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Evaluation of antioxidant activity of tobacco extracts by CUPRAC and H_2O_2 assay

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Tobacco (*Nicotiana tobacco* L.) is a plant containing biologically active substances (secondary metabolites) like phenolic acids, flavonoids, coumarins, saponins, carotenoids, etc. These compounds exhibit antioxidant activity. The aim of the study is to investigate the antioxidant activity of tobacco extracts by using cupric ion reducing antioxidant capacity (CUPRAC assay) and hydrogen peroxide scavenging assay (H_2O_2 assay). For the research purpose Bulgarian varieties of tobacco from Basmi variety group, grown conventionally or by organic production, and from conventionally grown Kabakulak and Virginia variety groups were used. 60 % methanolic extracts were obtained and the extracts were investigated for antioxidant activity by CUPRAC assay and H_2O_2 assay. The antioxidant activity determined by CUPRAC assay varied between 327.31±22.89 mM TE/g DM (variety Hanski 277, II cl.) and 751.45± 52.6 mM TE/g DM (variety Nevrokop, II cl.), while by H_2O_2 assay – between 226.14±14.46 mM TE/g DM (variety Hanski 277, II cl.) and 409.01±26.16 mM TE/g DM (variety Virginia 0842, II cl.). The obtained results indicated that the extracts from Basmi variety group grown conventionally or by organic production and the extracts obtained from tobaccos of the Virginia variety group, conventionally grown, have higher antioxidant activity compared to the tobacco extracts obtained from the Kabakulak variety group in both investigated methods. The results of the present study showed that 60 % methanolic tobacco extracts, obtained by Basmi, Virginia and Kabakilak variety groups, have significant antioxidant activity, determined by CUPRAC method and H_2O_2 assay.

Keywords: tobacco, tobacco extracts, antioxidant activity, CUPRAC assay, H_2O_2 assay

INTRODUCTION

The use of tobacco leaves for therapeutic purposes dates back years and increases in practice [1]. Tobacco as a medicinal plant synthesizes secondary metabolites (antioxidants) - polyphenols, coumarins, saponins, carotenoids, etc., which exhibit biological activity. The presence of biologically active substances in tobacco is a prerequisite for obtaining extracts with high biological (antioxidant) activity [2, 3].

Reactive oxygen species are unstable oxygen species with unpaired electron. They include radicals - superoxide radical ions ($O_2^{-\bullet}$), hydroxyl radicals ($HO^{\bullet}$), peroxyl radicals ($ROO^{\bullet}$), singlet oxygen radicals ($O_2^{\bullet}$) and non-radical species - hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) which are continuously generated under the physiological processes in the body. These unpaired electrons are called free radicals that can interact with important biomolecules like DNA, lipids, proteins which can destabilize molecules and damage the cell membrane by acquiring free electrons from a stable lipid membrane [4-6].

On the other hand, antioxidant molecules which are diverse in their nature, can prevent the oxidizing effect of free radicals by reducing them [5, 7].

According to the mechanism of action antioxidant assays may be classified into electron transfer (ET) based assays and hydrogen atom transfer (HAT) [4, 8, 9]. ET-based assays include FRAP and CUPRAC assays. Method for oxygen adsorption capacity of free electrons (ORAC method) and method for scavenging H_2O_2 radicals (H_2O_2 assay) belong to hydrogen atom transfer (HAT) methods. Finally, ABTS and DPPH methods are considered as borderline between ET- and HAT based assays) [4, 8, 9].

In each of the listed methods, only the antioxidant activity is examined in relation to the specific reaction for the respective method, and not the overall activity. For this reason no single assay is sufficient for reliable determination of antioxidant activity. The use of several different methods based on different types of reaction or the use of different radicals is recommended [4, 8-11]. Because of this, for a more complete characterization of tobacco extracts, it is necessary to apply different methods for determining their biological activity.

The aim of the study is to investigate the antioxidant activity of tobacco extracts by using cupric ion reducing antioxidant capacity (CUPRAC assay) and scavenging H_2O_2 radicals (H_2O_2 assay).

