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Scientific paper

Characterization of Biomolecules with Antibiotic Activity from Endophytic Fungi *Phomopsis Species*

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Abstract

Recently, growing interest is devoted to investigation of bioactive secondary metabolites of endophytic fungi. Thus, as an extension to our previous achievements related to antimicrobial potential of endophytic fungi, *Phomopsis species* isolated from conifer needles was selected as appropriately promising natural source for drug discovery. Its dichloromethane and ethanol extracts considerably inhibited growth of *Escherichia coli* and *Staphylococcus aureus*. Moreover, the individual compounds of dichloromethane extract have been separated, collected and purified using semi preparative liquid chromatographic analysis and comprehensively characterized using mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR). Based on their antimicrobial activity and unique structural characteristics in comparison with well-established drugs from the same therapeutic category, two dominant compounds (Z)-(Z)-2-acetoxyprop-1-en-1-yl-3-(3-((E)-3,4-dihydroxypent-1-en-1-yl)oxiran-2-yl)acrylate (denoted as 325-3) and (Z)-(Z)-2-acetoxyprop-1-en-1-yl 3-(3-((E)-4-hydroxy-3-oxopent-1-en-1-yl)oxiran-2-yl)acrylate (denoted as 325-5) were recognized as valuable leading structures for future discovery of novel antibiotics.

Keywords: Endophytic fungi; antibacterial activity; HPLC; GC-MS; NMR

1. Introduction

During evolution, plants have developed certain defending capabilities such as producing specific secondary metabolites in order to repel microorganisms, insects, or other animals that are feeding on them.[1] Moreover, plant tissues contain many types of microorganisms, which are referred to as endophytes.[2–4] During certain period of their life, endophytes colonize living internal tissues of their plant hosts without causing any symptoms.[2] Usually, an endophyte is specific for each host and is characteristic for conditions and geographical area in which its host develops.[5,6] Endophytic organisms seem to have mutualistic relation to their plant host by means that they preserve hosts from threats of pathogenic microorganisms, insects or other animals in return for their nutrition.[7,8] In order to facilitate the survival of a host plant, endophytes help host plant to overcome the invasion of pathogenic microorganisms by producing secondary metabolites.[9] There is also evidence that endophytes produce metabolites for plant growth regulation, productivity and phytoremediation.[10,11]

The literature search pointed out significant antibacterial and antimycotic activity of endophytic fungi *Phomopsis species*.[12–16] For example, secondary metabolites from fungus that was isolated from the leaves of tropical fruit tree *Garcinia dulcis* demonstrated activity on *Mycobacterium tuberculosis*.[17] Additionally, fungus isolated from marine-derived mangrove showed considerable potential for inhibition of *Candida albicans* and *Fusarium oxysporum*.[18] It is concluded that the fungus is susceptible to production of wide variety of secondary metabolites

and this fact is closely dependent on factors like type of plant host, plant organ, eventual cohabitance with other strains of microorganisms, climatic conditions, sessional fluctuations, habitat microclimate, temperature, etc.[5,19–21] Rakshith et al. state that the genus *Phomopsis* has been known to be a rich source of bioactive secondary metabolites of novel, diverse structure and function such as Phomopscichalasin, cytochalasin, convolvulanic acid, and isobenzofuranones, oblongolide, phomopsolide, Phomodiol, Phomoxanthones, and Xanthones dimer, phomoenamide, phomonitroester, deacetylphomoxanthone B, dicerandrol A, (1S,2S,4S)-p-menthane-1,2,4-triol, uridine, ethyl 2,4- dihydroxy-5,6-dimethylbenzoate and Phomopscilactone.[22] Those structures have been further tested and showed antibacterial, antifungal and antialgal activity.[23]

Thus, the main aim of this study was investigation of endophyte *Phomopsis species* isolated from conifer needles in Slovenia and its secondary metabolites responsible for the antimicrobial effect on selected representatives of G^- (*Escherichia coli*) and G^+ (*Staphylococcus aureus*) bacteria.

2. Experimental

2. 1. Chemicals and Reagents

For the purpose of executing microbiological tests, endophyte or bacterial sawing and endophyte extract preparation we have used: Potato dextrose agar – PDA, quality level 200, Sigma-Aldrich (Taufkirchen, Germany), LB broth, quality level 200, Sigma-Aldrich (Taufkirchen, Germany), Mueller-Hinton agar, quality level 100, Sigma-Aldrich (Taufkirchen, Germany), Dichloromethane, ≥ 99.0% purity, Sigma-Aldrich (Taufkirchen, Germany), Ethanol, ≥ 99.9% purity, Sigma-Aldrich (Taufkirchen, Germany), Methanol, ≥ 99.9% purity, Sigma-Aldrich (Taufkirchen, Germany) and Ampicillin USP standard substance, 99.3% purity, Sigma-Aldrich (Taufkirchen, Germany).

Furthermore, for the purpose of conducting all chromatographic analysis, GC-MS and NMR we have used: Acetonitrile, 99.99% purity, Sigma-Aldrich (Taufkirchen, Germany), Formic acid solution in water. 0.1% (v/v), Sigma-Aldrich (Taufkirchen, Germany), Helium gas, 99.999% purity, Sigma-Aldrich (Taufkirchen, Germany), Deuterochloroform, 99.9% purity, Sigma-Aldrich (Taufkirchen, Germany), Tetramethylsilane as the internal standard, > 99.99% purity, Sigma-Aldrich (Taufkirchen, Germany), DMSO, ≥ 99% purity, Sigma-Aldrich (Taufkirchen, Germany), Deuterochloroform, 99.9% purity, Sigma-Aldrich (Taufkirchen, Germany).

2. 2. Preparation of Endophytic Material

Endophytic fungi *Phomopsis species*, which is isolated from conifer needles, has been provided by the Department of Wood Science and Technology within Biotechnical Faculty, University in Ljubljana, Slovenia. Potato dextrose agar – PDA was used for fungal cultivation. PDA was prepared by dissolving 8.4 g of dry mixture in 200 mL of ultra-pure water, poured in Petri plates and left to solidify in sterile conditions. Afterwards, fungal sample was sawed on PDA plates and left to grow on room temperature (approximately 25 °C) for one month and then plate content was grinded and mixed using homogenizer Ultra Turrax (Ika, Staufen, Germany).

Two types of extraction solvents were tested. In accordance with previous literature records, dichloromethane and ethanol were selected due to their differences in polarity and ability to extract potential secondary metabolites.[24] Therefore, homogenization of fungal material was followed with the addition of 50 mL of dichloromethane and absolute ethanol separately and thorough mixing. Moreover, the mixtures were ultrasonicated for 15 minutes covered with thin foil and then left overnight at room temperature for extraction process to take place. Then, the mixtures were filtered through filter paper and solvent was removed using rotavapor set at 50 °C while the pressure was 700 mbar. Moreover, dichloromethane solution evaporated fully out of extract under reduced pressure, while ethanol extract sample had to be additionally left overnight on room temperature so that the rest of ethanol and remaining water could evaporate freely. Afterwards, both dry extracts were weighed and dissolved in approximately 5 mL of mixture of methanol and ultra-pure water (50:50, v/v) so that concentration of both extracts was approximately 8 mg/mL. This step was enhanced using ultrasonication for approximately 10 minutes. Afterwards, both samples were filtered through 0.22 μm nylon membranes (Agilent Technologies, Santa Clara, USA).

2. 3. Microdilution Assay

In order to determine antibiotic activity of prepared extracts from endophyte *Phomopsis species* that was isolated from conifer needles, cultures of *Escherichia coli* (strain DH5-α) and *Staphylococcus aureus* (strain ACTC 10788) obtained from permanent cultures, were used. LB broth for bacterial cultivation was prepared by dissolving 8 g of dry mixture in 400 mL of ultra-pure water. Bacterial suspensions were then prepared in a way that 20 mL of LB medium was added to the centrifuge container after which *E. coli* or *S. aureus* were seeded separately from the permanent culture. The centrifuge container was left over night and shaked in an incubator at 37 °C in order to multiply the bacteria.

Next, 2 mg of dichloromethane and 2 mg of ethanol dry extracts of the endophyte were dissolved separately in 1 mL of mixture of methanol and ultra-pure water (50:50, v/v) and used for initial antimicrobial activity testing within microdilution tests. Prior performing microdilution tests dissolved endophyte extract in mixture of methanol and ultra-pure water (50:50, v/v) was additionally concen-

trated using Speed Vac Plus (Savant, Waltham, MA, USA) to desired volume, required for microdilution assays.

