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Original Research Article

Phytochemicals in leaves and extracts of the variety "Plovdiv 7" of Bulgarian oriental tobacco (*Nicotiana tabacum* L.)

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ABSTRACT

This study aims to identify and analyze the non-volatile fraction (chemicals, polyphenols, phenolic acids, flavonoids, and triterpenes) and volatile fraction (essential oil and crude extracted aroma fractions) present in the leaves of "Plovdiv 7" oriental tobacco variety (*N. tabacum* L.). The results show that the nicotine content in the leaves reaches 2.3% (DW) and chemicals such as betulin (2340.7 µg/g), carnosic acid (845.2 µg/g) and ursolic acid (596.0 µg/g), are also present. In terms of free phenolic acids, chlorogenic acid (2545.0 µg/g), ferulic acid (1561.8 µg/g), and vanillic acid (4461.9 µg/g) are found to be dominant. The flavonoid profile is dominated by myricetin (134.2 µg/g), quercetin (334.1 µg/g), apigenin (493.0 µg/g) and luteolin (445.6 µg/g). In the volatile fraction, nineteen volatile components were identified (92.3%) where (*E*)-phytol (53.4%) was the major compound followed by solanone (6.8%), *cis*-5-butyl-4-methyldihydrofuran-2(3*H*)-one (6.4%) and dihydro-β-ionone (5.2%). In addition, the antimicrobial activity of "Plovdiv 7" tobacco leaf aroma extracts (concrete and resinoid extract) are tested against nine microbial strains. The results show a weak antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* bacteria, whereas no activity is recorded for the other seven microbial strains tested.

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1. Introduction

Cultural, or common tobacco (*Nicotiana tabacum* L.) belongs to the *Nicotiana* genus, *Solanaceae* family, and is an important cash crop in many countries around the world. Processed tobacco leaf is used for the production of various smoking and smokeless products, all of which are associated with long-term harmful effects on human health. On the other hand, the use of cultural tobacco and other *Nicotiana* species for ceremonial and therapeutical purposes goes back thousands of years. Tobacco leaves or extracts have been used in traditional and folk medicine due to their sedative, anesthetic, emetic, expectorant, diuretic, antispasmodic, anticonvulsant, anti-rheumatic, anti-inflammatory, wound healing and other properties (Kishore, 2014). To date, a vast number of bioactive compounds have been isolated from *N. tabacum* L. under different geographical coordinates (Shang et al., 2015; He et al., 2016; Shang et al., 2016; Shen et al., 2016; Xia et al., 2016).

There is an enormous amount of scientific information about the chemical composition of different types, varieties, cultivars and other selection sources of cultural tobacco (*N. tabacum* L.), as well as their technological properties and value. The number of identified chemical constituents of *N. tabacum* L. have reached over 4500 (Rodgman and Perfetti, 2013), representing various groups of chemicals. A substantial part of them (alkaloids, polyphenols, carotenoids,

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terpenes, saponines, etc.) are with proven biological activities, while others (aliphatic oxygen containing substances, monoterpenes, etc.) contribute to specific olfactory perceptions (Leffingwell, 1999; Rodgman and Perfetti, 2013).

Among these, the components of the polyphenol complex of tobacco leaves have been extensively studied as phytochemicals with both biological activity and technological importance, playing a decisive role in the development of leaf color and aroma.

The most characteristic polyphenols in tobacco leaves are chlorogenic acid (3-*O*-cafeoylquinic acid) and its isomers (5-*O*-cafeoylquinic, or neochlorogenic acid, 4-*O*-cafeoylquinic acid or cryptochlorogenic acid) as well as the glycosides rutin (quercetin-3-*O*-rutinoside), scopoletin, scopolin and kaempferol-3-*O*-rutinoside (Sheen, 1969; Leffingwell, 1999; Rodgman and Perfetti, 2013). A number of other phenolic acids are also identified in tobacco and tobacco smoke, i.e. coumaric, ferulic, caffeic, sinapic, *p*-hydroxybenzoic, *p*- and *o*-hydroxyphenylacetic, protocatechuic, *p*-hydroxyphenylpropionic acids (Snook et al., 1981). Dagnon and Dimanov (2007) and Dagnon et al. (2008) have studied the polyphenols and valeric acids derivatives in cured leaves of Bulgarian tobaccos and the respective smoke condensates, proposing a chemometric approach for evaluation of leaf quality and smoke aroma based on polyphenol content, as well as the option of using 3-methylvaleric acid as a chemical marker for distinguishing flue-cured Virginia from Burley and oriental tobaccos. A series of approaches and methods for isolation and quantitative determination of polyphenols in tobacco have been developed (Snook et al., 1981; Li et al., 2003; Chen et al., 2007; Wang et al., 2009; Gu et al., 2010; Xie et al., 2010; Yang et al., 2010).

