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Chemical Constituents in Leaves and Aroma Products of *Nicotiana rustica* L. Tobacco

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Abstract

Nicotiana rustica L. (Aztec tobacco) is the only *Nicotiana species*, except common tobacco (*N. tabacum* L.), which is cultivated for tobacco products. The leaves of *N. rustica*, however, accumulate various specialized metabolites of potential interest. Therefore, the objective of this study was to evaluate certain classes of metabolites (by HPLC and GC-MS) in the leaves, the essential oil (EO), concrete and resinoid of *N. rustica*. Three pentacyclic triterpenes were identified in the leaves (by HPLC): betulin (252.78 μg g^{-1}), betulinic (182.53 μg g^{-1}) and oleanolic (69.44 μg g^{-1}) acids. The dominant free phenolic acids in the leaves (by HPLC) were rosmarinic (4257.38 μg g^{-1}) and chlorogenic (1714.40 μg g^{-1}), and conjugated forms of vanillic (3445.71 μg g^{-1}), sinapic (1963.11 μg g^{-1}) and syringic (1784.96 μg g^{-1}). The major flavonoids in the leaves were luteolin (960.44 μg g^{-1}), apigenin (880.66 μg g^{-1}) and hyperoside (780.72 μg g^{-1}). The GC-MS profiling of the EO identified 19 components and the major ones were phytol (43.68 %), solanone (5.54 %), cis-5-butyl-4-methyldihydrofuran-2(3H)-one (5.23 %), dihydro-β-ionone (4.25 %), α-ionene (3.54 %) and β-damascenone (3.03 %). The major volatiles in the concrete were isoamyl alcohol (28.82 %), oxynicotine (9.02 %), phytol (7.80 %), 4-methyl-1-penthanol (6.33 %), cotinine (5.55 %) and 3-metyl-3-penthanol (4.09 %). Resinoid composition was dominated by nicotine (39.75 %), phytol (11.23 %), eicosane (4.88 %), diethyl phthalate (4.19 %), dibutyl phthalate (3.48 %) and solanone (3.27 %). Concrete and resinoid showed weak antibacterial activity. These results create grounds for considering *N. rustica* as a source to obtain aroma or other bioproducts.

Keywords: Essential oil; Extracts; *Nicotiana rustica* L.; Phytochemicals; Polyphenols; Triterpenes

1 Introduction

Nicotiana rustica L. (known also as "wild tobacco", "Aztec tobacco" or "makhorka") and *Nicotiana tabacum* L. (common tobacco) are the only two of the 76 *Nicotiana species* (Solanaceae) that are used for large-scale production of cured leaves intended for the manufacture of various tobacco products for human consumption (by smoking, chewing, snuffing). Reasonably, common tobacco (*N. tabacum* L.), the species being grown globally in a plethora of commercial types

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and varieties, has turned into one of the most studied plant materials in the world (Leffingwell, 1999; Rodgman & Perfetti, 2016). Considerably less is known about the chemical, technological and other characteristics of the other *Nicotiana species*.

N. rustica L. is an annual herbaceous plant, originating from Mexico and with natural habitats found in Peru and Bolivia. *N. rustica* L. was probably domesticated earlier than *N. tabacum*, and there is documented evidence of its historical use in religious rituals by the indigenous peoples of the Americas (Kishore, 2014; Winter, 2000). The plants are stout, 0.60 to 1.20 m high. The leaves are simple, broad-oval, petiolate and very dense. The flowers are pale-yellow to green. The vegetation period is comparatively short, 90 to 100 days, and the leaf yield may reach 1.5 to 2 tonnes per hectare (Knapp, Chase & Clarkson, 2004; Kostoff, 1941; Yadav, Rathi, Pednekar & Rewachandani, 2016).

Although considered naturalized worldwide, nowadays *N. rustica* is grown and used in small amounts, in a limited number of countries, e.g. Turkey, India, Russia, South America, Vietnam and other Asian countries, where it is represented by a number of regionally defined varieties. Apart from being used in domestic smoking products (cigarette-like "papirossi", cigars or water-pipe tobacco), *N. rustica* L. is included in chewing tobacco, and moist and dry snuffs (e.g. in some regions of India or Pakistan) (Djordjevic & Doran, 2009; Kishore, 2014; Kurucu, Kartal & Erenmemişoğlu, 1998). One of the driving forces of *N. rustica* L. cultivation nowadays is its high alkaloid level and its suitability for an industrial production of nicotine and nicotine-related products (Jassbi, Zare, Asadollahi & Schuman, 2017; Kurucu et al., 1998; Saitoh, Noma & Kawashima, 1985; Sisson & Severson, 1990). The content of nicotine (3-[(2S)-1-methylpyrrolidin-2-yl] pyridine), the basic native alkaloid of *N. rustica* L. may reach as high as 8 to 10 % (in single reported cases even 15 to 18 %), depending greatly on variety, environmental and cultivation conditions. Tatarchenko, Mokhnachev and Kasyanov (2003) have summarized data about *N. rustica* L., emphasizing that different varieties grown under identical conditions accumulate considerably different amounts of nicotine in the leaves, from 3.4 to 8.32 %. Sisson and Severson (1990) in a study on the alkaloid levels and composition of 64 *Nicotiana species*, reported 5.4 μg g^{-1} total-alkaloid content in freeze-dried green leaves from greenhouse-grown plants (of which 98.2 % were nicotine, 0.5 % nornicotine, 0.8 % anabasine, 0.5 % anatabine), and considerably higher content in leaves from field-grown plants at 25.6 μg g^{-1} (97.1 % nicotine, 0.7 % nornicotine, 0.5 % anabasine, 1.7 % anatabine). Kurucu et al. (1998) found substantial differences in the alkaloid levels (nicotine, nornicotine and anabasine) of *N. rustica* leaves depending on plant cultivation conditions (for example, nicotine levels varying from 7.71 % to 2.12 % in the tobacco from two different regions of Turkey). In a comprehensive review on the secondary metabolites from the genus *Nicotiana* and their biological activities, Jassbi et al. (2017) pointed out that nicotine biosynthesis and aerial accumulation in *Nicotiana species* may increase substantially in response to environmental and other factors, such as herbivore attack and leaf damage. Stanfill et al. (2015) determined 5.09 % nicotine level in a Brazilian nasal snuff (rapé) product made entirely of *N. rustica* tobacco without flavorings, while according to Furbee (2009) *N. rustica* leaves contained up to 18 % nicotine. Studies of floral volatiles from *N. rustica* L. (Raguso, Levin, Foose, Holmberg & McDade, 2003; Schlotzhauer, Horvat, Chortyk, Nottingham & Jackson, 1995) observe that nicotine has been present in major amounts in flower oil as well (over 20 %), a coherent result of the well-documented intensive synthesis of this secondary metabolite by the species (Jassbi et al., 2017; Sisson & Severson, 1990).

