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DUR E SHAHWAR

ANÁLISE TRANSCRIPTÔMICA PARCIAL DE *HIMATANTHUS DRASTICUS* E AVALIAÇÃO DE SUAS PROTEÍNAS DE LÁTEX: IMPLICAÇÕES PARA A VIABILIDADE CELULAR, IMUNOMODULAÇÃO E POTENCIAL TERAPÊUTICO

FORTALEZA
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Orientador: Prof. Dr. Márcio Viana Ramos

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RESUMO

O látex de *Himatanthus drasticus* contém diversos compostos bioativos com potencial terapêutico. No entanto, as pesquisas sobre seus constituintes, particularmente a fração proteica (HdLP) e suas atividades biológicas, ainda são limitadas. Além disso, não há dados genômicos ou transcriptômico disponíveis para esta planta em bancos de dados públicos. Este estudo busca preencher algumas dessas lacunas ao caracterizar parcialmente o perfil transcriptômico das folhas e casca, e analisar composição e os efeitos biológicos das proteínas do látex de *H. drasticus*. A fração proteica do látex de *H. drasticus* foi subfracionada por cromatografia de troca iônica (DEAE-Sepharose), e analisadas por eletroforese, e caracterizadas por espectrometria de massas. Ensaios de viabilidade celular *in vitro* foram realizados em linhagens celulares RAW264.7, L929 e C2C12 para avaliar a citotoxicidade do HdLP e suas subfrações. Experimentos *in vivo* em camundongos *Swiss* foram conduzidos para investigar o impacto do HdLP na hiperglicemia, infecção e imunomodulação. A análise transcriptômica identificou transcritos diferencialmente expressos entre folhas e casca, associados à regulação metabólica, respostas de defesa e síntese de compostos bioativos, fornecendo novos insights sobre o perfil molecular dessa planta. A análise cromatográfica do HdLP revelou a presença de duas subfrações distintas, Hd-1 e Hd-2. A espectrometria de massas (MS) identificou 27 proteínas/peptídeos na subfração Hd-1 e 26 na Hd-2. Os ensaios de viabilidade celular demonstraram efeitos distintos, dependentes da concentração: o HdLP reduziu a viabilidade das células RAW264.7 e L929, mas aumentou das células C2C12 na mesma faixa de concentração (2-31,3 µg/mL). O subfração Hd-1 promoveu consistentemente a viabilidade celular em quase todas as concentrações testadas (0,1-25 µg/mL), enquanto o Hd-2 exibiu uma resposta bifásica, aumentando a viabilidade em concentrações mais baixas (0,2-3,9 µg/mL) e reduzindo-a em concentrações mais altas (62,5-125 µg/mL). Os estudos *in vivo* indicaram que o HdLP não teve impacto significativo na hiperglicemia ou infecção, mas apresentou efeitos imunomoduladores, incluindo depleção de leucócitos e aumento da contagem de plaquetas, além de leve hepatotoxicidade. Este estudo representa um a primeira investigação transcriptômica de *H. drasticus*, proporcionando uma compreensão mais profunda da composição do HdLP e estabelecendo bases para futuras pesquisas sobre seus mecanismos de ação e potenciais aplicações em imunomodulação e medicina regenerative.

Palavras-chave: análise transcriptômica; hematopoiese; hiperglicemia; imunomodulação; proteínas do látex; potencial terapêutico.

ABSTRACT

The latex of *Himatanthus drasticus* contains diverse bioactive compounds with therapeutic potential. However, research on its constituents, particularly the small protein fraction (HdLP) and its biological activities, remains limited. Additionally, no genomic or transcriptomic data for this plant are available in public databases. This study aims to bridge some of these gaps by partially characterizing the transcriptomic profile of leaves and bark and analyzing the composition and biological effects of latex proteins of *H. drasticus.* The protein fraction of *H. drasticus* latex was sub-fractionated using ion exchange chromatography (DEAE-Sepharose), analyzed via electrophoresis, and characterized through mass spectrometry. *In vitro* viability assays were conducted on RAW264.7, L929, and C2C12 cell lines to evaluate the cytotoxicity of HdLP and its sub-fractions. Additionally, i*n vivo* experiments in *Swiss* mice were performed to assess the impact of HdLP on hyperglycemia, infection, and immunomodulation. Transcriptomic analysis identified differentially expressed transcripts between leaves and bark associated with metabolic regulation, defense responses, and synthesis of bioactive compounds, providing novel insights into the molecular landscape of this plant. Chromatographic analysis of HdLP showed the presence of two distinct sub-fractions, Hd-1 and Hd-2. Mass spectrometry (MS) analysis detected 27 proteins/peptides in subfraction Hd-1 and 26 in Hd-2. Cell viability assays demonstrated distinct concentration-dependent effects: HdLP reduced viability in RAW264.7 and L929 cells but increased it in C2C12 cells at the same concentration range (2-31.3 µg/mL). Hd-1 consistently promoted viability at almost all the tested concentrations (0.1-25 µg/mL), whereas Hd-2 displayed a biphasic response by increasing viability at lower concentrations (0.2-3.9 µg/mL) and reducing it at higher ones (62.5-125 µg/mL). *In vivo* studies indicated that HdLP had no significant impact on hyperglycemia or infection but exhibited immunomodulatory effects, including leukocyte depletion and increased platelet counts, along with mild hepatotoxicity. This study represents the first transcriptomic investigation of *H. drasticus*, provides a deeper understanding of HdLP composition, and lays the groundwork for future research into its mechanisms of action and potential applications in immunomodulation and regenerative medicine.

Keywords: transcriptomic analysis; hematopoiesis; hyperglycemia; immunomodulation; latex proteins; therapeutic potential.

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LIST OF ABBREVIATIONS AND ACRONYMS

µg	Microgram
µL	Microliter
µm	Micrometer
ABC	ATP-Binding Cassette
ANK_REP_REGION	Ankyrin Repeat Region
ANOVA	Analysis of Variance
AT	Adenine-Thymine
ATP	Adenosine Triphosphate.
BAS	Basophils
BCA	Bicinchoninic Acid Assay
BCRJ	Banco de Células do Rio de Janeiro
BHI	Brain Heart Infusion
bp	Base pair
BP	Biological Processes
C. procera	*Calotropis procera*
CA	California
CC	Cellular Components
CD	Cluster of Differentiation
CD-HIT	Cluster Database at High Identity with Tolerance
cDNA	Complementary DNA
CFU	Colony-Forming Unit.
cGMP	Cyclic Guanosine Monophosphate
CNS	Central Nervous System
CO_2	Carbon Dioxide
CONCEA	Conselho Nacional de Controle de Experimentação Animal
COX	Cyclooxygenase
CPM	Count Per Million
DBD_Tnp_Mut	DNA-Binding Domain of Mutator-Family Transposases
DEA	Differential Expression Analysis
DEAE	Diethylaminoethyl
DESeq	Differentially Expressed Sequence
DET	Differentially Expressed Transcript

dL	Deciliter
DMEM	Dulbecco’s Modified Eagle Medium
DNA	DeoxyRibonucleic Acid
DTT	Dithiothreitol
EGCG	Epigallocatechin gallate
EOS	Eosinophils
ESI	Electron-Spray Ionization
est	Expressed Sequence Tags
FastQC	Fast Quality Control
FBS	Fetal Bovine Serum
FHDHA	Hydroalcoholic fraction of *H. drasticus* latex
FJNB	n-Butanolic Fraction of Janaguba
$FoxP^{3+}$	Forkhead Box P3
g	Gram
GC	Gas Chromatography
GC	Guanine-Cytosine
GO	Gene Ontology
h	Hour
H&E	Hematoxylin and Eosin
H. brasiliensis	*Hevea brasiliensis*
H. drasticus	*Himatanthus drasticus*
HB	Hemoglobin
HCl	Hydrochloric Acid
Hd	*Himatanthus drasticus*
HDAC	Histone Deaetylase
HDHE	Hydroalcoholic Extract of *H. drasticus*
HdLP	*Himatanthus drasticus* Latex Proteins
HPLC	High Performance Liquid Chromatography
HSP	Heat Shock Protein
HT	Hematocrit
i.p	Intraperitoneal
IAA	Iodoacetamide
IC_{50}	Half Maximal Inhibitory Concentration

IDs	Identification Numbers
IEC	Ion Exchange Chromatography
IL	Interleukin
iNOS	Induced Nitric Oxide Synthase
JRL	Jacalin-Related Lectin
KATP channels	ATP-Sensitive Potassium Channels
kDa	Kilodalton
kg	Kilogram
KOBAS	KEGG Orthology Based Annotation System
LC	Liquid Chromatography
L-NAME	Nω-Nitro-L-Arginine Methyl Ester
LYM	Lymphocytes
M	Molar
mA	Miliampere
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MF	Molecular Functions
mg	Miligram
min	Minute
mL	Milliliter
mM	Milimolar
MO	Missouri
MON	Monocytes
MPO	Myeloperoxidase
Mr	Relative Molecular Mass
mRNA	Messenger RNA
MS	Mass Spectrometry
MSP	Major Sperm Protein
MW	Molecular Weight
NaCl	Sodium Chloride
NF-kB	Nuclear Factor Kappa-B
NH_4HCO_3	Ammonium Bicarbonate
nm	Nanometer

NMR	Nuclear Magnetic Resonance
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NP-SH	Non-Protein Sulfhydryl
OBC	Optimal Bacterial Concentration
ORFs	Open Reading Frames
OVA	Ovalbumin
PAGE	Polyacrylamide Gel Electrophoresis
PANTHER	Protein Analysis Through Evolutionary Relationships
PBS	Phosphate-Buffered Saline
PC	Principal Component
PCA	Principal Component Analysis
pH	Power of Hydrogen
PLA	Platelet Count
PRO	Plasma Protein
PTM	Post Translational Modification
RBC	Red Blood Cell
RIN	RNA Integrity Number
RNA	Ribonucleic Acid
RNA-Seq	RNA Sequencing
ROD	Rod Cells
rRNA	Ribosomal RNA
RSEM	RNA-Seq by Expectation–Maximization
S. Typhimurium	*Salmonella Typhimurium*
SBP	SQUAMOSA-Binding Protein
SDS	Sodium Dodecyl Sulfate
SEG	Segmented Neutrophils
SEM	Standard Error of the Mean
SisGen	Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado
SP	São Paulo
SPF	Specific pathogen-free
SS	Salmonella-Shigella

STAR	Spliced Transcripts Alignment to a Reference
STZ	Streptozotocin
TLC	Thin Layer Chromatography
TNF-α	Tumor Necrosis Factor-α
Tris	Tris(hydroxymethyl)aminomethane
TRPV1	Transient Receptor Potential Vanilloid 1.
USA	United States of America
v/v	Volume/Volume
WBC	White Blood Cell

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1 INTRODUCTION

1.1 Natural products: Promising alternatives to Synthetic drugs

Synthetic medicines offer fast-acting relief but are frequently associated with notable side effects. Their intricate manufacturing processes also contribute to high costs, limiting their accessibility for much of the global population (Karimi A *et al*., 2015). Therefore, the pharmaceutical industry has shown sustained interest in bio-based products derived from natural sources as potential alternatives to synthetic drugs (Atanasov AG *et al*., 2021).

In contrast to synthetic drugs, natural products are generally considered safer and more effective, often exhibiting fewer side effects and better compatibility with the body's metabolic processes. Their broad acceptance stems from cultural and social beliefs, as well as affordability and accessibility (Wangchuk P, 2018). Ongoing scientific investigations and clinical trials conducted by researchers and pharmaceutical companies have provided empirical support for their therapeutic potential. Furthermore, the isolation, refinement, and standardization of specific bioactive compounds have facilitated their integration into modern drug delivery systems (Chaachouay N & Zidane L, 2024).

Currently, over 50% of commercially available therapeutic drugs are either derived from or inspired by natural products (Biomed & Tech Talks, 2025). It is estimated that natural product-based or related drugs account for approximately 35% of the global pharmaceutical market. Among these, plant-derived compounds represent the largest portion (25%), followed by those obtained from microorganisms (13%) and animal sources (3%) (Calixto JB, 2019).

Medicinal plants alone form the foundation of nearly 25–50% of the drugs currently used in healthcare worldwide, and researchers continue to explore both known and exotic plant species in pursuit of novel bioactive compounds (Anand U *et al*., 2019; Salmerón-Manzano E *et al*., 2020). This ongoing scientific effort reflects the growing recognition of plant-based pharmaceutical strategies as a more efficient, cost-effective, and safer alternative to traditional methods that rely on animal cell cultures or microbial fermentation. By utilizing natural compounds from plants, these approaches not only underscore the vital role of medicinal flora in contemporary

medicine but also enhance the drug accessibility and expedite people's access to essential medications (Veeresham C, 2012; Singh MP & Gohil KJ, 2024).

1.2 Medicinal Plants

Herbal remedies continue to serve as a cornerstone of healthcare for approximately 75–80% of the global population (Wangchuk P *et al*., 2018; WHO, 2019, WHO 2023). With nearly 30,000 plant species documented for medicinal use, traditional plant-based therapies hold profound cultural significance and offer promising solutions for a wide spectrum of health conditions (Ekor M, 2014; Allkin B, 2017; Wangchuk, 2018). However, despite their widespread use, many medicinal plants remain underexplored scientifically, and the majority of traditional applications lack rigorous clinical validation.

Plants naturally produce secondary metabolites in response to environmental stressors such as drought, predation, and interspecies competition. Although these compounds originally evolved for survival, they have attracted considerable scientific interest due to their potent pharmacological properties and broad therapeutic potential (Yeshi *et al*., 2022). As global interest in natural medicine continues to rise, these bioactive substances are increasingly recognized as valuable resources in modern drug discovery.

Among the most studied secondary metabolites are alkaloids, flavonoids, terpenoids, phenolic compounds, and saponins. Flavonoids such as quercetin and kaempferol are well-known for their strong antioxidant and anti-inflammatory properties, making them effective in managing oxidative stress-related disorders (Panche *et al*., 2016). Likewise, terpenoids such as limonene and menthol exhibit notable antimicrobial, anticancer, and analgesic effects (Thoppil RJ & Bishayee A, 2011).

The advent of biotechnology has further enhanced the role of plants in medicine by enabling their use as bio-factories for pharmaceutical production (Paul M & Julian, 2011). The extraction of active plant ingredients dates back to the early 19th century with the isolation of morphine from *Papaver somniferum* (Brownstein MJ, 1993; Brook K *et al*., 2017). This milestone was followed by the discovery of several other pivotal compounds, including digitoxin, cocaine, pilocarpine, codeine, and quinine,

each of which played a transformative role in the development of early therpeutics (Chaachouay N & Zidane L, 2024; Mohammed AO *et al*., 2025).

Over the years, many plant-derived molecules have been thoroughly investigated and successfully commercialized. Notable examples include paclitaxel from *Taxus brevifolia* for cancer treatment, artemisinin from *Artemisia annua* for malaria, and silymarin from *Silybum marianum* for liver disorders (Tighe SP *et al.,* 2020; Gillessen A & Schmidt HH *et al*., 2020; Sharifi-Rad J *et al*., 2021; Roesch C *et al*., 2025).

In light of the growing interest in natural therapeutics, there is an urgent need to bridge traditional knowledge with modern scientific validation. Expanding research on medicinal plants and their bioactive compounds will not only deepen our understanding of plant-based healing but also accelerate the discovery of novel, effective, and sustainable treatments for contemporary health challenges.

1.3 Potential of Angiosperms in the Medicinal Field

Angiosperms, with their remarkable diversity and abundance of active compounds, play a central role in the field of medicinal plants. This group of land plants comprises 64 orders, 416 families, approximately 13,000 known genera, and 300,000 known species, providing a vast reservoir of bioactive substances for therapeutic exploration (Christenhusz MJM & Byng JW, 2016). Several phytochemicals extracted from angiosperms are already being utilized to treat various human conditions, including infections, inflammation, cancer, oxidative stress, gastric and neuro disorders, diabetes, wound healing, immune-related issues, and many more. Advancements in phytochemical research have paved the way for the discovery of novel bioactive compounds with unique mechanisms of action.

Recent studies continue to explore the immense potential of angiosperms in medicinal applications, with numerous findings highlighting their therapeutic value. A recent review by Liu W *et al*. (2023) explores the origins, production, and pharmacological effects of polyphenols. They have highlighted the cytotoxic effects of polyphenols on cervical, lung, and liver cancer cells, demonstrating mechanisms such as apoptosis induction, autophagy promotion, and metastasis suppression. Compounds like ellagic acid, ferulic acid, and epigallocatechin gallate (EGCG) have

shown potential in targeting these cancers by disrupting critical cellular pathways.

Panax ginseng, a member of the angiosperm family “Araliaceae”, has been the subject of multiple recent investigations for its neuroprotective and anti-inflammatory effects (Li J *et al*., 2021; Wu J *et al*., 2022; Zhang R *et al*., 2023). The studies revealed that *ginseng* saponins could mitigate neurodegeneration in Alzheimer’s disease models by enhancing neuronal survival and modulating inflammatory pathways. Similarly, saponins from *Panax ginseng* have been explored for their immunomodulatory and neuroprotective effects, providing potential therapeutic options for conditions such as neurodegenerative diseases (Cho H, 2012; Han Y *et al*., 2021).

Berberine, an isoquinoline alkaloid derived from *Berberis* species, has shown promising results in the management of type-2 diabetes and metabolic syndrome due to its ability to modulate glucose and lipid metabolism (Yin J *et al*., 2008).

Another significant example is the exploration of *Euphorbia* species, which belong to the Euphorbiaceae family and are known for their latex-producing capabilities. *Euphorbia* extracts have been shown to possess potent anticancer, antimicrobial, and anti-inflammatory properties, with promising therapeutic outcomes in treating skin cancers and infections (Mavundza E *et al*., 2022; Jiménez-González V *et al*., 2023; Zaghlol AA *et al*., 2024; Dwijayanti DR *et al*., 2024).

