

Comparison of structure and antioxidant activity of polysaccharides extracted from the leaves of *Plantago major* L., *P. media* L. and *P. lanceolata* L.

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Received April 14, 2017; Revised June 07, 2017

Dedicated to Acad. Ivan Juchnovski on the occasion of his 80th birthday

In the current study for the first time were investigated the chemical composition and antioxidant activity of polysaccharides isolated from three indigenous for Bulgaria species of *Plantago* genus - *Plantago major* L., *Plantago lanceolata* L. and *Plantago media* L. Crude polysaccharides were extracted from fresh leaves with water and dilute acid and their yield was between 0.64% and 2.79%. The chemical composition of water-extractable polysaccharides (WEPs) and total acid-extractable polysaccharides (TAEPs) of *Plantago* leaves was evaluated by HPLC analysis. The phytochemical data revealed the presence of branched heteropolysaccharides with different neutral/acidic monosaccharide ratio. The predominant monosaccharide unit of WEPs was galacturonic acid (62.64% - 70.58%). Additionally, there were registered small amounts of arabinose and rhamnose. In TAEPs among with galacturonic acid (36.93% - 41.46%), significant amounts of neutral monosaccharides as galactose (22.80% - 46.11%) and rhamnose (16.96% - 35.74%) were determined. Two types of analyses were used to evaluate the antioxidant activity of *Plantago* isolated polysaccharides: DPPH and FRAP assay. Based on DPPH method, WEPs exhibited stronger radical scavenging ability (29.39% - 40.08%) compared to TAEPs (19.44% - 24.15%). In parallel, WEPs showed greater rate of ferric reducing power (103.71 - 137.83 μ M TE/5 mg Ps) compared to TAEPs (34.63 - 117.66 μ M TE/5 mg Ps). Although lower than synthetic BHT, *Plantago* polysaccharides revealed antioxidant potential and could be further explored as promising natural antioxidants for the nutraceutical and pharmaceutical industries.

Key words: *Plantago major* L.; *Plantago media* L.; *Plantago lanceolata* L.; polysaccharides; antioxidant activity

INTRODUCTION

Plantago genus includes herbaceous plant species used worldwide as a remedy for wound healing, inflammations, respiratory disorders and digestive system affections [1-3]. The European Pharmacopoeia has approved for medical uses the leaves from *Plantago lanceolata* and seeds from *P. ovata*, *P. afra* and *P. indica* [4], while *P. major* leaves have been included in World Health Organization Monographs [5]. Fifteen *Plantago* species are native to the Bulgarian flora. Among them *Plantago major* L., *Plantago media* L. and *Plantago lanceolata* L. are widespread in the country and traditionally used by local people [6]. *P. major* and *P. lanceolata* leaves have been known as a rich source of biologically active compounds

like polysaccharides, phenolic acids, flavonoids, iridoid glycosides and vitamins [1-5].

Plant polysaccharides have emerged as an important class of bioactive natural products and widely used in pharmaceuticals, biomaterials, food additives and nutrition [7]. They and their derivatives have been found to possess diverse biological activities, such as immunostimulatory, antiinflammatory, antiviral, antitumor, radioprotective, hepatoprotective and antifatigue effects [7,8]. In addition, many studies elucidate that polysaccharides isolated from plants have antioxidant activity [7,9].

According to European Medicines Agency [10] and Kardošová [3] from *P. lanceolata* leaves have been isolated pectic polysaccharides, rhamnogalacturonan, arabino-galactan and α -D-glucan. Samuelsen *et al.* [2,11] reported in *P. major* leaves the presence of an acidic arabinogalactan and highly esterified pectic polysaccharide,

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composed mainly of arabinose, galactose, rhamnose and galacturonic acid. However, so far no thorough investigation about polysaccharide content of *P. media* leaves has been found in literature. Immunomodulatory and antimicrobial activity of *Plantago* leaves polysaccharides have been reported [2,12], but there is a lack of information about their antioxidant activity.

The aim of this study was to investigate the polysaccharide composition of three widely spread *Plantago* species in Bulgaria (*Plantago major* L., *Plantago media* L. and *Plantago lanceolata* L.) and to determine their antioxidant activity. The phytochemical analysis and antioxidant assessment provide useful information with regard to health promoting and functional quality of the studied medicinal plants.

