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Biologically Active and Volatile Compounds in Leaves and Extracts of *Nicotiana alata* Link & Otto from Bulgaria

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Abstract

The aim of the study was to identify the chemical composition of leaves, essential oil and extracts of two genotypes of *N. alata* Link & Otto (jasmine tobacco). HPLC analysis of triterpenes identified only betulin (251.12 µg/g) in the leaves of white flowers genotype, and betulin (284.30 µg/g) and betulinic acid (393.75 µg/g) – in that with pink flowers. Totally, 12 phenolic acids and 7 flavonoids were determined in the leaves. The most abundant free phenolic acid were chlorogenic (3796.21 and 2523.37 µg/g, respectively in white and pink forms) and other hydroxycinnamic acids (rosmarinic, sinapic, caffeic), and conjugated - vanillic acid (3077.34 and 4926.68 µg/g, respectively). The major flavonoids of both genotypes were: free - hyperosid (35.85 and 107.30 µg/g), and conjugated – apigenin (249.55 and 211.74 µg/g), luteolin, hesperetin and kaempferol. 19 components were determined (by GC/GC-MS) in the essential oils (representing 83.86 % and 67.09 % of oil content), among which the major were: phytol, solanone, cis-5-butyl-4-methyldihydrofuran-2(3h)-one, dihydro-β-ionone, α-ionene, β-damascenone, 1-methylnaphthalene. In the concretes were identified 19 components (82.03 % and 65.63 %, respectively), of which over 3 % were: isoamyl alcohol, oxynicotine, phytol, 4-methyl-1-penthanol, cotinine, 3-metyl-3-penthanol, 3-penthanone. The number of identified volatiles in the resinoids was 16 (94.93 % and 75.94 %), with major components: nicotine, phytol, eicosane, diethyl phthalate, dibutyl phthalate, solanone, furfuryl alcohol. Both extraction products showed moderate antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis*. Data from the study on *N. alata* leaf composition substantiates its potential use for obtaining specific phytoproducts.

Key words: Essential oil, *N. alata*, Polyphenols, Tobacco, Triterpenes

INTRODUCTION

The genus *Nicotiana* has 76 naturally occurring species, which are characterized by large polymorphism. Diversity is manifested in the existence of significant morphological, cytological and biochemical differences between species [1, 2].

Scientific information for chemical and technological characteristics of various commercial types and breeding varieties of cultivated tobacco *(N. tabacum* L., and to some extent – *N. rustica*) is huge. The identified chemical components of tobacco currently reach over 4500 [3], representing various classes with biological activity (alkaloids, polyphenols, carotenoids, terpenes, saponins, etc.) or specific aromatic descriptions (aromatic compounds, aliphatic oxygen-containing forms, monoterpenoids, etc.) [3, 4].

There is relatively little evidence for chemical characteristics of other *Nicotiana* species, and the focus has been mostly on their use as genetic material for breeding programs to create varieties with resistance to various adverse factors and phytopathogens [5].

N. alata Link & Otto (family Solanaceae, genus *Nicotiana,* section *Alatae*, chromosome number 2n=18 (Goodspeed) is a perennial branched herbaceous plant originating from Southern Brazil and Northern Argentina [1]. Natural habitats are found in Uruguay, Brasil, Paraguay, Argentina and other countries. The plant is typically 40 – 70 cm tall, environmentally undemanding although not tolerant to drought. It flowers from June to October-November, with long-tubed, white, yellowish-green, pink, purple or red flowers. Leaves are simple, spatulate, sessile, and are attached to the stem by distinctive winged petioles. Basal leaves can grow up to 30 cm long, while upper leaves are much smaller.

N. alata is grown only as ornamental plant, although some authors [6, 7] classify it as traditionally used as smoking tobacco, mainly for religious purposes. It is often referred to as "jasmine tobacco" or "flowering tobacco" due to its abundant, beautiful flowers and mild sweet nocturnal fragrance.

N. alata is now available in a wide range of varieties and hybrids, distinctive by flower appearance and fragrance. Reasonably, most of research on the species is focused on the chemical composition and properties connected to plant blossom.