Furthermore, *Papaver somniferum* (opium poppy) continues to be a crucial source of alkaloids such as morphine, which remain at the core of pain management therapies (Butnariu M *et al*., 2022). Recent advances in genetic and metabolic engineering of this plant have led to enhanced yields of morphine and other valuable alkaloids, advancing its pharmaceutical use.

These studies demonstrate the ongoing relevance of angiosperms in developing novel and effective treatments for a wide range of medical conditions. Also, these findings underscore the immense potential of secondary metabolites in addressing complex human diseases and encourage further exploration of medicinal plants for pharmaceutical development. While secondary metabolites from different plant parts, such as leaves and stems, have long been recognized for their diverse therapeutic potential, another significant aspect of medicinal plants lies in the therapeutic effects of their latex.

1.4 Laticifer cells and latex

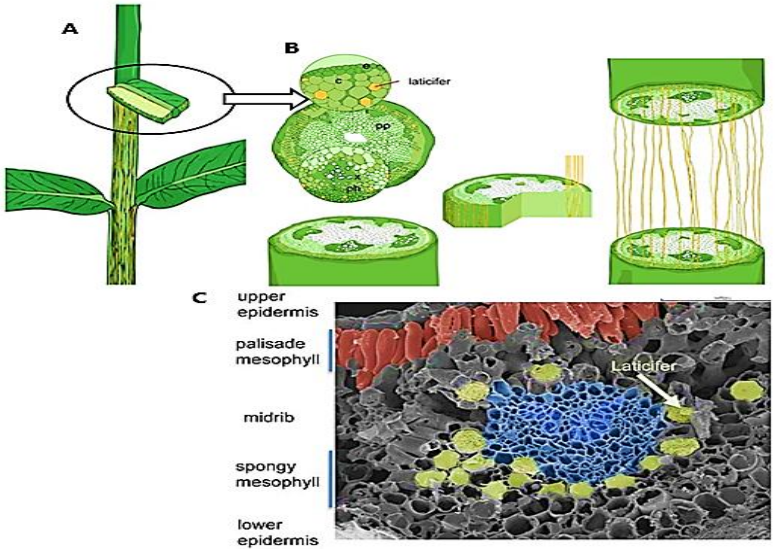
Plant latex, particularly from species with specialized laticifer cells, contains a rich array of bioactive compounds that exhibit a wide range of pharmacological activities. These compounds are increasingly being studied for their potential to treat various human diseases, further expanding the therapeutic applications of plant-derived substances.

More than 20,000 species across over 40 angiosperm families are known to produce latex. The most prominent families with latex-producing plants include Apocynaceae, Asteraceae, Euphorbiaceae, and Papaveraceae (Konno K, 2011; Abarca LFS *et al*., 2019). These plants have specialized elongated cells “laticifers” which form shortly after seed germination and extend throughout the plant body (Fig. 1).

These specialized cells produce a viscous emulsion known as “latex”. Most plants produce white-colored latex, while in some species, it can be found in other colors such as orange, scarlet, or yellow (Fig. 2). For example, *Euphorbia tirucalli* (Euphorbiaceae) produces yellow latex, while *Calotropis gigantea* (Apocynaceae) exudes milky white latex (Konno, 2011). Additionally, *Papaver somniferum* (opium poppy) has been reported to produce scarlet-colored latex in certain cultivars (Abarca LFS *et al*., 2019).

The biochemical composition of latex also varies across different tissues and organs within the same plant and among plants of different species. Despite variations in color and composition, latex in all species plays a crucial role in the plant's defense mechanism against pathogens and herbivores (Konno, 2011; MV Ramos *et al*., 2019; Freitas CDT *et al*., 2020; Takashima T *et al*., 2023). It contains a wide array of bioactive compounds such as alkaloids, terpenoids, cardenolides, resins, starches, tannins, rubber, gums, sugars, oils, and proteins (Abarca LFS *et al*., 2019; MV Ramos *et al*., 2019). These bioactive compounds are not only advantageous for the survival of plants but also offer potential health benefits for humans. Latex proteomes seem to vary among species and within the same species, as latex exuded from stems or leaves, for example, is expected to differ in protein content and abundance (Ramos MV *et al.,* 2019).

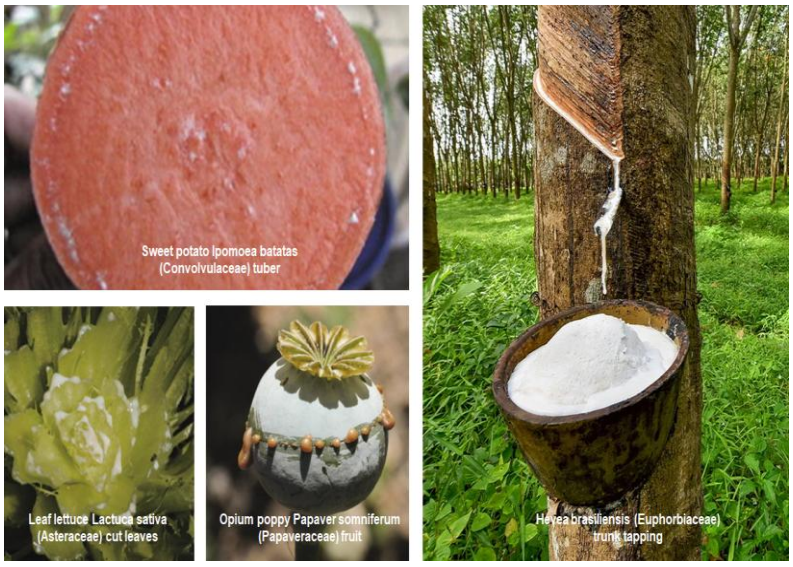
Figure 1: Disposition and abundance of laticifer cells along the hypocotyl axis



Source: Castelblanque L *et al*., 2016, (Supplementary data)

A) Laticifer cells or rows of laticifer cells disposed in parallel along the hypocotyl are shown in yellow. **(B**) c: cortex; e: epidermis; ph: phloem; pp: pith parenchyma; x: xylem. **(C**) At two magnification scales, a cross-section of the stem is shown where laticifer cells were artificially colored in yellow using Photoshop, the vascular bundles in blue, and the epidermis in pink. The different tissues surrounding the laticifer cells are indicated in the legends.

Figure 2: Latex exudation from different agricultural plants



Source: Google.

1.5 *Himatanthus drasticus* as a Medicinal Plant

H. drasticus, commonly known as Janaguba, is a latex-producing plant belonging to the Apocynaceae family and was previously classified as "*Plumeria drastica*" (Plumel MM, 1991; Spina AP, 2004; Spina AP, 2013; Spina AP, 2015). This tall plant can reach a height of 7 m and is characterized by its broad, leathery leaves reminiscent of latifolia varieties. Its flowers are arranged in corymbs, an indeterminate inflorescence type. In corymbs, flowers emerge from various points along the same stem but reach a uniform height due to the differing lengths of their pedicels (Fig. 3). This unique arrangement allows for an aesthetically pleasing display of blooms.

Figure 3: *Himatanthus drasticus*

Source: Google.

Geographically, *H. drasticus* is distributed across Guyana, Suriname, French Guiana, and several regions of Brazil, including the North, Northeast, Midwest, and Southeast (Mousinho KC *et al*., 2011; Oliveira MG *et al*., 2022). In Brazil, it is predominantly found in the states of Bahia, Ceará, Pará, Paraíba, Pernambuco, Piauí, Maranhão, Rio Grande do Norte, Alagoas, Sergipe, Roraima, Amazonas, and Minas Gerais (Lorenzi & Matos, 2008). It is known by different regional names across Brazil such as janaguba, tiborna, jasmim-manga, raivosa, pau-de-leite, joanaguba, and sucuuba (Plumel, 1991; Spina AP, 2004).

A study by Santos (2004) analyzed the lipid fraction derived from the ethanol extract of *H. drasticus* leaves and identified several bioactive compounds. These included methyl esters of various fatty acids, such as undecanoic, hexadecanoic,

octadecanoic, 9-octadecenoic, 9,12-octadecenoic, eicosanoic, and octanoic acids. Additionally, triterpene lupeol and steroids β-sitosterol and stigmasterol were also detected in this extract.

The HPLC-ESI-MS analysis of hydro-alcoholic extract of *H. drasticus* leaves identified the presence of plumericin, plumieride or isoplumericin, quercetin and its derivatives, rutin, and chlorogenic acid (Figueiredo CSSES *et al*., 2017).

Luz *et al.,* (2014) examined the hydroalcoholic extract and its fractions (ethyl acetate, n-butanol, hexane, and aqueous) obtained from the bark. They identified a wide range of bioactive compounds, including flavonoid glycosides, triterpenes, tannins, alkaloids, and steroids. These compounds were shown to exhibit biological and pharmacological activities. The study highlighted the antimicrobial, anti-inflammatory, antiviral, and antioxidant properties of coumarins present in the extracts, as well as the hypocholesterolemic and antifungal actions of saponins.

In the latex of *H. drasticus*, the presence of different phytochemicals such as phenols, flavonols, free steroids, derivatives of cinnamate and flavanones i.e lupeol cinnamate, α-amyrin and β-amyrin, triterpenes, tannins, and proteins has been confirmed by various studies (Lucetti DL *et al*., 2010; Mousinho KC *et al*., 2011; Almeida SCX *et al*., 2017; Santos GJL *et al*., 2018; Almeida SCX *et al*., 2019).

1.5.1 Pharmacological properties of H. drasticus

The first study to investigate the antitumor activity and acute oral toxicity of the crude methanolic extract from *H. drasticus* leaves was done by Sousa EL *et al.,* (2010), against Sarcoma-180 tumor in male Swiss albino mice. The extract demonstrated significant antitumor activity, with tumor growth inhibition rates of 67.7% (300 mg/kg) and 68% (400 mg/kg). It also showed low toxicity at doses of 50, 300, and 2000 mg/kg administered orally. Histopathological analysis revealed alterations in the tissues of the spleen, liver, kidneys, and lungs.

In another study, hydroalcoholic extract of *H. drasticus* (HDHE) leaves exhibits antimicrobial activity against *Klebsiella pneumoniae*, significantly reducing its viability and inhibiting biofilm formation. HDHE also reduced the proliferation of human peripheral blood mononuclear cells stimulated by lipopolysaccharides, with no significant cytotoxicity observed (Figueiredo CSSES *et al*., 2017).

A preliminary study on the phytochemical and biological properties of *H. drasticus* stem bark revealed that the ethanolic extract of its stem bark exhibited toxicity against brine shrimp but showed no antimicrobial activity against the tested (in vitro) pathogens (Escherichia coli, Enterobacter, and Streptococcus). However, the extract demonstrated antinociceptive effects in the writhing test in rats (Colares *et al*., 2008).

Several scientific studies have demonstrated the effectiveness of *H. drasticus* latex for treating ulcers, arthritis, gastritis, inflammations, and diabetes when taken orally, as well as wounds and skin conditions when applied topically (Ribeiro DA *et al*., 2014; Moura DF *et al*., 2020).

Preclinical research has shown that latex of *H. drasticus* possesses a range of therapeutic properties, including anti-ulcer and gastroprotective (Leite GO *et al*., 2009; Marques LM, 2012; Pinheiro RCP *et al*., 2013), antitumor (Mousinho KC *et al*., 2011), anti-nociceptive and anti-inflammatory (Lucetti *et al*., 2010; Matos MPV *et al*., 2013; Almeida SCX *et al*., 2019), antimicrobial (Figueiredo CSSES *et al*., 2017), anti-oxidant (Marques LM, 2012; Pinheiro RCP *et al*., 2013; Carmo LD, 2015), wound healing (Souza TFG, 2015) and hypoglycemic (Morais FS *et al*., 2020) activities.

Researchers are captivated by the remarkable therapeutic potential of *H. drasticus* latex. Its multifaceted therapeutic effects make it an invaluable natural resource for developing novel pharmacological agents and exploring its underlying bioactive compounds for potential clinical applications.

1.5.2 Clinical trials and applications of H. drasticus latex

In Brazil, the latex *H. drasticus* is widely utilized by local communities for its traditional medicinal properties, being employed to treat a range of health conditions. It is commonly found in public markets, where it is sold in bottles containing a mixture of crude latex and water in a 1:1 (v/v) ratio (Fig. 4). This preparation is typically stored at room temperature and used for the treatment of wounds, pain, cancer, anemia, inflammations, hemorrhoids, bacterial and parasitic infections, and various gastrointestinal, central nervous system (CNS), and endocrinal disorders (Santos GJL *et al*., 2018; Almeida SCX *et al*., 2017).

Various secondary metabolites present in *H. drasticus* latex have been studied widely, covering flavonols, phenols, derivatives of cinnamate, and flavanones e.g. lupeol cinnamate, α-amyrin and β-amyrin (Lucetti DL *et al*., 2010; Mousinho KC *et al*., 2011; Almeida SCX *et al*., 2017; Santos GJL *et al*., 2018; Almeida SCX *et al*., 2019).

Figure 4: *Himatanthus drasticus* latex

Source: Google.

Lucetti *et al*. (2010) investigated the anti-inflammatory, antinociceptive, and analgesic properties of lupeol acetate, a triterpene extracted from the latex of *H. drasticus*, using a carrageenan-induced edema model. The study revealed that lupeol acetate inhibited the release of myeloperoxidase (MPO) from activated neutrophils, reduced the production of iNOS by decreasing the number of iNOS-releasing cells at the edema site, and influenced the functioning of the opioid system.

Santos GJL *et al*. (2017) evaluated the wound-healing potential of *H. drasticus* latex in a mice model. The latex promoted significant wound contraction, granulation tissue formation, collagen fiber reorganization, and moderate expression of CD^{68+} and mast cells, indicating a mild inflammatory response. The wound-healing effects of latex were attributed to the presence of triterpenes (lupeol and α/β-amyrin).

In another study, Santos and coworkers investigated the phytochemical composition and antitumor effects of *H. drasticus* latex using ascitic and solid Sarcoma-180 tumor models in Swiss mice. Results of phytochemical analysis revealed the presence of flavanones, flavonols, phenols, free steroids, and cinnamoyl derivatives of α/β-amyrin and lupeol in the latex. The latex reduced oxidative damage by significantly lowering the serum malondialdehyde levels in the ascitic model. In the solid tumor model, it modulated immune responses by reducing the expressions of CD^{4+} and CD^{8+} in the paw and $CD^{4+,}$ CD^{8+}, $FoxP^{3+}$, and $HSP\text{-}60^{+}$ in popliteal lymph nodes. They linked the observed effects to the triterpenes identified in the latex, demonstrating its potential for modulation of oxidative stress and immune pathways (Santos GJL *et al*., 2018).

Almeida SCX *et al*., (2019) studied a triterpene-rich (FJNB) fraction of *H. drasticus* latex in acute nociception and inflammation models using Wistar rats and Swiss mice. FJNB (n-butanolic fraction of janaguba) demonstrated significant anti-inflammatory and antinociceptive effects, reducing paw edema by 25% in the carrageenan-induced paw edema test and inhibiting nociception by up to 52% (1st phase) and 67% (2nd phase) in the formalin test at a dose of 10 mg/kg. Additionally, FJNB inhibited the expression of iNOS, COX-2, TNF-α, HDAC, and NF-kB, highlighting its mechanism of action in modulating key inflammatory mediators.

In a study, phytochemical analysis of Hydroalcoholic fraction of *H. drasticus* latex (FHDHA) identified the compounds such as caffeic acid, 3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 15-demethylplumieride acid, protocatechuic acid, vanillic acid, catechin, plumieride, and α-ethyl glucoside, through mass spectrometry and ^{1}H, ^{13}C NMR analysis. Enzyme inhibition assays revealed that Hydroalcoholic (FHDHA, FHDHA1), and plumieride fractions exhibited strong, concentration-dependent inhibitory activity against α-amylase and α-glucosidase. The IC_{50} values for these fractions were comparable to acarbose, a standard inhibitor, indicating competitive inhibition for both enzymes. These findings suggest that the latex has compounds that may help to regulate glucose absorption by slowing carbohydrate digestion (Morais FS *et al*., 2020).

Another phytochemical analysis of *H. drasticus* latex using thin layer chromatography (TLC) and GC-MS revealed the presence of saponins, reducing sugars, steroids, terpenes, lupeol acetate, α/β-amyrin derivatives, and betulin. The latex showed toxicity towards S-180 cells at 50 and 100 µg/mL concentrations. No

adverse effects were observed in acute oral toxicity and mutagenicity tests conducted on mice treated with 2000 mg/kg of latex, however, the Comet assay indicated the presence of genotoxicity. While the dose tested was significantly higher than those traditionally used (10- to 35-fold), the findings highlight the need for caution, as no safe exposure level for genotoxic compounds is established (Moura DF *et al*., 2020).

In latex-producing plants, latex proteins play a crucial role in defending plants against various invading organisms, including viruses, bacteria, parasites, fungi, and herbivores (Nawrot R., 2017; Ramos MV *et al*., 2019). Therefore, plant latex proteins have vast therapeutic applications and are currently being used as immune-modulating agents (Nascimento DC *et al*., 2016; Sousa BF *et al*., 2020), anticoagulants (Siritapetawee J *et al*., 2020; Hamed MB *et al*., 2020; Kusuma CG *et al*., 2021), disinfectants (Lima-filho JV *et al*., 2010; Siritapetawee J *et al*., 2018; Sousa BF *et al*., 2020), anti-proliferative (Mousinho KC *et al.*, 2011; Hew CS *et al*., 2013; Villanueva J *et al*., 2015), anti-inflammatory (Kumar VL *et al*., 2015; MV Ramos *et al*., 2020; Tavares LS *et al*., 2021; Oliveira LES *et al*., 2022), and antioxidants (Reyes JARA AM *et al*., 2018; MV Ramos *et al*., 2020; Oliveira LES *et al*., 2022; Andrade GL *et al*., 2023; Freitas CDT *et al*., 2024).