EXPERIMENTAL

Materials and reagents

Plantago major and *Plantago lanceolata* mature leaves were collected from Thracian valley floristic region, Bulgaria (42°08'N, 24°44'E) and *Plantago media* leaves were collected from Rhodope Mountains floristic region, Bulgaria (41°75'N, 24°16'E), in the vegetative season of 2015. The botanical identification of plant species was carried out according to Tutin *et al.* [13] and Delipavlov and Cheshmedzhiev [6]. Assay kits of monosaccharides, galacturonic acid, bovine serum albumin, 2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), butylated hydroxytoluene (BHT) were purchased from Sigma Aldrich, US. Arabinogalactan was purchased from Megazyme, Ireland. All other chemicals and solvents used in this study were of analytical grade.

Polysaccharide extraction

Alcohol-insoluble residues (AIR) from *Plantago* leaves were used for isolation of polysaccharides. The procedure was previously reported in our study [14]. Polysaccharides were extracted from AIR with water and dilute hydrochloric acid according to the methodology described by Kratchanova *et al.* [15] with slight modifications.

Determination of the total neutral sugars, uronic acids and protein

The content of total neutral sugars was determined with a colorimetric phenol-sulfuric acid method [16], using glucose (20 - 100 µg/mL) as a

reference standard. Uronic acid analysis followed the method of Blumenkrantz and Asboe-Hansen [17], calibrated against a standard of galacturonic acid (25 - 150 µg/mL). Protein content in the isolated polysaccharides was determined by Bradford method with a bovine serum albumin (0 - 100 µg/mL) as a standard [18].

TFA hydrolysis

The crude water and total acid-extractable polysaccharides (50 mg) were hydrolysed with 2 M trifluoroacetic acid (TFA) at 121°C for 1 h in an autoclave [19].

HPLC analysis

The hydrolysis products composition were determined with HPLC system Konik-Tech, with RI Detector Shodex R1-101 and Tracer Excel ODSB 120/5 µm (150 x 0.4 mm) column, mobile phase water, flow rate 0.1 mL/min and 0.3 mL/min, temperature 30°C. The registered peaks of the samples were evaluated using reference monosaccharide standards.

Determination of antioxidant activity

The 2, 2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH) free radical scavenging activity was determined by using the method reported by Kao and Chen [20] with slight modifications. A 0.5 mM solution of DPPH in methanol was prepared and 0.2 mL of this solution was added to 1 mL of polysaccharide solutions (5 mg/mL). The radical scavenging activity was calculated by the following equation:

$$\% \text{ Inhibition} = \frac{AB - AA}{AB} \times 100$$

where AB was the absorption of the blank sample (t = 0 min) and AA - the absorption of the polysaccharide solution (t = 30 min) [21].

The ferric reducing antioxidant power (FRAP) was determined according to the method of Benzie and Strain [22] with some modifications. FRAP reagent (2.7 mL) was mixed with 0.3 mL of polysaccharide samples (5 mg/mL). The mixture was incubated at 37°C for 30 min in dark, and the absorbance was measured at 593 nm. The results were expressed as micromol Trolox equivalents (0 - 500 µM) per 5 mg polysaccharide (µM TE/5 mg Ps).

BHT (0.1 mg/mL) and arabinogalactan (ArG) (5 mg/mL) were used as controls. All data for antioxidant activity were expressed by triplicate measurements with standard deviation.

RESULTS AND DISCUSSION

Polysaccharide concentration, content of neutral sugars, uronic acids and proteins

The composition of the polysaccharides (Ps) isolated from the three studied *Plantago* species was presented in Table 1. The polysaccharide content after aqueous extraction was between 0.64% and 2.79%, with highest amount established in *P. media* leaves (2.79%). The highest concentration of polysaccharides in *P. major* leaves was detected by total acid extraction (2.47%), whereas the amount of the total-acid extractable polysaccharides in *P. media* and *P. lanceolata* were close-range: 1.46% and 1.22%, respectively. The obtained results correspond to these established by Olennikov *et al.* [23]. They have reported water-soluble polysaccharide content in *P. major* fresh leaves in the range between 1.5% and 3.3%. Kardošová has reported 1.5% concentration of crude water-extractable polysaccharide from *P. lanceolata* leaves per dry mass [3].

