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PROTEIN AND ALKALOID PATTERNS OF THE FLORAL NECTAR IN SOME SOLANACEOUS SPECIES

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The family *Solanaceae* includes several melliferous plants, which tend to produce copious amounts of nectar. Floral nectar is a chemically complex aqueous solution, dominated by sugars, but minor components such as amino acids, proteins, flavonoids and alkaloids are present as well. This study aimed at analysing the protein and alkaloid profile of the nectar in seven solanaceous species. Proteins were examined with SDS-PAGE and alkaloids were analyzed with HPLC. The investigation of protein profile revealed significant differences in nectar-protein patterns not only between different plant genera, but also between the three *Nicotiana* species investigated. SDS-PAGE suggested the presence of several Nectarin proteins with antimicrobial activity in *Nicotiana* species. The nectar of all tobacco species contained the alkaloid nicotine, *N. tabacum* having the highest nicotine content. The nectar of *Brugmansia suaveolens, Datura stramonium, Hyoscyamus niger* and *Lycium barbarum* contained scopolamine, the highest content of which was measured in *B. suaveolens*. The alkaloid concentrations in the nectars of most solanaceous species investigated can cause deterrence in honeybees, and the nectar of *N. rustica* and *N. tabacum* can be considered toxic for honeybees.

Keywords: Brugmansia – Datura – Hyoscyamus – Lycium – Nicotiana – nectar

INTRODUCTION

Solanaceae are a family comprising approximately 2800 woody and herbaceous plant species in tropical and temperate regions with major species diversity in Central and South America [16]. There is a significant number of melliferous plants in the family, some of which, e.g. *Nicotiana tabacum* L. [14, 50] and *Lycium barbarum* L. [37], are apiculturally significant, too. In most representatives of the *Solanaceae* family, the base of the ovary is surrounded by a ring-shaped nectary, which is characteristic also in the genera investigated in the present study, including *Datura, Hyoscyamus, Lycium* and *Nicotiana* [11, 26, 27, 29].

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Abbreviations: HPLC – high performance liquid chromatography; SDS-PAGE – sodium dodecyl sulphate polyacrylamid gel electrophoresis

Along with the dominant sugars sucrose, glucose and fructose, other carbohydrates can also be found in lower amounts in various floral nectars, e.g. arabinose, galactose, mannose, gentiobiose, lactose, maltose, melibiose, trehalose, melezitose, raffinose and stachyose [5]. Besides sugars, nectar may contain glycosides, phenolic compounds, amino acids, reducing acids, fragrance compounds, lipids, proteins, alkaloids, antibiotics and vitamins [6, 38, 49]. Some of these minor nectar-components, such as phenolic compounds [10–11, 30, 32], amino acids [10–12, 24, 30–32, 43], antioxidant and reducing organic acids [12, 20, 31], sesquiterpenes, aromatic alcohols and aldehydes [44], as well as lipids [10, 30, 31] were reported in the floral nectar of certain *Solanaceae* species.

The protein content of nectar has already been analyzed in some solanaceous taxa [12–13, 19–23, 31, 34, 40–41, 51, 53, 60, 63]. The amino acid sequence and functions of the so-called Nectarin proteins were determined in the floral nectar of the ornamental tobacco hybrid LxS8 (*Nicotiana langsdorffii* × *Nicotiana sanderae* var. LxS8) [19–21, 53]. These proteins were shown to be enzymes with antimicrobial activity playing a key role in protecting the gynoecium against microorganisms. Other enzymes including ribonucleases, desoxyribonucleases, a peroxidase, an endochitinase, the presumed fructokinase which can prevent microbial colonization in the nectar of *Petunia hybrida;* as well as ribonucleases, a peroxidase and a chitinase were identified in the nectar of *Nicotiana langsdorffii* × *Nicotiana sanderae* var. *LxS8* [40–41].

