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original scientific paper

Blackthorn Flower Extract Impact on Glycaemic Homeostasis in Normoglycemic and Alloxan-Induced Hyperglycaemic C57BL/6 Mice

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SUMMARY

Research background. The use of plants and their extracts in treatments of chronic diseases is widely known in traditional medicine. The aim of this study is to determine the effects of 10-day consumption of Prunus spinosa L. flower extract on blood glucose, glycaemic load, serum α-amlyase and serum insulin, in normoglycaemic and hyperglycaemic (alloxan) mice model.

Experimental approach. Normoglycemic and hyperglycemic (alloxan treated, 150 mg/kg body mass) C57BL/6 mice were treated daily, during 10 days, with Prunus spinosa L. flower extract by gavage. The sugar content within extract was determined by HPLC analysis. In mice, blood and serum blood glucose level and OGTT-test were determined by blood glucometer. Serum insulin was determined by ELISA assay and α-amlyase by colourimetric assay.

Results and conclusions. The Prunus spinosa L. flower extract increased glucose in normoglycaemic mice by 30 % after 1st and 5th day and by 17 % after 10th day of consumption in normoglycaemic mice. It is a consequence of released sugars because sugar analysis revealed 59.8 mg/L monosaccharides, mainly fructose (55.7 mg/L) and glucose (24.3 mg/L) within the extract. On the opposite, the extract consumption, reduced serum blood glucose in alloxan-induced hyperglycaemic mice by 29 % after 10 days of treatment. Oral glucose tolerance test also confirmed that in the hyperglycaemic group treated with Prunus spinosa L. flower extract glucose

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homeostasis was improved and showed decrease in blood glucose, since the blood glucose over the period of 120 min, glucose homeostasis is faster achieved after treatment with shows that in Prunus spinosa L. flower extract. Serum insulin increased by 49 % and serum alpha amylase by 46 % after 10 days of treatment with Prunus spinosa L. flower extract in hyperglycaemic group. Thus, it can be concluded that Prunus spinosa L. flower extract improved glucose tolerance, enhanced insulin secretion and lowered serum α-amylase activity.

Novelty and scientific contribution. The results examined for the first time the potential of Prunus spinosa L. flower extract in hyperglycaemia management.

Key words: hyperglycaemia, Prunus spinosa L. flower extract, oral glucose tolerance test, insulin, α-amylase

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease that is characterized by chronic impaired blood glucose levels and hyperglycaemia as a result of compromised insulin secretion or impaired insulin action (1). Physiologically and biochemically, in various tissues, the chronic hyperglycaemia can induce oxidative stress that terminate normal biological activities and cause a cascade of diabetic chronic complications (2).

The usage of plants and their extracts in many treatment of chronic diseases is widely known in traditional medicine but also has a big potential for the treatment of hyperglycaemia and diabetic complications especially by reducing the levels of oxidative stress on a cellular cytoplasmic level (1, 3-6). Recent scientific data support the fact that polyphenols including flavonoids, phenolic acids, lignans and stilbenes found in most plants can have a positive effect on many chronic diseases, and act like potent antioxidants and anti-inflammatory agents (4,5,7,8)

Prunus spinosa L. presents the rich source of phytochemicals, polyphenols, including phenolic acids and flavonoids, A-type proanthocyanidins, anthocyanins, flavanols and flavones, flavan-3-ols and has many antiinflammatory, diuretic, blood purifying, spasmylytic and antitumor activities (3,9,10,11). All those plant phenolic compounds have potent antioxidant capacity (11) and there is evidence that they can regulate hyperglycemic levels, adipocytokine gene expression and enhance some metabolic activities (12). The hypoglycemic effects of medicinal herbs on hyperglycemia include physiological mechanisms such as enhancing peripheral tissue insulin sensitivity, inhibition of digestive enzymes involved in carbohydrate breakdown and inhibition of glucose absorption in gastrointestinal tract (13,14). The aim of this study was to determine the effect of 10 days consumption of Prunus spinosa L. flower extract on blood glucose, glycemic load and
glycemic parameters, serum α-amylase and serum insulin, in alloxan model of hyperglycemic mice. Despite traditional use of Prunus spinosa L. as medicinal herb, there are no studies that investigated effect of Prunus spinosa L. flower extract on glycemic homeostasis. Obtained results might serve as a guide toward the design of nutraceutical polyphenol mixture as supportive therapy in the hyperglycemia treatment.

