GENOME WIDE IN-SILICO AND EXPRESSION ANALYSIS, QUANTIFICATION AND ANTIOXIDATIVE POTENTIAL OF YATEIN PHYTOCHEMICAL IN FLAX MICROGREENS UNDER ABIOTIC STRESS CONDITIONS

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in

Biochemistry

By

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DECLARATION

I, hereby declared that the presented work in the thesis entitled "**GENOME WIDE *IN-SILICO* AND EXPRESSION ANALYSIS, QUANTIFICATION AND ANTIOXIDATIVE POTENTIAL OF YATEIN PHYTOCHEMICAL IN FLAX MICROGREENS UNDER ABIOTIC STRESS CONDITIONS**" in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Gurmeen Rakhra, working as Assistant Professor in the School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

(Signature of Scholar)

Name of the scholar: Preedhi Kapoor

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "**GENOME WIDE *IN-SILICO* AND EXPRESSION ANALYSIS, QUANTIFICATION AND ANTIOXIDATIVE POTENTIAL OF YATEIN PHYTOCHEMICAL IN FLAX MICROGREENS UNDER ABIOTIC STRESS CONDITIONS**" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Biochemistry of School of Bioengineering and Biosciences is a research work carried out by Preedhi Kapoor, (12020525), is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

(Signature of Supervisor)

Name of supervisor: Dr. Gurmeen Rakhra

Designation: Assistant Professor

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Abstract

Flax (*Linum usitatissimum*) contains a wide range of essential nutrients, such as proteins, polyunsaturated fatty acids (PUFAs), phenolic compounds, fibers, flavonoids, and lignans. Lignans, among the several components in flax, are recognized for their antioxidant potential and plays a significant part in plant protection mechanisms. The biosynthesis of lignans in flax is primarily driven by pinoresinol-lariciresinol reductases (PLRs), with PLR2 enzymes playing a pivotal role in the reduction of lariciresinol to secoisolariciresinol. Notably, *PLR2s* are involved in the *in-vivo* biosynthesis of (-)-yatein, a key lignan, in the aerial parts of flax. These PLR2s are also implicated in enhancing stress tolerance, emphasizing their crucial function in the adaptive responses to abiotic stress conditions. Given the limited information on the genome-wide analysis of *PLR2s* in flax, we conducted an *in-silico* analysis of the *PLR2s* genes to characterize their structural properties and evolutionary patterns, thereby uncovering their potential functional roles in flax. The identification and characterization of *PLR2* genes was done using Phytozome database and 30 *PLR2* genes were identified encoding for 30 PLR2 proteins. These genes were subjected to ESTs analysis and 22 genes were selected out of 30 genes based on the similarity index. The molecular weights and isoelectric points (pI) of the identified proteins ranged from 27.69 kDa to 70.66 kDa and 5.20-9.60, respectively. Most of the PLR2s were found to have Nmr A like family domain which is responsible for nitrogen metabolite repression in fungi. The PLR2s were predicted to be primarily localized in cytoplasm (20), with a smaller number of genes identified in plastids (2). Motif analysis resulted in the identification of the 15 motifs, out of which 4 motifs were linked with NmrA like family domain. It was also observed that the number of introns in the open reading frames varied from 0-7 for majority of the genes and only one gene was lacking intron. Gene ontology (GO) enrichment analysis revealed that genes were involved in the regulation of NmrA like family, lignan biosynthesis, phenylpropanoid pathway biosynthesis etc. Cis-acting elements specific to hormones, stress responses, and developmental processes were identified within the *LuPLR2* genes, emphasizing their potential regulatory involvement in mediating key physiological and environmental responses. Phylogenetic analysis of

LuPLR2 genes across various species revealed three distinct subfamilies (I-III), with *LuPLR2s* exclusively positioned in subfamily III. Genes within the same subfamily, such as *Benincasa hispida* (*BhPLR2-like*), *Linum corymbulosum* (*LcPLR1*), *Linum flavum* (*LfPLR2*), and *Linum album* (*LaPLR1*), share functional similarities, including their involvement in lignan biosynthesis. Furthermore, the expression of these identified *LuPLR2s* were studied in flax microgreens under different abiotic stress conditions (cold, heat, NaCl induced salt stress and Polyethylene glycol induced drought stress) using qRT-PCR. It was found that expression levels of almost all genes demonstrated a considerable upregulation relative to control, with the striking exception of heat stress, where expression was predominantly downregulated. Also, quantification of yatein under abiotic stress conditions was performed using HPLC, revealing that yatein was most abundant under PEG-induced drought stress at 280 nm.

Yatein has emerged as a prominent focus of pharmacological study due to its therapeutic and anti-oxidative properties. To understand the role of yatein application in the anti-oxidative potential of flax microgreens under different, abiotic stress conditions were imposed to both untreated and yatein-treated flax microgreens. There was a significant enhancement in anti-oxidative enzyme activities (SOD, CAT, APX, GPX, GST, and GR) in yatein-treated flax microgreens comparable to untreated flax microgreens.

Therefore, this study offered valuable clues to gain knowledge about the roles of *LuPLR2s* in metabolic processes, stress responses, and evolutionary relationships through in-silico analysis and expression analysis. Moreover, this work emphasizes the critical role of yatein in driving the anti-oxidative capacity as well as enhancing flax resilience.

Acknowledgements

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Not all may be acknowledged, but no one is forgotten.

Preedhi Kapoor

Table of Contents

List of Tables

List of Figures

Abbreviations

ABA - Abscisic acid

ANOVA - Analysis of variance

AsA-GSH - Asada-halliwell

Bp - base pairs

BSA - Bovine serum albumin

CAT – Catalase

CDNB - 1-chloro-2,4-dinitrobenzene

CDS - coding sequences

CNGs - Cyanogenic glycosides

DAD - Diode array detector

DHA - Dehydroascorbic acid

DIRs- Dirigent proteins

DMSO - Dimethyl sulfoxide

DNPGS - 2,4-dinitrophenyl) glutathione-S

EDTA - Ethylene diamine tetra acetic acid

ESTs - Expressed sequence tags

GO - Gene ontology

GSH - Reduced glutathione

GSSG - Oxidized glutathione

GST - Glutathione-S-Transferase

H_2O_2 - Hydrogen peroxide

HMGA- 3-hydroxy-3-methylglutaric acid

HMM -Hidden Markov Model

HPLC -High-Performance Liquid Chromatography

IUPs/IDPs - Intrinsically unstructured (or disordered) proteins

JA - Jasmonic acid

LDG - lariciresinol diglucoside

MDHA – Monodehydroascorbate

MeJA - Methyl jasmonate

NADPH - Nicotinamide Adenine Dinucleotide Phosphate

NBT - Nitroblue tetrazolium

PEG - Polyethylene Glycol-6000

PINO- Pinoresinol

PLRs - Pinoresinol-lariciresinol reductases

PSMs - Plant secondary metabolites

PUFAs - Polyunsaturated fatty acids

qRT-PCR- Quantitative real time polymerase chain reaction

ROS - Reactive oxygen species

SA - Salicylic acid

SD - Standard deviation

SDG - Secoisolariciresinol diglucoside

SECO- Secoisolariciresinol

SOD - Superoxide Dismutase

CHAPTER 1: INTRODUCTION

Science is not only compatible with spirituality; it is a profound source of spirituality.

–**Marie Curie**

Flax (*Linum usitatissimum* L.), belonging to family Linaceae, is an indigenous and essential crop in India, and is also being used as a nutritional supplement in several parts of the world. Flax is cultivated in over 50 countries worldwide, with Canada, India, Russia, Kazakhstan, and China being the leading producers (Food, 2023; Stavropoulos et al., 2023). It is an annual herb with blue flowers and is typified by a flat, ovoid, and sharp seed comprising of an embryo with two cotyledons which are embedded by the endosperm (Saleem et al., 2020). It is a versatile crop, as reflected in its scientific name "usitatissimum" which translates to the "the most useful" because of its multiple uses (Mavroeidis et al., 2022). The crop yields two primary products: seeds and fibers. Flax fibers, known for their high cellulose content, are exceptionally strong and widely used in the textile industry (Goyal et al., 2014). On the contrary, flaxseed contain abundant biologically active substances, including high-quality proteins, polyunsaturated fatty acids (PUFAs), dietary fibers, and secondary metabolites like phenolics, flavonoids, and lignans. Flaxseeds are also rich in oil, comprising approximately 40% of their composition (Kolodziejczyk et al., 2012). Its oil is characterized by its high linolenic acid content, low levels of saturated fatty acids, and abundance of omega-3 and omega-6 fatty acids (Topnikova et al., 2022). These compounds contribute to the nutritional profile of flaxseed as a functional food with potential health benefits, including anti-oxidative and anti-inflammatory properties.

Despite the presence of variety of biologically active substances, flaxseed shows abundance of anti-nutritional components like cyanogenic glycosides (CNGs) and phytates. Therefore, the germination process has been utilized in flaxseed with the goal of obtaining microgreens that contain lower quantities of anti-nutrients (Bouajila et al., 2020; Kajla et al., 2017; Lemmens et al., 2019; Puccinelli et al., 2022; Sanmartin et al., 2020; Tavarini et al., 2019, 2021). These microgreens are young plants that fall between the stages of sprouts and baby greens and can be readily cultivated in urban and peri-urban areas, where space is often limited. They have a brief growth cycle and can be grown in the presence or absence of soil without needing additional inputs such as fertilizers or pesticides and are typically harvested within 7-14 days of germination (Bhaswant et al.,

2023). These edible young seedlings are an excellent source of vitamin C, an antioxidant that help guard the body against the damaging effects of free radicals (Bhaswant et al., 2023). In addition, certain microgreens have been shown to contain even more beta-carotene than carrots, which aid in reducing the risk of diseases, especially specific cancers and eye conditions (Singh et al., 2024). Microgreens are rich in various phytochemicals, including polyphenols such as phenolic acids, stilbenes, and lignans; terpenoids like carotenoids and tocopherols; and nitrogen-containing metabolites such as glucosinolates (Dereje et al., 2023). Flavonoids provide antimicrobial and insecticidal benefits, protect against UV radiation, and scavenge H_2O_2 (Hasnat et al., 2024). Phenolic acids exhibit allelopathic effects, act as astringents, and serve as signaling molecules (e.g., salicylic acid) (Mandal et al., 2017). Stilbenes help plants respond to pathogens and stress, lignans offer defense against diseases and pests, carotenoids protect against photooxidation, and tocopherols help preserve the integrity of long-chain PUFAs within cell membranes, while glucosinolates work to inhibit pathogen growth (Al-Khayri et al., 2023; Ražná et al., 2021; Sotelo et al., 2015; Traber & Atkinson, 2007; Viljanen et al., 2002).

Amongst all the components found in flax microgreens, lignans are well-known for their antioxidant properties and also known to have a crucial function in protecting plants (Chen et al., 2024). These are complex phenylpropanoid compounds, often found in dimeric or oligomeric forms (Hano et al., 2021). Their biosynthesis begins with the coupling and orientation of two coniferyl alcohol molecules by oxidases (e.g., laccase or peroxidase) in the presence of dirigent proteins (DIRs) leading to the formation of various pinoresinol enantiomers through the phenylpropanoid pathway (Barker, 2019; Kim & Sattely, 2022). With the help of the enzyme, PLR (pinoresinol-lariciresinol reductase), PINO (pinoresinol) gets transformed into lariciresinol and then it is converted into secoisolariciresinol (SECO), which is an essential precursor in the process of lignan metabolism (Markulin et al., 2019). This reduction by PLR is crucial, as it open pathways to various lignans, including furano, dibenzylbutane, dibenzylbutyrolactone, and aryltetrahydronaphthalene (Markulin et al., 2019). In flax, five *PLR* genes had been encoded, though only two had been studied extensively. *LuPLR1* catalyzes (+)-SECO

biosynthesis, leading to (+)-secoisolariciresinol diglucoside (SDG) in seeds, whereas *LuPLR2* directs the production of (-)-yatein in flax leaves (Corbin et al., 2017; C. Hano et al., 2006; Hemmati et al., 2010) (**Figure 1.1**). The PLR derived pathway, particularly leading to synthesis of dibenzylbutyrolactone lignans like yatein, is a focus of research due to the anti-oxidative and therapeutic properties of lignans. Yatein, in particular, has garnered research interest due to its multifaceted biological activities closely linked to its chemical structure. As a dimeric phenylpropanoid with notable hydroxyl and methoxy groups, yatein exhibits significant anti-oxidative potential by neutralizing free radicals, thereby reducing oxidative stress (Corbin et al., 2017). Various studies in the past have also demonstrated yatein's cytotoxic and antiviral activities, adding to its therapeutic promise. For instance, yatein extracted from plants such as *Austrocedrus chilensis* has shown potent anti-proliferative effects on murine myeloma cells, inducing cell death and disrupting cellular integrity (Donoso-Fierro et al., 2015). It was also known to exhibit antiviral activity against HSV-1 replication in HeLa cells in *Chamaecyparis obtuse,* where it suppressed viral gene expression and DNA replication (Kuo et al., 2006). A study by Ho et al., (2019), demonstrated the efficient properties of yatein isolated from *Calocedrus formosana* leaves' extract where it suppressed the proliferation of human lung adenocarcinoma A549 and CL1-5 cells by activating both apoptotic mechanisms (intrinsic and extrinsic). Notably, yatein also serves as a precursor of podophyllotoxin, a compound with antitumor properties used in clinical applications. This connection positions yatein as a significant focus of pharmacological research, with applications spanning multiple therapeutic properties including mitotoxic, neurotoxic, insecticidal, antimicrobial, anti-inflammatory, antispasmogenic, hypolipidemic, immune-suppressive, antioxidant, analgesic, and cathartic effects (Shah et al. 2021). The presence of yatein in flax microgreens highlights the nutritional and functional potential of these young plants, particularly under controlled growth conditions designed to enhance their antioxidant response to stress.

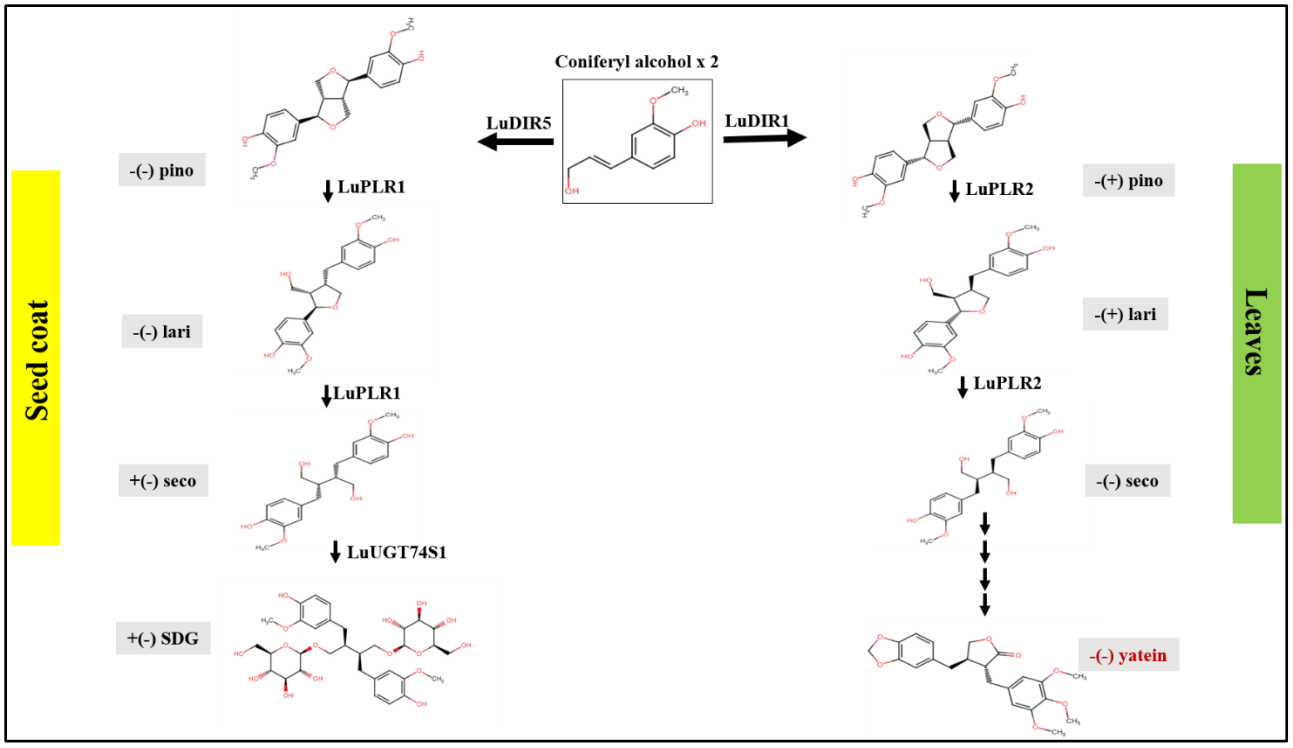

Figure 1.1: Proposed biosynthetic pathway of Yatein in Flax adopted from Corbin et al., (2017). Pino, pinoresinol; seco, secoisolariciresinol; lari, lariciresinol; SDG, secoisolariciresinol diglucoside; UGT, UDP-glucosyl transferase; PLR1, pinoresinol lariciresinol reductases 1; PLR2, pinoresinol lariciresinol reductases 2, DIR, dirigent protein oxidase complex.

PLRs exhibit differential expression across flax tissues and respond dynamically to environmental stress (Hemmati et al., 2010; Renouard et al., 2014). It was reported that mechanical wounding for 8 and 24 h in the leaves of flax caused enhanced gene expression of *PLR2*, thereby increasing the production of yatein (a lignan). Also, wounding stress applied to *Podophyllum hexandrum* resulted in an increased accumulation of transcripts of *PhPLR* at 3 h and 6 h but diffused later at 12 h of wounding (Wankhede et al., 2013). Furthermore, *PLRs* might help in the repair mechanism adopted by the plants during environmental cues rather than the protection mechanism (Xiao et al., 2015). In Indigowood (*Isatis indigotica*), the expression of *PLR1* (*IiPLR1*) was significantly upregulated following exposure to ultraviolet-B (UV-B) light, with peak expression observed 30 minutes after the UV-B light was turned off (Xiao et al., 2015). Yousefzadi et

al., (2012) also reported an elevated expression of *PLR* genes in *Linum album* under the exposure of blue light thereby resulting in an increased lignan production. Besides, lignan enhancement in *Linum album* cell cultures in the presence of *Fusarium graminearm* had been depicted (Tahsili et al., 2014). The insights into the above mentioned studies suggest that the abiotic stress induced biosynthesis of lignans results from the upregulation of *PLR* gene transcription.

Despite these advancements, research on the genetic and molecular mechanisms of yatein synthesizing genes (*LuPLR2s*) remains unexplored under abiotic stress in flax. Unraveling the functions of *PLR2s* in stress regulation remains a pivotal objective in understanding stress acclimation mechanisms. To this end, we proposed a comprehensive study to identify and characterize *PLR2* genes in flax microgreens for the first time using advanced bioinformatics approaches, aiming to uncover their functional significance. In this work, we systematically analyzed *PLR2s*, delving into their physicochemical properties, conserved domains and motifs, evolutionary relationships, gene structure, subcellular localization, intrinsic disorder tendencies, and secondary structural features using *in silico* tools. To further validate these computational findings, we conducted expression analysis of *PLR2s* in flax microgreens through real-time PCR under varying abiotic stress conditions. In addition, to further elucidate the concentration of yatein under different abiotic stress conditions, its quantification was performed using the highly sensitive and accurate High-Performance Liquid Chromatography (HPLC) technique. This approach enabled precise measurement of yatein levels, offering critical insights into its dynamic regulation and potential contribution to stress tolerance in flax microgreens. Furthermore, this study also explored the antioxidative role of yatein in flax microgreens to scavenge effectively the reactive oxygen species (ROS) across abiotic stress conditions.

The overarching goal of this research is to investigate the yatein's role and the genes (*PLR2s*) which are synthesizing yatein in enhancing the stress tolerance of flax microgreens under abiotic stress conditions. By examining their impact on antioxidative

defense mechanisms, this study seeks to uncover key molecular insights into stress resilience. The findings will provide a foundation for developing climate-resilient flax varieties with enhanced anti-oxidative properties, superior nutritional value, broader agricultural, and health applications.

CHAPTER 2: REVIEW OF LITERATURE

The only true wisdom in knowing is you know nothing.

–Socrates

2.1 Introduction

Flax, an annual herbaceous plant known for its extensive cultivation across many regions worldwide. Its Latin name, meaning “highly useful,” reflects its historical significance as a source of fiber and oil. Flaxseeds are rich in fats, with PUFAs accounting for approximately 70% of the total fat content (Sangiorgio et al., 2023). The chemical composition of flaxseeds is primarily influenced by factors including the variety, yielding period, geographic region, and post- processing techniques (Garros et al., 2018; Kajla et al., 2015). The cotyledons contain most of the lipids and proteins, while the hulls are the primary reservoir of carbohydrates. Additionally, flaxseeds are abundant in phenolic acids and micronutrients, including minerals like potassium, as well as tocopherol and niacin. Flaxseeds are highly valued in the food industry for their advantageous properties. When incorporated as components, they contribute distinct sensory qualities to food (Cichońska et al., 2021; Mueed et al., 2022). Adding flaxseeds to food items has been shown to enhance their nutritional profile, prolong shelf life, and improve sensory appeal for customers. However, the flaxseed content should be limited to no more than 20% to avoid adverse effects on texture, gluten performance, and overall consumer acceptance, particularly among those who may find an overly nutty flavor unappealing (Kaur et al., 2018). Flaxseed oil, is known for its exceptional functional properties, including its ability to inhibit oxidation and delay rancidity in products. However, its use must be carefully managed due to high susceptibility to oxidative degradation (Shadyro et al., 2020). Its gum, or mucilage valued for its probiotic properties and exceptional ability to maintain moisture and retain water. These qualities make it a valuable ingredient for certain beverages and for stabilizing the products based on porks (Kaur et al., 2018). It is preferred over other food gums as smaller amounts are needed to achieve similar textural properties (Dzuvor et al., 2018).

Flaxseeds are among the richest plant sources of lignans, a group of compounds found in most higher plants and classified as phytoestrogens. These (Lignans) perform a critical function in protecting plants and seeds from diseases, infections, and herbivores (Ionescu et al., 2021; Ražná et al., 2021) (**Figure 2.2**). Chemically, although their structural

models may vary, lignans are composed of two phenyl propane units (C6-C3) linked by a β-β' bond at C8. This configuration classifies them as diphenolic substances or phenylpropanoid dimers (Chhillar et al., 2021; Sainvitu et al., 2012). These phenylpropane units, referred to as "monolignols," consist of p-coumaryl, coniferyl, and synapyl alcohols, which vary in their degree of methoxylation on the aromatic rings. Predominantly found in the cell walls of plant tissues is Coniferyl alcohol (Toure & Xueming, 2010). Based on the bonding patterns of distinct phenyl propane units, different types of lignans can be produced. These categorized into 8 classes depend on their cyclic structure, carbon framework, as well as oxygen placement (Chhillar et al., 2021). The lignan content in flaxseeds in estimated to be 75-800 times greater than that found in grains, legumes, fruits, and vegetables (Kajla et al., 2015). Lignans have diverse applications due to their valuable nutritional properties, such as antioxidant, anti-inflammatory, and anticancer effects (Di et al., 2017).

Lignans are phenolic compounds with notable antioxidant properties, which are closely linked to their beneficial effects on human health (Rahman et al., 2022). SECO, and other types of lignans present in flaxseed acts as a precursor to mammalian estrogens. These are converted by anaerobic intestinal microflora into enterolignans, specifically enterodiol and enterolactone (Senizza et al., 2020). Lignans are now recognized for a wide array of health benefits including anticholestrol, antiviral, anticancer, antioxidant, athletic performance enhancement, antidiabetic, estrogenic and antiestrogenic, anti-inflammatory, anti-depressant, anti-bacterial, and antifungal properties (Almario et al., 2013; Cui et al., 2020; Domínguez-López et al., 2020; Draganescu et al., 2021; Y. Han et al., 2020; Ma et al., 2013; Mukhija et al., 2022; Oomah, 2001; Rodríguez-García et al., 2019) (**Figure 2.1**).The phenolic nature of lignans allows them to act as "scavengers" of hydroxyl radicals, offering substantial protection against diseases linked to free radicals produced under lipid oxidation, carbohydrates and proteins in the body. Free radicals like these have the ability to cause damage to membrane lipids, , nucleic acids, proteins, and tissues and potentially leading to conditions like cancer, respiratory diseases, neurological disorders, premature aging, and diabetes (Bhaswant et al., 2023). Lignans may aid in managing hormone-related

cancers because of their structural resemblance to estrogens that are found in mammals (Toure & Xueming, 2010). By reducing their accumulation in the blood, they can bind to estrogen receptors, reduce estrogen activity, thereby lowering cancer development (Gao et al., 2024). Moreover, their mechanism of action seems to be more intricate, possibly affecting protein synthesis & enzymes which are intracellular. In liver, they stimulate the formation of sex hormone that binds globulin and lowering free hormone levels in the plasma. Lignans may also share interaction with sex proteins that binds steroids and halt certain enzymes that are steroid-metabolizing, thereby serving a preventive function against cancers (breast & colon) (Sainvitu et al., 2012). It has been suggested that SDG may protect against breast cancer by controlling the zinc transporters' expression, as compared to normal cells, the levels of zinc are elevated in the affected cells. Additionally, enterolactone, a metabolite of SDG, has been shown to inhibit cancer cell proliferation, migration, and metastasis (Zhang et al., 2008). While significant progress in comprehending the mechanisms associated with lignans has been made, further research is necessary to resolve remaining questions and deepen knowledge about their role in health and disease prevention.

In plants, lignan formation begins with coniferyl alcohol, where 2 molecules of it joined to produce pinoresinol in the presence of DIR (Bekhit et al., 2018; Kajla et al., 2015; Kezimana et al., 2018). This compound subsequently leads to produce lariciresinol, which is further transformed into SECO. SECO undergoes dehydrogenation to yield matairesinol. SECO primarily exists in its glycosylated form, SDG, constituting major proportion of the lignans found in seeds of flax (Chhillar et al., 2021). Unlike other lignans found in their free form, SDG in flaxseed is encapsulated within a structure called the lignan complex (Kezimana et al., 2018). In this biopolymer, SDG are linked via 3-hydroxy-3-methylglutaric acid (HMGA), with a molecular mass of approximately 4000 Da. Of this mass, about 35% is composed of SDG, which consists of 5 SDG units and 4 HMGA units (43). **Table 2.1** displays the quantities of key lignans found in seeds of flax (Yeung, 2023).

Table 2.1 Major amount of lignans present in the seeds of flax

Lignans	**Amount (mg) /100g (flaxseed)**
Secoisolariciresinol (SECO)	257.60
Lariciresinol	11.46
Pinoresinol	8.64
Matairesinol	6.68

The differences in SDG and SECO levels found in flaxseed and other matrices can be attributed to factors like cultivar, geographic origin, harvest year, and the treatment the matrix undergoes prior to analysis (De Silva & Alcorn, 2019). The extraction process is crucial in lignan production, as alkaline and acidic treatments under harsh conditions can either damage the lignans or result in the formation of different types (Smeds et al., 2007). Therefore, an effective lignan revovery procedure is crucial for supplement or nutraceutical manufacturers to maximize lignan yields while minimizing the extraction and concentration of antinutrients or harmful substances (Waszkowiak et al., 2015). Flaxseeds contain beneficial compounds but also antinutritional factors like linatine, cyanogenic glycosides, and phytic acid (Kajla et al., 2015). However, recent research has shown that the germination process can significantly alter the biochemical composition of flaxseeds, reducing antinutrients while enhancing the bioavailability of essential nutrients and bioactive compounds, including lignans. This has sparked increasing interest in flax microgreens- young seedlings harvested early in development as a functional food. Flax microgreens are particularly promising because they may retain or even boost the nutritional and Antioxidative properties of mature seeds, making them an appealing addition to the diet and a growing area of scientific study.

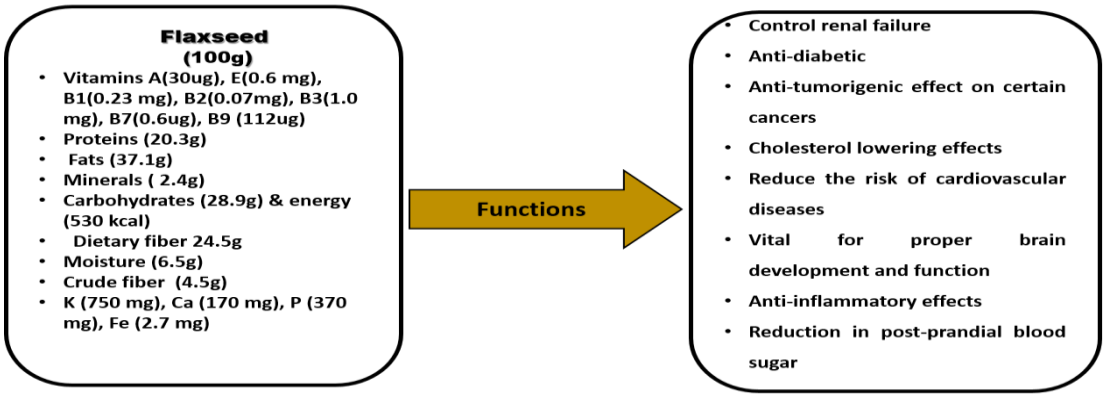

Figure 2.1 Composition of flaxseeds and its potential health benefits. K: Potassium, Ca: Calcium, P: Phosphorus and Fe: Iron

2.2 Microgreens on the rise:

In the 1980s, Americans began regularly incorporating immature vegetables into their meals. A chef's garden in the *United States* became the most notable source for these vegetables, cultivating and shipping them while still young. Since then, trendy restaurants had increasingly included these underutilized vegetables for their nutritional value and innovative appeal (Lone et al., 2024). These immature vegetables are collectively known as microgreens. Today, microgreens are grown in greenhouses worldwide. Since the term "functional foods" was introduced in 2000, microgreens have gained widespread recognition as a source of health and longevity. They are now commonly used in the agricultural sector to produce a variety of products (Moraru et al., 2022). Although microgreens have been around for decades and come from many different plant families, a universally accepted definition is still lacking. Various definitions of microgreens exist in scientific literature, but they are generally described as germinated seeds with fully developed true leaves and non-senescent cotyledons, harvested before root formation.

Microgreens are cultivated from a variety of commercial food crops, including vegetables, cereals, and herbs, and are defined by fully developed cotyledons, with or without partially expanded true leaves (Treadwell et al., 2020). The specific portion of the young stem, as well as leaves (true), is cut 7 to 21 days after germination (Galieni et al., 2020). These functional microvegetables typically range in height from 2 to 8 cm and are recognized for their unique sensory characteristics, and bright colours, though their size is small. They are also packed with a range of micronutrients, which differ depending on the plant species used for cultivation (Samuolienė et al., 2019). Due to their high levels of health-promoting phytonutrients, including antioxidants, vitamins, minerals, and phenolic compounds, microgreens are regarded as the next generation of "superfoods" or "functional foods." They are grown on both small and large scales using simple cultivation methods, alongside commercial crops and edible flowers (**Table 2.2**).

Table 2.2 Key conditions and requirements for cultivating microgreens

Parameters	**Requirements**	**References**
Seeds	▪ The quality of seeds and the seeding density per tray are crucial factors in producing high-quality microgreens. ▪ Seed treatment influences the germination rate and shoot weight.	(Lee et al., 2004)
Light	▪ An optimal photosynthetic photon flux (PPF) of 440 µmol/m²/s is required for achieving higher microgreen yields.	(Verlinden, 2020) (Zhang et al., 2020)
Growth medium	▪ Microgreens are mainly grown in soilless substrate systems that support enhanced growth. ▪ A nutrient based solution is provided, containing all the necessary components for growth.	(Kyriacou et al., 2016)
Treatment of pathogens	▪ Environmental conditions in which microgreens are cultivated can lead to various plant infections caused by	(Weber, 2017)

	microorganisms, potentially resulting in root and seedling rot. ▪ The use of calcium nitrate fertilizer along with liquid and nitrogen fertilizers boosts microgreen growth by 20%. ▪ Species such as *Trichoderma* are employed for the management of pathogens, and its use as a treatment for seeds also promotes growth of microgreens.	
Harvesting	▪ These could be harvested by cutting the plantws manually with scissors or a knife.	(Kyriacou et al., 2016)
Post-harvesting	▪ They are washed and then refrigerated to 5°C before being packaged in polythene bags to prevent contamination. ▪ Packaging in sterile conditions and following hygienic conditions help extend the microgreens' shelf life.	(Kou et al., 2013)

Due to their growing popularity, a wide range of vegetables from various plant families are now being used to produce microgreens (**Table 2.3**)(Di Bella et al., 2020; Diets, 1992; Marchioni et al., 2021; Tan et al., 2020). The study of various potential microgreen genotypes reveals a wide range of appeaeances, flavours, textures, phytochemical profiles, and nutritional values. Common species employed for microgreen production include cereals, oilseed plants, fiber plants and aromatic species (Michell et al., 2020). Microgreens are primarily recognized for their content of both macro and micronutrients. They are also abundant in biological phytochemicals, which have significant potential to improve human health and help prevent diseases. Microgreens contain higher concentrations of key biological components, such as, sugars, phylloquinones, ascorbic acid, α-tocopherol, carotenoids, β-carotene, anthocyanins, glucosinolates, and phenolic antioxidants, compared to mature plants. For instance, a comparison of red cabbage at the microgreen and mature stages revealed that the microgreen stage has higher levels of phylloquinone, β-carotene, and glucoraphanin than the mature stage (Choe et al., 2018). However, anthocyanins were more abundant in the

mature stage than in the microgreen stage. A detailed discussion is provided on the different types of bioactive phytochemicals found in various microgreens (**Table 2.3).**

Table 2.3 Different varieties of microgreens containing diverse range of nutrients and phytochemicals

Microgreens	**Growth duration**	**Nutrients**	**Properties**	**References**
Amaranth	10 days	Chlorophyll (a,b), anthocyanins, carotenoids, ascorbic acid	Antioxidant potential	(Rocchetti et al., 2020)**,** (Sarker & Oba, 2019)
Red beets	10 days	Polyphenols, betaxanthins, betacyanins	Antioxidant potential, gastrointestinal activity	(Sarker & Oba, 2019)
Spinach	20 days	Chlorophyll, lutein, β-carotene, phenols, ascorbic acid	Antioxidant activity	(Petropoulos et al., 2021)
Parsley	19 days	Polyphenols, α-tocopherols, ascorbic acid, β-carotene, lutein	Antioxidant potential	(Samuoliene et al., 2016)
Carrot	7-14 days	Polyphenols, Chlorophylls, α-tocopherols, anthocyanins, carotenoids	Antioxidant activity	(Paradiso et al., 2018)
Soybean	8 days	Phenolics, flavonoids	Antioxidant activity	(Zhang et al., 2020)
Cucumber	9 days	Phenolics, flavonoids, ascorbic acid	Antioxidant activity	(Yadav et al., 2019)
Jute	9 days	Phenolics, flavonoids, ascorbic acid	Antioxidant activity	(Yadav et al., 2019)

2.2.1 The impact of microgreens on health:

Food has played a crucial role in the development of human culture. It provides essential nutrients and energy necessary for growth, development, and survival (Chen et al., 2018). In addition to nourshing the body , food has also helped people in various societies prevent and address a range of health issues (Singh et al., 2020). The contemporary field of food science and nutrition represents human progress, with advancements stemming from the integration of knowledge in the field of food biotechnology. The developments are driven by empirical research. The focus on nutrition and diet seeks to prevent micronutrient deficiencies (such as vitamins and minerals) and assist in managing chronic conditions, including obesity (Cena & Calder, 2020). Metabolic disorders (Chronic), which impact the health of humans over extended periods, have long been a significant challenge in healthcare. While these diseases may not pose an immediate threat, they lead to long-term health complications and increase risk factors for individuals over time (Hotamisligil, 2006). Microgreens, small harvested vegetables packed with nutrients, minerals, and phytochemicals, have gained popularity for their diverse culinary applications (**Figure 2.2)** The amount of scientific research exploring the direct benefits of microgreens on health is limited, that presents a significant challenge. Thus, additional research is needed to better understand the individual and combined health effects of microgreens.

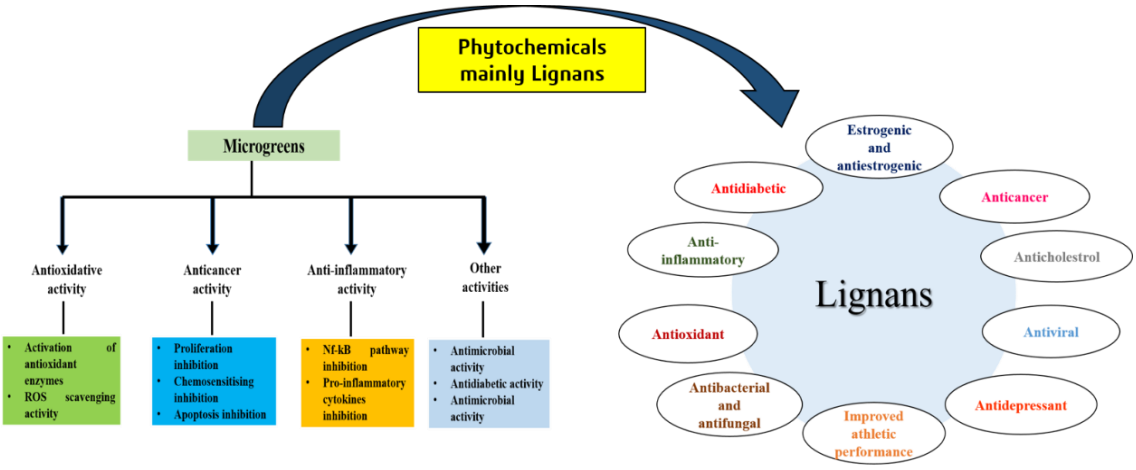

Figure 2.2: Therapeutical properties of microgreens and lignans

2.3 Expression patterns of *LuPLRs*: Main genes responsible for lignan biosynthesis

Lignans exhibit variations in stereochemistry and enantiomeric forms among lignans, which might affect not only species as well as also in different plant parts and stages of growth. There are unique trends in the expression of *PLR* genes in terms of time, space, and organs. Although, lignin composition differs for organs in Greater burdock, no *PLRs* have so far been cloned or characterized. It had been found that the seed coat of flax comprised of *LuPLR1* expression (Hano et al., 2006). Its distribution makes perfect sense due to the fact that the lignins concentrates within this tissue, which holds nearly all of the SECO that flax produces in its diglucosylated state (Hemmati et al., 2010). *AtPrR2* is restricted to the roots of Arabidopsis, whereas *AtPrR1* is mainly expressed in both the root as well as in the stem tissues (Nakatsubo et al., 2008; Zhao et al., 2015). In contrast, gibberellic acid (GA) exerts an opposing regulatory influence on *LuPLR1* expression (Corbin, Decourtil, et al., 2013). Hano et al., (2006) observed that in cell suspension of flax, the expression of *LuPLR1* increased when exposed to extract of *F. oxysporum*. However, lignin concentration may have even decreased, suggesting that MeJA could be influencing the incorporation of lignans into the excess lignin produced, or that a lack of precursors due to another effect of this biotic stress (possibly caused by insufficient availability) is occurring, or that the enzyme itself is being regulated. *Linum album* is capable of producing podophyllotoxin, which is the aryltetralin lignan that is utilized in the semi-synthesis of Etoposide®, a prominent cancer prevention drug that is categorized as important by the World Health Organization. Due to the growing demand for alternative sources of various substances, compounds such as MeJA, SA, and chitosan or fungal extracts have been shown to stimulate the production of podophyllotoxin when applied to cultures (Tashackori et al., 2018; Van Fürden et al., 2005; Tahsili et al., 2014; Yousefzadi et al., 2010). It was discovered that extract of *Fusarium* increased both the production of podophyllotoxin and the expression of *LaPLR* (Esmaeilzadeh Bahabadi et al., 2012, 2014). However, no direct correlation was observed between *PLR* expression and the synthesis of the final product. Despite a more than threefold increase in lignin production compared to control cultures after SA treatment, *LaPLR* expression did not increase (Yousefzadi et al.,

2010). These findings indicate that the regulation of the entire pathway is complex, involving differential control of gene expression through various signaling pathways that mediate hormonal regulation, as evidenced by research on flax. Wankhede et al., (2013) detected that in *Podophyllum hexandrum*, the *PhPLR* gene shows increased expression when the plant undergoes MeJA treatment, wounding stress, & UV radiation in the leaves.

