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Polyphenol content in tobacco (*N. tabacum* L.) and antioxidant activity

M. Docheva[1], D. Kirkova[1]*, L. Stoyanova[1,3], V. Dureva[1], R. Dimova[1], D. Dimitrova[1, 2], M. Bachvarova[2], E. Syuleyman[2]

[1]*Tobacco and Tobacco Products Institute, Agricultural Academy, Markovo 4108, Bulgaria*
[2] *University of Plovdiv "Paisii Hilendarski", Department of Organic Chemistry, 24 Tsar Asen Str., 4000 Plovdiv, Bulgaria*
[3]*University of Plovdiv "Paisii Hilendarski", Department of Chemical Technology, 24 Tsar Asen Str., 4000 Plovdiv, Bulgaria*

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The aim of the present work is to determine the content of polyphenols in tobacco varieties and to investigate the antioxidant activity in tobacco extracts. Bulgarian tobacco varieties from the Basmi and Kabakulak variety groups produced conventionally and biologically, were used. Two types of extracts were investigated - crude extracts obtained by four solvents - 100% CH_3OH, 60% CH_3OH, H_2O, and 96% C_2H_5OH, and purified extracts obtained by further purification of crude 60% methanolic extract using two techniques - solid phase extraction (SPE) and resin purification, Tobaccos from the variety group Basmi, conventionally grown, have a higher polyphenol content compared to the tobaccos from variety group Kabakulak. No significant difference was observed in the polyphenol contents of in conventionally and organically grown tobaccos of the same variety. The content of phenolic compounds in crude extracts by various solvents decreased in the following order: 60% CH_3OH>H_2O>100% CH_3OH>96% C_2H_5OH. The crude and purified tobacco extracts showed high antioxidant activity determined by ABTS, FRAP and DPPH assays. The total phenolic content and antioxidant activity of 60% crude methanolic extracts were close to the SPE purified and resin-purified tobacco extracts.

Key words: tobacco extracts, polyphenol content, total phenolic content, antioxidant activity

INTRODUCTION

Polyphenol compounds are secondary metabolites found mainly in plants [1]. They have a great variety of biological functions, some polyphenols act as antioxidants based on their ability to form delocalized unpaired electrons, stabilizing the formed phenoxyl radical after reaction with lipid radicals [2].

Tobacco (*N. tabacum* L.) is a plant containing a large number of chemical substances, including polyphenols. Depending on the type and variety of tobacco, the method of drying and storage, more than 4,500 individual chemical substances have been identified [3]. The cultivation of tobacco in Bulgaria over the last few years has been divided into two agricultural practices: in the conditions of organic production and conventional production [4].

The aim of the present work is to determine the polyphenol content in conventionally and organically grown Bulgarian tobacco varieties and to investigate the antioxidant activity in the obtained crude and purified extracts.

MATERIALS AND METHODS

Material

Dry leaves of tobacco variety Basmi– conventionally and organically (bio) grown and tobacco variety Kabakulak – conventionally grown, were used as a material. The cultivars are from the collection of the Tobacco and Tobacco Products Institute, Plovdiv, Bulgaria. The description of the analyzed tobaccos is presented in Table 1.

Reagents and equipment

2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 100 % CH_3OH, 96 % C_2H_5OH, gallic acid, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), sodium carbonate, hydrochloric acid, 2M Folin-Ciocalteu`s phenol reagent were purchased from Sigma-Aldrich, USA. All chemicals and solvents were of HPLC grade.
Liquid chromatograph equipped with a binary pump, UV/VIS detector and analytical column "Kromasil" C_{18}, 5 μm, 150 mm, Perkin Elmer LC 290, USA. UV/VIS spectrophotometer "Spectroquant Pharo 300".

