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Profiling antifungal metabolites of *Trichoderma atroviride* by GC-MS for *in vitro* biocontrol of *Fusarium graminearum* and *Rhizoctonia solani*

Madani Gargari M[1], Rahnama K[2*]and Shahiri Tabarestani M[3]

[1] *Department of Agriculture, Go.C., Islamic Azad University, Gorgan, Iran. Mitra.madanigargari@iau.ac.ir*
[2] *Department of Plant Protection, Faculty of Plant Production, Gorgan University of Agricultural Science and Natural Resources, Gorgan, Golestan, 4918943464, Iran.*
[3] *Department of Agriculture, Payame Noor University, Tehran, Tehran, 1955643183, Iran.*

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Abstract

The antifungal compounds of *Trichoderma* fungi hold significant potential for eco-friendly biological control of pathogenic fungi, offering sustainable alternatives to chemical fungicides. The present study aimed to identify secondary metabolites (SMs) of *T. atroviride* 6022 using Gas Chromatography–Mass Spectrometry and evaluate their antifungal activity against *Fusarium graminearum* and *Rhizoctonia solani*. A total of 372 different SMs were identified. Compounds with molecular weights between 100 and 300 g/mol constituted 87.90% of SMs. Simple hydrocarbons and organo-oxygens comprised 76.11% and 15.59% of SMs, respectively. The remaining 8.3% belonged to organo-sulfur, organo-boron, organo-chlorine, organo-nitrogen, organo-bromine, organo-silicon, organo-phosphorus, and organo-fluorine compounds. Gliotoxin, Gliovirin, Peptaibolin, Bis(2-ethylhexyl) phthalate, Dibutyl phthalate, Eicosane, Hexadecanoic acid, methyl ester, Naphthalene, Nerolidol, Phenanthrene, Styrene, Tetradecane, Toluene, 10-Hydroxy-2-decenoic acid, benzophenone, cyclopentadecane, heneicosane, indane, and naphtho[2,1-b]thiophene were identified as antifungal SMs of *T. atroviride* 6022. A 30% concentration of the *T. atroviride* extract inhibited the growth of *F. graminearum* and *R. solani* by 77.78% and 81.25%, respectively. Therefore, SMs play a crucial role in the antifungal properties of *T. atroviride*. Additionally, the similarity of SMs between genetically similar *Trichoderma* spp. is possible, and the ecological niche is an important factor in the diversity of SMs within the same fungal species.

Keyword – Biological control **–** Organic compounds **–** Plant diseases **–** Head blight **–** Sheath blight

Introduction

Fusarium graminearum and *Rhizoctonia solani* are considered among the most significant soil-borne plant pathogenic fungi globally (Doehlemann et al. 2017). The economic impact of *F. graminearum* damage is estimated to range from 200 to 400 million dollars annually in the global agricultural economy (Vander Lee et al. 2015). The first major epidemic of *F. graminearum* occurred in 1982 in the northern Great Plains of North Dakota, South Dakota, and Minnesota, affecting

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Corresponding Author: Kamran Rahnama – e-mail – rahnama@gau.ac.ir

approximately 4 million hectares of spring wheat and barley with an approximate loss of one billion dollars (Vaughan et al. 2016). Furthermore, trichothecene, deoxynivalenol (vomitoxin) and zearalenone (phytoestrogen) represent the primary mycotoxins produced by *F. graminearum*, capable of causing anorexia and abortions in livestock (Chen et al. 2020). Additionally, sheath blight, caused by *R. solani*, stands as one of the most devastating diseases affecting rice (Li et al. 2021) and potato (Kiptoo et al. 2021). The severity of *R. solani* in other plant hosts can lead to varying consequences, ranging from minor to major yield losses (from 25% to 100%), an increase in soil tare, and a decrease in the industrial quality of crops (Li et al. 2021). Pentachloronitrobenzene (PCNB) and triazoles are commonly used fungicides to control the damage caused by *R. solani* and *F. graminearum* on plants (Chen et al. 2020, Kiptoo et al. 2021). However, research indicates that dietary exposure to PCNB in rats can lead to liver damage (Kuai et al. 2020). Additionally, triazoles exhibit detrimental effects, such as disrupting freshwater ecosystem balance, exhibiting carcinogenic properties in animals and humans, inducing neurotoxicity or hepatotoxicity in livestock, and potentially influencing the expression of certain genes in mammals (Fang et al. 2019). Consequently, the use of these pesticides has resulted in irreversible environmental damage, underscoring the urgent necessity for biological control agents more than ever.

The *Trichoderma* genus encompasses more than 100 described species. The main benefits of *Trichoderma* spp. include biological control of pests in agriculture and producing biosynthetic enzymes for various industries (Zin & Badaluddin 2020). Mycoparasitism in *Trichoderma* spp. involves chemotropic growth, whereupon contact, the hyphae coil around the pathogen mycelia, initiating the secretion of cell-wall-degrading enzymes. This process ultimately leads to the destruction of the pathogen's cells, providing nutrition for *Trichoderma* spp. (Poveda 2021). In recent years, numerous secondary metabolites have been identified from *Trichoderma* spp., which exhibit species-specific differences in activity and structure (Lee et al. 2016). Researchers have generally reported epipolythiodioxopiperazines, peptaibols, pyrones, butenolides, pyridones, azaphilones, koninginins, steroids, anthraquinones, lactones, and trichothecenes as secondary metabolites of *Trichoderma* spp., exhibiting significant antifungal properties (Khan et al. 2020). These compounds have demonstrated efficacy against a range of fungal pathogens, including *Candida* spp., *Cryptococcus* spp., *Aspergillus* spp., *Scedosporium* spp., *Exophiala* spp. (Chen et al. 2010), *Pythium* spp., *Fusarium* spp., *Sclerotium* spp., *Verticillium* spp., and *Phytophthora* spp. (Khan et al. 2020). The antifungal compounds produced by *Trichoderma* fungi demonstrate promising potential for sustainable and effective biocontrol strategies against pathogenic fungi, offering valuable alternatives to conventional chemical fungicides and contributing to environmentally friendly agricultural practices. Despite considerable progress in identifying secondary metabolites of *T. atroviride* with potent antifungal activity against plant pathogenic fungi, isolates from diverse geographical areas and hosts exhibit distinct profiles. Moreover, limited research has been conducted on the biocontrol activity of *T. atroviride* against *F. graminearum* and *R. solani*. Therefore, this study aimed to identify the secondary metabolites (SMs) of *T. atroviride* 6022 using Gas Chromatography–Mass Spectrometry (GC-MS), a technique chosen for its high sensitivity and accuracy in detecting both volatile and semi-volatile compounds. *Trichoderma atroviride* isolate 6022 has demonstrated strong biocontrol potential against plant pathogens such as *Macrophomina phaseolina* and *Sclerotinia sclerotiorum* (Kia and Rahnama 2016, Tabarestani et al. 2017), primarily through the production of both volatile and non-volatile antifungal compounds. Previous studies have identified key metabolites, including iso-amyl-alcohol and stearic acid, as contributors to its antagonistic activity (Shahiri Tabarestani et al. 2016a, Shahiri Tabarestani et al. 2016b). The effectiveness of this isolate, supported by dual culture assays and metabolite profiling, suggests its potential as an eco-friendly alternative to chemical pesticides in agricultural systems (Akbari Oghaz et al. 2024, Akbari et al. 2019). Building on these findings, the present study aimed to not only identify the secondary metabolites produced by *T. atroviride* 6022 but also to evaluate their antifungal efficacy against *F. graminearum* CBS 131778 and *R. solani* AG4_S7 under in vitro conditions. By linking metabolite identification with antifungal bioactivity, this research provides a more

comprehensive understanding of the role of secondary metabolites in the biocontrol potential of *T. atroviride* 6022.

Materials & Methods

Preparation of *T. atroviride*

Trichoderma atroviride 6022 was obtained from the fungal collection at Gorgan University of Agricultural Sciences and Natural Resources. Its molecular sequeces for the internal transcribed spacer (ITS) and the large subunit rRNA (LSU) genomic regions have been deposited in the National Center for Biotechnology Information (NCBI) under accession numbers OQ780734.1 and OQ780715.1, respectively, by Akbari Oghaz et al. (2024). *Trichoderma atroviride* was cultured on potato dextrose agar (PDA) medium (Merck 110130), with a pH adjusted to 7 and then incubated for 3 days at 25 ± 2°C under dark conditions. Following robust growth, *T. atroviride* was inoculated into Erlenmeyer flasks containing Potato-Dextrose-Broth (PDB) medium (HiMedia M403) by introducing 5 mm disks of young mycelia (three days old) at pH 7. The cultured PDB was incubated at 25 ± 2°C under 12-hour light/dark cycle on a shaker (150 rpm) for three weeks. The cultured PDB was filtered using a 400-mesh sieve to separate the mycelium and spores from the medium. Following the method described by Siddiquee et al. (2012), an equal volume of ethyl acetate (Merck, CAS: 141-78-6, EC: 205-500-4) was added to the PDB medium and left for 12 hours to ensure the inactivation of any remaining fungal components (Siddiquee et al. 2012). The ethyl acetate phase was separated from the medium (water phase) using a Buchner vacuum filtration funnel. The resulting ethyl acetate extracts were then evaporated at 60°C using a rotary evaporator. Ethyl acetate was chosen for its efficiency in extracting diverse secondary metabolites, its ability to inactivate fungal components, and its widespread use in similar studies, while the rotary evaporator ensured gentle solvent removal under reduced pressure, preventing thermal degradation and preserving metabolite integrity. The evaporated extract was immediately resuspended in 100 mL of Merck HPLC-grade methanol, n-hexane, and n-butanol. and was either subjected to GC-MS analysis promptly or stored at -20°C in an ultra-low temperature freezer.

Identification of secondary metabolites

A Shimadzu GC (Japan), coupled with a QP5050A Mass Selective Detector (MSD), was utilized to analyze the secondary metabolites. The GC-MS system functioned with an electron ionization energy of 70 eV. Chromatographic separation was achieved using a non-polar capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness, DB-5MS or an equivalent, manufactured by Agilent Technologies). The temperature program for the oven was as follows: starting at 60 °C for 1 minute, increasing by 10 °C per minute to 300 °C, and holding at 300 °C for 5 minutes. The injector was set at 300 °C and operated in splitless mode, while the detector was maintained at 320 °C. Helium (99.999% purity, supplied by Linde Gas), served as the carrier gas at a flow rate of 1.0 mL/min. The mass spectrometer operated with an Ion source temperature of 200 °C, a scan range of 35–450 m/z, and a scan rate of 0.50 scans per second. A solvent delay of 3 minutes was implemented to eliminate solvent interference (Shahiri et al. 2016a) The system was calibrated using a standard tuning mixture (PFTBA, perfluorotributylamine, Sigma-Aldrich), following the manufacturer's calibration guidelines. Metabolites were putatively identified by comparing the obtained mass spectra with the National Institute of Standards and Technology (NIST23) database, with a match probability of ≥75%. To reduce ambiguity, the retention indices (RI) were also calculated and compared with literature values for further confirmation of the identified compounds. A positive control using a caffeine standard (Sigma-Aldrich) validated the GC-MS method, and a negative control, consisting of sterile media without fungal inoculation, was included to detect potential background noise or contamination. Each treatment, along with both controls, was injected in triplicate to ensure replicability and enhance the statistical validity of the results.

