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Exploration of Wild *Cymbopogon citratus* (DC.) Stapf. for Fingerprint Compounds in Volatile Oil

R. Karthik[1], J. Hemalatha[2], M. Vinutha[3] and K. J. Thara Saraswati[4]
[1,2,4]Department of Microbiology and Biotechnology, Bangalore University, Bengaluru - 560 056
[3]Department of Biotechnology and Genetics, Ramaiah College of Arts, Science and Commerce, Bengaluru - 560 054
e-Mail : www.kxrth8k@gmail.com

Abstract

The wild *Cymbopogon citratus* (DC.) Stapf. collected from Janabharathi campus, Bangalore University, Bengaluru was subjected to hydrodistillation method and essential oil extraction was carried out. The GC-MS analysis was performed and the chemical composition of the essential oil was determined. A total of 45 fingerprint compounds were found in the essential oil out of which geranial (53.07%), Trans-p-mentha-2-8-dienol (3.12%), Cis-p-mentha-2-8-dienol (2.24%), Verbenol (2.92%), Geranyl acetate (2.34%), Geraniol (2.17%), D-limonene (1.79%) and linalool (1.30%) were present in higher percentages. The essential oil biosynthesis of terpenoid compounds consisted of mono, di and sesquiterpenes. These terpenoid compounds play important role in plant development and serve as potential compounds showing varied bioactivities.

Keywords : Wild *Cymbopogon citratus,* Essential oil, GC-MS, Geranial, Terpenoid compounds

The genus *Cymbopogon* belongs to the tribe Andropoganeae of the family Poaceae (Gramineae). Around 140 species of *Cymbopogon* has been reported which are termed as aromatic grasses (Khanuja *et al.,* 2005). The essential oils of these plants have economical values in flavors, fragrances, cosmetics, perfumery, soaps, detergent, toiletry, tobacco and pharmaceutical industries (Ganjewala, D. *et al.,*2008). The essential oil of Cymbopogons have been extensively investigated and its chemical composition (Khanuja *et al.,* 2005 and Sidibe *et al.,* 2001).

The wild *Cymbopogon citratus* (DC.) Stapf. presently investigated is commonly called as West Indian lemongrass. It is a perennial grass which is most commonly grown and distributed in tropical regions (Francisco *et al.,* 2011). *C. citratus* is an economically important aromatic grass and one of the medicinal herbs with immeasurable pharmacological activities (Oladeji *et al*., 2019). The plant is cultivated due to its century-long record of extensive therapeutic applications in Ayurvedic medicines in most countries. (Aftab *et al.,* 2011 and Arreytarkang *et al.,* 2014).

Due to the therapeutic potential of *C. citratus* in antibacterial (Wannissorn *et al.,* 2005), antifungal (Nakagawa *et al.,* 2003), antiprotozoal (Holetz *et al.,* 2003), anti-carcinogenic (Puatanachokchi *et al.,* 2002), anti-inflammatory (Abe *et al.,* 2004) activities the plant is used to cure diabetes, gastrointestinal infection, anxiety, depression, pneumonia and malaria (Manvitha *et al.,* 2014, Chinsembu 2015 and Costa *et al.,* 2016).

During the study the essential oils mainly contained Monoterpenes as the major compounds such as citral, geraniol, citronellal, linalool, limonene, β-caryophyllene, geranyl acetate (Banthorpe DV *et al.,*1980). During the study citral is present as the major compound (53.07%) which are present in two isomeric form Geranial (Citral-A) and neral (Citral-B).

Material and Methods

Ecological Details and Plant Collection

The plant sample for the study was collected from the Janabharathi campus, Bangalore University, Departmentof Microbiology and Biotechnology. Bangalore University campus has an area of 1100 acre

The latitude 12°9496" N and longitude 77°5088" E, respectively with an area of 4.452 sq. kilometers. The average rainfall was 0.1 per cent. The wind flow was 8.1 miles per hour with a humidity of 60 per cent. The sample was collected on 10th January 2021.

