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Polyphenol content and antioxidant activity of aqueous/methanol extracts from different tobacco species (*Nicotiana*)

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The aim of the study was to characterize the polyphenol content in the leaves of different tobacco species (*N. alata* Link & Otto, *N. rustica* L., *N. tabacum* L.), as well as to obtain extracts enriched in phenolic acids and flavonoids, and to evaluate their radical-scavenging activity. The content of chlorogenic acid in the leaves varied from 2.44 ± 0.21 mg/g (*N. rustica*) to 7.10 ± 0.50 mg/g (*N. alata*), and that of rutin – from 0.55 ± 0.05 mg/g (*N. alata*) to 10.85 ± 0.80 mg/g (*N. rustica*). The content of flavonoids was in the range from 0.66 ± 0.12 mg/g (*N. alata*) to 11.51 ± 0.8 mg/g (*N. rustica*). Two types of extracts were obtained using a macroporous resin – enriched in phenolic acids (PA) and enriched in flavonoids (F). The concentration of phenolic acids in Extracts PA was from 6.33 ± 0.50 mg/g (*N. tabacum*) to 3.88 ± 0.20 mg/g (*N. rustica*), and the recovery of phenolic acids – from 71 % to 86 %. Extracts F from *N. rustica* and *N. tabacum* were with flavonoid content of 9.7 ± 0.7 mg/g and 6.15 ± 0.4 mg/g, respectively, and those from the two genotypes of *N. alata* – with only 0.43 ± 0.09 mg/g. The recovery of flavonoids was 72 ± 9 %. Extracts PA were maximally purified from flavonoids, and Extracts F – from phenolic acids. All extracts revealed high antioxidant activity, having IC_{50} values from 5.82 ± 0.97 μg/ml (*N. rustica*) to 14.56 ± 2.61 μg/ml (*N. tabacum*). The results from the study give grounds for regarding the investigated *Nicotiana* species as a promising plant material for obtaining purified natural extracts with potential use in biopharmacy.

Keywords: Polyphenols, Tobacco, Aqueous/methanol extracts, Antioxidant activity

INTRODUCTION

The taxonomy of the genus *Nicotiana*, family *Solanaceae*, includes more than 70 species [1], of which only *Nicotiana tabacum* L. (common tobacco), and to a much lesser extent – *N. rustica* L. (also known as Aztec tobacco, or wild tobacco), are commercially cultivated and widely used for the manufacture of various tobacco products for smoking, snuffing, or oral use. Tobacco is an important cash crop in many countries throughout the world, and it is considered one of the most thoroughly investigated plant materials, along with tea and coffee. More than 4500 individual chemical constituents are identified in the different types, varieties, and selection lines of tobacco, or at different stages of its vegetation and processing. A significant part of these substances are biologically active, e.g., polyphenols, alkaloids, terpenes, saponins, carotenoids, *etc*. [2, 3]. Currently, more than 15 polyphenol metabolites have been identified in *N. tabacum* L., representing the groups of phenolic acids and flavonoids. The major polyphenols in tobacco are chlorogenic acid (3-*O*-caffeoylquinic acid) and its isomers neochlorogenic acid (5-*O*-caffeoylquinic acid) and 4-*O*-caffeoylquinic acid, and the flavonoids rutin (quercetin-3-rutinoside) and kaempferol-3-rutinoside [4–6]. Chlorogenic acid and rutin (vitamin P) possess a wide spectrum of biological activities, e.g., antioxidant, antibacterial, anti-inflammatory, anti-mutagenic and antitumor properties [7, 8].

The available information about the phytochemical characteristics of other *Nicotiana* species (e.g., *N. affinis, N. rustica, N. alata grandiflora, N. longiflora, N. sylvestris, N. wigandioides, etc*.) is relatively limited, and the research has been focused mainly on their alkaloid content [1, 9, 10].

