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SOME VOLATILE METABOLITES PRODUCED BY THE ANTIFUNGAL-*TRICHODERMA ASPERELLUM* UZ-A4 MICROMYCETE

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ABSTRACT

The fungus of the genus *Trichoderma* is characterized by high biological activity and the ability to synthesize many compounds of great scientific interest. Among them, producers of amino acids, nucleic and organic acids, vitamins, surface-active substances, numerous hydrolases, various antibiotics and other substances were revealed. In this work there were studied volatile organic compounds produced by the fungus *Trichoderma asperellum* Uz-A4, isolated from the soil of the cotton field of the Bukhara region of Uzbekistan infected with phytopathogens. The antagonistic activity of the fungus about phytopathogens *Alternaria alternata, Aspergillus niger* and *Fusarium solani* was evaluated. *Trichoderma asperellum* Uz-A4 micromyzet showed high activity on the 4 th day against *Alternaria alternata* and *Fusarium solani* about *Aspergillus niger* on the 9th day. From the culture fluid of the fungus *Trichoderma asperellum* Uz-A4, 11 main substances have been isolated and identified. Mass - spectrometric analysis has shown that these are the substances of Phenylethylcohol; 5-hydroxymethylfurfural; Dehydroa Ceticacid; 1-Dodecanol; 2,4-di-tert-Butylphenol; Diethyl Suberate; n-hexadecanoic acid; 1-hexadecanol, 2-methyl; Phthalic Acid, Ethyl Pentadecyl Ester; Mono (2-Thylhexyl) Phthalate; Octadecanoic Acid. A chemical formula, molecular weight and the absorption spectrum of these substances have been determined. The results indicated that these secondary metabolites could be useful for biological control applications of *T. asperellum* Uz-A4 strain against diverse plant pathogens.

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INTRODUCTION

According to many scientists, more than 1.5 million microscopic fungal species are spread worldwide. However, approximately 10% of the fungi and 1% of them were studied on the spectra of secondary metabolites (Weber *et al.*, 2007). It is known that the largest amount of natural preparation, 45% of antibiotics obtained based on secondary metabolites are produced by fungi. In this case, the share of asidialmacromycetes is 11%, while the share of micromycetes belonging to the *Penicilium*, *Aspergillus*, *Trichoderma* and *Tolypocladium* is 33%. The representatives of make up almost 99% of metabolites used in medicine and agriculture (Zhu *et al.*, 2011).

Genus *Trichoderma* the secondary metabolites formed are important for agriculture some of which are noteworthy for their antifungal properties against phytopathogenic fungi (Daoubi *et al.*, 2009). *Trichoderma* metabolites are chemically diverse natural compounds of relatively low molecular weight produced primarily by microorganisms and plants. The secondary metabolites are biosynthezed pathways from primary

metabolites (i.e., polycetides or mevalonate pathways derived from acetyl coenzyme A or amino acids) and are synthesized by certain genes. The expression of these genes is controlled by one or more global regulators (Herbert, 1989). The secondary metabolites exhibit several biological activities related to the body's survival functions, such as competing against other micro and macro-organisms, symbiosis, and ion exchange. *Trichoderma* the production of the secondary metabolites in fungi is often interrelated with specific stages of morphological dynamics, during the active growth phase the metabolites increase and the number of species increases. The secondary metabolites exhibit several biological functions and play an important role in regulating interactions between organisms. The are phytotoxins (secondary metabolites produced by plants phytopathogenic microorganisms), mycotoxins (secondary metabolites produced by fungi that cause disease and death in humans and animals), pigments (metabolites that form color compounds with antioxidant activity), and antibiotics (natural microbiological resistance or secondary metabolites capable of destruction) (Keller *et al.*, 2005; Chiang *et al.*, 2009).

Among microorganisms, fungi of the *Trichoderma* are one of the most powerful biological agents in use today because they produce various metabolites against pathogenic microbes (Khan *et al.*, 2020; Ming *et al.*, 2012). *Trichoderma* lives in the soil and grows saprophytically on many substrates, such as tree bark, and plant roots and affects animals (a source of protein and enzyme-rich nutrients when added to feed) and plants (growth, development, microbiological protection) (Atanasova *et al.*, 2013; Holzlechner *et al.*, 2017). Some of the secondary metabolites produced by *Trichoderma* are important as drugs and a single compound (6-pentyl-a-piron) as a food flavor. Since the discovery of gliotoxin in the early 1930s, the extraction and study of metabolites from fungi of the *Trichoderma* genus have begun (Weindling and Emerson, 1936). Over the years, analytical studies have isolated more than 120 secondary metabolites from *Trichoderma* and determined their structures as well (Sivasithamparam and Ghisalberti, 1998; Reino *et al.*, 2008). However, it is difficult to determine the exact amount of secondary metabolites produced by *Trichoderma*, which can form more than 1000 compounds, depending on the characteristics of the strain, environmental conditions and the sensitivity of the detection method. In recent years, genetic and genomic studies have revealed that *Trichoderma* secondary metabolites form new types of metabolites, taking into account biosynthetic pathways, fungal metabolism and environmental interactions (Reithner *et al.*, 2007). These micromycetes produce several pharmaceutical and biotechnologically important secondary metabolites, including non-ribosomal peptides, terpenoids, pyrons, indolyl compounds, peptaibols, polycetides, sideophores, volatile and non-volatile terpenes (Contreras-Cornejo *et al.*, 2016; Vinale *et al.*, 2008; Velázquez-Robledo *et al.*, 2011; Müller *et al.*, 2013). One of them is a steroidal metabolite viridin, an antifungal compound isolated from various *Trichoderma sp* (*T. koningii, T. viride, T. virens*) (Golder and Watson, 1980; Singh *et al.*, 2005). This antibiotic secondary metabolite exhibits potent antagonism to microorganisms such as *Botrytis allii, Colletotrichum lini, Fusarium caeruleum, Penicillium expansum, Aspergillus niger* va *Stachybotrys atra* (Reino *et al.,* 2008).

After reviewing these, we set the goal of our study is to analyze the secondary volatile metabolites that form the fungus *Trichoderma* and their properties.

MATERIALS AND METHODS

Study area and Material Selection

The *Trichoderma* sp. 4 strain was selected from the soils of a cotton field infected with phytopathogenic diseases in the Bukhara region of the Republic of Uzbekistan in October 2019.

Fungi Identification

The soil samples were dried in air for 4 hours and the isolation of microorganisms was carried out by the method of serial dilutions. The inoculum was incubated at 28 +30°C for 5 days. Observation of the appearance of colonies was recorded for 3 to 5 days. Colonies with symptoms of *Trichoderma* in Petri dishes were isolated and kept clean for further study. Isolated strains were identified by classical methods based on morphology using the relevant literature (Park *et al.*, 2005). The isolated strains were deposited at the Institute of Microbiology of the Academy of Sciences of the Republic of Uzbekistan, where they were kept at a low temperature (4-5 °C).