Bacterial suspensions were diluted in LB broth in order to set the suitable optical density between 0.08 and 0.10 of the mixture by measuring the absorbance of a mixture at λ = 600 nm.[25,26] Assay was carried out on a microwell plate by executing tests in duplicates for extract activity as well as for positive and negative control. Activity of extract was tested in a mixture of 90 μL of *E. coli* or *S. aureus* suspension solution in LB broth and 10 μL of extract obtained as described above. Negative control was prepared using 90 μL of *E. coli* or *S. aureus* suspension solution in LB broth and 10 μL of mixture of methanol and ultra-pure water (50:50, v/v). As positive control, 90 μL *E. coli* or *S. aureus* suspension solution in LB broth was mixed with 1 μL of 100 mg/mL solution of ampicillin that was dissolved in 9 μL of 50% (v/v) solution of methanol in water. Plates were analysed instantly at absorbance of 600 nm and left overnight under incubation temperature of 37 °C for growing and to be measured also the next day in order to detect any changes in the bacterial growth. Changes in absorbance due to bacterial growth were used to calculate bacterial inhibition rate for each of the *Phomopsis species* extract types. The microdilution assay for determination of antibacterial activity was done for every prepared sample in duplicate and average result was calculated.

2. 4. Isolation and Chemical Structure Characterization of Components from Endophytic Fungal Material

Prior chromatographic and spectral analysis, 2 mg of dichloromethane dry endophyte extract was dissolved in 1 mL of methanol and ultra-pure water (50:50, v/v). Isolation and collection of components from endophyte extract was performed on Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Dreieich, Germany) equipped with DAD detector and binary pump. Separation was achieved on Hypersil Gold semi-preparative revered-phase HPLC column (C18, particle size 5 μm, length 150 mm, internal diameter 10 mm, Thermo Fisher Scientific, Dreieich, Germany) at 25 °C. Samples were maintained at 10 °C in autosampler prior analysis. Injection volume was 100 μL. A gradient elution program was composed of acetonitrile as mobile phase A and 0.1% (v/v) formic acid solution in water as mobile phase B. The gradient program started with 95% (v/v) mobile phase B. During 28 minutes, the percentage of mobile phase B was decreasing to 5% (v/v), following the return to the initial ratio in 0.05 minute. Column was then re-equilibrated, so the total run time was 40 minutes at a flow rate of 2 mL/min. Detection was performed simultaneously, by measuring the absorbance at 220 nm, 235 nm, 254 nm and 285 nm wavelength in order to assure recording of all possible components with different spectral properties.

Fraction collection procedure was performed on *Phomopsis species* dichloromethane dry extract dissolved in 50% (v/v) solution of methanol in water, using the aforementioned chromatographic method on Dionex Ultimate 3000 HPLC system with DAD detection. Five distinct peaks from the chromatogram at the retention times of approximately 12.88, 13.67, 14.40, 16.02 and 17.13 min have been manually collected in Eppendorf tubes. Solvent from separate peak fractions was removed using rotavapor set at 50 °C while the pressure was 700 mbar and dry peak residues have been used for further analytics.

Mass spectrometry (MS) analyses were done on Agilent Technologies 5975C MS system (Agilent Technologies, Santa Clara, USA) coupled with Agilent Technologies 6890N GC system (Agilent Technologies, Santa Clara, USA). Initial temperature of the oven of the GC system was 60 °C and maximum temperature was 325 °C. Front inlet of the system was set in mode to split ratio 10:1, while initial temperature was set at 200 °C, pressure was 14.47 psi and split flow was 10.0 mL/min. The method used Agilent Technologies HP-5 5% Phenyl Methyl Siloxane, Agilent 19091J-433 column, (internal diameter 0.25 mm, length 30.0 m, film thickness 0.25 mm, column format 7 inch, manufacturer Agilent Technolog Santa Clara, USA) and helium gas pumped at 1 mL/min flow rate. The fractions collected using semi-preparative HPLC analysis with the concentration of 0.1 mg/mL were introduced into GC-MS system with 1 μL injection volume and total run time was 44 minutes. The mode of ionization was electron impact operated at 70 eV. Low mass scan parameters for MS was 10, while high mass scan parameter was 550.

For acquiring nuclear magnetic resonance (NMR) spectra on Bruker Ascend 400 (400 MHz) spectrometer (Billerica, MA, USA), the fractions collected using semi-preparative HPLC analysis, were evaporated using Rotavapor R-114 (Büchi, Flawil, Switzerland) and dry content weighing about 5 mg was dissolved in 250 μL deuterochloroform. NMR spectra were recorded at 25 °C and chemical shifts were given in parts per million (δ) downfield from tetramethylsilane as the internal standard.

2. 5. Disc Diffusion Method for Determining the Antibiotic Potential

Firstly, the microbial cultures of *E. coli* and *S. Aureus* were diluted with saline solution until the density of 0.5 McFarland. Both cultures were seeded on separate Mueller-Hinton agar plates. Afterwards, 15 μL of collected peak fraction 3, 4 and 5 were added on separate places on seeded Mueller-Hinton agar plates, while 15 μL DMSO served as control on each plate. Then, plates were incubated in thermostat at 37 °C in the period from 16 to 24 h. Growth inhibition zone diameter readings are performed after incubation for each of the peak fractions.

3. Results and Discussion

3. 1. Evaluation of Endophytic Fungi Extract Antibiotic Activity

Within microdilution assay for antibiotic activity of dichloromethane extract against *E. coli* after 24 h, average absorbance value 0.4603 was for the negative control, and 0.1590 for the positive control. Dichloromethane extract of *Phomopsis species* showed absorbance value of 0.2812. Therefore, it was concluded that this extract demonstrated inhibition rate of 59.44%. The same set of microdilution test experiments were repeated using dry ethanol extract of the fungi and these also pointed out the presence of antibacterial activity. Moreover, it was observed that ethanol extract demonstrated 51.63% of inhibition rate against *E. coli*.

In order to further evaluate the relationship between extract concentration and the level of antibiotic activity on *E. coli*, a microdilution experiment was repeated using additional four lower volumetric ratios of dichloromethane extract, since better inhibition dependency was observed comparing to ethanol extract of fungus. In this experiment also negative control was used in the same volumetric ratios. Therefore, *Phomopsis species* dichloromethane extract samples were prepared so that the percentage of *Phomopsis species* dichloromethane extract solution in LB broth suspension of *E. coli* was approximately 10%, 5%, 2.5% or 1% (v/v). From the results, the concentration dependent antibiotic activity was observed. In specific, average absorbance value of 0.2812 was measured for 10% (v/v) extract, 0.3305 for 5% (v/v) extract, 0.3275 for 2.5% (v/v) extract and 0.3896 for 1% (v/v) extract. As expected, lower presence of extract or control influenced greater growth of *E. coli*. Furthermore, even the sample with the lowest ratio of *Phomopsis species* dichloromethane extract also demonstrated antibiotic effect comparing to the negative control, as can be concluded from the average absorbance value for extract (0.3896 at 1%, v/v) comparing to the average absorbance value for negative control (0.4603). Based on presented data, inhibition concentration of dichloromethane extract that eradicates 50% of bacteria (IC50) was calculated to be 6.95%.

Furthermore, the same procedure was performed once again for evaluation of antibacterial activity of endophytic fungi extracts against *S. aureus* after 24 h. It was noticed that ethanol extract has high inhibition rate of 92.80% on the growth of *S. aureus* during preliminary tests. On the other side, dichloromethane extract did not show notable antibacterial activity against *S. aureus*. Thus, we continued with microdilution assay with *Phomopsis species* ethanol extract. Samples for microdilution test were prepared so that *Phomopsis species* ethanol extract concentration in *S. aureus* final suspension was approximately 10%, 5% and 1% (v/v). From the results, the concentration dependent antibiotic activity was observed. In specific, average absorbance value of 0.1755 was measured for 10% (v/v) extract, 0.1805 for 5% (v/v) extract and 0.2010 for 1% (v/v) extract. Absorbance result for negative control was 0.4655 and for positive control 0.1530. Moreover, even sample with lowest tested dilution of 1% (v/v) of *Phomopsis species* ethanol extract has pronounced antibiotic activity comparing to both positive and negative control. Out of available data, IC50 value of *Phomopsis species* ethanol extract for *S. aureus* of 1.30% was calculated.

