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Research Article

Preliminary *in vitro* study of anti-oxidant activity and anti-diabetic potential of plant extracts from 4 herbal substances not traditionally used for treatment of diabetes mellitus

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Abstract

The focus of the presented study is the *in vitro* anti-oxidant activity and anti-diabetic potential of water extracts from the following four herbal substances, not traditionally used for treatment of diabetes mellitus – leaves of *Sambucus ebulus* L. and *Prunus mahaleb* L., and flowering stems of *Cichorium intybus* L. and *Satureja kitaibelii* Wierzb. ex Heuff. The water extracts are obtained through ultrasonication. The extract of *S. kitaibelii* stands out due to its highest values in all studied indicators – total phenolic content, scavenging potential (DPPH, ABTS) and α-glucosidase inhibitory activity which was six times higher than acarbose. The extract of *C. intybus* also showed significant α-glucosidase inhibitory activity compared to acarbose. The flowering stems of both species are promising sources of biologically active substances for blood sugar control in diabetes mellitus.

Keywords

medicinal plants, water extracts, DPPH, ABTS, α-glucosidase inhibitory activity

Introduction

Diabetes mellitus (DM) is a chronic endocrine disease that involves a complex of metabolic disorders which over time damages the heart, blood vessels, eyes, kidneys, and nerves. It affects a large part of the human population and generally causes one of the highest mortality rates according to WHO. Hyperglycemia is the main symptom of DM, which gradually leads to serious complications (Oguntibeju 2019). One way is through oxidative stress induced by the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) related with the hyperglycemia. The oxidative stress leads to disruption of the general cellular metabolism and cellular damage (especially for the pancreatic β-cells), genome and epigenome instability, inflammation, organ dysfunction, etc. (Kang et al. 2020). One of the best strategies to prevent hyperglycemia is using α-glucosidase inhibitory agents to control postprandial blood sugar levels along with anti-oxidative agents against oxidative stress. Some medicinal plants contain ingredients with such properties (Benalla et al. 2010; Fenercioglu et al. 2010; Kumar et al. 2011; Govindappa 2015). Polyphenols are secondary metabolites which are ubiquitous in plants and are considered to be in many ways beneficial in the prevention and management of DM, including anti-oxidative activity and α-glucosidase inhibitory capacity (Scalbert et al. 2005; Bahadoran et al. 2013; Costa et al. 2017; Sun et al. 2020).

The Bulgarian flora is famous for its vast diversity of medicinal plants and their rich resources which leads to the fact that the country is one of the biggest exporters of herbs in Europe (Lange 2002; Evstatieva et al. 2007). Many Bulgarian plants are used in treatment of diabetes according to traditional medicine. However, the market is dominated by phytoproducts in which the leading ingredients with hypoglycemic effect are mainly imported phytopreparations of foreign origin as extracts of *Curcuma longa*, *Cinnamomun zeylanicum*, *Zingiber officinale*, etc. The reasons for this are complex and some of them are positively related to the difficult accessibility of resources for some plants with small or scattered populations or lack of scientific evidence for the efficiency in therapy for others. That is why we tried a combined approach to screening herbal substances with anti-diabetic potential from the Bulgarian flora based on scientific evidence for the virtue of plants' active compounds and good availability of the resources in the country.

Satureja kitaibelii Wierzb. ex Heuff. is a Balkan endemic species found in the territory of the former Federal Republic of Yugoslavia, Bulgaria, and a small part of Romania (Velchev 1989; Euro + Med (2006-); Ciocârlan 2009; Đorđević et al. 2014; WCSP 2021). Phytochemically, the aerial parts of *S. kitaibelii* have been studied in only in Serbian populations in terms of the essential oil characteristics (Slavkovska et al. 2001; Đorđević et al. 2014; Dodoš et al. 2019) and relation between chemical composition and antioxidant potential, lipid peroxidation inhibition and antimicrobial activities of the extracts (Ćetković et al. 2007; López-Cobo et al. 2015; Gopčević et al. 2019). The extracts of *S. kitaibelii* are rich of phenolic compounds mainly phenolic acids and flavonoids (López-Cobo et al. 2015; Gopčević et al. 2019). There are a few studies that argue for antidiabetic and hypoglycemic properties of some other *Satureja* species, i.e. *S. cuneifolia* (Aydn et al. 1995) and *S. khuzestanica* (Abdollahi et al. 2003; Vosough-Ghanbari et al. 2008). In Bulgaria, *S. kitaibelii*, previously considered a subspecies of *S. montana*, has been a subject of a single study to establish the dynamics of the accumulation of essential oil in several forms of *S. montana* and *S. pilosa* (Genova 1980).

