"Investigation of Anti-obesity Potential of Heartwood Hydroalcoholic Extract of *Acacia suma* (Roxb) in High-Fat Diet Fed C57BL/6 Mice"

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DOCTOR OF PHILOSOPHY

IN

THE FACULTY OF PHARMACY

By

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Nikita Ningappa Kanbarkar

LIST OF ABBREVIATIONS

ACN:	Acetonitrile
ANOVA:	Analysis of variance
ALP:	Alkaline phosphatase
AP:	Adipose Tissue
API:	Ayurvedic pharmacopeia of India
ARC:	Arcuate nucleus
AMP:	Adenosine monophosphate
AMPK:	AMP-activated protein kinase
Aq.:	Aqueous
$AlCl_2$:	Aluminium chloride
BMI:	Body Mass Index
BAT:	Brown Adipose Tissue
CAT:	Catalase
CB:	Cannabinoid receptor type 1
CPCSEA:	Committee for the purpose of control and supervision of experiments on animals
CPT1:	Carnitine palmitoyl transferase 1
CRF:	Corticotrophin releasing factor
CREB:	Cyclic AMP response element-binding protein
ChEBI:	Chemical entities of biological interest
$CuSO_4$:	Cupric Sulfate
DMEM:	Dulbecco's modified eagle's medium
DMSO:	Dimethyl sulfoxide
DNS:	Dextrose/Sodium Chloride solution
DLPFC:	Dorsolateral prefrontal cortex
DPPH:	2, 2-diphenyl-1-picrylhydrazyl
EGC:	Epigallocatechin
ELISA:	Enzyme Linked Immunosorbent Assay
FAS:	Factors associates suicide/ Cell Apoptosis
FT:	Fisetin
FDA:	Food and drug administration
FOXO1:	Forkhead box protein O1
FRAP:	Ferric Reducing Antioxidant Power
GABA:	Gamma-aminobutyric acid
GLUT4:	Glucose transporter type 4
FFA:	Free fatty acid
HDL:	High density lipo-proteins
HFD:	High fat diet
HPLC:	High performance liquid chromatography
HPTLC:	High performance thin layer chromatography
HCl:	Hydrochloric acid

HNO_3:	Nitric acid
H_2SO_4:	Sulphuric acid
H_2O_2:	Hydrogen peroxide
hrs.:	Hours
HT:	Hydroxy tryptamine
$HgCl_2$:	Mercuric chloride
IAEC:	Institutional animal ethical committee
ICH:	International Council for Harmonization
ICMR-INDIAB:	Indian council of medical research- India diabetes
IBD:	Inflammatory bowel disease
IC_{50}:	Half maximal inhibitory concentration
KEGG:	Kyoto Encyclopedia of Genes and Genomes
KOH:	Potassium hydroxide
LDL:	Low density lipo-proteins
LOD:	Limit of detection
LOQ:	Limit of quantification
MCH:	Melanin concentrating hormone
NaOH:	Sodium hydroxide
Na_2CO_3:	Sodium bicarbonate
NOTCH1:	Neurogenic locus notch homolog protein 1
NCOA:	Nuclear co-activator 1
OD:	Optical density
OECD:	Organization for Economic Co-operation and Development
PCR:	Polymerase chain reaction
PDB:	Protein data base
PDA:	Photo diode array
POMC:	Pro-opiomelanocortin
PYY:	Peptide YY
PPAR γ:	Peroxisome Proliferator Activated Receptor Gamma
PPAR δ:	Peroxisome Proliferator Activated Receptor Delta
PVN:	Paraventricular nucleus of the hypothalamus
PVDF:	Polyvinylidene fluoride
µm:	Micron meter
mg/dL:	Milligram per deciliter
mg/kg:	Milligram per kilogram
Min:	Minutes
MTT:	3 - (4, 5-dimethylthiazol- 2 - yl)-2, 5-diphenyltetrazolium bromide
Nm:	Nanometer
NO:	Nitric Oxide
QT:	Quercetin
R^2:	Coefficient of regression
ROS:	Reactive oxygen species
R_t:	Retention time
RT-PCR:	Real Time-Polymerase chain reaction

RSD:	Relative standard deviation
SREBP:	Sterol regulatory element binding proteins
SGOT/AST:	Serum glutamic oxaloacetic transaminase/ Aspartate transaminase
SGPT/ALT:	Serum Glutamic Pyruvic Transaminase/ Alanine transaminase
SOD:	Superoxide dismutase
SEM:	Standard error of the mean
TBARS:	Thiobarbituric acid reactive substances
TC:	Total cholesterol
TCA:	Trichloroacetic acid
TLC:	Thin layer chromatography
TG:	Triglycerides
TNF-α:	Tumor necrosis factor
THF:	Tetrahydrofuran
TFC:	Total flavonoid content
TPC:	Total phenolic content
TTC:	Total tannin content
UCP:	Uncoupling protein
VLDL:	Very low density lipo-proteins
VMH:	Ventromedial hypothalamus
V/v:	Volume/volume
Vs:	Versus
WHO:	World Health Organization

ABSTRACT

Background: Obesity is defined as "Chronic complex diseases characterize with excessive adiposity that impairs health. BMI is a major indicator of obesity measured as weight in kilogram divided by height in meter-square. Other than obesity metabolic syndrome includes glucose intolerance, insulin resistance, lipid disturbance, hypertension and cardiac diseases.

Aim and objectives: The study aimed to investigate the anti-obesity effect of *Acacia suma* (Roxb.) in the experimental animal model. The objectives of this study are to predict the binding affinity of phytoconstituents from *Acacia suma* using in-silico approach and investigation of anti-obesity activity of heartwood hydroalcoholic extract of *Acacia suma* using 3T3-L1 cell line and in experimental animal model.

Methodology: The HPLC and HPTLC characterization of HAE of *Acacia suma* were performed to confirm the quality of procured plant material. Phytochemicals showing anti-lipase and anti-oxidant potential from plant was predicted by using molecular docking and network pharmacology. The inhibition of lipase, alpha amylase and alpha glucosidase potential was tested for extract. In-vitro study includes estimation of lipid content in differentiated 3T3-L1 cells. Extract was tested on high-fat diet-induced (60% fat) C57BL/6 mice as obesity screening model. Anthropometrical, biochemical, oxidative stress level and histopathological evaluation were made on C57BL/6 mice.

Results: The HPLC method development and validation analysis has explored the presence of epigallocatechin, fisetin and quercetin in extract. Moreover, HPTLC characterization had proved the presence of epigallocatechin in extract, HPTLC-DPPH assay was performed which confirms the anti-oxidant potential of extract, diinsininol was found with highest docking score by in-silico method. The strong anti-lipase activity along with alpha amylase and alpha glucosidase inhibition potential was analyzed for hydroalcoholic extract of *Acacia suma*. The extract treated groups had shown significant reduction in body weight, abdominal

circumference and body mass index in disease control group compared to the normal.

Conclusion: The high-fat diet-induced C57BL/6 mice obesity model had shown significant reduction in weight gain on dose dependent manner when treated with heartwood hydroalcoholic extract of *Acacia suma*.

Keywords: Obesity, *Acacia suma*, hydroalcoholic extract, lipase inhibition, anti-oxidants, C57BL/6

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Introduction

1. INTRODUCTION

Obesity is a preventable kind of chronic disease which negatively affect whole body functioning. Food metabolic dysfunction creates an imbalance in both food intake and energy expenditure.[1] Previous reports suggest that obese peoples are in large numbers in developed countries, but now obesity is affecting rapidly the developing countries as well.

Worldwide more 650 million persons were determined to be obese, while more than 1.9 billion adults were found to be overweight. In India, 135 million people were affected by obesity.[2] According to diabetes study the India the prevalence of generalized obesity and abdominal obesity were 135 and 153 million individuals respectively.[3]

Allopathic medications have been used to treat and relieve this immediate issue. In particular, Orlistat (Xenical), Phentermine-topiramate (Qsymia), Naltrexone-bupropion (Contrave), Liraglutide (Saxenda), and Semaglutide are available products that have gained regulatory approval. Few drugs have been banned due to their life-threatening adverse effects, including psychiatric disorders and strokes.[4] The mode of action of anti-obesity drugs is classified as digestive enzyme inhibitors, appetite suppressants, fat absorption inhibition, thermogenesis and increased fat mobilization etc.[5]

The pathogenesis of obesity suggests that obesity is a multifactorial polygenic disease that includes many metabolic pathways and genetic malfunction responsible for energy homeostasis. Many genes are associated with obesity: leptin, adiponectin, pro-opiomelanocortin, insulin, peptide YY, resistin and ghrelin etc. Leptin is a hormone produced by adipocytes that aid in storing energy, regulation, as well as fertility. Adiponectin act as homeostatic regulator of glucose, lipid, and energy

metabolism. Pro-opiomelanocortin (POMC) works by controlling eating habits and peptide YY (PYY) helps to manage the body weight by hypothalamic mechanism. Hence inactivity or mutation of any one of these gene leads to obesity.[6]

Advancement in technology to reside with sedentary lifestyle and consumption of unhealthy diet increases risk of overweight and obesity. The lack of exercise and the use of electronic devices may lead metabolic diseases. Few etiological factors are depicted in (figure1). Obesity not only affects health but also has potential effects on the social and economic well-being of an individual and community. Despite identifying obesity as a serious problem to date there is no such medicine available to cure obesity.

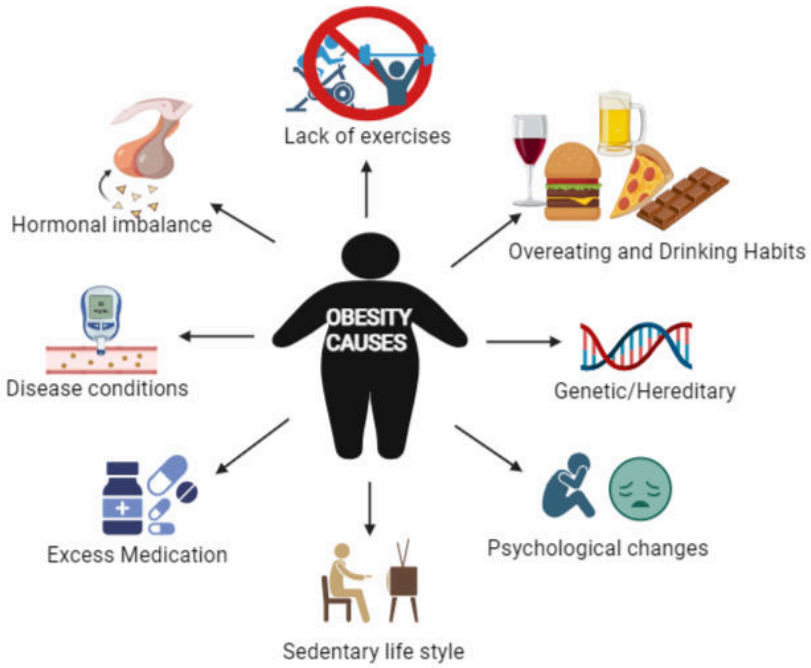


Figure 1: Etiological factors of obesity

Herbal medicines are now accepted all over the world, and market demands for these medicines have increased day by day. Natural health products or medicines are considered more effective and free from side effects also herbal medicines are identified and practised in ancient times to treat ailments. However, very few traditional medicines have been proven scientifically for their therapeutic use, and

many have to standardise to check their quality, and purity and control adulteration or use of fake medicines. India is blessed with well-recorded and practised knowledge of traditional medicines such as Ayurveda, Siddha, and the Unani system of medicines. Screening of these medicinal plants for improving the quality of life to serve effective and safe medicines is essential.[7]

According to traditional texts, the heartwood of *Acacia suma* was used to treat metabolic disorders and belongs to 'Fabaceae' family and known as Kadar or Shwet Khadir in Sanskrit. In India, it is found in Bengal, Bihar and Karnataka regions.[8] *Acacia suma* has bitter and thermogenic property also used for the treatment of epilepsy, diabetes, and obesity. In Ayurvedic texts, the heartwood of this plant is reported to use in the treatment of *Kusthaghna, Mukharoga, Raktadosa, Madhumeha, and Medodosa.*[9] The stem bark of the plant contains Gallocatechin-7-gallete, Quercetin, and 5, 4 dihydroxy-7, 3 dimethoxyflavone- 3-o-D- galactopyranoside whereas heart-wood contains β sitosterol, stigmasterol and oleanolic acid. Earlier studies, reported *Acacia suma* to have diuretic and laxative properties[10], anti-inflammatory and analgesic properties[11], anthelmintic activity[12], and wound healing properties.[13]

Considering the traditional use of heartwood of *Acacia suma* in metabolic disorders, no attempts were exercised to explain its influence on obesity. As an outcome, we stepped forward to study the anti-obesity potential of *Acacia suma* on experimental animal.

Chapter – 2

Aim and Objectives

2. AIM AND OBJECTIVES

Aim

To investigate the anti-obesity potential of *Acacia suma* [Roxb.] in the experimental animal model

Objectives

- To study the anti-obesity potential of heartwood hydroalcoholic extract of *Acacia suma* in 3T3-L1cells and in high fat diet fed C57BL/6 mice model.
- To assess the lipase inhibition, alpha amylase inhibition and alpha glucosidase inhibition of extract by in-vitro method.
- To predict the binding affinity of phytoconstituents from *Acacia suma* with targets related to obesity by in-silico approach.

Chapter – 3

Review of Literature

3. REVIEW OF LITERATURE

3.1. Background

Obesity is defined as "Chronic complex diseases characterize with excessive adiposity that impairs health. Also, it's a multifactorial disease caused due to obesogenic environments, psycho-social factors and genetic variants. In a few patients, it may occur due to excess medications, immobilization or genetic factor. BMI is a major indicator of obesity assessed as weight in kilogram by height in meter.[2,14] Other than obesity metabolic syndrome includes glucose intolerance, insulin resistance, lipid disturbance, hypertension and cardiac diseases.[15]

3.2. Epidemiology of Obesity

The global burden of metabolic diseases, mostly overweight and obesity has increased and the data collected from 199 countries represented that prevalence of obesity was 1.9 million during the year 2008 to 2013. Also increase in BMI was observed in men 29 to 37% and in women 30 to 38 % between the years 1980 to 2013.[16]

In the study conducted in India by ICMR-INDIAB-3 in 2015, reported that in women, BMI was found 23.6 and 21.2 kg/m^2 whereas; in men 22.7 and 20.9 kg/m^2 were observed in urban and rural areas respectively in four selected states of India. After translating these values for the whole country around 88 million overweight, 135 million with generalised obesity, 153 million with abdominal obesity and 107 million with combined obesity would be estimated in India.[3]

Being the second-largest country in population, India has third place in the most obese country in the world after America and China. The generalised obesity in south India was 46% compared to 35% in America. It was reported that all grades of obesity can cause a large number of deaths significantly[17] and predicted the level of overweight and obesity may reach 89% in males and 85% in females by the year

2030; resulting increased risk of heart diseases by 97%, cancer by 61% and type 2 diabetes by 21% which can directly affect on healthcare cost. Whereas reducing 5% BMI can save €495 expenditure spent on obesity-related healthcare costs by 2030.[18]

3.3. Classification of Obesity

As the grades of obesity increases the risk of obesity-related complications increases. Based on BMI obesity can be categorised as follows-

i. Normal range: 18.5-24.9 kg/m^2

ii. Overweight: 25.0-29.9 kg/m^2

iii. Grade-I Obesity: 30.0-34.9 kg/m^2

iv. Grade-II Obesity: 35.0-39.9 kg/m^2

v. Grade-III Obesity: ≥ 40 kg/m^2

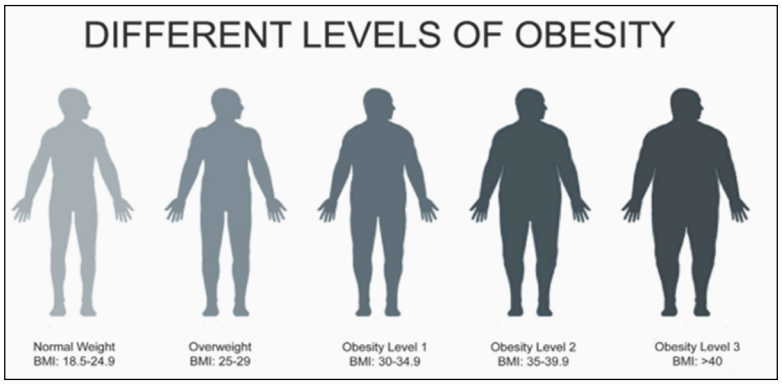


Figure 2: Classifications of obesity

3.4. Adipose tissue

Adipose tissue is the major endocrine gland and energy-storing reservoir of the body. Two types of human adipocytes are found; white adipose tissue (WAT) and brown adipose tissue (BAT). WAT consists of a large fat droplet and other organelles and brown adipose tissue had many fat droplets and more mitochondria shown in the (figure 3). Further BAT is having two types; Subcutaneous (WAT) and Visceral (WAT)[19]

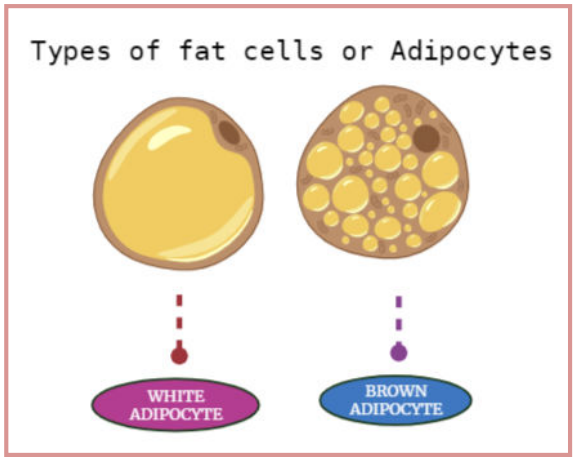


Figure 3: Types of adipose tissue

3.5. Functions of adipose tissue

Adipose tissues (AP) are distributed all over the body and act as an energy reservoir and are capable of storing more energy in the form of lipid droplets. AP act as an insulator by generating heat and maintaining body temperature hence called a homeostasis agent. It provides nutrients by lipolysis of triglycerides in the form of fatty acids and glycerol.[15]

Dysfunction of lipid storage capacity can cause deposition of lipid in circulation and it starts accumulating in the liver, heart, kidney, and muscles in the form of fatty acid and triglyceride and contributes to generate obesity and their comorbidities like insulin resistance, diabetes, cardiovascular diseases and bone diseases.[20]

Adipocytes have specialised secretary functions where it secrets lipid, proteins/ peptides, interleukin and cytokines, collagen, matrix metalloproteinase, and lipoprotein lipase, which regulates thermogenesis, angiogenesis, and immunity, inflammation, blood circulation and insulin secretion. Hence finding out genes or proteins which regulate the normal function of adipocytes become a newer concept for the treatment of obesity.[21]

The effects of increased adiposity on physiological and metabolic changes along with their mechanism were represented in (figure 4).[22]

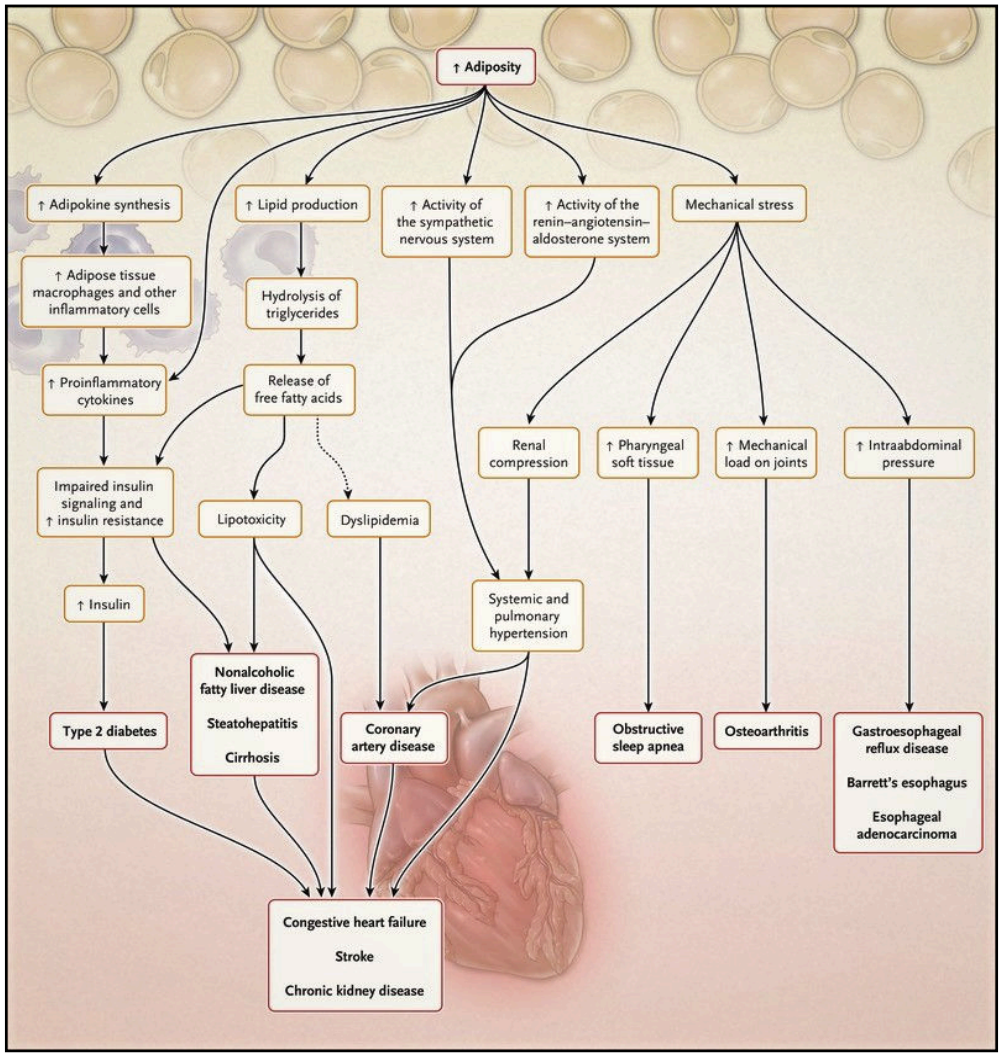


Figure 4: Schematic representation of mechanical, metabolic and physiological effects of excess adiposity on the body[22]

3.6. Genes/proteins involved in Obesity

Being polygenic or multifactorial disorder obesity is strongly influenced by the up-and-down regulation of genes. The genetic factors responsible for weight gain are as follows; Leptin is the specific gene that regulates adipose tissue expansion by hypothalamic act through a single transmembrane-domain-receptor, mutation of leptin genes can cause early onset of obesity.[23] Along with controlling body weight and energy storage it plays important role in fertility.

Adiponectin: it is an adipocytokine situated on chromosome 3q27 and regulates lipid, glucose and protein metabolism. Adiponectin is exclusively produced by adipose tissue it has a matrix-like protein with a homology of collagen.[24]

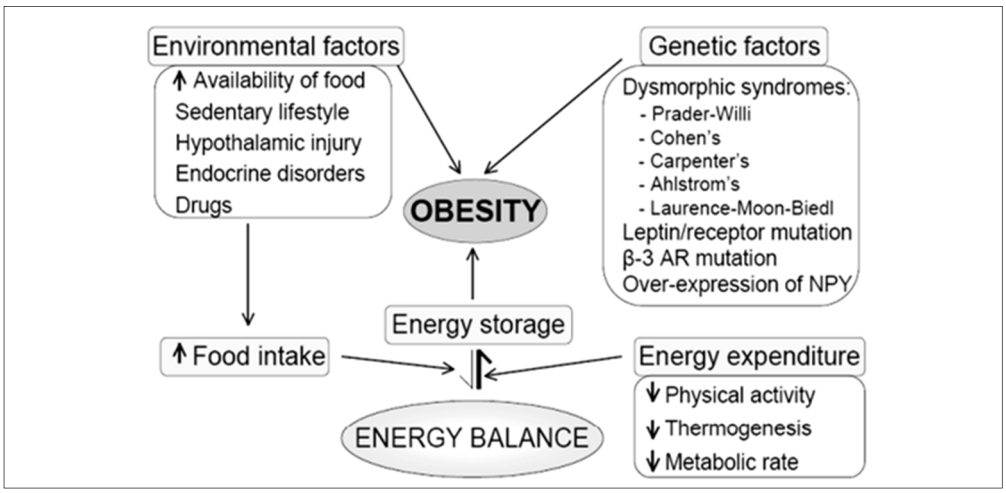


Figure 5: Correlation between energy intake, energy expenditure and energy balance[24]

Pro-opiomelanocortin (POMC) plays important role in human behaviour and pharmacologically proven activities including central and peripheral systems, adrenocortical function, regulating food intake and energy storage and the immunity system. Lack of POMC can cause obesity in rodents and humans, defective adrenal function and altered pigmentation. This hormone can be used in the treatment of obesity.[25,6]

Uncoupling proteins are found in the inner mitochondrial membrane and act as carrier proteins. Divided into four classes; UCP1: in BAT, UCP2: present in several skeleton muscles, UCP3: in BAT and skeleton muscles and UCP4: is located in the brain. Increased gene expressions of UCP give rise to type 2 diabetes and weight gain.[26]

Insulin over secretion of insulin generates obesity; insulin resistance has a marked effect on mitochondrial dysfunction, lipotoxicity and inflammation. Insulin reduces

blood glucose, promotes mitochondrial function regulates cell proliferation. Insulin resistance initiates many health issues; such as obesity, fatty liver, lipodystrophy, oxidative stress and inflammation. Also, PPAR γ contributes to insulin resistance; it controls lipid synthesis and fat storage.[27] Beta2 adrenergic receptor gene plays an important role in lipolysis found in human adipose tissue. The defective expression may lead to a significant effect on obesity and lipolysis function.[28] Resistin is the cysteine-rich protein that takes part in generation of type 2 diabetes mellitus and obesity. Resistin has a glucose homeostasis effect, studies have identified the highest level of resistin in obese patients.[29] Ghrelin is an orexigenic hormone that contains 28 amino acids which stimulate food intake. Over-expression of ghrelin promotes obesity and metabolic syndrome. Dieting can maintain ghrelin levels, in obese patients reduced level of ghrelin has been observed.[30]

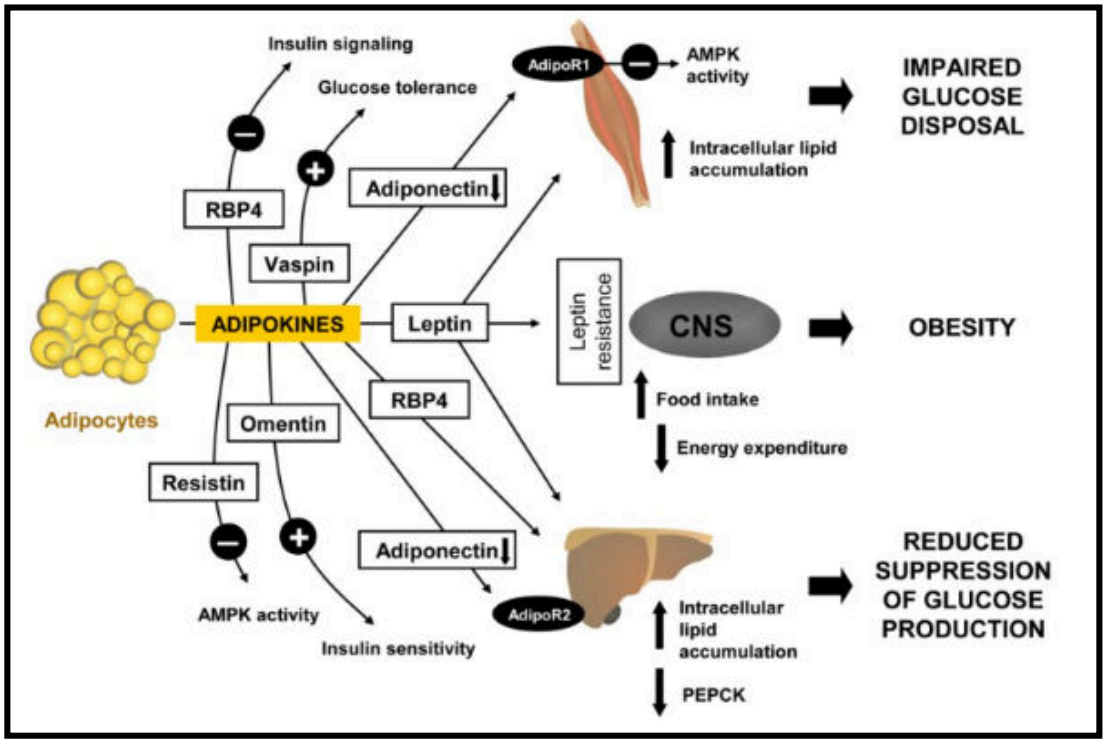


Figure 6: Role of adipocytes dysfunctioning in obesity[31]

3.7. Environmental conditions and obesity

Environment plays an important role in obesity. Easy attraction has been increased towards oily and spicy food, which contains high calories, high fat and cholesterol and also available at a cheap price. In today's stressed-out world very few peoples control their eating habits by maintaining food frequencies and time by having a healthy and nutritional diet. Eating fast food, junk food, and using electronic devices while eating these habits can affect the young generation and promoting these conditions leads to an imbalance between food intake and expenditures. Further, Several endocrine disorders and medication overuse, can all contribute to obesity.[32]

3.8. Food intake and obesity

Food intake is dependent on the behavioural patterns of human beings. The interaction between the satiety signal and the sensory signal produced in the hypothalamic region of the brain influences hunger and imaginary affection for food, its taste, smell and texture get activated. The satiety signals are connected to the orbitofrontal cortex which drives the brain to eat food and generates the eating pattern of the person. Hence it is necessary to always eat healthy and nutritional food.[33]