DNA Isolation and Purification

A 200 µl of the *Trichoderma* sp. 4 fungus sample was taken in a 1.5 ml plastic tube. Then 200 µl of 200 mM LiOAc, 1% SDS buffer was added, vortexed thoroughly and incubated at 70 °C for 5 minutes (Arnold *et al.*, 2011). The samples were then centrifuged at 15,000 g for 5 min. The liquid portion was taken to a new plastic

tube, an equal volume of 96% ethanol was added and vortexed. For DNA precipitation, the sample was stored at -20 °C for 1 h and centrifuged at 15,000 g for 5 min. The liquid in the tube was discarded and washed in 70% ethanol. The precipitate was dissolved in 100 µl of TE buffer and detected on a 0.8% agarose gel. Add another 100 µl of TE to the DNA dissolved in 100 µl of TE. 1 µl of RNAase was added, vortexed and incubated at 37 °C for 30 min. Then, 0.1 volume (20 µl) of NaAc and 1 volume of isopropanol (220 µl) were added. The sample was stored at -20°C for 1 hour and centrifuged at maximum speed for 10 minutes.The liquid in the tube was discarded and washed in 70% ethanol. The precipitate was dissolved in 50 µl of TE buffer and detected on a 0.8% agarose gel.

PCR Amplification of the ITS Fragment

Universal oligonucleotide primers of the internal transcribed spacer (ITS) gene were used for PCR amplification: ITS1-(TCCGTAGGTGAACCTGCGG), and ITS4-(TCCTCCGCTTATTGATATGC) (White *et al.*, 1990). PCR amplification of DNA samples isolated from bacterial strains was conducted in the GenPak® PCR MasterMix kit. In this case, the reaction was prepared in a total volume of 20 µl, consisting of 10 µl of Dilution, 8.2 µl of double-distilled water, 0.4 µl of primer (ITS1 and ITS4) and 1 µl of DNA samples. PCR amplification optimization initial denaturation at 94 °C for 3 minutes, denaturation at 94 °C for 40 seconds, primer annealing at 55 °C for 40 seconds, elongation at 70 °C for 90 seconds, final elongation at 70 °C for 7 minutes, + ∞ at 4 °C, repeated for 35 cycles. Amplicons were detected by electrophoresis on a 2% agarose gel stained with ethidium bromide.

PCR Product Purification and Sequencing

For sequencing, PCR products were cut from 2% agarose gel and purified using the QIAquick® Gel Extraction Kit manual. The amount of purified PCR products was measured in a NanoDrop device. Sequencing of the samples was performed using BigDye Terminator v.3.1 cycle sequencing kit and Applied Biosystems® Genetic Analyzers, 3130 series sequence (Thermo Fischer Scientific, USA). The sequence result of this *Trichoderma* sp 4 strain was aligned with the species in NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree for ITSof *Trichoderma* sp 4 was built via *MEGA-X* (versión*10.1.8)* software (Tamura *et al.*, 2007).

Dual Culture Analysis

A dual culture analysis. To determine their antagonism, the strains of *Alternaria alternata*, *Fusarium solani* and *Aspergillus niger*, which have a phytopathogenic effect, were studied against the strain *Trichoderma asperellum* Uz-A4. A block (6 mm in diameter) was taken from the antagonist and phytopathogens and placed in the CDA nutrient medium in the same way on both sides of the Petri dish (5.5 mm) oppositely. The experiment was repeated 3 times. Placed in a thermostat with a temperature of 25 °C. The radius of colony antagonism on the 7th day was measured and calculated by the following formula (Mao *et al.*, 2020).

$$Growth\ inhibition\ rate\ (\%) = \frac{Control\ colony\ radius - Treatment\ colony\ radius}{Control\ colony\ radius} \times 100$$

Growth Inhibition Rate Control Colony Radius Treatment Colony Radius Fermentation

T. asperellum Uz-A4 strain was grown on Mandel's agar medium (in a test tube) for 6 days, and its suspension at a concentration of 106–7 spores/ml was used as an equivalent material. Microscopic fungus modified by Mandels (Mandels *et al.*, 1962) (g/l: KH2PO4 – 1.0; (NH4)2HPO4 – 2.3; MgSO4 – 0.5; CaCl2 – 0.3; sucrose – 20; (pH 5.5)) were grown on nutrient medium in 500 ml Erlenmeyer flasks. in 250 ml of nutrient medium, on an orbital shaker (shakers IKA® KS 130) at a speed of 180 rpm, at a temperature of 28-30 °C for 14 days.

Filtration

On the 14th day of growth, the biomass of the *T. asperellum* Uz-A4 strain was isolated from the culture liquid by double filtration through filter paper (Whatman #1). The culture fluid extracted from the biomass was stored at 4 °C.

Extraction

The separated culture liquid was extracted 3 times in a separating funnel 3:1 in ethyl acetate (EtOAc). The extraction was repeated every 2 hours. The aqueous layer at the bottom of the separating funnel was removed. The extract with ethyl acetate was dried at a temperature of 40°C under a vacuum (in a rotary evaporator) (Stracquadanio *et al.*, 2020).

Analysis of VOCs

Unknown volatiles was detected in a YL 6900 GX/MS gas chromatography-mass spectrometric detector using a YL 6900 GX/MS (Young In Chromass, Korea) equipped with a DB-5MS column (30 m × 0.25 mm inner diameter, 0.25 µm film thickness) substances were identified. Oven temperature - initial - 80°C/3.0 min, heating rate – 15 °C/min to 250 °C, hold - 3.0 min, Helium was used as

carrier gas at a flow rate of 1.0 ml/min. Evaporator temperature – 280 °C, flow section - 1/20, analysis time - 17 min. organized the Liquid samples were injected into disparities using a 1 µl microsyringe. The temperature of the transmission line was 300 °C, the ionization voltage was –70 eV, and the ion source temperature was 230 °C. Scanning range - 30-350 a.m.u. The components were identified from the mass spectra of each component after comparison with the available spectral data in the MS library NIST 2017 (Guarrasi *et al.*, 2017).

RESULTS

The PCR product of the ITS part of *Trichoderma asperellum* Uz-A4 strain (ON534075) is about 600 bp (Figure 1), and when we compared it with the data in the NCBI BLAST database, it showed that this strain is 100% similar to about 100 species of *Trichoderma asperellum*. We analyzed the phylogenetic tree with the species *T.hamatum*, *T.atroviride*, *T.viride*, *T.harzianum* and *Protocrea pallida* as an outgroup. We named this strain (ON534075) *Trichoderma asperellum* Uz-A4 strain based on the molecular identification of the ITS part.

Phylogenetic tree of the ITS portion of *Trichoderma asperellum* strain Uz-A4 (blue) (Figure 2) and related species and *Protocrea pallida* as an outgroup. Antagonistic properties of *Trichoderma asperellum* Uz-A4 strain against phytopathogenic strains of *A. alternata* isolated from infected wheat (*Triticum aestivum)*, *A. niger* isolated from infected cucumber (*Cucumis sativus* L.) and *F. solani* isolated from bean (*Phaseolus* L.) were studied during the research.