In *H. drasticus*, although the therapeutic potential of latex is largely attributed to its secondary metabolites, the significance of latex proteins (HdLP) cannot be overlooked.

1.5.3 Therapeutic applications of H. drasticus latex proteins (HdLP)

There are a few studies involving the protein fraction of *H. drasticus* latex, known as HdLP, which have highlighted its potential therapeutic applications, particularly in cancer and immune-related research. These findings, although limited, form the foundation for further investigation of bioactive properties of HdLP and support its continued exploration as a candidate for novel therapeutic interventions.

The *in vitro* and *in vivo* antitumor activity of *H. drasticus* latex proteins (HdLP) was investigated alongside its effects on the immune system by Mousinho KC *et al*. (2011). While HdLP exhibited no significant cytotoxicity on cultured tumor cells, it demonstrated antitumor activity against Sarcoma-180 and Walker-256 carcinosarcoma (*in vivo* models) when administered intraperitoneally but was

ineffective when given orally. Histopathological analysis revealed minimal effects on liver and kidney tissues, suggesting low toxicity. HdLP also showed immunomodulatory properties by increasing the OVA-specific antibody production, relative spleen weight, and megakaryocyte colony incidence. This study suggested that the anticancer potential of HdLP may be linked to its immune-stimulating effects.

Marques (2012) highlighted the gastroprotective effect of HdLP in an ethanol-induced gastric lesion model in rats. HdLP administered intravenously at a dose of 5.0 mg/kg significantly reduced ethanol-induced gastric lesions by 83% and improved histopathological scores. The protective effect was linked to nitric oxide (NO) production and was inhibited when animals were pretreated with L-NAME (NOS inhibitor), glibenclamide (ATP-sensitive potassium channel blocker), or ODQ (guanylate cyclase inhibitor), suggesting the involvement of NO, cGMP, and KATP channels in the mechanism. However, pretreatment with COX inhibitor “indomethacin” or the TRPV1 antagonist “capsazepine” did not affect the gastroprotective action of HdLP. Additionally, HdLP restored depleted glutathione (NP-SH) levels in the gastric mucosa, highlighting its antioxidant properties. These findings demonstrated that HdLP exerts a gastroprotective effect through multiple mechanisms involving NO, cGMP, KATP channel activation, and antioxidant action.

Pinheiro *et al*. (2013) also confirmed the gastroprotective effect of HdLP (i.v. route), linking this activity to the modulation of the NO/cGMP/K-ATP pathway. This pathway enhances blood flow to the stomach walls and supports the mucosal immune system, thereby exhibiting an anti-ulcerogenic effect.

Matos *et al*., (2013) investigated the anti-inflammatory and antinociceptive effects of *H. drasticus* latex in inflammation models. Latex significantly inhibited carrageenan-induced neutrophil migration in rats, reducing neutrophil infiltration into the peritoneal cavity by 96% ($p<0.05$) and increasing plasma nitric oxide synthesis by 54% ($p<0.05$). Anti-inflammatory activity was attributed to the protein fraction of the latex, which retained its efficacy even after proteolysis or heat treatments at 100°C (30 min), demonstrating resistance to degradation. HdLP also exhibited dose-dependent antinociceptive effects, suppressing abdominal constrictions in acetic acid-treated mice and reducing paw-licking behavior in the formalin test. These results confirm the traditional claims of *H. drasticus* latex as an anti-inflammatory and analgesic agent,

highlighting the robust pharmacological potential of its protein fraction, which remains effective when administered orally due to its stability under adverse conditions.

Carmo (2015) studied the effects of HdLP in a zymosan-induced arthritis model in male Swiss mice. Results of this study showed that HdLP (50 mg/kg i.v.) significantly reduced leukocyte count, myeloperoxidase activity, and levels of inflammatory mediators (NO, IL-1β, and IL-6). Furthermore, HdLP treatment decreased swelling, synovial membrane damage, and improved nociceptive thresholds. These results indicated that HdLP demonstrates both antinociceptive and anti-inflammatory effects in the arthritis model, potentially by reducing the neutrophil migration and inflammatory mediator release.

In a recent study, Souza TFG *et al*., (2023), highlight the wound-healing potential of HdLP, a novel therapeutic property of *H. drasticus* latex. They evaluated the wound-healing properties of proteins extracted from HdLP in an excisional wound model. HdLP exhibited no cytotoxicity in murine fibroblasts (12.5–100 µg/mL) and did not induce dermal irritation. Wounds treated with HdLP (0.5%, 1.0%, and 2.0%) demonstrated enhanced healing compared to controls, including a commercial healing agent. HdLP promoted an accelerated inflammatory phase marked by IL-1β release, which facilitated earlier IL-10 secretion by macrophages during the proliferative phase. This contributed to fibroblast proliferation, collagen synthesis, and organized re-epithelialization, resulting in more mature, remodeled tissue. Histological analysis and inflammatory marker measurements confirmed that HdLP downregulated prolonged inflammation and improved tissue regeneration.

The research on latex proteins of *H. drasticus* (HdLP) remains limited, primarily due to the challenges associated with extracting these proteins. While some studies have been published, much of the available information exists in the form of theses, further reflecting the difficulty in obtaining and analyzing these compounds.

In addition to the limited studies on *H. drasticus*, there is also a significant gap in research due to the absence of genomic data for this plant. The lack of comprehensive genomic information further complicates the understanding of the molecular mechanisms underlying its therapeutic properties. This absence of genomic data, coupled with the challenges in the extraction of latex and its components, has hindered more in-depth studies into the bioactive compounds of *H. drasticus* and their potential applications.

Therefore, this study was designed to bridge the gap in current research by characterizing the protein profile of *H. drasticus* latex, investigating its biological activities, and evaluating its potential therapeutic applications, particularly in the context of immune modulation and cell viability. Additionally, transcriptomic analysis was incorporated to provide a deeper understanding of the molecular mechanisms underlying the therapeutic effects, thus contributing valuable genomic data for this plant.

2 OBJECTIVES

2.1 General Objective

Transcriptomic analysis of *Himatanthus drasticus* and evaluation of the effects of its latex proteins (HdLPs) on hyperglycemia, infection, and immune responses.

2.2 Specific Objectives

- To perform transcriptomic analysis of leaves and stems of *H. drasticus* to identify differentially expressed genes related to defense responses, and bioactive compound biosynthesis.
- To fractionate and characterize the protein fraction of *H. drasticus* latex.
- To assess the effects of HdLP and its sub-fractions on cell viability, proliferation, and cytotoxicity.
- To explore the impact of HdLP on hyperglycemia, infection, and immune responses in Swiss mice.
- To assess the hematological and histological effects of HdLP, and its fractions on the experimental model.
- To investigate the immunomodulatory effects of HdLP in animal models.

3 STUDY I: PARTIAL TRANSCRIPTOMIC ANALYSIS OF *H. DRASTICUS* LEAVES AND BARK

3.1 Introduction

H. drasticus is a plant species with no genomic and transcriptomic data available, despite its potential biological and pharmacological relevance. To expand the molecular understanding of this species, we conducted a partial transcriptomic analysis using RNA sequencing (RNA-Seq) of its leaf and stem tissues. This study represents the first RNA-Seq–based investigation of *H. drasticus*, providing a foundational overview of gene expression patterns in these tissues.

3.2 Materials and methods

3.2.1 Plant material

Fresh young leaves and stem samples were collected in triplicate from different *H. drasticus* plants, located in the vicinity of Edson Queiroz, Fortaleza, Ceará, Brazil. They were washed in distilled water, frozen in liquid nitrogen, and then crushed into powder using a pestle and mortar. A quantity from each sample was used for RNA extraction, and the remainder was stored at -80°C until the transcripts were analyzed. The plant species was confirmed by comparing the botanical material with samples deposited and registered under voucher number 40408 at Herbarium Prisco Bezerra, Department of Biology, Federal University of Ceará, Fortaleza, Brazil. Legal permission for accessing and use of the botanical material was registered in the SisGen platform under the identification A689147.

3.2.2 RNA extraction, library construction, and sequencing

Total RNA was extracted from 0.1 g of ground tissue per sample using the Concert "℠Plant RNA Reagent (Invitrogen, Darmstadt, Germany) following the manufacturer's instructions. The DNase digestion step was applied during RNA extraction to remove genomic DNA contamination using RNase-Free DNase Set (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA

purification was conducted by SV Total RNA Isolation System (Promega, Madison, USA). Total RNA quantity was estimated by absorbance at 260 nm in a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA), and the quality was verified in a 1.5% agarose gel stained with ethidium bromide. Furthermore, the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, USA) confirmed total RNA integrity and quantity. Total RNAs (1 µg) with RNA integrity number (RIN) values above 7 were used for library construction and sequencing for each sample.

Four cDNA libraries, consisting of leaves in biological triplicate, and a single library of bark were prepared and sequenced from 1 µg of total RNA by a sequencing facility (Macrogen, Inc., Seoul, South Korea). A strand-specific RNA-Seq library preparation was performed using the Illumina TruSeq stranded mRNA, following the manufacturer's instructions. A paired-end (2 × 150 bp) sequencing library was prepared and then sequenced on the NovaSeq6000 platform (Illumina, San Diego, CA, USA).

3.2.3 Reads quality check and de novo transcriptome assembly

The raw reads quality was assessed using the FastQC v.0.12.0 software (Andrews, 2010) for all samples before and after trimming. Then, raw reads containing adapters, low-quality bases (< Q20), and short sequences (≤ 75 bp) were trimmed by the Trimmomatic v.0.36 tool (Bolger *et al.,* 2014). Subsequently, any ribosomal RNA (rRNA) sequences were removed from the samples to align raw reads to the SILVA rRNA database (Quast *et al*., 2013) using the BBDuk program, which is a part of the bbmap tools (https://sourceforge.net/projects/bbmap/). To remove any insect (fly) contamination, raw reads were mapped to the *Drosophila melanogaster g*enome (reference), using STAR aligner v.2.7.11a (Dobin *et al*., 2013).

After trimming, filter reads were assembled using the default parameters of Trinity v.2.15.1 (Grabherr *et al.,* 2011). Furthermore, the reference transcriptome was submitted to the CD-HIT-est tool (Li and Godzik, 2006) to remove redundant transcript sequences with an identity greater than 90%. The high quality of the assembly was confirmed by TransRate (Smith-Unna *et al*., 2016).

3.2.4 Transcriptome annotation

The open reading frames (ORFs) within assembled transcripts were identified using the default parameters of TransDecoder v.5.7.1 (https://github.com/TransDecoder/TransDecoder). Then, predicted peptides from ORFs were aligned to Pfam and SwissProt databases using the HMMER and Blast-p tools, respectively. InterProScan was used to get GO IDs of predicted peptides against the PANTHER database.

3.2.5 Differential expression analysis

Filter reads were mapped to a non-redundant transcriptome to estimate transcript abundances using the Bowtie2 aligner (Langmead *et al*., 2009) and the RSEM v.1.2.0 (Li and Dewey, 2011) package. The expected counts were normalized using the CPM function from the edgeR package (Robinson *et al*., 2010) to filter low-expressed transcripts. Only the transcripts that presented a count-per-million (CPM) ≥ 2 in at least one sample were selected for differential expression analysis. Subsequently, the expected counts and length of remaining transcripts were provided to the DESeq2 package v. 1.30.0 (Love *et al*., 2014) to perform principal component analysis (PCA) and to infer differentially expressed transcripts (DET) between leaf and bark. Only transcripts presenting an adjusted p-value<0.05 and |log2 fold-change|≥ 1 were considered as DETs (Benjamini and Hochberg, 1995). Then, functional enrichment analysis of the DETs was performed using the GOseq R package v. 1.30.0 (Young *et al.,* 2010) and KOBAS software v. 3 (Kanehisa and Goto, 2000; Xie *et al*., 2011).

3.3 Results

3.3.1 Sequencing quality check

The RNA-Seq analysis yielded 42.06 – 59.85 million raw reads per cDNA library with an average read length of 151 bp (Table 1). Raw reads were filtered to obtain high-quality reads by removing reads containing adapter or poly (N) sequences, low-quality sequences (Q < 20) from 5′ and 3′ ends of the reads, and sequences shorter than 75 bp. After this first filtration, on average, 40.47 – 56.35 million clean reads were obtained for each sample. Then, additional filtration was performed to remove rRNAs that yielded, on average, 181.81 million reads of mRNAs. The percentages of Phred scores at the Q30 level (error probability less than 0.1%) ranged from 92.9 to 94.2%, and the GC content ranged from 45.2 to 47.9%.

Table 1 – RNA-Seq analysis of leaf and stem of *H. drasticus*.

Sample ID	Total bases (bp)	Total reads	GC (%)	AT (%)	Q20 (%)	Q30 (%)
Leaf-1	6,352,356,486	42,068,586	45.4	54.6	97.9	94.2
Leaf-2	8,998,338,244	59,591,644	45.5	54.5	97.0	92.9
Leaf-3	9,037,714,816	59,852,416	45.2	54.8	97.3	93.3
Stem-2	8,506,511,010	56,334,510	47.9	52.1	97.7	93.9

Source: self-authorship

- Sample ID: Sample name.
- Total Bases (bp): Total number of bases sequenced.
- Total Reads: Total number of reads. For ilumina paired-end sequencing, this value refers to the sum of read 1 and read 2.
- GC (%): ratio of GC content.
- AT (%): ratio of AT content.
- Q20 (%): ratio of bases that have a Phred quality score of over 20.
- Q30 (%): ratio of bases that have a Phred quality score of over 30.

3.3.2 Transcriptome assembly

The transcriptome assembly of *Himatanthus drasticus* was performed using 181,81 million reads of mRNAs, which produced 146,221 transcripts. After removing redundant transcript sequences, a total of 92,935 transcripts remained in the analysis. Basic statistics of the assembly evidenced the high quality of *H. drasticus* transcriptome (Table 2). The transcript length varied from 283 bp to 13,627 bp, while the N50 sizes of the assembly were around 1,666 bp.

Table 2. *De novo* transcriptome assembly statistics.

	CD-HIT-est Statistics
Total transcripts	92,935
Smallest transcript (bp)	283
Largest transcript (bp)	13,627
Mean transcripts length (bp)	1,130
N50 (bp)	1,666
GC content (%)	0.40502

Source: Self-authorship

The high quality of transcriptome assembly was also determined according to the number of reads from cDNA libraries that mapped back to the assemblies. In this study, the alignment rate ranged from 65% in the Leaf-2 library to 72% in the Leaf-1 library. A large proportion of reads mapping back to the assembly (i.e. above 60–70%) indicates proper quality sequence reconstruction and a representative transcriptome (Haas *et al*., 2013).

Among 92,935 transcripts from transcriptome assembly, only 74,451 transcripts presented ORFs, which were translated into the predicted peptide sequences. Thereafter, 33,099 (44.45%) and 48,285 (64.85%) predicted peptides were annotated using Pfam and SwissProt databases, respectively (Supplementary file 1 & file 2). In addition, the InterProScan software identified Gene Ontology (GO) terms for 29,397 predicted peptides (Supplementary file 3).

3.3.3 Differential expression analysis

To assess the variability and clustering of gene expression profiles between stem and leaf samples, a principal component analysis (PCA) was performed, revealing a clear separation of the samples along the principal components, with PC1 explaining 64% of the variance and PC2 explaining 19% (Fig. 5). To further validate the distinction in gene expression of stem and leaf samples, a hierarchical clustering heatmap was generated (Fig. 6). As expected, the overall expression showed a tissue-dependent pattern grouping replicates of leaves (Leaf-2 and Leaf-3). Although one leaf replicate (Leaf-1) clustered with a stem sample, the correlation between them was lower than leaf replicates.

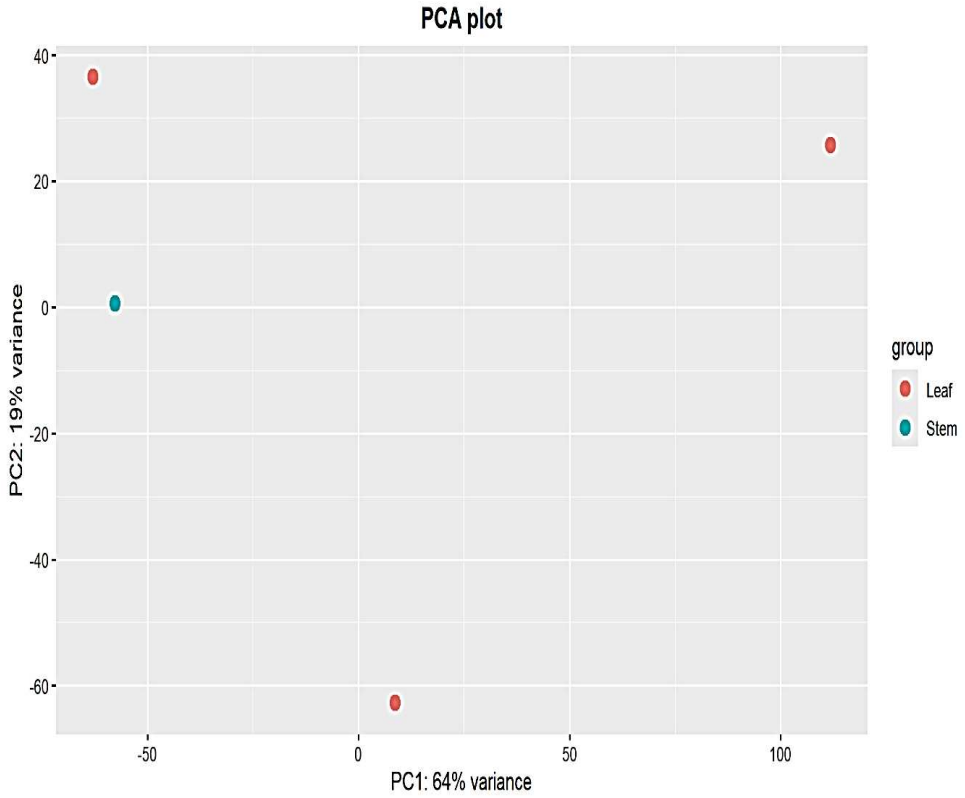
In stem replicates, the following transcripts presented the log2FoldChange > 10, indicating the higher expression of these genes in stem compared to leaf replicates.