MATERIALS AND METHODS

Study design in vivo: animals and diets

For this experiment a total of 24 male inbred C57BL/6 mice weighing 30 ± 1.5 g were obtained from the Department of Animal Physiology, Faculty of Science, University of Zagreb, Croatia. Animals had access to a standard laboratory diet and tap water ad libitum and received 12 h of light per day. The standardized diet was 4 RF 21, Mucedola (Settimo Milanese, Italy). The composition of the standardized pellet mouse feed included wheat, wheat straw, hazelnut skins, maize, soy bean, corn gluten feed, fishmeal, dicalcium phosphate, sodium chloride, whey powder, soy bean oil, yeast, and components and supplements: 12 % moisture, 18.5 % protein, 3 % fats, 6 % crude fibres, 7 % crude ash, E672 (vitamin A), E671 (vitamin E), E1 (Fe), E2 (I), E3 (Co), E4 (Cu), E5 (Mn), E6 (Zn). Maintenance and care of all experimental animals was performed according to the guidelines of the Republic of Croatia (15). The experimental procedures were approved by the Bioethics Committee of the Faculty of Science, University of Zagreb (16) and were conducted according to the Guidelines on in vivo experiments and accepted international standards (17).

The treatment of the animals and experimental design

Animals were randomly divided according to the treatment in four groups (Fig. 1). The first group named Control (C) is normoglycemic and untreated, receiving the phosphate buffer saline (PBS, Sigma-Aldrich Chemie GmbH, Germany) instead of the extract. The second group, Prunus spinosa L. flower extract (PSE) is a normoglycemic group treated with the extract. Furthermore, the third, hyperglycemic group, is the AL-alloxan treated. The fourth is the Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract (AL+PSE). Each group contained 6 animals in both the control and in the Prunus spinosa L. flower extract treatment groups. The experiment consisted of two connected segments regarding the blood glucose monitoring (Fig. 1). The first part of the experiment analysed the changes of the blood glucose, insulin and amylase levels after single Prunus spinosa L. flower extract dose (24h), 5 and 10 doses to determine the changes within those parameters over the period of 10 days. The second part of the experiment consisted of the glucose tolerance test (OGTT) on the same experimental days respectively, with the
aim to establish the dynamics of physiological blood glucose management after oral glucose challenge in the experimental groups over the time course of 120 min.

Fig 1. should be placed here.

Induction of experimental hyperglycemia with alloxan

To pharmacologically create chronic hyperglycemic in the animals within hyperglycemic group, the animals were treated with Alloxan (Sigma Chemical, St.Louis, USA). The hyperglycemia was induced by injecting the mice with alloxan (Sigma) at a dose of 150 mg/kg body mass intraperitoneally with syringe, five days prior to the beginning of the experimental treatments of groups with Prunus spinosa L. flower extract (Fig. 1). After 24 h following the alloxan injection, blood glucose level of mice were assessed and mice with levels >8.5 mmol/L, that stayed increased above that level over 5 days were considered to have hyperglycemia. After 5 consecutive days of hyperglycaemia (above the >8.5 mmol/L) mice within the hyperglycemia group were considered to be hyperglycemic and were included in the beginning of treatment experiment with P. spinosa flower extract, at the same time (day 0), together with normoglycemic groups (control-PBS saline and P. spinosa flower extract treated).

