Antidiabetic and antioxidant activities of two alpine species of *Swertia* L.

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Abstract

Species of *Swertia* L. from alpine areas are used for the treatment of various ailments in ayurvedic and Tibetan medicine. The present work has attempted to assess the antidiabetic and antioxidant activity of the methanol extracts from aerial parts of two species of *Swertia*, *S. barunensis* and *S. multicaulis* from alpine areas of Nepal. The study has also attempted to compare the liquid chromatography mass spectrometry (LC-MS) analysis of their methanol extracts. LC-MS analysis of respective extracts indicated the presence of several common phytoconstituents in the two species. The antidiabetic potential was determined by using glucosidase inhibition assay while antioxidant potential was determined by using DPPH radical scavenging assay. The results showed that *S. barunensis* extracts had higher antioxidant activity compared to *S. multicaulis* extracts, while the extracts of both species had similar antidiabetic activity. This shows the possibility of using *S. barunensis* as a substitute to *S. multicaulis*. Similarly, other alpine species of *Swertia* may also possess such activities and should be studied to unravel their therapeutic potential.

Keywords: Biological activity, Bitter gentians, Methanol extract, Nepal Himalaya, Phytochemistry

1. Introduction

Non-communicable diseases (NCDs) are increasing globally every year and account for over 70% of total deaths worldwide. In terms of the total number of deaths caused by NCDs, cancer and diabetes mellitus (DM) are among the most important ones as they stand respectively at 1st and 7th positions globally [1]. They are the 3rd and 7th major causes of death in Nepal as well [2]. Exposure to various risk factors is considered a major driving cause responsible for the high proportion of total deaths due to NCDs [3].

The approaches taken to cope with the increased incidence of NCDs vary based on the type of NCDs. The use of various medicinal plants and their products in various forms of alternative medicines especially in rural areas is one of those approaches. The Gentians belonging to the family Gentianaceae, are among the important medicinal plants, especially in alpine and sub-alpine areas. Several species of gentians like species of *Gentiana, Halenia, Lomatogonium, Swertia*, etc. are used in treating ailments related to the liver [4]. Among the species of alpine *Swertia*, *S. franchetiana* and *S. mussotii* are used for the treatment of liver disorders in traditional Tibetan Medicine [5] while *S. multicaulis, S. kingii* are used in treating bile disorders and associated problems in Sowa Rigpa system of traditional medicine [4].

Studies have shown a strong link between certain liver disorders like non-alcoholic fatty liver disease (NAFLD) and diabetes mellitus (DM). Patients with DM are more likely to develop NAFLD [6] and those with NAFLD are also likely to trigger DM [7]. Considering the ability of various species of *Swertia* to cure various ailments related to the liver, the present study aims to evaluate the extracts from aerial parts of two alpine species of *Swertia*, *S. barunensis* Chassot and *S. multicaulis* D.Don, in controlling hyperglycemia, one the symptoms of DM, through inhibition of two key enzymes of carbohydrate metabolism, α-amylase and α-glucosidase. Out of these two selected species, *S. multicaulis* is a medicinal plant distributed throughout the Himalayas with reported use in the treatment of various bile (liver) related problems [4] and in fever, cough and cold [8], while *S. barunensis* does not have reported any medicinal use. Studies on phytochemical screening and evaluation of biological activity of Swertia extracts are confined mostly to *S. chirayita* and its substitutes, [9-12] most of which are found from sub-tropical to temperate range. The species of *Swertia* from alpine areas like *S. mutlicaulis,* though routinely used in various traditional medicine in

Nepal [4, 8], have not been studied for their in-vitro biological activities. The present study thus aims to evaluate extracts of *S. barunensis* and S. *multicaulis* for their antidiabetic and antioxidant activity in vitro. At the same time, the extracts are also evaluated by Liquid chromatography Mass Spectrometry (LC-MS) to search for compounds with antidiabetic and antioxidant activities.

2. Materials and Methods

2.1 Sample collection

Plant materials consisted of aerial parts of two alpine species of *Swertia*, namely *S. barunensis* and *S. mutlicaulis* (Fig. 1), collected from open meadows in Jaljale area (ca. 4500 m asl), Sankhuwasabha district, East Nepal. The photographs of the species used for the study are shown in Fig. 1. The samples were collected along with voucher specimens from their natural habitats. *S. barnunensis* was identified based on information published in the protologue [13] while *S. multicaulis* was identified comparing with specimens deposited at Tribhuvan University Central Herbarium (TUCH). The voucher specimens of respective species are deposited at TUCH.

2.2 Extraction preparation

The samples were cleaned and air dried in shade until constant weight was obtained. The samples were then ground into fine powder using an electric grinder. The powdered mass was then subjected to extraction with 10 times the volume of Methanol (Fisher Scientific India Ltd) in an ultrasonic bath for 1 hour following Khanal et al. [10] with modification. The extract was filtered through Whatman no 1 filter paper and the filtrate was collected. The extraction process was repeated once again for the residue. After extraction, the mixture was filtered again and the filtrates obtained after extraction of samples of respective samples were mixed and evaporated under reduced pressure in a rotary evaporator. The dry extract was collected and stored at -20°C.

2.3 Evaluation of antidiabetic activity

The antidiabetic activity of methanol extracts of the selected species of *Swertia* was evaluated by using α-glucosidase inhibition assay following the protocol of Si et al. [14] with slight modification. The reaction medium consisted of a 10mM

Fig. 1: Photographs of *S. barunensis* Chassot (left) and *S. multicaulis* D.Don (right) in their natural habitat

solution of p-nitrophenyl, β-D-glucopyranoside (Sigma-Aldrich, Germany) in 0.1M potassium phosphate buffer (pH 6.4). The reaction mixtures were dispensed at the rate of 150µL per well. Two sets of three reactions each were prepared for each of the plant extract or control. To the one set (blank) 40 µL of 1M Na_2CO_3 solution was added to the reaction mixture while the next set was kept as such. To the reaction mixture, 10 µL of respective plant extracts or that of acarbose of different concentrations were added and the plates were pre-incubated at 30°C for 5 minutes. Then 10µL of α-glucosidase solution prepared in phosphate buffer (0.1 u/mL) was added to all the cells and the plate was incubated for the next 20 minutes. After incubation, 40µL of 1M Na_2CO_3 was added in all the wells except the blank. Negative controls were also prepared by taking the pure solvent instead of plant extract or acarbose. After that the control was prepared as above but by replacing the plant extract with pure methanol. Then the absorbance was taken at 405 nm in MultiScan (Thermo Fisher Scientific) Elisa Plate Reader. Percentage inhibition of enzyme activity was determined by using the following formula.

$$\% \text{ inhibition } \frac{\text{cotrol asorbance} - \text{sample absorbance}}{\text{cotrol asorbance}} \times 100$$

IC_{50} values of α-glucosidase inhibition by acarbose and plant extracts of two species of *Swertia* were calculated by using the following formula as mentioned in Sharifi-Rad et al. [15].

