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In vitro antioxidant and antidiabetic potential of herbal mixture traditionally used in treatment of *diabetes mellitus*

Original Article

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Abstract:

This work aimed to assess and compare the phytochemical composition, the free radical neutralization capacity, and antidiabetic potential of a traditional herbal mixture and its individual ingredients: *Apium graveolens*, *Polygonum aviculare, Hypericum perforatum*, and *Conyza canadensis*. Antioxidative capacity was evaluated using DPPH radical scavenging test. Antidiabetic activity was assessed using α-amylase inhibition assay. The results showed that there was a positive correlation between antioxidant and antidiabetic activity with total phenol ($p < 0.001$) and flavonoid content ($p < 0.001$) in all tested extracts. Herbal mixture had higher total phenol and flavonoid content than most of its individual ingredients. Its antioxidant capacity was excellent, even higher than standard, butylated hydroxytoluene ($p < 0.001$). Moreover, it showed significant antidiabetic potential (IC_{50} value 99.70±8.4 μg/ml). To conclude, the herbal mixture showed great potential for the future use as a dietary supplement in the therapy for diabetes.

Key words:
herbal mixture, antioxidative activity, antidiabetic activity, DPPH test, α-amylase inhibition assay

Apstrakt:

***In vitro* antioksidativni i antidijabetski potencijal biljne mešavine tradicionalno korišćene u lečenju dijabetesa**

Cilj ovog rada je procena i upoređivanje fitohemijskog sastava, kapacitet neutralisanja slobodnih radikala i antidijabetskog potencijala tradicionalne biljne mešavine i njenih pojedinačnih lekovitih komponenti: *Apium graveolens*, *Polygonum aviculare*, *Hypericum perforatum* i *Conyza canadensis*. Antioksidativni potencijal utvrđen je na osnovu DPPH testa. Antidijabetska aktivnost je procenjena upotrebom testa inhibicije α-amilaze. Rezultati su pokazali da postoji pozitivna korelisanost antioksidativne i antidijabetske aktivnosti sa ukupnim fenolima ($p < 0.001$) i flavonoidima ($p < 0.001$) kod svih testiranih ekstrakata. Biljna mešavina je imala veliki sadržaj ukupnih fenola i flavonoida u poređenju sa većinom svojih pojedinčanih biljnih komponenti. Dobar je antioksidans, čak i u poređenju sa standardom, butilisanim hidroksianizolom ($p < 0.001$). Pored toga, pokazuje značajan antidijabetski potencijal (IC50 vrednost 99.70±8.4 μg/ml) ($p < 0.001$). Može se zaključiti da biljna mešavina ima veliki potencijal kao dopuna terapija dijabetesa u budućnosti.

Ključne reči:
biljna mešavina, antioksidativna aktivnost, antidijabetska aktivnost, DPPH test, test inhibicije α-amilaze

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Introduction

Medicinal plants, because of their many pharmacological activities, have been used for medicinal purposes for centuries, in the treatment of a variety of diseases including Diabetes mellitus (Khairullah et al., 2020). Diabetes is a chronic metabolic disorder that may significantly reduce the quality of affected individuals' lives (Harding et al, 2019). A constant hyperglycaemic environment usually leads to the elevated production of reactive oxygen species (ROS) (Wang & Wang, 2017), which can contribute to the development of secondary diabetes complications, such as nephropathy, retinopathy, osteoporosis, cardiovascular and Alzheimer's disease (Volpe et al., 2018; Huang et al., 2018; Thomas et al., 2019; Schuett, 2020).

Many herbal medical supplements, taken

together with standard pharmacotherapy, may prevent secondary complications of diabetes (Li et al., 2015; Yu et al., 2015, Dash et al., 2018). Many polyphenols extracted from vegetative parts of plants have hypoglycaemic effects (Liang et al., 2017; Su et al., 2020; Zhao et al., 2020) and they are capable to bind to the free radicals and neutralise them. That way, they can protect the animal cells from oxidative damage, and inhibit the progression of many chronic diseases (Kandaswami & Middleton, 1994).

Phenolic acids and flavonoids are two groups of phenolic components in plants. They have many biological activities such as antioxidant, coronary and heart diseases protective, anti-inflammatory, anti-cancer, and antimicrobial ones (Bhandari et al., 2008). Moreover, they are capable to inhibit α-amylase, an important enzyme in the metabolism of carbohydrates. Namely, after the inhibition of this enzyme the blood sugar level decreases, so, they have a major role in the contribution to the standard pharmacotherapy for diabetes (Kooti et al., 2016).