Treatment with Prunus spinosa L. flower extract (PSE)

The treatments (PBS for control and Prunus spinosa L. extract in the normoglycemic and hyperglycemic treatment groups), were administered as single daily oral gavage doses in a volume of 0.2 mL. The Prunus spinosa L. flower extract treated groups were dosed with 25 mg of total phenolics in extract solution per kg of body mass of the animal. The preparation, analytical details and chemical compositions of extracts determinated by UPLC MS/MS are published previously (18) where it was shown that the extract contained different types of polyphenols including phenolic acids, flavan-3-ols, flavones and especially flavonols (kaempferol and quercetin glycosides) together with quantities present in Prunus spinosa L. dry flower and the administered Prunus spinosa L. extract solution. Prior to animal administration of the desired doses, the original extract was evaporated under reduced pressure and temperature of 45 °C to concentrate the solution of polyphenols and to remove excess alcohol. This reduced the amount of alcohol to minimum to be safe for mice. Before application to the mice the concentrated solution was re-dissolved and further diluted with water to achieve the final dose concentration of 25 mg TP GAE/kg of mice. The dose of Prunus spinosa L. extract of 25 mg of total phenolics per kg bm of C57BL/6 mouse was derived from per 0.2 mL of gavage volume. In our previous study (19) we applied the same dose as in this study but in different regime (single dose, 24 h, plasma bioavailability) where the list of components and doses of individual polyphenolics in the extract consumed by animals as well as the analysis of phenolic content in food.
pellet (regular food that both groups consumed as normal chow), are given in detail (19). The polyphenolic content was assessed by UPLC-MS/MS analysis in commercial Mucedola food pellets since the control group was fed pellets ad libitum (average 3500-4000 mg pellet/mouse/day). The same types of polyphenols were determined in pellets, but the TP content (as the sum of all determined polyphenolic compounds) was in negligible concentrations (4.64 μg/100 mg dm of pellet). A dominant polyphenolic compound which comprised almost 54 % of TP content within the feed pellets was flavonol isorhamethin-rutinoside (2.50 μg/100 mg dm of pellet) while other compounds were determined in the range from 0.005 to 0.175 μg/100 mg dm of pellet or undetected.

**Determination of sugar content in Prunus spinosa L. flower extract (PSE)**

The sugars were simultaneously analysed by a direct injection of the extracts, previously filtered through a 0.45-µm pore size membrane filter (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Chromatographic separation was performed using HPLC analysis with Agilent 1260 quaternary LC Infinity system (Agilent Technologies, Santa Clara, CA, USA) equipped with refractive index detector (RI), an automatic injector and ChemStation software. The separation of sugars (fructose, glucose and sucrose) was performed on a Cosmosil-5C18, Sugar-D (250×4.6 mm i.d.) column (Naclai Tesque, Inc., Kyoto, Japan). The solvent composition and the used gradient conditions were described previously by Bogdanov and Baumann (1988) (23). For isocratic elution, mobile phase A contained 80 % acetonitrile in water. Operating conditions were: constant flow rate 1.3 mL/min in 20 min, column temperature 30 °C, injection volume was 10µL and equilibration time 2 min. Detection was performed with refractive index detector. Identification of sugars was carried out by comparing retention times of the authentic standards (fructose, glucose and sucrose). The quantifications of sugars were made by the external standard method. All sugar standards, were dissolved methanol at a concentration of 50 mg/L. Working sugar standards solutions were prepared by diluting the initial solution to yield five concentrations in a range from 1 to 50 mg/L. Quantitative determination was carried out using the calibration curves of the standards:

fructose: \( y=70684x + 4030 \), \( R^2=0.99; \) /1/

\[ y=72170x+6655.3, \quad R^2=0.99; \] /2/ and

\[ y=71630x+1199.3, \quad R^2=0.00 \] /3/

**Collection of blood samples from the animals**

Blood samples were collected from the tail vain into EDTA tubes that was used for insulin and amylase assay, while a drop of whole blood was used for blood glucose assay. The blood samples
for insulin and amylase assay, were mixed thoroughly to prevent blood clotting and were centrifuged at 2000xg for 10 min in Centrifuge Micro 200R (Hettich, Germany).

