



King Saud University
Arabian Journal of Chemistry

www.ksu.edu.sa
www.sciencedirect.com



ORIGINAL ARTICLE

Traditional multi-herbal formula in diabetes therapy – Antihyperglycemic and antioxidant potential



Nebojša Salaj^{a,*}, Nebojša Kladar^{a,b}, Branislava Srđenović Čonić^{a,b},
Katarina Jeremić^a, Maja Hitl^a, Neda Gavarić^{a,b}, Biljana Božin^{a,b}

^a University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Hajduk Veljkova 3, 21000 Novi Sad, Republic of Serbia

^b University of Novi Sad, Faculty of Medicine, Center for Medical and Pharmaceutical Investigations and Quality Control, Hajduk Veljkova 3, 21000 Novi Sad, Republic of Serbia

Received 11 March 2021; accepted 17 July 2021

Available online 28 July 2021

KEYWORDS

Tea mixture;
Antioxidant;
Antihyperglycemic activity;
Chemometrics

Abstract Traditional medicinal plants are used as an additional form of type 2 diabetes therapy in which oxidative stress is one of the progression factors. The aim of the study was the evaluation of antihyperglycemic and antioxidant potential of tea mixture registered as food and marketed for treatment of type 2 *diabetes mellitus*. Chemical profiling of the extracts included quantification of total phenolics and flavonoids, while antihyperglycemic potential was estimated through determination of inhibition potential of α -amylase and α -glucosidase. Additionally, extracts were characterized for specific phenolic compounds by high performance liquid chromatography (HPLC). The *in vitro* antioxidant activity was estimated by application of DPPH, OH, NO and FRAP tests. The greatest anti- α -amylase potential is attributed to the *Phaseoli legumen*, while the highest potential to inhibit α -glucosidase was recorded for *Olivae folium*. Generally, screening of the phenolic compounds enabled us to conclude that a high level of correlation exists between anti- α -amylase potential and the quantified amounts of total phenolics, as well as gallic acid, while the chlorogenic acid is most probably responsible for the inhibition of α -glucosidase activity. Therefore, the high total ratio (40%, m/m) of *Olivae folium* and *Phaseoli legumen* in the evaluated tea mixture can

* Corresponding author at: University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Hajduk Veljkova 3, 21000 Novi Sad, Republic of Serbia.

E-mail addresses: nebojsa.salaj@mf.uns.ac.rs (N. Salaj), nebojsa.kladar@mf.uns.ac.rs (N. Kladar), branislava.srdjenovic-conic@mf.uns.ac.rs (B. Srđenović Čonić), katarina.jeremic@mf.uns.ac.rs (K. Jeremić), maja.bekut@mf.uns.ac.rs (M. Hitl), neda.gavarić@mf.uns.ac.rs (N. Gavarić), biljana.bozin@mf.uns.ac.rs (B. Božin).

Peer review under responsibility of King Saud University.



<https://doi.org/10.1016/j.arabjc.2021.103347>

1878-5352 © 2021 The Author(s). Published by Elsevier B.V. on behalf of King Saud University.
This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

be stated as fully justified. However, future *in vivo* research is required for further understanding of the antihyperglycemic activity mechanisms of secondary metabolites found in specified herbal components, as well as to evaluate their synergistic effect when applied in a form of tea mixture.

© 2021 The Author(s). Published by Elsevier B.V. on behalf of King Saud University. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Diabetes mellitus (DM) is a chronic progressive metabolic disease characterized by hyperglycemia which occurs as a consequence of defect in insulin secretion, insulin sensitivity, or both. The main types of diabetes are classified as: type 1, type 2, gestational *diabetes mellitus* and other specific types of DM. Type 2 *diabetes mellitus* (T2DM) is the most prevalent form of DM followed by the two pathophysiological defects in endocrine function – insulin resistance and impaired insulin secretion (American Diabetes Association, 2014). Namely, in response to hyperglycemia, various pathways leading to the oxidative stress increase are activated. The aforementioned includes production of mitochondrial reactive oxygen species (ROS), enhanced non-enzymatic glycosylation of proteins and favor of the hexosamine biosynthetic pathway (Kawahito et al., 2009). The overall elevated oxidative stress plays a key role in development of leading metabolic disturbances characteristic for T2DM – insulin resistance and a relative deficiency of insulin secretion (Evans et al., 2002).

Nine classes of oral prescription medicines currently approved for the treatment of T2DM are frequently associated with gastrointestinal adverse effects. One of the most popular pharmacological approaches in the management of postprandial hyperglycemia is reduction of dietary carbohydrate digestion, and consequent absorption, through the inhibition of key enzymes involved in their intestinal metabolism – α -amylase and α -glucosidase (Obloh et al., 2014).

The usage of traditional herbal remedies is widely adopted in treatment of different types of diabetes. More than 400 medicinal plants have been investigated for their beneficial effects in the treatment of T2DM, but only for a limited number was proved to be clinically efficient and safe. A wide range of biologically active constituents isolated from medicinal plants, such as phenolics, alkaloids, terpenoids and tannins, have been reported to display antihyperglycemic activity through α -amylase and α -glucosidase inhibition (Alam et al., 2014; Bahadoran et al., 2013; Salehi et al., 2019). This might represent a novel therapeutic strategy for the treatment of postprandial hyperglycemia, when taking into account relatively clinically insignificant and rare adverse effects, in contrast to conventional antihyperglycemic medicines (Salehi et al., 2019). However, currently popular traditional medicinal systems (phytotherapy, traditional Chinese medicine, Ayurveda) are based on holistic approach, which implies the use of medicinal plants mixtures, instead of single herbal components. Numerous studies have revealed the clinical importance of synergistic effects produced by application of multicomponent herbal mixtures in patients with diabetes, as well as potential mechanisms of action (Rather et al., 2013).

Thus, the aim of the current study was the evaluation of antihyperglycemic and antioxidant potential of tea mixture

registered as food and marketed as a remedy for treatment of type 2 *diabetes mellitus*, containing common wormwood herb (*Artemisia absinthium* L.), leaves of common nettle (*Urtica dioica* L.), black mulberry (*Morus nigra* L.), blueberry (*Vaccinium myrtillus* L.), silver birch (*Betula pendula* Roth, *Betula pubescens* Ehrh.), olive (*Olea europaea* L.) and common bean legumes (*Phaseolus vulgaris* L.). Furthermore, special emphasis was placed on the contribution of herbal mixture individual components to the investigated biological activity and elucidation of secondary metabolites contributing to the specified medicinal properties.

2. Materials and methods

2.1. Reagents and chemicals

2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, sulphanilamide, *N*-(1-naphthyl)-ethylenediamine dihydrochloride and iron(III)-chloride hexahydrate were obtained from Alfa Aesar (Haverhill, MA, USA). Folin-Ciocalteu (FC) reagent was purchased from Merck (Darmstadt, Germany). Ethanol, hydrochloric acid, trichloroacetic acid, hydrogen peroxide, sodium carbonate, potassium dihydrogenphosphate, potassium hydrogenphosphate and sodium nitroprusside were obtained from Lach-Ner (Brno, Czech Republic). Sodium bicarbonate, iron(II)-sulphate heptahydrate, acetic acid and methanol were purchased from POCH (Gliwice, Poland). Gallic acid, caffeic acid, *trans*-cinnamic acid, *p*-coumaric acid, chlorogenic acid, rosmarinic acid, ferulic acid, quercetin, rutin, quercitrin, aluminum-chloride, 2-deoxy-D-ribose, 2-thiobarbituric acid, ethylenediaminetetraacetate dihydrate, 2,4,6-tris(2-pyridyl)-*s*-triazine, Starch azure, α -amylase, α -glucosidase, glutathione (reduced) and *p*-nitrophenyl- α -D-glu copyranoside were obtained from Sigma Aldrich (St. Louis, MO, USA). All chemicals used in this study were of analytical grade, except for the reagents for chromatography analysis, which were of HPLC grade.

