DIVERSITY ANALYSIS OF *Murraya koenigii* (l.) Spreng IN KERALA THROUGH PHYTOCHEMICAL AND GENETIC PROFILING

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(2015-09-004)

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DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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By

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THESIS

Submitted in partial fulfilment of the requirement for the degree of

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DEPARTMENT OF PLANT BIOTECHNOLOGY
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KERALA, INDIA
2020

DECLARATION

I hereby declare that the thesis entitled **"Diversity analysis of *Murraya koenigii* (L.) Spreng in Kerala through phytochemical and genetic profiling"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Place: Vellayani
Date: 17/11/2020

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KSCSTE - Jawaharlal Nehru Tropical Botanic Garden and Research Institute
An institution of Kerala State Council for Science, Technology & Environment
National Centre of Excellence

CERTIFICATE

Certified that this thesis entitled **"Diversity analysis of *Murraya koenigii* (L.) Spreng in Kerala through phytochemical and genetic profiling"** is a record of research work done by **Ms. ASWANI SURESHBABU T. (2015-09-004)** under my guidance and supervision and that this has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Aswani Sureshbabu T.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

α	Alpha
β	Beta
γ	Gamma
δ	Delta
%	Percentage
° C	Degree Celsius
µg	Microgram
µL	Microlitre
µM	Micromolar
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
Ach	Acetylcholine
AchE	Anticholinesterase
AFLP	Amplified Fragment Length Polymorphisms
bp	Base pair
CAT	Catalase
cm	centimeter
DNA	Deoxyribonucleic acid
EDTA	Ethylene Diamine tetra acetic acid
et al.	et alia
EtBr	Ethidium bromide
FT-IR	Fourier Transform Infra-Red spectroscopy
g	Gram
GC	Gas Chromatography
GPX	Glutathione peroxidase
GRD	Glutathione reductase
GSH	Glutathione
LPO	Lipid peroxidation NO Nitric oxide assay
HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin Layer

ISSR	Inter Simple Sequence Repeats
ITS	Internal Transcribed Spacer
KAU	Kerala Agricultural University
Kb	Kilo bases
Kg	Kilogram
KSCSTE-JNTBGRI	KSCSTE-Jawaharlal Nehru Tropical Botanic Garden and Research Institute
L	Litre
L.	Linnaeus
m	Meter
M	Molar
mg	milligram
$MgCl_2$	Magnesium Chloride
min	Minute
mL	Millilitre
mm	Millimeter
mM	Millimolar
MS	Mass Spectrometry
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometer
NMR	Nuclear Magnetic Resonance spectroscopy
OD	Optical density
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PVP	Polyvinylpyrrolidone
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
rpm	revolutions per minute
s	second
SCAR	Sequence Characterized Amplified Region
SCoT	Start Codon Targeted Marker
SNP	Single Nucleotide Polymorphisms
SOD	Superoxide dismutase

SSR	Simple Sequence Repeats
Taq	*Thermus aquaticus*
TAE	Tris-acetate EDTA buffer
TE	Tris-EDTA buffer
TLC	Thin Layer Chromatography
Tm	Melting temperature
UV	Ultra violet
V	Volt
w/v	weight/volume
µg	Microgram

1. INTRODUCTION

Murraya koenigii (source plant of curry leaf), is one among the widely used spices world over. It belongs to the family *Rutaceae*. The plant is naturally found in the Indo Malay region, and is distributed usually in tropical and subtropical region (Handral *et al.* 2012). The plant is important for its leaves which are used for flavouring and spicing of food. The curry leaves have several medicinal properties such as anti-diabetic, antioxidant, antimicrobial, anti-inflammatory, anti-carcinogenic and hepatoprotective activity (Kirupa and Kariitha, 2015).

There are large numbers of bioactive compounds present in plants. The extraction of secondary metabolites from plants and their quantitative and qualitative evaluation are important for the discovery of new biomolecules that can be directly used by the pharmaceutical and agrochemical industries. Phytochemistry defines the metabolites found in the plants (Ingle *et al.* 2017). Curry leaves are the greatest source of carbazole alkaloids such as koenigine and mahanimbine. Carbazole alkaloids and triterpenes isolated from *M. koenigii* has showed anticancer and antioxidant properties (Singh *et al.* 2014). Fresh leaves of *M. koenigii* are the source of volatile and non-volatile compounds. The leaves yielded 0.5% essential oil by hydrodistillation method. The GC-MS analysis revealed that, the oil was found to contain cis-caryophyllene, dipentene, α-eudesmol, iso-caryophyllene and β-elemene as the major compounds. The oil composition suggests that the oil might find use in heavy type of soap as fixative, perfumes and detergent (Chowdhury, 2000).

Phytochemical analysis include the extraction and analysis of bioactive compounds present in the plant extracts by various analytical methods such as TLC (Thin Layer Chromatography), HPLC (High Performance Liquid Chromatography), GC (Gas Chromatography), HPTLC (High Performance Thin Layer Chromatography), and its detection through UV-Vis spectroscopy, Fourier Transform Infra-Red spectroscopy (FT-IR), Nuclear Magnetic Resonance spectroscopy (NMR), and Mass Spectrometry (MS) (Ingle *et al.* 2017). Diversity evaluation among the plant species is regularly conducted

using techniques like biochemical, morphological and molecular analysis. Molecular markers are considered as the vital tools for assessing plant diversity (Govindaraj *et al.* 2015). Inter Simple Sequence Repeat (ISSR), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Single Nucleotide Polymorphism (SNP), Ribosomal Internal Transcribed Spacer (ITS), Sequence - Characterized Amplified Region (SCAR), Simple Sequence Repeats (SSR) or Microsatellites, and Start Codon Targeted Polymorphism (SCoT) are the major molecular markers used for the analysis of genetic diversity of plants. Short conserved region adjoining the ATG start codon in plant genes are considered as the basis for the development of Start Codon Targeted (SCoT) plant DNA marker. Single 18-mer primers are used in polymerase chain reaction (PCR) of SCoT with an annealing temperature of 50˚C. Standard agarose gel electrophoresis is used in the separation of PCR amplicons. The specified method was also applied in rice with a collection genetically diverse genotypes as well as population raised by backcross (Devi *et al.* 2019).

The present study entitled "Diversity analysis of *M. koenigii* (L.) Spreng in Kerala through phytochemical and genetic profiling" has been undertaken with the major objective of selecting elite accessions of *M. koenigii* with respect to aroma compounds and essential oil content, to find the optimal season and maturity stage for harvest based on the organic volatile compounds of *Murraya koenigii* leaves. The evaluation of genetic diversity at intra species level has also been studied using SCoT marker. This study has also been made to discover variation of quality and quantity of volatile essential oil of elite lines of *M. koenigii* in Kerala through chemical profiling in different season as well as different places. The elite accessions selected can be used for large scale cultivation in the state.

2. REVIEW OF LITERATURE

2.1 INTRODUCTION

Our country possess a wealthy floristic diversity and has approximately 47,000 species of plants, out of which 7,500 are attributed with healthcare applications; while around 800 species are used for making ayurvedic formulations. A significant number of plants remain unknown regarding their pharmacological potentialities and may be used as repositories of possible pharmacologically active compounds for the production of new scaffolds to different ailments being resisted (Gahlawat *et al.* 2014). One of them is *Murraya koenigii,* also known as curry leaf, member of the Rutaceae family grows widely in Indian region. The plant has broad use in culinary purposes and amongst the major components in conventional Ayurvedic system possesses wide pharmacological properties as well (Kamat *et al.* 2015).

2.2 DISTRIBUTION OF *Murraya koenigii*

The family Rutaceae consists of 160 genera and nearly 2,000 species. The genus contains shrubs and small trees, distributed worldwide, especially in temperate and tropical regions. Rutaceae includes several economically important fruit trees as well as ornamental species. Some of the members have aromatic leaves, with oil glands on the surfaces. *Murraya koenigii* is native to Sri Lanka, India and other countries in south Asia. Curry leaf trees are naturalised in wastelands in India, except in the higher altitudes of the Himalayan region. *M. koenigii* is abundantly distributed in hills of Uttaranchal, Assam, Sikkim to Garhwal, Madhya Pradesh, Tamil Nadu, Andhra Pradesh, and Kerala. And later the plant reached South Africa, Malaysia and Reunion Island along with South Indian immigrants. It decorates almost all the houses of Southern India and is widely cultivated in Australia, China, Ceylon, and Burma (Rana *et al.* 2004).

2.3 TRADITIONAL USE OF *Murraya koenigii*

The application of curry leaves as a vegetable flavouring agent has been recorded in the early Tamil literature of the 1st to 4th centuries A.D. It is also mentioned in Kannada literature a few centuries later. The curry leaves are very closely related to South India, where the term curry derives from Tamil 'Kari' for spiced sauces. Kari-pattha is the alternative name for curry leaf in India (Singh *et al.* 2014).

The traditional medicine system in East Asia discusses the essential uses of this herb. Leaves of *M koenigii* are an important part of the Indian diet to increase appetite and digestion. *M koenigii* is used as a stimulant, anti-dysenteric and for the cure of diabetes mellitus. The leaves, root and bark possess, anthelmintic, analgesic, anticarminative, antiemetic, laxative and anti-carcinogenic properties (Khanum *et al.* 2000). . The leaves can be used externally for the cure of wounds, skin eruptions and for the treatment of bites with poisonous animals. Traditionally the leaves are used to minimize body pain, fatigue, nausea and itching. Pain associated with kidney disorders has been alleviated by the roots of *M. koenigii*. Leaves are also used to treat fresh cuts, dysentery, diarrhea, vomiting, bruises and dropsy. Fruits are known to possess nutritional as well as astringent properties. The small woody portions of *Murraya koenigii* are used to reinforce gums, and used to clean teeth as *datum.* It has also been used as an antiperiodic medicine (Bhandari, 2012). Roots are used for body pain while the stem bark is applied for the treatment of snakebite (Syamasundar *et al.* 2012).

2.4 PHARMACOLOGICAL PROPERTIES

Curry leaves have numerous remarkable pharmacological activities such as antimicrobial, anti-diabetic, cholesterol-reducing, antioxidant, anti-ulcer, cytotoxic, phagocytic and anti-diarrheal.

2.4.1 Antioxidant activity

Amongst the green leafy vegetables, highest antioxidant activity was found in *M. koenigii* (2691 µmol of ascorbic acid/ g sample) compared to *Trigonella, Centella,* and

Amaranthus species (Gupta and Prakash, 2009). Mitra *et al*. (2012) found that the water extracts of *M. koenigii* leaf offer substantial defense to rat cardiac tissue by reducing cadmium-induced oxidative stress mainly due to its antioxidant property. Changes in the amounts of decreased glutathione, lipid peroxidation, protein carbonyl content, increases in the activity of cardiac antioxidants and pro-oxidant enzymes revealed that the damage to the cadmium-induced tissue was caused by oxidative stress. It was previously reported that, dichloromethane and ethyl acetate fractions of the 70% acetone extract of the leaves of *Murraya koenigii* has increased Oil Stability Index (OSI) values considerably comparable to α-tocopherol and BHT.

The remarkable antioxidant activity of *Murraya koenigii* leaves was suggested correlated to its specific carbazole alkaloids, with antioxidant potential (Vasudevan and Parle, 2009). Roy *et al*. (2005) have examined the possibility that *M. koenigii* leaves may be an active and novel free radical quenching therapeutic agent in patients ailing with cancer and related ailments, who have encountered stress related to cadmium oxidative on an occupational basis.

2.4.2 Anti-diabetic activity

According to Dusane *et al*. (2012) extracts of *Murraya koenigii* leaves have showed pancreatic beta cell protection and functional pancreatic islets that produce insulin. Both histochemical and immunohistochemistry analyses indicated a productive role for insulin and a protective role for islets. In addition, *M. koenigii* extracts increased glucose levels by 6-phosphate dehydrogenase enzyme regulated muscle and hepatic glycogenesis, resulting in improved glucose consumption. Hyperglycemia levels have also declined due to pancreatic and intestinal glucosidase inhibitory activity of *M. koenigii* extracts.