Pyridine-, sterane- and tropane-alkaloids are characteristic for the *Solanaceae* family [1–2, 15, 42]. As early as the 1960s it was presumed that these alkaloids, as secondary metabolites, may be secreted into the nectar as well [25, 35, 39, 62]. The accumulation of allelochemicals (including alkaloids) occurring in nectar is presumed to be a result of selective secretion [48, 61]. Scopolamine and atropine could be isolated also from honeys originating from flowers of plants with high levels of these alkaloids [7]. In most cases the alkaloid-content of pollen and nectar is significantly lower than that of the leaves, flowers and other floral parts [17, 28], which does not allow the detection of these compounds in nectar samples with low-sensitivity analytical methods, as e.g. thin layer chromatography (TLC). This can be in the background of earlier failed attempts to detect alkaloids in nectar samples of solanaceous plants [4, 12, 31–32]. However, by using modern analytical techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC), a number of researchers have already succeeded in identifying as well as quantitatively determining the characteristic alkaloids in the floral nectar of various *Solanaceae* species [3, 13, 17, 28–29, 44, 57]. Some of these alkaloids, like hyoscyamine, scopolamine, tropin, nicotine and anabasine were reported to cause feeding deterrence to animal pollinators [28, 55, 57]. Alkaloids, along with some non-protein amino acids and phenolic components, belong to the most common nectar toxins [52].

The present study examines the protein profile of the floral nectar of *Nicotiana tabacum* L., *N. rustica* L., *N. alata* Link et Otto, *Cestrum* × *'Newellii'* (Veitch) Nicholson, *Lycium barbarum* L. and *Brugmansia suaveolens* Persoon; the nicotine content of the floral nectar in the above three *Nicotiana* species, as well as the atro-

pine- and scopolamine-content of the floral nectar samples of *L. barbarum, B. suaveolens, Datura stramonium* L. and *Hyoscyamus niger* L. In addition to providing new data about minor nectar components of species not investigated previously, the possible roles of the examined nectar-components are discussed.

MATERIALS AND METHODS

Investigated plant taxa

Investigated plant taxa included *Brugmansia suaveolens* Persoon, *Cestrum* × *'Newellii'* (Veitch) Nicholson, *Datura stramonium* L., *Hyoscyamus niger* L., *Lycium barbarum* L., *Nicotiana tabacum* L., *N. rustica* L. and *N. alata* Link et Otto. Voucher specimens of each investigated species were deposited at the herbarium of the Department of Plant Biology, University of Pécs, Hungary.

Nectar sampling

Floral nectar was extracted with glass capillary tubes bearing microlitre marks (CM Scientific Ltd., Silsden, United Kingdom) from flowers of 10–60 specimens (depending on the species) in the Botanical Garden of the University of Pécs, Hungary. Nectars were sampled on several different occasions between May 2005 and October 2006, during the flowering period of each species. The pooled nectar samples were stored in Eppendorf tubes at –20 °C until further use.

Determination of total protein concentration

Protein quantity determination was performed according to the procedure described in [18], using bovine serum albumin (BSA) as calibration standard.

SDS-PAGE separation of nectar proteins

The separation of nectar proteins was performed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to [47]. Fifteen µl of raw nectar per species was loaded in one pocket, using a 15% (w/v) separating gel in mini-gel system (Bio-Rad™). Protein molecular weight markers (Fermentas™) were used as standards. The proteins were made visible by using PageBlue™ Protein Staining Solution (Fermentas™). Individual protein bands were evaluated compared to molecular weight markers.

HPLC analysis of alkaloids in the nectar

HPLC analysis was performed in the months of February–April of 2007. Nectar samples were diluted twofold with 10 mM sodium phosphate buffer, then filtered with a syringe filter (Millex-HN, 0.45 μm, nylon, 33 mm, non-sterile; Merck Millipore, Darmstadt, Germany).

The high pressure liquid chromatographic (HPLC) analysis of various alkaloids was performed using a Shimadzu liquid chromatograph [two pumps (LC-10ADVP), degasser (DGU-14A), manual injector with a 20 μL loop, diode array detector (SPD-10AVP) and a computer data acquisition station]. The column used was a Phenomenex Synergy 10 μ Hydro-RP 80 (250 mm × 4.6 mm, average particle size 10 μm). All measurements were done at ambient temperature. Each properly prepared nectar sample was run three times in the HPLC-device.