The diversity of ethnobotanical, biological, phytochemical and pharmacological properties of a wide spectrum of medicinal plants from all over the world determines their increasing use in different fields of pharmacy, biotechnology, traditional and folk medicines (Aidi Wannes et al., 2017; Mohammadhosseini, 2017; Mohammadhosseini et al., 2017). A suitable approach to the utilization of the valuable metabolite profile and biologically active compounds of tobacco, similar to that applied to other plant bio-sources, consists of obtaining concentrated extraction products with targeted composition. Various plant extracts, essential oils, volatile fractions with biological activity obtained by different technologies (traditional or accelerated solvent extraction, microwave or ultrasound assisted extraction, solid-phase microextraction, supercritical fluid extraction, subcritical water extraction, etc.) have already found application in diverse areas of human life (Huie, 2002; Frezza et al., 2017; Volcan Maia et al., 2017). The choice of a specific application of plant extracts is governed by their characteristic chemical composition and biological activity -in perfumery, cosmetics and aromatherapy; as flavors, modifiers, preservatives and antioxidants in food industry; as human or animal therapeutics in pharmacy and medicine, as pesticides in agriculture, etc.

Many of the extracts obtained from tobacco contain nicotine, which partially limits their application in food industry or necessitates further purification to make them useful for skin-contacting perfumery, toiletries or cosmetics products. On the other hand, the presence of tobacco alkaloids, together with the other biologically active compounds in the extracts creates grounds for their possible use in specific cosmeceuticals, in biopharmacy, in the treatment of neurological or cognitive diseases, in agriculture (insecticides, semiochemicals), etc. (Akinpelu and Obuotor, 2000; Murcute et al., 2010; De Biasi, 2015).

Different aroma extraction products applied in perfumery and cosmetics, such as concrete, resinoid, absolute (by traditional solvent extraction) and their alternatives (by extraction with liquefied gasses, fractionated extraction, column chromatography or adsorbent purification, etc.) have been obtained from cured leaves of oriental tobaccos. Each one is characterized by a specific chemical composition reflecting the influence of extraction conditions, which therefore determines its biological value and specific scope of application.

The major components of the essential oil and different extracts from oriental and semi-oriental tobaccos from Serbia were reported as follows: neophytadiene (23%) and solanone (29.5%) -in the essential oil; a mixture of C_{25}-C_{35} alkanes (25-40%), agatholic acid (4.1-9.1%), neophytadiene (2.3-9%) and 8,13-*epoxy*-14-labden-12-ol (4.1-6.5%) -in CO_2 extracts; neophytadiene (4-31.9%) and a mixture of higher saturated hydrocarbons (8.4-60.4%) -in ether and ethylacetate extracts (Stojanovic et al., 2000; Alagić et al., 2002; Palic et al., 2002; Alagić et al., 2006; Radulovic et al., 2006). In the essential oil of semi-oriental tobaccos from Iraq, the major components were neophytadiene (23.9-47.7%), 1,2-dihydro-2,5,8-trimethylnaphtalene (4.5-14.4%), solanone (3.2-15.8%), β-damascenone (2.6-8.5%), megastigmatrienone isomers (1.8-5.3%), etc. (Popova et al., 2004).

Out of the 21 volatile components identified in the essential oil from Bulgarian oriental tobacco of the "Krumovgrad 90" variety, twelve were over 3%: abiet-8-en-18-oic acid (10.3%), solanone (7.0%), tetracosane (7.0%), dihydro-β-ionone (5.9%), δ-cadinene (4.7%), (*E*)-phytol (4.5%), heptacosane (3.7%), pentacosane (3.4%), hexacosane (3.4%), octacosane (3.3%), retinol A (3.3%), nonacosane (3.2%) (Popova et al., 2015). In the concrete of the same tobacco, 21 of the volatiles were over 1%, the major ones being palmitic acid (10.3%), 2,2-dimethyl-5,5-bis(2-methyl-2-propenyl)-1,3-dioxane-4,6-dione (3.4%), 4a,7,7,10a-tetramethyl-dodecahydrobenzo[f]chromen-3-one (3.4%). In the same study, the major components of the resinoid were found to be: α-linolenic acid (6.7%),

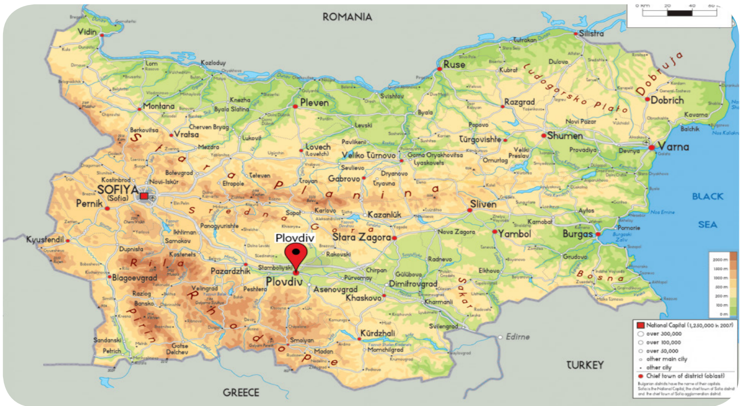

Fig. 1. Photograph of "Plovdiv 7" variety of Bulgarian oriental tobacco (*N. tabacum* L.) on the field and a map of the sampling region.

nicotine (5.7%), palmitic acid (5.3%), norambreinolide (3.4%), 5-hydroxymethylfurfural (2.6%), stigmasterol (2.5%), γ-sitosterol (2.5%), β-amyrin (2.4%), 1-eicosanol (2.4%), α-amyrin (2.2%), 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (2.1%), (*E*)-phytol (2.1%).