Most of the current information on the expression of *PLRs* is based on two PLRs from Flax. *PLR1* & *PLR2* in flax have a important function in producing (+)-SECO & (-)-SECO respectively. (-)-SECO ultimately contributes to the formation of yatein (Corbin et al., 2017). Both *PLR1* and *PLR2* of flax were found to be expressed in seeds as well as in roots, though *PLR2* had been identified in the aerial parts (Hano et al., 2006; Hemmati et al., 2010). The expression of *PLR1* rises during the early stages of seed formation, with higher levels observed in the earlier developmental phases compared to mature seeds. Furthermore, the lack of activity of *PLR1* gene promotor shown by the seed coats of the terminated seeds suggests that the embryo could control PLR gene expression (Hano et al., 2006; Zhao et al., 2015). Several cis-elements were detected in the gene promoter regions of *LuPLR* which are known to play role in its regulation, such as ABRE, MYB-PLANT, MYB, & W-box motifs (Hano et al., 2006; Kwon et al., 2001; Burlat et al., 2001; Morimoto & Satake, 2013). However, there is currently a gap in the published research regarding the transcription factors that regulate *PLR* expression. Corbin, Decourtil, et al., (2013) proved that a prenylation phenomenon assists to regulate via transcription triggered by ABA. As demonstrated in both flaxseeds, a significant association exists between ABA, and the regulation of the *LuPLR1*, as well as synthesis of SECO and flax cell suspension (Corbin, Renouard, et al., 2013; Renouard et al., 2012). At the developmental stage 2 (WS2), accumulation of ABA in seeds peaks when the seed is nearly fully developed (Renouard et al., 2012). *LuPLR2* is responsible for producing (-)-SECO, a lignan precursor that primarily accumulates in flax leaves (Hemmati et al., 2010; Von Heimendahl et al., 2005). Yatein production as well as *LuPLR2* expression in flax are enhanced by MeJA treatment and wounding, suggesting that lignans are of great importance in the defense mechanisms of plants (Corbin et al., 2017).

2.4 Comparative Analysis of Yatein with Other Similar Lignans

Among these lignans, yatein has emerged as a compound of notable importance, largely due to its unique structural attributes and potent biological activities that distinguish it from other members of its class (Gao et al., 2024). To fully appreciate the potential of yatein in therapeutic applications, it is essential to undertake a comparative analysis with other similar lignans such as podophyllotoxin, arctigenin, and sesamin, exploring both the similarities and differences that define their biological efficacy and pharmacokinetics.

Yatein shares this fundamental structural framework with other lignans, characterized by its dibenzylbutyrolactone core—a feature that it shares with podophyllotoxin, a lignan that has been extensively studied and utilized for its potent anticancer properties (Choudhary & Saraf, 2021). However, despite this structural similarity, yatein stands out due to its unique chemical features, particularly the presence of specific hydroxyl and methoxy groups. These groups are not merely structural nuances; they significantly influence the biological activity of yatein, particularly its antioxidant capacity. Research has shown that yatein exhibits antioxidant activity compared to other lignans like podophyllotoxin and arctigenin, a property that is largely attributed to its distinct functional groups which enhance its ability to scavenge free radicals and inhibit oxidative stress (Kim et al., 2019). This antioxidant activity positions yatein as a particularly promising compound for the development of health supplements and therapeutic agents aimed at mitigating oxidative stress-related conditions.

In addition to its structural uniqueness, yatein also exhibits distinct biological activities that differentiate it from other lignans. For instance, while podophyllotoxin is renowned for its cytotoxic properties and its role as a precursor in the synthesis of anticancer drugs like etoposide and teniposide, yatein offers a different therapeutic profile with a more favorable safety margin. Comparative studies have revealed that yatein, while sharing the anticancer potential of podophyllotoxin, exhibits a significantly lower toxicity profile, making it a safer option for therapeutic use (Shi et al., 2019). This reduced toxicity is a critical advantage in drug development, where the therapeutic index—the ratio of a

drug's toxic dose to its effective dose—plays a crucial role in determining the viability of a compound as a pharmaceutical agent. Furthermore, yatein's lower toxicity is complemented by its superior bioavailability. Bioavailability refers to the proportion of a drug that enters the circulation when introduced into the body and is thus available for therapeutic action. Yatein has been found to have better bioavailability compared to some other lignans, meaning that it is more easily absorbed and utilized by the body, which enhances its effectiveness as a therapeutic agent (Wang et al., 2021). This superior bioavailability, combined with its low toxicity, makes yatein an attractive candidate for further development as a natural therapeutic agent, particularly in the context of chronic diseases where long-term treatment is necessary.

Beyond its antioxidant and anticancer properties, yatein also exhibits other biological activities that further distinguish it from other lignans. For example, arctigenin, another lignan that shares some structural similarities with yatein, is known for its anti-inflammatory and antiviral activities. However, yatein's anti-inflammatory properties are coupled with a unique ability to modulate cellular signaling pathways that are not as prominently affected by other lignans. Studies have indicated that yatein can influence pathways related to cell proliferation and apoptosis, making it a compound of interest not only for its direct biological effects but also for its potential role in regulating complex biological processes (Pereira et al., 2020). This regulatory capacity suggests that yatein could be particularly useful in the treatment of diseases where inflammation and aberrant cell growth are key pathological features, such as in certain cancers and autoimmune disorders.

In addition to these comparative advantages, yatein's potential as a therapeutic agent is further supported by its ability to interact synergistically with other compounds. In the realm of natural product research, the concept of synergy—where the combined effect of two or more compounds is greater than the sum of their individual effects—is of great interest. Yatein has been shown to exhibit synergistic effects when used in combination with other lignans or phytochemicals, enhancing their collective biological activity. For instance, when combined with lignans like pinoresinol or matairesinol, yatein

has been observed to potentiate their antioxidant and anti-inflammatory effects, suggesting that it could be used as part of a combination therapy to achieve greater therapeutic outcomes (Kim et al., 2019). This synergistic potential not only broadens the scope of yatein's application but also underscores the importance of studying it in the context of complex biological systems where multiple compounds interact.

The distinctive features of yatein, from its structural attributes to its biological activities and pharmacokinetics, underscore the importance of studying it as a unique entity within the lignan class. While it shares many characteristics with other lignans, such as podophyllotoxin, arctigenin, and sesamin, yatein's specific combination of high antioxidant activity, low toxicity, superior bioavailability, and potential for synergy makes it a particularly valuable compound for further research and development. Moreover, the study of yatein contributes to the broader field of phytochemistry, where understanding the diversity of plant-derived compounds is essential for discovering new drugs and therapies. As research into yatein continues to evolve, it is likely that further insights will be gained into its mechanisms of action, therapeutic potential, and possible applications in various fields of medicine and health.

In conclusion, yatein is a lignan that stands out from its peers due to its unique structural and functional characteristics. While it shares the basic dimeric phenylpropanoid structure with other lignans, its specific functional groups endow it with higher antioxidant activity and a favorable safety profile, making it an attractive candidate for therapeutic use. Its superior bioavailability and stable pharmacokinetics further enhance its potential as a natural therapeutic agent. Additionally, yatein's ability to interact synergistically with other phytochemicals opens up possibilities for its use in combination therapies, potentially leading to more effective treatment options for a variety of conditions. As research progresses, yatein may emerge as a key player in the development of natural health products and pharmaceutical agents, contributing to the ongoing investigation of lignans as a valuable reservoir of biologically active components with significant therapeutic potential.

2.4.1 Challenges and Advancements in Yatein Research

Yatein, a dibenzylbutyrolactone lignan, has become an area of significant interest due to its promising biological activities, including its potential as an antioxidant, anti-inflammatory, and anticancer agent. However, the research into yatein has faced several critical challenges that have hindered the full exploration of its therapeutic and agricultural applications. Among these challenges, the low concentration of yatein in natural plant sources, difficulties in its extraction and characterization, and the complexity of its biosynthesis have posed significant barriers to progress. Despite these obstacles, recent advancements in biotechnology, analytical chemistry, and extraction techniques have made significant strides in overcoming these challenges, opening new avenues for research and application in various fields. This comprehensive review will explore the challenges faced in yatein research and the corresponding advancements that have propelled this area of study forward.

One of the most significant challenges in yatein research is its low natural abundance in plants. Yatein is typically found in very small quantities in species such as Podocarpus and Cupressaceae, making it difficult to obtain sufficient amounts for detailed study. This scarcity has historically limited the scope of research on yatein, as large quantities of the compound are required to conduct in-depth pharmacological and toxicological studies that are essential for its development as a therapeutic agent (Fang & Xie, 2020). The low yield of yatein from natural sources has also posed challenges for its commercial production, limiting its availability for potential use in the pharmaceutical and agricultural industries. However, recent advancements in extraction techniques have provided new solutions to this problem. Techniques such as ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE) have significantly improved the efficiency of yatein extraction, allowing for the isolation of larger quantities of the compound from plant materials. UAE, for instance, uses ultrasonic waves to enhance the penetration of solvents into plant tissues, thereby increasing the extraction yield of yatein and other bioactive compounds (Chen et al., 2020). SFE, on the other hand, employs supercritical CO2 as a solvent, which can selectively extract yatein based on its solubility under specific

pressure and temperature conditions. These advancements have not only increased the availability of yatein for research purposes but have also made it more feasible to explore its commercial applications.

Another significant challenge in yatein research has been the difficulty in accurately characterizing the compound due to its complex molecular structure. Yatein, like many other lignans, possesses a dimeric phenylpropanoid structure with various functional groups that can complicate its analysis. Traditional analytical techniques, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, have been widely used to study yatein, but these methods have limitations when it comes to providing detailed structural information, especially for complex molecules like yatein (Zhou et al., 2022). For example, conventional one-dimensional NMR spectroscopy can provide information about the types of atoms present and their immediate neighbors, but it may not always reveal the full spatial arrangement of these atoms within the molecule. Similarly, traditional mass spectrometry, while effective at determining molecular weight and identifying certain fragments, may not always be able to fully characterize the entire structure of complex lignans like yatein. However, advancements in analytical chemistry have introduced more sophisticated techniques that have significantly enhanced the ability to characterize yatein with greater precision. High-resolution mass spectrometry (HRMS), for example, offers higher accuracy in determining molecular weights and can resolve complex mixtures of isomers that were previously difficult to distinguish (Cui et al., 2020). Additionally, two-dimensional NMR spectroscopy techniques, such as COSY (Correlation Spectroscopy) and HSQC (Heteronuclear Single Quantum Coherence), have enabled researchers to obtain more detailed information about the spatial arrangement of atoms within the yatein molecule, allowing for a more comprehensive understanding of its structure. These advancements have not only improved the accuracy of yatein characterization but have also facilitated the study of its interactions with other molecules, which is crucial for understanding its biological activity.

The complexity of yatein's biosynthesis represents another major challenge in this area of research. Yatein is biosynthesized through the shikimic acid pathway, which

involves the coupling of coniferyl alcohol units followed by various enzymatic modifications. This biosynthetic pathway is not only complex but also tightly regulated, making it difficult to manipulate for enhanced production of yatein in plants (Gupta et al., 2021). The low natural abundance of yatein is partly due to the fact that the enzymes involved in its biosynthesis are often present at low levels in plants, and the pathway may compete with other metabolic processes that divert precursors away from lignan biosynthesis. However, advancements in biotechnology have provided new tools for overcoming these challenges. For example, the use of gene synthesis and genetic modification techniques has made it possible to study and manipulate the genes involved in yatein biosynthesis, such as *LuPLR2*, which encodes for the enzyme pinoresinol-lariciresinol reductase, a key enzyme in the lignan biosynthetic pathway (Wang et al., 2021). By overexpressing this enzyme or other related enzymes in transgenic plants, increase in the production of yatein could be possible, thereby making it more accessible for research and potential commercial applications. Additionally, advances in metabolic engineering have enabled the re-routing of metabolic pathways to favor lignan biosynthesis, further enhancing yatein production in plant systems.

The advancements in biotechnology have also opened up new possibilities for the production of yatein in non-plant systems. For example, microbial fermentation has emerged as a solid alternative for the formation of plant-derived products. By introducing the genes involved in yatein biosynthesis into microorganisms such as Escherichia coli or Saccharomyces cerevisiae, it is possible to produce yatein in a controlled environment, independent of the limitations posed by plant growth and cultivation (Shi et al., 2019). This approach not only provides a more sustainable and scalable method for yatein production but also allows for the potential creation of yatein analogs with enhanced or novel biological activities through combinatorial biosynthesis. The use of synthetic biology techniques, where entire biosynthetic pathways are reconstructed in microbial hosts, has further expanded the possibilities for producing yatein and other lignans at a commercial scale.

In addition to the challenges related to extraction, characterization, and biosynthesis, the study of yatein's biological activity has also faced hurdles, particularly in understanding its pharmacokinetics and pharmacodynamics. Yatein's low bioavailability and rapid metabolism in the body have been identified as significant obstacles to its development as a therapeutic agent. Bioavailability refers to the proportion of a drug that reaches systemic circulation in an active form after administration. For yatein, like many natural compounds, poor bioavailability is a major issue due to its low solubility in water and its rapid degradation by metabolic enzymes (Liu et al., 2019). This has made it challenging to achieve the necessary therapeutic concentrations of yatein in the body, limiting its effectiveness as a drug. However, recent advancements in drug delivery systems have provided potential solutions to these issues. For instance, the development of nanoparticle-based delivery systems has shown promise in enhancing the bioavailability of poorly soluble compounds like yatein. By encapsulating yatein in nanoparticles, it is possible to protect the compound from metabolic degradation and enhance its absorption in the gastrointestinal tract, thereby improving its bioavailability (Zhang et al., 2021). Additionally, the use of prodrugs, which are chemically modified versions of a drug that undergo conversion into the active compound within the body, has also been explored as a means of improving the pharmacokinetic profile of yatein. These strategies have the potential to overcome the limitations of yatein’s bioavailability and metabolism, making it a more viable candidate for therapeutic development.

Moreover, the understanding of yatein's pharmacodynamics—the study of the biochemical and physiological effects of the compound and its mechanisms of action—has been advanced through the use of systems biology approaches. By integrating data from genomics, proteomics, and metabolomics, researchers have been able to gain a more comprehensive understanding of how yatein interacts with biological systems at the molecular level. These findings will not only have contributed to a better understanding of yatein's mechanism of action but have also identified potential biomarkers for monitoring its therapeutic effects in clinical settings. The application of computational modeling and bioinformatics tools has further enhanced the ability to predict the interactions of yatein

with its molecular targets, enabling the design of more effective analogs and derivatives with improved pharmacological profiles.

In summary, while yatein research has faced significant challenges, particularly in terms of its extraction, characterization, and biosynthesis, recent advancements in technology and methodology have addressed many of these obstacles. The development of more efficient extraction techniques, such as ultrasound-assisted and supercritical fluid extraction, has increased the availability of yatein for research and commercial applications. Advances in analytical chemistry, including high-resolution mass spectrometry and two-dimensional nuclear magnetic resonance spectroscopy, have improved the ability to accurately characterize yatein and understand its complex structure. Furthermore, biotechnological innovations, such as genetic modification and microbial fermentation, have opened new avenues for the production of yatein and the study of its biosynthesis. Additionally, advancements in drug delivery systems and systems biology approaches have provided new insights into yatein's pharmacokinetics and pharmacodynamics, paving the way for its development as a therapeutic agent. As research continues to evolve, it is likely that yatein will play an increasingly important role in the fields of medicine and agriculture, with the potential to be used in a wide range of applications, from health supplements to novel drug therapies.

Understanding how environmental factors influence the production and activity of yatein is crucial for optimizing its use in therapeutic applications. As discussed in previous sections, the antioxidant activity of yatein is significantly influenced by abiotic conditions such as temperature, light, and water availability. By optimizing these environmental conditions during the cultivation of plants that produce yatein, it may be possible to enhance the yield and efficacy of this compound for therapeutic use. By this approach, the development of health supplements & therapies that harness the full potential of yatein as a natural antioxidant and anti-inflammatory phytochemical (Alam et al., 2020).

2.5 Identification of Gaps in the Current Literature on Yatein and *LuPLR2*

The exploration of yatein and *LuPLR2* in recent years has led to considerable advancements in understanding their biological significance, yet numerous gaps persist in the literature that hinder a comprehensive understanding of these compounds and their potential applications. Yatein, a lignan with promising therapeutic properties, and *LuPLR2*, a gene implicated in its biosynthesis, both hold significant potential in the fields of medicine and agriculture. However, the current state of research is limited by several critical gaps that need to be addressed to fully harness their benefits.

One of the most prominent gaps in the literature concerns the regulatory mechanisms governing the expression of *LuPLR2* under diverse environmental conditions. While it is well-documented that *LuPLR2* is upregulated in response to various abiotic stresses, including drought, salinity, and extreme temperatures, the specific signaling pathways and transcription factors involved in this regulation remain largely unexplored. Current studies, such as those by Hano et al., (2021), have laid the groundwork by identifying some stress-induced expression patterns of *LuPLR2*, yet the intricacies of its regulation are not fully elucidated. This lack of understanding is a significant bottleneck in the application of *LuPLR2* for enhancing crop resilience, as the precise molecular players and their interactions in the stress response pathways are critical for developing effective strategies to manipulate its expression for agricultural benefit.

Moreover, the literature is notably deficient in studies exploring the influence of environmental factors beyond abiotic stressors on the expression and function of *LuPLR2*. For instance, while light and temperature are known to play crucial roles in the development of plants, their impact on *LuPLR2* expression and the subsequent effects on yatein biosynthesis have not been thoroughly investigated. The current research predominantly focuses on drought, heat, cold and salinity, with limited attention given to how variations in light intensity or photoperiod, might modulate *LuPLR2* expression and, consequently, yatein production. This gap in knowledge not only limits our understanding

of the environmental adaptability of *LuPLR2* but also constraints the potential to optimize yatein production for therapeutic use.

Another critical gap in the current literature is the insufficient exploration of yatein's antioxidant potential under varying environmental conditions. While some studies have demonstrated that yatein exhibits significant antioxidant activity, the extent to which this activity is modulated by environmental factors remains unexplored. The antioxidant properties of yatein are particularly relevant in the context of its potential use as a therapeutic agent for oxidative stress-related diseases. There is a pressing need for more comprehensive studies that examine how factors such as light, temperature, and nutrient availability impact yatein's antioxidant activity. Such research is crucial for developing strategies to enhance yatein's stability and effectiveness in various therapeutic contexts.

Furthermore, the biosynthesis of yatein itself presents another area where the literature is lacking. Although the biosynthetic pathway of yatein has been partially elucidated, with key enzymes and intermediates identified, there remains a substantial gap in understanding the full spectrum of genetic and biochemical factors that regulate its production. For instance, the role of *LuPLR2* in yatein biosynthesis has been suggested but not conclusively demonstrated. Studies such as those by Corbin et al., (2017) have hinted at a possible link between *LuPLR2* expression and yatein production, but the exact mechanisms and interactions involved are still unknown. The lack of information is a significant obstacle to efforts focused at enhancing yatein production through genetic manipulation or biotechnological approaches. To fully capitalize on the therapeutic potential of yatein, it is essential to conduct more detailed studies that map out the entire biosynthetic pathway, identify all the regulatory elements involved, and determine how these elements can be manipulated to increase yatein yield.

In addition to the biosynthetic pathway, there is also a significant gap in the literature regarding the post-biosynthetic modifications of yatein. Post-biosynthetic modifications, such as glycosylation or methylation, can significantly alter the biological activity and stability of natural compounds. However, there is a dearth of studies examining

whether such modifications occur in yatein and, if they do, how they affect its therapeutic properties. Understanding these modifications could provide valuable insights into how yatein's biological activity can be optimized for specific applications, whether in medicine or agriculture. Addressing this gap will require a multidisciplinary approach, combining expertise in plant biology, biochemistry, and pharmacology to fully explore the potential of yatein and its derivatives.

Finally, another gap in the literature relates to the long-term ecological impacts of using yatein as a crop protection agent. While yatein's potential benefits in enhancing crop resilience are clear, there is little research on how its widespread use might affect the broader ecosystem. For instance, the impact of yatein on non-target organisms, soil health, and biodiversity is not well comprehended. This gap in knowledge hinders the safe and sustainable use of yatein in agriculture. Future research should focus on conducting comprehensive ecological assessments to evaluate the potential risks and benefits of yatein use in various agricultural contexts. Such studies will be crucial for developing guidelines and best practices for the application of yatein in a way that maximizes its benefits while minimizing any negative environmental impacts.

In conclusion, while significant progress has been made in understanding yatein and *LuPLR2*, the current literature is still marked by several critical gaps. These gaps, which include a limited understanding of the regulatory mechanisms governing *LuPLR2* expression, insufficient exploration of yatein's antioxidant potential under different environmental conditions, and a lack of studies on the agricultural applications of yatein, represent significant obstacles to fully realizing the potential of these compounds. Addressing these gaps will require a concerted research effort, drawing on expertise from a range of disciplines and employing a variety of methodological approaches. By filling these gaps, researchers can pave the way for new and innovative applications of yatein and *LuPLR2* in medicine and agriculture, ultimately contributing to the development of more resilient crops and more effective therapeutic agents.

Scope of the study

Flax (*Linum usitatissimum*) is a nutritionally rich plant recognized for its substantial concentration of bioactive substances, particularly lignans, which contribute to its anti-inflammatory, antioxidant, and stress-mitigating attributes. Lignans, a class of polyphenolic compounds, have a critical role in the protection of plants and have been extensively studied for their health benefits, including their potential in combating oxidative stress. Among the lignans found in flax, yatein is of particular interest due to its Antioxidative potential and possible involvement in the tolerance of environmental cues. The formation of lignans is regulated by specific gene families, such as *DIRs*, *PLRs*, and *O-methyltransferases*, which influence their accumulation and biological activity. However, the molecular regulation of yatein under abiotic stress conditions remains largely unexplored.

This study aims to investigate the genome-wide identification of yatein-biosynthetic genes (*PLR2s*) in flax microgreens using bioinformatics approaches. An investigation of the expression patterns associated with *PLR2s* across variety of stress (abiotic) conditions to understand their regulatory mechanisms. Additionally, the study quantifies yatein concentration in flax microgreens with the help of HPLC. Furthermore, antioxidative potential of yatein in flax microgreens under different abiotic stress conditions would be evaluated through antioxidative enzyme assays. By integrating computational, molecular, and antioxidative approaches, this research aims to enhance the understanding the role of yatein in stress tolerance, with potential application in crop improvement and plant biotechnology.

Research Objectives

1. Genome wide *In-silico* characterization of genes responsible for Yatein biosynthesis in Flax microgreens.
2. Expression analysis using Real time PCR and Quantification of Yatein phytochemical using HPLC under different abiotic conditions.
3. Effect of Yatein on Antioxidative potential of flax microgreens under different Abiotic stress conditions.

CHAPTER 3: MATERIALS AND METHODS

"Science is a way of thinking much more than it is a body of knowledge."
― **Carl Sagan**

3. Materials and methods

3.1 Gene identification of the PLR2 genes in the genome of L. usitatissimum

Using available genome assembly (*Phytozome genome ID: 200 • NCBI taxonomy ID: 4006*) in Phytozome database (https://phytozome-next.jgi.doe.gov/), members of pinoresinol lariciresinol reductases gene family in *L. usitatissimum* were identified (Goodstein et al., 2012; Song et al., 2025) (**Figure 3.1**). From Uniprot database (https://www.uniprot.org/), FASTA sequence of LuPLR2 was used to run as a query (BLASTX) against *L. usitatissimum* with an e-value cut-off (-1) with the help of Phytozome database (accessed 20 August, 2022) (Corbin et al., 2017). All the information including genomic sequences, transcript sequences, CDS (coding sequences), and peptide sequences about the *PLR2* genes in *L. usitatissimum* was downloaded. Subsequently, all the peptide sequences were obtained and the HMM (Hidden Markov Model) (http://hmmer.org/) was used to verify each member of the *PLR2* gene family and the members which were devoid of typical PLR2 domains were discarded (Finn et al., 2016; Zandawala et al., 2024) (**Figure 3.2**).

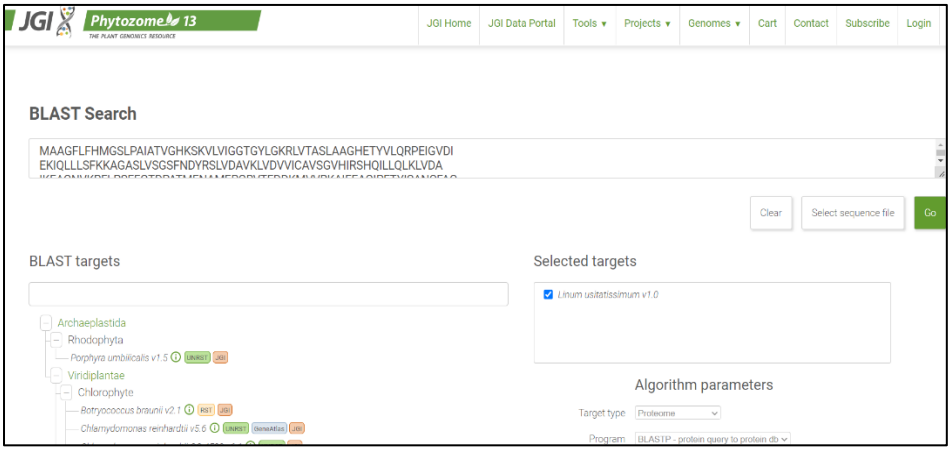

Figure 3.1: Retrieval of *LuPLR2* sequences through Phytozome database

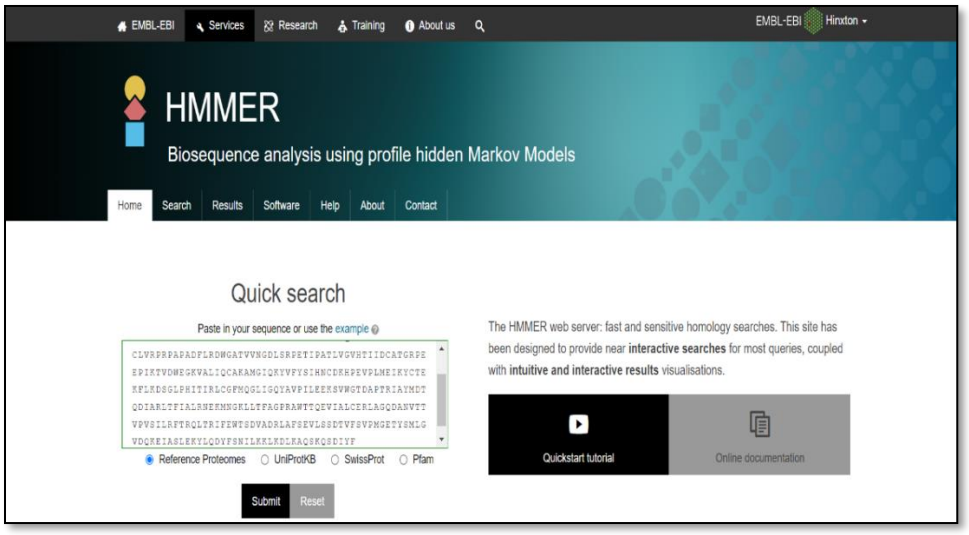

Figure 3.2: Domain analysis of LuPLR2 proteins through HMMER

Analysis of intrinsic disorder nature of proteins and their secondary structures within the *LuPLR2* genes

The web server PONDR-FIT (http://www.pondr.com/) (accessed 15 July, 2024) was used with the default parameters to predict the disordered nature of PLR2s (Ii, 2022; Xue, 2011). For determining the secondary structure of identified *PLR2s*, the GOR4 database (https://npsa-pbil. ibcp.fr/cgi-bin/secpred_gor4.pl) (accessed 17 July, 2024) was used. The protein sequence in FASTA format had been entered as query (Kloczkowski et al., 2002; Tasneem et al., 2023).

Expressed sequence tags (ESTs): ESTs analysis results in similarity index of standard genes with other existing genes using nucleotide BLAST and this was carried out using CDS of *PLR2* genes with the help of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (accessed 21 August, 2022) (Jongeneel, 2000; A. Kumar, 2016) (**Figure 3.3**).

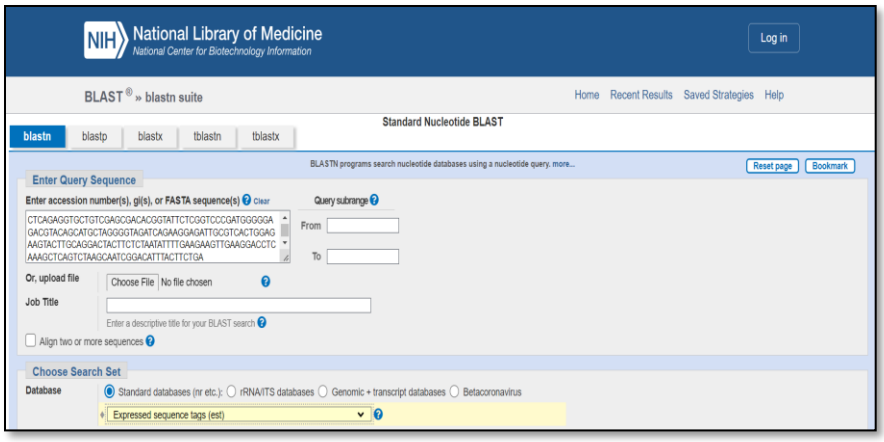

Figure 3.3: ESTs analysis of *LuPLR2*s through NCBI Blast nucleotide

Protein properties of *PLR2* genes in *L. usitatissimum*

Prediction of isoelectric points and molecular weights were done using ExPASy (http://expasy.org/) (accessed 24 August, 2022) (Gasteiger et al., 2003; Mohanta et al., 2022) (**Figure 3.4**). Subcellular sites were forecasted by LOCTREE 3 (https://rostlab.org/services/loctree3/) (Colinet et al., 2024; Goldberg et al., 2014) (accessed 24 August, 2022) (**Figure 3.5**).

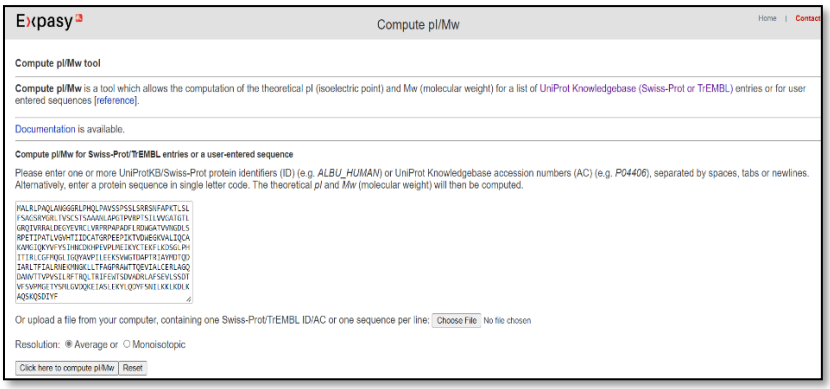

Figure 3.4: Prediction of Molecular weights and isoelectric points of LuPLR2 proteins by Expasy

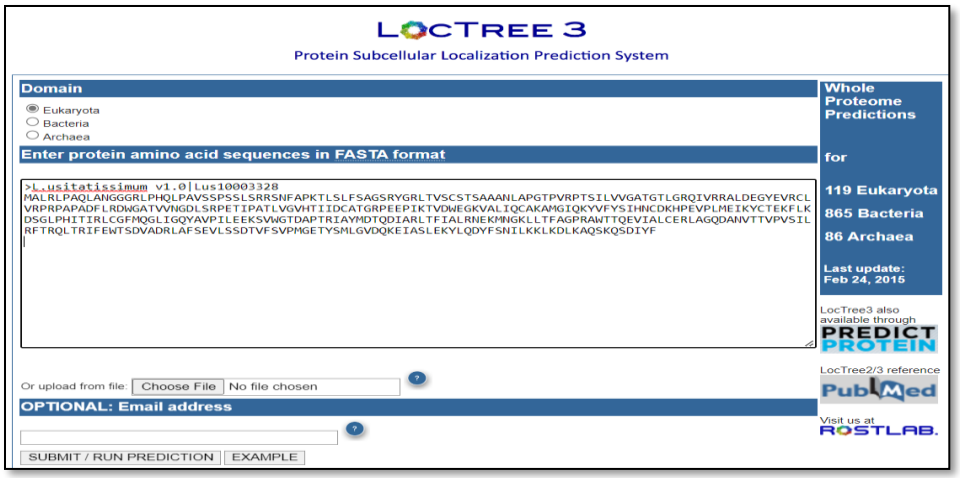

Figure 3.5: Prediction of sub-cellular localization of LuPLR2 proteins through LOC TREE3

Analysis of conserved motif domain and exon- intron arrangement

Analysis of motifs was performed with the help of database, MEME 5.4.0 (https://memesuite.org/meme/) with the default settings (motif= 10 default setting) (Aydinli et al., 2022; Bailey et al., 2009) (accessed 25 August, 2022) (**Figure 3.6**). The exon-intron structure was analysed by comparing the CDS sequences of the *PLR2* genes with the genome sequence and their structure analysis was performed with the help of a known web server (GSDS 2.0) (accessed 30 August, 2022) (Deng et al., 2024; Hu et al., 2015) (**Figure 3.7**).

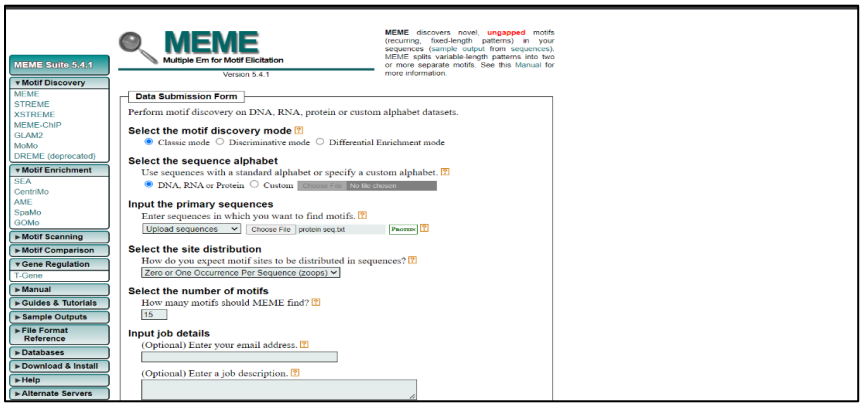

Figure 3.6: Motif analysis of *LuPLR2*s through MEME database

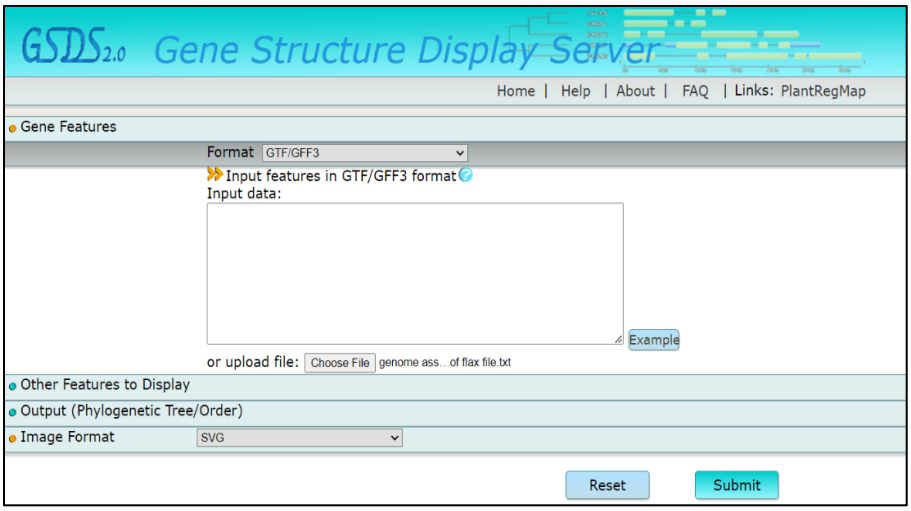

Figure 3.7: Exon-intron structure organization of *LuPLR2s* with the help of GSDS software

Gene ontology (GO) term analysis

The research conducted a GO enrichment analysis to find out the functions and molecular pathways of *LuPLR2* genes (Ge et al., 2020). The Shiny GO 0.77 web server (http://bioinformatics.sdstate.edu/go74/) was used to perform GO enrichment analysis by using the *LuPLR2* gene sequences (accessed 20 October, 2024) (**Figure 3.8**).