Sambucus ebulus L., *Cichorium intybus* L. and *Prunus mahaleb* L. have long history in folk medicine of the Mediterranean, South-East Europe and the Middle East. The taproots of chicory and the fruits and kernels of mahaleb cherry are famous for their hypoglycemic effect. Yet the most abundant plant parts of the three plants – the leaves of *Sambucus ebulus* and *Prunus mahaleb* and the leafy stems with inflorescences of *Cichorium intybus* are little studied. According to the research they all show high *in vitro* antioxidant activities (Aquil et al. 2006; Dalar and Konczak 2014; Meriç et al. 2014; Taghizadeh et al. 2015; Brieudes et al. 2016; Chandra and Jain 2016) which can be attributed mainly to the group of hydroxycinnamic phenolic acids and their derivatives (chlorogenic acid, caffeic acid, ferulic, p-coumaric, cichoric acid, etc.) and secondly to the flavonoid glycosides (Yeşilada 1992; Yeşilada 1997; Mulinacci et al. 2001; Mastelic et al. 2006; Bidel et al. 2007; Kaya et al. 2009; Jerkovic et al. 2011; Schwaiger et al. 2011; Ieri et al. 2012; Dalar and Konczak 2014; Brieudes et al. 2016; Cvetanovič et al. 2017; Li et al. 2018; Sytar et al. 2018; Senica et al. 2019).

The aim of our study is to quantify the total phenolic content in aqueous extracts of *Satureja kitaibelii* aerial parts, *Sambucus ebulus* leaves, *Prunus mahaleb* leaves and *Cichorium intybus* flowering stems in relation to their antioxidant potential and α-glucosidase inhibitory activity. To the best of the authors' knowledge, the α-glucosidase inhibitory activity of all mentioned plant substances has not been studied yet.

Material and methods

Chemicals and reagents

Folin-Ciocalteu reagent, Gallic acid, 2,2-diphenyl-2-picryl-hydrazyl-hydrate (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), acarbose, alpha-glucosidase from *Saccharomyces cerevisiae*, ρ-nitrophenyl-α-D-glucopyranoside (ρNPG) were obtained from Sigma-Aldrich (Darmstadt, Germany). All reagents were of analytical grade.

Plant material

All plant materials were gathered in the period July-August 2020, from protected area "Kailaka", near the town of Pleven, Middle Danube plain, Bulgaria. The studied plant substances were as follows: leaves of *Sambucus ebulus* and *Prunus mahaleb*, flowering stems of *Cichorium intybus* and *Satureja kitaibelii*. The plant community of the Balkan endemic species *Satureja kitaibelii* falls under the habitat type 6240* Sub-pannonic steppes according to the Habitat Directive 92/43/EEC (1992). The bedrock in the locality is limestone and the altitude is 200 m a.s.l. The taxonomic identification of the species follows Delipavlov and Cheshmedzhiev (2003). For *Satureja kitaibelii* additional references of Ball et al. (1972), Velchev (1989) and Ciocârlan (2009) are considered. All plant substances were air-dried at room temperature. The yield ratios of fresh to dry herbal substances for the four species are as follows: *Sambucus ebulus* 3.9:1, *Prunus mahaleb* 2.9:1, *Cichorium intybus* 2.6:1, and *Satureja kitaibelii* 2.3:1.

Preparation of plant extracts

The air-dried and grounded plant materials were extracted in triplicate with distilled water (1:10 w/v) in an ultrasonic bath (35 kHz) with gradual increase in temperature (T1 = 39 °C; T2 = 48 °C; T3 = 53 °C). The plant extracts were concentrated in a rotary vacuum evaporator, dried in vacuum drying oven and stored under freezing conditions. The yield of the extracts was calculated using the formula:

$$\text{yield (\%)} = \frac{\text{Weight dry extract}}{\text{Weight dry substance}} * 100 .$$

Determination of total phenolic content

Total phenolic content (TPC) was determined by the method of Singleton and Rossi (1965) with some modifications. To 0.2 mL of suitably pre-diluted extract 1.8 mL of dH_2O and 0.2 mL of Folin-Ciocalteu reagent were sequentially added. After five minutes 2 mL of 7% Na_2CO_3 were added and then the mixture was diluted up to 5 mL with dH_2O. The reaction continued in dark for 90 minutes. The absorbance was measured at 750 nm against dH_2O. The standard calibration curve was plotted using gallic acid (1–200 μg/mL). The total phenolic content was expressed as gallic acid equivalents per gram of dry extract (mg GA/g DE).