Evaluation of the antifungal activity of *T. atroviride* 6022 extract

Fusarium graminearum CBS 131778 (NCBI accession number: JX119004) was obtained from the Mohaghegh Ardabili University (Davari et al. 2013). *Rhizoctonia solani* AG4_S7 (NCBI accession number: JQ671210) was obtained from Tarbiat Modares University (Haratian et al. 2013). The plant pathogenic fungi were cultured on PDA medium and incubated at 25 ± 2 °C under dark conditions for three days. The filtered liquid medium of *T. atroviride* 6022 (FLMT) was mixed with PDA medium at a temperature of 40 ± 2 °C in ratios of 10%, 20%, and 30% before solidification. PDA without FLMT served as a control treatment. The treated PDA medium with FLMT was transferred to 9 cm Petri dishes, and plant pathogenic fungi were placed on the center of the Petri dishes using 4 mm discs of grown colonies. Incubation occurred at 25 ± 2 °C under dark conditions for four days. Three replicates were conducted for each treatment. The percentage inhibition of radial growth of plant pathogenic fungi, relative to their respective control treatments, was quantified using ImageJ software version 1.53s in 2D mode. The inhibition rate reflected the difference in the percentage of surface area covered by the pathogenic fungus in the treatment containing *Trichoderma* culture medium extract, compared to this surface area in the control treatment (without extract). The results are expressed as a percentage of inhibition. A Completely Randomized Design (CRD) was employed as the statistical analysis method. Analysis of variance (one-way ANOVA) was conducted using SPSS statistical software, version 21.0. Mean values were compared using Duncan's multiple range test subset for $\alpha = 0.05$. Statistical charts were generated using Microsoft Excel software, version 2211.

Results

Biological control of plant pathogenic fungi under *in vitro* conditions

Different concentrations of FLMT significantly inhibited the radial growth of *F. graminearum* and *R. solani* compared to the control treatment (Fig. 1). The differences among all treatments were significant in controlling *F. graminearum*, as illustrated in Fig. 1E. As expected, with increasing FLMT concentration, the growth inhibition rate of pathogenic fungi increased proportionally. The 30% FLMT inhibited the growth of *F. graminearum* by 77.78% (Fig. 1E). Furthermore, Duncan's multiple range test identified the antifungal activity of 30% FLMT against *F. graminearum* as the most effective among the other FLMT concentrations. The antifungal activity of 30% FLMT against *F. graminearum* was 23.54% higher than that of 20% FLMT and 48.83% higher than that of 10% FLMT (Fig. 1E). Additionally, *F. graminearum* in the control treatment reached the end of the petri dishes and did not exhibit any inhibition compared to the control treatment (Fig. 1A; Fig. 1B).

Similarly, 30% FLMT exhibited the highest antifungal activity against *R. solani*, with a rate of 81.25%, compared to the control treatment (Fig. 1F). Furthermore, significant differences among all FLMT concentrations in controlling *R. solani* were observed. In contrast, *R. solani* in the control treatment reached the end of the Petri dishes without any inhibition (Fig. 1C). The inhibitory activity of 30% FLMT against *R. solani* was 27.45% higher than that of 20% and 124.13% higher than that of 10% concentrations (Fig. 1F). Comparatively, *R. solani* was better controlled than *F. graminearum* under both 30% and 20% FLMT (Fig. 1E; Fig. 1F). However, the antifungal activity of 10% FLMT against *F. graminearum* was greater than its activity against *R. solani*. Consequently, *R. solani* exhibited a more pronounced biological response to the presence of secondary metabolites from *T. atroviride* compared to *F. graminearum* (Fig. 1E, F).

Identified SMs using n-hexane solvent

The GC-MS analysis revealed the presence of 250 different SMs in the liquid culture medium of *T. atroviride* using n-hexane solvent (Table 1). Of the identified SMs using n-hexane solvent, 9.6% had the lowest percentage of the area under the chromatograph line (%Area) (Fig. 2A; Table 1). Octane exhibited the highest %Area (15.719%) among the identified compounds using n-hexane solvent.

Additionally, the identified SMs of *T. atroviride* using n-hexane solvent displayed variations in molecular structure and weight (Fig. 2B; Table 1). Simple hydrocarbons constituted 81.6% of all identified SMs using n-hexane solvent (Fig. 2B). Organo-oxygen and organo-sulfur compounds accounted for 7.6% and 4%, respectively, of all identified compounds using n-hexane solvent (Fig. 2B). Other organic compounds, including organo-boron, organo-chlorine, organo-nitrogen, organo-bromine, and organo-silicon compounds, made up 0.8%, 0.8%, 0.4%, 0.4%, and 0.4%, respectively, of all identified SMs using n-hexane solvent (Fig. 2B).

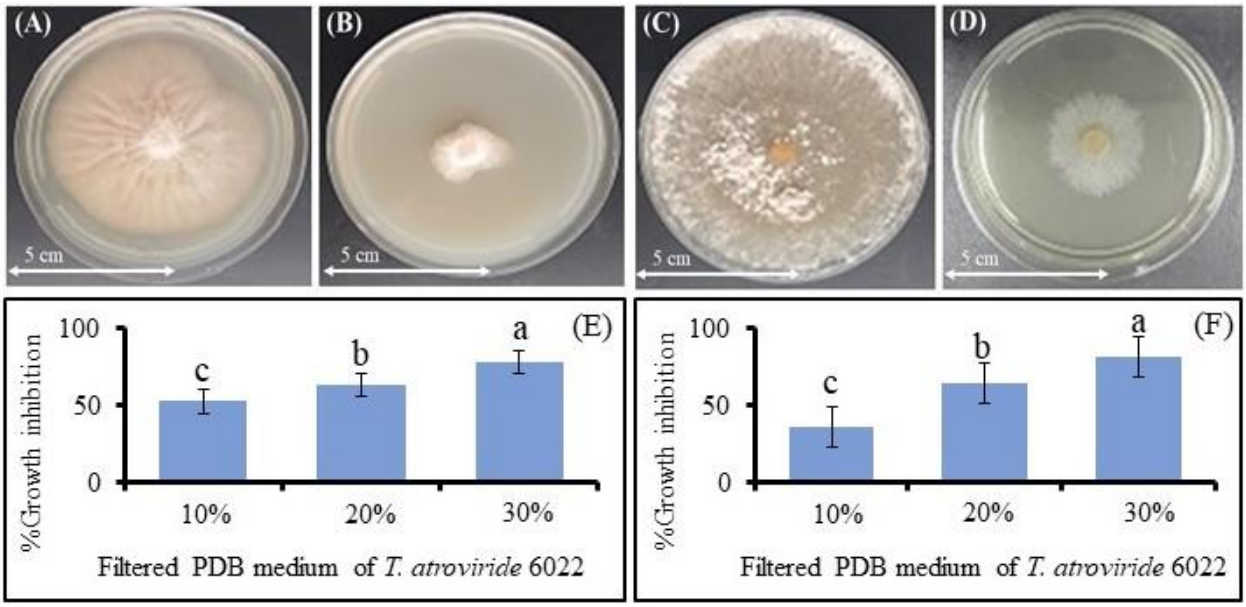

Fig. 1 – Investigation into the radial-growth inhibition activity of plant pathogenic fungi using filtered PDB medium from *T. atroviride* 6022 is presented as follows: (A) Growth of *F. graminearum* CBS 131778 on uninoculated PDA as the control treatment. (B) Growth of *F. graminearum* CBS 131778 on inoculated PDA with 30% filtered PDB medium from *T. atroviride* 6022. (C) Growth of *R. solani* AG4_S7 on uninoculated PDA as the control treatment. (D) Growth of *R. solani* AG4_S7 on inoculated PDA with 30% filtered PDB medium from *T. atroviride* 6022. (E) Grouping of treatments with different concentrations of filtered PDB medium from *T. atroviride* 6022 based on percentage inhibition of radial growth of *F. graminearum* CBS 131778 (P-value = 0.0001). (F) Grouping of treatments with different concentrations of filtered PDB medium from *T. atroviride* 6022 based on percentage inhibition of radial growth of *R. solani* AG4_S7 (P-value = 0.0001). Error bars represent the standard error, and bars sharing the same letters indicate no significant difference.

Moreover, 3.2% and 0.8% of the identified organic compounds using n-hexane solvent contained two and three elements, respectively, in their chemical structure (Fig. 2B; Table 1). Among organic compounds with two elements, 2-methyl-4-fluoro-1H-inden-1-one, pentadecyl heptafluorobutyrate, and tetracosyl heptafluorobutyrate featured fluorine elements in their structures, a characteristic not found among hydrocarbons with a single element (Table 1). Additionally, only phosphonic acid, dioctadecyl ester contained a phosphorus element in its structure, a feature absents in hydrocarbons with single or three elements (Table 1). In terms of molecular weight, SMs within the range of 100–200 g/mol constituted the highest percentage of identified compounds (73.2%) using n-hexane solvent (Fig. 2B; Table 1). Following this, SMs within the 200–300 g/mol weight range comprised 15.6% of all identified compounds using n-hexane solvent. Notably, 1-methyl-1,3-cyclopentadiene and methyl

dicyclopentadiene possessed the lowest weight among the identified SMs using n-hexane solvent, while 1,54-dibromo-tetrapentacontane exhibited the highest weight (Fig. 2B; Table 1).

Table 1 – Identified metabolites from *T. atroviride* (6022) using n-hexane solvent.