2.2. Herbal material and preparation of extracts

The commercially available tea mixture (marketed as “Tea used in diabetes”) purchased at pharmacy (Novi Sad, 2019) was used in this research. The composition of the tea mixture declared by the manufacturer is presented in Table 1.

Also, the individual herbal components were macroscopically separated from the tea mixture and were further evaluated for their biological potential and chemical profile. The extracts of tea mixture and individual herbal components were prepared in a form of decoct, as suggested by the manufacturer’s instructions available on the packaging (heating with boiling water for 15 min). The decocts were filtered, evaporated to dryness by heating, and the extraction yield (dry

Table 1 The composition of the tea mixture.

English common name	Latin name	Botanical name	Mass fraction (w/w, %)
common wormwood herb	<i>Absinthii herba</i>	<i>Artemisia absinthium</i> L., Asteraceae	5%
common nettle leaves	<i>Urticae folium</i>	<i>Urtica dioica</i> L., Urticaceae	5%
black mulberry leaves	<i>Mori nigrae folium</i>	<i>Morus nigra</i> L., Moraceae	10%
olive leaves	<i>Olivae folium</i>	<i>Olea europaea</i> L., Oleaceae	10%
blueberry leaves	<i>Myrtilli folium</i>	<i>Vaccinium myrtillus</i> L., Ericaceae	20%
silver birch leaves	<i>Betulae folium</i>	<i>Betula pendula</i> Roth, <i>Betula pubescens</i> Ehrh., Betulaceae	20%
common bean/bean shell legumes	<i>Phaseoli legumen</i>	<i>Phaseolus vulgaris</i> L., Fabaceae	30%

extract, d. e.) was quantified. The dried extracts were dissolved in distilled water for the purpose of biological potential evaluation and preliminary chemical characterization. In order to perform detailed chemical profiling by high-performance liquid chromatography (HPLC), the obtained dried extracts were dissolved in 70% (v/v) methanol.

2.3. Chemical characterization

2.3.1. Preliminary chemical characterization

The obtained extracts were preliminary chemically characterized in a term of total phenolic content (TPC) and total flavonoid content (TFC), according to the previously described method (Božin et al., 2013). The amounts of total phenolics and flavonoids were expressed as mg of gallic acid equivalents (GAE) per g of dry extract (mg GAE/g d.e.) and mg of quercetin equivalents (QE) per g of dry extract (mg QE/g d.e.), respectively, based on the previously obtained calibration curves for gallic acid and quercetin.

2.3.2. Detailed chemical profiling

The previously developed and validated high performance liquid chromatography (HPLC) method was used for identification and quantification of selected phenolic compounds (Salaj et al., 2020). The molecules of interest were separated using an Agilent Technologies 1100 liquid chromatographer equipped with diode array detector (Agilent Technologies, Waldbronn, Germany) on a reversed-phase Nucleosil C18 column (4.6 × 250 mm, 5 μm particle size;) held at 30 °C. The mobile phase consisting of 1% (v/v) aqueous formic acid (solvent A) and methanol (solvent B) was delivered in gradient mode as follows: 0 min, 10% B; 10 min, 25% B; 20 min, 45% B; 35 min, 70% B; 40 min, 100% B; 46 min, 10% B. The injected volume was 10 μL and the total running time was 48 min, using a variable flow-rate (0–10 min, 1 mL/min; 10–20 min, 0.8 mL/min; 20–30 min, 0.7 mL/min; 30–46 min, 1 mL/min). For the quantification of the selected compounds, chemical standards of gallic (GA), caffeic (CA), *trans*-cinnamic (CNA), *p*-coumaric (pQA), chlorogenic (CHA), rosmarinic (RA) and ferulic acid (FA), quercetin (Qe), rutin (R) and quercitrin (Qt) were dissolved in methanol and run under the same experimental conditions. The compounds of interest were monitored at 280 nm (GA, CA and CNA), 330 nm (pQA, CHA, RA, FA and Qe) and 350 nm (R and Qt) and identified by comparing the retention times and UV spectra of unknown peaks with those of corresponding reference standards. The results were expressed as micrograms per gram of dry extract (μg/g d.e.).

2.4. Evaluation of antioxidant activity

The potential of the obtained extracts to neutralize 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydroxyl (OH) and nitroso (NO) radicals was determined spectrophotometrically, as previously described (Božin et al., 2013). The results were expressed as IC₅₀ values (the concentration of sample required for neutralization of 50% of free radicals).

Furthermore, the ferric reducing antioxidant power (FRAP) of extracts was estimated according to a modified methodology of Benzie and Strain (Benzie and Strain, 1996; Lesjak et al., 2011). Ascorbic acid was used as a positive control, so the FRAP values were expressed as milligrams of ascorbic acid equivalents (AAE) per g of dry extract (mg AAE/g d.e.).

2.5. Evaluation of antihyperglycemic potential

The antihyperglycemic potential of the obtained extracts was evaluated through the α-amylase and α-glucosidase inhibition assays. Both of the tests were performed according to previously described and modified procedures from Sigma Aldrich (Kladar et al., 2017, 2015; Mun'im et al., 2013). Acarbose was used as a positive control. The results were expressed as percentage of α-amylase and α-glucosidase inhibition activity.

2.6. Statistical analysis

The obtained results were processed by the use of Microsoft Excel, v.2010 software package and Statistica v12.5 software (StatSoft Inc., Tulsa, OK, USA). All of the results were expressed as means of triplicate analyses corrected by standard deviation (SD). Based on the percentage of free radical neutralization, IC₅₀ values were determined by regression analysis. The obtained dataset was analyzed by means of univariate (one-way ANOVA followed by post-hoc Tukey test) and multivariate statistical methods (principal component analysis (PCA) and hierarchical cluster analysis (HCA)). Multivariate statistical methods were applied to a dataset determined by variables describing antioxidant potential (IC₅₀ values, FRAP value) and antihyperglycemic potential (Amy8000 and Glu40). The variables describing antihyperglycemic potential were derived as reciprocal values of a percentage of inhibition at a concentration of extract at which the activity of the tested enzyme was inhibited by more than 50% (for at least one of the extracts). Specifically, the selected concentration in the case of α-amylase was 8000 μg/mL (Amy8000), while in the case of α-glucosidase was 40 μg/mL (Glu40). The previously stated

derivatization of variables was performed in order to obtain the same mathematical nature as of IC_{50} values.

3. Results and discussion

3.1. Chemical characterization

3.1.1. Preliminary chemical characterization

The results of preliminary chemical characterization (Table 2) showed notable variability of extraction yield (ranging from 11.16 to 32.42%). Previously performed studies dealing with individual herbal components included in the examined tea mixture have shown great variability in terms of biological potential determination and chemical characterization. The aforementioned can be a consequence of several factors. Namely, we must not neglect the possibility of genetic variations in biological sources, as well as unavoidable and well-recognized influence of ecological factors, which both significantly affect the intensity of secondary metabolites biosynthesis in plants (Ferreira et al., 2007; Heimler et al., 2005; Jovančević et al., 2011; Kahkonen et al., 1999; Kostić et al., 2013; Lee et al., 2013; Pourmorad et al., 2006; Szentmihályi et al., 2010).

