Study of immunomodulatory potential of *Zanthoxylum oxyphyllum* Edgew. and *Z. acanthopodium* DC. from Sikkim Himalaya

A Thesis Submitted to

SIKKIM UNIVERSITY

In partial fulfilment of the requirement for the

Degree of Doctor of Philosophy

By

Sumiran Dewan

Department of Botany

Under the supervision of

Prof. Dhani Raj Chhetri, Department of Botany (Supervisor)

Dr. Bisu Singh, Department of Zoology (Co-supervisor)

School of Life Sciences

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Dedicated to

God, my master. I am but a simple actor in your grand play.

My parents. All that I am, all that I ever will be, has been for you, will be for you.

My brother. Some things are impossible, but those that are not, we shall achieve together.

My family, for blood is thicker than water.

My only best friend. “I am glad you are here with me. Here at the end of all things, Sam.” – J.R.R. Tolkien

DECLARATION

I declare that the Ph.D. thesis titled **"Study of immunomodulatory potential of *Zanthoxylum oxyphyllum* Edgew. and *Z. acanthopodium* DC. from Sikkim Himalaya"** submitted to **Sikkim University** in partial fulfilment for the requirement of the degree of Doctor of Philosophy in Botany is a bonafide research work carried out by me under the **supervision** of **Prof. Dhani Raj Chettri,** Department of Botany, and **co-supervision** of **Dr. Bisu Singh**, Department of Zoology, School of Life sciences, Sikkim University.

The thesis does not bear any content that has been submitted for a degree or diploma at any other University or Institution. Further the references used to supplement the research and the materials obtained have been duly acknowledged.

Sumiran Dewan
Registration No.: 20/Ph.D/BOT/04
Department of Botany
School of Life Sciences
Sikkim University, Gangtok

Place: Gangtok
Date:

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LIST OF ABBREVIATIONS

AA – Arachidonic acid

AAS Atomic absorption spectrophotometer

Ad – Adi

ALP - Alkaline phosphatase

ALT - Alanine aminotransferase

ANOVA – Analysis of variance

APC – Antigen presenting cells

APCs - Antigen presenting cells

ARDS – Acute-respiratory distress syndrome

As – Assamese

AST - Aspartate aminotransferase

BALF - Bronchoalveolar lavage fluid

BCR – B cell receptor

Bh – Bhutia

BHA - Butylated hydroxyanisole

BHT - Butylated hydroxytoluene

BLB - Bering Land Bridge

BSA – Bovine serum albumin

CAT – Catalase

CCL18 – CC chemokine ligand 18

CCL21 - C-C motif chemokine ligand 21**CCl4 -** Carbon tetrachloride

CCL5 - C-C motif chemokine ligand-5

CCR7 - C-C chemokine receptor 7

CD – Cluster of differentiation

CO_2 – Carbon dioxide

COPD - Chronic obstructive pulmonary disease

COVID-19 – Coronavirus disease 19

COX – Cyclooxygenase

CREB - Cyclic AMP-Responsive Element-Binding Protein

CRP - C-reactive protein

CXCL10 - C-X-C motif chemokine ligand 10)

CXCL8 – C-X-C motif chemokine ligand 8

DAMPs – Damage associated molecular patterns

DE – Diosgenin equivalent

DHA - docosahexaenoic acid

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

DPPH – 2,2-diphenyl-1-picrylhydrazyl

DW – Dry weight

Dz – Dzongkha

ECGC - Epigallocatechin gallate

ELISA – Enzyme linked immunosorbent assay

eNOS – Endothelial nitric oxide synthase

EPA - eicosapentaenoic

ERK - Extracellular signal-regulated kinase

FA – Fatty acid

Fc – Fragment Crystallizable region

FRAP – Ferric reducing antioxidant power

Ga – Garo

GAE – Gallic acid equivalent

GALT – Gut associated lymphoid tissue

GC-MS – Gas chromatography mass spectrometry

GE – Glucose equivalent

GG – Galactosaminoglycan

GM-CSF – Granulocyte macrophage colony stimulating factor

GM-CSF - Granulocyte-macrophage colony-stimulating factor

GRO/KC - Growth-regulated oncogene/keratinocyte chemoattractant

GSH – Glutathione

H_2SO_4 – Sulphuric acid

HA – Histamine antagonist

HCl – Hydrochloric acid

HMC-1 - Human mast cell line cells

HRBC – Human red blood cell

HRP – Horseradish peroxidase

HSCs- Hematopoietic stem cells

HTR-8/SVneo – Human trophoblast cell line Simian virus 40 large T antigen

HUVECs - Human umbilical vein endothelial cells

IBD - Inflammatory bowel disease

IC_{50} – 50 % Inhibitory concentration

ICAM-1 - Intercellular cell adhesion molecule 1

ICR - Institute of Cancer Research

IFN – Interferon

Ig – Immunoglobulin

IKK - IκB kinase

IL – Interleukin

iNOS – Inducible nitric oxide synthase

IκB - Inhibitor of nuclear factor kappa B

JAK - Janus kinase

Kh – Khasi

LOX – Lipoxygenase

Lp – Lepcha

LPS – Lipopolysaccharide

LT – Leukotrienes

LTB4 - Leukotriene B4

LX - Lipoxins

MAE – Microwave assisted extraction

MALT – Mucosa associated lymphoid tissue

MAPK - Mitogen-activated protein kinase

MCP-1 - Monocyte chemoattractant protein-1

MHC – Major histocompatibility complex

MIC – Minimum inhibitory concentration

Mo – Monpa

mRNA – Messenger ribonucleic acid

MSC – Mesenchymal stem cell

mTORC1 - Mammalian target of rapamycin complex 1

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MUFA – Monounsaturated fatty acid

MyD88 - Myeloid differentiation primary response gene 88

Na_2CO_3 – Sodium carbonate

NADPH – Nicotinamide adenine dinucleotide phosphate hydrogen

NALB - North Atlantic Land Bridge

$NaNO_2$ – Sodium nitrite

NaOH – Sodium hydroxide

NASH - Non-alcoholic steatohepatitis

NFAT - Nuclear factor of activated T cells

Nf-κB – Nuclear factor kappa B

NIST – National institute of standards and technology

NLR - Nucleotide-binding oligomerization domain like receptors

NLRP3 - Nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3

nNOS – Neuronal nitric oxide synthase

NO – Nitric oxide

NOD - Nucleotide-binding oligomerization domain

Np – Nepali

NSAID – Non steroidal anti-inflammatory drug

NSB - Non-specific binding

PAI-1 - Plasminogen activator inhibitor-1

PAMP - Pathogen associated molecular pattern

PBS – Phosphate buffered saline

PG - Propyl gallate

PG – Prostaglandins

PGE2 - Prostaglandin E2

PKM2 - Pyruvate kinase M2

PMA - Phorbol 12-myristate 13-acetate

POWO – Plants of the world online

PPP3 - Protein phosphatase 3

PRR - Pattern recognition receptors

PUFA – Polyunsaturated fatty acid

Px – Peroxidase

QE – Quercetin equivalent

RBC – Red blood cell

RNA – Ribonucleic acid

RNS – Reactive nitrogen species

RORγt - Receptor-related orphan nuclear receptor gamma

ROS – Reactive oxygen species

RPMI-1640 – Roswell-park memorial institute

SARS-CoV-2 - Severe acute respiratory syndrome coronavirus 2

SBLN - Serum bilirubin

SCFAs - short-chain fatty acids

SEM – Standard error of mean

SGOT – Serum glutamic-oxaloacetic transaminase

SGPT - Serum glutamic pyruvic transaminase

Sh – Sherpa

SOD – Superoxide dismurtase

SPF - Sunscreen protection factor

STAT - Signal transducer and activator of transcription

TAC – Total antioxidant capacity

TACE - TNF-α Converting Enzyme

TAE – Tannic acid equivalent

TBHQ - Tert-butylhydroquinone

TCR – T cell receptor

TD – Thymus dependent

TGF – Transforming growth factor

T_H - T helper cells

TI – Thymus independent

TLR - Toll like receptors

TMB - 3,3',5,5'-tetramethylbenzidine

TNF – Tumour necrosis factor

TX – Thromoxanes

TXB2 - Thromboxane B2

TYK2 - tyrosine kinase 2

UAE – Ultrasonic assisted extraction

UV – Ultraviolet

VCAM-1 - Vascular cell adhesion protein 1

VEGF - Vascular endothelial growth factor

WHO – World health organization

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Chapter 1

Introduction

The utilization of plants as a source of medicine has been ongoing since thousands of years. Through trial-and-error, ways of preparing traditional *Materia medica* have been passed down to our generation from ancient civilizations. The World Health Organization (WHO) states that 80 % of the world population, particularly in developing nations, are dependent on medicinal plants for their basic healthcare. Although traditional medicine was and is still often brushed off as superstition by many people, with advancement in science, it has been possible to conduct a fair, evidence-based investigation into the properties of plants and how they interact with human bodies, as well as the isolation of active principles from plants. Consequently, this has led to gradual acceptance of traditional medicine system leading to advancement in the discovery of plant derived drugs. The best example of plant derived drug in today's time is quinine, the anti-malarial drug, isolated from the bark of *Cinchona* species (Gurib-Fakim, 2006). Many plant-based components provide safer and more environmentally friendly substitutes for synthetic chemicals (Barbinta-Patrascu et al., 2024).

The Himalayan region is a treasure trove of medicinal plants. Many endemic and unique plants can be found in the Himalayan region owing to a great diversity in the environmental and edaphic factors as well as varied altitudinal range across the region. The medicinal plants found in these regions are utilized by much of the local population in traditional healthcare system. In fact, 65% of the Indian population rely on traditional medicinal practices for their primary healthcare. Out of 8000 species of angiosperms found in the Himalayan region, 1748 species have been reported to be in use as source of traditional medicines. The worldwide population has been gaining much interest in herbal remedies and natural sources of drugs, and the recent years has seen a surge in the investigation of plants for their pharmacological properties, such as anti-cancer, anti-diabetes, immunomodulatory, anti-microbial, and anti-inflammatory to name a few (Malik et al., 2015). Naturally, demand for medicinal plants across the globe has been going through the roof because not only do they ensure absence of side effects but also

because candidate species that have been tried and tested through traditional medicine for centuries, can be possibly safer.

The Indian Himalaya encompasses the states of Uttarakhand, Himachal Pradesh, Arunachal Pradesh, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, Tripura, Assam and West Bengal and Union territories of Jammu and Kashmir and Ladakh (Anonymous, 2015) of which, Sikkim is located between 27º 5'- 28º 10' N latitude and 88º 4' - 88º 58' E longitude, sharing its borders with Nepal in the West, Bhutan in the East, China in the North and West Bengal in the South (Lepcha et al., 2019). The state is a part of Himalayan biodiversity hotspot and maintains about 26 % of the country's total biodiversity (Das et al., 2012). Lepcha, Bhutia and Nepalese are three of the major tribal inhabitants in the state. All three tribes practice some form of traditional medicine. *Astilbe rivularis*, *Artemisia indica*, *Bergenia ciliata*, *Clematis buchananiana*, *Cynodon dactylon*, *Drymaria cordata*, *Eupatorium cannabinum, Litsea cubeba, Oroxylum indicum, Phytolacca acinose, Polygonum molle, Rhus chinensis, Rubus elliptics, Tupistra nutans, Zanthoxylum armatum* are some of the local plants frequently used by healers as decoctions or concoctions to alleviate diseases (Tamang et al., 2023).

The genus *Zanthoxylum* Linn. belongs to the family Rutaceae. They are deciduous shrubs with prickles as a distinct character (Brizicky 1962). Different species of the genus is found in the continents of Asia, America, and Africa. *Zanthoxylum* finds plethora of use in traditional medicinal practices particularly for treating rheumatism, toothache, malaria, sickle cell anaemia, microbial infections, inflammation, abdominal pains, wounds, diarrhoea and tumours (Okagu et al. 2021a). Of the 234 accepted species of *Zanthoxylum* distributed worldwide (POWO, 2024), Sikkim harbours seven of the species viz. *Z. acanthopodium* DC, *Z. armatum* DC, *Z. myriacanthum* Hook. f., *Z. ovalifolium* Wight, *Z. rhetsa* (Roxb.) DC (Dash and Singh, 2011), *Z. oxyphyllum* Edgew. (Grierson and Long, 1983) and *Z. tomentellum* Hook. f. (Hajra et al., 1997).

Although there is dearth of written reports on the traditional use of *Z. oxyphyllum* in Sikkim Himalaya, reports on the use of this species by other tribes particularly in Ilam (Nepal), Arunachal Pradesh and Assam are available. Flowers and fruits find their use as analgesic, anti-inflammatory (Chetry et al., 2018) anti-pyretic, in the treatment of snake bites (Uprety et al., 2016) and as anti-helminthic, blood purifier and appetizer (Medhi et al., 2014). *Z. acanthopodium* DC. is often found in the local markets, sold as spice. Fruits,

leaves and barks of this species find their use as hepato-protective agent (Chhetri et al., 2008) pain relievers, for treating stomach and dental problems (Purohit et al., 2014) anti-pyretic, for treating cholera and as anti-rheumatic (Chhetri et al., 2020).

Immunity in the body is ensured by a complex system of cells, organs and proteins that collectively form the immune system. Fundamentally, there are two types of immunity in our body namely- innate immunity and adaptive immunity. Innate immunity is readily available and provides a general, prompt defence against any infection, although lacking in specificity. Neutrophils, eosinophils, mast cells and basophils and natural killer cells are involved in innate immunity. Adaptive immunity also called acquired immunity, is activated after exposure to a pathogen (antigen) and provides antigen specific defence. T-lymphocytes and B-lymphocytes are the major cells involved in adaptive immunity. Both types of immunity work together intricately through cellular and humoral pathways to provide immunity as well as fight off infections (Parkin and Cohen 2001a). Modulation of the immune system either in a suppressive manner or augmentative manner is called immunomodulation and any product (natural or synthetic) that can bring about such manipulation in the immune system is called an immunomodulator. Thus immunomodulators may be immunosuppressants or immunostimulants (Saroj et al., 2012).

Besides other pharmacological activities like anti-cancer, anti-diabetes, anti-microbial, etc. phytochemicals have been reported to exert immunomodulatory activity in the recent years. Phenolics, flavonoids, alkaloids and terpenoids are some of the known immunomodulators. These phytochemicals generally modulate one or more component of the immune system which further brings about changes in the downstream pathways, however, each phytochemical works in its own capacity and there is no singular pathway to explain their mechanism. Resveratrol (phenolic compound) has been reported to modulate the immune system by inhibiting the generation of the enzyme inducible Nitric oxide synthase (iNOS) *in vitro*. Alkaloids like cocaine and diterpenoids like andrographolide, on the other hand have been shown to modulate release of cytokines (Zebeaman et al., 2023). Besides phytochemicals, micronutrients like vitamins and minerals also play a role in immunomodulation. Vitamin A, B_1, B_2, B_3, B_5, B_6, B_7, $_9$, B_{12}, C, D, E and K have all been shown to modulate the expression and release of cytokines, colony stimulating factors, enhance phagocytosis, and increase or decrease ROS (reactive

oxygen species). Minerals like zinc, selenium, iron, magnesium, copper and iodine also cause modulation of the immune system via regulation of cytokine release, anti-body release or expression of cluster of differentiation proteins (CD) (Mitra et al., 2022).

In recent years, researchers have studied the release of cytokines and reactive oxygen species from cells of the immune system to speculate immunomodulatory potential of plants (Amina et al., 2020). Cells of the immune system all originate in the bone marrow from hematopoietic stem cells. These cells are found in the circulatory and lymphatic system. However, there are certain cells that are not a part of the immune system yet exhibit functions similar to immune cells. These cells are podocytes (resident kidney cells), certain cells of the ear, stem cells and fibroblasts (Typiak and Żurawa-Janicka, 2024). Trophoblast cells too, despite not being part of the immune system, are proven to show immunological functions. These cells form the outer layer of blastocyst and express Pattern recognition receptors (PRR). PRR are proteins present on certain cells and are crucial part of innate immune response. PRRs recognize specific patterns present in pathogens called pathogen associated molecular pattern (PAMP) that activate downstream signalling pathways ultimately providing defence against foreign invaders. Toll like receptors (TLR), Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are a type of PRR. Trophoblast cells express TLR and NLRs. In response to bacterial and viral stimulation, TLR and NOD signalling have been proven to alter cytokine and chemokine production and expression of immunoregulatory genes and proteins in trophoblast cells. HTR8/SVneo and Swan71 are trophoblast cell lines that are being investigated to understand the immunological functions of trophoblast cells (Motomura et al., 2023). Therefore, although trophoblast cells are not a part of the immune system, they exhibit immunological functions through expression of PRRs, immunological genes and cytokines and can serve as model cell lines for investigating the immunomodulatory properties of medicinal plants.

Having established a fair background behind understanding traditional knowledge as a tool for selection of plants with potential medicinal properties, interaction between phytochemicals and the attenuation of signals responsible for certain diseases, and the importance of selecting the right model to test efficacy of selected medicinal plants, it is finally important to explain the need to search for sources of immunomodulatory drugs in modern times.

Inflammation is a response of the immune system, specifically the innate immune system, to infection or damage. Acute-inflammation is a defence mechanism that is necessary for getting rid of infection, repairing damage, healing, and altogether restoring homeostasis. Inflammatory stimulus is recognized by PRRs that in turn activate intracellular inflammatory pathways via mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-κB), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways that result in the regulation of downstream inflammatory cytokines. These cytokines in turn modulate the inflammatory response that results in vasodilation, increased capillary permeability and localization of leukocytes that finally resolve the infection/damage in the cells. Inflammatory mediators are short lived and normally, once the issue has been resolved, the inflammatory response ceases (Chen et al., 2017). However, sometimes inflammation can persist for prolonged periods in which case it becomes chronic.

Chronic inflammation in the body has been associated to diseases like asthma, rheumatoid arthritis, hepatitis, cardiac disease, Alzheimer's, epilepsy, depression and schizophrenia (Pahwa et al., 2024). COVID-19 infection, caused by novel SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is also followed by a hyperactive immune response, unrestrained inflammation, and a cytokine storm. This cytokine storm has been found to be the cardinal root of viral sepsis and inflammation-induced lung damage, pneumonitis, acute respiratory distress syndrome (ARDS), respiratory failure, shock, organ failure and death (Velikova et al., 2024).

Immunomodulatory drugs can be crucial in managing a variety of such medical conditions because of their ability to modulate the immune system's activity and plants with their diverse range of bioactive compounds are ideal templates as sources of such drugs. Considering COVID-19 (coronavirus disease 2019) situation also, direct targeting of the immune system can be an effective method for lessening the repercussions brought about by the virus in the body. Immunomodulatory drugs can prove to be useful for:

a. Autoimmune Diseases: Conditions like rheumatoid arthritis, lupus, and multiple sclerosis involve the immune system attacking the body's own tissues. Immunomodulatory drugs may help in suppressing this inappropriate immune response.

b. Chronic Inflammation: In diseases such as inflammatory bowel disease (IBD) and psoriasis, the immune system is overly active, leading to chronic inflammation. The anti-inflammatory drugs can help reduce inflammation and manage symptoms.
c. Cancer: Certain cancers can evade immune surveillance. Immunomodulatory drugs can be useful in enhancing the immune system's ability to recognize and destroy cancer cells.
d. Organ Transplantation: To prevent organ rejection, immunosuppressive drugs may be utilized to dampen the immune response against the new organ.
e. Allergies: In cases where allergies are severe and not manageable with conventional treatments, immunomodulatory drugs can help modify the immune response to allergens.
f. Infectious Diseases: Immunomodulatory drugs can help manage conditions where the immune system is either overactive or not responding effectively to infections.
g. COVID-19: mitigation of the SARS-CoV-2 induced immune hyperactivity and cytokine storm.

There are several drugs available in the market that are effective in modulating the immune system. However, it cannot be denied that synthetic drugs come with their own set of side effects and prolonged use can result in high blood pressure, obesity, diabetes, bone density loss, gastrointestinal issues, hepatotoxic problems, kidney related injury, cardiovascular problems, cerebral complications, respiratory tract issues, mitochondrial toxicity, an increased risk of infections and altered response to physical stress (Strzelec et al., 2023). Moreover, there has been an alarming increase in the inception of 'superbugs', which are multiple anti-biotic resistant microbes that make treatment of infections more difficult (Baral, 2023).

An alternative, more compatible source of immunomodulators is desirable and thus, plants are the best natural source. It is reported that worldwide, 150 plants have shown immunomodulatory activity (Zebeaman et al. 2023). Among these, *Panax ginseng* ('ginseng', family - Araliaceae), *Zingiber officinale* (ginger, family - Zingiberaceae), *Withania somnifera* ('ashwagandha' family- Solanaceae), *Astragalus membranaceus* ('Mongolian milkvetch', family - Leguminosae), *Ocimum sanctum* ('tulsi' family - Lamiaceae), *Morus alba* (mulberry, family - Moraceae), *Andrographis paniculate*

('kalmegh', family- Acantharean), *Curcuma longa* (turmeric, family- Zingiberaceae), *Cinnamomum zeylanicum*, (cinnamon, family - Lauraceae) and *Azadirachta indica*, ('neem', family - Meliaceae) are well known immunomodulators that have been used in traditional medicine since ancient times (Dasgupta et al., 2023). In an era marked by the increasing prevalence of autoimmune diseases, antibiotic resistance, and global pandemics, these plants offer a natural and potentially powerful means of enhancing and modulating the immune system. They are generally well-tolerated, can be used over extended periods, and have a long history of safe use in traditional medicine. Furthermore, the complexity of plant compounds allows for a more nuanced approach to immune modulation, potentially reducing the risk of over-suppression or over-stimulation of the immune system. Immunomodulatory plants could be used as adjuvants to enhance vaccine efficacy by priming the immune system for a stronger response. Immunoadjuvants are substances that modulate the immune response to antigens and as such can have great potential in applied immunotherapy. Many plant phytochemicals like lectin and delta form of inulin are already being studied for their adjuvant properties. Additionally, they have also found use in the clinical development of vaccines (Kumar et al., 2022). Immunomodulatory plants can also play a crucial role in boosting the body's natural defence mechanisms, reducing the need for antibiotics, and potentially reversing the trend of anti-biotic resistance. For example, *Astragalus membranaceus* is known for its immune-boosting properties, which can help the body fight infections more effectively, thus reducing the dependence on antibiotics (Qader et al., 2021). As scientific research continues to uncover the mechanisms behind their effects, immunomodulatory plants are likely to play an increasingly important role in healthcare, complementing conventional treatments and contributing to a more holistic approach to health and disease prevention.

Therefore, this research has been conducted keeping in mind the need of the hour for screening traditional medicinal plants for their ability to modulate the immune response. Immunomodulatory therapies are materializing as favourable avenues for alleviating inflammation associated diseases and it is important as researchers to provide relevant data in the scientific community. *Zanthoxylum* species are globally utilized for their medicinal properties and many researches have proven them to be effective against management of chronic diseases (Okagu et al., 2021). *Z. oxyphyllum* Edgew. and *Z. acanthopodium* DC. are two of the species that have scarcely been investigated. Both the species have been shown to exhibit anti-inflammatory activity *in vitro* (Chatterjee et al.,

2020; Yang et al., 2022). However, a significant research gap was observed regarding the immune response to these two plants, thus prompting the need to investigate their immunomodulatory potential. There is also no information on the phytochemical composition as well as the mineral content of the leaves and fruits of *Z. oxyphyllum* Edgew. and *Z. acanthopodium* DC.

To address these basic gaps, the present study was designed to focus on identifying the phytochemical and mineral content of the two species as well as to quantify the bioactive phytochemical groups. For immunomodulatory studies, an *in vitro* anti-oxidant and anti-inflammatory assay was conducted followed by effect of the methanolic extracts of *Z. oxyphyllum* Edgew. and *Z. acanthopodium* DC on inflammatory mediators in LPS stimulated HTR-8/SVneo cells. The research outcome to expected to provide a preliminary understanding on how the two plant extracts may potentially modulate immune response in the cells. The phytochemical analysis of the extracts is expected to enable the detection and quantification of bioactive compounds that may be responsible for the immunomodulatory potential of the plant extracts. Lastly, the findings could validate traditional uses of these plants in immune-related conditions and contribute to the development of new, plant-based immunomodulatory agents that are safe, effective, and potentially more accessible than existing therapies. Ultimately, by focusing on *in vitro* research, this study aims to fill the gaps in our understanding of immunomodulatory activities of these medicinal plants thereby providing a solid foundation for potential future research and clinical applications.

Chapter 2

Review of Literature

2.1. Human immune system: An introduction

The human body is constantly exposed to pathogenic microorganisms, allergens and toxins that pose potential health risks. However, diseases cannot easily manifest themselves because of the protection provided by our immune system. The human immune system is a vast network of lymphoid organs, white blood cells, antibodies and cytokines that identifies and checks pathogenesis, thus providing immunity. Immunity is the quality of the body to provide resistance against pathogens and other foreign substances and eliminate them, while simultaneously possessing tolerance for own healthy cells/molecules. The fundamental function of immune system, thus, is to check invasion of pathogens/toxins in the body, destroy and eliminate any foreign substances in the body, limit the deleterious effect of such pathogens/toxins, repair any damage, and maintain homeostasis. Immunity in the body is affected mainly by leukocytes (white blood cells). Depending on their mode of action immunity in higher vertebrates is divided into two types - innate and adaptive immunity (Figure 2.1) (Parkin and Cohen, 2001).

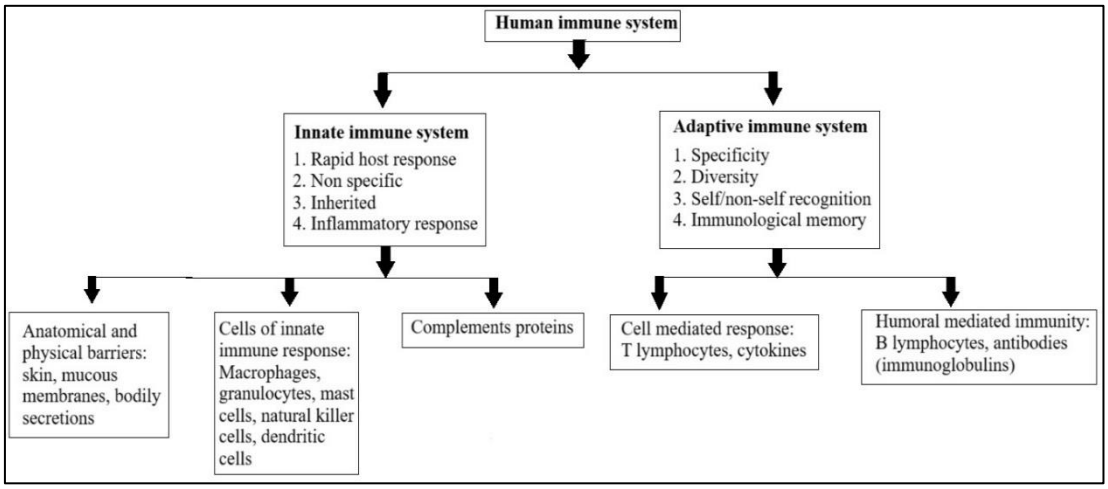

Figure 2.1. Flowchart showing classification and major functions of the different components of human immune system.

2.1.1. Cells of the immune system

All cells of the immune system arise from the pluripotent haematopoietic stem cells (HSC) in the bone marrow, which is a primary lymphoid organ. The HSCs give rise to two types of progenitor cells- common myeloid progenitor cells and common lymphoid progenitor cells. Common myeloid progenitor cells eventually give rise to erythrocytes (red blood cells), platelets, granulocytes (neutrophil, eosinophil, and basophil), mast cells, and macrophages. Common lymphoid progenitor cells give rise to T lymphocyte, B lymphocyte and natural killer cells. Dendritic cells are made by both type of progenitor cells. Once mature, these cells migrate from the bone marrow to other peripheral tissues and may reside in these tissues or circulate in the blood stream and lymphatic system (Figure 2.2) (Janeway et al., 2001).

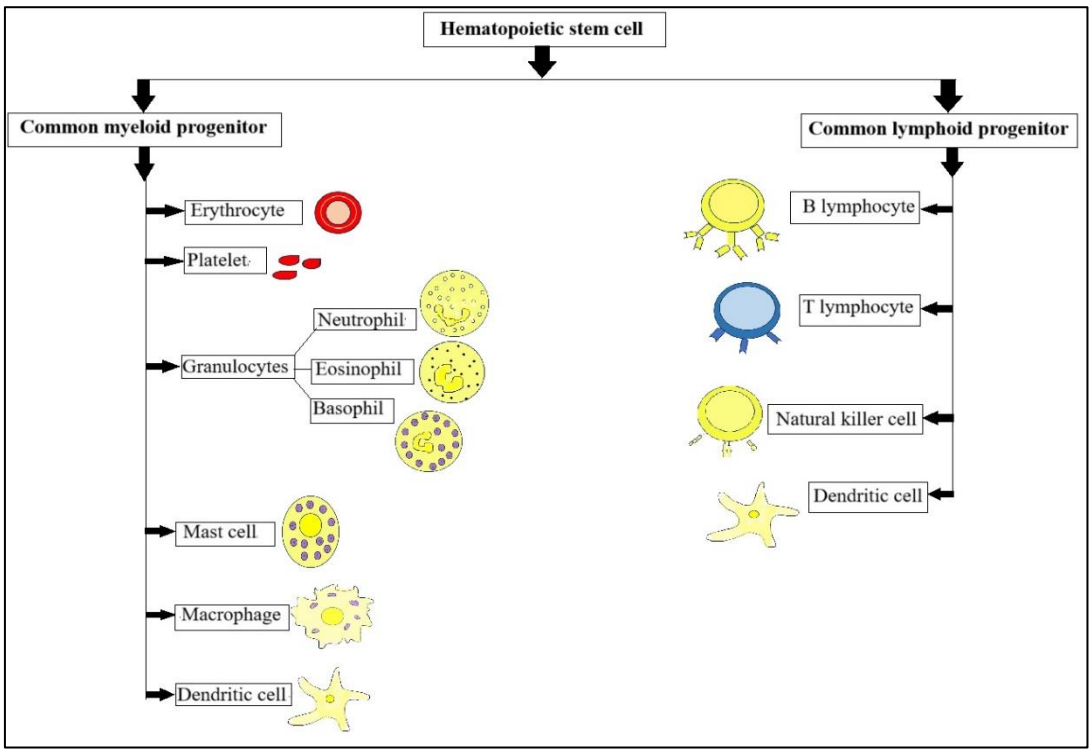

Figure 2.2. Different types of immune cells in human system.

The cells of myeloid lineage are all involved in innate immune response. Macrophages are long lived cells that can engulf and destroy pathogens (phagocytosis), induce inflammation and are antigen presenting cells (APCs) that present digested pieces of pathogens (antigens) to cells of adaptive immune response. Macrophages are also scavenger cells that assist in clearance of dead cells and debris. The granulocytes i.e.

neutrophil, eosinophil, and basophil are also phagocytes but are comparatively short lived. Among these, neutrophils are produced most abundantly. These cells have granules containing degradative enzymes and toxic proteins that are released upon activation. While neutrophils can phagocytose a variety of micro-organisms, eosinophils and basophils are chiefly involved in defence against large parasites and allergic reactions (Janeway et al., 2001). Mast cells are mononuclear granulocyte that enable dilation of the blood vessels during immune response. Upon activation, the cell granules release chemicals and enzymes like release histamine and proteases that elicit inflammation and innate immune response (Rao and Brown, 2008). Natural killer cells of lymphoid progenitor cell origin, are also involved in innate immune response. These cells kill virus infected cells and tumour cells. The lymphocytes (T cells and B cells) are antigen specific cells that comprise key components of the adaptive immune system. The T lymphocytes require a maturation process in the thymus and are thus named T lymphocytes. The B cells mature within the bone marrow itself. On encountering antigens, these cells are activated and differentiate into effector lymphocytes from naïve lymphocytes. The lymphocytes also differentiate into memory cells that provide immunological memory and consequently long-term immunity. These cells can recognize and bind to various antigens via antigen receptors on their surface. The dendritic cells are also phagocytes but they are chiefly specialized in presenting antigens to T lymphocytes thus establishing a pivotal link between innate and adaptive immune response (Janeway et al., 2001).

2.1.2. Innate immunity

The innate immunity, also termed as non-specific immunity, is inherited and is the first responder to a harmful stimulus. Its main function is to provide immediate defence to the host. Physical and anatomical barriers like skin, mucous membranes of the respiratory, digestive, reproductive, and urinary tract and bodily secretions (tears, saliva, sweat), along with effector cells (monocytes, macrophages, natural killer cells, neutrophils, eosinophils, basophils, mast cells), complement cytokines, and acute phase proteins form the chief components of the innate immune system. The innate immunity mainly recognizes and responds to antigens, which are any foreign particles, including pathogens. The main characteristics of innate immunity are its rapid host response, lack of specificity i.e. it provides the same defence against every antigen rather than a specified response against each, and the lack of immunological memory i.e. it does not recognize pathogens

that it may have encountered in the past. Inflammation is a typical response observed during activation of this type of immunity. Innate immunity also plays a vital role in the activation of adaptive immunity. However, due to lack of specificity, the innate immune system can sometimes damage normal self-tissues (Parkin and Cohen, 2001).

Effector cells of the innate immune system mainly respond to pathogens via recognition by germline encoded receptors called pattern recognition receptors (PRR). These receptors also enable the cells to differentiate between self and non-self. The three main types of PRRs expressed are Toll like receptors (TLRs), nucleotide oligomerization domain-like receptors (NLRs) or NOD like receptors and collectin family receptors. The structurally diverse PRRs recognize the Pathogen associated molecular patterns (PAMPs) which are molecular structures present on the pathogens. PAMPs can be bacterial lipopolysaccharides (LPS), flagellin, mannose residues, bacterial DNA, double stranded RNA, etc. DAMPs (damage associated molecular patterns), which are molecules released on lysis of damaged or dying cells are recognized by NOD like receptors. Another cell surface proteins are Major histocompatibility complex (MHC). MHC Class I are expressed by all nucleated cells. The inhibitory receptors expressed by natural killer cells recognize MHC Class I which helps them to differentiate between virus infected or altered cells, thus regulating the preferential activation of immune response. Recognition of pathogens by these receptors lead to downstream signalling and subsequent innate immune response. Macrophages and dendritic cells are also antigen presenting cells. These two cells express MHC Class II proteins through which they present digested antigens to cells of the adaptive immunity thus forming a bridge between the two types of immunity (Turvey and Broide, 2010).

Complements proteins are also a part of the innate immune system. They are about 30 different serum glycoproteins that circulate in the blood and are named C1-C9 based on the order of their discovery. These proteins are inactive zymogens that on encountering pathogens, are activated and initiate a cascade of reactions that facilitate elimination of the invading pathogen (Janeway et al., 2001).

2.1.3. Adaptive immunity

Adaptive immunity, also called acquired immunity is slower than innate immunity but tailored to target specific antigens. Chief characteristics of this type of immunity is its specificity and diversity against pathogens, and immunological memory wherein it can recognize and provide more enhanced and rapid response upon reinfection. The cells that express receptors for these pathogens persist in the body for a long time. The adaptive immunity functions with the help of lymphocytes. T lymphocyte originate in bone marrow and matures in the thymus, whereas B lymphocytes originate and mature in bone marrow and secrete antibodies and cytokines. The T-cell receptors (TCR) in T lymphocytes and antibodies, also called B-cell receptors (BCR) in B lymphocytes allow these cells to recognize antigens. Adaptive immunity is mediated by two type of responses – cellular immunity and humoral immunity. While T cells are the key players in cellular immunity essentially targeting intracellular pathogens such as viruses, some bacteria, and cancer cells, B cells are vital during the effector stage of humoral immunity primarily defending against extracellular pathogens, such as bacteria and viruses that are outside of cells. Upon encountering an antigen, and often with assistance from T cells, B cells can mature into plasma cells, which produce proteins called antibodies (immunoglobulins) that specifically target the antigens (Parkin and Cohen, 2001).

Based on the expression of surface glycoproteins, T cells are of two types - CD4+ (T helper cells) and CD8+ T (T cytotoxic cells). T helper (T_H) cells assist other memory B lymphocytes, CD8+ cells and macrophages by activating them and effecting downstream immune response. CD4+ cells themselves, are activated by antigens presented on MHC class II molecules by APCs which result in their prompt division into different types of T_H cells including memory T cells and subsequent release of cytokines leading the way for adaptive immune response. T cytotoxic cells or killer cells recognize antigens presented on MHC class I molecules and release cytotoxic molecules like granzymes and perforins from their granules that induce apoptosis in the target cells, mainly tumour cells or virus infected cells (Janeway et al., 2001).

The B lymphocytes can either directly recognize free soluble antigens without antigen presentation, or those antigens presented by the APCs. Activation of B lymphocytes cause their proliferation into plasma cells and memory B cells. The plasma cells release

immunoglobulin proteins also called antibodies which then attack the invading pathogens (Janeway et al., 2001).

2.1.3.1. Antibodies

Antibodies are glycoproteins released by effector B cells also called plasma cells that circulate in the blood and lymph. They are 'Y' shaped soluble glycoproteins made up of two identical short polypeptide chains and two identical long polypeptide chains held together by disulphide bonds. The short chain and long chain are also termed light chain and heavy chain respectively. The variable region of the chain also called fragment of antigen binding (Fab) is the region that binds with the antigen and makes up 1/3 of the total length of the protein. The N-terminal (amino terminal) to which the antigen binds, is present in this region. The constant region or fragment of crystallization (Fc region) binds with B lymphocyte and other cells and make up the rest of the entire length of protein. The C-terminal (carboxylic terminal) to which the cells bind, is present in this region. Based on the type of heavy chain, immunoglobulins are classified into five types, each represented by a lower-case Greek letter – IgM (μ heavy chain), IgG (γ heavy chain), IgA (α heavy chain), IgD (δ heavy chain) and IgE (ε heavy chain). The light chain can be only either of the two types – κ or λ. On binding with a specific binding region of the antigen, called epitopes, these antigens get labelled for destruction by other immune cells. IgM is the largest and the first antibody to be produced. It is the first responder to pathogen attack. IgG is the major antibody in blood and extracellular fluid. It is involved in opsonization of pathogens as well as activation of complement pathway and is the only immunoglobulin that can cross the placenta and provide protection to the foetus. IgA found in mucosal areas (gut, respiratory tract), protect against pathogens in mucosal surfaces. IgE is involved in allergic responses and parasitic infections and IgD plays a role in B cell activation (Janeway et al., 2001).

2.1.3.2. Cytokines

Each cell of the immune system performs it's own specific roles. To interact and communicate with each other, these cells secrete low molecular weight soluble proteins or glycoproteins called cytokines that are recognized by membrane receptors present in the cells. Cytokines are produced transiently, function at very low concentrations (picomolar to nanomolar) and play a non-enzymatic role in regulating cell activity. They

represent the fourth major category of intercellular signalling molecules, alongside neurotransmitters, hormones, and autacoids (Nathan and Sporn, 1991). Cytokines are named according to their properties and secreting cells, like lymphokines (produced by lymphocytes), monokines (from monocytes), chemokines (with chemotactic properties), and interleukins (produced by leukocytes and acting on other leukocytes) and can function in autocrine, paracrine, or endocrine way. A single cytokine can affect multiple cell types (pleiotropy), or different cytokines can also trigger a similar effect (redundancy). Often, they work in cascades, where one cytokine prompts the production of others, and they can interact in either synergistic or antagonistic ways. T_H cells and macrophages are the principal producers of cytokines (Zhang and An, 2007). Depending on their mode of action, cytokines can be pro-inflammatory, anti-inflammatory or exert both types of responses. They effect cell mobility, proliferation, differentiation, activation and destruction of tumour or damaged cells (Foster, 2001).

Commonly, cytokines are grouped into families namely, interleukin (IL) family, tumour necrosis factor (TNF) family, transforming growth factor (TGF) family and interferon (IFN) family (Wautier and Wautier, 2023). The interleukin family of cytokines is vast and can elicit both pro-inflammatory and anti-inflammatory responses. Some interleukins like IL-1β, IL-6, IL-31, IL-33, etc. are pro-inflammatory cytokines that up-regulate the inflammatory response on infection or injury, while others like IL-4, IL-10, IL-11, and IL-13 are anti-inflammatory cytokines that help control the response of pro-inflammatory cytokines, working alongside specific cytokine inhibitors and soluble receptors to modulate the immune system. TNF-α of the TNF family is also a pro-inflammatory cytokine that regulates the apoptotic pathways and activation of inflammation via Nf-κB pathway (Zhang and An, 2007). TGF family of cytokines effect proliferation and differentiation of many cell types and are also involved in wound healing, stimulation of extracellular matrix proteins and enhancing cell adhesion. The IFN family commonly inhibits replication of virus in normal cells and can affect increased expression of MHC Class I and II in immune cells. Chemokines are chemotactic proteins that guide immune cells to sites of infection or inflammation. They help release immune cells from the bone marrow and regulate processes like cell activation and survival. They are also involved in the coordination between innate and adaptive immune response (Wautier and Wautier, 2023).

2.1.4. Organs of immune system

Organs of the immune system are divided into primary lymphoid organs and secondary lymphoid organs. The primary lymphoid organs bone marrow and thymus where lymphocytes develop and mature. Bone marrow is the spongy tissue within bones that serves as the principal site for haematopoiesis (Janeway et al., 2001) and thymus is a small organ located within the rib-cage just above the heart that serves as the site of selection and maturation of naïve T lymphocytes as well as their subsequent categorization into CD4+ helper T cells and CD8+ cytotoxic T cells (Thapa and Farber, 2019).

The secondary lymphoid organs are where lymphocytes sustain and initiation of adaptive immune response takes place. Spleen, lymph nodes, mucosa associated lymphoid tissues (MALT) such as the tonsils, gut associated lymphoid tissue (GALT) like Peyer's patches, and appendix are secondary lymphoid organs (Morsink et al., 2020). Spleen is the largest lymphoid organ. It filters the blood to remove old and damaged red blood cells (RBCs), identifies foreign particles/pathogens, houses B and T lymphocytes and is also the site for differentiation of B lymphocytes into plasma (Lewis et al., 2019). Lymph nodes are bean shaped tissues distributed as clusters in areas such as the neck, armpits, groin, and along the major lymphatic vessels that filter lymphatic fluids for pathogens. Swelling of lymph nodes is indicative of infection or progression of a disease (Krishnamurty and Turley, 2020). The nasopharyngeal tonsils, palatine tonsils, and lingual tonsils form part of the MALT that are chiefly responsible for defence against pathogens that may enter through the nose or orally (Brandtzaeg, 2003). Peyer's patches provide immunity against pathogens that may enter the digestive tract and activate the circulating lymphocytes and macrophages. Additionally, these tissues also enable the distinction between hazardous pathogens and harmless pathogens, and help in maintaining the beneficial gut microflora (Heel et al., 1997). The appendix is largely accepted to play a part in the immune system by providing a sanctuary for those gut microflora that get driven out of the gut (Bollinger et al., 2007).

2.1.5. Mechanism of immune response

The innate and adaptive immune response work in tandem to generate an effective immune response. The physical and chemical barriers provide the first line of defence

against invading pathogens by preventing their entry into the body or trapping them and enabling the cells of immune system to destroy them. The epithelial cells of skin and mucosal tissues also release peptides like human β-defensins, lysozyme and cathelicidin, that exert antimicrobial activity against a vast number of bacteria, fungi, chlamydiae, and enveloped viruses (Tosi, 2005).

When these barriers are breached by invading pathogens, the innate immune system immediately responds to the invasion. Macrophages are phagocytic cells that serve as one of the bodies first responders to the site of invasion, playing a crucial role in initiating the immune response. Phagocytosis is a process by which phagocytic cells like macrophages recognize and engulg pathogens. Recognition of PAMPs via PRRs activates these cells to phagocytose the pathogens and release cytokines that recruit neutrophils and more macrophages. Upon engulfment, exposure of the pathogens to lysozymes and acid hydrolases within the phagocytes degrades their cell walls and proteins. These cells also facilitate release of reactive oxygen species like superoxide (O_2^-), hydrogen peroxide, hydroxyl radicals, and nitric oxide (NO) through a process called oxidative burst that damages and kills the pathogens. Larger pathogens like parasites are destroyed by a synchronized recruitment and release of lysosomal products by macrophages, neutrophils, and eosinophils. Upon activation of the TLRs, macrophages and dendritic cells also release pro-inflammatory cytokines like IL-1β, IL-6, IL-12, TNF-α and chemokines like CXCL8 that trigger and sustain inflammation. Inflammatory mediators cause vasodilation and attract more neutrophils and complement proteins to the site of infection along with increased temperature that is unfavourable for survival of many pathogens. Redness and swelling at the site of infection as well as fever are the observable characteristics of inflammation. Immature dendritic cells circulating in the skin epithelium or gastrointestinal mucosa actively ingest antigens via phagocytosis or micropinocytosis, which is a process by which extracellular antigens are non-specifically engulfed with large volumes of surrounding fluids. When these dendritic cells encounter pathogens, TLR signalling induces expression of a receptor called CCR7 (C-C chemokine receptor 7) that enables their maturation as well as increased sensitivity to chemokine CCL21 (C-C motif chemokine ligand 21) released by lymphoid tissue. This sensitivity causes migration of the now mature dendritic cells to lymph nodes. Mature dendritic cells can express increased levels of long lasting MHCs and co-stimulatory signals that enables them to present antigens to naïve T cells in a stable manner and release adhesion

molecules and chemokines like CCL18 that draw naïve T cells. Antigen presentation to T cells cause activation of adaptive immune response (Janeway et al., 2001).

The complement proteins also recognize invading pathogens. Upon recognition of an invading pathogen, an initial zymogen (C3) is activated. This activated protein in turn proteolyzes a downstream complement protein initiating a series of successive proteolysis and activation of other downstream zymogens ultimately activating an effector protein that either directly kills the pathogen or facilitates its phagocytosis through opsonization (coating pathogens with antibodies or other proteins to enable their detection by phagocytes). Complement proteins also interact with cells of the immune system to induce an inflammatory response (Janeway et al., 2001).

Adaptive immune response can be of two types; cell mediated response and humoral response. Presentation of antigens to mature naïve T cells in the lymphoid organs facilitates cell mediated adaptive immune response. Successful activation, survival, and differentiation of the T cells is induced by interaction of MHC-Class II, molecule B7 and secreted cytokines from dendritic cells with TCR, CD4 and CD28 in T cells. Activated T cells release IL-2 which binds to high affinity receptor in the T cell themselves and induce their proliferation and differentiation into effector T cells. While CD4+ cells differentiate into many subsets mainly T_H1, T_H2, T_H17 and T regulatory cells, all CD8+ cells differentiate into one type of cell i.e., CD8 cytotoxic T cells (Janeway et al., 2001). T_H1 cells are the main effector cells in cell mediated immunity. These cells release cytokines IFN-γ, GM-CSF, TNF-α and membrane bound signals that activate macrophages. On activation, macrophages increase their expression of CD40 and TNF receptors and secrete TNF-α which increases their antimicrobial activity and induces oxidative burst. Activated macrophages also increase their B7 and MHC Class II molecules to enable activation of more CD4+ cells. Effector cytokines and chemokines released by T_H1 cells further kill chronically infected macrophages, induce production of more macrophages in the bone marrow and recruitment of additional macrophages at the site of infection. T_H2 cells promote immune response to parasitic invasion. They secrete cytokines IL-4, IL-5, IL-9, and IL-13 that assist in B cell activation, proliferation and isotype switching. They also induce phagocyte independent inflammation via IgE antibody activation (Romagnani, 2000). T_H17 cells secrete IL-17A, IL-17F and IL-6 enabling recruitment of neutrophils to the infected site thus facilitating inflammation. T regulatory cells secrete cytokines IL-10

and TGF-β that exert suppressive effects to maintain balance and mitigate autoimmunity and chronic inflammation (Vignali et al., 2008). Antigens presented on MHC Class I by virus infected cells is recognized by TCR in CD8+ cells. On recognition, CD8+ cells induce apoptosis of the target cells by releasing perforin and granzymes which are delivered into the cytosol of the infected cells (Janeway et al., 2001).

The humoral immune response is mediated by B cells and secreted antibodies. Activation of B cell antibody responses to antigens occur by T cell dependent or T cell independent manner. Surface immunoglobulins of B cells (BCR) can recognize a wide range of antigens, including proteins, glycoproteins, polysaccharides, viruses, and bacteria. On detection of an antigen, B cells internalize them and process them into peptides that are presented on MHCII molecules to helper T cells. Helper T cells recognize the peptide-MHC Class II complex on B cells and provide signals through CD40-CD40 ligand interaction. In such cases, the antigens that require T cell involvement for antibody responses are called thymus dependent (TD) antigens. The interaction between peptide-MHC Class II complex on B cells and effector T cells leads to the secretion of B cell stimulatory cytokines IL-4, IL-5 and IL-6 by the T cells that induce proliferation and differentiation of B cells into plasma cells that secrete antibodies that act against pathogens and memory cells. For thymus-independent (TI) antigens, such as bacterial polysaccharides, B cells can be activated without T cell help. Two types of signalling are involved. First signalling is facilitated by direct recognition of microbial components or extensive cross-linking of BCRs. Second signalling is through recognition of antigens by TLRs. This type of B cell activation provides limited class switching and produces no memory cells. TI antigens provide a faster antibody response, contributing to protection against extracellular bacteria (Janeway et al., 2001). Particularly T_H2 cells are involved in humoral response. Further, they are known to release anti-inflammatory cytokines like IL-10 that can decrease the inflammatory response (Figure 2.3) (Libby, 2008).

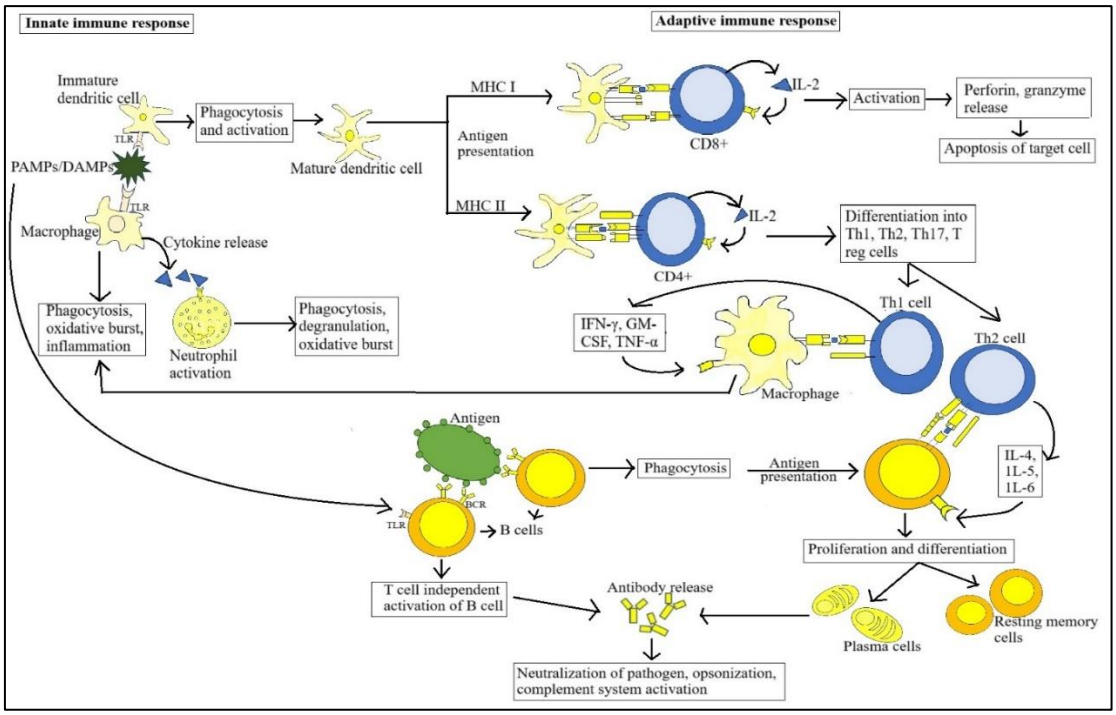

Figure 2.3. Diagrammatic representation of the responses of human immune cells with reference to innate and adaptive immunity.

2.1.6. Mechanism of inflammatory response

Inflammation is a protective immune response of the body to any infection or injury. Upon encountering invading pathogens, inflammatory response is initiated by cells of the innate immune system to rapidly kill pathogens and restore homeostasis. Such inflammatory response is termed acute inflammation. It is initiated when PRRs like TLRs recognize PAMPs and/or NLRs recognize DAMPs. Recognition of pathogens by TLRs initiates a common signalling pathway via activation of NF-κB. Under normal conditions, NF-κB is inhibited by a protein IκB (inhibitor of nuclear factor kappa B) and remains inactive. MyD88 (myeloid differentiation primary response gene 88) is an adaptor protein present in the cytosol. Upon TLR signalling, a MyD88-dependent signal transduction pathway induces phosphorylation of inhibitory IκB protein by IKK (IκB kinase) and IκB bound NF-κB is released. This transcription factor trans-locates to the nucleus where it binds to the target genes for transcription of pro-inflammatory cytokines. DAMP recognition on the other hand, signals NLRs to activate caspase-1 to convert cytokines into their active forms. The pro-inflammatory cytokines released are IL-1β, IL-6, and TNF-α among others. Along with chemokines and other co-stimulatory factors, the pro-inflammatory

cytokines elicit recruitment of effector cells like macrophages and neutrophils to the site of infection via chemotaxis where they phagocytose pathogens or kill them via oxidative burst. During acute inflammation, mast cells and resident macrophages also release histamine, leukotrienes and prostaglandins that promote vasodilation and vascular permeability (Ashley et al., 2012). Inflammation induces phospholipases to cleave arachidonic acid from the plasma membrane. This arachidonic acid is then converted to prostaglandins and leukotrienes (collectively called eicosanoids) by enzymatic activity of cyclooxygenase (COX) and lipoxygenase (LOX) respectively. The classic signs of inflammation i.e. pain, redness, swelling and fever are facilitated by these eicosanoids and histamine (Henderson, 1994; Ricciotti and FitzGerald 2011). When the invading pathogens have been expelled, there is a class switch in lipid mediator generation that promotes generation of pro-resolving mediators like lipoxins instead of pro-inflammatory mediators to initiate tissue repair and healing. Lipoxins along with other pro-resolving mediators like peptides and gaseous particles enable dismantling of chemokine and cytokine gradient to prevent leukocyte influx, induce apoptosis in neutrophils, facilitate phagocytosis of dead /damaged cells by macrophages, enhance recruitment of monocytes for wound healing and promote the movement of non-apoptotic cells to the lymphoid organs (Headland and Norling, 2015). A polarization of inflammatory response into pro-inflammatory or anti-inflammatory mechanism also takes place depending upon the type of pathogens encountered. T_H1 cells promote pro-inflammatory responses against intracellular parasites by releasing cytokines like IFN-γ, IL-2 and TNF-α. T_H2 cells provide protection against macro parasites and promote anti-inflammatory responses via release of cytokines like IL-4, IL-5, IL-10, and IL-13. T_H1 and T_H2 responses are mutually opposing, reflecting a balance between proinflammatory and anti-inflammatory mechanisms. This balance can be adjusted to influence the outcome of an infection (Ashley et al., 2012).

Failure to expel the invading pathogen or resolve the inflammatory response can lead to chronic inflammation. Chronic inflammation is a prolonged state of inflammatory condition which is detrimental for health and is associated with the pathogenesis of diseases like asthma, rheumatoid arthritis, hepatitis, cardiac disease, Alzheimer's, epilepsy, depression and schizophrenia (Pahwa et al., 2024).

2.2. Types of immunomodulators

As a means of better more pro-active approach for managing various diseases associated with chronic inflammation, immunomodulatory therapy has opened a broad avenue that can directly target the immune system. Immunomodulators can potentially target specific regulators in the immune system and modulate their response (Strzelec et al., 2023). Some immunomodulators being researched today have been discussed here to enable better understanding of how they function and their potential as immuno-therapeutic agents (Figure 2.4)

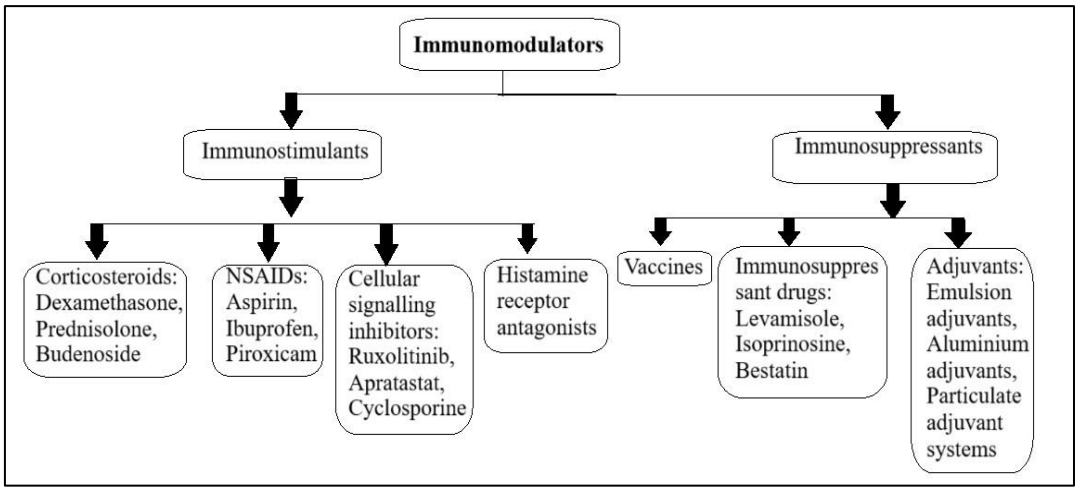

Figure 2.4. Different types of immunomodulators with examples.

2.2.1. Immunosuppressants

Immunosuppressants are agents that can suppress the immune response and can be applicable in treating hyperactive immune response as seen in organ transplantations and autoimmune diseases. Corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), histamine antagonists (HAs), cellular signalling inhibitors like Janus kinase (JAK) inhibitors, calcineurin inhibitors, TNF-α Converting Enzyme (TACE) inhibitors etc. are agents that have been studied for their potential use as immunosuppressants (Strzelec et al., 2023).

- Corticosteroids are endogenous glucocorticoids. Dexamethasone, prednisolone, and budesonide are common corticosteroids that find their use in the treatment of asthma, chronic obstructive pulmonary disease (COPD), acute respiratory disease

(ARD), allergies and even arthritis and lupus. The primary effect of corticosteroids is to inhibit transcription factors that produce pro-inflammatory mediators. They also inhibit the enzyme phospholipase A2 which is involved in the release of arachidonic acid and lysophospholipids from the cells membrane that further produce inflammatory mediators like prostaglandins and leukotrienes. Corticosteroids suppress inflammatory enzyme genes like cyclooxygenase-2 (COX-2) and iNOS, and pro-inflammatory cytokines such as TNF-α and interleukins. Conversely, they upregulate annexin A1 which is a protein that counters inflammatory response. Although corticosteroids are effective against a wide array of disease and can be administered in numerous ways, they do, undeniably have side effects depending on dose and duration of usage. Their notable side effects include hypothalamic-pituitary-adrenal axis suppression, high blood pressure, obesity, diabetes, and bone density loss among other (Williams, 2018).

- NSAIDs (non-steroidal anti-inflammatory drugs) such as aspirin, ibuprofen, naproxen, indomethacin, piroxicam, and paracetamol work by inhibiting cyclooxygenase (COX) enzyme that is responsible for producing prostaglandins from arachidonic acid. This in turn limits production of prostaglandin E2 (PGE-2) and prostacyclin (PGI-2) both of which are inflammatory mediators responsible for vasodilation. Hence, flow of other inflammatory mediators like histamine into the capillaries is limited. NSAIDs do not directly inhibit the accumulation of proinflammatory cells. However, they also carry risks such as gastrointestinal issues, liver toxicity, kidney damage, cardiovascular and cerebral complications, respiratory issues, and mitochondrial toxicity (Strzelec et al., 2023).
- Histamine is a strong inflammatory mediator. It can regulate the functions of granulocytes, mast cells, dendritic cells, natural killer cells, T helper and regulatory cells, CD8+ cells and B cells by binding to their histamine receptors. Histamine has been associated to the onset of various pathological diseases and therefore, the use of histamine receptor antagonists have found their use in immune-therapy as immunosuppressants (Sarasola et al., 2021). Four histamine receptors have been identified so far. H1 receptors have been found to regulate allergic inflammation, H2 receptors have been found to mediate gastrointestinal related functions, H3 receptors have been found to regulate neurotransmission,

and H4 receptors have been found to modulate cell of the immune system. Hence specific antagonists of these receptors are potentially being tested for their ability to modulate the downstream response. Deptropine is a long-established H1 receptor antagonist that is used for the treatment of asthmatic symptoms (Qu et al., 2021).