Determination of blood glucose levels in blood samples from the animals

The glucose levels in whole blood samples (a drop of whole blood) from the mice tail vein, was determined by blood glucometer that uses test strips to assess a glucose oxidoreductase mediated dye reaction, according to the instruction of the manufacturer (Medismart Sapphire blood glucose system, Lobeck Medical AG, Switzerland). The blood glucose levels were measured on the day of 1st, 5th and 10th and the same assay for glucose determination was applied in oral glucose tolerance test (OGTT) part of the experiment.

Determination of insulin levels in blood samples from the animals

Insulin was determined by ELISA, according to the manufacturer protocol Elabscience, Mouse INS (Insulin) Kit, USA (21). This ELISA kit used the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Mouse INS. Standards or samples were added to the micro ELISA plate wells combined with the specific antibody. Then a biotinylated detection antibody specific for Mouse INS and Avidin-Horseradish Proxidase (HRP) conjugate are added to each well. Only those wells that contained Mouse INS, biotinylated detection antibody and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution and the color turned yellow. The absorbance (A) was measured spectrophotometrically (Spectrophotometer Libra S22, Biochrom Ltd., United Kingdom) at a wavelength of 450±2nm.

Determination of α-amylase in blood samples from the animals

Amylase was determined by colorimetric assay, according to the manufacturer protocol Abcam Amylase assay kit (colorimetric), USA (ab 102523) (22). Amylase assay kit detects activity of α-amylase through a two step reaction. Alpha amylase cleaves the substrate ethylidiene-pNP-G7 to produce smaller fragments that are modified by α-glucosidase, causing the release of a chromophore that can be measured at absorbance A=405 nm. The assay can detect α-amylase content as low as 0.2 mU.

Oral glucose tolerance test (OGTT)

OGTT evaluates the ability to respond appropriately to a glucose challenge. The animals were fasted 6h before commencing the experiment. OGGT was performed by oral administration of glucose load of 2 mg/kg, in all groups. Blood samples were collected from the tail vein at 0, 15, 30, 60, 120
min after the oral glucose load. The glucose levels were measured using a blood glucose meter (Medismart Sapphire blood glucose system) according to the manufacturer instruction. Total glycemic response to OGTT were calculated from the areas under the curve (AUC). Baseline blood glucose concentrations were taken before the application of glucose and baseline measurements were done to establish glucose dynamics in untreated mice in order to evaluate the difference of hyperglycemic and normoglycemic groups. Day 0 measurement was conducted immediately after administrating the *Prunus spinosa* L. flower extract solution to establish the potential of the extract to modulate blood glucose and day 1 measurement was done. After the day 0 glucose challenge the following measurements are made at the same intervals, 0, 15, 30, 60 and 120 min.

**Statistical analysis**

All the data were expressed as mean values ± standard deviation (SD). SPSS statistic version 17 and GraphPad Prism were used for visualization of data (Figures) and statistical comparisons between groups by Kruskal Vallis (ANOVA) (23, 24).

**RESULTS AND DISCUSSION**

The *Prunus spinosa* L. flower extract is a rich source of polyphenols, with the most abundant identified by UPLC MS/MS profiling, quercetin and kaemferol glycosides, represented by kaempferol-pentoside and rhamnoside and quercetin-pentoside and also phenolic compounds belonging to the classes of hydroxycinnamic acids, and flavonol glycosides (18).