* To whom all correspondence should be sent:
E-mail: desislavaa894@gmail.com

Table 1. Description of analyzed tobaccos

Growth	Tobacco variety	Sort	Class	Harvest	Designation
Conventional	Basmi	Krumovgrad 58	I	2020	Sample A1
			II	2020	Sample A2
		Krumovgrad 78 C	I	2020	Sample B1
			II	2020	Sample B2
	Kabakulak	Han Tervel 39	I	2020	Sample C1
			II	2020	Sample C2
		Hanski 277	I	2020	Sample D1
			II	2020	Sample D2
Bio	Basmi	Krumovgrad 58	I	2019	Sample AB1
			II	2019	Sample AB2
		Krumovgrad 58	I	2020	Sample AB3
			II	2020	Sample AB4
		Nevrokop1146	I	2020	Sample NB1
			II	2020	Sample NB2

Methods

Preparation of crude extracts: Dry tobacco powder (0.2 g) was extracted with 10 ml H_2O, 100 % CH_3OH, 60 % CH_3OH, 96 % C_2H_5OH for 30 min on a shaker. The extracts were filtered by a syringe filter and used for further analysis.

Preparation of resin-purified tobacco extracts: Resin-purified tobacco extracts were prepared using the method previously reported by Docheva and Dagnon [5].

Preparation of purified tobacco extracts by solid phase extraction (SPE-purified tobacco extracts): SPE-purified tobacco extracts were prepared using the method previously reported by Dagnon and Edreva [6].

Determination of polyphenol content using HPLC: The quantitative determination of polyphenols in tobaccos was carried out by the method described by Dagnon and Edreva [6].

Determination of total phenolic contents (TPC) using the Folin-Ciocalteu (FC) method: The amount of TPC was based on the FC method [7] with some modifications [8]: 0.1 ml tobacco extract (water, 100 % methanolic, 60% methanolic and 96 % ethanolic extracts), 6 ml H_2O and 0.5 ml 0.2 M FC reagent were added. After 4 min 3.4 ml 7.5 % Na_2CO_3 is added. All the samples and the blank were stored in the dark for 2 h, and then the absorbance was measured at 765 nm against the blank sample - 0.1 ml solvent, 6 ml H_2O and 0.5 ml 0.2 M FC reagent. The concentration of the phenolic compounds in the extracts was calculated using gallic acid as standard, and the results were expressed as milligrams gallic acid equivalents per gram extract (mg GAE/g).

2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging method: The ABTS free radical scavenging activity was determined by the method previously reported by Kirkova *et al.* [9].

2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method: The DPPH radical scavenging activity was determined as reported by Docheva *et al.* [10].

Ferric reducing antioxidant power method (FRAP): The FRAP assay was conducted according to the original method reported by Benzie and Strain [11] and modified by Docheva *et al.* [12].

Antioxidant activity was determined using Trolox as standard and the results were calculated as mM TE/g. Assays were performed in triplicate and data were presented as mean values ± standard deviation.

RESULTS AND DISCUSSION

Polyphenol content in different tobaccos determined by HPLC

The polyphenol complex in tobacco mainly included phenolic acids and flavonoids. The main contribution to the amount of phenolic acids is due to chlorogenic acid (ChA), neochlorogenic acid (NChA) and cryptochlorogenic acid (CrChA), while to flavonoids – rutin (Rut) and nicotiflorin (NF). Gallic acid, caffeic acid, sinapic acid, coumarin, myricetin 3-*O*-galactoside, neoeriocitrin, (+)-catechin, scopoletin and others were found in smaller amounts [6]. Full extraction of the polyphenols in tobacco was achieved with 60 % CH_3OH [6].

The content of the main components in the polyphenol complex of the analyzed tobaccos is presented in Figure 1. The content of ChA varied widely - from 3.65±0.37 mg/g (Sample D2) to

20.15±2.02 mg/g (Sample NB1). Less variation was observed in the contents of NChA and CrChA.