- Cellular signalling inhibitors work by interrupting the signalling cascade thereby inhibiting cytokine activity and enabling the targeting of autoimmune and inflammatory disease suppression. JAK (Janus-kinase) are non-receptor tyrosine kinases that play a key role in cytokine signalling via the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway. In humans, there four types of JAK, JAK1, JAK2, JAK3, and tyrosine kinase 2 (TYK2). A JAK inhibitor called ruxolitinib is an FDA-approved inhibitor that can obstruct both JAK1 and JAK2 and is utilized for the treatment of myelofibrosis and polycythemia vera (Damsky et al., 2021). Calcineurin is also known as protein phosphatase 3 (PPP3). It is a calcium and calmodulin-dependent serine/threonine phosphatase protein, mainly involved in calcium-dependent signal transduction pathways like T cell activation. Calcineurin inhibitors like cyclosporine have a high affinity for cytoplasmic receptors called cyclophilin-1, competitively inhibiting the binding of calcineurin. This drug-receptor complex terminates the activation and dephosphorylation of nuclear factor of activated T cells (NFAT) thereby inhibiting an inflammatory response (Safarini et al., 2024). TNF-α Converting Enzyme (TACE) is a zinc metalloproteinase (MMP) belonging to the ADAMs (Members of A Disintegrin and Metalloproteinase family) that are type I transmembrane proteinases. TACE is responsible for the conversion of pro-TNF-α into mature TNF-α and its release into the blood thereby making it an attractive target for TNF-α-mediated inflammatory diseases like rheumatoid arthritis, sepsis, and cancer. Apratastat (TMI-005) compound is currently being researched as TACE inhibitor (Murumkar et al., 2020).

2.2.2. Immunostimulants

Contrary to immunosuppressants, immunostimulants are compounds that can boost the immune system and improve the resistance of the body to pathogenic infections. These compounds work by potentially enhancing the activity of immune cells through

increased oxidative activity, phagocytic activity and stimulating cytotoxic cells. Immunostimulants can interact with the immune cell receptors and find potential use in treating immunodeficiency, antibiotic resistance, allergic reactions, immunosuppressive effects of antibiotics and conditions like AIDS. Broadly, immunostimulators are categorized into two types: specific immunostimulants that can act as antigen to stimulate immune responses and non-specific immunostimulants that do not have antigen-like property but act to enhance the immune response to other potential antigens like adjuvants (Shahbazi and Bolhassani, 2016).

- Prime example of specific immunostimulants are vaccines. Vaccines are weakened/ inactive antigens that may be injected, conferred orally, or sprayed nasally into the body to induce an immune response. On exposure to this antigen, the body produces antibodies against it thereby creating immunological memory and preparing the body for future infection by the given pathogen (Sell, 2019)
- Immunostimulatory drugs like levamisole, isoprinosine and bestatin are non-specific immunostimulants that can modulate the immune cells and cytokines thereby enhancing the cellular and humoral immune response. Levamisole can potentially increase immunity by stimulating lymphocyte population and is frequently used in combination with 5-fluorouracil for treating rheumatoid arthritis. Isoprinosine was found to increase the level of cytokines like IL-1, IL-2, and IFN-γ as well as increase lymphocyte content and is used for treating Herpes simplex infections, Epstein-Barr, and Measles viruses. Bestatin has been found to enhance the production of granulocytes. However, there are many side effects associated to the use of these drugs including but not limited to rashes, nausea, increased uric acid level in serum and urine etc. (Shahbazi and Bolhassani, 2016).
- Adjuvants are also a type of immunostimulants because they can improve the immunogenicity of vaccines. Some classical adjuvants are aluminium adjuvants, emulsion adjuvants like MF59 and AS03, TLR agonist molecule-based adjuvants like AS04 and CpG ODN 101, and Particulate adjuvant system like AS01. Vaccines paired with adjuvants have been known to aid APC maturation and promote APC and CD4+ interaction. They also enhance

the production of more cytokines like IL-6, IL-10, IL-12, and TNF-α and co-stimulatory molecules like CD40, CD80 and CD86, inducing maturation of naïve T cells into active T cells (Zhao et al., 2023).

2.3. Plant based immunomodulators

Plants are rich in valuable phytochemicals that hold much potential as bioactive components. Indeed, many modern drugs like vinblastine, vincristine, capsaicin, paclitaxel and galantamine are all approved, plant-based drugs. The search for plant based immunomodulators has also been making much progress (Jantan et al., 2015), with some of the immunomodulatory phytochemicals being discussed further in this text (Figure 2.5)

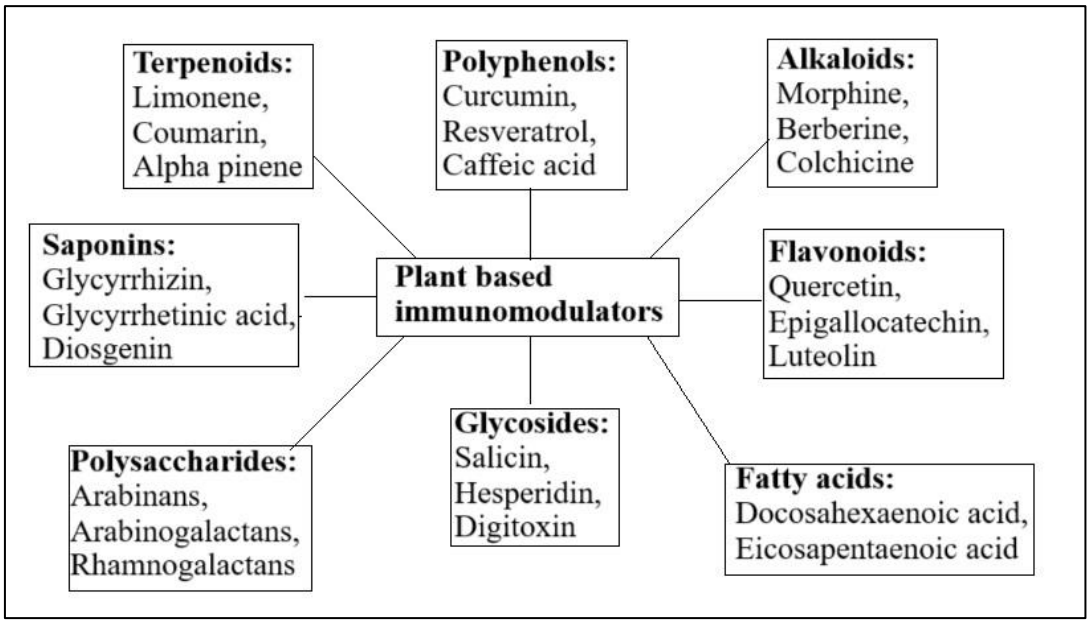

Figure 2.5. Different metabolites of plant origin having immunomodulatory properties.

2.3.1. Polyphenols

Polyphenols are aromatic secondary plant compounds with a phenol moiety. The amino acid phenylalanine is the precursor and all plant phenolic compounds are made from it through the shikimic acid and acetate-malonate pathways. In plants, phenolic compounds are chiefly responsible for protection against UV irradiation and protection from pathogenic attack. They are present as conjugated forms with primarily sugar residues but sometimes also with carboxylic and organic acids, amines, lipids, and other phenols

(Pandey and Rizvi, 2009). Polyphenols are known to impact the production of cytokines as well as pro-inflammatory genes expression. They exhibit pleiotropic effects in the response of immune system, inhibiting NF-κB, TLRs, xanthine oxidase, NADPH oxidase (NOX), phospholipase A2 (PLA2), COX and LOX, phosphatidylinositide 3-kinases/protein kinase B (PI3K/AkT), inhibitor of kappa kinase/c-Jun amino-terminal kinases (IKK/JNK), mammalian target of rapamycin complex 1 (mTORC1) and JAK/STAT. They can also regulate mitogen-activated protein Kinase (MAPk) and arachidonic pathways and upregulate the antioxidant enzymes like superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase (Px) (Yahfoufi et al., 2018).

Curcumin is a prime example of polyphenolic compound that shows many immunomodulatory activities *in vitro* and *in vivo*. *Curcuma longa* is a chief source of curcumin. *In vitro*, curcumin can inhibit inflammatory cytokines (TNF-α and IL-1) and mediators (prostaglandins and leukotriens) in human umbilical vein endothelial cells. *In vivo* studies in male adult rats showed that curcumin could upregulate Peroxisome proliferator-activated receptor gamma (PPARγ) which is a transcription factor that can regulate homeostasis of the immune system (Yahfoufi et al., 2018). Resveratrol is another stilbenoid polyphenol that is found in grapes and berries like blueberries, cranberries, and bilberries. In LPS induced RAW 264.7 (murine macrophage cells), resveratrol was found to reduce NF-κB activation and the expression of cyclooxygenase-2 (COX-2) while in AR42J cell line (rat epithelial tumour cells), it could exhibit anti-oxidant effect via the Myd88-dependent signalling pathway. Additionally, in U-937, Jurkat, HeLa and H4 cells lines, the compound was found to suppress TNF-α induced NF-κB activation, phosphorylation, and nuclear translocation of the p65 subunit of NF-κβ, activation of Mitogen-activated protein kinase (MAPK) kinase (MEK) and JNK (c-Jun N-terminal kinases). Moreover, it could also suppress NF-κ B-dependent reporter gene transcription. *In vivo*, resveratrol reduced T_{reg} cells and the production of TGF-β (transforming growth factor β), while enhancing the CD8+ cells and IFN-γ production in C3H/He mice (Malaguarnera, 2019). Phenolic acids like caffeic acid, ferulic acid and *p* coumaric acid which are widely found in most vegetables and fruits, were investigated for their immunomodulatory activity in splenocytes, peritoneal macrophages and red blood cells (RBCs) isolated from BALB/c mice. It was reported that these phenolic acids could enhance splenocyte natural killer cell activity, stimulate the CD8+ activity against B16-

F10 melanoma cells, inhibit phagocytic activity of macrophages and exerted anti-oxidant activities (Kilani-Jaziri et al., 2017).

2.3.2. Flavonoids

Flavonoids are the predominant class of polyphenols that are found in all plants. These compounds are known to modulate inflammatory mediators such as nitric oxide, prostanoids or leukotrienes. Quercetin is a flavonoid that is commonly found in vegetables like onions and broccoli and in fruits of the citrus family, grapes, berries, and cherries. In human cells like mast cells and THP-1 cells, quercetin has been reported to decrease the production of cytokines like TNFα, IL-1β, IL-6 and IL-8 by inhibiting the degradation of Nf-kappa-B inhibitor alpha (Iκ Bα) along with nuclear translocation of p65, as well as inhibit the phosphorylation of extracellular signal-regulated kinase (ERK) and JNK; while in murine cells like primary murine small intestinal epithelial cell, mouse BV-2 microglia and RAW264.7 macrophages, quercetin can suppress NFκB, NOS, COX-2, IKK, recruitment of the CREB (Cyclic AMP-Responsive Element-Binding Protein) binding protein (CBP/p300), phosphorylation and the activation of JNK/SAPK (stress activated protein kinases), ERK1/2, and p38 (Yahfoufi et al., 2018). The flavone compound, apigenin, exerted immunomodulatory activity by suppressing COX-2 expression in lupus T and B lymphocytes as well as APCs and promoting apoptosis in these cells. It could also reduce the activity of transcription factors NF-κB and AP-1 (Activator protein 1) in pancreatic β-cells. Besides their suppressive activity on transcription factors and inflammatory enzymes, flavonoids like quercetin, kaempferol, apigenin and catechin also can obstruct NO production and expression of iNOS in RAW264.7 cells (González-Gallego et al., 2014).

Epigallocatechin gallate (ECGC) also suppresses COX-2 expression in PC-3 cells. In murine peritoneal macrophages, it was found to inhibit the binding of NF-κB to DNA. Furthermore, EGCG has been discovered to have a protective function against autoimmune-induced tissue damage brought on by Sjogren's syndrome. It does this by working on p38 MAPK1 signalling pathway to shield human salivary glands from TNF-α-induced cytotoxicity. *In vivo*, EGCG blocks the activation of p38 MAPK in female ICR mice, hence inhibiting phorbol ester-induced activation of the transcription factors NF-κβ and CREB in mouse skin. Other flavonoid compounds like luteolin, genistein,

galangin and apocynin all exert anti-inflammatory and immunomodulatory activity *in vitro* by modulating the expression of transcription factors and enzymes like NF-κB, ERK, JNK, NOX and cytokines like TNF-α, IL-6, IL-1β, and IL-8 (Yahfoufi et al., 2018).

2.2.3. Glycosides

Glycosides are a large class of secondary metabolites characterized by the presence of a carbohydrate molecule that can be converted into a sugar (glycone) and a non-sugar component (aglycone or genin) by hydrolytic cleavage (Garcia et al., 2007). Based on the chemical nature of aglycone, glycosides are classified into many sub-categories, some of which will be discussed here.

Salicin, an alcoholic glycoside obtained from many species of *Salix* and *Populus* is known for its anti-inflammatory property. In LPS induced RAW264.7 cells D (−)-Salicin reduced the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 while increasing IL-10 concentration. It was also shown that D (−)-Salicin could suppress MAPKs and NF-κB signalling pathways. In LPS injected mice models too, suppression of MAPKs and NF-κB signalling pathways by D (−)-Salicin was observed (Li et al., 2015). Hesperidin is a flavone glycoside that is found profusely in citrus fruits. Hesperidin is widely known for its anti-inflammatory and anti-oxidant properties. *In vitro* studies in RAW 264.7 cells showed that hesperidin could significantly reduce prostaglandin E2 (PGE2) production. In other cell lines like HMC-1(human mast cell line cells) and HUVECs (human umbilical vein endothelial cells), it was found to inhibit production of inflammatory cytokines and TNF-α-induced Vascular cell adhesion protein 1 (VCAM-1) expression. In rat model of rheumatoid arthritis, it was observed that hesperidin could significantly reduce IL-1β, IL-6, TNF-α levels while increasing IL-10 secretion, T-lymphocyte proliferation, and IL-2 production. It was also found that hesperidin could reduce IL-4, IL-5, and IL-13 concentrations in bronchoalveolar lavage fluid (BALF) as well as inhibit B cell-dependent production of OVA-specific IgE in murine OVA-induced airway inflammation model (Parhiz et al., 2015).

Many cardiac glycosides can exhibit immunomodulatory activities. Digitoxin, which is commonly found in *Digitalis* species, could inhibit the differentiation of Th17 through suppression of IL-17 transcription potentially delaying the emergence of autoimmune

reactions in mice. In cystic fibrosis, the compound suppressed NFκB thereby downregulating IL-8. Like digitoxin, another cardiac glycoside digoxin, which is also found in *Digitalis* species, inhibited retinoic acid receptor-related orphan nuclear receptor gamma (RORγt) thereby suppressing Th17 differentiation in abdominal aortic aneurysm induced mice model. In another *in vivo* study, digoxin was able to suppress hypoxia-induced factor 1 α, NF-κB transcription, and ROS by binding to Pyruvate kinase M2 (PKM2) thus enabling its potential in treating non-alcoholic steatohepatitis (NASH) (Škubník et al., 2021).

2.2.4. Saponins

Saponins are a sub category of glycosides that have L-arabinose, D-xylose, D-glucose, D-glucuronic acid, D-galactose, L-rhamnose or D-fructose as the glycone and triterpenoid or steroid backbone as the aglycone moiety. They are classified accordingly based on the nature of their aglycones (Moghimipour and Handali, 2015). *Glycyrrhiza glabra* (liquorice) of family Fabaceae is a good source of triterpene saponin called glycyrrhizin and pentacyclic triterpenoid glycyrrhetinic acid, both of which are widely known for their anti-inflammatory, immunomodulatory and anti-oxidant potential among others. Researchers claim that the two compounds can bind directly to chief enzymes of the arachidonic acid metabolic pathway. A study showed that in alcoholic hepatitis rat models glycyrrhetinic acid could inhibit vascular endothelial growth factor (VEGF), granulocyte-macrophage colony-stimulating factor (GM-CSF), human growth-regulated oncogene/keratinocyte chemoattractant (GRO/KC) and intercellular cell adhesion molecule 1 (ICAM-1) secretion. In LPS stimulated RAW 264.7 cells, both glycyrrhizin and glycyrrhetinic acid inhibited free radical generation. Several independent studies have demonstrated that the two compounds are capable of inhibiting iNOS, TLRs, cytokines (mainly interleukins), MAPKs, Nf-κB, certain chemokines like CXCL10 (C-X-C motif chemokine ligand 10), C-C motif chemokine ligand-5 (CCL5) and CCL11 and COX-2 (Richard, 2021).

Diosgenin is another steroidal saponin that has demonstrated anti-inflammatory, anti-oxidant and anti-diabetic activity. Diosgenin can be found mostly in *Solanum* species and *Dioscorea* species. Diosgenin was shown to reduce NO production in LPS and IFN-γ stimulated RAW 264.7 cells and C57BL/6 male mice peritoneal macrophages possibly

via inhibition of iNOS. In the same study, it was shown that the compound was able to inhibit IL-1β and IL-6 production. Further analysis also demonstrated that there was decreased synthesis of p65 NF-κB protein while preventing the degradation of IkBα along with decrease in c-Jun and c-Fos protein levels as well. While no inhibition of MAPK and ERK1/2 was observed, JNK activity was significantly inhibited in diosgenin treated macrophages (Jung et al., 2010).

2.2.5. Alkaloids

Alkaloids are nitrogen containing secondary metabolites that are found in small amounts in most living beings. Atropine, Scopolamine, Cocaine, S-(-)-nicotine, morphine, codeine, quinine are all examples of alkaloids that are obtained from plants (Dalton et al., 2003).

Morphine belongs to a class of drugs called opioids or opiates that are used as pain killers. It is a natural alkaloid that is derived from the resin of poppy seeds (*Papaver somniferum*). Years of research provide evidence that morphine exerts immunomodulatory effect on both innate and cell-mediated immunity depending on the dose used. *In vivo*, it can have suppressive effect on NK cells, T cells, B cells, macrophages, and granulocytes. In macrophages, the inhibitory effect in their migratory and phagocytic activity along with promotion of apoptosis has been demonstrated. Further, it may also promote CD4+ differentiation to T_h2 cells causing omission of cell-mediated immune response. In concanavalin A (a lectin extracted from *Canavalia ensiformis*) induced lymph node T cells, the proliferation and production of IL-2 and IFN-γ was suppressed by morphine treatment. In tumour cells, activation of Nf-κB was inhibited thereby causing apoptosis of the cells. Another research showed that while low doses of morphine can promote activation of Nf-κB and TNF-α production, at higher doses, the same was found to be suppressed in murine macrophages thus indicating that the compound can have both immunostimulatory or immunosuppressive property depending on the dose used (Dinda et al., 2005). Similarly, another plant alkaloid-colchicine which is obtained from *Colchicum autumnale* and *Gloriosa superba*, is known to reduce inflammation and modulate innate immune response. In peripheral blood mononuclear cells from obese adults, the compound was shown to reduce total monocytes, NK cells, CD4+ T effector cells and total CD8+ cells while increasing the total population of dendritic cells, CD4+

T central memory cells, CD8+ T central memory cells and CD8+ CD38High cells. The authors also showed that changes in natural killer cell population could possibly arise from changes in COX-2, pulmonary surfactant-associated protein D, myeloperoxidase, proteinase 3, resistin and phosphodiesterase 5A, all of which are biomarkers of inflammation. Additional studies in mouse models have shown that colchicine directly affects T and B lymphocytes along with monocytes (Patel et al., 2023).

Berberine is another plant alkaloid that has immunomodulatory property. It is an isoquinoline alkaloid that has been found in plants of the Berberidaceae, Annonaceae, Papaveraceae, Ranunculaceae and Rutaceae family. It was reported that berberine suppresses TNF-α, IL-6 and Monocyte chemoattractant protein-1 (MCP-1). It can also downregulate the expression of COX-2 and inhibit MMP- 2 and -9. Berberine can also modulate the production of cytokines in various diseases. It was found to decrease expression of cytokines IL-1β, IL-6, IL-17, IFN-γ and TNF-α while increasing IL-10 and IL-22 in colitis. A decrease in IL-6, IL-17 and IFN-γ was observed for autoimmune encephalomyelitis as well as myocarditis while in autoimmune uveoretinitis, a decrease in IL-1β, IL-6 and IL-23 was observed. Additionally, berberine was reported to have beneficial modulatory effect on T_{reg}/T_H17 balance, dendritic cells, and macrophages (Ehteshamfar et al., 2020).

2.3.6. Terpenoids

Terpenoids are made up of isoprene (2-methyl-1,3-butadiene) units and are the most abundant secondary plant metabolites. Based on the number of isoprene units, terpenoids can be classified as hemiterpenes (single isoprene unit), monoterpene (two isoprene units), sesquiterpenes (three isoprene units), diterpene (four isoprene units), triterpene (six isoprene units), tetraterpenes (eight isoprene units) and polyterpenes (> four terpene or eight isoprene units) (Zwenger and Basu, 2008). Terpenoids can exert a wide range of modulatory effect in the immune system. Monoterpenes like limonene which is found mainly in *Citrus* species, have been shown to have immunomodulatory properties. In mouse primary splenocytes, limonene showed modulatory effect on IL-4, IL-5, and IL-10. While in $CD3^+$ $CD8^+$ T cells an inhibitory effect was observed for IFN-γ, IL-2, and TNF-α, in $CD3^+$ $CD4^+$ T cells, a similar inhibitory effect was observed for IFN-γ, IL-2, TNF-α, IL-4, and IL-13. In human NK cell line (KHYG-1 cells), limonene was shown to

increase IFN-γ production while in mouse primary splenocytes and in another model of LPS-induced pleurisy, the same had inhibitory effect on NO, IFN-γ and IL-4. Similarly, in human lymphocytes too, limonene inhibited the enzymes iNOS and COX-2 and production of PGE-2 and NO (Meeran et al., 2021). Other monoterpenes like coumarin, α-pinene and geraniol also showed various modulatory activity *in vitro*. In RAW 264.7 cells, coumarin was found to decrease the production of PGE2, TNF-α, NO, IL-6 and IL-β while geraniol treatment could inhibit NO generation. α-pinene was found to effectively modulate MAPK and NF-κB pathways in LPS induced mouse peritoneal macrophages. Similarly, in LPS induced THP-1 cells, α-pinene inhibited the nuclear translocation of NF-κB while in inflammatory stress induced human chondrocytes, inhibitory effect in the stimulation of IL-1β and activation of iNOS and catabolic MMP-1 genes was observed (Ghosh et al., 2019).

Artemisinin is a sesquiterpene that is obtained from *Artemisia annua*. Several studies have shown that it can inhibit nuclear translocation of NF-κB as well as decrease NO, PGE2, IL1β, IL-6 and IL-10 in LPS stimulated cells. *In vivo*, an artemisinin derivative - ethyl 2-[4-(12-β-artemisininoxy)] phenoxylpropionate, could down-regulate NF-κB and retinoic acid induced G gene/ Jun activating domain binding protein (Rig-G/JAB1) pathways in autoimmune encephalomyelitis induced in a mouse model. Ginkgolide is a diterpene obtained from the leaves of *Ginkgo biloba*. It has been shown that ginkgolide B could effectively block expression of IL-6, IL-1β and TNF-α in cerebral ischaemia-reperfusion induced mice while in asthma induced mice, MAPK/ERK pathway was suppressed. Moreover, in tetrachloromethane induced liver inflammation, an upregulation of IκBα was observed. From *Panax* species, the triterpenoid saponins ginsenosides are obtained which have broad utilization in treatment of inflammatory and neurodegenerative disorders and cancers. Ginsenoside Rg3 was found to effectively block COX-2 and NF-κB in Phorbol 12-myristate 13-acetate (PMA) treated mice and in TPA (tissue plasminogen activator) induced female ICR (Institute of Cancer Research) mice, the compound was demonstrated to reduce the expression of COX-2. Further, red ginseng extract was found to be effective in decreasing STAT3 phosphorylation and Th17 cells population in arthritis induced mice model (Ghosh et al., 2019).

2.3.7. Fatty acids

There is increasing evidence that suggesting that dietary fatty acids can also modulate the immune system thereby highlighting their importance in the management of health and diseases, particularly inflammatory and autoimmune conditions. MUFAs and PUFAs can be added to the diet from olive oil, avocados, and some nuts like walnuts. Fatty acids are essential in human diet and their incorporation has been associated to promote good health. MUFAs like palmitic acid and stearic acid, and PUFAs like oleic acid, linoleic acid, α-linolenic acid, arachidonic acid, eicosapentaenoic and docosahexaenoic are important fatty acids for human health. Humans lack the enzyme required to synthesize α-linolenic acid (ω-3) which in turn in necessary for synthesis of other PUFAs like eicosapentaenoic (EPA) and docosahexaenoic acid (DHA), therefore, plant products are main source of these essential fatty acids for humans. A positive correlation between consumption of oleic acid from olive oil and peanut oil and the reduced risk of developing asthma has been reported. Similar positive correlation was also found for improved glucose regulation. EPA and DHA have been found to exert anti-inflammatory effect *in vitro* and *in vivo*. One of the pathways via which they exert their activity has been found to be by binding to PPARs thereby down-regulating the NF-κB mediated downstream cytokines. Fatty acids also reduce inflammation through the production of specialized molecules called resolvins and neuroprotectins. When aspirin acetylates COX in the presence of DHA and EPA, there is an increase in the production of these molecules. During inflammation, fatty acids like arachidonic acid get cleaved from the plasma membranes by phospholipases. Arachidonic acid (AA) has been known to play a role in the creation of inflammatory prostanoids via the COX pathway, further accentuating inflammation. Aspirin-acetylated COX-2 increases resolvin production, which counteracts the inflammatory effects of AA-derived prostaglandins (Coniglio et al., 2023).

PUFAs, particularly DHA, reduce inflammation by altering plasma membrane properties, specifically by affecting lipid rafts. Lipid rafts are membrane domains rich in cholesterol, glycophospholipids, and receptors, involved in processes like endocytosis, cell signalling, and exocytosis. DHA has been shown to decrease lipid raft levels by up to 30%, which may contribute to its anti-inflammatory effects. Increased levels of MHC I was also observed upon DHA and EPA inclusion (Coniglio et al., 2023). In the eicosanoid

signalling cascade, DHA and EPA have been found to switch AA thereby reducing other inflammation inducing eicosanoids like PGE2, thromboxane B2 (TXB2), and leukotriene B4 (LTB4). Moreover, both the fatty acids influenced the expression of cytokines, defensive functions of macrophages, promoted phagocytic activity and reduced chemotaxis of monocytes in humans. Fatty acids have also been shown to modulate responses in the immune cells. Phagocytic activity in neutrophils were increased in mouse peritoneal neutrophils on DHA treatment. Similar increased phagocytic activity of goat polymorphonuclear leucocytes was observed on treatment with DHA or EPA. In dendritic cells of humans and mouse models, both the fatty acids inhibited MHC II activity and activation of mast cells via IgE was reduced. Saturated fatty acids (FAs) interact with Toll-like receptors (TLRs), particularly TLR2 and TLR4, to increase COX-2 expression and ERK phosphorylation (p-ERK), promoting inflammation. In contrast, omega-3 FAs like DHA and EPA suppress COX-2 and p-ERK, reducing inflammation. In immune cells like monocytes and macrophages, saturated FAs enhance reactive oxygen species (ROS) production in serum-deprived conditions, while EPA and DHA decrease this response. High-fat diets rich in saturated FAs raise endotoxin (LPS) levels and TLR4 activation, triggering pro-inflammatory pathways such as the NLRP3 (nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3) inflammasome, which produces cytokines like IL-1β and IL-18. Additionally, the PPAR-γ receptor, located in lipid rafts, modulates inflammation through Nf-κB–dependent pathways, promoting the synthesis of lipid mediators and cytokines. However, the suppression of NF-κB by EPA and DHA is independent of PPAR-γ (Al-Khalaifah, 2020).

2.3.8. Polysaccharides

Along with proteins, nucleotides and lipids, polysaccharides are also one of essential macromolecules. They are basically polycarbohydrates comprising glycosidic linkage between many units of monosaccharides. Many plant polysaccharides like arabinans, arabinogalactans, rhamnogalactans, rhamnogalacturonans and glucan have been found to influence the immune response (Paulsen, 2001). Plant polysaccharides have been shown to influence immune cells like macrophages, T and B lymphocytes, NK cells as well as the production of complement proteins and cytokines. There us much evidence suggesting that the effect of polysaccharide on the activity of macrophages vary depending on their type and source. Certain isolated plant polysaccharide like KMCP (arabinogalactan

isolated from *Ixeris polycephala*), BRP-4 (*Basella rubra* pectin-type polysaccharide), SF1, SF2 (Safflower polysaccharides) etc., were shown to stimulate the release of NO in mouse peritoneal macrophages and/or macrophage RAW264.7. Conversely however, other polysaccharides like GG (Galactosaminogalactan), SGG (urea soluble galactosaminogalactan), and a burdock (*Arctium lappa*) polysaccharide (BP) had inhibitory effect on the NO release in LPS stimulated macrophages. While the polysaccharide SBF inhibited cytokine release, CPE-II, a polysaccharide component of *Citrus unshiu* enhanced the TNF-α, IL-6 and IL-12 release in RAW264.7 cells. Another polysaccharide component AP-AU-1, obtained from *Alchornea cordifolia*, also supressed IL-1β, IL-6, IL-10, TNF-α and GM-CSF in human and mouse macrophages. It was reported that plant polysaccharides can exert their immunomodulating activity by binding receptors like TLR4, CD14, complement receptor 3 (CR3), scavenger receptor (SR), mannose receptor (MR), and Dectin-1 (belonging to C-type lectin receptor (CLR) family) in the macrophages (Meijerink et al., 2018).

The dietary β1,3-linked glucans found in mushrooms was found to influence myeloid cell maturation, cytokine release as well as increased respiration and production of ROS in phagocytic cells by binding to dectin-1 (Meijerink et al., 2018). AQP70-3 is an arabinofuranan isolated from *Akebia quinate*. In LPS induced RAW264.7 cells, the polysaccharide stimulated their phagocytic activity, enhance the release of NO, promote production of IL-6, IL-1β and TNF-α. In a zebrafish model also the compound enhanced release of NO and ROS (Wang et al., 2021).

Chronic inflammation in general has been associated to low fibre diet. The prevailing idea is that insufficient fibre intake hinders the development of a healthy, diverse gut microbiota, leading to reduced production of short-chain fatty acids (SCFAs) like butyrate. Reduced butyrate production can impact inflammatory pathways like NF-κB signalling as well as inhibition of deacetylase which in turn can affect gut-wall integrity. Butyrate also influences gene expression related to glutathione and uric acid metabolism thereby reducing oxidative stress. This negative feedback linked low intake of dietary fibre can affect gut-wall integrity and contribute to inflammation in conditions such as inflammatory bowel disease. A cross-sectional investigation of over 140 overweight Hispanic and African-American adolescents showed that the group with the highest fibre intake had significantly lower plasma markers of inflammation. A 36% and 43%

reduction in plasminogen activator inhibitor-1 (PAI-1) and resistin levels, respectively was observed in comparison to the group with lowest fibre intake. Similar results were noted for insoluble fibre. However, in another study, data from the Airwave Health Monitoring Study, involving 6,898 participants, showed an inverse relationship between fibre intake and inflammatory markers such as C-reactive protein (CRP), as well as body composition indicators like BMI, body fat percentage, and waist circumference. The study suggests that the reduction in inflammation (measured by CRP) could be mediated by changes in body composition rather than the direct effect of fibre. Although the fact that dietary fibre does seem to play some role in the inflammatory response, more conclusive on-ground research and intense analytical investigation needs to be conducted to establish the signalling cascades associated to dietary fibre and their impact on immune response (Barber et al., 2020).

2.3.9. Others

Besides plant secondary metabolites, polysaccharides and lipids, plant peptides have also garnered much attention in the recent years for their ability to modulate immune system. Plant based peptides are short proteins composed of 2-23 specific amino acids mainly, proline, arginine and lysine which remain inactive when present in the parent protein sequence but can be released by enzymatic hydrolysis. Different peptides can have different effects depending on their amino acid profile, chemical structures as well as peptide sequence. A study showed that a peptide (GYPMYPLPR) found in rice albumin (*oryzatensin*) induced phagocytic activity in immune cells. Another identified peptide (YGIYPR) from rice protein, was found to be effective in significantly enhancing the proliferation of RAW 264.7 macrophages. In RAW 264.7 cells a wheat germ globulin was found to promote release of pro-inflammatory cytokines as well as induce rapid multiplication of lymphocytes. From soybean (*Glycine max*), lunasin and lunasin-like peptides were found to inhibit pro-inflammatory mediators, especially peptide with 5 kDa molecular mass was found to be very effective in suppressing production of IL-1b, IL-6 and NO, as well as the expression of PGE2, COX-2, iNOS along with inhibition of p65 and p50 nuclear translocation and NF-kB transactivation (Ashaolu et al., 2024).

2.4. Genus *Zanthoxylum*: An introduction

Zanthoxylum, commonly known as prickly ash or toothache tree (locally called 'Timbur') is a diverse genus of flowering plants belonging to the family Rutaceae. It encompasses approximately 234 species of trees and shrubs (POWO, 2024) , many of which are found in tropical, subtropical, and temperate regions around the world, particularly in Asia, Africa, and the Americas. *Zanthoxylum* species are known for their distinctive aromatic qualities, with leaves, bark, and fruit that often contain essential oils. These plants are perhaps best known for their culinary and medicinal importance. The dried husks of some species, particularly *Zanthoxylum piperitum* and *Zanthoxylum simulans*, are used to produce Sichuan pepper, a key spice in Chinese cuisine. The sharp, numbing sensation produced by Sichuan peppercorns is a result of compounds like hydroxy-α-sanshool, which activates nerve endings in a unique way. In traditional medicine, various species of *Zanthoxylum* have been utilized to treat ailments ranging from toothaches (hence the name "toothache tree") to digestive disorders. Some species have also been investigated for their potential antimicrobial, antioxidant, and anti-inflammatory properties. Ecologically, *Zanthoxylum* species often serve as host plants for the larvae of various butterflies and other insects. The genus is also important in reforestation efforts in some tropical areas due to its hardiness and adaptability (Okagu et al., 2021).

Overall, *Zanthoxylum* is a highly versatile genus with significant culinary, medicinal, and ecological value, making it a subject of interest in various scientific fields.

2.4.1. *History*

In the first volume of Species Plantarum published in 1753, the genus *Zanthoxylum* was first officially described by Carl Linnaeus. The first species that was described was *Zanthoxylum trifoliatum* (Linné, 1753). The name *Zanthoxylum* is a derivation from ancient Greek words 'xanthos' meaning yellow and 'xylon' meaning wood (Quattrocchi 1999). The genus belongs to the family Rutaceae. It boasts an extensive fossil record, with the oldest fossils identified in Early Eocene deposits in England. Additional fossils of *Zanthoxylum* have been found in the Americas from the Late Oligocene onwards, and in Africa and Asia from the Miocene period onward (Appelhans et al. 2018). From Miocene epoch [23.03-5.3 million years ago (Steinthorsdottir et al., 2021)], 28 fossil

seeds of the now extinct *Zanthoxylum kristinae* was discovered in North Bohemia in Czech Republic (Holy et al., 2012). The genus shares a close phylogenetic relationship with the genera *Phellodendron*, *Tetradium*, and *Toddalia* due to similarities in their phytochemical compositions, and they are collectively classified as "proto-Rutaceae," one of the oldest lineages in the Rutaceae family. The migration of *Zanthoxylum* from the Old World to the Americas likely occurred during the Eocene, supported by fossil evidence from the Late Oligocene. Two key routes for this migration were the North Atlantic Land Bridges (NALB) and the Bering Land Bridge (BLB). The NALB was likely used during the Early Eocene, while the BLB became more plausible during the Oligocene due to the genus' adaptation to temperate climates. *Zanthoxylum* is unique in showing migrations via both routes. It also colonized Pacific islands, with *Z. mayu* diverging from South American species during the Eocene, before the Juan Fernández Islands formed (Appelhans et al. 2018).

Currently, the genus has pan-tropical distribution and can be found in all the continents, except Antarctica. Plants of the genus are generally dioecious and maybe shrubs, trees, or woody climbers. A most distinguishing character is their usually pinnate leaves, and thorns on their stems and main branches. Additionally, in several species, prickles can be found in the leaf rachis as well, occurring at stipule-like positions in pairs. The flowers usually grow in panicles and are functionally either male or female. They have four sepals and four petals, with the sepals remaining attached to the fruit. Male flowers feature four stamens positioned opposite the sepals, while female flowers contain up to five carpels that are mostly free, with styles that may be fused near the tip. The fruit generally consists of up to four follicles fused at the base, each holding a single seed nearly as large as the follicle itself and remain attached to the fruit via their funicle. The seeds are known for their shiny black pellicle (Appelhans et al. 2018).

2.4.2. Different species of genus Zanthoxylum

Worldwide, about 234 species of *Zanthoxylum* has been accepted by Plants of the World Online (POWO, 2024). All the species described have been listed in Table 2.1. World-wide percentage distribution of the species is presented in figure 2.6.

2.4.3. Species of Zanthoxylum found in Sikkim

The species of *Zanthoxylum* reported from Sikkim are *Z. acanthopodium* DC, *Z. armatum* DC, *Z. myriacanthum* Hook. f., *Z. ovalifolium* Wight, *Z. rhetsa* (Roxb.) DC (Dash and Singh, 2011), *Z. oxyphyllum* Edgew. (Grierson and Long, 1983) and *Z. tomentellum* Hook. f. (Hajra et al., 1997).

Table 2.1. Global distribution of different species of *Zanthoxylum* spp.

No.	Name of species	Native range of distribution
1.	*Zanthoxylum acanthopodium* DC.	Himalaya to Southern China and Western Malaysia
2.	*Zanthoxylum aculeatissimum* Engl.	Bolivia (La paz)
3.	*Zanthoxylum acuminatum* Sw.	Mexico to Tropical America
4.	*Zanthoxylum ailanthoides* Siebold & Zucc.	Southern China to temperate Eastern Asia and Philippines
5.	*Zanthoxylum albiflorum* Baker f.	New Caledonia
6.	*Zanthoxylum albuquerquei* D.R. Simpson	Peru
7.	*Zanthoxylum amamiense* Ohwi	Nansei-shoto (Ryukyu islands, Japan)
8.	*Zanthoxylum amapaense* (Albuq.) P.G.Waterm. (Brazil, Venezuela)	Eastern Venezuela to Western Brazil and French Guiana
9.	*Zanthoxylum americanum* Mill.	South-Eastern Canada to Central and Eastern U.S.A. and Mexico (Nuevo León, Tamaulipas)
10.	*Zanthoxylum amplicalyx* Reynel	Cuba
11.	*Zanthoxylum anadenium* (Urb. & Ekman) J. Jiménez Alm.	Haiti (Massif de la Hotte)
12.	*Zanthoxylum andamanicum* Kurz	Andaman Islands (India)
13.	*Zanthoxylum andinum* Reynel	Ecuador
14.	*Zanthoxylum anison* L.O.Williams	Guatemala
15.	*Zanthoxylum anthyllidifolium* Guillaumin	Southern Vietnam
16.	*Zanthoxylum apiculatum* (Sandwith) P. G. Waterman	Panama to Guyana and northern Bolivia
17.	*Zanthoxylum arborescens* Rose	Mexico, Nicaragua
18.	*Zanthoxylum armatum* DC.	Indian sub-continent to temperate Eastern Asia and Malaysia

19.	*Zanthoxylum asiaticum* (L.) Appelhans, Groppo & J.Wen	Ethiopia to Eswatini, West Indian Ocean, tropical and subtropical Asia
20.	*Zanthoxylum atchoum* (Aké Assi) P. G. Waterman	Côte d'Ivoire (Ivory Coast)
21.	*Zanthoxylum austrosinense* C. C. Huang	South-Eastern China (to South-Western Hubei)
22.	*Zanthoxylum avicennae* (Lam.) DC.	Southern China to Western and Central Malaysia
23.	*Zanthoxylum backeri* (Bakh. f.) T. G. Hartley	South-Western Java
24.	*Zanthoxylum bajarnandia* Wall. ex Hook.f.	Indian subcontinent
25.	*Zanthoxylum beecheyanum* K.Koch	Nansei-shoto (Daitojima), Ogasawara-shoto
26.	*Zanthoxylum bifoliolatum* Leonard	Hispaniola to Puerto Rico
27.	*Zanthoxylum bissei* Beurton	Eastern Cuba
28.	*Zanthoxylum bonifaziae* Cornejo & Reynel	Ecuador
29.	*Zanthoxylum bouetense* (Pierre ex Letouzey) P. G. Waterman	Gabon to Congo
30.	*Zanthoxylum brachyacanthum* F. Muell.	Eastern Queensland to North-Eastern New South Wales
31.	*Zanthoxylum brisasanum* (Cuatrec.) P. G. Waterman	Colombia to Peru
32.	*Zanthoxylum brisoferox* Reynel	Columbia
33.	*Zanthoxylum buesgenii* (Engl.) P.G.Waterman	Southern Nigeria to West Central tropical Africa
34.	*Zanthoxylum bungeanum* Maxim.	Himalaya to China
35.	*Zanthoxylum burkillianum* Babu	Arunachal Pradesh (India)
36.	*Zanthoxylum calcicola* C. C. Huang	China (South East Yunnan, South West Guizhou, western Guangxi)
37.	*Zanthoxylum campicola* Reynel	Colombia to Ecuador
38.	*Zanthoxylum canalense* (Guillaumin) P.G.Waterman	New Caledonia
39.	*Zanthoxylum capense* (Thunb.) Harv.	South tropical and Southern Africa
40.	*Zanthoxylum caribaeum* Lam.	Mexico to tropical America
41.	*Zanthoxylum caudatum* Alston	Sri Lanka
42.	*Zanthoxylum celebicum* Koord.	Sulawesi
43.	*Zanthoxylum chalybeum* Engl.	Belize, Guatemala, Honduras
44.	*Zanthoxylum chevalieri* P.G.Waterman	Western tropical Africa
45.	*Zanthoxylum chocoense* Reynel	North-west Colombia
46.	*Zanthoxylum chuquisaquense* Reynel	Bolivia

47.	*Zanthoxylum ciliatum* Engl.	Mexico to Northern Venezuela
48.	*Zanthoxylum claessensii* (De Wild.) P. G. Waterman	West Central tropical Africa
49.	*Zanthoxylum clava-herculis* L.	Central and South-Eastern United States to Eastern Mexico
50.	*Zanthoxylum coco* Gillies ex Hook.f. f. & Arn.	Argentina, Bolivia, Paraguay
51.	*Zanthoxylum collinsiae* Craib	China to Indo-China
52.	*Zanthoxylum comosum* (Theodor Carl Julius Herzog|Herzog) P.G.Waterman	Central Bolivia
53.	*Zanthoxylum compactum* (Huber ex Albuq.) P.G.Waterman	Venezuela to Western South America
54.	*Zanthoxylum complexum* Reynel	Ecuador
55.	*Zanthoxylum conspersipunctatum* Merr. & Perry	New Guinea
56.	*Zanthoxylum cucullatipetalum* Guillaumin	Vietnam
57.	*Zanthoxylum davyi* (I.Verd.) P.G.Waterman	Zimbabwe to South Africa
58.	*Zanthoxylum decaryi* H.Perrier	Madagascar
59.	*Zanthoxylum delagoense* P.G.Waterman	Southern Mozambique
60.	*Zanthoxylum deremense* (Engl.) Kokwaro	Malawi, Tanzania
61.	*Zanthoxylum dimorphophyllum* Hemsl.	Southern China to Indo-China, Taiwan
62.	*Zanthoxylum dinklagei* (Engl.) P.G.Waterman	Southern Nigeria to Western Central tropical Africa
63.	*Zanthoxylum dipetalum* H.Mann – Kāwa‘u	Hawaii
64.	*Zanthoxylum dissitum* Hemsl.	Central to Southern China to Hainan
65.	*Zanthoxylum djalma-batistae* (Albuq.) P.G.Waterman	Brazil
66.	*Zanthoxylum domingense* (Krug & Urb.) J.Jiménez Alm.	Haiti
67.	*Zanthoxylum dumosum* A.Rich.	Cuba
68.	*Zanthoxylum echinocarpum* Hemsl.	Southern China
69.	*Zanthoxylum ekmanii* (Urb.) Alain	Southern Mexico to Southern tropical America, Western Cuba
70.	*Zanthoxylum eliasii* D.M.Porter	Panama to Peru
71.	*Zanthoxylum esquirolii* H.Lév.	South Central China
72.	*Zanthoxylum fagara* (L.) Sarg.	Tropical and sub-tropical America
73.	*Zanthoxylum fauriei* (Nakai) Ohwi	South Korea, Japan
74.	*Zanthoxylum finlaysonianum* Wall.	Thailand
75.	*Zanthoxylum flavum* Vahl	Caribbean
76.	*Zanthoxylum foliolosum* Donn.Sm.	Mexico to Nicaragua

77.	*Zanthoxylum forbesii* T.G.Hartley	Sumatra
78.	*Zanthoxylum formiciferum* (Cuatrec.) P.G.Waterman	Colombia to Northern Peru
79.	*Zanthoxylum gardneri* Engl	Brazil
80.	*Zanthoxylum gentryi* Reynel	Western Colombia
81.	*Zanthoxylum gillespieanum* (A.C.Sm) A.C.Sm.	Fiji
82.	*Zanthoxylum gilletii* (De Wild.) P.G.Waterman	tropical Africa
83.	*Zanthoxylum glomeratum* C.C.Huang	China
84.	*Zanthoxylum grandifolium* Tul.	Colombia
85.	*Zanthoxylum haitiense* (Urb.) J.Jiménez Alm.	Haiti
86.	*Zanthoxylum hamadryadicum* Pirani	North-Eastern Brazil
87.	*Zanthoxylum harrisii* P.Wilson	Jamaica
88.	*Zanthoxylum hartii* (Krug & Urb.) P.Wilson	Jamaica
89.	*Zanthoxylum hawaiiense* Hillebr.	Hawaii
90.	*Zanthoxylum heitzii* (Aubrév. & Pellegr.) P.G.Waterm.	West Central tropical Africa
91.	*Zanthoxylum heterophyllum* (Lam.) Sm.	Mascarene islands
92.	*Zanthoxylum holtzianum* (Engl.) P.G.Waterman	Southern Somalia to Northern Mozambique
93.	*Zanthoxylum huangianum* Z.H.Chen & Feng Chen	South east China
94.	*Zanthoxylum huberi* P. G. Waterman	South Colombia to Venezuela (Bolívar) and Peru
95.	*Zanthoxylum humile* (E. A. Bruce) P. G. Waterman	Zimbabwe to Mozambique and Mpumalanga
96.	*Zanthoxylum impressinervium* Reynel	Colombia
97.	*Zanthoxylum impressocordatum Reynel*	Colombia
98.	*Zanthoxylum integrifoliolum* (Merr.) Merr.	Taiwan (Lan Yü) to Philippines
99.	*Zanthoxylum integrum* Aver.	Vietnam
100.	*Zanthoxylum iwahigense* Elmer	Philippines (Palawan)
101.	*Zanthoxylum jamaicense* P. Wilson	Jamaica
102.	*Zanthoxylum kallunkiae* Reynel	Ecuador
103.	*Zanthoxylum kauaense* A.Gray	Hawaii
104.	*Zanthoxylum khasianum* Hook.f.	Assam to China (West Yunnan)
105.	*Zanthoxylum kleinii* (R. S. Cowan) P. G. Waterman	Brazil
106.	*Zanthoxylum kwangsiense* (Hand. - Mazz.) Chun ex C. C. Huang	China (Chongqing, Southern Guizhou, Northern Guangxi)
107.	*Zanthoxylum laetum* Drake	China (Southern Yunnan to Guangdong) to Northern Vietnam

108.	*Zanthoxylum laurentii* (De Wild.) P.G.Waterman	Congo to Democratic Republic of Congo
109.	*Zanthoxylum leiboicum* C. C. Huang	China (South-Western Sichuan)
110.	*Zanthoxylum lemairei* (De Wild.) P.G.Waterman	Western tropical Africa to Uganda
111.	*Zanthoxylum lenticellosum* (Urb. & Ekman) J. Jiménez Alm.	Dominican Republic (Sierra de Ocoa)
112.	*Zanthoxylum lenticulare* Reynel	Colombia to North-Western Venezuela
113.	*Zanthoxylum lepidopteriphilum* Reynel	Ecuador to Peru, North-Western Venezuela
114.	*Zanthoxylum leprieurii* Guill. & Perr.	Tropical and Southern Africa
115.	*Zanthoxylum leratii* Guillaumin	New Caledonia
116.	*Zanthoxylum liboense* C. C. Huang	China (southern Guizhou)
117.	*Zanthoxylum limoncello* Planch. & Oerst.	Mexico to Colombia
118.	*Zanthoxylum lindense* (Engl.) Kokwaro	Eastern Tanzania
119.	*Zanthoxylum macranthum* (Hand.-Mazz.) C.C. Huang	South-Eastern Tibet to Southern China
120.	*Zanthoxylum madagascariense* Baker	Madagascar
121.	*Zanthoxylum magnifasciculatum* Reynel	Venezuela
122.	*Zanthoxylum magnifructum* Reynel	Colombia
123.	*Zanthoxylum mananarense* H. Perrier	Madagascar
124.	*Zanthoxylum mantaro* (J. F. Macbr.) J. F. Macbr.	Ecuador to Peru
125.	*Zanthoxylum maranionense* Reynel	Peru
126.	*Zanthoxylum martinicense* (Lam.) DC.	Caribbean to Northern Venezuela, Western South America
127.	*Zanthoxylum mauriifolium* Reynel	Ecuador
128.	*Zanthoxylum mayu* Bertero	Juan Fernández Islands
129.	*Zanthoxylum megistophyllum* (B. L. Burtt) T. G. Hartley	Solomon islands
130.	*Zanthoxylum melanostictum* Schltdl. & Cham.	Mexico to North-West Venezuela and Peru
131.	*Zanthoxylum mezoneurispinosum* (Aké Assi) W. D. Hawth.	Liberia to Ivory Coast
132.	*Zanthoxylum micranthum* Hemsl.	Central and East Central China
133.	*Zanthoxylum mildbraedii* (Engl.) P. G. Waterman	East central tropical Africa to South-Western Kenya
134.	*Zanthoxylum minahassae* Koord.	Sulawesi
135.	*Zanthoxylum molle* Rehder	Southern China

136.	*Zanthoxylum mollissimum* (Engl.) P. Wilson	Western and Southern Mexico to Colombia
137.	*Zanthoxylum monogynum* A. St. - Hil.	Eastern Bolivia to Brazil
138.	*Zanthoxylum motuoense* C. C. Huang	South-Eastern Tibet
139.	*Zanthoxylum multijugum* Franch.	China (North and Central Yunnan, Guizhou)
140.	*Zanthoxylum myriacanthum* Wall. ex Hook. f.	Eastern Himalaya to Southern China and Western and Central Malaysia
141.	*Zanthoxylum myrianthum* (A. C. Sm.) P. G. Waterman	Fiji (Vanua Levu)
142.	*Zanthoxylum nadeaudii* Drake	French Polynesia (Tubuai, Society Islands)
143.	*Zanthoxylum nashii* P. Wilson	Cuba to Hispaniola
144.	*Zanthoxylum nebuletorum* (Herzog) P. G. Waterman	Bolivia
145.	*Zanthoxylum nemorale* Mart.	Eastern Brazil
146.	*Zanthoxylum neocaledonicum* Baker f.	New Caledonia
147.	*Zanthoxylum nigrum* Mart.	Brazil (Bahia, Rio de Janeiro)
148.	*Zanthoxylum nitidum* (Roxb.) DC.	Tropical and Sub-tropical Asia to North-Eastern Australia
149.	*Zanthoxylum novoguineense* T. G. Hartley	New Guinea
150.	*Zanthoxylum oahuense* Hillebr.	Hawaii
151.	*Zanthoxylum oreophilum* (Guillaumin) P.G.Waterman	New Caledonia
152.	*Zanthoxylum ovalifolium* Wight	Tropical Asia to North-Eastern Queensland
153.	*Zanthoxylum ovatifoliolatum* (Engl.) Finkelstein	Angola to North-Western Namibia
154.	*Zanthoxylum oxyphyllum* Edgew.	Nepal to China (Western Yunnan) and Myanmar
155.	*Zanthoxylum palustre* Londoño-Ech. & Reynel	Colombia, Brazil
156.	*Zanthoxylum panamense* P. Wilson	Mexico (Chiapas) to Central America
157.	*Zanthoxylum pancheri* P. S. Green	New Caledonia
158.	*Zanthoxylum paniculatum* Balf. f.	Rodrigues (Mauritius)
159.	*Zanthoxylum paracanthum* (Mildbr.) Kokwaro	South-Eastern Kenya to Central Tanzania
160.	*Zanthoxylum paulae* (Albuq.) P. G. Waterman	The Guianas to Northern Brazil
161.	*Zanthoxylum pentandrum* (Aubl.) R. A. Howard	Trinidad to Northern Brazil
162.	*Zanthoxylum petenense* Lundell	Mexico (Veracruz) to Guatemala

163.	*Zanthoxylum petiolare* A. St. -Hil. and Tul.	Bolivia to Brazil and Northern Argentina
164.	*Zanthoxylum phyllopterum* (Griseb.) C.Wright	Cuba
165.	*Zanthoxylum piasezkii* Maxim.	China (Sichuan to Southern Shaanxi and Western Henan)
166.	*Zanthoxylum pilosiusculum* (Engl.) P. G. Waterman	Democratic Republic of Congo to Angola
167.	*Zanthoxylum pilosulum* Rehder and E. H. Wilson	China (Sichuan, Southern Gansu, Southern Shaanxi)
168.	*Zanthoxylum pimpinelloides* (Lam.) DC.	Cuba to Hispaniola
169.	*Zanthoxylum pinnatum* (J. R. Forst. and G. Forst.) W. R. B. Oliv.	Norfolk Island, Lord Howe Island, South-West Pacific
170.	*Zanthoxylum piperitum* (L.) DC.	China to South Korea, Japan
171.	*Zanthoxylum pluviatile* T.G.Hartley	New Guinea and Solomon Islands
172.	*Zanthoxylum pluvimontanum* Reynel & D.Tarazona	Ecuador
173.	*Zanthoxylum poggei* (Engl.) P. G. Waterman	West-Central tropical Africa
174.	*Zanthoxylum psammophilum* (Aké Assi) P. G. Waterman	Ivory coast
175.	*Zanthoxylum pseudoxyphyllum* Babu	India (Arunachal Pradesh to Assam)
176.	*Zanthoxylum pteracanthum* Rehder and E. H. Wilson	China (Western Hubei)
177.	*Zanthoxylum pucro* D. M Porter	Panama to North-Western Colombia
178.	*Zanthoxylum punctatum* Vahl	Caribbean
179.	*Zanthoxylum quassiifolium* (Donn. Sm.) Standl. and Steyerm.	Mexico (Veracruz, Chiapas) to Guatemala
180.	*Zanthoxylum quinduense* Tul.	Western South America to North-Western Venezuela
181.	*Zanthoxylum renieri* (G. C. C. Gilbert) P. G. Waterman	Democratic Republic of Congo to Uganda
182.	*Zanthoxylum retroflexum* T. G. Hartley	Sumatera, Borneo
183.	*Zanthoxylum retusum* (Albuq.) P. G. Waterman	Eastern Brazil
184.	*Zanthoxylum rhetsa* (Roxb.) DC.	Tropical Asia to Northern Australia
185.	*Zanthoxylum rhodoxylon* (Urb.) P.Wilson	Jamaica
186.	*Zanthoxylum rhoifolium* Lam.	Tropical and sub-tropical America
187.	*Zanthoxylum rhombifoliolatum* C. C. Huang	China (Chongqing, Guizhou)

188.	*Zanthoxylum riedelianum* Engl.	Mexico to Southern tropical America
189.	*Zanthoxylum rigidum* Humb. and Bonpl. ex Willd.	Western South America to Paraguay
190.	*Zanthoxylum rubescens* Planch. ex Hook.	Western tropical Africa to South-Western Kenya and Angola
191.	*Zanthoxylum sambucirhachis Reynel*	Ecuador
192.	*Zanthoxylum sarasinii* Guillaumin	New Caledonia
193.	*Zanthoxylum scandens* Blume	Assam (India) to Nansei-shoto and Western Malaysia
194.	*Zanthoxylum schinifolium* Siebold and Zucc.	Central and Eastern China to temperate Eastern Asia
195.	*Zanthoxylum schlechteri* Guillaumin	New Caledonia
196.	*Zanthoxylum schreberi* (J. F. Gmel.) Reynel ex C. Nelson	Mexico to Venezuela and Bolivia, Caribbean
197.	*Zanthoxylum setulosum* P. Wilson	Costa Rica to North-Western Venezuela
198.	*Zanthoxylum simulans* Hance	China, South Korea, Taiwan
199.	*Zanthoxylum spinosum* (Sw.) Sw.	Florida to Caribbean
200.	*Zanthoxylum sprucei* Engl.	Ecuador to Peru and Western Brazil
201.	*Zanthoxylum stelligerum* Turcz.	Central and Eastern Brazil
202.	*Zanthoxylum stenophyllum* Hemsl.	Central and East central China
203.	*Zanthoxylum subspicatum* H. Perrier	Madagascar
204.	*Zanthoxylum syncarpum* (Tul.) Tul. ex B. D. Jacks.	Venezuela, Eastern Brazil
205.	*Zanthoxylum taediosum* A. Rich.	Cuba
206.	*Zanthoxylum tambopatense* Reynel	Peru
207.	*Zanthoxylum tenuipedicellatum* (Kokwaro) Vollesen	South east Tanzania to north east Mozambique
208.	*Zanthoxylum tetraphyllum* (Urb. and Ekman) J. Jiménez Alm.	Haiti (Massif de la Selle)
209.	*Zanthoxylum tetraspermum* Wight and Arn.	Southern India, Sri Lanka
210.	*Zanthoxylum thomense* (Engl.) A. Chev. ex P. G. Waterman	Gabon and Gulf of Guinea Island
211.	*Zanthoxylum thorncroftii* (I.Verd.) P.G.Waterman	Limpopo to Mpumalanga
212.	*Zanthoxylum thouvenotii* H.Perrier	Madagascar
213.	*Zanthoxylum tidorense* Miq.	Maluku Islands
214.	*Zanthoxylum timoriense* Span.	Lesser Sunda Islands (Timor)
215.	*Zanthoxylum tingana* Reynel	Ecuador

216.	*Zanthoxylum tingoassuiba* A. St. - Hil.	Eastern and Southern Brazil
217.	*Zanthoxylum tomentellum* Hook.f.	Nepal to China (North-Western Yunnan) and Northern Myanmar
218.	*Zanthoxylum tragodes* (L.) DC.	Caribbean
219.	*Zanthoxylum trijugum* (Dunkley) P. G. Waterman	Central Tanzania to Zambia
220.	*Zanthoxylum tsihanimposa* H. Perrier	Madagascar
221.	*Zanthoxylum undulatifolium* Hemsl.	China (North-Eastern Yunnan to Southern Shaanxi)
222.	*Zanthoxylum unifoliolatum* Groppo & Pirani	Brazil
223.	*Zanthoxylum usambarense* (Engl.) Kokwara	Ethiopia to Rwanda
224.	*Zanthoxylum usitatum* Pierre ex Laness.	Northern Vietnam
225.	*Zanthoxylum venosum* Leonard	Hispaniola
226.	*Zanthoxylum verrucosum* (Cuatrec.) P. G. Waterman	Colombia to Ecuador
227.	*Zanthoxylum vinkii* T. G. Hartley	New Guinea
228.	*Zanthoxylum viride* (A. Chev.) P. G. Waterman	West tropical Africa to Congo
229.	*Zanthoxylum vitiense* A. C. Sm.	Fiji (Viti Levu, Vanua Levu)
230.	*Zanthoxylum wutaiense* I. S. Chen	Taiwan (Pingdong)
231.	*Zanthoxylum xichouense* C. C. Huang	China (South-Eastern Yunnan)
232.	*Zanthoxylum yakumontanum* (Sugim.) Nagam.	Japan (Yakushima)
233.	*Zanthoxylum yuanjiangensis* C. C. Huang	China (Southern Yunnan)
234.	*Zanthoxylum zanthoxyloides* (Lam.) Zepern. and Timler	Western tropical Africa

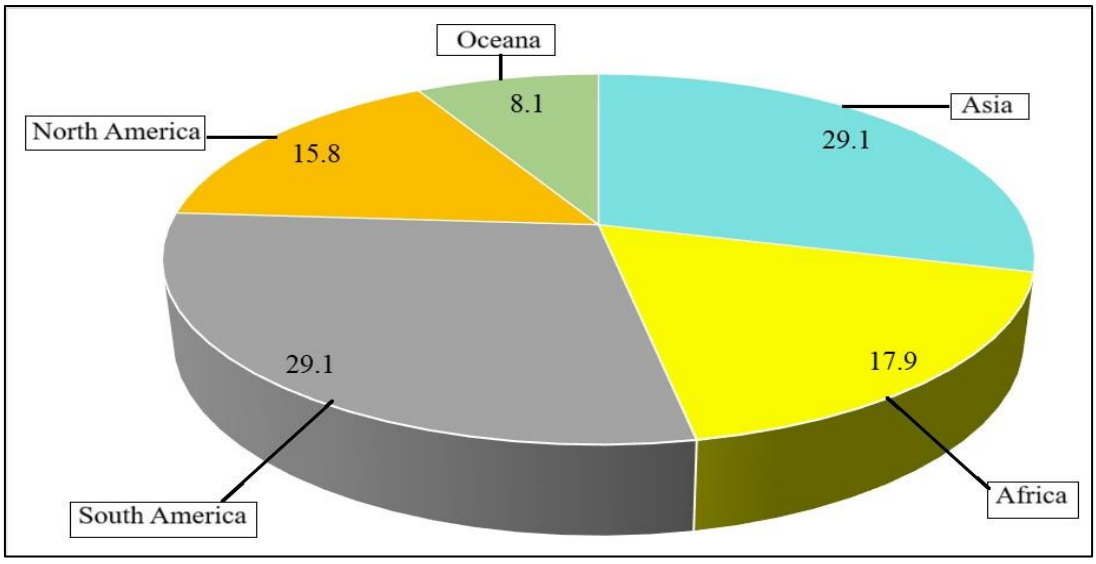

Figure 2.6. Percentage wise global distribution of *Zanthoxylum* species.

