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Secondary metabolites from tobacco and different natural herbs, extracted by maceration with polar solvents

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Tobacco (*N. tabacum* L.) is an annual plant belonging to the family *Solanaceae*, genus *Nicotiana*. The first information of the use of tobacco as a medicinal plant data back to 1492. After the isolation of the alkaloid nicotine from tobacco leaves in 1828 and the establishment of its addictive effect, the medical world ceased to use tobacco as a treatment. Several centuries later, scientific research has resumed and it is again directed towards a detailed study of the biologically active substances in tobacco and its alternative use in medicine, bio-engineering and biotechnology. Organic tobacco is a new industrial plant product that is grown on certified bio fields, without using conventional fertilizers and plant protection preparations. The nicotine content is significantly lower than that of tobacco grown under conventional conditions. It is of interest to study the quantitative and qualitative composition of some secondary metabolites in organic tobacco extracts, like total phenolic content, obtained by maceration with different polar solvents, and the comparison with selected medicine plants extracts - thyme, hawthorn, horsetail, nettle, and dandelion. Our research found that bio tobacco has a high content of phenolic acids close to the content of phenolic acids in thyme and is higher than that of the medicinal plants horsetail, dandelion, hawthorn and nettle. Bio tobacco extracts have high antioxidant activity determined by ABTS, DPPH, HPSA, FRAP, CUPRAC methods, similar to that of thyme extracts activty. Bio tobacco extracts have significantly higher antioxidant activity than hawthorn, nettle, horsetail and dandelion extracts.

Keywords: bio tobacco, medical herbs, total phenolic content, antioxidant activity

INTRODUCTION

The plants are able to synthesize many different secondary metabolites [1,2]. Secondary metabolites are directly related to its adaptive properties to the environment [3]. The secondary metabolites such as phenolic acids, flavonoids, and terpenoids are responsible for the antioxidant potential in medicinal plants, which is expressed in the ability to bind to free radicals and "neutralize" their action [1].

The utilization of medicinal plants in folk and official medicine dates back centuries. In connection with the growing potential for the use of medicinal plants, there is an interest in scientific research related to the qualitative and quantitative composition of biologically active substances in medicinal plants, including their antioxidant activity, preclinical and clinical research on the quality and action [3].

Tobacco (*N. tabacum* L.) is an annual plant belonging to the family *Solanaceae*, genus *Nicotiana*. The traditional use of tobacco is to make tobacco products for smoking. As a plant tobacco is also characterized by a large number of secondary metabolites - alkaloids, polyphenols, coumarins, isoflavonoids, carotenoids, terpenes, etc. [4-9]. The plant dates back to 1492. After the isolation of the alkaloid nicotine from tobacco leaves in 1828 and the establishment of its addictive effect, the medical world ceased to use tobacco as a treatment [10]. Several centuries later, scientific research has resumed and it is again directed towards a detailed study of the biologically active substances in tobacco and its alternative use in medicine [11]. Heretofore, more than 15 individual components of the phenolic acids and flavonoids have been identified in tobacco and tobacco waste. The largest amounts are phenolic acids - chlorogenic, neochlorogenic and cryptochlorogenic acids, and flavonoids - rutin and nicotiflorin. Caffeic acid, scopoletin, scopolin, quercetin were found in relatively smaller amounts [12, 13].

At the Tobacco and Tobacco Products Institute, Bulgaria a procedure for growing tobacco on a certified bio field has been developed for several years. There are already data on the main chemical composition of the Krumovgrad 58 bio tobacco variety, where the nicotine content is significantly lower compared to growing the same tobacco variety under conventional conditions [14].

In previous research, the polyphenolic content of different types, varieties, and lines of conventionally

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grown tobacco was investigated [18]. It is of interest to study the polyphenolic content of bio tobacco and compare its phenolic profile with that of medicinal plants from Bulgaria. For this purpose, we selected 5 herbs from different genera and families - thyme, nettle, hawthorn, horsetail and dandelion.

MATERIALS AND METHODS

Plant material: The plant materials, hawthorn fruits (*Crataegus monogyna*), horsetail (*Equisetum arvense*), thyme (*Thymus spp*), nettle leaves (*Urtica dioica* L.) and dandelion root (*Taraxacum officinale*), were purchased from the commercial network from Plovdiv region, Bulgaria. Tobacco was grown in a certified experimental bio field - Gotse Delchev, of the Tobacco and Tobacco Products Institute.

