METABOLITE PROFILING OF SWEET ALOES (*EUPHORBIA NERIIFOLIA*) AND POTENTIAL ROLES IN TRADITIONAL ASTHMA THERAPY

A thesis submitted to the committee of Graduate Studies in partial fulfillment of the requirements for the Master of Arts Degree in the Faculty of Arts and Science

TRENT UNIVERSITY
Peterborough Campus, Ontario, Canada

Sustainability Studies M.A Graduate Programme
May 2023

ABSTRACT

METABOLITE PROFILING OF SWEET ALOES (*EUPHORBIA NERIIFOLIA*) AND POTENTIAL ROLES IN TRADITIONAL ASTHMA THERAPY

by Malaika Persaud

Heated leaf extracts of *Euphorbia neriifolia* (sweet aloes) are used traditionally in Guyana for asthma therapy, but the phytoconstituents have not been studied and phytohormones are generally unexplored in *Euphorbia* species. Phytoconstituents in asthma-effective traditional heated extracts were analyzed and compared with unheated leaves and latex extracts as screens to identify candidate compounds for asthma treatment. Analysis employed untargeted and targeted mass spectrometry-based metabolomics. The untargeted approach revealed thousands of features in samples with higher abundance in heated extracts and confirmation of 33 metabolite identities (confidence levels 2 and 3) which are known to have antioxidant and anti-inflammatory properties. Highly accumulated and unique features were detected in latex, and those characteristic compounds may reflect the expanded therapeutic uses of *E. neriifolia* globally. A targeted metabolomic analysis identified and quantified several cytokinins, including aromatic kinetin, and acidic phytohormones. Traditional heating improved the content of several cytokinins with known therapeutic potential, indicating they may be candidates for asthma management.

Keywords: *Euphorbia neriifolia*, sweet aloes, asthma, cytokinins, metabolomics, hormonomics, phytochemicals, phytoconstituents, phytohormones, traditional remedies, secondary metabolites.

PREFACE

Natural products were the primary source of medication growing up with a grandmother of indigenous background. Her knowledge of plants and concoctions which she used for healing purposes, oftentimes for illnesses that primary healthcare services failed to render aid was intriguing. My interest was especially piqued by the treasured prickly cactus-like plant that occupied a prominent position at our home. It was a plant known only by its vernacular name from knowledge passed on from her father, a man from an indigenous tribe in the North-West District of Guyana. Although I wanted to explore further, not much information was available at that time. It would be long before the scientific name was discovered, nevertheless care for the plant was always emphasized. This study is dedicated to the memory of my mother and grandmother who encouraged the use of this natural product over the years.

ACKNOWLEDGMENTS

Firstly, thanks to the Almighty for the will and strength to persevere. With sincerest gratitude, I acknowledge my co-supervisors Dr. Emery and Dr. Narine. I am eternally grateful to Dr. Neil Emery for his constant infectious positivity and whose extensive knowledge of plant biology and guidance contributed significantly to the advancement of this work. The invaluable support of Dr. Suresh Narine and his work with CGX-FEC, the University of Guyana, and Trent University to bring the program to fruition is applauded. I respect you both greatly.

To my colleagues and members of the Emery Lab, I extend heartfelt appreciation. Each of you has been a gem on this journey. Special thanks to Peter Andreas, Kimberly Molina Bean, Vedanti Ghatwala, Zeynab Azimychetabi, Dr. Ainsely Lewis, and Dr. Anna Kisiala. For external lab support, thanks to Rob Hayman, the Guyana team, Dr. Laziz Bouzidi, and Dr. Tamanna Sultana.

Finally, the consideration, patience, and love of my family cannot be overlooked. You are my rock!

Sincerest thanks to you all!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

SUMMARY OF ABBREVIATIONS

ABA	Abscisic acid
AR	Aromatics
BA	N6-benzyladenine
BAR	N^6-benzyladenosine
CK	Cytokinins
cZ	cis-Zeatin
cZR	cis-Zeatin riboside
DZ	Dihydrozeatin
DZR	Dihydrozeatin riboside
EIC	Extracted ion chromatogram
FB	Free base form of cytokinin
GA	Gibberellic acid
GLUC	Glucosides
IAA	Indole-3-acetic acid
iP	N^6-isopentenyladenine
iPR	N^6-isopentenyladenosine
IS	Internal standard
JA	Jasmonic acid
MeS	Methylthiolated form of cytokinins
m/z	Mass to charge value
ng	Nanograms
NT	Nucleotide forms of cytokinins
O-G	O-glucoside forms of cytokinins
LE	Latex extracts
PRM	Parallel reaction monitoring
R	Ribosides

RB	Riboside fraction
SA	Salicylic acid
SE	Simple extract
SPE	Solid phase extraction
TE	Traditional extract; extract from heated leaf; heated extract
tZ	trans-zeatin
tZR	trans-zeatin riboside

GLOSSARY

Cytokines — Small proteins produced by various cell types in animals with a crucial role in helping to regulate the immune system, inflammation, and response to injuries or infections. Dysregulation of cytokines can contribute to diseases, including autoimmune diseases, inflammatory disorders and cancer. Cytokines are structurally and functionally unrelated, but they are often incorrectly used in literature in reference to cytokinins.

Cytokinins — A class of “plant” hormones or phytohormones that are involved in regulating growth and development processes. Cytokinins have over time been detected in all life forms including mammal systems and have effects on them regardless of whether they are endogenously produced.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Natural products and asthma

Asthma is a chronic non-communicable respiratory disease that affects millions of people worldwide with a high prevalence in children (Asher & Pearce, 2014; Stoodley et al., 2019). Complex interactions between various mediators; like cytokines and chemokines, and cell types including mast cells and eosinophils, result in airway hyperactivity characterized by bronchoconstriction or increased inflammation (Polosa & Blackburn, 2009; Sahiner et al., 2011; Sawale et al., 2017). Oxidative stress and reactive oxygen species in addition to the cytokine triggers are commonly implicated (Lambrecht et al., 2019; Michaeloudes et al., 2022; Sahiner et al., 2011). The characteristic symptoms of asthma are cough, wheezing, shortness of breath, and tightness in the chest (Stoodley et al., 2019) for which natural products have been traditionally used to ameliorate (Q. Li et al., 2020).

The etiology of asthma remains unknown as evidence indicates complexity (Polosa & Blackburn, 2009) with theories ranging from environmental to genetics and more recent gender implications with hormonal factors (Asher & Pearce, 2014; Bulkhi et al., 2020). Asthma guidelines recommend consistent medical follow-up to devise the best therapeutic outcomes as often cases are uncontrolled. Current therapies include multi-drug use of short-acting beta-agonists (SABAs), and long-acting beta-agonists (LABAs) in addition to inhaled corticosteroids (ICS) (Chipps et al., 2022; Horak et al., 2016). Treatment with these drugs is laden with side effects that pose challenges generally and with

children as numerous organ systems are affected that may be irreversible upon discontinuing medication use (Buttgereit et al., 2005). Hypertension, glaucoma, neuropsychiatric disorders, osteoporosis, aseptic necrosis of the femur, myopathies and skin thinning are some issues reported (Buttgereit et al., 2005). Moreover, the condition is uncontrolled in many patients. Therefore, exploration of safer alternatives that are potentially sourced from natural products of indigenous and local cultures is warranted.

Natural products, used for centuries to cure diseases, could be considered the cornerstone of modern pharmaceutical development. In fact, synthetic analogs for therapeutic equivalent drugs to approximately 121 natural products have not been forthcoming (Thorat & Bolli, 2017). Consequently, some natural drugs crossed the realm of alternative medicine to acceptance into the conventional medicine arena. The extraction of the cardiac glycoside, digoxin from foxglove in 1930, almost two centuries after its first recorded use by the physician William Withering in Europe (Scalese & Salvatore, 2017), was a breakthrough for congestive heart failure treatment. Another example is the major drug used in chronic venous insufficiency, Daflon (Ramelet, 2001), which constitutes the purified flavonoid fraction from the plant extract from the epicarp of *citrus aurantium var amara*. This supports the importance of the study of natural products and conservation of knowledge prevalent in Indigenous and local cultures for treating diseases. In light of the gravity of asthma, there is a necessity for the study of plants with professedly anti-asthmatic properties. Some studies indicate *Adianthum aethipiocum, Acalypha indica, Euphorbia hirta* and

Euphorbia tirucalli (Dogra et al., 2015) among plants used for asthma but do not include *E. neriifolia*. Some studies show *E. neriifolia* use in other territories (Mali & Panchal, 2017; Sawale et al., 2017). Sweet aloes (*Euphorbia neriifolia)* from Guyana are used traditionally to treat asthma in babies and children (DeFilipps et al., 2004) with purportedly no side effects, but no local studies are known that evaluate its constituents, efficacy or safety.

1.2 Application of Indigenous Knowledge to Asthma Treatment

Western Scientific methods involve testing and validation while Indigenous Knowledge represents another way of seeing the world through ages of observation and practice (Mazzocchi, 2006). The Indigenous peoples extended their interaction with the natural environment and learned through time in a manner that could be compared to Western knowledge. Together, both methods work to improve healthcare. Traditional remedies and folklore medicine in Guyana may be attributed to the fusion of beliefs and practices of Amerindian people (otherwise termed the Native or Indigenous people of Guyana) with the culture of enslaved Africans, or East Indians (who arrived as indentured labourers from India), and perhaps a little influence from other ethnic groups that form the multicultural society that came about following colonialism. Whatever the origin, numerous remedies exist for medical conditions. These include the use of sweet aloes or *E. neriifolia*, for skin conditions and asthma (DeFilipps et al., 2004; Grimmond et al., 2002; West et al., 2018).

The use of sweet aloes was observed mainly from Indigenous people among whom the passage of knowledge has reportedly been through oral means

over generations. Sweet aloes, typically grown in the North-West District of Guyana (Van Andel, 2000) present a unique case as they are grown in a limited area. The latex exuded from the plant is used to treat skin conditions while the extract from the heated leaves of the species is traditionally used to treat wheezing and asthma in babies and children (DeFilipps et al., 2004; Deborah A Lachman-White et al., 1992). The decades-old local literature presents scanty information on the species in Guyana. In contrast, reported uses of *Euphorbia* species over centuries include a plethora of pharmacological actions in other countries. The medicinal properties of *E. neriifolia,* known globally include analgesic, anemia correction, anti-asthmatic, anti-convulsant, anti-diabetic, anti-inflammatory, anti-microbial, anti-plasmodial, anti-oxidant, anti-tumor, bronchitis, cholesterol-lowering, contraceptive, coughs, emetic, pneumonia and as poisons or anti-venom (Ernst et al., 2015; Mali & Panchal, 2017; Sultana et al., 2022; Thorat & Bolli, 2017).

For treating asthma, the traditional remedy made by the indigenous people in Guyana involves heating the leaves of *E. neriifolia* for a short time over a flame or on a hot plate typically at temperatures which would be approximately above 200 degrees Celsius. The liquid is squeezed from the leaf and a pinch of salt is then added to the expressed liquid (Deborah A Lachman-White et al., 1992). An estimated amount of approximately one-quarter of a teaspoon is given to infants and increased in older children. In contrast to other cultures like India (Chaudhary et al., 2023) where milky latex is used, only the traditional extract form from *E. neriifolia* is used for asthma in Guyana. Moreover, mainly the leaves are reported

as used in therapies that include nail infections, fevers, and diabetes (Van Andel, 2000) but, the form of application for these conditions is unclear.

The use of heated leaves in Guyana for the traditional treatment of asthma was practiced for at least a century. This was observed from the fact that this remedy was passed through generations from my great-grandfather of an indigenous tribe in the North-West District to my grandmother born in 1929. Yet the chemical basis remained unexplored resulting in little knowledge of the phytoconstituents in the local species and scientific validation. While literature implies the use of latex, though purported to be toxic (Salehi et al., 2019) few reports of phytoconstituents exist (Benjamaa et al., 2022) as it relates to asthma. The species was found to be one of the less researched of the genus (Ernst et al., 2015). Additionally, global research has yet to focus on compounds, including phytohormones, which may hold therapeutic potential for asthma. Furthermore, limited phytochemical studies on traditional extract of *E. neriifolia* in its heated form to derive constituents exist. Antimicrobial and anthelmintic activity studies were conducted on extracts from heated leaves (Raghuwanshi et al., 2013), but most phytochemical studies to derive the constituents were qualitative and untargeted. To address the mentioned gaps, I studied sweet aloes using the heated, traditional remedy and compared it to extracts from unheated leaves and latex, to obtain a general and targeted chemical profile that may relate to possible efficacious application for the therapy of asthma.

Many parts of the plant are used including roots for snake bites, scorpion stings and as antispasmodics (Mali & Panchal, 2017). Some uses of the sample

types to be investigated in this study are shown in Table 1. The application of *E. neriifolia* has been shown to be extensive as its use covers many systems and conditions in other cultures. The list presents a snippet of the potential of the species reported globally.

Table 1: Euphorbia sample types and their traditional uses

Sample Type	Definitions	Source	Uses in Guyana	Uses in other countries	Source
Simple Leaf Extract (SE)	Content extracted from leaf tissue at normal temperature	Leaf	No known use	Earaches, wounds, CNS depressant, aphrodisiac, adjunct in Covid 19 treatment	(Pramanik et al., 2022; Sultana et al., 2022)
Traditional Extract (TE)	Clear, light yellowish liquid obtained from squeezing heated leaves in a process that mimics the traditional mode of preparation of the remedy	Leaf	Wheezing, asthma, coughs, colds	Earache, epilepsy	(Deborah A Lachman-White et al., 1992; Sawale et al., 2017; Sultana et al., 2022)
Latex Extract (LE)	Milky white exudate from damaged plant parts, broken leaves, and stem	Latex	Rashes	Asthma, bronchitis, syphilis, leprosy, gonorrhea, rashes, tumors, healing cracks on soles of feet.	(Mali & Panchal, 2017; Sawale et al., 2017; Sultana et al., 2022)

1.3 Sweet Aloes

1.3.1 Biological Classification

E. neriifolia (sweet aloes) belongs to the Euphorbia genus of the Euphorbiaceae family. It is known globally by names like snuhi, thohar, som chao, Dog's Tongue and Indian Spurge among others (Sultana et al., 2022). The genus is the largest of the Euphorbiaceae family and the third largest of angiosperms (Ernst et al.,

2015; Salehi et al., 2019). Like the family, Euphorbia contains plants of very diverse structures that are well noted for their application in medicine (Thorat & Bolli, 2017). The genus *Euphorbia* comprises approximately 2000 species (Ernst et al., 2015; Magozwi et al., 2021; Salehi et al., 2019). Some plants are endemic to areas while others have been introduced to other territories which may account for the wide distribution of the species in temperate, tropical, and sub-tropical regions (Magozwi et al., 2021). The genus is divided into four sub-genera: *Chamayesce, Esula pers, Euphorbia* and *Rhizanthium* with the last mostly localized to Africa and the others having cosmopolitan distribution (Ernst et al., 2015). *E. neriifolia* is mostly found in warmer climates on rocky ground and can grow to 20 feet (Mali & Panchal, 2017).

Figure **1**: *E. neriifolia* (sweet aloe) grown in the greenhouse at Trent University

1.3.2 Morphology

The range of diversity in structure and appearance of plants in the Euphorbia genus manifests in forms from herbs to large trees and laticifer formation type (Castelblanque et al., 2021). A common characteristic is the milky latex which perhaps gives *E. neriifolia* its name “milk hedge” in India. Latex is known to be toxic and a skin irritant and is exuded upon damage to the plant parts (Salehi et al., 2019). *E. neriifolia* is a cactus-like, fleshy, xerophytic, erect, prickly succulent with a unique flower structure. It is purported to be leafless for most of the year except for monsoons in India when new leaves appear (Thorat & Bolli, 2017). Contrary to this, leaves were present from the onset of growth in this thesis through winter and summer consistent with what occurs in Guyana year-round. Leaves are green, thick, leathery and arranged in rows of five and develop at nodes. Flowers develop at the top of leaves and spiny thorns are found at nodes at the base of the leaves. Differences at the level of chromosomes were noted in morphotypes of the species from Ahmedabad and Chandigarh and were attributed to possible hybridization (Choda, 1978). This level of study was not done in this research but note is made of differences.

Figure **2**: *E. neriifolia* flowers shown on left and prickly spined stems on the right

1.3.3 Distribution

Several species of the genus *Euphorbia* are distributed in temperate and tropical areas (Benjamaa et al., 2022). *E. neriifolia* is found mainly in tropical and subtropical areas where they are grown as ornamental hedges (Sultana et al., 2022). It is native to Southeast Asia and is found in hilly areas in India, Burma, Bangladesh, Malaysia, New Guinea, and Vietnam (Bigoniya & Rana, 2005; Sultana et al., 2022). In Guyana, it is normally grown in the North-West District (Van Andel, 2000) as shown in Figure **3**.

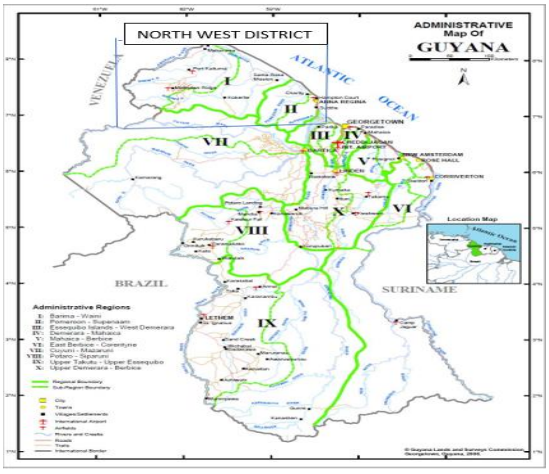

Figure **3**: North-West District depicted at the top of the Map of Guyana.

1.3.4 Potential Impact – *Euphorbia* as a model natural product resource

The number of medicinal roles of the plant globally indicates great potential for *E. neriifolia* for therapeutic applications beyond current uses in Guyana. Differences in geographic location and environmental factors were demonstrated to affect the metabolic profile of *Cannabis sativa* and *Lycium barbarum* from different regions of China (C.-R. Li et al., 2022; Y. Li et al., 2022; Liu et al., 2022). Studies proved climate affected endogenous hormone levels in grapes (Benjamaa et al., 2022; Karimi et al., 2020). No study was found that focused on metabolic differences in *E. neriifolia* species grown in different parts of the world. With asthma prevalence greater than 10% in Guyana (Kellman et al., 2020) and in countries like China (X. Li et al., 2020) research focused on sweet aloes could prove beneficial to determine if environmental factors have impacted the constituents. Additionally, of the *Euphorbia* species assessed by IUCN in 2015, 70% of 243 species were deemed at risk of extinction (Salehi et al., 2019) but no indication exists of *E. neriifolia* endangered status in Guyana. The socio-economic impacts could be further explored, in addition to higher level education and health indigenization. Although, the focus of this study is on phytochemistry, note is made of the above considerations.

1.4 Phytochemistry and application to healthcare

Phytochemistry and investigation of metabolites have been a major focus for finding pharmacologically active components for disease management from *E. neriifolia*. Secondary metabolites are the primary targeted bioactive

compounds. Chemical composition of extracts could vary depending on the species, part of the plant and extraction method used (Salehi et al., 2019). The secondary metabolites sorted by chemical class yield groups of flavonoids, alkaloids, terpenoids, tannins, cardiac glycosides, saponins, steroids, limonoids and phenolics (Thorat & Bolli, 2017). They are used for numerous purposes including asthma, for which metabolites were the main compounds studied (Nalban et al., 2019; Park et al., 2010). Flavonoids comprise approximately 4000 phenolic compounds in classes including flavanols, the largest group typically found in fruits vegetables tea and red wine (e.g. fisetin, quercetin, kaempferol and myricetin), flavones (e.g. luteolin and apigenin), flavanones (naringenin), anthocyanidins, chalcones and the subclass isoflavones which are considered phytoestrogens (Harborne & Williams, 2000; Kumar & Pandey, 2013; Sharma & Janmeda, 2017). They exist as O-glycosides, C-glycosides or glucuronosides and are known to have inhibitory or inducing effects on enzyme systems which subsequently lead to the inhibition of histamine thus preventing inflammation (Harborne & Williams, 2000; Kumar & Pandey, 2013). Synthesis of flavonoids occur via the phenylpropanoid pathway and studies suggest that high flavonoid levels are beneficial to asthma (Knekt et al., 2002). Considering the isoflavone group of flavonoids are known for estrogenic activity (Harborne & Williams, 2000), this would present a negative mechanism in asthma considering theories on hormonal implication (Bulkhi et al., 2020). This may be a potential factor for the use of *E. neriifolia* primarily in children or pre-pubescent individuals.

1.4.1 Secondary Metabolites

Extensive studies have been conducted on Euphorbia species within the last few years to explore roles in diseases like cancer (Betancur-Galvis et al., 2002; Cruz et al., 2020; Mazur et al., 2022; Ramsay et al., 2011) and more recently COVID-19 (Pramanik et al., 2022). The primary focus on each species was on metabolites in plant tissues: leaf, stem, root, or latex (Chaudhary & Janmeda, 2022; Kumar et al., 2021; Palit et al., 2016; Salehi et al., 2019) but not in heated extracts form as per traditional asthma treatment. Studies on asthma in rat and mouse models were done which proved efficacious (Bigoniya & Rana, 2005; Xia et al., 2018). However, these studies used powdered air-dried leaves with simple tests to confirm presence of compound classes but did not identify specific compounds. Despite taking cues from traditional therapies, no study used the traditional “quailing” method as in Guyana to check phytochemical profiles. Most studies employed the use of shade-dried leaves or air-dried leaves (Sawale et al., 2017). A mouse-model study to prove efficacy of *E. neriifolia* used a milder application that involved heating leaves in an oven at a lower temperature of 80 C for a longer time than the traditional method in Guyana (Raghuwanshi et al., 2013). Neither the phytohormone profile nor the use of *E. neriifolia* in asthma was considered. In addition, estrogen and testosterone levels are indicated in pathogenesis and expression of asthma (Bulkhi et al., 2020; Tam et al., 2011) but no study investigated the possible therapeutic role of phytoestrogens or phyto-androgens and few studies examine the agonistic activity of plant metabolites on receptors (Basu et al., 2019). Therefore, an

untargeted approach was used to investigate sweet aloes / *E. neriifolia* sample types to obtain a general metabolite screen before filtering for a targeted approach.