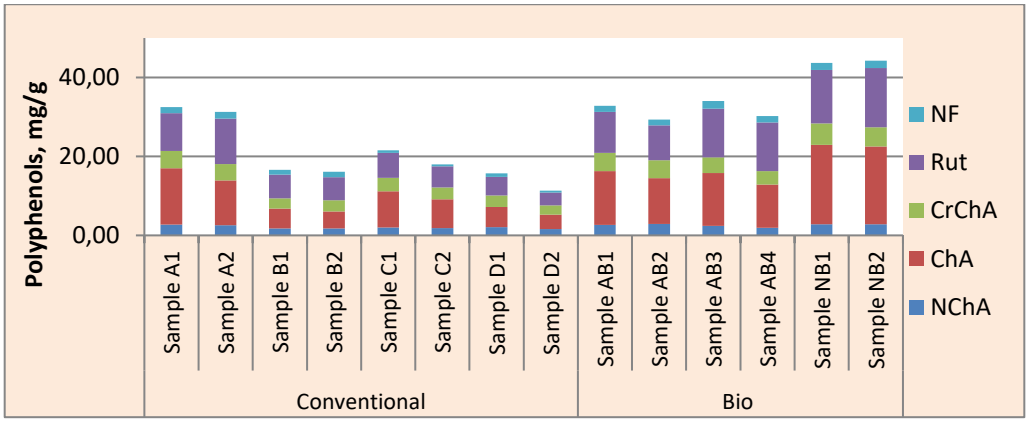

Figure 1. The main components in the polyphenol complex in tobaccos, mg/g

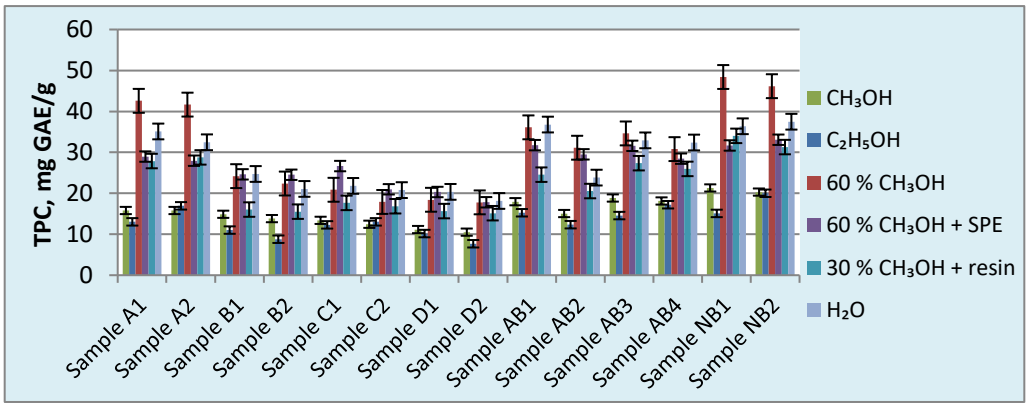

Figure 2. Total phenolic content in tobacco extracts, mg GAE/g

The content of Rut varied between 3.27±0.33 mg/g (Sample D2) and 14.94±1.49 mg/g (Sample NB1). The amount of NF did not exceed 1.91±0.19 mg/g (Sample AB3 and Sample NB1). The polyphenol content in variety Krumovgrad 58 - Sample A1 and Sample A2 (average 32.49±0.89 mg/g) was twice as much as that in Krumovgrad 78 C - Sample B1 and Sample B2 (average 16.36±0.38 mg/g). The tobacco variety Han Tervel 39 - Sample C1 and Sample C2 (average 19.77±2.51 mg/g) was with higher polyphenol content compared to the variety Hanski 277 – Sample D1 and Sample D2 (average 13.50±3.10 mg/g).

Tobaccos from variety group Basmi, conventionally grown (Samples A1, A2, B1 and B2), were characterized with relatively higher polyphenolic content (average 24.11±8.97 mg/g) to the tobaccos of variety group Kabakulak, conventionally grown, Samples C1, C2, D1 and D2 (average 16.63±4.29 mg/g).

Polyphenol content in the organic tobacco variety Nevrokop was average 43.96±0.45 mg/g and was higher than in the organic tobacco variety Krumovgrad 58 (average 31.90±3.13 mg/g). No significant difference in the polyphenol contents in the variety Krumovgrad 58, conventional production and organic production was observed. Statistically, no difference was noticed in the polyphenol contents between the different classes of tobacco from the same variety, as well as between the different harvests – see Fig. 1.