2.4.4. Taxonomic position of Z. oxyphyllum and Z. acanthopodium

- Taxonomic position of *Z. oxyphyllum* (POWO, 2024)

  Kingdom - Plantae
  Phylum - Streptophyta
  Class - Equisetopsida
  Subclass - Magnoliidae
  Order - Sapindales
  Family - Rutaceae
  Genus - *Zanthoxylum*
  Species - *Zanthoxylum oxyphyllum* Edgew.

- Taxonomic position of *Z. acanthopodium* (POWO, 2024)

  Kingdom - Plantae
  Phylum - Streptophyta
  Class - Equisetopsida
  Sub-class - Magnoliidae
  Order - Sapindales
  Family - Rutaceae
  Genus - *Zanthoxylum*
  Species - *Zanthoxylum acanthopodium* DC.

2.4.5. Morphology of Z. oxyphyllum and Z. acanthopodium

Z. oxyphyllum maybe climbing shrubs or scandent and upto 5 m in height. The branchlets are terete, usually armed with scattered, retrorse or rarely psudo-stipular, straight and brownish prickles that maybe 1.5 cm in length. Leaves are imparipinnate and up to 30 cm long. The petiole and rachis are grooved above. Leaflets maybe 5-29 or up to 25 but rarely, 3-foliate leaves can occur on the same branchlets. These leaflets are alternate to opposite, ovate-lanceolate or oblong with the younger ones being linear-lanceolate, acute to cuneate or obtuse to rounded at base, acuminate at apex, acumen retuse at tip, crenate to glandular-serrate along margins, 2,-10 x 1 – 4 cm, chartaceous or coriaceous, glabrous and glossy above. The midnerve is prickly from beneath and secondary nerves are 5-16 in paris and spreading. Petiolules are 3-7 mm long. Inflorescence are terminal and axillary, 3-12 cm

in length, glabrous to puberulent with the peduncle often being prickly. Flowers are arranged in umbels or clusters of cymes and 5-7 mm long. Males flowers have linear-lanceolate bracts that are 1.5-2 mm long. The pedicels are glabrous to puberulent and 3-6 mm in length. Sepals are four, ovate-triangular or rarely rounded, obtuse, 1-1.5 mm long and glabrous or ciliate with an apical gland. Petals are four in number, ovate-elliptic, 3-5 mm long, fleshy, obtuse and the mid-nerve is prominent with spreading lateral veinlets. Stamens are 4,6-7 mm long with oblong anthers that are 1-1.5 mm long and yellowish. Disk is pulvinate and 1-1.5 mm high. Pistillodes are 1 or 2 in number and ca 1.5 mm high. The female flowers have same bracts, sepals, petals and disk as in male flowers. Staminodes are absent. Gynoecium is 5-carpellate with ovoid carpels that are compressed, 4-5 mm long and glandular-punctate. Styles are coherent, ca 2 mm long. Stigma is cohering to a peltate disk and capitate. Fruiting pedicel is 5 -1 0 mm long. Follicles are 4-2 in number with 0, 1, 2, or 3 persistent abortive carpels with each follicle being sub-globose, apiculate, 5-6 mm across and pustular. The seeds are sub-globose, ca 4 mm across and black (Hajra et al., 1997) (Figure 2.7). Synonyms of *Z. oxyphyllum* have been specified in the official website of Plants of the World Online (POWO, 2024) as:

Homotypic Synonyms:

1. *Fagara oxyphylla* (Edgew.) Reeder and S. Y. Cheo

Heterotypic Synonyms:

1. *Zanthoxylum alpinum* C. C. Huang
2. *Zanthoxylum taliense* C. C. Huang
3. *Zanthoxylum tibetanum* C. C. Huang

Local names:

Nepali- Laharey timbur

Lepcha- Siritak dangji

Figure 2.7. *Z. oxyphyllum*: a) habit, b) flowers, c) leaves, d) fruits

Z. acanthopodium is a shrub or small tree that can grow up to 6 m in height. The branchlets are cylindric, armed with pseudo-stipular, or rarely scattered, straight or incurved, compressed, reddish brown prickles about 25 mm in length which may be ferruginous-pubescent. Barks are grey-brownish and lenticellate. Leaves are three, foliate or imparipinnate and up to 25 cm long. Petiole and rachis are armed with compressed prickles on both sides, ferruginous-pubescent to glabrate, narrowly to broad winged. These wings are 2.5-6 mm broad and present on either side. Leaflets are 3-15, opposite, subsessile, ovate-lanceolate to elliptic-lanceolate or oblong, obtuse to cuneate and slightly oblique at base, acute to acuminate or rarely obtuse at apex, subentire to glandular-serrate along margins, 1- 11 x 0.5 – 3 cm, glossy green above and light greenish beneath, chartaceous, glabrous to ferruginous-pubescent especially on lower surface. Midnerve is armed with flattened prickles above and beneath. Secondary nerves are prominent, in 5-30 pairs and reticulate. Inflorescence is axillary, paniculate and up to 2 cm in length. They are dense and ferruginous-pubescent. Male flowers are ca 3 mm long with minute bracts that can be ca 0.5 mm long and hairy. Pedicels are slender, 1.5-3 mm long and pubescent. Perianth is uniseriate or irregularly seriate, with the segments being 5-8 in number,

lanceolate or ligulate, acute or obtuse, 1 – 1.5 mm long, greenish or yellow and hairy. Stamens are 4-6 in number with linear filaments, ca 0.8 mm high. Pistillodes are 2-3 (-5). Female flowers are ca 2 mm long, with ca 1.5 mm long and pubescent pedicels. Perianth and disk are the same as in male flowers. Staminodes are absent. Ovary is 2-5 carpellate with each carpel being 1.5 mm long, ovoid, glandular-punctate and hairy or glabrous. Style is divergent, articulated at about 3 mm below globose stigma and ca 0.5 mm long. Follicles are 2-3 and carpels are 4-1 or 0 and caducous, abortive, ovoid-sub-globose, ca 4 mm across, reddish and pustular. Seeds are ca 3 mm across, black and shiny (Hajra et al., 1997) (Figure 2.8). Synonyms of *Z. acanthopodium* have been specified in the official website of Plants of the World Online (POWO, 2024) as:

Homotypic Synonyms:

1. *Fagara acanthopodium* (DC.) M. Hiroe

Heterotypic Synonyms:

1. *Zanthoxylum acanthopodium* var. *oligotrichum* Z. M. Tan
2. *Zanthoxylum acanthopodium* var. *timbor* Hook.f.
3. *Zanthoxylum acanthopodium* var. *villosum* C. C. Huang
4. *Zanthoxylum alatum* Wall.
5. *Zanthoxylum timbor* Wall.

Local names

Nepali- Bokey timbur

Lepcha- Kunupat

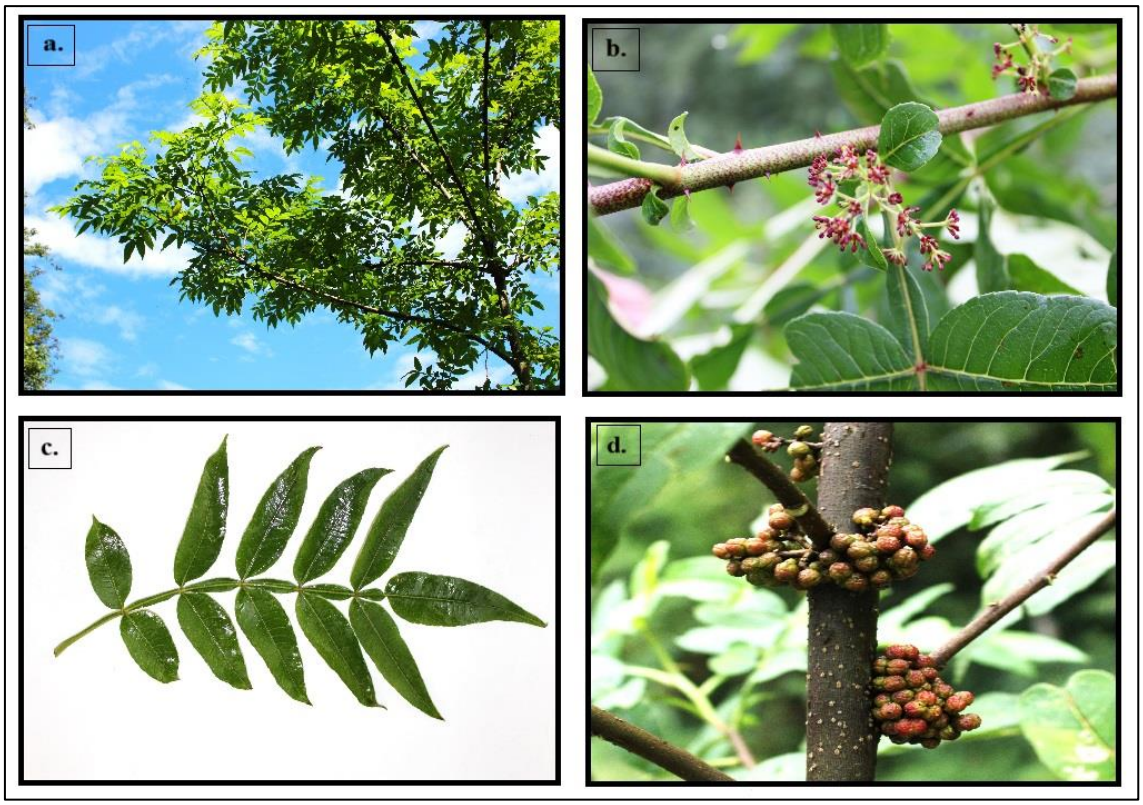

Figure 2.8. *Z. acanthopodium*: a) habit, b) flowers, c) leaves, d) fruits

2.4.6. Geographical distribution of Z. oxyphyllum and Z. acanthopodium

The native range of distribution of *Z. oxyphyllum* is Assam (India), south central China, eastern Himalaya, Myanmar, Nepal, and Tibet (POWO, 2024). Chukka, Thimphu, Punakha, Tongsa, upper Mo Chu, upper Kuru Chu and upper Kulong Chu districts in Bhutan in wooded valleys and riverbank shingles and altitudes ranging between 1980-2745 m (Grierson and Long, 1983). In India, the species is found in Sikkim, Darjeeling in West Bengal (Grierson and Long, 1983), lower Dibang valley in Arunachal Pradesh (Gupta and Mandi, 2013), Mokokchung in Nagaland (Ayangla et al., 2016), Jonai, Boko and Goalpara in Assam (Talukdar and Tayung, 2021), Khasi and Garo hills in Meghalaya (Momim et al., 2016) and Manipur (Hajra et al., 1997) (Figure 2.9).

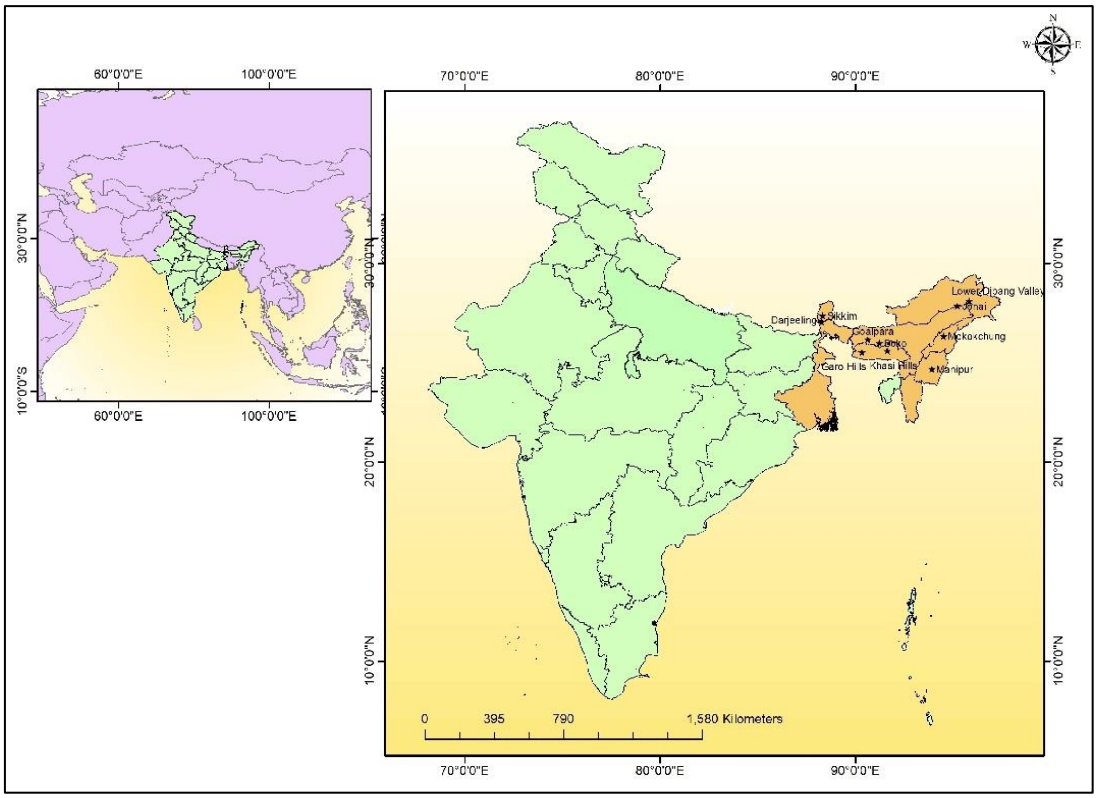

Figure 2.9. Distribution of *Z. oxyphyllum* Edgew. in India (Map courtesy: Mr. Phu Tshering Bhutia, not to scale).

Z. acanthopodium is distributed in Assam (India), Bangladesh, south central and south-east China, East Himalaya, Laos, Malaya, Myanmar, Nepal, Thailand, Tibet, and Vietnam (POWO, 2024). It is also found in Phuntsholing, Chukka, Deothang, Punakha, Tongsa and Mongar districts in Bhutan (Grierson and Long, 1983). In India, it can be found in Mizoram, Manipur (Hajra et al., 1997), Darjeeling in West Bengal (Grierson and Long, 1983), Sikkim (Dash and Singh, 2011) and Shillong in Meghalaya (Majumder et al., 2014) (Figure 2.10).

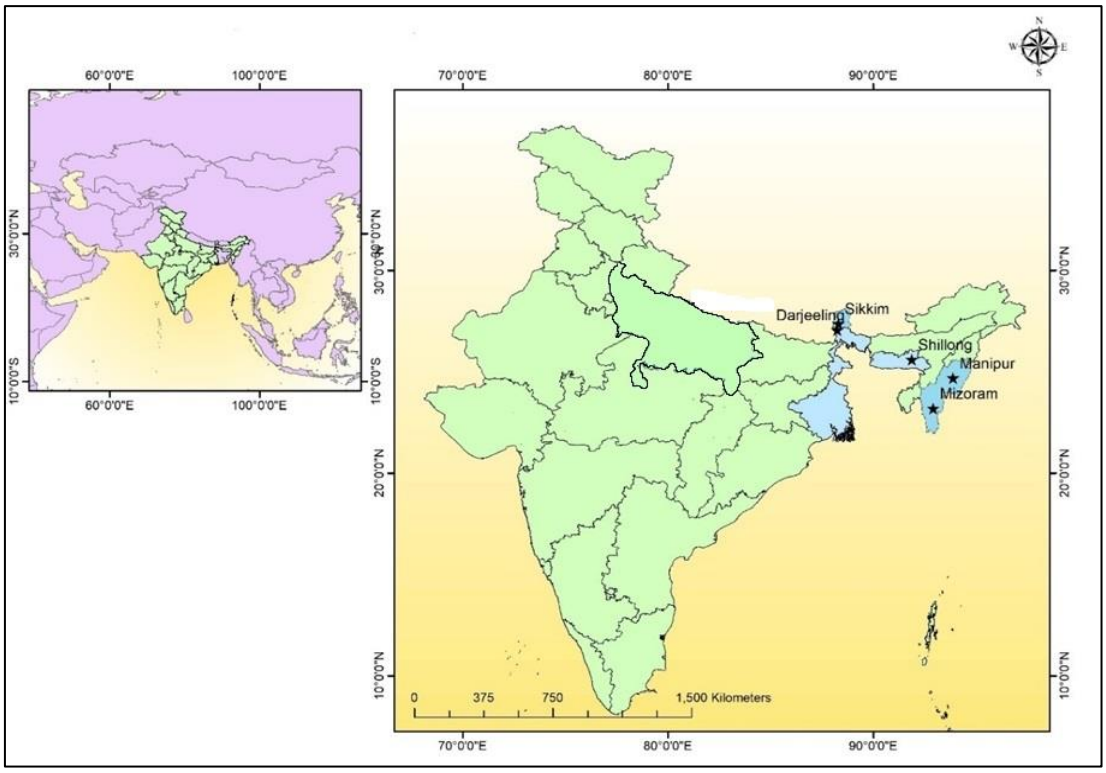

Figure 2.10. Distribution of *Z. acanthopodium* DC. in India (Map courtesy: Mr. Phu Tshering Bhutia, not to scale).

2.4.7. Local traditional uses of Z. oxyphyllum and Z. acanthopodium

The *Zanthoxylum* species have been used in traditional and local medicines as well as food source by ethnic populations around the globe. Traditionally, different parts of the plant species including fruits, seeds, leaves, and barks in various concoctions or as single components, have been reported to find their uses in the treatment of microbial infections, sickle cell anaemia, malaria, inflammation associated ailments, dental problems, and dispelling parasites from the body to name a few. In addition, it is well known that various secondary metabolites isolated from the species have shown potential as anti-oxidants, anti-inflammatory agents, anti-diabetic agents, anti-obesity agents etc. (Okagu et al., 2021). The stem-bark of *Z. acanthopodium* is used in Darjeeling Himalaya as an antipyretic agent (Rai, 2002) and the fruit of the same plant is chewed directly as antihepatopathic in Sikkim Himalaya (Chhetri *et al.,* 2008). *Z. oxyphyllum* and *Z. acanthopodium* also find their use in alleviating various ailments particularly by the tribes residing in north-eastern states of India (Table 2.2).

Table 2.2: Traditional uses, locality of use and parts used of *Z. oxyphyllum* and *Z. acanthopodium* in different parts of Himalaya

Plant species	Region	Vernacular name	Parts used	Traditional uses	Reference
Z. oxyphyllum	Nepal	Ban Timur, Lekh Timur (Np)	Fruit and flower	Paste from immature fruits used to relieve toothache; used for treating pain, tumour, fever, cholera, and snake bite	Uprety et al., 2016
	West Bengal: Nepali	Lahare timur, Bhainsi timbur (Np)	Bark and fruit	Appetizer; pickles used as source of food and as liver tonic	Rai, 2002; Saha, 2015
	Meghalaya (Jaintia)	Jaiur-blai (Kh), Mecheng (Ga)	Fruit, shoot leaf and seed	Pickled/cooked as food source by local tribes	Sawian et al., 2007; Momin et al., 2016
	Arunachal Pradesh: Adi, Monpa, Apatani	Onger/hibe (Ad), Mechme (Mo)	Leaf and Fruit	Treatment of stomach problems, asthma, bronchitis, and rheumatism	Kala, 2005; Chetry et al., 2018; Tsering et al., 2022
	Assam: Bodo Kachari	Mejenga (As)	Leaf, tender shoot and fruit,	Tender shoots eaten for blood purification, mitigating leukoderma, toothache, and dispelling tapeworms	Buragohain et al., 2011; Bhuyan, 2015; De, 2016
Z. acanthopodium	Nepal: Lepcha	Boke timur (Np); Gee (Sh), Thingne (Dz)	Leaf, seed, fruit, and bark	Used for curing toothache, expelling worms, relieving indigestion, fever and cholera	Bhattarai, 2013; Uprety et al., 2016
	Bhutan	-	Fruit	Used as medicine and condiment	Uprety et al., 2016
	Arunachal Pradesh: Monpa, Apatani	Yerchengma (Mo)	Leaf and fruit	Used for treatment of inflammation in liver and stomach, dysentery and stomach-ache	Kala, 2005; Tsering et al., 2022

	Sikkim: Bhutia Limboo Lepcha	Bokey timbur (Np) Narik (Bh)	Fruit and bark	Used for treating fever, cholera, indigestion, rheumatism, toothache, stomach-ache, gastritis, cold, as an appetizer, mouth-freshener, anthelminthic, anti-leech agent and treating hepatopathy	Chhetri et al., 2008; Badola and Pradhan, 2013; Lepcha et al., 2019; Chhetri et al., 2020
	West Bengal: Lepcha Nepali	Bokay timbur (Np), Kunupat (Lp)	Fruit, seed, bark,	Used for relieving gastritis, stomach colic, liver disorder, as anti-biotic, as an aromatic tonic for fever and cholera and as a remedy for toothache and hepatopathy	Rai, 2002; Chhetri et al., 2008

Abbreviations: Np = Nepali; Kh = Khasi; Ga = Garo; Ad = Adi; Mo = Monpa; As = Assamese; Sh = Sherpa; Dz = Dzongkha; Bh = Bhutia; Lp = Lepcha

2.5 Phytochemistry of *Zanthoxylum* species

The genus *Zanthoxylum* has a rich and unique phytochemical composition including terpenoid compounds, alkaloids and polyphenols.

2.5.1. Terpenes

Different types of terpenoid compounds, particularly monoterpenes, diterpenes and sesquiterpenes from *Zanthoxylum* species have been identified. Monoterpenes like α-fenchene, d-limonene, 2,6-dimethyl-2,4,6-octatriene, 3-carene and α-Thujene were identified in *Z. bungeanum*, *Z. asiaticum*, *Z. leprieurii*, *Z. rhetsa*, *Z. caribaeum*, *Z. zanthoxyloides* and *Z. fagara*. Sesquiterpenes like germacrene A, B, and D, germacrene D-4-ol, β-caryophyllene, α-humulene, germacrene, bicyclogermacrene, sabinene, isoledene, α-copaene, α-gurjunene, β-cedrene, α-curcumene, α-cadinene, α-calacorene, cadalene, valencene, β-patchoulene, β-elemene, (Z)-α-bisabolene, cis-α-bergamotene, α-muurolene, α-muurolol, spathulenol, globulol, epi-cubenol, caryophyllene oxide, cedrol,

α-acorenol, patchouli alcohol, bulsenol, α-bisabolol, germacrone, (2Z, 6E)-farnesol, cubebol, α-cadinol, gleenol, α-eudesmol, β-acorenol, 8-cedrene-13-olβ, (Z,E)-famesol, (Z,Z)-farnesol, β-acoradienol, α-selinene, α-cyperone, (-)-juniper camphor, E-carveol, nerol, cuminaldehyde, carvone, pipertitone, geraniol, phellandral, allo-aromadendrene, lupeol, (Z)-3-hexenal, undecan-2-one, citral, (E)-linalool oxide, hexanal, (Z)-3-hexenol and geranyl isobutyrate were identified in *Z. rhoifolium*, *Z. fagara*, *Z. caribaeum*, *Z. syncarpum*, *Z. rhetsa*, *Z. rubescens*, *Z. gilletii*, *Z. riedelianum*, *Z. armatum*, *Z. syncarpum*, *Z. zanthoxyloides*, *Z. schreberi*, *Z. elephantiasis*, *Z. clava-herculis*, *Z. tingoassuiba*, *Z. petiolare*, *Z. asiaticum*, *Z. sprucei*, *Z. quinduense*, *Z. lemairei* and *Z. flavum.* Diterpenes like myrcene, α-phellandrene, β-phellandrene, trans-β-ocimene, p-cymenene, terpinolene, camphene, (Z)-β-ocimene, (E)-β-ocimene, limonene, α-pinene, β-pinene, α-terpinene, p-cymene, geranyl acetate, γ-terpinene, citronellal and pentadecane were identified in *Z. rhetsa*, *Z. asiaticum*, *Z. leprieurii*, *Z. gilletii*, *Z. fagara*, *Z. caribaeum*, *Z. armatum*, *Z. zanthoxyloides*, *Z. bungeanum* and *Z. rhoifolium* (Mutinda et al., 2023). Additionally, isobauerenol, phenylethanol, bornylacetate, linalool, friedelin, transcaryophyllene, β-Amyrenone and β-Amyrin have also been isolated from the genus (Okagu et al., 2021).

In an analysis of the leaves of *Z. acanthopodium* collected from Manipur it was found that ield the leaves contain 58 compounds consisting of 75.6% of the oil obtained through hydrodistillation. The study showed that oxygenated monoterpenes constituted linalool (14.3%) and α-terpineol (1.5%), the oxygenated sesquiterpenes was composed of α-cadinol (2.2%), nerolidol (1.7%) and epi-α-cadinol (1.4%) being the main compounds while β-caryophyllene (3.0%) and germacrene D (1.9%) were the major sesquiterpene hydrocarbons (Rana and Blazquez, 2008).

2.5.2. Alkaloids

Several classes of alkaloids have been identified from the genus *Zanthoxylum*. Some unsaturated aromatic amides like N-methyl,N-(3 ,4- methylenedioxyphenylethyl), 3', 4-dimethoxycinnamoylamide, N-(3,4-methylenedioxyphenylethyl)-3'-4'-dimethoxycinnamoylamide and N-(3,4-dimethoxyphenylethyl), 3', 4' methylenedioxycinnamoylami-de were isolated from the stem bark of *Z. rubescens*. Benzophenanthridines alkaloids like (-)-6-acetonyldihydrochelerythrine, nornitidine, arnottianamide, nitidine, dihydronitidine, oxynitidine, fagaronine, dihydroavicine,

chelerythrine, dihyrochelerythine, norchelerythrine, oxychelerythrine, fagaridinel, rutaceline, sanguinarine, toddaline, 8-methoxydihydrochelerythrine, skimmianine, avicine, γ- fagarine, aculeatin, robustine, des-N-methylchelerythrine, oxyavicine and zanthoxyline were isolated from *Z. quinduense*, *Z. asiaticum*, *Z. bungeanum*, *Z. rubescens*, *Z. zanthoxyloides*, *Z. leprieurii*, *Z. paracanthum*, *Z. gillettii*, *Z. lemairie*, *Z. usambarense*, *Z. chalybeum*, *Z. fagara*, *Z. ailanthoides*, *Z. rhoifolium*, *Z. armatum*, *Z. rhetsa*, *Z. nitidum*, *Z. flavum*, *Z. usambarense*, *Z. thomense*, *Z. capense*, *Z. madagascariense*, *Z. austrosinense*, *Z. tsihanimposa*, Z. decaryi, *Z. avicennae* and *Z. integrifoliolum*. Quinoline alkaloids like N- methylflindersine, 4-methoxy-1-methyl-2-quinolone, integriquinolone, edulitine, 3-dimethylallyl-4-methoxy-2-quinolone, 8-methoxy-n-methylflindersine and zanthodioline were isolated from *Z. asiaticum*, *Z. tsihanimposa*, *Z. avicennae*, *Z. zanthoxyloides* and *Z. rhetsa*. Pyranoquinoline alkaloids like simulenoline, zanthosimuline, huajiaosimuline, peroxysimulenoline, decarine and benzosimuline were isolated from *Z. bungeanum*, *Z. austrosinense, Z. syncarpum*, *Z. ailanthoides* and *Z. integrifoliolum*. Aporphine alkaloids like bis-(4-hydroxy-2-keto-l-methyl-3-quinolinyl) methylene, tembetarine and magnoflorine were isolated from *Z. bungeanum*, *Z. rubescens*, *Z. zanthoxyloides*, *Z. gillettii*, *Z. leprieurii*, *Z. armatum* and *Z. anodynuin*. (Mutinda et al., 2023).

Most acridone alkaloids like 1-Hydroxy-10- Methylacridon-9-one, 1-hydroxy-3-methoxy-10-methyl-9-acridone, Hydroxy-1,3-dimethoxy-10-methyl-9-acridone, 3-hydroxy-1, 5,6-trimethoxy-9-acridone, 3-hydroxy-1,4- dimethoxy-10-methyl-9-acridone (helebelicine A), 1-hydroxy-3-methoxy-10-methyl-9-acridone, 4-hydroxyzanthacridone oxide (2,4'), 4-hydroxyzanthacridone, 4-methoxyzanthacridone, 3,4,5,7-tetrahydroxy-1-methoxy-10-methyl-9-acridone, 1,6-dihydroxy-3-methoxy-9-acridone, 3-hydroxy-1,2-dimethoxy-10-methyl-9-acridone (helebelicine B), 1,3-dihydroxy-4-methoxy-10-methyl-9-acridone, Tegerrardin A, 3-hydroxy-1-methoxy-10-methyl-9-acridone, 1-hydroxy-3-methoxy-10-methyl9-acridone, 1-hydroxy-2,3-dimethoxy-10-methyl-9-acridone, 1,2-dihydroxy-3-methoxy-10-methyl-9-acridone, 2,4′-hydroxyzanthacridone oxide and 1-hydroxy-2,3-dimethoxy-9-acridone were isolated from roots, stems and fruits of *Z. leprieurii* and *Z. zanthoxyloides*. Some aromatic amides have also been identified in the genus. Fagaramide, herclavine, lemairamin, lemairamide, N-isobutyl-cinnamamide, isoarnottianamide, piperlonguminine, rubemamine, rubemamide, rubescenamin, dioxamide, dioxamin, zanthosinamide, dihydroalatamide, zanthomamide, (-)-tembamide

and syncarpamide were identified in *Z. gilletii*, *Z. zanthoxyloides*, *Z. rubescens*, *Z. lemairie*, *Z. thomense*, *Z. lemairie*, *Z. ailanthoides*, *Z. tessmannii*, *Z. armatum*, *Z. syncarpum* and *Z. rhetsa*. Besides the different types of alkaloids described above, other types of alkaloids like quaternary alkaloids, N-cyclohexylamides, amide trans-cinnamoylamide, liphatic amides, canthinones, furoquinoline alkaloid, carbazole alkaloids, tetrahydroberberine alkaloids, benzylisoquinoline alkaloids, aporphine alkaloids, pyranoquinoline alkaloids and benzo[h]quinoline alkaloid have been identified in the genus (Mutinda et al., 2023).

2.5.3. Polyphenols

Several polyphenolic compounds, especially flavonoids, coumarins and lignans have been isolated from many *Zanthoxylum* species. Coumarins and their derivatives like umbelliferone, scopoletin, scoparone, xanthotoxin, imperatorin, bergapten, marmesin, collinin, 8-methoxyanisocoumarin H, acetoxyschinifolin, 7-(60 R-hydroxy-30,70 -dimethyl-20 E,70 -octadienyloxy) coumarin, 7-[(E)30,70-dimethyl-60-oxo-20,70-octadienyl]oxy-coumarin, 7-[(E)-70-hydroxy-30,70-dimethy-locta-20,50- dienyloxy]-coumarin, schinilenol, schinindiol, aurapten, auraptene, 8-methoxyanisocoumarin, pimpinelline, isopimpinellin, xanthyletin, osthol, xanthoxyletin, xanthotoxin, 6-formyl-7- methoxycoumarin, isoimperatorin, oxypeucedanin, psoralen, dipetaline, alloxanthoxyletin, toddasin, toddanol, toddanone, toddanone, toddacoumaquinone, toddacoumalone, toddalenone, 8- formyllimettin, norbraylin, trimethoxycoumarin, luvangetin and toddasiatin were isolated from *Z. armatum*, Z. *ailanthoides*, *Z. zanthoxyloides*, *Z. thomense*, *Z. rhetsa*, *Z. nitidum*, *Z. avicennae*, *Z. leprieurii*, *Z. tessmanni*, *Z. gillettii*, *Z. lemairie*, *Z. syncarpum*, *Z. flavum*, *Z. asiaticum*, *Z. schinifolium*, *Z. lemairie* and *Z. americanum* (Mutinda et al., 2023). Additionally, 8-formylalloxanthoxyletin, (Z)-avicennone, avicennol, avicennol methyl ether, cis-avicennol methyl ether, avicennin, aesculetin dimethyl ether, 7,8,9-trimethoxycoumarin, 7,8-dimethoxycoumarin, isoscopletin, zhebeiresinol, tetracosyl ferulate, schinicoumarin, acetoxyaurapten, epoxycollinin, schininallylol, epoxyaurapten, hydrangetin, acetoxycollinin, anisocoumarin, platydesmine, amottianamide, tetracosyl ferulate and 7-[(E)-7′ -hydroxy-3′ ,7′ dimethylocta-2′ ,5′ -dienyloxy] coumarin were also isolated from *Z. avicennae*, *Z. gilletii*, *Z. nitidum* and *Z. bungeanum* (Okagu et al., 2021).

Ligans and their derivatives like 2,3-Di(3'4' methylenedioxybenzyl)-2-buten-4-olide0, sesamin, syringaresinol, yangambin, 4'0 demethyl magnolin, kobusin, matairesinol, arctigenin, arctigenin methyl ether, hinokinin, savinin, asarinin, horsfieldin, fargesin, planispine A, pinoresinol-di-3,3-dimethylallyl, L -planinin, phylligenin, (+)-yangambin, eudesmin, (-)-hibalactone, (-)- methylpluviatolide, (-)-kaerophylin, (-)-hinokinin, (-)-cubebin, (7′S,8′S)-bilagrewin, (7′S,8′S)-5-demethoxybilagrewin, (7′S,8′S)-5-O-demethyl-4′-O-methylbilagrewin, (7′S,8′S)-nocomtal, (–)-5-methoxybalanophonin, wutaiensol methyl ether, demethoxywutaiensol methyl ether, methyl wutaiensate, methyl 7-hydroxyanodendroate, methyl 7-methoxyanodendroate, wutaifuranol, wutaipyranol, 7-methoxywutaifuranol, 7-methoxywutaifuranal, methyl wutaifuranate and methyl 7-methoxybenzofuran-5-carboxylate were isolated from *Z. lemairie, Z. zanthoxyloides*, *Z. leprieurii*, *Z. tessmanni*, *Z. gillettii*, *Z. thomense*, *Z. rhetsa*, *Z. paracanthum*, *Z. americanum*, *Z. bungeanum*, *Z. elephantiasis*, *Z. armatum*, *Z. petiolare*, *Z. schreberi*, *Z. avicennae*, *Z. integrifoliolum* and *Z. wutaiense* (Mutinda et al., 2023). Further, Zanthpodocarpins A, B, C-H, (1R,2R,5R,6S)-2-(3,4Dimethoxyphenyl)-6-(3,4dihydroxyphenyl)-3,7ioxabicyclo[3.3.0]octane, magnone A, rel-(1R,5R,6S)-6-(4-hydroxy-3Methoxyphenyl)-3,7dioxabicyclo[3.3.0]octan-2-one, dimethoxysamin, pinoresinol, medioresinol and piperitol-3,3-dimethylallyl ether were isolated from *Z. bungeanum*, *Z. armatum*, *Z. nitidum* and *Z. zanthoxyloides* (Okagu et al., 2021).

Flavonoids and their derivatives like quercitrin, isoquercitrin afzelin, datiscin, neohesperidin, eriocitrin, hyperoside, hesperidin, hesperetin, hyperin, diosmetin, vitexin, myricetin, myricitrin, rutin, quercetin and quercetin-3-Oglucopyranoside have been found in *Z. zanthoxyloides*, *Z. bungeanum* and *Z. nitidum* (Okagu et al., 2021). Moreover, dihydrofisetin, lemairones A, lemairones B, apigenin, kaempferol, kaempferol-7-O-glucoside, kaempferol-3-O-α-L-rhamnoside, tambulin, 3,5 -diacetyltambuline, 3,5 -diactyltambulin, ternatin, diosmin, quercitrin (quercetin-3-O-rhamnoside), quercetin-3-O-β-D-galactoside, quercetin-3-O-b-D-glucoside, quercetin-3-O-α-L-rhamnoside, arbutin and isorhamnetin 3-O-a-L-rhamnoside have been isolated from *Z. asiaticum*, *Z. lemairei*, *Z. armatum*, *Z. integrifoliolum*, *Z. syncarpum* and *Z. bungeanum* (Mutinda et al., 2023).

Other phenolic compounds and phenolic acids like 2-methoxy-4hydroxylphenyl-1-O-α-L-rhamnopyranosyl-(1′ ′ →6′)β-D-glucopyranoside, cuspidiol, dihydrocusidiol, caffeic

acid, chlorogenic acid, hydrocuspidiol, vanillic acid, trans cinnamic acid and Zanthoxylum tannins 1H-[1,2,4]-oxadiazolo-[4,3α]-quinoxalin-1-one were isolated from *Z. armatum*, *Z. quinduense* *Z.* zanthoxyloides, *Z. gilletii* and *Z. bungeanum* (Okagu et al., 2021).

2.5.4. Fatty acids and sterols

Some fatty acids like dodecanoic acid, tetradecanoic acid, quinic acid, n-hexadecanoic acid, 9,12-octadecadienoic acid (Z, Z)-, oleic acid, palmitic acid, protocatechuic acid, myristic acid, 4-(3-Methylbut-2-enyloxy) benzoic acid, nonanoic acid and 3,4,5-trimethoxyphenol were isolated from *Z. rhetsa*, *Z. zanthoxyloides*, *Z. armatum*, *Z. elephantiasis*, *Z. bungeanum*, *Z. paracanthum* and *Z. nitidum* (Mutinda et al., 2023). Additionally, the fatty amides hydroxy-α-sanshool and hydroxy-β-sanshool have been isolated from *Z. bungeanum* and *Z. nitidum* (Okagu et al., 2021). Sterols like stigmast-5-en-3-one, stigmastenol and 3β-glucositosterol were isolated from *Z. elephantiasis* and *Z. schreberi* (Mutinda et al., 2023) while β-sitosterol and β-sitostenone were isolated from *Z. budrunga* and *Z. bungeanum* (Okagu et al., 2021).

2.5.5. Others

Other compounds like alcohols, aldehydes, ketones, ether compounds, plasticizer compounds, chromene and sugars have also been isolated from some species of the genus. Alcohols like 4-[(6,7-Dimethoxy-1,2,3,4-tetrahydro-1-isoquinolinyl) methyl] phenol, (Z)-2-pentenol, 1-hexanol, menth-2-en-1-ol, 1-Butanol, 2-butoxyethanol and 1-Octanol were identified in *Z. rhetsa*, *Z. bungeanum* and *Z. rhoifolium* (Mutinda et al., 2023). From the stem wood of *Z. nitidum* aldehydes like (E)-4-(4-hydroxy-3-methylbut-2-enyloxy)benzaldehyde, (Z)-methyl 3-(4-((E)-4-hydroxy-3-methylbut-2-enyloxy)phenyl)acrylate, 4-hyroxybenzaldehyde and syringaldehyde were identified (Chen et al., 2011).

Ketones like 3-buten-2-one, 4-(2,6,6- trimethyl-1-cyclohexen-1-yl), 2-tridecanone and 2-undecanone were identified in *Z. rhetsa*, *Z. bungeanum*, *Z. armatum* and *Z. syncarpum* (Mutinda et al., 2023). Ether compounds (butane, 1,1-diethoxy-, pentane, 1,1-diethoxy- and propane, 1,1,3-triethoxy-) and plasticizer compounds (1,2-Benzenedicarboxylic acid, butyl octyl ester and1,2-Benzenedicarboxylic acid, diisooctyl ester) were identified from the spines of *Z. rhetsa* (Lalitharani et al., 2010).

Chromenes like (E)-3-(2,2-dimethyl-2H-chromen-6-yl)prop-2- enal and cleomiscosin D were found in *Z. avicennae* stem wood and sugars like saccharose, D-glucose, rhamnose, galactose, glucose, sucrose and L-rhamnose were identified in *Z. leprieurii*, *Z. armatum*, *Z. bungeanum* and *Z. sprucei* (Mutinda et al., 2023).

2.6. Pharmacology of *Zanthoxylum* species

From a pharmacological perspective, *Zanthoxylum* species are a valuable bioresource given that they possess a wide range of phytochemicals. Moreover, the presence of these phytochemicals contributes certain pharmacological properties to the species thereby making them potent anti-microbial agents, anti-inflammatory agents, anti-oxidants, anti-diabetic, anti-pyretic and anti-cancer to name a few.

2.6.1. Anti-oxidant activity

DPPH radical scavenging assay is a common method for investigation of anti-oxidant property of plant samples. *Zanthoxylum* species exhibit anti-oxidant activity at various capacity, depending on the plant part studied, method of extraction along with the species studied. Phuyal et al. (2020) showed that *Z. armatum* fruits (cultivated and wild) had an IC_{50} value of 40.62 µg/ml and 45.62 µg/ml respectively in DPPH assay while the same for bark extracts were 63.39 µg/ml and 67.82 µg/ml respectively, in comparison to the standard reference ascorbic acid which had an IC_{50} value of 36.22 µg/ml (Phuyal et al., 2020). Fresh fruits of *Z bungeanum* on the other hand, showed an IC_{50} value of 82.71 µg/ml for DPPH radical scavenging assay (Jing et al., 2021). The ethanolic fruit extract of *Z. acanthopodium* on the other hand, exhibited an IC_{50} value of 17.9745 mg/ml (Syaputri et al., 2022). The n-butanol fractions of *Z. zanthoxyloides* leaves had IC_{50} values between the range of 21.38- 29.43 mg/ml in comparison to the standard reference ascorbic acid (20.62 mg/ml) for DPPH radical scavenging assay. For ferric reducing anti-oxidant power (FRAP) assay and total anti-oxidant capacity assay, the IC_{50} values were between 36.70- 52.87 µg/ml and 31.80- 202.03 µg/ml respectively in comparison to 32.51 µg/ml and 37.22 µg/ml respectively for ascorbic acid (Ayoka et al., 2020).

Moreover, hydroxy sanshools found in many *Zanthoxylum* species, were shown to be effective in reducing oxidative stress in the colon cancer - HCT-116 cells. It was demonstrated that these compounds could increase the activity of anti-oxidant enzymes

like catalase, superoxide dismutase and glutathione peroxidase while decreasing malondialdehyde concentration. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the activation of genes responsible for producing antioxidant enzymes. Under normal conditions, Keap1 (Kelch-like ECH-associated protein 1) binds to Nrf2, leading to its degradation through the ubiquitin-proteasome system, maintaining redox balance. However, during oxidative stress, elevated reactive oxygen species (ROS) trigger the dissociation of Nrf2 from Keap1, preventing Nrf2 degradation. The freed Nrf2 then moves into the nucleus, where it activates the expression of its target antioxidant genes. The hydroxy sanshools increased the expression of Nrf2 as well its downstream anti-oxidant protein HO-1, while reducing Keap1 protein thereby proving to be potent anti-oxidants (Chen et al., 2024).

2.6.2. Antibacterial/ antiviral activity

The methanolic fruit, seed, and bark extracts of *Z. armatum* were showed effective anti-bacterial activity against *Staphylococcus aureus* with a zone of inhibition (ZOI) of 20.72 mm and 18.10 mm for wild and cultivated fruits respectively, 17.83 mm and 16.33 mm for wild and cultivated seeds respectively, 17.01 mm and 16.44 mm for wild and cultivated bark respectively (Phuyal et al., 2020a). *Z. limonella* also showed various levels of anti-bacterial activity against different strains of bacteria. Against *Mycobacterium tuberculosis* H37Rv, the plant had a minimum inhibitory concentration (MIC) of 200 µg/ml. Essential oil from the same species was also effective against *Clostridium perfringens* DMST 15191 with ZOI of 27.0 mm, *Salmonella typhimurium* TISTR 292 with ZOI of 20.5 mm, *Campylobacter jejuni* DMST 15190 with ZOI of 18 mm, *Bacillus cereus* with ZOI of 20.5 mm and *Staphylococcus aureus* with ZOI of 21.5 mm (Supabphol and Tangjitjareonkun, 2014).

The compound dihydrochelerythrine extracted from *Z. rhetsa* demonstrated anti-bacterial activity against *Staphylococcus aureus* SK1 with MIC values of 8 g/ml and against *Escherichia coli* TISTR 780 with MIC value of 16 g/ml (Maduka and Ikpa, 2021). *Z. bungeanum* essential oil inhibited *S. aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus laterosporus laubach*, and *E. coli* with MIC values of 5.0, 1.25, 2.5, 1.25, and 2.5 mg/ml respectively (Zhang et al., 2017). While ethyl acetate portion of *Z. nitidum* stems inhibited *Escherichia coli* (MIC value 46.9 µg/ml) and *Salmonella* (MIC value 187.5 µg/ml), n-

butanol portion of the roots showed inhibition against *Candida albicans* with MIC value of 187.5 µg/ml (Lu et al., 2020)

Additionally, *Zanthoxylum* species have also been shown to exert anti-viral activity to various degrees. The methanolic fruit extracts of *Z. armatum* showed anti-viral activity against *Herpes simplex* virus type 1(HSV-1) and influenza virus A with cytotoxicity concentration 50 (CC_{50}) value of > 100 mg/ml and 36 mg/ml respectively. Further, the aqueous leaf extracts also exhibited anti-protozoal activity against *Giardia lamblia* and *Plasmodium berghei* (Phuyal et al., 2019). Against hepatitis-B virus (HBV), isolated phytochemicals from *Z. nitidum* roots, namely, 6-methoxy-5,6-dihydronitidine and 5-methoxydictamnine showed inhibitory percentage of 43.3% and 49.3%, respectively which was higher in comparison to the reference standard used lamivudine (29.6%) (Lu et al., 2020). From *Z. schinifolium*, an isolated alkaloid called collinin also exhibited anti-viral activity against HBV via prevention of HBV DNA synthesis with effective dose 50 (ED_{50}) value, infectious dose 50 (ID_{50}) value and selectivity index (SI) value of 68.3 µg/ml, 17.1 µg/ml and 3.99 respectively. Moreover, *Z. ailanthoides*, *Z. integrifoliolum* and *Z. japonica* were also found to exert significant anti-HIV activity (Wen et al., 2024). Further, an isolated compound Tembamide from *Z. limonella* also exerted anti-viral activity against HIV with EC_{50} value of < 0.1 µg/ml (Supabphol and Tangjitjareonkun, 2014).

2.6.3. Anti-fungal activity

Z. limonella showed anti-fungal activity against pathogenic fungi like *Cladosporium cucumerinum* (MIC value 25 µg/ml) and *Pyricularia oryzae* (MIC value 6.25 µg/ml). Essential oil from the species also showed inhibition against yeasts like *Rhodotorula glutinis* (ZOI value 42.3 mm, MIC value 1 mg/ml), *Schizosaccharomyces pombe* (ZOI value 34.0 mm, MIC value 2 mg/ml) and *Hanseniaspora uvarum* (ZOI value 33.3 mm, MIC value 1 mg/ml) (Supabphol and Tangjitjareonkun, 2014). Similarly, *Z. bungeanum* leaves were also found to be effective against pathogenic fungi *Botrytis cinerea* (IC_{50} value 11.82 mg/ml), *Piricularia oryzae* (IC_{50} value 12.31 mg/ml), *Physalospora piricola* (IC_{50} value 39.48 mg/ml), *Glomerella cingulate* (IC_{50} value 13.00 mg/ml) and *Venturia pyrina* (IC_{50} value 33.22 mg/ml). The essential oil from the species showed anti-fungal activity against phytopathogenic fungi namely, *Alternaria solani* (IC_{50} value 0.44

mg/ml), *Botryodiplodia theobromae* (IC_{50} value 0.48 mg/ml), *Fusarium oxysprum* f.sp. *cucumerinum* (IC_{50} value 0.43 mg/ml), *Fusarium oxysprum* f.sp. *niverum* (IC_{50} value 0.48 mg/ml), *Bipolaris maydis* (IC_{50} value 0.24 mg/ml), *Leptosphaeria maculans* (IC_{50} value 0.13 mg/ml), *Magnaporthe grisea* (IC_{50} value 0.28 mg/ml), *Rhizoctonia cerealis* (IC_{50} value 0.27 mg/ml), *Rhizoctonia solani* (IC_{50} value 0.24 mg/ml), *Venturia pirina* (IC_{50} value 0.41 mg/ml) and *Verticillium dahlia* (IC_{50} value 0.32 mg/ml) (Zhang et al., 2017).

In a separate study, it was shown that *Z. armatum* strongly inhibited certain fungi like *Candida albicans*, *Aspergillus flavus* and *Fusarium solani* with percentage inhibition of mycelial growth of 66.67 %, 55.33 % and 46.33 % respectively. Additionally, essential oil from the leaves demonstrated anti-fungal activity against crop pathogens like *Alternaria alternata*, *Alternaria brassicae* and *Curvularia lunata* with antifungal indices of 35.6 %, 14.5 % and 42.0 % respectively (Phuyal et al., 2019)

2.6.4. Antipyretic activity

In vivo studies were conducted to test anti-pyretic potential of fruit and leaf extracts of *Z. armatum* in pyrexia induced mice models. It was reported that both the extracts were effective in significantly reducing the temperature in the mice with the pyrexia control activity being at par with the reference drug paracetamol (Alam et al., 2020). The stem bark of *Z. zanthoxyloides* also reduced temperature in a time dependent manner in pyrexia induced albino Wistar rats (Tougoma et al., 2021). Additionally, ethanolic extracts and fractions of *Z. asiaticum* root was shown to exert anti-pyretic activity in yeast-induced hyperthermic female albino rat models comparable to that of the standard drug paracetamol (Mutinda et al., 2023).

2.6.5. Hepatoprotective effects

The ethanol extract from the leaves of *Zanthoxylum armatum* was investigated for its in-vivo hepatoprotective effects against liver damage induced by carbon tetrachloride (CCl4) in Wistar albino rats. Silymarin (100 mg/kg body weight) was used as the reference control. Oral administration of the extract at a dose of 500 mg/kg significantly ($P < 0.001$) reduced the elevated levels of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), serum bilirubin (SBLN), and inflammation, helping to restore normal hepatic enzyme

levels. Additionally, the hepatoprotective effect of the ethanol extract of *Zanthoxylum armatum* bark was studied in CCl4-induced liver toxicity in male Wistar rats. A mixture of 1:1 CCl4 and olive oil significantly elevated serum markers such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), ALP and total bilirubin. Oral administration of the extract at doses of 100, 200, and 400 mg/kg daily for 7 days resulted in a significant reduction of these elevated parameters. Notably, the dose of 400 mg/kg exhibited hepatoprotective activity comparable to silymarin at 25 mg/kg (Phuyal et al., 2019). On tetrachloromethane-induced induced experimental liver damage in Kunming mice, water extract of *Z. nitidum* effectively decreased the level of serum alanine aminotransferase, AST and liver malondialdehyde while increasing the lover SOD activity (Lu et al., 2020).

2.6.6. Anti-diabetic activity

Alkalmydes isolated from the fruits of *Z. schinifolium* were found to be effective in reducing streptozotocin (STZ) induced diabetes in Sprague–Dawley rats. The animals were given different doses (3, 6, and 9 mg/kg of body weight) of alkamydes for 28 days. With the increase in dose, a significant decrease in relative weights of kidney and liver along with fasting blood glucose, and fructosamine levels was observed. An improvement in the body weight and overall glucose tolerance was also observed. There was a rise in the liver and muscle glycogen and plasma insulin levels and reduction of the histopathological changes in pancreas with the higher doses exerting effects comparable to that of the standard drug glibenclamide. The alkamydes also facilitated decrease in the expression of phosphoenolpyruvate caboxykinase and glucose-6-phosphatase (key gluconeogenesis enzymes) while increasing the the glycolysis enzyme (glucokinase) in the liver. An improved level of pancreatic duodenal homeobox-1, glucokinase, and glucose transporter 2 in the pancreas was observed as well. Additionally, alkamydes increased transient receptor potential cation channel subfamily V member 1 and decreased cannabinoid receptor 1 expressions in the liver and pancreas as opposed to glibenclamide (You et al., 2015).

In vitro assays of α-amylase inhibitory activity and α-glucosidase inhibitory activity on *Z. rhetsa* root bark showed that the plant showed a dose dependent inhibitory activity with IC_{50} value of 81.45 µg/ml for inhibition of α-amylase and 115.67 µg/ml for inhibition of

α-glucosidase as compared to the reference standard acarbose which had IC_{50} values of 14.05 µg/ml and 27.78 µg/ml respectively. Further *in vivo* studies on alloxan induced Swiss-albino mice model demonstrated that the extract could reduce the blood glucose levels (Barman et al., 2024). The anti-diabetic potential of leaf, fruit, and bark extract of *Z. armatum* was also assessed in a separate study. *In vitro* α-glucosidase inhibitory activity showed significant inhibition of the enzyme by all three extracts with IC_{50} values of 47.87 for leaf, 21.82 for bark and 31.62 for fruit. *In vivo* studies in alloxan induced diabetic albino mice further demonstrated that all extracts were effective in reducing the blood glucose level with the activity of leaf extract being comparable to that of standard drug Glibenclamide (Alam, 2018).

2.6.7. Anti-inflammatory activity

Stems of *Z. rhetsa* showed significant HRBC membrane protection as well as inhibition of protein denaturation activity. In another independent study, it was shown that essential oil from the plant could significantly inhibit the production of NO in LPS induced RAW 264.7 cells, with an IC50 value of 16.42 ng/ml (Maduka and Ikpa, 2021). The essential oil from *Z. bungeanum* exhibited notable protection against dimethylbenzene-induced ear oedema and acetic acid-induced pain in rat model. Further, water extract from the plant also showed 65.76% inhibition of oedema weight and 51% inhibition of writhing responses in rat models. Similarly, alkaloids from *Z. bungeanum* were also found to inhibit hot-plate-induced pain and dextran-40-induced itch-scratch responses in rat models. In a separate study, it was shown that methanolic extracts of *Z. bungeanum* pericarps inhibited expression of iNOS mRNA in LPS-stimulated J774.1 macrophages and LPS/IFN-γ-stimulated mouse peritoneal exudate macrophages (Zhang et al., 2017). Fruit and stem bark extracts of *Z. armatum* was also found to inhibit carrageenan induced paw oedema in Wister rats (Phuyal et al., 2019). Similarly, ethanolic root extract of *Z. nitidum* showed 63.45 % inhibition of xylene-induced ear oedema in mice at a dose of 150 mg/kg. On the other hand, 150 and 75 mg/kg doses of root extracts from the plant showed inhibitory rates of 52.94 % and 52.00 % respectively, on acetic acid induced vascular permeability augmentation of mice abdominal cavity. In a separate study, it was shown that alkaloids from *Z. nitidum* suppressed the levels of TNF-α and IL-8 in the serum of ulcerative colitis rats while nitidine chloride isolated from the roots also effectively reduced expression of TNF-α, IL-1β and IL-6 in LPS induced RAW 264.7

macrophages. Additionally, the compound also showed inhibition of NF-κβ transcription as well as MAPK phosphorylation (Lu et al., 2020).

Decarine is a compound derived from *Z. myriacanthum* that has also exhibited anti-inflammatory activity. It was found to notably decrease NO, TNF-α and IL-1β in LPS induced RAW264.7 and THP-1 cells. *Z. piperitum* on the other hand was shown to be effective in reducing 12-oxo-tetradecanol-13-acetate induced ear oedema in mice. Further, the plant was also found to suppress the expression of enzymes iNOS and COX-2, inhibit production of ROS and hypoxanthine adenine phosphoribosyltransferase 1 (HPRT1) and increase the production of ribosomal protein L8 (RPL8) in LPS-induced RAW 264.7 cells. In 2,4-dinitrobenzene bovine serum albumin (DNP-BSA) induced RBL-2H3 cells, *Z. coreanum* volatile oil was found to inhibit IL-4 release while in LPS activated RAW 264.7 macrophages, the same was found to suppress the release of TNF-α, IL-6 and NO as well as the expression of iNOS and COX-2. The volatile oil was further demonstrated to down-regulate transcription of NF-κβ, expression of NF-κβp65 protein, phosphorylation of JNK, ERK and p38 proteins while increasing the IkB-α concentration (Wen et al., 2024).

2.6.8. Gastroprotective activity

The total alkaloids of *Zanthoxylum nitidum* has been shown to demonstrate a protective effect against gastric ulcers in experimental rat models. At doses of 2 and 2.5 g/kg, the alkaloids significantly reduced ulcer indices in ulcers induced by alcohol, stress, and pyloric ligation ($P < 0.01$). In the pyloric ligation-induced model as well, these doses decreased pepsin activity and malondialdehyde levels, while increasing superoxide dismutase, nitric oxide (NO), and prostaglandin E2 (PGE2) levels ($P < 0.05$). These biochemical changes likely explain the protective effect on ulcers. Additionally, water extracts from various parts of the plant, including roots, stems, leaves, and above-ground portions, at different dosages, were effective in reducing ulcer indices caused by hydrochloric acid and ethanol in rats. Overall, *Z. nitidum* exhibits potent anti-gastric ulcer activity, likely through modulation of oxidative stress and gastric mucosal protection mechanisms (Lu et al., 2020). Ethanolic extract of *Z. zanthxyloides* root bark has also been reported to reduce ulcer index by 71 % at 250 mg/kg and 85 % at 500 mg/kg. The extract exerted this gastroprotective effect by inhibiting cholinergic, nicotinic and

histaminic receptors. The water extract of *Z. bungeanum*, administered at doses of 2.5, 5.0, and 10 g/kg (based on the mass of the crude herb), exhibited significant inhibitory effects on various experimentally induced gastric ulcers in mice (Zhang et al., 2017). Similarly, the ethanol extract of *Zanthoxylum rhoifolium* stem bark exhibited antiulcer activity by protecting the gastric mucosa through its antioxidant properties and preventing damage from free radical-induced oxidation. Additionally, it activated KATP channels and enhanced the availability of nitric oxide (NO), contributing to its ulcer-protective effects (Okagu et al., 2021).