Scavenging effect on 2,2-diphenyl-1-picryl hydrazyl radical (DPPH)

The radical scavenging ability was determined according to the method of Mensor et al. (2001). Samples with different concentrations were obtained from the initial extracts. To 2.5 mL of every sample 1 mL of 0.3 mM alcoholic DPPH solution was added. The absorbance was measured at 518 nm against a blank sample with ethanol after a stay of the samples for 30 minutes in dark. Solutions of various concentrations of rutin were used as a positive controls. Antiradical activity was expressed as IC_{50} – the concentration of the extracts that causes 50% inhibition of the radical formation. The IC_{50} is calculated by a linear graph equation that expresses the relationship between percentages of inhibitory activity and extract/standard concentration.

Scavenging effect on 2,2 azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)

The method of Alara et al. (2019) was used with minor modifications. The ABTS radical was generated by mixing ABTS (7.0 mM in dH_2O) and potassium persulfate (2.45 mM in dH_2O) in equal amounts. The reaction continued for 16 h at room temperature in dark. Prior to analysis, 2.0 mL of the generated radical ($ABTS^{\bullet}$) was diluted in ethanol (1:30; v/v) until obtaining a final absorbance of 0.7 ÷ 0.8 at 734 nm. After this preparation, 1710 μL ABTS solution was mixed with 90 μL of the sample extracts with various concentrations sequentially. The mixtures were kept in dark for 7 minutes and then the absorbencies were measured at 734 nm against a blank sample with ethanol. Solutions of various concentrations of rutin were used as positive controls. Antiradical activity was expressed as IC_{50} values.

Determination of α-glucosidase inhibitory activity

The method of Dong et al. (2012) was employed with slight modification. The reaction mixture of 100 μL 0.1 M phosphate buffer (pH 6.8), 20 μL enzyme solution and 40 μL of extracts or acarbose at different concentration was incubated in each well at 37 °C for 5 min. After incubation 40 μL 0.75 mM ρNPG solution was added to initiate the enzyme reaction. The released p-nitrophenol was monitored at 405 nm each 5 min for a total time of 20 min by a multimode microplate reader Mithras LB 943 MF (Berthold Technologies, Germany) in 96 micro well against a blank sample without enzyme. One unit of enzyme activity was defined as the amount of enzyme that released 1 mM of p-nitrophenol per minute under the assay conditions. The control was the enzyme reaction without inhibitors and the positive control was acarbose. The results of alpha-glucosidase inhibitory activity were expressed as percentages of inhibitory activity and relative enzyme activity. The IC_{50} value was calculated in manner described above.

Statistical analysis

Statistical analyses were performed using Microsoft Excel 2010. All analyzes were carried out in triplicate and the results were expressed as a mean ±SD.

Results and discussion

Determination of total phenolic content and radical scavenging activity

Aqueous extracts of medicinal plants are mixtures of multiple components, which may contain both primary and secondary metabolites in various concentrations. The large group of phenolic compounds are secondary plants metabolites, which possess many health benefits, such as anti-inflammatory, antimicrobial, antioxidant, antidiabetic, etc. (Cory et al. 2018).

The extraction yields, total polyphenolic content and antiradical capacity of the extracts against DPPH and ABTS expressed as IC_{50} values are presented in Table 1. The yield of the extracts, using ultrasound-assisted extraction with water varies from 45 to 50% in aqueous extract of *Cichorium intybis* (CIW). According to study published by Gupta et al. (2012) water based extraction with ultrasound leads to a 20% increase in extract yield. In addition to the high yield, the efficiency of the extraction, is directly correlating with the extract biological functions. The ultrasound assisted extraction is conducted in

Table 1. Yield, content of total polyphenols (TPC) and IC_{50} values of DPPH and ABTS assays of the studies aqueous plant extracts.