Peak No.	Metabolite	Molecular formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
1	Methyl- Cyclopentane	C_6H_{12}	84.162	5.439	6.119	91	96-37-7
2	3-methyl-2-Pentene	C_6H_{12}	84.16	5.601	1.029	99	10273-90-2
3	1-methyl-1,3-Cyclopentadiene	C_6H_8	80.13	5.682	0.059	89	96-39-9
4	3,3-dimethyl- Pentane	C_7H_{16}	100.2	5.884	0.039	80	562-49-2
5	1- Hexene	C_6H_{12}	84.16	6.103	4.639	77	592-41-6
6	2,3-dimethyl- Pentane	C_7H_{16}	100.2	6.184	0.279	85	565-59-3
7	3-methyl- Hexane	C_7H_{16}	100.2	6.288	0.649	89	589-34-4
8	Cis- 1,3-dimethyl- Cyclopentane	C_7H_{14}	98.19	6.49	0.629	93	2532-58-3
9	Heptane	C_7H_{16}	100.2	6.808	0.449	83	142-82-5
10	2-Heptene	C_7H_{14}	98.19	6.975	0.039	91	592-77-8
11	2,2-dimethyl- Hexane	C_8H_{18}	114.23	7.351	0.049	87	590-73-8
12	Methyl- Cyclohexane	C_7H_{14}	98.19	7.501	0.329	98	108-87-2
13	2,5-dimethyl- Hexane	C_8H_{18}	114.23	7.628	0.069	98	592-13-2
14	2,4-dimethyl- Hexane	C_8H_{18}	114.23	7.697	0.179	85	90622-56-3
15	Ethyl- Cyclopentane	C_7H_{14}	98.19	7.795	0.099	97	1640-89-7
16	4-methyl-1-Heptene	C_8H_{16}	112.21	7.951	0.089	83	13151-05-8
17	1-ethyl-2-methyl- Cyclopentane	C_8H_{16}	112.21	8.113	0.049	83	930-89-2
18	1,2,3-trimethyl- Cyclopentane	C_8H_{16}	112.21	8.194	0.079	94	2613-69-6
19	3,4-dimethyl-3-Hexene	C_8H_{16}	112.21	8.39	0.059	95	19550-88-0
20	2,3-dimethyl- Hexane	C_8H_{18}	114.23	8.546	0.159	99	584-94-1
21	2-methyl- Heptane	C_8H_{18}	114.23	8.737	0.289	84	592-27-8
22	Toluene	C_7H_8	92.14	8.806	0.549	76	108-88-3
23	3-methyl- Heptane	C_8H_{18}	114.23	9.06	3.789	75	589-81-1
24	Cis-1,3-dimethyl- Cyclohexane	C_8H_{16}	112.21	9.28	0.409	79	638-04-0
25	1-ethyl-3-methyl- Cyclopentane	C_8H_{16}	112.21	9.661	5.249	82	2613-66-3
26	Octane	C_8H_{18}	114.23	10.088	15.719	97	111-65-9
27	2-Octene	C_8H_{16}	112.21	10.423	0.209	87	111-67-1
28	Cis- 1,4-dimethyl- Cyclohexane	C_8H_{16}	112.21	10.481	0.149	87	589-90-2
29	2,2,5-trimethyl- Hexane	C_9H_{20}	128.25	10.66	0.059	95	3522-94-9
30	2,4-dimethyl- Heptane	C_9H_{20}	128.25	10.775	0.069	79	2213-23-2
31	Cis- 1-ethyl-2-methyl- Cyclopentane	C_8H_{16}	112.21	10.851	0.109	88	930-89-2
32	2,6-dimethyl- Heptane	C_9H_{20}	128.25	10.978	0.119	83	1072-05-5
33	Cis- 1,2-dimethyl- Cyclohexane	C_8H_{16}	112.21	11.093	0.079	76	2207-01-4
34	propyl- Cyclopentane	C_8H_{16}	112.21	11.174	0.399	87	2040-96-2
35	Ethyl- Cyclohexane	C_8H_{16}	112.21	11.249	0.379	92	1678-91-7
36	1,1,3-trimethyl- Cyclohexane	C_9H_{18}	126.24	11.359	0.149	93	3073-66-3
37	1,1,4-trimethyl- Cyclohexane	C_9H_{18}	126.24	11.428	0.049	99	7094-27-1
38	1-ethyl-2-methyl- Cyclohexane	C_9H_{18}	126.24	11.532	0.049	81	3728-54-9
39	3-ethyl-2-methyl- Hexane	C_9H_{20}	128.25	11.636	0.069	93	16789-46-1
40	3,3,4-trimethyl- Hexane	C_9H_{20}	128.25	11.792	0.039	95	16747-31-2
41	1,2,4-trimethyl- Cyclohexane	C_9H_{18}	126.24	11.867	0.099	82	2234-75-5
42	2,3-dimethyl- Heptane	C_9H_{20}	128.25	11.965	0.109	79	3074-71-3
43	3-ethyl-4-methyl- Hexane	C_9H_{20}	128.25	12	0.099	82	3074-77-9
44	3,4-dimethyl- Heptane	C_9H_{20}	128.25	12.069	0.109	92	922-28-1
45	4-ethyl- Heptane	C_9H_{20}	128.25	12.115	0.079	79	2216-32-2
46	Ethyl- Benzene	C_8H_{10}	106.16	12.202	0.319	85	100-41-4
47	2-methyl- Octane	C_9H_{20}	128.25	12.254	0.139	86	3221-61-2
48	Xylene	C_8H_{10}	106.16	12.491	0.669	84	1330-20-7
49	4-methyl-5-Undecene	$C_{12}H_{24}$	168.32	12.676	0.039	98	143185-91-5
50	1,2,3-trimethyl- Cyclohexane	C_9H_{18}	126.24	12.733	0.159	87	1678-97-3
51	1,2,4-trimethyl- Cyclohexane	C_9H_{18}	126.24	12.774	0.089	88	2234-75-5

Table 1 – Continued.

Peak No.	Metabolite	Molecular formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
52	2,2-dimethyl- Octane	$C_{10}H_{22}$	142.28	12.999	0.109	94	15869-87-1
53	2,3-Dimethyl-3-heptene	C_9H_{18}	126.24	13.074	0.069	75	59643-74-2
54	1-Ethyl-4-methylcyclohexane	C_9H_{18}	126.24	13.178	0.089	98	3728-56-1
55	Styrene	C_8H_8	104.15	13.265	0.089	84	100-42-5
56	1,3-dimethyl- Benzene	C_8H_{10}	106.16	13.369	0.319	84	108-38-3
57	3,3,5-trimethyl- Heptane	$C_{10}H_{22}$	142.28	13.478	0.079	87	7154-80-5
58	Nonane	C_9H_{20}	128.2	13.571	0.579	88	111-84-2
59	4-ethyl-3-Octene	$C_{10}H_{20}$	140.27	13.464	0.039	79	53966-51-1
60	Cis-2-Nonene	C_9H_{18}	126.24	13.75	0.059	87	6434-77-1
61	1-Ethyl-4-methylcyclohexane	C_9H_{18}	126.24	13.906	0.069	88	3728-56-1
62	3,4-dimethyl- Octane	$C_{10}H_{22}$	142.28	13.998	0.069	77	15869-92-8
63	2,5-Dimethylpyrroline	C_6H_9N	95.14	14.05	0.049	91	625-84-3
64	2,3-dimethyl- Octane	$C_{10}H_{22}$	142.28	14.142	0.079	94	7146-60-3
65	2,4,6-trimethyl- Heptane	$C_{10}H_{22}$	142.28	14.2	0.119	83	2613-61-8
66	4-Dimethyl cyclooctene	$C_{10}H_{18}$	138.25	14.316	0.039	76	13151-98-9
67	3,5-dimethyl- Octane	$C_{10}H_{22}$	142.28	14.414	0.069	96	15869-93-9
68	2,5-dimethyl- Octane	$C_{10}H_{22}$	142.28	14.46	0.079	82	15869-89-3
69	(1-methylethyl)- Benzene	C_9H_{12}	120.19	14.524	0.189	96	98-82-8
70	bis(2-Ethylhexyl) ether	$C_{16}H_{34}O$	242.44	14.685	0.079	77	10143-60-9
71	Propyl- Cyclohexane	C_9H_{18}	126.24	14.755	0.089	93	1678-92-8
72	2,6-dimethyl- Octane	$C_{10}H_{22}$	142.28	14.801	0.099	79	2051-30-1
73	Cis-3-Nonene	C_9H_{18}	126.24	14.864	0.089	82	20237-46-1
74	1,2,3,5-tetramethylcyclohexane	$C_{10}H_{20}$	140.27	14.928	0.159	83	3726-36-1
75	5,6-dimethyl- Undecane	$C_{13}H_{28}$	184.36	15.095	0.109	93	17615-91-7
76	1-Octene	C_8H_{16}	112.24	15.39	0.079	98	111-66-0
77	1,1,2,3-tetramethyl Cyclohexane	$C_{10}H_{20}$	140.27	15.471	0.049	88	6783-92-2
78	4-ethyl- Octane	$C_{10}H_{22}$	142.28	15.604	0.509	81	15869-86-0
79	5-methyl- Nonane	$C_{10}H_{22}$	142.28	15.771	0.419	75	15869-85-9
80	4-methyl- Nonane	$C_{10}H_{22}$	142.28	15.823	0.109	84	17301-94-9
81	2-methyl- Nonane	$C_{10}H_{22}$	142.28	15.904	0.189	79	871-83-0
82	1-ethyl-2,4-dimethyl- Cyclohexane	$C_{10}H_{20}$	140.27	15.991	0.049	79	61142-69-6
83	3-ethyl- Octane	$C_{10}H_{22}$	142.28	16.048	0.119	92	5881-17-4
84	3-methyl- Nonane	$C_{10}H_{22}$	142.28	16.187	0.769	94	5911-04-6
85	1,1-dimethyl- Cyclopentane	C_7H_{14}	98.19	16.285	0.059	75	1638-26-2
86	Cis- 1-methyl-4-(1-methylethyl)- Cyclohexane	$C_{10}H_{20}$	140.27	16.337	0.099	83	6069-98-3
87	1-ethyl-2-methyl- Benzene	C_9H_{12}	120.19	16.568	0.109	92	611-14-3
88	Cyclotetradecane	$C_{14}H_{28}$	196.37	16.718	0.189	99	295-17-0
89	1-hexyl-3-methyl- Cyclopentane	$C_{12}H_{24}$	168.32	16.788	0.379	81	61142-68-5
90	Ethyl Cyclohexane	C_8H_{16}	112.21	16.845	0.519	96	1678-91-7
91	Tert-butyl- Benzene	$C_{10}H_{14}$	134.22	17.03	0.219	95	98-06-6
92	1,2,3-trimethyl- Benzene	C_9H_{12}	120.19	17.065	0.129	75	526-73-8
93	Decane	$C_{10}H_{22}$	142.28	17.365	4.369	93	124-18-5
94	4-Decene	$C_{10}H_{20}$	140.27	17.434	0.059	87	19689-18-0
95	1,2-dipropyl- Cyclopentane	$C_{11}H_{22}$	154.29	17.504	0.069	90	91242-57-8
96	(2-methylbutyl)- Cyclopentane	$C_{10}H_{20}$	140.27	17.538	0.069	86	53366-38-4
97	3,7-dimethyl-1-Octene	$C_{10}H_{20}$	140.27	17.596	0.059	78	4984-01-4
98	1-Heptacosanol	C27H56O	396.73	17.833	0.039	93	2004-39-9
99	2,5,6-trimethyl- Decane	$C_{13}H_{28}$	184.36	17.885	0.059	95	62108-23-0
100	4-methyl-Decane	$C_{11}H_{24}$	156.31	17.989	0.089	89	2847-72-5
101	1-methyl-2-propyl- Cyclohexane	$C_{10}H_{20}$	140.27	18.081	0.089	75	4291-79-6
102	1,3,5-trimethyl- Benzene	C_9H_{12}	120.19	18.133	0.079	84	108-67-8
103	1-propenyl- Benzene	C_9H_{10}	118.18	18.208	0.179	90	637-50-3
104	3a,4,7,7a-tetrahydro-4,7-Methano-1H-indene	$C_{10}H_{12}$	132.2	18.33	0.149	91	26472-00-4
105	Butyl-Cyclohexane	$C_{10}H_{20}$	140.27	18.428	0.069	79	1678-93-9
106	Pentyl-Cyclopentane	$C_{10}H_{20}$	140.27	18.526	0.079	82	3741-00-2
107	1H-Indene, 2,3-dihydrodimethyl-	$C_{11}H_{14}$	146.22	18.618	0.409	96	53563-67-0
108	1-(2-methylpropyl)- Cyclohexane	$C_{10}H_{20}$	140.27	18.722	0.049	82	1678-98-4

Table 1 – Continued.

