Metabolomics and Camphor Biosynthetic Pathway Analysis of *Ocimum kilimandscharicum*

THESIS

Submitted to
Academy of Scientific & Innovative Research
for the award of the degree of

Doctor of Philosophy

In

BIOLOGICAL SCIENCES

By

Ms. Priyanka Singh

Registration Number: 10BB11J26110

Under the guidance of

Dr. Ashok P. Giri **(Research Supervisor)**

Dr. H. V. Thulasiram **(Research Co-supervisor)**

CSIR-National Chemical Laboratory,
Pune -411008, India

August 2016

Dedicated to My Family

The Love of a Family Is *Life's* Greatest Blessing!

सीएसआईआर - राष्ट्रीय रासायनिक प्रयोगशाला
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
डॉ. होमी भाभा मार्ग, पुणे - 411 008. भारत
CSIR - NATIONAL CHEMICAL LABORATORY
(Council of Scientific & Industrial Research)
Dr. Homi Bhabha Road, Pune - 411 008, India

Dr. Ashok P. Giri,
Sr. Scientist, Plant Molecular Biology Unit,
Division of Biochemical Sciences, CSIR-National Chemical Laboratory,
Tel: 91 20 25902710, E-mail: ap.giri@ncl.res.in

CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled *Metabolomics and Camphor Biosynthetic Pathway Analysis of Ocimum kilimandscharicum* submitted by Ms. *Priyanka Singh* to Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of *Doctor of Philosophy in Biological Sciences,* embodies original research work under our supervision/guidance. We further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material obtained from other sources has been duly acknowledged in the thesis. Any text, illustration, table etc., used in the thesis from other sources, have been duly cited and acknowledged.

Ms. Priyanka Singh

(Student)

Dr. H. V. Thulasiram

(Co-supervisor)

H. V. THULASIRAM
Ph.D., Scientist
Division of Organic Chemistry
National Chemical Laboratory
Dr. Homi Bhabha Road, Pune - 411 008

Dr. Ashok P. Giri

(Supervisor)

Dr. Ashok P. Giri
Senior Scientist
Biochemical Sciences Division
National Chemical Laboratory
Pune - 411 008.

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the thesis entitled "**Metabolomics and Camphor Biosynthetic Pathway Analysis of *Ocimum kilimandscharicum***" submitted by me for the Degree of Doctor of Philosophy to Academy of Scientific & Innovative Research (AcSIR) is the record of work carried out by me at Biochemical Sciences Division, CSIR- National Chemical Laboratory, Pune - 411008, India, under the supervision of Dr. Ashok P. Giri (research supervisor) and Dr. H. V. Thulasiram (research co-supervior). The work is original and has not formed the basis for the award of any degree, diploma, associateship, and fellowship titles in this or any other university or other institute of higher learning. I further declare that the material obtained from other resources has been duly acknowledged in the thesis.

Singh 22/08/2016

Priyanka Singh
Plant Molecular Biology Group
Biochemical Sciences Division
CSIR-National Chemical Laboratory
Dr. Homi Bhabha Road, Pashan
Pune – 411008
Maharashtra, India

ACKNOWLEDGEMENTS

The task of devoting oneself to a journey in pursuit of unanswered questions is not an easy one. This journey, called Ph.D., cannot be travelled in solitude. My Ph.D. is dedicated to all those who believed in my dreams and through their constant support, acumen, appreciation and constructive critisism made this journey one of the most challenging and enriching experiences of my life. The satisfaction of solving the most difficult puzzles, the pride in self belief, the art to never give up, the ability to take calculated risks and to imagine what lies beyond was all, but a small part of my scientific research. However, it would not have been possible without the painstaking efforts of several people.

Firstly, I would like to express my heartfelt grattitude to my guide, ***Dr. Ashok P. Giri****, for his unparalled contributions in mentoring me as a "student of science" and shaping my Ph.D. and career. His enthusiasm for research was both contagious as well as truly inspiring. His encouragement for testing even the weirdest of ideas set him apart. He imbibed in me the ability to think out of the box, to take ownership of my work, to seek multiple evidences to test one idea and spend substantial amount of time in analysing the results from one experiment. He taught me to be patient with work and with people. His confidence in me was my biggest moral support even when I was treading the roughtest roads in my academic life. Thanks Ashok for the endless guidance, insightful discussions, morale boosting sessions and mood uplifting lab celebrations. Indeed I am honoured to have a guide and mentor like you.*

I would like to thank ***Dr. H.V. Thulasiram****, my coguide for providing me extremely valuable inputs in form of his ideas, time and funds. He*

expanded my knowledge and understanding in the field of chemical sciences. Indeed, the discussions with him were mind provoking and extremely useful in understanding the chemistry behind all the biology. My association with Dr. Thulasiram was truly enlightening and helped in giving the right direction to my research. I would like to thank ***Dr. Mahesh J. Kulkarni*** *for helping me in my work related to diabetes. He greatly aided in the smooth functioning of all experiments and analysis of the results. He helped me in formulating and testing new hypothesis relevant to my research and reviewed my work periodically. I would also like to thank* ***Dr. Sachin Agawane*** *and* ***Dr. Ramanamoorthy Boppanna*** *for helping me with the mouse model studies in my diabetes research. Their enthusiasm and willingness to work was admirable. I take this opportunity to thank my doctoral advisory committee members* ***Dr. V. Ravi Kumar*** *and* ***Dr. Asish Kumar Bhattacharya*** *for evaluating my work in depth and providing valuable feedback enabling fine refinement of my research goals. I would like to thank* ***Dr. Vidya Gupta*** *for being an outstanding woman scientist and a source of motivation to all the budding women scientists. Her critical analysis of my work from time to time during presentations and seminars helped me in strengthening faith in my research and boosting my confidence. A very special thanks to* ***Prof. Asaph Aharoni*** *for hosting me at the Weizmann Institute of Science, Israel and providing me with an opportunity of a lifetime. His outlook towards science was awe-inspiring, one of its kind and of instrumental in remoulding my research attitude. I am greatly indebted to both Ashok and Asaph for making it possible for me to experience the work culture on an international platform and working with people from 14 different nationalities. It was not only an unforgettable academic experience but also a very rewarding personal experience!*

I take this opportunity to thank ***Dr. Oren Tzfaldia, Dr. Sachin Punekar, Dr. Arvind Korwar, Mr. Mahmud shaikh, Mr. Garikapatti Swamy, Mr. Anurag Shukla, Ms. Priya Sarate, Dr. Rakesh Joshi, Mr. H. J. Ramesha*** *and* ***Dr. Raviraj Kalunke*** *who helped me in planning experiments and analysing data. I would like to thank all labmates especially* ***Mr. Amey Bhide, Mr. Atul Anand, Mr. Rahul Tanpure, Dr. Yojana Chikate, Dr. Neha Mahajan, Dr. Neha Khandelwal, Dr. Meena Pandey*** *for their cherished friendships, helping with daily lab work and providing moral and emotional support throughout my PhD.*

I take this opportunity to thank ***Director CSIR-NCL*** *for providing me an opportunity to work in one of the most esteemed institutes in India and providing extensive infrastructure. I also wish to thank the* ***chair*** *and* ***all faculty members*** *of Division of Biochemical Sciences, CSIR-NCL, for their constant support and cooperation and* ***CSIR*** *for research fellowship.*

At the end I would like to thank my parents without whom none of this would have been possible. Their blessings and good wishes have always been like a protective viel surrounding me. This PhD is dedicated to my mother ***Mrs. Rekha Singh****, who stands at the tallest pedestal in my heart; my father,* ***Mr. Anil Kumar Singh****, who is an epitome of love and sacrifice for me; my brother,* ***Dr. Aditya Kumar Singh****, who is a symbol of hardwork and perseverance; my sister-in-law,* ***Dr. Shikha Singh****, who stands tall against all odds; my* ***grandparents*** *who taught me to smile in the face fear and rejection, my husband and soulmate* ***Mr. Rohan Johri****, who holds my hand till eternity for all the good and bad; and finally* ***God****, who brought such wonderful family, friends, collegeues and mentors in my life.*

Priyanka Singh

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	degree centigrade
µL	Microlitre
µM	Micromolar
4CL	4-Coumarate-CoA Ligase
AAT	Alcohol Acyl Transferase
ABC	ATP- Binding Cassette
ADT	Aerogenate Dehydratase
AGE	Advanced Glycation End Products
AGT	Anthocyanidin 3-O-Glucoside 5-O-Glucosyltransferase
ANNOVA	Analysis Of Variance
ANS	Anthocyanidin Synthase
ATP	Adenosine Triphosphate
BCS	β-Caryophyllene Synthase
bdh	Borneol Dehydrogenase
BERS	Bergamotene Synthase
BIS	Bisabolol Synthase
bppd	Bornyl Diphosphate Diphosphatase
bpps	Bornyl Diphosphate Synthase
BSA	Bovine Serum Albumin
C3H	*p*-Coumarate 3-Hydroxylase
C4H	Cinnamate-4-Hydroxylase
CAAT	Coniferyl Alcohol Acetyl Transferase

CAD	Cinnamyl Alcohol Dehydrogenase
CDS	Cadinene Synthase
CCMT	Cinnamate Carboxyl Methyltransferase
CCR	Cinnamoyl-CoA Reductase
CEL	Carboxyethyl Lysine
CHI	Chalcone Isomerase
CHS	Chalcone Synthase
CM	Chorismate Mutase
CML	Carboxy Methyl Lysine
CO_2	Carbon Dioxide
COMT	Caffeoyl O-Methyl Transferase
CPS	9-Epi-Caryophyllene Synthase
CS	Chorismate Synthase
CVOMT	Chavicol O-Methyl Transferase
Cyp	Cytochrome *p*450 Oxidoreductase
DCM	Dichloromethane
DFR	Dihydroflavonol 4-Reductase
DHQD	3-Dehydroquinate Dehydratase
DMAPP	Dimethyl Allyl Pyrophosphate
DMSO	Dimethyl Sulphoxide
DTPs	Diterpene synthases
DXR	1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase
DXS	1-Deoxy-D-Xylulose 5-Phosphate Synthase

EF1α	Elongation Factor-1-alpha
EGS	Eugenol Synthase
EME	Eugenol Methyl Ether
EOMT	Eugenol-O-Methyltransferase
ERF	Ethylene Responsive Factor
HESI	Heated Electrospray Ionization
F3H	Flavanone 3-Hydroxylase
FA	Fatty Acids
FAR	Farnesene Synthase
FES	Fenchol Synthase
FPP	Farnesyl Diphosphate
FPPS	Farnesyl Diphosphate Synthase
GAS	Germacrene-A Synthase
GC	Gas Chromatography
GDS	Germacrene- D synthase
GES	Geraniol Synthase
GGPPS	Geranylgeranyl Diphosphate Synthase
GPP	Geranyl Diphosphate
GPPS	Geranyl Diphosphate Synthase
GPPS.LSU	Geranyl Diphsophate Synthase Large Subunit
GPPS.SPS	Solanesyl Diphhosphate Synthase
GPPS.SSU	Geranyl Diphsophate Synthase Small Subunit
GSAR	Glycation Sensitive Amino acid Residues

GST	Glutathione S-Transferase
GTS	Gamma-Terpinene Synthase
HbA1c	Glycated Hemoglobin
HCA	Heirarchial Cluster Analysis
HCT	Hydroxycinnamoyl Transferase
HDS	4-hydroxy-3-methylbut-2-enyl Diphosphate Synthase
HMGR	3-Hydroxy-3-Methylglutaryl-CoenzymeA Reductase
HMGS	3-Hydroxy-3-Methylglutaryl-CoenzymeA Synthase
HPPD	4-Hydroxyphenylpyruvate Dioxygenase
HRP	Horse Radish Peroxidase
IPI	Isopentenyl Diphosphate Isomerase
IPP	Isopentenyl Pyrophosphate
KS	Ent-Kaurene Synthase
LC	Liquid chromatography
LIM	Limonene Synthase
LIS	Linalool Synthase
LMS	Limonene-Myrcene Synthase
m/z	Mass by Charge
MATE	Multi-Antimicrobial Extrusion Protein
MCT	2-C-Methyl-D-Erythritol 4-Phosphate cytidylyltransferase
MDS	2-C-Methyl-D-Erythritol 2,4-Cyclodiphosphate Synthase
MeJa	Methyl Jasmonate
MEK	4-Diphosphocytidyl-2-C-Methyl-D-Erythritol Kinase

MEP	Methylerythritol Phosphate
MeV	Multiexperiment Viewer
MS	Mass Spectrometry
MSA	Mouse Serum Albumin
MTPs	Monoterpene Synthases
MVA	Mevalonic Acid
MVK	Mevalonate Kinase
MYS	Myrcene Synthase
NADH	Reduced Nicotinamide Adenine Dinucleuotide
NES/LIS	Nerolidol/Linalool Synthase
NES	Nerolidol Synthase
NGS	Next Generation Sequencing
NMR	Nuclear Magnetic Resonance
Ob4CL	*Ocimum basilicum* 4-Coumarate-CoA Ligase
OE	Gene Overexpression
OE_BDH	*bdh* overexpression
OE_GPPS	*gpps* overexpression
PAGE	Polyacrylamide Gel Electrophoresis
PAL	Phenylalanine Ammonia-Lyase
PAT	Prephanate Aminotransferase
PCR	Polymerase Chain Reaction
PHD	Plant Homeo Domain
PIN	Pinene Synthase

PNPG	*para*-Nitrophenyl-α-D-Glucopyranoside
PPO	Polyphenol Oxidase
PUR	Pulegone Reductase
PVDF	Polyvinylidene Difluoride
RAGE	Receptor for Advanced Glycation End Product
RAS	Rosmarinic Acid Synthase
RNA	Ribonucleic acid
RNAi	Gene silencing
RNAi_BDH	*bdh* silencing
RNAi_GPPS	*gpps* silencing
RPKM	Reads Per Kilobase per Million
SAM	S-Adenosyl-L-Methionine
SCS	1,8-Cineole Synthase
SDS	Sodium Dodecyl Sulphate
SDSS	Sequence Detection System software
SES	Selinene Synthase
STPS	Sesquiterpene Synthases
STZ	Streptozotocin
TBS	Tris Buffered Saline
TES	Terpinolene Synthase
TLC	Thin Layer Chromatography
UV-A	Ultraviolet-A
ZIS	Zingiberene Synthase

Table of contents

CHAPTER 2

CHAPTER 4

Preface

Ocimum species present a wide array of diverse metabolites possessing immense medicinal and economic value. The importance of this genus is undisputable and exemplified in the ancient science of Chinese and Indian (Ayurveda) traditional medicine. The key to this medicinal potential of genus *Ocimum* might lie in the vast array of secondary metabolites and phytochemicals including terpenoids, phenylpropanoids, flavonoids, phenolic compounds etc. present in various plant parts. Unlike several other plant species of *Artemisia*, *Salvia*, *Catharanthus*, *Taxus*, *Mentha*, etc. that are largely exploited, detailed characterization and identification of important metabolites from *Ocimum* species remained unexplored. Also, molecular pathways leading to the production, storage, transport and metabolism of these compounds are poorly understood. We believe that a better understanding of the multi-level regulation of biosynthesis of intermediates and metabolites, coupled with an understanding of their bioactivity, will help us harness the inherent diversity of *Ocimum* species optimally.The present study aims to explore *Ocimum* metabolome for important metabolites and study their biosynthesis, storage and transport in plant.

Chapter 1 includes a detailed survey of literature related to *Ocimum* species and their importance in the plant kingdom. It also discusses our present understanding of the metabolite diversity present across different species and factors responsible for the complex chemical evolution. Based on our comprehensive understanding of the genus and

available metabolomic and genetic resources, the chapter concludes with the objectives of the work.

Chapter 2 comprises exploring medicinal applications of *Ocimum* metabolites. The study revealed eugenol as an effective inhibitor of Advanced Glycation End products (AGEs) using biophysical, biochemical, proteomic and *in vivo* mice model studies. Eugenol was found to have a dual mode of action in combating diabetes; it lowered blood glucose by inhibiting α-glucosidase and prevented AGE formation by binding to ε-amine group on lysine, protecting it from glycation, offering potential use in diabetic management.

Chapter 3 deals with exploring agro- based application of *Ocimum* metabolites. Defense metabolites including camphor, limonene and β-caryophyllene were found to be effective bio-pesticides, using feeding- choice assays, analysis of insect growth and mortatlity data as well as studying changes in tuning of primary and secondary metabolism in plant.

Chapter 4 deals with understanding the biosynthesis, transport and storage of important *Ocimum* metabolites revealed in our previous studies. Integrating transcriptomics with metabolomics not only helped us in dissecting the camphor and eugenol biosynthetic pathway from camphor basil, it also revealed stringent metabolite partitioning betweeen aerial and root tissues. The molecular mechanism underlying metabolite partitioning and its probable biological relevance to the plant was further explored.

Chapter 5 gives a comprehensive summary, highliting key findings from the work done during the Ph.D. tenure, finally culminating into major conclusions and future prospects.

CHAPTER 1

General Review of Metabolite Diversity and Complex Chemical Evolution in Genus *Ocimum*

1.1 Introduction

Genus *Ocimum* belonging to family Lamiaceae comprises between 50 to150 species.[1] The difference in the estimates of species number is partly attributed to reasons like taxonomic revisions and generic description of the genus amongst others. It was first described by Linnaeus in 1753 in the book *Species Plantarum*.[2] The name *Ocimum basilicum* was derived from the Greek word *Okimon* (smell) and *basilikon* (royal), referring to its royal fragnance. While in India the *Ocimum* plant is considered sacred and worshipped, in other parts of the world it is hailed as the "queen of herbs" because of its strong aromatic appeal and culinary usage. With the establishment of ancient medicinal practises in India (Ayurveda) and China (Traditional Chinese Medicine), *Ocimum* was recognised as a medicinal herb with great healing powers. Ocimum kilimandscharicum or camphor basil is a commercially and medicinally important specie which grows in rich moist well drained soils and ambient temperature.

Main centres of diversity for *Ocimum* include tropical and subtropical regions of Africa, India and South America.[3] With the exception of *O. tenuiflorum* and *O. gratissimum* that are indegenous to India, most species are native to Africa including O. *kilimandscharicum* and found in wild population.[4] Although *Ocimum* species are known to abound in medicinally important metabolites, only few species have been thoroughly profiled. Our knowledge about most other species remains limited. All species are identifiable by the presence of a large amount of signature metabolite(s) along with several other metabolites in relatively minute quantities. The diversity of metabolites produced by *Ocimum* plants is indeed enormous. Specific functions and/or necessity for production of such diverse and complex chemical compounds by the plant remain elusive. Interestingly, what we know is

certain *Ocimum* species are either "terpenoid-rich" or "phenylpropanoid-rich". However, factors determining the direction of flux are largely unknown. Terpenoids are formed from the mevalonic acid (MVA) pathway in the cytoplasm and the methylerythritol phosphate (MEP) pathway in the plastid.[5] Phenylpropanoid pathway starts with the amino acid phenylalanine and eventually results in the formation of phenylpropenes such as eugenol, chavicol, anethole etc., along with intermediates for biosynthesis of lignin, rosmarinic acid, anthocyanins etc. These pathways have been well characterized in related genera including *Salvia*, *Mentha* and *Lavandula* [6-12] but not in such details in any *Ocimum* species. However, with the influx of next generation sequencing data[13, 14] along with metabolomics, proteomics and phylogeny studies,[15-19] now it seems possible to gain a deeper insight into the perplexing diversity. The present review aims at providing a comprehensive overview of the evolutionary, environmental and internal factors that may have resulted in pathway diversification and extensive chemical evolution across *Ocimum* species.

1.1.1 Importance of studying genus *Ocimum*

The unequivocal importance of genus *Ocimum* was established more than 5,000 years back with the advent of ancient traditional medicinal practises in India and China. Thereafter, there have been several reports of important bioactivities of *Ocimum* species; tissue extracts and metabolites there in (**Table 1.1**).[20-81] Although most species in this genus are associated with some or the other bioactivity, the exact compound or group of compounds, responsible for the said bioactivity remains elusive in most cases (**Table 1.1**). Basil also finds extensive application in the food, flavor, and fragnance industry, and the essential oil serves as a major source of economic wealth to the country. The plant is easy

to grow and propagate, and adapts well to extreme environmental conditions including high precipitation, long dry spells and high temperature. Some species are capable of vegetative propagation through stem cuttings like *O. kilimandscharicum*, which makes commercial cultivation less tedious and more cost effective. Several *Ocimum* species grow as wild plants in various parts of the world. Since there has been no significant domestication of this wild medicinal plant, its genetic diversity has been preserved in nature, making the system more interesting to explore. Furthermore, presence of different basil types/cultivars rich in diverse metabolites provides a unique system for studying secondary metabolic pathways. In addition, glandular trichomes accord the opportunity to study the biosynthesis and regulation of these pathways at the level of a single cell. *Ocimum* thus presents an attractive system to explore, particularly from the point of view of secondary metabolism.

1.1.2 Overview of extensive diversity within genus *Ocimum*

Although genus *Ocimum* boasts of 50 – 150 species, metabolite data for very few species is available. For example, *O. obovatum* and *O. labiatum* are well tested for therapeutic properties (**Table 1.1**); however, their chemical composition remains unknown. *Ocimum* species abound in diverse secondary metabolites including terpenoids, phenylpropanoids, rosmarinic acid, flavonoids and phenolics. **Figure 1.1** shows representative examples of structurally diverse classes of secondary metabolites found across genus *Ocimum*. These mainly include monoterpenes (example, camphor, eucalyptol, α-pinene, β-ocimene, terpinolene), sesquiterpenes (example, farnesene, β-caryophyllene, germacrene D) and phenylpropanoids (example, eugenol, eugenol methyl ether, chavicol, methyl chavicol, methyl cinnamate). Few metabolites like germacrene D and β-caryophyllene are

commonly found across most species in the genus; however, others like camphor and eugenol have a specie- specific distribution. Higher terpenes (C20 and above) and alkaloids have not been well charaterized from any *Ocimum* species.

Table 1.1: Bioactivities of *Ocimum* species tissue extracts/purified compound(s)

sp.	**Bioactivity**	**Extract/Compound**
Ok	Free radical scavanging[20]	Leaf essential oil, camphor, mixture of 1,8-cineole and limonene
	Anticancer[20, 21]	Leaf essential oil;[20] 50% alcoholic aqueous leaf extract[21]
	Anti-inflamatory[20]	Leaf essential oil, camphor, mixture of 1,8-cineole and limonene
	Insecticidal[22]	DCM leaf extract, camphor, limonene and β-caryophyllene
	Antidiarrhoel[23]	Aqueous leaf extract
	Antimicrobial[24]	Essential oil, borneol, bornyl acetate, camphor, caryophyllene oxide, 1,8-cineole, limonene, linalool, α-pinene, β-pinene, spathulenol
	Antiplasmodial[25]	DCM plant extract
	Antioxidant[26,27]	Methanolic extracts of leaves,[26, 27] and callus[26]
	Radioprotective[21]	50% alcoholic aqueous leaf extract
	Mosquito repellent[28]	Plant essential oil, dry plant material
Olb	Antioxidant[29]	Ethanolic leaf extract, labdane (isolated diterpenoid)
	Anti-inflammatory[29]	Ethanolic leaf extract, labdane diterpenoid
Ola	Antimicrobial[24,30,31]	Essential oil extract; Ethanolic extract of various plant parts; methanol, aqueous and n-hexane extracts
	Mosquito-repellent[32]	Volatiles from fresh, dried and smoking dried leaves
	Antioxidant[27,33]	Plant essential oils and methanolic extracts[27]
	Anti-inflammatory[34]	Aqueous and ethanolic leaf extracts
	Hepatoprotective[35]	Aqueous and methanolic leaf extracts

	Analgesic[36]	Aqueous and ethanolic plant extracts
Oo	Anitmicrobial[37]	Leaf essential oil
Ot	Antidiabetic[38]	60% ethanolic leaf extract
	Anti-hyperlipidemic[38]	60% ethanolic leaf extract
	Anti-oral toxicity effect[38]	Hydroalcoholic leaf extract
	Antioxidant[39]	Methanolic extracts of leaf, inflorescence, stem and callus
	DNA damage protective[40]	Anthocyanin extracts
	Antibacterial[41,42]	Essential oil
	Anticancer[43]	Aqueous and ethanolic leaf extracts
	Antiglycation[44]	Methanolic and water extracts and their fractions (DCM, ethyl-acetate, *n*-butanol, water)
	Antistress[45]	OciBest (whole plant extract in gelatin capsules)
	α-amylase inhibitory[46]	Isopropanol extract
	Mosquito repellent[47]	Plant essential oil
	Antiherpes[48]	Methanol and DCM extracts
	Ameliorative potential[49]	Methanol extracts, Saponin- rich fraction
Oa	Free Radical Scavenging[50]	Ethanol, butanol and ethyl-acetate extracts from leaves
	Anti-inflammatory Activity[51]	Essential oil, linalool, 1,8-cineole
	Anti-herpes[48]	Methanol and DCM extracts
	Antimircobial[52]	Plant essential oil
Oba	Antiherpes[48]	Methanol and DCM extracts
	Anti-inflammatory [53,54]	Whole plants;[53] Ethanol-water (25%) extract of leaves[54]
	Antiplasmodial[55]	Plant ethanolic extracts (leaf, stem, root,flower)
	Antioxidant and Antimicrobial[56-60]	Essential oil extracted *via* hydrodistillation;[56] plant extracts prepared using ethanol, butanol, chloroform,water, ethyl acetate;[58] essential oil, linalool, eugenol[59] acetone and ethanol extracts[60]
	Antimalarial[61]	Leaf essential oil
	Anticancer[62,63]	Plant methanolic extract;[62] petroleum ether soluble and insoluble methanolic extracts, ursolic acid
	Larvicidal activity[64,65]	Leaf essential oil[64]

	Antituberculosis[66]	Methanolic extract of leaves, fruits and flowers; bacilicin
	Preventing ischemia, reperfusion-induced cerebral damage and motor dysfunctions[67]	Ethyl-acetate extract of leaves
	Antihypertensive effects[68]	Aqueous plant extract
	Vasorelaxant and anti-platelet aggregation[69]	Aqueous plant extract
	Antigiardial activity[70]	Plant essential oil, linalool, eugenol
	Antiviral[71]	Aqueous and ethanolic plant extracts, apigenin, linalool, ursolic acid
Og	Protection of liver from oxidative stress and fibrosis[72]	Polyphenol extract
	Antioxidant and Antimutagenic[73]	Leaf aqueous extract
	Antitrypanosomal and antiplasmodial[74]	Crude ethanol extract, essential oil and pure compounds
	Free radical scavenging and antioxidant[75]	Aqueous extract, methanol extract and eugenol
	Prevention against Liver Fibrosis[76]	Aqueous leaf extract
	Antimicrobial[77,78,79]	Plant essential oil;[77,78] eugenol, methyl eugenol;[77] hexane and methanol extracts alone and in combination with aminoglycosides[79]
	Corrosion Inhibition[80]	Seed extract
	Cerebroprotection[81]	Ethanolic plant extract

*****Ok (*O*. *kilimandscharicum*), Olb (*O*. *labiatum*), Ola (*O*. *lamiifolium*), Oo (*O*. *Obovatum*), Ot (*O*. *tenuiflorum*), Oa (*O*. *americanum*), Oba (*O*. *basilicum*), Og (*O*. *gratissimum*), DCM (dichlorormethane)

Since most *Ocimum* species have not been profiled for their metabolites, the possibility that the genus represents much more diversity than what we percieve now is realistic. As mentioned previously, each species is characterized by a distinct metabolic fingerprint and

presence of a signature compound(s) as the major fraction. Although metabolite profiling *via* conventional techniques such as gas chromatography (GC) has been routinely employed, advanced analytical techniques including liquid chromatography (LC), mass spectrometry (MS) and nuclear magnectic resonance (NMR) have not been reported, which help in gaining a better understanding of the global distribution of metabolites and pathway intermediates.

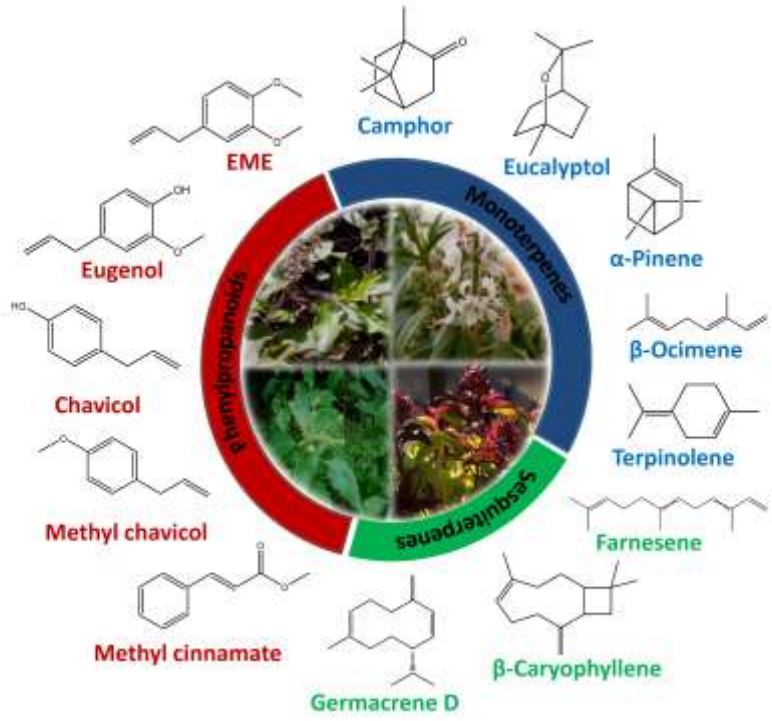


Figure 1.1: Representative examples of structurally diverse classes of secondary metabolites *viz*. monoterpenes, sesquiterpenes and phenylpropanoids found across genus *Ocimum*

Till now only 12 *Ocimum* species have been analysed for their essential oil composition (**Fig. 1.2**).

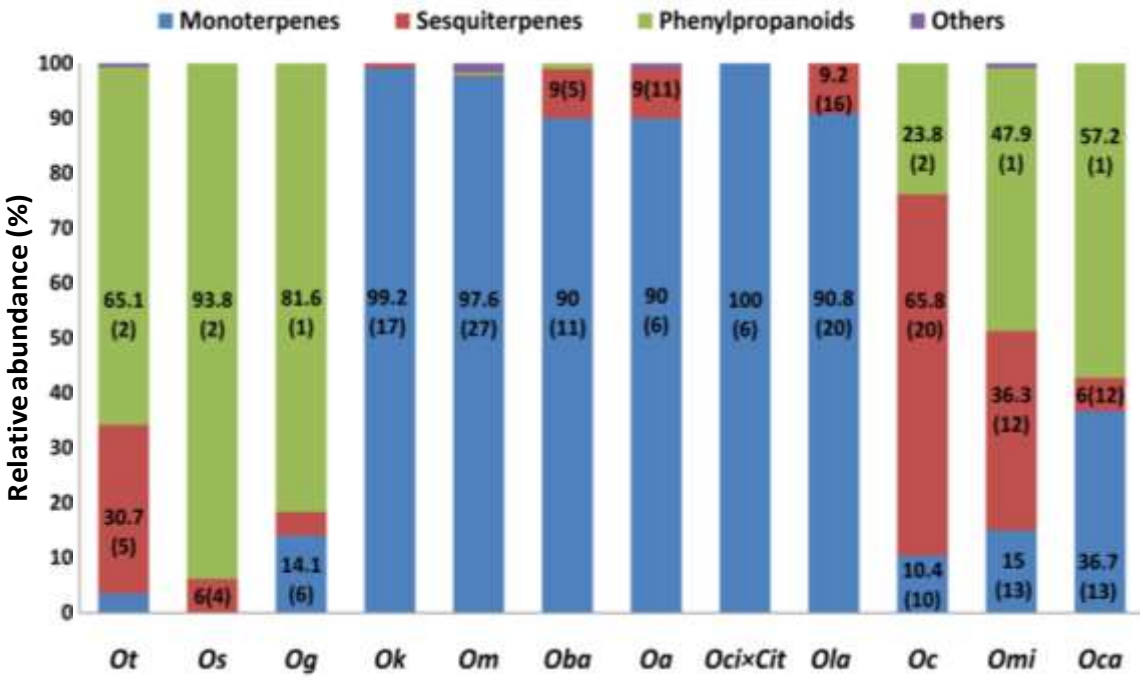


Figure 1.2: Overview of diversity across *Ocimum* species. Numbers in parenthesis indicates number of compounds; numbers outside parenthesis indicate percentage of metabolite in total volatile fraction. (*Ot*, *O. tenuiflorum*; *Os*, *O. selloi*; Og, *O. gratissimum*; *Ok*, *O. kilimandscharicum*; *Om*, *O. minimum*; *Oba*, *O.basilicum*; *Oa*, *O. americanum*; *Oci x Cit*, *Ocimum x Citriodorum*; *Ola*, *O. lamiifolium*; *Oc*, *O. campechianum*; *Omi*, *O. micranthum*; *Oca*, *O. canum*)

Overall, they can be classified as having (i) high phenylpropanoid content, (ii) high terpenoid content, and (iii) similar/comparable amounts of phenylpropnoids and terpenoids. High phenylpropanoid content group contains about 60 to 90% phenylpropanoids and includes *O. gratissimum*,[82] *O. tenuiflorum*[83] and *O. selloi*.[84] High terpenoid containing group, includes *O kilimandscharicum*,[85] *O. minimum*,[86] *O. basilicum*,[87] *O. americanum*,[88] *Ocimum* × *citriodorum*[89] and *O. lamiifolium*[90] contain approximately 40 to 75% terpenoids. The third group includes *O. campechianum*,[91] O.

micranthum[92] and *O. canum*[93] which show approximately equal amount of phenylpropanoids and terpenoids. Interestingly, terpenoids unlike phenylpropanoids, show a universal presence in varying amount in all *Ocimum* species.

Signature compounds known in *Ocimum* species are as follows: camphor in *O. kilimandscharicum* (56%), citral in *O. americanum* (47%), eugenol in *O. gratissimum* (82%) and *O. micranthum* (47%), eugenol methyl ether in *O. tenuiflorum* (62%), linalool in *O. basilicum* (48%), methyl chavicol in *O. selloi* (93%) and *O. canum* (53%), geranyl acetate in *O. minimum* (70%), sabinene in *O. lamiifolium* (33%) and geranial in *Ocimum* × *citriodorum* (43%) (**Fig. 1.2**). In plant kingdom, metabolite diversity is commonly found at the level of family or genus, but such vivid diversity at the level of species and subtypes (within species) makes genus *Ocimum* occupy a special niche in nature.

1.2 Potential evolutionary events influencing metabolite diversity *via* pathway modulation

Ocimum genome has evolved as a result of dramatic series of events including polyploidy, aneuploidy, chromosomal duplications/translocations/deletions etc., [16, 94, 95] which led to unprecedented diversification of species in Africa, India and South America. The ability to cross-pollinate and hybridize further led to the emergence of subtypes within species, which were capable of interbreeding and producing hybrids. For example, *Ocimum×citriodorum* is a hybrid between *O. americanum* and *O. basilicum* and has a strong lemony scent.[18] *O. americanum* originated from *O. canum* and *O. basilicum*.[96] The African blue basil subtype (*O. kilimandscharicum*) is evolved as a hybrid between *O. kilimandscharicum* and *O. basilicum* and abounds in camphor, linalool and eucalyptol.

Interestingly, the hybrids display significantly different metabolite profile than their parents including new metabolites that are not found in the parents, indicating co-dominance, epistatis or interaction of genes.[89] As reported in several other plant genera, ploidy levels also affect essential oil production, resulting in a greater accumulation of essential oils in polyploids than that in diploids.[95, 97-99] All these events taken together might have led to greater genetic diversity and continuous expansion of gene pool, yielding new species/subtypes/varieties over a short period.

During the course of evolution, there may have been events that led to terpenoid and phenylpropanoid pathway diversification across different *Ocimum* species. It is interesting to note that species abounding in phenylpropanoids also have an active terpenoid pathway and vice versa. This suggests that all species evolved from an ancestor, which harbored active genes for both the pathways. However, differential expression and regulation of pathway genes determined the final chemical composition in each species.[5, 100] Other factors like plant habit may also have influenced the selection of one pathway over the other. For example, it has been suggested that the sanctum group has evolved to produce phenolic compounds because of its perennial woody habit, whereas the basilicum group has evolved to produce terpenoid-rich compounds owing to its annual herbaceous habit.[4] Evolution of gene coding regions also had a profound impact on the diversity of *Ocimum* species metabolites. For example, *O. basilicum* fenchol synthase and myrcene synthase, and geraniol synthase and linalool synthase are 95% and 81% similar, respectively; however, they catalyse the formation of very different products. These genes most probably evolved as a result of gene duplication events and acquired mutations leading to functional differentiation, [100] eventually contributing to metabolite diversity. Few pathway

genes involved in the biosynthesis of selected metabolites have been reported and characterized from *Ocimum* and few other genera (**Table 1.2**).

Table 1.2: Genes involved in biosynthesis of major secondary metabolites in basil

Compound	**Gene (abb.) (org.)**	**Reaction catalysed**
Eugenol	Eugenol synthase (EGS) (*O. basilicum* and *F. annanasa*)	coniferyl acetate to eugenol
	Coumaryl CoA Ligase (4CL) (*O. tenuiflorum*)	hydroxycinnamic acids to Coenzyme A (CoA) esters
	R2R3-MYB transcription factor (EOBII) (*F. annanasa*)	transcription factor regulating structural genes in phenylpropanoid pathway
Eugenol methyl ether	Eugenol O-methyl transferase (EOMT) (*O. basilicum*)	eugenol to eugenol methyl ether
Methyl chavicol	Chavicol O-methyl transferase (CVOMT) (*O. basilicum*)	chavicol to methyl chavicol
Camphor	bornyl diphosphate synthase (BPPS) (S. *officinalis*)	geranyl diphosphate to bornyl diphosphate
	Borneol dehydrogenase (BDH) (*S. officinalis* and *L. intermedia*)	borneol to camphor
Eucalyptol (1,8- cineole)	1,8-cineole synthetase (S. *officinalis*)	neryl diphosphate to 1,8-cineole
Linalool	Linalool synthase (LIS) (*O. basilicum*)	GPP to linalool
Terpinolene	Terpinolene synthase (TES) (*O. basilicum*)	GPP to terpinolene (as major product) and α-pinene and limonene (as side products)
Fenchol	Fenchol synthase (FES) (*O. basilicum*)	GPP to fenchol (as major product) and α-pinene and limonene (as side products)
Myrcene	Myrcene synthase (MES) (*O. basilicum*)	GPP to myrcene
Cadinene	Cadinene synthase (CDS) (*O. basilicum*)	FPP to γ-cadinene (as major product) and Muurola 3,5-diene (as side product)

Selinene	Selinene synthase (SES) (*O. basilicum*)	FPP to α & β- selinene (major product); β-elemene and nerolidol (side product)
Zingiberene	Zingiberene synthase (ZIS) (*O. basilicum*)	FPP to α-zingiberene (major product); α-bergamotene, nerolidol, β-farnesene and β-bisabolene (side product)
Germacrene-D	Germacrene-D synthase (GDS) (*O. basilicum*)	FPP to Germacrene-D
Geraniol	Geraniol synthase (GES) (*O. basilicum*)	GPP to geraniol
Amyrin (triterpene)	2,3-oxidosqualene cyclase (AS1 and AS2) (*O. basilicum*)	2,3-epoxy-2,3-dihydrosqualene to α/β- amyrin
General phenyl propanoid pathway	Production of anthocyanin pigment 1 (PAP1) (*A. thaliana*)	transcriptional regulator of floral scent
	p-coumaroyl shikimate 3'-hydroxylase (CS3'H) (*O. tenuiflorum*)	p-coumaroyl 5-O- shikimate to caffeoyl 5-O- shikimate
	Caffeic acid O-methyl transferase (COMT) (*O. basilicum*)	caffeate to ferrulate
	Caffeic acid O-methyl transferase (COMT) (*O. tenuiflorum*)	caffeate to ferrulate
	Cinnamyl alcohol dehydrogenase (CAD) (*O. tenuiflorum*)	cinnamyl alcohol to cinnamyldehyde
	Cinnamyl alcohol dehydrogenase (CAD) (*O. basilicum*)	cinnamyl alcohol to cinnamyldehyde
	Cinnamate-4-hydroxylase (C4H) (*O. tenuiflorum*)	cinnamic acid to 4-coumaric acid
	Cinnamate-4-hydroxylase (C4H) (*O. basilicum*)	cinnamic acid to 4-coumaric acid

Genes like eugenol synthase involved in catalysing the final step of eugenol production has been well charaterized (**Table 1.2**). However, most genes present upstream in the eugenol biosynthetic pathway remain functionally uncharacterized despite availability of

huge transcriptomic databases. Genes from camphor biosynthesis pathway have been well characterized from related genera like *Salvia* and *Lavandula*, however, there are no reports from genus *Ocimum* (**Table 1.2**). Modifying enzymes like chavicol and eugenol O-methyltransferases also have been well characterized (**Table 1.2**). Information about transcription factors responsible for controlling biosynthesis of these metabolites and the transporter proteins responsible for long distance transport from source to sink tissue in *Ocimum* species also remains scarce. Genes reported from yet another important category of compounds, flavones and flavonoids, have been listed in **Table 1.2**. Overall, information about the biosynthesis, transport and storage of these metabolites, at the genetic level is very scarce and need to be further probed. Several other factors during species diversification and naturalization in other parts of the world have been discussed briefly, which help us in explaining the mystery behind the complex chemical evolution and pathway diversification.

1.3 Factors regulating secondary metabolite flux and chemical diversity in *Ocimum* species

Metabolite diversity observed at the level of species in genus *Ocimum* is dependent on several internal and external factors (**Fig. 1.3**). Some of the known factors responsible for regulating terpenoid and phenylpropanoid pathways are discussed.

