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Identification and quantification of hepatotoxic pyrrolizidine alkaloids in the Ethiopian medicinal plant *Solanecio gigas* (Asteraceae)

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The pyrrolizidine alkaloid content of *Solanecio gigas* (Vatke) C. Jeffrey (Asteraceae), an Ethiopian medicinal plant widely used for the treatment of colic, diarrhea, gout, otitis media, typhoid fever, and noted for its wound dressing and antiabortifacient activities was studied. The flower and leaf extracts contained 0.19% and 0.14% alkaloids (dry weight), respectively. GLC-MS analysis indicated that all the alkaloids in the flowers are pyrrolizidine alkaloids (PAs), whereas the leaves contain other type of alkaloids with PAs occurring in low concentrations. Roughly, 80% and 90% of the total PAs in the flowers and the leaves, respectively, were shown to occur as *N*-oxides. Eighteen alkaloids were detected in the flower extract with the retronecine type twelve-membered macrocyclic diesters integerrimine, senecionine and usaramine comprising 82% of the total PA content. Analysis of the PA profile of the leaves indicated that it has a simpler pattern than the one observed for the flowers. Only five PAs were detected in the leaves with integerrimine making up about 50% of the total PAs. Quantification of the PA content by GLC showed that the flowers and leaves contain 3321.21 and 84.84 μg per 10 g of dried plant material, respectively. These results indicate that users of this herb are at high risk of poisoning since the most toxic twelve membered macrocyclics of the retronecine type are the dominant PAs in the plant.

1. Introduction

Solanecio gigas (Vatke) C. Jeffrey (Asteraceae) is a giant rosette herb or shrub with soft woody stems growing to 4 m high. The leaves are large and shallowly lobed with dull yellow flower heads which are clustered in large terminal panicles (Tadesse 2004). All parts of the plant, but especially the flowers, have an unpleasant or mushy odour. Although it is mainly found along stream banks in wet evergreen forests, it also grows as a hedge-plant in church compounds and house-gardens in villages and is very common throughout the highlands of Ethiopia.

S. gigas is endemic to Ethiopia where it is well known by various vernacular names, which include "Yeshkoko-Gomen", "Abezenta" and "Nobe". It is also one of the most widely used plants in Ethiopian traditional medicine. The aboveground whole plant (stems, leaves and flowers) are used for the treatment of colic, diarrhea, gout, otitis media, typhus, for wound dressing, as an anti-abortifacient and to improve the mental faculty (Desta 1994; Getahun 1998; Abebe et al. 2003). Extracts of the roots are also employed against typhoid fever (Fichtl and Adi 1994). Despite its wide application in traditional medicine, however, there appears to have been no report in the literature concerning the chemical constituents of the plant. To date, the only published reports available on *S. gigas* are investigation of the ecophysiology of niche occupation of the plant in a small mountain valley in afromontane forest valley of central Ethiopia (Lüttge et al. 2001), and the activity of its leaf extracts against a limited number of bacteria and fungi (Desta 1994).

S. gigas belongs to the tribe Senecioneae, which contains genera such as *Cacalia*, *Crassocephalum*, *Emilia* and *Senecio*, all of which are adept in the biosynthetic elaboration of hepatotoxic pyrrolizidine alkaloids (PAs) (Cava 1968; Asada 1985; Cheng 1986; Mattocks 1986). The wide application of *S. gigas* as a herbal medication and its close taxonomical similarities with the extensively studied *Senecio* species prompted our interest in the scientific investigation of this herb. The aim of the present study is therefore to check if PAs are present in this medicinal herb and if so, to quantify the level of alkaloids in flowers and leaves of the plant.

2. Investigations, results and discussion

2.1. Alkaloid profiles

Acid-base partition of the hydroalcoholic extract of the flowers and leaves of *S. gigas* with and without zinc treatment allowed us to determine the content of free PAs and N-oxides in the plant parts. Thus, the proportions of PAs present as N-oxides in the flowers and leaves were found to be 92% and 81% of the total PA content, respectively.

