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Research Article

Total phenolic content and antioxidant activity of extracts obtained from tobacco waste seeds, grown under organic production

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Abstract: This study aimed to determine the total phenolic content of Oriental tobacco waste seeds, grown under organic production, and to evaluate their antioxidant activity by extraction with different solvents under different conditions. The extraction was performed with H_2O, 99.9% CH_3OH, 60% CH_3OH, and 96% C_2H_5OH under maceration and ultrasonic extraction at 20°C and 40°C. All solvents were used in a volume of 4 mL, 5 mL, and 6 mL. The total phenolic content varied between 0.89 mg/g GAE (maceration; sample/solvent ratio 0.1g/5mL, C_2H_5OH) and 5.85 mg/g GAE (maceration; sample/solvent ratio 0.1g/6mL, C_2H_5OH). Ethanolic and 60% methanolic extracts had the highest antioxidant activity as determined by the DPPH method; 60% methanolic and water extracts had the highest antioxidant activity as determined by the ABTS method; while methanolic and 60% methanolic extracts had the highest antioxidant activity as determined by the FRAP method. In addition, the content of nicotine in tobacco seed extract was not detected.

1. INTRODUCTION

Tobacco (*Nicotiana tabacum* L.) is a plant from the genus *Nicotiana*, family Solanaceae, and its leaves are widely used for smoking, chewing, and sniffing. *Nicotiana tabacum* is cultivated as an economically important crop all over the world. In Bulgaria, tobacco is produced conventionally according to standard agro-technological practices for the respective variety. In 2016, the Tobacco and Tobacco Products Institute at the Agricultural Academy in Sofia certified a biofield at the Experimental Tobacco Station Department - Gotse Delchev, where tobacco cultivation began under organic farming conditions. Organic production (farming) includes cultivation practices without the use of conventional techniques and plant protection preparations. This is a new worldwide direction that aims at achieving sustainable and ecological agricultural practices (Bozukov *et al.*, 2019; Raei & Aghaei-Gharachorlou, 2015).

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Tobacco plants produce extremely large quantities of seeds that can be collected per hectare area and may change depending on the place, the type of tobacco plant, and weather conditions. The quantity obtained, for example in Türkiye and Macedonia, is from 600 up to 2500 kg per hectare (Usta, 2005). Tobacco seed is also part of the tobacco cultivation process. Seeds are not collected as a commercial product; they are collected for cultivation (Usta *et al*., 2011). Tobacco seeds can be considered waste under certain circumstances, typically when they are no longer needed for cultivation and are discarded. Research into the quality of the tobacco seed by Tomov *et al.* (1983) shows a certain influence of the size of tobacco seeds on their sowing qualities. Seeds smaller than 0.5 mm were of reduced quality and were recommended as unfit for sowing. Kochev (2008) examined the aerodynamic characteristics of tobacco seeds, techniques, and principles of separation for granulation purposes and identified tobacco seeds smaller than 0.5 mm in size as waste.

Tobacco seeds can give two main products, namely cake and oil. The former is rich in proteins (18 – 41%), amino acids (aspartic acid about 2%, glutamic acid 5 – 6%, arginine over 3% as well as serine, isoleucine, threonine, valine, and others not exceeding 2%), and fiber 11 – 12% (Abbas Ali *et al*., 2008; Frega *et al*., 1991; Rossi *et al*., 2013). On the other hand, the oil content of the seed is between 30% – 50%, rich in unsaturated fatty acids, sterols, and tocopherols (Popova *et al.*, 2018; Zlatanov *et al.*, 2007). Polyphenolic compounds such as gallic acid, 3,4-dihydroxybenzoic acid, and catechin are also found in tobacco seeds (Özcan *et al*., 2023). Data on the content of rutin 0.22 – 0.64 mg/g and chlorogenic acid 0.98 – 2.66 mg/g was found in the literature for Virginia and Burley tobacco seeds. These compounds as secondary metabolites in tobacco seeds have been the subject of study regarding their synthesis as a result of abiotic factors. The phenolic acids of plant extracts can be determined as a total phenolic content (TPC). Plant phenolic compounds are normally soluble in polar organic solvents such as water, ether, chloroform, ethyl acetate, methanol, and ethanol. Methanol, ethanol, and water are mostly used for dissolving phenolics for analytical purposes from different parts of the plant (Alara *et al.*, 2021). The extraction of phenols can be carried out by ultrasound under supercritical CO_2 extraction or microwave-assisted extraction (Banozic *et al.*, 2020). The total phenolic content of tobacco seeds was reported to be 1.24 – 2.26 mg GAE/g when the polyphenols were extracted with CH_3COCH_3 and 1.20 – 1.44 mg GAE/g using 80% C_2H_5OH (Xie *et al*., 2011). Polyphenols are chemical substances with proven antioxidant properties (Kumar *et al*., 2023).