The major emphasis for therapy so far in *Euphorbia* species has been on flavonoids and terpenes (Chang et al., 2022; Magozwi et al., 2021; Palit et al., 2016) reported to reduce chronic illness. Although the mechanisms of action are not completely understood, the roles of these metabolites are well-established. As such, an opportunity exists for the exploration of phytohormones like cytokinins (CKs) or abscisic acid (ABA) for potential therapeutic value.

1.4.2 Phytohormones in therapy

This thesis undertakes a targeted mass spectrometric analysis of plant hormones in *Euphorbia neriifolia* to explore their potential as therapeutic compounds. Phytohormones, first defined in 1948 to differentiate them from animal hormones (Ňorbová et al., 2021), are signaling molecules found in plants that regulate their growth and development processes. They are grouped as cytokinins like kinetin (K) and zeatin (Z); acidic hormones like auxins, gibberellins (GAs), abscisic acid (ABA), salicylic acids (SA), and brassinosteroids. Cytokinins, not to be confused with cytokines, are adenine-derived compounds substituted at the N^6 position with an isoprenoid or aromatic side-chain (Fathy et al., 2022) that regulate plant growth and adaptation to environmental conditions and are classified as isoprenoids or aromatics (Kisiala et al., 2019; Voller et al., 2010).

Isoprenoid CKs are more abundant in nature while aromatics are limited to very few plant species (Voller et al., 2010). The aromatics include the first cytokinin discovered in 1955, kinetin (K a.k.a 6-furfuryl adenine) and its riboside, kinetin riboside (KR) and n^6-benzyl adenine (BA), and its corresponding riboside N^6-benzyadenosine (BAR) (Hauserova et al., 2005). Through various processes, the interconversion of cytokinin molecules yields fractions of free bases, ribosides, nucleotides, glucosides (Hauserova et al., 2005), and Methylthiolated derivatives (Figure **4**).

Cytokinins and their derivatives were demonstrated to be neuroprotective, immunomodulatory, affect the division of mammalian cells by promoting or inhibiting cell division and inducing cell differentiation among other effects (Bowie et al., 2018; S. W. Kim et al., 2020; Lee et al., 2012). Kinetin, in particular, has generated significant interest in human therapy within the past few decades in oxidative stress, DNA repair and neurodegenerative disorders like Huntington's disease (Del Mondo et al., 2023; Fathy et al., 2022; Jablonska-Trypuc et al., 2016; Mielcarek & Isalan, 2021; Rajabi et al., 2012) and slowing aging of human cells. Benzyladenine (BA) influences oxidative stress parameters (Jablonska-Trypuc et al., 2016) and the riboside is noted for high toxicity to cell lines (Fathy et al., 2022). N^6-isopentenyladenosine (iPR) also demonstrates cytotoxicity.

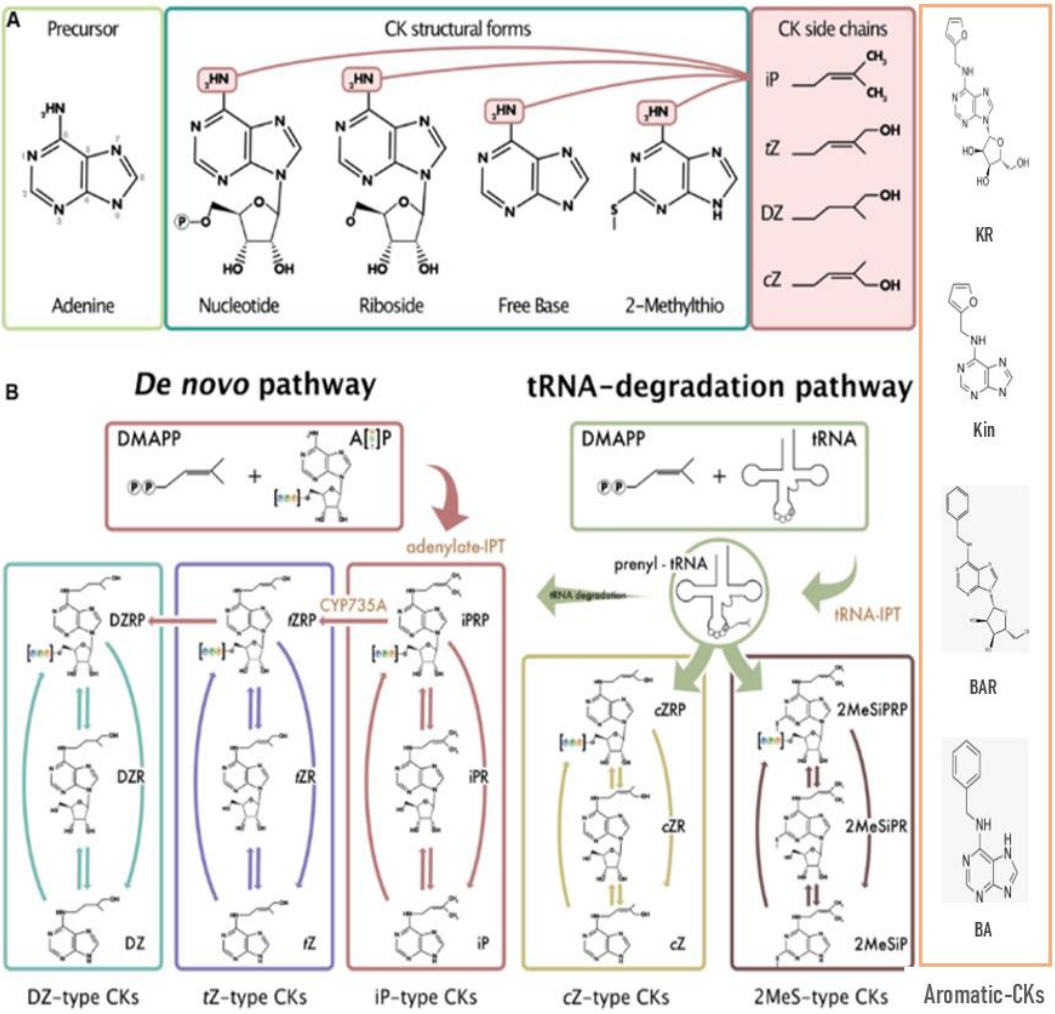

Figure **4**: Interconversion of cytokinin forms. Adapted from (Aoki et al., 2020). Kinetin (KIN), kinetin riboside (KR) and N^6-Benzyladenine and N^6-benzyladenosine structures are shown but are not known to be part of the normal pathway shown for the isoprenoids.

These isoprenoid fractions include further cytokinin types: zeatin – cis and trans forms (cZ and tZ), dihydrozeatin (DZ) and isopentenyladenine (iP). Zeatin and zeatin ribosides have received increased interest for gerontomodulatory effects including reducing UV potentiated aquaporin 3 downregulation thereby reducing photoaging in human skin keratinocytes (Ji et al., 2010; Rattan &

Sodagam, 2005). The O-glucoside form of zeatin was reported to have a potential role in neurodegenerative diseases (Del Mondo et al., 2023). This was different in cancer (Voller et al., 2010) where O-Glucosides were reported as functionally less active on cell lines. Cytokinins have effects on mammalian cells where they can be taken up and changed in addition to having applications in cancer, aging, and skin conditions (Aoki et al., 2019; Fathy et al., 2022; Othman et al., 2016; Seegobin et al., 2018). In plants, free base and riboside molecules are credited as active fractions while nucleotides are considered inactive. Tissue lines showed sensitivity to iPR, cZR, KR, BAR and CK-NTs (Voller et al., 2010). The CK-FBs, MeS, O-Glucs and N-Glucs possess little or no activity in human cell lines compared to NT and RB fractions that exhibit pharmacologic effects in cancer and Huntington's (Voller et al., 2010). KR is converted to KRNT as triphosphate (KTP) by adenine phosphoribosyltransferase (APRT) enzyme. This molecule then exerts its effects on DNA repair and reactive oxygen species clean up (Bowie et al., 2018). Considering this, cytokinin and phytohormone studies in the genus only targeted exogenous applications of hormones for effects on plant growth or in vitro regeneration to date, leaving the area of endogenous hormone use unexplored.

The application of acidic hormones (i.e., (ABA), (GAs), indole-3-acetic acid (IAA), jasmonic acid (JA), and salicylic acid (SA)) in human health were explored. Following the debate over the endogenous presence of ABA in mammals and proof (Le Page-Degivry et al., 1986), current research points to the roles of ABA in humans in stimulating glucose uptake for diabetic control as well as a marker

for asthma and chronic obstructive pulmonary disease (Ashraf et al., 2021; Hoang et al., 2021; Kooshki et al., 2021; Magnone et al., 2020; Sturla et al., 2017). Antimicrobial properties were noticed for three enantiomers of GAs where GA_3 showed good activity with lower for GA_7 and GA_9 (Toner et al., 2021). Additionally, anti-inflammatory properties were detected (Reihill et al., 2016). Recent studies point to the cell regeneration properties of IAA (Cernaro et al., 2015) and anti-inflammatory properties of IAA in mouse models for ankylosing spondylitis (Shen et al., 2022). Jasmonic acid is also indicated in anti-aging skin applications, cancer and anti-inflammation processes (Henriet et al., 2017). The role of the non-steroidal anti-inflammatory agent aspirin is potentiated by the potent metabolite SA (S. W. Kim et al., 2020). The role of aspirin in health has been well documented since its discovery as it remains widely used for blood thinning and cardiovascular diseases (Jones et al., 2021). All the acidic hormones have therefore been shown to have some impact on human disease. Given the global use of *E. neriifolia* in multiple health conditions, the effect of hormones and their role have been under investigated.

1.4.3 Research and metabolomic approaches

Beyond a targeted search, such as the exploration of phytohormones, there are many other types of compounds that might explain the therapeutic value of heated Euphorbia extract. Improved technology using the field of metabolomics can be applied with fewer limitations to obtain a snapshot of the state of biological analytes when investigating natural products (Barchet, 2013;

Jez et al., 2021). Metabolomics is an emerging field of study concerned with systematic identification and quantification of small molecules or metabolites in samples (Idle & Gonzalez, 2007). It looks at deciphering small molecule enigmas through scientific inquiry much similar to genetics as the aim is detecting and characterizing small molecules with a view of their involvement in nature (Barchet, 2013). Untargeted metabolomics presents a broad screen of metabolites and helps detect pathway changes. It could ultimately help determine metabolites that may be novel since it presents the entire metabolome. Emphasis can then be placed on a more targeted approach to compounds with suspected high therapeutic potential using other branches of metabolomics such as hormonomics; an area concerned with identifying hormones (i.e. phytohormones and related analogues) in biological samples (Giebelhaus et al., 2022).

To date, sweet aloes from Guyana remain unexplored with sparse dated information from ecological sources and surveys of medicinal plants that briefly mention the plant but are devoid of phytochemical evaluation. While studies exist from other countries on a limited number of phytochemicals in *E. neriifolia* (Chaudhary & Janmeda, 2022; Kumar et al., 2021), hormonomics investigation of the species has not been documented. Furthermore, review papers make quick mention of heated extracts and latex for asthma therapy, but analytical work appears nonexistent. To my knowledge, no study focused on a comprehensive phytoconstituent investigation of secondary metabolites and hormones, nor delved into the range of compounds with antioxidant and anti-inflammatory

properties specifically for *E. neriifolia* (i.e., sweet aloes) in Guyana. Metabolomics allows for multiple foci which was exploited in this research.

1.4.4 Hypothesis and Objectives

The established use of traditionally heated extract of sweet aloes / *E. neriifolia* leaves predominantly for asthma in Guyana raises the question as to its constituents, while use of latex in Indigenous cultures in other countries would infer there is valuable phytoconstituents in the tissue type for exploration. Therefore, the present research on *E. neriifolia* (sweet aloes) was designed to (a) screen for phytoconstituents that may have therapeutic potential in asthma therapy and (b) quantify endogenous phytohormone levels within different sample types as used indigenously compared to simple leaf extract. I hypothesized that the heated traditional extracts would contain higher concentrations and numbers of hormones and chemical constituents, that could potentially lead to improvement in asthma therapy.

The objectives of this study were to:

1. Comparatively evaluate the phytochemicals and phytohormones in simple extract (SE), traditional extract (TE) and extracted latex (LE) samples
    a. Determine the number of features present and those upregulated or down-regulated between sample types.
    b. Holistically explore the pathways of compounds in *E. neriifolia*
2. Build a list of phytochemicals based on potential use in asthma therapy for semi-targeted metabolomic analysis.

3. Determine the presence of phytohormones with human therapy potential among sample types and their quantification.
4. Calculate a preliminary ingredient list per teaspoon of traditionally heated leaf extract.

This was achieved by combining a wide screen for metabolites with anti-inflammatory and antioxidant properties via an untargeted and semi-targeted metabolomic approach together with a quantitative, targeted determination of phytohormone levels on the three sample types.

CHAPTER 2: MATERIALS AND METHODS

2.1 *Euphorbia neriifolia* – Plant Material

Sweet aloes plant stems were obtained from Guyana at geographic coordinates 6°43’46” N 58°14’17” W and transported to Canada following the regulatory and phytosanitary procedures. Four stems were planted one per pot in soaked soil in Sunshine Professional Growing Mix (Mix #1 Sungrow Horticulture, Canada). Growth occurred under 16 h light photoperiod, 60-80% RH, 24-27 °C, 8 h dark photoperiod, 60-80 % RH, at 19 – 22 °C in the Aurora Greenhouse (Conviron, Canada) (Morrison et al., 2015) at Trent University from September 2021 to June 2022.

2.2 Sample Preparation of Plant Material

Leaves were randomly sampled from three plants to make biological replicates, (n=5) for simple fresh leaf extract (SE) and (n=5) for traditionally heated leaf extract (TE). Extracted Latex (LE) samples (n=5) exudates were collected from leaves detached from stems and at points of stems after separating leaves and cutting stem. Approximately 0.100 g fresh weight of leaf tissues (SE), were cut and placed in 2 mL safe lock centrifuge tubes while 0.100 g (LE) samples were collected in 5 mL tubes. Leaves for TE samples were heated to 250 °C for 15 seconds and then squeezed to yield the liquid traditional extract (TE). This procedure mimics the preparation normally performed when being prepared for children by Guyanese indigenous persons as per the indigenous traditional method (D.A. Lachman-White et al., 1992). The TE

samples were collected into separate 5 mL tubes and centrifuged for 10 minutes at 5000 rpm after which 2 mL were transferred to individual 15 mL tubes. (SE), (LE), and (TE) samples were flash-frozen in liquid nitrogen and SE was stored at -80 °C until further processing. The LE and TE samples were freeze-dried (LabConco Free Zone lyophilizer; Kansas City, MO, USA) and stored at -20°C.

2.3 Solid phase extraction for metabolite and phytohormone purification

Phytohormone and other metabolite extractions were carried out in a sequential extraction process to obtain different phytohormone fractions using modified methods previously published (Kisiala et al., 2019; Simura et al., 2018) to facilitate the multi-extraction of 39 cytokinins and acidic hormones: (Abscisic acid (ABA), gibberellins (i.e., GA_1. GA_4, GA_7, GA_9, GA_{20}), indole-3-acetic acid (IAA), jasmonic acid (JA) and salicylic acid (SA)) from a single plant sample. To enable phytohormone quantification through the isotope dilution technique, internal standards (IS) of deuterated phytohormones were added to each sample constituting 1 mL 50% ice-cold acetonitrile (ACN): (60.1 ng ABA ([$^2H_4$] ABA) (PBI, Saskatoon, Canada), 10 ng each of acidic phytohormones IAA, and SA (OLChelmm, Olomouc, Czech Republic), 20 ng each of gibberellins (i.e., GA_1. GA_4, GA_7, GA_9, GA_{20}) and 10 ng each of deuterated cytokinin standards consisting of aromatic, methylthiolated, and glucoside forms. The CKs scanned for are as follows (Table **2**): benzyladenine (BA), benzyladenine riboside (BAR), kinetin (KIN), cis-zeatin (cZ), cis-zeatin riboside (cZR), cis-zeatin-9-glucoside (cZ9G), cis-zeatin nucleotide (cZNT), cis-zeatin O-glucoside (cZOG), cis-zeatin riboside-O-glucoside (cZROG), dihydrozeatin (DZ), dihydrozeatin nucleotide

(DZNT), dihydrozeatin-O-glucoside (DZOG), dihydrozeatin riboside (DZR), dihydrozeatin riboside-O-glucoside (DZROG), dihydrozeatin-9-N-glucoside (DZ9G), isopentenyladenine (iP), isopentenyladenine nucleotide (iPNT), isopentenyladenine-9-glucoside (iP9G), isopentenyladenosine (iPR), 2-methylthio-isopentenyladenine (2MeSiP), 2-methylthio-isopentenyladenosine (2MeSiPR), 2-methylthio-zeatin (2MeSZ), 2-methylthio-zeatin riboside (2MeSZR), trans-zeatin (tZ), trans-zeatin riboside (tZR), trans-zeatin-9-glucoside (tZ9G), trans-zeatin nucleotide (tZNT), trans-zeatin O-glucoside (tZOG), and trans-zeatin riboside-O-glucoside (tZROG) (Bean et al., 2021). While JA wasn't added as an internal standard, ([$^{2}H_{4}$] ABA) was used as a standard for JA quantification. METLIN (Domingo-Almenara et al., 2019) was used to confirm JA in plant samples based on the protonated monoisotopic mass.

SE samples containing two zirconium beads (Comeau Technique Ltd., Montréal, Québec, Canada), were homogenized using the Retsch 300 ball mill grinder (Haan, Germany) at 25 Hz for 5 min, in a 4 °C cold room. All samples (i.e., SE, TE, and LE), were vortexed and then refrigerated at -20 °C for overnight passive extraction.

The samples were transferred on ice from the -20 °C freezer and the TE and LE samples were centrifuged at 5000 rpm for 5 minutes, while the SE samples were done at 10,000 rpm for 10 minutes (ThermoScientific Sorvall ST 16 centrifuge; ThermoFisher Scientific, San Jose, CA, USA). The supernatants were collected into 2 mL tubes and combined with subsequent supernatants collected upon washing samples with two sets of 500 µL of cold ACN and treated

as before. Three method blank samples were prepared and extracted similarly to plant samples. The supernatants and blank samples were vortexed and centrifuged at 10000 rpm for 5 mins.

HLB cartridges (VIOLET™ 200mg/6 mL, 40 µm; Canadian Life Sciences; Peterborough, Canada) were sequentially preconditioned with methanol and ddH_2O water. Columns were equilibrated with 50% ACN before loading supernatants followed by 2 mL of 30% aqueous ACN. Each extract was divided into two samples; one set was for CKs and the other fraction for derivatization of acidic phytohormones (method in following section; 2.4) and other metabolites. All samples were evaporated to dryness overnight at ambient temperature in a speed vacuum centrifuge concentrator (Thermo Savant UVS 400a; ThermoFisher Scientific, Berlin, Germany).

Table **2**: Endogenous and isotope labeled phytohormones scanned for using the Q-Exactive Orbitrap mass spectrometer and their classifications, abbreviations, and their associated deuterated internal standard via isotopic dilution technique.

Endogenous CK phytohormones	Abbreviation	^{2}H-labelled Internal Standards
Free bases (FB)		
N^6-isopentenyladenine	iP	$^2H_6$iP
trans-zeatin	tZ	$^2H_3$DZ
cis-zeatin	cZ	$^2H_3$DZ
Dihydrozeatin	DZ	$^2H_3$DZ
Ribosides (RB)		
N6-isopentyadenosine	iPR	$^2H_6$[9R]iP
trans-zeatin riboside	tZR	$^2H_5$[9R]tZ
cis-zeatin riboside	cZR	$^2H_5$[9R]tZ
Dihydrozeatin riboside	DZR	$^2H_3$[9R]DZ
Nucleotides (NT)		
N^6-isopentyadenosine-5'-monophosphate	iPRP	$^2H_6$[9RMP]iP
trans-zeatin riboside-5'-monophosphate	tZRP	$^2H_6$[9RMP]DZ
cis-zeatin riboside -5'-monophosphate	cZRP	$^2H_6$[9RMP]DZ
Dihydrozeatin riboside-5'-monophosphate	DZRP	$^2H_6$[9RMP]DZ
Glucosides (GLUC)		
isopentenyladenine-7-glucoside	iP7G	$^2H_6$iP
isopentenyladenine-9-glucoside	iP9G	$^2H_6$iP
trans-zeatin-O-glucoside	tZOG	$^2H_5$tZOG
cis-zeatin-O-glucoside	cZOG	$^2H_5$tZOG
Dihydrozeatin-O-glucoside	DZOG	$^2H_7$DZOG
trans-zeatin-O-glucoside riboside	tZROG	$^2H_5$tZROG
cis-zeatin-O-glucoside riboside	cZROG	$^2H_5$tZROG
Dihydrozeatin-O-glucoside riboside	DZROG	$^2H_7$DZROG
trans-zeatin-9-glucoside	tZ9G	$^2H_5$tZ9G
cis-zeatin-9-glucoside	cZ9G	$^2H_5$tZ9G
Dihydrozeatin-9-glucoside	DZ9G	$^2H_3$DZ9G
Methylthiols (2MeS)		
2-Methylthio- N^6-isopentayladenine	2MeSiP	$^2H_6$2MeSiP
2-Methylthio-N^6-isopentenyladenosine	2MeSiPR	$^2H_6$2MeSiPR
2-Methylthio-zeatin	2MeSZ	$^2H_5$2MeStZ
2-Methylthio-zeatin riboside	2MeSZR	$^2H_5$2MeStZR
Aromatics		
Kinetin	KI	$^2H_7$BA
N^6-benzyladenine	BA	$^2H_7$BA
N^6-benzyladenosine	BAR	$^2H_7$[9R]BA
Acidic Phytohormones		
Abscisic acid	ABA	[$^2H_4$] ABA
Indole-3-Acetic Acid	IAA	[$^2H_5$] IAA
Jasmonic acid	JA	[$^2H_6$] JA (not added)
Salicylic acid	SA	[$^2H_4$]SA
Gibberellin 1	GA_1	[$^2H_4$]GA_1
Gibberellin 4	GA_4	[$^2H_2$]GA_4
Gibberellin 7	GA_7	[$^2H_2$]GA_7
Gibberellin 9	GA_9	[$^2H_2$]GA_9
Gibberellin 20	GA_{20}	[$^2H_2$]GA_{20}

2.3.1 Derivatization of acidic phytohormones

The dried phytohormone fraction containing metabolites from the initial HLB extraction was used to derivatize acidic phytohormones, (modified from (Kojima et al., 2009). Dried samples were reconstituted with these reagents added in order: 75 µL of 1-propanol (Fisher Scientific; Ottawa, Ontario, Canada), 20 µL of dd$H_2$0 water, 5 µL of 500 mM bromocholine (Fisher Scientific; Ottawa, Ontario, Canada) in 70% ACN and 1 µL of triethylamine (Fisher Scientific; Ottawa, Ontario, Canada). These mixed samples were vortexed and tubes with punched holes were suspended in a water bath at 80 °C for 130 minutes, then moved immediately to ice and dried under the speed vacuum concentrator for 3 hours (Kojima et al., 2009).