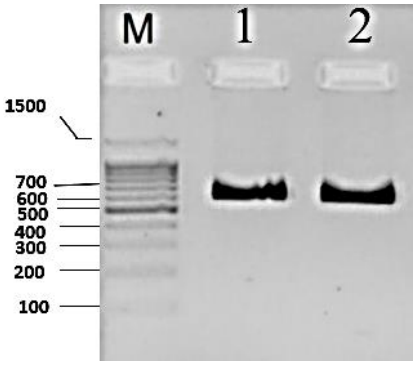


Figure 1. PCR amplification of the ITS part of the fungus *Trichoderma asperellum* Uz-A4 M-100+ DNA ladder, 1,2-PCR products of *Trichoderma asperellum* Uz-A4.

T. asperellum Uz-A4 strain showed 77% antagonism against the *A. alternata* strain in 7 days, stopped the growth of the phytopathogenic fungus, multiplied on its colonies, changed the color of the colony, and acted as a super parasite. As *A. niger* strain is a relatively strong phytopathogen, *T. asperellum* Uz-A4 strain produced 55% antagonism, 97% antagonistic zones were formed compared to *F. solani* strain, surrounded *F. solani* hyphae and growth was observed in the hyphae (Figure 3).

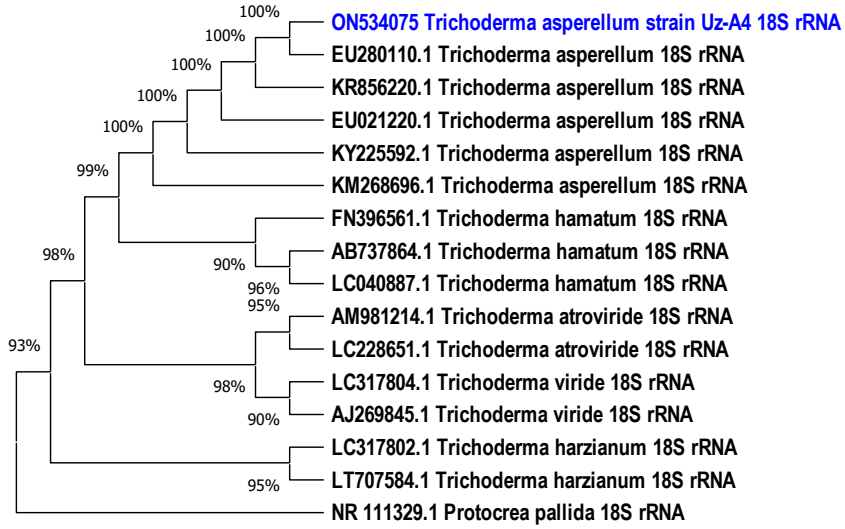
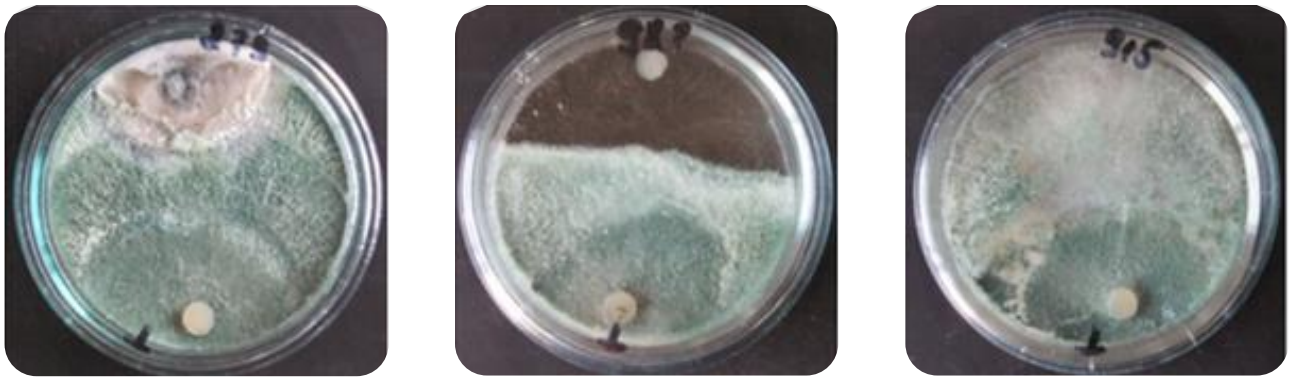


Figure 2. Phylogenetic analysis of *Trichoderma asperellum* Uz-A4 strain.



A. alternata *A. niger* *F. solani*

Figure 3. Anti-fungal properties of *T. asperellum* Uz-A4 strain about phytopathogenic strains.

To detect volatiles in the secondary metabolites, the *T. asperellum* Uz-A4 strain was first grown in liquid culture and was filtered and separated from the biomass. Mass spectral gas chromatography (GC-MS) analysis was performed on the extracted liquid culture (Figure 4).

Phenylethyl alcohol, 5-Hydroxymethylfurfural, dehydroacetic acid, 1-Dodecanol, 2,4-di-tertbutyl phenol, diethyl suberate, n-hexadecanoic acid, 1-hexadecanol, 2-methyl-, phthalic acid, ethyl pentadactyl ester, mono(2-ethylhexyl) phthalate, octadecanoic acid were identified compared to GC-MS library base depending on the absorption rate of some spectra of the chromatogram (Table 1).

When the liquid culture of the fungus *T. asperellum* Uz-A4 was extracted and volatile substances were detected, the presence of the substance-phenyl ethyl alcohol was demonstrated. The chromatogram showed that the absorption rate of this substance was 6,393 minutes (Figure 5). Although phenyl ethyl alcohol is a volatile substance by nature, it further enhances the antagonistic ability of fungi of the *Trichoderma* genus. Phenylethyl alcohol have been shown in studies to inhibit the growth of *F. Incarnatum*from 21,68% to 74.29% (Intana *et al.*, 2021).

5-Hydroxymethylfurfural secondary metabolite showed an absorption rate of 7,672 minutes (Figure 6). It is known from the literature that 5-Hydroxymethylfurfural is a furfural sugar compound and is involved in the formation of enzymes (β-glucanase, cellulose) of fungi of the *Trichoderma* genus.

