decrease energy usage (Gil-martín et al., 2022).

Kusumastuti (2021) examined the application of Soxhlet extraction in the fashion and textile

industry, focussing on the extraction of natural dyes from various plant sources. Kusumastuti's work investigates enhancements in Soxhlet extraction techniques, focussing on different temperatures and solvents, to create natural dye powders tailored for textile applications. This study emphasises the versatility of Soxhlet extraction within the textile sector, showcasing its efficacy in effectively obtaining natural dyes for fashion and textile uses.(Kusumastuti, 2021)

Zhang (2018) highlights the versatility of Soxhlet extraction in isolating bioactive compounds from natural sources, particularly from plants. Zhang's review emphasises the important role of natural products in drug development and explores different techniques for extraction and isolation, such as Soxhlet extraction, in the field of natural product studies.Thus, it highlights the essential function of Soxhlet extraction in tapping into nature's pharmacy and harnessing bioactive compounds for pharmaceutical and nutraceutical applications.(Zhang et al., 2018)

Kasiramar (2018) examines the efficacy of Soxhlet extraction for acquiring plant metabolites with varying solubilities in water, while also addressing its environmental impacts, especially concerning the use of volatile organic solvents. This study emphasises the flexibility of Soxhlet extraction and its possible environmental impacts. In a complementary study, Khaw (2017) highlights recent advancements designed to enhance the efficiency of Soxhlet extraction, emphasising its significance in analytical chemistry for extracting organic compounds from solid or semi-solid matrices using volatile organic solvents. These studies provide important insights into the effectiveness of Soxhlet extraction and its significance in analytical chemistry, while also considering issues related to environmental sustainability and solvent use (Kasiramar et al., 2019) ,(Khaw et al., 2017).

2.7 PHYTOCHEMICALS

Recent studies by Kalita in 2022 summarised natural chemicals, specifically polysaccharides with hypolipidemic activity; she extensively detailed their occurrence, chemistry, and molecular mechanisms. The natural polysaccharides, along with certain biopolymers such as proteins and nucleic acids, can create diverse structures that may help prevent chronic metabolic conditions like dyslipidaemia. Sulfated polysaccharides demonstrate a significant capacity for lowering lipid and cholesterol levels (Kalita et al., 2022).

Shabir (2022) highlighted that natural antioxidants sourced from plants exhibit considerable inhibitory effects on the free radicals present in living organisms during active metabolization. The overproduction of free radicals heightens the likelihood of neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease, and multiple sclerosis. This investigation sought to evaluate the ethnopharmacological impacts of Urtica dioica (UD), Matricaria chamomilla (MC), and Murraya koenigii (MK) in mitigating rotenone-induced toxicity in wild-type Drosophila melanogaster (Oregon R+) across biochemical, cellular, and behavioural dimensions. The analysis of the ethanolic extract of M. koenigii, conducted through HPLC, identified approximately 24 components, including apigenin, myricetin, fumaric acid, rutin, isorhamnetin, kaempferol, quercetin, coumaric acid, and chlorogenic acid.(Shabir et al., 2022) Prajapathi (2021) assessed the antipsychotic properties of Lagenaria siceraria (Molina) (Cucurbitaceae) fruits, commonly referred to as 'bottle gourd.'The chloroform fraction of the methanolic extract (CFME) and the acetone fraction of the methanolic extract (AFME) are utilised as extracting solvents to maximise the yield of phytochemicals.The findings indicated the presence of saponins, flavonoids, tannins, and phytosterols, which are linked to their antipsychotic properties.(Prajapati et al., 2021).

A study conducted by Kejariwal et al demonstrated a novel approach highlighting the significance of the antioxidation potential of polysaccharides from M. koenigii, utilising hot water, cold water, and alkali-extracted fractions of these polysaccharides.The purified fraction of the polysaccharide comprised 87% sugar and contained less than 7% protein. Therefore, it is established that all polysaccharides derived from Murraya koenigii hold equal significance as natural antioxidants and are utilised in the food industry (Kejariwal, 2021).

Liu carried out a study focused on exploring the inhibition potentials and mechanisms of significant biflavones derived from G. biloba on pancreatic lipase (PL), which is a crucial target in the regulation of lipid absorption. The findings unequivocally indicated that all examined biflavones in G. biloba, such as isoginkgetin, bilobetin, ginkgetin, and sciadopitysin, exhibited strong to moderate inhibitory effects on PL, with IC50 values spanning from 2.90 μM to 12.78 μM. Additional studies on inhibition kinetic analyses and docking simulations revealed that

isoginkgetin, bilobetin, and ginkgetin exhibited strong potency as PL inhibitors (Ki b 2.5 μM), forming significant interactions with the catalytic triad of PL through hydrogen bonding. The findings presented compelling evidence for elucidating the hypolipidemic effects of G. biloba. Furthermore, the newly identified PL inhibitors from G. biloba may act as lead compounds for the advancement of biflavonoid-type PL inhibitors (P.K. Liu et al., 2018).

In 2017, Prabhachandh conducted a study on the phytochemicals present in Murraya koenigii leaf extract from coastal and urban areas, concluding that both environments are abundant in phenolics, flavonoids, saponins, terpenoids, tannins, and alkaloids.The total saponin content of M. koenigii was compared between urban and coastal areas, revealing a higher value of 2.71 ± 0.02 mg/g in the coastal area, in contrast to 2.33 ± 0.01 mg/g in the urban area. The study indicates that Murraya koenigii possesses a considerable amount of antioxidant properties. The protective effect of Murraya koenigii leaves is largely attributed to phytochemicals, including flavonoids and phenols. (Prabhachandh & Babychan, 2017) Tomar performed a comparative efficacy analysis using various solvents including ethanol, methanol, acetone, DMSO, and water. The study revealed that the concentration of hydrolysable tannin in M. koenigii leaf extract was notably highest in IPA for both old and young leaves, measuring 46.3 mg and 46.7 mg GAE/g, respectively. The findings indicate that most phytochemicals are effectively extracted using distilled water, while methanol serves as an efficient solvent for tannin extraction. Additionally, it was observed that higher temperatures result in reduced extraction efficiency of tannins (Tomar et al., 2017).

A study conducted by Rajan (2014) examined the anti-obesity effects of seven different solvent extracts (n-hexane, toluene, dichloromethane, ethyl acetate, absolute methanol, 80% methanol, and deionised water) derived from germinated brown rice (GBR) on pancreatic lipase activity. The GBR extract utilising hexane demonstrated the most significant inhibitory effect (13.58 ± 0.860%) at a concentration of 200 μg/ml, followed by the hexane extract at 100 μg/ml (9.98 ± 1.048%). In contrast, the ethyl acetate extract exhibited the least effect (2.62 ± 0.677%) at a concentration of 200 μg/ml on pancreatic lipase activity. The findings indicate that GBR extracts, particularly those derived from the least polar and intermediate polar solvents, demonstrate an inhibitory effect on pancreatic lipase, reduce fat accumulation by inhibiting adipocyte differentiation, and promote lipolysis in adipocytes.(Rajan and colleagues, 2014)

A comparable study selected an intake of 50 g/day of curry leaf, which may yield over 4 mg of quercetin, with the expectation of contributing to the reduction of cardiovascular disease in vivo. (Ashokkumar et al., 2013) Studies have also been conducted on the influence of coumarin derivatives on lipid metabolism, including esculetin and fucoxanthin. Furthermore, organosulfur compounds, phytosterols, and polyunsaturated fatty acids exhibit an anti-obesity effect.

The analysis of spectral data from the isolated alkaloids resulted in the identification of mahanimbine, koenimbine, clausazoline-K, and koenigicine.(Birari et al., 2010) All these compounds underwent a pancreatic lipase inhibition assay, where mahanimbin demonstrated the highest level of inhibition, thereby confirming its lipid-lowering capability. In a study conducted by Chakrabarty, the compound Mahanimbin did not demonstrate a reduction in glucose levels (Chakrabarti, 2009); however, Mahanimbin at a concentration of 30 mg/kg/day of body weight significantly lowered cholesterol and triacylglycerols during the experimentation (Birari et al., 2010).

Graf (2005) discusses the extensive array of flavonoids and their subclasses, such as flavonols, flavones, and flavanones. The diverse agricultural practices influencing their concentration in foods pose a significant challenge for the creation of comprehensive food composition databases for these compounds. Studies in epidemiology and various other investigations indicate a negative correlation with the risk of certain types of cancer, cardiovascular diseases, and other chronic conditions. The existing knowledge concerning the bioavailability and biotransformation of these flavonoids is quite limited. Numerous investigations have demonstrated a consistent pattern of inverse correlations between flavonoid intake and the incidence of cardiovascular disease, with

findings indicating that quercetin may inhibit LDL oxidation. Furthermore, a daily intake of 4 mg of quercetin has demonstrated a 21% decrease in mortality related to cardiovascular disease (Graf et al., 2005).

Adebajo (2000) isolated minor furocoumarins from Murraya koenigii seeds, specifically Xanthotoxin and phellopterin, which are claimed to contribute to the stomachic, febrifuge, antivomiting, antidysenteric, tonic, antiperiodic, stimulant, and carminative properties of Murraya koenigii seeds. The authors have highlighted the significance of the two most potent carbazole alkaloids present in the methanolic extract of M. koenigii leaves: girinimbiol and girinimbine. These compounds have been shown to possess hepatoprotective and hypoglycemic properties. Girinimbine and girinimbilol demonstrated the highest activity, with IC (50) values recorded at 1.08 and 1.20 microg/ml, respectively. The acetylation process resulted in an enhancement of the activity of mahanimbilol and girinimbilol to 1.08 and 0.60 microg/ml, respectively. (Adebajo & Reisch, 2000)

Adipocyte PDE activity was evaluated at 30' pH 7.4 in the presence of cyclic AMP, informed by established findings on the inhibition of PDE (phosphodiesterase enzyme) by flavonoids across various tissues, as reported by Kuppuswamy and his colleague. Catechin, epicatechin, fustin, and taxifolin demonstrated a stimulatory effect on PDE when compared to the standard IBMX, a well-known PDE inhibitor. It was concluded that the absence of C in the structure leads to the stimulation of PDE, as observed in the case of catechin.Due to their inhibitory effects on phosphodiesterase with cyclic AMP as the substrate, quercetin, luteolin, scutellarein, phloretin, and genistein have been categorised as lipolytic flavonoids (Kuppusamy & Das, 1992). Mukherjee and his team documented the extraction and structural analysis of a novel carbazole alkaloid, mukonicine, derived from the leaves of Murraya koenigii Spreng, as a continuation of their pursuit for further alkaloids. Mahanimbine and girinimbine exhibited numerous similarities with this mukonicine.(Mukherjee and colleagues, 1983)

2.8 ANTIOXIDANTS

Luevano (2023), In the quest for new, effective, safe, and cost-efficient anticancer medicines from the Rutaceae family, indigenous to the Mediterranean area, the cytotoxic, anti-hemolytic, and antioxidant activities of Ruta chalepensis L. (Rutaceae) extract, as well as its fractions and isolated components, were studied. The results align with several studies, indicating that R. chalepensis and the flavonoid rutin contained within it provide protection to red blood cells against oxidative stress caused by free radicals. To far, no structured preclinical or clinical studies including these drugs have been performed with human subjects. The AAPH radical-induced haemolysis protection experiment serves as an ex vivo model to illustrate the notable antioxidant effects of some natural products, since it induces lipid peroxidation in normal red blood cells. Luevano attributes this protective effect to the polyphenol concentration, indicating that polyphenols interact with the components of the erythrocyte membrane via hydrogen bonds, therefore preventing the oxidation of membrane proteins and lipids. The experiment assessed the effectiveness of cytotoxic treatment, concentrating on the methanolic extract, to ascertain its antioxidant activity. The results demonstrated its capacity to scavenge 1,1-diphenyl-2-picryl hydrazyl (DPPH) and 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals, indicating antioxidant activity with IC50 values of 89.90 µg/mL and 130.06 µg/mL, respectively.Joel H. Elizondo-Luévano et al., 2023

In 2021, Sonter conducted an evaluation of the bioactive constituents, antioxidant capabilities, and anti-inflammatory effects of leaf extracts from Murraya paniculata, a plant historically used in the Indian medical system, sourced from Amarkantak in Madhya Pradesh. The inquiry included phytochemical analysis, assessments of antioxidant capacity, membrane stabilisation, and antibacterial activity. The methanol extract exhibited the greatest levels of flavonoids and phenolics, whereas the acetone extract shown significant ABTS inhibitory action, with an IC50 value of 555.18 ± 1.68 µg/mL. The hexane extract had the greatest H2O2 radical scavenging action, with an IC50 value of 509.84 ± 3.03 µg/mL. The aqueous extract exhibited a haemolysis rate of 19.4 ± 0.66% for red blood cells and had a protective efficacy of 80.5 ± 0.66% against haemolysis induced by hypotonic solutions at elevated concentrations. The fractions derived from the hexane extract demonstrated a greater zone of inhibition than the crude extract. The principal

constituents identified in these fractions were cyclohexane (40.11%) and 3-(6-Methoxy-3-methyl-2-benzofuranyl) Cyclohexanone (13.68%), as determined by GC–MS/MS analysis. The findings validate the traditional use of M. paniculata and highlight its potential for further exploration in pharmaceutical development initiatives.

Zin et al. (2021) conducted a comparative examination of three extraction procedures to assess their impact on the antioxidant capabilities of Murraya koenigii. This research sought to evaluate the antioxidant capabilities and flavonoid content of M. koenigii leaves by solvent-assisted extraction (SAE), microwave-assisted extraction (MAE), and ultrasonic-assisted extraction (UAE). The assessment of antioxidant capabilities was performed using both qualitative and quantitative approaches using high-performance liquid chromatography (HPLC). The leaves acquired using the UAE approach had a significant reaction in the 2,2-diphenyl-2-picryl-hydrazyl (DPPH) test, achieving an inhibition percentage of 78.00±1.00. In experiments using ferric thiocyanate (FTC) and thiobarbituric acid (TBA), the M. koenigii leaves with the lowest absorbance were found to have the strongest antioxidant activity, with the UAE-extracted leaves showing an absorbance of 0.01±0.00. The analysis for total phenolic content (TPC) indicated that the MAE technique yielded the highest total phenolic content, recorded at 120.60±14.81 mg GAE/g sample. The total flavonoid content (TFC) test showed that the MAE technique produced the highest total flavonoid content of 93.38±4.33 mg QE/g sample. The leaves extracted with MAE showed the greatest concentrations of gallic acid, catechin, epigallocatechin gallate, rutin, and kaempferol (mg/L), whereas the SAE extraction technique produced the highest concentrations of p-coumaric acid, myricetin, and quercetin (mg/L). This study's results indicate that M. koenigii leaves extracted via UAE demonstrated superior antioxidant activities compared to those extracted through MAE and SAE, thereby offering significant insights into the influence of various extraction methods on the antioxidant properties of M. koenigii.(Zin, 2021)

Abeysinghe and his colleagues conducted a phytochemical investigation to evaluate the total polyphenol and flavonoid content, as well as the in vitro antioxidant and antibacterial capabilities of Sri Lankan cultivars of Murraya koenigii and Micromelum minutum leaves. This research assessed the proximate composition, in vitro antioxidant activity, total phenolic content (TPC), flavonoid content (TFC), and antibacterial efficiency of both species using standardised

procedures. The findings indicated significant disparities in nutritional content, antioxidant capacity, and antibacterial properties between the two species. M. koenigii exhibited a markedly enhanced nutritional profile in terms of ash, crude protein, and fat when assessed on a dry weight basis. The in vitro antioxidant capacity of M. koenigii and M. minutum was evaluated using the DPPH radical scavenging test, resulting in IC50 values of 107.2 μg/mL for M. koenigii and 208.4 μg/mL for M. minutum.Abeysinghe et al. (2021b)

In 1990, Benzie and Strain performed an assessment of the FRAP assay, a method that originated in the late 1800s. The advantages of the FRAP assay were emphasised in comparison to other methods for evaluating total antioxidant power, particularly its simplicity, speed, cost-effectiveness, and robustness. The FRAP assay utilises antioxidants as reductants in a colorimetric framework linked to redox reactions. The FRAP assay stands out from many indirect radical scavenging tests that seek to quantify total antioxidant capacity, as it does not include a lag phase in its measurements. This assay necessitates no sample pretreatment, upholds consistent stoichiometric factors, shows linearity over an extensive range, displays remarkable reproducibility, and provides high sensitivity. Moreover, the FRAP assay is accessible as it does not demand specialised equipment or advanced expertise, and it does not impose strict requirements on timing and reaction conditions. This method is suitable for a wide range of intricate biological fluids, including plasma, serum, saliva, tears, urine, cerebrospinal fluid, exudates, transudates, and also for aqueous and ethanolic extracts derived from drugs, foods, and plants, along with both simple and heterogeneous solutions of pure antioxidants. The FRAP assay can be performed with any automated analyser that offers blank-corrected readings at 593 nm at designated intervals after the sample and reagent have been mixed.(Benzie & Strain, 1999) Okhawa et al. (1979) developed a standardised approach for quantifying lipid peroxide levels in animal tissues by examining their interaction with thiobarbituric acid. A ten percent (w/v) homogenate of tissue was mixed with sodium dodecyl sulphate, an acetate buffer at pH 3.5, and an aqueous solution of thiobarbituric acid. The mixture underwent heating at 95°C for a duration of 60 minutes, followed by the extraction of the resulting red pigment utilising a combination of n-butanol and pyridine. The concentration was subsequently assessed by measuring the absorbance at 532 nm. Tetramethoxypropane was utilised as the external standard, and the quantification of lipid peroxide levels was expressed in nanomoles of malondialdehyde. "This approach was utilised

to evaluate the lipid peroxide concentrations in the livers of rats subjected to carbon tetrachloride toxicity." (Ohkawa et al., 1979)

2.9 THE ROLE OF NATURAL PRODUCTS IN MITIGATING THE ADVERSE EFFECTS OF ANTIPSYCHOTIC DRUGS

A comprehensive review by Kanagasundaram (2021) underscores the inadequacy of current strategies for managing dyslipidemia, necessitating the development of innovative and effective treatment approaches. The study found that conventional lipid-lowering medications often fall short, primarily due to their short-term administration, which fails to yield significant improvements in lipid profiles. Among 48 studies analyzed, only 26 placed lipid management as a primary focus, while 22 considered it secondary. This highlights the potential benefits of targeted interventions for patients with schizophrenia who suffer from metabolic complications. In a similar vein, Mazareel (2020) explored the implications of psychotropic medications on obesity and metabolic health, revealing that dyslipidemia often goes untreated and that the physical health of these patients is frequently overshadowed by the need to manage psychotic symptoms. McDonell (2011) further emphasized the insufficient integration of psychiatric and medical care, which hinders effective metabolic management.

In their 2021 study, George et al. demonstrated the efficacy of naringin in alleviating clozapine-induced adverse effects in rats, particularly concerning weight gain, metabolic irregularities, and agranulocytosis. The researchers induced schizophrenia in rats using ketamine, followed by the administration of both clozapine and naringin. Results indicated that naringin significantly increased total leukocyte counts, preventing agranulocytosis, and offered metabolic benefits by lowering serum total cholesterol and triglyceride levels. Furthermore, naringin effectively mitigated weight gain and reduced serum glucose levels, thus preventing hyperglycemia associated with clozapine treatment.

Loubna K et al. (2020) reported that herbal remedies account for 3–5% of all intoxication cases in Morocco, with a concerning 17% resulting in fatalities. Their research focused on Hammada scoparia (Pomel), an often-overlooked herb with significant phytotherapeutic properties. Investigating both acute and subacute toxicities of its aqueous extract in rodent models, they found an oral LD50 of less than 5000 mg/kg, classifying it as slightly toxic. The 30-day subacute toxicity

study indicated liver toxicity, corroborated by hematological, biochemical, and histological analyses.

Reddy et al. (2019) explored the neuroprotective effects of an aqueous extract from leaves administered at varying doses against paraquat-induced Parkinsonism in rats. Results demonstrated that treated animals outperformed controls in behavioral and locomotor assessments. A methanolic extract's neuroprotective potential was also evaluated in a global cerebral ischemia model, with significant cognitive enhancements observed in treated groups.

Li et al. (2017) investigated the effects of artesunate on hepatic steatosis and elevated plasma triglycerides induced by clozapine. Over six weeks, various groups of rats received saline, clozapine, artesunate, or a combination of both. The clozapine group exhibited heightened triglyceride levels and hepatic damage markers, which were mitigated in those receiving both clozapine and artesunate.

Concerns regarding clozapine's safety were raised by Abdel Hamid et al. (2017), who examined the protective effects of Captopril, an angiotensin-converting enzyme inhibitor with antioxidant properties, against clozapine-induced myocarditis. The four-week study involved various treatment groups, with significant histopathological improvements and reduced oxidative stress observed in those co-administered Captopril and clozapine.

Patil et al. (2012) demonstrated that Murraya koenigii leaves could reverse haloperidol-induced orofacial dyskinesia in animal models. The study assessed antioxidant enzyme levels and lipid peroxidation, revealing significant improvements in the locomotor effects and restoration of antioxidant defenses in haloperidol-treated rats. The same year, they also investigated the potential of curry leaves for managing diabetes, linking oxidative stress to tardive dyskinesia.

Desai et al. (2012) explored the hepatoprotective effects of Murraya koenigii L. leaf extract in rats subjected to CCl4-induced hepatotoxicity. Their results indicated significant reductions in liver damage markers and improvements in antioxidant levels, suggesting that the extract has substantial hepatoprotective potential.

Miranda-Velasquez et al. (2010) investigated the hypocholesterolemic properties of Cnidoscolus chayamansa, with significant cholesterol reductions observed only in aqueous extracts, indicating alternative pathways for cholesterol metabolism.

Goicoechea et al. (2008) evaluated various sampling techniques for measuring Creatine Kinase (CK) levels in rats, revealing that collection methods significantly affected CK activity due to stress-related factors, leading to recommendations for more reliable sampling techniques.

Dhanapakiam et al. (2008) explored the incorporation of coriander seeds into the diet of rats on a high-fat regimen, noting a hyperlipidaemic effect linked to increased plasma LCAT activity, which aids in cholesterol conversion. Luo et al. (2008) studied a stilbene-rich extract from Cajanus cajan, reporting significant reductions in the atherogenic index in hypercholesterolemic mice, suggesting potential benefits in lipid metabolism.

Lastly, the impact of Azadirachta indica leaf extract on serum lipid profiles in normal and streptozotocin-induced diabetic rats was investigated by Chattopadhyay & Bandyopadhyay (2005). The extract significantly decreased total cholesterol, LDL, VLDL, triglycerides, and total lipids, indicating its potential role in addressing cardiovascular risks associated with hyperglycemia.

2.10 RESEARCH GAP

While the therapeutic potential of Murraya koenigii (curry leaf) has been widely recognized, particularly for its traditional medicinal applications, there remains a significant gap in the understanding of its specific bioactive metabolites in the context of managing hyperlipidemia induced by clozapine. Clozapine, although an effective antipsychotic, is notorious for its adverse effects on metabolic health, including dyslipidemia, which poses serious cardiovascular risks to patients. Current literature primarily focuses on the phytochemical composition of Murraya koenigii, with limited studies investigating its targeted effects on clozapine-induced metabolic disturbances.

Moreover, the existing research on the antioxidant properties of Murraya koenigii lacks comprehensive evaluations of the specific contributions of various extracts (methanolic, chloroform, and aqueous) and their respective phytoconstituents. Although preliminary studies have suggested the presence of beneficial compounds such as phenolics, flavonoids, saponins, and terpenoids, quantitative assessments of these metabolites across different extraction methods are scant. Furthermore, the correlation between the antioxidant capacity of these extracts and their ability to mitigate hyperlipidemia remains unexplored.

The assessment of the biochemical impacts of Murraya koenigii extracts on antioxidant enzyme levels—such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)—in the brain of clozapine-treated rats is particularly underrepresented in the literature. This oversight underscores a critical need for research aimed at elucidating the neuroprotective mechanisms of Murraya koenigii in the context of clozapine-induced dyslipidemia.

Thus, the proposed research aims to fill these gaps by systematically investigating the bioactive metabolites of Murraya koenigii and their potential to ameliorate clozapine-induced hyperlipidemia, while also providing insight into the underlying biochemical pathways involved. This study will not only enhance the understanding of Murraya koenigii's therapeutic efficacy but also pave the way for developing complementary strategies to mitigate the metabolic side effects of antipsychotic medications.

2.11 NEED FOR THE STUDY

The metabolic implications of antipsychotic medication extend beyond mere pharmacological effects, often influenced by individual lifestyle choices and the accessibility of healthcare services. Understanding how metabolism interacts with various antipsychotic drugs is crucial for optimizing therapeutic strategies. A nuanced comprehension of these metabolic disparities can guide clinicians in selecting appropriate medications, enabling a more precise risk-benefit analysis tailored to individual patient needs. Moreover, it is essential to investigate whether these metabolic responses vary regionally, as cultural and lifestyle factors can significantly influence health outcomes.

In light of the dyslipidemia frequently associated with antipsychotic therapy—a significant concern that often goes untreated—it becomes imperative to explore comprehensive risk management strategies. Integrating lifestyle modifications into treatment plans, such as self-management techniques that empower patients and their families to engage actively in monitoring their health, is vital. For instance, the American Heart Association's 2013 guidelines advocate for the use of lipid-lowering agents like statins based on a person's cardiac risk profile. Yet, the

intersection of antipsychotic medications and effective management of dyslipidemia has not been thoroughly examined in clinical practice, leaving a substantial gap in therapeutic knowledge.

Given the variability in pharmacological effects derived from different plant constituents, herbal alternatives represent a promising avenue for addressing these concerns. Murraya koenigii, or curry leaf, is a notable example of a plant rich in secondary metabolites such as alkaloids, terpenoids, flavonoids, glycosides, and phenolics—compounds recognized for their diverse biological activities, including antioxidant and hypolipidemic effects. Harnessing these beneficial properties through appropriate solvent extraction techniques can maximize the yield of phytochemicals, providing a natural adjunct to pharmacological interventions.

The ease of access and preparation of herbal medicines enhances their appeal, particularly for psychiatric patients who may benefit from complementary therapies. Simple consumption methods—such as decoctions, dry leaf powders, or incorporating leaves into culinary practices—can promote adherence and safety. However, it is essential to address the potential for adverse events linked to herbal treatments, particularly those related to adulteration or inherent toxicity in certain alkaloids.

Despite the promising pharmacological profile of Murraya koenigii, the specific hypolipidemic effects of its leaves in the context of clozapine-induced hyperlipidemia remain underexplored in recent literature. The study addresses a critical gap that has been identified by Kanagasundaram (2021) and Mazareel (2020), highlighting the inadequacy of current lipid lowering strategies, especially in patients with Schizophrenia who often receive insufficient metabolic care. Also, alternative therapeutic agents like Naringin, Withania Somnifera, Reserpine, Cajanus Cajan have been tested for therapeutic potential of phytochemicals in mitigating side effects of psychotropic medications and lipid abnormalities. This study is significant in promoting the safe and evidence based inclusion of herbal remedies in psychiatric and metabolic care, moving towards more personalised treatment models.

2.12 AIM AND OBJECTIVES

Aim: To evaluate the ameliorative effect of the bioactive metabolites derived from *Murraya Koenigii* (curry leaf) extract on Clozapine induced hyperlipidemia in Wistar rats

Objectives:

1) Collection, Identification, and Preparation of methanolic, chloroform and aqueous extract of Murraya koenigii Leaves.
2) To ascertain various phytoconstituents present in methanolic, chloroform and aqueous extract of Murraya koenigii leaves by using preliminary phytochemical tests
3) To determine quantitatively Phenolic, Flavonoid, Saponin, Tannin and Terpenoid content in aqueous and methanolic extracts of Murraya koenigii leaves.
4) To determine antioxidant and anti-hyperlipidemic ability methanolic, chloroform and aqueous extract of Murraya Koenigii leaves using in-vitro models
5) To evaluate the anti-hyperlipidemic effect of Murraya Koenigii Leaves against clozapine-induced dyslipidemia in Wistar rats
6) To study the effects of chloroform extract of Murraya koenigii leaves on SOD, CAT, GPx levels in Clozapine treated rat brain.

CHAPTER 3
MATERIALS AND METHODS

3.1 SAMPLE COLLECTION AND PROCESSING

Collection Site 1: Murraya koenigii was handpicked from Cortalim, a picturesque village in South Goa, located at coordinates 15°07'04.8'N, 74°04'37.2'E (Fig. 9).

Collection Site 2: The second collection site was Cansaulim, also in South Goa, positioned at 15.3368°N, 73.8978°E.

Collection Site 3: A sample of the leaves was gathered from Garca Branca, a botanical garden situated in Loutolim, Goa, with coordinates 15.3511°N, 73.9771°E (Fig.10).

These collection sites are neighboring villages, separated by a distance of approximately 10-15 kilometers. After harvesting, the leaves were thoroughly washed with distilled water to eliminate any impurities. "Care was taken to separate the leaves from any weeds and to remove all foreign matter." The cleaned leaves were then air-dried for 3 to 4 weeks before being finely ground using a mechanical blender (Fig 11). All ground materials were stored in sterile containers in a dry environment for future use (Gupta et al., 2010).

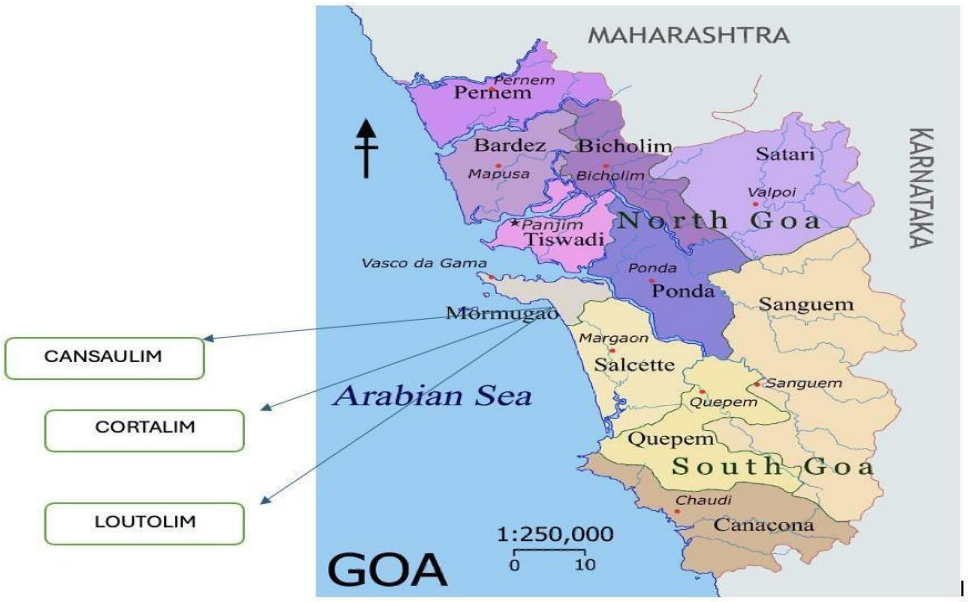

Figure 9 Maps of South Goa showing collection site (source: Map of India).

Figure 10 Botanical Garden collection site

Figure 11 Shade drying of leaves

3.2 IDENTIFICATION OF MURRAYA KOENIGII

The identification process of *Murraya koenigii* involved both taxonomical and molecular approaches, ensuring comprehensive validation of the species.

3.2.1 TAXONOMICAL AND MORPHOLOGICAL IDENTIFICATION

To facilitate taxonomical classification, a herbarium specimen was prepared in accordance with the guidelines provided by the Botanical Survey of India. A branch containing leaves and flowers was treated with mercuric chloride/methanol and pressed between sheets of paper for a duration of 15 days to achieve optimal drying. Once dried, the specimen was affixed to a herbarium sheet, and a detailed label was attached, specifying habitat, collection location, and flower type (Parvatiya & Anusandhan, 2021).

Both live and herbarium samples of *Murraya koenigii* were deposited in the Botany Department at Goa University for expert identification. A certificate of identification was obtained from the eminent botanist, Prof. Janardhanan, who serves as the Head of the Botany Department at Goa University, confirming the morphological and taxonomical features of the plant.

3.2.2 MOLECULAR IDENTIFICATION USING RBCL GENE SEQUENCING

a) DNA Isolation

The genomic DNA extraction was conducted according to the methodology developed by Nisha et al. (2018). A 1-gram leaf sample was pulverised in liquid nitrogen and then added to 10 mL of 2X CTAB buffer. The procedure, which included grinding with liquid nitrogen, successfully compromised the cell walls (Joshi & Ramesh, 2018). Genomic DNA was isolated via the Cetyl Trimethyl Ammonium Bromide (CTAB) technique described by Saghai Maroof et al. (1984).

The lysis solution consisted of 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB (w/v), and 10 mM β–mercaptoethanol (recently added). The material was homogenised and then centrifuged at 12,000 rpm for 10 minutes to remove cellular debris. Subsequently, the mixture was vortexed and incubated at 60°C for 30 minutes, after which it was treated with an equal amount of chloroform: isoamyl alcohol (24:1). The supernatant was obtained by centrifugation at 10,000 rpm for 15 minutes at ambient temperature, thereafter transferred to a sterile autoclaved centrifuge tube, and treated with 1/10 volume of 3M sodium acetate (pH 5.2) and 1/2 volume of 5M NaCl.

DNA was precipitated with 0.6 volume cold isopropanol and pelleted by centrifugation at 10,000 rpm for 10 minutes at 4°C. The supernatant was removed, and the DNA pellet was rinsed twice with 70% ethanol. Subsequent to air drying, the DNA was reconstituted in 500 µL of 1X TE buffer. Bovine Pancreatic RNAase (5 mg/mL) was included into the DNA solution to eliminate RNA contaminants and incubated at 37°C for one hour. A further extraction using chloroform: isoamyl alcohol (24:1) was conducted, and the aqueous phase was transferred to a fresh tube, where sodium acetate (3M) and cooled isopropanol were included for DNA precipitation at -20°C for one hour. The DNA pellet was air-dried and then reconstituted in 50 µL of sterile water (Rogers & Bendich, 1988).

DNA quantification was performed with a spectrophotometric technique with a UV-Vis

spectrophotometer. A 5 µL DNA sample was diluted in 3000 µL of a mildly alkaline buffer (10 mM Tris·Cl, pH 7.5) inside a cuvette. Absorbance was quantified at 260 nm and 280 nm, and DNA concentrations were determined using the formula:

$$\text{DNA concentration } (\mu g/mL) = \frac{50 \times OD_{260}}{1000}$$

An absorbance of 1 unit at 260 nm corresponds to 50 µg of genomic DNA per *mL*.

b) Polymerase Chain Reaction (PCR)

To identify the presence of the rbcL gene, a PCR-based approach was employed. Genomic DNA was extracted from the biological sample using the standard isolation protocol. Specific primers designed for conserved regions surrounding the rbcL gene were utilized in the PCR setup, which included a high-fidelity DNA polymerase (Mshiywa et al., 2023). The PCR cycling conditions were optimized for rbcL gene amplification, consisting of denaturation, annealing, and extension steps.

The resulting PCR products were analyzed by gel electrophoresis to confirm the presence and size of the amplified rbcL gene fragment under UV light. Purification of the PCR product and subsequent DNA sequencing were performed for enhanced accuracy in identifying the targeted gene.

The amplified product was sequenced using the ABI PRISM 3730 Genetic Analyzer (Applied Biosystems). The sequences of the rbcL primers used were:

- **rbcLa-F:** (5'-ATGTCACCACAAACAGAGACTAAAGC-3')
- **rbcLa-R:** (5′-GTAAAATCAAGTCCACCRCG-3')

PCR Steps

⇒ **DNA Denaturation:** *The double-stranded DNA is separated into single strands.*

⇒ **Annealing:** *Primers bind or hybridize to complementary sequences on the single-stranded DNA.*

⇒ **DNA Polymerase Elongation:** *The enzyme synthesizes a new strand of DNA using the existing strand as a template. This rapid replication process exponentially increases the number of DNA copies.*

⇒ **PCR Master Mix Preparation:** *The PCR reaction was performed in a 20 µL mixture containing 10 µL Takara mix, 1 µL of 10 µM forward and reverse primers, and 100-200 ng of template DNA (N. J. Shah, 2019).*

⇒ **Electrophoresis and Amplification:** *A sample of 5-10 µL was mixed with one-sixth volume of 6X loading buffer and analyzed on an agarose gel. The gels were stained with fluorescent dye for visualization under UV light.*

The final PCR conditions included: 1 cycle at 95 °C for 3 minutes, 40 cycles at 94 °C for 45 seconds, 55 °C for 45 seconds, and 72 °C for 1 minute, followed by 1 cycle at 72 °C for 7 minutes. The amplified rbcL PCR products, approximately 750 bp in size, were sent to Eurofins Genomics (Bangalore, India) for sequencing (Kumar et al., 2018).

c) Phylogenetic Analysis

The rbcL gene sequences were compared to sequences available in GenBank using the BLASTN program (Liebert et al., 2000) and aligned using CLUSTALW software (Thompson et al., 1994). Phylogenetic trees were constructed using the Maximum Parsimony method as described by Nei and Kumar (2000). All analyses were conducted using the MEGA11 package (Kumar et al., 2021).

3.3 DRY AND WET YIELD OF MURRAYA KOENIGII

The determination of the yield of *Murraya koenigii* involved measuring both the dry and wet weights of the samples. A precise weighing balance was employed to obtain accurate measurements of the plant material before and after the Soxhlet extraction process. The yield calculation was based on the following formula, adapted from Cojandaraj (2016):

$$\text{Yield } (\%) = \left(\frac{\text{Weight of Residue}}{\text{Initial Sample Weight}} \right) \times 100$$

This formula facilitates the comparison of the yield differences between the dry and wet samples following incubation.

3.4 EXTRACTION

The extraction of bioactive metabolites from *Murraya koenigii* was conducted using the Soxhlet extraction method, utilizing a range of solvents including distilled water, methanol, and chloroform. This process involved the sequential extraction of metabolites from non-polar to polar solvents, maximizing the extraction efficiency of the desired compounds.

Soxhlet Extraction Process

- ⇒ **Preparation of Plant Material**: *Approximately 25 grams of dried and ground Murraya koenigii leaves were placed in a porous bag or thimble made of strong filter paper, which was then positioned within the Soxhlet extractor.*
- ⇒ **Assembly Setup**: *The Soxhlet apparatus was assembled on a heating mantle. The appropriate extracting solvent was added to a round-bottom flask.*
- ⇒ **Heating the Solvent**: *The solvent was heated, allowing it to vaporize and subsequently condense in the condenser. The hot extracting solvent then dripped into the thimble containing the plant material, facilitating the extraction process.*
- ⇒ **Siphoning**: *Once the liquid level in the extraction chamber reached the top of the siphon tube, the contents were siphoned back into the round-bottom flask. This cycle allowed for continuous extraction of the phytoconstituents from the plant material.*
- ⇒ **Extraction Cycles**: *The extraction process was conducted for ten reflux cycles, ensuring thorough extraction of the bioactive compounds.*
- ⇒ **Concentration of Extracts**: *The organic solvents used in the extraction were concentrated to dryness under reduced pressure using a rotary evaporator. Specific temperature settings were maintained: 50°C to 55°C for methanol, 40°C to 45°C for chloroform, and 80°C to 87°C for the aqueous extract.*
- ⇒ **Storage of Extracts**: *The resulting extracts were air-dried and stored in a refrigerator for further analysis. Additionally, the isolates were desiccated to preserve their stability and were kept in a freezer for future applications, as suggested by Ganeshan et al. (2007) and Aspé & Fernández (2011). The final residues obtained from the extraction were retained for qualitative and quantitative analyses of their phytochemical constituents.*

This systematic approach ensures a comprehensive extraction of bioactive elements from *Murraya koenigii*, laying the groundwork for further investigation into its potential applications.