2.3.2 Solid phase extraction - sequential elution of CK fractions: free base, riboside, methylthiolated and nucleotide forms

Samples of the supernatants for CK sequential elution dried overnight using a speed vacuum centrifuge. Residues were reconstituted in 1 mL of 1M formic acid to promote complete protonation of CKs, vortexed and centrifuged at 5000 rpm – 10000 rpm for 5 minutes. The redissolved residues were run through mixed-mode cation exchange SPE cartridges (IRIS™ MCX 200mg/6.0 mL, 40 µm; Canadian Life Sciences, Peterborough, Canada) sequentially preconditioned using MeOH and 1M formic acid (HCOOH) as previously described (Farrow & Emery, 2012). Nucleotides were eluted first with 0.35M Ammonium hydroxide (NH_4OH) while free base, methylthiolated, glucoside and riboside cytokinin forms

eluted together using 0.35M Ammonium Hydroxide in 60% Methanol (NH_4OH: MeOH (40:60 v/v)); all separating according to polarity and cationic properties (Kisiala et al., 2019)). All collected fractions were dried by the speed vacuum centrifuge.

As nucleotide CK forms cannot be detected with this mass spectrometry methodology, they were dephosphorylated (Kisiala et al., 2019). Samples prepared for CK nucleotide extraction were redissolved in 1.0 mL of 0.1 M ethanolamine and vortexed before being phosphatased. Twelve (12) µL of phosphatase enzyme (New England BioLabs Ltd., Pickering, Canada) were added to the samples, vortexed, incubated at 37 °C overnight, and dried for 6 hours in a speed vacuum centrifuge. The samples were redissolved in 1.5 mL of ddH_2O water, vortexed, and centrifuged for 10mins at 10 000 rpm. C18 SPE cartridges (C18, 6 cc, 500 mg; Canadian Life Sciences, Peterborough, ON, Canada) were preconditioned with methanol and ddH_2O before samples were introduced and allowed to flow through the column by gravity. CK NT elution was done with 1.25mL of 80% MeOH and collected samples were speed vacuumed overnight.

Dried samples belonging to all fraction types (CK fractions and acidic phytohormones) were redissolved in 300 µL of starting conditions constituting AcOH, ACN, and ddH_2O in a ratio of 0.08:5:94.92 (v:v:v) respectively. Samples were vortexed and centrifuged at 5000 rpm for 10 minutes before being transferred to 2 mL clear vials with 350 µL glass inserts and stored at -20 °C until mass spectrometric analysis.

2.4 Ultrahigh-pressure liquid chromatography-mass spectrometry analysis of phytohormones and metabolites

The phytohormone samples were analyzed using a Q Exactive orbitrap mass spectrometer in (ThermoScientific; Waltham, Massachusetts, USA) equipped with heated electrospray ionization source (HESI-II) coupled to a Thermo Dionex Ultimate 3000 UHPLC (ThermoScientific; San Jose, USA) (Kisiala et al., 2019). The chromatographic separation of CKs was accomplished with HGP-3400RS dual pump and WPS-3000 autosampler equipped with a Kinetex C18 column (2.1 i.d x 50 mm, 2.6 µm particle size, Phenomenex, Torrace, U.S.A) operated at an approximate room temperature of 22 ºC. The instrument control was achieved with Chromeleon 6.8 Chromatography Data System software (ThermoScientific; Ottawa, Canada).

All phytohormone fractions were eluted with component A comprising dd$H_2$0 with 0.08% CH_3COOH and component B comprising CH_3CN with 0.08% CH_3COOH, at a flow rate of 0.5 mL/min. The CK fractions were eluted with a multi-step gradient. Starting condition consisting of 5% B, was held at 0.5 min increasing linearly to 45% B over 4.5 min, followed by an increase to 95% B over 6.5 min; 95% B was held constant for 1 min before returning to starting conditions for 2 min for column re-equilibration. Total run time was for CKs was 8.2 mins with an injection volume of 25 µL (Bean et al., 2021; Kisiala et al., 2019).

A full scan in positive ionization mode was done for the acidic phytohormones and untargeted metabolites with a run time of 15 minutes on the eluted analytes in the Q Exactive Orbitrap high-resolution mass spectrometer

with modifications as per (Kisiala et al. 2019). A mass range of m/z 100 to 700 was used at a resolution of 140,000 at m/z 200 full width at half minimum (FWHM), with automatic gain control (AGC) target of 3×10^6, and maximum injection time (IT) of 524 ms. A representative sample from the replicates was used for data-dependent tandem mass spectrometry (ddms^2). Samples deriving from the HLB extraction for acidic phytohormones and metabolites, the full scan was done at a resolution of 70 000 with an AGC target of 5×10^4. For fragmentation, it was done at a resolution of 17 500 with an AGC target of 5×10^5. The fragmentation was triggered at a loop count of 10 (top 10 most intense peaks per scan), with a precursor isolation window of 1 m/z. The maximum IT was 64 ms.

For the cytokinin samples, acquisition was performed in positive ion mode, and data were acquired via parallel reaction monitoring (PRM) as per Kisiala and associates (Kisiala et al., 2019). PRM data were acquired at resolution of 35 000 fwhm at m/z 200. PRM parameters consisted of an automatic gain control (AGC) of 3×10^6 and a maximum IT of 128 ms. The precursor isolation window width was m/z 1.2. The normalized collision energy (NCE) was individually optimized for each compound by stepwise increments, where at least 10% of the unfragmented precursor ion was retained.

2.5 Data Analysis

2.5.1 Data analysis for mass spectrometry-based metabolomics

The LC-MS raw data acquired during sample analysis via UHPLC–MS were exported as mzXML files using the MSConvert module in Proteowizard 3.022

(Adusumilli & Mallick, 2017; Kessner et al., 2008) and preprocessed using the multigroup option in XCMS Online (Gowda et al., 2014; Huan et al., 2017) for peak detection, retention time correction, and alignment of the metabolites detected in the UHPLC–MS analysis. Peak detection was performed using centWave peak detection (∆m/z = 10 ppm; minimum peak width, 10 s; maximum peak width, 60 s) and mzwid = 0.015, minfrac = 0.5, bw = 10 for alignment of retention time. The obiwarp method was used for retention time correction. The signal-to-noise ratio was set to 10:1. The Kruskal-Wallis non-parametric test was used for statistical analysis.

2.5.2 Statistical analysis and differential metabolite selection for comparative analysis

The processed data in the form of an Excel worksheet file derived from XCMS Online, was separated into csv files corresponding to sample type (i.e., SE (unheated), TE (heated) and LE (latex extract) samples. These sample types were put in folders according to pairwise comparisons (i.e., SE vs TE, and SE extract vs LE) then zipped together. The zipped folder was uploaded to MetaboAnalyst 5.0 (Pang et al., 2022) and submitted to the Statistical Analysis (one factor) module. Mass and retention time tolerances of 0.005 m/z and 30 seconds were chosen respectively. For data filtering, the inter-quantile range function was used. For sample normalization, normalization by sum was chosen. Log transformation was chosen for data transformation, and pareto scaling was chosen as the option for data scaling. This was done to reduce the skewness of the data and reduce the mask effects (Zandonadi et al., 2023).

Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were performed on the normalized datasets (Zandonadi et al., 2023). These statistical methods project the variables to a new space to determine which variables in the complex data set are responsible for observations being classified into groups or categories. The Variable Importance in Projection (VIP) arising from PLS-DA analysis was used to decipher features responsible for the separation of sample types. Features refer to distinct signals detected from mass spectrometric analysis generated by different molecules (metabolites) present in the biological sample that are characterized by their mass-to-charge ratio (m/z) and retention time. At this stage the identity of the metabolite that generated the signal is unknown thus feature is used instead of metabolite. Univariate statistics (i.e., t-test) was applied to calculate the statistical significance of m/z values in treatment comparisons (i.e., the SE (unheated control) vs TE (heated) and the unheated control (SE) vs the milky latex (LE).

Volcano plot analyses were performed to determine significantly changed features (i.e., up-or-downregulated features) occurring in the TE and LE compared to the control sample SE. As volcano plots provide a summary of t-test and fold change (FC) analysis, a $\log_2 FC > 1$ or $\log_2 FC < -1$ was chosen with a false detection rate (FDR) < 0.05. Figures for volcano plots were regenerated using VolcaNoseR (Goedhart & Luijsterburg, 2020).

2.5.3 Untargeted metabolite identification

2.5.3.1. Database query using quality EICs generated from XCMS Online

For the respective treatments (SE, TE, and LE), the single module was selected in XCMS online to verify quality peaks from the extracted ion chromatograms (EICs) generated from XCMS Online. These EICs images were visually identified, and the corresponding m/z (i.e., m/z medium stylized as mzmed as generated by XCMS Online) that is within the EIC m/z extracted range was selected from the .xlsx file downloaded from XCMS online processing.

To look at the chemodiversity of features, Venn diagrams were used as a visualization tool. All features detected using the single module in XCMS online, and all quality EICs were subjected under Venn diagram analysis. Duplicate m/z values were automatically removed using InteractiVenn (Heberle et al., 2015).

The m/z values which would be the observed masses arising from quality XCMS-generated EICs were queried against the theoretical masses of different databases such as: Pathos (Leader et al., 2011), MetaboQuest (http://tools.omicscraft.com/MetaboQuest/), and CEU Mass Mediator 3.0 (Gil-de-la-Fuente et al., 2019). Putative metabolites that were unique, and shared between the milky latex, the unheated extract (SE) and the heated extracts (TE) were determined, and as a cut-off, metabolites with KEGG IDs were selected. As indicated, putative metabolites refer to potential assignments for previously generated features based on matching their characteristics, (best match basis), to known metabolites in databases or reference libraries. This does not signify a definitive confirmation. Only $[M+H]^+$ adducts were considered for annotation.

2.5.3.2. KEGG functional and pathway analyses modules in MetaboAnalyst 5.0

In addition, using the m/z, retention time (rt) and p-values of all detected aligned features generated by XCMS Online multimode module was put in a .csv file to be analyzed using functional analysis to query whether there may be metabolites of interest in *E. neriifolia*. Annotation was performed with a 5 ppm mass tolerance and the mummichog algorithm (Li et al., 2013) with a 0.05 p-value cut-off (Piasecka et al., 2022) with *Arabidopsis thaliana* reference metabolome in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Braga et al., 2022). Annotated compounds were then screened via the pathway and enrichment analyses modules in MetaboAnalyst 5.0.

Metabolites over-represented on the pathway were ranked using hypergeometric testing, with correction done using the Benjamini-Hochberg false discovery rate (FDR) (Piasecka et al., 2022). The KEGG IDs that would be generated using functional analysis were used for determining putative compounds. Putative metabolites were identified according to the metabolite annotation level guidelines (levels 1 – 5) previously published (Schrimpe-Rutledge et al., 2016; Tsugawa et al., 2021).

The KEGG IDs of the matched compounds were submitted to the pathway analysis module in MetaboAnalyst 5.0, which uses the hypergeometric test (Banimfreg et al., 2022) and the latest KEGG version of the *A. thaliana* pathway library (Braga et al., 2022). It should be noted that the exact m/z value of the compounds were inputted into the MetaboAnalyst 5.0 and that peak quality was

not determined. The Pathway analysis would give an idea of the putative compounds based on exact mass within a 5 ppm error margin.

2.5.3.3 Tandem mass spectrometry (MS^2) data processing

To find matches for compounds fragmented using the $ddms^2$ methodology, different bioinformatics tools were used to cover as much matches as possible. The mzMine 2.5.3 analysis pipeline (Olivon et al., 2017; Pluskal et al., 2010) was used to look for MS^1 features corresponding to MS^2 fragments. Data acquired from samples analyzed using $ddMS^2$ were converted to the mzXML format using the MSConvert option in Proteowizard, as mentioned previously. The peaks were aligned using the parameters previously published involving mass detection, chromatogram building, smoothing, and deconvolution, deisotoping, alignment and gap filling (Reveglia et al., 2022). All data (for MS^1 and MS^2) was used and stored in .mgf format.

SIRIUS (v. 5.6.3) was used for further molecular annotation of fragmentation patterns from $ddms^2$ data (Duhrkop et al., 2019; Ludwig, Fleischauer, et al., 2020; Ludwig, Nothias, et al., 2020). The aligned MS^2 spectra in .mgf format that was exported from mzMine was imported into SIRIUS (v. 5.6.3). The settings were as follows: Orbitrap was chosen as the instrument with the option to scope MS/MS isotopes. The MS^2 mass deviation was set to 10 ppm, with 10 candidates chosen and one candidate per ion. Positive ionization adducts $[M+H]^+$, was chosen, with no constraints on tree or compound timeouts.

Elements existing within biological molecules (i.e., C, H, O, N, S, P) were allowed in formula assignment. All databases were selected for querying. The default settings of Zodiac were used for SIRIUS molecular formula ranking, with the defaults for CSI: FingerID interface, and CANOPUS for compound class prediction. For candidates, those with a Zodiac score greater than 90%, and a similarity (SIRIUS) score greater than 75% were chosen. Not all compounds were identified by name, and within the SIRIUS module, under the structures tab, the compound with rank 1 was queried using the corresponding InChiKey in PubChem if the name was not shown, or if the name was unfamiliar.

UHPLC-MS/MS raw data were processed with MS-DIAL 5.1 (Tsugawa et al., 2015). Automatic feature detection was performed between retention times of 0 and 15 min and for masses within 100 – 700 Da (i.e., m/z) for mass signal extraction in positive ionization mode. MS^1 and MS^2 tolerance were set to 0.01 and 0.025 Da, respectively, in profile mode. Minimum feature height, mass slice width and the sigma window value were all set to the default of 1000 (AU; arbitrary units), 0.1 Da, and 0.5 respectively (Hu et al., 2019). Alignment parameters for samples was done at 0.015 Da for mass tolerance and 0.05 min for the retention time tolerance for MS1. Database matches were done with the parameters of MS^1 and MS^2 at 0.01 and 0.05 Da respectively (instructions as previously published (Perez de Souza et al., 2019)). Databases downloaded and chosen for level 2 matching were as follows: ESI(+)-MS/MS from authentic standards, ESI(+)-MS/MS from standards+bio+in silico, MassBank and MassBank-EU, ReSpect, GNPS, CASMI2016, MetaboBASE, RIKEN PlaSMA

authentic standards, RIKEN PlaSMA bio-MS/MS (MSI level 1, 2, 3, or 4), Fiehn/Vaniya natural product library, and BMDMS-NP from the MS-DIAL website (<http://prime.psc.riken.jp/compms/msdial/main.html> accessed March 1, 2023).

2.5.4. Semi-targeted metabolite identification

A list of putative therapeutic metabolites was assembled based on purported anti-inflammatory or antioxidant properties in plants of ethnobotanical significance (Table 3). Their identification in samples SE, TE and LE was accomplished via semi-targeted screening for the specified metabolites in XCalibur 4.3 through identification of fragmentation patterns using full scan and $ddMS^2$ data. This was done by extracting the corresponding m/z values for each therapeutic metabolite (i.e., Table 3), and the chromatographic peak areas were manually integrated for the five biological replicates from each sample type. Fold changes for therapeutic compounds were calculated as the ratio of average peak areas of [Treatment]/[Control], where the treatment was considered either TE or LE, and the control was the SE. Regarding metabolite annotation, these are at level confidence 3 according to the Metabolomics Standards Initiative (Sumner et al., 2007).

Table **3**: Therapeutic metabolites reported in literature from other members of the *Euphorbia sp*. that were investigated using the semi-targeted approach.

Metabolite	Chemical formula	M	M+H	References
4-OH benzoic acid	$C_7H_6O_3$	138.0311	139.039	(Ertas et al., 2015)
Bridelionoside D	$C_{19}H_{36}O_8$	392.2405	393.2483	(Djouwoug et al., 2021)
Caffeic acid	$C_9H_8O_4$	180.0417	181.0495	(Yener et al., 2018)
Chrysin	$C_{15}H_{10}O_4$	254.0573	255.0652	(Ertas et al., 2015)
Coumarin	$C_9H_6O_2$	146.0362	147.0441	(Ertas et al., 2015)
Ethyl linoleate	$C_{20}H_{36}O_2$	307.2631	308.271	(Sudha T & R., 2013)
Ferulic acid	$C_{10}H_{10}O_4$	194.0573	195.0652	(Yener et al., 2018)
Fisetin	$C_{15}H_{10}O_6$	286.0472	287.055	(Ertas et al., 2015)
Hyperoside	$C_{21}H_{20}O_{12}$	464.0949	465.1028	(Ertas et al., 2015)
Myricetin	$C_{15}H_{10}O_8$	318.037	319.0448	(Djouwoug et al., 2021; Ertas et al., 2015)
Neriifolin	$C_{30}H_{46}O_8$	534.3187	535.3265	(Sultana et al., 2022)
Prostratin	$C_{22}H_{30}O_6$	390.2037	391.2115	(Sultana et al., 2022; Tang et al., 2012)
Protocatechuic acid	$C_7H_6O_4$	154.026	155.0339	(Ertas et al., 2015; Yener et al., 2018)
Quercetin	$C_{15}H_{10}O_7$	302.0421	303.0499	(Sultana et al., 2022)
Rutin	$C_{27}H_{30}O_{16}$	610.1528	611.1607	(Ertas et al., 2015; Sultana et al., 2022; Yener et al., 2018)
Sapintoxin	$C_{30}H_{37}NO_8$	539.2514	540.2592	(Sobottka et al., 2016)

2.5.5. Quantification of endogenous phytohormones via the isotopic dilution method.

Phytohormone levels in different sample types derived from *E. neriifolia* (i.e., SE, TE and LE) were reported as CK groups: (aromatics, free bases, ribosides, nucleotides, glucosides, and methylthiols) and as CK types (iP-,tZ-, cZ- and DZ-types) as per (Bean et al., 2021). The processing and quantification of all detected phytohormones was done using the Processing Setup and Quan Browser modules in XCalibur 4.3 (Kisiala et al., 2019). To do so, all endogenous phytohormone peaks were identified using their protonated monoisotopic mass compared to the retention times of the deuterated phytohormone internal standards (IS) in full scan mode. Plant phytohormone concentrations were

calculated using relative quantification through the direct comparison of analyte peak areas relative to IS using the following formula: phytohormone concentration [pmol/gFW] = (((peak area of analyte/peak area of IS) × mass of IS)/MW × 1000)/mass of sample; where FW − mass of the tissue, mass of IS = (10 ng, 20 ng or 60.1ng) as per used in method, MW −molecular mass of CK [g] (Kisiala et al., 2019). All plant sample types were analyzed in quintuplicate. Based on annotation levels due to the guidelines of the Metabolomics Standards Initiative (Schrimpe-Rutledge et al., 2016; Sumner et al., 2007), all phytohormones reported are at level 1 in identification.

CHAPTER 3: RESULTS AND DISCUSSION

3.0 General Overview

E. neriifolia is a species in the Euphorbia genus from the family Euphorbiaceae used in indigenous cultures. Extracts from heated leaves of *E. neriifolia* are traditionally given to babies and children in Guyana to treat wheezing in asthma while the latex is indicated for rashes (DeFilipps et al., 2004), but the chemistry of the local species is unknown and unexplored. While latex is used for asthma in indigenous cultures in India (Sultana et al., 2022), this has not been a reported practice in Guyana. Furthermore, information on the heated leaf extract is sparse globally. Therefore, the three sample types; traditional heated extract (referred to as either heated or TE), the latex extract (LE) and the simple leaf extract (SE) were investigated comparatively within the context of asthma therapy with SE used as a control.

TEs were prepared in a manner to mimic the traditional method of preparation which involved using the liquid collected from squeezing heated leaves. Extracts of SE, TE and LE were subjected to phytochemical and phytohormone extraction procedures as previously described in the method section. These yielded suitable fractions for metabolites, acidic hormones and cytokinins to be separated and detected through UHPLC-MS/MS to determine their presence in each sample type and further identification.

A broad survey of the features of heated (TE) vs control (SE) and latex (LE) vs control (SE) was done to give an overall view of sample types using an untargeted metabolomic approach. Here features are largely unidentified and

therefore only allow for identification at confidence levels 4 or 5 (Schrimpe-Rutledge et al., 2016; Sumner et al., 2007). For increased specificity, a semi-targeted list of metabolites compiled from literature was used to identify features based on comparison to databases using m/z values and fragmentation patterns, raising the confidence to levels 2 and 3 and leading to putative metabolite identification. Finally, complete identification of hormones via a targeted approach with a confidence level 1 was achieved with the use of isotopically labeled standards (Sumner et al., 2007). Identification at the confidence level 1 relates to complete identification without uncertainty.

Metabolomic investigation using an untargeted approach provided a broad overview of the contents of the biological analyte. The non-discriminatory approach captured all observable features within the sample types. This revealed the overall remarkable potential of the species. Features seen at this level included all metabolites irrespective of categorization as phytochemicals or phytohormones as all form part of the global screen. Significant effort and tools were required for identification of compounds using untargeted and semi-targeted compared to a targeted approach. The targeted approach, which is precise in determination leads to confidence level 1 but can be prohibitive due to the high cost of acquiring isotopically labeled standards for investigative purposes. Therefore, only phytohormones were determined at this level.

Multiple studies showed the efficacy of phytohormones in treating human diseased states (Jablonska-Trypuc et al., 2016; Othman et al., 2016; Shen et al., 2022) yet profiling of hormones in species of the *Euphorbia* genus remains

unexplored in relation to therapeutics. The functions of cytokinins remain elusive but the class attracts attention particularly as a result of the role of kinetin and zeatin and their therapeutic potential (Naseem et al., 2020). Generally, focus on secondary metabolites to treat medical conditions appears to overshadow the exploration of the role of phytohormones which appears true for the application of *Euphorbia* species. Since secondary metabolites have been studied in various species of Euphorbia, though less in *E. neriifolia*, background information was available which was different for the phytohormones for the species in the different sample types. Phytohormones are gaining attention but primarily investigated phytohormones are the older more known types like abscisic acid, salicylic acid, auxins, gibberellins, and jasmonates (Pereira-Marostica et al., 2019; Toner et al., 2021). Here, the phytohormone profiles for three sample types of *E. neriifolia* are presented for the first time. The results of the study are presented with cytokinins shown first then the acidic hormones.