The analysis of scopolamine and atropine was performed according to the procedure of [33, 46], with modifications. Scharlau HPLC grade methanol and 10 mM sodium phosphate buffer, pH 3.0 were used. The flow rate used was 1.00 mL/min. The eluent contained 35% methanol. The components were detected at 228 nm. The limit of detection (LOD) was $1.08 \cdot 10^{-3}$ mg/mL and $1.28 \cdot 10^{-3}$ mg/mL for scopolamine and atropine, respectively. The concentration limit of quantification (LOQ) was $3.28 \cdot 10^{-3}$ mg/mL and $3.87 \cdot 10^{-3}$ mg/mL for scopolamine and atropine, respectively.

Nicotine was determined according to the method of Tambwekar et al. [58], with minor changes. Scharlau HPLC grade methanol and 10 mM sodium phosphate buffer, pH 6.8 were used. The flow rates used were 1.00 mL/min. The eluent contained 65% methanol. The components were detected at 261 nm. LOD was $2.10 \cdot 10^{-3}$ mg/mL while LOQ was determined as $6.23 \cdot 10^{-3}$ mg/mL.

LCMS solution (Shimadzu) software was used to control the chromatographic system and for data processing.

RESULTS

Protein content of the floral nectar

The analysis of nectar proteins revealed that each of the investigated plant taxa contained proteins in their floral nectar, although in highly variable amounts (Table 1). Concentrations varied not only between different genera, but also within the *Nicotiana* genus. In accordance with the concentration values, the strongest protein bands in the SDS-PAGE gel can be seen in *N. rustica* (Fig. 1), which contained an order of magnitude higher concentration of nectar proteins compared to *N. tabacum*. Further analysis of the protein pattern with SDS-PAGE included the taxa with protein concentrations exceeding 50 μg/mL.

Analysis by SDS-PAGE revealed the presence of one major protein band of 60 kDa in both *N. alata* and *N. rustica* (Fig. 1). Three less prominent bands can be seen

Table 1
Nectar protein concentrations (μg/mL) of various solanaceous species, measured according to Bradford [18], using bovine serum albumin (BSA) as calibration standard

Genus name	*Brugmansia*	*Cestrum* ×	*Lycium*	*Nicotiana*		
Species name	*suaveolens*	*newellii*	*barbarum*	*alata*	*rustica*	*tabacum*
Concentration (μg/mL)	88	44	131	84.5	265.5	21

Data are from a single series of measurements.

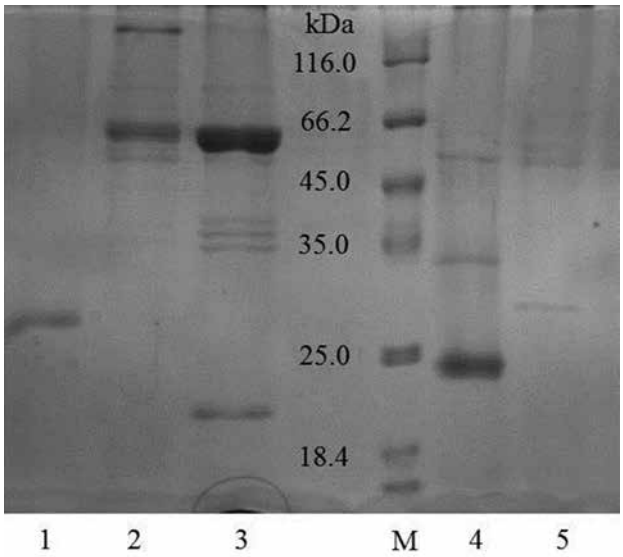

Fig. 1. SDS-PAGE separation of nectar proteins. Lane 1: *B. suaveolens,* Lane 2: *N. alata,* Lane 3: *N. rustica,* M: Protein Molecular Weight Markers (from above to below): 116.0 kDa, 66.2 kDa, 45.0 kDa, 35.0 kDa, 25.0 kDa, 18.4 kDa, Lane 4: *L. barbarum,* Lane 5: *C.* × *'Newellii'*. The nectar samples were run at least three times and, in each case, we received similar protein profiles. In each analysis, 15 μL raw nectar was loaded in gel