Specifically, the quantification of total phenolics in the examined samples has shown lower total phenolic content in common wormwood herb (*Absinthii herba*), black mulberry leaves (*Mori nigrae folium*) and olive leaves (*Olivae folium*) extracts, compared to the results of previously performed studies on the same type of extracts (Ferreira et al., 2007; Kostić et al., 2013; Lee et al., 2013). On the other hand, common nettle leaves (*Urticae folium*), blueberry leaves (*Myrtilli folium*), silver birch leaves (*Betulae folium*) and common bean legumes (*Phaseoli legumen*) extracts were characterized by a higher content of total phenolics, compared to the previous studies of these plant species (Heimler et al., 2005; Mišan et al., 2011; Pourmorad et al., 2006; Szentmihályi et al., 2010). The lower total flavonoid content was quantified in common wormwood herb, black mulberry, common nettle and olive leaves extracts (Heimler et al., 2005; Kostić et al., 2013; Lee et al., 2013; Pourmorad et al., 2006; Szentmihályi et al., 2010). Generally, the differences recorded for quantities of total phenolics and flavonoids, when compared to previously published papers, might be attributed to the geographical origin of the plant

material, as well as the prolonged heated assisted extraction applied for extraction in the current research.

3.1.2. Detailed chemical profiling

Detailed chemical profiling (Fig. 1, Table 3) gives insight into variations of specific phenolic compounds in the examined tea mixture and its individual components. Gallic and ferulic acid were the predominant components present in *Betulae folium* extract. Hydroxycinnamic acids, such as caffeic and *p*-coumaric acids, as well as quercetin were the most abundant in *Mori nigrae folium* extract. *Trans*-cinnamic acid and flavonoid glycosides - rutin and quercitrin were the dominant compounds found in *Phaseoli legumen* extract, while chlorogenic and rosmarinic acids were the main phenolic components of the *Olive folium* extract.

The conducted epidemiological studies have shown that dietary intake of phenolic-rich food corresponds to a lower incidence of the chronic degenerative diseases, such as cancer, diabetes type 2, Alzheimer's disease and cardiovascular diseases (Gutiérrez-Grijalva et al., 2016). Previous studies have reported beneficial effects of flavonoids, such as quercetin, on endothelial cell functions, and these effects are mediated via adenosine monophosphate-activated protein kinase (AMPK) pathway, key sensor of cellular energy homeostasis (Shen et al., 2012). Also, numerous studies have shown that antiplatelet effect of flavonoids may be a result of the thromboxane A2 receptor antagonism and inhibition of thromboxane formation (Shu-Huey et al., 1991).

3.2. Antioxidant potential

The obtained results of free radicals scavenging activity of tea mixture and its individual components against DPPH and OH radical (Fig. 2) suggest variations in the antioxidant potential of the examined extracts. The IC_{50} values obtained in DPPH assay ranged from 2.05 $\mu\text{g/mL}$ (*Phaseoli legumen* extract) to 30.48 $\mu\text{g/mL}$ (*Mori nigrae folium* extract). Previously was observed that *Phaseoli legumen* extract possesses the highest DPPH free radical scavenging activity (Boateng et al., 2008). Significant ($p < 0.05$) negative correlations between DPPH radical scavenging activity (IC_{50} value) of the examined extracts and total phenolic content

Table 2 Total phenolic content, total flavonoid content and extraction yield in the investigated extracts.

Sample	TPC (mg GAE/g d.e.) ^a mean \pm SD ^c	TFC (mg QE/g d.e.) ^b mean \pm SD	Extraction yield (%)
Tea mixture	61.50 \pm 1.01	8.33 \pm 0.15	11.38
<i>Absinthii herba</i>	85.83 \pm 3.56	6.31 \pm 0.28	18.72
<i>Urticae folium</i>	48.11 \pm 7.12	7.43 \pm 0.27	16.95
<i>Mori nigrae folium</i>	59.69 \pm 7.18	9.36 \pm 0.01	23.71
<i>Myrtilli folium</i>	25.18 \pm 1.09	4.70 \pm 0.49	16.27
<i>Betulae folium</i>	80.15 \pm 3.27	13.04 \pm 0.40	19.55
<i>Phaseoli legumen</i>	181.34 \pm 5.83	31.78 \pm 1.89	32.42
<i>Olivae folium</i>	98.74 \pm 1.89	4.97 \pm 0.27	11.16

^a mg GAE/g d.e. – mg of gallic acid equivalents per g of dry extract.

^b mg QE/g d.e. – mg of quercetin equivalents per g of dry extract.

^c Values represent mean \pm standard deviation of triplicate analysis (n = 3).

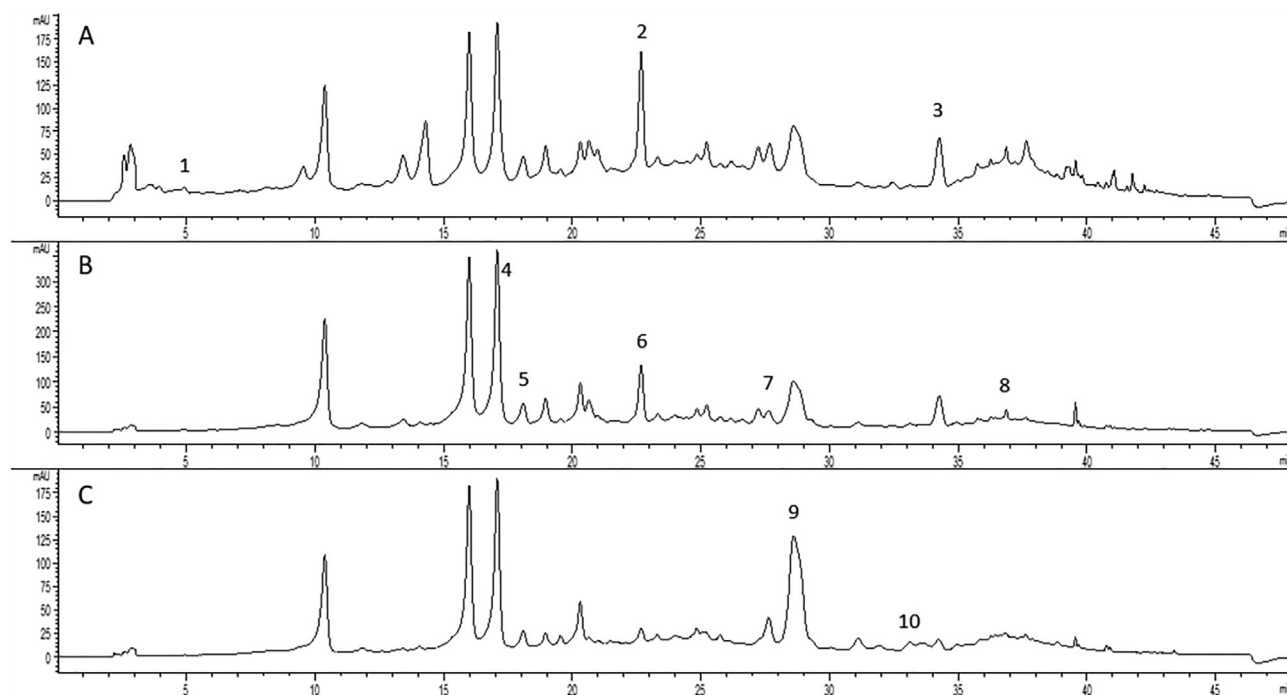


Fig. 1 HPLC-DAD chromatogram of tea mixture extract with detection at 280 nm (A), 330 nm (B), 350 nm (C): 1 – gallic acid, 2 – caffeic acid, 3 – *trans*-cinnamic acid, 4 – *p*-coumaric acid, 5 – quercetin, 6 – chlorogenic acid, 7 – rosmarinic acid, 8 – ferulic acid, 9 – rutin, 10 – quercitrin.