Peak No.	Metabolite	Molecular formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
109	3-butenyl-Benzene	$C_{10}H_{12}$	132.2	18.797	0.039	90	768-56-9
110	1-methyl-4-(2-propenyl)- Benzene	$C_{10}H_{12}$	132.2	18.844	0.039	77	3333-13-9
111	Indene	C_9H_8	116.16	18.924	0.769	87	95-13-6
112	1,3-diethyl-Benzene	$C_{10}H_{14}$	134.22	18.994	0.099	99	141-93-5
113	Para tolyl acetaldehyde	$C_9H_{10}O$	134.2	19.063	0.109	93	104-09-6
114	2-Tolyloxirane	$C_9H_{10}O$	134.2	19.184	0.099	81	2783-26-8
115	2-ethyl-1,4-dimethyl- Benzene	$C_{10}H_{14}$	134.2	19.323	0.079	85	1758-88-9
116	2-methyl- Decane	$C_{11}H_{24}$	156.31	19.386	0.069	86	90622-57-4
117	3,7-dimethyl- Nonane	$C_{11}H_{24}$	156.31	19.612	0.059	83	17302-32-8
118	3-Phenyl-1-butanol	$C_{10}H_{14}O$	150.22	19.641	0.079	97	2722-36-3
119	3a,4,5,6,7,7a-Hexahydro-4,7-methanoindene	$C_{10}H_{14}$	134.2	19.848	2.399	77	4488-57-7
120	1-methyl-2-(1-methylethyl)- Benzene	$C_{10}H_{14}S$	166.29	19.935	0.169	88	54576-42-0
121	1-methyl-3-(1-methylethyl)- Benzene	$C_{10}H_{14}$	134.2	20.004	0.069	84	535-77-3
122	(2-methyl-1-propenyl)- Benzene	$C_{10}H_{12}$	132.2	20.137	0.129	99	768-49-0
123	Methyl dicyclopentadiene	C_6H_8	80.13	20.345	0.159	95	96-38-8
124	Undecane	$C_{11}H_{24}$	156.31	20.559	0.329	79	1120-21-4
125	Octahydro-4,7-Methano-1H-indene	$C_{10}H_{16}$	136.23	20.674	0.159	89	6004-38-2
126	2-methyl-Bicyclo[2.2.1]hept-2-ene	C_8H_{12}	108.18	20.871	0.159	97	694-92-8
127	1-methyl-4-(1-methylethyl)- Benzene	$C_{10}H_{12}$	132.2	20.94	0.069	98	1195-32-0
128	3,6-dimethyl-Decane	$C_{12}H_{26}$	170.33	21.125	0.079	80	17312-53-7
129	1-ethyl-2,3-dimethyl- Benzene	$C_{10}H_{14}$	134.22	21.285	0.089	97	933-98-2
130	2-butenyl- Benzene	$C_{10}H_{12}$	132.2	21.396	0.069	80	1560-06-1
131	1,2,3,3a,4,6a-hexahydro-1-Pentalenol	$C_8H_{12}O$	124.18	21.593	0.109	75	5549-09-7
132	4-ethenyl-1,2-dimethyl-Benzene	$C_{10}H_{12}$	132.2	21.662	0.089	75	27831-13-6
133	2-ethenyl-1,4-dimethyl-Benzene	$C_{10}H_{10}$	130.19	21.749	0.039	85	1758-88-9
134	Trans-1-Methyl-2-propylcyclohexane	$C_{10}H_{20}$	140.266	21.87	0.079	78	42806-77-9
135	Octahydro-2-methylene-4,7-Methano-1H-indene	$C_{11}H_{16}$	148.24	21.945	0.349	94	50745-90-9
136	2,3-dihydro-4-methyl-1H-Indene	$C_{10}H_{12}$	132.2	22.049	0.219	99	824-22-6
137	4-ethyl- Decane	$C_{12}H_{26}$	170.33	22.147	0.079	87	1636-44-8
138	4-methyl-Tricyclo[5.2.1.0(2,6)]dec-4-ene	$C_{11}H_{16}$	148.24	22.245	0.769	90	1005046-58-1
139	1-methyl- 1H-Indene	$C_{10}H_{10}$	130.19	22.384	0.879	89	767-59-9
140	2-Methyl-1H-indene	$C_{10}H_{10}$	130.19	22.557	0.419	75	2177-47-1
141	3a,4,7,7a-Tetrahydrodimethyl-4,7-methanoindene	$C_{12}H_{16}$	160.25	22.73	0.049	77	26472-00-4
142	1,2,3,4-tetrahydro- Naphthalene	$C_{10}H_{12}$	132.2	22.805	0.279	92	119-64-2
143	1,4-Dihydronaphthalene	$C_{10}H_{10}$	130.19	22.892	0.379	75	612-17-9
144	(1,1-dimethylbutyl)- Benzene	$C_{12}H_{18}$	162.27	22.123	0.129	90	1985-57-5
145	3-Cyclohexenecarbonyl chloride	C_7H_9CLO	144.6	23.239	0.059	75	54417-92-4
146	Naphthalene	$C_{10}H_8$	128.17	23.574	1.939	95	91-20-3
147	Dodecane	$C_{12}H_{26}$	170.33	23.735	1.529	95	112-40-3
148	Cyclopentanecarboxylic acid, 3-methylene-, methyl ester (9CI)	$C_8H_{12}O_2$	140.18	23.978	0.079	76	37575-80-7
149	1,6-dimethyl- decahydro-Naphthalene	$C_{12}H_{22}$	166.3	24.018	0.299	98	1750-51-2
150	2,6-dimethyl- Undecane	$C_{13}H_{28}$	184.36	24.099	0.099	93	17301-23-4
151	Decahydro-2-methyl- Naphthalene	$C_{11}H_{20}$	152.28	24.238	0.049	83	2958-76-1
152	6,7-Dimethyl-3H-isobenzofuran-1-one	$C_{10}H_{10}O_2$	162.18	24.376	0.239	76	569-31-3
153	2,6-dimethyl- decahydro- Naphthalene	$C_{12}H_{22}$	166.3	24.474	0.529	88	1618-22-0
154	1,2-dimethyl- decahydro-Naphthalene	$C_{12}H_{22}$	166.3	24.809	0.069	84	3604-14-6
155	Trans-syn-Tricyclo[7.3.0.0(2,6)]-8-dodecene	$C_{12}H_{18}$	162.27	24.919	0.059	96	30767-91-0
156	1-Tert-butyl-4-ethynylcyclohexane	$C_{12}H_{22}$	166.3	25.023	0.119	98	1565550-13-1
157	2-Methyl-4-fluoro-1H-inden-1-one	$C_{10}H_7FO$	162.16	25.127	0.159	86	52045-42-8
158	1,3-Cycloheptadiene	C_7H_{10}	94.15	25.208	0.079	93	4054-38-0
159	2-methyl- Dodecane	$C_{13}H_{28}$	184.36	25.531	0.049	84	68551-19-9
160	3-methyl- Dodecane	$C_{13}H_{28}$	184.36	25.733	0.079	95	17312-57-1
161	6-[(Z)-1-Butenyl]-1,4-cycloheptadiene	$C_{11}H_{16}$	148.24	25.814	0.099	89	33156-92-2
162	4-methyl-4-phenyl-2-Pentanone	$C_{12}H_{16}O$	176.25	25.866	0.109	82	7403-42-1

Table 1 – Continued.

Peak No.	Metabolite	Molecular formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
163	Benzene, 1,2,4-trimethyl-5-(1-methylethyl)-	$C_{12}H_{18}$	162.27	26.063	0.069	82	10222-95-4
164	cis-anti-cis-Tricyclo[7.3.0.0(2,6)]dodecan-7-one	$C_{12}H_{18}O$	178.27	26.253	0.149	99	29782-49-8
165	Cyclotridecane	$C_{13}H_{26}$	182.35	26.363	0.039	76	295-02-3
166	Bicyclo(3.3.1)non-2-ene	C_9H_{14}	122.21	26.478	0.059	93	6671-66-5
167	Tridecane	$C_{13}H_{28}$	184.36	26.565	0.169	82	629-50-5
168	2-methyl- Naphthalene	$C_{11}H_{10}$	142.2	26.796	0.219	89	91-57-6
169	5-methyl-5-phenyl-2-Hexanone	$C_{12}H_{16}O$	176.25	27.362	0.159	94	14128-61-1
170	1-Chloro-2-methyl-2-phenylpropane	$C_{10}H_{13}CL$	168.663	27.518	0.119	87	515-40-2
171	2-butyldecahydro- Naphthalene	$C_{14}H_{26}$	194.36	27.634	0.099	83	3730-08-3
172	2,3-dimethyl-2-butyl- Borane	$C_{12}H_{30}B2$	196	27.703	0.099	95	22784-01-6
173	2-hexyl-1-Decanol	$C_{16}H_{34}O$	242.44	27.951	0.409	95	2425-77-6
174	Sulfurous acid, hexyl pentadecyl ester	$C_{21}H_{44}O_3S$	376.6	28.049	0.209	86	3381-25-7
175	2,3-Dimethyldodecane	$C_{14}H_{30}$	198.39	28.188	0.449	85	6117-98-2
176	10-methyl- Eicosane	$C_{21}H_{44}$	296.6	28.327	0.459	94	54833-23-7
177	1,54-dibromo- Tetrapentacontane	$C_{54}H_{108}Br_2$	917.2	28.379	0.169	76	852228-22-9
178	3-methyl- Tridecane	$C_{14}H_{30}$	198.39	28.529	0.809	75	6418-41-3
179	Ethylpropylcyclopentane	$C_{10}H_{20}$	140.27	28.615	0.169	84	2040-96-2
180	2,6,10-trimethyl- Dodecane	$C_{15}H_{32}$	212.41	28.714	0.169	77	3891-98-3
181	(-)-trans-Pinane	$C_{10}H_{18}$	138.25	29.002	0.179	94	33626-25-4
182	Behenic alcohol	$C_{22}H_{46}O$	326.60	29.054	0.189	95	661-19-8
183	Phosphonic acid, dioctadecyl ester	$C_{36}H_{75}O_3P$	587	29.089	0.199	75	3135-18-0
184	1-Pentadecene	$C_{15}H_{30}$	210.4	29.193	0.729	88	13360-61-7
185	Tetradecane	$C_{14}H_{30}$	198.39	29.378	2.709	98	629-59-4
186	3,5,24-trimethyl- Tetracontane	$C_{43}H_{88}$	605.2	29.441	0.589	92	55162-61-3
187	Tritetracontane	$C_{43}H_{88}$	605.2	29.718	0.459	82	7098-21-7
188	2,7-dimethyl- Naphthalene	$C_{12}H_{12}$	156.22	29.851	3.119	79	582-16-1
189	Pentadecyl heptafluorobutyrate	$C_{19}H_{31}F_7O_2$	424.4	29.955	0.369	83	959261-23-5
190	2-Methylbenzo[b]thiophene	C_9H_8S	148.22	30.059	0.309	97	1195-14-8
191	Hexamethyl- Benzene	$C_{12}H_{18}$	162.27	30.481	0.649	76	87-85-4
192	1-chloro-Octadecane	$C_{18}H_{37}CL$	288.94	30.573	0.419	77	3386-33-2
193	1,6-dimethyl-Naphthalene	$C_{12}H_{12}$	156.22	30.793	0.619	84	575-43-9
194	2,6,10,14-tetramethyl- Heptadecane	$C_{21}H_{44}$	296.6	30.914	1.509	94	18344-37-1
195	Cyclopentadecane	$C_{15}H_{30}$	210.4	31.047	0.189	91	295-48-7
196	3-methyl- Tetradecane	$C_{15}H_{32}$	212.41	31.116	0.499	95	18435-22-8
197	3,7-dimethyl- Nonane	$C_{11}H_{24}$	156.31	31.376	0.509	79	17302-32-8
198	1-Hexadecene	$C_{16}H_{32}$	224.42	31.665	0.429	84	629-73-2
199	Pentadecane	$C_{15}H_{32}$	212.41	31.878	2.039	82	629-62-9
200	2-(1-methylethyl)-Naphthalene	$C_{13}H_{14}$	170.25	32.375	0.349	97	2027-17-0
201	2,5,7-trimethyl- Benzo[b]thiophene	$C_{11}H_{12}S$	176.28	32.427	0.209	87	16587-65-8
202	2,4,6- trimethyl - Azulene	$C_{13}H_{14}$	170.25	32.514	0.369	94	48134-26-5
203	2,3,6-trimethyl- Naphthalene	$C_{13}H_{14}$	170.25	32.652	1.909	93	829-26-5
204	1,4,5,6,7-pentamethyl-2,3-dihydro-1H-indene	$C_{14}H_{20}$	188.31	32.762	0.249	83	16204-67-4
205	dodecahydro-9b-Boraphenalene	$C_{12}H_{21}B$	176.11	32.837	0.309	77	16664-33-8
206	6-methyl- Pentadecane	$C_{16}H_{34}$	226.44	32.993	0.149	79	10105-38-1
207	2,6,10-trimethyl- Tetradecane	$C_{17}H_{36}$	240.5	33.137	0.159	91	14905-56-7
208	4-methyl- Pentadecane	$C_{16}H_{34}$	226.44	33.224	0.359	89	2801-87-8
209	2-methyl- Pentadecane	$C_{16}H_{34}$	226.44	33.345	0.259	80	1560-93-6
210	6-chloro-N-ethyl-2,4-diamine -1,3,5-Triazine	$C_5H_8CLN_5$	173.6	34.004	0.139	81	1007-28-9
211	1-Eicosanol	$C_{20}H_{42}O$	298.55	34.102	0.089	95	629-96-9
212	Hexadecane	$C_{16}H_{34}$	226.44	34.212	0.629	91	544-76-3
213	Tetracosyl heptafluorobutyrate	$C_{28}H_{49}F_7O_2$	550.7	34.356	0.199	85	959085-66-6
214	2-ethyl-5,7-dimethyl-Benzo[b]thiophene	$C_{12}H_{14}S$	190.31	34.893	0.099	85	18428-05-2
215	4,6,8-Trimethylazulene	$C_{13}H_{14}$	170.25	35.037	0.089	79	941-81-1
216	6-Pentyl-2H-pyran-2-one	$C_{10}H_{14}O_2$	166.22	35.124	0.079	80	27593-23-3
217	5-hydroxy-2-decenoic acid	$C_{10}H_{18}O_3$	186.25	35.24	0.089	79	51154-96-2