Many studies have shown the presence of a beneficial synergistic effect of bioactive phytochemicals when used together as polyherbal mixtures (Sun et al., 2015; Chien et al., 2016; Madić, 2019; Savych, 2021). Having in mind this and the fact that even 10.5% of the human population was diagnosed with diabetes in 2021 (Cho et al., 2018; Sun et al, 2022), many contemporary studies are increasingly turning to the development of new supplements based on traditionally used polyherbal mixtures that will contribute to the amelioration of both primary and secondary complications of diabetes (Pan et al., 2013).

This study aimed to evaluate the phytochemical composition, antioxidant, and *in vitro* antidiabetic properties of aqueous extract of the other one of the traditionally used antidiabetic polyherbal mixture, as well as aqueous extracts of its individual constituents: celery (*Apium graveolens* L., Apiaceae) lives, centaury common knotgrass (*Polygonum aviculare* L., Polygonaceae), St. John' wort (*Hypericum perforatum* L., Hypericaceae), and horseweed (*Conyza canadensis* L. Cronquist, Asteraceae) areal parts.

Materials and Methods

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Butylated hydroxytoluene (BHT), Quercetin hydrate (QuE), D-maltose monohydrate, potato starch, porcine pancreatic α-amylase were purchased from Sigma Aldrich (A3176), 3,5-dinistrosalicylic acid 97 + % from Alfa Aesar (USA), dimethyl sulfoxide (DMSO) from Acros Organics (Belgium), Gallic acid anhydrous (GAE) from Merck (USA), Folin-Ciocalteu's reagent from Carlo Erba Reagents (Spain). All the other chemicals used were analytical-grade reagent chemicals.

Plant material and extract preparation

Plant material, i.e., roots of *P. aviculare* L., and aerial parts of *H. perforatum* L., *A. graveolens* L. and *C. canadensis* (L.) Rupr. were collected in South-Eastern Serbia (Sićevo Gorge, Stara planina Mt., Niška Banja, and Gornja Koritnica, respectively) during 2021, taxonomically identified and the voucher specimens were deposited at the herbarium collection of the Faculty of Sciences and Mathematics, University of Niš under the following accession numbers: 16421, 16422, 16423 and 16424, for *P. aviculare*, *H. perforatum*, *A. graveolens* and *C. canadensis*, respectively. Plant material was dried at room temperature in dark for 2–3 weeks, and the herbal mixture was prepared according to the traditionally used recipe (Životić & Životić, 1979). 10 g of each plant material and the herbal mixture were separately boiled in 100 ml of distilled water until half of the liquid evaporated. The obtained aqueous extract was then filtered, and the solvent was totally removed using a vacuum evaporator (IKA RV10 Rotary Evaporator with HB10 Bath, Gaithersburg).

Determination of total phenolic content

The total phenolic content (TPC) of extracts was determined spectrophotometrically by the Folin-Ciocalteu method as previously described (Madić, 2021). 0.3 ml of tested extract (1 mg/ml, diluted in methanol) was mixed with 1.5 ml Folin-Ciocalteu's reagent (diluted in methanol 1:10 (v/v)) and 1.2 ml of Na_2CO_3 (7.5%). After 2 h of incubation time in the dark at room temperature, the absorbance was measured at 740 nm (UV-1650PC, Shimadzu 1650, Europe). The total phenolic concentration was calculated from a gallic acid (GAE) calibration curve (10–100 mg/l, diluted in methanol). Data were expressed as gallic acid equivalents (GAE)/g of the extract.

Determination of total flavonoid content

The total flavonoid content (TFC) was determined as previously described by Madić et al. (2021). 1 ml of tested extract (1 mg/ml, diluted in methanol) was mixed with 6.4 ml of distilled water (dH_2O), 0.3 ml of $NaNO_2$ (5%), 0.3 ml of $AlCl_3$ (10%) and 2 ml of NaOH (1 M). After 30 min of incubation period in the dark at room temperature, absorbance was measured at 510 nm. The total flavonoid content in extracts was calculated from a quercetin hydrate (QuE) calibration curve (10–100 mg/l, diluted in

methanol) and expressed as quercetin equivalents (QuE)/g of dry extract.