($r = -0.95$) as well as total flavonoid content ($r = -0.74$) were recorded. Furthermore, it was noticed that higher ability of DPPH neutralization was found for *Phaseoli legumen*, *Urticae folium*, *Betulae folium* and *Olivae folium* extracts, while *Absinthii herba* extract showed higher IC_{50} values, compared to the results of previous studies (Goldsmith et al., 2015; Nikolova et al., 2011; Otles and Yalcin, 2012; Sidaoui et al., 2016; Zou and Chang Sam, 2014). In the tested concentration range, *Myrtilli folium* extract showed no potential to neutralize 50% of DPPH radicals. The ability of *Mori nigrae folium* extract to scavenge DPPH was similar to the results of previous studies (Memon et al., 2010). The comparison of the *in vitro* obtained results concerning free radical scavenging assays may represent an issue due to the differences in applied analytical protocols which results in generating of different concentrations of free radicals. Therefore, the use of positive control – synthetic antioxidants greatly contributes to the credibility of the results obtained in the assessment of antioxidant activity. Regarding this, it is necessary to highlight the remarkable antioxidant effect of *Phaseoli legumen* extract, when compared to the antioxidant effect of quercetin dihydrate and propyl gallate, determined under the same experimental conditions (Kladar et al., 2017).

Furthermore, statistically significant differences were recorded in the results obtained for neutralization potential of DPPH radicals exhibited by examined herbal extracts and ascorbic acid ($F(8) = 2917.37$, $p = 0.00$). The post-hoc Tukey test has demonstrated that the differences in antioxidant potential do not exist only in the case of *Absinthii herba* ($p = 0.14$) and *Phaseoli legumen* ($p = 1.00$) extracts, when compared to ascorbic acid, thus confirming their strong antioxidant potential.

The potential of the examined extracts to neutralize OH radical resulted in the IC_{50} values ranging from 21.98 $\mu\text{g/mL}$ (*Olive folium*) to 132.95 $\mu\text{g/mL}$ (*Myrtilli folium*). Propyl gallate, a positive control, exhibited slightly higher ability to neutralize hydroxyl radicals in comparison to the examined *Olive folium* extract. A significant correlation ($p < 0.05$) between the neutralization potential of OH radical (IC_{50} values) and total phenolic content quantified in the examined extracts was noticed ($r = -0.81$).

None of the tested samples showed the ability to neutralize 50% of the generated NO radicals in the tested concentration range, indicating a low ability of reactive nitrogen species neutralization.

The *Phaseoli legumen* extract exhibited the highest ability to reduce Fe^{3+} in the FRAP assay (337.83 ± 3.09 mg AAE/g d. e.), while generally the results of FRAP assay (Fig. 3) significantly correlated ($p < 0.05$) with the quantified amount of total phenolics in the extracts ($r = 0.87$). The recorded FRAP values of the *Absinthii herba*, *Urticae folium* and *Phaseoli legumen* extract were higher when compared to the results of the previously conducted studies (Boateng et al., 2008; Kőszegi et al., 2020; Orhan et al., 2010).

3.3. Antihyperglycemic potential

Phenolic compounds have been highlighted as bioactive metabolites with significant health promoting benefits, especially in prevention of chronic non-communicable diseases. The inhibition of α -glucosidase and α -amylase, enzymes responsible for dietary carbohydrates metabolism, is one of the mechanisms by which phenolics reduce postprandial hyperglycemia.

Table 3 Detailed chemical characterization of tea mixture and its individual components.

Content ($\mu\text{g/g d.e.}$) ^a	Sample	Tea mixture	<i>Absinthii herba</i>	<i>Urticae folium</i>	<i>Mori nigrae folium</i>	<i>Myrrilli folium</i>	<i>Betulae folium</i>	<i>Phaseoli legumen</i>	<i>Olivae folium</i>
GA ^b		11.56 \pm 1.73	137.85 \pm 20.68	< LOD ^c	106.50 \pm 15.98	< LOD	1140.15 \pm 171.02	56.60 \pm 8.49	< LOD
CA		694.80 \pm 34.74	37.29 \pm 1.86	615.15 \pm 30.76	909.25 \pm 45.46	30.93 \pm 1.55	174.81 \pm 8.74	362.85 \pm 18.14	644.00 \pm 32.20
CNA		296.40 \pm 32.60	7.50 \pm 0.83	23.34 \pm 2.57	358.50 \pm 39.44	< LOD	< LOD	956.12 \pm 105.17	7.46 \pm 0.82
pQA		2713.78 \pm 271.38	2437.56 \pm 243.76	224.46 \pm 22.45	3891.625 \pm 3891.63	191.50 \pm 19.15	720.63 \pm 72.06	7281.30 \pm 728.13	192.10 \pm 19.21
Qe		165.78 \pm 11.60	433.65 \pm 30.36	33.90 \pm 2.37	1812.75 \pm 126.89	< LOD	214.74 \pm 15.03	329.49 \pm 23.06	38.90 \pm 2.72
CHA		200.80 \pm 10.04	61.50 \pm 3.08	287.10 \pm 14.36	73.25 \pm 3.66	139.60 \pm 6.98	348.41 \pm 17.42	340.69 \pm 17.03	416.30 \pm 20.82
RA		354.88 \pm 21.29	308.94 \pm 18.54	< LOD	< LOD	< LOD	< LOD	< LOD	2221.00 \pm 133.26
FA		292.13 \pm 17.53	122.20 \pm 6.73	17.73 \pm 1.06	272.25 \pm 16.34	< LOD	892.29 \pm 53.34	82.15 \pm 4.93	273.10 \pm 16.39
R		3266.63 \pm 261.33	< LOD	< LOD	15415.75 \pm 1233.26	97.40 \pm 7.79	19944.30 \pm 1595.54	30250.00 \pm 2420.00	< LOD
Qt		204.35 \pm 10.22	< LOD	< LOD	1174.00 \pm 58.70	< LOD	3123.00 \pm 156.15	3332.83 \pm 166.64	327.30 \pm 16.37

^a Each value is the mean \pm expanded measuring uncertainty ($k = 2$).

^b Quantified compounds: GA – gallic acid, CA – caffeic acid, CNA – *trans*-cinnamic acid, pQA – *p*-coumaric acid, CHA – chlorogenic acid, RA – rosmarinic acid, FA – ferulic acid, Qe – quercetin, R – rutin, Qt – quercitrin.

^c LOD – limit of detection.

Based on the results of α -amylase inhibitory activity (Fig. 4), it can be concluded that several analyzed samples (*Absinthii herba*, *Urticae folium*, *Betulae folium* and *Phaseoli legumen*) have shown dose-dependent increase in inhibitory effect of α -amylase. Furthermore, *Betulae folium* and *Phaseoli legumen* extracts exhibited the strongest ability of inhibiting α -amylase, at concentration of 8000 $\mu\text{g/mL}$. Also, α -amylase inhibitory activity (Amy8000) significantly correlated ($p < 0.05$) with the total flavonoid content ($r = -0.94$) and GA content ($r = -0.71$). The *Phaseoli legumen* extract showed an inhibition rate over 50% at concentration greatly higher (8000 $\mu\text{g/mL}$) than the IC_{50} value of positive control (acarbose, $\text{IC}_{50} = 4.9 \mu\text{g/mL}$). However, common bean is a medicinal plant, widely studied as *in vitro* and *in vivo* α -amylase inhibitor. Phaseolamin, an active compound of *P. vulgaris*, is a glycoprotein present in white kidney beans and known as non-competitive inhibitor of mammalian salivary and pancreatic α -amylase. Also, water extract of *P. vulgaris* (Phase 2®) is registered as a dietary supplement on the market (De Gouveia et al., 2014; Etxeberria et al., 2012).