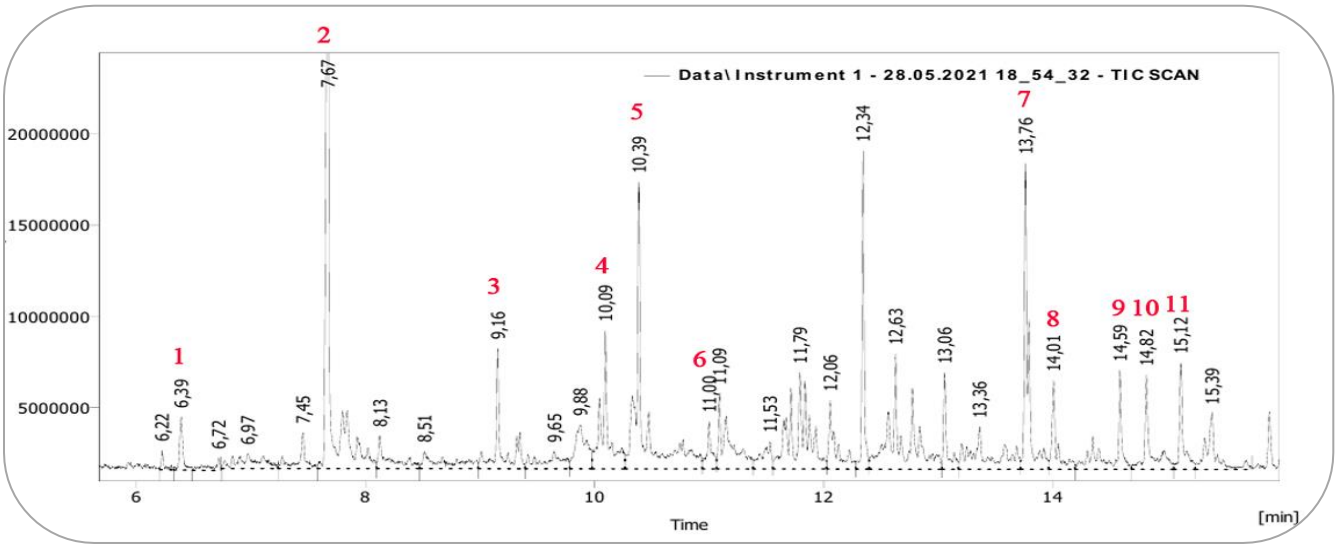


Figure 4. *T. asperellum* Uz-A4 fungal strain culture extract GC chromatogram; 1) Phenylethylalcohol; 2)5-Hydroxymethylfurfural; 3) Dehydroaceticacid; 4) 1-Dodecanol; 5) 2,4-Di-tert-butylphenol; 6) Diethyl suberate; 7) n-Hexadecanoic acid; 8) 1-Hexadecanol, 2-methyl; 9) Phthalic acid, ethyl pentadecyl ester; 10) Mono(2-ethylhexyl) phthalate; 11) Octadecanoic acid.

Table 1. Determination of spectra obtained in *T. asperellum* Uz-A4 fungal strain cultures extract GC with database in MS library

S. No.	Metabolite names	Chemical formula	Molecular mass g/mol	Absorption rate (min)
1	Phenylethylalcohol	$C_8H_{10}O$	122,16	6,93
2	5-Hydroxymethylfurfural	$C_6H_6O_3$	126,11	7,672
3	Dehydroaceticacid	$C_8H_8O_4$	168,14	9,155
4	1-Dodecanol	$C_{12}H_{26}O$	186,33	10,094
5	2,4-Di-tert-butylphenol	$C_{14}H_{22}O$	206,32	10,388
6	Diethyl suberate	$C_{12}H_{22}O_4$	230,30	11,001
7	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256,42	13,764
8	1-Hexadecanol, 2-methyl	$C_{17}H_{36}O$	256,5	14,007
9	Phthalic acid, ethyl pentadecyl ester	$C_{25}H_{40}O_4$	404,6	14,588
10	Mono(2-ethylhexyl) phthalate	$C_{16}H_{21}O_4$	277,33	14,817
11	Octadecanoic acid	$C_{18}H_{36}O_2$	284,48	15,118

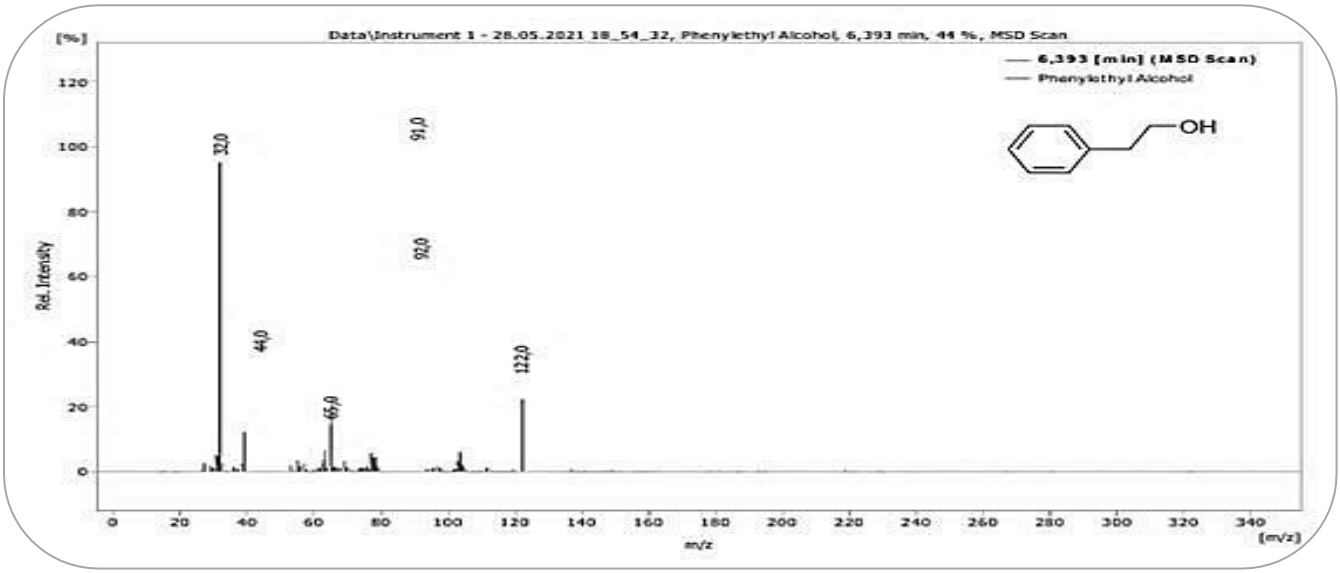


Figure 5. Phenylethylalcohol chromatogram.

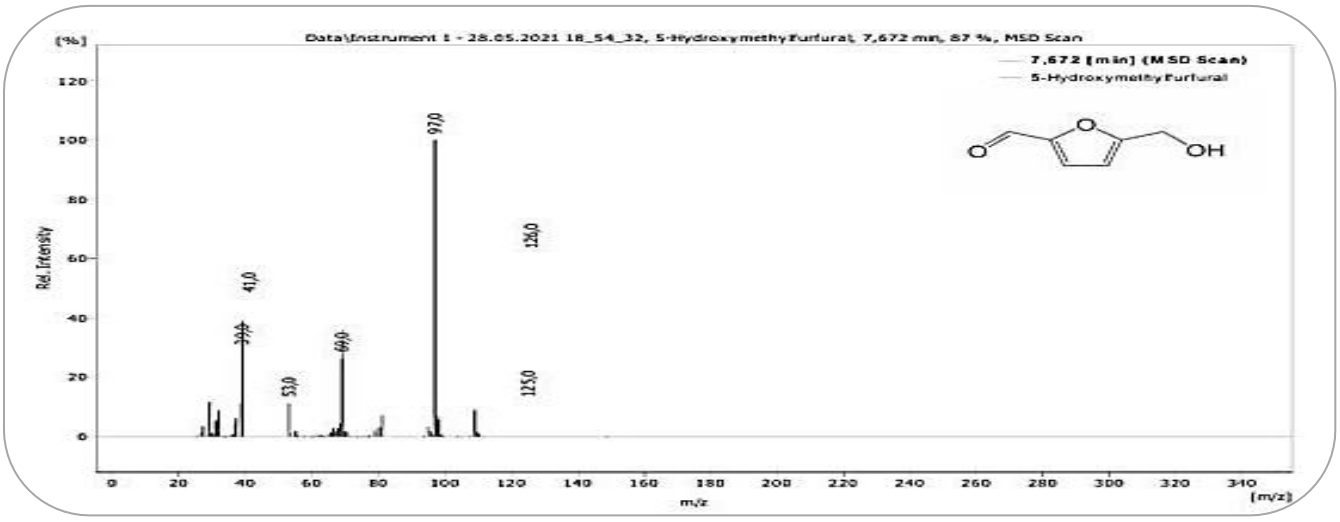


Figure 6. Hydroxymethyl furfural chromatogram.

