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Effect of arbuscular mycorrhizal fungi inoculation on the antioxidant property and volatile organic compounds emission by *Cymbopogon citratus* L.

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Abstract

Arbuscular mycorrhizal (AM) fungi influence host plants in the synthesis of many secondary metabolites with pharmaceutical importance. The effect of AM fungi on metabolite production in the *Cymbopogon citratus* is less studied. The present study investigates the changes influenced by the two AM fungi *Glomus mosseae* and *Glomus fasciculatum* on the chemical composition of *C. citratus*. The bioactive compounds produced in treated and control plants were tested for antioxidant activity and further characterized by Gas Chromatography-Mass Spectrometry, High-Performance Liquid Chromatography techniques. It was found that AM fungal inoculation in mixed culture conditions showed increased radical scavenging activity with minimum IC_{50} value of 19.08µg/mL and compound production at 90 days of treatment. In the treated plant extract the metabolites were present at a higher concentration as compared with control plants. The GC-MS results are supported by the HPLC spectrum which showed a similar outcome. This study proves that inoculation of AM fungi in combination is more beneficial in influencing the production of bioactive compounds than the pure culture.

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Introduction

The arbuscular mycorrhizal (AM) fungi are obligating plant symbiotic microorganisms that are known to supply phosphorus in addition to supplementing a vast range of nutrients and secondary metabolites to their host plants (Smith *et al.*, 2014). They are the most commonly occurring mycorrhizal association and are recognized by the formation of unique structures called arbuscules and vesicles (Choi *et al.*, 2018). They are ubiquitous in their distribution and are observed in nearly all plants (Harrison, 2015).

The symbiotic association between these organisms and the roots of the host plants is very important in terms of nutrition supply, plant protection and in finally the overall growth and yield of the plant (Kumar *et al.*, 2020). The most important aspect of these AM fungi is the improved uptake of immobile nutrients like Cu, P, Zn etc., (Bonfante and Genre, 2015). These nutrients are absorbed by the AM fungi from the soil and translocated to the host plant (Harrier and Watson, 2004).

AM fungi also aid in the enhancement of chlorophyll levels in the leaves of the associated plant (Wipf *et al.*, 2019), and improve plant tolerance to many abiotic and abiotic stresses (Gutjahr and Parniske, 2013). The complex hyphal network of AM fungi significantly contributes to enhancing soil aggregation and thus helps soil conservation (Bergera and Gutjahr, 2021). Therefore inoculation of AM fungi to medicinally important plant during the early stage of development is an alternative approach for improving plant growth and yield (Chen *et al.*, 2018).

In recent years, medicinal plants have gained greater importance owing to their tremendous application in the medicines, pharmaceutical, cosmetic and fragrance industries (Rui-Ting *et al.*, 2021). Therefore, there is a need for intensive research to develop the quality and quantity of drugs and food produced from medicinal and crop plants in a shorter period and at a lower cost by using AM fungi (Duc *et al.*, 2021). The arbuscular mycorrhizae (AM) have gained importance in improving the performance of many economically important plants (Moustakas *et al.*, 2020).

There has been a steady increase in the cultivation of medicinal plants to maintain a continuous supply to support the increasing demand for drugs (Song *et al.*, 2019). But, to meet this demand corresponding research on AM fungi and their association with medicinal plants have received very little attention (Pandey *et al.*, 2018). Therefore, immense exploration in this aspect is essential for a better understanding.

The *Cymbopogon citratus* is cultivated on a large scale in the tropics and subtropics because of its diverse uses in cosmetics, food and flavour industries, agriculture and pharmaceutical industries (Oladeji *et al.*, 2019). *C. citratus* possesses a strong lemony odour because of the aldehyde citral present in high content (Majewska *et al.*, 2019). The plant is highly nutritious, and rich in vitamins, minerals and macronutrients like protein and carbohydrates (Subramaniam *et al.*, 2020). The plant possesses antimicrobial compound citral which gives a strong lemon-like aroma (Sousa *et al.*, 2021). The essential oil from lemongrass and medicinal tea from the plant has been traditionally used as a therapeutic agent for antioxidant (Patiño-Ruiz *et al.*, 2020), antimicrobial (Trang *et al.*, 2020), anti-inflammatory (Valková *et al.*, 2022) and antiparasitic (Boeira *et al.*, 2020) purposes.

The plant is rich in phytochemical constituents like alcohols, aldehydes, esters, tannins, saponins, flavonoids, alkaloids, phenolics, anthraquinones, ketones and long-chain hydrocarbons (Valkova *et al.*, 2022). The lemongrass oil consists of citral, eugenol, isoeugenol, linalool, limonene, citronellol, nerol, burneol, α-terpineol, luteolin, quercetin, apigenin and kaempferol (Borges *et al.*, 2021). Hydroxycinnamic acids like chlorogenic and p-coumaric acid derivatives and flavones like apigenin and luteolin derivatives are the phenolic compounds identified from the leaves of *C. citratus*. The plant also contains electrolytes, minerals, condensed-type tannins and triterpenes (Cherian *et al.*, 2020). The rich phytochemicals found in *C. citratus* were reported with many pharmacological properties such as antihypertensive, antidiabetic and anticancer activities (Hacke *et al.*, 2020).