Mahanimbine was isolated by column chromatography of the petroleum ether extract of *M. koenigii*. Anti-diabetic activity was carried out on streptozotocin-induced Wistar rats with mahanimbine at doses of 50 mg/kg and 100 mg/kg. The hypoglycemic effect of mahanimbine follows the mechanism either by raising pancreatic insulin secretion from β

cells in islets of Langerhans or by enhancing peripheral glucose uptake. Mahanimbine showed remarkable alpha amylase inhibitory effect along with acarbose (Dineshkumar *et al.* 2010).

2.4.3 Vasodilating activity

Water extracts from *Murraya koenigii* leaves has shown a dose-dependent negative chronotropic effect on the frog cardiovascular system, which may be attributed to the direct action on the heart and blood vessels. K^+ ion concentration was also observed to be very small due to flame photometry, suggesting no participation of K^+ ions. The water extract of the leaves has a vasodilatory effect free from histaminergic, muscarinic, and ß-adrenergic receptors as it improved the number of drops/ minute in the frog hind limb perfusion study and also does not contain an antagonistic activity of the adrenergic receptor. The water extract of the leaves demonstrated significant impact at a concentration of 1 mg/ml (Kadam *et al.* 2011). Ethanolic extract of the leaves *Murraya koenigii* demonstrated, on isolated frog heart, dose dependent strong positive ionotropic effect. The reaction to *Murraya koenigii* 62.5-1000 µg was not influenced either by propanolol theophylline, sildenafil, or imidazole. Changes in sodium and potassium concentrations were not altered. The findings indicated that *Murraya koenigii* had a beneficial effect on ionotropy, likely by the supply of calcium from extra cell sites (Shah *et al.* 2006).

2.4.4 Hypocholesterolemic activity

Crude ethanol extract of plant leaves of *M. koenigii* was found to have a hypocholesterolemic activity in aged mice. The result was verified by the discovery of a dose-dependent reduction in cholesterol levels in elder mice. A dose of 500 mg/kg was determined to be effective than a dose of 300 mg/kg which was equivalent to simvastatin, the normal cholesterol-reducing drug (Tembhurne *et al.* 2010).

2.4.5 Antiulcer Activity

Antiulcer function of water and diethyl ether extracts *Murraya koenigii* have been observed in the reserpine-induced gastric ulcer experiment in albino rats. The plant extract has shown to be efficient against gastric ulceration and the safety was comparable to ranitidine (Shirwaikar *et al.* 2006). Sharma *et al.* (2011) has explained the anti-ulcer activity of aqueous extract of *M. koenigii* leaves using model of acute gastric lesions induced by aspirin, ethanol or cold restrain stress and pylorus ligation in rats. These studies confirmed that the water extract of *Murraya koenigii* leaves can act as good antiulcer agent.

2.4.6 Anti-diarrhoeal potential

The bioassay guided fractionation of *M. koenigii* seeds hexane extract culminated in the isolation of carbazole alkaloids such as koenine, koenimbine and kurryam. Within the isolated compounds, koenimbine and kurryam showed important inhibitory action against castor oil-mediated diarrhoea and PGE2 mediated intestinal pooling experimented on rats (Mandal *et al.* 2010).

2.4.7 Hepatoprotective

Hydro-ethanolic extracts of *Murraya koenigii* leaves at doses varying from 200- 600 mg/kg body weight demonstrated a substantial reduction in the levels of aspartate aminotransferases, alkaline phosphatase, alanine aminotransferases, and bilirubin content in CCl_4-treated hepatotoxic rats. In addition, rats treated with *Murraya koenigii* have resulted in a dose-dependent rise in hepatic reduced glutathione, catalase, superoxide dismutase, and decreased lipid peroxidation. Study showed that CCl_4-induced decrease in cellular antioxidants was prohibited with *M. koenigii* extract with minimal hepatocyte damage in experimental rats, thus suggesting the hepatoprotective potential ability by the polyphenol content in *M. koenigii* extract (Desai *et al.* 2012).

Prolonged consumption of alcohol is one of the most common and significant cause of liver failure leading to fatality. Since there are no dependable hepatoprotective drugs, the existing problem gets complicated. This makes the patient prefer and adopt an alternative medicinal strategy for treating and managing hepatic complications (Shivashankara *et al.* 2012). Carbazole alkaloids and tannins obtained from water extracts exhibited excellent hepatoprotective activity against ethanol-induced hepatotoxicity compared to the regular medication L-ornithine L-aspartate (LOLA) (Sathaye *et al.* 2011 and Shivashankara *et al.* 2012).

2.4.8 Analgesic and anti-nociceptive effects

The leaves methanol extract had an analgesic effect in the hot plate model and the formalin-induced paw licking reaction in rats. The ability may be related to the prevention of sensitization of nociceptors, to the regulation of sensitized nociceptors or to the blocking of nociceptors at the peripheral and central levels. Methanol extracts have been obtained at varying doses, i.e. 100 mg/ml, 200 mg/ml, 400 mg/ml. Amongst, 400 mg/ml findings shows positive results (Gupta *et al.* 2010).

2.4.9 Wound healing effect

Anand *et al.* (2011) reported the wound healing property of *M. koenigii*. The experiment was done by using male albino rats with alcoholic extract of the leaves of *Murraya koenigii*. In the excision, the wound healing model showed that there was a reduction in the wound region in three groups used for wound healing operation. The incision model shows a substantial improvement in the tensile strength of the 12-day old wound by the application of *Murraya koenigii* therapy. Thus, the leaves of *M. koenigii* have been shown to have considerable healing effect on wounds.

2.4.10 Skin pigmentation

The composition of the volatiles present in the leaf of *Murraya koenigii* possesses sun protection effect. It proved that the sun screen cream specifications were fulfilled

according to the official approval guidelines, but the SPF (Sun Protection Factor) for curry leaf oil cream formulation indicated a minimal skin safety against sunlight and erythema. Cream has been shown to be helpful in the maintenance of normal pigmentation of skin or can be applied as antioxidants (Patil *et al.* 2010).

2.4.11 Anticancer property

Girinimbine, a carbazole derived from the stem bark of *Murraya koenigii,* induced apoptosis in HepG2 cells considerably, suggesting the need for further study of preclinical hepatocellular carcinoma in human models (Narasimhan *et al.* 1975).

The findings of the research done by Bhattacharya *et al* (2010) has been provided the proof of the presence of the death receptor which mediated the extrinsic pathway of apoptosis in mahanine-induced anticancer activity in MOLT-3 cells. The average number of neoplasms in the colon and intestines was substantially small as shown by the histological and morpho analyses in animals treated with *Murraya koenigii* (Khan *et al.* 1996).

2.4.12 Antimicrobial property

Panghal *et al.* (2011*)* analyzed 21 species, using the methanol extracts, for *in vitro* antibacterial effect against multi resistant bacteria which includes Gram negative as well as Gram positive strains. The report has showed that *Staphylococcus epidermidis* was potentially inhibited by *Murraya koenigii.* Acetone extract of fresh *M. koenigii* leaves yielded mahanimbine, murrayanol and mahanine as carbazole alkaloids. All possess mosquitocidal and antimicrobial properties (Rahman *et al.* 2005) and displayed inhibition activities of topoisomerase I and II (Ito *et al.* 2006). Benzoisofuranone compounds, carbazole alkaloids and steroids have also been isolated from *M. koenigii*.

2.4.13 Memory enhancing

Tembhurne and Sakarkar (2010) found that the alcoholic extract of *M. koenigii* leaf suppressed cholesterol in mice and showed significant brain acetylcholinesterase enzyme

inhibition, and thus increased the content of acetylcholine in brain homogeneity which eventually improved memory in aged rats. In studies conducted by Mani *et al. (*2012), alkaloidal extracts of the leaves of *Murraya koenigii* (20 and 40 mg/kg), enhanced the effects of protective antioxidants properties like reduced glutathione (GSH), glutathione peroxidase (GPX), superoxide dismutase (SOD), glutathione reductase (GRD), catalase (CAT), and in brain homogenate. Additionally, it demonstrated a reduction in lipid peroxidation (LPO) and nitric oxide assay (NO). There also was an enhancement in the acetylcholine (Ach) levels and lowering of anticholinesterase (AchE) activity. All of these demonstrate the potential beneficial role of *M. koenigii* in neuroprotection against neurodegenerative diseases such as Alzheimer‘s disease.

2.5 PHYTOCHEMISTRY OF *Murraya koenigii*

Proximate analysis of the leaves of *Murraya koenigii* revealed the presence of nicotinic acid, vitamin C, fiber, minerals, proteins, carbohydrates, carotene, vitamin A, and calcium. Fresh leaves of *Murraya koenigii* have 9.7-13.1% ash, 61.8-66.2% moisture, 14.6-18.9% sugar, 2.1-12.5% protein, 1.4-1.8% acid insoluble ash, 1.4-1.8% methanol soluble extractives and aqueous extractive value ranged from 27.3-33.5% (Gupta *et al.* 2010). The fruit pulp contains 17% of total soluble solids, 5% of moisture, 0.2% of non-reducing sugar, 10% of total sugar, 13% of vitamin C, 10% of reducing sugar, 2% of protein, 0.1% of phosphorus, 2% of total ash, 0.2% of calcium, 0.8% of potassium, 0.1% of iron, 0.00057% of tannin, and 0.2% of magnesium (Jain *et al.* 2017; Bonde *et al. 2011*). Seeds of *M. koenigii* consist of furocoumarin lactones, phospholipids, carbazole alkaloids, glycolipids, and terpinene. The plant is also reported to have 4% of total lipids in which 5% are glycolipids, 85% neutral lipids and 10% phospho-lipids. Free fatty acids (10.2%), triacyl glycerols (73.9%) and small amounts of diacylglycerols and monoacylglycerols were also found.

2.5.1 Volatile chemical profiling

Murraya koenigii is remarkably appreciated for its distinct aroma property. There are reports on the quantitative and qualitative characteristics of the aromatic leaves (Padmakumari, 2009). In 1981, MacLeod and Pieris reported that essential oil of curry leaf plant contains monoterpene (16.0 %) and sesquiterpene hydrocarbons (80.0 %). Mallavarapu *et al.* (2000) have been analyzed the oils from fruits and leaves of curry leaf plant by GC/GC/MS. The major constituents of the leaf oil and fruit oil were β-phellandrene (50.1%) and α-pinene (48.1%) respectively.

Analysis of the leaf oil of *Murraya koenigii* collected from southern Nigeria has shown an essential oil constitution of mostly of sesquiterpenoids (89.1 %). The major compounds of the oil were caryophyllene epoxide (6 %), α-cadinol (7 %), bicyclogermacrene (10 %), β-caryophyllene (20 %), α -humulene (5 %) and β-selinene (6 %) (Onayade and Adebajo, 2000). Walde, *et al* (2006) studied oil from leaf and flowers collected from Hyderabad and reported that the major components were hydrocarbons belonging to mono-and sesquiterpene category. They noticed that the chemical constituents of plant essential oils vary regionally. Wong, *et al* (1996) found that the major constituent of flower oil includes β-ocimene (18.0 %), β-caryophyllene (24.2 %), and linalool (8.0 %). Rao *et al* (2011) investigated the chemical diversity of wild and cultivated *M. koenigii* leaf essential oils obtained from different Indian locations. GC and GC-MS analysis of essential oils consolidated 90 compounds, yielding 94 - 99% of the oil. α-Pinene and β-pinene were found in the volatiles of *M. koenigii* that grow wild, in high yields. In cultivated samples, β-phellandrene (14–50%), (E)-caryophyllene (26%-31%), α-pinene (13–35%), and α-selinene (9%, 10%) were the predominant volatile constituents. They reported that the fragrant profiles of the volatile oils were significantly different, and identified (Z,E)-farnesol, hexadecanoic acid, tetradecanoic acid, 1,10-di-epi-cubenol, cada-1,4-diene, γ-eudesmol, (Z,Z)-farnesol piperitone, and α–muurolol for the first time from the plant. The seeds contain terpinene, terpinen-4-ol, linalool and ocimene (Sathaye *et al.* 2011; Kesari *et al.* 2007). Chemical variation has

been found in curry leaf essential oils of various origins. Essential oils from China, Malaysia, and northern India are abundant in α- or β-pinenes (Rana *et al.* 2004). Syamasundar, et al. (2012) have stated that essential oils from Nigeria, Sri Lanka and southern India were abundant in sesquiterpenes with β-caryophyllene as the main constituent. It is further found that concentrations of monoterpenes are higher than those of sesquiterpenes in Western Ghats.