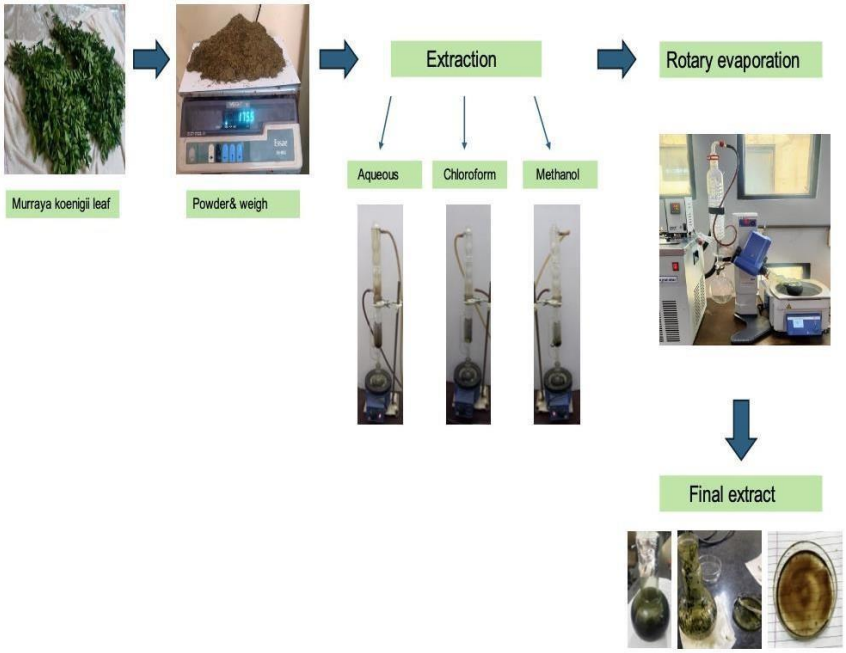

Figure 12 Technique of Drying, powdering, Soxhlet extraction and Rotary evaporation

3.5 PHYTOCHEMICAL SCREENING

3.5.1 QUALITATIVE TESTS

A comprehensive phytochemical analysis was conducted on the collected samples of *Murraya koenigii* to identify the presence of key bioactive compounds. The tests targeted major phytochemicals including alkaloids, flavonoids, anthraquinones, coumarins, glycosides, phenols, saponins, and carbohydrates, following methodologies outlined by Igara et al. (2016) and Phuyal et al. (2019).

a. Detection of Alkaloids

To detect alkaloids, a solution was prepared by mixing 0.355 g of corrosive sublimate with 5 g of potassium iodide in water. This solution was combined with the **Murraya koenigii** leaf extract

and 1% HCl. The resultant filtrate was treated with Mayer's reagent, which yielded a turbid or precipitated solution with a green hue, indicating the presence of alkaloids (Shaikh & Patil, 2020).

b. Detection of Anthraquinones

One gram of powdered **Murraya koenigii** leaves was dissolved in 20 mL of chloroform in a dry test tube and boiled in a steam bath for five minutes. After cooling and filtration, the percolate was mixed with an equal volume of 10% ammonia solution. The presence of anthraquinones was confirmed by shaking the mixture and observing a vibrant pink color in the upper aqueous layer. A reference was prepared using 10 mL of 10% ammonia solution mixed with 5 mL of chloroform (Deyab et al., 2016).

c. Determination of Flavonoids

To assess flavonoid presence, 0.5 g of each extract was subjected to boiling with distilled water and then filtered. To 2 mL of the filtrate, few drops of a 10% ferric chloride solution were introduced. A green-blue or violet hue indicates the presence of phenolic hydroxyl groups. (Y.-L. Cheng et al., 2016).

d. Detection of Coumarin

The addition of a few drops of alcoholic sodium hydroxide to 2 mL of the test solution resulted in a yellow color, confirming the presence of coumarin (Dias et al., 2020).

e. Test for Glycosides

A solution was prepared by amalgamating 2 mL of the extract with glacial acetic acid, a drop of ferric chloride, and concentrated sulphuric acid. The emergence of a reddish-brown pigment at the boundary of the two layers, along with a blue-green hue in the top layer, signified the existence of glycosides. (Visweswari et al., 2013).

f. Phenol Test

To evaluate phenolic chemicals, 1 mL of the extract was combined with 2 mL of deionised water, thereafter including 0.5 mL of sodium carbonate and 0.5 mL of Folin-Ciocalteau's reagent. A blue-green tint indicated the existence of phenols. (Samar et al., 2022).

g. Saponin Test

A sample of 0.5 g was mixed with 5 mL of distilled water in a test tube, warmed, and then agitated vigorously. The formation of stable foam was indicative of saponins (Y. Wang et al., 2022).

h. Steroids Test

To detect steroids, 2 mL of the extract was mixed with 2 mL of chloroform, followed by the careful addition of 2 mL of concentrated sulfuric acid. The development of a cherry color in the lower chloroform layer indicated the presence of steroids (R et al., 2017).

i. Tannins Test

Approximately 0.5 g of the extract was boiled in 20 mL of purified water and then filtered. To the filtrate, 1 mL of the extract was added along with 1 mL of 5% FeCl3. The emergence of a brownish-green coloration confirmed the presence of tannins (Dey & Ghosh, 2010).

j. Terpenoids Testing

To test for terpenoids, 1 mL of the extract was combined with 2 mL of chloroform and carefully layered with 1.5 mL of concentrated sulfuric acid. The appearance of a reddish-brown color at the interface indicated the presence of terpenoids (Xu et al., 2023).

3.5.2 QUANTITATIVE TESTS

The following biomolecules were quantified in the extracts using established quantitative methods:

a. Phenolic Estimation

The total phenolic content was quantified using the Folin-Ciocalteau colorimetric (FCR) technique as outlined by Singleton & Rossi (1965). Two hundred microlitres of Murraya koenigii extract were added to screw-cap test tubes, followed by the incorporation of 1.0 mL of Folin-Ciocalteau reagent (diluted 1:1 with water) and 1.0 mL of 7.5% sodium carbonate solution. The tubes were vigorously combined using a vortex mixer and incubated for two hours. Absorbance was quantified at 760 nm using a spectrophotometer (Beckman, USA). The total phenolic content was quantified in milligrams of Gallic acid equivalents (GAE) per gram of dried material, use a

standard calibration curve derived from several concentrations of Gallic acid solution prepared from a 1 mg/mL stock solution. (Tofighi et al., 2016).

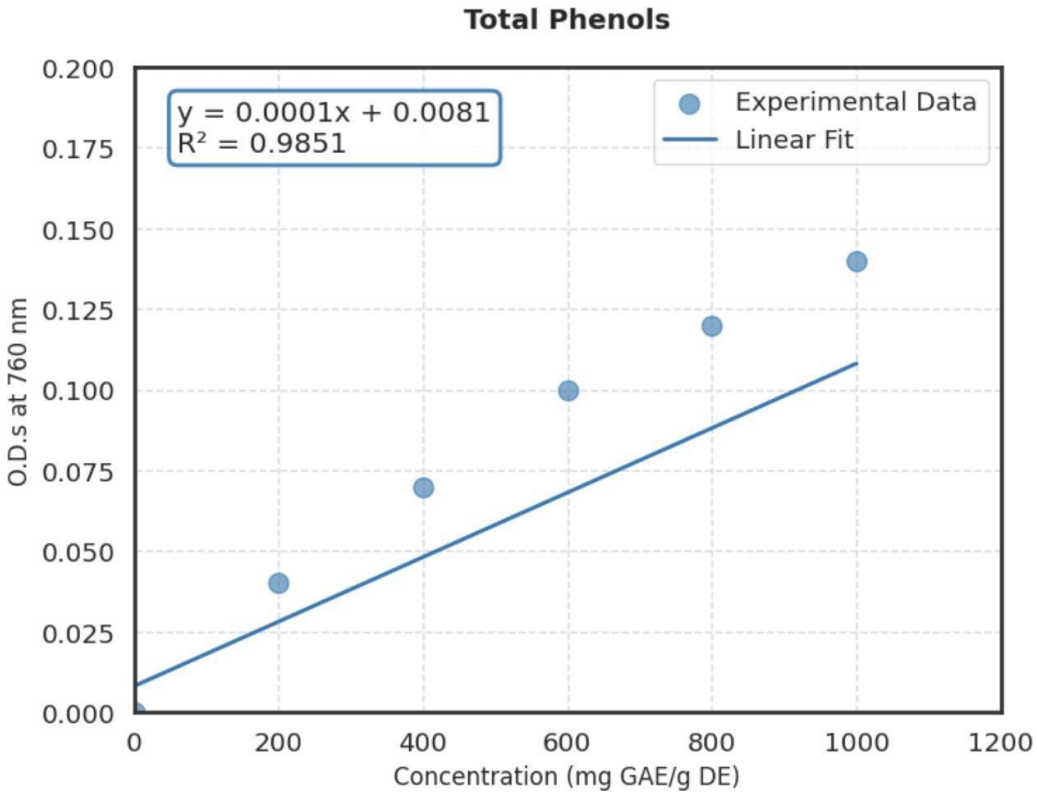

Figure 13 Standard Gallic acid curve for estimation of total phenols

$$\text{Total Phenolic Content} = \left(\frac{\text{Amount of Gallic Acid (mg)}}{\text{Mass of Sample (g)}} \right) \times \text{Volume of Extract (mL)}$$

b. Flavonoid Analysis

The assessment of total flavonoid content was conducted using the aluminum chloride colorimetric method, as described by Lin and Tang (2007). This method is well-established for quantifying flavonoids and was further supported by the methodology of Lamison and Carnet (1990).

In this analysis, aliquots of 1.5 mL of the sample extracts were mixed with an equal volume of a 2% aluminum chloride hexahydrate solution (2 g dissolved in 100 mL of methanol). The mixture

was then vigorously shaken to ensure thorough mixing, and after allowing it to incubate for 10 minutes, the absorbance was measured at a wavelength of 510 nm.

Quercetin was utilized as the reference standard, with a calibration curve established using concentrations ranging from 0.05 to 0.45 mg/mL. The total flavonoid content of the plant extracts was subsequently expressed in terms of milligrams of Quercetin equivalents (QE) per gram of dried extract, following the approach outlined by Saptarini and Herawati (2019). This method provides a reliable estimation of the flavonoid concentration, highlighting the potential bioactive properties of the Murraya koenigii extracts.

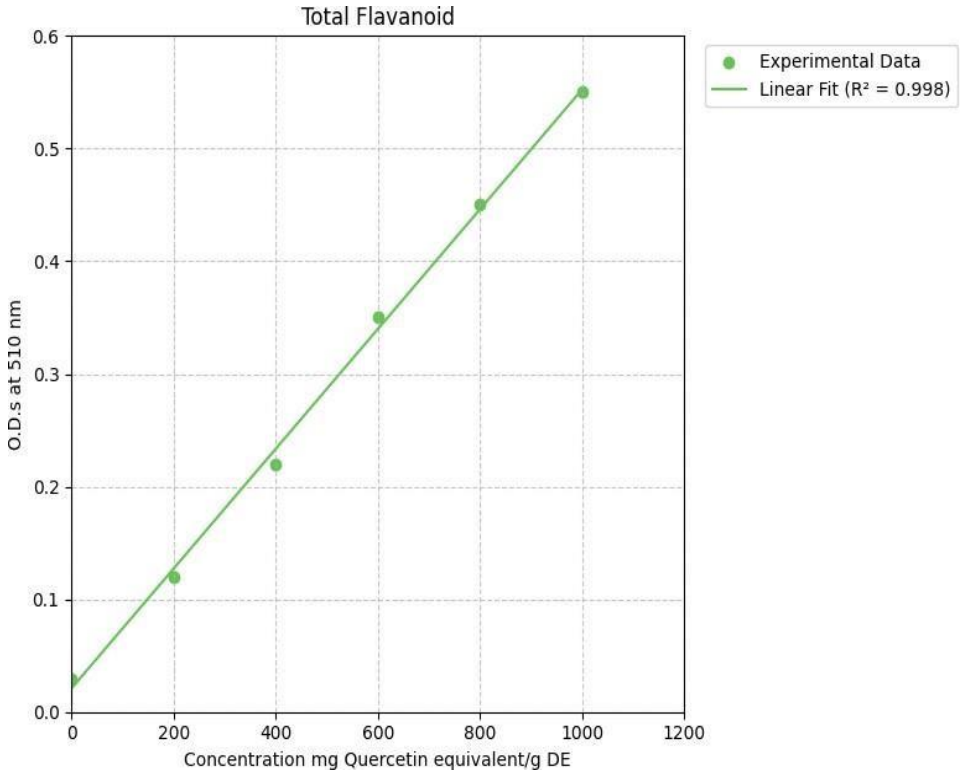

Figure 14 Standard Quercetin graph for estimation of Flavanoid content

$$\text{Total Flavonoid Content} = \frac{\text{Concentration of Quercetin} \times \text{Volume of Extract}}{\text{Mass of Extract}}$$

(c) Determination of Glycosides

The quantitative evaluation of cardiac glycosides in each plant extract was performed using a modified technique derived from Solich et al. (1992). Precisely, 100 µL of each extract was amalgamated with 1 mL of newly formulated Baljet's reagent, which comprises 95 mL of 1% picric acid and 5 mL of 10% NaOH. Following one hour of incubation, the mixture was diluted with 2 mL of distilled water, and the absorbance was assessed at 495 nm using a Shimadzu UV/VIS spectrophotometer (model UV 1800, Kyoto, Japan). A standard curve was established using digoxin values from 12.5 to 100 mg/L. The total glycoside concentration was quantified as milligrammes of digoxin per gramme of dried extract, as specified by Tofighi et al. (2016) and Solich et al. (1992).

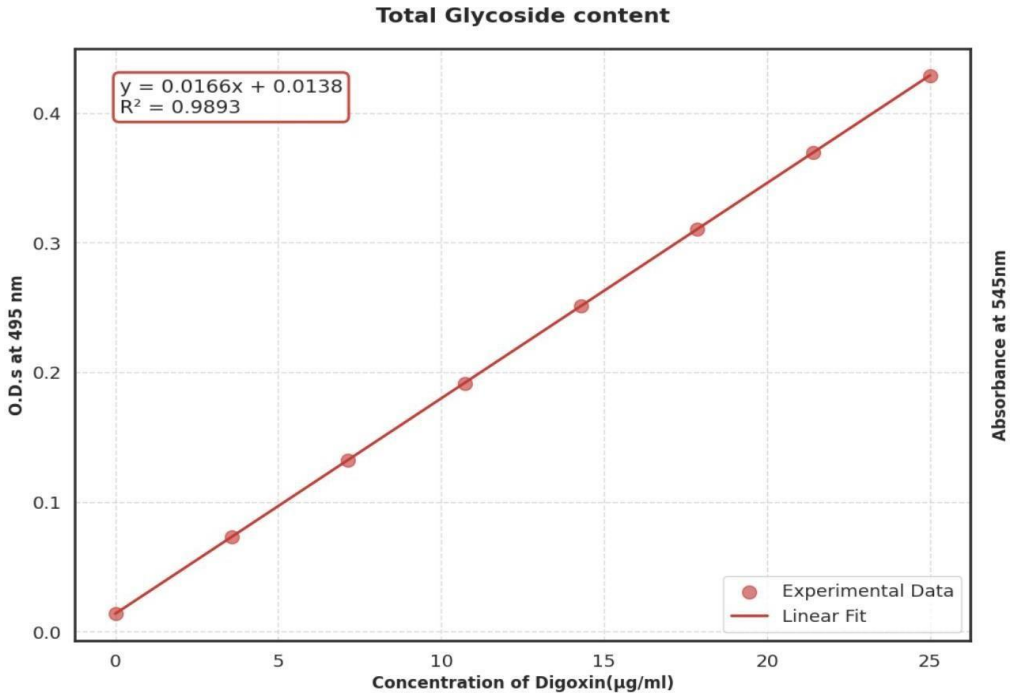

Figure 15 Standard Digoxin curve for glycoside estimation

(d) Saponins

For the saponin analysis, color development reagents were prepared as follows: Solution A comprised 50 mL of concentrated sulfuric acid and 50 mL of ethyl acetate, while Solution B

contained 0.5 mL of p-anisaldehyde and 99.5 mL of ethyl acetate. A test tube was filled with 2 mL of diluted saponin solution, followed by the addition of 1 mL each of Solutions A and B. The test tube was sealed with a glass stopper and mixed thoroughly, then allowed to develop color for 10 minutes in a water bath at 60 °C. After cooling for 10 minutes at room temperature, the absorbance of the developed solution was measured at 430 nm. Ethyl acetate was used as a control; a test tube containing 2 mL of ethyl acetate served as the reagent blank. Diosgenin dissolved in ethyl acetate was employed as the standard for saponin quantification (Prabhachandh & Babychan, 2017).

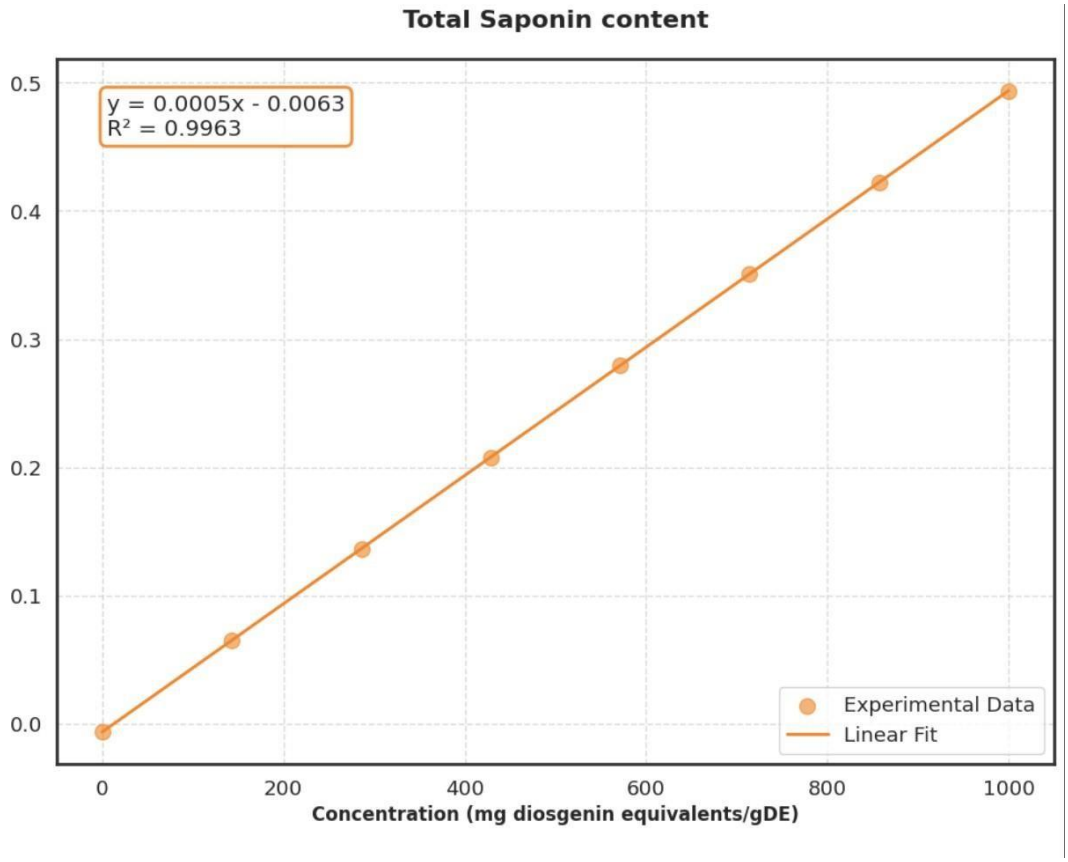

Figure 16 Standard Diosgenin curve for saponin estimation

$$\text{Saponins} = \frac{\text{Concentration of diosgenin} \times \text{Amount of saponins extracted}}{\text{Weight of crude powder}} \times 100$$

(e) Terpenoids

The total terpenoid content was assessed using the method outlined by Ghorai et al. (2012). In this process, 200 µL of dissolved methanol was added to a 2 mL microcentrifuge tube containing 1.5 mL of chloroform and 20 mg of Murraya extract. The mixture was vortexed thoroughly and allowed to rest for three minutes. Subsequently, 100 µL of concentrated sulfuric acid (H_2SO_4) was introduced into each tube, which were then incubated in the dark at room temperature (30 °C) for 1.5 to 2 hours. At the end of the incubation period, a reddish-brown coloration indicated the presence of terpenoids. The supernatant was carefully decanted without disturbing the precipitate, and 1.5 mL of 95% (v/v) methanol was added. The mixture was vortexed until the residue was fully dissolved, and the absorbance was measured at 545 nm, using 95% (v/v) methanol as the blank (Ghorai et al., 2012).

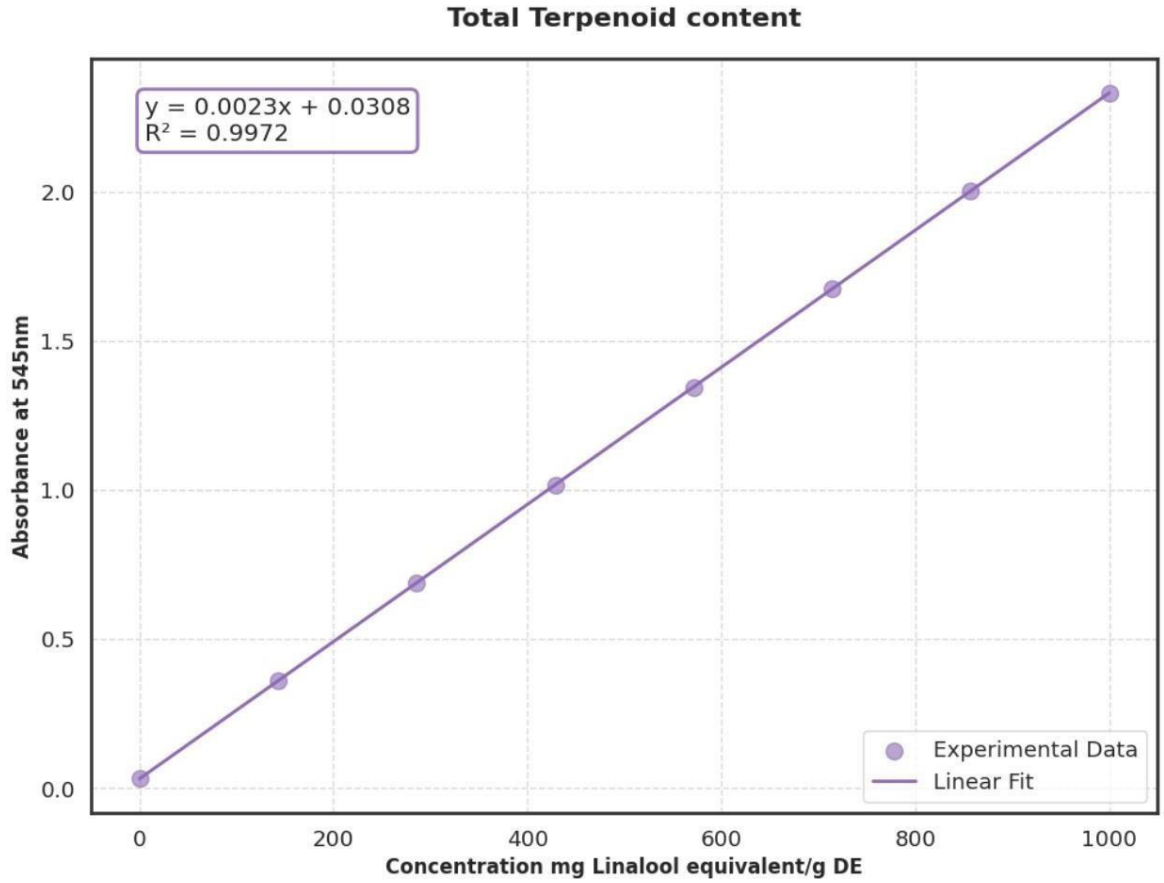

Figure 17 Total terpenoid content derived from standard curve of Linalool

$$\text{Terpenoids} = \frac{\text{Concentration of linalool} \times \text{Amount of terpenoids extracted}}{\text{Weight of extract}} \times 100$$

(f) Total Tannin Content

The tannin concentration of the plant extract was measured with the methodology outlined by Siddhuraj and Manian (2007). Five hundred microlitres of the extract were amalgamated with one hundred milligrammes of polyvinyl polypyrrolidone and five hundred microlitres of distilled water in individual test tubes. The mixes were incubated at 4 °C for four hours. Following centrifugation at 5,000 rpm for 5 minutes, 20 µL of the supernatant was obtained. The supernatant included just simple phenolics, devoid of tannins, which had precipitated with the polyvinyl polypyrrolidone. The phenolic concentration of the supernatant was quantified at 725 nm, reported as gallic acid equivalents (GAE) on a dry matter basis. The tannin content was determined via the formula:

$$\text{Tannins} = \text{Total Phenol} - \text{Free Phenol}$$

3.6 ANTIOXIDATIVE ACTIVITY

3.6.1 SCAVENGING CAPACITY OF DPPH RADICAL

The free radical scavenging activity of Murraya koenigii was assessed in vitro using the 1,1 - diphenyl-2-picryl hydrazyl (DPPH) technique, as outlined by Von Gadow et al. (1997). A 2 mL aliquot of a 6×10^{-5} M methanolic DPPH solution was combined with 50 µL of a 20 mg/mL methanolic sample solution. Absorbance measurements started promptly, with a continuous recording of the decline in absorbance at 515 nm for a duration of 16 minutes at ambient temperature. A methanolic solution of pure quercetin at a concentration of 1 mg/mL served as a control. The DPPH radical scavenging activity % was determined using the method outlined by Yen and Duh (1994):

$$\text{IP} = \text{Abs (control)} - \text{Abs (test)} / \text{Abs}(control) \times 100$$

3.6.2 ABTS SCAVENGING ACTIVITY

The sample's antioxidant capacity was evaluated using the ABTS radical cation decolorisation technique, as described by Zheleva-Dimitrova et al. (2010) and Miller & Rice-Evans (1996). A 7

mM ABTS solution in water was made, and ABTS*+ was produced by combining it with 2.45 mM potassium persulfate, allowing the combination to rest in the dark for 12-16 hours. The ABTS*+ solution was diluted with 100% ethanol to get an absorbance of 0.7 (±0.02) at 734 nm, thereafter equilibrated at 30 °C. A combination of 50 µL of a seaweed extract (20 mg/mL) and 2 mL of the diluted ABTS*+ solution was formulated, and the absorbance was recorded at 734 nm precisely 6 minutes after mixing. Solvent blanks were used in each test, and measurements were performed in triplicate using concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/mL for enhanced reliability (Zheleva-Dimitrova et al., 2010).

$$\text{IP} = \text{Abs (control)} - \text{Abs (test)} / \text{Abs}(control) \times 100$$

3.6.3 FRAP ASSAY

The Ferric Reducing Antioxidant Power (FRAP) assay was performed according to Benzie and Strain (1996) with modifications. Stock solutions included a 300 mM acetate buffer (3.1 g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_4O_2$, pH 3.6), a 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, and a 20 mM $FeCl_3 \cdot 6H_2O$ solution. A fresh working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ solution, and 2.5 mL of $FeCl_3 \cdot 6H_2O$ solution, then warmed to 37 °C before use. Leaf extracts (150 µL) were reacted with 2850 µL of FRAP solution in the dark for 30 minutes. The absorbance of the colored product, a ferrous tripyridyltriazine complex, was measured at 593 nm. The experiment included concentrations of 0.2, 0.4, 0.6, 0.8, and 1 mg/mL for reliable determinations (Benzie & Strain, 1999).

$$\text{IP} = \text{Abs (control)} - \text{Abs (test)} / \text{Abs}(control) \times 100$$

3.6.4 LIPID PEROXIDATION

Lipid peroxidation, triggered by the $FeSO_4$-ascorbate system in sheep liver homogenate, was quantified using the thiobarbituric acid reactive substances (TBARS) technique, as delineated by Ohkawa et al. (1979). The reaction mixture included 0.1 mL of 25% sheep liver homogenate in Tris-HCl buffer (20 mM, pH 7.0; KCl 30 mM; $FeSO_4$ $(NH_4)_2SO_4 \cdot 7H_2O$ 0.06 mM) and differing amounts of synthesised peptides, resulting in a final volume of 0.5 mL. The mixture was incubated

at 37 °C for one hour. After incubation, 0.4 mL was extracted and subjected to sodium dodecyl sulphate (8.1%), thiobarbituric acid (0.8%), and trichloroacetic acid (20%). The mixture was subjected to heating, cooling, and centrifugation, and the absorbance of the butanol-pyridine layer was measured at 532 nm (Ningappa et al., 2008; Ohkawa et al., 1979). The concentrations evaluated were 0.2, 0.4, 0.6, 0.8, and 1 mg/mL.

$$IP = Abs\ (control) - Abs\ (test) / Abs\ (control) \times 100$$

3.7 IN-VITRO ANTIHYPERLIPIDEMIC ASSAY

3.7.1 PANCREATIC LIPASE INHIBITORY ACTIVITY

The inhibitory activity of pancreatic lipase was assessed by quantifying the hydrolysis of p-nitrophenyl butyrate (p-NPB) to p-nitrophenol, following a method established in prior research (Jeong et al., 2014). An enzyme solution of 0.1 mg/ml was prepared by reconstituting porcine pancreatic lipase with 0.1 M Tris-HCl buffer at pH 8. Subsequently, 5 µl of the test sample was combined with 90 µl of enzyme buffer and incubated for 15 minutes at 37°C. Following the incubation period, 5 µl of 10 mM p-nitrophenylbutyrate (p-NPB) was introduced to the enzyme mixture, and the reaction was permitted to continue for an additional 15 minutes at 37°C. Following the incubation period, the absorbance was assessed at 405 nm utilising a UV-Vis spectrophotometer. The absorbance values recorded at 405 nm offer insights into how Murraya koenigii samples inhibit pancreatic lipase activity, which is essential for fat breakdown, thereby contributing important information for evaluating their potential anti-hyperlipidemic properties. The absorbance was compared with controls and a blank that consisted of buffer instead of the leaf sample. The concentrations of the sample will be set at 0.2, 0.4, 0.6, 0.8, and 1 mg/ml. (Rajan et al., 2014; Rani et al., 2019)

$$PI = \frac{(Abs.\ of\ control\ with\ \text{porcine lipase} - Abs.\ of\ test)}{(Abs\ of control\ with\ \text{lipase} - Abs\ of\ control\ without\ \text{lipase}) \times 100}$$

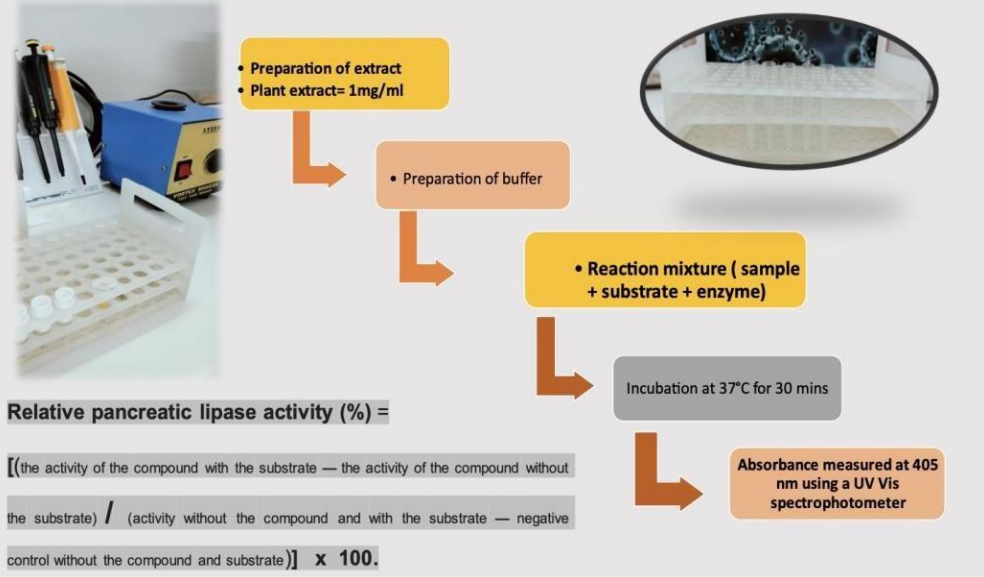

Figure 18 Procedure of Pancreatic lipase inhibition assay

3.8 ANIMAL EXPERIMENTATION

3.8.1 PROCUREMENT OF ANIMALS

Wistar rats weighing between 220-350 g were selected for this study. The animals were procured from Lacsmi Biofarms, Pune, and housed in husk-lined polypropylene cages. They were maintained under controlled environmental conditions: a 12-hour light/dark cycle, temperature of 25 ± 2°C, and relative humidity of 55 ± 10% (Figure 19-20). The rats were provided a standard pellet diet along with free access to water. The study received approval from the Institutional Animal Ethics Committee (IAEC) of Goa University, Taleigao Plateau, Goa, India (Approval No: GUZ/IAEC/23-24/N1, Project Code No. IAEC/23-24/N1).

3.8.2 ACUTE TOXICITY STUDIES

The acute oral toxicity evaluation was performed on nine female Wistar rats. Testing adhered to the Organisation for Economic Cooperation and Development (OECD) norms, particularly OECD Guideline 423 for acute oral toxicity. The body weight of each rat was individually recorded before to the experiment to ascertain suitable dosage, with quantities modified according to 10 mL/kg of body weight. Throughout the trial, the rats were given standard pellet meal and had unrestricted access to reverse osmosis (RO) water. The subjects were acclimatised to laboratory settings for seven days before to the study's initiation and were categorised into three groups for acute toxicity assessment and five groups for sub-acute evaluation. During the experiment, the rats were kept in an environment at 24°C with a 12-hour light/dark cycle.

Figure 19 Rats housed in Polypropylene cages

Figure 20 Animal house, Goa university

An acute oral toxicity study was conducted following the OECD test guideline 423 - Acute toxic class method (OECD guideline, 2002). Three groups of three young, healthy adult female Wistar rats, weighing between 240 and 350 grammes, were established for the study. The chloroformic extracts of *Murraya Koenigi* were administered orally in a single dose, reaching a maximum of 2000 mg/kg body weight. Body weight was measured prior to dosing and subsequently recorded weekly until the experiment concluded. The body weights of rats from each group were recorded using a weighing balance (Fig 21) prior to and throughout the 28-day treatment period on the 0th, 7th, 14th, and 28th days. Lethality and unusual clinical signs were recorded on the day of dosing

every 4 hours and continued to be monitored for 13 days thereafter. A blood sample will be collected for biochemical and routine analyses. At the conclusion of the experiment, significant pathological alterations were noted through the surgical excision of the liver and kidney, which will be weighed (Fig 22) and subsequently sent for histopathological analysis.

The calculation of relative organ weight (ROW) for each animal will be performed using the formula ROW(%) = (Organ weight/body weight) × 100.The liver and kidney fragments were fixed in a 10% formalin solution, subsequently embedded in paraffin wax, sectioned, and stained with haematoxylin and eosin for visualisation under an optical microscope. Images were captured using a camera microscope.

Figure 22 Rat weight

Figure 21 Weight of liver

3.8.2.2 HEMATOLOGY AND BIOCHEMISTRY INVESTIGATIONS

At the end of each trial (on the 15th day for acute oral toxicity studies), the rats were anesthetized using a mixture of Alcohol, Chloroform, and Ether (ACE) in a 1:2:3 ratio. Blood samples (4 mL) were collected via cardiac puncture using disposable syringes. Blood was stored in K2EDTA tubes for hematological analysis, including hemoglobin (HGB), white blood cell count (WBC), and differential counts (neutrophils, lymphocytes, monocytes, eosinophils, and basophils). Biochemical parameters (urea, creatinine, total protein, albumin, ALP, ALT, and AST) were assessed using blood stored in plain tubes. Blood samples in plain tubes were left at room temperature for 15-20 minutes to allow clotting, then centrifuged at 5000 rpm for 20 minutes to obtain serum. Following blood collection, the rats were sacrificed by cervical dislocation.

3.8.2.3 HISTOPATHOLOGICAL OBSERVATIONS

After blood collection, the rats were sacrificed, and essential organs (liver and kidneys) were excised via a midline incision. The organs were cleaned of surrounding fat, blotted dry, and weighed to calculate the relative organ weight (ROW) using the formula:

$$\text{ROW} = \frac{\text{Absolute organ weight}}{\text{Body weight at sacrifice}} \times 100$$

Histopathological examination was conducted on the liver and kidneys. Samples were preserved in 10% buffered formalin, regularly processed, and embedded in paraffin wax. Thin slices (5 µm) were prepared, stained with haematoxylin and eosin, and analysed under a light microscope by a pathologist unaware of the research groups.

3.8.3 EFFECT OF *MURRAYA KOENIGII* ON CLOZAPINE-INDUCED ADVERSE EFFECTS

3.8.3.1 EXPERIMENTAL DESIGN

Clozapine was administered orally at a dose of 10 mg/kg BW for 14 days to induce lipid abnormalities, weight gain, and behavioral changes. Different doses of chloroformic extracts of *Murraya koenigii* (MK) were administered orally 1 hour after clozapine treatment. The extracts and standard drug were suspended in distilled water. The groups were as follows:

- **Group I**: Control (10 mL/kg normal saline)
- **Group II**: Clozapine (10 mg/kg BW)
- **Group III**: Clozapine (10 mg/kg BW) + Statin (5 mg/kg BW after 1 hour)
- **Group IV**: Clozapine (10 mg/kg BW) + MK (100 mg/kg BW after 1 hour)
- **Group V**: Clozapine (10 mg/kg BW) + MK (200 mg/kg BW after 1 hour)
- **Group VI**: Clozapine (10 mg/kg BW) + MK (400 mg/kg BW after 1 hour)
- **Group VII**: Clozapine (10 mg/kg BW) + MK (800 mg/kg BW after 1 hour)

3.8.3.2 BLOOD INVESTIGATIONS

Baseline fasting blood samples were collected from all rats before the experiment began. Additional blood samples were collected weekly via cardiac puncture over the 28-day treatment period. Glucose, renal function tests (RFT), liver function tests (LFT), and lipid profiles were assessed at 0, 14, and 28 days to monitor the effects of treatments.

Figure 23 Blood collection by cardiac puncture method

3.8.3.3 BODY WEIGHT ANALYSIS

The body weight of each rat was measured using a precision balance on days 1, 7, 14, and 21 to monitor changes throughout the study (Ityodugh et al., 2019).

3.8.3.4 BLOOD CHEMISTRY ANALYSIS

On days 0, 14, and 28, blood samples were collected via cardiac puncture(Fig 23) under mild ether anesthesia to assess various biochemical parameters. Blood glucose, total cholesterol,

triglycerides, HDL cholesterol, SGOT, SGPT, ALP, GGT, CPK, creatinine, urea, total protein, and albumin levels were measured using commercial kits according to manufacturer protocols. Analysis was conducted with a fully automated analyzer (CORALYSER MINI, manufactured by Tulip Diagnostics), as shown in Fig. 24.

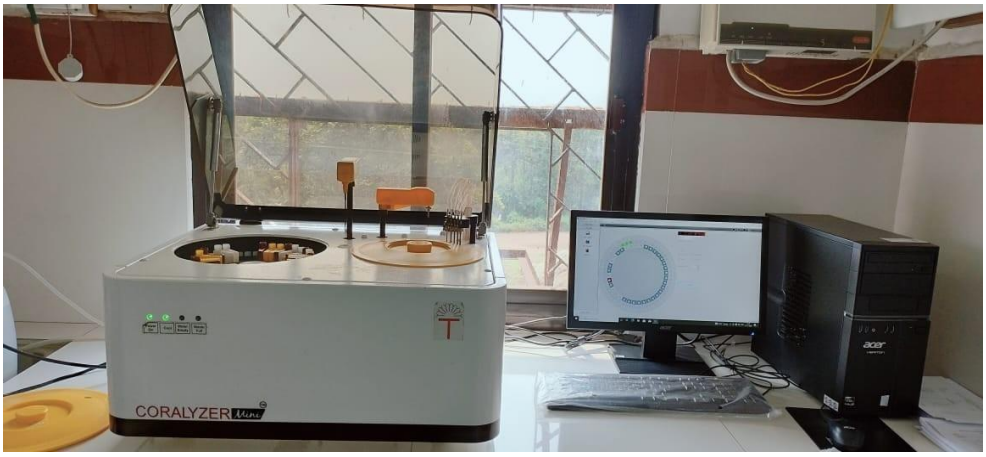

Figure 24 Fully automated biochemistry analyser

3.8.3.4.1 BIOCHEMICAL ESTIMATIONS

Estimation of Glucose

To determine glucose levels, 10 µl of serum was mixed with 1 ml of glucose working reagent (phosphate buffer, pH 7.0 – 170 mmol/l, glucose oxidase – 15000 IU/I, peroxidase – 1500 IU/I, 4-aminoantipyrine – 0.28 mmol/l, phenol – 16 mmol/l) and incubated for 7 minutes at 37°C. Glucose oxidase (GOD) catalyzes the conversion of glucose to gluconic acid, producing hydrogen peroxide, which reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to form a red quinoneimine dye. Absorbance was measured at 505 nm.

$$\text{Glucose (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \right) \times 100$$

Estimation of Cholesterol

For cholesterol estimation, 10 µl of serum was mixed with 1 ml of working reagent (buffer, pH 7.5 – 100 mmol/l, cholesterol oxidase ≥ 100 IU/l, cholesterol esterase ≥ 150 IU/l, peroxidase ≥ 500 IU/l, 4-aminoantipyrine – 0.5 mmol/l, phenol ≥ 10 mmol/l) and incubated for 5 minutes at 37°C. Cholesterol esterase catalyses the hydrolysis of cholesterol esters to liberate free cholesterol, which is then oxidised to cholest-4-en-3-one by cholesterol oxidase. In the presence of peroxidase, hydrogen peroxide reacts with 4-aminoantipyrine and phenol to produce a red dye, with absorbance measured at 510 nm.

$$\text{Total Cholesterol (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}\right) \times 200$$

Estimation of Triglycerides

To estimate triglycerides, 10 µl of serum was combined with 1 ml of working reagent (buffer, pH 7.2 – 50 mmol/l, lipase ≥ 2000 IU/l, glycerol kinase ≥ 300 IU/l, glycerol phosphate oxidase ≥ 1000 IU/l, peroxidase ≥ 500 IU/l, ATP – 1 mmol/l, chromogen - 2 mmol/l) and incubated for 10 minutes at 37°C. Triglycerides are hydrolysed by lipase to yield glycerol, which is then phosphorylated by glycerol kinase and oxidised to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidises the chromogen to a red compound, which is quantified at 510 nm.

$$\text{Total Triglyceride (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}\right) \times 200$$

Estimation of HDL Cholesterol

HDL cholesterol was determined by precipitating VLDL and LDL with polyethylene glycol. The supernatant containing HDL was assayed using the CHOD/PAP method.

$$\text{HDL Cholesterol (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}\right) \times 25 \times 2$$

Step 1: Preparation of Supernatant

Reagent	Volume (ml)
Precipitating Reagent (L3)	0.1

Sample	0.1

- **Procedure**: Mix the reagents well and incubate at room temperature for 5 minutes. Then, centrifuge at 2500-3000 rpm to obtain a clear supernatant.

