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Research Article

Isolation of cis-2-methylpalmitolein-9 acid from the fungus *Trichoderma asperellum Uz-A4* and its Spectroscopic analysis

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Abstract
Today, the use of natural and non-harmful substances is one of the main and a pressing issue in all industries. In this regard, most researchers are focused on extracting the necessary components from natural sources and raw materials. At the same time, the purpose of the present practical work is based on the extraction of biologically active substances from microbodies (fungi). In this exploratory study, the extraction of secondary metabolites from the strain Trichoderma asperellum Uz-A4 was performed. Fungal biomass was prepared and extracted using alcoholic extraction, and the resulting extract was fractionated by column chromatography using various solvents. In the chloroform: methanol 9:1 system step of the fractionation process, a high content of cis-2-methylpalmitolein-9 acid was formed, along with a number of secondary metabolites. For the first time, this unsaturated fatty acid was isolated from the strain of *Trichodema asperellum* Uz-A4. When the structure of this substance was isolated as a pure substance and its structure was studied by other spectroscopic assays, it turned out to be cis-2-methylpalmitoleic acid.

Keywords: Antifungal, *cis-2-methylpalmitolein-9 acid,* Extract, Fungus, Metabolism, Spectrospic*, Trichoderma asperellum*

INTRODUCTION

Palmitoleic acid is a fatty acid with good bioactivity, possessing several important properties that can be utilised in both the nutritional and pharmaceutical fields. The production of volatile organic metabolites (VOCs) is a manifestation of the action properties of Trichoderma spp. One of them is the resistance to the growth and development of pathogenic fungi or bacteria. In addition to showing an average inhibition value of 64.49% in *T. asperellum* (T4) and 61.59% in *T. harzianum* (T12), 98 volatile metabolites were identified by GC-MS analysis. For example, phenylethyl alcohol, several phenolic isomers, benzene derivatives, D-limonene, octadecanoic acid methyl ester, hexadecanoic acid, toluene, eicosamine and eicosamine-like volatiles with antifungal activity were identified (Reghmit *et al.*, 2024). Additionally, in an experiment conducted on another Trichoderma strain, two new antifungal metabolites, designated as cytosporones Y (1) and Z (2), were identified in the broth prepared from Trichoderma sp. FKI-6626. MS and NMR studies of the identified substances were carried out and both metabolites were identified. Both compounds were found to have antifungal activity against 4 species of Aspergillus, which are causative agents of aspergillosis (Azami *et al*., 2024). Fatty acids in general are important molecules that perform the main function in most organisms and play a role as signaling agents in plant-plant, plant-microbial and plant-environment interactions (Raffaele *et al*., 2009). Several studies have shown that fatty acids and

their derivatives directly inhibit the growth of plant pathogens in the rhizosphere and improve the environment of the plant rhizosphere to reduce the occurrence of diseases found in crops and improve crop growth (Davis *et al*., 1997; Liu *et al*., 2012 and Liu *et al*., 2008). Palmitic acid (PK) is a saturated long-chain fatty acid compound that is common in beans, sunflowers, and cotton seeds (Lukonge *et al*., 2007; Perez-Vich *et al*., 2016; Zhao *et al*., 2019). In addition, it is found in high levels in exudates and residues of many plant roots (Guo *et al*., 2010; Pan *et al*., 2013). Previous studies have demonstrated that PK can inhibit the growth of plant-based pathogens and promote the growth of seedlings (Abdel-Naime *et al*., 2019; Ding *et al*., 2019). This fatty acid (9-hexadecenoic acid or palmitic acid) has also been detected in *T. hamatum* filtrate (peak area-3.96%), initially identified as an indicator of antimicrobial activity (Khairillah *et al*., 2021 and Ghavam *et al*., 2021). Additionally, palmitic acid has been detected in the filtrate of T. asperellum and is utilised as a herbal feed supplement as well as in the treatment of certain diseases (Singh *et al*., 2021). *T. asperellum* culture fluids have significant cytotoxic effects against plant pathogens (Stracquadanio *et al*., 2020) and palmitelaid acid has also been identified as a biomarker of T. virens against cotton fungus disease caused by Rhizoctonia solani (Gajera *et al*., 2020), both of which are *T. sp.*is detected in the extract of. To date, 75 species of the genus *Trichoderma* have been studied genetically, and work in this regard continues with consistency (Druzhinina *et al*., 2011). Having familiarised with the above sources, the present study aimed to determine that cis-2-methylpalmitolein-9 acid, one of the secondary volatile metabolites produced by the fungus of the Trichoderma family, has an important antifungal function.