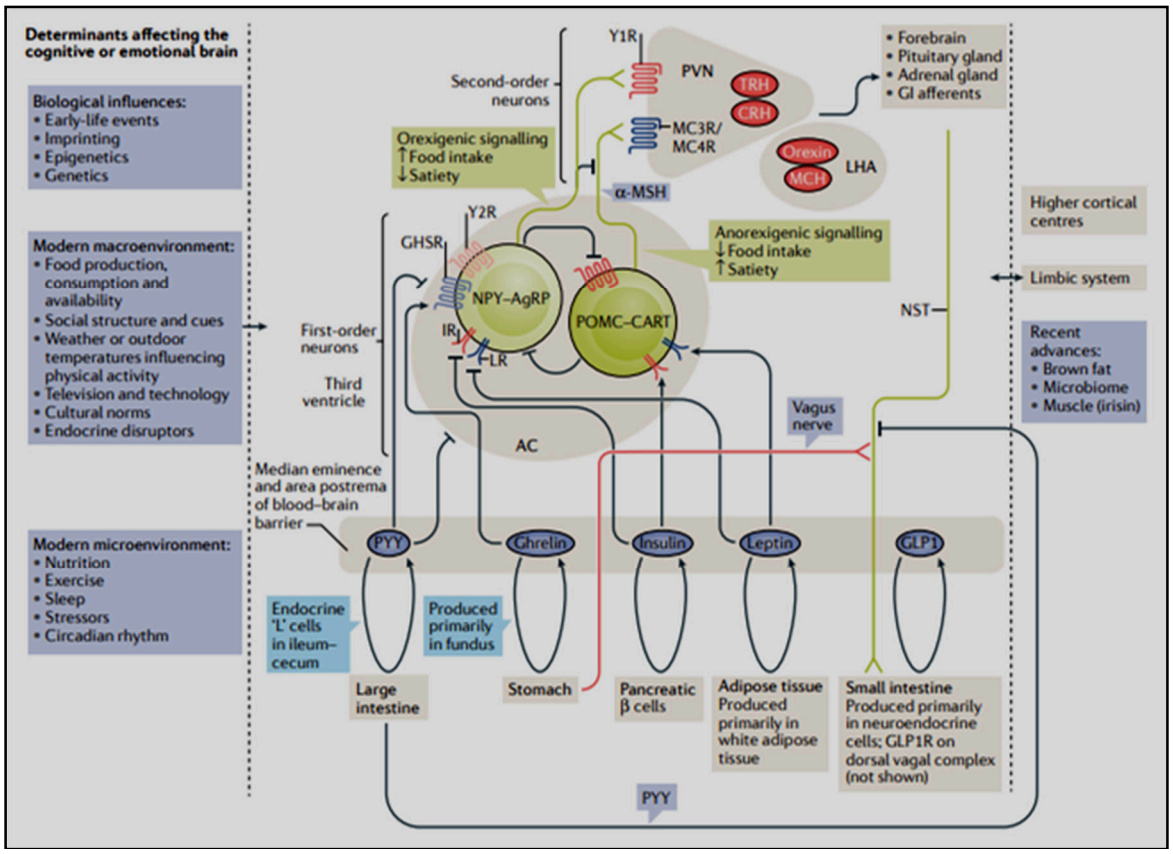


Figure 7: Neuronal and hormonal pathways influence food intake and satiety in the brain. The neuro–hormonal pathways, gut hormones and adiposity signals reciprocally interact between the hypothalamus, brainstem, higher cortical areas and limbic system to control appetite regulation**. α-MSH:** α-melanocyte-stimulating hormone; **CRH:** Corticotropin-releasing hormone; **GHSR:** Growth hormone secretagogue receptor; **GI:** Gastrointestinal; **GLP1:** Glucagon-like peptide 1; **GLP1R:** GLP1 receptor; **IR:** insulin receptor; **LHA:** lateral hypothalamic area; **LR:** Leptin receptor; **MCH:** Melanin-concentrating hormone; **MC3R:** Melanocortin receptor 3; **NST:** Nucleus of the solitary tract; **PVN:** Paraventricular nucleus; **PYY:** Peptide YY; **TRH:** Thyrotropin-releasing hormone; **Y1R:** Y1 receptor; **Y2R:** Y2 receptor.[34]

3.9. Role of anti-oxidants in obesity

Adipokines such as insulin, leptin, adiponectin interleukin, and TNF-alpha are secreted from adipose tissue causes a significant disruption in obese people. An increased level of inflammatory adipokines triggers the mitochondrial function to produce ROS (reactive oxidative species) and dysfunctional mitochondria generate an abnormally high amount of ROS, which can be controlled by brown adipose tissue by thermogenesis. Anti-oxidants like vitamin E, polyphenols, carotenoids, heme oxygenaes-1 and superoxide dismutase control the oxidative stress by reducing the elevated level of inflammatory mediators.[35]

The amount of anti-oxidants present in the human body is measured by estimating the plasma concentration of anti-oxidant markers such as vitamin E and C, retinol, carotenoids, zinc level and antioxidant enzyme concentration.[36] The pathways responsible for oxidative stress and antioxidant defence mechanism have been described in (figure 8).

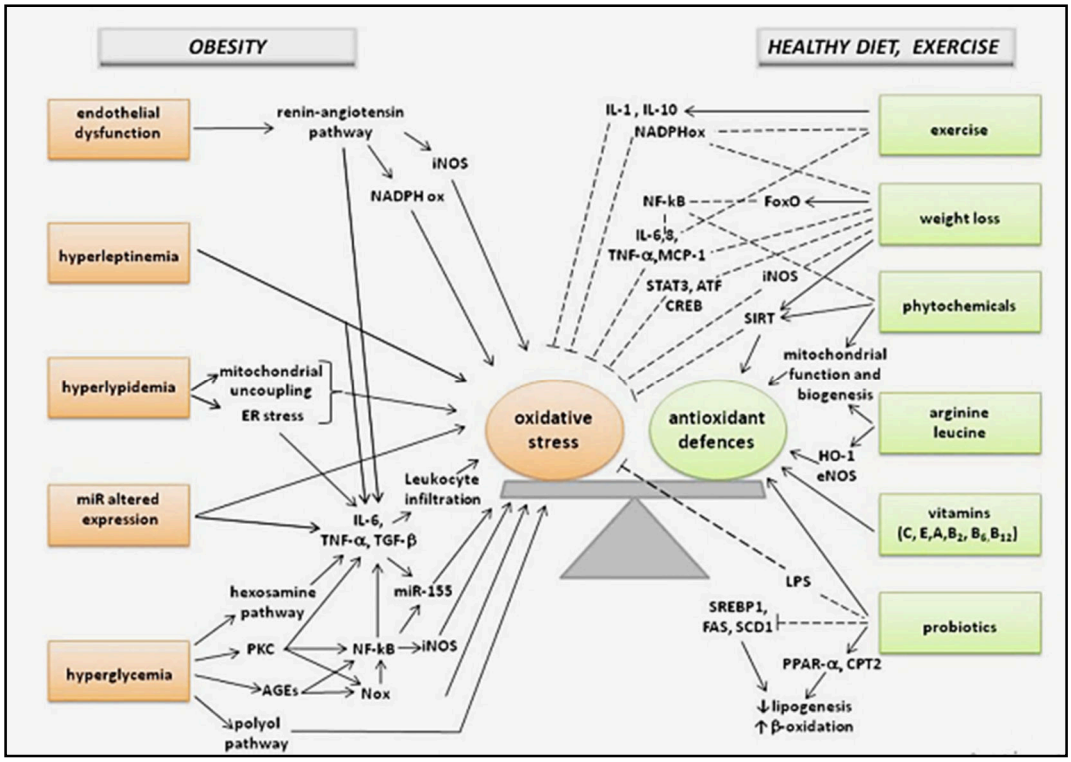


Figure 8: Relationship between oxidative stress and antioxidant defences mechanism. **AGEs:** Advanced glycation end products; **ATF:** NF-κB, activating transcription factor; **CPT2:** Carnitine palmitoyltransferase 2; **CREB:** Cyclic AMP response element binding; **ER:** Endoplasmic reticulum; **FAS:** Fatty acid synthase; **FoxO:** Forkhead box, sub-group O; **HO-1:** Heme oxygenase-1; **iNOS:** Inducible nitric oxide synthase; **LPS:** Lipopolysaccharide; **MCP-1:** Monocyte chemotactic protein-1; **miR:** MicroRNA; **NF-κB:** Nuclear factor-κB; **Nox:** NADPH oxidase; **PKC:** Protein kinase C; **PPAR-α:** Peroxisome proliferator-activated receptor-α; **SCD1:** Stearoyl-CoA desaturase-1; **SIRT:** Sirtuin; **SREBP1:** Sucrose responsive element binding protein1; **STAT3:** Signal transducer and activator of transcription 3; **TGF-β:** Transforming growth factor-β; **TNF-α:** Tumor necrosis factor-α.[36]

3.10. Obesity-related co-morbidities

Obesity is associated with several co-morbidities, overweight or obese people are at risk of having cardiac diseases, insulin resistance, diabetes mellitus, glucose intolerance, dyslipidaemia, arthritis, gout, stroke, sleep apnoea and certain cancer can also cause due to obesity.[37] Increased BIM is a sign of increased risk of cardiac diseases.[38,39] Diabetes is the major risk factor of obesity with similar

pathophysiological pathways hence it is also called 'Diabesity'. The expected number of diabetes patients by 2040 is around 642 million.[31]

Dysfunctioning of lipid metabolism causes an increase in serum concentrations of HDL, LDL, VLDL, triglycerides and cholesterol. Obese people are more vulnerable for developing dyslipidemia.[40] Obesity has a significant impact on arthritis, disability of joints movement increased risk of joint damage and inflammation which contributes to osteoarthritis.[41] Weight gain and gout have a positive association, obese patients are more likely to cause gout compared to non-obese people.[42] Around 20% of cancer cases occur due to obesity. A list of cancers caused due to obesity includes liver, breast, colon, renal, thyroid, gallbladder, leukaemia and prostate cancer. Physical exercise to reduce increased weight improves the quality of life in cancer patients.[43]

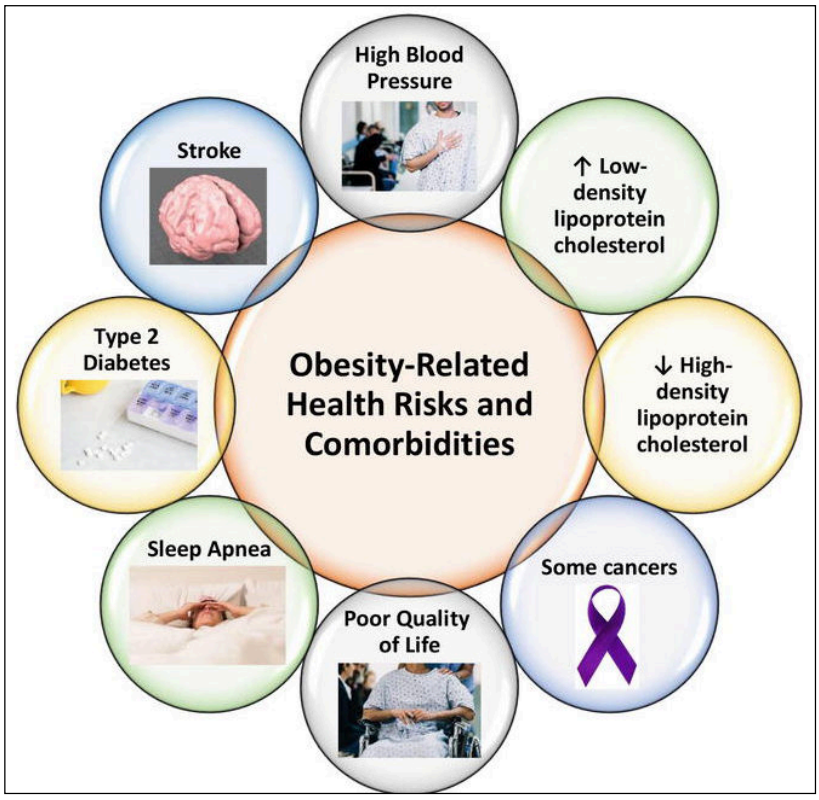


Figure 9: Comorbidities and risk factors of obesity [44]

3.11. Current pharmacotherapy for obesity treatment

Non-pharmacological treatment for obesity

1. Diet interventions- Diet control is the first treatment for obesity. Eating fruits and boiled vegetables change diet intake in the long-term gives can reduce body weight significantly.
2. Mental behaviour - Psychological thinking to promote weight loss. Complete control of the intake of unhealthy food. Positive thoughts endorse lifestyle to reduce mental stress.
3. Physical exercise - Increased level of hormones i.e. leptin, insulin, and ghrelin increase satiety and hunger which can be regulated by exercise.
4. Manipulation of DLPFC (Dorsolateral Prefrontal Cortex) - It regulates consistent eating habits in the short interval by directing DLPFC functions in the brain which involved eating habits and patterns.

Table 1: Pharmacological treatments on obesity[45]

Drugs name	Mechanism of Action	Adverse effect
Orlistat (Xenical)	Lipase inhibitor, Decreases lipid absorption	Faecal inconveniency, oily stools, oily spotting, Vitamin A, D, E, and K deficiency.
Lorcaserin (Belviq)	5HT 2CR agonist, Reduces food intake	Constipation, dry mouth, fatigue, headache
Phentermine (Qsymia)	Nor-epinephrine agonists, GABA agonists, suppress appetite	Anxiety, constipation, dry mouth, insomnia, depression
Liraglutide (Saxenda)	Decrease gastric emptying time and increases satiety	Nausea, anxiety, dyspepsia, vomiting
Naltrexone (Contrave)	Dopamine and norepinephrine reuptake inhibitor increase satiety and suppresses appetite	Nausea, headache, dry mouth, dizziness, constipation

Recently, FDA has withdrawn Lorcaserin from the market for the clinical assessment of cancer risk. Other anti-obesity agents listed in the above table are costly and are reported to have some adverse effects.[46]

3.12. Banned anti-obesity medicines

The long-term consumption of medicines for the obesity treatment had shown serious side effects. Due to lack of safety and efficacy, few anti-obesity medicines are removed out from the market to stop their use. Some of them are listed below in (Table 2).

Table 2: List of anti-obesity drugs banned by the FDA[47]

Drug name	Mechanism of action	Side effects
Amphetamine Dexamphetamine Methamphetamine	Appetite suppression	Cardiac vascular dysfunction, cognitive dysfunction, anxiety
Phentermine Diethylpropion Phenylpropanolamine	Appetite suppression	Increased heart rate, blood pressure and insomnia, constipation
Fenfluramine	Appetite suppression	Hypertension
Dexfenfluramine	Appetite suppression	Pulmonary hypertension
Sibutramine	Appetite suppression	Risk of heart attack and stroke
Rimonabant	CB1 receptor antagonist	Depression and anxiety
Aminorex	Appetite suppression	Pulmonary hypertension
Mazindol	Appetite suppression	Anxiety, insomnia

3.13. Pharmacological screening models of obesity

The top cause of death around the globe is obesity. Proper drug pharmacological screening is essential due to the critical need for safe and reliable medications for the management and prophylaxis of obesity. Non-clinical screening models can help to determine the drug's lethal level and establish the optimal dose. Few animal models have been listed below in (Table 3).

Table 3: Pharmacological screening models of obesity[48,49,50]

Models	Details
Diet-induced models	Diet-induced obesity models are the most commonly used screening models for anti-obesity drugs. High-fat diet, Cafeteria diets and high sucrose diet. The inducing procedure of obesity using HFD is easy and takes short time to induce obesity and increase insulin levels. These models are cost-effective but had poor standardization.
Chemical agents	Administration of Gold Thioglucose (30-40mg/kg) can cause necrosis and lesions on the ventromedial portion of the hypothalamus in mice and induce obesity. Monosodium glutamate cause destruction of the ventromedial hypothalamic site and arcuate nuclei and generates obesity.
Non-human primate models	Includes macaques, rhesus monkeys, and baboons which exhibit obesity similar to human obesity along with co-morbidities. These models are costly and are having lack approved facilities to limit their use of these models.
Surgical model	Acute injury of the arcuate nucleus (ARC), PVN, ventromedial hypothalamus (VMH) and ovariectomy are examples of surgical models. These kinds of obesity models are hard to perform and need surgical expertise.
Genetic models of obesity	
Monogenic models	The mutation of the leptin-melanocortin gene pathway causes an increased body weight in mice three times higher than unaffected mice. POMC knockout, melanocortin-4-receptor knockout mice. Wistar Kyoto rats are an example of monogenic models of obesity.
Polygenic models	When more than two genes get mutated or gene functions get modify is called polygenic models. Example: New Zealand obesity mouse, Kuo Kondo, M16, Spiny Mice and Sand rat. These are the more similar models compared to human obesity. These models are used frequently as anti-obesity screening models.
Transgenic models	Transgenic models are associated with insulin resistance, hyperplasia and high insulin level. Examples: Over expression of corticotrophin-releasing factor (CRF) and serotonin 5-HT-2c in mice, Increased level of GLUT4 glucose transporters, melanin-concentrating hormone (MCH), Beta-3- adrenergic, Neuropeptide-Y mice. To evaluate the mechanism of a particular drug these models are used.

3.14. Ethnomedicinal plants having anti-obesity potential

In the global market utilisation of medicinal plant and their products keep on rising day by day. Plant-based herbal products and pharmaceutical supplements are accepted by people as health care solutions. The world's 80% population believe and uses plant-based natural products with or without assuring their side effects and toxicity criteria. This rational use of herbal remedies can cause serious side effects due to a lack of adequate information hence standardisation of plants in qualitative and quantitative aspects is necessary to understand the beneficial effects of plant origin products in the treatment of diseases and disorders.[51]

However, the source of modern medicine has occurred by traditional medicine such as Ayurvedic Pharmacopeia of India (API), *Charaka Samhita*, *Sushruta Samhita*, and other ancient practised knowledge.[52] Some of the pre-clinical anti-obesity screening of plants were listed in the below table 4.

Table 4: List of medicinal plants reported for anti-obesity potential

Plant name	Study parameters	Observation
Acacia polyphenol [53]	The study was planned for a seven-week study; Body weight, plasma glucose, insulin level and gene expressions of PPARα, PPARδ, CPT1, ACO and UCP3 genes were performed.	This study concluded that increased gene expression of energy expenditure-related genes and reduced-fat levels in the liver.
Morinda citrifolia [54]	In this research work, Gene expression of PPAR-γ, SREBP-1c, FAS, G6P, ChREBP and fetuin-A was studied.	End of the study it was observed that down-regulation of PPAR-γ, and SREBP-1c in the liver and up-regulation of PPAR-α in white adipose tissue.
Panax ginseng [55]	Anthropometrical parameters such as body weight, liver and epididymal adipose tissue weight and food efficiency. RT-PCR was done for PPAR γ, aP2 and leptin genes.	Decreased serum levels of FFA, TC, TG, LDL, cholesterol, glucose, leptin and insulin. Also decreased levels of PPAR γ, aP2 and leptin were reported.

Terminalia paniculata [56]	The study parameters are body weight, body composition, blood glucose, insulin, tissue and serum lipid profiles, atherogenic index, liver markers, and gene expression of leptin, adiponectin, FAS, PPAR γ, AMPK-1alpha and SREBP-1c was studied.	Biochemical, histological and molecular studies demonstrated antiadipogenic and anti-obesity activities of *Terminalia paniculata*.
Curcuma longa [57]	It was a 12-week study performed on a high-fat diet-induced rat model. Body weight gain, blood glucose, insulin, HDL, and Cholesterol were studied. Gene expression of fatty acid synthase, acetyl-CoA carboxylase, adipocyte protein 2, and lipoprotein lipase was performed.	Fermented *Curcuma longa* extract prevents obesity by suppressing adipogenesis and promoting lipolysis. Suppressed adipocyte differentiation and lipogenesis were observed with decreased mRNA expressions of fatty acid synthase, acetyl-CoA carboxylase, adipocyte protein 2, and lipoprotein lipase.

Recently one study has been conducted on marine source plants. The ethanolic extract of *Ecklonia cava* the brown algae were tested on albino rats for the screening of anti-obesity potential. The study has reported a marked reduction in adipose tissue weight along with a decreased level of liver-damaging biomarkers observed. The gene expression of adipogenic genes like PPAR gamma, FAS and SREBP was found significantly reduced in extract-treated groups.[58]

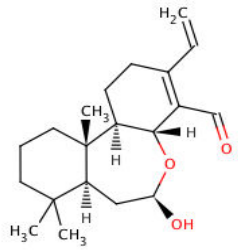
3.15. Plant profile

***Acacia suma* (Roxb) Buch. -Ham.**

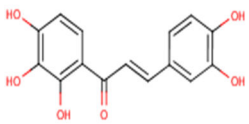
- *Acacia suma* [Roxb] is commonly known as; English- White cutch tree, white catechu; Hindi- Safed Khair; Sanskrit- Somavalkh, Sweta khadirah and Kadarah; Marathi- Paandharaa Khair; Kannada- Kandaraha and Mugali.
- Family - Fabaceae
- Chemical Constituents – Diaboline, β sitosterol, stigmasterol and oleanolic acid and 3 β acetate and saponin.

- Distribution - Karnataka, Bengal, Bihar region and Tamil Nadu.
- Medicinal Properties - Bitter, Astringent, Thermogenic, revulsive, depurative and Anthelmintic.
- Ethnomedicinal used- Leprosy, leucoderma, skin diseases, ulcer, epilepsy, rheumatism, diabetes and obesity.
- In the Ayurvedic Pharmacopoeia of India (API) the plant is reported for the treatment of Kusthaghna (skin illnesses), Mukharoga (mouth disorders), Raktadosa (anemia), Madhumeha (diabetes), and Medodosa (Excessive fat).
- Parts used – Stem Bark, Heartwood[8,9]
- The other secondary metabolites were retrieved from the ChEBI online tool and are listed below (https://www.ebi.ac.uk/chebi/).

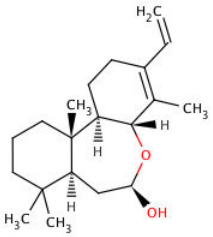
(5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-dien-7-ol-17-al



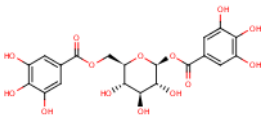
Okanin



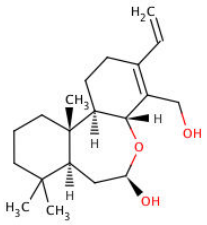
(5S,7R, 8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-dien-7-ol



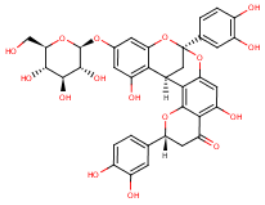
1,6-bis-O-galloyl-β-D-glucose



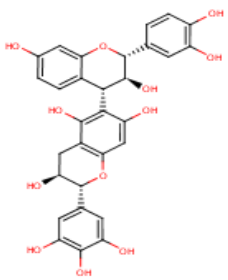
(5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol



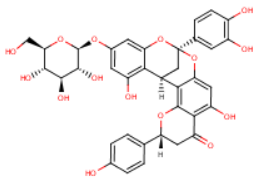
Diinsininol



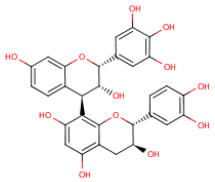
Fisetinidol-(4α,6)-gallocatechin



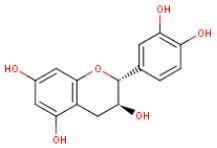
Diinsinin



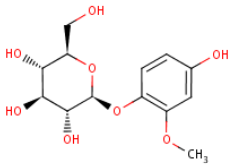
Epirobinetinidol-(4β,8)-catechin



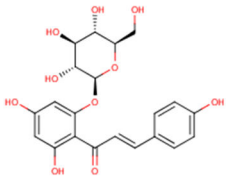
(+)-Catechin



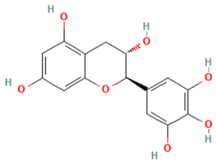
4-hydroxy-2-methoxyphenyl 1-O-β-D-glucopyranoside



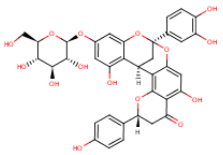
Phlorizin Chalcone



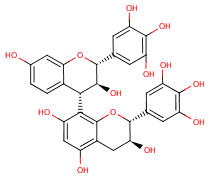
Gallocatechin



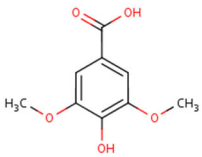
4-coumaric acid methyl ester



Robinetinidol-(4α,8)-gallocatechin



Syringic acid



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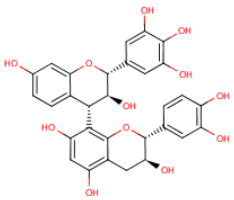
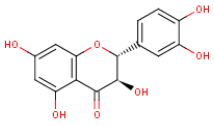
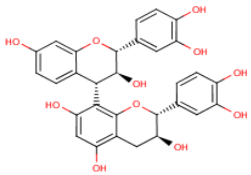


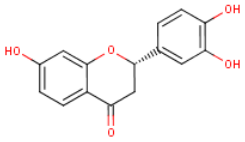
Taxifalin



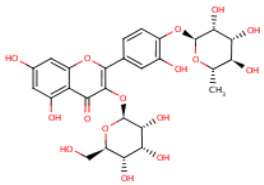
Fisetinidol-(4α,8)-catechin



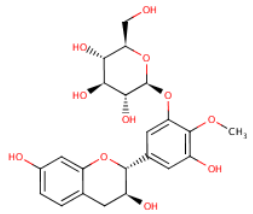
Butin



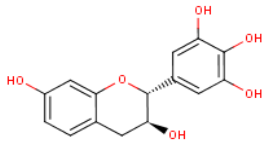
Quercetin 4'-O-α-L-rhamnopyranosyl-3-O-β-D-allopyranoside



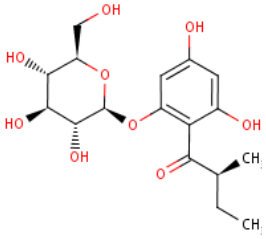
4'-O-methylrobinetinidol 3'-O-β-D-glucopyranoside



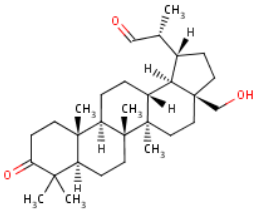
Robinetinidol



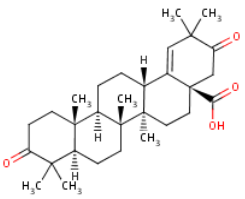
Multifidol glucoside



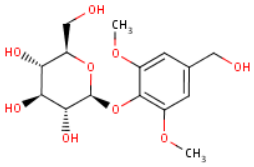
(20R)-28-hydroxylupen-30-al-3-one



3,21-dioxoolean-18-en-28-oic acid



3,5-dimethoxy-4-hydroxybenzyl alcohol-4-O-β-D-glucopyranoside



Auriculoside

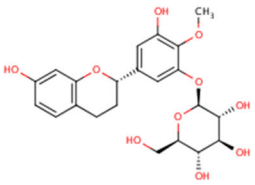


Figure 10. Heartwood of *Acacia suma* [Roxb]

Figure 11. Heartwood powder of *Acacia suma* [Roxb]

Table 5: Taxonomical nomenclature of *Acacia suma*

Plant Profile	
Kingdom	Plantae
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Fabales
Family	Fbaceae
Genes	*Acacia*
Species	*A. Polyacantha*

3.16. Earlier research activities on *Acacia suma*

Table 6: List of earlier research activities on *Acacia suma*

Activities	Plant part used	Findings
Diuretic and Laxative activity[10]	Stem bark	The aq. extract of *Acacia suma* bark (400 mg/kg) resulted from sig. increase in the urine volume, and urinary concentration of Na^+, K^+ and Cl^- ions in mice.
Analgesic, anti-inflammatory and antipyretic[11]	Stem bark	Ethanolic extract of stem bark of *Acacia suma* has shown analgesic, antipyretic and anti-inflammatory activity at 200 and 400 mg/kg doses.
Anthelmintic activity[12]	Stem bark	Gallocatechin-7-gallete, Quercetin, and 5,4 dihydroxy-7,3 dimethoxyflavone- 3-o-D-galactopyranoside is present in the stem bark extract of *Acacia suma*.
Antihyperglycemic and Antihyperlipidemic [59]	Stem bark	Methanolic extract of *Acacia suma* had shown significant effect as anti-hyperglycemic and anti-hyperlipidemic in a dose-dependent manner on male wistar rats.
Wound healing activity[13]	Leaves	Excision and incision wound models were used to test the wound healing activity of chloroform, ethanol, and aqueous extracts of *A. suma* leaves. Ethanol and aq. extracts showed a faster rate of wound healing as compared to chloroform extract.
Glucose-Lowering Efficacy[60]	Root	The streptozotocin-induced anti-diabetic activity of extracts was evaluated on adult Wistar rats using petroleum ether, chloroform, ethanol and water extract. The ethanolic extract was found significantly effective on glucose lowing potential in a dose-dependent manner.

3.17. Need for the study

Obesity is initiated because of disturbance in food consumption and energy expenditure and is associated with serious chronic conditions such as diabetes, hypertension and atherosclerosis. It is the most neglected and preventable kind of disease, due to lack of physical exercise, sedentary lifestyle, personal habits and hormonal imbalance that leads the obesity in human beings.

The FDA-approved drugs - Amphetamine, Rimonabant, Sibutramine and Phentermine have been withdrawn as are associated with several side effects, while orlistat is currently the only FDA-approved drug in use.

In Ayurvedic Pharmacopoeia of India, the heartwood of *Acacia suma* is duly mentioned for the treatment of *Kusthaghna* (Skin diseases), *Mukharoga* (Mouth diseases), *Raktadosa* (Anemia), *Madhumeha* (Diabetes) and *Medodosa* (Excessive fatness). As an outcome, the research aims to explore the anti-obesity potential of *Acacia suma* [Roxb] in experimental animal.