DPPH radical scavenging assay

The DPPH radical scavenging assay was used to evaluate the antioxidant activity of tested extracts (Blois, 1958). The dried aqueous extracts were dissolved in 100% methanol. 0.3 ml of different concentrations of each extract, i.e., 5, 10, 15, 25, 50, 75, 100 µg/ml were taken and mixed with 2.7 ml of DPPH radical methanol solution (0.04 mg/ml). The experiment was performed in the dark at room temperature for 30 minutes. The absorbance was measured at 517 nm. 0.3 ml of methanol with DPPH radical methanol solution was taken as a control. The radical scavenging activity (DPPHsa) was calculated by the formula:

DPPHsa (%)=[(Abs(control)-Abs(sample)/ Abs(control)]x100

Butylated hydroxytoluene (BHT) was used as a positive control.

α-amylase inhibition assay

The α-amylase inhibition assay was done by a slightly modified method of Radulović et al. (2013). Potato starch (1% w/v) was diluted in 0.02 M sodium phosphate buffer (enriched with 6.7 mmol/l sodium chloride, pH 6.9) and stabilized by heating at 70 °C for 15 minutes. Five concentrations of each extract, i.e., 10, 25, 50, 75, and 100 µg/ml were prepared by diluting the stock solution in dimethyl sulfoxide (DMSO). 1 ml of each extract was mixed with 1 ml of pancreatic α-amylase (1U/ml) (A3176, Sigma Aldrich). After 20 minutes of preincubation period at 37 °C, 1 ml of the preincubated mixture was mixed with 1 ml of potato starch and incubated at 37 °C for 20 minutes. 1 ml of DNSA colour reagent was added to each tested sample, boiled for 5 minutes, cooled down, and diluted in 9 ml of dH_2O. In the end, the generation of maltose was calculated from a maltose calibration curve at the wavelength of 540 nm. α -amylase inhibition activity was calculated as follows:

Inhibition (%)=[(Ac-As)/Ac]*100

where Ac was the absorbance of the positive control, and As absorbance of the tested extract.

Statistical analysis

Statistical analysis was done using GraphPad Prism 5 (GraphPad Software, La Jolla California USA). All experiments were done in triplicate and data were expressed as the mean±standard deviation. The differences between the controls and the individual dosage groups of the tested extract were analysed by the one-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. The relation between total phenol, total flavonoid content, IC_{50} of DPPH scavenging activity, and IC_{50} of the α-amylase inhibition potential of tested extracts was assessed using Pearson's correlation coefficient. Statistical differences were accepted if p was less than 0.05.

Results and discussion

The ethnopharmacological values of polyherbal preparation and its individual ingredients, four medicinal plants (Životić & Životić, 1979) were tested through evaluation of their phytochemical composition, antioxidant, and antidiabetic effects.

The yield of extraction, the total phenolic (TPC) and flavonoid content (TFC) of tested extracts are shown in **Tab. 1**. The yield of extraction was positively correlated to flavonoid content of extract (r = 0.93; $p<0.05$). The highest TPC and TFC values were found in *H. perforatum* extract (523.33±5.77 mg of GA/g of extract, and 113.3±0.56 mg of QuE/g of extract, respectively), while the *A. graveolens* extract showed the lowest values of both TPC and TFC (16.67±5.77 mg of GA/g of extract, and 45.47±0.81mg of QuE/g of extract, respectively).

The results of the DPPH and α-amylase inhibition tests showed that all tested extracts had some level of antioxidant and *in vitro* antidiabetic activity. Those

Table 1. Yield, total phenolic and total flavonoid content of tested extracts. Statistically significant different from herbal mixture (*) and tested extracts according to Tukey's Multiple Comparison Test ($p<0.001$)

Extract	Extraction yield (%)	TPC (mg of GA/g of extract)	TFC (mg of QuE/g of extract)
herbal mixture	26.69	209.67±2.31	102.87±5.12
P. aviculare	24.43	107.98±2.64*	98.57±1.23
H. perforatum	12.52	523.33±5.77*	113.3±0.56*
A. graveolens	82.35	16.67±5.77*	45.47±0.81*
C. canadensis	29.96	237.01±2.6*	112.6±0.35*

Comparison Test ($p<0.001$)

activities were concentration-dependent (p<0.001) (**Fig. 1, Fig. 2**).

The lowest DPPH IC_{50} and IC_{50} of α-amylase inhibition potential values were observed in *H. perforatum* (5.54±1.34 μg and 51.12±10.44 μg) extract and the highest ones in *A. graveolens*' extract (161.28±11.54 μg and 268.81±16.69 μg), as shown in **Fig. 1** and **Fig. 2**. The polyherbal mixture's extract had similar total flavonoid content as *C. canadensis*' one, but higher than its other two individual ingredients, *P. aviculare* and *A. graveolens*.