Samples	Yield of extract, %	TPC, mg GAE/g DE	DPPH	ABTS
			IC_{50}, mg/mL	
Rutin	-	-	0.028	0.194
SKW	45.57	160.10 ± 6.47	0.087	0.33
SEW	46.52	108.59 ± 3.11	0.328	0.70
CIW	50.42	86.45 ± 1.54	0.55	1.41
PMW	46.30	15.29 ± 0.24	11.92	55.13

TPC: total phenolic content; GAE: gallic acid equivalents; SKW: extract of *Satureja kitaibelii*; SEW: extract of *Sambucus ebulus*; CIW: extract of *Cichorium intybis*; PMW: extract of *Prunus mahaleb*.

lower temperature than conventional methods such as infusion and decoction. That contributes directly to the greater extend for the preservation of thermally unstable components. Additionally, this method is appropriate for increase in polyphenolic yields in plant extracts according to (Dai and Mumper 2010).

Folin-Ciocalteu method is based on oxidation-reduction reactions between the reagents used and phenolic compound. The electron transfer measures the reducing capacity of components in plant extracts (Noreen et al. 2017). The extract that exhibited the highest total phenolic content was SKW (160.10 mg GAE/g DE), followed by extracts of SEW, CIW and PMW. TPC concentration of *Satureja kitaibelii* in our research correlates with the results of Gopčević et al. (2019) for the Serbian population of the same species. They reported 158.85 ± 15.02 mg GA/g in extract of stem, leaves and flowers The TPC obtained by them using ultrasound assisted extraction method were higher than the conventional methods of extraction which in above-mentioned case was bimaceration.

In the present study total phenolic content of dwarf elder's extract was 108.59 ± 3.11 mg GAE/ g DE. In previous studies, the estimated concentration of polyphenolic compounds varies from 43.47 mg GAE/g to 116.3 mg GAE/g extract (Meriç et al. 2014; Topuzović et al. 2016; Cvetanović et al. 2017). Cvetanović et al. (2017) reported 116.3 mg chlorogenic acid equivalent in subcritical aqueous extract of *Sambucus ebulus* leaves with Serbian origin. Topuzović et al. (2016) reported 43.47 mg GAE per gram aqueous extract of leaves obtained by 24 h infusions. In conclusion, numerous environmental factors as climatic and atmospheric conditions in combination with technological parameters such as extraction method, temperature, extraction time, type and polarity of the solvent used, lead to significant differences in the concentration of total polyphenols in *Sambucus ebulus* (Cvetanović 2020).

The investigated aqueous extract of *Cichorium intybus* had a total phenolic content of 86.45 ± 1.54 mg GAE/ g DE. Abbas et al. (2015) showed a similar result of 85 mg GAE/ g DE in hydro-alcoholic extract. According to Taghizadeh et al. (2015) total phenolic content in *Prunus mahaleb* strongly depends on genotypes and ranging from 7.25 to 23.13 mg GAE/g DE. The result obtained in our study is 15.29 mg GAE/g DE and is in the middle of the given range.

To evaluate *in vitro* antioxidant effects of plant extracts, two methods against organic radicals, were used. These tests are the most accepted models for screening the free radical scavenging activity of any plant extracts. The samples exhibited a concentration-dependent radical inhibitory activity in both analyses. The DPPH assay is based on single electron transfer (SET) colorimetric reaction. There is high positive correlation between concentration of phenolic content and DPPH inhibitory activity because of similar mechanism of the methods (Moharram and Youssef 2014). The lower concentration of extracts shows more potential antiradical activity and measurement of the reducing ability of components in plant extracts. The lowest IC_{50} value was obtained by SKW (0.087 mg/mL) where is the highest concentration of total phenols (160.10 ± 6.47 mg GAE/g DE). This trend is maintained in the analysis of all investigated plant extracts. The identified IC_{50} concentration of *Satureja kitaibelii* extract in our study correspond to those reported by Gopčević et al. (2019) – 71.20 ± 9.55 µg/mL.

