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Bioactive metabolites of rhizosphere fungi associated with *Cymbopogon citratus* (DC.) Stapf

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Abstract
Rhizosphere soil samples of commercially explored aromatic and medicinal plant, *Cymbopogon citratus* (DC.) Stapf (Var. OD-19) were collected from Anand Agriculture University, Gujarat. A total of 62 fungal isolates representing 9 genera and 15 different species were isolated and identified. The most dominant fungal species were *Aspergillus niger* (20.96%), *Aspergillus terreus* (12.90%), *Aspergillus flavus* (9.67%), *Rhizopus oryzae* (8.06%), *Rhizopus nigricans* (8.06%), *Mycelia sterila* (8.06%), *Aspergillus fumigatus* and *Trichoderma viride* (6.64%) followed by *Rhizopus stolonifer* (3.22%), *Penicillium sps*.(3.22%), *Curvularia lunata* (3.22%), *Fusarium oxyporum* (3.22%), *Mucor racemosus* (3.22%), *Curvularia inaequalis* (1.61%) and *Alternaria tenuis* (0.62%). These fungal species were cultured on Potato Dextrose broth and bioactive compounds were extracted using ethyl acetate and identified by using GC-MS. Out of 62 fungal isolates *Trichoderma viride* was found to synthesize Citral, in Potato Dextrose Broth supplemented with 2 g/L geranyl pyrophosphate (GPP) as an universal precursor of monoterpenes. Besides Citral, the two other important compounds were isolated include 1, 2-Benzenediol and 2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)-respectively. The results indicated that rhizosphere fungi isolated from *Cymbopogon citratus* could be a potential source for isolation and purification of bioactive compounds which may have potential pharmaceutical applications. This is the first report of the rhizosphere fungi isolated from *Cymbopogon citratus* synthesizing Citral and other bioactive compounds.

Keywords: Citral, *Cymbopogon citratus*, GC-MS, *Trichoderma viride*

Introduction
Medicinal plants are a rich source of bioactive metabolites (Toussaint *et al.* 2007) [34], and are considered to be safe environmentally compared to the synthetic chemicals for the treatment number of human ailments (Nema *et al.* 2013) [18]. The cost of production, side effects and the development of resistance by disease causing agents against synthetic chemicals, necessitated the use of chemicals derived from medicinal plants for human and animal applications. In view of these, there is a need for sustainable cultivation and continuous production of naturally available resources for variety of applications. *Cymbpogon citratus* (*Lemon grass*) is an important medicinal and aromatic plant due to its high medicinal value. The oil extracted from *C. citratus* is used for skin care and cosmetic products, such as soaps, deodorants, shampoos, lotions, and tonics (Lawless, 1995) [15]. Citral is the main active compound extracted from lemon grass used in perfumery industries and has strong antimicrobial and pheromonal effects in insects (Robacker & Hendry, 1977) [27].

The role of microbes in plant growth, nutrient availability, disease resistance, yield and quality of medicinally important compounds is demonstrated in medicinal plants. Nowadays there are increasing research in the interaction between medicinal plant and their rhizosphere microbes for the improvement of medicinal plants. Hiltner (1904) [9] introduced the term rhizosphere for the soil zone just adjacent to plant roots. Fungal abundance is 10–20 times higher in the rhizosphere than in the bulk soil (Morgan *et al.* 2005) [16]. The rhizosphere microbes play an important role in improving medicinal values of medicinal plants. The inoculation of rhizosphere fungi is a sustainable technology to enhance the quantity and quality of the medicinal plant compounds. Overall the study will help to explore the possibilities of enhancing the active contents in the selected medicinal plant. This interest is also linked to environmental concerns for reduced use of chemicals as well as an appreciation for utilization of biological and organics in agriculture.

Meager work has been done in area of screening of rhizosphere fungi and bioactive compounds isolated from these rhizosphere fungi associated with selected important medicinal plant.

Materials and Methods

1. Collection of rhizosphere soil samples

Collection of soil samples from rhizosphere region of *C. citratus* was done using the method (Dongmo and Oyeyiola 2006) [6] in the month of August.