Plant Identification

DNA Barcoding Studies and Phylogenetic Analysis was Carried out to Identify the Species

Total Genomic DNA was isolated from the plant sample using Plant Genomic DNA Mini-spin kit. DNA was amplified using the plant specific selective universal region oligo primers (rbcL and matK) (Ashok *et al.*, 2017). 50 µl of PCR reaction mixture contained 50 mg of gDNA, 100ng of each forward and reverse primers, 2 µl of 10 mM dNTPs mix, 5 µl of 10X Taq Polymerase buffer, 3U of Taq polymerase enzyme and made up with PCR grade water. The PCR program was as follows: an initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 1min, annealing temperature standardized at 60°C, extension temperature at 72°C for 2 min and final extension was at 72°C for 10 min. PCR product was run on one per cent agarose gel in 1X TAE buffer and the products were purified using Nucleo-pore, Genetix Biotech PCR clean up kit and purified fragments were sequenced. The sequenced data was edited using Bio edit tool. The experiment was repeated thrice for validation of reproducibility of the barcode sequence.

Isolation of Total Cellular DNA and Primer Designing for Barcode Loci Amplification

Fresh and young leaves of the wild plant were taken and subjected to total extraction of cellular DNA using CTAB method. The corresponding gene sequences of the genus *Cymbopogon* were retrieved from NCBI Gene-Bank data domain for precisely designing the specific primers for the amplification of three barcoding loci and ITSI and 2 spacers. PCR primer pairs were mapped out from the conserved regions using software primer 3.0 (version 0.4.0). (Bishoyi *et al*., 2017).

Barcode Amplification, Sequencing, Validation and Data Analysis

Two chloroplast loci and one nuclear DNA locus (ITS region) of the isolated DNA from the fresh young leaves were amplified using primers that were designed. The PCR reaction mixture contained the template DNA, buffer, MgCl2, dNTPS, designed primer and DNA polymerase. The PCR program that was set involved 35 cycles, each cycle starting from an initial stage of denaturation at 90 °C for 5 minutes, followed by annealing stage at 60 °C for 1 minute, extension stage at 70 °C for 2 minutes and final extension at 72 °C for 10 minutes. The PCR products were purified and sequenced. (Bishoyi *et al.,* 2017). Sanger sequencing of amplicons were carried out using BDT v3.1 Cycle sequencing kit on Abi 3730xl Genetic Analyzer. Annotation software were used to annotate the sequenced data. Validation of the designed primers and sequenced data was done by repeating the experiment twice fromthe starting DNA isolation step to the sequencing step. The PCR products were also subjected to 1.6 per cent agarose gel for the visualization of the amplified products. The gel was pictured with a Gel Doc XR + (Biorad). Annotated contig barcode sequences were subjected to BLAST (NCBI domain) for the verification and were finally submitted to GenBank of NCBI. The DNA sequences were aligned automatically using the program CLUSTALW in OMEGA 6.0 and constructed NJ derived phylogenetic tree.

Essential Oil Studies

Extraction : Clevenger apparatus was used for oil extraction. The fresh plant (root, leaf, stem, inflorescence) was collected from the site was used for essential oil extraction. Whole plant is taken for the oil extraction. Leaves, stem, roots and inflorescence were separated and are washed under tap water followed by distilled water to remove dust particle. The plant was dried at ambient temperature for 2 days in the laboratory to remove the moisture content and the dried plants were cut into small pieces, weighed and used for extraction

of essential oil. The plant materials were subjected to hydro-distillation using Clevenger type apparatus for 3 hours. The oil was dried over anhydrous sodium sulphate and was stored in sealed vials in the refrigerator for further analysis. Amount of essentail oil extracted is calculated by using the formula given below.

$$\text{Essential oil extration (\%)} = \frac{\text{Amount of essential oil recovered (ml)}}{\text{Amount of crop biomass distilled (g)}} \times 100$$