Proven that a majority of all medicinal plants are having mycorrhizal interaction, the effects of these AM fungal organisms may be exploited for the increased production of bioactive metabolites (biofertilizer effect) or the production of new pharmaceutical compounds. The present study aimed to assess the effect of AM fungal inoculants on the bioactive compounds produced by *C. citratus*.

Materials and methods

The two commercial cultures *Glomus mosseae* and *Glomus fasciculatum* were collected from the Centre for Natural Biological Resources and Community Development (CNRBCD), Bangaluru, Karnataka to study the effect of AM fungi on *C. citratus* and bioactive compound production. Plantlings of *C. citratus* were procured from the Indian Institute of Horticultural Research (ICAR) Research Institute, Hessarghatta, Bengaluru, Karnataka.

Experimental design and plant growth conditions

The fifteen days old plantlings were transferred to pots filled with a sterilized substrate (600g of sterilized soil and 200g sterilized farmyard manure 3:1w/w) and were inoculated with 5% AM fungal inoculum (Asensio *et al.*, 2012). The effect of AM fungi on the plants was tested in different treatments viz., pure cultures of *G. mosseae* and *G. fasciculatum* and in mixed culture. Plants were grown in a greenhouse condition for 30, 60 and 90 days and were watered regularly. Each treatment included two replicates and each replication had fifteen plants. At the end of the predetermined period, all plants were removed and shade-dried (Hart *et al.*, 2015).

Solvent extraction

The crude extracts were prepared by the Soxhlet extraction method as described by Ghadiri *et al.*, (2020). About 20gm of powdered aerial plant material was packed in a thimble and extracted with 250mL of methanol as a solvent for extraction. The extraction process was for 24h or till the solvent in the siphon tube of the extractor became colourless. The extracts were taken in a beaker and placed in a hot water bath at 30-40^{o}C till all the solvent was evaporated.

DPPH radical scavenging assay

The free radical scavenging activity of the host plant treated with AM fungi for 30, 60 and 90 days was carried out and compared with the respective uninoculated control plant extracts. The solvent extracts were taken in triplicates in different concentration ranges (100µL, 200µL, 400µL, 600µL, 800µL and 1000µL) in each test tube and the volume was made up to 1mL. To this about 3mL of 0.1mM DPPH (1,1-Diphenyl-2-picryl hydrazyl) was added. The mixture was shaken well and incubated in dark for 30min. The absorbance was measured at 517nm using an Anatech UV-Vis Spectrophotometer. The radical scavenging activity was calculated by the formula

$$\text{Scavenging activity (\%)} = \frac{(Ac - As)}{Ac} \times 100$$

Where Ac indicates control and As is the absorbance of the sample (Ichikawa *et al.*, 2019).

Gas Chromatography - Mass Spectrometry analysis

The crude extract from plants inoculated with pure culture and mixed culture of AM fungi for different time duration were subjected to characterization by GC-MS analysis to compare the metabolites produced. The Agilent GC-MS apparatus (GC: 7890A; MSD5975C) with a silica HP-5 capillary column (30m–0.25mm, an ID film thickness of 0.25mm) coupled directly with a single quadrupole MS was used. The peaks detected in the GC chromatogram were assigned as a particular compound through mass spectral data analysis software and NIST MS library data, 2010 (Shi *et al.*, 2020).

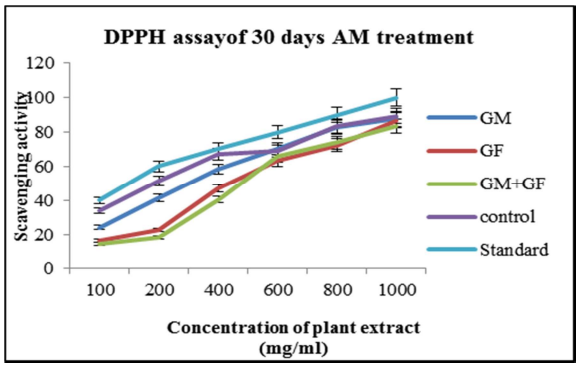
High-performance liquid chromatography analysis

The HPLC analysis of the crude methanol extracts from AM inoculated and control plants were performed using the instrument Agilent Technologies, Model: 1200 Infinity Series. The mobile phase was prepared using acetonitrile and water in the ratio of 50:50 (v\v). The column used for the assay was C18 with a flow rate of 1mL/min and the detector wavelength was set at 273nm. The volume of the sample injected was 20µL and the temperature was set at 25°C. The total run time was 30min (Gissawong *et al.*, 2019).