Together with other *Nicotiana* species, *N. alata* is a model plant in bioengineering - in experiments for the production of vaccines against virus diseases, agents against cancer diseases, blood substitutes, therapeutic proteins, immunoglobulins, protease inhibitors, defensins, biodegradable plastics, industrial enzymes, solvents [8]. From the flowers of *N. alata* is isolated the immunoreactive protein NaD1 - a plant defensin that displays powerful inhibitory activity against several filamentous pathogens [9 – 12]. The essential oil obtained from *N. alata* flowers is highly appreciated in perfumery, with an odor profile

described as "summery hay and leather scent with dry tonality" (https://www.fragrantica.com/notes/Tobacco-blossom-346.html). The scent emitted nocturnally by petal lobes is described as "terpenoid-rich (e.g., sabinene, β-myrcene, limonene, trans-β-ocimene, 1,8-cineole) and benzenoid-rich (e.g., methyl benzoate, methyl salicylate)" [13].

N. alata is regarded a species that does not synthesize nicotine, which is partly explained by the fact that it is indeed the most low-in-alkaloids species of the family. Sisson and Severson [14], provided data for alkaloid levels and composition in freeze-dried green leaves of 64 *Nicotiana* species, all of which contained a measurable alkaloid fraction (at least 10 $\mu g.g^{-1}$). They found that the total-alkaloid content in leaves from greenhouse-grown plants of *N. alata* was 0.2 $\mu g.g^{-1}$ (of which – 68.8 % nicotine, 9.5 % nornicotine, 21.3 % anabasine, 0.4 % myosmine), while that in leaves from field-grown plants was only 0.04 $\mu g.g^{-1}$ (100 % nicotine). Data about other chemical constituents in the leaves of *N. alata* are hardly available.

The strive for expanding the scope of plant sources available for obtaining aroma or biologically active extracts reasonably placed the *Nicotiana* species in the light of our scientific attention, and in 2015 the Tobacco and Tobacco Products Institute (Markovo, Bulgaria) set up the experimental growing of some uncommon to the country *Nicotiana* species, including *N. alata* Link. & Otto.

Therefore, the aim of this study is to identify the chemical composition of cured leaves of *Nicotiana alata* Link & Otto from Bulgaria, as well as that of the essential oil and extraction products obtained from them.

MATERIALS AND METHODS

Plant material

The study was carried out with two genotypes of *Nicotiana alata* Link & Otto – with white and pink flowers. Plants were grown in 2016 on the experimental fields of Tobacco and Tobacco Products Institute (part of Bulgarian Agrarian Academy), situated in the region of Plovdiv, southern Bulgaria. The soil was hummus-carbonate (rendzina), with organic matter content - 2.31 %; total nitrogen content - 0.212 %; mobile forms of phosphorus P_2O_5 – 14.85 mg/100g soil; available potassium K_2O - 67.5 mg/100 g soil; pH – 8.2. The vegetation period was June – September, 2016, having an average temperature of 22 °C and an average amount of rainfall of 44.5 mm. Due to drought susceptibility of the species, additional irrigation was carried out, twice during vegetation. All leaves were successively collected (picked by hand at maturity), and then sun cured in strings. Cured leaves were stored in an air-conditioned environment (in cardboard boxes) until processing.

In the sample preparation step, leaves were oven-dried (40 °C; 6 h), ground by a laboratory mill and sieved. For the determination of polyphenols and triterpenes, ground samples were additionally finely powdered by a laboratory homogenizer. The moisture content (%) was determined by drying (103±2 °C) to constant weight [15].

Determination of nicotine, reducing carbohydrates, nitrogen, mineral substances

The basic groups of chemicals in cured tobacco leaves were determined by standardized analytical methods: total alkaloids (as nicotine) – ISO 15152:2003, reducing carbohydrates – ISO 15154:2003; total nitrogen – BSS 15836:1988; mineral substances – ISO 2817: 1999.

Determination of polyphenols

Sample preparation. Extraction of phenolic compounds was done with 0.5 g and 1 g of powdered sample and 70 % methanol (Sigma, Germany), in an ultrasonic bath at 70 °C for 3 hours. The extracted biomass was separated by filtration and the procedure was repeated twice more. The combined extract was evaporated to dryness on a rotary evaporator. The residue was dissolved in methanol and used for HPLC analyses after filtration with 0.45 μm syringe filter. The extraction of conjugated phenolics was done by the same procedure, only that a solution of 2M HCl in methanol was used.