2.5.2 Non-volatile chemical profiling

Preliminary phytochemical studies of *M. koenigii* leaves using chromatography method showed the presence of volatile oil in petroleum ether fraction while of alkaloids and fixed oil were found in the benzene fraction. The fractions were investigated by thin layer chromatography using the solvent systems toluene: ethyl acetate, and ethyl acetate: methanol: water solvent system with separate detection reagents for the identification of phyto-constituents. Acetone, methanol chloroform, and benzene extracts showed the presence of alkaloids. The petroleum ether extract was positive for volatile oil, while fixed oil was present in the benzene extract. The next study revealed the moisture content as 15%, total ash as 73.3%, and acid soluble ash as 74%, acid insoluble ash as 26%, water soluble ash as 89.5%, water insoluble ash as 10.5% and sulphated ash as 80%, Several potent antioxidant compounds were identified and confirmed by structure elucidation through various spectroscopic methods (Sharma and Kumar, 2019).

Muthumani *et al.* (2009) extracted compounds such as alkaloids, flavonoids and sterols from *M. koenigii* leaves using petroleum ether, ethanol, ethyl acetate, chloroform and water as solvents. Isomahanimbicine , mahanimbine, koenimbidine and murrayacine were isolated from petroleum ether extract (Patidar, 2011). The leaves also contain carbazole alkaloids such as, girinimbine, murrayastine, pyrayafoline, koenigin, koenidine, murrayaline, iso-mahanimbine, koenin and koenimbine and triterpenoid alkaloids like cyclomahanimbine and tetrahydromahanimbine. While stem bark has carbazole alkaloids such as murrayazoline, mahanimbine, murrayacine, murrayazolidine, girinimbine, xynthyletin and koenioline.

The chemical components of the bark of *Murraya koenigii* are primarily carbazole carboxylic acid, carbazole alkaloids, glycolipids, phospholipids and galactoside of coumarin. The alcohol extract of the stem bark showed the presence of koeniginequinone A and koeniginequinone B. Chemical structures of the isolated compounds were confirmed as 7-methoxy-3-methyl carbazole-1,4-quinone and 6,7-dimethoxy-3-methyl carbazole-1,4- quinone (Chakraborty *et al.* 1997). Bhattacharya *et al.* (1994) identified methyl- 2-methoxy carbazole-3-carboxylate, 1-hydroxy-3-methyl carbazole, mukonal, a probable biogenetic intermediate of pyranocarbazole alkaloid and murrayazolinol (a minor carbazole alkaloid). The reports revealed the presence of compounds mahanimbine, mahanimbinol, girinimibine, girinimbine and mahanimbilol (possible biogenetic precursors of girinimbine and mahanimbine) from *Murraya koenigii* stem bark. Presence of mukeic acid (1- methoxycarbazole- 3-carboxylic acid) has been reported from the stem (Gahlawat *et al.* 2014). Presence of the umbelliferone, osthol and coumarin galactoside, marmesin- 1‘-O-β-D-galactopyranoside has been reported in the ethanolic extract of the stem bark. Hexane extract showed the presence of 3- (1, 1-dimethyl allylxanthyletin).

The roots of *M. koenigii* include several kinds of bioactive compounds. Srivastava *et al.* (1993) isolated marmesin-1‖-O-rutinoside, murrayagetin and murrayanol. Chihiro *et al.* (1993) identified monomeric and binary carbazole alkaloids such as mukoenine-A, B, C and murrastifoline-F, bismurrayaquinone and bi koeniquinone A from stem and root bark. Mukoline and mukolidine were isolated from the benzene extract of the roots of the plant Roots also possessed girinimbine and koenoline (1- methoxy-3- hydroxy methyl carbazole) (Jain *et al.* 2017).

Bakar *et al.* (2007) isolated mahanimbine, girinimbine, murrayanine and murrayafoline-A from stem bark and roots by spectroscopic methods and comparison of spectral data with that of published data.

2.6 GENETIC DIVERSITY OF *Murraya koenigii*

Knowledge on variety of medicinal and aromatic plant species is essential for the development and proper functionalisation; hence, a great deal of attention has been paid to their production, conservation and quality assessment (Bimbiraite *et al.* 2008). Diversity on genetic matter is the pivot of diversity amongst habitats, ecosystems and species, as described at the Earth Summit in Rio de Janeiro (CBD, Article 2). Every individual is genetically unique by nature. The assessment of intra-species and inter species diversity is performed by various morphological, biochemical and molecular analyses (Govindaraj *et al.* 2014).

A systematic analysis of the variation in molecular genetics among the germplasm may be helpful in assessing how morphologically dependent taxonomic diversification reveal genomic differentiation patterns. Recently, polymerase chain reaction (PCR) marker systems has emerged as quicker and require a few plant material for DNA extraction. Khatik and Joshi (1983) reported that the modified CTAB system yielded consistently positive results in terms of both the consistency and quantity of DNA (A260/280) relative to the Dellaporta process.

DNA polymorphism is the existence of different DNA sequences within individuals, groups, or populations. Various types of polymorphisms of DNA can be traced by a number of techniques; many of the techniques employs restriction of fragment length polymorphisms (RFLPs) with polymerase chain reactions (PCRs), directed amplification of mini satellite DNA (DAMD), random amplification of polymorphic DNA (RAPDs), and inter-simple sequence repeats (ISSRs) (Parker *et al.* 1998).

Verma and Rana (2013) have reported remarkable thoughts into the genetic diversity among both the wild and cultivated accessions of *Murraya koenigii* and affirmed the possible application of the markers as tools to detect degree of genetic variability. The ISSR was most effective relative to the RAPD and DAMD markers showing 96.7 % polymorphism in cultivated and wild collections of *Murraya koenigii*. The genetic

structure of plant populations illustrates the dynamics of numerous factors, including the longstanding evolutionary history of the species (distribution shifts, population isolation and habitat fragmentation), genetic drift, gene flow, selection and mating system (Schaal *et al.* 1998). The genetic polymorphism identified by these markers is consistent with the observation that natural populations of out breeding species are predicted to have greater genetic diversity than inbreeding species (Apte *et al.* 2006). In comparison, the high genetic diversity observed in *M. koenigii* may have been retained from generation to generation. The preservation of genetic polymorphism in natural populations can also reflect the mechanism of adaptation to environmental heterogeneity. Fingerprinting of DNA is known to produce markers at a fast rate, unchanged by the conditions and have been clearly used to assess genetic diversity and relationship at cultivar, population and species levels in several medicinal and aromatic plants (Verma and Rana, 2013). The variable level of genetic diversity detected in the present investigation could be useful in the prospecting of the diverse accessions in *Murraya koenigii*.

3. MATERIALS AND METHODS

The present study entitled "Diversity analysis of *Murraya koenigii* (L.) Spreng in Kerala through phytochemical and genetic profiling" was carried out at the Division of Phytochemistry and Phytopharmacology, KSCSTE-Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, during 2019-2020 academic year. Details regarding the experiments are explained in this chapter.

3.1 Chemicals and equipment

Chemicals used were ethanol, anhydrous sodium sulphate, PVP (polyvinyl pyrollidone), agar, TAE, ethidium bromide (Molecular Grade). Equipment like Clevenger apparatus, weighing balance, water bath, centrifuge, nanospectrophotometer, GC-MS (Shimadzu Gas Chromatograph Mass Spectrometer (QP2020C NX), agarose gel electrophoresis system and gel document system (UVP EC3 chemi HR 410 imaging system) were used for this study.

3.2 Plant material

Fresh matured leaves of 5 accessions and tender leaves of 9 accessions of *Murraya koenigii* were collected from various geographical locations in Kerala for chemical and genetic diversity analysis respectively. For seasonal variation study, fresh matured leaf samples were taken from Kozhikode during summer, rainy and winter seasons. Also matured and young leaves of one accession were compared for chemical constitution.

3.3 Phytochemical studies

3.3.1 Volatile chemical profiling

a) Isolation of essential oil

Fresh leaves (250 g each) were chopped into small portions and hydro-distilled for 3 hr in a Clevenger-type apparatus. The essential oil collected was dried using sodium sulphate and stored in 4° C prior to further analysis.

b) Essential oil analysis

i) GC- MS analysis

The essential oils were analyzed by injecting 1 µL of the diluted essential oil in diethyl ether (1:10 dilution) to Shimadzu Gas Chromatograph Mass Spectrometer (QP2020C NX) fitted with a cross bond 1, 4-bis (dimethylsiloxy) phenylene dimethyl polysiloxane Rxi-5 Sil MS capillary column (30 m x 0.32 mm, film thickness 0.25 µm) fixed with Shimadzu single quadrupole 8030 series mass selective detector. The injector temperature was 240°C, and the oven temperature was at 60-250°C at the rate 3°C/minute. The ion source temp of the mass detector was 240°C, and the interphase temp was 260°C.

ii) Determination of RRI

Relative retention indices (RRI) of volatile oil constituents were calculated on the HP-5 column using C8-C30 straight chain alkanes as standards (Aldrich Chemical Company, USA).

RRI=100[(RT-Hn)/ (Hn+1-Hn)]+100n

Where RT is the retention time of the constituent, Hn and Hn+1 are retention times of reference hydrocarbons with n and (n+1) carbon respectively.

iii) Identification of the components

The constituents were identified by MS library (NIIST 17, Wiley 275), in comparison with their relative retention indices (RRI) calculated with respect to homologous of n-alkanes and compared with literature data and published Mass spectra (Adams., 2007).

iv) Dendrogram construction

To find the variation between different accessions, Hierarchical Cluster Analysis (HCA) was performed using SPSS ver. 16.0 software. The data obtained from GC-MS analysis was compiled in excel and dendrogram was plotted by using the SPSS ver. 16.0 software.

3.4. Genetic diversity analysis

Genetic diversity analysis of *Murraya koenigii* was studied by using SCoT (Start Codon Targeted) markers.

3.4.1 Selection of molecular marker

For the analysis of genetic polymorphism and differentiation, SCoT markers already available in JNTBGRI were used.

Table 3.1 List of selected SCoT markers

Sl. No	Primer	Sequence
1	SCoT 3	CAACAATGGCTACCACCG
2	SCoT 5	CAACAATGGCTACCACGT
3	SCoT 10	CAACAATGGCTACCAGCC
4	SCoT 15	ACGACATGGCGACCGCGA
5	SCoT 16	ACCATGGCTACCACCGAC

3.4.2 DNA Isolation

The genomic DNA was isolated from the leaves of nine accessions of *Murraya koenigii* collected from different places of Kerala by using CTAB method. Young leaves were collected and cleaned by wiping with sterile distilled water and the DNA isolation was commenced as per the following procedure;

Before the beginning of isolation, the CTAB buffer was kept warm by keeping at 65˚C in a water bath.

- 150 mg of fresh leaf sample was weighed and grinded to fine powder using liquid nitrogen.
- The tissue powder was quickly transferred into a clear autoclaved 2mL centrifuge tube and then 1mL of pre-warmed (65˚C) CTAB buffer was added, mixed well.
- After that 4µL proteinase K was added and mixed well again by inversion for 1 minute.

- Mixture was incubated at 70˚C for 30 minute with gentle mixing by gentle inversion of tubes.
- Centrifuged the tubes after incubation at 13,000 rpm for 5minute.
- The supernatant was transferred to a clean centrifuge tube and phenol: chloroform: isoamyl alcohol in the ratio 25: 24:1 was added.
- It was mixed by inversion and centrifuged for 5 minutes at 10,000 rpm.
- The supernatant was poured to a new clean tube and add chloroform: isoamyl alcohol (24:1) mixture and mixed by inverting tube by 20-25 times to form an emulsion.
- Centrifuged the mix for 10 minutes at 13,000 rpm and repeated once.
- The supernatant was carefully decanted and poured to a new tube and was precipitated by adding equal amount of isopropyl alcohol
- The sample was mixed with gentle inversion and incubated at -80˚Cfor minimum of an hour.
- After incubation the sample was centrifuged at 13,000 rpm for 15 minutes.
- Add 100µL 100% ethanol and 5mL sodium acetate and centrifuge it in10, 000 rpm for 5 minutes.
- The supernatant was discarded and pellet was washed twice or thrice with 100µL 70% ethylalcohol at 10,000 rpm for 5 minutes.
- Decanted the supernatant and air dried the DNA pellet at room temperature until whitish pellet become transparent.
- Pellet was resuspended in 100 µL Tris-EDTA buffer or deionized water.