Cured leaves of *N. rustica* L. are also a traditionally recognized source for obtaining citric acid, as its average content of 7 to 8 % may reach as high as 18 % if appropriate cultivation and post-harvest practices are applied (Tatarchenko et al., 2003).

In terms of the assessment of *N. rustica* L. leaf quality as a material for smoking and smokeless tobacco products, a number of basic indices of the chemical composition have been determined, e.g. ash (13.7 - 19.4 %), reducing substances (1.20 - 1.80 %), soluble carbohydrates (in trace amounts), total nitrogen (4.10 - 4.60 %), ammo-

nia (0.71 - 0.79 %) and organic acids (15.90 - 24.40 %) (Tatarchenko et al., 2003). Hristeva and Nikolov (2006) evaluated nine varieties of *N. rustica* L. as genetic donors in selection procedures aimed at enhancing the smoking quality of common tobacco, and provided the following data about their chemical characteristics: ash (13.08 - 15.87 %), reducing substances (6.77 - 15.00 %), soluble carbohydrates (6.13 - 14.06 %), total nitrogen (1.51 - 2.12 %), total alkaloids (0.78 - 2.87 %), nicotine (0.30 - 1.44 %), chlorogenic acid (0.43 - 0.83 %) and rutin (0.73 - 1.31 %).

In the last two decades the focus of phytochemical research on tobacco has been on the analysis of biologically active secondary metabolites such as plant volatiles, terpenes, carotenoids, sterols, saponins, phenolics and secondary alkaloids, and the results reveal the clear potential of *N. tabacum* L. and some other *Nicotiana species* in this aspect (Andersson, Wennstrom & Gry, 2003; Budzianowski, 2014; Chowański et al., 2016; Jassbi et al., 2017; Kodama, Fujimori & Kato, 1984; Rodgman & Perfetti, 2016; Zhou, Li, Feng & Li, 2013). The distinctive phytochemical profile of *Nicotiana species* justifies their processing by different extraction techniques (traditional, accelerated, microwave or ultrasound assisted solvent extraction, supercritical extraction with liquefied gasses and subcritical water extraction) (Huie, 2002). Extracts from *N. rustica* L. leaves and flowers revealed biological and pharmacological activities, such as antimicrobial, anti-proteolytic, antioxidant and insecticidal (Bakht, Azra & Shafi, 2013; Digrak, Alma & Ilçim, 2001; Ibrahim, Aliyu, Abusufiyanu, Bashir & Sallau, 2011; Jassbi et al., 2017).

Driven by these contemporary trends in tobacco science, in 2015 the Tobacco and Tobacco Products Institute in Bulgaria set up a research programme for the investigation of tobacco species that are not common to Bulgaria, including *N. rustica* L. and *N. alata* Link & Otto.

Therefore, the objective of the current study is to evaluate certain classes of biologically active secondary metabolites in the leaves of *N. rustica* L. tobacco, experimentally grown in Bulgaria, and to identify the chemical composition of different aroma products (essential oil, concrete and resinoid).

2 Materials and Methods

2.1 Materials

Plant material

Tobacco (*N. rustica* L. var. rustica) was grown on the experimental fields of the Tobacco and Tobacco Products Institute (a branch of the Bulgarian Agricultural Academy), in the region of Plovdiv, South Bulgaria ($42^o04'55.2"$N $24^o42'16.8"$E). Soil characteristics were: hummus-carbonate (rendzina) type, organic matter (by Turin) 2.31 %; total nitrogen (by Kjeldahl) 0.212 %; mobile forms of phosphorus P_2O_5 (by Egner - Reem) 14.85 mg 100 g^{-1} soil; available potassium K_2O 67.5 mg 100 g^{-1} soil and pH 8.2. The vegetation period (June - September 2016) was characterized by an average temperature of 22^oC and an average rainfall of 44.5 mm, during which two additional irrigations were carried out. Fresh leaves were picked by hand at maturity. The leaves were stringed and sun-cured in the open air for about two weeks, until the characteristic leaf coloration (green with bronze or brown hues) developed and the mid-rib became brittle. Until processing, leaf material was stored in cardboard boxes in an air-conditioned warehouse. For the preparation of analytical samples, the leaves were oven-dried (40^oC; 6 h) and ground in a laboratory mill. A portion of the ground sample, intended for polyphenol and triterpene analysis, was further powdered by a laboratory homogenizer. The moisture content (%) was determined by drying to constant weight at 103 ± 2^oC, and all results have been presented on a dry weight (DW) basis.

Chemicals

HPLC grade methanol and acetonitrile, as well as phenolic acid and flavonoid standards were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Germany).

2.2 Determination of leaf chemical composition

Nicotine, reducing carbohydrates, nitrogen, and ash content in the leaves

The chemical indices were determined by standardized analytical methods: nicotine ISO 15152:2003; reducing carbohydrates ISO 15154:2003; total nitrogen BDS 15836:1988 and mineral substances (ash) ISO 2817:1999. All analyses were done in triplicate and the respective mean values have been presented.

HPLC analysis of polyphenols in the leaves

Portions of 0.5 g homogenized leaf samples were taken and subjected to triple sonicated extraction with 70 % methanol at 70 ^{o}C for 3 hours. The combined extract was evaporated at 60 ^{o}C, the residue was dissolved in methanol and the solution (filtered through a 0.45 μm syringe filter) was transferred to the HPLC unit. For the extraction of conjugated phenolics, 2M HCl in methanol was used, and the other conditions were the same (Marchev, Georgiev, Ivanov, Badjakov & Pavlov, 2011).

The HPLC system used for the determination of phenolic acids and flavonoids consisted of a Waters 1525 Binary Pump (Waters, Milford, MA, USA), UV-VIS detector (Waters 2487 Dual λ Absorbance Detector (Waters, Milford, MA, USA) and SUPELCO Discovery HS C18 column (5 μm, 250 mm × 4.6 mm, operated at a temperature of 26 ^{o}C), and Breeze 3.30 software.