Spectrophotometric determination of TPC in tobacco

The TPC determined by FC method is presented in Figure 2. It is noteworthy that the TPC shows higher content than the phenolic acids determined with HPLC (Figs. 1 and 2). These results can be explained with the selectivity of the definable components – with HPLC only chlorogenic acid and its isomers can be identified. The TPC determined the entire range of phenolic acids and some flavonoids [13]. The differences were also due to the different standards that were used – Rut for HPLC and gallic acid for spectrophotometric analysis.

Figure 2 shows that solvents with different polarities extract different amounts of the TPC. The quantity of TPC decreased in the following order: 60% CH_3OH >H_2O>100 % CH_3OH >96 % C_2H_3OH. The TPC in the extracts obtained with 60 % CH_3OH,

varied between 17.80±1.25 mg GAE/g (Sample D2) and 48.40±3.39 mg GAE/g (Sample NB1) and were close to the TPC in the water extracts - 18.13±0.54 mg GAE/g (Sample D2) and 37.50±2.63 mg GAE/g (Sample NB2). The TPC in 100 % methanolic extracts (from 10.51 ±0.73 mg GAE/g – Sample D2 to 21.31±1.48 mg GAE/g – Sample NB1) showed approximately twice as low results compared to the 60 % methanolic extracts (from 17.79 ±1.26 mg GAE/g – Sample D2 to 48.40±5.52 mg GAE/g – Sample NB1) and water extracts (from 18.13 ±1.29 mg GAE/g – Sample D2 to 36.37±4.31 mg GAE/g – Sample NB1). The ethanolic extracts were characterized with the lowest TPC (from 8.80 ±0.62 mg GAE/g - Sample B2 up to 17.20±1.20 mg GAE/g - Sample AB4).

The selective extraction with solvents of different polarity was used to extract not only the target compounds, but also other substances with similar polarity. Two techniques to purify extracts containing phenolic compounds were applied - solid-phase extraction (SPE) and resin purification.

The TPC of tobacco extracts, obtained by SPE ranged from 17.80±1.27 mg GAE/g (Sample D2) to 33.10±2.32 mg GAE/g (Sample NB2), while the TPC of the resin-purified tobacco extracts varied from 15.10 ±1.06 mg GAE/g (Sample D2) to 34.00±2.38 mg GAE/g (Sample NB). The TPC was comparable to that of extracts obtained by extraction with 60% CH_3OH – see Fig. 2.

It is notable that purified extracts from Samples A1, A2, NB1 and NB2 obtained by SPE and resin purification showed a reduction in total phenolic content of about 15 mg GAE/g, compared to the 60 % methanolic extracts.

The investigated crude and purified extracts from Bulgarian tobacco varieties had a significantly higher content of phenolic acids compared to tobacco extracts from Pakistan, where the TPC varied between 4.85±0.08 mg GAE/g and 24.82±0.07 mg GAE/g [13]. In tobacco leaves from Tunisia, the polyphenols varied between 14.46 mg GAE/g and 23.05 mg GAE/g [14].

Antioxidant activity of tobacco extracts

Three different methods (ABTS, DPPH, and FRAP) were applied to identify the antioxidant activity of tobacco crude and purified extract. DPPH and ABTS are stable free radicals that can determine the free radical scavenging capacity of antioxidants [15]. The FRAP method can measure the antioxidant and reduction abilities of plant extracts according to their ability to reduce Fe^{3+} to Fe^{2+} [16].