IC50 = EXP (LN(conc.>50%) – ((pi>50% - 50)/(pi>50% - pi<50%) × LN(conc>50%/ conc<50%)))

Where, EXP is exponential and LN is natural Logarithm, conc. is the concentration and pi is the percentage inhibition.

2.4 Evaluation of antioxidant activity

Antioxidant activity was evaluated by comparing the ability of methanol extracts to scavenge the 0.2 mM solution of 1,1- diphenyl-2 picryhydrazyl (DPPH) following the protocol of Singh et al. [16]. After evaluating the radical scavenging activity of plant extracts and ascorbic acid at various concentrations, the IC50 value was calculated in the same manner as that used for the evaluation of α-glucosidase inhibition activity.

2.5 Liquid chromatography mass spectrometry (LC-MS)

LC-MS analysis of crude extracts of *S. barunensis* and *S. multicaulis* was carried out in Shimadzu LCMS system consisting of Shimadzu prominence LC and Shimadzu MS 2020 systems using Shim-pack XR-ODS 3×50 mm, id 2.2 µm columns. The data were analyzed by using LabSolutions (Shimadzu, Japan). The samples were prepared by mixing 150 µL of sample solution (1mg/mL) and 600 µL water. The mixture was vortexed well, filtered and then 5 µL of it was injected into the system. Other parameters used in running the LCMS systems are as follows:

Mobile phase A: 0.1% formic acid in water
Mobile phase B: 0.1% formic acid in acetonitrile
Flow rate: 0.3 mL/min
Column oven temp: 30°C
Run time: 35 min
uv: 315 nm

LC parameter:

Time (min)	Proportion of phase B (%)
0.01	5
20	95
30	95
30.01	5

MS parameter:
Ionization source: ESI in positive mode
Acquisition mode: Scan
Start m/z: 35
End m/z: 750
DL temp.: 250 °C
Nebulizing gas flow: 1.5 L/min
Heat block temp: 200 °C
Drying gas flow: 15 L/min

2.6 Data Analysis

All data were taken in triplicates and one-way analysis of Variance (ANOVA) was carried out to find out the statistical significance of the differences in IC_{50} value of DPPH radical scavenging activity and glucosidase inhibition activity. All analyses were done by using Microsoft Excel 2013 (Microsoft Corporation, USA).

3. Results

The results of the investigation on antidiabetic (α-glucosidase inhibition) activity, antioxidant activity and LCMS profiles of crude methanolic extracts of *S. barunensis* and *S. multicaulis* are as follows.

The α-glucosidase inhibiting activity of crude methanol extracts of *S. barunensis* and *S. multicaulis* were also found to be significantly lower (IC_{50} values of 0.61±0.01 and 0.65±0.02 mg/mL, respectively) than that of acarbose (IC_{50} value of 0.48±0.04 mg/mL) at $p<0.05$ (Fig. 2). The differences in IC_{50} value of α-glucosidase inhibition between the two species of *Swertia* were not statistically significant ($p<0.05$).

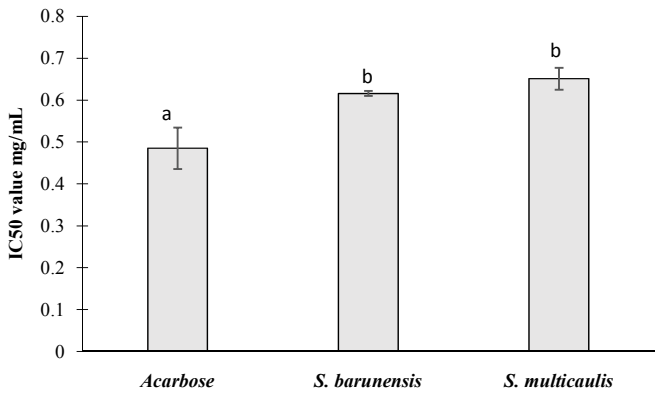

Fig. 2: α-glucosidase inhibition activity of methanol extracts of *S. barunensis* and *S. multicaulis* compared with that of acarbose (The bars with different letters are significantly different $P<0.05$)

Antioxidant activity of crude methanol extracts of aerial parts of *S. barunensis* and *S. multicaulis* (Fig. 3) were much lower (IC_{50} values of 43.74 ± 2.48 and 51.94 ± 1.73 µg/mL, respectively) compared to that of ascorbic acid (IC_{50} values of 23.62 ± 0.90 µg/mL). The difference of means in IC_{50} values of Ascorbic acid with that of either species was statistically significant ($P<0.05$). Similarly, the difference of means between IC50 values of two species of *Swertia* was also statistically significant ($P<0.05$).