The concentration of the neutral sugars in total acid-extractable polysaccharides (TAEPs) in all samples showed twice higher content of neutral sugars (58.05% - 62.54%) compared to water-extractable polysaccharides (WEPs) (29.11% - 37.15%). These results could be attributed to acid hydrolysis of the bond linkages between polysaccharides and cell wall constituents in dilute hydrochloric acid [15, 24]. The water extraction on the other hand is assumed to be non-destructive [15, 24], which can explain the registered lower amount of neutral sugars in water-extractable fractions. The

amount of uronic acids was higher for WEPs (62.29% - 69.82%) compared to TAEPs (36.14% - 41.10%). The ratio between uronic acids and neutral sugars was 2:1 for WEPs and 1:1 for TAEPs. Similar ratio uronic acids to neutral sugars in consequently obtained water and acid-extractable polysaccharides from leek has been reported by Kratchanova *et al.* [15]. They have established 73.6% polyuronic content and 18.4% neutral sugars for WEPs and 27.5% polyuronic content and 71.1% neutral sugars for acid-extractable polysaccharides.

The protein content of all investigated samples was low (0.55% - 2.14%), which indicated a high purity of the extracted polysaccharides. Samuelsen *et al.* have reported protein content in WEPs from *P. major* leaves up to 1.8% [11], while Kardošová has determined significantly higher amount of protein content in *P. lanceolata* leaves WEPs (5.6%) [3].

Monosaccharide composition

HPLC profiles of the polysaccharide hydrolysis products have been shown in Fig. 1. Based on the monosaccharide analysis, WEPs from *P. major* and *P. lanceolata* leaves were found to be mainly composed of galacturonic acid (GalA) - from 62.64% to 70.58% (Table 2). The amount of detected arabinose (Ara) was from 37.36% to 29.42% and rhamnose (Rha) was registered only in traces (Fig. 1A, 1E). Olennikov *et al.* have also determined galacturonic acid as the main monomer in *P. major* water-soluble polysaccharides [23]. Samuelsen *et al.* have reported that in *P. major* leaves was present only galacturonic acid (39.0% - 71.7%) [11], while Kardošová has reported 35.8% galacturonic acid and 21.9% glucuronic acid in WEPs from *P. lanceolata* leaves [3].

Similarly to our results, Kardošová has determined in WEPs from *P. lanceolata* leaves

Table 1. Chemical composition of WEPs and TAEPs from *P. major*, *P. media* and *P. lanceolata* leaves.

Sample	Ps concentration ± SD (g/100 g fresh leaves)	Neutral sugars ± SD (%)	Uronic acid ± SD (%)	Protein ± SD (%)
<i>P. major</i> WEPs	1.84 ± 0.24	37.15 ± 0.28	62.29 ± 1.10	0.56 ± 0.02
<i>P. major</i> TAEPs	2.47 ± 0.26	61.72 ± 3.22	36.14 ± 1.62	2.14 ± 0.31
<i>P. media</i> WEPs	2.79 ± 0.19	34.88 ± 2.10	64.45 ± 2.08	0.67 ± 0.02
<i>P. media</i> TAEPs	1.46 ± 0.32	58.05 ± 1.54	41.10 ± 2.32	0.85 ± 0.05
<i>P. lanceolata</i> WEPs	0.64 ± 0.08	29.11 ± 0.88	69.82 ± 2.46	1.07 ± 0.12
<i>P. lanceolata</i> TAEPs	1.22 ± 0.11	62.54 ± 1.15	36.91 ± 0.77	0.55 ± 0.01

arabinose (26.0%) as main neutral monosaccharide [3], while Samuelsen *et al.* have reported almost equal amounts of arabinose (8.8% - 24%), galactose (8% - 34%) and xylose (11% - 22%) in different fractions of *P. major* WEPs [11].

The presented results suggested that *P. major* and *P. lanceolata* WEPs have been composed of ramified rhamnogalacturonan I (RG I), which main chain was constructed by GalA residues, rarely alternated with Rha units. The registered significant amount of Ara assumed arabinan type side chains, which branch points were the Rha units in RG I. The predominant monosaccharide components in *P. media* WEPs were GalA and Rha, with 1.86 GalA:Rha ratio. The results suggested the presence of more branched heteropolysaccharides, in which main chain every two residues of GalA were alternated with Rha. From HPLC profile of *P. media* WEPs a peak with a retention time of 10.74 min was observed, which probably corresponded to polysaccharide side chains with a degree of polymerization (DP) > 2 (Fig. 1C).