ABTS method. Antioxidant activity of tobacco extracts, determined by ABTS method, is shown in Figure 3. The average antioxidant activity of crude 60% methanolic extracts obtained from tobacco variety Kabakulak (Samples C1, C2, D1 and D2) was 157.1±6.3 mM TE/g - lower than the antioxidant activity of tobacco variety Basmi ecotype Krumovgrad tobacco extracts – average 259.3±19.8 mM TE/g. No significant difference in the antioxidant activity of tobaccos from the variety group Basmi, conventionally (251.7±19.8 mM TE/g) and organically (264.4±19.8 mM TE/g) grown was observed. The antioxidant activity of the water extracts varied between 135±9.45 mM TE/g (Sample D2) and 363.9±25.47 mM TE/g (Sample A2) and was similar to that of 60% methanol extracts. It is notable that water extracts obtained from conventionally produced (Samples A1 and A2) and from biologically produced (Samples AB3, AB4, NB1, and NB2) tobaccos had higher activity than 60 % methanolic extracts, despite the lower total phenolic content (Figure 3).

The antioxidant activity of the crude methanolic extracts varied from 61.70±4.93 mM TE/g (Sample D1) to 97.30±6.32 mM TE/g (Sample NB1). The ethanolic extracts had the lowest antioxidant activity, although approximately the same amount of TPC with the methanolic extracts. In crude tobacco extracts obtained from conventionally produced tobacco and organic tobacco no significant difference in antioxidant activities was observed (Fig. 3).

The antioxidant activity of the SPE-purified tobacco extracts ranged from 124.00±8.68 mM TE/g (Sample D2) to 239.00±16.73 mM TE/g (Sample NB2) and was higher than that. of the resin-purified extracts (from 87.90±6.15 mM TE/g Sample D2 to 180.00±12.60 mM TE/g Sample NB1). The antioxidant activity of the SPE-purified tobacco extracts was not significantly different from the crude 60% methanolic extracts. The data obtained indicate that the purification of the tobacco extracts did not affect the increase of the antioxidant activity.

DPPH method. Antioxidant activity determined by DPPH assay showed the highest activity in crude 60% methanolic extracts (from 91.3±6.4 mM TE/g Sample D2 to 263.60±18.40 mM TE/g Sample NB1), followed by methanolic extracts (between 31.30±2.20 mM TE/g Sample D2 and 166.10±11.60 mM TE/g Sample NB1) and ethanolic extracts (ranging from 19.10±1.30 mM TE/g Sample D2 to 71.69±5.00 mM TE/g Sample NB1) – see Fig. 4.

The antioxidant activity of purified SPE tobacco extracts ranged from 96.00±6.70 mM TE/g (Sample D2) to 241.90±16.80 mM TE/g (Sample NB1), and it was close to the antioxidant activity of crude 60% methanolic extracts.

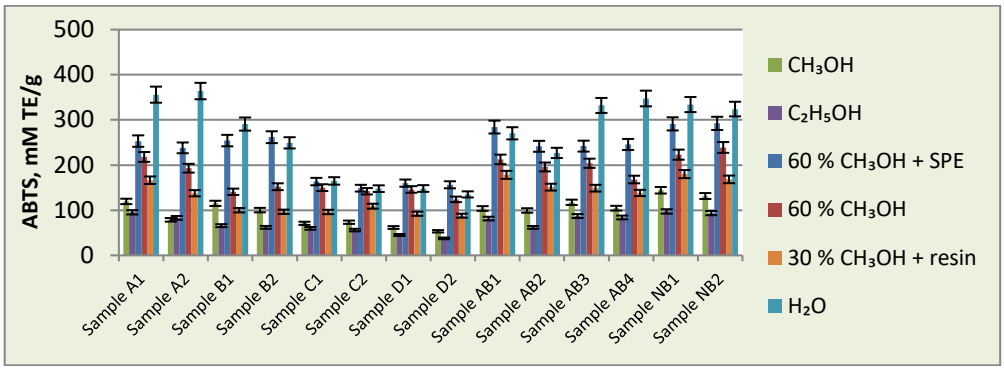

Figure 3. Antioxidant activity of tobacco extracts, determined by ABTS assay, mM TE/g