2.6.9. Anti-cancer activity

The essential oil of *Zanthoxylum bungeanum* has demonstrated notable anti-tumour effects both *in vitro* and *in vivo*. At 4 mg/ml, it inhibited the growth of H22 (mouse hepatoma) cells with a 76% inhibition rate after 72 hours. In mouse models, the same at doses of 10, 25, 50, and 100 mg/kg suppressed tumour growth with inhibition rates of 20.15%, 40.03%, 60.25%, and 62.58%, respectively. *In vitro* also inhibition of the proliferation of various cancer cell lines, including cervical tumour cell - HeLa (IC50 = 11.2 mg/ml), adeno-carcinomic human alveolar basal epithelial cells - A549 (IC50 = 6.26 mg/ml), myelogenous leukaemia cell line - K562 (IC50 = 1.37 mg/ml) and rat adrenal medullary tumor cell line - PC12 (0.5–2.0 mg/ml) was observed. Additionally, inhibition of PC-3 (human prostate cancer cell line), HEp-2 (human epithelial cell line) and MCF-7 cells (human breast cancer cell line), with IC50 values ranging from 0.021% to 0.04%. The proliferation of HaCaT (human keratinocytes) cells was also significantly reduced in a dose- and time-dependent manner (IC50 = 0.024% at 48 hours). It was shown that the anti-cancer effect of *Zanthoxylum bungeanum* essential oil was linked to the activation of apoptotic pathways, with increased expression of cleaved caspase-8, -9, -3, poly (ADP-ribose) polymerase (PARP), Bcl-2-associated X-protein (BAX), and decreased Bcl-2 levels. Moreover, hyperoside, a compound isolated from *Z. bungeanum* leaves, showed inhibitory effects on the human intestinal cells - SW620 cells, with IC50 values of 72.35 μM, 36.41 μM, and 19.51 μM at 24, 48, and 96 hours, respectively. Its mechanism of action was shown to be via increased ROS production, reduced mitochondrial membrane potential ($\Delta\Psi m$), upregulation of apoptotic proteins like cytochrome c and caspases, and downregulation of antioxidant enzymes such as glutathione peroxidase (GSH Px) and catalase (CAT) (Zhang et al., 2017).

The antitumor-promoting potential of the methanolic fruit extract of *Zanthoxylum limonella* was evaluated using an in vitro Epstein-Barr virus early antigen (EBV-EA) activation assay in Raji lymphoid cells, induced by 12-O-tetradecanoylphorbol-13-acetate (TPA, 40 ng/mL). The fruit extract exhibited a strong inhibitory effect, with inhibition exceeding 70% at a concentration of 200 µg/ml (Supabphol and Tangjitjareonkun, 2014). The chloroform fraction of *Z. rhesta* bark was also found to be cytotoxic to B16-F10 melanoma cells while the silver nanoparticles from its seed coat showed cytotoxicity against A549 cell line (Maduka and Ikpa, 2021).The anti-cancer potential of compounds from *Zanthoxylum nitidum* was evaluated against three human cancer cell lines: MCF-7, NCI-H460 (human lung cancer cells), and SF268 (human glioblastoma cells). Liriodenine showed strong activity with IC50 values of 2.19, 2.38, and 3.19 mg/ml respectively. Another compound, 7-methoxy-8-demethoxynitidine, demonstrated potent cytotoxic effects against multiple cancer cell lines (KB, MCF-7, LNCaP, HepG2, LU-I), with IC50 values between 10.3 and 12.6 µmol/l. The STAT3 signaling pathway, a crucial target in cancer treatment, was also investigated. Screening of *Z. nitidum* root compounds identified 8-methoxydihydrochelerythrine as a potent and selective inhibitor of the STAT3 pathway (IC50 = 11.56 µmol/l). This compound inhibited STAT3 phosphorylation and target gene expression, leading to growth inhibition in human carcinoma cells with constitutive STAT3 activation (IC50 = 3.14 - 4.72 µmol/l) (Lu et al., 2020). Total alkaloid extracts from *Z. ailanthoides*, *Z. simulans* and *Z. chalybeum* were also shown to have inhibitory activity at a range of 60.71 %–93.63 % against SGC7901 (gastric tumor cell), HeLa, colon tumor cell HT29 and liver tumor cell Hep G2 (Wen et al., 2024).

2.6.10. Other activities

Besides the other pharmacological effects mentioned earlier, the *Zanthoxylum* species have also shown potential as analgesic, larvicidal agent, mosquito repellent, photo-protective and anti-diarrhoeal to name a few. The essential oil and petroleum ether extract from the fruits of *Zanthoxylum limonella* were tested as mosquito repellents against *Aedes albopictus*. In a mustard and coconut oil base, the fruit oil provided the longest-lasting protection, with a 30% concentration in mustard oil offering 296–304 minutes of repellence. The undiluted fruit oil provided complete protection for 2 hours against *Aedes aegypti*, *Culex quinquefasciatus*, and *Anopheles dirus*. Additionally, the essential oil was

highly effective against the fourth instar larvae of *A. aegypti*, achieving 50% and 95% mortality (LC50 and LC95) at concentrations of 24.61 and 55.81 ppm within 24 hours (Supabphol and Tangjitjareonkun, 2014). The seed oil of *Zanthoxylum armatum*, vanillin, and the fruit oil of *Zanthoxylum piperitum* were found to enhance repellent activity against female *Aedes aegypti* mosquitoes for 445 minutes at a concentration of 0.57 mg/cm² on a mustard oil 404 minutes in a coconut oil base (Phuyal et al., 2019). Another study showed that monoterpenes (piperitone, 4-terpineol, and linalool) from *Z. bungeanum* showed notable ant repellent activity (Zhang et al., 2017).

The methanolic extract of *Zanthoxylum rhetsa* stem bark was evaluated for analgesic and antidiarrheal activities in mice. Administered orally at doses of 250 and 500 mg/kg, the extract significantly reduced acetic acid-induced abdominal writhing (indicating analgesic activity) and castor oil-induced diarrhoea (suggesting antidiarrheal effects) (Maduka and Ikpa, 2021). *Z. armatum* also was shown to provide a protection of 60 % in castor-oil-induced diarrhoea in mice, at a concentration of 1000 mg/kg. Similarly, essential oil from leaves of the plant demonstrated spasmolytic effect on rabbit jejunum smooth muscle (Phuyal et al., 2019). The stem and root extracts of *Z. nitidum* were found to significantly decrease the number of painful twists as well as improve the pain threshold in Kunming mice thereby implying their strong analgesic property (Lu et al., 2020).

Bark extract of *Z. rhetsa* was also demonstrated to exert photo protective effects. The ethyl acetate fraction, used as an active ingredient in two sunscreen cream formulations (F1 and F2) showed sunscreen protection factor (SPF value) of 3.60 and 6.90 respectively. Additionally, the ethyl acetate fraction and hesperidin significantly suppressed the expression of NF-κβ, MMP 1, MMP 3, and MMP 9 in UVB treated human dermal fibroblasts indicating their potential as sunscreen and anti-photo aging agents (Maduka and Ikpa, 2021). Further, it was found that *Z. bungeanum* could exert anti-depressive effect on behavioural despair models. In post-stroke depression in brain tissue of rats, the fruit extracts upregulated norepinephrine and serotonin (Zhang et al., 2017).

Research Objectives

Based on the literature gap found on the genus *Zanthoxylum* spp. from Sikkim Himalaya mainly with respect to its phytochemical, immunomodulatory and antioxidant activity, the broad aim of the thesis was to provide baseline information to the existing literature.

The specific objectives proposed for the research were:

1. **Phytochemical screening of the leaves and fruits of *Z. oxyphyllum* and *Z. acanthopodium* from Sikkim Himalaya**

2. **To study the *in vitro* immunomodulatory activity of crude leaf extracts of the *Z. oxyphyllum* and *Z. acanthopodium***

3. **To study the *in-vitro* immunomodulatory activity of the crude fruit extracts *Z. oxyphyllum* and *Z. acanthopodium***

Chapter 3

Materials and Methods

3.1. Collection of plant samples

Collection of plant samples were done from different locations within Sikkim Himalayan region. A research permit was obtained from Forests, Environment and Wildlife Management Department, Gangtok, Sikkim. A separate permit was also obtained from Sikkim Himalayan Zoological Park, Gangtok, Sikkim. For each species, three different locations were selected. Only undamaged mature leaves and ripened fruits were collected. *Z. oxyphyllum* leaf and fruit samples were collected from Sikkim Himalayan Zoological Park and two separate sites from FM danra region in Gangtok. *Z. acanthopodium* leaf and fruit samples were collected from Salep Tanki area and FM danra region in Gangtok, and GTA office area in Darjeeling. The plant sample was identified at the Department of Botany, Sikkim University and the voucher specimens have been deposited to the departmental herbarium with accession numbers 749 and 677 for *Z. oxyphyllum* and *Z. acanthopodium* respectively.

Table 3.1. Sites of *Z. oxyphyllum* Edgew. and *Z. acanthopodium* DC. sample collection in Sikkim and Darjeeling Himalaya.

(R=biological replicate)

Z. oxyphyllum Edgew.	*Z. acanthopodium* DC.
R-1 FM danra, Gangtok: 27° 21' 27.4788" N, 88° 37' 41.8692" E	R-1 Salep Tanki road side, Gangtok: 27° 21' 14.796" N, 88° 37' 31.1376" E
R-2 FM danra, Gangtok: 27° 20' 54" N, 88° 36' 50" E	R-2 GTA office, Darjeeling: 27° 1' 49.944" N, 88° 15' 33.516" E
R-3 Sikkim Himalayan Zoological Park: 27° 21' 23.6838" N, 88° 37' 51.78" E	R-3 FM danra, Gangtok: 27° 21' 27.4788" N, 88° 37' 41.8692" E

3.2. Extraction of plant samples

Samples were collected by 10.00 AM and immediately brought to the laboratory where they were thoroughly rinsed under tap water, in sodium hypochlorite solution (1%) and a final rinse was done with distilled water. Any unwanted twigs and branches were removed. The samples were oven dried in Genetix programmable drying oven (Model-GX-A5055A) at 60°C until they were completely dry and a constant weight was observed on weighing. The dried samples were ground in a waring blender and sieved. 5 gm of the powdered material was extracted in 50 ml 80% methanol via ultrasonic sonication in Labman digital ultrasonic sonicator for 60 minutes at 50°C. The extraction process was repeated three times with the same sample and filtered through Whatman filter No.1 filter paper. The filtrate was evaporated in Heidolph rotary evaporator until the solvent evaporated to obtain a crystallized, dark green coloured leaf extracts and a burnt maroon coloured fruit extract. The extracts were stored at 4°C until further study.

3.3. Phytochemical analysis of the plant samples

3.3.1. Preliminary qualitative analysis

Preliminary chemical tests were carried out from crude powder and/or methanolic extract to identify different phytoconstituents as follows:

3.3.1.1. Alkaloids

200 mg of methanolic extract of each plant sample was weighed accurately and dissolved in 10ml of ethanol and then filtered using filter paper. 1ml of the filtrate was then mixed with 6 drops of Wagner's reagent. A creamish, brownish-red or orange precipitate indicated the presence of alkaloids (Parekh and Chanda, 2007)

For the preparation of Wagner's reagent, 1.27 gm of iodine and 2 gm of potassium iodide were weighed in a digital balance, dissolved in 5ml of water and the final volume made up to 100ml with distilled water.

3.3.1.2. Carbohydrates

Benedict's reagent (alkaline solution of cupric citrate complex) test was used for determination of carbohydrates. 5 ml of reagent was mixed with 2 mg of powdered samples and boiled for 2 minutes. A greenish-yellow colouration indicated low presence of carbohydrate while red colouration indicated a high amount (Gupta et al., 2013).

3.3.1.3. Phenols

The extract (500 mg) was dissolved in 5 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds (Soloway and Wilen, 1952).

3.3.1.4. Flavonoids

The crude powder and methanolic extract were treated with a few drops of diluted sodium hydroxide separately. Formation of intense yellow colour which turned colourless on addition of few drops of diluted HCl indicated presence of flavonoids (Parekh and Chanda, 2007).

3.3.1.5. Glycosides

Keller-Kiliani test was performed for the presence of cardiac glycosides. The crude powder and methanolic extract was each treated with 1ml mixture of 5% $FeCl_3$ and glacial acetic acid (1:99 v/v). To this solution, few drops of concentrated H_2SO_4 was added. Appearance of greenish blue colour within few minutes indicated the presence of cardiac glycosides (Parekh and Chanda, 2007).

3.3.1.6. Steroids

Liebermann-Burchard reaction test was conducted for detection of steroids. Briefly, 200 mg of plant powder was mixed with 2 ml acetic anhydride and a few drops of concentrated sulphuric acid. A blue-green ring indicated the presence of terpenoids (Parekh and Chanda, 2007).

3.3.1.7. Protein

Ninhydrin test was conducted to determine presence of proteins. 2 mg of plant extracts were dissolved in 2 ml of distilled water. To this 1 ml of Ninhydrin reagent was added and boiled in water bath for 5 minutes. A blue or purple colour indicated the presence of free amino acids (Yemm et al., 1955).

3.3.1.8. Tannins

About 0.5gm of the extracts were boiled in 10ml water in a test tube and then filtered. A few drops of 0.1% ferric chloride were added to the filtrate. A greenish black colouration and precipitation indicated presence of tannins (Parekh and Chanda, 2007)

3.3.2. Quantitative analysis

3.3.2.1. Estimation of pigments

Chlorophyll a, Chlorophyll b and carotenoids were determined. 50 mg fresh tissues were homogenized in mortar and pestle with 5 ml 95 % ethanol and centrifuged at 5000 rpm for 10 minutes. The supernatant was collected and absorbance was immediately measured at 664 nm, 649 nm and 470 nm. The following formula was used for the final calculations and the results were expressed as mg/g fresh weight (Sumanta et al. 2014)

$Ch\text{-}a = 13.36 * A_{664} - 5.19 * A_{649}$

$Ch\text{-}b = 27.43* A_{649} - 8.12 * A_{664}$

Carotenoids = (1000 * A_{470} - 2.13 * Ch a - 97.63 * Ch b)/209

For estimation of anthocyanins, 1 g of fresh tissue was homogenized with a mixture of 20 ml n-propanol, hydrochloric acid, and water in the ratio 18:1:81 and heated in boiling water bath for 10 minutes, followed by incubation at 4°C for 24 hours. The extract was filtered and then measure at 535 and 650 nm in spectrophotometer. The following formula was used for calculation of total anthocyanins (Shan et al., 2009):

Anthocyanins = A_{535} – 0.22 * A_{650}

3.3.2.2. Estimation of crude protein content

The protein content in plant samples were estimated with Bradford method (Spencer and Davie, 2002) with slight modifications. For protein extraction, 100 mg of the plant tissues was homogenized in 5 ml of distilled water, centrifuged at 10,000 rpm for 20 minutes and the supernatant was collected as crude protein samples. Bradford reagent was prepared by dissolving 10 mg Coomassie brilliant blue in 10 ml methanol and 50 ml 85% phosphoric acid. To 1 ml of crude protein sample 3 ml Bradford reagent was added and incubated at room temperature for 5 minutes. The tubes were gently vortexed and absorbance was measured at 595 nm in a UV-Vis spectrophotometer. BSA (bovine serum albumin) was used as the standard reference. Total soluble protein was presented as µg/mg protein.

3.3.2.3. Estimation of total phenolic content

Total phenolic content in the extracts were assessed using the method described by Singleton and Rossi (1965) with slight modifications. Briefly 0.5 ml extracts were mixed with 0.5 ml of 1000-fold Folin–Ciocalteu reagent and dark incubated for 5 minutes at room temperature. After that 1 ml of freshly prepared 2% Na_2CO_3 solution was added and mixed well. After 10 min of dark incubation, the absorbance of the blue colour that developed was read at 730 nm using UV-Vis spectrophotometer (Shimadzu). Gallic acid was used as the phenolic standard and the concentrations of total phenolic compounds were presented as µg/ gallic acid equivalent mg (µg GAE/mg).

3.3.2.4. Estimation of total flavonoid content

The total flavonoid content of all the extracts were quantified according to the colorimetric method described by Zishen et al., (1999) with minor modifications. About 250µl of different extract was mixed with 1.25ml distilled water and 75µl of 5% $NaNO_2$. After 5 minutes of incubation at room temperature, 0.15ml of 10% $AlCl_3$ was added and mixed well. The mixture was incubated for 6 minutes at room temperature after which 0.5ml of 1mM NaOH was added. Finally, the volume was made up to 2.5ml with 275µl of distilled water and mixed well. The absorbance was measured at 510 nm after 30 minutes of incubation. The flavonoid content was determined from a quercetin standard curve and results were expressed as µg/mg of quercetin equivalent (QE) mg (µg/ QE mg).

3.3.2.5. Estimation of total tannin content

To 1 ml of each extract was added 3 ml of 0.1 $FeCl_3$ in 0.1 N HCl followed immediately by 3 ml of 0.008 M $K_3Fe(CN)_6$ and incubated for 10 minutes at room temperature. The absorbance was measured at 760 nm. Tannic acid was used as the standard reference and the result was expressed as µg/ tannic acid equivalent (TAE) mg (Gupta and Verma, 2011).

3.3.2.6. Estimation of total saponin content

In a test tube, 0.25 ml of the each methanolic extract was taken. To it was added 0.25 ml (8% w/v) vanillin and 2.5 ml of (72 %) H_2SO_4. The mixture was incubated at 60°C for 10 minutes at room temperature followed by immediately cooling in ice water bath for 10 minutes. The final absorbance was taken spectrophotometrically at 560 nm. Diosgenin was used as the standard the final result was expressed as µg/ diosgenin equivalent (DE) mg (Le et al., 2018).

3.3.2.7. Estimation of total polysaccharide content

1 ml of 5 % phenol was added to 1 ml of each methanolic extract. To this mixture was added 5 ml concentrated H_2SO_4 and incubated for 10 minutes at room temperature. The absorbance was taken spectrophotometrically at 488 nm. Glucose was used as standard and the result was expressed as µg/ glucose equivalent (GE) mg (Bhatti et al., 2013).

3.4. *In vitro* anti-oxidant studies of plant extracts

3.4.1. DPPH radical scavenging assay

To prepare the DPPH (2,2-Diphenyl-1-picrylhydrazyl) solution, 24 mg of DPPH was dissolved in 100 ml methanol (95%) and the absorbance was adjusted to 1 ± 0.02 at 517 nm. Briefly, to 1 ml various concentrations of methanolic extracts (50-250 µg/ml) or the standard (gallic acid) was added 3 ml of 0.5 mM DPPH solution, vortexed and incubated for 30 minutes at room temperature in the dark. The absorbance was measured at 517 nm (Umamaheswari and Chatterjee, 2008). The disappearance of DPPH radical was calculated as percentage scavenging activity with the following formula:

% scavenging activity = $[(A_c - A_s) / A_c] * 100$

Where, A_c = absorbance of control, A_s = absorbance of sample

2.4.2. Ferric reducing antioxidant power assay (FRAP)

Ferric reducing anti-oxidant activity was done according to a protocol described by Bhalodia et al. (2013) with slight modifications. 2.5 ml (0.2 M) phosphate buffer and 2.5 ml potassium ferricyanide was serially added to 1 ml of various concentrations of methanolic extracts (50-250 µg/ml) and incubated at 50°C for 20 minutes. Trichloroacetic acid (2.5 ml) was added to this mixture and centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and to it was added with 2.5 ml distilled water followed by 0.5 ml (0.1 %) ferric chloride and incubated for 10 minutes. The final absorbance was measured at 700 nm. Gallic acid was used as the reference standard.

2.4.3. Total antioxidant capacity (TAC)

The total anti-oxidant activity was assayed using the Phosphomolybdate method (Umamaheswari and Chatterjee, 2008). 1 ml of various concentrations of methanolic extracts (50-250 µg/ml) and 3 ml of reaction mixture (0.6 M H_2SO_4, 28mM sodium phosphate, 4 mM ammonium molybdate) was incubated in a water bath at 95°C for 90 minutes and cooled to room temperature. The absorbance was taken at 695 nm. Gallic acid was used as the standard reference.

3.5. *In vitro* anti-inflammatory studies of plant extracts

3.5.1. HRBC membrane stabilization assay

Membrane stabilization assay was performed according to Shinde et al. (1999). A total of 2 ml blood sample was collected from the investigator who had not taken any non-steroidal anti-inflammatory drug for the last 15 days. The blood was mixed with 2.0 ml of Alsever's solution and centrifuged at 3000 rpm for 10 minutes. The packed RBC pellet obtained was washed with normal saline (0.9%) three times over. The resolved RBC pellet was reconstituted as 10% (v/v) suspension with iso-saline (pH 7.2) which served as the HRBC stock suspension.

The reaction mixture contained 1ml of plant extract or the standard reference drug (diclofenac sodium) prepared in distilled water in the concentrations of 1, 2, 3, 4 and 5 mg/ml, 2.0 ml of hyposaline (0.24 % NaCl), 1 ml of PBS (0.15 M, pH 7.4) and 0.5 ml HRBC suspension (10%). The mixture was incubated for 30 minutes at 37 °C and then centrifuged at 2500 rpm for 5 minutes. The absorbance of the collected supernatant was measured at 560 nm. The HRBC membrane protection was calculated by the formula:

% protection = $100-[(OD_1/OD_2) * 100]$

Where, OD_1= optical density of the test sample, OD_2= optical density of the control

3.5.2. Protection of protein denaturation assay

Protection of protein denaturation assay was done according to Anyasor et al. (2019). Concentrations of standard reference drug (diclofenac sodium) and methanolic plant extracts were prepared in methanol in the concentration range of 1-5 mg/ml. The reaction mixture consisted of 2 ml 1% w/v BSA, 2 ml of different concentrations of extract or standard drug and PBS (pH 6.4) and incubated for 30 minutes at 37°C. The temperature was increased to 70°C for 10 minutes to induce protein denaturation. After cooling to room temperature, the absorbance was measured at 660 nm. PBS was used as control. The percentage inhibition of protein denaturation was calculated using the formula:

% inhibition of protein denaturation = $100 * [1- (A_2/A_1)]$

where A_1 = absorbance of the control, A_2 = absorbance of the test sample.

3.6. Estimation of mineral content

For estimation of mineral content, the plant samples were sent to Indian Institute of Horticultural Research-ICAR, Bengaluru.

3.6.1. Estimation of nitrogen content

Nitrogen content of plant samples was estimated by Kjeldhal's method. Briefly, 100 mg powdered samples were added into digestion tubes with 1.1. g salt-catalyst mixture and 4 ml of concentrated sulphuric acid and mixed. The tubes were placed on aluminium heating block preheated to 300°C and boiled for 60 minutes for digestion after which the mixture was then removed from the block and cooled to room temperature. The digest was diluted with distilled water up to 50 ml and mixed thoroughly. Ammonium in the digest was finally estimated by making an aliquot of alkaline with 10 N sodium hydroxide followed by a steam distillation analysis. The nitrogen estimated was presented as percentage dry weight (Humphries, 1956).

3.6.2. Estimation of potassium

Potassium content was estimated in a flame photometer using method described by Chapman and Pratt, (1962) and plotted against at known potassium standard curve. The potassium content was presented as percentage dry weight.

3.6.3. Atomic absorption spectrophotometric analysis of macro and micronutrients

The concentration of mineral nutrients (Mg, Ca, Mn, Zn, Cu, Fe) were determined using Atomic Absorption Spectrophotometer by Wet digest method.

An acid mixture of Nitric acid and Perchloric acid in the proportion 10:4 was prepared. 0.5g of powdered plant sample was taken in a of 250ml conical flask. To this sample about 10 ml acid mixture was added and the conical flask was kept in a hot plate for heating in an open and ventilated space. The red fumes of nitrous oxide were allowed to escape till white fumes started appearing. The white fumes were allowed to escape for a minute and the heating was stopped, the mixture was cooled to room temperature. 20 ml of distilled water was added to the colourless extract and filtered through Whatman no.1 filter paper.

The colourless extract was taken in a 50 ml volumetric flask and the volume was make up with distilled water. The sample thus becomes ready.

An atomic absorption spectrophotometer (AAS 280 FS Agilent Technologies, Santa Clara, USA) was used for the quantification of selected macro-elements (Ca and Mg) and micro-elements (Mn, Zn, Cu, and Fe) concentrations in the samples with Atomic Absorption Spectrophotometer (Piper, 1966; AOAC, 1990).

3.7. Gas chromatography–mass spectrometry (GC-MS) analysis of plant extracts

GC-MS analysis the plant extracts were determined using Agilent 5977B EI/CI MSD as per the method described by Kadhim et al. (2016). The temperature was set at 40°C, raised to 250°C at the rate of 5°C/minute. The run time was 30 minutes. Ionization of sample components were performed on EI mode (70 eV) with helium as the carrier gas at a flow rate of 1.0 ml/min. 0.5 ml of sample was injected in split mode of 20:1. The mass spectrum scan range was set at 29.0 to 500 (m/z)**.** Interpretation of mass spectrum of GCMS was done using the database of National Institute Standard and Technology (NIST). The mass spectrum of phytochemicals was compared with the spectrum of known compounds stored in the NIST library.

3.8. Cell culture studies

To test the *in vitro* immunomodulatory potential of plant extracts, human trophoblast cell line – HTR-8/SVneo cell line kindly provided by Dr. H. Yasmin (Cooch Behar Panchanan Barma University) was used. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 0.2 % sodium bicarbonate and 10 ml penicillin-streptomycin antibiotic. 10 % heat inactivated foetal bovine serum was added to the media only before conducting an assay. All media used were syringe filtered with 0.20 µm Minigen syringe filters. The cells were grown in CO_2 incubator at 37 °C and 5 % CO_2. All further experiments were conducted only after 70 % confluence of cells were observed in the T75 flasks, at passage number 13-14.

Different concentrations of the plant extracts and standard reference drug (diclofenac sodium) were reconstituted in RPMI-1640 media for further studies.

3.8.1. MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay for cell viability

MTT assay was performed, to check the toxicity of plant extracts on HTR-8/SVneo cells. Flat bottomed 48-well plate was used for this assay. Plant extracts and reference drug at concentrations of 5, 15, 30, 50, 70 and 100 µg/ml were used. Briefly, cells were seeded at a density of 1 x 10^5 cells per well and 100 µl of each concentration of plant extract/reference drug was added to the designated well. The plates were incubated in CO_2 incubator at 37 °C and 5 % CO_2 for 24 hours. Separate set of plates with equal volume of cells and plant extracts were incubated for 48 hours under similar conditions. After incubation, old media was discarded and cells were washed and each well was washed with 200 µl of 1X phosphate buffered saline (PBS). The plates were centrifuged in Thermo Scientific ST 40R centrifuge at 3000 rpm for 10 minutes. The PBS was discarded carefully without disturbing cell pellet and 200 µl of freshly made MTT solution was added to each well. The plates were kept for 4 hours dark incubation at 37 °C. After incubation, the plates were centrifuged again at 3000 rpm for 10 minutes, the supernatant was discarded and 200 µl of DMSO was added to stop the reaction with thorough mixing. Absorbance was measured at 570 nm in a Synergy LX multimode reader (Kumar et al., 2018).

3.8.2. Treatment of cells for further studies

Cells were seeded at 1 x 10^5 cell/well in 48 well plates. After 70 % confluency was observed, 10 µg/ml lipopolysaccharide (LPS) from *E. coli* (O55:B5) dissolved in RPMI media was used to induce inflammation in the cells. After 12 hours, the cells were treated with 15 µg/ml and 30 µg/ml of each plant extract and diclofenac sodium and incubated for another 12 hours. Cells without LPS activation or treatment were designated as negative control [C (-)] and cells with LPS activation without treatment were designated as positive control [C (+)]. The supernatants were collected and stored at -80 °C for further analysis. Before each assay, a two times dilution of each supernatant was prepared.

3.8.3. NO release assay

The cell supernatants were analysed for nitrite release as indicator of NO production using Griess reagent kit from Thermo-fisher (G7921) according to the manufacturer's instructions. The Greiss reagent was prepared by mixing equal volumes of N-(1-naphthyl)

ethylenediamine (Component A) and sulfanilic acid (Component B). For the assay, 20 µl of Greiss reagent, 150 µl of nitrite containing supernatant and 130 µl deionized water was added to each designated well and incubated for 30 minutes at room temperature. A mixture of 20 µl Greiss reagent and 280 µl deionized water was taken as blank. The absorbance was measured at 548 nm in a multimode reader. Standard curve was made with a given nitrite solution. The nitrite released was presented as µg/ml (Guevara et al., 1998).

3.8.4. Estimation of cytokines by Enzyme-Linked Immuno Sorbent Assay (ELISA)

Release of pro-inflammatory cytokine IL-6 and TNF-α and anti-inflammatory cytokine IL-4 in the cell supernatants were analysed using ELISA kits (Sigma-Aldrich). The assay was carried out according to the manufacturer's instructions. All the reagents required were prepared beforehand. A standard curve of IL-6 was made at from1.37 pg/ml to 1000 pg/ml. the reagents were kept a room temperature and vortexed prior to using. 100 µl of each diluted supernatant was added to designated well, covered, and incubated for 2.5 hours at room temperature with gentle shaking. The supernatant was discarded and washed four times with 1 X wash buffer. Then 100 µl of biotinylated detection antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking. The solution was discarded and the plate was washed again for four times with 1 X wash buffer. This was followed by addition of 100 µl of HRP (Horseradish Peroxidase)-streptavidin to each well and incubation for 45 minutes at room temperature with gentle shaking. The solution was discarded and the plate was washed again four times with 1 X wash buffer. Then 100 µl of TMB (3,3',5,5'-tetramethylbenzidine) solution was added to each well, covered and incubated for incubated for 30 minutes with gentle shaking. Finally, 50 µl of stop solution was added to each well and the absorbance was measured at 450 nm in multimode reader.

3.9. Cyclooxygenase-1 (COX-1) inhibitory assay

To check inhibitory effect of plant extracts on COX-1, COX (ovine/human) inhibitor screening assay kit from Cayman Chemicals was used and the assay was done according the manufacturer's instructions. All reagents were prepared in advance. Briefly, 100 µl ELISA buffer was added to the wells designated as NSB (non-specific binding), 50 µl of ELISA buffer was added to wells designated as B_0 (blank), 50 µl of different

concentrations of standard (for graph) was added to designated wells, and 50 µl each concentration of plant extract/reference standard was added to designated wells. Then 50 µl of $PGF_{2\alpha}$ (prostaglandin) AChE tracer was added to each well followed by addition of 50 µl $PGF_{2\alpha}$ ELISA antiserum. The plate was covered and incubated for 18 hours at 4°C. Following incubation, the solution was discarded and the plate was washed 5 times with wash buffer. 200 µl of Ellman's reagent was added to each well and the plate was covered and incubated in dark for 120 minutes with gentle shaking. The absorbance was measured at 412 nm in ELISA plate reader.

3.10. Lipoxygenase (LOX) inhibitor assay

To check inhibitory effect of plant extracts on LOX, LOX inhibitor screening assay kit from Cayman Chemicals was used and the assay was done according the manufacturer's instructions. All reagents were prepared in advance. 90 µl of 15-LO and 10 µl each concentration of plant extract was added to each well and incubated for 5 minutes. Then 10 µl of arachidonic acid was added to each well followed by incubation of 10 minutes with gentle shaking. After incubation 100 µl of chromogen was added to all wells and incubated again for 5 minutes with gentle shaking. The absorbance was measured at 500 nm in ELISA plate reader. Nordihydroguaiaretic acid (NDGA) was used as the reference standard.

3.11. Statistical analysis

All statistical analysis was done using Sigmaplot 13. All graphs were made using Microsoft Excel. Experiments were carried out in triplicates and presented as mean ± standard error of mean (SEM). One way analysis of variance followed by post hoc Tukey test was conducted for *in vitro* anti-inflammatory assays. For cell culture studies, two tailed student's t test was used for calculating the significant difference between cells treated with plant extracts/diclofenac sodium and C (+). Symbols used for P values < 0.05 = *, <0.01 = ** and < 0.001 = ***. One way analysis of variance was used to test significant difference between the cells treated with plant extracts and cells treated with diclofenac sodium, followed by Holm-Sidak post hoc test. A P value of < 0.05 was considered statistically significant for one-way ANOVA. Heat map and principal component analysis was generated using Metaboanalyst 6.0.

Chapter 4

Results and Discussions

The present work which embodies research on a very niche topic in the sense that it involves Sikkim Himalayan biodiversity which is an important component of the Himalayan biodiversity hotspot (Lepcha et al., 2019). Naturally it is imperative that in the region the diversity of plants is extremely varied, along with many endemic and rare plants that show great diversity in a small horizontal or vertical distance. Moreover, the major ethnic groups in the area, the Nepalese, Lepchas and Bhutias have their own rich ethnomedicinal traditions (Chhetri et al., 2005).

The plants of the genus *Zanthoxylum* have been in use across the Himalaya in the traditional system of medicine for different ailments including pain relief, fever, cholera, liver inflammation (Uprety et al., 2016; Kala, 2005; Tshering et al., 2022). etc. Moreover, some species of *Zanthoxylum* including *Z rhetsa* (stems), *Z. bungeanum* (essential oil), *Z. armatum* (fruits, stem bark), *Z. nitidum* (roots), *Z. coreanum* (essential oil), exhibited potent anti-inflammatory activity (Maduka and Ikpa, 2021; Zhang et al., 2017; Phuyal et al., 2019; Lu et al., 2020; Wen et al., 2024).

Despite the sizeable number of reports as mentioned above, there is a dearth of information regarding the immunomodulatory studies on the genus *Zanthoxylum* from this part of the world. In addition, all the analytical works so far undertaken was from different climatic and geographical regions offering different niche, different edaphic factors, and different ecotypes.

Under the backdrop of such underpinnings the present work was undertaken involving two hitherto less researched *Zanthoxylum* spp., viz, *Z. oxyphyllum* and *Z. acanthopodium* in order to validate ethnic knowledge, create baseline data and valorise the generated information, the outcome of the study as presented below:

4.1. Extraction yield percentage

In any plant species, efficiency of extraction is important to obtain a wide spectrum of natural compounds with variable biological properties. Selection of solvents and method of extraction is of paramount importance to extract maximum amount of active biochemicals. For extraction of a wide range of phytochemicals in their active form, methanol is an optimum solvent (Dhawan and Gupta 2016; Ajibade et al., 2013; Basma et al. 2011). Methanol has high polarity and can therefore be used as a typical organic solvent to dissolve a number of polar substances (Ott et al., 2012), including both hydrophilic and lipophilic phytochemicals. Therefore, in the present research, hydro-methanol (80% methanol) was used a solvent of extraction.

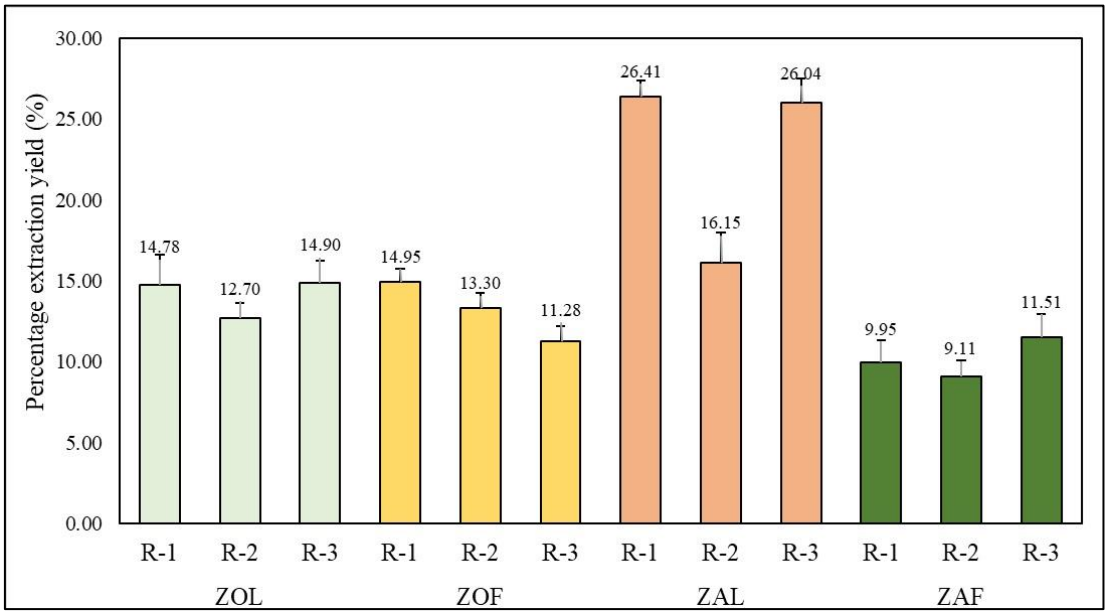

Figure 4.1: Extraction yield (%) of *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations in aqueous methanol. (Data presented as ± SEM, n = 3).

ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits, ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

In the *Zanthoxylum* spp., all plant samples showed considerable extraction yield (Figure 4.1). In the present study, *Z. oxyphyllum* leaves showed extraction yield percentage between the range of 12.7 – 14.9 ± 0.7 %, while the same for the fruits was between 11.3 – 15.0 ± 1.1 %. In a previous study by Ayangla et al. (2016), the ethanolic leaf extracts of *Z. oxyphyllum* showed an extraction yield percentage of 9.85 % while for the fruits the extraction yield was 14.1 % (Ayangla et al., 2016). This slight variation might be

attributed to the climatic factors, edaphic factors and the solvent used. *Z. acanthopodium* leaves in the present study, had extraction yield between the range of 16.1 – 26.4 ± 3.7 %, the highest among all the extracts examined, while the same for fruits was 9.1 – 11.5 ± 0.7 %, the lowest among all the extracts examined. Other species of *Zanthoxylum* like *Z. armatum* leaves had an extraction yield of 7.78 % in ethanol, while *Z. rhetsa* leaves and seeds had extraction yield of 9.23%, and 14.21% respectively (Ayangla et al., 2016). Therefore, it may be hypothesized that in this case, in addition to other factors mentioned, maybe ultrasound assisted extraction system is a better method for extraction when compared to the extraction in aqueous ethanol.

4.2. Qualitative phytochemical screening

Qualitative phytochemical screening of the plant samples was done to detect the presence of phytochemicals. Preliminary screening of plant samples provides an overview of the phytochemical composition of the samples being studied thereby enabling estimation of their possible pharmacological properties (Shaikh and Patil, 2020). The phytochemicals screened were detected in all the plant extracts in moderate (+) to abundant levels (++) (Table 4.1). This result agrees with a previous study, where *Zanthoxylum* species tested positive for alkaloids, carbohydrates, phenols, flavonoids, proteins, glycosides, coumarins and tannins (Ayangla et al., 2016).

Table 4.1. Phytohemical constituents detected in *Z. oxyphyllum* Edgew. and *Z. acanthopodium* DC leaves and fruits.

		ZOL			ZOF			ZAL			ZAF		
No.	Phytochemical	R-1	R-2	R-3	R-1	R-2	R-3	R-1	R-2	R-3	R-1	R-2	R-3
1.	Alkaloids	+	-	+	+	+	+	+	+	+	+	+	+
2.	Carbohydrates	+	+	+	++	++	++	+	+	+	++	++	+
3.	Phenols	++	++	++	+	++	++	+	+	++	++	++	++
4.	Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
5.	Glycosides	+	+	+	++	+	++	-	+	+	+	+	+
6.	Steroids	+	+	+	+	+	+	+	+	+	+	+	+
7.	Proteins	+	+	+	+	+	+	-	+	+	+	+	+
8.	Tannins	+	+	+	+	+	+	+	+	+	+	+	+

5.3. Quantitative phytochemical analysis

5.3.1. Estimation of pigments

Chlorophyll is the major photosynthetic pigment found in plants and certain bacteria and can be of many types. They are made up of a porphyrin ring with a magnesium ion chelated to the ring, and an attached hydrocarbon tail. Chlorophyll a and b are the most common types of chlorophyll found in plants. In recent years, it has been elucidated that chlorophylls can exert several pharmacological activities including but not limited to anti-oxidant, anti-inflammatory, neuro-protective, protection against endocrine disruptors, anti-obesity, anti-cancer, anti-mutagenic and anti-toxic (Martins et al., 2023). It was shown that chlorophyll a and b were both successful in inhibiting TNF-α gene expression in HEK293 Cells (Human Embryonic Kidney 293 cells) (Subramoniam et al., 2012). Therefore, in the present study, estimation of chlorophyll a and b content in the plant samples was considered of relevance.

The total chlorophyll a and b content as well as carotenoids and anthocyanin content were spectrophotometrically estimated in all the fresh plant samples. As expected, chlorophyll a content was higher in the leaves of both the species in comparison to the fruits. While *Z. oxyphyllum* leaves had chlorophyll a content between the range of 1.62 - 2.36 ± 0.22 mg/g FW, in *Z. acanthopodium* leaves, the same was found to be slightly less at 1.77 – 1.99 ± 0.07 mg/g FW. In the fruits of both the species, significantly lower amounts of chlorophyll a were found. *Z. oxyphyllum* fruits had between 0.10 – 0.18 ± 0.02 mg/g FW and *Z. acanthopodium* fruits had 0.12 - 0.30 ± 0.05 mg/g FW of chlorophyll a (Figure 4.2).

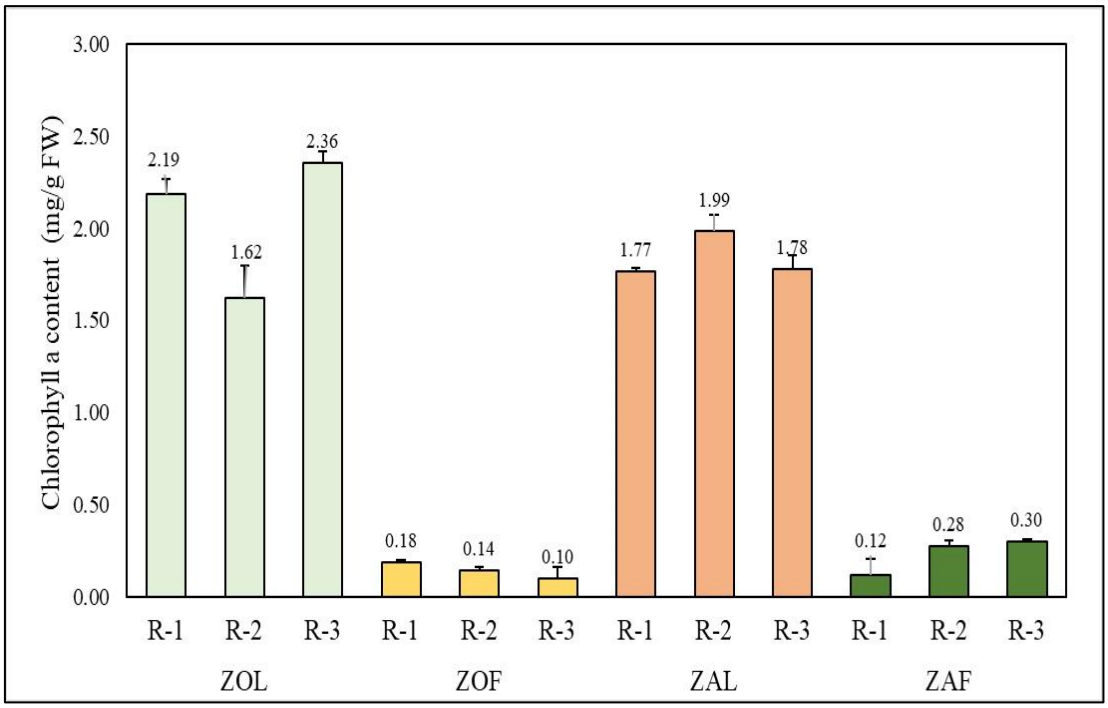

Figure 4.2. Chlorophyll a content (mg/g FW) in *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).

ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

In contrast, chlorophyll b content in the leaf samples were less in comparion to chlorophyll a. As observed for chlorophyll a, the content was more in leaves of both the species than the fruts. In *Z. oxyphyllum* leaves, chlorophyll b content was between the range of 0.63 – 0.93 ± 0.09 mg/g FW while in *Z. acanthopodium* leaves, the same was found to be 0.72 – 1.37 ± 0.21 mg/g FW. In the fruits however, the chlorophyll b content was observed to more than chlorophyll a content. Fruits of *Z. oxyphyllum* had chlorophyll b between 0.12 – 0.20 ± 0.02 mg/g FW while fruits of *Z. acanthopodium* contained 0.13 – 0.27 ± 0.04 mg/g FW (Figure 4.3).

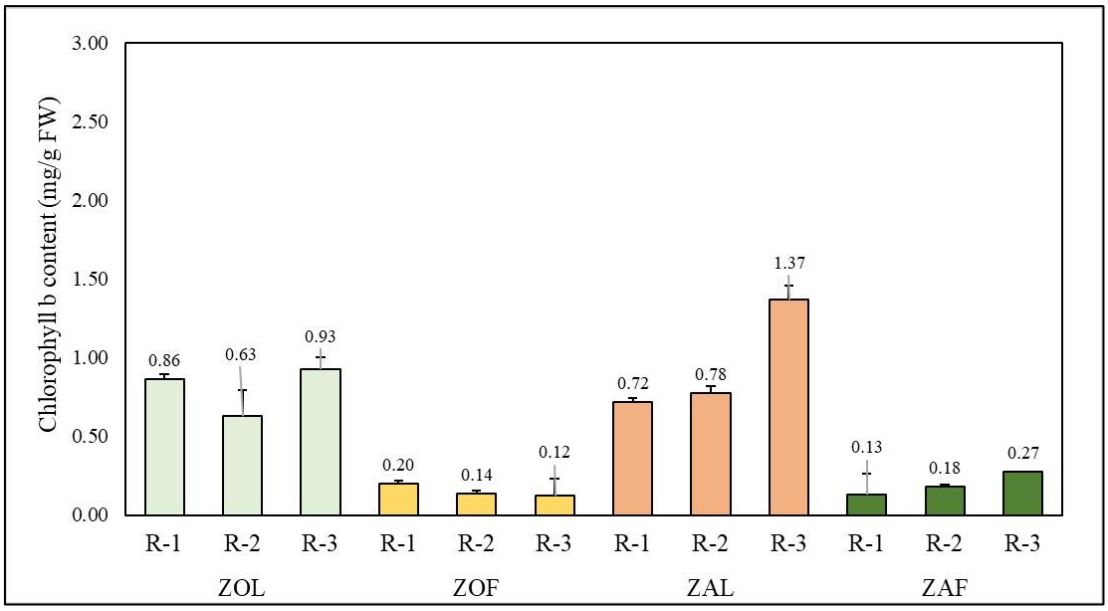

Figure 4.3. Chlorophyll b content (mg/g FW) in *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).

ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

When compared to other medicinal plants from different geographical regions, chlorophyll content in *Z. oxyphyllum* and *Z. acanthopodium* leaves and fruits were observed to be lower. In *Phyllanthus amarus*, chlorophyll a was found to be 13.96 µg/ml and chlorophyll b was found to be 7.56 µg/ml. Similarly, in *Adhotoda vasica*, *Tridax procumbens*, *Calotropis gigantean*, *Phyllanthus emblica*, *Gymnema sylvestre*, *Catharanthus roseus* and *Psoralea corylifolio* chlorophyll a was found to be 11.86, 11.72, 12.96, 31.77, 11.50, 30.86 and 13.13 µg/ml respectively, while chlorophyll b was found to be 12.51, 11.58, 6.49, 29.88, 5.22, 22.50 and 7.17 µg/ml respectively (Pallavaram, 2015).

However, when compared to other Rutaceae plants like *Citrus reticulata* peel which contained 0.0241 ± 0.0004 µg/g FW chlorophyll a and 0.0436 ± 0.0007 µg/g FW chlorophyll b (Kashyap et al., 2020), and *Z. rhetsa* fruits from the same geography had a total chlorophyll content of 0.123 ± 0.012 mg/g (Sundriyal and Sundriyal, 2001). However, the chlorophyll content in the two *Zanthoxylum* species studied was higher.

Carotenoid content in the leaves of *Z. oxyphyllum* and *Z. acanthopodium* were found to be almost comparable, with *Z. oxyphyllum* leaves having 0.37 – 0.58 ± 0.06 mg/g FW and *Z. acanthopodium* leaves having 0.33 – 0.53 ± 0.06 mg/g FW. In comparison, fruit from both the species contained only negligible amount of carotenoid content. While *Z. oxyphyllum* fruits had only 0.01 – 0.03 ± 0.01 mg/g FW carotenoids, *Z. acanthopodium* fruits had a slightly higher range of 0.02 – 0.08 ± 0.02 mg/g FW (Figure 4.4). Carotenoids are a class of isoprenoid pigments that are also involved in photosynthesis and are responsible for conferring the orange-red colour to plant parts. They are excellent anti-oxidants and therefore play a role in enhancing the immune response. Consumption of carotenoids has also been associated to lower risk of cancer (Bendich and Olson, 1989).

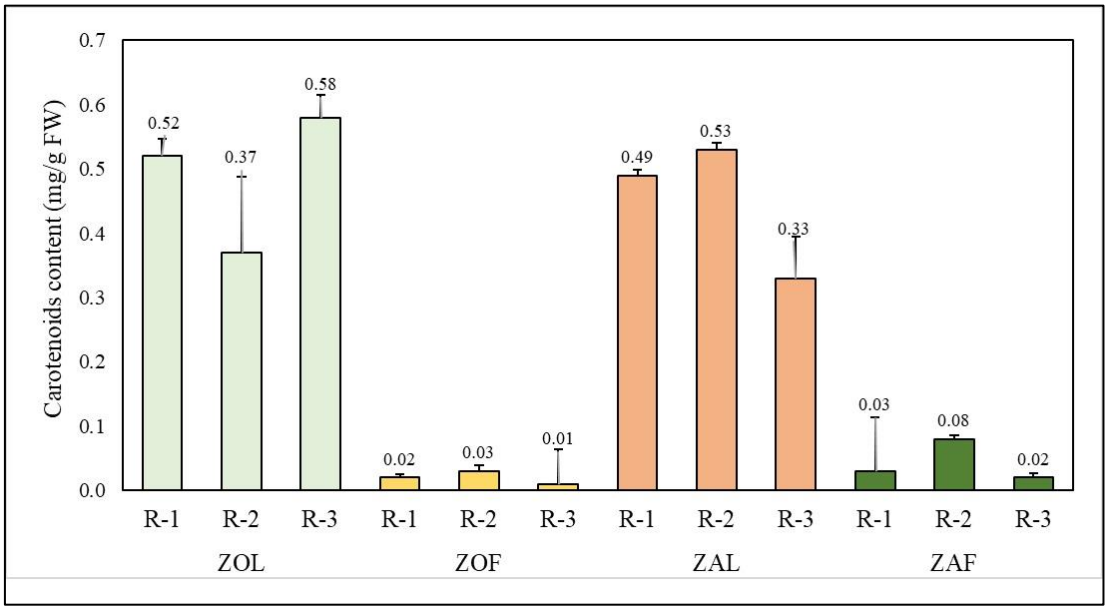

Figure 4.4. Carotenoid content (mg/g FW) in *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).

ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

It was reported that in flavedo of *Citrus sinensis*, total carotenoid content was 12.6 mg/100 g FW which is less than the amount observed in leaves of both *Z. oxyphyllum* and *Z. acanthopodium*. Moreover, in the pulp of the same orange, the total carotenoid present was 1.4 mg/100 g FW, which is less than the amount observed in fruits and leaves of both species examined in the present study. In comparison to Washington navel orange (159 mg/kg DW) and Tarocco orange (63.7 mg/kg DW) (Saini et al., 2022) however,

carotenoid content in both the *Zanthoxylum* species studied was lower. In *Ficus roxburghii*, carotenoid content was reported to be 0.68±0.10 mg GAE/ g which is comparable to that observed for the leaves of both *Zanthoxylum* species studied (Pandey et al., 2018). In comparison to *Hippophae rhamnoides* however, which contained 1180 to 1710 mg/kg of total carotenoids (Singh and Gupta, 2021), both *Zanthoxylum* species studied was observed to have less amount of total carotenoids. Thus it may be said that the present samples possessed an appreciable amount of carotenoids when compared with other medicinal plants from the same or different family.

Anthocyanins are water soluble flavonoids that give blue to red pigmentation to fruits and vegetables. These secondary metabolites are known to exert antimicrobial, anti-inflammatory, anti-mutagenic and anti-proliferative, anti-viral and anti-carcinogenic activities and their consumption has been associated to lowered risk of diseases related to immune system (Liu et al., 2021). In the present study, different plant samples contained varying amounts of anthocyanins (Figure 4.5). Fruits of *Z. oxyphyllum* contained the highest amount of anthocyanin ranging between 0.38 – 0.77 ± 0.12 mg/g FW. This was followed by *Z. oxyphyllum* leaves and *Z. acanthopodium* fruits with 0.11 – 0.48 ± 0.11 mg/g FW and 0.14 – 0.45 ± 0.09 mg/g FW anthocyanins respectively. The least amount was detected in *Z. acanthopodium* leaves with 0.16 – 0.27 ± 0.04 mg/g FW anthocyanins. In an earlier report, it was shown that *Z. bungeanum* contained total anthocyanin content between the range of 206.81 mg/100 g - 301.16 mg/100g which is comparatively very high (Jing et al., 2021b).

When compared to *Z. rhetsa* fruits, which was reported to have anthocyanin content of 0.896 ± 0.32 mg/100 g (Sundriyal and Sundriyal, 2001), fruits and leaves of both *Z. oxyphyllum* and *Z. acanthopodium* had higher anthocyanin content. In fact, the anthocyanin content detected in both the *Zanthoxylum* species studied, was more than fruits of *Rubus ellipticus* (3.81 ± 0.008 mg/100 g), *Diploknema butyracea* (0.615 ± 0.001 mg/100 g), *Eleagnus latifolia* (1.58 ± 0.26 mg/100 g) and *Machilus edulis* (0.391 ± 0.013 mg/100 g), which are all wild edible fruits found in the Sikkim Himalayas (Sundriyal and Sundriyal, 2001). The foregoing discussion showed that in both the *Zanthoxylum* spp., the function of anthocyanin-based protection of the plants was taken up by the fruits rather than the leaves. Moreover, maybe the insect pollination of *Zanthoxylum* relies more on aroma than on vivid colouration.

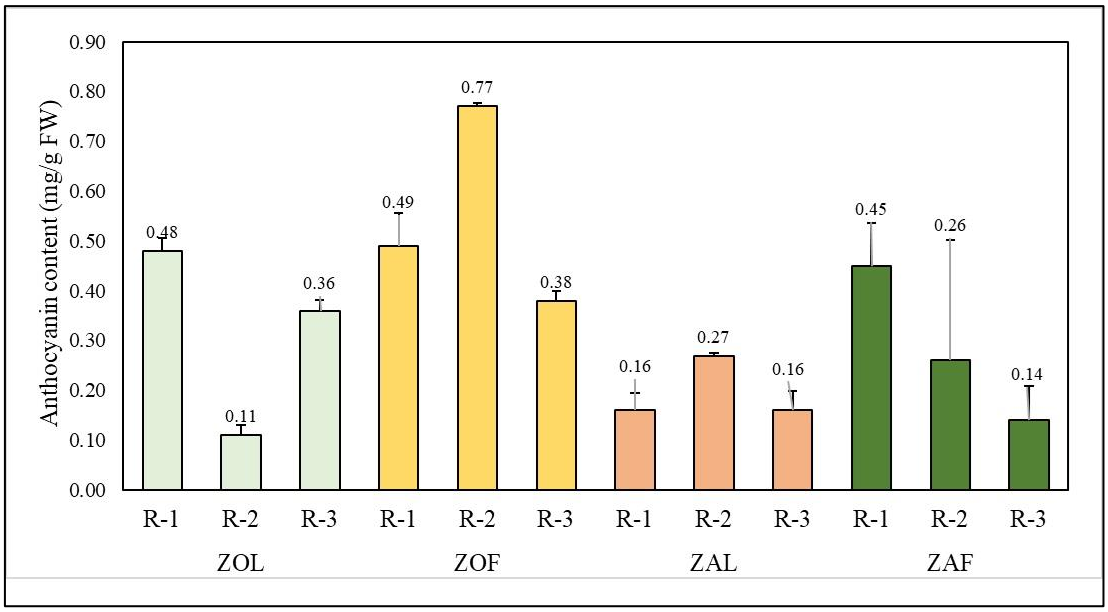

Figure 4.5. Anthocyanin content (mg/g FW) in *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).

ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

5.3.2. Estimation of crude protein content

Crude protein content was found to be within a comparable range for both the species. In *Z. oxyphyllum* leaves, the crude protein content was between the range of 77.52 – 119.60 ± 12.22 mg/g FW while in the fruits the same was found to be 96.62 – 102.38 ± 1.86 mg/g FW. In *Z. acanthopodium* leaves, it was lowest at 63.68 – 94.83 ± 9.05 mg/g FW and in *Z. acanthopodium* fruits it was 94.85 – 108.67 ± 4.23 mg/g FW (Figure 4.6). The crude protein contents of plant samples in the present study is comparable to the content reported in *Z. zanthoxyloides*, which had a value of 11.00 ± 0.31 % (Olushola-Siedoks et al., 2020). However, in *Z. acanthopodium* fruits found in Indonesia, the protein content was higher (16.2 %) (Satria et al., 2023) than the value observed in the present study. It is known that plants show great variability in their morphology, physiology and genetics as adaptation to different kinds of environmental conditions (Cavallini and Natali 1991). Therefore, in the present study, it can be assumed that because of the wide variation of geographical conditions between Sikkim Himalayan region and Indonesian islands, an intraspecific variation in the protein content was observed.

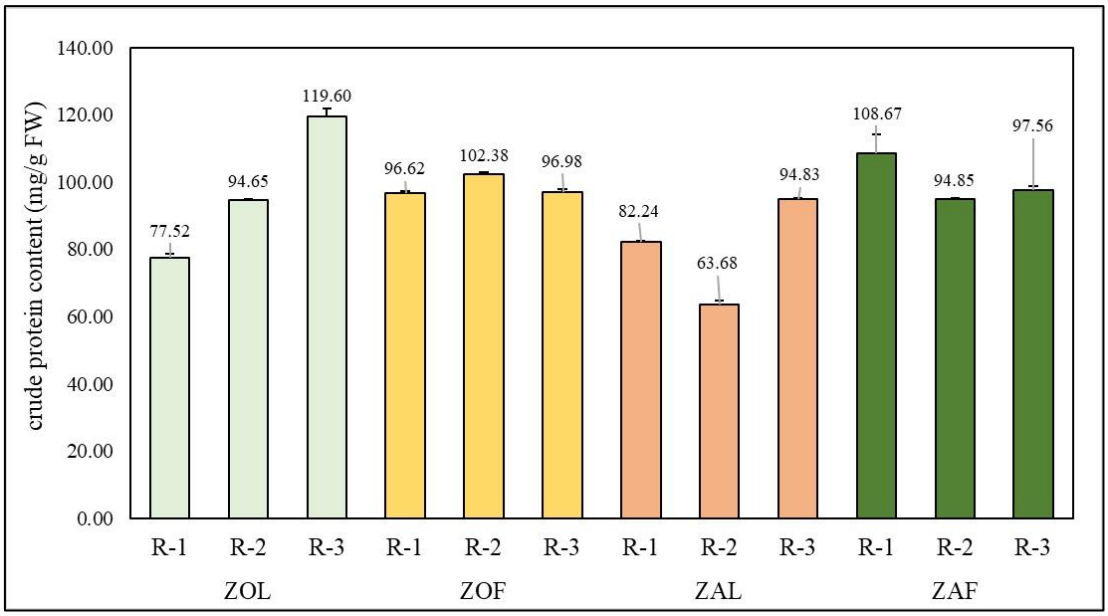

Figure 4.6. Crude protein content (mg/g FW) in *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).

ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

In other more widely available species of *Zanthoxylum* like *Z. armatum* as well, the crude protein content was observed to be higher (19.95 ± 0.41 %) than the values observed in the present study (Phuyal et al., 2020c). The protein content range of all the plant samples in the current study was comparable to other wild fruits of Sikkim Himalaya like *Hippophae rhamnoides* which was reported to have protein content of 10.32 % and *Cucumis melo* with a protein content of 8.29 % (Sundriyal and Sundriyal, 2001). However, when compared to other more popular sources of plant protein like wild mushrooms - *Russula gnathangensis* and *Ramaria thindii* which were reported to have protein content of 185 µg/ml and 164 µg/ml (Paul and Das, 2021), leaves and fruits of both the species of *Zanthoxylum* studied had less protein.