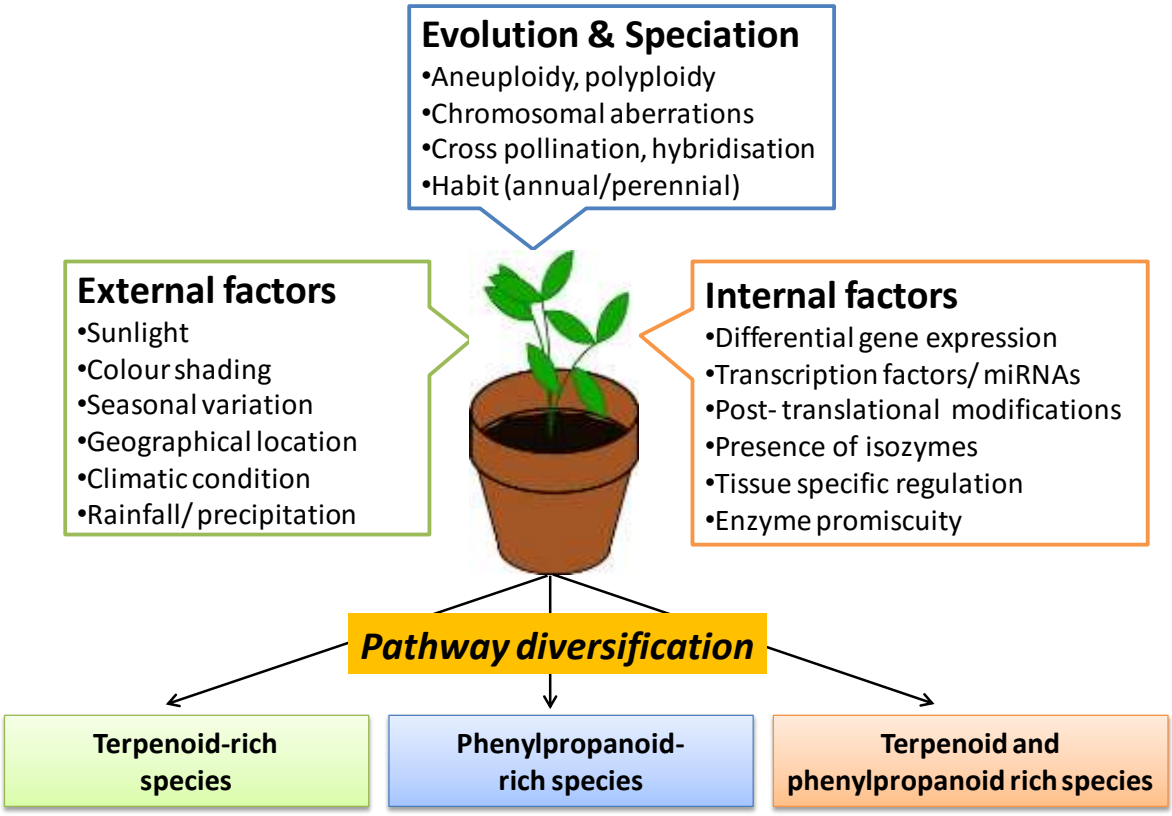


Figure 1.3: Factors responsible for chemical diversity; and terpenoid and phenylpropanoid pathway diversification in *Ocimum* species

1.3.1 Differential gene expression of enzymes in phenylpropanoid and terpenoid pathways

Gene expression plays an important role in diverting metabolic flux toward either the terpenoid or the phenylpropanoid pathway.[5, 13,100] In particular increased expression of terminal enzymes in the terpenoid pathway and reduced expression of phenylpropanoid entry point enzymes such as phenylalanine ammonia-lyase (PAL) has been observed in *O. basilicum var*. SD, rich in terpenoids. In another variety, *O. basilicum var*. EMX, however, the expression level of general phenylpropanoid pathway enzymes, PAL and 4-coumarate-CoA ligase (4CL) was found to be significantly higher corresponding to

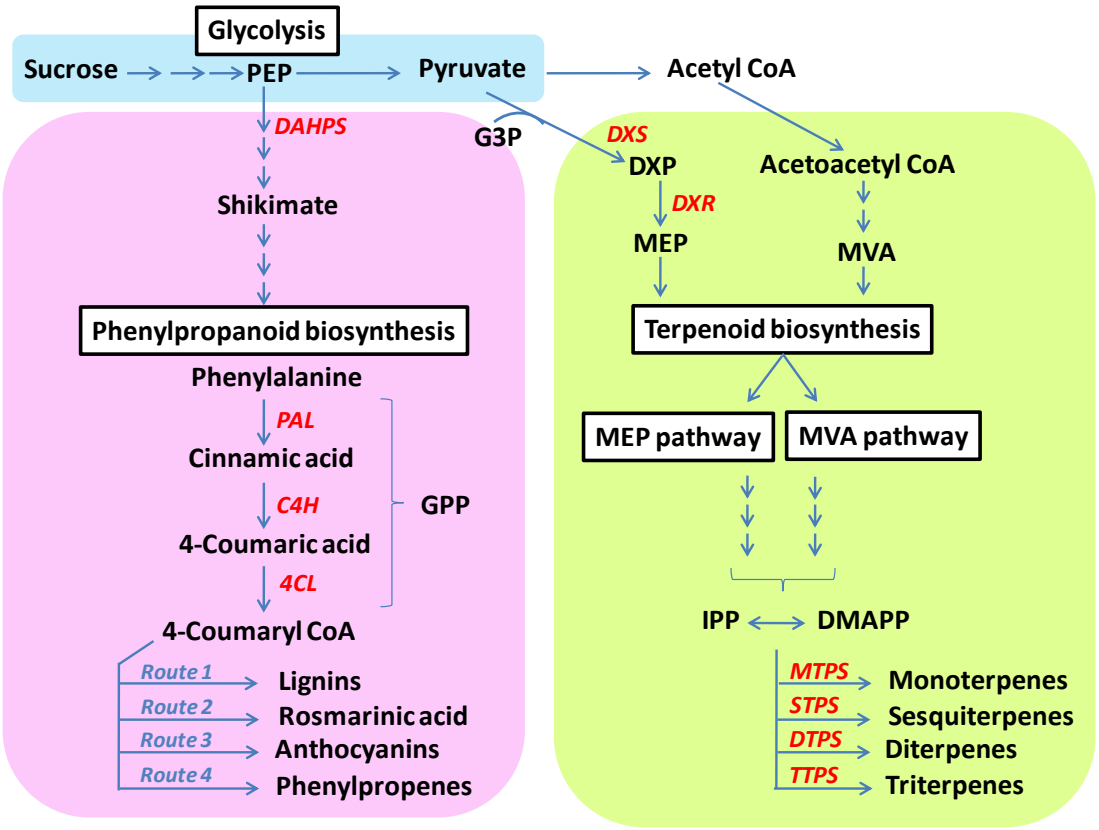


Figure 1.4: Major regulatory checkpoints in phenylpropanoid and terpenoid pathways. Enzymes potentially governing the direction of flux have been marked in red. (PEP, Phosphoenol pyruvate; G3P, Glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, Methylerythritol phosphate; MVA, Mevalonic acid; IPP, Isopentenyl pyrophosphate; DMAPP, Dimethylallyl pyrophosphate; GPP, General phenylpropanoid pathway; DAHPS, 3-Deoxy-D-arabinoheptulosonate 7-phosphate synthase; PAL, Phenylalanine ammonia-lyase; C4H, Cinnamate-4-hydroxylase; 4CL, 4-Coumarate-CoA ligase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; MTPS, Monoterpene synthases; STPS, Sesquiterpene synthases; DTPS, diterpene synthases; TTPS; Triterpene synthases)

higher phenylpropanoid content.[5] These results were supported by next generation sequencing data of *O. tenuiflorum* and *O. basilicum*.[14] *O. tenuiflorum* rich in phenylpropanoids, shows much higher expression of general phenylpropanoid pathway enzymes including PAL, cinnamate-4-hydroxylase (C4H) and 4CL, reads per kilobase per million (RPKM) = 91.47, 34.53 and 9.52 respectively; compared to *O. basilicum* rich in terpenoids, RPKM = 11.3, 11.83 and 5.65 respectively. In *O. basilicum*, however, the entry point enzymes of the MEP pathway, representing the cytosolic pathway for terpenoid synthesis, including 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) was more (RPKM = 50.58) compared to *O. tenuiflorum* (RPKM = 15.69).[14] Thus, overexpressing the entry point enzymes at major metabolic branching points also helps in directing the flux towards either phenylpropanoid or terpenoid pathway.[122-124] Evidently, differential expression of enzymes strategically present at pathway branch points might play a crucial role in determining flux regulation (**Fig. 1.4**).

1.3.2 Enzyme promiscuity

One of the major reasons for metabolite diversity observed in *Ocimum* species is the promiscuity of terpene synthases. These enzymes are capable of accepting a substrate and yielding a major product as well as multiple side products. For instance, Iijima *et al* characterized eight terpene synthases from three cultivars of *O. basilicum*.[100, 114] *In vitro* recombinant protein assays using geranyl diphosphate (GPP) as substrate for putative monoterpene synthases and farnesyl diphosphate (FPP) as substrate for putative sesquiterpene synthases was performed. Terpinolene synthase gave terpinolene as the major product and α-pinene, limonene and an unidentified monoterpene as the side products. Fenchol synthase produced fenchol and limonene as major products and α-

pinene and an unidentified monoterpene as the side products. Cadinene synthase produced γ-cadinene as the major product and muurola 3, 5-diene as the side product. Selinene synthase produced selinene as the major product and β-elemene and nerolidol as side products. In contrast, myrcene synthase and geraniol synthase exclusively produced myrcene and geraniol as end products.[100] In another study by Major *et al*, using bornyl diphosphate synthase (producing camphene as the side product), it was proven that electrostatically guided dynamics determined end product formation.[110] Current evidence suggests that enzyme promiscuity may play an important role in contributing to the diversity across *Ocimum* species.

1.3.3 Transcription factors

Transcriptional regulation of secondary metabolism in plants for flavonoids (particularly anthocyanins), alkaloids (including nicotine, indole alkaloids and benzylisoquinolines) and terpenoids has been widely reported.[125-127] Recently, PAP1 transcription factor was shown to enhance the production of both terpenoids and phenylpropanoids in rose plant.[116] Deep sequencing of *O. tenuiflorum* and *O. basilicum* revealed the presence of 40 transcription factor families including MYB, WRKY, bHLH, HB, NAC, bZIP etc. which are known regulators of secondary metabolism in plants.[14] A recent study performed using the red and green forma of *O. tenuiflorum* suggested light-mediated regulation of anthocyanin accumulation.[128] It was observed that when red forma seedlings grown under natural lighting conditions, were transferred to a special greenhouse which cuts off the UV-A and UV-B radiation, the leaves turned green within 20 days. Further investigation revealed the role of transcription factors, bHLH and WD40, in downregulating the terminal enzymes of anthocyanin biosynthesis including flavonone-3′-hydroxylase,

leucoanthocyanidine dioxygenase and dihydroflavonol reductase, responsible for red coloration. In another study by Misra *et al*, transcription factors belonging to APETALA2/Ethylene responsive factor (ERF), WRKY, plant homeo domain (PHD) and zinc finger families were upregulated in methyl jasmonate (MeJa)-treated *O. basilicum* plants, suggesting their possible role in regulating secondary metabolism in *Ocimum* species.[115] Thus, available data suggests transcription factors are also key regulators of terpenoid and phenylpropanoid pathway in *Ocimum* species and provide a more stringent control over the direction of flux.

1.3.4 Post-translational modifications

Post-translational modifications including phosphorylation, ubiquitination and arginine monomethylation of phenylpropanoid and terpenoid pathway enzymes such as phosphoglucomutase, glucose-6-phosphate isomerase, phosphoglycerate mutase, PAL and chavicol O-methyl transferase (CVOMT) were observed in basil glandular trichomes. Post translation modifications help in explaining situations where the mRNA level does not match with the metabolite or protein level. For example, the enzyme CVOMT is responsible for methylating chavicol. *O. basilicum var*. SD produces negligible amount of methylchavicol. However, the mRNA and protein levels for this enzyme were found to be very high. In contrast, very little enzyme activity and metabolites were detected. It was observed that this enzyme was ubiquinated providing a valid explanation for the discrepencies in mRNA, protein, enzyme activity and metabolite level. Ubiquitination leads to a rapid degradation of CVOMT post translation,[5] resulting in decreased formation of methyl chavicol. In another example, PAL, catalyzing the first committed step in phenylpropanoid biosynthesis, is phosphorylated in *O. basilicum var*. SD, rich in

monoterpenes; however, other basil varieties (SW, MC, and EMX-1), rich in phenylpropanoids, lack PAL phosphorylation.[5] It has been reported earlier that phosphorylation results in the reduction of PAL activity.[129,130] Above examples suggest that post translation modifications provide an additional regulatory step in determining the expression of key enzyme activities in secondary metabolic pathways in *Ocimum* species.

1.3.5 Presence of isozymes

Phenylpropanoid pathway produces substrates for synthesis of several important secondary metabolites. PAL, C4H and 4CL catalyse the initial few steps leading to the formation of coumaryl CoA. Latter represents a branching point, from which different end products including phenylpropenes, lignins, flavonoids and rosmarinic acid can be synthesized. Thus, 4CL represents a crucial step in pathway regulation and diversification. In recent work by Rastogi *et al*, it was reported that *O. basilicum* 4CL has 5 different isoforms.[103] RNAi experiments involving the silencing of a specific isoform, Oba4CL, led to a reduced production of phenylpropanoids without affecting lignin and sinapic acid content. Thus, only one of the isoforms of 4CL was involved in the synthesis of phenylpropenes. This also represents the commitment of a specific isoform of an enzyme to a specific biosynthetic pathway at a very initial step. Presence of such pathway-committed isoforms keeps the pathway finely tuned and delicately balanced in basil.

1.3.6 External factors

Being species native to the tropics, *Ocimum* plants are always subjected to severe environmental conditions including excessive heat, rainfall, humidity, dryness etc. Adaptability, thus, is the key to survival. It has been reported that external environmental

factors, including the type of light, radiation, season, geographic conditions etc., influence essential oil composition. Same *Ocimum* specie show altered metabolic profile under different environmental factors. Red and blue shading conditions in *O. selloi* showed decline in level of phenylpropanoids and elevated level of in comparison with plants grown in full light.[84] Plants grown under blue shading had more number of metabolites than plants subjected to full light and red shading. Decreased accumulation of methyl chavicol was observed in plants cultured under colored netting, accompanied by an increase in α-copaene, germacrene D and bicyclogermacrene content.[84] This suggests a chemical defense strategy of plants against less favorable growth conditions. Similar kind of study was performed with *O. basilicum* cultivated in soil covered by colored mulches which demonstrated that size and aroma of leaves as well as the concentration of soluble phenols greatly improved.[131] Seasonal variation of essential oil composition was observed in *O. basilicum* and *O. tenuiflorum*.[83] To show the effect of geographic conditions on essential oil composition, *O. gratissimum* and *O. campechianum* were grown in Chocó Department (Columbia) and Ecuador region that resulted in different chemical composition.[91] Similarly, *O. basilicum* and *O. gratissimum* grown in Benin, Cameroon, Congo and Gabon vary in chemical composition.[90] *O.gratissimum* plants grown in Columbia showed altered metabolite profile as compared with those grown in Europe.[91,132-135] This data indicates external factors including climatic conditions and geographical variations might be influencing the chemical profile of *Ocimum* species.

1.3.7 Developmental and tissue specific regulation

During cinnamic acid and methylcinnamate (MC) formation from phenylalanine, activity of two enzymes, PAL and S-adenosyl-L-methionine: cinnamate carboxyl

methyltransferase (SAM:CCMT) shows an important regulatory control point.[136] In different developemental stages of *O. basilicum*, the relation between MC content, PAL and SAM:CCMT activity was examined. SAM: CCMT activity showed correlation with MC content in young leaves.[136] Likewise, eugenol-O-methyltransferase (EOMT) is responsible for methylation of eugenol to form methyleugenol in one of the final steps of phenylpropanoid pathway. The expression pattern of EOMT positively correlated with the amount of eugenol/isoeugenol and methyleugenol in different developmental stages of all the analyzed chemotypes.[137] Along with development-specific regulation of metabolite accumulation, some metabolites in *Ocimum* species also show tissue-specific regulation. For example, analysis of trichome, leaf, stem and root shows a strong association between eugenol content and Ob4CL expression in *O. basilicum*.[103]

1.3.8 MicroRNA mediated regulation

Based on *O. basilicum* EST data set, the function of miRNAs and their targets was predicted using *in silico* approach.[138] Four miRNA families miR164c, miR5658, miR414 and miR5021 were evaluated for their potential targets. These miRNA families showed regulatory role during stress-metabolite response. Although this study was based upon computational evaluation, further *in planta* experimentation is required to determine the critical role of miRNAs during secondary metabolism in *Ocimum* species.[138]

1.4 Future Applications

Ocimum acts as a reservoir of several important secondary metabolites found in nature, thereby making it a very attractive system to explore. Although the genome of *Ocimum* has not yet been sequenced, the recent influx of next generation sequencing data of various tissues such as trichomes and leaves, has helped us in understanding various

factors that are responsible for regulating the formation of phenylpropanoids and terpenoids in *Ocimum* species. Using the current information, we can genetically engineer *Ocimum* species to overexpress the desired metabolites by redirecting the metabolite flux.[139-142] This knowledge can also be used for breeding new chemotypes producing interesting spectra of essential metabolites. Since these metabolites impart flavor and aroma, and possess medicinal properties, they can be heterologously expressed in plants, which are routinely used raw in our diet, such as tomato, thereby increasing their flavor and nutritive value. The expression of *O. basilicum* α-zingiberene synthase under the control of polygalacturonase promotor led to the unexpected accumulation of 15 sesquiterpenes and 10 monoterpenes, which were not present in the non-transformed fruit.[143] In a separate study, the expression of *O. basilicum* geraniol synthase under the same promoter led to the accumulation of geraniol and its derivatives, which had profound impact on tomato flavor as well as aroma.[144] Moreover, expressing terpene synthase genes from *Ocimum* in food crops will impart greater resistance against pathogens and pests. Till date, it is not well established whether there is a cross talk between the phenylpropanoid and terpenoid pathways. The glandular trichomes present in several *Ocimum* plants provide a very exciting isolated single-celled system to unravel the exchange, if any, of upstream intermediates between these two pathways. Thus, *Ocimum* species find useful applications in industrial, culinary, medicinal as well as scientific research areas, asserting their important position in the plant kingdom.

1.5 Objectives of the work

The experimental work in this thesis revolves around exploring the *Ocimum* metabolome for metabolites of medicinal, agricultural and commercial importance and understanding their biosynthesis, transport and storage *in planta*. The major objectives of the work are as follows:

- Understanding metabolite diversity present across genus *Ocimum* and factors responsible for complex chemical evolution
- Exploring medicinal applications of *Ocimum* metabolites using biophysical, biochemical, proteomic and *in vivo* mice model studies
- Elucidating agro- based application of *Ocimum* metabolites (defense metabolites as effective bio-pesticides)
- Next generation sequencing (using Illumina platform) and global untargeted metabolomics (using LC- Orbitrap) of different tissues of camphor basil to understand putative genes and metabolites involved in synthesis of commercially and medicinally important metabolites like camphor and eugenol.
- Dissecting camphor biosynthetic pathway from *O. kilimandscharicum* by cloning and *in planta* functional characterization (gene silencing and overexpression) of pathway genes including geranyl diphosphate synthase and borneol dehydrogenase.
- Understanding the mechanism underlying metabolite partitioning of eugenol and camphor in camphor basil to understand their synthesis, transport and storage in plant.

1.6 References

(1) J.E. Simon, J. Quinn and R.G. Murray, in *Advances in new crops*, ed. J. Janick and J.E. Simon, Timber Press, Portland, 1990, Basil: A source of essential oils, 484.

(2) C. Linnaeus, in *Species Plantarum*, Laurentii Salvii, Holmiae (Stockholm), 1753, *Ocimum*, 597.

(3) P.D. Cantino, R.M. Harley and S.J. Wagstaff, in *Advances in Labiate Science*, ed. R. M. Harley and T. Reynolds, Royal Botanic Gardens, Kew, 1992, Genera of Labiatae: Status and Classification, 502.

(4) M. K. Khosla, Study of inter-relationship, phylogeny and evolutionary tendencies in genus *Ocimum*. *Ind J. Genet*., 1995, **55**, 71.

(5) Z. Xie, J. Kapteyn and D.R. Gang, A systems biology investigation of the MEP/terpenoid and shikimate/phenylpropanoid pathways points to multiple levels of metabolic control in sweet basil glandular trichomes. *Plant J*., 2008, **54**, 349.

(6) D. Yang, X. Du, X. Liang, R. Han, Z. Liang, Y. Liu, F. Liu and J. Zhao, Different roles of the mevalonate and methylerythritol phosphate pathways in cell growth and tanshinone production of *Salvia miltiorrhiza* hairy roots. *PLoSOne*, 2012, **7**, e46797.

(7) A. Marchev, C. Haas, S. Schulz, V. Georgiev, J. Steingroewer, T. Bley and A. Pavlov, Sage *in vitro* cultures: a promising tool for the production of bioactive terpenes and phenolic substances. *Biotechnol. Lett*, 2014, **36**, 211.

(8) G.W. Turner and R. Croteau, Organization of monoterpene biosynthesis in *Mentha*. Immunocytochemical localizations of geranyl diphosphate synthase, limonene-6-hydroxylase, isopiperitenol dehydrogenase, and pulegone reductase. *Plant Physiol*, 2004, **136**, 4215.

(9) R. B. Croteau, E. M. Davis, K. L. Ringer and M. R. Wildung, (−)-Menthol biosynthesis and molecular genetics. *Naturwissenschaften*, 2005, **92,** 562.

(10) R. Rios-Estepa, G. W. Turner, J. M. Lee, R. B. Croteau and B. M. Lange, A systems biology approach identifies the biochemical mechanisms regulating monoterpenoid essential oil composition in peppermint. *PNAS*, 2008, **105**, 2818.

(11) A. Lane, A. Boecklemann, G. N. Woronuk, L. Sarker and S. S. Mahmoud, A genomics resource for investigating regulation of essential oil production in *Lavandula angustifolia*. *Planta*, 2010, **231,** 835.

(12) G. Woronuk, Z. Demissie, M. Rheault and S. Mahmoud, Biosynthesis and therapeutic properties of *Lavandula* essential oil constituents. *Planta Med*., 2011, **77**, 7.

(13) D. R. Gang, J. Wang, N. Dudareva, K. H. Nam, J. E. Simon, E. Lewinsohn and E. Pichersky, An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. *Plant Physiol*., 2001, **125**, 539.

(14) S. Rastogi, S. Meena, A. Bhattacharya, S. Ghosh, R. K.Shukla, N.S. Sangwan, R. K. Lal, M. M. Gupta, U. C. Lavania, V. Gupta, D. A. Nagegowda and A. K. Shasany, De novo sequencing and comparative analysis of holy and sweet basil transcriptomes. *BMC Genomics*, 2014, **15**, 588.

(15) F. Bast, P. Rani and D. Meena, Chloroplast DNA phylogeography of holy basil (*Ocimum tenuiflorum*) in Indian subcontinent. *Scientific World J*., *2014*, **2014,** doi:10.1155/2014/847482

(16) K. Carović-Stanko, Z. Liber, V. Besendorfer, B. Javornik, B. Bohanec, I. Kolak and Z. Satovic, Genetic relations among basil taxa (Ocimum L.) based on molecular markers, nuclear DNA content, and chromosome number. *Plant Syst*. *Evol*., 2010, **285**, 13.

(17) A. J. Paton, D. Springate, S. Suddee, D. Otieno, R. J. Grayer, M. M. Harley, F. Willis, M. S. J. Simmonds, M. P. Powell and V. Savolainen, Phylogeny and evolution of basils and allies (Ocimeae, Labiatae) based on three plastid DNA regions. *Mol*. *Phylogenet*. *Evol*., 2004, **31**, 277.

(18) A. Paton and E. Putievsky, Taxonomic problems and cytotaxonomic relationships between and within varieties of *Ocimum basilicum* and related species (Labiatae). *Kew Bull*., 1996, **51**, 509.

(19) A. P. Singh, S. Dwivedi, S. Bharti, S. Srivastava, V. Singh and S. P. S. Khanuja, Phylogenetic relationships as in *Ocimum* revealed by RAPD markers. *Euphytica*, 2004, **136**, 11.

(20) V. T. de Lima, M. C. Vieira, C. A. Kassuya, C. A. Cardoso, J. M. Alves, M. A. Foglio, J. E. de Carvalho and A. S. Formagio, Chemical composition and free radical-scavenging, anticancer and anti-inflammatory activities of the essential oil from *Ocimum kilimandscharicum*. *Phytomedicine*, 2014, **21**, 1298.

(21) J. Monga, M. Sharma, N. Tailor and N. Ganesh, Antimelanoma and radioprotective activity of alcoholic aqueous extract of different species of *Ocimum* in C57BL mice. *Pharm*. *Biol*., 2011, **49**, 428.

(22) P. Singh, R. H. Jayaramaiah, P. Sarate, H. V. Thulasiram, M. J. Kulkarni and A. P. Giri, Insecticidal potential of defense metabolites from *Ocimum kilimandscharicum* against Helicoverpa armigera. *PloS One*, 2014, **9**, e104377.

(23) R. V. Sarin, S. Narwal and P. A. Bafna, Anti-diarrhoeal activity of aqueous extract of *Ocimum kilimandscharicum*. *J*. *Ethnopharmacol*., 2013, **148**, 223.

(24) D. Runyoro, O. Ngassapa, K. Vagionas, N. Aligiannis, K. Graikou and I. Chinou, Chemical composition and antimicrobial activity of the essential oils of four *Ocimum* species growing in Tanzania. *Food Chem*., 2010, **119**, 311.

(25) B. O. Owuor, J. O. Ochanda, J. O. Kokwaro, A. C. Cheruiyot, R. A. Yeda, C. A. Okudo and H. M. Akala, *In vitro* antiplasmodial activity of selected Luo and Kuria medicinal plants. *J*. *Ethnopharmacol*., 2012, **144**, 779.

(26) H. Song, P. Kumar, G. Arivazhagan, S. I. Lee, H. M. Yoon, I. H. Kim, H. J. Kwon, J. M. Kim and F. L. Hakkim, Antioxidant property of leaves and calluses extracts of in-vitro grown 5 different *Ocimum* species. *J*. *Plant Biotechnol*, 2012, **39**, 146.

(27) F. L. Hakkim, G. Arivazhagan and R. Boopathy, Antioxidant property of selected *Ocimum* species and their secondary metabolite content. *J*. *Med*. *Plant*. *Res*, 2013, **2**, 250.

(28) E. J. Kweka, F. W. Mosha, A. Lowassa, A. M. Mahande, M. J. Mahande, C. P. Massenga, F. Tenu, E. E. Lyatuu, M. A. Mboya and E. A. Temu, Longitudinal evaluation of *Ocimum* and other plants effects on the feeding behavioral response of mosquitoes (Diptera: Culicidae) in the field in Tanzania. *Parasit*. *Vectors*, 2008, **1**, 1.

(29) P. Kapewangolo, J. J. Omolo, R. Bruwer, P. Fonteh and D. Meyer, Antioxidant and anti-inflammatory activity of *Ocimum labiatum* extract and isolated labdane diterpenoid. *J*. *Inflamm*., 2015, **12**, 1.

(30) E. Lulekal, J. Rondevaldova, E. Bernaskova, J. Cepkova, Z. Asfaw, E. Kelbessa, L. Kokoska and P. Van Damme, Antimicrobial activity of traditional medicinal plants from ankober district, north shewa zone, amhara region, Ethiopia. *Pharm*. *Biol*., 2014, **52**, 614.

(31) D. Damtie and Y. Mekonnen, *In vitro* evaluation of the antibacterial activities of the methanol, aqueous and n-hexane extracts of *Ocimum lamiifolium* from Ethiopia. *Afr. J. of Pharm. Pharmacol*, 2015, **9**, 91.

(32) F. F. Dube, K. Tadesse,G. Birgersson, E. Seyoum, H. Tekie, R. Ignell and S. R. Hill, Fresh, dried or smoked? Repellent properties of volatiles emitted from ethnomedicinal plant leaves against malaria and yellow fever vectors in Ethiopia. *Malar. J.*, 2011, **10**, 1.

(33) M. J. Mukazayire, J. C. Tomani, C. Stevigny, J. C. Chalchat, F. Conforti, F. Menichini and P. Duez, Essential oils of four Rwandese hepatoprotective herbs: Gas chromatography–mass spectrometry analysis and antioxidant activities. *Food Chem*., 2011, **129**, 753.

(34) W. Mequanint, E. Makonnen and K. Urga, In vivo anti-inflammatory activities of leaf extracts of *Ocimum lamiifolium* in mice model. *J. Ethnopharmacol*., 2011, **134**, 32.

(35) M. J. Mukazayire, V. Allaeys, P. B. Calderon, C. Stévigny, M. J. Bigendako and P. Duez, Evaluation of the hepatotoxic and hepatoprotective effect of Rwandese herbal drugs on *in vivo* (guinea pigs barbiturate-induced sleeping time) and *in vitro* (rat precision-cut liver slices, PCLS) models. *Exp. Toxicol. Pathol*., 2010, **62**, 289.

(36) E. Makonnen, A. Debella, D. Abebe and F. Teka, Analgesic properties of some Ethiopian medicinal plants in different models of nociception in mice. *Phytother. Res*., 2003, **17**, 1108.

(37) Y. Naidoo, C. T. Sadashiva, N. Kasim, A. Nicholas and G. Naidoo, Chemical Composition and Antimicrobial Activity of the Essential Oil of *Ocimum obovatum* E. Mey. Ex Benth. (Lamiaceae). *J. Essent. Oil Bear. Pl*., 2014, **17**, 142.

(38) S. Parasuraman, S. Balamurugan, P. V. Christapher, R. R. Petchi, W. Y. Yeng, J. Sujithra and C. Vijaya, Evaluation of Antidiabetic and Antihyperlipidemic Effects of

Hydroalcoholic Extract of Leaves of *Ocimum tenuiflorum* (Lamiaceae) and Prediction of Biological Activity of its Phytoconstituents. *Pharmacogn*. *Res*., 2015, **7**, 156.

(39) F. L. Hakkim, C. G. Shankar and S. Girija, Chemical composition and antioxidant property of holy basil (*Ocimum sanctum* L.) leaves, stems, and inflorescence and their *in vitro* callus cultures. *J*. *Agric*.*Food Chem*., 2007, **55**, 9109.

(40) B. Sarkar, D. Kumar, D. Sasmal and K. Mukhopadhyay, Antioxidant and DNA damage protective properties of anthocyanin-rich extracts from *Hibiscus* and *Ocimum*: a comparative study. *Nat*. *Prod*. *Res*., 2014, **28**, 1393.

(41) P. Phanthong, P. Lomarat, M. T. Chomnawang and N. Bunyapraphatsara, Antibacterial activity of essential oils and their active components from Thai spices against foodborne pathogens. *Science Asia*, 2013, **39**, 472.

(42) M. Stefan, M. M. Zamfirache, C. Padurariu, E. Trută and I. Gostin, The composition and antibacterial activity of essential oils in three *Ocimum* species growing in Romania. *Open Life Sci*., 2013, **8**, 600.

(43) K. Karthikeyan, P. Gunasekaran, N. Ramamurthy and S. Govindasamy, Anticancer activity of *Ocimum sanctum*. *Pharm*. *Biol*., 1999, **37**, 285.

(44) F. M. Mahomoodally, A. H. Subratty, A. Gurib-Fakim and M. I. Choudhary, Antioxidant, antiglycation and cytotoxicity evaluation of selected medicinal plants of the Mascarene Islands. *BMC Complement*. *Altern*. *Med*., 2012, **12**, 1.

(45) R. C. Saxena, R. Singh, P. Kumar, M. P. S. Negi, V. S. Saxena, P. Geetharani, J. J. Allan and K. Venkateshwarlu, Efficacy of an extract of *Ocimum tenuiflorum* (OciBest) in the management of general stress: A double-blind, placebo-controlled study. *Evid*. *Based Complement*. *Alternat*. *Med*., 2011, **2012**, 1.

(46) P. Sudha, S. S. Zinjarde, S.Y. Bhargava and A.R. Kumar, Potent α-amylase inhibitory activity of Indian Ayurvedic medicinal plants. *BMC Complement. Altern. Med.*, 2011, **11**, 1.

(47) O. Chokechaijaroenporn, N. Bunyapraphatsora and S. Kongchuensin, Mosquito repellent activities of *Ocimum* volatile oils. *Phytomedicine*, 1994, **1**, 135.

(48) R. Yucharoen, S. Anuchapreeda and Y. Tragoolpua, Anti-herpes simplex virus activity of extracts from the culinary herbs *Ocimum sanctum L.*, *Ocimum basilicum L.* and *Ocimum americanum L. Afr. J. Biotechnol.*, 2013, **10**, 860.

(49) G. Kaur, A. Bali, N. Singh and A. S. Jaggi, Ameliorative potential of *Ocimum sanctum* in chronic constriction injury-induced neuropathic pain in rats. *An. Acad. Bras. Ciênc.*, 2015, **87**, 417.

(50) B. T. Aluko, O. I. Oloyede and A. J. Afolayan, Polyphenolic contents and free radical scavenging potential of extracts from leaves of *Ocimum americanum L. Pak. J. Biol. Sci.*, 2013, **16**, 22.

(51) A. N. Yamada, R. Grespan, A. T. Yamada, E. L. Silva, S. E. Silva-Filho, M. J. Damião, M. M. de Oliveira Dalalio, C. A. Bersani-Amado and R. K. N. Cuman, Anti-inflammatory activity of *Ocimum americanum L.* essential oil in experimental model of zymosan-induced arthritis. *Am. J. Chin. Med.*, 2013, **41**, 913.

(52) S. Thaweboon and B. Thaweboon, *In vitro* antimicrobial activity of *Ocimum americanum L.* essential oil against oral microorganisms. *Southeast Asian J. Trop. Med. Public Health*, 2009, **40**, 1025.

(53) U. Szymanowska, U. Złotek, M. Karas and B. Baraniak, Anti-inflammatory and antioxidative activity of anthocyanins from purple basil leaves induced by selected abiotic elicitors. *Food Chem*., 2015, **172**, 71.

(54) N. P. Yadav, R. Khatri, D. U. Bawankule, A. Pal, K. Shanker, P. Srivastava, A. K. Gupta and D. Chanda, Topical anti-inflammatory effects of *Ocimum basilicum* leaf extract in the phorbol-12, 13-dibutyrate model of mouse ear inflammation. *Planta Med*., **75**, PA72

(55) S. J. Inbaneson, R. Sundaram and P. Suganthi, *In vitro* antiplasmodial effect of ethanolic extracts of traditional medicinal plant *Ocimum* species against *Plasmodium falciparum*. *Asian Pac*. *J.Trop*. *Med*., 2012, **5**, 103.

(56) D. Beatović, D. Krstić-Milošević, S. Trifunović, J. Šiljegović, J. Glamočlija, M. Ristić, and S. Jelačić, Chemical Composition, antioxidant and antimicrobial activities of the essential oils of twelve *Ocimum basilicum L.* cultivars grown in Serbia. *Rec*. *Nat*. *Prod*., 2015, **9**, 62.

(57) L. Vlase, D. Benedec, D. Hanganu, G. Damian, I. Csillag, B. Sevastre, A. C. Mot, R. Silaghi-Dumitrescu and I. Tilea, Evaluation of antioxidant and antimicrobial activities and phenolic profile for *Hyssopus officinalis*, *Ocimum basilicum* and *Teucrium chamaedrys*. *Molecules*, 2014, **19**, 5490.

(58) B. Kaurinovic, M. Popovic, S. Vlaisavljevic and S. Trivic, Antioxidant capacity of *Ocimum basilicum L*. and *Origanum vulgare L*. extracts. *Molecules*, 2011, **16**, 7401.

(59) I. H. N. Bassolé, A. Lamien-Meda, B. Bayala, S. Tirogo, C. Franz, J. Novak, R. C. Nebié and M. H. Dicko, Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha x piperita L*. and *Ocimum basilicum L*. essential oils and their major monoterpene alcohols alone and in combination. *Molecules*, 2010, **15**, 7825.

(60) K. R. Durga, S. Karthikumar and K. Jegatheesan, Isolation of potential antibacterial and antioxidant compounds from *Acalypha indica* and *Ocimum basilicum*. *Afr*. *J*. *Plant Sci*., 2010, **4**, 160.

(61) P. A. Ntonga, N. Baldovini, E. Mouray, L. Mambu, P. Belong and P. Grellier, Activity of *Ocimum basilicum*, *Ocimum canum*, and *Cymbopogon citratus* essential oils against *Plasmodium falciparum* and mature-stage larvae of *Anopheles funestus* ss. *Parasite*, 2014, **21**, 1.

(62) K. H. Al-Ali, H. A. El-Beshbishy, A. A. El-Badry and M. Alkhalaf, Cytotoxic activity of methanolic extract of *Mentha longifolia* and *Ocimum basilicum* against human breast cancer. *Pak*. *J*. *Biol*. *Sci*., 2013, **16**, 1744.

(63) A. K. Qamar, A. Dar, B. S. Siddiqui, N. Kabir, H. Aslam, S. Ahmed, S. Erum, S. Habib and S. Begum, Anticancer activity of *Ocimum basilicum* and the effect of ursolic acid on the cytoskeleton of MCF-7 human breast cancer cells. *Lett*. *Drug Des*. *Discov*., 2010, **7**, 726.

(64) M. Govindarajan, R. Sivakumar, M. Rajeswary and K. Yogalakshmi, Chemical composition and larvicidal activity of essential oil from *Ocimum basilicum (L.)* against *Culex tritaeniorhynchus*, *Aedes albopictus* and *Anopheles subpictus* (Diptera: Culicidae). *Exp*. *Parasitol*., 2013, **134**, 7.

(65) K. Murugan, P. Murugan and A. Noortheen, Larvicidal and repellent potential of *Albizzia amara Boivin* and *Ocimum basilicum Linn* against dengue vector, *Aedes aegypti* (Insecta: Diptera: Culicidae). *Bioresour*. *Technol*., 2007, **98**, 198.

(66) B. S. Siddiqui, H. A. Bhatti, S. Begum and S. Perwaiz, Evaluation of the antimycobacterium activity of the constituents from *Ocimum basilicum* against *Mycobacterium tuberculosis*. *J. Ethnopharmacol*., 2012, **144**, 220.

(67) K. S. Bora, S. Arora and R. Shri, Role of *Ocimum basilicum L.* in prevention of ischemia and reperfusion-induced cerebral damage, and motor dysfunctions in mice brain. *J. Ethnopharmacol*., 2011, **137**, 1360.

(68) A. Umar, G. Imam, W. Yimin, P. Kerim, I. Tohti, B. Berké and N. Moore, Antihypertensive effects of *Ocimum basilicum L.* (OBL) on blood pressure in renovascular hypertensive rats. *Hypertension Res*., 2010, **33**, 727.

(69) S. Amrani, H. Harnafi, D. Gadi, H. Mekhfi, A. Legssyer, M. Aziz, F. Martin- Nizard and L. Bosca, Vasorelaxant and anti-platelet aggregation effects of aqueous *Ocimum basilicum* extract. *J. Ethnopharmacol*., 2009, **125**, 157.

(70) I. de Almeida, D. S. Alviano, D. P. Vieira, P. B. Alves, A. F. Blank, A. H. C. Lopes, C. S. Alviano and S. R. Maria do Socorro, Antigiardial activity of *Ocimum basilicum* essential oil. *Parasitol. Res*., 2007, **101**, 443.

(71) L. C. Chiang, L. T. Ng, P. W. Cheng, W. Chiang and C. C. Lin, Antiviral activities of extracts and selected pure constituents of *Ocimum basilicum*. *Clin. Exp. Pharmacol. Physiol*., 2005, **32**, 811.

(72) Y. H. Chen, Y. W. Chiu, J. C. Shyu, C. C. Tsai, H. H. Lee, C. C. Hung, J. M. Hwang, J. Y. Liu and W. H. Wang, Protective Effects of *Ocimum gratissimum* Polyphenol Extract on Carbon Tetrachloride-Induced Liver Fibrosis in Rats. *Chinese J. Physiol*., 2015, **58**, 55.

(73) D. C. Gontijo, L. C. Fietto and J. P. V. Leite, Phytochemical Assessment And Antioxidant, Antimutagenic And Toxicological Activity Of *Ocimum gratissimum L*. Leaf aqueous extract. *Rev*. *Bras*. *Plantas Med*., 2014, **16**, 874.

(74) G. H. Bénédicta, K. Kpoviessia, S. D. S. Kpoviessia, E. Y. Ladekan, F. Gbaguidi, M. Frédérichd, M. Moudachiroub, J. Quetin-Leclercqc, J.C. Accrombessia and J. Beroc, *In vitro* antitrypanosomal and antiplasmodial activities of crude extracts and essential oils of *Ocimum gratissimum* Linn from Benin and influence of vegetative stage. *J*. *Ethnopharmacol*., 2014, **155**, 1417.

(75) S. K. Mahapatra and S. Roy, Phytopharmacological approach of free radical scavenging and anti-oxidative potential of eugenol and *Ocimum gratissimum Linn*. *Asian Pac*. *J.Trop*. *Med*., 2014, **7**, S391.

(76) Y. W. Chiu, P. Y. Chao, C. C. Tsai, H. L. Chiou, Y. C. Liu, C. C. Hung, H. C. Shih, T. J. Lai and J. Y. Liu, *Ocimum gratissimum* is effective in prevention against liver fibrosis *in vivo* and *in vitro*. *Am*. *J*. *Chin*. *Med*., 2014, **42**, 833.

(77) R. K. Joshi, Chemical composition, *in vitro* antimicrobial and antioxidant activities of the essential oils of *Ocimum gratissimum*, *O*. *sanctum* and their major constituents. *Indian J*. *Pharm*. *Sci*., 2013, **75**, 457.

(78) A. Katara, C. K. Pradhan, P. Singh, V. Singh and M. Ali, Volatile Constituents and Antimicrobial Activity of Aerial parts of *Ocimum gratissimum Linn*. *J*. *Essent*. *Oil Bear*. *Pl*., 2013, **16**, 283.

(79) E. F. F. Matias, F. A. V. Santos, J. M. F. Silva, C. E. S. Souza, S. R. Tintino, G. M. M. Guedes, C. R. Medeiros, M. F. B. Braga, T. S. Almeida, J. G. M. Costa, I. R. A. Menezes

and H. D. M. Coutinho, Screening the in vitro modulation of antibiotic activity of the extracts and fractions of *Ocimum gratissimum L. Afr. J. Microbiol. Res*., 2012, **6**, 1902.

(80) M. O. Nkiko and J. T. Bamgbose, Corrosion inhibitive effect of *Ocimum gratissimum* extract on zinc-aluminium alloy in hydrochloric acid. *Portugaliae Electrochimica Acta*, 2011, **29**, 419.

(81) K. S. Bora, R. Shri and J. Monga, Cerebroprotective effect of *Ocimum gratissimum* against focal ischemia and reperfusion-induced cerebral injury. *Pharm. Biol*., 2011, **49**, 175.

(82) A. C. M. de Santana, G. S.Pereira, C. M. Boaventura, A. P. T. Uetenabaro, L. C. D. B. Costa and R. A. de Oliveira, Rupture of glandular trichomes in *Ocimum gratissimum* leaves influences the content of essential oil during the drying method. *Revista Brasileira de Farmacognosia*., 2014, **24**, 524.

(83) S. R. Vani, S. F. Cheng and C. H. Chuah, Comparative study of volatile compounds from genus *Ocimum. Am. J. Appl. Sci*., 2009, **6**, 523.

(84) L. C. Costa, J. E. Pinto, E. M. Castro, E. Alves, L. F. Rosal, S. K. Bertolucci, P. B. Alves and T. S. Evangelino, Yield and composition of the essential oil of *Ocimum selloi Benth*. cultivated under colored netting. *J.Essent. Oil Res*., 2010, **22**, 34.