This data supported the hypothesis that biomolecules from investigated endophytic fungi have the potential to become candidates for new potential drugs as demonstrated bioactivity could represent valuable contribution to current antibacterial therapy with a proper chance for dealing with growing trend of bacterial resistance. Based on presented data and having in mind future industrial exploitation of prepared extracts, we have further analysed the components of *Phomopsis species* dichloromethane extract using spectral and chromatographic tools, due to its stability and facilitated dry extract preparation comparing to the ethanol one. Furthermore, based on the previous research of the group[24] we expected that more potent secondary metabolites could be extracted from the fungal material using dichloromethane as extraction solvent.

Nevertheless, *Phomopsis species* dry ethanol extract secondary metabolites could be a subject for further analytical research using proposed methods in this article, that were developed for characterization of components of *Phomopsis species* dichloromethane extract.

3. 2. Active Biomolecules Isolation and Chemical Structure Elucidation

Dichloromethane dry extract dissolved in 50% (v/v) solution of methanol in water has been further analysed using semi-preparative HPLC method. Total number of five peaks was inspected in the chromatogram at the retention times of 12.88, 13.67, 14.40, 16.02 and 17.13 min, respectively (Figure 1). The compounds related to all five peaks seemed to have relatively similar spectral properties (Figure 2), so it was possible to record all of them in a single chromatogram acquired at 235 nm detection wavelength. As expected, due to differences in chromatographic systems (e.g. the composition of mobile phase and chemistry of stationary phase, technical characteristics of used instruments) employed during sample collection and following GC-MS analysis, the previously reported retention times were different from those in recorded total ion chromatogram (Figure 3 and 8). Despite this, reliable peak-to-peak tracking was accomplished with the same elution order and all relative retention times preserved. Although there was a risk that the first two eluting peaks belong to compounds with activity that was measured for the whole extract, we deliberately omitted them in further work by means of preparative chromatography and fraction collections for chemical structure elucidation. Authors have invested effort to chromatographically resolve

all peaks for further processing. However, the purity of fractions was serious limitation as well as concentration level of a compound in a collected fraction. Due to this, background noise was too high and we could not perform accurate data analysis except for last three eluting peaks.

In line with fraction collection, we have performed disc diffusion method for determining the antibiotic potential of fractions as verification of bioactivity in order to ensure that ascribed bioactivity of whole extract could be related to all collected fractions. Tested concentration of peak fraction 3 and 4 was 1 mg/mL, while fraction of peak 5 had the concentration 3 mg/mL. The results revealed that there was no activity on any of the microorganisms tested for peak fraction 4. For peak fraction 3, a 6 mm diameter inhibition zone was observed on *S. aureus* plate, while no antimicrobial activity was observed on *E. coli* plate. Furthermore, for peak fraction 5, a 7 mm diameter of the inhibition zone was observed against *S. aureus* and 4 mm diameter of the inhibition zone was noted on *E. coli* plate. The test pointed out that only peaks with elution order 3

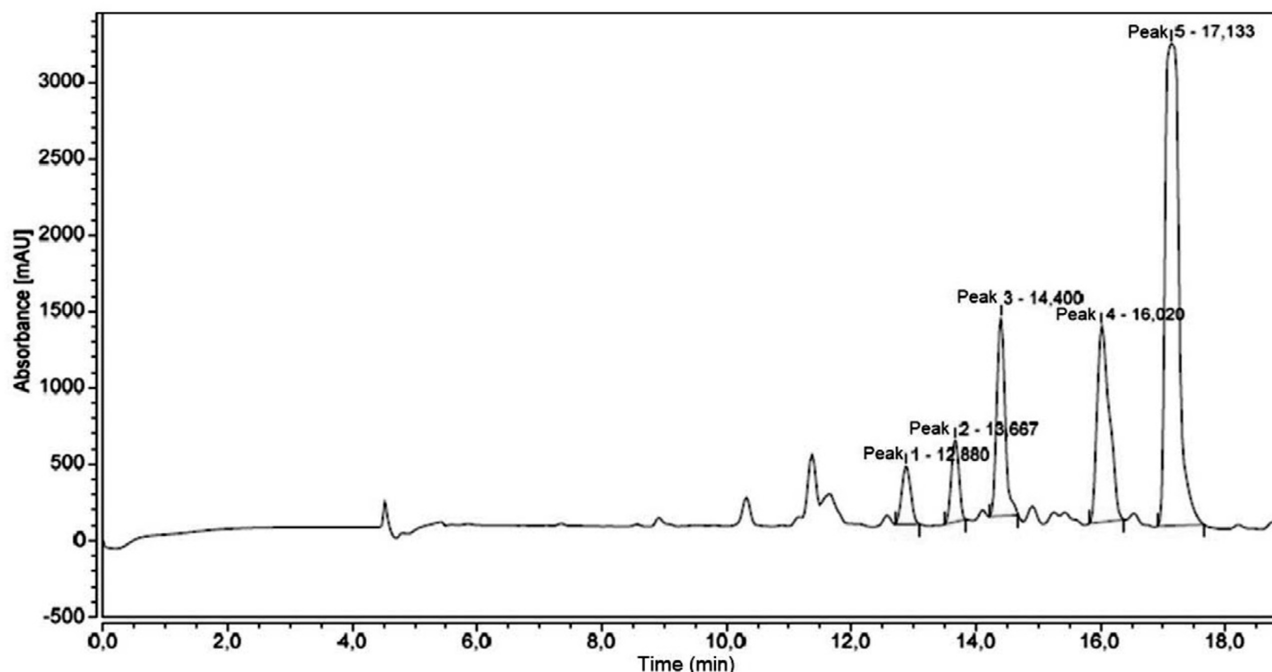

Figure 1. Chromatogram of *Phomopsis species* dichloromethane dry extract dissolved in mixture of methanol and water 50:50 (v/v) from semi-preparative HPLC analysis

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) spectral analysis of compounds 325-3 and 325-5

	Compound 325-3		**Compound 325-5**	
Position	δ_H (*J* in Hz)	δ_c	δ_H (J in Hz)	δ_c
1		162.33		161.95
2	6.24 (d, *J* = 9.7 Hz, 1H)	125.12	6.25 (d, *J* = 9.7 Hz, 1H)	124.12
3	7.00 (dd, *J* = 9.7, 5.5 Hz, 1H)	140.89	7.10 (dd, *J* = 9.7, 5.9 Hz, 1H)	141.00
4	5.38 (dd, *J* = 5.5, 3.0 Hz, 1H)	63.41	5.66 (dd, *J* = 5.8, 2.8 Hz, 1H)	63.20
5	5.10 (s, 1H)	78.50	5.98 (dd, *J* = 4.7, 2.8 Hz, 1H)	76.85
6	5.89 (dd, *J* = 16.0, 5.8 Hz, 1H)	124.73	H-6/H-7: 6.42 (d, *J* = 4.7 Hz, 2H) *	142.97
7	6.01 (dd, *J* = 15.5, 5.2 Hz, 1H)	134.72	H-6/H-7: 6.42 (d, *J* = 4.7 Hz, 2H) *	124.54
8	3.93 (s, 1H)	76.22		202.04
9	3.62 (s, 1H)	70.60	4.36 (q, *J* = 7.1 Hz, 1H)	73.14
10	1.17 (d, *J* = 6.3 Hz, 3H)	11.98	1.40 (d, *J* = 7.1 Hz, 3H)	19.59
1’	6.90 (d, *J* = 6.0 Hz, 1H)	140.89	6.86 (dd, *J* = 7.3, 1.7 Hz, 1H)	139.57
2’		127.60		127.53
3’	1.80 (s, 3H)*	18.84	1.79 (s, 3H)*	14.54
1”		166.74		166.50
2”	1.81 (s, 3H)*	14.54	1.80 (s, 3H)*	12.00
OH	C-8/C-9: 2.22 (bs, 1H); 2.16 (bs, 1H)		C-9: 3.36 (bs, 1H)	