5.3.3. Estimation of total phenolic content

Dietary polyphenols exert significant immunomodulatory effects by influencing various immune cells and pathways. Polyphenols reduce inflammation in conditions like inflammatory bowel disease by inducing regulatory T cells (Tregs) in the gut, suppressing pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), promoting apoptosis, and reducing DNA damage. Their potential extends to preventing and treating autoimmune diseases like type 1 diabetes, rheumatoid arthritis, and multiple sclerosis by regulating signaling pathways, limiting demyelination, and suppressing inflammatory responses. Polyphenols also mitigate allergic and autoimmune reactions by inhibiting autoimmune T cell proliferation and downregulating pro-inflammatory cytokines, including interleukin-6 (IL-6), IL-1, and interferon-γ (IFN-γ) (Shakoor et al., 2021).

In the present study, the total phenolic content was found to be highest in *Z. acanthopodium* leaf extracts with a range of 110.78 – 184.44 ± 21.45 µg GAE/ mg. This was followed by fruit extracts of both *Z. oxyphyllum* and *Z. acanthopodium* with a range of 41.22 – 93.91 ± 15.21 µg GAE/ mg and 69.42 – 84.03 ± 4.22 µg GAE/ mg resepctively. The least amount of total phenolic content was observed in *Z. oxyphyllum* leaf extracts which ranged between 39.72 – 63.07 ± 6.77 µg GAE/ mg (Figure 4.7).

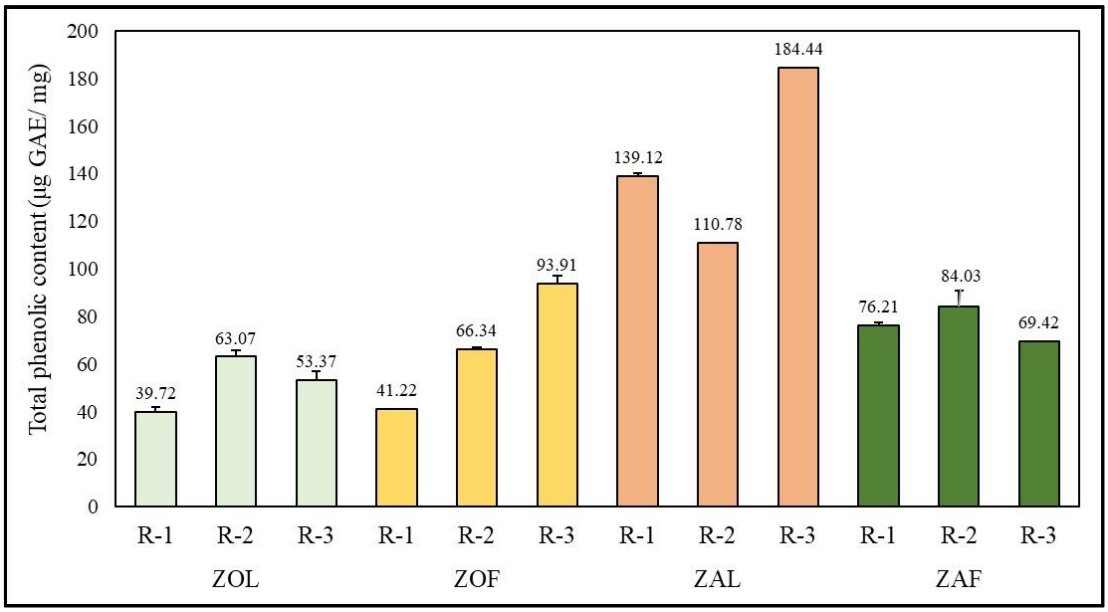

Figure 4.7. Total phenolics content (µg GAE/ mg) in methanolic extracts of *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).

ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

It was reported previously that in the essential oil of fruits of *Z. acanthopodium*, the total phenolic content was 3.81 mg GAE/g which is relatively low as compared to its methanolic extract counterpart in the present study (Rahmi et al., 2023). On the other hand, the total phenolic content in methanolic extract of *Z. oxyphyllum* leaves in the present investigation agrees to a previous study where the same was found to have a total phenolic content of 55.37 ± 1.21 81 mg GAE/g (Chelleng et al., 2021). In *Hippophae salicifolia*, a high altitude wild medicinal plant found in Sikkim Himalaya, the total phenolic content in the methanolic extracts of pulp, leaves and seeds was reported to be 123.5 ± 1.2 mg GAE/g, 156.7 ± 1.8 mg GAE/g and 98.7 ± 1.5 mg GAE/g respectively. In comparison, only leaf extracts of *Z. acanthopodium* was close to the phenolic content observed in *Hippophae salicifolia* (Saikia and Handique, 2013). *Z. armatum* is another locally available species of *Zanthoxylum* with great market value. It was reported that this species had total phenolic content of 185.02 ± 2.15 GAE/g in wild fruits, 226.3 ± 1.14 GAE/g in cultivated fruts, 167.74 ± 2.63 GAE/g in wild seeds, 137.72 ± 4.21 GAE/g in cultivated seeds, 185.15 ± 1.22 GAE/g in wild bark and 171.13 ± 6.73 GAE/g in cultivated bark (Phuyal et al., 2020), all of which are greater than the values observed for

all the extracts examined in the present study. Plant phenolics profers anti-inflammatory activity by reducing the production of pro-inflammatory cytokines, cyclooxygenase, etc. Hence, such effects in *Zanthoxylum* species is not out of place.

5.3.4. Estimation of total flavonoid content

Flavonoids are a sub class of plant polyphenols that exert multiple pharmacological benefits. They have been reported to contain antioxidant, anti-diabetic, anti-viral, anti-allergic, anti-carcinogenic and immunomodulatory properties by maintaining a balance between Th1 and Th2 cells (Gandhi et al., 2018). In the present study, the highest flavonoid content was observed in the leaf extracts of *Z. acanthopodium* with a range of 22.65 – 27.33 ± 1.53 µg QE/mg. This value supports the previous result where the highest amount of total phenolic content was also found in *Z. acanthopodium* leaf extracts. *Z. oxyphyllum* leaf extracts had the second highest flavonoid content ranging between 13.83 – 15.34 ± 0.46 µg QE/mg. In the fruit extracts of both the species, flavonoid content was found to be similar with *Z. oxyphyllum* fruit extracts having 4.32 – 6.09 ± 0.51 µg QE/mg and *Z. acanthopodium* fruit extracts having 4.39 – 5.69 ± 0.43 µg QE/mg (Figure 4.8). In a previous study, the total flavonoid content in the methanolic leaf extracts of *Z. oxyphyllum* was slightly higher at 22.55 ± 0.87 mg RE/ g (Chelleng et al., 2021). In another study, it was shown that essential oil of *Z. acnathopodium* fruits had 11.42 mg QE/g of total flavonoid content which is more in comparison to the value observed for fruit methanolic extract in the present study (Rahmi et al., 2023).

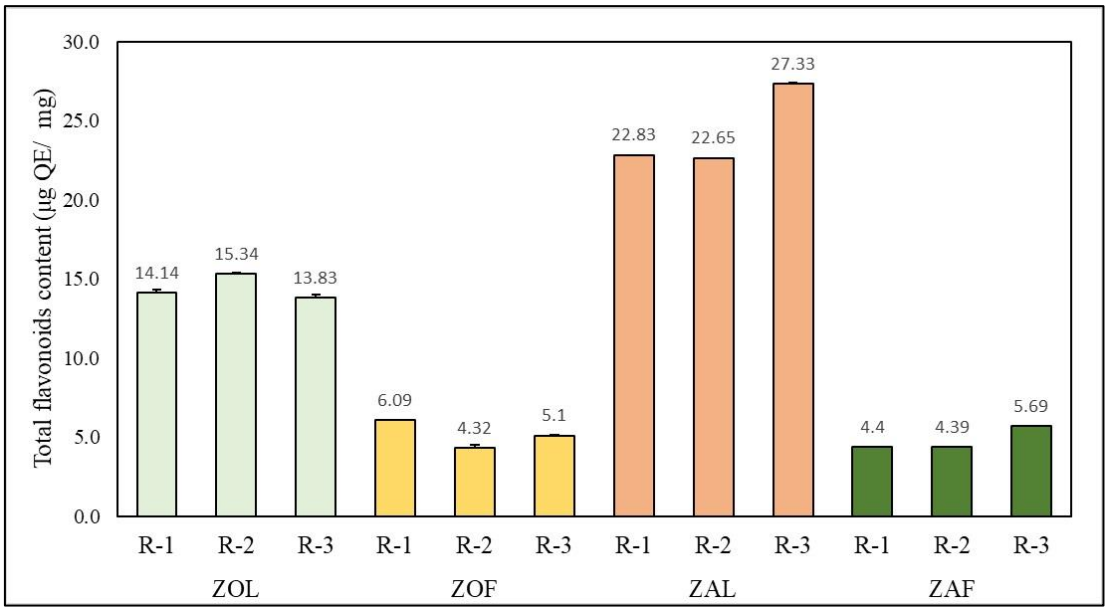

Figure 4.8. Total flavonoids content (µg GAE/ mg) in methanolic extracts of *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).
ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Total flavonoid content observed in leaf and fruit extracts of both the species in the present study was comparatively lower in comparison to different parts of *Z. armatum*, which had a value of 103.7 ± 1.39 QE/g, 92.71 ± 3.14 QE/g and 91.27 ± 3.13 QE/g in wild fruits, seeds and bark respectively and 135.17 ± 2.02 QE/g, 76.58 ± 4.18 QE/g and 111.2 ± 3.67 QE/g in cultivated fruits, seeds and bark respectively (Phuyal et al., 2020). The wide variation in the the flavonoid content may be accounted for by the genetic differences as well as edaphic factor variation in the Eastern Himalay with that of Western Nepal in case of *Z. armatum*. In ccomparison to another local ethnomedicinal plant *Litsea cubeba* which was reported to have total flavonoid content of 3.8 ± 0.042 mg RE/ g (Subba and Rai, 2018) however, all extracts in the present study had higher flavonoid content.

5.3.5. Estimation of total tannin content

Tannins are sub class of polyphenols that are found in plants as complexes with proteins, polysaccharides, and alkaloids. Tannins are reported to have many beneficial effects including *in vitro* immunomodulation (Behl et al., 2021). In the present study, total tannin content in all the extracts was observed to be more than that of total flavonoid content.

Following the trend observed for total phenolic and total flavonoid content, the highest total tannin content was found to be in extract of *Z. acanthopodium* leaf with a range of 61.40 – 124.70 ± 20.51 µg TAE/mg. *Z. acanthopodium* fruit extracts had the second highest content ranging between 32.20 – 71.69 ± 11.59 µg TAE/mg. Fruit and leaf extracts of *Z. oxyphyllum* had comparatively less amount with the leaf extract showing a wide range of 5.61 – 30.62 ± 7.39 µg TAE/mg and the fruit extracts having 24.12 – 40.37 ± 5.20 µg TAE/mg (Figure 4.9).

In *Z. armatum*, the tannin content reported was less (2.69 ± 0.07 mg of GAE/g) as compared to all the extracts in the present study (Phuyal et al., 2020c). In *Z. tetraspermum* on the other hand, the total tannin was reported to be 0.80 % g DW (Narayanasamy and Ragavan, 2012). While the total tannin content in *Z. oxyphyllum* extracts were comparable to an ethnomedicinal plant *Equisetum diffusum* (37.8 ± 0.051 mg GAE/g), the total tannin content of *Z. acanthopodium* extracts were comparable to another ethnomedicinal plant *Betula alnoides* (62 ± 0.034 mg GAE/g) (Subba and Rai, 2018). Further, in comparison to other wild edible plants, the tannin content in all the extracts in the present study was much higher. The tannin content reported in *Aralia armata*, *Macropanax dispermus*, *Fagopyrum dibotrys*, *Heracleum wallichii*, *Tupistra clarkei*, and *Rumex nepalensis* was 0.68 ± 0.06 %, 0.09 ± 0.02 %, 0.34 ± 0.017 %, 0.04 ± 0.003 %, 0.26 ± 0.007 % and 0.29 ± 0.003 % respectively (Seal et al., 2023). Thus, the tannin content in the *Zanthoxylum* species studied is appreciable in comparison to other Himalayan medicinal plants.

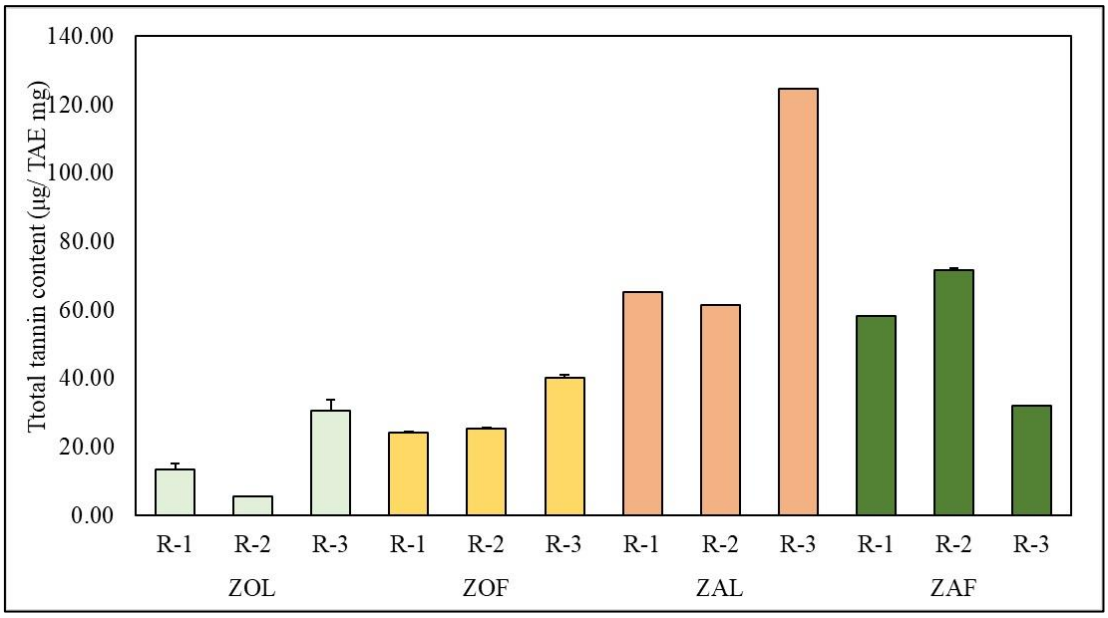

Figure 4.9. Total tannin content (µg GAE/ mg) in methanolic extracts of *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).

ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

5.3.6. Estimation of total saponin content

Saponins are a sub class of terpenoids and is present in abundance in most plants, especially pulses. In the recent years, saponins have been reported to exert properties such as anti-inflammatory, hypo-cholesterolemic and immune-stimulatory activities (Singh et al., 2017). In the present study, extracts of *Z. acanthopodium* were found to have higher total saponin content in comparison to *Z. oxyphyllum* extracts. While saponin content in *Z. acanthopodium* leaf extract was between 8.12 – 16.89 ± 2.73 µg DE/mg, the same in *Z. acanthopodium* fruit extract was 8.53 – 23.70 ± 4.30 µg DE/mg. Between the two *Z. oxyphyllum* samples, lead extract had more saponin at 7.65 – 10.60 ± 0.85 µg DE/mg and lowest amount was found in fruit extracts at 0.57 – 2.34 ± 0.85 µg DE/mg (Figure 4.10).

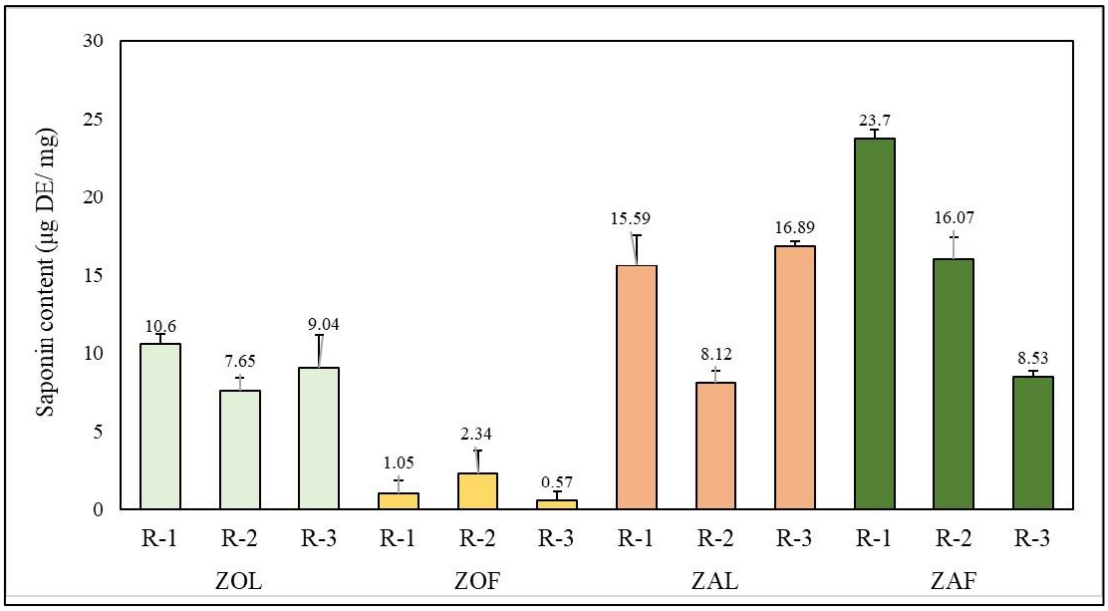

Figure 4.10. Total saponin content (µg GAE/ mg) in methanolic extracts of *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).
ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Saponin content reported in *Z. armatum* leaf (21.57 ± 0.12 mg/g), fruit extract (28.60 ± 0.10 mg/g) and bark extract (14.78 ± 0.10 mg/g) (Ibrar et al., 2017) was comparable to *Z. acanthopodium* extracts in the present study. Among other wild medicinal plants found in Sikkim Himalaya, saponin content reported in *Aralia armata* (3.86 ± 0.03 %), *Fagopyrum dibotrys* (4.92 ± 0.02 %), *Heracleum wallichii* (2.54 ± 0.02 %) and *Tupistra clarkei* (3.88 ± 0.01 %) was more than all the extracts investigated in the present study and saponin content reported in *Macropanax dispermus* (0.09 ± 0.003 %), and *Rumex nepalensis* (0.05 ± 0.004 %) was less (Seal et al., 2023).

5.3.7. Estimation of total polysaccharide content

Polysaccharides are long chain carbohydrate molecules composed of monosaccharide units. They are also referred to as dietary fibres. The most common plant polysaccharides are glycogen, cellulose and starch. Plant polysaccharides have been known to exert a wide range of beneficial activities like antitumor, anticoagulant, antioxidative, antiviral, antihyperlipidemic and antihepatotoxic. In addition, they also have the potential to modulate immune responses (Sindhu et al., 2021). In the present study, *Z. oxyphyllum*

fruit extracts showed the widest and highest range of polysaccharide content between 101.17 – 451.71 ± 103.48 µg GE/mg. Fruit and leaf extracts of *Z. acanthopodium* had polysaccharides ranging from 214.23 – 333.69 ± 36.92 µg GE/mg and 183.72 – 354.65 ± 52.78 µg GE/mg respectively. The lowest content was observed for *Z. oxyphyllum* leaves with values of 108.28 – 264.83 ± 47.68 µg GE/mg (Figure 4.11).

It was reported that in *Spondias axillaris* and *Eriolobus indica*, two wild fruits found in Sikkim Himalaya, the total polysaccharide content was 87.07 ± 0.01 % and 89.06 ± 0.52 % respectively (Pandey et al., 2018), which is higher than all the extracts investigated in the present study. In another locally available wild fruit, *Ficus roxburghii*, as well, the total polysaccharide content was reported to be 90.81 ± 0.44 % (Pandey et al., 2018) which is more than the value found for all extracts in the present study. However, the total polysaccharide content in *Z. oxyphyllum* leaf extract is comparable to some common Indian vegetables, such as *Beta vulgaris* (23.7 % DW), *Alium cepa* (14.8 % DW), *Solanum tuberosum* (12.5 % DW), *Musa sapientum* (26.4 % DW) and *Lycopersicum esculentum* (21.0 % DW).

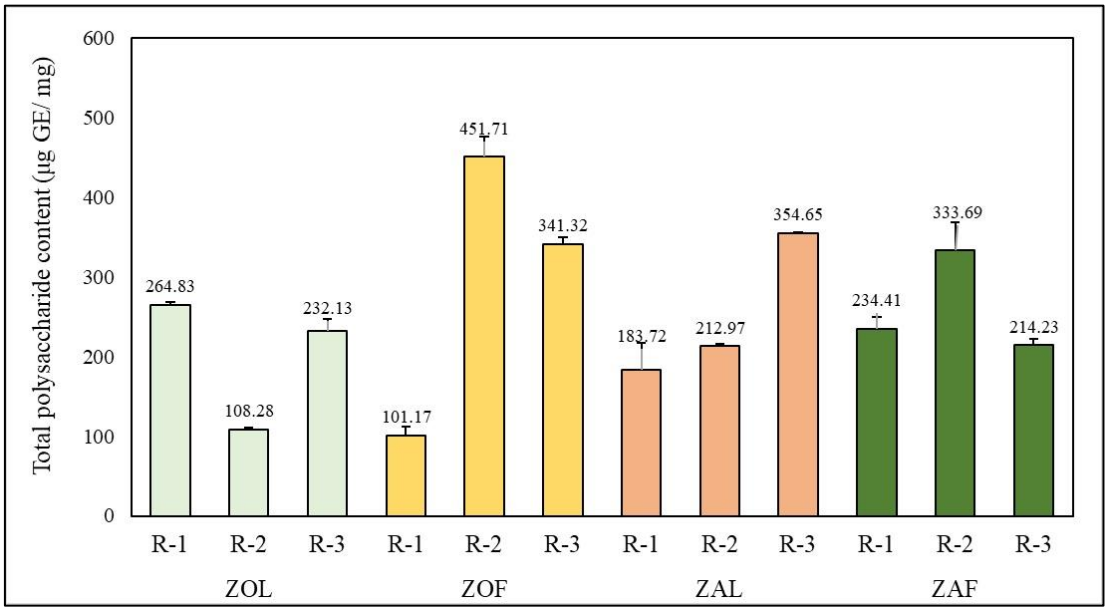

Figure 4.11. Total polysaccahride content (µg GAE/ mg) in methanolic extracts of *Z. oxyphyllum* Edgew. leaves and fruits from three different locations and *Z. acanthopodium* DC leaves and fruits from three different locations (Data presented as ± SEM, n = 3).
ZOL = Z. oxyphyllum Edgew. leaves, ZOF = Z. oxyphyllum Edgew. fruits, ZAL = Z. acanthopodium DC. leaves, ZAF = Z. acanthopodium DC. fruits; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Similarly, the total polysaccharide content in *Z. acanthopodium* leaf and fruit extracts is also comparable to some vegetables like *Trigonella foenum grecum* leaves (35.3 % DW), *Peucedanum graveoleus* (34.6 % DW), *Lagenaria vulgari* (32.3 % DW), *Vicia faba* (31.0 % DW), *Cyamopsis tetragona loba* (35.2 % DW), *Phaseoleus vulgaris* (35.4 % DW) and *Solanum menongena* (33.6 % DW). Lastly, the highest range of polysaccharide range detected in *Z. oxyphyllum* fruit extract, can be compared to *Daucus carota* (39.5 % DW), *Brassica oleracea capitata* (39.6 % DW), *Raphanus sativus* (37.4 % DW), *Brassica oleracea botyritis* (45.9 % DW), *Dolichos lablab* (43.4 % DDW) and *Abelmoschus esculentus* (41.2 % DW) (Khanum et al., 2000).

5.4. *In vitro* anti-oxidant studies of plant extracts

Oxidative stress, a concept increasingly studied in medical sciences, arises from an overproduction of reactive oxygen species (ROS) in cellular mitochondria. This imbalance, often caused by factors such as UV radiation and environmental pollutants, disrupts the equilibrium between antioxidants and pro-oxidants. Oxidative stress is implicated in numerous degenerative conditions, including carcinogenesis, inflammation, diabetes, hypertension, atherosclerosis, Alzheimer's and Parkinson's diseases, aging, and cardiovascular disorders. Under normal conditions, ROS production and elimination are balanced, but when this balance is disrupted, oxidative damage occurs, significantly impacting human health (Gulcin and Alwasel, 2023).

Antioxidants are substances that inhibit or prevent oxidation, even at low concentrations, by donating electrons to neutralize free radicals and minimizing oxidative damage in biological processes. They are vital in maintaining the balance between oxidants and antioxidants in metabolism and have applications in food and pharmaceutical industries. They prevent lipid peroxidation, preserve food, and drug quality, and extend shelf life. While synthetic antioxidants like BHT (Butylated hydroxytoluene), PG (propyl gallate), BHA (butylated hydroxyanisole) and TBHQ (tert-butylhydroquinone) are widely used due to their cost-effectiveness and high reactivity, they are associated with potential health risks, including carcinogenesis, skin allergies, fatty liver, and gastrointestinal issues. Concerns over their safety have prompted efforts to replace them with natural antioxidants, which are less toxic, biodegradable, and safer for long-term use (Gulcin and Alwasel, 2023).

Natural antioxidants, commonly found in fruits, vegetables, herbs, and spices, are rich in bioactive compounds such as tannins, catechins, phenolics, and flavonoids. Popular sources include tea, cinnamon, cloves, rosemary, and linden. These plant-based antioxidants help reduce the risk of diseases and mitigate degenerative disorders, making them a preferred choice for health-conscious consumers (Gulcin and Alwasel, 2023).

ROS accumulation has been known to trigger inflammation via activation of immune cell receptors and the subsequent release of inflammatory mediators. Antioxidants play a critical role in eliminating these ROS thereby modulating immune responses by reducing oxidative stress, which is closely linked to inflammation and immune dysfunction (Liu et al., 2011). *In vitro* anti-oxidant assays provide a quantitative measure of the ability of plant extracts to neutralize free radicals, which can help predict their potential to protect immune cells from oxidative damage. Therefore, in the present study, assessment of the anti-oxidant capacity of the extracts was considered necessary to provide insight into their potential role in supporting immune health. Plants often contain multiple bioactive compounds with both antioxidant and immunomodulatory effects (Wang et al., 2017). Hence, a preliminary *in vitro* antioxidant assay was expected to help identify whether the observed immunomodulatory activity of the extracts in the later studies was related to their antioxidant properties. Antioxidants can directly influence immune signalling pathways and cytokine production (Ajith et al., 2017). *In vitro* anti-oxidant assays like DPPH (1,1-diphenyl-2-picrylhydrazil) radical scavenging assay, ferric reducing anti-oxidant power assay (FRAP) and total anti-oxidant capacity (TAC) are quick, inexpensive, and easy to perform, making them an ideal first step to screen extracts before moving on to more complex and resource-intensive cell culture studies.

5.4.1. DPPH radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazil (DPPH) radical, discovered in 1922, is a stable free radical widely used for evaluating antioxidant activity. This method, initially developed by Blois in 1958 and refined by Brand-Williams in 1995, measures the ability of antioxidants to neutralize DPPH radicals through hydrogen atom donation. The reaction leads to a colour change from violet to pale yellow as the radical is reduced to its hydrazine form (DPPH-H), which is monitored using UV-vis spectroscopy at 517 nm. The reaction's simplicity and speed have made the DPPH test a standard tool for assessing

the antioxidant capacity of pure compounds, herbal extracts, and phenolics (Gulcin and Alwasel, 2023).

The IC_{50} value, or "inhibitory concentration," represents the concentration of an antioxidant required to scavenge 50% of initial DPPH radicals within a specific time frame. Also referred to as EC_{50} in some contexts, this measure is widely used in biochemistry to compare the radical-scavenging abilities of different antioxidants. A lower IC_{50} value indicates a stronger antioxidant activity. Due to its simplicity and practicality, IC_{50} is a standard method for evaluating and quantifying the effectiveness of antioxidants in neutralizing DPPH radicals (Gulcin and Alwasel, 2023).

When looking at the antioxidant activity of a compound, a straightforward relationship between dose and effect can often be seen in the form of a linear-no-threshold model. In this model, the response increases steadily with the dose. This type of response is called a linear or monotonic correlation because it shows a consistent, predictable relationship (Barreiro-Sisto et al., 2024). In the present study, all the extracts showed a monotonic correlation for DPPH radical scavenging activity.

At 50 µg/ml concentration, leaf extracts of *Z. oxyphyllum* showed a scavenging percentage of between 5.05 – 14.83 % which increased with the dose and showed highest scavenging activity of 34.00 – 83.69 % at 250 µg/ml concentration (Figure 4.12). The IC_{50} value for the same was observed to be between 172.21 – 352.63 ± 53.75 µg/ml. The fruit extract of *Z. oxyphyllum* also showed a similar range of scavenging activity with 50 µg/ml concentration showing 11.83 – 24.65 % activity and the highest scavenging activity being 51.98 – 80.64 % at 250 µg/ml (Figure 4.13). The IC_{50} value of the same was calculated as 143.03 – 229.00 ± 26.97 µg/ml, less than the value for *Z. oxyphyllum* leaf extracts.

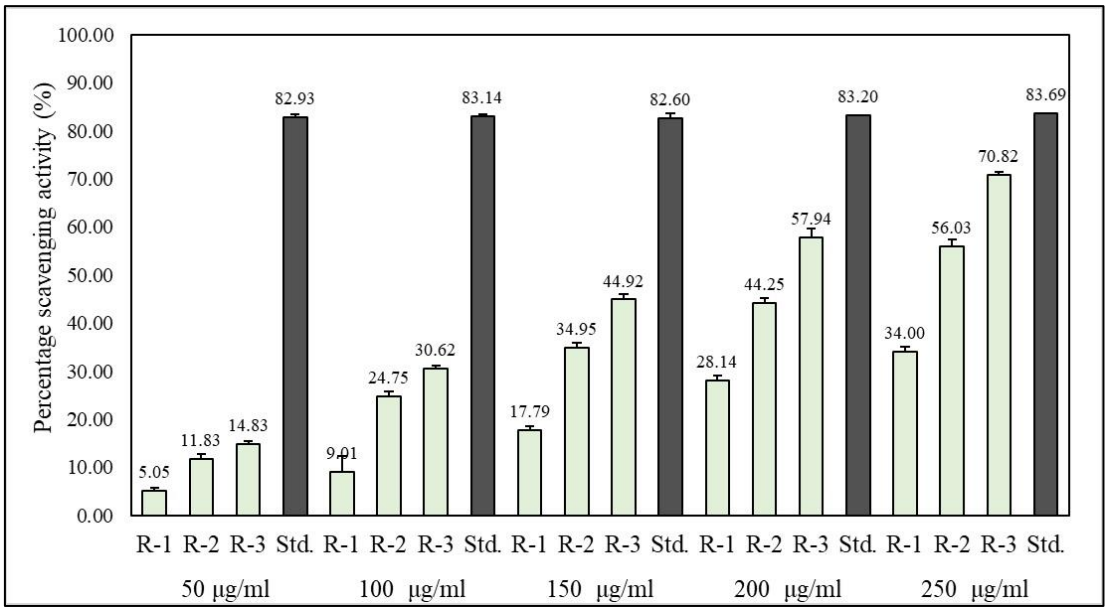

Figure 4.12. DPPH radical scavenging activity of methanolic extracts of *Z. oxyphyllum* Edgew. leaves (Data presented as ± SEM, n = 3).
Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

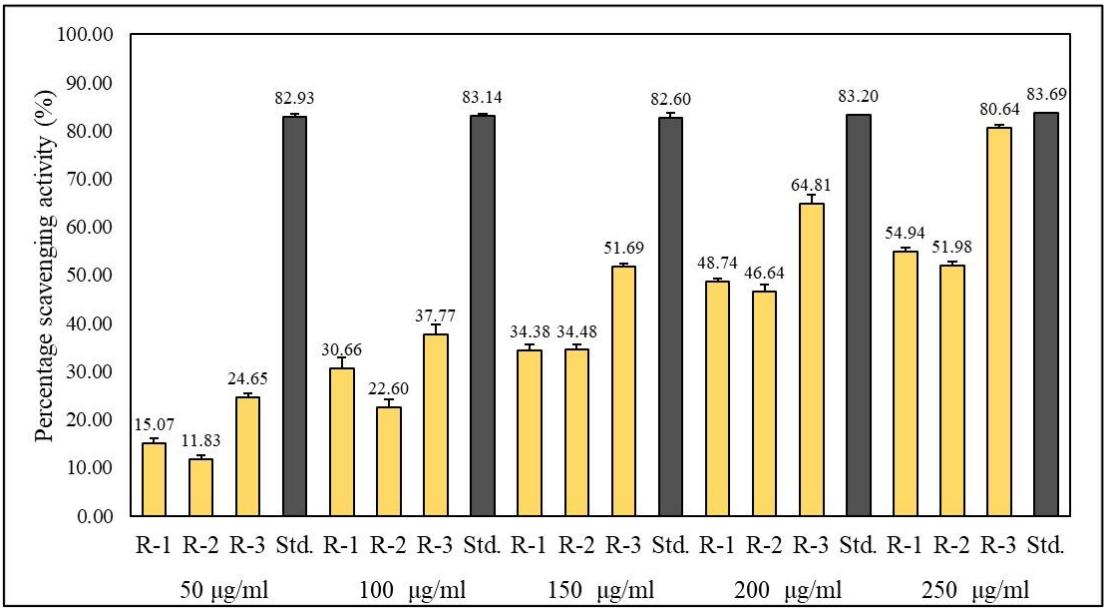

Figure 4.13. DPPH radical scavenging activity of methanolic extracts of *Z. oxyphyllum* Edgew. fruits (Data presented as ± SEM, n = 3).
Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Among all the extracts studied, *Z. acanthopodium* leaf extracts showed the highest capacity for DPPH radical scavenging. At 50 µg/ml concentration, the extracts had a

percentage activity of 37.53 – 72.44 % which is like the activity shown by *Z. oxyphyllum* leaf extract at a higher concentration of 250 µg/ml. In fact, with increasing concentration, the extracts showed activity comparable to that of the reference standard gallic acid which is a naturally occurring strong, antioxidant compound. At the highest concentration of 250 µg/ml, the extract showed a percentage scavenging activity of between 75.19 – 83.93 % (Figure 4.14). The IC_{50} value calculated was 10.32 – 98.52 ± 25.47 µg/ml. The fruit extracts of *Z. acanthopodium* on the other hand, exhibited the least percentage scavenging activity with a value of 3.96 – 9.25 % at 50 µg/ml, increased to a value of 30.85 – 65.62 % at 250 µg/ml (Figure 4.15). The IC_{50} value calculated for the same was 192.67 – 354.16 ± 47.47 µg/ml. In comparison, the reference standard gallic acid had an IC_{50} value of 11.73 ± 0.92 µg/ml.

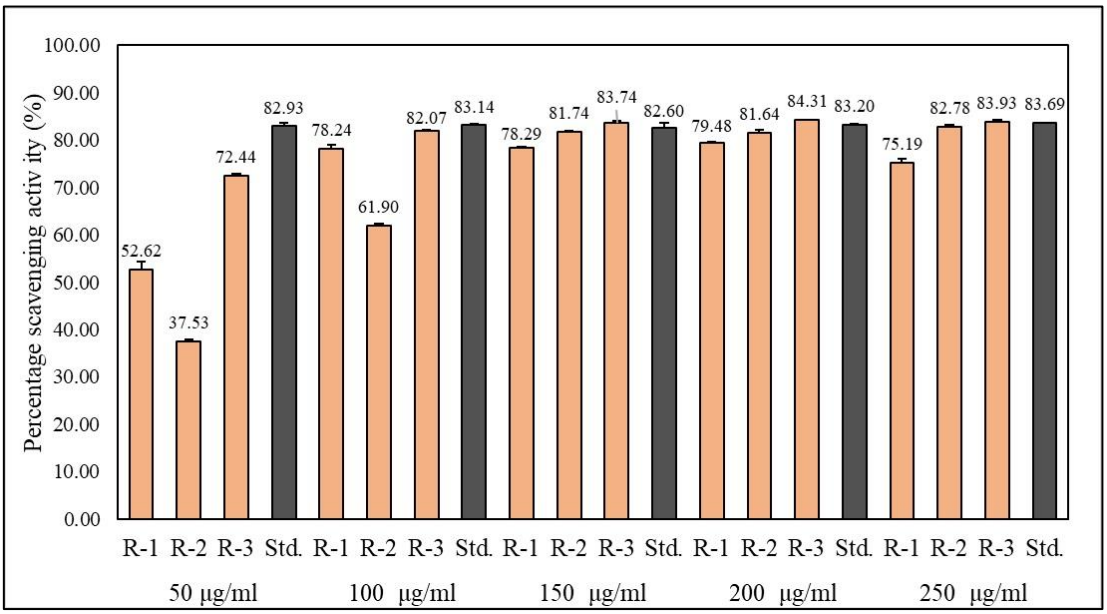

Figure 4.14. DPPH radical scavenging activity of methanolic extracts of *Z. acanthopodium* DC. leaves (Data presented as ± SEM, n = 3).

Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Zanthoxylum species are known to exert antioxidant potential to various degrees. The wild and cultivated fruits of *Z. armatum* was reported to have strong IC_{50} values of 45.62 µg/ml, 86.75 µg/ml and 67.82 µg/ml for wild fruits, seeds and bark extract respectively, while the same for cultivated fruits, seeds and bark extracts were 40.62 µg/ml, 94.49 µg/ml and 63.39 µg/ml respectively (Phuyal et al., 2020c). Polyphenols are the main secondary metabolites responsible for conferring antioxidant properties to plants

(Hollman, 2001). In the study by Phuyal et al. (2020), the total phenolics and flavonoids content of all the extracts studied was comparatively higher in comparison to the values observed in the present study. Hence, the higher IC_{50} values of the *Z. oxyphyllum* anf *Z. acanthopodium* extracts than that of *Z. armatum* extracts can be justified.

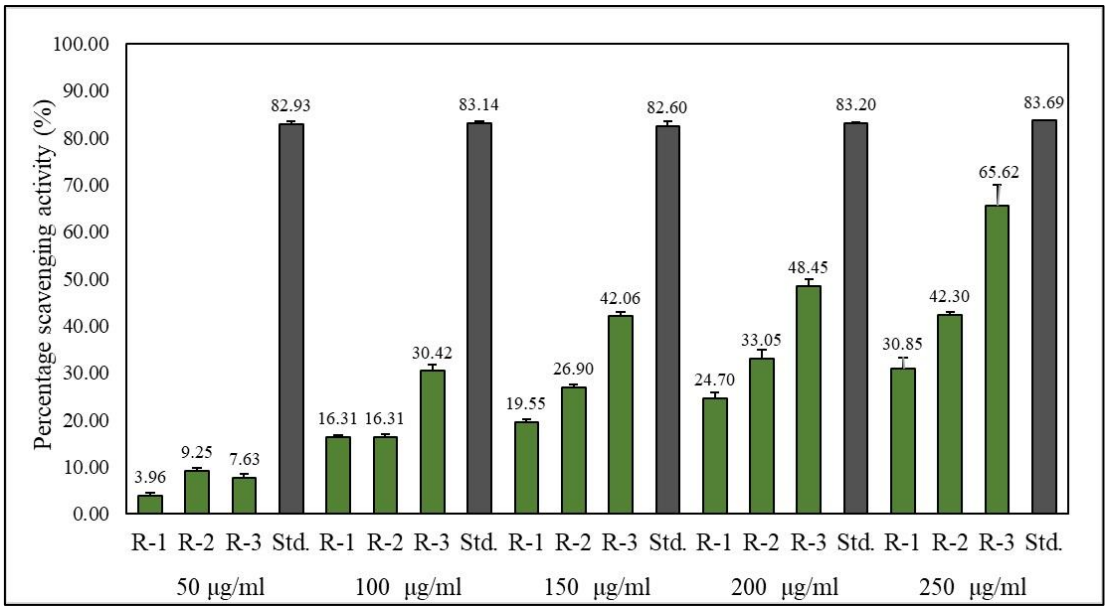

Figure 4.15. DPPH radical scavenging activity of methanolic extracts of *Z. acanthopodium* DC. fruits (Data presented as ± SEM, n = 3).
Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Other local, wild medicinal plants found in Sikkim Himalaya were reported to have comparable IC_{50} values. Subba and Rai (2018) reported that *Betula alnoides*, *Equisetum diffusum*, *Litsea cubeba*, *Quercus lamellosa*, *Stephania glabra*, *Zingiber zerumbet* had IC_{50} values of 200.1 ± 0.754 µg/ml, 253.1 ± 0.07 µg/ml, 288.18 ± 0.32 µg/ml, 143.3 ± 0.314 µg/ml, 149.15 ± 0.324 µg/ml and 305.2 ± 0.243 µg/ml respectively (Subba and Rai, 2018). *Paris polyphylla* is another high value medicinal plant found in Sikkim Himalaya that has cytotoxic properties and is traditionally used for treating cancer. It was reported that the IC_{50} value of DPPH radical scavenging activity of its rhizome extracts was 2.55 ± 0.04 - 2.01 ± 0.16 mg/ml (Lepcha et al., 2019) which is comparatively very high from the extracts investigated in the present study. In comparison to *Rhus chinensis* however, which was reported to have an IC_{50} value of 1.04 ± 0.16 µg/ml, all the extracts in the present study had higher IC_{50} values (Chhetri et al., 2020). Nevertheless, it can be

concluded that all the extracts studied showed low to moderate DPPH radical scavenging potential.

5.4.2. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was used to investigate the antioxidant power of the extracts. The principle of this method is based on the reduction of a ferric-tripyridyltriazine complex to its ferrous, coloured form in the presence of antioxidants. In this type of assay, the antioxidant activity is exerted by the compounds via electron donation rather than hydrogen atom donation, both of which is an indication of anti-oxidant potential of a compound/ plant extract. A higher absorbance is related to higher antioxidant power and vice versa (Guo et al., 2003). In the present study, all the extracts showed various capacities for ferric reducing antioxidant power in a dose dependent manner signifying the consistent reduction of Fe^{3+} to Fe^{2+} and thereby indicating the reduction potential of the plant extracts. At lower concentrations of 50 µg/ml, both *Z. oxyphyllum* extracts showed negligible reducing power. At the highest concentration (250 µg/ml), the extracts showed considerable activity, with *Z. oxyphyllum* leaf extracts having an absorbance of 0.32 – 0.46 and the same for fruit extract being 0.20 – 0.35 (Figures 4.16 and 4.17).

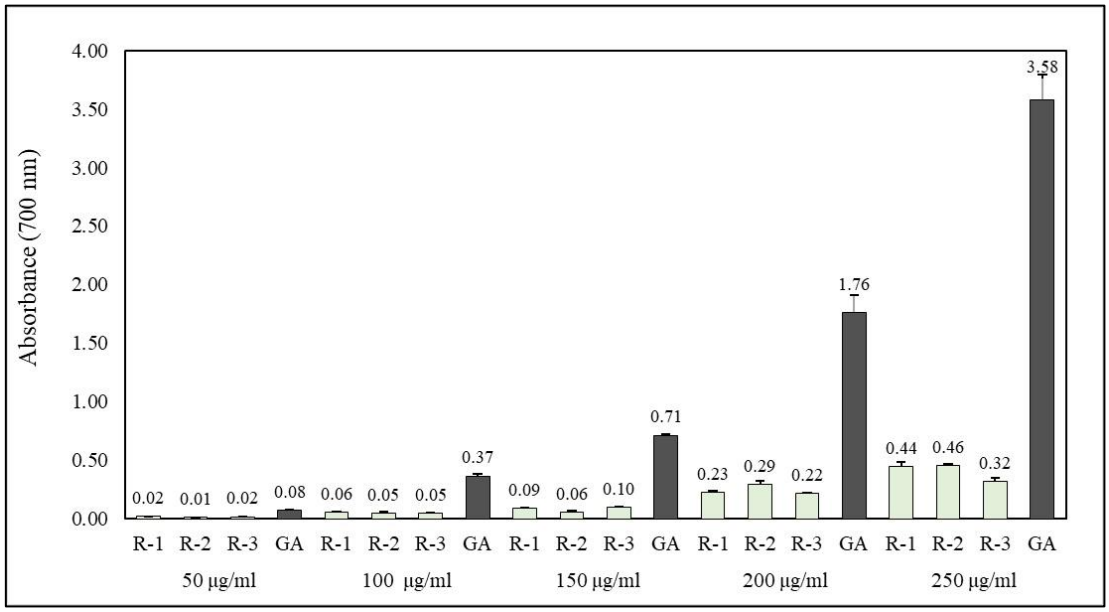

Figure 4.16. FRAP activity of methanolic extracts of *Z. oxyphyllum* Edgew. leaves (Data presented as ± SEM, n = 3).

Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Z. acanthopodium extracts showed better reducing antioxidant power in comparison. While the leaf extract showed absorbance of 0.10 - 0.27 at 50 µg/ml, the same at 250 µg/ml was 0.60 – 1.12. Similarly, at 50 µg/ml the fruit extract had absorbance of 0.07 – 0.28 and at 250 µg/ml, the same was 0.67 – 0.87 (Figures 4.18 and 4.19). In fact, at lower concentrations of 50 µg/ml and 100 µg/ml, both the *Z. acanthopodium* extracts showed absorbance comparable to that of the reference standard gallic acid. At 50 µg/ml gallic acid had absorbance of 0.08 and at 100 µg/ml the absorbance was 0.37, both of which were comparable to *Z. acanthopodium* extracts. However, at higher concentrations, none of the extracts studied showed absorbance comparable to that of the standard.

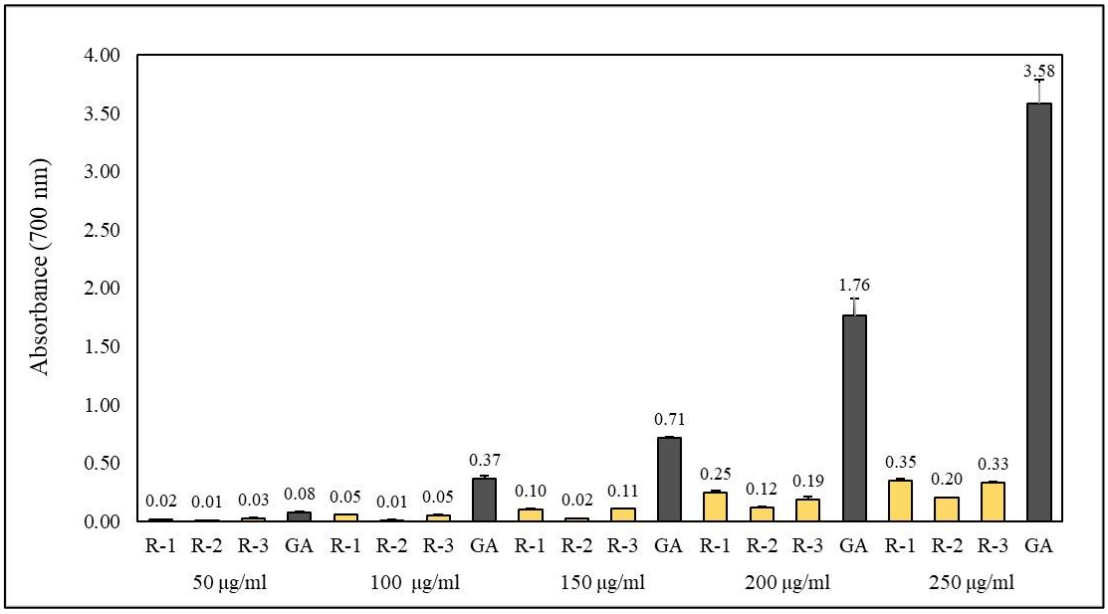

Figure 4.17. FRAP activity of methanolic extracts of *Z. oxyphyllum* Edgew. fruits (Data presented as ± SEM, n = 3).
Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

When compared to *Z. armatum*, the ferric reducing power of all the extracts was relatively lower. At 7.81 µg/ml, the methanolic leaf extract had an absorbance of 0.265 ± 0.06 which is more than the value observed for all the extracts in the present study at 50 µg/ml. At 250 µg/ml concentration the *Z. armatum* extracts had absorbance value of 1.411 ± 0.46 while the values observed for all the extracts in the present study was relatively less (Kanwal et al., 2015). In a separate study, the methanolic extracts of *Z. armatum* stem showed comparatively less reducing power particularly compared to *Z. acanthopodium* extracts, with absorbance of 0.602 ± 0.008 at 400 µg/ml (Mukhija and Kalia, 2014).

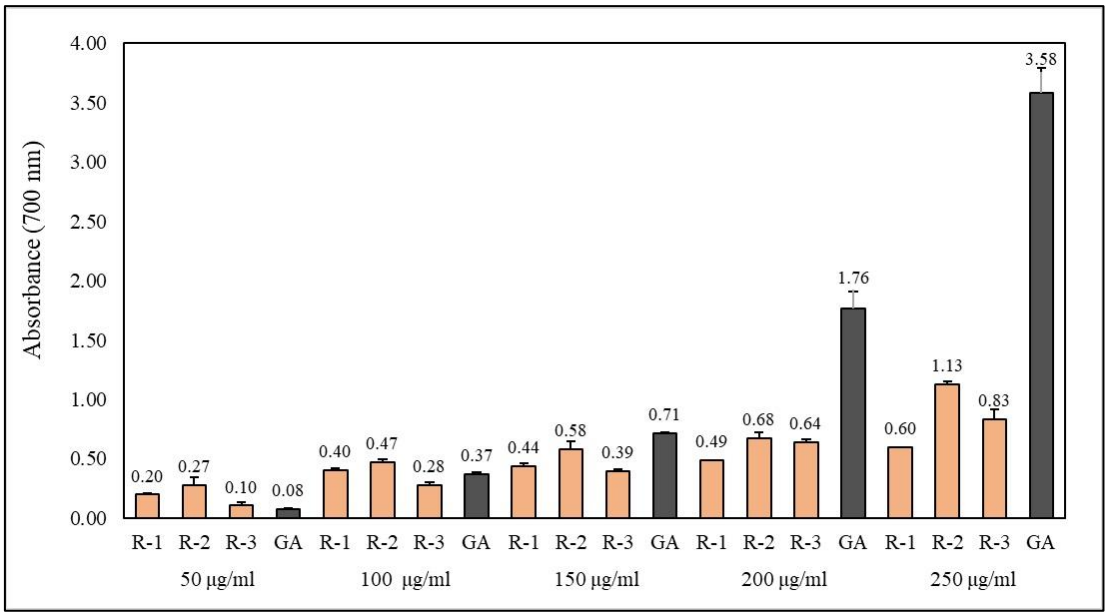

Figure 4.18. FRAP activity of methanolic extracts of *Z. acanthopodium* DC. leaves (Data presented as ± SEM, n = 3).

Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

In comparison to other ethnomedicinal plants, both *Z. oxyphyllum* and *Z. acanthopodium* extracts showed equal or better reducing power. In a previous study it was reported that methanolic leaf and bark extract of *Hippophae salicifolia* showed reducing power comparable to that of *Z. acanthopodium* extracts with absorbance of 0.44 and 0.46 respectively at 200 µg/ml concentration (Goyal et al., 2011). The roots of wound healing plant *Amaranthus spinosus* however, showed less reducing power with absorbance of less than 0.5 even at 1000 µg/ml concentration (Barku et al., 2013) in comparison to all the extracts examined in the present study. Therefore, along with DPPH radical scavenging potential, all the extracts studied also showed various degrees of ferric reducing potential.

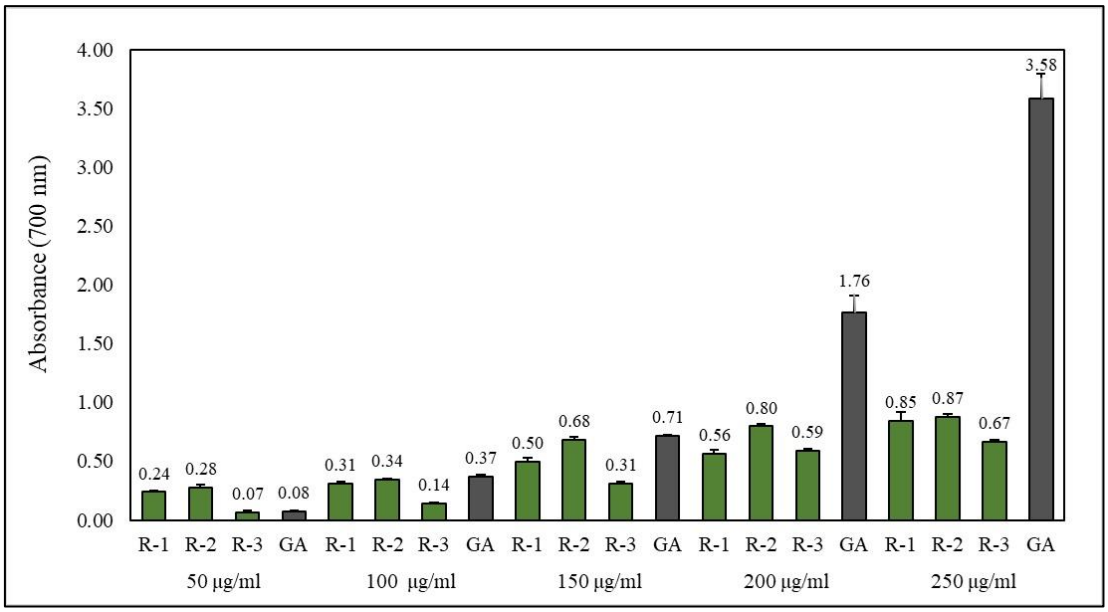

Figure 4.19. FRAP activity of methanolic extracts of *Z. acanthopodium* DC. fruits (Data presented as ± SEM, n = 3).

Std. = reference standard gallic acid; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

5.4.3. Total antioxidant capacity

To test the total antioxidant capacity of the plant extracts, phosphomolybdate assay was used. The principle behind the assay is the reduction of phosphomolybdate ion by an antioxidant to form a green phosphate/Mo(V) complex. The total antioxidant capacity was expressed as gallic acid equivalents. A higher absorbance was indicative of higher antioxidant activity. In the present study, *Z. oxyphyllum* fruit extracts showed the highest capacity for reducing phosphomolybdate ion with value of 48.84 – 72.51 ± 4.56 µg GAE/ 100 µg. Leaf extracts of the plants showed comparable total antioxidant activity with *Z. oxyphyllum* leaf extract having value of 37.07 – 52.73 µg GAE/ 100 µg and the same for *Z. acanthopodium* leaf being 33.75 – 43.03 µg GAE/ 100 µg. Among the extracts, least total antioxidant capacity was exerted by *Z. acanthopodium* fruit extracts with value ranging from 18.82 - 20.90 µg GAE/ 100 (Figure 4.20).

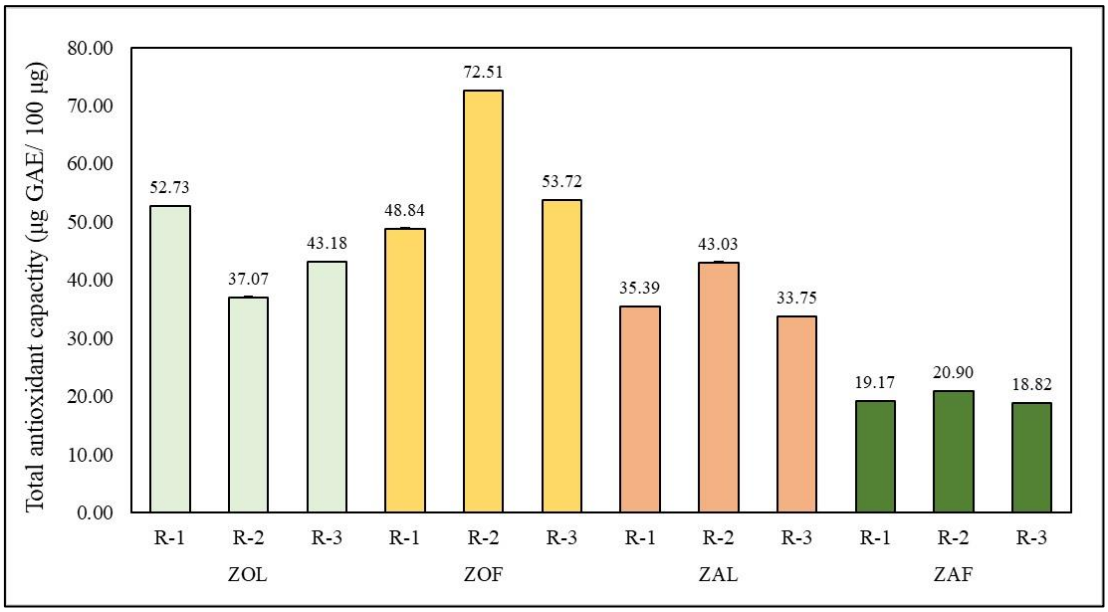

Figure 4.20. Total antioxidant capacity of methanolic extracts of *Z. oxyphyllum* leaf and fruit and *Z. acanthopodium* DC. leaf and fruit (Data presented as ± SEM, n = 3).
R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

It was reported that ethyl acetate, acetone, and chloroform extracts of *Bauhinia variegata* leaves had total antioxidant capacities of 257.5, 163.6 and 251.7 µg propyl gallate equivalent (PGE)/g of extract respectively while stem extracts of *Tinospora cordifolia* exhibited antioxidant capacity equivalent to 400-500 mg PGE/g (Mishra et al., 2011). All the extracts examined in the present study had higher total antioxidant capacity than the two species reported. In fact, both leaf and fruit extracts of *Z. oxyphyllum* showed total antioxidant capacity similar to that reported for *Piper longum* fruit extracts which showed very high total antioxidant capacity ranging from 408.6 - 604.4 mg PGE/g (Mishra et al. 2011). In comparison to the Rutaceae medicinal plant *Aegle marmelos* leaf extracts which were reported to have an antioxidant capacity ranging from 0.32 ±0.13 - 49.19 ±1.56 α-tocopherol equivalent (µg/mL) also both *Z. oxyphyllum* and *Z. acanthopodium* extracts in the present study had higher antioxidant capacity. *Cocculus hirsutus* leaf extracts were also reported to show comparatively lower antioxidant capacity ranging between 0.21 ±0.10 - 16.03 ±0.89 α-tocopherol equivalent (µg/mL) in the same study (Goswami et al., 2018). Moreover, the total antioxidant capacity of leaf extracts of *Hippophae rhamnoides* and *H. tibetana* was also reported to be relatively less than all the extracts investigated in the present study (Haq et al., 2022). A relatively higher total antioxidant activity in both

the *Zanthoxylum* samples indicates a greater synergism among the different anti-oxidants present in them.

Overall, all the extracts in the present study, exhibited potential anti-oxidant activity *in vitro*. None of the extracts were significantly more potent than the reference standard gallic acid. However, both *Z. oxyphyllum* and *Z. acanthopodium* extracts showed anti-oxidant potential equivalent to many other, more widely known ethnomedicinal plants. Reports suggest that much of the anti-oxidant potential exerted by plants is strongly correlated to their secondary metabolite content, particularly phenolics and flavonoids (Sundriyal and Sundriyal, 2001). Phenolic compounds are primary antioxidants that prevent or slow down lipid oxidation by neutralizing free radicals, which reduces the formation of harmful byproducts like aldehydes and ketones. Their effectiveness depends on the number and arrangement of hydroxyl groups. Phenolics donate hydrogen to free radicals, forming more stable radicals that interrupt oxidation. Stronger antioxidant activity occurs when hydrogen transfer is easier, as in α-tocopherol, which has a lower reduction potential than peroxyl radicals. The phenoxyl radical formed is stabilized by electron delocalization in the aromatic ring, and substitutions at certain positions improve its stability by blocking further reactions (Shahidi and Ambigaipalan, 2015). In the present study, considerable amounts of total phenolics were detected in all the extracts, especially *Z. acanthopodium* leaf extract which interestingly showed better DPPH radical scavenging activity as well as ferric reducing antioxidant power than the rest suggesting a positive correlation between the polyphenol group and radical scavenging ability as well as ferric ion reducing ability. Flavonoids, being a sub group of polyphenols was also found in the highest amount in *Z. acanthopodium* leaf extracts thus justifying its highest DPPH radical scavenging activity and ferric ion reducing activity among the samples studied.

5.5. *In vitro* anti-inflammatory studies of plant extracts

In vitro anti-inflammatory assays are good preliminary indicators of immunomodulatory potential since inflammation is a key part of the immune response. These assays reveal a plant's ability to regulate inflammatory pathways, which often overlap with mechanisms involved in immune modulation. They are simple, controlled, and cost-effective for screening bioactive compounds. Hence, HRBC membrane stabilization assay and heat induced protein denaturation assay was conducted.