The polyherbal mixture's extract showed great antioxidative potential even when used in the lowest tested concentration, and much higher than two of its ingredients. i.e., extract of *P. avicularae* and *A. graveolens*. Additionally, in a slightly higher concentration polyherbal mixture' extract was efficient in α-amylase inhibition, even more than *A. graveolens*' extract and similar to *C. canadensis*' and *P. avicularae*' ones. These results are in concordance with previous research, where polyherbal mixtures had higher biological activities than most of their individual components (Madić et al, 2019).

For evaluating the antioxidant capacities of tested medicinal herbs extracts, we compared their DPPH scavenging activities to the activity of the standardly used antioxidant agent, i.e., BHT. In the DPPH test, the radical scavenging activity of all the tested herbal extracts and BHT increased in a concentration-dependent manner as shown in **Fig.**

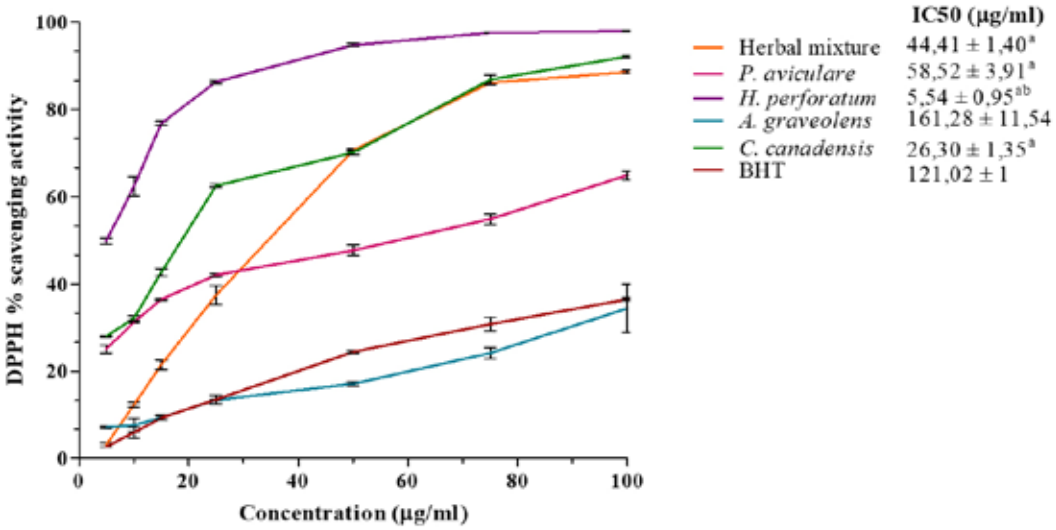

Fig. 1. DPPH scavenging activity and IC_{50} values of tested extracts. Statistically significant different between BHT (**a**), herbal mixture (**b**) and tested extracts according to Tukey's Multiple Comparison Test (p<0.001)

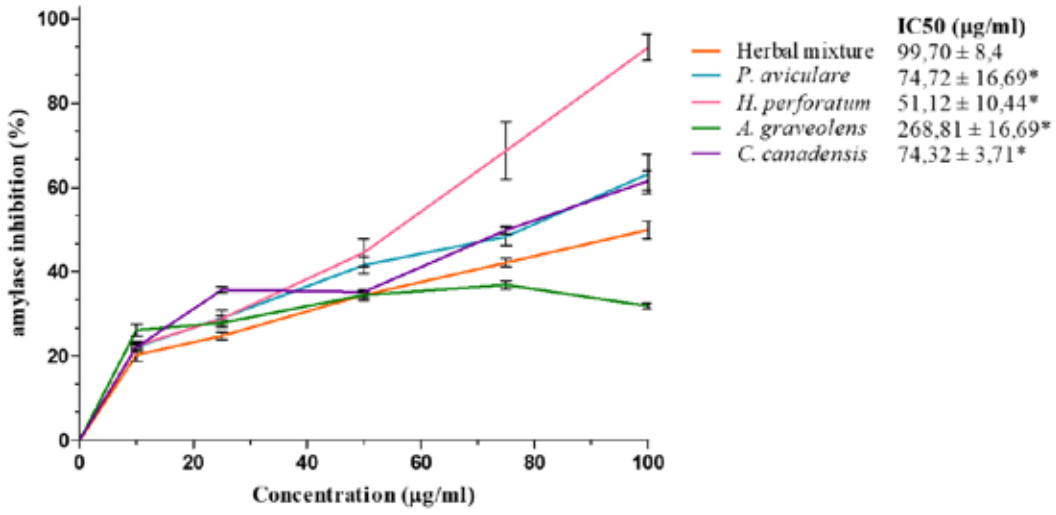

Fig. 2. The α-amylase inhibition assay and IC_{50} values of tested extracts. Statistically significant different between herbal mixture (*), and tested extracts according to Tukey's Multiple Comparison (p<0.001)

1. All the tested extracts except *A. graveolens,* had statistically higher (p<0.001) antioxidant activity than BHT (**Fig. 1**).

