

## UPLC-HRESI-MS and GC-MS analysis of the leaves of *Nicotiana glauca*

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Accepted October 27, 2020  
Published online January 18, 2021

The alkaloid-rich fraction obtained by fractionation of the crude methanolic extract of the leaves of wild tobacco tree *Nicotiana glauca* Graham (Solanaceae) was analyzed using UPLC-MS and GC-MS. Anabasine, a piperidine alkaloid, was identified as the major constituent with approximately 60 % (*m/m*) of the alkaloid-rich fraction. In addition to anabasine, six secondary metabolites were identified using high-resolution UPLC-MS. Anabasine was quantified in the leaves to be 1 mg g<sup>-1</sup> dry plant material. The GC-MS analysis revealed five compounds with anabasine as the major component, while nicotine was not detected. Moreover, GC-MS was used for the analysis of the volatile oil that was obtained by hydro-distillation from the leaves of *N. glauca*. The volatile plant oil was found to be rich in oxygenated sesquiterpenes (*e.g.*,  $\beta$ -bisabolol) and carboxylic acids and esters (*e.g.*, ethyl linoleate and hexadecanoic acid), whereas anabasine was not detected.

**Keywords:** *Nicotiana glauca*, tobacco tree, UPLC-HRESI-MS, GC-MS, anabasine, nicotine, volatile oil, methanolic extract

Several plant species are known to biosynthesize nicotine and other tobacco-like alkaloids, some of which are reported to be toxic (1). Although there are more than sixty *Nicotiana* species, it is shown that two are toxic to humans, namely *Nicotiana tabacum* L. "tobacco" and *Nicotiana glauca* Graham "tobacco tree" (Solanaceae) (1). Whereas nicotine is the major pyridine alkaloid in tobacco, anabasine, which is a piperidine alkaloid, is the major one in the tobacco tree (Fig. 1) (2).

The ingestion of *N. glauca* is associated with toxic and teratogenic effects in cattle, pigs, sheep and goats. The toxicity signs are palatoschisis and multiple congenital contracture deformities (1, 3–6). Accidental ingestion has led to multiple cases of intoxication and death in humans (3, 7–12). Anabasine is responsible for the acute toxicity associated with the

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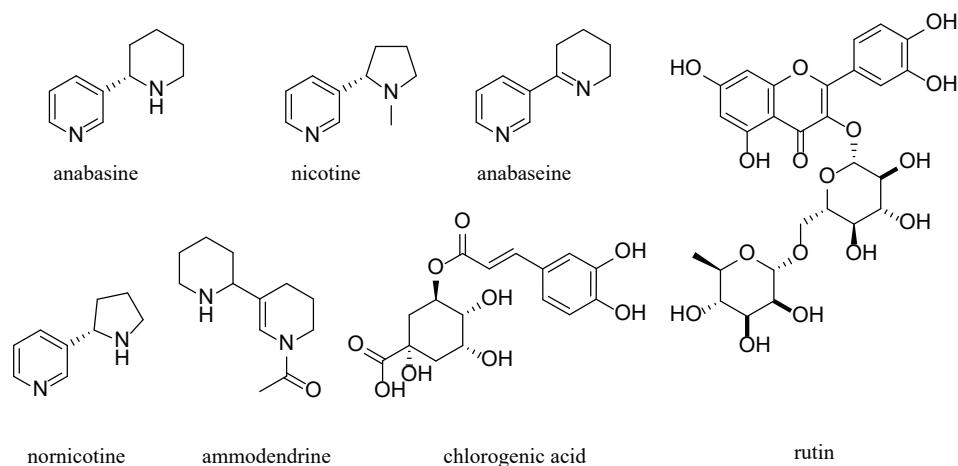


Fig. 1. Structures of anabasine and nicotine, the major alkaloids in tobacco and tree tobacco, and other secondary metabolites identified in *N. glauca* using UPLC-HRESI-MS.

ingestion of the leaves of *N. glauca* (1). Two patients were found dead after ingesting sufficient amounts of the leaves of the anabasine-containing tobacco tree plant, *N. glauca*. The patients were brought to the emergency suffering from paralysis, increased salivation, severe muscle weakness, respiratory depression, increased blood pressure, disturbed vision, nausea, vomiting and diarrhea (13). Anabasine, an agonist of nicotinic acetylcholine receptor (nAChRs), is more potent than nicotine by several folds. This action on the nAChRs at the neuromuscular junction could lead to paralysis and respiratory failure (1, 12). In the recent report, *N. glauca* was found to affect skeletal muscle homeostasis. Hence, the reported traditional use of *N. glauca* as a remedy in hormone replacement therapy could worsen menopausal symptoms (14).