The *Prunus spinosa* L. flower extract contained significant amount of sugar (Fig. 2), accounting for the total of 59.8 mg/L. The analysis of individual sugar types within the extract revealed that the most dominant sugar in *Prunus spinosa* L. flower extract was the fructose with 55.7 % (33.3 mg/L) of the total concentration of sugar content. It was followed by glucose 40.3 % (24.3 mg/L) and low ratio of sucrose with only 3.8 % (2.3 mg/L) of total sugar content. Therefore, the *Prunus spinosa* L. flower extract carbohydrate contained 96 % monosaccharides and only 3.8 % disaccharides.

The results in Fig. 3. show serum blood glucose levels in the experimental groups of C57BL/6 mice and the effects of *Prunus spinosa* L. flower extract intake on blood glucose in normoglycemic and hyperglycemic (alloxan) mice after a single (1st day) or five (5th day) and ten (10th day) repeated doses. The *Prunus spinosa* L. flower extract intake resulted in slightly but significantly (p ≤0.05) increased blood glucose in normoglycemic treated mice (*Prunus spinosa* L. flower extract group) compared to the untreated control mice (C-group, control) but only on the 1st (Fig. 3.a) and 5th (Fig. 3.b) experimental day. At the end of the experiment, (Fig. 3.b) and ten received doses, despite the
Prunus spinosa L. flower extract treatment, the blood glucose levels equalized with control (Control-group levels) where no significant differences between the groups were found (Fig. 3.c).

When the Prunus spinosa L. flower extract was administered to the hyperglycemic mice, there was a significant (p ≤0.05) reduction of blood sugar concentration (compared to AL-alloxan treated hyperglycemic group) after five (Fig. 3.b) and ten (Fig. 3.c) received Prunus spinosa L. flower extract doses (5th and 10th experimental day, Fig. 3.b and 3.c, respectively). This signifies that consumption of Prunus spinosa L. flower extract in Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract showed improvement in hyperglycemia lowering after at least 5 or 10 repeated daily consumptions of Prunus spinosa L. flower extract. Interestingly, the single dose (1st experimental day, Fig. 3.a) was not sufficient to reduce the hyperglycemia significantly (comparison of Alloxan challenged group and Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract group).

The oral glucose tolerance test (OGTT) in Fig. 4, shows the 2 h (120 min) dynamics of blood glucose adaptation. The blood glucose levels are shown as the pharmacokinetic (PK) curves (Fig. 4. a-e) in all treatment groups after consumption of glucose solution. The effects of simultaneous Prunus spinosa L. flower extract intake in normoglycemic-glucose challenged (Control and Prunus spinosa L. flower extract group) and hyperglycemic-glucose challenged mice Alloxan and Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract are shown separately on different experimental days. The baseline blood glucose concentration without glucose treatment in all groups, but with a single Prunus spinosa L. flower extract dose in normoglycemic and hyperglycemic groups (Fig. 4. a and f), treatment groups challenged with oral glucose ingestion prior to Prunus spinosa L. flower extract treatment (Fig.4 b and g) and receiving a single (1st day, 24h after Prunus spinosa L. flower extract intake (Fig 4. c and h) or five (5 consecutive Prunus spinosa L. flower extract intake, (Fig.4 d and i) and ten (10 consecutive Prunus spinosa L. flower extract intake, (Fig 4., e and j) repeated Prunus spinosa L. flower extract doses (5th and 10th day, respectively). It could be noticed (Fig. 4. a-e) that combined treatment Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract improves glucose homeostasis and shows decrease in blood glucose level as compared to alloxan group. The pharmacokinetic (PK) curves on (Fig. 4. a-e) are analysed for differences on their corresponding areas under the curves (AUC, Fig. 4. f-j). For analysis simplicity, statistical significance was calculated only for AUC values (Fig. 4, f-j), which indicate whole glucose excursion after glucose loading, over a period of 120 min for each respected group. There was a significant difference between hyperglycemic group treated with Prunus spinosa L. flower extract compared to Alloxan treated hyperglycemic group (p=0.0002) for day 0 (Fig. 4. g) and day 5th (Fig. 4.i). There was a significant difference between Prunus spinosa L. flower extract and Control group
for baseline treatment (p=0.0405) and days 1\textsuperscript{st} (Fig. 4.h) (p=0.0007) and 10\textsuperscript{th} (Fig. 4.j) (p=0.004). Cumulatively the blood glucose level over the period of 120 min seen from AUC graphs, shows that in \textit{Prunus spinosa} L. flower extract group, glucose homeostasis is achieved faster, even after 10 days of treatment.