Bulgaria is a country with century long traditions in producing high-quality oriental tobacco, recognized on the international tobacco market as an essential component of the blends for different tobacco products. Over 80% of the country's tobacco production and export is oriental tobacco of the ecotype "Krumovgrad". At the same time, the traditions established in the selection of oriental tobacco varieties and the diversity of available varieties with unique phytochemical characteristics offer far bigger choices of plant materials and many more opportunities for obtaining natural phytoproducts with distinctive chemical composition, properties and applications. The "Plovdiv 7" variety is a Bulgarian high-quality oriental tobacco with unique phytochemical characteristics, and is considered as one of these plant selection achievements, which provide alternatives in the "Basma" tobacco variety group not only in terms of smoking properties, but also as a raw material for obtaining extraction products.

Therefore, the study is focused on the investigation of tobacco from a Bulgarian variety of oriental tobacco, i.e. "Plovdiv 7" (*N. tabacum* L.). The average yield under normal agrotechnical conditions is 1600-2000 kg/ha dry leaf. The optimal range of the basic chemical characteristics of cured leaves is: nicotine 1.0-1.2%, soluble carbohydrates 10-14%, and total nitrogen 1.7%. The variety is recognized by the Executive Agency for Variety Testing, Field Inspection and Seed Control and has been included in the 2012 Official Variety List of Bulgaria.

The aim of the study was to analyze the active phytochemicals in the leaves of "Plovdiv 7" oriental tobacco variety and to identify the chemical composition of the essential oil obtained by hydro-distillation and other crude aroma fractions extracted from leaves. The study also aimed to evaluate the antimicrobial activity of crude extracted aroma fractions against nine bacterial strains.

2. Experimental

2.1. Plant material

The material for the study -Bulgarian oriental tobacco (*N. tabacum* L.) of variety "Plovdiv 7", was provided by the Tobacco and Tobacco Products Institute (a branch of the Bulgarian Agricultural Academy). A representative sample of the tobacco material (ID number 42PL-16) has been deposited at the Tobacco and Tobacco Products Institute. The tobacco (Fig. 1) was grown on the Institute's experimental fields in the region of Plovdiv, South Bulgaria (42°04'55.2"N 24°42'16.8"E), on humus-carbonate (rendzina) soil characterized by: organic matter content (by Turin) -2.3%; total nitrogen content (by Kjeldahl) -0.2%; mobile forms of phosphorus P_2O_5 (by Egner -Reem) -14.9 mg/100g soil; available potassium K_2O (by Milcheva) -67.5 mg/100g soil; soil reaction (pH in H_2O) -8.2 (Arinushkina, 1970). The average temperature during the vegetation period (June -September 2016) was 22 °C, with an average rainfall of 44.5 mm. The matured leaves were picked by hand, then cured following the traditional technology for oriental tobacco. Cured leaves were de-stringed, classified and stored in cardboard boxes. The variety is created by intervarietal hybridization of line "Red color No 171" and variety "Tekne-Chervenakovski". Botanically, it belongs to the "Basma" variety group, ecotype "Ustina". The plants are 100-115 mm high, with an average number of usable leaves of 25-30. The leaves are elliptic, smooth, with average size of 25 by 11.5 cm (14^{th} leaf). Cured leaves are light yellow to golden yellow with orange hues, medium bodied.

For the purpose of the phytochemical analyses, the tobacco leaves were oven-dried (40 °C; 6 h) and ground. The moisture content of tobacco (%) was determined by drying to constant weight (103 ± 2 °C), and all results have been presented on a dry weight basis (Stoyanova et al., 2007).

2.2. Chemicals

All chemicals were used as supplied, without further purification. HPLC grade methanol and acetonitrile, as well as phenolic acid and flavonoid standards were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Germany).

2.3. Determination of leaf chemical composition

2.3.1. Basic chemical constituents

The basic indices of the chemical composition of cured tobacco leaves were determined by standardized analytical methods: nicotine -ISO 15152:2003; reducing carbohydrates -ISO 15154:2003; total nitrogen -BSS 15836:1988; mineral substances -ISO 2817:1999. All analyses were performed in triplicate, and the mean values with the respective variation have been presented.

2.3.2. Polyphenols

2.3.2.1. Sample preparation

The primary ground tobacco was further finely powdered by a laboratory homogenizer, and 0.5 g or 1.0 g samples were taken. The extraction of phenolic substances was carried out with 70% methanol in an ultrasonic bath at 70 ºC for 3 hours. After filtration, the extraction procedure was repeated twice. The resulting extracts were combined, evaporated (to dryness) at 60 ºC on a rotary evaporator, and then dissolved in methanol. The solution was filtered (through a 0.45 µm syringe filter) and transferred to the HPLC unit (Marchev et al., 2011b). For the extraction of conjugated phenolic substances, HCl (2.0 M) in methanol was used, and the other conditions were the same.