The determination of phenolic acids was done according to Marchev, Georgiev, Ivanov et al. (2011). The mobile phases were: Phase A 2 % acetic acid and Phase B 0.5 % acetic acid:acetonitrile = 1:1 (v/v). The gradient elution profile was (Phase B): 0 - 30 min increase from 5 % to 35 % at 0.8 mL min^{-1}; 30 - 45 min from 35 % to 70 % at 0.4 mL min^{-1}; 45 - 50 min from 70 % to 80 % at 1.2 mL min^{-1}; 50 - 60 min from 80 % to 100 % at 1.2 mL min^{-1}; 60 - 65 min decrease from 100 % to 5 % at 0.8 mL min^{-1} and 65 - 70 min maintained to equilibrate the column. The standards used for building the calibration curves were gallic, protocatechuic, salicylic, chlorogenic, vanillic, caffeic, syringic, ferulic, sinapic, p-coumaric and cinnamic acids. Detection was performed at wavelengths of 280 nm and 320 nm.

The mobile phases in the gradient elution of flavonoids were: Phase A 2 % acetic acid and Phase B methanol. The gradient elution profile was set up as follows (Phase B): 0 - 10 min increase from 30 % to 50 % at 1.0 mL min^{-1}; 10 - 15 min held at the same flow rate; 15 - 16 min increase to 52 % at a flow rate of 0.8 mL min^{-1}; 16 - 30 min increase to 80 % at the same flow rate; 30 - 35 min decrease to 30 % at 1.0 mL min^{-1} and then maintained to 40 min to equilibrate the column (Marchev, Georgiev, Ivanov et al., 2011). Myricetin, kaempferol, quercetin, hesperidine and apigenin were used as standards for plotting the calibration curves. Detection wavelengths were 308 nm and 380 nm.

The determination of the quercetin glycosides rutin and hyperoside was carried out under the following conditions: 2 % acetic acid (Phase A) and acetonitrile (Phase B) used as mobile phases, and elution gradient set up to the profile of 0-15 min 20 % Phase B; 15-17 min 50 % Phase B and 17-20 min 20% Phase B (Ivanov et al., 2014). Rutin and hyperoside were used as standards to build the calibration curves. The detection was carried out at 370 nm.

HPLC analysis of triterpenes in the leaves

Samples of 1.0 g finely ground tobacco were subjected to threefold extraction with acetone (each for 30 min), in an ultrasonic bath, at hydro module 1:20 (w/v) and temperature 45^{o}C. The solvent in the combined extract was evaporated at 60^{o}C, using a rotary vacuum evaporator, and the residue was transferred to 1 mL methanol, filtered through a 22 μm filter and analyzed by HPLC. The identification of triterpenes was carried out on the same HPLC system as described for polyphenols. The mobile phase was an aqueous solution of potassium dihydrogen phosphate (pH 2.8): methanol = 12:88 (v/v), the flow rate was: 0 - 18 min at 0.8 mL min^{-1}; 18 - 19 min at 0.6 mL min^{-1}; 19 - 30 min at 0.6 mL min^{-1}; 30 - 31 min at 0.8 mL min^{-1} and 31 - 40 min at 0.8 mL min^{-1}. The detection wavelength

was 210 nm. The determination of triterpenes was against a standard curve, with carnosic acid, betulin, betulinic, ursolic and oleanolic acid (97 %) (Extrasynthese, France) used as standards (Marchev, Georgiev, Badjakov et al., 2011).

2.3 GC-MS profiling of aroma products (essential oil, concrete and resinoid)

One and the same plant material can be processed by different methods, thus obtaining aroma products with specific chemical composition, olfactory, biological and other properties. Common aroma products obtained from essential oil-bearing and medicinal plants include essential oils, concretes, absolutes, resinoids, oleoresins and tinctures (Bauer, Garbe & Surburg, 2001). The flow chart (Fig. 1) illustrates the basic principles of obtaining the aroma products investigated in this study, essential oil, concrete and resinoid.

Essential oil was obtained by hydrodistillation of 100 g dry tobacco in a laboratory Clevenger-type glass apparatus of the British Pharmacopoeia, modified by Balinova and Diakov (Stoyanova, Georgiev & Atanasova, 2007). The distilled essential oil was dried over anhydrous sulfate and stored at 4^{o}C until analysis.

Resinoid was obtained by two-stage, static batch extraction of 100 g tobacco with 95 % ethanol. The extraction conditions were: hydro module (raw material:solvent) 1:10 (w/v); duration of the first and second stage of extraction, 2.5 h and 2 h, and temperature 70^{o}C. The solvent was evaporated using a rotary vacuum evaporator, at a temperature 55^{o}C (Stoyanova et al., 2007).

Concrete was obtained by two-stage, static batch extraction of 100 g tobacco with petroleum ether (FILLAB, Bulgaria) under the following conditions: hydro module (raw material:solvent) 1:10 (w/v); duration of the first and second extraction stage, 1 h and 0.5 h, and temperature 30^{o}C. The solvent was evaporated using a rotary vacuum evaporator at a temperature of 35 ^{o}C (Stoyanova et al., 2007).

The GC-MS was carried out with an Agilent 5975C MSD system coupled to an Agilent 7890A gas chromatograph (Agilent Technologies Inc., Santa Clara, CA). Agilent J&W HP-5MS column (0.25 μm, 30 m x 0.25 mm) was used with helium as a carrier gas (1.0 mL min^{-1}). The operational conditions were: oven temperature 35^{o}C for 3 min and 5^{o}C/min to 250^{o}C for 3 min, total run time 49 min; injector temperature 260^{o}C; ionization voltage 70 eV; ion source temperature 230^{o}C; transfer line temperature 280^{o}C; solvent delay 4.25 min and mass range 50-550 Da. The MS was operated in scan mode. One μL of the sample diluted with n-hexane (10 %, v/v) was injected into the GC-MS system at split ratio 30:1. The GC analysis was carried out using an Agilent 7890A GC system and FID temperature of 270^{o}C. In order to obtain the same elution order with GC-MS, simultaneous triplicate injections were done by using the same column and the same operational conditions.

The identification of compounds was made by comparing their mass spectra with those from mass spectra libraries (Adams, 2001) and by comparing the literature and estimated Kovat's (retention) indices that were determined using mixtures of homologous series of normal alkanes from C_8 to C_{40} in hexane, under the conditions described above. The percentage ratio of volatile components was computed using the normalization method of the GC/FID peak areas.