(85) S. K.Singh, A. Anand, S. K. Verma, M. A. Siddiqui, A. Mathur and S. Saklani, Analysis of phytochemical and antioxidant potential of *Ocimum kilimandscharicum Linn. Int. J. curr. Pharm. Res*., 2010 **3**, 40.

(86) M. Özcan and J. C. Chalchat, Essential oil composition of *Ocimum basilicum L*. and *Ocimum minimum L.* in Turkey. *Czech J. Food Sci*., 2002, **20**, 223.

(87) D. Ioannidis, B. Lynda and C. B. Johnson, UV-B is required for normal development of oil glands in *Ocimum basilicum L.* (Sweet Basil). *Ann. Botany*, 2002, **90,** 453.

(88) R. Parida, S. I. Sandeep, B. K. Sethy, S.Sahoo and S. Mohanty, Chemical composition, antioxidant and antimicrobial activities of essential oil from lime basil (*Ocimum americanum*): a potent source for natural antioxidant. *Int J Pharm Pharm Sci,* 2014, **6,** 481.

(89) A. S. D. Costa, M. D. F. Arrigoni-Blank, M. A. A. P. D. Silva, M. F. Alves, D. D. A. Santos, P. B. Alves and A. F. Blank, The impact of hybridization on the volatile and sensorial profile of *Ocimum basilicum L. Scientific World J.*, 2014, **2014**, 1.

(90) F. Tchoumbougnang, P. A. Zollo, F. Avlessi, G. A. Alitonou, D. K. Sohounhloue, J. M. Ouamba and C. Menut, Variability in the chemical compositions of the essential oils of five *Ocimum* species from tropical African area. *J. Essent. Oil Res.*, 2006, **18**, 194.

(91) N. P. Benitez, E. M. M. León and E. E. Stashenko, Eugenol and methyl eugenol chemotypes of essential oil of species *Ocimum gratissimum L.* and *Ocimum campechianum Mill.* from Colombia. *J. Chromatogr. Sci.,* 2009, **47**, 800.

(92) G. Sacchetti, A. Medici, S. Maietti, M. Radice, M. Muzzoli, S. Manfredini, E. Braccioli and R.Bruni, Composition and functional properties of the essential oil of Amazonian basil, *Ocimum micranthum Willd.*, Labiatae in comparison with commercial essential oils. *J. Agric. Food Chem.*, 2004, **52**, 3486.

(93) J. C. Nascimento, L.C. Barbosa, V. F. Paula, J. M. David, R. Fontana, L. A. Silva and R. S. França, Chemical composition and antimicrobial activity of essential oils of *Ocimum canum Sims.* and *Ocimum selloi Benth. Anais da Academia Brasileira de Ciências*, 2011, **83**, 787.

(94) O. U. Edet and P. O. Aikpokpodion, Karyotype Analysis of *Ocimum basilicum* in South-Eastern Nigeria. *Am. J. Plant Sci*., 2014, **5**, 126.

(95) R. B. Bose and J. K. Choudhury, A Comparative Study of the Cytotaxonomy, Pallynology, Physiology of 'Diploid'and 'Polyploid'Plants of *Ocimum kilimandscharicum Guerke* and their Yield of Raw Material and Volatile Contents. *Caryologia*, 1962, **15**, 435.

(96) P. Pushpangadan and S. N. Sobti, Cytogenetical studies in the genus *Ocimum*. Origin of *O. americanum*, cytotaxonomical and experimental proof. *Cytologia*, 1982, **47**, 575.

(97) T. Zderkiewicz, Content of oil in different stages of ripe fruits of diploid and tetraploid cumin (*Carum carvi L.*). *Acta Agrobot*., 1971, **24**, 121.

(98) H. Dijkstra and G. J. Speckmann, Autotetraploidy in caraway (*Carum carvi L.*) for the increase of the aetheric oil content of the seed. *Euphytica*, 1980, **29**, 89.

(99) E. K. Janaki Ammal and S. N. Sobti, The origin of the Jammu mint. *Curr. Sci*., 1962, **31**, 387.

(100) Y. Iijima, R. Davidovich-Rikanati, E. Fridman, D. R. Gang, E. Bar, E. Lewinsohn and E. Pichersky, The biochemical and molecular basis for the divergent patterns in the biosynthesis of terpenes and phenylpropenes in the peltate glands of three cultivars of basil. *Plant Physiol*., 2004, **136**, 3724.

(101) G. V. Louie, T. J. Baiga, M. E. Bowman, T. Koeduka, J. H. Taylor, S. M. Spassova, E. Pichersky and J. P. Noel, Structure and reaction mechanism of basil eugenol synthase. *PloS One*, 2007, **2**, e993.

(102) I. Aragüez, S. Osorio, T. Hoffmann, J. L. Rambla, N. Medina-Escobar, A. Granell, M. A. Botella, W. Schwab and V. Valpuesta, Eugenol production in achenes and

receptacles of strawberry fruits is catalyzed by synthases exhibiting distinct kinetics. *Plant Physiol*., 2013, **163**, 946.

(103) S. Rastogi, R. Kumar, C. S. Chanotiya, K. Shanker, M. M. Gupta, D. A. Nagegowda and A. K. Shasany, 4-Coumarate: CoA ligase partitions metabolites for eugenol biosynthesis. *Plant Cell Physiol*., 2013, pct073.

(104) L. Medina-Puche, F. J. Molina-Hidalgo, M. Boersma, R. C. Schuurink, I. López-Vidriero, R. Solano, J. M. Franco-Zorrilla, J. L. Caballero, R. Blanco-Portales and J. Muñoz-Blanco, An R2R3-MYB Transcription Factor Regulates Eugenol Production in Ripe Strawberry Fruit Receptacles. *Plant Physiol*., 2015, **168**, 598.

(105) D. R. Gang, N. Lavid, C. Zubieta, F. Chen, T. Beuerle, E. Lewinsohn, J. P. Noel and E. Pichersky, Characterization of phenylpropene O-methyltransferases from sweet basil facile change of substrate specificity and convergent evolution within a plant O-methyltransferase family. *Plant Cell*, 2002,**14**, 505.

(106) E. Lewinsohn, I. Ziv-Raz, N. Dudai, Y. Tadmor, E. Lastochkin, O. Larkov, D. Chaimovitsh, U. Ravid, E. Putievsky, E. Pichersky and Y. Shoham, Biosynthesis of estragole and methyl-eugenol in sweet basil (*Ocimum basilicum L*). Developmental and chemotypic association of allylphenol O-methyltransferase activities. *Plant Sci*., 2000, **160**, 27.

(107) R. Croteau and F. Karp, Biosynthesis of monoterpenes: preliminary characterization of bornyl pyrophosphate synthetase from sage (*Salvia officinalis*) and demonstration that geranyl pyrophosphate is the preferred substrate for cyclization. *Arch*. *Biochem*. *Biophys*., 1979, **198**, 512.

(108) M. L. Wise, T. J. Savage, E. Katahira and R. Croteau, Monoterpene synthases from common sage (*Salvia officinalis*) cDNA isolation, characterization, and functional expression of (+)-sabinene synthase, 1, 8-cineole synthase, and (+)-bornyl diphosphate synthase. *J*. *Biol*. *Chem*., 1998, **273**, 14891.

(109) D. A. Whittington, M. L. Wise, M. Urbansky, R. M. Coates, R. B. Croteau and D. W. Christianson, Bornyl diphosphate synthase: structure and strategy for carbocation manipulation by a terpenoid cyclase. *Proc*. *Natl*. *Acad*. *Sci*. *USA*, 2002, **99**, 15375.

(110) D.T. Major and M. Weitman, Electrostatically Guided Dynamics The Root of Fidelity in a Promiscuous Terpene Synthase?.*J*. *Am*. *Chem*. *Soc*., 2012, **134**, 19454.

(111) R. Croteau, C. L. Hooper and M. Felton, Biosynthesis of monoterpenes: Partial purification and characterization of a bicyclic monoterpenol dehydrogenase from sage (*Salvia officinalis*). *Arch*. *Biochem*. *Biophys*., 1978, **188**, 182.

(112) L. S. Sarker, M. Galata, Z. A. Demissie and S. S. Mahmoud, Molecular cloning and functional characterization of borneol dehydrogenase from the glandular trichomes of *Lavandula x intermedia*. *Arch*. *Biochem*. *Biophys*., 2012, **528**, 163.

(113) R. Croteau and F. Karp, Biosynthesis of monoterpenes: partial purification and characterization of 1, 8-cineole synthetase from *Salvia officinalis*. *Arch*. *Biochem*. *Biophys*., 1977, **179**, 257.

(114) Y. Iijima, D. R. Gang, E. Fridman, E. Lewinsohn and E. Pichersky, Characterization of geraniol synthase from the peltate glands of sweet basil. *Plant Physiol*., 2004, **134**, 370.

(115) R. C. Misra, P. Maiti, C. S. Chanotiya, K. Shanker and S. Ghosh, Methyl jasmonate-elicited transcriptional responses and pentacyclic triterpene biosynthesis in sweet basil. *Plant Physiol*., 2014, **164**, 1028.

(116) M. M. B. Zvi, E. Shklarman, T. Masci, H. Kalev, T. Debener, S. Shafir, M. Ovadis and A. Vainstein, PAP1 transcription factor enhances production of phenylpropanoid and terpenoid scent compounds in rose flowers. *New Phytol*., 2012, **195**, 335.

(117) A. K. Upadhyay, A. R. Chacko, A. Gandhimathi, P. Ghosh, K. Harini, A. P. Joseph, *et al*., Genome sequencing of herb Tulsi (*Ocimum tenuiflorum*) unravels key genes behind its strong medicinal properties. *BMC Plant Biol*., 2015, **15**, 212.

(118) A. Berim, J. Park and D. R. Gang, Unexpected roles for ancient proteins: flavone 8- hydroxylase in sweet basil trichomes is a Rieske- type, PAO- family oxygenase. *Plant J*., 2014, **80**, 385.

(119) A. Berim and D. R. Gang, Characterization of two candidate flavone 8-O-methyltransferases suggests the existence of two potential routes to nevadensin in sweet basil. *Phytochem*., 2013, **92**, 33.

(120) A. Berim and D. R. Gang, The roles of a flavone-6-hydroxylase and 7-O-demethylation in the flavone biosynthetic network of sweet basil. *J*. *Biol*. *Chem*., 2013, **288**, 1795.

(121) A., Berim, D. C. Hyatt and D. R. Gang, A set of regioselective O-methyltransferases gives rise to the complex pattern of methoxylated flavones in sweet basil. *Plant Physiol*., 2012, **160**, 1052.

(122) N. J. Bate, J. Orr, W. T. Ni, A. Meromi, T. Nadlerhassar, P. W. Doerner, R. A. Dixon, C. J. Lamb and Y. Elkind, Quantitative relationship between phenylalanine ammonia-lyase levels and phenylpropanoid accumulation in transgenic tobacco identifies a rate-determining step in natural product synthesis. *Proc*. *Natl Acad*. *Sci*., 1994, **91**, 7608.

(123) L. Carretero-Paulet, I. Ahumada, N. Cunillera, M. Rodriguez-Concepcion, A. Ferrer, A. Boronat and N. Campos, Expression and Molecular Analysis of the ArabidopsisDXR Gene Encoding 1-Deoxy-d-Xylulose 5-Phosphate Reductoisomerase, the First Committed Enzyme of the 2-C-Methyl-d-Erythritol 4-Phosphate Pathway. *Plant Physiol*., 2002, **129**, 1581.

(124) M. Rodriguez-Concepcion, Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells. *Phytochem*. *Rev*., 2006, **5**, 1.

(125) Z. X. Yu, J. X. Li, C. Q. Yang, W. L. Hu, L. J. Wang and X. Y. Chen, The jasmonate-responsive AP2/ERF transcription factors AaERF1 and AaERF2 positively regulate artemisinin biosynthesis in *Artemisia annua L*. *Mol*. *Plant*, 2012, **5**, 353.

(126) S. Mishra, V. Triptahi, S. Singh, U. J. Phukan, M. M. Gupta, K. Shanker and R. K. Shukla, Wound induced tanscriptional regulation of benzylisoquinoline pathway and characterization of wound inducible PsWRKY transcription factor from *Papaver somniferum*. *PLoS ONE*, 2013, **8**, e52784

(127) M. A. Naoumkina, X. He and R. A. Dixon, Elicitor-induced transcription factors for metabolic reprogramming of secondary metabolism in *Medicago truncatula*. *BMC Plant Biol*, 2008, **8**, 1.

(128) P. Vyas, I. Haque, M. Kumar and M. Mukhopadhyay, Photocontrol of differential gene expression and alterations in foliar anthocyanin accumulation: a comparative study using red and green forma *Ocimum tenuiflorum*. *Acta Physiol*. *Plant*., 2014, **36**, 2091.

(129) E. G. Allwood, D. R. Davies, C. Gerrish, B. E. Ellis and G. P. Bolwell, Phosphorylation of phenylalanine ammonia- lyase: evidence for a novel protein kinase and identification of the phosphorylated residue. *FEBS Lett*., 1999, **457**, 47.

(130) G. P. Bolwell, A role for phosphorylation in the down-regulation of phenylalanine ammonia-lyase in suspension-cultured cells of French bean. *Phytochemistry*, 1992, **31**, 4081.

(131) J. H. Loughrin and M. J. Kasperbauer, Light reflected from colored mulches affects aroma and phenol content of sweet basil (*Ocimum basilicum L.*) leaves. *J. Agr. Food Chem*., 2001, **49**, 1331.

(132) M. G. de Vasconcelos, A. A. Craveiro, F. J. Abreu, M. I. L. Machado and J.W. Alencar, Chemical variation during daytime of constituents of the essential oil of *Ocimum gratissimum* leaves. *Fitoterapia,* 1999*,* **70,** 32.

(133) C. M. M. Freire, M. O. M. Marques and M. Costa. Effects of seasonal variation on the central nervous system activity of *Ocimum gratissimum L.* essential oil. *J. Ethnopharmacol*., 2006*,* **105,** 161.

(134) E. Guenther, in *The Essential oils*., van Nostrand Co. Inc., New York, USA, Volume III, V, 1995, 427.

(135) R. Hiltunen and Y. Holm, *Basil the genus Ocimum*, Harwood Academic Publishers, 1999.

(136) Z. Hao, Biosynthesis of methylcinnamate in *Ocimum basilicum* L., Purdue University, 1998.

(137) I. K. Renu, I. Haque, M. Kumar, R. Poddar, R. Bandopadhyay, A. Rai and K. Mukhopadhyay, Characterization and functional analysis of eugenol O-methyltransferase gene reveal metabolite shifts, chemotype specific differential expression and developmental regulation in *Ocimum tenuiflorum L. Mol. Biol. Rep*., 2014, **41**, 1857.

(138) N. Singh and A. Sharma, *In-silico* identification of miRNAs and their regulating target functions in *Ocimum basilicum*. *Gene*, 2014, **552**, 277.

(139) B. M. Lange and A. Ahkami, Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes—current status and future opportunities. *Plant Biotech*. *J*., 2013, **11**, 169.

(140) N. Dudareva, A. Klempien, J. K. Muhlemann and I. Kaplan, Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytol*., 2013, **198**, 16.

(141) M. J. Fischer, S. Meyer, P. Claudel, M. Bergdol and F. Karst, Metabolic engineering of monoterpene synthesis in yeast. *Biotechnol*. *Bioeng*., 2011, **108**, 1883.

(142) N. Misawa, Pathway engineering for functional isoprenoids. *Curr*. *Opin*. *Biotechnol*., 2011, **22**, 627.

(143) R. Davidovich-Rikanati, E. Lewinsohn, E. Bar, Y. Iijima, E. Pichersky and Y. Sitrit, Overexpression of the lemon basil α- zingiberene synthase gene increases both mono- and sesquiterpene contents in tomato fruit. *Plant J*., 2008, **56**, 228.

(144) R. Davidovich-Rikanati, Y. Sitrit, Y. Tadmor, Y. Iijima, N. Bilenko, E. Bar, B. Carmona, E. Fallik, N. Dudai, J. E. Simon, E. Pichersky and E. Lewinsohn, Enrichment of tomato flavor by diversion of the early plastidial terpenoid pathway. *Nature Biotechnol*., 2007, **25**, 899.

CHAPTER 2

Potential dual role of eugenol in inhibiting advanced glycation end products (AGEs) in diabetes:

Proteomic and mechanistic insights

2.1 Introduction

Diabetes mellitus (DM) is a metabolic disorder of multiple etiologies characterized by elevated levels of blood glucose resulting from defects in insulin production, insulin action, or both. Pharmacological treatment of diabetes includes the use of oral anti-diabetic agents that aid in controlling hyperglycemia. These drugs either promote insulin secretion, insulin sensitivity; decrease the hepatic glucose output or aid in absorption of glucose. An important class of drug molecules, effective in management of diabetes is α-glucosidase inhibitors. These regulate blood glucose level by inhibiting digestion of oligosaccharides/ carbohydrates like maltose, maltotriose, dextrins, sucrose etc. into glucose.[1] Examples of α-glucosidase inhibitors includes acarbose,[2] miglitol,[3] voglibose[4] etc.

Apart from these drugs, inhibition of advanced glycation end products (AGEs) is considered as a useful therapeutic strategy in management of diabetes. AGEs are formed by a series of non-enzymatic reactions between reducing sugars and amine group of proteins.[5, 6] Upon glycation, proteins tend to lose their structure and function[7]. AGEs bind to receptor for AGEs (RAGE), spawn reactive oxygen species, and downstream signaling contributes basically in elicitation of pro-inflammatory response.[8]

AGEs and AGE-RAGE axis has been implicated in various disease pathophysiologies including vascular and diabetic complications.[5, 6] Growing evidences on involvement of AGEs in disease has made them attractive therapeutic targets. Thus molecule(s) that inhibit the formation of AGEs are effective in management of diabetes. Consequently extensive research effort has been devoted to develop anti-AGE therapeutics. These

includes (i) preventing the formation of AGEs, example, ascorbic acid,[9] aspirin, [10] metformin,[11] etc. (ii) de-glycation of Schiff bases/Amadori products involving transglycation approach using drugs like hydralazine,[12] (iii) reversal of AGE induced modifications like cross links, example, phenacylthiazolium bromide (PTB),[13] and (iv) preventing the body from ill effects of AGE formation, example, resveratrol and curcumin.[14,15] Combination of oral drugs, and oral drugs with insulin has been used for better control of diabetes and diabetic vascular complications. For example, combination therapy with repaglinide and rosiglitazone has been verified to be safe and effective in diabetes treatment.[16] Similarly, combination of voglibiose with glibenclamide or gliclazide [17]; or miglitol with metformin[18] offer better glycemic control.

Therapeutically important genus *Ocimum*, comprising herbaceous members belonging to family Lamiaceae, is considered a boon for medicinal chemists.[19] Leaf extracts of several species have a long and successful history of being used in ancient folk medicine; having antioxidant,[20] antistress,[21] anticancer,[22] radiation protection,[23] antifungal,[24] insecticidal[25] and several other bioactivities. The key to this medicinal potential of genus *Ocimum* might lie in the vast array of secondary metabolites and phytochemicals including terpenoids, phenylpropanoids, flavonoids, phenolic compounds etc. present in various plant parts. *Ocimum tenuiflorum* (former *O. sanctum*) leaf extracts have been shown to have hypoglycemic effects by induction of insulin secretion from perfused pancreas, isolated islets and clonal pancreatic β-cells; [26, 27] however, the principle compound and its mechanism of action are poorly understood. In view of this, we demonstrate the anti-diabetic activity of eugenol from *O. gratissimum* using *in vitro* and *in vivo* approaches. Here we have shown, eugenol isolated from *O. gratissimum* to have a potential dual

effector role in diabetes control; it acts as an effective α-glucosidase inhibitor as well as a glycation inhibitor, mimicking the effect of combination therapy. Although both synthetic and natural anti-diabetic therapeutics are available, latter seems to be the obvious choice owing to its low toxicity and lesser side effects. Thus, identifying anti-AGE lead molecules of natural origin would provide a significant thrust to diabetes research in future.

2.2 Materials and method

2.2.1. Chemicals and plant material

All chemicals were procured from Sigma-Aldrich (St Louis, MO, USA), otherwise mentioned. Yeast α-glucosidase and *p*-nitrophenyl-α-D-glucopyranoside were procured from SRL and Himedia (Mumbai, MS, India), respectively. Three species namely *O. kilimandscharicum*, *O. tenuiflorum* and *O. gratissimum* were grown under the following greenhouse conditions: temperature, 28 to 30°C; humidity, 35 to 40%; light conditions, 16h light, 8h dark. After harvesting, tissue was immediately subjected to further extraction procedure.

2.2.2. Gas chromatography-mass spectrometry (GC-MS) analysis of *Ocimum* plant tissues

Extractions were performed as described earlier.[25] Leaf tissue (1g) was mixed in 10mL dichloromethane (DCM) and kept for 18 to 24h at 28ºC. The extract was filtered and incubated for 2h at -20°C to allow lipid precipitation. DCM extract was filtered again, concentrated under vacuum on a rotary evaporator and subjected to GC and GC-MS analyses as reported previously.[25]

2.2.3. Purification and NMR characterization of major metabolites from *Ocimum* species

Large-scale metabolite extraction was performed using 10g leaves as mentioned earlier.[25] Thin-layer chromatography (TLC) was performed on silica gel G-coated plates (0.25mm for analytical) developed three times in 5% petroleum ether in ethyl acetate. Compounds were visualized under UV light (254nm) or by spraying with a solution of 3% anisaldehyde, 2.8% H_2SO_4, 2% acetic acid in ethanol followed by heating for 1 to 2 min. Purification of major compounds was performed by flash chromatography using 240-400 mesh silica gel columns and petroleum ether-ethyl acetate gradient mixture as the eluent. NMR (^1H and ^{13}C) for purified compounds was carried out on Bruker DRX-500 (500MHz), Bruker AC-200 (200MHz) spectrometers in $CDCl_3$. Chemical shifts were reported in parts per million, with respect to tetramethylsilane as the internal standard.

2.2.4. BSA-AGE fluorescence assay

Stock solutions (100mM) of aminoguanidine hydrochloride, ocimene, α-pinene, terpinolene, farnesene, β-caryophyllene, camphor, eugenol, eugenol methyl ether (EME) and eucalyptol were prepared in 30% DMSO and vortexed for 15 min for uniform mixing. For extract preparation, dichloromethane extract was concentrated to dryness under vacuum on a rotary evaporator, re-dissolved in 30% DMSO and vortexed for 15 min.

The reaction was set up as described earlier.[28] BSA glycation reaction was carried out by incubating 1mL of 50mg mL^{-1} BSA in 0.1M phosphate buffer (pH 7.4) and 0.5M dextrose monohydrate containing 5mM sodium azide as bacteriostat at 37°C for 7 days with extracts and 15mM of above mentioned compounds. DMSO (30%) and aminoguanidine were used as solvent and positive control, respectively. The BSA glycation was monitored

at 370/440nm by using Varioskan Flash 4.00.53 spectrofluorometer (Thermo Scientific, Waltham, MA, USA). Percent inhibition of glycation was calculated by using the formulae; (C-T)/C×100 where C is the relative fluorescence intensity of glycated BSA in absence of an inhibitor and T is the relative fluorescence intensity of glycated BSA in presence of an inhibitor.

2.2.5. Blind docking and probability analysis

Blind docking and probability analysis of eugenol with mouse serum albumin (MSA) was performed as described earlier.[29]

2.2.6. Intrinsic fluorescence assay

BSA (50mg mL^{-1}) in phosphate buffer (50mM, pH 7.4) was incubated at 37^{o}C for 2h with different concentrations of eugenol (1-50mM) dissolved in 30% DMSO. Intrinsic fluorescence was monitored using spectrofluorometer (excitation: 280nm, emission: 300-450nm).

2.2.7. Circular dichorism analysis of BSA and BSA-eugenol complexes

BSA (50mg mL^{-1}) in phosphate buffer (50mM, pH 7.4) was incubated at 37^{o}C for 2h with varying concentrations of eugenol (5, 10 and 25mM) dissolved in 30% DMSO. 0.02mg mL^{-1} concentration of protein was used to measure the CD spectra. All the CD spectra were recorded at room temperature (24^{o}C) using JASCO J-815 CD spectropolarimeter (Jasco Inc., Easton, MD, USA) over wavelength ranging from 250-190nm.

2.2.8. Animal experiments

Ethics statement: All animal experiments were approved by Institutional Animal Ethics Committee of National Centre for Cell Sciences, Pune, MS, India. The experimental protocols were carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals, India

Twenty healthy male balb/c mice, 6 to 8 weeks old, weighing 20 to 25g were used for experiments. Mice were maintained in standard polyvinyl cages under the following conditions: temperature, 24 to 26°C; humidity, 35 to 40%; light conditions, 16h light, 8h dark and fed on pellet diet and water *ad libitum*. Streptozotocin (STZ; 45 mg/kg body weight, 100 µL) was administered intraperitoneally in citrate buffer (50mM, pH 4.5) to mice for 5 consecutive days to induce diabetes. Mice were monitored for a period of 15 days for establishment of stable hyperglycemic condition. During this time parameters like weight, water intake, physical appearance, animal behavior, urine output and blood glucose were measured routinely. Mice which displayed stable hyperglycemic condition were chosen for experiments and divided in three groups: Group I, STZ control; Group II, Vehicle control; Group III, eugenol- treated mice. 3 mice per control group and 9 mice per treated group were taken. 100µL intraperitoneal injection of eugenol (100mg/kg body weight) in vehicle (Ethanol: Tween80: Saline = 1:1:18) twice a week for two weeks was administered. Time between consecutive injections was 3 days.

2.2.9. Estimation of blood glucose and HbA1c levels

Blood glucose and HbA1c levels were measured using Bayer's CONTOUR blood glucose meter and Bayer A1C Now Kit (Bayer, Leverkusen, NRW, Germany), respectively according to the manufacturer's instructions.

2.2.10. α-glucosidase inhibition assay and kinetics

α-glucosidase inhibition assay was performed as described earlier.[30] 100µL eugenol in varying concentration (2.5 to 12.5mM; prepared in 30% DMSO), 50µL of 5mM *p*-nitrophenyl- α-D-glucopyranoside (PNPG) and 50µL yeast α-glucosidase (0.25U/mL) were mixed and incubated at 37^{o}C for 30 min. Reaction was terminated by addition of 2mL of 200mM Na_2CO_3. Amount of *p*-nitrophenol released was measured using a spectrophotometer at 405nm. Mode of inhibition of yeast α-glucosidase by eugenol was determined by measuring enzyme activity with increasing concentration of PNPG in the presence and absence of eugenol at different concentration.[30] Type of enzyme inhibition was determined using Lineweaver-burke plot analysis using Michelis-Menten kinetics.

2.2.11. Plasma collection and insulin measurement

Plasma was collected on the last day of experiment (day 45) and stored at -80°C until further use. Plasma insulin measurements were performed at Department of Biochemistry, King Edward Memorial (KEM) Hospital, Pune, MS, India.

2.2.12. Tissue processing for histopathology

Mice were sacrificed on the last day of experiment (day 45) by cervical dislocation. Part of spleen, liver, heart, lungs, kidney, pancreas and brain were fixed in 10% formalin for histopathological analysis. Tissues were processed in a Leica TP 1020 tissue processor

and embedded in paraffin blocks using Leica EG 1160 paraffin embedder. The paraffin blocks were cut into sections of 4mm using a Microm HM 360 microtome. The slides were stained with hemotoxylin and eosin using a Microm HMS-70 stainer. Permanent slides were made and evaluated for histopathological changes under Olympus BX51 microscope.

2.2.13. Western blotting

Western blotting was performed in biological duplicates and technical triplicates for plasma samples. *In vitro* BSA-AGE assay was performed in duplicate and technical triplicates of each sample. Protein (5µg), in each case, was resolved on 12% SDS-PAGE, transferred onto polyvinylidene difluoride membrane (PVDF) membranes and blocked overnight at 4°C with 5% membrane blocking agent prepared in TBS. The membranes were incubated with primary antibody Anti-AGE (Abcam) at a dilution of 1:2000 for 1h, followed by 1:5000 secondary antibody (Goat Anti-Rabbit IgG) conjugated with horseradish peroxidase (HRP) for 30 min. Protein bands were visualized using WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, USA) and documented by using Syngene DYVERSITY gel doc system (Syngene, Cambridge, UK).

2.2.14. In-gel trypsin digestion and LC-MS analysis of *in vitro* samples

In gel trypsin digestion was performed a described earlier.[31] Tryptic peptides were analyzed by nano LC-MS^E (MS at elevated energy) using a Nano Acquity UPLC system (Waters Corporation, Milford, MA) online coupled to a Q-TOF, SYNAPT-HDMS (Waters Corporation) as described by Cheng et al.[32] LC-MS^E data were processed using Protein Lynx Global Server 2.4 (PLGS; Waters Corporation). Search was performed

against UniProt-P02769 (BSA) sequence database. Glycation modifications of lysine, amadori (162.05Da), CML (+58.0Da) and CEL (+72.02Da) were considered as additional variable modifications. Glycation modifications identified by PLGS were manually validated as described by Bhonsle *et al*.[33, 34]

2.2.15. In-solution trypsin digestion and LC-MS/MS analysis of plasma proteins

In solution trypsin digestion was performed as described earlier.[31] Peptides were desalted using Zip tip C18 (Millipore, Billerica, MA, USA), concentrated by vacuum centrifuge and stored at -20°C until further use. Peptides (5µl injections containing 3.5µg of peptides) were loaded on Eskigent C18 reverse phase column (100*0.3mm, 3µm, 120Å) with 97% of mobile phase A (100% water, 0.1% formic acid) and 3% of mobile phase B (100% acetonitrile, 0.1% formic acid) at 8µl/min flow rate. The peptides were separated at 8µl/min flow rate for 100 min linear gradient of 3% to 50% mobile phase B. After 100 min the gradient was raised to 90% B for 9 min and the column was re-equilibrated to 3% mobile phase B for 11 min. All samples were analyzed on Triple TOF 5600 mass spectrometer (Sciex; Concord, ON, Canada) as described by Jones *et al*.[35] The samples were acquired in positive and high-sensitivity mode using Electrospray ionization (ESI) method. The acquired MS dataset was processed using the Proteome Discoverer software (Version 1.4.1.14, Thermo Fisher Scientific, Bremen, Germany). SEQUEST HT search engine was used for peptide identification. Data was searched against UniProt P07724 (mouse serum albumin) sequence database. Ion search parameters used included peptide precursor and fragment mass tolerance- 10ppm and 0.5Da respectively with 2 missed cleavages and 1% FDR. Glycation modifications of lysine, Amadori (162.05Da), CML (+58.0Da) and CEL (+72.02Da) were considered as additional variable modifications.

2.2.16. Statistical Analysis

GC-MS analyses of leaf tissue, *in vitro* BSA-AGE assay/s for extracts and compounds and IC_{50} assays for eugenol and aminoguanidine were performed in triplicates and values were represented as mean ± standard deviation. Unpaired t-test was performed for blood glucose, plasma insulin and HbA1c measurements. Western blotting for plasma samples was performed in biological duplicates and technical triplicates. One-way ANOVA followed by unpaired t-test was performed for blot density analysis. Unpaired t-test suggested significant differences between data at $p<0.0001$ (indicated as '****'), $p<0.001$ (indicated as '***'), $p<0.01$ (indicated as '**') and $p<0.05$ (indicated as '*'). NS represents non-significant difference in data.

2.3. Results and discussion

2.3.1. Chemical profiling unravels terpene and phenylpropanoid abundance in *Ocimum* species

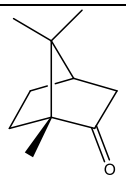
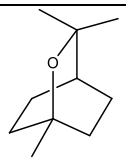
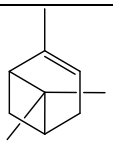
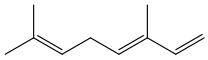
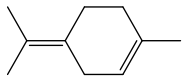
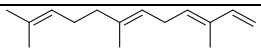
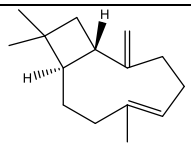
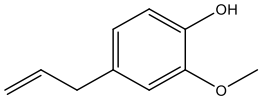
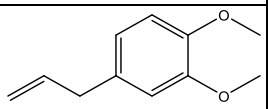
GC-MS based chemical profiling of leaf tissue of three *Ocimum spp*. revealed that each species was rich in a specific set of compounds representing a distinct metabolic fingerprint. Results indicated predominance of monoterpenes, sesquiterpenes and phenylpropanoids. Hydrocarbons including dodecane, dodecene, heptene, octane derivatives etc. were detected in minor quantities. The entire list of compounds identified is provided in **Table 2.1**. **Table 2.2** provides a selected subset of major metabolites screened for antiglycation activity using BSA-AGE assay.

Table 2.1: GC-MS-based chemical profiling of leaf tissue of *Ocimum kilimandscharicum*, *Ocimum tenuiflorum* and *Ocimum gratissimum*

Compound name	*O. kilimandscharicum*	*O. tenuiflorum*	*O. gratissimum*
Monoterpenes			
Pinene	0.92 ± 0.2	ND	ND
β- Ocimene	ND	ND	4.94 ± 0.3
Borneal	0.74 ± 0.003	ND	ND
Borneol	ND	1.2 ± 0.06	ND
Camphene	3.59 ± 0.01	ND	ND
Camphor	**47.33 ± 0.3**	ND	ND
Eucalyptol	19.85 ± 0.16	ND	ND
Limonene	4.97 ± 0.01	ND	ND
Myrtenol	0.99 ± 0.15	ND	ND
Terpineal	0.28 ± 0.002	ND	ND
Terpineol	0.25 ± 0.03	ND	ND
Terpinolene	0.41 ± 0.01	ND	ND
Thujanol	2.78 ± 0.01	ND	0.5
Cis-thujene	ND	ND	0.24
Sesquiterpenes			
α- Caryophyllene	0.45 ± 0.02	ND	ND
α- Copaene	0.56 ± 0.01	3.33 ± 0.12	1.54
α- Humulene	ND	0.88 ± 0.04	ND
β- Bourbonene	ND	1 ± 0.05	0.39
β- Caryophyllene	3.68 ± 0.02	14.5 ± 0.09	2.87 ± 0.03
β- Cubebene	0.39 ± 0.004	2.33 ± 0.01	0.66 ± 0.05
β- Elemene	0.25 ± 0.002	0.89 ± 0.06	0.36 ± 0.05
δ- Cadinene	0.18 ± 0.01	3.11 ± 0.15	0.36 ± 0.001
Elemol	ND	1.72 ± 0.08	ND
Farnesene	0.69 ± 0.23	ND	ND
Germacrene D	5.19 ± 0.05	5.83 ± 0.35	9.52 ± 0.15
Germacrene-D-al	0.09 ± 0.01	ND	0.21
Murrolene	ND	ND	0.10 ± 0.01
Others			
Eugenol	ND	ND	**78.25 ± 0.4**
Eugenol Methyl Ether	ND	**60.41 ± 0.75**	ND
Dodecane	0.19 ± 0.002	0.37± 0.02	ND
Dodecene	0.18 ± 0.002	0.43 ± 0.01	ND
Heptene	0.33 ± 0.02	ND	ND
Octane derivative	0.10 ± 0.03	0.46 ± 0.12	ND
Tetradecane	ND	0.23 ± 0.01	ND

^ND (not detected)

Table 2.2: *Ocimum spp*. metabolites used for BSA-AGE assay

Class of metabolite	Name	Structure	% composition		
			Ok	***Ot***	***Og***
A. Monoterpene	Camphor		47.33 ± 0.3	ND	ND
	Eucalyptol		19.85 ± 0.16	ND	ND
	α-Pinene		0.92 ±0.20	ND	ND
	β-Ocimene		ND	ND	4.94 ± 0.3
	Terpinolene		0.41 ± 0.01	ND	ND
B. Sesquiterpene	Farnesene		0.69 ± 0.23	ND	ND
	β-caryophyllene		3.68 ± 0.02	14.5 ± 0.09	2.87 ± 0.03
C. Phenylpropanoid	Eugenol		ND	ND	78.25 ± 0.4
	Eugenol methyl ether		ND	60.41 ± 0.75	ND

^ND (not detected), *Ok* (*O*. *kilimandscharicum*), *Ot* (*O*. *tenuiflorum*), *Og* (*O*. *gratissimum*)

2.3.2. *In vitro* inhibition of AGEs by metabolites from *Ocimum* species

Leaf and inflorescence extracts from three *Ocimum* species and major metabolites therein including camphor, eucalyptol, eugenol, eugenol methyl ether (EME), ocimene, α-pinene, terpinolene, β-caryophyllene and farnesene were evaluated for their *in vitro* anti-glycation activity using BSA-AGE fluorescence assay.

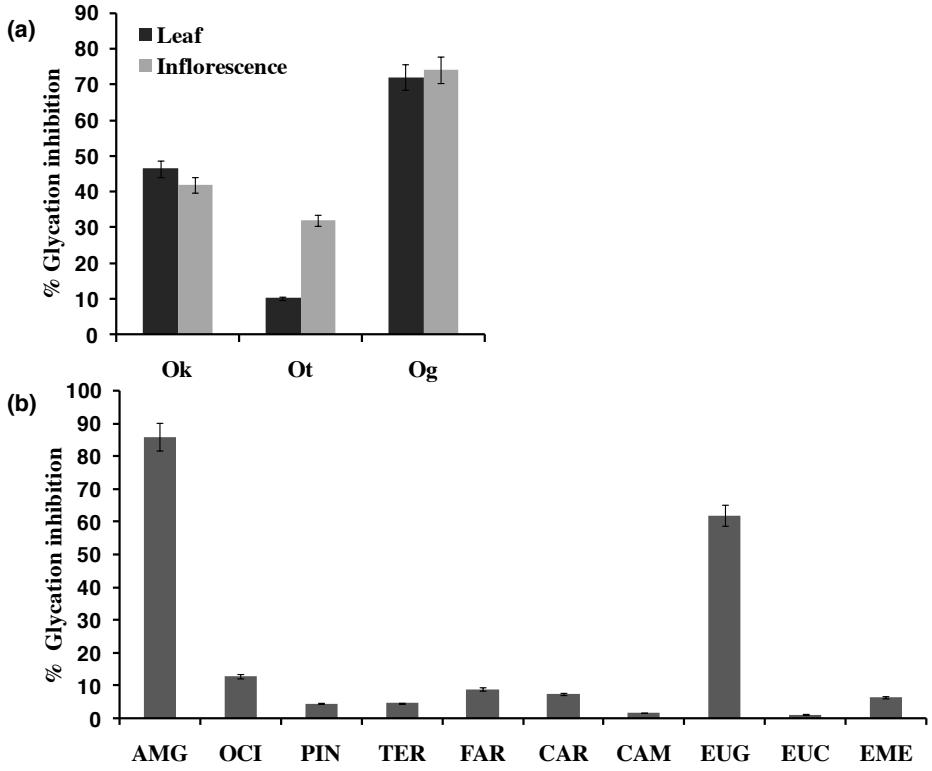


Figure 2.1: *In vitro* BSA-AGE inhibition assay. Glycation inhibition shown by (a) leaf and inflorescence extracts of *O*. *kilimandscharicum* (Ok), *O*. *tenuiflorum* (Ot), *O*. *gratissimum* (Og) and (b) standard compounds, aminoguanidine (AMG), ocimene (OCI), pinene (PIN), terpinolene (TER), farnesene (FAR), β- caryophyllene (CAR), camphor (CAM), eugenol (EUG), eucalyptol (EUC) and eugenol methyl ether (EME).

Maximum inhibition of glycation was observed with *O*. *gratissimum* extract, which is rich in eugenol. Inflorescence and leaf extracts of *O*. *gratissimum* inhibited the formation of AGEs by 74% and 72%, respectively (**Fig. 2.1a**). *O*. *tenuiflorum* leaf extracts rich in EME showed least (10%) inhibition of glycation (**Fig. 2.1a**). *O*. *kilimandscharicum* leaf and inflorescence extracts, rich in camphor and eucalyptol (**Table 2.1**) displayed significant inhibition of AGE formation, 46% and 42%, resp. (**Fig. 2.1a**). Of all the metabolites assessed, eugenol displayed highest, 58% inhibition of glycation (**Fig. 2.1b**). Other metabolites did not inhibit AGE formation significantly. Based on these studies, eugenol, the major metabolite present in *O*. *gratissimum* was considered for *in vivo* studies. Inhibitory concentration required to inhibit 50% AGE formation (IC_{50}) for eugenol was 10mM (**Fig. 2.2b**) while for aminoguanidine hydrochloride was 1mM (**Fig. 2.2a**).

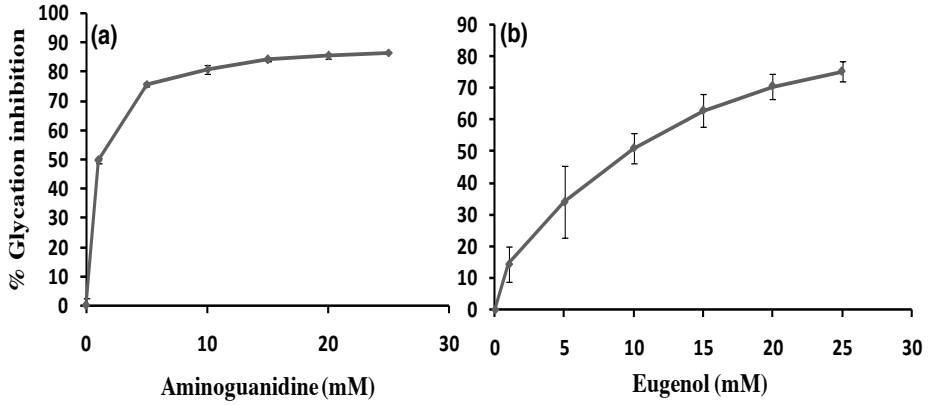


Figure 2.2: Glycation inhibition assays by aminoguanidine and eugenol. IC_{50} values for AGE inhibition by (a) Aminoguanidine and (b) Eugenol. Values represent mean±standard deviation (n=3).

Since anti-glycation activity of extracts was significantly higher than that of individual metabolites, it was hypothesized that either of the following possibilities might exist: (i)

anti-glycation activity of tissue extracts maybe due to synergistic action of a group of metabolites rather than a single metabolite or (ii) the metabolites present *in planta* are structurally modified, imparting them enhanced anti-glycation potential. To test this hypothesis, major metabolites (eugenol, EME and camphor) were purified and characterized by NMR analysis.