2.3.2.2. HPLC analyses

The phenolic acids and flavonoids were determined on a Waters HPLC system (Waters, Milford, MA, USA), equipped with a Binary Pump (Waters 1525), UV-VIS detector (Waters 2487 Dual λ Absorbance Detector) and SUPELCO Discovery HS C_{18} column (5 µm, 250 mm×4.6 mm, operated at a temperature of 26 ºC).

2.3.2.3. Phenolic acids

Two mobile phases were used in the gradient elution: Phase A -2.0% acetic acid in water, and Phase B -0.5% acetic acid in water-acetonitrile (1:1, v/v). The gradient was set as follows (Phase B): 0-30 min -increase from 5% to 35% at 0.8 mL/min; 30-45 min -from 35% to 70% at 0.4 mL/min; 45-50 min -from 70% to 80% at 1.2 mL/min; 50-60 min -from 80% to 100% at 1.2 mL/min; 60-65 min -decrease from 100% to 5% at 0.8 mL/min, 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 (Sigma). The detection wavelengths were 280 nm and 320 nm (Marchev et al., 2011b).

2.3.2.4. Flavonoids

The mobile phases in the gradient elution were: Phase A (2.0% acetic acid in water) and Phase B (methanol). The gradient was set up as follows (Phase B): 0-10 min-increase from 30% to 50% at 1.0 mL/min; 10-15 min-held on at the same flow rate; 15-16 min-increase to 52% at flow rate of 0.8 mL/min; 16-30 min-increase to 80% at the same flow rate; 30-35 min -decrease to 30% at 1.0 mL/min, and then maintained to 40 min to equilibrate the column. Myricetin, kaempferol, quercetin, hesperidine and apigenin (Sigma-Aldrich) were the standards used for building the calibration curves. The detection wavelengths were 308 nm and 380 nm (Marchev et al., 2011b).

The quercetin glycosides rutin and hyperoside were determined by using the following mobile phases in the gradient elution: Phase A (2.0% acetic acid in water) and Phase B (acetonitrile). The elution gradient setup was as follows: 0-15 min 20% Phase B; 15-17 min 50% Phase B; 17-20 min 20% Phase B. Rutin and hyperoside (Sigma-Aldrich) were used as standards to build the calibration curves. The detection was carried out at 370 nm (Ivanov et al., 2014).

2.3.3. Triterpenes

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 (raw material:solvent)=1:20 (w/v) and temperature 45 ºC. The solvent in the combined extract was evaporated on a rotary vacuum evaporator (at 60 ºC) and the residue was transferred to 1 mL methanol, filtered through a 22 µm filter and analyzed by HPLC (Marchev et al., 2011a).

The determination of triterpenes was carried out on the same HPLC system (Waters, Milford, MA, USA) as described for the phenolic acid and flavonoid analyses. The mobile phase was an aqueous solution of potassium dihydrogen phosphate (pH 2.8):methanol=12:88 (v/v), with flow rate gradient as follows: 0-18 min at 0.8 mL/min; 18-19 min at 0.6 mL/min; 19-30 min at 0.6 mL/min; 30-31 min at 0.8 mL/min, and 31-40 min at 0.8 mL/min. The detection wavelength was 210 nm. The determination of triterpene compounds was against a previously built standard curve, with carnosic acid, betulin, betulinic, ursolic and oleanolic acid (97%) (Extrasynthese, France) used as standards (Marchev et al., 2011a).

2.4. Obtaining and analysis of essential oil and aroma extraction products

2.4.1. Essential oil

Essential oil was obtained by hydrodistillation of one hundred gram portion of dry tobacco leaves in a laboratory Clevenger-type glass apparatus of the British Pharmacopoeia, modified by Balinova and Diakov (Stoyanova et al., 2007). The distilled essential oil was dried over anhydrous sodium sulfate (Merck) and stored at 4 °C until analysis. The mean yield of essential oil was 0.3 ± 0.01% (v/w) based on three replicate distillations of plant material.

2.4.2. Extraction products

Resinoid was obtained by two-stage, static batch extraction of one hundred gram portion of ground leaves with 95% ethanol (FILLAB, Bulgaria). The extraction conditions were as follows: hydro module (raw material:solvent) -1:10 (w/v); duration of the first and second stage of extraction -2.5 h and 2 h; temperature 70 °C. The solvent was evaporated on a rotary vacuum evaporator, at a temperature of 55 °C (Stoyanova et al., 2007).

The concrete was obtained by two-stage, static batch extraction of one hundred gram portion of ground leaves 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; temperature 30 °C. The solvent was evaporated on a rotary vacuum evaporator at water bath temperature of 35 °C (Stoyanova et al., 2007).

The mean concrete and resinoid yields based on three replicate extractions were 1.9 ± 0.07% (w/w) and 20.4 ± 0.19% (w/w), respectively.