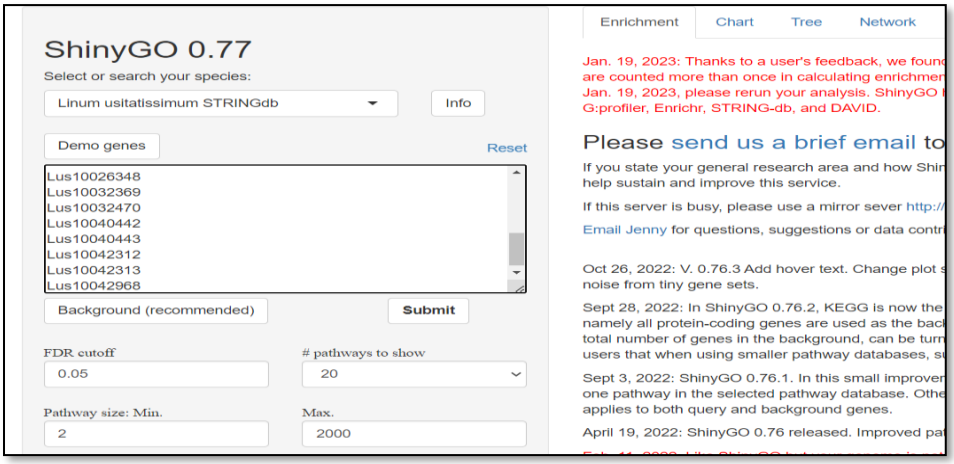

Figure 3.8: Gene ontology of *LuPLR2*s through Shiny GO 0.77 database

Prediction of cis-regulatory elements in the promotor regions of *LuPLR2* genes

To identify the cis elements, 5' upstream flanking sequences (2000bp upstream) were retrieved from Phytozome database. For the analysis of cis elements, these sequences were run in PlantCARE database (Lescot et al., 2002) (Accessed 25 October, 2024) (**Figure 3.9**). For better understanding, these elements were displayed using TB tools database (Chen et al., 2023).

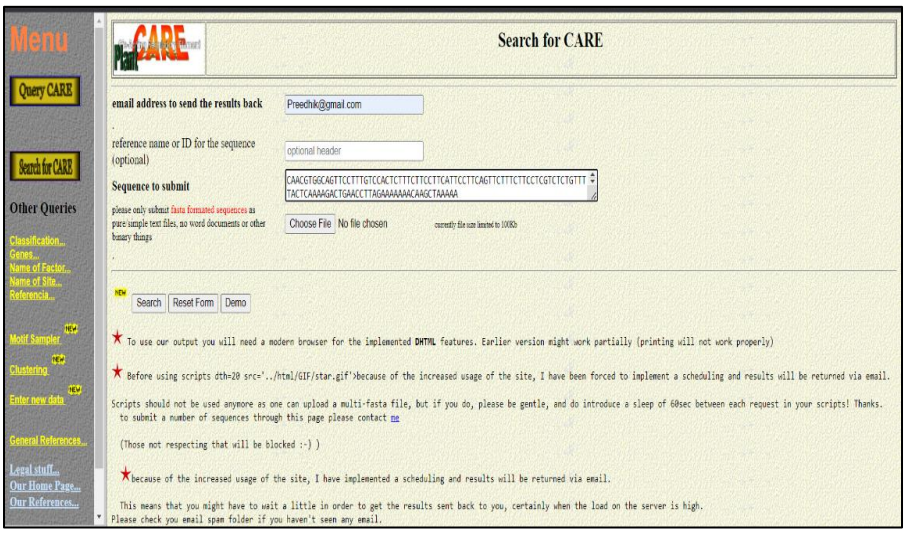

Figure 3.9: Analysis of cis elements of *LuPLR2s* through PlantCARE database

Phylogenetic analysis of *PLR2* genes in *L. usitatissimum*

The muscle tool in MEGA (version 11) (accessed 30 October, 2024) was utilized to align the complete amino acid sequences of PLR proteins from *Vitis riparia*, *Gossypium hirsutum, Populus trichocarpa, Populus euphratica, Vitis vinifera*, *Populus nigra*, *Hibiscus syriacus, Populus alba, Linum album, Salix purpurea, Theobroma cacao, Linum flavum, Ziziphus jujube, Diospyros lotus, Linum corymbulosum, Durio zibethinus, Gossypium raimondii, Gossypium arboretum, Lycium ferocissimum, Lycium barbarum, Solanum dulcamara, Solanum verrucosum, Capsicum baccatum, Punica granatum, Capsicum chinense, Benincasa hispida, Solanum pennellii, Alnus glutinosa, Lotus japonicas, Solanum stenotomum, Prosopis cineraria, Camellia sinensis)* and *Linum usitatissimum*, applying the default settings. Phylogenetic tree construction was performed with the help

of MEGA version 11 using the neighbor-joining algorithm with the default parameters (Neighbor-Joining; Bootstrap 1000) under the maximum likelihood (ML) approach (Tamura et al., 2021). The resulting tree was generated in Newick format and then visualized using the iTOL platform (https://itol.embl.de/) (Letunic & Bork, 2024).

3.2 *Expression analysis using Real time PCR and Quantification of yatein phytochemical using HPLC under different abiotic conditions.*

3.2.1 Plant growth and stress conditions

Flax microgreens (local variety) were grown in CSIR-IIIM, Jammu, India, under regulated conditions of 24 ± 2 ℃ with a 16-hour light and 8-hour dark period for 10 days in a growth chamber (Pervical Scientific, USA), with the growing media that consisted of coco peat. Different abiotic stress conditions [Salt stress: 5 and 50 mM of Sodium Chloride (NaCl), Heat stress: 30 ºC after germination, Cold stress: 4 ºC after germination, Drought stress: Polyethylene Glycol-6000 (PEG) (5% & 20%)] were imposed on flax microgreens on 10th day of germination for 8 and 24 h. The control seedlings were maintained under normal conditions with a regular water supply, serving as a comparison to stressed seedlings. The control as well as stressed seedlings were harvested at 11th day and were kept at -80 ° C for further experimentation.

3.2.2 Expression analysis of Flax *LuPLR2* genes under abiotic stress conditions:

RNA isolation: By following the method of Gani et al., (2021), the total RNA was collected from 50-100 mg leaves of both stressed as well as control flax microgreens for qRT-PCR based expression studies by using the Trizol method. In liquid nitrogen, the tissue was processed to achieve fine powdered consistency, after which powdered tissue (100 mg) was dissolved in 500 µl of Trizol. Then, it was homogenized and again 500 µl of trizol was added to Eppendorf tubes. All the tubes were incubated at room temperature for 5 min and then centrifuged at 12,000 rpm for 10-20 min. The supernatants were transferred into fresh

Eppendorf tubes and 0.2 ml of chloroform was added to all the tubes. All the tubes were vortexed and incubated for 3-5 min. Again the tubes were centrifuged at 12,000 rpm for 10-20 min and the supernatants were transferred into fresh tubes. 500 µl of isopropanol was added to all the tubes. The tubes were incubated on ice for 10-15 min and then centrifuged at 12,000 rpm for 15-20 min. The supernatants were discarded and 1 ml of ethanol was added to the tubes and centrifuged at 10,000 rpm for 5 min. The pellets were air-dried, dissolved in DEPC water and were stored at -80 ºC. The quality and pureness of all the isolated RNA were checked through Agarose gel electrophoresis and Nanodrop 2000 Spectrophotometer (Thermoscientific™).

DNase treatment: In order to eradicate any remaining DNA contamination, about 4 micrograms of isolated from each tissue was then subjected to RNase-free DNase treatment, which was performed with the help of TURBO DNA-free™ Kit (Thermo Scientific) (Kundan et al., 2022).

cDNA libraries: The synthesis of cDNA was performed using 2 micrograms of RNA (DNase-treated) in accordance with the standard protocol of the Revert Aid H Minus Ist Strand cDNA manufacturing Kit (available from Thermo Scientific).

Quantitative real-time (qRT)-PCR analysis of LuPLR2 genes:

Primer designing: For the designing of primers, 15 genes were selected out of 22 genes on the basis of domain similarity as that of standard sequence. From the CDS sequences, primers of the 15 *LuPLR2* genes was designed with the help of Oligocalc server (http://biotools.nubic.northwestern.edu/OligoCalc.html) on the basis of melting temperature and GC content (Kundan et al., 2022) (**Table 3.1**). The specificity of primers were checked through semi-quantitative PCR (data not shown).

Table 3.1 Primers of the *LuPLR2s* designed through Oligocalc server

S.no.	Gene	FP	Bp	GC	Tm	RP	Bp	GC	Tm	Amplicon size
1.	Lus10003328	AGTGCCTATTCTCGAG GAGAA	21	48	59.5	AAGTCTCTCACAC AATGCTATC	22	41	58.4	193
2.	Lus10007599	GGTTCACAGTCATCAT CGTG	20	50	58.4	GGTACGGATTCAA GTCCAGC	20	55	60.5	186
3.	Lus10010403	TATGGAGATGGCAAC GTCAAAG	22	45	60.1	GAAGTTGGTTGCC AGAGAGC	20	55	60.5	181

4.	Lus10012143	TTTCACAGCTCGGAACCCTA	20	50	58.4	CTTTGAGTCATCACATTCTCTG	22	41	58.4	184
5.	Lus10012145	TGGCAACGTCAAAGTGGTGTA	21	48	59.5	TCAGTCTTTTGAAGTTGGTTGC	22	41	58.4	183
6.	Lus10012147	TTCTTCCTTCACGTGACCATG	21	48	59.5	GGGACAACACGTTTTTGGGC	20	55	60.5	195
7.	Lus10022632	CCTACATGGACACCCAGGAT	20	55	60.5	GGTGAACCTCAGGATCGAGA	20	55	60.5	191
8.	Lus10023557	GAGGCATCGGAAATTTACCCT	21	48	59.5	ATCTGCTATCGCTGCCCTCT	20	55	60.5	195
9.	Lus10023558	TCAAATCTCCACCTCGAGACA	21	48	59.5	CGTCACAATCTCGTTCATCGA	21	48	59.5	182
10.	Lus10026348	AGGGACAAAGTAGTCATTCTCG	22	45	60.1	CCCAATCTTTTTCTCCCACAGA	22	45	60.1	189
11.	Lus10032470	ACATCAACCAGCTTGCCCAC	20	55	60.5	AACGTCGGTTGGCTGGTTGA	20	55	60.5	194
12.	Lus10040442	TTGGGAGACGGCAATGCTAAA	21	48	59.5	TCTGGAACATAGACCCTTTTCA	22	41	58.4	200
13.	Lus10042312	GCAACCCAAAAGATGATCCAAG	22	45	60.1	GTATCACATTCATTGGAGCTGC	22	45	60.1	192
14.	Lus10042313	GGAACCGCCAAAGCTGTGTA	20	55	60.5	GCTCCTCGGGAACATAAATCT	21	48	59.5	196
15.	Lus10042968	TCTAAACATCAACCAGCTTGCC	22	45	60.1	GACTGGTTGAGGTTGTAGTTGA	22	45	60.1	189
16.	LuETIF5A (a Eukaryotic Translation Initiation Factor 5A)	TGCCACATGTGAACCGTACT	20	50	Greater than 58	CTTTACCCTCAGCAAATCCG	20	50	Greater than 58	-

In addition, quantitative real time polymerase chain reaction (qRT-PCR) was performed on a Step One Plus^{TM} Real-Time PCR System (Applied Biosystems) with SYBR Green mix (Thermo Scientific, USA) by adhering to the procedure described in Gani et al., (2021). Each 10 µl reaction mixture consisted of 5 µl of 2X SYBR Green mix containing ROX dye, 1 µl of diluted cDNA template, 3.6 µl of DNase/RNase-free deionized water, and 0.2 µl each of gene-specific primers (forward and reverse). The reactions were conducted in 3 technical replicates. Thermal cycling conditions included an initial denaturation for 10 min at 95℃, followed by 40 cycles for 15 seconds at 95℃ and for 1 min at 60℃. Ct values for the reference gene (LuETIF5A) and for the test genes were recorded for analyzing the expression levels after performing qRT-PCR. These values were utilized to estimate the relative fold-change in gene expression levels relative to the reference gene with the help of $2^{-\Delta\Delta Ct}$ method as explained by Livak & Schmittgen, (2001). The results were represented as the mean of three replicates, with values expressed as mean ± Standard deviation (SD). Statistical significance was analyzed using Student's t-test.

3.2.3 Quantification of yatein phytochemical under abiotic stress conditions using HPLC:

***Sample collection*:** For the HPLC analysis, flax microgreens subjected to different abiotic stress conditions for 24 h were selected. Dry weight equivalent to 300 mg of stressed and control microgreens were shade dried for almost 20-25 days and were finely powdered with the help of pestle and mortar.

Extraction procedure: The quantification of yatein in the leaves of flax microgreens was conducted following the method described by Rakesh et al., (2021). The finely powdered microgreens (stressed and control) were transferred into the conical flasks and 100 % ethanol (50 ml) was added to each conical flask. The flasks were placed on an orbital shaker set at 150 rpm for 30 minutes at room temperature. The mixture was then filtered using Whatman filter paper, and then, the filtrate was centrifuged for 10 minutes at 10,000 rpm. The clear supernatant was collected in Eppendorf tubes and were kept at -20ºC for HPLC. Before HPLC analysis, the samples were filtered through a 0.45µM nylon membrane.

Quantification of Yatein phytochemical by HPLC: The yatein quantification was performed using a reverse-phase HPLC system (Agilent 1100 series) equipped with a C-18 column (Zorbax Eclipse Plus C18, 4.6×150 mm×5µm) coupled with a diode array detector (DAD) detector. The mobile phase consisted of solvent A (0.2% acetic acid in water) and solvent B (methanol), with its composition varying throughout the run in accordance to a non-linear gradient at a controlled flow (Corbin et al., 2017). The injection volume was 20µl, and the detection wavelength was set at 280nm. Yatein standard (yatein) with an HPLC-purity of 98% was obtained from Chem Faces, China.

Table 3.2 Gradient at a controlled flow rate

Gradient	Time (in minute)	%B
	0	60
	1.5	60
	7	90
	13	100

3.3. *Effect of Yatein on Antioxidative potential of flax microgreens under different abiotic stress conditions*

Flax microgreens (local variety) were cultivated in CSIR-IIIM, Jammu, India, under regulated conditions of 24 ± 2 ℃ with a 16-hour light and 8-hour dark period in a growth chamber (Pervical Scientific, USA). The growing media consisted of coco peat (Set 1) and coco peat mixed with yatein [Set 2, i.e., 5mg of yatein in 1M of Dimethyl sulfoxide (DMSO)]. On both sets of microgreens abiotic stress conditions were implemented on the10th day for 24 h as mentioned in section 3.2.1 and on 11th day leaves of microgreens were cut with the help of scissors after 24 h. Leaves were stored at -80 ℃ analyzing the antioxidative enzyme activities (**Figure 3.10**). Yatein was procured from Centre of Biomedical Research, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Rae Bareli Road, Lucknow.

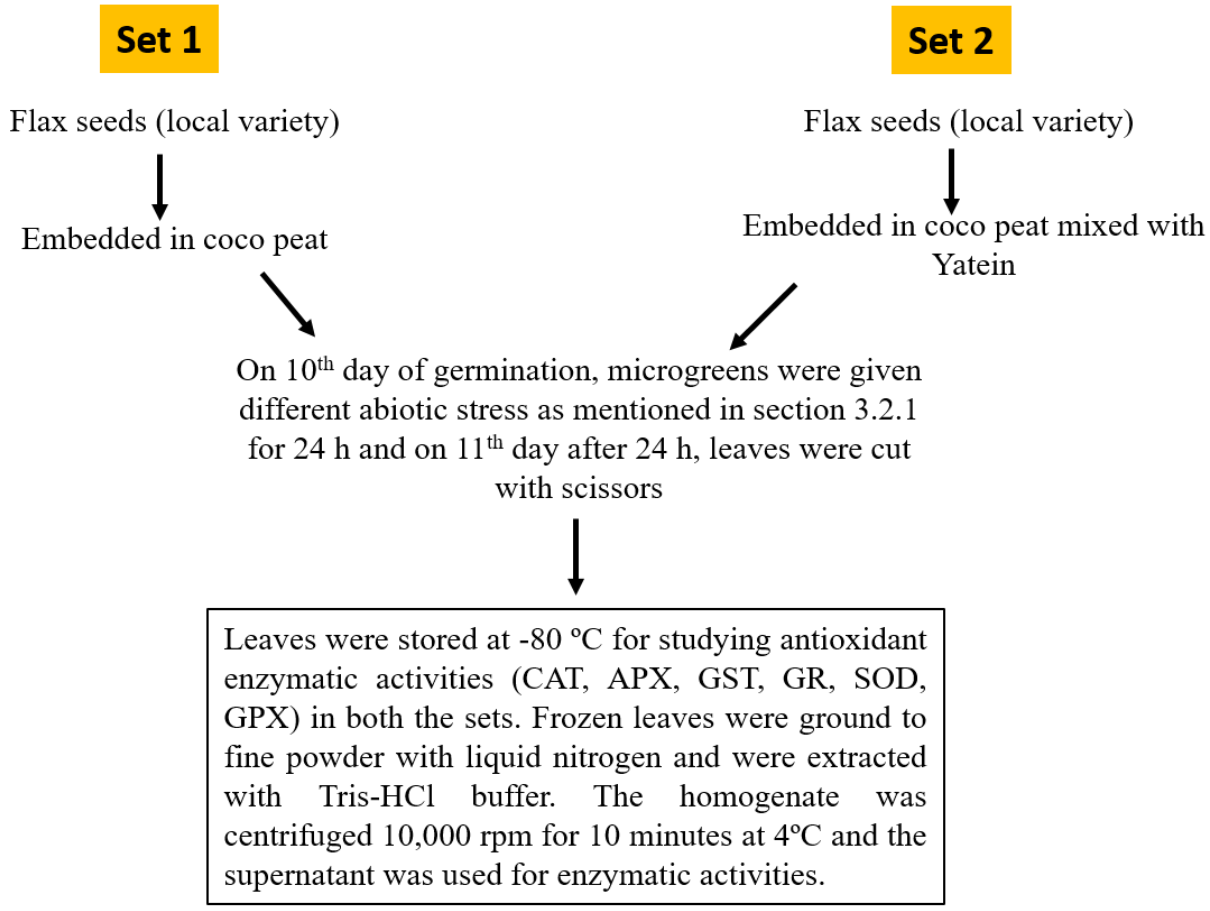

Figure 3.10 Flowchart representing the methodology of the third objective

3.3.1 Protein estimation

In the supernatant, the total soluble protein content was estimated by Bovine serum albumin (BSA) as a standard, following the method described by Arunima & Verulkar, (2022); LOWRY et al., (1951). BSA solutions of varying concentrations (40-200 µg) were prepared by diluting the BSA stock solution (1 mg ml^{-1}) with distilled water. For each test tube, 2 ml of analytical reagent was added, and the contents were thoroughly mixed. For 10 minutes, the tubes were kept at room temperature, after which the addition of 0.2 ml of Folin-Ciocalteu Reagent (FCR) was done. The mixtures were then incubated at 37°C for 30 minutes. The optical density (O.D.) of the samples was estimated at 660 nm using a double-beam UV-VIS spectrophotometer. A standard calibration curve was created by plotting absorbance values against the corresponding

BSA concentrations. The absorbance of the test samples was measured, and the protein concentration was calculated using the standard curve.

3.3.2 Antioxidant studies:

Superoxide Dismutase (SOD) activity

SOD activity was determined following the method of Kunos, (2022); Thomas, (2002), with slight modifications. The reaction mixture included 50 mM Tris-HCl buffer (pH 7.0), 3 µM EDTA, 14.5 mM methionine, 2.25 mM Nitroblue tetrazolium (NBT), and 60 µM riboflavin, and 240 µg of enzyme extract. The whole reaction began by exposing the tubes for 15 minutes under fluorescent lamps. Then by keeping the tubes in the dark space for some time (10 minutes), this reaction in the tubes was halted, and the absorbance was measured at 560 nm using a double-beam UV-VIS spectrophotometer. 1 unit of SOD activity is estimated as the amount of SOD protein required to inhibit 50% of the photoreduction of NBT to blue formazan.

Enzyme activity

Reduction = (Absorbance of sample/ Absorbance of control)* 100 = y

Inhibition (X) = 100-y

50% inhibition = 1 unit

X% inhibition = 1/50*X = Z units

Specific activity [Units (mg protein)$^{-1}$ = Z units (mg protein)$^{-1}$

Catalase (CAT) activity

CAT activity was estimated using the method described by Ait Barka, (2001); Hou, (2023), which evaluates the conversion of hydrogen peroxide (H_2O_2) into water and oxygen. The reaction mixture (1ml) comprised of 10mM hydrogen peroxide, 50 Mm Tris-HCl buffer (pH 7.0240), and 240 µg of enzyme extract. This was performed by monitoring the lowering of hydrogen peroxide at 240 nm over 5 minutes using a double beam UV-VIS spectrophotometer. CAT activity (1 unit) was described as the enzyme'

amount used to catalyze the oxidation of 1mmol of H_2O_2/min. This activity was quantified as mmol of hydrogen peroxide which was oxidized per mg of protein (min^{-1} mg $protein^{-1}$), with an extinction coefficient (ε) of 39.4 mM^{-1} cm^{-1}.

$$\text{Specific activity (mmoles } min^{-1} \text{ (mg Protein)}^{-1}) = \frac{\text{Change in Absorbance } min^{-1} * \text{Total reaction volume}}{\text{Extinction coefficient} * \text{Sample volume} * \text{mg protein}}$$

Glutathione-S-Transferase (GST) activity

This was measured following the protocol of Hussain et al., (2022); Rakhra et al., (2015), which involves the GST-catalyzed reaction between reduced glutathione (GSH) and GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB) to form conjugate (2,4-dinitrophenyl) glutathione-S (DNPGS). The change in absorbance was taken at 340 nm, and monitored at 5 minutes using an UV- VIS spectrophotometer. Reaction mixture (3ml) contained 50mM Tris-HCl buffer (pH 7.0), 1mM Ethylene diamine tetra acetic acid (EDTA), 1 mM CDNB, 1mM GSH, and 240 µg of enzyme extract. One unit of GST activity is the amount of enzyme catalyzing the formation of 1 mmol of DNPGS per minute. Using an extinction coefficient (ε) of 9.6 mM^{-1} cm^{-1}, the specific activity was quantified as millimole of conjugate formed per minute per mg of protein (min^{-1} mg $protein^{-1}$),

$$\text{Specific activity (mmoles } min^{-1} \text{ (mg Protein)}^{-1}) = \frac{\text{Change in Absorbance } min^{-1} * \text{Total reaction volume}}{\text{Extinction coefficient} * \text{Sample volume} * \text{mg protein}}$$

Ascorbate peroxidase activity (APX)

This activity was determined by the the formation of monodehydroascorbate, through the hydrogen peroxide dependent oxidation of ascorbate Ait Barka, (2001); P. Kumar, (2022). The reaction mixture (1ml) included 50mM Tris-HCl buffer (pH 7.0), 0.2 mM

ascorbic acid, 20 µM H_2O_2, 0.2mM EDTA, and 240 µg of enzyme extract. The change in optical density (OD) was monitored at 290 nm for 5 minutes using a double-beam UV-VIS spectrophotometer. 1 unit is described as the millimole of ascorbate oxidized/min, and specific activity is described as the mmol of ascorbate oxidized per minute per mg of protein (min^{-1} mg $protein^{-1}$) with extinction coefficient (ε) of 2.8 mM^{-1} cm^{-1}.

$$\text{Specific activity (mmoles } min^{-1} \text{ (mg Protein)}^{-1}) = \frac{\text{Change in Absorbance } min^{-1} * \text{Total reaction volume}}{\text{Extinction coefficient} * \text{Sample volume} * \text{mg protein}}$$

Guaiacol peroxidase (GPX) activity

GPX activity was assessed by the method outlined by Lu et al., (2025); Rakhra et al., (2015) employing guaiacol as a substrate and measuring absorbance at 470 nm. Reaction mixture (1ml) comprised of 50 mM Tris-HCl (pH 7.0), 10 mM guaiacol, and 5 Mm H_2O_2, and extract of enzyme (240 µg). The GPX activity was observed at 470 nm by tracking the formation of the tetraguaiacol for 5 minutes using a double beam UV-VIS spectrophotometer. One unit of enzyme activity is the mmol of tetraguaiacol produced per minute. This was quantified in terms of specific activity which was quantified as the millimole of tetraguaiacol formed or hydrogen peroxide reduced min^{-1} mg $protein^{-1}$ (ε= 26.6 mM^{-1} cm^{-1}).

$$\text{Specific activity (mmoles } min^{-1} \text{ (mg Protein)}^{-1}) = \frac{\text{Change in Absorbance } min^{-1} * \text{Total reaction volume}}{\text{Extinction coefficient} * \text{Sample volume} * \text{mg protein}}$$

Glutathione Reductase (GR) activity

This activity was determined by coupling the oxidation of NADPH (Nicotinamide Adenine Dinucleotide Phosphate) to the lowering of oxidized glutathione (GSSG) with the measurement of this reduction conducted at 340 nm, as described by Shahid et al., (2020); Thomas, (2002). 50 mM Tris-HCl buffer (pH 7.0), 1 mM GSSG, 0.15 mM NADPH, and 3 mM $MgCl_2$ were used in the reaction mixture (1ml) as well as 240 µg of enzyme extract was also added. The change in absorbance at 340 nm was recorded at 340 nm for 5 minutes using a double-beam UV-VIS spectrophotometer. One unit of enzyme activity is estimated as mmol of NADP+ formed per minute. This activity was quantified as the millimole of $NADP^+$ formed or NADPH oxidized min^{-1} mg $protein^{-1}$ (ε= 6.2 mM^{-1} cm^{-1}).

$$\text{Specific activity (mmoles min}^{-1}\text{ (mg Protein)}^{-1}) = \frac{\text{Change in Absorbance min}^{-1} * \text{Total reaction volume}}{\text{Extinction coefficient} * \text{Sample volume} * \text{mg protein}}$$

Statistical analysis: This study utilized data comprising the means and standard errors from three replicates (n = 3) to assess result accuracy. The significance between the untreated control and untreated samples under abiotic stress was determined by one-way analysis of variance (ANOVA) was performed, followed by Tukey's test with a significance level of $p \leq 0.05$. Subsequently, one-way ANOVA, followed by Tukey's test was also applied to yatein-treated control and yatein treated samples under abiotic stress at a significance level of $p \leq 0.05$. Lastly, to determine the significant between untreated and yatein-treated groups, Student's t-test was employed.

CHAPTER 4: RESULTS

"Anyone who has never made a mistake has never tried anything new."
-Albert Einstein

4.1 Identification of Flax PLR2 gene family members

In the present study, the FASTA sequence of the protein of interest (*LuPLR2*) was retrieved from Uniprot database to run as a query against the *Linum usitatissimum* (flax) in Phytozome database. The FASTA sequence was subjected to BLAST-X and 30 *PLR2* genes were found on basis of similarity index that encode for 30 different PLR2 proteins in flax. Genomic sequences, transcript sequences, CDS sequences, and peptide sequences were downloaded for all the genes.

4.1.1 Intrinsic disordered nature and secondary structure analysis of PLR2 proteins:
Intrinsically unstructured (or disordered) proteins' (IUPs/IDPs), are common in eukaryotic proteomes and perform major functions in cellular processes, particularly transcriptional regulation and cell signaling. The investigation of these proteins using PONDR database revealed the presence of disordered nature amongst all the identified proteins (**Table 4.1**). *Lus10012146* was found to be highly disordered with 41.33% disorder nature while *Lus10026350* was having least percentage of disorder *i.e.,* 5.48%. Additionally, the secondary structure prediction of the identified PLR2s using the GOR database indicated the presence of random coils, α-helices, and β-strands. The prediction showed random coils in each protein, suggesting that these proteins lack well-defined ordered secondary structures (**Table 4.2**). *Lus10007599* exhibits a high number of α-helices and β-strands, specifically, 195 and 109, which enhances the binding of PLR2s to their common sites.

Table 4.1 Predicted percentage of Intrinsic Disorder in *PLR2* genes of *Linum usitatissimum* (Lus) based on PONDR analysis

S.No.	Protein ID	Predicted residues	No. of residues disordered	% Disordered	No. of disordered regions	Longest disordered region	Average prediction score
1.	Lus10003328	338	83	21.39	5	29	0.2755
2.	Lus10004028	340	61	17.94	5	23	0.2162
3.	Lus10007599	628	130	20.70	10	30	0.2694
4.	Lus10009821	320	64	20.00	6	26	0.2574
5.	Lus10010403	314	40	12.74	5	21	0.2261
6.	Lus10012143	314	51	16.24	6	19	0.2428
7.	Lus10012145	312	54	17.31	8	20	0.2416
8.	Lus10012146	75	31	41.33	4	19	0.4978
9.	Lus10012147	328	60	18.29	4	30	0.2215
10.	Lus10021709	195	48	24.62	4	23	0.2569
11.	Lus10021740	338	45	13.31	6	13	0.2039
12.	Lus10022632	387	86	22.22	5	30	0.2817
13.	Lus10023096	352	74	21.02	8	26	0.2446
14.	Lus10023097	176	48	27.27	3	41	0.2973
15.	Lus10023557	460	181	39.35	8	83	0.4209
16.	Lus10023558	310	60	19.35	6	20	0.2868
17.	Lus10024472	245	87	35.51	6	22	0.3487
18.	Lus10026348	305	38	12.46	4	24	0.2087
19.	Lus10026350	310	17	5.48	5	5	0.1753
20.	Lus10026351	311	57	18.33	5	16	0.2474
21.	Lus10032369	289	45	15.57	6	22	0.2067
22.	Lus10032470	372	88	23.66	7	26	0.2766
23.	Lus10035016	327	26	7.95	2	20	0.2254
24.	Lus10035058	342	73	21.35	5	29	0.2866
25.	Lus10040442	305	60	19.67	7	20	0.2499
26.	Lus10040443	295	44	14.92	5	16	0.2548
27.	Lus10042311	311	58	18.65	6	15	0.2489
28.	Lus10042312	261	49	18.77	6	10	0.2516
29.	Lus10042313	265	30	11.32	2	24	0.2026
30.	Lus10042968	266	58	21.80	5	26	0.2697

Table 4.2 Secondary structure prediction of PLR2 proteins in *Linum usitatissimum* (Lus) using the GOR4 database

S.No.	Gene ID	AA	No. of coils	No. of helix	No. of sheets
1.	Lus10003328	388	204	111	73
2.	Lus10004028	340	157	98	85
3.	Lus10007599	628	324	195	109
4.	Lus10009821	320	169	101	50
5.	Lus10010403	314	135	124	55
6.	Lus10012143	314	141	111	62
7.	Lus10012145	312	126	117	69
8.	Lus10012146	75	46	4	25
9.	Lus10012147	328	148	114	66
10.	Lus10021709	195	111	52	32
11.	Lus10021740	338	170	85	83
12.	Lus10022632	387	208	104	75
13.	Lus10023096	352	171	103	78
14.	Lus10023097	176	87	48	41
15.	Lus10023557	460	201	185	74
16.	Lus10023558	310	141	88	81
17.	Lus10024472	245	126	78	41
18.	Lus10026348	305	167	82	56
19.	Lus10026350	310	154	80	76
20.	Lus10026351	311	169	62	80
21.	Lus10032369	289	135	93	61
22.	Lus10032470	372	184	111	77
23.	Lus10035016	327	164	96	67
24.	Lus10035058	342	197	74	71
25.	Lus10040442	305	161	68	76
26.	Lus10040443	295	147	66	82
27.	Lus10042311	311	163	71	77
28.	Lus10042312	261	129	88	44
29.	Lus10042313	265	146	69	50
30.	Lus10042968	266	137	73	56

4.1.2 EST analysis : EST analysis results in similarity index of standard genes with other existing genes using nucleotide BLAST (**Table 4.3**). ESTs analysis were done for all the identified genes and this resulted in similarity index of standard gene with other existing genes using nucleotide BLAST. 22 genes were selected out of 30 genes, in accordance to their similarity index (more than 95 % for all the hits obtained), which were based on EST analysis.

Table 4.3 ESTs analysis of *PLR2* genes of Flax

Gene ID	QUERY ID	QUERY LENGTH	RESULT SEQUENCE/QUERY ID	LENGTH OF MATCHING SEQUENCE	SUBJECT START-END	QUERY START-END	STRANDS	GAPS	GAP%	IDENTITY %
Lus10003328	lcl|Query_48899	1167	JG172467.1	826	1-721	445-1165	+/+	0	0%	100%
			JG186662.1	765	1-721	446-1166	+/+	0	0%	99%
			JG178292.1	578	1-541	450-990	+/+	0	0%	99%
			JG181798.1	879	1-841	154-994	+/+	0	0%	99%
			JG177835.1	864	1-841	200-1039	+/+	1	0%	99%
			JG109792.1	642	642-162	685-1165	+/-	0	0%	99%
			JG066070.1	832	1-721	445-1165	+/+	0	0%	99%
			JG068860.1	905	1-900	154-1054	+/+	1	0%	99%
			JG070888.1	573	1-537	155-695	+/+	4	0%	99%
			JG065657.1	793	1-661	450-1110	+/+	0	0%	96%
			JG171018.1	850	1-661	450-1110	+/+	0	0%	96%
			JG071133.1	802	1-781	154-934	+/+	0	0%	96%
			JG223953.1	849	1-661	450-1109	+/+	1	0%	96%
			JG173178.1	814	1-661	450-1110	+/+	0	0%	96%
			JG222771.1	321	1-301	154-454	+/+	0	0%	96%
			JG072696.1	926	1-901	154-1054	+/+	0	0%	96%
			JG070684.1	719	1-661	450-1110	+/+	0	0%	96%
			JG230635.1	904	2-902	158-1058	+/+	0	0%	96%
			JG065718.1	768	1-661	449-1109	+/+	0	0%	96%
			JG070150.1	701	1-657	154-814	+/+	4	0%	95%

Lus10004028	lcl|Query_25985	1023	JG242399.1	705	705-105	388-988	+/-	0	0%	100%
			JG097238.1	687	687-87	415-1015	+/-	0	0%	100%
			JG191835.1	743	Range 1: 743-263 Range 2: 220-40	158-638 827-1007	+/- +/-	0 0	0% 0%	100% 100%
			JG210115.1	517	517-97	589-1009	+/-	0	0%	100%
			JG204621.1	679	Range 1:679-319 Range 2: 277-97	277-637 825-1005	+/- +/-	0 0	0% 0%	100% 98%
			JG083413.1	312	312-132	796-976	+/-	0	0%	100%
			JG081738.1	498	Range1:498-318 Range2: 280-100	461-641 825-1005	+/- +/-	0 0	0% 0%	100% 100%
			JG211728.1	209	1-61	912-972	+/+	0	0%	100%
			JG247773.1	757	757-97	343-1003	+/-	0	0%	99%
			JG242520.1	757	757-97	344-1004	+/-	0	0%	99%
			JG098354.1	707	707-107	398-998	+/-	0	0%	99%
			JG098124.1	692	692-92	412-1012	+/-	0	0%	99%
			JG237383.1	687	687-87	415-1015	+/-	0	0%	99%
			JG199479.1	612	612-72	480-1020	+/-	0	0%	99%
			JG243232.1	495	495-75	587-1007	+/-	0	0%	99%
			JG100571.1	629	Range 1: 629-269 Range2: 228-108	279-639 879-999	+/- +/-	0 0	0% 0%	99% 99%
			JG242642.1	704	704-104	372-972	+/-	0	0%	99%
			JG238488.1	691	691-91	385-985	+/-	0	0%	99%
			JG235090.1	710	710-110	390-990	+/-	0	0%	99%

			JG236387.1	707	707-107	398-998	+/-	0	0%	99%
			JG090606.1	690	690-90	415-1015	+/-	0	0%	99%
			JG196137.1	658	658-59	416-1016	+/-	1	0%	99%
			JG055038.1	660	660-120	462-1002	+/-	0	0%	99%
			JG084698.1	323	323-83	782-1022	+/-	0	0%	99%
			JG238489.1	747	747-87	329-989	+/-	0	0%	99%
			JG048015.1	723	723-63	353-1013	+/-	0	0%	99%
			JG232673.1	678	678-78	388-988	+/-	0	0%	99%
			JG090035.1	680	680-140	425-965	+/-	0	0%	99%
			JG241946.1	419	Range1: 274-94 Range2: 419-299	Range1: 825-1005 Range2: 534-654	+/- +/-	1 0	0% 0%	99% 100%
			JG230808.1	592	592-112	514-994	+/-	0	0%	99%
			JG246618.1	718	718-118	388-988	+/-	0	0%	99%
			JG041819.1	691	691-91	391-991	+/-	0	0%	99%
			JG236813.1	695	695-95	398-998	+/-	0	0%	99%
			JG233596.1	692	692-92	415-1015	+/-	0	0%	99%
			JG243371.1	578	578-38	430-970	+/-	0	0%	99%
			GW866675.1	894	1-781	238-1018	+/+	0	0%	99%
			JG242051.1	594	594-114	515-995	+/-	0	0%	99%
			JG243781.1	575	575-95	516-996	+/-	0	0%	98%
			JG236927.1	593	593-113	516-996	+/-	0	0%	98%
			JG081845.1	70	70-10	660-720	+/-	0	0%	97%
			JG095202.1	232	232-112	870-990	+/-	0	0%	97%
Lus10007599	lcl|Query_8265	1887	GO511141.1	702	189	1023	+/+	0	0%	100%
			JG152477.1	706	706-166	1293-1833	+/-	0	0%	99%
			JG238236.1	675	675-135	371-911	+/-	1	0%	99%
			JG232954.1	715	715-175	375-915	+/-	1	0%	99%
			JG096801.1	569	569-149	523-943	+/-	1	0%	99%
			JG241703.1	704	704-104	346-945	+/-	1	0%	99%

			JG249601.1	725	725-185	362-902	+/-	1	0%	99%
			JG248970.1	730	730-190	375-915	+/-	1	0%	99%
			JG095717.1	713	713-173	375-915	+/-	1	0%	99%
			JG241117.1	729	729-189	376-916	+/-	1	0%	99%
			JG242857.1	701	701-161	404-994	+/-	1	0%	99%
			JG251327.1	597	597-177	508-928	+/-	1	0%	99%
			JG231605.1	717	717-177	377-917	+/-	1	0%	99%
			JG243251.1	714	714-174	377-917	+/-	1	0%	99%
			JG237800.1	722	722-182	383-923	+/-	1	0%	99%
			JG240247.1	671	671-191	423-902	+/-	2	0%	99%
			JG093135.1	680	680-200	425-905	+/-	1	0%	99%
			JG249335.1	669	669-189	438-918	+/-	1	0%	99%
			JG236360.1	728	728-68	280-940	+/-	1	0%	99%
			JG077939.1	451	451-31	507-927	+/-	1	0%	99%
			JG236562.1	728	728-188	368-907	+/-	2	0%	99%
			JG235005.1	720	720-182	369-909	+/-	3	0%	99%
			JG251273.1	672	672-132	373-913	+/-	1	0%	99%
			JG232705.1	716	716-176	375-915	+/-	1	0%	99%
			JG234278.1	582	582-162	508-928	+/-	1	0%	99%
			JG237972.1	377	377-197	728-908	+/-	1	0%	99%
			JG091800.1	371	371-191	734-913	+/-	2	0%	99%
			JG230993.1	691	691-91	314-914	+/-	1	0%	99%
			JG091068.1	659	659-179	429-909	+/-	1	0%	99%
			JG245398.1	671	670-190	436-916	+/-	1	0%	99%
			JG236963.1	653	653-173	441-921	+/-	1	0%	99%
			JG218102.1	872	Range1: 336-816 Range2: 53-233	1151-1629 943-1123	+/+ +/+	3 0	0% 0%	99% 99%
			JG143529.1	441	441-201	651-891	+/-	1	0%	99%
			JG244738.1	731	731-191	375-915	+/-	1	0%	99%
			JG236801.1	424	424-184	668-908	+/-	1	0%	99%
			JG245007.1	585	585-165	508-928	+/-	1	0%	99%
			JG239938.1	443	443-203	662-902	+/-	1	0%	99%
			JG085440.1	437	437-197	668-908	+/-	1	0%	99%
			JG139797.1	135	132-12	724-844	+/-	0	0%	98%
			JG241837.1	436	436-196	668-908	+/-	1	0%	98%
			JG218398.1	136	15-135	943-1063	+/+	0	0%	98%