Table 2.3: NMR spectroscopic data of purified compounds

Compound (Mol. Wt.)	Purity (%)	^{1}H NMR	^{13}C NMR
Eugenol (164 g/mol)	99.9	(200 MHz, $CDCl_3$); δ (ppm): 3.30–3.33(d, 2H, H-7), 3.87 (s, 3H, H-10), 5.03 (br s, 1H, H-9), 5.08 -5.12 (m, 1H, H-9), 5.5 (br s, 1H, 1-OH), 5.85-6.05 (m, 1H, H-8), 6.69 (m, 2H, H-3, 5), 6.83-6.87 (m, 1H, H-6).	(50 MHz, $CDCl_3$); δ(ppm): 39.86 (C-7), 52.82 (C-10), 111.05 (C-3), 114.20 (C-6), 115.49 (C-9), 121.14 (C-5), 131.89 (C-4), 137.79 (C-8), 143.86 (C-1), 146.39 (C-2)
Eugenol Methyl Ether (178 g/mol)	98.2	(200 MHz, $CDCl_3$); δ(ppm): 3.85 (s, 3H, H-10), 3.86 (s, 3H, H-11), 3.31-3.34 (d, 2H, H-7), 5.03 (br s, 1H, H-9), 5.08 (m, 1H, H-9), 5.85-6.05 (m, 1H, H-8), 6.70 (br s, 1H, H-3), 6.73-6.74 (m, 1H, H-5), 6.78-6.82 (m, 1H, H-6).	(50 MHz, $CDCl_3$); δ(ppm): 39.77 (C-7), 55.75 (C-10), 55.89 (C-11), 111.18 (C-3), 111.79 (C-6), 115.57 (C-9), 120.35 (C-5), 132.59 (C-4), 137.66 (C-8), 147.32 (C-1), 148.84 (C-2).
Camphor (152 g/mol)	98	(200 MHz, $CDCl_3$); δ(ppm): 2.29-2.42 (m, 1H, H-3), 0.84 (s, 3H, H-8), 0.91 (s, 3H, H-9), 0.96 (s, 3H, H-10), 1.25-1.47 (m, 2H, H-4), 1.65-1.80 (m, 2H, H-5), 1.89-2.09 (m, 2H, H-2).	(50 MHz, $CDCl_3$); δ(ppm): 9.22 (C-10), 19.12 (C-8), 19.76 (C-9), 27.02 (C-4), 29.89 (C-5), 43.02 (C-3), 43.28 (C-2), 46.77 (C-7), 57.69 (C-6), 200.98 (C-1).

Results indicated that there were no structural difference between metabolites present *in planta*, in comparison with standard compounds procured from Sigma-Aldrich (**Table 2.3**). Since these metabolites present *in planta* were not structurally modified, it can be

suggested that the increased antiglycation activity of *Ocimum* tissue extracts is probably due to synergistic effect of a group of metabolites. However, further investigation is required to acertain the hypothesis.

2.3.3. Eugenol shows increased binding affinity for surface lysine residues on mouse serum albumin but does not alter the protein secondary structure

Based on *in vitro* BSA-AGE assay, eugenol was observed to be a potent inhibitor of AGEs compared to EME. Structurally, the difference between eugenol and EME is that, in eugenol a hydroxyl group is present at the para position (4'-OH); however, in EME this group is masked by the presence of a methyl group. We speculate the anti-glycation activity of eugenol might be due to the presence of free 4'-OH group. Previous studies support the fact that, presence and position of hydroxyl group determines the activity of flavonoids.[36] We imply that masking of active hydroxyl group in EME is responsible for decline in antiglycation activity of the compound. To gain more insight into eugenol-protein interaction, blind docking study of eugenol with mouse serum albumin (MSA) was performed. Out of many surface-exposed lysine, blind docking results indicate that eugenol preferentially binds to Lys-236 and Lys-375. In **Fig. 2.3**, surface exposed lysine residues have been marked in red, and binding of multiple eugenol molecules to single MSA molecule is depicted.

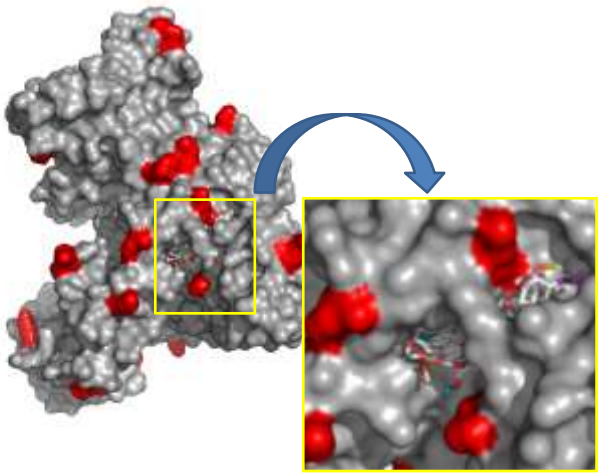


Figure 2.3: *In silico* analysis of interactions between eugenol and MSA. Blind docking and probablity analysis of eugenol with MSA. Surface exposed lysine have been marked in red. Inset depicts binding of several eugenol molecules to surface lysines on MSA.

Eugenol shows stronger binding (average binding energy, 6 Kcal/mol) with surface exposed lysines as compared to AMG (average binding energy, 4.3 Kcal/mol).

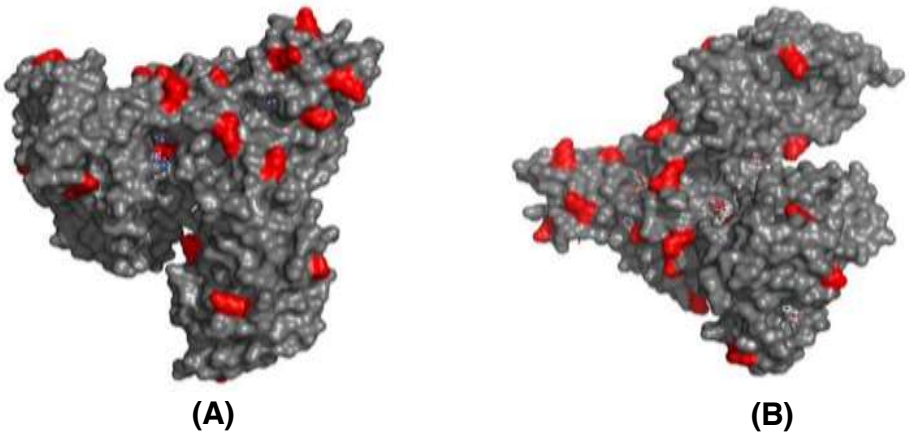


Figure 2.4: Glycation modifications depicting surface exposed lysine residues (A) glycated BSA with AMG (B) glycated BSA with EME

EME also showed strong binding affinity for MSA (**Fig. 2.4**), however, it did not display good inhibition of glycation *in vitro*. Thus, preliminary evidence suggests that the 4'-OH group of eugenol is potentially capable of binding to the amine group of lysine residues on protein molecule and competitively inhibiting the binding of sugar.

Intrinsic fluorescence assay and circular dichorism (CD) were performed to understand the nature of interaction between eugenol and BSA.

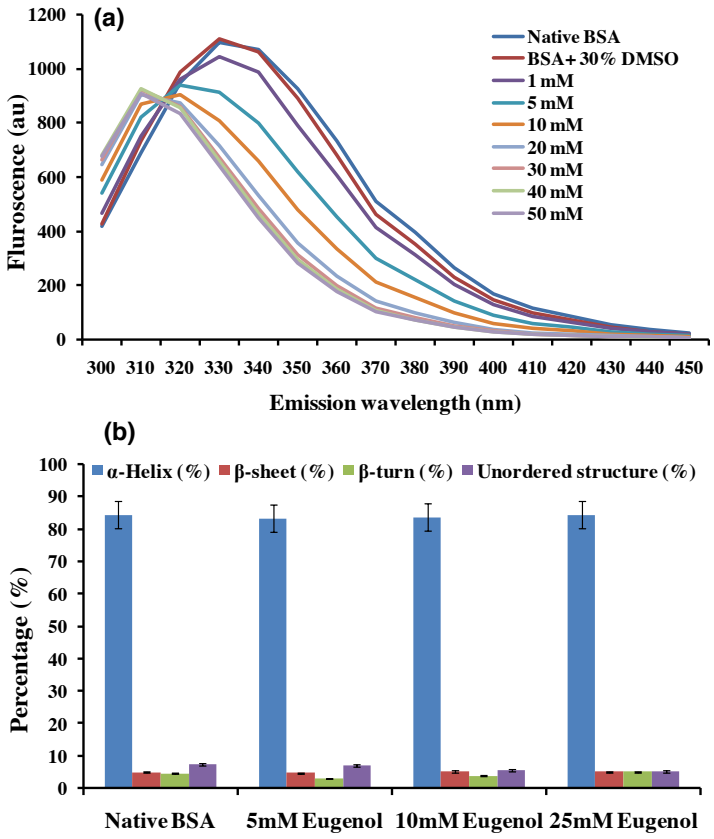


Figure 2.5: Biophysical analysis of BSA and eugenol interaction (a) Fluorescence quenching of BSA by eugenol (b) CDPro analysis of native BSA and BSA treated with 5, 10 and 25mM eugenol.

In the intrinsic fluorescence assay, it was evident that eugenol binds to BSA in a concentration dependent manner (**Fig. 2.5a**).As the concentration of eugenol was increased, a consistent decrease in intrinsic fluorescence intensity was documented. CD of BSA incubated with eugenol was performed to understand the effect of binding of eugenol on the secondary structure of BSA. However, the secondary structure of BSA (**Fig. 2.5b**) remained unchanged (84.3 ± 1.9 %, α-helix; 4.85 ± 0.2%, β-sheet; 4.45 ± 1.9%, β-turn; 7.4 ± 0.2% unordered). The interaction did not induce any transition from α-helix to β-sheet or *vice versa*. in the structure of protein. Hence we conclude that eugenol binds to BSA but the binding does not cause any significant change in its secondary structure.

2.3.4. Eugenol administration affects blood biochemical parameters

The effect of administration of eugenol on blood glucose, HbA1c and insulin was investigated. Since hyperglycemia is known to be the foremost cause of diabetic complications, molecule(s) that lower blood glucose are frontrunners for management of diabetes. Mice belonging to Group I (STZ control) and group II (vehicle control) did not show any significant decrease in blood glucose (396 mg/dL and 353 mg/dL, respectively). Mice belonging to Group III (eugenol- treated mice) exhibited a 38% decrease in blood glucose, with levels dropping from 420 mg/dL to 262mg/dL, on an average (two-tailed *p-value*, 0.0042). Lowering blood glucose level helps subsequently in lowering formation of AGEs. Administration of eugenol showed significant decrease in blood glucose level (**Fig. 2.6a**). Although the blood glucose levels decreased, it did not lead to a severe hypoglycemic condition. Decrease in blood glucose may be attributed to the inhibition of α-glucosidase activity (**Fig. 2.7**), as discussed in subsequent section. Furthermore, the blood glucose levels increased when the treatment was withdrawn.

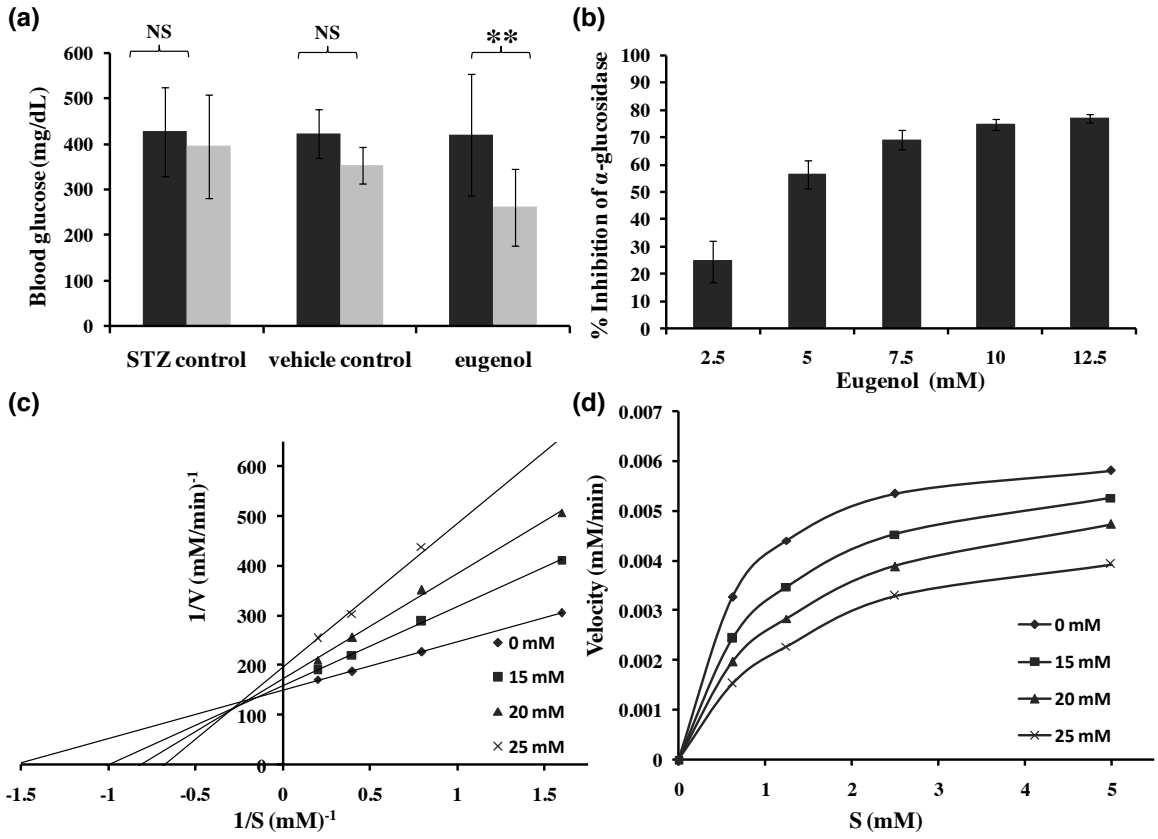


Figure 2.6: Kinetic studies of alpha-glucosidase inhibition by eugenol (a) Blood glucose measurement after intraperitoneal administration of eugenol (n=8) in STZ- induced balb/c mice. Unpaired t-test suggested significant differences between data at $p<0.01$(indicated as '**') and $p<0.05$ (indicated as '*'). NS represents non-significant difference in data. (b) α-glucosidase inhibition assay. Inhibition kinetics depicted via (c) Lineweaver-burke plot and (d) Michaelis- Menten plot showing mixed inhibition of α-glucosidase by eugenol.

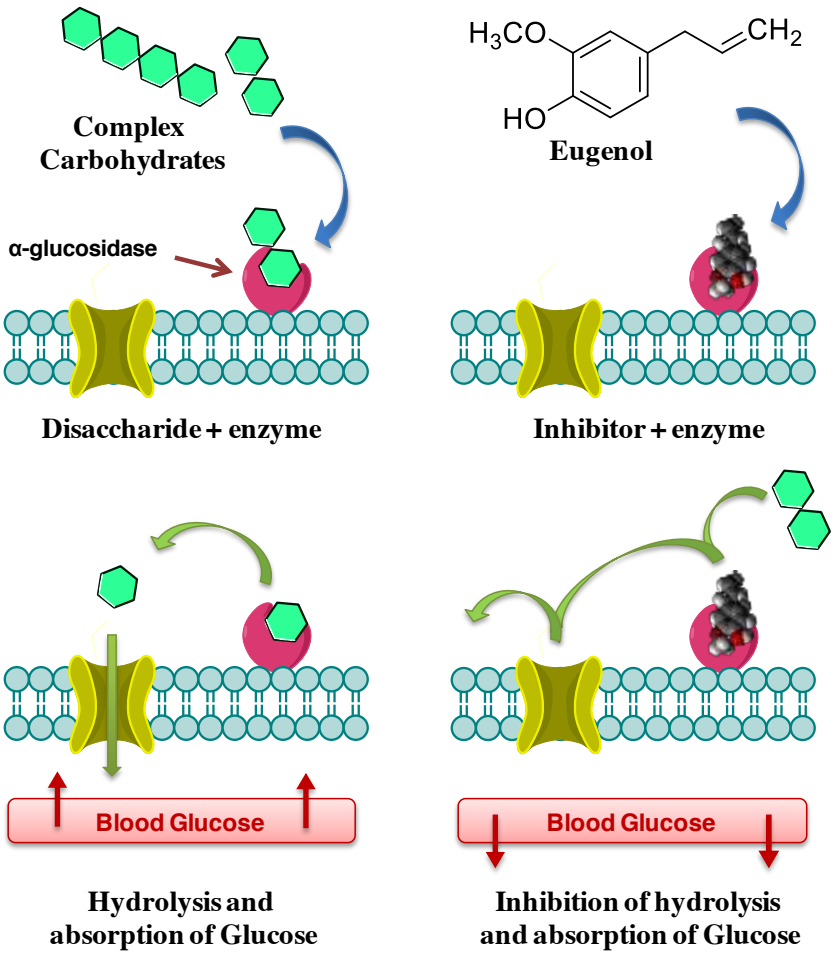


Figure 2.7: Inhibition of α-glucosidase by eugenol slows carbohydrate metabolism resulting in decrease in blood glucose

HbA1c essentially serves as a marker for monitoring glycemic status over a period of three months. We did not notice any significant change in HbA1c levels between different groups of mice: Group I (9.9), Group II (10.7), Group III (8.9), values in the parenthesis represent average for each group of treatment (**Fig. 2.8a**). The possible reason behind no significant change in HbA1c level may be due to the short duration of study (45 days). HbA1c levels are known to changes over a period of 3 to 4 months. Since our study was restricted to a short period of 45 days, no drastic changes in HbA1c levels were observed.

No significant change in the insulin level was evident between the groups (**Fig. 2.8b**) suggesting that eugenol lowers the blood glucose by inhibiting glucosidase activity.

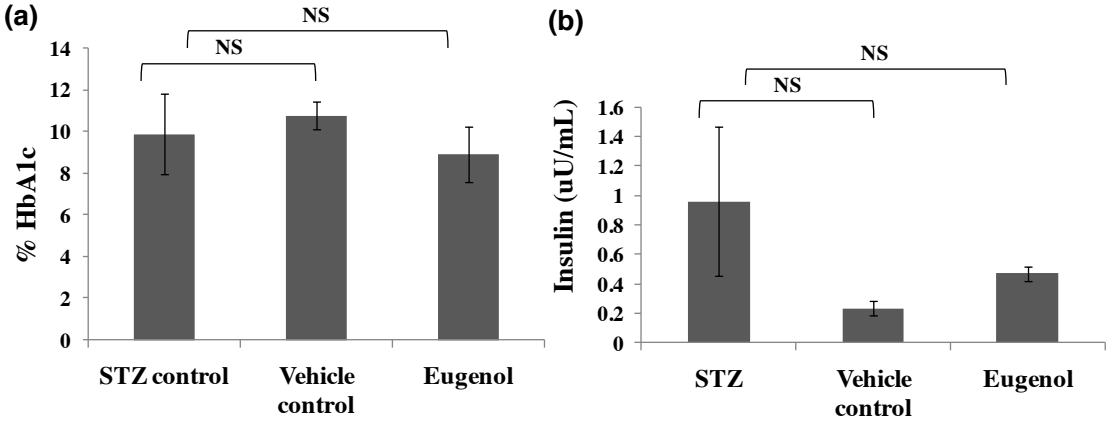


Figure 2.8: Analysis of blood biochemical parameters. Measurement of (a) HbA1c, (b) plasma insulin. Unpaired t-test suggested significant differences between data at $p<0.05$ (indicated as '*'). NS represents non-significant difference in data.

2.3.5. Mixed inhibition of α-glucosidase by eugenol might lead to decrease in blood glucose

We speculate that, the decrease in blood glucose may be due to inhibition of intestinal α-glucosidase. α-Glucosidase is localized in brush border epithelium of small intestine and catalyzes the conversion of oligosaccharides/carbohydrates like maltose, maltotriose, dextrins, sucrose etc. into glucose. α-Glucosidase inhibitors are known to lower blood glucose level by slowing carbohydrate metabolism.[2,3,4] Eugenol inhibited yeast α-glucosidase in a concentration dependent manner (**Fig. 2.6b**) with IC_{50} value around 5mM. Lineweaver-Burke plot analysis revealed mixed type of inhibition; thus, eugenol can bind to both enzyme and enzyme-substrate complex (**Fig. 2.6c & d**). **Fig. 2.7** shows schematic

representation of inhibition of α-glucosidase by eugenol. Hence, eugenol, an α-glucosidase inhibitor can be a potential candidate in the treatment and management of diabetes.

2.3.6. Eugenol treated mice display significantly less histopathological lesions

Histopathology was performed to understand the gross and microscopic effect of eugenol on different tissues (spleen, liver, heart, lungs, kidney, pancreas and brain). Spleen, heart and lung tissues of mice belonging to all three groups did not show significant abnormal lesions. However, brain, pancreas, kidney and liver tissue displayed moderate to severe histopathological lesions depending upon the treatment administered.

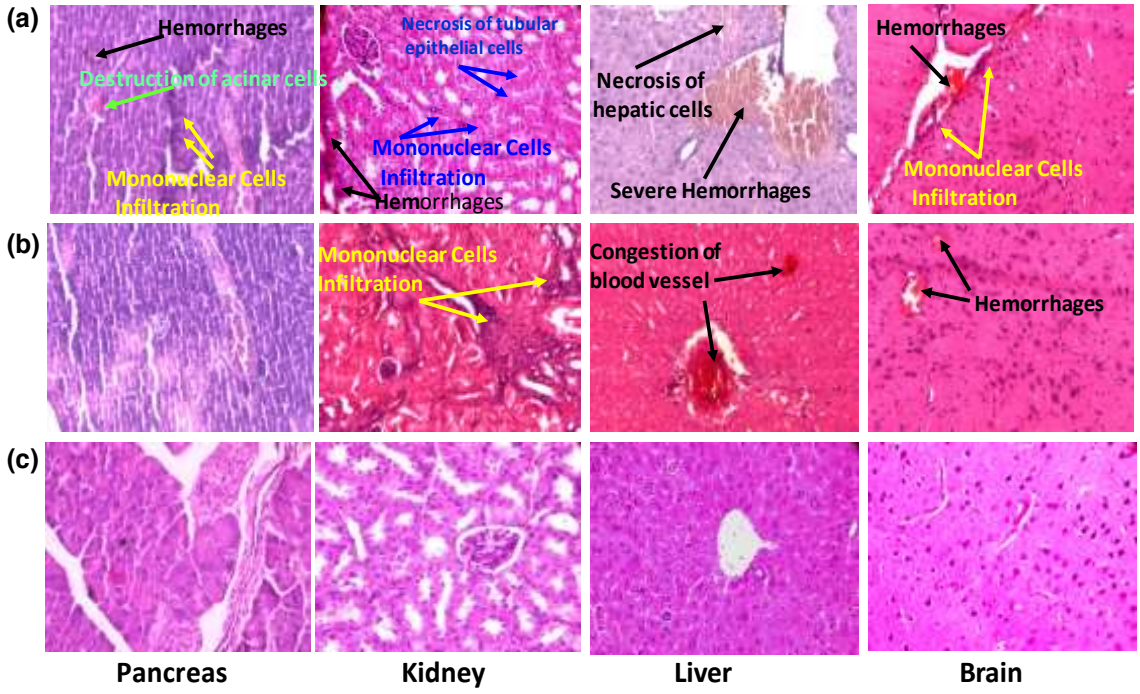


Figure 2.9: Histopathological examination of pancreas, kidney, liver and brain tissues of (a) STZ control (b) vehicle control (c) eugenol treated mice.

Mice belonging to Group I revealed most severe lesions (**Fig. 2.9a**) followed by those belonging to Group II (**Fig. 2.9b**). In both these groups, brain tissue revealed mononuclear

cell proliferation and focal hemorrhages. Pancreas exhibited focal destruction of acinar cells, hemorrhages and mononuclear cell infiltration. Kidney sectioning exposed necrosis of tubular epithelial cells, focal hemorrhages and mononuclear cell infiltration. Liver reflected severe hemorrhages and focal necrosis of hepatic cells. Occurrence of these severe lesions may be due to oxidative stress related cell injury caused by hyperglycemia in these groups. Occurrence of aforementioned lesions was significantly less in mice belonging to Group III (**Fig. 2.9c**), however, mild degeneration was observed in hepatocytes. Results indicate that eugenol treated mice displayed healthier histopathology than diabetic mice emphasizing therapeutic effect of eugenol against hyperglycemia.

2.3.7. Western blot analysis shows *in vitro* and *in vivo* inhibition of AGEs by eugenol

Western blot analysis of *in vitro* BSA-AGE assay samples and *in vivo* plasma samples showed significant differences in eugenol-treated and un-treated glycated BSA (**Fig. 2.10a & c**) as well as eugenol-treated and un-treated STZ mice plasma (**Fig. 2.10b & d**) following statistical analysis of blot density data. The results clearly indicate significant reduction in binding of glucose in presence of eugenol, showing western blot relative density, 0.46 for eugenol-treated glycated BSA compared to control with p=0.0008 (p<0.001 indicated by ***) in BSA-AGE samples (**Fig. 2.10c**) and 0.64 for eugenol-treated STZ mice with p=0.0011 (p<0.01 indicated by **) in *in vivo* plasma samples (**Fig. 2.10d**).

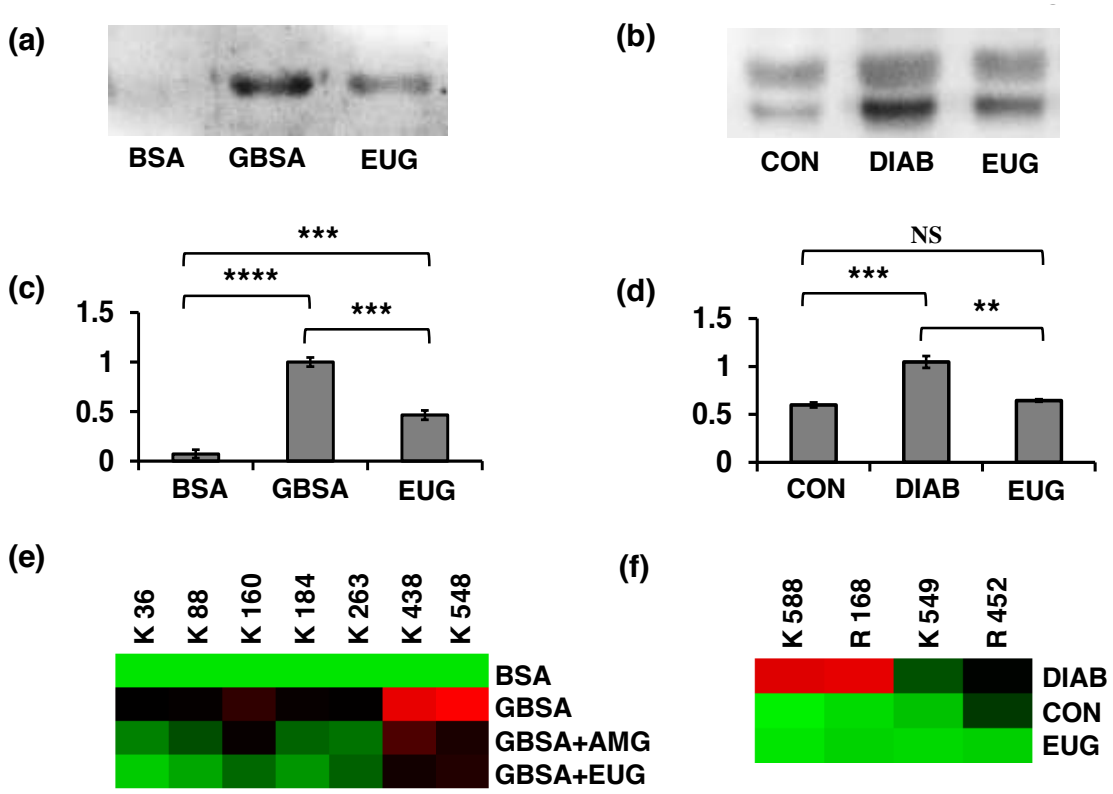


Figure 2.10: Proteomic analysis of *in vitro* and *in vivo* samples for AGE formation. Western blot using anti-AGE antibody & blot density analysis of *in vitro* BSA-AGE assay samples (a) & (c) and *in vivo* plasma samples (b) & (d) for probing AGE formation. One way ANOVA followed by unpaired t-test suggested significant differences between data at $p<0.01$ (indicated as '**'), $p<0.001$ (indicated as '***') and $p<0.0001$ (indicated as '****'). NS represents non-significant difference in data. Heat map showing extent of AGE induced modifications on specific lysine and arginine residues in (e) *in vitro* BSA-AGE assay and (f) plasma protein, identified by LC-MS^E. Heat map generated using Multi Experiment Viewer (MEV) software. (GBSA, glycated BSA; AMG, aminoguanidine; EUG, eugenol-treated sample; CON, control healthy mice; DIAB, STZ control plasma)

One of the reasons for lesser accumulation of AGEs in plasma could be because of decreased level of blood glucose, which can be attributed to α-glucosidase inhibitory activity of eugenol. Further, eugenol can also inhibit formation of AGEs by competitively inhibiting the binding of sugar to proteins both *in vivo* and *in vitro*, as discussed previously. Thus, both these factors result in overall lesser accumulation of AGEs.

2.3.8. LC-MS analysis reveals lesser extent of AGE modification on peptides upon eugenol treatment

LC-MS analysis was performed to understand the sites of AGE modification for both *in vitro* BSA-AGE assay and *in vivo* plasma protein especially mouse serum albumin (MSA). Heatmap analysis showing extent of AGE modification on glycated peptides, including glycation sensitive amino acid residues (GSAR) containing peptides of MSA is depicted in **Fig. 2.10f**. It was noticed that AGE modified peptides were found to decrease in response to eugenol treatment and were found to be highest in plasma of STZ treated diabetic mice. The MS/MS spectra of AGE modified peptides are provided in **Table 2.4** and **Fig. 2.11**. Glycation sensitive sites R168 and R452, [12, 37] which are suggested to be markers for type II diabetes[37] significantly showed lesser extent of glycation in response to eugenol treatment. The fact that extent of AGE-modification is less on peptides containing GSAR residues, makes eugenol a convincing candidate for early inhibition of glycation. Similarly, eugenol showed significantly lesser AGE modification in *in vitro* glycated BSA sites K36, K88, K160, K184, K263, K438 and K548 in comparison with glycated BSA and positive control AMG (**Fig. 2.10e**). The MS/MS spectra for these sites are provided in **Table 2.5** and **Fig. 2.12**). Thus, LC-MS analysis of glycated peptides reveals that

eugenol, contributes significantly in reducing the extent of glycation, both *in vitro* and *in vivo* eventually resulting in lesser accumulation of AGEs.

Table 2.4: Average precursor ion intensity and other information of AGE modified and corresponding unmodified peptides in *in vivo* plasma samples.

	Mod Site	Peptide Start-end	Peptide sequence	Peptide m/z Da	Peptide MH+ Da	PCS	Avg. XCorr	STZ-control (APII)	Vehicle control (APII)	Eug (APII)
1	588	585-602	AAD**K***DT**C***FSTEGPNLVTR	715.3	2143.97	+3	4.44	2.29e4	1.60e4	2.24e4
2	UM	585-602	AADKDTCFSTEGPNLVTR	661.3	1981.92	+3	6.39	2.44e4	5.81e5	4.41e5
3	168	153-168	ENPTTFMGHYLHEVA**R***	688.6	2063.95	+3	2.10	2.06e4	2.20e4	7.88e3
4	UM	153-168	ENPTTFMGHYLHEVAR	634.6	1901.90	+3	5.16	2.16e4	3.10e5	9.20e4
5	549	549-558	**K***QTALAELVK	421.5	1262.71	+3	3.71	1.20e5	8.41e4	5.78e4
6	UM	549-558	KQTALAELVK	367.5	1100.66	+3	4.72	3.51e5	7.05e5	7.78e5
7	452	439-452	APQVSTPTLVEAA**R***	801.4	1601.83	+2	1.03	1.49e4	8.56e4	8.92e4
8	UM	439-452	APQVSTPTLVEAAR	720.4	1439.79	+2	4.46	1.5e4	9.64e4	1.50e5

^ UM, Unmodified; * Indicates modified amino acid side chain; PCS, Peptide Charge State; APII, Average Precursor Ion Intensity.

Table 2.5: Extent of AGE modification on peptides *in vitro*. Values in the table represent the average cumulative intensity ratio (CIR) of AGE modified peptides to their unmodified form

Site	K36	K88	K160	K184	K263	K438	K490	K548
BSA	0	0	0	0	0	0	0	0
glycated BSA	11.91 ± 02.56	14.52 ±02.32	47.09 ±10.78	16.36 ±04.40	12.70 ±01.61	184.28 ± 46.10	09.44 ±02.47	199.82 ±47.55
glycated BSA+AMG	04.91 ± 00.83	06.93 ±00.52	16.46 ±03.63	06.07 ±01.08	05.46 ±00.56	68.79 ± 14.04	03.47 ±00.43	30.61 ±03.44
glycated BSA+EUG	02.03 ± 00.11	03.43 ±00.69	05.90 ±00.96	04.08 ±00.23	06.19 ±01.32	24.66 ± 06.62	05.47 ±01.08	36.52 ±03.20

^ Bovine Serum Albumin, BSA; Aminoguanidine hydrochloride, AMG; Eugenol, EUG; lysine, K; Arginine, R.

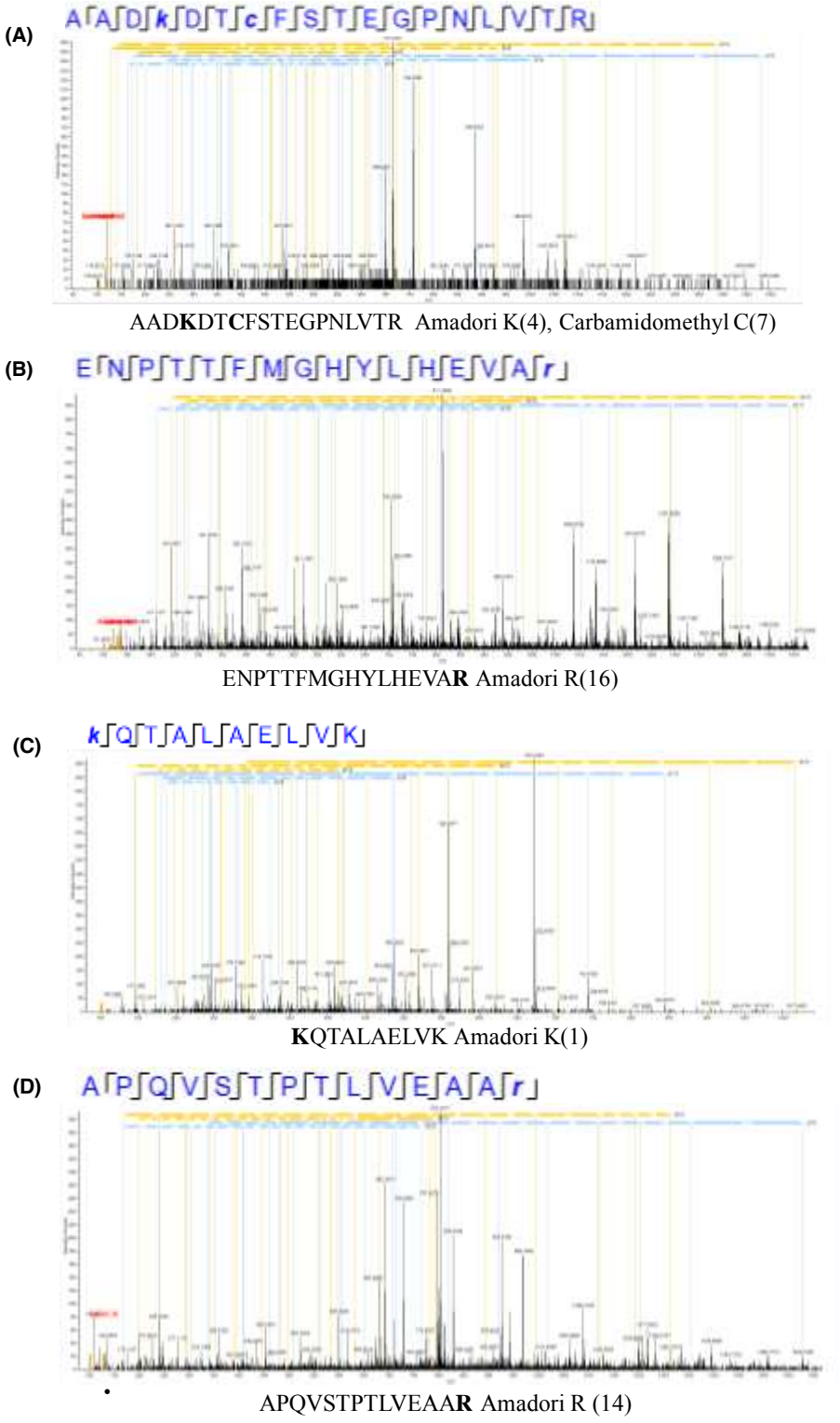
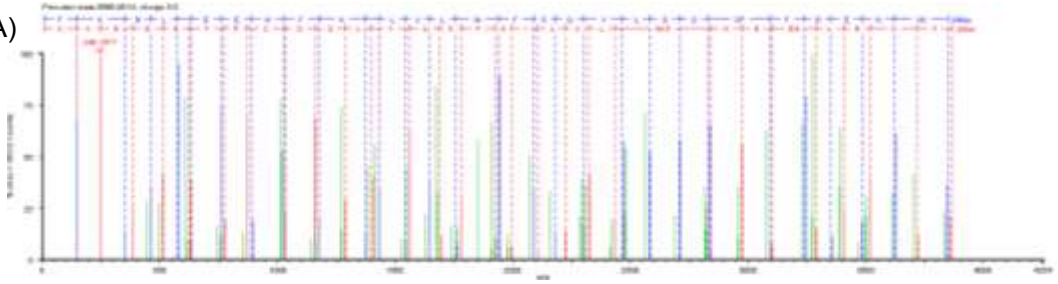


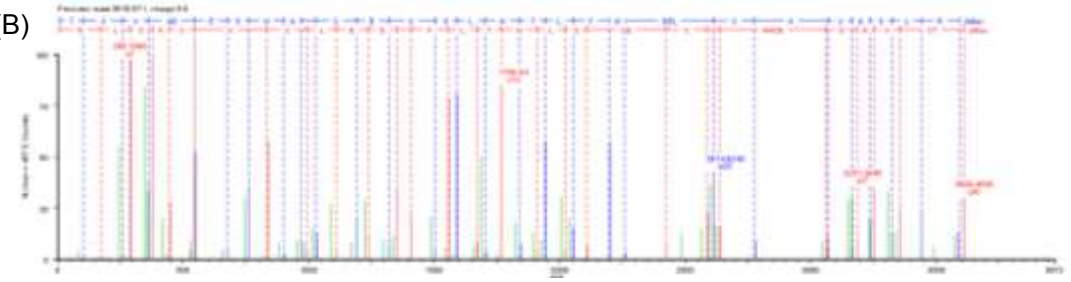
Figure 2.11: MS/MS spectra annotation of AGE modified peptides of MSA at (A) K588 (B) R168 (C) K549 (D) R452; K, Lysine; R, Arginine.

(A)



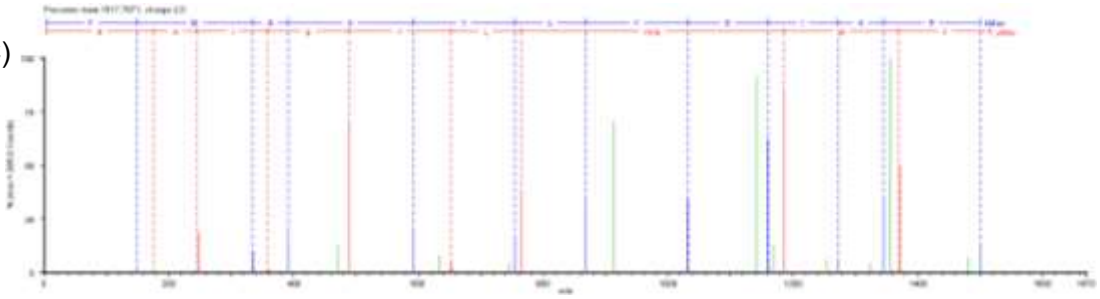
(R)FKDLGEEHFKGLVLIAFSQYLQQCPFDEHVK(L) CEL (2), CEL (10), Carbamidomethyl C (24)

(B)



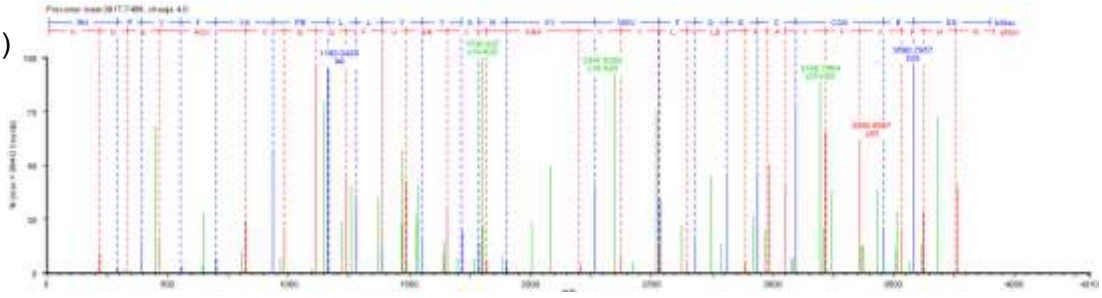
(K)TCVADESHAGCEKSLHTLFGDELCKVASLR(E) Carbamidomethyl C (2), Carbamidomethyl C (11), CML (13), Carbamidomethyl C (24), AMADORI (25)

(C)



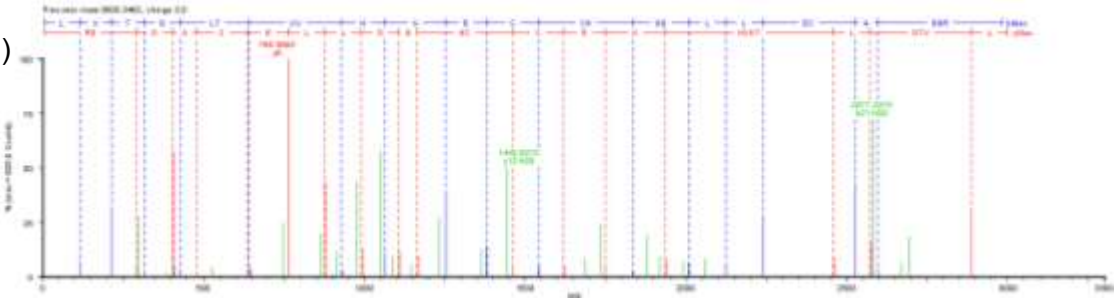
(K)FWGKYLYEIAR(R) CEL (4)

(D)



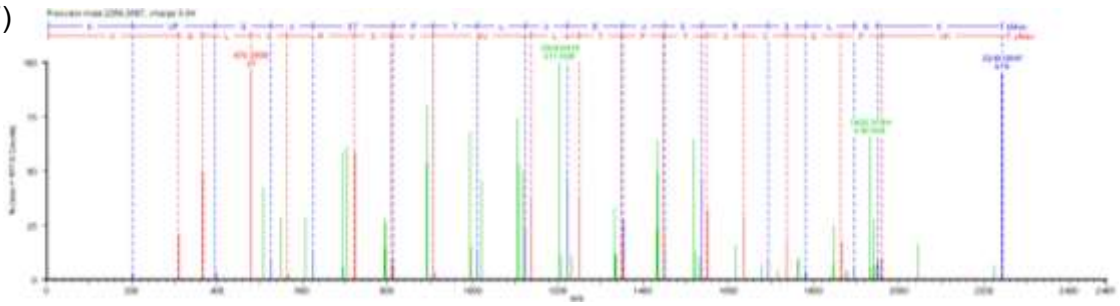
(R)RHPYFYAPELLYYANKYNGVFQECCQAEDK(G) CEL (16), Carbamidomethyl C (24), Carbamidomethyl C (25), CEL (30)

(E)



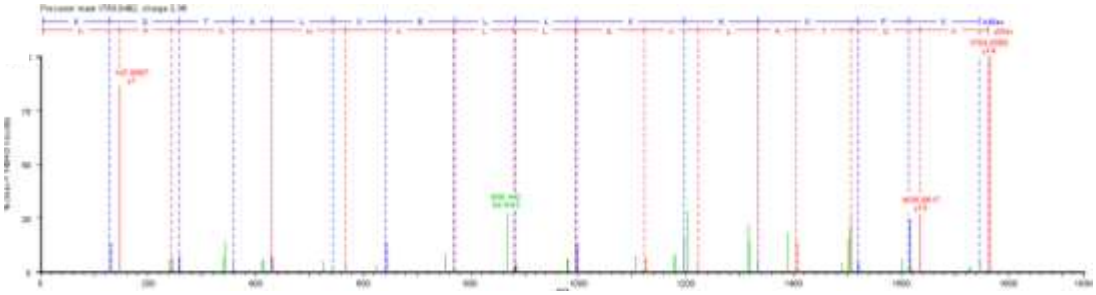
(K)LVTDLTKVHKECCHGDLLECADDR(A) CML (7), CML (10), Carbamidomethyl C (12), Carbamidomethyl C (13), Carbamidomethyl C (20)

(F)



(R)KVPQVSTPTLVEVSRSLGK(V) CEL (1), AMADORI (19)

(G)



(K)KQTALVELLKHKPK(A) CEL (10), CML (12)

Figure 2.12: MS/MS spectra annotation of AGE modified peptides of BSA at (A) K36 (B) K88 (C) K160 (D) K184 (E) K263 (F) K438 (G) K548

2.4 Conclusions

We identified eugenol, a phenylpropanoid, as a potent inhibitor of AGEs both by *in vitro* and *in vivo* studies.