Tobacco, horsetail, thyme, nettle leaves, dandelion root were ground into a fine powder, while hawthorn fruits were crumbled to a particle size of 2-3 mm.

Chemicals: All chemicals are of analytical grade quality and were purchased from Honeywall and Sigma Aldrich (USA).

Instruments: Spectrophotometer "Spectroquant Pharo 300", UV/Vis (Merck, USA)

Preparation of plant extracts: Dry plant powder (0.1 g) was extracted with 10 ml solvent (60 % methanol, 70 % ethanol, water and acetone) for 1h, 2h, 4h, 12h, 72h and 92h on static maceration. The extracts were filtered by a syringe filter and used for further analysis.

Determination of total phenolic content (TPC) by the Folin-Ciocalteu (FC) assay: The TPC of 60 % methanolic, 70 % ethanolic, aqueous and acetonic extracts was assessed using the FC method [16], with some modification [17]: 0.1 ml of plant extract (water, 60 % methanolic, 70% ethanolic and acetonic extract), 6 ml water and 0.5 ml 0.2 M FC reagent were placed into a test tube. After 4 minutes 3.4 ml of 7.5 % Na_2CO_3 was added. All samples and the blank were stored in the dark for 2 hours and then measured at 765 nm against the blank sample. 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).

Determination of antioxidant activity

1,1-diphenyl-2-picryl-hydrazyl (DPPH) assay. The DPPH scavenging capabilities of 60 % methanolic and 70 % ethanolic were evaluated using the free radical method that is reported by Docheva *et al*. [18]. This method measures the reaction of the antioxidants with stable DPPH free radicals. The absorbance is measured at 515 nm against a methanol.

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) assay. The ABTS radical cation ($ABTS^{\bullet+}$) scavenging activities of the 60 % methanolic and 70 % ethanolic solutions were evaluated according to the original method of Re *et al.* [19] with slight modifications by our previous study [20]. The $ABTS^{\bullet+}$ cation radical developed from its reaction with ABTS (7 mM) in H_2O and $K_2S_2O_8$ (2.45 mM) at room temperature in the dark for 14-16 h. The absorbance at 734 nm was measured for each sample relative to methanol.

Hydrogen peroxide scavenging activity (HPSA) assay. HPSA of 60 % methanolic and 70 % ethanolic extracts was determined using 0.2 M phosphate buffer (PB, pH = 7.4) and H_2O_2 (2 mM dissolved in PB). 0.1 ml plant extracts, 0.6 ml H_2O_2 and 3.3 ml PB were placed into a test tube. After 10 minutes in the dark, the absorbance at 230 nm was measured for each sample.

Ferric reducing antioxidant power (FRAP) assay. The reducing power of the ferric (Fe^{3+}) ions of both extracts was measured using Benzie`s method [21] with slight modification reported by Docheva *et al*. [22]. The basis of this method is the reduction of Fe^{3+} to ferricyanide in stoichiometric excess relative to the antioxidants. In this method, the absorbance measurements of the samples are obtained at 593 nm against a blank.

Cupric reducing antioxidant power (CUPRAC) assay. The reducing power of the cupric ions (Cu^{2+}) of aqueous alcoholic extracts was determined according to Apak *et al*. [23] with slight modifications: to a test tube were added 1 ml $CuCl_2$ solution (10 mM dissolved in water), 1 ml of neocuproine alcoholic solution (7.5 mM dissolved in ethanol) and 1 ml of ammonium acetate buffer solution (1 M, pH=7), followed by mixing; 0.1 ml of herbal extract and 1 ml of water were added (total volume, 4.1 ml) and mixed well. The absorbance was measured at 450 nm for each sample after 30 min in the dark [17].

Antioxidant activity was determined using Trolox as reference standard and results were calculated as mM TE/g. The results of each sample were represented as the mean ± standard deviation of three independent replicates.