Galactose (Gal), GalA and Rha were the monosaccharide components determined in TAEPs of the three investigated *Plantago* species (Fig. 1B, 1D, 1F). The content of GalA was about 40% for all TAEPs (Table 2). The amount of Rha (35%) in

P. media TAEPs was twice higher compared to *P. major* and *P. lanceolata* TAEPs (17%). The results suggested the presence of RG I polysaccharide with galactan type side chains. *P. media* TAEPs with GalA:Rha ratio 1.16 resulted to be the polysaccharides with the most branched structure among all investigated samples.

Antioxidant activity

The use of more than one method is recommended to give a comprehensive prediction of antioxidant efficacy [25]. Two types of analyses were used to evaluate the antioxidant activity of *Plantago* leaves isolated polysaccharides: DPPH (measures the ability to scavenge free radicals) and FRAP assay (measures the ability to reduce Fe³⁺ to Fe²⁺ by donating an electron). In the present work we study for the first time the antioxidant activity of polysaccharides from *Plantago* leaves. The results from DPPH and FRAP assays were summarized in Table 3. Based on DPPH method, *Plantago* leaves WEPs showed stronger antioxidant activity (29.39% - 40.08%) compared to TAEPs (19.44% - 24.15%). These may be due to the higher content of galacturonic acid in WEPs.

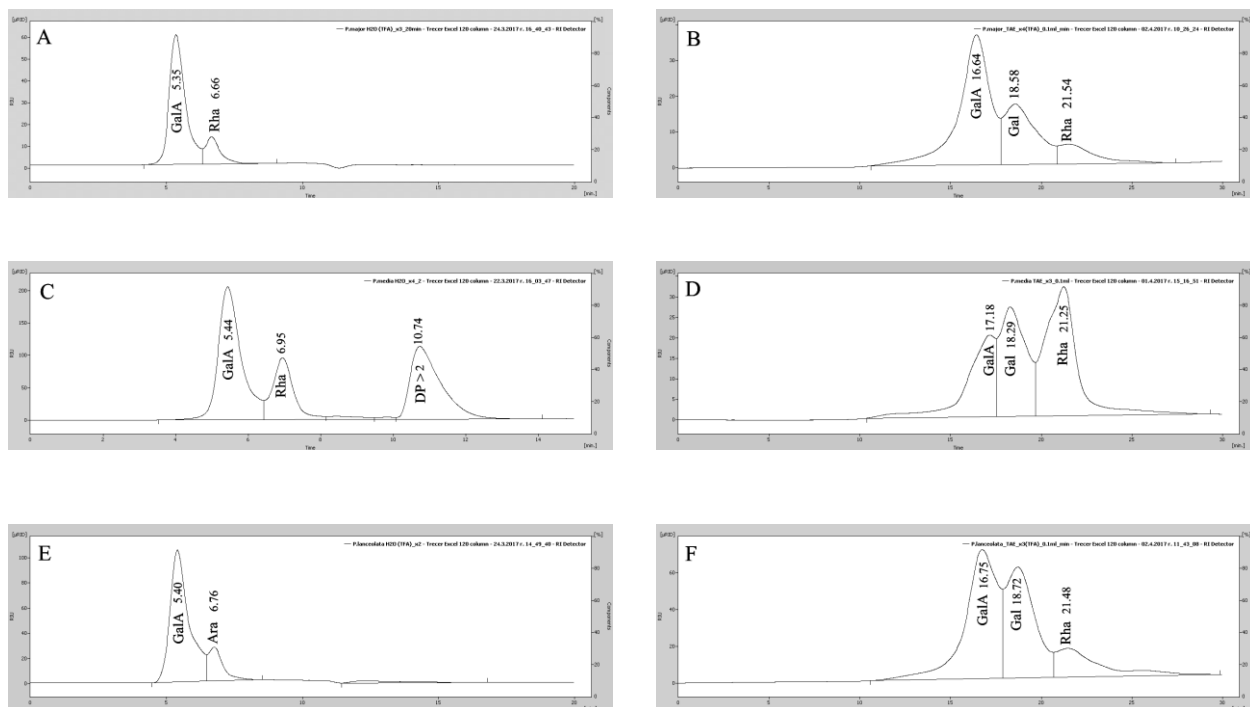


Fig.1. HPLC profiles of polysaccharide hydrolysis products: (A) *P. major* WEPs; (B) *P. major* TAEPs; (C) *P. media* WEPs; (D) *P. media* TAEPs; (E) *P. lanceolata* WEPs; (F) *P. lanceolata* TAEPs.