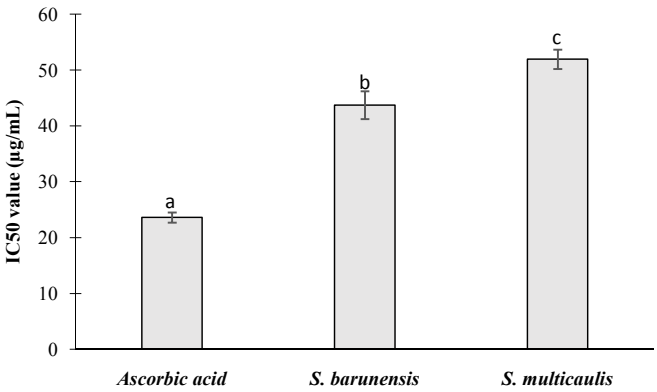

Fig. 3: IC_{50} value (µg/mL) of DPPH radical scavenging activity of ascorbic acid and methanolic extracts of *S. barunensis* and *S. multicaulis* (Bars with different letters are significantly different $P<0.05$)

LC-MS chromatogram of crude methanolic extracts of *S. barunensis* yielded 7 major peaks while that of *S. multicaulis* yielded nine major peaks. Among these peaks five peaks (retention time values of ca. 6.8, 9.2, 15. 2, 21.1 and 33.8 minutes) were common to both species, while the remaining five peaks (two from *S. barunensis* and three from *S. multicaulis*) were unique to respective species (Fig. 4). The summarized peak table of the two species and the tentative identity of the compounds based on m/z values of the compounds seen in LCMS chromatogram and the molecular mass of known compounds reported from other species is presented in Table 1. The details of LC-MS profiles are given in the supplementary file.

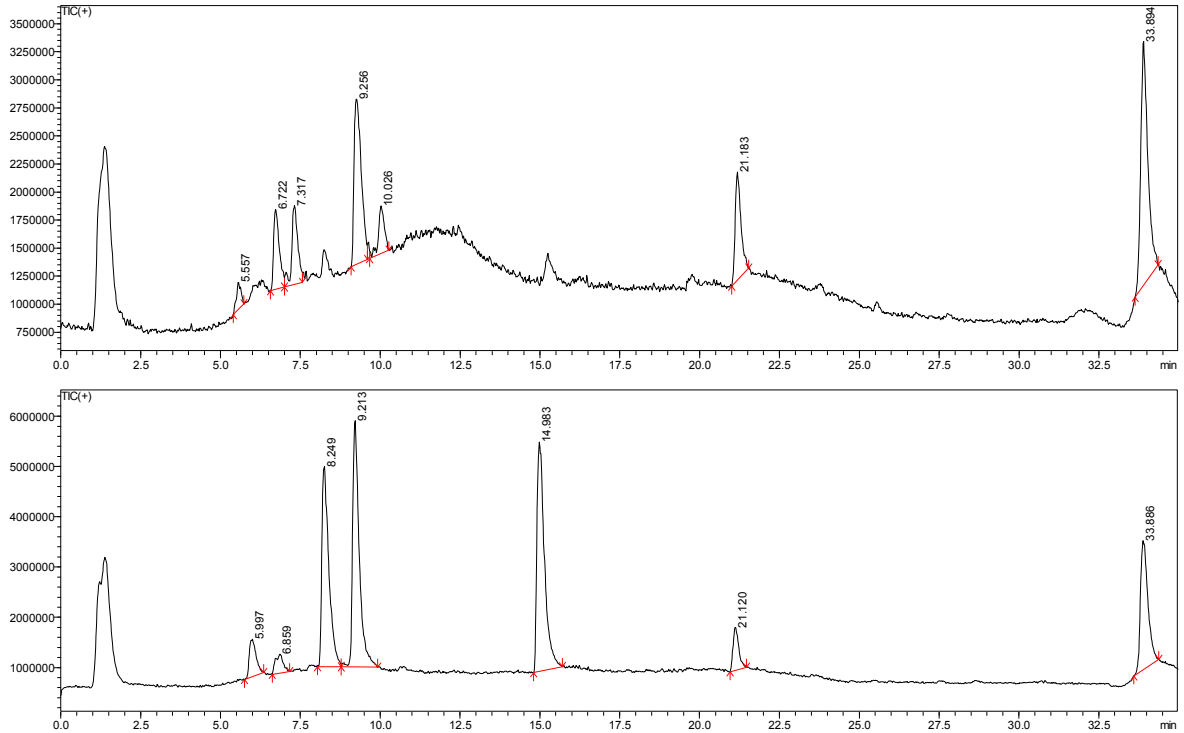

Fig. 4: LCMS chromatogram of extracts of *S. barunensis* (above) and *S. multicaulis* (below)

Table 1: Summarized peak of methanol extracts of *S. barunensis* (SBA) and *S. multicaulis* (SMU), and putative compounds based on molecular mass of compounds reported from species of *Swertia*

Rt (mins)	m/z ratio of major peaks	SBA	SMU	Reported compounds from different species of *Swertia*
5.5	363		√	
5.8	471	√		Maslinic acid ($C_{30}H_{48}O_4$) from *S. mussotii* [17] or its isomer
5.9	227	√		Gentiocrucine B/D ($C_{10}H_{13}NO_5$) isolated from *S. angustifolia* and *S. macrosperma* [18] or its isomer
6.7	375		√	Loganic acid or epi-loganic acid ($C_{16}H_{24}O_{10}$) reported from *S. mussotii* [17] or its isomer
7.3	359		√	De-oxy loganic acid isolated from *S. mussotii*, (Cao et al. 2015) or Swertiajaposide B/E ($C_{16}H_{24}O_{9;}$) isolated from *S. japonica* [19] or its isomer
8.2	453	√	√	Swertia lactone C/D ($C_{30}H_{46}O_3$) from S. petiolata [20] and *S. speciosa* [21] or its isomer
9.2	340	√	√	1,5-dihydroxy-3-(-2-oxopropyl)-6-methoxycarbonylxanthone ($C_{18}H_{14}O_7$ reported from *S. elata* [22] or its isomer
9.2	680/679	√	√	Demethylswertipunicoside ($C_{32}H_{24}O_{17}$) reported from *S. punicea* [23] or isomers
10.0	303		√	1-hydroxy-3,5,8-trimethoxyxanthone or 1-hydroxy-3,7,8-trimethoxyxanthone ($C_{16}H_{14}O_6$) [24]
15.2	412	√	√	
21.1	466	√	√	1-O-β-D glucopyranosyl-3,8-dihydroxy-4,5-dimethoxyxanthone ($C_{21}H_{23}O_{12}$) reported from *S. bimaculata* [25] or its isomer
33.8	152	√	√	

4. Discussion

Extracts from species of *Swertia* like *S. chirayita* and *S. nervosa* have been studied for their effect on inhibition of α-glucosidase enzyme in vitro [9]. Similarly, studies involving inhibition of α-amylase by extracts of *S. chirayita* [11, 12] and *S. cordata* [11] have also been carried out. These studies have shown not only the species-specific and solvent specific [9, 11] differences but parts-specific differences [11] in inhibitory potential of *Swertia* extracts against these enzymes.