2.4 Antimicrobial activity of aroma products

The antimicrobial activity was determined against the Gram-positive bacteria *Staphylococcus aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633, the Gram-negative bacteria *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Salmonella abony* NTCC 6017, the yeasts *Sacharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10231, and the molds *Aspergillus niger* ATCC 16404. The test-microorganisms were obtained from the National Bank of Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria) and were deposited in the microbial culture collection of the Department of Biotechnology and Food Technology, Razgrad Branch, Angel Kanchev University of Russe, Bulgaria. The antimicrobial activity was studied by the agar diffusion cup method using 8 mm cups

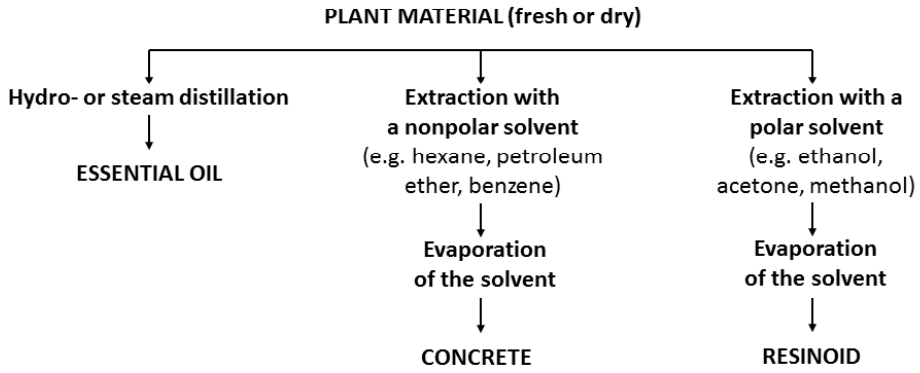

Figure 1: Principal scheme to obtain essential oil, concrete and resinoid from plant materials

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. The cultivation was carried out at 37^{o}C for 24 h (bacteria), at 27^{o}C for 24 h (yeasts) and for 72 h (molds), and the diameter of the inhibition zones was measured (Zaika, 1988). Blank dishes, with only solvent applied were also included as a negative control, in order to make the necessary corrections due to solvent activity. All tests were performed in triplicate.

2.5 Statistical analysis

All experiments were performed at least three times. All data were presented as mean ± standard deviation (SD). Statistical significance was assessed by Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. Differences between means were considered statistically significant if $p < 0.05$.

3 Results and Discussion

3.1 Chemical composition of tobacco leaves

Basic chemical indices of tobacco

Leaf chemical composition is one of the decisive criteria in the evaluation of the quality of any tobacco type cultivated for commercial purposes (i.e. with the purpose to yield leaf material with certain, typical chemical, physical and sensory features). The assessment of the quality level and the typical nature of tobacco leaf material through its chemical composition are based on the complex consideration of some recognized, key indicators (Leffingwell, 1999; Stanfill et al., 2015). These include: the total alkaloid content (expressed as nicotine, free and/or ionized); the total content of reducing sugars (or alternatively of soluble carbohydrates); the total nitrogen content (or alternatively protein nitrogen); and ash (i.e. the total mineral matter, expressed as pure ash, corrected for sand and other stuck impurities), and are determined by standardized methods (by the ISO or national bodies). As it has been stated previously, *N. rustica* has been historically cultivated for tobacco products intended for human consumption, and is still an important cash crop in certain regions of the world. On the other hand, the species remains relatively

uncommon to Bulgaria and there is scarce data about its leaf chemical composition relevant to this particular geographical origin. Therefore, the first step in the current study was the determination of the basic leaf chemical indices of *N. rustica* tobacco experimentally grown in the country, in an attempt to assess its quality and compliance with the tobacco type profile (Table 1).

An analysis of the numerical data in Table 1 reveals that the chemical composition of the studied plant material can be considered as typical for *N. rustica*, with a chemical profile described by some authors (Tatarchenko et al., 2003) as resembling that of cigar tobacco which combines high alkaloid, nitrogen and ash levels with very low or trace amounts of soluble carbohydrates. Nevertheless, there were some characteristic leaf features, obviously influenced by environmental and other factors of the growing area or by the applied agricultural practices, which affected plant metabolism. The content of nicotine was reasonably high (3.09 %), but still closer to the lower limit of the characteristic 2 to 8 % nicotine range reported for commercial *N. rustica* tobacco (Tatarchenko et al., 2003). Similarly, the total nitrogen content was insufficiently high (2.68 %), being about a half of that found in the leaves of high quality *N. rustica* (Tatarchenko et al., 2003). On the other hand, leaves accumulated substantially higher amounts of reducing carbohydrates (3.82 %) than the typical range cited for the species by the same authors (0.5 - 1 %). These findings suggest that, in case of an interest in a commercially-oriented production of *N. rustica* in the country, the growing conditions (e.g. agricultural practices, soil, plant nutrition, curing and breeding) should be optimized so as to realise the full potential of the species in terms of leaf quality. It should be regarded, as well, that some of the analyzed leaf chemical indicators represent plant metabolites that are extractible and biologically active, and their levels would contribute to the composition and properties of plant-derived crude or processed extracts.

Table 1: Chemical indices of *N. rustica* L. leaves

Index	Content (% DW [a])
Nicotine	3.09 ± 0.03 [b]
Reducing sugars	3.82 ± 0.02
Total nitrogen	2.68 ± 0.01
Ash	17.52 ± 0.15

[a] DW: dry weight basis
[b] data expressed as mean (n=3) ± standard deviation

Triterpenes and polyphenols

The analysis of the two groups of biologically active metabolites, triterpenes and polyphenols, resulted in the qualitative and quantitative profile of *N. rustica* L. leaves as presented in Table 2 and Table 3.

Three pentacyclic triterpenes of the oleanane type were identified in the leaves, among which dominated the terpene alcohol betulin (50.08 % of the total triterpenoid content). The presence of ursolic and carnosic acids were not detected. All three triterpene secondary metabolites have well documented biological and pharmacological activities (Parikh et al., 2014; Patlolla & Rao, 2012).

In the group of free phenolic acids, cinnamic acid derivatives comprising mainly rosmarinic and chlorogenic acid were dominant. In the group of conjugated phenolic acids (determined after acid hydrolysis), hydroxybenzoic acid derivatives (vanillic acid, syringic acid) were dominant. The dominant free and conjugated flavonols were myrecitin and quercetin, respectively. Flavons were mainly in a conjugated form. In the group of quercetin glycosides, only the free form of hyperosid was identified, while the conjugated form of hesperetin was dominant in flavon glycosides. This profile of polyphenol metabolites in the leaves of *N. rustica* L. substantially differs from the profile established in *N. tabacum* L. leaves from Bulgaria, reflecting species distinctiveness.