Table 2. Monosaccharide composition of WEPs and TAEPs from *P. major*, *P. media* and *P. lanceolata* leaves (expressed as % of the total carbohydrates).

Sample	Ara (%)	Rha (%)	Gal (%)	GalA (%)	GalA/Rha
<i>P. major</i> WEPs	37.36	traces	-	62.64	-
<i>P. major</i> TAEPs	-	16.96	46.11	36.93	2.18
<i>P. media</i> WEPs	-	35.12	-	64.88	1.85
<i>P. media</i> TAEPs	-	35.74	22.80	41.46	1.16
<i>P. lanceolata</i> WEPs	29.42	traces	-	70.58	-
<i>P. lanceolata</i> TAEPs	-	17.33	45.55	37.12	2.14

Uronic acids have been considered to be potent antioxidants, which is attributed to the fact that their carbonyl group, similarly to phenolic acids, was attached to a ring molecule [26]. Among the isolated polysaccharides, the most predominant one for its scavenging ability was *P. media* WEPs (40.08%). Arabinogalactan, used as a control, showed close-range scavenging ability (21.19%) to TAEPs (19.44% - 24.15%). The lower antioxidant capacity of TAEPs could be attributed to both the low amount of galacturonic acid and the significant galactose content. According to the investigation of Meng *et al.* [27] the galactose content did not correlated to the polysaccharide antioxidant activity. Our results were in accordance to the investigations of Wang *et al.* [28], who reported a better scavenging ability of different acidic polysaccharide fractions from *Lycium barbarum* L. fruit (52.5% - 84.9%) compared to neutral polysaccharides (38.1%).

In parallel, the same tendency was observed for the FRAP values of the investigated polysaccharides. WEPs exhibited greater rate of ferric reducing power (103.71 - 137.83 $\mu\text{M TE}/5 \text{ mg Ps}$) compared to TAEPs (34.63 - 117.66 $\mu\text{M TE}/5 \text{ mg Ps}$). *P. lanceolata* WEPs had the most pronounced ferric reducing power - 137.83 $\mu\text{M TE}/5 \text{ mg Ps}$, which was in accordance to the highest value of galacturonic acid (70.58%) in *P. lanceolata* WEPs among all isolated polysaccharides. In addition, FRAP values of arabinogalactan (56.28 $\mu\text{M TE}/5 \text{ mg ArG}$) were in close-range to those of TAEPs.

The antioxidant potential of *Plantago* polysaccharides was compared to the synthetic BHT (Table 3). Although the registered values by DPPH and FRAP methods were lower, still *Plantago* polysaccharides showed significant potential as natural antioxidants. In comparison Kardošová and Machová have investigated the effects of rhamnogalacturonan (RG), obtained from

P. lanceolata var. *libor* leaves, on inhibition of lipid peroxidation and reported 45.3% antioxidant activity for 0.227 mM RG [29]. A few data of antioxidant activities of *Plantago* seeds isolated polysaccharides have been reported.

Table 3. Assessment of antioxidant activity of *P. major*, *P. media* and *P. lanceolata* WEPs and TAEPs.

Sample	DPPH \pm SD (%)	FRAP \pm SD ($\mu\text{M TE}/5 \text{ mg Ps}$)
<i>P. major</i> WEPs	35.35 \pm 0.58	103.71 \pm 0.69
<i>P. major</i> TAEPs	19.44 \pm 0.87	34.63 \pm 0.23
<i>P. media</i> WEPs	40.08 \pm 1.75	132.40 \pm 2.11
<i>P. media</i> TAEPs	24.15 \pm 1.03	94.65 \pm 1.10
<i>P. lanceolata</i> WEPs	29.39 \pm 1.50	137.83 \pm 2.57
<i>P. lanceolata</i> TAEPs	24.07 \pm 0.75	117.66 \pm 0.98
<i>Controls:</i>		
Arabinogalactan (ArG)	21.19 \pm 0.13	56.28 \pm 0.15 $\mu\text{M TE}/5 \text{ mg ArG}$
BHT	93.49 \pm 0.06	556.10 \pm 0.09 $\mu\text{M TE}/0.1 \text{ mg BHT}$