3.4.3 Agarose Gel Electrophoresis

To check the DNA integrity of samples agarose gel electrophoresis was used. This technique separates the DNA by the difference of its size and charge in the presence of an electric field through an agarose gel medium. 4µl of DNA from each sample was loaded in 0.8% agarose gel. The gel was run at 70V with 1X tank TAE buffer in horizontal gel electrophoresis unit for about 2 hours. When the electric field was

applied then the loaded DNA samples were moved towards the positive charged electrode cathode through the polymerized agarose gel. In this technique the pore size of the agarose gel act as a sieve and the movement of the DNA are directly proportional to the size of the pores. The genomic DNA bands in the gel were visualized by Ethidium bromide staining under the gel documentation system.

3.4.4 Nano photometer analysis

Quality and quantity of isolated DNA samples were checked by using Nano photometer (Implen, Germany). 1.5µl of sterile distilled water was used as blank. Nano photometer needed just 1.5µl of DNA for measuring the optical density and the specific parameters. The instrument provides information on the absorbance of nucleic acid at 280nm, 260nm and 230nm as well as the concentration and the purity.

3.4.5 PCR amplification of genomic DNA with the selected primers

Polymerize chain reaction (PCR) of the DNA samples was done with the selected SCoT markers. SCoT markers have only single primer because it provides start codon targeted sequence amplification.

Table 3.2 PCR reaction mix components

Sl.No.	Reagents	Volume
1.	Water	5.3µl
2.	Taq PCR master mix	7.5µl
3.	SCoT primer (2.8mM)	1.2µl
4.	Template DNA	1µl
5.	Total volume	15.0µl

Amplification was carried out in Applied Biosystems Veriti Thermal Cycler (Thermo Fisher Scientific Corporation, USA).

Table 3.3 Temperature profile for PCR

Stages	Step	Cycle	Time	Temperature
Stage 1	Initial denaturation	1	5 minutes	94˚C
	Denaturation	35	1 minute	94˚C
Stage 2	Annealing	35	1 minute	50˚C
Stage 3	Initial extension	35	2 minutes	72˚C
	Final extension	35	10minutes	72˚C
	Store			4˚C

The amplified products were resolved in 1.5% agarose gel with Ethidium bromide and were analyzed using gel documentation system (UVP EC3 Chemi HR 410 imaging system, UK). A 100 bp (Origin, Kerela) DNA ladder was loaded along with the samples to compare the size of resultant bands.

4. RESULTS

This diversity analysis study of *Murraya koenigii* was accomplished by analyzing morphological, phytochemical and genetic variations of leaf samples from different locations of Kerala.

4.1 PLANT COLLECTION

Five accessions of *M. koenigii* were used for bio-geographical variation study (**Table 4.1; Figure 4.1; Plate 4.1**) and nine accessions of tender leaves were used for genetic variation study (**Table 4.7; Figure 4.1**).The samples were collected from across the state, ranging from south (Thiruvananthapuram) to north (Kannur), during winter season of 2020. Three accessions of fresh matured leaves of the same plant were collected during winter summer and rainy seasons (January, April and June respectively) from Kozhikode for seasonal variation studies. The results were tabulated and are given in **Table 4.2.**Young and mature leaves of one accession from Trivandrum were collected for studying chemical variation of maturity stage. The results were tabulated and are given in **Table 4.3.**

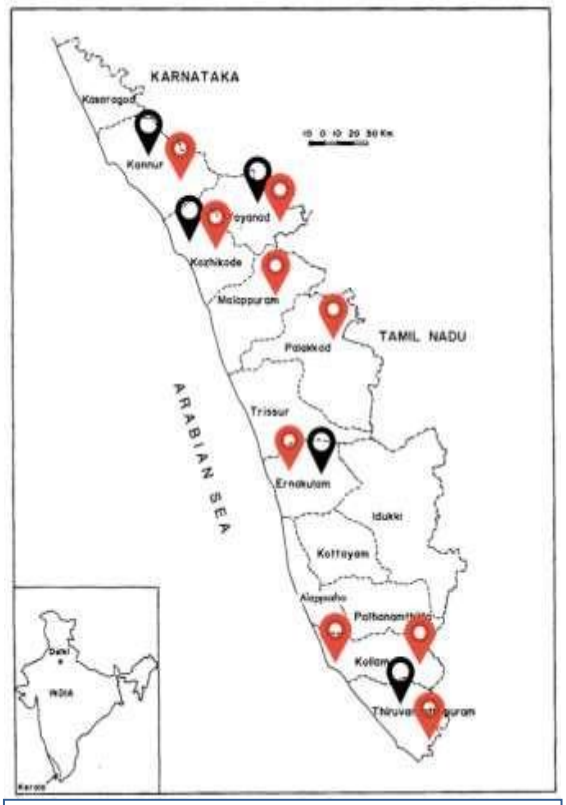


Figure 4.1 Plant collection locations for
-Biogeographical variation study
- Genetic diversity study

4.2. MORPHOLOGICAL STUDY

Morphological examination of *M. koenigii* in its habitat was carried out (**Plate 4.1).** The plant is aromatic deciduous, 6 meter in height, 15-40 cm in diameter. The main stem is dark green to brownish in colour and leaves are 15-30 cm in length, bipinnately compound. Each leaves having 11-25 leaflets on rachis present alternatively. Matured leaves are dark green and young leaves are pale green in colour.

Plate 4.1 Accessions of *Murraya koenigii* collected from **A**: Kannur (MkL-Kn); **B**: Wayanad (MkL-Wy); **C**: Kozhikode (MkL-Kz); **D**: Ernakulam (MkL-Ek); **E**: Thiruvananthapuram (MkL-Tv)

4.3 PHYTOCHEMICAL ANALYSIS

Volatile chemical analysis of the collected samples was performed using gas chromatography.

4.3.1. Volatile chemical profiling

4.3.1.1 Volatile oil yield and characteristics

The essential oil yield is given in **Table 4.1**. Density of *M. koenigii* leaf oil was lesser than water and showed characteristic pale yellow colour with typical aroma.

Table 4.1 Collection location and oil yield of *Murraya koenigii* accessions from different biogeographic locations.

Sl. No	Collection location	Sample code	Oil yield (% v/w)
1	Kannur	MkL-Kn	0.40
2	Kozhikode	MkL-Kz	0.13
3	Wayanad	Mkl-Wy	0.26
4	Ernakulam	MkL-Ek	0.13
5	Thiruvananthapuram	MkL-Tv	0.23

(MkL- *Murraya koenigii* Leaf, Kn- Kannur, Kz-Kozhikode, Wy-Wayanad, Ek-Ernakulam, Tv-Thiruvananthapuram)

Table 4.2 Seasonal variation in oil yield of *Murraya koenigii* leaf collected from Kozhikode.

Sl.No.	Season	Sample code	Yield (% v/w)
1	Winter(January)	MkL-KzW	0.33
2	Summer (April)	MkL-KzS	0.16
3	Rainy (June)	MkL-KzR	0.48

(MkL-*M. koenigii* Leaf, Kn- Kannur, Kz-Kozhikode, W- Winter, S- Summer, R- Rainy)

Table 4.3 Variation in oil yield of *Murraya koenigii* leaf in different maturity stages collected from Thiruvananthapuram

Sl. No.	Maturity stage	Sample code	Oil yield (% v/w)
1	Mature leaf	MkL-TvM	0.23
2	Young leaf	MkL-TvY	0.10

(MkL- *Murraya koenigii* Leaf, TvM-Thiruvananthapuram mature leaf, TvY – Thiruvanathapuram young leaf)

4.3.2. Analysis of essential oil

A total of 52 components were identified from the leaf essential oil samples (**Table 4.4**). GC-MS analysis of oils showed that sesquiterpines were the major class of compounds present in leaf samples **(Figure 4.1)**.

Table 4.4 Leaf essential oil constituents identified from different accessions of *Murraya koenigii* for bio-geographical variation study

Sl. No	**RRI_{cal}**	**RRI_{Lit}**	**Compound**	**MkL-Kn**	**MkL-KzW**	**MkL-Wy**	**MkL-Ek**	**MkL-Tv**
1	924	924	α-Thujene	1.7	-	-	-	-
2	932	932	α-Pinene	3.9	1.2	1.6	0.3	15.2
3	948	946	Camphene	-	-	-	-	0.6
4	978	976	β-Pinene	4.5	-	-	-	5.3
5	988	988	Myrcene	2.1	1.3	1.9	0.5	1.4
6	1006	1002	α-Phellandrene	1.4	3.2	4.1	0.8	2.5
7	1016	1014	α-Terpinene	1.8	-	-	-	-
8	1023	1020	p-Cymene	5.7	0.7	-	-	0.6
9	1028	1024	β-Phellandrene	8.8	18.5	19.3	5.0	17.8
10	1033	1032	Z-β-Ocimene	-	-	-	0.6	-
11	1046	1044	E-β-Ocimene	1.0	11.6	4.9	7.7	6.6
12	1056	1054	γ-Terpinene	19.0	-	0.3	-	0.5
13	1083	1085	Terpinolene	3.2	-	-	-	0.4

14	1098	1095	Linalool	0.6	0.6	-	0.6	0.4
15	1122	1118	cis-p-Menth-2-en-1-ol	-	0.4	-	-	-
16	1122	1125	exo-2-Norborneol acetate	-	-	-	-	0.4
17	1140	1136	trans-p-Menth-2-en-1-ol	-	-	0.8	-	-
18	1161	1165	Lavandulol	-	-	-	0.2	1.0
19	1177	1174	Terpinene-4-ol	0.5	-	0.3	-	-
20	1193	1186	α-Terpineol	-	-	1.0	-	0.6
21	1207	1207	trans Piperitol	-	-	0.5	-	-
22	1282	1288	Lavandulyl acetate	1.3	0.7	1.1	0.3	1.0
23	1372	1374	α-Copanene	-	-	0.8	-	
24	1386	1389	β-Elemene	-	-	-	1.9	0.6
25	1420	1419	E-Caryophyllene	15.0	26.1	26.7	27.6	17.9
26	1425	1430	β-Copaene	-	0.5	0.7	-	-
27	1430	1432	trans-α-Bergamotene	0.8	-	0.3	-	0.8
28	1433	1439	Aromandrene	0.8	-	-	-	-
29	1451	1452	α-Humulene	4.4	7.0	7.3	7.7	5.2
30	1465	1471	4,5-di-epi-Aristolochene	-	-	-	0.4	-
31	1471	1475	γ-Muurolene	-	0.8	-	0.5	-
32	1483	1483	α-Amorphene	-	3.4	-	-	3.1
33	1485	1489	β-Selinene	3.6	-	4.0	8.4	-
34	1495	1498	α-Selinene	5.4	6.0	6.1	13.1	5.5
35	1500	1508	Germacrene A	-	-	-	1.5	-
36	1513	1511	¤-Amorphene	-	1.6	1.5	-	0.5
37	1513	1520	7-epi-α-Selinene	-	-	-	0.4	-
38	1552	1559	Germacrene B	-	-	-	0.2	-
39	1558	1561	E-Nerolidol	1.0	0.6	0.4	1.0	1.2
40	1573	1577	Spathulenol	0.8	0.4	-	-	0.5
41	1578	1582	Caryophyllene oxide	1.7	2.9	1.8	1.7	1.9
42	1579	1590	Globulol	1.7	-	-	-	0.8
43	1600	1600	Rosifoliol	0.9	-	-	-	-
44	1602	1608	Humulene epoxide-II	-	0.7	-	0.4	-
45	1620	1627	1-epi-Cubenol	-	-	1.7	-	-
46	1628	1635	cis-Cadin-4-en-7-ol	-	-	-	1.0	-
47	1630	1639	Caryophylla-4-	0.5	0.9	0.5	-	-