Dehydroacetic acid showed an absorption rate of 9,155 minutes on the GC chromatogram (Figure 7). This substance is currently used in the storage and packaging of fruit and vegetables as well as cosmetics (Saravanakumar *et al.*, 2018). The volatility of 1-Dodecanol was relatively high, resulting in an absorption rate of 10,094 minutes on the chromatogram (Figure 8).

It is known from the literature that 1-Dodecanolis an organic product by nature and is a fatty alcohol. As metabolites of the microorganism, the fungi *Aspergillus niger, Rhizopus oryzae, Aspergillus terreus, Trichoderma viride, Aspergillus flavus* micromycetes were also extracted from ethyl acetate and found to be metabolites in GC-MS (Shaikh and Mokat, 2017).

2,4-Di-tert-butylphenolGC showed an absorption rate of 10,388 minutes when analyzed(Figure 9). 2,4-Di-tert-butylphenolis a metabolite with cytotoxicity, insecticidal, nematidic activity, antagonistic properties (Zhao *et al.*, 2020; Shobha *et al.*, 2020; Chen and Dai, 2015). In a study on *T. asperellum* Uz-A4 extraction, diethylsuberate GC produced an absorption rate of 11,001 minutes in the analysis (Figure 10).

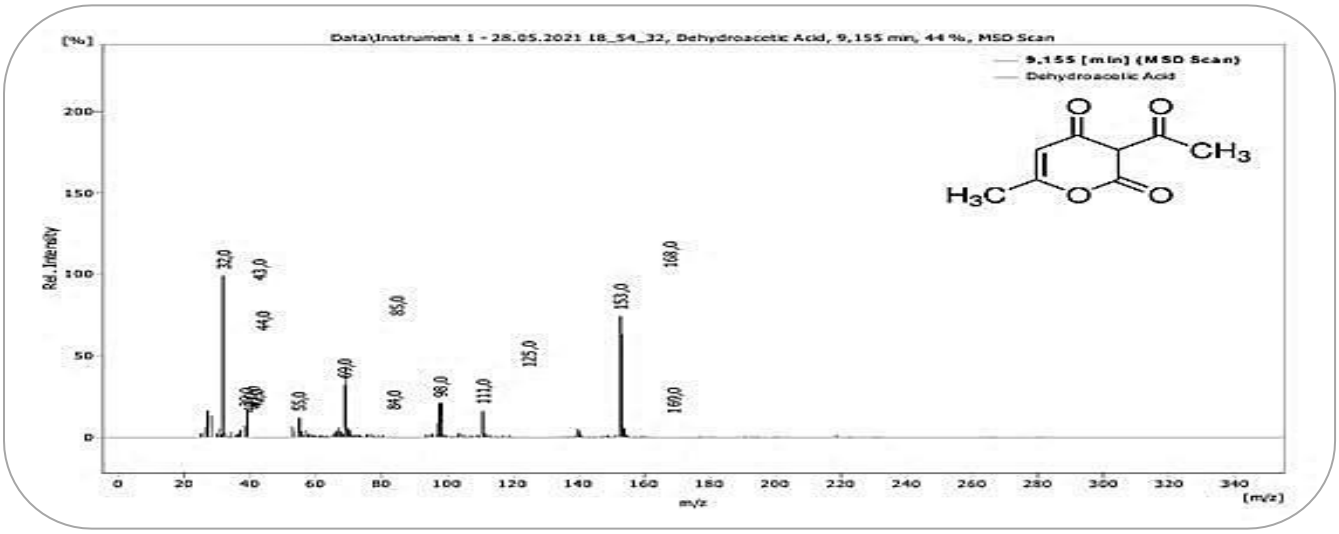


Figure 7. Dehydroacetic acid chromatogram.

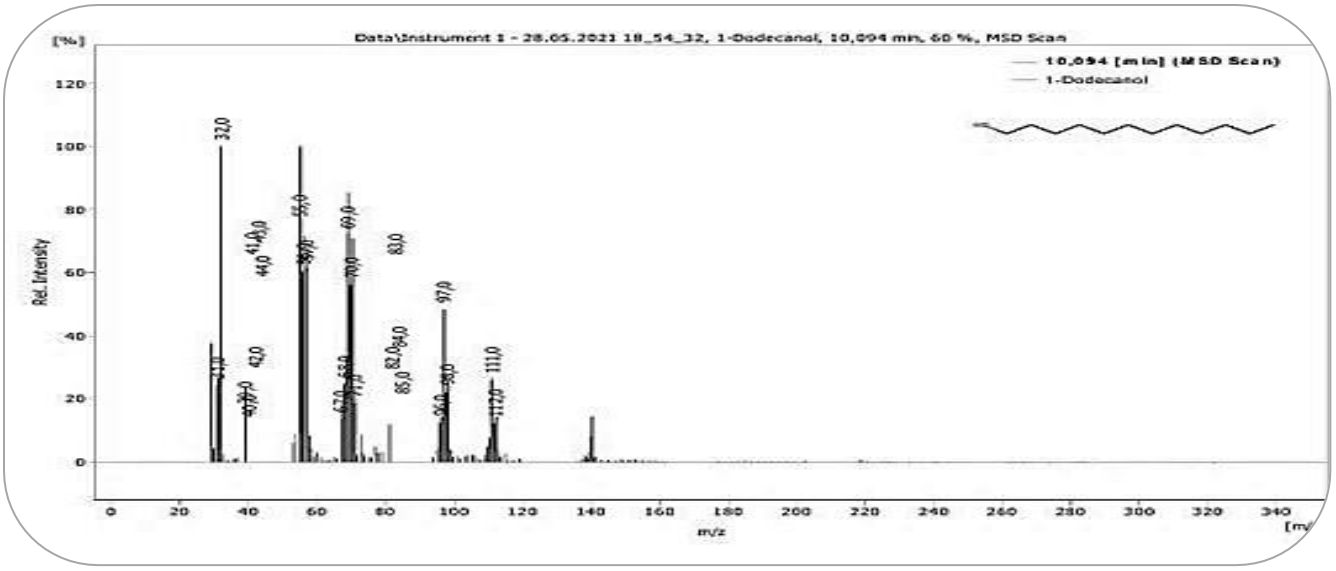


Figure 8. 1-Dodecanolchromatogram.