There was positive correlation between TFC and DPPH radical scavenging activity (r=0.98; p<0.05) as well as between TFC and α-amylase inhibition activity (r=0.96; p<0.05). Antioxidative and α-amylase inhibition activities of tested extracts were positively correlated (r=0.94; p<0.05), as shown in **Fig. 1** and **Fig. 2**. This is in concordance with previous studies where both medicinal herbs' antioxidative potential and polyherbal mixtures' one were directly correlated to the amount of their phytochemicals (Torabian et al., 2008; Fratianni et al., 2019). Besides the direct positive correlation between polyphenols and the antioxidative activity, it is well known that these bioactive substances are potent inhibitors of digestive enzymes such as α-amylase (Ali et al., 2006; Demir et al., 2019; Ganesan & Xu, 2019; Kuar et al., 2021). This is an important aspect of the application of polyphenol-enriched diets because it is possible to prevent hyperglycaemia and stabilise blood sugar in diabetic patients (Ali et al., 2006; Demir et al., 2019; Ganesan & Xu, 2019) by inhibiting this enzyme (Zhang et al., 2020).

Previous studies showed that tested medicinal plants, when used individually, have great potential for inhibition of digestive enzymes responsible for breaking down carbohydrates, the main cause of hyperglycaemia, i.e., the primary complication of diabetic patients, as well as great potential for binding and neutralising the free radicals, which excess is characteristic in secondary complications of diabetes. Numerous studies indicated the high antioxidant capacity of *H. perforatum* (Fathi & Ebrahimzade, 2013; Oliveira et al., 2017), which was once again confirmed by our study; however, its effect in the before mentioned medicinal herbs combination was previously unknown. Moreover, of all the tested extracts, the highest α-amylase inhibitory activity had the extract of *H. perforatum*, which is in concordance with previous studies that showed that couple of other species of *Hypericum* genus, such as *H. perfoliatum*, *H. humifusum* and *H. lydium*, had strong inhibitory activity against this enzyme (Bejaoui et al., 2017; Eurygur et al., 2019).

The results of this study are in agreement with studies of El-Akhal et al. (2021), and Abood & Kadhim (2021) which acknowledged a high amount of polyphenols such as epicatechin, catechin, quercetin, apigenin, luteolin, quercetin, quercetin, apigenin, p-coumaric acid, caffeic acid present in the aqueous extract of *C. canadensis*. According to Lateef et al. (2018) even the essential oil of this medicinal herb had high DPPH radical scavenging capacities.

Compared to the other tested extracts, the lowest antioxidative and anti-α-amylasis activities had *A. graveolens*' one, while previous studies showed at least some level of these activities (Neagu et al., 2019; Khoshnamvand et al., 2020; Suleman, et al., 2020; Al Aboody, 2021), owing to the presence of polyphenols such as cyanidin, chlorogenic acid, apigenin, apiin, rutin, kaempferol, caffeic acid, p-coumaric acid, ferulic acid (Jung et al., 2011; Helaly et al., 2015; Liu et al., 2016; Sui et al., 2016; Preicina et al., 2018).

Compared to the herbal mixture, extract of *P. aviculare* also had lower antioxidant and anti-amylase activities, however, according to previous research, when used in higher concentrations, it showed some level of both free-radical scavenging (Mahmoudi et al., 2021) and anti-amylase (Cai et al., 2020) activities, most probably due to the presence of many phenols such as quinic acid, gallic acid, protocathuic acid, catechin, caffeic acid, epicatechin, and quercetin (Salinitro et al., 2020; Wu et al., 2021).

To the best of our knowledge, this is the first report on the phytochemical composition, antioxidant, and antidiabetic effects of this polyherbal mixture traditionally used as a remedy for diabetes.

Conclusions

Diabetes is a major health problem and economic burden all over the world. Because of that, the results presented in this study are important since they represent the initial screening in search for the best candidates able to contribute to the amelioration of both primary and secondary diabetes complications. Namely, great anti-α-amylase and antioxidant activities shown by the herbal mixture extract, even when used in low concentrations, suggest a strong conclusion that it might be used as a dietary supplement. However, more comprehensive in vivo studies are needed.

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