Gas Chromatography and Mass Spectrometry (GC-MS)

GC-MS analysis of the essential oil sample was conducted on Shimadzu GC-MS QP-2010 plus instruments. QP-2010 plus is equipped with column RTX-5MS (length; 30 m and film thickness. ID:0.25 mm). The initial temperature of the column when programmed was at around 40 °C with a hold of 2 minutes then at a Ramp rate of 5 °C to 280 °C for 0 minutes followed by 20 °C to 300 °C for 2 minutes. The injector had a temperature of about 250 °C. Helium was used as the carrier gas which had a gas flow rate of 0.7ml / min with a split ratio of 1:1000.

RESULTS AND DISCUSSION

Plant Identification

The wild *Cymbopogon* presently investigated was subjected to DNA Barcoding and essential oil obtained from the plant was studied by using GCMS analysis and is identified as *Cymbopogon citratus*. The plant sample gave around approximately 1 ml of oil sample. The oil color was pale yellow. The oil sample was collected from a wild ecotype.

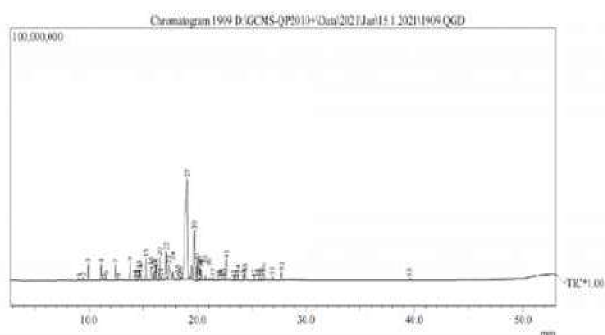

Fig. 1: GC-MS chromatogram of wild *Cymbopogon citratus*

Dna Bar Coding

Out of three loci (rbcL, matK, and ITS spacers 1 and 2), only rbcL loci were amplified successfully and evolutionary analysis was conducted in Clustal Omega using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree (Fig. 2). The evolutionary distances were computed using the Maximum composite likelihood method and the units of the number of base substitutions per site. The phylogeny indicates that the studied plant sample is very closely grouped underclad of *Cymbopogon* sp. This result supports the study of NCBI BLAST leading to confirmation of the species as *Cymbopogon citratus* and was submitted the same in NCBI GenBank under the accession number OK094428

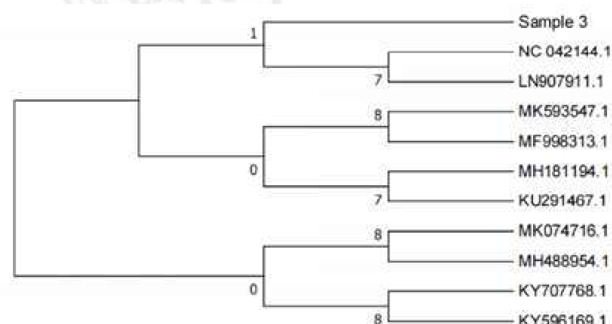

Fig. 2 : Phylogenetic tree constructed based on rbcL gene nucleotide sequences of *Cymbopogon* species

Essential Oil Analysis

The essential oil compounds were classified as monoterpenes, sesquiterpenes, diterpenes, ketones, steroids. A total of 45 compounds were obtained which were classified based on their structural details (Table 1). The oil contained majorly mono-terpenes about 70.46 per cent, sesquiterpenes of 1.11 per cent, diterpenes of 1.53 per cent. Compounds like ketones 5.44 per cent, carboxylic acids 0.88 per cent, esters 0.14 per cent were also present . The bioactivity of these compounds reported by earlier research articles has been listed (Table 2).