2.4.3. Gas chromatography (GC)

The GC analysis was performed on an Agilent 7890A gas chromatograph equipped with an FID detector and HP-5 MS column (0.25 μm, 30 mx0.25 mm). The temperature gradient of the column for the analysis of the essential oil was set as follows: 40 °C for 3 min, then 5 °C/min to 300 °C and held on for 5 min, run time 60 min, split ratio 60:1, gas flow 1.0 mL/min, MS source: 230 °C, MS Quad: 150 °C. The temperature gradient of the column for the analysis of the extracts was set as follows: 100 °C for 2 min, then 15 °C/min to 180 °C, held on for 1 min, then 5 °C/min to 300 °C, held on for 10 min, run time 42.3 min, splitless mode, gas flow 1.2 mL/min, MS source: 230 °C, MS Quad: 150 °C.

2.4.4. Gas chromatography-mass spectrometry (GC-MS)

The GC-MS analysis was carried out on an Agilent 5975C gas chromatograph equipped with an MS detector. The carrier gas was helium, the column and temperature set ups were as for the GC analysis, MS spectral correlations were done using Wiley, NBS, NIST 08 and own library, as well as published data (Adams, 2001).

2.4.5. Antimicrobial activity

The antimicrobial activity of the extracts was determined against a set of test microorganisms including: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Salmonella abony* NTCC 6017, *Saccharomyces cerevisiae* ATCC 9763, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404, and *Fusarium moniliforme*. The test microorganisms were obtained from the National Bank of Industrial Microorganisms and Cell Cultures (Sofia, Bulgaria) and have been deposited in the microbial culture collection of the Department of Biotechnology and Food Technology, Razgrad Branch, University of Russe, Bulgaria. The antimicrobial activity was studied 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. The cultivation was carried out at 37 °C for 24 h (bacteria), at 27 °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 used as a negative control in order to make the necessary corrections due to solvent activity. Ciprofloxacin (CPH 5μg/disk, HiMedia Laboratories Ltd., India) and fluconazole (FLC 25 μg/disk, HiMedia Laboratories Ltd., India) were used as positive controls. All tests were performed in triplicate.

3. Results and Discussion

3.1. Basic chemical indices of "Plovdiv 7" tobacco variety

The cured tobacco leaves were characterized by the basic chemical indices typically recognized as determinative for their quality as tobacco raw material intended for smoking -nicotine, carbohydrates, nitrogenous compounds, and ash (data shown in

Table 1

Basic quality chemical indices (% DW) of cured leaves of "Plovdiv 7" tobacco variety (n=3).

Group of compounds	Content in leaves (DW%)
Nicotine	2.3 ± 0.02
Reducing sugars	8.4 ± 0.07
Total nitrogen	1.9 ± 0.01
Mineral matter (ash)	14.7 ± 0.13

Table 2
Phenolic acids and flavonoids in leaves of "Plovdiv 7" tobacco variety (*N. tabacum* L.) (n=3).

Compounds	Free	Conjugated (after acid hydrolysis)
	μg/g DW	μg/g DW
Rosmarinic acid	3137.1 ± 30.1	518.7 ± 5.0
Caffeic acid	209.6 ± 2.1	244.3 ± 2.3
Chlorogenic acid	2545.0 ± 24.6	29.8 ± 0.2
p-Coumaric acid	420.9 ± 4.1	315.3 ± 3.1
Sinapic acid	117.8 ± 1.0	773.0 ± 7.5
Ferulic acid	1561.8 ± 14.2	422.4 ± 4.2
Cinnamic acid	61.3 ± 0.5	33.9 ± 0.3
Gallic acid	243.6 ± 2.3	99.6 ± 0.9
3,4-Dihydroxybenzoic acid	77.2 ± 0.7	293.3 ± 2.8
2-Hydroxybenzoic acid	673.3 ± 6.7	49.2 ± 0.4
Vanillic acid	439.0 ± 4.2	4461.9 ± 4.3
Syringic acid	181.0 ± 1.7	435.1 ± 4.2
Myricetin	184.7 ± 1.7	134.2 ± 1.3
Hesperetin	42.3 ± 0.4	118.3 ± 1.1
Quercetin	31.8 ± 0.3	334.1 ± 3.3
Apigenin	NF	493.0 ± 4.8
Luteolin	53.7 ± 0.5	445.6 ± 4.4
Rutin	NF	NF
Hyperoside	247.8 ± 2.5	NF
Kaempferol	20.2 ± 0.1	294.1 ± 2.8

NF-Compound not found

Table 1). At the same time, these indices include phytochemicals that are extractible and biologically active.

The results revealed a relatively higher-nicotine profile of the tobacco material, both regarding the typical nicotine levels of Bulgarian oriental tobaccos of the "Basma" group and the variety's optimal characteristics (nicotine 1.0-1.2%, maximum 2.0%). On the other hand, the level of the balancing reducing sugars was significantly lower than the optimal values for the variety (10-14%). These deviations were also true in comparison with data from a field trial by Kalinova and Yanev (2015), in which the same variety was used as a control under the same soil conditions (crop years 2013 and 2014). These results obviously reflect the influence of other biological factors, such as meteorological conditions, time of harvest, curing, etc.