The presentation of results of this comparative investigation of the three sample types TE, LE and SE will be approached in the following manner.

(a) The broad indication of the features detected through the untargeted metabolomic approach is presented beginning with a Venn diagram of all detected features followed by an examination of the characteristics of the features in a volcano plot. See Venn diagram Figure **5** and, volcano plot Figure **6** which show the comparative features in SE and TE samples and SE and LE respectively. The Venn diagram method was chosen to get a clear representation of the features in each type of extract as well as see the

relationship and similarities between the extracts. It is a simple, effective means to visualize the number of features and potential metabolites in a specific sample type or shared between two or all three types of extracts. This method does not provide information with respect to accumulation of a feature, whether it is higher or lower in one extract type over another thus the volcano plot was used. The volcano plots give an overview of the number of features with higher or lower accumulation in compared samples.

(b) Subsequently, results from the semi-targeted approach employed to reveal specific putative metabolites from a pre-determined list are presented. To reiterate, the focus was on detecting molecules with antioxidant and anti-inflammatory properties, assembled from literature sources, that may be essential to asthma therapy. Identification is based on comparison to established information in databases with respect to mass-to-charge values and fragmentation patterns. Mass spectrometric data of samples are uploaded and compared with mass spectral databases. Since the use of standards is not employed in this process, the compounds are putative of possible matches instead of direct ones and thus reported as levels 2 to 4 (Sumner et al., 2007).

Finally, the targeted approach is presented which identifies and quantifies the hormones in SE, TE and LE sample types using isotopically labeled standards. The identification of compounds in this approach are at a confidence level of 1 indicating great accuracy and specificity (Schrimpe-Rutledge et al., 2016).

3.1.1. Untargeted metabolomics screen for putative metabolites

As a first approach to determine the chemical potential of *E. neriifolia* sample types, a Venn diagram analysis was done using m/z values found within the extracted ion chromatograms (EIC) range generated from XCMS Online with InteractiVenn (Heberle et al., 2015) to visualize chemo-diversity. An overview of the number of features and distribution among sample types was obtained. This data is represented in Figure **5** as a triple Venn diagram showing (13,704) features detected in SE, TE and LE samples either as unique per sample type or shared between and among sample types.

Less than 5000 features were detected in the SE compared to almost 6000 in TE and over 5000 in LE (Figure **5** and APPENDIX A). Approximately three percent (3%) of features or 415 are shared among all three sample types at the intersection (∩), i.e., SE ∩ TE ∩ LE, while 4.16 %, 3.97%, and 2.61% are shared between SE ∩ TE only, SE ∩ LE only and TE ∩ LE only respectively. The lower number of shared features between TE and LE emphasizes the difference in the extract types. This trend was consistent in same order for total shared features between SE and TE, SE and LE and TE and LE at 7.18%, 7 ,% and 5.64%. These percentages represent the shared features in the entire intersections for each pair of samples. For single and unique features per sample type, TE showed more unique features uncommon to the other types with (4531), followed by LE then SE.

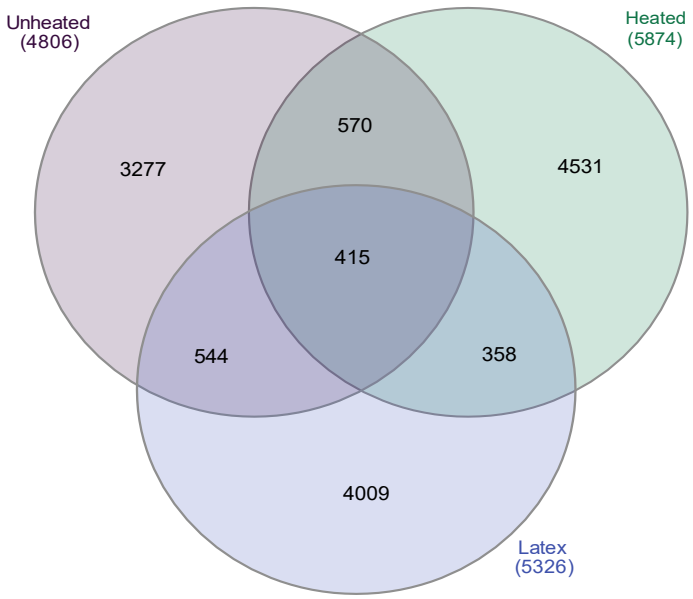

Figure **5**: Venn diagram depicts the number of m/z metabolites in simple unheated leaf extract (SE), traditional (TE) and latex extracts (LE).

TE presents 22% and 10% more features than SE and LE respectively. LE shows an almost 11% higher number of features than SE. The vast number of features shown in all sample types together attest to the significant potential of *E. neriifolia* and its implication for multiple conditions including malaria, syphilis, gonorrhea, hypertension, diabetes, hyperlipidemia, blood disorders and psychological conditions among multiple traditional uses globally (Chang et al., 2012; Chaudhary et al., 2023). This pharmaceutical value of *E. neriifolia* is further evidenced by recent use in patients hospitalized with Covid-19 (Pramanik et al., 2022), where patients treated with *E. neriifolia* leaves as an adjunct to standard of care improved faster than patients given standard of care regimen only. Although that study cannot be deemed statistically significant due to a small

number of participants, the findings were nevertheless encouraging. That method of use employed in the study would be similar to using SE. Therefore, while findings of more features in one sample type can be taken as greater potential of one over the other as there are possibly more metabolites with potential activity, taken in isolation may not necessarily be definitive in terms of pharmacologically relevant activity. Each sample type could have different applicable roles in therapy. The difference in chemical composition of latex compared to TE and SE was expected, but the high number of features detected was surprising considering latex is exuded with specific functionality in plant defense from the laciferous system (Benjamaa et al., 2022; Mazur et al., 2022). Possibility exists that this attribute is responsible for latex being deemed the most valuable component of *Euphorbia* species (Salehi et al., 2019). Though, with the reported toxicity, commercial use as paints and rubber are probably more likely responsible for this categorization (Pisano et al., 2016; Salehi et al., 2019) since no reference seen alluded to this extensive number of features in the sample type previously. The possibility exists for employing LE in different treatments than other sample types, resulting from chemical uniqueness of LE. Even though the TE samples presented a higher number of features per the hypothesis, it was expected to possess significantly more than LE.

As a second approach, TE and LE were examined to determine up-regulated or down-regulated features relative to the control. Volcano plot analyses were performed with VolcaNoseR (Goedhart & Luijsterburg, 2020) to generate volcano plots that incorporate a summary of t-test and fold change

analysis. This data is represented in Figure **6** A which shows greater up-regulation in the TE compared to SE and Figure **6** B that shows LE and SE. In Figure **6**, each dot represents one feature. Pink dots show the higher accumulation of features in the TE and LE compared to SE samples. The cyan dots indicate that those features accumulate less in TE and LE compared to SE which is used as the standard since it is obtained directly from the leaf tissue and thought to be less active. The grey dots indicate features in common with no significant difference in expression all using parameters of $log_2FC>1$ or $log_2FC<-1$ and false detection rate of <0.05. While it is noted that accumulation of features and concentrations may vary depending on factors including the plant's growth stage and environmental conditions, still no study has been found with which to compare these findings. Further analysis can be conducted to test various parameters.

The volcano plot (Figure **6** A) indicates that of 1622 features affected, 16.5 % (268) in TE presented with lower abundance, otherwise termed downregulated for this purpose and 83.5 % had higher abundance or upregulation than SE. This contrasts with SE and LE Figure **6** B where 40.3% to 59.7% of 1808 features had lower and higher abundance in LE respectively. Abundance could be attributed to the treatment (whether heated as in TE or unheated as in SE and LE) resulting in greater or lesser extraction of metabolites. Upregulation and downregulation should be understood in the context that metabolites are observed in samples being compared and refers to the abundance of a metabolite per sample. Therefore, when one feature/metabolite is shown at elevated levels in one

sample type, conversely the same feature is lower in the control sample to which the comparison is made (TE or LE and the control SE).

Simplistically, in Figure **6** A and Figure **6** B, one pink dot represents a feature with greater levels in either TE or LE compared to SE while a cyan dot represents a feature that is less. Conversely, cyan dots express features with greater accumulation in SE while pink dots indicate features with lower amounts compared to either TE or LE.

The features in LE are interesting. Most of what is known on latex results from research on few species related to rubber production like the “rubber tree” *Hevea brasiliensis* from which natural rubber is made and *E. characias* from which many diterpene compounds were characterized (Pisano et al., 2016). Despite hypotheses on possible functions based on presence of concentrated defense substances, the specific functions of laciferous systems and latex remain unknown (Mazur et al., 2022). Proteomics formed a major research component of *H. brasiliensis* due to allergic sensitization that occurs with rubber/latex use in humans (Medda et al., 2011; Pintus et al., 2010) and continues to enjoy the spotlight with *E. characias*. Pathogenesis related (PR) proteins were the most identified proteins in latex (Gracz-Bernaciak et al., 2021; Mazur et al., 2022). Enzymes, carbohydrates and minerals continue to be studied (Bottier, 2020). The continued focus on these constituents results in a lack of a comprehensive broader range of the compounds in latices. Proteins, enzymes and minerals were not targeted in this thesis for which some of the elevated features could possibly be explained.

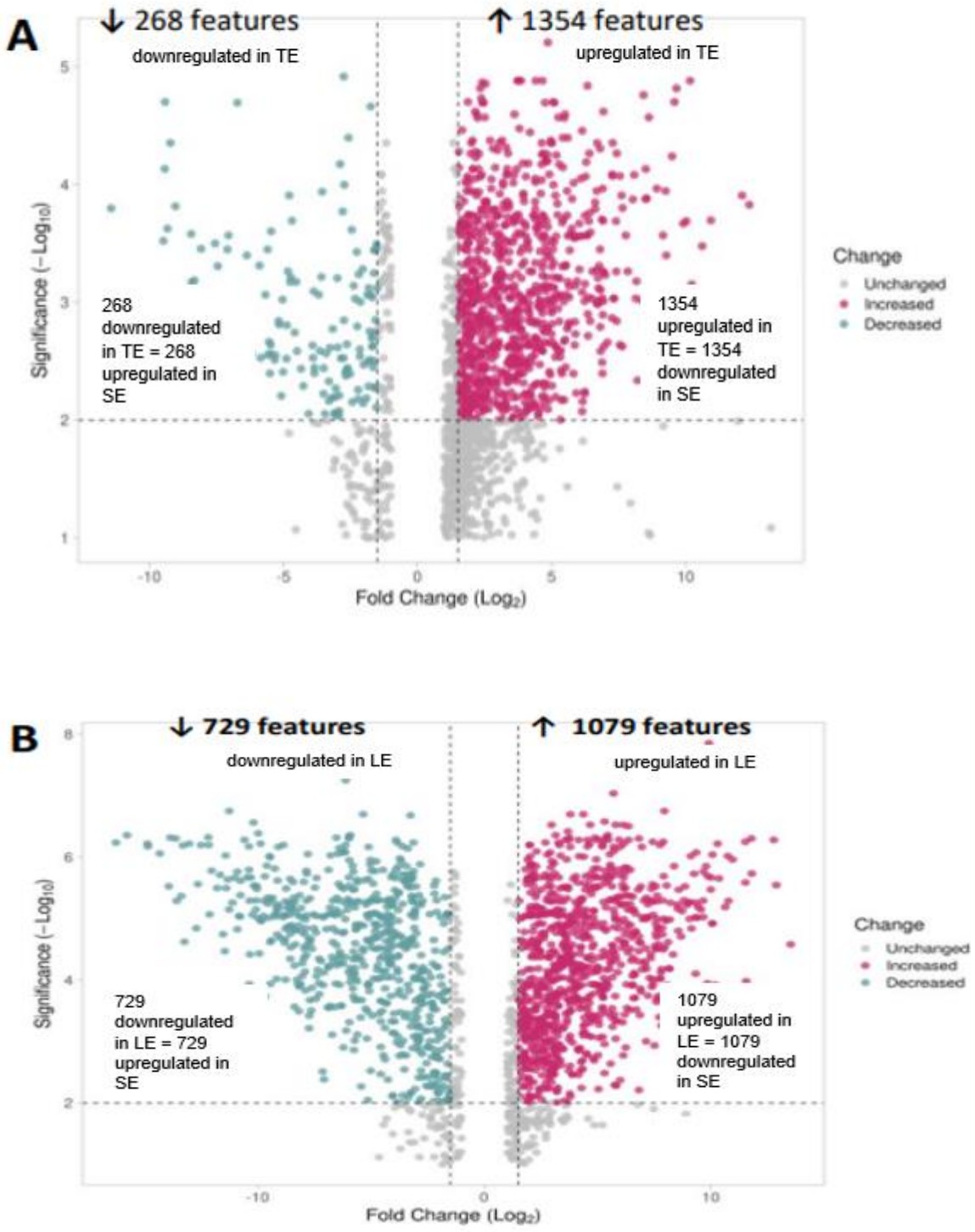

Figure **6** A and Figure **6** B**:** Volcano plots showing up or down-regulated features *(i.e., increased (pink) or decreased (cyan)). (A) shows (SE) versus (TE) and (B) shows (SE) versus (LE) extracts. The threshold of fold-change values is at $log_2FC>1$ or FC<-1 for features of interest.

Secondary metabolites like flavonoids, alkaloids and terpenoids, are credited with being more biologically active and dominant constituents of *Euphorbia* (Magozwi et al., 2021; Salehi et al., 2019), thus the next step was to investigate major pathways expressed to determine if these secondary metabolites were consistent with literature. Features from XCMS Online including m/z, retention time and p-values were used to determine metabolites in Kyoto Encyclopedia of Genes and Genomes (KEGG) (Braga et al., 2022) database with *Arabidopsis thaliana* reference metabolome. Annotated compounds were screened and mapped via the pathway enrichment module in MetaboAnalyst 5.0. This was done to reveal the major metabolic pathways in which the detected features are involved and to confirm presence of the principal characteristic constituents of *Euphorbia.* The pathway analysis is shown in Figure **7**. The finding is consistent with literature as features involved in the pathways of highest significance belong to classes of terpenes and flavonoids. Additionally, flavonoid formation is perpetuated by the phenylpropanoid pathway which is shown as the most significant (Kumar & Pandey, 2013).

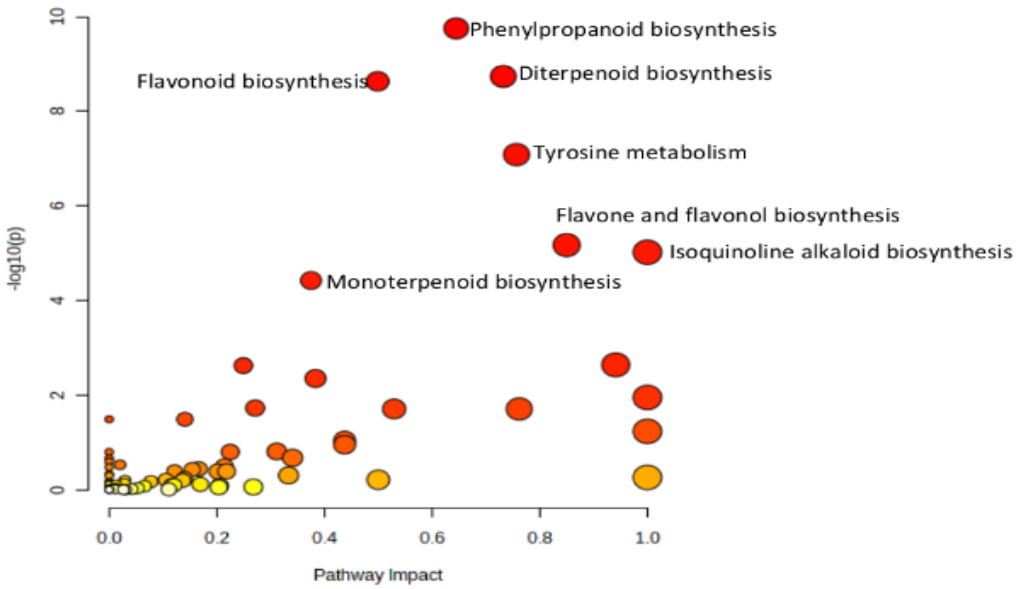

Figure **7**: Pathway analysis of putative metabolites found using the mummichog algorithm within MetaboAnalyst 5.0 analysis mode from all detected features for all sample types. It shows most prevalent pathways from SE, TE and LE collectively.

It warrants attention that up to this point the untargeted approaches previously shown using the Venn diagram, volcano plot and pathway analyses produced an overall view of *E. neriifolia* constituents. The process was non-selective since the global screen used all the data returned from XCMS Online with regards to the m/z values and therefore includes all metabolites irrespective of categorization as phytochemicals or phytohormones. Despite some shared features shown in the Venn diagram, *E. neriifolia* sample types were shown to be distinctly different from each other. This can be adequately seen in the principal component analysis (PCA) in Figure **8**. The PCA transforms original variables into a new set of uncorrelated variables called principal components (PCs). Each PC is a linear combination of the original variables and the first few typically

account for most of the variation in the data. This was used to visualize the overall structure and clustering patterns in the data, identify outliers, and detect trends in the sample types (Richardson, 2009). The plots show comparisons of TE vs SE and LE vs SE respectively.

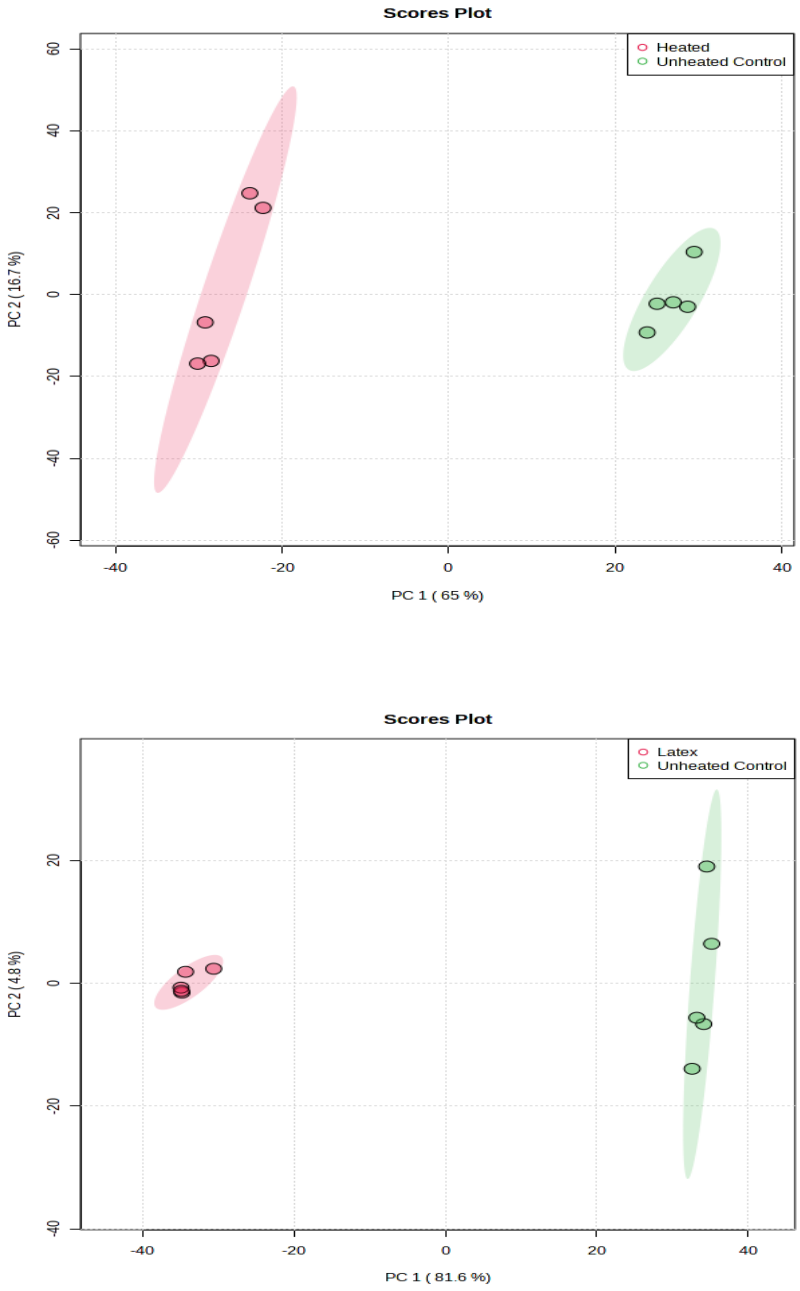

Figure **8**: PCA comparing (A) TE to SE and (B) L to SE. PCA gives an overview of the separation of sample types and indicates distinct sample differences.

The PCA, together with Partial Least Squares Discriminant Analysis (PLS-DA) – a multivariate statistical method used in metabolomics studies to analyze and classify data based on differences in pre-defined groups were used to identify variables or features responsible for the separation between the sample types. Fifteen top features were determined most responsible for the divergence of samples of TE vs SE and LE vs TE by the Variance in Importance Projection (VIP) emanating from the PLS-DA (see APPENDIX B). Eight were identified at a confidence level of 5.

From this point forward, the untargeted approach was narrowed to primarily focus on screening specific features in sample types in relation to asthma therapy. The differences between the sample types determined as major features responsible for divergence by the VIP were checked in MetaboQuest for putative matches using the m/z values in the VIP (see APPENDIX B). One m/z value that occurred at 301.03333 on the VIP was shown to be involved in the separation in both plots. Putative matches for this value were returned as pseudopurpurin or emodic acid, both anthraquinones with formula $C_{15}H_8O_7$ and similar exact masses at 300.027. Anthraquinones are noted for their wide range of activities including anti-oxidant and anti-inflammatory properties (Marković et al., 2018). Emodic acid or emodin displayed suppressant effects on immunoglobulin E (IgE) -mediated anaphylaxis (Lu et al., 2011). Suppression of this cytokine is beneficial to asthma and other allergic diseases. Emodin is also known for its laxative effects and literature indicate the use of *E. neriifolia* as a laxative (Sultana et al., 2022). Only one feature was upregulated in LE relative to

SE. Interestingly, in both VIPs the feature at 301.03333 was lower in abundance in TE and LE relative to SE. This metabolite is at a confidence level of 5. More research would be required for complete identification.