\textbf{Fig 4. should be placed here.}

The results in Fig. 5 (a-c) show levels of insulin and alpha amylase in serum of normoglycemic (Control) and hyperglycemic (Alloxan) mice experimental groups and the effects of \textit{Prunus spinosa} L. flower extract intake (in normoglycemic \textit{Prunus spinosa} L. flower extract group and hyperglycemic group treated with \textit{Prunus spinosa} L. flower extract) after single (1\textsuperscript{st} day) or five (5\textsuperscript{th} day) and ten (10\textsuperscript{th} day) repeated doses. The \textit{Prunus spinosa} L. flower extract intake did not affect amylase activity until the 5\textsuperscript{th} experimental day but resulted in significantly lower serum α-amylase activity (p<0.0001) in \textit{Prunus spinosa} L. flower extract group compared to control group until the 10\textsuperscript{th} (Fig. 5.c) experimental day. This means that 10 repeated doses of daily consumption of \textit{Prunus spinosa} L. flower extract had the potential to inhibit amylase activity. Alloxan significantly increased the α-amylase in hyperglycemic group compared to normoglycemic control group. However, when the \textit{Prunus spinosa} L. flower extract was administrated to the hyperglycemic mice there was a significant reduction of α-amylase activity (p<0.0001) compared to Alloxan group on the 1\textsuperscript{st} (Fig. 5.a) and 10\textsuperscript{th} (Fig. 5.c) experimental days. The results in Fig. 5 (d-f) show levels of blood insulin. When the \textit{Prunus spinosa} L. flower extract was administrated to normoglycemic mice the insulin level was significantly higher (p=0.0017) compared to the normoglycemic control, on day 5. Alloxan group has a significantly lower insulin levels compared to control group for 1\textsuperscript{st} (p=0.0004) (Fig. 5.d) and 10\textsuperscript{th} (p=0.013) (Fig. 5.f) experimental days. However, when the \textit{Prunus spinosa} L. flower extract was administrated to hyperglycemic mice there was a significant rise of serum insulin compared to alloxan group for 1\textsuperscript{st} (Fig. 5.d) (p=0.0005) and 10\textsuperscript{th} (Fig. 5.f) (p=0.018) experimental days.

\textbf{Fig 5. should be placed here.}

The consumption of \textit{Prunus spinosa} L. flower extract, both short term (in min, shown with OGTT) or repeatedly in long term (during 10 days) can modulate blood glucose levels and in long term might influence the α-amylase and insulin levels.