3.2. Triterpenes in the leaves of "Plovdiv 7" tobacco variety

The following biologically active triterpenes were determined in the leaves of "Plovdiv 7" tobacco variety: betulin (2340.7 μg/g), carnosic acid (845.2 μg/g) and ursolic acid (596.0 μg/g). These results showed that the dominating alcohol betulin constituted 61.9% of the quantified triterpenes, while the level of ursolic acid was 1.4 times as low as that of carnosic acid. No acids of the lupane and oleanane type (betulinic acid, oleanolic acid) were identified.

3.3. Polyphenol composition of the leaves of "Plovdiv 7" tobacco variety

The polyphenol content found in the leaves of "Plovdiv 7" tobacco variety is presented in Table 2. The data showed that the group of free phenolic acids was dominated by cinnamic acid derivatives, largely represented by chlorogenic and ferulic acids, whereas the benzoic acid derivatives, with vanillic acid as their major representative, dominated the group of conjugated phenolic acids (determined after acid hydrolysis).

The group of conjugated flavonols was dominated by myricetin and quercetin, whereas apigenin and luteolin were predominant in that of conjugated flavons. Quercetin glycosides and flavon glycosides were found in very low concentrations.

The results about the polyphenol profile of "Plovdiv 7" tobacco variety comply with the conclusions drawn by a number of authors that the total polyphenol content of and the respective individual compounds in tobacco depend greatly on the *N. tabacum* L. type, plant origin, environmental conditions, season, drying, storage, and other factors (Leffingwell, 1999; Dagnon and Dimanov, 2007; Dagnon et al., 2008; Wang et al., 2008; Wang et al., 2010; Docheva and Dagnon, 2015). There is a relatively limited number of sources providing data useful for making comparative analysis about the content of phenolic acids other than chlorogenic acid and its derivatives neochlorogenic acid and 4-*O*-cafeoilquinic acid, in Bulgarian oriental tobaccos.

Referring to the polyphenol complex of the most popular and widely grown oriental tobacco in Bulgaria -"Krumovgrad 90" variety, as established in our previous work (Popova et al., 2011, 2015), a conclusion could be drawn that "Plovdiv 7" had a different and specific profile regarding the biologically active polyphenols. Nevertheless, the total amount of polyphenols (phenolic acids and flavonoids) was within the typical range of other Bulgarian oriental tobacco varieties (Docheva and Dagnon, 2015). Among the major quantitative

Table 3
Volatile composition (%) of essential oil and extracts from tobacco.

No.	Name	RI	Essential oil	Concrete	Resinoid
1	Acetic acid	673	nd	nd	3.0
2	3-penthanone	701	nd	3.4	nd
3	Ethylmethyl ketone	733	nd	0.8	nd
4	Isoamyl alcohol	760	nd	35.2	nd
5	2-Methyl-1-butanol	762	nd	2.1	nd
6	2-Hexanol	812	nd	1.1	nd
7	Furfural	838	nd	nd	2.7
8	4-Methyl-1-penthanol	843	nd	7.7	nd
9	3-Metyl-3-penthanol	846	nd	5.0	nd
10	Furfuryl alcohol	865	nd	nd	3.5
11	Isoamyl acetate	885	nd	0.7	nd
12	2-Methylbutyric acid	898	nd	2.1	nd
13	α-Pinene	939	0.1	0.2	nd
14	Benzaldehyde	965	nd	1.1	nd
15	β-Pinene	979	0.2	nd	nd
16	Myrcene	997	0.3	nd	nd
17	6-Methyl-5-hepten-2-ol	1003	nd	0.2	nd
18	Trimethylpyrazine	1008	nd	nd	1.7
19	Limonene	1030	0.3	0.6	3.3
20	Eucalyptol (1,8-cineole)	1032	0.1	0.5	0.8
21	Benzyl alcohol	1041	0.3	nd	nd
22	Linalool	1103	0.4	nd	nd
23	α-Ionene	1256	4.3	nd	nd
24	Linalyl acetate	1259	0.7	nd	nd
25	2-Methylnaphthalene	1295	3.1	nd	nd
26	1-Methylnaphthalene	1312	3.4	nd	nd
27	*cis*-5-Butyl-4-methyldihydrofuran-2(3*H*)-one	1344	6.4	nd	nd
28	Nicotine	1366	0.2	0.4	43.6
29	Solanone	1374	6.8	1.7	4.0
30	Oxynicotine	1396	nd	11.0	nd
31	β-Caryophyllene	1419	0.2	nd	nd
32	Dihydro-β-ionone	1443	5.2	nd	nd
33	β-Farnesene	1448	nd	nd	3.0
34	Dimethyl phthalate	1460	3.1	nd	nd
35	β-Damascenone	1390	3.7	nd	nd
36	Diethyl phthalate	1602	nd	nd	5.1
37	Farnesylacetone	1922	nd	nd	1.5
38	(*E*)-phytol	1960	53.4	9.5	13.7
39	Dibutyl phthalate	1972	nd	nd	4.3
40	Cotinine	1981	nd	6.8	0.5
41	Eicosane	2000	nd	nd	6.0
42	Isopropyl palmitate	2026	nd	nd	2.8
	Sum of identified,%	-	**92.2**	**90.1**	**99.5**