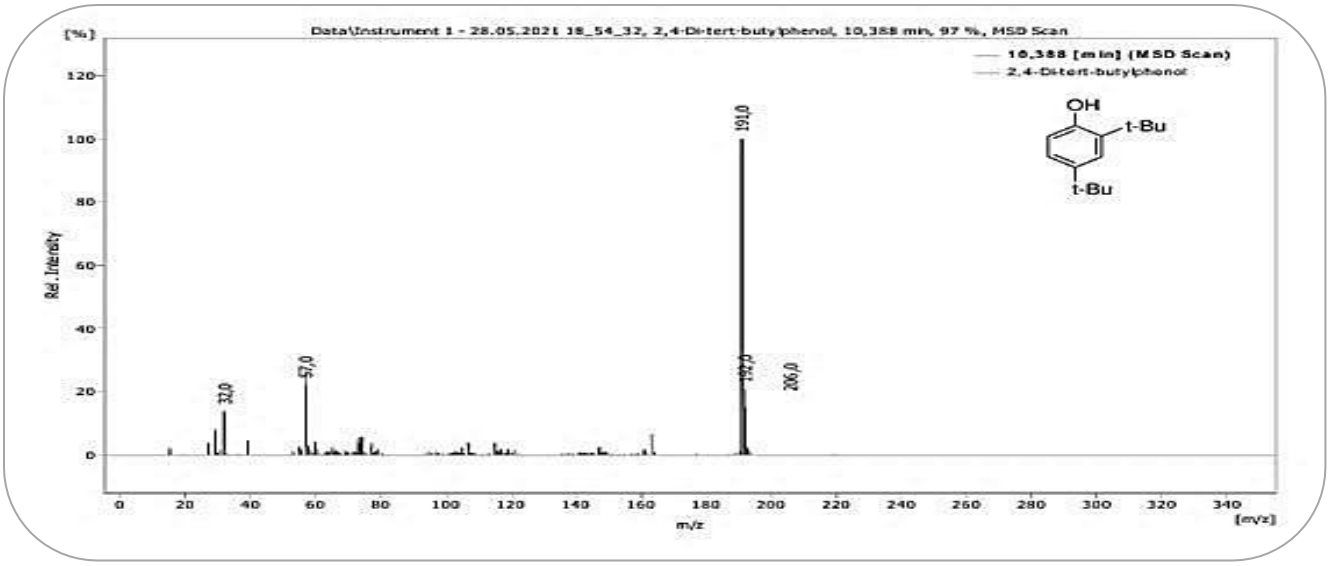


Figure 9. 2,4-Di-tert-butylphenol chromatogram.

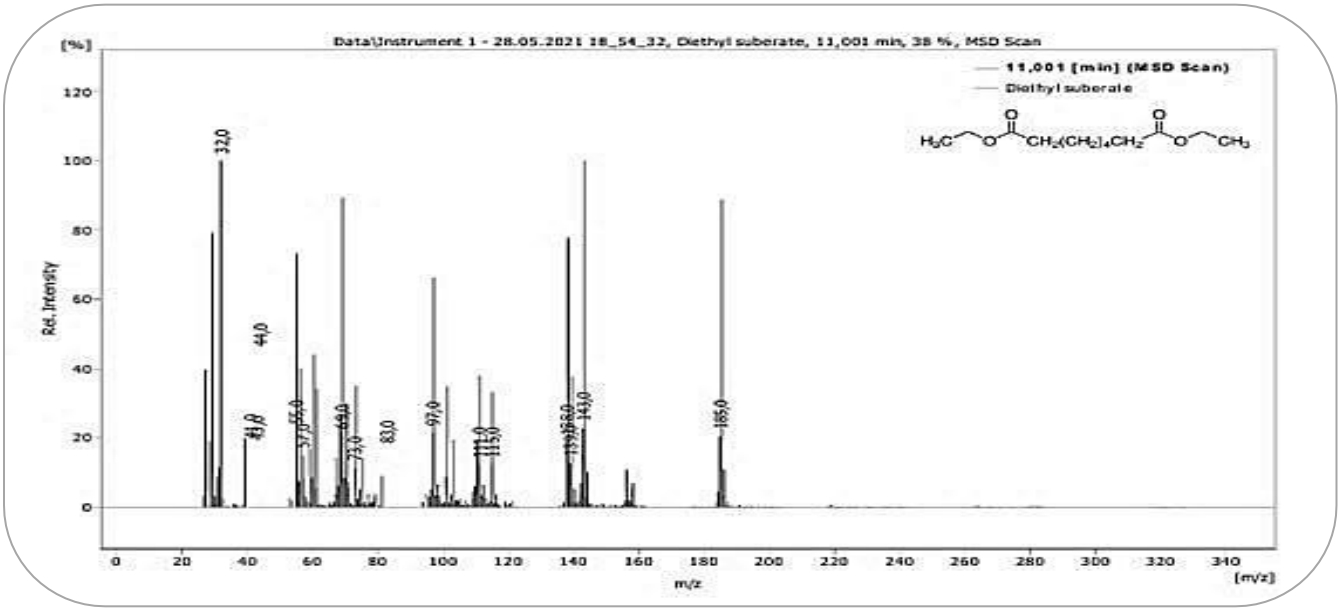


Figure 10. Diethyl substrate chromatogram.

n-Hexadecanoic acidGC showed an absorption rate of 13,764 minutes when analyzed (Figure 11).The presence of n-hexadecanoic acid (6.17%) in the analysis of the metabolite *T. atroviridi* GC-MS. Due to the fatty acid content, its antioxidant and antibacterial properties have been known (Saravanakumar *et al.*, 2018).

1-Hexadecanol produced a 14,007-minute absorption rate on a 2-methyl GC chromatogram (Figure 12). Among the secondary metabolites of *Trichoderma*, the antioxidant content of this substance is high (Ali, 2021).

Phthalic acid, ethyl pentadecyl ester showed an absorption time of 14,588 minutes (Figure 13). This substance is characterized by the fact that it is an active enzymatic and bioactive substance among secondary metabolites (Bahaa *et al.*, 2019).