The features in the VIP were checked in the annotated single-class metabolite list to check their electron ion chromatographs. Forty percent of the features in the VIP for both groups TE vs SE and LE vs SE were contained in the top two thousand features. So, more features responsible for main differences were found to occur outside of the main peak features, i.e., features for which electron ion chromatograms were available. Most of these features were further unmatched in the databases and therefore returned no putative metabolites occurring at the m/v values or were unrelated to inflammatory or antioxidant properties. The TE vs SE VIP also revealed possible putative compounds with properties for cardiac and bone tissue consistent with would be pertinent to other reported uses of *E. neriifolia*.

The feature at 413.30264 in the SE vs TE VIP returned a putative match for a metabolite dihydrodiosgenin. Dihydrodiosgenin, a steroidal saponin found in several plants in the *Dioscorea* genus, fenugreek and yam, is indicated in anti-inflammatory disorders such as acute pancreatitis, lung injury and asthma (Parama et al., 2020; Shen et al., 2018). Confirmation of this metabolite is at level 4 in TE sample only which means mainly mass and structure confirmed (Sumner et al., 2007).

The m/z values from total screen were then subjected to database searches in metaboQuest. Characteristic phytoconstituents of *Euphorbia* were

identified in *E. neriifolia* which included afzelin, kaempferol namely kaempferol-3-O-rutinoside, kaempferol-3-O-α-rhamnoside, and Beta-amyrin. These are expected as confirmed in (Mali & Panchal, 2017; Sultana et al., 2022) and were identified at a confidence level 2 with matching of m/z values and fragmentation pattern through databases. All exhibit anti-inflammatory properties. Terpenes like linalool and 1,8 cineole were indicated to possess anti-inflammatory properties with a steroid-saving effect for asthma (Juergens et al., 2003). 1,8 cineole reduced various cytokine levels of IL-1B, IL-4, IL-6 IL-13 and IL-17 as well as TNF-α and PGE2 (T. Kim et al., 2020). These metabolites were shown in global screen at m/z 153.1267 but were not confirmed to level 2.

Enzymes and proteins identified in the untargeted approach were disregarded as they were not a focus of this thesis with respect to asthma therapy. This could be a future consideration as evidence indicate the large amount of these constituents in latex (Gracz-Bernaciak et al., 2021). Secondary metabolites noted in LE include general classes: terpenes, alkaloids, proteins, enzymes, phenolics and cardenolides. Triterpenoids namely cycloartenol, lupeol and lanosterol are reported to have higher concentrations in latex than other tissues. These latex constituents are representative of multiple families (Gracz-Bernaciak et al., 2021; Pintus et al., 2011) but may differ according to species. Lupeol and lanosterol were detected but not confirmed to level 2.

Four other metabolites, namely rutin, luteolin, chlorogenin and isatin were confirmed at a confidence level of 2. See Table **4**. Mass spectra for these metabolites can be found in APPENDIX C. Eudesmin, a lignan shown to possess

neuroprotective properties with application in Alzheimer's disease, also indicates antibacterial and anti-inflammatory activity (Castillo et al., 2022). This metabolite was detected with a confidence level of 2. It occurred in metabolites with the $[M+Na]^+$ adducts which is outside the previously set strict detection criteria of $[M+H]^+$ adducts only for a narrower focus and so is not reported in the table. Eudesmin was detected only in the traditional sample and is noteworthy since the stipulated criteria would have caused exclusion of putative compounds with possible therapeutic potential to be identified. This was however necessary since using more adducts returned an overwhelming number of putative metabolites initially.

Isatin, a versatile indole derivative from plants has been shown to possess antiallergic properties in addition to cytotoxic, antimalarial, antiviral and antimicrobial pharmacological activity (Cheke et al., 2022; Kandasamy et al., 2010). It is also a metabolite of the neurotransmitter serotonin and has been shown to positively affect dopamine levels in conjunction with antiparkinsonian agents (Faro et al., 2020). Given the traditional use of *E. neriifolia* for brain stimulation isatin may be a contributor. Luteolin and kaempferol-3-O-α-rhamnoside were both found in SE and TE. Isatin was not detected in TE but was the only compound in this group seen in LE. All compounds were detected in SE except a purine nucleoside, N^6-threonylcarbamoyladenosine which was only seen in TE. Although not unexpected as this t^6A nucleoside is conserved in tRNAs across kingdoms, its presence only in the heated sample warrants further exploration. It was noted as a potential biomarker for Covid-19 disease

(Nagayoshi et al., 2022). This finding was interesting due to the similarity in respiratory distress Covid-19 and asthma and the results of detected cytokinins that follow.

Table **4**: Level 2 metabolite annotations as generated by MS-DIAL. Compounds were matched using various public databases in positive ionization mode as described on RIKEN's MS-DIAL website. Only M+H adducts were considered.

Family	Metabolite	Molecular Formula	Observed m/z	Fragments	Sample Type
Flavones, flavanols and flavonoid3-O-glycosides	Luteolin	$C_{15}H_{10}O6$	287.0538	287.0541, 90.9570	TE, SE
	Kaempferol-7-neohesperidoside	$C_{27}H_{30}O_{15}$	595.1629	287.0541, 85.0281	SE
	Kaempferol-3-O-alpha-rhamnoside	$C_{21}H_{20}O_{10}$	433.1111	287.0541, 85.0281	TE, SE
	Rutin (Rutoside)	$C_{27}H_{30}O_{16}$	611.1585	303.0490, 216.1097, 85.0281	SE
Quinic acids and derivatives	Chlorogenic Acid	$C_{16}H_{18}O_9$	355.101	163.0384, 111.0435	SE
Indoline	Isatin	$C_8H_5NO_2$	148.0387	148.0386, 92.0490, 58.8082	SE, LE
Secondary alcohol	Beta-amyrin(ai)	$C_{30}H_{50}O$	427.3924	409.373169, 191.179489, 427.391724, 137.129868, 428.389130	TE
purine nucleoside	N^6-threonycarbamoyl adenosine	$C_{15}H_{20}N_60_8$	413.1389	281.0985, 162.0400, 136.0615	TE

Selected compounds from the global screen that showed up in multiple databases were checked in literature and those having anti-inflammatory properties detected at a confidence level of 3 are shown in Table **5**. Roseoside or vomilfoliol 9-O-B-D glucopyranoside has been noted in multiple databases and

has been detected in *E. heteradena* (ÖKSÜZ et al., 2002). No studies were found to show its presence in *E. neriifolia* and its actions however are indicated as anti-inflammatory in a study on *Chaenomeles speciosa* (Yao et al., 2020). The phorbol ester 12-deoxyphorbol-13-angelate was detected only in the latex (LE) sample which is congruent with other studies for the species for the presence of phorbol esters (Tsai et al., 2016). However, no studies were found that indicated its presence in *E. neriifolia*. The chemical formula $C_{35}H_{34}O_6$ is the same as for ingenol mebutate a compound known for its use in actinic keratoses and both are tetracylic compounds with similar structures (Ramsay et al., 2011). These compounds are identified as being the same (Zhao et al., 2022). No study has reported its presence in this species to date. Interestingly, 12-deoxyphorbol-13-angellate has the same mass as budesonide as indicated on PubChem. Budesonide, a synthetic glucocorticoid, is a potent medication in asthma therapy that was used in children 5 – 12 years (Hopp et al., 2023). The ringed structures of budesonide and the phorbol compound are interesting because of structural similarity in the number of rings. The use of oral budesonide for eosinophilic esophagitis would suggest possible use of latex metabolites for this condition, but risk assessment for safety considering toxicity must be determined. Overall, most compounds in the untargeted global screen were detected in SE than the other sample types. Table 5 shows the level 3 metabolites.

Table **5**: Level 3 metabolite annotations of selected anti-inflammatory compounds in all sample types as generated by SIRIUS 5.6.3. A zodiac score (over 95%) and a similarity score (over 75%) were chosen as cut offs for this table.

Compound	Sample Types	Experimental Mass	Formula	Zodiac Score [%]	Similarity [% SIRIUS Score]	R. T.	Fragments	Ontology: Compound classes/biochemical pathways
Heriguard	SE, TE	355.1011	$C_{16}H_{18}O_9$	100	100	3.22	181.0486; 163.0382; 145.0285; 135.0441	Quinic acids and derivatives; phenylpropanoids
3-O-Feruloylquinic acid	SE, TE	369.1172	$C_{17}H_{20}O_9$	100	98.13	3.94	177.0543; 145.0282; 137.0229	Quinic acids and derivatives; phenylpropanoids
Roseoside, (vomifoliol 9-O-B-D glucopyranoside	All	387.1998	$C_{19}H_{30}O_8$	100	96.09	4.99	207.1372; 189.1274; 179.1439; 123.0793; 149.0954; 95.0856	Alkyl glycosides; Terpenoids
12-Deoxyphorbol 13-angelate	LE	431.2413	$C_{25}H_{34}O_6$	99.95	90.1	10.03	313.1790; 295.1677; 277.1596; 267.1719; 179.0848; 83.0490	Tetracyclic diterpenoids; terpenoids; tigliane and ingenane diterpenoids
Reinutrin	SE, TE	435.0907	$C_{20}H_{18}O_{11}$	97.085	99.69	6.33	303.0505; 115.0387; 73.0280; 61.0285	Flavonoid-3-O-glycosides; flavonoids
Trifolin (Kaempferol 3-O-rutinoside)	SE, TE	449.1057	$C_{21}H_{20}O_{11}$	93.467	99.69	6.41	287.0524; 145.0483; 127.0393; 85.0282	Flavonoid-3-O-glycosides; flavonoids
Hirsutrin	SE	465.1016	$C_{21}H_{20}O_{12}$	98.291	100	6.16	303.0504; 145.0499; 109.0283; 97.0283	Flavonoid-3-O-glycosides; flavonoids
Mikwelianin	SE	479.0809	$C_{21}H_{18}O_{13}$	99.44	98.3	6.28	303.0504; 159.0288; 141.0172; 131.0338; 113.0225	Flavonoid-3-O-glucuronides; flavonoids; phenylpropanoids
methyl 2-[(2S)-3-oxo-2-[(Z)-pent-2-enyl]cyclopentyl]acetate	SE, TE	225.1477	$C_{13}H_{20}O_3$	100	96.13	11.32	193.1209; 81.0330; 175.1107; 147.1159; 83.0849	Jasmonic acids; cyclic ketones; octadecanoids
Coumarin	SE	147.0433	$C_9H_6O_2$	100	93.42	14.11	103.0535; 91.0538; 85.0281; 68.9966	Pyranones and derivatives; 1-benzopyrans
Phyllanthusiin E	SE	293.0296	$C_{13}H_8O_8$	100	78.16	5.18	247.0223; 219.0281; 191.0334	Gallotannins; phenolic acids; phenylpropanoids
Eriojaposide B	SE	517.2615	$C_{25}H_{40}O_{11}$	93.166	75.71	7.51	149.0956; 105.0180; 191.1416	Terpene glycosides; Terpenoids (Megastigmanes)

3.1.2. Semi- targeted metabolites identification

A more focused approach was employed following the broad untargeted approach. Here, metabolites with inflammatory and antioxidant properties were assembled from literature on medicinal plants for investigation rather than researching the plethora of putative matches returned from the global screen to determine if these were main characteristics. The term semi-targeted is used since it involves targeted identification of specific metabolites from the untargeted global screen or full scan data instead of investigating what shows up. Additionally, manual inspection of individual extracted ion chromatograms (EIC) was done in each sample to ensure acceptable quality m/z peaks with minimal signal-to-noise ratios or interference to further reduce features from the original two thousand peaks. Some examples are shown in APPENDIX E. This yielded a more manageable sample set with almost 90% fewer features than the triple Venn diagram from the untargeted screen Figure **5**. This reduced the putative candidate pool from the original 13,704 to 1,675 or roughly 12% Figure **9**. A list of m/z values is shown at APPENDIX J.

This reduced feature set was used for detection and identification of secondary metabolites with known characteristic properties in asthma therapy in a semi-untargeted approach as previously indicated. Confirmation of the results was accomplished at a confidence of level 2 (Sumner et al., 2007). A greater number of metabolites of interest were detected in TE and SE than LE.

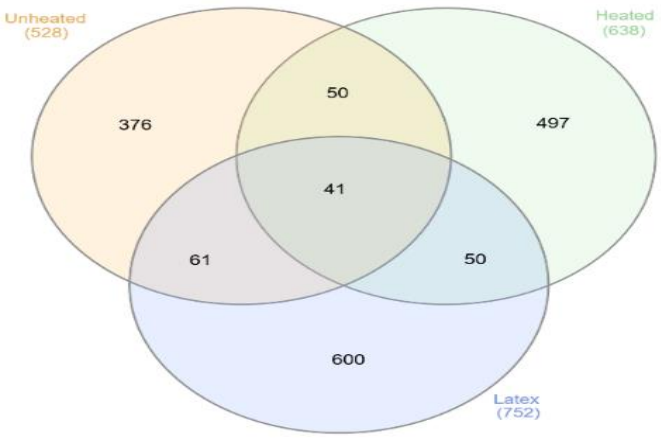

Figure **9**: Venn diagram shows reduced features from the global screen after visual inspection of EICs to obtain the highest quality peaks for putative compound evaluation. Features reduced to approximately 12%.

Fourteen compounds from the assembled list for semi-targeted identification were detected (see Figure **10**). Protocatechuic acid, an ingredient in grapes, onions and green tea which attenuates airway inflammation (Q. Li et al., 2020) was detected in only TE and caffeic acid with DNA damage protection and antioxidant properties (Owumi et al., 2022) was identified only in LE. Both compounds are phenolic acids with a carboxylic group (Kakkar & Bais, 2014). Supplementation with protocatechuic acid in piglets decreased serum levels of TNF-α, IL2 and IL-6 levels (Hu et al., 2020). The reduction of IL-6 in humans could be a potential mechanism of action in improving asthma symptoms. Caffeic acid was shown to possess airway regulatory functions to improve asthmatic conditions (Ma et al., 2016) but further studies would be required to prove efficacy and safety in asthma for either metabolite. This shows that different compounds with similar functions are expressed in the sample types. Although neither of these was present in the SE, ferulic acid, belonging to the same group

(Hu et al., 2020) was detected in that sample type and has been shown to prevent airway remodeling by inhibiting TH2 cytokines (Brugiolo et al., 2017).

Latex, deemed to have pro-inflammatory and tumor-promoting activity and toxicity associated with phorboid compounds with ingenane, tigliane and daphnane diterpene derivatives (Wu et al., 2009), shows fewer compounds with anti-inflammatory properties from the assembled list. While a lower number of the semi-targeted metabolites were found in SE and LE than in TE (Figure **10**), lower ability to act against inflammation cannot be predicted since the list is not exhaustive and other compounds were not captured. Further investigation would be needed. The list of compounds is shown in Table **6** and the peak intensities for the metabolites are shown in APPENDIX D.

Neriifolin and prostratin levels were statistically higher in LE than in TE and SE. The presence of neriifolin was not surprising in LE as it is consistent with literature (Yadav et al., 2012). In contrast, prostratin, a tigliane-type phorbol ester isolated from *E. fischeriana* and assessed for its anti-HIV properties (Barrero et al., 2011; Jian et al., 2018), was unexpected in TE and SE samples of sweet aloes even at low does due to phorbol toxicity. An attempt to confirm its identification to level 1 from the current level 4 could be pursued in future. Interestingly, the known characteristic metabolites quercetin, rutin, and myricetin showed higher levels in SE but more metabolites, 7 from 14 (Figure **10**) were higher in TE than SE and LE. Latex shows quite different overall profile with 3 dominant compounds; caffeic acid, neriifolin and prostratin and 11 compounds that are either not present or only at trace levels.

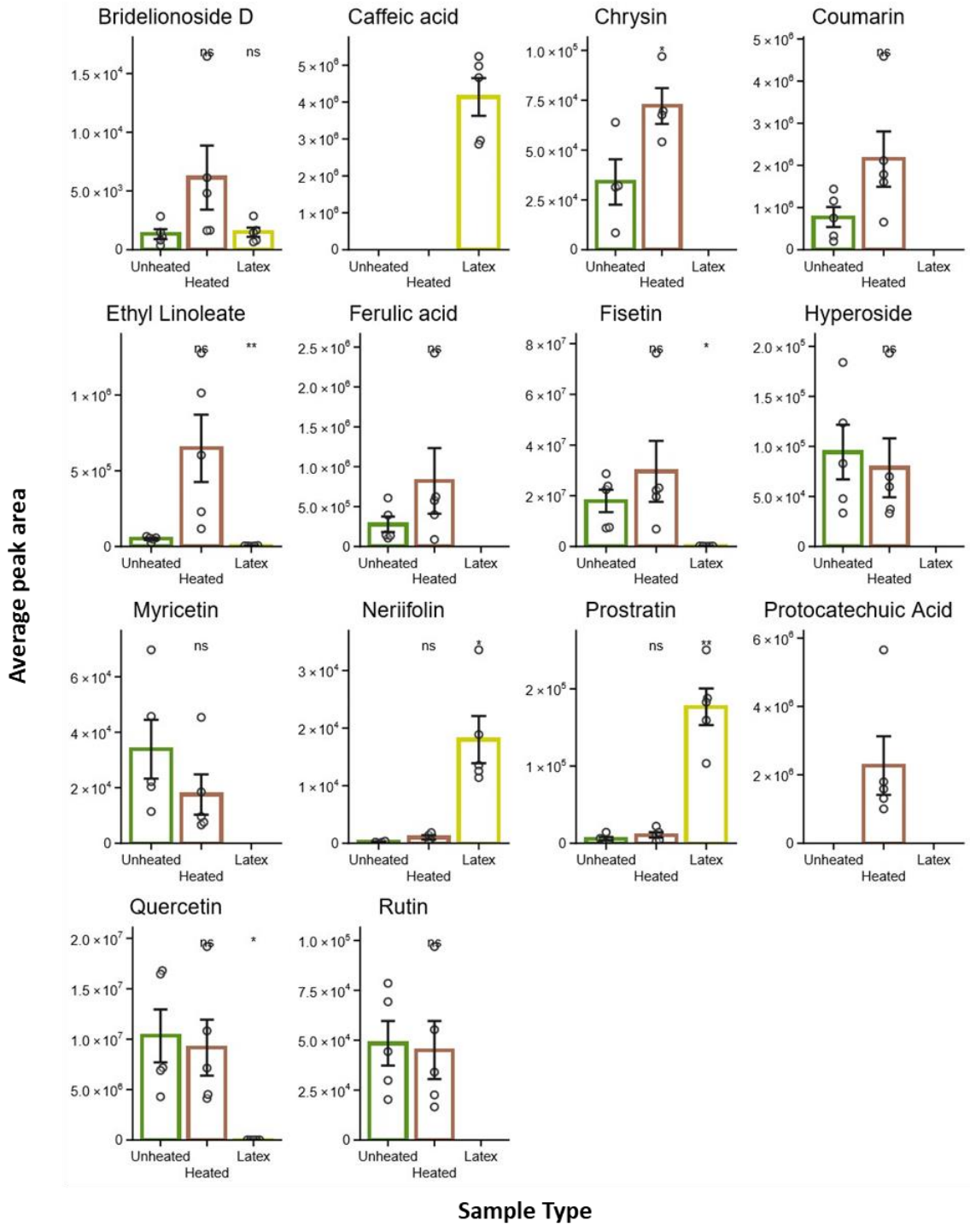

Figure **10**: Quantities of therapeutic metabolites as shown by average peak areas according to sample type (n=5). The star symbol signifies statistical difference between the referenced SE and the investigated TE and LE samples. The term “ns” means not significant. N.B unheated refers to the simple extract SE and heated to the traditional extract. Compounds are detected at confidence level 3 (Sumner et al., 2007).

Table **6**: Metabolites with antioxidant and / anti-inflammatory properties from list assembled from literature (Table **3**) to devise a more focused identification of compounds from the broad global screen in XCalibur 4.3.

Metabolite	Chemical formula	M	M+H	Detected in Sample
Bridelionoside D	$C_{19}H_{36}O_8$	392.2405	393.2483	ALL
Caffeic acid	$C_9H_8O_4$	180.0417	181.0495	LE
Chrysin	$C_{15}H_{10}O_4$	254.0573	255.0652	SE, TE
Coumarin	$C_9H_6O_2$	146.0362	147.0441	SE, TE
Ethyl linoleate	$C_{20}H_{36}O_2$	307.2631	308.271	ALL
Ferulic acid	$C_{10}H_{10}O_4$	194.0573	195.0652	SE, TE
Fisetin	$C_{15}H_{10}O_6$	286.0472	287.055	ALL
Hyperoside	$C_{21}H_{20}O_{12}$	464.0949	465.1028	SE, TE
Myricetin	$C_{15}H_{10}O_8$	318.037	319.0448	SE, TE
Neriifolin	$C_{30}H_{46}O_8$	534.3187	535.3265	ALL
Prostratin	$C_{22}H_{30}O_6$	390.2037	391.2115	ALL
Protocatechuic acid	$C_7H_6O_4$	154.026	155.0339	TE
Quercetin	$C_{15}H_{10}O_7$	302.0421	303.0499	ALL
Rutin	$C_{27}H_{30}O_{16}$	610.1528	611.1607	SE, TE

3.2.1 Targeted Identification of Cytokinin (CK) and acidic type phytohormones

The traditional (TE), latex (LE) and simple extracts (SE) were investigated for cytokinins. Samples were spiked with isotopically labelled standards (IS) which allowed mass spectrometric detection and quantification based on comparison of relative peak area of endogenous compound to authentic standards. The detection and identification of these compounds attain confidence level 1 due to the use of authentic standards as full identification occurs (Sumner et al., 2007). This class of hormones has been implicated in improvement of human health conditions with pharmacological activity of phytohormones occurring at femtomolar levels with observable variation in activity at increased levels in some instances (Kisiala et al., 2019; Naseem et al., 2020; Voller et al., 2010). However, endogenous phytohormones have not been explored in *E. neriifolia* nor even the genus previously. There were observable variations in the proportion and amount of the six cytokinin fractions in the different sample types (Table **7**). Almost twice the number of cytokinins were detected in SE (13) compared to LE (7). The highest number occurred in TE with 22. No literature was available for comparison as this is the first exploration of cytokinins in the species.