			JG077934.1	297	297-57	559-799	+/-	0	0%	97%
			JG237212.1	481	481-181	611-911	+/-	1	0%	97%
			JG232910.1	688	688-148	371-894	+/-	18	3%	97%
			JG249801.1	187	187-7	668-848	+/-	0	0%	96%
Lus10009821	lcl|Query_13031	963	JG237221.1	677	677-197	446-926	+/-	0	0%	100%
			JG237546.1	626	626-206	523-943	+/-	0	0%	99%
Lus10010403	lcl|Query_21961	945	JG246334.1	719	719-179	364-904	+/-	0	0%	100%
			JG218823.1	283	1-121	802-922	+/+	0	0%	100%
			JG234153.1	186	186-66	802-922	+/-	0	0%	100%
			JG237335.1	281	281-161	802-922	+/-	0	0%	100%
			JG097376.1	271	271-151	814-934	+/-	0	0%	100%
			JG090464.1	752	752-152	317-917	+/-	0	0%	99%
			JG236018.1	620	620-140	449-929	+/-	0	0%	99%
Lus10012143	lcl|Query_56599	945	JG232993.1	758	758-38	192-912	+/-	0	0%	100%
			JG228859.1	369	1-361	393-753	+/+	0	0%	100%
			JG090550.1	727	727-127	341-941	+/-	0	0%	99%
			JG243769.1	641	641-161	439-919	+/-	0	0%	99%
			JG227690.1	887	1-841	40-880	+/+	1	0%	99%
			JG227867.1	881	1-841	40-880	+/+	0	0%	99%
			JG226946.1	854	1-841	40-878	+/+	2	0%	99%
			JG096807.1	509	509-149	573-933	+/-	0	0%	99%
			JG235703.1	737	737-17	213-933	+/-	0	0%	99%
			JG242485.1	718	718-178	364-904	+/-	0	0%	99%
			JG096361.1	705	705-165	366-906	+/-	0	0%	99%
			JG166606.1	647	647-47	303-903	+/-	0	0%	99%
			JG098278.1	275	275-35	648-888	+/-	0	0%	99%
			JG100408.1	611	611-131	411-891	+/-	0	0%	99%
			JG230941.1	574	574-154	495-915	+/-	0	0%	99%
			JG221804.1	180	1-121	485-605	+/+	0	0%	99%
			JG241432.1	597	597-177	468-888	+/-	0	0%	98%
Lus10012145	lcl|Query_23415	939	JG249000.1	722	722-182	340-880	+/-	0	0%	99%
			JG234760.1	708	708-168	352-892	+/-	0	0%	99%
			JG247508.1	613	613-313	639-939	+/-	0	0%	99%
			JG234571.1	720	720-180	340-880	+/-	0	0%	99%
			JG240429.1	629	629-149	432-912	+/-	0	0%	99%
			JG233437.1	607	607-127	454-934	+/-	0	0%	99%
			JG095425.1	711	Range1:711-531 Range2:	559-739 768-888	+/- +/-	0 0	0% 0%	99% 99%

					236-116					
Lus10012147	lcl|Query_316789	987	JZ368914.1	494	375	355	+/+	0	0%	100%
			JG847607.1	731	501	355	+/+	0	0%	100%
			JG850674.1	549	255	355	+/+	0	0%	100%
			JG858343.1	605	499	355	+/+	0	0%	100%
			EV495147.1	504	350	355	+/+	0	0%	100%
			ES826606.1	624	351	355	+/+	0	0%	100%
			ES810478.1	1357	37	355	+/+	0	0%	100%
			ES840187.1	1271	258	355	+/+	0	0%	100%
			ES841146.1	961	322	355	+/+	0	0%	100%
			ES816117.1	1078	139	355	+/+	0	0%	100%
			ES820172.1	805	370	355	+/+	0	0%	100%
			ES801081.1	1146	450	355	+/+	0	0%	100%
			ES803169.1	677	354	355	+/+	0	0%	100%
			ES805694.1	1226	361	355	+/+	0	0%	100%
			DW488810.1	604	225	355	+/+	0	0%	100%
			DW488809.1	604	379	355	+/+	0	0%	100%
			DW483917.1	561	207	355	+/+	0	0%	100%
			DW238429.1	677	188	355	+/+	0	0%	100%
			DT574220.1	915	346	355	+/+	0	0%	100%
			JG236360.1	728	Range1: 728-428	280-580	+/-	0	0%	96%
			JG230993.1	691	Range1: 691-451	314-554	+/-	0	0%	96%
			JG232910.1	688	Range1: 686-506	373-553	+/-	0	0%	96%
			JG241703.1	704	Range1: 704-464	346-586	+/-	0	0%	96%
			JG251273.1	672	Range1: 672-492	373-553	+/-	0	0%	96%
			JG249601.1	725	Range1: 725-485	362-602	+/-	0	0	95%
Lus10021740	lcl|Query_51277	1017	JG214073.1	764	12-552	471-1011	+/+	0	0%	100%

			JG023580.1	651	651-231	577-997	+/-	0	0%	99%
Lus10022632	lcl|Query_25303	1164	JG065657.1	793	1-661	447-1107	+/+	0	0%	100%
			JG171018.1	850	1-661	447-1107	+/+	0	0%	100%
			JG223953.1	849	1-661	447-1106	+/+	1	0%	99%
			JG173178.1	814	1-661	447-1107	+/+	0	0%	99%
			JG070684.1	719	1-661	447-1107	+/+	0	0%	99%
			JG065718.1	768	1-661	446-1106	+/+	0	0%	99%
			JG071133.1	802	1-781	151-931	+/+	0	0%	99%
			JG072696.1	926	1-901	151-1051	+/+	0	0%	99%
			JG230635.1	904	2-902	155-1055	+/+	0	0%	99%
			JG070150.1	701	1-657	151-811	+/+	4	0%	99%
			JG222771.1	321	1-301	151-451	+/+	0	0%	98%
			JG172467.1	826	1-721	442-1162	+/+	0	0%	96%
			JG186662.1	765	3-663	445-1105	+/+	0	0%	96%
			JG066070.1	832	1-721	442-1162	+/+	0	0%	96%
			JG178292.1	578	1-541	447-987	+/+	0	0%	96%
			JG109792.1	642	642-162	682-1162	+/-	0	0%	96%
			JG177835.1	864	3-843	199-1038	+/+	1	0%	96%
			JG067000.1	270	1-241	151-391	+/+	0	0%	96%
			JG068860.1	905	1-900	151-1051	+/+	1	0%	95%
			JG181798.1	879	1-841	151-991	+/+	0	0%	95%
			JG070888.1	573	1-537	152-692	+/+	4	0%	95%
Lus10023096	lcl|Query_22421	1059	CP014518.1	4410241	2956659	27	+/+	0	0%	100%
			AP024525.1	4413566	2870410	27	+/+	0	0%	100%

			CP027627.1	26636119	Range1: 21763099-21762739 Range2: 21764402-21764042 Range4: 21762600-21762480 Range5: 21764605-21764545	501-861 118-478 915-1035 1-61	+/- +/- +/- +/-	1 0 0 0	0% 0% 0% 0%	99% 100% 100% 100%
			CP027629.1	17699753	Range1: 3323197-3323557 Range3: 3324189-3324309 Range4: 3324429-3324549 Range5: 3322993-3323053	118-478 757-877 912-1032 1-61	+/+ +/+ +/+ +/+	0 1 0 0	0% 0% 0% 0%	94% 96% 96% 96%
Lus10023557	lcl|Query_390487	1383	JG089829.1	542	541-181	997-1357	+/-	0	0%	100%
			GW864238.1	553	Range1:1-121 Range2: 248-308	1177-1297 1314-1374	+/+ +/+	0 0	0% 0%	100% 100%
			JG286961.1	590	589-229	997-1357	+/-	0	0%	99%

			JG162389.1	483	482-122	997-1357	+/-	0	0%	99%
			JG272594.1	625	609-249	991-1350	+/-	0	0%	99%
			JG159775.1	592	548-188	991-1350	+/-	1	0%	99%
			JG290538.1	592	591-231	1007-1367	+/-	0	0%	99%
			JG266819.1	635	600-240	991-1346	+/-	5	1%	98%
			JG054527.1	678	Range1: 649-469 Range2: 378-258	1021-1201 1223-1343	+/- +/-	0 0	0% 0%	98% 95%
			JG025142.1	666	Range1: 666-486 Range2: 402-282	1028-1208 1223-1343	+/- +/-	0 0	0% 0%	97% 95%
			JG147174.1	715	649-349	1021-1321	+/-	0	0%	97%
			JG162789.1	764	724-424	1021-1321	+/-	0	0%	97%
			JG140210.1	660	614-314	1021-1321	+/-	0	0%	97%
			JG164270.1	678	604-304	1021-1321	+/-	0	0%	97%
			JG130615.1	562	562-322	1116-1356	+/-	0	0%	97%
			JG136929.1	506	506-326	1150-1330	+/-	0	0%	96%
			JG154359.1	758	692-392	1021-1321	+/-	0	0%	96%
			JG261106.1	640	604-304	1021-1321	+/-	0	0%	96%
			JG140966.1	700	634-334	1021-1321	+/-	0	0%	96%
			JG271532.1	640	604-304	1021-1321	+/-	0	0%	96%
			JG261296.1	761	697-398	1021-1321	+/-	1	0%	96%

			JG237826.1	601	601-361	1116-1356	+/-	0	0%	96%
			JG144198.1	615	582-282	1021-1321	+/-	0	0%	96%
			JG089504.1	686	650-350	1021-1321	+/-	0	0%	96%
			JG232456.1	656	600-300	1021-1321	+/-	0	0%	96%
			JG048971.1	701	635-335	1021-1321	+/-	0	0%	96%
			JG123524.1	679	605-305	1021-1321	+/-	0	0%	96%
			JG145899.1	162	114-54	1021-1081	+/-	0	0%	96%
			JG019416.1	499	499-319	1152-1332	+/-	0	0%	96%
			JG100820.1	724	650-350	1021-1321	+/-	0	0%	96%
			JG265031.1	607	607-187	448-868	+/-	2	0%	95%
Lus10023558	lcl|Query_60177	933	JG286222.1	640	640-220	469-889	+/-	0	0%	99%
			JG053694.1	568	568-208	542-902	+/-	0	0%	99%
			JG249188.1	722	722-242	408-888	+/-	0	0%	99%
			JG248684.1	697	697-217	411-891	+/-	0	0%	99%
Lus10024472	lcl|Query_53737	738	JG275016.1	710	710-350	328-688	+/-	0	0%	100%
Lus10026348	lcl|Query_31975	918	JG093299.1	745	745-145	287-887	+/-	0	0%	100%
			JG245397.1	602	602-182	444-864	+/-	0	0%	100%
			JG027408.1	454	454-154	574-874	+/-	0	0%	100%
			JG246234.1	583	583-163	444-864	+/-	0	0%	99%
			JG243195.1	684	684-144	363-902	+/-	1	0%	99%
			JG233531.1	617	617-197	444-864	+/-	0	0%	99%
			JG251481.1	716	716-116	314-914	+/-	0	0%	99%
			JG216082.1	272	5-185	719-899	+/+	0	0%	98%
			JG234862.1	269	269-29	627-867	+/-	0	0%	97%
			JG265660.1	267	267-147	745-865	+/-	0	0%	97%
			JG215102.1	459	12-312	600-900	+/+	0	0%	96%
			JG061034.1	456	12-312	600-900	+/+	0	0%	96%
			EX720330.1	481	2-302	600-900	+/+	0	0%	96%
			JG218027.1	863	12-852	61-901	+/+	0	0%	96%
			JG151249.1	342	341-281	816-876	+/-	0	0%	96%
			JG224071.1	796	7-487	401-881	+/+	0	0%	96%

			JG225855.1	789	1-481	407-887	+/+	0	0%	96%
			JG178870.1	637	1-481	408-888	+/+	0	0%	96%
			JG072251.1	620	1-481	410-890	+/+	0	0%	96%
			JG227033.1	674	1-481	410-890	+/+	0	0%	96%
			JG225674.1	768	1-481	410-890	+/+	0	0%	96%
			JG244835.1	661	661-181	387-867	+/-	0	0%	96%
			JG041549.1	516	516-276	678-918	+/-	0	0%	96%
			JG241619.1	725	725-125	317-917	+/-	0	0%	96%
			JG087631.1	727	727-187	320-860	+/-	0	0%	96%
			JG231588.1	726	726-186	320-860	+/-	0	0%	96%
			JG243301.1	725	725-185	320-860	+/-	0	0%	96%
			JG245875.1	725	725-185	321-861	+/-	0	0%	96%
			JG217962.1	376	12-192	680-860	+/+	0	0%	96%
			JG246171.1	744	744-144	302-902	+/-	0	0%	96%
			JG238765.1	673	670-190	382-862	+/-	0	0%	96%
			JG223006.1	776	1-481	410-890	+/+	0	0%	96%
			JG246261.1	729	729-189	321-861	+/-	0	0%	96%
			JG264491.1	471	471-171	574-874	+/-	0	0%	96%
			JG288988.1	719	716-176	334-874	+/-	0	0%	96%
			JG227107.1	786	1-481	407-886	+/+	0	0%	96%
			JG223110.1	786	1-481	407-887	+/+	0	0%	96%
			JG094508.1	742	742-322	444-864	+/-	0	0%	95%
			JG249066.1	724	724-184	322-862	+/-	0	0%	95%
			JG221262.1	723	1-421	462-882	+/+	0	0%	95%
			JG223768.1	724	1-421	462-882	+/+	0	0%	95%
			JG091817.1	656	655-295	532-892	+/-	0	0%	95%
			JG234990.1	659	659-299	534-894	+/-	0	0%	95%
			JG242068.1	527	526-166	520-880	+/-	0	0%	95%
			JG119466.1	461	388-148	661-901	+/-	0	0%	95%
			JG235582.1	658	658-299	532-892	+/-	1	0%	95%
Lus10032369	lcl|Query_14767	870	JG204328.1	794	Range1: 381-81	568-868	+/-	0	0%	97%
			JG111816.1	680	Range1: 379-79 Range2: 680-620	568-868 438-498	+/- +/-	0 0	0% 0%	96% 97%
			JG094094.1	711	Range1:392-92	568-868 420-480	+/-	0	0%	96%

					Range2: 711-651		+/-	0	0%	98%
			JG202971.1	693	Range1:392-92 Range2:693-633	568-868 438-498	+/- +/-	0 0	0% 0%	96% 97%
			JG206819.1	752	Range1: 407-107 Range2: 751-691	568-868 391-451	+/- +/-	0 0	0% 0%	95% 97%
Lus10032470	lcl|Query_229919	1119	JG056846.1	656	656-182	611-1091	+/-	6	1%	96%
Lus10040442	lcl|Query_62759	918	JG265031.1	607	607-187	448-868	+/-	0	0%	99%
Lus10040443	lcl|Query_40343	888	JG291504.1	596	Range1: 525-165 Range2: 548	500-860 446	+/- +/-	0 0	0% 0%	99% 100%
Lus10042312	lcl|Query_312189	786	CV478569.1	738	1-241	535-774	+/+	1	0%	99%
Lus10042313	lcl|Query_30151	798	JG218027.1	863	129-849	58-778	+/+	0	0%	100%
			JG241619.1	725	725-125	197-797	+/-	0	0%	100%
			JG246171.1	744	744-144	182-782	+/-	0	0%	100%
			JG087631.1	727	727-187	200-740	+/-	0	0%	100%
			JG231588.1	726	726-186	200-740	+/-	0	0%	100%
			JG243301.1	725	725-185	200-740	+/-	0	0%	100%
			JG245875.1	725	725-185	201-741	+/-	0	0%	100%
			JG224071.1	796	7-487	281-761	+/+	0	0%	100%
			JG225855.1	789	1-481	287-767	+/+	0	0%	100%
			JG178870.1	637	1-481	288-768	+/+	0	0%	100%
			JG072251.1	620	1-481	290-770	+/+	0	0%	100%
			JG227033.1	674	1-481	290-770	+/+	0	0%	100%
			JG225674.1	768	1-481	290-770	+/+	0	0%	100%
			JG215102.1	459	12-312	480-780	+/+	0	0%	100%
			JG061034.1	456	12-312	480-780	+/+	0	0%	100%
			EX720330.1	481	2-302	480-780	+/+	0	0%	100%
			JG041549.1	516	516-276	558-798	+/-	0	0%	100%
			JG217962.1	376	12-192	560-740	+/+	0	0%	100%
			JG216082.1	272	1-181	595-775	+/+	0	0%	100%
			JG246261.1	729	729-189	201-741	+/-	0	0%	99%
			JG244835.1	661	661-181	267-747	+/-	0	0%	99%
			JG223006.1	776	1-481	290-770	+/+	0	0%	99%

			JG094508.1	742	742-322	324-744	+/-	0	0%	99%
			JG242068.1	527	527-167	399-759	+/-	0	0%	99%
			JG091817.1	656	656-296	411-771	+/-	0	0%	99%
			JG234990.1	659	659-299	414-774	+/-	0	0%	99%
			JG264491.1	471	471-171	454-754	+/-	0	0%	99%
			JG288988.1	719	719-179	211-751	+/-	0	0%	99%
			JG238765.1	673	673-193	259-739	+/-	0	0%	99%
			JG227107.1	786	1-481	287-766	+/+	1	0%	99%
			JG223110.1	786	1-481	287-767	+/+	0	0%	99%
			JG221262.1	723	1-421	342-762	+/+	0	0%	99%
			JG223768.1	724	1-421	342-762	+/+	0	0%	99%
			JG249066.1	724	724-184	202-742	+/-	0	0%	99%
			JG235582.1	658	658-299	412-772	+/-	1	0%	99%
			JG221836.1	360	1-301	268-568	+/+	0	0%	99%
			JG088269.1	590	590-170	366-786	+/-	0	0%	99%
			JG151249.1	342	341-281	696-756	+/-	0	0%	99%
			JG265660.1	267	267-147	625-745	+/-	0	0%	99%
			JG119466.1	461	388-148	541-781	+/-	0	0%	99%
			JG281344.1	365	365-185	561-741	+/-	0	0%	98%
			JG027408.1	454	454-154	454-754	+/-	0	0%	96%
			JG093299.1	745	745-145	167-767	+/-	0	0%	96%
			JG245397.1	602	602-182	324-744	+/-	0	0%	96%
			JG246234.1	583	583-163	324-744	+/-	0	0%	95%
			JG243195.1	684	684-144	243-782	+/-	1	0%	95%
			JG251481.1	716	714-114	196-796	+/-	0	0%	95%
			JG233531.1	617	617-197	324-744	+/-	0	0%	95%
Lus10042968	lcl|Query_36671	801	JG056846.1	656	647-167	308-788	+/-	0	0%	100%

4.1.3 Properties of LuPLR2 proteins: Physiochemical properties of PLR2 proteins were analysed in which molecular weight ranged from 27.69 kDa to 70.66 kDa and pI value ranged from 5.20-9.60 (**Table 4.4**). Lus10024472 was found to be smallest protein with amino acid length of 245 and Lus10007599 was the largest protein amongst all with 628 amino acid length. In terms of localization (subcellular), LOCTREE3 analysis revealed that most of the genes were located in cytoplasm (20) while some were found to be present in plastids (2). Pfam and HMMER databases were used for domain analysis of all the genes. Out of 22 genes, 5 genes had multiple domains while 17 had single domains. The conserved domain analysis of *PLR2* genes demonstrated remarkable uniformity with most of the genes containing a single domain belonging to NmrA like family domain (**Table 4.4**).

Table 4.4 Physiochemical properties of PLR2 proteins in flax: Molecular weights (MW), isoelectric point (pI value), Coding sequence (CDS), amino acids (AA), Domains, subcellular localization

S.no.	Gene Id	Molecular weight (kDa)	pI	CDS	AA	Domain SD/MD	Subcellular Localization
1.	Lus10003328	42.759	8.93	1167	388	SD	Plastid
2.	Lus10004028	37.70808	5.77	1023	340	SD	cytoplasm
3.	Lus10007599	70.66526	6.22	1887	628	MD	cytoplasm
4.	Lus10009821	35.12244	6.46	963	320	SD	cytoplasm
5.	Lus10010403	35.2574	5.76	945	314	SD	cytoplasm
6.	Lus10012143	35.29341	5.64	945	314	SD	cytoplasm
7.	Lus10012145	35.03219	5.34	939	312	SD	cytoplasm
8.	Lus10012147	36.50096	6.22	987	328	SD	cytoplasm
9.	Lus10021740	37.23795	8.16	1017	338	SD	cytoplasm
10.	Lus10022632	42.5811	9.02	1164	387	SD	plastid
11.	Lus10023096	38.33078	5.61	1059	352	SD	cytoplasm
12.	Lus10023557	51.21092	9.60	1383	460	SD	cytoplasm
13.	Lus10023558	34.50371	7.05	933	310	SD	cytoplasm
14.	Lus10024472	27.69767	5.88	738	245	SD	cytoplasm
15.	Lus10026348	33.38787	5.32	918	305	MD	cytoplasm
16.	Lus10032369	32.12463	5.86	870	289	SD	cytoplasm

17.	Lus10032470	40.61839	5.83	1119	372	SD	cytoplasm
18.	Lus10040442	33.72063	8.27	918	305	SD	cytoplasm
19.	Lus10040443	32.73163	8.57	888	295	MD	cytoplasm
20.	Lus10042312	29.19415	5.20	786	261	MD	cytoplasm
21.	Lus10042313	29.52978	5.36	798	265	SD	cytoplasm
22.	Lus10042968	28.92198	5.45	801	266	MD	cytoplasm

4.1.4 Conserved motif analysis: Protein sequence motifs are signatures of protein families and can often be used as tools for the prediction of protein function (**Figure 4.1**). Motif analysis for PLR2 proteins was performed using web server MEME 5.4.0 (https://memesuite.org/meme/) (Bailey et al., 2009), identified 15 different motifs, out of which four motifs were associated with NmrA family which were validated by performing domain analysis of individual motifs (**Table 4.5**).

Table 4.5 Motif analysis of PLR2 proteins in flax

S.No	MOTIF	MOTIF SEQUENCE	WIDTH of motif	PUTATIVE FUNCTION OF MOTIF (HMMER)	No. of genes having this motif
1.	Motif 1	TIKAVEDPRTLNKTVYJRPPENVLSFNEL VAJWEKKIGKTLEKVYVPEEEFLKLIRE	57	NmrA- like family	14
2.	Motif 2	FDEKMVVRRAIEEAGIPFTYISANCFAGY FLGNLAQPGAJSPPRDKVIILGDGNVKA VYVDEDDIATY	68	NmrA- like family	11
3.	Motif 3	LEAGHPTYVLVRPETGLDIEKLQLLLSFK KAGAHLVEGSFNDHESLVDAVKLVDVV ICTVS	61	NmrA-like family	9
4.	Motif 4	KSKVLVIGGTGYJGKRJVEAS	21	-	18
5	Motif 5	FGVEASELYPDVKYTTVDEYL	21	-	15
6.	Motif6	IKEAGNVKRFJPSEFGNDPDR	21	-	12
7.	Motif 7	NVGLALGHSVFVEGCQTNFEI	21	-	15
8.	Motif 8	HTIIDCATGRPEEPIKTVDWEGKVALIQC AKAMGIQKYVFYSIHNCDKHPEVPLMEI KYCTEKFLKDSGLPHITIRLCGFMQGLIG QYAVPILEEKSVWGTDAPTRIAYMDTQ DIARLTFIALRNEKMNGKLLTFAGPRAW TTQEVIALC	150	NmrA like-family	2

9.	Motif 9	NGYTVHATLRSLDDKAKVGLLTSLPNA DTNLILFKADIYNPDQFZSAIDGCHFVFH VAYPLQHQSDSATYKDRIEAMAEGSKRI AESCVKSGTVKRLIYTASVMAASPLFDD GSEYGPTVDESCWTPLHVSFQYSDPFT	139	-	2
10.	Motif 10	HVEDVCEALVFCMEKKTPLKGRFVCAA GTLSVREIATYIRDRHPELHVDATLMGE GGKEIEVDNSKLKKMGFSYKYDTRGIIE ESLECAKRLGALPD	97	-	2
11.	Motif 11	FTRQLTRIFEWTSDVADRLAFSEVLSSDT VFSVPMGETYSMLGVDQKEIASLEKYLQ DYFSNILKKLKDLKAQSKQSDIY	80	-	2
12.	Motif 12	GGHQILLQLKLVEA	14	-	12
13.	Motif 13	ZGYGFRNLVRPKPAPADPLRDWGATILN GDJKDPELIPKTJ	41	-	4
14.	Motif 14	MGDALEPGRET	11	-	5
15.	Motif 15	GDVDDHESLVKAIKQVDIVIS	21	-	5

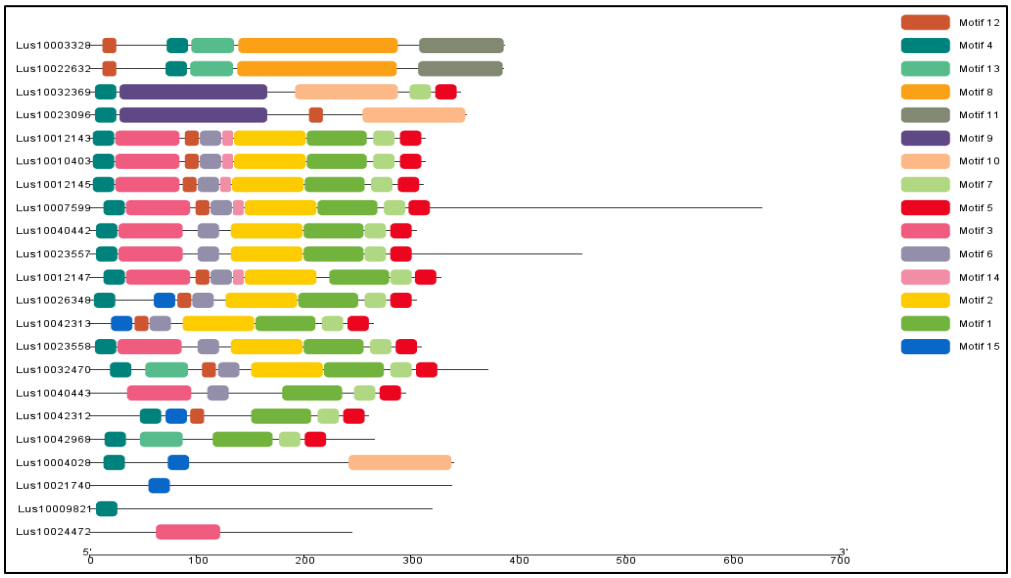

Figure 4.1: **Distribution of conserved motifs in PLR2 proteins of flax**

4.1.5 Analysis of exons-introns: Analysis of the exons-introns organization within *LuPLR2* gene family uncovered unique structural patterns. Gene structure was displayed and analysed using the GSDS2.0 server (**Figure 4.2)**. Maximum number of exons (12) and introns (11) were present in *Lus10007599* while no intron was present in *Lus10024472* (**Table 4.6**). No UTRs regions were reported.

Table 4.6 No. of exons and introns present in *PLR2* genes

Gene Id	No. of Exons	No. of introns
Lus10003328	8	7
Lus10004028	4	3
Lus10007599	12	11
Lus10009821	6	5
Lus10010403	4	3
Lus10012143	4	3
Lus10012145	4	3
Lus10012147	4	3
Lus10021740	5	4
Lus10022632	8	7
Lus10023096	4	3
Lus10023557	10	9
Lus10023558	5	4
Lus10026348	4	3
Lus10032369	4	3
Lus10032470	5	4
Lus10040442	5	4
Lus10040443	6	6
Lus10042312	5	4
Lus10042313	4	3
Lus10042968	4	3
Lus10024472	1	0

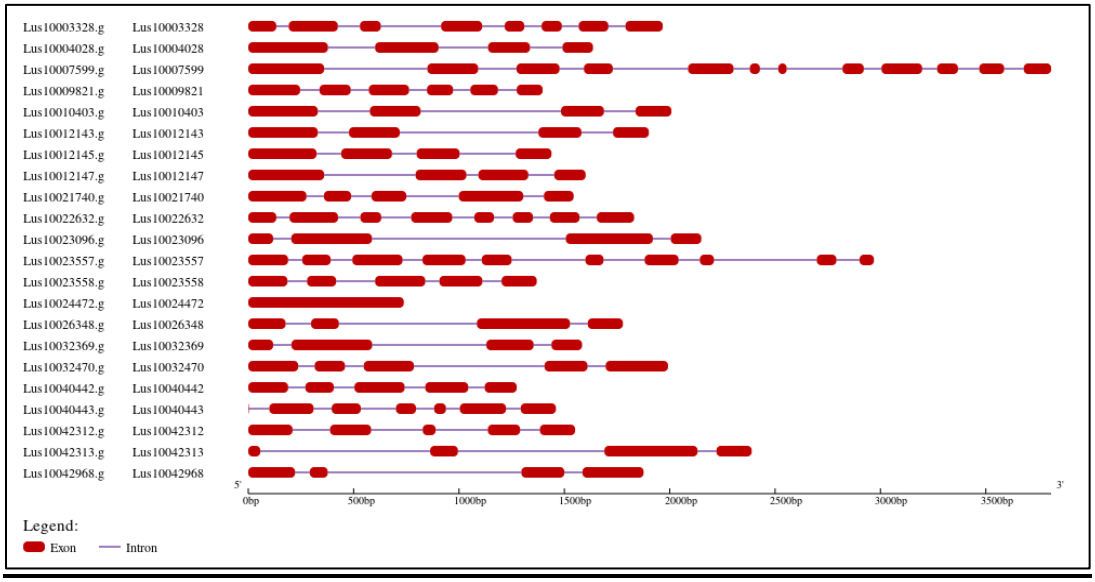

Figure 4.2: The exon/intron organization of *PLR2* genes in flax

4.1.6 GO annotation: The GO enrichment analysis conducted in this study provided insights into the functions of *LuPLR2* genes. Specifically, these genes were involved in the regulation of Nmr A-like family, in the biosynthesis of lignan and their metabolic processes, biosynthesis of various secondary metabolites, NADP(H) binding, oxidoreductase activity, phenylpropanoid biosynthesis process and its metabolic process, aromatic compound biosynthetic process, oxidation-reduction process, secondary metabolic process (**Table 4.7, Figure 4.4**). These genes were also found to be associated with NAD dependent epimerase/dehydratase family, and 3-beta hydroxysteroid dehydrogenase/isomerase family (**Figure 4.3**).

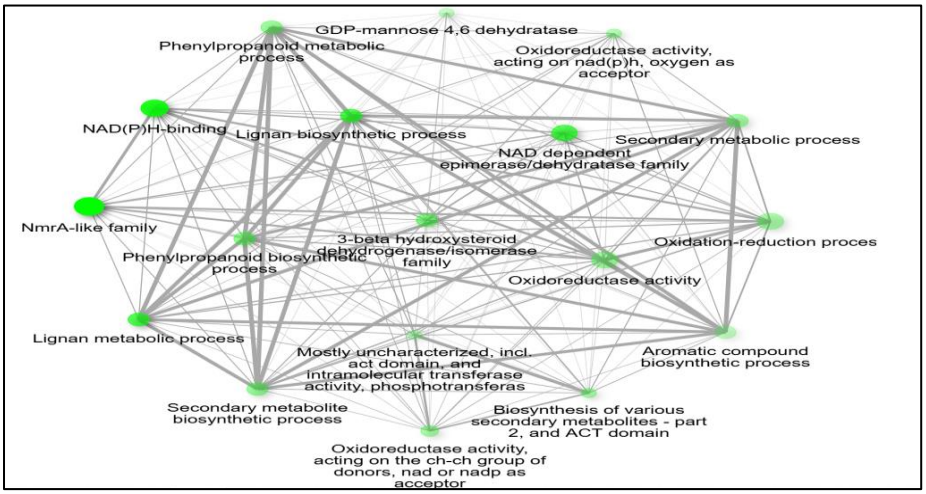

Figure 4.3: An interactive plot illustrates nodes and edges connecting multiple functional pathways. Darker nodes indicate more significant gene sets, larger nodes represent bigger gene sets, and thicker edges signify overlapping genes.