Step 2: Cholesterol Assay

Addition Sequence	Blank (ml)	Standard (ml)	Test (ml)
Working Reagent (L1 + L2)	1.0	1.0	1.0
Distilled Water	0.05	-	-
HDL Standard	-	0.05	-
Supernatant	-	-	0.05

- **Procedure**: Mix the contents well and incubate at 37°C for 15 minutes. Measure the absorbance of the Standard and Test samples against the Blank within 60 minutes.

Determination of LDL and VLDL Cholesterol

LDL and VLDL cholesterol levels were calculated using Friedewald's formula (1972).

$$\text{LDL Cholesterol} = \text{Total Cholesterol} - (\text{HDL} + \text{VLDL})$$

$$\text{VLDL Cholesterol} = \frac{\text{Triglycerides}}{5}$$

Estimation of Urea

Urea concentration was measured by the urease-GLDH method, with absorbance read at 340 nm.

Addition Sequence for Urea Assay

Addition Sequence	Standard (S) (ml)	Test (T) (ml)
Working Reagent	1.0	1.0
Urea Standard / Test Sample	0.01	0.01

- **Note**: Pipette the specified volumes into clean, dry test tubes labeled for Standard (S) and Test (T) as indicated.

$$\text{Urea (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \right) \times 40$$

Estimation of Creatinine

In an alkaline media, picric acid interacts with creatinine to produce an orange-hued complex with alkaline picrate. The colour intensity produced at a particular duration is exactly proportional to the concentration of creatinine in the sample analysed at

520nm.

Pipette out into a clean test tube labelled standard or test

Addition sequence	(S) / (T)
Picric Acid reagent(L1)	0.5ml
Buffer reagent (L2)	0.5ml
Creatinine Standard / Sample	0.1ml

Mix well and read the absorbance for standard and test after exactly 30 seconds.

$$\text{Creatinine (mg/dl)} = \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of standard}}\right) \times 2$$

Estimation of SGOT

To 100 µl of the serum, 1 ml of SGOT working reagent (tris buffer, pH 7.7 – 80 Mm, L-aspartate – 200 Mm, NADH – 0.15 Mm, LDH ≥ 200 IU/I, α- ketoglutarate -12 mM, MDH ≥ 300 IU/I) was added and mixed thoroughly. The assay mixture was immediately transferredto the thermostat cuvette and stop watch was started simultaneously. The first reading was recorded at 60^{th} sec and subsequently three more readings with 30 sec interval. The conversion of NADH to NAD during the conversion of oxaloacetate to L- malate is proportional to the concentration of SGOT in serum and is measured at 340 nm.

$$\text{Activity of SGOT (IU/l)} = \triangle \text{ Absorbance/ min} \times 1749$$

Estimation of SGPT

To 100 µl of the serum, 1 ml of SGPT working reagent (tris buffer, pH 7.7 – 80 Mm, L-alanine –

200 Mm, NADH – 0.15 Mm, LDH ≥ 200 IU/I, α- ketoglutarate -12 mM, MDH ≥ 300 IU/I) was added and mixed thoroughly. The assay mixture was immediately transferred to the thermostated cuvette and stop watch was started simultaneously. The first reading was recorded at 60^{th} sec and subsequently three more readings with 30 sec interval. The conversion of NADH to NAD during the conversion of pyruvate to L-malate is proportional to the concentration of SGPT in serum and is measured at 340 nm. The calculation was doneusing the below formula.

$$\text{Activity of SGPT (IU/l)} = \triangle \quad \text{Absorbance/ min} \times 1749$$

Estimation of ALP

To 20 ml of the serum, 1 ml of ALP working reagent (containing diethanolamine buffer,pH 9.8 – 1 mol/l, p-ntrophenyl phosphate – 10mmol/l, magnesium chloride – 0.5 mmol/l) was added and mixed thoroughly. The assay mixture was then transferred immediately to the thermostated cuvette and stop watch was recorded at 60^{th} sec and subsequently, three more readings with 30 sec interval. ALP eleaves p –nitrophenyl phosphate into p-nitrophenyl phosphate into p-nitrophenol and phosphate p-nitrophenol is a yellow colour compound in alkaline medium and hence the absorbance is measured at 405nm which is directly proportional to the ALP activity.

$$\text{ALP (IU/l)} = \text{Absorbance/min} \times 2720$$

3.8.3.4.2 ESTIMATION OF CREATINE KINASE (CK)

Creatine kinase was measured kinetically, monitoring the increase in absorbance due to NADPH production.

Creatine Kinase (CK) is primarily found in muscle and brain tissues. Elevated CK levels can indicate muscle dystrophy, myocardial infarction (MI), cardiovascular disease (CVD), or electrical shocks. Other causes of increased CK include intramuscular injections, strenuous exercise, or surgery.

Assay Method:

The CK activity is quantified with the UV Kinetic technique. CK facilitates the reaction between creatine phosphate and ADP, yielding creatine and ATP. The ATP produced is used with glucose and catalysed by hexokinase to generate glucose-6-phosphate. This chemical converts NADP to NADPH in the presence of glucose-6-phosphate dehydrogenase. The rate of NADP reduction to NADPH is quantified by a rise in absorbance, which is directly proportional to CK activity in the sample.

Reference Range:

- 24–195 U/L

Reagents

- **L1 (Enzyme Reagent):** Imidazole buffer (pH 6.1), glucose (25 mmol/L), magnesium acetate (12.5 mmol/L), EDTA (2 mmol/L), N-acetyl cysteine (25 mmol/L), NADP (2.4 mmol/L), hexokinase (>6.8 U/mL).
- **L2 (Starter Reagent):** ADP (15.2 mmol/L), D-Glucose-6-phosphate dehydrogenase (>8.8 U/mL), creatine phosphate (250 mmol/L), AMP (25 mmol/L), diadenosine pentaphosphate (103 µmol/L).

Working Reagent Preparation:

Mix 4 parts of L1 and 1 part of L2. This mixture is stable for 10 days if stored at 2 –8°C. Alternatively, 0.8 mL of L1 and 0.2 mL of L2 can be used to prepare 1 mL of working reagent per assay.

Note: Ensure the working reagent's absorbance is below 0.800 against distilled water at 340 nm.

CK Assay Procedure

1. **Label test tubes:** Prepare separate tubes for Standard (S) and Test (T).
2. **Incubate** at 37°C before beginning the assay.

Addition Sequence	**Standard (S) / Test (T)**
Working Reagent (L1 + L2)	1.0 mL
Incubate (37°C)	**1 min**
Sample	0.02 mL

- **Measure:** After the addition of the sample, proceed to measure absorbance at 340 nm to determine CK activity.

Mix well and read the absorbance for test immediately at 340nm. Note the initial absorbance A, after 1 min & repeat the absorbance reading after every 1,2 &3 mins. calculate the mean absorbance change per min

Activity of CK(U/l) = $\triangle$ Absorbance/ min × 8095

3.8.3.5 HEMATOLOGICAL ANALYSIS

Blood samples were withdrawn by cardiac puncture under mild ether anesthesia on the 0^{th}, 14^{th}, and 28^{th} day of the study for estimating blood parameters like Hemoglobin, total WBC count, Differential count (*Neutrophils, Lymphocytes, Basophils, Eosinophils, Monocytes*), RBC count, and Platelets as per standard protocols in a automated cell counter.(Fig 25)

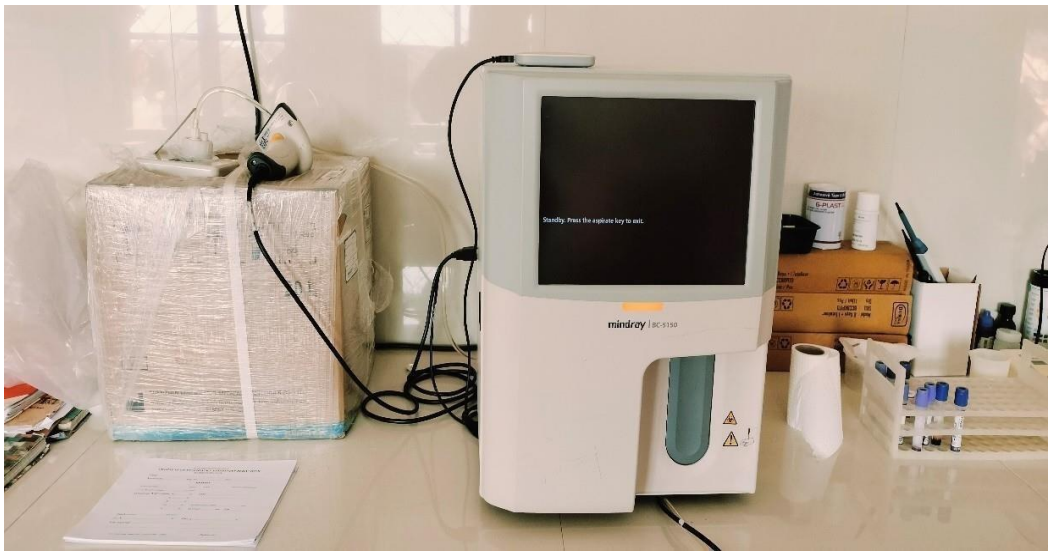

Figure 25 Automated cell counter for estimating Blood hematology parameters (MINDRAY)

3.8.3.6 HISTOPATHOLOGICAL INVESTIGATIONS

Following Wadaan's (2009) protocol, the animals were humanely sacrificed on the twenty-first day using cervical dislocation. At the study's conclusion, all rats were euthanized, dissected (Fig 26), and organs including the liver, adipose tissue, kidneys, and brain were collected. These organs were then rinsed in normal saline and fixed in 10% formalin (Fig 27a).

3.8.3.6.1 Tissue Processing Procedure:

Tissue samples underwent dehydration, clearing, and paraffin infiltration. Ethyl alcohol served as the primary dehydrating agent, although acetone and isopropyl alcohol could also be used. After dehydration, tissues were transferred to a paraffin solvent and embedded in paraffin to create tissue blocks (Fig 27b). Sections were then cut to 6 µm thickness using a Spencer-type rotating microtome.

3.8.3.6.2 Staining Process:

Sections were affixed to slides and stained by sequential immersion in the following reagents:

1. Xylol (2 changes) – 3 minutes each

2. Acetone – 3 minutes
3. Alcohol 95% – 3 minutes
4. Hematoxylin stain – 20 minutes
5. Running water – 20 minutes
6. Eosin stain – 5 minutes
7. Alcohol 95% (3 changes) – 3 minutes each
8. Acetone (2 changes) – 3 minutes each
9. Xylol (2 changes) – 3 minutes each

After staining, slides were mounted with DPX (Diphenyl Phthalate Xylene) and cover slips were placed carefully to avoid air bubbles (Kumar et al., 2022).

3.8.3.6.3 Microscopy and Preservation:

The stained sections were examined under a light microscope (Fig 28). Additionally, a portion of the brain tissue was preserved for homogenization by deep freezing at -20°C, stored in foil packets (Noeman et al., 2011).

Figure 26 Dissection of rat

a: Organ stored in Formalin b : Tissue embedded in paraffin wax

Figure 27 Preparation of Organs for histopathological examination

Figure 28 Microscope with camera for histopathological examination

3.8.3.7 ANTIOXIDANT STATUS OF BRAIN

The whole brain was excised, incubated on cooled ice for purification, weighed, and thereafter stored at -20°C until analysis. A rat brain tissue homogenate was produced at a ratio of 1 g of wet

tissue to 10 volumes (w/v) of 0.05 M ice-cold phosphate buffer (pH 7.4) and homogenised using a Teflon homogeniser.Kumari et al. (2016) Tissue homogenates were subjected to centrifugation at 15,000 ×g at 4°C for 30 minutes, after which the supernatant was filtered and preserved at −20°C until analysis (Khan et al., 2012). 0.2 mL of homogenate was used for the determination of TBARS. The residual homogenate was centrifuged at 15,000 rpm at 4°C for 60 minutes, and the supernatants were used for SOD estimation. (Chitra et al., 2017)

CATALASE (CAT) ESTIMATION

CAT activity was assessed using a reaction solution comprising 2.5 ml of 50 mmol phosphate buffer (pH 5.0), 0.4 ml of 5.9 mmol H2O2, and 0.1 ml of tissue homogenate. The absorbance changes of the reaction solution at 240 nm were measured after one minute. Khan et al. (2012) One unit of catalase activity is defined as an absorbance change of 0.01 units per minute per milligramme of protein.

$$CAT = \{[2.3 \times log \frac{OD\ WWnWWtWWal}{OD\ ooWWnal}/\Delta t*100]/0.693\}\ /mg\ of\ protein$$

GLUTATHIONE PEROXIDASE ASSAY

The activity of glutathione peroxidase was evaluated using the technique established by Mohandas et al. (1984). The reaction mixture included 1.49 ml of phosphate buffer (0.1 mol; pH 7.4), 0.1 ml of EDTA (1 mmol), 0.1 ml of sodium azide (1 mmol), 0.05 ml of glutathione reductase (1 IU/ml), 0.05 ml of GSH (1 mmol), 0.1 ml of NADPH (0.2 mmol), 0.01 ml of H2O2 (0.25 mmol), and 0.1 ml of homogenate, resulting in a total volume of 2 ml. The depletion of NADPH at 340 nm was documented at 25°C. Enzyme activity was determined as nmol NADPH oxidised per minute per milligramme of protein, using a molar extinction coefficient of $6.22 \times 10^3\ M^{-1}\ cm^{-1}$.(Khan et al., 2012; Goyal, 2011)

SUPEROXIDE DISMUTASE ASSAY

The assessment of SOD was conducted by the suppression of the production of the NADH-phenazine methosulphate-nitroblue tetrazolium formazan complex. The estimation of SOD activity was conducted using the technique described by Kakar et al. The reaction mixture comprised: 0.1 ml of phenazine methosulphate (186 µmol), 1.2 ml of sodium pyrophosphate buffer (0.052 mmol; pH 7.0), and 0.3 ml of the supernatant obtained from centrifugation (1500 × g for 10 minutes followed by 10,000 × g for 15 minutes) of the homogenate. The enzymatic reaction commenced with the addition of 0.2 ml of NADH (780 µmol) and was terminated after 1 minute by the addition of 1 ml of glacial acetic acid. The quantity of chromogen produced was quantified by assessing colour intensity at 560 nm. Results are presented in units per milligramme of protein.Khan et al. (2012)

DETERMINATION OF MALONDIALDEHYDE (MDA) CONTENT

The TBARS test is used to quantify the concentration of malondialdehyde (MDA), providing an estimate of lipid peroxidation levels. The assay for lipid peroxidation was performed following a modified method described by Iqbal et al. (2016).

Assay Procedure:

The reaction mixture, totaling 1.0 ml, consisted of the following components:

- 0.58 ml of phosphate buffer (0.1 mol; pH 7.4)
- 0.2 ml of the homogenate sample
- 0.2 ml of ascorbic acid (100 mmol)
- 0.02 ml of ferric chloride (100 mmol)

The solution was incubated at 37°C in a shaking water bath for one hour. To halt the reaction, 1.0 ml of 10% trichloroacetic acid was introduced. Subsequently, 1.0 ml of 0.67% thiobarbituric acid was added, and the tubes were immersed in a boiling water bath for 20 minutes. Subsequent to boiling, they were placed in a crushed ice bath and centrifuged at 2500 × g for 10 minutes.

Measurement:

The formation of TBARS in each sample was assessed by measuring the optical density of the supernatant at 535 nm using a spectrophotometer, against a reagent blank (Chitra et al., 2017). Results were expressed as nmol of TBARS per minute per mg of tissue at 37°C, using a molar extinction coefficient of 1.56 × 10^5 M^-1 cm^-1.

Calculation of MDA:

$$\text{MDA (mmol)} = \frac{(\text{Abs. at } 532 - \text{Abs. at } 600) \times \text{Volume of reaction mixture}}{\text{mg protein} \times \text{Volume of the sample}} \times 1000$$

Where:

- Abs. at 532 is the absorbance measured at 532 nm
- Abs. at at 600 is the absorbance measured at 600 nm.
- The coefficient of absorbance is 1.53 mM.

3.9 STATISTICAL ANALYSIS

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 25. Descriptive statistics are presented as mean ± standard deviation (SD). To evaluate the significance of variance among groups, one-way analysis of variance (ANOVA) was conducted, followed by Dunnett's multiple comparison test. A p-value of less than 0.05 ($p < 0.05$) was considered indicative of significant differences between the experimental conditions.

CHAPTER 4
RESULTS AND DISCUSSION

Clozapine is recognized as an atypical antipsychotic used in the treatment of schizoaffective disorder and schizophrenia, particularly in cases where other treatments have proven ineffective (Henderson et al., 2000; Lindenmayer et al., 2003). However, its use has been constrained by metabolic side effects and the risk of agranulocytosis in patients with schizophrenia. Metabolic complications associated with clozapine therapy include weight gain, glucose intolerance, visceral obesity, hypertension, and dyslipidemia (Mookhoek et al., 2011). Dyslipidemia is a significant risk factor for cardiovascular diseases, as it contributes to the development of atherosclerosis, potentially leading to coronary heart disease (CHD) and stroke (Kopin & Lowenstein, 2017).

The primary pharmacological strategy for managing dyslipidemia involves the use of HMG CoA reductase inhibitors, commonly known as statins. However, statins are associated with notable side effects, including hyperglycemia and peripheral neuropathy, which often discourage adherence to

this medication (Reynaldi et al., 2021). As a result, there is an increasing demand for alternative treatments, particularly herbal remedies, to address dyslipidemia.

Murraya koenigii, belonging to the class Magnoliospida and the family Rutaceae, has garnered research attention for its bioactive properties. Studies focus on extracting bioactive compounds using aqueous, methanol, and chloroform solvents, highlighting the phytochemical, antioxidant, and antihyperlipidemic effects observed in both in vitro and in vivo models. This investigation aims to explore the therapeutic potential of Murraya koenigii in mitigating weight gain, agranulocytosis, and hyperlipidemia in animal models treated with clozapine.

4.1 IDENTIFICATION OF *MURRAYA KOENIGII*

4.1.1 AUTHENTICATION

The plant sample was identified as *Murraya Koenigii L.Spreng* belonging to class *Magnoliospida* and genus *Murraya J.Koenig* ex L. by phycologist Professor. Dr. Janardanan, Head of Botany Department. A specimen along with herbarium(Fig 29) was submitted to the Botany department of Goa University, Goa and a certificate of identification was procured.

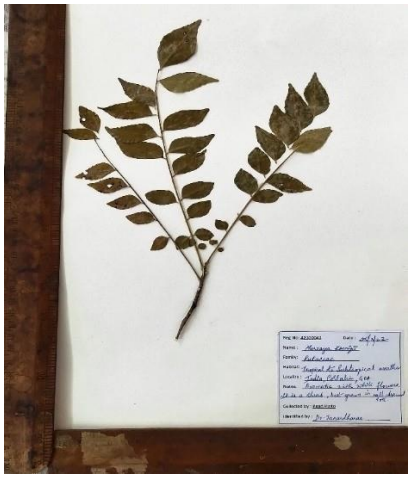

Figure 29 Herbarium of Murraya koenigii species collected from Cortalim submitted to Botany department, Goa University for taxonomical identification

4.1.2 PHYLOGENETIC ANALYSIS

4.1.2.1 ISOLATED GENOMIC DNA

Genomic DNA was isolated from the Murraya koenigii plant samples as shown in the Fig 30. DNA concentration results indicated that 102 µg/sample have a purity of 1.82 (Table1)

Table 1 DNA Quantification

Sample code	Concentration (µg/ml)	Purity (A260/280)
Leaf Sample	102	1.82

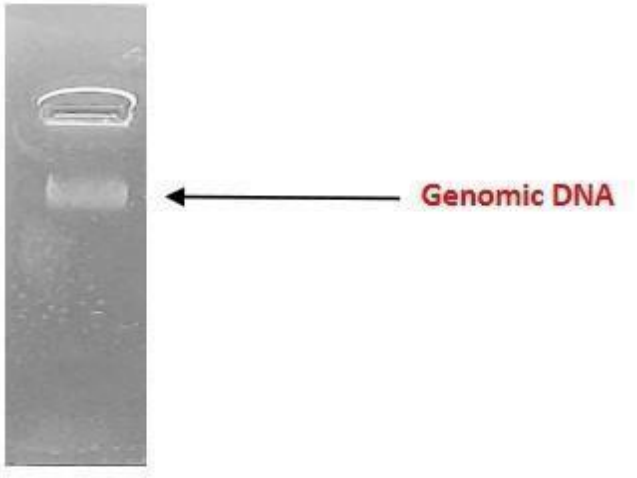

Figure 30 Genomic DNA of the selected plant sample

4.1.2.2 AMPLICIFICATION AND ELECTROPHORESIS

The extracted genomic DNA underwent amplification of the rbcL gene, which was successfully amplified and visualized using 1.5% agarose gel electrophoresis (Figure 31). The anticipated amplicon size was approximately 750 kb, and this size was confirmed in the resulting amplicon. The amplified product was subsequently purified utilizing the Exo-sap method and analyzed using an ABI Prism gene sequencer. The sequencing process yielded approximately 750 base pairs from the forward primer.

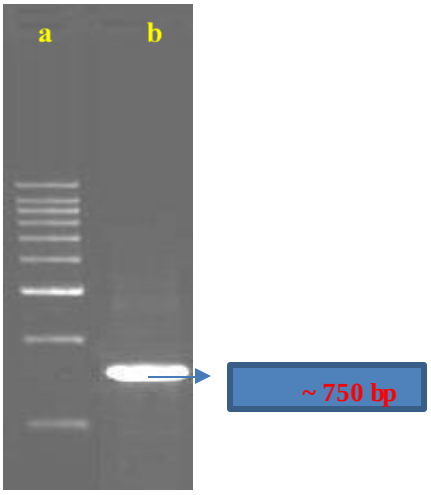

Figure 31 PCR amplification profile of selected plants sample

Conditions: 1.5% agarose gel electrophoresis

(Lane a: 1kb DNA Ladder; b: Sample)

1 KB DNA Ladder (bp):10000, 8000, 7000, 6000, 5000, 4000, 3000, 2000, 1000, 500.

4.1.2.3 SEQUENCING RESULTS FOR THE MURRAYA KOENIGII SAMPLE

The 750bp consensus sequence obtained upon initial analysis using NCBI- nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi#) were found to belong to Murraya koenigii with 99.46% similarity and 100% query cover.

Murraya Koenigii (#KY563317)

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TATAAATTGACTTATTATACTCCTGACTATGTAACCAAAGATACTGATATCTTGGCAGCA

TTCCGAGTAACTCCTCAGCCCGGAGTTCCACCCGAGGAAGCGGGGGCTGCGGTAGCTGCG

GAATCTTCTACTGGTACCTGGACAACGGTGTGGACCGATGGGCTTACCAGCCTTGATCGT

TACAAAGGGCGATGCTACAACATTGAGCCCGTTGCTGGAGAAGAGAATCAATATATATGT

TATGTAGCTTACCCGTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACATGTTTACTTCC

ATTGTGGGTAATGTATTTGGTTTCAAAGCACTGCGCGCTCTACGTCTAGAGGATCTACGA

ATCCCTCCTGCGTATTCTAAAACTTTCCAAGGCCCGCCTCACGGCATCCAA-GTTGAGAG

AGATAAATTGAACAAGTATGGCCGTCCCCTGTTAGGATGTACTATTAAACCTAAACTGGG

GTTATCCGCTAAGAATTATGGTAGGGCGGTTTATGAATGTCTACGTGGTGGACTTGACTT

TACCAAAGATGATGAGAACGTGAATTCCCAACCATTTATGCGTTGGAGGGACCGTTTCGT

ATTTTGTGCGGAAGCACTTTATAAAGCGCAAGCTGAAACAGGTGAAATCAAAGGTCATTA

CTTGAATGCTACTGCAGGTACATGCGA

Murraya Paniculata (#LC794905)

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TACCAAAGATGATGAGAACGTGAACTCCCAACCATTTATGCGTTGGAGGGACCGTTTCTT

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Feroniella Oblata (#LC794898)

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Acicarpha Tribuloides #NC_084373

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 # MW722359_Murraya_exotica

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CTTGAATGCTACTGCAGGGACATGCGA

 # LC794908_Triphasia_trifolia

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#LC794903 Micromelum minutum

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#LC794878_Aegle_marmelos

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#Pinto (sample)

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Sequencing Analysis

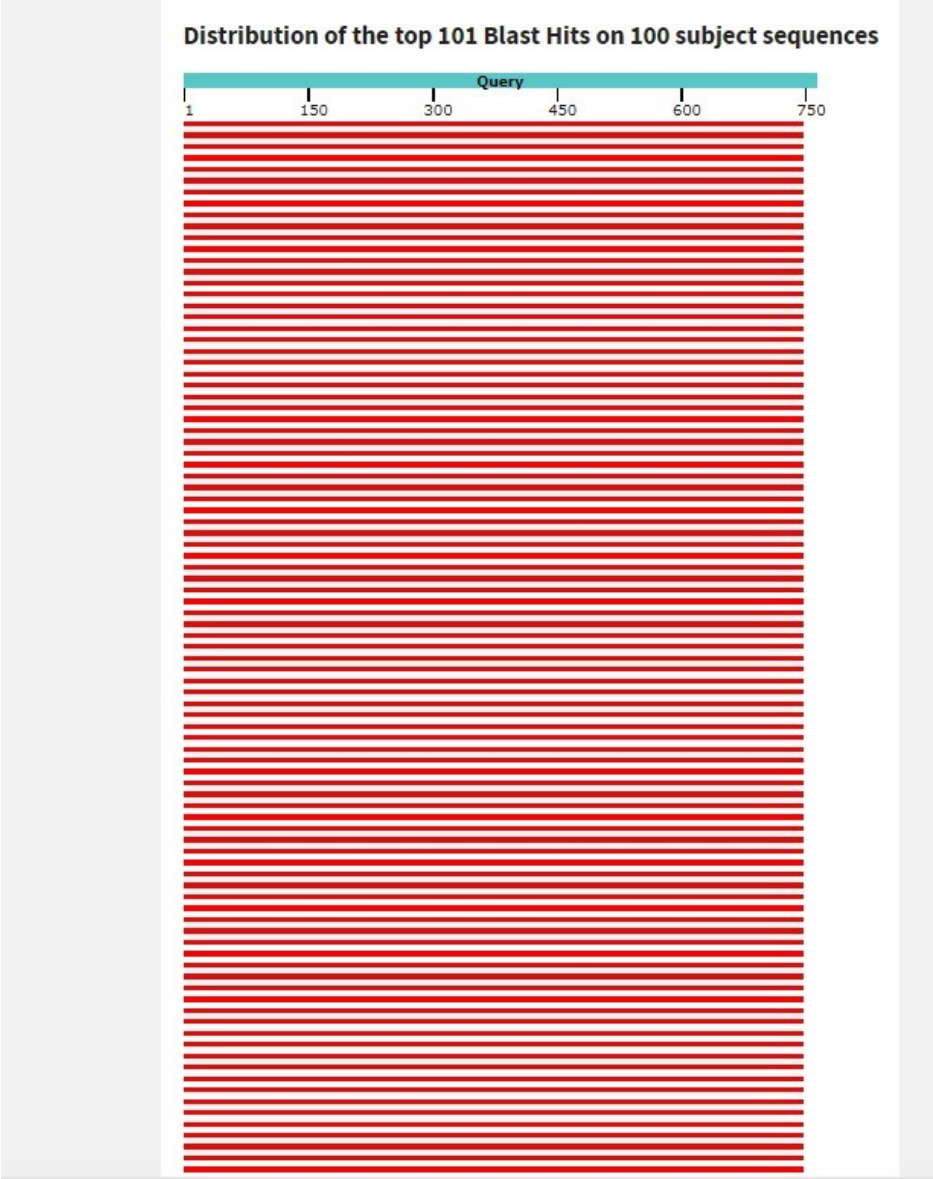


Figure 32 BLAST Analysis of Murraya koenigii

4.1.2.4 PHYLOGENETIC ANALYSIS

The sequences analyzed using the BLAST program on the NCBI website revealed sequence similarities with existing plant rbcL gene sequences of up to 99.46%. Closely related species rbcL gene sequences were identified and subsequently aligned using CLUSTAL W. The aligned sequences were further analyzed using the Maximum Parsimony method. The resulting phylogenetic tree, derived from the sequences obtained from the selected plant, is illustrated in Fig. 33.

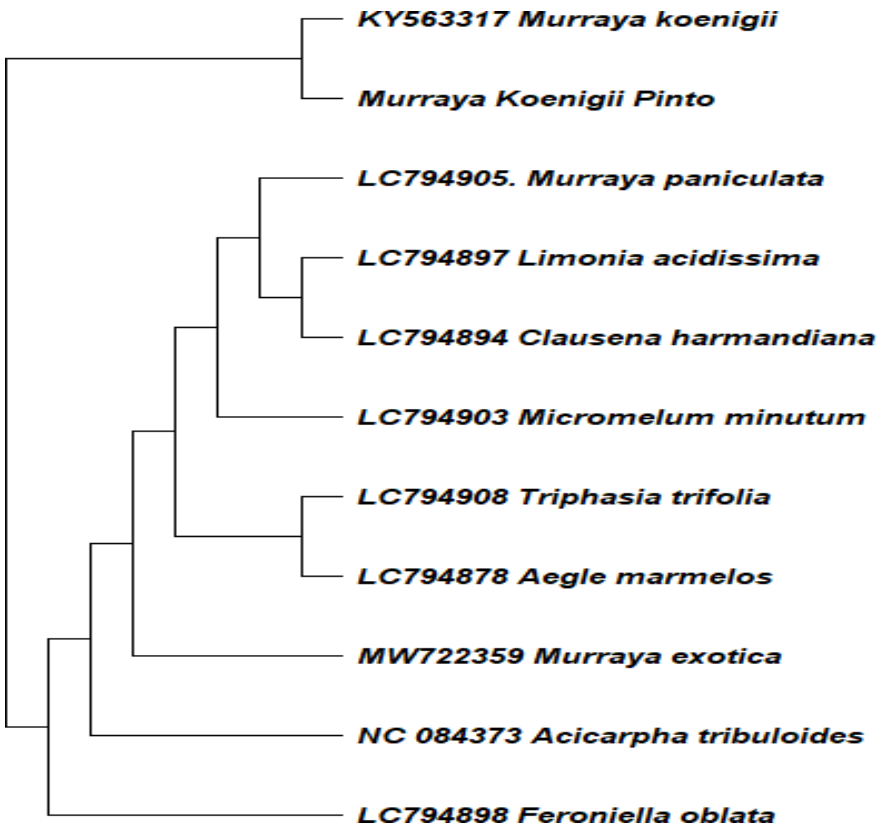


Figure 33 Maximum-Likelihood phylogenetic tree of Family Rutaceae and related sequences

Based on the BLAST analysis (Figure 32) and phylogeny analysis clearly revealed that the given plant sample belonged to the taxa Murraya koenigii. Numerous research studies have been undertaken to investigate various terrestrial plant species, yielding substantial insights into their habitats and origins. Similar to other prominent horticultural crops, this spice has not received the recognition it warrants concerning its potential for crop enhancement. Given i ts adaptability to diverse edapho-climatic conditions and its nutraceutical advantages, there exists considerable potential to identify genotypes that exhibit strong adaptability to sodic soils, which could serve as an alternative crop in saline-affected areas. (Ganapathi et al., 2022). Curry leaf exhibits genetic variation independently in terms of plant growth, productivity, and vulnerability to biotic influences. The findings of the study indicated that the relatively low variance observed in the plant's genetic background is attributed solely to genetic and

environmental factors. A phylogenetic tree illustrates the evolutionary relationships among specific species or gene lineages and remains a crucial instrument in contemporary biological research(Zou et al., 2024). To determine the relatedness among species, researchers employed methods such as DNA profiling and sequencing, utilizing molecular markers including the ribulose-bisphosphate carboxylase gene (rbcL) and 18S rRNA. The phylogenetic tree generated from the sequence data and the rBcL marker exhibited a significantly high maximum likelihood probability value when compared to other genetic markers. The results of this study hold considerable value for the pharmaceutical sector, facilitating DNA-based species identification of the Murraya koenigii plant and identifying potential adulterants during its collection(Bare et al., 2024). The findings of this study will be useful in establishing the molecular confirmation and differentiation of the species Murraya koenigii.

4.2 TOTAL YIELD OF MURRAYA KOENIGII

4.2.1 DRY YIELD OF MURRAYA KOENIGII

The weight of the Murraya koenigii leaves was measured by utilizing a weighing balance. The samples of leaves from 3 locations weighing of 500 gms, 450 gms and 400 gms were taken and its dry weight was measured, then it was kept at 37-degree Celsius incubation for 24 hours. 87 gms, 75 gms and 70 gms of dry yield was obtained. A mean weight obtained from Murraya koenigii was 77± 8.7 gms while mean percentage of dry yield obtained was 17.4 %, 16.6%. and 17.5 % respectively. (Table 2)

Table 2 Dry yield of Murraya koenigii

Wet weight(gm)	Period of drying	Dry weight(gm)	Yield percentage
500	37º C incubation for 24 hours	87±0.2	17.4%
450		75±0.5	16.6%
400		70±0.3	17.5%

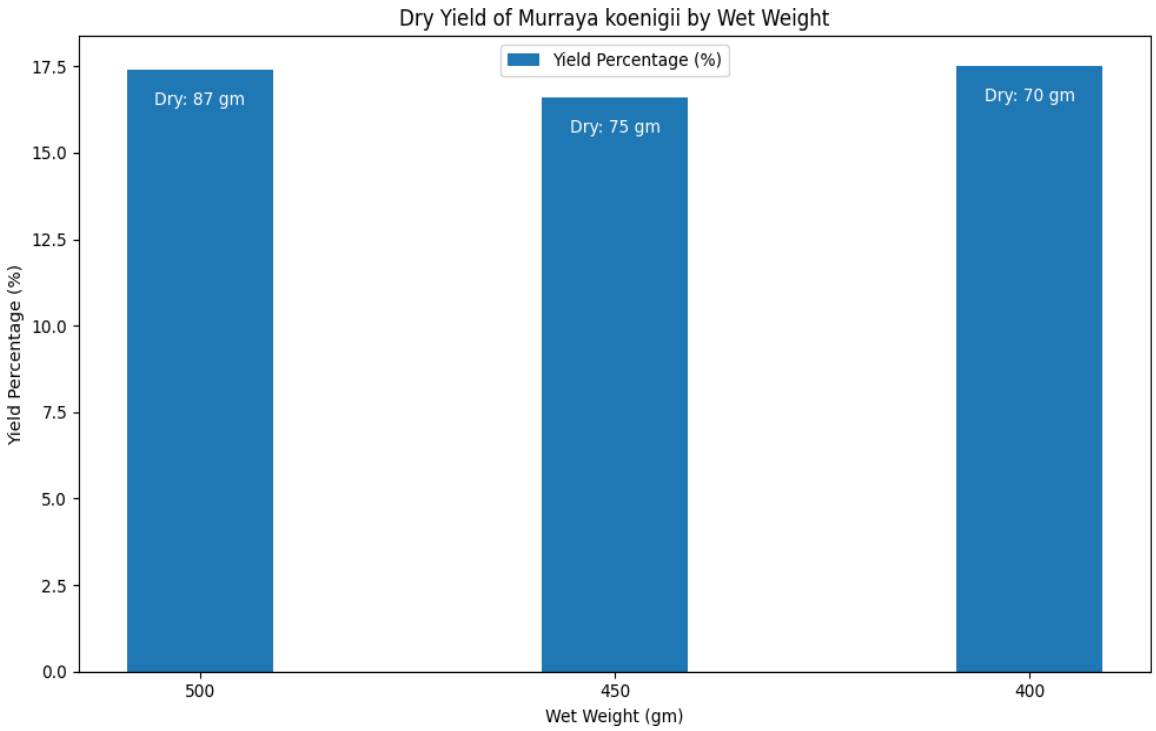


Figure 34 Dry yield derived from wet weight of Murraya koenigii

4.2.2 Total yield on solvent extraction of Murraya koenigii

The selection of a solvent for the extraction of bioactive compounds should prioritize low toxicity, the ability to evaporate at relatively low temperatures, and optimal physiological permeability. Additionally, the solvent should not cause dissociation of the extract, should enhance the stability of crude metabolites, and must not facilitate their degradation (Elmasry, 2023). The leaves of Murraya koenigii were subjected to Soxhlet extraction, utilizing 25 grams of dried material placed in a thimble. The extraction process was conducted over a duration of 10 hours, incorporating eight reflux cycles, with methanol, chloroform, and water serving as the solvents. The resulting extracts were subsequently evaporated under vacuum with a rotary evaporator, air-dried, and stored at 4ºC for future applications. (Longo & Rakesh, 2008.). The yield of crude extract fromdried Murraya leaves using methanol, chloroform and water were 2.75, 1.56, and 3.2 grams respectively.The yield used in this case was found to give a mean ± SD of 2.67 ± 0.09 (yield 10.6%), 1.5 ± 0.05 (yield 6.0%) and 3.2 ± 0.02 (yield 12.8 %) from 25 gms (Table 3). The crude employing the aqueous solvent allowed the total yield of 3.2 grams; this was relatively greater than methanolic extract. On the other handthe yield obtained from

the chloroform extract was relatively low.