In SE, no aromatics (AR), two FBs, all RBs, 3 NTs, 4-Glucs, and 3 acidic hormones were detected. Notably, aromatic CKs were detected only in heated samples together with 3 FBs, all RBS, NTs, Glucs, 1 MeS but no acidic

hormones. Latex extracts showed the fewest with 1 FB, all RBs, 2 NTs and 1 acidic hormone (Table **7**).

Table **7**: Concentration of phytohormones in three sample types extracts of *E. neriifolia.* These were reported as the mean ± the standard error.

Hormone class	Compound	SE	TE	LE
		[pmol / g fwt]	[pmol / g fwt]	[pmol / g fwt]
Cytokinins				
Aromatic CKs	BA	-	-	-
	BAR	-	0.29 ±0.11	-
	KIN	-	42.39 ±18.95	-
Freebase CKs	iP	2.32 ±0.12	1.16 ±0.1	2.46 ±0.13
	tZ	0.7 ±0.32	1.5 ±0.38	-
	cZ	-	0.64 ±0.09	-
	DZ	-	-	-
Ribosides - CKs	iPR	16.7 ±1.95	10.5 ±3.08	2.65 ±0.48
	tZR	1.85 ±0.35	1.25 ±0.39	0.17 ±0.05
	cZR	1.24 ±0.19	1.35 ±0.54	4.13 ±0.48
	DZR	14.23 ±2.97	0.41 ±0.19	10.84 ±1.16
Nucleotides - CKs	iPNT	261.62 ±52.27	19.2 0.98	-
	tZNT	10.2 ±3.39	4.44 ±0.92	0.25 ±0.08
	cZNT	8 ±2.97	45.13 ±1.57	1.92 ±0.44
	DZNT	-	0.08 ±0.02	-
Glucosides -CKs	iP9G	-	0.14 ±0.04	-
	tZ9G	-	0.38 ±0.21	-
	tZOG	8.2 ±2.76	18.62 ±3.1	-
	cZOG	-	2.78 ±0.55	-
	DZOG	-	0.39 ±0.07	-
	tZROG	22.29 ±14.37	41.9 ±16.37	-
	cZROG	17.42 ±4.16	28.9 ±10.12	-
	DZROG	3.66 ±1.59	10.65 ±3.81	-
Methylthiols -CKs	MeSZ	-	-	-
	MeSZR	-	0.86 ±0.34	-
Acidic Hormones	ABA	61.74 ±11.37	-	-
	GA7	-	-	0.53 ±0.09
	IAA	381.11 ±88.38	-	-
	JA	220.51 ±35.71	-	-
TOTAL		**1,031.79**	**232.96**	**22.95**

Of the six possible cytokinin classes, four were detected in the SE compared to TE where all six were detected and only three in LE. SE contained the highest concentration of total CKs, followed by TE while LE had much lower than both. The FB form of CKs was notably low relative to other CK forms in all sample types. MeS in trace amount and AR-CKs were detected in TE unlike other sample types lacking them while LE additionally was deficient in Glucs (Figure **11** A).

The differences extended to the percentage composition of the specific fractions among the sample types. SE comprised 76% NTs, 14% Glucs, 9.2% RBs and 0.8% FBs. In TE, NT forms (29%), Glucs (44.5%), RBs (5.8%) Glucs, FB (1.4%) with AR being 42.68% and trace amount of MeS (Figure **11** B). NTs are prone to degradation into RBs (Kudo et al., 2010) which could possibly explain the significant change of NTs between SE and TE from 76% to 29%. The RB level in TE was still lower than SE but the ratio of RBs to NTs in SE and TE increased from 0.12 to 0.2 respectively. Therefore, differences in CK levels could partially be a function of degradation and / or aggressive extraction of components in TE that were otherwise available in SE. Notably higher levels of R-O-Glucs were present in TE. This was probably due to interconversion via glycosylation of free base to O-Glucs to a lesser but more likely as a result of the extraction method.

LE samples on the other hand, despite having lower total CKs compared to SE and LE, showed distinct difference with higher percentage composition of the active CK forms in plants at 77.5 % RBs and 10.7% FBs (Figure **11** B).

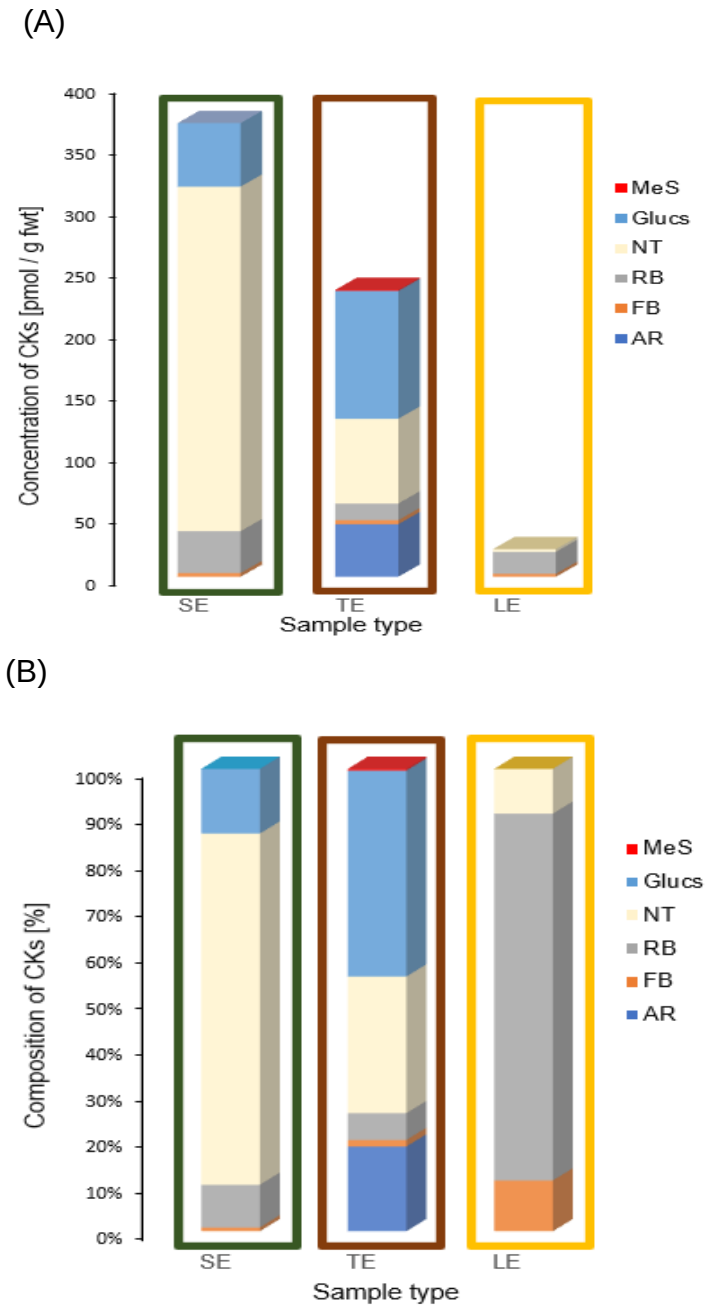

Figure **11**: Concentration of CKs in sample types of *E. neriifolia*. (A) shows the amount in each sample type. (B) shows the percentage composition of the fractions per sample type (B). The green, brown and yellow borders represent simple extract (SE), traditional extract (TE) and Latex extract (LE).

Latex is reported to contain terpenes, alkaloids, proteins and sugars which serve different roles in plant defense and physiology (Gracz-Bernaciak et al.,

2021). However, the concept of involvement of cytokinins in local and long distance signaling with respect to its presence in xylem sap (Kudo et al., 2010) makes the finding of CKs in LE intriguing. In the absence of literature for comparison, long distance signaling of CKs in latex may be a less common phenomenon, as latex is specialized, thick fluid exuded by the specific plant cells, laticifers at site of injury (Mazur et al., 2022). But, laticifers run throughout the plant system (Mazur et al., 2022) so it is possible that the cytokinins contribute to some long-distance signaling functions by movement of latex within the plant. As indicated, latex exuded when a plant is damaged (Sultana et al., 2022), offers protection from invading pathogens, insects or herbivores to prevent further damage (Benjamaa et al., 2022). The specific role and significance of cytokinins in latex and their involvement in signaling is not well established and the biological roles lack universal consensus (Pintus et al., 2010). The role of cytokinins in plants is more understood therefore release at the site of the wound could potentially contribute to local signaling events to promote cell division, tissue repair and thus wound healing. Although this could be a possible mechanism more research would be required to fully understand their potential functions. With respect to treatment of human diseased states like skin infections for which latex is implicated, the thick substance may first act as a barrier and cytokinins may offer similar protection in would healing. The role in asthma does however require further elucidation.

3.2.1.1 Free bases, ribosides and nucleotide CKs

Free base cytokinin iP and tZ types are the most active and important isoprenoid components in plants (Kudo et al., 2010; Miyawaki et al., 2006; Sakakibara, 2006). NT CK forms are detected at higher levels in SE and TE samples compared to LE while LE samples show more FB than NT. CK-NTs demonstrate a wide range of activity: anti-hypertensive, antiviral, antipsychotic and anti-inflammatory (S Drenichev et al., 2016). SE exhibited greater levels of iPNT and tZNT than TE, but TE showed more cZNT and DZNT. Higher levels of cZNT at 45.13 pmol/g fwt in TE compared to 8 and 1.92 pmol/g fwt in SE and LE respectively could indicate other potential applications. The trend iPR and iP for SE and TE are both approximately in proportions 1:8 within sample types. While DZ was undetected, the corresponding ribosides were present. Overall, a higher number of CK phytohormones at varying levels are present in TE than SE and LE (Figure **12**). For reference, weights of replicates and peak areas are recorded in APPENDIX F, G and H. LE, often referred to as sap or milky sap (Mazur et al., 2022), contained higher levels of DZR and cZR compared to LE and TE. The amount of these two ribosides were also higher than both iPR and tZR within the same LE sample. Evidence indicate iP-type cytokinins like iPR are the major forms of CKs in phloem sap and tZR is the major one found in xylem sap (Kudo et al., 2010) which differs from the pattern seen in LE. Already shown to be unique from SE and TE, LE shows differences from plant sap components. Hence the interchangeable use of latex and sap in literature should be reconsidered in the context of Euphorbia as the terms may be confusing.

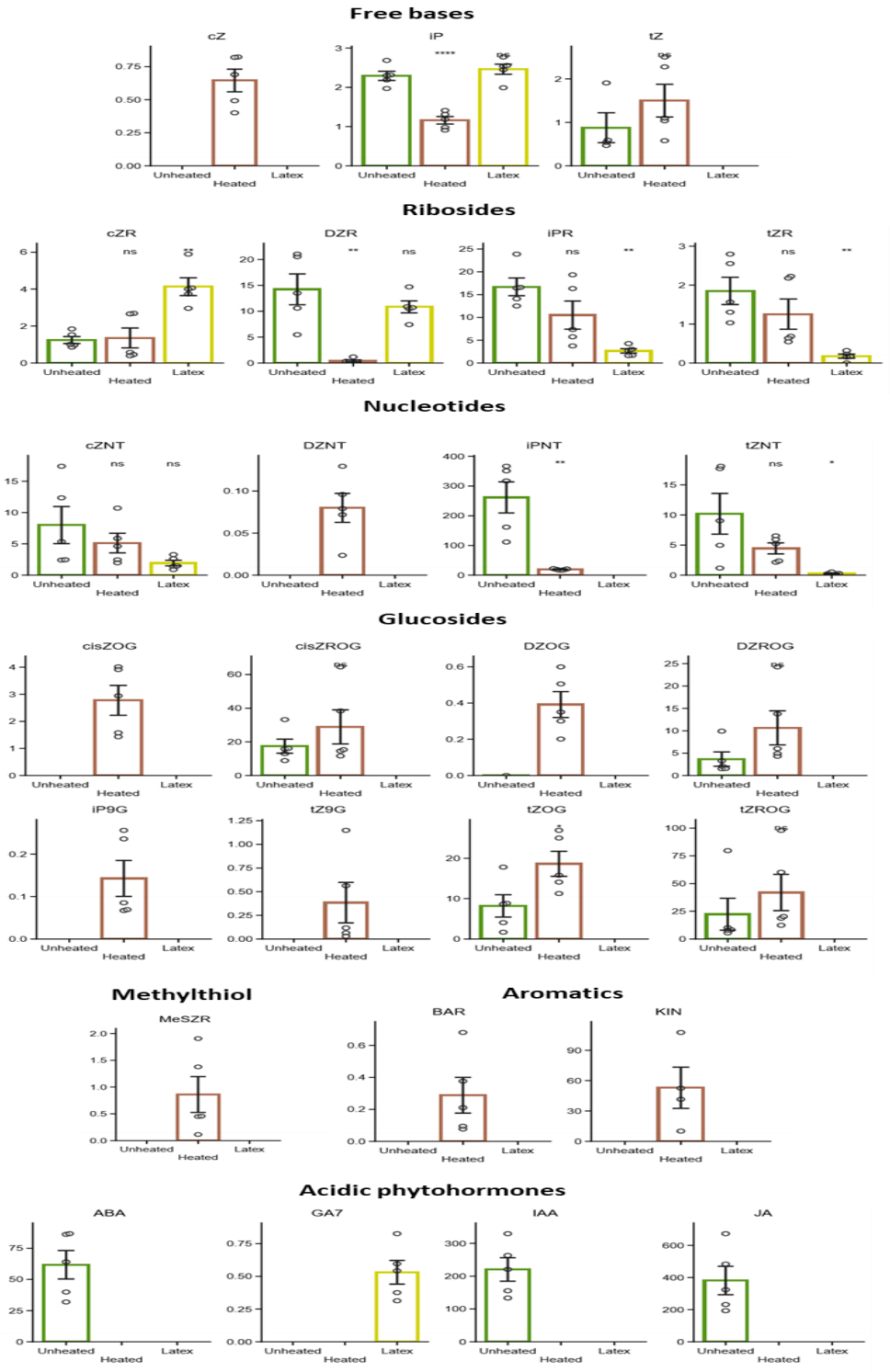

Figure **12:** CK and acidic phytohormone levels in SE, TE and LE samples with replicates (n=5). The star symbol (*) signifies statistical difference between the referenced control SE (unheated in green) and the investigated treatment types TE (heated in brown) or LE (latex in yellow). The term “ns” means not significant as it relates to difference between the SE and TE or SE and LE.

Generally, DZ was detected as RB and NT forms in samples. Patterns of RB detection levels varied among the samples as SE showed higher levels of iPR>DZR>tZR>cZR compared to TE with iPR>cZR>tZR>DZR and LE with DZR>cZR>iPR>tZR. This was quite curious because the hypothesis predicted presence of higher levels of active phytohormones in TE with greater potential in therapy. However, the dose-dependent activity of the molecules is also a consideration in pharmacological response which would necessitate further investigation as evidence with respect to hormones and asthma is unavailable.

3.2.1.2 Aromatics CKs and their action in humans – kinetin

Given their limited distribution in nature but significant health applications of aromatic CKs (Voller et al., 2010), presence of aromatics in a species could signal major potential. Despite incomplete understanding of the biogenesis of aromatics and cellular targets in humans (Othman et al., 2021), they are important as studies show improvement in health conditions with their application. The possibility therefore exists that these fractions play a role in the beneficial effects of the species. Kinetin, at a level of 42.39 pmol/g fwt was detected in the traditional extract and comprised the bulk of the aromatic cytokinins in the samples. While it was not reported to be directly involved in treatment of asthma, kinetin has been studied for both antioxidant and anti-inflammatory properties (Lappas, 2015; Lee et al., 2012; Souza et al., 2023), which could have positive effects on asthma resulting from the same triggers. Evidence suggest the activation of A2a-R receptors cause increased intracellular

cAMP levels without augmenting inositol triphosphate levels unlike with activation of A1, A2B and A3 receptors (Polosa & Blackburn, 2009). This leads to anti-inflammatory effects, bronchodilation and tissue repair. Therefore, inhibition of pro-inflammatory cytokines and agonistic effect on A2a-R receptors (Lee et al., 2012; Othman et al., 2021) could be potential mechanisms of action by which kinetin can exert its pharmacological effect in asthma.

Additionally, given known effects of kinetin in DNA and tissue repair, receptor damage and epithelial tissue dysfunction (Lappas, 2015; Puddicombe et al., 2000) which occur in asthma could be potentially improved by kinetin. To reiterate, the aetiology of asthma is largely unknown and genetic factors are implicated. Therefore the possibility for action of kinetin at the genome level based on its involvements in genetic splicing for familial dysautonomia (Hims et al., 2007) could potentially be mode of action. The action of kinetin in humans has gained much attention but has not been directed to respiratory disease, further studies would be needed to establish a direct connection between kinetin and the treatment or management of asthma.

BAR, like kinetin, has demonstrated effects in oxidative stress (Jablonska-Trypuc et al., 2016) and was confirmed as one of only three cytokinin ribosides with cytotoxic properties (Voller, 2021). The other two being iPR and KR. These contradict the study by Gonzalez et al that indicate positive effects of cZR (Gonzalez et al., 2021). Similarly, new information and research could determine application to chronic respiratory issues.

3.2.1.3 Glucoside and Methylthiolated CKs

Glucs and MeS types of cytokinins are mostly seen as inactive. The greater number of storage O-glucosides (O-Glucs) forms present in the TE compared to LE and complete non-detection in LE was another remarkable occurrence. Given that hormones play a role in regulation of plant growth (Kojima et al., 2009) and latex forms part of the lactiferous system responsible manly for plant defense (Mazur et al., 2022), it is not surprising that CK storage forms are absent from latex samples. It appears however that the heating process allowed for more extraction of these metabolites. A noteworthy finding is that larger quantities of riboside type O-Glucs were exhibited for further elucidation. Trans-Z types are the most abundantly occurring isoprenoids (Voller et al., 2010) and higher levels of trans-zeatin O-Glucs were found in TE. The presence of riboside forms of zeatin O-Glucs and difference in tZOG and tZROG for example, at almost twice the concentration of SE in TE again points to potential extraction by heating.

MeS forms were only detected in TE samples. The expression of methylthiolated CK riboside fraction, MeSZR in TE alone (Table **7**) in low amounts and consistent with literature, warrants more exploration as their origin, biological activity and functions are poorly understood (Gibb et al., 2020). The data remains inconclusive about MeSiP and MeSiPR which formed part of the initial investigation. There was good recovery of the IS in all samples of all sample types but signal to noise interference resulted in poor quality spectra and masking of endogenous compounds.

The MeS forms showed variation among individual replicates. This pattern was observed with other metabolites analyzed among each of the sample types. The patterns show the divergence of samples with respect to metabolite concentration in replicates irrespective of similar growth parameters. (See Figure **12**). The differences in metabolite content in each replicate of sample types can be seen in the plots.

3.2.1.4 Acidic hormones

The perceived value of phytohormones in therapy has been increasing and their roles as nutraceuticals are under investigation (Daliu et al., 2020; S. W. Kim et al., 2020; Magnone et al., 2020). Detection of hormones was noticeable in SE from *Euphorbia neriifolia.* There was good recovery of isotopically labelled standards (IS) in the SE for compounds screened. ABA, IAA and JA were exhibited in this sample type, (APPENDIX I). IS for JA was not added to samples, the peak areas in the analytes were intense in all replicates and detection was within 5 ppm error using ABA as the standard and METLIN for confirmation. GA_7 was detected in LE. The standard recovery in LE was poor but much worse in TE samples. Since the IS were added just prior to extraction which followed the heating process by a few stages, direct heat application cannot explain the poor standard recovery. A change in matrix or other factor must be explored to determine cause. As a side note IAA and JA derivatives were indicated as putative compounds in heated samples in the global screen. These were not explored further since preference was for confidence level 1 for the hormones in

light of possession of standards. This provided a rationale, however, for potentially altering the methodology for future detection.

Furthermore, latex samples were not heated but showed a similar result in poor standard recovery for some hormones, signifying the differences in the matrices compared to the simple leaf sample. Refer to APPENDIX I. Interestingly, the appearance of GA in latex could be an indication of its use in asthma as it is stated that it acts by inducing the anti-inflammatory A20 protein in lung epithelia tissues (S. W. Kim et al., 2020)

The contents of a teaspoon of traditional extract were calculated and the ingredient information is presented below (Table **8**). The list comprises quantities of the phytohormones determined through targeted metabolomics and names of metabolites identified through untargeted and semi-targeted metabolomic approaches. Quantification of phytohormones was possible due to use of internal standards. More analysis is required to determine other metabolites to develop a more comprehensive profile for the traditional and other extract types.

Table **8**: Chemical profile assembled to represent contents of a 5 ml teaspoon of traditional extract. This was put together combining compounds detected with untargeted, semi-targeted and targeted metabolomics.

PHYTOHORMONES	CONCENTRATION (PMOL)		METABOLITES
BAR	1.49		bridelionoside D
KIN	218.10		chrysin
iP	5.97		coumarin
tZ	7.72		ethyl linoleate
cZ	3.29		feroylquinic acid
iPR	54.02		ferulic acid
tZR	6.43		fisetin
cZR	6.95		heriguard
DZR	2.11		hyperoside
iPNT	98.78		kaempferol
tZNT	22.84		luteolin
cZNT	232.19		methyl jasmonate
DZNT	0.41		myricetin
iP9G	0.72		nerifolin
tZ9G	1.96		prostratin
tZOG	95.80		protocatechuic acid
cZOG	14.30		quercetin
DZOG	2.01		Roseoside
tZROG	215.58		rutin
cZROG	148.69		
DZROG	54.79		
MeSZR	4.42		

CHAPTER 4: CONCLUSION AND FUTURE WORK

This thesis was undertaken to comparatively evaluate the phytoconstituents of traditional heated leaf extract, latex and simple extracts of *E. neriifolia* and their potential roles with respect to use of the species in asthma therapy. The simple extract was used as a control for comparison as it is thought to have less therapeutic impact. The presence of phytochemicals and phytohormones was investigated using UH-PLC MS-MS untargeted and targeted metabolomic approaches to determine and develop a chemical profile. A greater number and more upregulated features were detected in the traditional extracts indicating the availability of possibly increased metabolites from which potential therapeutic benefit could be derived and biologically active metabolites for treating inflammatory diseases were identified. Identification of characteristic compounds such as quercetin, myricetin and kaempferol indicate sweet aloe constituents are consistent with *E. neriifolia* species reported globally, which would imply alternative applications of sweet aloes beyond the few current uses for asthma, rashes, cough and colds ailments in Guyana.