Chapter – 4

Materials and Methods

4. MATERIALS AND METHODS

4.1. List of materials and chemicals required in the project

S. No	Material Name	Procurement source
1.	Epigallocatechin	Yarrow pharma, Mumbai, India
2.	Fisetin	Sigma-Aldrich
3.	Quercetin	ACTIN Pharmaceuticals, China.
4.	*p*-nitrophenyl butyrate (PNPB)	Sigma-Aldrich
5.	Acetonitrile (ACN)	Merck, India
6.	HPLC analytical-grade water	Merck Mumbai, India
7.	3T3 adipocyte cell line	National Centre for Cell Sciences, Pune
8.	Pre-coated TLC silica gel plates	Merck, Germany
9.	High Fat Diet	VRK Nutrition Solution, Pune-India
10.	Orlistat	PELSON pharmaceuticals, Hyderabad
11.	DPPH reagent	Sigma-Aldrich
12.	Gallic acid	Sigma-Aldrich

4.2. List of instruments used in this project

S. No.	Instruments Name	Company Details
1.	UV Spectrophotometer	UV-1800, Shimadzu Japan
2.	Rotary evaporator	Buchi Evaporator
3.	Lyophilizer	Lyophilizer CHAIST, ALPHA 1-2 LO Plus
4.	Incubator	New Brunswick, galaxy 170 R
5.	Inverted microscope	Labomed
6.	Microplate reader	*LISAPlus* / Elisa Epoch Biotek, USA
7.	HPLC instrument	Dionex P680
8.	HPTLC instrument	CAMAG Linomat V

4.3. STUDY FLOW CHART

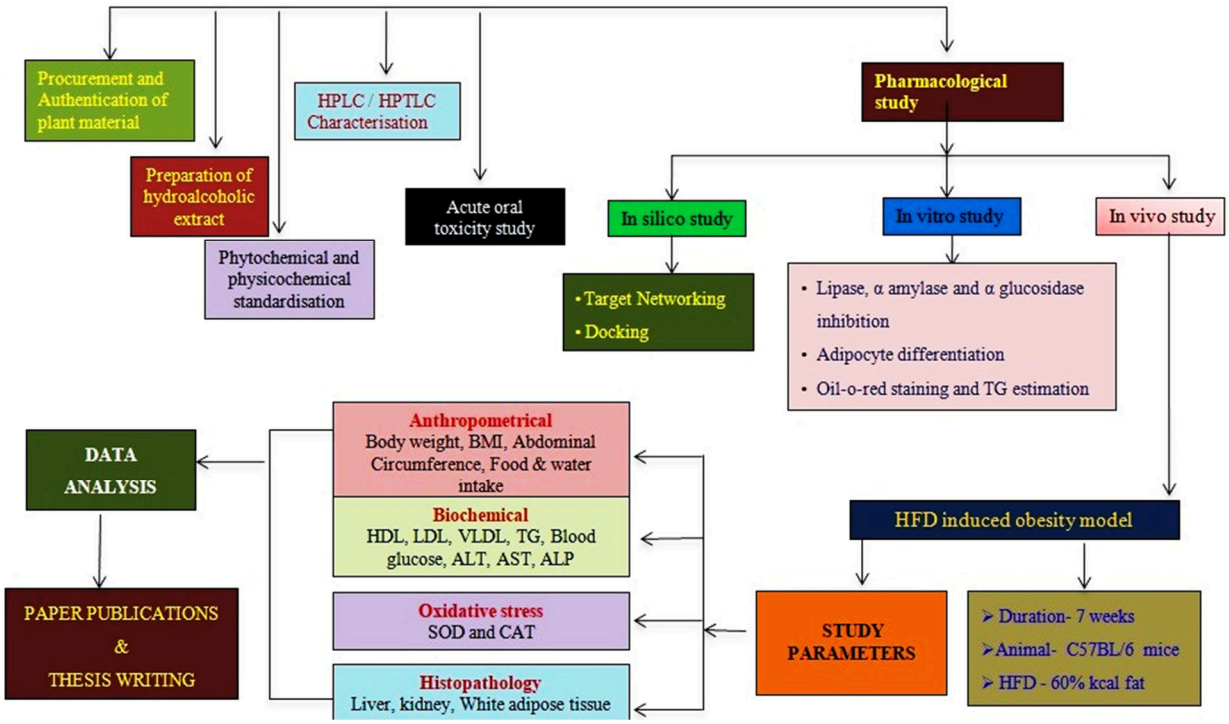


Figure 12: Schematic representation of study

4.4. Collection and authentication of plant material

The heartwood of *Acacia suma* was procured from an authenticated source. It was procured and authenticated by Dr K. Madhava Chetty, Plant taxonomist, Assistant professor, Department of Botany, Shri Venkateshwara University, Tirupati – 02; with plant specimen number **0661**.

4.5. Maceration process

The *Acacia suma* heartwood powder was subjected for maceration process for 7 days using ethanol and water in the ratio (70:30) to obtained hydroalcoholic extract.[61]

4.6. Standardization of plant material

Determination of ash value, moisture content, extractive value, pH, and Fluorescence analysis was determined to ensure the quality of plant material.[62]

Ash value determination

It essential to identify low-grade products or crude drugs consist of soil and sand, such drugs are exhausted kinds of materials and can be tested for water-soluble ash and acid-insoluble ash.[62]

i) Determination of total ash

The coarse and dried form test material was weighed for 2 g in a silica crucible and placed in the incinerator at 500°C till it converts into ash. The crucible was weighed again after cooling and total ash content (%) was calculated.

Total ash = Ash weight / initial weight of powder (g) × 100

ii) Determination acid insoluble ash

Using 45 ml of dilute HCl, the ash obtained from total ash was boiled for 5 minutes. The mixture was filtered through ash-free filter paper, and the resulting residue was washed with hot water. The filter paper has been ignited and the weight was recorded after it had completely dried. The acid-insoluble ash (%) was determined.

Acid insoluble ash = Acid insoluble ash weight / initial weight of powder (g) × 100

iii) Determination water-soluble ash

Similarly, the ash obtained from total ash was boiled for 5 min. using 45 ml of distilled water. The mixture was filtered using ash-less filter paper and obtained residue was washed with hot water. After complete drying the filter paper ignited and weight was noted. The water-soluble ash (%) was calculated.

Water soluble ash = water soluble ash weight / initial weight of powder (g) × 100

Determination moisture content

Estimation of moisture content is a % loss in weight (w/w). Crude materials containing water or volatile matter can be measured by this method. Initially a glass-

stopper bottle weight was noted. Accurately weighed 2 gm of test material was placed in the bottle, again weight of bottle and test material was noted. The bottle loaded with test material was placed into the oven. The material was dried till to get constant weight readings. After cooling the bottle final weight noted to determine the moisture content (%).[63]

Moisture content (%) = Loss in weight / weight of powder (g) × 100

Determination of extractive values

The plants material are consist of number of metabolites which are responsible for their pharmacological activities in the form of primary and secondary metabolites. These metabolites can be extracted with different solvents using various extraction procedures. The number of bioactive components retrieved out of from specific quantity of plant material using various solvents is known as extractive value.[63]

i) Alcohol soluble extractive

The 2.5 g of coarse powder of test material was macerated with 100 ml of ethanol in 250 ml of conical flask for 24 h. After shaking frequently for 6 h the flask was allowed to stand for 18 h. It was then filtered and 25 ml of the filtrate was evaporated to dryness in flat bottomed flask, dried at 105 ± 1°C and weighed. The percentage of alcohol-soluble extractives was calculated.

ii) Water soluble extractive

The 2.5 g of coarse powder of test material was macerated with 100 ml of chloroform-water in 250 ml of conical flask for 24 h. After shaking frequently for 6 h the flask was allowed to stand for 18 h. It was then filtered and 25 ml of the filtrate was evaporated to dryness in flat bottomed flask, dried at 105 ± 1°C and weighed. The percentage of water-soluble extractives was calculated.

Determination of pH

The pH value of aqueous solution of plant material (1% w/v) was determined.

Fluorescence analysis

Fluorescence analysis was carried out by mixing the powder of test material with various reagents like HCl, H_2SO_4, HNO_3, NaOH and Ammonia. The presence of fluorescence was observed under UV chamber at 254 nm, 366 nm and visible light.

4.7. Phytochemical evaluation

Phytochemical screening was performed to confirm presence of various phytoconstituents.[63]

Table 7: List of phytochemical tests

Phytochemical test	Procedure
Test for Carbohydrates	**Molisch's Test:** To 2-3 ml of test sample few drops of alpha naphthol solution were added and shaken. Then concentrated sulphuric acid was added from the sides of test tubes to observed with the formation of a violet ring at the junction of two liquids **Benedict's Test:** Mixed an equal volume of Benedict's and test solution in a test tube. heat in boiling water bath for 5mins **Fehling's Test:** 1 ml Fehling's A and 1 ml Fehling's B solutions, combined, boiled for 1 minute. Add an equal amount of test solution. Kept for 5 min. on boiling water bath. **Borfoed's Test:** Borfoed's reagent and test solution were mixed in equal parts. Cool it after 1-2 minutes of heating in a boiling water bath.
Test for Proteins	**Biuret's Test:** test solution of 3ml added with 4% NaOH and a few drops of 1% $CuSo_4$ solution **Millon's Test:** Mixed 3ml of the test solution with 5ml Millon's reagent **Xanthoprotein Test:** A white precipitate was noticed when 1 mL of concentrated sulphuric acid was added to 3 ml of extract. **Precipitation test** a) With alcohol b) 5% $HgCl_2$ solution c) 5% $CuSO_4$ solution d) 5% Ammonium Sulphate
Test for Amino acids	**Ninhydrin Test:** heated 3 ml of the test solution and 3 drops

	of 5% Ninhydrin solution in a boiling water bath for 10 min. the purple or bluish colour appears. **Tyrosine Test:** heated 3 ml test solution and 3 drops of Millon's reagent. The solution turns into dark red colour. **Cysteine Test:** To 5 ml test solution added a few drops of 40% NaOH and 10 % lead acetate solution. Boil, a black precipitate of lead acetate was formed.
Test for Steroids	**Salkowski test:** To 2 ml of extract, added 2 ml chloroform and 2 ml concentrated sulphuric acid. mixed well chloroform layer appears red and acid layer showed greenish-yellow fluorescence **Libermann-Burchard Test:** To 2 ml test solution, a few drops of acetic anhydride were added. The solution was boiled and cooled and 2 drops of concentrated sulphuric acid was added from the side test tube. The brownish ring at the junction of two layers and green colour in the upper layer for steroids was observed for triterpenoids deep red colour in the lower layer was observed. **Sulphur Powder Test:** In a 3 mL test solution, a small amount of sulphur powder was added. The presence of steroids and triterpenoids is confirmed by the presence of sulphur powder at the bottom.
Test for Saponin Glycosides	**Foam Test:** To 2 ml test solution, 2 ml of water was added, shaken vigorously and observed for persistent foam.
Test for Cardiac Glycosides	**Baljet's Test:** To 2 ml test solution 1 ml of sodium picrate solution was added and observed for yellow to orange colour. **Legal's Test:** To 2 ml test solution, 1 ml of pyridine and 1 mL of alkaline sodium nitroprusside solution were added and observed for pink to red colour. **Keller Killiani Test:** To 2 ml of extract add glacial acetic acid 1 drop of 5% ferric chloride and concentrated sulphuric acid. reddish brown colour appears at the junction of the two liquid layers and the upper layer bluish green
Test for Anthraquinone Glycosides	**Modified Borntragers test:** To 2 ml test solution, 2 ml of dilute sulphuric acid and 2 ml of 5% ferric chloride solution were added, boiled for 5 min, the solution was filtered while hot, then cooled and the filtrate was shaken gently with an equal volume of chloroform. The lower layer of chloroform was separated and shaken with half of its volume of dilute ammonia and observed ammonical layer for rose-pink to red colour. **Borntragers test:** To 2 ml test solution, 1 ml of dilute sulphuric acid was added, boiled for 5 min, the solution was

	filtered while hot, then cooled and the filtrate was shaken gently with an equal volume of chloroform. Chloroform at the lower layer was separated and shaken with half of its volume of dilute ammonia solution and the ammonical layer was observed for rose-pink to red colour.
Test for Flavonoids	**Shinoda Test:** To 2 ml test solution add 2 ml of 95% ethanol, five drops of concentrated hydrochloric acid and 0.5 g of magnesium turnings. The solution turns to pink, crimson red or occasionally green to blue colour within a few minutes. **Alkaline reagent test:** The addition of an increasing amount of sodium hydroxide to the residues shows yellow colouration which decolourises after the addition of acid. **Zinc Hydrochloride Test:** To the 2 ml test solution, a mixture of zinc dust with concentrated hydrochloric acid was added and red colour after a few minutes was observed.
Test for Alkaloids	**Dragendorff's Test:** To 2-3 ml filtrate, add few drops Dragendorff's reagent. The orange-brown precipitate was formed. **Mayer's Test:** 2-3 ml filtrate with a few drops of Mayer's reagent gave precipitate **Hager's Test:** To 2 ml test solution, a few drops of Hager's reagent were added and observed for yellow colour precipitate. **Wagner's Test:** To 2 ml test solution, a few drops of Wagner's reagent were added and observed for reddish-brown colour precipitate.
Tannins and Phenolic Compounds	To 2-3 ml of alcoholic extract add a few drops of the following reagents 5% Ferric chloride Lead acetate solution Gelatine solution Potassium dichromate

4.8. Qualitative chemical analysis of the crude extract

a) Estimation of phenolic content

The total phenolic content of the plant extracts was determined spectrophotometrically. The one ml of Folin-Ciocalteu's reagent was added to 1 ml of sample and mixed thoroughly. Further, 4 ml of sodium carbonate was added and volume adjusted to 10 ml of distilled water. The mixture was allowed to stand for 2h

at room temperature. The content was centrifuged at 2000rpm for 5 min and the absorbance of the supernatant was measured at 760 nm. A standard curve was obtained using different concentrations of gallic acid. The total phenolic content was expressed in terms of mg of gallic acid equivalents per gram of plant extract.[64]

b) Estimation of flavonoid content

Total flavonoid content was measured by colorimetric assay. One ml of extracts was added into a 10 ml volumetric flask, in which 0.3 ml of 5% $NaNO_2$ was be added. After 5 minutes, 0.3 ml of 10% $AlCl_3$ and 2 ml of 1 M NaOH were added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against the prepared reagent blank at 510 nm. The total flavonoid content of the extracts was expressed as mg of quercetin equivalents per gram of extract.[65]

c) Estimation of tannin content

A colorimetric assay has been used to assess the total tannin content (TTC). The final concentration was made with 10 mg of HAE *Acacia suma* mixed in 10 ml of water. Iron (III) chloride (20 mM) and potassium ferricyanide (8 mM) were prepared in hydrochloric acid (0.1 M), and the final volume was topped off with distilled water to make 7 ml. Tannic acid was used as the reference standard while plotting the calibration curve. At 700 nm, absorbance was measured. Tannic acid was measured as mg per gram of total tannin content.[65]

4.9. HPLC characterisation of *Acacia suma*

4.9.1. Experimental setup for HPLC

HPLC prominence instrument system (Shimadzu version 1.25; LC-20AD, Japan) furnished with prominence diode array detector, pump, autosampler, degasser, rheodyne injection and C18 column oven was used for the analysis at 40 °C

temperature. At a flow rate of 1 ml/min, the optimised mobile phase ACN: Phosphate buffer (0.1 % OPA; 30:70 v/v) was adjusted with pH 2.7 and filtrated through a PVDF filter membrane (0.45 m). The sample analysis was done with 20 µl injection volume.

4.9.2. Preparation of standards for simultaneous estimation

Stock solutions of ECG, FT and QT were individually prepared at a concentration of 1 mg/mL in methanol. The standard preparation of EGC, FT and QT were prepared in the concentration range of 2.5-160 µg/mL, by the dilution of stock solution using the HPLC grade water. All standard preparations were stored in an amber-coloured and tightly-stopper volumetric flask at 4 °C, before the analysis.

4.9.3. Sample preparation

Acacia suma extract (10 mg) was dissolved in 10 ml of distilled water and sonicated for five minutes to improve dissolution.

4.9.4. Validation of optimized HPLC method

The HPLC method validation parameters includes system suitability, linearity, accuracy, precision, LOD, LOQ, robustness, and ruggedness. The method specificity was validated in full compliance with ICH guidelines to ensure that the HPLC methodology is appropriate and reliable for the proposed work.[66,67]

4.10. HPTLC method development and validation

4.10.1. Sample preparation

HAE of *Acacia suma* (10mg) was sonicated for 5 minutes with 10ml of distilled water before being diluted to make different concentrations.

4.10.2. Preparation of stock solution of standard

The stock solution of epigallocatechin (1mg/ml) was used to prepare different concentrations (7, 8, 9, 10, 11, 12µg/ml) using methanol. 5µl of the standard solution was used for 8 mm band application on HPTLC plate.

4.10.3. Calibration curve of epigallocatechin

In methanol, a working stock solution of epigallocatechin was prepared. A linear relation in both peak area concentration and peak height was investigated utilising seven concentrations ranging from 1-5µg/ml.

4.10.4. HPTLC instrumentation conditions

The plate was saturated with mobile phase (THF: Toluene: Acetic Acid: Water; 16:8:2:1 (v/v)) mobile phase. The plate was left in the chamber until the increasing solvent level reached 7 cm. The sample application was done with CAMAG-Linomat V automated spray on band applicator equipped with a 100 µl syringe and operated with the following settings: band length 8 mm, application rate 150 nl/sec, the distance between 11.4 mm, distance from the plate side edge1.5 cm and distance from the bottom of the plate 2 cm. CAMAG TLC Scanner 4 was used for densitometric scanning to quantify the bands using *VISIONCATS* software (Linomat V).[68,69]

4.10.5. HPTLC method validation

The method validation was done by considering parameters like linearity, accuracy; precision and reproducibility the data were expressed in terms of % RSD as per the ICH guidelines.

4.10.6. HPTLC-DPPH antioxidant assay

The pre-developed chromatographic plate was immersed in an ethanolic solution that contains 0.2 percent w/w DPPH. Excess DPPH solvent was removed by drying for 10 minutes in the dark at room temperature. Lemon yellow spots were observed and densitometric scanning was done after post-derivatization at 540 nm.[70,71,72]

4.11. In-silico anti-oxidant activity

The *Acacia suma* phytochemicals was performed by retrieving list of phytochemicals from ChEBI online tool. Utilizing online tool, Swiss Target

Prediction, fifteen compounds were identified as Cytochrome P450 inhibitors depending on their Canonical SMILES. The mmff94 force field was used for minimization and the file was saved (.pdbqt); using online tool MolSoft and ADVERSEPred each compound was tested for drug likeness score and side effects respectively.

4.11.1. Preparation of protein and ligand for docking

Cytochrome P450 in complex with a peptide (PDB: 1OG5) was downloaded from the RCSB protein data bank. The protein structure was minimised using protein preparation wizard (Schrodinger, LLC).[73] LigPrep creates an energy-minimized structure with multiple tautomers and stereoisomers used for molecular docking. Molecular docking was performed using the Schrodinger Suite software's GLIDE docking module. Glide docking generates Kcal/mol as glide docking scores.

4.12. In-vitro anti-oxidant assays

DPPH scavenging activity

The DPPH solution (0.1mM) 3.5ml in ethanol mixed with different concentrations of sample in the range of (7.8µg/ml - 250µg/ml) kept in the dark for twenty minutes. At 517nm, the absorbance of the solution was measured. The following equation was used to calculate the per cent DPPH radical scavenging.[74]

% Inhibition = (Absorbance of blank − Absorbance of test) ⁄ Absorbance of blank × 100

H_2O_2 radical scavenging activity

H_2O_2 (40 mM) prepared in phosphate buffer saline at 7.4 pH. H_2O_2 solution (1.5ml) was mixed with various concentrations 50-300µg/ml of test extract. After wrapping the test tubes with aluminium foil for 10 minutes, the absorbance at 230nm

was measured. Ascorbic acid was used as a standard. The percent H_2O_2 scavenging activity calculated using formula.[75]

% Inhibition = (Absorbance of blank − Absorbance of test) / Absorbance of blank × 100

Nitric oxide assay

A volume of 0.5 mL of 10 mM sodium nitroprusside in PBS was mixed with 1 ml different extract concentrations incubated at 25° C for 1h. After that 1ml of Griess reagent was added. The produced colour has been UV spectrophotometer assessed at 540nm Vs a blank sample. The percentage inhibition of nitric oxide radicals was calculated using the following formula.[74]

% Inhibition = (Absorbance of blank − Absorbance of test) / Absorbance of blank × 100

Total antioxidants assay

The different concentrations (50-300µg/ml) of HAE of *Acacia sum* was mixed with 3ml of reagent mixture (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes were wrapped in aluminium foil and placed in a 95°C boiling water bath for 90 minutes. After cooling, the absorbance of the solution was analysed at 695nm. The total antioxidant activity was expressed as of ascorbic acid equivalents.[76]

Lipid peroxidation activity

The egg yolk homogenate (250µl) prepared in 10% distilled water was used for lipid peroxidation assay. 250µl egg homogenate mixed with various concentrations of extract (50-300µg/ml). Further, 25µl $FeSO_4$, (0.07 M) was added and incubated for 30 min. After incubation, 750µl acetic acid (pH 3.5) and 750µl Thiobarbituric Acid (TBA) prepared in 1.1% Sodium dodecyl sulphate and 25µl 20% TCA were added,

centrifuged and then heated for 60 minutes in a boiling water bath. After cooling, 3ml of 1-butanol was added and centrifuged for 5 minutes at 2000 rpm. At 532nm, the absorbance of the organic upper layer was measured.[77]

Ferric reducing power assay

The extract 0.5ml (50-300µg/ml) was incubated at 50°C for twenty minutes with 1.5ml of PBS pH 6.6 and 1.5ml of Potassium ferricyanide (1%). Further 1.5 ml of trichloroacetic acid (TCA) was added and 10 minutes centrifuged at 3000 rpm. 1.5 ml of upper layer solution mixed with 1.5ml of distilled water. Finally, 300 µl of $FeCl_3$ added and again centrifuged for 5 minutes at 3000 rpm. The absorbance of the solution was measured at 700nm, and a graph of absorbance v/s concentrations was plotted.[71]

Pharmacological Studies

4.13. In-silico anti-lipase activity

Phytoconstituents from *Acacia suma* were identified by using ChEBI online tool. Lipase inhibitors were predicted and molecular docking of lipase inhibitors was performed using Autodock software.

4.13.1. Identification of anti-lipase phytoconstituents from *Acacia suma*

The Swiss Target Prediction had been used to identify the Phospholipase A2 group IIA inhibitors by querying the SMILES of each molecule. Drug like-ness score and side effects of each phytochemical were noted using online tools MolSoft and ADVERPred database.[78] DIGEP-Pred[79] was used to predict the up-and-down regulation of genes. Further gene enrichment analysis was performed to identify KEGG Kyoto Encyclopedia of Genes and Genomes pathways, the interaction between genes was analysed by using STRING[80] online software. Network construction was made with command 'Network analyzer' using Cytoscape 3.7.1.[81]

4.13.2. Molecular docking of lipase inhibitors

The compounds structures were retrieved from PubChem in .sdf format and converted into .pdb using Discovery Studio 2019. Compounds were minimised using mmff94[82] force field and converted into .pdbqt. Similarly, target was (PDB: 3U8B) retrieved from the RCSB database. The compounds showing lipase inhibition potential are docked with phospholipase A2 group IIA using autodock4[83] to know there docking score.

4.14. In-vitro: Enzymatic assays

4.14.1. Lipase inhibitor assay

Pancreatic lipase enzyme was used to perform lipase inhibition assay using p-nitrophenyl butyrate (PNPB) as substrate. Six concentrations of hydroalcoholic extract of *A. suma* were prepared from stock solution (10 mg in 10 ml) by serial dilution method. Orlistat, a well-established lipase inhibitor was taken as standard drug. 25µl of test solution and similar volume of standard drug solution were incubated with 50µl of enzyme, 100µl of buffer solution and 25µl of PNPB solution for 30 min at 37 °C. PNPB Hydrolysis was estimated at 400 nm using ELISA plate reader by repeating the assay in triplicates. Percent (%) inhibition was calculated by using following formula[84]

% Inhibition = (Absorbance of blank − Absorbance of test) ⁄ Absorbance of blank × 100

4.14.2. Assessment of alpha amylase inhibition

The 1ml of PBS was mixed with 0.5 ml of different concentrations of samples and standard solution and 200µl of α-amylase and 200µl of 5mg/ml starch was added and incubated for 10 min. at room temperature. Starch was taken as control. 400µl of DNS solution was added to stop the reaction and boiled for 5min and cooled. The

absorbance was measured at 540 nm. Metformin was used as standard.[85] The % inhibition of enzyme was calculated using the following formula

% Inhibition = (Absorbance of blank − Absorbance of test) / Absorbance of blank × 100

4.14.3. Assessment of alpha glucosidase inhibition

The p-nitrophenyl α-D-glucopyranoside (PNPG) was used as substrate, 3 mM PNPG was dissolved in 50 mM PBS (pH 6.5). Different concentrations for sample were prepared at in 5% DMSO. 30µl of each concentration was added with 36µl phosphate buffer and 17µl of PNPG and incubated for 5 min at 37°C. Further, 17µl of -glucosidase was added and incubated at 37°C for 15 minutes. To stop the reaction after incubation, 100µl of Na_2CO_3 (267 mM) was added. At 405 nm, the absorbance was measured. As a standard drug, acarbose was used. The formula was used to calculate the percentage inhibition as follows[86]

Inhibition Percentage = Optical density of blank-Optical density of sample/Optical density of blank×100.

4.15. In-vitro: cell line study

4.15.1. MTT cytotoxicity assay

The Preadipocytes are seeded at 37ºC in 96-well flat-bottom microplate in 95% humidity and 5% CO_2. The various concentrations of extract were added into cells and incubated for 48 h. Further cells were washed with PBS for two times. MTT solution of 20µl was added and incubated at 37ºC. 100µl of DMSO was added to dissolve the formazan crystals after 4h and absorbance was recorded at 570 nm using a microplate reader. % cell viability was calculated compared to control cells using formula[87],

% cells viability = Optical density of sample / Optical density of control ×100

4.15.2. Adipocyte differentiation

The preadipocytes (3T3-L1) were seeded with high glucose DMEM, 10% FBS at 37°C and 5% CO_2. The adipocyte differentiation was induced in two days of post-confluence preadipocytes incubated with cocktail solution containing 10µg/ml insulin, 2.5µl dexamethasone and 0.5 mM 3-isobutyl-1-methyl-xanthine along with plant extract for 48 h. The lipid content in 3T3-L1 cells were estimated as described in the previous study.[88]

4.15.3. Lipid accumulation staining assay

After cell differentiation, adipocyte staining was done using oil o red stain along with cell lipid accumulation was measured. The cells were fixed with 10% formalin for 1 h and stained using oil o red stain for 1 h. The cells were washed with 60% isopropanol and observed under light microscopes. Lipid accumulation was quantified by measuring the absorbance at 492 nm.[89]

4.16. In- vivo: HFD induced anti-obesity screening model

4.16.1. Animal study and ethical approval

The Institutional animal ethical committee (IAEC) of KLE College of Pharmacy, Belagavi (Reg. No. 221/ Po/ Re/ S/ 2000/ CPCSEA) has approved the use of C57BL/6 male mice to perform a designed experimental study. Throughout the animal study, CPCSEA guidelines were followed. The experimental animals are procured from the National Institute of Biosciences, Dhangwadi; Pune. The mice were placed in clean polypropylene cages with room temperature 22 ± 2 ^{0}C for 12 hrs. light and dark cycles.[90]

ANIMAL STUDY FLOW CHART

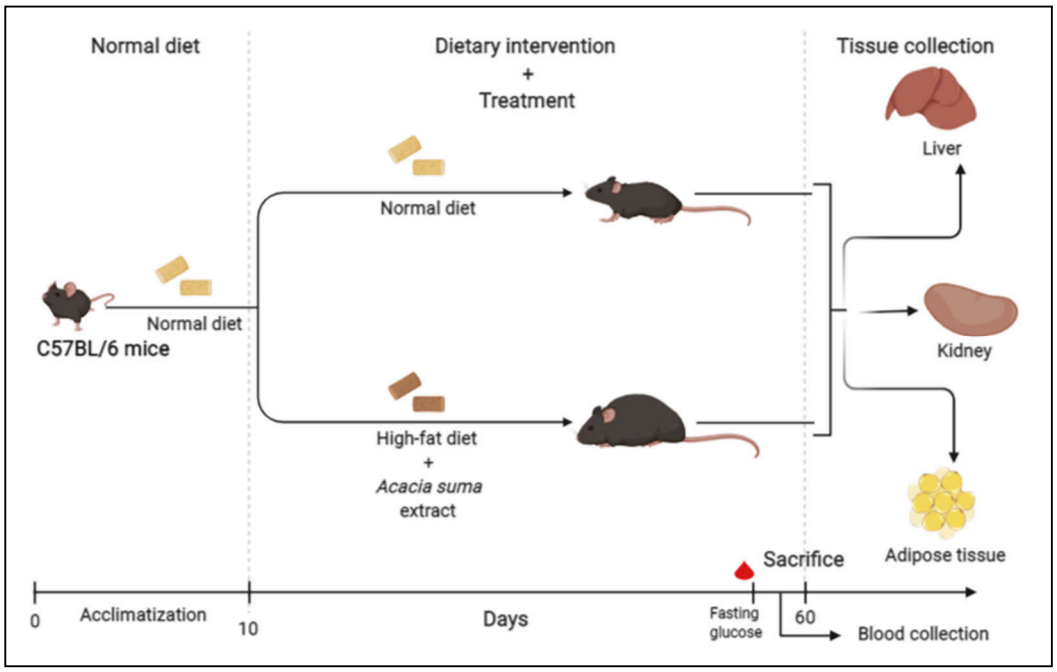


Figure 13: Animal study flow chart

4.16.2. Acute toxicity study of extract

The toxicity study was conducted by following 423 Guidelines specified by OECD 2008. Three female mice were grouped as test and three female mice as control. The 2000 mg/kg dose was calculated and administered orally using water as vehicle. Clinical signs were observed continuously such as behaviour, neurological and autonomic profiles of mice 30 minutes, up to 24 hours, followed by 14 days to confirm any lethality, morbidity and death are been observed for changes in fur, eyes, and mucous membranes. Every animal was monitored on a routine basis for morbidity and mortality.[91]

4.16.3. HFD induced obesity screening model

The male mice (C57BL/6) (five-six weeks old) of 16-18 gm weight were purchased from the National Institute of Biosciences, Dhangwadi, Pune. The 60% fat containing HFD was purchased from VRK Nutritional Solution, Pune; the certificate of analysis of HFD has been attached in the annexure. Three mice were kept in each

polypropylene cage. The mice were acclimatized for one week and randomly divide into six groups. **Group I-** Normal; receives a normal diet, **Group II-** Obese; receives a High-fat diet containing 60% fat, **Group III-** Standard; receives orlistat 10mg/kg and **Group IV, V** and **VI** receive HAE of *Acacia suma* 100mg/kg, 200mg/kg and 400mg/kg respectively. Each grouped animal has given easy access to food (high fat & low fat) and water *ad libitum* for 7 weeks.[53] Food intake and body weight was measured weekly. After the end of the study, on 50th day followed by overnight fasting, blood glucose measured and blood sample was collected by cardiac puncher. The liver, kidney and adipose tissue are collected and preserved in buffered formalin (10%) for histopathological investigation.