Undoubtedly, over the last years, the interest in the obtaining and application of extracts from traditional or exotic medicinal plants has been constantly growing, and tobacco is a part of this tendency as well. It has been established that the extracts enriched in phenolic acids and flavonoids obtained from different types or varieties of cultivated tobacco have high radical-scavenging activity [5].

Due to the favorable impact of social, geographical and historical factors, Bulgaria has gained recognition over the centuries, as a producer and exporter of high-quality aromatic oriental tobacco. In the country there are traditions and

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distinctive achievements in the selection, development and improvement of oriental tobacco varieties. Parallel to this, consistent research aimed at the obtaining of diverse tobacco-derived bio-products has been carried out. As a part of its research program, the Tobacco and Tobacco Products Institute (Markovo, Bulgaria) started in 2015 the experimental growing of some *Nicotiana* species that had not been cultivated in the country, such as *N. alata* Link & Otto and *N. rustica* L.

The aim of this work was to determine the content of polyphenols in the leaves of different species of wild and cultivated tobacco (*N. alata* Link. & Otto, *N. rustica* L., and *N. tabacum* L.), as well as to obtain extracts enriched in phenolic acids and flavonoids and to evaluate their radical-scavenging activity.

MATERIALS AND METHODS

Plant material

The cured leaves of three tobacco species – *N. alata* Link & Otto, *N. rustica* var. *rustica* and *N. tabacum* L were used as the material in the study. One of them – *N. alata* Link & Otto, was represented by two genotypes (with white and pink petals, flowers at full blossom). Common tobacco – *N. tabacum* L., was represented by Plovdiv 7 (Pd7) variety, a Bulgarian variety of oriental tobacco, belonging to the Basma variety group, Ustina ecotype.

The plant material was provided by the Tobacco and Tobacco Products Institute, part of the Bulgarian Agricultural Academy. All plants were grown in 2016 on the experimental fields of the Institute, situated in the region of Plovdiv, South Bulgaria, under identical agro-ecological and meteorological conditions. The agrochemical characteristics of the soil were as follows: hummus-carbonate (rendzina) type; organic matter content (by Turin) – 2.31 %; total nitrogen content (by Kjeldahl) – 0.21 %; mobile forms of phosphorus P_2O_5 (by Egner-Reem) – 14.85 mg/100 g; available potassium K_2O (by Milcheva) – 67.5 mg/100 g; soil reaction (pH in H_2O) – 8.2 [11]. The vegetation period (June – September) was characterized by an average monthly temperature of 22 °C and an average amount of rainfall – 44.5 mm.

Tobacco leaves were hand-picked at maturity, stringed and sun-cured (48 h) following the traditional technology for oriental tobacco - oven-dried (40 °C; 6 h). Cured leaves were stored in cardboard boxes in an air-conditioned environment to avoid undesirable changes before processing and were finely milled at room temperature before analysis. The tobacco powder was stored in plastic bags in the dark.

Methods

Preparation of extracts enriched in phenolic acids and flavonoids: In order to obtain extracts enriched in phenolic acids or flavonoids we used the polymeric adsorbent (macroporous resin) Amberlite XAD 7, and adapted the method described by Docheva and Dagnon [12]. Samples of 0.5 g of dry tobacco powder were subjected to ultrasound-assisted extraction of polyphenols with 10 ml of 60% CH_3OH for 15 min. The extract was filtered and 3 g of the polymeric adsorbent Amberlite XAD 7 were added. The adsorption of polyphenols on the macroporous resin was carried out for 2 h under static conditions. The extracts enriched in phenolic acids (Extracts PA) and in flavonoids (Extracts F) were obtained by consecutive desorption of the absorbed polyphenols. First we desorbed the phenolic acids by using 70 ml of 30% CH_3OH (thus obtaining Extracts PA), and then – the flavonoids by using 65 ml of 100% CH_3OH (Extracts F). Both desorption steps were carried out under dynamic conditions on a mechanical shaker for 15 min. The content of polyphenols was determined in an aliquot of the obtained extracts by HPLC.