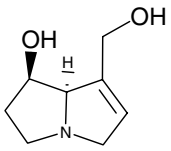
Table 1: Alkaloids identified in *S. gigas* leaves and flowers by GLC-MS

Alkaloid	RI	$[M]^+$	Characteristic ions m/z (relative abundance)
Retronecine **(1)**	1449	155	155 (23), 138 (2), 11 (61), 94 (20), 80 (100), 68 (16), 53 (11)
Unknown A **(2)**	2231	321	321 (4), 222 (25), 144 (28), 136 (4), 121 (86), 120 (44), 119 (4), 108 (43), 93 (16), 83 (100), 59 (23), 55 (68)
Unknown B **(3)**	2248	335	335 (1), 256 (33), 228 (5), 160 (5), 136 (13), 121 (65), 120 (100), 119 (15), 106 (25), 94 (13), 93 (10), 80 (9), 55 (5)
Unknown C **(4)**	2259	337	337 (30), 208 (19), 136 (32), 120 (100), 119 (74), 93 (71), 80 (21), 55 (18)
Unknown D **(5)**	2278	333	333 (1), 222 (13), 208 (4), 194 (7), 177 (8), 153 (5), 122 (16), 121 (90), 120 (56), 108 (40), 93 (14), 83 (100), 55 (48)
Unknown E **(6)**	2297	337	337 (6), 222 (26), 138 (44), 136 (79), 121 (78), 120 (100), 119 (72), 95 (99), 93 (71), 80 (28), 55 (22)
Senecionine **(7)**	2308	335	335 (14), 246 (19), 220 (18), 138 (45), 136 (96), 120 (100), 119 (81), 109 (27), 95 (50), 94 (70), 93 (72), 80 (33), 67 (14), 53 (25)
Seneciphylline **(8)**	2318	333	333 (6), 246 (9), 138 (34), 136 (71), 120 (100), 119 (82), 95 (57), 94 (82), 93 (64), 80 (31), 67 (18), 53 (15)
Bulgarsenine **(9)**	2352	337	337 (4), 246 (2), 211 (35), 140 (100), 138 (63), 123 (39), 122 (68), 96 (33), 95 (35), 82 (95), 53 (16)
Spartioidine **(10)**	2354	333	333 (1), 289 (6), 246 (11), 166 (79), 138 (39), 137 (77), 136 (64), 120 (100), 119 (73), 108 (32), 95 (61), 94 (65), 93 (61), 80 (32), 55 (40)
Integerrimine **(11)**	2362	335	335 (6), 291 (12), 248 (12), 220 (21), 153 (6), 138 (46), 136 (87), 121 (77), 120 (99), 119 (100), 109 (24), 95 (68), 93 (85), 80 (31), 53 (25)
Neoplatyphylline **(12)**	2386	337	337 (5), 220 (3), 211 (36), 180 (8), 140 (100), 138 (47), 123 (45), 122 (74), 108 (9), 96 (26), 95 (15), 82 (92), 55 (16)
Sarracine **(13)**	2411	337	337 (3), 237 (16), 222 (17), 140 (47), 139 (31), 138 (100), 123 (65), 122 (65), 95 (38), 82 (70), 55 (22)
Neosarracine **(14)**	2434	337	337 (1), 237 (29), 222 (20), 140 (36), 139 (33), 138 (100), 123 (37), 122 (32), 108 (63), 95 (56), 82 (50), 55 (26)
Unknown F **(15)**	2436	337	337 (9), 319 (43), 308 (28), 155 (23), 136 (35), 124 (23), 120 (30), 119 (74), 111 (73), 94 (29), 93 (11), 80 (100), 68 (13), 55 (23)
Unknown G **(16)**	2468	337	337 (10), 319 (52), 308 (30), 280 (5), 155 (24), 136 (24), 120 (17), 119 (80), 11 (70), 106 (50), 94 (28), 93 (11), 83 (28), 80 (100), 68 (15), 55 (23)
Unknown H **(17)**	2476	369	369 (6), 338 (7), 226 (6), 155 (33), 138 (100), 136 (24), 120 (16), 119 (13), 94 (52), 93 (83), 80 (30), 55 (14)
Unknown I **(18)**	2521	337	337 (1), 288 (2), 155 (8), 138 (65), 137 (44), 136 (29), 119 (12), 94 (39), 93 (100), 80 (17), 55 (10)
Neosenkirkine **(19)**	2554	365	365 (4), 337 (9), 294 (17), 266 (19), 250 (23), 222 (18), 211(21), 168 (26), 151 (100), 123 (54), 110 (64), 96 (40), 94 (21), 81 (50)
Unknown J **(20)**	2593	339	339 (3), 72 (14), 207 (8), 153 (19), 138 (65), 137 (76), 136 (58), 121 (17), 120 (10), 119 (13), 109 (15), 94 (52), 93 (100), 80 (23), 67 (12), 55 (15)
Usaramine **(21)**	2604	351	351 (11), 246 (5), 220 (13), 138 (36), 136 (84), 121 (73), 120 (96), 119 (100), 93 (73), 80 (29), 53 (23)