3.2 Chemical composition of aroma products (essential oil, concrete and resinoid)

Beside the identification of phytochemicals in the cured leaves of *N. rustica* L. tobacco from

Table 2: Triterpenes in *N. rustica* L. leaves

Compound	Content (μg g^{-1} DW [a])
Carnosic acid	nd [b]
Betulin	252.78 ± 2.11 [c]
Betulinic acid	182.53 ± 1.51
Oleanolic acid	69.44 ± 0.55
Ursolic acid	nd

[a] DW: dry weight basis
[b] nd: not detected
[c] data expressed as mean (n=3)
± standard deviation

Bulgaria, our goal was also the characterization of the chemical composition of the essential oil (obtained by hydrodistillation) and that of two concentrated, ready-to-use aroma products obtained by solvent extraction, concrete and resinoid. We hypothesized that the comparative assessment of these three aroma and biologically active products would reveal distinct differences in their volatile profiles, due to the influence of temperature, pH of the medium, extractant nature and other factors relating to the transformations of plant metabolites during hydrodistillation and extraction.

The essential oil of *N. rustica* was a light brown liquid, and the final concentrated extraction products (concrete and resinoid) were dark brown waxy masses, all with specific tobacco odor. In these characteristics they did not differ from the respective aroma products obtained from common tobacco described elsewhere (Georgiev & Stoyanova, 2006; Popova et al., 2015). The yield of concrete was 1.50 ± 0.01 % (w/w) and that of resinoid was 15.62 ± 0.09 % (w/w). These results were sufficiently high, in a parallel with the yields of concrete (0.25 - 3.0 %) and resinoid (8 - 27 %) from common aromatic plants (Georgiev & Stoyanova, 2006). On a yield basis, the results suggest the potential of *N. rustica* leaves as a source to obtain aroma products by solvent extraction. The yield of essential oil, however, was considerably lower (0.09 ± 0.00 %, v/w), thus supporting some previous observations about only trace amounts of essential oil in *N. rustica* leaves (Tatarchenko et al., 2003). For comparison, leaves of common tobacco were found to contain between 0.2 % and 1.5 % essential oil (Alagić, "Selekcija", Palić, Stojanović & Nikolić, 2002; Tatarchenko et al., 2003; Zhang, Gao, Zhang, Liu & Ye, 2012), and in single cases of highly aromatic Bulgarian oriental varieties up to 2-2.5 % (Georgiev & Stoyanova, 2006).

Data from the identification of the volatile composition of the essential oil, concrete and resinoid are presented in Table 4.

GC-MS analysis identified 19 components in the essential oil of *N. rustica* L. leaves (constituting 75.49 % of the total oil content). Six of the identified volatiles were in quantities over 3 %: (E)-phytol (43.68 %), solanone (5.54 %), cis-5-butyl-4-methyldihydrofuran-2(3H)-one (5.23 %), dihydro-β-ionone (4.25 %), α-ionene (3.54 %) and β-damascenone (3.03 %). These results reveal an essential oil that is not significantly different from the one of Oriental type *N. tabacum* L. (Popova et al., 2015). *N. rustica* L., however, has not been referenced much as a source of essential oil or other aroma products, and it is hard to make parallels to previous results about leaf oil chemical composition. The established low oil accumulation also probably does not favor *N. rustica* as a promising aromatic plant. Overall, data in Table 4 showed that, in terms of aroma-active compounds, *N. rustica* essential oil could not be associated with distinct major compounds, responsible for its aroma profile. As previously reported, tobacco essential oil (*N. tabacum* L.) is different from other plant essential oils, which always contain a set of special profile-shaping components (Alagić et al., 2002; Zhang et al., 2012). One possible explanation of these results may be related to the particular distillation conditions of tobacco oil (i.e. distillation from a strongly acidified medium, pH 2.00), which stimulate the acidic hydrolysis of certain classes of plant metabolites (including carotenoids).

Nineteen compounds were identified in the concrete, representing 73.83 % of its composition. Six of them were in quantities over 3 % and could be considered as major components: isoamyl alcohol (28.82 %), oxynicotine (9.02 %), (*E*)-phytol (7.80 %), 4-methyl-1-penthanol (6.33 %), cotinine (5.55 %) and 3-metyl-3-penthanol (4.09 %). It is hard to draw comparisons, as to the

Table 3: Polyphenols in *N. rustica* L. leaves

No	Compounds	Free (μg g^{-1} DW [a])	Conjugated (μg g^{-1} DW)
	Phenolic acids		
1	Rosmarinic acid	4257 ± 100 [b]	394.6 ± 1.1
2	Chlorogenic acid	1714 ± 40	130.7 ± 1.0
3	Sinapic acid	725.5 ± 1.7	1963 ± 59
4	Ferulic acid	286.7 ± 1.2	388.5 ± 1.2
5	Caffeic acid	260.3 ± 1.2	nd [c]
6	*p*-Coumaric acid	99.64 ± 0.88	372.6 ± 1.2
7	Cinnamic acid	9.54 ± 0.08	110.2 ± 0.7
8	2-Hydroxybenzoic acid	1376 ± 30	343.3 ± 1.2
9	Syringic acid	287.7 ± 1.3	1785 ± 66
10	Vanillic acid	75.41 ± 0.51	3446 ± 100
11	3,4-Dihydroxybenzoic acid	34.41 ± 0.28	142.6 ± 1.0
12	Gallic acid	nd	67.72 ± 0.51
	Flavonoids		
1	Myricetin	107.5 ± 0.6	79.80 ± 0.50
2	Kaempferol	14.81 ± 0.09	94.75 ± 0.66
3	Quercetin	24.41 ± 0.11	389.4 ± 1.2
4	Apigenin	nd	880.7 ± 19.5
5	Luteolin	29.17 ± 0.12	960.4 ± 20.8
6	Hyperoside	780.7 ± 0.7	nd
7	Hesperetin	70.93 ± 0.49	567.8 ± 1.5

[a] DW: dry weight basis
[b] data expressed as mean (n=3) ± standard deviation
[c] nd: not detected

best of our knowledge no detailed research on the yield or composition of concrete from *N. rustica* L. from Bulgaria or other regions has been published.

In the resinoid, a total of 16 compounds were identified (85.44 % of its composition). The major constituents (over 3 %) were: nicotine (39.75 %), (E)-phytol (11.23 %), eicosane (4.88 %), diethyl phthalate (4.19 %), dibutyl phthalate (3.48 %) and solanone (3.27 %). The technology of obtaining, together with the high native alkaloid level in the leaves, make *N. rustica* L. resinoid a higher-nicotine product, with a more specific possible application. For example, a suitable one could be its inclusion in hair treatment products, as there is evidence for a favorable effect of tobacco alkaloids on hair growth (Murkute, Sahu, Mali & Rangari, 2010).