between 35.0 and 45.0 kDa, and a stronger one halfway between 18.4 and 25.0 kDa in *N. rustica*. The predominant protein in *B. suaveolens* occurs between 25 and 35 kDa, whereas in *L. barbarum* slightly below 25 kDa (Fig. 1).

Alkaloid content of the floral nectar

The HPLC analysis of floral nectar samples revealed that each investigated taxon contained the alkaloid specific to the given plant genus, confirming that the alkaloids

Table 2
Alkaloid concentration of floral nectar samples

Plant	Alkaloid	Concentration (µg/mL±SE)
Nicotiana alata	nicotine	0.79±0.09
Nicotiana rustica		2.53±0.14
Nicotiana tabacum		5.89±0.40
Hyoscyamus niger	scopolamine	2.92±0.13
Lycium barbarum		24.28±4.89
Datura stramonium		99.01±3.20
Brugmansia suaveolens		149.80±6.01

Data are means±standard error (SE) calculated from three measurements. The quantity of atropine remained below the concentration limit of quantification (LOQ = 3.87 µg/mL).

characteristic to various organs of a plant are secreted into the nectar, as well. Accordingly, nicotine was detected in the nectar samples of all three *Nicotiana* species, while scopolamine was identified in the samples from four other solanaceous genera (Table 2). Characteristic chromatograms of nicotine and scopolamine are demonstrated by Figs 2 and 3, respectively.

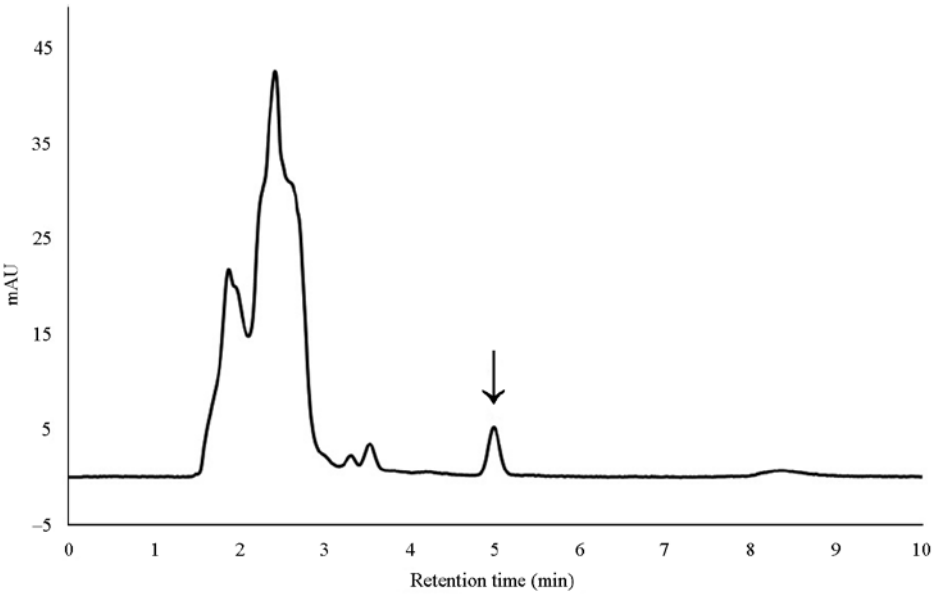

Fig. 2. Chromatogram of nicotine in *Nicotiana tabacum* nectar sample, t_R = 4.99 min (arrow)