TABLE 1

Classification of essential oil compounds into chemical group

Compounds	Area %	R. Time	Mol. Weight	Mol. formula
Monoterpenes				
Tricyclene	0.30	9.083	136.23	$C_{10}H_{16}$
alpha-Pinene	0.37	9.460	136.23	$C_{10}H_{16}$
D-Limonene	1.79	12.438	136.23	$C_{10}H_{16}$
beta-Ocimene	0.22	12.724	136.23	$C_{10}H_{16}$
Linalool	1.30	14.647	154.25	$C_{10}H_{18}O$
Myrtanal	0.78	14.738	152.2334	$C_{10}H_{16}O$
Citronellal	0.50	14.738	154.25	$C_{10}H_{16}O$
Verbenol	2.92	16.571	152.23	$C_{10}H_{16}O$
Carvyl acetate	0.59	18.418	194.27	$C_{12}H_{12}O_2$
Citral Alpha	8.74	19.708	152.23	$C_{10}H_{16}O$
Geraniol	2.17	19.456	154.25	$C_{10}H_{18}O$
Bornyl acetate	0.26	20.210	196.29	$C_{12}H_{20}O_2$
Geranic acid	0.19	21.973	168.23	$C_{10}H_{16}O_2$
Geranyl Acetate	2.19	22.666	196.29	$C_{12}H_{20}O_2$
Trans-p-Mentha -2,8-dienol	3.12	15.291	152.23	$C_{10}H_{16}O$
cis-p-Mentha -2,8-dien-1-ol	6.1	15.723	152.2334	$C_{10}H_{16}O$
Chrysanthenyl acetate	0.18	23.590	194.27	$C_{12}H_{18}O_2$
Pulegone	0.15	14.301	152.23	$C_{10}H_{16}O$
Neral (Citral Beta)	44.33	19.068	152.23	$C_{10}H_{16}O$
Sesquiterpenes				
Caryophyllene	0.65	23.769	204.35	$C_{15}H_{24}$
beta.-Chamigrene	0.17	25.226	204.35	$C_{15}H_{24}$
beta-lonyl acetate	0.19	25.623	236.35	$C_{15}H_{24}O_2$
Cuparene	0.28	25.887	202.33	$C1_5H_{22}$
Elemol	0.46	26.896	222.37	$C_{15}H_{26}O$
Caryophyllene Oxide	1.11	27.769	220.35	$C_{15}H_{24}O$
Diterpenes				
Beta-Cyclocitral	1.26	16.131	152.23	$C_{10}H_{16}O$
Steviol	0.27	39.568	318.4	$C_{20}H_{30}O_3$
Ketones				
Sulcatone	1.77	11.094	126.20	$C_8H_{14}O$
2-Nonanone	0.18	14.373	142.24	$C_9H_{18}O$
2-Undecanone	1.27	20.338	170.29	$C_{11}H_{22}O$
Dodecanal	0.15	23.321	184.32	$C_{12}H_{24}O$
4-nonanone	2.07	13.786	142.24	$C_9H_{18}O$
Carboxylic Acids				
Isoeugenol	0.88	24.367	164.20	$C_{10}H_{12}O_2$
Fatty Acid Ester				
Isopentyl octanoate	0.14	24.271	2140.34	$C_{13}H_{26}O_2$
UN-grouped				
Bicyclo [2.2.1] heptane, 2, 2 -dimethyl-3 methylene-, (1S)-	1.83	9.912	272.5	$C_{20}H_{32}$ -
Cyclohexene, 2-ethenyl-1,3, 3-trimethyl-	0.14	14.536	150.26	$C_{11}H_{18}$
Bicyclo [3.3.0] oct-2-en -7-one, 6-methyl-	0.37	16.683	136.19	$C_9H_{12}O$
Carane, 4,5 -epoxy-, trans	3.70	17.110	152.2334	$C_{10}H_{16}O$
1,3-Methano -5bH-cyclobuta [cd] pentalen-5b-ol, octahydro-	1.40	18.204	150.22	$C_{10}H_{14}O$
3-Cyclohexen-1 -one, 2-isopropy l-5-methyl	0.54	19.326	152.23	$C_{10}H_{16}O$
2-Isopropenyl-5 -methylhex-4-enal	0.16	20.136	154.25	$C_{10}H_{18}O$
trans-2- [2'- (2" -Methyl-1" -propenyl) cyclopropyl] propan-2-ol	0.17	22.409	154.2493	$C_{10}H_{18}O$
Selina-6-en-4-ol	0.78	26.132	222.37	C15H26O
(1S,4R)-p-Mentha -2,8-diene, 1-hydroperoxide	0.77	20.724	168.23	C10H16
(2R,4R)-p-Mentha -[1(7),8]-diene, 2-hydroperoxide	0.45	21.409	168.23	C10H16O2