Specific phytoconstituents from various species of *Swertia* have been studied for their antidiabetic activity and at least 12 different compounds, mostly the xanthones, have been validated to possess antidiabetic activity [26]. Among these, one pentaoxygenated xanthone (1,7,8-trihydroxy-3,4-dimethoxyxanthone; m/z=289), and five tetra oxygenated xanthones (1,3,5,8-tetramethoxyxanthone, m/z=316.3; mangiferin, m/z=421; 1-O-(β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl)-3,8-dihydroxy-4,5-dimethoxyxanthone, m/z= 597; 1-O-(β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl)-8-hydroxy-3,4,5-trimethoxyxanthone, m/z=611 and 8-O-(β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl)-1-hydroxy-3,4,5- trimethoxyxanthone, m/z=611) have been shown to exhibit antidiabetic activity through inhibition of α-glucosidase [9, 25, 27]. The presence of compounds with m/z values of 290, 421 and 612 as seen in the LCMS profiles of extracts of both *S. barunensis* and *S. multicaulis* and that of compound with m/z value of 597 in *S. barunensis* indicates the possibility of the presence of respective α-glucosidase inhibitors or their isomers in the extracts of these two species. However, a detailed analysis involving activity-guided isolation of different compounds from these extracts is necessary to ascertain the role of these compounds in the overall α-glucosidase inhibition activity of extracts of different species.

Studies on antioxidant activity of extracts of different species of *Swertia* have also revealed species specific [9-11, 28] as well as solvent specific [9, 11] and parts-specific [11] differences in antioxidant potential. However, none of these studies have involved species from alpine region. Similar species patterns in antioxidant potential of extracts has also been reported in present study. Studies involving antioxidant potential of isolated compounds rather than the crude extracts have also extracts have also been carried out for different species of *Swertia* like *S. decussata* [29], *S. japonica* [30], *S. mussotii* [31], *S. chirayita* [32]. These and several other studies have led to the identification of various compounds, mainly xanthones responsible for antioxidant activity in extracts of *Swertia* included the individual compounds. Luo et al. [31] reported varying degree of antioxidant activity of 25 different xanthones isolated from *S. mussotii* alone.

Bellidifolin ($C_{14}H_{10}O_6$; m/z = 274.22), one of the most common xanthones found in several species of *Swertia* is also reported to show antioxidant activity *in vitro* [30, 32]. Similarly, Xanthones like 8-O-β-D-glucopyranosyl-1,2-dihydroxy-6-methoxyxanthone (m/z=435) and 3-O-demethylswertipunicoside ($C_{32}H_{24}O_{17}$; m/z=679) are also known for their antioxidant activity [33]. Presence of the compound with an m/z value of 679/680 in the extracts of both species in the present study indicates towards the presence of 3-O-demethylswertipunicoside in these plants as well, and it may be the cause behind relatively high *in-vitro* antioxidant activity of extracts of these two species.

5. Conclusion

Evaluation of biological activity of methanol extracts of *S. barunensis* and *S. multicaulis* revealed comparable results in terms of antidiabetic and antioxidant activities. LC-MS analysis of the extracts of both species also showed many common peaks, indicating the presence of similar compounds. This study indicates the therapeutic potential of *S. barunensis*, one of the endemic plant species from Nepal, and its potential use as substitutes of *S. multicaulis*.

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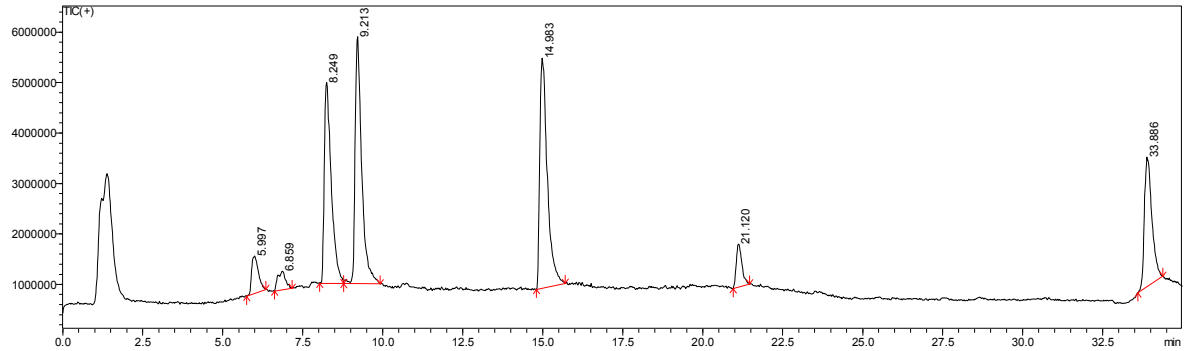
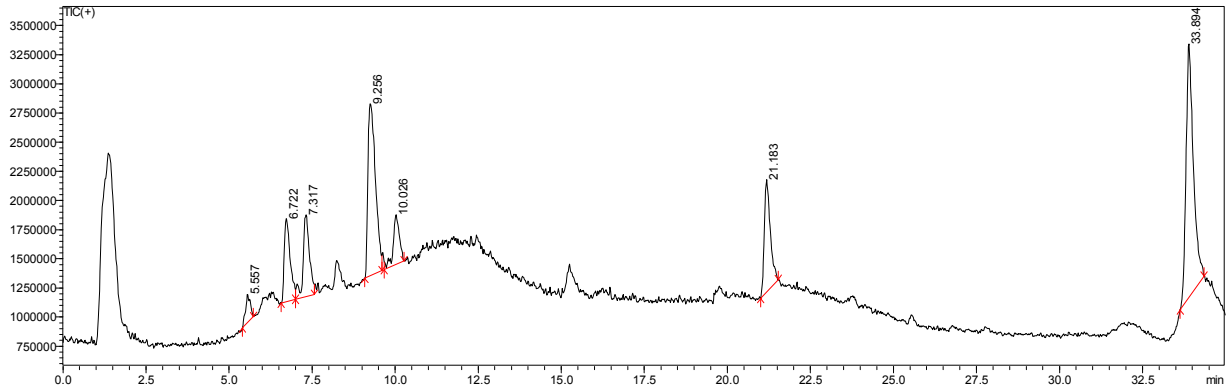
Supplementary files