RI -retention index, nd -not detected

differences of certain interest in the distribution of phenolic acids was the significantly lower content of chlorogenic acid, the major phenolic in tobacco (approx. 4 times as low) -2545.0 μg/g DW in "Plovdiv 7" and 10536.5 μg/g DW in "Krumovgrad 90" leaves (Popova et al., 2011, 2015). Similar differences were found for cinnamic acid -61.3 μg/g in "Plovdiv 7" and 479.1 μg/g in "Krumovgrad 90" leaves, and proto-catechuic acid -77.2 μg/g and 1731.2 μg/g, respectively. Those were compensated by higher levels of sinapic (117.8 μg/g), ferulic (1561.8 μg/g) and vanillic (434.0 μg/g) acids in "Plovdiv 7" (the respective values for "Krumovgrad 90" were 38.1 μg/g, 145.5 μg/g and 145.5 μg/g) (Popova et al., 2011, 2015).

3.4. Chemical composition of the essential oil and extracts of "Plovdiv 7" tobacco variety

The cured tobacco leaves were further processed to determine the content and composition of essential oil (obtained by hydrodistillation) and of two aromatic products, obtained by solvent extraction -concrete and resinoid. Tobacco (*N. tabacum* L.) concrete and resinoid are the final, ready-to-use aromatic extraction products traditionally applied to perfumery and cosmetics. The three products chosen, which are aroma and biologically active, were obtained and analyzed in the study in order to create grounds for a comparison between the transformations of volatile metabolites occurring during hydrodistillation and extraction, due to the influence of temperature, extractant nature, etc.

The essential oil distilled from "Plovdiv 7" tobacco leaves was a yellowish-brown liquid, and the final concentrated extraction products were dark brown semi-solid masses, all with specific tobacco odor.

The identified volatile components of the oil and extracts from "Plovdiv 7" oriental tobacco variety are presented in Table 3.

Tobacco concrete and resinoid are produced in a number of countries, but their composition and biological value differ according to the variety, type, country of origin, agro-climatic, curing, fermentation, storage and other conditions. In Bulgaria, the traditions in obtaining tobacco extraction products were established as early as the first half of the 20th century, but only for "Dzhebel Basma" oriental tobacco. Recently, "Krumovrgad" group of varieties has also been studied in a similar direction (Popova et al., 2015). "Plovdiv 7" as

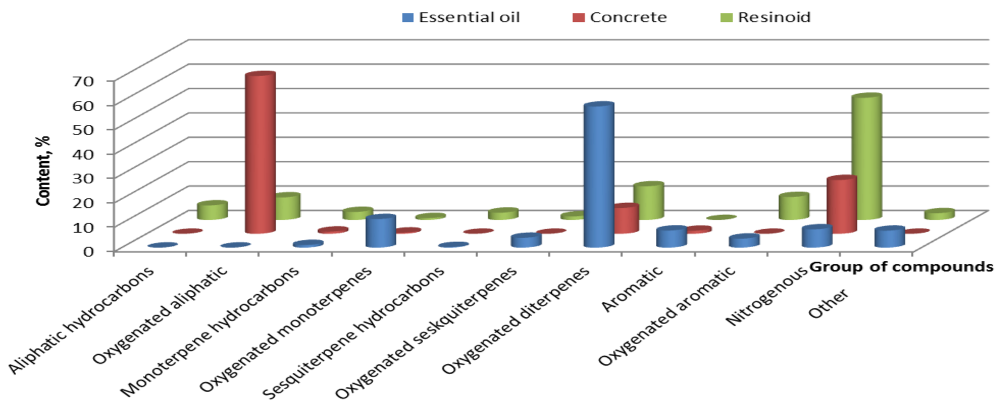

Fig. 2. Groups of components in essential oil, concrete and resinoid from tobacco leaves (% of the identified).

a less popular variety reasonably justifies our interest in its investigation not only as a tobacco material intended for direct human consumption (smoking and smokeless products) but also as a plant source for obtaining biologically and aroma active natural extraction products of their own profile and value.

The results about the volatile compounds in "Plovdiv 7" tobacco extracts (Table 3) confirm those suggestions, revealing clear differences from data about extracts obtained from other oriental tobaccos (Popova et al., 2015).