Table 8: Grouping and treatment of animals

Groups	Groups Name	Treatment [*P.O*]	Total
I	Normal	Normal Diet	36
II	Obesity	High Fat Diet	
III	Standard	Orlistat 10 mg/kg + HFD	
IV	HAE + *Acacia suma*	100 mg/kg + HFD	
V	HAE + *Acacia suma*	200 mg/kg + HFD	
VI	HAE + *Acacia suma*	400 mg/kg + HFD	

(n=6; each group)

4.16.4. Evaluation of study parameters

- **Anthropometrical parameters**

The body weight of experimental mice was measured on weekly bases for 7 weeks. And the nutritional intake i.e. food intake and water intake were measured daily.

- **Lipid profile/ Biochemical analysis**

Animals fasted overnight at the end of the study, and after inducing anesthesia, they were sacrificed by cervical dislocation; cardiac puncher was made for blood collection. The blood sample was centrifuged at 3000 rpm and obtained serum was

used for estimation of glucose, triglycerides, cholesterol, HDL, LDL, and VLDL level. AST, ALT and APL using Erba Mannheim diagnostic kits were also calculated.

- **Oxidative stress parameters**

Liver weight was noted and tissue homogenate was prepared with one part of liver tissue and nine part of 0.1 M ice cold PBS. The mixture was centrifuged at 4°C for 5000 rpm and supernant was stored at -80°C, the supernant was then used for the assessment of sodium dismutase activity and catalase activity.

Sodium dismutase (SOD)

The liver tissue homogenate was used to perform sodium dismutase activity as explained by Marklund and Marklund, 1974. The 20 µl liver homogenate was mixed with 2ml buffer with (pH 8.2), 0.6 ml of EDTA and 0.3 ml of pyrogallol. The absorbance of resultant mixture was recorded at 420 nm on spectrophotometer. The SOD activity was expressed as U/g of tissue.[92]

Catalase activity (CAT)

The assessment of catalase activity was done by following previously reported procedure with some changes.[93] To the 20 µl liver homogenate added 2 ml of PBS (pH 7.0) and 1 ml of H_2O_2 solution. The absorbance was measured at 240 nm using spectrophotometer. The CAT activity was expressed as U/g of tissue.

- **Histopathological analysis**

The liver, kidney and adipose tissue was collected for histopathological study. The small piece of organ was fixed with buffered formalin (10%). A thin section (5 µm) of tissue was stained by using H&E staining agents (hematoxylin and eosin).[94] The slide was observed under microscope at 40X to examine pathological changes in the respective tissue. The histopathological score was given based on the changes

occurred in liver, kidney and adipose tissue as ('-' Nil; '+' minimal; '++' mild; '+++' moderate) which indicates levels of changes.

4.16.5. Statistical data analysis

The degree of significance was described in terms of Mean ± Standard deviation or Standard Error of Mean.[95] The cumulative body weight, cumulative food intake and cumulative energy intake were measured by means of two away ANOVA using by Bonferroni post-test.[96] Differences between means for total water intake, abdominal circumference, change in body weight, BMI, liver weight, SOD, CAT and lipid profile test were analysed for the statistical significance by a one-way analysis of variance with Tukey post hoc test.[97] Statistical significance of research data were analysed by GraphPad Prism version 5.0.

Chapter – 5

Results

5. RESULTS

In the current study, various standardisation parameters were included such as preliminary phytochemical screening, determination of physicochemical properties, fluorescence analysis and quantitative estimation of phytoconstituents along with HPLC and HPTLC fingerprinting for the characterization of the heartwood of *Acacia suma*.

5.1. Processing of hydroalcoholic extract

The coarse powder of *Acacia suma* hydroalcoholic extract had a yield of **9.75** %

5.2. Evaluation of physicochemical parameters

Table 9: Physicochemical analysis of heartwood powder of *Acacia suma*

Parameters	Result
Color	Yellowish-brown
Odor	Characteristic
Taste	Bitter
Yield of extract	9.75 %
Water soluble extract	7.2 ± 0.8
Alcohol soluble extract	1.06 ± 0.61
Total ash	3.33 ± 0.76
Acid insoluble ash	1.83 ± 0.58
Water-soluble ash	3.16 ± 1.04
Loss on drying	5.5 ± 0.5
Ph	5.8 ± 0.3

5.3. Fluorescence Analysis

Fluorescence analysis of *Acacia suma* heartwood powder was carried out with various reagents HCl, HNO_3, H_2SO_4, NaOH, and Ammonia.

Table 10: Fluorescence analysis of heartwood powder of *Acacia suma*

Reagents	Visible light	254 nm	366 nm
Powder	Pale white	Whitish	Black
Powder + water	Pale white	Whitish-yellow	Black
Powder + HCl	Yellowish- green	Greenish-yellow	Black
Powder + H_2SO_4	Brownish-yellow	Greenish-yellow	Black
Powder + HNO_3	Greenish-yellow	Greenish-yellow	Black
Powder + KOH	Pale-Yellowish	Yellowish	Black
powder + NaOH	Yellowish	Yellowish-brown	Black
Powder + Ammonia	Pale yellow	Greenish-yellow	Black

5.4. Phytochemical screening of extract

Table 11: Phytochemical screening of HEA of *Acacia suma*

Phytochemical test	Inference
Test for Carbohydrates	
Molisch's Test	+
Benedict's Test	+
Fehling's Test	+
Borfoed's Test	+
Test for Proteins	
Biuret's Test	+
Millon's Test	+
Xanthoprotein Test	+
Precipitation test a) With alcohol	+
b) 5% $HgCl_2$ solution	+
c) 5% $CuSO_4$ solution	+
d) 5% Ammonium Sulphate	+
Test for Amino acids	
Ninhydrin Test	+
Tyrosine Test	-

Cysteine Test	-
Test for Steroids	
Salkowski Test	+
Libermann-Burchard Test	+
Sulphur Powder Test	+
Test for Saponin Glycosides	
Foam Test	+
Test for Cardiac Glycosides	
Baljet's Test	-
Legal's Test	-
Keller Killiani Test	-
Test for Anthraquinone Glycosides	
Modified Borntragers test	-
Borntragers test	-
Test for Flavonoids	
Shinoda Test	+
Alkaline reagent test	+
Zinc Hydrochloride Test	+
Test for Alkaloids	
Dragendorff's Test	+
Mayer's Test	+
Hager's Test	+
Wagner's Test	+
Tannins and Phenolic Compounds	
5% Ferric chloride	+
Lead acetate solution	+
Gelatine solution	+
Potassium dichromate	+

5.5. Quantitative estimation of phytoconstituents

The total phenolic content, tannin content and flavonoid content were determined as equivalent to gallic acid (GAE/g), tannic acid (TAE/g) and quercetin

(QE/g), respectively. The standards calibration curve equation was used to express the results. The calibration curve equation for total phenolic content is (y = 0.0015x + 0.0211, R2 = 0.989), for total tannin content is (y = 0.0025x + 0.0399, R2 = 0.990) and for total flavonoid content is (y=0.0003x+0.0016, R2=0.997). The extract had revealed maximum phenolic content than tannin content and flavonoid content represented in (Table 12).

Table 12: Quantitative estimation of phytoconstituents

Phytochemicals content	
Total Phenolic Content	571.49 ± 27.3 mg (GAE/g)
Total Tannin Content	165.9 ± 8.6 mg (TAE/g)
Total Flavonoid Content	92.3± 15.2 mg (QE/g)

5.6. HPLC standardisation of *Acacia suma*

The HPLC method development and validation were performed on *Acacia suma*. Epigallocatechin, fisetin and quercetin were used as marker compounds. The Shimadzu HPLC instrument is equipped with a prominence system (LC-20AD, Japan) equipped with SPD-M20A diode array detector (PDA), LC- 20AD pump, autosampler, DGU-20A5 degasser, rheodyne injection with 20µl loop and column oven. The chromatographic analysis was carried out using a C18 column at a column temperature of 40 °C. The optimized mobile phase was used as ACN: Phosphate buffer (0.1% OPA) 30:70 v/v ratio, pH 2.7 was adjusted with sodium hydroxide, at a flow rate of 1 mL/min.

5.6.1. System suitability by HPLC

Table 13: System suitability study by HPLC

Parameters	EGC		FT		QT	
	Mean ± SD	% RSD	Mean ± SD	% RSD	Mean ± SD	% RSD
tR (min.)	3.51 ± 0.002	0.04	6.98 ± 0.001	0.06	12.1 ± 0.03	0.24
Peak area	195410 ± 3020.6	1.55	4935729 ± 39946	0.81	3179821 ± 7201	0.23
Plate count	4531 ± 60.5	1.33	7620 ± 78.4	1.03	8293.5 ± 79.01	0.95
Tailing factor	1.33 ± 0.01	0.79	1.31 ± 0.00	0.18	1.3 ± 0	0.15

SD: Standard deviation, tR: Retention time, RSD: relative standard deviation

5.6.2. Linearity by HPLC

Table 14: Linearity

Standard compounds	Concentration Range (µg/ml)	Slope	Intercept	R^2	LOD (µg/ml)	LOQ (µg/ml)
EGC	2.5-160	6802	35670	0.999	0.076	0.231
FT	2.5-160	143642	276370	0.999	0.012	0.038
QT	2.5-160	90074	158303	0.999	0.010	0.031
		At Isosbestic Point 287 nm				
EGC	2.5-160	1605	28455	0.999	0.054	0.162
FT	2.5-160	38239	54749	0.999	0.008	0.025
QT	2.5-160	29016	36200	0.999	0.007	0.021

5.6.3. Precision and Accuracy by HPLC

Table 15: Precision and accuracy study

Standards	Active content (µg/ml)	Intraday (n=3)		Interday (n=3)					
		Found ± SD (µg/ml)	% RSD	1st day		2nd day		3rd day	
				Found ± SD (µg/mL)	% RSD	Found ± SD (µg/mL)	% RSD	Found ± SD (µg/mL)	% RSD
EGC	5	4.97 ± 0.02	0.48	4.98 ± 0.09	1.82	4.95 ± 0.01	0.20	4.89 ± 0.08	1.72
	10	10.06 ± 0.11	1.06	10.15 ± 0.10	0.97	9.75 ± 0.10	0.99	9.86 ± 0.11	1.07
	20	20.10 ± 0.18	0.91	20.03 ± 0.02	0.10	20.05 ± 0.24	0.69	20.08 ± 0.12	0.58
FT	5	4.93 ± 0.01	0.27	5.06 ± 0.07	1.43	5.03 ± 0.02	0.3	4.99 ± 0.04	0.88
	10	10.04 ± 0.06	0.60	9.83 ± 0.11	1.12	9.69 ± 0.18	1.83	10.01 ± 0.19	1.93
	20	20.06 ± 0.06	0.31	19.94 ± 0.04	0.19	19.96 ± 0.05	0.23	19.90 ± 0.07	0.35
QT	5	5.02 ± 0.10	1.90	4.53 ± 0.02	0.51	4.54 ± 0.02	0.44	4.57 ± 0.40	0.88
	10	10.07 ± 0.03	0.58	10.18 ± 0.11	1.12	10.32 ± 0.10	0.97	10.26 ± 0.14	1.33
	20	20.12 ± 0.13	0.63	19.76 ± 0.08	0.38	19.93 ± 0.16	0.79	19.89 ± 0.10	0.51

RSD: relative standard deviation, SD: Standard deviation

5.6.4. Recovery of drug by HPLC

Table 16: Recovery studies of drug

Standards	Active content (µg/ml)	Level (%)	Spiked quantity (µg/ml)	Recovered quantity (µg/ml)	Recovery (%)	RSD (%)
EGC	10	50	6.23 ± 0.08	6.12 ± 0.10	98.20	0.67
	10	100	8.53 ± 0.12	8.51 ± 0.06	99.80	1.19
	10	150	11.25 ± 0.08	11.10 ± 0.02	98.40	0.82
FT	10	50	7.40 ± 0.09	7.55 ± 0.01	101.75	0.54
	10	100	9.52 ± 0.10	9.85 ± 0.00	102.8	1.05
	10	150	12.37 ± 0.06	12.63 ± 0.06	101.81	0.92
QT	10	50	7.40 ± 0.04	7.52 ± 0.06	101.72	1.19
	10	100	9.67 ± 0.01	9.65 ± 0.05	99.72	0.43
	10	150	12.40 ± 0.02	12.54 ± 0.10	101.13	0.69

% RSD: Percent relative standard deviation

5.6.5. Determination of isosbestic point by UV spectrophotometer

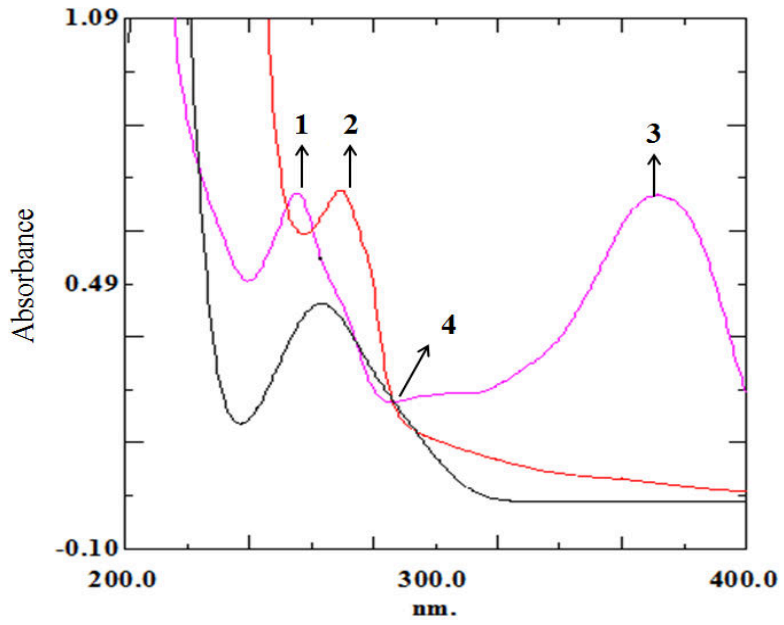


Figure 14: Isosbestic point of 1) EGC, 2) FT, 3) QT and 4) Isosbestic point

5.6.6. Determination of isosbestic point for EGC, FT and QT by HLPC

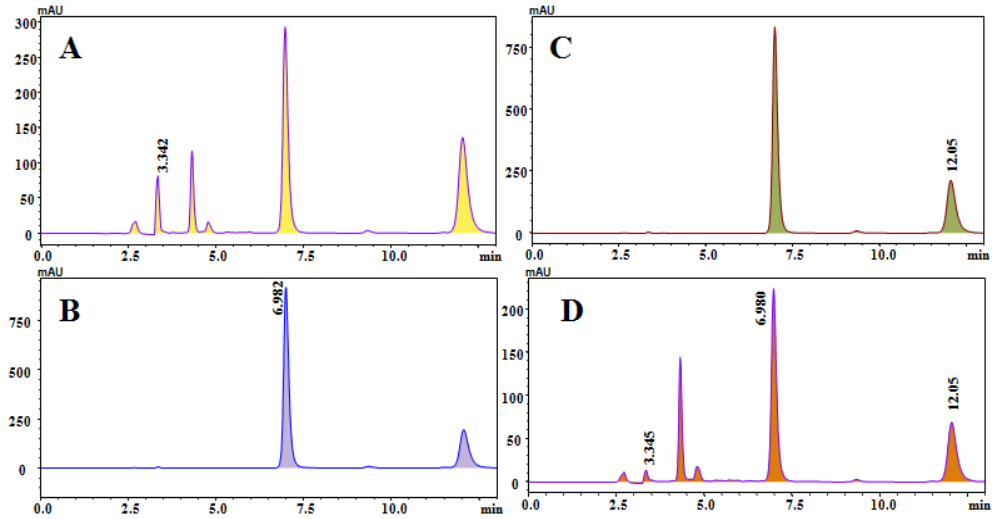


Figure 15: HPLC chromatograms. A) EGC (269 nm), B) FT (359 nm), C) QT (370 nm), D) Isosbestic point (287 nm)

5.6.7. Optimized HPLC method applicability of *Acacia suma*

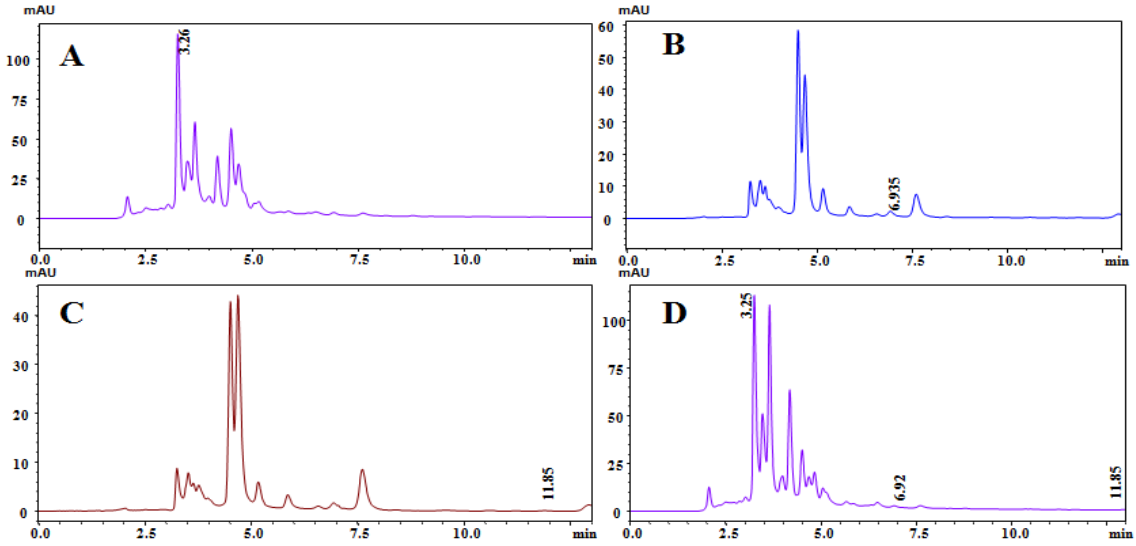


Figure 16: HPLC Chromatogram of *Acacia suma*: A) EGC; B) FT; C) QT; D) Isosbestic point

5.6.8. Estimation of drug content in *Acacia suma* by HPLC

Table 17: Drug content estimation by HPLC

Standards	EGC	FT	QT
Drug content (mg/g)	107.2 ± 0.22	1.71 ± 0.03	1.72 ± 0.07

5.7. High-performance thin layer chromatography fingerprinting of *Acacia suma*

5.7.1 Determination of calibration curve

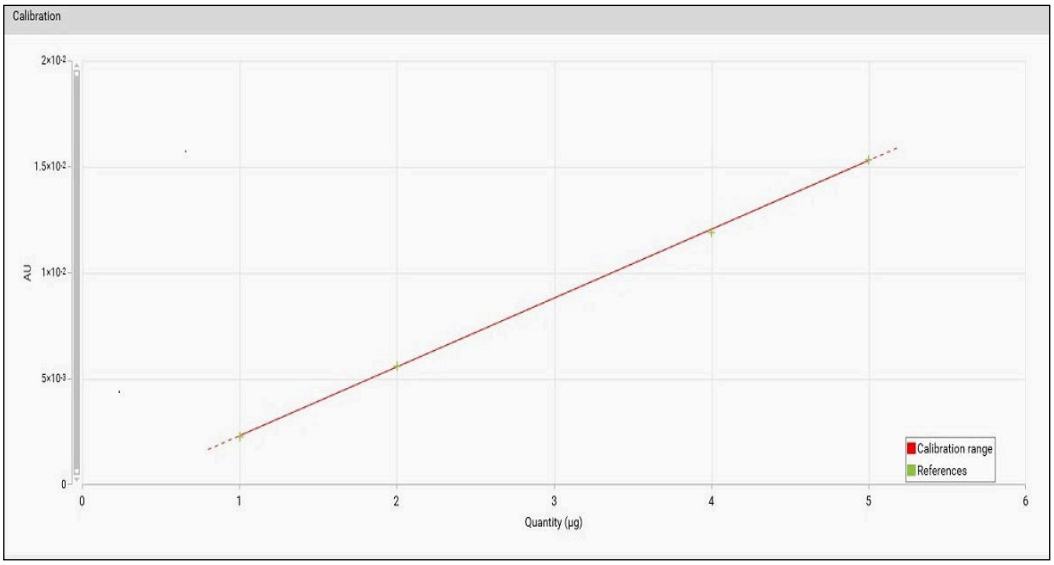


Figure 17: HPTLC calibration curve of epigallocatechin

Table 18: HPTLC calibration curve parameters of epigallocatechin

Parameters	Values
Linearity range	1 - 5 (µg/ml)
Linearity equation	$Y= 3.245\times10^{-9}x- 9.437\times10^{4}$
Correlation coefficient	0.9999
Coefficient of variance	0.76%
Regression mode	Linear

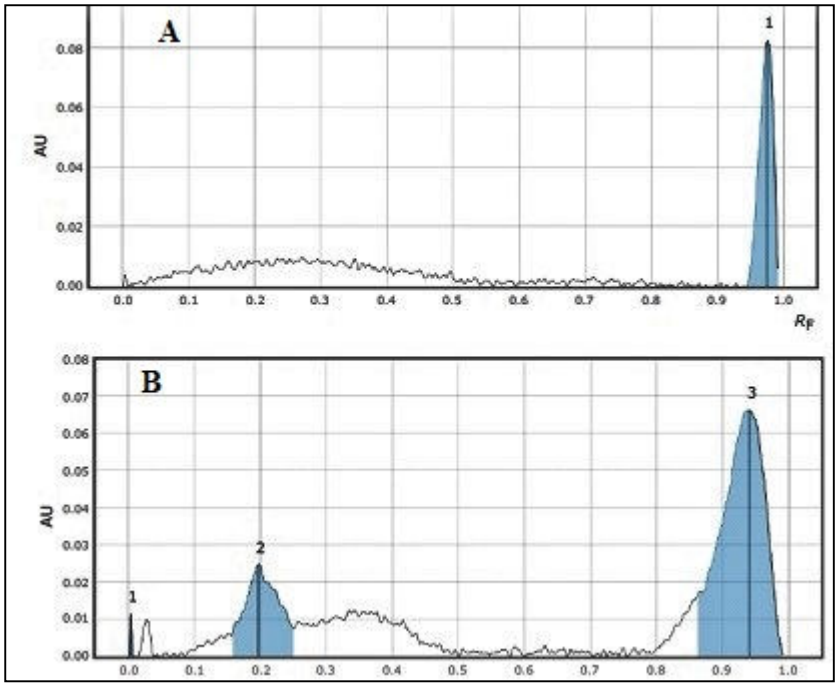


Figure 18: HPTLC densitogram of [A] EGC [B] EGC in *Acacia suma*

5.7.2. HPTLC fingerprinting of *Acacia suma*

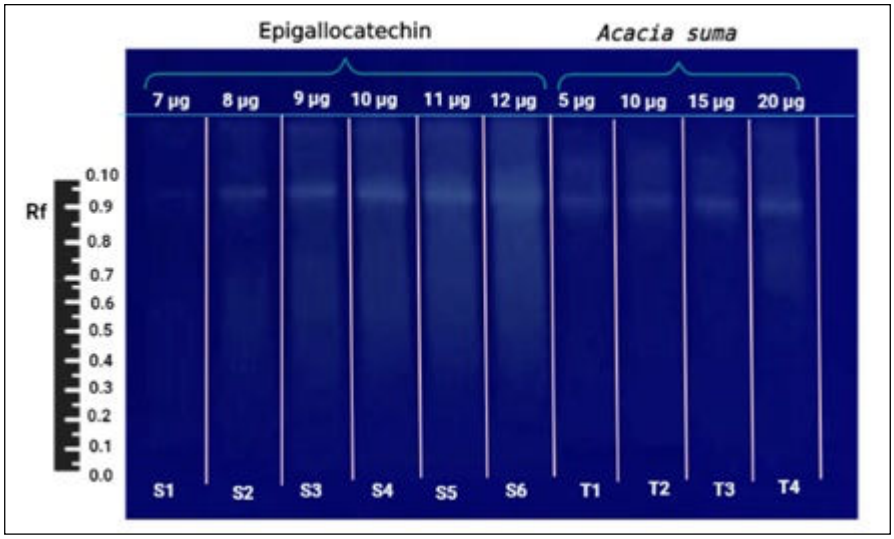


Figure 19: HPTLC fingerprint profiles of *Acacia suma*

Table 19: Percent recovery of epigallocatechin by HPTLC

Levels	Area	% Recovery
80 %	0.00145	82.16%
100 %	0.00207	102.7 %
120 %	0.00406	122.4%

5.7.3. HPTLC-DPPH Anti-Oxidant Assay

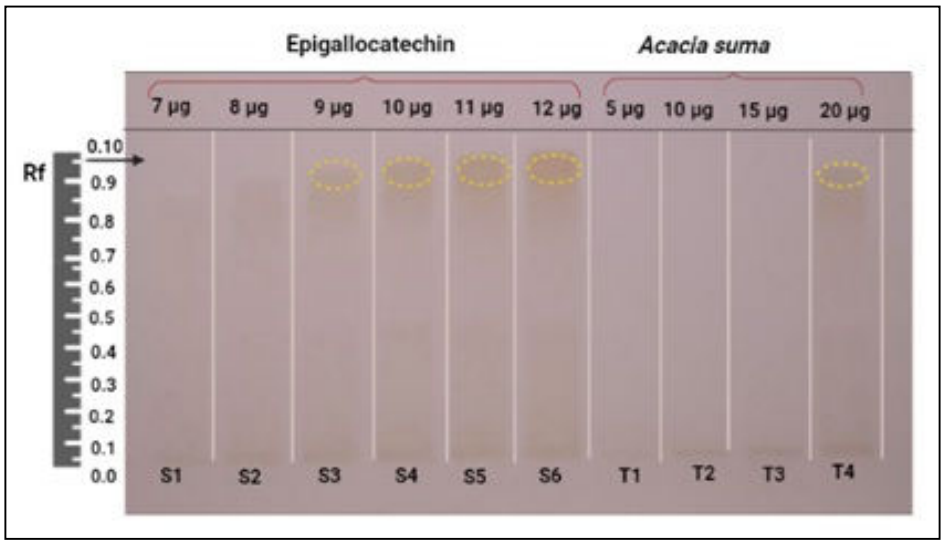
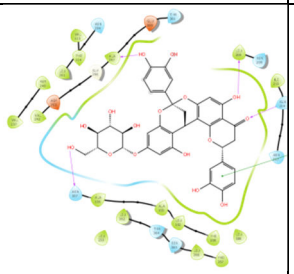
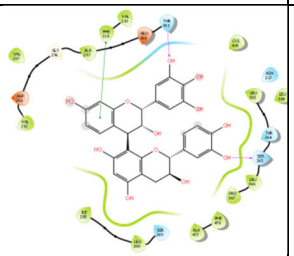
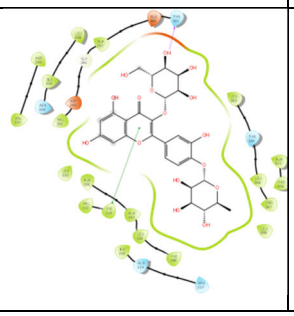
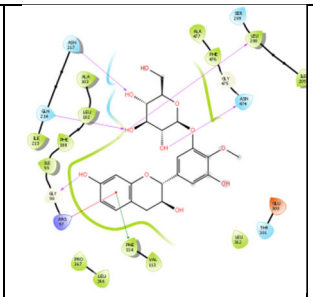
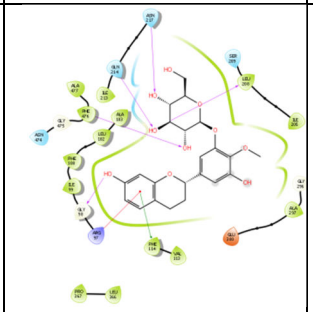
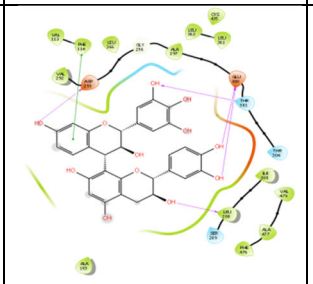
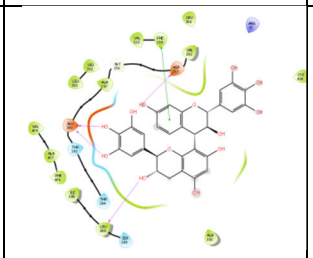
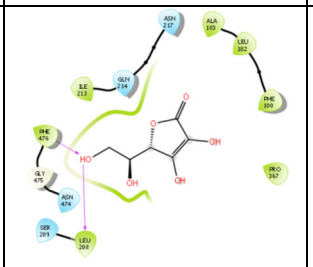
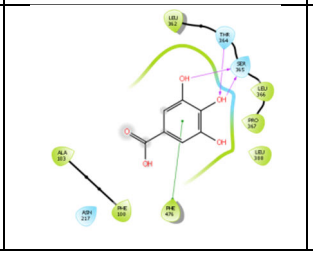