Heat application could be attributed with causing increased extraction of phenolic and other metabolites (Roshanak et al., 2016). This advocates for incorporating Indigenous methods into scientific evaluations of natural products to accurately identify components in traditional therapies (Mazzocchi, 2006). Traditional knowledge intertwined with western methods could therefore add significant benefit in healthcare as extract types were shown to be very different

and varying in metabolite profiles. The presence of phytohormones of the aromatic cytokinin type in the traditional remedy is a catalyst for further testing of the sample types. This finding was interesting as aromatics like kinetin are found to have limited distribution in nature as indicated previously. Pharmacological activities of natural products may be derived from collective contributions of phytoconstituents from different categories. Therapeutic benefit for asthma could potentially be a function of both phytochemicals and phytohormones but further research is required to link phytohormones such as kinetin with asthma roles.

There is considerable scope for future work on *E. neriifolia*. From sixty-two metabolites comprising thirty-three secondary metabolites and twenty-nine hormones; forty-three were detected in simple extract, forty-four in the traditional / heated extract and fifteen in the latex. The strict criterion of using $[M+H]^+$ adducts only and KEGG IDs to make this study manageable would eliminate metabolites with adducts outside this range. Much more room exists for analysis within the pre-defined conditions. This could then be expanded to include other adducts like [M+Na] in the future to identify compounds excluded by previous criteria. The shared metabolites between traditional and latex samples only and the other intersections as well as unique ones could be explored further APPENDIX J.

Additionally, plants were grown in a controlled environment and samples used excluded the leaves at the apex and the bottom most parts of plants. The data can be used as a baseline and further research can be conducted to compare samples from natural habitats, phytochemical differences between older

and younger leaves or with leaves at the top of plant in addition to varying temperatures at which traditional extract is collected. In relation to the theories on hormonal influence as it relates to asthma, specific investigation for isoflavones among sample types could be another potential area of focus. In the interest of safety concerns, potential toxic chemicals in the latex can be explored as well as stability testing of traditional extract. Finally, confirmation of levels 2 to 5 compounds of interest to elevate them to level 1 (Sumner et al., 2007) could be beneficial but financial requirement for isotopically labelled standards may be prohibitive.

Overall, *E. neriifolia* contains thousands of features with potential therapeutic activity in each extract type, but more features occur in the traditional extract. The possibility exists that reported benefits in asthma are not exclusively the result of a single metabolite or only secondary metabolites but could be attributed to effects of phytohormones or synergy between groups. Promotion of use as per traditional remedy may be an immediate means to wider utilization in primary healthcare. Further research on the species could pave the way for wider use of an alternative remedy for chronic respiratory and other disorders.

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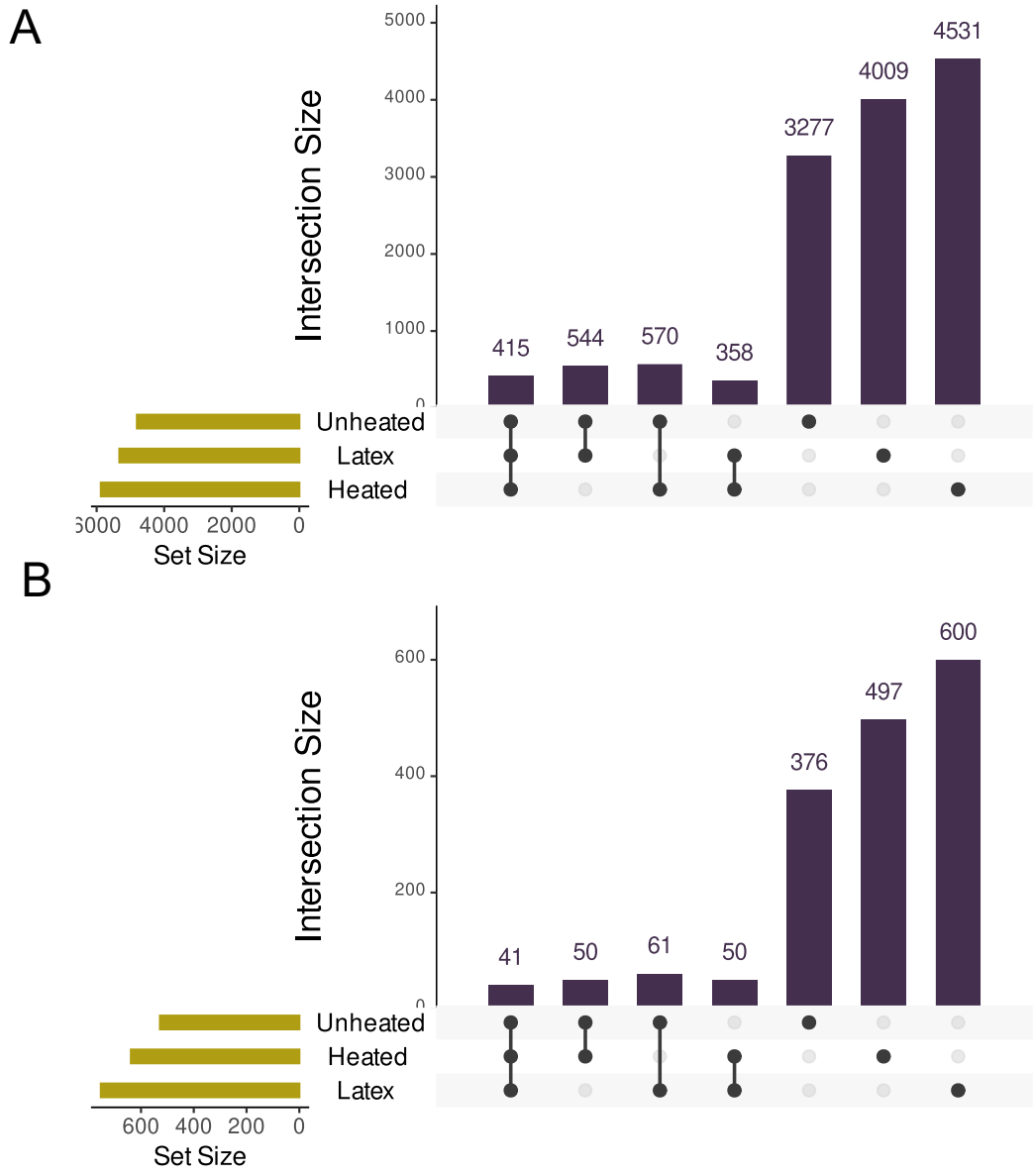
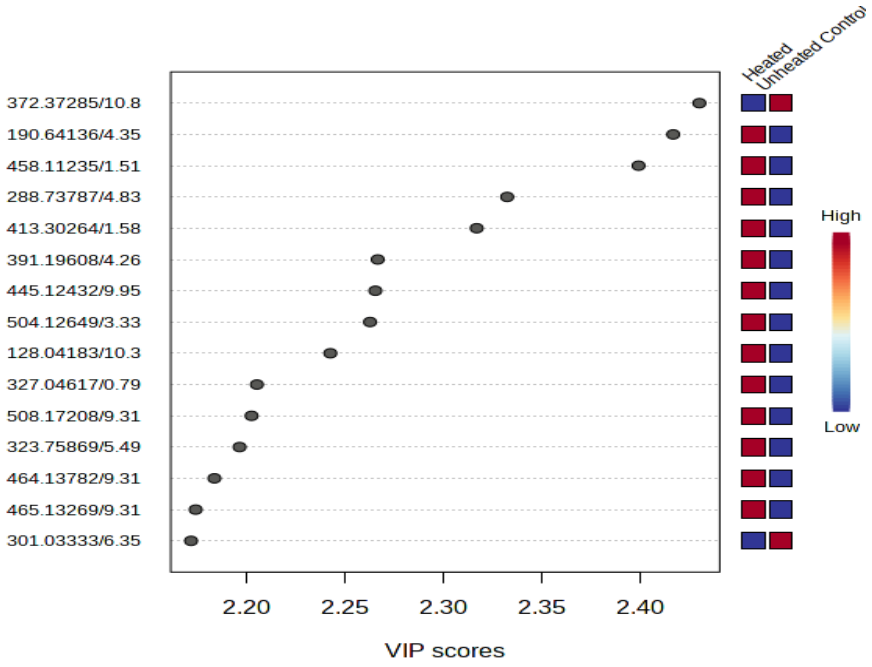
Appendices

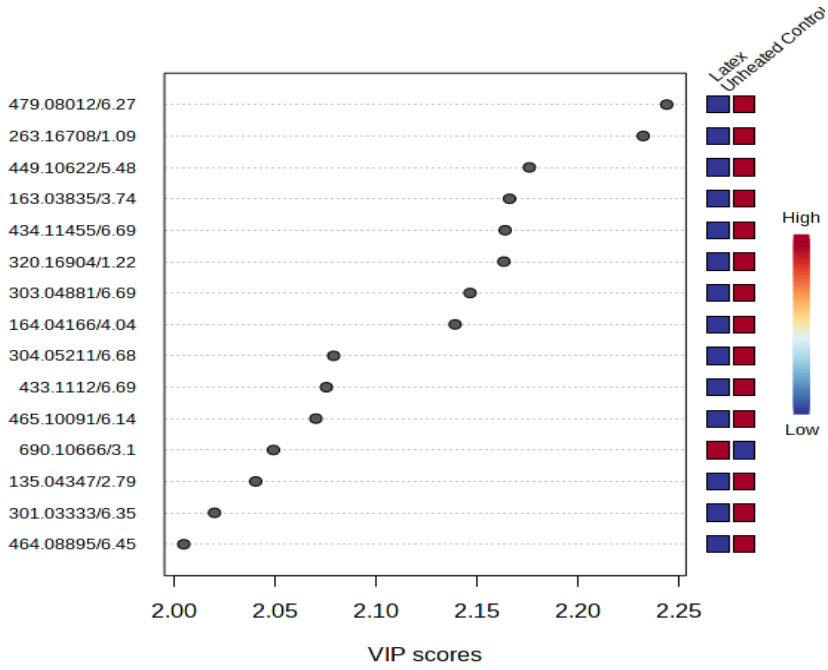
APPENDIX A



Up-Set plots of simple extract (unheated), traditional extract (heated) and latex extract samples showing shared and unique features in (A) all unfiltered aligned peaks generated from XCMS online and (B) quality peaks from extracted ion chromatograms (EIC).

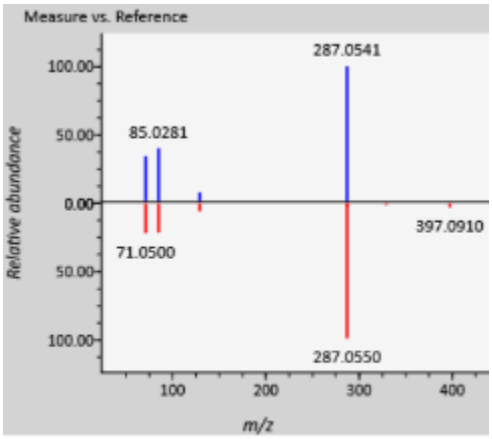
APPENDIX B



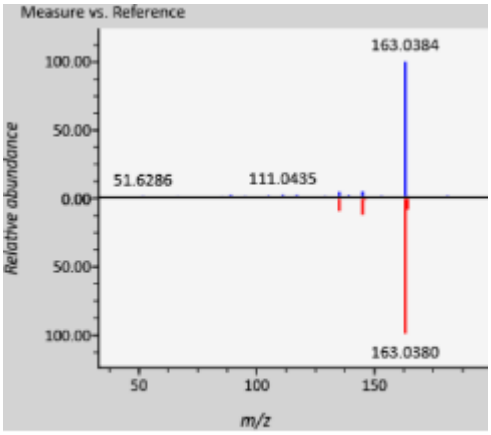


Variable importance of projection (VIP) comparing (A) simple to traditional and (B) simple to latex extracts. VIP plots generated from PLS-DA data show features that cause separation between sample types as in PCA. The 15 most significant features are displayed. More than 60% of features for both groups were not in top 2000 features so EICs could not be compared. The 60% however mostly corresponded with compounds with cardiac, bone and other therapeutic applications.

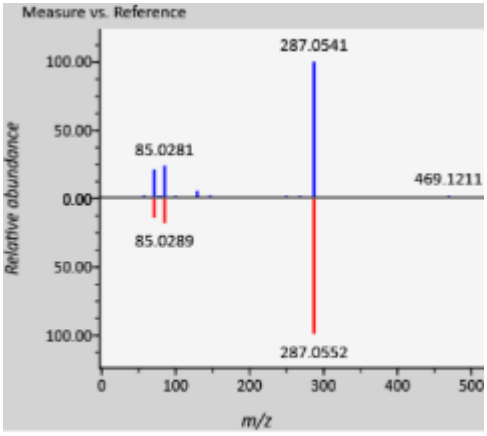
APPENDIX C



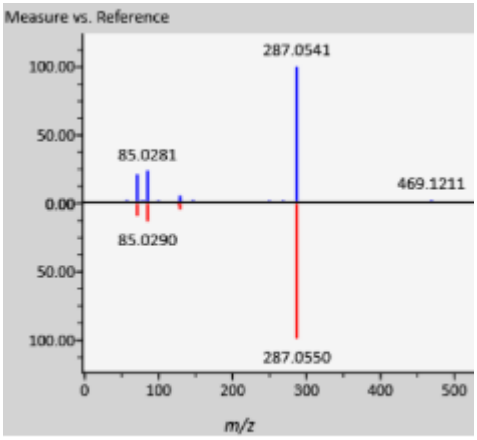
Afzelin



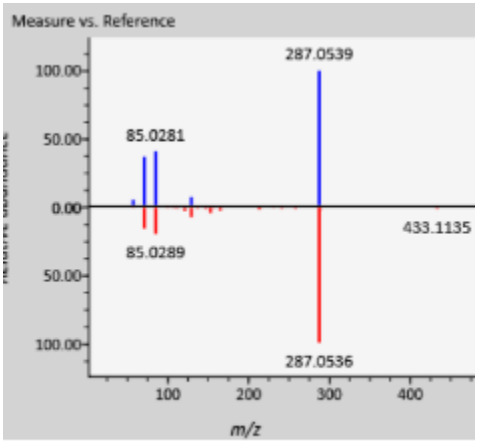
Chlorogenic acid



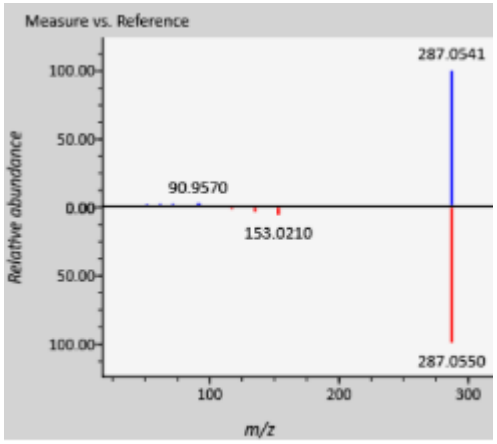
Kaempferol-7-neohesperidoside



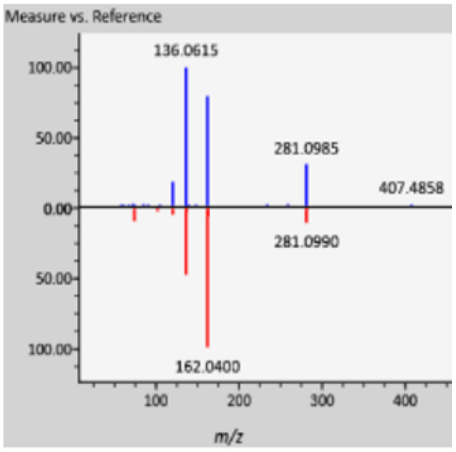
Kaempferol-3-O-rutinoside



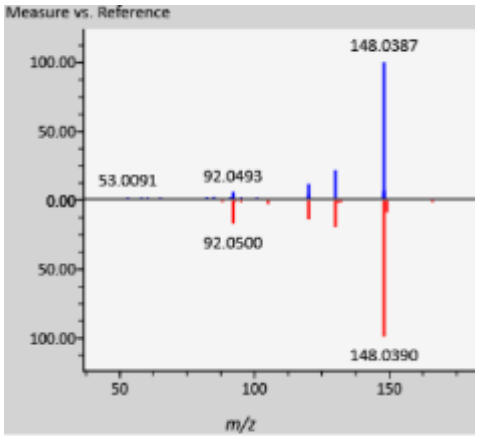
Kaempferol-3-O-alpha-L-rhamnoside



Luteolin



N6-Threonylcarbamoyladenosine



Isatin

Mass spectra of putative compounds identified at level 2. Blue lines represent fragments detected in *E. neriifolia* samples while red lines indicate the matched fragments in reference databases.

APPENDIX D

Table: Semi-targeted metabolite intensities

Filename	Ethyl linoleate	Bridelionoside D	Myricetin	Neriifolin	Prostratin	Quercetin	Protocatechuic acid	Rutin	Caffeic acid	Ferulic acid	Hyperoside	Fisetin	Coumarin	Chrysin
SE1	24814.96	369.94	11418.33	27.47	1474.95	4286794.84	0.00	20242.62	0.00	104191.94	33437.86	7256946.74	326155.47	8491.20
SE2	48947.29	1121.36	69697.91	164.80	3686.07	16794666.24	0.00	78537.30	0.00	393366.16	183927.74	22428091.06	1158250.52	63912.04
SE3	56969.13	817.68	20409.05	181.82	14249.01	7168709.82	0.00	29839.24	0.00	144881.68	47777.48	23834954.87	753764.14	31389.17
SE4	66783.80	1474.62	22198.28	0.00	4019.26	6892228.14	0.00	44299.31	0.00	136426.59	82957.06	7576154.98	198891.85	32139.99
SE5	55141.05	2844.88	45742.23	349.24	6228.64	16458443.56	0.00	69289.23	0.00	608437.30	123530.59	28699369.98	1439859.42	0.00
TE1	603516.75	6140.65	6622.88	0.00	10607.40	4117851.45	1585235.13	22584.11	0.00	87465.53	37136.52	6896455.15	655306.13	0.00
TE2	1013718.67	4813.98	45384.10	706.11	22066.80	19176306.95	5653932.65	96908.56	0.00	2423921.07	193215.33	76251056.20	4587991.09	96984.78
TE3	117054.61	1649.65	7573.86	1389.96	2793.53	7133525.96	1004862.56	33976.79	0.00	398076.18	59723.45	22167055.67	1788313.69	69599.31
TE4	229189.39	1628.00	9637.34	93.23	4424.77	4516416.97	1310704.95	16585.03	0.00	576103.83	33116.81	23098399.79	1604592.79	54082.72
TE5	1276652.95	16455.50	18507.62	1845.01	14062.02	10820499.17	1797203.53	55279.19	0.00	623670.16	69833.79	19647766.72	2116569.27	67652.55
LE1	6297.68	2874.13	0.00	11408.16	187883.53	8237.55	0.00	0.00	5238391.83	0.00	0.00	211355.52	0.00	0.00
LE2	1739.64	1646.19	0.00	13561.44	182762.27	11767.33	0.00	0.00	4663155.70	0.00	0.00	119640.74	0.00	0.00
LE3	3784.13	797.75	0.00	12545.85	159361.36	2265.75	0.00	0.00	2959934.49	0.00	0.00	89572.87	0.00	0.00
LE4	4181.10	665.49	0.00	33602.23	250569.71	3167.92	0.00	0.00	4975383.63	0.00	0.00	181666.37	0.00	0.00
LE5	3497.42	1466.65	0.00	18892.99	103486.60	2130.47	0.00	0.00	2858848.99	0.00	0.00	147871.93	0.00	0.00

APPENDIX E

EIC of two features deemed responsible for divergence / separation Simple Extract and Traditional Extract samples by the Variance Importance of Projection. (A) and (B) were higher in abundance than TE and TE spectra fell outside the 2000 peak range and were unavailable for visual inspection