There is little information about the antioxidant activity of extracts from tobacco seeds, especially for those grown under organic production and considered as waste products. In this regard, the scientific interest of the group is directed to determine the total phenolic content in tobacco seeds, considered as waste and grown under organic production. The scope is also to evaluate their antioxidant activity by extracting them with different solvents under different conditions. The aim of this study is therefore to investigate the total phenolic content and antioxidant activity of extracts obtained from tobacco waste seeds, grown under organic production. The data from this study will add to the information on the influence of organic production on the accumulation of phenolic compounds in tobacco seeds. Tobacco seeds do not contain nicotine, a toxic alkaloid with a high content in the tobacco leaves. The results obtained for the antioxidant activity of the compounds extracted from tobacco seeds can outline prospects for their use in the pharmaceutical and food industry as nicotine-free products with health benefits.

2. MATERIAL and METHODS

2.1. Samples

Tobacco seeds from the Bulgarian oriental tobacco variety Krumovgrad 58 were used. The tobacco was grown under organic production conditions in a certified experimental field Gotse Delchev at the Tobacco and Tobacco Products Institute, Plovdiv, Bulgaria under "Technology for organic tobacco production" developed at the Institute of Tobacco and Tobacco Products

(Bozukov, 2018). The seeds were collected and mechanically fractionated with sieves with a diameter of 0.6 mm. Three fractions were separated according to the techniques and principles for separation for granulation purposes described by Kochev (2008) – over 0.6 mm, between 0.5 – 0.6 mm, and under 0.5 mm. Seeds with a size under 0.5 mm were unsuitable for transplanting and considered as waste. The tobacco waste seeds (TWS – under 0.5 mm) were grounded and used for the preparation of extracts.

2.2. Reagents

2,2-diphenyl-1-picrylhydrazyl - DPPH (CAS No: 1898-66-4), 2,2'-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) - ABTS (CAS No: 30931-67-0), methanol (CAS No: 67-56-1), Gallic acid (CAS No: 149-91-7), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), ethanol (CAS No: 64-17-5), sodium carbonate (CAS No: 497-19-8), hydrochloric acid (CAS No: 7647-01-0), 2,4,6-tripyridyl-s-triazine – TPTZ (CAS No: 3682-35-7), 2M Folin-Ciocalteu`s phenol reagent were purchased from Sigma-Aldrich, USA. All chemicals and solvents were for HPLC grade.

2.3. Equipment

UV/VIS Spectrophotometer “Spectroquant Pharo 300” was equipped with a xenon flash lamp, a grating monochromator with a step motor with a wavelength range of 190 – 1000 nm ±1 nm accuracy. The photometric measurement was performed by a photodiode light sensor with a measuring range of A= -3.300 to A= +3.300 (Merck KGaA, Germany). Spectrophotometer was used for the determination of TPC and antioxidant activity by DPPH, ABTS, and FRAP methods.

An automatic autoanalyzer (SEAL Analytical - AA3, Germany) was used for the determination of the content of total alkaloids as nicotine by 460 nm. AA3 is equipped with a multi-speed peristaltic pump and a digital photometer (24-bit high-resolution A/D converter, linear range 0-1.8 (ABS)), detection resolution: 0.1 ug/L, autosampler, and AACE software package.

The ultrasonic bath “Elma Transsonic T 460/H” with a frequency of 460 H was used for ultrasonic extraction (Elma Schmidbauer GmbH, Germany).

2.4. Methods

2.4.1. *Preparation of tobacco seed extracts (TSE)*

Samples from grounded TWS were weighed (0.1 g) on an analytical balance and placed into conical flasks for extraction. The extraction process was performed with four types of solvent – H_2O, 99.9% CH_3OH, 60% CH_3OH, and 96% C_2H_5OH under different conditions – maceration for 24 h, ultrasound extraction for 20 min at 20^oC, and ultrasound extraction for 20 min at 40^oC. All solvents were used in a volume of 4 mL, 5 mL, and 6 mL. The proportion of the sample-solvent ratio was based on the previous research of the study group on the method for the extraction of polyphenols from tobacco leaves (Docheva *et al*., 2018). The extracts were filtered: An aliquot of the extracts obtained was subjected to analysis for total phenolic content using the Folin–Ciocalteu method and antioxidant activity using the DPPH free radical scavenging method (DPPH method), ABTS radical scavenging method (ABTS method), and Ferric reducing antioxidant power assay (FRAP method).

2.4.2. *Determination of the content of total alkaloids as nicotine - Continuous-flow analysis method*

An extract of the TWS was prepared with 5% acetic acid and the total alkaloids (as nicotine) content of the extract was determined by reaction with sulphanilic acid and cyanogen chloride. Cyanogen chloride was generated *in situ* by the reaction of potassium cyanide and chloramine - T. The color developed was measured at 460 nm (ISO 15152:2003).

2.4.3. *Determination of total phenolic content using the Folin-Ciocalteu method*

The amount of total phenols (TPC) was based on the Folin–Ciocalteu (FC) method (Singleton & Rossi, 1965) with some modification. 0.1 mL TSE, 6 mL H_2O, and 0.5 mL 0.2 M Folin–Ciocalteu reactive were placed into the test tube. After 4 min 3.4 mL 7.5% Na_2CO_3 was added. All the samples were stored in the dark for 2 hours and then were measured at 765 nm. The concentration of the phenolic compounds in the extracts was calculated using Gallic acid as standard, and the results were expressed as milligrams of Gallic acid equivalents per gram extract (mg GAE/g).