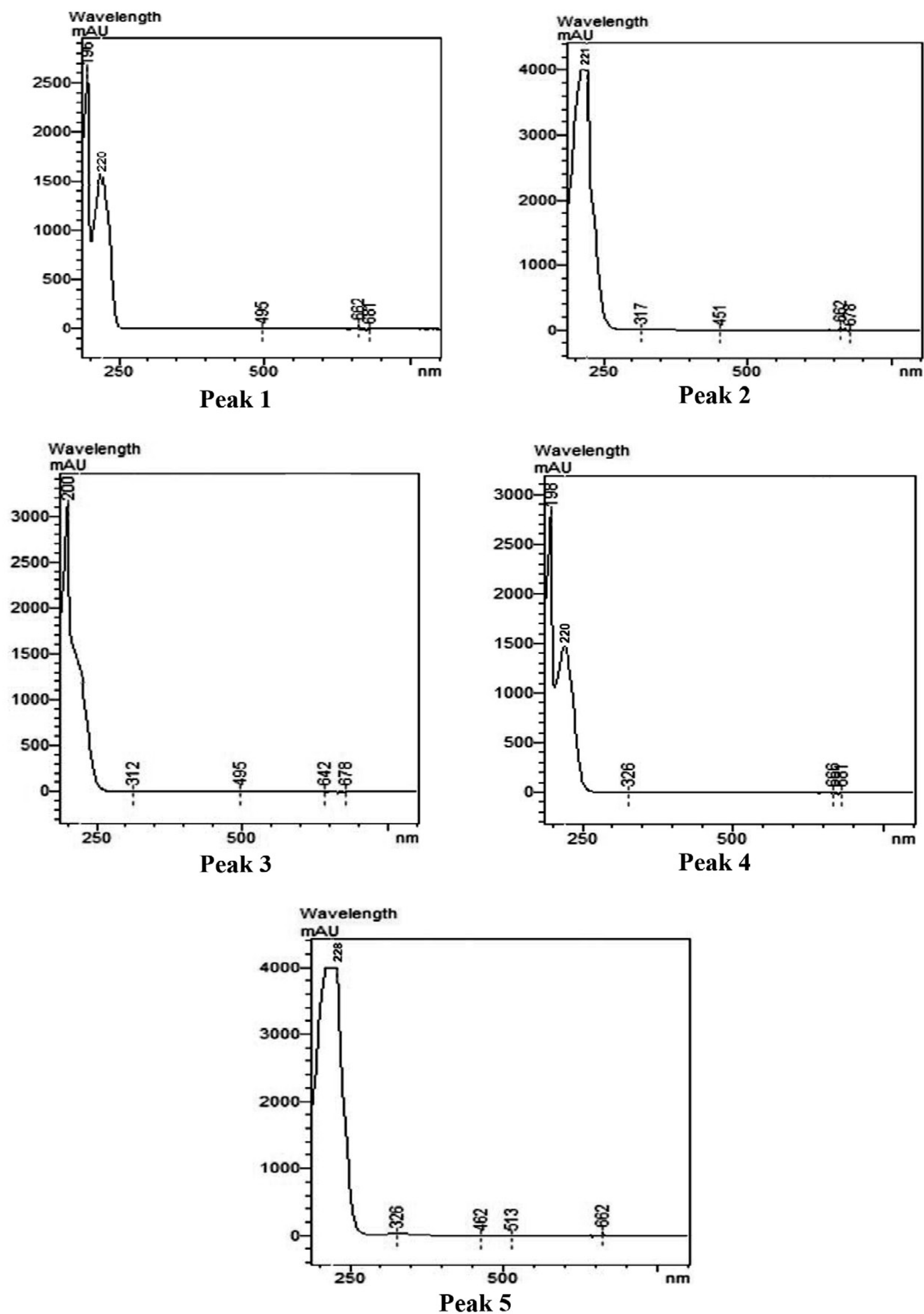

Figure 2. Absorption spectra of compounds corresponding to five peaks chromatographed using dichloromethane dry extract dissolved in methanol and water 50:50 (v/v)