Results and discussion

DPPH radical scavenging Assay (1, 1-Diphenyl-2-picryl hydrazyl)

The DPPH radical scavenging assay revealed that extracts from *C. citratus* treated with AM fungi in mixed culture for 90 days, exhibited the highest scavenging activity with an IC_{50} value of 19.08µg/mL followed by *G. fasciculatum* with an IC_{50} value of 20.86µg/mL. The uninoculated control plant extracts at all-time duration of 30, 60 and 90 days showed an average IC_{50} of 21.96µg/mL. The scavenging activity was increased significantly at 60 and 90 days AM treated plant extracts (Fig. 1).



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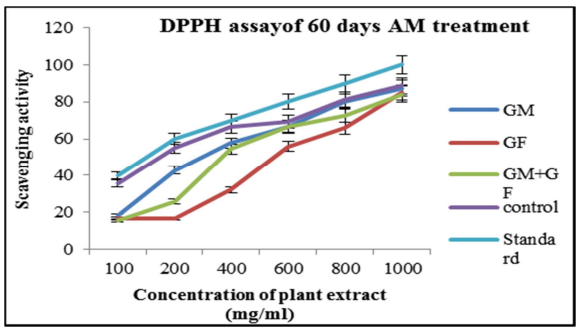

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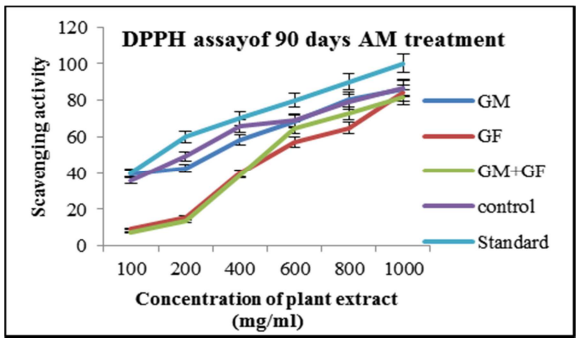

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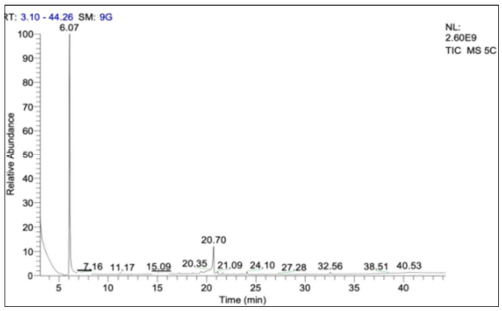
Fig. 1. Antioxidant activity of AM inoculated and control *C. citratus* extracts. a- 30 days treatment, b- 60 days treatment, c- 90 days treatment.

Gas Chromatography-Mass Spectrometry analysis 30 Days AM fungal treatment

In the GC-MS analysis, distinct phytochemical profiles were observed in the plant extracts tested from 30 days of AM fungal treatment. Twelve different compounds were identified in plant extract treated with the monoculture of *G. mosseae*.



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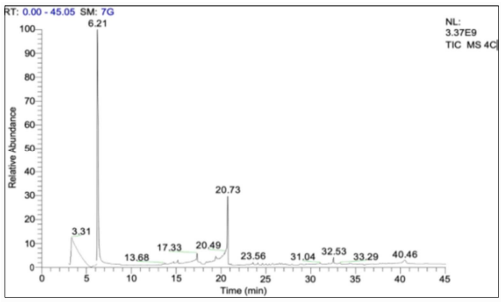

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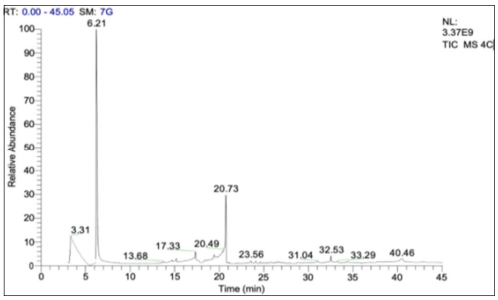

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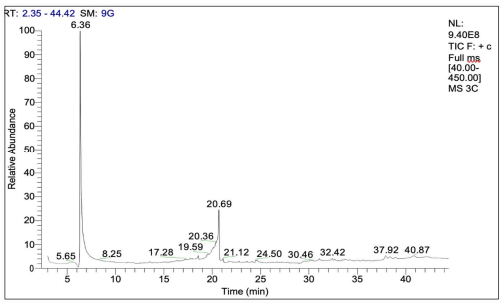

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Fig. 2. GC-MS Chromatogram of 30 days treated *C. citratus* extracts with a-*G. mosseae*, b-*G. fasciculatum* c-mixed culture, d-control.