Tree tobacco is a tree-like shrub that can grow to a height of 6 m. The leaves are glaucous, ovate or elliptic, up to 13 cm long, with yellow tubular flowers. The plant is native to South America but naturalized in warm-temperature parts of the world (15). *N. glauca* was reported to be of value for the treatment of jaundice, bruises and inflamed throat (16). In Jordan, the plant grows in areas like Jordan Valley and along roadsides in Central Jordan to northern parts (16, 17).

Due to the volatility of the piperidine alkaloids, GC-MS was mainly used in qualitative and quantitative analysis of secondary metabolites in *N. glauca*. Several papers also reported UV, PDA or MS-based HPLC quantitative analysis of anabasine and/or other secondary metabolites in *N. glauca* (8–10, 18, 19).

In this study, a new method was developed based on ultra-performance liquid chromatography-high-resolution mass spectrometry (UPLC-HRESI-MS) for the qualitative analysis of the secondary metabolite constituents of *N. glauca*. Since no previous phytochemical work was pursued on this Jordan-naturalized highly poisonous plant, GC-MS qualitative and quantitative analysis was also conducted. This research project represents the first comprehensive UPLC-MS vs. GC-MS study of *N. glauca* of Jordan.

## EXPERIMENTAL

*General experimental design*

In this study, the methanolic extract and the essential oil of the dried leaves of *N. glauca* were studied extensively for their secondary metabolite constituents. A schematic presentation of the phytochemical studies experimental design is shown in Fig. 2.

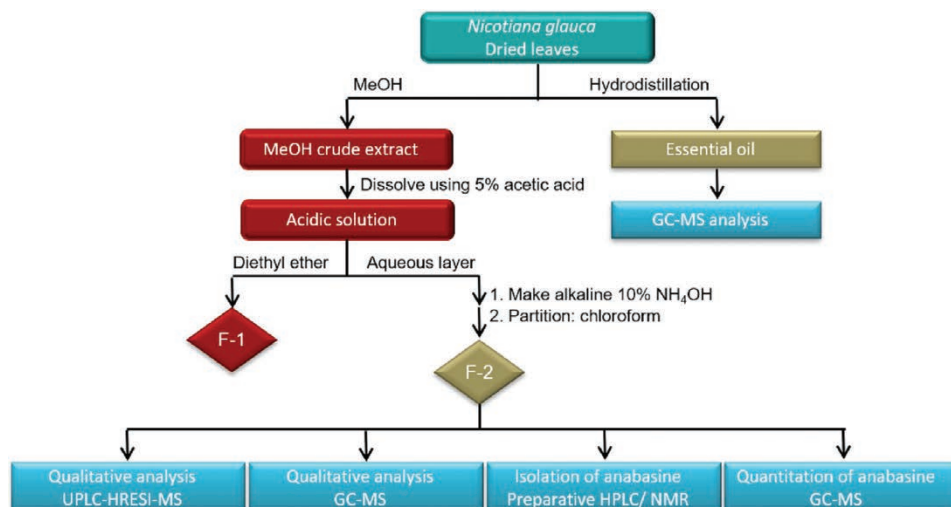


Fig. 2. Schematic representation of the experimental design.

*Methanolic extract of N. glauca*

*Plant collection, identification, extraction and fractionation.* – *Nicotiana glauca* leaves were collected in April 2013 along the street sides of the Irbid-Amman highway. Dr. Mohammed Gharaibeh, a plant taxonomist from the Faculty of Agriculture at Jordan University of Science and Technology (JUST), identified the collected plant material. A voucher specimen of *N. glauca* was deposited at the Faculty of Pharmacy Plant Museum, JUST, Irbid, Jordan. The raw plant material was chopped into small pieces, then dried in the shade away from direct sunlight, at room temperature. After that, the plant material was subjected to grinding into a fine powder using a mortar and pestle to yield ~55 g dry material. The powdered material was soaked with 2 L HPLC grade methanol (Fisher Chemicals, USA) in a 2-L Erlenmeyer flask, sealed with parafilm, kept in a dark place for 72 h with intermittent shaking. The solvent was decanted and then evaporated under vacuum to yield ~9 g methanolic extract.

An aliquot of the *N. glauca* crude methanolic extract (~4.5 g) was dissolved in 500 mL of 5% acetic acid and partitioned with diethyl ether ( $3 \times 100$  mL). The acidic aqueous extract was then made basic using 10%  $\text{NH}_4\text{OH}$  and then partitioned with  $\text{CHCl}_3$  ( $3 \times 200$  mL). Diethyl ether (F-1) and chloroform (F-2, alkaloid-rich fraction) layers were evaporated to

dryness under vacuum to yield 140.9 and 141.1 mg, resp. (Fig. 2). Diethyl ether was used for defatting the extract and hence F-1 was discarded from further analysis.