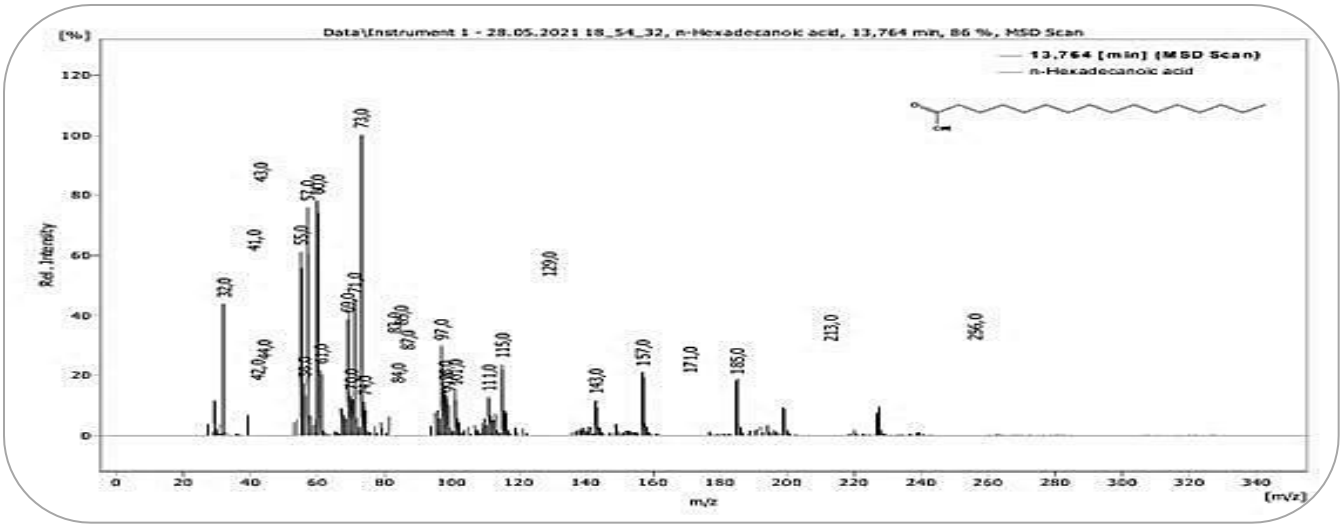


Figure 11. n-hexadecanoic acid chromatogram.

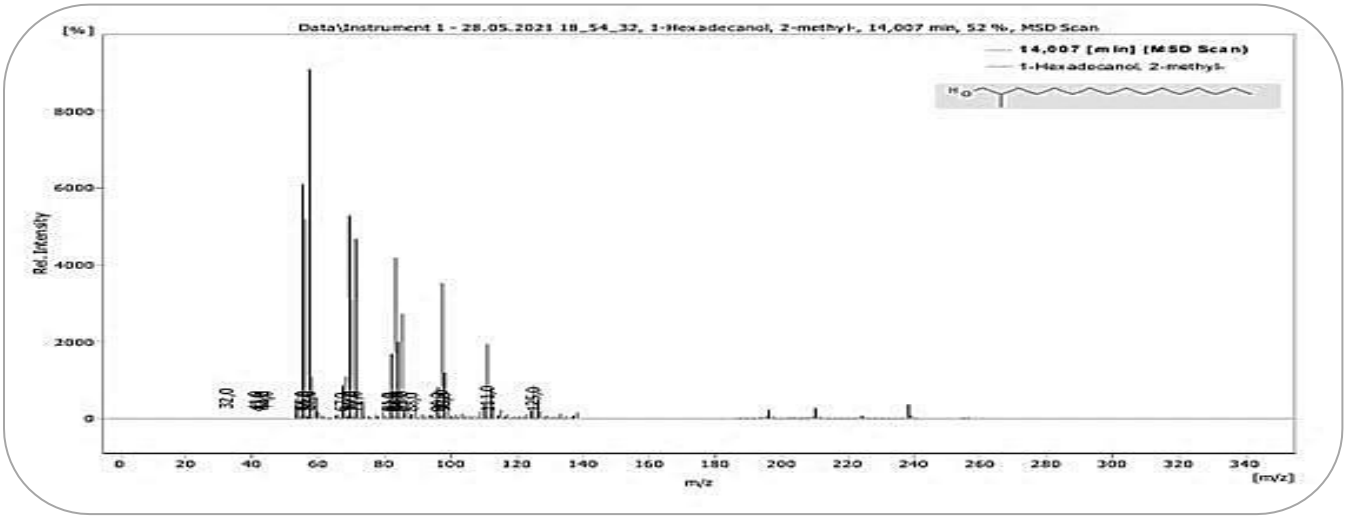


Figure 12. 1-hexadecanol, 2-methylchromatogram.

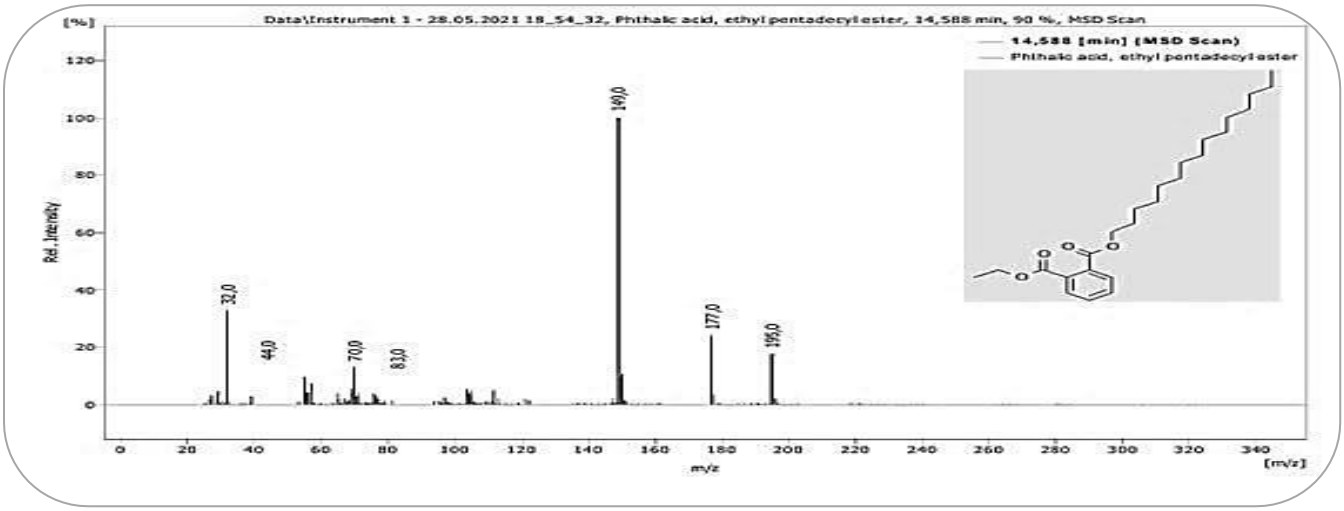


Figure 13. Phthalic acid, ethyl pentadecyl ester chromatogram.

Mono(2-ethylhexyl) phthalate volatile metabolite showed an absorption rate of 14,817 minutes when *T. asperellum* Uz-A4 liquid culture was extracted with ethyl acetate (Figure 14). It is known from the literature that this substance is also actively involved for antifungal properties (Yang *et al.*, 2013). Octadecanoic acid showed an absorption rate of 15,118 minutes in GC analysis (Figure 15). Octadecanoic acid metabolite is a metabolite involved in the formation of carbohydrate containing substances (Kaushik *et al.*, 2020).