Terpenoid Biosynthesis

Monoterpene Biosynthesis : Monoterpenes are the major compounds present in the essential oil of wild *Cymbopogon citratus*. The synthesis of terpenoid mainly occurs through Geranyl diphosphate (GPP). The monoterpenes pathway GPP gets converted into tricyclene due to the presence of tricyclene synthase with a release of diphosphate molecule. D-limonene is synthesized

TABLE 2
Bioactivity of the compounds

Compound Name	Bio-Activity	Reference
Tricyclene	Anti Microbial Activity	Essien (2011)
Alpha. -Pinene	Respiratory track infections, Flavor, Fragrance, Antibacterial, Insecticidal	Allenspach (2021)
D-Limonene	Cancer prevention, Immune modulation, Anti -prolife -rative	Jessica A Miller (2011)
Beta-ocimene	Antimicrobial and antioxidant	Lopes-Lutz, Daise *et al.* (2008)
2-Nonanone	Anticancer and antimicrobial effects.	Mahmoud Eman A *et al.* (2020)
Linalool	anti-inflammatory, anticancer, anti-hyperlipi demic, antimicrobial, antinoceptive, analgesic, anxiolytic, antide pressive and neuroprotective	Pereira, Irina, *et al.* (2018)
Bicyclo[3.1.1] heptane-2 -carboxaldehyde, 6,6-dimethyl-	Bactericidal activity	Tonial, Fabiana, *et al.* (2020)
Trans-p-Mentha-2, 8-dienol	Anti Microbial Activities	Bassole, I. H. N., *et al.* (2011)
Beta-Cyclocitral	Plant growth Hormone, helps in root developments and branching.	Dickinson, Alexandra J., *et al.* (2019)
Citronellal	Anti-fungal properties	Lee, Sun-Og, *et al.* (2007)
Verbenol	NF	
Decanal	Anti-Salmonella Agents and Antioxidant Activity	Shareef, Hasanain Khaleel *et al.* (2016)
Neral	Anti-Microbial Anti-inflammatory, Anti- Fungal	Zielinska, Aleksandra *et al.* (2018)
Geraniol	antioxidant and anti-inflammatory properties	Maczka (2020)
Citral	Anti-Microbial, Anti-inflammatory, Anti- Fungal	Zielinska, Aleksandra *et al.* (2018)
Bornyl acetate	Anti-Inflammatory Functions	Yang, He *et al.* (2014)
2-Undecanone	Anticancer and antimicrobial effects.	Mahmoud, Eman, A *et al.* (2020)
Geranic acid	Tyrosinase Inhibitor in lemon grass	Masuda, Toshiya, *et al.* (2008)
Geranyl Acetate	Anti-Inflammatory, Anxiolytic, Antimicrobial, Diuretic, Antiseptic, Anti-cancerous	Chahal, K. K., *et al.* (2018)
Dodecanal	antibacterial, antifungal and anti-oxidative	Mandal, Shyamapada (2015)
Caryophyllene	anticancer activities, affecting growth and proliferation of numerous cancer cells	Fidyt, Klaudyna, *et al.* (2016)
Isoeugenol	antioxidant, antibacterial activities	Zhang, Liang-Liang *et al.* (2017)
Caryophyllene oxide	anticancer activities, affecting growth and proliferation of numerous cancer cells	Fidyt, Klaudyna, *et al.* (2016)
Steviol	antitumor,antibacterial, antihyperglycemic, anti-inflammatory activities	Wang Mingying *et al.* (2018)