HPLC analysis. Identification and quantification of phenolic acids and flavonoids were performed by using Waters 1525 Binary Pump HPLC system (Waters, Milford, MA, USA), equipped with Waters 2484 dual Absorbance Detector (Waters, Milford, MA, USA) and Supelco Discovery HS C18 column (5 μm, 25 cm × 4.6 mm), operated under control of Breeze 3.30 software.

Phenolic acids. A gradient elution by using mobile phase of Solvent A (2 % acetic acid) and Solvent B (0.5 % acetic acid : acetonitrile, 1:1 v/v) was performed, following the procedure described by Marchev et al. [16]. The gradient was set up as follows: 0-30 min Solvent B increased from 5% to 35% at a flow rate of 0.8 mL/min; 30-45 min Solvent B increased to 70% at a flow rate of 0.4 mL/min; 45-50 min Solvent B increased to 80% at a flow rate of 1.2 mL/min; 50-60 min Solvent B increased to 100% at a flow rate of 1.2 mL/min; 60-65 min Solvent B dropped down to 5 % at a flow rate of 0.8 mL/min and was hold on up to 70 min to equilibrate the column. Gallic, protocatechuic, salicylic, chlorogenic, vanillic, caffeic, syringic, ferulic, sinapic, p-coumaric and cinnamic acids (Sigma, Germany) were used as standards to build calibration curves. The detection was carried out at 280 nm.

Flavonoids. The gradient elution was performed by using mobile phase of Solvent A (2% acetic acid) and Solvent B (methanol). The gradient was set up as follows: 0-10 min Solvent B increased from 30% to 50% at a flow rate of 1.0 mL/min; 10- 15 min hold on at the same flow rate; 15-16 min Solvent B increased to 52% at a flow rate of 0.8 mL/min; 16-30 min Solvent B increased to 80% at the same flow rate; 30-35 min Solvent B dropped down to 30 % at a flow rate of 1.0 mL/min and hold on up to 40 min to equilibrate the column [16]. Myricetin, kaempferol, quercetin, hesperidine and apigenin (Sigma, Germany) were used as standards to build calibration curves. Detection wavelength was 380 nm.

The quercetin glycosides rutin and hyperoside were analyzed on the same HPLC system by using mobile phase of Solvent A (2% acetic acid) and Solvent B (acetonitrile). The gradient of elution was setup as follows: 0-15 min 20%

Solvent B; 15-17 min 50% Solvent B; 17-20 min 20% Solvent B [17]. Rutin and hyperoside (Sigma-Aldrich, Germany) were used as standards to build calibration curves. The detection was carried out at 370 nm.

Determination of triterpenes

Sample preparation. 1 g finely powdered tobacco was subjected to threefold extraction with acetone (biomass:solvent, 1:20, w/v) in an ultrasonic bath, each for 30 min at 45 °C. The combined extract was evaporated on a rotary vacuum evaporator and the residue was transferred to 1 cm^3 methanol [18].

HPLC analysis. The determination of triterpenes was carried out on the same HPLC system (Waters, Milford, MA, USA) as described for phenolic acids and flavonoids. The mobile phase was potassium dihydrogen phosphate (pH 2.8) : methanol = 12:88 (v/v), flow rate was 0.80 mL/min and the detection wavelength was 210 nm. Quantification was done by a previously built standard curve.

Obtaining and analysis of essential oil and aroma extraction products

Essential oil was obtained by hydrodistillation in a laboratory glass apparatus of the British Pharmacopoeia, modified by Balinova and Diakov [15], dried over anhydrous sulfate and stored in at 4 °C.

Resinoid was obtained by extraction with 95 % ethanol (FILLAB, Bulgaria) under the following conditions: static, batch mode; twofold extraction for 2,5 h and 2 h; temperature 70° C; raw material : solvent – 1:10, w/v. The solvent was evaporated on a rotary vacuum evaporator at temperature 85 °C [15].