			(12),8(13)-dien-5-ol					
48	1637	1640	epi-α-Muurolol	-	0.5	1.4	-	-
49	1640	1644	α-Muurolol	-	0.8	-	-	-
50	1647	1652	α-Eudesmol	-	-	-	0.7	-
51	1653	1658	Selin-11-en-4- α- ol	3.3	4.4	4.6	9.0	4.1
52	1656	1665	Intermedol	-	0.4	-	-	-
Total				95.4	96.4	93.6	97.3	89.9
Monoterpene-Hydrocarbons				53.1	36.5	32.1	12.9	45.9
Monoterpene- Oxygenated				2.4	1.7	3.7	1.1	3.4
Total Monoterpene				55.5	34.8	35.8	18	49.3
Sesquiterpine-Hydrocarbons				30	45.4	47.4	64.6	30.6
Sesquiterpine- Oxygenated				9.9	13	10.4	14.8	10.3
Total Sesquiterpine				39.9	58.4	57.8	79.3	40.6

(MkL- *Murraya koenigii* Leaf, Kn- Kannur, Kz-Kozhikode, Wy-Wayanad, Ek-Ernakulam, Tv-Thiruvananthapuram)

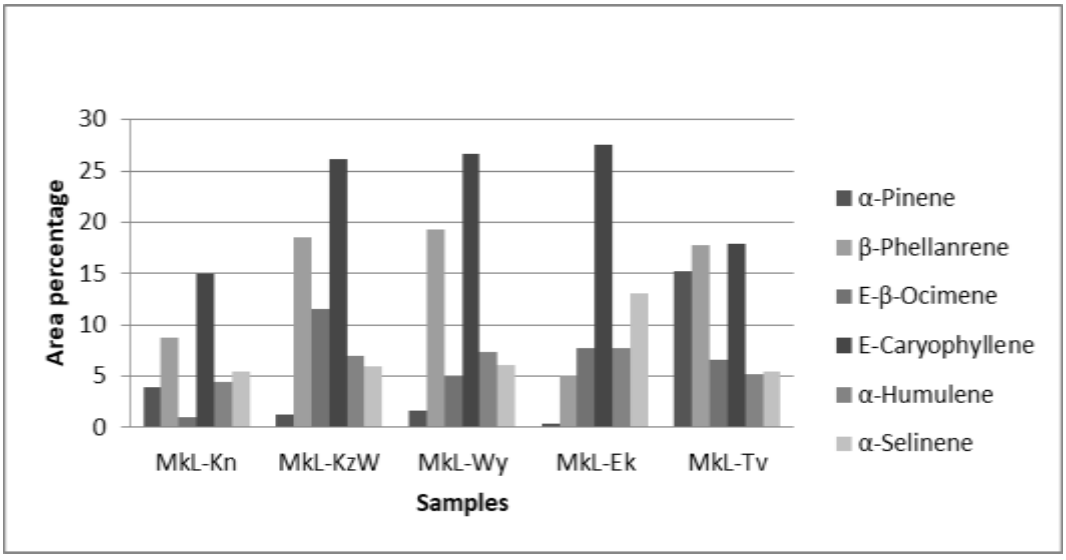
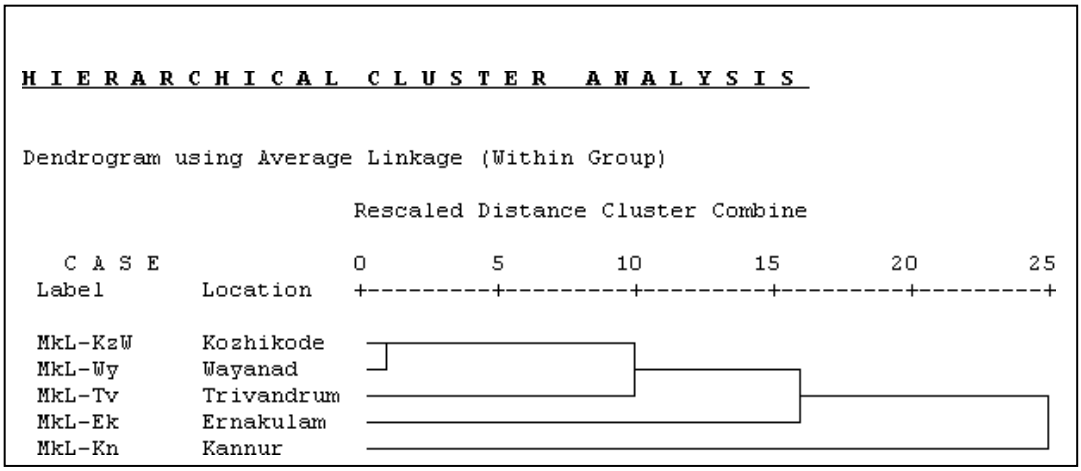


Figure 4.2 Major compounds of *Murraya koenigii* leaf essential oil



Proximity Matrix

Case	Squared Euclidean Distance				
	1:MkL-Kn	2:MkL-KzW	3:MkL-Wy	4:MkL-Ek	5:MkL-Tv
1:MkL-Kn	.000	808.960	720.680	805.430	662.440
2:MkL-KzW	808.960	.000	86.140	376.630	331.040
3:MkL-Wy	720.680	86.140	.000	336.090	341.840
4:MkL-Ek	805.430	376.630	336.090	.000	692.370
5:MkL-Tv	662.440	331.040	341.840	692.370	.000

This is a dissimilarity matrix

Figure 4.3 Dendrogram and proximity table of M*urraya koenigii* based on leaf essential oil composition

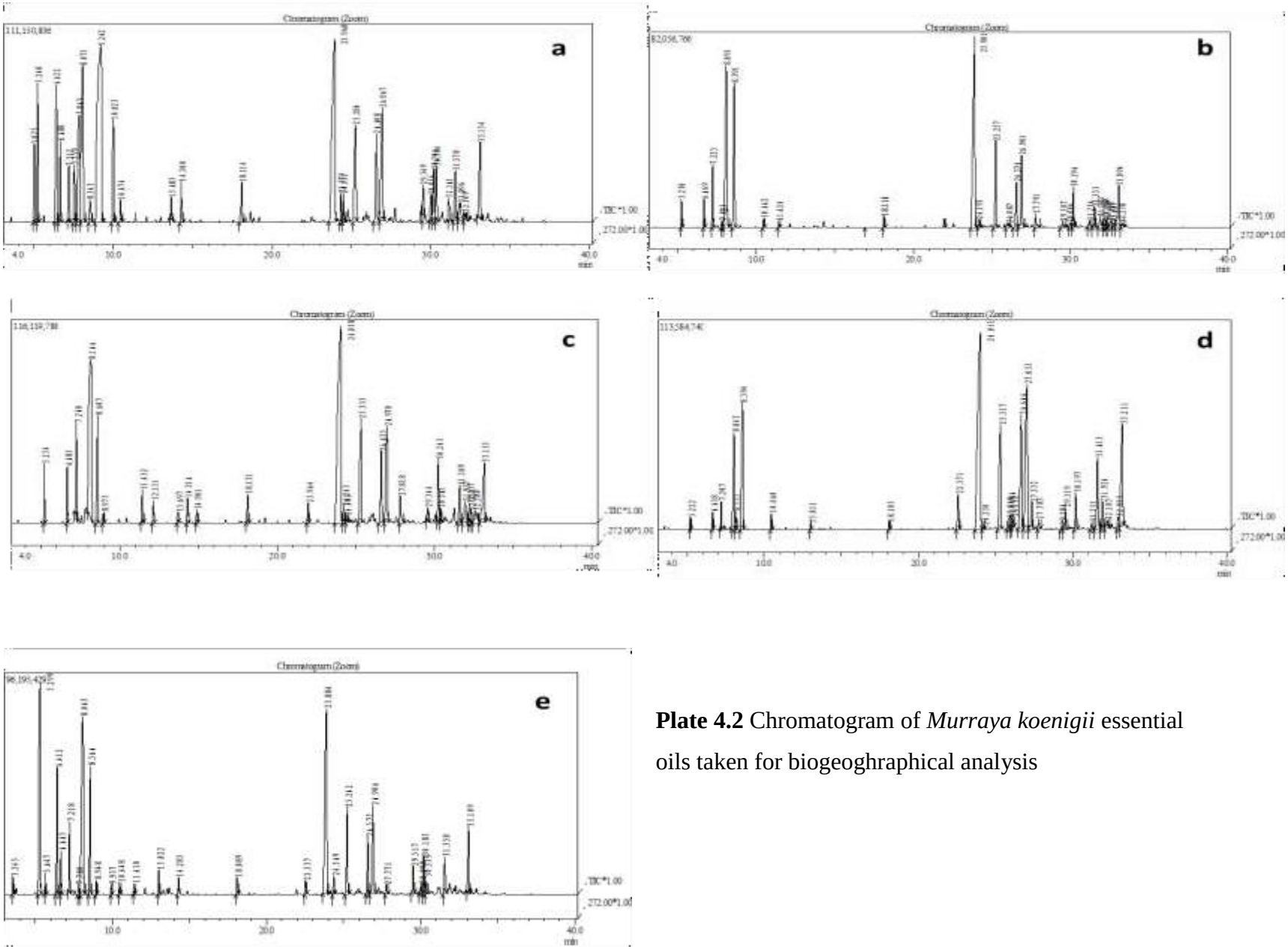


Plate 4.2 Chromatogram of *Murraya koenigii* essential oils taken for biogeoghraphical analysis

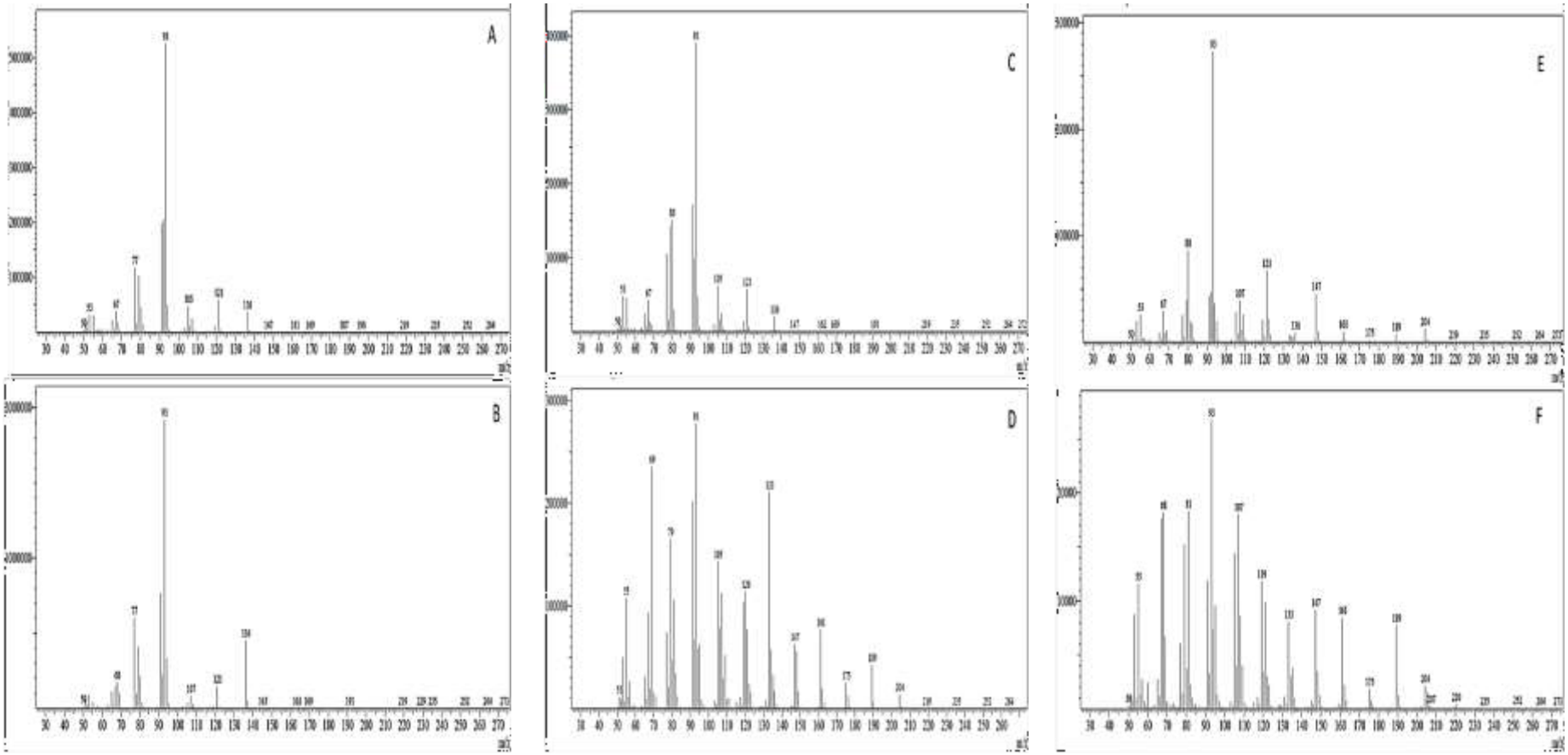


Plate 4.3 Mass spectrum of major compounds. **A)** α-Pinene; **B)** β-Phellanrene; **C)** E-β-Ocimene; **D)** E-Caryophyllene; **E)** α-Humulene and **F)** α-Selinene