2. Isolation and culturing of of rhizosphere mycoflora by serial dilution plate technique

10 gm of soil sample were taken for serial dilution. The rhizosphere fungi were isolated by serial dilution plate method (Johnson & Curl, 1972) [11]. Petri plates were incubated at 26°C in an incubator for seven days. The fungal colonies expressed after incubation were isolated individually. Isolated fungi were identified using fungal mannuals (Barnett & Hunter, 1972; Khulbe, 2001; Raper & Thom, 1949; Nelson, Toussoun & Marasas, 1983; Onions, Allsopp & Eggins, 1981) [1, 25, 14, 17].

3. Mass culturing of fungi and extraction of bioactive compounds

250 ml Erlenmeyer flask containing 100 ml of potato dextrose broth, supplemented with 2 g/L geranyl pyrophosphate (GPP) was inoculated with the fungal isolates (Zebec, 2016). The inoculated flasks were kept at room temperature for 21 days under stationary conditions with intermittent shaking. After incubation, the broth culture was filtered and the mycelia and culture filtrate was separated. To the culture filtrate, equal volume of ethyl acetate was added, mixed well for 10 min. and kept for 5 min. till the separation of aqueous and solvent layers. The upper ethyl acetate layer containing the active compounds was separated using separating funnel. Further, the fungal mycelium was ground to a fine paste using a Mortar and Pestle in 10 ml of ethyl acetate and the mixture was filtered through cheese cloth. Both mycelial and culture filtrate extracts were pooled together and evaporated to complete dryness in hot air oven maintained at 80ºC. The extract residue was dissolved in 5 ml of Dimethyl sulfoxide (DMSO) and stored at 4ºC for GC-MS analysis (Haque *et al.* 2005 and Radji *et al.* 2011) [8, 23].

4. Gas Chromatography Mass Spectrometry (GC-MS) Analysis

The fungal extracts from 62 fungal isolates in DMSO, were subjected to GC- MS analysis to identify the bioactive compounds. The HP-5 fused silica capillary column (Length – 30 m; Film thickness- 25 µm I.D - 0.2 mm) was used. The temperature program was as follows: the initial temperature was 50ºC and was heated for 5 min and then it was heated up to 240º C at the rate of 3ºC per min. (Farhang *et al*. 2013) [7]. The identification of components was accomplished using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrums of the Unknown compounds were compared with the spectrum of the known compounds stored in the NIST library.

Results and Discussion

1. Isolation and Identification of Rhizosphere fungi

A total 62 rhizosphere fungal isolates belonging to 9 genera were isolated and identified. The most abundant fungal species were found are *Aspergillus niger* (20.96%), *Aspergillus terreus* (12.90%), *Aspergillus flavus* (9.67%), *Rhizopus oryzae* (8.06%), *Rhizopus nigricans* (8.06%), *Mycelia sterila* (8.06%), *Aspergillus fumigatus* and *Trichoderma viride* (6.64%) followed by *Rhizopus stolonifer* (3.22%), *Penicillium* sps. (3.22%), *Curvularia lunata* (3.22%), *Fusarium oxyporum* (3.22%), *Mucor racemosus* (3.22%), *Curvularia inaequalis* (1.61%) and *Alternaria tenuis* (0.62%). Present experiment was repeated three times.

Fungal colonisation of medicinal and aromatic plants has been reported widely. Panwar and Tarafdar (2006) [20] identified 5 genera of AM fungi in the rhizosphere of 3 medicinal plant species (*Leptadenia reticulata, Mitragyna parvifolia, Withania coagulans*). Rhizosphere soil of the medicinal plants (*Ocimum sanctum* and *Centella asiatica*) reported the presence of 16–17 species of fungi (Sagar and Kumari 2009) [28]. Khamna *et al*., 2009 [13] obtained a total of 445 actinomycete isolates from 16 medicinal plant rhizosphere soils. Among them, 23 Streptomyces isolates showed activity against phytopathogenic fungi. Srivastava and Kumar (2013) [31] investigated rhizosphere fungal population in the roots surrounding area of *Abutilon indicum, Amaranthus polygamus, Achyranthes aspera, Argemone maxicana* and *Aloe vera.* All these species found to grow in wild and northern plains of India. Total number of 37 species of rhizosphere fungi was isolated and maximum numbers of fungi were found in this region as compare to the non-rhizosphere region. Maximum number of fungal species were found in *Abutilon indicum* (11) followed by *Aloe vera* (9), *Achyranthus aspera* (9), *Amaranthus polygamus* (8) and *Argemone maxicana* (7). Dai *et al.* (2009) [4] reported *Fusarium* spp. and *Verticillium* spp. in *Dioscorea zingiberensis, Atractylodes lancea, Euphorbia pekinensis, Ophiopogon platyphyllum* and *Pinellia ternate*. *Acaulospora scrobiculata* and *Glomus aggregatum* was reported in *Andrographis paniculata* (Radhika and Rodrigues, 2011) [22].