Concrete was obtained by extraction with petroleum ether (FILLAB, Bulgaria) under the following conditions: static, batch mode; twofold extraction for 1 h and 0,5 h; temperature 30° C; raw material : solvent – 1:10, w/v. The solvent was evaporated on a rotary vacuum evaporator at water bath temperature 35° C [15].

GC analysis. The system used consisted of Agilent 7890A chromatograph equipped with FID detector and HP-INNOWax Polyethylene Glycol column (60 m x 0,25 mm; film thickness 0,25 μm). The conditions of analysis were set up as follows: 70 °C - 10 min, 70 to 240 °C - 5 °C/min, 240 °C – 5 min; 240 to 250 °C - 10 °C/min, 250 °C – 15 min; carrier gas - helium, at 1 mL/min constant flow; injector - split, 250 °C, split ratio 50:1.

GS/MS Analysis. The conditions were set up as follows: Agilent 5975C gas chromatograph, carrier gas helium, column and temperature were the same as for the GC analysis, detectors: FID, 280 °C, MSD, 280 °C, transfer line.

Antimicrobial activity

The study included: Gram-positive bacteria - *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633; Gram-negative bacteria - *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NTCC 6017; yeasts - *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 1023; molds - *Aspergillus niger* ATCC 16404 and *Fusarium moniliforme*. All test-microorganisms were obtained from the National Bank of Industrial Microorganisms and Cell Cultures – Sofia, Bulgaria and are deposited in the microbial culture collection of Department of Biotechnology and Food Technology, Branch – Razgrad, University of Russe "A. Kanchev", Bulgaria. The antimicrobial activity was determined by the agar diffusion cup method, using 8 mm cups and 50 μL of the samples. The respective media were soybean-casein digest agar medium (Biolife) – for bacteria, and Sabouraud dextrose agar (Biolife) – for yeasts and molds. Cultivation was done at 37 °C for 24 h (bacteria), at 27 °C for 24 h (yeast) and at 27 °C for 72 h (molds), after which the diameter of the inhibition zones was measured. Results were corrected for inhibition due to solvent activity.

Statistical analysis

All experiments were done in at least a threefold repetition. Data are presented as mean ± standard error of the mean. Statistical significance was assessed by either Student's-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Differences between means were considered statistically significant if $p > 0.05$.

RESULTS AND DISCUSSION

The results from the identification of the chemical composition of cured leaves of *N. alata* are presented in Table 1. These are chemical indexes that typically characterize the quality of leaf tobacco [4], but are also representative of phytochemicals in the plant material that define its status as a matrix for further analysis.

Table 1. Chemical indexes of cured leaves (*N. alata*)

Plant material	Content in leaves (% DW)			
	Nicotine	Reducing carbohydrates	Total nitrogen	Mineral matter (ash)
Variation with white flowers	<LOQ[a)]	4.98±0.02	2.50±0.01	22.96±0.11
Variation with pink flowers	0.40±0.00	4.78±0.02	2.24±0.01	22.09±0.10

[a)] LOQ (limit of quantification) = 0.01 %

As data in the table suggests, there were no significant differences between the two types of plant material (leaves from plants with white or pink flowers). *N. alata* leaves were characterized by a minimal amount of nicotine and low reducing sugars content, but the share of inorganics and total nitrogen on a dry weight basis was relatively big. That composition identifies a clear difference of the wild species in comparison with *N. tabacum* varieties. In the table, the value for the nicotine content of the genotype with pink flowers (0.40 %) was higher than that reported by Sisson and Severson [14] and Winter [7] – 0.02 % or 0.10 %, which could be explained by the analytical approach of the current study, in which tested were cured (not freeze-dried green) leaves and the applied standard method determined the content of total alkaloids as nicotine.

Table 2. Polyphenols in *N. alata* leaves
(NAWF – *N. alata* variation with white flowers, NAPF – *N. alata* variation with pink flowers)