The distribution of the identified chemical constituents of essential oil, concrete and resinoid, by groups of chemicals (percentage of the identified) reveal the distinctive profile of each of the three aroma products. The essential oil was dominated by oxygenated diterpenes (57.86 %), followed by oxygenated monoterpenes (11.70 %) and nitrogenous compounds (7.51 %). The most abundant chemical constituents of the concrete belonged to the group of oxygenated aliphatics (64.72 %), followed by nitrogenous compounds (22.05 %) and oxygenated diterpenes (10.56 %). The major part of the resinoid was constituted by nitrogenous compounds (52.49 %, and mainly alkaloids), followed by diterpene (13.14 %), aromatic (8.98 %) and oxygenated aliphatic (8.84 %) compounds. The results show that some of the aroma-active classes of compounds (e.g. oxygenated terpenes and aromatic compounds) were found in the oil, but not in the concrete and res-

Table 4: Volatile composition of the aroma products from *N. rustica* L. leaves

No	Compound	RI [a]	Content (%)		
			Essential oil	Concrete	Resinoid
1	Acetic acid	673	nd [b]	nd	2.45 ± 0.02 [c]
2	3-Penthanone	701	nd	2.77 ± 0.02	nd
3	Ethylmethyl ketone	733	nd	0.68 ± 0.00	nd
4	Isoamyl alcohol	760	nd	28.82 ± 0.23	nd
5	2-Methyl-1-butanol	762	nd	1.70 ± 0.01	nd
6	2-Hexanol	812	nd	0.94 ± 0.00	nd
7	Furfural	838	nd	nd	2.21 ± 0.02
8	4-Methyl-1-penthanol	843	nd	6.33 ± 0.05	nd
9	3-Methyl-3-penthanol	846	nd	4.09 ± 0.03	nd
10	Furfuryl alcohol	865	nd	nd	2.89± 0.02
11	Isoamyl acetate	885	nd	0.59 ± 0.00	nd
12	2-Methylbutyric acid	898	nd	1.69 ± 0.01	nd
13	α-Pinene	939	0.10 ± 0.00 [d]	0.20 ± 0.00 [d]	nd
14	Benzaldehyde	965	nd	0.93 ± 0.00	nd
15	β-Pinene	979	0.14 ± 0.00	nd	nd
16	β-Myrcene	997	0.28 ± 0.00	nd	nd
17	6-Methyl-5-hepten-2-ol	1003	nd	0.17 ± 0.00	nd
18	Trimethylpyrazine	1008	nd	nd	1.40 ± 0.01
19	Limonene	1030	0.28 ± 0.00 [d]	0.47 ± 0.00 [e]	2.68 ± 0.02 [f]
20	Eucalyptol (1,8-cineole)	1032	0.11 ± 0.00 [d]	0.37 ± 0.00 [e]	0.65 ± 0.00 [f]
21	Benzyl alcohol	1041	0.23 ± 0.00	nd	nd
22	Linalool	1103	0.34 ± 0.00	nd	nd
23	α-Ionene	1256	3.54 ± 0.02	nd	nd
24	Linallyl acetate	1259	0.59 ± 0.00	nd	nd
25	2-Methylnaphthalene	1295	2.51 ± 0.02	nd	nd
26	1-Methylnaphthalene	1312	2.76 ± 0.02	nd	nd
27	cis-5-Butyl-4-methyldihydrofuran-2(3H)-one	1344	5.23 ± 0.04	nd	nd
28	Nicotine	1366	0.13 ± 0.00 [d]	0.32 ± 0.00 [e]	39.75 ± 0.12 [f]
29	Solanone	1374	5.54 ± 0.03 [d]	1.39 ± 0.01 [e]	3.27 ± 0.02 [f]
30	Oxynicotine	1396	nd	9.02 ± 0.08	nd
31	β-Caryophyllene	1419	0.19 ± 0.00	nd	nd
32	Dihydro-β-ionone	1443	4.25 ± 0.04	nd	nd
33	β-Farnesene	1448	nd	nd	2.46 ± 0.01
34	Dimethyl phthalate	1460	2.56 ± 0.02	nd	nd
35	β-Damascenone	1390	3.03 ± 0.02	nd	nd
36	Diethyl phthalate	1602	nd	nd	4.19 ± 0.03
37	Farnesylacetone	1922	nd	nd	1.20 ± 0.00
38	(E)-Phytol	1960	43.68 ± 0.18 [d]	7.80 ± 0.07 [e]	11.23 ± 0.07 [f]
39	Dibutyl phthalate	1972	nd	nd	3.48 ± 0.01
40	Cotinine	1981	nd	5.55 ± 0.05 [d]	0.43 ± 0.00 [e]
41	Eicosane	2000	nd	nd	4.88 ± 0.03
42	Isopropyl palmitate	2026	nd	nd	2.27 ± 0.01
	Total identified	-	75.47	73.83	85.44
By groups of compounds (% of the identified)					
	Aliphatic hydrocarbons	-	-	5.71	
	Oxygenated aliphatic compounds	-	64.72	8.84	
	Monoterpene hydrocarbons	1.06	0.91	3.14	
	Oxygenated monoterpenes	11.70	0.50	0.76	
	Sesquiterpene hydrocarbons	0.25	-	2.88	
	Oxygenated sesquiterpenes	4.01	-	1.40	
	Oxygenated diterpenes	57.86	10.56	13.14	
	Aromatic compounds	6.98	1.25	-	
	Oxygenated aromatic compounds	3.70	-	8.98	
	Nitrogenous compounds	7.51	22.06	52.49	
	Other compounds	6.93	-	2.66	
	Total	100	100	100	

[a] RI: retention (Kovat's) index
[b] nd: not detected
[c] data expressed as mean (n=3) ± standard deviation
[d–f] means with different superscripts in a row differ significantly ($p < 0.05$)

inoid (or present in much smaller amounts). Apparently this distribution is related to the different pathway of obtaining the respective aroma products and reflects the influence of factors such as temperature (being respectively 30^{o}C in concrete extraction, 70^{o}C in resinoid extraction and steam temperature in essential oil distillation), duration of treatment (1.5 h in the extraction of concrete and 3 h in the hydrodistillation of the oil), and solvent polarity (nonpolar solvent in the case of concrete and polar for resinoid). The identification of individual aroma-active compounds in the essential oil, such as α-ionene, dihydro-β-ionone and β-damascenone, seems to be related to the degradation of leaf carotenoids, as influenced by the high temperature and occurrence of acidic hydrolysis during hydrodistillation, previously reported for common tobacco and other plant materials (Leffingwell, 1999; Winterhalter & Rouseff, 2001). Such carotenoid-related compounds were not identified in *N. rustica* concrete and resinoid, whereas the latter contained more hydrocarbons and their oxygenated derivatives due to the selective extraction by the solvents, which gave them the characteristic waxy texture.