- TRINITY_DN4219_c0_g1_i11
- TRINITY_DN21688_c0_g1_i1
- TRINITY_DN56725_c0_g1_i1
- TRINITY_DN9738_c1_g1_i12
- TRINITY_DN13853_c0_g1_i1

These transcripts codify Lipoxygenase, Homeobox protein transcription factors, Endochitinases, ATP-binding cassette sub-family C, and Jacalin-Related Lectin 3 (JRL3), respectively.

Overall, the differential expression analysis showed 2709 differentially expressed transcripts (DETs) between tissues (leaf and stem). Among them, 2362 transcripts were expressed more in leaves (Supplementary file 4), and 347 transcripts were expressed more in stems (Supplementary file 5).

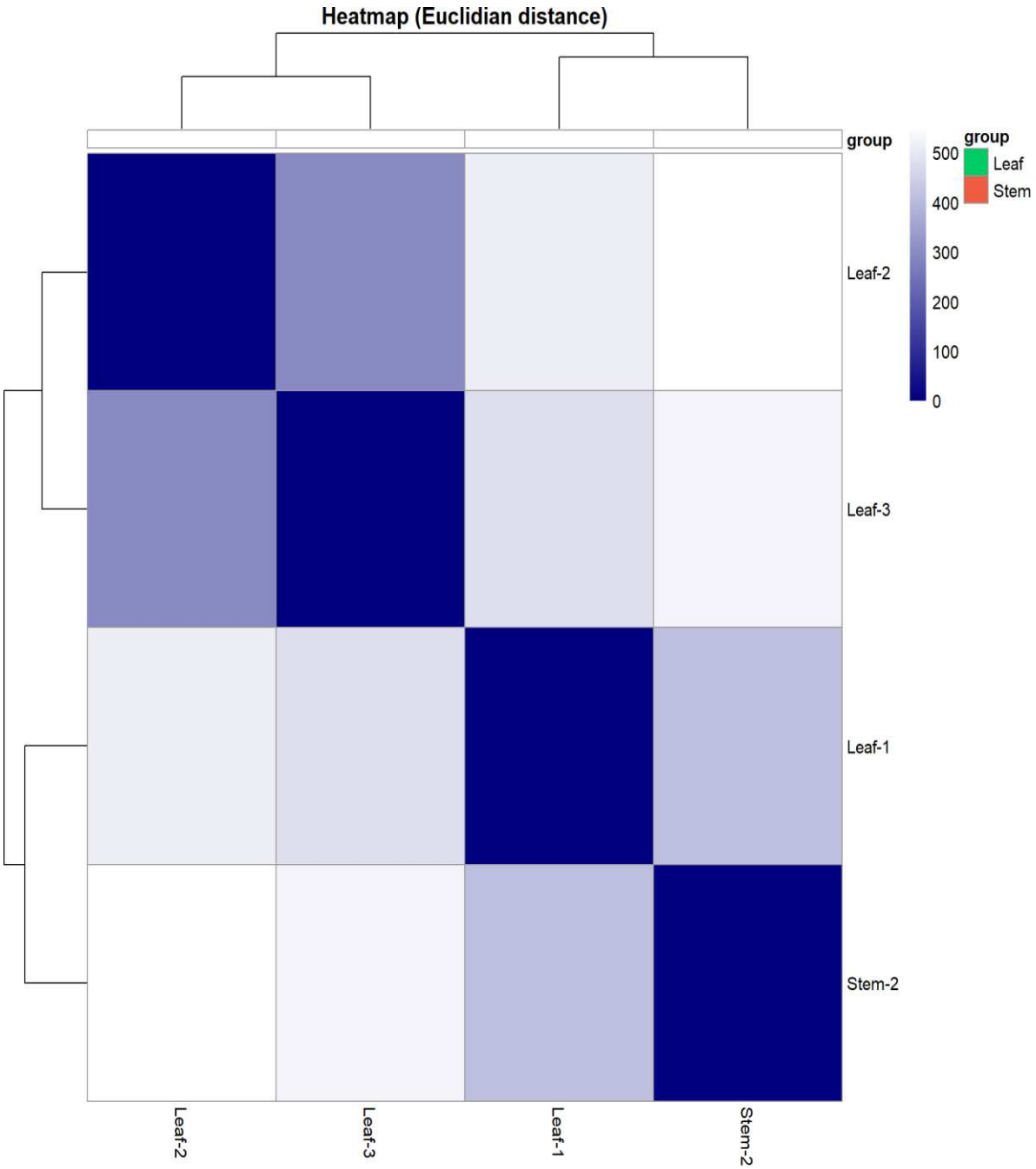
Figure 5: Principal component analysis of bark and leaf samples.



Source: Self-authorship

Principal component (PC) analysis plot displaying the clustering of all four samples based on gene expression profiles. PC1 and PC2 account for 64% and 19% of the variability, respectively, with a clear separation between the two tissue types.

Figure 6: Hierarchical Clustering Heatmap of leaf and bark samples based on Euclidean distance.



Source: Self-authorship

On the other hand, the transcripts given below had log2FoldChange < -10, indicating their higher expression in leaves than in stem replicates.

- TRINITY_DN9574_c0_g1_i1
- TRINITY_DN1091_c0_g2_i3
- TRINITY_DN11108_c0_g1_i4
- TRINITY_DN649_c0_g1_i13
- TRINITY_DN4086_c0_g1_i12
- TRINITY_DN2115_c0_g1_i2
- TRINITY_DN4834_c0_g1_i16

These transcripts codify Uridine kinase, Oligopeptide transporter YGL114W-related, Malate and Lactate dehydrogenase, Homeobox protein transcription factors, Thioredoxin-dependent peroxide reductase, Lecithin-cholesterol acyltransferase-related, and Protein phosphatase 2C, respectively.

3.3.4 Functional annotation

Functional annotation of the DETs between tissues (leaf and stem) was carried out to decipher the general profile related to the biological functions represented in the transcriptome of *H. drasticus* (Fig. 7). Gene Ontology (GO) terms were assigned after BLASTX search against the UniProt SwissProt database and the transcripts were classified into biological processes (BP), molecular functions (MF), and cellular components (CC) (Supplementary file 6).

In the biological process (BP) category, several processes were significantly overrepresented. Highly significant overrepresentation was observed in categories “photosynthesis, light harvesting (GO:0009765, GO:0009768, GO:0015979), translation (GO:0006412), response to light stimulus (GO:0009416), cell fate commitment (GO:0045165), proton motive force-driven ATP synthesis (GO:0015986), photosynthetic electron transport in photosystem I (GO:0009773), aerobic respiration (GO:0009060), photosystem II assembly (GO:0010207), glycine decarboxylation via glycine cleavage system (GO:0019464), photosynthetic electron transport chain (GO:0009767), abaxial cell fate specification (GO:0010158), mitochondrial electron

transport: cytochrome c to oxygen (GO:0006123), multicellular organism development (GO:0007275), and regulation of secondary shoot formation (GO:2000032)". These categories had p-values ≤ 0.001, ranging from 10^{-18} to 10^{-5}. Furthermore, an additional 84 categories demonstrated statistically significant p-values (< 0.05) and were also deemed overrepresented (Supplementary file 6).

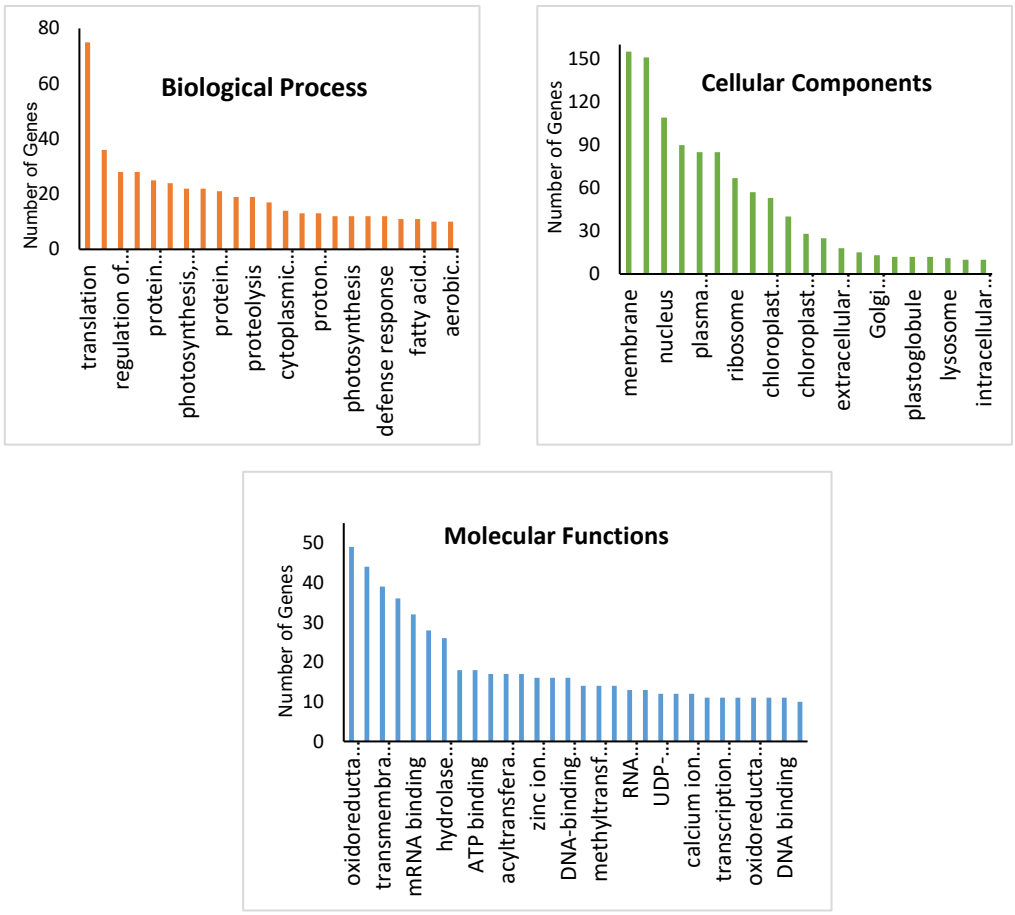
Several processes demonstrated strong overrepresentation in the cellular component (CC) category. Notably, the categories "chloroplast-thylakoid (GO:0009535, GO:0009507, GO:0009579, GO:0009543, GO:0009570), ribosome (GO:0005840, GO:0022625, GO:0022627, GO:0015935), plastoglobule (GO:0010287), photosystem I (GO:0009522, GO:0009538), chitin-based extracellular matrix (GO:0062129), apoplast (GO:0048046), photosystem II (GO:0009523, GO:0009654), glycine cleavage complex (GO:0005960), mitochondrial respiratory chain complex IV (GO:0005751), proton-transporting ATP synthase complex, catalytic core F1 (GO:0045261), and respiratory chain complex I (GO:0045271)" exhibited highly significant p-values ≤ 0.001, ranging from 10^{-23} to 10^{-5}. Additionally, 20 more categories with p-values < 0.05 were also recognized as statistically significant (Supplementary file 6).

And in the Molecular Function (MF) category, the following terms demonstrated significant overrepresentation with p-values ≤ 0.001, ranging from 10^{-23} to 10^{-5}, "structural constituent of ribosome (GO:0003735), acyltransferase activity, transferring groups other than amino-acyl groups (GO:0016747), proton-transporting ATP synthase activity, rotational mechanism (GO:0046933), structural constituent of chitin-based larval cuticle (GO:0008010), oxidoreductase activity (GO:0016491), glycine binding (GO:0016594), oxidoreductase activity (GO:0016620, GO:0016616), proton-transporting ATPase activity (GO:0046961), C-4 methylsterol oxidase activity (GO:0000254), and guanylate kinase activity (GO:0004385)". In addition to the aforementioned categories, 74 more categories with p-values < 0.05 were also found to be statistically significant (Supplementary file 6).

Along with gene ontology analysis, pathway enrichment highlights key metabolic processes in the stem and leaf of *H. drasticus*, suggesting its potential for various therapeutic applications. The analysis of gene expression in the stem (Fig. 8A) and leaves (Fig. 8B) of *H. drasticus* reveals a significant enrichment of metabolic

pathways associated with diverse therapeutic potentials, particularly those associated with immune-modulatory, neuroprotective, and metabolic regulation activities.

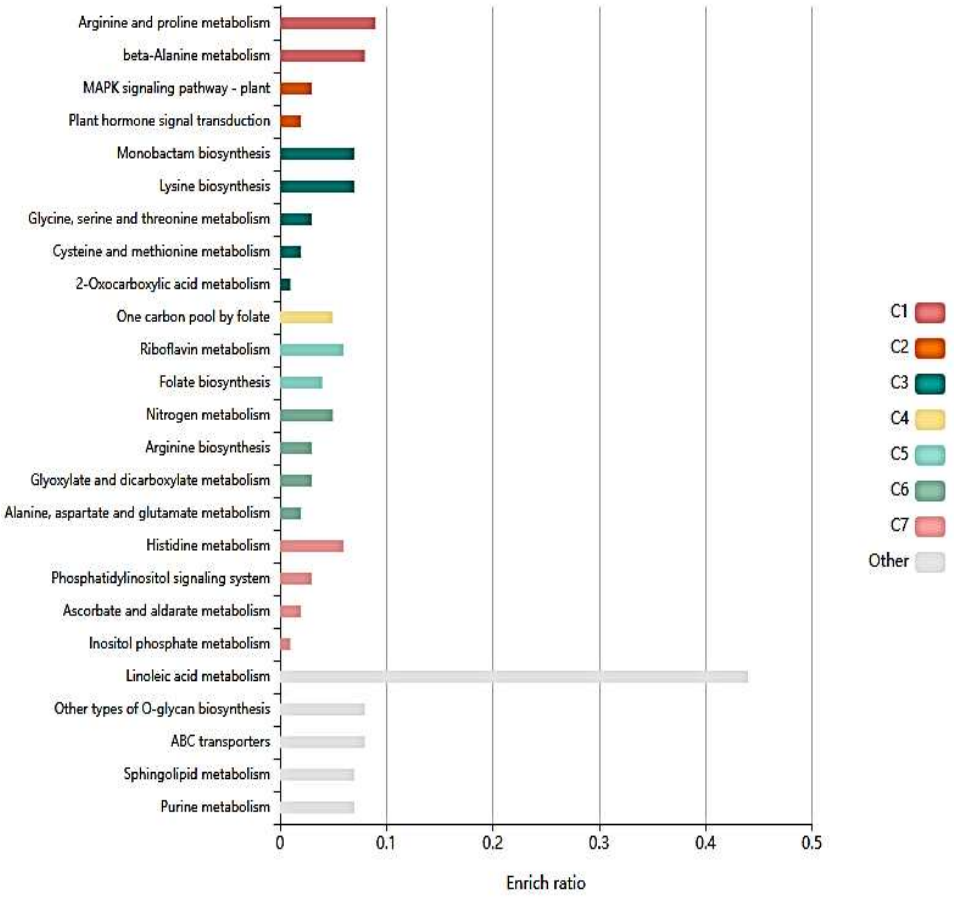
Figure 7: Gene ontology (GO) enrichment analysis of DETs between leaf and stem samples of *H. drasticus*.



Source: Self-authorship

GO: Gene ontology, BP: Biological processes, CC: Cellular components, MF: Molecular function. The Gene Ontology (GO) analysis of differentially expressed transcripts (DETs) between *H. drasticus* stem and leaf revealed key biological processes, cellular components, and molecular functions associated with highly expressed genes. The BP analysis highlights the dominant functions of translation, transmembrane transport, and photosynthesis-related activities. The CC analysis shows a strong presence of genes associated with membranes, cytoplasm, nucleus, and chloroplasts. Meanwhile, MF analysis indicates genes involved in oxidoreductase activity, RNA binding, transmembrane transporter activity, and DNA-binding transcription factor activity.