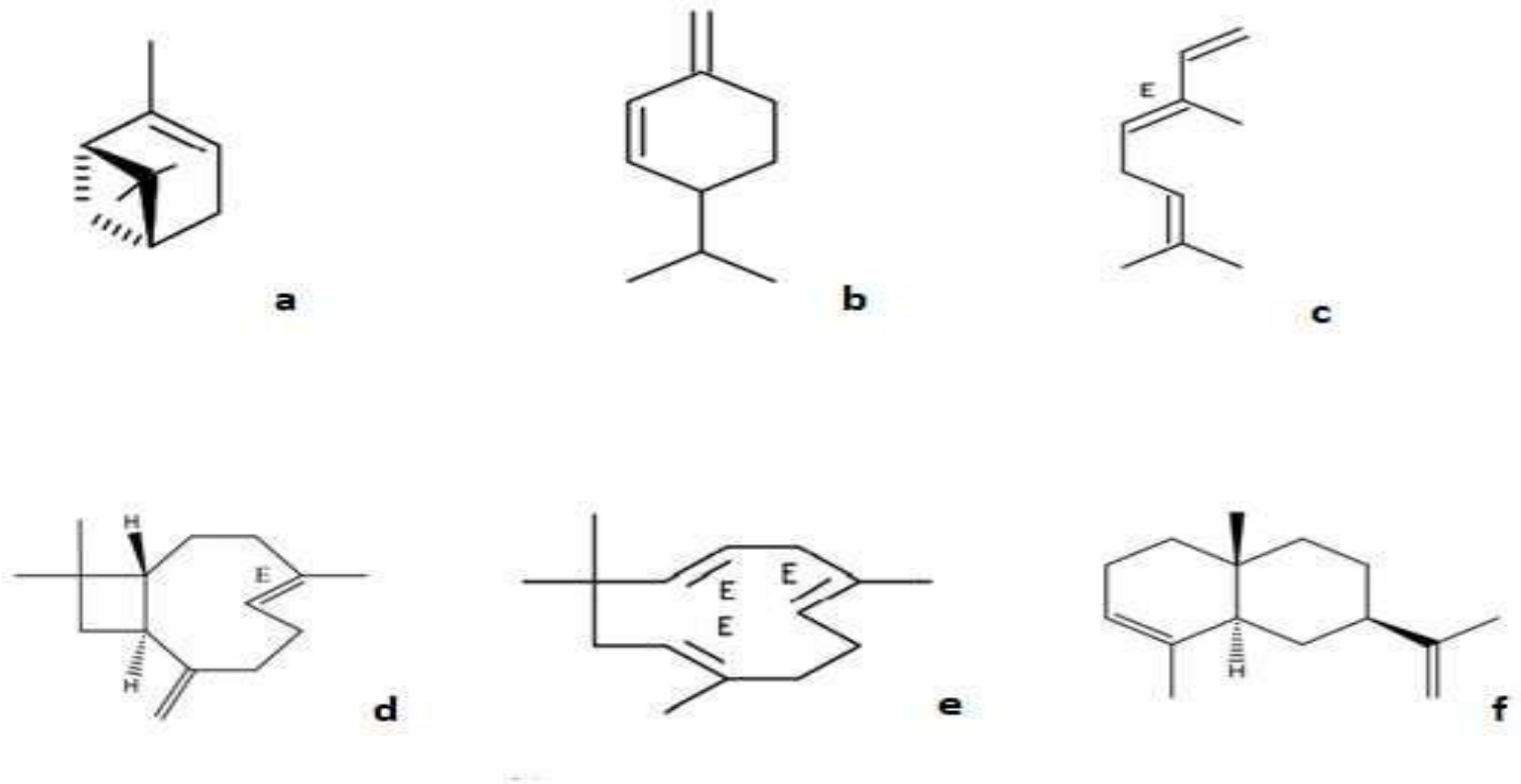


Plate 4.4 Chemical structure of major compounds **a)** α-Pinene, **b)** β-Phellanrene, **c)** E-β-Ocimene, **d)** E-Caryophyllene, **e)** α-Humulene, **f)** α-Selinene

Table 4.5 Essential oil **c**hemical composition of *Murraya koenigii* leaves collected at different seasons

Sl. No.	RRIcal	RRILit	Compound	MkL-KzW	MkL-KzS	MkL-KzR
1.	932	932	α-Pinene	1.2	3	2.7
2.	970	961	Sabinene	-	0.4	-
3.	988	988	Myrcene	1.3	3.2	2.4
4.	1006	1002	α-Phellandrene	3.2	7	7
5.	1016	1014	α-Terpinene	-	-	0.3
6.	1023	1020	p-Cymene	0.7	0.7	-
7.	1028	1024	β-Phellandrene	18.5	31	30
8.	1046	1044	E-β-Ocimene	11.6	10.7	7.4
9.	1056	1054	γ-Terpinene	-	-	0.3
10.	1098	1095	Linalool	0.6	1.1	0.6
11.	1122	1118	cis-p-Menth-2-en-1-ol	0.4	1.1	1.1
12.	1140	1136	trans-p-Menth-2-en-1-ol	-	0.6	-
13.	1139	1141	Camphor	-	-	0.6
14.	1161	1165	Lavandulol	-	0.4	-
15.	1177	1174	Terpinene-4-ol	-	0.5	0.3
16.	1193	1186	α-Terpineol	-	2.2	1.3
17.	1206	1207	trans-Piperitol	-	-	0.3
18.	1282	1288	Lavandulyl acetate	0.7	0.6	0.3
19.	1372	1374	α-Copaene	-	-	0.7
20.	1386	1389	β-Elemene	-	0.6	-
21.	1420	1419	E-Caryophyllene	26.1	18.5	22.5
22.	1425	1430	β-Copaene	0.5	0.4	0.7
23.	1444	1448	cis-Muurrolo-3,5-diene	-	-	0.3
24.	1451	1452	α-Humulene	7	5	6.7
25.	1471	1475	γ-Muurolene	0.8	1.6	-
26.	1483	1483	α-Amorphene	3.4	-	2.2
27.	1494	1492	¤-Selinene	-	-	4.8

28.	1495	1498	α-Selinene	6	3.2	-
29.	1500	1508	Germacrene A	-	0.4	-
30.	1513	1511	¤-Amorphene	1.6	0.7	1
31.	1558	1561	E-Nerolidol	0.6	-	-
32.	1573	1577	Spathulenol	0.4	-	-
33.	1578	1582	Caryophyllene oxide	2.9	1.4	0.7
34.	1578	1590	Globulol	-	-	0.3
35.	1602	1608	Humulene epoxide-II	0.7	-	-
36.	1620	1627	1-epi-Cubenol	-	0.7	0.7
37.	1628	1635	cis-Cadin-4-en-7-ol	-	0.6	0.5
38.	1630	1639	Caryophylla-4-(12),8(13)-dien-5-ol	0.9	-	-
39.	1637	1640	epi-α-Muurolol	0.5	0.4	-
40.	1640	1644	α-Muurolol	0.8	-	0.8
41.	1653	1658	Selin-11-en-4- α- ol	4.4	2	2.3
42.	1656	1665	Intermedol	0.4	-	-
Total				95.2	98	98.8
Monoterpene-Hydrocarbons				36.5	56	50.1
Monoterpene- Oxygenated				1.7	6.5	4.5
Sesquiterpine-Hydrocarbons				45.4	30.4	38.9
Sesquiterpine- Oxygenated				11.6	5.1	5.3

(MkL- *Murraya koenigii* Leaf, KzW- Kozhikode Winter season, KzS- Kozhikode Summer season, KzR- Kozhikode Rainy season)

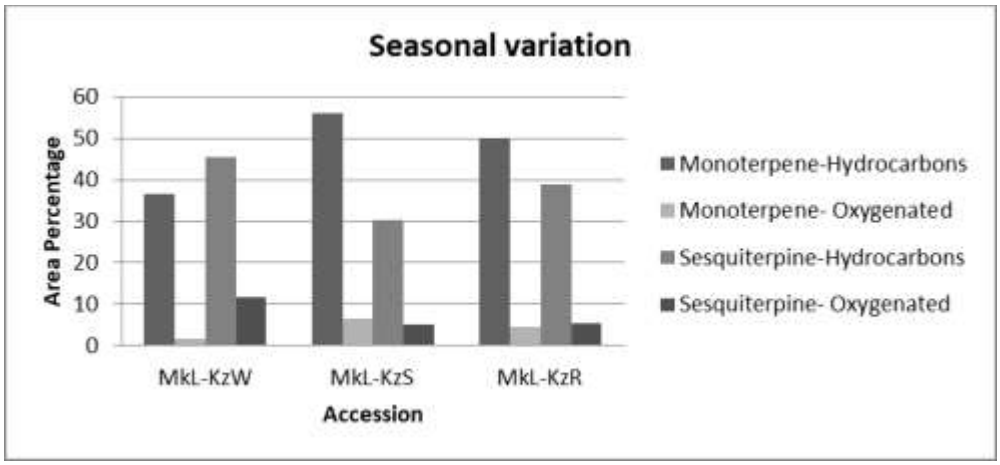


Figure 4.4 Major classes of compounds in *Murraya koenigii* in seasonal variation study

Table 4.6 Leaf essential oil components of *Murraya koenigii* collected at different maturity stages

Sl. No	RRI_{cal}	RRI_{Lit}	Compound	MkL-Tv	MkL-TvY
1.	932	932	α-Pinene	0.3	5.6
2.	948	946	Camphene	0.6	-
3.	970	961	Sabinene	-	0.2
4.	978	976	β-Pinene	5.3	1.2
5.	988	988	Myrcene	1.4	1
6.	1006	1002	α-Phellandrene	2.5	3.5
7.	1023	1020	p-Cymene	0.6	-
8.	1028	1024	β-Phellandrene	17.8	35.5
9.	1033	1032	Z-β-Ocimene	-	0.2
10.	1046	1044	E-β-Ocimene	6.6	8.1
11.	1056	1054	γ-Terpinene	0.5	0
12.	1098	1095	Linalool	0.4	0.2
13.	1122	1125	exo-2-Norborneol acetate	0.4	-
14.	1161	1165	Lavandulol	0.4	-
15.	1193	1186	α-Terpineol	0.6	-

16.	1282	1288	Lavandulyl acetate	1	-
17.	1386	1389	β-Elemene	0.6	2.7
18.	1420	1419	E-Caryophyllene	17.9	27.3
20.	1451	1452	α-Humulene	5.2	6.7
21.	1481	1489	β-Selinene	-	1.1
22.	1483	1483	α-Amorphene	3.1	-
23.	1494	1492	δ–Selinene	-	2.4
24.	1495	1498	α-Selinene	5.5	2
25.	1513	1511	δ-Amorphene	0.5	-
26.	1558	1561	E-Nerolidol	1.2	0.3
27.	1573	1577	Spathulenol	0.5	-
28.	1578	1582	Caryophyllene oxide	1.9	-
29.	1602	1608	Humulene epoxide-II	-	0.3
30.	1647	1652	α-Eudesmol	4.1	1.5
Total				78.9	99.8
Monoterpine Hydrocarbon				35.6	55.3
Monoterpine Oxygenated				2.8	0.2
Total monoterpines				38.4	55.5
Sesquiterpine Hydrocarbon				32.6	42.2
SesquiterpineOxygenated				7.7	2.1
Total sesquitrpines				40.3	44.3