2.4.4. *DPPH free radical scavenging method (DPPH)*

The DPPH radical (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was carried out as reported by Docheva *et al*. (2014). A solution of $DPPH^{\bullet}$ reagent was prepared daily with a concentration of 0.12 mM (0.0048 g $DPPH^{\bullet}$ was dissolved in 100 mL CH_3OH). Two mL from the DPPH solution was placed in a vessel and 2 mL of TSE was added. The mixtures were placed in the dark for 30 min at room temperature. The absorbance was measured at 515 nm using a spectrophotometer. The results were presented as Trolox equivalents per gram extract (mM TE/g).

2.4.5. *ABTS radical scavenging method (ABTS)*

The ABTS (2,2´-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) free radical scavenging activity was determined by the method previously reported by Kirkova *et al.* (2020). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reaction between 7 mM ABTS in water and 2.45 mM $K_2S_2O_8$ in water, stored in the dark at room temperature for 12–16 h. $ABTS^{\bullet+}$ solution was diluted with CH_3OH to an absorbance of 0.70±0.02 at 734 nm. The mixture of $ABTS^{\bullet+}$ and TSE (1:9 v/v) was placed in a vessel in the dark at room temperature and after 10 min absorbance was measured at 734 nm. The results were presented as Trolox equivalents per gram extract (mM TE/g).

2.4.6. *Ferric reducing antioxidant power method (FRAP)*

The method is based on the reduction of the ferric tripyridyl-s-triazine complex (Fe^{3+} TPTZ) to a ferro-form (Fe^{2+} TPTZ), which has an intense blue color with an absorption maximum of 593 nm. The FRAP assay was conducted according to the original method reported by Benzie and Strain (1966) and modified by Docheva *et al*. (2020). FRAP reagent was prepared by 0.3 M acetate buffer (pH 3.6), ferric chloride dissolved in water, and 0.0312 g TPTZ dissolved in 10 mL 0.04 M HCl mixing in ratio 10:1:1 v/v/v. For the assay 0.05 mL TSE, 0.15 mL distilled water, and 1.5 mL FRAP reagent were mixed. Blue color became visible and after 15 min in the dark, the absorbance of the sample was measured at 593 nm. The results were presented as Trolox equivalents per gram extract (mM TE/g).

2.4.7. *Calculation of relative antioxidant activity index (RACI)*

The RACI is the mean value of the standard scores transformed from the initial data generated by different methods, with no unit limitation and no variance between methods. The following equitation analyzed the relative antioxidant capacity index (RACI):

$$\mathrm{RACI} = \frac{x - \mu}{\sigma}$$

Where x – represents the raw data, μ – the mean value, σ – the standard deviation of the assays (Gorjanovic´ *et al.*, 2013).

2.4.8. *Statistics*

All experimental procedures were done in triplicate. The quantitative data were expressed as mean ± standard deviation (SD). Samples were divided into three groups: maceration, ultrasound 20ºC, and ultrasound 40ºC while One-way ANOVA and Duncan`s test were employed to identify differences in the means of each group. Statistics were analyzed using

IBM SPSS Statistics (version 25, IBM Corp.) and significance was declared at $p<0.05$. Correlation was performed using MS Excel Correlation Data Analysis.

3. RESULTS

3.1. Total Phenolic Content (TPC)

The TPC in TSE, prepared by different solvents under maceration with different sample/solvent ratios, is presented in Table 1. The highest TPC was achieved in TSE with maceration, obtained by C_2H_5OH, ratio 0.1 g/6 mL (5.85±0.63 mg/g GAE) and followed by extract, obtained by 60% CH_3OH, ratio 0.1 g/5 mL (2.34±0.28 mg/g GAE). The lowest TPC was reported for extraction with C_2H_5OH, ratio 0.1 g/4 mL (0.80±0.11 mg/g GAE). There wasn`t a significant difference in the TPC of the TSE with CH_3OH between the sample/solvent ratios.

Under ultrasound extraction at 20°C for 20 min, the TPC varied between 0.96±0.09 mg/g GAE (0.1 g/4 mL H_2O) and 2.12±0.68 mg/g GAE (0.1 g/ 4 mL 60% CH_3OH). It is noteworthy that the TPC in TSE was higher when the extraction was in the ratio 0.1g/4 mL and 0.1g/5 mL solvent CH_3OH, 60% CH_3OH, and C_2H_5OH, compared to the sample/solvent ratio 0.1g/6 mL. The highest TPC in TSE obtained by ultrasound extraction at 40°C in sample/solvent ratio 0.1 g/5 mL 60% CH_3OH (2.71±1.06 mg/g GAE) and 0.1 g/4 mL 60% CH_3OH (2.62±0.55 mg/g GAE) was reported. There were differences in the TPC in TSE, obtained by ultrasonic extraction at 20°C and 40°C. TPC obtained with CH_3OH and 60% CH_3OH was higher as the temperature rose. Generally, 60% CH_3OH extracted the largest amount of total phenols in all the extraction methods described.