*Isolation of anabasine from N. glauca alkaloid-rich fraction (F-2).* – Alkaloid-rich fraction F-2 was purified using preparative HPLC. This was done using a Varian Prostar HPLC and a Phenomenex Gemini-NX C<sub>18</sub> (250 × 21.2 mm, 5 μm) preparative column (Phenomenex). The mobile phase used was a gradient system consisting of acetonitrile/water (acidified with 0.1 % formic acid). The gradient started at 0:100 to 5:95 over 15 min at a flow rate of 21.24 mL min<sup>-1</sup>.

An aliquot of the alkaloid-rich fraction F-2 (~50 mg) was dissolved in 300 μL of dioxane/methanol (1:1). The 300-μL sample was then injected into the preparative HPLC column. After preparative HPLC, we got a pure compound, which turned to be anabasine. The structure of the compound was elucidated using UPLC-HRESI-MS and NMR (<sup>1</sup>H and <sup>13</sup>C).

<sup>1</sup>H and <sup>13</sup>C nuclear magnetic resonance data were collected using a JEOL ECS-400 NMR spectrometer operating at 400 MHz for <sup>1</sup>H and 100 MHz for <sup>13</sup>C and equipped with a high sensitivity JEOL Royal probe and a 24-slot autosampler (JEOL Ltd., Japan). Residual solvent signals were utilized for reference. For <sup>1</sup>H NMR and <sup>13</sup>C NMR, chloroform-D was used as a solvent at the temperature of 17.1 and 18.1 °C, resp.

*UPLC-HRESI-MS analysis of N. glauca alkaloid-rich fraction (F-2).* – For UPLC-HRESI-MS analysis, the method used by El-Elimat *et al.* (20) was used with modification. Briefly, a solution at the concentration of 2 mg mL<sup>-1</sup> of F-2 fraction was prepared using a 1:1 mixture of methanol and dioxane (150 μL). HRMS data were collected using a Thermo LTQ Orbitrap XL mass spectrometer (Thermo Fisher, USA). The instrument was controlled and the data was collected and analyzed using Thermo Scientific Xcalibur 2.1 software. A Waters Acquity UPLC system along with a BEH (2.1 × 50 mm; 1.7 μm) column at 40 °C was used (Waters Corp., USA). The mobile phase consisting of acetonitrile:water (acidified with 0.1 % formic acid) was used for gradient elution starting with 0 % acetonitrile then increasing linearly to 5 % within 3 min, then to 100 % in 4 min. The total run time was 7.5 min and the flow rate used was 0.3 mL min<sup>-1</sup>.

*GC-MS analyses of N. glauca alkaloid-rich fraction (F-2).* – Qualitative analysis of the alkaloid-rich fraction (F-2) was done using GC-MS along with the NIST database. GC-MS was carried out using a Shimadzu QP2010S (Shimadzu, Japan) equipped with electron impact ionization and a single quadrupole mass analyzer, along with an AOC-20S auto-sampler and AOC-20i autoinjector. GC column used was a Phenomenex Zebron ZB-SMS (30 m × 0.25 mm, 0.5 μm) (Phenomenex, USA). The GC conditions were as follows: column oven temperature was set at 70 °C, injection temperature was 250 °C, carrier gas was He, the pressure was 59.5 kPa, flow rate and column flow rate were 11.2 and 0.97 mL min<sup>-1</sup>, resp. The column oven temperature was set at 70 °C for 2 min, it was then increased to 250 °C over 8 min, followed by a hold of 5 min. The total programmed time was 29.5 min. The MS was set as the following: ion source and interface temperature were set at 250 °C, with a solvent cut time of 2 min, scan speed was 2500 amu s<sup>-1</sup> and mass range of *m/z* 40–400 was used. Additionally, NIST searchable database was used for compounds' identification.

GC-MS was also used for the quantitative analysis of anabasine in the alkaloid-rich fraction F-2. A linear calibration curve ( $R^2 = 0.9822$ ) of anabasine was prepared in five concentrations (62.5, 125, 250, 500 and 1000 μg mL<sup>-1</sup>) of anabasine authentic standard (≥ 97 %, Sigma Aldrich, USA).

### *Analysis of the essential oil*

*Plant material and oil distillation.* – *N. glauca* leaves were collected in two consecutive days as two separate collections. The two samples were air-dried separately in a well-ventilated area and then ground into a powder. About 500 g of each collection of the ground plant material was subjected to essential oil hydrodistillation using a Clevenger-type apparatus (JSGW, India) for a total time of 4 h. The isolated essential oils ( $n = 2$ ) of each collection were dried over anhydrous sodium sulfate (Analar, UK), transferred into amber glass bottles, and stored at 4 °C until required for analysis.