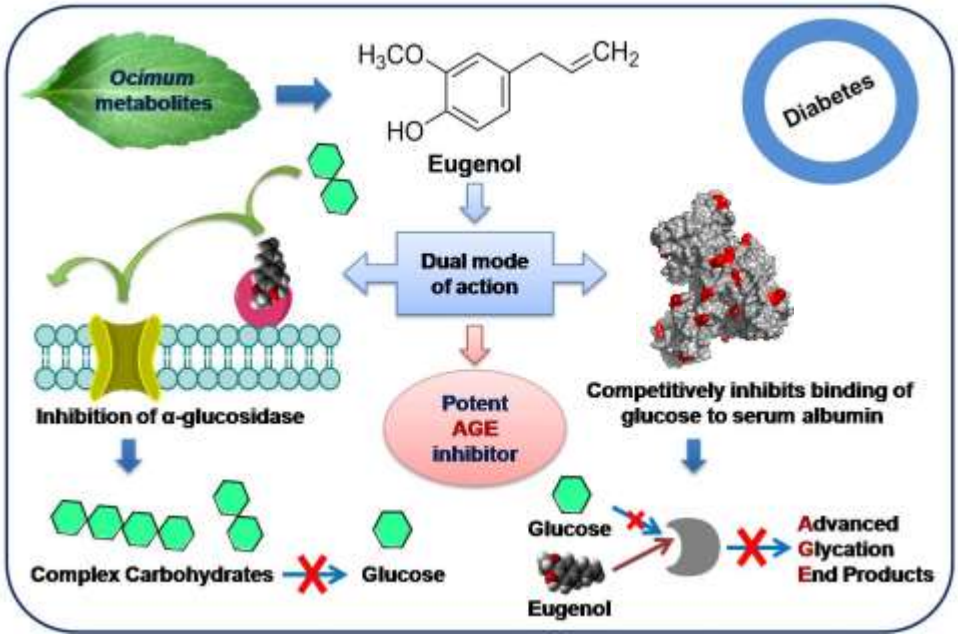


Figure 2.13: Schematic presentation of proposed potential dual role of eugenol in inhibiting AGEs. Leaf photograph courtesy, R.H.J. (co-author).

We propose that eugenol exerts potential dual mode of action in combating AGEs (**Fig. 2.13**). It might inhibit intestinal α-glucosidase and block the conversion of complex carbohydrates to glucose, resulting in lower blood glucose level and subsequent reduction in AGE formation. Also, eugenol competitively inhibits the binding of sugar to serum albumin by binding to amine group of surface exposed lysine residues *via* its reactive 4'-OH group. The above observations were strongly supported by biophysical, biochemical, proteomic and histopathological studies. Thus, here we report eugenol, isolated from *O. gratissimum*, as a natural, FDA-approved non-toxic potent inhibitor of AGEs that can be used in management of diabetes.

2.5 References

(1) F. A. Van De Laar, P.L. Lucassen, R.P. Akkermans, E. H. Van De Lisdonk, G. E. Rutten and C. Van Weel, α-Glucosidase inhibitors for patients with type 2 diabetes: results from a Cochrane systematic review and meta-analysis. 2005, *Diabetes Care*, **28**, 154.

(2) A. J. Scheen, Clinical efficacy of acarbose in diabetes mellitus: a critical review of controlled trials. *Diabetes Metab*., 1998, **24**, 311.

(3) N. Fukaya, K. Mochizuki, Y. Tanaka,T. Kumazawa, Z. Jiuxin, M. Fuchigami and T. Goda, The alpha-glucosidase inhibitor miglitol delays the development of diabetes and dysfunctional insulin secretion in pancreatic beta-cells in OLETF rats. *Eur*. *J*. *Pharmacol*., 2009, **624**, 51.

(4) K. Matsumoto, M. Yano, S. Miyake, Y. Ueki, Y. Yamaguchi, S. Akazawa and Y. Tominaga, Effects of voglibose on glycemic excursions, insulin secretion, and insulin sensitivity in non-insulin-treated NIDDM patients. *Diabetes Care*, 1998, **21**, 256.

(5) M. Brownlee, Advanced protein glycosylation in diabetes and aging. *Annu*. *Rev*. *Med*., 1995, **46**, 223.

(6) M. Brownlee, The pathobiology of diabetic complications. *Diabetes*, 2005, **54**, 1615.

(7) M. Luthra and D. Balasubramanian, Nonenzymic glycation alters protein structure and stability. *J*. *Biol*. *Chem*., 1993, **288**, 18119.

(8) M. J. Kulkarni, A. M. Korwar, S. Mary, H. S. Bhonsle and A. P. Giri, Glycated proteome: From reaction to intervention. *Proteomics Clin*. *Appl*., 2013, **7**, 155.

(9) J. A. Vinson and T. B. Howard, Inhibition of protein glycation and advanced glycation end products by ascorbic acid and other vitamins and nutrients. *J*. *Nutr*. *Biochem*., 1996, **7**, 659.

(10) P. Urios, A. M. Grigorova-Borsos and M. Sternberg, Aspirin inhibits the formation of pentosidine, a cross-linking advanced glycation end product, in collagen. *Diabetes Res*. *Clin*. *Pract*., 2007, **77**, 337.

(11) D. Ruggiero-Lopez, M. Lecomte, G. Moinet, G. Patereau, M. Lagarde and N. Wiernsperger, Reaction of metformin with dicarbonyl compounds. Possible implication in the inhibition of advanced glycation end product formation. *Biochem*. *Pharmacol*., 1999, **58**, 1765.

(12) S. K. Kesavan, S. Bhat, S. B. Golegaonkar, M.G. Jagadeeshaprasad, A. B. Deshmukh, H. S. Patil *et al*., Proteome wide reduction in AGE modification in streptozotocin induced diabetic mice by hydralazine mediated transglycation. *Sci*. *Rep*., 2013, **3,** 2941.

(13) S. Vasan, X. Zhang, X. Zhang, A. Kapurniotu, J. Bernhagen, S. Teichberg, An agent cleaving glucose derived protein crosslinks *in vitro* and *in vivo*. *Nature*, 1996, **382**, 275.

(14) K. Mizutani, K. Ikeda and Y. Yamori, Resveratrol inhibits AGEs- induced proliferation and collagen synthesis activity in vascular smooth muscle cells from stroke-prone spontaneously hypertensive rats. *Biochem*. *Biophys*. *Res*. *Commun*., 2000, **274**, 61.

(15) Y. Tang, Curcumin eliminates the effect of advanced glycation end-products (AGEs) on the divergent regulation of gene expression of receptors of AGEs by interrupting leptin signaling. *Lab*. *Invest*., 2014, **94**, 503.

(16) P. Raskin, J. McGill, M. F. Saad, J. M. Cappleman, W. Kaye, N. Khutoryansky and P. M. Hale, Combination therapy for Type 2 diabetes: repaglinide plus rosiglitazone. *Diabetic Med*., 2004, **21**, 329.

(17) K. Matsumoto, Y. Sera, Y. Abe, T. Tominaga, Y. Ueki and S. Miyake, Combination therapy of alpha-glucosidase inhibitor and a sulfonylurea compound prolongs the duration of good glycemic control. *Metabolism*, 2002, **51**, 1548.

(18) J. L. Chiasson and L. Naditch, The synergistic effect of miglitol plus metformin combination therapy in the treatment of type 2 diabetes. *Diabetes Care*, 2001, **24**, 989.

(19) P. Prakash and N. Gupta, Therapeutic uses of *Ocimum sanctum* Linn (Tulsi) with a note on eugenol and its pharmacological actions: a short review. *Indian J. Physiol. Pharmacol*., 2005, **49**, 125.

(20) F. L. Hakkim, G. Arivazhagan and R. Boopathy, Antioxidant property of selected *Ocimum* species and their secondary metabolite content. *J. Med. Plants Res*., 2008, **2**, 250.

(21) K. P. Bhargava and N. Singh, Anti-stress activity of *Ocimum sanctum* Linn. *Indian J. Med. Res*., 1981, **73**, 443.

(22) K. Karthikeyan, P. Gunasekaran, N. Ramamurthy and S. Govindasamy, Anticancer activity of *Ocimum sanctum*. *Pharmac. Biol*., 1999, **37**, 285.

(23) P. Uma Devi, A. Ganasoundari, B. Vrinda, K. K. Srinivasan and M. K. Unnikrishnan, Radiation protection by the *Ocimum* flavanoids orientin and vicenin: mechanisms of action. *Radiat. Res*., 2005, **154**, 455.

(24) A. Khan, A. Ahmad, N. Manzoor and L. A. Khan, Antifungal Activities of *Ocimum sanctum* essential oil and its lead molecules. *Nat*. *Prod*. *Commun*., 2010, **5**, 345.

(25) P. Singh, R. H. Jayaramaiah, P. Sarate, H. V. Thulasiram, M. J. Kulkarni and A. P. Giri, Insecticidal Potential of Defense Metabolites from *Ocimum kilimandscharicum* against *Helicoverpa armigera*. *PLoS ONE*, 2014, **9**, e104377.

(26) R. R. Chattopadhyay, Hypoglycemic effect of *Ocimum sanctum* leaf extract in normal and streptozotocin diabetic rats. *Indian J*. *Exp*. *Biol*., 1993, ***31***, 891.

(27) J. M. A. Hannan, L. Marenah, L. Ali, B. Rokeya, P. R. Flatt and Y. H. A. Abdel-Wahab, *Ocimum sanctum* leaf extracts stimulate insulin secretion from perfusd pancreas, isolated islets and clonal pancreatic beta-cells. *J*. *Endocrinol*., 2006, **189**, 127.

(28) A. M. Korwar, G. Vannuruswamy, M. G. Jagadeeshaprasad, R. H. Jayaramaiah, S. Bhat, B. Regin *et al*, Development of diagnostic fragment ion library for glycated peptides of human serum albumin: Targeted quantification in prediabetic, diabetic and microalbuminuria plasma by parallel reaction monitoring, SWATH and MSE. *Mol*. *Cell Proteomics*, 2015, **14**, 2150.

(29) K. A. Jones, P. D. Kim, B. B. Patel, S. G. Kelsen, A. Braverman, D. J. Swinton *et al*, Immunodepletion plasma proteomics by tripleTOF 5600 and Orbitrap elite/LTQ-Orbitrap Velos/Q exactive mass spectrometers. *J*. *Proteome Res*., 2013, **12**, 4351.

(30) W. Bors and C. Michel, Chemistry of the antioxidant activity of polyphenols. *Ann*. *N Y Acad*. *Sci*., 2002, **957**, 57.

(31) M. Zhang, W. Xu and Y. A. Deng, New Strategy for Early Diagnosis of Type 2 Diabetes Mellitus by standard- free, label- free LC- MS/MS quantification of glycated peptides. *Diabetes*, 2013, **62**, 3936.

(32) K. Yanagisawa, Z. Makita, K. Shiroshita, T. Ueda, T. Fusegawa, S. Kuwajima *et al*, Specific fluorescence assay for advanced glycation end products in blood and urine of diabetic patients. *Metabolism*, 1998, **47**, 1348.

(33) Y. M. Kolekar, G. Vannuruswamy, S. B. Bansode, B. Santhakumari, H. V. Thulasiram and M. J. Kulkarni, Investigation of antiglycation activity of isoprenaline. *RSC Adv*., 2015, **5**, 25051.

(34) B. Elya, K. Basah, A. Mun'im, W. Yuliastuti, A. Bangun and E. K. Septiana, Screening of α-glucosidase inhibitory activity from some plants of apocynaceae, clusiaceae, euphorbiaceae, and rubiaceae. *J*. *Biomed*. *Biotechnol*., 2011, **2012**, 1.

(35) H. S. Bhonsle, A. M. Korwar, S. S. Kote, S. B. Golegaonkar, A. D. Chougale, M. L. Shaik et al, Low plasma albumin levels are associated with increased plasma protein glycation and HbA1c in diabetes. *J*. *Proteome Res*., 2012, **11**, 1391.

(36) F. Y. Cheng, K. Blackburn, Y. M. Lin, M. B. Goshe and J. D. Williamson, Absolute protein quantification by LC/MS (E) for global analysis of salicylic acid-induced plant protein secretion responses. *J*. *Proteome Res*., 2009, **8**, 82.

(37) H. S. Bhonsle, A. M. Korwar, K. K. Suresh, S. D. Bhosale, S. B. Bansode and M. J. Kulkarni, "Zoom-ln"-A targeted database search for identification of glycation modifications analyzed by untargeted tandem mass spectrometry. *Eur*. *J*. *Mass Spectrom*., 2012, **18**, 475.

CHAPTER 3

Insecticidal potential of defense metabolites from *O. kilimandscharicum* against *Helicoverpa armigera*

3.1 Introduction

Members of genus *Ocimum* have a unique blend of secondary metabolites which imparts them great medicinal properties as well as a peculiar flavor and taste.[1] Several members of *Ocimum* are known to possess antioxidant,[2] antistress,[3] anticancer,[4] radiation protection,[5] antifungal,[6] antidiabetic,[7] insecticidal,[8] properties and other important bioactivities. *Ocimum* species abound in a diversity of secondary metabolites including terpenes, phenylpropanoids, phenolics etc., some of which may be involved in defensive roles. However, defense metabolites from these species have not been characterized, although, the insecticidal activity of the plant leaves against storage pests is reported.[8] Different species of *Ocimum* greatly differ in the composition of their secondary metabolites and may offer variable levels of resistance to specific insect pests. *Ocimum kilimandscharicum,* also known as camphor basil, is a relatively unexplored tropical plant species widely distributed in East Africa, India and Thailand. The species possesses a rich reservoir of secondary metabolites such as camphor, eucalyptol, limonene, geramacrene D and β-caryophyllene. These metabolites are reported to have insecticidal properties.[9, 10, 11] Thus, *O. kilimandscharicum* is an attractive system for studying potential insecticidal molecules.

Usually, insect infestation results in the reprogramming of both primary and secondary metabolism in plants. The roles of secondary metabolites in plant defense have been extensively studied and well documented.[12] However, the changes in primary metabolism that occur during infestation are equally important. Primary metabolites provide building blocks and energy molecules, all of which are required for defense pathways to function. Primary metabolites such as carbohydrates, proteins and lipids are also affected

significantly during insect infestation. For example, the deposition of a plant polysaccharide callose is crucial for induced plant defense in rice and *Arabidopsis*.[13, 14] Similarly, large amount of callose deposition is evident in *O. basilicum* after phloem injury.[15] Plant proteins such as chitinases, enzyme inhibitors, and lectins have been well characterized and are known to aid in defense by repelling insects, inhibiting their feeding, or impairing their digestive or neural systems.[16, 17] Lipids or fatty acids (FAs) have direct and indirect roles in plant defense and function to provide biosynthetic precursors for cuticular components and jasmonic acid.[18] The fuel for producing secondary metabolites is derived from primary metabolites in the form of isopentenyl pyrophosphate (IPP), adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADH), etc.

Plant secondary metabolites are involved in several defense-related and other functions such as (i) prevention of herbivore and pathogen attack, (ii) attraction of pollinators and symbionts,[19] and (iii) plant-plant communication.[20] The diverse pool of secondary metabolites in genus *Ocimum* probably offers great resistance to biotic stresses. Unlike synthetic insecticides, plant-based bio-insecticides provide an organic, low-risk, environmentally friendly approach toward the management of insects in agriculture. Moreover, most of the terpenes and phenylpropanoids are ingredients of several medicinal formulations, and therefore their toxicity for mammals could be minimal.[21] The basil plant contains many useful secondary metabolites, which may prove to be important for the formulation of cost-effective bio-insecticides.

Helicoverpa armigera (Lepidoptera: Noctuidae) is a devastating insect pest that feeds on several economically important crop plants such as cotton, tomato, maize, chickpea, pigeon pea, etc.[22, 23]. *O. kilimandscharicum* is a non-host plant for *H. armigera*. Our

earlier studies revealed the developmental and digestive flexibility in *H. armigera* fed on various diets.[24, 25] *H. armigera* regulates its enzyme levels to obtain better nourishment from its diet and avoid toxicity due to nutritional imbalance. Previous studies showed that ethyl acetate extracts of *O. canum* flowers and acetone extracts of *O. tenuiflorum* (previously *O. sanctum*) possess antifeedent and larvicidal characteristics, enabling them to act against *H. armigera*.[26] However, our knowledge of the interactions between *O. kilimandscharicum* and *H. armigera* is limited. The current study documents the changes in levels of primary and secondary metabolites in *O. kilimandscharicum* after *H. armigera* infestation. Furthermore, we have analyzed the responses of *H. armigera* larvae after feeding on *O. kilimandscharicum* metabolites.

3.2 Materials and methods

3.2.1 Insect culture

H. armigera larvae were maintained on chickpea flour-based artificial diet under laboratory conditions (28 ± 2 ºC and 75% relative humidity). The composition of the artificial diet was as follows: (A) 50 g chickpea flour, 5 g wheat germ, 12 g yeast extract, 3.5 g casein, 0.5 g sorbic acid, and 1 g methyl paraben in 150 mL distilled water, (B) 0.35 g choline chloride, 0.02 streptomycin sulphate, 2 g ascorbic acid, 0.15 g cholesterol, becadexamin multivitamin multi–mineral capsule (GlaxoSmithKline Pharmaceuticals Limited), 200 mg vitamin E, 1mL formaldehyde, 0.3 g bavistin, 30 mL distilled water; and (C) 6.5 g agar in 180 mL distilled water. 'A' and 'B' were mixed together and molten agar 'C' was added to that mixture .[27]

3.2.2 Plant maintenance

O. *kilimandscharicum* and tomato plants (*var*. Abhinav) were grown in the greenhouse. The conditions in the greenhouse were as follows: temperature, 28 to 30 °C; humidity, 35 to 40%; light conditions, 16 h light, 8 h dark.

3.2.3 Feeding-choice assay

One gram each of *O*. *kilimandscharicum* and tomato leaves were arranged in plastic Petri plates (15 cm diameter) opposite each other on moist filter paper. Second-instar *H*. *armigera* larvae were randomly transferred to the Petri plates (6 larvae/plate; n=5). The amount of tissue remaining was noted each day at the same time for four days. The insects' preference for a particular tissue type was proportional to the amount of tissue consumed. Greater consumption indicated greater preference in the choice assay (**Fig. 3.1A**).

3.2.4 Growth and mortality data

H. *armigera* second- instar larvae were allowed to feed on artificial diet, tomato and *O*. *kilimandscharicum* plants individually. Five larvae per plant and 10 plants each of *O*. *kilimandscharicum* and tomato were infested with the larvae. Plants were covered with polythene bags, which were pierced with holes to allow respiration and maintained under the following greenhouse conditions: temperature, 28 to 30 °C; humidity, 35 to 40%; light conditions, 16 h light, 8 h dark. For feeding on artificial diet, 50 larvae were maintained in vials (1 larvae/vial) containing equal amount of artificial diet. Percentage larval mortality and average increase in body mass were recorded every alternate day for 8 days.

3.2.5 Biochemical and metabolite study

Second-instar *H*. *armigera* larvae were allowed to feed on *O*. *kilimandscharicum* plants (6 larvae/plant), 12 plants, for 6 days. Controls plants with no insects were also maintained.

Control and test plants were covered with polythene bags, which were pierced with holes to allow respiration and maintained under the following greenhouse conditions: temperature, 28 to 30 °C; humidity, 35- 40%; light conditions, 16 h light, 8 h dark. Tissues were collected from the plants (local and systemic leaves, stem and roots) and larvae (whole larvae) after 12h, 24 h, day 3 and day 6 and stored at -80^{o}C till further use. The plant extracts for gas chromatography- mass spectrometry (GC-MS) were prepared using freshly harvested tissue that is described in further section.

3.2.6 Estimation of carbohydrates, proteins, and lipids from plant tissues

The plant tissues (local and systemic leaves, stem and roots) collected at different time intervals (12h, 24 h, day 3 and day 6) were analyzed for carbohydrates, proteins, and lipids. Total protein content was determined by the Kjeldahl method.[28] The phenol sulfuric acid method[29] was used to estimate total carbohydrate content using glucose as a standard. Total lipid content was measured using the sulpho–phospho–vanillin method.[30] All the assays were performed in duplicate and repeated thrice.

3.2.7 *H. armigera* enzyme activity assays

Whole larval tissues (100 mg) were homogenized in 300 μL of 0.02 M sodium-phosphate buffer (pH 6.8) containing 10 mM NaCl for 2 h at 4 ºC. The homogenate was then centrifuged at 13,000× g for 30 min at 4 ºC. The supernatant was collected, stored at -20 ºC and used as crude enzyme source.

Total protease activity from *H. armigera* larvae fed on *O. kilimandscharicum* plants was assayed using azocasein as substrate.[31] Trypsin activity assays were performed as

described by Tamhane *et al*.[32] One protease unit was defined as the amount of enzyme in the assay that causes an increase in absorbance by one optical density under the given assay conditions. Amylase activity from the gut of *H. armigera* larvae was analyzed by the dinitrosalycylic acid method,[33] as described by Kotkar *et al*.[24] One amylase unit was defined as the amount of enzyme required to release 1 µM maltose/minute at 37 °C under the given assay conditions. Lipase activity from gut homogenates was estimated using the p164 nitrophenyl palmitate assay.[34] One unit of lipase activity was defined as the amount of enzyme that causes an increase of one optical density under the given assay conditions. All the assays were performed in duplicate and repeated thrice.

3.2.8 Extraction and analysis of metabolites

Plant tissue (1 g) (local and systemic leaves, stem and roots) was mixed in 10 mL dichloromethane (DCM) and kept for 18 to 24 h at 28 ºC. The extract was filtered and incubated for 2 h at -20 °C to allow lipid precipitation. DCM extract was filtered again, concentrated under vacuum on a rotary evaporator and subjected to GC and GC-MS analysis.

GC analyses were carried out on an Agilent 7890A instrument equipped with a hydrogen flame ionization detector and an HP-5 capillary column (30 m X 0.32 mm X 0.25 µm, J and W Scientific). Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. The column temperature was raised from 70 °C to 110 °C at 2 °C min^{-1}, then raised to 180 °C at 3 °Cmin^{-1} and finally to a temperature of 220 °C with a 10 °C min^{-1} rise; here it was held for 2 min. Injector and detector temperatures were 230 °C and 250 °C, respectively. GC-MS was performed on a HP 5975C mass selective detector interfaced with a HP 7890A gas chromatograph. GC-MS analyses were performed under similar conditions

using an HP-5 MS capillary column (30 m X 0.32 mm X 0.25 µm, J and W Scientific) with helium as the carrier gas. Compounds were identified by comparing the retention time and mass fragmentation pattern of the standards of major constituents and also by comparing acquired mass spectra and retention indices with NIST/NBS and the Wiley mass spectral library (software version 2.0, Dec. 2005).

3.2.9 *H. armigera* larvae fed on specific compounds

O. kilimandscharicum leaf extract and candidate compounds (camphor, limonene, **β**-caryophyllene, procured from Sigma, St. Louis, MO, USA) were dissolved in 30% dimethyl sulfoxide (DMSO) and incorporated in artificial diet at final concentration of 10, 100 and 1000 ppm. Diet prepared with equivalent amount of 30% DMSO was used as control. Larvae (20 per diet) were maintained individually in vials. . Percentage larval mortality and average larval body mass were recorded every alternate day up to pupation. Pupal deformities were also recorded.

3.2.10 Statistical analysis

Significant differences between diet treatments were determined using two way ANOVA followed by Tukey's multiple comparison test for figure 3.1B, 3.2A, 3.2B, 3.2C, 3.7A, 3.7B. One way ANOVA followed by Tukey's multiple comparison test was performed to analyze the statistical differences between data in figure 3.4. Unpaired T test was used to compare data from two treatments i. e. tomato and *O. kilimandscharicum* in figure 3.1C and to compare metabolic changes in local and systemic tissue in supplementary figure 3.3A, 3.3B and 3.3C. One way ANOVA and Unpaired t-test data was considered to be significantly different within the treatments if the F-value obtained was higher than the critical F-value at $p<0.001$, $p<0.01$, $p<0.05$. Small letters are used to

indicate statistically different groups of treatments.. NS represents non-significant difference within the treatments and/or in the respective day.

3.3 Results and discussion

3.3.1 *O. kilimandscharicum* defense compounds deter larvae from feeding, adversely affecting their growth and development

Feeding- choice assays showed that *H. armigera* larvae consumed significantly less *O. kilimandscharicum* leaf tissue than tomato (**Fig. 3.1A**). By the end of the fourth day, larvae had consumed all the tomato leaves and showed lower preference for *O. kilimandscharicum* leaves. Such resistance to feeding on *O. kilimandscharicum* leaves by *H. armigera* larvae clearly indicates the presence of defense compounds, which strongly deter larval feeding. It was also observed that larvae fed on *O. kilimandscharicum* plants showed significant growth impairment as well as an increase in mortality.

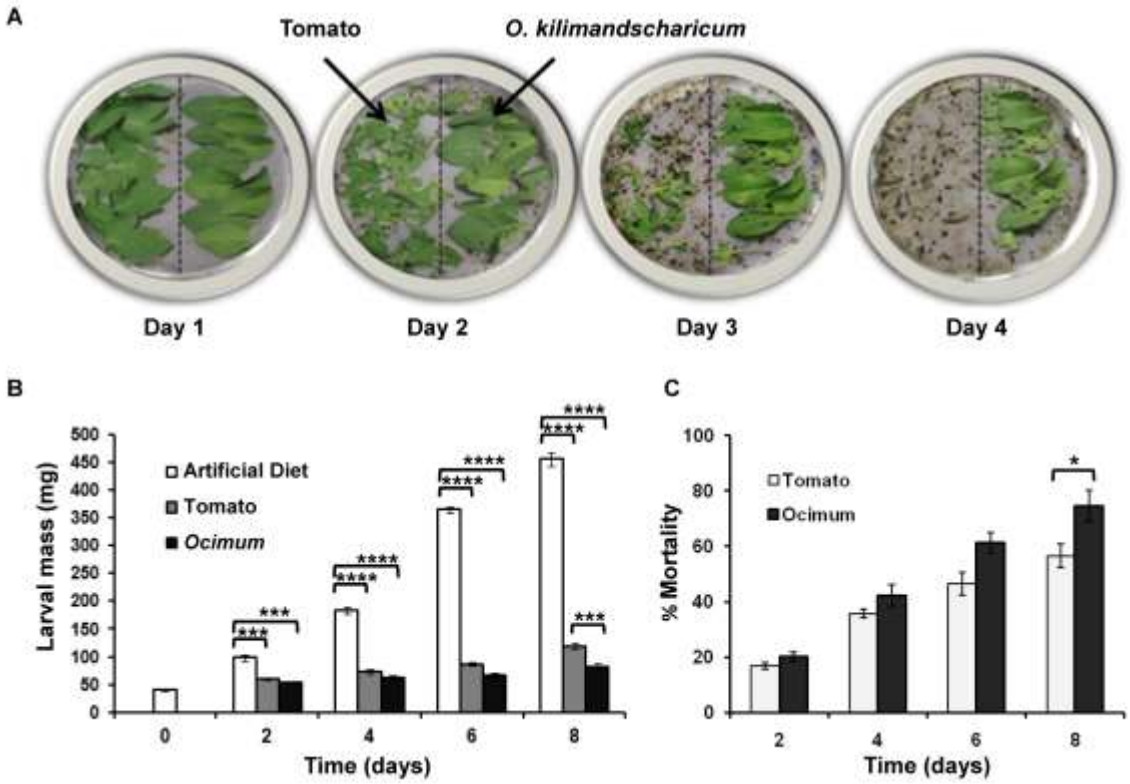


Figure 3.1. Performance of *H*. *armigera* feeding on tomato and *O*. *kilimandscharicum* leaves. **A.** Feeding assay showing feeding preference of *H*. *armigera* second-instar larvae for tomato over *O*. *kilimandscharicum* **B.** average larval mass and **C.** percentage mortality of larvae fed on artificial diet, tomato and *O*. *kilimandscharicum*. Two way ANOVA followed by Tukey's multiple comparisons test (Figure 3.1B) and Unpaired T test (Figure 3.1C) suggested significant difference between the data at $p<0.0001$(indicated as ****), $p<0.001$(indicated as '***'), $p<0.05$(indicated as '*').

These results are statistically supported by two way ANOVA followed by Tukey's multiple comparison test and Unpaired T test respectively **(Fig. 3.1B and 3.1C)**. Results of the two way ANOVA show a statistically significant interaction between treatments and larval mass at various days for a total variance of 28.27% at $p<0.0001$ **(Table 3.1 and Fig. 3.1B)**.

Table 3.1. Two way analysis of variance for performance of *H*. *armigera* on various days feeding on tomato and *O*. *kilimandscharicum* leaves.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	195388	8	24424	F (8, 24) = 113.6	P < 0.0001
Time	201904	4	50476	F (4, 24) = 234.8	P < 0.0001
Diets (AD, TO, OC)	290117	2	145058	F (2, 6) = 2400	P < 0.0001
Subjects (matching)	362.7	6	60.45	F (6, 24) = 0.2812	P = 0.9402
Residual	5160	24	215		
Total	692931	44			

DF = Degrees of freedom, SS = Sum of squares, MS = Mean square, n = numerator, d = denominator, p = probability of significance, $\alpha = 0.05$

The average body mass of larvae fed on *O*. *kilimandscharicum* was consistently lower on day 2, 4 and 6 than larvae fed on tomato but on day 8 it was significantly lower (at $p<0.001$ '***'). As expected, control (artificial) diet fed larvae showed significantly higher body mass (at $p<0.0001$ '****') as compared to *O*. *kilimandscharicum* and tomato fed larvae on all days **(Fig. 3.1B).** No significant difference was observed in the mortality of *O*. *kilimandscharicum* and tomato fed larvae on day 2, 4 and 6. Although tomato is a host plant for *H*. *armigera*, it is known that larvae prefer to feed on the tomato fruit. Mortality of larvae fed on tomato leaves may be attributed to the presence of defense proteinaceous molecules like proteinase inhibitors or secondary metabolites. Results clearly indicate that the insects were unable to counteract the action of potential defense metabolites (**Fig. 3.1C**). Overall growth in *H*. *armigera* larvae fed on *O*. *kilimandscharicum* was slowed, possibly owing to the presence of defense metabolites.

3.3.2 Changes in protein, carbohydrate and lipid content in *O. kilimandscharicum* upon insect attack

Different parts of *O*. *kilimandscharicum* plant were analyzed after larval infestation over a period of six days to estimate the changes in total carbohydrate, protein and lipid content. Two way ANOVA followed by Tukey's multiple comparisons test showed significant interaction for the total varience of 13.32%, 27.23% and 31.11% at $p<0.0001$ between the up and down regulation of primary metabolites (i.e. protein, carbohydrate and lipase respectively) in different tissue (leaf, stem and root respectively) and the days of infestation (**Table 3.2 and Fig. 3.2**). Protein content in *O*. *kilimandscharicum* leaves increased significantly during 12 and 24 h following insect infestation (**Fig. 3.2A**). Moreover, protein content increased in systemic leaves compared to in local leaves (**Fig. 3.2A**). However, the protein content decreased progressively as time increased. A similar trend was observed in stem and root tissues. The early increase in protein content might be a part of induced plant defense. A similar trend in lipid content was observed in all tissues. The carbohydrate content in *O*. *kilimandscharicum* plants increased two-fold in the first 24 h following infestation as compared to the carbohydrate content in uninfested plants (**Fig. 3.2B**), and subsequently remained the same pattern. The sudden increase in carbohydrate content confirms previous reports, which state that sugars play an important role in induced- defense by acting as important signaling molecules.[35, 36]

Table 3.2. Two way analysis of variance for macromolecular content of *O. kilimandscharicum* leaves, stem and root on various days of *H. armigera* infestation.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Protein					
Interaction	485.4	8	60.68	F (8, 12) = 106.0	P < 0.0001
Time	1820	4	455.1	F (4, 12) = 795.2	P < 0.0001
Tissue (Leaf, stem, root)	1325	2	662.4	F (2, 3) = 311.0	P = 0.0003
Subjects (matching)	6.39	3	2.13	F (3, 12) = 3.722	P = 0.0422
Residual	6.867	12	0.5723		
Total	3644	29			
Carbohydrate					
Interaction	75461	8	9433	F (8, 36) = 199.3	P < 0.0001
Time	86421	4	21605	F (4, 36) = 456.6	P < 0.0001
Tissue (Leaf, stem, root)	113466	2	56733	F (2, 9) = 4273	P < 0.0001
Subjects (matching)	119.5	9	13.28	F (9, 36) = 0.2806	P = 0.9759
Residual	1704	36	47.32		
Total	277170	59			
Lipid					
Interaction	446.9	8	55.86	F (8, 36) = 40.37	P < 0.0001
Time	794.9	4	198.7	F (4, 36) = 143.6	P < 0.0001
Tissue (Leaf, stem, root)	137.2	2	68.58	F (2, 9) = 78.34	P < 0.0001
Subjects (matching)	7.878	9	0.8753	F (9, 36) = 0.6325	P = 0.7617
Residual	49.82	36	1.384		
Total	1437	59			

Degrees of freedom, SS = Sum of squares, MS = Mean square, n = numerator, d = denominator, p = probability of significance, $\alpha = 0.05$

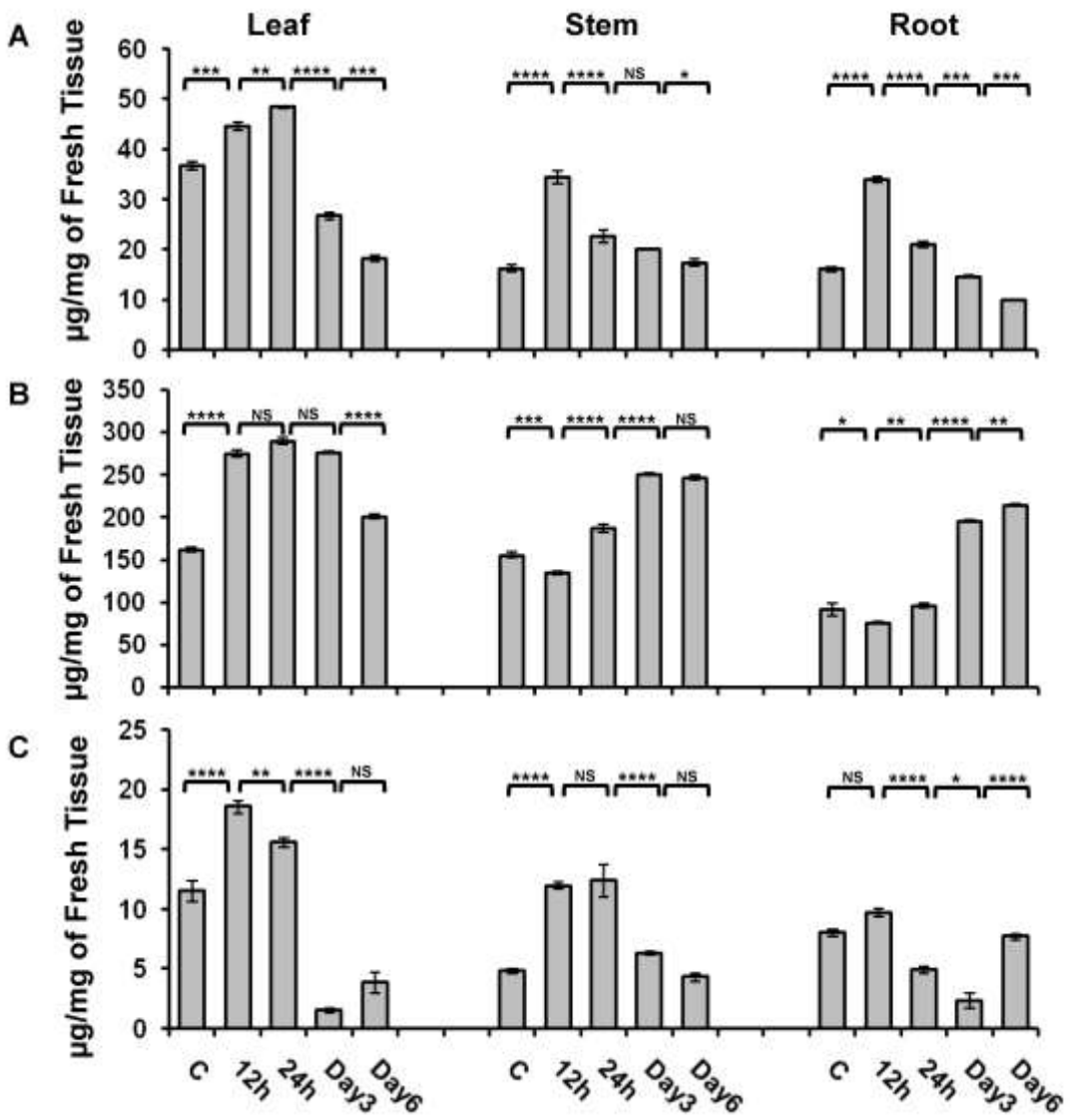


Figure 3.2. Protein, carbohydrate and lipid content of *O*. *kilimandscharicum* leaves *following H*. *armigera* feeding. Changes in the levels of **A.** total proteins **B.** total carbohydrates **C.** total lipids in leaves, stems and roots of tomato and *O*. *kilimandscharicum* at 12h, 24h, day 3, and day 6 post-infestation by *H*. *armigera* second-instar larvae. Two way ANOVA followed by Tukey's multiple comparisons test suggested significant difference between the data at. $p<0.001$(indicated as '***'), $p<0.01$ (indicated as '**'), $p<0.05$(indicated as '*'). One color represents data from respective day. NS

represents group with non-significant difference in that particular day. Error bars represent Mean ± SD of 4 independent sets of tissue samples.

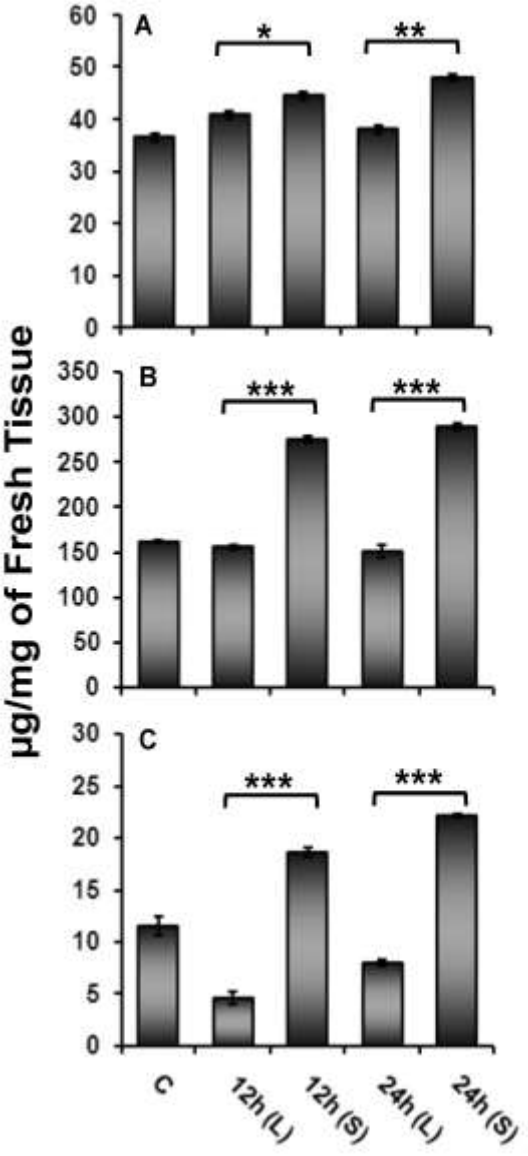


Figure 3.3: Changes in protein, carbohydrate and lipid content in local versus systemic leaves of *O. kilimandscharicum* following *H. armigera* feeding. Changes in levels of **A.** total proteins **B.** total carbohydrates **C.** total lipids in local (L) versus systemic (S) leaf tissue in *O. kilimandscharicum* at 12 and 24h post-infestation by *H. armigera* second-instar larvae. Unpaired t test suggested significant difference between the local and systemic tissue analysis data at. $p<0.001$(indicated as '***'), $p<0.05$(indicated as '*'). Error bars represent Mean ± SD of 4 independent sets of tissue samples.