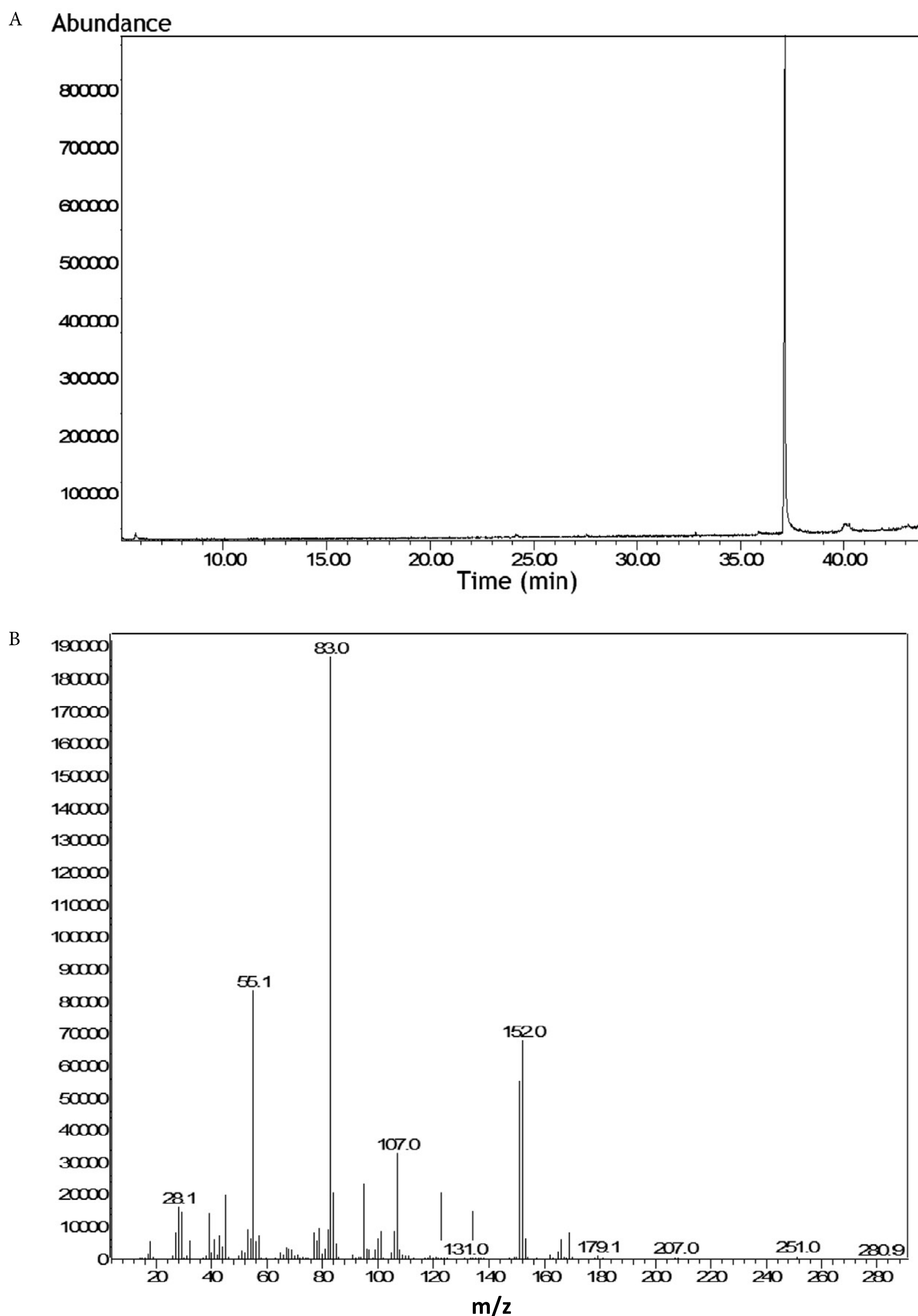

Figure 3. Total ion chromatogram (A) and GC/MS spectra (B) of compound 325-3

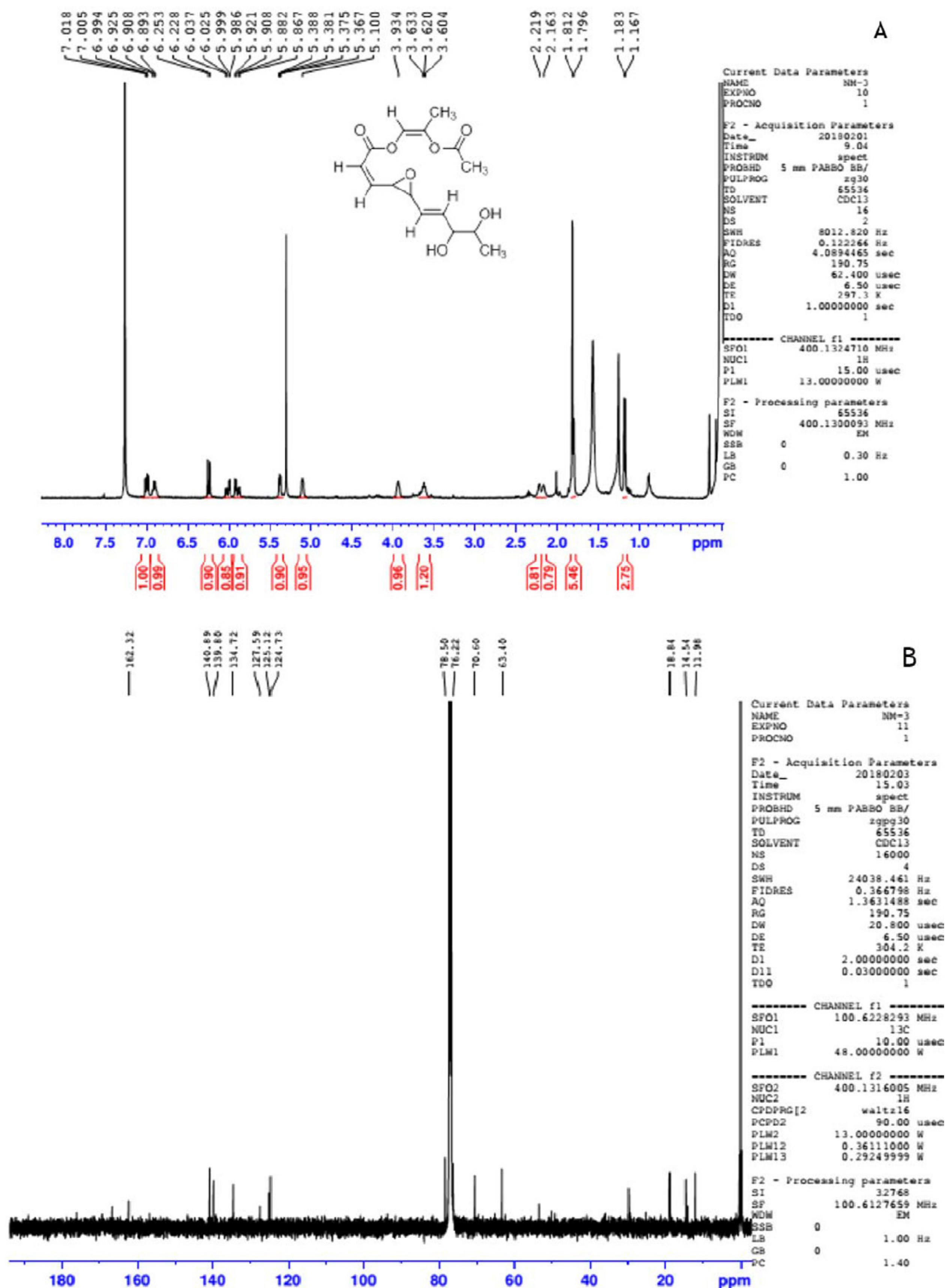

Figure 4. ^{1}H NMR spectra (A), ^{13}C NMR spectra (B) for toluene standard and compound 325-3