Table 3 Yield of Soxhlet extraction with different solvents

SAMPLE QUANTITY IN GM	SOLVENTS	YIELD IN GM (MEAN ± SD)	YIELD %
25	methanol	2.67± 0.09	10.60%
25	chloroform	1.5 ± 0.05	6.00%
25	water	3.2 ± 0.02	12.8%

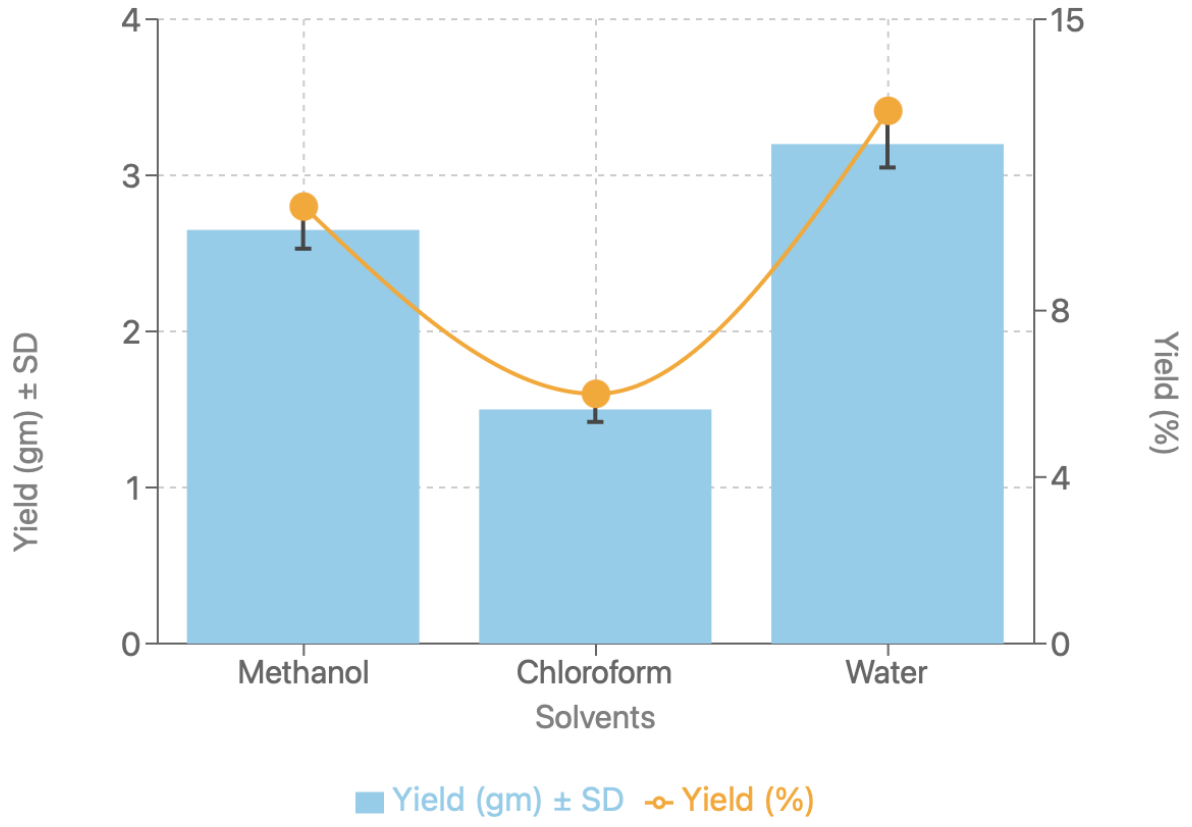


Figure 35 Yield of Soxhlet extraction with different solvents

The findings of this research underscore the significant impact of extraction methods and environmental conditions on optimizing the yields of bioactive compounds from Murraya koenigii extracts. Elements such as photoperiod, light intensity and quality, temperature, soil composition, and water availability play a crucial role in the synthesis of phytochemicals.

Consequently, employing various extraction techniques, including the use of different solvent compositions or fractionation with two immiscible solvents, will be beneficial in developing superior phytopharmaceuticals with a higher concentration of biologically active substances. (Satyavarapu et al., 2023). By employing Soxhlet extraction with different temperatures, retention times, and solvents, the study assesses the effectiveness of these methods in extracting bioactive constituents (Sablania et al., 2019). This study provides valuable insights into the extraction and identification of bioactive compounds from Murraya koenigii, highlighting the importance of optimizing extraction methods and conditions to improve both yield and effectiveness (Isabel & Isabel, 2021).

4.3 PRELIMINARY PHYTOCHEMICAL ANALYSIS

The phytochemical constituents of Murraya koenigii were established in all crude extracts that were extracted from methanol, chloroform and water solvents after condensation in a rotary evaporator. The concentration of the bioactive ingredients alkaloids, saponins, tannins, terpenoids, flavonoids, phenols, quinones, glycosides, and steroids was analyzed using the technique developed by Velavan and colleagues (Velav, 2015).

4.3.1 QUALITATIVE EXAMINATION

Preliminary phytochemical analyses of the leaf extracts from Murraya koenigii have identified thepresence of saponins, flavonoids, coumarins, tannins, terpenoids, glycosides, and phenols (Fig. 36,37,38). Specifically, alkaloids and steroids could not be identified in any of the three groups of extracts (Table 4). Some people classify phytochemicals, also known as phytonutrients, as secondary plant products that probably have health-promoting effects. But, it should be noted that phytochemicals vary in kinds and intensity in specific genera of plants. As a result of the evaluated data, it can be state that conducting phytochemical evaluations is crucial for setting pharmacopoeia standards.

Table 4 Phytochemical analysis of Murraya koenigii

Constituents	**Results**		
	Methanol (Solvent)	**Chloroform (solvent)**	**Aqueous**
Alkaloids	Absent	Absent	Absent
Anthraquinones	Present	Present	Present
Coumarins	Present	Present	Present
Flavonoids	Present	Present	Present
Glycosides	Present	Present	Present
Phenol	Present	Present	Present
Saponin	Present	Present	Present
Steroids	Absent	Absent	Absent
Tannins	Present	Present	Present
Terpenoids	Present	Present	Present
Total Phytochemicals present	**8**	**8**	**8**
Total Phytochemicals absent	**2**	**2**	**2**

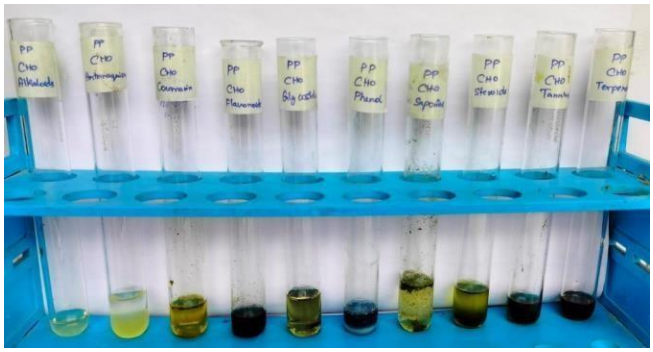


Figure 36 Phytochemical analysis of Murraya koenigii Chloroformic extract

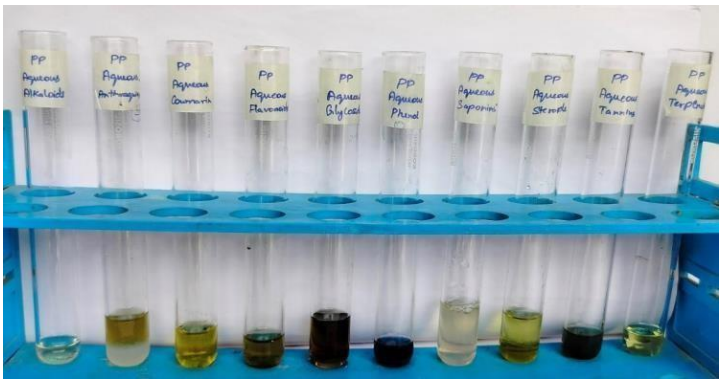


Figure 37 Phytochemical analysis of Murraya koenigii Aqueous extract

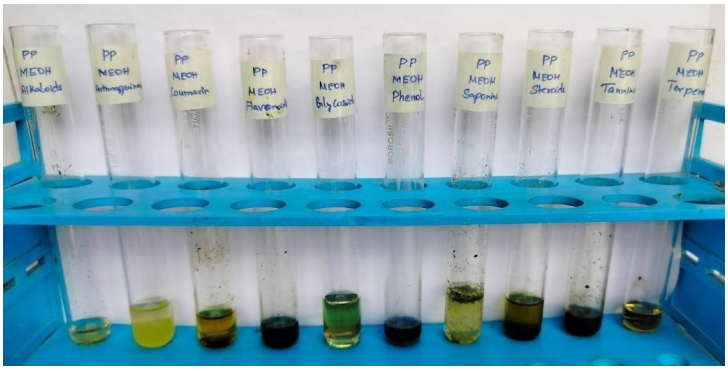


Figure 38 Phytochemical analysis of Murraya koenigii Methanolic extract

4.3.2 QUANTITATIVE EXAMINATION

The samples were analyzed for the presence of bioactive compounds utilizing established methods to assess total phenolics, flavonoids, glycosides, saponins, tannins, and terpenoids. (Table 5)

Table 5 Results of quantitative estimations of bioactive compounds present in the crude extracts of Murraya koenigii.

S.NO.	ASSAY	UNIT	Aqueous Extract	Methanol Extract	Chloroform Extract
1	Total Flavonoids Content	mg Quercetin equivalent/DE	392.3 ±0.57	334.6 ± 0.57	369 ± 1.73
2	Total Glycosides Content	mg Digoxin equivalent DE	362.6 ± 1.15	435.2 ± 2.3	244 ± 3.4
3	Total Content Phenol	mg Gallic acid equivalent/g DE	855.3 ± 0.41	581.6 ± 0.57	422 ± 0.40
4	Total Content Saponin	mg Diosgenin equivalent/g DE	157.1 ± 1.02	319 ± 0.50	301.6 ± 0.32
5	Total Content Tannins	mg Gallic acid equivalent/g DE	520 ± 4.65	948.2 ± 0.34	773.5 ± 0.5
6	Total Terpenoids Content	mg Linalool equivalent/g DE	238.2 ± 0.64	678 ± 0.95	428.6 ± 1.38

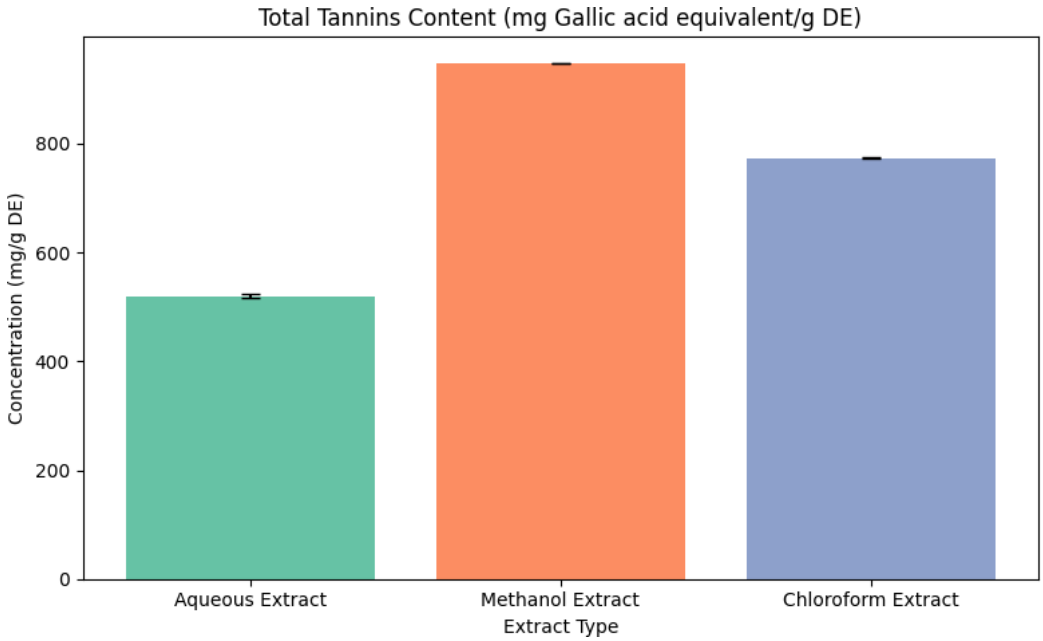


Figure 39 Total Tannins Content (mg Gallic acid equivalent/DE)

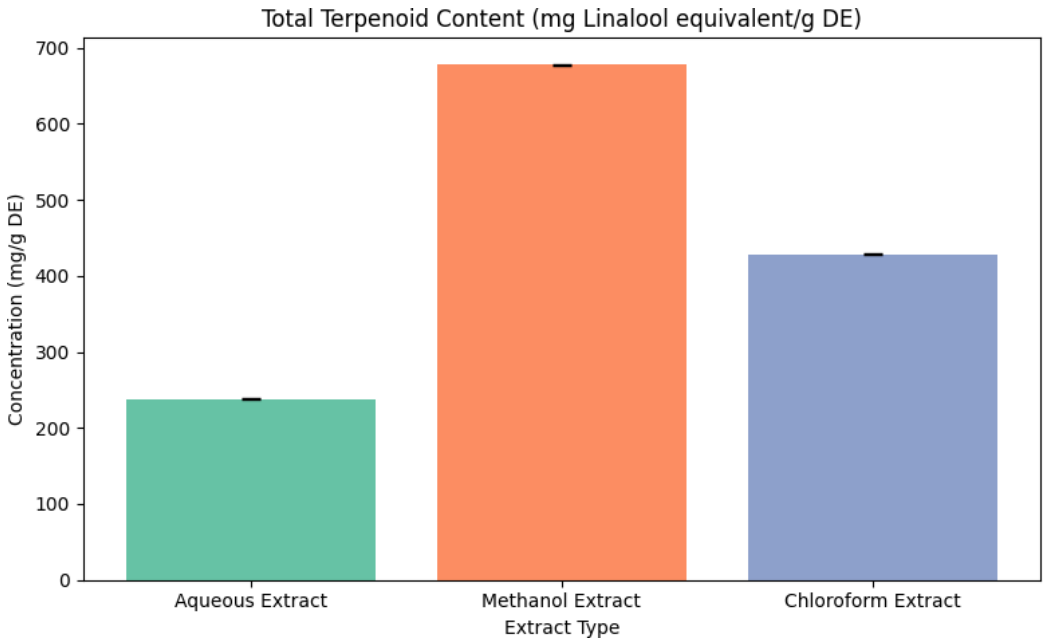


Figure 40 Total Terpenoids Content (mg Linalool Equivalent/g DE)

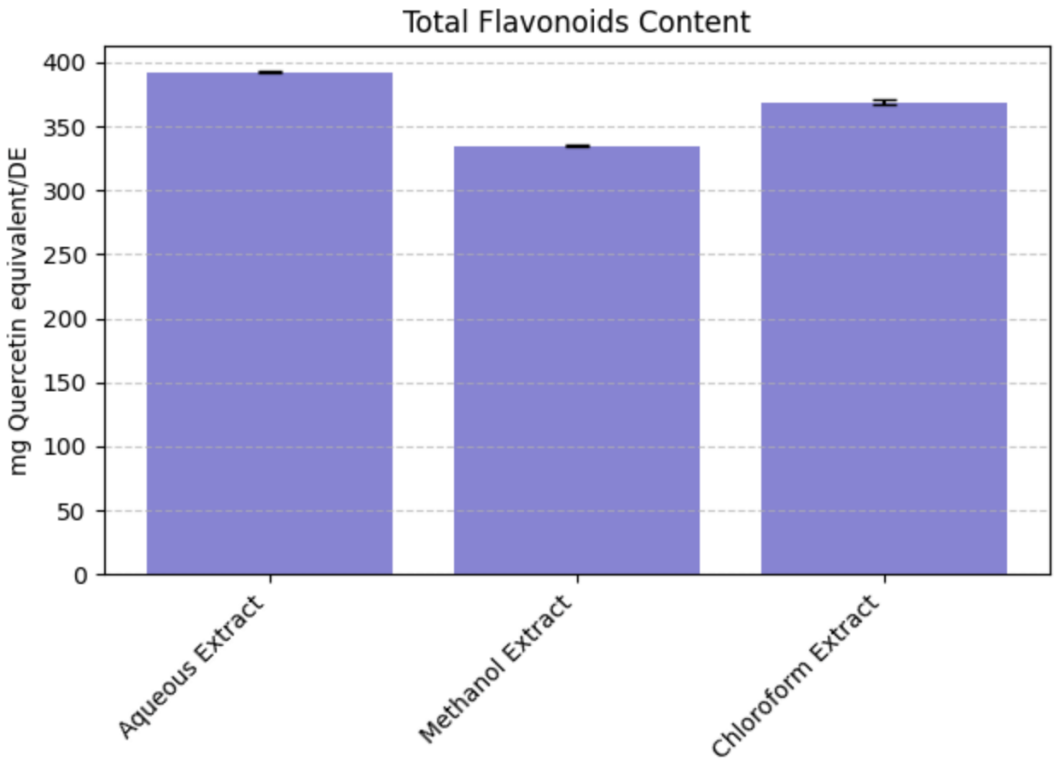


Figure 41 Total Flavonoids content(mg Quercetin equivalent/DE)

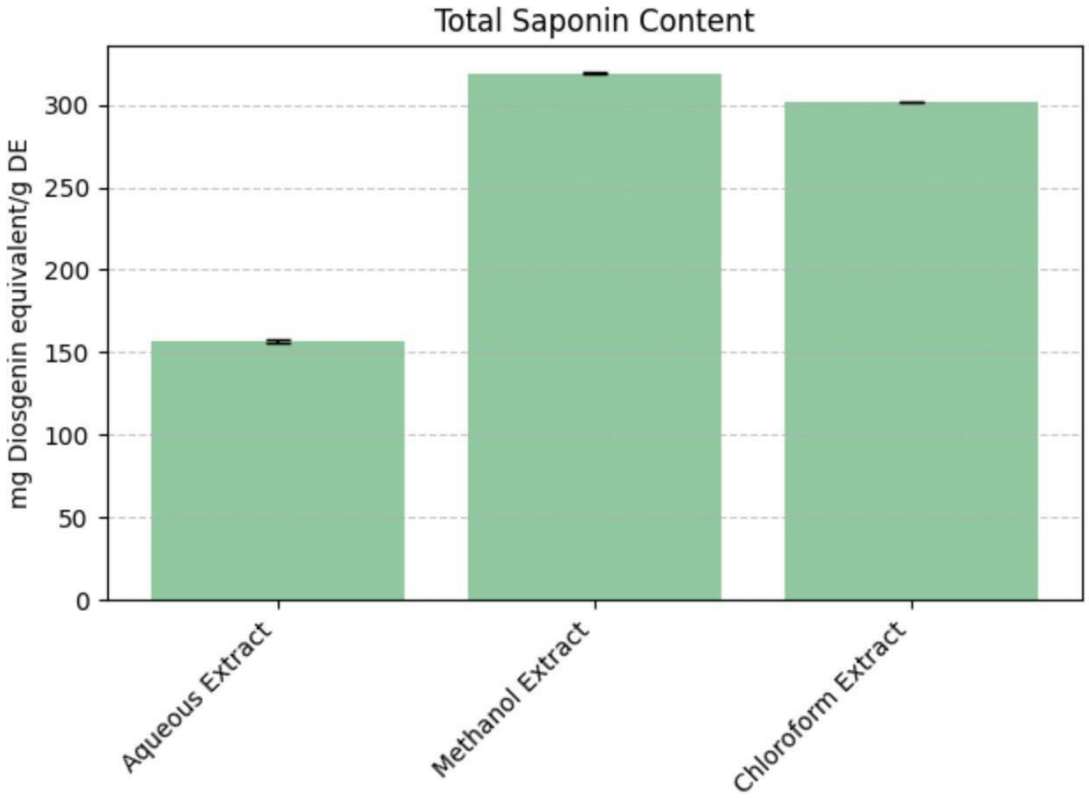


Figure 42 Total Saponin Content(mg Diosgenin equivalent/DE)

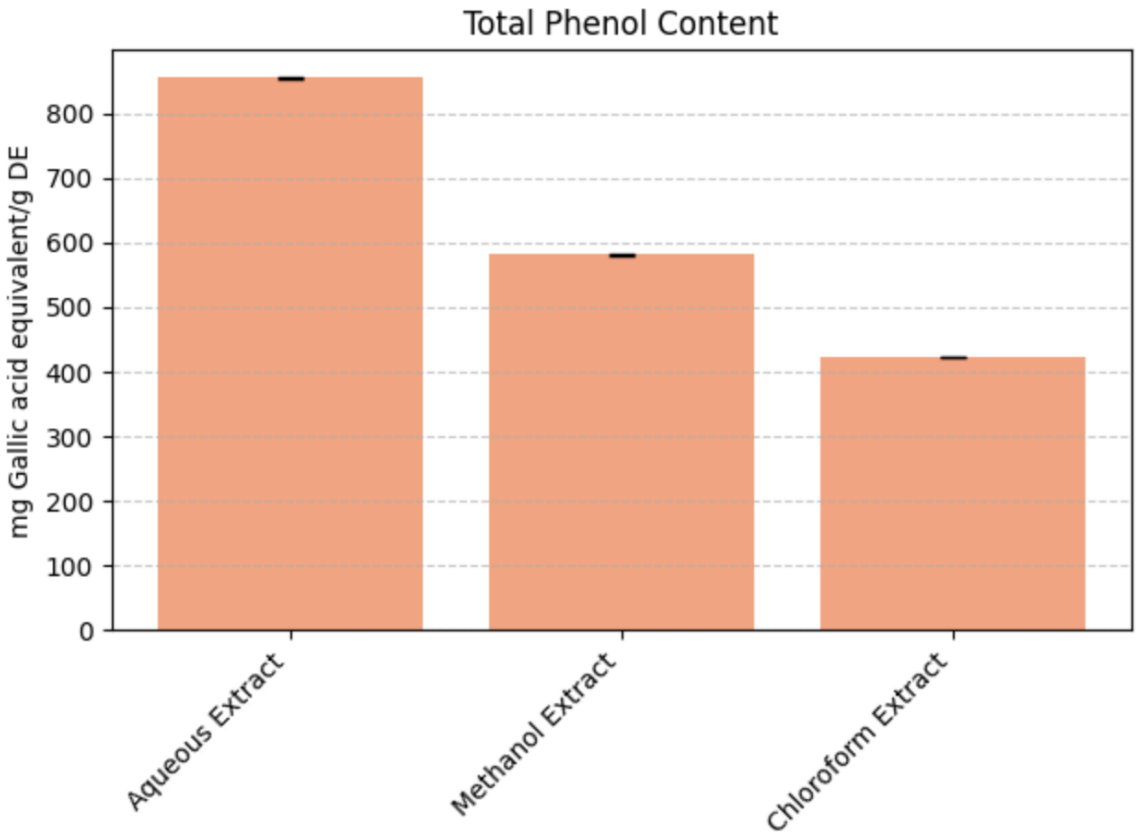
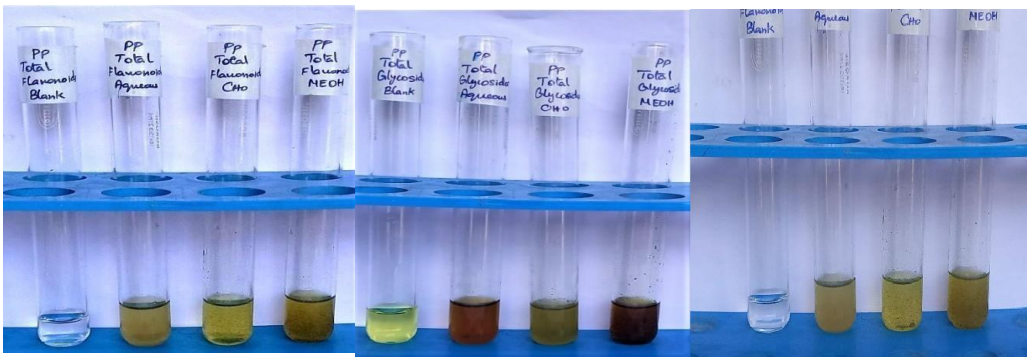
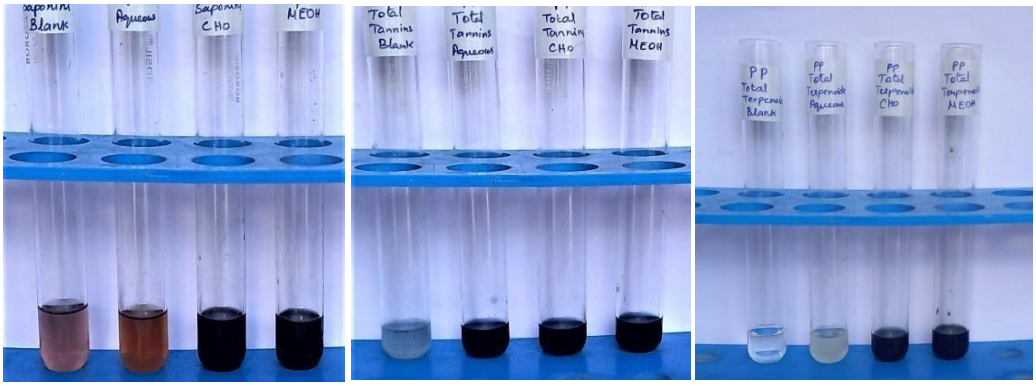


Figure 43 Total Phenol Content(mg Gallic acid equivalent/DE)

The study explores the use of various solvents for extraction, specifically methanol, aqueous, and chloroform, to address the high polarity of the compounds found in the leaves of Murraya Koenigii. This plant exhibits significant variability in its phytochemical composition, indicating that no single solvent can effectively extract all of the compounds present.(Salomi & Manimekalai, 2016). The results further show that these solvents are well chosen for the quantitative analysis of the different phytochemicals isolated from the leaves(Banerjee et al., 2017). The concentration of identified compounds is different for the three solvents, meaning that each solvent is capable of extracting phytochemicals differently.



(a): Total Flavanoids (b): Total Glycoside (c): Total Saponins



(d): Total Phenols (e): Total Tannins (f): Total Terpenoids

Figure 44 Quantitative estimation of Phytochemicals of Murraya koenigii.

4.3.2.1 ESTIMATION OF TOTAL PHENOLS

The phenolic content in the aqueous, methanol, and chloroform extracts of *Murraya koenigii* was quantified as 855.3 ± 0.41, 581.6 ± 0.57, and 422 ± 0.40 mg GAE/g of sample, respectively (Fig 44 d ,Table 5). The total phenolic content was assessed utilizing the standard gallic acid curve equation. Phenolic compounds are well-recognized for their chemopreventive properties, particularly due to their antioxidant activity (María et al., 2018), and their role in promoting cardiovascular health (Kant Upadhyay, 2021). The redox properties of these compounds enable them to function as effective antioxidants, primarily owing to the presence of hydroxyl groups that enhance their ability to neutralize free radicals. This correlation suggests that the concentration of phenolics could serve as a reliable indicator for the rapid assessment of antioxidant activity. A

positive linear relationship has been established between phenolic content and the antioxidant potential across various herbs (Zheng & Wang, 2001). Their significant role in combating free radicals and mitigating oxidative damage highlights the importance of these compounds in health maintenance (Faizal et al., 2009; Rahman et al., 2017). Due to their antioxidative properties and health benefits, crude extracts rich in phenolics from fruits, herbs, vegetables, and cereals are increasingly utilized in the food industry (Baba & Malik, 2015). Cai et al. demonstrated that the addition of trace elements, including Co, Ag, and Cd, significantly enhances the synthesis of phenolic acids (Cai et al., 2013). Further exploration of phenolic compounds could yield valuable insights into their therapeutic applications derived from *M. koenigii* in human health (Fraga et al., 2019).

4.3.2.2 TOTAL FLAVONOID CONTENT

The total flavonoid content in the aqueous, methanol, and chloroform extracts of *Murraya koenigii* was determined to be 392.3 ± 0.57, 334.6 ± 0.57, and 369 ± 1.73 mg Quercetin equivalent per DE, respectively (Table 5, Fig. 44a). This was assessed using the standard quercetin curve equation. Flavonoids represent a diverse group of phytonutrients found in many plant-derived foods, recognized for their significant antioxidant, anti-inflammatory, and health-promoting properties. Their unique chemical structure—characterized by two benzene rings connected by a heterocyclic pyran ring—is critical for their classification and biological activities (Ribeiro et al., 2023). Flavonoids are particularly adept at neutralizing a variety of oxidizing agents, such as singlet oxygen and numerous free radicals associated with various diseases. They inhibit the formation of reactive oxygen species, bind to trace elements that promote free radical generation, eliminate reactive species, and enhance the efficacy of protective antioxidants (Baba & Malik, 2015). Research has shown that flavonoids and phenolic compounds can inhibit pancreatic lipase activity in pigs by interacting with the enzyme-substrate complex, thereby reducing lipid absorption (Villa-Ruano et al., 2013). These plant-derived compounds may act as effective antioxidants that prevent LDL oxidation due to their phenolic and flavonoid constituents (Gajaria et al., 2015). Although polyphenols and flavonoids exhibit moderate inhibition of pancreatic lipase (P. K. Liu et al., 2018), β-sitosterol has been linked to significant reductions in triglycerides, total cholesterol, LDL, and VLDL levels, along with increases in HDL, as demonstrated in multiple animal studies (Vinuthan

et al., 2015). These compounds can effectively lower plasma cholesterol levels by reducing the intestinal absorption of dietary cholesterol. Furthermore, studies suggest that rats on a high-fat diet exhibit improved fat mobilization via norepinephrine-induced lipolysis in adipocytes (Saad et al., 2021), indicating that polyphenols may impede the absorption of carbohydrates and lipids in the small intestine by inhibiting α-amylase activity and the absorption of palmitic acid (Han et al., 2003; Saad et al., 2021).

4.3.2.3 TOTAL SAPONINS

The saponin content in the aqueous, methanol, and chloroform extracts of *Murraya koenigii* was quantified as 157.1 ± 1.02, 319 ± 0.50, and 301.6 ± 0.32 mg Diosgenin equivalent/g DE, respectively (Table 5, Fig. 44c). The total saponin content was evaluated using the standard diosgenin curve equation. Saponins typically consist of a sapogenin core linked to a sugar moiety (Szabo, 2020). High concentrations of saponins are known to reduce lipid absorption (Bajes et al., 2020). Comparative studies on total saponin content from herbs cultivated in urban versus coastal regions revealed that coastal plants had higher concentrations than their urban counterparts (Prabhachandh & Babychan, 2017). This finding underscores the importance of environmental factors in influencing the phytochemical composition of plants. Naturally occurring saponins can inhibit lipid accumulation in liver parenchymal cells and regulate fat accumulation in adipose tissue (Variya et al., 2018). They reduce cholesterol levels by forming low-solubility complexes with cholesterol, thus hindering its absorption in the intestines. Additionally, saponins obstruct cholesterol absorption by interacting with bile acids to form micelles and have demonstrated antihypertriglyceridemic effects by promoting triglyceride elimination through fecal excretion (Elekofehinti, 2014). A study by Chen et al. revealed that diosgenin, a steroidal saponin derived from *Dioscorea opposita*, can increase GSH and GPx levels, which are reduced due to doxorubicin treatment in mice (Chen et al., 2015). The ongoing research into saponins from terrestrial plants emphasizes their potential in drug discovery and development.

4.3.2.4 TOTAL TERPENOIDS

The terpenoid content in the aqueous, methanol, and chloroform extracts of *Murraya koenigii* was quantified as 238.2 ± 0.64, 678 ± 0.95, and 428.6 ± 1.38 mg Linalool equivalent/g DE, respectively (Table 5, Fig. 44f). The total terpenoid content was determined using the standard linalool curve

equation. The biological efficacy of plant extracts can largely be attributed to secondary metabolites, including alkaloids, flavonoids, and terpenoids. These compounds are primarily produced by plants as a defense mechanism against microbial threats and environmental stressors (C. Li et al., 2023). Many plants generate a thick resin primarily composed of volatile terpenes, and the concentrations of these metabolites can be significantly influenced by environmental factors such as photoperiod, light intensity, temperature, soil composition, and water availability (Yang et al., 2018). Insects can convert terpenes from their plant-based diets into growth hormones and pheromones (Szafranek et al., 2012). Terpenoid levels can vary significantly between leaf and bark tissues and may fluctuate in response to various environmental and biological stressors (Ghorai et al., 2012).

4.3.2.5 TOTAL TANNIN CONTENT

The tannin content in the aqueous, methanol, and chloroform extracts of *Murraya koenigii* was assessed as 520 ± 4.65, 948.2 ± 0.34, and 773.5 ± 0.5 mg GAE/g of sample, respectively (Table 5). The methanol extract displayed the highest tannin concentration, followed by the chloroform and aqueous extracts, as shown in Fig. 44e. Tannins are naturally occurring polyphenolic compounds found in plants and can be classified into two main categories: hydrolysable and condensed tannins. Comparative analyses of various solvents, including ethanol, methanol, acetone, DMSO, and water, indicated that the highest hydrolysable tannin concentration in *M. koenigii* leaf extracts was obtained using isopropanol for both mature and young leaves (Kalita et al., 2022; Tomar et al., 2017). Gallotannin, a type of hydrolysable tannin commonly known as tannic acid, consists of a glycosidic core linked to a galloyl unit. Ellagitannins have been associated with promoting vascular health (He, 2022). Gallotannins can be further categorized into simple and complex galloyl glucoses (Torres-León et al., 2017). Tannins have been shown to exert cholesterol-lowering effects by inhibiting the absorption of free fatty acids in the intestine, which occurs through binding with mucosal proteins and intestinal epithelial cells within the small intestine (Vinarova et al., 2015). Furthermore, tannins have demonstrated the ability to lower glucose levels by inhibiting the activities of α-amylase and α-glucosidase (Sieniawska, 2015). Regulating the antioxidant status of pancreatic β-cells is crucial for delaying the onset of insulin-dependent diabetes mellitus (Li et al., 2005). The compound 1,2,3,4,6 -penta-O-galloyl-β-D-glucose has exhibited potential as an α-glucosidase inhibitor, while the hydrolysable tannin gallic

acid has demonstrated inhibitory effects on key metabolic enzymes (Pal et al., 2022). Further exploration of the influence of solvents and extraction techniques on tannin yield could provide valuable insights into optimizing extraction processes for enhanced therapeutic applications.

4.3.2.6 Total Glycoside content

To determine the total content of cardiac glycosides, digoxin can be utilized as a standard, with absorbance measured at 495 nm. Various concentrations of digoxin (20, 40, 60, 80, 100 µg/ml) serve as the standard. The total cardiac glycoside content can subsequently be reported in mg of digoxin equivalent per gram of dry weight. A calibration curve can be established using standard digoxin solutions within the range of 10-50 µg/ml, which should demonstrate linearity, achieving a regression coefficient of 0.9893.

The glycoside content results from the aqueous, methanol, and chloroform extracts of Murraya koenigii were measured at 362.6 ± 1.15, 435.2 ± 2.3, and 244 ± 3.4 mg digoxin/g of sample, respectively (Table 5). The methanolic extract exhibited the highest glycoside content, followed by the aqueous extract, with the chloroform extract showing the lowest levels, as illustrated in Fig 44 b. Glycosides are composed of a sugar moiety, termed the glycone, and a non-sugar moiety known as the aglycone or genin. The exploration of C-glycosides dates back to the 19th century(Franz and Grun, 1983). The first compounds identified for extraction were aloin (barbaloin) and scoparin in 1851, succeeded by bergenin in 1880 and vitexin in 1898. Glycosides play a vital role in the treatment of cardiac failure and arrhythmias, which is why they are classified as cardiac glycosides (Zeece et al., 2020). These cardiac glycosides function by enhancing the availability of calcium to cardiac fibers and inhibiting sodium-potassium-adenosine triphosphatase, leading to increased intracellular sodium and decreased intracellular potassium levels. By the end of 2021, researchers had successfully isolated and characterized a total of 754 C-glycosides and their derivatives from a variety of plant sources. Currently, 66 functional C-glycosyltransferases (CGTs) have been identified in plants, which provide sustainable and efficient approaches for the synthesis of C-glycosides(Hua et al, 2022). The importance of plant-derived C-glycosides is increasingly recognized in the pharmaceutical, agricultural, and food sectors, with several of these compounds being developed for clinical applications. For instance, puerarin is

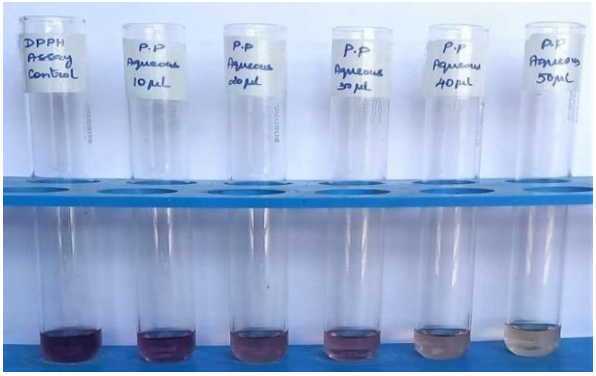
employed in the management of cardiovascular diseases. With the progress in sensitive and structurally informative techniques, particularly LC/MS and NMR, future discoveries of novel structures are expected. (Zhang et al., 2022).

4.4 ANTIOXIDANT ACTIVITY

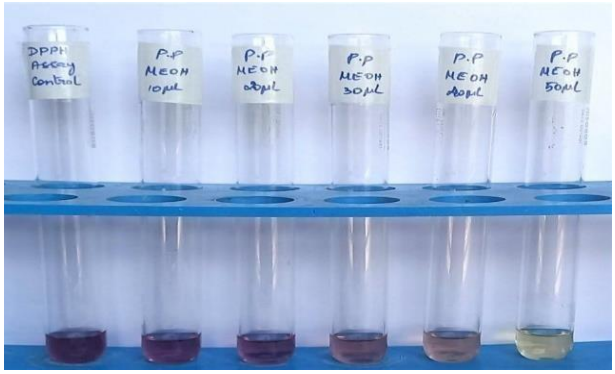
A direct correlation has been established in the research regarding the total phenolic content (TPC), total flavonoid content (TFC), and the antioxidant activity of M. koenigii leaf extracts. The protective effects of these plants are likely due to the presence of tannins, flavonoids, and phenolic compounds, as indicated by the preliminary phytochemical analysis of the chloroform and methanol extracts of the leaves. Previous research has demonstrated that M. koenigii exhibited more pronounced activities in the conducted assays when compared to M. minutum (Abeysinghe et al., 2021).

4.4.1 DETERMINATION USING THE 1, 1-DIPHENYL-2-PICRILHYDRAZYL ASSAY.

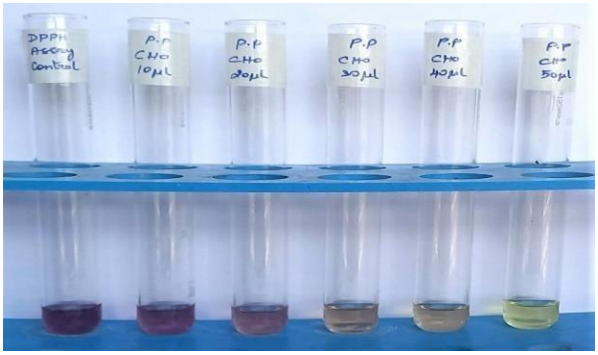
The ability of the DPPH radical to be eliminated was observed spectrophotometrically at the concentrations mentioned below for the experiment.



a) Aqueous extract



b) Methanolic extract



c) Chloroformic extract

Figure 45 DPPH assay with different concentrations of all three extracts of Murraya koenigii

CONC (mg/ml)	Ascorbic acid (%Inhibition)	Aqueous (%)	Methanolic (%)	Chloroformic (%)
0.2	56.12±1.0	3.89±0.09	20.43±0.57	13.72±0.15
0.4	72.17±1.0	5.06±0.05	21.63±0.50	21.23±0.50
0.6	87.77±1.6	15.3±0.08	27.6±0.64	45.3±0.46
0.8	93.27±1.5	36.7±0.12	37.2±0.56	50.4±0.57
1	98.01±1.0	43.2±0.12	63.6±0.51	66.5±0.46
IC50	**0.45**	**0.67**	**1.98**	**0.67**

Table 6 In- Vitro DPPH of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

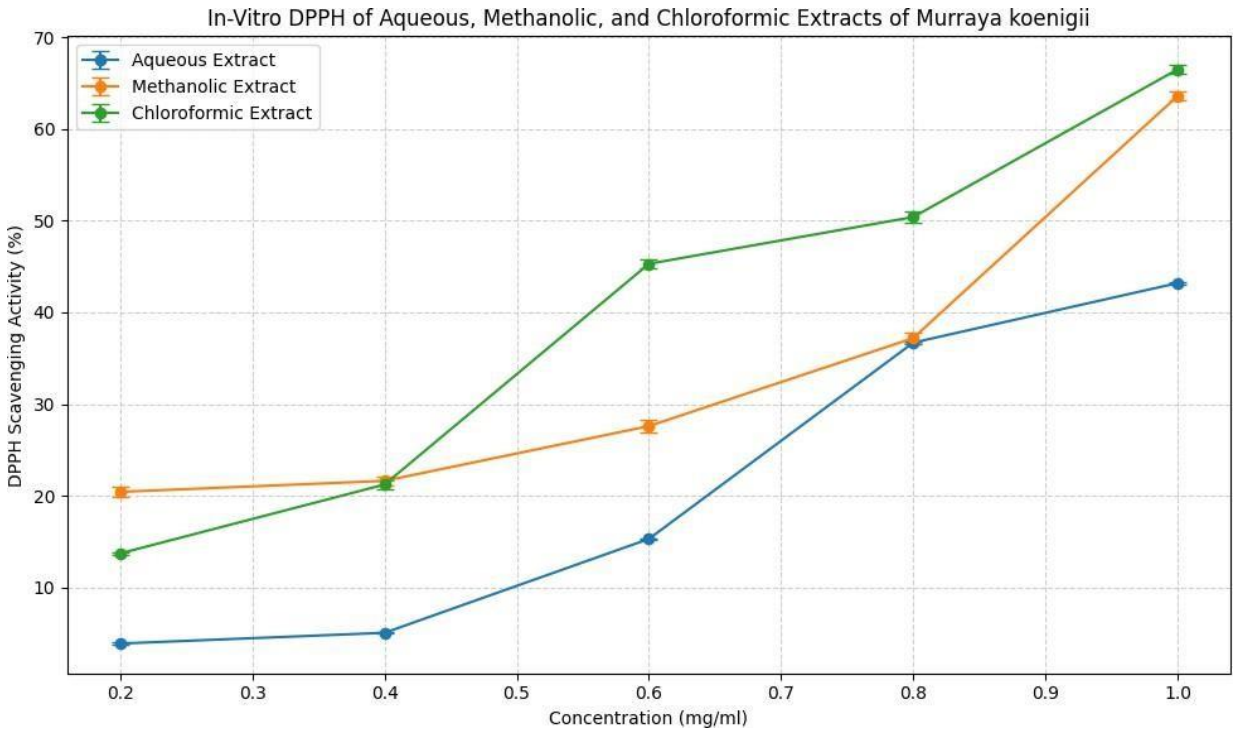


Figure 46 Graphical representation of DPPH radical scavenging activity using aqueous, methanol and Chloroform extract of Murraya koenigii

The results showed that the highest possible amount of DPPH, measuring 66.5±0.46 was obtained from the chloroformic extract of Murraya koenigii. On the other hand, the methanolic extract of Murraya koenigii yielded a slightly lower amount of DPPH+, measuring 63.6±0.51 followed by aqueous extract giving 43.2±0.12. The DPPH (1,1-diphenyl-2-picrylhydrazyl) assay is widely recognized as an effective method for assessing antioxidant capacity, as it evaluates the ability of compounds to neutralize DPPH radicals, which are highly reactive molecules characterized by an unpaired electron. Research conducted by Parithy et al. (2021) indicates that M. koenigii leaves processed via ultrasonic- assisted extraction (UAE) exhibited a strong reaction in the DPPH assay followed by those subjected

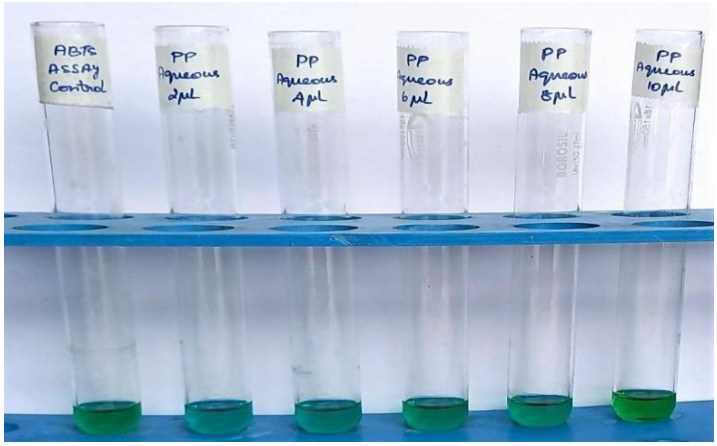
to microwave-assisted extraction (MAE) and solvent-assisted extraction (SAE) (Parithy et al., 2021). To further elucidate the antioxidant potential of M. koenigii extracts, IC50 values for both M. koenigii and M. minutum methanol extracts were assessed,with M. koenigii exhibiting greater activity than M. minutum at a specified antioxidant concentration.. Variations in the maturation stages of leaves and environmental growth conditions across different geographical locations may account for these differences. Consequently, it is clear that the antioxidant properties of M. koenigii are linked to various phenolic compounds (Abeysinghe et al., 2021).