Figure 20: HPTLC-DPPH fingerprinting of EGC and *Acacia suma*

5.8. Molecular docking score of Cytochrome P450 inhibitors

Table 20: Molecular docking score of Cytochrome P450 inhibitors from *Acacia suma*

Compound name	Glide g score	2D Structure	NHB	HBD
Diinsininol	-13.151		20	Ala297, Val292, Val237, Met240, Leu201, Phe114, Val113, Ala106, Leu362, Leu233, Ala103, Leu102, Phe100, Leu366, Pro367, Leu388, Phe476, Ala477, Ile213, Leu208
Epirobinetinidol -(4β,8)-catechin	-11.632		14	Val113, Phe114, Ala297, Val237, Val292, Ile205, Leu208, Ala477, Phe476, Pro367, Leu366, Leu362, Leu388, Cys435
Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside	-11.379		20	Leu208, Ala297, Met240, Ile205, Val237, Val297, Lel233, Ala106, Val113, Phe114, Ala103, Leu102, Phe100, Ile213, Leu388, Pro367, Leu366, Phe476, Ala477, Leu362

4'-O-methylrobinetinidol 3'-O-β-D-glucopyranoside	-10.955		14	Ala103, Leu102, Phe100, Ile213, Ile99, Pro367, Leu366, Phe114, Val113, Leu362, Ala477, Phe476, Leu208, Ile205
Auriculoside	-10.765		14	Ala477, Phe476, Ile213, Ala103, Leu102, Phe100, Ile99, Phe114, Val113, Pro367, Leu366, Ala297, Leu208, Ile205
Robinetinidol-(4α,8)-catechin	-10.76		14	Val113, Phe114, Val292, Leu366, Ala297, Leu362, Leu361, Cys435, Ile205, Val479, Leu208, Ala477, Phe476, Ala103
Robinetinidol-(4α,8)-gallocatechin	-10.669		14	Cys435, Leu366, Val292, Phe114, Val113, Ala297, Leu362, Leu361, Val479, Ala477, Phe476, Ile205, Leu208, Ala103
Ascorbic acid	-7.506		7	Leu208, Phe476, Ile213, Ala103, Leu102, Phe100, Pro367
Gallic acid	-7.119		7	Ala103, Phe100, Phe476, Leu388, Pro367, Leu366, Leu362

NHB number of hydrogen bond, *HBD* hydrogen bond donor

5.8.1. Adverse effects and drug likeness score of Cytochrome P450 inhibitors

Table 21: Adverse effect of Cytochrome P450 inhibitors from *Acacia suma*

Phytochemical name	Adverse effect	*P*a	*P*i
Diinsininol	Nephrotoxicity	0.358	0.109
	Hepatotoxicity	0.337	0.314
Epirobinetinidol-(4β,8)-catechin	-	-	-
Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside	-	-	-
4'-O-methylrobinetinidol 3'-O-β-D-glucopyranoside	Nephrotoxicity	0.412	0.079
Auriculoside	Nephrotoxicity	0.369	0.103
Robinetinidol-(4α,8)-catechin	-	-	-
Robinetinidol-(4α,8)-gallocatechin	-	-	-
Fisetinidol-(4α,8)-catechin	-	-	-
Okanin	Hepatotoxicity	0.579	0.155
	Cardiac failure	0.441	0.086
Fisetinidol-(4α,6)-gallocatechin	-	-	-
Butin	Hepatotoxicity	0.692	0.103
(5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-dien-7-ol-17-al	-	-	-
3,21-dioxoolean-18-en-28-oic acid	-	-	-
(5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol	-	-	-
(20R)-28-hydroxylupen-30-al-3-one	-	-	-
Ascorbic acid	-	-	-
Gallic acid	Hepatotoxicity	0.699	0.101
	Nephrotoxicity	0.307	0.150

Pa- Probable activity, *Pi*- Probable inactivity

5.9. Evaluation of in-vitro antioxidant potential of *Acacia Suma*

Scavenging of 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical

Radical scavenging activity of HAE of *Acacia suma* towards stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was analysed against reference anti-oxidant ascorbic acid and IC_{50} was found 81.46 ± 2.72µg/ml represented in (figure 21).

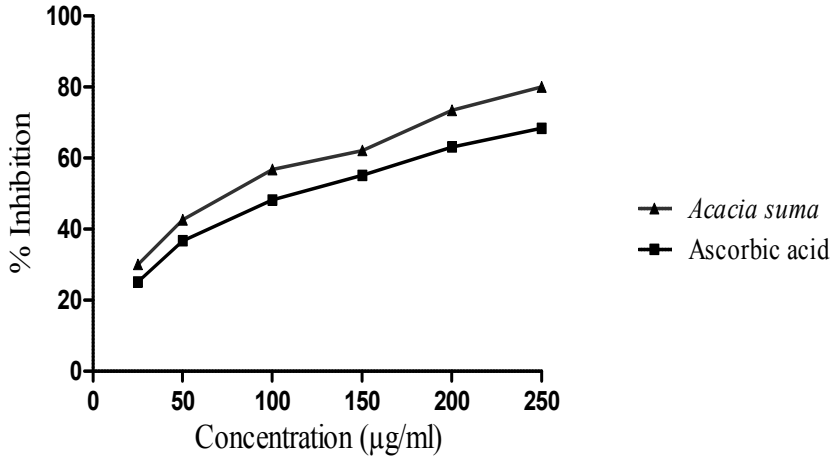


Figure 21: DPPH radical scavenging potential of *Acacia suma*

Hydrogen peroxide (H_2O_2) scavenging assay

The assay was applied to determine potential of sample to eliminate free radicals. Hydrogen peroxide (H_2O_2) scavenging activity of *Acacia suma* had shown increased percentage of scavenging with increase in concentration and IC_{50} found as 61.39 ± 1.85µg/ml against ascorbic acid (figure 22).

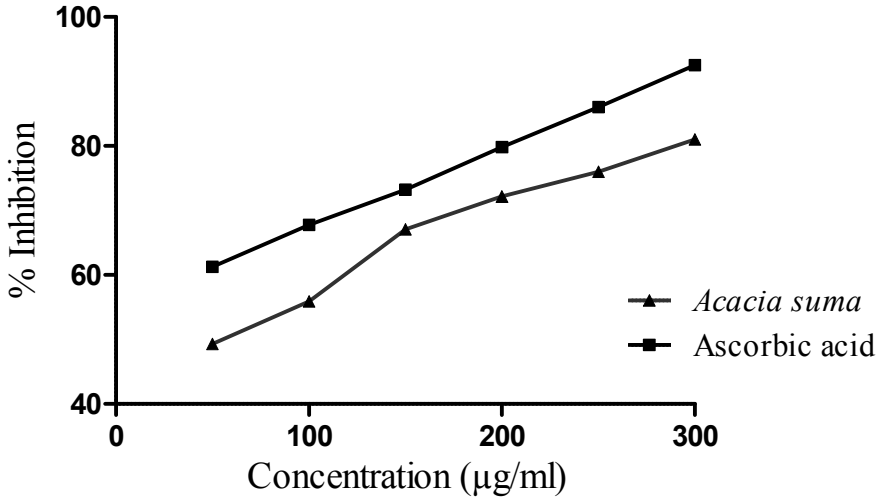


Figure 22: Hydrogen peroxide scavenging assay

Scavenging of nitric oxide

The test sample has observed to have concentration dependent activity it was compared with ascorbic acid as standard with IC_{50} 21.30 ± 2.26 µg/ml and percentage inhibition has represented in (figure 23).

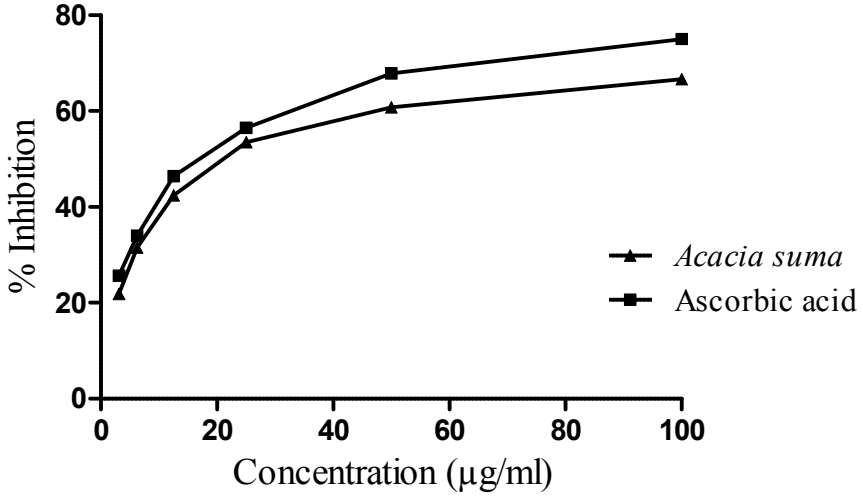


Figure 23: Nitric oxide assay

Total antioxidants capacity (TAC)

Total anti-oxidant capacity of the crude extract and was determined by the phosphomolybdenum method using reference as ascorbic acid. The IC_{50} found as 55.13 ± 2.86µg/ml. The graph was plotted against percentage inhibition vs concentration depicted in (figure 24).

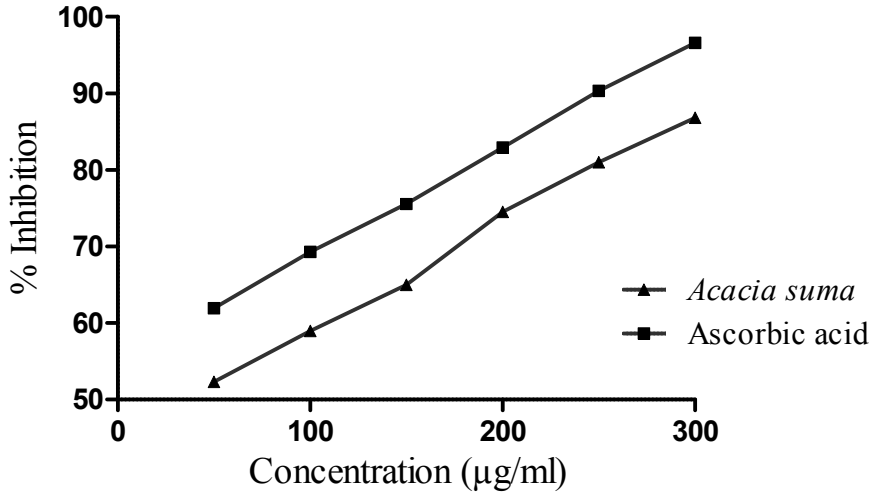


Figure 24: Total anti-oxidant assay

Lipid peroxidation assay

The hydroalcoholic extract of *Acacia suma* inhibits lipid peroxidation induced by ferrous sulphate in egg yolk homogenate in a concentration-dependent manner with IC_{50} 77.03 ± 2.47 µg/ml. Where, Ascorbic acid was used as a reference drug and percentage inhibition has represented in (figure 25).

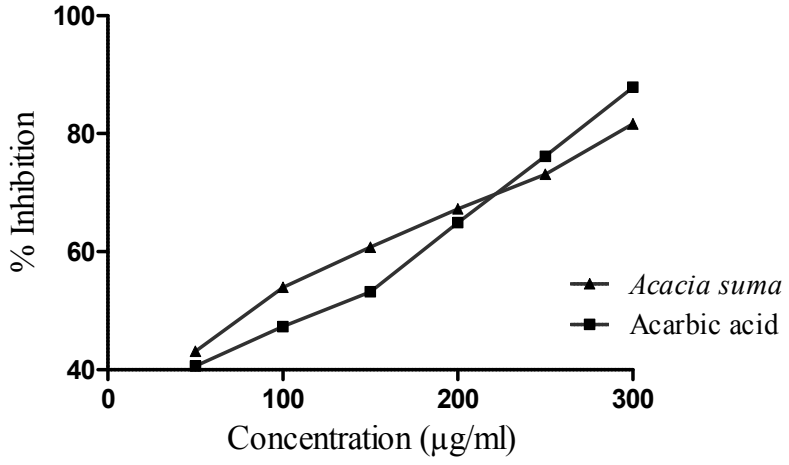


Figure 25: Lipid peroxidation assay

Ferric reducing power assay

Reducing power of *Acacia suma* indicates a high potential in hydrogen-donating ability which could react with free radicals to convert them into more stable products thereby terminating radical chain reactions. *Acacia suma* has shown concentration-dependent values and compared with ascorbic acid used as a reference drug represented in (figure 26).

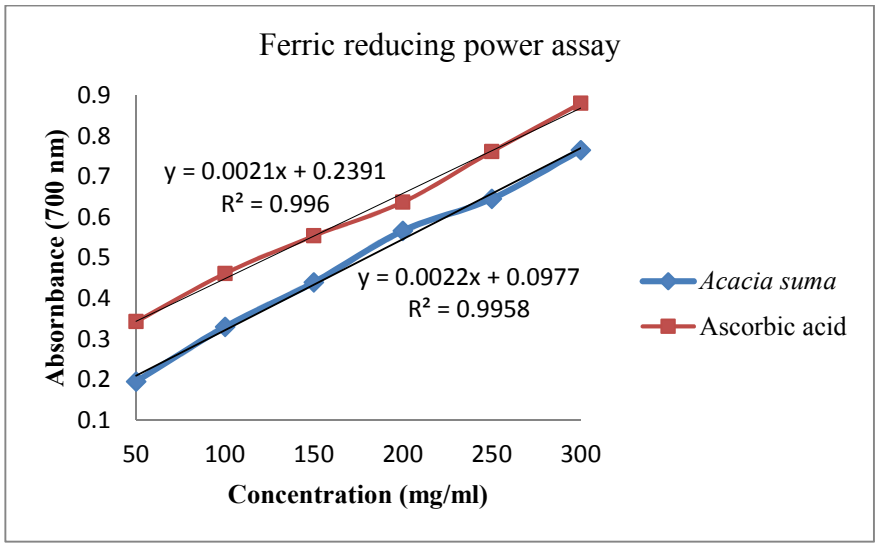


Figure 26: Ferric reducing power assay

5.10. Pharmacological evaluation for anti-obesity activity

5.10.1 Lipase inhibition assay: molecular docking

The anti-lipase activity of *Acacia suma* (Phospholipase A2 group IIA inhibitors)

Table 22: Drug likeness score of lipase inhibitors from *Acacia suma*

Phytochemicals	PubChem CID	Molecular formula	Molecular weight (g/mol)	NHBA	NHBD	MolLogP	DLS
(5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol	54672538	$C_{20}H_{32}O_3$	320.24	3	2	320.24	-0.68
Fisetinidol-(4α,6)-gallocatechin	21192716	$C_{30}H_{26}O_{12}$	578.14	12	10	3.29	0.60
Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside	71306323	$C_{27}H_{30}O_{16}$	610.15	16	10	-2.32	0.73
Orlistat	3034010	$C_{29}H_{53}NO_5$	495.7	5	1	8.82	-0.62

NHBA: Number of hydrogen bond acceptor, NHBD: Number of hydrogen bond donor, DLS: Drug likeness score

Side effects of lipase inhibitors from *Acacia suma*

Table 23: Side effects of lipase inhibitors from *Acacia suma*

Phytochemicals	Side effect	*Pa*	*Pi*
(5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol	No side effect	-	-
Fisetinidol-(4α,6)-gallocatechin	No side effect	-	-
Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside	• Hepatotoxicity	0478	0.207
	• Nephrotoxicity	0.288	0.17
Orlistat*	• Hepatotoxicity	0.983	0.006
	• Nephrotoxicity	0.97	0.004
	• Myocardial infraction	0.304	0.19

*FDA approved Marketed lipase inhibitor, *Pa*- Probable activity, *Pi*- Probable inactivity

Gene enrichment analysis

Table 24: Gene enrichment analysis of lipase inhibitors form *Acacia suma*

Phytochemicals	Pathway	Description	Gene count	Gene codes	False discovery rate
(5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol	hsa04931	Insulin resistance	6	CREB3L1, FOXO1, MAPK8, PPARGC1A, PPP1CB, RPS6KA3	0.0247
	hsa04211	Longevity regulating pathway	5	CAMKK2, CREB3L1, FOXO1, PPARGC1A, SESN1	0.0425
Fisetinidol-(4α,6)-gallocatechin	hsa04919	Thyroid hormone signalling pathway	7	ITGAV, KAT2B, KRAS, MYH6, NCOA1, NOTCH1, PFKFB2	0.0258
Quercetin 4'-O-α-L-rhamnopyranosyl-3-O-β-D-allopyranoside	hsa05224	Breast cancer	9	ERBB2, HES1, KRAS, LRP6, NCOA1, NOTCH1, PGR, WNT11, WNT7A	0.0125

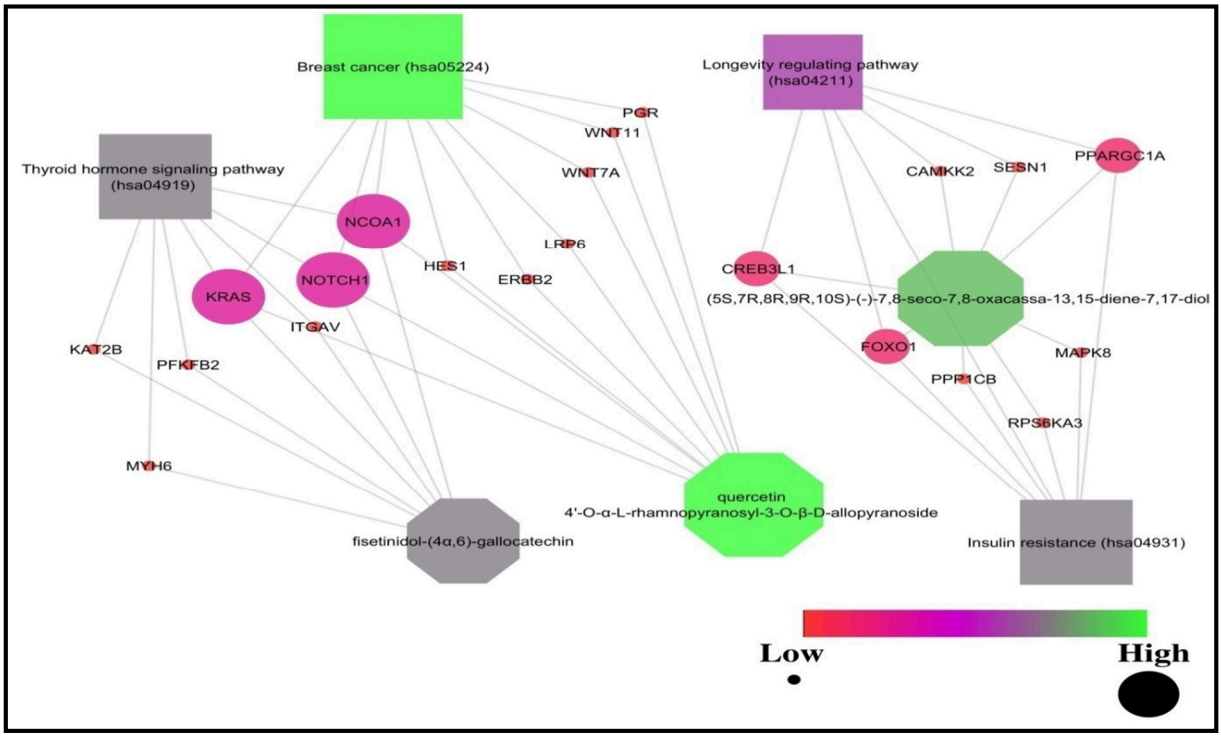


Figure 27: Network construction of phytoconstituents, targeted genes and disease pathways

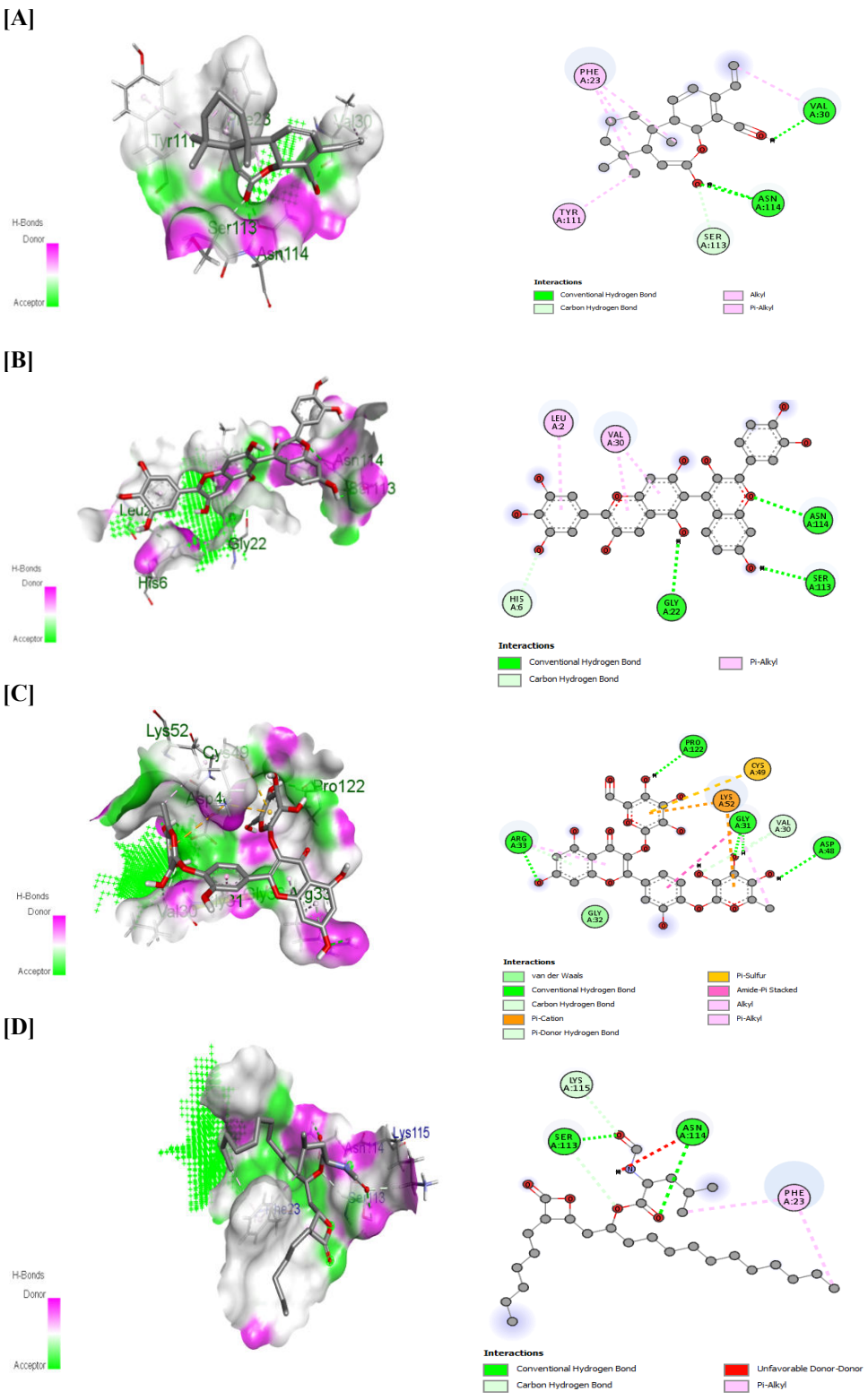


Figure 28: 2D & 3D Interaction between A. 5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol, B. Fisetinidol-(4α,6)-gallocatechin, C. Quercetin 4'-

O-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside and D. Orlistat with phospholipase A2 group IIA Inhibitors.

Docking score or binding affinity of lipase inhibitors

Table 25: The binding affinity of lipase inhibitors

Phytochemicals	Binding Affinity	NHB	HBD
5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol	-5.9	2	ASN114, VAL30
Fisetinidol-(4α,6)-gallocatechin	-7.2	3	GLY22, SER113, ASN114
Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside	-7.6	5	AR433, PRO122, GLY31, ASP48, CYS49
Orlistat*	-5.2	2	SER113, ASN114

*FDA approved Marketed lipase inhibitor

5.10.2 Enzyme inhibition assays

Lipase inhibition assay

The HEA of *Acacia suma* had shown highest pancreatic lipase inhibition activity using PNPB as substrate and orlistat was used as standard anti-lipase agent. The IC_{50} was found as 46.07µg/ml and 35.79µg/ml for HAE of *Acacia suma* and orlistat respectively. The graph was plotted against % inhibition and concentration (µg/ml) of extract represented in (figure 29).

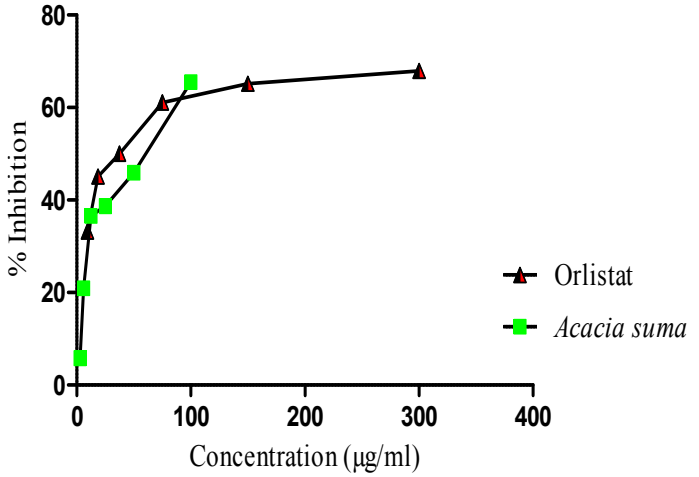


Figure 29: Lipase inhibition activity of *Acacia suma*

Alpha amylase inhibition

Acacia suma had shown maximum alpha amylase activity was compared to Metformin, α-amylase inhibitor. The 50% inhibitory concentration was found as 12.5µg/ml and 52.09µg/ml for HAE of *A. suma* and Metformin respectively. The graph has plotted against % inhibition and concentration (µg/ml) represented in (figure 30).

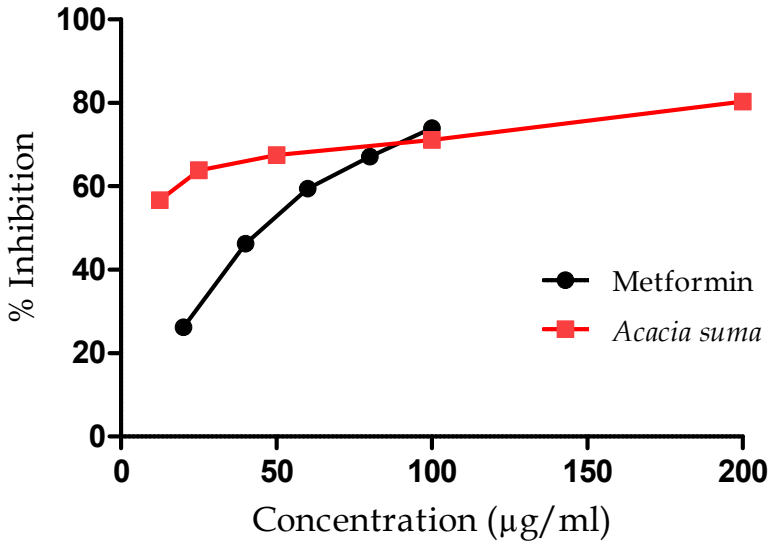


Figure 30: Alpha amylase inhibition assay

Alpha glucosidase inhibition

Acacia suma had shown maximum alpha amylase activity was compared to Acarbose, The IC_{50} was found as 93.79µg/ml and 79.74µg/ml for HAE of *A. suma* and Acarbose respectively. The graph has plotted against % inhibition and concentration (µg/ml) represented in (figure 31).

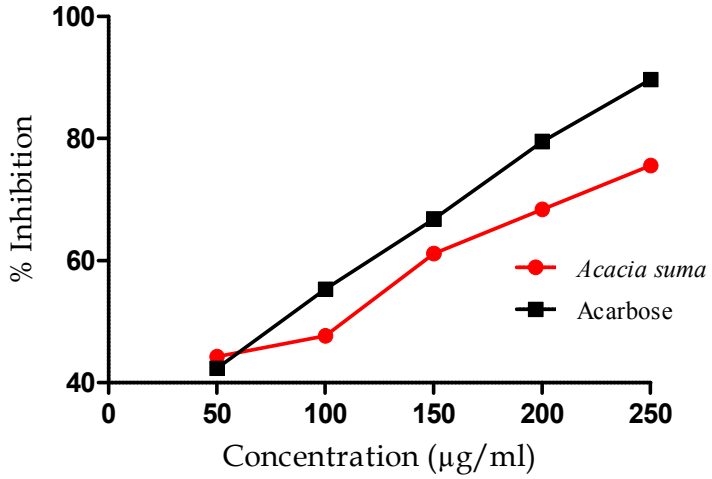


Figure 31: Alpha glucosidase inhibition assay

5.10.3 In-vitro cell line study: MTT assay

The effect of plant extract was tested for cell viability using 3T3 adipocyte cells. The extract was found safe and no-toxic on the normal adipocytes. The graph was plotted against % cell viability and concentration (µg/ml) represented in (figure 32).