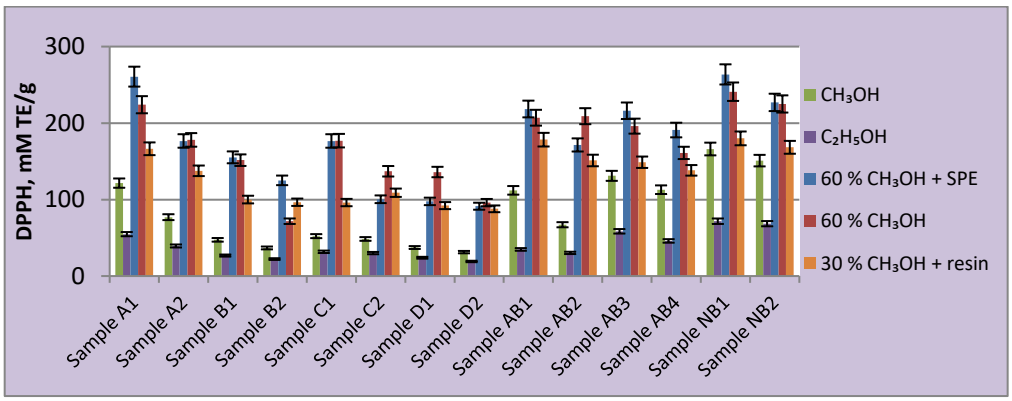

Figure 4. Antioxidant activity of tobacco extracts, determined by DPPH assay, mM TE/g

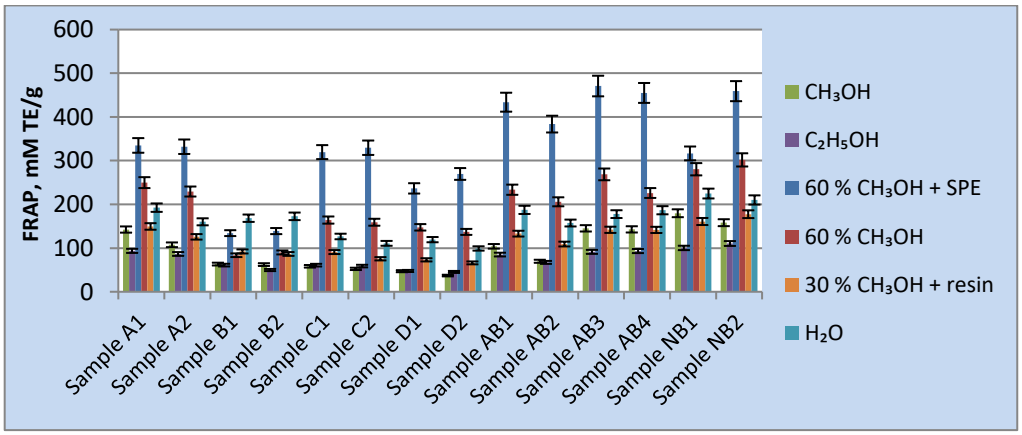

Figure 5. Antioxidant activity of tobacco extracts, determined by FRAP assay, mM TE/g

It is noteworthy that resin-purified extracts obtained from tobaccos of variety group Kabakulak (Sample C1, Sample C2, Sample D1 and Sample D2) had lower antioxidant activity compared to the SPE-purified extracts. The obtained data can be explained by the fact that through the purification with resin, some of the substances contained in tobacco, which have the ability to react with free radicals, were removed. In the resin-purified extracts obtained from conventionally and organically produced variety group Basmi, the antioxidant activity ranged from 60.00±4.20 mM TE/g (Sample B2) to 215.90±15.10 mM TE/g (Sample NB2) and was close to resin-purified extracts (Fig. 4).

The crude and purified tobacco extracts, obtained from variety group Basmi tobaccos, grown under organic conditions (Samples AB1, AB2, AB3, AB4, NB1, and NB2), showed higher antioxidant activity determined by the DPPH method compared to that of conventionally grown tobaccos from variety groups Basmi and Kabakulak.

FRAP method. The results of FRAP assay are presented in Figure 5. The antioxidant activity of crude tobacco extracts decreased in the following order: 60% CH_3OH, H_2O, 100 % CH_3OH and C_2H_5OH. Despite approximately the same amount of TPC in the 60 % methanolic extracts and in the water extracts (Fig. 2), the difference in antioxidant activity was between two and three times (Fig. 5).