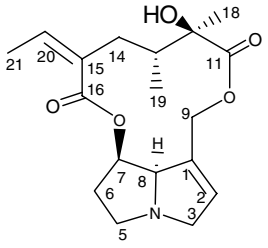
As shown in Table 1, GLC-MS analysis of the reduced alkaloid extract of the flowers of *S. gigas* demonstrated the presence of eighteen alkaloids of which ten were identified by comparison of their RIs, molecular masses and mass fragmentation patterns with defined reference data from our PA database or in some cases with reference compounds. Six of the identified compounds were unsaturated alkaloids. Of these, four were twelve-membered macrocyclic esters based on retronecine. One of the alkaloids detected was a twelve-membered macrocyclic but with the necine base otonecine (N-methylated azacyclooctan-4-one ring system). The unsaturated necine base retronecine was also detected in the sample as a minor component. The remaining four alkaloids were saturated alkaloids based on platynecine base. Two of them were open chain diesters and the remaining two twelve-membered macrocyclics.

Eight alkaloids were also present in the sample in relatively low concentrations. As the RIs and fragmentation patterns of these alkaloids were not consistent with the data of any of the alkaloids documented in the literature or in our database, they were identified as unknown B (RI = 2248), unknown C (RI = 2259), unknown E (RI = 2297), unknown F (RI = 2436), unknown G (RI = 2468), unknown H (RI = 2476), unknown I (RI = 2521) and unknown J (RI = 2593). The precise structures of these alkaloids could not be determined from the data obtained by GLC-MS analysis. However, some important structural features were obtained from their MS fragmentation patterns. Inspection of the mass spectra of these alkaloids (Table 1) revealed that although their RIs and/or molecular masses are different from each other, they all contain peaks with m/z 136, 120, 119, 93. Perusal of literature unveils that in a number of cases the presence of these four fragment ions has served as a basis for identification of the retronecine nucleus of alkaloids with a twelve-membered macrocyclic ring (Bhacca and Sharma 1968; Crout 1969; Coucourakis and Gordon-Gray 1970). In addition to these peaks, the other fragment ions in the region m/z 155-55 of the spectra of these alkaloids, which are due largely to fragments of the pyrrolizidine ring, are strikingly similar with those of retronecine (Table 1). It has been documented that the main fragmentation pathways of the macrocyclic diesters leave the necine base until a late stage (Neuner-Jehle et al. 1965; Atal 1966). Thus, it can be concluded that all the unknown alkaloids that were detected in the flower extracts of *S. gigas* are most likely twelve membered macrocyclic diesters of retronecine.

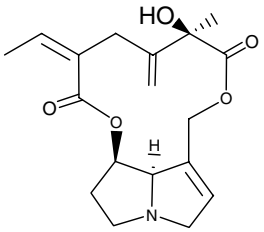
The flower extract of *S. gigas* is dominated by the unsaturated twelve membered macrocyclic diesters. Integerrimine, senecionine and usaramine were the three major PAs that comprised more than 80% of the total