Compounds	Free		Conjugated (after acid hydrolysis)	
	NAWF	*NAPF*	*NAWF*	*NAPF*
	Phenolic acids (µg/g dry biomass)			
Gallic acid	NF[a]	NF	40.28	87.60
3,4-diOH Benzoic acid	NF	NF	179.20	56.88
2-OH Benzoic acid	475.45	265.11	222.53	22.33
Chlorogenic acid	3796.21	2523.37	304.18	56.53
Vanillic acid	391.77	348.93	3077.34	4926.68
Caffeic acid	106.26	125.40	624.76	698.97
Syringic acid	905.92	341.31	712.93	486.98
p-Coumaric acid	78.06	59.93	263.86	247.24
Sinapic acid	185.67	154.84	680.44	582.81
Ferulic acid	77.85	55.76	408.22	303.45
Cinnamic acid	100.55	5.12	172.18	29.98
Rosmarinic acid	249.95	352.76	588.46	584.52
	Flavonoids (µg/g dry biomass)			
Myricetin	49.08	61.51	42.95	48.13
Hesperetin	49.54	57.78	63.48	101.53
Quercetin	26.49	30.19	30.03	72.20
Luteolin	48.58	30.88	49.54	454.48
Kaempferol	20.87	18.95	51.57	57.02
Apigenin	NF	NF	249.55	211.74
Hyperosid	35.85	107.30	-	-

[a]NF – compound not found

Considering the identification of the chemical composition of *N. alata* leaves with regard to phytochemicals with well-established biological activities, there were determined the contents of triterpenes and polyphenols (free and conjugated phenolic acids and flavonoids).

Two pentacyclic triterpenes were found in *N. alata* leaves - betulin and betulinic acid. In the white flower genotype identified was only betulin (251.12 µg/g dry biomass), while that with pink flowers contained both betulin (284.30 µg/g) and betulinic acid (393.75 µg/g). Other pentacyclic triterpenes of oleanane or ursane type were not found (e. g., oleanolic acid, ursolic acid), neither was carnosic acid. Betulin and betulinic acid are naturally occurring triterpenes isolated from the bark of several trees and bushes (principally the white birch (*Betula pubescens*), possessing diverse biological and pharmacological activities. Betulin is known to be one of the first isolated natural products (reported in 1788), with antitumor and antiviral activity [19]. Betulinic acid is considered the most biologically active molecule of the family, having antiretroviral, antimalarial and anti-inflammatory effects, being a selective inhibitor of human melanoma and presenting anti-HIV activity [20, 21].

The results about the content of phenolic acids and flavonoids in the leaves of *N. alata* are shown in Table 2. Data revealed that in both genotypes the group of free phenolic acids was dominated by chlorogenic acid and other hydroxycinnamic acids (rosmarinic, sinapic, caffeic), but in quantities close to the latter were found 2-OH benzoic, vanillic and syringic acids (hydroxybenzoic acids). Among the conjugated phenolic acids dominated the group of hydroxybenzoic acids (mostly vanillic acid). Data about free flavonoids showed higher levels of the flavon glycoside hyperosid, while in the conjugated flavonoids group dominated flavones (apigenin and luteolin), flavanones (hesperetin) and flavonols (kaempferol). There were some differences in the polyphenol content between the two genotypes (e.g., in the content of the free syringic and cinnamic acids, the conjugated 2-OH benzoic, 3.4-diOH benzoic, chlorogenic and cinnamic acids, free hyperosid and conjugated luteolin), that could be attributed to differences in plants metabolism.

The leaf material of *N. alata* was used to obtain essential oils (by hydrodistillation) and two concentrated aromatic products (by solvent extraction) – concrete and resinoid. The latter two are the final, ready-to-use extraction products obtained from cured and fermented common tobacco (*N. tabacum* L.) that are widely applied in perfumery and cosmetics. By this approach of obtaining and analyzing three aroma and biologically active products, a comparison could be made between volatile metabolites synthesized by the plant (essential oil) and their transformations due to temperature, solvent nature, etc. during the extraction process (concrete and resinoid).

Data from the identification of the chemical composition of the three products (volatile compounds) are presented in Table 3.

The yield of essential oil (0.04 %) was much lower than that of oriental tobacco (*N. tabacum*) from Bulgaria, but the yields of concrete (approx. 1.60 %) and resinoid (approx. 10 %) were in comparable proportions [22].

There were no differences in the appearance of the concentrated extraction products obtained from the two genotypes – the concretes were semi-solid dark-brown, and the resinoids – light-brown wax-like masses, with specific odor.