The DPPH assay serves as an effective method for assessing and comparing the antioxidant capabilities of different species within the same family. In research focused on the ethnopharmacological effects of three plant species, M. koenigii displayed the most pronounced antioxidant properties, attributed to its elevated TPC and total flavonoid content (TFC) (Shabir et al., 2022).

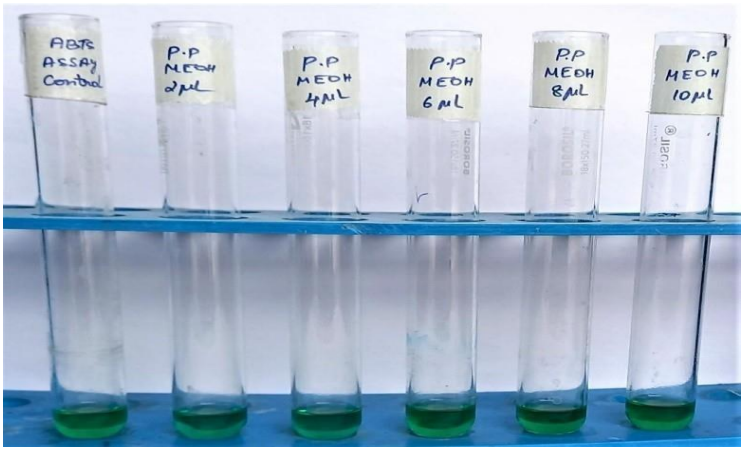
The DPPH assay is an important tool for measuring the antioxidant capacity of plants, offering valuable insights into their potential as natural antioxidant sources for various applications, such as food preservation and nutraceutical development.

4.4.2 MEASUREMENT METHOD THAT USES 2, 2 AZINO BIS (3-ETHYLBENZ THIAZOLINE) -6-SULFONIC ACID ASSAY

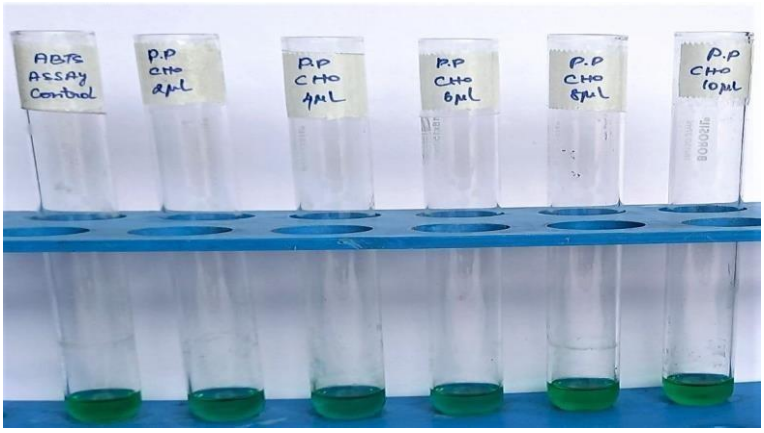
In the experiment, various concentrations of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) ranging from 0.2 mg/ml to 1 mg/ml were used. The radical cation ABTS+ was generated using potassium persulfate. The results showed that the highest possible amount of ABTS+, measuring 93.6 %, was obtained from the chloroformic extract of Murraya koenigii. On the other hand, the methanolic extract of Murraya koenigii yielded a slightly lower amount of ABTS+, measuring 85.49% followed by aqueous extract giving 61.45%. These findings indicate that the chloroformic extract had a higher capacity to generate ABTS+ compared to the other extract. The different concentrations of ABTS used in the experiment likely influenced the amount of ABTS+ generated (Fig.47).



(a) Aqueous extract



(b) Methanolic extract



(c) Chloroformic extract

Figure 47 ABTS assay with different concentrations of all three extracts of Murraya koenigii

Table 7 In-Vitro ABTS assay of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

CONC (mg/ml)	Ascorbic acid (%Inhibition)	Aqueous (%Inhibition)	Methanolic (%Inhibition)	Chloroformic (%Inhibition)
0.2	57.18±3.0	22.7 ± 0.07	29 ± 0.5	89.46 ± 0.04
0.4	69.13±3.7	28.5 ± 0.11	35.76 ± 0.11	89.6 ± 0.05
0.6	82.36±2.5	39.5 ± 0.09	63.89 ± 0.05	89.86 ± 0.59
0.8	89.47±4.7	48.1 ± 0.05	79.12 ± 0.05	91.4 ± 0.41
1	97.3±8.6	61.7 ± 0.3	85.7 ± 0.5	93.6 ± 0.5
IC50	**0.65**	**2.38**	**0.55**	**1.64**

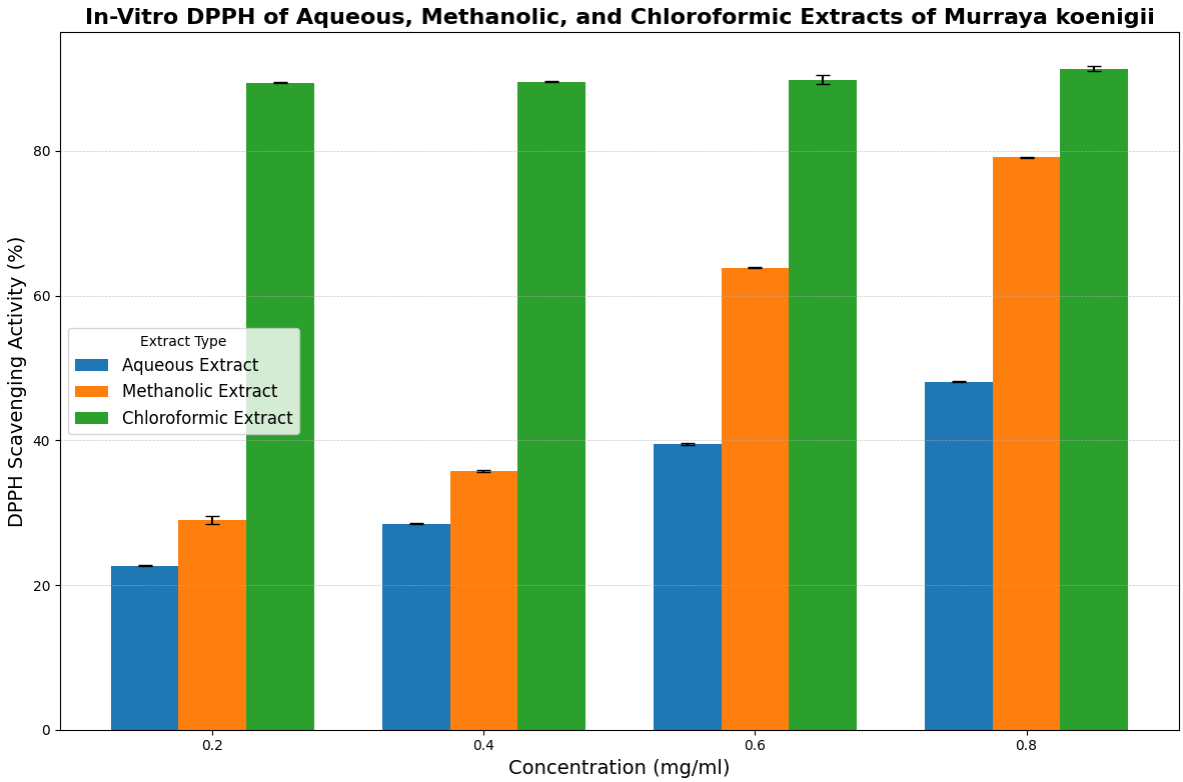


Figure 48 Results of ABTS assay of aqueous, methanolic and chloroformic extract of MK

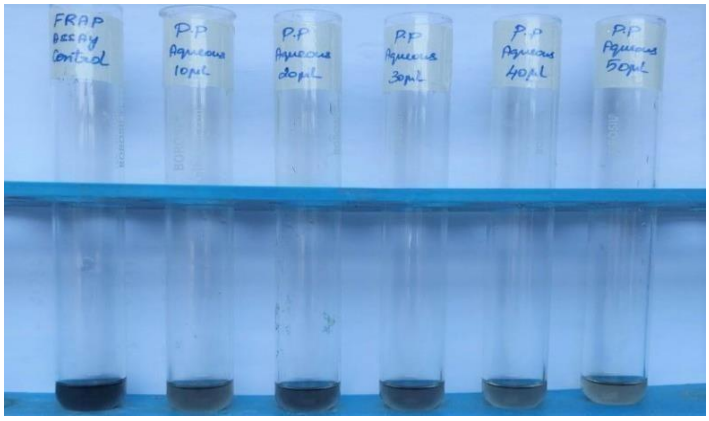
The ABTS assay serves as an essential instrument for analyzing the antioxidant potential of plant extracts, offering valuable insights into their viability as natural antioxidant sources for diverse applications, such as food preservation and nutraceutical development.

For instance, a study hexane and acetone extracts of Murraya koenigii were examined to possess superior antioxidant properties relative to the standard (Sonter et al., 2021). Additionally, the influence of infusion temperature on free radical scavenging capacity was evaluated using both DPPH and ABTS assays and findings suggested that higher infusion temperatures enhance the concentration of phenolic compounds, thereby positively impacting the free radical scavenging ability(Chang et al,2020).

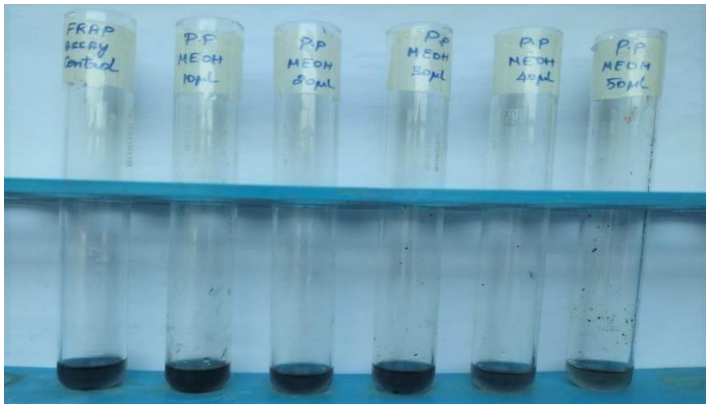
4.4.3 ANTIOXIDANT ASSESSMENT USING THE FERRIC REDUCING ANTIOXIDANT POWER ASSAY

In the FRAP assay, aqueous, methanolic and chloroformic extract gave 21.6 ± 0.4, 32.7 ±

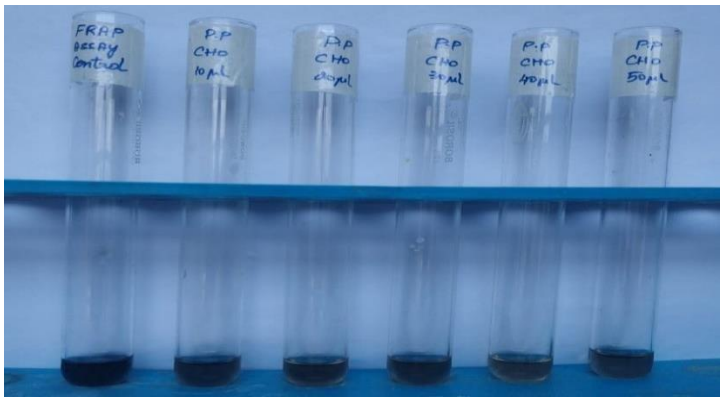
0.11, 37.7 ± 0.34 % respectively. The reaction entails the conversion of Fe3+—TPTZ (iron [III]-2,4,6-tripyridyl-S-triazine) to Fe2+—TPTZ through the action of an antioxidant compound. Usually the reducing power enhances with increased concentration similar pattern was demonstrated in our study. This suggests that Murraya koenigii contains hydrophilic polyphenolic compounds responsible for its enhanced reducing power (Velavan et al., 2007). The activities of CEMK and MEMK were markedly higher in comparison to AEMK. Vitamin C served as the standard at a concentration of 100 µg/ml, yielding an absorbance of 0.514.



(a) Aqueous extract



(b) Methanolic extract



(c) Chloroformic extract

Figure 49 FRAP assay with different concentrations of all three extracts of Murraya koenigii

Table 8 In- Vitro FRAP of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

CONC (mg/ml)	**Ascorbic acid(%)**	**Aqueous (%)**	**Methanolic (%)**	**Chloroformic (%)**
0.2	52.3 ± 0.21	2.9 ± 0.01	16.23 ± 0.57	3.16 ± 0.10
0.4	62.5 ± 0.05	5.28 ± 0.05	22.93 ± 0.57	7.3 ± 0.41
0.6	76.7 ± 0.167	10.3 ± 0.46	29.65 ± 0.25	19.14 ± 0.07
0.8	82.6 ± 0.12	17.6 ± 0.5	31.67 ± 0.51	31.7 ± 0.52
1	98.7 ± 0.08	21.6 ± 0.4	32.7 ± 0.11	37.7 ± 0.34
IC50	**3.64**	**0.75**	**0.43**	**0.65**

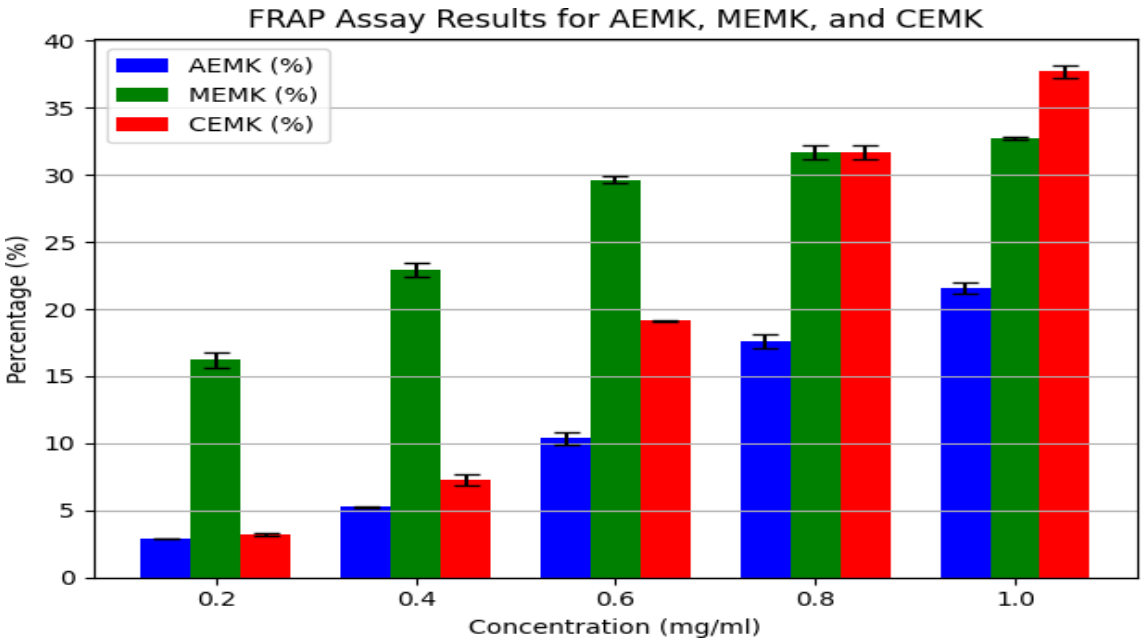
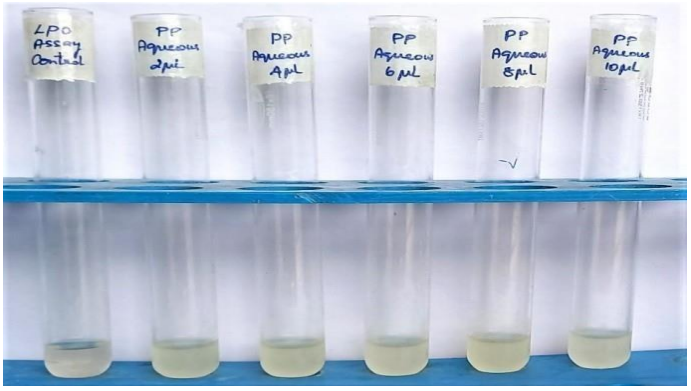


Figure 50 Graphical representation of FRAP assay using Aqueous, Chloroform and methanol extract of Murraya koenigii

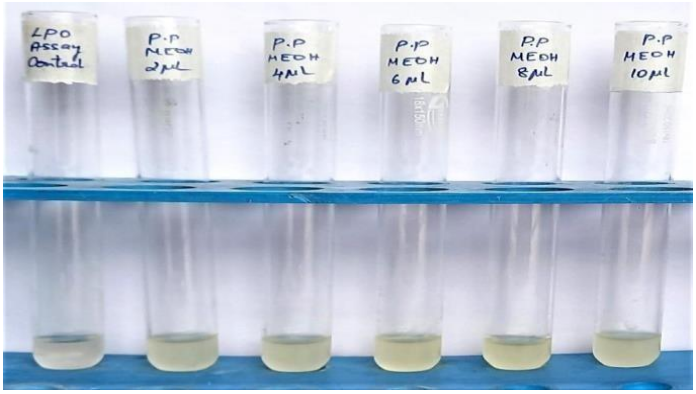
The literature indicates that the FRAP method is effective in assessing the total antioxidant capacity of fresh biological fluids, including plant homogenates and pharmacological plant products (Gohari et al., 2011). Ourfindings align with those of Sonter et al. (2021), who evaluated the total reducing capacity of Murraya extracts using both FRAP and reducing power assays and methanolic extract recorded highest value compared to acetone, water, chloroform, and hexane. The reducing power of a compound or extract reflects its ability to donate electrons. Our research clearly demonstrates that the methanolic extract of the leaves possesses a significant scavenging capacity, indicating a strong correlation between the high antioxidant capacity of the methanol extract and its total phenolic and flavonoid content (i.e., greater absorbance corresponds to enhanced antioxidant properties). A significant linear relationship was observed between total phenolic content and FRAP values. However, medicinal plant infusions that possess significant antioxidant potential and elevated antioxidant capacity can be regarded as valuable dietary sources of potent antioxidants (Katalinic et al., 2006). It is also observed that infusions prepared at the elevated temperature displayed an antioxidant capacity that was more than twice as high, as assessed exclusively by the FRAP method. (Katalinic et al., 2006)

4.4.4 ASSAY FOR SCAVENGING LIPID PEROXIDATION

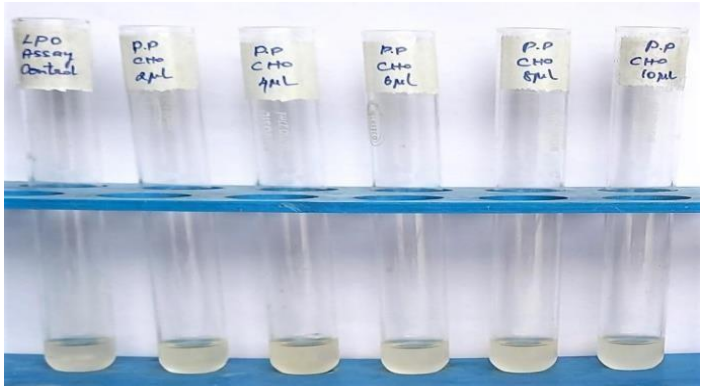
The investigation into the oxidation degradation of lipids was conducted utilizing aqueous, methanol, and chloroform extracts of Murraya koenigii. The findings indicated that the aqueous extract demonstrated alipid oxidation degradation level of 1.58±0.01, whereas the methanolic extract showed a degradation of 2.59±0.02. In contrast, the chloroform extract exhibited the most significant degradation at 14.07±0.05.These results imply that the chloroform extract possesses a superior ability to inhibit lipid oxidation when compared to both the methanol and aqueous extracts, as evidenced by the greater percentage of degradation recorded. It is likely that the varying concentrations of the extracts employed in the study affected the degree of lipid oxidation degradation observed(Fig 51 a,b,c).



(a) Aqueous extract



(b) Methanolic extract



(c) Chloroformic assay

Figure 51 Lipid peroxidation assay with different concentrations of all three extracts of Murraya koenigii

Table 9 In-Vitro LPO scavenging potential of Aqueous, Methanolic, and Chloroformic Extracts

CONC (mg/ml)	Ascorbic acid (%)	Aqueous (%)	Methanolic (%)	Chloroformic (%)
0.2	7.57 ± 0.02	0.13 ± 0.02	0.29 ± 0.02	1.73 ± 0.05
0.4	11.9 ± 0.6	0.28 ± 0.007	1.09 ± 0.03	3.5 ± 0.01
0.6	17.2 ± 0.81	0.73 ± 0.03	1.89 ± 0.05	5.26 ± 0.05
0.8	21.01 ± 0.5	1.16 ± 0.02	2.18 ± 0.03	11.09 ± 0.06
1	28.33 ± 0.1	1.58 ± 0.01	2.59 ± 0.02	14.07 ± 0.05
IC50	**2.63**	**0.87**	**0.52**	**0.74**

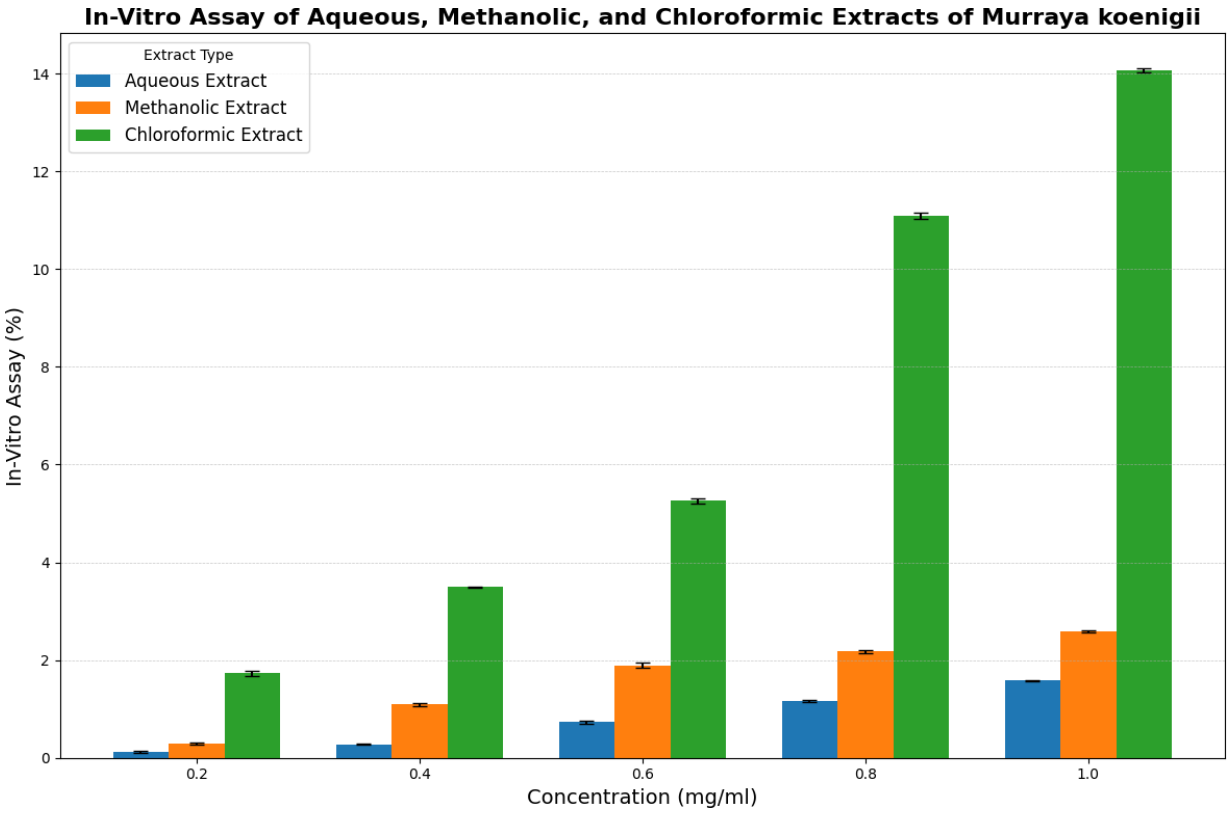


Figure 52 Graphical representation of Lipid peroxidation activity using aqueous, Chloroformic and methanolic extract of Murraya koenigii

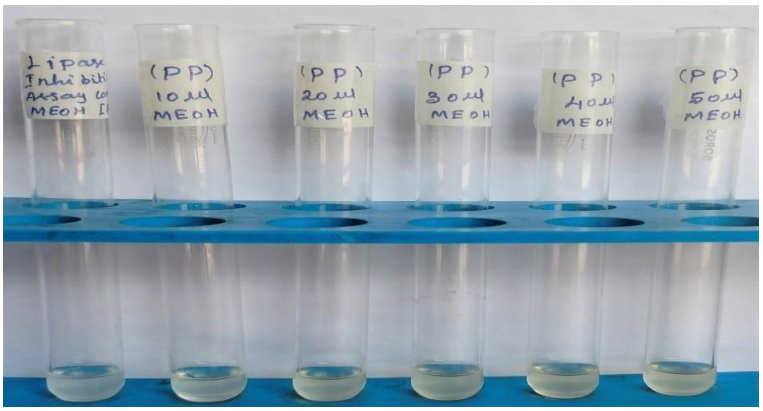
Lipid peroxidation of unsaturated fatty acids is frequently utilized as a marker for heightened oxidative stressand subsequent cytotoxic effects (Anwer et al., 2007). The objective of Temburne's research was to assessthe antioxidant efficacy of ethanolic extracts from Murraya koenigii leaves (MKL) by measuringThiobarbituric Acid Reactive Substances (TBARS) as an indicator of lipid peroxidation. The oraladministration of MKL extract resulted in a reduction of TBARS levels, signifying a decreased rate of lipidperoxidation. Consequently, our current findings, along with existing phytochemicalliterature (Iyer and Uma, 2008; Chakrabarty et al., 1997), suggest that the antioxidant carbazole alkaloids present in M. koenigii may play a role in stabilizing glycemic levels(Tembhurne & Sakarkar,

2010),Additionally, Saponin named diosgenin was found to lower serum levels of markers indicativeof cardiotoxicity, as well as cardiac levels of thiobarbituric acid reactive substances (TBARS) and reactive oxygen species (ROS) (C. Chen et al., 2015).

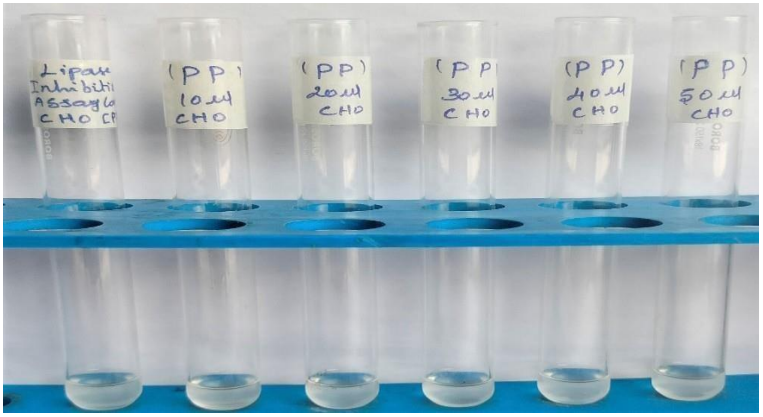
The hydroalcoholic extract of A. aspera was selected for investigation regarding its effects on haloperidol- induced parkinsonism in a Wistar albino model. Administered at a dosage of 400 mg/kg, the HA extract demonstrated a significant reduction (*$p \leq 0.01$) in the parkinsonism effects observed in the Wistar albinorats. The lower levels of lipid peroxidation (LPO) in the brains of the drug-treated groups, along with the increased activities of both enzymatic and non-enzymatic antioxidants, indicate that the extract effectively reduces oxidative stress(Chitra et al., 2017).

4.5 INVIVO ANTI HYPERLIPIDEMIC ACTIVITY OF MURAYYA KOENIGII - PANCREATIC LIPASE INHIBITION ASSAY

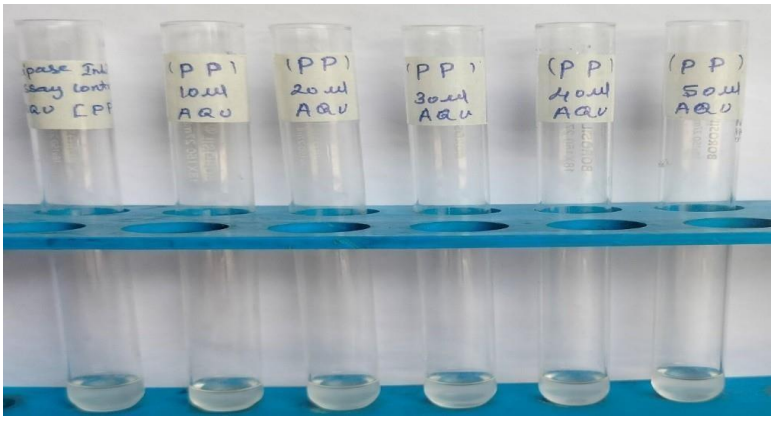
The activity of inhibition against pancreatic lipase enzyme was investigated using aqueous, methanol as well as chloroform extracts obtained from Murraya koenigii. The results demonstrated that the aqueous extract exhibited an inhibitory activity of 36.8±0.75% against pancreatic lipase, followed by methanolic extract that gave 51.8±0.04 % against pancreatic lipase, while the chloroform extract displayed highest inhibitionactivity of 53.4±0.04 % when compared to each other. In addition to that, the studied extracts' IC50 values were compared with the standard antilipase compound Orlistat, which has an IC50 value of 12.38 ± 2.3 µg/mL (Jaradat, 2017).The findings suggest that all extracts of Murraya koenigii possess the ability to inhibit pancreatic lipase enzyme, with the chloroformic extract showing a higher inhibitory potential compared to the methanol and aqueous extracts across the tested range of concentrations (Fig56,57,58). The observed differences in inhibitory activity could be attributed to differences concentrations of bioactive Compounds found in the extracts. Further investigations are necessary to determine and isolate the specific compounds responsible for this activity



(a) Methanolic extract



(b) Chloroform extract



(c) Aqueous extract

Figure 53 Pancreatic Lipase Inhibition assay of different extracts of Murraya koenigii

Table 10 : Optical density at 405 nm and % inhibition calculation with Aqueous, chloroform and methanol extract of Murraya koenigii

Concentration (mg/ml)	**O.D of Test (Aqueous)**	**O.D of Control**	**O.D Control - O.D Test**	**% Inhibition**
Control (no sample)	0.453	0.453	0	0.00
0.2	0.411	0.453	0.042	9.27
0.4	0.399	0.453	0.054	11.92
0.6	0.362	0.453	0.091	20.09
0.8	0.324	0.453	0.129	28.48
1	0.288	0.453	0.165	36.42
Concentration (mg/ml)	**O.D of Test (Chloroform)**	**O.D of Control**	**O.D Control - O.D Test**	**% Inhibition**
Control (no sample)	0.453	0.453	0	0.00
0.2	0.353	0.453	0.1	22.08
0.4	0.304	0.453	0.149	32.89
0.6	0.275	0.453	0.178	39.29
0.8	0.251	0.453	0.202	44.59
1	0.211	0.453	0.242	53.42
Concentration (mg/ml)	**O.D of Test (Methanol)**	**O.D of Control**	**O.D Control - O.D Test**	**% Inhibition**
Control (no sample)	0.453	0.453	0	0.00
0.2	0.361	0.453	0.092	20.31
0.4	0.337	0.453	0.116	25.61
0.6	0.318	0.453	0.135	29.80
0.8	0.275	0.453	0.178	39.29
1	0.218	0.453	0.235	51.88

Table 11 Pancreatic lipase activity of standard Orlistat.

Standard	Concentration (µg/ml)	OD at 410 nm	% Inhibition
Blank	-	0.00	-
Control	-	0.453	-
ORLISTAT	200	0.704	89.1
	400	0.727	92
	600	0.750	95
	800	0.758	96
	1000	0.774	98

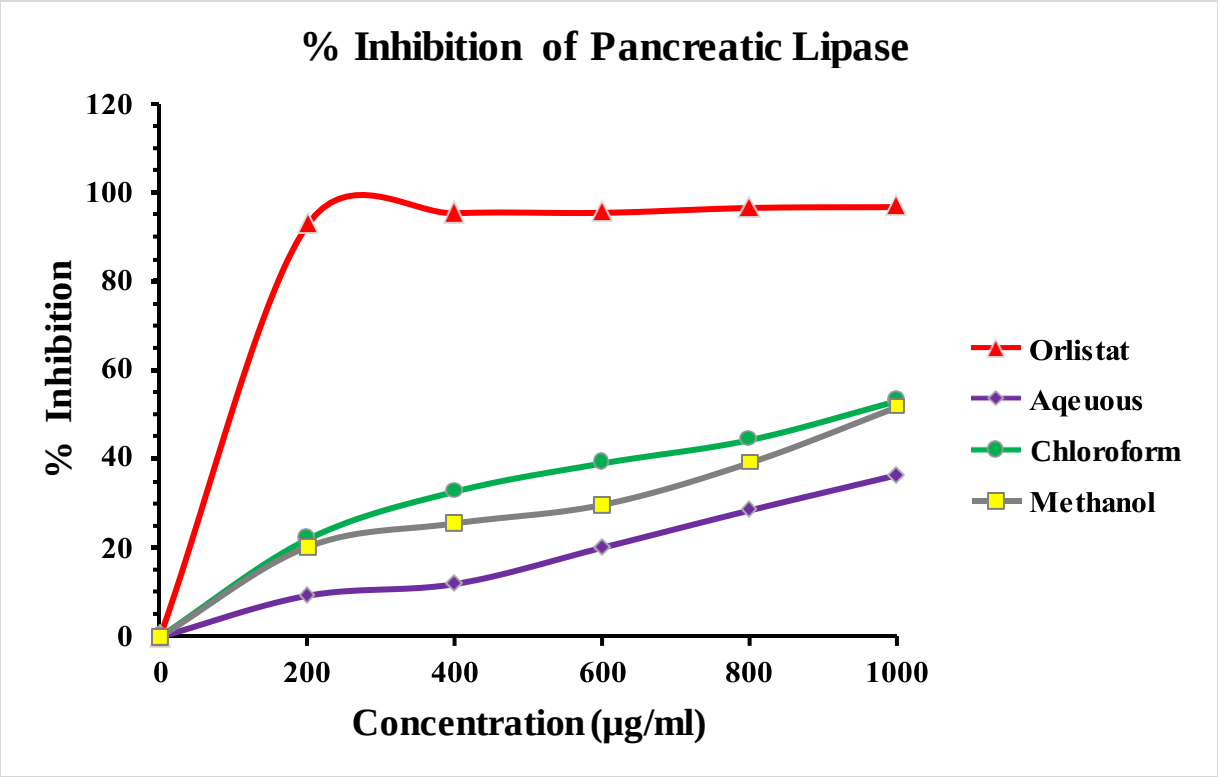


Figure 54 Graphical representation of Pancreatic lipase inhibition assay of chloroformic, methanolic and aqueous extracts in comparison with standard orlistat.

Table 12 In- Vitro Pancreatic Lipase Inhibition assay of Aqueous, Chloroformic and Methanolic extracts of Murraya koenigii

Conc (mg/ml)	Orlistat (%Inhibition)	Aqueous (%Inhibition)	Chloroform (%Inhibition)	Methanol (%Inhibition)
0.2	89.1 ± 0.13	8.53 ± 1.2	22.00 ± 0.01	20.30 ± 0.00
0.4	92 ± 0.05	12.30 ± 0.33	32.80 ± 0.00	25.60 ± 0.00
0.6	95 ± 0.01	18.50 ± 1.3	39.30 ± 0.015	29.80 ± 0.00
0.8	96 ± 0.16	27.60 ± 1.29	44.60 ± 0.01	39.29 ± 0.05
1	98 ± 0.17	36.80 ± 0.75	53.40 ± 0.04	51.80 ± 0.04
IC 50	0.73	0.82	2.77	3.45

The chloroformic extract of Murraya koenigii showed an activity of 53.42% at a concentration of 1 mg/ml, proving to be most effective in inhibiting pancreatic lipase followed by methanolic extract that reported 51.88% . The aqueous extract does show inhibitory activity of 36.42% but to a lesser extent. The results demonstrated that M.K extracts inhibits pancreatic lipase in a concentration dependent manner; the inhibition % for chloroformic extract ranged from 22% ± 1.2 to 53.42% ± 1.5 from the lowest to the highest concentration (1000 µg/ml). The chloroformic extract in vitro inhibition of pancreatic lipase could be attributed to the presence of polyphenols and flavonoid compounds.

Our findings align with the data reported by Birari et al. (2009), Rani et al., and Gaur et al., supporting the notion that Murraya koenigii may serve as a viable alternative to synthetic medications for addressing dyslipidemia. The increasing global interest in traditionally utilized edible plants is noteworthy. The phytochemicals found in these plant extracts may contribute to their pancreatic lipase activity, as indicatedby Ong et al. (2014). Numerous prior studies have demonstrated that flavonoids and phenolic compounds exhibit inhibitory effects by interacting with the enzyme-substrate complex, thereby reducing the rate of lipid absorption (Jaradat, 2017). Phatak et al. (2019) attributed the antihyperlipidemic effects of Murraya koenigii to the presence of bioactive compounds such as saponins, alkaloids, and flavonoids. However, the mere presence of these phytochemicals does not guarantee lipid reduction; rather, it is their concentration that significantly influences their anti-lipidemic properties (Zheng et al., 2012). Polyphenols have been suggested to induce conformational changes in the lipase enzyme structure, with tyrosine and tryptophan being the primary amino acids involved in binding(Oluwagunwa et al., 2021).