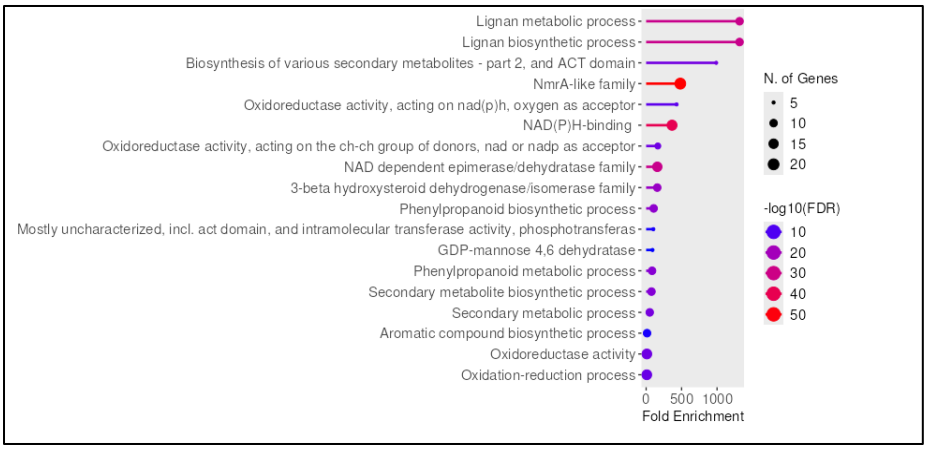

Figure 4.4: Gene ontology terms displaying the number of genes (x-axis) across various pathways in relation to fold enrichment (y-axis)

Table 4.7 Role of *LuPLR2* genes in the functions of various processes

S.No.	Fold Enrichment	nGenes (no. of genes involved in specific pathway)	Pathway Genes	Pathway	Genes
1.	481.9401	20	82	NmrA-like family (nitrogen metabolism and pathogenicity)	Lus10003328, Lus10004028, Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10021740 Lus10022632 Lus10023096 Lus10023557 Lus10023558 Lus10026348 Lus10032369 Lus10032470 Lus10040442 Lus10040443 Lus10042312 Lus10042313 Lus10042968
2	365.122	17	92	NAD(P)H-binding	Lus10003328 Lus10004028 Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10021740 Lus10022632 Lus10023096 Lus10023557 Lus10023558 Lus10026348 Lus10032369 Lus10040442 Lus10040443 Lus10042313
3	1317.303	10	15	Lignan metabolic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
4	1317.303	10	15	Lignan biosynthetic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
5	157.6559	15	188	NAD dependent epimerase/dehydratase family (metabolic pathways)	Lus10003328 Lus10004028 Lus10009821 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10021740 Lus10022632 Lus10023096 Lus10023557 Lus10026348 Lus10032369 Lus10040442 Lus10042968
6	156.8218	10	126	3-beta hydroxysteroid dehydrogenase/isomerase family (production of steroid hormones)	Lus10003328 Lus10004028 Lus10010403 Lus10012143 Lus10021740 Lus10023096 Lus10023557 Lus10026348 Lus10032369 Lus10040442
7	106.2341	10	186	Phenylpropanoid biosynthetic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
8	83.72689	10	236	Phenylpropanoid metabolic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
9	75.41811	10	262	Secondary metabolite biosynthetic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313

10	50.79575	10	389	Secondary metabolic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
11	987.9773	5	10	Biosynthesis of various secondary metabolites - part 2, and ACT domain	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147
12	10.48947	16	3014	Oxidoreductase activity (oxidation or reduction)	Lus10004028 Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10021740 Lus10023096 Lus10023557 Lus10023558 Lus10026348 Lus10032369 Lus10040442 Lus10040443 Lus10042312 Lus10042313
13	164.6629	7	84	Oxidoreductase activity, acting on the CH-CH group of donors, NAD or NADP as acceptor	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023558 Lus10040443
14	9.545674	16	3312	Oxidation-reduction process	Lus10004028 Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10021740 Lus10023096 Lus10023557 Lus10023558 Lus10026348 Lus10032369 Lus10040442 Lus10040443 Lus10042312 Lus10042313
15	449.0806	5	22	Biosynthesis of various secondary metabolites - part 2, and basic region leucin zipper	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147
16	95.39091	7	145	Oxidoreductase activity, acting on the CH-CH group of donors	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023558 Lus10040443
17	429.5553	5	23	Oxidoreductase activity, acting on NAD(P)H, oxygen as acceptor	Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
18	13.0426	10	1515	Aromatic compound biosynthetic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
19	101.8533	5	97	Mostly uncharacterized, incl. act domain, and intramolecular transferase activity, phosphotransferases	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147
20	89.81612	5	110	GDP-mannose 4,6 dehydratase (glycoconjugates biosynthesis)	Lus10004028 Lus10009821 Lus10021740 Lus10023096 Lus10032369
21	192.7761	4	41	Dihydrokaempferol 4-reductase activity	Lus10004028 Lus10021740 Lus10023096 Lus10032369

22	11.18887	10	1766	Organic cyclic compound biosynthetic process	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147 Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
23	141.1396	4	56	Polysaccharide biosynthesis protein	Lus10004028 Lus10021740 Lus10023096 Lus10032369
24	138.6635	4	57	RmlD substrate binding domain (dTDP-rhamnose biosynthetic process)	Lus10009821 Lus10021740 Lus10023096 Lus10032369
25	47.96006	5	206	Oxidoreductase activity, acting on nad(p)h	Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
26	92.9861	4	85	Male sterility protein (cytoplasmic male sterility)	Lus10004028 Lus10021740 Lus10023096 Lus10032369
27	219.5505	3	27	Flavanone 4-reductase activity (biosynthesis of anthocyanins)	Lus10004028 Lus10023096 Lus10032369
28	48.789	4	162	Flavonoid biosynthetic process	Lus10004028 Lus10021740 Lus10023096 Lus10032369
29	987.9773	2	4	Response to hydroperoxide (redox reactions)	Lus10023558 Lus10040443
30	131.7303	3	45	Anthocyanin-containing compound biosynthetic process	Lus10004028 Lus10023096 Lus10032369
31	45.95243	4	172	Flavonoid metabolic process	Lus10004028 Lus10021740 Lus10023096 Lus10032369
32	790.3818	2	5	Response to sorbitol, and NmrA-like family	Lus10032470 Lus10042968
33	107.7793	3	55	Anthocyanin-containing compound metabolic process	Lus10004028 Lus10023096 Lus10032369
34	85.91107	3	69	Flavonoid biosynthetic process, and anthocyanin biosynthesis	Lus10004028 Lus10023557 Lus10042312
35	232.4652	2	17	Mixed, incl. rhamnogalacturonan endolyase activity, and synaptotagmin-like mitochondrial-lipid-binding domain	Lus10023096 Lus10032369
36	20.1628	4	392	Oxidoreductase activity, acting on the CH-OH group of donors, nad or nadp as acceptor	Lus10004028 Lus10021740 Lus10023096 Lus10032369
37	11.93209	5	828	Plasmodesma (intercellular communication process)	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147
38	11.93209	5	828	Symplast (transport of water and nutrients)	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147

39	179.6322	2	22	DNA helicase complex (DNA replication)	Lus10023558 Lus10040443
40	11.4217	5	865	Cell-cell junction	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147
41	11.34302	5	871	Anchoring junction	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147
42	164.6629	2	24	Positive regulation of leaf senescence	Lus10023558 Lus10040443
43	164.6629	2	24	Positive regulation of leaf development	Lus10023558 Lus10040443
44	17.25724	4	458	Oxidoreductase activity, acting on CH-OH group of donors	Lus10004028 Lus10021740 Lus10023096 Lus10032369
45	146.367	2	27	Remorin, C-terminal region , and response to sorbitol (cellular signal transduction processes)	Lus10032470 Lus10042968
46	146.367	2	27	Photosystem II assembly	Lus10003328 Lus10022632
47	34.26511	3	173	Ketoreducatse (KR) domain (polyketides biosynthesis)	Lus10004028 Lus10023096 Lus10032369
48	9.939409	5	994	Cell junction	Lus10007599 Lus10010403 Lus10012143 Lus10012145 Lus10012147
49	109.7753	2	36	Mixed, incl. photosystem II assembly, and alkane catabolic process	Lus10003328 Lus10022632
50	26.94483	3	220	Pigment biosynthetic process	Lus10004028 Lus10023096 Lus10032369
51	98.79773	2	40	Mixed, incl. lignan metabolic process, and corpus callosum development	Lus10026348 Lus10042313
52	91.90486	2	43	Mixed, incl. photosystem ii assembly, and photosynthetic electron transport in photosystem i	Lus10003328 Lus10022632
53	22.71212	3	261	Pigment metabolic process	Lus10004028 Lus10023096 Lus10032369
54	75.99825	2	52	Mixed, incl. lipid transport, and rhamnogalacturonan endolyase activity	Lus10023096 Lus10032369
55	63.74047	2	62	Regulation of leaf senescence	Lus10023558 Lus10040443
56	58.11631	2	68	Mixed, incl. remorin, c-terminal region , and calmodulin-binding	Lus10032470 Lus10042968

57	47.61336	2	83	Circadian rhythm - plant, and DNA photolyase activity	Lus10023558 Lus10040443
58	43.42757	2	91	Mixed, incl. photosynthesis, light reaction, and protein of unknown function (duf1118)	Lus10003328 Lus10022632
59	39.51909	2	100	Photosystem ii	Lus10003328 Lus10022632
60	36.93373	2	107	Mixed, incl. circadian rhythm - plant, and phototropism	Lus10023558 Lus10040443
61	658.6515	1	3	Triphosphatase activity	Lus10024472
62	658.6515	1	3	These sequences are functionally identified as members of the adenylate cyclase family, which catalyses the conversion of ATP to 3,5-cyclic AMP and pyrophosphate.	Lus10024472
63	32.66041	2	121	Photosystem	Lus10003328 Lus10022632
64	31.87023	2	124	Translation initiation factor activity	Lus10003328 Lus10022632
65	31.87023	2	124	Regulation of leaf development	Lus10023558 Lus10040443
66	31.87023	2	124	ATPase complex	Lus10023558 Lus10040443
67	31.36436	2	126	Mixed, incl. remorin, c-terminal region , and cupin	Lus10032470 Lus10042968
68	30.87429	2	128	Mixed, incl. lipid transport, and endoplasmic reticulum membrane organization	Lus10023096 Lus10032369
69	30.63495	2	129	Mixed, incl. photosynthesis, light reaction, and quinone binding	Lus10003328 Lus10022632
70	395.1909	1	5	Prenol kinase activity, and sesquiterpenoid and triterpenoid biosynthesis	Lus10021740
71	395.1909	1	5	Chalcone-flavanone isomerase, and naringenin 3-dioxygenase activity	Lus10004028
72	27.06787	2	146	Translational initiation	Lus10003328 Lus10022632
73	27.06787	2	146	Photosynthesis, light reaction	Lus10003328 Lus10022632
74	26.17158	2	151	Mixed, incl. circadian rhythm - plant, and response to blue light	Lus10023558 Lus10040443

75	329.3258	1	6	Regulation of protein complex stability, and photosystem ii assembly	Lus10003328
76	329.3258	1	6	Flavanone 4-reductase activity, and zinc finger, c3hc4 type (ring finger)	Lus10032369
77	282.2792	1	7	CYTH domain (triphosphate tunnel metalloenzyme functions)	Lus10024472
78	246.9943	1	8	Transposition	Lus10009821
79	246.9943	1	8	Saccharopine dehydrogenase NADP binding domain (lysine catabolism process)	Lus10026348
80	197.5955	1	10	Domain of unknown function (DUF1995)	Lus10007599
81	18.55356	2	213	Translation factor activity, RNA binding	Lus10003328 Lus10022632
82	17.96322	2	220	Positive regulation of multicellular organismal process	Lus10023558 Lus10040443
83	17.33293	2	228	Translation regulator activity, nucleic acid binding	Lus10003328 Lus10022632
84	164.6629	1	12	Anthocyanin biosynthesis	Lus10023557
85	16.67472	2	237	Translation regulator activity	Lus10003328 Lus10022632
86	151.9965	1	13	Mixed, incl. chalcone-flavanone isomerase, and naringenin 3-dioxygenase activity	Lus10004028
87	16.13024	2	245	Positive regulation of developmental process	Lus10023558 Lus10040443
88	109.7753	1	18	Mostly uncharacterized, incl. pathogenesis-related protein bet VI family, and pronephros development	Lus10040442
89	103.9976	1	19	Mixed, incl. triphosphatase activity, and inorganic h+ pyrophosphatase	Lus10024472
90	13.53394	2	292	Photosynthesis	Lus10003328 Lus10022632

91	11.62326	2	340	Regulation of shoot system development	Lus10023558 Lus10040443
92	73.1835	1	27	Mixed, incl. prenylation, and prenylcysteine methylesterase activity	Lus10021740
93	48.19401	1	41	Flavonoid biosynthetic process, and epidermal cell fate specification	Lus10004028
94	8.704646	2	454	Thylakoid membrane (light-dependent reactions of photosynthesis)	Lus10003328 Lus10022632
95	42.95553	1	46	Sterol biosynthetic process, and lanosterol synthase activity	Lus10009821
96	42.95553	1	46	Mostly uncharacterized, incl. protein of unknown function (duf1644), and regulation of animal organ morphogenesis	Lus10040442
97	8.553916	2	462	Photosynthetic membrane (light reaction of photosynthesis)	Lus10003328 Lus10022632
98	8.165101	2	484	Chloroplast thylakoid membrane (light-dependent reactions of photosynthesis)	Lus10003328 Lus10022632
99	8.165101	2	484	Plastid thylakoid membrane (light reactions)	Lus10003328 Lus10022632
100	2.923875	5	3379	Plasma membrane (communication and signalling of cells)	Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
101	37.99913	1	52	Sterol biosynthetic process, and squalene-hopene cyclase n-terminal domain	Lus10009821
102	7.967559	2	496	Regulation of translation	Lus10003328 Lus10022632
103	7.779349	2	508	Regulation of cellular amide metabolic process	Lus10003328 Lus10022632
104	7.658739	2	516	Chloroplast thylakoid (light reactions of photosynthesis)	Lus10003328 Lus10022632
105	7.599825	2	520	Plastid thylakoid (light reactions)	Lus10003328 Lus10022632
106	7.044401	2	561	Response to oxidative stress	Lus10023558 Lus10040443

107	6.384344	2	619	Post-transcriptional regulation of gene expression	Lus10003328 Lus10022632
108	24.3945	1	81	Steroid biosynthetic process, and lanosterol synthase activity	Lus10009821
109	5.803097	2	681	Thylakoid (light reactions of photosynthesis)	Lus10003328 Lus10022632
110	19.95914	1	99	Mostly uncharacterized, incl. haus complex, and plant protein of unknown function (duf863)	Lus10040442
111	5.333211	2	741	Generation of precursor metabolites and energy	Lus10003328 Lus10022632
112	2.36528	5	4177	Cell periphery (mitosis, gene expression)	Lus10023557 Lus10026348 Lus10040442 Lus10042312 Lus10042313
113	5.06655	2	780	Chloroplast stroma (carbon cycle)	Lus10003328 Lus10022632
114	5.021486	2	787	Plastid stroma (carbon cycle)	Lus10003328 Lus10022632
115	4.837098	2	817	Regulation of multicellular organismal development	Lus10023558 Lus10040443
116	4.682357	2	844	Cellular protein-containing complex assembly	Lus10003328 Lus10022632
117	13.91517	1	142	Mixed, incl. acid phosphatase activity, and terpenoid backbone biosynthesis	Lus10021740
118	4.290889	2	921	Membrane protein complex	Lus10003328 Lus10022632
119	4.190784	2	943	Translation	Lus10003328 Lus10022632
120	4.195233	2	942	Regulation of multicellular organismal process	Lus10023558 Lus10040443
121	4.151165	2	952	Peptide biosynthetic process	Lus10003328 Lus10022632
122	3.987799	2	991	Protein-containing complex assembly	Lus10003328 Lus10022632
123	3.863059	2	1023	Formation of organelle sub-compartment	Lus10003328 Lus10022632
124	3.686482	2	1072	Amide biosynthetic process	Lus10003328 Lus10022632
125	3.649039	2	1083	Peptide metabolic process	Lus10003328 Lus10022632
126	3.537967	2	1117	Regulation of developmental process	Lus10023558 Lus10040443
127	3.380589	2	1169	Protein-containing complex subunit organization	Lus10003328 Lus10022632
128	3.141422	2	1258	Regulation of cellular protein metabolic process	Lus10003328 Lus10022632

129	6.698151	1	295	DNA recombination	Lus10009821
130	3.04227	2	1299	Regulation of protein metabolic process	Lus10003328 Lus10022632
131	2.984826	2	1324	Cellular amide metabolic process	Lus10003328 Lus10022632

4.1.7 Analysis of conserved Cis elements:

The analysis of promotor regions in *LuPLR2* genes revealed a diverse array of regulatory motifs critical for transcriptional control. Among these, TATA-box (72) and CAAT-box (47) were identified as the dominant cis-elements, functioning as core promotor elements and were abundantly found in *Lus10007599* and *Lus10042313*, respectively (**Figure 4.5**). Box-4, G-Box, and I-box were also detected, which were categorized as light-responsive elements, indicating the involvement of *LuPLR2* genes in photosynthetic regulation. Furthermore, a range of stress-responsive elements were identified, highlighting their roles in adapting to environmental challenges. For example: ARE confers responsiveness to anaerobic stress. MBS regulates drought responses by interacting with MYB transcription factors. DRE1 and LTR are associated with responses to cold and dehydration stress. STRE governs transcription under heat, osmotic, and oxidative stress conditions. AP-1 is engaged in the gene expression involved in variety of stimuli, involving stress, cytokines and growth factors. This study also uncovered several hormonal-responsive elements, including ABRE, AT-ABRE, GARE-motif, P-box, TATC-box, TGA-element, AuxRR-core, Sp-1, and ERE, suggesting that *LuPLR2* genes are influenced by hormonal signaling pathways such as abscisic acid, gibberellin, and auxin. Promotor motifs linked to tissue-specific or developmental regulation-such as O2-site, HD-Zip-3, GCN_4 were also identified. These elements are likely involved in the spatial and temporal expression of *LuPLR2* genes. Notably, the presence of WUN-motifs, which are activated during wounding, underscores the role of these genes in triggering defense mechanisms. Pathogen-defense motifs, such as W-box and as-1, were observed, suggesting participation in salicylic-acid and jasmonic acid-mediated defense pathways. In addition, Circadian elements were also seen, important for controlling gene expression in response to environmental changes. Other regulatory motifs, including MYB and MYC- binding sites, were identified, pointing to their roles in secondary metabolism and abiotic stress tolerance. The detection of Gap-box and Box-II, associated to specific signaling and metabolic pathways, further emphasizes the intricate regulation of *LuPLR2* genes.

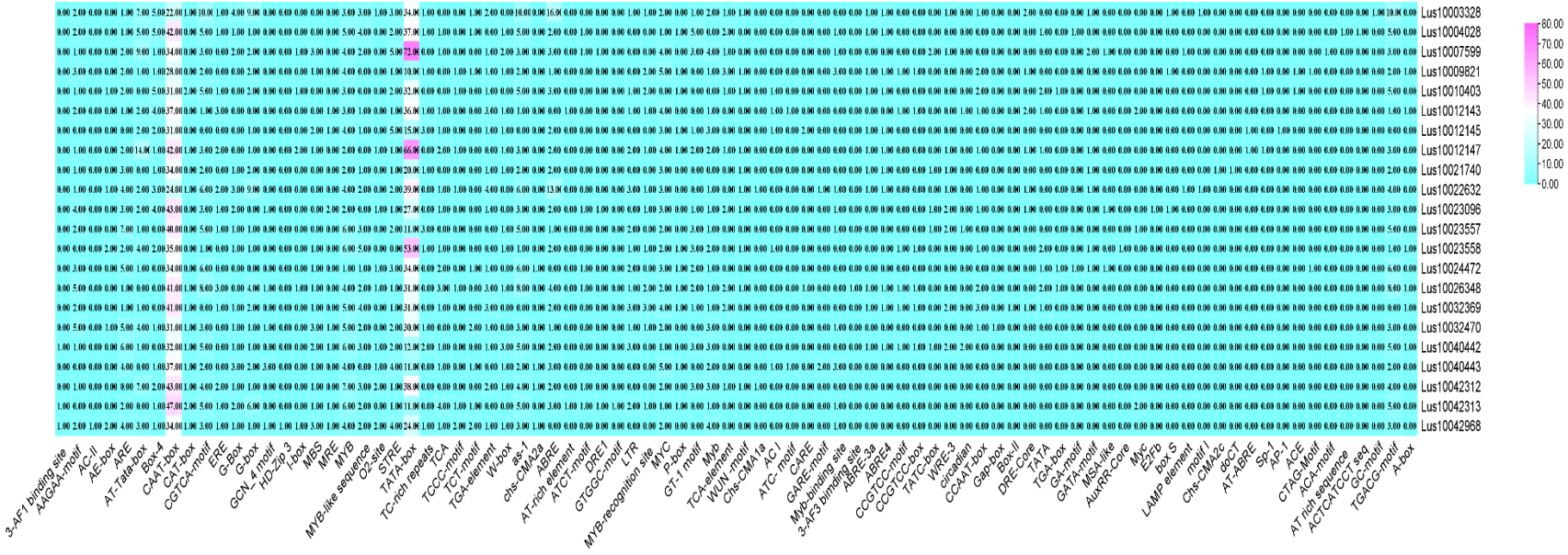

Figure 4.5: Representation of Cis-elements identified in *LuPLR2s* via TBtools software

4.1.8 Phylogenetic analysis : Phylogenetic analysis was conducted to uncover conserved patterns by examining relationships among *PLR2* genes and other species. In this study, 22 identified *LuPLR2* genes from Phytozome and other *PLR* genes (*PLR2, PLR1* and *PLR-like* genes) from various plants/genera *(Vitis riparia, Gossypium hirsutum, Populus trichocarpa, Populus euphratica, Vitis vinifera, Populus nigra, Hibiscus syriacus, Populus alba, Linum album, Salix purpurea, Theobroma cacao, Linum flavum, Ziziphus jujube, Diospyros lotus, Linum corymbulosum, Durio zibethinus, Gossypium raimondii, Gossypium arboretum, Lycium ferocissimum, Lycium barbarum, Solanum dulcamara, Solanum verrucosum, Capsicum baccatum, Punica granatum, Capsicum chinense, Benincasa hispida, Solanum pennellii, Alnus glutinosa, Lotus japonicas, Solanum stenotomum, Prosopis cineraria, Camellia sinensis)* were systematically classified into different clades (**Figure 4.6**). To improve the clarity and provide a comprehensive understanding of the phylogenetic relationships, a specific color scheme was used for each clade. Red color represents clade I, pink color for clade II, blue for clade III. Clade I and clade II contains the genes from other species whereas clade III contains one *BhPLR2-like*, all the identified *PLR2* genes of flax and other three *PLR* genes from different species of flax, namely, *LcPLR1, LfPLR2*, and *LaPLR1*.

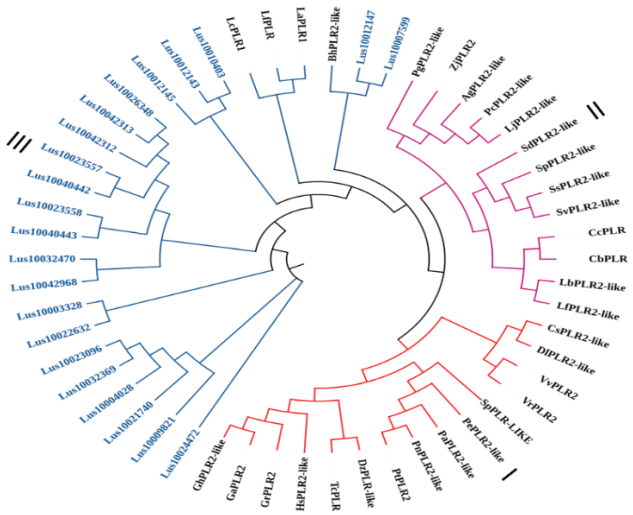

Figure 4.6: Phylogenetic tree of the *PLR2s* of different plants

4.2 Expression analysis using Real time PCR and Quantification of yatein phytochemical using HPLC under different abiotic conditions

4.2.1 Expression analysis of *LuPLR2* genes using qRT-PCR

An analysis of the expression *LuPLR2* genes across various stress (abiotic) conditions was carried out through **qRT-PCR** in the leaves of flax microgreens. The stress treatments included salinity stress (5 mM and 20 mM NaCl), PEG-induced drought stress (5% and 20% PEG), cold stress (4℃), and heat stress (30℃). Out of the total 22 *LuPLR2* genes identified through Phytozome database, 15 genes were selected based upon domain similarity with the domain of the standard gene. After this, 8 genes were selected from the set of 15 genes for expression analysis based on primer specificity using semi quantitative PCR (data not shown). Gene expression was quantified as a relative change at 8 h and 24 h following the stress treatments (**Figure 4.7**). Across all stress conditions, transcript levels of nearly all genes exhibited a significant upregulation compared to the control, with the notable exception of heat stress where expression was generally downregulated (**Table 4.8**).

Lus10003328 displayed the highest expression under control conditions, suggesting a potential baseline role in normal physiological states. Under **cold stress at 8 h,** *Lus10042313*, *Lus10040442*, and *Lus10026348* depicted higher expression as compared to control. It is noteworthy here that among these, the highest upregulation (2.2- fold) was exhibited by ***Lus10040442***. On the other hand, *Lus10010403, Lus10022632*, *Lus10003328, Lus10012145*, and *Lus10007599* were found to have lower expression compared to control. During **cold stress at 24 h,** the genes *Lus10042313, Lus10040442*, *Lus10012145*, and *Lus10026348* exhibited significant upregulation, with ***Lus10040442*** demonstrating the highest expression level (3.2- fold) relative to control. Conversely, *Lus10010403, Lus10003328, Lus10022632,* and *Lus10007599* exhibited marked downregulation under the same conditions. Under **heat stress at 8 and 24 h,** all the genes were found to be downregulated in comparison to control. Under **5 mM NaCl at 8 h,** as compared to control, *Lus10042313, Lus10040442,* and *Lus10026348* exhibited upregulation, with ***Lus10042313*** showing the highest upregulation (1.2-fold). In contrast, *Lus10010403, Lus10003328,*

Lus10022632, Lus10012145, and *Lus10007599* exhibited reduced expression levels compared to control under the same conditions. Under **5 mM NaCl at 24 h, *Lus10012145*** reported the highest expression as compared to control (1.2- fold) whereas all other genes including *Lus10010403, Lus10042313, Lus10003328, Lus10022632, Lus10040442, Lus10026348,* and *Lus10007599* were found to be downregulated. Under **20 mM NaCl at 8 h,** *Lus10042313, Lus10040442,* and *Lus10026348* demonstrated increased expression levels relative to the control. Notably, the most significant upregulation (3.4- fold) was exhibited by ***Lus10042313***. However, the remaining genes, such as, *Lus10010403, Lus10003328, Lus10022632, Lus10012145*, and *Lus10007599* were observed to be downregulated. Under **20 mM NaCl at 24 h,** *Lus10007599, Lus10042313 Lus10022632, Lus10010403, Lus10012145, and Lus10026348* exhibited upregulation as compared to control. Notably, the highest upregulation (2.3- fold) was depicted by ***Lus10007599***. On the contrary, *Lus10003328,* and *Lus10040442* were found to be downregulated. Under **5% PEG at 8 h,** *Lus10010403, Lus10042313, Lus10012145, Lus10040442,* and *Lus10026348* revealed upregulation as compared to control. Amongst the upregulated genes, ***Lus10042313*** was the most highly expressed (2.1- fold). In contrast, *Lus10003328, Lus10022632,* and *Lus10007599* exhibited lower expression compared to control under the same conditions. Under **5% PEG at 24 h,** *Lus10010403, Lus10012145,* and *Lus10026348* exhibited upregulation amongst all the genes as compared to control. It is noted that the maximum upregulation (1.8- fold) was shown by ***Lus10012145***. Conversely, *Lus10042313, Lus10003328, Lus10040442, Lus10022632,* and *Lus10007599* depicted marked downregulation in comparison to control. Under **20% PEG at 8 h,** *Lus10010403, Lus10042313, Lus10026348, Lus10040442,* and *Lus10012145* demonstrated upregulation in comparison to control. Specifically, ***Lus10026348*** revealed maximum expression (3.1-fold) among all the genes. All other genes including *Lus10003328, Lus10022632,* and *Lus10007599* were observed to be downregulated. During **20% PEG at 24 h,** *Lus10012145,* and *Lus10010403* genes exhibited upregulation with the highest expression depicted by ***Lus10012145*** (1.88-fold). However, all other genes including *Lus10042313, Lus10003328, Lus10022632, Lus10040442, Lus10026348,* and *Lus10007599*

demonstrated downregulation relative to the control.

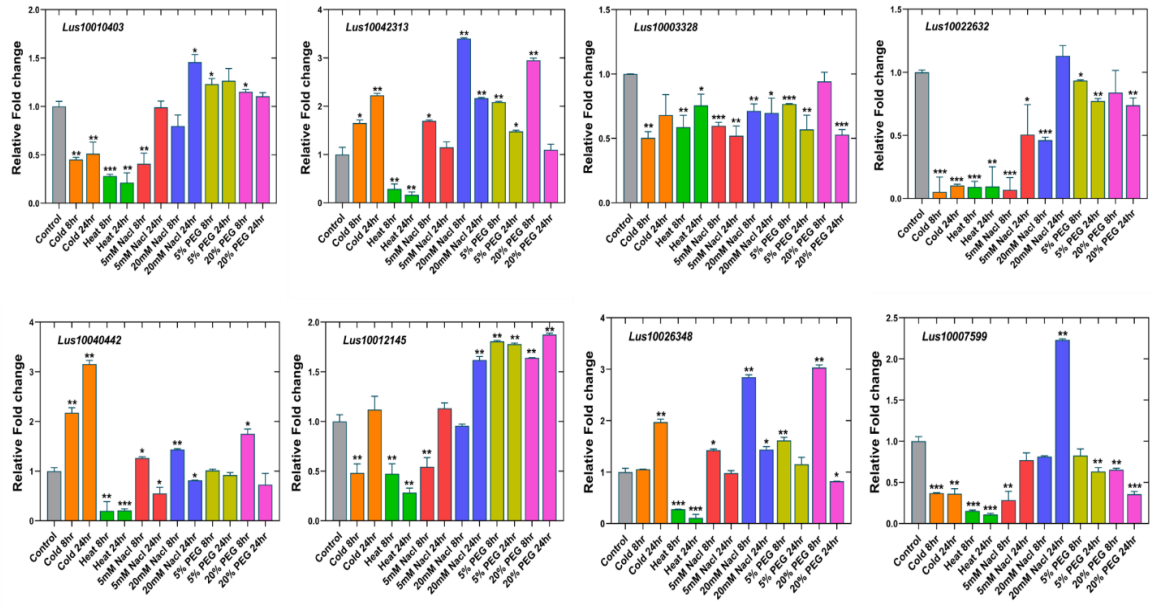

Figure 4.7: The expression of selected *LuPLR2s* in the leaves of flax microgreens has been examined using real-time quantitative PCR experiment. The outcomes displayed the mean of replicates (n=3), representing mean of ± Standard deviation (SD) values. The Student's t-test was implemented in order to evaluate their statistical significance. At p-values less than 0.001, less than 0.01, and less than 0.05, respectively, the asterisks * **P, * *P, and *P are used to indicate the significance of the fold change.

Table 4.8 showing the upregulation and downregulation of *PLR2* genes under different stress and treatment conditions in flax microgreens analyzed through qRT-PCR with respect to control.

Abiotic stress conditions	**Treatment**	**Upregulated genes**	**Downregulated genes**
Heat	Heat 30°C 8 h	-	*All PLR2 genes*
	Heat 30°C 24 h	-	*All PLR2 genes*
Cold	Cold 4°C 8 h	*Lus10042313, Lus10040442, Lus10026348*	*Lus10010403, Lus10022632, Lus10003328, Lus10012145,*

			Lus10007599
	Cold 4°C 24 h	*Lus10042313, Lus10040442, Lus10012145, Lus10026348*	*Lus10010403, Lus10003328, Lus10022632, Lus10007599*
NaCl	5mM 8 h	*Lus10042313, Lus10040442, Lus10026348*	*Lus10010403, Lus10003328, Lus10022632, Lus10012145, Lus10007599*
	5mM 24 h	*Lus10012145*	*Lus10010403, Lus10042313, Lus10003328, Lus10022632, Lus10040442, Lus10026348, Lus10007599*
	20mM 8 h	*Lus10042313, Lus10040442,* Lus10026348	*Lus10010403, Lus10003328, Lus10022632, Lus10012145, Lus10007599*
	20mM 24 h	*Lus10007599, Lus10042313 Lus10022632, Lus10010403, Lus10012145, Lus10026348*	*Lus10003328, Lus10040442*
Drought	PEG 5% 8 h	*Lus10010403, Lus10042313, Lus10012145, Lus10040442, Lus10026348*	*Lus10003328, Lus10022632, Lus10007599*
	PEG 5% 24 h	*Lus10010403, Lus10012145, Lus10026348*	*Lus10042313, Lus10003328, Lus10040442, Lus10022632, Lus10007599*
	PEG 20% 8 h	*Lus10010403, Lus10042313, Lus10026348, Lus10040442, Lus10012145*	*Lus10003328, Lus10022632, Lus10007599*
	PEG 20%, 24 h	*Lus10012145, Lus10010403*	*Lus10042313, Lus10003328, Lus10022632, Lus10040442, Lus10026348, Lus10007599*

4.2.2 Quantification of yatein phytochemical by HPLC

HPLC is among the most commonly utilized techniques for analyzing various compounds in plants. HPLC technique was used to identify and quantify yatein phytochemical in ethanolic extracts of flax microgreens under different abiotic stress conditions. The quantification was performed using a standard calibration method. The quantities of the compound detected in the analyzed samples are presented in **Table 4.9**. The retention time was found to be approximately 8.5 min for all the samples (**Figure 4.8**). In the ethanolic extracts of flax microgreens prepared under different abiotic stress conditions, yatein was found to be most prevalent in 20% PEG induced drought stress conditions (5.332 mg/gm), followed by 5% PEG (4.408 mg/gm) at 280 nm.

Table 4.9 Concentration of Yatein in the ethanolic extracts of Flax microgreens

S.No.	Abiotic stress treatments	Concentration of Yatein (mg/gm)
1.	Control	BLQ
2.	5 mM NaCl	0.744
3.	20mM NaCl	0.715
4.	5% PEG	4.408
5.	20% PEG	5.332
6.	Heat stress	0.582
7.	Cold stress	0.651

BLQ:BelowQuantification

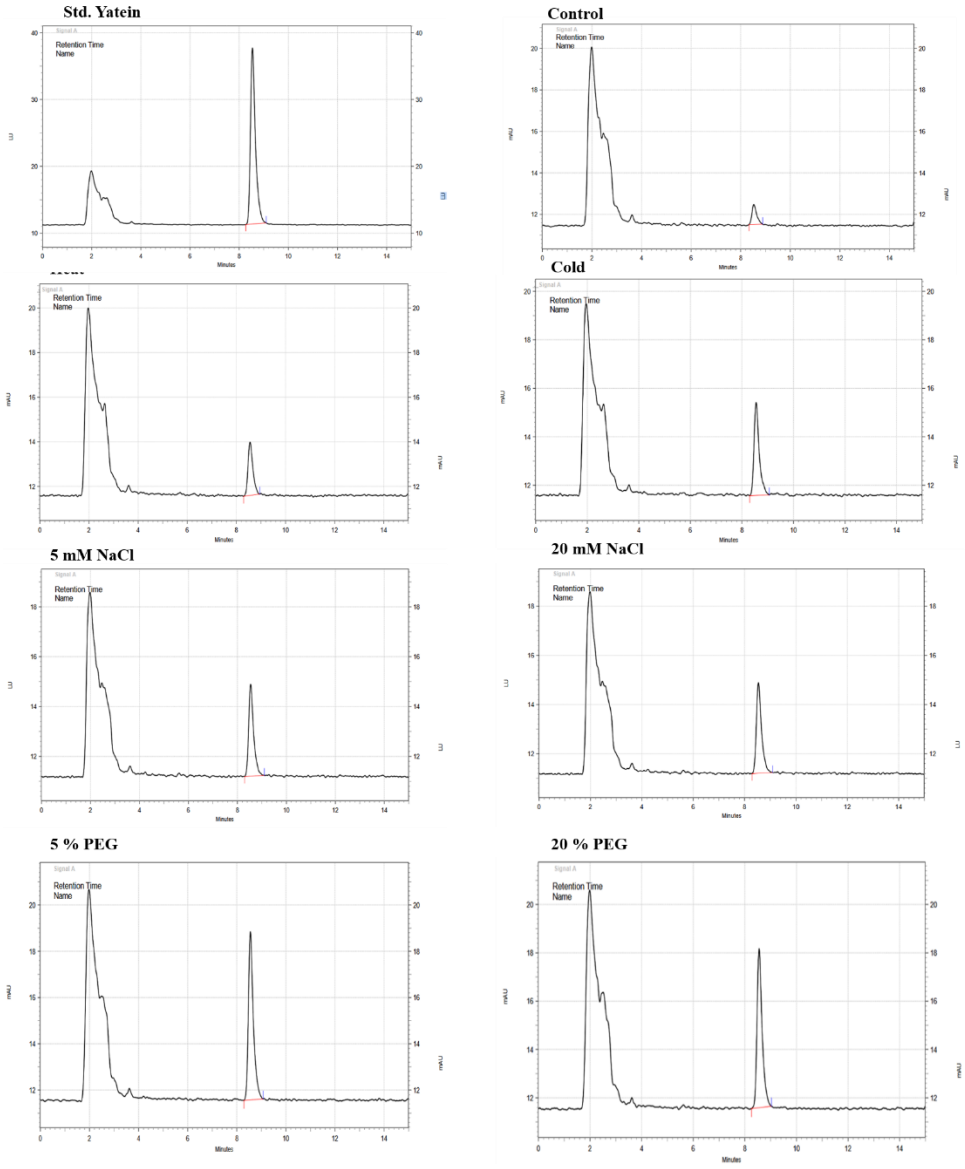

Figure 4.8 Chromatograms of Yatein concentration in the leaves of flax microgreens under different abiotic stress conditions

4.3 Effect of Yatein on Antioxidative potential of flax microgreens under different abiotic stress conditions

To investigate the activity of enzymatic antioxidants, abiotic stress conditions were applied to the both untreated and yatein-treated flax microgreens. This approach aimed to assess how yatein influenced the anti-oxidative response of flax microgreens under abiotic stress conditions. By comparing the enzymatic activity levels between the treated and untreated groups, the study sought to elucidate the protective or modulatory role of yatein in mitigating damage induced by abiotic stress.

4.3.1 Protein Estimation: The concentration of protein in the samples was estimated with the help of standard (BSA). After determining the protein concentration, 240 µg of supernatant was taken for studying Antioxidative activities.

4.3.2 Changes in SOD activity: SOD activity was evaluated between untreated and yatein-treated flax microgreens under various stress conditions. In untreated microgreens, highest SOD activity was observed under 5% PEG stress, followed by 5 mM NaCl as compared to control. Moderate SOD activity was recorded under cold stress. The lowest APX activity under 20 mM NaCl, 20% PEG, as well as heat stress was recorded relative to other stress conditions, though still greater than control untreated microgreens [**Figure 4.9 (a)**]. Under yatein treatment, microgreens demonstrated relatively higher SOD activity across all the stress conditions compared to yatein-treated control. The highest SOD activity was recorded under 5% PEG, followed by 5 mM NaCl, 20% PEG and 20 mM NaCl when compared to yatein-treated control. In contrast, cold and heat stress conditions exhibited the lowest SOD activity, though still higher than the control yatein treated samples [**Figure 4.9 (b)**]. Comparing yatein-treated and untreated flax microgreens, yatein treatment significantly enhanced SOD activity under all the stress conditions. Among these, 5% PEG induced the highest SOD activity in yatein-treated microgreens [**Figure 4.9 (c)**].

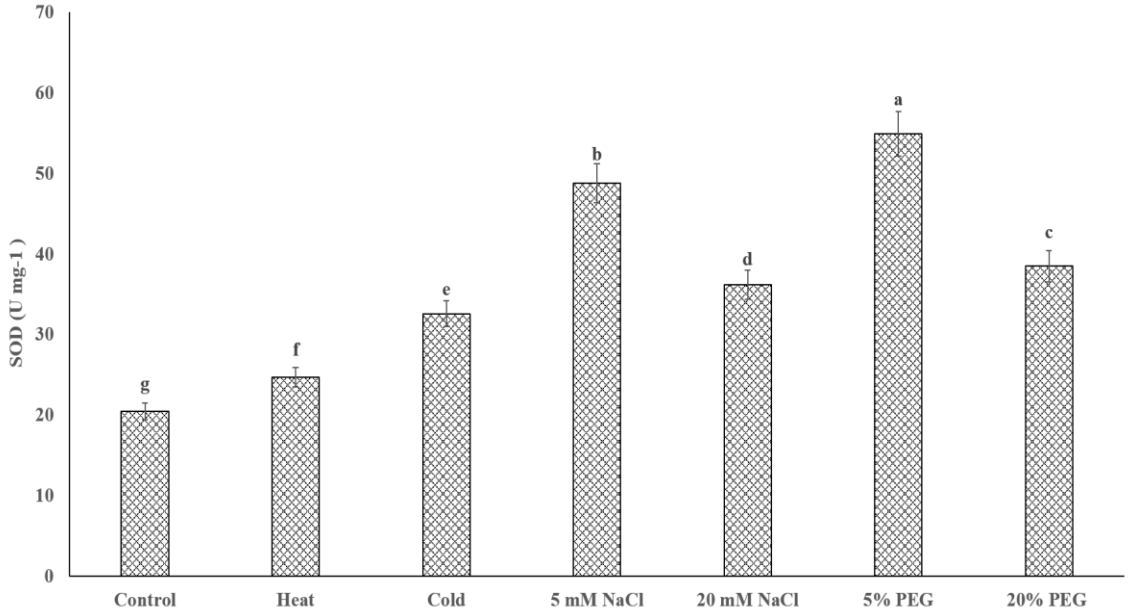

Figure 4.9 (a) SOD activity of Untreated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

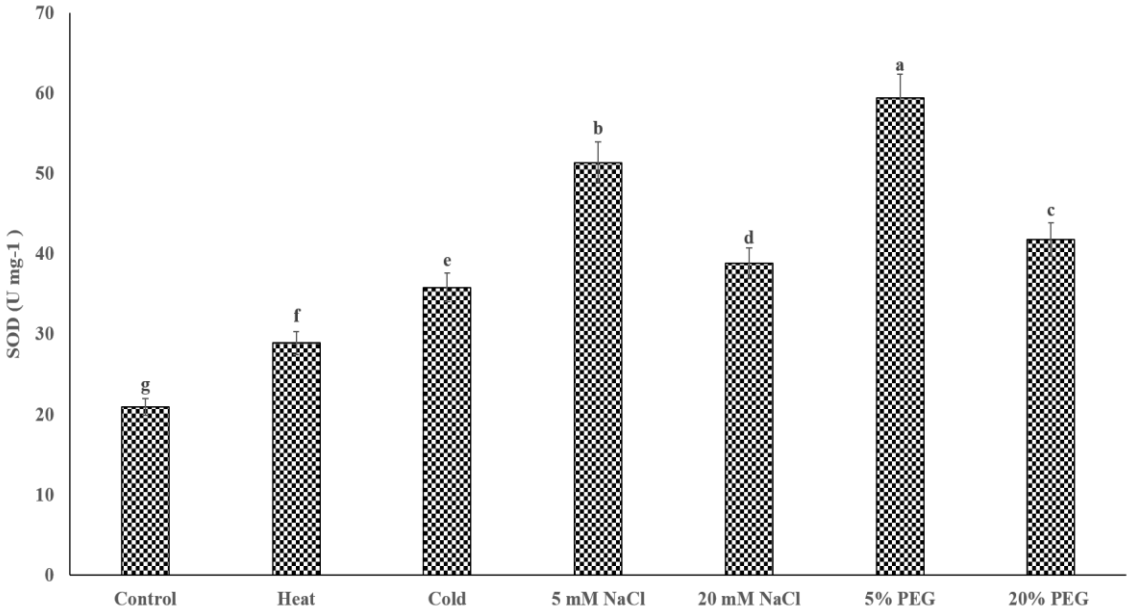

Figure 4.9 (b) SOD activity of Yatein-treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

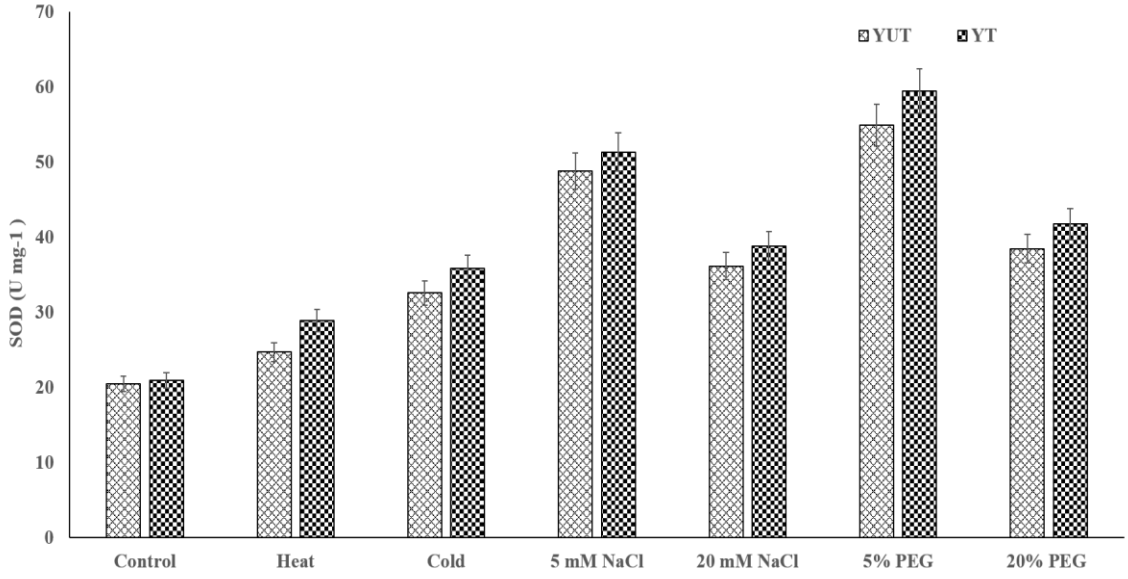

Figure 4.9 (c) SOD activity of Untreated and Yatein- treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. Student's t-test was employed for measuring the statistical significance with the asterisks * **P, * *P, and *P used to indicate the significance at p-values less than 0.001, less than 0.01, and less than 0.05, respectively

4.3.3 Changes in the CAT activity: CAT activity was examined between untreated and yatein treated flax microgreens under various stress conditions. In untreated microgreens, the highest CAT activity was observed under cold stress, whereas moderate increase observed under 20mM NaCl as compared to control. The lower SOD activity under 5 mM NaCl as well as PEG (5% and 20%) was observed when compared to other stress conditions, but still higher than control untreated sample. In contrast, heat stress led to the lowest CAT activity among the other stress conditions though it remained slightly higher

than in control untreated flax microgreens [**Figure 4.10 (a)**]. Yatein-treated samples exhibit enhanced CAT activity across all stress conditions as compared to control yatein treated sample, with the highest levels detected under cold stress, followed by 20% PEG treatment. A moderate increase was noted under salt stress (20 mM and 5 mM NaCl) and 5% PEG, whereas heat stress exhibited the lowest CAT activity among the other stress treatments, though higher than the yatein-treated control [**Figure 4.10 (b)**]. The yatein treated flax microgreens significantly boosted CAT activity compared to untreated flax microgreens, with the highest CAT activity observed under cold stress [**Figure 4.10 (c)**].