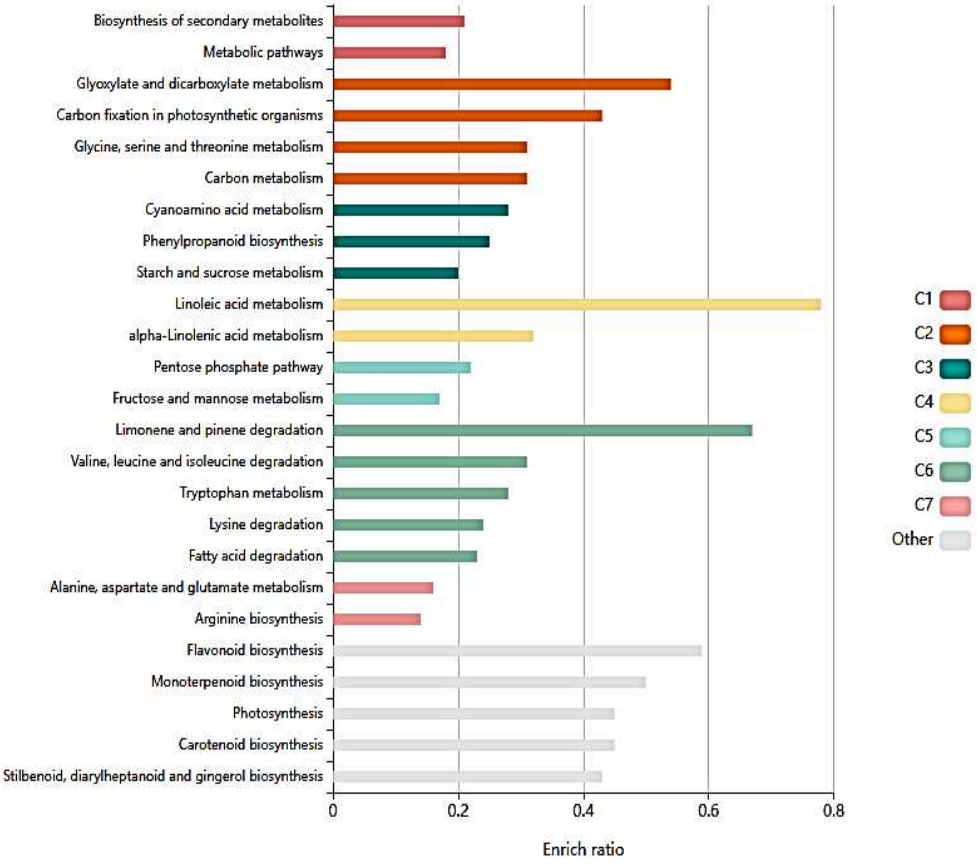
Figure 8A: Metabolic pathways enriched in the stem of *H. drasticus*



Source: Self-authorship

Metabolic pathways enrich in the stem. Each row represents an enriched function, and the length of the bar represents the enrichment ratio, which is calculated as "input gene number"/ "background gene number". The color of the bar represents different clusters. For each cluster, if there are more than 5 terms, the top 5 with the highest enrichment ratio were displayed.

Figure 8B: Metabolic pathways enriched in the leaves of *H. drasticus*



Source: Self-authorship

Metabolic pathways enriched in the leaves. Each row represents an enriched function, and the length of the bar represents the enrichment ratio, which is calculated as "input gene number"/ "background gene number". The color of the bar represents different clusters. For each cluster, if there are more than 5 terms, the top 5 with the highest enrichment ratio were displayed.

3.4 Discussion and Conclusion

The transcriptomic analysis of *Himatanthus drasticus* revealed significant differential gene expression between leaf and stem tissues, reflecting tissue-specific specialization in metabolic and defense-related processes consistent with the distinct physiological roles of each organ.

The transcripts highly expressed in the stem are predominantly associated with inflammation, defense responses, cellular signaling, cancer, infection, and wound healing (Leitner *et al*., 2007; Nagelin *et al*., 2009; Sutherland *et al*., 2014; Van Moerkercke *et al*., 2015; Yang *et al*., 2016; Bhutia *et al*., 2019; Yamada & Sato, 2021; Feng *et al*., 2021). Since the latex of H. drasticus is secreted from the stem, this expression profile provides valuable molecular evidence supporting the therapeutic properties observed for the latex. The high expression of genes involved in lipid oxidation, lectin activity, chitin degradation, and secondary metabolite transport suggests that the stem serves as a biosynthetic hub for bioactive compounds that contribute to the latex's anti-inflammatory, antimicrobial, antifungal, and wound-healing activities. Moreover, the upregulation of transcription factors related to stress regulation and developmental control indicates that the stem actively coordinates the synthesis and accumulation of these bioactive molecules within the latex.

For instance, the high expression of lipoxygenase in the stem suggests its role in the synthesis of oxylipins, including jasmonic acid, which are key mediators of defense signaling and inflammation regulation. This finding supports the anti-inflammatory and wound-healing properties previously observed in the latex extracted from the stem. Similarly, JRL3, known for its carbohydrate-binding activity, is implicated in pathogen recognition and defense, suggesting that latex proteins may possess antimicrobial and immune-enhancing properties. The abundant expression of endochitinases further supports this hypothesis, as these enzymes degrade chitin, a major structural component of fungal cell walls, indicating potential antifungal activity of the latex. In addition, ABC transporters play essential roles in the transport of secondary metabolites and xenobiotics, contributing to the chemical diversity and bioactivity of the latex. Homeobox transcription factors, also upregulated in the stem, regulate developmental and stress-responsive pathways, possibly modulating the biosynthesis and storage of bioactive compounds within the latex.

Pathway enrichment analysis further reinforces this connection, revealing significant activation of amino acid metabolism pathways, including arginine, proline, lysine, and histidine metabolism, which are closely associated with immune modulation and inflammation control (Phang *et al*., 2008; Feng *et al*., 2013; Asosingh *et al*., 2020; Kelly & Pearce, 2020). The enrichment of phosphatidylinositol signaling and inositol phosphate metabolism points to potential neuroprotective and cell-signaling functions (Berridge, 2016; Desale & Chinnathambi, 2021). Similarly, the presence of riboflavin and folate metabolism pathways suggests contributions to antioxidant defense and hematopoietic regulation (Koury & Ponka, 2004; Mikkelsen & Apostolopoulos, 2019; Sobral *et al*., 2024). The enrichment of ascorbate and aldarate metabolism further supports the stem's role in antioxidant and cytoprotective activities (Gęgotek & Skrzydlewska, 2022; Liang & Song, 2024). Collectively, these transcriptomic signatures provide a molecular basis for the wide-ranging therapeutic potential of the stem and its excretions.

Complementary to the stem-specific expression patterns, the transcripts highly expressed in the leaves showed association with processes related to metabolic regulation, oxidative stress response, inflammation, neurological disorders, and cancer (Lammers & Lavi, 2007; Kumar *et al*., 2009; Rousset *et al*., 2011; Lu & Holmgren, 2014; Mansouri *et al*., 2017). The metabolic pathways enriched in leaf tissues further support their therapeutic potential, particularly through antioxidant, anti-inflammatory, neuroprotective, and metabolic regulatory activities. Notably, pathways such as flavonoid and carotenoid biosynthesis are strongly linked to antioxidant defense, helping to reduce oxidative stress and prevent cellular damage (Frandsen & Narayanasamy, 2018; Andarwulan *et al*., 2021; Shen *et al*., 2022). In addition, the presence of linoleic acid and α-linolenic acid metabolism pathways highlights their anti-inflammatory potential, which may be beneficial in chronic inflammatory conditions (Anand & Kaithwas, 2014). Moreover, the amino acid metabolism pathways, including glycine, serine, and threonine metabolism, are associated with neuroprotective effects, supporting cognitive function and potentially contributing to the management of neurodegenerative diseases (Myint & Sun, 2023).

Gene Ontology (GO) analysis revealed significant enrichment in biological processes (BP) such as photosynthesis, translation, response to stimuli, cell fate commitment, and secondary metabolite regulation, highlighting the plant's complex

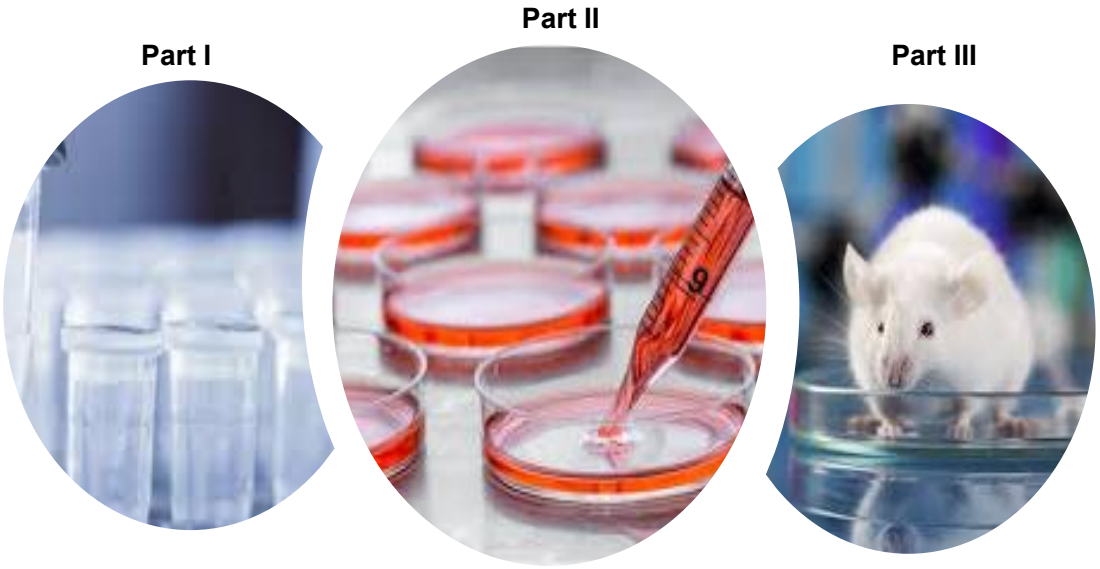
adaptive and defense mechanisms. Cellular component (CC) categories emphasized the roles of chloroplasts, thylakoids, ribosomes, and extracellular matrices, suggesting the participation of both structural and secretory components in the synthesis and distribution of bioactive molecules. Molecular function (MF) analysis revealed enrichment of oxidoreductase, acyltransferase, ATPase, and glycine-binding activities, linking these enzymes to antioxidative, anti-inflammatory, and metabolic processes.

This study provides the first comprehensive link between transcriptomic data and the therapeutic potential of *H. drasticus*, offering a molecular framework to explain its diverse biological effects. The high expression of defense and metabolism-related genes in the stem underscores its role as a reservoir of bioactive proteins and secondary metabolites, while the leaf transcriptome reflects metabolic specialization and antioxidant potential. These findings not only advance the pharmacological understanding of *H. drasticus* but also support its future exploration as a promising source of bioactive compounds for therapeutic applications.

4 STUDY II: EVALUATION OF TOXICITY & THERAPEUTIC POTENTIAL OF *H. DRASTICUS* LATEX PROTEINS

4.1 Introduction

Despite the extensive research on the bioactive compounds of *H. drasticus*, the protein fraction of its latex has remained largely unexplored. Understanding the composition of the protein fraction of *H. drasticus* latex (HdLP) is essential as it may hold the potential for novel therapeutic applications, especially given its distinct biological effects. The characterization of these proteins will not only fill a critical knowledge gap in the molecular profile of *H. drasticus* but also provide a foundation for future research into their mechanisms of action and possible contributions to immunomodulation, regenerative medicine, and other biomedical fields. In this study, which was divided into three parts, we aimed to characterize the proteins present in the HdLP fraction of *Himatanthus drasticus* latex through HdLP fractionation, in vitro toxicity assessment, and evaluation of in vivo biological effects, to better understand its complex biological roles and support the exploration of its full therapeutic potential.



4.2 PART I: Fractionation and characterization of HdLP

4.2.1 Materials and methods

4.2.1.1 Latex Processing

The freshly collected latex of *H. drasticus* was processed using the method of (Mousinho KC *et al*., 2011) with some modifications. Initially, the latex was centrifuged (10,000 x g) for 30 min at 4ºC. This procedure allowed the precipitation of insoluble fractions rich in isoprene polymers (rubber). The supernatant obtained was exhaustively dialyzed against distilled water for 72 h, at 25ºC to remove hydrophilic metabolites of small molecular size (Mr < 8 kDa). The fraction retained inside the dialysis membrane was centrifuged again to remove any leftover rubber under the conditions previously described, and the supernatant was recovered and lyophilized. This rubber-free fraction, rich in soluble macromolecules, was named “HdLP” (*H. drasticus* latex proteins) and subjected to ion-exchange chromatography (IEC) for fractionation after measuring the protein concentration by Bradford assay.

4.2.1.2 Fractionation and characterization of HdLP

For sub-fractionation of HdLP, ion-exchange chromatography (IEC) was performed. The DEAE-Sepharose fast flow matrix equilibrated with 50 mM sodium acetate buffer (pH 5.0) was selected as the stationary phase. The sample was prepared by dissolving 70 mg of HdLP in 5 mL of 50 mM Sodium Acetate buffer (pH 5.0) and centrifuged (10,000 x g, 15 min, 4°C) to remove undissolved material. The resulting supernatant (5 mL ± 0.5) was loaded into the column. After washing the unbound material, the bound proteins were eluted using the same buffer with an increased NaCl concentration (250 mM). Both subfractions (5 mL/tube) were collected at a flow rate of 1 mL/min, and all the elutes were monitored at 280 nm. The protein subfractions obtained were subsequently dialyzed and freeze-dried for further evaluation.

For the analysis of protein profile and quality of HdLP and subfractions, one-dimensional SDS-PAGE was performed (Laemmli 1970). The 5% polyacrylamide prepared in 500 mM Tris-HCl (pH 6.8) buffer was used as the stacking gel while the

12.5% polyacrylamide made in 3000 mM Tris-HCl (pH 8.8) was used as the separating gel. The samples were dissolved in 60 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, and 1% sodium dodecyl sulfate (SDS), in the absence of 2-mercaptoethanol. The protein concentrations used were determined by the Bradford method (1976). The marker solution used for estimation of molecular weight was comprised of α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30.00 kDa), ovalbumin (45.00 kDa), bovine serum albumin (66.00 kDa), and phosphorylase-β (97.00 kDa).

The runs were completed under a constant supply of 40 mA, and a voltage of 100 V per gel, in an average duration of 2 h at 25°C. The 25 mM Tris-HCl (pH 8.3) containing 192 mM glycine and 0.1% SDS was used as a running buffer. After the electrophoretic run, protein bands were observed by staining the gel with a solution of 0.05% Coomassie Brilliant Blue R-250 in distilled water, acetic acid, and methanol (4:1:5; v:v:v) for 4 h, followed by the removal of dye with the same solution devoid of dye.

4.2.1.3 Digestion of proteins for LC-MS

The protein sub-fractions extracted as described above were subjected to trypsin digestion. For each sub-fraction, 10 µg of protein, as determined by the BCA (bicinchoninic acid assay) method, was used. Samples (100 µg of protein/sample) were first treated with a 0.2% RapiGest SF (Waters, Milford, USA) in 50 mM NH_4HCO_3. The mixtures were incubated at 80°C for 15 min to facilitate protein denaturation. Subsequently, 5 µL of 100 mM dithiothreitol (DTT) was added to reduce the disulfide bonds, followed by incubation at 60°C for 30 min.

Next, 5 µL of 300 mM iodoacetamide (IAA) was added for cysteine alkylation, preventing the reformation of disulfide bonds. The samples were kept in the dark at room temperature for 30 min to ensure complete alkylation. Trypsin (1 µg, Promega) was then added, and the digestion was carried out at 37°C for approximately 16 h. Finally, the samples were centrifuged at 13,000 × g for 30 min at 6°C, and the supernatants were collected for subsequent mass spectrometry analysis.

4.2.2 RESULTS

4.2.2.1 Ion Exchange Chromatography and Electrophoresis

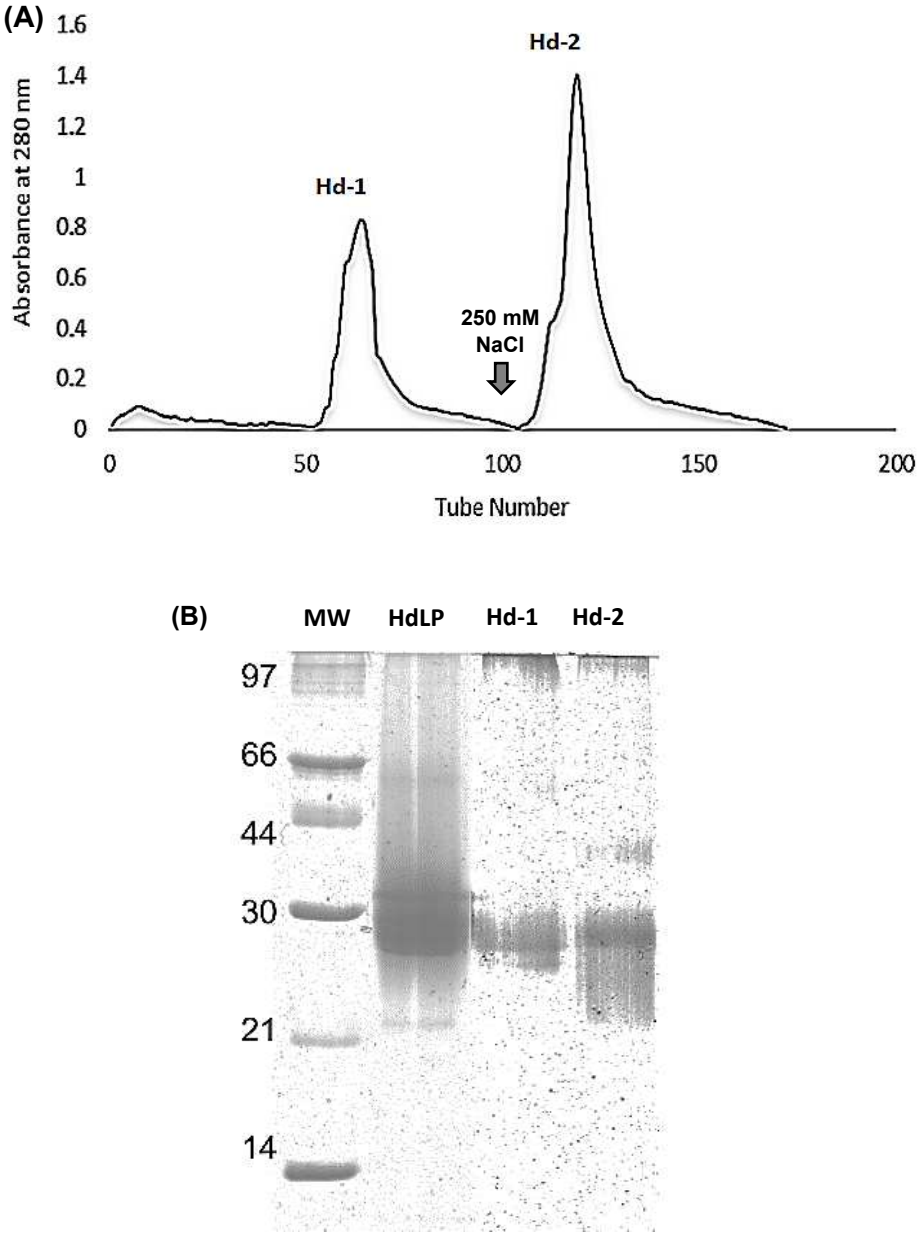
Results of chromatography and one-dimensional SDS-PAGE showed the presence of two different sub-fractions, designated as Hd-1 and Hd-2, in HdLP under the experimental conditions applied (Fig. 9A).