Determination of polyphenols in the plant material: A sample of 0.1 g of powdered tobacco was extracted with 5 ml of 60% CH_3OH for 15 min in an ultrasonic bath. The extract was filtered through a mechanical filter under vacuum. The polyphenols were purified by solid-phase extraction through a C-18 cartridge, and then the solution was filtered through a 0.45 μm membrane filter. The content of polyphenols was determined in an aliquot of the solution by HPLC according to the validated method by Dagnon and Edreva [13].

The separation of polyphenols was carried out on a chromatograph (Perkin Elmer Ltd., England) equipped with a UV/VIS detector at a single wavelength of 340 nm and an analytical column Kromasil C18, 150 mm, 5 μm, 4.6 mm i.d. (Supelco Park, PA, USA). The mobile phases were: phase A – CH_3OH:1% CH_3COOH = 5:95, and phase B – CH_3OH:1% CH_3COOH = 85:15. The gradient elution setup was: 100 % A 0 min; 15 min to 80% A; 35 min to 45 % A; 40 min to 45 % A. The flow rate of the eluent was 1.0 ml/min, and the volume of the injected sample – 10 μl.

Determination of polyphenols in the extracts: The content of polyphenols in an aliquot of the extracts was determined by Dagnon and Edreva [13]. The content of polyphenols was calculated from the peak area on a calibration curve in the

concentration range of 0.005 mg/ml to 0.1 mg/ml rutin and chlorogenic acid. The calculation took into account the sample quantity and the final volume of the sample.

Determination of the radical scavenging activity of extracts enriched in flavonoids (DPPH• method): In order to evaluate the free radical scavenging activity (RSA) by the DPPH• method, we evaporated the extracts enriched in flavonoids on a rotary vacuum evaporator, weighed the solid residue and dissolved it in CH_3OH.

A fresh solution of DPPH• with a concentration of 0.12 mM (0.0024 g of DPPH• dissolved in 50 ml of CH_3OH) was prepared. Then we mixed 2 ml of the DPPH• solution with 2 ml of the extract solutions in different concentrations (0.95 μg/ml; 1.9 μg/ml; 3.8 μg/ml; 7.6 μg/ml; 15 μg/ml; 30 μg/ml, and 60 μg/ml). The resultant solutions were incubated for 30 min in the dark at room temperature. The absorbance of the samples was read at 515 nm on a spectrophotometer. The radical scavenging activity of the samples was calculated by the equation:

$$RSA\ (\%) = [(A_0 - A_b)/A_0] \times 100,$$

where A_0 is the absorbance of the control blank (2 ml of DPPH• solution mixed with 2 ml of CH_3OH), and A_b is the absorbance of the respective sample.

For the interpretation of data the half-maximum inhibitory concentration IC_{50} (μg/ml) was used, defined as the concentration of the extract that achieves 50 % scavenging of DPPH•. The respective IC_{50} value was calculated by interpolation of a graph based on a linear regression model. The concentrations of the samples were plotted on the abscissa, and the mean values of RSA (%) calculated by the equation from three repetitions – on the ordinate.

RESULTS AND DISCUSSION

Polyphenols in the leaves of the studied tobacco species

The individual composition of the polyphenol complex of the two uncommon to the country and not cultivated *Nicotiana* species (*N. alata* and *N. rustica*) was similar to that of the commercial oriental tobacco (*N. tabacum*). The major components were represented by chlorogenic acid, neochlorogenic acid and 4-*O*-caffeoylquinic acid, and the flavonoids rutin (quercetin-3-rutinoside) and kaempferol-3-rutinoside. The observed differences between the samples were mainly quantitative.