Table 3. Chemical composition of *N. alata* essential oil and extracts
(NAWF – *N. alata* variation with white flowers, NAPF – *N. alata* variation with pink flowers)

№	Compound	RI	Essential oil		Concrete		Resinoid	
			NAWF	*NAPF*	*NAWF*	*NAPF*	*NAWF*	*NAPF*
1	Acetic acid	673	nd[a]	nd	nd	nd	2.72	2.18
2	3-Penthanone	701	nd	nd	3.08	2.46	nd	nd
3	Ethylmethyl ketone	733	nd	nd	0.76	0.61	nd	nd
4	Isoamyl alcohol	760	nd	nd	32.02	25.62	nd	nd
5	2-Methyl-1-butanol	762	nd	nd	1.89	1.51	nd	nd
6	2-Hexanol	812	nd	nd	1.04	0.83	nd	nd
7	Furfural	838	nd	nd	nd	nd	2.45	1.96
8	4-methyl-1-penthanol	843	nd	nd	7.03	5.62	nd	nd
9	3-metyl-3-penthanol	846	nd	nd	4.54	3.63	nd	nd
10	Furfuryl alcohol	865	nd	nd	nd	nd	3.21	2.57
11	Isoamyl acetate	885	nd	nd	0.66	0.53	nd	nd
12	2-Methylbutyric acid	898	nd	nd	1.88	1.50	nd	nd
13	α-Pinene	939	0.11	0.09	0.22	0.18	nd	nd
14	Benzaldehyde	965	nd	nd	1.03	0.82	nd	nd
15	β-Pinene	979	0.16	0.13	nd	nd	nd	nd
16	β-Myrcene	997	0.31	0.25	nd	nd	nd	nd
17	6-Methyl-5-hepten-2-ol	1003	nd	nd	0.19	0.15	nd	nd
18	Trimethylpyrazine	1008	nd	nd	nd	nd	1.56	1.25
19	Limonene	1030	0.31	0.25	0.52	0.42	2.98	2.38
20	1,8-cineole	1032	0.12	0.10	0.41	0.33	0.72	0.58
21	Benzyl alcohol	1041	0.25	0.20	nd	nd	nd	nd
22	Linalool	1103	0.38	0.30	nd	nd	nd	nd
23	α-Ionene	1256	3.93	3.14	nd	nd	nd	nd
24	Linallyl acetate	1259	0.66	0.53	nd	nd	nd	nd
25	2-Methylnaphthalene	1295	2.79	2.23	nd	nd	nd	nd
26	1-Methylnaphthalene	1312	3.07	2.46	nd	nd	nd	nd
27	cis-5-Butyl-4-methyl-dihydrofuran-2(3H)-one	1344	5.81	4.65	nd	nd	nd	nd
28	Nicotine	1366	0.14	0.11	0.36	0.29	44.17	35.34
29	Solanone	1374	6.15	4.92	1.54	1.23	3.63	2.90
30	Oxynicotine	1396	nd	nd	10.02	8.02	nd	nd
31	Beta-Caryophyllene	1419	0.21	0.17	nd	nd	nd	nd
32	Dihydro-β-ionone	1443	4.72	3.78	nd	nd	nd	nd
33	β-Farnesene	1448	nd	nd	nd	nd	2.73	2.18
34	Dimethyl phthalate	1460	2.84	2.27	nd	nd	nd	nd
35	β-Damascenone	1390	3.37	2.70	nd	nd	nd	nd
36	Diethyl phthalate	1602	nd	nd	nd	nd	4.66	3.73
37	Farnesylacetone	1922	nd	nd	nd	nd	1.33	1.06
38	Phytol	1960	48.53	38.82	8.67	6.94	12.48	9.98
39	Dibutyl phthalate	1972	nd	nd	nd	nd	3.87	3.10
40	Cotinine	1981	nd	nd	6.17	4.94	0.48	0.38
41	Eicosane	2000	nd	nd	nd	nd	5.42	4.34
42	Isopropyl palmitate	2026	nd	nd	nd	nd	2.52	2.02
	Sum of identified, %		83.86	67.09	82.03	65.62	94.93	75.94
	Yield, %		0.04	0.04	1.68	1.35	10.27	9.50