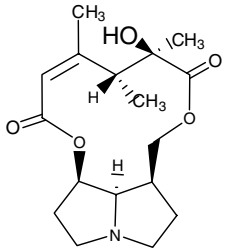
Retronecine **(1)**



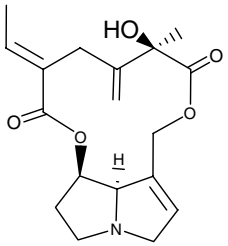
Senecionine **(7)**



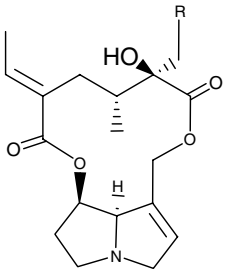
Seneciphylline **(8)**



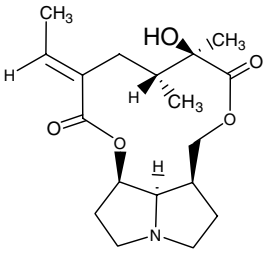
Bulgarsenine **(9)**



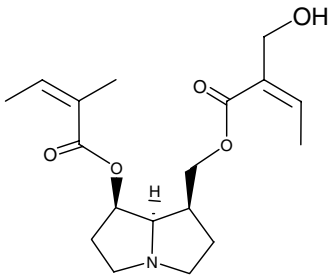
Spartioidine **(10)**



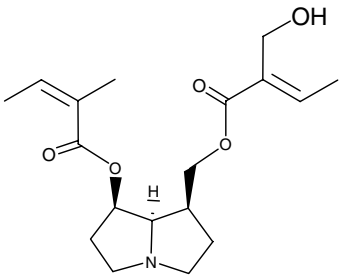
R=H Integerrimine **(11)**
R=OH Usaramine **(21)**



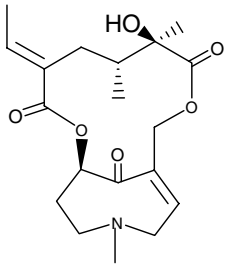
Neoplatyphylline **(12)**



Sarracine **(13)**



Neosarracine **(14)**



Neosenkirkine **(19)**

PA content of the extract (Table 2). Senecionine and integerrimine are isomers having indistinguishable mass spectra; identifications were based on their RIs and comparisons with authentic standards of each compound. Usaramine (hydroxyintegerrimine) was identified on the basis of its distinct mass spectrum which contains intense peaks at m/z 119 and 120. Moreover, it was retained on GC much longer than senecionine or integerrimine (Witte et al. 1993).

The four saturated PAs that were found in the flower sample were bulgarsenine, neoplatyphylline, sarracine and neosarracine. Although the four alkaloids have the same molecular weight, the mass fragmentation patterns of the latter two compounds are similar but significantly different from those of the former two. All these alkaloids were detected as very minor components and they accounted for roughly 2.5% of the total PA content (Table 2).

The leaf extracts of *S. gigas* showed a simpler PA pattern than the one observed for the flower extracts although alkaloids other than PAs, which are not the focus of this study were also detected. Only five PAs were found in detectable levels as shown in Table 2. Three of them were positively identified as twelve-membered macrocyclic diesters of the retronecine type with integerrimine and spartioidine making up more than 70% of the total PA content. It is interesting to note that the flowers contain seneciphylline while its *E*-isomer spartioidine occurs in the leaves. The saturated platynecine-based alkaloid neoplatyphilline was detected as a minor component making up about 5% of the total PAs present in the leaves.

Table 2: Profiles and concentrations of PAs in the leaves and flowers of *S. gigas* as quantified by GLC

Alkaloid	RI	Alkaloid composition (% relative abundance)*	
		Leaves	Flowers
Retronecine **(1)**	1449	ND	tr
Unknown A **(2)**	2231	4.72	ND
Unknown B **(3)**	2248	ND	1.34
Unknown C **(4)**	2259	ND	0.36
Unknown D **(5)**	2278	18.52	ND
Unknown E **(6)**	2297	ND	0.87
Senecionine **(7)**	2308	ND	22.76
Seneciphylline **(8)**	2318	ND	1.35
Bulgarsenine **(9)**	2352	ND	0.33
Spartioidine **(10)**	2354	21.31	ND
Integerrimine **(11)**	2362	50.47	54.48
Neoplatyphylline **(12)**	2386	4.98	1.43
Sarracine **(13)**	2411	ND	0.36
Neosarracine **(14)**	2434	ND	0.24
Unknown F **(15)**	2436	ND	2.13
Unknown G **(16)**	2468	ND	1.67
Unknown H **(17)**	2476	ND	3.15
Unknown I **(18)**	2521	ND	3.68
Neosenkirkine **(19)**	2554	ND	0.41
Unknown J **(20)**	2593	ND	0.37
Usaramine **(21)**	2604	ND	5.08
Total PAs (μg/10 g dry weight)		84.84	3321.21

* Total alkaloid 100%
ND = Not detected; tr = traces (amounts less than < 0.1% of total PA)

Two alkaloids identified as unknown A (RI = 2231) and unknown D (RI = 2278) were also detected in the leaf extract of *S. gigas*. These components accounted for 4.72% and 18.52% of the total PA content, respectively. Further structural analysis of these alkaloids was not possible due to the small amount of total PAs present in the leaves. However, the compounds have typical mass fragmentation patterns of PAs showing peaks at m/z 136, 121–119, and 93, characteristic fragments for the retronecine part of retronecine-based alkaloids (Neuner-Jehle et al. 1965; Rashkes 1978).