Table 1 – Continued.

Peak No.	Metabolite	Molecular formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
218	2,6,10-trimethyl-Pentadecane	$C_{18}H_{38}$	254.49	35.32	0.149	89	3892-00-0
219	4-methyl-Hexadecane	$C_{17}H_{36}$	240.47	35.517	0.069	77	25117-26-4
220	Undecyl- Cyclopentane	$C_{16}H_{32}$	224.43	35.592	0.049	77	6785-23-5
221	N-[4-bromo-n-butyl]-2-Piperidinone	$C_9H_{16}BrNO$	234.13	35.638	0.069	98	195194-80-0
222	3-methyl- Hexadecane	$C_{18}H_{38}$	254.5	35.817	0.079	83	6418-43-5
223	1-ethyl-3,5-diisopropyl- Benzene	$C_{14}H_{22}$	190.32	36.21	0.049	77	15181-13-2
224	(2S)-2-{2-[(2S)-2-acetamido-4-methylpentanamido]-2-methylpropanamido}-N-(1-{[(2S)-1-hydroxy-3-phenylpropan-2-yl]carbamoyl}-1-methylethyl)-4-methylpentanamide	$C_{31}H_{51}N_5O_6$	589.8	36.32	0.039	98	219637-06-6
225	Heptadecane	$C_{17}H_{36}$	240.5	36.452	0.149	99	629-78-7
226	2,6,10,14-tetramethyl- Pentadecane	$C_{19}H_{40}$	268.52	36.585	0.069	96	1921-70-6
227	Tetra(1-propynyl)silane	$C_{12}H_{12}Si$	184.07	36.683	0.049	99	20143-21-9
228	(1R,7S,8S,11R)-7-hydroxy-11-(hydroxymethyl)-15-methyl-12,13-dithia-9,15-diazatetracyclo[9.2.2.01,9.03,8]pentadeca-3,5-diene-10,14-dione	C13H14N2 O4S2	326.4	37.076	0.079	97	67-99-2
229	4-(1,1-dimethylethyl)-2-methyl- Benzenethiol	$C_{11}H_{16}S$	180.31	37.203	0.039	87	15570-10-2
230	4-methyl-Heptadecane	$C_{18}H_{38}$	254.5	37.7	0.039	91	26429-11-8
231	2-methyl-Heptadecane	$C_{18}H_{38}$	254.5	37.81	0.049	95	72123-30-9
232	3-methyl- Heptadecane	$C_{18}H_{38}$	254.49	37.994	0.039	96	6418-44-6
233	Naphtho[2,1-b]thiophene	$C_{12}H_8S$	184.26	38.306	0.039	85	233-02-3
234	bis-1,1'-(1-butenylidene) Benzene	$C_{16}H_{16}$	208.29	38.485	0.039	98	1726-14-3
235	Octadecane	$C_{18}H_{38}$	254.49	38.595	0.119	93	593-45-3
236	2,6,10,14-tetramethyl- Hexadecane	$C_{20}H_{42}$	282.5	38.815	0.069	89	638-36-8
237	17-Pentatriacontene	$C_{35}H_{70}$	490.93	38.884	0.039	99	6971-40-0
238	6-propyl- Tridecane	$C_{16}H_{34}$	226.44	39.456	0.039	95	55045-10-8
239	2-methyl- Nonadecane	$C_{20}H_{42}$	282.5	40.074	0.039	98	52845-07-5
240	1-Methyldibenzothiophene	$C_{13}H_{10}S$	198.29	40.426	0.039	90	31317-07-4
241	2-methyl-Heptadecane	$C_{18}H_{38}$	254.5	40.501	0.039	87	72123-30-9
242	Nonadecane	$C_{19}H_{40}$	268.52	40.634	0.079	92	629-92-5
243	n-Hexadecanoic acid	$C_{16}H_{32}O$	256.42	41.783	0.049	89	57-10-3
244	2-methyl-Nonadecane	$C_{20}H_{42}$	282.5	41.875	0.039	81	52845-07-5
245	Eicosane	$C_{20}H_{42}$	282.5	42.574	0.109	88	112-95-8
246	Heneicosane	$C_{21}H_{44}$	296.6	44.434	0.059	81	629-94-7
247	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	282.5	45.15	0.039	89	2027-47-6
248	Docosane	$C_{22}H_{46}$	310.6	46.272	0.059	83	629-97-0
249	Tricosane	$C_{23}H_{48}$	324.6	48.367	0.059	91	638-67-5
250	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6	55.881	0.069	99	117-81-7

*: National institute of standards and technology (version 23). **: Chemical abstracts service.

Identified SMs using methanol solvent

The number of identified SMs using methanol solvent, compared to n-hexane solvent, showed a decrease of 58.4% (Table 1; Table 2). In total, 104 SMs were identified from the liquid culture medium of *T. atroviride* using methanol solvent, with simple hydrocarbons accounting for 61.53% of the identified SMs (Fig. 3B; Table 2). Although among the identified SMs using methanol solvent, organo-oxygen (24.03%), organo-nitrogen (0.96%), organo-sulfur (2.88%), organo-boron (1.92%), and organo-chlorine (0.96%) compounds were identified, organo-bromine and organo-silicon compounds were not detected (Fig. 3B; Table 2). Organic compounds with two elements constituted about 0.96% of the identified SMs using methanol solvent (Fig. 3B). Among organic compounds with two elements, pentadecyl trifluoroacetate specifically contained the fluorine element, which was not found in the structure of other organic compounds with one or three elements (Fig. 3B; Table 2). Only 8-hydroxy-

14-(2-hydroxy-3,4-dimethoxyphenyl)-4,10-dioxa-15,16-dithia-11,18diazapentacyclo[11.3.2.0^{1},^{11}.0^{3},^{5}.0^{3},^{9}] octadec-6-ene-12,17-dione, among all identified SMs using methanol solvent, had three elements in its molecular structure (Table 2).

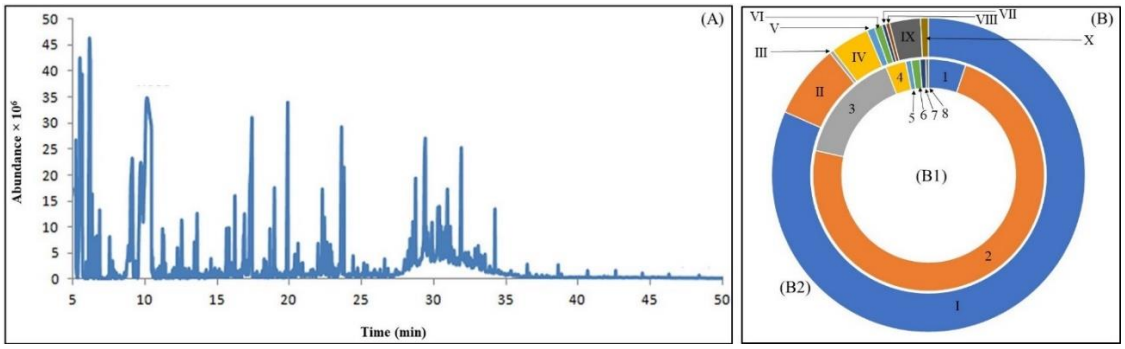

Fig. 2 – The compounds identified from cultured *T. atroviride* 6022 using n-hexane solvent are outlined as follows: (A) Chromatograph depicting the identified compounds utilizing a capillary column with nonpolar stationary phases. (B) Doughnut charts illustrating the diversity of the various identified compounds. The inner chart (B1) presents diversity based on molecular weight categories [1: 0–100 g/mol; 2: 100–200 g/mol; 3: 200–300 g/mol; 4: 300–-400 g/mol; 5: 400–500 g/mol; 6: 500–600 g/mol; 7: 600–700 g/mol; 8: 900–1000 g/mol]. The outer chart (B2) represents diversity based on molecular structure categories [I: Simple hydrocarbons; II: Organo-oxygen compounds; III: Organo-nitrogen compounds; IV: Organo-sulfur compounds; V: Organo-boron compounds; VI: Organo-chlorine compounds; VII: Organo-bromine compounds; VIII: Organo-silicon compounds; IX: Organic compounds with two elements; X: Organic compounds with three elements].

Similar to the conditions observed with identified SMs using n-hexane solvent, organic compounds with weight ranges between 100–200 and 200–300 g/mol comprised the largest number of identified SMs using methanol solvent, accounting for 59.61% and 28.84%, respectively (Fig. 3B; Table 2). However, SMs with a molecular weight greater than 605.2 g/mol were not found among the identified SMs using methanol solvent (Fig. 3B; Table 2). Among the identified SMs using methanol solvent, 3,5,24-trimethyl-tetracontane had the highest weight at 605.2 g/mol, while 1-butanol had the lowest weight at 74.121 g/mol (Table 2). The 9-acetylphenanthrene, with 0.103%, had the lowest %Area, while naphthalene, with 10.603%, had the highest %Area among the identified SMs using methanol solvent (Fig. 3A; Table 2).