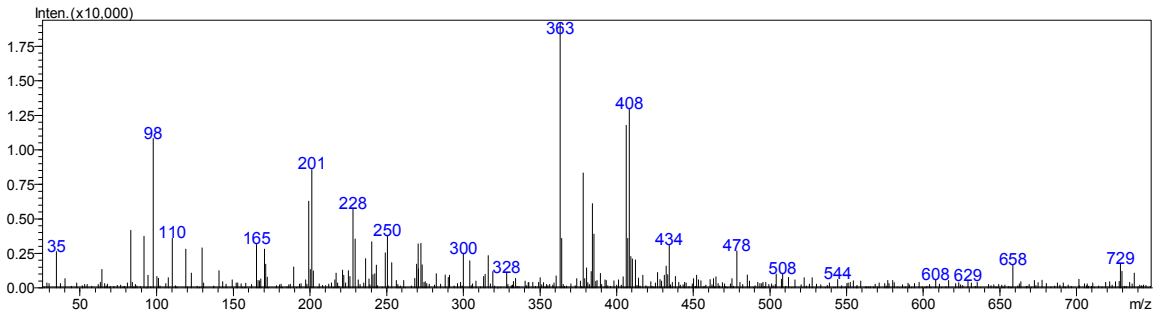


TIC of SBA

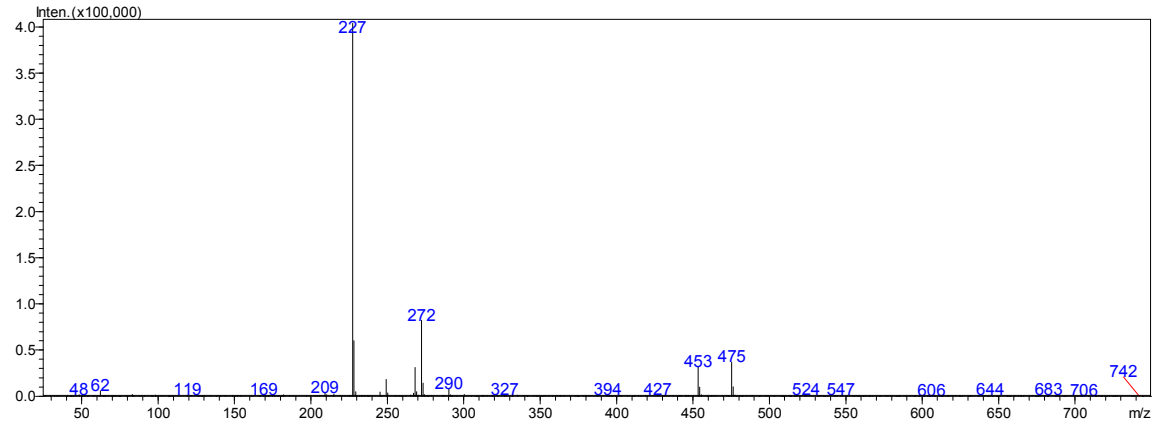


TIC of SMU

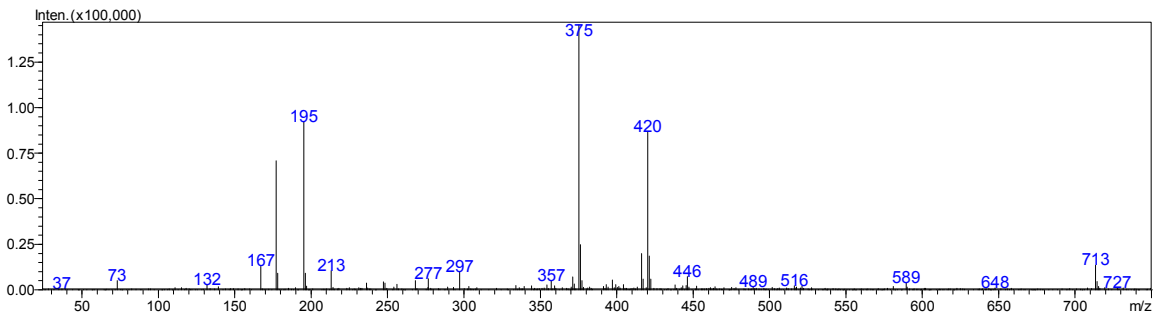
Peak table SMU		Peak table SBA	
Peak#	**Ret. Time**	**Peak#**	**Ret. Time**
1	5.557	1	5.997
2	6.722	2	6.859
3	7.317	3	8.249
4	9.256	4	9.213
5	10.026		
6	15.2	5	14.983
7	21.183	6	21.12
8	33.894	7	33.886



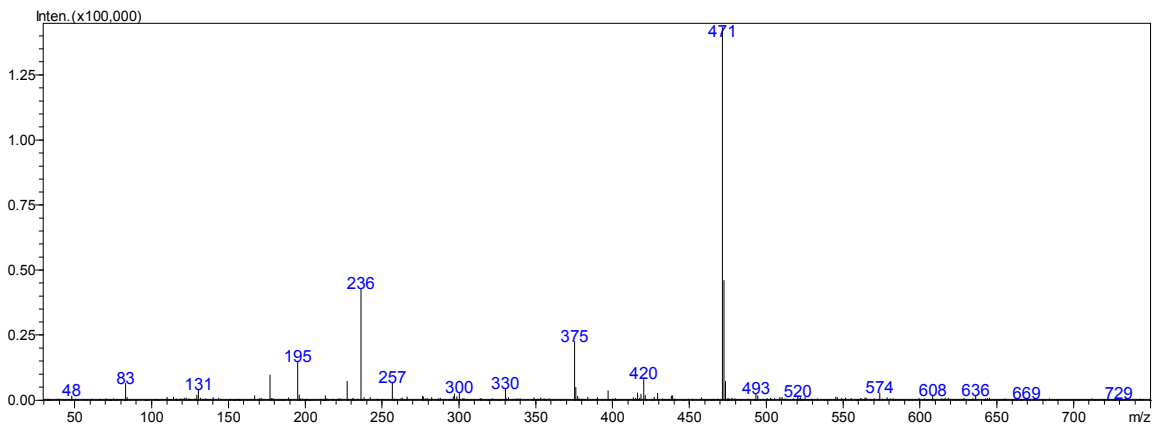
SMU#: 1 Scan(E+) Ret. Time : [5.533->5.567]-[5.400<->5.733] Scan# : [333->335]-[325<->345]