However, with a decrease in aerial tissues, the carbohydrates might relocate to the roots; this could explain the significant increase in the carbohydrate content of the root tissue on

the sixth day (**Fig. 3.2B**). Schwachtje et al. [37] reported that *Nicotiana attenuata* plants divert their resources to less vulnerable tissues within the plant such as roots as a part of their defense strategy. We observed significantly more carbohydrates accumulation in systemic leaf tissue than in local tissue (**Fig. 3.3B**). This could be the plant's way to protect its non-damaged plant parts by mobilizing resources and defense compounds. It was previously demonstrated that after a plant is injured or wounded by herbivore attack, local tissues signal systemic tissues to increase the plant's defense activity.[38, 39] From these observations, it can be hypothesized that *O. kilimandscharicum* adopts a carbohydrate-mediated defense strategy to combat insect infestation, a strategy that exists at the level of primary metabolism. The lipid content of *O. kilimandscharicum* leaves increased significantly during 12 and 24 h following infestation and then gradually declined (**Fig. 3.2C**). Furthermore, insect infestation was found to be responsible for the accumulation more lipids in systemic leaves as compared to local leaves (**Fig. 3.3C**). According to earlier reports, both 16- and 18-carbon fatty acids are known to modulate basal, effector-triggered and systemic immunity in plants. A sudden increase of lipid content in leaves of *O. kilimandscharicum* indicated the onset of secondary metabolite formation as a part of plant defense. Although basil is rich in secondary metabolites, no such details are available for the fatty-acid derived plant defense in *O. kilimandscharicum*.

3.3.3 *H. armigera* regulates its digestive enzymes after feeding on *O. kilimandscharicum*

One way ANOVA followed by Tukey's multiple comparisons test suggested significant difference between the expression of protease, amylase and lipase in insect gut on 12h, 24h, day 3 and 6. The total protease activity of larvae fed on *O. kilimandscharicum* was

measured at various time intervals. Initially, protease activity was found to decrease beginning at 12 h after feeding and continuing to the third day of feeding (**Fig. 3.4A**); however, protease activity increased dramatically on the sixth day of feeding. The initial decrease in protease activity can be attributed to the increased expression of inhibitory proteins in *O*. *kilimandscharicum*. The digestive track of insect is enriched with cocktail of proteases to utilize plant proteins and obtain amino acids for nutrition from plants. Moreover, plant defensive proteins also play a significant role in modulating the expression of insect proteases. Therefore, the higher protease activity observed on the sixth day after feeding might be indicative of the attempts of *H*. *armigera* larvae to obtain more nutrition from the ingested plant food. The plants produced antifeedent and antinutritive compounds that might be responsible for significant differences in amylase, protease and lipase activities in *H*. *armigera* larvae fed on *O*. *kilimandscharicum*. Amylase activity was examined during all the feeding assays (**Fig. 3.4B**). The amylase activity found in larvae correlated with the carbohydrate content of *O*. *kilimandscharicum*, which remained significantly high. Possibly, *H*. *armigera* maintains its amylase activity to better utilize the higher amount of carbohydrates from *O*. *kilimandscharicum*.[24, 25]

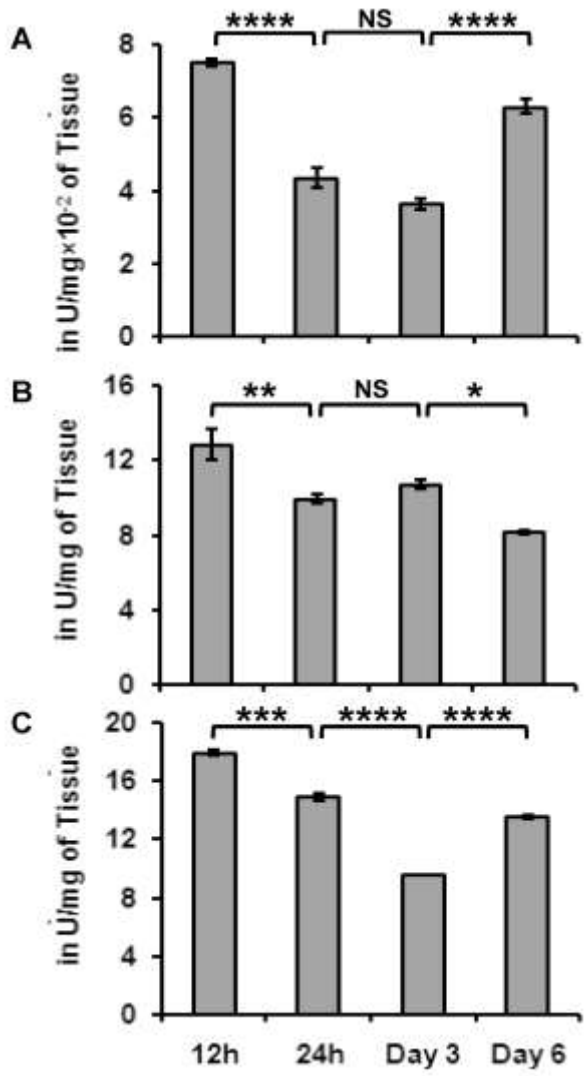


Figure 3.4. Digestive enzymes of *H*. *armigera* larvae fed on *O*. *kilimandscharicum* leaves. Changes in the levels of **A.** protease **B.** amylase **C.** lipase activity of *H*. *armigera* second-instar larvae fed on *O*. *kilimandscharicum* plants at 12h, 24h, day 3 and day 6. One way ANOVA followed by Tukey's multiple comparisons test suggested significant difference between the data at. $p<0.001$(indicated as '***'), $p<0.01$(indicated as '**'), $p<0.05$(indicated as '*'). Results are an average of three independent experiments conducted in duplicate. Error bars represent Mean ± SD.

A gradual decline in lipase activity was observed in larvae fed on *O*. *kilimandscharicum* from 12 h after feeding to the third day (**Fig.3.4C**). Lipase activity measured in *H*. *armigera* larvae fed on *O*. *kilimandscharicum* was correlated with lipid content in the plant. The current study is consistent with our previous findings which revealed the differential expression of proteases, amylases and lipases in *H*. *armigera* in response to different diets.[25]

3.3.4 Compounds associated with secondary metabolism are central to *O. kilimandscharicum* defense

Consistently increasing accumulations of monoterpenes, sesquiterpenes, phenylpropanoids and hydrocarbons were evident in the leaves of *O*. *kilimandscharicum* from 12 h to day 3 after insect infestation (**Fig. 3.5**). The maximum defense response was elicited on the third day, when levels of all the metabolites were higher. However, the accumulation of metabolites decreased progressively towards day 6. When a few leaves are left, plants mobilize their resources in the direction of their stems and roots. However, metabolite accumulation in systemic leaf tissues was higher than in local tissues in 12 and 24 h after insect attack (**Fig. 3.5**).

Changes in the levels of metabolites observed in the stem follow a pattern similar to that in leaves (**Fig. 3.6**). The defense response was high on the third day, so the plant mobilized all its reserves in the roots, and hence fewer metabolites were detected in the stem on the sixth day. Generally, the stem contains fewer metabolites than leaves. Our results suggest that the stem seems to play the role of translocator. The stem transports metabolites from roots to leaves during the initial defense response and channels

metabolite reserves to the roots during later stages of infestation when the aerial tissues are consumed.

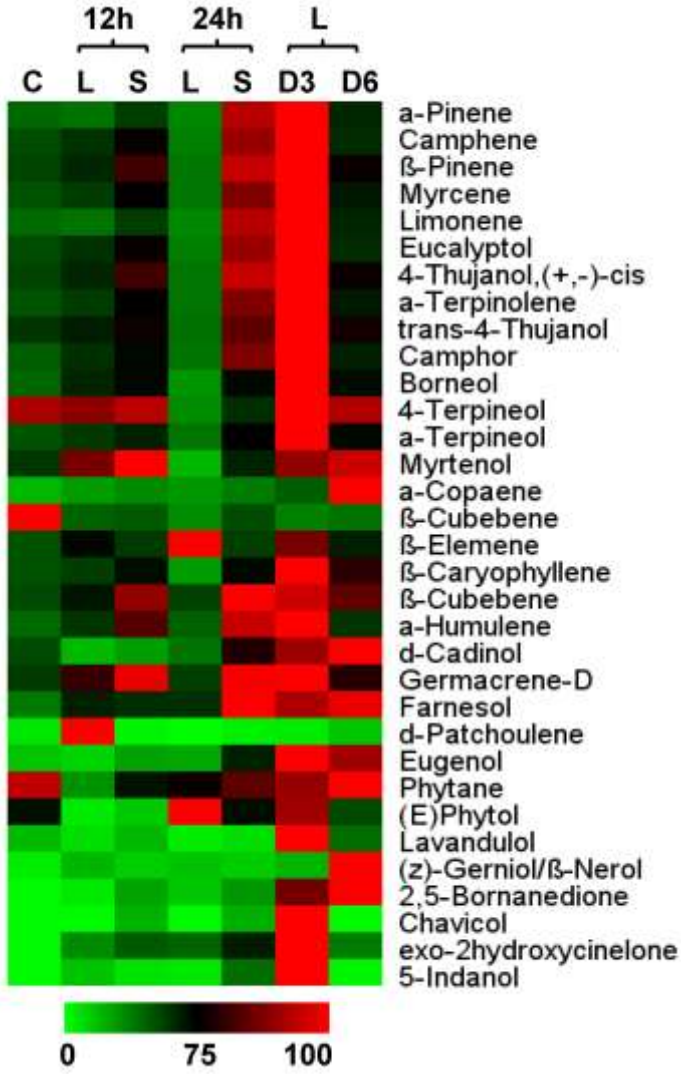


Figure 3.5. Metabolic changes in leaves of *O*. *kilimandscharicum* following *H*. *armigera* infestation. Heat map representing relative expression of a sub-set of volatiles elicited in leaf tissue during *O*. *kilimandscharicum-H*. *armigera* interaction; comparison between metabolite profiles of local (L) and systemic (S) leaf tissue in *O*. *kilimandscharicum*, 12h

and 24h after feeding by *H. armigera*, and also on days 3 (D3) and 6 (D6), compared to control (C) plants

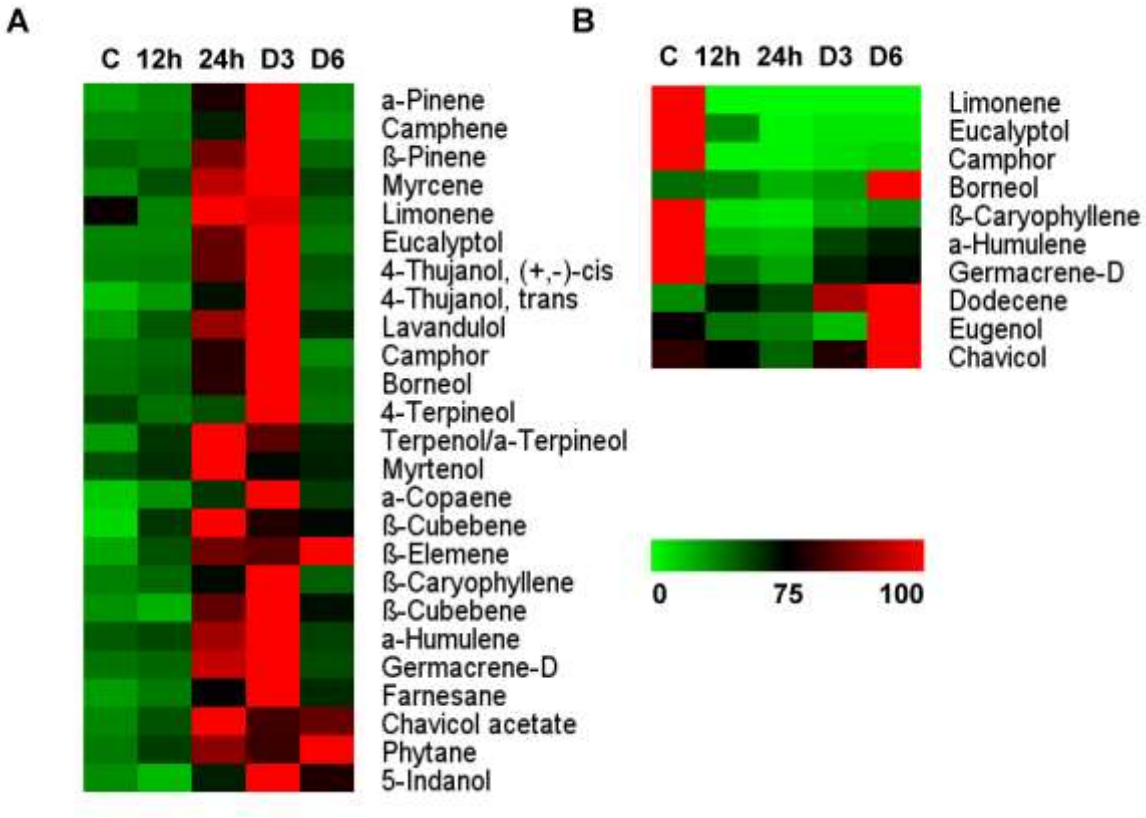


Figure 3.6. Metabolic changes in stems and roots of *O. kilimandscharicum* following *H. armigera* infestation. Heat map representing relative expression of a sub-set of volatiles elicited in **A.** stems and **B.** roots during *O. kilimandscharicum- H. armigera* interaction at 12h, 24h, and on days 3 (D3) and 6 (D6) as compared to control (C) plants

Few compounds were detected in roots, such as camphor, eucalyptol, limonene, eugenol, geramacrene D and humulene. Levels of these metabolites decreased initially (12 h post-infestation) and were minimal at 24 h (**Fig. 3.6B**). This probably happened because defense metabolites present in the roots were mobilized to the leaves, which need to be protected from the insect feeding and damage. However, the metabolite concentration in

roots gradually increased between days 3 and 6. The metabolic pool might be channeled back to the roots if the aerial parts are destroyed.

3.3.5 *O. kilimandscharicum* metabolites cause severe pupal deformities in *H. armigera*

To measure the insecticidal performance of individual defense metabolites from *O. kilimandscharicum*, feeding assays were carried out with *H. armigera* second instar larvae. Results of two way ANOVA show a statistically significant interaction for the total variance of 9.95% at $p<0.0001$ between the days of infestation and growth of larvae fed on leaf extract, camphor, limonene, β-caryophyllene, artificial diet **(Fig. 3.7A, Table 3.3)** and also for mortality (for the total variance of 5.96 % at $p=0.0009$) **(Fig. 3.7B, Table 3.3)**. Growth was retarded in all larvae fed on the diet supplemented with selected metabolites on all days. Artificial diet fed larvae showed significantly more larval mass as compared to larvae fed on the other diets at day 4, 6, and 8 (at $p<0.0001$) (**Fig. 3.7A**). Larvae fed on the selected metabolites exhibited different percentage mortality. Larvae fed on limonene-based diet showed significantly more mortality (forming separate group 'b') as compared to other three diets on day 2 and 4 (at $p<0.01$, $p<0.0001$) whereas β-caryophyllene showed significantly more mortality compared to other diets (forming separate group 'c') on day 6 and 8 (at $p<0.05$, $p<0.01$, $p<0.0001$). Significantly less growth and high mortality in four diets (leaf extract, camphor, limonene, β-caryophyllene) fed larvae indicates gradual effect of these compounds on insect survival **(Fig. 3.7B)**. Additionally, pupal deformities were evident in the insects fed on camphor and *O. kilimandscharicum* leaf extract (**Fig. 3.7C**).

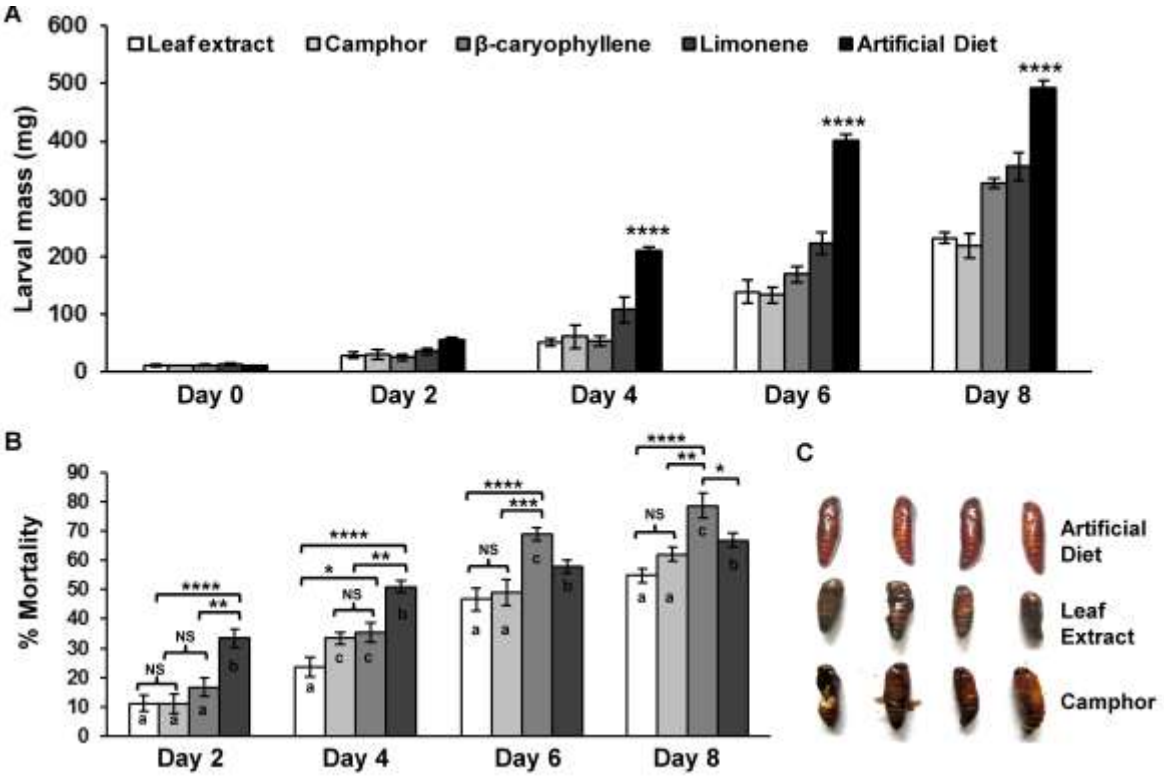


Figure 3.7. Antibiosis to *H*. *armigera* following exposure to *O*. *kilimandscharicum* leaf extract and selected compounds. **A.** Average larval mass and **B.** percentage of mortality of *H*. *armigera* second-instar larvae fed on artificial diet supplemented with *O*. *kilimandscharicum* leaf extract, camphor, limonene, β-caryophyllene at 100 ppm (day 0 to 8) and **C.** pupal deformities and death for larvae fed on diet supplemented with *O*. *kilimandscharicum* extract and camphor. Two way ANOVA followed by Tukey's multiple comparisons test between the treatment and larval mass (A) at different time points suggested significant interaction at $p<0.0001$. Significant difference in data at $p<0.0001$ (indicated by ****), at $p<0.001$ (indicated by ***), at $p<0.01$ (indicated by **), at $p<0.05$ (indicated by *). Small letters in (B) represents results for Tukey's post hoc test. Similar alphabets in column represent group of diets showing non-significant (NS) difference in

mortality while different alphabets represents diets exhibiting statistically different mortality in that particular day. NS represents non-significant difference in mortality.

Table 3.3. Two way analysis of variance for growth inhibition and percentage mortality of *H. armigera* upon exposure to *O. kilimandscharicum* leaf extract and selected metabolites on various days

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Growth inhibition					
Interaction	140388	16	8774	F (16, 40) = 32.79	P < 0.0001
Time	1.04E+06	4	259175	F (4, 40) = 968.6	P < 0.0001
Treatments	210262	4	52566	F (4, 10) = 41.55	P < 0.0001
Subjects (matching)	12651	10	1265	F (10, 40) = 4.728	P = 0.0002
Residual	10704	40	267.6		
Total	1.41E+06	74			
% Mortality					
Interaction	1246	9	138.4	F (9, 24) = 4.838	P = 0.0009
Time	16021	3	5340	F (3, 24) = 186.6	P < 0.0001
Treatments	2732	3	910.5	F (3, 8) = 33.80	P < 0.0001
Subjects (matching)	215.5	8	26.94	F (8, 24) = 0.9415	P = 0.5019
Residual	686.7	24	28.61		
Total	20900	47			

DF = Degrees of freedom, SS = Sum of squares, MS = Mean square, n = numerator, d = denominator, p = probability of significance, $\alpha = 0.05$

Our results clearly show that these metabolites can directly affect insect growth, survival and pupation, and hence can be used as potent insecticides.

3.4 Conclusion

O. kilimandscharicum elicited a strong defense response to counteract *H. armigera* larval infestation. The defense-associated metabolites such as monoterpenes, sesquiterpenes and

phenylpropanoids were upregulated. The growth and development of *H. armigera* larvae was significantly retarded when they fed on *O. kilimandscharicum* leaves as compared to tomato leaves. Initially, primary metabolism in *O. kilimandscharicum* was drastically affected by insect infestation as was evident from the increased concentration of carbohydrates. Moreover, metabolites such as camphor, β-caryophyllene, terpinolene and limonene increased greatly during infestation. This increase might be attributed to the plant's strong insecticidal properties.[40, 41] Importantly, selected compounds from *O. kilimandscharicum* leaves were also able to retard larval growth and induce pupal deformities in *H. armigera*. We conclude that defense metabolites from *O. kilimandscharicum* possess strong insecticidal activity even at lower concentrations revealed by present study and corroborated by earlier reports. [40, 41]

3.5 References

(1) P. Prakash and N. Gupta, Therapeutic uses of *Ocimum sanctum* Linn (Tulsi) with a note on eugenol and its pharmacological actions: a short review. *Indian J. Physiol. Pharmacol*., 2005, **49**, 125.

(2) F. L. Hakkim, G. Arivazhagan and R. Boopathy, Antioxidant property of selected *Ocimum* species and their secondary metabolite content. *J. Med. Plants Res*., 2008, **2**, 250.

(3) K. P. Bhargava and N. Singh N, Anti-stress activity of *Ocimum sanctum* Linn. *Indian J. Med. Res*., 1981, **73**, 443.

(4) K. Karthikeyan, P. Gunasekaran, N. Ramamurthy and S. Govindasamy, Anticancer activity of *Ocimum sanctum*. *Pharmaceutical Biology*, 1999, **37**, 285.

(5) P. Uma Devi, A. Ganasoundari, B. Vrinda, K. K. Srinivasan and M. K. Unnikrishnan, Radiation protection by the *Ocimum* flavanoids orientin and vicenin: mechanisms of action. *Radiat*. *Res*., 2005, **154**, 455.

(6) A. Khan, A. Ahmad, N. Manzoor and L. A. Khan, Antifungal Activities of *Ocimum sanctum* essential oil and its lead molecules. *Nat*. *Prod*. *Commun*., 2010, **5**, 345.

(7) M. R. I. Khan, M. A. Islam, M. S. Hossain, M. Asadujjaman, M. I. I. Wahed, B. M. Rahman *et al*., Antidiabetic effects of the different fractions of ethanolic extracts of *Ocimum sanctum* in normal and alloxan induced diabetic rats. *J*. *Sci*. *Res*., 2010, **2**, 158.

(8) J. O. Ogendo, M. Kostyukovsky, U. Ravid, J. C. Matasyoh, A. L. Deng, E. O. Omolo *et al*, Bioactivity of *Ocimum gratissimum* L oil and two constituents against five insect pests attacking stored food products. *J*. *Stored Prod*. *Res*., 2008, **44**, 328.

(9) V. Rozman, I. Kalinovic and Z. Korunic, Toxicity of naturally occurring compounds of Lamiaceae and Lauraceae to three stored- product insects. *J*. *Stored Prod*. *Res*., 2006, **43**, 349.

(10) A. Liska, V. Rozman, I. Kalinovic, M. Ivecic and R. Balicevic, Contact and fumigant activity of 1, 8-cineole, eugenol and camphor against *Tribolium castaneum* (Herbst). *Julius-Kühn-Archiv*, 2010, **425**, 716.

(11) L. Qiantai and S. Yongcheng, Studies on effect of several plant materials against stored grain insects. *Proceedings of the Seventh International Conference on Stored-Product Protection*, 1998, **1**, 836.

(12) R. N. Bennett and R. M. Wallsgrove, Secondary metabolites in plant defence mechanisms. *New Phytol*., 1994, **127**, 617.

(13) P. Hao, C. Liu, Y. Wang, R. Chen, M. Tang, B. Du *et al*. Herbivore-induced callose deposition on the sieve plates of rice: An important mechanism for host resistance. *Plant Physiol*., 2008, **146**, 1810.

(14) R. Maor and K. Shirasu, The arms race continues: battle strategies between plants and fungal pathogens. *Curr*. *Opin*. *Microbiol*., 2005, **8**, 399.

(15) A. C. U. Furch, J. B. Hafke and A. J. E. van Bel, Plant and stimulus-specific variations in remote-controlled sieve-tube occlusion. *Plant Signal*. *Behav*., 2008, **3**, 858.

(16) J. A. Zavala, A. P. Patankar, K. Gase, D. Hui, I. T. Baldwin, Manipulation of endogenous trypsin proteinase inhibitor production in *Nicotiana attenuate* demonstrates their function as anti-herbivore defences. *Plant Physiol*., 2004, **134**, 1181.

(17) H. Chen, C. G. Wilkerson, J. A. Kuchar, B. S. Phinney and G. A. Howe, Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. *Proc*. *Natl*. *Acad*. *Sci*. *USA*, 2005, **102**, 19237.

(18) A. Kachroo, J. Shanklin, E. Whittle, L. Lapchyk, D. Hildebrand, P. Kachroo, The *Arabidopsis* stearoyl-acyl carrier protein-desaturase family and the contribution of leaf isoforms to oleic acid synthesis. *Plant Mol*. *Biol*., 2006, **63**, 257.

(19) J. B. Harborne, Secondary Metabolites: Attracting Pollinators: els., 2001.

(20) J. K. Holopainen, Multiple functions of inducible plant volatiles. *Trends Plant Sci*., 2004, **9**, 529.

(21) M. B. Isman, Plant essential oils for pest and disease management. *Crop Prot*., 2000, **19**, 603.

(22) G. P. Fitt, The ecology of Heliothis species in relation to agroecosystems. *Ann*. *Rev*. *Entomol*., 1989, **34**, 17.

(23) C. N. K. Rajapakse and G. H. Walter, Polyphagy and primary host plants: oviposition preference versus larval performance in the lepidopteran pest *Helicoverpa armigera*. *Arthropod–Plant Interact*., 2007, **1**, 17.

(24) H. M. Kotkar, P. J. Sarate, V. A. Tamhane, V. S. Gupta and A. P. Giri, Responses of midgut amylases of *Helicoverpa armigera* to feeding on various host plants. *J*. *Insect Physiol*., 2009, **55**, 663.

(25) P. J. Sarate, V. A. Tamhane, H. M. Kotkar, N. Ratnakaran, N. Susane, V. S. Gupta *et al*. Developmental and digestive flexibilities in the midgut of a polyphagous pest, the cotton bollworm, *Helicoverpa armigera*. *J*. *Insect Sci*., 2012, **42**, 1.

(26) C. Kamaraj, A. Abdul Rahuman and A. Bagavan, Screening for antifeedant and larvicidal activity of plant extracts against *Helicoverpa armigera* (Hübner), *Sylepta derogate* (F.) and *Anopheles stephensi* (Liston). *Parasitol*. *Res*., 2008, **103**, 1361.

(27) S. Nagarkatti and A. Prakash, Rearing *Heliothis armigera* (Hubn) on artificial diet. Commonwealth Institute of Biological Control, Bangalore, Technical Bulletin, 1974, **17**, 169.

(28) J. Kjeldahl, A new method for the estimation of nitrogen in organic compounds. *Z Anal*. *Chem*., 1883, **22**, 366.

(29) M. DuBois, K. Gilles, J. Hamilton, P. Rebers and F. Smith, Colorimetric method for determination of sugars and related substances. *Anal*. *Chem*., 1956, **28**, 350.

(30) N. Zöllner and K. Kirsch K, über die quantitative Bestimmung von Lipoiden (Mikromethode) mittels der vielen natürlichen Lipoiden (allen bekannten Plasmalipoiden) gemeinsamen Sulfophosphovanilin- Reaktion. *Z Ges*. *Exp*. *Med*., 1962, **135**, 545.

(31) F. M. Brock, C. W. Frosberg and J. G. Buchanan-Smith, Proteolytic activity of rumen microorganisms and effect of proteinase inhibitors. *Appl. Environ. Microbiol.*, 1982, **44**, 561.

(32) V. A. Tamhane, N. P. Chougule, A. P. Giri, A. R. Dixit and M. N. Sainani, *In vivo* and *in vitro* effect of *Capsicum annum* proteinase inhibitors on *Helicoverpa armigera* gut proteinases. *Biochimica. Et. Biophysica. Acta.*, 2005, **1722**, 156.

(33) P. Bernfeld, Amylases, α and β. *Methods in Enzymology*, 1955, **1**, 149.

(34) U. K. Winkler and M. Stuckman, Polysaccharide enhancement of exolipase formation by *S. Marcescens. J. Bacteriol.*, 1979, **138**, 663.

(35) F. Rolland, E. Baena-Gonzalez and J. Sheen, Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annu. Rev. Plant Biol.*, 2006, **57**, 675.

(36) T. Roitsch and M. C. González, Function and regulation of plant invertases: sweet sensations. *Trends Plant Sci.*, 2004, **9**, 606.

(37) J. Schwachtje, P. E. H. Minchin, S. Jahnke, J. T. van Dongen, U. Schittko, I. T. Baldwin, SNF1-related kinases allow plants to tolerate herbivory by allocating carbon to roots. *Proc Natl Acad Sci USA*, 2006, **103**, 12935.

(38) J. León, E. Rojo and J. J. Sánchez-Serrano, Wound signaling in plants. *J. Exp. Bot.*, 2001, **52**, 1.

(39) S. Dinant and P. Suárez-López, Multitude of signal molecules acting long-distance via Phloem. Signaling and Communication in Plants, Springer series, 2012, Chapter 6, 89.

(40) A. J. Bekele, D. O. Ofori and A. Hassanali, Products derived from the leaves of ocimum kilimandscharicum (Labiatae) as post- harvest grain protectants against the infestation of three major stored product insect pests. *Bull*. *Entomol*. *Res*., 1995, **85**, 361.

(41) J. Bekele and A. Hassanali, Blend effects in the toxicity of the essential oil constituents of *Ocimum kilimandscharicum* and *Ocimum kenyense* (Labiateae) on two post-harvest insect pests. *Phytochemistry*., 2001, **57**, 385.

CHAPTER 4

Integrating transcriptomics with metabolomics reveals tissue- specific metabolic partitioning in *O. kilimandscharicum*

4.1 Introduction

Metabolite partitioning refers to the vectorial transport and accumulation of primary and/or secondary metabolites in an organ-, tissue-, cell- or organelle-specific manner. Plants are known to habor several intricately interwoven metabolic pathways, sharing substrates, intermediates and products among themselves. In view of this complexity, the strategy of partitioning and compartmentation may be to circumvent metabolic interference between diverse classes of metabolites leading to their optimal biosynthesis.[1-5] Partitioning of metabolites is also important to strike a compromise between allocation of resources towards growth and/or defense.[6-9] Partitioning defense-related metabolites to floral organs, roots or trichomes helps to enhance their defense potential and enables them to ward off predators effectively. Metabolite partitioning or redistribution may take place under normal as well as stress conditions, enabling the plant to achieve a steady state as postulated in the theory of functional balance.[10] Under normal growth conditions, partitioning of sulphur between biosynthesis of amino acids and glucosinolates for defense is controlled by the activity of adenosine-5'-phosphosulfate-3'-phosphokinase (APS kinase).[11] Partitioning of carbohydrates between source (leaves) and sink (roots) tissue has been extensively studied in tomato.[12-16] Upon experiencing drought, partitioning and reallocation of carbon was confirmed between shoot and root system in maize plants.[8] High atmospheric CO_2 levels resulted in increased partitioning of carbon to photosynthetic and phenylpropanoid pathway in oil palms.[17] Hence, partitioning of metabolites is crucial for optimal and prompt reverberation of growth, development, defense and stress-related simuli.

Broadly, metabolite partitioning can occur *via* two major underlying mechanisms; (1) differential or tissue- specific expression of pathway genes or (2) transport of metabolite from source to sink tissue. Tissues accumulating more amount of a metabolite mostly show a greater expression of pathway genes and thus, a more active pathway for production of that metabolite. In several cases, the expression of pathway entry point, rate-limiting or committed step catalysing enzymes, plays a critical role in manipulating the metabolic flux. For example, overexpression of myo-inositol oxygenase, the entry point enzyme for ascorbate biosynthesis led to 2 to 3- fold increase in ascorbate levels in *Arabidopsis* leaves.[18] The bean *pal2* gene, encoding phenylalanine ammonia lyase, catalyzing the first step in phenylpropanoid synthesis was overexpressed in transgenic tobacco, which resulted in significant increase in hydroxycinnamic esters in leaves. However, only a small increase in lignin content in stem and no significant change in leaf rutin (flavonoid) content was observed.[19] In another example, enhancement of artemisinin biosynthesis was achieved by (1) overexpression of entry point enzyme of general terpenoid pathway (*dxr*) as well as by (2) co-overexpression of artemisinin pathway-specific genes (*cyp71av* and *cpr*) in the whole plant.[20] On the other hand some tissues tend to accumulate certain metabolites in enormous quantities, however, they lack the pathway enzymes for its biosynthesis. In such cases, the metabolite is synthesized in source tissue and transported to the sink tissue *via* proteins called transporters. An interesting and extensively studied example is that of long distance transport of sucrose *via* phloem. Sucrose is further unloaded in the sink organs and utilized as a source of energy and carbon.[21] Long distance transport of nicotine alkaloids, synthesized in roots and transported *via* xylem to the vacoules of the leaf mesophyll cells has been deeply

investigated and involves jasmonate- mediated signalling.[22] Several ABC (ATP- Binding Cassette) transpoters have been implicated to be involved in transportation of secondary metabolites including alkaloids, terpenoids, phenolics etc.[23] In order to understand the phenomena of partitioning, one needs to understand changes taking place at the level of genes as well as metabolites in that pathway. Integrating metabolomics with transcriptomics helps us gain a holistic view into pathway genes, intermediates, transporters, transcription factors etc. that may be involved in synthesis, transport or storage of the said metabolite.

Ocimum species contain numerous secondary metabolites including terpenes, phenylpropanoids, flavonoids, phenolics etc.[24] These metabolites are distributed in a highly tissue- specific manner in the plant. *Ocimum* species thus provide, an attractive model system for studying the mechanism of metabolite partitioning and its underlying biological significance. The biosynthesis, transport and storage of most of these essential metabolites remains unknown. However, our understanding of this genus has increased in the past few years due to the availability of genomic, transcriptomic and metabolomic data sets on different species.[25-27] Here, we consider the case of *O. kilimandscharicum*, also called camphor basil, as a representaive member of genus *Ocimum* to study metabolite partitioning. The present study investigates the biosynthesis of two major metabolites, camphor and eugenol, in *Ocimum kilimandscharicum*. Further we understand the mechanism and biological significance of stringent partitioning of these metabolites between the aerial and underground system.

4.2 Materials and methods

4.2.1 Chemicals and reagents: All chemicals and reagents were procured from Sigma Aldrich unless mentioned otherwise.

4.2.2 Plant growth conditions: *O. kilimandscharicum* plants were grown in the greenhouse under the following conditions: temperature, 28 to 30 °C; humidity, 35 to 40%; light conditions, 16 h light, 8 h dark.

4.2.3 Gas Chromatography – Mass Spectrometry: GC-MS was performed as described earlier.[28] Briefly, plant tissue (1 g) was mixed in 10 mL dichloromethane (DCM) and kept for 18 to 24 h at 28 ºC. The extract was filtered and incubated for 2 h at -20 °C to allow lipid precipitation. DCM extract was filtered again, concentrated under vacuum on a rotary evaporator and subjected to GC and GC-MS analysis. GC analyses were carried out on an Agilent 7890A instrument equipped with a hydrogen flame ionization detector and an HP-5 capillary column (30 m X 0.32 mm X 0.25 μm, J and W Scientific). Nitrogen was used as the carrier gas at a flow rate of 1 mL/min. The column temperature was raised from 70 °C to 110 °C at 2 °C min^{-1}, then raised to 180 °C at 3 °Cmin^{-1} and finally to a temperature of 220 °C with a 10 °C min^{-1} rise; here it was held for 2 min. Injector and detector temperatures were 230 °C and 250 °C, respectively. GC-MS was performed on a HP 5975C mass selective detector interfaced with a HP 7890A gas chromatograph. GC-MS analyses were performed under similar conditions using an HP-5 MS capillary column (30 m X 0.32 mm X 0.25 μm, J and W Scientific) with helium as the carrier gas. Compounds were identified by comparing the retention time and mass fragmentation pattern of the standards of major constituents and also by comparing

acquired mass spectra and retention indices with NIST/NBS and the Wiley mass spectral library (software version 2.0, Dec. 2005).

4.2.4 Transcriptome profiling: Tissues (flowers, young leaves, roots and trichomes from inflorescence) were collected from *O. kilimandscharicum* plants growing in green house at CSIR-National Chemical Laboratory. Sigma Spectrum Plant RNA Isolation kit was used for RNA isolation. Quality of RNA was checked on Nanodrop. Transcriptome sequencing using Illumina Next Generation Sequencing (NGS), *de novo* assembly, sequence clustering, sequence annotation, gene ontology assignment and transcript abundance measurement was performed at Genotypic Technology, Bangalore, India.

4.2.5 Global untargeted metabolomics using LC-Orbitrap

4.2.5.1 Metabolite extraction: Extractions were performed as described earlier.[29] Briefly, 100 mg tissue [Young leaves, mature leaves, stem, seeds, buds, flower, petal, sepal, anther and pistil (A+P), trichomes from young leaves, trichomes from stem and trichomes from inflorescence] was ground to a fine powder using liquid nitrogen. 600 µL ice cold extraction buffer (98.875% methanol with 0.125% formic acid) was added and vortexed immediately for 10 s. Sonication was performed at 40 mHz for 15 mins in a sonicating water bath and centrifuged at 20,000*g* for 10 mins. The supernatent was transferred to a fresh 1.5 mL eppendorf and pellet was re-extracted using 400 µL extraction buffer. Supernatent was pooled and filtered through a 0.2 µM PVDF filter and stored at -80°C until further use.

4.2.5.2 Liquid Chromatography-Orbitrap instrument set up: All samples were run in triplicates and analyzed using thermoscientific hybrid quadrapole orbitrap mass spectrometer as described earlier.[29] All samples were acquired in negative mode using Heated Electrospray Ionization (HESI) method. Samples (injection volume, 10 µL) were loaded on the C18 reverse phase column (150 mm × 4.6 mm; particle size, 3µM). Data was acquired in full scan mode with m/z window 66.7 to 1000 Da. HRMS was set at: resolution, 70,000; AGC target, 1e6; maximum inject time, 120; sheath gas flow rate, 45; auxillary gas flow rate, 10; sweap gas flow rate, 2; spray voltage, 2.80 kV; spray current, 24.70 µA; capillary temperature, 300°C; S- lens RF level, 45; auxillary gas heater temperature, 390°C. Chromatographic runs of 30 mins including washing and re-conditioning of column were employed with a mobile phase flow rate of 1mLmin^{-1}.Two eluents were used as mobile phase; eluent A was 0.1% formic acid in ultrapure water and eluent B was 0.1% formic acid in acetonitrile. The inlet file was programmed according to the following gradient settings:

Time (min)	%A	%B
0	95	5
20	25	75
25	25	75
26	95	5
30	95	5

4.2.5.3 LC-MS data preprocessing and analysis: Xcaliber raw files (.raw) were converted to .mzXML format using default parameters in proteowizard.[30] Analysis was

performed with XCMS using R program, [31] and Maven.[32] For performing analysis using XCMS, R program was launched and XCMS package was loaded. The directory was changed to select the folder containing the .mzXML files. Data files (Young leaves, mature leaves, stem, seeds, buds, flower, petal, sepal, an ther and pistil were uploaded, peaks were grouped, retention time was corrected, data was re-grouped after retention time correction and the report was generated as an excel sheet (as per the published and optimized protocol for orbitrap acquired data). Statistical analysis was performed and mass/charge's (m/z's) detected in all three replicates were only considered for further analysis. Differentially accumulated m/z's were sorted and hierarchial clustering analysis (HCA) was performed. Trichomes from young leaves, trichomes from stem and trichomes from inflorescence were analysed separately using the same steps as above and differentially accumulated m/z's were subjected to heatmap analysis. Maven was used for analysing the intermediates from eugenol biosynthesis pathway using default parameters.

4.2.6 Heatmap and HCA analysis

The HCA analysis for 710 differentially accumulated m/z's in (Young leaves, mature leaves, stem, seeds, buds, flower, petal, sepal, anther and pistil) was performed using Multiexperiment Viewer software (MeV). All heatmap analyses were also pereformed using Multiexperiment Viewer (MeV) software.

4.2.7 Real time analysis for *gpps* and *bdh*

Total RNA was extracted from young leaves, flowers, trichomes of inflorescence and root of *O. kilimandscharicum* using Sigma Spectrum Plant RNA Isolation Kit. 2 µg RNA was used to synthesis cDNA using the Superscript III first strand cDNA synthesis kit from Invitrogen. Expression of *gpps* and *bdh* was analyzed across all tissues using qPCR

(7900 HT Applied Biosystems, USA) and SYBR Green PCR Master Mix (2X) (Roche). The PCR (reaction volume, 10 µL) was set up by mixing 5 µL SYBR Green Master mix, gene specific forward and reverse primers and 25ng cDNA as template.Elongation factor 1 alpha (EF1a) was used as the reference gene. The thermal cycling conditions were as follows: initial hold (50°C, 2 min); initial denaturation (95°C, 10 min); 40 amplification cycles (95°C, 15 s; 60°C, 1 min) and finally followed by additional stage (60°C, 15 s; 95°C, 15 s and 37°C, 2 min). Data was analyzed using SDS software (version 4.0) and relative quantitation of gene expression was performed using ΔΔCT method.

4.2.8 *A. tumefaciens* mediated *in planta* transient silencing and overexpression:

4.2.8.1 Vector construction and agroinfiltration

For *A. tumefaciens*- mediated transient overexpression (OE) and silencing (RNAi) of *bdh* and *gpps*, the genes were amplified from *O. kilimandscharicum* cDNA (from young leaves) using specific forward and reverse primers in PCR. The amplified PCR fragment was cloned in pGEMT Easy and gene sequence was confirmed by nucleotide sequencing. For making overexpression constructs, the amplicon was cloned into binary vector *p*RI101AN using *Xba* I and *Sac* I- mediated restriction digestion. For making silencing constructs, sense and antisense fragments were cloned sequentially on either side of the wheat starch branching intron between SalI/KpnI and BamHI/SacI restriction sites of binary vector pRI101AN, respectively. Correct orientation of amplicons was confirmed by nucleotide sequencing. Overexpression and silencing constructs were transformed in *A. tumefaciens* strain GV3101 and agroinfiltration was performed using a 5 mL syringe barrel on the abaxial surface of *O. kilimandscharicum* leaves. Normal untreated plants

(control or uninduced) and plants infiltrated with *A. tumefaciens* GV3101 harboring empty pRI101AN vetor (Empty vector) acted as controls. Plants were maintained in the greenhouse under the following conditions: temperature, 16 to 18 °C; humidity, 35 to 40%; light conditions, 16 h light, 8 h dark. For overexpression analysis, local leaf tissue was collected on day 4 for real time and metabolite analysis. For silencing analysis, local as well as upper systemic leaf tissue was collected on day 8 for semi-quantitative PCR and metabolite analysis.