In 2016, Thombre *et al*. recorded 11 species of rhizosphere fungi isolated from *Santalum album*. Out of 11 species 10 species were belongs to class Hyphomycetes namely *Aspergillus niger*, *Aspergillus terricola*, *Aspergillus terreus*, *Aspergillus funiculosus*, *Aspergillus fumigatus*, *Aspergillu srestrictus*, *Aspergillus flavus*, *Aspergillus flavipes*, *Fusarium oxyporum*, *Penicillium* spp. and *Mycelia sterilia* belongs to Basidomycetes. During the investigation *Aspergillus niger*, *Aspergillus terricola* and *Penicillium* spp. were frequently observed and recorded during study. Shaikh and Nadaf (2013) [29] isolated and identified the rhizosphere fungi of local aromatic rice varieties viz., Basmati 370, Jiri, Ambemohar Tambda, Ambemohar 157, Indrayani, and Raibhog. The fungi, *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Curvularia lunata, Curvularia inaequalis, Alternaria tenuous, Rhizopus nigricans, Rhizopus oryzae, Trichoderma viride, Trichoderma lignorum harz, Nigrospora oryzae, Mucor racemosus, Phoma* spp. And *Fusarium oxyporum* were predominant in most of the tested varieties. Rhizosphere soil of the medicinal plants (*Ocimum sanctum* and *Centella asiatica*) showed the presence of 16–17 species of fungi (Sagar and Kumari 2009) [28].

Fourteen common cultivars of tree peony (*Paeonia suffruticosa*) rhizosphere was colonised by AM fungi (Shi *et al*. 2013) [30]. AM fungi (colonised by *Glomus intraradices* and *Glomus mosseae*) have improved salinity and plant growth tolerance by various mechanisms in *Bacopa monnieri*, an important medicinal plant (Khaliel *et al*. 2011) [12]. Seventeen species of AM fungi and fungal colonisation structures (hyphal coils, hyphae and vesicles) were present in roots of *Huangshan magnolia* (*Magnolia cylindrica*) (Yang *et al*. 2011) [35]. The species were from the genera *Glomus* (8 species), *Acaulospora* (6 species), *Scutellospora* (2 spp.) and *Gigaspora* (1 spp). Sundar *et al.*, 2011 [32] reported 21 AM fungal species in roots of the medicinal plants such as

Indigofera aspalathoides, *Eclipta prostrata* and *Indigofera tinctoria*. Five genera of AM fungi were identified in the rhizosphere of 3 medicinal plant species *viz.*, *Mitragyna parvifolia*, *Withania coagulans* and *Leptadenia reticulate* by Panwar and Tarafdar, 2006 [20].

There was an enormous variation in the AM fungi spore population and root colonization in the rhizosphere of ten medicinal plant species *viz.*, *Aloe barbadensis*, *Emblica officinalis*, *Mimosa pudica*, *Rauvolfia tetraphylla*, *Centella asiatica*, *Sapindus trifoliatus*, *Euphoria longan*, *Rauwolfia serpentina*, *Smilax* sp. and *Trachyspermum copticum*, in spite of their growth in similar climatic conditions (Hussain and Srinivas 2013) [10]. Khamna *et al*. (2009) [13] reported a total of 445 actinomycete isolates from 16 medicinal plant rhizosphere soils. The AM fungal community in the rhizosphere of *Phellodendron amurense* showed three general groups of *Glomus*, *Hyponectria* and *Scutellospora* respectively (Cai *et al*. 2009) [2].