The lipolysis process within adipose tissue fat cells is initiated by fluctuations in cAMP levels, which subsequently activate protein kinase A and its substrates, including hormone-sensitive lipase and perilipin (Boccellino & Angelo, 2020). Among these, hormone-sensitive lipase plays a crucial role in the mobilizationof fats. Additionally, calcium and colipase are known to enhance the stability of pancreatic lipase by maintaining the association of the heterodimer. Furthermore, the use of herbal drugs such as Murraya koenigii has been shown to induce the

dissociation of this enzyme heterodimer (Bello et al., 2017). This hypothesis aligns with the current study's findings, which indicate that extracts from less polar solvents exhibit a slightly superior lipolytic effect compared to those from polar solvents(Effect et al., 2021) .

Numerous edible plants have the potential to serve as alternatives to Orlistat in obesity management, as they exhibit anti-lipase properties (Jaradat, 2017). The findings indicate that the chloroform extract of Murraya koenigii may function as a natural inhibitor of pancreatic lipase, positioning it as a novel candidate in the treatment of obesity. These plants can be safely incorporated into daily diets, utilized as natural supplements for the prevention or treatment of obesity, and employed in managing hyperlipidemia.

A comparative analysis of pancreatic lipase inhibition was conducted using three different extracts from the leaves of Murraya koenigii. The chloroform extract demonstrated the highest efficacy, suggesting its potential as a powerful anti-obesity agent in addressing hyperlipidemia. It is hypothesized that the presence of tannins, saponins, and flavonoids in this plant significantly contributes to its anti-hyperlipidemic effects. Ongoing research aims to identify the bioactive phytochemicals responsible for the anti-obesity effects of Murraya koenigii, with the goal of elucidating the inhibition mechanisms and clinical applications associatedwith this plant.

4.6 ANIMAL EXPERIMENTATION

In this study, Wistar rats of either sex weighing between 240-350 grams were obtained from the LACSMI BIOFARMS, Pune, India. The rats were housed in propylene cages covered with husk and maintained undergood conditions, including a 12 -hour light/dark cycle at a temperature of 25±2 ^{o}C and a relative humidity of55±10%. Throughout the experiment, the rats were given a regular pellet meal and had free access to water. The Institutional Animal Ethics Committee (IAEC) of Goa University(Zoology), Goa, India, authorized the experimental procedure with the protocol number (Approval no: GUZ/IAEC/23-24/N1, Project code no.IAEC/23-24/N1).

4.6.1 ACUTE TOXICITY STUDY

Acute oral toxicity Limit test was performed according to the OECD test guideline 423- Acute

toxic class method (OECD guideline, 2002) following the annexure 2d. According to the guidelines established by the OECD, when available data indicates that mortality is improbable at the maximum starting dose of 2000 mg/kg body weight, a limit test should be performed. This limit test is primarily applicable in scenarios where prior information suggests that the test substance is likely to be non-toxic, meaning it exhibits toxicity only at doses exceeding regulatory limits. A limit test at a single dose level of 2000 mg/kg body weight can be conducted using six animals (three per step), adhering to the protocol outlined in annexure 2d. Young healthy female Wistar rats (240-350g, b. wt.) were divided into three groups of comprising of three animals each. The Murraya Koenigii chloroformic extracts were administered once orally with the maximum dose of 2000 mg/kg body weight. The findings of this study indicated that the chloroformic extract does not induce acute toxicological effects, and no fatalities occurred among the rats.

4.6.1.1 GENERAL SIGNS AND BEHAVIOURAL ANALYSIS

Animals fed with 2000 mg/kg body weight /day of chloroformic extract did not cause mortality or any clinical signs of acute toxicity within 24 h and within a 14-day period. Although the locomotor activities of the mice fed with 2000 mg/kg body weight/day were slightly reduced and decreased food intake or anorexia was observed on day 1 post treatment. Nevertheless, this group of animals recovered quickly and no mortality was found up to 14 days. The group receiving 200 mg/kg body weight/day of chloroformic extract did not show any behavioral changes. No change in skin, eye colour, tremors, urination fluctuation, ataxia, diarrhea was noted in both the groups. LD50 of the test drug was found to be greater than 2000 mg/kg b. wt, and was found to be safe when administered once orally to fasting female Wistar rats (Table 13)

Table 13 General Appearance And Behavioral Observations Of Acute Toxicity Study For Control And Treated Groups

OBSERVATION	G1 (CONTROL)			G2 (2000mg/kg/bw)			G3 (200mg/kg/bw)		
	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14	Day 1	Day 7	Day 14
Ataxia/Convulsions/ Tremors	×	×	×	×	×	×	×	×	×
Temperature	×	×	×	×	×	×	×	×	×
Anorexia	×	×	×	✓	×	×	×	×	×
Urination	×	×	×	×	×	×	×	×	×
Rate of Respiration	×	×	×	×	×	×	×	×	×
Change in skin/eye color	×	×	×	×	×	×	×	×	×
Hypoactivity	×	×	×	✓	✓	×	×	×	×
Diarrhea	×	×	×	×	×	×	×	×	×
General health- Nasal discharge, Salivation, Dyspnea	×	×	×	×	×	×	×	×	×
Death	×	×	×	×	×	×	×	×	×

4.6.1.2 EFFECT ON BODY WEIGHT

The baseline body weight of all the rats included in the acute toxicity study group were in the range of 220-320 gms. On day 7 and at the end of the experimental period, the body weight of the three

group of rats were significantly reduced in contrast to the control group of rats (Table 14). Although, the control and treated rats appeared healthy at the end of the experiment, there was anorexia observed in the rats treated with maximum dose 2000 mg/kg bw on day 1 post dosage. According to the data obtained, there was no weight loss in any of the groups. The percentage changes in body weight of the chloroformic extract-treated groups were not substantially different compared to the control rats ($p > 0.05$). Generally, variations in the body weight of animals are utilized as indicators of potential adverse drug effects (Kharchoufa et al., 2020). The body weight gain of female rats receiving 200 mg/kg body weight was lower than that of the control group, with gains of 16 grams compared to 23 grams in the control group. This weight reduction may be linked to a decrease in appetite, likely due to a diminished food efficiency ratio (Adebajo et al., 2006). Changes in organ weight can reflect changes in body weight, so it's common to calculate the relative organ weight (organ to body weight ratio). However, this doesn't fully control for the dependence on body weight(Lazic, Semenova, and Williams 2020). Additionally, the relative weight of organs can indicate possible organ damage (Lazic et al., 2020). Analysis was done based on the wet weight (absolute weight) and body weight at necropsy, the organ-to-body weight ratio (relative weight) was calculated. The absolute weights of the liver and kidneys did not show any difference between the normal controls and treated groups($p > 0.05$) (Table 14 and 15).

Table 14 Body weight of the animals of the acute toxicity study group

GRP	Treatment	Body Weight (grams)		
		Day 1	**Day 7**	**Day 14**
I	CONTROL (N.S)	287.33 ± 43.14	298.33 ± 47.24	310.66 ± 54.37
II	MK Chloroformic extract (2000 mg/kg b.wt.)	271.66 ± 37.50	278.00 ± 36.36	290.00 ± 34.64
III	MK Chloroformic extract (200 mg/kg b.wt.)	238.66 ± 10.01	246.00 ± 11.53	254.33 ± 14.97

Values expressed in Mean±SD; n=3

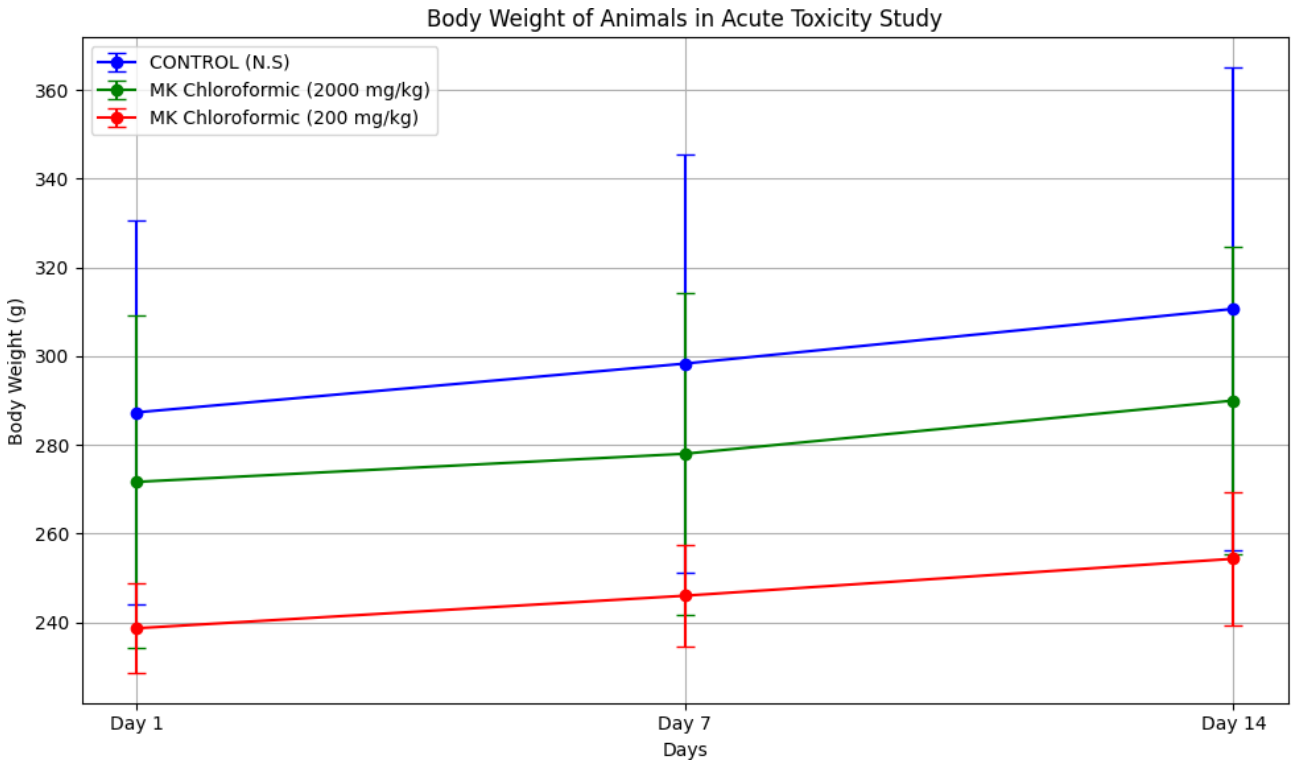


Figure 55 Body weight of Animal in Acute Toxicity Study

Table 15 Absolute and Relative organ weight of rats in acute oral toxicity study

PARAMETERS	**G1 (CONTROL)**	**G2 (2000 mg/kg/bw)**	**G3 (200 mg/kg/bw)**
ABSOLUTE ORGAN WEIGHTS			
LIVER (gms)	10.7 ± 0.40	10.5 ± 0.30	10.5 ± 0.05
RT. KIDNEY (gms)	0.94 ± 0.04	0.89 ± 0.02	0.89 ± 0.01
RELATIVE ORGAN WEIGHTS			
BODY WT (gms)	310.6 ± 54.3	290 ± 34.64	261 ± 10.5
LIVER (gms)	3.51 ± 0.60	3.59 ± 0.28	4.03 ± 0.15
RT. KIDNEY (gms)	0.30 ± 0.04	0.30 ± 0.03	0.34 ± 0.02

*Values expressed as a mean ± standard deviation. *p-value less than 0.05, ($p < 0.05$) significant value*

4.6.1.3 EFFECT OF THE EXTRACT ON SERUM BIOCHEMICAL MARKERS IN ACUTE ORAL TOXICITY TESTING

Table 16 shows the biochemical parameter data for the control and chloroformic extract-treated groups in the acute oral toxicity test. There was no significant difference found in the biochemical parameters between groups ($p > 0.05$). Murraya koenigii chloroformic extract attenuated the increase in urea concentration in blood at the 2000mg/kg dose however, chloroformic extract 200 mg/kg treatment did not produce any significant change as compared to normal controls. There were no profound variations in glucose, creatinine, liver enzymes, total protein, albumin, and lipid profile in the treated group.

Table 16 Biochemical characteristics of rats in acute oral toxicity tests

PARAMETERS	UNITS	G1(CONTROL)	G2(2000mg/kg)	G3(200 mg/kg)
GLUCOSE	mg/dl	98 ± 2.51	106± 11.06	96± 7.0
UREA	mg/dl	32 ±7.57	33.66± 4.04	36± 5.2
CREATININE	mg/dl	0.5 ±0.06	0.48± 0.06	0.46± 0.11
SGOT(AST)	U/L	163 ±36.9	191± 6.02	187± 9.29
SGPT(ALT)	U/L	72 ±4.04	98.66± 12.50	68.6± 7.23
ALP	U/L	141 ±28.5	131.3± 8.02	156± 20.4
T. PROTEIN	gm/dl	7.03 ±0.20	7.06± 0.05	7.3± 0.15
ALBUMIN	gm/dl	3.66 ±0.23	3.4± 0.05	3.9± 0.11
CHOLESTROL	mg/dl	46 ±4.0	52± 3.0	50.3± 5.5
TRIGLYCERIDES	mg/dl	76 ±4.58	82± 5.19	88± 8.5
HDL CHOL	mg/dl	16 ±5.56	22.3± 2.09	23± 5.56

Values expressed as a mean ± standard deviation

4.6.1.4 EFFECT OF EXTRACT ON HEMATOLOGICAL PARAMETERS IN ACUTE ORAL TOXICITY TESTS

Blood parameters which includes Total white blood cell count, Differential count of Neutrophils, Lymphocytes and eosinophils, Hemoglobin, Platelet count, RBC count and Packed cell volume in rats treated with acute MK dose of 2000 mg/kg bw and 200 mg/kg bw along with control group(N.S) were analyzed and results shown in table 17. There was no significant difference in hematological parameters between groups ($p > 0.05$; Table 17). The results showed that all hematological indicators, including hemoglobin (HB) and total white blood cell count, were within normal limits in both the control and MK -treated groups. In the ANOVA test, there is no significant correlation between the groups in acute test ($p > 0.05$).

Table 17 Hematological parameters of the rats in acute oral toxicity tests

PARAMETERS	G1 (NS 10ml/kg)	G2 (2000mg/kg/bw)	G3 (200mg/kg/bw)
TOTAL WBC (10^9/L)	10.6 ±1.03	11.7±7.37	9.2 ±1.24
NEUTROPHILS (%)	13.9 ±1.84	17.3±2.08	27.8 ±2.25
LYMPHOCYTES (%)	85.9 ±1.81	81.9 ±2.58	71.3 ±2.25
EOSINOPHILS (%)	0.16±0.05	0.7 ±0.52	0.86 ±0.23
HEMOGLOBIN (gm%)	13.9±0.15	13.7 ±0.49	14.7 ±1.21
PLATELETS (109/L)	9.0 ±0.63	8.0±0.54	9.0±1.39
RBC (10^{12}/L)	8.13 ±0.18	8.25 ±0.61	8.59±0.98
PCV (%)	41.6 ±0.52	41.4 ±1.15	45.0±3.86

Values expressed as a mean ± standard deviation

4.6.1.5 HISTOPATHOLOGICAL EXAMINATION OF LIVER AND KIDNEYS.

Table 18 Gross Histopathological Examination Of Liver And Kidneys.

STEP	TREATMENT	ORGANS	OBSERVATIONS
I	N.SALINE (10 ml/kg b.wt)	LIVER, KIDNEY	Liver shows normal architecture. (Fig 56a) Kidney shows normal architecture. (Fig 57a)
II	MK extract - (2000 mg/kg b.wt.)	LIVER, KIDNEY	Liver tissue shows infiltration of lymphocytes. (Fig 56b) Kidney tubules show slight oedema, no necrosis. (Fig 57b)
III	MK extract - (200 mg/kg b.wt.)	LIVER AND KIDNEY	No abnormality observed in liver (Fig 56c) and kidneys (Fig 57c).

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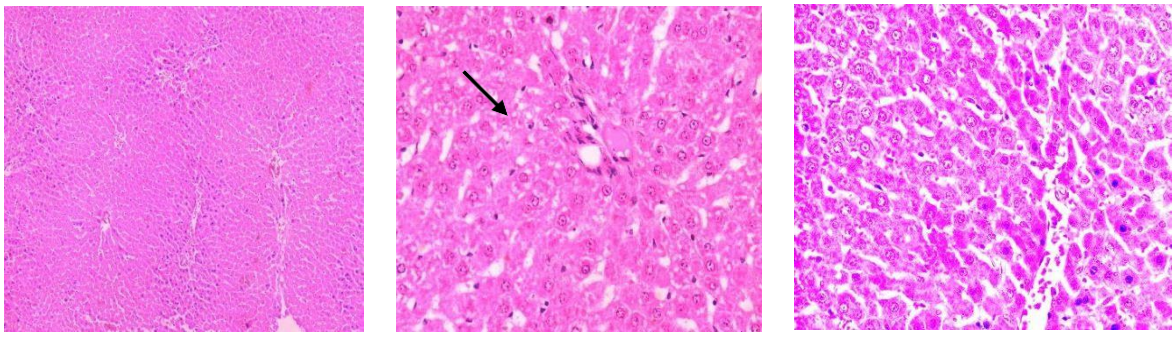
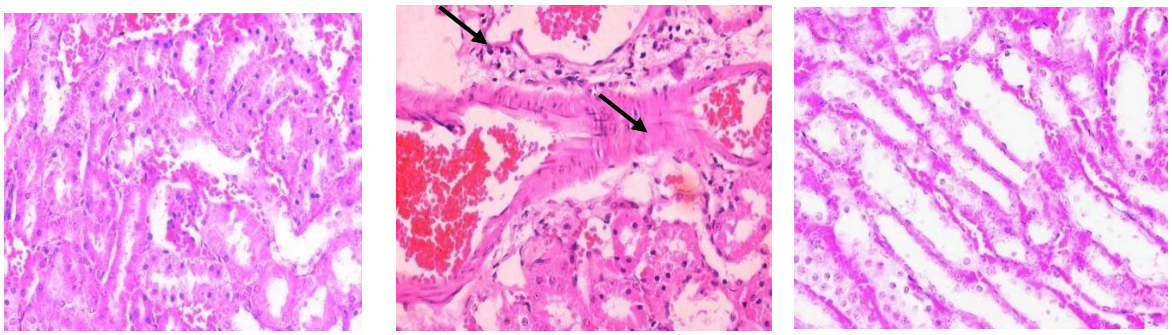


(a) **(b)** **(c)**

Figure 56 Effect of acute oral dose of chloroformic extract of Murraya koenigii on Liver. The number on the images represent the treatment groups. (a)Control rats,(b)rats treated with2000 mg/kg bw,(c)rats treated with 200 mg/kg bw.



(a) (b) (c)

Figure 57 Effect of acute oral dose of chloroformic extract of Murraya Koenigii on Kidney. The number on the images represent the treatment groups. (a)Control rats, (b)rats treated with2000 mg/kg bw, (c)rats treated with 200 mg/kg bw. Arrow Points at lymphocytic infiltration and edema.

In accordance with OECD guideline 423, the outcomes of this test facilitate the grading and classification of the substance using the Globally Harmonised System of Classification and Labelling of Chemicals. Consequently, the MK chloroformic extract is categorized as category 5, indicating a low risk of acute toxicity, which represents the lowest toxicity classification (Guideline et al., 2001). Murraya koenigii crude extract was tolerated at a dose of 2000 mg/kg body weight when administered as a single dose (Ramaswamy et al., 2012). Consequently, the present study was designed to examine the acute toxicity of the chloroform extract in rats, initiating with a dose of 200 mg/kg, which is considered effective for evaluating toxic effects in this investigation. In our investigation, the treated rats with acute dose of 2000mg/kg bw Chloroformic extract of Murraya koenigii developed an increase in total white blood cell count but statistically insignificant as the WBC count may vary and the normal reference range being so vast.(Delwatta et al. 2018) Histological examination of kidneys and liver organs treated acutely with 2000 mg/kg bw showed slight edema in the kidney and simultaneously infiltration of lymphocytes was observed in liver, although the normal architecture was maintained. Mild hypo activity was also noted with 2000 mg/kg bw of the extract. However, there was no mortality observed at any dose levels. The chloroformic extract of Murraya koenigii was found to be safe upto 2000mg/kg of body weight of Wistar rats(Maximum tolerated dose- MTD by the acute toxicity mode study as per OECD guidelines. Based on this as well as previous literature, four dose levels were selected for

the assessment of anti-hyperlipidemic activity of the Murraya koenigii chloroformic extract as shown in the table.

Table 19 Selection of effective dose

Sample	Maximum Tolerated Dose (mg/kg)	Selected Max Effective Dose (mg/kg)	Second Dose (Double the Selected Dose) (mg/kg)	Third Dose (Double the Previous Dose) (mg/kg)	Fourth Dose (Double the Previous Dose) (mg/kg)
Chloroform extract of Murraya koenigii	2000	100	200	400	800

4.6.2 PHARMACODYNAMIC STUDY

4.6.2.1 EFFECT OF TREATMENT ON BODY WEIGHT

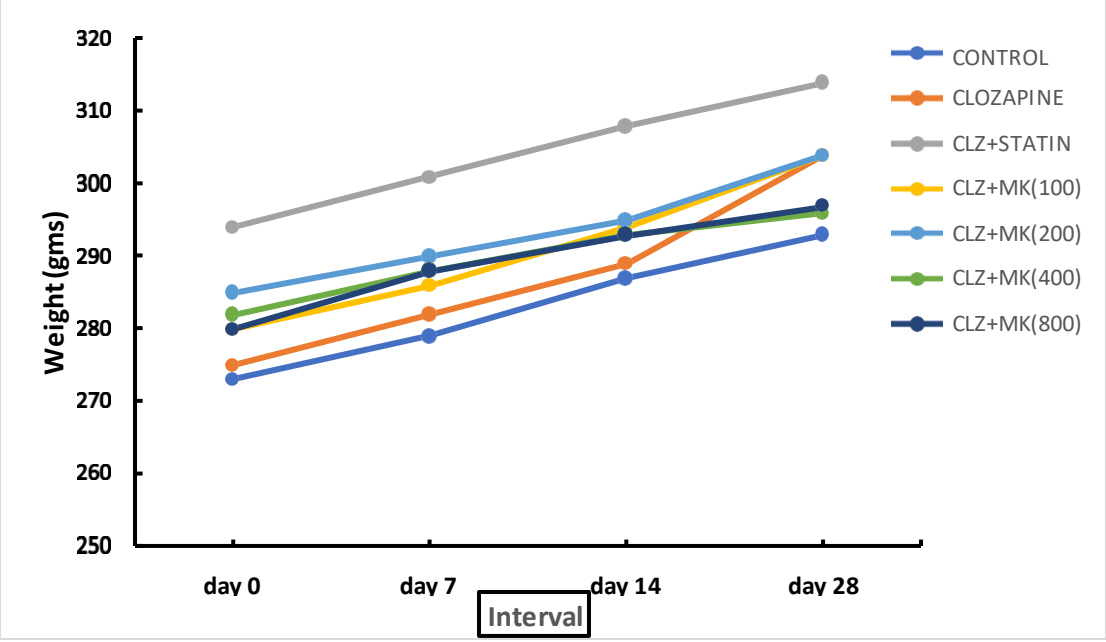


Figure 58 Effects of different doses of chloroformic extract of Murraya koenigii treatment on body weight of rats.

Table 20 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Body weight. Values represent the Mean± SEM of six rats in each group (n=6). Statistical analysis was performed using one-way analysis of variance. *= p<0.05 compared to the clozapine control

INTERVAL	GRP 1 (N.SALINE)	GRP 2 (CLZ)	GRP 3 (CLZ+ STAT)	GRP 4 (CLZ + MK100)	GRP 5 (CLZ + MK100)	GRP 6 (CLZ + MK100)	GRP 7 (CLZ + MK100)
DAY 0	273.3 ± 21.3	275.0 ± 22.46	294.5 ± 12.2	280.3 ± 21.7	285.0 ± 18.36	282.16 ± 20.4	280.3 ± 26.2
DAY 7	279.0 ± 20.4	282.16 ± 21.8	301.0 ± 11.66	286.8 ± 22.1	290.0 ± 18.4	288.3 ± 19.69	288.1 ± 26.8
DAY 14	287.3 ± 18.2	289.16 ± 21.1	308.5 ± 10.67	294.5 ± 22.0	295.0 ± 17.36	293.8 ± 20.06	293.6 ± 25.7
DAY 28	293.3 ± 18.3	304.0 ± 20.58	314.16 ± 10.1	304.0 ± 22.02	304.5 ± 17.2 *	296.8 ± 20.6 *	297.5 ± 26.4 *

The baseline body weight of all the rats included in the groups for evaluation of anti hyperlipidemic property study were noted as in table 19 in the range of 250-350 gms. From initial treatment on day 1 and till day 14, there was no significant difference between groups in body weight. Day 14 onwards clozapine-treated rats showed a significant increase in body weight upto 30 gms as compared to all other groups .Day 28 revealed that the Standard group III , Treatment group VI and VII showed no significant weight gain throughout the next 14 days of treatment(Table 20). It is very important to note that the amelioration in the increment of the body weight was observed in the groups that received CEMK 400 mg/kg bw (Group VI) and CEMK 800 mg/kg bw (Group VII). According to the Table19 the percentage increase of body weight after treatment with Statin as well as Murraya koenigii in each group from Group III onwards were in the order of 5.6%, 9.5%, 9.5%, 3.0%, and 3.9% respectively which are significant also. Hence from this it is appear that high and medium dose is able to protect the body from weight gain because of the drug clozapine even better than standard drug statin.

Birari et al. (2010) demonstrated the anti-obesity and lipid-lowering properties of dichloromethane (MKD), ethyl acetate (MKE), and MKM extracts in a model of obesity induced by a high-fat diet. Continuous administration of MKD and MKE at a dosage of 300 mg/kg/day via oral route for two weeks resulted in a significant reduction ($p \leq 0.05$) in weight gain compared to the weight gain observed in rats solely fed a high-fat diet (Birari et al., 2010). A similar decrease in body weight was noted following treatment with the extracts, with weights decreasing from 62.3 ± 0.6 g on day 0 to 57.2 ± 0.9 g on day 10. No significant adverse effects were reported in any of the animals following treatment with either the extract or the vehicle. Autopsy results indicated that animals in the herbal treatment group exhibited some degree of gut adhesion, suggesting local irritation following the extract administration (Xie et al., 2006). Conversely, Reynaldi et al. (2021) found no significant change in the average body weight of high-fat diet-fed rats ($p = 0.576$) when treated with crude Murraya koenigii extract at doses of 200, 300, and 400 mg/kg body weight. The curry leaf extract is known to contain flavonoids, tannins, and saponins (Pinto et al., 2024). Flavonoids may enhance the activity of the lipoprotein lipase enzyme, facilitating the hydrolysis of triglycerides into free fatty acids, thereby reducing triglyceride levels in the body. Alkaloids have been shown to diminish the activity of pancreatic lipase, which can lead to a reduction in triglycerides absorbed from the small intestine (Oluwagunwa et al., 2021). Saponins exhibit antihypertriglyceridemic effects by promoting the excretion of triglycerides through feces (Vinarova et al., 2015a). Research conducted by Tembhurne et al. confirmed that administration of curry leaf extract at a dose of 300 mg/kg body weight for 15 days in male Wistar rats significantly lowered triglyceride levels (Tembhurne & Sakarkar, 2012). In contrast, Li et al. (2017) investigated the potential effects of artesunate on metabolic changes induced by clozapine in rats treated with saline. The treatment had no effect on weight gain and caused no hyperglycemia, hyperinsulinemia(Li et al. 2017)

4.6.2.2 EFFECT ON BIOCHEMICAL PARAMETERS.

4.6.2.2.1 EFFECT ON TREATMENT OF BLOOD GLUCOSE

Table 21 Effect of different doses of chloroformic extract of Murraya koenigii leaves on fasting glucose levels in clozapine treated rats. The results are presented as mean± SD (n=6)

Group	GRP 1 (N.S)	GRP 2 (CLZ)	GRP 3 (CLZ + Statin)	GRP 4 (CLZ + MK100)	GRP 5 (CLZ + MK200)	GRP 6 (CLZ + MK400)	GRP 7 (CLZ + MK800)
Glucose (mg/dl)	93 ± 3.6	99 ±1.52	91 ± 3.78	90 ± 3.7	98 ± 2.0	97 ± 4.58	94 ± 4.0

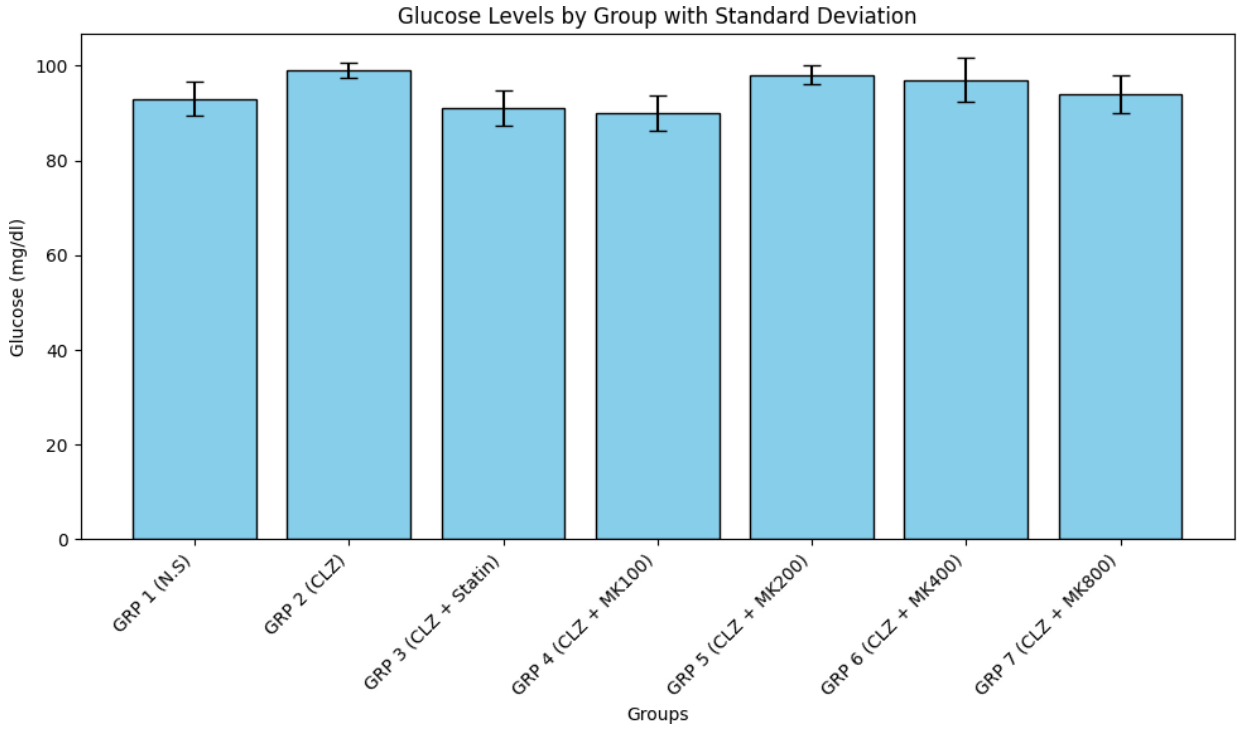


Figure 47 Effects of treatment on blood glucose, Values represent the Mean± SEM of six rats in each group (n=6).

On day 0, the blood glucose levels of all rats in the study were found to be within the normal range, as they had not received any drug treatment. However, a slight increase in blood glucose was noted in the rats administered clozapine on the 28th day. A decrease was observed in groups III (91 ± 3.78) and IV (90 ± 3.7), respectively. It is crucial to highlight that all treated groups demonstrated improvements in blood glucose levels, although all values remained within the normal range established for rats. A comparable trend in glucose levels was reported by Li et al. (2017), where treatment with Artesunate did not affect weight gain and did not induce hyperglycemia or hyperinsulinemia in rats treated with clozapine. Chronic administration of the atypical antipsychotic clozapine has been linked to an increased risk of glucose homeostasis deterioration, resulting in hyperglycemia and insulin resistance. Sadik et al., conducted a study in which a group

received clozapine orally at a dosage of 10 mg/kg body weight daily for six weeks, exhibited hyperglycemia, hyperinsulinemia, and insulin resistance compared to the control group. This disruption in glucose regulation was associated with non-significant changes in body weight. Thus, concluding that clozapine administration leads to hyperglycemia and insulin resistance in a manner mostly independent of weight gain, and may be attributed to an increase in hepatic phosphorylase activity and increased expression level of G6Pase.(El-seweidy et al. 2014)

4.6.2.2.2 EFFECT ON RENAL FUNCTION TESTS

Urea and Creatinine were estimated in rats of group received clozapine drug, standard group receiving clozapine and statin, normal as well as in treatment groups.

Table 22 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Urea and Creatinine in clozapine treated rats The results are presented as mean±SD of six rats in each group (n=6). Statistical analysis was performed using one-way analysis of variance with post-hoc testing. *= *p<0.05*.

Treatment Group	GRP 1 (N.S)	GRP 2 (CLZ)	GRP 3 (CLZ + Statin)	GRP 4 (CLZ + MK100)	GRP 5 (CLZ + MK200)	GRP 6 (CLZ + MK400)	GRP 7 (CLZ + MK800)
UREA (mg/dl)	43 ± 4.7	50 ± 2.5 *	44 ± 5.0	42 ± 4.9	42 ± 3.0	41 ± 3.7	41 ± 3.6
CREAT (mg/dl)	3.7 ± 0.05	3.4 ± 0.0	3.6 ± 0.03	3.6 ±0.02	3.4 ± 0.04	3.3 ± 0.0	3.4 ± 0.0

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Blood urea nitrogen (BUN) and creatinine (Cr) are widely recognized indicators of kidney function and the structural integrity of renal tissues (Mahipal & Pawar, 2017). Our investigation clearly shows an increase in the mean urea level in the group II(Clozapine) in comparison to the normal control group I(Table 21). "In our investigation, the elevated urea levels observed in rats treated with clozapine indicated renal toxicity." This increase in BUN levels may be attributed to damage inflicted on the kidney tubules, as evidenced by significant alterations in kidney tissues when compared to the control group. Administration of CEMK at low doses of 100 and 200 mg/kg body

weight resulted in a significant reduction ($P < 0.01$) in urea levels among the rats in comparison to the clozapine group(Grp II). However, no significant changes in the creatinine values were observed between the controls and treated group as $p>0.05$. One of clozapine's unrecognized potential side effects is renal insufficiency and nephritis. Numerous case reports indicate that some patients experience a decline in kidney function, necessitating the cessation of clozapine treatment, even when there is a significant therapeutic response to a low dosage (Davis & Kelly, 2019).

In a separate investigation, mice were subjected to a high-fat diet (HFD) for a duration of 10 weeks. The clozapine group was administered 2 mg/kg/day of oral clozapine for 8 weeks concurrently with the HFD. The clozapine group exhibited significantly elevated serum levels of creatinine and blood urea nitrogen (BUN), with increases of 2.3-fold and 2.0-fold, respectively. The onset of renal damage is associated with a reduction in antioxidant enzymes within the kidneys; this diminished antioxidant activity may lead to necrosis and compromised renal function. Consequently, it can be inferred that prolonged use of clozapine contributes to kidney damage, which is characterized by the infiltration of inflammatory cells into the renal interstitium and heightened levels of key nephropathy markers, including BUN and creatinine. The protective effects of M. koenigii were shown to induce significant dose-dependent reductions in serum urea and creatinine levels, also confirmed through histological examinations of the kidneys from these subjects revealed comparable tissue regeneration attributed to the aqueous extract(Yankuzo et al., 2011).

Table 23 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Protein and Albumin content in clozapine treated rats. The results are presented as mean±SD (n=6)

Treatment Group	GRP 1 (N.S)	GRP 2 (CLZ)	GRP 3 (CLZ + Statin)	GRP 4 (CLZ + MK100)	GRP 5 (CLZ + MK200)	GRP 6 (CLZ + MK400)	GRP 7 (CLZ + MK800)
Total Protein (mg/dl)	7 ± 0.2	6.5 ± 0.2	6.9 ±0.15	6.9 ± 0.1	6.5 ± 0.3	6.5 ± 0.15	6.6 ± 0.1
Albumin (mg/dl)	3.6 ±0.23	3.2 ±0.05	3.4 ± 0.2	3.5 ± 0.2	3.3 ± 0.15	3.1 ± 0.1	3.1 ± 0.1

Total protein and albumin estimations are a part of liver function tests. Total Protein and serum albumin concentrations (Table 23) in the Murraya koenigii treated groups did not show any significant variations (P > 0.05) when compared with the control group. Similarly, the clozapine group(II) also did not show marked difference in both total protein and albumin values in comparison to control group (I). The values for protein and albumin were well within the normal test range(Delwatta et al. 2018).

4.6.2.2.3 EFFECT ON LIVER ENZYMES

Table 24 Effect of different doses of chloroformic extract of Murraya koenigii leaves on liver enzymes (SGOT, SGPT, ALP, GGT) in clozapine treated rats. The results are presented as mean±SD (n=6). Statistical analysis was performed using one-way analysis of variance with post-hoc testing *=$p<0.05$

	GRP 1 (N.S)	GRP 2 (CLZ)	GRP 3 (CLZ + Statin)	GRP 4 (CLZ + MK100)	GRP 5 (CLZ + MK200)	GRP 6 (CLZ + MK400)	GRP 7 (CLZ + MK800)
SGOT (IU/L)	150 ± 26.5	179 ± 25.7 *	149 ± 21.5	160 ± 26.5	158 ± 22.0	169 ± 16.8	153 ± 25.3 *
SGPT (IU/L)	78 ± 13.6	87 ± 10.0 *	82 ± 7.3	85 ± 6.6	89 ± 12.6	75 ± 7.0	65 ± 13.4 *
ALP (IU/L)	75 ± 28.5	73 ± 27.5	88 ± 11.1	79 ± 22.1	66 ± 25.5	69 ± 28.7	78 ± 19.3
GGT (IU/L)	9.9 ± 1.8	11.0 ± 1.26 *	10.7 ± 1.17	7.9 ± 0.80 *	7.6 ± 1.4 *	8.2 ± 1.33 *	9.8 ± 1.05 *

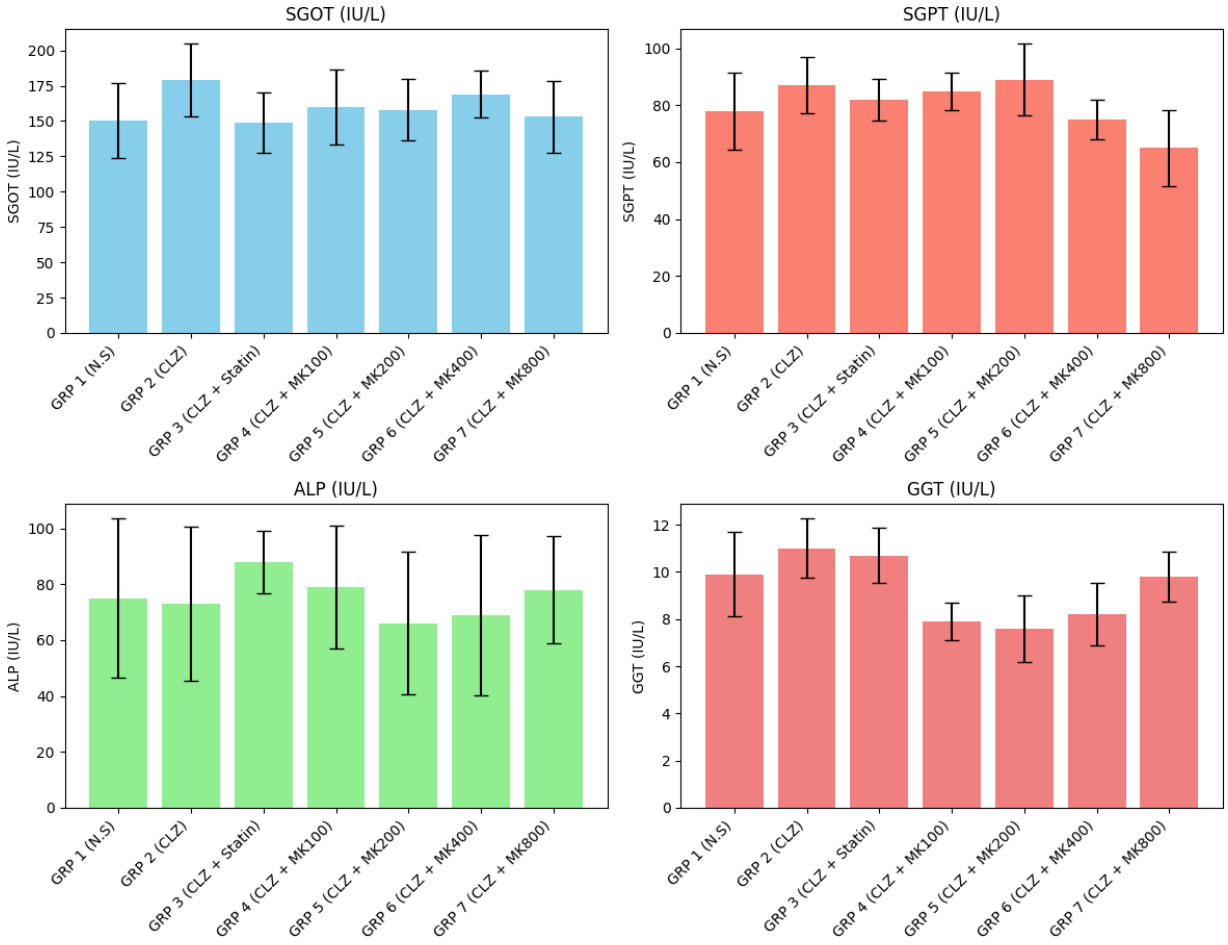


Figure 60 Effect of different doses of chloroformic extract of Murraya koenigii leaves on liver enzymes (SGOT, SGPT, ALP, GGT) in clozapine treated rats. The results are presented as mean±SD (n=6)

The liver enzymes were estimated in rats of group received clozapine drug, standard receiving statin, normal as well as in Murraya koenigii treated groups. The investigation clearly indicated that Grp II showed higher level of SGOT and SGPT, while protection was observed in the last two higher doses of CEMK . The protection against liver damage could not be achieved with lower doses of CEMK as it was probably overshadowed by the clozapine drug toxicity. The findings suggested that the extract ameliorate the clozapine induced liver enzyme damage but at a higher dose of 800 mg/kg bw.