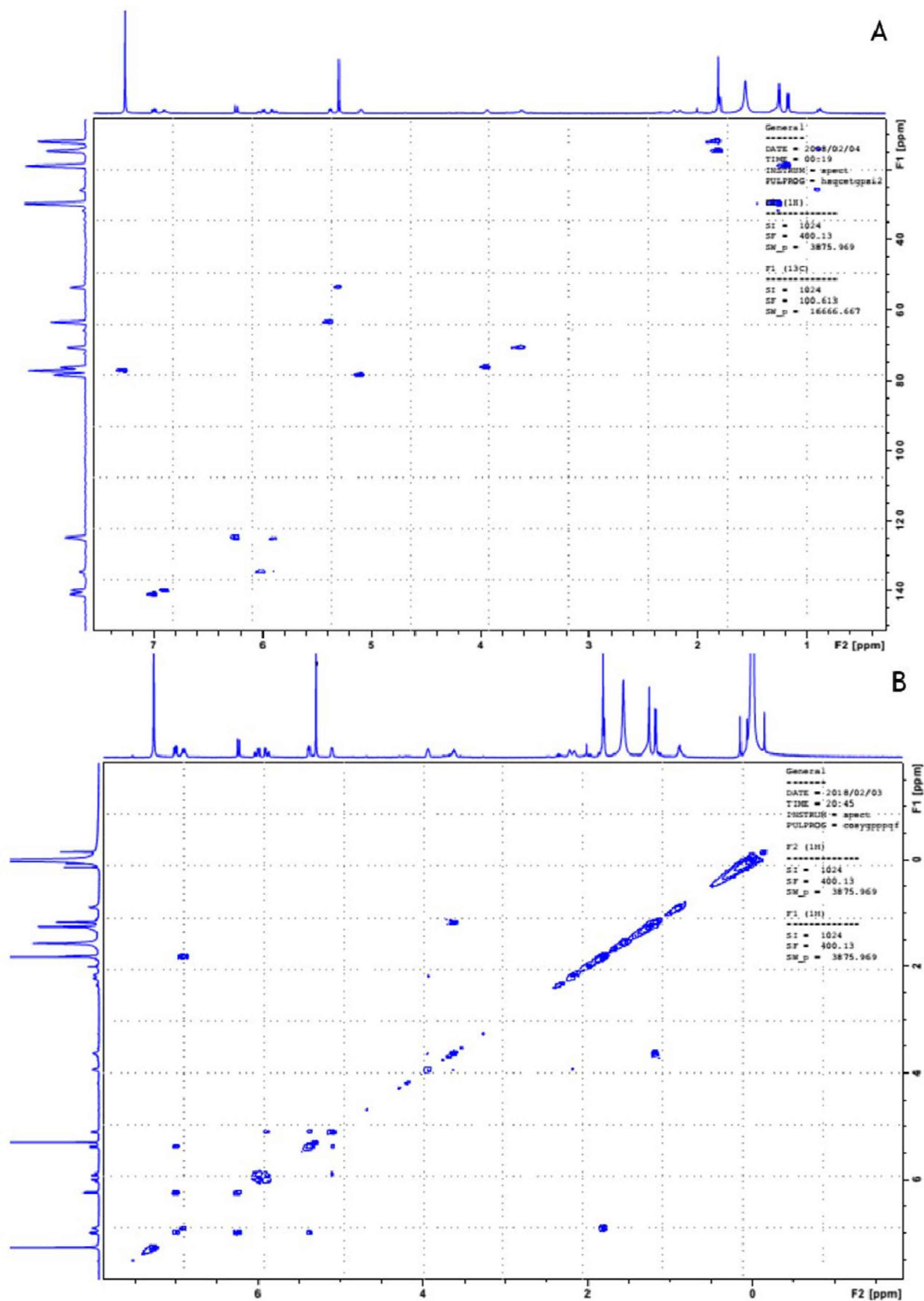

Figure 5. HSQC (A) and COSY (B) spectra of compound 325-3

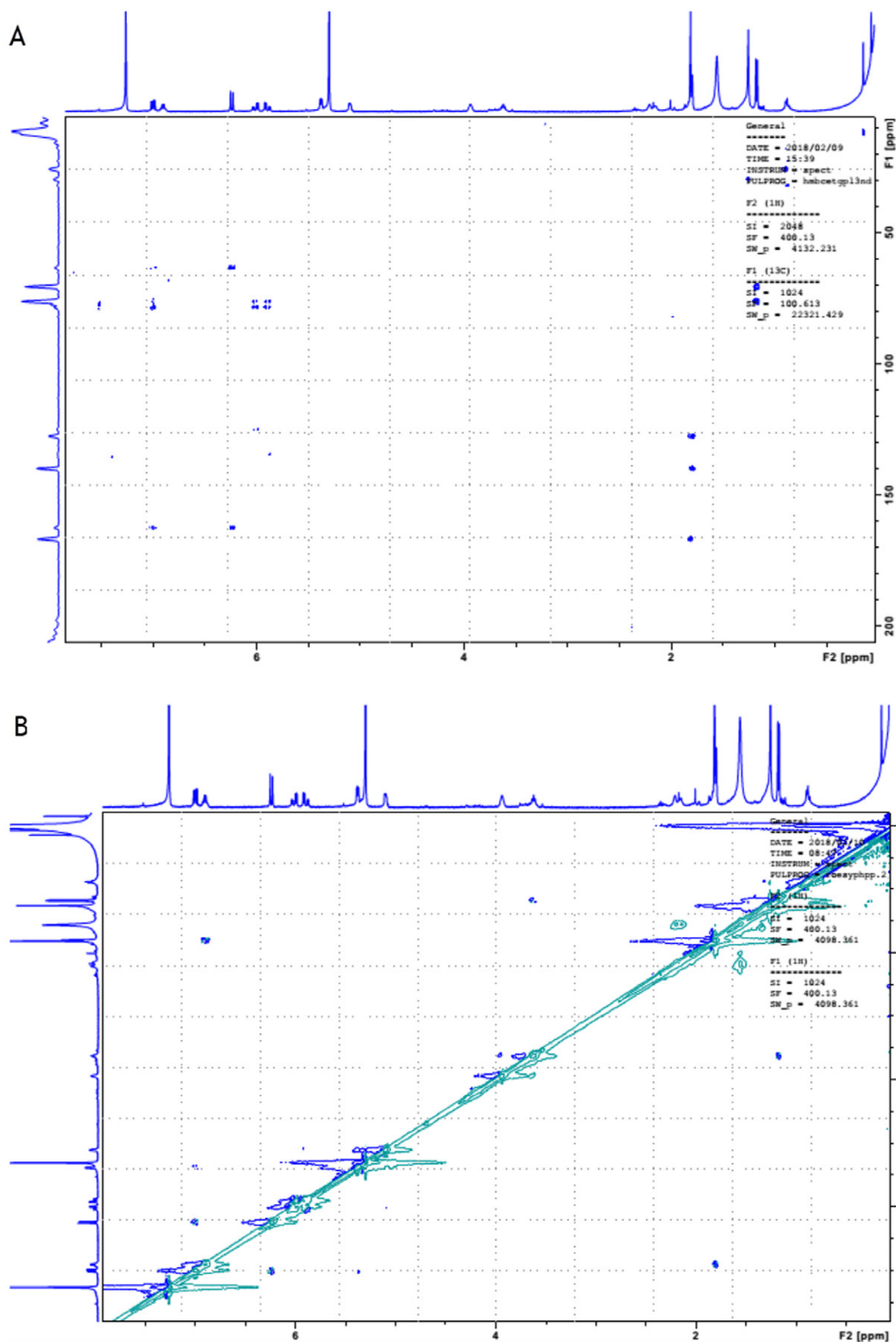

Figure 6. HMBC (A) and NOESY (B) spectra of compound 325-3

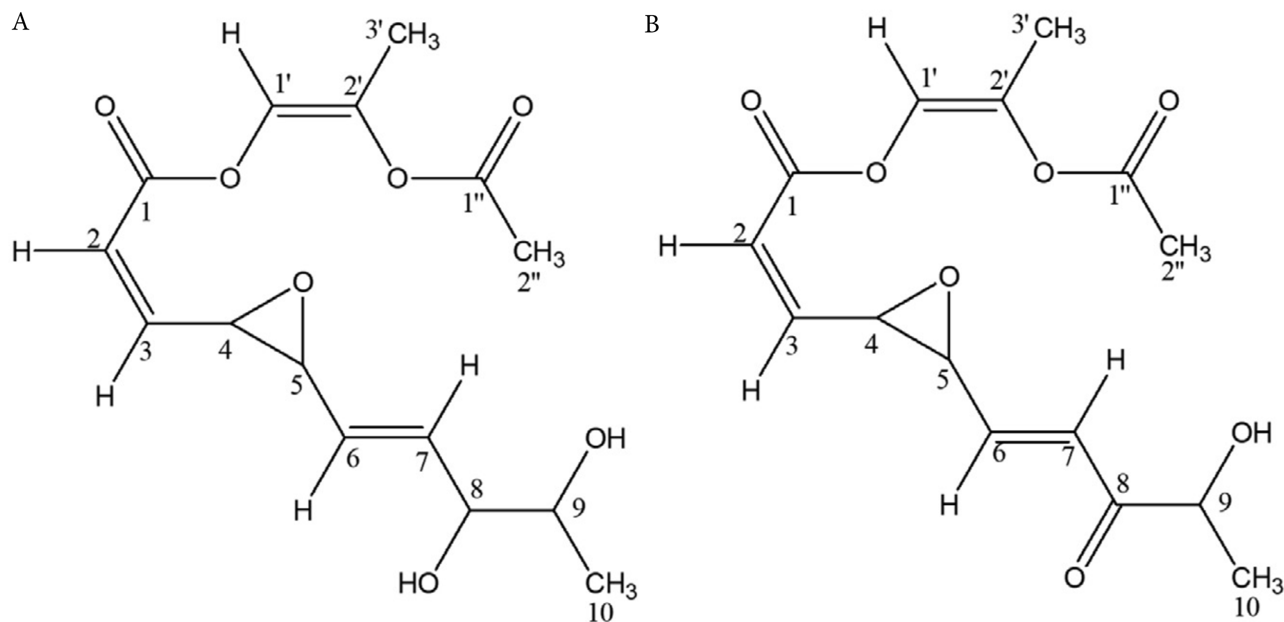

Figure 7. Molecular structures of compounds 325-3 (A) and 325-5 (B)

and 5 showed bioactivity. Therefore, after fraction collection and weighing of evaporated fractions, it has been noticed that dry mass of the collected peak fractions 3 and 5 was at least 5 mg, which was dissolved with deuterochloroform in cuvette up to volume of 250 μL. The final concentration was high enough to perform suitable NMR analysis. The compounds corresponding to peak 3 and 5 were denoted as 325-3 and 325-5 in further text, respectively.

Compound that corresponded to peak 3 in the chromatogram and denoted as 325-3, was obtained as a colourless oil with the molecular formula of $C_{15}H_{20}O_7$ (recorded by GC-MS method as ion [M+H-OH-CH_3] = 281, calc. 313.1282, Figure 3). The numbers of hydrogen and carbon atoms observed in the ^{1}H- and ^{13}C-NMR spectra recorded in $CDCl_3$ (Table 1) were in agreement with the molecular formula. The ^{1}H NMR (Figure 4A) data indicated the existence of five sp^2 methines (H-2, H-3, H-6, H-7 and H-1'), four oxygenated sp^3 methines (H-4, H-5, H-8 and H-9), two hydroxyl and three methyl groups (H-10, H-3' and H-2"). The ^{13}C NMR (Figure 4B) and HSQC spectra (Figure 5A) indicated 15 carbons, which were classified into six olefinic carbons (C-2, C-3, C-6, C-7, C-1' and C-2'), four oxygenated sp^3 methine carbons (C-4, C-5, C-8 and C-9), three sp^3 methyl carbons (C-10, C-3' and C-2") and two carbonyl carbons (C-1 and C-1"). The ^{1}H–^{1}H COSY of compound 325-3 (Figure 5B) showed all expected bonds between hydrogens on adjacent carbons from H-2 to H-10 and from H-1' to H-3'. The HMBC correlations (Figure 6A) from H-2 to C-1, H-3' to C-2' and H-2" to C-2' also proved suggested structure. The Z geometries of C-1'/C-2' and C-2/C-3 double bonds were elucidated by the NOESY cross-peaks between H-2 and H-3 and H-1′ and H-3′ (Figure 6B). Also, the absence of the NOESY cross-peak between H-6 and H-7 indicated E configuration of the C-6/C-7 double bond. Additionally, configurations of all double bonds were confirmed by magnitudes of the coupling constants in ^{1}H-NMR spectra. Thus, the structure of compound 325-3 was finally elucidated as (Z)-(Z)-2-acetoxyprop-1-en-1-yl-3-(3-((E)-3,4-dihydroxypent-1-en-1-yl)oxiran-2-yl)acrylate (Figure 7A). Geometric isomers of this compound were not detected.