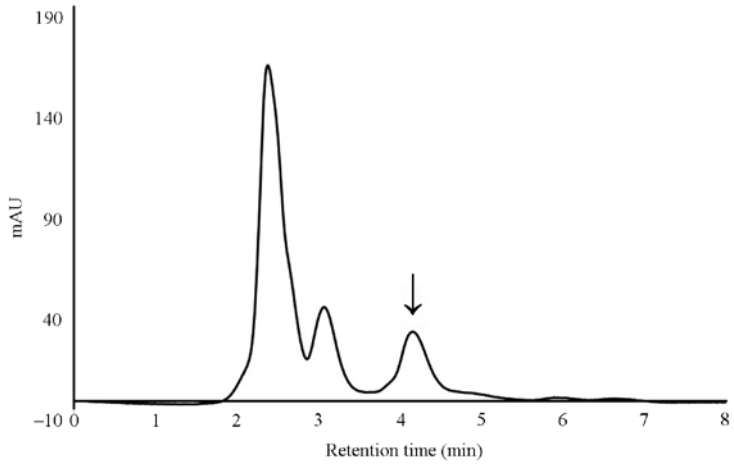

Fig. 3. Chromatogram of scopolamine in *Brugmansia suaveolens* nectar sample, t_R = 4.15 min (arrow)

DISCUSSION

Floral nectar protein concentrations 47.0, 42.2, 20.5 and 25.7 µg/mL had been measured for *N. attenuata*, *N. tabacum*, *Datura aurea* and *Cestrum purpureum*, respectively, in the same order of magnitude [13, 63] as in our study. Evaluating various *Nicotiana* taxa, *N. rustica* had higher nectar protein concentrations, compared to either the other two species involved in our study (Table 1), or to *Nicotiana* species investigated previously [13, 23, 63].

The 5 major proteins in the floral nectar of the ornamental tobacco *N. langsdorffii* ×*N. sanderae* var. LxS8, labelled Nectarin 1 to 5 were found to accumulate up to the concentrations of 240–250 µg/mL, which equals approximately the total protein concentration [19, 23, 60]. This concentration is much closer to the values measured by us in *N. rustica* (265.5 µg/mL) than to the nectar protein concentration of *N. alata* (84.5 µg/mL), even though a considerable part of the gene pool of *N. alata* can be found in the LxS8 outcrossing ornamental tobacco, since *N.* × *sanderae* is a hybrid of *N. alata* and *N. forgetiana*.

From the investigated solanaecous taxa, *Nicotiana* species share the highest number of identities in their pattern of protein bands, which can be attributed to their taxonomic proximity, and the *Nicotiana* genus significantly differs from the other 3 solanaceous species investigated.

Brugmansia×*candida* had been described as a Nectarin I (NEC1)-positive plant species [19]. In the case of loading crude nectar (without heat treatment) into the gel, NEC1 was found to migrate in a monomer form with a molecular weight of 29 kDa and as an oligomer molecule with a weight of 165 kDa. In the current study, however, only the 29 kDa band corresponding to this protein could be detected in *B. suaveolens* (Fig. 1).

Our results are congruent with those of [19] who claimed that *N. alata* is a NEC1-positive plant species. The uppermost band (165 kDa) in Lane 2 in Fig. 1 can be identified as the oligomer form of NEC1 with superoxide dismutase activity. Other nectar protein bands of *Nicotiana* species investigated in the present study also correspond to Nectarin proteins. The more powerful band slightly below the 66.2 kDa marker in both *N. alata* and *N. rustica* is the Nectarin IV (NEC4) protein with 60 kDa molecular weight and poligalacturonase inhibitor function [53] and it is a tandem of the Nectarin V (NEC5) protein with glucose oxidase activity and a molecular weight of 61–65 kDa, described by Carter [21]. The protein band faintly visible in *N. rustica* between 45 and 35 kDa can be considered to be the Nectarin III (NEC3) protein with bifunctional monodehydroascorbate reductase and carbonic anhydrase activities and a molecular weight of 40 kDa, as described by Carter and Thornburg [20]. The protein at the same height with the 35 kDa molecular weight marker can be identified as the Nectarin II (NEC2) protein with a molecular weight of 35 kDa which is to be qualified as the degradation product of NEC3.