from GPP in the presence of limonene synthase by the release of diphosphate molecule. Limonene gets converted to pulegone through various intermediate reactions first the synthesis of trans-isopiperitenone occurs by limonene 3-monooxygenase of the class Oxidoreductases followed by trans-Isopiperitenol gets converted into Isopiperitenone by isopiperitenol dehydrogenase of the class Oxidoreductases. Isopiperitenone is converted into cis-Isopulegone in presence of Isopiperitenone reductase of the class Oxidoreductases. cis-Isopulegone takes an intermediate form of pulegone enzyme involved is unclear. Linalool is synthesized from GPP in the presence of linalool synthase of the class Lyases. Beta-ocimene is synthesized from GPP in the presence of Beta-ocimene synthase. Alpha-Pinene is obtained from GPP in presence of alpha-pinene synthase of the class Lyases, and the further reaction of alpha-pinene leads to the formation of Verbenol in the presence of Cytochrome P450 Reductase and NADPH as a co-factor. Geraniol is obtained from GPP in presence of geranyl diphosphate diphosphatase of the class Hydrolases. In presence of NADPH as a co-factor citronellol is formed and the enzyme

involved is unknown. In the presence of an unknown oxidized electron carrier citronellol is converted into citronellal and the enzyme involved is unknown. Geraniol in presence of geraniol dehydrogenase gets converted to geranial, geranial takes an intermediate form of neral and the enzyme responsible for this reaction is not known. Geranial in presence of di-oxygen molecules and water as co-factor in the presence of aldehyde oxidase / indole-3-acetal dehyde oxidase forms Geranic acid. Geranial acetate is formed from geraniol in the presence of acetyl-CoA as a co-factor and acetyl CoA acetyltransferase. The pathway for the reaction invloved is represented in (Fig. 3).

Sesquiterpene Synthesis

Sesquiterpenes occur through MVA (Mevalonate) pathway. Farnesyl diphosphate (FDP) is a major molecule responsible for the synthesis of sesquiterpenes. FDP is synthesized from GPP + IPP (geranyl-diphosphate + isopentenyl pyrophosphate), in the presence of Farnesyl diphosphate synthase. FDP in the presence of sesquiterpene synthase enzyme. Beta-chamigrene is formed. FPD gets converted into (2Z,6E) - hedycaryol in the presence of a water molecule and the enzyme responsible for this reaction is not known. (2Z,6E)-hedycaryol further reacts to form elemol and the enzyme responsible for the reaction is unknown. FDP forms Caryophyllene in the presence of Caryophyllene synthase. Cuprenene is synthesized from FDP in the presence of sesquiterpene synthase and Cuprenene in the presence of an unknown oxidized electron carrier forms cuparene. The pathway for the reaction invloved is represented in (Fig. 4)

Diterpene Synthesis

Steviol is a diterpene molecule synthesized from ent-kaurene, GGPP in the presence of ent-kaur-16-ene synthase results in the formation of ent-kaurene. ent-kaurene is converted into ent-kaurenol, which is futherd conveted intoent-kaurenal, then it is converted into ent-kar-16-en-19-oate which is finally converted into steviol throught the whole reaction (an oxidized [NADPH-hemoprotein reductase]) acts as the co-factor for the reaction where a dioxygen moleclue is converted as a water molecules. The pathway for the reaction invloved is represented in (Fig. 5).

Discussion : The essential oil obtained was subjected to GC-MS analysis and a young leaf of the plant sample was provided to perform DNA-barcoding. From the reports obtained the plant was identified as *Cymbopogon citratus*. The plant has a wide variety of secondary metabolites which have unique properties.