*GC-MS analysis of the essential oil.* – The GC-MS analysis of *N. glauca* leaf oil was performed using a Varian Chrompack CP-3800 GC/MS/MS-200 (Varian, USA). The instrument was equipped with a split-splitless injector. The column used was a DB-5 GC column (30 m × 0.25 mm, 0.25 μm). The GC-MS/MS conditions were as reported previously by Hudaib *et al.* (21). Briefly, the injector, detector, and the transfer-line temperature were set at 250, 160, and 230 °C, resp., with a split ratio of 1:10. The individual oil components were separated from each other using a linear temperature program. The temperature was increased from 60 to 250 °C at an increasing rate of 3 °C min<sup>-1</sup>. After that, the temperature was held for 5 min at 250 °C. Using a full scan mode and electron ionization (EI) at 70 eV, the mass detector range was set to scan ions between 40 and 400 *m/z*. Samples were analyzed in duplicate. Using the exact chromatographic conditions, a mixture of *n*-alkanes (C<sub>8</sub>–C<sub>20</sub>), a certified analytical standard solution (40 mg L<sup>-1</sup> in hexane, Sigma-Aldrich, USA), was injected into the GC-MS. For each separated component of the oil, Kovats retention index was calculated based on retention times by applying the Van Den Dool equation (22). The oil components were then tentatively identified by comparing their calculated Kovats retention indices with those reported in the literature (23) and by matching their MS spectra with those of commercially available libraries of MS spectra, namely WILEY, NIST and ADAMS-2007.

## RESULTS AND DISCUSSION

### *Phytochemical studies of the methanolic extract of N. glauca*

*UPLC-HRESI-MS analysis of N. glauca alkaloid-rich fraction (F-2).* – UPLC-HRESI-MS method was used for the qualitative analysis of the crude MeOH extract of *N. glauca*. The runtime was short (7.5 min) and effective to detect compounds of low molecular mass as those found in *N. glauca*. The method was implemented successfully for the analysis of the alkaloid-rich fraction of *N. glauca*, where seven compounds were identified (Fig. 1). The compounds identified were: anabasine, anabaseine, nicotine, nornicotine, ammodendrine, rutin and chlorogenic acid. The identification was based on multiple strategies, mainly HRMS data, total ion chromatogram (TIC) *vs.* single ion chromatogram (SIC) analysis, retention time, polarity, and by comparison with authentic reference standards (Figs. 3–5 and S1-S10). Anabaseine, ammodendrine and chlorogenic acids are reported here for the first time in this species.

*GC-MS analysis of N. glauca alkaloid-rich fraction (F-2).* – GC-MS analysis of the alkaloid-rich fraction (F-2) along NIST searchable library identified 5 main compounds with anabasine

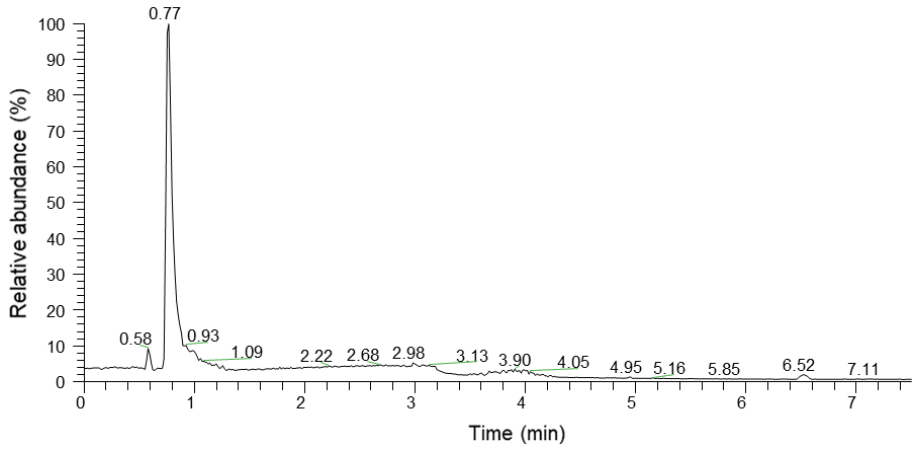


Fig. 3. (+)-ESI base peak chromatogram of the alkaloid-rich fraction (F-2) of *N. glauca* leaves extract.