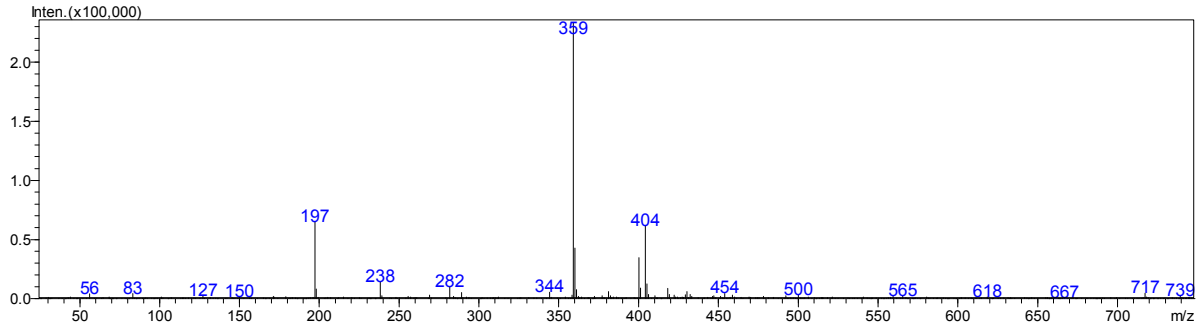
SBAt#: 1 Scan(E+) Ret. Time : [5.983->6.017]-[5.750<->6.350]



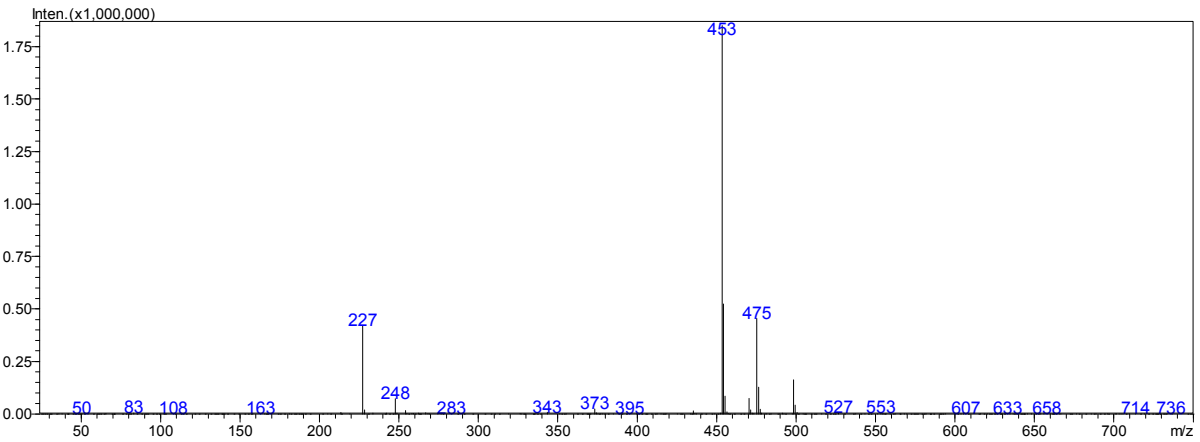
SMU#: 1 Scan(E+) Ret. Time : [6.700->6.733]-[6.567<->7.000] Scan# : [403->405]-[395<->421]



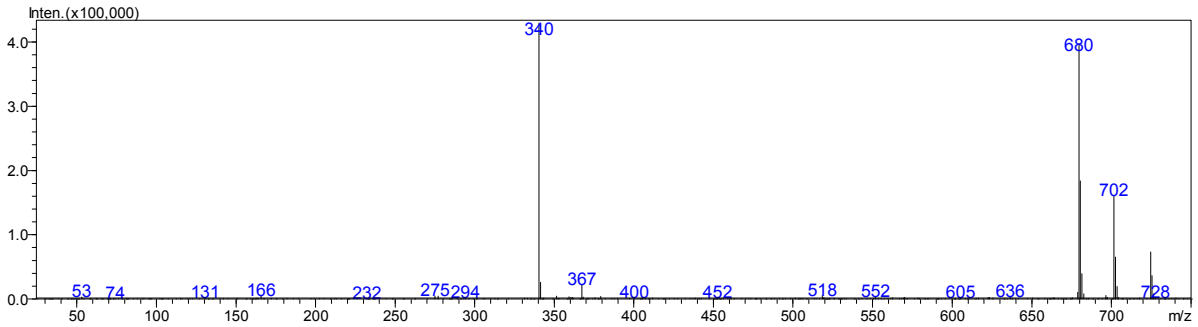
SBA#: 1 Scan(E+) Ret. Time : [6.850->6.883]-[6.617<->7.167]



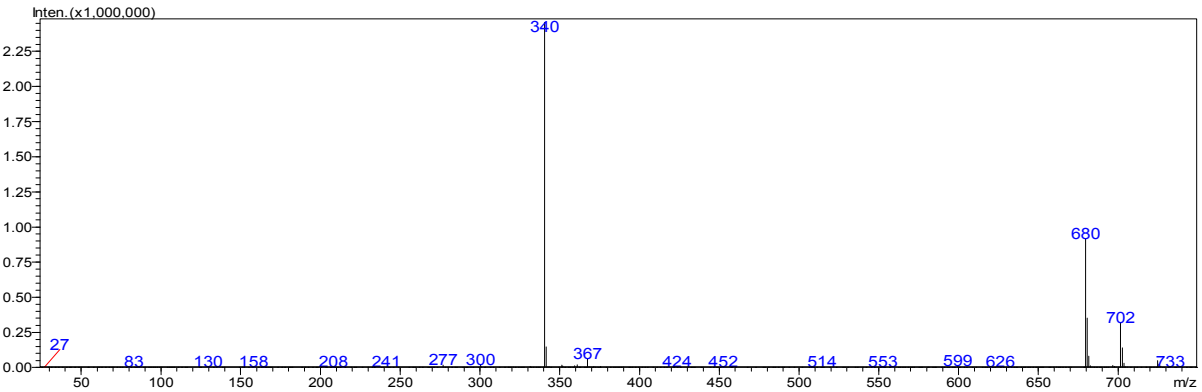
SMU#: 1 Scan(E+) Ret. Time : [7.300->7.333]-[7.000<->7.567] Scan# : [439->441]-[421<->455]