The qualitative and quantitative composition of the studied tobacco species is presented in Table 1. Data revealed significant variations in the contents of all components of the polyphenol complex. The differences in the content of the two components having major contribution to the total polyphenol content – chlorogenic acid and rutin – were well defined. The content of chlorogenic acid varied from 2.44 ± 0.21 mg/g (*N. rustica*) to 7.10 ± 0.50 mg/g (*N. alata*), and that of rutin – from 0.55 ± 0.05 mg/g (*N. alata*) to 10.85 ± 0.80 mg/g (*N. rustica*). As data suggested, the *N. alata* species was characterized by a particularly low content of flavonoids (0.66 ± 0.12 mg/g, average for the two samples) in comparison with the other tobaccos studied, and a comparatively high amount of phenolic acids (7.2 ± 0.5 mg/g). The highest total content of polyphenols was found in the oriental tobacco (Pd7) – 16.8 mg/g, and the contents of phenolic acids (8.6 ± 0.6 mg/g) and flavonoids (8.2 ± 0.6 mg/g) were statistically identical.

The content of polyphenols in the sample was within the range characteristic for Bulgarian oriental tobacco [14].

Table 1. Polyphenols (mg/g) in the leaves of different tobacco species (n=3)

Tobacco	Components of the polyphenol complex (mg/g)					Ref.
	Phenolic acids			Flavonoids		
	Neochlorogenic acid	Chlorogenic acid	4-*O*-caffeoyl-quinic acid	Rutin	Kaempferol-3-rutinoside	
N. alata (1)[a]	0.04 ± 0.003	7.10 ± 0.50	0.47 ± 0.05	0.55 ± 0.05	0.20 ± 0.01	
N. alata (2)[b]	0.14 ± 0.01	6.10 ± 0.40	0.55 ± 0.06	0.48 ± 0.05	0.09 ± 0.01	
N. rustica	0.86 ± 0.07	2.44 ± 0.21	1.23 ± 0.14	10.85 ± 0.80	0.66 ± 0.07	
N. tabacum (Pd7)	1.46 ± 0.15	4.80 ± 0.30	2.32 ± 0.21	6.40 ± 0.50	1.80 ± 0.15	
Djebel Basma 1	1.44±0.11	8.03±0.6	3.18±0.2	8.52±0.60	1.39±0.11	[14]
Basma 79	1.28±0.10	5.10±0.4	3.12±0.2	6.71±0.50	1.47±0.11	[14]
Krumovgrad 90	1.41±0.09	4.80±0.3	2.53±0.17	6.80±0.50	1.92±0.13	[14]

[a] *N. alata* genotype with white flowers; [b] *N. alata* genotype with pink flowers

Polyphenols in the extracts

In compliance with the aim of the study, two types of extracts were obtained from the respective tobacco species – one enriched in phenolic acids (Extracts PA) and one enriched in flavonoids (Extracts F). The task was completed by carrying out a sequential separation of the polyphenol components, which was based on their different solubility in the eluent during desorption from the macroporous resin.This specific approach of two-stage desorption allows the targeted maximum amount of phenolic acids and minimum of flavonoids to be achieved in Extracts PA, and *vice versa* – in Extracts F. Therefore, the two types of extracts with different concentrations of the biologically active polyphenols could be regarded as individual products for targeted use in phytopharmaceutical formulations.

a) Extracts enriched in phenolic acids

Fig. 1 presents data about the total content of phenolic acids in the Extracts PA and in the leaves of the respective tobaccos. The analytical results reveal that the highest concentration of phenolic acids was achieved in the extract obtained from the Pd7 variety of oriental tobacco – 6.33 ± 0.50 mg/g, while the lowest – in the extract obtained from *N. rustica* – 3.88 ± 0.20 mg/g. There could be observed a proportionate correlation between the content of phenolic acids in the extracts and in the respective tobacco material. The recovery of phenolic acids was in the range from 71% to 86 %, for the different samples. These data were in compliance with previous research, which found that the average recovery of phenolic acids in extracts obtained by a similar extraction procedure from different Bulgarian cultivars of oriental tobacco was 76% [12]. Extracts PA were maximally purified from flavonoids. The extracts from *N. alata* (samples 1 and 2) were found to contain no flavonoids, while in those from *N. rustica* and *N. tabacum* only a minimal recovery of flavonoids was detected (8 %) despite the considerable flavonoid amount in the initial raw material – an average of 9.8 ± 2.3 mg/g (Table 1).