Table 1. Total phenolic content (TPC, mg/g GAE) in tobacco waste seed extracts obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1g/6 mL
TPC, mg/g GAE	Maceration/24 h	H_2O	0.98±0.32^{ab}	1.46±0.43^{bc}	0.94±0.03^{ab}
		CH_3OH	1.11±0.20^{abc}	1.36±0.26^{abc}	1.06±0.13^{abc}
		60% CH_3OH	1.12±0.35^{abc}	2.34±0.28^{d}	1.56±0.10^{c}
		C_2H_5OH	0.80±0.11^{a}	0.89±0.08^{ab}	5.85±0.63^{e}
	Ultrasound 20°C/20 min	H_2O	0.96±0.09^{a}	1.12±0.20^{a}	1.02±0.09^{a}
		CH_3OH	1.48±0.11^{abc}	1.72±0.34^{abc}	1.16±0.39^{ab}
		60% CH_3OH	2.12±0.68^{c}	1.32±0.26^{ab}	1.18±0.16^{ab}
		C_2H_5OH	1.96±0.56^{bc}	1.09±0.36^{a}	1.05±0.10^{a}
	Ultrasound 40°C/20 min	H_2O	1.00±0.09^{a}	1.31±0.49^{a}	1.19±0.04^{a}
		CH_3OH	1.14±0.24^{a}	1.39±0.23^{a}	2.02±1.05^{ab}
		60% CH_3OH	2.62±0.55^{b}	2.71±1.06^{b}	1.31±0.10^{a}
		C_2H_5OH	1.06±0.01^{a}	1.09±0.54^{a}	1.52±0.51^{a}

Note: ^{a}Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different ($p<0.05$), n = 3.

3.2. Antioxidant Activity (AO)

The AO activity of TWS extracts was determined using the DPPH-assay, ABTS-assay, and FRAP-assay.

3.2.1. DPPH assay

The antioxidant activity of the TSE was analyzed with the DPPH assay for the three of the solvents (60% CH_3OH, CH_3OH, and C_2H_5OH). The radical scavenging activity of extracts, obtained under maceration, varied between 1.86 ±0.12 mM TE/g (0.1 g/4 mL CH_3OH) and 13.94±0.30 mM TE/g (0.1 g/6 mL C_2H_5OH) (Table 2). The maceration method obtained the highest antioxidant activity with C_2H_5OH as a solvent. The results for methanolic extracts did

not depend on the sample/solvent ratio. This ratio affected the extracts obtained by 60% CH_3OH as a solvent.

Radical scavenging activity for the extracts, obtained by ultrasound at 20°C, varied between 1.07±0.80 mM TE/g (0.1 g/4 mL CH_3OH) and 8.49±0.32 mM TE/g (0.1 g/6 mL 60% CH_3OH), while at 40°C - between 1.25±0.10 mM TE/g (0.1 g/4 mL CH_3OH) and 10.83±0.44 mM TE/g (0.1 g/6 mL C_2H_5OH). The extracts, obtained with 60% CH_3OH and C_2H_5OH, had higher antioxidant activity, determined by the DPPH method, compared to the CH_3OH. Methanolic extracts showed between 3- and 4 times lower antioxidant activity (an average of 2.14 mM TE/g) compared to other extracts (an average of 9.22 mM TE/g). 60% CH_3OH used for extraction with ultrasound at 20°C was the most effective based on the results. It is noteworthy that the radical-scavenging activity of extracts, obtained under maceration, had a higher activity than those obtained by ultrasound extraction despite the lower content of phenolic acids. The values for ultrasound extraction at 40°C, 0.1g/6 mL sample/solvent ratio are higher than those for ultrasound extraction at 20°C and are close to the obtained with maceration.

Table 2. Antioxidant activity of tobacco seed extracts, determined by the DPPH method, obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1g/6 mL
DPPH, mM TE/g	Maceration/24 h	CH_3OH	1.86±0.12^{a}	2.16±0.05^{a}	2.41±0.08^{a}
		60% CH_3OH	7.26±0.43^{c}	8.71±0.53^{d}	10.23±0.45^{e}
		C_2H_5OH	6.19±0.76^{b}	8.96±1.29^{d}	13.94±0.30^{f}
	Ultrasound 20°C/20 min	CH_3OH	1.07±0.80^{a}	2.40±0.87^{ab}	2.10±0.44^{ab}
		60% CH_3OH	6.09±0.83^{cd}	6.58±0.40^{d}	8.49±0.32^{e}
		C_2H_5OH	5.83±0.99^{cd}	4.87±1.03^{c}	2.7±0.83^{b}
	Ultrasound 40°C/20 min	CH_3OH	1.25±0.10^{a}	2.01±0.45^{ab}	2.11±0.04^{ab}
		60% CH_3OH	3.92±0.89^{ab}	5.64±0.40^{b}	9.48±0.99^{c}
		C_2H_5OH	3.39±0.26^{ab}	5.33±0.57^{b}	10.83±0.44^{c}

Note: ^{a}Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different (p<0.05), n = 3.