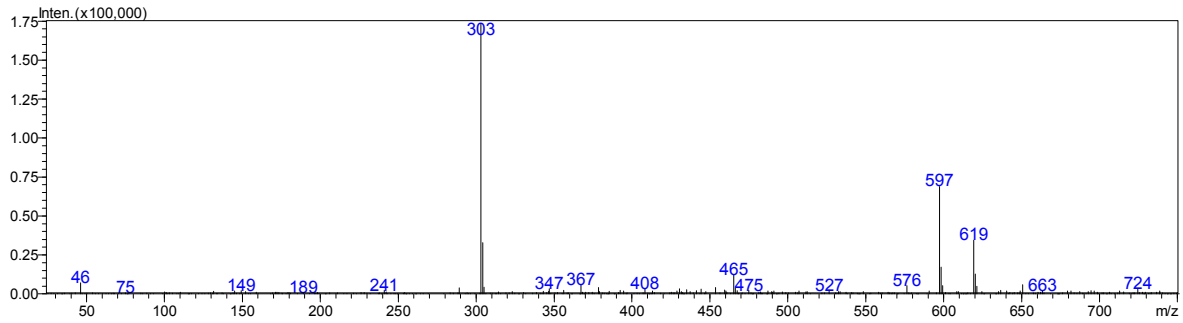
SBA#: 1 Scan(E+) Ret. Time : [8.233->8.267]-[8.033<->8.783]



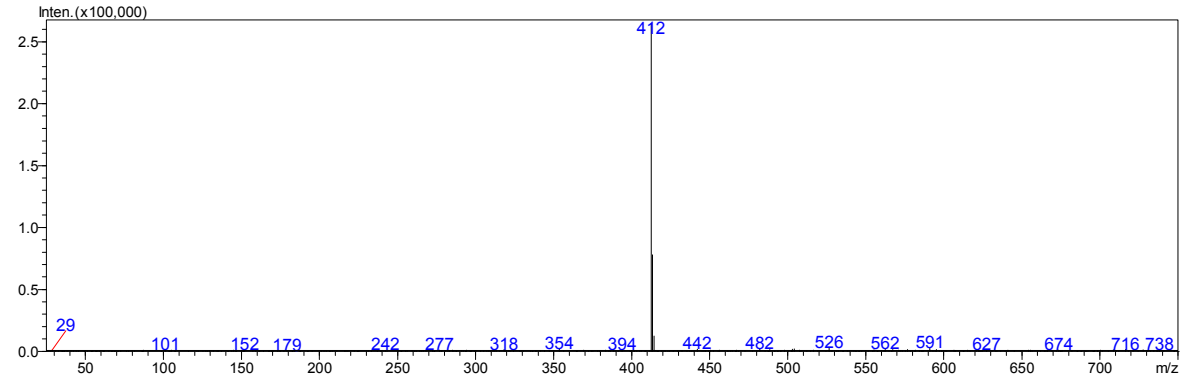
SMU#: 1 Scan(E+) Ret. Time : [9.233->9.267]-[9.083<->9.600] Scan# : [555->557]-[546<->577]



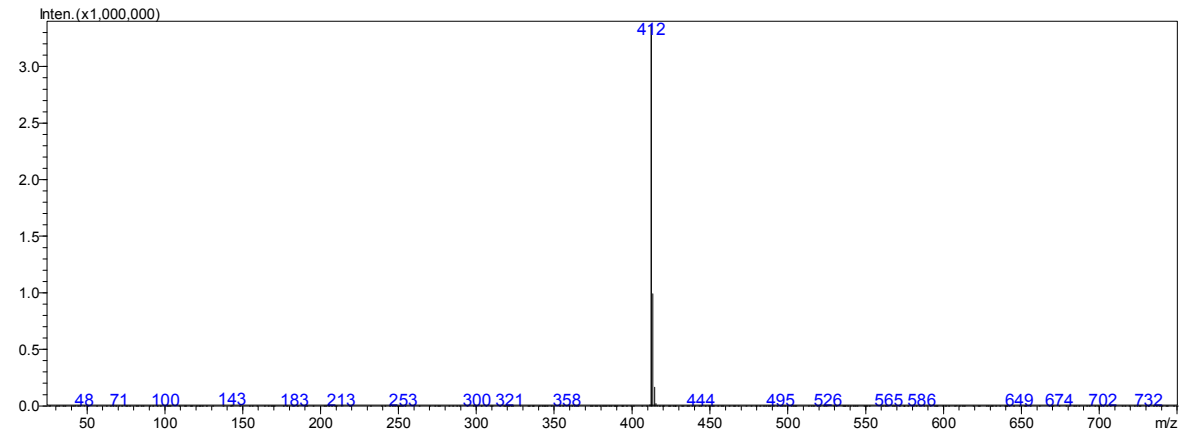
SBA#: 1 Scan(E+) Ret. Time : [9.200->9.233]-[8.783<->9.917]



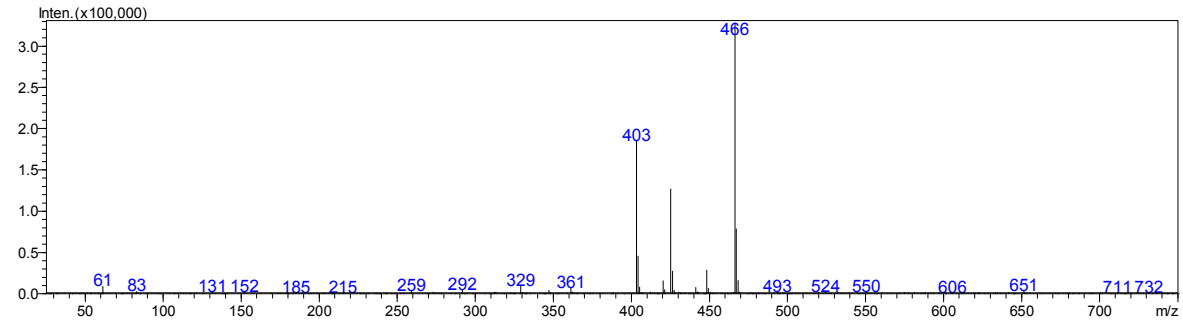
SMU#: 1 Scan(E+) Ret. Time : [10.017->10.050]-[9.667<->10.267] Scan# : [602->604]-[581<->617]