3.3 Antimicrobial activity

Two of the obtained aroma products, concrete and resinoid, were tested for antimicrobial activity against a set of test-microorganisms. They inhibited only the growth of the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*. Their activity, however, was weak to moderate, with diameters of the inhibition zones from 9.8±0.01 mm to 11.5±0.03 mm. The rest of the tested bacteria, yeasts and molds were not sensitive to the concrete and resinoid from *N. rustica* L. leaves. Similar findings of limited or no inhibition activity of ethanol and n-hexane extracts from *N. rustica* L. leaves on bacteria were reported by Bakht et al. (2013).

4 Conclusions

The study reveals the potential of *N. rustica* L. leaves as a plant material that is rich in biologically active compounds, expanding therefore its importance beyond the production of smoking or smokeless tobacco products. The findings justify its consideration as a valuable source for obtaining aroma products of the types traditionally used in cosmetics or other areas (essential oil, concrete, resinoid). Different classes of secondary metabolites with biological activity, alkaloids, triterpenes, phenolic acids and flavonoids, have been identified in the leaves of the tobacco species that is not traditional to Bulgaria. The analysis of the chemical composition of the distilled leaf essential oil and the two concentrated extraction products (concrete and resinoid) revealed their specific volatile profile. To the best of our knowledge, these are the first data about obtaining concrete and resinoid from *N. rustica* L. and their chemical identification, especially with regard to the one grown in Bulgaria. The results from the study create grounds for directing the respective aroma products to specific applications and for future investigations aimed at expanding the species' importance.

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References

Adams, R. P. (2001). *Identification of essential oil components by gas chromatography/quadrupole mass spectroscopy*. (R. P. Adams, Ed.). Carol Stream, USA: Allured Publishing Corporation.

Alagić, S., "Selekcija", I. S., Palić, R., Stojanović, G. & Nikolić, M. (2002). Chemical composition and antimicrobial activity of the essential oil of the oriental tobacco yaka. *Journal of Essential Oil Research*, *14*(3), 230–232. doi:10 . 1080 / 10412905 . 2002.9699832. eprint: https://doi.org/10.1080/10412905.2002.9699832

Andersson, C., Wennstrom, P. & Gry, J. (2003). Nicotine alkaloids in solanaceous food plants. *TemaNord*, *531*, 1–37.

Bakht, J., Azra & Shafi, M. (2013). Antimicrobial potential of different solvent extracts of tobacco (*nicotiana Rustica*) against gram negative and positive bacteria. *Pakistan Journal of Botany*, *45*(2), 643–648.

Bauer, K., Garbe, D. & Surburg, H. (2001). Common fragrance and flavor materials. *Preparation, Properties and Uses. Weinheim: Wiley–VCH*.

Budzianowski, J. (2014). Tobacco-a producer of recombinant interferons. *Przeglad lekarski*, *71*, 639–43.

Chowański, S., Adamski, Z., Marciniak, P., Rosiński, G., Büyükgüzel, E., Büyükgüzel, K., ... Bufo, S. A. (2016). A Review of Bioinsecticidal Activity of Solanaceae Alkaloids. *Toxins*, *8*(3). doi:10.3390/toxins8030060

Digrak, M., Alma, M. H. & Ilçim, A. (2001). Antibacterial and Antifungal Activities of Turkish Medicinal Plants. *Pharmaceutical Biology*, *39*(5), 346–350. doi:10.1076/phbi.39.5.346.5903. eprint: https://doi.org/10.1076/phbi.39.5.346.5903

Djordjevic, M. V. & Doran, K. A. (2009). Nicotine content and delivery across tobacco products. In J. E. Henningfield, E. D. London & S. Pogun (Eds.), *Nicotine psychopharmacology* (pp. 61–82). doi:10.1007/978-3-540-69248-5_3

Furbee, B. (2009). Clinical neurotoxicology e-book: Syndromes, substances, environments, expert consult-online and print. In M. R. Dobbs (Ed.), (Chap. Neurotoxic plants, pp. 523–542). Elsevier Health Sciences.

Georgiev, E. & Stoyanova, A. (2006). A guide for the specialist in aromatic industry. *Plovdiv, Bulgaria*.

Hristeva, T. & Nikolov, E. (2006). Interspecies distance with basic chemical and technological signs of tobacco - nicotiana rustica. papers presented at science in globalization in 21st century. stara zagora: Union of scientists. In *International science conference, held at the union of scientists, stara zagora* (Vol. 1, pp. 152–156). Retrieved from https://nrs.nacid.bg/register/search

Huie, C. W. (2002). A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants. *Analytical and Bioanalytical Chemistry*, *373*(1-2), 23–30. doi:10.1007/s00216-002-1265-3

Ibrahim, M. A., Aliyu, A. B., Abusufiyanu, A., Bashir, M. & Sallau, A. B. (2011). Inhibition of *Naja nigricolis* (reinhardt) venom protease activity by *Luffa egyptiaca* (mill) and *Nicotiana rustica* (linn) extracts. *Indian Journal of Experimental Biology*, *49*(7), 552–554.

Ivanov, I., Vrancheva, R., Marchev, A., Petkova, N., Aneva, I., Denev, P., ... Pavlov, A. (2014). Antioxidant activities and phenolic compounds in Bulgarian *Fumaria* species. *Int. J. Curr. Microbiol. App. Sci*, *3*(2), 296–306. Retrieved from https://www.ijcmas.com/vol-3-2/Ivan%5C%20G.%5C%20Ivanov,%5C%20et%5C%20al.pdf

Jassbi, A. R., Zare, S., Asadollahi, M. & Schuman, M. C. (2017). Ecological roles and biological activities of specialized metabolites from the genus nicotiana. *Chemical Reviews*, *117*(19), 12227–12280. doi:10.1021/acs.chemrev.7b00001

Kishore, K. (2014). Monograph of tobacco *(Nicotiana tabacum). Indian Journal of Drugs*, *2*, 5–23.

Knapp, S., Chase, M. W.-. & Clarkson, J. J. (2004). Nomenclatural changes and a new sectional classification in *Nicotiana* (Solanaceae). *Taxon*, *53*(1), 73–82. doi:10.2307/4135490

Kodama, H., Fujimori, T. & Kato, K. (1984). Glucosides of ionone-related compounds in several *Nicotiana* species. *Phytochemistry*, *23*(3), 583–585. doi:10.1016/S0031-9422(00)80386-6

Kostoff, D. (1941). Cytogenetics of the genus nicotiana. karyosystematics, genetics, cytology, cytogenetics and phylogenesis of tobaccos. *Cytogenetics of the genus Nicotiana. Karyosystematics, genetics, cytology, cytogenetics and phylogenesis of tobaccos*.