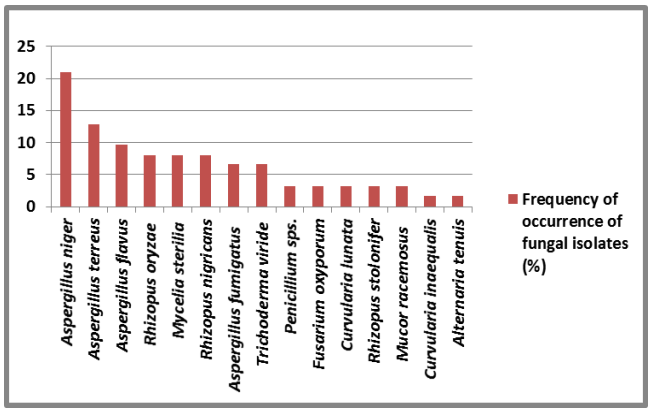

Fig 1: Percentage of fungal isolates per species

2. Gas Chromatography Mass Spectrometry (GC-MS) Analysis: GC-MS analysis was carried out to identify the possible compounds in the bioactive fraction of *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Rhizopus oryzae*, *Fusarium oxyporum* and *Trichoderma viride* extracts.

The GC-MS analysis showed the presence of 3-Methylbut-3-en-2-ol, Toluene, Methane, sulfinylbis-, Benzeneethanol, Benzene, 1,3-bis (1,1-dimethylethyl)-, 1-dodecanol, 1-Dodecene in *Aspergillus niger* extract**,** 3-Methylbut-3-en-2-ol, Toluene, Benzeneethanol, 1-Dodecene, 1-dodecanol, (trans)-2-nonadecene in *Rhizopus oryzae extract.* In *Aspergillus terreus* ethyl extract showed presence of 3-Methylbut-3-en-2-ol, 1-Undecanol, 1-Dodecene, 1-dodecanol. Important biologically active compounds namely Nerol and Citral with 3-Methylbut-3-en-2-ol, Toluene, Phenylethyl Alcohol, 1-Dodecene, 1-dodecanol were found to be present in *Trichoderma viride* ethyl extract. *Mycelia sterilia* showed presence of 1, 2-Benzenediol, 2,6-Octadien-1-ol, 3,7-dimethyl-, (Z)- while as *Aspergillus flavus* showed presence of 3-Methylbut-3-en-2-ol, Toluene, 1-Dodecene, 1-dodecanol. *Fusarium oxyporum* was found to be synthesized 3-Methylbut-3-en-2-ol, Methane, sulfinylbis-, 1-dodecanol and 1-Dodecene. Figure 2 shows the GC-MS chromatogram of ethyl acetate extracts of *Trichoderma viride* and Fig 3 shows the MS spectrum of Citral isolated from *Tichoderma viride* extract. In ethyl acetate extract of given fungus, a total of 3 major peaks were observed on the chromatogram. Among the possible compounds identified, given major groups of compounds detected were 1,2-Benzenediol, which is an aromatic compound comes under class phenol. Second major peak was confirmed as 2, 6-Octadien-1-ol, 3,7-dimethyl-, (Z)- which is a is a monoterpene used in perfumery. Third major and important peak was found as Citral which is either a pair, or a mixture of terpenoids with the molecular formula $C_{10}H_{16}O$. Citral is an aroma compound which is used in perfumery and flavoring. It has strong antimicrobial qualities, and pheromonal effects in insects.