ABTS method was reported to use both mechanisms – hydrogen atom transfer (HAT) and SET (Prior et al. 2005). Between 2.33 (*Sambucus ebulus*) and 4.6 times (*Prunus mahaleb*) higher concentrations of extracts are required to obtain 50% inhibition of the ABTS radical in comparison with DPPH. Positive control of rutin (quercetin-3-rhamnosyl glucoside) which is a natural flavone derivative, was used in the antiradicals' analysis. The scavenging effect of samples decreased in the following order: rutin > SKW > SEW > CIW > PWM. The minimum IC_{50} concentration was obtained by SKW (0.33 mg /mL) which is 1.7 times higher than the positive control.

Evaluation of α-glucosidase inhibitory activity

Many experimental studies exhibited exacerbated relationship between oxidative stress and diabetes by measuring markers of oxidative stress (Yang et al. 2011; Ullah et al. 2016). The high antiradical activity demonstrated in this experimental research is the reason for evaluating *in vitro* antidiabetic potential of the plants. Alpha-glucosidase inhibitors play a key role in early phase of the treatment of type 2 diabetes. The decrease in the activity of the α-glucosidase leads lower postprandial blood glucose. Acarbose is used as an oral antihyperglycemic agent (Laar 2008). The most prominent gastrointestinal side effects such as diarrhea and flatulence occurring often in patients population treated with acarbose during the course of therapy, make the substances from natural origins a suitable alternative due to expected lack of side effects (Kumar and Sudha 2012).

The α-glucosidase inhibition correlates with the increasing the concentration of tested plant extracts. Their inhibitory activity was compared as a percentage as shown in Table 2. The two different concentrations of each extract tested were observed side by side. The lowest concentration of positive control acarbose of 1 mg/mL

Table 2. Alpha-glucosidase inhibition activity of plant extracts with different concentrations.

Samples	Concentration of extract, mg/mL	Inhibition of α-glucosidase, %
Acarbose	1	5.47 ± 0.42
	7.5	53.39 ± 0.54
SKW	0.695	28.22 ± 0.64
	5.56	92.02 ± 1.14
SEW	0.647	12.77 ± 0.15
	6.47	16.60 ± 0.24
CIW	0.563	11.91 ± 0.39
	5.63	48.01 ± 0.49
PMW	0.699	9.65 ± 0.15
	6.99	17.38 ± 0.78

demonstrated 5.47% inhibitory activity. After increase the concentrated 7.5 times (7.5 mg/mL) the inhibition raised up to 53.39%. The two concentrations of SKW 0.695 mg/mL and 5.56 mg/mL showed inhibition rates of 28.22% and 92.02%, respectively, which clearly exceeded the inhibitory activities of acarbose. To the best of our knowledge this is the first report of α-glucosidase inhibitory activity of *Satureja kitaibelii* which shows that the plant has a good potential as a blood glucose regulating agent. At lower tested concentrations, the aqueous extract of chicory showed an inhibition of 11.91% which increased to 48.01% with a tenfold increase in concentration. Both values obtained for the leafy flowering stems of *Cichorium intybus* are comparable to the performance of acarbose. Dalar and Konczak (2014) reported a pronounced inhibitory activity of the leaf extract against α-glucosidase (IC50:4.25 ± 0.08 mg/ml). Anti-diabetic activity of the whole plant and the fruit (cypsella) were proved through *in vivo* analysis (Pushparaj et al. 2007; Draz et al. 2010; Chandra et al. 2018). Some other studies research directly the anti-diabetic and related activities of the roots (Kanj et al. 2019), leaves (Mathusamy et al. 2008; Ahmed 2009; Brieudes et al. 2016) or aerial parts (Azay-Milhau et al. 2013) of *C. intybus*. Our original results are the first evidence for α-glucosidase inhibitory activity of the upper parts of the flowering stems of *C. intybus*. The properties of the stems of this plant are very poorly studied according to the literature and data on their antidiabetic potential is practically lacking. Probably this is due to their dryness, hollowed pith and significant participation of transport and support tissues, which can lead to the assumption that they do not possess biological activity. Our results show that the chemical composition of the flowering upper part of the stems of needs further research in order to be explained the significant α-glucosidase inhibitory activity which could not be attributed only to the heads in different flowering stage and the few small leaves on this part of the stem. Moreover, the most abundant and easy to collect part of *C. intybus* as a perennial herbaceous plant is the flowering top part of the stem. On the other hand, SEW and PMW showed only moderate activities with 16.60% and 17.38% inhibition rate. Samples with the lower tested concentrations showed higher inhibition potential described in percentages compared to the positive control – acarbose.