The results of α -glucosidase inhibitory activity (Fig. 5) of the *Absinthii herba*, *Urticae folium*, *Mori nigrae folium*, *Betulae folium* and *Olive folium* extracts have showed concentration dependent increase in α -glucosidase inhibitory activity. Among all of the tested extracts, *Olive folium* extract exhibited the highest inhibitory effect on α -glucosidase (87.42% of inhibition) at concentration of 40 $\mu\text{g/mL}$, which is in agreement with previous studies (Othman et al., 2009). Inhibitory activity of the *Olive folium* extract was found to be especially significant when compared to the IC_{50} value of acarbose ($\text{IC}_{50} = 41 \mu\text{g/mL}$), while the previous studies identify oleoic acid and oleuropein as strong inhibitors of α -glucosidase (Etxeberria et al., 2012). *Phaseoli legumen* extract was found to be the most efficient inhibitor of α -glucosidase activity (46.79% of inhibition) at concentration of 80 $\mu\text{g/mL}$. The results were consistent with the results of the previously conducted studies (Mojica et al., 2015). The α -glucosidase inhibitory activity (Glu40) significantly negatively correlated ($p < 0.05$) with the CHA content ($r = -0.79$).

The α -glucosidase inhibitory activity of *Olive folium* and *Phaseoli legumen* extracts may be attributed to the high content of specific phenolic compounds, which supports previous studies where phenolics have been described as potent α -amylase and α -glucosidase inhibitors. Generally, phenolic compounds have been widely investigated as antidiabetic agents. Phenolic acids, such as CA, GA, CHA and RA are easily oxidized to quinones. The mechanism of action of these intermediary products could lay in the interaction with free thiol and amino nucleophilic groups of carbohydrates-hydrolyzing enzymes. The results of the previously conducted *in vivo* studies showed that some hydroxycinnamic derivatives, such as FA, effectively suppresses glycaemia by elevating glucokinase activity, increasing the levels of plasma insulin and production of glycogen in diabetic rats. Also, CHA, FA and CA are able to reduce bioavailability of carbohydrates by inhibiting intestine sodium-dependent glucose transporter (Bahadoran et al., 2013). The antidiabetic effect of CHA is mainly attributed to the stimulation of glucose transport *via* AMPK activation in skeletal muscles (Salehi et al., 2019). Furthermore, recent studies have revealed a relationship between hydroxyl groups amount in the flavonoid structure and inhibitory activity against α -amylase and α -glucosidase. Specifically,

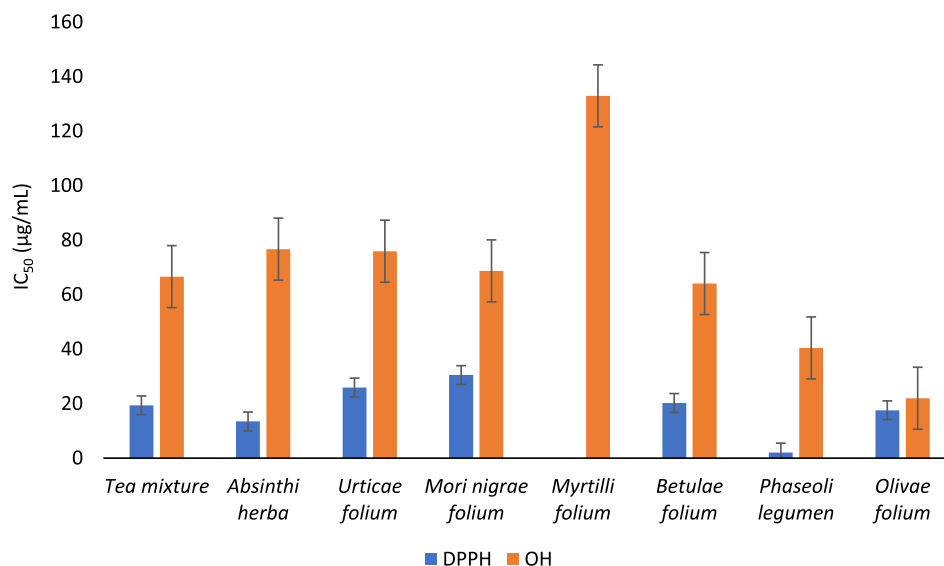


Fig. 2 The free radicals scavenging activity of tea mixture and its individual components against DPPH and OH radical.

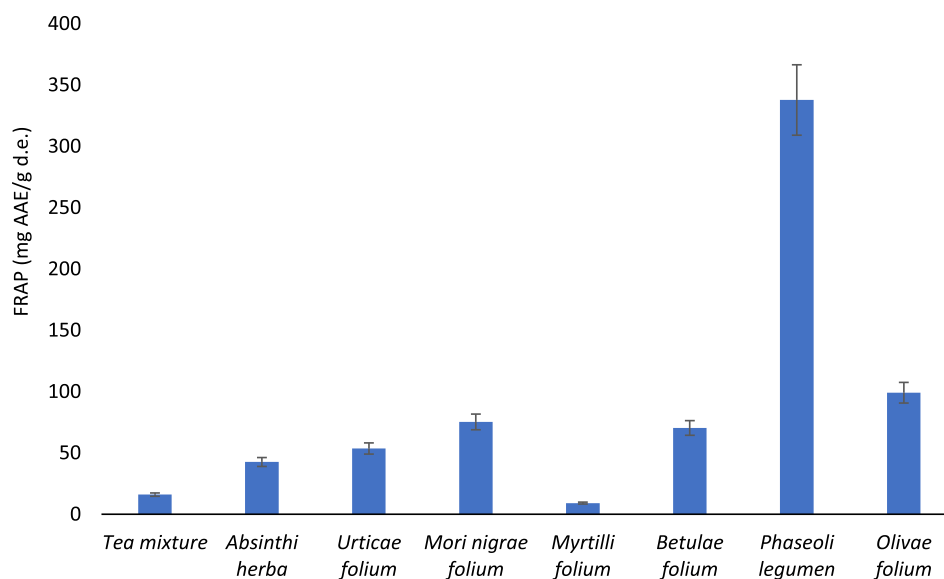


Fig. 3 FRAP values of the analyzed samples.

it was noticed that flavonoids containing more than three OH groups exhibited higher inhibitory activity against pancreatic α -amylase (De Souza Schmidt Gonçalves et al., 2010). Quercetin is a flavonoid of the flavonol class present in a variety of medicinal plants. It exhibits anti-inflammatory effect on pancreatic β -cells through the ability to inhibit aldose reductase and also enhances expression and translocation of insulin-independent GLUT4 transporter resulting in increased glucose uptake in myocytes. Also, quercetin has demonstrated strong radical scavenging activity against hydroxyl, superoxide, and peroxy radicals, the major free radicals in T2DM (Alam et al., 2014). Rutin (quercetin-3-O-rutinoside) is a widely distributed flavonoid glycoside among plants and has important pharmacological effects. It was found to alter the activities

of gluconeogenic and glycolytic enzymes and improves the translocation of GLUT 4 transporter (Salehi et al., 2019).