Nineteen components representing 92.3% of the total oil content were identified in the essential oil. Nine major constituents (over 3%) of the oil were found: (*E*)-phytol (53.4%), solanone (6.8%), *cis*-5-butyl-4-methyldihydrofuran-2(3*H*)-one (6.4%), dihydro-β-ionone (5.2%), α-ionene (4.3%), β-damascenone (3.7%), 1-methylnaphthalene (3.4%), dimethyl phthalate (3.1%), 2-methylnaphthalene (3.1%). The rest of the components were in quantities below 1%. The results about the volatile profile of the essential oil of "Plovdiv 7" variety were in conformity with the findings from other studies on cultural tobacco (*N. tabacum* L.), considering the fact that tobacco essential oil is generally different from the oils of other aromatic plants, in which several specific profile-shaping components are present (Stojanovic et al., 2000; Alagić et al., 2002; Palic et al., 2002; Zhu et al., 2005; Alagić et al., 2006; Radulovic et al., 2006; Popova et al., 2015). The essential oil yield (0.3%) was slightly lower than that from "Krumovgrad 90" oriental tobacco (0.4%) (Popova et al., 2015), but still fell within the range typical of different tobacco types and origins (0.2 -1.5%) (Georgiev and Stoyanova, 2006)

As seen from the data in Table 3, nineteen components were identified in the concrete, representing 90.2% of the total content. Twelve of them were in concentrations over 1% and the remaining 7 were under 1%. The major constituents (over 3%) were: isoamyl alcohol (35.2%), oxynicotine (11.02%), (*E*)-phytol (9.5%), 4-methyl-1-penthanol (7.7%), cotinine (6.8%), 3-metyl-3-penthanol (5.0%), 3-penthanone (3.4%). The concrete yield (1.9%) was close to that obtained under similar conditions from "Krumovgrad 90" variety of oriental tobacco (1.6%) (Popova et al., 2015).

In the resinoid, sixteen components representing 99.4% of the total content were identified. Two of them were in concentrations under 1% and the remaining 14 constituents were in concentrations over 1.0%. The major constituents (over 3%) were: nicotine (43.6%), (*E*)-phytol (3.7%), eicosane (6.0%), diethyl phthalate (5.1%), dibutyl phthalate (4.3%), solanone (4.0%), furfuryl alcohol (3.5%), limonene (3.3%), β-farnesene (3.0%). The resinoid yield (20.4%) was lower than that obtained under similar conditions for "Krumovgrad 90" oriental tobacco (25.7%) (Popova et al., 2015), but higher than the typical range for fermented oriental tobacco cited in earlier references (12-17%) (Georgiev and Stoyanova, 2006).

Similar to tobacco essential oil, these results confirm that in the extraction products from tobacco, the dominant or profile-shaping components could hardly be specified, as has been found in studies on other *N. tabacum* L. representatives (Stojanovic et al., 2000; Alagić et al., 2002; Palic et al., 2002; Zhu et al., 2005; Alagić et al., 2006; Radulovic et al., 2006; Popova et al., 2015). The differences in the yields of essential oil and extracts from those previously reported for oriental tobacco of other varieties and origins (Stojanovic et al., 2000; Alagić et al., 2002; Palic et al., 2002; Alagić et al., 2006; Georgiev and Stoyanova, 2006; Radulovic et al., 2006; Popova et al., 2015) were probably due to the genetic factor (variety) as well as to the soil and climatic conditions influencing plant development and metabolism.

The relative distribution of major groups of volatiles in the essential oil and extracts of "Plovdiv 7" oriental tobacco (expressed as percentage of the identified) is shown in Fig. 2.

Oxygenated diterpenes (57.9%), followed by oxygenated monoterpenes (11.7%) and nitrogenous compounds (7.5%), were predominant in the essential oil. The concrete was dominated by oxygenated aliphatic forms (64.7%), followed by nitrogenous compounds (22.1%) and oxygenated diterpenes (10.6%). The most abundant components in the resinoid were the nitrogenous compounds (mainly alkaloids, 50.1%) followed by oxygenated diterpene (13.8%), aromatic (9.4%) and aliphatic (9.3%) compounds.

3.5. Antimicrobial activity of the tobacco extracts

The concentrated extraction products obtained from tobacco leaves (concrete and resinoid) were tested for antimicrobial activity. The results showed a low to moderate antimicrobial activity compared to the positive control (ciprofloxacin) only against the Gram-positive bacteria *Staphylococcus aureus* (diameters of inhibition zones 12.5 ± 0.2 mm and 11.7 ± 0.1 mm, respectively for concrete and resinoid) and *Bacillus subtilis* (diameter of inhibition zones -11.5 ± 0.1 mm and 12.3 ± 0.2 mm, respectively), while the other seven test-microorganisms were not sensitive.

4. Concluding remarks

Data about phytochemicals with biological activity in leaves from "Plovdiv 7" Bulgarian oriental tobacco variety (*N. tabacum* L.) have been provided in this study for the first time. The tobacco leaves were found to contain different triterpenes and polyphenols (phenolic acids, flavonoids). Two concentrated extraction products (concrete and resinoid) have been obtained, which along with the distilled essential oil, have been characterized in terms of volatile compounds content. Each of the products has revealed a specific volatile composition distinguishing it from the concretes and resinoids from other tobaccos (oriental, Virginia, Burley). These findings underlie the consideration of the tobacco variety studied as a suitable resource for obtaining aroma and biologically active products for perfumery, cosmetics, and other applications. Therefore, the data about the phytochemicals in "Plovdiv 7" Bulgarian oriental tobacco variety could be of interest not only on a national basis but for other tobacco growing countries, as well.

Conflict of interest

The authors declare that there is no conflict of interest.

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