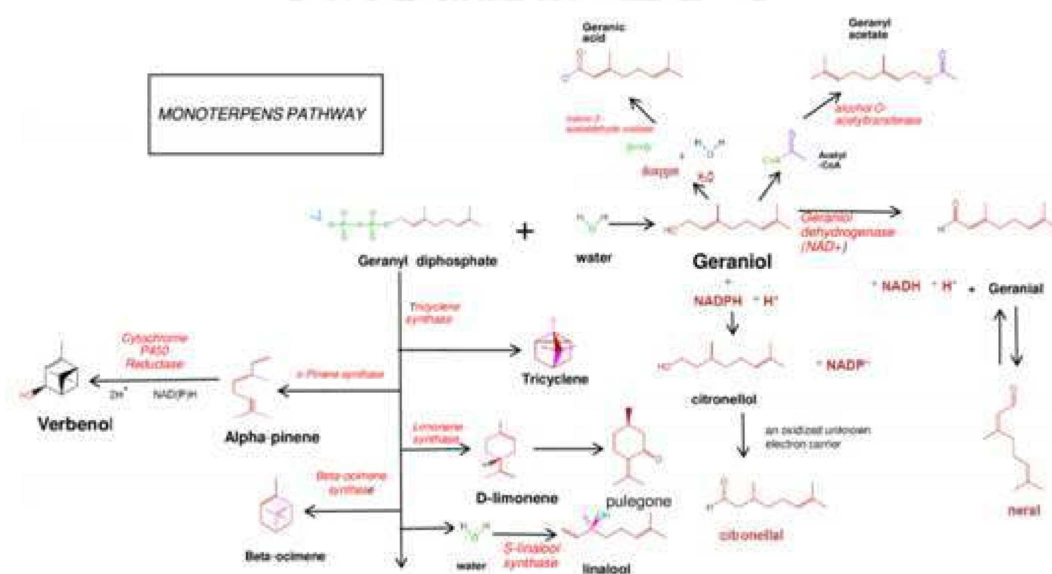

Fig. 3 : Overview of pathways for Monoterpenes in Wild *Cymbopogon citratus*

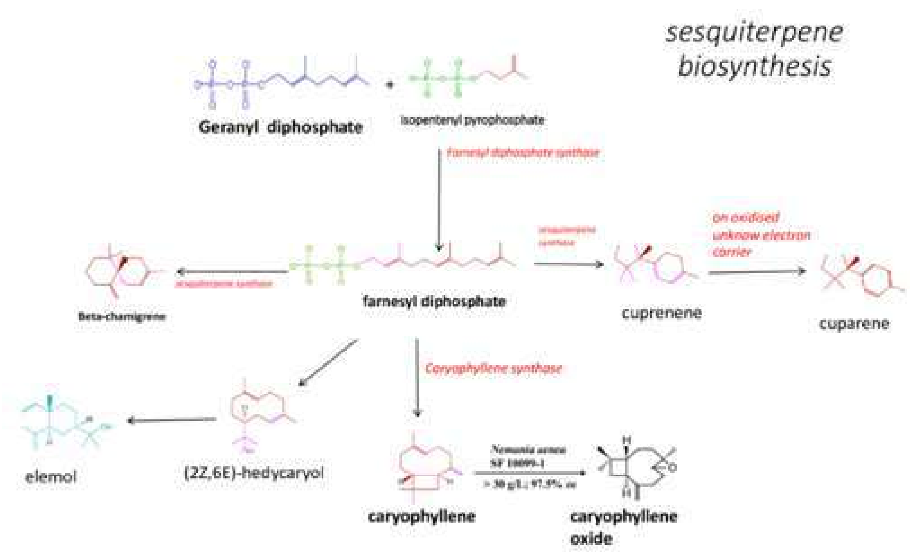

Fig. 4 : Overview of the pathways for Sesquiterpenes in Wild *Cymbopogon citratus*