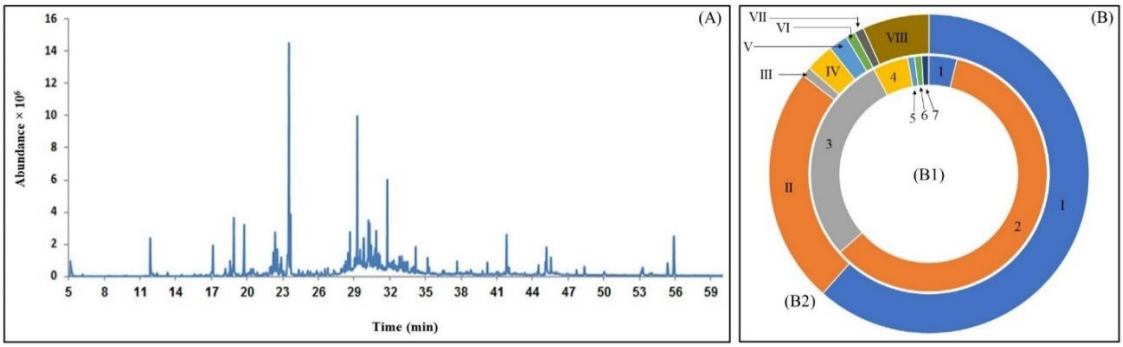

Fig. 3 – The compounds identified from cultured *T. atroviride* 6022 using methanol solvent are detailed as follows: (A) Chromatograph displaying the identified compounds utilizing a capillary column with nonpolar stationary phases. (B)Doughnut charts illustrating the diversity of the various identified

compounds. The inner chart (B1) showcases diversity based on molecular weight categories [1: 0–100 g/mol; 2: 100–200 g/mol; 3: 200–300 g/mol; 4: 300–400 g/mol; 5: 400–500 g/mol; 6: 500–600 g/mol; 7: 600–700 g/mol]. The outer chart (B2) represents diversity based on molecular structure categories [I: Simple hydrocarbons; II: Organo-oxygen compounds; III: Organo-nitrogen compounds; IV: Organo-sulfur compounds; V: Organo-boron compounds; VI: Organo-chlorine compounds; VII: Organic compounds with two elements; VIII: Organic compounds with three elements].

Table 2 – Identified metabolites from *T. atroviride* 6022 using the methanol solvent.

Peak No.	Metabolite	Molecular Formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
1	Acetic acid, ethyl ester	$C_4H_8O_2$	88.11	5.162	1.283	95	141-78-6
2	1-Butanol	$C_4H_{10}O$	74.121	6.19	0.143	91	71-36-3
3	2-Furanmethanol	$C_5H_6O_2$	98.1	11.867	1.743	90	98-00-0
4	Ethylbenzene	C_8H_{10}	106.167	12.144	0.143	87	100-41-4
5	Xylene	C_8H_{10}	106.16	12.439	0.503	99	1330-20-7
6	propyl-Benzene	C_9H_{12}	120.2	15.598	0.193	94	103-65-1
7	3-methyl-Nonane	$C_{10}H_{22}$	142.28	16.123	0.133	76	5911-04-6
8	1,2,3,5-Tetramethylbenzene	$C_{10}H_{14}$	134.221	17.019	0.323	84	527-53-7
9	Decane	$C_{10}H_{22}$	142.29	17.146	1.543	80	124-18-5
10	1-propenyl-Benzene	C_9H_{10}	118.176	18.185	0.573	91	637-50-3
11	3a,4,7,7a-tetrahydro-4,7-Methano-1H-indene	$C_{10}H_{12}$	132.2	18.295	0.183	90	933-60-8
12	Indane	C_9H_{10}	118.176	18.589	0.843	75	496-11-7
13	Indonaphthene	C_9H_8	116.16	18.896	2.753	87	95-13-6
14	1,2-diethyl-Benzene	$C_{10}H_{14}$	134.22	18.976	0.113	95	25340-17-4
15	3a,4,5,6,7,7a-hexahydro-, endo-4,7-methanoindene	$C_{10}H_{14}$	134.22	19.768	2.573	79	4488-57-7
16	2-ethyl-1,4-dimethyl-Benzene	$C_{10}H_{14}$	134.22	19.924	0.163	81	1758-88-9
17	(2-methyl-1-propenyl)-Benzene	$C_{10}H_{12}$	132.2	20.126	0.283	87	768-49-0
18	1-methyl-4-(1-methylethyl)-Benzene	$C_{10}H_{12}$	132.2	20.201	0.223	86	1195-32-0
19	methyl dicyclopentadiene	C_6H_8	80.13	20.322	0.603	80	26519-91-5
20	(1,1-dimethylpropyl)-Benzene	$C_{11}H_{16}$	148.245	20.495	0.553	86	2049-95-8
21	1-methyl-4-(2-propenyl)- Benzene	$C_{11}H_{14}$	146.23	21.39	0.163	85	3333-13-9
22	2-octynoic acid	$C_8H_{12}O_2$	140.18	21.581	0.203	82	5663-96-7
23	2-ethenyl-1,3-dimethyl- Benzene	$C_{10}H_{12}$	132.2	21.656	0.193	96	2039-90-9
24	Tricyclo[5.2.1.0(2,6)]decane, 4-methyl-	$C_{11}H_{18}$	150.26	21.933	0.533	88	50745-90-9
25	1-ethenyl-4-ethyl-Benzene	$C_{10}H_{12}$	132.2	22.032	0.473	82	3454-07-7
26	(1-methyl-2-cyclopropen-1-yl)-Benzene	$C_{10}H_{10}$	130.186	22.367	3.893	91	65051-83-4
27	1,2,3,4-tetrahydro-Naphthalene	$C_{10}H_{12}$	132.2	22.788	0.643	83	119-64-2
28	1,4-Dihydronaphthalene	$C_{10}H_{10}$	130.19	22.875	0.993	78	612-17-9
29	(1,1-dimethylbutyl)-Benzene	$C_{12}H_{18}$	162.27	23.1	0.293	84	1985-57-5
30	Naphthalene	$C_{10}H_8$	128.17	23.412	10.603	94	91-20-3
31	Dodecane	$C_{12}H_{26}$	170.33	23.666	2.453	97	112-40-3
32	2,4-dimethyl-1-(1-methylpropyl)-Benzene	$C_{12}H_{18}$	162.27	24.359	0.353	80	1483-60-9
33	(1,1,2-trimethylpropyl)-Benzene	$C_{12}H_{18}$	162.27	24.671	0.253	76	26356-11-6
34	5-cyclopropylidene- Pentanoic acid, methyl ester	$C_{10}H_{16}O_2$	168.23	25.341	0.253	77	2177-77-7
35	1-Phenyl-1-hexanone	$C_{12}H_{16}O$	176.26	25.861	0.253	84	942-92-7
36	5-methyl-Tridecane	$C_{14}H_{30}$	198.39	26.248	0.253	89	25117-31-1
37	Tridecane	$C_{13}H_{28}$	184.37	26.554	0.253	92	629-50-5
38	2-methyl-Naphthalene	$C_{11}H_{10}$	142.2	26.796	0.663	99	91-57-6

Table 2 – Continued.

Peak No.	Metabolite	Molecular Formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
39	5-methyl-5-phenyl-2-Hexanone	$C_{13}H_{18}O$	190.28	27.356	0.153	87	14128-61-1
40	1-(dodecyloxy)-2-nitro-Benzene	$C_{18}H_{29}NO_3$	307.4	27.934	0.423	92	83027-71-8
41	(2S)-2-{2-[(2S)-2-acetamido-4-methylpentanamido]-2-methylpropanamido}-N-(1-{[(2S)-1-hydroxy-3-phenylpropan-2-yl]carbamoyl}-1-methylethyl)-4-methylpentanamide	$C_{31}H_{51}N_5O_6$	589.8	28.026	0.233	75	219637-06-6
42	4-methyl-Tridecane	$C_{14}H_{30}$	198.39	28.159	0.603	95	26730-12-1
43	10-methyl-Eicosane	$C_{21}H_{44}$	296.6	28.298	0.553	89	54833-23-7
44	Diethylmethyl-Borane	$C_5H_{13}B$	83.97	28.355	0.283	99	7397-46-8
45	3-methyl-Tridecane	$C_{14}H_{30}$	198.39	28.494	1.193	94	6418-41-3
46	2,6,10-trimethyl-Dodecane	$C_{15}H_{32}$	212.41	28.662	1.813	98	3891-98-3
47	2(E)-Tetradecene	$C_{14}H_{28}$	196.37	29.054	1.233	83	1652-97-7
48	Cyclotetradecane	$C_{14}H_{28}$	196.37	29.158	0.603	81	295-17-0
49	Tetradecane	$C_{14}H_{30}$	198.39	29.274	6.263	78	629-59-4
50	3,5,24-trimethyl-Tetracontane	$C_{43}H_{88}$	605.2	29.378	0.793	84	55162-61-3
51	5-Phenylbicyclo[2.2.1]hept-2-ene	$C_{13}H_{14}$	170.25	29.453	0.673	76	6143-30-2
52	1-ethyl-Naphthalene	$C_{12}H_{12}$	156.22	29.516	1.933	76	1127-76-0
53	Methoxyacetic acid, 2-pentadecyl ester	$C_{18}H_{36}O_3$	300.5	29.678	0.753	79	959264-10-9
54	1,7-dimethyl-Naphthalene	$C_{12}H_{12}$	156.22	29.817	8.113	88	575-37-1
55	3,5-dimethyl-Benzo[b]thiophene	$C_{10}H_{10}S$	162.25	30.048	0.753	87	1964-45-0
56	8-hydroxy-14-(2-hydroxy-3,4-dimethoxyphenyl)-4,10-dioxa-15,16-dithia-11,18-diazapentacyclo[11.3.2.0^{1},^{11}.0^{3},^{5}.0^{3},^{9}]octadec-6-ene-12,17-dione	$C_{20}H_{20}N_2O_8S_2$	480.5	30.14	0.683	94	83912-90-7
57	3-(1-methylethyl)-1H-pyrazolo[3,4-B]pyrazine	$C_8H_{10}N_4$	162.19	30.446	1.473	89	19868-88-3
58	2,6,11-trimethyl-Dodecane	$C_{15}H_{32}$	212.41	30.492	0.323	93	31295-56-4
59	4,8-dimethyl-Tridecane	$C_{15}H_{32}$	212.41	30.539	0.613	84	55030-62-1
60	1,2-Dicyclohexylethane	$C_{14}H_{26}$	194.36	30.654	1.253	83	3321-50-4
61	Undecane	$C_{11}H_{24}$	156.31	30.862	2.793	98	1120-21-4
62	3-methyl-Tetradecane	$C_{15}H_{32}$	212.41	31.081	0.853	81	18435-22-8
63	1,4-dimethyl-Naphthalene	$C_{12}H_{12}$	156.22	31.162	1.203	86	571-58-4
64	5,9,9-trimethyl -Spiro[3.6]deca-5,7-dien-1-one	$C_{13}H_{18}O$	190.28	31.307	0.303	88	81532-19-6
65	Hexadecane	$C_{16}H_{34}$	226.44	31.353	1.353	91	544-76-3
66	Pentadecyl trifluoroacetate	$C_{17}H_{31}F_3O_2$	324.4	31.63	0.483	88	959010-23-2
67	Pentadecane	$C_{15}H_{32}$	212.41	31.803	3.273	98	629-62-9
68	1-(chloromethyl)-Naphthalene	$C_{11}H_9Cl$	176.64	31.948	0.513	78	86-52-2
69	6-Pentyl-2H-pyran-2-one	$C_{10}H_{14}O_2$	166.22	32.144	1.033	77	27593-23-3
70	Trimethyl(2,6 ditert. butylphenoxy)silane	$C_{17}H_{30}OSi$	278.5	32.185	0.513	77	78721-87-6
71	1,4,6-trimethyl Naphthalene	$C_{13}H_{14}$	170.25	32.346	3.413	92	2131-42-2
72	2,5,7-trimethyl- Benzo[b]thiophene	$C_{11}H_{12}S$	176.28	32.392	0.343	83	16587-65-8
73	Dodecahydro-9b-Boraphenalene	$C_{12}H_{21}B$	176.12	32.808	0.653	96	16664-33-8
74	2-methyl-Pentadecane	$C_{16}H_{34}$	226.44	33.328	0.303	79	1560-93-6
75	2,3,6-trimethyl- Naphthalene	$C_{13}H_{14}$	170.25	33.501	0.693	81	829-26-5
76	1-Hexadecene	$C_{16}H_{32}$	224.42	34.015	0.313	97	629-73-2
77	1-methyl-1-(1-methylethyl)-2-nonyl-Cyclopropane	$C_{16}H_{32}$	224.42	34.344	0.273	89	41977-40-6

Table 2 – Continued.