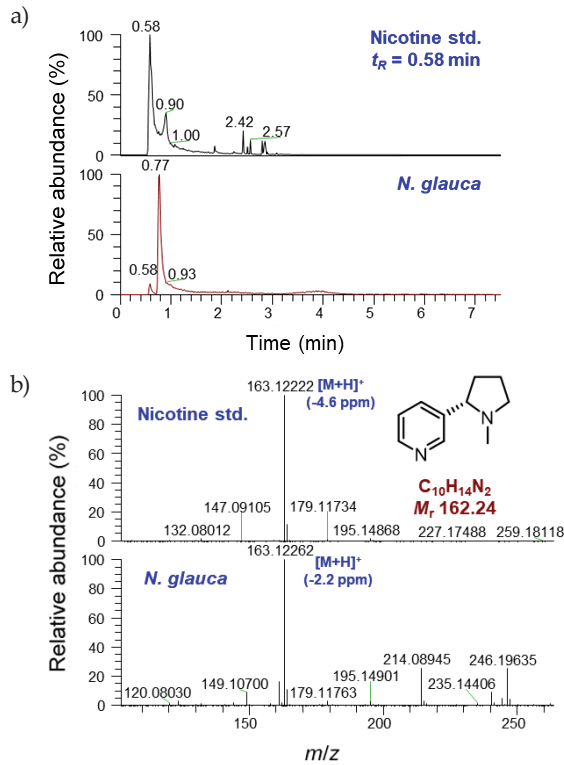


Fig. 4. a) (+)-ESI SIC of *N. glauca* ( $m/z$ : 163) of nicotine standard and *N. glauca* extract. b) (+)-ESI HRMS of nicotine standard and in *N. glauca*.

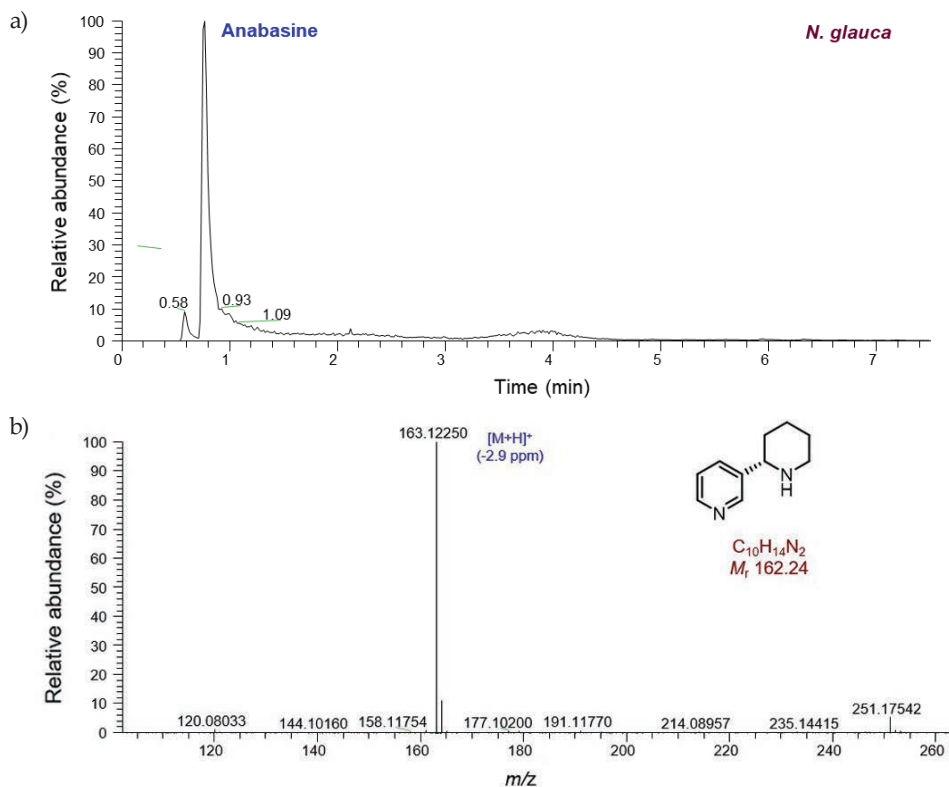


Fig. 5. MS detection of anabasine in *N. glauca* extract: a) (+)-ESI SIC of *N. glauca* extract (anabasine *m/z*: 163), b) (+)-ESI HRMS of anabasine in *N. glauca* extract.

being the major one, namely: 5-pentanolactam, anabasine, 1,2,3,4-tetrahydro-pyridine-2,5-dicarbonitrile, 1,2,3,5,9b-penta-azacyclopenta[ $\alpha$ ]naphthalen-4-ol, and 1-ethynyl-1-isocyanocyclohexane that eluting at 11.5, 17.2, 17.6, 18.4 and 18.9 min, resp. (Figs. 6, 7 and S11-S17).