MATERIALS AND METHODS

Technology for preparing fungal extract

A strain of *Trichoderma asperellum* Uz-A4, isolated from the rhizosphere of corn roots growing in the Tashkent region of the Republic of Uzbekistan, was grown in Mandeléa agar medium (in a test tube) for 6 days and used as an ectopic material from a suspension at a concentration of 10^6-7 spores/mL.

The fungus of *T. asperellum* Uz-A4 was grown in 250 ml nutrient medium in 500 ml volume Erlenmeyer tubes under feed conditions, in a shaker at a speed of 180 cycles/min (IKA® KS 130 shakers), for 14 days at a temperature of 24-26°C, and separated from its cultural fluid biomass by filtering. The separated biomass and cultured liquid were extracted. The total weight of the extract sum was 9.25g. The resulting extraction sum was adsorbed to 400 g Silpearl silicagel. The adsorbent was placed in a Columnar chromatographic column. When columnar chromatography using a chloroform:methanol system (9:1) was performed, 7.3 g of cis -methylpalmitolein-9 acid (I) was isolated, and its structure was studied (Jurakulova *et al*., 2021; Kamolov *et al*., 2021). Chloroform:methanol 9:1 fraction with thin layer and gaseous liquid chromatography (Fig. 1)

Metabolite analysis methods

Volatile metabolites were analyzed by gas chromatography and mass spectrometry using a YL 6900 GX/MS (Young In Chromass, Korea) equipped with a DB-5MS column (30 m × 0.25 mm inner width and 0.25 µm film thickness). The initial temperature of the apparatus was 80 °C, and the heating rate was 15 °C/min to 250 °C. The holding time was 3.0 min, and helium was used as the carrier gas at a flow rate of 1.0 mL/min. The volatilization temperature was 280°C, the flow rate was 1/20,