The protein profile of *N. rustica* is the most similar to that of *N. alata*, however, the bands of the NEC2, NEC3 and the NEC4/NEC5 doublet are sharper and more clearly visible in *N. rustica*. The presumed NEC1 is positioned elsewhere in *N. rustica* compared with *N. alata*. The molecular weight of the protomer subunit of Nectarin I is 22.5 kDa [19], and in the case of *N. rustica* a protein band is visible with a molecular weight similar to this between 25 and 18.4 kDa.

As far as the quantity of nectar-alkaloids is concerned, to date there are only a few exact data related to the *Solanaceae* family. The total alkaloid concentration in the nectar of *Atropa belladonna, B. aurea* (yellow flowers), *B. aurea* (red flowers) and *N. tabacum* was determined as 273, 91.7, 7.34 and 0.166 µg/g, respectively [28]. The concentration of scopolamine was 57.04 µg/g and 4.57 µg/g in yellow flowered and red flowered *B. aurea*, respectively. In contrast, we measured higher concentrations of scopolamine in the nectar of *B. suaveolens* (Table 2). The concentration range of scopolamine and atropine was determined as 11.00–400.04 µg/mL and 0.19–37.00 µg/mL in various *Datura* species *(D. innoxia, D. metel, D. meteloides, D. tatula),* respectively, in a previous study [17]. The current study detected scopolamine for the first time in the floral nectar of *D. stramonium* and its concentration fitted within the range determined in Boros et al. [17]. We reported for the first time the presence of scopolamine in the floral nectar of *Hyoscyamus niger* and *Lycium barbarum*. The above data suggest that the concentration of alkaloids in the nectar can vary within wide ranges, not only within the same genus, but also in different varieties of the same species.

Previous studies determined 0.1–0.5 µg/mL and 0.6–5 µg/mL as the concentration range of nicotine and anabasine, respectively, in the nectar of *N. glauca* [57]; whereas in *N. tabacum* 0.33 µg/mL was measured as the concentration of nicotine and anabasine [3]. In the nectar of *N. attenuata* the concentration of nicotine was 3 ± 0.35 µg/mL [13]. The mean concentrations of nicotine measured by us in the nectar of *N. alata* and *N. rustica* were within the range of 0.79–2.5 µg/mL which was found to be attractive and even addictive for honeybees [55], whereas nicotine concentrations

in *N. tabacum* nectar slightly exceeded 5 μg/mL, which can already have aversive effect. In a different study, adult honeybee workers and even their larvae were reported to tolerate naturally occurring nectar nicotine-concentrations (0.1–5 μg/mL), with no disadvantageous effect on their emergence success and survival [45]. The same authors claimed that a nicotine concentration below 4.8 μg/mL did not cause deterrence in honeybee foraging behaviour. Due to their detoxificating enzymes, honeybees show physiological plasticity against pesticides and special floral metabolites [56, 59]. However, a higher nicotine concentration (50 μg/mL) was found to cause the post-emergence mortality of the larvae [55]. In another set of honeybee feeding experiments [28] the ED_{50} value (the concentration of alkaloid which caused 50% of the maximal possible effect) for both nicotine and scopolamine was determined as 0.3 μg/mL, i.e. already such low concentrations of the above alkaloids were found to cause deterrence. For pyridine alkaloids a dose-dependent deterrence was reported by [57]. According to the results of Detzel and Wink [28] the alkaloid concentrations measured in all the nectars in our study (Table 2) would have a negative effect on the foraging behaviour of honeybees; and even if the data of Singaravelan et al. [54] are taken into consideration, at least one of the three *Nicotiana* species investigated would deter honeybees from feeding on its nectar. The LD_{50} value for nicotine in the nectar was determined as 2 μg/mL [28], which means that, according to our data, the nectar of both *N. rustica* and *N. tabacum* could be toxic for honeybees.

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