Compound 325-5 was also obtained as a colourless oil, whose molecular formula was determined as $C_{15}H_{18}O_7$ (recorded by GC-MS method as ion [M+H-OH-CH3] = 279, calc. 311.1125, Figure 8). A comparison of ^{1}H spectra (Figure 9A) showed that compound 325-5 shared the same spectroscopic characteristics with compound 325-3, with one oxygenated sp^3 methine and one hydroxyl group less. In the ^{13}C NMR spectra of compound 325-5 (Figure 9B), three carbonyl carbon signals and three oxygenated sp^3 methines were detected, suggesting that compound 325-5 was oxidation product of compound 325-3. The ^{1}H–^{1}H COSY (Figure 10B) showed bonds between hydrogens on adjacent carbons from H-2 to H-7 and from H-9 to H-10 indicated carbonyl group at C-8. The HMBC correlations (Figure 11A) from H-7 to additional carbonyl group also supported this assumption. Configurations of all double bonds were found to be the same as of compound 325-3, based on the analysis of NOESY spectrum (Figure 11B) and magnitudes of the coupling constants. Based on this, the structure of compound 325-5 was determined as (Z)-(Z)-2-acetoxyprop-1-en-1-yl 3-(3-((E)-4-hydroxy-3-oxopent-1-en-1-yl)oxiran-2-yl)acrylate (Figure 7B). Geometric isomers of this compound were not detected.

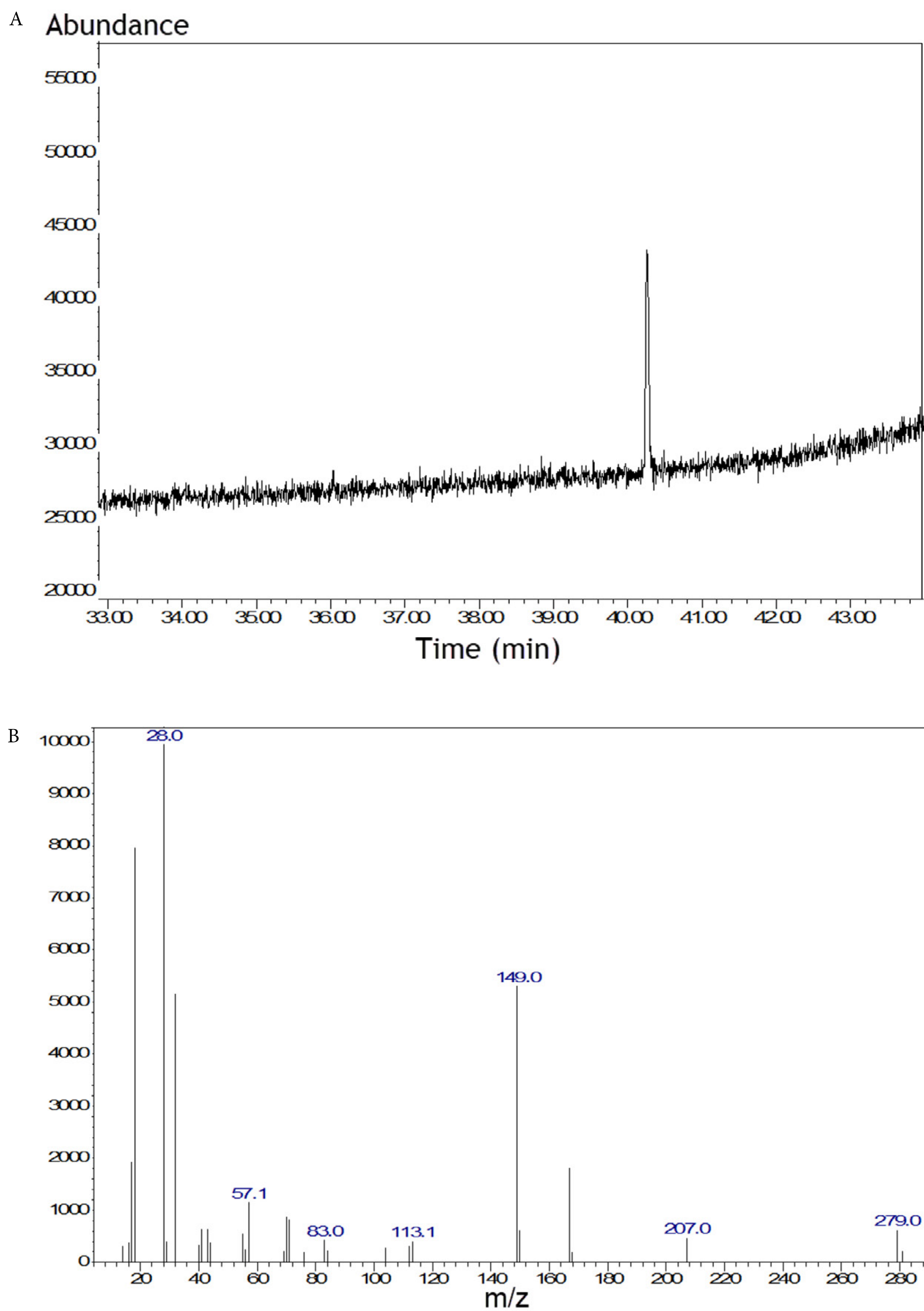

Figure 8. Total ion chromatogram (A) and GC/MS spectra (B) of compound 325-5

Figure 9. ^{1}H NMR spectra (A), ^{13}C NMR spectra (B) for toluene standard and compound 325-5