3.4. Chemometric evaluation of the tea mixture biological potential

The principal component analysis (PCA) was applied to evaluate the dataset describing antioxidant and antihyperglycemic activity of tea mixture and its individual components. The performed PCA indicates that the first two extracted components (PCA1 and PCA2) describe more than 90% of the samples' variability. The variability described by PCA1 mostly correlates with the results describing antioxidant and anti- α -glucosidase potential, while in the case of PCA2, those are

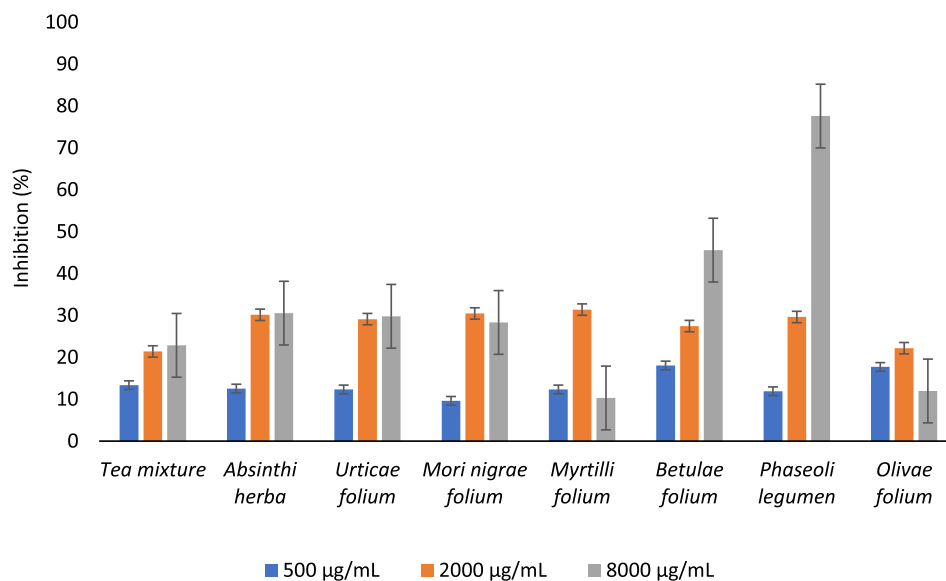


Fig. 4 The α -amylase inhibitory activity of the analyzed samples.

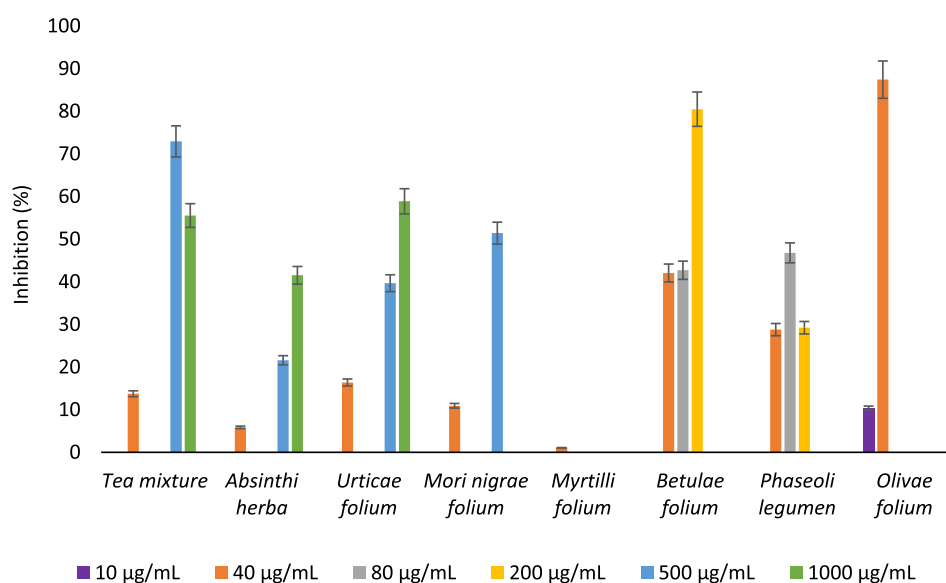


Fig. 5 The α -glucosidase inhibitory activity of the analyzed samples.

the variables describing antihyperglycemic potential (Fig. 6a). The position of the analyzed samples in the space defined by the first and the second principal component axes indicates on *Myrtille folium* sample located in the negative part of PCA1, based on a lower antioxidant and antihyperglycemic activity (Fig. 6b). On the other hand, the position of *Olivea folium* and *Phaseoli legumen* samples in the positive part of PCA1 suggests stronger antioxidant activity, while these samples further differentiate in the space defined by PCA2, based on the stronger anti- α -glucosidase and anti- α -amylase potential, respectively.

Additionally, the results of HCA (Fig. 7) were confirming the separative grouping of *Myrtille folium* extract as a separate cluster, based on modest antioxidant potential, while all of the

other samples belong to one large cluster, which further differentiates *Phaseoli legumen* and *Olivea folium* extracts as separate subclusters.

4. Conclusion

The performed study enabled evaluation of the potential health beneficial effects of tea mixture classified as food product, but also marketed as complementary herbal remedy in treatment of diabetes. Namely, it was observed that the high total phenolic content of specific components mostly contributes to the antioxidant potential (neutralization of DPPH and OH radical, FRAP value) of the examined tea mixture. Furthermore, if we consider the individual components found

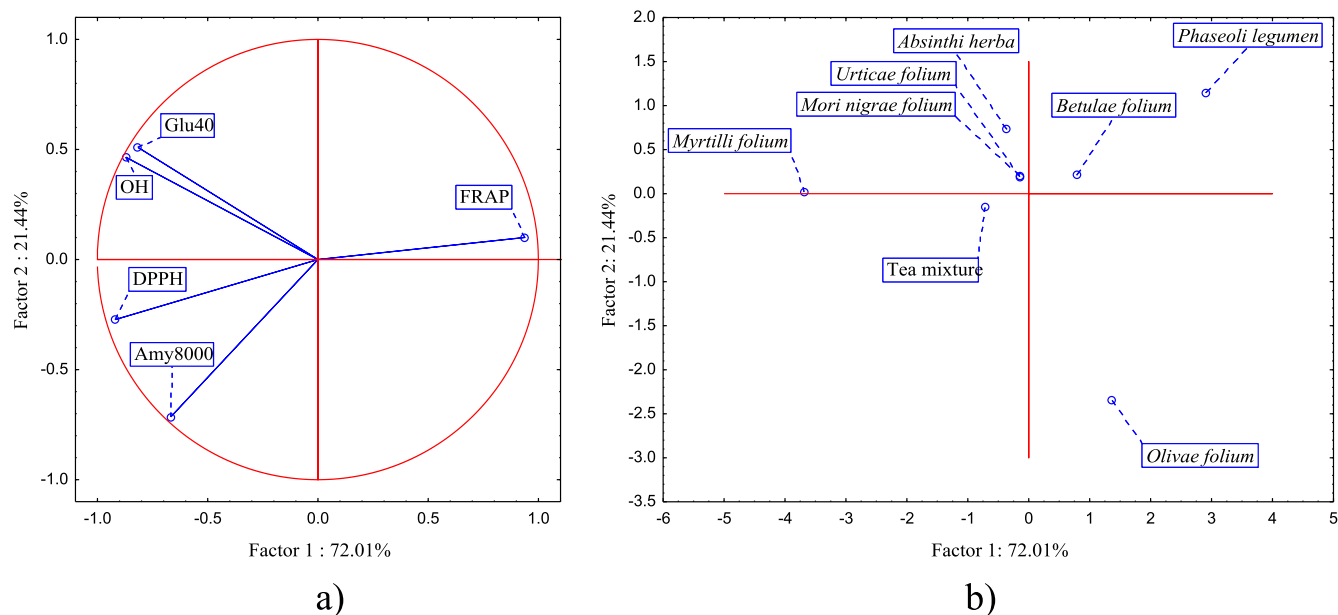


Fig. 6 PCA. (a) Loading plot of the first two principal components (PCA1 and PCA2) and (b) the position of the analyzed samples - cases in the space defined by the first two principal components.