4.2.8.2 Analysis of transient transgenics local and systemic leaves

Plants transiently overexpressing *gpps* (OE_GPPS) and *bdh* (OE_BDH) were analyzed for the extent of gene overexpression using real time PCR (decribed above). The effect of gene overexpression on metabolite profile was studied using GC-MS (described above) of infiltrated local leaf tissue compared to control treatments (control and empty vector plants). For silencing studies, hairpin formation was checked in local leaf tissue as well as tissues of control treatments using intron- specific primers in *gpps* (RNAi_GPPS) and *bdh* (RNAi_BDH) transient transgenics. The effect of hairpin formation on gene expression was analysed by semiquantitative PCR using gene specific primers. GPPS- and BDH- local as well as systemic leaves and control leaves (control and empty vector plants) were used as template. To study the effect of gene silencing on metabolite profile GC-MS of infiltrated local and control leave tissues was performed and compared as described above.

4.2.9 Co-expression Analysis

Co-expression analysis was performed using *egs1* and *4cl4* as bait genes using CoExpNetviz.[33] Gene correlation matrix was prepared using RPKM values from the NGS data. The network was vizualized and edited in cytoscape (v3.5.1).

4.3 Results and Discussion

4.3.1 *Ocimum* species display stringent metabolite partitioning between aerial and root tissue

GC-MS analysis of tissues across all examined *Ocimum* species revealed strict partitioning of metabolites between the aerial shoot system including young leaves, mature leaves, inflorescence and flowers; and the underground root system (**Fig. 4.1**). In *O*. *kilimandscharicum* (*Ok*), camphor, a monoterpene, accumulated predominantly in the aerial tissues (Young leaf, 54.6%; Mature leaf, 51.03%; Inflorescence, 58.04%; Flower, 60.5%) while eugenol, a phenylpropanoid, was detected between 0 to 0.1% in these tissues (**Fig. 4.1**). In contrast, roots largely accumulated eugenol (56.6%) as compared to camphor (16%). On the other hand, in *O*. *gratissimum* (*Og*) eugenol accumulated in aerial tissues (Young leaf, 71.2%; Mature leaf, 80.1%; Inflorescence, 63.7%; Flower, 74.8%) and roots accumulated borneol (57%), a monoterpene, as the most abundant metabolite (**Fig. 4.1**). Thus, tissue specificity in accumulation of metabolites was independent of their chemical nature (terpene or phenylpropanoid). Chemotypes belonging to the same *Ocimum* specie also show partitioning of metabolites. Both *O*. *basilicum* chemotype I (*Oba I*) and *O*. *basilicum* chemotype II (*Oba II*) accumulate methyl cinnamate in their aerial parts (40 to 63%). However, *O*. *basilicum* chemotype I accumulates large quantities of estragole (32.1%) (**Fig. 4.1**) and *O*. *basilicum* chemotype II accumulates

eugenol (44.4%) in their roots (**Fig. 4.1**). Thus, metabolite partitioning also occurs at the sub- specie level. Interestingly, in the above example, eugenol, estragole and methyl cinnamate are all phenylpropanoids, and despite belonging to the same chemical class of compounds are still stringently partitioined. In another example, *O*. *tenuiflorum* (*Ot*), also called purple ruffles, partitions eugenol methyl ether, a

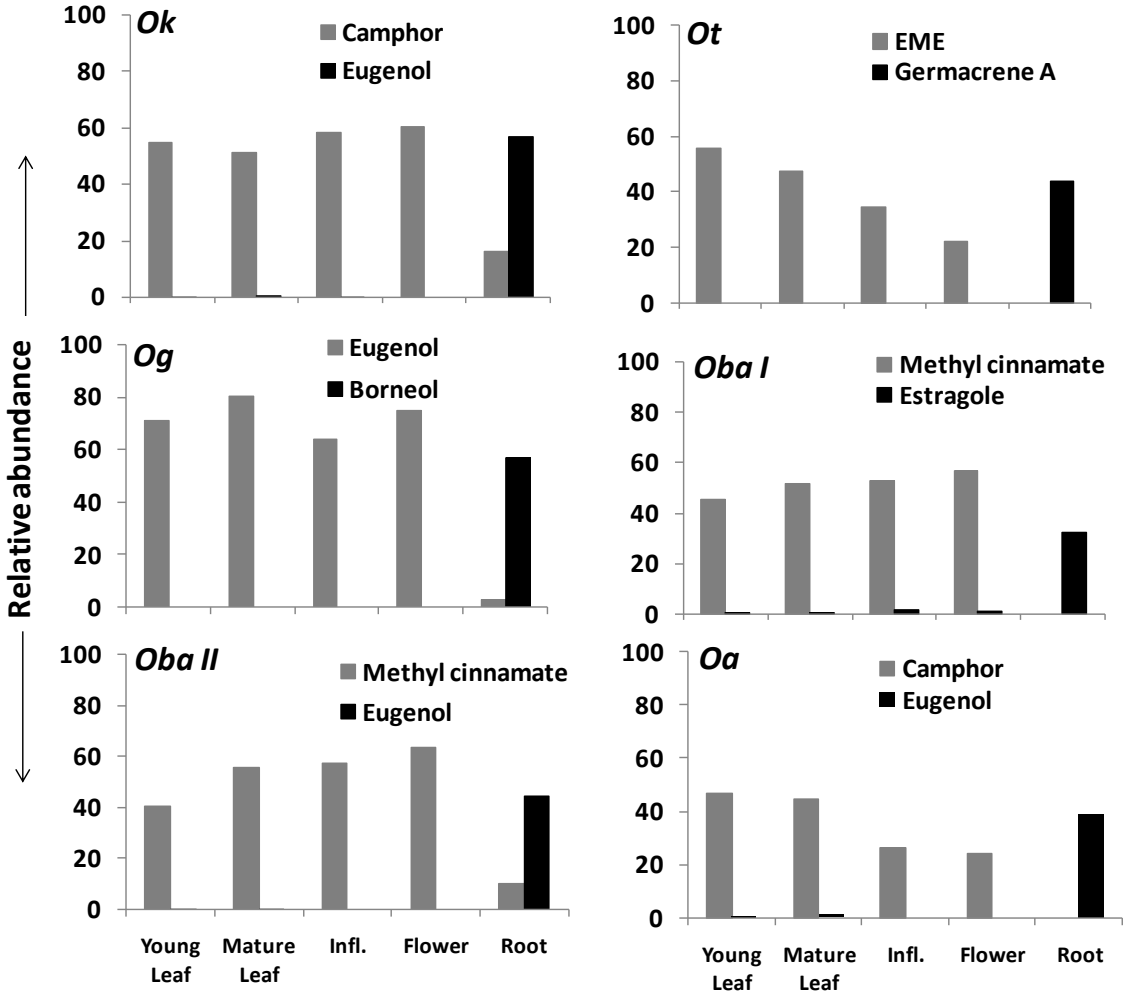


Figure 4.1: Tissue- specific metabolite partitioning observed in young leaves, mature leaves, inflorescence (Infl.), flower and root of *O*. *kilimandscharicum* (*Ok*), *O*. *gratissimum* (*Og*), *O*. *basilicum* chemotype I (*ObaI*), *O*. *tenuiflorum* (*Ot*), *O*. *basilicum* chemotype II (*Oba II*) and *O*. *americanum* (*Oa*)

phenylpropanoid in the aerial tissues (Young leaf, 55.6%; Mature leaf, 47.4%; Inflorescence, 34.7%, Flower, 21.9%) and germacrene A, a sesquiterpene in the roots (44%) (**Fig. 4.1**). Like *O. kilimandscahricum*, *O. americanum* (*Oa*) also partitions camphor to the aerial tissues (Young leaf, 46.6; Mature leaf, 44.6; Inflorescence, 26.3%, Flower, 24.3%) and eugenol to the roots (38.9%) (**Fig. 4.1**). Thus, the phenomenon of metabolite partitioning was found to be active across several species in genus *Ocimum*, and was independent of the chemical nature of metabolite or the type of plant tissue.

4.3.2 Transcriptomic data reveals tissue-specific expression of terpenoid and phenylpropanoid pathway genes in *O. kilimandscharicum*

Among all the *Ocimum* species examined, *O. kilimandscharicum* was further studied as a representative example to understand the basic mechanism underlying metabolite partitioining and its biological relevance to the plant. Next generation sequencing of flowers, young leaves, trichomes of inflorescence and roots was performed. Analysis including transcript assembly statistics, transcript annotation, gene ontology and metabolic pathway prediction revealed significant diversity among the tissue transcriptomes. Differential expression of general phenylpropanoid pathway enzymes and their isoforms including phenylalanine ammonia lyase (PAL), cinnamate-4- hydroxylase (C4H) and coumarate-CoA ligase (4CL) was evident (**Fig. 4.2A**). These enzymes play a crucial role in diverting the metabolic flux towards synthesis of phenylpropanoids and related compounds. Although *O. kilimandscharicum* roots primarily accumulate eugenol to a large extent, eugenol synthase (*egs*), the gene for the enzyme catalysing the final step in eugenol biosynthesis, converting coniferyl acetate to eugenol, was conspicuous by its absence. *Okegs* was infact expressed mainly in the young leaves (Reads per kilobase per

million, RPKM = 89.3). The absence of eugenol synthase in roots leads to the speculation that eugenol is produced in the young leaves or their trichomes (source tissue) and is subsequently transported to the roots (sink tissue).

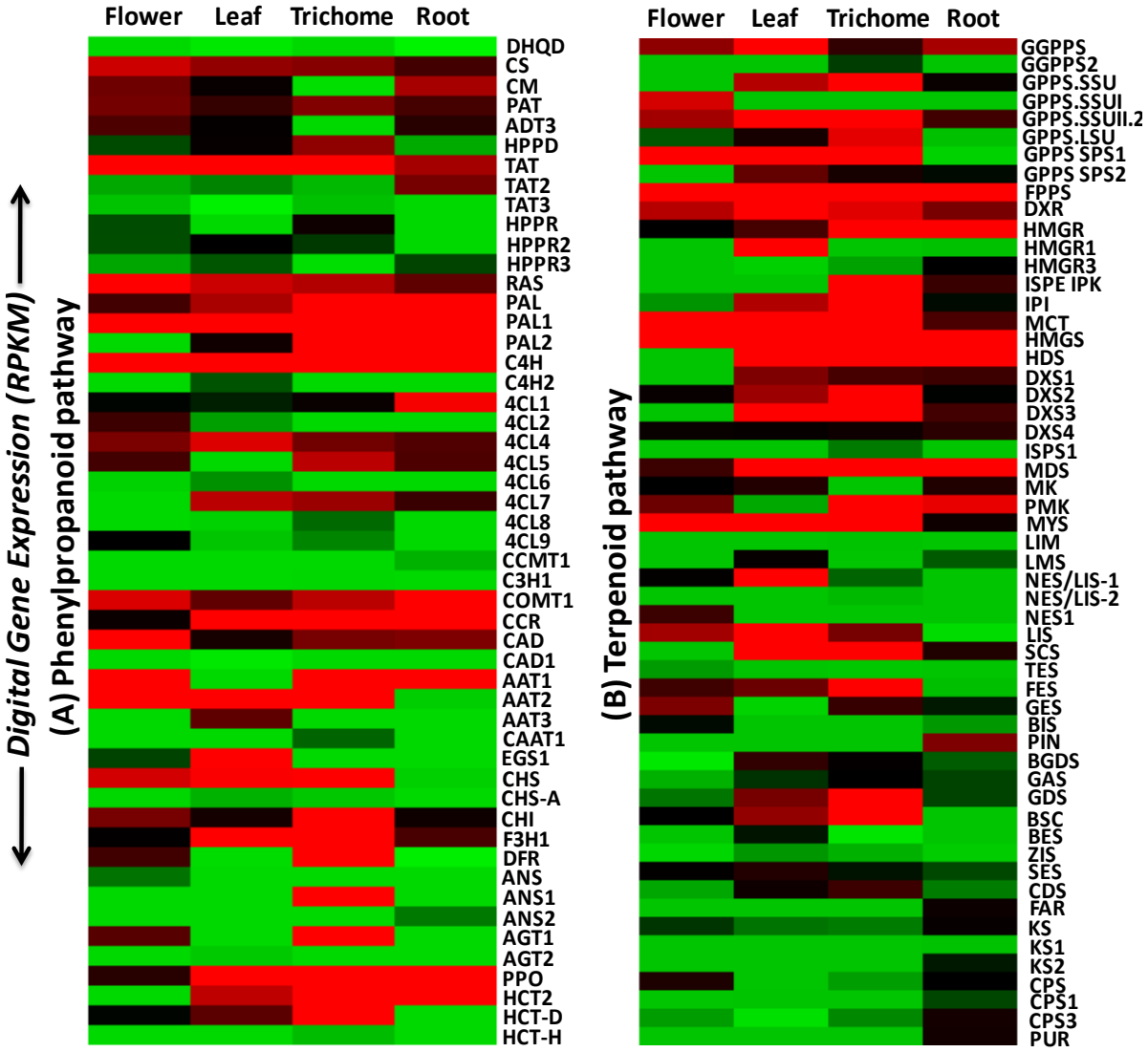


Figure 4.2: Differential expression of genes involved in (A) Phenylpropanoid pathway and (B) terpenoid pathway in flowers, trichomes of inflorescence, roots and young leaves in *O. kilimandscharicum*.

Genes involved in terpenoid biosynthesis revealed a similar pattern (**Fig. 4.2B**). Aerial tissues (young leaf, inflorescence, flower) showed higher expression of geranyl diphosphate synthase (entry point enzyme for monoterpene biosynthesis) as well as downstream monoterpene synthases (MTPSs). In contrast, roots showed higher expression of geranylgeranyl diphosphate synthase (entry point enzyme for diterpene biosynthesis) as well as downstream diterpene synthases (DTPSs). Thus, preliminary evidence from NGS data suggests that partitioning of metabolites between aerial and root tissue in *O. kilimandscharicum* can be attributed to both; differential expression of pathway genes as well as transport from source to sink tissue.

4.3.3 Global untargeted metabolomics reveals tissue- specific accumulation of metabolites in *O. kilimandscharicum*

In order to get a comprehensive overview of tissue- specific distribution of metabolites and pathway intermediates, global untargeted metabolomics of aerial tissues was performed (**Fig. 4.3A**). The number of putative metabolites detected in each tissue are as follows: Young leaf (2238), mature leaf (2248), stem (2213), bud (2295), flower (2367), anther and pistil (2235), petal (2287), sepal (2307), seed (2311). Maximum number of putative metabolites were detected in flower followed by seed and sepal, and least in stem. Overall, a total of 2589 putative metabolites were detected, out of which 1878 were uniformly present across all nine tissues (**Fig 4.3B**). Over 700 putative metabolites were variably distributed, that is, detected in one to eight tissues. Interestingly, 110 putative metabolites showed highly tissue specific accumulation, that is, they were uniquely detected in only one tissue. Of these 59 putative metabolites were detected in seed, 15 in anther and pistil, 11 in stem, 9 in petal, 5 in young leaf, 3 each in sepal, bud,

mature leaf and 2 in flower (**Fig. 4.3C**). HCA analysis for the 710 differentially detected putative metabolites shows clustering of tissues according to their metabolite profiles (**Fig. 4.3D**). It was observed that all tissues are not only rich in metabolites but also have their own unique metabolic fingerprint. However, whether these metabolites are synthesized in the tissue or transported from a source tissue needs to be further understood.

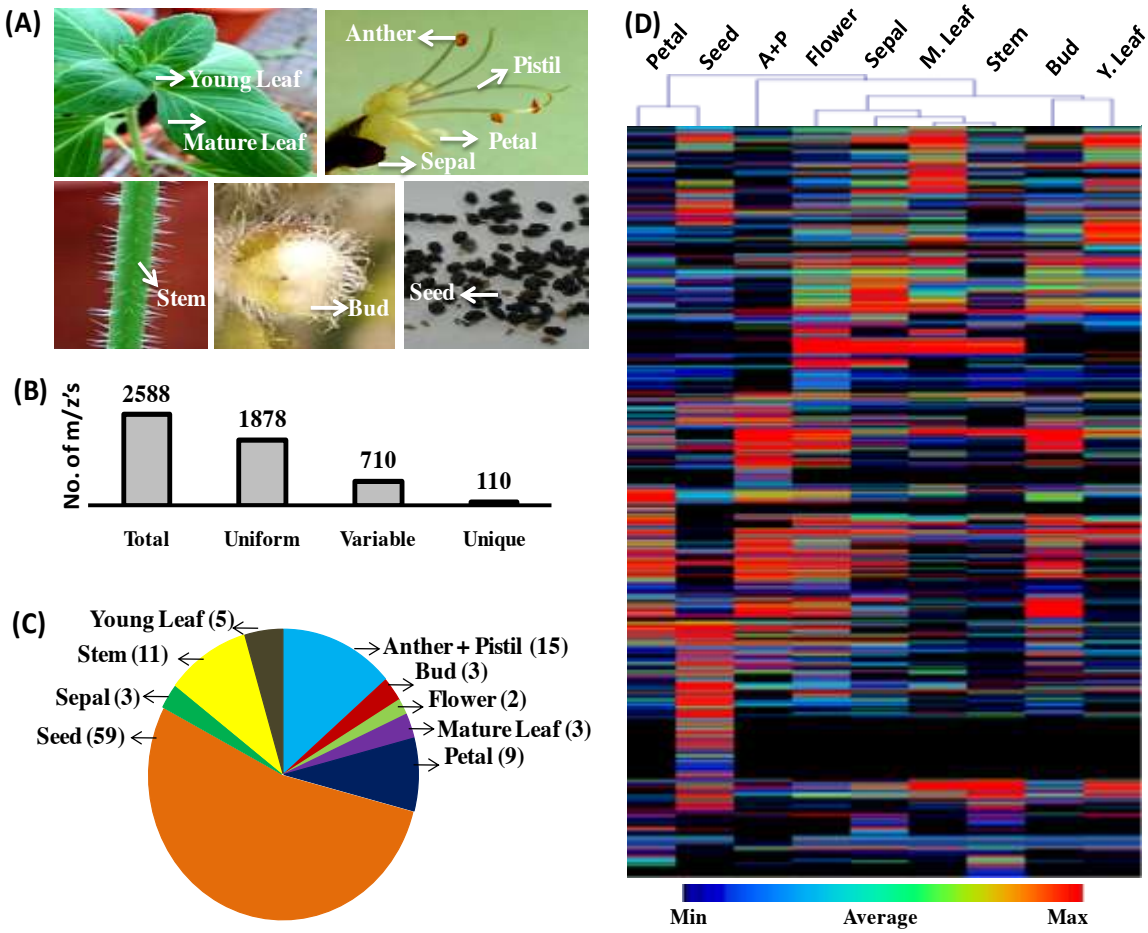


Figure 4.3: LC- Orbitrap based global untargeted metabolomics. (A) tissues used for metabolomic analysis (B) Bar graph representing total number, uniformly-, variably- and uniquely- occuring putative metabolites (C) Pie chart representing distribution of highly

tissue-specific (unique) putative metabolites (D) Heirarchial clustering analysis (HCA) of 710 variably occuring putative metabolites

In a separate analysis using trichomes from inflorescence (Infl.), stem and young leaves, we observed that trichomes accumulated much lesser putative metabolites (619) than other aerial parts suggesting functional specialization of metabolism (**Fig. 4.4A**).

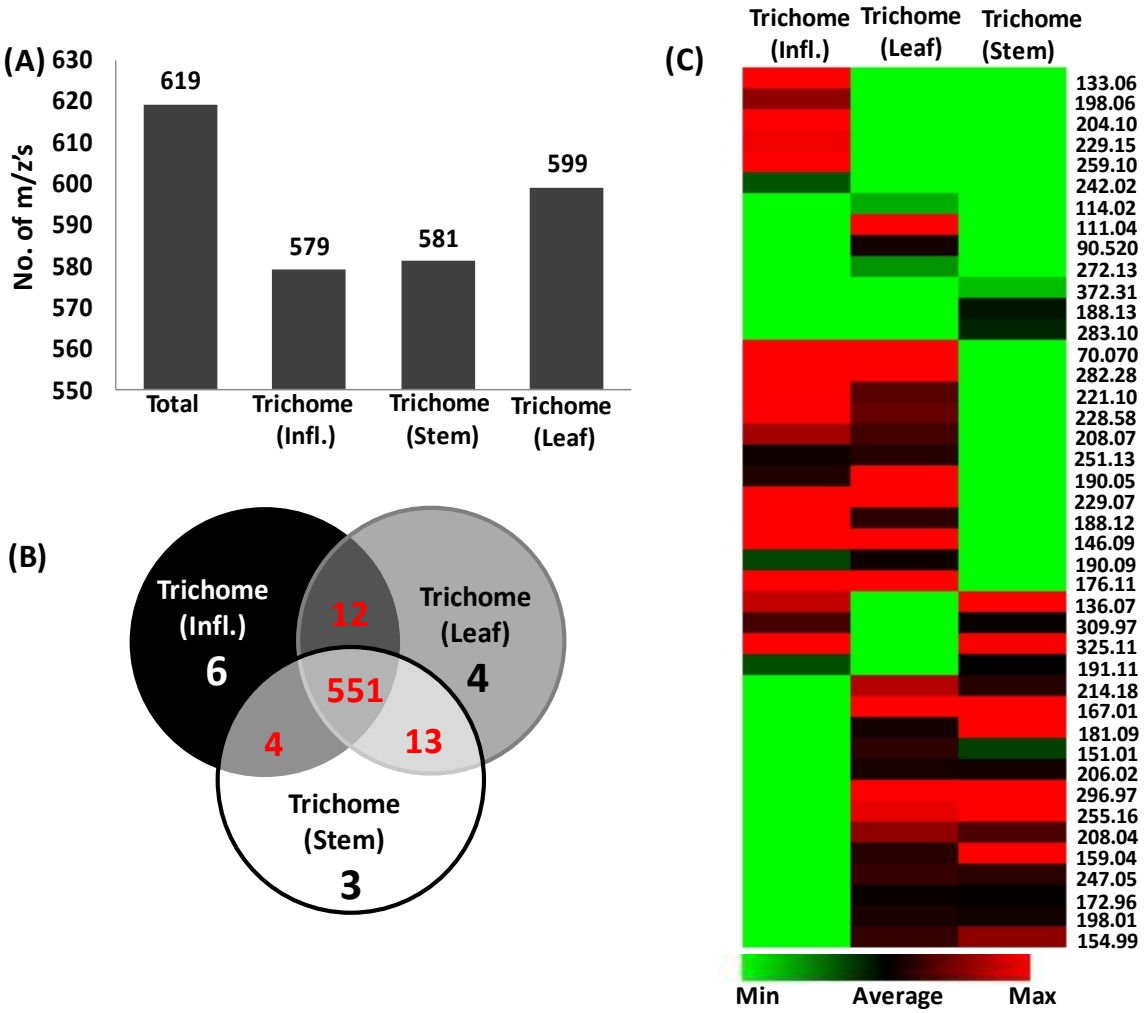


Figure 4.4: LC-Orbitrap based trichome metabolomics. (A) Bar graph representing number of putative metabolites in trichomes of inflorescence, leaves and stem (B) Venn diagram showing distribution of uniformly occuring and unique or tissue-specific

putative metabolites (C) Heatmap showing distribution of 42 tissue-specific putative metabolites

Number of putative metabolites detected in each tissue are as follows: Trichomes of inflorescence (579), trichomes of leaves (599) and trichomes of stem (581). Of these 551 were uniformly present in all three trichome types (**Fig. 4.4B**). Trichomes of different parts also exhibited differential metabolite profile (**Fig. 4.4C**). Thus, global untargeted metabolomics helps us gain a deeper insight into the perplexing complexity and unique metabolite fingerprints of *O. kilimandscharicum*. Eugenol and camphor pathway- specific metabolites and intermediates have been discussed below in revelant sub-sections.

4.3.4 Camphor biosynthesis: Differential expression of both *gpps* and *bdh* is putatively responsible for partitioning of camphor

The putative camphor biosynthesis pathway starting from IPP/DMAPP comprised of 4 steps catalysed by 4 different enzymes: geranyl diphosphate synthase (*gpps*), bornyl diphosphate synthase (*bpps*), bornyl diphosphate diphosphatase (*bppd*) and borneol dehydrogenase (*bdh*) (**Fig. 4.5A**).[34-37] GPPS, catalysing the first step in the pathway, that is, conversion of IPP/DMAPP into GPP; and BDH, catalyzing the final step, that is, conversion from borneol to camphor were cloned from *O. kilimandscharicum*. GC-MS analysis revealed that aerial tissues including young leaves, mature leaves, inflorescence (Infl.), flower and stem accumulated large quantities of camphor (**Fig. 4.5B**). However, borneol, the precursor for camphor was not detected. This leads to the hypothesis that maybe in aerial tissues the efficency of conversion from borneol to camphor was high and hence no borneol was detected. This can be attributed to the high expression of *bdh* in aerial tissues. In roots, however, the conversion from borneol to camphor was not as

efficient, which was evident by the presence of significant amounts of both the precursor (borneol) as well as the product (camphor) (**Fig. 4.5B**).

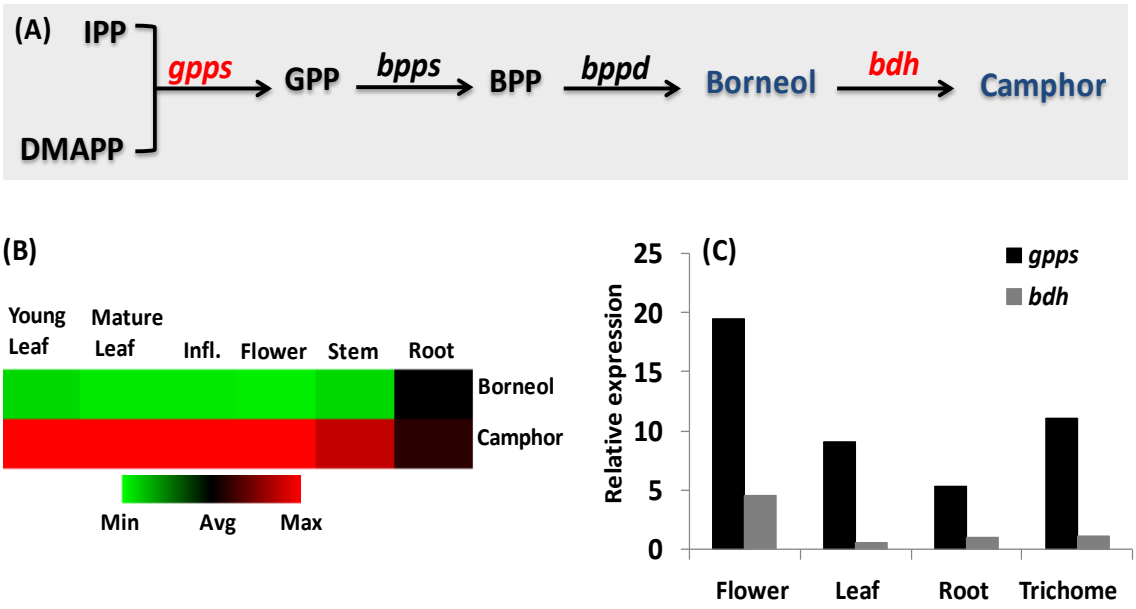


Figure 4.5: (A) Schematic representation of putative camphor biosynthetic pathway in *O. kilimandscharicum*. Genes marked in red are cloned and characterized to show their involvement in camphor biosynthesis [Isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), geranyl diphosphate synthase (*gpps*), bornyl diphosphate synthase (*bpps*), bornyl diphosphate diphosphatase (*bppd*), borneol dehydrogenase (*bdh*)] (B) GC-MS based tissue- specific profiling of pathway metabolites (borneol and camphor) in young leaves, mature leaves, inflorescence, stem and roots in *O. kilimandscharicum*.(C) Bar graph representing real time analysis for *gpps* and *bdh* across young leaves, flower, trichomes and roots

Higher expression of *gpps* was uniformly apparent in all aerial tissues including flower, young leaf and trichome as compared to roots. While the expression of *bdh* was significantly higher only in flower compared to other tissues (**Fig. 4.5C**). Thus, roots

show lower expression levels of both the pathway entry point enzyme (*gpps*) as well as the terminal enzyme (*bdh*). Although young leaves also show relatively lower expression level of *bdh*, the expression of *gpps* was significantly higher, which may be responsible for diverting the metabolic flux towards camphor production in leaves. Thus, the partitioning of camphor to aerial tissues can be attributed to differential expression of putative pathway genes (*gpps* and *bdh*).

4.3.5 Transient gene silencing and overexpression studies reveal role of *gpps* and *bdh* in camphor biosynthesis

To ascertain the role of *gpps* and *bdh* in camphor biosynthesis, both genes were functionally characterized via *Agrobacterium* mediated transient gene silencing (RNAi) and overexpression (OE) studies. The constructs designed for RNAi are depicted in **Fig. 4.6A**. For gene silencing, formation of hairpin structure is essential. Hairpin formation in the RNAi_GPPS and RNAi_BDH plants (in local leaf tissue) was confirmed by semi-quantitative PCR using intron-specific primers (**Fig. 4.6B**). Absence of intron in uninduced (control) and empty vector infiltrated plants acted as negative control (**Fig. 4.6B**). Following hairpin formation, *gpps* and *bdh* were successfully silenced, in both the local as well as systemic tissue of RNAi_GPPS and RNAi_BDH plants respectively as revealed by expression analysis (**Fig 4.6C**). For both *gpps* and *bdh*, the gene expression in uninduced (control) and empty vector infiltrated plants remained unaltered. To understand the effect of gene silencing on metabolite levels, GC-MS of infiltrated local leaf tissue was performed. In the RNAi_BDH plants, the relative abundance of camphor decreased significantly from 58.4% to 35.6% (**Fig. 4.6D**). Similarly, in the RNAi_GPPS

plants, the relative abundance of most monoterpenes including camphor, eucalyptol, terpinolene, thujanol, borneol, terpineol and myrtenol decreased significantly (**Fig. 4.6E**).

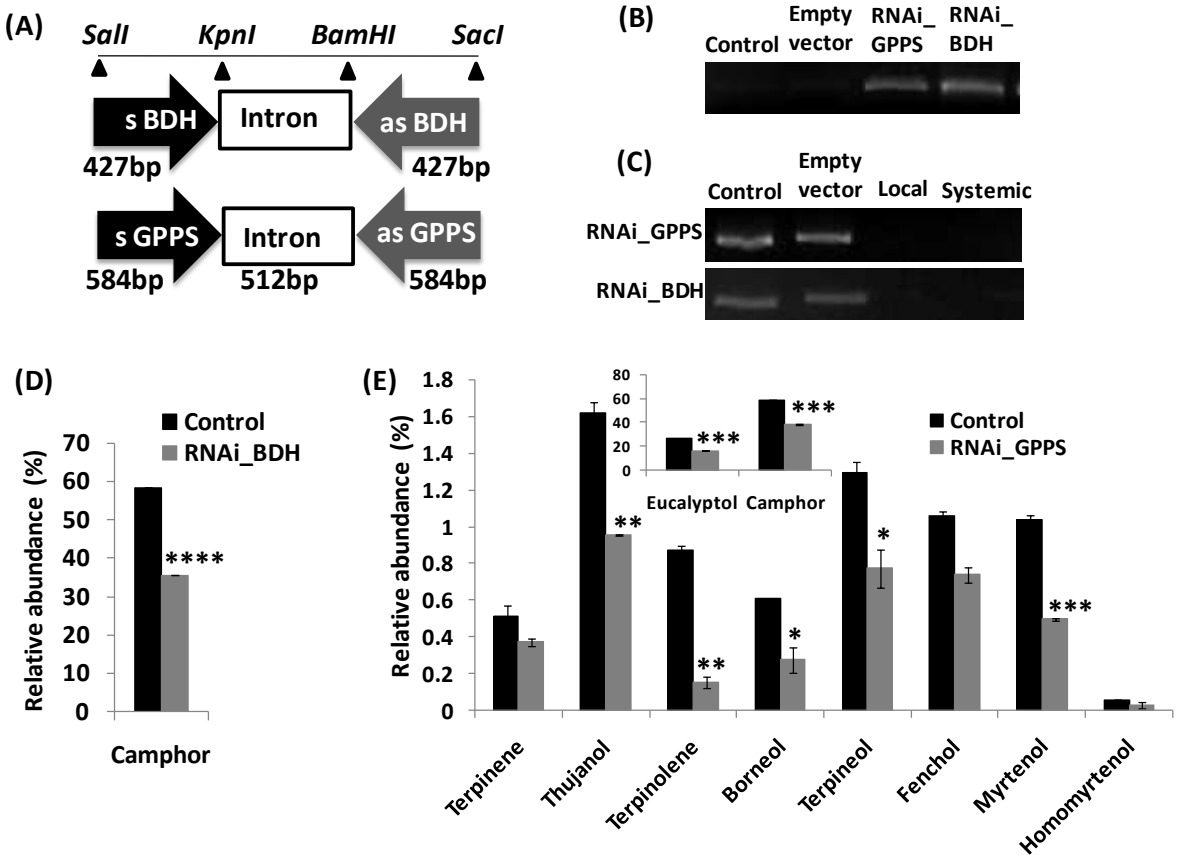


Figure 4.6: *A. tumefaciens*- mediated gene silencing (RNAi) of geranyl diphosphate synthase (*gpps*) and borneol dehydrogenase (*bdh*) in *O. kilimandscharicum* (A) construction of RNAi vectors using gene- specific sense (s) and antisense (as) sequences; (B) semi-quantitative PCR for detecting hairpin formation in uninduced leaves (control), leaves infiltrated with empty vector and local leaf tissue of RNAi_GPPS and RNAi_BDH plants; (C) semi-quantitative PCR showing RNAi- mediated gene silencing in local and systemic leaves of RNAi_GPPS and RNAi_BDH plants; GC-MS based volatile profile for (D) RNAi_BDH, and (E) RNAi_GPPS plants. Unpaired t-test suggested significant

differences between data at $p<0.0001$ (indicated as '****'), $p<0.001$ (indicated as '***'), $p<0.01$ (indicated as '**') and $p<0.05$ (indicated as '*').

Thus, gene silencing studies proved that *gpps* and *bdh* play key role in camphor biosynthesis and silencing either gene positively affects the production of camphor in plant.

Overexpression of *gpps* (OE_GPPS) and *bdh* (OE_BDH) was confirmed, 1.5 and 1.8 fold resp., by gene expression analysis compared to uninduced (control) plants (**Fig. 4.7A, B**).

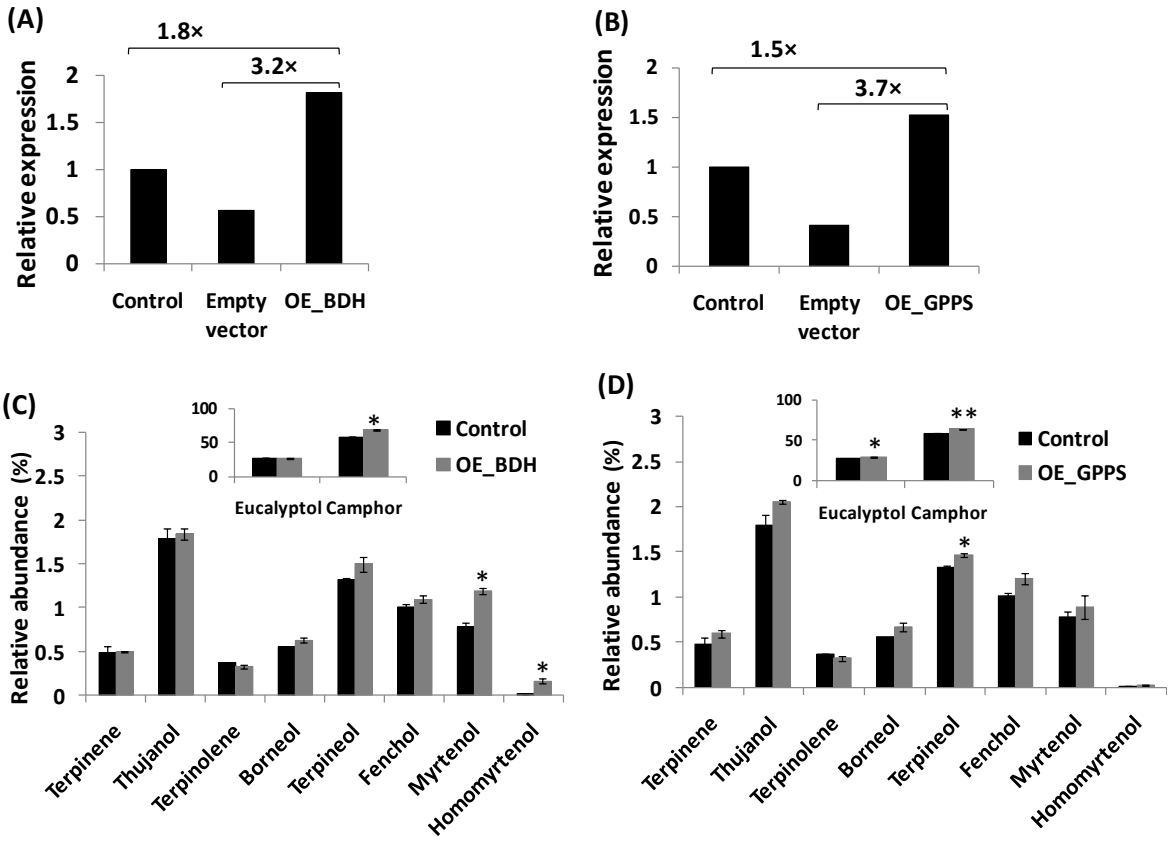


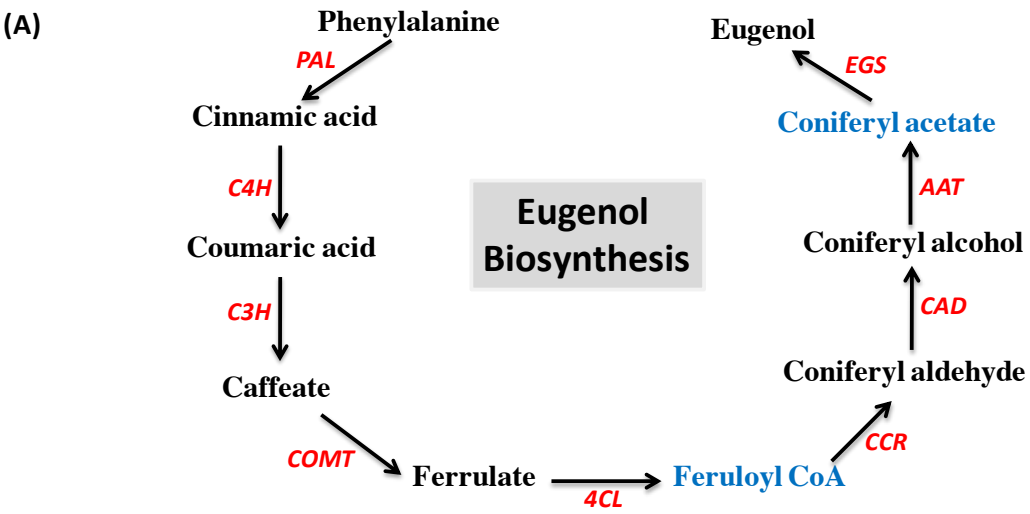
Figure 4.7: *A. tumefaciens*- mediated gene overexpression (OE) of geranyl diphosphate synthase (*gpps*) and borneol dehydrogenase (*bdh*) in *O. kilimandscharicum* (A) Real time PCR analysis for detecting gene expression in (A) OE_BDH and (B) OE_GPPS plants

compared to uninduced (control) and empty vector infiltrated leaves ; GC-MS based volatile profile for (C) OE_BDH, and (D) OE_GPPS plants. Unpaired t-test suggested significant differences between data at $p<0.0001$ (indicated as '****'), $p<0.001$ (indicated as '***'), $p<0.01$ (indicated as '**') and $p<0.05$ (indicated as '*').

Transient overexpression of BDH in leaf tissue resulted in significant increase in camphor content from 59 to 68.4% (**Fig. 4.7C**). The amount of other monoterpenes also increased, however, the increase was not significant. Further, transient overexpression of *gpps* resulted in significant increase in amount of major monoterpenes including camphor, eucalyptol and terpinolene (**Fig. 4.7D**). Although, the amount of other monoterpenes also increased, the increase was not found to be significant. Thus, gene overexpression studies support the findings of RNAi studies and confirm that indeed both *gpps* and *bdh* are involved in camphor biosynthesis thereby validating the proposed putative pathway. Thus, we dissected the camphor biosynthesis pathway from *O. kilimandscharicum* and showed that partitioning of camphor to the aerial plant parts is due to differential expression of pathway genes (*gpps* and *bdh*).

4.3.6 Eugenol biosynthesis and partitioning: Transport from source (leaves) to sink (root)

In *O. kilimandscharicum* plants, roots partition and accumulate large quantities of eugenol (56%). In order to understand the mechanism underlying partitioning of eugenol in roots, the genes and metabolites (pathway intermediates) involved in eugenol biosynthesis were analysed **(Fig. 4.8A**).



(B)

Metabolite	m/z
Cinnamic acid	149.0595
4-Coumarate	165.0545
Caffeate	181.0495
Ferrulate	195.0654
Coniferyl aldehyde	179.0702
Coniferyl alcohol	181.0859
Eugenol	165.0909

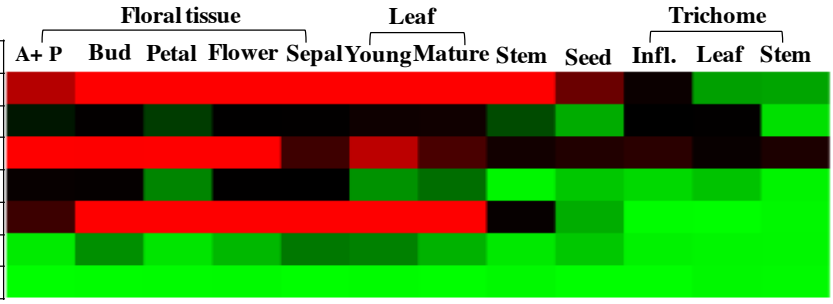


Figure 4.8: (A) Eugenol biosynthetic pathway from *Ocimum* species. Enzymes marked in red and metabolites/intermediates marked in black were detected in NGS and global metabolomic analysis respectively. Metabolites marked in blue could not be detected on the LC-Orbitrap. (B) Heat map showing accumulation of eugenol pathway intermediates and their corresponding mass/charge (m/z) ratio in aerial parts including anther and pistil (A+P), buds, petals, flowers, sepals, young leaves, mature leaves, stem, seeds and, trichomes of young leaves, inflorescence and stem

The eugenol biosynthetic pathway has been previously reported from *Ocimum*.[26] The pathway starts with the amino acid phenylalanine and through a sequential series of nine enzyme catalyzed steps gets converted to eugenol. All genes involved in eugenol

biosynthesis reported previously were detected in *O. kilimandscharicum*. NGS analysis revealed highest expression of *egs* in young leaves (RPKM, 89.3) followed by flowers (RPKM, 5.08). However, *egs* expression was not detected in roots. This result positively co-relates with the data from global untargeted metabolomics which shows greater accumulation of pathway intermediates like cinnamic acid, 4-coumarate, caffeate, ferrulate and coniferyl aldehyde in young leaves and organs like anther and pistil, bud, petal, flower and sepals (**Fig. 4.8B**). Integrating data from transcriptomics and metabolomics reveals that *O. kilimandscharicum* roots lack eugenol synthesis machinery. Hence we propose that eugenol is synthesized in the aerial tissue including leaves and/or their trichomes and transported to the roots *via* transporters.