Acta Chim. Slov. **2020,** *67,* 445–461

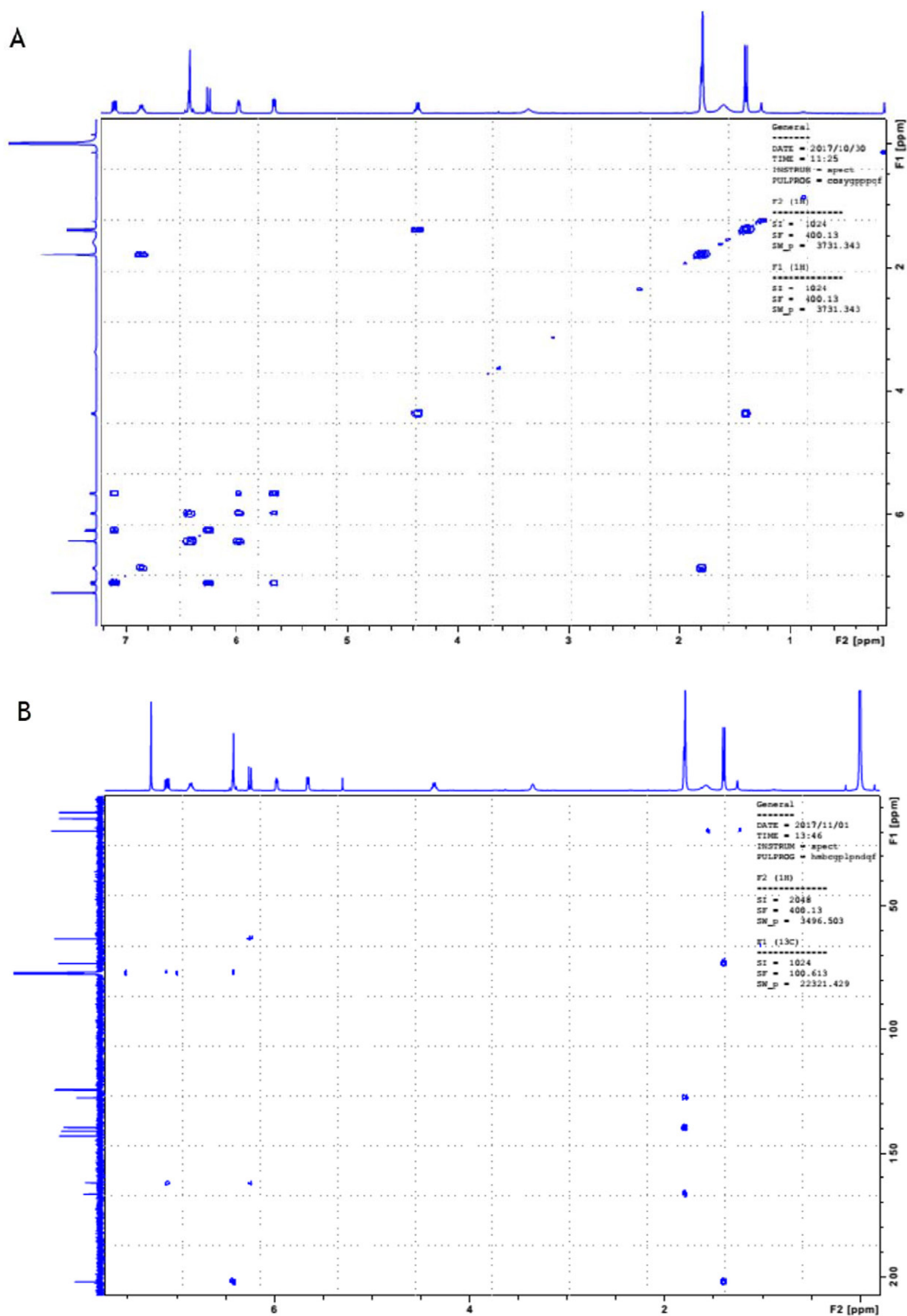

Figure 10. HSQC (A) and COSY (B) spectra of compound 325-5

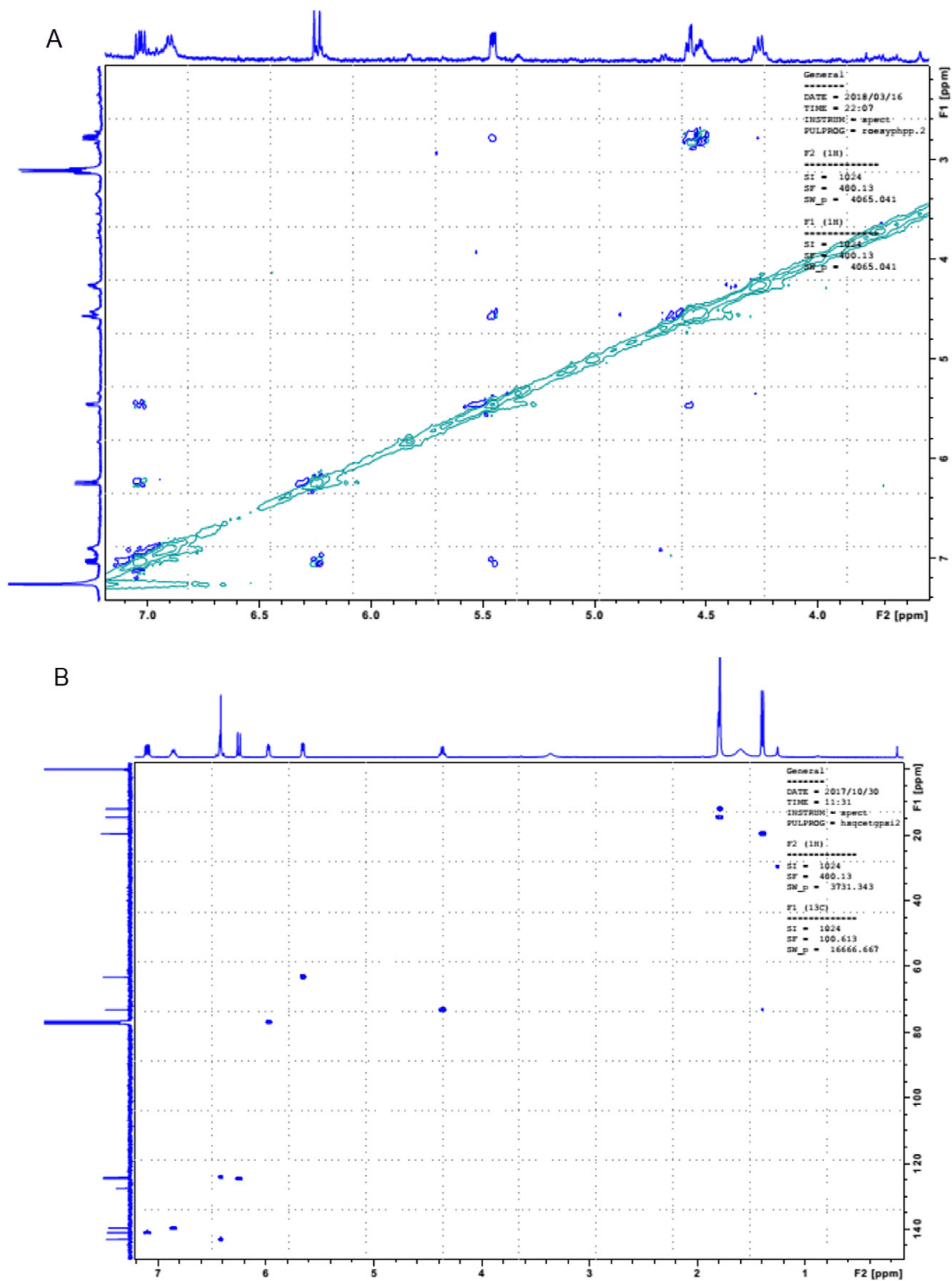

Figure 11. HMBC (A) and NOESY (B) spectra of compound 325-5

Phomopsis sp. was already recognized as potent producer of bioactive compounds. However, it can be noticed that *Phomopsis sp*. is able to produce a variety of known secondary metabolites with great differences in chemical compositions. Among them, relatively small molecule which is similar to compounds resulted from our study, is phomoenamide, a metabolite of *Phomopsis sp.* PSU-D15 isolated from leaves of *Garcinia dulcis (Roxb.) Kurz.* that has moderate activity against *M. tuberculosis*. [27] Another endophytic fungi, *Culvularia geniculate* obtained from limbs of *Catunaregam tomentosa*, produces five metabolites known as culvularides A-E, with similar structures to molecules from *Phomopsis species* we have characterized. For cuvularides A-E antifungal activity against *Candida albican*s has been determined.[28] Therefore, the authors consider that the future perspective of this research could be directed towards spreading the antimicrobial assay evaluation in accordance with mentioned indices from literature records.

4. Conclusions

Within this scientific article, antibiotic potential of endophyte *Phomopsis species* isolated from conifer needles has been determined against bacteria *Escherichia coli* and *Staphylococcus aureus*. Dominant compounds potentially responsible for antimicrobial activity have been discussed. Based on their antimicrobial activity and unique structural characteristics in comparison with well-established drugs from the same therapeutic category, two dominant compounds (Z)-(Z)-2-acetoxyprop-1-en-1-yl-3-(3-((E)-3,4-dihydroxypent-1-en-1-yl)oxiran-2-yl)acrylate (denoted as 325-3) and (Z)-(Z)-2-acetoxyprop-1-en-1-yl 3-(3-((E)-4-hydroxy-3-oxopent-1-en-1-yl)oxiran-2-yl)acrylate (denoted as 325-5) were characterized. Moreover, based on their specific properties, these biomolecules could serve as leading structures for further antibiotic drug discovery. The evaluation of relationship between the chemical structure and the intensity of antibacterial activity may provide guidelines for development of series of new derivatives and further improvement of bioactivity.

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Povzetek

V zadnjem desetletju je vse več zanimanja namenjenega raziskovanju bioaktivnih sekundarnih metabolitov endofitnih gliv. Kot nadaljevanje naših predhodnih raziskav na področju endofitnih gljiv s protimikrobnim potencialom, smo izbrali in izolirali vrsto *Phomopsis sp.* iz iglic iglavcev. Diklorometanski in etanolni ekstrakt *Phomopsis sp.* sta znatno zavirala rast bakterij *Escherichia coli* in *Staphylococcus aureus*. Spojine diklorometanskega izvlečka so bile ločene, zbrane in očiščene s pomočjo tehnike semi-preparativne visoko zmogljivostne tekočinske kromatografije (HPLC) ter okarakterizirane s tehnikama masne spektrometrije (MS) in jedrske magnetne resonančne spektroskopije (NMR). Na podlagi protimikrobne aktivnosti in edinstvenih strukturnih značilnosti, v primerjavi z uveljavljenimi zdravilnimi učinkovinami iz iste terapevtske kategorije, sta bili dve prevladujoči spojini (Z)-(Z) -2-acetoksiprop-1-en-1-il-3- (3- ((E) -3,4-dihidroksipent-1-en-1-il)oksiran-2-il)akrilat (označen kot 325-3) in (Z)-(Z)-2-acetoksiprop-1-en-1-il-3-(3-((E)-4-hidroksi-3-oksopent-1-en-1-il)oksiran-2-il)akrilat (označen kot 325-5) prepoznani kot obetavni spojini za nadaljnje raziskave na področju novih naravnih antibiotičnih učinkovin.

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