5.5.1. HRBC membrane stabilization assay

To test the anti-inflammatory potential of *Z. acanthopodium* and *Z. oxyphyllum* extracts, human red blood cell stabilization assay was conducted. HRBC membrane stabilization assay is an advanced non-animal approach that has have proven to be effective and has significantly reduced the reliance on animal testing in evaluating medicines and chemicals. Inflammation is a vital defence mechanism against harmful stimuli like infections, burns, toxins, and allergens. However, excessive, or uncontrolled inflammation can lead to chronic conditions such as heart disease, septic shock, and rheumatoid arthritis. Lysosomal enzymes released during inflammation contribute to tissue damage through lipid peroxidation and degradation of macromolecules. Stabilizing lysosomal membranes is crucial in controlling the inflammatory response by preventing the release of damaging enzymes and proteases from activated neutrophils.

The human red blood cell (HRBC) membrane shares similarities with the lysosomal membrane. Therefore, the stabilization of HRBC membranes under hypotonic conditions is used as an *in vitro* model to evaluate the anti-inflammatory potential of drugs or plant extracts. Stabilization in this context suggests the ability of the plant extracts being studied to protect lysosomal membranes, thereby limiting tissue damage and inflammation (Sharma et al., 2018). The standard anti-inflammatory drug, diclofenac sodium was used as reference. In the present study, all the extracts showed membrane stabilization percentage to various degrees.

The leaf extracts of *Z. oxyphyllum* exerted a dose dependent membrane stabilization activity. At lower concentration of 1 mg/ml, the extracts showed percentage activity of 32.19 – 65.05 ± 9.56 % which increased with increasing concentration (Figure 4.21). The extracts exhibited activity on par with that of the standard drug (diclofenac sodium) at

higher concentrations, where the percentage protection observed for the extracts was 76.97 – 87.40 ± 3.35 % at 4 mg/ml and 81.23 – 88.29 ± 2.08 % at 5 mg/ml was and the same for diclofenac sodium was 78.96 ± 0.33 % and 83.50 ± 1.96 % at 4 mg/ml and 5 mg/ml respectively. A one-way ANOVA showed that there was no significant difference between the activity exerted by the extracts and diclofenac sodium with *P* values of 0.80, 0.25 and 0.52 for *Z. oxyphyllum* leaf extracts R-1, R-2 and R-3 respectively.

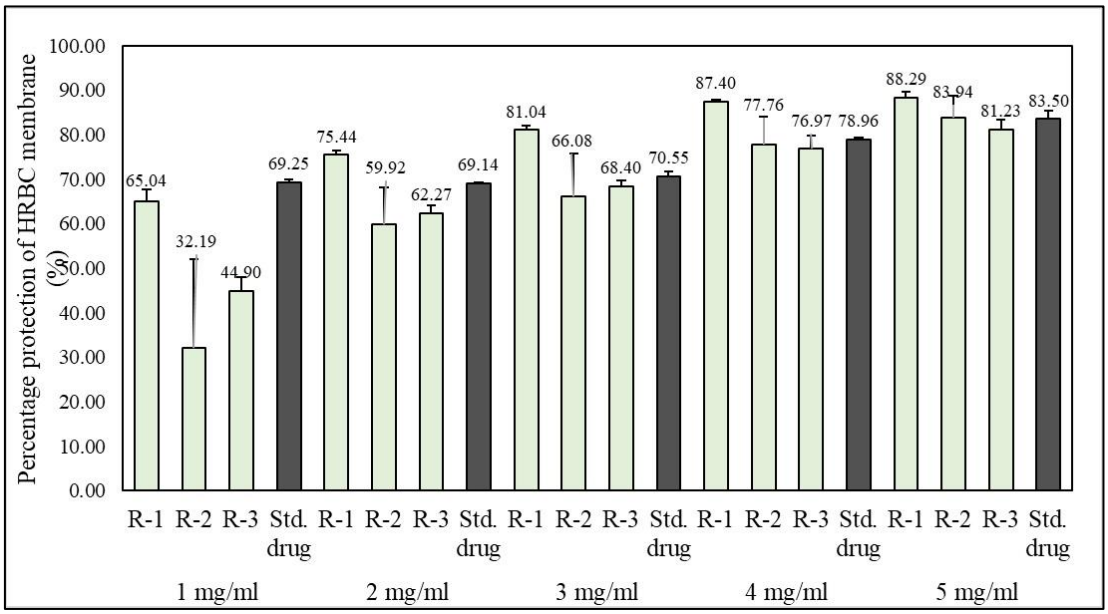

Figure 4.21. HRBC membrane stabilization activity of *Z. oxyphyllum* leaf extracts (Data presented as ± SEM, n = 3).
Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Interestingly, a biphasic response was observed for *Z. oxyphyllum* fruit extracts. At lower concentration of 1 mg/ml, a high percentage protection of 69.56 – 88.30 ± 15.10 % was observed. However, this activity decreased at increasing concentrations, with the lowest activity observed at 5 mg/ml with values ranging between 54.38 – 57.78 ± 1.06 % (Figure 4.22).

Such bi-phasic response has also been termed as hormesis. Hormesis describes a situation where a compound has opposite effects depending on its dose: low doses stimulate beneficial effects, while high doses can inhibit or even cause harm. Two key features of this hormetic response curve have been observed. Low doses were shown to typically enhance biological responses by 30–60% compared to normal level and the stimulatory

effects observed were within a specific range, usually 5–10 times lower than the dose at which the effect switched to inhibition (Jodynis-Liebert and Kujawska, 2020).

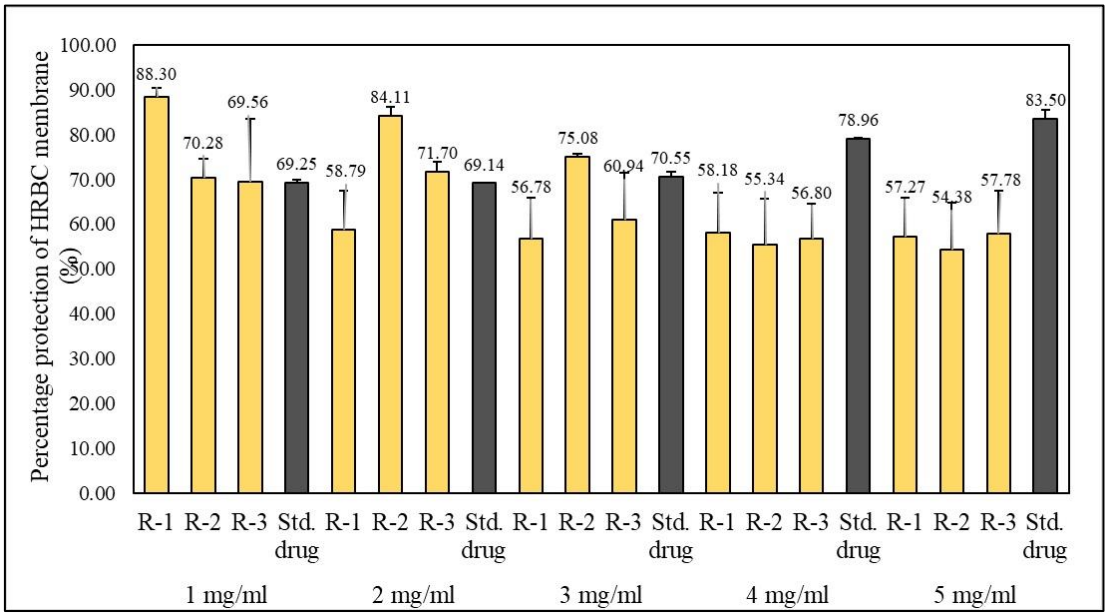

Figure 4.22. HRBC membrane stabilization activity of *Z. oxyphyllum* fruit extracts (Data presented as ± SEM, n = 3).
Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Further, hormetic responses are independent of the biological system being studied, whether it involves microbes, plants, animals, or humans. It is also independent of the specific biological outcome being measured, the level of organization (cell, organ, or whole organism), or whether the experiments are conducted *in vitro* or *in vivo*. Furthermore, hormesis can be triggered by various agents and is not tied to a single mechanism of action. This universality makes hormesis a broadly applicable concept across different fields of biology (Calabrese et al., 2024). However, there is still much insufficiency when it comes to understanding the actual mechanisms underlying a hormetic response (Barreiro-Sisto et al., 2024). Nevertheless, hormesis is a universal phenomenon and have been observed for many plant based products (Calabrese et al., 2024).

In the present study, a similar hormetic pattern was observed for *Z. oxyphyllum* fruit extracts. Many phytochemicals, like resveratrol, curcumin, and sulforaphane have been reported to exhibit hormesis (Jodynis-Liebert and Kujawska, 2020). Therefore, it is possible that certain phytochemicals present in the *Z. oxyphyllum* fruit extract, either

independently or as a complex with certain minerals, may be responsible for this response. The result obtained in the present study also agrees to a previous *in vivo* investigation where the ethanolic leaf extract of *Z. oxyphyllum* at minimum dose of 30 mg/kg the extract showed highest inhibition of carrageenan induced paw oedema at 88.37 %, however at higher dose of 100 mg/kg, this inhibition was reduced to 61.25 % (Chatterjee et al., 2020). The widely known Indian medicinal plant *Moringa oleifera* with proven anti-oxidant, anti-inflammatory and chemoprotective potential was also shown to exert such hormetic responses (Calabrese et al., 2024). In the present study, a one-way ANOVA showed that the HRBC membrane stabilization activity of *Z. oxyphyllum* fruit extracts did not differ significantly from that of diclofenac sodium, with *P* values calculated as 0.27, 0.11 and 0.27 for R-1, R-2 and R-3 respectively.

The leaf extracts of *Z. acanthopodium*, like *Z. oxyphyllum* leaf extracts, also exerted a dose dependent protection percentage. At lower concentration of 1 mg/ml, the percentage protection ranged between 30.72 – 55.78 ± 4.37 %. At the highest dose of 5 mg/ml, the percentage protection was also observed to be the highest with values ranging between 66.16 – 79.33 ± 4.37 % (Figure 4.23). When compared to the standard drug however, *Z. acanthopodium* leaf extracts exhibited significantly lower membrane stabilization activity. A one-way ANOVA showed *P* values of 0.32, <0.01 and <0.01 for R-1, R-2, and R-3 respectively.

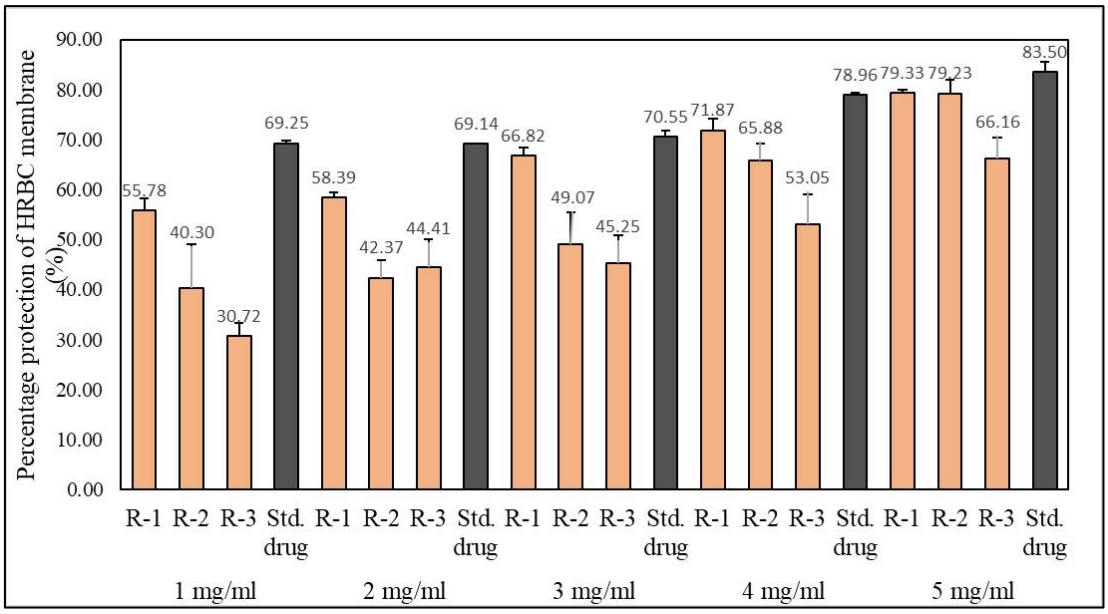

Figure 4.23. HRBC membrane stabilization activity of *Z. acanthopodium* leaf extracts (Data presented as ± SEM, n = 3).

Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

A similar dose dependent activity was also observed for *Z. acanthopodium* fruit extracts. At 1 mg/ml, the extracts exhibited the least percentage of protection with values of 30.71 – 55.78 ± 8.72 %, while the highest values were observed at the highest concentration of 5 mg/ml (66.16 – 79.33 ± 2.54 %) (Figure 4.24). In comparison to diclofenac sodium however, the extracts exhibited significantly lower percentage protection with *P* values of 0.30, 0.07 and <0.01 for R-1, R-2, and R-3 respectively.

Phytochemicals in plants are thought to exert their medicinal effects through common mechanisms, despite their ability to target structurally and functionally unrelated proteins. These compounds may influence the physical and chemical properties of cell membranes, such as fluidity, micro viscosity, elasticity, order, and permeability. Flavonoids have been shown to exhibit a wide range of pharmacological activities. While their precise modes of action are not fully understood, they are believed to interact with functional proteins, including enzymes, receptors, and ion channels, as well as modify lipid bilayers to alter membrane properties. This dual interaction may contribute to their bioactivity and therapeutic potential (Tsuchiya, 2015).

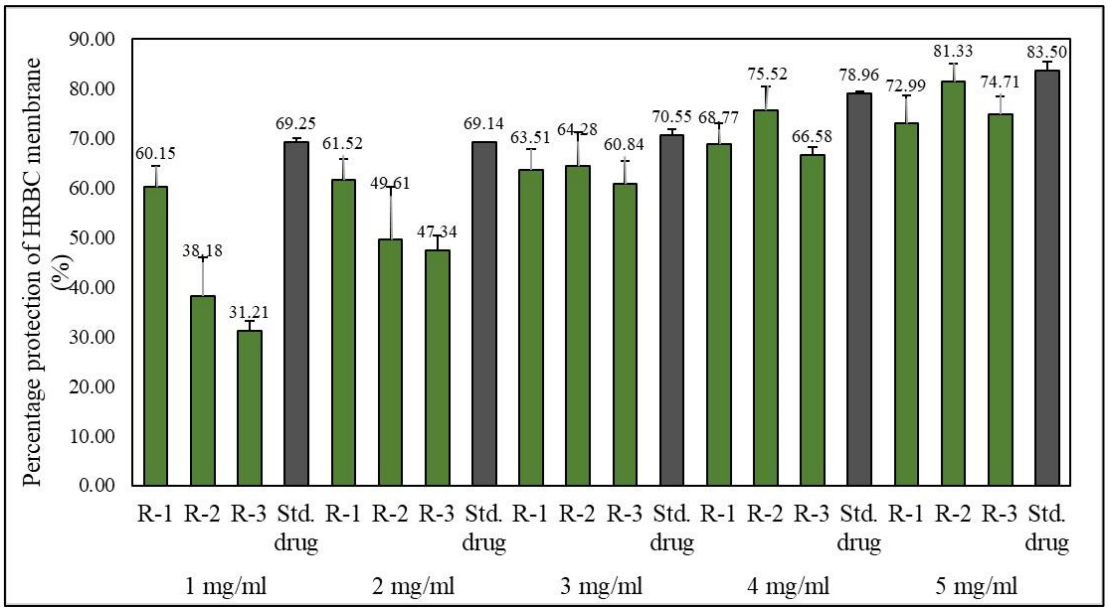

Figure 4.24. HRBC membrane stabilization activity of *Z. acanthopodium* fruit extracts (Data presented as ± SEM, n = 3).

Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

In addition, tannins and saponins have also been reported to bind to cations and other biomolecules, enhancing the stability of the erythrocyte membrane and further preventing cell lysis (Debnath et al., 2013). Therefore, in the present study as well, the potential anti-inflammatory mechanism of plant extracts may possibly involve binding to erythrocyte membranes. This interaction could alter the surface charge of the cells, reducing their ability to aggregate, thereby promoting mutual repulsion of similarly charged cells and hence preventing heat induced hemolysis (Debnath et al., 2013).

It was observed that both the extracts of *Z. oxyphyllum* exhibited better HRBC membrane stabilization activity than *Z. acanthopodium* extracts. In the earlier anti-oxidant studies, it had been observed that *Z. oxyphyllum* extracts showed lower DPPH radical scavenging and FRAP activity in comparison to *Z. acanthopodium* extracts. While *in vitro* anti-inflammatory assays like HRBC membrane stabilization assays are primarily biological processes whereby different phytochemicals may interact with RBC membranes consequently altering the surface charges of the cells (Debnath et al., 2013), anti-oxidant activity typically involves neutralizing free radicals or reactive oxygen species (ROS) to prevent oxidative stress which is primarily a chemical process (Gulcin and Alwasel, 2023). This difference in mechanism of action as well as the different methods used for

investigating anti-inflammatory and anti-oxidant activities may be responsible for the lack of correlation observed between the two assays in the extracts studied, particularly *Z. oxyphyllum* extracts. Further, bioactive compounds differ in their structural and functional properties and a compound or group of compounds that can exert anti-inflammatory activity may not necessarily be potent anti-oxidant and/or vice versa (Lila and Raskin, 2005).

A similar investigation on another *Zanthoxylum* species, *Z. rhetsa* showed that the ethanolic stem bark extract could exert moderate membrane stabilization activity of 29.92 %, 39.92 %, 56.26 % and 74.12 % at 20, 60, 80 and 100 µg/ml concentrations respectively (Parthiban et al., 2017). *Citrus nobilis* methanolic peel extract however, showed better membrane stabilization activity than both *Z. oxyphyllum* and *Z. acanthopdodium* extracts in the present study with its activity ranging between 46.76 % to 89.67% at 50 to 200 mg/ml concentration (Malik et al., 2021). The activity of the extracts in the present study was comparable to other local medicinal plants like *Clematis buchananiana* and *Tupistra nutans* which demonstrated membrane stabilization activity of 59.2 ± 1.6 %, 64.7 ± 0.2 %, 70.7 ± 1.3 %, 76.4 ± 0.7 % and 82.5 ± 0.3 %, and 53.7 ± 9.7 %, 57.7 ± 10.0 %, 64.0 ± 11.6 %, 67.1 ± 11.9 % and 70.9 ± 9.9 % respectively at 1, 2, 3, 4, and 5 mg/ml concentrations (Singh et al., 2020). Further, the membrane stabilization activity of the extracts in the present study was also comparable to that of a native medicinal plant of Sikkim Himalayan region, *Rheum nobile*, which exerted percentage protection of 54.87 ± 0.08 %, 59.37 ± 0.03 %, 63.25 ± 0.06 %, 67.87 ± 0.03 % and 73.12 ± 0.06 % at 1, 2, 3, 4 and 5 mg/ml respectively (Subba et al., 2023).

5.5.2. Protein denaturation assay

Protein denaturation involves the loss of a protein's secondary and tertiary structures due to external factors like heat, strong acids or bases, concentrated salts, or organic solvents. This process often renders proteins biologically inactive and is a key contributor to inflammation. During the acute inflammatory response, neutrophils and monocytes are drawn to the site by chemotactic signals to ingest and break down foreign materials, with denatured proteins playing a significant role in triggering this response (Dhami et al., 2019). Therefore, the ability of plant extracts to prevent heat induced protein denaturation is another simple and reproducible method of analysing the *in vitro* anti-inflammatory efficacy of any product.

In the present study, it was observed that the leaf extract of *Z. oxyphyllum* leaf extracts exerted a bi-phasic activity. At 1 mg/ml concentration, the extracts showed protection percentage of 77.39 – 94.75 ± 5.01 % while at higher concentration of 4 mg/ml and 5 mg/ml, this activity decreased to 18.99 – 31.67 ± 3.66 % and 5.61 – 37.14 ± 9.45 % respectively (Figure 4.25). This bi-phasic activity was not observed for the leaf extracts in HRBC membrane stabilization assay. A one-way ANOVA showed that there was no significance difference in the activity between the *Z. oxyphyllum* leaf extracts and the standard drug – diclofenac sodium (*P* values 0.90, 0.63 and 1.00 respectively for R-1, R-2 and R-3).

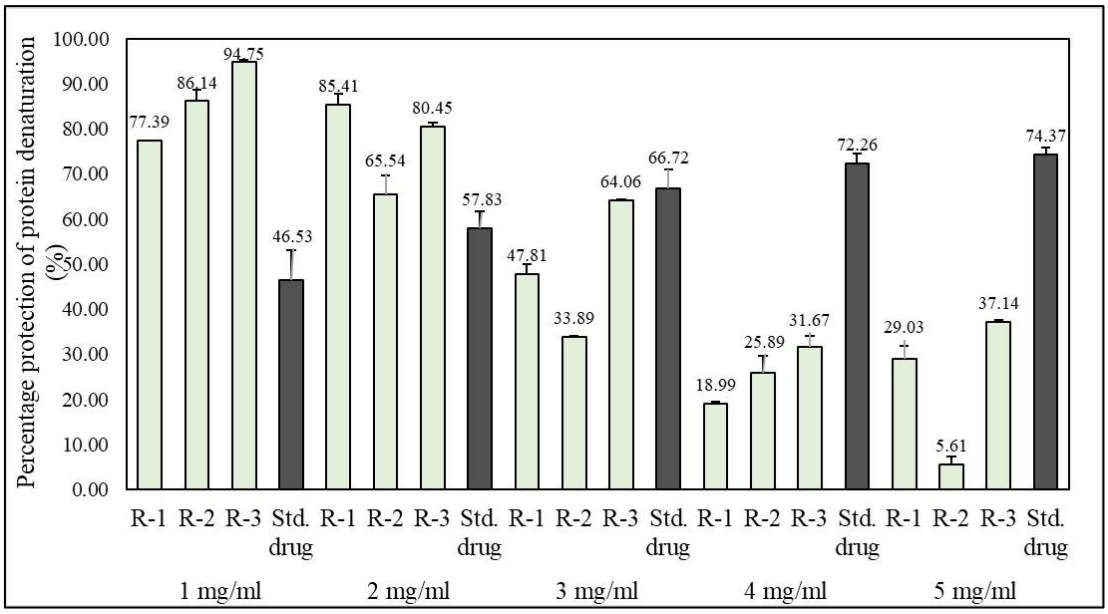

Figure 4.25. Protection of protein denaturation activity of *Z. oxyphyllum* leaf extracts (Data presented as ± SEM, n = 3).
Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Unlike the observation made for HRBC membrane stabilization assay, the fruit extracts of *Z. oxyphyllum* did not exert a bi-phasic activity for protection of protein denaturation assay. At lower concentration of 1 mg/ml, the extracts exerted a percentage protection of 53.24 – 77.33 ± 7.16 % while the same for diclofenac sodium was 46.53 ± 6.50 %. This percentage protection activity was observed to have a direct correlation with the concentration, the activity increasing with increasing concentration. At the highest concentration of 5 mg/ml, the extracts showed a percentage protection of 79.27 – 83.37 ± 1.19 % while the same for diclofenac sodium was observed to be 74.37 % (Figure 4.26).

A one-way ANOVA between the extracts the standard drug showed that there was no significant difference in the activity between the two with *P* values of 0.07, 0.57 and 0.71 respectively for R-1, R-2 and R-3.

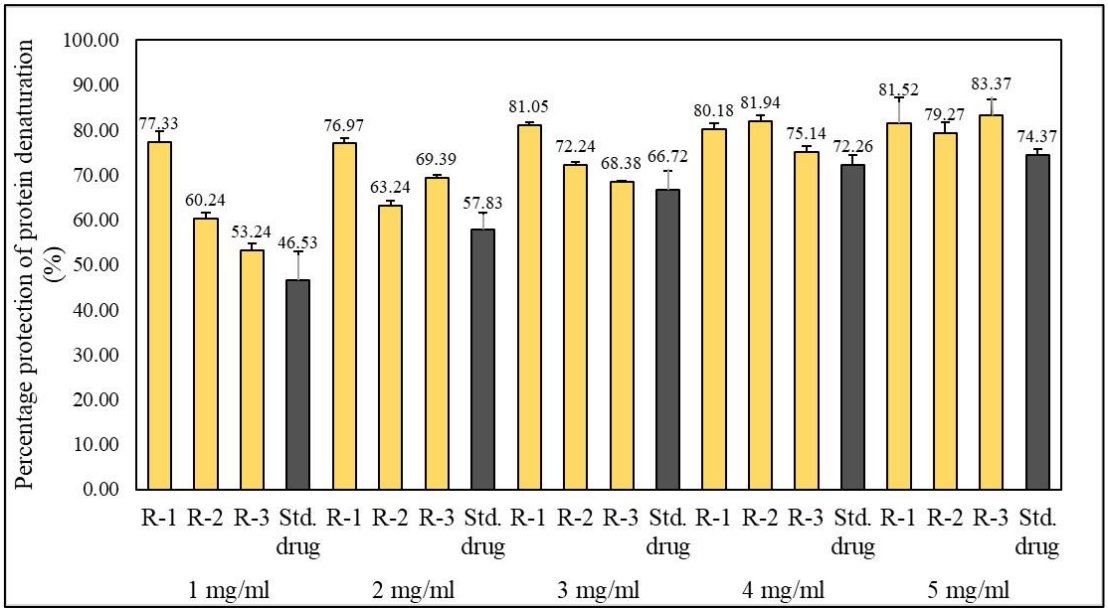

Figure 4.26. Protection of protein denaturation activity of *Z. oxyphyllum* fruit extracts (Data presented as ± SEM, n = 3).
Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

The leaf extracts of *Z. acanthopodium* showed a dose dependent activity. The protection percentage for all concentrations was observed to be lower than that of diclofenac sodium. At the lowest concentration of 1 mg/ml, the extracts showed percentage protection between 21.67 – 36.99 ± 4.44 %, which was comparatively lower than diclofenac sodium (46.43 ± 6.50 %). At the highest concentration of 5 mg/ml, the activity of the extracts was increased to 67.48 – 71.85 ± 1.33 % (Figure 4.27). A one-way ANOVA showed that the activity of *Z. acanthopodium* was on par with that of diclofenac sodium with the *P* values being 0.28, 0.88 and 0.38 for R-1, R-2 and R-3 respectively. Similarly, the fruit extracts of *Z. acanthopodium* also exerted a dose dependent activity that increased with increasing concentration (Figure 4.28). At 1 mg/ml concentration, the percentage protection was 10.91 – 15.24 ± 1.25 % and at the highest concentration of 5 mg/ml same was observed to be 39.02 – 46.14 ± 2.17 %.

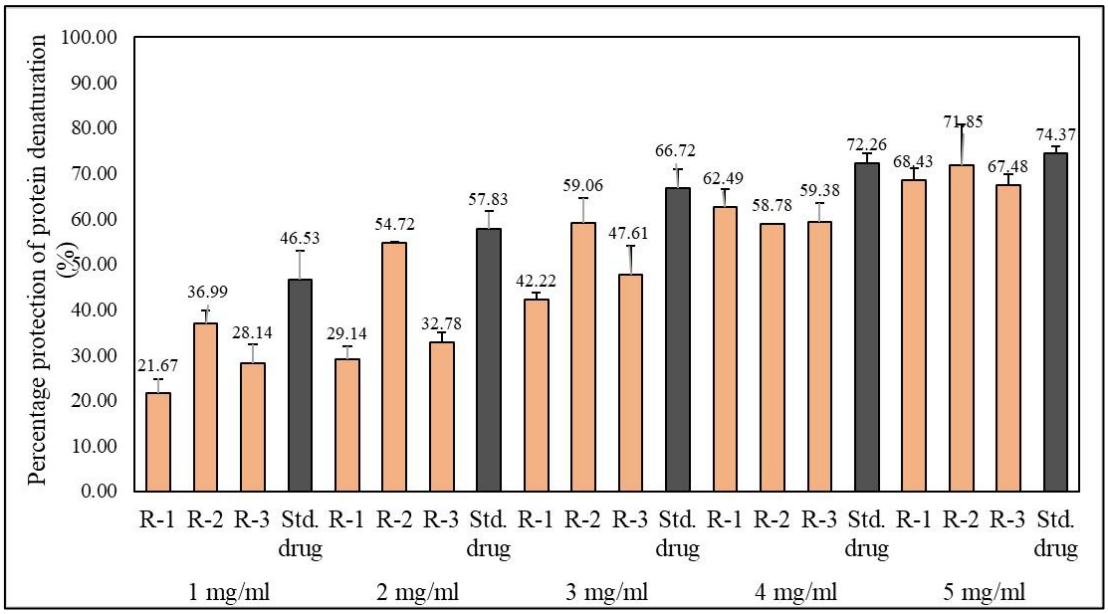

Figure 4.27. Protection of protein denaturation activity of *Z. acanthopodium* leaf extracts (Data presented as ± SEM, n = 3).

Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

A comparison among all four extracts studied i.e. *Z. oxyphyllum* extracts and *Z. acanthopodium* extracts revealed that the least protection percentage was exhibited by *Z. acanthopodium* fruit extracts. Further it was observed that the activity exerted by the fruit extracts was significantly lower than that of the standard drug with *P* values < 0.01 for all biological replicates. Therefore, the *Z. oxyphyllum* extracts showed better protection of protein denaturation activity in comparison to *Z. acanthopodium* extracts.

Certain other *Zanthoxylum* species like *Z. rhetsa* and *Z. armatum* were also shown to exert similar protection of protein denaturation. The ethanolic extract of *Z. rhetsa* bark was reported to exert a protection percentage of 24 %, 29 %, 48 % and 56 % for 20, 60, 80 and 100 µg/ml concentrations respectively (Parthiban et al., 2017). Similarly, *Z. armatu*m bark essential oil was also reported to exhibit a concentration dependent protection percentage ranging from 3.125 ppm to 100 ppm with IC_{50} values ranging from 16.40 ± 0.48 to 35.9 ± 0.12 (Dhami et al., 2019)..

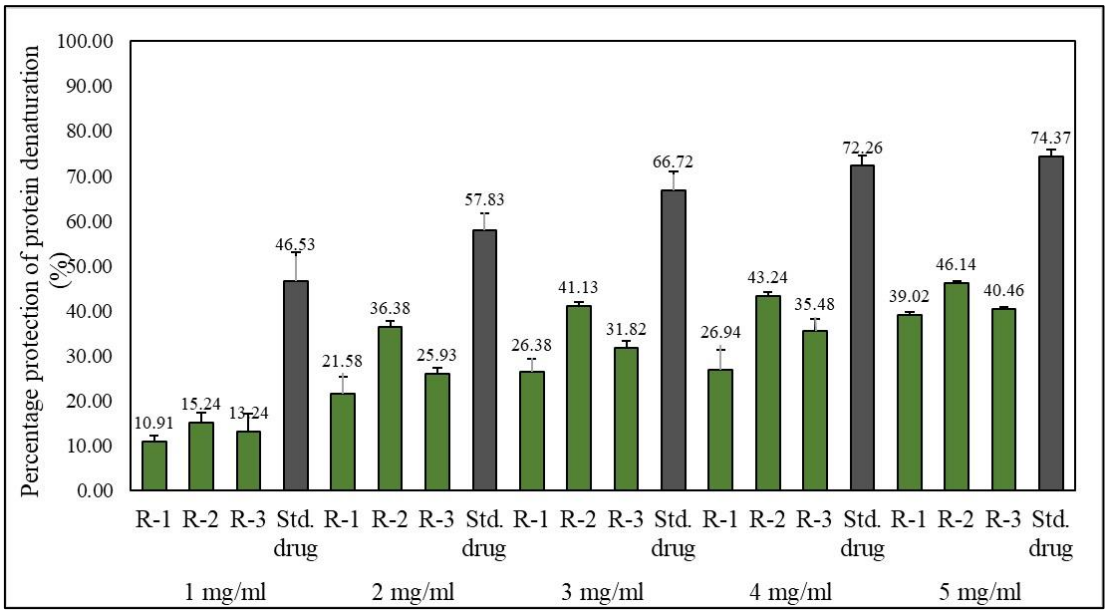

Figure 4.28. Protection of protein denauturation activity of *Z. acanthopodium* fruit extracts (Data presented as ± SEM, n = 3).

Std. = reference standard diclofenac sodium; R-1 = biological replicate 1, R-2 = biological replicate 2, R-3 = biological replicate 3

Another *Zanthoxylum* species *Z. zanthoxyloides* also exhibited a concentration dependent protection activity. It was reported that the hydro-ethanolic fruit extract showed a denaturation inhibition percentage of 73.04 ± 1.16 % to 84.54 ± 0.67 %, the bark extract showed activity between 75.75 ± 1.70 % to 83.19 ± 0.58 % and the root extract exhibited activity of 78.07 ± 3.13 % to 92.95 ± 1.10 from 31.25 µg/ml to 1000 µg/ml concentration (Togola et al., 2023), which is comparable to that observed in the present study. It was also shown that all these *Zanthoxylum* species exerted protection percentage on par with that of the standard drug. Compared to another native ethnomedicinal plant *R. nobile*, which was reported to exert a protection percentage of 21.8 ± 0.03 % to 79.98 ± 0.03 % from 1 to 5 mg/ml concentration (Subba et al., 2023), while *Z. oxyphyllum* extracts showed better inhibition of protein denaturation, *Z. acanthopodium* fruit extracts exerted a lower protection activity.

The observation made for both HRBC membrane stabilization and protein denaturation assay showed that all the extracts investigated in the present study possessed *in vitro* anti-inflammatory capacity to various degrees. These preliminary studies presented a fair idea on the potential immunomodulatory capacity of all the extracts. It was also observed that

Z. oxyphyllum extracts showed activity on par with that of diclofenac sodium when compared to *Z. acanthopodium*.

Genetics and environmental factors play crucial roles in determining the biological activities of plants. Intraspecific variation, encompassing the genomic and phenotypic diversity within and across populations, is fundamental to plants' evolutionary adaptation to environmental changes and stress responses. Environmental conditions and genetic makeup significantly influence the quality and yield of phytochemicals, contributing to the diverse pharmacological activities observed in medicinal plants (Batubara et al., 2020). A one-way ANOVA amongst the biological replicates revealed that there was no significant intraspecific variation in the *in vitro* anti-inflammatory activity. Since, the plant biological replicates were all collected from locations with similar environmental conditions and altitudinal range, an absence of significant difference in their *in vitro* anti-inflammatory activity could be expected. Based on this observation a further streamlining of the extracts was done for further *in vitro* immunomodulatory assays, GC-MS and element analysis to ensure the efficiency, accuracy, and reliability of results while minimizing unnecessary resource use without compromising data quality as well as to avoid redundancy of data.

5.6. Gas-chromatography and mass spectrometry analysis of plant extracts

Gas-chromatography and mass spectrometry analysis was done to identify the phytoconstituents present in the extracts. The GC-MS phytochemical profile of each extract and their reported biological activity are presented in Tables 4.2, 4.3, 4.4 and 4.5. Interestingly, hexadecanoic acid, methyl ester was found to be the most abundant compound in all the extracts. In *Z. oxyphyllum* leaf extract the most abundant compounds were hexadecanoic acid, methyl ester (71.65 %) followed by 1-tridecene (43.59 %), trans-2-methyl-4-n-pentylthiane, S,S-dioxide (33.87 %) and 1-hexadecanaminium and N,N,N-trimethyl-, octadecenoate (30.46 %). In *Z. oxyphyllum* fruit extract hexadecanoic acid, methyl ester (39.66 %) was followed by methyl 9,10-octadecadienoate (32.01 %) and hydrazinecarbothioamide (11.66 %). In *Z. acanthopodium* leaf extract, hexadecanoic acid, methyl ester (66.73 %) was followed by trans-2-methyl-4-n-pentylthiane, S,S-dioxide (38.04 %), 1-tridecene (32.67 %) and oxirane, [[4-(1,1-dimethylethyl)phenoxy]methyl]- (21.19 %). Finally, in *Z. acanthopodium* fruit extract hexadecanoic acid, methyl ester (73.46 %) was followed by 1-tridecene (34.44 %), 1-

hexadecanaminium, N,N,N-trimethyl-, octadecenoate (31.64 %) and trans-2-methyl-4-n-pentylthiane, S,S-dioxide (30.32 %). Some of the phytochemicals detected were reported to possess bioactive potential.

Table 4.2. Phytochemicals detected in *Z. oxyphyllum* leaf extracts

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
1.	4.439	Benzyl (1,2,3-thiadiazol-4-y)carbamate	2.95	$C_{10}H_9N_3O_2S$	Not reported
2.	4.974	Benzene, (isothiocyanatomethyl)-	0.53	C_8H_7NS	Anti-bacterial, anti-fungal and anti-inflammatory, anticancer (Dinh et al., 2021)
3.	5.056	Benzyl-diseryl phosphate	8.75	$C_{13}H_{19}N_2O_8P$	Not reported
4.	5.168	4-Methoxybenzyl isothiocyanate	3.45	C_9H_9NOS	Antimicrobial (Swart et al., 2002)
5.	5.592	Arsenous acid, tris(trimethylsilyl) ester	1.96	$C_9H_{27}AsO_3Si_3$	Antiviral, antithyroid, anticataract (Alexander et al., 2022)
6.	5.988	Borinic acid, diethyl-	0.83	$C_4H_{11}BO$	Acidifier, arachidonic acid Inhibitor, increases aromatic amino acid decarboxylase activity, inhibits production of uric acid, urine acidifier (Perumal et al., 2021)
7.	6.153	Silane, hexyl-	0.5	$C_6H_{16}Si$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
8.	6.541	4-[4-Nitrophenyl]-1,2,3-thiadiazole	4.07	$C_8H_5N_3O_2S$	Not reported
9.	6.891	1-Cycloocten-5-yne, (Z)-	2.19	C_8H_{10}	Not reported
10.	7.236	3-Methoxyphenyl isothiocyanate	1.38	C_8H_7NOS	Cholinesterase inhibitor, antioxidant, and anti-inflammatory activity (Burcul et al., 2018)
11.	7.412	Spiro[2.4]heptane, 1-ethenyl-5-(1-propenylidene)-	3.41	$C_{12}H_{16}$	Not reported
12.	7.663	Isobutyl acrylate	1.64	$C_7H_{12}O_2$	Not reported
13.	8.007	Tetrazole, 1-(4-fluorophenyl)-5-(1,3-diphenyl-2-imidazolidinylmethyl)-	0.75	$C_{23}H_{21}FN_6$	Not reported
14.	8.642	Methyl 11,12-tetradecadienoate	0.85	$C_{15}H_{26}O_2$	Not reported
15.	10	(S)-9-[(S)-2-(Hydroxymethyl)pyrrolidin-1-yl]-3-methyl-3,4-dihydro-2H-benzo[b][1,4,5]oxathiazepine 1,1-dioxide	0.65	$C_{14}H_{20}N_2O_4S$	Not reported
16.	11.142	1,3,2-Dioxaborolane, 2-ethyl-4,5-dimethyl-	1.94	$C_6H_{13}BO_2$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
17.	11.831	Ethyl 2-((dibutoxyphosphoryl)oxy)-3,3,3-trifluoropropanoate	1.36	$C_{13}H_{24}F_3O_6P$	Not reported
18.	12.315	Aluminum, triethyl-	2.42	$C_6H_{15}Al$	Not reported
19.	12.456	benzo[b][1,4,5]oxathiazepine 1,1-dioxide	0.38	$C_{15}H_{24}N_2O_4S$	Not reported
20.	12.825	2-n-Butylthiolane, S,S-dioxide	0.81	$C_8H_{16}O_2S$	Not reported
21.	13.923	1,2-Oxaborolane, 2-ethyl-4,5-dimethyl-	0.9	$C_7H_{15}BO$	Not reported
22.	14.357	Octanoic acid, methyl ester	2.37	$C_9H_{18}O_2$	Not reported
23.	15.26	Silane, cyclohexyldimethoxymethyl-	3.47	$C_9H_{20}O_2Si$	Not reported
24.	17.058	Boric acid, ethyl-, didecyl ester	2.51	$C_{22}H_{47}BO_2$	Not reported
25.	17.345	Cyclohexylmethylsilane	1.58	$C_7H_{16}Si$	Not reported
26.	17.557	2-Ethylthiolane, S,S-dioxide	19.05	$C_6H_{12}O_2S$	Not reported
27.	18.1	Decane, 3,8-dimethyl-	2.92	$C_{12}H_{26}$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
28.	18.186	Heptane, 2,2,3,3,5,6,6-heptamethyl-	6.59	C14H30	Not reported
29.	18.501	Undecane	14.14	$C_{11}H_{24}$	Anti allergic, anti inflammatory (Choi et al., 2020), immunosuppressant (Baky et al., 2021)
30.	18.924	2-Propyl-1-pentanol, chlorodifluoroacetate	1.28	$C_{10}H_{17}ClF_2O_2$	Not reported
31.	19.012	Bis(2-ethylhexyl) hydrogen phosphite	1.6	$C_{16}H_{35}O_3P$	Not reported
32.	19.09	Nonane, 5-propyl-	4.44	$C_{12}H_{26}$	Not reported
33.	19.222	Decane, 2,3,5,8-tetramethyl-	3.04	$C_{14}H_{30}$	Not reported
34.	19.412	2-Isopropyl-5-methyl-1-heptanol	2.59	$C_{11}H_{24}O$	Antimicrobial (Uyan et al., 2020)
35.	19.765	Dodecane, 1-fluoro-	9.5	$C_{12}H_{25}F$	Anti-bacterial, anti-tumor, anti-cancer, antioxidant, anti-microbial, anti-HIV, anti-viral, anti-cervical cancer (Arif et al., 2022)
36.	19.961	Methyl 8-methyl-nonanoate	2.81	$C_{11}H_{22}O_2$	Not reported
37.	20.259	Cyclopentyl-methyl-phosphinic acid, 2-isopropyl-5-methyl-cyclohexyl ester	6.52	$C_{16}H_{31}O_2P$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
38.	20.259	cis-2-Methyl-4-n-butylthiane, S,S-dioxide	5.14	$C_{10}H_{20}O_2S$	Not reported
39.	20.259	2-Azido-2,4,4,6,6,8,8-heptamethylnonane	4.59	$C_{16}H_{33}N_3$	Not reported
40.	21.438	trans-2,4-Dimethylthiane, S,S-dioxide	4.66	$C_7H_{14}O_2S$	Glutathione-S-Tansferase inhibitor, increases glutathinone-S- transferase (GST) activity, decreases oxaloacetate transaminase activity, Catechol-O-Methyl-Transferase-Inhibitor, Decreases Glutamate Oxaloacetate Transaminase, Decreases Glutamate Puruvate Transaminase, Glucosyl-Transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, transdermal, smart drug, adrenocortical stimulant (Hassan Mohammad et al., 2021)
41.	21.654	1-Tridecene	43.59	$C_{13}H_{26}$	Not reported
42.	21.802	trans-2-Methyl-4-n-butylthiane, S,S-dioxide	4.4	$C_{10}H_{20}O_2S$	Not reported
43.	21.86	Tridecane	14.32	$C_{13}H_{28}$	Not reported
44.	22.895	Heptylcyclohexane	3.32	$C_{13}H_{26}$	Not reported
45.	23.067	Oxirane, diethylboryloxymethyl-	19.89	$C_7H_{15}BO_2$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
46.	23.239	2-Azido-2,4,4,6,6-pentamethylheptane	4.18	$C_{12}H_{25}N_3$	Antitumor, anti-inflammatory (Addai et al., 2022)
47.	23.33	Propanoic acid, 2,2-dimethyl-, anhydride with diethylborinic acid	11.2	$C_9H_{19}BO_2$	Not reported
48.	23.994	C51Dodecane, 2,2,11,11-tetramethyl-	16.96	$C_{16}H_{34}$	Not reported
49.	24.715	Oxirane, [[4-(1,1-dimethylethyl)phenoxy]methyl]-	16.65	$C_{13}H_{18}O_2$	Anti-inflammatory, analgesic, antipyretic (Mahmoud and Alshammari, 2022)
50.	26.804	Tetradecyl trifluoroacetate	6.91	$C_{16}H_{29}F_3O_2$	Not reported
51.	27.261	Tetradecane, 1-chloro-	2.09	$C_{14}H_{29}Cl$	Not reported
52.	27.634	(1S,6S,7R,10S)-10-Isothiocyanato-4-cadinene	3.91	$C_{16}H_{25}NS$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
53.	28.131	2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol	21.36	$C15_5H_{26}O$	Not reported
54.	28.379	3,7 Dimethyloctyl propylphosphonofluoridate	7.15	$C_{13}H_{28}FO_2P$	Not reported
55.	9.745	21-Decanol, 2-hexyl-	11.26	$C_{16}H_{34}O$	Antimicrobial (Ferdosi et al., 2023)
56.	30.014	Chloroacetic acid, dodecyl ester	3.55	$C_{14}H_{27}ClO_2$	Not reported
57.	30.303	Isobutyl tetradecyl ether	2.79	$C_{18}H_{38}O$	Not reported
58.	30.422	Trihexadecyl borate	3.27	$C_{48}H_{99}BO_3$	Not reported
59.	30.683	Pentadecane, 7-(bromomethyl)-	0.68	$C_{16}H_{33}Br$	Not reported
60.	30.901	trans-2-methyl-4-n-pentylthiane, S,S-dioxide	33.87	$C_{11}H_{22}O_2S$	Glutathione-S-Transferase-Inhibitor, Catechol-O-Methyl-Transfearse inhibitor, Myo-neuro-stimulator, Nitric Oxide Synthatase inhibitor, NO scavenger, Stimulates Morepinephrine production, Stimulates Sympathetic nervous system, decrease glutamate oxaloacetate transaminase, decrease glutamine pyruvate transaminase, Glycosyl transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, smart drug, adrenal supporter (Hassan Mohammad et al., 2021)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
61.	32.6	N-(1H-Tetrazol-5-yl)decanamide	12.31	$C_{11}H_{21}N_5O$	Not reported
62.	33.081	Dodecyl heptyl ether	0.67	$C_{19}H_{40}O$	Not reported
63.	33.62	Hexadecanoic acid, methyl ester	71.65	$C_{17}H_{34}O_2$	Antibacterial (Shaaban et al., 2021), insect repellent (Gaikwad and Nalawade, 2020)
64.	33.773	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,methyl ester	14.39	$C_{18}H_{28}O_3$	Antifungal, antioxidant (Kumar et al., 2016)
65.	34.034	Eicosane, 10-methyl-	3.74	$C_{21}H_{44}$	Antioxidant (Rhetso et al.,2020)
66.	36.895	Methyl 12,13-tetradecadienoate	17.48	$C_{15}H_{26}O_2$	Not reported
67.	37.372	1-Hexadecanaminium, N,N,N-trimethyl-, octadecanoate	30.46	$C_{37}H_{77}NO_2$	Not reported

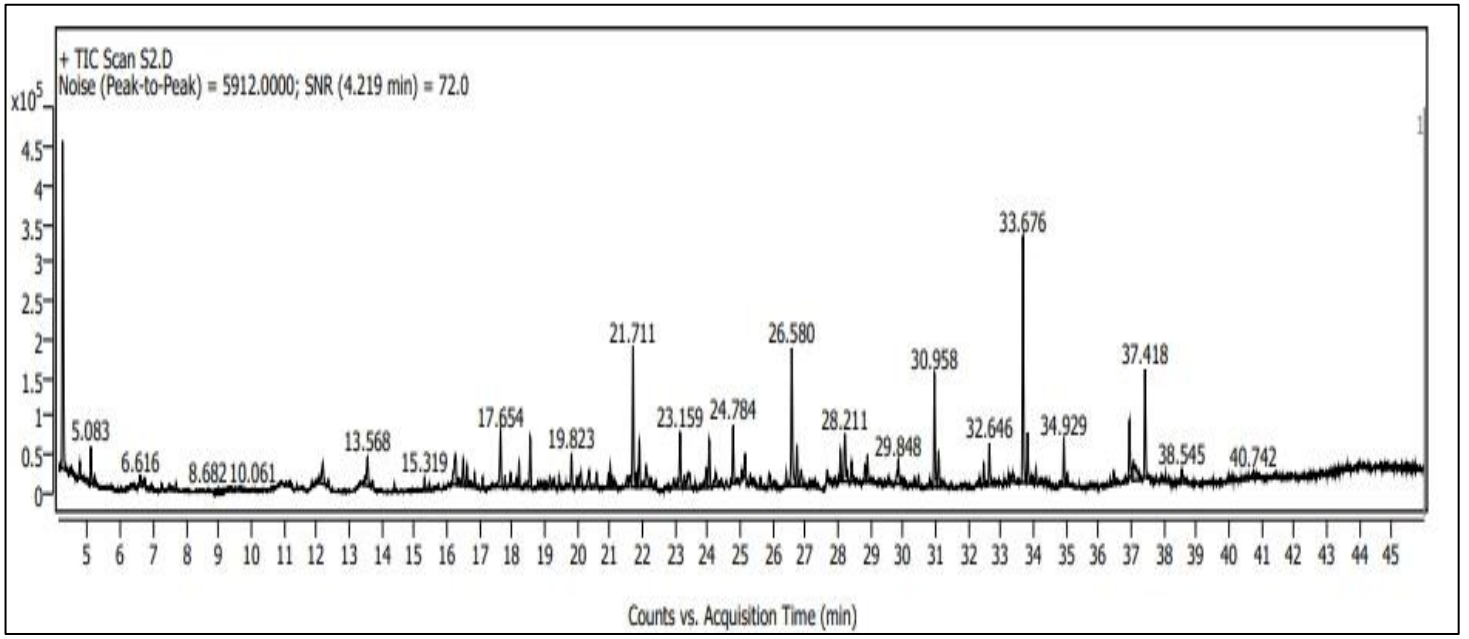

Figure 4.29. GC-MS chromatogram of *Z. oxyphyllum* leaf extract

Table 4.3. Phytochemicals detected in *Z. oxyphyllum* fruit extract

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
1.	4.25	Hydrazinecarbothioamide	11.66	CH_5N_3S	Chemothrapeutic (Marenco et al., 2012), antitumor, antiprotozoal, antiviral (Ambhore et al., 2019)
2.	4.48	Benzyl (1,2,3-thiadiazol-4-y)carbamate	0.35	$C_{10}H_9N_3O_2S$	Not reported
3.	5.01	Benzene, (isothiocyanatomethyl)-	0.05	C_8H_7NS	Anti-bacterial, anti-fungal, anti-inflammatory, anticancer (Dinh et al., 2021)
4.	5.1	Benzyl-diseryl phosphate	1.18	$C_{13}H_{19}N_2O_8P$	Not reported
5.	5.22	4-Methoxybenzyl isothiocyanate	0.37	C_9H_9NOS	Antimicrobial (Swart et al., 2002)
6.	6.02	Borinic acid, diethyl-	0.1	$C_4H_{11}BO$	Acidifier, arachidonic acid inhibitor, increases aromatic amino acid decarboxylase activity, inhibits production of uric acid, urine
7.	6.61	Benzene, 1-isocyanato-3-methoxy-	0.52	$C_8H_7NO_2$	Not reported
8.	6.94	1-Cycloocten-5-yne, (Z)-	0.1	C_8H_{10}	Not reported
9.	7.49	Isobutyl acrylate	0.7	$C_7H_{12}O_2$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
10.	12.19	Hexane, 3,3-dimethyl-	2.02	C_8H_{18}	Not reported
11.	12.35	Aluminum, triethyl-	0.28	$C_6H_{15}Al$	Not reported
12.	13.73	Heptane, 2,3,5-trimethyl-	0.22	$C_{10}H_{22}$	Not reported
13.	14.38	Octanoic acid, methyl ester	0.27	$C_9H_{18}O_2$	Not reported
14.	14.61	Labetalol di-n-butylboronate	0.06	$C_{27}H_{38}B_2N_2O_3$	Not reported
15.	15.32	Silane, cyclohexyldimethoxymethyl-	0.28	C9H20O2Si	Not reported
16.	16.85	2-Azido-2,4,4,6,6-pentamethylheptane	0.33	$C_{12}H_{25}N_3$	Antitumor, anti-inflammatory (Addai et al., 2022)
17.	17.1	Borane, diethyl(decyloxy)-	0.22	$C_{14}H_{31}BO$	Antifungal, antibacterial, anticancer (Soni et al., 2023)
18.	17.64	2-Ethylthiolane, S,S-dioxide	2.08	$C_6H_{12}O_2S$	Not reported
20.	18.558	Undecane	1.45	$C_{11}H_{24}$	Anti allergic, anti inflammatory (Choi et al., 2020), immunosuppressant (Baky et al., 2021)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological actvity
21	18.95	2-Propyl-1-pentanol, chlorodifluoroacetate	0.13	$C_{10}H_{17}ClF_2O$	Not reported
22	19.04	Sulfurous acid, 2-ethylhexyl undecyl ester	0.12	$C_{19}H_{40}O_3S$	Not reported
23	19.17	Undecane, 5-methyl-	0.54	$C_{12}H_{26}$	Not reported
24	19.28	Decane, 2,3,5,8-tetramethyl-	0.3	$C_{14}H_{30}$	Not reported
25	19.45	2-Isopropyl-5-methyl-1-heptanol	0.29	$C_{11}H_{24}O$	Antimicrobial (Uyan et al. 2020)
26	19.67	Allyl n-octyl ether	0.12	$C_{11}H_{22}O$	Antibacterial (Sajeesh and Parimelazhagan, 2014)
27	19.83	Nonane, 3-methyl-5-propyl-	1.11	$C_{13}H_{28}$	Not reported
28	19.99	Methyl 8-methyl-nonanoate	0.42	$C_{11}H_{22}O_2$	Not reported
29	20.36	Cyclopentyl-methyl-phosphinic acid, 2-isopropyl-5-methyl-cyclohexyl ester	0.71	$C_{16}H_{31}O_2P$	Not reported
30	20.6	cis-2-Methyl-4-n-butylthiane, S,S-dioxide	0.51	$C_{10}H_{20}O_2S$	Not reported
31	22.07	2-Azido-2,4,4,6,6,8,8-heptamethylnonane	0.64	$C_{16}H_{33}N_3$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological actvity
32.	22.97	Heptylcyclohexane	0.53	$C_{13}H_{26}$	Not reported
33.	23.16	Oxirane, diethylboryloxymethyl-	2.45	$C_7H_{15}BO_2$	Not reported
34.	24.05	Dodecane, 2,2,11,11-tetramethyl-	1.74	$C_{16}H_{34}$	Not reported
35.	24.78	Ethyl 4-t-butylbenzoate	2.86	$C_{13}H_{18}O_2$	Not reported
36.	26.41	trans-2,4-Dimethylthiane, S,S-dioxide	0.44	$C_7H_{14}O_2S$	Glutathione-S-Tansferase inhibitor, increases glutathinone-S-transferase (GST) activity, decreases oxaloacetate transaminase activity, Catechol-O-Methyl-transferase-inhibitor, decreases glutamate oxaloacetate transaminase, decreases glutamate pyruvate transaminase, glucosyl-transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, transdermal, smart drug, adrenocortical stimulant (Hassan Mohammad et al., 2021)
37.	26.58	trans-2-Methyl-4-n-butylthiane, S,S-dioxide	5.3	$C_{10}H_{20}O_2S$	Not reported
38.	26.75	Tetradecyl trifluoroacetate	0.58	$C_{16}H_{29}F_3O_2$	Not reported
39.	27.387	2-Bromotetradecane	0.07	$C_{14}H_{29}Br$	Not reported
40.	27.908	3-n-Hexylthiolane, S,S-dioxide	0.36	$C_{10}H_{20}O_2S$	Antioxidant, anticancer (Onyeaghala et al., 2015), antimicrobial potential (Ralte et al., 2022)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological actvity
41.	28.08	Cyclohexylmethylsilane	1.62	$C_7H_{16}Si$	Not reported
42.	29.56	Tridecanol, 2-ethyl-2-methyl-	0.44	$C_{16}H_{34}O$	Not reported
43.	29.85	1-Decanol, 2-hexyl-	1.39	$C_{16}H_{34}O$	Antimicrobial (Ferdosi et al., 20203)
44.	29.97	9-Methyl-Z-10-pentadecen-1-ol	0.34	$C_{16}H_{32}O$	Not reported
45.	30.47	Trihexadecyl borate	0.42	$C_{48}H_{99}BO_3$	Not reported
46.	30.96	trans-2-methyl-4-n-pentylthiane, S,S-dioxide	4.3	$C_{11}H_{22}O_2S$	Glutathione-S-transferase-inhibitor, catechol-O-methyl-transfearse inhibitor, myo-neuro-stimulator, nitric oxide synthatase inhibitor, NO scavenger, stimulates norepinephrine production, stimulates sympathetic nervous system, decrease glutamate oxaloacetate transaminase, decrease glutamine pyruvate transaminase, glycosyl transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, smart drug, adrenal supporter (Hassan Mohammad et al., 2021)
47.	32.64	N-(1H-Tetrazol-5-yl)decanamide	1.63	$C_{11}H_{21}N_5O$	Not reported
48.	33.1	Dodecyl heptyl ether	0.15	$C_{19}H_{40}O$	Not reported
49.	33.68	Hexadecanoic acid, methyl ester	39.66	$C_{17}H_{34}O_2$	Antibacterial (Shaaban et al., 2021), insect repellent (Gaikwad and Nalawade, 2020)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological actvity
50	33.82	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,methyl ester	1.75	$C_{18}H_{28}O_3$	Antifungal, antioxidant (Kumar et al., 2016)
51	34.08	Eicosane, 10-methyl-	0.4	$C_{21}H_{44}$	Antioxidant (Rhetso et al., 2020)
52	35.04	Chloroacetic acid, pentadecyl ester	0.64	$C_{17}H_{33}ClO_2$	Acidifier, arachidonic acid inhibitor, increase aromatic amino acid decarboxylase activity (Sharmla et al., 2020)
53	36.46	1-Dodecanol, 2-octyl-	0.55	$C_{20}H_{42}O$	Not reported
54	36.82	Methyl 9,10-octadecadienoate	32.01	$C_{19}H_{34}O_2$	Catechol-O-methyl-transferase inhibitor, methyl donor, methyl guanidine inhibitor (Hassan Mohammad et al., 2021)
55	37.05	Methyl 12,13-tetradecadienoate	2.45	$C_{15}H_{26}O_2$	Not reported
56	37.42	1-Hexadecanaminium, N,N,N-trimethyl-, octadecanoate	9.54	$C_{37}H_{77}NO_2$	Not reported
57	44.02	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	2.68	$C_{14}H_{44}O_6Si_7$	Acidulant, acidifier, control of fever, increase aromatic amino acid decarboxylase activity, anti- uric acid, urinary-acidulant (Rehman et al., 2024)