[a] nd – not detected

In the essential oil, a total of 19 components were identified, constituting respectively 83.86 % and 67.09 % of the total oil content for the two genotypes. In the essential oil of *N. alata* form with white flowers 7 major components (in concentration above 3 %) were identified: phytol (48.53 %), solanone (6.15 %), cis-5-butyl-4-methyldihydrofuran-2(3h)-one (5.81 %), dihydro-β-ionone (4.72 %), α-ionene (3.93 %), β-damascenone (3.37 %) and 1-methylnaphthalene (3.07 %). In the essential oil of the pink flowers form over 3 % were 5 components: phytol (38.82 %), solanone (4.92 %), cis-5-butyl-4-methyldihydrofuran-2(3h)-one (4.65 %), dihydro-β-ionone (3.78 %) и α-ionene (3.14 %). The remaining two - β-damascenone and 1-methylnaphthalene, were in slightly lower, but still comparable concentrations – 2.70 % and 2.46 %, respectively. As anticipated, no significant differences were found between the two *N. alata* genotypes grown side-by-side, but the results offer much more detailed data about the essential oil profile of plant forms with different petal color, that may nuance their olfactory properties. These results comply with conclusions from previous research on tobacco essential oil (*N. tabacum)* stating that it is generally different in composition from other essential oils, which usually contain a limited number of special, profile-shaping aroma compounds [22 – 26].

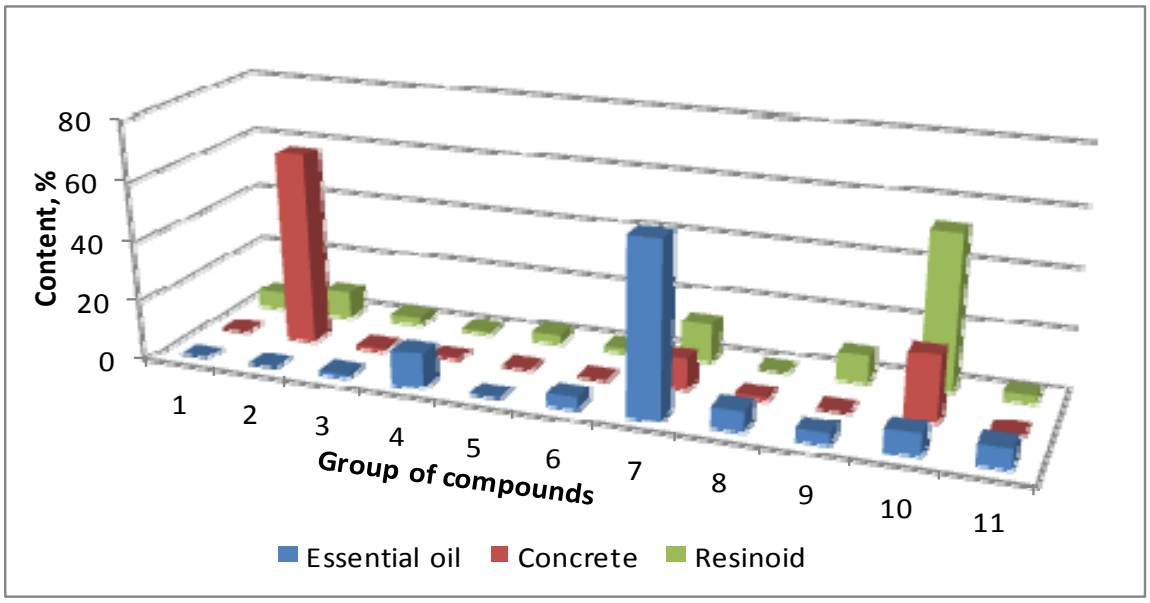

Fig. 1. Groups of components in essential oil, concrete and resinoid from *N. alata* leaves (% of the identified) (1 – Aliphatic hydrocarbons; 2 – Aliphatic oxygenated compounds; 3 – Monoterpene hydrocarbons; 4 – Monoterpene oxygenated compounds; 5 – Sesquiterpene hydrocarbons; 6 – Seskquiterpene oxygenated compounds; 7 – Diterpene oxygenated compounds; 8 – Aromatic; 9 – Aromatic oxygenated compounds; 10 – Nitrogenous compounds; 11 – Other compounds)