2.2. Toxicological implications

It is now well recognised that PAs cause acute toxicity, chronic toxicity and genotoxicity in large variety of animal species. Whilst acute poisoning causes massive hepatotoxicity and hemorrhagic necrosis, chronic poisoning takes place mainly in the liver, lungs and blood vessels (Mattocks 1986). Like most genotoxic compounds, PAs require metabolic activation to exert genotoxicity (Fu et al. 2002). One of the most important pathways responsible for PA intoxication is hydroxylation at the C-3 or C-8 position of the necine base to form 3- or 8-hydroxy necine derivatives followed by dehydration, to form the corresponding dehydropyrrolizidine (pyrrolic) derivatives (Yang et al. 2001). For such metabolic activation to take place, 1,2 unsaturation, an esterified allylic hydroxyl group at C_9 and an esterified alcoholic hydroxyl group at C_7 are essential features.

PAs of the retronecine, otonecine and platynecine types occur in the flowers, whereas the leaves contain PAs which are based on retronecine and platynecine necine bases. As indicated above, the platynecine type alkaloids cannot undergo oxidation to pyrrolic metabolites because of the absence of a double bond at 1,2 position and therefore are not toxic. On the other hand, the retronecine type alkaloids are abundant in nature and are well known to be highly toxic because of their potential to be converted to pyrrolic intermediates. Although literature reports on the metabolic activation of otonecine type PAs and the induced hepatotoxicity are rather scarce, Lin and co-workers identified a dehydroretronecine metabolite from an otonecine type PA confirming that these type of alkaloids is also enzymatically converted to metabolites which are similar to those formed from retronecine type PAs (Lin et al. 1998, 2000). As a result of this conversion, these class of alkaloids is also potentially toxic.

A study conducted by Frei et al. (1992) has shown that senecionine, one of the major alkaloids in the flowers of *S. gigas*, is among the alkaloids with the highest mutagenic activity. Similarly, in rats senecionine, seneciphylline and usaramine were reported to have an acute lethal dose of less than 0.1 mmol/kg body weight (Culvenor et al. 1976).

GLC quantification of PAs in the extracts of *S. gigas* using senecionine as an external standard revealed that in 10 g of dried plant material the flowers and leaves contain 3321.21 and 84.84 μg of PAs, respectively. In both organs the predominant PAs contain a 1,2-unsaturated necine skeleton. The health hazard that may arise as a result of consuming this herb is therefore apparent. According to Culvenor (1983), a dose of 0.7–1.5 mg/kg per day of the most toxic twelve membered macrocyclic alkaloids such as retronecine can cause liver necrosis, liver fibrosis, and cirrhosis within 2 weeks of intake. The toxic dose for children could go down to 0.01–0.06 mg/kg per day because of the greater sensitivity of children to the effects of this type of alkaloids (Jago 1970). It has also been reported that a single intake of 10 to 20 mg of an alkaloid or alkaloid mixture can cause acute toxicity with enlargement of liver cells and liver nuclei, disturbances of liver metabolism, inhibition of mitosis due to DNA blocking and fatty degeneration (Roeder 1995). However, long-term administration of smaller doses (less than 10 μg) may cause liver cirrhosis and carcinomas with exposure to the most toxic PAs. Although, PAs do not accumulate in any parts of the body, the prolonged use of these compounds may increase the chance for developing liver diseases (Mattocks 1986). The World Health Organization in 1989 suggested that the lowest intake rate of PAs that reportedly caused veno-occlusive disease in a human was just 0.015 mg/kg of body weight per day (Dharmananda 2001).

In light of the above, several governments and industrial agencies have proposed or set regulations to limit the exposure of the public to these alkaloids. For example in Belgium the limit of PAs has been proposed to be 1 μg/g of herb; the German Health Administration has limited the daily intake of PAs to 1 μg with limited administration of a total of six weeks per year. Likewise, the American Herb Products Association has recommended that all botanicals containing PAs to be used only for external application; they should not even be applied to broken or abraded skin (Dharmananda 2001).