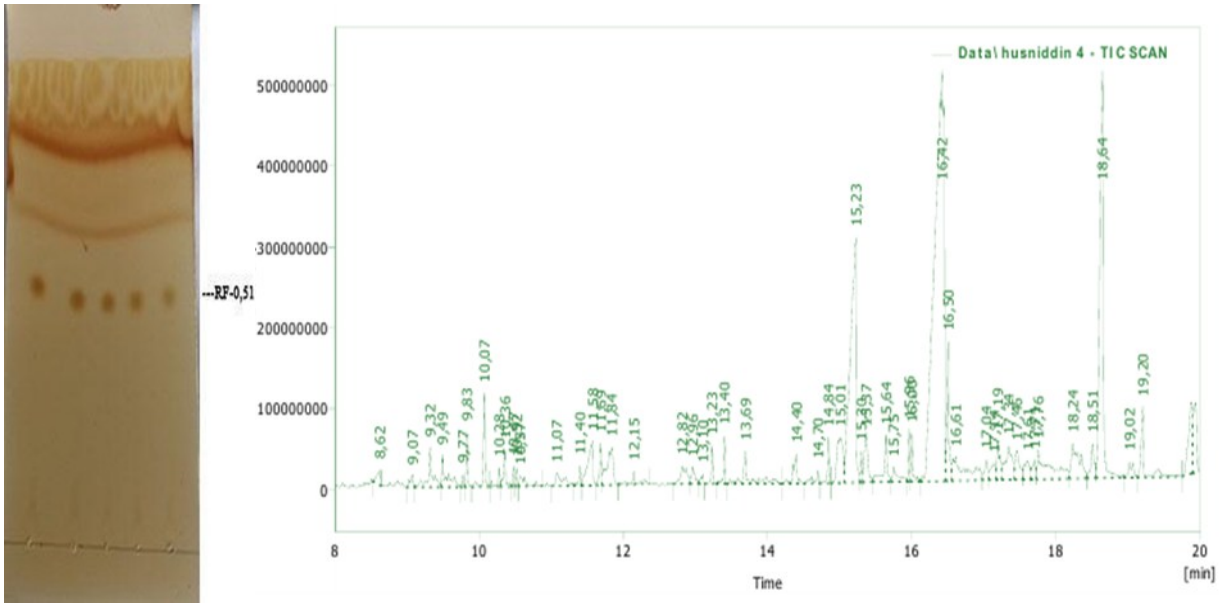

Fig. 1. *Thin layer and gaseous liquid chromatography*

and the analysis time was 17 min. Liquid samples were injected into the syringe using a 1 µl microsyringe. The transfer line temperature was 300°C, the ionization voltage was –70 eV, and the temperature of the ion generating section was 230°C. The scanning range was 30-350 a.m.u. The substances were identified by comparing their spectra with those of the available spectral data in the NIST 2017 MS database. YaMP spectra were obtained in deuteropyridine and deuterochloroform (δ, m.d., 0-TMS) in the UNITY plus 400 instrument (Kamolov *et al*., 2021).

RESULTS AND DISCUSSION

IQ spectrum of cis-2-methylpalmitolein-9 acid

The extract from biomass was obtained by fractionating a chloroform:methanol system in a 9:1 ratio, using 500 µg of the extracted metabolite I with a concentration of 500 µg/mL. An IQ spectrum in tablet form was obtained. The present study investigated metabolite I using IQ spectra (Fig. 2). It is well established that physical-chemical research methods provide valuable data in the study of organic substance structure and the composition of isolated metabolites. The absorption lines of the C-H bond bonded to the sp^2 hybrid C atom in the 3008 cm^{-1} aliphatic chain were observed in the IQ spectrum of palmitoleic acid. In the range 2919-2853 cm^{-1} absorption lines of the C-H bond bound to the sp^3 hybrid C atom were observed. In the field range, we can see absorption lines formed by the deformation oscillations of the O-H bond in the carboxyl group.

PMR spectrum of cis-2-methylpalmitolein-9 acid

The PMR spectrum of the substance is given (Fig. 3). In the PMR analysis of the substance, R-CH3 in the sp^3 hybrid methyl group C-16 knocks out 3H triplet signals at 0.880 m.h. of carbon-bound hydrogen. Multiple signals of 10 methylene-CH_2-group hydrogens can be observed at 1,255 mHz and 1,309 mHz. C-11 and C-8 emit quartet signals at 1,629 m.h. and 2,018 m.h. because they bind to the carbon-containing methylene-CH_2- group. Jump to search Hydrogen signals located in the -OH group in the carboxyl group produce a singlet signal at 5,344 m.h.

500 µg of cis-2-methylpalmitolein-9 acid was dissolved in methanol and placed in a nuclear magnetic tube. 1H-NMR and 13C-NMR analysis of the sample Bruker spectrometer (400 MHz) (Bruker, Germany) (Van Leeuwen *et al*., 2008) was performed for analysis.

GDP C^{13} spectrum of cis-2-methylpalmitolein-9 acid

YaMR C13 spectroscopic (nuclear magnetic resonance) methods were used to determine the cis-2-methylpalmitolein-9 acid obtained from this fraction (Fig. 4).

In the analysis of the substance on GDP C13, the sp^3 hybridized R-CH3 carbon signal was observed at 14,256 m.h. (million contributions), and the R-CH2-R carbon signal in the methylene group was observed at 27.279 mHz to 29.896 mHz. (14 carbon signals for the presence of 14 methylene). Specific signals were observed at 129 m.h. and 130 m.h. of the two carbons C=C. The signal was observed at 180 m.h. of R-COOH carbon, which belongs to the carboxyl group.