b) Extracts enriched in flavonoids

The content of flavonoids in the extracts obtained by the second stage of desorption varied considerably between the samples, being proportionate to their initial content in the respective tobacco material (Fig. 2). The highest content of flavonoids was found in the extract obtained from *N. rustica* tobacco – 9.7 ± 0.7 mg/g, followed by that in the extract from *N. tabacum* (Pd7) – 6.15 ± 0.4 mg/g. These are, respectively, the tobaccos characterized by the highest level of flavonoids (Table 1). The content of flavonoids in the extracts obtained from the two genotypes of *N. alata* (samples 1 and 2) was only 0.43 ± 0.09 mg/g on the average, which corresponds well to their lesser amount in the initial plant material (at an average of 0.66 ± 0.12 mg/g).

The recovery of flavonoids from tobacco was approximately equal for all samples – mean 72 ± 9 %, despite the distinctive differences in the content of flavonoids in the plant materials. In all extracts enriched in flavonoids, the detected content of phenolic acids was on the minimum – mean 0.33 ± 0.06 mg/g (e.g., recovery 5 %).

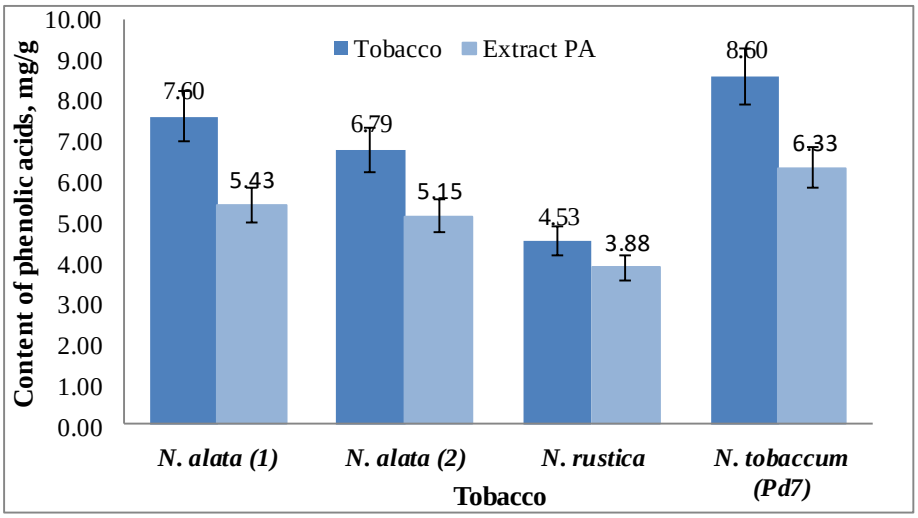

Fig. 1. Content of phenolic acids (mg/g) in the leaves of the studied tobaccos and in the obtained extracts enriched in phenolic acids

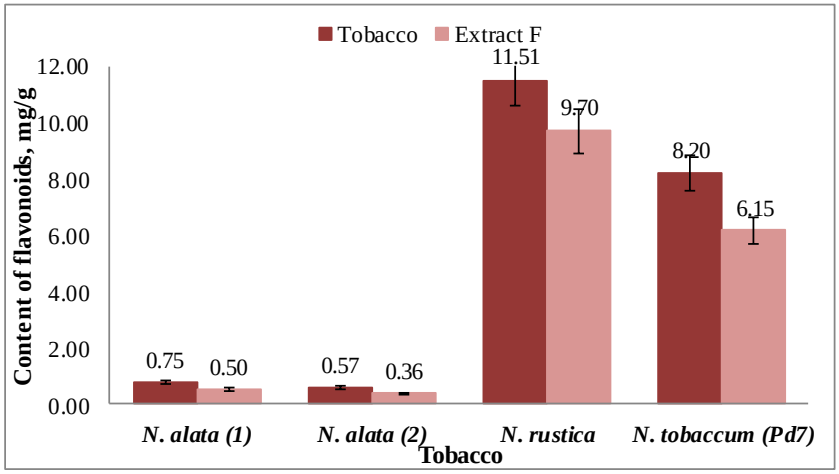

Fig. 2. Content of flavonoids (mg/g) in the leaves of the studied tobaccos and in the obtained extracts enriched in flavonoids

Those data were not significantly different from the findings by a previous research on Bulgarian oriental and Virginia tobacco (*N. tabacum*) cultivars, in which the mean recovery of phenolic acids was 92 %, and that of flavonoids – 6 % [12, 14].