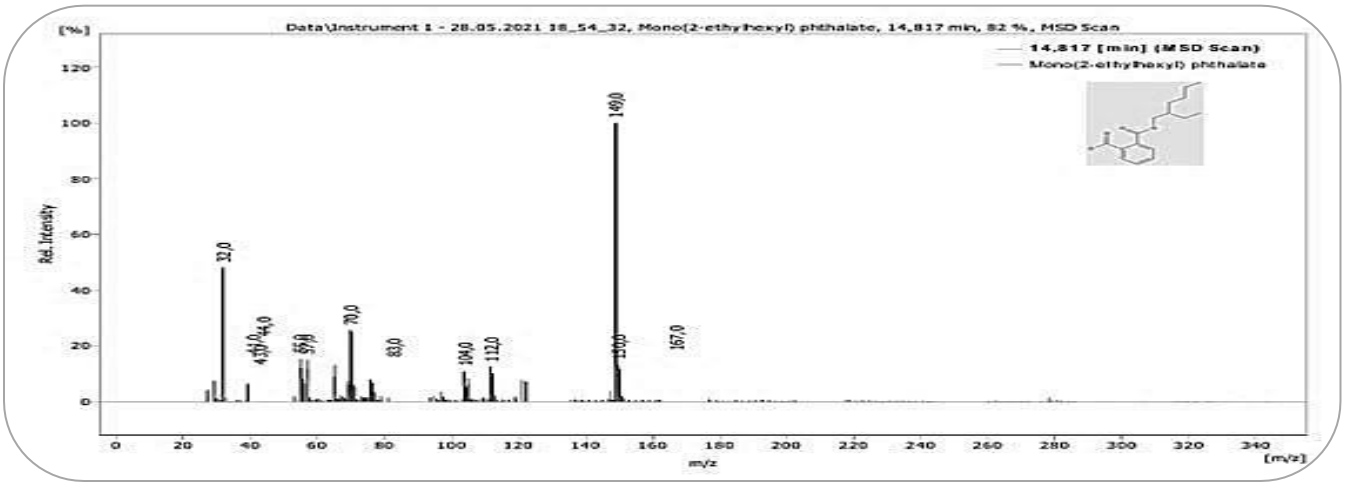


Figure 14. Mono(2-ethylhexyl) phthalate chromatogram.

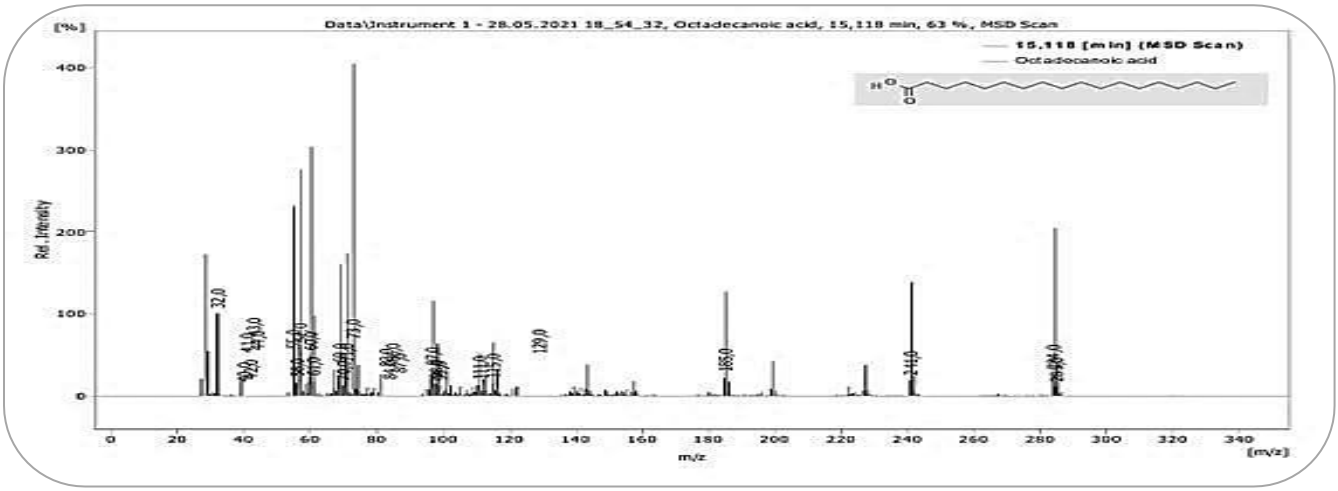


Figure 15. Octadecanoic acidchromatogram.

DISCUSSION

Biopreparations based on Trichoderma are used in vegetable growing, melon growing, greenhouses, horticulture, viticulture and growing various ornamental plants and trees. This micromycete protects plants from phytopathogens. Increases seed germination capacity, enhances plant growth, increases metabolism, expands leaf plate surface, and improves soil structure. In recent years, when we got acquainted with the volatile metabolites identified in *Trichoderma* species, it was proposed as a biological protection agent that reduces the effects of plant diseases (Sunpapao *et al.*, 2018; Andriamialisoa *et al.*, 2004; Wonglom *et al.*, 2019).

The biological activity of *Trichoderma* fungi provides an important advantage in the competition with pathogens in terms of growth and development speed, competition for habitat and food. *Trichoderma* develops in the hyphae of phytopathogenic fungi, and wraps and destroys the cell walls with the help of lytic enzymes. As a result, it continues its life form as a hyperparasite. In

addition, along with mycoparasitism, it also produces antibiotics. The limits the activity of pathogenic microorganisms (Harman, 1996). In figure 3, we can observe the development of the *T. asperellum* Uz-A4 strain in the hyphae of pathogenic microorganisms after antagonistic development against the *F.solani* strain.

The authors also noted the production of volatile and non-volatile antibiotics by *Trichoderma* species in the biocontrol of plants, the formation of antagonistic properties against phytopathogenic microorganisms (Ubalua and Oti, 2007). Volatile metabolites identified in our studies are metabolites that affect the antagonistic properties of the strain.

In recent years, in connection with the rapid development of biotechnology, interest in microscopic fungi of the genus *Trichoderma,* which attract the attention of researchers in connection with their practical significance for obtaining biologically active substances, plant protection products and as an active destructor of plant polysaccharides. It is known that *Trichoderma* emits various metabolites: growth factors (auxins, cytokines and ethylene), organic acids, intracellular amino acids, vitamins and over 100 antibiotics (Benítez *et al.*, 2004). "TopShield" (New York), "Trichodex" (Israel), "Sternifag SP", "Trichodermin", "SellovirdineV-G20x", "Gliokladin", "Viridin" (Russia), "Fungilex J", "Fekord-2012-S" (Belarus) and other biological drugs were developed by international scientists based on fungi belonging to the *Trichoderma* (Reithner *et al.,* 2007).

CONCLUSIONS

The results of this study showed that *T. asperellum* Uz-A4 micromycet fungi have high biological activity. The 11 main metabolites in the culture fluid are Phenylethyl Alcohol, 5-Hydroxymethyl furfural, Dehydroacetic Acid, 1-Dodecanol, 2,4-Di-tert-butylphenol, Diethyl suberate, n-Hexadecanoic acid, 1-Hexadecanol 2-methyl, , eth ester, Mono (2-ethylhexyl) phthalate was found to be present. Secondary metabolites were found to have cytotoxicity, insecticidal, nematidic activity, and antimicrobial antagonistic properties. The studies we conducted had demonstrated that *T. asperellum* Uz-A4 strain could produce several important metabolites with high biological activity.

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DATA AVAILABILITY

The data supporting this study's findings are available on request from the corresponding author.

ETHICAL STATEMENT

This study did not engage in any human or animal testing.

AUTHOR CONTRUBUTIONS

All the authors have contributed equally to the research and compiling the data as well as editing the manuscript.

CONFLICT OF INTEREST

The authors have not declared any conflict of interest.

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