HMBC spectrum of cis-2-methylpalmitolein-9 acid

Our next result is the HMBC spectrum of 2-methylpalmitolein-9-acid, which shows the correlation effects of geminal and vicinal carbons and hydrogens (Fig. 5).

The spin-spin interaction of the proton of the -CH3 radi-

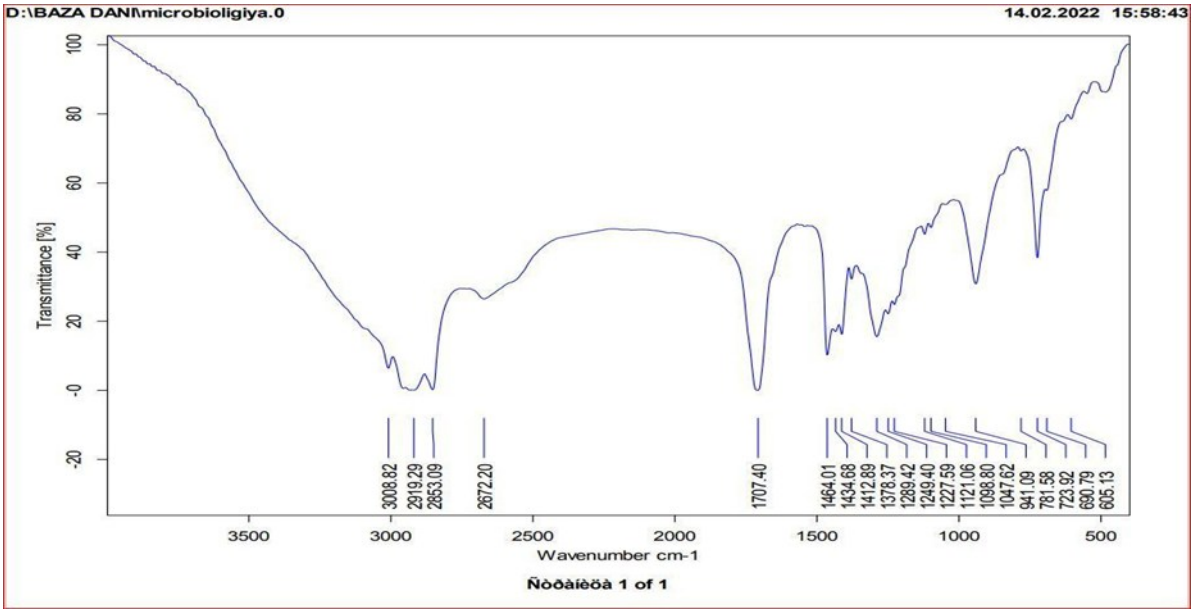

Fig. 2. *IQ spectrum of cis-2-methylpalmitolein-9 acid*

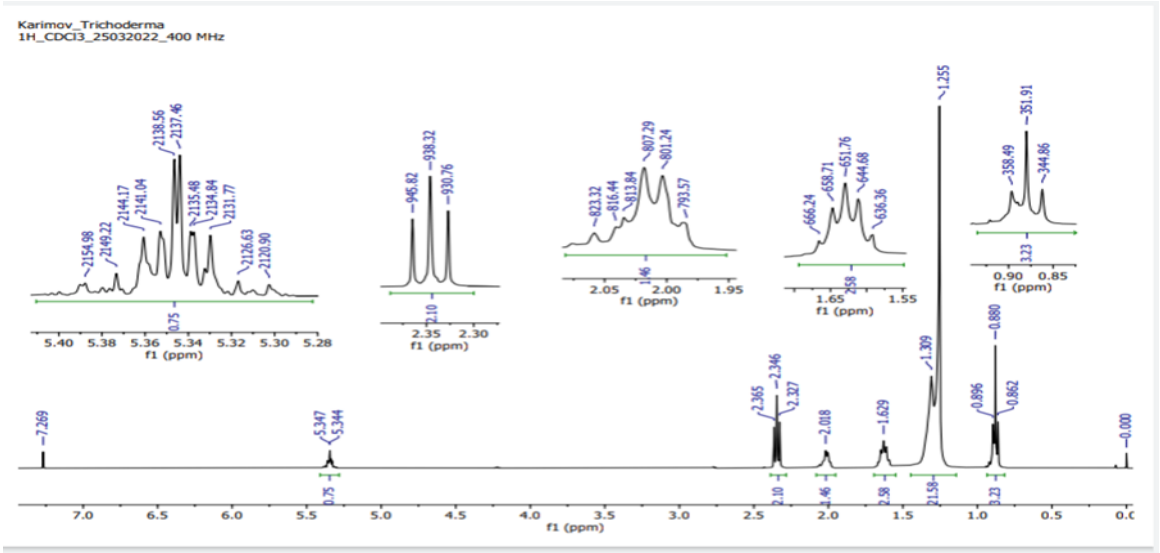

Fig. 3. *PMR spectrum of cis-2-methylpalmitolein-9 acid*

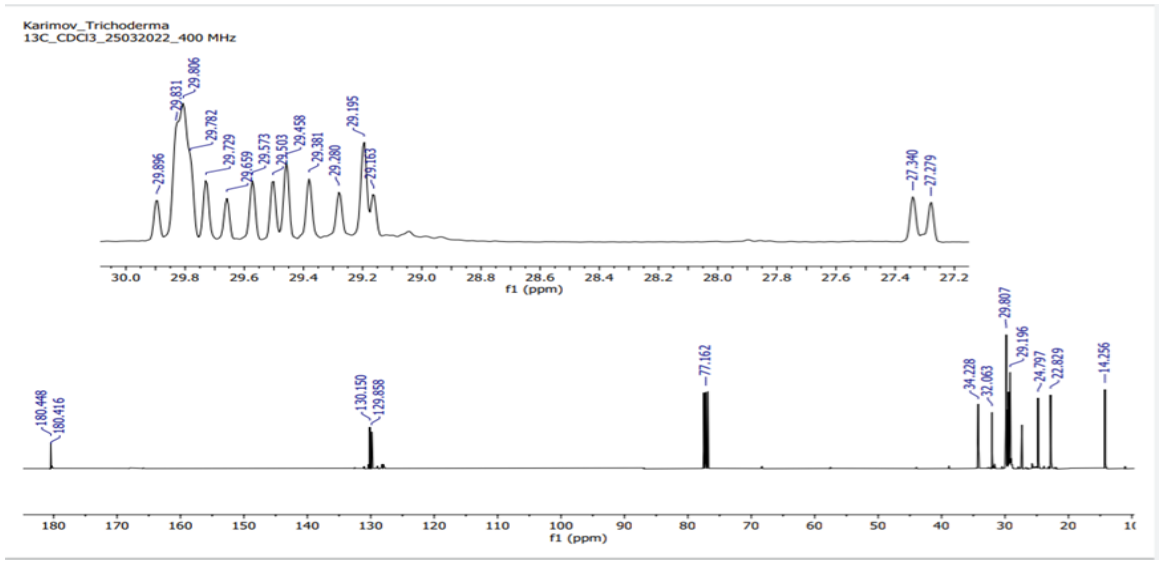

Fig.4. *GDP C^{13} spectrum of cis-2-methylpalmitolein-9 acid*

cal at 1.629 m.p. in the HMBC spectrum of 2-methylpalmitolein-9 acid with the carbonyl group at carbon C-1 indicates that it is vicinal to the carbonyl group. The interaction of the proton at carbon C-2 at 2.346 m.p. in the HMBC spectrum with the C=C carbon at 130.162 m.p. indicates that the protons are located in the double bond. The interaction of the signal at 5.347 m.p. specific to the proton in the -OH group of 2-methylpalmitolein-9 acid with the signal at 27.216 m.p. specific to the C-2 carbon indicates that the -OH group is vicinal.