The major compounds identified in these extracts were 3,3,4,4-Tetrafluoro-1,5-hexadiene (29.04%), Benzene, (2-methyl cyclopropyl) (5.34%), 2,4-Difluorophenyl hydrazine (0.63%), Ethanone, 1-(2-hydroxy-5-methylphenyl) (16.03%), Decanoic acid (3.05%). About eighteen different chemical constituents were identified from the extract of the plants treated with the monoculture of *G. fasciculatum*. The major compounds identified in these extracts were 1,7-dideoxy-d-mannoheptulose1,7-bis-benzylamine (5.18%), Phen-1,4-diol, 2,3-dimethyl-5-trifluoromethyl- (7.23%), Bicyclo (4.4.0) dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl) (3.09%). Palmiticacid, methyl ester (2.94%). In the mixed culture inoculated plant extract, twenty different compounds were recorded. Some of the compounds include 2,5-Octadecadiynoic acid, methyl ester (61.03%). Phenol 3-(1,1-dimethylethyl)-4-(11.06%), Bicyclo (4.4.0) dec-2-ene-4-ol, 2-methyl-9-(prop-1-en-3-ol-2-yl) (2.94%), Palmitic acid, methyl ester (3.90%). 6-hydroxy-4-methyl, dimethyl acetal, acetate (2.94%), 4-Cyano-3,5-dimethylpheno (1.10%). Different effects of inoculants were also observed for volatile compounds like 2,5-Octadecadiynoic acid, methyl ester, and 3-Nitro-2-butanol which was found to be significantly higher in plants treated with mixed culture as compared to monoculture and control plants (Fig. 2).

60 Days AM fungal treatment

The GC-MS analysis of phytochemicals from the 60 days AM fungal treated plant extracts revealed the presence of sixteen different compounds in plant treated with the monoculture of *G. mosseae*. Furfuryl alcohol (10.74%) was an additional compound at a higher concentration and some of the other major compounds identified in this extract were Diethyl Phthalate (3.90%), 3,12,25-Tris(acetyloxy) cholestan-7-yl acetate (0.87%), (Z)-Cinnamic acid (0.518%). About nineteen different chemical constituents were identified from the extract of the plants treated with the monoculture of *G. fasciculatum*. Butanedioic acid (2.94%) was the additional compound produced along with the other compounds identified. In the mixed culture inoculated plant extract, twenty-three different compounds were recorded. Changes in the compounds produced include 4-Hexenal, 6-hydroxy-4-methyl, dimethyl acetal, acetate (14.03%), 2-Myristynoyl pantetheine (4.30%), 2-Undecanone (1.94%), 2-Myristynoyl pantetheine (1.90%). The concentration of the compounds was found to be higher in the 60 days AM treated (monoculture and mixed culture) plant extracts compared to control plants (Fig. 3).

90 Days AM fungal treatment

After subjecting 90 days of AM fungal-treated plant extracts to GC-MS analysis, it was found that the spectrum was similar to 60 days treated plant extract. Further in the monoculture, *G. mosseae* treated plants, it was noted that concentrations of Ethanone, 1-(2-hydroxy-5-methylphenyl) (23.03%), Decanoic acid (5.05%) were slightly higher as compared with 30 days plant extracts.

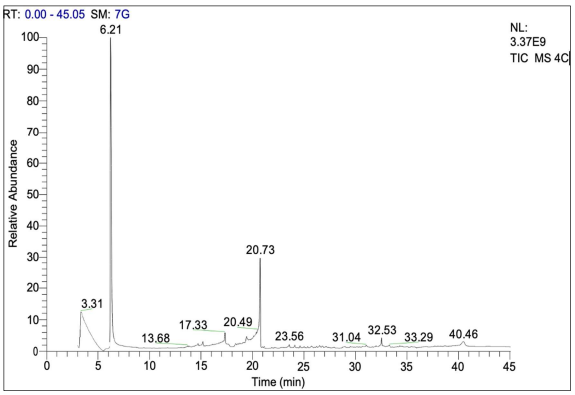
In the plants treated with the monoculture of *G. fasciculatum,* the percentage of Butanedioic acid increased from 2.94 to 8.34%. In the mixed culture inoculated plant extract, twenty-five different compounds were recorded. Changes in the compounds produced include Oxime-, methoxy-phenyl- (1.90%), 2H-1-Benzopyran/Edulan (0.96%), and Acrolein (1.06%) (Fig. 4).

High-performance liquid chromatography analysis

30 Days AM fungal treatment

The HPLC chromatogram of AM fungal-treated *C. citratus* plants revealed a significant difference in the peak produced. Twelve peaks were identified in plant extract treated with the monoculture of *G. mosseae* corresponding to the twelve chemical components detected in GC-MS analysis. Four major peaks were recorded at a retention time of 2.47min, 3.26min, 3.48 min and 4.128min. About thirteen peaks were recorded from the extract of the plants treated with the monoculture *G. fasciculatum*.