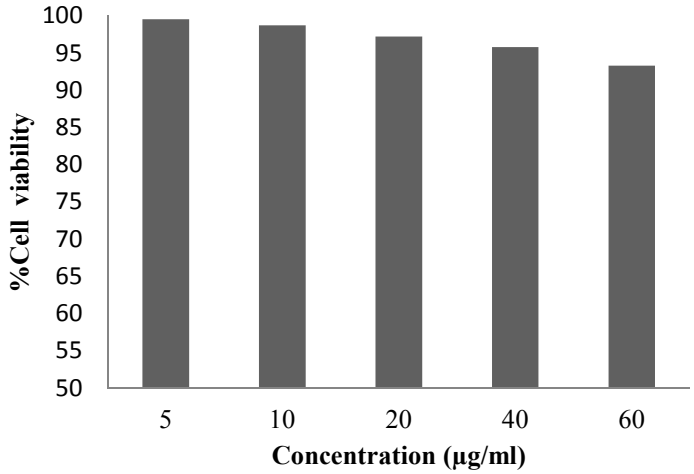


Figure 32: Percent cell viability of 3T3 cells

Oil O red staining assay

When adipocytes treated with cocktail solution; in induction group shown large number of mature adipocyte cells compared to control. The presence of lipid droplets was found less in extract treated cell compared to cocktail-induced cells represented in (figure 33).

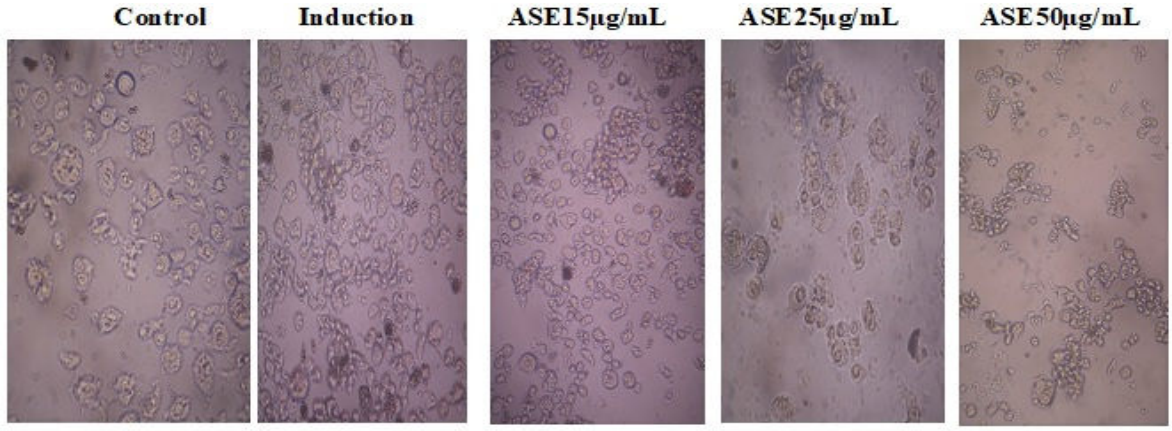


Figure 33: 3T3 cells morphology with oil-o-red stain

Triglyceride estimation of 3T3 cells

The TG was estimated by oil-o-red staining of differentiated 3T3 adipocyte cells. The absorbance of control, induction and treated cells were measured. The Absorbance of induction group has significantly increased ($p < 0.001$); (0.909 ± 0.018) compared to control (0.500 ± 0.174). The absorbance of ASE15µg/ml ($p < 0.05$); (0.8030 ± 0.028) ASE25µg/ml ($p < 0.001$); (0.691 ± 0.018), ASE50µg/ml ($p < 0.001$); (0.440 ± 0.019) were significantly decreased compared to induction group cells.

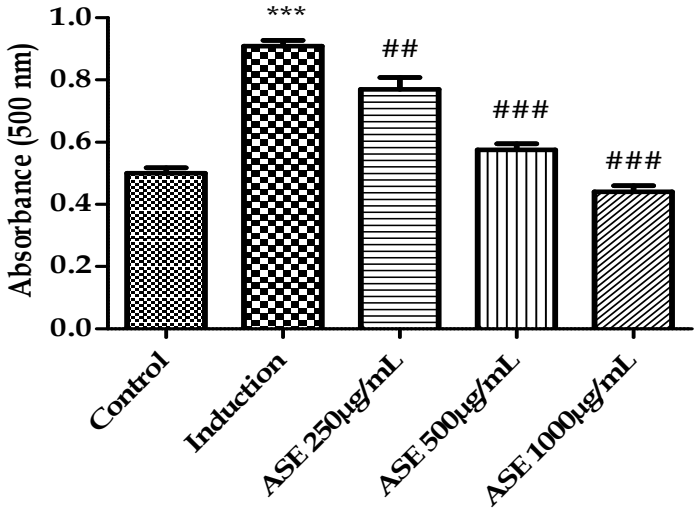


Figure 34: Triglyceride estimation in 3T3-L1; The degree of significance was described in terms of Mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. incubated.

5.10.4 In-vivo animal study

Toxicity study in mice

In the acute oral toxicity study, 2000 mg/kg test dose of *Acacia suma* has not cause any mortality during 14 days observation period. Mice have not shown any signs of toxicity or change in general behaviour it was compared to the control group. The summary of the observations is depicted (table 26).

Table 26: Acute toxicity of *Acacia suma* in C57BL/6 mice

Parameters	Vehicle control	*Acacia suma* (2000mg/kg)
Skin and fur	Normal	No Change
Eyes and Mucous membrane	Normal	No Change
Respiratory system	Normal	No Change
Circulatory system	Normal	No Change
Autonomic nervous system	Normal	No Change
Central nervous system	Normal	No Change
Somatomotor activity	Normal	No Change
Behavioural pattern	Normal	No Change
Tremor	Normal	No Change

Convulsions	Normal	No Change
Salivation	Normal	No Change
Diarrhoea	Normal	No Change
Lethargy	Normal	No Change
Sleep	Normal	No Change
Coma	Normal	No Change
Death	-	-

Table 27: Effect of *Acacia suma* on body weight during acute toxicity study

Body weight(g)	Control	Treatment group
Day 1	16.32 ± 014	16.49 ± 0.52
Day 7	17.50 ± 0.50	17.33 ± 0.13
Day 14	18.93 ± 0.29	18.73 ± 0.35

Morphological observations of oral acute toxicity

Morphological changes were observed with no gross charges in control and treatment group.

5.11 Investigation of anti-obesity potential of *Acacia suma* on HFD induced mice

Cumulative body weight (g)

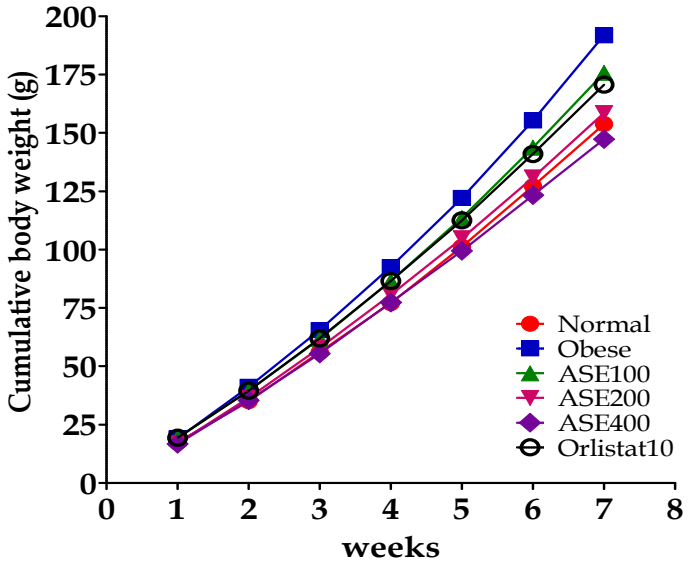


Figure 35: Cumulative body weight of mice; The cumulative body weight of mice has been increased weekly. Significant difference was observed in HFD (60%) fed

animals and normal diet (10%) ($p< 0.001$). The difference measured by two-way ANOVA with Bonferroni test in terms of mean ± SEM.

Cumulative food intake (gm)

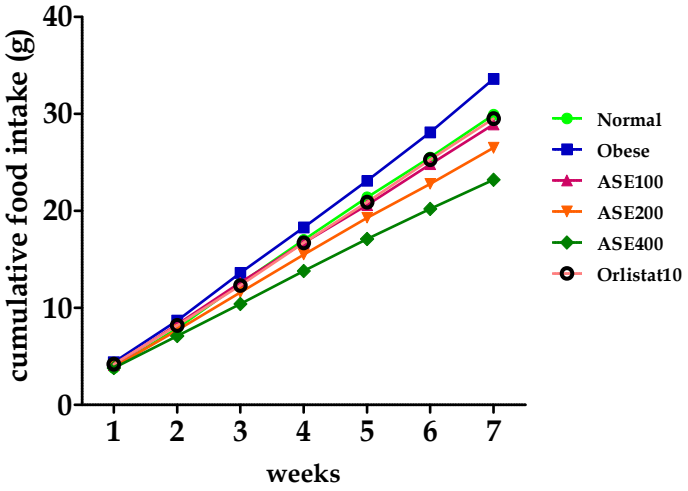


Figure 36: Cumulative food intake; The cumulative food intake of mice was found increased weekly. The difference was observed between HFD fed animals and normal diet fed animals found non-significant. The difference assessed by two-way ANOVA with Bonferroni test in terms of mean ± SEM.

Cumulative energy intake (kcal/J)

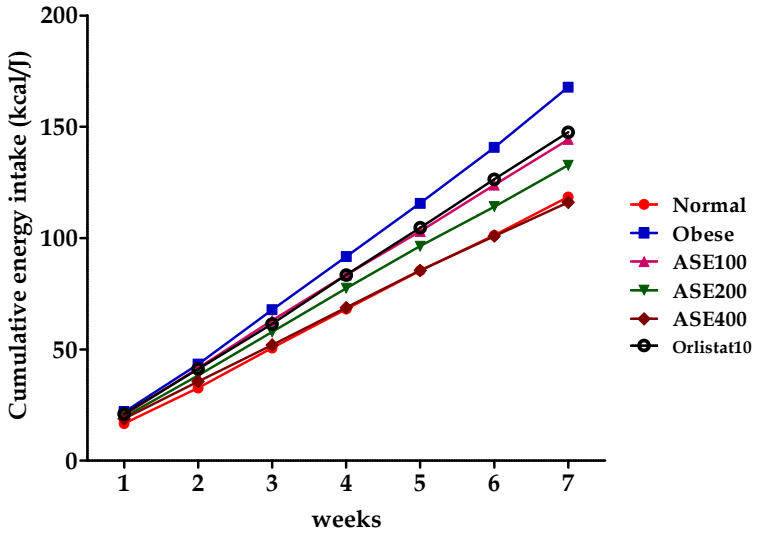


Figure 37: Cumulative energy intake; The cumulative energy intake of mice was increased weekly. The difference was observed between HFD (60%) fed animals and normal diet (10%) found non-significant results for four weeks and significant on 5th ($p<0.05$), 6th ($p < 0.01$) and 7th ($p < 0.001$) weeks. The difference assessed by two-way ANOVA with Bonferroni test in terms of mean ± SEM.

Total water intake (ml)

The total water intake by mice was measured where, in HFD induced obesity control group water intake has significantly decreased ($p< 0.001$); (127.0 ± 4.68 ml) compared to normal (156.4 ± 2.48 ml). And in treatment groups the water intake has significantly increased ($p< 0.001$); ASE200 mg/kg (156 ± 2.27 ml); ASE400 mg/kg (178.7 ± 2.17 ml) and orlistat10 mg/kg (161.0 ± 4.30 ml) compared to obesity control. Whereas ASE100 mg/kg shown non-significant results.

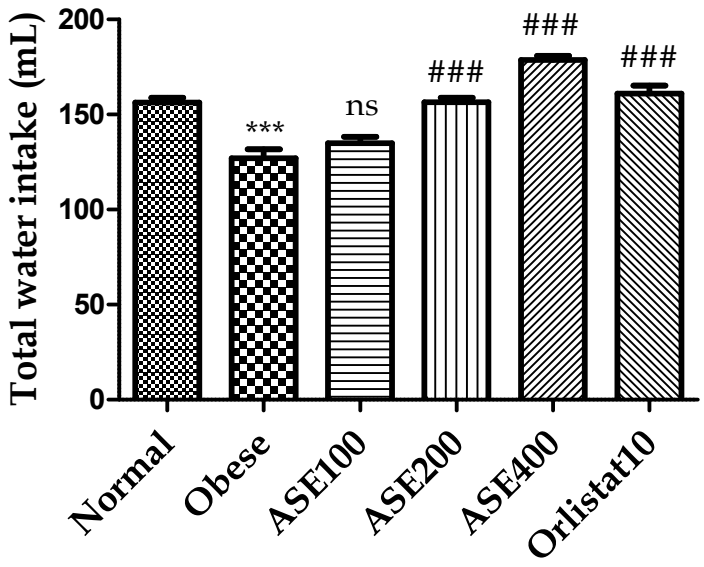


Figure 38: Total water intake (ml); statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Change in body weight gain (g)

The change in mice body weight of high-fat diet-induced obesity control group has significantly increased ($p< 0.001$); (17.52 ± 0.22 g) compared to normal (9.24 ± 0.26 g). Whereas in treatment groups change in body weight has significant decreased ($p< 0.001$); ASE100 mg/kg (12.61 ± 0.21 g), ASE200 mg/kg (10.60 ± 0.31g); ASE400 mg/kg (7.30 ± 0.31g) and orlistat10 mg/kg (10.43 ± 0.15g) compared to obesity control.

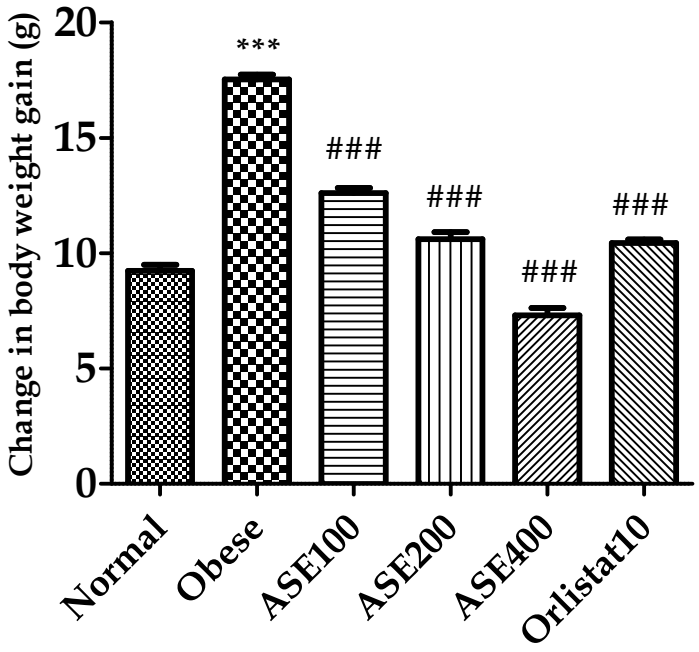


Figure 39: Effect of ASE on change in body weight; statistical significance was expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Abdominal circumference (cm) on 7th week

Abdominal circumference was measured on 7th week. High-fat diet-induced obesity control group had shown significantly increased ($p< 0.001$) abdominal circumference (8.17 ± 0.13 cm) compared to normal (6.313± 0.11 cm). Whereas treatment groups had shown significant decreased ($p< 0.001$) abdominal circumference as, ASE100 mg/kg (7.72 ± 0.076 cm), ASE200 mg/kg (7.32 ± 0.157 cm); ASE400 mg/kg (6.69 ± 0.065cm) and orlistat10 mg/kg (7.26 ± 0.058 cm) compared to obesity control.

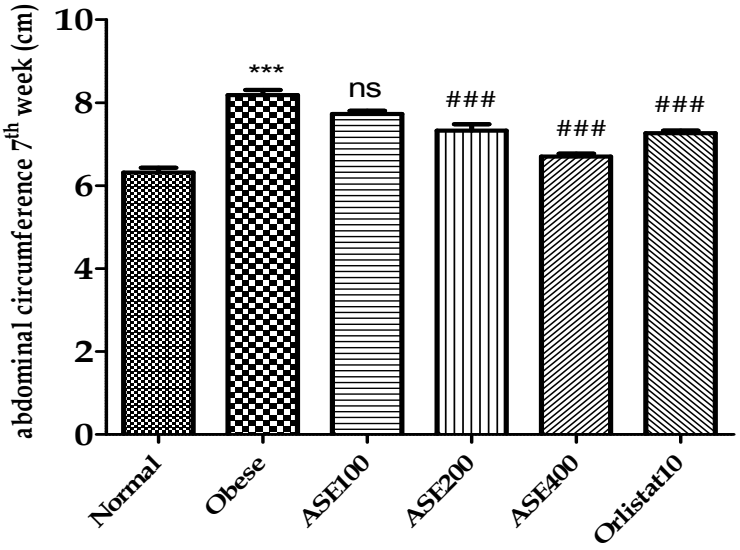


Figure 40: Effect of ASE on abdominal circumference (cm); Statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Body mass index (BMI) gcm^{-2}

The BMI was measured on 7th week; the high-fat diet-induced obesity control group has shown significantly increased BMI (0.617 ± 0.0029 gcm^{-2}) ($p< 0.001$) compared to normal (0.449 ± 0.0025 gcm^{-2}). Whereas treatment groups had shown significant decreased BMI $p< 0.001$; ASE100 mg/kg (0.584 ± 0.0035 gcm^{-2}), ASE200 mg/kg (0.491 ± 0.0024 gcm^{-2}); ASE400 mg/kg (0.438 ± 0.0029 gcm^{-2}) and orlistat10 mg/kg (0.529 ± 0.0037 gcm^{-2}) compared to obesity control.

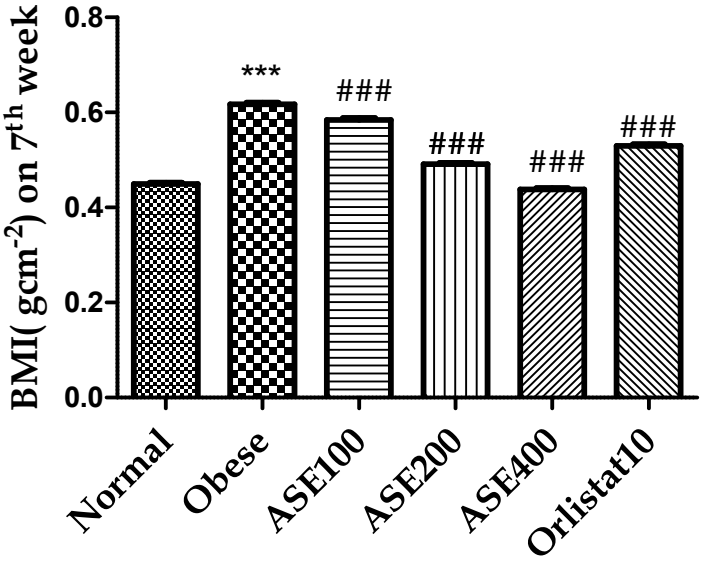


Figure 41: Effect of ASE on BMI (gcm^{-2}) on 7th week, statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Liver weight (g) of mice

The obesity control group has shown significant increased (1.60 ± 0.026 g) ($p< 0.001$) in liver weight was compared with normal (1.027 ± 0.045 g). The ASE100 mg/kg group had shown significant reduction $p<0.01$ (1.38 ± 0.031 g) in liver weight compared to obese. Also, ASE200 mg/kg (1.216 ± 0.047); ASE400 mg/kg (1.038 ± 0.034) and orlistat10 mg/kg (1.29 ± 0.031) had shown reduced liver weight compared to obese.

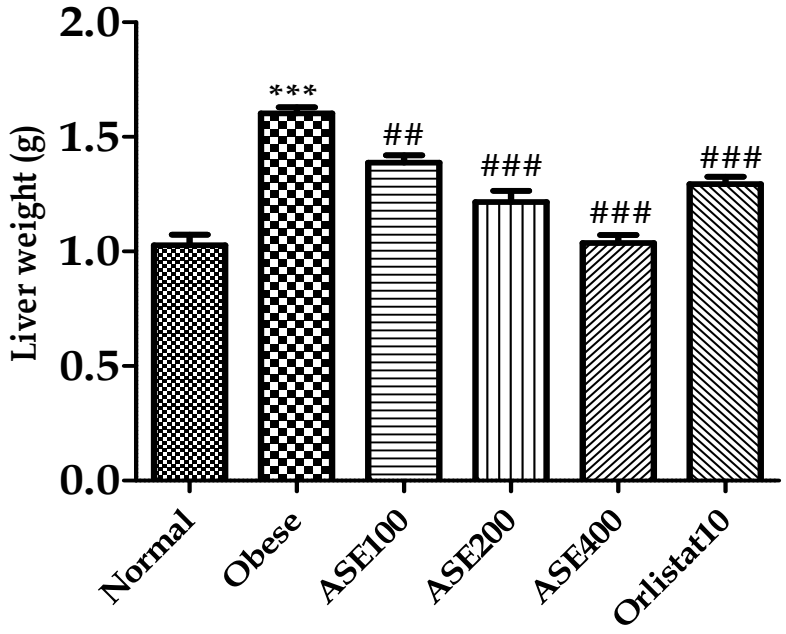


Figure 42: Effect of ASE on liver weight, (n=6 in each group); Statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Estimation of Alanine transaminase (ALT)

Significantly increased ALT level in HFD fed group (115.5 ± 0.83 U/L) was observed compared to normal (55.3 ± 0.96 U/L). Whereas, ASE100 mg/kg (108.3 ± 0.89 U/L), ASE200 mg/kg (76.8 ± 1.10 U/L), ASE400 mg/kg (67.51 ± 1.53 U/L) and orlistat10 mg/kg (91.95 ± 1.07 U/L) had shown decreased ALT level compared to obese group.

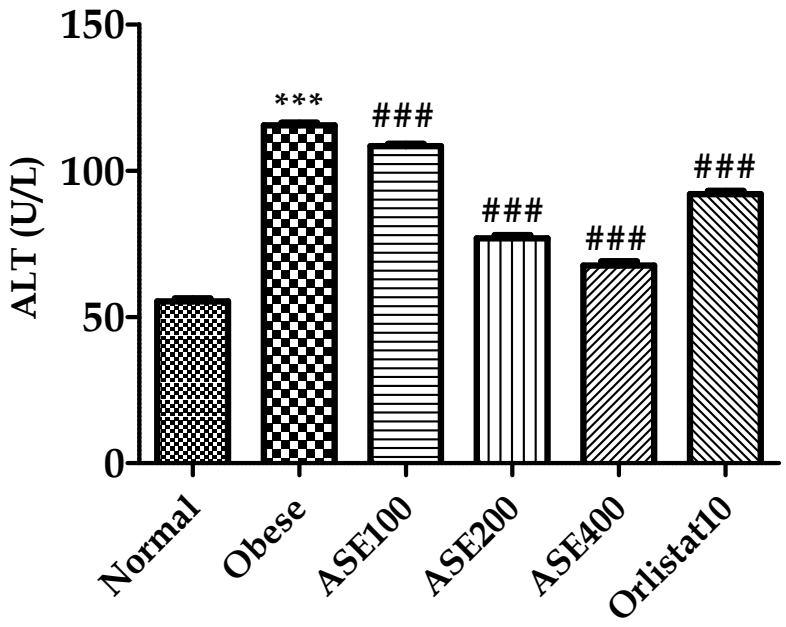


Figure 43: Effect of ASE ALT (U/L), (n=6 in each group); statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Estimation of aspartate transaminase (AST)

The AST has significantly increased in HFD fed obese group (142.6 ± 1.72 U/L) compared to normal (107.7 ± 1.07 U/L). Whereas, ASE100 mg/kg (116.4 ± 1.40 U/L), ASE200 mg/kg (100.1 ± 1.26 U/L), ASE400 mg/kg (73.9 ± 0.98 U/L) and orlistat10 mg/kg (123.7± 1.88 U/L) had shown decreased AST level compared to obese group.

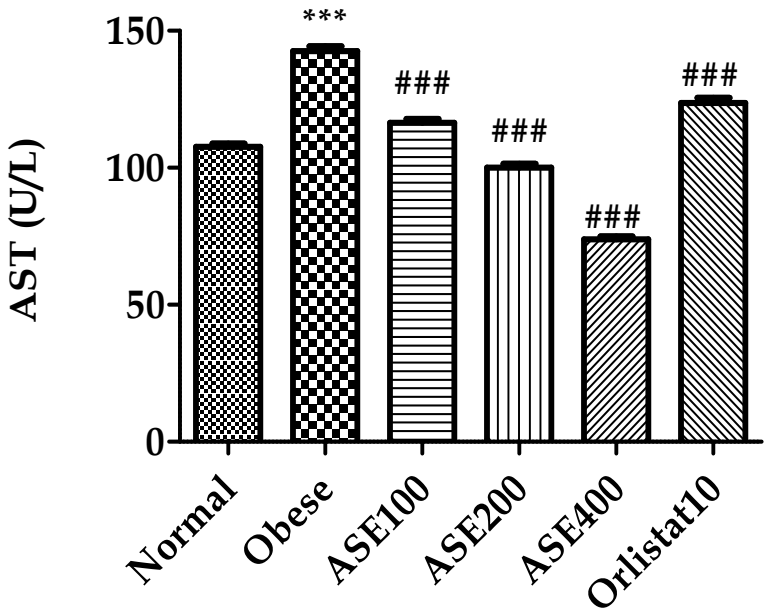


Figure 44: Effect of ASE on AST (U/L) (n=6 in each group); statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Estimation of alkaline phosphatase (ALP)

The ALP has significantly ($p < 0.001$) increased in obesity control group (129.6 ± 3.95 U/L) compared to normal (49.10 ± 3.96 U/L). Whereas, ASE100 mg/kg (94.86 ± 2.28 U/L), ASE200 mg/kg (92.58 ± 3.73 U/L), ASE400 mg/kg (75.11± 4.0 U/L) and orlistat10 mg/kg (98.38 ± 2.96 U/L) had shown decreased ALP level compared to obese group.

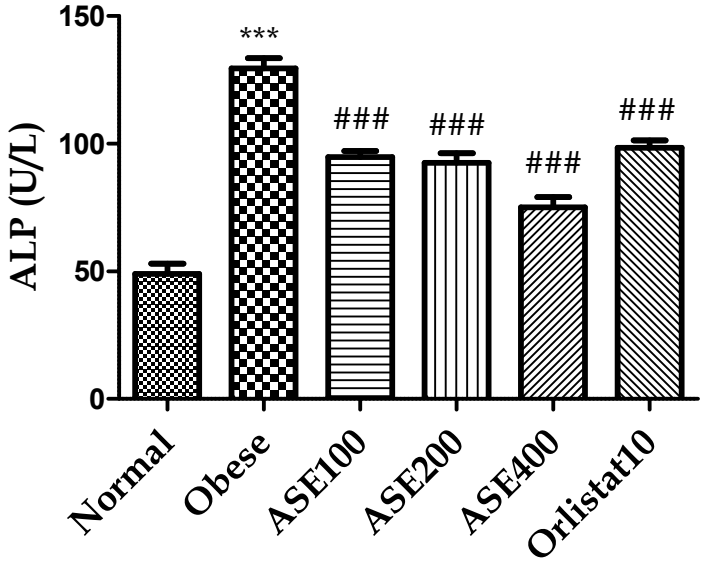


Figure 45: Effect of ASE on ALP (U/L), (n=6 in each group); statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Estimation of fasting glucose (mg/dL)

On overnight fasting, mice blood glucose level was measured. We observed significantly decrease ($p < 0.001$) blood glucose level in treatment groups; ASE100 mg/kg (129.3 ± 1.14 mg/dL), ASE200 mg/kg (113.3 ± 0.88 mg/dL), ASE400 mg/kg (105.2 ± 1.47 mg/dL) and orlistat10 mg/kg (124.0 ± 1.36 mg/dL) was compared with obese group. Whereas fasting blood glucose of obesity control group (138.5 ± 0.61 mg/dL) has significant increased ($p< 0.001$) compared to normal (96.33 ± 0.88 mg/dL).

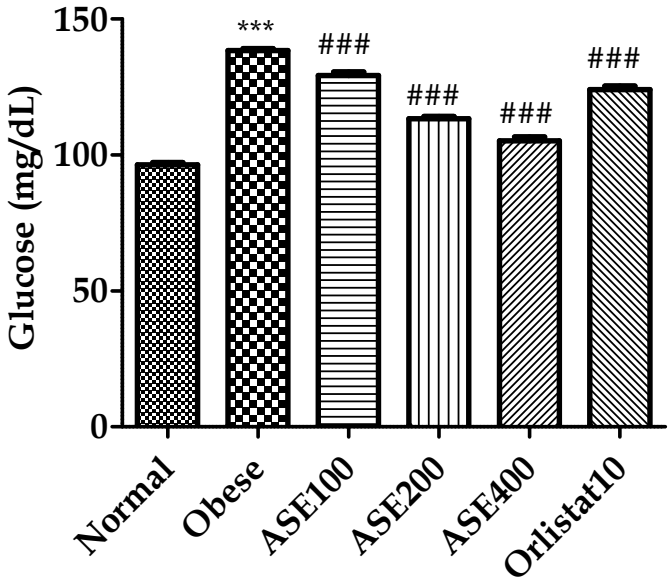


Figure 46: Effect of ASE on blood glucose, (n=6 in each group); statistical significance were expressed in terms of mean ± SEM where, $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

Oxidative stress

Superoxide dismutase (SOD)

The significant increased level of SOD ($p < 0.001$) was found in ASE200 mg/kg (153.7 ± 2.6), ASE400 mg/kg (229.5 ± 4.07) and orlistat10 mg/kg (127.8 ± 10.9) compared to obese. Whereas significant reduction ($p < 0.001$) in SOD was observed in obese group (78.8 ± 3.07) compared to normal (252.0 ±7.95). However, ASE100 mg/kg had shown nonsignificant result compared to obese group represented in (figure 47).