The anabasine content in an alkaloid-rich fraction of the *N. glauca* leaves was also quantified using GC-MS using the calibration curve of an authentic reference standard of anabasine and found to be 1 mg g<sup>-1</sup> dry plant material.

*Isolation of anabasine from N. glauca alkaloid-rich fraction (F-2).* – Anabasine was successfully isolated from the alkaloid-rich fraction (F-2) of *N. glauca*. The described reversed-phase prep-HPLC yielded two sub-fractions. The first fraction was found to be impure and was hence discarded from further analysis. Sub-fraction 2 that eluted at a retention time of ~6.5 min yielded anabasine (29.91 mg) (Fig. 8). The chemical structure of anabasine was elucidated by the means of HRESI-MS and NMR data (Fig. 9). The spectroscopic and spectrometric data matched those reported in the literature for anabasine (24, 25). Anabasine was found to be the major constituent of approximately 60 % (*m/m*) in F-2 fraction.

As reported above, *N. glauca* leaf extract was analyzed by the means of UPLC-HRESI-MS and GC-MS. In both methods, anabasine was identified as the major constituent. In the



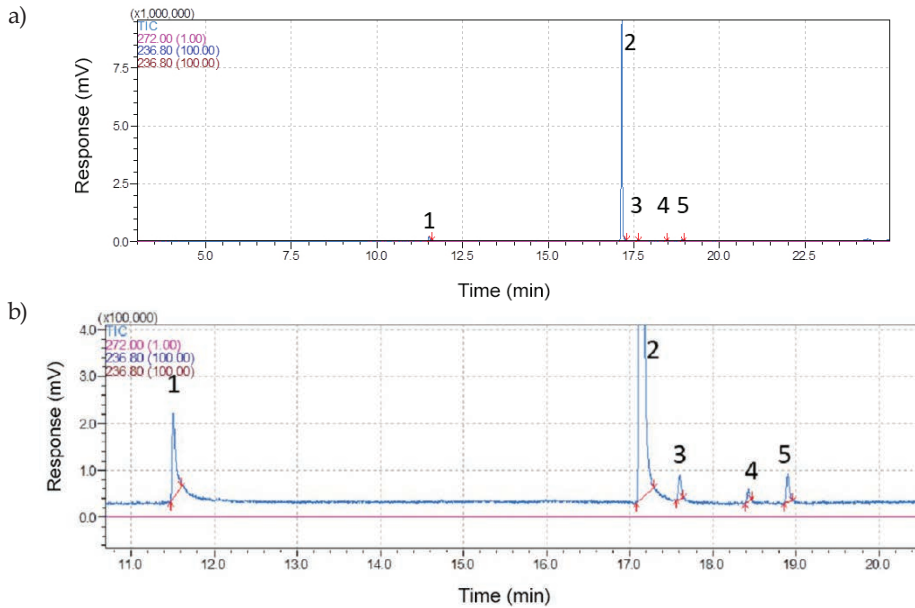


Fig. 6. a) GC-MS TIC chromatogram of the alkaloid-rich fraction (F-2) of *N. glauca*. Five peaks were detected in the chromatogram at the following retention times: 11.5 (1), 17.2 (2), 17.6 (3), 18.4 (4), and 18.9 (5) min, which were identified as 5-pentanolactam, anabasine, 1,2,3,4-tetrahydro-pyridine-2,5-dicarbonitrile, 1,2,3,5,9b-pentaaza-cyclopenta[*a*]naphthalen-4-ol and 1-ethynyl-1-isocyano-cyclohexane, resp. b) Zoom display of a).

literature, *N. glauca* was reported to contain the following main secondary metabolites: (*S*)-(-)-anabasine, (*R*)-(+)-anabasine, (*S*)-(-)-nicotine, nor nicotine, myosmine, anatabine, isoni-cotine, 4-methoxycinnamoyl ethylenediamine,  $\beta$ -sitosterol, chlorinated amides, scopoletin,

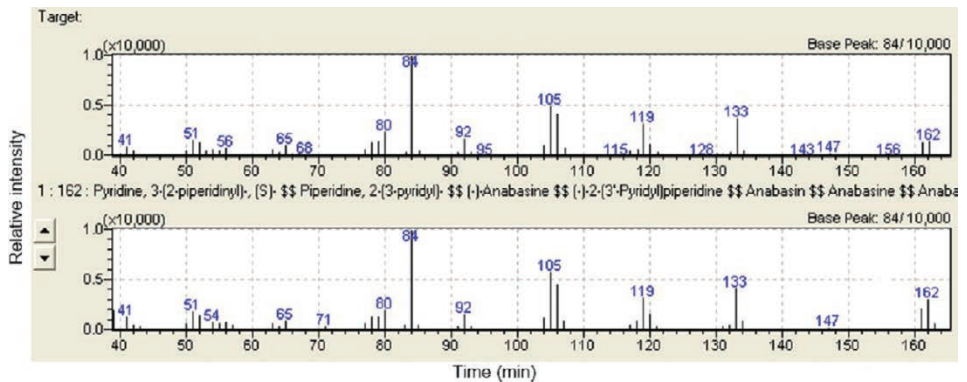


Fig. 7. GC-MS EI MS spectrum of the peak eluted at 17.2 min and hit #1 of the library search (NIST05): anabasine.