The major peaks were identified in these extracts at an RT of 2.4min, 3.2min, 3.5min, 3.7min and 18.4min. In the mixed culture inoculated plant extracts, thirteen peaks were recorded. Some of the major peaks were recorded at 2.4min, 3.264min, 3.7min and 18.4min (Fig. 5).



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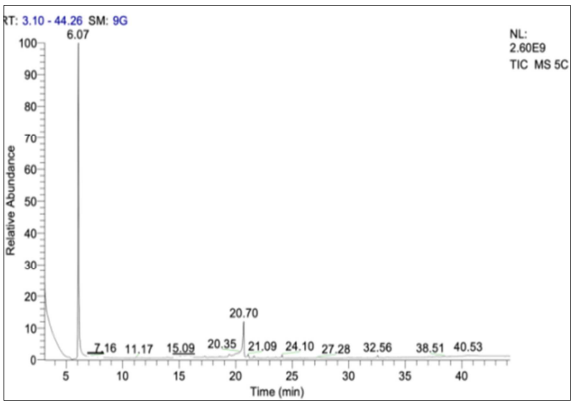

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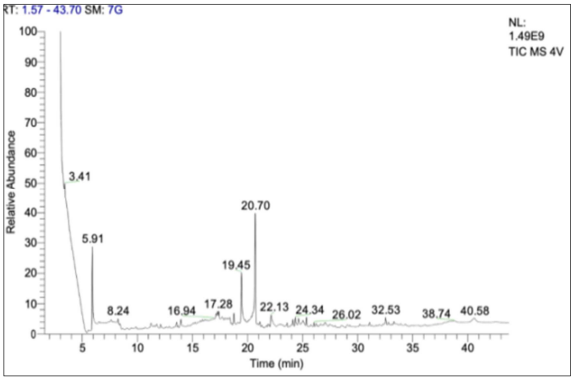

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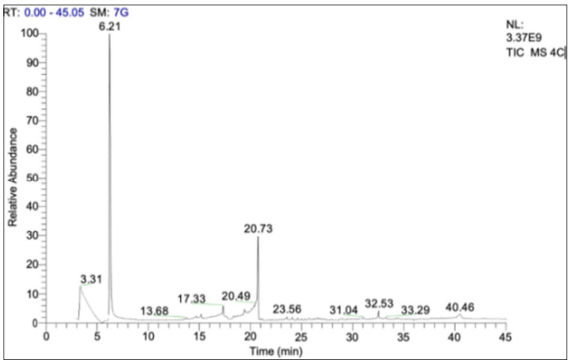

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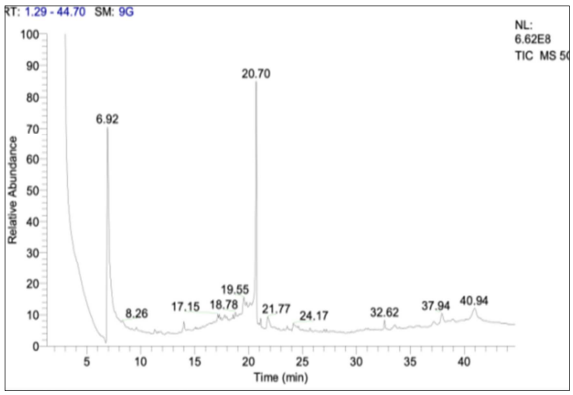
Fig. 3. GC-MS Chromatogram of 60 days treated *C. citratus* extracts with a-*G. mosseae*, b-*G. fasciculatum* c-mixed culture, d-control.



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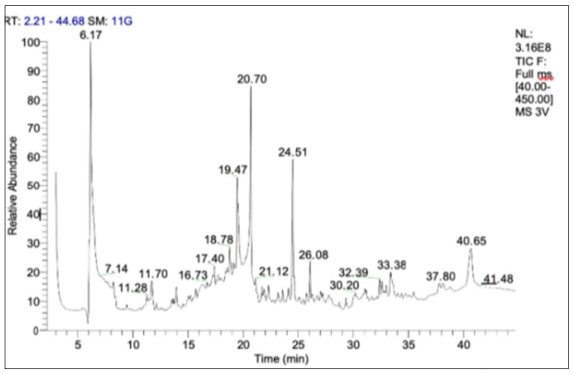

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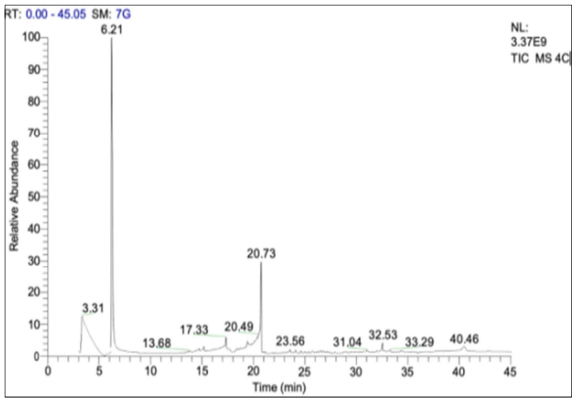