There was no significant difference in the values of ALP obtained between the groups as the reference range is quite variable(Delwatta et al. 2018). It would be right to state that liver damage was not initiated expressed by nonincrement in some enzyme activities such as ALP.

The liver enzyme GGT(Gamma glutamyl transferase) was estimated in rats of group received clozapine drug, standard receiving statin, normal as well as in Murraya koenigii treated group . Our results clearly indicated that Grp II showed higher level of GGT while protection was observed in the almost all the doses of Murraya koenigii. The protection against liver damage was observed to such a level where the figures almost were close to normal control group. The findings suggested that the extract ameliorate the clozapine induced liver enzyme damage even at a lower dose of 100 mg/kg bw. Our results are in accordance with Desai et al, who investigated the effect of Murryaa koenigii dose on hepatotoxic rats and found that different doses (200, 400 and 600 mg/kg body weight) showed significant decrement in activity levels of alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase and total protein(Desai et al. 2012)

The administration of curry leaves at a dosage of 300 mg/kg body weight once daily for one month resulted in reductions of SGOT and SGPT levels by 21.7% and 25.0%, respectively(Kesari et al. 2007)

The liver serves as the primary organ for drug metabolism, making it crucial to understand how drugs impact its integrity. Clozapine undergoes extensive metabolism in the liver through the isoenzymes of the mixed-function oxidase cytochrome P450 system, resulting in the formation of its active metabolite, norclozapine (desmethylclozapine) (Prior et al., 1999). The resulting hepatic damage can be evaluated by measuring serum levels of marker enzymes such as ALT, AST, and ALP, along with bilirubin, which indicate hepatotoxicity as they leak from hepatocytes into the bloodstream (S. N. Desai et al., 2012). Drugs administered intraperitoneally, along with their metabolites, quickly achieve peak concentrations in the liver (Ferno et al., 2009). While treatment with drugs can yield favorable results for mood and anxiety disorders, they may also lead to hepatic toxicity and other adverse effects (Barakauskas et al., 2010; Polydoro et al., 2004). In light of this discussion, another study was conducted to examine the effects of clozapine on liver toxicity through an animal model of depression, compared to controls and the findings revealed a significant increase liver SGPT of chronically isolated rats when compared to vehicle-treated controls (Zlatković et al., 2014). Based on our findings and the presence of polyphenols in the

leaves of Murraya koenigii, we conclude that the chloroformic extract of this plant has the potential to protect the liver from oxidative damage, even at lower doses.

4.6.2.2.4 EFFECT OF TREATMENT ON LIPID PROFILE

Lipid profile was evaluated on the final day of the experimental study period, depicted in Table 19. Group I served as normal control and group II was given clozapine ,while Group IV ,V ,VI and VII were given Murraya koenigii extract in the dose 100,200,400 and 800 mg/kg bw respectively. Group III was given treatment of standard drug, Atorvastatin correspondingly. Highly significant hypocholesterolemic effect ($p < 0.001$) was observed in Groups VI and VII when compared to group II. Group IV and Group V revealed highly ameriolated significant effect ($p < 0.001$) on TG and VLDL-C. But such an effect on HDL-C in comparison to group II that served as hyperlipidemic control was not achieved by Murrya koenigii treated groups. Groups VI and VII showed highly significant hypolipidemic effect ($p < 0.001$) on LDL-C as compared to Group II even better than the standard (STATIN) group III, whereas the lower doses (100 and 200 mg/kg bw of CEMK were able to bring about a significant decrease in LDL cholesterol.

Table 25 Effects of treatment on serum Lipid profile, Values represent the Mean± SEM of six rats in each group (n=6). Statistical analysis was performed using one-way analysis of variance with post-hoc testing. * *denotes statistical significance p<0.05*

Treatment Group	GRP 1 (N.S)	GRP 2 (CLZ)	GRP 3 (CLZ + Statin)	GRP 4 (CLZ + MK100)	GRP 5 (CLZ + MK200)	GRP 6 (CLZ + MK400)	GRP 7 (CLZ + MK800)
CHOLESTEROL (mg/dl)	53 ±4.58	67 ±2.64*	57 ±3.51*	55 ±3.6*	52 ±4.16*	48 ±1.0*	45 ±3.51*
TRIGLYCERIDE (mg/dl)	74 ±4.36	99.3 ±12.0*	70 ±5.3*	64.6 ±4.8*	63.8 ±3.9*	62 ±4.3*	61.8 ±3.91*
HDL CHOL (mg/dl)	22 ±1.2	20.2 ±0.5*	20.8 ±1.3	20.8 ±1.8	21.2 ±1.7*	21.2 ±1.0*	21.6 ±1.4*

VLDL (mg/dl)	14.8 ±0.8	19.8 ±2.3*	13.8 ±1.1*	12.9 ±0.9*	12.7 ±0.8*	12.4 ±0.86*	12.2 ±0.78*
LDL (mg/dl)	14.8 ±1.9	27.4 ±4.3*	21.6 ±3.8	18.5 ±3.4*	18.3 ±2.8*	14.5 ±3.0*	8.4 ±3.7*

4.6.2.2.4.1 EFFECT ON TOTAL CHOLESTEROL

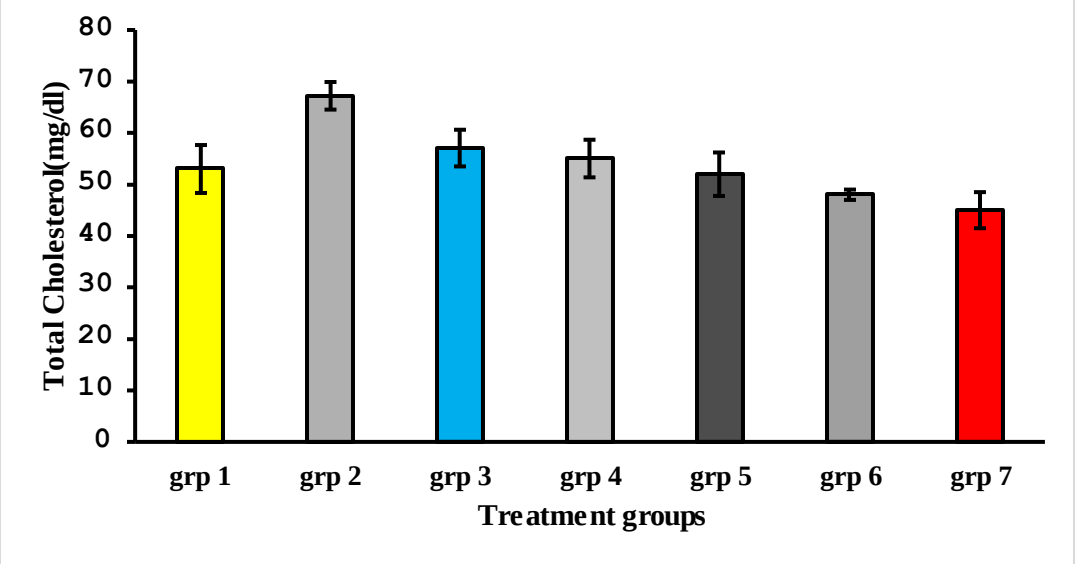


Figure 61 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Total Cholestrol in clozapine treated rats. The results are presented as mean±SD (n=6) .

The investigation indicated that GII exhibited elevated cholesterol levels, while a dose-dependent protective effect was observed, with the highest dose approaching the protection level of the normal group of animals.

The changes in the lipidemic profile due to CLZ therapy have been recorded (Koro et al., 2002; Atmaca et al., 2003), and recent assessments have investigated the effects of Quit therapy on lipid metabolism and the related risk of hyperlipidaemia (Meyer & Koro, 2004). The negative impacts associated with dyslipidaemia resulting from CLZ encompass a significant elevation in serum triglycerides (hypertriglyceridemia) and an increase in total cholesterol levels (hypercholesterolaemia). The dyslipidemic effects associated with hypertriglyceridemia seem to

be more significant compared to those impacting cholesterolemia in the context of CLZ (Wirshing et al., 2002; Atmaca et al., 2003).

The results of our study are consistent with the research conducted by Phatak et al. (2012), which examined the antihyperlipidemic effects of Murraya koenigii leaves using methanolic and aqueous extracts. Their findings indicated a reduction in serum lipid parameters, specifically total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C), as well as a decrease in atherogenic risk, linked to the bioactive compounds present in the extracts. A similar lipid-lowering effect has been noted with different extraction solvents, such as chloroform (Birari et al., 2010), dichloromethane, ethyl acetate (Tembhurne & Sakarkar, 2012), and ethanol (Kesari et al., 2007), alongside the aqueous and methanol extracts highlighted by Phatak et al. (2019). Additionally, the stem bark extract of Murraya koenigii has shown a hypolipidemic effect comparable to that of the leaves (Kant Upadhyay, 2021; Mandal et al., 2010). In a study from 1996, Khan et al. investigated the biochemical impacts of incorporating curry leaf into the diets of Albino rats. The subjects were provided with a standard laboratory diet that included 20% coconut oil and 10% curry leaf feed over a period of 90 days, making up 10% of their body weight. The intervention led to a reduction in total serum cholesterol and LDL, as well as very low-density lipoprotein (VLDL), while simultaneously increasing high-density lipoprotein (HDL) levels. Xie et al. (2006) also documented the hypocholesterolemic effects of Murraya koenigii extract in diabetic ob/ob mice, utilising intraperitoneal injections of 80 mg/kg of curry leaf extract over a period of ten consecutive days. Previous studies have mainly concentrated on the antihyperlipidemic properties of Murraya koenigii leaves in relation to lipid profiles. The hypolipidemic effects of CEMK offer significant advantages for patients undergoing treatment with clozapine, demonstrating beneficial outcomes across various dosage levels.

4.6.2.2.4.2 EFFECT ON SERUM TRIGLYCERIDES

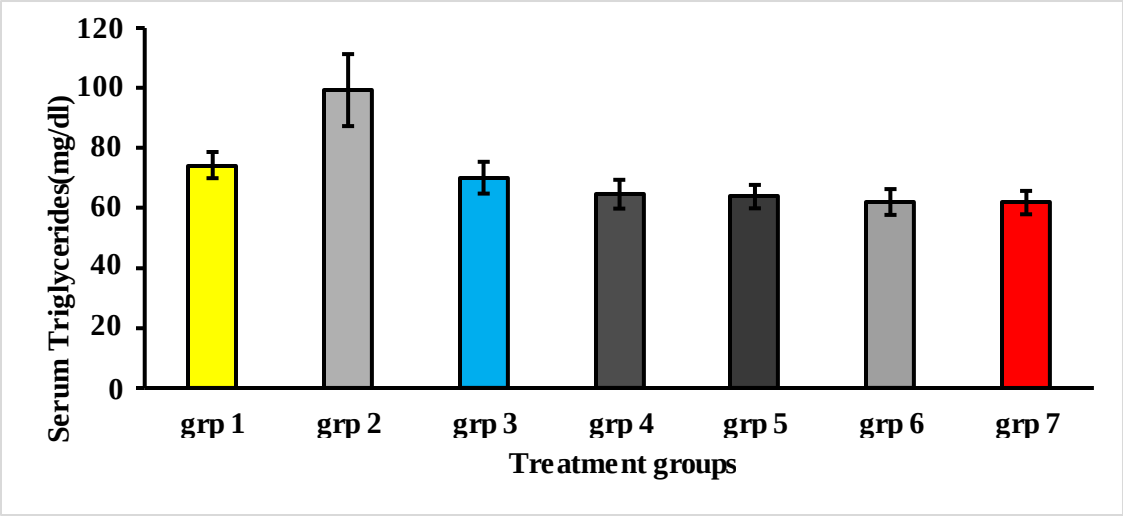


Figure 62 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Serum Triglycerides in clozapine treated rats. The results are presented as mean±SD (n=6) .

The investigation clearly indicated that GII exhibited elevated levels of triglycerides, while the protective effect of Murraya koenigii extract was observed in a dose-dependent manner.This is associated with the standard group receiving statins, which demonstrates a comparable lipid-lowering effect.

Triglycerides are created by converting most of the fats found in the body. These fats are carried through the bloodstream and stored in different tissues. Elevated levels of total cholesterol and triglycerides can lead to the thickening of blood vessel walls, thereby heightening the risk of coronary heart disease and stroke.

Insulin resistance, frequently linked to antipsychotic therapy, generally leads to increased synthesis of fatty acids and triacylglycerides in hepatocytes. The administration of atypical antipsychotics significantly increases the synthesis of complex lipids, such as phospholipids, triacylglycerides, and free fatty acids, in primary hepatocytes obtained from mice and rats (Lauressergues et al., 2011, 2010). Furthermore, acute exposure of rats to clozapine leads to a notable increase in phospholipid and triglyceride concentrations in the liver (Ferno et al., 2009). Atypical antipsychotics, as noted earlier, facilitate lipogenesis, resulting in the buildup of triacylglycerides, while concurrently suppressing lipolysis in adipose tissue. Adipocytes from rats administered

olanzapine demonstrated hypertrophy, leading to increased cell sizes (Minet-Ringuet et al., 2007), potentially heightening their vulnerability to rupture. The rupture may enable the release of adipocyte contents, including triacylglycerides, into the bloodstream, which could increase the risk of dyslipidaemia (Monteiro et al., 2006). Additionally, research indicates that the administration of atypical antipsychotics in rats exhibiting dyslipidaemia correlates with an increase in SREBP1 gene expression within both liver and adipose tissues (Ferno et al., 2009; Jassim et al., 2012; Minet-Ringuet et al., 2007; Skrede et al., 2012). These results suggest that the elevation of SREBP1 gene expression may play a crucial role in the mechanisms through which antipsychotics contribute to dyslipidaemia (Gonçalves et al., 2015).

Curry leaf extract contains a high concentration of flavonoids, alkaloids, tannins, and saponins (Baskaran et al., 2011). Flavonoids increase the activity of the lipoprotein lipase enzyme, which aids in the hydrolysis of triglycerides into free fatty acids, thus lowering triglyceride levels in the body. Alkaloids reduce the activity of pancreatic lipase, subsequently leading to a decrease in the absorption of triglycerides from the small intestine. Saponins demonstrate the ability to reduce elevated triglyceride levels by facilitating the elimination of triglycerides via faecal excretion (Elekofehinti, 2014). The study by Tembhurne et al. showed that giving curry leaf extract at a dosage of 300 mg/KgBW for 15 days to male Wistar rats led to a notable decrease in triglyceride levels (Sakarkar DM, Tembhume SV, 2017).

4.6.2.2.4.3 EFFECT ON HDL CHOLESTROL

The investigation noted that GII showed slightly lower level of HDL cholesterol while increase in the levels were observed in dose dependent manner as with the highest dose the protection level was found closer to the normal group of animal.

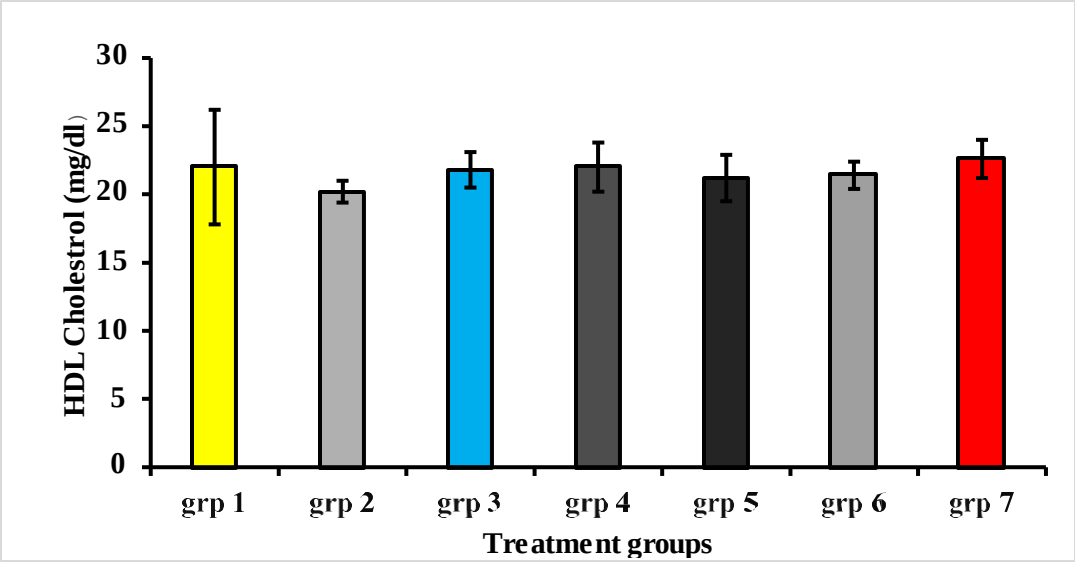


Figure 63 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Serum HDL Cholestrol levels in clozapine treated rats. The results are presented as mean±SD (n=6) .

Mental illnesses with a dyslipidemic state posing lower HDL cholesterol values are inconsistent in literature. some studies indicated lower HDL cholesterol and elevated triglyceride levels, while others did not. Notably, one study reported significantly reduced HDL levels and increased triglyceride levels (Osborn et al., 2008). However, other studies did not find significant differences in HDL (Saari et al., 2005).

Off-label lipid-lowering agents also demonstrated efficacy in improving triglycerides and total cholesterol levels, with statistically significant results (Kanagasundaram et al., 2021). An analysis of multiple studies revealed that antipsychotic switching or add-on strategies were particularly effective in enhancing lipid parameters that are often dysregulated in schizophrenia, specifically triglycerides and HDL cholesterol (Newcomer et al., 2007). Feeding the rats with aqueous extract of 300 mg/kg body weight increased the HDL-cholesterol level by 16 and 29.4% in normal and diabetic rats, respectively, as compared with their initial values.

In the research conducted by Phatak et al., both AEMK and MEMK demonstrated hypolipidemic effects in rats fed a high-fat, high-fructose diet (HFFD). The HFFD regimen over a 14-week period

was associated with increased levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-C) when compared to the normal control group. Both AEMK and MEMK played a significant role in substantially reducing TC and LDL-C levels. Therefore, further comprehensive studies are necessary to validate the hypolipidemic properties of curry leaves as a complementary therapy in human clinical trials. If confirmed, this could serve as an adjunct to current hypolipidemic medications.

4.6.2.2.4.4 EFFECT ON VLDL CHOLESTROL

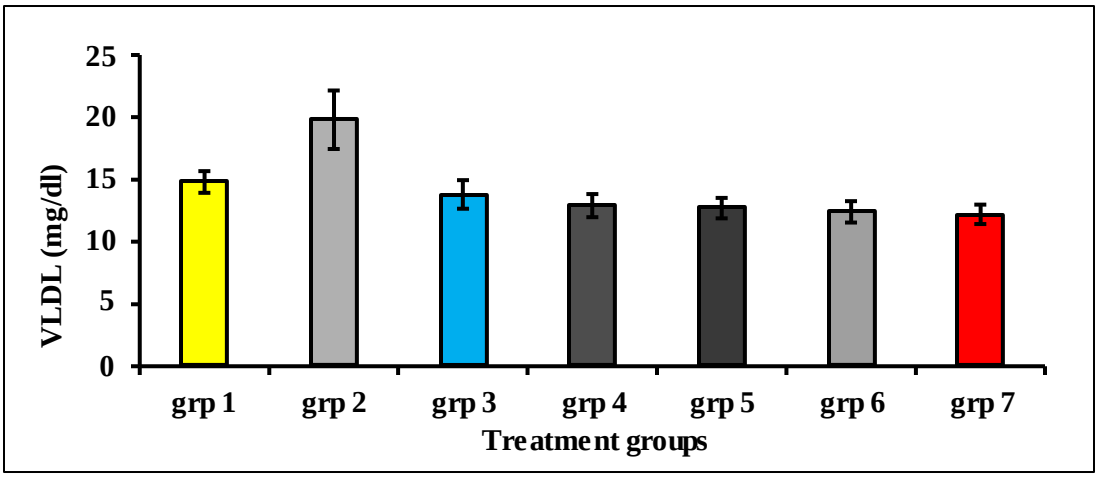


Figure 64 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Serum VLDL Cholestrol levels in clozapine treated rats. The results are presented as mean±SD (n=6) .

Highly significant hypocholesterolemic effect ($p < 0.01$) was observed in Groups VI and VII when compared to group II. Group IV and Group V revealed significant effect ($p < 0.05$) on TG, VLDL-C as compared to Group II almost nearing the effect produced by standard drug (STATIN) group III.

Lipoprotein cholesterol encompasses a category of protein complexes found in circulation that facilitate the transport and distribution of cholesterol throughout the body. This category includes low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, and very low-density lipoprotein (VLDL) cholesterol. VLDL accounts for 10–15% of serum cholesterol and consists of cholesterol, triglycerides, apo B-100, apo C-1, apo C-II, apo C-III, and apo E (Xue et al., 2014).

A study was conducted to investigate the acute effects of antipsychotics on lipid levels and other aspects of the metabolic profile. In this research, mice received an intraperitoneal injection of clozapine. The administration of clozapine quickly triggered direct transcriptional changes in the liver, affecting genes that regulate transcription factors, including sterol regulatory element-binding proteins, peroxisome proliferator-activated receptors, and liver X receptors. This process promoted hepatic lipid accumulation by enhancing lipogenesis, as these genes play a crucial role in fatty acid biosynthesis, independent of food consumption and weight gain. Consequently, there was an increase in triglyceride levels(Fernø et al. 2009; Jassim et al. 2012)

4.6.2.2.4.5 EFFECT ON LDL CHOLESTROL

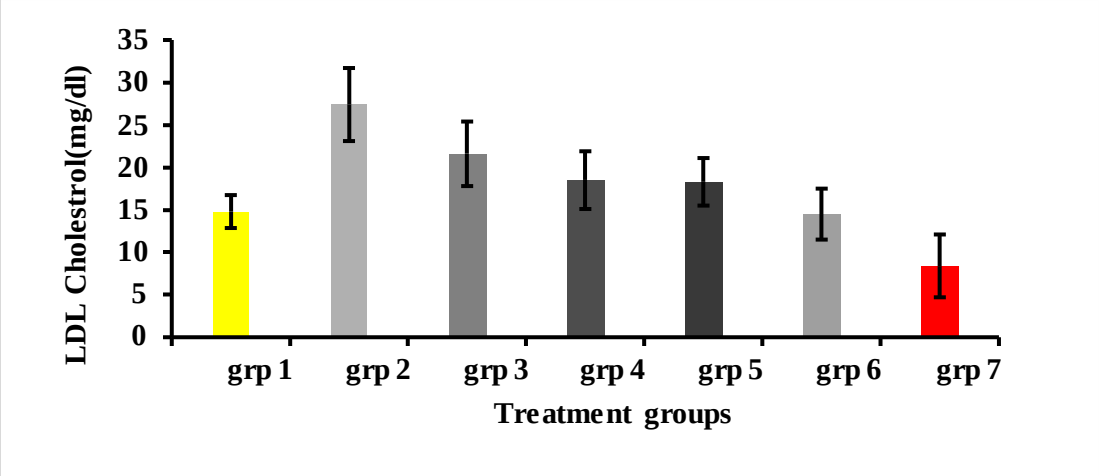


Figure 65 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Serum VLDL Cholestrol levels in clozapine treated rats. The results are presented as mean±SD (n=6) .

Our study revealed a significant increase in LDL cholesterol levels in the clozapine-treated group, highlighting the drug's metabolic side effects. Notably, the chloroformic extract of *Murraya koenigii* demonstrated a dose-dependent reduction in LDL levels, indicating a protective lipid-lowering effect. These findings align with prior studies, such as Jiang et al. (2020), which observed similar improvements in triglyceride and total cholesterol profiles. Reducing LDL and triglycerides by 40 mg/dL is linked to a 20% and 4–5% decreased risk of cardiovascular disease, respectively (Marston et al., 2019), reinforcing the clinical relevance of our results.

Despite the prevalence of dyslipidemia in psychiatric populations, it often goes untreated due to the prioritization of mental health symptoms (Falissard et al., 2002; Millar, 2008). Statins remain the standard treatment but are associated with adverse effects like hyperglycemia and neuropathy, limiting their use. Traditional remedies such as *Murraya koenigii* offer a promising alternative, supported by their bioactive compounds. Flavonoids may inhibit HMG-CoA reductase, alkaloids can reduce fat absorption by inhibiting pancreatic lipase, and saponins bind cholesterol in the gut. Tannins also reduce fat absorption by interacting with mucosal proteins and forming barriers in the intestine. These phytochemicals collectively support the therapeutic potential of curry leaves in managing antipsychotic-induced dyslipidemia.

Clozapine is primarily metabolized by CYP1A2, CYP3A4, CYP2D6 enzymes in the CYTP450 Complex in the liver. Murraya koenigii is found to have compounds like Flavanoids, Terpenoids, Tannins, and Saponins, some of which may modulate the CYT P enzymes. There is a need to explore the potential for drug – drug interactions involving these enzymes, emphasizing the need for close monitoring of CLZ plasma levels when patients are on other medications that affect CYTP isoforms.

Though considered safe in food amounts, concentrated extracts might influence clozapine metabolism or interact at the level of oxidative stress and lipid metabolism. As of current evidence,there is no direct clinical study confirming that Curry leaf alters the therapeutic efficacy of Clozapine. However, based on its antioxidant and anti-inflammatory properties, Murray koenigii protects against Clozapine induced metabolic side effects. These may be beneficial and may compliment Clozapine.

4.6.2.2.5 EFFECT OF TREATMENT ON CREATINE KINASE

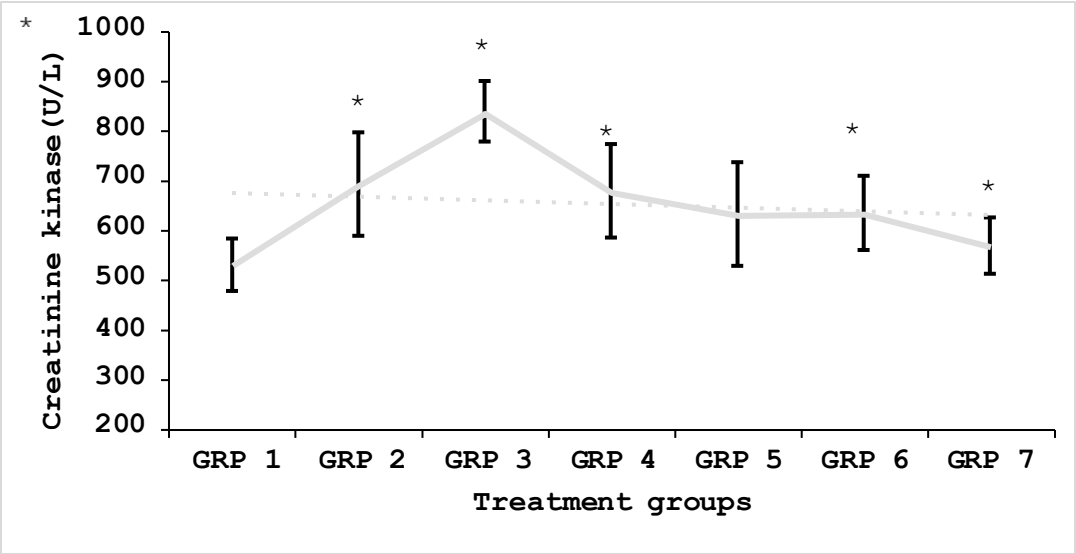


Figure 66 Effect of different doses of chloroformic extract of Murraya koenigii leaves on Creatine kinase levels in clozapine treated rats. The results are presented as mean±SD (n=6) Statistical analysis was performed using one-way analysis of variance with post-hoc testing. *= p<0.05

Myocarditis and cardiomyopathy are serious conditions that can be life-threatening. These complications appear to occur more frequently with clozapine compared to other atypical antipsychotics, although they remain relatively uncommon. The risk of myocarditis is heightened during the initial three months of clozapine therapy, while cardiomyopathy can manifest at any point during treatment(Flanagan et al., 2020).

Most strategies aimed at managing dyslipidemia necessitate a minimum duration of six months to effectively lower the incidence of cardiovascular disease (CVD) events (Kopin & Lowenstein, 2017).

Creatine kinase (CK) activity in rat serum or plasma is commonly utilized to assess myopathic conditions, and evaluate the myotoxic effects of various drugs(Masters et al. 1995; Pierno et al. 2006). However, in rat studies there could be variations in the levels of CK depending upon the site of blood collection and use of anesthetic agents. Although,it was established that ether does not significantly elevate CK levels (M. Goicoechea et al., 2008). The results obtained in our study were 530 ,691.5, 837, 678, 631.5, 633.8, and 568 IU/L in Grp I, II, III, IV, V, VI, and VII respectively. The elevated CK levels observed may be attributed to the cardiac puncture method and the ether anesthesia applied in our study. Our findings revealed significantly higher CK levels

in the clozapine group and in group III, which received statin as an adjunct treatment (Fig 53). Previous studies have shown that Fluvastatin and Atorvastatin can bring about notable increase in plasma CK levels(upto 2118 ± 202 mU/ml), due to potential release of myoglobin, LDH, CK, or creatinine from damaged (Pierno et al., 2006).

4.6.2.2.5 EFFECT OF TREATMENT ON ANTIOXIDANT ENZYMES IN THE BRAIN

The levels of antioxidant enzymes, and TBARS contents in the brain of all the experimental groups is shown in table 26. Administration of CEMK significantly ($p < 0.001$) altered the concentration of SOD, CAT, GPx and TBARS in a dose-dependent way. In contrast, The standard group III that received statin at 5 mg/kg b.w. almost reduced the concentration of TBARS in brain tissue($p < 0.05$). SOD, CAT, and GPx activities were significantly decreased in clozapine-treated group when compared to normal control group and were again restored significantly on treatment with CEMK at 100 and 200 mg/kg doses. TBARS was significantly increased in Clozapine-treated group of mice when compared to control group. CEMK reduced the TBARS level. CEMK at 400 and 800 mg/kg bw doses could replenish the antioxidant enzymes furthermore and improve the AO status of brain significantly.

Table 26 Effects of Clozapine and Combination Treatments on Antioxidant Enzyme Activities and Lipid Peroxidation.The results are presented as mean±SD (n=6) Statistical analysis was performed using one-way analysis of variance with post-hoc testing. *= p<0.05

Groups (n=6)	Dose (mg/kg)	SOD (µ/mg protein)	CAT (µ/mg protein)	GPx (µ/mg protein)	LPO (n moles/mg protein)
Grp I	Normal saline	3.4 ± 0.1	0.28 ± 0.03	8 ± 0.16	5.1 ± 0.09
Grp II	Clozapine (10mg/kg bw)	2.3 ± 0.2*	0.24 ± 0.03	6 ± 0.47*	8.3 ± 0.14*
Grp III	Clozapine (10) + Statin (5mg/kg)	2.7 ± 0.1c	0.32 ± 0.01	6.6 ± 0.2*	3.5 ± 0.13*
Grp IV	Cloz (10) + CEMK (100)	2.7 ± 0.0*	0.3 ± 0.01*	6.4 ± 0.24*	5.1 ± 0.29*
Grp V	Cloz (10) + CEMK (200)	3.5 ± 0.0*	0.32 ± 0.0*	8.2 ± 0.27*	4.4 ± 0.26*
Grp VI	Cloz (10) + CEMK (400)	4.1 ± 0.1*	0.33 ± 0.01*	10.1 ± 0.3*	4 ± 0.24*
Grp VII	Cloz (10) + CEMK (800)	4.4 ± 0.4*	0.36 ± 0.0*	11.7 ± 0.4*	3.3 ± 0.23*

Various hypotheses have been proposed regarding clozapine-induced cardiotoxicity. Abdel-Wahab and Metwally (2014), along with Killian et al. (1999), suggested that myocarditis resulting from clozapine may be attributed to a type I IgE-mediated acute hypersensitivity reaction. A plausible explanation for this hypothesis is that clozapine is bioactivated in myocardial tissue to form a chemically reactive nitrinium ion metabolite, which can lead to cellular damage, lipid peroxidation, and the production of free radicals, as discussed by Williams et al. (2003). Therefore, it was suggested that Murraya koenigii (L.) Spreng. leaf extract may have the potential to attenuate clozapine-induced cardiotoxicity which may arise as a result of the damage caused by free radical formation(Sandamali et al. 2020). Many previous studies have also shown that compounds with antioxidant property may bring down the cardiac biomarkers in rats(Kant Upadhyay 2021).

During oxidative stress, numerous morphological and functional alterations are observed in the pathogenesis of many central neuronal disorders. Studies show that prolonged treatment with antiparkinson drugs such as dopamine agonist, dopamine replenishment therapy, and monoamine oxidase inhibitors leads to severe side effects and decrease in the sensitivity for the therapy (Nellore et al.,2015). Further, typical neuroleptic drugs such as chlorpromazine, haloperidol, and reserpine use in schizophrenia lead to decrease in dopamine content and state of catalepsy. Previous studies have shown that dopamine receptors in the striatum are involved in the neuroleptic-induced catalepsy(Pemminati, Nair, and Dorababu 2007).Treatment with antipsychotic Haloperidol also decreased the brain antioxidant enzymes(Chitra et al. 2017). Similar effect of Haloperidol on brain antioxidant enzymes was studied by Patil et al., who investigated the impact of the alcohol extract of M. koenigii leaves (EEMK) and its alkaloid fraction (AMK) on levels of antioxidant defense enzymes. Both EEMK and AMK were found to significantly counteract the reduction in forebrain SOD and CAT levels induced by haloperidol, while also markedly decreasing LPO and restoring the diminished GSH levels resulting from chronic haloperidol administration (Patil et al., 2012). Murraya koenigii aqueous extract showed potential in replenishing the antioxidant enzyme levels even at lower doses in the brain of Paraquat treated animals(Reddy et al., 2020).

Free radicals are highly reactive with specific cellular components and have cytotoxic properties (Ravindranath & Reed, 1990), and neuronal loss in the striatum has been reported in animals treated chronically with neuroleptics (Nielsen & Lyon, 1978). It has been observed that mitochondria swell in response to clozapine, potentially serving as a protective mechanism against oxidative damage caused by the heightened production of mitochondrial reactive oxygen species (mROS) from the respiratory chain. Numerous studies have indicated that clozapine stimulates the generation of reactive oxygen species (Walss et al., 2008; Fehsel et al., 2007).Other studies have shown that minor oxidative stress induces mitochondrial swelling and the formation of a mitochondrial ‘‘firewall’’ which prevents propaga- tion of Mros (Jou et al.,2008). Treatment with Murraya koenigii extract increased the activity of these enzymes by quenching the free radicals. The observed reduction in lipid peroxidation (LPO) levels in the brains of subjects treated with the extract, along with enhanced activities of both enzymatic and non-enzymatic antioxidants, implies that the extract mitigates oxidative stress. However, the precise mechanism through which M. koenigii protects against oxidative damage induced by clozapine remains uncertain. It has been documented that M. koenigii activates various antioxidant defense enzymes, leading to increased levels of superoxide dismutase (SOD), glutathione reductase (GSH), and catalase (CAT) (Sathaye et al., 2011; Yankuzo et al., 2011). Additionally, another proposed mechanism of action for M. koenigii involves its ability to scavenge free radicals, particularly as an effective hydroxyl radical (OH^-) scavenger (Rao et al., 2007). Consequently, one or more of these mechanisms may play a role in alleviating clozapine-induced oxidative damage (Patil et al., 2012). The protective influence of M. koenigii against oxidative stress is likely attributed to its content of polyphenols, and flavonoids, all of which are recognized for their antioxidant capabilities (Rehana, 2017).

Flavonoids function as antioxidants through multiple mechanisms, including the scavenging of free radicals, metal chelation, and the inhibition of specific enzymes such as NADPH oxidase in human neutrophils, mitochondrial succinoxidase, and NADH oxidase. Additionally, they impede microsomal cytochrome P-450 (Fe^{2+})-dependent enzymatic reactions due to their metal-chelating properties (Miura, 1998).

4.6.2.3 EFFECT OF TREATMENT ON HAEMATOLOGICAL PARAMETERS

4.6.2.2.5 EFFECT OF TREATMENT ON TOTAL WBC COUNT

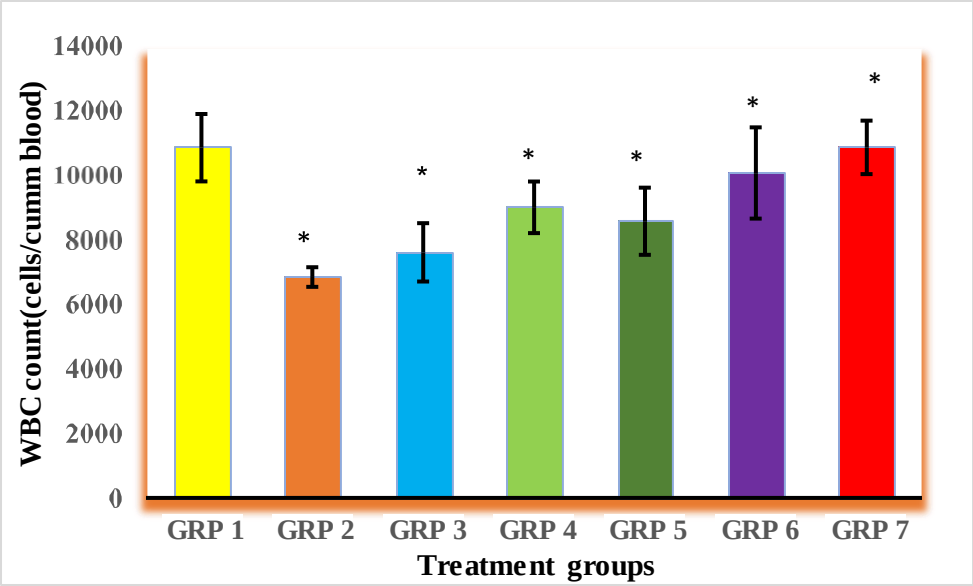


Figure 67 Effect of Clozapine/Murraya koenigii treatment on White blood cell counts in a 28 day study. data represented as mean± SD.Statistical analysis was performed using one-way analysis of variance with post-hoc testing, ∗ = p<0.05

The obtained results reveal that white blood cell counts of the experimental groups I, II, III, IV, V,VI, and VII were as follows 11150, 7183, 7916, 9800, 9565, 10450, 11048 respectively, clearly indicating the decrease in WBC counts in the clozapine group .It is observed that CEMK is able to maintain a healthy WBC count along side clozapine treatment. Clozapine was initially launched as an antipsychotic medication in the 1970s; however, a series of fatalities associated with the drug's potential to cause agranulocytosis resulted in its withdrawal in numerous countries. Subsequent research in the 1980s demonstrated its exceptional effectiveness in treating treatment-resistant schizophrenia (TRS), which affects approximately 30% of individuals diagnosed with the condition. Following this discovery, clozapine was reintroduced for this specific indication, but its administration necessitates regular hematologic monitoring due to the associated risks(Howes et al. 2017; Remington et al. 2016). One of the most significant, albeit rare, and potentially life-threatening hematological adverse effects is agranulocytosis, which increases susceptibility to

infections. Additional blood disorders that may occur include leukopenia, neutropenia, anemia, leukocytosis, and eosinophilia.