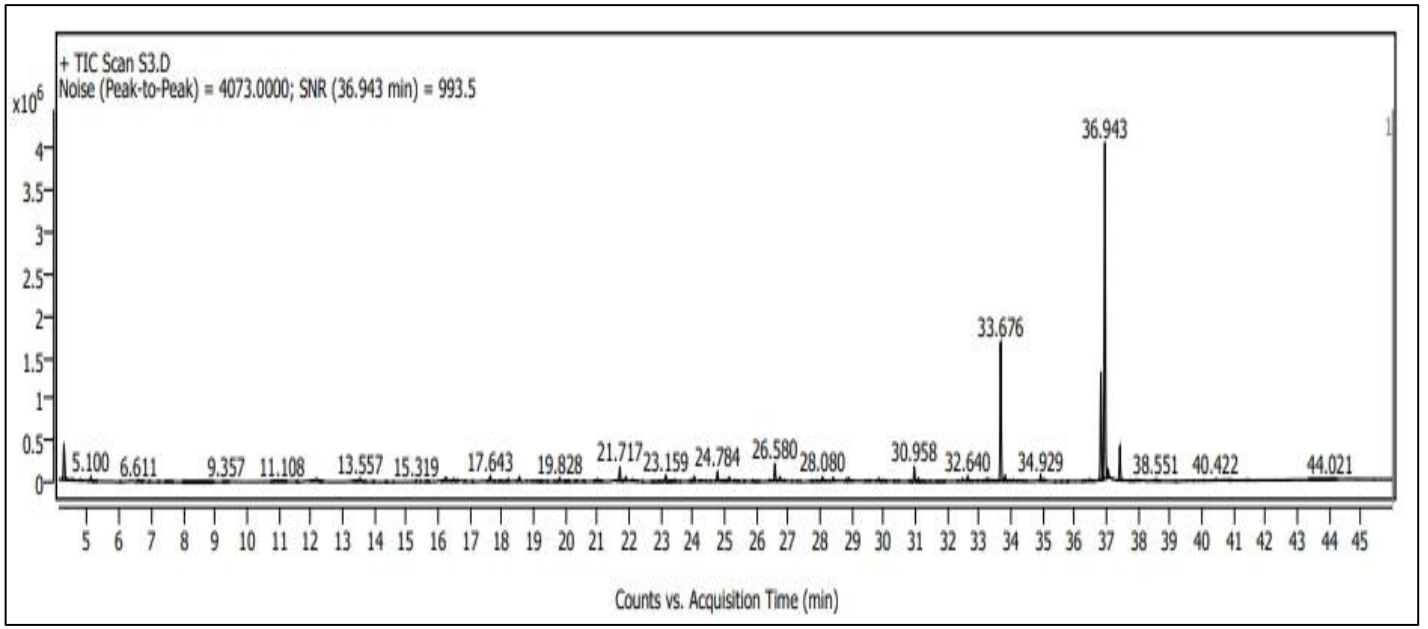

Figure 4.30. GC-MS chromatogram of *Z. oxyphyllum* fruit extract

Table 4.4. Phytochemicals detected in *Z. acanthopodium* leaf extracts

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
1.	4.402	Benzyl (1,2,3-thiadiazol-4-y)carbamate	2.78	$C_{10}H_9N_3O_2S$	Not reported
2.	4.946	Benzene, (isothiocyanatomethyl)-	1.17	C_8H_7NS	Anti-bacterial, anti-fungal, anti-inflammatory, anticancer properties (Dinh et al., 2021)
3.	5.043	Benzyl-diseryl phosphate	10.39	$C_{13}H_{19}N_2O_8P$	Not reported
4.	5.174	4-Methoxybenzyl isothiocyanate	3.97	C_9H_9NOS	Antimicrobial (Swart et al., 2002)
5.	5.735	2-Bromo-N-[(1-(cyclohexylmethyl)-1H-1,2,3-triazol-4-yl)methyl]-Nmethylbenzenesulfonamide	1.4	$C_{17}H_{23}BrN_4O_2S$	Not reported
6.	6.616	Adamantane, 1-isothiocyanato-3-methyl-	8.05	$C_{12}H_{17}NS$	Not reported
7.	6.725	.beta.-l-Rhamnopyranoside, phenyl-2,3-O-ethylboranediyl-4-Obenzyl-	4.06	$C_{21}H_{25}BO_5$	Not reported
8.	6.914	n-Dodecylpyridinium chloride	1.83	$C_{17}H_{30}ClN$	Antibacterial (Hrenovic et al., 2008)
9.	7.263	Borinic acid, diethyl-, 1-cyclododecen-1-yl ester	2.61	$C_{16}H_{31}BO$	Not reported
10.	8.545	Methyl 13,14-octadecadienoate	1.05	$C_{19}H_{34}O_2$	Not reported
11.	11.42	Methyl 11,12-octadecadienoate	0.64	$C_{19}H_{34}O_2$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
12.	15.33	9-Borabicyclo[3.3.1]nonane, 9-(benzoyloxy)-	3.9	$C_{15}H_{19}BO_2$	Not reported
13.	16.8	2-Azido-2,4,4,6,6-pentamethylheptane	4.04	$C_{12}H_{25}N_3$	Antitumor, anti-inflammatory (Addai et al., 2022)
14.	17.37	Methyl 10,11-tetradecadienoate	0.85	$C_{15}H_{26}O_2$	Antibacterial (Oli et al., 2024)
15.	17.56	2-Ethylthiolane, S,S-dioxide	18.9	$C_6H_{12}O_2S$	Not reported
16.	17.75	1-Hexanol, 5-methyl-2-(1-methylethyl)-	2.92	$C_{10}H_{22}O$	Not reported
17.	18.18	Heptane, 2,2,3,3,5,6,6-heptamethyl-	7.03	$C_{14}H_{30}$	Not reported
18.	18.51	Undecane	14.26	$C_{11}H_{24}$	Anti allergic, anti inflammatory (Choi et al., 2020), immunosuppressant (Baky et al., 2021)
19.	18.92	2-Propyl-1-pentanol, chlorodifluoroacetate	1.49	$C_{10}H_{17}ClF_2O_2$	Not reported
20.	19.09	Nonane, 5-propyl-	4.52	$C_{12}H_{26}$	Not reported
21.	19.28	Decane, 2,3,5,8-tetramethyl-	3.28	$C_{14}H_{30}$	Not reported
22.	19.41	2-Isopropyl-5-methyl-1-heptanol	3.02	$C_{11}H_{24}O$	Antimicrobial (Uyan et al. 2020)

No.	Retention time	Compound name	Area percenta	Molecular formula	Biological activity
23.	19.75	Dodecane, 1-fluoro-	10.79	$C_{12}H_{25}F$	Anti-bacterial, anti-tumor, anti-cancer, antioxidant, anti-microbial, anti-HIV, anti-viral, anti-cervical cancer (Arif et al., 2022)
24.	20.37	Cyclopentyl-methyl-phosphinic acid, 2-isopropyl-5-methyl-cyclohexyl ester	7.18	$C_{16}H_{31}O_2P$	Not reported
25.	20.89	2-Azido-2,4,4,6,6,8,8-heptamethylnonane	4.36	$C_{16}H_{33}N_3$	Not reported
26.	20.98	cis-2-Methyl-4-n-butylthiane, S,S-dioxide	10.44	$C_{10}H_{20}O_2S$	Not reported
27.	21.48	trans-2-Methyl-4-n-butylthiane, S,S-dioxide	2.31	$C_{10}H_{20}O_2S$	Not reported
28.	21.66	1-Tridecene	32.67	$C_{13}H_{26}$	Not reported
29.	21.87	Tridecane	9.5	$C_{13}H_{28}$	Not reported
30.	22.91	Cyclohexylmethylsilane	2.31	C_7H16Si	Not reported
31.	23.06	Oxirane, diethylboryloxymethyl-	17.85	$C_7H_{15}BO_2$	Not reported
32.	23.62	2,6,10-Trimethyltridecane	1.29	$C_{16}H_{34}$	Not reported
33.	24	Dodecane, 2,2,11,11-tetramethyl-	17.81	$C_{16}H_{34}$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
34.	24.7	Oxirane, [[4-(1,1-dimethylethyl)phenoxy]methyl]-	21.19	$C_{13}H_{18}O_2$	Anti-inflammatory, analgesic, antipyretic effects (Mahmoud and Alshammari, 2022)
35.	26.38	trans-2,4-Dimethylthiane, S,S-dioxide	2.31	$C_7H_{14}O_2S$	Glutathione-S-tansferase inhibitor, increases glutathinone-S-transferase (GST) activity, decreases oxaloacetate .transaminase activity, catechol-O-methyl-transferase-inhibitor, decreases glutamate oxaloacetate transaminase, decreases glutamate pruvate transaminase, glucosyl-transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, transdermal, smart drug, adrenocortical stimulant (Hassan Mohammad et al., 2021)
36.	26.52	trans-2-methyl-4-n-pentylthiane, S,S-dioxide	38.04	$C_{11}H_{22}O_2S$	Glutathione-S-transferase-inhibitor, catechol-O-methyl-transfearse inhibitor, myo-neuro-stimulator, nitric oxide synthatase inhibitor, NO scavenger, stimulates norepinephrine production, stimulates sympathetic nervous system, decrease glutamate oxaloacetate transaminase, decrease glutamine pyruvate transaminase, glycosyl transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, smart drug, adrenal supporter (Hassan Mohammad et al., 2021)
37.	26.83	Tetradecyl trifluoroacetate	4.7	$C_{16}H_{29}F_3O_2$	Not reported
38.	28.53	1-Chloro-2-dodecyloxyethane	1.42	$C_{14}H_{29}ClO$	Not reported
39.	29.55	Pentafluoropropionic acid, hexadecyl ester	2.76	$C_{19}H_{33}F_5O_2$	Not reported
40.	29.75	1-Decanol, 2-hexyl-	11.96	$C_{16}H_{34}O$	Antimicrobial (Ferdosi et al., 20203)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
41.	30.44	Trihexadecyl borate	2.1	$C_{48}H_{99}BO_3$	Not reported
42.	30.71	Pentadecane, 7-(bromomethyl)-	2.76	$C_{16}H_{33}Br$	Not reported
43.	31.05	Trichloroacetic acid, undecyl ester	8.39	$C_{13}H_{23}Cl_3O_2$	Not reported
44.	31.91	Bis(2-ethylhexyl) hydrogen phosphite	2.18	$C_{16}H_{35}O_3P$	Not reported
45.	32.61	N-(1H-Tetrazol-5-yl)decanamide	10.75	$C_{11}H_{21}N_5O$	Not reported
46.	33.11	Dodecyl heptyl ether	1.11	$C_{19}H_{40}O$	Not reported
47.	33.62	Hexadecanoic acid, methyl ester	66.73	$C_{17}H_{34}O_2$	Antibacterial (Shaaban et al., 2021), insect repellent (Gaikwad and Nalawade, 2020)
48.	33.77	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-,methyl ester	12.77	$C_{18}H_{28}O_3$	Antifungal, antioxidant (Kumar et al., 2016)
49.	34.04	Pentadecane, 8-hexyl-	4.16	$C_{21}H_{44}$	Not reported
50.	35.04	Chloroacetic acid, pentadecyl ester	3.93	$C_{17}H_{33}ClO_2$	Acidifier, arachidonic acid inhibitor, increase aromatic amino acid decarboxylase activity (Sharmla et al., 2020)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
51.	36.91	Methyl 12,13-tetradecadienoate	16.88	$C_{15}H_{26}O_2$	Antioxidant (Aburagaegah et al., 2024)
52.	37.38	1-Hexadecanaminium, N,N,N-trimethyl-, octadecanoate	27.34	$C_{37}H_{77}NO_2$	Not reported
53.	40.74	Phosphoric acid, diundecyl ethyl ester	1.96	$C_{24}H_{51}O_4P$	Not reported
54.	42.99	Arsenous acid, tris(trimethylsilyl) ester	1.36	$C_9H_{27}AsO_3Si_3$	Antiviral, antithyroid, anticatract (Alexander et al., 2022)
55.	44.37	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	0.44	$C_{14}H_{44}O_6Si_7$	Acidulant, Acidifier, control of fever, increase aromatic amino acid decarboxylase activity, anti- uric acid, urinary-acidulant (Rehman et al., 2024)

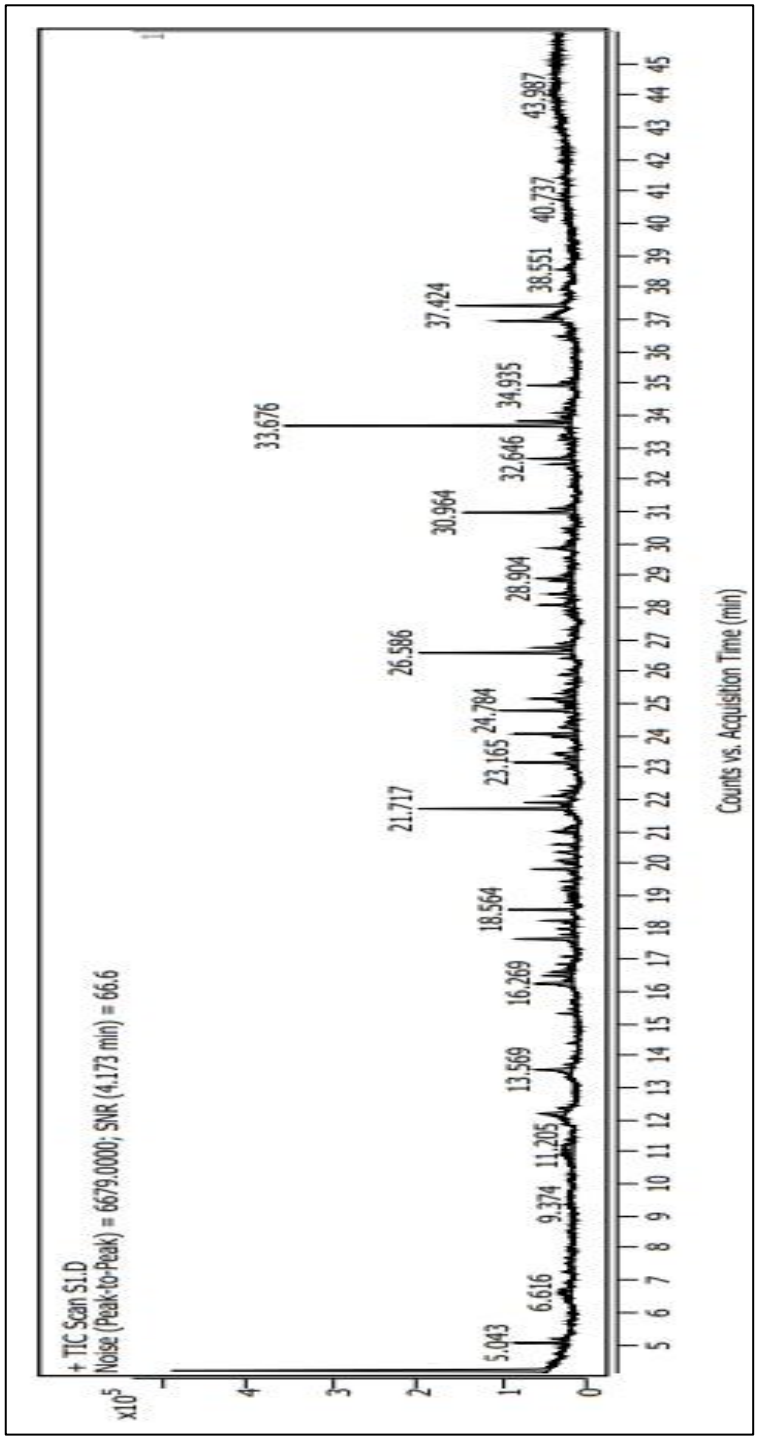

Figure 4.31. GC-MS chromatogram of *Z. acanthopodium* leaf extract

Table 4.5. Phytochemicals detected in *Z. acanthopodium* fruit extract

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
1.	4.99	Benzene, (isothiocyanatomethyl)-	0.95	C_8H_7NS	Anti-bacterial, anti-fungal, anti-inflammatory, anticancer properties (Dinh et al., 2021)
2.	5.08	4-(2-Isothiocyanatoethyl)phenol	8.22	C_9H_9NOS	Antibacterial, antioxidant (Thiruvangoth et al., 2024)
3.	5.2	2-Methoxybenzyl isothiocyanate	1.6	C_9H_9NOS	Not reported
4.	6.39	.beta.-l-Rhamnopyranoside, phenyl-2,3-O-ethylboranediyl-4-Obenzyl-	4.79	$C_{21}H_{25}BO_5$	Not reported
5.	6.59	Benzene, 1-isocyanato-3-methoxy-	7.41	$C_8H_7NO_2$	Not reported
6.	6.67	Benzyl (1,2,3-thiadiazol-4-y)carbamate	4.28	$C_{10}H_9N_3O_2S$	Not reported
7.	6.88	1-Cycloocten-5-yne, (Z)-	1.92	C_8H_{10}	Not reported
8.	7.5	Spiro[2.4]heptane, 1-ethenyl-5-(1-propenylidene)-	4.29	$C_{12}H_{16}$	Not reported
9.	7.68	Isobutyl acrylate	1.76	$C_7H_{12}O_2$	Not reported
10.	8.02	Arsenous acid, tripropyl ester	0.63	$C_9H_{21}AsO_3$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
11.	8.459	Boron, (3,3-dimethylbutyl)(Nmethylenemethanamine)bis(trifluoromethyl)-, (t-4)-	0.72	C10H18BF6N	Not reported
12.	8.648	Propanedioic acid, dimethyl ester	1.35	$C_5H_8O_4$	Acrocylindropepsin inhibitor, chymosin inhibitor, saccharopepsin inhibitor, aspartate 4-decarboxylase inhibitor, phobic disorders treatment (Sitio and Akmal, 2024), anti-tumour, anti-oxidant (Mohammed et al., 2016)
13.	12.1	Hexane, 3,3-dimethyl-	9.31	C_8H_{18}	Not reported
14.	12.85	2-n-Butylthiolane, S,S-dioxide	0.26	$C_8H_{16}O_2S$	Not reported
15.	13.72	Heptane, 2,4,6-trimethyl-	2.24	$C_{10}H_{22}$	Not reported
16.	13.96	Borinic acid, diethyl-	0.65	$C_4H_{11}BO$	Acidifier, arachidonic acid inhibitor, increases aromatic amino acid decarboxylase activity, inhibits production of uric acid, urine acidifier (Perumal et al., 2021)
17.	14.39	Octanoic acid, methyl ester	2.38	$C_9H_{18}O_2$	Not reported
18.	14.6	Cyclopentasiloxane, decamethyl-	0.35	$C_{10}H_{30}O_5Si_5$	Cytotoxicity against colon cancer cellsc(HCT116) and breast cancer cells (MCF-7), antiviral activity against avian influenza H5N1 virus (Chetehouna et al., 2024)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
19.	15.32	9-Borabicyclo[3.3.1]nonane, 9-(benzoyloxy)-	3.83	$C_{15}H_{19}BO_2$	Not reported
20.	16.56	cis-2-Methyl-4-n-butylthiane, S,S-dioxide	5.61	$C_{10}H_{20}O_2S$	Not reported
21.	17.06	Borane, diethyl(decyloxy)-	2.15	$C_{14}H_{31}BO$	Antifungal, antibacterial, anticancer (Soni et al., 2023)
22.	17.35	Cyclohexylmethylsilane	0.85	$C_7H_{16}Si$	Not reported
23.	17.58	2-Ethylthiolane, S,S-dioxide	16.78	$C_6H_{12}O_2S$	Not reported
24.	18.09	Decane, 3,8-dimethyl-	3.12	$C_{12}H_{26}$	Not reported
25.	18.18	Heptane, 2,2,3,3,5,6,6-heptamethyl-	6.76	$C_{14}H_{30}$	Not reported
26.	18.39	Aluminum, triethyl-	0.68	$C_6H_{15}Al$	Not reported
27.	18.5	Undecane	13.99	$C_{11}H_{24}$	Anti-inflammatory, anti-allergic (Choi et al., 2020),
28.	18.93	2-Propyl-1-pentanol, chlorodifluoroacetate	1.93	$C_{10}H_{17}ClF_2O_2$	Not reported
29.	19.045	Sulfurous acid, 2-ethylhexyl undecyl ester	2.31	$C_{19}H_{40}O_3S$	Not reported
30.	19.073	Nonane, 5-propyl-	6.24	$C_{12}H_{26}$	Not reported
31.	19.285	Decane, 2,3,5,8-tetramethyl-	4.34	$C_{14}H_{30}$	Not reported
32.	19.394	Propanoic acid, 2,2-dimethyl-, anhydride with diethylborinic acid	3.52	$C_9H_{19}BO_2$	Not reported

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
33.	19.77	Nonane, 3-methyl-5-propyl-	10.41	$C_{13}H_{28}$	Not reported
34.	19.99	Methyl 8-methyl-nonanoate	3.56	$C_{11}H_{22}O_2$	Not reported
35.	20.36	Cyclopentyl-methyl-phosphinic acid, 2-isopropyl-5-methyl-cyclohexyl ester	6.71	$C_{16}H_{31}O_2P$	Not reported
36.	21.49	trans-2-Methyl-4-n-butylthiane, S,S-dioxide	1.69	$C_{10}H_{20}O_2S$	Not reported
37.	21.65	1-Tridecene	34.44	$C_{13}H_{26}$	Pheromone, allomone (Arrabal et al., 2010)
38.	21.88	Tridecane	6.55	$C_{13}H_{28}$	Not reported
39.	22.03	2-Azido-2,4,4,6,6,8,8-heptamethylnonane	9.71	$C_{16}H_{33}N_3$	Not reported
40.	22.43	3,7-Dimethyl-1-octyl methylphosphonofluoridate	1.44	$C_{11}H_{24}FO_2P$	Not reported
41.	23.07	Cyclohexane, octyl-	19.01	$C_{14}H_{28}$	Not reported
42.	23.86	5-Nonanol, trimethylacetate	0.96	$C_{14}H_{28}O_2$	Not reported
43.	23.99	Dodecane, 2,2,11,11-tetramethyl-	15.81	$C_{16}H_{34}$	Not reported
44.	24.74	Oxirane, [[4-(1,1-dimethylethyl)phenoxy]methyl]-	11.34	$C_{13}H_{18}O_2$	Anti-inflammatory, analgesic, antipyretic (Mahmoud and Alshammari, 2022)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
45	25.05	1-Pentadecene	2.89	$C_{15}H_{30}$	Not reported
46	26.05	1-Dodecanol, 3,7,11-trimethyl-	26.054	$C_{15}H_{32}O$	Not reported
47	26.38	trans-2,4-Dimethylthiane, S,S-dioxide	2.57	$C_7H_{14}O_2S$	Glutathione-S-tansferase inhibitor, increases glutathinone-S-transferase (GST) activity, decreases oxaloacetate transaminase activity, catechol-O-methyl-transferase-inhibitor, decreases glutamate oxaloacetate transaminase, decreases glutamate pyruvate transaminase, glucosyl-transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, transdermal, smart drug, adrenocortical stimulant (Hassan Mohammad et al., 2021)
47	26.67	Cetene	12.61	$C_{16}H_{32}$	Not reported
49	26.8	2-Azido-2,4,4,6,6-pentamethylheptane	6.48	$C_{12}H_{25}N_3$	Antitumor, anti-inflammatory (Addai et al., 2022)
50	27.67	n-Dodecylpyridinium chloride	3.66	$C_{17}H_{30}ClN$	Antibacterial (Hrenovic et al., 2008)
51	28.01	Oxirane, diethylboryloxymethyl-	10.24	$C_7H_{15}BO_2$	Not reported
52	28.76	2-Octen-1-ol, 7-[(tetrahydro-2H-pyran-2-yl)oxy]-, (E)-	16.83	$C_{13}H_{24}O_3$	Not reported
53	29.73	1-Decanol, 2-hexyl-	12.38	$C_{16}H_{34}O$	Antimicrobial (Ferdosi et al., 20203)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
54.	30.29	Isobutyl tetradecyl ether	3.11	$C_{18}H_{38}O$	Not reported
55.	30.42	Trihexadecyl borate	3.54	$C_{48}H_{99}BO_3$	Not reported
56.	30.71	Pentadecane, 7-(bromomethyl)-	0.24	$C_{16}H_{33}Br$	Not reported
57.	30.91	trans-2-methyl-4-n-pentylthiane, S,S-dioxide	30.32	$C_{11}H_{22}O_2S$	Glutathione-S-transferase-inhibitor, catechol-O-.methyl-transfearse inhibitor, myo-neuro-stimulator, nitric oxide synthatase inhibitor, NO scavenger, stimulates norepinephrine production, stimulates sympathetic nervous system, decrease glutamate oxaloacetate transaminase, decrease glutamine pyruvate transaminase, glycosyl transferase inhibitor, increases glyoxalate transamination, reverse transcriptase inhibitor, smart drug, adrenal supporter (Hassan Mohammad et al., 2021)
58.	31.59	11,13-Dimethyl-12-tetradecen-1-ol acetate	3.57	$C_{18}H_{34}O_2$	Antimicrobial (Getahun et al., 2023)
59.	31.73	4-n-Hexylthiane, S,S-dioxide	3.2	$C_{11}H_{22}O_2S$	Not reported
60.	32.6	N-(1H-Tetrazol-5-yl)decanamide	11.46	$C_{11}H_{21}N_5O$	Not reported
61.	33.07	Dodecyl heptyl ether	1.55	$C_{19}H_{40}O$	Not reported
62.	33.62	Hexadecanoic acid, methyl ester	73.46	$C_{17}H_{34}O_2$	Antibacterial (Shaaban et al., 2021), insect repellent (Gaikwad and Nalawade, 2020)

No.	Retention time	Compound name	Area percentage (%)	Molecular formula	Biological activity
63	33.77	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	12.69	$C_{18}H_{28}O_3$	Antifungal, antioxidant (Kumar et al. 2016)
64	34.04	Eicosane, 10-methyl-	3.53	$C_{21}H_{44}$	Antioxidant (Rhetso et al.,2020)
64	34.55	Octadecane, 1-(ethenyloxy)-	0.9	$C_{20}H_{40}O$	Not reported
66	35.36	3-Ethyl-3-methylnonadecane	0.72	$C_{22}H_{46}$	Not reported
67	36.31	Bis(2-ethylhexyl) hydrogen phosphite	1.69	$C_{16}H_{35}O_3P$	Not reported
68	36.79	Methyl 12,13-tetradecadienoate	6.16	$C_{15}H_{26}O_2$	Not reported
69	37.15	Heneicosyl heptafluorobutyrate	0.47	$C_{25}H_{43}F_7O_2$	Not reported
70	37.37	1-Hexadecanaminium, N,N,N-trimethyl-, octadecanoate	31.64	$C_{37}H_{77}NO_2$	Not reported
71	38.01	Octyl tetradecyl ether	1.24	$C_{22}H_{46}O$	Not reported
72	39.44	Phosphoric acid, diundecyl ethyl ester	0.33	$C_{24}H_{51}O_4P$	Not reported
73	39.97	Caprolactone oxime, (NB)-O-[(diethylboryloxy)(ethyl)boryl]-	0.73	$C_{12}H_{25}B_2NO_2$	Not reported
74	40.61	2-Methyltetracosane	3.68	$C_{25}H_{52}$	Antioxidant (Ramya et al., 2015)
75	44.62	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	1.68	$C_{14}H_{44}O_6Si_7$	Acidulant, acidifier, control of fever, increase aromatic amino acid decarboxylase activity, anti- uric acid, urinary-acidulant (Rehman et al., 2024)

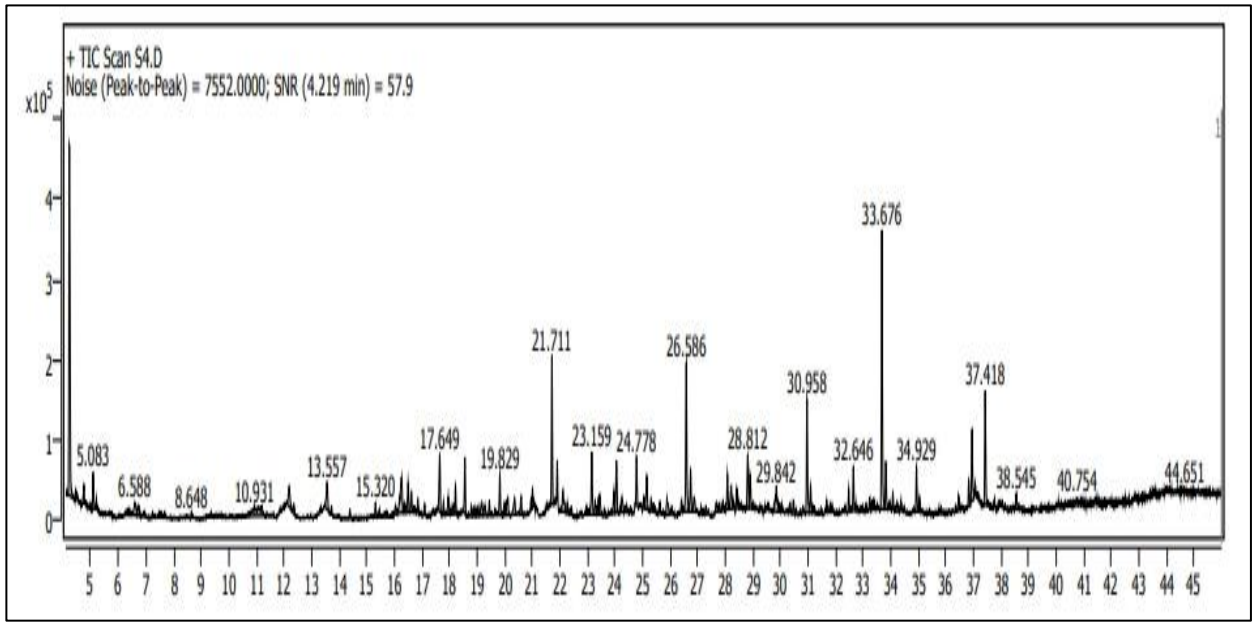

Figure 4.32. GC-MS chromatogram of *Z. acanthopodium* fruit extract

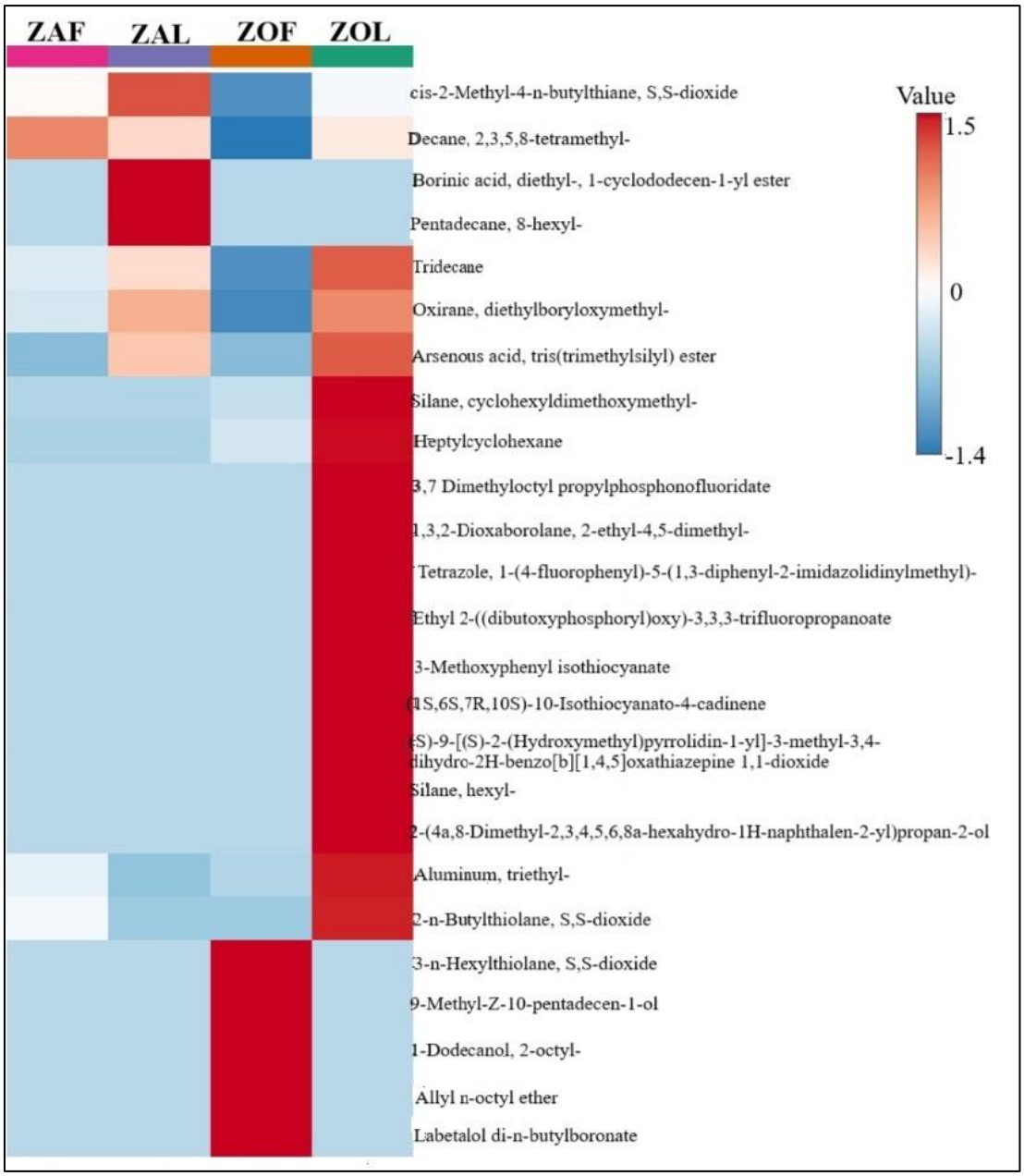

Figure 4.33. Heat map of top distinct phytochemicals in *Z. oxyphyllum and Z. acanthopodium* leaf and fruit extracts based on their unusually abundant or low area percentage.

ZOL = *Z. oxyphyllum* Edgew. leaves, ZOF = *Z. oxyphyllum* Edgew. fruits, ZAL = *Z. acanthopodium* DC. leaves ZAF = *Z. acanthopodium* DC. fruits

A heat map of top twenty-five phytochemicals based on their least or most abundance in the plant extracts revealed a sharp distinction (Figure 4.33). Certain compound like cis-2-Methyl-4-n-butylthiane, S,S-dioxide was detected in most abundance in *Z. acanthopodium* leaf extract (deep red colouration) at 10.44 %, medium amounts in *Z. acanthopodium* fruit extract at 5.61 % and *Z. oxyphyllum* leaf extract at 5.14 % (neutral

colour) and least amount in *Z. oxyphyllum* fruit extract (deep blue colour) at 0.51 %. Similarly, decane, 2,3,5,8-tetramethyl- was detected in *Z. acanthopodium* fruit extract in highest abundance (4.34 %). In the leaf extracts of *Z. acanthopodium* and *Z. oxyphyllum* the light pink colour (closer to red spectrum) indicated a slightly less amount of 3.28 % and 3.04 % respectively, while in *Z. oxyphyllum* fruit extract the least abundance was detected (deep blue colour) of 0.3 %. Tridecane was absent in *Z. oxyphyllum* fruit extract but present in the other three extracts at various percentage. Heptylcyclohexane and silane, cyclohexyldimethoxymethyl- were present in the leaf and fruit extracts of *Z. oxyphyllum* only and absent in *Z. acanthopodium* extracts.

3,7 Dimethyloctyl propylphosphonofluoridate, 1,3,2-Dioxaborolane, 2-ethyl-4,5-dimethyl-, Tetrazole, 1-(4-fluorophenyl)-5-(1,3-diphenyl-2-imidazolidinylmethyl)-, Ethyl 2-((dibutoxyphosphoryl)oxy)-3,3,3-trifluoropropanoate, 3-Methoxyphenyl isothiocyanate, (1S,6S,7R,10S)-10-Isothiocyanato-4-cadinene, (S)-9-[(S)-2-(Hydroxymethyl)pyrrolidin-1-yl]-3-methyl-3,4-dihydro-2H-benzo[b][1,4,5]oxathiazepine 1,1-dioxide, Silane, hexyl-, and 2-(4a,8-Dimethyl-2,3,4,5,6,8a-hexahydro-1H-naphthalen-2-yl)propan-2-ol were detected distinctly in *Z. oxyphyllum* leaf extract only, whereas 3-n-Hexylthiolane, S,S-dioxide, 9-Methyl-Z-10-pentadecen-1-ol, 1-Dodecanol, 2-octyl-, Allyl n-octyl ether and Labetalol di-n-butylboronate were detected distinctly in *Z. oxyphyllum* fruit extract only.

11,13-Dimethyl-12-tetradecen-1-ol acetate, 1-Dodecanol, 3,7,11-trimethyl-, 1-Pentadecene, 2-Methoxybenzyl isothiocyanate, 2-Methyltetracosane, 2-Octen-1-ol, 7-[(tetrahydro-2H-pyran-2-yl)oxy]-, (E)- and 4-(2-Isothiocyanatoethyl)phenol were compounds detected distinctly in *Z. acanthopodium* fruit extract only in high percentage area. Lastly, 1-Chloro-2-dodecyloxyethane, 1-Hexanol, 5-methyl-2-(1-methylethyl)-, 2,6,10-Trimethyltridecane, 2-Bromo-N-[(1-(cyclohexylmethyl)-1H-1,2,3-triazol-4-yl)methyl]-Nmethylbenzenesulfonamide, Adamantane, 1-isothiocyanato-3-methyl-, Borinic acid, diethyl-, 1-cyclododecen-1-yl ester, Methyl 10,11-tetradecadienoate, Methyl 11,12-octadecadienoate and Methyl 13,14-octadecadienoate were some compounds detected distinctly in *Z. acanthopodium* leaf extract only.

In the petroleum ether extract of *Z. acanthopodium* leaves, the phytochemical profile was different from the present study. Paulownin was the most abundant compound by area percentage (24.31 %), followed by 1H,3H-Furo[3,4-c]furan, 1,4-bis(3,4-

dimethoxyphenyl)tetrahydro- (16.49 %) and Stigmast-5-en-3-ol, (3.BETA.)- (10.74 %) (Devi et al., 2015). When compared to other *Zanthoxylum* species too, the phytocompounds detected in the extracts in the present study were different. In the hexane extract of *Z. armatum* fruits, the 2-Hydroxy cylclopenta decanone was the most abundant compound by area percentage with a value of 27.30 %, followed by palmitic acid (6.99 %) and piperitone (6.71 %) (Kayat et al., 2016). Many of the phytocompounds detected have been reported from other medicinal plants. Hexane, 3,3-dimethyl- was reported in *Physalis peruviana* (Prastiyanto et al., 2023), methyl 8-methyl-nonanoate was reported in *Azadirachta indica* oil (Kaur et al., 2022), cyclopentasiloxane, decamethyl- was reported in *Moringa oleifera* (Olaoye et al., 2024) and mango (Jaleel et al., 2021), trichloroacetic acid, undecyl ester was reported in *Ziziphusspina-christi* leaves (Mathkoor et al., 2023), tetradecyl trifluoroacetate was reported from *Vaucheria karachiensis* (Mohansundaram et al., 2020). However, identical compounds as in the present study has not so far been reported in other *Zanthoxylum* species.

In the GC-MS analysis, hexadecenoic acid, methyl ester was the most prominent phytochemical present. In all the samples, some known anti-inflammatory compounds viz, 3-methoxyophenyl isothiocyanate, undecane, 2-azido-2,4,4,6,6-pentamethyl heptane and oxirane, [[4-(1,1-dimethylethyl)phenoxy]methyl]- were found to be present except in case of *Z. oxyphyllum* fruits where the oxirane derivative was lacking. Several other detected compounds that had not hitherto been reported for their biological activities may be an interesting area of future research.

A principal component analysis of the phytochemical content of the extracts revealed well separated data points indicating a clear distinction in the phytochemical profile among the leaf and fruit extracts of *Z. oxyphyllum* and *Z. acanthopodium* (Figure 4.34).

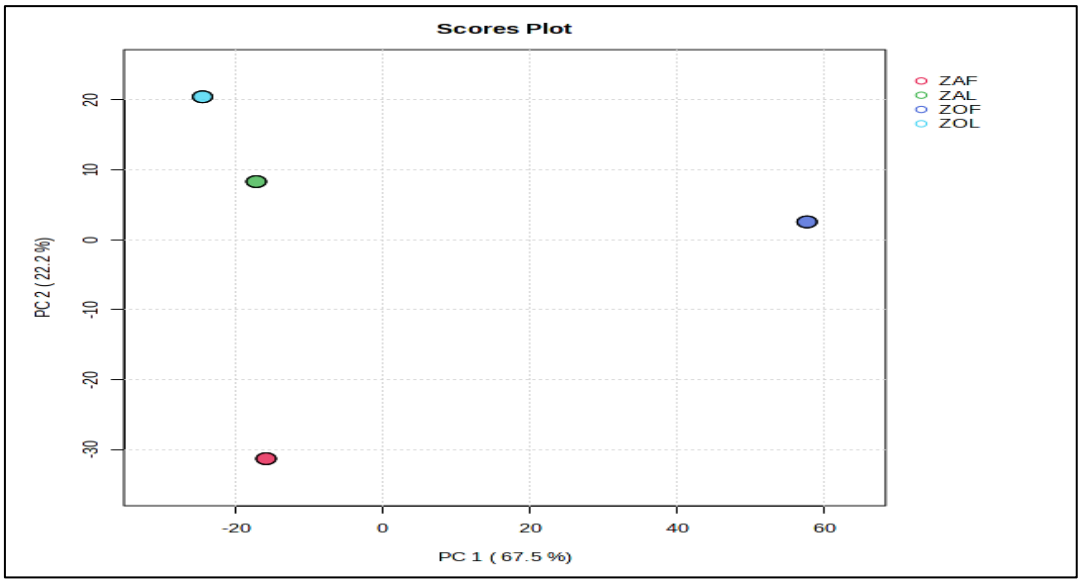

Figure 4.34. Principal component analysis of *Z. oxyphyllum and Z. acanthopodium* leaf and fruit extracts

ZOL = *Z. oxyphyllum* Edgew. leaves, ZOF = *Z. oxyphyllum* Edgew. fruits, ZAL = *Z. acanthopodium* DC. leaves ZAF = *Z. acanthopodium* DC. fruits

5.7. Element content analysis of plant samples

Along with regulatory cellular functions as coenzyme, cofactor for enzymes, and as signalling molecules, minerals also play a crucial role in the optimum functioning of immune system. Minerals are involved in the response of immune cells to cytokines after TLR stimulation, in the development, differentiation, and proliferation of lymphocytes, chemotaxis of granulocytes, maturation of immune cells and humoral and cellular immune response to name a few (Weyh et al., 2022). In the present study, all the plant samples contained considerable amounts of minerals. Nitrogen was present in comparable amount in all the samples with *Z. oxyphyllum* leaves and fruits containing 2.28 ± 0.25 % DW and 2.63 ± 0.25 % DW respectively. The same in *Z. acanthopodium* leaves and fruits were 2.74 ± 0.32 % DW and 2.38 ± 0.10 % DW respectively. Potassium was highest in *Z. acanthopodium* fruits and leaves at 0.15 % DW and 0.12 % DW respectively, whereas in the *Z. oxyphyllum* leaves and fruits the content was slightly lower at 0.09 % DW and 0.07 % DW respectively. In *Z. oxyphyllum* leaves and fruits, magnesium content was 0.20 ± 0.00 % DW and 0.44 ± 0.01 % DW respectively while in *Z. acanthopodium* leaves and fruits, the same was 0.74 ± 0.24 % DW and 0.33 ± 0.10 % DW respectively.

Calcium content was considerably high in *Z. oxyphyllum* fruits and *Z. acanthopodium* leaves at 2.14 ± 0.05 % DW and 3.37 ± 0.14 % DW respectively whereas in *Z. oxyphyllum* leaves and *Z. acanthopodium* fruits, the content was lower at 0.16 ± 0.01 % DW and 0.29 ± 0.04 % DW respectively (Figure 4.35).

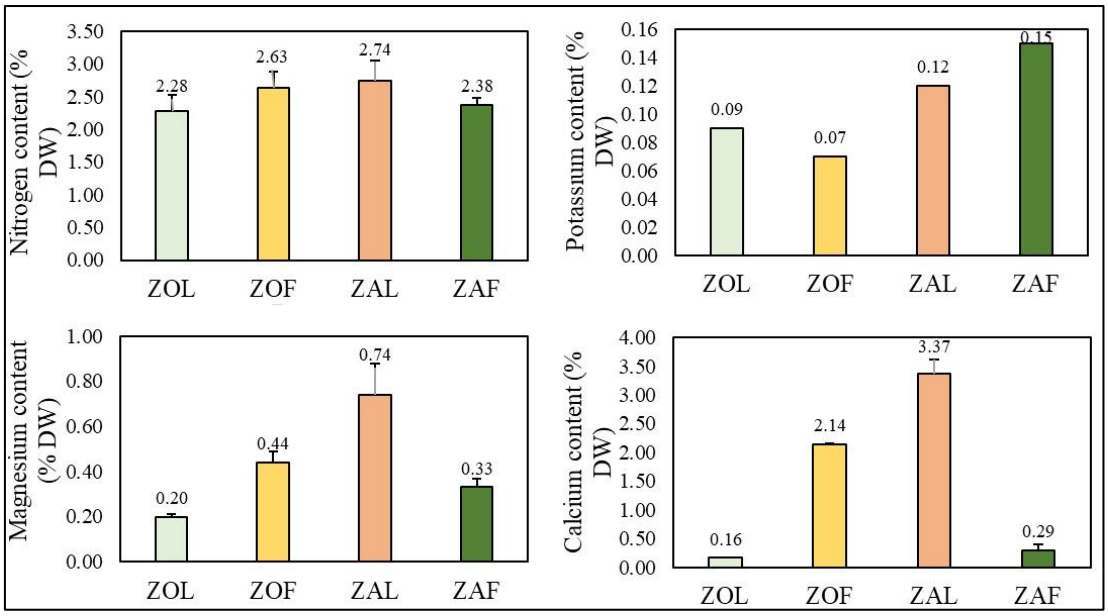

Figure 4.35. N, K, Mg and Ca content in *Z. oxyphyllum* and *Z. acanthopodium* leaves and fruits presented as percentage dry weight.

ZOL = *Z. oxyphyllum* Edgew. leaves, ZOF = *Z. oxyphyllum* Edgew. fruits, ZAL = *Z. acanthopodium* DC. leaves ZAF = *Z. acanthopodium* DC. fruits

Manganese, zinc, copper, and iron were present in trace amounts in all the samples. Manganese was relatively highest in *Z. oxyphyllum* fruits at 128.40 ± 3.10 mg/1000 g whereas in the leaves the content was lower at 9.45 ± 2.05 mg/1000 g. In *Z. acanthopodium* leaves, magnesium content was 50.05 ± 0.95 mg/1000 g however, in the fruits it was not detected. Zinc content in *Z. oxyphyllum* leaves and fruits was 35.20 ± 1.60 mg/1000 g and 65.35 ± 2.75 mg/1000 g respectively while in *Z. acanthopodium* leaves and fruits, the same was 70.00 ± 0.80 mg/1000 g and 44.40 ± 1.70 mg/1000 g respectively. Copper content was highest in *Z. acanthopodium* leaves with value of 18.55 ± 0.55 mg/1000 g. In the rest of the samples i.e. *Z. acanthopodium* fruits and *Z. oxyphyllum* leaves and fruits, copper content was 5.60 ± 0.40 mg/1000 g, 4.30 ± 1.20 mg/1000 g and 6.50 ± 0.30 mg/1000 g respectively. Iron content was higher in *Z. acanthopodium* leaves and fruits at 155.75 ± 17.45 mg/1000 g and 105.70 ± 20.70

mg/1000 g respectively. In the leaves and fruits of *Z. oxyphyllum* the same was 58.10 ± 10.90 mg/1000 g and 91.35 ± 3.55 mg/1000 g respectively (Figure 4.36).

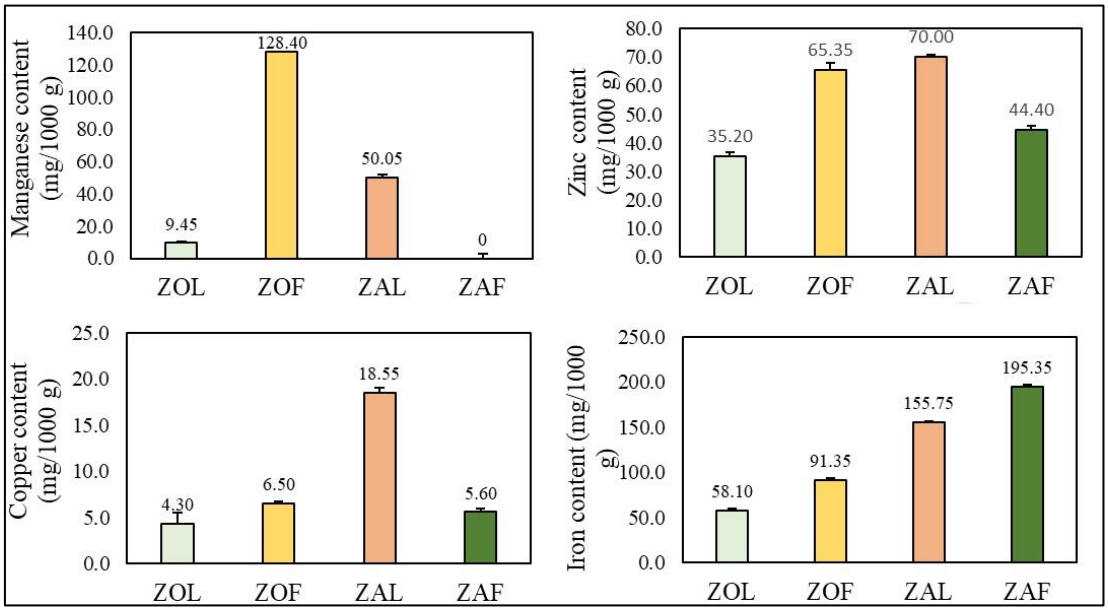

Figure 4.36. Mn, ZN, Cu and Fe content in *Z. oxyphyllum* and *Z. acanthopodium* leaves and fruits presented as mg/1000 g.

ZOL = *Z. oxyphyllum* Edgew. leaves, ZOF = *Z. oxyphyllum* Edgew. fruits, ZAL = *Z. acanthopodium* DC. leaves ZAF = *Z. acanthopodium* DC. fruits

Mineral contents were reported in considerable amounts from other *Zanthoxylum* species as well. In *Z. ovalifolium* the potassium, calcium, magnesium and nitrogen content was 1.22 %, 0.57 %, 0.029 % and 3.5 % respectively and iron, manganese, zinc and copper content was 74.50 ppm, 220.70 ppm, 33.30 ppm and 36.40 ppm respectively (Pavani et al., 2020). It was reported that in *Z. armatum* leaves calcium content was 0.226 ± 0.03 mg/100 g, magnesium content was 4.948 ± 0.21 mg/100 g, iron content was 0.0795 ± 0.02 mg/100 g, potassium content was 0.19 ± 0.011 mg/100 g, zinc was 0.026 ± 0.001 mg/100 g and manganese was 0.015 ± 0.002 mg/100 g, whereas in the fruits calcium content was 0.48 ± 0.031 mg/100 g, magnesium content was 3.07 ± 0.03 mg/100 g, iron content was 0.068 ± 0.001 mg/100 g, potassium content was 1.91 ± 0.003 mg/100 g, zinc was 0.072 ± 0.002 mg/100 g and manganese content was 0.012 ± 0.001 mg/100 g (Alam et al., 2019). In the stem bark of *Z. zanthoxyloides*, potassium, calcium, iron, magnesium and zinc was reported to be comparatively very high at 1012.9924 ± 0.0002 ppm, 6055.5591 ± 0.0003 ppm, 1093.8837 ± 0.0008 ppm, 1478.5064 ± 0.0004 ppm, 216.8516 ± 0.0007 ppm (Olushola-Siedoks et al., 2020).

Most of these mineral elements are important from immunomodulatory point of view. Of these, zinc is essential for the development of immune cells like T cells, B cells and macrophage. In addition, it also modulates cytokine production (Weyh et al., 2022). Magnesium plays important role in immune cell signalling. As such, it is important for the immune response of the cells (Weyh et al., 2022). Calcium plays role as a secondary messenger and helps in T and B lymphocyte activation. Adequate potassium level is important for reducing inflammation as it activates inflammatory response through ion channels. Manganese acts as co-factor for enzymes involved in the production of inflammatory mediators (Cannas et al., 2020). Iron supports proliferation and differentiation of immune cells especially T lymphocytes. Copper is specially required for mounting anti-oxidant defence and immune cell proliferation (Cannas et al., 2020). Nitrogen is the fundamental molecule that modulates the immune system by producing cytokines, antibodies, nitric oxide and it maintains balance between pro-inflammatory and anti-inflammatory responses (Ochoa and Magee, 1997).

Thus, mineral elements play important role in immunomodulation by the regulation of inflammation, immune cell activation, cytokine production etc. A balance in these minerals is essential for immunity, thereby justifying this study.

5.8. *In vitro* immunomodulatory studies of plant extracts

5.8.1. Culture of HTR-8/SVneo cells

The HTR-8/SVneo cell line is a heterogenous, immortalized human extra villous trophoblast cell line of non-cancerous origin, containing a mixed population of trophoblast and stromal/mesenchymal cells (Abou-Kheir et al., 2017). The cell line is widely used as a model for studying immune responses, inflammation during pregnancy and placental functions (Zhang et al., 2023; Deng et al., 2024). Trophoblast cells are known to express toll like receptors (TLRs) that can recognize viral/bacterial elements and cause a local inflammatory response, enabling them to release not only cytokines but various pro-inflammatory chemokines that can possibly lead to the recruitment of maternal immune cells and ‘educate’ these cells to perform a necessary innate immune response (Fest et al., 2007; Torricelli et al., 2009; Lu et al., 2024). Hence, HTR-8/SVneo cell line is a reliable *in vitro* model for investigation of immunomodulatory potential of

medicinal plants. Treatment with lipopolysaccharide (LPS) of *E. coli* has been demonstrated to cause inflammation in HTR-8/SVneo cells subsequently followed by the release of inflammatory cytokines (Zhang et al., 2021). In addition, LPS treatment also leads to release of anti-inflammatory cytokine IL-4 in human primary trophoblast cells (Torricelli et al., 2009). Figures of the cells at 0 hour, at 50 % confluency and at 90 % confluency have been given as figures 4.37, 4.38 and 4.39 respectively.

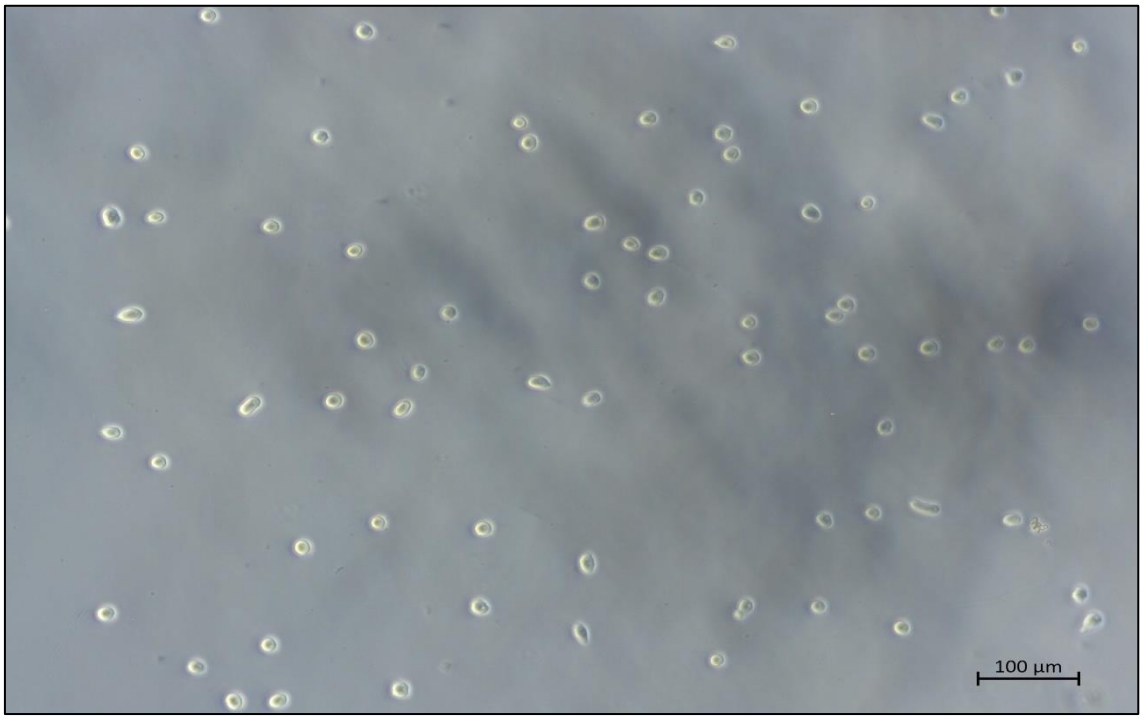

Figure 4.37. HTR-8/SV neo cells at 0 hour

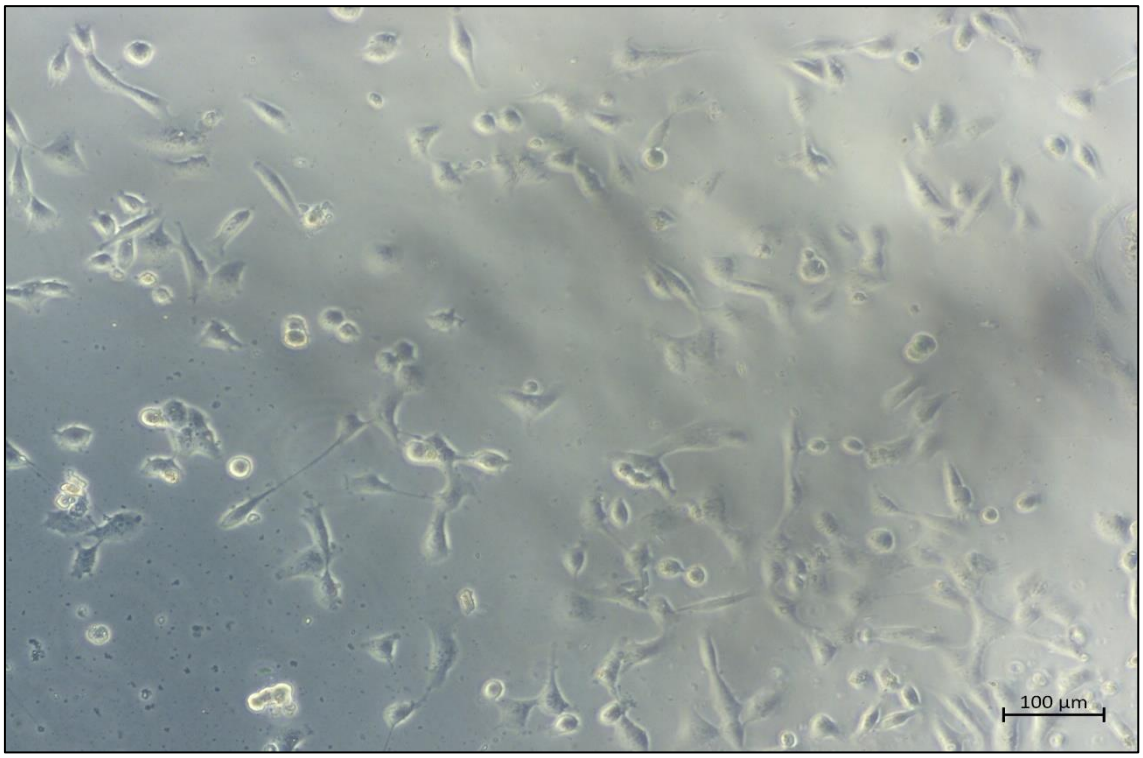

Figure 4.38. HTR-8/SV neo cells at 50 % confluent

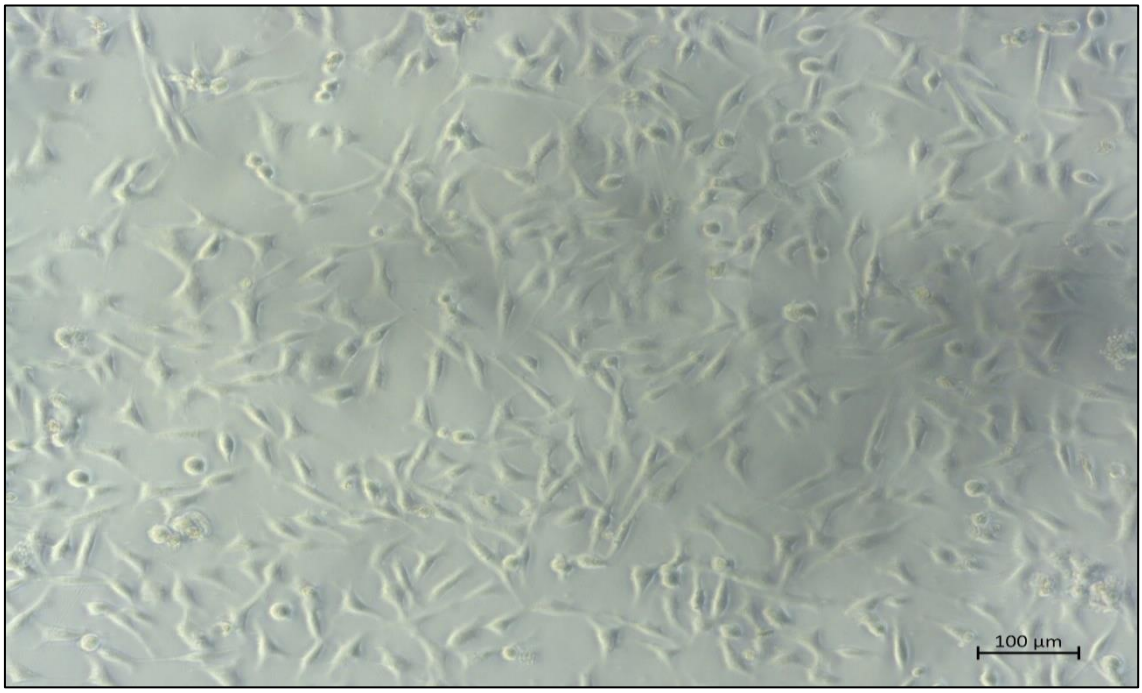

Figure 4.39. HTR-8/SV neo cells at 90 % confluent

5.8.2. MTT assay of HTR-8/SVneo cells

The MTT assay, based on the reduction of the yellow, water-soluble tetrazolium salt [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide], relies on the activity of dehydrogenase enzymes in live, metabolically active cells. These enzymes convert the tetrazolium salt into blue/magenta, water-insoluble formazan crystals. The concentration of dissolved formazan crystals, which directly correlates with the number of metabolically active cells, can be measured using a spectrophotometer or ELISA reader. The MTT assay is a straightforward and fast colorimetric method that provides quantitative results (Karakaş et al., 2017).