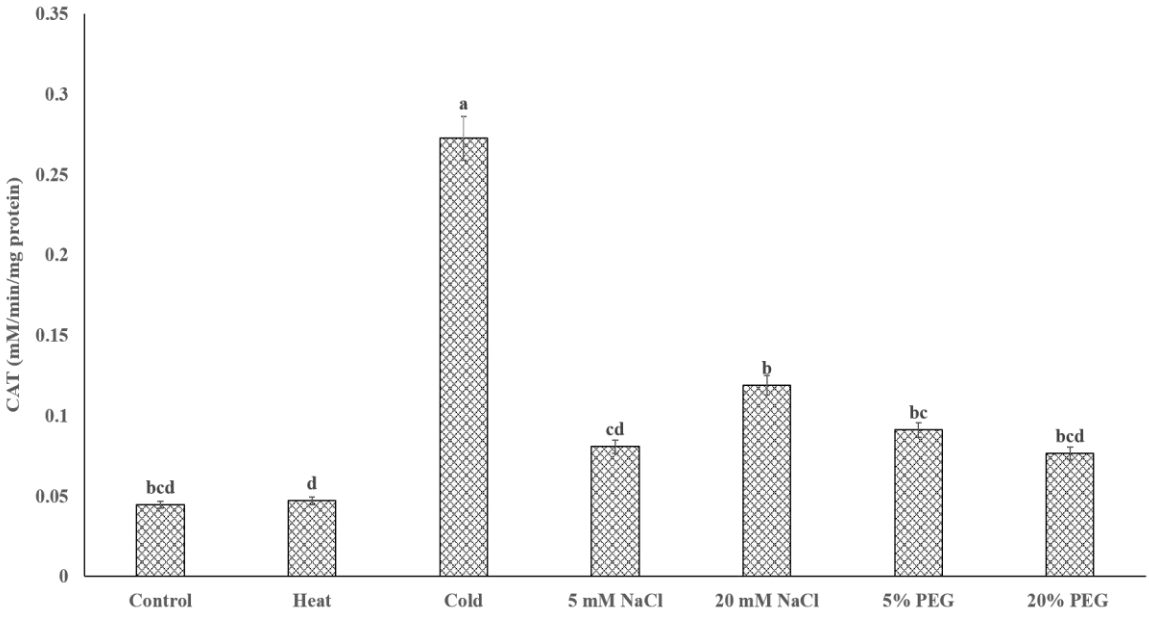

Figure 4.10 (a) CAT activity of Untreated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

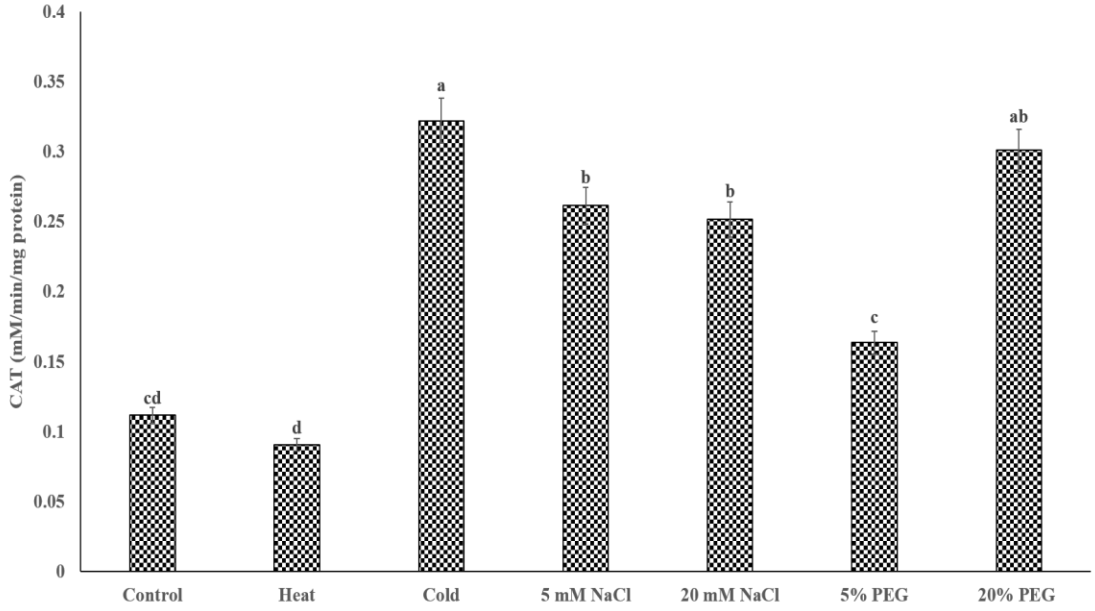

Figure 4.10 (b) CAT activity of Yatein-treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

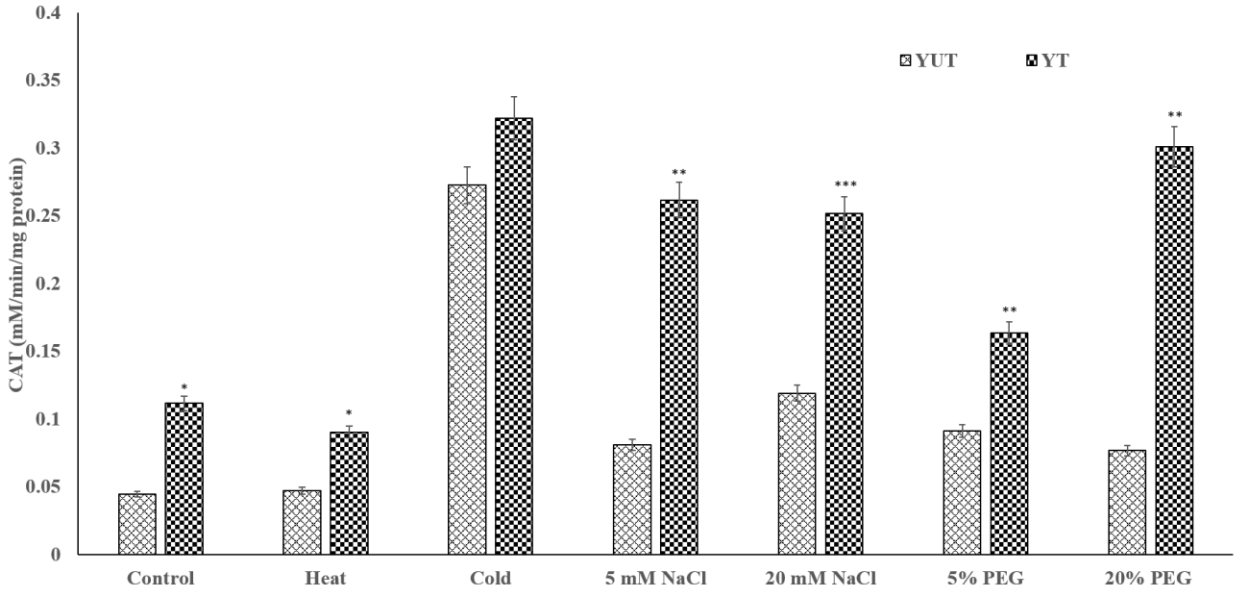

Figure 4.10 (c) CAT activity of Untreated and Yatein- treated flax microgreens at

different stress conditions. The data presented is an average of triplicates ± SD. Student's t-test was employed for measuring the statistical significance with the asterisks * **P, * *P, and *P used to indicate the significance at p-values less than 0.001, less than 0.01, and less than 0.05, respectively

4.3.4 Changes in the GST activity: GST activity was assessed between untreated and yatein-treated flax microgreens under different stress conditions. In untreated microgreens, GST activity increased under all the stress conditions when compared to control. The maximum GST activity was noted under heat and cold stress compared to untreated control. A moderate rise was observed under 20 % PEG treatment. Lower activity was recorded under 5 and 20 mM NaCl, whereas lowest activity was noted in 5% PEG as compared to other stress conditions, although slightly higher than the control untreated flax microgreens [**Figure 4.11 (a)]**. In contrast, yatein treatment significantly enhanced GST activity across all stress conditions compared to the yatein treated control. The highest GST activity was observed under heat stress. The other stress treatments, including cold, 20 mM NaCl, and 20% PEG, also exhibited moderate enhancement, while 5 mM NaCl and 5% PEG exhibited lower GST activity as compared to other stress conditions but still greater than yatein-treated control [**Figure 4.11 (b)]**. The yatein-treated samples exhibited substantially higher GST activity compared to untreated samples, with the highest activity recorded under heat stress [**Figure 4.11 (c)]**.

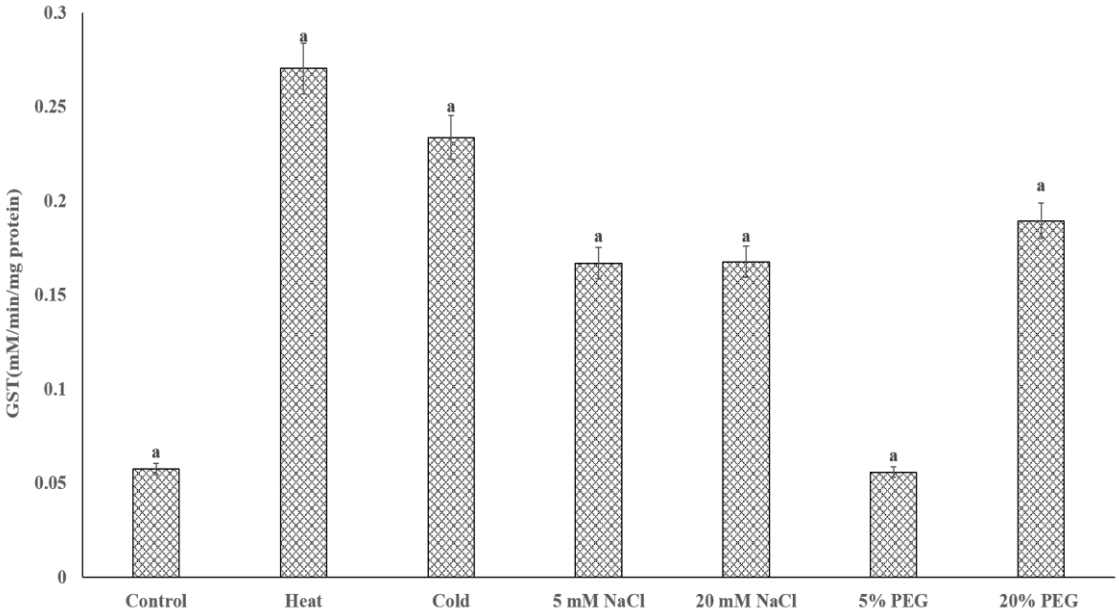

Figure 4.11 (a) GST activity of Untreated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

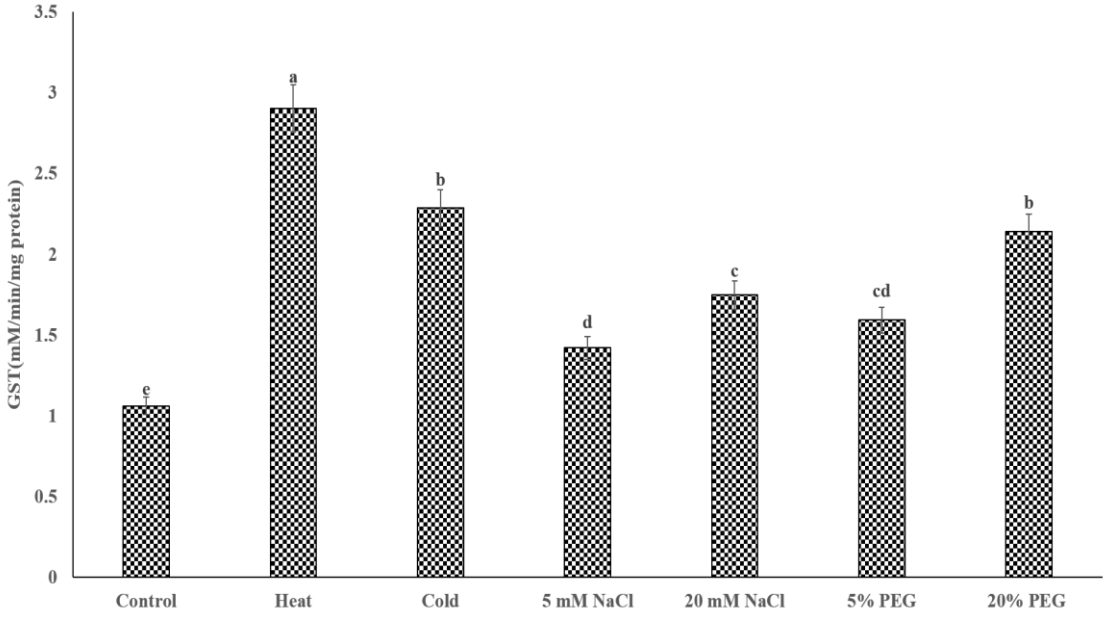

Figure 4.11 (b) GST activity of Yatein-treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are

designated by distinct letters displaying differences that are significant (p<0.05), which are measured with the use of One Way ANOVA (Tukey test)

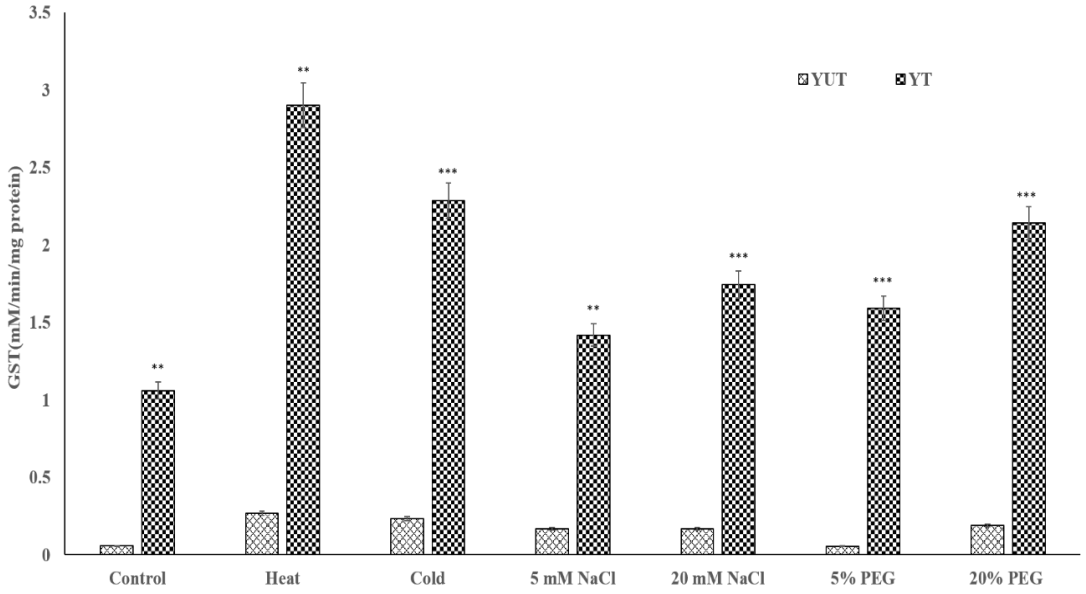

Figure 4.11 (c) GST activity of Untreated and Yatein- treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. Student's t-test was employed for measuring the statistical significance with the asterisks * **P, * *P, and *P used to indicate the significance at p-values less than 0.001, less than 0.01, and less than 0.05, respectively

4.3.5 Changes in the APX activity: APX activity was evaluated between untreated and yatein-treated flax microgreens under various stress conditions was examined. In untreated samples, notably highest APX activity was observed under 20% PEG stress, with 20 mM NaCl and 5% PEG treatments also showing elevated levels when compared to control untreated sample. Cold stress led to a moderate increase, while heat stress and 5 mM NaCl resulted in relatively lower APX activity compared to other stress conditions but remained higher than the untreated control [**Figure 4.12 (a)**]. Yatein-treatment significantly boosted APX activity across all stress conditions, except for heat stress in which APX activity was almost similar to yatein-treated control. The most notable increase was observed under

20% PEG stress while cold stress and 20 mM NaCl also exhibited substantial enhancements. Meanwhile, 5% PEG and 5 mM NaCl also exhibited moderate rise in the APX activity relative to other stress treatments, although still greater than yatein-treated control [**Figure 4.12 (b)**]. A comparative analysis demonstrated that yatein-treated samples had higher APX activity than untreated samples, with the highest activity recorded under 20% PEG stress [**Figure 4.12(c)**].

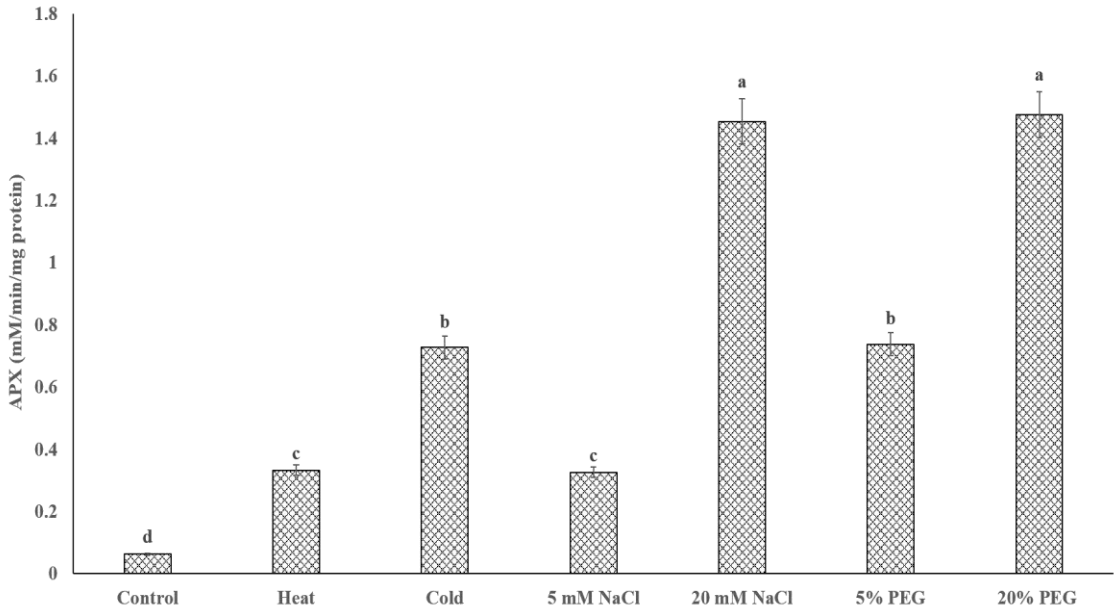

Figure 4.12 (a) APX activity of Untreated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

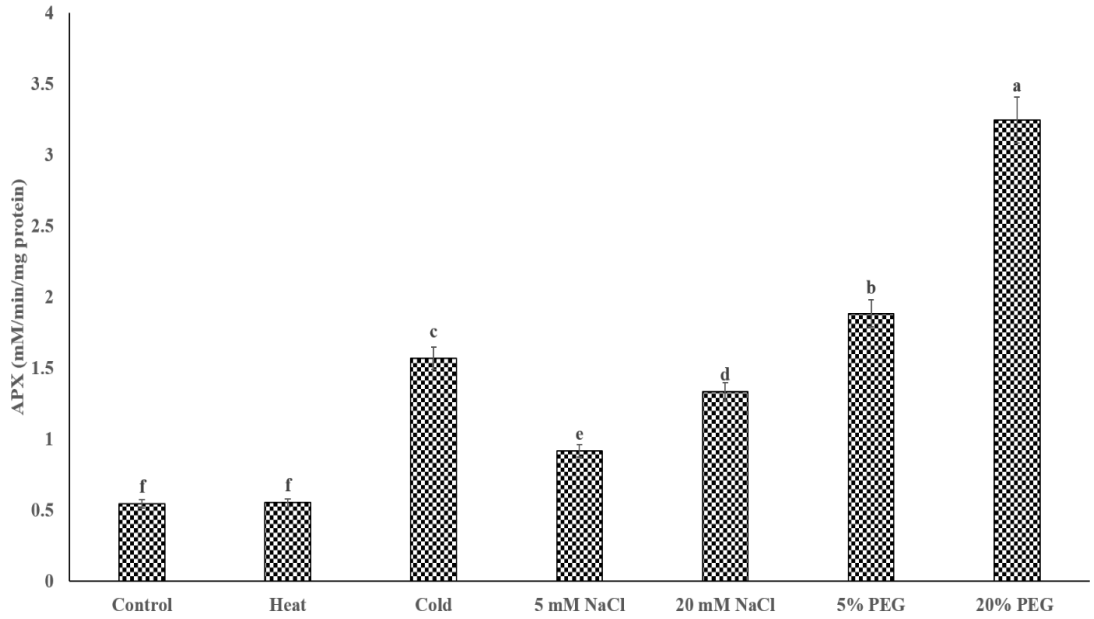

Figure 4.12 (b) APX activity of Yatein-treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

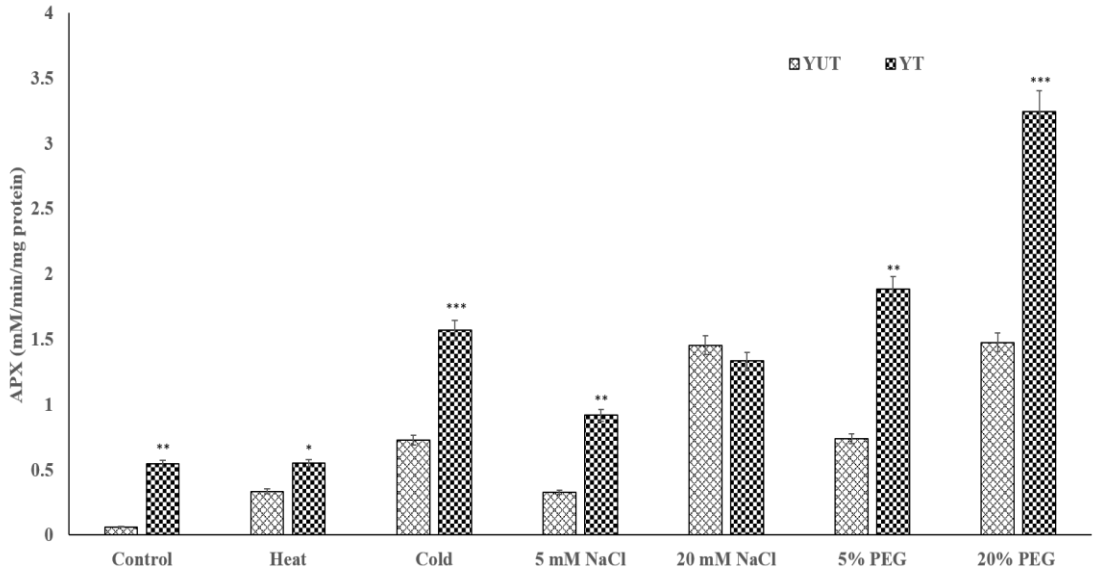

Figure 4.12 (c) APX activity of Untreated and Yatein- treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. Student's t-test was employed for measuring the statistical significance with the asterisks * **P, * *P, and *P used to indicate the significance at p-values less than 0.001, less than 0.01, and less than 0.05, respectively

4.3.6 Changes in GPX activity: GPX activity was analysed between untreated and yatein-treated flax microgreens under different stress conditions. In untreated group, the GPX activity varied across stress treatments, with the highest activity recorded under 20 mM NaCl treatment as compared to control. Heat stress resulted in moderate GPX activity, while cold stress, salt stress (5 mM and 20 mM NaCl), PEG treatments (5% and 20%) exhibited comparatively lower activity, still higher than the untreated control sample [**Figure 4.13 (a)]**. In yatein-treated flax microgreens, GPX activity increased significantly under all stress conditions, with the highest levels recorded under cold stress, surpassing all other conditions. Additionally, stress induced by heat, 5 and 20 mM NaCl as well as 5% and 20% PEG led a notable elevation in GPX activity compared to the control sample treated with yatein. [**Figure 4.13 (b)**]. A comparative analysis revealed that yatein-treated samples exhibited higher GPX activity than untreated counterparts across all stress conditions [**Figure 4.13 (c)**].

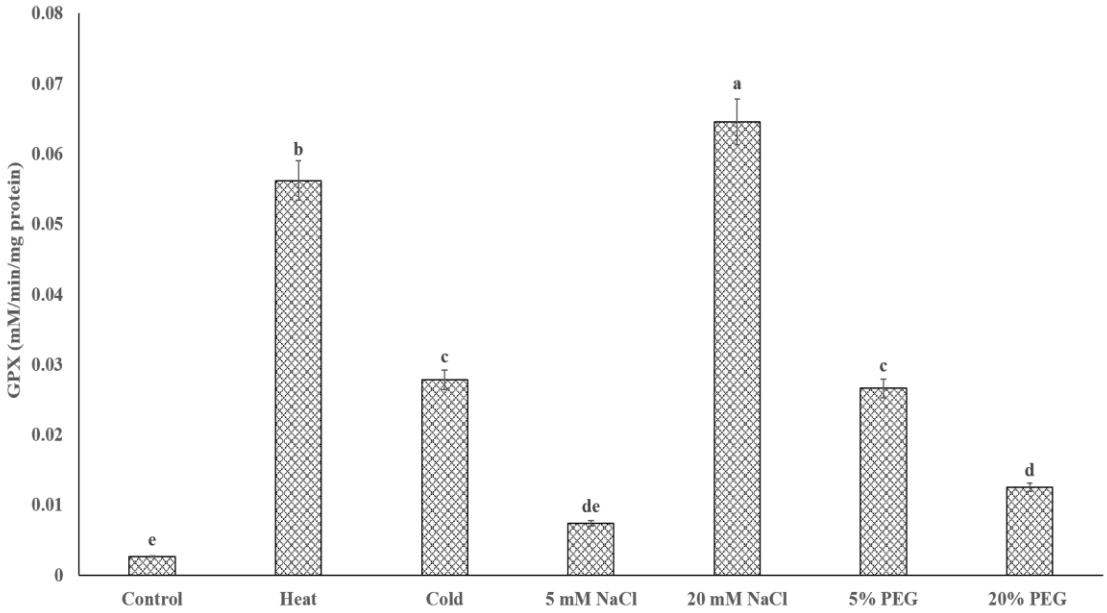

Figure 4.13 (a) GPX activity of Untreated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

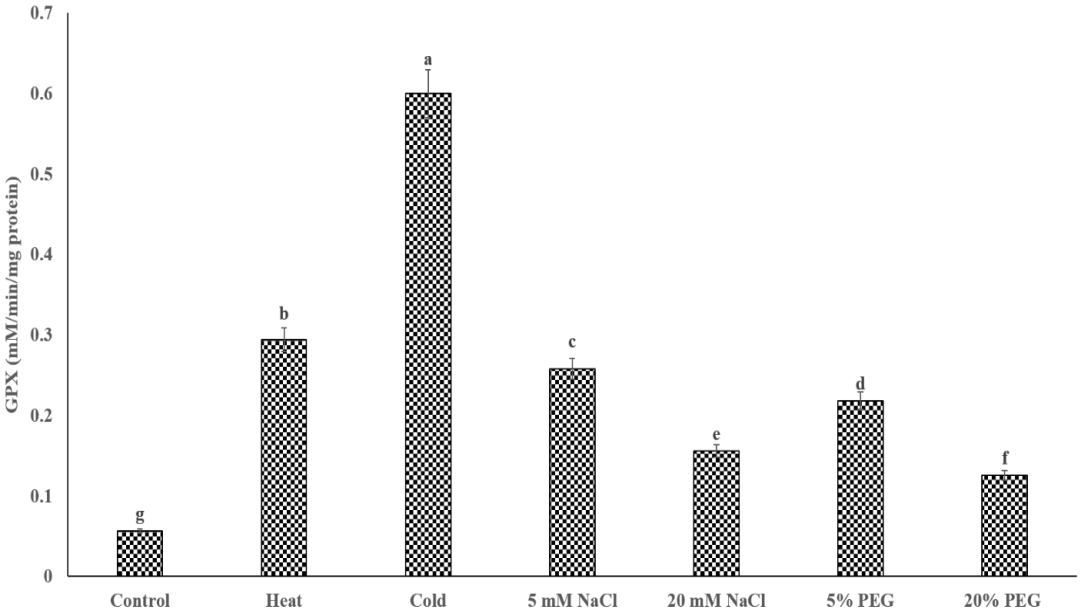

Figure 4.13 (b) GPX activity of Yatein-treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are

designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

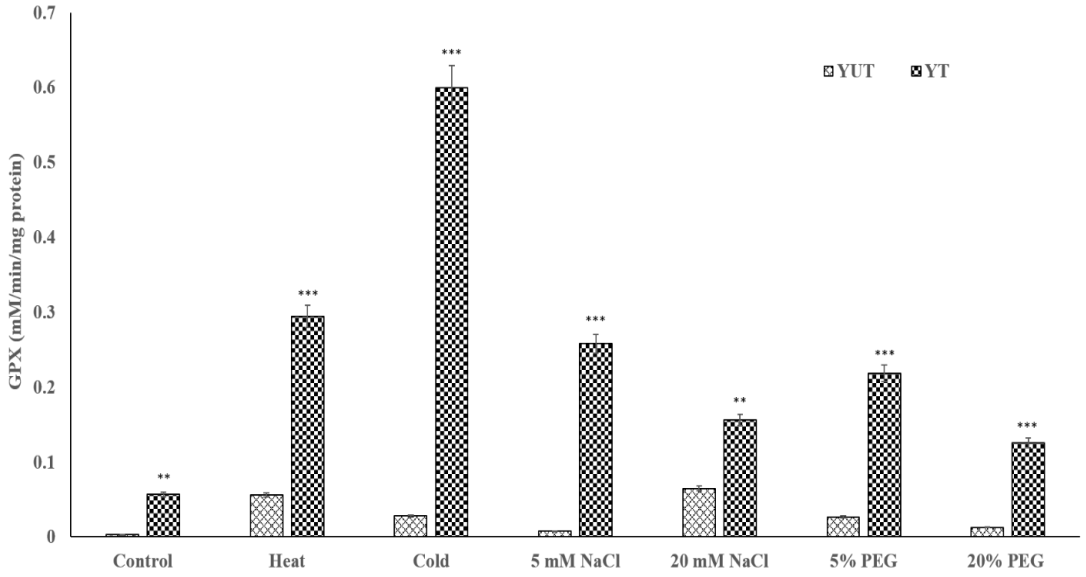

Figure 4.13 (c) GPX activity of Untreated and Yatein- treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. Student's t-test was employed for measuring the statistical significance with the asterisks * **P, * *P, and *P used to indicate the significance at p-values less than 0.001, less than 0.01, and less than 0.05, respectively

4.3.7 Changes in GR activity: GR activity was examined between untreated and yatein-treated flax microgreens under various stress conditions. In untreated group, GR varied significantly, with the highest levels observed under 5% PEG treatment, followed closely by 20 mM NaCl when compared to control untreated samples. Moderate activity was recorded under cold stress, 5 mM NaCl and 20 % PEG stress compared to 5% PEG and 20 mM NaCl, though higher than control untreated microgreens. In contrast, heat stress resulted in the lowest activity among all the stress conditions, although still greater than

control untreated flax microgreens [**Figure 4.14 (a)**]. In yatein-treated flax microgreens, GR activity showed the most substantial increase under heat stress, far exceeding all other conditions. Significant enhancements were also observed under 20 mM NaCl and PEG treatments (both 5% and 20%) when compared to control yatein-treated microgreens. Meanwhile, cold-stress and 5 mM NaCl yatein-treated group also showed moderate rise GR activity relative to the yatein-treated control sample [**Figure 4.14 (b)**]. The yatein-treated microgreens consistently exhibited higher GR activity than their untreated counterparts across all stress conditions [**Figure 4.14 (c)**].