(MkL- *Murraya koenigii* Leaf, Tv- Thiruvananthapuram matured leaf, TvY- Thiruvananthapuram young leaf)

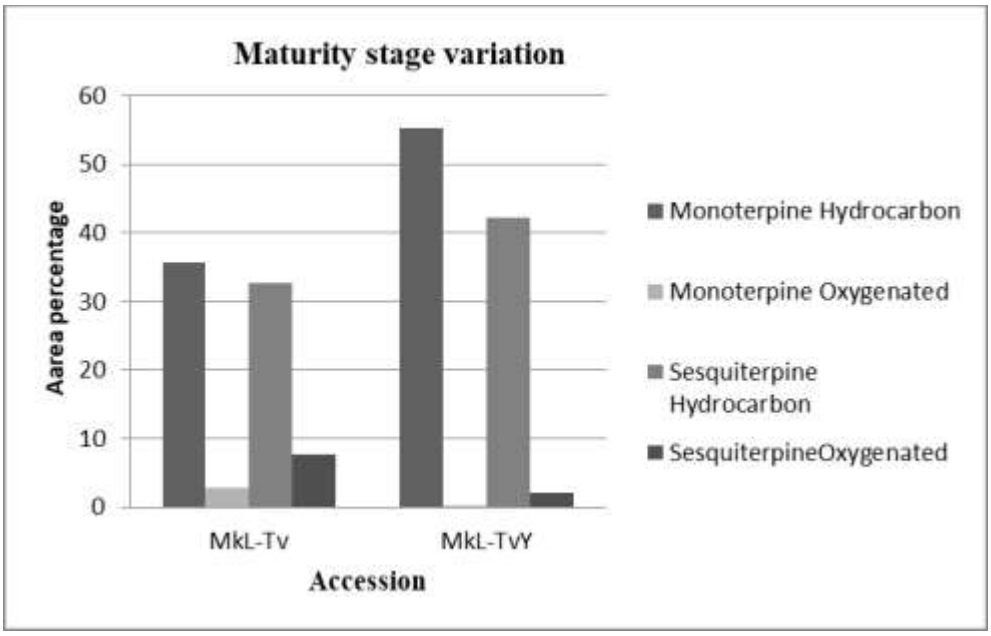


Figure 4.5 Major class of compounds in *Murraya koenigii* in Maturity stage variation study

4.4 GENETIC DIVERSITY ANALYSIS

4.4.1 Isolation of genomic DNA

The genomic DNA was isolated from eight accessions of *Murraya koenigii* using conventional CTAB method. The DNA samples were checked and quantified using a Nano photometer (**Table 4.7**) to confirm their purity and concentration. The total DNA yield ranged from 698 ng/µl to 1723 ng/µl with A260/280 ratio ranging from 1.0 to 2. The A260/230 ratio ranged from 0.4 to 1.9. 40 ng/µl dilutions of the DNA samples were made based on the total DNA yield which was used for further PCR amplification and marker analysis using SCoT primer. The total DNA (**Figure 4.6**) samples were also checked on 0.8% agarose gel for their yield and intactness.

Table 4.7 Quality and quantity of isolated DNA of *Murraya koenigii* samples

Sl. No	Sample code	Collection location	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
1	KLM-1	Karicode (Kollam)	1211	2	1.9
2	KLM-2	Oyoor (Kollam)	874	2	1.7
3	TVM	Mukkunada (Thiruvananthapuram)	744	2	1.7
4	MLP	Areekode (Malappuram)	698	1.8	1.8
5	PLK	Chunkam (Palakkad)	1723	1.2	0.4
6	NDR	Naduvannur (Kozhikode)	769	1.9	1.8
7	OMY	Omassery (Kozhikode)	1073	1.1	0.5
8	WYD	Pulpally (Wayanad)	1723	1.0	0.5
9	KNR	Sreekandapuram (Kannur)	1644	1.2	0.4

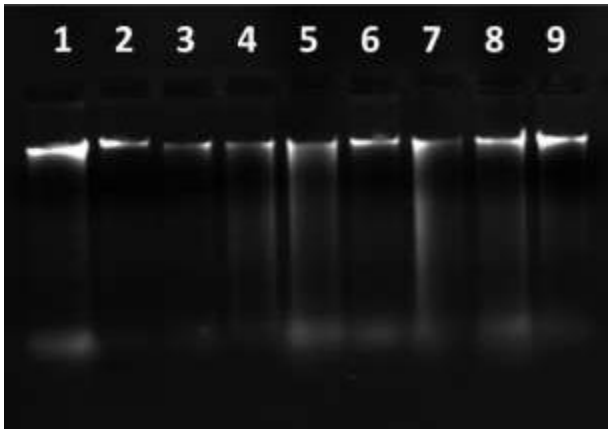


Figure 4.6 Gel image of DNA isolated from *Murraya koenigii* sample collected from various places in Kerala

4.4.2 Screening of SCoT markers

Five SCoT primers (SCoT 3, SCoT 8, SCoT 10, SCoT 15 and SCoT 16) were analysed for PCR optimization, characterisation, and their amplification within 9 different accessions of *Murraya koenigii*. Out of the 5 primers screened, only 3 (SCoT 10, SCoT 15 and SCoT 16) showed polymorphic and reproducible amplification profiles in limited samples and yielded 45 blurred and bright bands from 50 bp to 1100 bp in size. However, the number of bands ranged from one to seven, with a mean of 3 bands per primer, SCoT 10 showcased maximum number (5) of banding patterns and while SCoT15 showed least number (1) of banding (**plate 4.5**). While, primers SCoT 3 and SCoT 8 were unable to produce any amplification with *M. koenigii* genomic DNA. The results showed that a low polymorphism could be disclosed by SCoT markers in *M. koenigii* accessions.

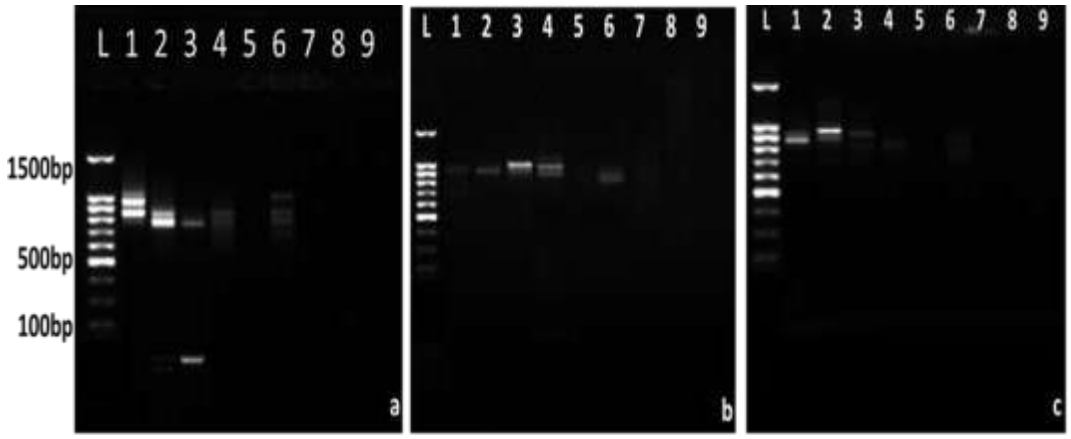


Plate 4.5 Amplified product of SCoT primers - a) SCoT primer 10, b) SCoT primer 15 and

c) SCoT primer 16

Molecular profiling using SCoT marker does not produce significant amplification throughout the samples. Since genetic diversity analysis was performed using only 5 accessions that showed amplification. The three primers selected (SCoT 10, SCoT 15 and SCoT 16) generated 28 countable and reproducible bands. The number of bands generated

per primer ranged from 9 (SCoT 10 and SCoT 15) to 10 (SCoT 16). Out of the generated 28 bands, 26 (92.85%) were polymorphic and 2 (7.15%) were monomorphic characters. The percentage of polymorphism per primer ranges from 88.88% to 100%. The primer which showed highest number of polymorphic bands was SCoT 15 (9 bands out of 9 bands) and the minimum number of polymorphic bands was SCoT 10 (8 bands out of 9).

Table 4.8 Number of scorable polymorphic bands of each primer

Primer ID	**Total no. of bands**	**No.of polymorphic bands**	**No.of monomorphic bands**	**Percentage of polymorphism**
SCoT 10	9	8	1	88.88%
SCoT 15	9	9	0	100%
SCoT 16	10	9	1	90%

5. DISCUSSION

Murraya koenigii belonging to Rutaceae family is used as a leafy spice for culinary, an ayurvedic medicine, and in perfumery industry. The present study gives an insight into the diversity of chemical constituents of different accessions of *M. koenigii*.

5.1 Field study

a) Bio-geographical variation: Plant samples were collected across Kerala regionally. The collection location varies from south Trivandrum to north Kannur. It is observed that the plant is distributed uniformly irrespective of biogeography. Majorly the plant grows in homestead area and it reaches up to 6 meters heights.

b) Seasonal variation: For seasonal variation studies the leaf samples were collected from the same plant during winter, summer and rainy season in a year. Lush growth was observed during rainy season. Leaf shedding was observed during summer season.

c) Maturity stage variation: Field study for maturity stage variation was conducted and matured and young leaf samples were collected from the same plant. Matured leaves were dark green in colour compared to pale in color for the young leafs.

5.2 Morphological study

Murraya koenigii is a small tree, 4-6 m long, having a trunk up to 40 cm (16 in) in diameter. The pungent leaves were pinnate, with 11-21 leaflets of 2-4 cm length and 1-2 cm width (Hema *et al.* 2011). The leaf samples collected from different places in the present study showed no variation in morphology except for Kannur sample (MkL-Kn). The leaves of MkL-Kn sample were greater in the size and more oval in shape compared to other samples.

5.3 Phytochemical study

5.3.1 Volatile chemical profiling

5.3.1.1 Essential oil yield and characteristics

Leaf oil of fresh leaves collected for bio-geographical variation studies were isolated using Clevenger type apparatus and the oil yield varies from 0.13%v/w to 0.40%v/w and have an average yield of 0.23%v/w with pale yellow characteristic colour and pungent typical aroma. The oil from Kannur have higher yield (0.40%v/w). Rao *et al*, (2011) found that the essential oil yield of *M. koenigii* from various place of India ranged from 1–2 ml/Kg plant material. So the yield in the present study is higher than the previous reports.

The essential oil of seasonal variation study yielded 0.16%v/w, 0.33%v/w and 0.48%v/w in winter, summer and rainy seasons respectively. Essential oil yield (v/w%) of *M. koenigii* in North India was reported to differ from 0.15to 0.18 % and 0.12 to 0.14% in two plants collected in different seasons (Verma *et al.* 2012). So the present study shows higher oil yield compared to the previous study. The study revealed that essential oil content is higher in rainy season. Acccording to Verma *et al.* (2012) spring and autumn seasons could be suitable for collection of their Leaves.

The study of variation of chemical compounds in essential oil were checked by hydrodistillation of one accession of both matured leaves and younger leaves with other accession. The young leaves and mature leaves yielded 0.10%v/w and 0.23% v/w essential oil respectively. In this study the matured leaves produced higher yield compared to the younger leaves

5.3.1.2 GC-MS analysis of essential oils

In this phytochemical study individual compounds present in the isolated essential oils were identified by GC-MS. The analysis revealed that E-caryophyllene, β-phellandrene, E-β-

ocimene, α-humulene, α-selinene and α-pinene are the major compounds present in the essential oil.

a) Variation of volatile compounds for different bio-geographical accessions

The samples collected for bio-geographical variation analysis showed some differences in the major compounds and the percentage of the common compounds. *M. koenigii* sample collected from Kannur (MkL-Kn) was found to have γ-Terpinene (19%) as major compound followed by E-caryophyllene (15.0%) and β-phellandrene (8.8%). In sample collected from Kozhikode (MkL-KzW) E-caryophyllene (26.1%) was the major compound followed by β-phellandrene (18.5%) and E-β-ocimene (11.6%). E-caryophyllene (26.7%) and β-phellandrene (19.3%) were found higher in MkL-Wy accession taken from Wayanad district. MkL-Ek accession collected from Ernakulam showed higher concentration of compounds E-caryophyllene (27.6%), α-selinene (13.1%) and selin-11-en-4-α-ol (9.0%). The sample collected from Trivandrum showed higher concentration of E-caryophyllene (17.9%), β-phellandrene (17.8%) and α-pinene (15.2%).