Radical-scavenging activity of the extracts enriched in flavonoids (Extracts F)

Previous research [15] has found that the use of acetone and water as solvents, when applying the DPPH$^{\bullet}$ method, results in incorrect (lower) values of IC_{50}. Taking into consideration that finding, we investigated the radical-scavenging activity only of the extracts enriched in flavonoids, which were obtained by desorption with 100 % methanol.

Table 2 presents data about the content of flavonoids in the respective tobacco sample and the established radical-scavenging activity (IC_{50}) of the extracts. The high antioxidant activity of the extract obtained from *N. rustica* (IC_{50} = 5.82 ± 0.97 µg/ml) corresponds to the high content of flavonoids in the extract (9.7 ± 0.6 mg/g), and comes close to the radical-scavenging activity of the pure reference compound rutin (IC_{50} = 3.56 ± 0.5 µg/ml). Although the extract obtained from the Pd7 oriental tobacco was also characterized by a high content of flavonoids (6.15 ± 0.4 mg/g), it revealed the weakest radical-scavenging activity (IC_{50} = 14.56 ± 2.61 µg/ml). On the other hand, the extracts obtained from *N. alata* (samples 1 and 2) contained approximately equal amounts of flavonoids – mean 0.43 mg/g, but demonstrated significantly different radical-scavenging activities – IC_{50} = 6.71 ± 1.01 µg/ml and IC_{50} = 12.10 ± 2.07 µg/ml, respectively (Table 2). These findings about the radical-scavenging activity could be explained by the presence of biochemicals in the extracts other than flavonoids, which have hydroxyl groups and might be able to react with the free radical. The established radical-scavenging activity of the extracts obtained from *N. alata*, *N. rustica* and *N. tabacum* (variety Pd7) in our study was significantly higher than that of the extracts obtained by two different methods of purification – by adsorption resin and by column chromatography – from the Bulgarian oriental tobacco varieties Djebel Basma 1 and Krumovgrad 90, latter being respectively IC_{50} = 31 µg/ml, and IC_{50} = 40 µg/ml [12, 16].

The results from the study clearly reveal that the enriched-in-flavonoids extracts obtained from wild and cultivated tobacco species possess greater radical-scavenging activity compared to that of the five most active medicinal plants in Bulgaria – curly dock (*Rumex crispus* L.; IC_{50}=40.09 µg/ml), black raspberry (*Rubus occidentalis* L.; IC_{50}=45.23 µg/ml), monk's-rhubarb (*Rumex alpinus* L.; IC_{50}=46.69 µg/ml), sun spurge (*Euphorbia helioscopia* L.; IC_{50}=49.52 µg/ml), and red raspberry (*Rubus idaeus* L.; IC_{50}=50.52 µg/ml) [17].

CONCLUSIONS

A comparative study of the polyphenol content in three tobacco species (*N. alata* Link & Otto, *N. rustica* L. and *N. tabacum* L.) was completed. The results from the HPLC analysis of polyphenols revealed that the leaves of the wild tobacco species (*N. alata* and *N. rustica)* share similar qualitative composition with the cultivated tobacco (*N. tabacum)*, represented in the study by the Bulgarian oriental variety Plodviv 7. There are, however, substantial quantitative differences in the polyphenol profile between the tobacco species. The two types of extracts obtained after fractionation on the microporous resin Amberlite XAD7 showed high levels of phenolic acids and flavonoids, respectively.