3.2.2. *ABTS assay*

Another stable free radical cation, ABTS, was used to evaluate the antioxidant activity of TSE. The results were analyzed based on the influence of the extraction method on the same principle as the DPPH assay. The highest antioxidant activity under maceration, determined by ABTS assay, was reported in the extracts, obtained by H_2O and 60% CH_3OH (Table 3). The antioxidant activity in H_2O TSE varied between 3.82±2.85 mM TE/g (0.1 g/5 mL) and 6.16±1.55 mM TE/g (0.1 g/6 mL), while in 60% CH_3OH extracts it varied between 5.13±0.44 mM TE/g (0.1 g/6 mL) and 5.97±0.42 mM TE/g (0.1 g/5 mL). The antioxidant activity for ethanolic and methanolic extracts was less than 1.22±0.37 mM TE/g as the lowest antioxidant activity was reported in ethanolic extracts.

Antioxidant activity of TSE, obtained by ultrasound extraction at 20°C and 40°C for 20 min, varied between 0.98±0.47 mM TE/g 0.1g/4 mL CH_3OH and 6.03±1.16 mMTE/g 0.1g/4 mL 60% CH_3OH (Table 3). The antioxidant activity with ultrasound 20°C performed with 60% CH_3OH depends on the sample/solvent ratio, and the highest result was achieved with 0.1g/4 mL. The ultrasound extraction with H_2O did not depend on the temperature and the results were close to high, between 4.32±0.36 and 5.57±1.29 mM TE/g for ultrasound 20°C and 4.89±0.34 - 5.01±0.04 mM TE/g for ultrasound 40°C.

The highest antioxidant activity, determined by the ABTS method by maceration and ultrasonic extraction, was reported in the extraction with 60% CH_3OH and H_2O, and lower for CH_3OH and C_2H_5OH (Table 3).

Table 3. Antioxidant activity of tobacco seed extracts, determined by ABTS method, obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1/6 mL
ABTS, mM TE/g	Maceration/24 h	H_2O	5.21±0.53^{bc}	3.82±2.85^{b}	6.16±1.55^{c}
		CH_3OH	1.13±0.22^{a}	1.22±0.37^{a}	1.15±0.15^{a}
		60% CH_3OH	5.17±0.61^{bc}	5.97±0.42^{c}	5.13±0.44^{bc}
		C_2H_5OH	1.22±0.62^{a}	0.20±0.32^{a}	0.15±0.29^{a}
	Ultrasound 20°C/20 min	H_2O	4.85±0.28^{cd}	5.57±1.29^{cd}	4.32±0.36^{cd}
		CH_3OH	0.98±0.47^{a}	1.43±1.42^{ab}	1.15±0.48^{a}
		60% CH_3OH	6.03±1.16^{d}	5.15±0.88^{cd}	3.84±0.37^{bcd}
		C_2H_5OH	1.46±0.60^{ab}	3.32±0.25^{abc}	1.41±1.16^{ab}
	Ultrasound 40°C/20 min	H_2O	4.89±0.34^{bc}	5.01±0.04^{bc}	4.95±2.05^{bc}
		CH_3OH	1.59±0.42^{a}	1.02±0.42^{a}	1.27±0.37^{a}
		60% CH_3OH	5.99±1.18^{c}	4.96±0.26^{bc}	5.45±1.30^{c}
		C_2H_5OH	3.13±2.32^{ab}	1.32±0.59^{a}	1.68±1.45^{a}

Note: ^{a}Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different (p<0.05), n = 3.

3.2.3. *FRAP assay*

The antioxidant activity of TSE, obtained by different sample/solvent ratios (H_2O, CH_3OH, 60% CH_3OH, and C_2H_5OH) under different extraction conditions by the FRAP method, is presented in Table 4. The antioxidant activity determined by FRAP-assay varied between 2.07±0.14 mM TE/g, (0.1g/4 mL C_2H_5OH) by maceration and 7.60±0.53 mM TE/g (0.1g/4 mL 60% CH_3OH) by ultrasound extraction at 40°C. The extracts, obtained upon maceration and ultrasound extraction by CH_3OH and 60% CH_3OH, had slightly higher antioxidant activity, compared to the C_2H_5OH and H_2O extracts (Table 4). Solvent/sample ratios 0.1 g/4 mL and 0.1 g/5 mL ultrasound extraction at 20°C and 40°C represent better results with CH_3OH and 60% CH_3OH than 0.1 g/6 mL. The results obtained with H_2O by maceration represent significantly different results in 0.1 g/6 mL sample/solvent ratio, close to that obtained with ultrasound extraction at 20°C.

Table 4. Antioxidant activity of tobacco seed extracts, determined by method, obtained with different solvents and conditions.