Based on the aforementioned recommended limits, the PA content of *S. gigas* surely provides reasons for concern at the human consumption of this plant. *S. gigas* is also one of the most important plants that contribute to the honeybee flora of Ethiopia (Fichtl and Adi 1994). Thus, honey produced from flowers of *S. gigas* may contain PAs further contributing to exposure of consumers to PAs.

It is therefore necessary for the regulatory bodies to set a regulation that prevents ingestion of the herb, in any form, or at least minimize its use as far as possible. It is also recommended that honey originating from flowers of *S. gigas* should be evaluated for its PA content.

3. Experimental

3.1. Plant material and a reference alkaloid

Leaves and flowers of *S. gigas* were collected on 22 April 2006, from Kebena area, northern part of Addis Ababa at an altitude 2400 m and were identified by Ato Melaku Wondafrash (the National Herbarium, Department of Biology, Addis Ababa University), where a voucher specimen (#2759) was deposited for future reference. Standard senecionine was purchased from Roth (Carl Roth GmbH + Co, Karlsruhe, Germany).

3.2. Alkaloid extraction

About 20 g (accurately weighed) of each of the dried and powdered plant parts were extracted (3×24 h) with of 80% methanol (150 ml) in a shaker. The combined extracts were evaporated to dryness under reduced pressure, dissolved in 40 ml of 1 M HCl and filtered. The filtrate was washed with dichloromethane until the washings were colourless. The acidic extract was divided into two equal portions. Zinc dust was added to one of the portions, stirred for 12 h and centrifuged. Similarly, the unreduced portion was also left aside for 12 h. The two portions were then treated identically as follows. Each portion was filtered and made alkaline (pH 9) with concentrated ammonia and applied onto columns packed with Chem Tube Hydromarix (Varian, Inc., Palo Alto, CA, USA). The alkaloids were eluted with dichloromethane (300 ml). The organic solvent was evaporated under reduced pressure to yield alkaloid extracts. The alkaloid extract obtained from zinc reduction represents total PAs as tertiary bases and the amount of free PAs in the plant were obtained from the unreduced alkaloid extracts. The percentage of N-oxides in the sample was calculated according to the following equation (Stelljes and Seiber 1990).

$$\% \text{ N-oxides} = \frac{\text{Total PAs} - \text{Free PAs}}{\text{Total PAs}}$$

3.3. GLC analysis

Quantification of PAs was performed using Varan 3400 gas chromatograph equipped with 30 m × 0.25 μm OV-1 bonded with 0.25 μm thick layer column (Ohio Valley, Marietta, Ohio, USA) and FID detector. Carrier gas (helium) flow rate was 1.0 ml/min. Injector temperature was 250 °C and the oven temperature was programmed from 100 °C to 300 °C at 4°/min after an initial 1-min-hold and a 6 min final hold. All PAs eluted within 40 min. PeakSimple 2000 chromatography data system (SRI Instruments, California, USA) was used for recording and integrating of the chromatograms. Senecionine was used as external standard for quantification. It was assumed that all the PAs responded to the detector identically to senecionine.

3.4. GLC-MS analysis

The analyses were carried out on Helwett-Packard gas chromatograph (GC 5890 II, Helwett-Packard GmbH, Bad Homburg, Germany) equipped with OV-1 bonded capillary column (30 m, i.d.: 250 μm, film thickness: 0.25 μm) (Ohio Valley, Marietta, Ohio, USA). The capillary column was directly coupled to a quadrupole mass spectrometer (SSQ 7000, Thermo-Finnigan, Bremen, Germany). Samples were injected (1 μl) with split mode (split ratio, 1 : 20). The injector temperature was 250 °C. Helium carrier gas flow rate was 1.0 ml min^{-1} with a pressure of 14 psi. The column temperature programme was 100–300 °C, 6 °C min^{-1} and/or 100–300 °C, 4 °C min^{-1}. All the mass spectra were recorded with the following condition: filament emission current, 200 μA; electron energy, 70 eV; ion source, 175 °C; mass range, 60–150; scan time, 0.5 s.

3.5. Alkaloid identification

Individual PAs were identified by comparing their RIs, molecular masses and mass fragmentation patterns with defined reference data from our PA database or the literature (Witte et al. 1993; El-Shazly et al. 1996, 1998, 1999; Asres et al. 2003) and in some cases with reference compounds. RIs were calculated using co-chromatographed standard hydrocarbons (C_8–C_{28}).

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