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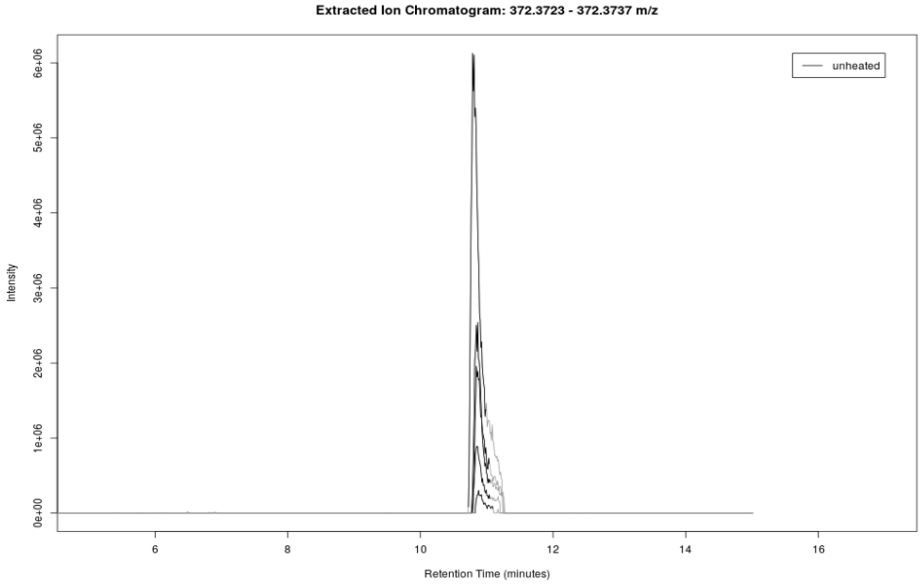
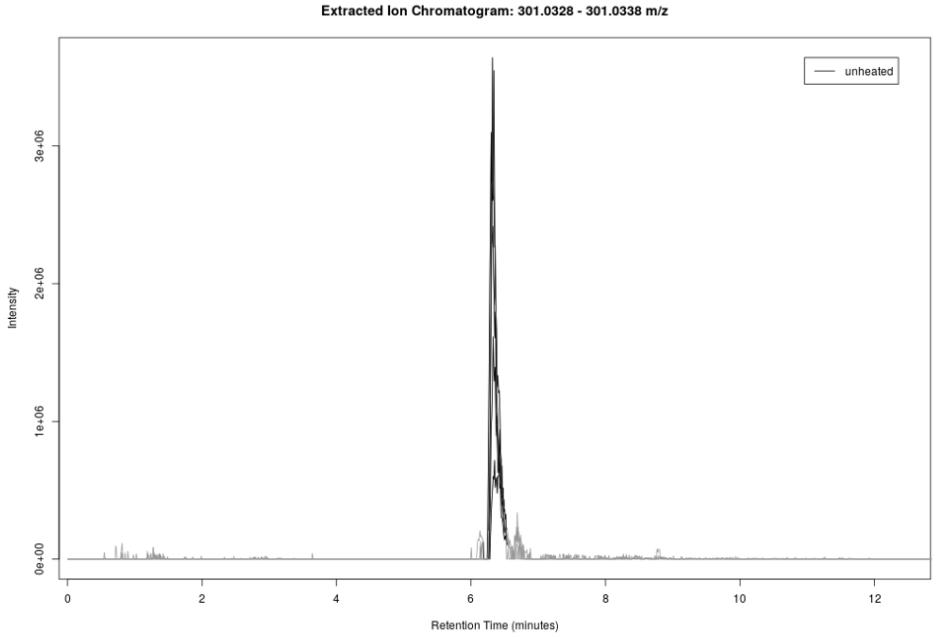
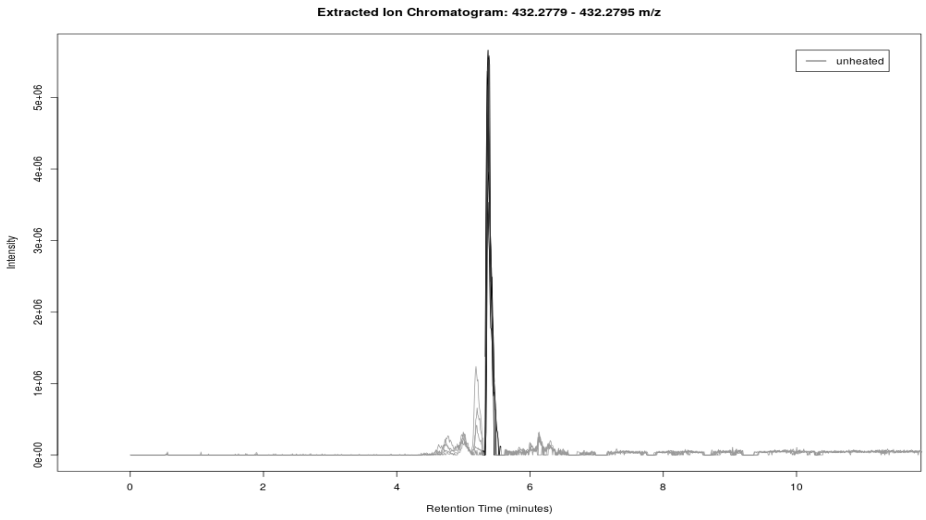
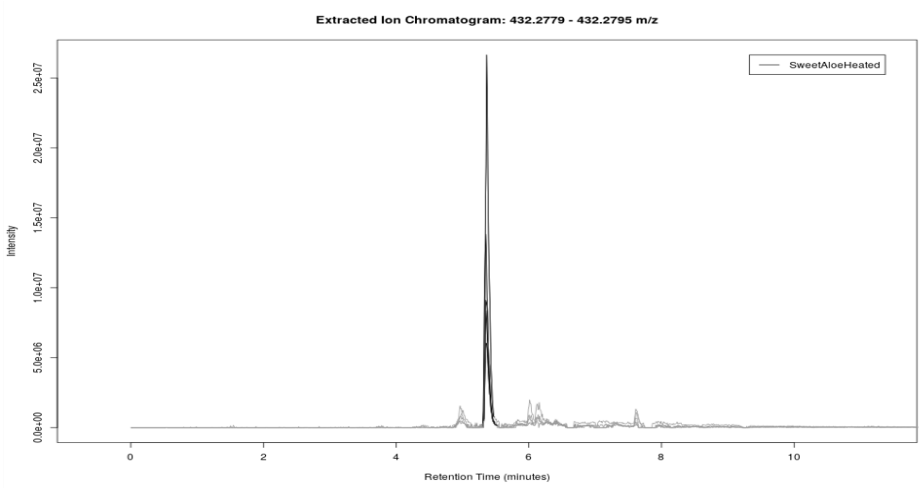
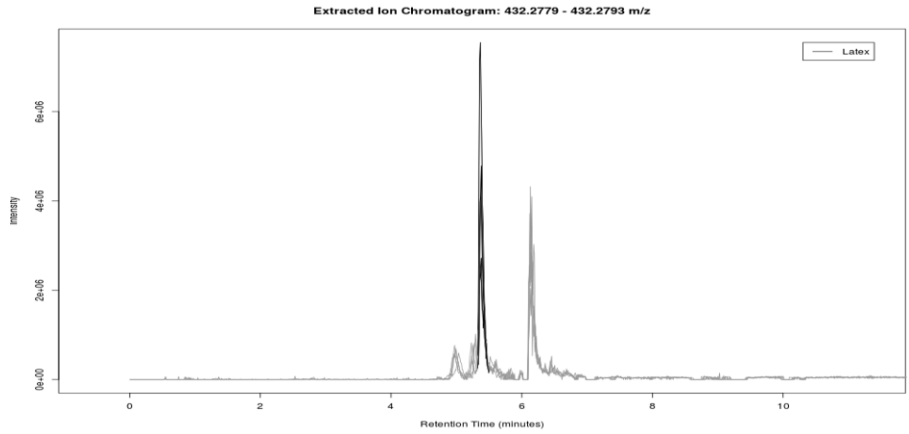




Predominant peak for investigation due to similar m/z value for principal metabolite of a long-acting beta-agonist salmeterol, observed in all three sample types. Six thousand mass spectra and corresponding m/z values and presence per sample are available for further examination.







APPENDIX F

Weights of replicates (n=5) for sample types used in extraction and quantification of cytokinin phytohormones (A) and for acidic hormones (B).

(A)

Name	Tissue weight for phytohormones (g.fwt)
SE1	0.0578
SE2	0.0583
SE3	0.0611
SE4	0.0590
SE5	0.0579
TE1	1.3257
TE2	1.2743
TE3	1.0686
TE4	1.1714
TE5	1.0400
LE1	0.0661
LE2	0.0603
LE3	0.0750
LE4	0.0655
LE5	0.0706

(B)

Name	Tissue weight for acidic hormone extraction (g.fwt)
SE1	0.0434
SE2	0.0437
SE3	0.0459
SE4	0.0442
SE5	0.0434
TE1	0.9943
TE2	0.9557
TE3	0.8014
TE4	0.8786
TE5	0.7800
LE1	0.0496
LE2	0.0453
LE3	0.0562
LE4	0.0492
LE5	0.0530

APPENDIX G

Peak areas of isotopically labelled standards and endogenous compounds. “D-” before the phytohormone abbreviation represents deuterated standard peak areas. Inclusion criteria: Presence of endogenous phytohormone in at least n=3 per sample type and peak areas of at least two replicates of the three must have been greater than 10000.

SAMPLE	ST-DZOG	D-TZ	tZ	cZOG	DZ	D-DZ	D-DZOG	TZOG	DZOG	tZ9G	D-Z9G	D-DZ9G	cZ	cZ9G	KIN	D-DZROG
SE1	684318.6	1394128	831.548	NF	NF	3512742	1074358	6104.581	NF	NF	1572065	335375.9	NF	NF	NF	913058
SE2	618999.4	1379786	954.5557	NF	NF	2949994	885755.7	12142.46	NF	NF	1282227	258097.1	NF	NF	NF	706843.1
SE3	635806	1871004	4780.663	NF	NF	4455760	1157643	2479.276	NF	NF	1835869	406088.7	NF	NF	NF	1179623
SE4	624769.1	1494018	1143.301	NF	NF	3538335	1091963	25018.38	NF	NF	1615268	331088.8	NF	NF	NF	829100
SE5	654394	1751788	0	NF	NF	4805912	1005905	8510.862	NF	NF	1516609	304673.1	NF	NF	NF	1030964
TE1	360185.6	1325392	22339.48	73063.39	16417.51	2851777	511742.6	490300.2	15600.86	28998.88	499378.7	95337.37	15432.11	2.36E-12	49086.92	351476.6
TE2	291338.6	1466458	42490.95	20252.19	4130.559	2867082	471541.5	199579	8077.754	3163.435	555641.8	115287.4	20155.37	NF	13617.82	540903.2
TE3	335836.4	1250316	66676.02	40280.99	2182.663	2885110	509312.5	216176.5	4204.827	1803.102	697234.1	139866.4	20197.02	2E-15	30372.25	430380.1
TE4	602355.9	1672254	107509.6	42472.43	NF	3628519	821880.4	308072.6	11095.97	1254.342	963974.2	201943.8	35311.57	NF	99392.51	598081.7
TE5	463989.4	1687822	42216.96	72000.51	16288.82	3959495	854250.7	460264.5	17228.36	27557.84	1233474	235990.7	31540.37	4.07E-12	68852.56	701615
LE1	380179.8	1006812	NF	NF	NF	2534083	625200.6	NF	NF	NF	892406.1	180824	NF	NF	NF	591126.9
LE2	562795.3	1275485	NF	NF	NF	3087955	835687.3	NF	NF	NF	1285076	267767.6	NF	NF	NF	753924
LE3	373492.7	1484499	NF	NF	NF	3241215	745633.5	NF	NF	NF	1329971	274805.3	NF	NF	NF	912505.7
LE4	310536.7	951088.6	NF	NF	NF	2253189	504308.3	NF	NF	NF	764801.3	167060.1	NF	NF	NF	557833.7
LE5	236020.7	762754.6	NF	NF	NF	1831823	385592.6	NF	NF	NF	467896.9	89883.63	NF	NF	NF	474478.8

Peak areas of isotopically labelled standards and endogenous compounds cont'd.

SAMPLE	cZROG	D-ZROG	t-ZROG	DZROG	D-tZR	tZR	DZR	D-DZR	cZR	D-iP	iP	iP9G
SE1	81074.33	1725839	27921.33	4156.686	8506026	17855.41	96933.66	13975635	16702.26	15041280	40952.48	2138.222
SE2	38740.94	1459084	36433.71	4343.423	7488515	24008.96	124863.4	9011757	13202.98	13343637	42464.72	0
SE3	115697.5	2280340	72776.87	5818.689	9452727	51867.62	202545.4	11410927	37478.12	15891221	43246.75	767.6049
SE4	182557.5	1814010	437636.3	24977.02	7637542	44269.15	78135.42	9325203	24200.65	15954322	43978.86	741.5942
SE5	84932.57	2196103	50930.13	10084.04	9426187	25042.64	53790.75	11580309	19424.43	16714084	38749.89	1707.776
TE1	2560974	582751.2	3887223	583396	4082505	423545.6	28109.76	6584901	511302.7	11434774	301831.7	141801.8
TE2	887052.4	929956.6	1138743	213300.7	6787760	208263.7	41923.57	8965348	174122.5	13563739	320739.7	42383.88
TE3	653583.1	775215.2	862462	115911.7	5350139	128148.7	10595.35	6385251	91854.02	9798046	300424.8	26446.45
TE4	778985.4	1112813	827608.5	155529.4	5130917	114992.1	34551.27	8203337	88658.49	13382180	417934.3	48829.65
TE5	2859053	1395802	4486221	518535	5838127	465026.1	170109.9	7506583	567766	12537810	314519.8	112343.1
LE1	NF	1133326	NF	NF	5948044	3187.219	43932.79	11489314	40906.93	14181758	46665.2	NF
LE2	NF	1439396	NF	NF	5902473	2089.223	69083.61	8453121	50825.29	14193099	48277.76	1509.671
LE3	NF	1588068	NF	NF	9132591	3692.23	91870.91	10291114	96351.05	13499195	40976.48	0
LE4	NF	1104379	NF	NF	5181558	3.88E-12	56782.27	8001266	70378.66	14934002	50464.77	NF
LE5	NF	755130.7	NF	NF	4408122	3509.172	67234.31	7242949	40905.66	13440269	49345.87	1218.102

Peak areas of isotopically labelled standards and endogenous compounds cont'd.

SAMPLE	D-iPR	2MeSZ	D-MeSZ	iPR	D-2MeSZR	2MeSZR	D-BAR	BAR	D-2MeSiP	D-2MeSiP
SE1	14532378	2108.808	6470099	358139.7	2058701	1873.792	1007867	NF	12786644	4952891
SE2	11087711	NF	5185738	361908.7	1831373	NF	641406	NF	9831205	5414437
SE3	14094298	5058.652	6531411	695768.5	1793672	NF	914211.1	NF	14196337	7710025
SE4	14803735	2004.478	6472296	418039.2	2174310	4046.149	898835.1	NF	13459459	6065284
SE5	16361714	4187.022	5752338	532214.4	1815211	NF	909628.3	NF	13945257	8542700
TE1	9659990	4682.407	3321869	8309894	925221	67119.31	698052.9	22535.49	11662173	5088787
TE2	11600850	2894.228	4887389	3651597	1553205	35452.96	775114.6	13291.52	13179380	6319411
TE3	8528252	4128.422	3036952	1149859	696169.9	3369.376	559226.4	1650.683	7696721	4044181
TE4	11331252	1810.827	4872081	2561122	1341380	28512.05	696255.4	6142.654	12909010	6695292
TE5	10617700	13300.6	4254211	6052273	1232819	97109.22	596750.9	2096.876	11239750	4781804
LE1	12412601	2283.391	5586860	51918.57	1838373	1727.339	844333.3	NF	11424133	4804231
LE2	12129620	1123.768	5652218	45239.18	3778426	2700.303	1234352	NF	10319765	4461414
LE3	12698667	NF	4830849	91983.11	2214624	7520.964	782175.7	NF	10233882	4350196
LE4	13497174	4508.677	5414874	94557.7	1816008	4276.001	836762	NF	11408484	4191992
LE5	11816222	NF	5094359	124519.3	3169981	8999.967	1430916	NF	10469567	4894105

APPENDIX H

Concentration of aromatic, freebase and riboside CKs [pmol/g fwt] in individual replicates of three sample types.

SAMPLE	CKS - AROMATIC			CKs -FREE BASE				CK- RIBOSIDES			
Name	BA	KIN	BAR	iP	tZ	cZ	DZ	iPR	tZR	cZR	DZR
SE1	-	-	-	2.32	0.47	-	-	12.51	1.03	0.97	13.50
SE2	-	-	-	2.69	0.54	-	-	16.44	1.57	0.86	20.55
SE3	-	-	-	2.19	1.91	-	-	23.88	2.55	1.85	21.04
SE4	-	-	-	2.30	0.59	-	-	14.09	2.80	1.53	10.60
SE5	-	-	-	1.97	-	-	-	16.58	1.31	1.01	5.47
TE1	-	41.56	0.68	0.98	0.58	0.40	-	19.34	2.23	2.69	0.21
TE2	-	10.11	0.38	0.91	1.04	0.49	-	7.35	0.69	0.57	0.32
TE3	-	52.56	0.08	1.41	2.28	0.69	-	3.74	0.64	0.46	0.10
TE4	-	107.70	0.21	1.31	2.50	0.82	-	5.74	0.54	0.39	0.23
TE5	-	-	0.09	1.19	1.10	0.82	-	16.33	2.18	2.66	1.17
LE1	-	-	-	2.45	-	-	-	1.68	0.23	2.96	7.42
LE2	-	-	-	2.77	-	-	-	1.61	0.17	4.06	10.49
LE3	-	-	-	1.99	-	-	-	2.71	0.15	4.00	10.70
LE4	-	-	-	2.54	-	-	-	3.00	0.00	5.90	10.88
LE5	-	-	-	2.56	-	-	-	4.25	0.32	3.74	14.71

Concentration of nucleotides, glucosides and methylthiolated CKs [pmol/g fwt] in three sample types.

SAMPLE	NUCLEOTIDES				GLUCOSIDES								METHIOL
Name	iPNT	tZNT	cZNT	DZNT	DZOG	DZROG	tZROG	cZROG	tZOG	cZOG	tZ9G	iP9G	MeSZR
SE1	351.15	1.16	2.41	-	-	1.53	5.45	15.82	4.04	-	-	-	-
SE2	366.68	4.96	2.46	-	-	2.05	8.34	8.87	8.82	-	-	-	-
SE3	161.76	17.72	12.36	-	-	1.56	10.16	16.16	1.67	-	-	-	-
SE4	111.47	18.10	17.41	-	-	9.91	79.67	33.23	17.80	-	-	-	-
SE5	317.04	9.04	5.35	-	-	3.28	7.80	13.01	8.63	-	-	-	-
HE1	16.46	5.24	5.87	0.10	0.60	24.29	97.98	64.55	26.92	4.01	1.15	0.26	1.38
HE2	17.72	2.15	2.45	0.02	0.35	6.00	18.71	14.58	14.10	1.43	0.12	0.07	0.45
HE3	20.78	5.95	4.62	0.07	0.20	4.89	20.28	15.36	15.79	2.94	0.06	0.07	0.11
HE4	19.16	2.36	1.98	0.08	0.30	4.31	12.36	11.64	11.26	1.58	0.03	0.09	0.46
HE5	21.87	6.52	10.71	0.13	0.51	13.78	60.18	38.35	25.01	3.91	0.56	0.24	1.91
LE1	-	0.11	1.37	-	-	-	-	-	-	-	-	-	-
LE2	-	0.26	2.60	-	-	-	-	-	-	-	-	-	-
LE3	-	0.52	0.90	-	-	-	-	-	-	-	-	-	-
LE4	-	0.28	3.28	-	-	-	-	-	-	-	-	-	-
LE5	-	0.11	1.47	-	-	-	-	-	-	-	-	-	-

APPENDIX I

(A) Deuterated (IS) peak areas and endogenous peak areas of analytes in replicates (n=5) of three sample types for quantification of acidic phytohormones.

Name	Peak area (IS)	Peak area endogenous	Peak area endogenous	Peak area (IS)	Peak area endogenous	Peak area (IS)	Peak area endogenous
	2HABA	ABA	JA	2HIAA	IAA Area	2HGA7	GA7
SE1	36418092.08	277238.42	2671777.35	4760046.43	482640.78	28388984.62	
SE2	4341724.93	71883.72	446661.49	812148.39	204976.69	6218160.20	
SE3	28464752.73	497557.58	1480395.61	4021374.90	502660.01	6023333.64	
SE4	46889689.59	584042.44	1681848.16	6715801.22	1148750.02	8344634.23	
SE5	13846119.49	84481.28	406920.69	2557310.82	511764.59	2974520.01	
TE1					8321373.96	142454.83	
TE2					2082432.28	-	
TE3					1124340.81	-	
TE4					1599234.18	20369.63	
TE5					7159128.04	92632.68	
LE1	29647347.15				545882.05	22741350.89	10115.54
LE2	25418278.38				237103.89	22196117.27	13718.28
LE3	21034151.36				377394.65	19641156.54	10886.05
LE4	23206881.23				276997.45	36181189.48	11055.50
LE5	23139158.23				415873.76	27548390.48	7562.83

(B) Acidic phytohormones in replicates (n=5) for three sample types.

	ABA	JA	IAA	GA7
SE1	39.91	483.48	133.45	
SE2	86.12	672.65	329.58	
SE3	86.67	324.16	155.60	
SE4	64.03	231.79	220.77	
SE5	31.96	193.48	263.13	
LE1				0.54
LE2				0.83
LE3				0.60
LE4				0.38
LE5				0.31

APPENDIX J

Mass-to-charge values of features at peaks detected with good quality EICs. Values are for shared features among sample types.

SE/TE/LE	SE/TE	SE/LE	TE/LE
117.0694	105.0330	105.0694	119.0680
118.0646	115.0538	121.1007	134.1040
119.0851	131.0850	133.1007	164.1064
134.1040	149.0956	143.0850	192.1375
147.1162	150.0988	149.0955	197.1528
159.1162	158.1084	171.1372	206.1248
165.1268	163.1111	172.1689	212.2364
173.1319	172.0961	180.1740	225.1265
174.1352	175.1474	183.1162	225.1476
184.2053	177.0539	183.1372	228.1950
189.1267	186.2210	192.1457	230.2470
197.1528	187.0958	197.1528	256.2625
207.1372	191.1423	205.1216	268.2624
208.1405	197.1528	217.1425	292.2009
211.1320	229.1789	222.1479	293.1523
226.1792	229.1983	230.2469	295.2256
241.1940	241.2026	240.2313	309.2411
259.2047	242.2832	241.2346	310.2445
267.1733	244.1897	244.2262	311.1629
272.2573	254.2467	249.1838	311.1842
277.2150	257.1889	252.1606	314.1818
291.2306	287.0538	256.2624	316.2834
295.1679	295.2256	267.1216	327.2517
295.2256	298.2727	274.2729	328.2548
296.2288	310.1483	275.2762	330.2628
309.2412	314.2677	292.2008	344.2782
310.2444	319.1277	292.2340	345.2620
313.2358	319.2831	295.2256	346.2656
327.2518	324.2154	296.1713	354.2622
331.2466	330.2627	299.2568	359.2967
331.2467	335.2179	311.2930	365.2074
363.3091	346.2079	313.1785	366.1250
392.2525	347.1840	313.2359	372.2153

Mass-to-charge values of features at peaks detected with good quality EICs cont'd.

SE/TE/LE	SE/TE	SE/LE	TE/LE
	351.1917	314.3039	378.1742
	383.1512	317.2864	378.2641
	395.2910	327.2513	387.1916
	395.3979	329.1732	392.1897
	396.3306	333.2524	393.1500
	399.2490	342.2030	400.1585
	400.2523	343.2061	401.2647
	403.0772	343.2940	414.2133
	404.2263	344.1535	441.3280
	406.2422	345.2619	445.2906
	424.1948	345.2815	450.2107
	425.1554	346.2848	477.3778
	451.1116	358.2937	495.3874
	451.3613	370.3663	497.2768
	465.0910	371.3696	512.4140
	484.3828	381.3390	528.2782
	505.1319	387.1998	555.3517
	510.2676	388.1906	577.3685
	522.9994	393.2842	600.4654
	534.3466	394.2876	602.4723
	544.2729	403.1789	
	556.4400	405.1138	
	595.1637	407.1685	
	597.2521	407.3353	
	601.4691	415.2523	
	607.3091	418.2539	
	642.3454	419.2240	
	645.4949	420.2210	
		431.2412	
		447.2401	
		458.2368	
		463.3408	
		510.2159	
		557.4434	
		688.5169	
		690.5233	