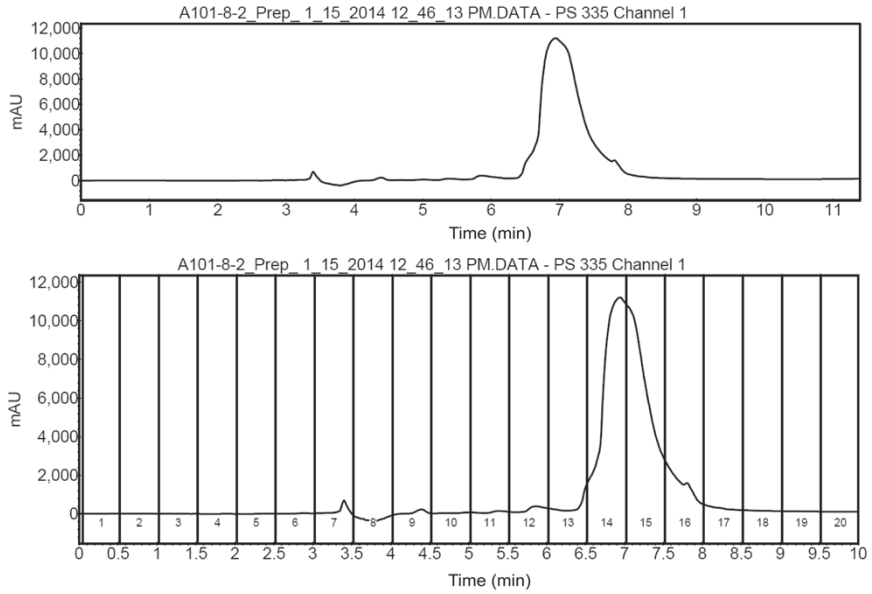


Fig. 8. Preparative HPLC chromatogram of the alkaloid-rich fraction of *N. glauca*. mAU – milli absorbance unit-

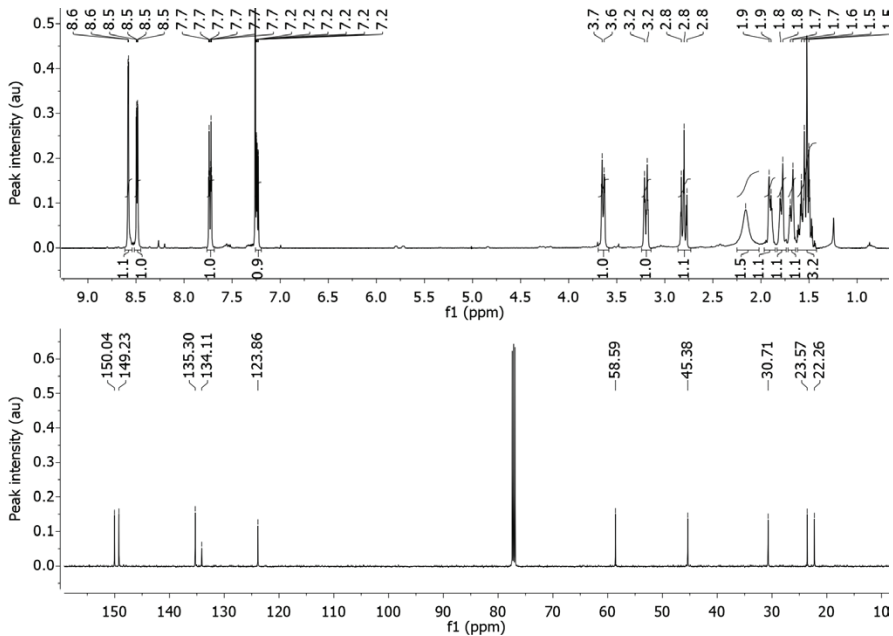


Fig. 9.  $^1\text{H}$  NMR spectrum of anabasine (400 MHz,  $\text{CDCl}_3$ ).

rutin, and  $\beta$ -sitosterol-3-*O*- $\beta$ -D-glucopyranoside (3, 18, 26). The chemical profile of *N. glauca* from Jordan was found to be comparable to those in the literature with anabasin as the major constituent. However, there are differences in the chemical profile regarding minor compounds, namely, myosmine, anatabine, isonicotinic acid, 4-methoxycinnamoyl ethylenediamine,  $\beta$ -sitosterol, chlorinated amides, scopoletin,  $\beta$ -sitosterol-3-*O*- $\beta$ -D-glucopyranoside, anabaseine, ammodendrine and chlorogenic acids.