The obtained data show that 60% methanolic extracts also contain other substances, which are capable of reducing Fe^{3+} to Fe^{2+}. The antioxidant activity of crude methanolic and ethanolic extracts was approximately the same, which is comparable with TPC.

It is noteworthy that the difference in the antioxidant activity of the SPE-purified tobacco extracts from 83.60±5.80 mM TE/g (Sample B1) to 301.80±21.10 mM TE/g (Sample NB2) was twice as high as the resin-purified tobacco extracts from 66.10±4.60 mM TE/g (Sample D2) to 177.50±12.40 mM TE/g (Sample NB2), despite approximately the same TPC. Tobacco extracts obtained from tobacco variety group Basmi organically grown (Samples AB1, AB2, AB3, AB4, NB1, and NB2) exhibited higher activity, tested by FRAP method, than conventionally produced tobaccos from the variety group Basmi and Kabakulak.

CONCLUSION

Tobaccos from the variety group Basmi (Krumovgrad and Nevrokop) had higher content of phenolic compounds than tobaccos from the variety group Kabakulak (Han Tervel 39 and Hanski 277). No statistically significant difference between conventionally and organically produced tobaccos of variety group Basmi and between classes was observed. Crude 60% methanolic and SPE-purified tobacco extracts, obtained from Bulgarian tobacco varieties conventionally and organically grown, showed strong antioxidant potential.

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REFERENCES

1. O. Alara, N. Abdurahman, C. Ukaegbu, *Curr. Res. Food Sci.,* **4 (3),** 200 (2021).
2. M. Andersen, K. Markham, Flavonoids: chemistry, biochemistry and applications, Boca Raton, FL: CRC Press, Taylor & Francis Group, 2006.
3. A. Rodgman, A. Perfetti, The Chemical Components of Tobacco and Tobacco Smoke, 2nd edn., CRC Press; Boca Raton, Florida, 2013.
4. M. Kasheva, H. Bozukov, M. Docheva, Y. Kochev, D. Kirkova, *BJCS*, **58 (4)**:48 (2021).
5. M. Docheva, S. Dagnon, *C. R. Acad. Bulg. Sci.***, 68 (2),** 183 (2015).
6. S. Dagnon, A. Edreva, *Beitr. Tabakforsch. Int.,* **20 (5)**: 355 (2003).
7. V. Singleton, J. Rossi, *Am. J. Enol. Vinic.,* **16 (48)**:144 (1965).
8. D. Bojilov, S. Manolov, B. Bozadzhiev, J. Stremski, I. Ivanov, *J. Int. Sci. Publ.,* **6**, 314 (2018).
9. D. Kirkova, S. Statkova-Abeghe, M. Docheva, Y. Stremski, S. Minkova, *Bulg. Chem. Commun.*, **52** (Special issue D), 196 (2020).
10. M. Docheva, S. Dagnon, S. Statkova-Abeghe, *Nat. Prod. Res.,* **28 (17),** 1328 (2014).
11. I. Benzie, J. Strain, *Analyatical Biochemistry*, **239,** 70 (1996).
12. M. Docheva, Y. Kochev, D. Kirkova, A. Stoilova, *Bulg. Chem. Commun.,* **52** (Special Issue D), 149 (2020).
13. X. Zou, B. Amrit, A. Rauf**,** M. Saeed, Y. Al-Awthan, M. Al-Duais, O. Bahattab, M. Khan, H. Suleria**,** *ACS Omega,* **6 (39),** 25361 (2021).
14. S. Nasr, S. Aazza, W. Mnif, M. Miguel, *J. Appl. Pharm. Sci,* **4 (08),** 23 (2014).
15. Molnar, M.; I. Jerković, D. Suknović, B. Rajs, K Aladić, D. Šubarić, S. Jokić, *Molecules*, ***22*** **(3),** 348 (2017).
16. J. Nilsson, D. Pillai, G. Onning, C. Persson, A. Nilsson, B. Akesson, *Mol. Nutr. Food Res.*, **49,** 239 (2005).