Ye *et al.* [30] and Yin *et al.* [31] have investigated the possible antioxidant effect of the polysaccharides obtained from aqueous extracts of dried seeds from *P. asiatica*. According to Ye *et al.* DPPH radical scavenging activity of the investigated polysaccharide increased from 25.6% to 81.4%, when the concentration of the polysaccharides increased from 0.15 to 0.75 mg/mL [30]. On the other hand, Yin *et al.* reported 50.8% DPPH radical scavenging effects of *P. asiatica* seeds WEPs at a concentration of 1 mg/mL, which ability to scavenge free radicals did not increase at higher concentrations [31].

CONCLUSION

In the present study for the first time were investigated and quantified the polysaccharide composition and antioxidant activity of *P. major*,

P. media and *P. lanceolata* leaves from Bulgaria. The phytochemical data revealed the presence of branched heteropolysaccharides with uronic acids:neutral sugars ratio varying from 2:1 for WEPs to 1:1 for TAEPs. The WEPs from *Plantago* leaves were composed mainly from galacturonic acid and minor amounts of arabinose and rhamnose, while in TAEPs galacturonic acid, galactose and rhamnose were detected. Based on DPPH and FRAP methods, *Plantago* isolated polysaccharides showed significant antioxidant activity. Among the investigated polysaccharides *P. media* WEPs exhibited the strongest radical scavenging ability (40.08%) and *P. lanceolata* WEPs showed the greater ferric reducing power (137.83 μ M TE/5 mg Ps). According to the results stated above, it could be concluded that *P. major*, *P. media* and *P. lanceolata* leaves are promising natural sources of biologically active polysaccharides which can be developed as new antioxidants for applications in pharmaceutical and food industries.

Acknowledgements: The authors are grateful to Medical University – Plovdiv for the financial support through Project SDP – 03/2015.

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СРАВНЕНИЕ НА СТРУКТУРАТА И АНТИОКСИДАНТНАТА АКТИВНОСТ НА ПОЛИЗАХАРИДИ, ИЗОЛИРАНИ ОТ ЛИСТА НА *Plantago major* L., *P. media* L. И *P. lanceolata* L.

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Постъпила на 18 април 2017 г.; Коригирана на 2 юни 2017 г.

(Резюме)

В настоящата работа за пръв път са изследвани химичния състав и антиоксидантната активност на полизахариди, получени от *Plantago major* L., *Plantago media* L. и *Plantago lanceolata* L., растящи в България. Полизахаридите са изолирани от свежи листа посредством водна и киселинна екстракция, като получените добиви варират от 0.64% до 2.79%. Химичният състав на водно-екстрахируемите полизахариди (ВЕПЗ) и тотално киселинно-екстрахируемите полизахариди (ТКЕПЗ) е анализиран посредством високоефективна течна хроматография. Фитохимичният анализ показва наличие на разклонени хетерополизахариди с различно съотношение между неутрални и кисели захари. В състава на ВЕПЗ основна монозахаридна единица е галактуроновата киселина (62.64% - 70.58%). Доказани са ниски количества неутрални захари като арабиноза и рамноза. В състава на ТКЕПЗ наред с галактуроновата киселина (36.93% - 41.46%), са установени значителни количества неутрални монозахариди като галактоза (22.80% - 46.11%) и рамноза (16.96% - 35.74%). Антиоксидантната активност на изолираните полизахариди е установена посредством два метода: DPPH и FRAP анализ. Резултатите от DPPH определянето показваха, че ВЕПЗ притежават по-силна радикал-улавяща способност (29.39% - 40.08%) спрямо ТКЕПЗ (19.44% - 24.15%). Аналогични са данните за FRAP анализа: по-силна редуцираща способност при ВЕПЗ (103.71 - 137.83 $\mu\text{M TE}/5 \text{ mg ПЗ}$) в сравнение с ТКЕПЗ (34.63 - 117.66 $\mu\text{M TE}/5 \text{ mg ПЗ}$). Полизахаридите от род *Plantago* показват значителна антиоксидантна активност, въпреки пониските установени стойности спрямо синтетичния антиоксидант ВНТ. Получените данни за изолираните полизахариди биха могли да послужат като бъдеща перспектива за разработване на природни антиоксиданти за хранителната и фармацевтична промишленост.