The SDS-PAGE showed a predominant protein band around 30 kDa in all three samples (HdLP, Hd-1 & Hd-2), which suggests that the major proteins present in these fractions have molecular weights of ~30 kDa (Fig. 9B).

4.2.2.2 LC-MS analysis

Due to the absence of a specific protein database for *H. drasticus*, protein identification was performed using publicly available datasets from *C. procera* and *H. brasiliensis* latex proteins as references. LC-MS analysis revealed that the proteins identified in HdLP subfractions exhibited structural and functional similarities to those found in *C. procera* and *H. brasiliensis*, as determined by their UniProt accession numbers. The analysis identified 27 proteins/peptides in subfraction Hd-1 (Table 3A) and 26 in subfraction Hd-2 (Table 3B). Most of the proteins/peptides detected in HdLP subfractions included chitinases, proteases, and proteins involved in the regulation of different metabolic activities, along with some unknown functions.

Figure 9: Ion exchange chromatography (A) & gel electrophoresis (B).



Source: Self-authorship

(A) Ion exchange chromatography in DEAE-Sepharose Fast Flow column at pH 5.0 of *H. drasticus* latex proteins. 70 mg of HdLP per 5 mL of Na-acetate buffer loaded in the column, Hd-1 and Hd-2 were eluted by increasing the ionic strength by adding 0.05 M and 0.25 M of NaCl to the elution buffer, respectively. **(B)** Protein profiles of HdLP, Hd-1, and Hd-2 were revealed by one-dimensional SDS-PAGE. MW-Molecular weight markers: 97.0 kDa phosphorylase β, 66.0 kDa albumin, 45.0 kDa ovalbumin, 30.0 kDa carbonic anhydrase, 20.1 kDa trypsin inhibitor, and 14.4 kDa α-lactalbumin.

Table 3A: Mass spectrometric analysis of subfraction Hd-1 of HdLP

Accession Number	Plant	Protein/Peptide	Function
A0A0A0Q2K8; M9QMC9	*C. procera*	Procerain B	Cysteine-type endopeptidase activity, proteolysis involved in protein catabolic processes
A0A2S1P2U9	*C. procera*	Photosystem I assembly protein Ycf3	Photosystem I assembly
A0A6A6KVU7; A0A6A6L3A1	*H. brasiliensis*	Uncharacterized protein	Male meiosis II
A0A6A6LIG1	*H. brasiliensis*	MSP domain-containing protein	Chitin catabolic process, cell wall macromolecule catabolic process, defense response to fungus
A0A6A6LSP7	***H. brasiliensis***	**Chitin-binding type-1 domain-containing protein**	**Chitin catabolic process, cell wall macromolecule catabolic process, defense response to fungus**
A0A6A6LLT4	*H. brasiliensis*	Glycoside hydrolase family 19 catalytic domain-containing protein	carbohydrate metabolic process, Chitin catabolic process, cell wall macromolecule catabolic process, defense response to fungus
A0A6A6MBP6	*H. brasiliensis*	EF-hand domain-containing protein	Calcium ion binding.
A0A6A6MD41	*H. brasiliensis*	DNA helicase	ATP binding, DNA binding, 3'-5' DNA helicase activity, hydrolase activity, recombinational repair
A0A6A6MI11	*H. brasiliensis*	ANK_REP_REGION domain-containing protein	Unknown
A0A6A6MPZ7	*H. brasiliensis*	DBD_Tnp_Mut domain-containing protein	Unknown
A0A6A6MR65	*H. brasiliensis*	Matrin-type domain-containing protein	RNA binding, Zn ion binding, mRNA splicing via spliceosome.
A0A6A6MT87	*H. brasiliensis*	SBP-type domain-containing protein	DNA binding, metal ion binding.
A0A6M6A649	*C. procera*	CPCpB	Proteolysis involved in protein catabolic processes, cysteine-type endopeptidase activity
A0A6M6A6I6	*C. procera*	CPCpD	Proteolysis involved in protein catabolic processes, cysteine-type endopeptidase activity
A0A067XG70	*C. procera*	Osmotin: antifungal laticifer protein	Defense response
A0A0K2G552	*C. procera*	Osmotin-like protein	Defense response
A0A165EL94	*C. procera*	Procerain	Proteolysis involved in protein catabolic processes, cysteine-type endopeptidase activity
A0A482E9S2	*C. procera*	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic	Acetyl-CoA carboxylase activity, carboxyl-or carbamoyl-transferase activity, ATP binding, Zn ion binding, fatty acid biosynthetic process, malonyl-CoA biosynthetic process.
A0A482EA80; A0A2S1P2V8	*C. procera*	Translation initiation factor IF-1, chloroplastic	Ribosome binding, rRNA binding, translation initiation factor activity
A0A6A6KQ46; A0A6A6KSW8; A0A6A6KV80; A0A6A6L3S4; A0A6A6LJ25	*H. brasiliensis*	Uncharacterized protein	Unknown

Table 3B: Mass spectrometric analysis of subfraction Hd-2 of HdLP

Accession Number	Plant	Protein/Peptide	Function
A0A0A0Q2K8; M9QMC9	*C. procera*	Procerain B	Cysteine-type endopeptidase activity, proteolysis involved in protein catabolic processes
A0A2S1P2U9	*C. procera*	Photosystem I assembly protein Ycf3	Photosystem I assembly
A0A6A6KVU7; A0A6A6L3A1	*H. brasiliensis*	Uncharacterized protein	Male meiosis II
A0A6A6LIG1	*H. brasiliensis*	MSP domain-containing protein	Chitin catabolic process, cell wall macromolecule catabolic process, defense response to fungus
A0A6A6LLT4	*H. brasiliensis*	Glycoside hydrolase family 19 catalytic domain-containing protein	carbohydrate metabolic process, Chitin catabolic process, cell wall macromolecule catabolic process, defense response to fungus
A0A6A6MBP6	*H. brasiliensis*	EF-hand domain-containing protein	Calcium ion binding.
A0A6A6MD41	*H. brasiliensis*	DNA helicase	ATP binding, DNA binding, 3'-5' DNA helicase activity, hydrolase activity, recombinational repair
A0A6A6MI11	*H. brasiliensis*	ANK_REP_REGION domain-containing protein	Unknown
A0A6A6MPZ7	*H. brasiliensis*	DBD_Tnp_Mut domain-containing protein	Unknown
A0A6A6MR65	*H. brasiliensis*	Matrin-type domain-containing protein	RNA binding, Zn ion binding, mRNA splicing via spliceosome.
A0A6A6MT87	*H. brasiliensis*	SBP-type domain-containing protein	DNA binding, metal ion binding.
A0A6M6A649	*C. procera*	CPCpB	Proteolysis involved in protein catabolic processes, cysteine-type endopeptidase activity
A0A6M6A6I6	*C. procera*	CPCpD	Proteolysis involved in protein catabolic processes, cysteine-type endopeptidase activity
A0A067XG70	*C. procera*	Osmotin: antifungal laticifer protein	Defense response
A0A0K2G552	*C. procera*	Osmotin like protein	Defense response
A0A165EL94	*C. procera*	Procerain	Proteolysis involved in protein catabolic processes, cysteine-type endopeptidase activity
A0A482E9S2	*C. procera*	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta, chloroplastic	Acetyl-CoA carboxylase activity, carboxyl-or carbamoyl-transferase activity, ATP binding, Zn ion binding, fatty acid biosynthetic process, malonyl-CoA biosynthetic process.
A0A482EA80; A0A2S1P2V8	*C. procera*	Translation initiation factor IF-1, chloroplastic	Ribosome binding, rRNA binding, translation initiation factor activity
A0A6A6KQ46; A0A6A6KSW8; A0A6A6KV80; A0A6A6L3S4; A0A6A6LJ25	*H. brasiliensis*	Uncharacterized protein	Unknown

4.2.3 Discussion and Conclusion

The mass spectrometry analysis of HdLP sub-fractions has revealed several important findings regarding its protein composition. Interestingly, both sub-fractions showed similar proteins, except one protein (A0A6A6LSP7), which was present only in Hd1, but in DEAE chromatography, they eluted differently, probably due to differences in protein charge, isoforms, post-translational modifications (PTMs), or slight conformational changes, affecting their interaction with the DEAE column.

Notably, proteins involved in proteolysis and defense responses, such as cysteine-type endopeptidases (Procerain B) and antifungal proteins (Osmotin), (Freitas CDT *et al*., 2007; Singh AN *et al*., 2010; Ramos MV *et al*., 2013; Molik ZA *et al*., 2025) were detected. These proteins are essential for various physiological functions, including defense mechanisms against pathogens, protein catabolism, and stress response. Furthermore, the presence of proteins like chitinase and glycoside hydrolase suggests a role in the breakdown of cell wall macromolecules, possibly indicating the plant's role in protecting from fungal infections.

The identification of proteins involved in metabolic processes, such as acetyl-CoA carboxylase, translation initiation factors, and DNA helicases, also points to potential broader biological functions of these latex proteins, including cellular energy regulation, protein synthesis, and DNA repair. Several uncharacterized proteins were detected, indicating a need for further investigation into their potential functions, which may reveal novel bioactivities.

This protein profile provides a foundation for further studies to explore the specific roles of these proteins in therapeutic applications, including their contribution to the bioactivity of latex proteins. The information gained from this analysis also paves the way for future transcriptomic and proteomic studies that could further elucidate the molecular mechanisms underlying the therapeutic effects of latex.

4.3 PART II: *In vitro* cytotoxicity assays

4.3.1 Materials and methods

In vitro assays were conducted using three distinct cell lines: RAW264.7 (macrophages), L929 (fibroblasts), and C2C12 (myoblasts), to evaluate the biological effects of HdLP and sub-fractions: Hd1 and Hd2. These cell lines were chosen for their ability to represent a broad spectrum of biological functions, allowing for a comprehensive evaluation of the potential therapeutic and cytotoxic effects of HdLP in various biological contexts.

4.3.1.1 Culturing of cells

The RAW264.7 macrophages, L929 fibroblasts, and C2C12 myoblasts were obtained from the Banco de Células do Rio de Janeiro (BCRJ), Brazil. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) High Glucose supplemented with 10% fetal bovine serum (FBS). Cells were cultured at 37°C in a humidified atmosphere with 5% CO_2. Cells were passed at approximately 80% confluency using standard trypsinization techniques and plated at an appropriate density to ensure exponential growth at the time of the assay. Cell viability was assessed using a resazurin-based assay. Resazurin is a blue, non-fluorescent dye that is reduced by viable cells to the pink-colored, fluorescent compound “resorufin”, providing a quantitative measure of cell viability.

4.3.1.2 Preparation of cell plates

Cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 100 µL of DMEM supplemented with 10% FBS. Plates were incubated overnight to allow cell attachment and recovery. The following day (after 24 h), cells were treated with serial dilutions of stock samples: HdLP (500 µg/mL), Hd-1 (50 µg/mL), and Hd-2 (500 µg/mL), prepared in DMEM with 10% FBS. A lower concentration of Hd-1 was used due to its limited yield following extraction and purification, which may reflect its

lower abundance within the latex protein fraction or partial loss during chromatographic separation.

The first dilution was prepared by mixing 50 µL of the stock solution with 50 µL of DMEM, resulting in a 1:2 dilution. Subsequent 1:2 serial dilutions were performed in the same manner to achieve a range of concentrations. The 50 µL of each diluted sample was added to the respective wells, ensuring a final volume of 100 µL per well. Control wells received 100 µL of DMEM only and no tested samples.

4.3.1.3 Resazurin assay

Following 72 hours of treatment, 10 µL of resazurin solution (0.1 mg/mL in phosphate-buffered saline) was added to each well. Plates were returned to the incubator and incubated for 3 h under standard culture conditions (37°C, 5% CO_2), to allow the viable cells to reduce resazurin to the fluorescent product, resorufin. Fluorescence was measured using a microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The fluorescence intensity is directly proportional to the number of metabolically active cells, providing a quantitative assessment of cell viability.

4.3.1.4 Data analysis

Cell viability was expressed as a percentage of the untreated control, calculated using the formula:

$$Cell\ viability\ (\%) = \left(\frac{Fluorescence\ of\ treated\ cells}{Fluorescence\ of\ control\ cells}\right) \times 100$$

4.3.1.5 Statistical analysis

Data was analyzed using One-way ANOVA to determine the significance of differences between treated and control groups. Statistical significance was set at $p<0.05$.

4.3.2 Results

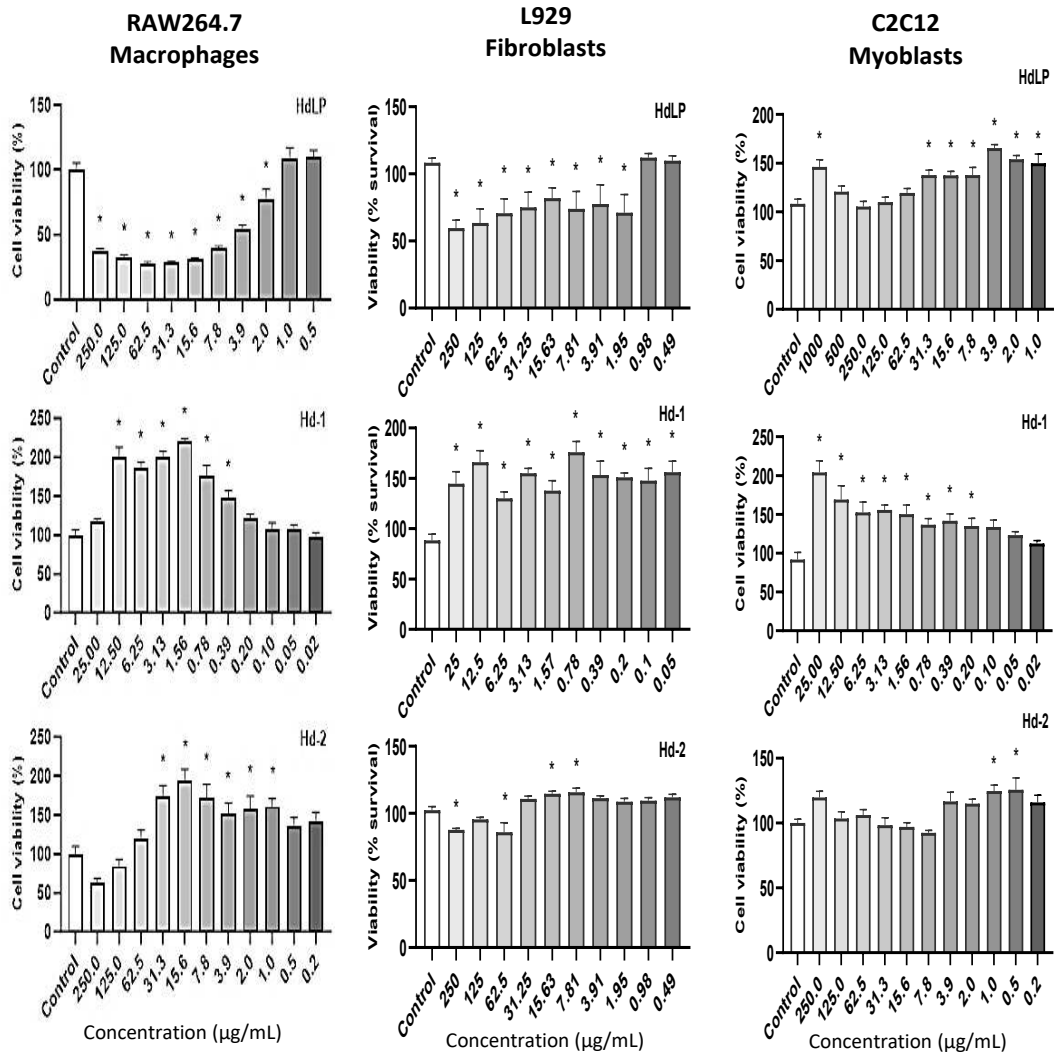
Notably, distinct concentration-dependent responses were observed across the cell lines tested. In RAW264.7 cells (Fig. 10), HdLP treatment decreased cell viability at concentrations ranging from 2 to 250 µg/mL. Conversely, Hd-1 treatment increased the viability at concentrations 0.39 to 12.5 µg/mL, with viability comparable to the control at 25 µg/mL. Interestingly, Hd-2 treatment exhibited a biphasic response, with increased viability at 1 to 31.3 µg/mL concentrations, while decreased viability at higher concentrations (125 and 250 µg/mL).