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EXPERIMENTAL

Material

Dry leaves of Bulgarian tobacco varieties from Basmi group (variety Krumovgrad 58, and variety Nevrokop - grown conventionally or under organic production), Kabakulak group (variety Han Tervel 39, and Hanski 277, conventionally grown) and Virginia group (variety Virginia 514 and Virginia 0842, conventionally grown – II cl.) were used as a material. The cultivars are from the collection of the Tobacco and Tobacco Products Institute, Plovdiv, Bulgaria.

Chemicals

All chemicals were of analytical grade quality and were purchased from Honeywell and Sigma Aldrich (USA).

Instrument

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

Preparation of plant extract

Dry tobacco powder (0.2 g) was extracted with 60 % methanol (10 ml), for 30 min on a mechanical shaker. The extracts were filtered by a syringe filter and were used for further analysis.

In vitro antioxidant activity

- *CUPRAC assay*. The reducing power of the cupric ions (Cu^{2+}) of 60 % methanolic tobacco extracts was determined according to Apak *et al*. [12] with slight modifications: 1 ml $CuCl_2$ solution (10 mM dissolved in water), 1 ml neocuproine alcoholic solution (7.5 mM dissolved in ethanol), 1 ml ammonium acetate buffer solution (1 M, pH=7), 0.1 ml tobacco extract and 1 ml water are successively added and well mixed. The absorbance was measured at 450 nm for each sample after 30 min in the dark [13]. Trolox was used as a standard in the range of 22.5 mM to 375 mM. The results are expressed as mM TE/g DM.
- *H_2O_2 assay*. For scavenging H_2O_2 radicals (H_2O_2 assay) 60 % methanolic tobacco extracts are analyzed 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 placed in a test tube. After 10 min in the dark, the absorbance at 230 nm is measured for each sample. Trolox is used as a standard in the range of 0.09 mM to 3.00 mM. The results are expressed as mM TE/g DM.

Determination of total phenolic contents (TPC) using the Folin-Ciocalteu method (FC)

The determination of the amount of TPC was based on the FC method with some modification [13, 14]: 0.1 ml 60 % methanolic tobacco extract, 6 ml water and 0.5 ml 0.2 M FC reagent are placed into a test tube. After 4 min 3.4 ml 7.5 % Na_2CO_3 is added. All the samples and the blank are stored in the dark for 2 h and then are measured at 765 nm against a blank sample. The concentration of the phenolic compounds in the extracts is calculated using gallic acid as a standard. The results are expressed as mg GAE/g DM.

Statistical analysis

All experiments were performed at least three times. The results were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Total phenolic content (TPC)

Medicinal plants are a source of a wide variety of natural products, such as phenolic acids and flavonoids which possess antioxidant properties [3]. Total phenolic content in tobacco extracts, determined by Folin-Ciocalteu method, is presented in Table 1.

Table 1 shows that tobaccos from the Basmi variety group, organic and conventional production, are characterized by the highest TFC (average 43.01±5.33 mg GAE/g DM), followed by the tobaccos from the Virginia variety group – average 34.25±2.64 mg GAE/g DM. Tobaccos from the Kabakulak variety group have twice lower content of phenolic acids compared to Basmi and Virginia tobaccos – about 17.80±0.12 mg GAE/g DM. There is no significant difference in TPC between organic and conventional production.

Antioxidant activity

Two *in vitro* model systems were used to evaluate scavenging activity in tobacco extracts – CUPRAC method and H_2O_2 method, which have different mechanisms.

CUPRAC assay

CUPRAC transfer ET-based method permits overall quantification of all kinds of antioxidants. ET-based spectrophotometric assays measure the capacity of an antioxidant by the reduction of a

chromogenic oxidant (probe) which changes color when reduced. The degree of color change (either an increase or decrease of absorbance at a given wavelength) is correlated with the concentration of antioxidants in the sample [10].

Antioxidant activity of tobacco extracts, evaluated by the CUPRAC method is presented in Fig. 1. It is obvious that the highest activity is manifested by the tobacco extracts obtained from organic production (Krumovgrad 58 and Nevrokop 1146, respectively, 609.14±42.60 mM TE/g and 648.20±44.20 mM TE/g) and tobacco Nevrokop 1146 conventional production – 751.45±52.6 mM TE/g, followed by the extracts from Virginia tobaccos, conventional production 695.64±48.65 mM TE/g (Virginia 0842) and 662.16±46.58 mM TE/g (Virginia 514) and the extract obtained from Krumovgrad 58, conventional production - 517.06±36.19 mM TE/g. Weaker activity is displayed by extracts obtained from the tobaccos of the variety group Kabakulak - Han Tervel 430.55±30.13 mM TE/g and Hanski 277 - 327.31±22.89 mM TE/g.