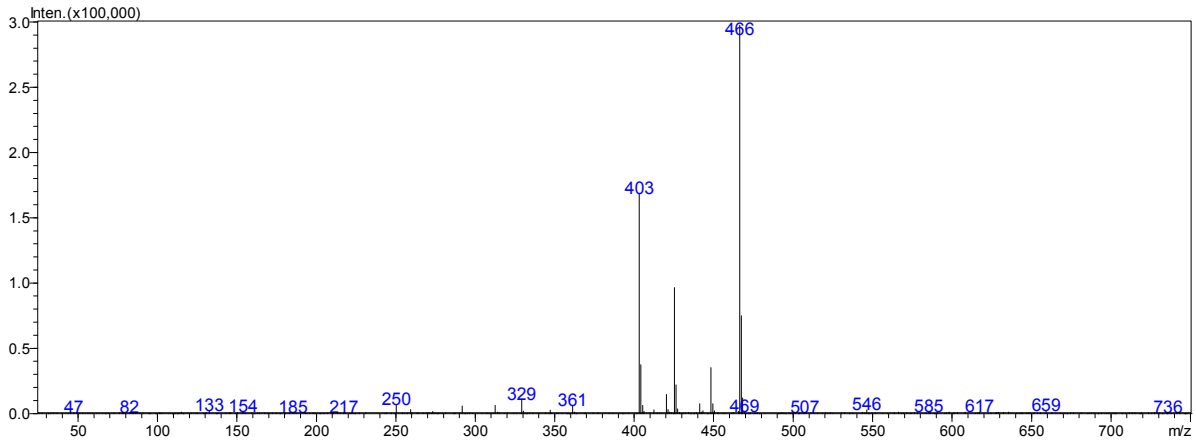
SMU#: 1 Scan(E+) Ret. Time : [15.250->15.283]-[15.117<->15.567] Scan# : [916->918]-[908<->935]



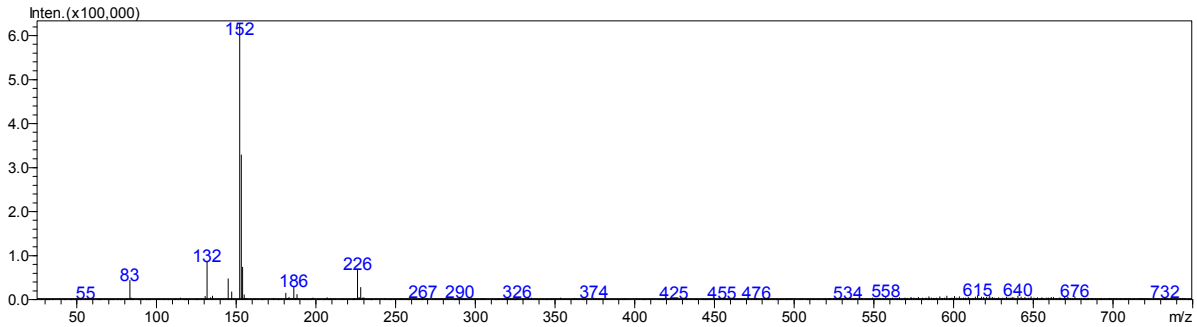
SBA#: 1 Scan(E+) Ret. Time : [14.967->15.000]-[14.800<->15.700]



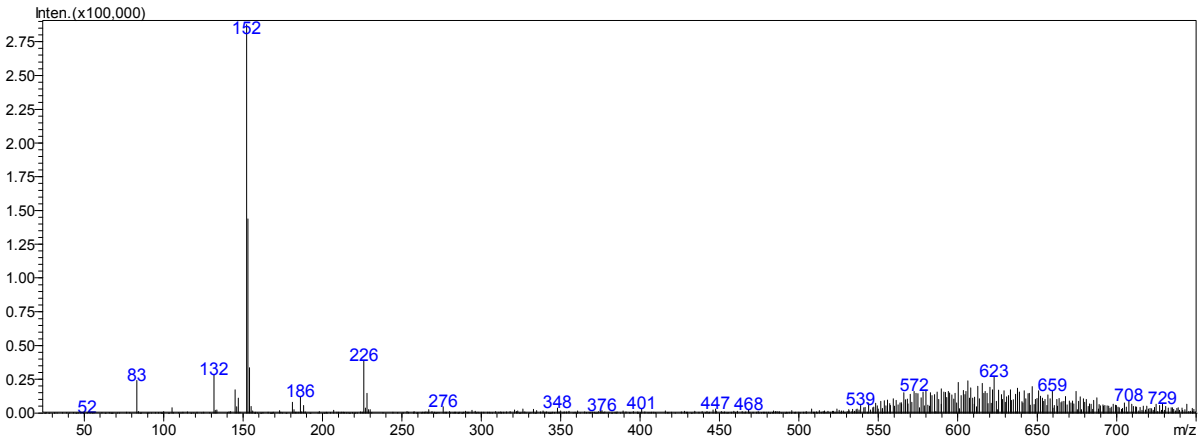
SMU#: 1 Scan(E+) Ret. Time : [21.167->21.200]-[21.000<->21.533] Scan# : [1271->1273]-[1261<->1293]



SBA#: 1 Scan(E+) Ret. Time : [21.100->21.133]-[20.950<->21.467]



SMU#: 1 Scan(E+) Ret. Time : [33.883->33.917]-[33.633<->34.350] Scan# : [2034->2036]-[2019<->2062]



SBA#: 1 Scan(E+) Ret. Time : [33.867->33.900]-[33.600<->34.383]

Legend: SBA- *S. barunensis*; SMU- *S. multicaulis*