In L929 cells (Fig. 10), HdLP treatment decreased the cell viability at concentrations ranging from 1.95 to 250 µg/mL. On the other hand, Hd-1 treatment increased the viability across all concentrations tested. In contrast, Hd-2 treatment decreased viability at higher concentrations (250 and 62.5 µg/mL) but increased the viability at lower concentrations (15.63 and 7.81 µg/mL).

In C2C12 cells (Fig. 10), HdLP treatment increased cell viability at concentrations ranging from 1 to 31.3 µg/mL. Hd-1 treatment exhibited a concentration-dependent increase in viability, with enhanced viability observed at concentrations ranging from 0.2-25 µg/mL. However, Hd-2 treatment exhibited increased viability at concentrations ranging from 0.5 to 1 µg/mL.

Although Hd-1 showed no cytotoxicity within the tested concentration range, it is possible that higher concentrations could affect cell survival. The limited amount of Hd-1 obtained after purification restricted the concentration range tested; therefore, potential inhibitory or cytotoxic effects at higher doses cannot be excluded.

Figure 10: Viability of cells treated with HdLP, Hd1, and Hd2 (resazurin assay)



Source: Self-authorship

The graph illustrates fluorescence intensity readings at an excitation wavelength of 540 nm and an emission wavelength of 590 nm, reflecting viable cell populations of RAW264.7 macrophages, L929 fibroblasts, and C2C12 myoblasts, post-treated with varying concentrations of *H. drasticus* latex proteins: HdLP, Hd-1, and Hd-2. Error bars depict the standard error of the mean (SEM), with n=4 independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (*p < 0.05 vs. control)."

4.3.3 Discussion and Conclusion

The differential effects of Hd-1 and Hd-2 on cell viability indicate that variations in protein composition, abundance, and molecular interactions play a crucial role in their bioactivity. Mass spectrometry analysis revealed the presence of proteolytic enzymes such as Procerain, CPCpB, and CPCpD in both sub-fractions, which are known cysteine-type endopeptidases with potential cytotoxic effects. The biphasic response observed for Hd-2, characterized by increased viability at low concentrations and decreased viability at higher doses, suggests that these proteases may be more abundant or active in Hd-2, contributing to its cytotoxicity at elevated levels.

In contrast, Hd-1 consistently promoted cell viability, particularly in macrophages, which may be attributed to a higher relative abundance of protective proteins such as Osmotin and Osmotin-like proteins. These proteins are associated with stress tolerance, cell protection, and immune modulation, potentially contributing to the cytoprotective effects observed. The distinct biological profiles of Hd-1 and Hd-2, therefore, reflect a balance between cytotoxic and cytoprotective components within the latex protein fraction.

The overall cell viability responses to *H. drasticus* latex proteins (HdLP) and their sub-fractions align with previous findings from other members of the Apocynaceae and Euphorbiaceae families, including *Calotropis procera* and *Hevea brasiliensis*. Latex from *C. procera* has been reported to exert cytotoxic effects on various cancer cell lines, primarily due to its cysteine proteases such as procerain and procerain B, which induce cell death through proteolytic degradation of cellular components (Choedon *et al*., 2006). Conversely, *H. brasiliensis* latex promotes fibroblast proliferation and accelerates wound healing, effects attributed to protein constituents that stimulate cellular growth and tissue regeneration (Penhavel *et al*., 2016).

In the present study, HdLP reduced viability in RAW264.7 macrophages and L929 fibroblasts but enhanced viability in C2C12 myoblasts, revealing a clear cell type-dependent response. This dualistic behavior mirrors that reported for latex proteins from related species, which can display either cytotoxic or proliferative effects depending on cell type and concentration. The transcriptomic data further support

these observations, as stem tissues, the source of the latex, showed elevated expression of genes encoding enzymes such as lipoxygenase, homeobox transcription factors, and endochitinases.

These enzymes are involved in inflammation, defense, and tissue repair processes. For instance, lipoxygenases are key mediators in inflammatory pathways and have been implicated in both cancer progression and resolution (Wisastra & Dekker, 2014), potentially explaining the decrease in cell viability in certain cell types, while other proteins may promote cell proliferation and wound healing in others.

These findings emphasize that the biological effects of HdLP and its sub-fractions are both cell-type and concentration-dependent, reflecting the chemical complexity and multifunctional nature of *H. drasticus* latex proteins. The results also demonstrate the importance of protein fractionation for isolating distinct bioactive components, paving the way for the identification of specific molecules with therapeutic potential. Further studies focusing on molecular mechanisms and signaling pathways will be essential to clarify the balance between cytotoxic and cytoprotective effects and to exploit these proteins for biomedical applications.

4.4 PART III: *In vivo* assays

4.4.1 Experimental design

This experimental approach was designed to:

• *Experiment I*: evaluate the protective effects of HdLP against hyperglycemia and lethal *Salmonella* infection in an animal model.

• *Experiment II*: assess the potential hematological and histopathological impacts of HdLP, Hd1, and Hd2 in an experimental model.

***Experiment I*:** To investigate the effects of HdLP on the immune system of infected hyperglycemic animals, hyperglycemia was induced using Streptozotocin (STZ). The most effective induction protocol was selected based on hyperglycemia severity, animal well-being, and survival rates. Peripheral blood glucose levels were monitored every two days using a portable glucometer. Animals with blood glucose levels exceeding 220 mg/dL were classified as hyperglycemic and subsequently assigned to different experimental groups.

To establish the optimal bacterial concentration for an acute inflammation assay, three different concentrations (10^2, 10^4, 10^6 CFU/ mL) of *S. Typhimurium* suspension were tested in diabetic animals. The ideal concentration was identified as the lowest lethal dose capable of inducing 100% mortality within 3-5 days post-infection, providing a suitable timeframe for further analysis (Lima-Filho *et al*., 2010).

Following hyperglycemic induction, animals were assigned to 4 groups. Aside from the control group, the three different doses (30, 60, and 90 mg/kg) of HdLP were given intraperitoneally to three distinct animal groups. These treatments were administered 24 hours prior to the infection with the optimal bacterial dose selected for inducing infection.

***Experiment II*:** To evaluate the potential hematological and histological effects, particularly related to the immune system, animals were randomly assigned to four groups: a control group and three experimental groups, each receiving a different protein treatment. The three experimental groups were given a single intraperitoneal

dose (30 mg/kg) of HdLP, Hd1, or Hd2 while the control group was given the vehicle solution only. After five days of treatment, the animals were euthanized for blood and tissue analysis.

4.4.2 Materials and methods

4.4.2.1 Animals

Specific pathogen-free (SPF) Swiss male mice (7-8 weeks old) were provided by the Central Animal House of the Federal University of Ceara, Brazil. The mice were housed under standard laboratory conditions throughout the study period with a 12-h light/dark cycle, controlled air temperature (25°C), and free access to fresh water and standard food (obtained from Purina, Paulínia, SP, Brazil). All animal experiments were conducted in compliance with the standards of the National Council for the Control of Animal Experimentation (CONCEA), after approval by the Ethics Committee for Animal Experimentation of the Federal University of Ceará, under protocol number: 2742280720, as well as per international rules for Animal Experimentation.

4.4.2.2 Experiment I

4.4.2.2.1 Induction of hyperglycemia

The animals were made hyperglycemic using streptozotocin (STZ), Sigma-Aldrich®, St. Louis, MO, USA. STZ was freshly dissolved in a citrate buffer (0.1 M, pH 4.5) and administered intraperitoneally. Mice received five consecutive daily injections of STZ at a dose of 40 mg/kg body weight after 4 h fasting. Blood glucose levels were monitored using a glucometer, with levels above 200 mg/dL indicating successful hyperglycemia induction. The animals (~20%) that did not develop hyperglycemia were euthanized under anesthesia by exsanguination, per standard procedure approved by the ethical committee.

4.4.2.2.2 Treatment with HdLP

Eleven days after the last STZ injection, hyperglycemic mice were randomly divided into four groups (6 animals per group), each group receiving a different treatment regimen. HdLP was administered intraperitoneally at doses of 30, 60, and 90 mg/kg body weight. Mice were treated once with HdLP. The control group received a vehicle (PBS) only. After 24 h of HdLP administration, the infection was induced in

animals by using OBC (i.p) of *Salmonella* determined earlier. The animals were observed for 7 days.

4.4.2.2.3 Induction of infection

*Salmonella enterica serovar Typhimurium (*genus *Salmonella):* a rod-shaped, flagellate, facultative anaerobic, and gram-negative bacterium, is a primary enteric pathogen infecting both humans and animals. To develop systemic infection in the current study model, *S. Typhimurium* (strain C5) was used, provided by Dr. Pietro Mastroeni from the University of Cambridge, England. The bacteria taken from *Salmonella-Shigella* media (SS media) were activated in Brain Heart Infusion (BHI) broth (37°C for 18 h) and then cultured in BHI agar for another 24 h at 37°C.

After 24 h of the final HdLP treatment, diabetic mice were challenged with a lethal dose of *S. Typhimurium*. Bacteria were cultured in BHI agar overnight, and the bacterial concentration was adjusted to 10^6 CFU/mL. Diabetic mice were infected intraperitoneally with 10^6 CFU/mL of *S. Typhimurium* in 100 µL of phosphate-buffered saline (PBS).

4.4.2.2.4 Survival analysis

Following infection, hyperglycemic mice were monitored for survival over seven days. Daily observations were conducted to assess the signs of morbidity and mortality, including weight loss, lethargy, and behavioral changes. The time of death for each animal was recorded, and survival rates were plotted as Kaplan-Meier survival curves.

4.4.2.2.5 Statistical analysis

Survival data were analyzed using the log-rank test to compare survival curves between different treatment groups. A p-value of <0.05 was considered statistically significant.

4.4.2.3 Experiment II

The mice were treated with HdLP, Hd1, and Hd2 to assess their effects on hematological parameters and tissue histology of experimental animals, with a particular *focus* on immune-related responses. HdLP, Hd1, and Hd2 were administered intraperitoneally at a dose of 30 mg/kg body weight once, while the control group received the vehicle solution only.

4.4.2.3.1 Hematology and Histology

Blood samples were collected from mice after five days of treatment with HdLP, Hd1, and Hd2. Blood was obtained via retro-orbital puncture under anesthesia, a standard procedure by the ethical committee.

After blood collection, mice were euthanized, and tissues (pancreas, liver, spleen, kidneys, and thymus) were harvested for histological analysis. Tissues were fixed in 10% formalin, embedded in paraffin, sectioned at 5 µm, and stained with hematoxylin and eosin (H&E). Sections were examined under a light microscope for histological changes.

4.4.3 Results

4.4.3.1 Results of Experiment I

Hyperglycemia was successfully induced in mice using five doses of STZ (40 mg/kg). Average peripheral blood glycemia was higher than 220 mg/dL two days after the last STZ injection. After 10 days, more than 70% of the animals showed stable levels of blood sugar higher than the selected threshold. The treatments did not change the survivability of animals; all treated animals succumbed to the infection within 3 to 5 days (Fig. 11).

Mice treated with 60 mg/kg survived one day longer than those treated with 30 and 90 mg/kg, although there was no statistically significant difference in survival rates between the two dosages. For this reason, and to save resources and time, their body samples were not analyzed in this study. The possible reasons behind the death of all animals can be one of the following:

- The tested proteins have no effect against hyperglycemia and infection,
- They may show some effect at higher doses,
- The selected animal model was not suitable for this kind of study.

To understand more precisely the reason behind this response, further studies are required using higher protein doses and different variants of mice.

4.4.3.2 Results of Experiment II

I. Hematological Analysis

Hematological analysis revealed significant alterations in mice treated with *H. drasticus* latex protein fractions HdLP, Hd-1, and Hd-2 compared to the control group (Table 4).

White blood cell (WBC) count was significantly decreased in HdLP-treated mice ($1.67 \pm 0.03 \times 10^6$ cells/μL), Hd-1-treated mice ($2.6 \pm 0.27 \times 10^6$ cells/μL), and Hd-2-treated mice ($2.88 \pm 0.41 \times 10^6$ cells/μL) compared to controls ($6.88 \pm 0.58 \times 10^6$ cells/μL). Differential leukocyte counts revealed a significant decrease in lymphocytes (LYM) in all treatment groups compared to controls. Platelet count (PLA) was significantly increased in HdLP-treated mice ($747.67 \pm 62.83 \times 10^3$ cells/μL), Hd-1-

treated mice (934.75 ± 83.86 × 10^3 cells/μL), and Hd-2-treated mice (955.25 ± 14.29 × 10^3 cells/μL) compared to controls (630.67 ± 62.83 × 10^3 cells/μL).

Furthermore, plasma protein (PRO) levels were significantly decreased in HdLP-treated mice (4.47 ± 0.07 g/dL), Hd-1-treated mice (4.40 ± 0.52 g/dL), and Hd-2-treated mice (4.55 ± 0.15 g/dL) compared to controls (6 ± 0.23 g/dL).

These results indicate potential systemic effects of *Himatanthus drasticus* latex protein fractions, Hd-1, and Hd-2 on hematopoiesis and immune function in mice, warranting further investigation into their underlying mechanisms and potential clinical implications.

II. Histological Analysis

Histological examination of liver tissue revealed mild degeneration in animals treated with HdLP, as well as in those treated with Hd-1 and Hd-2 (Fig**.** 12). Notably, animals administered Hd-2 exhibited focal inflammation in addition to degenerative changes.

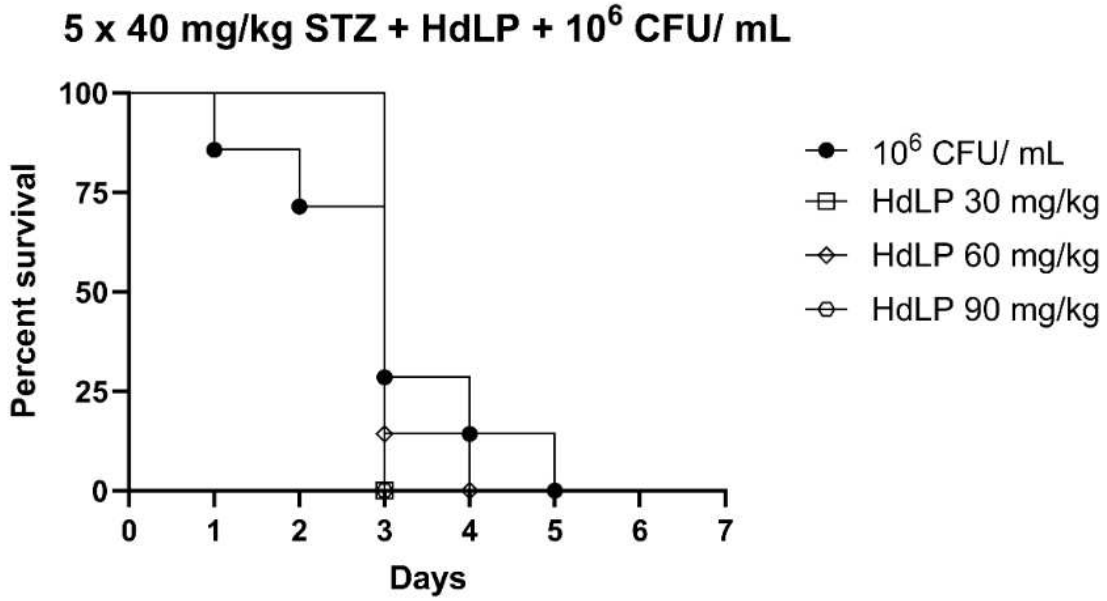
In contrast, histological analysis of spleen specimens indicated light lymphoid depletion in animals treated with HdLP, while those receiving Hd-1 displayed megakaryocytosis (Fig. 13). Interestingly, no histological abnormalities were observed in the pancreas, kidneys, or thymus across all treatment groups.

These findings suggest a potential hepatotoxic effect associated with the administration of HdLP and its fractions, characterized by mild degenerative alterations in the liver, while Hd-2 treatment additionally elicited focal inflammatory responses.

Conversely, effects on the spleen varied between treatments, with HdLP inducing lymphoid depletion and Hd-1 promoting megakaryocytosis, indicative of distinct immunomodulatory effects. The absence of histological findings in the pancreas, kidneys, and thymus suggests minimal systemic toxicity under the conditions tested.

Further investigations are warranted to elucidate the underlying mechanisms driving these observed histological changes and their implications for the overall safety profile of *H. drasticus* latex protein fractions.

Figure 11: Percent survival of diabetic mice treated with HdLP and subjected to lethal *Salmonella* infection.



Source: Self-authorship

Hyperglycemia was induced with 5 doses of 40 mg/kg of STZ. After successful induction, animals were treated with three different concentrations of HdLP (30, 60, 90 mg/kg), followed by infection with 10^6 CFU/mL of *Salmonella* Typhimurium suspension. Animals were observed for up to 7 days after infection. Survival rates were plotted, and differences were calculated via the log-rank test ($p < 0.05$).