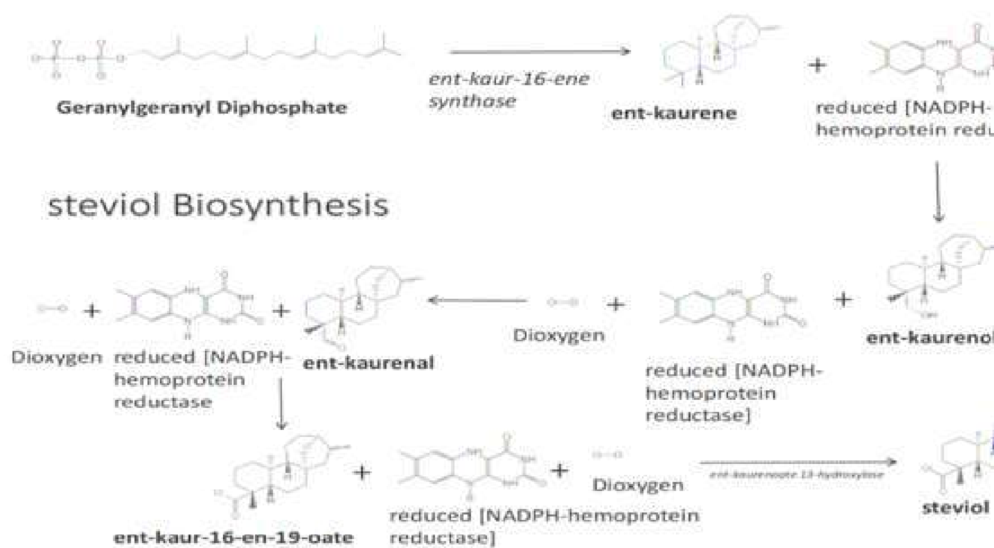

Fig. 5 : Overview of the pathways for steviol in wild *Cymbopogon citratus*

Antibacterial activity was seen in the citral compound (Neral + Geranial) which is isolated from the leaves of *Cymbopogon citratus*, they are most effective against gram positive and negative bacteria species (Soares *et al.,* 2103). Lemongrass is used to deal with pain and anxiety (Ademuyiwa *et al.,* 2015 and Viana *et al.,* 2000). In the ancient time during surgical operations, they were used as analgesic or pain reliever (Soares *et al.,* 2103; Nishijima *et al.,* 2014; Tavares *et al.,* 2015). They have some resistance to the pathogenic fungal cells causing disorder in proper secretion of mycotoxins during the storage of grains and other food substances (Nguefack *et al.,* 2012, Kpoviessi *et al.,* 2014). It has antifungal properties againts fungal infections like athlete's foot, ringworm, jock itch and yeast infections (Boukhatem *et al.,* 2014). The secondary metabolite Citral Citronellal Myrcene which is isolated has show antimalarial activity against plasmodium species (Kpoviessi *et al.,* 2014; Melariri 2010). The leaves of the plant were used to prepare tea since they regulated glucose, lipid

and fat level in the blood serum which prevented obesity and hypertension (Shah *et al.,* 2011). *Cymbopogon citratus* has antioxidant activity caffeoylquinic acid, flavonoids, chlorogenic acids, phenolic acids, swertiajaponin, and isoorientin. They induce Cu2+ which is mainly responsibly diminishing the LDL (Low-Density Lipoprotein) (Campos *et al.,* 2014). The essential oil of *Cymbopogon citratus* has been applied to control pathogens and insects (Sessou *et al.,* 2012).

Cymbopogon citratus is a perennail grass with various biological activities due to its essential oil compounds which are present. They are used as flavoring agent in food industries and beverage companies. The plant has medicinal properties like antibacterial, antifungal, anti-inflammatory, anticancer, analgesic, antiseptic and antinociceptive and antioxidant properties. Due to its various beneficial properties, the plant has good economical importance.

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