RESULTS AND DISCUSSION

Determination of TPC in plant extract by different solvents

Optimizing the method for extraction of phenolic compounds (TPC, TFC, and TTC) from medicinal plants has important and significant meaning to

future biomolecules for human health, pharmaceutical, and medicinal research. The extraction techniques, extraction time and the solvent selection are the main challenges toward the development of the technique for the qualitative and quantitative analysis of TPC in plant materials [24, 25].

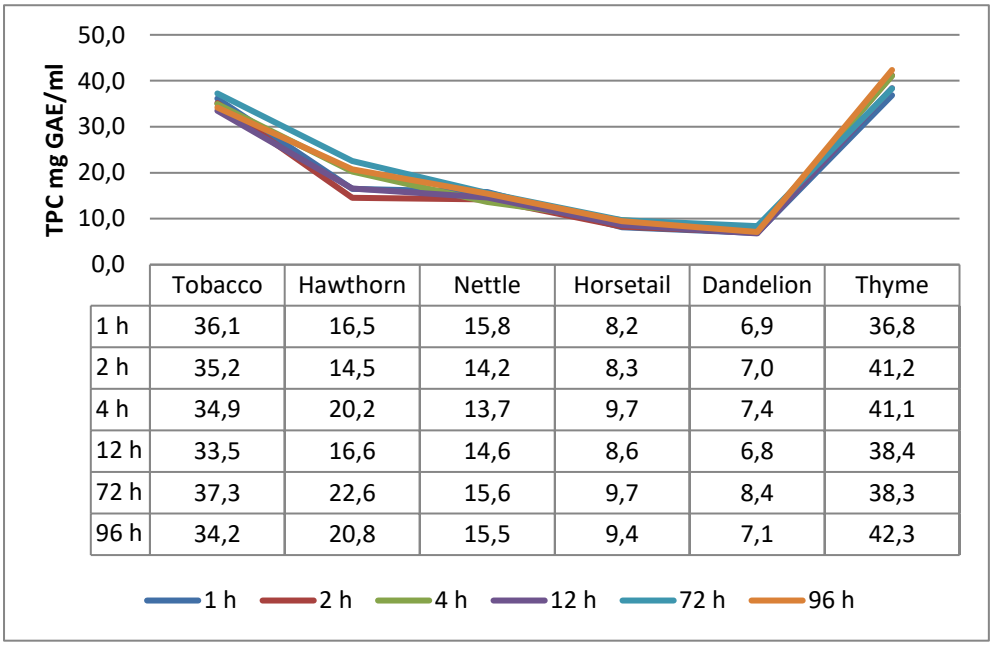
Effect of extraction time by maceration

The most utilized classical methods for extraction of TPC are maceration, solid-liquid extraction, Soxhlet extraction, etc. [25]. In order to investigate the effect of extraction time on total phenolic content yield, the extraction time studied was from 1 to 96 h. A solvent of 60% methanol was used, which has been proven to ensure complete extraction of the total phenolic content in tobacco [26]. As shown in Figure 1 the maximum yield of TPC for tobacco, nettle, horsetail, dandelion, thyme is 1 h. whereas, for the complete extraction of TPC from hawthorn fruit, 72 hours of static maceration are required because of the response surface.

Effect of extraction solvent

The most common solvents used for the extraction of phenolic compounds from plant materials are methanol, ethanol, acetone, and their various aqueous mixtures of various concentrations [25, 27]. Hence, for an optimization method for TPC extraction from tobacco, hawthorn, nettle, horsetail, dandelion and thyme, four solvents with different polarities were used - 60 % methanol, 70 % ethanol, water and acetone. The effect of solvents in the extraction of the target compounds was investigated by the FC method for determination of TPC. The highest total phenolic content was reported in the extracts obtained by 60 % methanol (6.9±0.4 mg GAE/g dandelion – 36.8±2.5 mg GAE/g thyme), followed by 70 % ethanol (5.5 mg GAE/g dandelion – 37.6±2.6 mg GAE/g thyme) and water (25.5±1.7 mg GAE/g bio tobacco II class – 5.4±0.3 mg GAE/g dandelion). The expected lowest amount of phenolic compounds was extracted with pure acetone.