Major compound present in the sample collected from Kannur district was identified asγ-Terpinene (19%). In other samples major compound was identified as E-caryophyllene (22.7%) followed by β-phellandrene (16.1%).In previous studies the major components present in *M. koenigii* collected from Western Himalayas were reported as α-pinene, sabinene, (E)-caryophyllene, β-pinene, terpinen-4-ol, γ-terpinene, α-terpinene, (E)-nerolidol, α-humulene, α-thujene, β-elemene, β-selinene and myrcene (Verma *et al.* 2013) and from south Indiaβ-phellandrene (30.2%) and E-caryophyllene (24.2%) (Raina *et al.* 2002).

The phytochemical diversity based on the distribution of volatile constituents among the 5 accessions studied were checked by statistical analysis based on Hierarchical Cluster Analysis (HCA) model using SPSS statistics 16.0 software (SPSS, Inc.). The accessions

were classified based on their similarity in chemical composition by hierarchical cluster analysis by creating dendrogram. From the statistical analysis, sample taken from Kannur (MkL-Kn) has been identified as very diverse from others. The accession from Wayanad and Kozhikode were similar in chemical composition and they were very close relatives. Both of these were relative to the Trivandrum accession. On the basis of the major and minor chemical constituents, all five samples showed remarkable variations associated with bio-geographical area. This study supports the phytochemical diversity of *M. koenigii* in various biogeographical areas of Kerala.

b) Variation of volatile compounds for accessions collected in different seasons

Study on seasonal variation in essential oil of *M. koenigii* showed some difference in the chemical constituents and quantity. Accessions were collected from Kozhikode during winter, summer and rainy seasons. The major compounds remain same as β-phellandrene, E-β-ocimene and E-caryophyllene, but the quantity varied considerably. The amount of monoterpines was higher and sesquiterpines were lesser in summer season compared to other seasons. Due to the presence of significant amounts of β-phellandrene (Monoterpine-hydrocarbon) and β-caryophyllene (Sesquiterpine-hydrocarbon), intense characteristic aroma was produced normally (Raina *et al.* 2002). Diferent studies on pungent plants have also showed that the essential oil constitution may vary significantly throughout the seasons (Verma *et al.* 2010). In rainy season significant amount of oil yield was observed because of the lush growth. According to this study rainy season was found as good season for harvest with higher oil content.

c) Variation of volatile compounds accessions collected during different maturity stages

Maturity stages also have an effect on the chemical composition of essential oils. Numbers of compounds identified from the essential oil of young leaves were less than that of mature

leaves. Concentrations of major compounds were higher in young leaves compared to mature leaves.

The present study revealed that most of the essential oil constituents were found to vary with respect to the change of season and maturity stage. Thus, the essential oil quality and composition of *M. koenigii* can be associated with the expression of various genes at various stages of plant growth or with environmental factors resulting from seasonal changes and their altitude or biogeography. Several experiments on aromatic plants have also shown that the content of volatile oils can differ remarkably over a year (Verma *et al.* 2010).

5.4 Genetic diversity analysis

The present study used SCoT primers for comparing diversity of genetic matters among 9 accessions of *Murraya koenigii*. ScoT marker technique is simple, low cost, quick, efficient and repeatable, requiring a few DNA in addition with nil prior DNA sequence knowledge. The above marker compounds are very simple to construct on the basis of the ATG sense, which is a very conserved region surrounding the translation initiation codon, so the ScoT marker experiment refers to the functional genes and their corresponding functionalities (Xiong *et al.* 2011). Unlike ISSR, RAPD, and AFLP marker system, SCoT is a gene targeted marker with multi-locus nature and can generate more information with biological traits and useful in elevated genetic polymorphism.

However, the present study could not generate good quality DNA suitable for molecular fingerprinting techniques using the conventional CTAB method. Due to limited time period it was not possible to perform other DNA isolation methods. Molecular profiling of nine accessions of *Murraya koenigii* using SCoT markers produced amplicons of expected size in the limited samples only. The non-amplification of the remaining samples was due to the unavailability of high quality DNA. Isolation of good quality DNA devoid of contaminants like polyphenols and protein is an essential prerequisite for molecular fingerprinting. The

present study focused on genetic diversity analysis between those accessions of *Murraya koenigii* that produced unambiguous and reproducible bands.

In the present study, 9 accessions of *M. koenigii* were screened using 5 SCoT markers, out of which 3 SCoT markers produced unambigous and reproducible bands in only 5 accessions which were collected from Kollam, (acc. nos. 1 & 2), Thiruvananthapuram (acc. no. 3), Malappuram (acc. no. 4) and Kozhikode (acc. no. 6). It was interesting to note that none of the 5 accessions analyzed by 3 primers showed exactly similar banding pattern (Plate 5). A previous study conducted by Verma and Rana (2013) using ISSR, RAPD, and DAMD primers reported 95.80, 96.07, and 96.73% polymorphism, respectively; whereas the present study,the 3 primers generated 26 polymorphic bands out of the total 28 bands, showing a polymorphic ratio of 88.88% to 100% with a mean polymorphism of 92.96% within 5 accessions of *Murraya koenigii.* A detailed analysis using more number of accessions and genetic markers would able to reveal exact picture of genetic variability of this important plant available in the State.

6. SUMMARY

The research work entitled "Diversity analysis of *Murraya koenigii* (L.) Spreng in Kerala through phytochemical and genetic profiling" was carried out at the Phytochemistry and Phytopharmacology Division, and Biotechnology and Bioinformatics Division of KSCSTE-JNTBGRI, Palode, during 2019-2020. Objectives of the study were to select elite accessions of *M. koenigii* with respect to aroma compounds and essential oil content, to find the optimal season and maturity stage for harvest based on the volatile organic compounds in the leaves and to evaluate genetic diversity at intra species level using SCoT marker.

Murraya koenigii, commonly known as curry leaf, is one among the spices belonging to the family Rutaceae. The plant is found naturally in Sri Lanka, India, and other South Asian countries. It had been used for culinary purposes as well as Ayurvedic medicines for centuries traditionally.

Five accessions of fresh matured leaves and nine accessions of tender leaves *of Murraya koenigii* were collected from different geographical locations of Kerala for phytochemical and genetic diversity analysis respectively. For seasonal variation study fresh matured leaf samples were taken from Kozhikode during summer, rainy and winter seasons. Also, one accession of matured and young leaves was compared for chemical constitution. Collected accessions were hydrodistilled to get essential oil. Yield of essential oil for bio-geographical variation, maturity stage variation and seasonal variation studies varied from 0.13%v/w to 0.40%v/w, 0.10%v/w to 0.23%v/w and 0.16%v/w to 0.48%v/w respectively.

The chemical constituents of isolated essential oil were analyzed using Shimadzu Gas Chromatograph Mass Spectrometer (QP2020C NX), coupled with Shimadzu single quadrupole 8030 series mass selective detector. The constituents were identified by using MS library (NIIST 17, Wiley 275) and comparing their relative retention indices (RRI) with respect to homologous of n-alkanes and compared with literature data and published mass spectra. From the study the major compounds were identified as E-caryophyllene, β-

phellandrene, E-β-ocimene, α-humulene, α-selinene and α-pinene. Sesquiterpines were the major class of compounds for all accessions excluding the sample collected from Kannur. The Kannur accession was observed as elite accession with higher yield and the components of essential oil had higher monoterpines content and typical aroma. In this accession γ-terpinene was reported as major compound. The essential oil of seasonal variation study yielded 0.16%v/w, 0.33%v/w and 0.48%v/w in winter, summer and rainy seasons respectively. Rainy season yielded more oil because of lush growth. Among the samples collected at various stages, matured leaves yielded 0.23%v/w and young leaves 0.10%v/w essential oil. Essential oil of young leaves contained less number of compounds compared to mature stage. This observation lead to the conclusion that the compounds present in the younger stages are converted to other compounds during the time of development, depending on the ecological interactions. The compound Z-β-ocimene, a potential insect attractant chemical, was identified in the young leaves, but not in the mature leaves.

The diversity in chemical constituents was statistically analyzed using SPSS software and concluded that the Kannur accession was entirely different in chemical composition from other samples and the Kozhikode sample and Wayanad sample were found as very close relatives compared to Ernakulam and Thiruvananthapuram.

Genetic diversity was checked by SCoT-PCR amplifications of the DNA isolated from young leaf samples taken from various locations of Kerala. However, in the present study, the DNA isolated was of less quantity and quality. Five SCoT primers (SCoT 3, 8, 10, 15 and 16) were used to check the polymorphism and diversity. The present study used 9 accessions of *Murraya koenigii* which were screened using 5 SCoT markers, out of which 3 SCoT markers produced unambiguous and reproducible bands in 5 accessions. Due to the presence of higher content in the polyphenol contaminants the amplification in all DNA samples were not getting uniformly. The 3 primers generated 26 polymorphic bands out of the total 28 bands, showing a polymorphic ratio of 88.88% to maximum value of 100% with

an average polymorphism of 92.96% within 5 accessions of *Murraya koenigii*. None of the 5 accessions analyzed by 3 primers showed exactly similar banding pattern.

The study revealed that *Murraya koenigii* shows variation in chemical constitution of essential oil with respect to biogeography, season and maturity stages. The changes may be related to the expression of different genes at various maturity stages of the plant. Change of environmental conditions during seasons and the biogeography like altitude may also be contributing to the gene expression pattern, which needs further investigations at molecular level.

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8. APPENDICS

APPENDIX I

CTAB Buffer

Tris HCl – 100mM

(pH 8) EDTA –

20mM (pH 8) NaCl

– 1.4M

CTAB – 2%

APPENDIX II

TE Buffer

Tris - 10mM

EDTA – 1Mm

APPENDIX III

TAE Buffer

Tris Base – 242g

Glacial Acetic Acid – 57.1 mL

0.5 M EDTA - 100ml

APPENDIX IV

Tracking Dye

Bromophenol Blue – 0.25%

Xylene Cyanol – 0.25%

Glycerol - 30%

Water – 69.5%

APPENDIX V

70% ethanol

Ethanol – 70 mL

Water –30

9. ABSTRACT

The research work entitled “Diversity analysis of *Murraya koenigii* (L.) Spreng in Kerala through phytochemical and genetic profiling” was carried out at Phytochemistry and Phytopharmacology Division, and Biotechnology and Bioinformatics Division of KSCSTE-JNTBGRI, Palode, during 2019-2020.

Murraya koenigii (L.) Spreng.is the leafy spice plant commonly known as curry leaf. The plant, belonging to the family *Rutaceae*, is native to India, Sri Lanka and other South Asian countries. It is widely used for culinary purposes and medicinal purposes in Ayurveda. To find the diversity within species through phytochemical and genomic analysis level, different accessions were collected from various biogeographical regions, seasons and different maturity stages. The essential oil was isolated from the samples by hydrodistillation using Clevenger type apparatus, and the oils were analyzed using GC-MS.

The results show considerable variation in the oil yield in different seasons, maturity stages and biogeography. Total oil yield varies from 0.10%v/w to 0.48%v/w. From this study, the major compounds were identified as E-caryophyllene, β-phellandrene, E-β-ocimene, α-humulene, α-selinene and α-pinene. Accession collected from Kannur was distinct from other accessions in statistical analysis. Seasonal variation analysis revealed that the oil yield was higher in the rainy season. Further, mature leaves possess a higher amount of essential oil compared to younger leaves. Genetic diversity was checked by isolating DNA from samples taken from various locations of Kerala and their diversity is analyzed by SCoT Primer PCR amplification. The SCoT markers showed an average of 92.96% polymorphism. This study provides a diversity profile of *Murraya koenigii* in Kerala. It can be further elaborated by analyzing more number of plant samples and soil samples from different places to develop a variety of high volatile content yield for farmers.