Peak No.	Metabolite	Molecular Formula	Weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
78	1,4,5-trimethyl- Naphthalene	$C_{13}H_{14}$	170.25	34.454	0.183	85	2131-41-1
79	Benzophenone	$C_{13}H_{10}O$	182.22	35.309	0.413	89	119-61-9
80	Heptadecane	$C_{17}H_{36}$	240.47	36.447	0.233	79	629-78-7
81	2,6,10,14-tetramethyl- Pentadecane	$C_{19}H_{40}$	268.5	36.585	0.213	91	1921-70-6
82	Nerolidol	$C_{15}H_{26}O$	222.37	37.665	0.513	75	7212-44-4
83	3(Z)-Tetradecene	$C_{14}H_{28}$	196.37	37.983	0.133	93	41446-67-7
84	1-Octadecene	$C_{18}H_{36}$	252.5	38.451	0.143	92	112-88-9
85	Octadecane	$C_{18}H_{38}$	254.5	38.589	0.123	91	593-45-3
86	2,6,10,14-tetramethyl- Hexadecane	$C_{20}H_{42}$	282.5	38.82	0.293	98	638-36-8
87	Phenanthrene	$C_{14}H_{10}$	178.23	38.907	0.153	87	85-01-8
88	(1-methyldodecyl)- Benzene	$C_{19}H_{32}$	260.46	41.021	0.163	86	4534-53-6
89	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	41.142	0.153	95	112-39-0
90	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	41.829	1.673	82	57-10-3
91	Sulfurous acid, diethyl ester	$C_4H_{10}O_3S$	138.19	41.927	0.313	83	623-81-4
92	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.34	42.037	0.253	99	84-74-2
93	9-Acetylphenanthrene	$C_{16}H_{12}O$	220.26	43.348	0.103	99	2039-77-2
94	10,13-Octadecadienoic acid, methyl ester	$C_{19}H_{34}O_2$	294.5	44.422	0.153	80	13481-95-3
95	cis-13-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.49	44.509	0.343	98	56554-47-3
96	9,12 (Z,Z)-Octadecadienoic acid	$C_{18}H_{32}O_2$	280.445	45.092	0.553	76	60-33-3
97	9(E)-Octadecenoic acid	$C_{18}H_{34}O_2$	282.46	45.173	1.513	86	112-79-8
98	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	45.554	0.883	78	57-11-4
99	Hexadecanamide	$C_{16}H_{33}NO$	255.44	45.993	0.133	94	629-54-9
100	9(Z)-Octadecenamide	$C_{18}H_{35}NO$	281.48	50.042	0.263	87	301-02-0
101	10-Hydroxy-2-decenoic acid	$C_{10}H_{18}O_3$	186.25	53.143	0.303	86	14113-05-4
102	9(Z)-octadecenal	$C_{18}H_{34}O$	266.46	53.264	0.593	90	2423-10-1
103	butyl 9,12-octadecadienoate	$C_{22}H_{40}O_2$	336.6	55.361	0.913	92	2025317-03-5
104	Bis(2-ethylhexyl) phthalate	$C_{24}H_{38}O_4$	390.6	55.892	2.723	95	117-81-7

*: National institute of standards and technology (version 23). **: Chemical abstracts service.

Identified SMs using n-butanol solvent

The number of identified SMs using n-butanol solvent was 92.8% and 82.69% lower than the number of identified SMs using n-hexane and methanol solvents, respectively (Table 3). In total, 18 SMs were identified using n-butanol solvent, in which no simple hydrocarbons were present (Fig. 4B; Table 3). Almost 77.77% of the identified SMs using n-butanol solvent were organo-oxygens, and the remaining 22.22% belonged to organic compounds that contained two different elements in their chemical structure (Fig. 4B; Table 3). Among the organic compounds with two elements, nitrogen and silicon elements were observed, both associated with oxygen (Table 3). Approximately 55.55% of the identified SMs using n-butanol solvent had a molecular weights between 200 and 300 g/mol, while 16.66% SMs fell within the weight range between 0–100 and 100–200, respectively (Fig. 4B; Table 3). The compound with the lowest molecular weight belonged to 1-butanol (74.121 g/mol), which also had the highest %Area at 91%. However, the highest molecular weight belonged to linoleic acid, butyl ester, with 392.7 g/mol. The 9(E)-octadecenoic acid and methyl ester had the lowest %Area at 0.61% (Table 3).

Table 3 – Identified metabolites from *T. atroviride* 6022 using n-butanol solvent.

Peak No.	Metabolite	Molecular formula	Molecular weight (g/mol)	Time (min)	%Area	%Match against NIST*	CAS ** registry No.
1	1-Butanol	$C_4H_{10}O$	74.121	5.982	91.001	88	71-36-3
2	2-methyl-1-Butanol	$C_5H_{12}O$	88.15	9.291	3.341	75	137-32-6
3	1-Pentanol	$C_5H_{12}O$	88.15	9.869	0.491	93	71-41-0
4	3-methyl - 4-Heptanone	$C_8H_{16}O$	128.21	14.847	0.451	95	15726-15-5
5	2-ethylhexanal	$C_8H_{16}O$	128.21	15.765	0.461	77	123-05-7
6	Butanoic acid, butyl ester	$C_8H_{16}O2$	144.21	17.111	0.231	80	109-21-7
7	n-Hexadecanoic acid	$C_{16}H_{32}O$	256.42	41.801	0.241	93	57-10-3
8	9(E)-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296.49	44.503	0.061	95	1937-62-8
9	9,12 (Z,Z)-Octadecadienoic acid	$C_{18}H_{32}O_2$	280.45	45.081	0.121	98	2197-37-7
10	9(E)-Octadecenoic acid	$C_{18}H_{34}O_2$	282.46	45.156	0.421	77	112-79-8
11	Octadecanoic acid	$C_{18}H_{36}O_2$	284.5	45.543	0.191	98	57-11-4
12	Hexadecanamide	$C_{16}H_{33}NO$	255.44	45.982	0.151	90	629-54-9
13	Octahydro-4a-methyl-7-(1-methylethyl)-2(1H)-Naphthalenone	C14H24O	208.34	49.932	0.091	81	54594-42-2
14	9(Z)-Octadecenamide	C18H35NO	281.5	50.036	0.221	80	301-02-0
15	Linoleic acid, butyl ester	C24H44O2Si	392.7	53.137	0.091	76	920753-80-6
16	9(Z)-Octadecenal	C18H34O	266.5	53.253	0.221	83	2423-10-1
17	Bis(2-ethylhexyl) phthalate	C24H38O4	390.6	55.886	2.101	84	117-81-7
18	10-methyl-5,10-dihydro-11H-dibenzo[b,e][1,4]diazepin-11-one	C14H12N2O	224.26	60.824	0.121	88	5814-41-5

*: National institute of standards and technology (version 23). **: Chemical abstracts service

Discussion

In this study, GC-MS analysis of FLMT demonstrated the considerable potential of *T. atroviride* to produce various SMs. Overall, we have identified 372 SMs. Given that most volatile organic compounds are non-polar, it is expected to identify a large number of SMs using n-hexane solvent (Siddiquee et al. 2012). However, few SMs were detected in the FLMT with n-butanol solvent. As reported by Siddiquee et al. (2012), most of the non-polar and semi-polar compounds are directed towards the organic phase. Therefore, the aqueous phase includes organic compounds with high polarity (Shahiri Tabarestani et al. 2016b). Given that the column used in this research was of a non-polar type, the low identification of compounds using n-butanol solvent can be explained. Other researchers have also demonstrated that most identified volatile organic compounds are lipophilic, resulting in low solubility in water (Lee et al. 2016, Martnez-Padrn et al. 2018, Shahiri Tabarestani et al. 2016a, Siddiquee et al. 2012). Additionally, we observed that SMs with a molecular weight range of 100–200 g/mol were the most abundant, with the majority identified by n-hexane solvent. Compounds with molecular weights between 200 and 300 g/mol and those between 0 and 100 g/mol, were next in frequency, accounting for 21.23% and 5.37%, respectively. Other identified SMs with different molecular weights constituted the remaining 6.72% of the compounds. Similarly, recent studies have reported the identification of the majority of SMs produced by *T. atroviride* within a molecular weight range between 100 and 300 g/mol (McMullin et al. 2017, Víglaš & Olejníková 2019). Martínez-Medina et al. (2014) and Lee et al. (2016) have indicated that the bio-potential of a native *Trichoderma* species is influenced by various abiotic factors such as average temperature, soil type, and humidity, underscoring the importance of the collection site for a *Trichoderma* isolate. Recent research has documented varying numbers of compounds in different isolates of *T. atroviride*, including 21 (Keszler et al. 2000), 25 (Stoppacher et al. 2010), 41 (Martnez-Padrn et al. 2018), 53 (Saravanakumar et al. 2018), 62 (Stracquadanio et al. 2020), 74 (Polizzi et al. 2011), 141 (Lee et al. 2016), and 278 (Siddiquee et al. 2012) compounds reported to date. Therefore, to the best of our knowledge, this study is the first to

report 372 SMs from a native *T. atroviride* isolate obtained from cucurbit rhizosphere fields in Golestan, Iran (Akbari Oghaz et al. 2024, Akbari et al. 2019).