The HMBC spectrum of cis-2-methylpalmitolein-9-acid is presented, which describes the correlation effects between the carbon and hydrogen atoms of the CH2 Groups in the geminal and vicinal states (Fig. 6).

HSQC spectrum of cis-2-methylpalmitolein-9 acid

Below is the HSQC spectrum of cis-2-methylpalmitolein -9-acid, which shows the correlation effects of geminal carbon and hydrogen (Fig. 7).

The spin-spin interaction of the -CH_3 radical proton, which showed a signal at 0.878 m.p. in the HSQC spectrum of 2-methylpalmitolein-9 acid, with the signal at 14.104 m.p. of the C-16 carbon indicates that it is located at the C-16 carbon. The spin-spin interaction of the -CH_3 radical proton, which showed a signal at 1.626 m.p. in the HSQC spectrum of 2-methylpalmitolein-9 acid, with the signal at 24.731 m.p. of the C-17 carbon indicates that it is located at the C-17 carbon. The interaction of the signal at 2.010 m.p. of the proton located at the C-2 carbon with the signal at 27.222 m.p. indicates that the protons are located at the C-2 carbon. The signal at 2.342 m.p., which is characteristic of the proton located in the C=C group of 2-methylpalmitolein-9 acid, with the signal at 34.196 m.p., which is characteristic of the C-8 carbon. The interaction with the signal indicates that it is located in the geminal state at the C-8 carbon.