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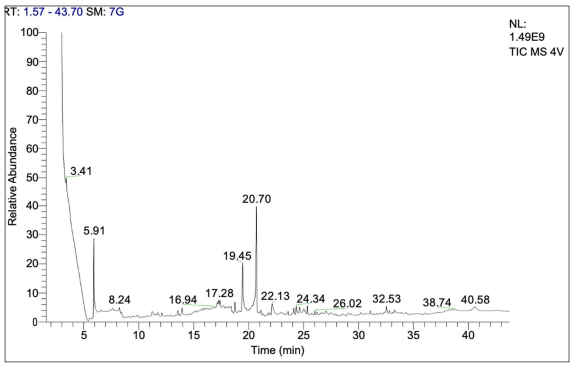

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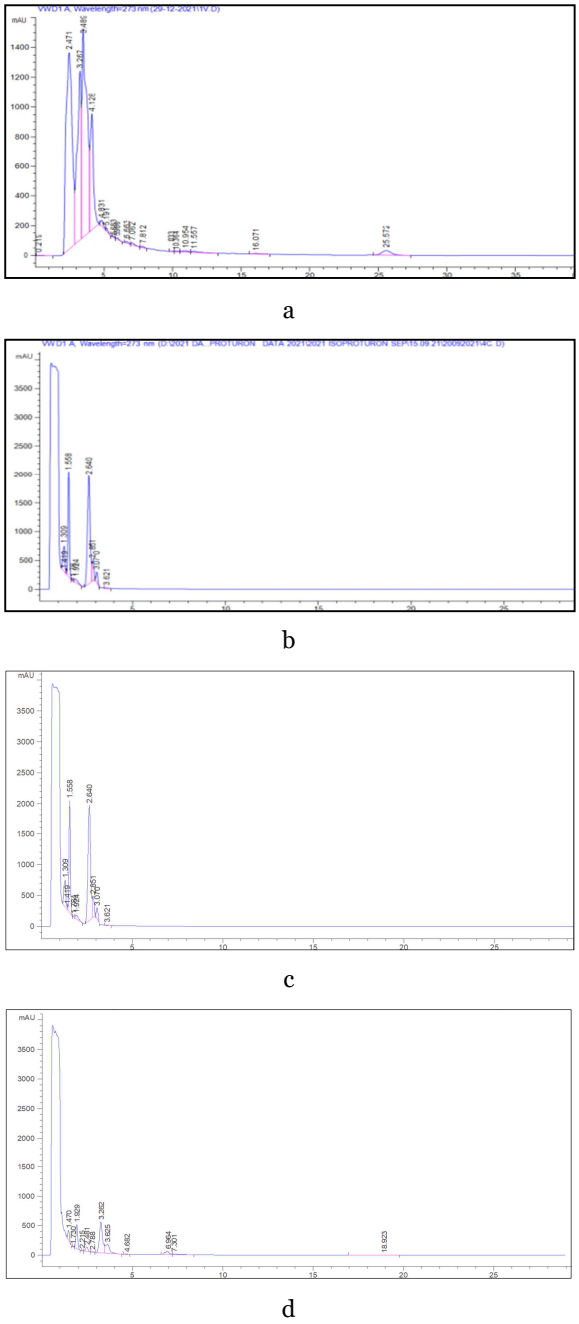
Fig. 4. GC-MS Chromatogram of 90 days treated *C. citratus* extracts with a-*G. mosseae*, b-*G. fasciculatum* c-mixed culture, d-control.