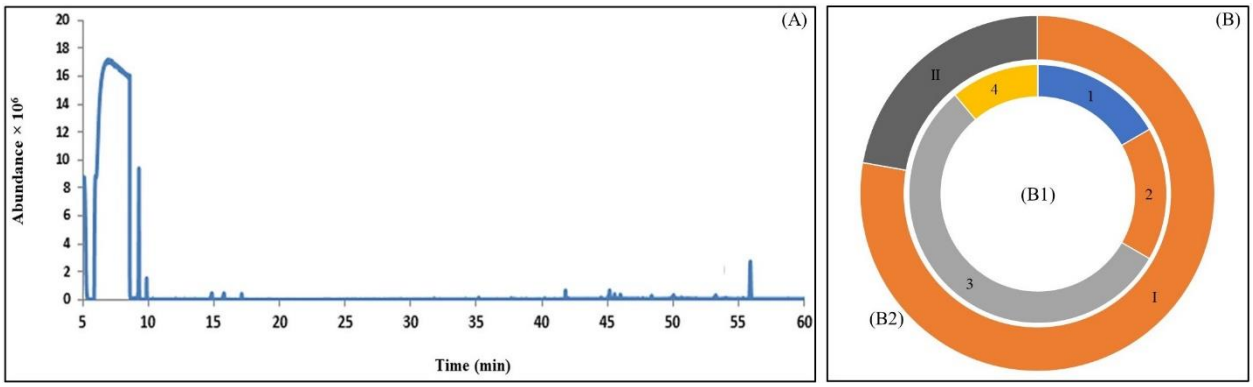

Fig. 4 – The compounds identified from cultured *T. atroviride* 6022 using n-butanol solvent are presented as follows: (A) Chromatograph illustrating the identified compounds through a capillary column with nonpolar stationary phases. (B) Doughnut charts portraying the diversity of the various identified compounds. The inner chart (B1) illustrates the diversity based on molecular weight categories [1: 0-100 g/mol; 2: 100-200 g/mol; 3: 200-300 g/mol]. The outer chart (B2) depicts the diversity based on molecular structure categories [I: Organo-Oxygen compounds; II: Organic compounds with two elements].

Biocontrol capability and antifungal compounds

Our research demonstrated that the use of FLMT could inhibit the growth of *R. solani* and *F. graminearum* under in vitro conditions. In line with our findings, Yörük et al. (2022) and Cabrera et al. (2020) reported the effective antagonistic properties of *T. atroviride* against *F. graminearum*. Additionally, El-Benawy et al. (2020) showed the promising biocontrol potential of *T. atroviride* against *R. solani*. Consistent with previous studies, we attribute the success of this biocontrol property to the secondary compounds produced by *T. atroviride* (Cabrera et al. 2020, El-Benawy et al. 2020). Among the identified organo-oxygen compounds in this research, 6-pentyl-2H-pyran-2-one and 5-hydroxy-2-decenoic acid were identified using both n-hexane and methanol solvents. These compounds belong to the class of pyrones, which have been used as flavoring agents and have been reported for their antifungal and plant growth-promoting activities (Vinale et al. 2008). Consistent with our results, pyrones have been identified in *T. atroviride* IMI206040 (Khan & Javaid 2020, Khan et al. 2020). The antifungal activity of pyrones from *Trichoderma* spp. has been reported against *C. albicans*, *Penicillium* spp., *C. neoformans*, *A. fumigatus*, *Armillaria mellea*, *Phytophthora* spp., and *Botrytis* spp. (Hill et al. 1995, Parker et al. 1997, Tarus et al. 2003, Vinale et al. 2008). This study identified peptaibolin ($C_{31}H_{51}N_5O_6$) among SMs. Peptaibolin belongs to the peptaibols classification. These are linear peptides consisting of α,α-dialkylated amino acids, isovaline, α-amino isobutyric acid (Aib), an acetylated N-terminus, and a C-terminal amino alcohol. Peptaibols have antimicrobial and resistance-inducing properties on plants against environmental stresses (Daniel & Rodrigues Filho 2007). Peptaibols have been reported from *T. viride*, *T. koningii*, *T. pseudokoningii*, *T. harzianum* and *T. virens* with high antifungal properties against *R. solani*, *F. oxysporum*, *V. dahlia* and *B. cinerea* (Khan et al. 2020). However, to the best of our knowledge, there is no existing report on the identification of peptaibols from *T. atroviride*. We identified gliotoxin and gliovirin using n-hexane and methanol solvents, respectively. These two compounds are classified as epipolythiodioxopiperazines (ETPs), which are characterized by a diketopiperazine ring derived from a peptide, and they possess high reactive potential (Jiang & Guo 2011). Gliotoxin has been previously isolated and identified from *T. lignorum*, *T. viride*, and *T. hamatum* (Brian 1944, Hussain et al. 1975, Weindling & Emerson 1936). Notably, gliotoxins play significant roles in the biocontrol of

various pathogens, including *R. bataticola*, *Macrophomina phaseolina*, *P. deharyanum*, *P. aphanidermatum*, *S. rolfsii*, and *R. solani* (Singh et al. 2005). Another member of ETPs, gliovirin, is primarily produced by *T. virens* and *T. longibrachiatum* (Nakano et al. 1990, Stipanovic & Howell 1982), contributing to the biocontrol of *R. solani* (Nakano et al. 1990) and *P. ultimum* (Howell & Stipanovic 1983). To date, no reports exist on the identification of ETPs from isolates of *T. atroviride*, further underscoring the novelty of our findings.

Among the antifungal metabolites identified in this study, 10-hydroxy-2-decenoic acid (10-HDA), a compound derived from royal jelly, exhibited notable antifungal properties. Its efficacy has been particularly demonstrated against *Staphylococcus aureus*, where it not only inhibited biofilm formation but also significantly reduced the viability of the pathogen (Gao et al. 2022). Its broad antimicrobial spectrum suggests potential as a natural treatment for various infections (Yang et al. 2018). Similarly, benzophenone and its derivatives have shown notable antifungal efficacy, especially a benzophenone-rich extract from Brazilian red propolis, which effectively targeted non-albicans *Candida* species with MIC values between 0.654 and 2.617 µg/mL (Fasolo et al. 2020). This extract's ability to cause significant cell damage positions benzophenone as a candidate for treating mucocutaneous fungal infections (Al-Ghorbani et al. 2013). Moreover, bis(2-ethylhexyl) phthalate (DEHP), isolated from *T. atroviride*, exhibited antifungal activity against various pathogens, including *C. albicans*, emphasizing its potential as a natural bioactive compound (Lotfy et al. 2018, Lotfy et al. 2018, Madani Gargari et al. 2021). Cyclopentadecane derivatives, such as mangromicins A and B from *Lechevalieria aerocolonigenes*, have shown promise as antifungal agents, demonstrating antitrypanosomal activity while maintaining low cytotoxicity (Nakashima et al. 2014). Dibutyl Phthalate (DBP) has also demonstrated substantial antifungal activity against *R. solani* and *C. albicans* (Ahsan et al. 2017, Roy et al. 2006, Shafeian et al. 2022). Eicosane, derived from *Streptomyces* species, effectively inhibits *F. oxysporum* and *C. albicans* (Beema Shafreen et al. 2022, Bhat et al. 2024), with observed morphological changes in fungi, potentially indicating its antifungal mechanism. Heneicosane, sourced from *Plumbago zeylanica*, has shown significant activity against *Aspergillus fumigatus* (Vanitha et al. 2020). Hexadecanoic acid, Methyl Ester from *Annona muricata* leaves, displayed variable antifungal effects among different pathogens, indicating potential for herbal-based antifungal treatment (Abubacker & Deepalakshmi 2013).

Indane derivatives and naphthalene derivatives have also exhibited strong antifungal activities (Erdoğan et al. 2023, Waheed & Mustafaa 2022, Zheng et al. 2023). Naphtho[2,1-b]thiophene derivatives and nerolidol have shown promising activities, with the latter enhancing the efficacy of traditional antifungals like fluconazole (Fonseca Bezerra et al. 2020). Additionally, phenanthrene and its derivatives have demonstrated significant antifungal activities against various pathogens (Faidallah et al. 2013, Zhao et al. 2018). Styrene and tetradecane have also exhibited antifungal properties, highlighting their potential roles in developing antifungal materials and biocontrol agents (Abbasova 2022, Pavirhra & Lalitha 2020). These antifungal metabolites have been identified and reported from different *Trichoderma* species. For instance, bis(2-ethylhexyl) phthalate has been isolated from *T. atroviride* (Madani Gargari et al. 2021), *T. viride* (Nakamiya et al. 2005, Narwade et al. 2024), and *T. harzianum* (Shahiri Tabarestani et al. 2016a, Shahiri Tabarestani et al. 2016b). Similarly, dibutyl phthalate was reported from *T. harzianum* (Shahiri Tabarestani et al. 2016, Shahiri Tabarestani et al. 2016), while eicosane was found in *Trichoderma* sp. (Hadibarata et al. 2007). Hexadecanoic acid, methyl ester has been documented in *T. pseudokoningii* (Khan et al. 2020) and *T. hamatum* (Oviya et al. 2022a, Oviya et al. 2022b). Additionally, naphthalene has been observed in *T. viride* (Mohammed et al. 2015), *T. asperellum* (Zafra & Cortés-Espinosa 2015, Zafra et al. 2015), and *T. harzianum* (Yu et al. 2021), while nerolidol was identified in *T. atroviride* (Wang et al. 2024) and *T. brevicompactum* (Shi et al. 2021). Phenanthrene was found in *T. asperellum* (Zafra et al. 2015), and styrene in *T. reesei* (de Oliveira et al. 2016). The compound tetradecane was reported from *T. harzianum*, *T. viride* (Pavirhra & Lalitha 2020), *T. koningii* (Gajera et al. 2020), and *T. hamatum* (Oviya et al. 2022a). Prior research has identified

T. viride and *T. harzianum* as microorganisms capable of producing gliotoxin, gliovirin, and peptaibolin (Khan & Javaid 2020, Khan et al. 2020). Toluene has also been detected in *T. virens* (Tabarestani et al. 2016). However, there are no reports available on the production of 10-Hydroxy-2-decenoic acid, benzophenone, cyclopentadecane, heneicosane, indane, and naphtho[2,1-b]thiophene by *Trichoderma* species.

Conclusion

This research has demonstrated that *T. atroviride* 6022 is capable of producing 19 different antifungal metabolites, including gliotoxin, gliovirin, peptaibolin, bis(2-ethylhexyl) phthalate, dibutyl phthalate, eicosane, hexadecanoic acid, methyl ester, naphthalene, nerolidol, phenanthrene, styrene, tetradecane, toluene, 10-hydroxy-2-decenoic acid, benzophenone, cyclopentadecane, heneicosane, indane, and naphtho[2,1-b]thiophene. The literature review emphasizes the significance of the ecological niche in influencing the diversity and quantity of secondary compounds produced by *T. atroviride*. The study further establishes the crucial role of these secondary compounds in the antifungal properties exhibited by *T. atroviride* 6022 against *F. graminearum* CBS 131778 and *R. solani* AG4_S7 under *in vitro* conditions. Consequently, *T. atroviride* 6022 emerges as a promising microorganism with a bright future in the development of biological fungicides. However, the practical application of these findings in real-world agricultural settings should be explored. For instance, the potential of *T. atroviride* 6022 as a biocontrol agent in crop protection could be compared to existing antifungal treatments currently used in agriculture, such as synthetic fungicides. This would provide a clearer understanding of its advantages in terms of environmental safety, cost-effectiveness, and sustainability. Moreover, future studies should assess how the application of *T. atroviride* in field conditions compares to laboratory-based in vitro results, including its performance under various environmental stresses.

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Author Contributions

All authors contributed to the study conception and design. Software analyzing, data curation, visualization, investigation and writing the original draft were performed by Mitra Madani Gargari. Kamran Rahnama contributed to the administration of the project, supervision, validation, funding acquisition, review and editing of the original draft. Maede Shahiri Tabarestani contributed to conceptualization, optimizing the tests, methodology, reviewing and editing of the original draft. All authors reviewed and approved the final version of the manuscript.

Data Availability

The raw data supporting the findings of this study will be made available upon reasonable request to the corresponding author.

Declarations

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical Approval and Consent to Participate

This article does not contain any studies with human participants or animals performed by any of the authors. All authors approved to participate in this research work and in the manuscript.

Consent for Publication

All authors approved this manuscript to be published.

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