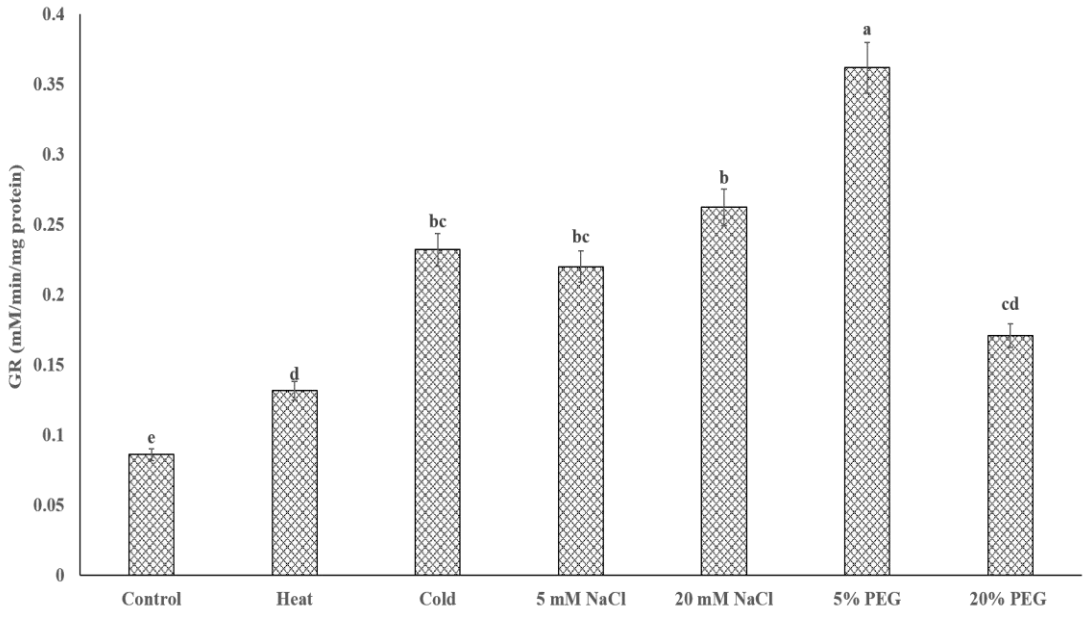

Figure 4.14 (a) GR activity of Untreated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

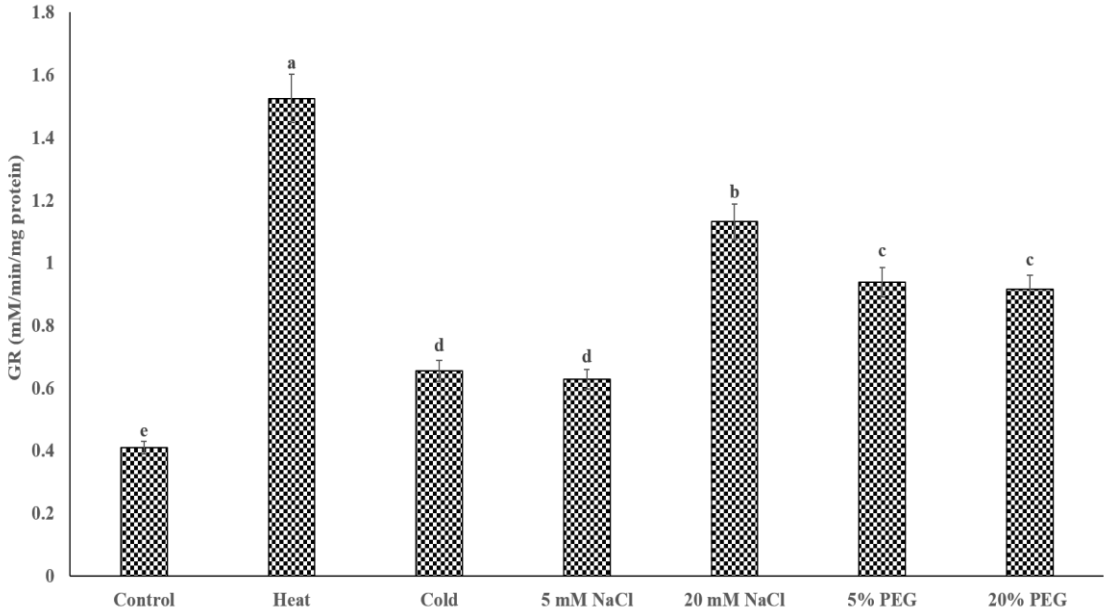

Figure 4.14 (b) GR activity of Yatein-treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. The bars are designated by distinct letters displaying differences that are significant ($p<0.05$), which are measured with the use of One Way ANOVA (Tukey test)

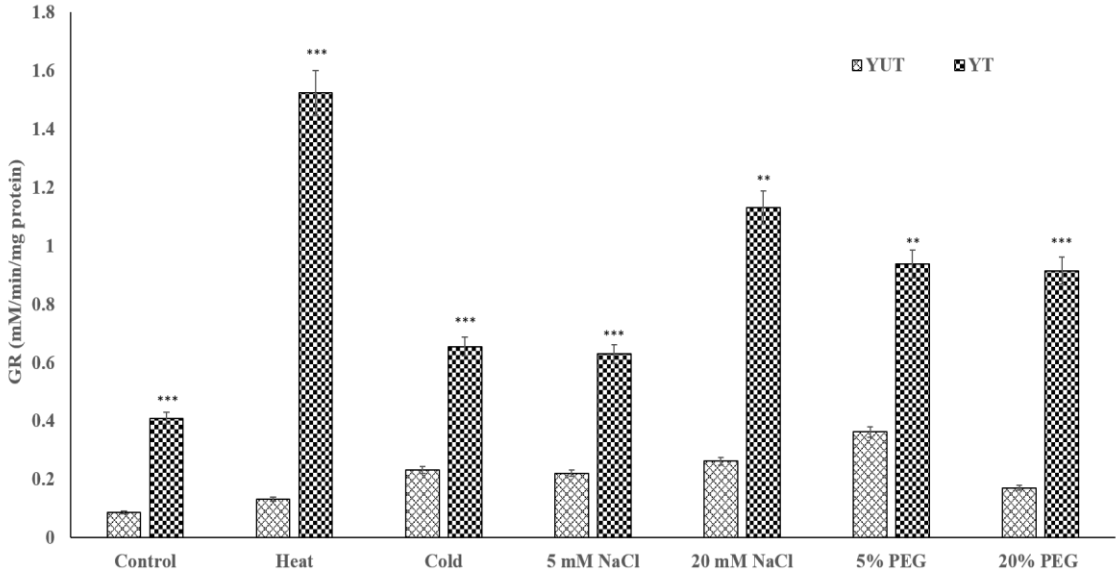

Figure 4.14 (c) GR activity of Untreated and Yatein- treated flax microgreens at different stress conditions. The data presented is an average of triplicates ± SD. Student's t-test was employed for measuring the statistical significance with the asterisks * **P, * *P, and *P used to indicate the significance at p-values less than 0.001, less than 0.01, and less than 0.05, respectively

CHAPTER 5: DISCUSSION

"It's pretty confusing."
"Good. Be confused. Confusion is where inspiration comes from." – **Robyn Mundell**

5.1 Identification of Flax PLR2 gene family members

Lignan biosynthesis has been extensively studied, and the proposed pathway is well-established and widely recognized (Ford et al., 2001). Lignans originate from the phenylpropanoid precursor, coniferyl alcohol, with their formation involving biomolecular phenoxy radical coupling as a key step in monolignol polymerization. . Lignan synthesis begins with the coupling of two coniferyl alcohol molecules, a reaction catalyzed by an oxidase (such as laccase or peroxidase) in conjunction with a DIR, resulting in the biosynthesis of pinoresinol (Kapoor et al., 2023). It is believed that DIR proteins have an impact on the synthesis of particular enantiomers of pinoresinol ensuring that the coniferyl alcohol molecules are placed particular orientation throughout the coupling phase (Kapoor et al., 2023). *PLR* facilitates the transformation of pinoresinol into lariciresinol, followed by its subsequent transformation into –(+)/-(-) SECO, and then -(-) SECO, subsequently leading to the formation of yatein in lignan biosynthesis (Markulin et al., 2019).

However, the lack of prior reports on the genome wide study of *PLR2* genes in flax underscores a significant research gap in understanding their roles and functions within this important crop. In our study, we aimed to address this gap by conducting an in-depth genome-wide investigation of the *PLR2s* in flax. From Phytozome database, a total of 30 *LuPLR2* genes were identified from Phytozome database in the flax leaves, marking a significant step in understanding the genetic landscape of this important crop. A number of *PLR2* genes had been identified in other organisms like *Arabidopsis* (*AtPrR2*), *Linum flavum (LfPLR2), Taiwania cryptomeriodes (TcPLR2.2), Thuja plicata* (*TpPLR2)* and *Tsuga heterophylla* (*ThPLR2)* (Chiang et al., 2019; Fujita et al., 1999; Gang et al., 1999; Nakatsubo et al., 2008; Shiraishi et al., 2016). This may indicate that *PLR2* genes must have endured persistent family-specific amplification throughout evolution. The domain analysis among the 30 *PLR2* genes exhibited a significant uniformity since most of the genes marked the presence of Nmr A-like family domain. This domain work as a negative transcriptional regulator that is involved in the post-translational modification of the transcription factor AreA. It exhibits an unexpected resemblance to the short-chain

dehydrogenase/reductase (SDR) family, showing the closest structural relationship to UDP-galactose 4-epimerase (Stammers et al., 2001). Nmr A-like family domain has also been identified as a component of the regulatory system responsible for nitrogen metabolite repression in fungi (*Asperigillus nidulans*) (Núñez-Corcuera et al., 2008). The detection of conserved domains elucidates the evolutionary relationships and probable beneficial functions of *LuPLR2* genes in the biology of plants.

The investigation of 30 LuPLR2 proteins also marked the presence of disordered nature amongst all the identified proteins (**Table 4.1**). All the proteins have disordered regions ranging from 5-10, indicating the availability of binding sites for common partners. Studies conducted on plant model organisms had revealed the presence of disorder in the usual processes that involve temporary interactions with multiple partners (Hsiao, 2022). A study on *Arabidopsis* revealed that the intrinsic disorder of certain proteins enabled them to engage in temporary interactions with various molecular partners involved in essential processes such as cell cycle regulation, DNA and RNA metabolism, signaling, and RNA splicing. Many of these processes were crucial for detecting and responding to environmental cues. These responses include mechanisms for light sensing, protein folding through chaperones, reactions to abiotic stresses, and pathways of secondary metabolism that help plants cope with environmental conditions (Pietrosemoli et al., 2013). Disordered regions in the proteins were also seen in PLR1_Tp, where loops (connecting the helices and strands) were disordered in the crystal structure and these serve as the insertion sites for PLR1_Tp (Min et al., 2003). These loops, like those in IDPs, provide the necessary flexibility for interacting with multiple binding partners. A group of proteins in plants, known as GRAS (for GIBBERELLIC ACID INSENSITIVE, REPRESSOR of GAI, and the SCARECROW) family, heavily depend on disorder for their functioning (Sun et al., 2012). These proteins had a significant role in the growth of plants as well as participated in cascades of signal transduction which are associated with hormonal response. Composition of amino acids determines whether a protein is organized with a stable 3-D structure or is intrinsically disordered (Trivedi & Nagarajaram, 2022). High net charge and low mean hydrophobicity cause IDPs/IDRs to unfold and expand due to strong electrostatic

repulsions and insufficient compaction force. In a study, crystal structures of PLR2_Tp had been analyzed and one of the residues (268) was found to become glycine upon symmetric substitutions (Min et al., 2003). The incorporation of glycine at position 268 is thought to increase the flexibility of the binding site, therefore facilitating the conformational flexibility of the protein to enhance the binding of pinoresinol. Also, one of the dehydrins in wheat was found to be rich in glycine in the total amino acid composition indicating the role of IDPs in wheat to protect cellular structures and lipid membranes from dehydration (Ridhi et al., 2023; Szlachtowska and Rurek, 2023).

Moreover, the secondary structure of the identified 30 PLR2s was determined and it revealed the presence of random coils, α-helices, and β-strands in all the proteins (**Table 4.2**). The prediction showed random coils in each protein, suggesting that these proteins lack well-defined ordered secondary structures. The inherent lack of structure in proteins enable them to function efficiently in a wide range of physiochemical conditions without any limitation of structural constraints (Ridhi et al., 2023). Disorder-to-order transitions in IDPs occur when IDRs fold into specified structures, such as α-helices or β-strands, following binding with a partner or during functional activation (Harini et al., 2023). The presence of α-helices and β-strands were found in all discovered proteins which suggested that α-helices as well as β-strands played a role in stabilizing proteins and membrane structure under abiotic stress (Sulatskaya et al., 2021). These secondary structures assure that plants are able to retain their cellular integrity, regulate gene expression, stabilize cellular components, and successfully adapt to environmental cues such as heat stress, drought stress, salinity stress, and cold stress (Zhang et al., 2020). For instance, the tumour suppressor protein p53 exhibits intrinsically disordered areas that undergo a conformational change, adopting α-helices or β-strands (Xue et al., 2013). A study on plant transcription factors (TFs) from the NAC family (NO APICAL MERISTEM, ATAF, CUP-SHAPED COTYLEDON), which are crucial in various processes such as stress response, plant defense, and development, revealed that they possess a conserved N-terminal DNA-binding domain and intrinsically disordered (variable) C-terminal region. (Sun et al., 2013). Upon binding to multiple partners, these proteins adopted a localized α-helix structure in

their C-terminal region. A similar process was observed in other plant transcription factors, such as those in the basic leucine zipper (bZIP) family, where interactions with multiple partners occur through disordered regions (Salladini et al., 2020).

ESTs are single-pass sequences of roughly 200-800 base pairs (bp) produced from randomly chosen cDNA clones. They serve as valuable tools for gene identification and the validation of gene predictions, as they reflect the expressed segments of a genome (Bogdewic, 2000; Pandian et al., 2017). In our study, 22 genes were screened out of 30 genes based upon the similarity index of more than 95% (**Table 4.3**). ESTs have been utilized in diverse applications, such as phylogenetics, proteomics, transcript profiling and microarray design (Haider & Pal, 2013; Li et al., 2009).

Furthermore, the physicochemical properties (**Table 4.4**) of 22 *LuPLR2* genes were observed in which molecular weight (MW) ranged from 27.69 kDa to 70.66 kDa and isoelectric point (pI) value ranged from 5.20-9.60. Comprehending MW and pI yield insights into a protein's composition along with potential associations. Proteins having distinct molecular weights and isoelectric points may serve specific functions in biological processes, including enzymes, transport proteins, or structural elements (Vascon et al., 2020). Most of these proteins were found to be localized in cytoplasm whereas some were found to be present in plastids. Likewise, dirigent proteins involved in lignan biosynthesis like PLR2 had also been found to be localized in chloroplast, cytoplasm, nucleus, and vacuolar regions in pear (*Pyrus bretschneideri*), barrel medick (*Medicago truncatula*), and pepper (*Capsicum annum* L.) (Cheng et al., 2018; Khan et al., 2018; Song & Peng, 2019). The maximum localization of LuPLR2s in cytoplasm suggests that they might be involved in signal transduction, energy production, antioxidant defense, and the formation of secondary metabolites (Isah, 2019). Conversely, proteins within plastids are crucial to photosynthesis, carbon fixation, synthesis of vital components like fatty acids, amino acids, pigments, and ROS detoxification (Przybyla-Toscano et al., 2018). Together, they may regulate metabolic and stress-response pathways, essential for sustaining functioning of cells under stress from environmental factors. Conserved motifs frequently signify regulatory components that influence transcriptional regulation, impacting gene expression

levels and configurations (Amjad et al., 2024). The examination of conserved motifs among the 22 *LuPLR2* genes in flax indicate the prevalence of motif 4 in most of the genes, implying critical functional components in these genes (**Figure 4.1**). The detection of similar motifs clarifies potentially regulatory systems and related mechanisms for regulation across *LuPLR2* genes, emphasizing their involvement in flax growth and development, responses to stress, and other biological activities. In flax, Corbin et al., (2018) studied 44 DIRs, consisting of 10 distinct motifs. Among these, the 7th motif was found to be engaged in the production of (+)/(-) enantiomers of pinoresinol. Exon-intron structure of genes are crucial in regulating gene expression and functionality (Jacob & Smith, 2017). In the case of *LuPLR2* gene family, the exon-intron organization within *LuPLR2* gene family revealed significant variation in gene structure, which provide insights into the functional diversification and evolutionary history of these genes (**Figure 4.2**). The presence of 12 exons and 11 introns in *Lus10007599* suggests a more complex gene structure, which might be associated the regulatory flexibility or alternative splicing, allowing the production of diverse transcripts and protein isoforms. This complexity performs a huge function in enabling adaptive responses to environmental stimuli, particularly under abiotic stress conditions (Scarrow et al., 2021). In contrast, the complete absence of introns in *Lus10024472* is indicative of a streamlined gene structure, potentially favoring rapid transcription and translation. This aligns with findings by Xu et al. (2021) in which *DIRs* in Mungbean (*Vigna radiata*) were reported to have had a classical structure, i.e., some genes had both exons and introns and some were intronless. Such intronless genes are often associated with functions requiring quick responses, such as defense mechanisms or stress-related pathways (Negi et al., 2016). The variations between these two extremes highlights the functional plasticity within *LuPLR2* gene family.

The GO enrichment analysis of the *LuPLR2* gene family highlights their involvement in multiple key biological processes and molecular functions, accentuating their diverse and critical roles in flax. The enrichment in processes such as lignan biosynthesis and secondary metabolite biosynthesis is particularly notable, given the importance of these pathways in plant defense, growth, and adaptation to abiotic stress conditions. Lignans are

known for their anti-oxidative properties and role in structural reinforcement, suggesting that *LuPLR2* genes may contribute significantly to stress tolerance mechanisms (Corbin et al., 2017). The association of *LuPLR2* genes with NADP(H) binding and oxidoreductase activity further supports their role in redox homeostasis and metabolic regulation (Von Heimendahl et al., 2005) (**Figure 4.4**). These functions are essential for managing oxidative stress induced by abiotic factors such as drought, salinity, and extreme temperatures. The enrichment in phenylpropanoid biosynthesis and its associated metabolic processes aligns with the production of phenolic compounds, which are essential for antioxidative defense and signal transduction in stress response (Sharma et al., 2019). Additionally, the involvement of *LuPLR2* genes with the Nmr A-like family and NAD-dependent epimerase/dehydratase family highlights their potential regulatory roles in metabolic pathways. The identification of 3-beta hydroxysteroid dehydrogenase/isomerase family suggests a link to sterol metabolism, which plays a role in membrane stability and cellular signaling under abiotic stress conditions (Simard et al., 2005).

In our work, the promotor regions of *PLR2* gene family were also examined. The cis-acting elements within gene regions called promotors are essential for modulating gene expression and establishing functional specialization (Hernandez-Garcia & Finer, 2014). The various discovered regulatory cis elements of the 22 *LuPLR2* genes offer significant insights into the complex mechanisms regulating their transcription (**Figure 4.5**). The prevalence of TATA-box (72) and CAAT-box (47) in *Lus10007599* and *Lus10042313*, respectively, emphasize their importance in activating transcriptional processes. The specific enrichment of cis elements including MYB recognition sites, MYC, W-box, ARE, DRE1, LTR, WUN-motif, and STRE indicate their possible functions in precisely modulating gene regulation, especially in response to stress (Abdullah et al., 2022). Besides, various hormonal responsive elements were identified, namely, ABRE, AT-ABRE, GARE-motif, P-box, TATC-box, TGA-element, AuxRR-core, and ERE, indicating that *LuPLR2* genes are governed by hormonal signaling pathways, including, abscisic acid, gibberellin, and auxin. Promotor elements associated with tissue-specific or developmental regulation-such as O2-site, HD-Zip-3, GCN_4 were also identified in this study. These

elements are likely to be engaged in the spatial and temporal expression of *LuPLR2* genes. The presence of similar cis elements (ABRE and MYB2) involved in the biosynthesis of lignans have also been reported previously in the promotor region of *LuPLR1* in flaxseed (Corbin et al., 2013). Further, numerous cis-acting elements associated with tissue-specific expression, hormone control, and responses to biotic and/or abiotic stressors had been identified in the promotor region (1,207 bp) of *LuPLR2* gene in flax leaves (Corbin et al., 2017). Similarly, putative cis-acting regions associated with stress responses, including wounding, pathogen attack, or elicitation, were discovered in many flax *DIR* promotor sequences (Corbin et al., 2018). Moreover, in *Isatis indigotica*, the promotor region of *DIR2* contained various cis-elements related to stress response and binding sites of MYB transcription factors which denoted the regulatory processes for *DIR2* expression (Chen et al., 2024). The occurrence and distribution of these cis-acting elements illuminates their role in plant defense pathways and stress adaptation and offer significant insights into the molecular processes governing the functioning of *LuPLR2* genes (Han et al., 2018).

Phylogenetic examination of *LuPLR2* genes across several species revealed three subfamilies (I-III), with *LuPLR2* genes confined to subfamily III (**Figure 4.6**). Members of the same subfamily may exhibit analogous functions (Von Heimendahl et al., 2005). For example, *BhPLR2-like, LcPLR1, LfPLR2,* and *LaPLR1* present in the same subfamily with *LuPLR2s* are involved in the biosynthesis of lignans and also catalyze the enantioselective conversion of (+)-pinoresinol into (+)-lariciresinol and (+)-lariciresinol into (-)-secoisolariciresinol (Bayindir et al., 2008; VonHeimendahl et al., 2005; Corbin et al., 2017). Therefore, it was found that all *PLR2* genes are closely associated with *Linum corymbulosum, Linum flavum, Linum album, and Benincasa hispida,* that evidenced that the flax and its other species as well as *B. hispida* were most closely associated among all other genes of plants. The evolutionary pattern of these genes determine the significance of the gene family in plant defense, stress response, and secondary metabolite diversity, reflecting both conservation and specialization across species.

5.2 Expression analysis using Real time PCR and Quantification of yatein phytochemical using HPLC under different abiotic conditions

The *PLR* gene family is of fundamental significance in governing plant development as well as responding to environmental cues particularly via the biosynthesis of lignans. The biosynthesis of lignans in flax is regulated by PLR enzymes which have opposite stereospecificity and this regulation is driven primarily by distinct gene expression patterns rather than solely by differences in the catalytic efficiencies by these isoforms. Among the PLR enzymes, PLR2s are crucial for the growth of plants and exhibit significant responsiveness to stress conditions. In order to clarify the functions of *LuPLR2* in flax under abiotic stress, we carried out the expression profiling of this gene family using qRT-PCR. This study highlights the need for an in-depth expression analysis of *PLR2* genes to uncover their contributions to lignan biosynthesis and plant adaptation. We analyzed the expression patterns of identified *LuPLR2s* in flax microgreens under control conditions and four abiotic stress treatments at 8 h and 24 h intervals. Under control conditions, *Lus10003328* exhibited the highest expression, indicating its potential role in maintaining baseline physiological functions. In accordance with our study, Corbin et al. (2017) found that the expression of *LuPLR2* expression was maximum young leaves (20 times more than normal) which is coherent with the flax yatein content (Hemmati et al., 2010). This elevated expression suggests potential regulation by auxin, which is actively produced during the development of leaf primordia (Aloni et al., 2003). In our study, the *PLR2* genes were also found to respond to abiotic stimuli in flax microgreens, including cold (4℃), heat (30℃), 5mM and 20 mM NaCl, 5% and 20% PEG suggesting that these genes are related to plant stress resistance. Under all stress conditions, transcript levels of nearly all genes demonstrated a considerable upregulation comparative to the control, with the striking exception of heat stress, where expression was predominantly downregulated. The expression analysis revealed that drought and salinity stresses markedly upregulated *LuPLR2* genes, particularly under 20% PEG and 20 mM NaCl conditions, whereas in heat stress, the reduced expression of *LuPLR2* genes was noted reinforcing the hypothesis that

the elevated temperatures inhibit the lignan biosynthesis. Additionally, certain *LuPLR2* genes also exhibited an overall upregulation under cold stress conditions. Despite the understanding of the importance of lignans including yatein in stress responses, there are no well-defined studies to substantiate the lignan biosynthesis genes (*PLRs*) expression in plants across abiotic stress. However, it has been demonstrated that these *PLRs* and related genes are involved in the process of biotic stress adaptation in a variety of plant species. For instance, in Velvet ash *(Fraxinus velutina Torr.)*, the expression of *FvPLR1*, a crucial gene engaged in lignan formation, had been found to be significantly higher in the phloem of trees infested by *Agrilus planipennis* compared to non-infested trees. Consequently, lignan levels in the phloem of infested trees increased by 290.96% and this enhanced lignan production contributes to improved resistance in Velvet ash, offering valuable insights into the defense mechanism of trees against wood borer infestations (Liu et al. 2022). Likewise, Ralph et al. (2007), observed that the *DIRs* expression, associated with lignan biosynthesis, was stimulated in spruce by a stem-boring insect. In the light of the fact that lignans play a part in defending plants against herbivores and diseases, it is hypothesized that such type of attacks could accelerate the genes' transcription that are involved in the production of lignans (Filipe and Borges, 2014). Some studies have also documented an increased expression of *PLRs* and their related genes in response to hormonal stress and growth regulators. For example, the application of MeJA significantly enhanced *LuPLR2* gene expression, accompanied by increased yatein concentration in the leaves of flax plants (treated). In contrast, no such effect was observed with salicylic acid, indicating that the stress response of *LuPLR2* was regulated through jasmonate signaling rather than salicylic acid pathways (Corbin et al., 2017). Another study reported that treatment with exogenous ABA upregulated the transcription of the *LuPLR1* & boosted the production of SDG in the flaxseed, highlighting the potential for increased lignan accumulation (Renouard et al. 2012). Additionally, stress-inducible expression (wounding, methyl jasmonate and UV-C) in *Podophyllum hexandrum* (*PhPLR),* and other genes associated with podophyllotoxin (PTOX) biosynthesis, namely, *PhSDH* (Secoisolariciresinol dehydrogenase), and dirigent protein oxidase (*PhDPO*) were observed in Himalayan Mayapple (Wankhede et al., 2013).

Interestingly, the patterns of expression of the genes involved in lignan formation were reported to be upregulated in response to various stress conditions in almost all the reported studies, closely aligning with our findings.

HPLC is a highly regarded and widely used method in phytochemical research for analyzing plant compounds, including yatein, a dibenzylbutyrolactone lignan. This technique is essential for identifying and quantifying compounds within complex plant extracts, providing valuable insights into chemical profiles and the metabolic responses of plants to environmental stress. In the present study, HPLC was employed to identify and measure yatein levels in ethanolic extracts of flax microgreens exposed to varying concentrations of abiotic stress. The analysis revealed that yatein consistently exhibited a retention time of approximately 8.5 minutes, facilitating its reliable identification at a detection wavelength of 280 nm. Quantification was achieved using a calibration curve, allowing precise measurement of yatein concentration in the extracts. The highest yatein concentration (5.332 mg/g) was recorded under 20% PEG-induced stress, suggesting an upregulation of the compound in response to increased drought stress. In contrast, a slightly lower concentration (4.408 mg/g) was observed in samples treated with 5% PEG, indicating that yatein production in flax microgreens is influenced by drought in a concentration-dependent manner. This reveals the potential role of abiotic stress, particularly drought, in enhancing the biosynthesis of phytochemicals like lignans. Yatein, as a secondary metabolite, may contribute to the adaptive mechanisms of plants or protective responses under adverse conditions. Moreover, the observed variability in yatein production under different stress treatments offers critical insights for researchers aiming to optimize conditions for the biosynthesis of this compound, whether for its pharmacological applications or other uses.

Consistent with our results, Corbin et al. (2017) studied that the yatein production was significantly higher in mechanically damaged leaves as compared to control samples assessed through HPLC, suggesting that mechanical stress could trigger lignan biosynthesis as part of plant's protective system. Likewise, Berim et al. (2005); Van

Fürden et al. (2005); Yousefzadi et al. (2010) previously studied the accumulation of lignans (in vitro) in *Linum album* and *Linum nodiflorum* through MeJA-dependent pathways. Also**,** exogenous stress hormonal treatments (50 µM of salicylic acid) to flax cell culture increased the biosynthesis of lignans (SDG), and lariciresinol diglucoside (LDG) and neolignans by 2 to 4 times in comparison to control (Nadeem et al. 2019). Jalal et al. (2022) demonstrated an increased production of SDG in callus at 50℃ exposed for 20 minutes under heat and cold stress as compared to control. Similarly, tetraploids of *Isatis indigotica* exhibited higher levels of lariciresinol (lignan) production compared to diploids, which enhanced the root development and increased resistance to drought and salt stress (Xiao et al., 2020). Another study by Calzone et al., (2023) investigated two pomegranate cultivars (Wonderful and Parfianka) that well responded against the NaCl stress by the formation of cinnamic acid derivatives involved in lignan production. Besides, Anjum et al. (2017) detected the impact of various doses of UV-C radiations which were found to be more efficient in producing lignans, neolignans and other biochemical indicators. Amongst the UV-C dosages, 3.6kJ/m^2 led to increased lignan (SDG, LDG) synthesis in cell cultures maintained in photoperiodic conditions. Additionally, cell cultures exposed to UV-C also gained significantly larger quantities of flavonoids and antioxidant-phenolics (Anjum et al., 2017). Notably, most of the studies aligned with our results depicting that the environmental cues can enhance the production of lignans in plants, providing a reliable approach for evaluating their phytochemical levels.

These results hypothesize that the expression patterns of *LuPLR2* genes are correlated with yatein accumulation under various abiotic stress conditions revealing a sophisticated interplay between gene regulation and secondary metabolite biosynthesis in flax microgreens. These findings substantiate the hypothesis that *LuPLR2* transcriptional regulation is integral to yatein production, particularly under salinity and drought stresses, where the upregulation of lignan biosynthesis appears to be a critical adaptive response. The dynamic interplay between gene expression and metabolite accumulation likely involves ROS-mediated signaling, given the known antioxidant properties of lignans. Abiotic factors generate oxidative stress might activate ROS-scavenging mechanisms,

including lignan biosynthesis. The upregulation of *LuPLR2* genes under abiotic stress suggests a feedback mechanism where oxidative cues enhance lignan biosynthesis to bolster plant resilience. Conversely, heat stress, which suppresses both *LuPLR2* expression and yatein accumulation, might represent a scenario where metabolic resources are diverted away from secondary metabolism towards survival strategies such as protein stabilization and heat shock responses.

5.3 Effect of Yatein on Antioxidative potential of flax microgreens under different abiotic stress conditions

To keep the balance between ROS synthesis and quenching, plants normally produce toxic oxygen metabolites at low levels which is crucial for plant development and growth. However, there is rapid increase in intracellular ROS concentrations because of environmental stresses which can disturb this balance and such imbalances trigger phytotoxic responses (Mandal et al., 2022). Plants are affected by the accumulation of excessive ROS, which presumably induces oxidative damage to DNA and impedes enzyme activity during protein oxidation, ultimately leading to programmed cell death (PCD) (Feng, Jia, et al., 2023). Nevertheless, it is improbable that plants can revert to a normal state under abiotic stress solely through their self-regulatory mechanisms; therefore, in response to abiotic stress, exogenous substances are considered enhancers that strengthen the existing antioxidant systems in plants (Feng et al., 2024; Feng, Gao, et al., 2023). Yatein, a naturally occurring lignan with potential anti-oxidative properties, was used exogenously in this study in order to mitigate stress-induced oxidative damage in flax microgreens. There is no well-defined study till date to report the effect of exogenous application of lignans including yatein on the antioxidative machinery in the plants under various stress conditions. This study addresses the gap by analyzing the anti-oxidative enzyme activities in untreated and yatein-treated flax microgreens exposed to abiotic stresses.

SOD is a metalloenzyme superoxide dismutase which is pivotal in defending aerobic organisms against oxidative stress induced by ROS. As the primary line of defense, SOD facilitates the dismutation of O^{-}_{2} into hydrogen peroxide and water. Its upregulation is closely linked to mitigating damage caused by stress, performing a major part in the adaptation of plants (Fujii et al., 2022; J. Xu et al., 2014). In untreated flax microgreens, as compared to the control, highest SOD activity was observed under 5% PEG-induced drought stress and 5 mM NaCl-induced salt stress [**Figure 4.9 (a)**]. Various studies in the past have also reported increased SOD activity in response to different environmental cues. In *Cicer arietinum, Beta vulgaris, and Brassica juncea*, the SOD activity had been reported to increase, leading to improved increased leaf production. The SOD activity was also found to be increased in *Olea europaea*, *Oryza sativa*, *Brassica juncea*, *Brassica rapa* cultivars under water stress, reflecting its vital role in combating drought-induced oxidative damage (Sen & Alikamanoglu, 2013; Sharma & Dubey, 2005; Verma et al., 2019). Moreover, Tibetan wild *Hordeum vulgare* genotypes exhibited significantly higher SOD activity during the anthesis stage under drought stress (Ahmed et al., 2013). Additionally, in wheat, SOD had been implicated in protecting photosystem II from oxidative damage, particularly during water stress (Deeba et al., 2012). In our study, the SOD activity was found to be moderate under cold, 20% PEG and 20 mM NaCl, whereas minimal activity was detected under heat stress in the untreated plants. The present study depicting an increase in the SOD activity under cold stress corroborates the previous findings, where cold-stressed tolerant cultivars of rice, barley, and tobacco exhibited differential SOD activity in roots and shoots, with roots generally showing high SOD activity (Mutlu et al., 2013; S. C. Xu et al., 2010). Further supporting our observations, expression profiling of SOD genes in water lilies depicted their responsiveness to heat, saline, cold, cadmium chloride, and copper sulphate stress (Khan et al., 2023). The variability in SOD activity across species and stress conditions underscores its critical role in oxidative damage mitigation and overall plant survival under stress.

In our study, the yatein-treated samples consistently exhibited higher SOD activity compared to their untreated counterparts under all the stress conditions, indicating the

antioxidative role of yatein [**Figure 4.9 (c)**]. Among all the stress conditions, the SOD activity was observed to be the highest under 5% PEG. Further supporting this, Parvin et al., (2020) demonstrated that the exogenous vanillic acid (VA) alleviated osmotic and ionic toxicity in salt-stressed tomato seedlings by increasing the relative water content and proline levels. Additionally, VA enhanced the SOD, and CAT activity in salt-treated seedlings, leading to decreased ROS production, lipoxygenase activity, and membrane damage.

Catalase is a key antioxidant enzyme that plays a crucial role in mitigating oxidative stress by breaking down H_2O_2 into water and oxygen. In plants, catalase is responsible for scavenging H_2O_2 generated during processes such as mitochondrial electron transport, β-oxidation of fatty acids, and photorespiratory oxidation (Anwar et al., 2024). In our study, in untreated microgreens, CAT activity was the highest under cold stress, indicating an enhanced ability to degrade H_2O_2 into water and oxygen, a critical function for mitigating ROS damage (Mittler, 2002; Zandi & Schnug, 2022). Moderate activity was observed under salt stress and drought stress, with relatively lower activity in heat stress compared to control [**Figure 4.10 (a)**]. These findings align with a study, where increased CAT activity was reported in the varieties of flax under cold stress (Ghoreishi et al., 2017). Moreover, in *Nicotiana tabaccum* and *Triticum aestivum*, CAT gene family was found to be upregulated under abiotic stress conditions (Liu et al., 2023; Tyagi et al., 2021). Similarly, CAT activity was found to increase under salt and drought stress conditions in *Cleome* species (Ajithkumar & Panneerselvam, 2014), flax (Khan et al., 2010), and olive plants (Sofo et al., 2005). This highlights the catalase role in plant stress tolerance, suggesting its variable activity under abiotic stress as a key mechanism for protecting cellular integrity and mitigating oxidative damage.

On the other hand, yatein treated samples generally exhibited higher CAT activity than the untreated samples under all the abiotic stress conditions, emphasizing the role of yatein in enhancing antioxidative defense [**Figure 4.10 (c)**]. As compared to control, the CAT activity was moderately higher under cold stress and 20 % PEG stress conditions in

the treated flax microgreens. Concomitant with our findings, exogenous cinnamic acid was also reported to enhance the activities of antioxidant enzymes, including SOD, CAT, GPX, and APX in cucumber leaves under chilling stress while reducing the contents of H_2O_2, MDA and O_2^- radicals as compared to non-treated stressed plantlets (Q. Li et al., 2011). Further supporting our observations, exogenous chlorogenic acid effectively mitigated the decline in chlorophyll concentrations and maximized potential Photosystem II efficiency in methyl-viologen mediated oxidative stress in the leaves of apple. It also significantly reduced membrane damage and lipid oxidation while enhancing the activity of antioxidant enzymes such as peroxidase (POD), CAT, and polyphenol oxidase (Mei et al., 2020).

GSTs represent a large and diverse family of multifunctional enzymes that respond to ROS (O^-_2 and H_2O_2). These enzymes catalyze the conjugation of electrophilic xenobiotic compounds, such as, alkenes, esters, epoxides, and alkyl and aryl halides with the tripeptide glutathione (Gülçin et al., 2016; Lee, 2024). Additionally, GSTs contribute to tyrosine metabolism, vacuolar sequestration of anthocyanins, hormone regulation, apoptosis regulation, and plant responses to stress conditions (Dixon et al., 2002; Waadt et al., 2022). In this study, in untreated flax microgreens, GST activity was significantly elevated under cold stress, followed by a moderate increase under heat, 20% PEG stress, and salt stress suggesting their role in sustaining redox homeostasis and maintaining metabolic functions during oxidative stress [**Figure 4.11 (a)**]. Aligned with our study, under drought stress, elevated GST activity had also been observed in drought-tolerant cultivars of *Triticum aestivum* (Rakhra et al., 2015). Also, C. Xu & Huang, (2008) reported higher GST activity in the thermos-tolerant *Agrostis scabra* grass cultivar compared to thermos-sensitive *Agrostis stonolifera*, thus providing thermos-tolerance to the former. Also, a GST gene *(CsGSTU8)* in tea plants exhibited a significant increase in expression under drought conditions, defining its function in the plant's reaction in the conditions of drought (Y. Zhang et al., 2021). Additionally, in *Phaseolus vulgaris*, GST gene family showed upregulation across both drought and salt stress, enabling *P. vulgaris* to acclimatize stressful conditions (Anik et al., 2024). This emphasize its crucial role of GSTs in

alleviating oxidative stress and sustaining cellular homeostasis, underscoring their importance in plant defense mechanisms under diverse abiotic stress conditions.

Conversely, in yatein-treated flax microgreens, GST activity was higher compared to untreated samples under all environmental constraints, indicating that yatein enhanced GST-mediated detoxification in flax microgreens [**Figure 4.11 (c)**]. The highest GST activity was observed under heat stress, where yatein treatment significantly enhanced activity compared to all other groups. Yatein treated flax microgreens under cold, 20 mM NaCl, 5 mM NaCl, 5% PEG, and 20% PEG stress also showed a notable increase in GST activity. In accordance to our results, the application of Vanillic acid upregulated the GST, SOD, and GR activity in salt-stressed tomato seedlings (Parvin et al., 2020).

In the Asada-halliwell (AsA-GSH) cycle, APX is a crucial enzyme for scavenging intracellular ROS and safeguarding plant cells. It utilizes an electron donor (AsA) to convert hydrogen peroxide into water, simultaneously producing two molecules of monodehydroascorbate (MDHA). In untreated flax microgreens, the highest APX activity was observed under 20% PEG stress, followed by 20 mM NaCl and 5% PEG treatments, while moderate activity was observed under cold stress [**Figure 4.12 (a)**]. These findings align with the role of APX in mitigating oxidative stress under osmotic and saline stress conditions, as demonstrated in transgenic tobacco plants harboring APX from Arabidopsis, which displayed enhanced tolerance to salt, drought, and PEG stress (Eltelib et al., 2012; Koussevitzky et al., 2008; Wang et al., 2005). Similarly, increased APX activity in pea under saline conditions had been reported in chloroplast-localized APX (chlAPX), providing protection against ROS generated in organelles like mitochondria and peroxisomes (Eltelib et al., 2012). The relatively lower APX activity under heat stress observed in our study is consistent with findings where APX activity decreased rapidly after heat shock treatment, potentially limiting its role in high-temperature adaptation in cucumber leaves (Gao et al., 2010). Under cold stress, moderate APX activity observed in our experimental set up aligns with findings in tolerant maize where enhancement in the APX activity was seen in response to low temperatures (Caverzan et al., 2012). Furthermore, the antioxidant enzymes, including, SOD, CAT, APX, GPX,

monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) were found to be upregulated in response to abiotic stress (cold, heat, salt, and light) in Arabidopsis (Filiz et al., 2019).

On the contrary, in yatein-treated flax microgreens, APX activity was higher compared to untreated samples under all the stress conditions, suggesting that yatein enhanced APX activity, thereby strengthening the antioxidative defense system in flax microgreens [**Figure 4.12 (c)**]. APX activity increased progressively under 20% PEG, cold and 20 mM NaCl treatment whereas 5% PEG and 5 mM NaCl showed moderate enhancement. Aligned with our study, in *Nitraria tangutorum*, SA application significantly suppressed the increase in electrolyte leakage and MDA content in the leaves of NaCl-treated seedlings. Additionally, SA treatment enhanced the accumulation of proline, soluble sugars, and soluble proteins. The activities of antioxidant enzymes, including SOD, CAT, APX, and POD were upregulated under NaCl stress and were further boosted by SA treatment (Liu et al., 2016). Moreover, exogenous SA in cadmium –exposed flax upregulated the activities of APX and POX (Belkadhi et al., 2014). Also, in submerged rice, SOD and APX activities were found to be increased with the application of protocatechuic acid and vanillic acid (Xuan & Khang, 2018).