	Extraction	Solvent	Sample/solvent ratios		
			0.1g/4 mL	0.1g/5 mL	0.1g/6 mL
FRAP, mM TE/g	Maceration/24h	H_2O	2.98±0.36^{abc}	3.14±0.25^{abc}	4.58±1.67^{bcd}
		CH_3OH	5.13±2.24^{cd}	4.41±0.63^{abcd}	6.26±3.10^{de}
		60% CH_3OH	7.49±1.22^{e}	7.53±1.65^{e}	5.08±0.68^{cd}
		C_2H_5OH	2.07±0.14^{a}	2.41±0.22^{ab}	2.46±0.11^{ab}
	Ultrasound 20°C/20 min	H_2O	3.33±0.40^{a}	4.43±0.72^{abcd}	4.33±0.07^{abcd}
		CH_3OH	5.93±1.81^{cd}	5.69±0.88^{bcd}	3.99±1.35^{ab}
		60% CH_3OH	5.46±0.27^{bcd}	6.02±1.78^{d}	4.47±0.14^{abcd}
		C_2H_5OH	4.14±0.53^{abc}	3.07±0.16^{a}	3.34±0.14^{a}
	Ultrasound 40°C/20 min	H_2O	3.61±0.09^{a}	3.96±0.17^{ab}	3.65±0.83^{a}
		CH_3OH	6.60±0.48^{bc}	5.48±2.36^{b}	5.26±2.66^{b}
		60% CH_3OH	7.60±0.53^{c}	7.19±1.39^{c}	5.07±0.82^{b}
		C_2H_5OH	3.95±1.01^{ab}	4.98±0.31^{b}	4.73±1.37^{b}

Note: ^{a}Values marked by the same letter in the same row and in one group (Maceration, Ultrasonic 20°C and Ultrasonic 40°C are not significantly different (p<0.05), n = 3.

3.3. Correlation Between TPC and AO Activity

The correlation between TPC and the antioxidant activity of extracts, obtained by the same extragents, is presented in Table 5. Table 5 shows that there were weak correlations between

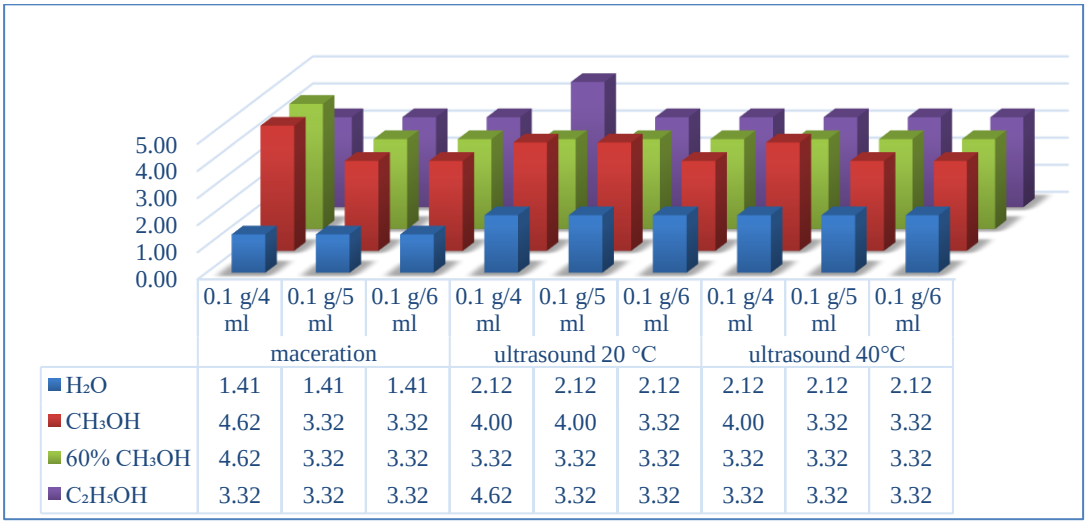
TPC and antioxidant activity. The highest correlation between DPPH assay and TPC was reported in extracts, obtained by C_2H_5OH – R^2=0.5468, followed by DPPH assay and TPC was reported in extracts, obtained by 60 % CH_3OH - R^2=0.3409. The correlation between the antioxidant activity by ABTS-assay and the TPC was not observed. The highest correlation was established in H_2O extracts (R^2=0.3124). The correlation between FRAP-assay and TPC was not observed, similar to the DPPH and ABTS-assay. R^2 varied between R^2=0.0021 (CH_3OH) and R^2=0.3238 (60% CH_3OH).

Table 5. Linear regression between total phenolic content (mg/g GAE) and antioxidant capacity by DPPH assay, ABTS assay, and FRAP assay.