Bio tobacco has a high TPC, which is close to the TPC of thyme and higher than that of the medicinal plants horsetail, dandelion, hawthorn and nettle. The TPC of horsetail and dandelion extracts obtained by extraction with 60% MeOH, 70% EtOH and H_2O is three times lower than that of thyme and bio tobacco extracts and about two times lower than hawthorn and nettle extracts.



	Tobacco	Hawthorn	Nettle	Horsetail	Dandelion	Thyme
1 h	36,1	16,5	15,8	8,2	6,9	36,8
2 h	35,2	14,5	14,2	8,3	7,0	41,2
4 h	34,9	20,2	13,7	9,7	7,4	41,1
12 h	33,5	16,6	14,6	8,6	6,8	38,4
72 h	37,3	22,6	15,6	9,7	8,4	38,3
96 h	34,2	20,8	15,5	9,4	7,1	42,3

Figure 1. Optimization for extraction of TPC by maceration with 60% MeOH

Table 1. TPC of tobaccos and medicinal plants, obtained by different extractants, (mg GAE/g)

Plants	60 % MeOH	70 % EtOH	Acetone	H_2O
Bio tobacco I cl.	36.1±2.5	27.8±1.9	2.0±0.1	22.6±1.5
Bio tobacco II cl.	35.2±2.4	20.8±1.4	2.1±0.1	25.5±1.7
Hawthorn	22.6±1.5	14.5±1.0	2.8±0.2	10.6±0.7
Nettle	15.8±1.1	10.8±0.7	nd.	12.4±0.8
Horsetail	8.2±0.5	5.6±0.3	1.4±0.1	6.2±0.4
Dandelion	6.9±0.4	5.5±0.3	nd.	5.4±0.3
Thyme	36.8±2.5	37.6±2.6	3.6±0.2	25.1±1.7

Phenolic acids as secondary metabolites are mainly formed as a result of biotic or abiotic stress [3]. In this regard, the amount of phenolic acids varies in the same medicinal plant grown under different environmental conditions. Therefore, the comparison of the amount of total phenolic content is mainly informative in character. However, research shows that polar solvents such as butanol, methanol, ethanol or water-alcohol mixtures extract a greater amount of phenolic acids from hawthorn, dandelion, thyme compared to non-polar solvents – diethyl ether, hexane [28-30].

Determination of antioxidant activity of plant extracts

Phenolic compounds are very important plant constituents with free radical scavenging ability, because of their hydroxyl groups. It has been established that phenolic compounds are the major plant compounds with antioxidant activity, and this activity is due to their redox properties. They are a class of antioxidant agents that can absorb and neutralize free radicals [31]. For a better characterization of the extracts, different methods are used to determine the antioxidant activity, based on different reaction mechanisms - transfer of both a hydrogen atom and an electron includes the ABTS, HPSA and DPPH methods; transfers of one electron includes the CUPRAC and FRAP methods [32, 33].

The antioxidant activity of only 60 % methanolic and 70 % ethanolic extracts are investigated, because of the highest TPC. All the extracts show high antioxidant activity determined by methods of transfer of both a hydrogen atom and an electron – ABTS, DPPH and HPSA. The highest antioxidant activity of 60 % methanolic extracts is reported by the ABTS method (from 80.3±5.8 mM TE/g - dandelion to 590.6±41.3 mM TE/g - thyme), followed by HPSA (from 42.2±2.7 mM TE/g - dandelion to 349.7±25.5mM TE/g - tobacco I class) and DPPH method (from 36.7±2.5 mM TE/g - dandelion to 428.0±29.9mM TE/g - thyme), illustrated in Table 2.

It is noteworthy that the extracts from different plants have different activity in relation to the different methods of transfer of both a hydrogen atom and an electron. Bio tobacco extracts exhibited equally high antioxidant activity determined by ABTS and HPSA; hawthorn, horsetail and dandelion - almost twice as much high activity determined by ABTS method compared to DPPH and HPSA, nettle – highest activity by HPSA, while thyme has highest activity determined by ABTS method.

The antioxidant activity determined by the ABTS method was higher compared to the DPPH method in the study of extracts of nettle [34] and thyme [35].

A significant difference in antioxidant activity was observed in transfer of one electron methods – FRAP and CUPRAC assays. The antioxidant activity determined by the CUPRAC method varies from 99.9±7.0 mM TE/g (dandelion) to 868.6±60.8 mM TE/g (thyme), and is twice higher than the antioxidant activity determined by the FRAP method - from 23.6±1.65 mM TE/g dandelion to 401.8±28.1mM TE/ g – thyme.