4.3.7 Co-expression analysis suggests that transporter(s) might be involved in partitioning of eugenol in roots

In order to understand the entire machinery involved in biosynthesis, transport and partitioning of eugenol in *O. kilimandscharicum*, co-expression analysis of eugenol synthase (*egs1*) and coumarate-CoA ligase (*4cl4*) was performed. Co-expression analysis revealed several candidate genes including *pal1*, *4cl5*, *4cl6*, *4cl1*, *c4h2*, *aat3*, *comt*, *cad1*and *ccr* to be putatively involved in eugenol biosynthesis (**Fig. 4.9**). Of these *4cl6, comt, cad, c4h2, aat3* showed positive co-expression pattern with bait genes, while *4cl5, 4cl1, ccr* and *pal1* showed negative coexpression. Although eugenol biosynthesis has been reported from other *Ocimum* species,[26] co-expression analysis is essential to understand the involvement of specific isoforms in eugenol biosynthesis. For example, the enzyme coumarate Co-A ligase (*4cl*) has atleast ten isoforms in *O. kilimandscharicum*. Previously, *Rastogi et al* have reported the involvement of a very

specific isoform, *os4cl*, in eugenol biosynthesis.[38] Other isoforms of the same enzyme participate in formation of lignins, flavonoids or other classes of phenolic compounds. Co-expression analysis thus helps us understand the isoforms whose expression pattern across several tissues is similar to the expression of genes involved in eugenol biosynthesis.

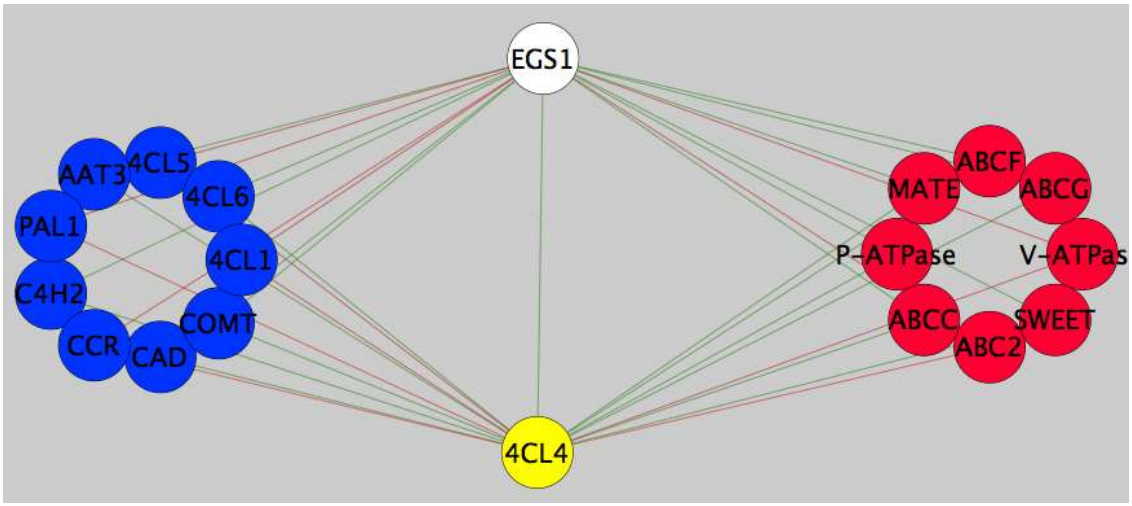


Figure 4.9: Gene co-expression network of EGS1 and 4CL4 using CoExpNetViz. Co-expression analysis depicting genes co-expresssed with EGS1 and 4CL4 (baits), including eugenol biosynthesis- related genes (marked in blue) and putative transporters (marked in red). Candidate genes positively co-expressing with bait genes are connected by green lines while genes showing negative correlation in expression are connected by red lines.

Several genes including those for ABC, MATE and sugar transporters were also co-expressed with eugenol biosynthesis machinery indicating their possible involvement in transport of eugenol towards the sink (**Fig. 4.9**). Different classes of ABC transporters including ABCC, ABC2, ABCF, ABCG were co-expressed with EGS1 and 4CL4. Of

these while ABCC, ABCF and ABCG showed positive co-expression pattern to the bait genes, ABC2 showed negative co-expression pattern. It is well known that ABC and MATE transporters are associated with transport of secondary metabolites in plants.[23,39,40] Also potentially long distance transport of phenylpropanoids is achieved by glycosylating the phenolic intermediates first.[41,42] The sugar transporter SWEET was positively co-expressed along with other transporters. Other co-expressed transporters include V-ATPases and P-ATPases, which may transport eugenol along the proton gradient. However, these candidate genes need to be functionally characterized using silencing and overexpression approaches to confirm their role in eugenol biosynthesis and transport. Co-expression analyis thus helped us retrieve candidate genes that may be putatively responsible for transport and metabolite partitioning in *O. kilimandscharicum*.

4.4 Conclusion

Metabolite partitioning was evident across several species of genus *Ocimum*. **Figure 4.10** gives a diagrammatic representation of metabolite partitioning and its underlying potential mechanisms. We attempted to understand mechanism of partitioining of camphor and eugenol in *O. kilimandscharicum* by integrating trascriptomics, global untargeted metabolomics and volatile profiling. We dissected the camphor biosynthetic pathway and proved that partitioning of camphor to the aerial tissues was attributed to tissue- specific expression of pathway entry point enzyme (GPPS) and terminal enzyme (BDH). Interestingly, the partitioining of eugenol to roots was attributed to transport from source (young leaves) to sink (roots) tissue in lieu of absence of *egs* expression in roots. We believe that partitioning of metabolites by the plant may have a defense related role, expecially because metabolites similar in chemical nature (example two

phenylpropanoids in *O*. *basilicum*) were also found to be partitioined in the same plant. We hypothesize that eugenol stored in roots, forms the second

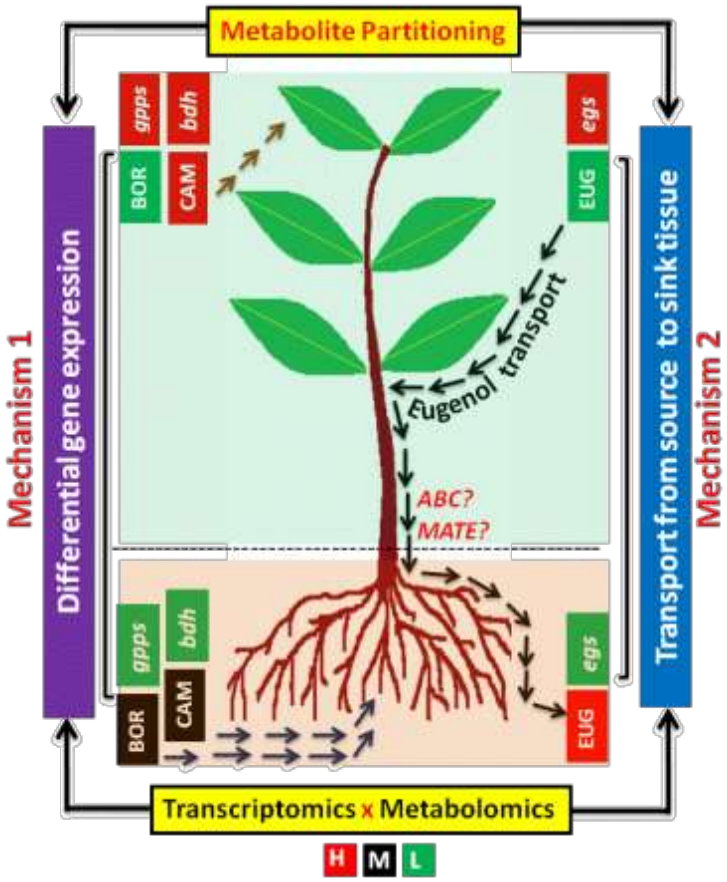


Figure 4.10: Diagrammatic representation of mechanism underlying metabolite partitioning in *O*. *kilimandscharicum*. [Borneol (BOR), camphor (CAM), eugenol (EUG), geranyl diphosphate synthase (*gpps*), borneol ehydrogenase (*bdh*), eugenol synthase (*egs*); high (H), medium (M),and low (L) expression or accumulation]

line of defense in *Ocimum*. It helps in combating insects and pests in later days of attack (example, day 3 onwards). It is slowly mobilized from roots to the aerial tissues upon insect attack as reported by us in our previous studies.[28] Since the composition of defense metabolite fraction changes constantly in the aerial parts, it provides an advantage to the plant to resist insect and pest attack. On the contrary, such mechanism offers less chance

to insect pests for adaptation to plant defenses. This may be the reason why *Ocimum* is not susceptible to pest attack compared to other plants as it is not only rich in secondary metabolites, but is also capable of changing the composition of defense fraction by remobilizing metabolites from root thereby illiciting a more effective defense response. Metabolite partitioning, however, may have other unforseeable consequences too.

4.5 References

(1) J. E. Lunn, Compartmentation in plant metabolism. *J.Exp. Bot.*, 2007, **58**, 35.

(2) A. Tiessen and D. Padilla-Chacon, Subcellular compartmentation of sugar signaling: links among carbon cellular status, route of sucrolysis, sink-source allocation, and metabolic partitioning. *Front.Plant Sci.*, 2013, **3**, 306.

(3) D. A. Nagegowda, Plant volatile terpenoid metabolism: biosynthetic genes, transcriptional regulation and subcellular compartmentation. *FEBS Lett.*, 2010, **584**, 2965.

(4) E. Ikonen, Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.*, 2008, **9**, 125.

(5) S. Gerdes, C. Lerma-Ortiz, O. Frelin, S. M. Seaver, C. S. Henry, V. de Crécy-Lagard and A. D. Hanson, Plant B vitamin pathways and their compartmentation: a guide for the perplexed. *J. Exp. Bot.*, 2012, **63**, 5379.

(6) P. D. Coley, J. P. Bryant and F. S. Chapin, Resource availability and plant antiherbivore defense. *Science*, 1985, **230**, 895.

(7) P. D. Coley, Effects of plant growth rate and leaf lifetime on the amount and type of anti-herbivore defense. *Oecologia*, 1988, **74**, 531.

(8) A. O. Lavinsky, P. C. Magalhães, R. G. Ávila, M. M. Diniz and T. C. de Souza, Partitioning between primary and secondary metabolism of carbon allocated to roots in four maize genotypes under water deficit and its effects on productivity. *Crop J*., 2015, **3**, 379.

(9) N. E. Havko, I. T. Major, J. B. Jewell, E. Attaran and G. A. Howe, Control of carbon assimilation and partitioning by jasmonate: An accounting of growth–defense tradeoffs. *Plants*, 2016, **5**, 7.

(10) R. Brouwer, Nutritive influences on the distribution of dry matter in the plant. *Neth*. *J*. *Agr*. *Sci*., 1962, **10**, 399.

(11) S. Kopriva, S. G. Mugford, P. Baraniecka, B. R. Lee, C. A. Matthewman and A. Koprivova, Control of sulfur partitioning between primary and secondary metabolism in *Arabidopsis*. *Front*. *Plant Sci*., 2012, **3**, 163.

(12) S. Osorio, Y. L. Ruan and A. R.Fernie, An update on source-to-sink carbon partitioning in tomato. *Front*. *Plant Sci*., 2014, **5**, 516.

(13) L. C. Ho, The mechanism of assimilate partitioning and carbohydrate compartmentation in fruit in relation to the quality and yield of tomato. *J*. *Exp*.*Bot*., 1996, **47**, 1239.

(14) Z. Gao, M. Sagi and S. H. Lips, Carbohydrate metabolism in leaves and assimilate partitioning in fruits of tomato (*Lycopersicon esculentum* L.) as affected by salinity. *Plant Sci*., 1998, **135**, 149.

(15) A. C. Worrell, J. M. Bruneau, K. Jummerfelt, M. Boersig and T. A. Voelker, Expression of a maize sucrose phosphate synthase in tomato alters leaf carbohydrate partitioning. *Plant Cell*, 1991, **3**, 1121.

(16) C. C. De Groot, L. F. M. Marcelis, R. Van den Boogaard and H. Lambers, H. Growth and dry-mass partitioning in tomato as affected by phosphorus nutrition and light. *Plant Cell Environ*., 2001, **24**, 1309.

(17) M. H. Ibrahim and H. Z. Jaafar, Impact of elevated carbon dioxide on primary, secondary metabolites and antioxidant responses of *Eleais guineensis Jacq*. (Oil Palm) seedlings. *Molecules*, 2012, **17**, 5195.

(18) A. Lorence, B. I. Chevone, P. Mendes and C. L. Nessler, Myo-Inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. *Plant Physiol*., 2004, **134**, 1200.

(19) P. A. Howles, V. J. Sewalt, N. L. Paiva, Y. Elkind, N. J. Bate, C. Lamb and R. A. Dixon, Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. *Plant Physiol*., 1996, **112**, 1617.

(20) L. Xiang, L. Zeng, Y. Yuan, M. Chen, F. Wang, X. Liu *et al*, Enhancement of artemisinin biosynthesis by overexpressing dxr, cyp71av1 and cpr in the plants of *Artemisia annua* L. *Plant Omics*, 2012, **5**, 503.

(21) F. Ludewig and U. I. Flügge, Role of metabolite transporters in source-sink carbon allocation. *Front*. *Plant Sci*., 2013, **4**, 231.

(22) T. Shoji, Y. Yamada and T. Hashimoto, Jasmonate induction of putrescine N-methyltransferase genes in the root of *Nicotiana sylvestris*. *Plant Cell Physiol*., 2000, **41**, 831.

(23) K. Yazaki, ABC transporters involved in the transport of plant secondary metabolites. *FEBS Lett*., 2006, **580**, 1183.

(24) P. Singh, R. M. Kalunke and A. P. Giri, Towards comprehension of complex chemical evolution and diversification of terpene and phenylpropanoid pathways in *Ocimum* species. *RSC Adv*., 2015, **5**, 106886.

(25) A. K. Upadhyay, A. R. Chacko, A. Gandhimathi, P. Ghosh, K. Harini, A. P. Joseph *et al*, Genome sequencing of herb Tulsi (*Ocimum tenuiflorum*) unravels key genes behind its strong medicinal properties. *BMC Plant Biol*., 2015, **15**, 1.

(26) D. R. Gang, J. Wang, N. Dudareva, K. H. Nam, J. E. Simon, E. Lewinsohn and E. Pichersky, An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. *Plant Physiol*., 2001, **125**, 539.

(27) S. Rastogi, S. Meena, A. Bhattacharya, S. Ghosh, R. K. Shukla, N. S. Sangwan *et al*, *De novo* sequencing and comparative analysis of holy and sweet basil transcriptomes. *BMC genomics*, 2014, **15**, 1.

(28) P. Singh, R. H. Jayaramaiah, P. Sarate, H. V. Thulasiram, M. J. Kulkarni and A. P. Giri, Insecticidal potential of defense metabolites from *Ocimum kilimandscharicum* against *Helicoverpa armigera*. *PloS one*, 2014, **9**, e104377.

(29) R. C. De Vos, S. Moco, A. Lommen, J. J. Keurentjes, R. J. Bino and R. D. Hall, Untargeted large-scale plant metabolomics using liquid chromatography coupled to mass spectrometry. *Nat*. *Protoc*., 2007, **2**, 778.

(30) D. Kessner, M. Chambers, R. Burke, D. Agus and P. Mallick, ProteoWizard: open source software for rapid proteomics tools development. *Bioinformatics*, 2008, **24**, 2534.

(31) C. A. Smith, E. J. Want, G. O'Maille, R. Abagyan and G. Siuzdak, XCMS: processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal*. *Chem*., 2006, **78**, 779.

(32) M. F. Clasquin, E. Melamud and J. D. Rabinowitz, LC-MS data processing with MAVEN: a metabolomic analysis and visualization engine. *Curr*. *Protoc*. *Bioinform*., 2012, **37**, 14.11.

(33) O. Tzfadia, T. Diels, S. De Meyer, K. Vandepoele, A. Aharoni and Y. Van de Peer, CoExpNetViz: comparative co-expression networks construction and visualization tool. *Front*. *Plant Sci*., 2015, **6**, 1194.

(34) R. Croteau, C. L. Hooper and M. Felton, Biosynthesis of monoterpenes: Partial purification and characterization of a bicyclic monoterpenol dehydrogenase from sage (*Salvia officinalis*). *Arch*. *Biochem*. *Biophysics*, 1978, **188**, 182.

(35) R. Croteau and F. Karp, Biosynthesis of monoterpenes: preliminary characterization of bornyl pyrophosphate synthetase from sage (*Salvia officinalis*) and demonstration that geranyl pyrophosphate is the preferred substrate for cyclization. *Arch*. *Biochem*. *Biophys*., 1979, **198**, 512.

(36) R. Croteau and F. Karp, Biosynthesis of monoterpenes: hydrolysis of bornyl pyrophosphate, an essential step in camphor biosynthesis, and hydrolysis of geranyl pyrophosphate, the acyclic precursor of camphor, by enzymes from sage (*Salvia officinalis*). *Arch*. *Biochem*. *Biophys*., 1979, **198**, 523.

(37) L. S. Sarker, M. Galata, Z. A. Demissie and S. S. Mahmoud, Molecular cloning and functional characterization of borneol dehydrogenase from the glandular trichomes of *Lavandula x intermedia*. *Arch*. *Biochem*. *Biophys*., 2012, **528**, 163.

(38) S. Rastogi, R. Kumar, C. S. Chanotiya, K. Shanker, M. M. Gupta, D. A. Nagegowda and A. K. Shasany, 4-Coumarate: CoA ligase partitions metabolites for eugenol biosynthesis. *Plant and cell physiology*, 2013, pct073.

(39) J. Ehlting, N. Mattheus, D. S. Aeschliman, E. Li, B. Hamberger, I. F. Cullis *et al*, Global transcript profiling of primary stems from *Arabidopsis thaliana* identifies candidate genes for missing links in lignin biosynthesis and transcriptional regulators of fiber differentiation. *Plant J*., 2005, **42**, 618.

(40) C. J. Liu, Y. C. Miao and K. W. Zhang, Sequestration and transport of lignin monomeric precursors. *Molecules*, 2011, **16**, 710.

(41) J. Le Roy, B. Huss, A. Creach, S. Hawkins and G. Neutelings, Glycosylation is a major regulator of phenylpropanoid availability and biological activity in plants. *Front*. *Plant Sci*., 2016, **7**, 735.

(42) T. Vogt, Phenylpropanoid biosynthesis. *Mol*. *Plant*, 2010, **3**, 2.

CHAPTER 5

Summary and Conclusion

This chapter provides a comprehensive summary of the work done, with major conclusions from each chapter, and future prospects of the work undertaken.

Chapter 1 presents the vast metabolic diversity existent in genus *Ocimum*. It provides cumulative information about the potential reason/s (evolutionary, environmental and molecular) for the complex chemical evolution across *Ocimum* species. We conclude that genes for both terpenoid and phenylpropanoid biosynthesis are present in all *Ocimum* species. However, several factors including genetic background, habit, ploidy levels, hybridization, differential gene expression, transcriptional and post translational modifications, isozymes etc. have played a major role in metabolic pathway diversification making *Ocimum* species either terpene- or phenylpropanoid- rich (**Fig. 5.1**). Additionally, the presence of many terpene synthases in single species and each one's ability to synthesize diverse metabolites from a single sustrate has further complicated the chemical evolution process.

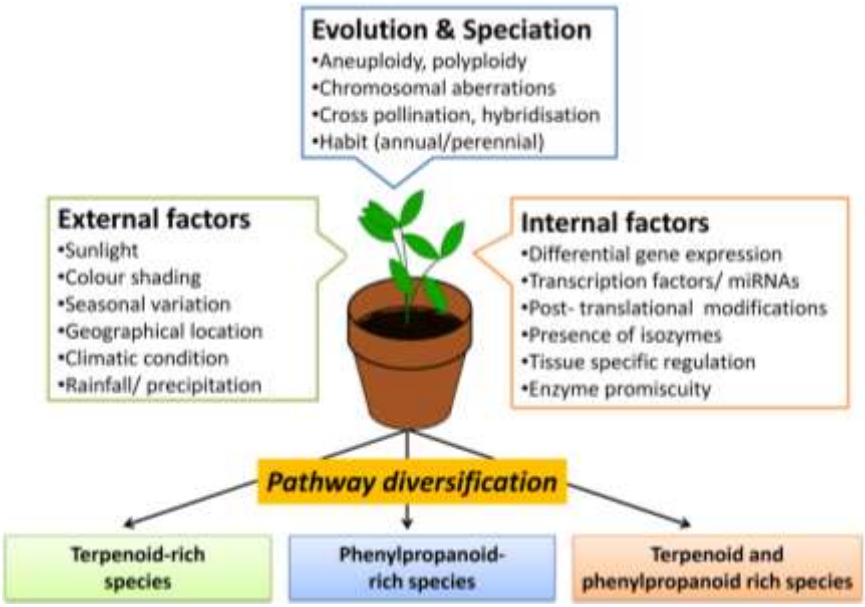


Figure 5.1 Factors responsible for chemical diversity; and terpenoid and phenylpropanoid pathway diversification in *Ocimum* species

Chapter 2 dealt with exploring the *Ocimum* metabolome for medicinally important metabolites. We identified eugenol, a phenylpropanoid, isolated from *O*. *gratissimum*, as a natural, FDA-approved non-toxic potent AGE inhibitor. We propose that eugenol has a potential dual mode of action (**Fig. 5.2**). It inhibits intestinal α-glucosidase and block conversion of complex carbohydrates to glucose, resulting in lowering blood glucose level. In addition, eugenol also competittively inhibits the binding of sugar to serum albumin by binding to amine group of surface exposed lysine residues *via* its reactive 4' -hydroxyl group. Thus, here we report eugenol, a potent inhibitor of AGEs that can be used in management of diabetes.

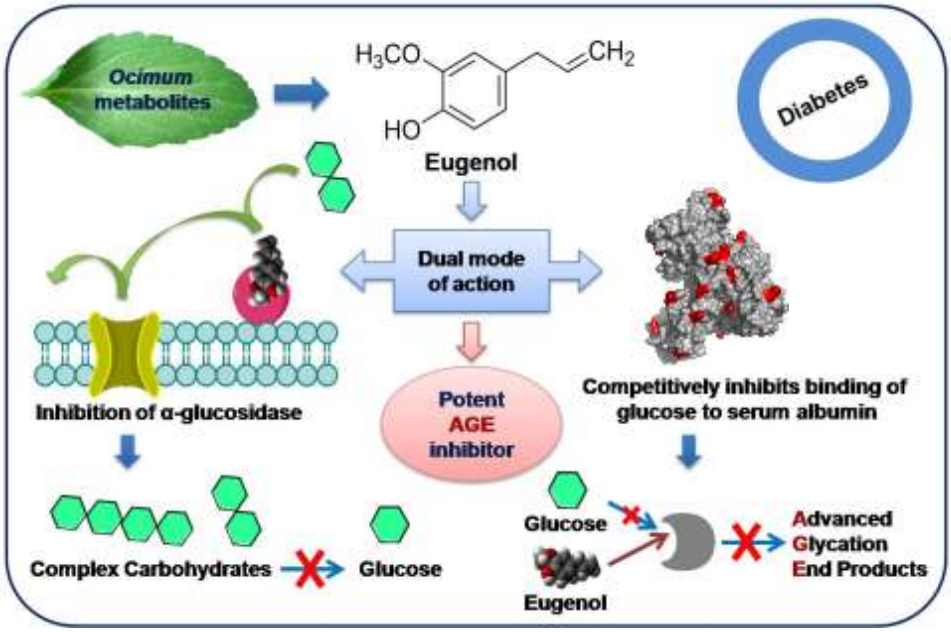


Figure 5.2 Schematic presentation of proposed potential dual role of eugenol in inhibiting AGEs.

In **Chapter 3,** we explored the *Ocimum* metabolome for potential insecticidal metabolites that may have agricultural applications. The interaction between a non-host

plant known for its strong defense (*O*. *kilimandscharicum*) and a devastating insect pest (*H*. *armigera*) was studied. The plant elicited a strong defense response to counteract larval infestationby upregulating metabolites including monoterpenes, sesquiterpenes and phenylpropanoids thereby adversely affecting larval growth and development (**Fig. 5.3**). Importantly, selected compounds from *O*. *kilimandscharicum* leaves including β-caryophyllene, terpinolene and limonene were also able to retard larval growth and induce pupal deformities in *H*. *armigera* when incorporated in artificial diet in ppm quantities. We conclude that *O*. *kilimandscharicum* plant and its defense metabolites possess strong insecticidal activity and can be used for controlling pest population in agricultural fields.

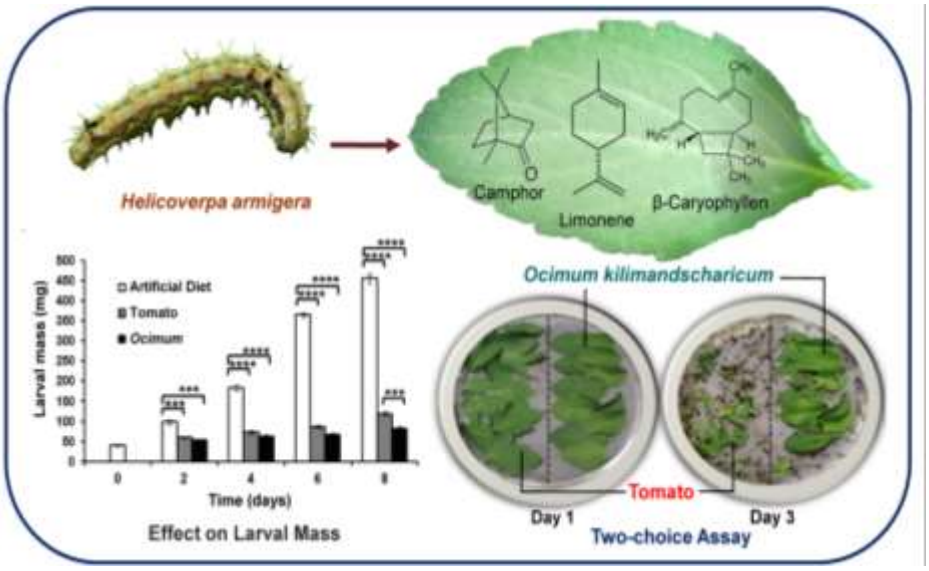


Figure 5.3 Schematic representation of antibiosis to *H*. *armigera* upon interacting with *O*. *kilimandscharicum* leaf and its defense related metabolite fraction.

Finally, in **Chapter 4** we dissect the mechanism for biosynthesis, storage and transport of medicinally and agriculturally important metabolites including camphor and eugenol. We elucidated the camphor biosynthetic pathway from camphor basil and proved the role of

gpps and *bdh* in regulating tissue- specific camphor levels. By integrating information from transcriptomics and metabolomics, we also studied how these metabolites are partitioned between the aerial and root system in the plant (**Fig. 5.4**). While partitioning of camphor in the aerial tissues was attributed to differential gene expression of the pathway entry point (*gpps*) as well as terminal enzyme (*bdh*); partitioning of eugenol in roots was mediated *via* long distance transport from leaves evident by absence of *egs* in roots. Co-expression analysis revealed candidate transporter genes that may aid in eugenol mobilization including ABC, MATE and SWEET transporters. We believe that metabolite partitioning may have specific defense- related implications in the *Ocimum* plant.

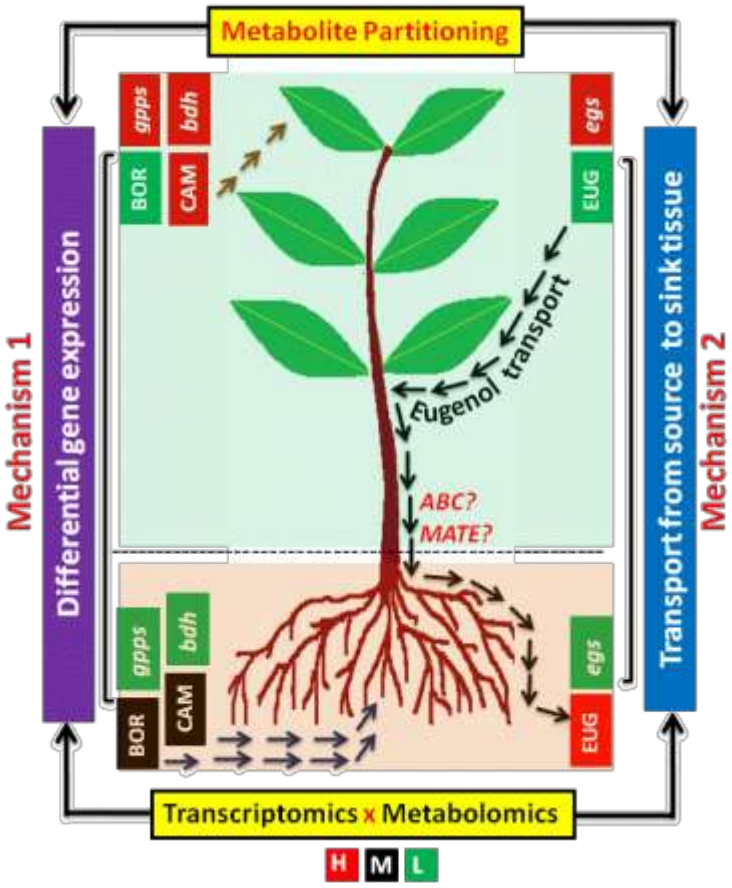


Figure 5.4 Mechanism underlying metabolite partitioning in *O. kilimandscharicum*.

The present work lays a strong foundation for further studies on genus *Ocimum*. *Ocimum* metabolome can be further tapped for other medicinally-, agriculturally- or commercially-important metabolites. Understanding the mode of action of medicinally essential metabolites, at molecular level, can help in management of several diseases. Also, these metabolites can be chemically derivatized to enhance their bioactivity and improve their potency. Understanding the *in planta* biosynthesis and transport of these metabolites *via* functional characterization of candidate genes, like transporters, is also important.It would further aid in engineering these pathways, in heterologous plant or bacterial systems, enabling large scale production of important metabolites.

LIST OF PUBLICATIONS

(1) M. Itkin, U. Heinig, O. Tzfadia, A. J. Bhide, B. Shinde, P. Cardenas, S. E. Bocobza, T. Unger, S. Malitsky, R. Finkers, Y. Tikunov, A. Bovy, Y. Chikate, P. **Singh**, I. Rogachev, J. Beekwilder, A. P. Giri and A. Aharoni, Biosynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. *Science*, 2013, **341**, 175.

(2) **P. Singh**, R. H. Jayaramaiah, P. Sarate, H. V. Thulasiram, M. J. Kulkarni and A. P. Giri, Insecticidal potential of defense metabolites from *Ocimum kilimandscharicum* against *Helicoverpa armigera*. *PloS One*, 2014, e104377.

(3) **P. Singh,** R. M. Kalunke and A. P. Giri, Towards comprehension of complex chemical evolution and diversification of terpene and phenylpropanoid pathways in *Ocimum* species. *RSC Adv*., 2015, **5**, 106886.

(4) **P. Singh,** R. H. Jayaramaiah, S. B. Agawane, G. Vannuruswamy, A. M. Korwar, A. Anand, V. S. Dhaygude, M. L. Shaikh, R. S. Joshi, R. Boppana, M. J. Kulkarni and A. P. Giri, Potential dual role of eugenol in inhibiting advanced glycation end products in diabetes: Proteomic and mechanistic insights. *Sci*. *Rep*., 2016, **6**, 18798.

(5) A. Anand, R. H. Jayaramaiah, S. D. Beedkar, **P. Singh**, R. S. Joshi, F. A. Mulani, B. Dholakia, S. A. Punekar, W. N. Gade, H. V.Thulasiram and A. P. Giri, Comparative functional characterization of eugenol synthase from four different *Ocimum* species: implications on eugenol accumulation. *BBA-Proteins and proteomics*, 2016.

(6) **P. Singh,** R. M. Kalunke, O. Tzfaldia, A. Shukla, H. V. Thulasiram and A. P. Giri Integrating transcriptomics with metabolomics reveals tissue- specific metabolite partitioning involving long distance transport in *Ocimum kilimandscharicum*. (Manuscript submitted)

(7) V. D. Anumone, **P. Singh**, A. P. Giri., M. V. Badiger**.** Eugenol incorporated polyurethane hydrogels as potent anti-diabetic polymers (Manuscript under preparation)

LIST OF PATENTS

1. A method for inhibition of α-glucosidase and advanced glycation end products (AGEs) in diabetes using eugenol (INV-2015-117)

PRIYANKA SINGH

E-Mail: chandnisingh22@gmail.com
Mobile No.: +919881732285

~ Biotechnology, Genetic Engineering, Medicinal Chemistry, Drug Development, Transcriptomics, Metabolomics, Mass Spectrometry~

PROFILE SUMMARY

A research enthusiast holding **Ph.D. in Biotechnology** from **CSIR- National Chemical Laboratory, Pune** with **6 years of research** and **2 years of teaching experience**.

- Publications in **high impact factor journals** (*Science*, *Nature Sci. Rep*).
- Demonstrated expertise in **genetic engineering, molecular biology, pathway engineering, drug development, transcriptomics, metabolomics, mass spectrometry, transgenic technology**.
- Worked in international research institutes and displayed excellent **spirit for team work and troubleshooting ability**.
- Proficient in **development & implementation of research plans** and **methodologies**.
- Experience in **mentoring research interns, trainees** and **students**.
- An **effective communicator** & **team leader** with strong analytical and organizational abilities with **flexible, detail oriented attitude.**

AREAS OF EXPERTISE

Biotechnology and Genetic Engineering

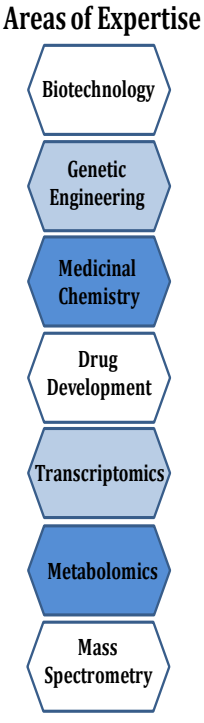
- Dissecting biosynthetic pathways of commercially/medicinally important metabolites
- Cloning and bacterial expression of candidate genes
- Developing transient transgenics by gene overexpression & silencing (RNAi) technique
- Real time analysis of gene expression

Medicinal Chemistry and Drug Developement

- Identifying potent medicinal metabolites from plants using *in vitro* and *in vivo* assays
- Isolation, purification and NMR characterization of potential drug molecules from plants
- Biosphysical characterization of interaction of drug with proteins
- Enzyme inhibition and kinetics upon drug binding
- Histopathologic examination for effect of drug administration on tissues
- Proteomic analysis including western blotting and LC-MS analysis using Triple- TOF and LC-Orbitrap to understand drug induced modifications at specific residues.

Transcriptomics

- Analysing data generated from next generation sequencing using Illumina platform.
- Performing co-expression and digital gene expression analysis



Metabolomics

- Handling LC-Orbitrap for global untargeted metabolomics followed by data analysis using XCMS (R program), XCMS online, Maven, Metlin etc.
- Handling gas chromatography- mass spectrometry (GC-MS) for volatile analysis

Mass- spectrometry

- Handling LC-Orbitrap, HDMS-Synapt, Triple-TOF, MALDI-TOF and GC-MS for several analyses

RESEARCH AND TEACHING EXPERIENCE

Since July'10: CSIR- National Chemical Laboratory, Pune, as PhD Researcher

Growth Path:

Jul'10-Jul'12: CSIR- Junior Research Fellow

Since Jul'12: CSIR- Senior Research Fellow

PhD thesis title

Metabolomics and Camphor Biosynthetic Pathway Analysis of *Ocimum kilimandscharicum* (Camphor basil)

Highlights:

- Publications in high impact factor journals like *Science* and *Nature Scientific Reports*.
- 4 full-length research papers published in peer-reviewed journals
- 3 manuscripts under preparation
- 1 patent filed
- Handled and mentored 6 research interns towards dissertation thesis
- Attended 3 international conferences
- Organising member for international conference on GMO Food Safety at CSIR-NCL

Jul'12 – Dec'12: Weizmann Institute of Science, Rehovot, Israel as Invited Researcher

Worked as an **Invited Researcher** at the **Weizmann Institute of Science, Rehovot, Israel** in the lab of Prof. Asaph Aharoni, Dept. of Plant Sciences on "*Elucidation of Biosynthetic pathway of Steroidal Glycoalkaloids in Solanaceous plants*".

Highlights:

- Collaborated with scientists from different institutes in Israel, India and Netherlands
- Published work in the prestigious *Science* Magazine (Impact Factor 33.6)

Jun'08 – Nov'09: L.A.D. College, Nagpur, Maharashtra as Full- time Post Graduate Lecturer

Employed as a ***lecturer*** **(for PG/M.Sc. Biotechnology)** at Dept. of Biotechnology, L.A.D. College, Nagpur on full time contract basis.

Highlights:

- Conducted **lectures** and **biotechnology practicals** for post- graduation students
- Helped in forming the teaching timetable and **conducting regular exams, seminars** and **presentations**.
- Active **organising member & hands-on-instructor** for **"BIOtechnique"** – *a workshop on techniques in Molecular Biology, Biophysical Chemistry and Immunology*, organized by R.B. School of Biotechnology, L.A.D. College, Nagpur
- Participated (**as faculty**) in presentation of paper titled "**Biotechnological approach for aroma production using agro-industrial waste by *Ceratocyctis*",** at NCOAT-NIRMITI-2009 (National conference)
- Participated in scientific deliberations/poster presentation at, " ***Herbotech 2007*** ", a **DST & ICMR** sponsored National seminar on **"Green treasure: An Interdisciplinary approach: Prospects and promises for human welfare"** organized by Dharampeth M.P. Deo Memorial Science College , Nagpur

Jan'08 – Apr'08: University of Delhi, South Campus, New Delhi as Research Intern

- Worked as **Research Intern** at the **University of Delhi, South Campus, New Delhi** in the lab of Dr. Saurabh Raghvanshi, Dept. of Plant Molecular Biology (DPMB) on **"*Detailed analysis of argonaute proteins involved in microRNA biogenesis*"**

PUBLICATIONS AND PATENTS

- Itkin M., Heinig U., Tzfadia O., Bhide A. J., Shinde B., Cardenas P., Bocobza S. E., Unger T., Malitsky ., Finkers R., Tikunov Y., bovy A., Chikate Y., **Singh P.**, Rogachev I., Beekwilder J., Giri A. P., Aharoni A. (2013) Biosynthesis of antinutritional alkaloids in solanaceous crops is mediated by clustered genes. *Science*, 341, 175-179. (Impact Factor - 33.6)
- **Singh, P.**, Jayaramaiah, R. H., Sarate, P., Thulasiram, H. V., Kulkarni, M. J., Giri, A. P. (2014) Insecticidal Potential of Defense Metabolites from *Ocimum kilimandscharicum* against *Helicoverpa armigera*. *PloS One*, e104377. (Impact Factor - 3.2)
- **Singh, P.**, Kalunke, R. M., & Giri, A. P. (2015) Towards comprehension of complex chemical evolution and diversification of terpene and phenylpropanoid pathways in *Ocimum* species. *RSC Advances*, 5, 106886-106904. (Impact Factor - 3.84)
- **Singh, P.**, Jayaramaiah, R.H., Agawane, S.B., Vannuruswamy, G., Korwar, A.M., Anand, A., Dhaygude, V.S., Shaikh, M.L., Joshi, R.S., Boppana, R., Kulkarni, M.J., Giri A.P. (2016) Potential Dual Role of Eugenol in Inhibiting Advanced Glycation End Products in Diabetes: Proteomic and Mechanistic Insights. *Scientific reports*, 6, 18798. (Impact Factor - 5.6)
- **Singh, P.**, Kalunke, R., Giri A.P. Integrating transcriptomics with metabolomics reveals tissue- specific metabolite partitioning in camphor basil. (Manuscript under preparation)
- Anand, A., Jayaramaiah, R.H., Bidkar, S., **Singh**, **P.**, Joshi, R.S. , Gade, W. N., Thulasiram, H. V., Giri, A. P. Phenylpropanoid abundance of *Ocimum* spp. is regulated by upstream hydroxylase and acyltransferase.(Manuscript under preparation)
- Anumone V.D., **Singh P.** Eugenol incorporated polyurethane hydrogels as potent anti-diabetic polymers (Manuscript under preparation)
- A method for inhibition of α-glucosidase and advanced glycation end products (AGEs) in diabetes using eugenol (Patent filed)

EDUCATION

- **Xth** from Bhartiya Vidya Bhavan, Nagpur CBSE in 2001 (88 %)
- **XII** from Bhartiya Vidya Bhavan, Nagpur CBSE in 2003 (86 %)
- **B.Sc. (Biotechnology)** from L.A.D. College, R.T.M. Nagpur University, Nagpur in 2006 (81 %)
- **M.Sc. (Biotechnology)** from L.A.D. College, R.T.M. Nagpur University, Nagpur in 2008 (67 %)
- **Ph.D. (Biotechnology)** from CSIR-National Chemical Laboratory, Pune in 2016 (Coursework CGPA 8.9)

TRAINING/COURSES UNDERTAKEN

- Special training on **Plant Molecular Biology and Tissue Culture Techniques** at Biotechnology section of Central Institute of Cotton Research (CICR), Nagpur, Maharashtra (M.S.)
- **On-job training programme** at Rainbow Medinova Diagonistic Services, Nagpur, Maharashtra (M.S.)
- **Certificate course in german language** from R.T.M. University, Nagpur, Maharashtra (M.S.)
- **C and C++ computer programming courses** from Aptech Computer Education, Nagpur, Maharashtra (M.S.)

EXTRACURRICULAR ACTIVITIES

- Spreading **diabetes awareness** & importance of **hydroponic method of crop cultivation** among **rural belts of India** as a part of CSIR 800-linked AcSIR project

PERSONAL DETAILS

Date of Birth:	22nd February 1985
Languages Known:	Hindi, English, Marathi, German
Permanent Address:	"Shantiniketan", 20-21, Mashruwala Marg, Dharampeth extension, Cement Road, Nagpur- 440010 Maharashtra