Table I. Chemical composition of the essential oil hydrodistilled from the leaves of *N. glauca* by GC-MS<sup>a</sup>

Code	RI (exp)	RI (lit)	Compound	Content (%)
1	991	985	2-Pentylfuran	0.63
2	1002	1002	2- $\delta$ -Carene	0.79
3	1124	NA	1-Methyl,3-cyclohexene-1-carboxaldehyde	1.01
4	1158	1154	(2 <i>E</i> , 6 <i>Z</i> )-Nonadienal	0.50
5	1165	1162	2 <i>E</i> -nonen-1-al	1.07
6	1210	1202	<i>n</i> -decanal	1.02
7	1296	NA	Dihydroedulan	1.38
8	1326	NA	2,4-Decadienal	1.10
9	1383	1385	$\beta$ -Damascenone	1.77
10	1422	1419	<i>trans</i> -Caryophyllene	0.78
11	1483	1489	$\beta$ -Ionone	2.36
12	1515	1510	Tridecanal	1.23
13	1521	1523	$\delta$ -Cadinene	1.04
14	1542	1539	$\alpha$ -Cadinene	0.52
15	1552	1550	$\alpha$ -Agarofuran	1.02
16	1563	NA	Isoaromadendrene epoxide	0.82
17	1567	NA	5 $\beta$ ,7 $\beta$ H,10 $\alpha$ -eudesm-11-en-1 $\alpha$ -ol	0.71
18	1587	1583	Caryophyllene oxide	0.54
19	1616	NA	<i>trans</i> -Epinephrine	6.23
20	1628	1624	Epi- $\gamma$ -eudesmol	0.54
21	1692	1686	$\alpha$ -Bisabolol	9.02
22	1719	1715	Longifolol	4.27
23	1780	NA	Hexadecenal	1.60
24	1835	1876	Hexadecenol	1.22
25	1843	NA	2-Pentadecanone-6,10,14-trimethyl	0.54
26	1889	NA	9,17-Octadecadienal	10.83
27	1896	NA	Ethyl linoleate	34.79
28	1922	-	Unknown	2.29
29	1979	NA	Hexadecanoic acid	10.38

NA – not applicable, RI – retention index

<sup>a</sup> Anabasin was non-detectable (or in trace amount) in the analyzed samples and was only traceable *via* the SIM technique.

### GC-MS analysis of the essential oil

The chemical composition of volatile oil hydrodistilled from the leaf of *N. glauca* has been analyzed by the means of GC-MS using Kovats retention index. A total of 29 compounds were identified (Table I). The volatile oil was found to be rich in oxygenated sesquiterpenes (*e.g.*,  $\beta$ -bisabolol) and carboxylic acids and esters (*e.g.*, ethyl linoleate and hexadecanoic acid) (Table I). However, anabasine was undetectable in the studied leaf volatile oil (Table I). Schlotzhauer *et al.* (27) reported the analysis of the volatile oil flowers of *N. rustica* and of *N. forgetiana* by means of capillary GC and GC-MS. Of the 17 compounds that were identified in *N. rustica* oil, nicotine and aromadendrene were found to be the major ones. On the other hand, a total of 14 compounds were identified in *N. forgetiana* oil with farnesyl acetone and  $\beta$ -pinene detected in the highest concentrations.

### CONCLUSIONS

UPLC-MS and GC-MS analysis identified anabasine, a piperidine alkaloid, as the major constituent in the leaves of *Nicotiana glauca* Graham (Solanaceae). The GC-MS analysis revealed five compounds with anabasine as the major component, while nicotine was not detected. This may be in line with the speculated high toxicity of *N. glauca* due to anabasine.

Moreover, GC-MS was used for the analysis of the volatile oil that was obtained by hydrodistillation from the leaves of *N. glauca*. The volatile plant oil was found to be rich in oxygenated sesquiterpenes (*e.g.*,  $\beta$ -bisabolol) and carboxylic acids and esters (*e.g.*, ethyl linoleate and hexadecanoic acid), whereas anabasine was not detectable.

*Acknowledgements.* – The authors acknowledge the overhead and logistic support from Jordan University of Science and Technology. The high-resolution mass spectrometry data were acquired in the Triad Mass Spectrometry Laboratory at the University of North Carolina at Greensboro.

Supplementary material available upon request.

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