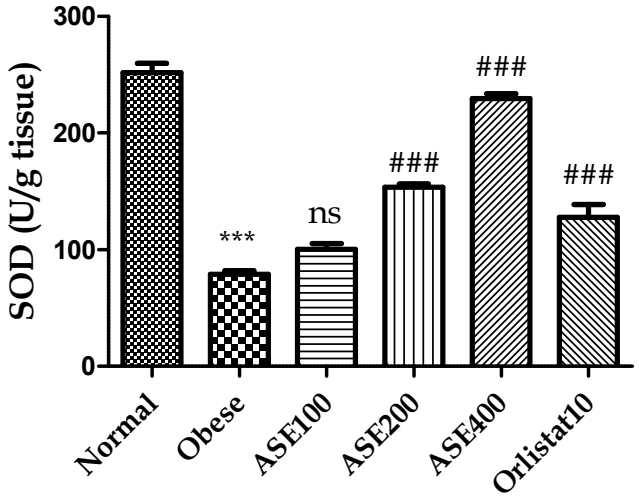


Figure 47: Effect of ASE on Superoxide dismutase (SOD), (n=6 in each group); statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$,$^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control. And ns refers to non-significant.

Catalase activity (CAT)

The significantly increased ($p < 0.001$) CAT level in plasma was found in ASE200 mg/kg (126.5 ± 4.37), ASE400 mg/kg (172.8 ± 8.14) and orlistat10 mg/kg (158.8 ± 4.77) compared to obese (81.9 ± 5.27). Whereas significant reduction ($p< 0.001$) were observed in obesity control group (81.9 ± 5.27) compared to normal (204.9 ± 6.57). ASE100 mg/kg had shown nonsignificant result compared to obese group represented in figure 48.

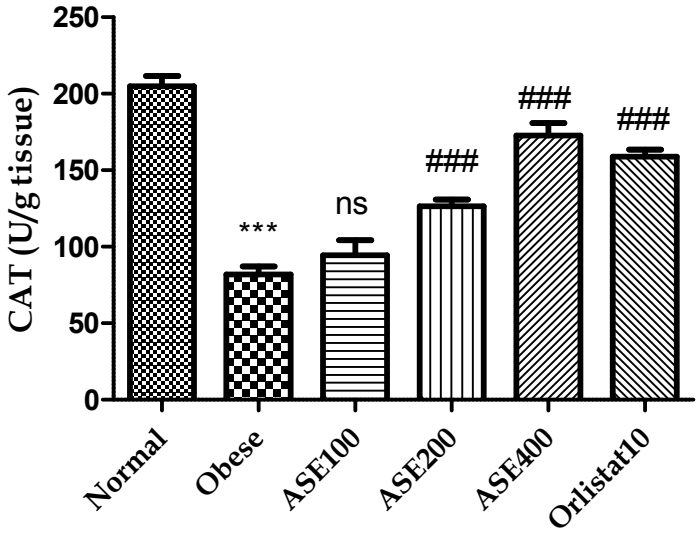


Figure 48: Effect of ASE on Catalase (CAT), (n=6 in each group); statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control. And ns refer to non-significant.

Histopathological analysis of epididymal adipose tissue

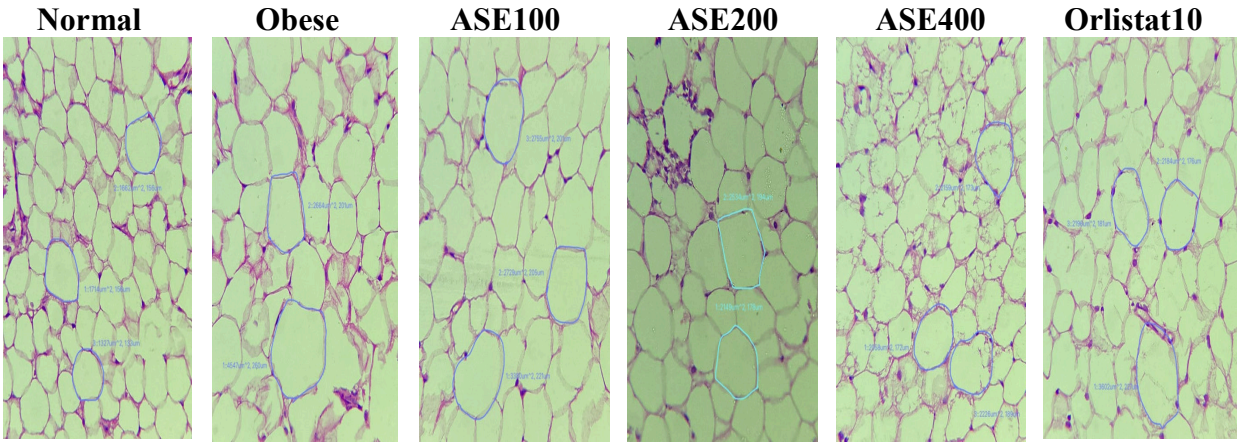


Figure 49: Effect of ASE on adipose tissue observed under 40X magnification**.** Significantly decreased adipocyte size and adipocyte number in extract-treated groups whereas congestion, inflammation and necrosis were observed in disease control group.

Estimation of average number of adipocytes

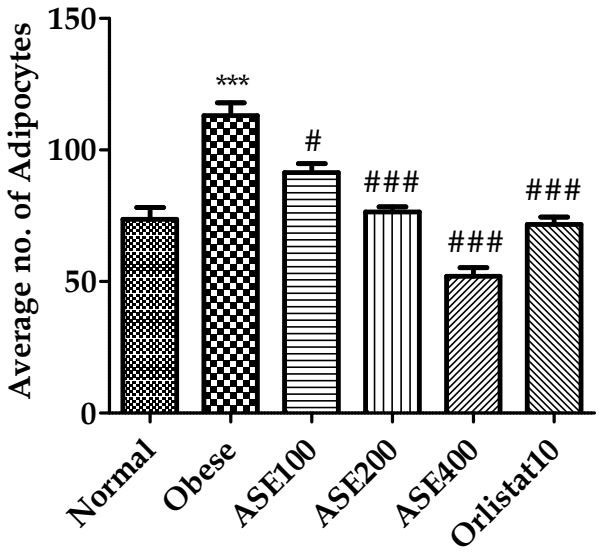


Figure 50: Average number of adipocytes in five circles of 400 µm diameter. Statistical significance were expressed in terms of mean ± SEM where $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obese. The average number of adipocytes was significantly increased in obese group ($p< 0.001$;

113.0 ± 4.93) compared to normal (73.6 ± 4.41). Whereas significant reduction in adipocytes were seen in ASE100 mg/kg ($p< 0.05$, 91.3 ± 3.48), ASE200 mg/kg ($p< 0.001$, 76.3 ± 2.02), AS400 mg/kg ($p< 0.001$, 52.0 ± 3.21) and orlistat10 mg/kg ($p< 0.001$, 71.6 ± 2.72) compared to obese group.

Histopathological analysis of liver tissue

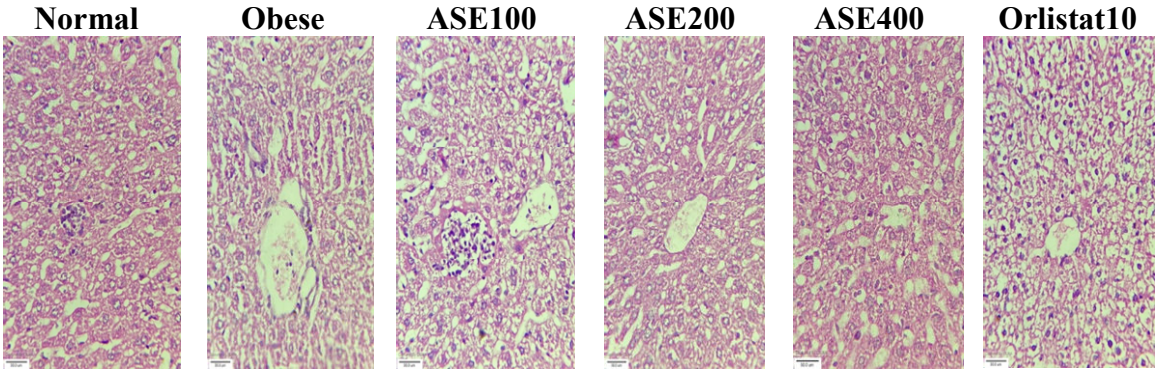


Figure 51: Effect of ASE on liver tissue (40X); Obesity control group had shown venous congestion, inflammation, spotty necrosis was observed. The extract treated groups ASE100, ASE200 and ASE400 had shown improvement in regeneration of liver cells with noticeable less histological score was observed.

Histopathological analysis of kidney

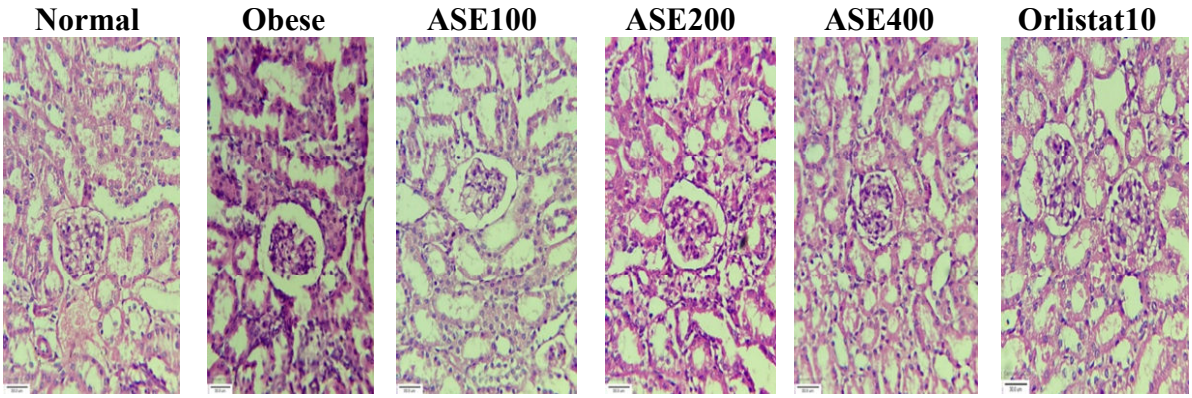


Figure 52: Effect of ASE on kidney tissue (40X). Significant decreased tubular congestion, glomerular congestion, cytoplasmic vacuoles and glomerular basement membrane thinking in ASE100, ASE200 and ASE400 and orlistat10 treated group compared with obese group. Whereas irregular thickening of glomerular membrane, Mesangial matrix expansion was observed in HFD fed obese group.

Table 28: Estimation of lipid profile

Parameters (mg/dL)	Normal	Obese	ASE100	ASE200	ASE400	Orlistat10
HDL	33.63 ± 0.45	76.76 ± 0.37***	70.93 ± 1.64^{ns}	65.98 ± 2.057$^{\#\#}$	46.83 ± 1.795$^{\#\#\#}$	62.83 ± 3.198$^{\#\#\#}$
LDL	11.95 ± 0.52	25.02 ± 0.56***	23.77 ± 0.851^{ns}	21.03 ± 0.695$^{\#\#}$	16.05 ± 0.605$^{\#\#\#}$	21.3 ± 0.876$^{\#\#}$
VLDL	17.76 ± 0.31	36.03 ± 0.38***	30.37 ± 0.39$^{\#\#\#}$	27.85 ± 0.19$^{\#\#\#}$	23.41 ± 0.35$^{\#\#\#}$	29.83 ± 0.36$^{\#\#\#}$
TG	88.79 ± 1.55	180.1 ± 1.92***	151.8 ± 1.96$^{\#\#\#}$	139.3 ± 0.96$^{\#\#\#}$	117.1 ± 1.75$^{\#\#\#}$	149.2 ± 1.80$^{\#\#\#}$
TC	121.0 ± 1.41	227.0 ± 0.73***	193.0 ± 0.96$^{\#\#\#}$	173.5 ± 0.76$^{\#\#\#}$	154.5 ± 0.67$^{\#\#\#}$	185.2 ± 1.72$^{\#\#\#}$

Data represented as the mean ± SEM (n=6); Level of significance were expressed in terms of $^{*}p<0.05$, $^{**}p < 0.01$, $^{***}p< 0.001$ vs. Normal control and $^{\#}p<0.05$, $^{\#\#}p< 0.01$, $^{\#\#\#}p< 0.001$ vs. Obesity control.

In lipid profile estimation, significant reduction in HLD level were observed in ASE400 mg/kg ($p< 0.001$) and ASE200 mg/kg ($p< 0.01$) but ASE100 mg/kg had shown non- significant results also orlistat10 mg/kg had shown significant ($p< 0.001$) decreased level of HDL compared to obese group. The obesity control group had shown significantly increased level of HDL ($p< 0.001$) compared to normal. Similarly, there was significant reduction ($p< 0.001$) in LDL, VLDL, TG and TC in ASE400 mg/kg, compared to obese group. Whereas significant increased ($p< 0.001$) level of LDL, VLDL, TG and TC were observed in obese group compared to normal.

Chapter – 6

Discussion

6. DISCUSSION

The current study, *Acacia suma* heartwood has selected by considering its traditional claims, in the treatment of metabolic diseases, previously reported activities and availability of crude plant material. The study focuses on investigation of the anti-obesity potential of *Acacia suma* in HFD induced experimental animal model initiated through plant material authentication and standardization.

Physicochemical properties like moisture content, ash value estimation, extractive value estimation, pH and fluorescence analysis were evaluated along with phytochemical screening and HPLC and HPTLC characterisation. Evaluation of these parameters helps to ensure the quality of test material and verify any adulteration with the product. The endangered plants are vulnerable to adulteration[98], ash value indicates the quality and purity of the natural product.[99] Moisture content ensure the rate of degradation and extractive value assure possible availability of phytochemicals in the crude drug[100] variations in the above parameters lead to adulterations of species or it might be low-quality products. In the present work all, physicochemical parameters are in the recommended range for the *Acacia suma* in API.

Further qualitative and quantitative screening of plant material was performed, presence of carbohydrates, proteins, amino acids, steroids, flavonoids and phenols were observed. The quantitative estimation phytochemical including total phenol content, total flavonoid content and total tannin content were found as 571.49 ± 27.3 mg (GAE/g), 92.33 ± 15.2 mg (QE/g) and 165.9 ± 8.63 mg (TAE/g) respectively. Phenolic content was found high which may support the anti-obesity potential of the drug. Polyphenols help in modulating pathways and are responsible for energy metabolism. Consumption of fruits and vegetables containing polyphenols provides

several health benefits; beverages like tea, coffee, chocolates and wine are also rich sources of polyphenols described below (figure 53).[101]

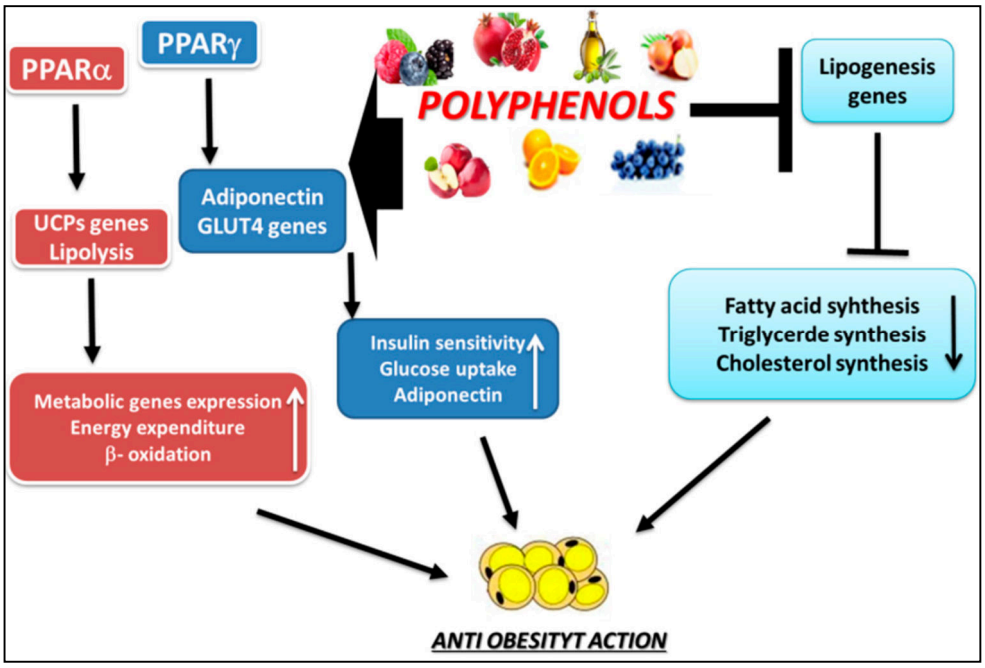


Figure 53: Sources of polyphenols and their anti-obesity action

High-performance liquid chromatography (HPLC) characterisation was performed for *Acacia suma* using simultaneous estimation of epigallocatechin, fisetin and quercetin where retention time 3.51, 6.98 and 12.1 minute was observed for QT, FT and EGC were at 278 nm respectively. The HPLC technique has revealed the presence of QT FT and EGC, in *Acacia suma*; which are previously reported for having anti-obesity potential.[102,103,104] These phytochemicals were predicted by in-silico anti-lipase activity. Further, the applicability of the optimised HPLC method was investigated for HAE of *Acacia suma* and observed the presence of three marker compounds in extract. However, earlier studies on other *Acacia* species i.e. *Acacia catechu* reported the presence of these phytochemicals.[105]

The HPTLC offers advanced separation along with visual detection of compounds on pre-coated TLC plate. HPTLC Characterisation was performed using epigallocatechin (EGC) as a marker compound. The developed chromatogram was

found specific with standardized solvent system THF: Toluene: Acetic Acid: Water [16:8:2:1 (v/v)] at R_F value 0.944. Adopting HPLC and HPTLC standardisation techniques ensures the quality of procured plant material to serve effective pharmacological activity.

Lipid accumulation in the adipose tissue generates reactive oxygen species that trigger the decreased activity of antioxidants, reduced level of antioxidants causes over-expression of adiponectin, cytokines and interleukin 6 which develops inflammation.[106] To relate the anti-obesity potential of extract we have been explore anti-oxidant activity of *Acacia suma* extract testing for various assays; including DPPH radical scavenging assay, hydrogen peroxide assay, nitric oxide assay, total antioxidant assay and lipid peroxidation assay with IC_{50} value 81.46 ± 2.72, 61.39 ± 1.85, 21.30 ± 2.26, 55.13 ± 2.86 and 77.03 ± 2.47 µg/mL respectively.

Antioxidants present in plant extract scavenge DPPH radicals and donates hydrogen atom[107] The formation of free radicals due to the removal of hydrogen atoms causes lipid peroxidation and damage membrane lipids. Peroxidation leads to the breakdown of hydrocarbon, ketones and aldehydes which affect the metabolic pathways, antioxidants prevent these reactions and stop the production of free radicals[108] high antioxidant activity was measured by a low IC_{50} value, in the present study, nitric oxide assay had shown low IC_{50}, implies that HAE of *A. suma* promotes scavenging of peroxynitrite anions.[74] Blocking or maintaining ROS production contributes to the healthy physiology of the body and improves antioxidant potential.[109]

Cytochrome P450 was selected as a gene/protein to find out its inhibitors from targeted phytochemicals using computational tools, where diinsininol has been predicted as a cytochrome P450 inhibitor with a docking score of -13.15 was

compared with gallic acid and ascorbic acid. Cytochrome P450 plays an important role in metabolism, homeostasis and detoxification; changes or mutation in this enzyme leads to the occurrence of various diseases.[110]

The pharmacology anti-obesity activity of *Acacia suma* was assessed by employing molecular docking, in-vitro assays and in-vivo animal model. The in-silico study includes molecular docking and network pharmacology was initiated by targeting lipase inhibitors from the *Acacia suma* phytoconstituents. Three compounds were identified as lipase inhibitors by gene enrichment analysis and KEGG pathway; (5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol; Fisetinidol-(4α,6)-gallocatechin and Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside. These phytochemicals from *Acacia suma* have been predicted to regulate lipase inhibition by insulin resistance pathway, breast cancer pathway, thyroid signalling pathway and longevity regulating pathway. The predicted docking score of compounds was higher compare to orlistat; the range of docking score found as Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside > Fisetinidol-(4α,6)-gallocatechin > (5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol > orlistat i.e. -7.6, -7.2, -5.9, -5.2 respectively.

Lipase or triacylglycerol hydrolase is an enzyme; hydrolyse triglycerides from glycerol and fatty acids. Pancreatic lipase enzyme releases into the stomach and helps for the digestion and abortion of lipids[111] produced by plants[112], microorganisms[113], fungi[114] and animals. The mechanism behind lipase inhibitors that to inhibit digestion and absorption of lipids present in ingested food stuff which further stop the storage of lipid (triglycerides/fat) in adipose tissue as described in the following figure.[115]

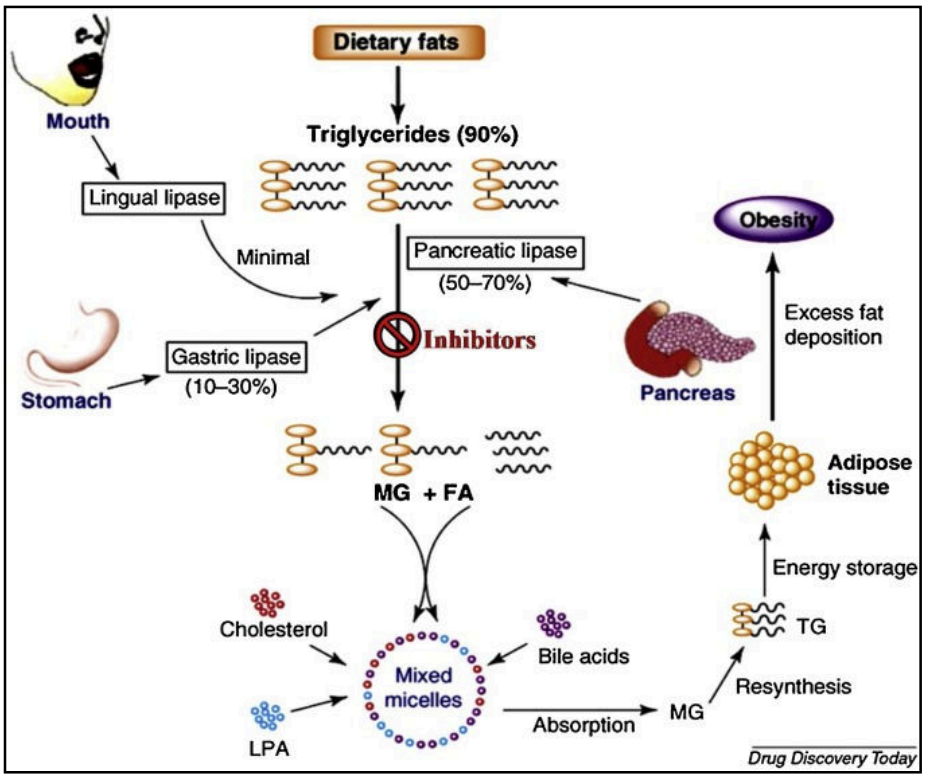


Figure 54: Pathways responsible for the anti-lipase activity

The hydroalcoholic extract has revealed strong lipase inhibition potential with IC_{50} 47.05µg/ml; inhibition of lipase is one of the mechanism to contribute as an anti-obesity agent.[116] Orlistat is a known drug that comes under the anti-obesity class and acts by inhibiting the pancreatic lipase enzyme.[117] Administered orally and prescribed under the brand name Alli (60 mg) and Xenical (120 mg) per day. The plant has also been reported for its glucose lowing property hence α amylase and α glucosidase inhibition potential were assessed with IC_{50} 12.5µg/ml and 93.79µg/ml respectively. The alpha-amylase and alpha-glucosidase inhibitors are agents inhibits the hydrolysis of carbohydrates and reduce the postprandial hyperglycemia.[85,86]

After in vitro enzymatic assays, the study further proceeded with the analysis of lipid accumulation inhibition potential of HAE of *Acacia suma* on 3T3-L1 adipocyte cells by employing adipocyte differentiation and oil-o-red staining assays. Adipogenesis can be evaluated by a 3T3-L1 cells line, inducing differentiating

cocktail solution i.e. insulin, dexamethasone and isobutylmethylxanthine.[118,119] 3T3-L1 adipocyte cell lines have been widely used to study anti-adipogenesis.[120,121] The cells treated with extract had shown a significant reduction in lipid accumulation based on concentration compared to control and induced groups. The plant *Acacia suma* had shown anti-adipogenic potential by suppressing 3T3-L1 cell growth.[89]

The lipase inhibitors predicted by In-silico approach are found to be regulating various pathways i.e. (5S,7R,8R,9R,10S) -(-)-7, 8-seco-7, 8-oxacassa-13, 15-diene-7, 17-diol has expressed insulin resistance pathway by modulating PPARGC1A, CREB3L1 and FOXO1 genes. Whereas Fisetinidol-(4α,6)-gallocatechin and Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside had commonly modulated KRAS, NCOA1and NOTCH1genes through thyroid signalling pathway and longevity regulating pathway respectively.

The peroxisome proliferator-activated receptors (PPAR) are nuclear receptors; key functional regulator gene is the peroxisome proliferator-activated receptor gamma (PPARG). PPARG plays a vital role in the maintenance, function and differentiation of adipocytes; it controls cell proliferation of various tissues and organs such as the colon, breast and bladder. Mutation in the functional activity of PPARG leads to uncontrolled cell growth or cancer of particular tissue or organ. PPARG actively work on inflammation, metabolism and cancer also it is the master key gene of adipogenesis and insulin sensitivity[122,123] Obesity is a measure of an increased number of white adipocytes or increased size of white adipocytes termed as hyperplasia and hypertrophy of white adipose tissue respectively.[124]

Interestingly, cAMP-responsive element-binding (CREB) proteins promote peroxisome proliferator-activated receptors gamma (PPARG) and together initiate 3T3-L1 cell differentiation.[125] Gene FOXO1 belongs to the FOXO family and

significantly participates in adipocyte differentiation and obesity[126] KRAS is mainly present in 3T3-L1 cells, inhibition of KRAS reduces adipocyte differentiation and lipid accumulation.[127] NOTCH1 promotes fat accumulation in high-fat diet-induced animal models whereas over expression of NCOA1enhances PPARG activation resulting in adipocyte proliferation and differentiation.[128,129] All the above genes are predicted by the in-silico lipase inhibition study. The listed gene's function may have been modulated by *Acacia suma* resulting significant reduction in adipocyte differentiation and lipid accumulation.

Considering, the favourable response of in-silico and in-vitro findings on lipase inhibition and reduced adipogenesis and lipid accumulation; the work progressed towards in-vivo experimental study, where we had selected HFD induced C57BL/6 mice to investigate the anti-obesity potential of *Acacia suma*.

Oral acute toxicity study assessment of the hydroalcoholic extract was planned on female C57BL/6 mice for 14 days as per OECD guidelines 423[91]; 2000 mg/kg body weight dose was calculated for mice and administered orally. No toxic symptoms were found, and no mortality or morbidity was found during 14 days duration. It indicates that test extract has no observed adverse effect level and hence drug was found non-toxic at $1/5^{th}$, $1/10^{th}$ and $1/20^{th}$ doses. Different doses are calculated (100, 200 and 400) mg/kg according to body weight of mice for anti-obesity screening. Previously conducted studies on leaves and stem bark of *Acacia suma*, authors had mentioned no sign of toxicity and selected 200 or 400 mg/kg doses for their activities.[10,11]

Increased food intake is directly proportional to sensory stimulation of food consumption; food intake can be promoted by the appearance of food, taste, odour and desire to complete satiety. Obesity can be induced by environmental factors, mental

stress and food intake frequencies.[130] A high-fat diet or cafeteria diet positively induces obesity in C57BL/6 mice resulting in increasing visceral fat, glucose tolerance and insulin resistance. The purpose of adopting C57BL/6 mice is to understand the physiology of obesity in human beings; C57BL/6 mice are a good screening model to mimic metabolic disorders like obesity and diabetes; when animals fed with high-fat diet induces obesity, hyperglycemia, hyperlipidaemia, hyperinsulinemia and hypertension.[131] Hence this model was utilised by many researchers as a screening model for diet-induced obesity and diabetes studies.[95,132,133]

The present research work was investigated by following various parameters; anthropometrical parameters, nutritional parameters, biochemical parameters, oxidative stress and histopathology of liver, kidney and adipose tissue were studied. In anthropometrical parameters; Body weight, abdominal circumference and BMI were measured. Body weight gain is an important factor to assess obesity by comparing the obesity control group induced with HFD containing 60% fat with normal control or treatment groups. Previously, it was reported that BMI of rats in the obese group is 0.53 gm/cm2 and were compared with a normal control group with having BMI of 0.57 gm/cm^2. Further, in another study it was reported that the normal range of BMI 0.45 to 0.68 gm/cm^2 for male and 0.45 to 0.50 gm/cm^2 for female.[134] In our study, the average BMI of the obese group on the 7th week was found 0.631± 0.017gm/cm^2 whereas in the normal group was 0.474 ± 0.010 gm/cm^2. We have observed significantly increased body weight, abdominal circumference and BMI in HFD fed control group was compared with the normal. Also, based on concentration ASE-treated groups demonstrated a decreased body weight significantly.