Method	Solvent			
	H_2O	CH_3OH	60% CH_3OH	C_2H_5OH
DPPH	-	0.0223	0.3409	0.5468
ABTS	0.3124	0.0106	0.2863	0.2193
FRAP	0.0505	0.0021	0.3238	0.1743

3.4. Evaluation of Relative Antioxidant Capacity Index (RACI)

To achieve a more reliable comparison between the TSE and Relative Antioxidant Capacity Index (RACI) was determined. As can be seen in Figure 1, the highest values for RACI (4.62) were observed in the extracts, obtained by maceration 0.1g /4 mL CH_3OH and 60% CH_3OH and by ultrasound extraction at 20°C, 0.1g /4 mL CH_3OH. Aqueous extracts had the lowest RACI values because they were studied only by the ABTS and FRAP assays. For extraction under maceration, the RACI value for water extracts was 1.41, while extraction by ultrasound was twice as high – 2.12. The remaining extracts occupy intermediate values of the RACI value – 3.32. It is noteworthy that there was no difference in the RACI coefficient, or in the total antioxidant activity of the extracts obtained by the different extraction methods.



	0.1 g/4 ml	0.1 g/5 ml	0.1 g/6 ml	0.1 g/4 ml	0.1 g/5 ml	0.1 g/6 ml	0.1 g/4 ml	0.1 g/5 ml	0.1 g/6 ml
	maceration			ultrasound 20 °C			ultrasound 40°C		
H_2O	1.41	1.41	1.41	2.12	2.12	2.12	2.12	2.12	2.12
CH_3OH	4.62	3.32	3.32	4.00	4.00	3.32	4.00	3.32	3.32
60% CH_3OH	4.62	3.32	3.32	3.32	3.32	3.32	3.32	3.32	3.32
C_2H_5OH	3.32	3.32	3.32	4.62	3.32	3.32	3.32	3.32	3.32

Figure 1. Relative antioxidant capacity index (RACI) of tobacco seed extract.

4. DISCUSSION and CONCLUSION

The results of the study expressed data on the total phenolic content of the TWS and the antioxidant activity of the TSE obtained from the TWS. The Post Host Test (Duncan`s test) was provided to check the differences between the mean values obtained for each of the analyzed extracts in the group and to determine the statistical similarity between them. The expected result is organic production to imply higher levels of synthesized secondary metabolites such as polyphenolic compounds as it is based on the biological defense of plants against harmful environmental factors. Phenolic acids are health-beneficial compounds, bioactive components

that are capable of delaying or inhibiting the oxidation processes that occur under the influence of atmospheric oxygen or reactive oxygen species (Rodgman & Perfetti, 2013). The phenolic acids of plant extracts can be determined as a total phenolic content (TPC). Xie *et al.* (2011) reported the total phenolic content of tobacco seeds to be 1.24 – 2.26 mg GAE/g when polyphenols were extracted with acetone and 1.20 – 1.44 mg GAE/g using 80% ethanol. The solvents used in the present scientific study showed better results than those reported in the literature (Table 1). The higher results obtained for TPC could be a result of the organic production of the tobacco seeds, considering that polyphenol synthesis depends on the growing conditions and is a response to the abiotic and biotic stresses (Corso *et al.*, 2020). Generally, 60% CH_3OH extracted the higher amount of total phenols in all the three described extraction methods in the study. The results agree with other investigators, which concluded that the highest yield of phenolics from different tobacco plants was observed with extraction with 60% CH_3OH (Dagnon & Edreva, 2003; Docheva *et al.*, 2014; Sun *et al*., 2015). On the other hand, the content of phenolic acids in Bulgarian oriental tobaccos Krumovgrad 988 and Krumovgrad 90 were 6.38 mg/g and 8.04 mg/g respectively (Docheva *et al.*, 2014). Comparing the content of TPC in tobacco leaf extracts with that in TSE, it can be concluded that tobacco seed extracts have 5 – 6 times lower content of phenolic compounds. The TSE was also analyzed for nicotine content. The result was negative, which is an advantage over the extracts obtained from the leaves. There is a similarity between the values obtained for TPC in TSE to that of aqueous extracts, obtained from medicinal plants. Research on water extracts of seven medicinal plants used in phytotherapy (wild yams, dandelion, leuzea, asparagus, Benedectine thistle, cotton thistle, and sarsaparilla) shows that the total phenols were between 0.87 mg GAE/g and 7.16 mg GAE/g (Angelova & Petkova, 2019). Tobacco plants are also known as medical plants and further analyses for individual polyphenol composition will be needed for better comparison.

The antioxidant activity of the TSE was analyzed with three methods, namely DPPH, ABTS, and FRAP methods. These methods were chosen, because they are rapid, robust, and accurate for systematically assessing the total antioxidant capacity of extracts from plant materials on a large scale (Piluzza & Bullitta, 2011). The combination of the DPPH and ABTS methods allows a more complete characterization of the antioxidant properties of the TSE due to the different mechanisms of elimination of $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, while the FRAP method determines the ability of the substances in the TSE to reduce Fe^{+3} to Fe^{+2}.