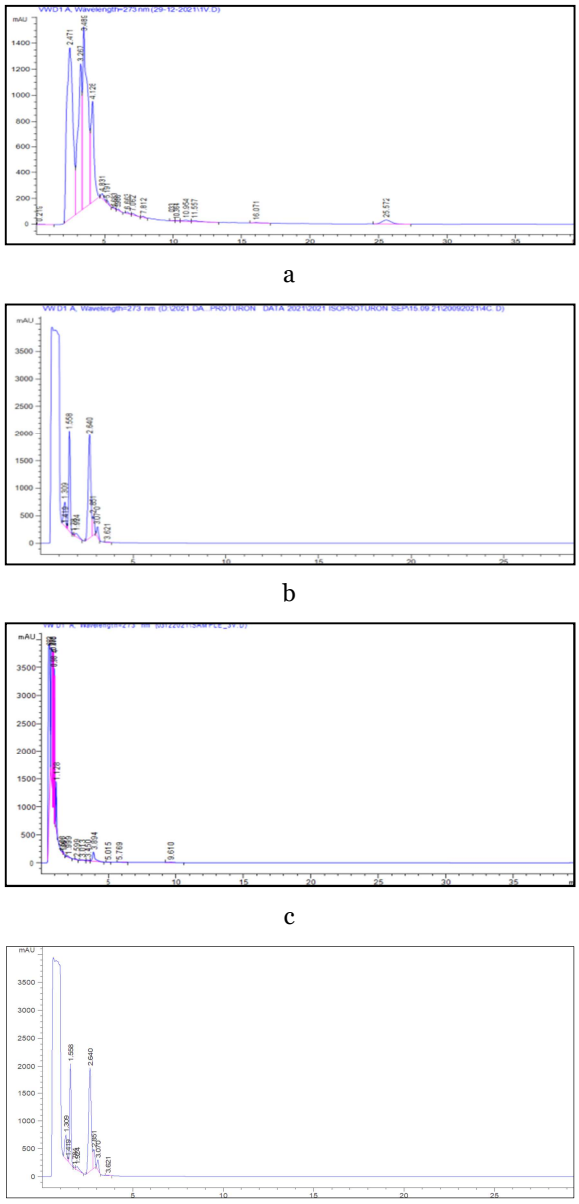


a b c d

Fig. 5. HPLC chromatogram of 30 days treated *C. citratus* extracts with a-*G. mosseae*, b-*G. fasciculatum* c-mixed culture, d-control.

60 Days AM fungal treatment

The HPLC spectrum of crude methanol extract from control and 60 days AM fungi-treated plant samples showed a difference in peak position as shown in fig. 2. In plant extract treated with the monoculture of *G. mosseae,* fifteen peaks with four major peaks at RT of 0.6min, 0.78min, 0.87min and 0.98min were produced. From extracts of *G. fasciculatum,* five major peaks along with thirteen minor peaks were noted. The RT of the major peaks was recorded at 2.471min, 3.267min, 3.489min, 4.12min and 25.57min (Fig. 6).



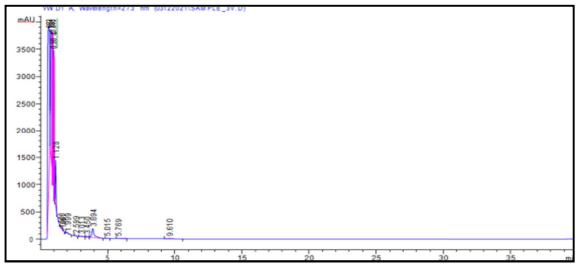
a b c d

Fig. 6. HPLC chromatogram of 60 days treated *C. citratus* extracts with a-*G. mosseae*, b-*G. fasciculatum* c-mixed culture, d-control.

90 Days AM fungal treatment

The HPLC analysis of 90 days AM fungal treated and control plants showed significant changes in the compounds produced as compared to control plant extracts. Among the twenty-one, peaks detected four major peaks were recorded at RT of 0.5min, 0.71min, 0.7min, and 1.1min. The chromatogram of *G. fasciculatum* treated plants produced thirteen minor

peaks and three major peaks at RT 2.4min, 3.26min, and 3.78min. About twenty-eight minor peaks and four major peaks at RT 5.48min, 6.03min, 18.4min and 3.78min were seen in plants treated with mixed culture, as shown in Fig. 7.



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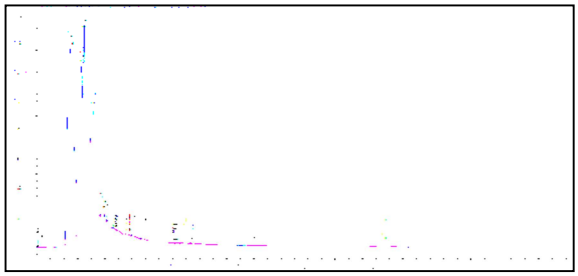

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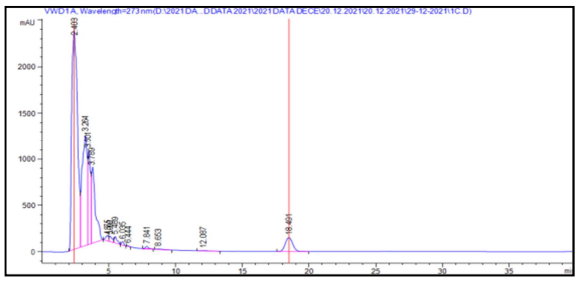

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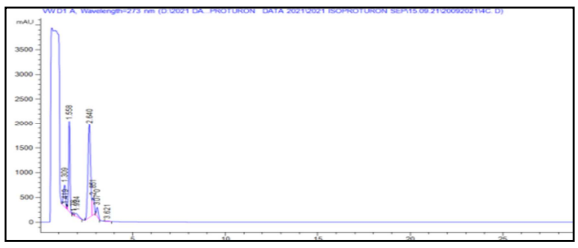

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Fig. 7. HPLC chromatogram of 90 days treated *C. citratus* extracts with a-*G. mosseae*, b-*G. fasciculatum* c-mixed culture, d-control.