The antioxidant activity determined by the CUPRAC method of tobacco extracts is higher than that of dandelion extracts (99.9±7.0 mM TE/g) and lower than that of thyme extracts (868.6±60.8 mM TE/g) obtained under the same conditions in our earlier studies [15].

Due to the aggressive action of H_2O_2, it is extremely important to find substances that have the ability to bind to H_2O_2 and neutralize its action. The peroxide radical scavenging method (H_2O_2 method) is related to the ability of antioxidants such as flavonoids, simple phenolic acids, hydroxycinnamic acids and other to trap peroxide radicals with a simple, inexpensive universal colorimetric procedure [11].

Table 1. Total phenolic content in Bulgarian varieties of tobaccos grown under organic and conventional production, mg GAE/g DM

Variety group	Type of production	Variety	TPC
Basmi	Organic	Krumovgtad 58, II cl.	36.11±2.11
		Nevrokop, II cl.	46.14±2.36
	Conventional	Krumovgtad 58, II cl.	41.66±2.08
		Nevrokop, II cl.	48.13±2.42
Kabakulak	Conventional	Han Tervel 39, II cl.	17.89±1.45
		Hanski 277, II cl.	17.72±1.45
Virginia	Conventional	Virginia 514, II cl.	36.12±2.11
		Virginia 0842, II cl.	32.38±2.08

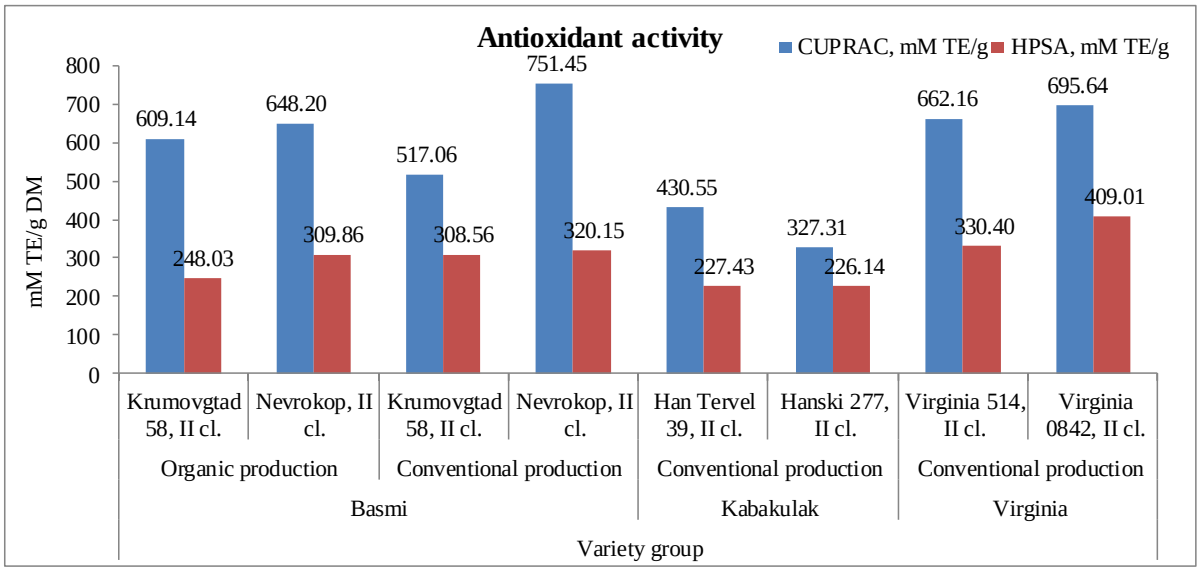

Fig. 1. Antioxidant capacities of tobacco extracts evaluated by CUPRAC method and H_2O_2 assay

The extracts from Virginia variety tobaccos are characterized by highest ability to neutralize peroxide radical group - 409.01±26.15 mM TE/g DM (Virginia 0842) and 330.4±21.12 mM TE/g DM (Virginia 514). Tobacco extracts obtained from Kabakulak varietal group are characterized by almost twice as low activity – 226.14±14.46 mM TE/g DM (variety Hanski 277) and 227.43±15.24 mM TE/g DM (Han Tervel 39) compared to the Virginia tobaccos.