Kurucu, S., Kartal, A. & Erenmemişoğlu, A. (1998). HPLC analysis of nicotiana rustica

and chewing tobacco (maraş powder) alkaloids. *FABAD Journal of Pharmaceutical Sciences*, *23*, 61–4. Retrieved from http://dergi.fabad.org.tr/pdf/volum23/Issue2/3.pdf

Leffingwell, J. (1999). Leaf chemistry-basic chemical constituents of tobacco leaf and differences among tobacco types. (pp. 265–284). doi:10.13140/2.1.5173.6645

Marchev, A., Georgiev, V., Badjakov, I., Kondakova, V., Nikolova, M. & Pavlov, A. (2011). Triterpenes production by rhizogenic callus of *Salvia Scabiosifolia Lam*. obtained via *Agrobacterium Rhizogenes* mediated genetic transformation. *Biotechnology & Biotechnological Equipment*, *25*(sup1), 30–33. doi:10.5504/BBEQ.2011.0109. eprint: https://doi.org/10.5504/BBEQ.2011.0109

Marchev, A., Georgiev, V., Ivanov, I., Badjakov, I. & Pavlov, A. (2011). Two-phase temporary immersion system for *Agrobacterium rhizogenes* genetic transformation of sage (*Salvia tomentosa Mill.) Biotechnology Letters*, *33*(9), 1873–1878. doi:10.1007/s10529-011-0625-5

Murkute, A., Sahu, M., Mali, P. & Rangari, V. (2010). Development and evaluation of formulations of microbial biotransformed extract of tobacco leaves for hair growth potential. *Pharmacognosy Research*, *2*(5), 300–303. doi:10.4103/0974-8490.72328. eprint: http://www.phcogres.com/article.asp?issn=0974-8490;year=2010;volume=2;issue=5;spage=300;epage=303;aulast=Murkute;t=6

Parikh, N. R., Mandal, A., Bhatia, D., Siveen, K. S., Sethi, G. & Bishayee, A. (2014). Oleanane triterpenoids in the prevention and therapy of breast cancer: Current evidence and future perspectives. *Phytochemistry Reviews*, *13*(4, SI), 793–810. doi:10.1007/s11101-014-9337-5

Patlolla, J. M. R. & Rao, C. V. (2012). Triterpenoids for cancer prevention and treatment: Current status and future prospects. *Current Pharmaceutical Biotechnology*, *13*(1), 147–155.

Popova, V., Gochev, V., Girova, T., Iliev, I., Ivanova, T. & Stoyanova, A. (2015). Extraction products from tobacco–aroma and bioactive compounds and activities. *Current Bioactive Compounds*, *11*(1), 31–37. doi:10.2174/1573407211011508041500 16

Raguso, R. A., Levin, R. A., Foose, S. E., Holmberg, M. W. & McDade, L. A. (2003). Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. *Phytochemistry*, *63*(3), 265–284. doi:10.1016/S0031-9422(03)00113-4

Rodgman, A. & Perfetti, T. A. (2016). *The chemical components of tobacco and tobacco smoke*. CRC press.

Saitoh, F., Noma, M. & Kawashima, N. (1985). The alkaloid contents of sixty nicotiana species. *Phytochemistry*, *24*(3), 477–480. doi:10.1016/S0031-9422(00)80751-7

Schlotzhauer, W. S., Horvat, R. J., Chortyk, O. T., Nottingham, S. F. & Jackson, D. M. (1995). Comparison of the volatile flower oils of *Nicotiana rustica* and *N. forgetiana*. *Journal of Essential Oil Research*, *7*(3), 265–269. doi:10.1080/10412905.1995.9698517

Sisson, V. A. & Severson, R. F. (1990). Alkaloid composition of the *Nicotiana* species. *Beitrage zur Tabakforschung International/Contributions to Tobacco Research*, *14*(6), 327–339.

Stanfill, S. B., Oliveira da Silva, A. L., Lisko, J. G., Lawler, T. S., Kuklenyik, P., Tyx, R. E., ... Watson, C. H. (2015). Comprehensive chemical characterization of rape tobacco products: Nicotine, un-ionized nicotine, tobacco-specific n '-nitrosamines, polycyclic aromatic hydrocarbons, and flavor constituents. *Food and Chemical Toxicology*, *82*, 50–58. doi:10.1016/j.fct.2015.04.016

Stoyanova, A., Georgiev, E. & Atanasova, T. (2007). A handbook for laboratory practice in essential oils. *Acad. Publ. House of the University of Food Technologies, Plovdiv*.

Tatarchenko, I. I., Mokhnachev, I. G. & Kasyanov, G. I. (2003). Chemistry of subtropical and food products: A handbook for students. In *Academia, moscow* (pp. 5–79). Retrieved from https://www.elibrary.ru/item.asp?id=20796343

Winter, J. C. (2000). *Tobacco use by native north americans : Sacred smoke and silent killer*. Includes bibliographical references (p. 387-434) and index. Norman : University of Oklahoma Press. Retrieved from https://trove.nla.gov.au/work/6546660

Winterhalter, P. & Rouseff, R. (2001). Carotenoid-derived aroma compounds: An introduction. (pp. 1–17). doi:10.1021/bk-2002-0802.ch001

Yadav, R., Rathi, M., Pednekar, A. & Rewachandani, Y. (2016). A detailed review on Solanaceae family. *European Journal of Pharmaceutical and Medical Research*, *3*(1), 369–378. Retrieved from http://www.ejpmr.com/admin/assets/article_issue/1451902449.pdf

Zaika, L. L. (1988). Spices and herbs: Their antimicrobial activity and its determination. *Journal of Food Safety*, (2), 97–118. doi:10.1111/j.1745-4565.1988.tb00511.x

Zhang, X., Gao, H., Zhang, L., Liu, D. & Ye, X. (2012). Extraction of essential oil from discarded tobacco leaves by solvent extraction and steam distillation, and identification of its chemical composition. *Industrial crops and products*, *39*, 162–169. doi:10.1016/j.indcrop.2012.02.029

Zhou, R., Li, D.-L., Feng, G.-L. & Li, G.-Y. (2013). A new sesquiterpene glucoside from *Nicotiana rustica L. Natural Product Research*, *27*(14), 1261–1264. doi:10.1080/14786419.2012.725398