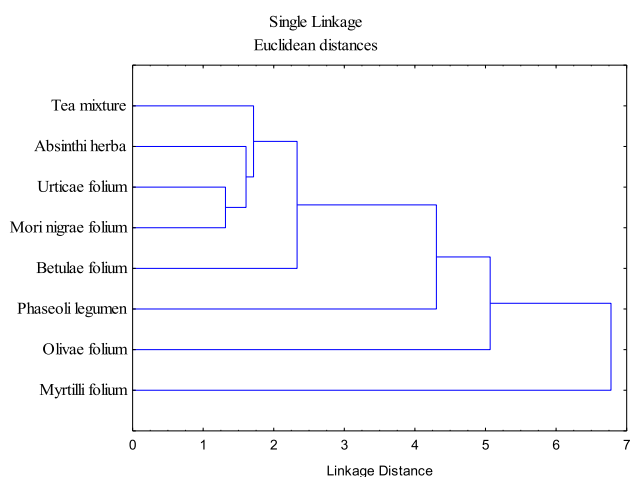


Fig. 7 Cluster diagrams of the analyzed samples based on antioxidant and antihyperglycemic activity.

in the mixture, the greatest anti- α -amylase potential is attributed to the *Phaseoli legumen*, while the highest potential to inhibit α -glucosidase was recorded for *Olivae folium*. Generally, screening of the tested compounds enabled us to conclude that a high level of correlation exists between anti- α -amylase potential and the quantified amounts of total phenolics, as well as gallic acid, while the chlorogenic acid is most probably responsible for the inhibition of α -glucosidase activity. The antihyperglycemic activity seems to be almost exclusively exerted by the *Phaseoli legumen* and *Olive folium* extracts, which further implicates an insignificant inhibition potency of the other herbal components. Therefore, the high total ratio (40%, m/m) of *Olivae folium* and *Phaseoli legumen* in the evaluated tea mixture can be stated as fully justified. However, future *in vivo*

research is required for further understanding of the antihyperglycemic activity mechanisms of secondary metabolites found in specified herbal components, as well as to evaluate their synergistic effect when applied in a form of tea mixture. This will allow us to make progress and further steps in potential development of functional food products and nutraceuticals.

Acknowledgements

This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (grant number: 451-03-68/2020-14/200114).

References

- Alam, M.M., Meerza, D., Naseem, I., 2014. Protective effect of quercetin on hyperglycemia, oxidative stress and DNA damage in alloxan induced type 2 diabetic mice. *Life Sci.* 109, 8–14. <https://doi.org/10.1016/j.lfs.2014.06.005>.
- American Diabetes Association, 2014. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 37, 81–90.
- Bahadoran, Z., Mirmiran, P., Azizi, F., 2013. Dietary polyphenols as potential nutraceuticals in management of diabetes: a review. *J. Diabetes Metab. Disord.* 12.
- Benzie, I., Strain, J., 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: The FRAP assay. *Anal. Biochem.* 239, 70–76. <https://doi.org/10.1006/abio.1996.0292>.
- Boateng, J., Verghese, M., Walker, L.T., Ogotu, S., 2008. Effect of processing on antioxidant contents in selected dry beans (*Phaseolus* spp. L.). *LWT - Food Sci. Technol.* 41, 1541–1547. <https://doi.org/10.1016/j.lwt.2007.11.025>.
- Božin, B., Kladar, N., Grujić, N., Anačkov, G., Samojlik, I., Gavarić, N., Čonić, B.S., 2013. Impact of origin and biological source on chemical composition, anticholinesterase and antioxidant properties of some St. John's wort species (*Hypericum* spp., Hypericaceae) from the central Balkans. *Molecules* 18, 11733–11750. <https://doi.org/10.3390/molecules181011733>.