In ethyl acetate extract of *Monochaetia kansensis isolated from Rhododendron* sp. showed presence of 2- tetradecene (22.00%), cyclodecane (19.39%), phenol, 2, 4-bis (1, 1-dimethyl ethyl) (9.78%), E-15-Heptadecenal (8.70%) and 1-octadene (12.29%) (Yogeswari, 2012) [37]. Rajalakshmi and Mahesh (2014) [24] reported that ethyl acetate extract of *Aspergilus terreus* isolated from the rhizosphere of medicinal plant showed presence of Cyclooctasiloxane, Hexadecamethyl, Heptasiloxane, hexadecamethyl- 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester, 9,12-Octadecadienoic acid (Z,Z)-, 9,12-Octadecadienoic acid, Linolsaeure,10,12-Hexadecadien-1-ol, Docosane (cas) n-Docosane, Hexacosane (CAS) n-Hexacosane, Tetracontane and n-Hexacosane. GCMS analysis of volatile metabolites in the ethyl acetate extract of the *Colletotrichum gloeosporioides* isolated from *Phlogacanthus thyrsiflorus* revealed the presence of Phenol,2,4-bis (1,1-dimethylethyl), 1-Hexadecene, 1-Hexadecanol, Hexadecanoic acid, octadecanoic acid methyl ester and 1-Nonadecene (Devi and Singh, 2013) [5]. The GC-MS results of ethyl acetate extract of *Fusarium proliferatum* FP85 revealed different metabolites such as 3-ethyl-2, 5-dimethyl-; Pyridine, 3-butyl-; Pyrazine, Stearic acid; Palmitic acid; Adipic acid; Oleic acid; Piperitenone oxide; butyl isobutyl ester and Phthalic acid, (Rasekhi, 2014) [26]. The ethyl acetate extract of *Aspergillus terreus* MP15 isolated from *Swietenia macrophylla* leaf showed major compound, di-n-octyl phthalate with 80% matching factor (Yin *et al.*, 2015) [36]. GC-MS analysis of ethyl extract of *Irpex lacteus isolated from Ocimum sanctum* showed presence of 8 compounds in the ethyl acetate extract were (Azaphenanthrene, 2-methyl-3-(2-phenylethenyl) (47.67%), Cycloheptasiloxane, tetradecamethyl) (22.18%), (Linolelaidic acid, methyl ester) (22.35 %), Androst-5-en-17-carboxylicacid (20.14%), (68.64%) Androst-5-en-17-carboxylic acid. N-Trifluoroacetyl-histidine, methyl ester or Methyl 3-(1H-imidazol5yl) 2-[(trifluoroacetyl) amino] propanoate and Thiocarbamic acid, N,N-dimethyl, S-1,3-diphenyl-2-butenyl ester, (Chaudhary and Tripathy, 2015) [3].

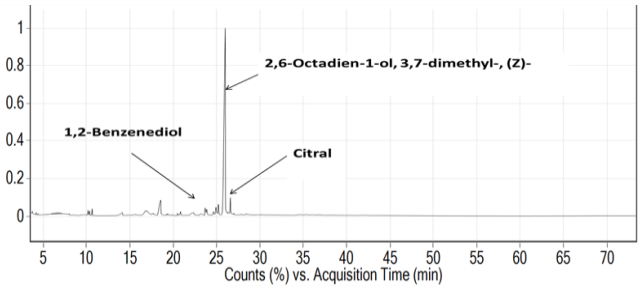

Fig 2: GC-MS chromatogram of *Tichoderma viride* shows citral peak after 21 days incubation in a PDB medium at 28 ºC.

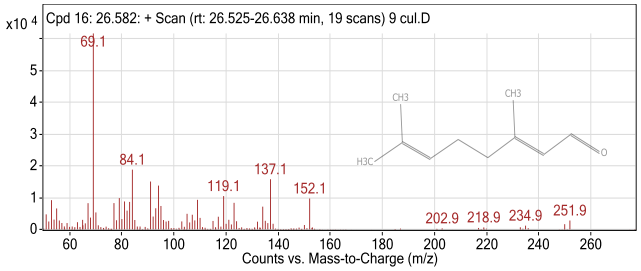

Fig 3: MS spectrum of Citral isolated from *Trichoderma viride* extract

In the present report for the first time completely optimized protocol for qualitative analysis of bioactive compounds from rhizosphere fungi of *Cymbopogon citratus* using GC-MS has been developed. This is the first report of isolation of Citral from the rhizosphere fungi, *Trichoderma viride*. The study highlights the active role of rhizosphere fungi in synthesizing this potential pharmacological bioactive compound. It is very likely that the rhizosphere associated with selected medicinal plant may harbor the potential microbes that might contribute in the synthesis of Citral. However, selecting and inoculating specific and efficient rhizosphere fungi for selected medicinally important plant are essential for the cultivation of medicinal plants in order to obtain the high-quality active metabolites. Therefore, further research is recommended to better understand the diversity and function of rhizosphere fungi and their uses in agricultural practices of medicinal plants.

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