GPX, a heme-containing protein, plays a critical role in detoxifying ROS by utilizing aromatic electron donors like guaiacol and pyrogallol to reduce H_2O_2, thereby mitigating oxidative damage (Bela et al., 2022; Das & Roychoudhury, 2014). The observed variations in GPX activity across different stress treatments in the untreated group reflect the dynamic role of enzyme in modulating oxidative stress responses [**Figure 4.13 (a)**]. GPX activity was notably highest under 20 mM NaCl treatment, consistent with its function in mitigating salinity- induced ROS accumulation. Moderate activity under heat stress and comparatively lower levels under cold stress and other stress conditions suggest that GPX may play a stress-specific role, with its efficacy varying according to the type and severity of stress (Chawla et al., 2013; W. U. Khan et al., 2024). This aligns with earlier studies where drought-tolerant cultivars of *Brassica napus* and *Oryza sativa* exhibited

higher GPX activity, highlighting the enzyme's vital role in sustaining cellular redox homeostasis under water-deficit stress (Dolatabadian et al., 2008; V. Kumar et al., 2009). Further supporting our observations, overexpression of GPX genes in transgenic plants demonstrated improved tolerance to various environmental conditions. For example, the *Nelumbo nucifera* transcript (*NnGPX*) showed significant upregulation in response to cold, heat, mechanical damage, and salt stress, while its overexpression in rice resulted in enhanced salt tolerance (Diao et al., 2014). Additionally, in *Citrullus lanatus* transcripts (*CIGPX*) and were markedly upregulated in response to salt, drought, and cold stress, as well as abscisic acid (ABA) treatment at various time points, indicating their role in abiotic stress adaptation and ABA-mediated signaling (Zhou et al., 2018). Furthermore, in *Nymphaea colorata* (*NcGPX)*, the GPX gene family were found to be upregulated in response to multiple abiotic stress conditions (Khan et al., 2024). The consistency of our findings with reports where both tolerant and sensitive varieties of wheat and rice exhibited increased GPX activity under salt stress reinforces the enzyme's universal role in oxidative stress mitigation (Alamgir Hossain et al., 2013; Mandhania et al., 2006).

Conversely, in yatein-treated flax microgreens, GPX activity was higher than untreated groups under all the stress conditions, indicating an enhancement of enzymatic antioxidative capacity under yatein treatment [**Figure 4.13 (c)**]. GPX activity peaked significantly under cold stress, in the yatein-treated group, displaying the highest activity among all the conditions, indicating its pivotal role in neutralizing lipid hydroperoxides and safeguarding membrane integrity. Under heat, 5 mM NaCl, and 20% PEG stress conditions, moderate increase was observed in GPX activity in yatein-treated samples compared to untreated ones. In accordance with our study, application of phenolic acids like SA decreased the harmful effects of drought stress by strengthening the total soluble proteins, chlorophylls a and b as well as peroxidase activity. The positive effects of SA in mitigating the adverse effects of drought stress may be attributed to enhanced stomatal regulation, preservation of leaf chlorophyll content, improved water use efficiency, and stimulation of root growth (Anosheh et al., 2012). Moreover, Yadu et al., (2017) reported

that in salt-stressed pea seedlings, exogenous SA enhanced the activities of SOD, CAT, GPX, and APX.

GR (a flavoprotein oxidoreductase) catalyzes the reduction of GSSG to GSH using NADPH. This process is crucial for regenerating antioxidant ascorbic acid (AsA) within the AsA-GSH cycle, by using MDHA and dehydroascorbic acid (DHA), thereby converting GSH to GSSG (Gondim et al., 2012; Mishra et al., 2023). By maintaining a high cellular GSH/GSSG ratio, GR ensures an adequate supply of reduced GSH, a key factor in mitigating oxidative stress and sustaining cellular redox homeostasis under environmental circumstances (Surya et al., 2008; Venkateswarlu et al., 2012). The observed variations in GR activity across different stress conditions in the untreated group emphasize its critical role in plant stress responses and adaptation [**Figure 4.14 (a)**]. The highest GR activity under 5% PEG treatment, and 20 mM NaCl, suggested a robust activation of the enzyme under drought and saline stress conditions. Moderate activity under cold and 5mM NaCl stress, alongside relatively lower activity under heat and 20% PEG, indicates that the involvement of GR in stress tolerance is influenced by both the type and severity of stress encountered. In the context of PEG-induced drought stress, the increased GR activity observed aligns with the previous studies highlighting its role in enhancing drought tolerance through elevated GSH levels and reduced GSSG levels, as seen in *Ctenanthe setosa* (Bian & Jiang, 2009). The correlation between high GSH levels and water regulation in leaves underscores the role of enzyme in preserving water content under water-deficit conditions (Jiang & Zhang, 2002). Similarly, GR activity performed a critical function in encountering ionic toxicity and osmotic inhibition under salt stress, with increased activity as demonstrated in a variety of crops, including tomato, rice, soybean, wheat, and Arabidopsis (Hasanuzzaman et al., 2013, 2017). These findings are consistent with our observations, further supporting the role of GR in mitigating oxidative stress induced by salt and drought conditions. Furthermore, in *Zea Mays*, increased activity of APX, GST, GR, and DHAR was observed, highlighting their essential role in protecting cells against ROS and metabolic disturbances during salt stress (Methela et al., 2024). Interestingly, GR activity had also been linked to thermotolerance. For instance, elevated

GSH levels and increased GR activity under high temperatures had been observed in wheat and maize, suggesting a role for GR in maintaining thermotolerance (Nahar et al., 2015b; Sumithra et al., 2006). The moderate activity of GR observed under cold stress in our study aligns with previous findings where cold-induced GR activity was linked to enhanced antioxidant defense and stress tolerance in French bean seedlings, rice, and eastern white pine (Doty, 2007; Hasanuzzaman et al., 2013). The capability of the enzyme to sustain high GSH levels & counteract ROS accumulation positions it as a key player in plant stress resilience.

On the other hand, flax microgreens treated with yatein, showed higher GR activity than their untreated groups, reflecting the role of yatein in boosting antioxidative defense mechanisms under stress [**Figure 4.14 (c)**]. Under heat stress conditions, GR exhibited the highest activity, showing a significant peak in GR activity compared to untreated microgreens, highlighting the strong protective effect of yatein under this specific stress. The improved GR activity under heat stress is consistent with findings by Nahar et al., (2015a), where exogenous GSH in mung bean seedlings enhanced tolerance to heat stress by regulating antioxidant systems, enhancing endogenous GSH levels, and detoxifying methylglyoxal (MG). This suggests that yatein may similarly bolster endogenous GSH, enabling effective ROS scavenging and improving cellular resilience. Moderate increase in GR activity were observed under cold and 20% PEG stress compared to their respective untreated groups in our study. This result parallels with the findings in chickpea seedlings exposed to osmotic stress where ellagic acid applied exogenously upregulated SOD, GR, CAT activity (El-Soud et al., 2013). Stress conditions such as 20 and 5 mM NaCl, 5% PEG depicted comparable results, with yatein treatment consistently increasing GR activity. Enhanced GR activity in yatein-treated samples suggests improved GSH recycling, thereby amplifying the plant's ability to mitigate oxidative damage induced by abiotic stress.

To conclude, the anti-oxidative enzyme activities in untreated flax microgreens reflect their natural defense strategies against abiotic stress. The significant enhancement observed in yatein-treated microgreens suggests that yatein may act through mechanisms

such as upregulating the expression of genes encoding anti-oxidative enzymes, modulating redox signaling pathways, or directly scavenging ROS. These mechanisms likely to contribute to improved enzymatic efficiency, including enhanced activity of SOD, CAT, and peroxidases, thereby decreasing oxidative damage. This highlights the potential of yatein as a functional phytochemical for enhancing stress resilience and nutritional quality in flax microgreens.

CHAPTER 6: SUMMARY AND CONCLUSION

If we knew what it was we were doing, it would not be called research, would it?"
– **Albert Einstein**

Flaxseeds are an exceptionally rich source of lignans and the concentration of lignans is 40 to 800 times higher than that found in other lignan-containing plant species. It also serves a model crop for exploring stress-resilient traits and bioactive compounds. Flax lignans are predominantly synthesized by PLR enzymes and these PLRs facilitate the conversion of PINO into SECO. Flax contains two enantioselective PLR enzymes, LuPLR1 and LuPLR2. Amongst these, LuPLR2 is exclusively expressed in the stems and leaves, suggesting its role in promoting yatein accumulation, potentially linked to plant defense mechanisms. Although *PLR2s* are known to play a significant role in lignan biosynthesis and stress responses in various plant species, research specifically focusing on these genes in flax remains limited. There is paucity of studies investigating their genomic characteristics, expression patterns, and functional implications under different environmental conditions. This gap in knowledge hinders a comprehensive understanding of their contribution to lignan biosynthesis, particularly in response to abiotic stress, which is crucial for optimizing the medicinal and agronomic potential of flax. Therefore, to investigate and understand the role of *LuPLR2* genes in flax, a genome-wide identification and analysis of *PLR2* genes was performed using the available genome assembly (*Phytozome genome ID: 200 • NCBI taxonomy ID: 4006*) from Phytozome database (https://phytozome-next.jgi.doe.gov/). The primary objectives of this study were:

1. Genome wide *In-silico* characterization of genes responsible for Yatein biosynthesis in Flax microgreens.

2. Expression analysis using Real time PCR and Quantification of Yatein phytochemical using HPLC under different abiotic conditions.

3. Effect of Yatein on Antioxidative potential of flax microgreens under different Abiotic stress conditions.

Methodology

A search for *PLR2* genes belonging to PLR family in flax was conducted using the Phytozome database. The CDS, peptide sequences, genomic sequences and transcript sequences were retrieved for all genes from the Phytozome database. Intrinsic disorder nature and secondary structures were predicted from PONDR and GOR database, respectively. EST analysis was performed using BLASTN against the EST database for Linum usitatissimum. Domain analysis was performed using HMMER and Pfam database. Localization analysis of PLR2 proteins was conducted using LOCTREE3 database, while the predicted molecular weights and isoelectric points were calculated with the Compute_PI server. Gene structure analysis was visualized with the GSDS 2.0 web server and conserved motifs were identified through MEME 5.4.0. Multiple sequence alignment of peptide sequences was conducted using the MUSCLE tool in MEGA11 database, and a phylogenetic tree was constructed with MEGA11 employing the neighbor-joining method with 1000 bootstrap replicates, which was further visualized using the i-TOL platform. Promotor region analysis, including the identification of cis-acting elements, was carried out using the PLANTCARE database. Gene ontology enrichment analysis was done using Shiny GO 0.77 web server.

Expression analysis of identified *LuPLR2* genes in flax microgreens was performed with the help of qRT-PCR under different abiotic stress conditions. The study also quantified yatein levels in flax microgreens grown under abiotic stress conditions using HPLC technique. Antioxidative assays were performed in untreated and yatein treated flax microgreens under different abiotic stress conditions as to know the anti-oxidative role of yatein in flax microgreens.

Salient findings

A total of 30 *PLR2* genes were identified in *Linum usitatissimum* through in-silico analysis. Almost all the proteins revealed disordered nature and the presence of secondary structures were seen among all the proteins. ESTs analysis was done on the basis of

similarity percentage and 22 out of 30 proteins were selected for further analysis. Domain analysis marked the presence of NmrA like family domain in most of the proteins. Motif analysis revealed that 4 motifs were associated with NmrA like family. Subcellular localization analysis depicted most of the PLR2s were localized in cytoplasm (20) while few were found in plastids (2). The molecular weights and pI ranged from 27.69 kDa to 70.66 kDa and 5.20-9.60, respectively. Phylogenetic analysis revealed that the PLR2s were associated with the formation of lignans. Gene structure analysis highlighted the organization of exons and introns, with only 1 gene lacking intron while 21 genes had both exons and introns. Gene ontology analysis revealed the involvement of most *PLR2* genes in the regulation of NmrA family like as well as in the lignan biosynthetic pathway. Promotor analysis identified various cis-acting elements, categorized into stress-responsive, hormone-responsive, and developmental cis elements.

Expression profiling revealed differential regulation of *PLR2* genes in flax microgreens under abiotic stress factors (salt, drought, and extreme temperatures), suggesting their active involvement in stress response mechanisms. Quantification of yatein in flax microgreens under abiotic stress conditions demonstrated a significant increase in yatein accumulation, particularly in response to drought and salinity stress, suggesting that yatein plays a necessary role in the adaptive response of the plant to abiotic stress. Antioxidative assays exhibited enhanced activity of key antioxidant enzymes, such as SOD, CAT, APX, GPX, and GR, in yatein-treated stressed microgreens. This confirms the role of yatein in activating antioxidative defense mechanisms, contributing to improved stress tolerance in flax microgreens.

The findings provide critical insights into the genetic and molecular basis of abiotic stress resilience in flax and offer promising avenues for genetic engineering and agronomic interventions to enhance the nutritional and pharmacological value of flax microgreens under environmental challenges.

Conclusion

- This study provides a comprehensive understanding of the *LuPLR2* gene family in *Linum usitatissimum,* elucidating their genomic structure, functional attributes, and regulatory mechanisms.
- Through in-silico analysis, key insights were gained into the intrinsic disorder properties, secondary structure, sub-cellular localization, and conserved domains of LuPLR2 proteins. The identification of stress-responsive and regulatory motifs in promotor regions highlights the involvement of genes in abiotic stress adaptation**.**
- Furthermore, the quantification of yatein in flax microgreens revealed an increase in yatein production under abiotic stress conditions. Besides this, the yatein-treated flax microgreens exhibited enhanced activity of anti-oxidative enzymes, like CAT, SOD, APX, GPX, GST, and GR. These results will further add to the role of yatein in modulating anti-oxidative pathways and improving stress tolerance.
- However, the findings are specific to flax microgreens and selected stress conditions, and further validation may be necessary to extend the applicability of the results to mature plants or other species.

Future aspects

Research on the relationships between environmental factors and *LuPLR2s*' gene expression has provided valuable insights into how plants adapt to their environment and cope with stress. One of the most urgent areas of future research is to gain the valuable insights into the development of stress-tolerant plants and the enhancement of plant resilience by understanding the connections between IDPs and PLR2s. While structural characterization techniques like X-ray crystallography, and NMR spectroscopy are classical approaches for elucidating protein structures, their application to PLR2 family enzymes involved in yatein biosynthesis may be limited due to challenges in crystallizing plant-derived proteins, particularly those with intrinsically disordered regions. Future research could instead focus on integrated computational and experimental approaches such as cryo-electron microscopy (cryo-EM), Molecular dynamics stimulations, Advanced metabolomics (UHPLC-QTOF, LC-MS/MS) etc. to dig deep into the structural dynamics of PLR2s, which can elucidate the roles of disordered regions of PLR2s in abiotic stress conditions.

Moreover, multi-omics integration, advanced sequencing, and computational-reannotation will validate gene structural features like exons, introns, and UTRs more precisely. Future validation of GO enrichment analysis and pathway associations for *LuPLR2* will depend on functional genomics, metabolic assays, structural biology, and molecular biology experiments. Besides, the validation of *LuPLR2* promotor analysis will combine experimental promotor dissection, TF-DNA interaction studies, and expression profiling under specific conditions. Such approaches will ensure that in-silico gene predictions reflect biologically confirmed functions as well as transform sequence-based predictions into functional regulatory maps.

In addition, the exploration of the regulatory mechanisms that control *LuPLR2* expression under various stress conditions is still missing in flax. In our study, we observed that the *LuPLR2s* were known to be upregulated across different abiotic conditions.

However, the precise signaling channels/pathways as well as transcription factors involved in this process remain largely unidentified, highlighting a gap in current understanding. This could be achieved through traditional breeding techniques, where plants with favorable regulatory traits are selectively bred, or through genetic modification, as well as specific genes involved in the regulation of *LuPLR2s* are altered to optimize yatein production.

Further research is needed to understand how yatein can be used to enhance crop resilience and protect plants from abiotic stress. Additionally, research could focus on developing crop varieties that have been genetically modified to produce higher levels of yatein, thereby enhancing their natural defenses against environmental stressors. The continued exploration of the impact of abiotic stress on secondary metabolite production in plants holds promise for advancing our understanding of plant biology and for developing innovative approaches to improve agricultural productivity and sustainability.

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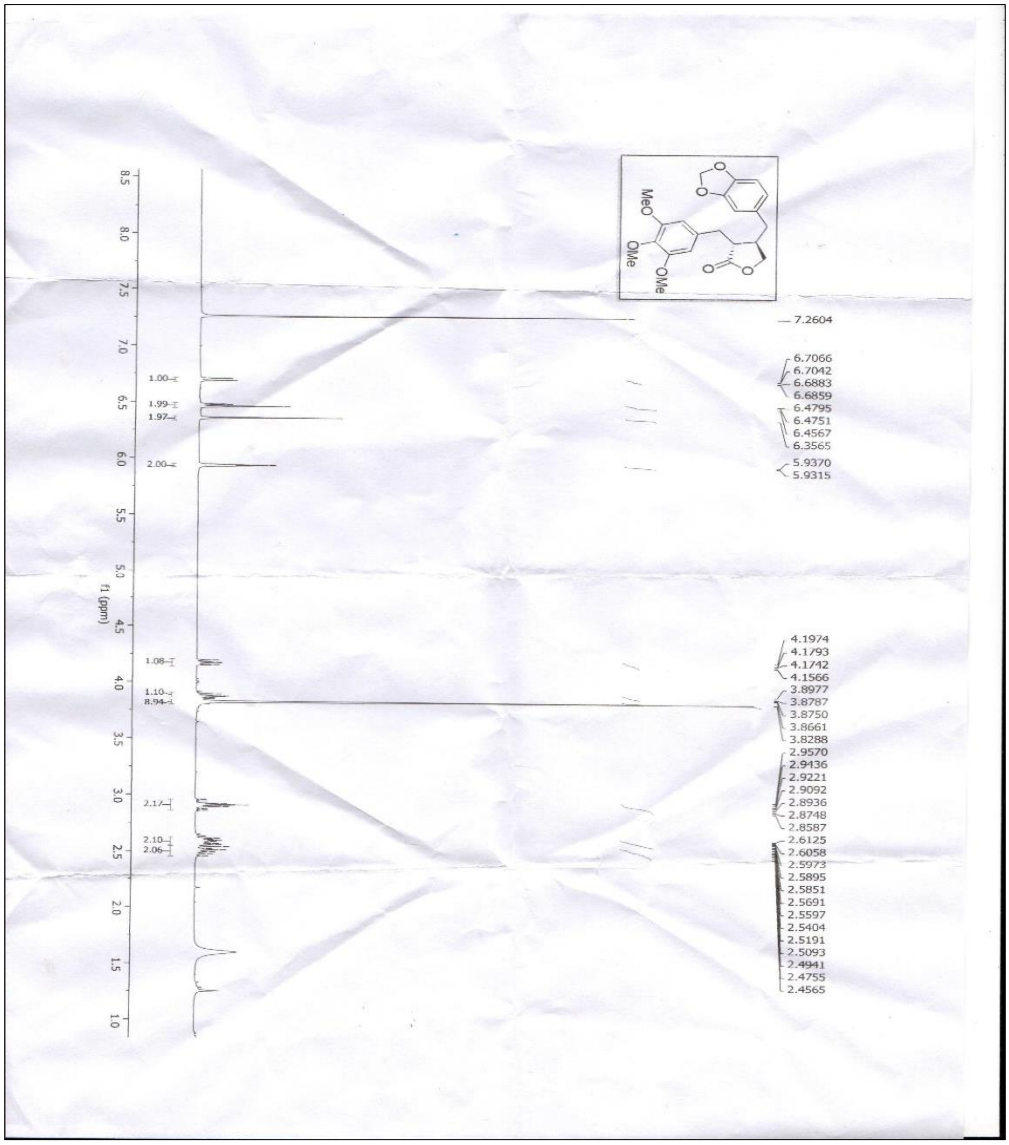
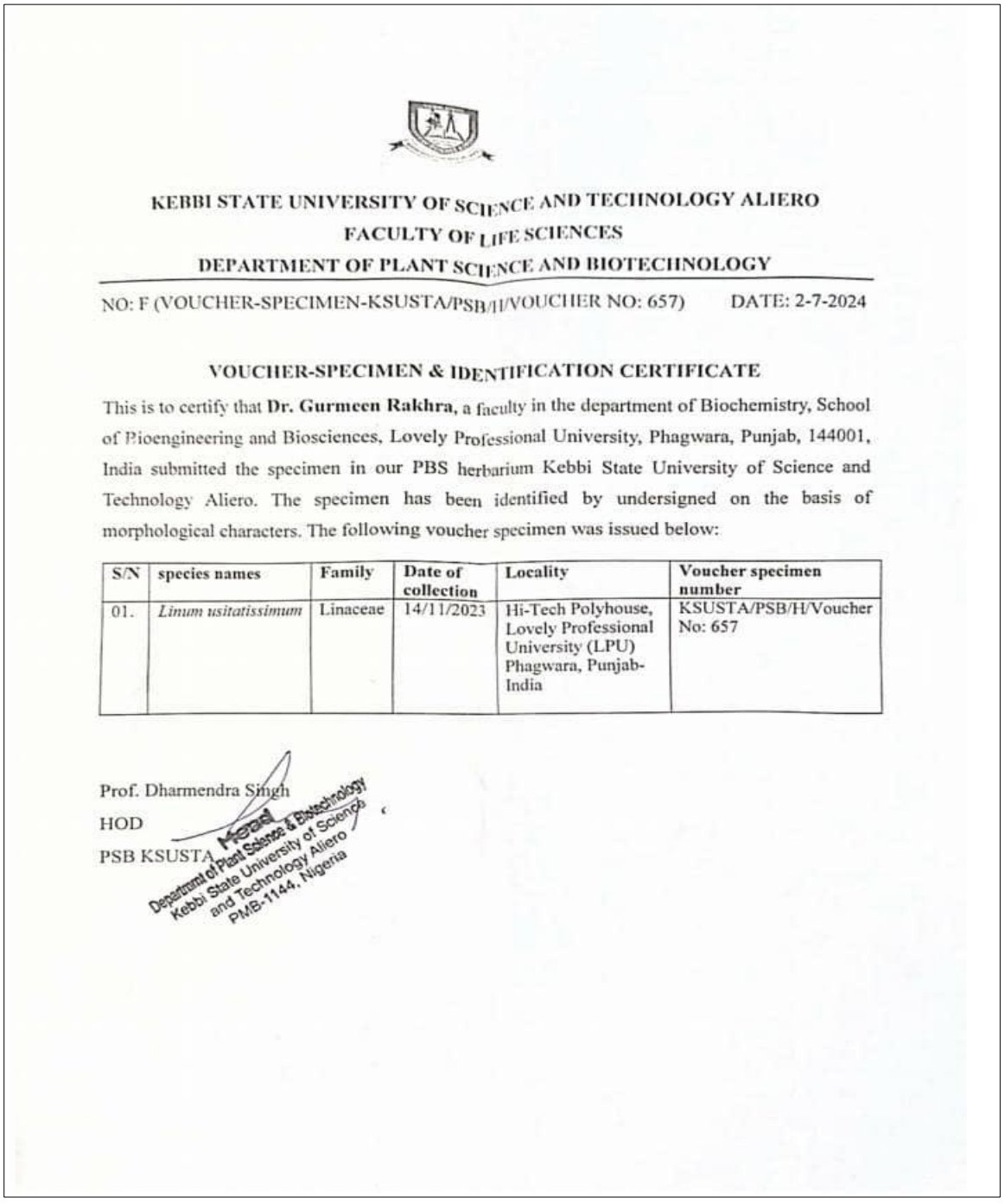
Appendix

- NMR report of the Yatein procured from Centre of Biomedical Research, Sanjay Gandhi Post-Graduate Institute of Medical Sciences, Rae Bareli Road, Lucknow, for conducting the Antioxidative studies



- Identification of the Flax Microgreens: Their identity was authenticated by a Professor (Dr. Dharmendra Singh) in Plant Taxonomy, Kebbi State University of Science and Technology Aliero (KSUSTA), with voucher specimen number (KSUSTA/PSB/H/ Voucher No: 657), deposited in the herbarium of the institute.



KEBBI STATE UNIVERSITY OF SCIENCE AND TECHNOLOGY ALIERO

FACULTY OF LIFE SCIENCES

DEPARTMENT OF PLANT SCIENCE AND BIOTECHNOLOGY

NO: F (VOUCHER-SPECIMEN-KSUSTA/PSB/H/VOUCHER NO: 657) DATE: 2-7-2024

VOUCHER-SPECIMEN & IDENTIFICATION CERTIFICATE

This is to certify that **Dr. Gurmeen Rakhra**, a faculty in the department of Biochemistry, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, 144001, India submitted the specimen in our PBS herbarium Kebbi State University of Science and Technology Aliero. The specimen has been identified by undersigned on the basis of morphological characters. The following voucher specimen was issued below:

S/N	species names	Family	Date of collection	Locality	Voucher specimen number
01.	*Linum usitatissimum*	Linaceae	14/11/2023	Hi-Tech Polyhouse, Lovely Professional University (LPU) Phagwara, Punjab-India	KSUSTA/PSB/H/Voucher No: 657

Prof. Dharmendra Singh

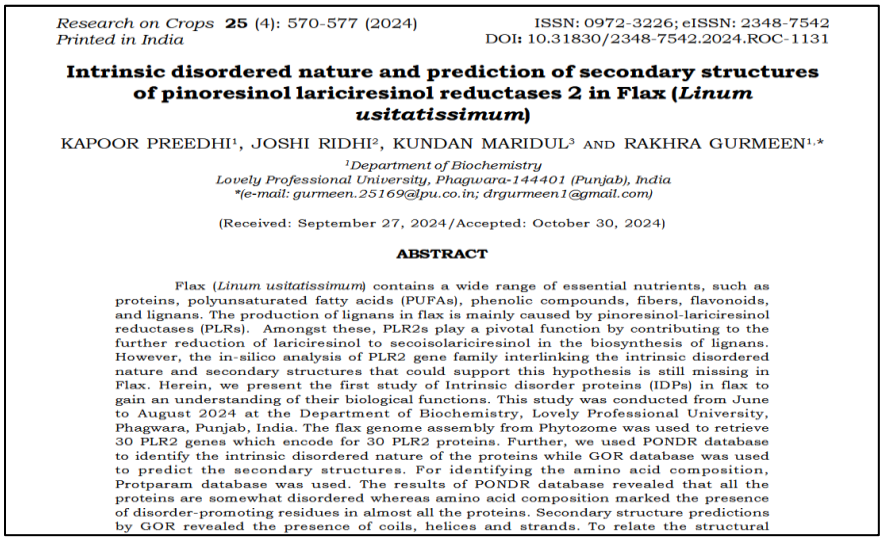
HOD

PSB KSUSTA

Department of Plant Science & Biotechnology
Kebbi State University of Science and Technology Aliero
PMB-1144, Nigeria

List of publications

- **Intrinsic disordered nature and prediction of secondary structures of pinoresinol lariciresinol reductases 2 in Flax (*Linum usitatissimum*)**



Research on Crops **25** (4): 570-577 (2024) ISSN: 0972-3226; eISSN: 2348-7542
Printed in India DOI: 10.31830/2348-7542.2024.ROC-1131

Intrinsic disordered nature and prediction of secondary structures of pinoresinol lariciresinol reductases 2 in Flax (*Linum usitatissimum*)

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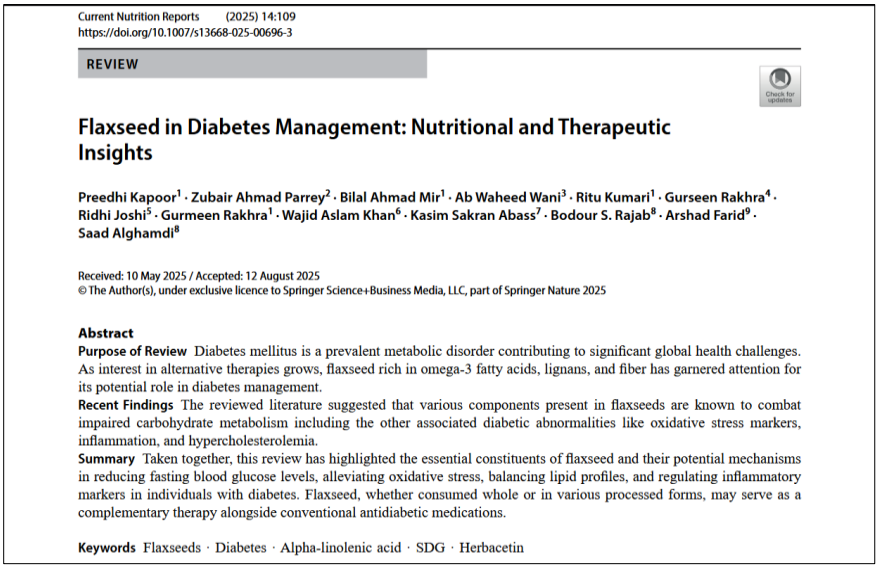
ABSTRACT

Flax (*Linum usitatissimum*) contains a wide range of essential nutrients, such as proteins, polyunsaturated fatty acids (PUFAs), phenolic compounds, fibers, flavonoids, and lignans. The production of lignans in flax is mainly caused by pinoresinol-lariciresinol reductases (PLRs). Amongst these, PLR2s play a pivotal function by contributing to the further reduction of lariciresinol to secoisolariciresinol in the biosynthesis of lignans. However, the in-silico analysis of PLR2 gene family interlinking the intrinsic disordered nature and secondary structures that could support this hypothesis is still missing in Flax. Herein, we present the first study of Intrinsic disorder proteins (IDPs) in flax to gain an understanding of their biological functions. This study was conducted from June to August 2024 at the Department of Biochemistry, Lovely Professional University, Phagwara, Punjab, India. The flax genome assembly from Phytozome was used to retrieve 30 PLR2 genes which encode for 30 PLR2 proteins. Further, we used PONDR database to identify the intrinsic disordered nature of the proteins while GOR database was used to predict the secondary structures. For identifying the amino acid composition, Protparam database was used. The results of PONDR database revealed that all the proteins are somewhat disordered whereas amino acid composition marked the presence of disorder-promoting residues in almost all the proteins. Secondary structure predictions by GOR revealed the presence of coils, helices and strands. To relate the structural

- **Flaxseed in Diabetes Management: Nutritional and Therapeutic Insights**



Current Nutrition Reports (2025) 14:109
https://doi.org/10.1007/s13668-025-00696-3

REVIEW

Flaxseed in Diabetes Management: Nutritional and Therapeutic Insights

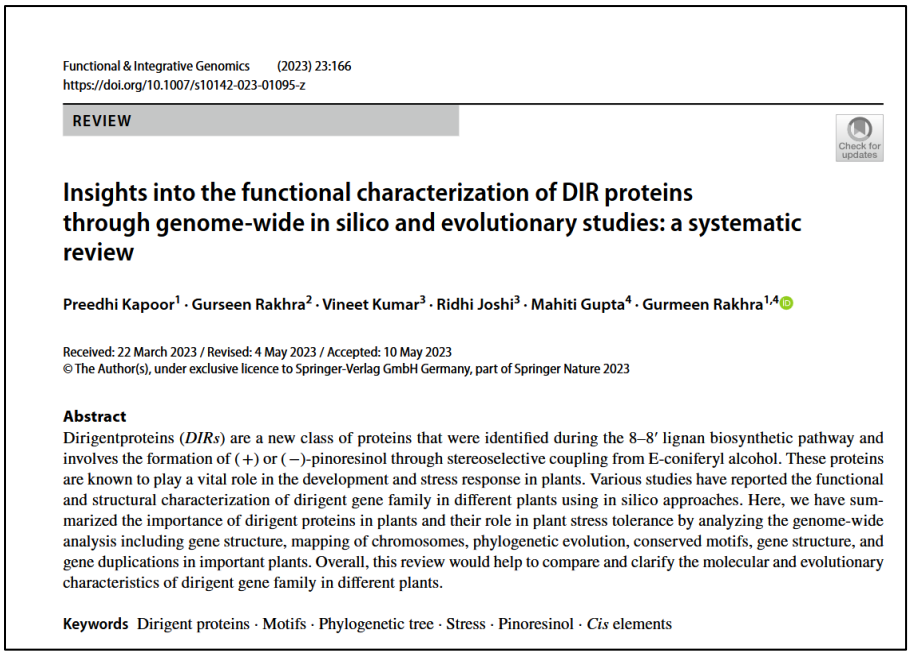
Preedhi Kapoor[1] · Zubair Ahmad Parrey[2] · Bilal Ahmad Mir[1] · Ab Waheed Wani[3] · Ritu Kumari[1] · Gurseen Rakhra[4] · Ridhi Joshi[5] · Gurmeen Rakhra[1] · Wajid Aslam Khan[6] · Kasim Sakran Abass[7] · Bodour S. Rajab[8] · Arshad Farid[9] · Saad Alghamdi[8]

Received: 10 May 2025 / Accepted: 12 August 2025

Abstract
Purpose of Review Diabetes mellitus is a prevalent metabolic disorder contributing to significant global health challenges. As interest in alternative therapies grows, flaxseed rich in omega-3 fatty acids, lignans, and fiber has garnered attention for its potential role in diabetes management.
Recent Findings The reviewed literature suggested that various components present in flaxseeds are known to combat impaired carbohydrate metabolism including the other associated diabetic abnormalities like oxidative stress markers, inflammation, and hypercholesterolemia.
Summary Taken together, this review has highlighted the essential constituents of flaxseed and their potential mechanisms in reducing fasting blood glucose levels, alleviating oxidative stress, balancing lipid profiles, and regulating inflammatory markers in individuals with diabetes. Flaxseed, whether consumed whole or in various processed forms, may serve as a complementary therapy alongside conventional antidiabetic medications.

Keywords Flaxseeds · Diabetes · Alpha-linolenic acid · SDG · Herbacetin

- **Insights into the functional characterization of DIR proteins through genome-wide in silico and evolutionary studies: a systematic review**



Functional & Integrative Genomics (2023) 23:166
https://doi.org/10.1007/s10142-023-01095-z

REVIEW

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Insights into the functional characterization of DIR proteins through genome-wide in silico and evolutionary studies: a systematic review

Preedhi Kapoor[1] · Gurseen Rakhra[2] · Vineet Kumar[3] · Ridhi Joshi[3] · Mahiti Gupta[4] · Gurmeen Rakhra[1,4]

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Abstract
Dirigentproteins (*DIRs*) are a new class of proteins that were identified during the 8–8′ lignan biosynthetic pathway and involves the formation of (+) or (−)-pinoresinol through stereoselective coupling from E-coniferyl alcohol. These proteins are known to play a vital role in the development and stress response in plants. Various studies have reported the functional and structural characterization of dirigent gene family in different plants using in silico approaches. Here, we have summarized the importance of dirigent proteins in plants and their role in plant stress tolerance by analyzing the genome-wide analysis including gene structure, mapping of chromosomes, phylogenetic evolution, conserved motifs, gene structure, and gene duplications in important plants. Overall, this review would help to compare and clarify the molecular and evolutionary characteristics of dirigent gene family in different plants.

Keywords Dirigent proteins · Motifs · Phylogenetic tree · Stress · Pinoresinol · *Cis* elements

❖ **Other publications:**

- **Synergistic effects of hydrogen sulfide and nitric oxide in enhancing salt stress tolerance in cucumber seedlings**
- **Unlocking the versatility of nitric oxide in plants and insights into its molecular interplays under biotic and abiotic stress**

List of conferences:

- **"4th International Conference: Global Efforts on Agriculture, Forestry, and Environment and Food Security (GAFEF-2022)".**

- **"6th International Conference: Strategies and Challenges in Agricultural and Life Science for Food Security and Sustainable Environment (SCALFE-2023)".**

- **“International Conference on Innovation and Intellectual Property Rights”.**

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Certificate of Participation

Presented to **Ms. Preedhi Kapoor** of **Lovely Professional University** in recognition of his/her participation in **“International Conference on Innovation and Intellectual Property Rights”** held on **18-19th April, 2022** organized by Division of Research and Development, Lovely Professional University, Punjab.

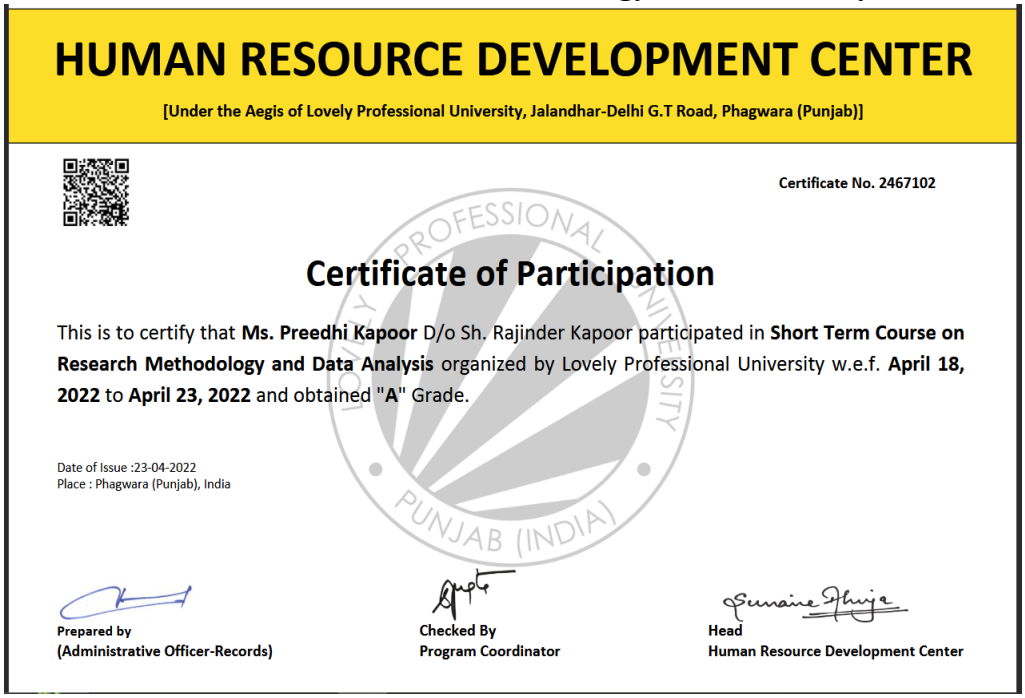
Date of Issue: 13-05-2022
Place: Phagwara (Punjab), India

Prepared by
(Administrative Officer-Records)

Convener
Dr. Runjhun Tondon
(IAIPR 2022)

Dr. Chander Prakash
CD, Division of Research and Development

- **“Short Term Course on Research Methodology and Data Analysis”.**



HUMAN RESOURCE DEVELOPMENT CENTER

[Under the Aegis of Lovely Professional University, Jalandhar-Delhi G.T Road, Phagwara (Punjab)]

Certificate No. 2467102

Certificate of Participation

This is to certify that **Ms. Preedhi Kapoor** D/o Sh. Rajinder Kapoor participated in **Short Term Course on Research Methodology and Data Analysis** organized by Lovely Professional University w.e.f. **April 18, 2022** to **April 23, 2022** and obtained "**A**" Grade.

Date of Issue :23-04-2022
Place : Phagwara (Punjab), India

Prepared by
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Checked By
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Human Resource Development Center