The baseline of the enzyme activity is shown as 100% for the non-inhibited enzyme reaction (Fig. 1A, B). The relative enzyme activity of α-glucosidase decreases within the range from 7% to 64% in dose-dependent manner by adding increasing concentrations of SKW from 0.7 to 5.6 mg/mL (Fig. 1A). The most active amongst the tested plants' extracts including positive control is SKW. This is also confirmed by comparing IC_{50} concentrations (Table 3). Therefore, 1.18 mg/mL from SKW extract was needed to achieve 50% inhibition of enzyme, which is approximately 6 times lower than acarbose (6.86 mg/mL).

After the studies accomplished, it could be concluded that only SKW could be applied as potential alternative of synthetic inhibitors against the digestive action of α-glucosidase.

Table 3. Concentrations of acarbose and *S. kitaibelii* extracts resulting in 50% inhibition of α-glucosidase activity.

Samples	IC_{50}, mg/mL
Acarbose	6.86
SKW	1.18

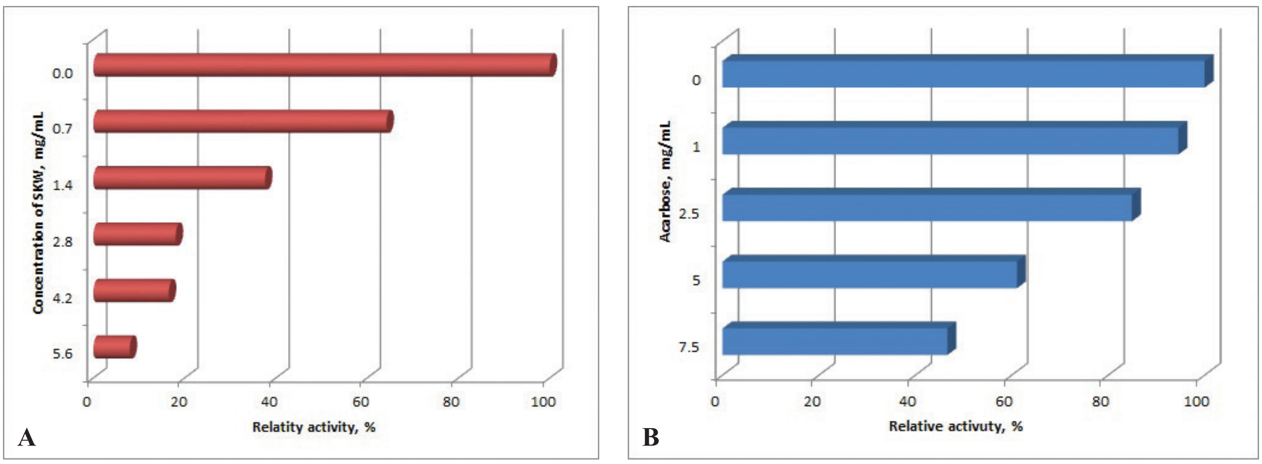

Figure 1. Relative enzyme activity of α-glucosidase by adding different concentration of (**A**) SKW and (**B**) acarbose IC_{50} values and relative enzyme activity were calculated for the samples which exhibited higher than 50% inhibition of α-glucosidase.

Conclusion

To the best of our knowledge this is the first report for *in vitro* α-glucosidase inhibitory activity of extracts from flowering stems of *Satureja kitaibelii*, flowering stems of *Cichorium intybus* and leaves of *Prunus mahaleb*, as well as the first such report for the Bulgarian population of *Sambucus ebulus*. The original data for the antioxidant activity of the extracts from flowering stems of *S. kitaibelii* and leaves of *P. mahaleb* collected from Bulgaria is also presented for the first time. The extract of *S. kitaibelii* stands out due to its highest values in all studied indicators – total phenolic content, scavenging potential (DPPH, ABTS) and α-glucosidase inhibitory activity. It showed prominent inhibitory activity against α-glucosidase nearly 6 times stronger than acarbose, followed by the extract of *C. intybus* with comparable to acarbose inhibitory activity. The flowering stems of both species, *S. kitaibelii* and *C. intybus*, are promising for further research as a natural sources of biologically active substances for blood sugar control in diabetes mellitus as safe analogues of the available at present oral medications.

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