Patients receiving clozapine must regularly monitor their leukocyte counts due to the potential risk of agranulocytosis (Ojong & Allen, 2013). This requirement can be attributed, in part, to alterations in neutrophil kinetics and their release from the bone marrow, as well as the suppression of neutrophil oxidative functions (Patel et al., 2019). Furthermore, activated neutrophils oxidize clozapine to form a nitrenium ion, which may irreversibly bind to neutrophils, thereby affecting their kinetics and contributing to agranulocytosis (Ng et al., 2014). The administration of naringin in conjunction with clozapine has been shown to counteract this effect, likely due to its antioxidant properties that inhibit lipid peroxidation and help maintain cellular integrity (George et al., 2021). Studies indicate that curry leaf extract has the ability to mitigate heavy metal(lead)-induced alterations in blood tissue but the same extract in combination with melatonin provides a better protection in the situation. (Ghosh et al. 2014)

4.6.2.2.5 EFFECT OF CLOZAPINE AND MURRAYA KOENIGII ON HB, RBC, NEUTROPHILS, LYMPHOCYTES, AND PLATELET COUNTS

Table 27 Effect of different doses of Murraya koenigii on othere hematology parameters. Data represented as mean± SD. Statistical analysis was performed using one-way analysis of variance with post-hoc testing, *=p<0.05

Treatment Group	GRP 1 (N.S)	GRP 2 (CLZ)	GRP 3 (CLZ + Statin)	GRP 4 (CLZ + MK100)	GRP 5 (CLZ + MK200)	GRP 6 (CLZ + MK400)	GRP 7 (CLZ + MK800)
Hemoglobin (gm%)	14.8± 1.0	15.4 ± 0.2	15.0 ± 0.6	15.2 ± 0.5	14.5 ± 0.9	14.6 ± 0.6	14.3 ± 0.4
Lymphocytes (%)	75 ± 4.3	80.6 ± 2.3	78.3 ± 1.5	77 ± 1.0	78 ± 2.0	76 ± 1.0	75.3 ± 0.5
Neutrophils (%)	24 ± 3.0	19.6 ± 1.6*	22.3 ± 1.6	22.5 ± 1.0	22.5 ± 1.8	23 ± 1.3*	23.3 ± 1.6*
RBC (10^{12}/L)	8.6 ± 0.4	8.4 ± 0.4	8.6 ± 0.3	8.4 ± 0.4	8.3 ± 0.3	8.6 ± 0.4	8.5 ± 0.4
Platelets (10^{9}/L)	9.6 ± 1.8	9.8 ± 0.4	10.0 ± 1.7	9.7 ± 1.3	9.8 ± 0.9	9.9 ± 1.1	9.3 ± 1.8

Approximately 0.8% of patients receiving clozapine treatment experience agranulocytosis (Alvir, 1993), which is identified by a neutrophil count of less than 0.5 × 10^9/L, placing these individuals at a heightened risk for severe and often fatal infections. The 2005 clozapine guidelines define neutropenia as an absolute neutrophil count (ANC) falling below 1500, occurring in roughly 3% of patients undergoing treatment (Atkin et al., 1996). Nevertheless, the implementation of mandatory monitoring of hematological parameters has led to a reduction in the incidence of agranulocytosis and has enhanced patient safety (Honigfeld et al., 1998). Rather than developing agranulocytosis, the majority of patients treated with clozapine exhibit an increase in the circulation of immature neutrophils and a rise in the total neutrophil count (Pollmacher et al., 1997). This neutrophilia is linked to an immune response and an elevation in inflammatory cytokines, such as IL-6 (Pollmacher et al., 2000). Additionally, prior research has indicated that clozapine stimulates an increase in the release of neutrophils from the bone marrow, as well as an overall increase in neutrophil count in rabbits, alongside a reduction in neutrophil half-life (Iverson et al., 2010).

The exact mechanism by which clozapine induces neutrophilia remains unclear, similar to the situation with clozapine-induced neutropenia. Nevertheless, several hypotheses have been put forward to elucidate the relationship between clozapine and the cells of the hematopoietic system. One of the most widely accepted theories characterizes this interaction as a dichotomous process, suggesting both direct and indirect effects that yield opposing outcomes. This theory posits that clozapine directly stimulates the production of reactive oxygen species, leading to an increased expression of pro-apoptotic genes such as p53, Bax-α, and Bik. Concurrently, the elevated release of cytokines, including TNF-α, IL-2, IL-6, and G-CSF, indirectly promotes the expression of anti-apoptotic proteins, which facilitate the differentiation and maturation of myelocytes. Consequently, leukocytosis arises from the dominance of anti-apoptotic factors, resulting in an overall increase in the total leukocyte count, particularly in neutrophils (Capllonch et al., 2018; Fehsel et al., 2005). Another hypothesis suggests that clozapine may trigger an inflammatory response in blood cells, a phenomenon that appears to be especially pronounced during the initial month of treatment (Liu et al., 2005).

The protective effects of Murraya koenigii leaves in vivo against the detrimental impacts of lead on hematological parameters have been highlighted. The MKCE group exhibited significantly improved levels of hemoglobin and white blood cell counts ($p<0.001$). Furthermore, MKCE appears to offer potential cardioprotective benefits against lead-induced anemia, cardiovascular diseases, and ischemic heart disease by mitigating hemotoxicity and restoring thrombocytic indices, positioning it as a promising candidate for lead chelation (Phatak & Matule, 2016). Comparable findings were reported in another study, which demonstrated that curry leaf extract can alleviate heavy metal-induced changes in blood tissue (Ghosh et al., 2014).

4.6.2.3 EFFECT OF TREATMENT ON HISTOPATHOLOGY OF LIVER, KIDNEY, BRAIN AND ADIPOSE TISSUE

Photomicrographs of the sections of the liver, kidneys, and adipose tissue of the rats treated orally with clozapine drug and different doses of chloroformic extract of Murraya koenigii for 28 days showed some histological changes, such as lymphocytic infiltration, slight oedema in the kidney, congestion of blood vessels, some vacuolated cells in brain and glycogen deposits in liver. The observations are listed in the table

Table 28 Gross pathological observations of Liver, Kidney, Brain and Adipose tissue

GROUP	TREATMENT	ORGANS	OBSERVATION
GRP 1	Normal saline - 10 ml/kg bw	LIVER	Normal architecture (fig 68 a)
		KIDNEY	Normal architecture (fig 69 a)
		BRAIN	Normal cells (fig 70 a)
		ADIPOSE	Normal adipocytes (fig 71 a)
GRP 2	Clozapine - 10 mg/kg bw	LIVER	Lymphocytic infiltration, glycogen granules in the cytoplasm, slight congestion (fig 68 b)
		KIDNEY	Congested blood vessels, rest normal architecture (fig 69 b)
		BRAIN	Vacuolated cells and slight edema (fig 70 b)
		ADIPOSE	Increase in size of few adipocytes (fig 71 b)
GRP 3	Clozapine 10 mg + statin 5 mg/kg bw	LIVER	Lymphocytic infiltration (fig 68 c)
		KIDNEY	Congested blood vessels (fig 69 c)

		BRAIN	Normal architecture (fig 70 c)
		ADIPOSE	Normal architecture maintained (fig 71 c)
GRP 4	Clozapine 10 mg + CEMK 100 mg/kg bw	LIVER	Lymphocytic infiltration and glycogen deposits seen (fig 68 d)
		KIDNEY	Congested blood vessels seen (fig 69 d)
		BRAIN	Normal architecture (fig 70 d)
		ADIPOSE	Increase in size of adipocytes rarely seen (fig 71 d)
GRP 5	Clozapine 10 mg + CEMK 200 mg/kg bw	LIVER	Few glycogen deposits seen (fig 68 e)
		KIDNEY	Congested blood vessels seen (fig 69 e)
		BRAIN	Normal architecture maintained (fig 70 e)
		ADIPOSE	Normal architecture maintained (fig 71 e)
GRP 6	Clozapine 10 mg + CEMK 400 mg/kg bw	LIVER	Normal architecture maintained (fig 68 f)
		KIDNEY	Normal architecture maintained (fig 69 f)
		BRAIN	Normal architecture maintained (fig 70 f)
		ADIPOSE	Normal architecture maintained (fig 71 f)
GRP 7	Clozapine 10 mg + CEMK 800 mg/kg bw	LIVER	Normal architecture maintained (fig 68 g)
		KIDNEY	Normal architecture maintained (fig 69 g)
		BRAIN	Normal architecture maintained (fig 70 g)
		ADIPOSE	Normal architecture maintained (fig 71 g)

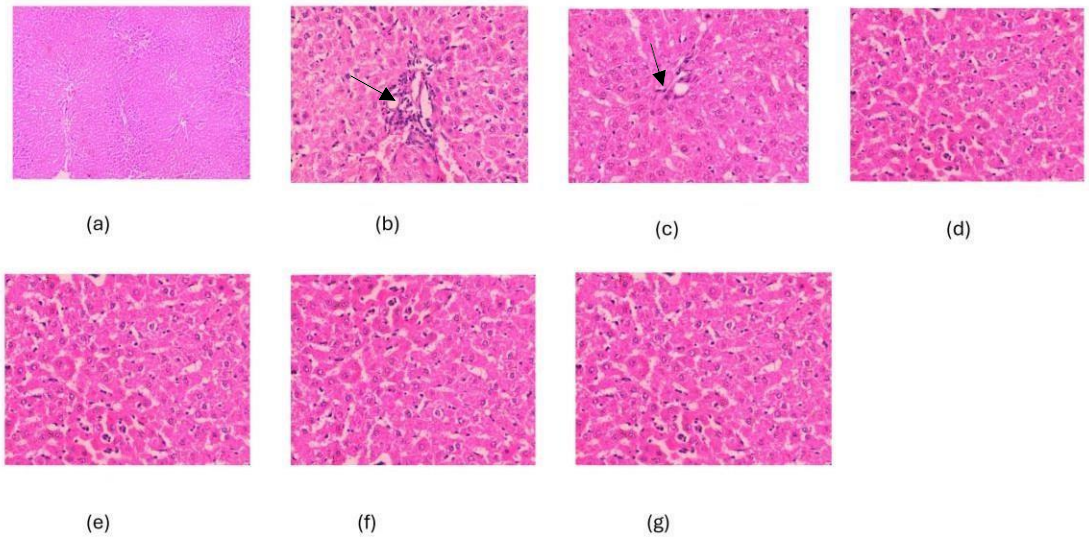


Figure 48 Effect of Clozapine and CEMK on liver histology in rats. Histological sections were visualized by staining with hematoxylin and eosin(H&E) observed under optical microscope(OLYMPUS Microscope)with 40x magnification (a)Control rats,(b)rats treated with clozapine,(c)rats treated with statin- standard group,(d)rats treated with CEMK(100mg/kg),(e) rats treated with CEMK(200mg/kg),(f) rats treated with CEMK(400mg/kg),(g) rats treated with CEMK(800mg/kg).

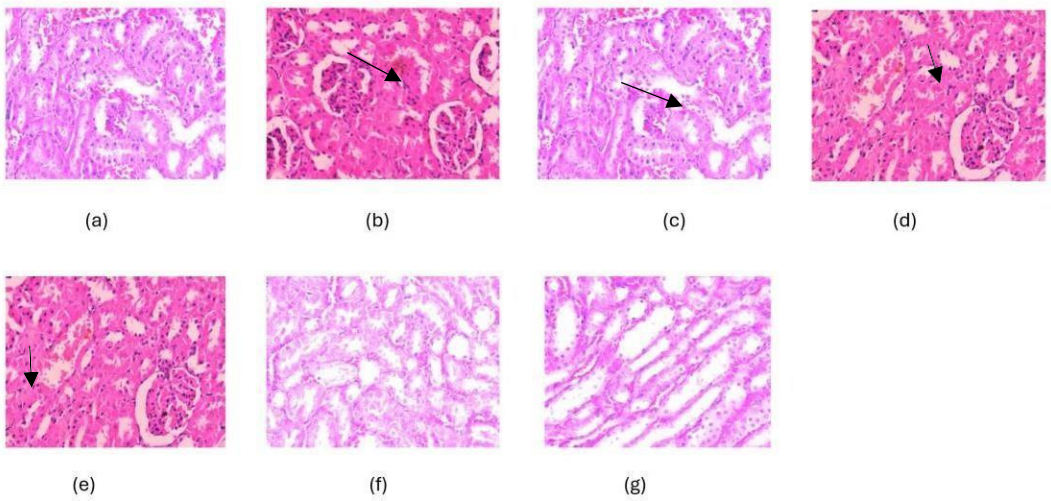


Figure 49 Effect of Clozapine and CEMK on Kidney histology in rats. The number on the images represent the treatment groups. (a)Control rats,(b)rats treated with clozapine,(c)rats treated with statin- standard group,(d)rats treated with CEMK(100mg/kg),(e) rats treated with CEMK(200mg/kg),(f) rats treated with CEMK(400mg/kg),(g) rats treated with CEMK(800mg/kg).

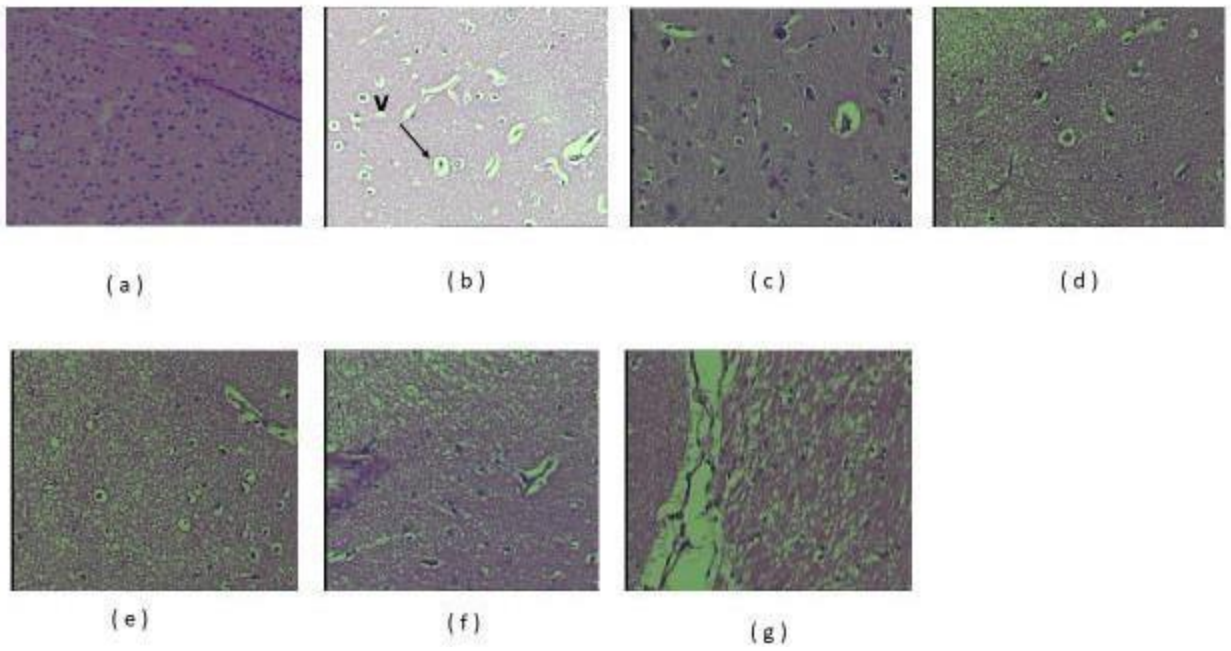


Figure 50 Effect of Clozapine, statin and CEMK on cortex region of brain in rats. The number on the images represent the treatment groups. (a)Control rats,(b)rats treated with clozapine,(c)rats treated with statin- standard group,(d)rats treated with CEMK(100mg/kg),(e) rats treated with CEMK(200mg/kg),(f) rats treated with CEMK(400mg/kg),(g) rats treated with CEMK(800mg/kg).

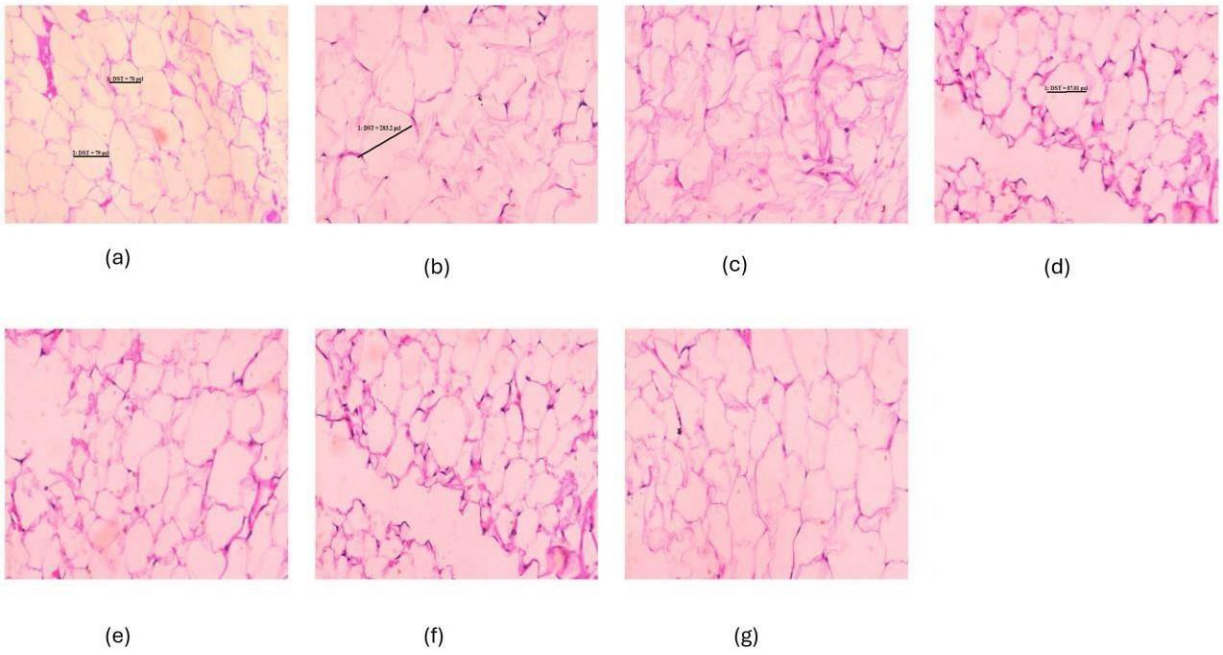


Figure 51 Effect of Clozapine, statin and CEMK on adipose tissue in rats. The number on the images represent the treatment groups. (a)Control rats,(b)rats treated with clozapine,(c)rats treated with statin- standard group,(d)rats treated with CEMK(100mg/kg),(e) rats treated with CEMK(200mg/kg),(f) rats treated with CEMK(400mg/kg),(g) rats treated with CEMK(800mg/kg). V stands for vacuolated cells in the brain. Adipocyte size, indicated by the arrowhead, in the form of fat cells is present in adipose tissues of clozapine treated group.

Recent studies have demonstrated that reducing inflammation and the infiltration of monocytes into muscle and liver tissues can mitigate the progression of Metabolic Syndrome (MetS) in a murine model (Martinez et al., 2011). The results indicate that various cell types may be vulnerable to a proinflammatory state induced by clozapine, which could lead to cellular dysfunction. In human patients, this condition may be aggravated by the infiltration of monocytes into tissues, resulting in increased local inflammation (Heart et al., 2013). Clozapine exerts its neuroprotective effects through histological modifications and the reduction of acetylcholinesterase (AChE), monoamine oxidase (MAO), and c-Fos levels. Additionally, clozapine addresses functional and cellular impairments linked to the schizophrenic effects induced by Dizocilpine (Andrabi et al., 2020).

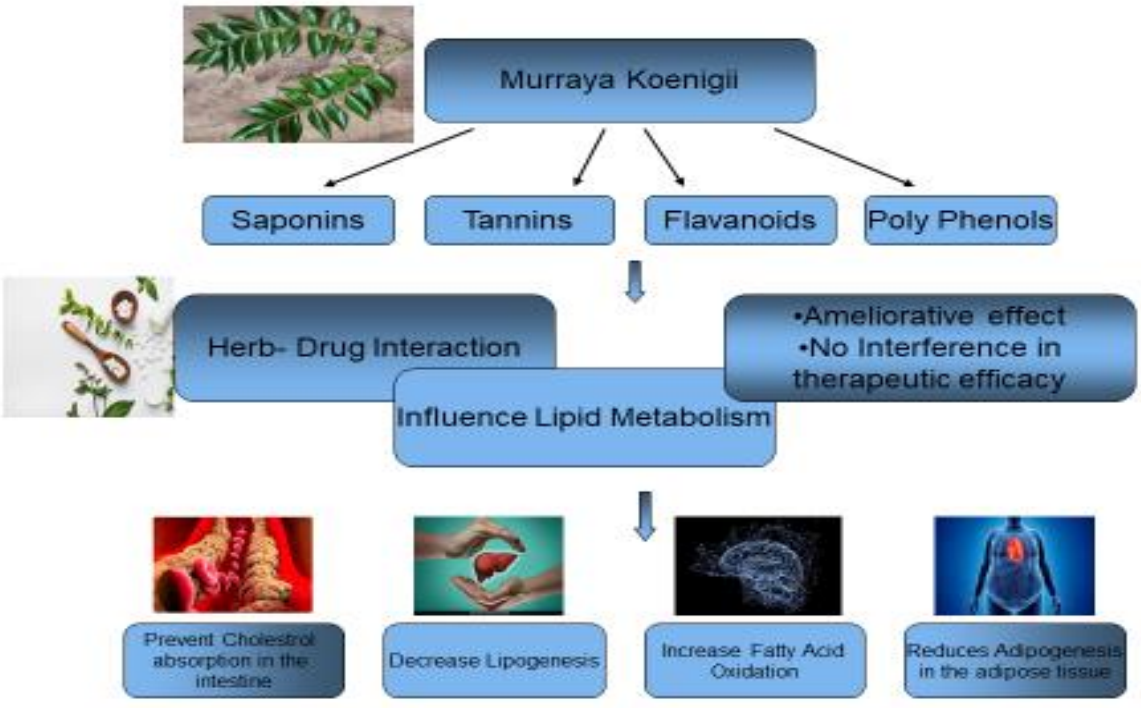
Histopathological assessments were performed to examine the impact of clozapine, statins, and Murraya koenigii on the prefrontal cortex of the brain. The findings indicated that brains subjected

to clozapine treatment displayed a limited presence of vacuolated cells. While clozapine caused only minor histological alterations in the prefrontal cortex, Murraya koenigii significantly alleviated these changes, as demonstrated by the maintenance of cortical structure. Comparable outcomes were observed in the livers of diabetic rats treated with Murraya (25 ml/kg/bw), which exhibited reduced cellular damage, resulting in gaps and inflammatory infiltration of lymphocytes and Kupffer cells. In contrast, the liver of diabetic rats administered Murraya (50 ml/kg/bw) extract showed signs of recovery from the diabetic state. The diabetic control group exhibited necrosis in the cortex, whereas diabetic rats treated with Murraya (25 ml/kg/bw) displayed a healing effect on the necrotic areas. A reduction in the number of vacuoles was noted when compared to the diabetic control rats. The cortex of diabetic rats treated with Murraya (50 ml/kg) exhibited an architecture akin to that of the control rats (Thorat et al., 2010).

The investigation into the impact of saponins on the expression of obesity-related genes in the liver and adipose tissue of mice subjected to a high-fat diet (HFD) represents a significant advancement in the field. The findings revealed notable effects on daily food consumption, food efficiency ratio, liver mass, and adipose tissue volume in HFD-induced obesity, with these effects being sustained over time during ad libitum conditions. The data suggest that saponins can effectively reduce body weight gain, liver mass, and adipose tissue volume (Chen et al., 2017; N. Li et al., 2021). Additionally, certain findings indicate that the increased expression of the lipogenic gene stearoyl-CoA desaturase 1 (SCD1) in skeletal muscle may play a role in the disruption of lipid metabolism and the advancement of obesity (Hulver et al., 2005), leading to accumulation in adipose tissues. Furthermore, clozapine (10 μM) was shown to elevate the expression levels of stearoyl-CoA desaturase-1 (SCD1) and SREBP-1 during both early (day 3) and late (day 7) phases of differentiation in human adipose-derived stem cells (ASC) (Chen et al., 2017).

Collectively, these studies indicate that certain SGAs promote the expression of SREBP-1 and its downstream lipogenic targets, albeit to varying extents. Overall, these studies show that some SGAs in- crease the expression of SREBP-1 and its downstream lipogenic targets, although to different levels, depending on the nuclear translocation of SREBP-1 and the subsequent modulation of adipogenesis (Chen et al.,2017)

Tan et al. reported that olanzapine treatment, resulted in morphological changes in the subcutaneous white adipose tissue (WAT) of female Wistar rats. This treatment led to an increase in the number of undifferentiated adipose cells in this tissue, detectable as early as the third day of administration, in a manner that was both dose- and time-dependent, yet independent of any body weight gain (Tan et al., 2010; Horska et al., 2016). Furthermore, Minet-Ringuet et al. demonstrated that male Sprague-Dawley rats subjected to a diet supplemented with second-generation antipsychotics (SGAs) for five weeks, did not experience weight gain but exhibited increased adiposity in both subcutaneous and visceral fat depots (Minet-Ringuet et al., 2007; Pouzet et al., 2003).



CHAPTER 5: CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The present study investigates the adverse side effects of the antipsychotic drug clozapine, focusing particularly on its impact on lipid metabolism, which may contribute to cardiovascular mortality among patients undergoing chronic treatment. The findings highlight that while clozapine is essential for managing symptoms of psychotic disorders, it induces significant dyslipidemia characterized by elevated levels of cholesterol and triglycerides. This lipid derangement is linked to the therapeutic efficacy of clozapine, as the drug enhances cholesterol synthesis to support neuronal health and stabilize mental states. However, this increase in lipids also poses serious health risks, necessitating effective management strategies to mitigate associated complications such as weight gain and increased cardiovascular risk.

In response to these challenges, the study explores the potential of **Murraya koenigii** (curry leaf) as a natural intervention. Through a series of experiments involving phytochemical analysis, in vitro antioxidant and hypolipidemic assessments, and in vivo studies in clozapine-induced hyperlipidemic rats, the research establishes that the chloroformic extract of M. koenigii exhibits superior antioxidant(DPPH radical scavenging assay IC50=0.67 microgram/ml) and significantly reduced serum Cholestrol, LDL-Chol and Triglycerides in a dose dependent manner($p < 0.05$). Furthermore, the acute toxicity studies revealed a favorable safety profile for M. koenigii, as no mortality or behavioural abnormalities were seen upto 2000 mg/kg, suggesting that it may serve as a viable adjunct therapy in clinical settings.

The ameliorative effects of M. koenigii were significant, as the treatment effectively normalized lipid profiles and restored antioxidant levels in the context of clozapine-induced oxidative stress(SOD,CAT and GPx were increased). Additionally, the restoration of white blood cell counts indicates its potential to mitigate agranulocytosis, a serious side effect associated with clozapine therapy. Histopathological examinations further corroborated the protective role of M. koenigii, demonstrating improvements in liver and kidney architecture, along with reduced adipocyte hypertrophy in adipose tissues of treated rats.

In summary, the results provide strong preclinical evidence for the use of Murraya koenigii as a natural remedy to counteract the lipid disturbances and oxidative stress induced by clozapine, supporting its role as a functional food that can enhance the health and well-being of patients undergoing long term antipsychotic treatment.

5.2 RECOMMENDATIONS FOR FUTURE RESEARCH

Building upon the findings of this study, several avenues for future research can be explored:

1. **Mechanistic Studies**: Further investigations into the molecular mechanisms by which M. koenigii exerts its hypolipidemic and antioxidant effects are warranted. Understanding the specific signaling pathways and gene expression changes associated with the bioactive compounds of M. koenigii could elucidate its therapeutic mechanisms.
2. **Isolation of Active Compounds**: Future studies should aim to isolate and characterize specific phytochemicals within M. koenigii, such as flavonoids, saponins, and tannins, to assess their individual contributions to lipid regulation and antioxidant activity. Advanced techniques such as high-performance liquid chromatography (HPLC) or mass spectrometry could provide insights into the specific active ingredients. This can enhance our understanding of which compounds are most effective in mitigating clozapine-induced side effects.
3. **Clinical Trials**: To establish the efficacy and safety of M. koenigii in clinical practice, well-designed clinical trials are essential. These studies should involve patients receiving clozapine therapy to evaluate the real-world benefits of incorporating M. koenigii into their treatment regimens.
4. **Formulation Development**: There is potential for developing dietary supplements or functional food products based on M. koenigii extracts. Such formulations could provide patients with a convenient and palatable means to address dyslipidemia and oxidative stress associated with antipsychotic treatment.
5. **Exploration of Other Herbal Remedies**: While this study focuses on M. koenigii, there is a vast array of herbal medicines that may offer similar protective benefits. "Comparative studies involving multiple plant species known for their lipid-lowering and antioxidant properties could lead to a broader understanding of alternative therapies in managing side effects of antipsychotic medications."
6. **Long-term Studies**: Investigating the long-term effects of M. koenigii supplementation in patients on clozapine could provide insights into the sustained benefits and safety over extended periods, especially concerning cardiovascular health and metabolic outcomes.
7. **Broader Pharmacological Interactions**: Understanding how M. koenigii interacts with other pharmacological agents, particularly other antipsychotics or medications for

comorbid conditions, will be essential in developing comprehensive treatment strategies that optimize patient outcomes.

5.3 FUTURE SCOPE OF THE STUDY

This study highlights the potential of **Murraya koenigii** (curry leaf) as an effective natural remedy for mitigating the adverse effects of clozapine, particularly regarding dyslipidemia and oxidative stress. Given the findings of this research, several avenues for future exploration can be proposed:

8. **Mechanistic Studies**: Investigating the underlying mechanisms by which **M. koenigii** exerts its effects on lipid metabolism and oxidative stress is crucial. Future studies could explore how the extracts influence gene expression and modulate signaling pathways associated with lipid synthesis and oxidative stress responses in both in vitro and in vivo models.
9. **Long-term Safety and Efficacy Trials**: While the acute toxicity study indicates a favorable safety profile for the chloroformic extract, long-term studies are necessary to evaluate chronic toxicity, potential side effects, and the overall efficacy of **M. koenigii** in managing dyslipidemia and weight gain in patients undergoing clozapine treatment.
10. **Clinical Trials**: To establish the therapeutic potential of **M. koenigii** in clinical settings, randomized controlled trials should be conducted. Further investigation into the psychopharmacological effects of **M. koenigii** may also reveal additional benefits for mental health, particularly neurodegeneration associated with psychiatric disorders.
11. **Formulation Development**: There is an opportunity to develop practical formulations (e.g., capsules, powders, or functional foods) containing **M. koenigii** extracts that could be easily integrated into the dietary regimens of patients.
12. **Exploration of Synergistic Effects**: Future research could explore the synergistic effects of **M. koenigii** with other natural products or conventional medications used in the management of psychiatric disorders. Understanding these interactions could lead to enhanced therapeutic strategies with fewer side effects.

In conclusion, the findings of this study serve as a foundation for further research into the multifaceted benefits of **M. koenigii**. The exploration of its potential not only contributes to the management of clozapine-related side effects but also positions it as a valuable natural resource in the broader context of health and wellness.

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ANNEXURE 1

गोंय विद्यापीठ
ताळगांव पठार,
गोंय – ४०३ २०६
~~फोन : +९१–८३२–२४५११८४/२४५२८८९~~
फॅक्स : +०९१–८३२–२४५११८४/२४५२८८९

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(Accredited by NAAC with Grade 'A')

Malapati K Janarthanam, Ph.D.
Senior Professor
Department of Botany

25/03/2022

CERTIFICATE

This is to certify that the plant provided in the form of herbarium sheet by Ms. Pearl Pinto, Research Scholar at Lovely Professional University, Phagwara is identified by me as *Murraya koenigii* (L.) Spreng. which is a synonym of *Bergera koenigii* L. (Rutaceae).

The identification of the given specimen is done at her request towards her project on "Ameliorative effect of *Murraya koenigii* on Clozapine induced Dyslipidemia".

25/03/2022
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ANNEXURE 2

PAPER 1

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Research Article

Effect of Stratified Doses of Murraya Koenigii on Brain Antioxidant Status of Wistar Rats Treated with Clozapine.

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Abstract
Clozapine is considered as the most potent antipsychotic medication for managing refractory schizophrenia. However, Clozapine may contribute to oxidative stress within the forebrain. Given that Murraya koenigii leaves are abundant in flavonoids and phenolic compounds, they possess significant free radical scavenging capabilities. Leaves were collected from southern parts of Goa, processed for extraction using Soxhlet extraction method with three solvents namely chloroform, methanol and water. The antioxidant property was evaluated in vitro using DPPH, ABTS, FRAP assays along with determination of MDA levels in all the three extracts. This study aims to examine possible outcome of Murraya koenigii on the Clozapine-induced oxidative stress in rats given saline, Clozapine, and Clozapine plus chloroformic extract of Murraya koenigii for 28 days. The brain was isolated and homogenized for the determination of Superoxide dismutase, Catalase, Glutathione peroxidase and lipid peroxidation assay. Our findings confirm the existence of hydrophilic polyphenolic compounds that contribute to the enhanced reducing capacity, along with elevated levels of flavonoids that are responsible for the ABTS and DPPH scavenging activities of the chloroform extract. In the clozapine-treated control group, oxidative stress was evident, characterized by a significant raise in MDA levels, simultaneously decreasing the levels of SOD, CAT and GPx when compared to the normal control. The CEMK-treated group exhibited an antioxidant effect, significantly reducing MDA levels and increasing antioxidant enzyme levels in comparison to the clozapine control subject in a dose dependent manner. So, this investigation makes it clear that the chloroform extract of Murraya koenigii leaves might significantly reduce the high levels of oxidative stress markers induced by the antipsychotic drug clozapine.

Keywords: Clozapine, Murraya koenigii, Antioxidant, Superoxide dismutase, Catalase, Glutathione Peroxidase, Lipid peroxidation, Flavanoids

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Pancreatic Lipase inhibition assay of various extracts of leaves of Murraya Koenigii in southern areas of Goa

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Abstract. The objective of the study was to assess the lipase inhibitory activities of chloroformic, methanolic and aqueous extracts from the commonly available *Murraya koenigii* (L.) Spreng leaves(Rutaceae) in southern villages of Goa, for potential use in the treatment of obesity. Extracs of the leaves of this plant were evaluated for lipase inhibitory activity using porcine pancreatic lipase (PPL: triacylglycerol lipase) and p-nitrophenyl butyrate in an in vitro assay. Among the three extracts screened, chloroformic extract exhibited the highest pancreatic lipase inhibitory activity of 53.42%, followed by methanolic extract (51.88%) and aqueous extract (36.42%), respectively. Chloroformic extract has not been screened for its pancreatic lipase inhibition assay. All the Crude extracts of leaves of Murraya *koenigii* (L.) Spreng leaves (Rutaceae) have potential as pancreatic lipase inhibitory agents. . Chloroformic extract was found to be most effective and hence can be used as a potent anti-obesity agent to combat hyperlipidemia.

Keywords: Murraya koenigii, pancreatic lipase inhibitory activity, Soxhlet extraction, p-nitrophenyl butyrate, anti-obesity agent

1 Introduction

Obesity is one of the leading causes for metabolic disorders that is an outcome of imbalance between food intake, physical activity, metabolic rate or could be drug induced. Several approaches have been implied for the treatment of obesity targeting at specific mechanisms, which include lipase inhibition, suppressive effect on food intake, stimulatory effects on energy expenditure, inhibition of adipocyte differentiation and the regulatory effect on lipid metabolism [1] The irregularities seen with respect to lipid levels such as increases in total and low-density lipoprotein (LDL) cholesterols, low concentrations of high-density lipoprotein (HDL) cholesterols, and high triglyceride levels is termed as Dyslipidemia. Drug induced dyslipidemia in particular increases the risk of cardiovascular disease and metabolic dysfunction[2]. Research shows that improving antioxidant status and arresting the accumulation of lipids in the hepatocytes improves blood lipid profile. [3]–[5] This is possibly the most effective way for combating Cardiovascular disorders and liver disorders. [6]
Currently there are plenty of therapeutic drugs but with limited efficacy and undesirable side effects. One of the most widely studied approach is the inhibition of pancreatic lipase [1]. Natural plant sources can interrupt the lipase as well as adipocyte activity ,thus, bring about inhibition of fat absorption and/or fat accumulation in the body [7]
The pancreatic lipase enzyme is a crucial enzyme in the human digestive system for breaking down dietary fat. Interfering with fat absorption along the gastrointestinal tract is one of the potential ways for treating obesity[8]. Pancreatic lipase has a major role in digestion of triglycerides. Pancreatic lipase inhibitors are substances that reduce the activity of the enzyme in the small intestine, primarily by decreasing fat absorption. Inhibition of pancreatic lipase activity is the most widely studied approach to find potential anti- obesity agents[9].

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MINI-REVIEW ARTICLE

Protective Effects of *Murraya koenigii*: Focus on Antihyperlipidemic Property

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Abstract: In the current scenario, discovery of natural bioactive components can be considered as a major development in treating common ailments. One of the medicinally important herbs is Murraya koenigii. The biological functions are promoted by the leaves, fruits, roots, and bark of this beautiful plant. It is the carbazole alkaloids that promote most of the medicinal properties and contribute to the anti-oxidative properties as well. Terpenoids, Flavonoids, Saponins and Phenols isolated from different parts of the plant have unique hypocholestrolemic and antidiabetic activities. Among commonly used alternative therapies, plant sterols present in *M. koenigii* may help to reduce cholesterol and triglyceride levels, in turn managing heart diseases. Experimental animal studies are proving the hypolipidemic ability of *M. koenigii*. Possible mechanisms involved in exhibiting such an amazing hypolipidemic ability can be attributed to the phytochemicals, some of which can reduce the absorption of cholesterol in the intestines or accelerate the catabolism of fats. In contrast, others can inhibit the enzyme HMG CoA reductase. *M. koenigii* can inhibit pancreatic lipase. Such a response could be due to the presence of carbazole alkaloids like Mahanimbin, Isomahanine, Murrayacinine, Koenimbine, Mahanimboline, Murrayazolinine, Girinimbine *etc*. These enzymes can be selected for the pharmaceutical mediation of hypocholesterolemia agents. A triumph over the production of lipids in the hepatic cells is achieved upon feeding *M. koenigii*, thereby bringing about a drastic fall in triglyceride levels. The present review provides a better understanding of the major components of *M. koenigii* against dyslipidemia that could serve as an herbal alternative while treating other pathological conditions. Although various extracts of *M. koenigii* have numerous medical applications, an extensive investigation of their toxicity, along with more clinical trials and standardization of protocols, is required to produce modern drugs from these leaf extracts.

Keywords: *Murraya koenigii*, dyslipidemia, anti-hyperlipidemic, anti-obesity, phytochemicals, pancreatic lipase, review.

1. INTRODUCTION

A medical disorder known as dyslipidemia is characterized by elevated levels of LDL cholesterol, triglycerides, and serum cholesterol along with a subsequent decline in High-density cholesterol (HDL) cholesterol. Dyslipidemia is a determinant of cardiovascular disease [1]. Blood flow across blood arteries is hampered by elevated cholesterol levels. This hypercholesterolemic condition imposes increased reactive oxygen species (ROS) emission, which amplifies lipid peroxidation and poses numerous hazards to the cardiovascular system. Additionally, it makes the patient's oxidative stress and atherosclerosis worse [2]. Obesity-related blood pressure, arteriosclerosis, blood vessel inflammation, and diabetic cardiomyopathy (DCM) afflict most people today. This is a more severe and prevalent diabetic consequence that raises mortality rates in the blood pressure association [3]. Synthetically manufactured drugs utilized to treat dyslipidemia belong to a class of HMG CoA reductase inhibitors (statins). Peripheral neuropathy, hyperglycemia and hepatotoxicity are some of the statin side effects that may make many people reluctant to take them.

So, naturally occurring herbs can be used as alternatives to treat dyslipidemia. Among commonly used alternative therapies for controlling lipids, plant-based diets have been shown to have some effectiveness [4]. The major component of any plant is sterol. These plant sterols may be indicated to fight high cholesterol, facilitate fat mobilization due to suppressed lipogenesis or bring about a depressed lipid deposition, which in turn works best for the prevention and management of heart diseases [2]. *A common herb, Murraya koenigii (Linn) Spreng, contains several chemical components that work together to produce a particular pharmacodynamic re-*

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