Table 2. Content of flavonoids (mg/g) and radical-scavenging activity (μg/ml) of tobacco extracts, enriched in flavonoids (n=3)

Tobacco/plants	Flavonoids in the extract (mg/g)	IC_{50} (μg/ml)	Ref.
N. alata (1)[a]	0.50 ± 0.06	6.71 ± 1.01	
N. alata (2)[b]	0.36 ± 0.01	12.10 ± 2.07	
N. rustica	9.70 ± 0.60	5.82 ± 0.97	
N. tabacum (Pd7)	6.15 ± 0.40	14.56 ± 2.61	
-	Rutin	3.56 ± 0.50	
Djebel Basma 1	8.48±0.72	31.95±5.75	12, 16
Krumovgrad 90	6.12±0.53	39.05±7.03	12, 16
Rumex crispus L.	-	40.09	17
Rubus occidentalis L.	-	45.23	17
Rumex alpinus L.	-	46.69	17
Euphorbia helioscopia L.	-	49.52	17
Rubus idaeus L.	-	50.52	17

[a]*N. alata* genotype with white flowers; [b]*N. alata* genotype with pink flowers

The enriched-in-flavonoids extracts obtained from the untraditional tobacco species – *N. alata* and *N. rustica* – possessed radical-scavenging activity that was two to three times higher than that of the cultivated tobacco (*N. tabacum*). The observed differences in the IC_{50} values support the assumption that the radical-scavenging activity depends not only on the content of flavonoids in the extracts but also on the presence of the accompanying biochemicals with antioxidant properties.

The results from the study give grounds for regarding the investigated *Nicotiana* species as a promising plant material for obtaining purified natural extracts with potential use in biopharmacy.

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ПОЛИФЕНОЛИ И АНТИОКСИДАНТНА АКТИВНОСТ НА ВОДНО-МЕТАНОЛНИ ЕКСТРАКТИ ОТ РАЗЛИЧНИ ВИДОВЕ ТЮТЮНИ

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(Резюме)

Целта на изследването е да се характеризира съдържанието на полифеноли в листата на различни видове тютюн (*Nicotiana alata* Link & Otto, *N. rustica* L., *N. tabacum* L.), както и да се получат екстракти, обогатени на фенолни киселини и флавоноиди и да се определи тяхната радикал-улавяща активност. Съдържанието на хлорогенова киселина в листата варира от 2.44 ± 0.21 mg/g (*N. rustica*) до 7.10 ± 0.50 mg/g (*N. alata*), а това на рутина – от 0.55 ± 0.05 mg/g (*N. alata*) до 10.85 ± 0.80 mg/g (*N. rustica*). Съдържанието на флавоноиди е в границите от 0.66 ± 0.12 mg/g (*N. alata*) до 11.51 ± 0.8 mg/g (*N. rustica*). Два вида екстракти са получени с помощта на полимерен адсорбент (смола) – обогатени на фенолни киселини (PA) и на флавоноиди (F). Концентрацията на фенолните киселини в екстрактите PA е от 6.33 ± 0.50 mg/g (*N. tabacum*) до 3.88 ± 0.20 mg/g (*N. rustica*), а добивът на фенолни киселини – от 71 % до 86 %. Екстрактите F от *N. rustica* и *N. tabacum* са с флавоноидно съдържание съответно 9.7 ± 0.7 mg/g и 6.15 ± 0.4 mg/g, а тези от двата генотипа на *N. alata* – със само 0.43 ± 0.09 mg/g. Добивът на флавоноиди е 72 ± 9 %. Екстрактите PA са максимално пречистени от флавоноиди, а екстрактите F – от фенолни киселини. Всички екстракти показват висока антиоксидантна активност, със стойности на IC_{50} от 5.82 ± 0.97 μg/ml (*N. rustica*) до 14.56 ± 2.61 μg/ml (*N. tabacum*). Резултатите от проучването дават основание да се счита, че изследваните видове Nicotiana са обещаващ растителен материал за получаване на пречистени натурални екстракти с потенциално приложение в биофармацията.