Genus '*Acacia*' has more than 1200 species, with various secondary plant metabolites which are a rich source of polyphenols.[135] In HPLC characterisation, the

presence of epigallocatechin, fisetin and quercetin was confirmed. Further, epigallocatechin has been reported for dose-dependent manner inhibition of adipogenesis, by enhancing transcription of adiponectin and uncoupling protein 1 (UCP 1)[104], also reported for lipase inhibition potential.[136] Fisetin can exert anti-adipogenesis by inhibiting the PPAR gamma gene in 3T3-L1 cell lines.[137] It was observed that preadipocytes when expose to quercetin results in decreased adipocyte differentiation by activating the AMPK signalling pathway.[102] Further, cyclic AMP is a major intracellular mediator for thermogenesis. *Acacia suma* has been reported for having thermogenic property.[8] In thermogenesis brown fat and beige fat plays any important role.[138] Brown adipocytes are responsible for generating heat by uncoupling protein 1 (UCP1) and act as thermogenic mediator to expand energy and contribute as another therapeutic pathway to deal with metabolic diseases.[139]

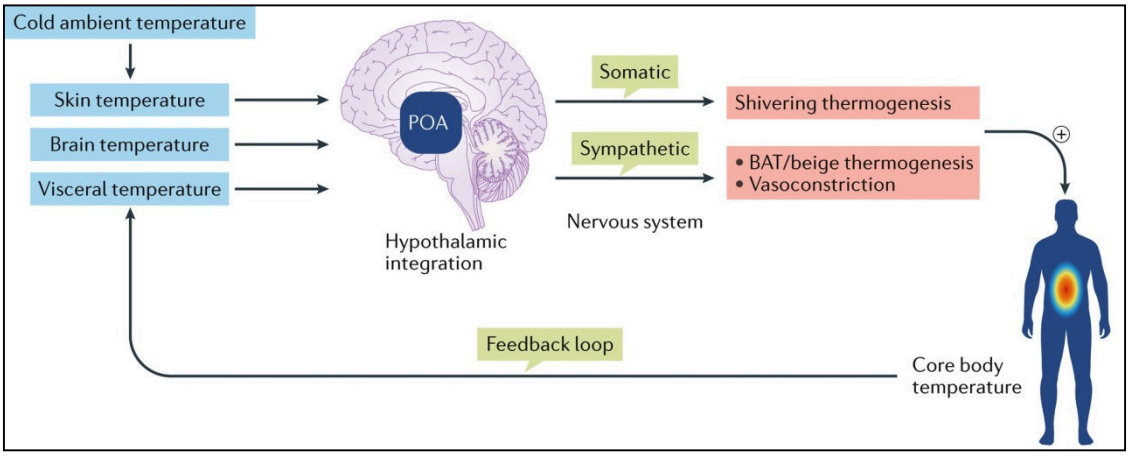


Figure 55: Relationship between thermogenesis and cold response.[139] Sympathetic and somatic nervous system outflow from the POA stimulates shivering as well as non-shivering thermogenesis, thus pre-emptively counteracting cold ambient temperatures.

Increased weight gain is directly proportional to the nutritional intake of the animals.[140] In nutritional parameters; food intake, energy intake, and water intake were evaluated. The study revealed significantly decreased food intake and energy intake in the *Acacia suma* extract-treated group was compared with normal and obesity control groups. Reduced food intake leads to slow down the metabolic rate and suppresses appetite.[141,142]

The body has its inbuilt antioxidant power to deal with reactive oxygen species (ROS) which defence against oxidative stress and protect against cancer, heart disease, obesity, diabetes and other chronic diseases[143] and was studied by sodium dismutase and catalase estimation in liver tissue. Reduces activity of SOD and CAT were observed in control group compared to the normal. The *Acacia suma* may have liver protective effect; had shown enhanced antioxidant activity of sodium dismutase and catalase in liver tissue of treatment groups were compared with the obese group.[144,145]

Over consumption of a high-fat diet causes oxidative stress and leads to inflammation and elevates concentration of liver functions marker enzymes SGOT (AST), SGPT (ALT) and ALP evidenced and lead to liver dysfunction due to fat deposition.[146,147] We observed an increased level of these enzymes in the high-fat diet-fed obesity control group compared with other groups.

The effect of *Acacia suma* extract on lipid profile was analysed by estimating total triglycerides, total cholesterol, high-density lipid, and low-density lipid and very-low-density lipid using serum sample of mice[148] however, study findings suggested that significant reduction in serum TG, TC, HDL, LDL and VLDL content in the *Acacia suma* extract-treated group compared to the HFD fed obese group and normal group.

Histopathology of liver, kidney and adipose tissue was performed. Extract treatment groups had signified reduced liver weight and lipid accumulation in liver cells and the obesity control group was observed with excess lipid content and significantly increased liver weight; indicating that extract may have suppressed genes responsible for lipid synthesis[149] Liver histopathology suggested venous and sinusoidal congestion, inflammation, necrosis and degeneration of hepatic cells

however, the histological scores are found less in extract treated groups compared with obese group mice.[150] In kidney histology suggested tubular and glomerular congestion along with enlargement of the glomerular area in the high-fat diet received obese group, whereas extract-treated groups had shown better morphology with no significant changes were observed, it may be due to the reno-protective effect of the extract was believed.[151] HFD fed mice observed with renal toxicity, change in renal function cause lipotoxicity and promote oxidative stress, necrosis and inflammation.[152]

The morphology of adipocytes was found congested, inflammation and necrosis and large lipid droplets were seen in obese group animals. Whereas, better morphological conditions observed in treatment groups compared to obese group animals. The significantly decreased adipocyte size and adipocyte number in extract-treated groups were observed in adipose tissue histopathology. Lipid synthesis was manifestly increased in the HFD induced obese group animals.

Above-discussed study findings indicated that heartwood hydroalcoholic extract of *Acacia suma* have a significant influence on HFD induced obesity. This plant serves a potential anti-obesity effect were studied.

Hypothetical Mechanism of Action of *Acacia suma* Heartwood

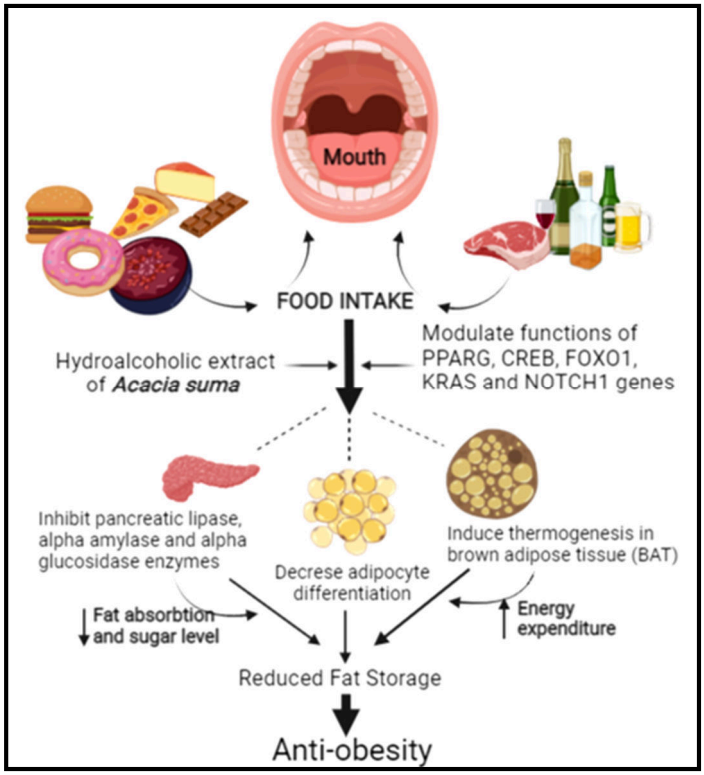


Figure 56: Hypothetical anti-obesity mechanism of *Acacia suma* heartwood

Chapter – 7

Conclusion

7. CONCLUSION

The in-silico results predicted the lead molecules as anti-lipase and anti-oxidant agents. The quantitative estimation of phytochemicals revealed presence of high amount of phenol, flavonoid and tannin content which could support the anti-oxidant and anti-obesity potential of plant extract.

The plant standardisation has confirmed the quality and purity of plant material by HPLC and HPTLC method to confirm the presence of epigallocatechin, fisetin and quercetin in extract. The in-silico approach has explored the possible pathways and gene responsible for anti-obesity effect of extract.

The in-vitro assays evident pancreatic lipase inhibition, alpha amylase inhibition and alpha glucosidase inhibition potential of plant extract which cloud the mechanism of action for revealing anti-obesity potential. Further, HFD induced mice obesity model had shown significant reduction in weight gain, abdominal circumference and body mass index on dose dependent manner.

Hence pre-clinical investigation of heartwood hydroalcoholic extract of *Acacia suma* proved anti-obesity potential on high fat diet fed mice model.

Chapter – 8

Summary

8. SUMMARY

The anti-obesity activity of *Acacia suma* [Roxb.] heartwood was observed and it could be due to one of the mechanisms i.e. lipase inhibition potential. The plant contains rich source of catechins and it could be responsible to reduce free radicals by inhibiting Cytochrome P450. The *Acacia suma* contains phenols, flavonoids and tannins these are the primary phytochemicals and could be these phytochemicals possess anti-obesity activity.

The three major phytochemicals are (**5S,7R,8R,9R,10S)-(-)-7,8-seco-7,8-oxacassa-13,15-diene-7,17-diol; Fisetinidol-(4α,6)-gallocatechin and Quercetin 4'-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside** have been revealed the lipase inhibition potential. Whereas, **Diinsininol** have been predicted for the highest docking score as anti-oxidant agent from *Acacia suma,* along with alpha amylase inhibition potential and alpha glucosidase inhibition potential and all these mechanisms could be combinedly act to prove anti-obesity pharmacological action of *Acacia suma* heartwood.

Chapter – 9

Limitations and Prospective

9. LIMITATIONS AND PROSPECTIVE

The molecular docking and network pharmacology assessment was followed for the first time to identify phytochemicals having anti-lipase potential from *Acacia suma* heartwood. The anti-lipase potential of extract was determined by in-vitro enzymatic assay. However, these phytochemicals cloud be isolated and quantified from the extract.

The *Acacia suma* heartwood has revealed anti-obesity potential and was evaluated by in-vivo animal model but further the effect of extract on various obesity causing biomarkers or genes needs to be evaluated and it is the prospective of current research findings.

Chapter – 10

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Chapter – 11

Annexure

11.ANNEXURE

- **Plant authentication certificate**

SRI VENKATESWARA UNIVERSITY
TIRUPATI-517 502, A.P., INDIA

Dr. K. MADHAVA CHETTY
M.Sc., M.Ed., M.Phil., Ph.D., PG DPD
Plant Taxonomist (IAAT: 357)
Assistant Professor
Department of Botany

Mobile :+91-9490486654
Phone: +91-877-2233685 (R)
E-mail: madhavachetty@gmail.com

Date: 27.12.2019

AUTHENTICATION CERTIFICATE

This is to certify that the following plant species for pharmacognostical / pharmaceutical /pharmacological / phytochemical investigation research work is identified and their botanical name and family name is given.

Botanical Name	Voucher number	Family
Acacia suma (Roxb.) Buch.-Ham. ex Voigt.	0661	Mimosaceae

Authenticated by
K. Madhava Chetty
(K. Madhava Chetty)
DR. K. MADHAVA CHETTY
M.Sc., M.Ed., M.Phil., Ph.D., PG DPD
ASSISTANT PROFESSOR
DEPARTMENT OF BOTANY
SRI VENKATESWARA UNIVERSITY
TIRUPATI-517 502, A.P. India

- **Institution Animal Ethical Committee Approval**

  After receiving ethical clearance from Institutional Animal Ethical Committee (IAEC) of KLE College of pharmacy Belagavi; the animal experiment was carried out of this research project with the registration number,

  Reg.No.221/Po/Re/S/2000/CPCSEA.

KLE College of Pharmacy
A Constituent Unit of
KLE Academy of Higher Education and Research
(Deemed to be University)
DEPARTMENT OF PHARMACOLOGY
JNMC Campus, Nehru Nagar, Belagavi – 590 010, Karnataka, India
BELAGAVI

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
Reg.No.221/Po/Re/S/2000/CPCSEA

Date: 17/03/2021

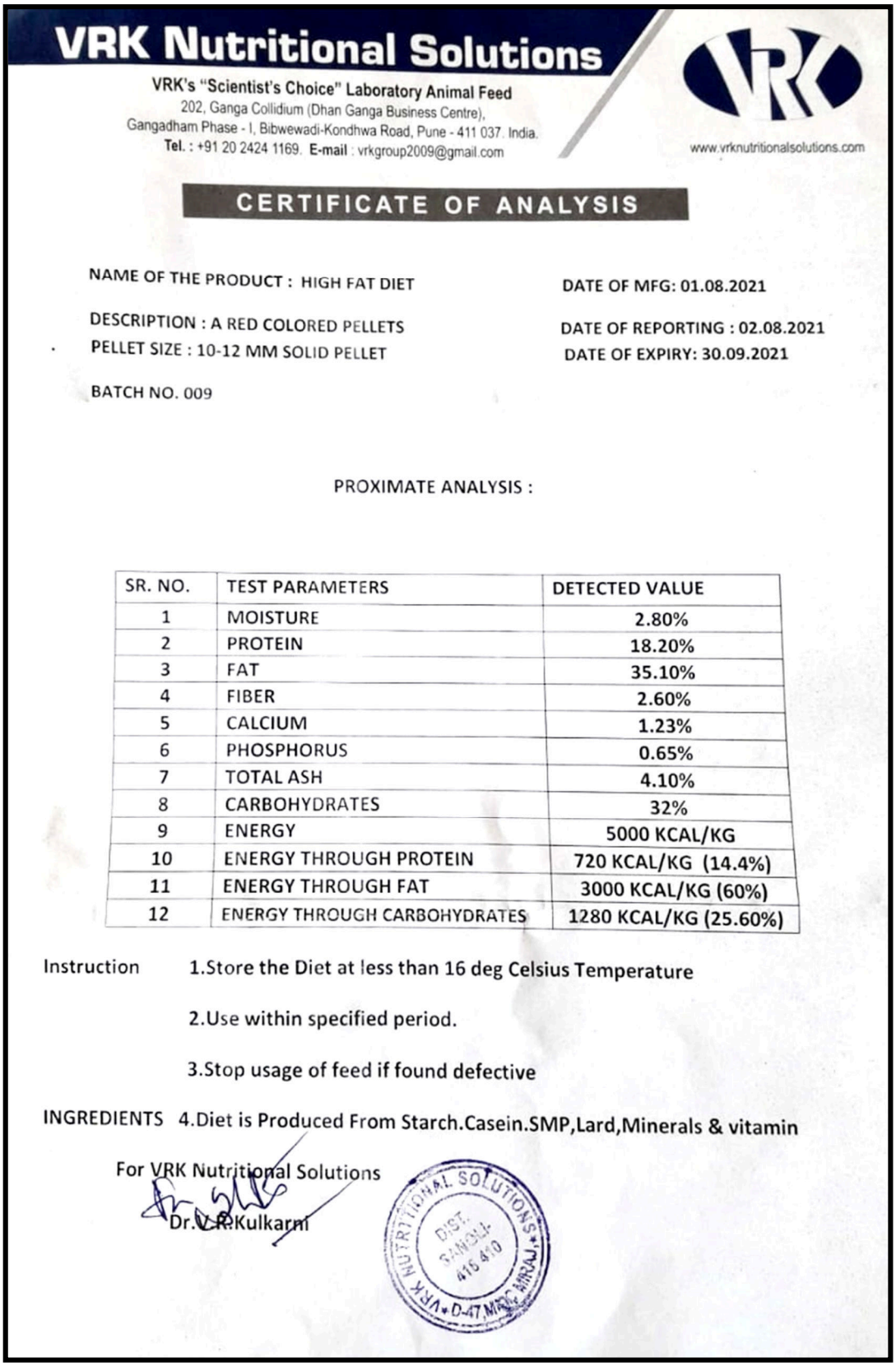
CERTIFICATE

This is to certify that the project proposal no ...16... entitled," Investigation of Anti-obesity potential of heart wood hydroalcoholic extract of *Acacia suma* (Roxb) in high fat diet fed C57BL/6 mice" submitted by Dr./ Mr. / Ms., Nikita N. Kanbarkar under the guidance of Dr. Sanjay Mishra has been approved/recommended by the IAEC of KLE College of Pharmacy, Belagavi, Reg.No.221/Po/Re/S/2000/CPCSEA in its meeting dated 13/03/2021, resolution No. 30 has been sanctioned ....42.... ~~Rats~~/ ~~Mice~~/ ~~Rabbits~~/~~Guinea~~ pig (animals) sex male ---- under this proposal for a duration of next.....months.

You are hereby informed to strictly adhere to the protocol submitted for approval. Further you are required to keep the account of animals used for the project in specified Performa, Form D.

Authorized by	Name	Signature	Date
Member Secretary:	Dr. N.A. Khalid	MEMBER-SECRETARY Institutional Animal Ethical Committee, KLES's College of Pharmacy, BELGAUM - 590010	17/03/2021
Main Nominee of CPCSEA:	Dr. Vinod Kumar	CPCSEA Nominee Institutional Animal Ethics Committee KLES's College of Pharmacy, BELGAUM.	17-3-2021

- **High Fat Diet (HFD) compositions and analysis certificate**



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CERTIFICATE OF ANALYSIS

NAME OF THE PRODUCT : HIGH FAT DIET

DATE OF MFG: 01.08.2021

DESCRIPTION : A RED COLORED PELLETS

DATE OF REPORTING : 02.08.2021

PELLET SIZE : 10-12 MM SOLID PELLET

DATE OF EXPIRY: 30.09.2021

BATCH NO. 009

PROXIMATE ANALYSIS :

SR. NO.	TEST PARAMETERS	DETECTED VALUE
1	MOISTURE	2.80%
2	PROTEIN	18.20%
3	FAT	35.10%
4	FIBER	2.60%
5	CALCIUM	1.23%
6	PHOSPHORUS	0.65%
7	TOTAL ASH	4.10%
8	CARBOHYDRATES	32%
9	ENERGY	5000 KCAL/KG
10	ENERGY THROUGH PROTEIN	720 KCAL/KG (14.4%)
11	ENERGY THROUGH FAT	3000 KCAL/KG (60%)
12	ENERGY THROUGH CARBOHYDRATES	1280 KCAL/KG (25.60%)

Instruction 1.Store the Diet at less than 16 deg Celsius Temperature

2.Use within specified period.

3.Stop usage of feed if found defective

INGREDIENTS 4.Diet is Produced From Starch.Casein.SMP,Lard,Minerals & vitamin

For VRK Nutritional Solutions

Dr.V.R.Kulkarni

VRK NUTRITIONAL SOLUTIONS DIST. SANGLI 416 410 D-47 MIDC MIRAJ

PUBLICATIONS

1. **Kanbarkar N, Mishra S**, **Khanal P.** Beneficial effect of phospholipase A2 group IIA inhibitors from Acacia suma in obesity: an in silico and in vitro study. ***Advances in traditional medicine***. 2020; 20(4):599-608. **(IF:1.86)**
2. **Kanbarkar N, Mishra S, Dodmani S & Kurangi B.** Simultaneous estimation of epigallocatechin, fisetin, and quercetin in Acacia suma and its potential against postprandial hyperglycemia. ***International Journal of Ayurvedic Medicine,*** 2022,13(1), 41–50.
3. **Kanbarkar N, Mishra S, Nandanwadkar S, Alegaon S.** Assessment of anti-oxidant activity and quantification of epigallocatechin in Acacia suma heartwood by HPTLC-DPPH fingerprinting method. ***Chemical Papers***. 2022 8:1-4. **(IF: 2.09)**

(Front page of each article has been attached)

PRESENTATIONS

1. **Nikita Kanbarkar, Sanjaykumar Mishra.** "**Theme: Phytomedicine for human health: Scope, Challenges and emerging trends"** Organized by 24th Annual convention and National conference of Society of Pharmacognosy at VJ's College of Pharmacy, Rajamahendravaram, Andhra Pradesh. Received **Best poster presentation** award [21st and 22nd Feb. 2020].

2. **Nikita Kanbarkar, Sanjaykumar Mishra.** E-conference poster presentation, themed as "**Alternative to animal testing's: Replacement, Reduction, Refinement, Rehabilitation, Reuse and Recreation strategies to address current scenario"** Organized by Laureate institute of Pharmacy, Kathog, Kangra [H.P.] Received **1st Prize** [July 2020].

3. **Nikita Kanbarkar, Sanjaykumar Mishra. Theme: Ethnopharmacology and medicinal plants-Approach towards product development** Organized by 8th International Congress of Society for Ethnopharmacology India. Titled as **"Beneficial effect of Phospholipase A2 group IIA inhibitors from *Acacia suma* in obesity: an in silico and in vitro study"** [27th to 29th August 2021].

(Certificates are attached)

Erratum

Advances in Traditional Medicine
https://doi.org/10.1007/s13596-020-00456-4

RESEARCH ARTICLE

Beneficial effect of phospholipase A2 group IIA inhibitors from *Acacia suma* in obesity: an in silico and in vitro study

Nikita Kanbarkar[1] · Sanjay Mishra[1,2] · Pukar Khanal[2]

Received: 6 March 2020 / Accepted: 4 May 2020

Abstract

Acacia suma Roxb. (Fabaceae) is Ayurvedic medicine distributed in Karnataka, Bengal and Bihar region. Phytoconstituents of *A. suma* were retrieved from ChEIB databases and queried for phospholipase A2 group IIA inhibitors. The present study is an effort to find out a novel therapeutic solution for the management of obesity disorders. Out of 29 reported compounds three were identified in modulating phospholipase A2 group IIA inhibitor their drug likeness score andprobable gene expression was identified. Docking study was performed using autodock4.0 to predict binding affinity of phytoconstituents with phospholipase A2 group IIA inhibitor and compared with clinically proven drug 'Orlistat' as lipase inhibitor. The respected pathway to show networking between phytochemicals and target were analyse by kyoto encyclopedia of genes and genomes pathway analysis for regulated genes. Further, in silico findings were validated for hydroalcoholic extract of *A. suma* by in vitro lipase inhibition assay. Molecular docking result revealed the presence of three flavonoid compounds for lipase inhibition activity namely: (1) (5S,7R,8R,9R,10S)-(−)-7,8–seco-7,8–oxacassa-13,15-diene-7,17-diol (2) Fisetinidol-(4α,6)-gallocatechin and (3) Quercetin4′-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside. However, Quercetin4′-*O*-α-L-rhamnopyranosyl-3-*O*-β-D-allopyranoside was predicted to possess the highest docking score i.e. − 7.6 kcal/mol with phospholipase A2 group IIA. The in vitro findings revealed significant anti-lipase activity with IC_{50} value − 46.07 µg/ml. Hence, the in silico and in vitro approaches has presented strong binding affinity and significant lipase inhibition activity respectively which supports anti-obesity potential of heart wood hydroalcoholic extract of *A. suma*.

Keywords *Acacia suma* · Catechin · Lipase inhibition · In silico study · Obesity

Abbreviations

ADMET	Absorption, distribution, metabolism, excretion and toxicity
API	Ayurvedic pharmacopeia of India
BMI	Body mass index
ChEBI	Chemical entities of biological interest
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and drug administration
KEGG	Kyoto encyclopedia of genes and genomes
PCIDB	Phytochemical interactions DB
PDB	Protein Data Bank
PNPB	*P*-nitrophenyl butyrate
RCSB	Research collaboratory for structural bioinformatics
SMILES	Simplified molecular-Input line-entry system
STRING	Search tool for the retrieval of interacting genes/proteins
WHO	World Health Organization

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Introduction

Obesity is the outcome of an imbalance between energy intake and energy expenditure. BMI [body mass index] ≥ 30 kg/m^2 is measured as obesity (Pradeepa et al. 2015). It is the key player to contribute for metabolic syndrome associated with complications such as diabetic mellitus, non-alcoholic fatty liver, arteriosclerosis and cancer (Fruh 2017). According to World Health Organisation, more

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Simultaneous estimation of epigallocatechin, fisetin, and quercetin in *Acacia suma* and its potential against postprandial hyperglycemia

Research Article

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Abstract

Anti-diabetic activity of the plants may follow one or the other mechanism such as their ability to restore the pancreatic tissue function or increase in insulin output or inhibiting the intestinal absorption of glucose or inhibiting the pancreatic enzymes like alpha-amylase, alpha-glucosidase, etc. The alpha-amylase and alpha-glucosidase inhibition assays were performed to analyze the anti-diabetic potential of *Acacia suma.* RP-HPLC method was developed and validated for simultaneous estimation of Epigallocatechin (EGC), Fisetin (FT), and Quercetin (QT) in hydroalcoholic extract (HAE) of *Acacia suma* and formulations. The simultaneous estimation was performed using the Luna C18 column with 20µl sample injection volume. Whereas, acetonitrile and phosphate buffer (0.1%, 30:70 v/v, pH 2.7) was used for resolution as mobile phase with column temperature 40 ^{o}C and flow rate 1 ml/min. The IC_{50} for alpha-amylase inhibition and alpha-glucosidase inhibition activity was found to be 12.5µg/ml and 93.79µg/ml respectively was compared with standards. The developed method was found simple, specific, precise and linear with regression coefficient (R^2 = 0.999) over the selected range of concentration (2.5-160 µg/mL) having detection limits 0.076, 0.012, 0.010 µg/mL and quantification limits 0.231, 0.038, 0.031 µg/mL for EGC, FT and QT respectively. In-vitro alpha-amylase and alpha-glucosidase inhibition assay support the traditional claim of the plant in the treatment of metabolic disorders. The established RP-HPLC method demonstrates the precise and easy determination of EGC, FT, and QT and is effectively studied by a gradient elution system.

Key Words: *Acacia suma,* Anti-diabetic, Epigallocatechin, Fisetin, RP-HPLC, Quercetin.

Introduction

Diabetic Mellitus is rapidly increasing worldwide. The deficiency of insulin leads to people suffering from diabetes and causes an elevated level of glucose in the blood. Anti-diabetic activity of the plants may follow one or the other mechanism such as their ability to restore the pancreatic tissue function or increase in insulin output or inhibiting the intestinal absorption of glucose or inhibiting the pancreatic enzymes like alpha-amylase, alpha-glucosidase, etc. (1).

The polyphenolic compounds and their estimation can be helpful in the exploration of the pharmacological effects of medicinal plants. Phenols are the major group of nutritional compounds of plants and are classified as secondary metabolites such as tannins and flavonoids. Compounds like catechins, resveratrol, quercetin, procyanidins, and anthocyanins are taken into consideration by researchers for investigating their potentials against inflammation, metabolic disorder, viruses, cancer, and other ailments (2, 3). The characterization of medicinal plants and derived medicines utilizing marker compounds or biochemical profiling of bioactive has become essential as some of which displays significant pharmacological actions. This phenomenon has directed to a faster and improved evaluation of the quality of plant products (4). One of the tools playing an essential part towards characterizing the plant material quality used in medicine is the simultaneous determination of marker compounds using HPLC.

Accurate identification and analyte quantification are particularly linked to the separation of the analyte. Because of this phenomenon, the RP-HPLC method is previously recognized to separate and measure the active constituents from complex matrices such as food products, beverages, plant extracts, and its formulations (5, 6). To ensure the presence of polyphenols in *Acacia suma* and Ayurvedic formulations, we aim to develop the RP-HPLC method for simultaneous determination of Epigallocatechin (EGC), Fisetin (FT), and Quercetin (QT).

Genus *Acacia* is known for the most prominent source of polyphenols, belongs to the family Leguminosae (Fabaceae) and approximately 800 species are documented globally (7). The heartwood of *Acacia suma* (Roxb) Buch.-Ham., commonly known as

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ORIGINAL PAPER

Assessment of anti-oxidant activity and quantification of epigallocatechin in *Acacia suma* heartwood by HPTLC-DPPH fingerprinting method

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Abstract

Research on natural anti-oxidant agents increased due to their wide range of applications on various diseases. HPTLC-DPPH is the novel technique where anti-oxidant potential of complex herbal extract can be detected on TLC plate. In thin-layer chromatography, silica gel plates (60 F_{-254}) were used as a stationary phase along with THF/Toluene/Acetic Acid/Water [16:8:2:1 (v/v)] as a mobile phase. Cytochrome P-450 inhibitors were predicted by in-silico method as anti-oxidant agent. HPTLC method development and validation were performed on CAMAG HPTLC system along with quantitative estimation of epigallocatechin in hydroalcoholic extract of Acacia suma. HPTLC method had ensured no change in R_F value (0.944) at wavelength 269 nm and revealed presence of epigallocatechin in *Acacia suma* is 404.2 mg/g. Yellow coloured bands on TLC plate were observed with DPPH reagent to confirm radical scavenging property. The in vitro DPPH radical scavenging assay, hydrogen peroxide assay, nitric oxide assay, total anti-oxidant assay and lipid peroxidation assay were performed, and IC_{50} values are 81.46 ± 2.72, 61.39 ± 1.85, 21.30 ± 2.26, 55.13 ± 2.86 and 77.03 ± 2.47 µg/ml, respectively. Additionally, in-silico data predicted 'Diinsininol' as cytochrome P450 inhibitor with docking score -13.51 kcal/mol. The study findings evident that hydroalcoholic extract of *Acacia suma* had significant anti-oxidant potential which was confirmed by in vitro assays, molecular docking study and HPTLC-DPPH anti-oxidantapproaches.

Keywords *Acacia suma* · Anti-oxidants · Cytochrome P450 · Epigallocatechin · HPTLC · Polyphenols

Introduction

Oxidative stress damages DNA, protein, lipid due to formation of free radical, and to fight against reactive oxidative species (ROS), body has scavengers such as glutathione, superoxide dismutase and catalase (Halliwell et al. 19). Phenolic compounds are secondary metabolites of plants and act as anti-oxidants by following various pathways; free radical scavenging, oxygen radical absorbance, and chelation of metal ions (Upadhyay et al. 39). The electron reduction potential of phenolic radicals is lower than electron reduction potential of oxygen radicals and hence phenolic compounds are excellent oxygen radical scavengers (Grace et al. 16).

The plant *Acacia suma* has distributed in Karnataka, Bihar, Bengal and Tamil Nadu states of India, commonly known as '*White catechu* or *Sweta khadirah*', and belongs to family Fabaceae. Traditionally, it is used to treat metabolic syndromes, skin diseases, ulcer and epilepsy and has been documented in API (Ayurvedic Pharmacopiea

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