- De Gouveia, N.M., Alves, F.V., Furtado, F.B., Scherer, D.L., Mundim, A.V., Espindola, F.S., 2014. An in vitro and in vivo study of the α -amylase activity of phaseolamin. *J. Med. Food* 17, 915–920. <https://doi.org/10.1089/jmf.2013.0044>.
- De Souza Schmidt Gonçalves, A.E., Lajolo, F.M., Genovese, M.I., 2010. Chemical composition and antioxidant/antidiabetic potential of Brazilian native fruits and commercial frozen pulps. *J. Agric. Food Chem.* 58, 4666–4674. <https://doi.org/10.1021/jf903875u>.
- Etxeberria, U., De La Garza, A.L., Campin, J., Martinez, J.A., Milagro, F.I., 2012. Antidiabetic effects of natural plant extracts via inhibition of carbohydrate hydrolysis enzymes with emphasis on pancreatic alpha amylase. *Expert Opin. Ther. Targets* 16, 269–297.
- Evans, J.L., Goldfine, I.D., Maddux, B.A., Grodsky, G.M., 2002. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. *Endocr. Rev.* 23, 599–622. <https://doi.org/10.1210/er.2001-0039>.
- Ferreira, I.C.F.R., Barros, L., Soares, M.E., Bastos, M.L., Pereira, J. A., 2007. Antioxidant activity and phenolic contents of *Olea europaea* L. leaves sprayed with different copper formulations. *Food Chem.* 103, 188–195.
- Goldsmith, C.D., Vuong, Q.V., Sadeqzadeh, E., Stathopoulos, C., Roach, P., Scarlett, C., 2015. Phytochemical properties and anti-proliferative activity of *Olea europaea* L. leaf extracts against pancreatic cancer cells. *Molecules* 20, 12992–13004. <https://doi.org/10.3390/molecules200712992>.
- Gutiérrez-Grijalva, E.P., Ambriz-Pérez, D.L., Leyva-López, N., Castillo-López, R.I., Heredia, J.B., 2016. Review: Dietary phenolic compounds, health benefits and bioaccessibility. *Arch. Latinoam. Nutr.* 66, 87–100.
- Heimler, D., Vignolini, P., Dini, M.G., Romani, A., 2005. Rapid tests to assess the antioxidant activity of *Phaseolus vulgaris* L. dry beans. *J. Agric. Food Chem.* 53, 3053–3056.
- Jovančević, M., Balijagić, J., Menković, N., Šavikin, K., Zdunić, G., Janković, T., Dekić-Ivanković, M., 2011. Analysis of phenolic compounds in wild populations of bilberry (*Vaccinium myrtillus* L.) from Montenegro. *J. Med. Plants Res.* 5, 910–914.
- Kahkonen, M.P., Hopia, A.I., Vuorela, H.J., Rauha, J.-P., Pihlaja, K., Kujala, T.S., Heinonen, M., 1999. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* 47, 3594–3962.
- Kawahito, S., Kitahata, H., Oshita, S., 2009. Problems associated with glucose toxicity: role of hyperglycemia-induced oxidative stress. *World J. Gastroenterol.* 15, 4137–4142.
- Kladar, N., Mrdanović, J., Anačkov, G., Šolajić, S., Gavarić, N., Srdenović, B., Božin, B., 2017. *Hypericum perforatum*: synthesis of active principles during flowering and fruitification - novel aspects of biological potential. *Evid.-Based Complement. Altern. Med.* 2017. <https://doi.org/10.1155/2017/2865610>.
- Kladar, N., Srdenović, B., Grujić, N., Bokić, B., Rat, M., Anačkov, G., Božin, B., 2015. Ecologically and ontogenetically induced variations in phenolic compounds and biological activities of *Hypericum maculatum* subsp. *maculatum*. *Hypericaceae*. *Brazilian J. Bot.* 38, 703–715. <https://doi.org/10.1007/s40415-015-0177-3>.
- Kostić, D.A., Dimitrijević, D.S., Mitić, S.S., Mitić, M.N., Stojanović, G.S., Živanović, A.V., 2013. Phenolic content and antioxidant activities of fruit extracts of *Morus nigra* L (Moraceae) from Southeast Serbia. *Trop. J. Pharm. Res.* 12, 105–110.
- Kőszegi, K., Békássy-Molnár, E., Koczka, N., Kerner, T., Stefanovits-Bányai, É., 2020. Changes in total polyphenol content and antioxidant capacity of stinging nettle (*Urtica dioica* L.) from spring to autumn. *Period. Polytech. Chem. Eng.* 64, 548–554. <https://doi.org/10.3311/ppch.14338>.
- Lee, Y.J., Thiruvengadam, M., Chung, I.M., Nagella, P., 2013. Polyphenol composition and antioxidant activity from the vegetable plant *Artemisia absinthium* L. *Aust. J. Crop Sci.* 7, 1921–1926.
- Lesjak, M.M., Beara, I.N., Orčić, D.Z., Anačkov, G.T., Balog, K.J., Francišković, M.M., Mimica-Dukić, N.M., 2011. *Juniperus sibirica* Burgsdorf. As a novel source of antioxidant and anti-inflammatory agents. *Food Chem.* 124, 850–856. <https://doi.org/10.1016/j.foodchem.2010.07.006>.
- Memon, A.A., Memon, N., Luthria, D.L., Bhangar, M.I., Pitafi, A.A., 2010. Phenolic acids profiling and antioxidant potential of mulberry (*Morus laevigata* W., *Morus nigra* L., *Morus alba* L.) Leaves and fruits grown in Pakistan. *Polish J. Food Nutr. Sci.* 60, 25–32.
- Mišan, A.Č., Mimica-Dukić, N.M., Mandić, A.I., Sakač, M.B., Milovanović, I.L., Sedej, I.J., 2011. Development of a rapid resolution HPLC method for the separation and determination of 17 phenolic compounds in crude plant extracts. *Cent. Eur. J. Chem.* 9, 133–142. <https://doi.org/10.2478/s11532-010-0126-8>.
- Mojica, L., Meyer, A., Berhow, M.A., de Mejía, E.G., 2015. Bean cultivars (*Phaseolus vulgaris* L.) have similar high antioxidant capacity, in vitro inhibition of α -amylase and α -glucosidase while diverse phenolic composition and concentration. *Food Res. Int.* 69, 38–48.
- Mun'im, A., Katrin, Azizahwati, Andriani, A., Mahmudah, K.F., Mashita, M., 2013. Screening of α -Glucosidase Inhibitory Activity of Some Indonesian Medicinal Plants. *Int. J. Med. Arom. Plants* 3, 144–150.
- Nikolova, M., Evstatieva, L., Nguyen, T.D., 2011. Screening of plant extracts for antioxidant properties. *Bot. serbica* 35, 43–48.
- Oboh, G., Isaac, A.T., Akinyemi, A.J., Ajani, R.A., 2014. Inhibition of key enzymes linked to type 2 diabetes and sodium nitroprusside induced lipid peroxidation in rats' pancreas by phenolic extracts of avocado pear leaves and fruit. *Int. J. Biomed. Sci.* 10, 210–218.
- Orhan, I.E., Belhatab, R., Şenol, F.S., Gülpınar, A.R., Hoşbaş, S., Kartal, M., 2010. Profiling of cholinesterase inhibitory and antioxidant activities of *Artemisia absinthium*, *A. herba-alba*, *A. fragrans*, *Marrubium vulgare*, *M. astranicum*, *Origanum vulgare* subsp. *glandulosum* and essential oil analysis of two *Artemisia* species. *Ind. Crops Prod.* 32, 566–571. <https://doi.org/10.1016/j.indcrop.2010.07.005>.
- Othman, A., Amer, M., Mogib, M., Samaha, R., 2009. Effects of the methanolic extracts of *Zizyphus spina christii*, *Olea europaea* and *Morus alba* leaves in Streptozotocin- induced diabetic rats. *Egypt. J. Hosp. Med.* 37, 759–771.
- Otles, S., Yalcin, B., 2012. Phenolic compounds analysis of root, stalk, and leaves of nettle. *Sci. World J.* 2012.
- Pourmorad, F., Hosseinimehr, S.J., Shahabimajd, N., 2006. Antioxidant activity, phenol and flavonoid contents of some selected Iranian medicinal plants. *African J. Biotechnol.* 5, 1142–1145.
- Rather, M.A., Bhat, B.A., Qurishi, M.A., 2013. Multicomponent phytotherapeutic approach gaining momentum: Is the “one drug to fit all” model breaking down? *Phytomedicine* 21, 1–14.
- Salaj, N., Kladar, N., Srdenović Čonić, B., Jeremić, K., Barjaktarović, J., Hitl, M., Gavarić, N., Božin, B., 2020. Stabilization of sunflower and olive oils with savory (*Satureja kitaibelii*, *Lamiaceae*). *J. Food Nutr. Res.* 59, 259–271.
- Salehi, B., Ata, A., Kumar, N.V.A., Sharopov, F., Ramírez-Alarcón, K., Ruiz-Ortega, A., Ayatollahi, S.A., Fokou, P.V.T., Kobarfard, F., Zakaria, Z.A., Iriti, M., Taheri, Y., Martorell, M., Sureda, A., Setzer, W.N., Durazzo, A., Lucarini, M., Santini, A., Capasso, R., Ostrander, E.A., Atta-ur-Rahman, Choudhary, M.I., Cho, W.C., Sharifi-Rad, J., 2019. Antidiabetic potential of medicinal plants and their active components. *Biomolecules* 9, 551. <https://doi.org/10.3390/biom9100551>.
- Shen, Y., Croft, K.D., Hodgson, J.M., Kyle, R., Lee, I.L.E., Wang, Y., Stocker, R., Ward, N.C., 2012. Quercetin and its metabolites improve vessel function by inducing eNOS activity via phosphorylation of AMPK. *Biochem. Pharmacol.* 84, 1036–1044.
- Shu-Huey, T., Wun-Chang, K., Feng-Nien, K., Che-Ming, T., 1991. Inhibition of platelet aggregation by some flavonoids. *Thromb. Res.* 64, 91–100.

- Sidaoui, F., Igueld, S.B., Yemmen, M., Mraih, F., Barth, D., Trabelsi-Ayadi, M., Cherif, J.K., 2016. Chemical and functional characterization of Tunisian *Artemisia absinthium* volatiles and non-volatile extracts obtained by supercritical fluid procedure. *Int. J. Pharm. Clin. Res.* 8, 1178–1185.
- Szentmihályi, K., Gere, A., Szöllösi-Varga, I., Blázovics, A., Jasztrab, S., Ladó, K., Then, M., 2010. Inhibition of lipid peroxidation of herbal extracts (obtained from plant drug mixtures of *Myrtilli folium*, *Phaseoli fructus sine seminibus* and *Salviae folium*) used in type 2 diabetes mellitus. *Acta Biol. Hung.* 61, 45–51.
- Zou, Y., Chang Sam, K., 2014. Antioxidant and antiproliferative properties of extract and fractions from small red bean (*Phaseolus vulgaris* L.). *J. Food Nutr.* 1, 1–11.