To check the cytotoxicity of the extracts on HTR-8/SVneo cells, MTT assay was conducted after 24 hours and 48 hours. A concentration range of 5, 15, 30, 50, 70 and 100 µg/ml of each extract was studied and a percentage viability of ≥ 80 % was considered acceptable. It was observed that all the extracts were non-cytotoxic to the cells at all concentrations. After 24- and 48-hours treatment, the cells showed a viability of above 80 % for all the extracts (Tables 4.6 and 4.7). Further, after 48 hours of treatment, a proliferative effect was observed in the cells with some group of cells showing > 100 % viability. Certain medicinal plants, due to the presence of many beneficial phytocompounds have been reported to have proliferative effects on non-cancerous cell lines. Natural compounds like those found in blueberries, green tea, catechin, carnosine, and vitamin D3 have been shown to promote the proliferation of bone marrow stem cells. Dietary fatty acids, such as oleic and linoleic acids, also support the growth of hematopoietic stem cells. *In vitro* studies indicated that supplementation with plant extracts could enhance mesenchymal stem cell (MSC) proliferation and differentiation. For example, using 1–100 µg/ml of citrus extracts stimulated human bone marrow MSC proliferation, whereas 50 µg/ml of the flavonone - naringin could boost rat BM-MSC growth. Additionally, fucoidan, derived from the brown algae *Laminaria japonica*, promoted the proliferation of human MSCs at concentrations of 0.1–10 µg/ml (Saud et al., 2019). Additionally, *Ageratina pichinchensis* was also shown to exert proliferative effect on normal human foetal lung cell line - MRC-5 (Romero-Cerecero et al., 2011).

In contrast, after 24 hours treatment diclofenac sodium was found to be cytotoxic to the cells at concentration of above 50 µg/ml and after 48 hours of treatment, this cytotoxicity was observed at concentration of above 15 µg/ml.

For further studies, two concentrations (15 and 30 µg/ml) of each plant extract and standard drug were selected.

Table 4.6. Percentage viability of HTR-8/SVneo cells after 24 hours treatment with plant extracts/standard drug presented as mean ± SEM (n=3).

	Mean percentage viability (%) of cells after 24 hours				
Concentration (µg/ml)	ZAL	ZAF	ZOL	ZOF	Std. drug
5	84.255 ± 3.191	88.097 ± 2.410	81.169 ± 4.069	81.540 ± 6.360	96.550 ± 8.075
15	80.694 ± 6.046	98.219 ± 2.599	91.280 ± 8.096	91.280 ± 2.367	100.524 ± 1.529
30	88.004 ± 2.290	87.254 ± 4.323	88.868 ± 1.862	88.868 ± 0.404	82.560 ± 1.184
50	82.755 ± 2.624	94.658 ± 1.937	85.900 ± 3.572	90.909 ± 5.454	82.636 ± 7.162
70	84.442 ± 0.375	90.347 ± 3.490	85.065 ± 0.968	88.312 ± 1.162	67.061 ± 0.406
100	83.411 ± 1.400	85.567 ± 2.004	86.735 ± 0.649	92.486 ± 1.033	74.158 ± 0.839

Table 4.7. Percentage viability of HTR-8/SVneo cells after 48 hours treatment with plant extracts/standard drug presented as mean ± SEM (n=3).

	Mean percentage viability (%) of cells after 48 hours				
Concentration (µg/ml)	ZAL	ZAF	ZOL	ZOF	Std. drug
5	95.735 ± 1.159	95.100 ± 3.358	101.756 ± 8.703	80.499 ± 1.756	106.443 ± 2.450
15	90.018 ± 2.769	99.819 ± 1.582	95.379 ± 1.368	103.604 ± 3.179	96.189 ± 4.991
30	100.363 ± 9.710	95.009 ± 2.860	93.900 ± 4.025	106.470 ± 5.195	55.445 ± 1.270
50	99.909 ± 6.238	96.552 ± 1.417	97.227 ± 1.521	100.739 ± 2.002	46.824 ± 1.906
70	91.924 ± 4.311	93.920 ± 1.287	99.445 ± 3.527	104.667 ± 1.245	41.016 ± 1.180
100	86.298 ± 8.964	80.036 ± 6.433	103.697 ± 2.991	104.713 ± 1.399	38.838 ± 2.541

ZOL = Z. oxyphyllum Edgew. leaf extract, ZOF = Z. oxyphyllum Edgew. fruit extract, ZAL = Z. acanthopodium DC. leaf extract, ZAF = Z. acanthopodium DC. fruit extract, Std. drug = diclofenac sodium

5.8.3. Effect of extracts in NO release from HTR-8/SVneo cells

Nitric oxide in cells is generated either by the constitutive enzymes endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) through calcium signalling or a third, inducible form of NOS (iNOS) which is expressed in cells by an outside stimuli like bacterial lipopolysaccharide or cytokines like TNF-α (Coleman, 2001). High levels of NO released can form a toxic reactive nitrogen species (RNS) peroxynitrite under aerobic conditions in the cells. Among the three NOS, eNOS and iNOS have been found to be expressed in trophoblast cells of human placenta (Du et al., 2021), thus the effect of the plant extracts to on its release in the inflammation induced cells was tested as one of the indicators of their immunomodulatory potential.

In the present study, no significant increase in the release of NO between C (-) and C (+) cells was observed. This observation agrees with a previous report by Singh et al. (2020), where 0.1 µg/ml, 1 µg/ml or10 µg/ml of LPS treatment for 24 hours did not cause significant increase in the release of NO in HTR-8/SVneo cells (Singh et al., 2020)

whereas, other compounds like the isoquinoline alkaloid - berberine hydrochloride and polyfluoroalkyl substances enhanced iNOS expression and the subsequent release of NO in trophoblast cells possibly via extracellular signal-regulated kinase (ERK) pathway (Du et al, 2021; Yang et al., 2023). It is known that LPS induced release of inflammatory mediators is a result of TLR-4 ligation (Lu et al., 2008) however, through the observation made in the present study, it can be hypothesized that in HTR-8/SVneo cells TLR-4 ligation of LPS induces the release of regulatory cytokines but not inflammatory mediators like NO and hence there was no induction of iNOS. Alternatively, prior to elevated NO production, induction of iNOS requires an initial lag phase consistent with the mRNA synthesis (Coleman, 2001) and there is insufficient report on how long this lag phase can last in HTR-8/SVneo cells.

Among the plant extracts, only *Z. oxyphyllum* fruit extract was found to reduce the level of NO in the cells. In fact, the reduction of NO concentration by both concentrations (15 µg/ml and 30 µg/ml) of ZOF treated cells was even significantly less than the basal concentration in the C (-) cells (*P* value 0.01 for both) (Figure 4.40). While NO level in the C (-) cells i.e. cells in which there was no LPS activation and no plant extract/standard drug treatment was 25.49 ± 0.40 µg/ml, the same in *Z. oxyphyllum* fruit extract treated cells were 21.28 ± 0.39 µg/ml and 20.50 ± 0.34 µg/ml for 15 µg/ml and 30 µg/ml concentrations respectively. Similar suppressive effect was also observed for cells treated with 30 µg/ml of diclofenac sodium (*P* value 0.02) which had NO level of 21.68 ± 0.90 µg/ml. One way ANOVA revealed that both concentrations of *Z. oxyphyllum* fruit extract (15 µg/ml and 30 µg/ml) treated cells had significantly less levels of NO in comparison to 15 µg/ml concentration of diclofenac sodium with *P* values of 0.02 and 0.01 respectively.

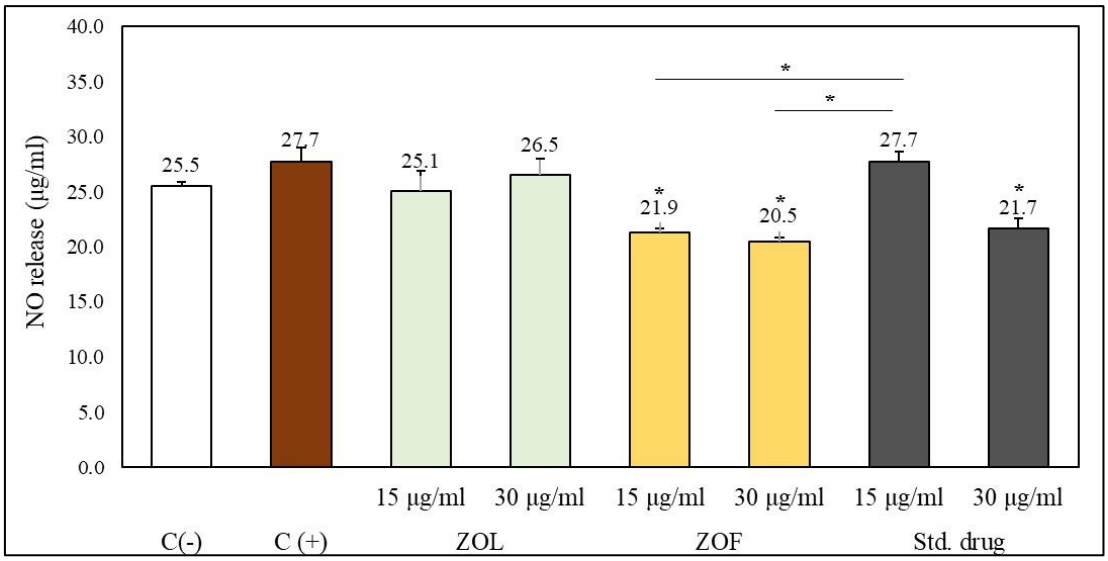

Figure 4.40. Effect of *Z. oxyphyllum* leaf and fruit extracts on NO release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZOL = *Z. oxyphyllum* Edgew. leaf extract, ZOF = *Z. oxyphyllum* Edgew. fruit extract, Std. drug = diclofenac sodium; *C (-) = Control (no LPS treatment, no plant extract/drug treatment), C (+) = Control (LPS treatment only); * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 representing significant difference of plant extract/drug treated cells from C(+) and/or significant difference between plant extract treated cells and standard drug treated cells.*

Zanthoxylum species are known to scavenge NO. Santhanam et al. (2013) reported that ethyl acetate extract of *Z. rhetsa* bark successfully scavenged NO *in vitro* with IC_{50} value of 50 ± 0.35 µg/ml (Santhanam et al., 2013). Similarly, methanol, chloroform, and petroleum ether extracts of *Z. armatum* leaves were also shown to scavenge NO *in vitro* with IC_{50} values of 105.0 ± 1.64 µg/ml, 157.60 ± 1.99 µg/ml, and 185.30 ± 2.48 µg/ml respectively (Karmakar et al., 2015). Since no significant increase in NO was induced by LPS treatment in the HTR-8/SVneo cells, the ability of *Z. oxyphyllum* fruit extract to reduce NO level in the cells can be attributable to their ability to directly scavenge NO rather than suppression of iNOS.

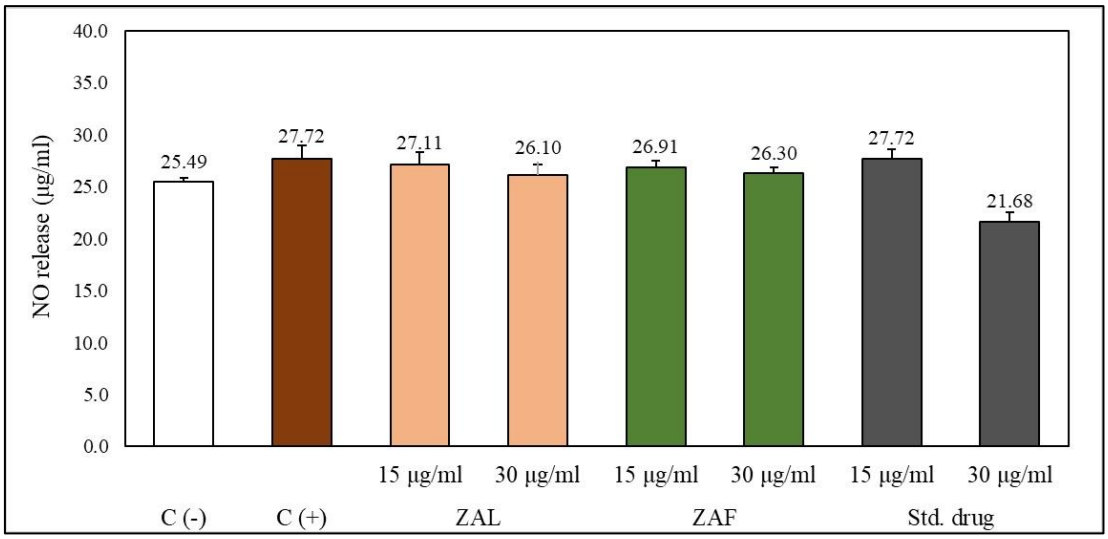

Figure 4.41. Effect of *Z. acanthopodium* leaf and fruit extracts on NO release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZAL = *Z. acanthopodium* DC. leaf extracts ZAF = *Z. acanthopodium* DC. fruit extract, Std. drug = diclofenac sodium; *C (-) = Control (no LPS treatment, no plant extract/drug treatment), C (+) = Control (LPS treatment only); * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 representing significant difference of plant extract/drug treated cells from C(+) and/or significant difference between plant extract treated cells and standard drug treated cells.*

In comparison, *Z. oxyphyllum* leaf extract (Figure 4.40) and *Z. acanthopodium* leaf and fruit extracts (Figure 4.41), were either ineffective in reducing the NO level or lowered the same only in negligible quantity (*P* value > 0.05 for all). The NO level in *Z. oxyphyllum* leaf extract treated cells were 25.09 ± 1.81 µg/ml and 26.51 ± 1.46 µg/ml for 15 µg/ml and 30 µg/ml concentrations respectively. The same for *Z. acanthopodium* leaf extract was 27.11 ± 1.23 µg/ml and 26.10 ± 1.07 µg/ml and for fruit extract was 26.91 ± 0.61 µg/ml and 26.30 ± 0.61 at 15 µg/ml and 30 µg/ml concentrations respectively.

Conversely, other species of *Zanthoxylum* could effectively reduce NO level in LPS induced cells. In J774.1 macrophages and mouse peritoneal exudate macrophages the methanolic extract of *Z. bungeanum* decreased the release of NO via inhibition of iNOS mRNA expression (Tezuka et al., 2001). Similarly, the *Z. schinifolium* essential oil also suppressed the expression of iNOS in RAW 264.7 murine macrophage cells (Lee et al., 2009). Fruit essential oil of *Z. nakai* and isobutylamides from *Z. nitidum* exhibited similar suppression of iNOS in LPS induced macrophage cells (Guo et al., 2018; Qin et al., 2020). It is worth noting that ethanolic extract of *Z. acanthopodium* fruits showed suppressive activity towards iNOS expression in LPS stimulated RAW 264.7 cells while in the present

study both concentrations of *Z. acanthopodium* fruit extract showed only negligible (*P* value 0.59 and 0.36 respectively) inhibitory activity towards NO release in HTR-8/SVneo cells (Yanti et al., 2011). It is reasonable to state that *Z. acanthopodium* fruit and leaf extracts have better iNOS suppressing property than direct scavenging of NO *in vitro* and hence the contrasting result obtained in the present study. However, among all the extracts investigated in the present study, *Z. oxyphyllum* fruit extract treated cells exhibited the most significant reduction in NO with its activity being even significantly better than the standard drug.

5.8.4. Effect of extracts in IL-6 release from HTR-8/SVneo cells

Interleukin 6 (IL-6) is a cytokine rapidly produced in response to infections and tissue injuries, playing a critical role in acute phase responses, hematopoiesis, and immune regulation. While its expression is tightly controlled, dysregulated IL-6 production contributes to chronic inflammation, autoimmunity, and various diseases. IL-6 induces acute-phase protein synthesis, modulates T-cell differentiation (promoting Th17 and inhibiting Treg), and disrupts immune tolerance. Persistent IL-6 dysregulation is linked to conditions like rheumatoid arthritis, Castleman's disease, and systemic lupus erythematosus. Targeting IL-6 or its receptor has shown therapeutic potential, reducing disease severity in experimental models and offering a novel strategy for immune-mediated diseases (Tanaka et al., 2014). HTR-8/SVneo cells are known to express pattern recognition receptors like toll-like-receptors 4 (TLR-4) which recognize LPS, thus activating downstream signalling pathways that result in the release of pro-inflammatory cytokine like IL-6 through Nf-κB pathway (Lu et al., 2024).

Unlike its effect on NO release in the cells, LPS caused significant upregulation of pro-inflammatory cytokine IL-6 in the cells (*P* value 0.02). While the baseline level of IL-6 in the C (-) cells was 1151.69 ± 81.46 pg/ml, the same in C (+) cells i.e., LPS activated cells without extract/drug treatment, was significantly higher at 1832.71 ± 45.01 pg/ml. In the present study, both *Z. oxyphyllum* extracts were effective in significantly suppressing the level of IL-6 in the LPS activated cells. *Z. oxyphyllum* leaf extract treated cells had IL-6 level of 1520.91 ± 97.38 pg/ml and 1634.77 ± 44.42 pg/ml at 15 µg/ml and 30 µg/ml concentrations respectively, both of which was significantly lower than C (+) cells (*P* value = 0.04 for both concentrations). *Z. oxyphyllum* fruit extract treated cells also exhibited significantly lower levels of IL-6 at 1060.85 ± 20.92 pg/ml and 1036.54 ±

69.98 pg/ml for 15 µg/ml and 30 µg/ml concentrations respectively. One-way ANOVA revealed that while *Z. oxyphyllum* fruit extract activity was on par with that of 30 µg/ml of diclofenac sodium, in comparison to 15 µg/ml of diclofenac sodium, the extract was significantly better at suppression of IL-6 in the cells with *P* values < 0.001 for both concentrations (Figure 4.42).

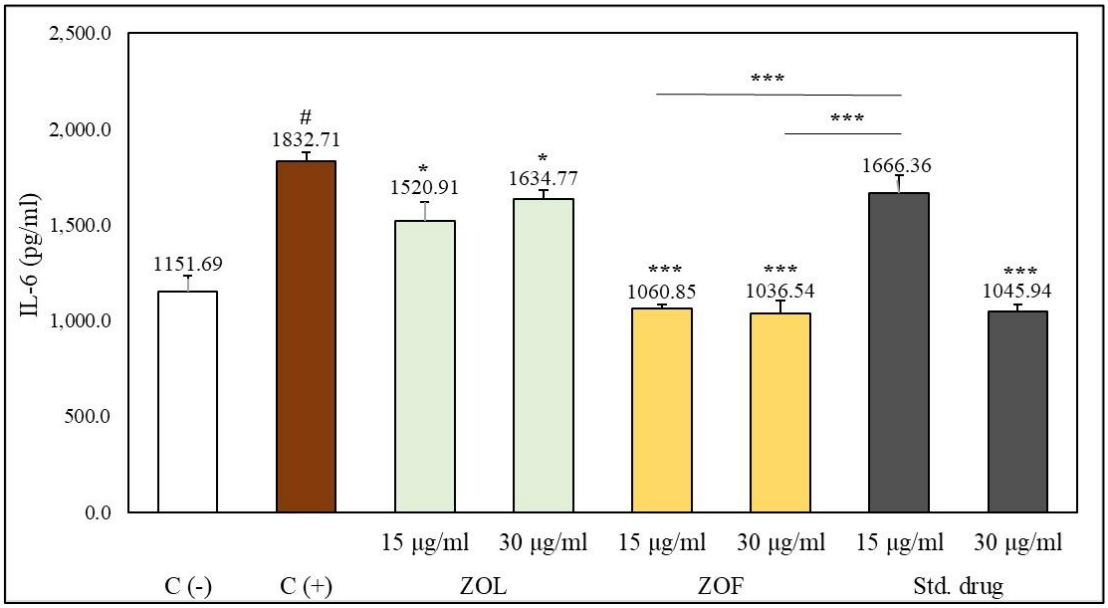

Figure 4.42. Effect of *Z. oxyphyllum* leaf and fruit extracts on IL-6 release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZOL = *Z. oxyphyllum* Edgew. leaf extract, ZOF = *Z. oxyphyllum* Edgew. fruit extract, Std. drug = diclofenac sodium; *C (-) = Control (no LPS treatment, no plant extract/drug treatment), C (+) = Control (LPS treatment only); # = p value < 0.05 representing significant difference of C(+) from C(-); * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 representing significant difference of plant extract/drug treated cells from C (+) and/or significant difference between plant extract treated cells and standard drug treated cells.*

In comparison, *Z. acanthopodium* extracts showed lower IL-6 suppression activity. The leaf extract at 15 µg/ml did not significantly reduce IL-6 level in the cells with the value being 1694.25 ± 49.49 pg/ml. However, at 30 µg/ml concentration, a significant suppression was observed with IL-6 level of 1550.26 ± 77.25 pg/ml (*P* value 0.03). The fruit extract on the other hand did not exert significant reduction with IL-6 levels being 1607.62 ± 93.73 pg/ml and 1593.54 ± 72.66 pg/ml at 15 µg/ml and 30 µg/ml concentrations respectively (Figure 4.43).

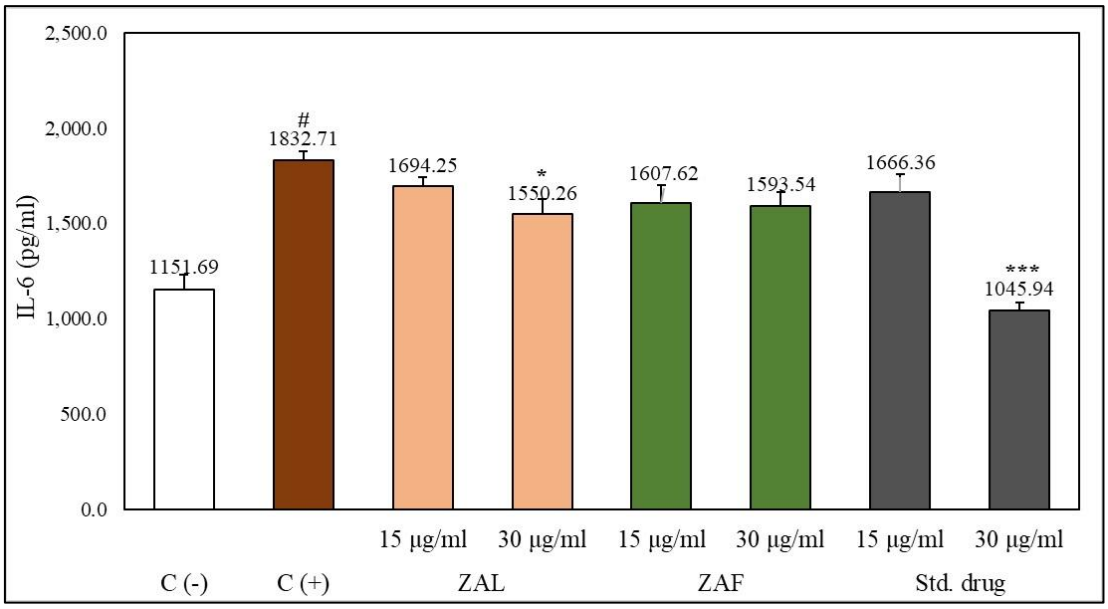

Figure 4.43. Effect of *Z. acanthopodium* leaf and fruit extracts on IL-6 release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZAL = *Z. acanthopodium* DC. leaf extracts ZAF = *Z. acanthopodium* DC. fruit extract, Std. drug = diclofenac sodium; *C (-) = Control (no LPS treatment, no plant extract/drug treatment), C (+) = Control (LPS treatment only); # = p value < 0.05 representing significant difference of C(+) from C(-); * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 representing significant difference of plant extract/drug treated cells from C (+) and/or significant difference between plant extract treated cells and standard drug treated cells.*

Previous studies reported the suppressive ability of *Z. acanthopodium* fruit extract on IL-6 and TNF-α release in RAW 264.7 cells (Yanti et al., 2011), pre-eclampsia Wistar mice (Barus et al., 2020) and *Streptococcus sanguinis* infected mice fibroblast cells (Lelyana et al., 2021). However, in the present study, the same observation was not made. As mentioned earlier, genetics and environmental factors play an important role in regulating the biological activity of plants (Batubara et al., 2020). Since *Z. acanthopodium* fruit extract activity from previous report were from Indonesia (with a predominantly tropical climatic zone), it is possible that there may be significant variation in their genetic composition and consequently phytochemical composition from the plants from Sikkim Himalayan region with a predominantly temperate climatic zone. Moreover, the models studied and dose concentration were also different than the present study, therefore a variation in the results obtained was expected.

Other species of *Zanthoxylum* have also been reported to inhibit IL-6 in different cell lines. Seed oil of *Z. bungeanum* inhibited the expression of IL-6 in the human lung epithelial cell line - BEAS-2B cells possibly by suppression of TLR4/MyD88/NF-*κ*B

signalling pathway (Hou et al., 2021). In a different study, *Z. coreanum* also suppressed IL-6 release in LPS induced RAW 264.7 cells via the NF-κB and MAPKs signalling pathways (Zuo et al., 2020). Similarly, *Z. rhetsa* showed IL-6 suppressive activity by preventing the phosphorylation of IκBα in the NF-κB signalling pathway in RAW 264.7 cells (Santhanam et al., 2020). Further, *Z. armatum* also significantly inhibited IL-6 in LPS induced mouse peritoneal macrophage (Noreen et al., 2019). Therefore, it is possible that in the present study the plant extracts, especially *Z. oxyhphyllum* extracts exerted IL-6 suppression activity via modulation of NF-κB signalling pathway.

5.8.5. Effect of extracts in TNF-α release from HTR-8/SVneo cells

Tumour necrosis factor-alpha (TNF-α) is a cytokine with diverse effects on various cell types and plays a central role in regulating inflammatory responses. While essential for normal immune function, excessive or dysregulated TNF-α production contributes to the development of inflammatory and autoimmune diseases. Conditions such as rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis, psoriasis, and non-infectious uveitis are linked to abnormal TNF-α secretion, highlighting its significance in disease pathogenesis. TNF-α also stimulates the production of other inflammatory molecules, making it a key target for understanding and managing these disorders (Jang et al., 2021). HTR-8/SVneo cells are also known to release TNF-α upon LPS stimulation via Nf-κB pathway (Lu et al., 2024).

In the present study, activation with LPS caused significant increase in the TNF-α level in the C (+) cells (*P* value 0.03). While the TNF-α content in C (-) cells was 283.08 ± 24.31 pg/ml, the same in C (+) cells was significantly higher at 431.47 ± 36.09 pg/ml. Suppressive effects of the extracts on TNF-α were also observed. The leaf extract of *Z. oxyphyllum* showed significant suppression of the cytokine at 15 µg/ml concentration with TNF-α level of 300.63 ± 3.65 pg/ml and *P* value of 0.02. The same at 30 µg/ml concentration did not exert significant suppression with TNF-α level being 303.43 ± 41.82 pg/ml. However, consistent with observation made for NO and IL-6 release, *Z. oxyphyllum* fruit extract showed significant suppression of TNF-α in the cells for both concentrations (*P* values 0.01 and 0.02 respectively). While TNF-α at 15 µg/ml concentration was 242.34 ± 9.99 pg/ml, the same for 30 µg/ml concentration was 261.57 ± 22.41 pg/ml (Figure 4.44). The activity of the fruit extract was also on par with that of

the standard drug with there being no significant difference in the activity between the two treatment groups.

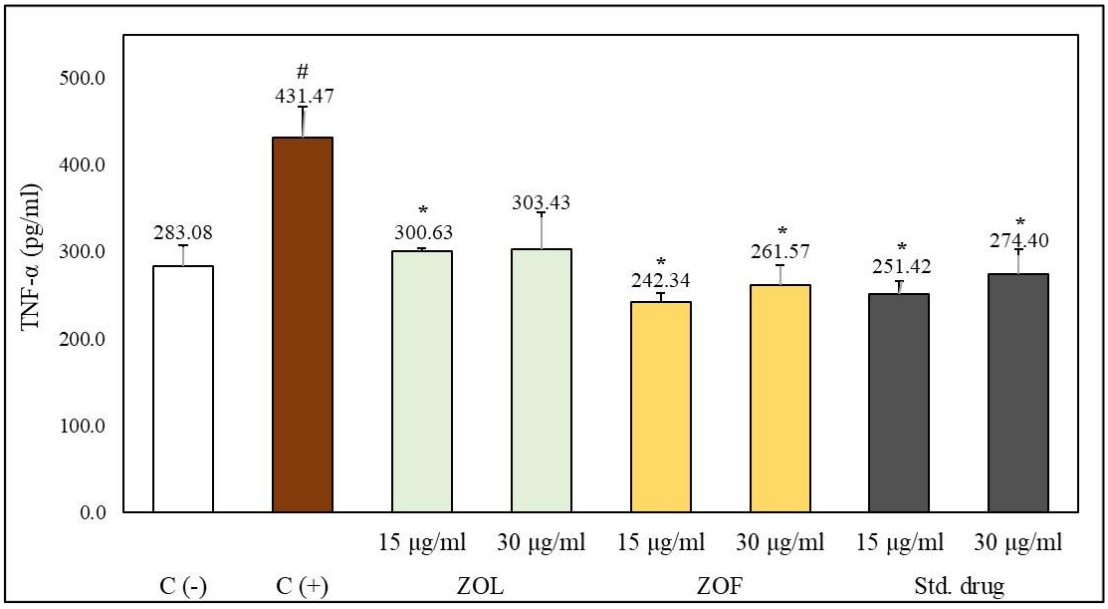

Figure 4.44. Effect of *Z. oxyphyllum* leaf and fruit extracts on TNF-α release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZOL = *Z. oxyphyllum* Edgew. leaf extract, ZOF = *Z. oxyphyllum* Edgew. fruit extract, Std. drug = diclofenac sodium; *C (-) = Control (no LPS treatment, no plant extract/drug treatment), C (+) = Control (LPS treatment only); # = p value < 0.05 representing significant difference of C(+) from C(-); * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 representing significant difference of plant extract/drug treated cells from C (+) and/or significant difference between plant extract treated cells and standard drug treated cells.*

A similar trend was also observed for *Z. acanthopodium* extracts with the leaf extract showing significant suppression at one concentration only and the fruit extract exerting significant suppression at both concentrations. At 15 µg/ml concentration of the leaf extract, no significant suppression was observed with TNF-α level being 337.93 ± 30.65 pg/ml, however at 30 µg/ml the same was significantly lower at 272.63 ± 12.84 pg/ml (*P* value = 0.01). In the cells treated with fruit extract, the TNF-α level was significantly suppressed with the values being 275.61 ± 16.24 pg/ml and 229.84 ± 23.96 pg/ml at 15 µg/ml and 30 µg/ml concentrations respectively (Figure 4.45). *P* value for the same was 0.02 and 0.01 respectively. Like *Z. oxyphyllum* fruit extract, the activity of Z. acanthopodium fruit extract was also on par with that of the standard drug.

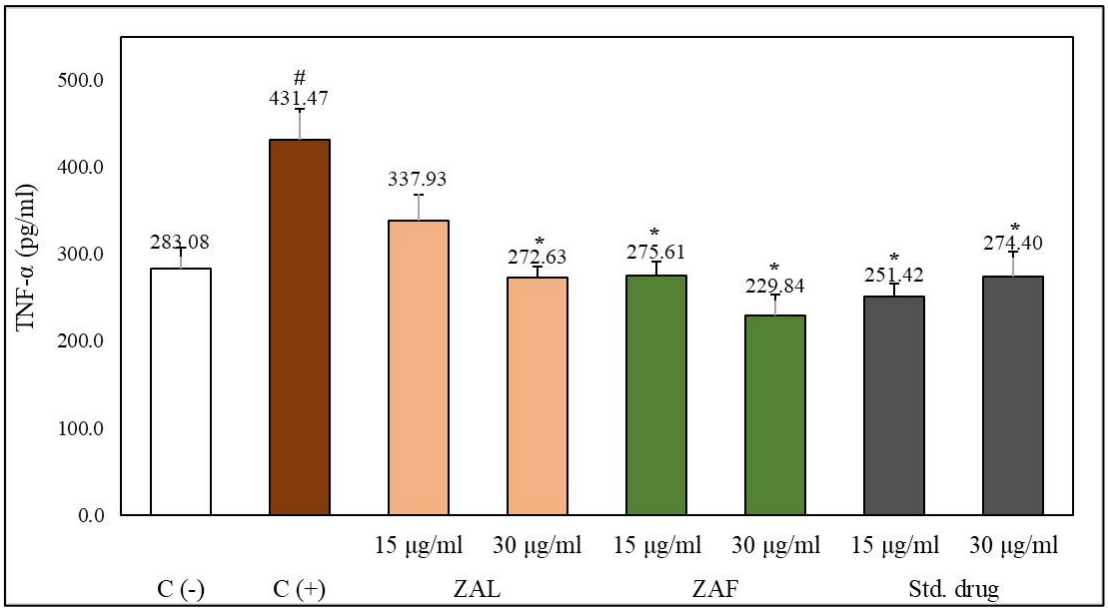

Figure 4.45. Effect of *Z. acanthopodium* leaf and fruit extracts on TNF-α release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZAL = *Z. acanthopodium* DC. leaf extracts ZAF = *Z. acanthopodium* DC. fruit extract, Std. drug = diclofenac sodium; *C (-) = Control (no LPS treatment, no plant extract/drug treatment), C (+) = Control (LPS treatment only); # = p value < 0.05 representing significant difference of C(+) from C(-); * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001 representing significant difference of plant extract/drug treated cells from C (+) and/or significant difference between plant extract treated cells and standard drug treated cells.*

It was reported that compounds 2α-methyl-2β-ethylene-3β-isopropyl-cyclohexan-1β, 3α-diol and phenol-O-β-D-arabinopyranosyl-4′-(3″, 7″, 11″, 15″-tetramethyl)-hexadecan-1″-oate isolated from *Z. armatum* methanolic fruit extract showed significant suppression of TNF-α in LPS induced mouse peritoneal macrophage (Noreen et al., 2019). It is possible that certain phytocompounds present in the extracts in the present study may also be similarly responsible for their suppressive effect on TNF-α. Further the seed oil of *Z. bungeanum* inhibited the expression TNF-α in the human lung epithelial cell line - BEAS-2B cells in a dose dependent manner possibly via TLR4/MyD88/NF-*κ*B signalling pathway (Hou et al., 2021). *Z. coreanum* essential oil on the other hand was shown to suppress TNF-α in LPS induced human leukemia monocytic cell line - THP-1 cell line by preventing phosphorylation of IKK and IκB (Zuo et al., 2020). Therefore, it can be assumed that in the present study, the extracts showed suppression of TNF-α in the cells possibly via modulation of NF-*κ*B signalling pathway or phosphorylation of IKK and IκB.

5.8.6. Effect of extracts in IL-4 release from HTR-8/SVneo cells

IL-4 is a pleotropic cytokine that has immunoregulatory functions and inhibitory effect on the LPS induced pro-inflammatory cytokine release. A proper balance of pro-inflammatory and anti- inflammatory cytokines is crucial for the reduction of inflammation in the cells (Opal and DePalo, 2000; Aggarwal et al., 2019). Although there are no previous reports on LPS causing IL-4 release from HTR-8/SVneo cells, it was reported that LPS from *E. coli* (serotype 0111:B4) at 10 ng/ml, 100 ng/ml and 100 ng/ml caused significant release of IL-4 in the isolated human trophoblast primary cells (Torricelli et al., 2009). Since HTR-8/SVneo cells are an immortalized human trophoblast cell line derived from first-trimester placental tissue (Abou-Kheir et al., 2017), it was assumed that LPS stimulation would result in the release of the anti-inflammatory cytokine in the cells.

A significant release of IL-4 post LPS induction was observed in the HTR-8/SVneo cells in our investigation (*P* value <0.001). While in the C (-) cells, the IL-4 level was 3.21 ± 0.35 pg/ml, the same in C (+) cells was 13.39 ± 0.73 pg/ml. As observed for previous assays, the *Z. oxyphyllum* extracts, especially fruit extract showed significant suppression of IL-4 in the cells. At 15 µg/ml concentration, the leaf extract showed significant suppression with IL-4 level at 9.56 ± 0.79 pg/ml (*P* value 0.02), while the same at 30 µg/ml was 11.24 ± 1.38 pg/ml which was not significantly different from C (+). The fruit extract on the other hand showed significant suppression with IL-4 levels 10.54 ± 0.46 pg/ml and 10.24 ± 0.39 at 15 µg/ml and 30 µg/ml respectively (*P* values 0.03 and 0.02 respectively) (Figure 4.46). The activity of the fruit extract was on par with that of the standard drug. The *Z. acanthopodium* extracts did not cause significant suppression of IL-4 in the cells. In the leaf extract treated cells IL-4 level was 13.18 ± 0.52 pg/ml and 12.523 ± 0.13 pg/ml at 15 µg/ml and 30 µg/ml respectively. The same in fruit extract treated cells were 13.77 ± 0.27 pg/ml and 11.52 ± 0.51 pg/ml respectively (Figure 4.47).

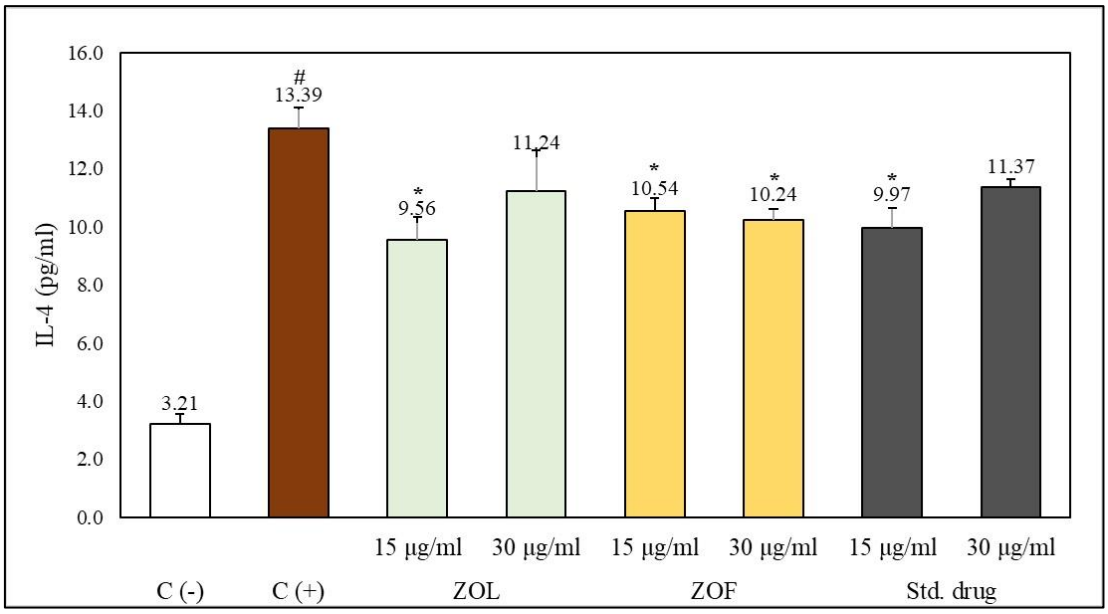

Figure 4.46. Effect of *Z. oxyphyllum* leaf and fruit extracts on IL-4 release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZOL = *Z. oxyphyllum* Edgew. leaf extract, ZOF = *Z. oxyphyllum* Edgew. fruit extract, Std. drug = diclofenac sodium

For all the plant extract treated cells, the level of IL-4 was significantly more than the basal level i.e. C (-) despite suppression exerted by *Z. oxyphyllum* extracts. Further, none of the extracts exhibited a cumulative effect on the anti-inflammatory cytokine. *Z. acanthopodium* extracts exerted a selective suppression by suppressing only pro-inflammatory cytokines IL-6 and TNF-α while showing no modulation of the anti-inflammatory cytokine IL-4. Although *Z. oxyphyllum* extracts showed significant suppression of IL-4, the suppression was not comparable to that at C (-) level.

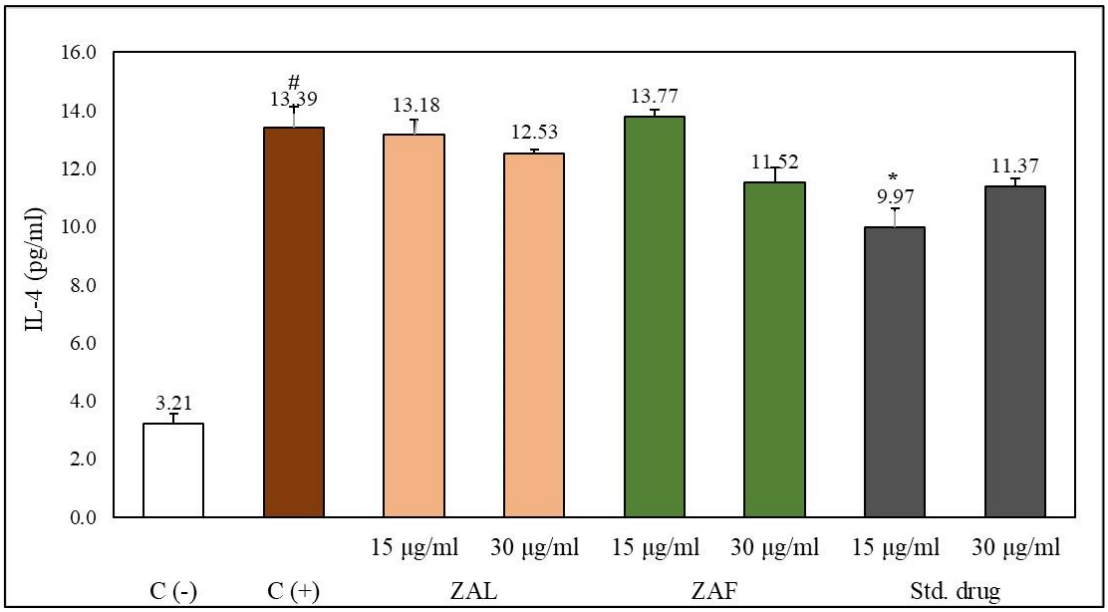

Figure 4.47. Effect of *Z. acanthopodium* leaf and fruit extracts on IL-4 release in HTR-8/SVneo cells (Data presented as ± SEM, n = 3).

ZAL = *Z. acanthopodium* DC. leaf extracts ZAF = *Z. acanthopodium* DC. fruit extract, Std. drug = diclofenac sodium

It was reported that *Zanthoxylum* species or its isolated bioactive compounds can have a suppressive effect on IL-4 in rat RBL-2H3 cells and BALB/c mice as a potential anti-allergic agent (Lee and Lim, 2010; Wang et al., 2016; Guo et al., 2018;). Similar reports on suppression of IL-4 are have been reported from other medicinal plants, but the research have all focused on allergic models and the potential anti-allergic effects of the plants (Gandhi et al., 2020). Therefore, effect of plant extracts on IL-4 release may be subject to the model being studied.

The results obtained in the cell culture studies was consistent with *in vitro* anti-inflammatory studies where *Z. oxyphyllum* extracts exerted better anti-inflammatory activity than *Z. acanthopodium* extracts. In both the studies, activity of *Z. oxyphyllum* extracts was also significantly better than diclofenac sodium or on par with it. It is also clear that all extracts exerted immunomodulatory activity in HTR-8/SVneo cells via suppression of pro-inflammatory cytokines. In the *in vitro* total anti-oxidant assay, it was observed that *Z. oxyphyllum* fruit extract had the highest range of total anti-oxidant capacity which is also consistent with its NO scavenging activity in HTR-8/SVneo cells.

5.7. *In vitro* inhibition of inflammatory enzymes

Cyclooxygenase (COX) and lipoxygenase (LOX) are inflammatory enzymes involved in the production of eicosanoids from arachidonic acid. Eicosanoids are a class of signalling molecules that are involved in immunological responses like initiating and resolving inflammation. These molecules include prostaglandins (PG), thromboxanes (TX), leukotrienes (LT), and lipoxins (LX). While PG and TX production depends on COX enzymes, LOX enzymes are responsible for production of producing pro-inflammatory leukotrienes and anti-inflammatory lipoxins. Eicosanoids have been associated with the pathogenesis of various inflammatory diseases (Leval et al., 2002), thus modulation of these enzymes may provide additional insights into the potential of plant extracts as immunomodulators.

Cyclooxygenases are of two types in the human body, COX-1 and COX-2. COX-1 is a constitutively expressed enzyme found in most cells, but more abundantly in stomach and platelets and COX-2 is an inducible form that is expressed in response to inflammatory stimuli. COX-1 plays a key role in thromboxane biosynthesis and activation of platelets. Earlier, the isoform COX-2 was reported to be involved in inflammation related complications, however, recent advancements have revealed that COX-1 is also involved in the pathogenesis of many diseases including atherosclerosis (Perrone et al., 2010). Lipoxygenases are distinctly classified into 5-, 8-, 12-, and 15-lipoxygenases (Wisastra and Dekker, 2014). The 15-LOX-1 pathway plays a significant role in inflammatory respiratory disorders, including asthma, rhinitis, and COPD, by producing proinflammatory eoxins in eosinophils, mast cells, and nasal polyps in allergic individuals. Its metabolites, such as 15-HETE, contribute to Th1-allergic inflammation, particularly in cases involving allergens and viral stimuli like dsRNA. This has positioned 15-LOX-1 as a potential therapeutic target for treating virus-associated asthma and other respiratory conditions characterized by Th1-driven immune responses. Additionally, in airway epithelial cells, elevated 15-LOX-1 levels interact with phosphatidylethanolamine-binding protein-1, sustaining MAPK/ERK pathway activation and further highlighting its role in respiratory inflammation (Sadeghian and Jabbari, 2016).

In the present study it was observed that *Z. oxyphyllum* extracts exerted better COX-1 inhibition activity in comparison to *Z. acanthopodium* extracts. While *Z. oxyphyllum* leaf extract had an IC_{50} value of 0.37 µg/ml, the same for fruit extract was 0.52 µg/ml. For *Z.*

acanthopodium leaf extract, the value was 1.73 µg/ml and for the fruit extract the value was 2.43 µg/ml. The standard drug diclofenac sodium had an IC_{50} value of 0.31 µg/ml. Inhibitory potential of *Z. oxyphyllum* leaf extract was comparable to that of the standard drug however, other extracts exerted lower inhibitory potential in comparison. All the extracts also inhibited 15-LOX to various degrees. *Z. oxyphyllum* extracts exerted a comparable level of inhibition with IC_{50} values of 13.75 µg/ml for leaf extract and 14.88 µg/ml for the fruit extracts. Interestingly, *Z. acanthopodium* leaf extract exerted the most 15-LOX inhibition activity with the lowest IC_{50} values of 2.19 µg/ml while contrastingly the fruit extract exhibited the least inhibition with an IC_{50} values of 18.71 µg/ml. The same for standard diclofenac sodium was 15.74 µg/ml (Table 4.8).

Table 4.8. IC_{50} values of *Z. oxyphyllum* and *Z. acanthopodium* leaf and fruit extracts for inhibition of inflammatory enzymes

	IC_{50} values (µg/ml)		
No.	Variable	COX-1	15-LOX
1.	ZOL	0.37	13.75
2.	ZOF	0.52	14.88
3.	ZAL	1.73	2.19
4.	ZAF	2.43	18.71
5.	Diclofenac sodium	0.31	15.74

ZOL = *Z. oxyphyllum* Edgew. leaf extract, ZOF = *Z. oxyphyllum* Edgew. fruit extract, ZAL = *Z. acanthopodium* DC. leaf extracts ZAF = *Z. acanthopodium* DC. fruit extract Std. drug = diclofenac sodium

The plant extracts in the present study showed inhibition of inflammatory enzymes comparable to that of other *Zanthoxylum* species. It was reported that the ethanol extract of *Z. chalybeum* showed inhibition of COX-1 with an IC_{50} value of 24.89 µg/m while no inhibition of 15-LOX was observed (Schultz et al., 2021). *Z. capense* was also shown to exhibit 15-LOX inhibition with IC_{50} value of 14.92 µg/ml (Adebayo et al., 2015). Certain phytochemicals can be responsible for the dual inhibition of COX and LOX. Chebulagic

acid, a tannin isolated from *Terminalia chebula* fruits was reported to inhibit 5-LOX, COX-1 and COX-2 with IC_{50} values of 2.1 ± 0.057 µM, 15 ± 0.288 µM, and 0.92 ± 0.011 µM respectively. A steroid compound called stigmasta-7,22-diene-3-one isolated from *Isodon rugosus* was also reported to exert dual LOX/COX inhibition with IC_{50} values of 3.36 and 4.72 µg/ml respectively. Several flavonoids like mangiferin, (2E)-3-(4-hydroxy-3-pentylphenyl)-1-(2-hydroxy-4,5-di((*E*)-prop-1-enyl)phenyl)prop-2-en-1-one, glabridin, naringenin-7-*O*-*β*-D-glucuronide, etc. also exhibited potential LOX/COX inhibition. Besides, these phytochemicals, alkaloids from *Tinospora cordifolia* and terpenoids like 16-hydroxy-cleroda-4(18), 13-dien-16,15-olide, 3*α*,16*α*-dihydroxy-cleroda-4(18),13(14)*Z*-dien-15,16-olide and 16*α*-hydroxy-cleroda-3,13(14)*Z*-dien-15,16-olide from the seeds of *Polyalthia longifolia* were all reported to exert LOX/COX inhibition to various degrees. Phenolics, resins, glycosides and other types of phytochemicals have been reported to show inhibition of inflammatory enzymes (Mukhopadhyay et al., 2023). In the present study, the plant extracts contained several bioactive compounds. Inhibitory activity exerted by the plant extracts can be attributed to these phytochemicals.

Chapter 5

Summary and Conclusions

In the present study, it was clear that both the species investigated possessed much pharmacological potential. The extraction method of sonication in hydro-methanol proved to be a good extraction method since the extraction yield percentage for leaf and fruit of both the species was found to be in good amounts. The phytochemical analysis revealed that both *Z. oxyphyllum* and *Z. acanthopodium* extracts contained many phytochemical groups in considerable amounts. Phytochemical groups with proven immunomodulatory activity like phenolics, flavonoids, tannins, polysaccharides, carotenoids, anthocyanins, photosynthetic pigments and saponins were available in considerable amounts in the all the extracts studied. A survey of relevant literature showed that the phytochemical groups detected in the extracts was comparable to that of many other more widely known medicinal plants. *Z. oxyphyllum* and *Z. acanthopodium* investigated in the present study are wild, lesser-known species of *Zanthoxylum* that was the focus of the present study since, despite their use as traditional medicine, their pharmacological potential remained largely underexplored. The distribution of these two species in Sikkim Himalayan region is such that they can be easily accessed by local population residing in rural areas. The present study only provides a preliminary information on the immunomodulatory potential of the two species studied. However, by laying the foundation, the present research aims to popularize *Z. oxyphyllum* and *Z. acanthopodium* too as potential immunomodulatory agents that should not be overlooked.

Through GC-MS analysis it was revealed that the two species contain many phytocompounds that were unreported from other *Zanthoxylum* species. Some of the phytocompounds detected were reported to show many biological activities. Interestingly, the most abundant compound in all four extracts was a common compound named hexadecanoic acid, methyl ester which is a fatty acid methyl ester compound with reported biological properties. However, a principal component analysis revealed that the phytochemical profile of each extract was distinct. Considerable amounts of minerals were also detected in both the plant species in varying amounts.

The *in vitro* anti-oxidant potential of the two species was moderate. It was clear that *Z. acanthopodium* extracts showed better DPPH radical scavenging and ferric ion reducing activity. However, for phosphomolybdate reducing assay, *Z. oxyphyllum* extracts showed comparatively better activity. It was evident that each species showed anti-oxidant affinity for different types of reactive oxygen species.

The preliminary *in vitro* anti-inflammatory analysis revealed that both the species had HRBC membrane stabilization ability and prevented heat induced protein denaturation. In fact, *Z. oxyphyllum* extracts showed better *in vitro* anti-inflammatory potential with its activity being on par with that of diclofenac sodium. While *Z. acanthopodium* extracts exerted a dose dependent activity, *Z. oxyphyllum* extracts did not show this correlation. It is possible that certain phytocompounds in *Z. oxyphyllum* may exhibit hormesis at higher concentrations and this universal phenomenon had been recorded for many other medicinal plants including *Curcuma longa*.

Upon stimulation with lipopolysaccharide of *E. coli*, inflammation was induced in HTR-8/SVneo cells which released inflammatory cytokines. Therefore, HTR-8/SVneo cells proved to be a good model to investigate immunomodulatory potential of the medicinal plant extracts.

The LPS induction did not cause a marked increase in the release of nitric oxide in the cells. While *Z. oxyphyllum* leaf extract and *Z. acanthopodium* leaf and fruit extracts did not cause any significant change in the NO content in the cells, *Z. oxyphyllum* fruit extract and diclofenac sodium treatment caused its significant suppression. It is possible that certain compounds distinctly present in *Z. oxyphyllum* fruit extract can directly scavenge NO with great specificity. The NO content in *Z. oxyphyllum* fruit extract treated cells was in fact significantly lower than that of diclofenac sodium treated cells indicating its better ability at modulation than even the standard drug.

Stimulation with LPS caused significant increase in the pro-inflammatory cytokine IL-6 in the cells. *Z. oxyphyllum* leaf and fruit extracts showed much potential in the suppression of IL-6 in the cells. Consistent with NO assay result, the fruit extract of *Z. oxyphyllum* showed the most potency with its activity being significantly better than that of diclofenac sodium. In fact, the fruit extract completely suppressed IL-6 release in the cells since the IL-6 level in fruit extract cells was comparable to the base-line level of non LPS activated,

non-extract/drug treated cells. *Z. acanthopodium* leaf extract exerted significant suppression of IL-6 at a higher concentration however, *Z. acanthopodium* fruit extract did not exert any suppression. In the preliminary *in vitro* anti-inflammatory study, it was observed that *Z. acanthopodium* fruit extract showed the least activity in comparison to other extracts and this observation was consistent with IL-6 release assay as well where *Z. acanthopodium* fruit extract did not cause its modulation in the cells.

A significant increase in TNF-α level was also observed in the cells. Significant suppression of TNF-α was observed for cells treated with all the four extracts. Specifically, the fruit extract of each species exerted better suppression in comparison to their leaf extract counterparts. Activity of the fruit extracts was also on par with that of standard drug. Moreover, the level of TNF-α in the fruit extracts treated cells was comparable to the base-line level, indicating that they could potentially exert complete suppression of inflammation induced TNF-α in the HTR-8/SVneo cells. Another inference was that *Z. acanthopodium* fruit extract showed great specificity regarding the pro-inflammatory cytokine suppression since it exerted no suppression for IL-6 but significantly suppressed TNF-α, while *Z. oxyphyllum* fruit extract did not show this specificity and suppressed both the pro-inflammatory cytokines.

The anti-inflammatory cytokine IL-4 was also significantly released in the cells upon LPS treatment. *Z. acanthopodium* extracts showed no suppression of IL-4 in the cells. This further indicates that *Z. acanthopodium* can potentially modulate inflammatory response specifically via suppression of pro-inflammatory cytokines, rather than via modulation of inflammatory mediator NO or cumulative effect on the release of IL-4. *Z. oxyphyllum* extracts on the other hand showed significant suppression of IL-4 in the cells. Therefore while *Z. acanthopodium* can be explored further for its selective suppression of pro-inflamamatory cytokines, indentification of individual phytocompounds responsible for pro-inflammatory cytokine suppression and anti-inflammatory cytokine suppression must be done in case of *Z. oxyphyllum*.

Further investigation on effect of the extracts on inflammatory enzymes revealed that all the extracts potentially inhibited COX-1 and 15-LOX *in vitro*. While *Z. oxyphyllum* extracts showed better COX-1 inhibitory activity, *Z. acanthopodium* leaf extract showed better 15-LOX inhibitory activity. Consistent with earlier results, *Z. acanthopodium* fruit extract exerted the least inhibitory activity for both the enzymes among all four extracts.

This result further cements the potential of both the species as immunomodulatory agents. It is evident that between the two species, *Z. oxyphyllum* extracts showed more potential as immunosuppressant whereas *Z. acanthopodium* extracts showed greater target specificity for inflammatory mediators and the varying degree of activity among the extracts may be possible due to their distinct phytochemical profile.

The research presented explored the pharmacological validation of two underexplored medicinal plants *Z. oxyphyllum* and *Z. acanthopodium* from Sikkim Himalaya. The plants of this group have been reported to have several traditional uses as medicine. The present study bridges the gap between traditional knowledge and explores pharmacological potential through the rigours of modern scientific approach. The findings highlighted the value of rich biodiversity of Sikkim Himalaya in the bioprospecting for bioactive compounds, particularly immunomodulators. The use of human trophoblast cell line (HTR-8/SVneo) as the model system to assess immunomodulatory potential of the plant was innovative and scientifically justified. This thesis contributes to the research on the search for plant based therapeutic agents using natural resources from the Himalaya.

For any given research, there is always room for improvement. Hence, for actualization of a direct impact in the society, future focus will be on identification and isolation of bioactive components, pathway studies, therapeutic targeting, and pharmacokinetic studies of *Z. oxyphyllum* and *Z. acanthopodium*. Nevertheless, the present study established that the leaf and fruit extracts of *Z. oxyphyllum* and *Z. acanthopodium* exhibited immunomodulatory potential *in vitro* via suppression of inflammatory mediators. In the future, the present research may lead to the development of *Zanthoxylum* spp. based immunomodulatory drugs that may address immune related disorders like chronic inflammation, autoimmune diseases, COVID-19 associated cytokine storms, etc.

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GLOSSARY

Allergen - a substance that can cause an allergic reaction in people with sensitive immune systems

Analgesic – a drug that is used to relieve pain

Anti-inflammatory – a substance that is used to reduce inflammation

Anti-oxidant – a substance that can inhibit oxidantion

Apoptosis – process of programmed cell death

Cytokine – a protein made by immune/non-immune cells that is involved in immune fucntion

Essential oil - a concentrated hydrophobic liquid that contains volatile compounds; present in plants

Fatty acids - a carboxylic acid with an aliphatic chain, which is either saturated or unsaturated; important component of lipids

Follicles – (of a plant) dry fruit that develops from a single carpel, or ovary, of a flower and contains multiple seeds

Glabrous – smooth and hairless (of a plant organ)

Glycoproteins – a protein with s sugar molecule attached to it

Hepato-protective – ability to protect liver from damage

Herbarium - a collection of preserved plant specimens and related data that is used for scientific study

Immunostimulant - any substance that can boost the immune system's ability to fight infection and disease

Immunosuppressant - drugs that reduce the body's immune response

Inflammation – natural, protective mechanism of the body in response to injury, infection, or other harmful substances

Inflorescence - a group of flowers on a plant stem that is made up of a main branch or a system of branches.

Lymphoid - a term that refers to lymphocytes, a type of white blood cell, or to tissues where lymphocytes develop.

Myeloid - bone marrow or to a type of blood cell that originates in bone marrow

NSAIDSs - a class of medications that can relieve pain, reduce inflammation, and lower fevers

Pathogen -a microorganism that can cause disease in a host, such as a virus, bacteria, fungi, or protozoa

Prickles - a short, pointed outgrowth on the bark or epidermis of a plant; a small thorn

Pyrexia – fever; a condition where the body temperature rises above the normal range

Scandent – having a climbing habit

Trophoblast - a thin layer of cells that surrounds an embryo and plays a vital role in implantation and placentation