It is noteworthy that the difference in antioxidant activity between the FRAP and CUPRAC method for hawthorn, dandelion and thyme 60 % methanolic extracts is between two and three times, while for tobacco and nettle extracts – more than four times. This shows that there are substances in the methanolic extracts of tobacco and dandelion that have the ability to reduce Cu^{2+} to a greater extent than Fe^{3+}.

Table 2. Antioxidant activity of tobaccos and medicinal plant extracts, obtained by 60 % MeOH and 70 % EtOH, mM TE/g

Extract	DPPH	ABTS	HPSA	FRAP	CUPRAC
60 % MeOH					
Bio tobacco I cl.	262.5±18.5	367.9±25.7	349.7±25.5	197.0±13.7	801.7±56.1
Bio tobacco II cl.	249.3±17.4	344.0±24.1	318.7±23.0	192.3±13.4	807.3±56.3
Hawthorn	105.5±7.3	208.8±14.6	172.1±9.8	113.0±7.91	333.9±23.3
Nettle	107.8±7.3	148.5±10.3	197.8±13.6	95.0±6.6	470.6±32.9
Horsetail	54.0±3.7	86.3±6.0	113.7±7.9	30.1±2.1	103.3±7.2
Dandelion	36.7±2.5	80.3±5.8	42.2±2.7	23.6±1.65	99.9±7.0
Thyme	428.0±29.9	590.6±41.3	312.2±22.4	401.8±28.1	868.6±60.8
70 % EtOH					
Bio tobacco I cl.	260.2±18.2	284.2±19.8	483.2±33.8	182.1±12.7	865.9±60.6
Bio tobacco II cl.	213.2±14.9	296.1±20.7	400.8±28.0	172.6±12.0	759.8±53.1
Hawthorn	122.4±8.5	223.3±15.6	217.1±15.2	113.8±7.9	339.4±23.7
Nettle	64.5±4.5	130.1±9.1	195.2±13.6	56.5±4.0	238.8±16.7
Horsetail	28.0±1.9	70.3±4.9	109.9±7.7	22.7±3.9	127.4±8.9
Dandelion	31.8±2.2	72.3±5.1	60.6±4.2	17.0±1.2	113.5±77.9
Thyme	353.3±24.7	556.3±38.9	477.0±33.3	372.0±26.0	1064.0±74.2

The antioxidant activity of the ethanolic extracts is similar to that of the methanolic extracts – shown in Table 2. It is noteworthy that the antioxidant activity determined by ABTS method of bio tobacco ethanolic extracts (average 290.2 mM TE/g) is by about 60 mM TE/g lower than the methanolic extracts (average 355.9 mM TE/g). Antioxidant activity of tobacco and thyme ethanolic extracts determined by the HPSA method is by about 100 mM TE/g higher compared to the methanolic extracts. Bio tobacco methanolic and ethanolic extracts have similar high antioxidant activity to thyme extracts determined by ABTS, DPPH, HPSA, FRAP, CUPRAC methods, which is related to the high TPC. The lowest antioxidant activity is reported for the methanolic and ethanolic extracts of dandelion and horsetail, which have the lowest TPC.

CONCLUSION

The total phenolic content and antioxidant activity of bio tobacco extracts and of medicinal plants hawthorn, thyme, dandelion, nettle and horsetail are investigated. The highest yield of TPC by maceration for 1 hour is achieved. An exception is hawthorn fruit, which requires a maceration of 72 hours. No significant differences in TPC when extracted with 60 % methanol and 70 % ethanol are observed. Bio tobacco has a high content of phenolic acids and high antioxidant activity determined by ABTS, DPPH, HPSA, FRAP, CUPRAC methods, which is close to the content of phenolic acids and antioxidant activity in thyme.

Tobacco extracts are characterized with higher TPC and antioxidant activity compared to the medicinal plants horsetail, dandelion, hawthorn and nettle. The 70 % ethanol is a suitable solvent for extracting the maximum amount of phenolic compounds, and the obtained extracts can be used as potential antioxidants.

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