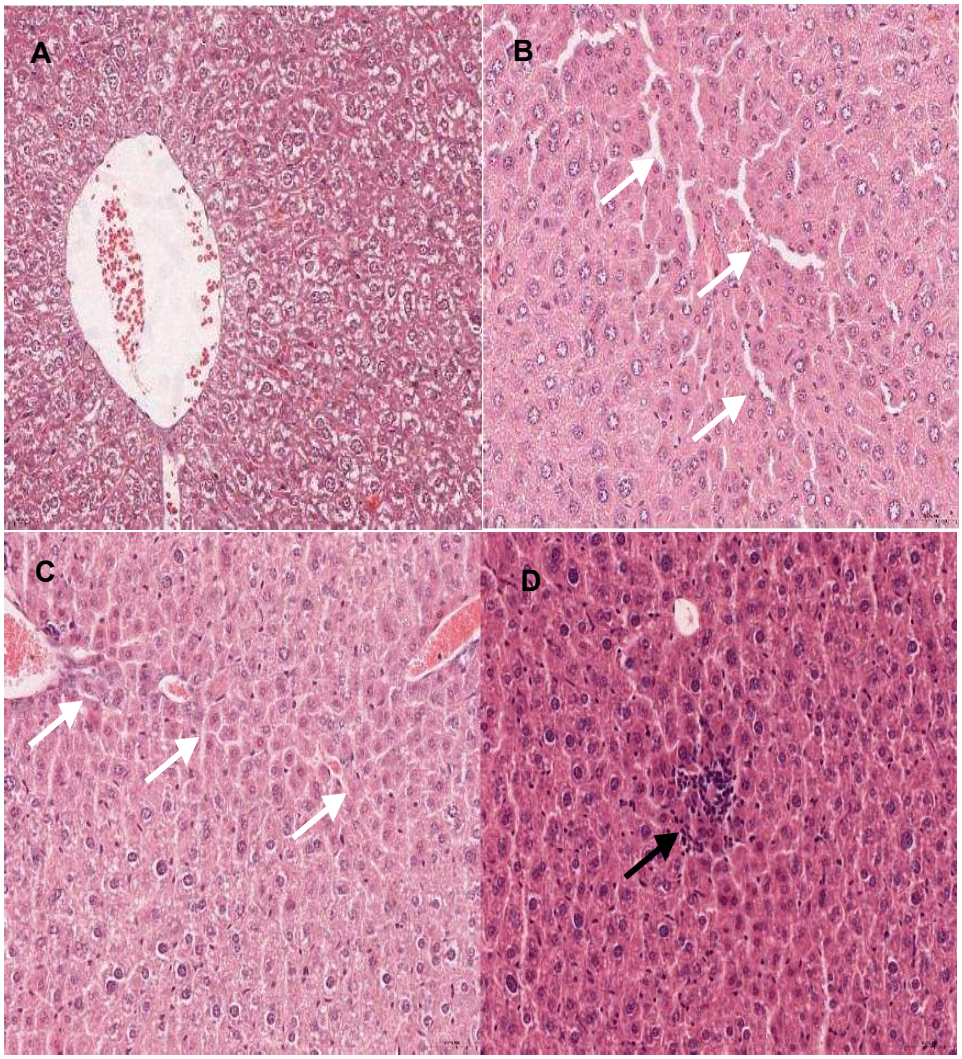
Table 4: Hematological analysis of mice following treatment with 30 mg/kg of *H. drasticus* latex protein fractions.

Parameters	Units	Control	HdLP	Hd-1	Hd-2
RBC	$\times 10^6$ cells/ uL	8.59 ± 0.22	9.92 ± 0.11	9 ± 0.67	8.98 ± 0.21
HB	g/ L	137.29 ± 3.16	154 ± 3.46	137 ± 7.99	137.75 ± 3.33
HT	%	40.95 ± 1.16	46 ± 1.15	42.5 ± 1.94	42.25 ± 0.58
MCV	μm^3	47.67 ± 0.59	46.33 ± 0.71	47.45 ± 1.56	47.08 ± 1.14
MCHC	%	33.59 ± 1.12	33.43 ± 0.09	32.15 ± 0.51	32.55 ± 0.65
WBC	$\times 10^6$ cells/ uL	6.88 ± 0.58	1.67 ± 0.03*	2.6 ± 0.27*	2.88 ± 0.41*
ROD	$\times 10^6$ cells/ uL	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEG	$\times 10^6$ cells/ uL	0.51 ± 0.14	0.17 ± 0.04	0.82 ± 0.15	1.04 ± 0.28
LYM	$\times 10^6$ cells/ uL	5.23 ± 0.25	1.46 ± 0.08*	1.73 ± 0.15*	1.68 ± 0.23*
EOS	$\times 10^6$ cells/ uL	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01	0.03 ± 0.02
BAS	$\times 10^6$ cells/ uL	0 ± 0	0 ± 0	0 ± 0	0 ± 0
MON	$\times 10^6$ cells/ uL	0.01 ± 0.01	0.01 ± 0.01	0.04 ± 0.02	0.08 ± 0.04
PLA	$\times 10^3$ cells/ uL	630.67 ± 62.83	747.67 ± 62.83	934.75 ± 83.86	955.25 ± 14.29*
PRO	g/ dL	6 ± 0.23	4.47 ± 0.07*	4.40 ± 0.52*	4.55 0.15*

Source: Self-authorship

Hematological parameters of mice treated with 30 mg/kg of *H. drasticus* latex protein fractions. The table presents hematological parameters including RBC: Red Blood Cells; HB: Hemoglobin; HT: Hematocrit; MCV: Mean Corpuscular Volume; MCHC: Mean Corpuscular Hemoglobin Concentration; WBC: White Blood Cells; ROD: Rod Cells; SEG: Segmented Neutrophils; LYM: Lymphocytes; EOS: Eosinophils; BAS: Basophils; MON: Monocytes; PLA: Platelets; PRO: Proteins. Data are expressed as mean ± standard error (SEM) of three independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test (*$p < 0.05$ vs. control).

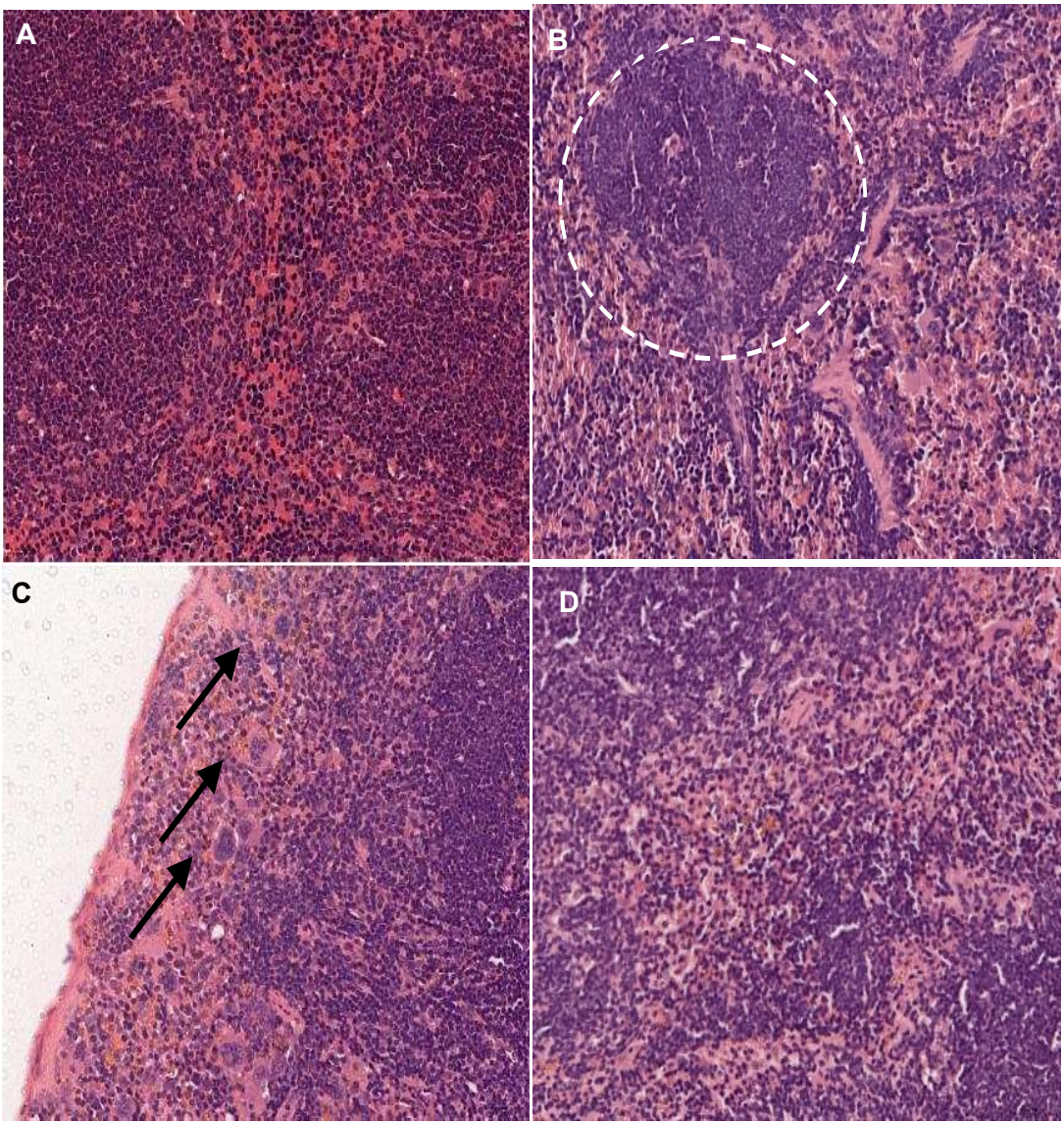
Figure 12: Histological analysis of liver tissue from mice treated with *H. drasticus* latex protein fractions.



Source: Self-authorship

Liver histology of mice treated with *H. drasticus* latex protein fractions. Representative histological images of liver tissue from mice treated with (A)Control, (B) HdLP, (C) Hd-1, and (D) Hd-2. Animals treated with HdLP, Hd-1, and Hd-2 exhibit mild degeneration (white arrow), while Hd-2 treatment also shows focal inflammation (black arrow). Hematoxylin and eosin staining, magnification 40x.

Figure 13: Histological analysis of spleen tissue from mice treated with H. drasticus latex protein fractions.



Source: Self-authorship

Spleen histology of mice treated with *H. drasticus* latex protein fractions. Representative histological images of spleen tissue from mice treated with (A) Control, (B) HdLP, (C) Hd-1, and (D) Hd-2. HdLP-treated animals show light lymphoid depletion (white line), while Hd-1 treatment results in megakaryocytosis (black arrow): Hematoxylin and eosin staining, magnification 40x.

4.4.4 Discussion and Conclusion

The first study highlighting the pharmacological potential of latex proteins reported their anti-inflammatory effects in three different animal models (Alencar, 2004). Since then, latex proteins have been associated with the modulation of various clinically relevant inflammatory processes, including severe bacterial infections. Additionally, they have been shown to mitigate the side effects of antimetabolic drugs used in cancer therapy (Alencar *et al*., 2017).

Among these proteins, proteases and osmotin have emerged as key candidates in promoting physiological homeostasis in animals experiencing metabolic or physiological disturbances.

The benefits of *H. drasticus* latex for treating diabetes have been widely claimed locally, but no consolidated information or scientific evidence supports it.

A recent study by Morais FS *et al*. (2020) demonstrated the inhibitory effect of *H. drasticus* latex on enzymes associated with type 2 diabetes, indicating its potential anti-diabetic properties. This activity was attributed to secondary metabolites, particularly plumieride, found in the hydroalcoholic fraction (FHDHA) of latex. Several other studies have explored the pharmacological properties of *H. drasticus* latex, particularly its anti-inflammatory and wound-healing effects. For instance, research has demonstrated that the latex of *H. drasticus* accelerates the healing process of excisional wounds by modulating the inflammatory phase and promoting complete re-epithelialization (Souza TF *et al*., 2023). Studies by Matos *et al*., (2013) and Santos GJL *et al*., (2018) have also confirmed the potential immunomodulatory effects of *H. drasticus* latex and its derivatives. However, direct studies on the effects of this latex specifically in diabetic and infected animal models remain limited.

Latex is a highly complex substance containing soluble molecules, subcellular structures, and suspended rubber particles, which readily precipitate in aqueous media. Its soluble phase exhibits a diverse molecular composition that varies significantly among species. While latex possesses both beneficial pharmacological properties and potential toxic effects, fractionation is crucial for isolating toxic compounds and chemically investigating specific fractions. This approach enhances our understanding of latex compounds' biological activities and ensures safer use in folk medicine, regardless of the intended curative purpose.

In our study, although multiple low-dose Streptozotocin (STZ) injections successfully induced hyperglycemia (Experiment I), the rubber-free protein fraction (HdLP) from *H. drasticus* latex did not improve survival outcomes or alleviate hyperglycemia in infected hyperglycemic animals. However, in experiment II, hematological and histological analyses of healthy animals indicated that HdLP and its sub-fractions may possess immunomodulatory properties.

Several studies support the immunomodulatory properties of HdLP, however, there is no evidence to date supporting its hypoglycemic effects.

According to the MS results of HdLP characterization in this study, both its sub-fractions contain proteins similar to the latex proteins of *C. procera* and *H. brasiliensis*. While the latex proteins of these plants have shown positive effects in hyperglycemia and infection models (Araújo LA *et al*., 2018; Oliveira KA *et al*., 2019; Sousa BF *et al*., 2020; Oliveira KA *et al*., 2021; Tavares LS *et al.,* 2021), HdLP did not exhibit similar effects.

The lack of hypoglycemic and infection-modulating effects in HdLP, despite containing proteins similar to those found in *C. procera* and *H. brasiliensis*, could be due to differences in protein composition, post-translational modifications, or structural variations that alter their biological activity. Post-translational modifications, such as glycosylation or phosphorylation, can significantly influence protein function, potentially enhancing or diminishing their bioactivity. Additionally, latex is a complex mixture of proteins, enzymes, and secondary metabolites that may act synergistically or antagonistically. HdLP may lack synergistic interactions between key bioactive proteins or contain inhibitory factors that counteract potential therapeutic effects. The protective effects observed in *C. procera* and *H. brasiliensis* could be due to the presence of co-factors or stabilizing molecules that enhance protein function, which might be absent or less effective in *H. drasticus* latex. Furthermore, the differences in experimental models, dosage, and the physiological response of test animals could also account for the variations in therapeutic efficacy.

Further research is necessary to explore these aspects, optimal dosing strategies, alternative animal models, and the identification of active compounds within the latex that may confer protective effects against diabetes and infection. These future investigations will be crucial in determining whether *H. drasticus* latex and HdLP can be effectively utilized in diabetes-related infections and immune modulation.

5 STUDY RESTRICTIONS

The study presents several limitations that should be addressed in future research.

- There is currently no accurate method to effectively purify HdLP from the latex of *H. drasticus*, as the presence of impurities hampers the protein extraction process, leading to a significantly reduced yield of proteins after multiple elutions.

To resolve this, future approaches could focus on optimizing the purification process by improving the separation of impurities from the desired proteins. This could involve refining the extraction conditions to enhance selectivity and efficiency, exploring alternative techniques to target specific proteins, and minimizing the loss of protein yield. Additionally, further investigation into the biochemical properties of the latex and proteins may reveal better strategies for isolating HdLP with higher purity and quantity.

- Regarding protein dosing, the doses used may not be optimal for the mice's physiology, leading to rapid mortality following *Salmonella* infection.

To address this, conducting a dose-response experiment could help determine the threshold that maximizes immune response without overwhelming the animals.

- The *Salmonella* infection model may be too harsh for hyperglycemic mice, leading to quick mortality due to their compromised immune systems.

Using a less virulent strain of *Salmonella* or choosing a different pathogen with a more gradual disease progression could provide a better window for the immune response to develop.

- The use of Specific Pathogen-Free (SPF) *Swiss* male mice in this study appears to be an unsuitable model for investigating the protective effects of *H. drasticus* latex proteins against infection in hyperglycemic conditions. SPF mice are bred in highly controlled environments, resulting in underdeveloped immune systems with limited exposure to pathogens. This immunological naivety may render them more vulnerable to infections, particularly in a hyperglycemic state where immune function is already compromised. The rapid mortality observed post-*Salmonella* infection suggests that these mice lacked the necessary immune resilience to withstand the

infection, making it difficult to assess the potential protective effects of the tested proteins.

As an alternative, I propose using outbred or conventionally raised mice, such as CD-1 or non-SPF Swiss mice, which have more diverse microbiomes and robust immune responses due to natural exposure to environmental antigens.

- Finally, the variability in protein composition, particularly the concentration of cysteine peptidases in the HdLP fraction, may limit its effectiveness, as previous studies have linked the hypoglycemic and immune-boosting effects of latex proteins to the presence of these enzymes.

Modifying protein extraction methods, such as using affinity chromatography specifically targeting peptidases, or conducting enzyme-specific assays to measure peptidase content more accurately, could improve the purity and therapeutic potential of the protein.

These adjustments could help overcome the current limitations and refine the study for more reliable results.

6 GENERAL CONCLUSION

In conclusion, this study showed that *Himatanthus drasticus* latex proteins may not be effective for improving hyperglycemia or combating infections, but they exhibit significant immunomodulatory effects. Overall, the findings support the potential use of *H. drasticus* latex proteins in wound healing and inflammation models. However, further research is needed to isolate the active components and better understand their mechanisms of action for more targeted therapeutic applications.

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APPENDIX A

Supplementary file 1
https://drive.google.com/file/d/1DjDiOVyWDjnGywn0RsczztJFF_j_wy5b/view?usp=drive_link

Supplementary file 2
https://drive.google.com/file/d/1jZmEc5eGUWlygC5mSH3Syx0f8bCzDaH/view?usp=drive_link

Supplementary file 3
https://drive.google.com/file/d/1lLw12fpJA-UosFOyRWsROeaI_ap3mIR/view?usp=drive_link

Supplementary file 4 (for leaves)
https://drive.google.com/drive/u/1/folders/1ZT2PSlcSI1mtlJUdcozndpttgtD6U8cC

Supplementary file 5 (for stem)
https://drive.google.com/drive/u/1/folders/1ZT2PSlcSI1mtlJUdcozndpttgtD6U8cC

Supplementary file 6
https://drive.google.com/drive/u/1/folders/1wuuOcWJHY8dl3gkC4gkbcDtM0QziwHUU