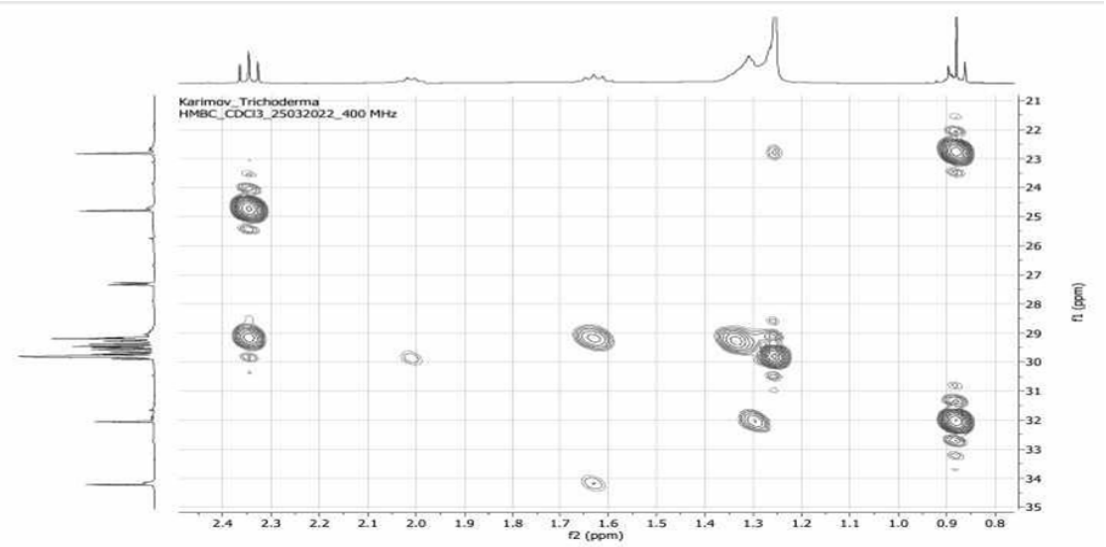

Fig. 5. *HMBC spectrum of cis-2-methylpalmitolein-9 acid*

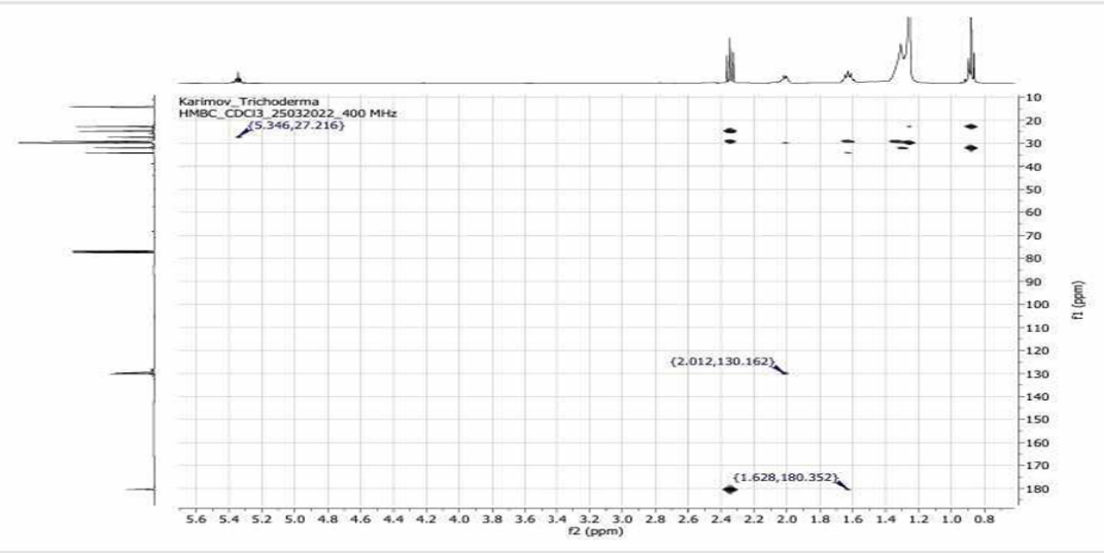

Fig. 6. *HMBC spectrum of cis-2-methylpalmitolein-9 acid*

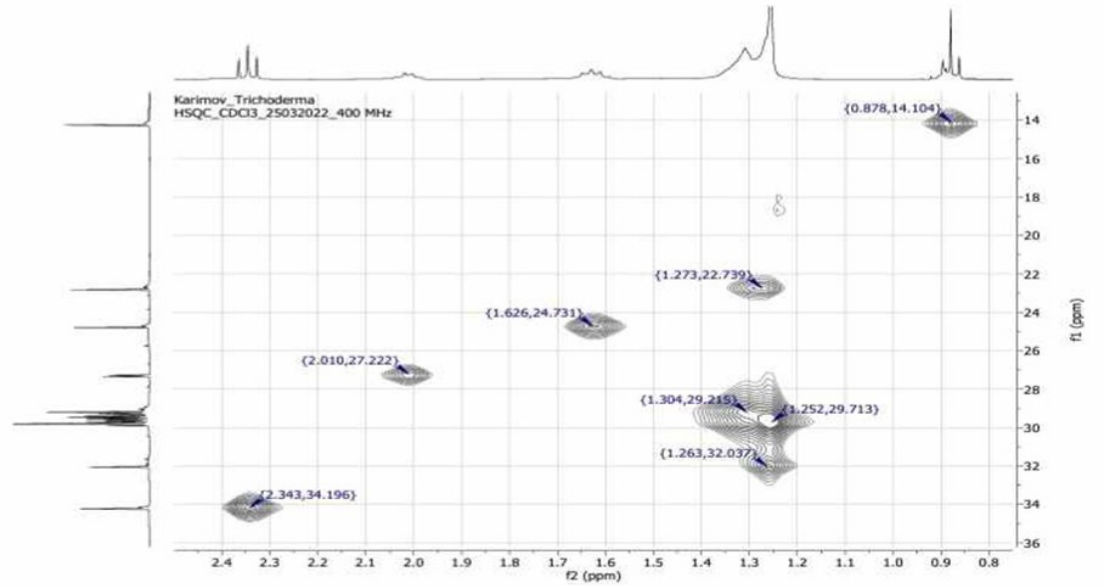

Fig. 7. *HSQC spectrum of cis-2-methylpalmitolein-9 acid*

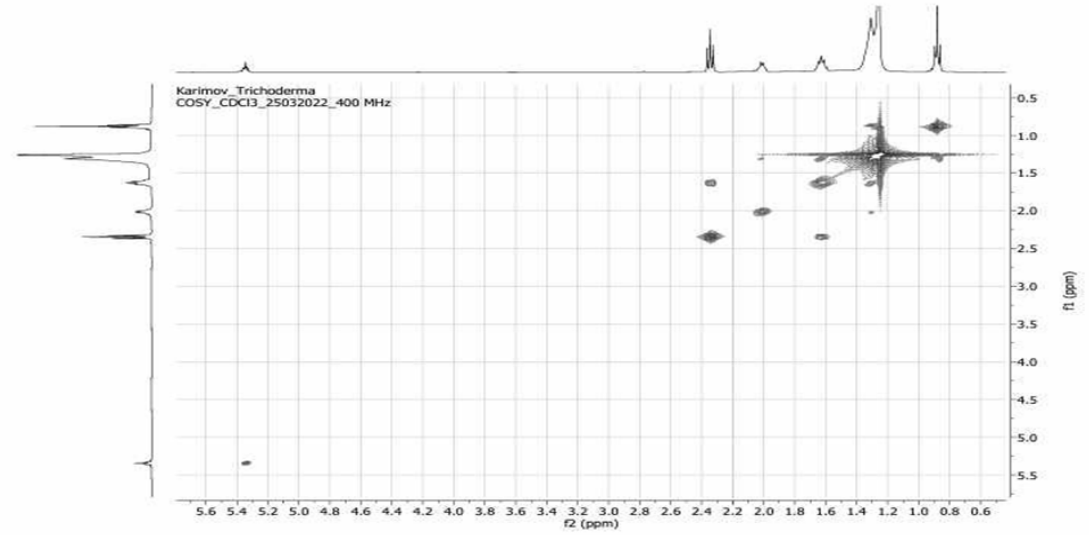

Fig. 8. *COSY spectrum of cis-2-methylpalmitolein-9 acid*

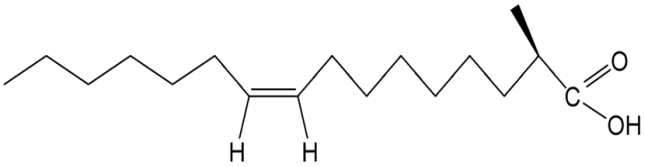

Fig. 9. *Structure of Cis-2-methylpalmitolein-9 acid*

COSY spectrum of cis-2-methylpalmitolein-9 acid

The COSY spectrum of cis-2-methylpalmitolein-9-acid is given, which describes the correlation effects of protons in the geminal and vicinal states (Fig. 8).

The interaction of the signal at 1.629 m.p. of the proton located at carbon C-17 with the signal at 2.010 m.p. of the proton located at carbon C-2 indicates that they are geminal to each other.

According to the obtained spectroscopic studies, the structural formula of cis-2-methylpalmitolein-9 acid was determined (Fig. 9).

Among the secondary metabolites isolated from the strain of *Trichoderma asperellum* Uz-A4, the unsaturated fatty acid cis-2-methylpalmitolein-9 was considered to be isolated for the first time. Scientific research in these areas has shown that various volatile compounds, including fatty acids (Zeiad *et al*., 2023; Ivan *et al*., 2023), as well as 43 other volatile compounds (Nitish *et al*., 2017). Although representatives of different classes of organic compounds have also been identified in research studies, the fact that the same unsaturated acid has not been identified indicates that this case differs from similar studies.

Conclusion

In the present study, the compound cis-2-methylpalmitolein-9 acid was isolated from the biomass of the fungal strain *T. asperellum Uz-A4* and separated in the chloroform: methanol (9:1) fraction. The structure of this substance was determined based on GLX, IR, NMR 13C and PMR, HMBC, HSQC, COSY spectroscopic analyses. The substance was the most abundant (7.3 g) in terms of quantity.

Conflict of interest

The authors declare that they have no conflict of interest.

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