The highest antioxidant activity was recorded in the DPPH method, followed by the ABTS-method and the FRAP method. The DPPH assay is one of the widely used methods and, together with the ABTS assay, is commonly used to measure the total antioxidant activity of various biological samples by measuring radical scavenging through electron donation (Birasuren *et al.*, 2013). The results for DPPH and ABTS methods (Tables 2 and 3) agreed with the data reported by Xie *et al.* (2011), who reported the antioxidant activity of extracts from tobacco seed flour from Maryland variety by the following methods: DPPH radical scavenging capacity (RDSC) of extracts of seed flour 2.65 – 4.27 µmol Te/g; radical cation ABTS•+ scavenging capacity – 3.42 – 6.89 µmol Te/g; and oxygen radical absorbance capacity (ORAC) – 44 – 74 µmol Te/g. Comparability was observed between antioxidant activity in the extracts, investigated by the DPPH method and the ABTS method with CH_3OH solvent. The antioxidant activity of the C_2H_5OH extracts and 60% CH_3OH extracts was shown to be lower in the ABTS method compared to the DPPH method. Sargi *et al*. (2013) reported similar relation that the extracts of golden flax (3.38 ±0.09 Mmol TEAC g^{-1}), brown flax (3.70 ±0.09 Mmol TEAC g^{-1}) had higher antioxidant activity (ABTS assay) than the results obtained with the same extracts with the DPPH method (golden flax 1.16 ±0.04 Mmol TEAC g^{-1}; brown flax 1.56 ±0.01 Mmol TEAC g^{-1}).

FRAP assay is commonly used to study the antioxidant capacity of plant materials. Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The simple and reliable FRAP assay measures the reducing potential of an

antioxidant reacting with a ferric-TPTZ (Fe(III)-TPTZ) complex and producing a colored ferrous-TPTZ (Fe(II)-TPTZ) complex by a reductant at a low pH (Munteanu & Apetrei, 2021). The extracts, obtained by maceration with CH_3OH and C_2H_5OH, had higher antioxidant activity with the FRAP assay compared to the ABTS assay, while ethanolic extracts had lower antioxidant activity compared to the DPPH assay. The antioxidant activity of TSE is higher compared to the extracts of seeds, rich in Omega-3 – Chia (2.86 ± 0.10 Mmol TEAC g^{-1}), golden flax (0.33 ± 0.10 Mmol TEAC g^{-1}), brown flax (0.76 ± 0.01 Mmol TEAC g^{-1}), white perilla (4.01 ± 0.29 Mmol TEAC g^{-1}), and brown perilla (5.24± 0.09 Mmol TEAC g^{-1}), investigated by FRAP-assay (Sargi *et al*., 2013).

The results obtained for the correlation between TPC and AO activity (Table 5) can be discussed with different qualitative and quantitative compositions in the extracts that suppress or enhance the antioxidant activity. The results obtained by Docheva *et al.* (2020) for tobacco extracts are similar; the researcher concluded that there were some other chemical components in the tobacco extracts, other than phenolics, which were variety-dependent and influenced the DPPH radical scavenging activity. Youn *et al*. (2019) established a weaker correlation between the FRAP value and the concentrations of TPC in extracts, obtained from *Dendropanax morbifera LEV.*, while Angelova and Petkova (2019) found a positive linear correlation between TPC and antioxidant activity (DPPH and FRAP) – R> 0.9 of 7 extracts of medicinal plants.

Figure 1 reveals that the RACI values of all TSE are positive, which indicates that the extracts have a high relative antioxidant capacity. The results for RACI value in the present study are higher than those reported for nettle leaf extracts - RACI value between −1.589 and +1.108 reported (Knežević *et al.*, 2019).

A comparison between the AO activity of the TSE and extracts from tobacco leaves shows that the antioxidant activity of TSE was almost 10 times lower. Docheva *et al.* (2020) established that the antioxidant activity of tobacco extracts, obtained by 60% CH_3OH varied between 76.50±7.12 $mMFe^{2+}/g$ (Myumunovo seme) and 46.66±4.23 $mMFe^{2+}/g$ (Djebel basma 1). The difference in antioxidant activity between ethanolic seed extracts and ethanolic leaf tobacco extracts (between 7.89±0.85 $mMFe^{2+}/g$ DM – Djebel basma 1 and 17.14±1.72 $mMFe^{2+}/g$ DM Myumunovo seme) was even more than 10 times greater.

The total result for antioxidant activity is a prerequisite for further studies using other methods. The paucity of published information on the content of polyphenols in tobacco seeds and their antioxidant activity gives scope for future scientific research in the field of utilization of this type of source. In conclusion, the study extended the information on the accumulation of phenolic compounds in tobacco seeds and the antioxidant activity of extracts derived from them. Maceration with 60% CH_3OH as a solvent could be an appropriate method for obtaining polyphenolic extracts from tobacco waste seeds, with antioxidant activity. Further studies will be necessary to expand the knowledge of the influence of organic production on the accumulation of polyphenolic compounds. Based on the research carried out, tobacco seeds offer the opportunity to obtain extracts of polyphenolic compounds suitable for potential applications in the pharmaceutical and food industries and the possibility of utilizing tobacco waste seeds.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Liliya Stoyanova: Fundings, Conception, Materials, Data collection and processing, Analysis and Interpretation. **Maria Angelova-Romova**: Analysis and Interpretation, Supervision, and Writing **Margarita Docheva**: Design, Literature review. **Desislava Kirkova**: Design, Supervision.

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