In the concrete obtained from both *N. alata* genotypes were identified 19 components (respectively - 82.03 % and 65.63 % of the total content). Of them, 7 were present in concentrations above 3 % in the form with white flowers, and 6 – in that with pink flowers: isoamyl alcohol (respectively – 32.02 % and 25.62 %), oxynicotine (10.02 % and 8.02 %), phytol (8.67 % and 6.94 %), 4-methyl-1-penthanol (7.03 % and 5.62 %), cotinine (6.17 % and 4.94 %), 3-metyl-3-penthanol (4.54 % and 3.63 %), 3-penthanone (3.08 % and 2.46 %). It is hard to stipulate about the specificity of the aroma profile of the concrete of *N. alata* grown in Bulgaria and the influence of regional factor, as to our best knowledge, no such detailed investigation of *N. alata* leaves, and particularly of leaf concrete, has been performed previously. The presence of skin irritating nicotine and related compounds (oxynicotine, cotinine) in the concretes suggests that they might be used in perfume, natural cosmetic or aromatherapy formulations only after purification or processing to absolute.

In the resinoid, the number of identified volatiles was 16 – constituting respectively 94.93 % and 75.94 % of the total yield from the studied genotypes. In the resinoid obtained from *N. alata* form with white flowers, 7 of the identified compounds were over 3 %, and in that from the pink flowers form – 5, as follows: nicotine (44.17 % and 35.34%, respectively), phytol (12.48 % and 9.98 %), eicosane (5.42 % and 4.34 %), diethyl phthalate (4.66 % and 3.73 %), dibutyl phthalate (3.87 % and 3.10 %), solanone (3.63 % and 2.90 %), furfuryl alcohol (3.21 % and 2.57 %). Similar to the concrete from *N. alata* leaves, these, to our knowledge, are first data revealing the specific aroma components of species' leaf resinoids. Reasonably, the composition of *N. alata* leaf resinoid differed substantially from those obtained under the same conditions from Bulgarian varieties of common tobacco (*N. tabacum*) [22].

There were observed no differences in the distribution of volatiles by major groups of chemical compounds between the two genotypes studied. The volatiles profile by groups of compounds (percentage of the identified) of essential oil, concrete and resinoid is presented on Fig. 1.

Essential oil contained mostly diterpene oxygenated compounds (57.87 %), followed by monoterpene oxygen containing (11.70 %) and nitrogenous (7.50 %) compounds. Concrete was dominated by aliphatic oxygen containing forms (64.72 %), followed by nitrogenous (22.05 %) and diterpene oxygen containing (10.57 %) compounds. In the resinoid dominated nitrogenous compounds (mostly alkaloids – 52.51 %), followed by diterpene (13.15 %), aromatic (8.99 %) and aliphatic (8.83 %) oxygen containing forms.

Based on the results from identification of the chemical composition of the obtained extraction products (concrete and resinoid*)* from *N. alata* leaves, and the presence of biologically active substances, the next step was the investigation of their antimicrobial activity. In the tests, there were no differences between the studied genotyped on a product base, all showing moderate antimicrobial activity only against the Gram-positive bacteria *Staphylococcus aureus* (with diameter of inhibition zones in the range from 9.1 mm to 13.4 mm) and *Bacillus subtilis* (diameter of inhibition zones – from 9.3 mm to 10.4 mm). The other seven microorganisms tested (*Escherichia coli ATCC 873; Pseudomonas aeruginosa; Salmonela abony NCTC 601; Candida albicans ATCC 1023; Saccharomyces cerevisiae; Aspergillus niger; Fusarium moniliforme)* were not sensitive to either concrete or resinoid from *N. alata* leaves.

CONCLUSIONS

Data achieved by the study provide for the first time information about biologically active and other components in the leaves of *N. alata* grown in Bulgaria – alkaloids, reducing sugars, total nitrogen, triterpenoids, free and conjugated phenolic acids and flavonoids. Data obtained by GC-MS analysis identifies the chemical composition of *N. alata* leaf essential oil and the concentrated extraction products concrete and resinoid, common to perfumery and cosmetics. All these results create grounds for expanding the use of *N. alata* beyond merely ornamental purposes, as well as for taking benefit from other parts of the plant, beside flowers, for obtaining specific phytoproducts.

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