The tobacco extracts, obtained from conventionally and organically produced Basmi variety group, occupy an intermediate position. Antioxidant activity, determined by H_2O_2 assay varied between 248.03 ±15.89 mM TE/g DM (Krumovgrad 58, organic production) and 320.15±20.47 mM TE/g DM (Nevrokop, conventional production). No significant difference in antioxidant activity was observed between the extracts obtained from conventional and biological tobacco production - Fig. 1.

The antioxidant activity determined by the H_2O_2 method of tobacco extracts is higher than antioxidant activity of dandelion extracts (42.2±2.7 mM TE/g) [15].

Correlation between TPC and antioxidant activity

To observe the dependence between TPC and AOA, the correlation dependence of the two free radical scavenging activity methods for tobacco extracts was investigated. Figures 2 and 3 present the correlation between TFC in tobacco extracts and antioxidant activity evaluated by CUPRAC assay and H_2O_2 assay.

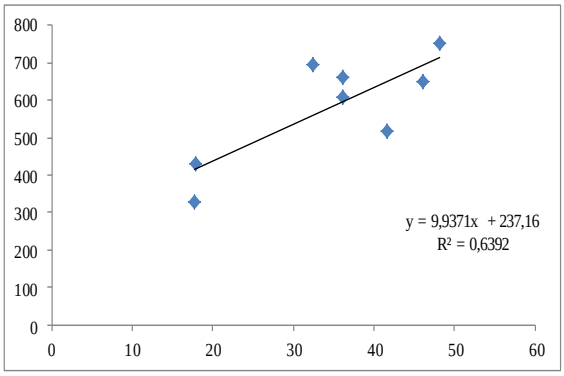

Fig. 2. Correlation between TPC and antioxidant activity, evaluation by CUPRAC assay

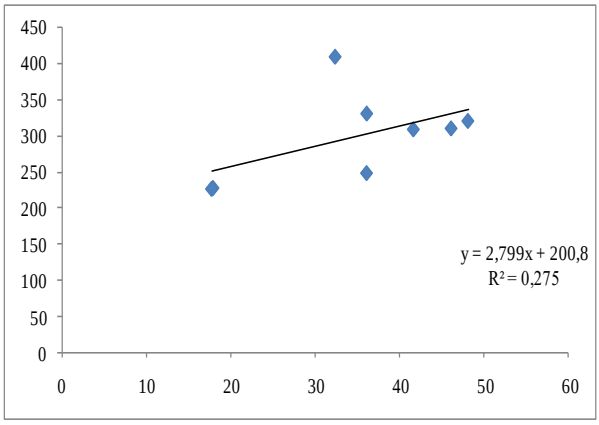

Fig. 3, Correlation between TPC and antioxidant activity, evaluation by H_2O_2 assay

As it is seen from Figs. 2 and 3, a better correlation is observed between TPC and CUPRAC method (R^2=0.639) than between TPC and H_2O_2 method (R^2=0.275).

CONCLUSION

Conventionally and organically grown Bulgarian tobaccos were used. Since it has been accepted nowadays that both hydrogen atom transfer (HAT)- and electron transfer (ET)-based assays are needed to give a reliable estimate of the antioxidant activity of plant extracts, in this work antioxidant activity of 60 % tobacco extracts was assayed by CUPRAC method (ET-based assay) and H_2O_2 method (HAT-based assay).

The results of the present study showed that:

- Tobacco extracts from Basmi and Virginia varietal groups, both conventional and organic production, have a higher antioxidant activity compared to the extracts from tobaccos from the Kabakulak varietal group in both investigated methods.
- The antioxidant activity of tobacco extracts determined by electron transfer methods (CUPRAC-method) is higher than the activity determined by hydrogen atom transfer methods (H_2O_2). Tobacco extracts have a higher ability to reduce Cu^{+2} to Cu^{+} compared to their ability to scavenge peroxide radicals.
- There is a better linear correlation between TPC and CUPRAC method, compared to the correlation between TPC and H_2O_2 method.

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