Discussion

The arbuscular mycorrhizal fungi have proven to influence the metabolism of the associated host plant. To fully understand the effects of AM fungi on chemical components produced, it is necessary to systematically study the crude extracts using different analytical techniques. This study evaluates the chemical composition of the extract of the *C. citratus* plant using GC-MS and HPLC analysis. The GC-MS analysis of *C. citratus* plant extracts revealed the existence of 4-Tetrafluoro-1,5-hexadiene (29.04%), Decanoic acid (3.05%), Bicyclo [4.4.0] dec-2-ene-4-ol, 2-methyl-9- (prop-1-en-3-ol-2-yl)- (27.41%) Palmitic acid, methyl ester (2.94%). This shows that inoculation of AM fungi considerably changes the yield and composition of the compounds. The presence of an increased number of compounds from mixed AM-treated plant extract proves that AM fungi in combination favour diversity of phytochemical compound production.

According to the work conducted by Fontana *et al.* (2009), volatile organic compounds and glycosides of *Plantago lanceolate* L. were assessed in mycorrhizal and non-mycorrhizal plant samples. It was noted that In contrast, mycorrhizal infection increased the emission of -3-hexenyl acetate in untreated *P. lanceolate*.

Hassan *et al.*, in 2010, investigated the essential oil content and composition and nutrient acquisition of *Ocimum basilicum* inoculated with AM fungi G. fasciculatum, *G. etuonicatum*, *G. intraradices* and non-mycorrhizal control plants. It was noted that AM-treated plant samples had a significantly higher content of essential oil. The inoculation of *G. fasciculatum* increased the essential oil yield as compared to other AM fungi. The analysis of essential oil by GC-MS revealed that the highest relative abundance was recorded for linalool as the major compound in the essential oil of basil leaves. The methyl chavicol profile was increased considerably upon AM fungal inoculation. In the study conducted by Wisnu (2018), proved that *Travis* Wetland manuka inoculated with AM fungi produced 41 compounds as detected in GC-MS peaks and some of the compounds identified were maltol, β-elemene, α–selinene, β-selinene, trans-calamenene, grandiflorone and flavonoid. Sorghum (*Sorghum bicolor*) plants inoculated with either *G. mosseae* or *G. intraradices*, AM-treated plants produced more alcohols, alkenes, acids and ethers but fewer linear-alkanes and AM fungi also affected the morphological traits in the host roots (Kumar *et al.*, 2021).

Karagiannidis *et al.* (2011) reported that mycorrhizal oregano and mint plants had a considerably high content of essential oils and nutrients. It was also noted that the composition of the essential oil also differed from AM-treated plants and control plant extract as tested by GCMS analysis. When the growth was considered the AM fungal-treated plant samples showed better growth than that of the control plants. These results propose that the use of mycorrhizal fungi aid host plants to grow in low-fertility soils reduces fertilizer inputs and increases essential oils produced in the plant.

Viola tricolor L. a valuable medicinal plant was inoculated with different AM fungi. It was noted that the mycorrhizal colonization and arbuscule formation intensity was higher when *F. mosseae* and *R. irregularis* were treated in monoculture than in mixed culture. The plants treated with *R. irregularis* had higher concentrations of P, Mg, Ca, hydroxybenzoic acid and rutin, in comparison to the control. It was also reported that inoculation of mixed culture of AM fungal species increased the concentration of Cu, Mg and rutin (Zubek *et al.*, 2015). The study by Chen *et al.* (2013), reported the potential of *F. mosseae* inoculated cucumber seedlings which produced more secondary metabolites like phenols, flavonoids and lignin. Antioxidant activity was also at a higher percentage in the mycorrhizal plants than in non-mycorrhizal plant extracts. When the extracts were subjected to GC-MS analysis large increments were observed in glucose-6-phosphate dehydrogenase, shikimate dehydrogenase, phenylalanine ammonia-lyase, polyphenol oxidase, guaiacol peroxidase, caffeic acid peroxidase and chlorogenic acid peroxidase.

Conclusion

The symbiotic association which exists between the plant and the AM fungi helps the host plants to acclimatise to many biotic and abiotic stress in the environment. The AM fungi help in plant growth, nutrient uptake and stress responses. Results from the present study show that inoculation of AM fungi to a medicinally important plant like C. citratus has the potential to alter the chemical components especially volatile organic compounds in the host plants. They also prove that it may be possible to use mycorrhizae to affect the quality and quantity of the essential oil produced by the host plants.

Conflict of interest

The authors declare no conflict of interest

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