When taken under normoglycemic conditions (healthy non-hyperglycemic individuals) the extract shows the ability to slightly but significantly raise blood sugar concentration, as shown in the results for \textit{Prunus spinosa} L. flower extract group compared to the control group (Fig. 4.). In available literature there are examples that certain plant extracts shows similar properties. For example, Tourkey (25) reported significantly higher level of glucose in mice treated with \textit{Moringa oleifera} aqaeus extract. The observed short term glucose elevation in normoglycemia could be explained
by significant presence of sugars in Prunus spinosa L. flower extract. On the other hand, relative normalization of blood glucose balance in hyperglycemia could be attributed to richness in polyphenol molecules and contributed to polyphenolic content in Prunus spinosa L. flower extract. Flavonoids are characterized by their basic skeleton arranged in the form C6-C3-C6, two aromatic rings A and B linked by a unit of three carbon atoms. Hydroxyl group and sugars are very common flavonoid skeleton substituents, and they increase the water solubility of flavonoids. Flavonoids exist naturally as glycosides (26, 27). The sugar content analysis showed that Prunus spinosa L. flower extract contains a significant amount of simple sugars, monosaccharides up to approximately 97 % of total sugars. Nevertheless, the results shows that in Prunus spinosa L. flower extract treated groups on 1st and 5th day glucose levels tend to be slightly higher than in the control group although never reaching pathologic hyperglycemia as in alloxan treated group. On metabolic and physiologic point of view nutritional characteristic of food and beverages that are rich in available carbohydrates is that their consumption induce postprandial hyperglycemia. The liberated glucose is absorbed within the intestinal enterocytes by the specific transporters (27). The fast absorption of glucose activates the regulatory mechanisms of glucose homeostasis. Besides free sugars present in the Prunus spinosa L. flower extract (Fig. 2), some of the sugars which covalently bind to phenolic compounds might be liberated during digestion and absorption, which overall led to the rise in blood serum glucose level. It was observed that despite the slight increase of blood glucose taken under normal metabolic conditions, the Prunus spinosa L. flower extract in hyperglycemic mice promotes faster recovery and improved glucose homeostasis, as shown in Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract groups compared to Alloxan groups Furman et al. (28) gave a review on medicinal plants extract that express similar acute hypoglycemic effect in diabetic rodents, for example Pycnanthus angolensis (rich in terpenoids), Phyllanthus sellowianus (rich in flavonoids), Gentiana olivieri (rich in flavons), and Cinnamonum zeylanicum (rich in cinnamaldehyde). Most researchers used polyphenol rich-plant extract along with other bioactive substances to adress their numerous antidiabetic mechanisms, which may include lowering blood glucose, inhibition of polysaccharide digestion, inhibition of hepatic gluconeogenesis, stimulation of peripheral glucose uptake, modulation of intestinal microbiome, antioxidant effect, and stimulation of GLP-1 secretion (4, 28, 29). Such protective effects are usually prescribed to the richness of phenolic molecules in plant extracts. It is known that flowers of blackthorn contains complex of flavonoid, derivates of flavonol: kaempferol, quercetin and their glycosides bound with arabinose, rhamnose, xylose (30). In Prunus spinosa L. flower extract, individual polyphenols like kaempferol, quercetin are glycosylated (10). Olsewska et al (10) showed compounds such as kaempferol 3-O-α-L-arabinofuranoside, kaempferol 3-O-(2”-E-p-coumaroyl)-α-L-arabinofuranoside, kaempferol 3-O-β-D-xylopyranoside, kaempferol 7-O-α-L-rhamnopyranoside and kaempferol 3-O-α-L-rhamnopyranoside. In the Prunus
spinosa L. flower extract that we have used in this work the composition and concentrations of glycosylated polyphenols is also known for 28 polyphenolic compounds analysed by UPLC-MS/MS (19). Flavonol glycosides were shown to be the major polyphenolic class in blackthorn flowers, especially quercetin and kaempferol as kaempferol rhamnosyl-hexoside, quercetin-pentosyl-hexoside, kaempferol-pentosylhexoside kaempferol-pentoside, quercetin-rhamnoside, kaempferol-rhamnosid, quercetin acetylhexoside, kaempferol acetylhexoside (19). In previous experiment with single dose, we have shown that a significant number of those glycosides can be traced by UPLC-MS/MS method in plasma of mice (19) and in organs such as intestine, liver and kidney (11).

Beside the longterm effect of Prunus spinosa L. flower extract on blood glucose it is very important to know the short-term dynamic of adaptation of blood glucose after consumption of glucose solution. The oral glucose tolerance test (OGTT) was used here to evaluate blood glucose homeostasis, intake of blood glucose inside tissues and also indirectly evaluate glucose absorption in time of 2 h (120 min). Fig. 4 shows the blood glucose levels during 120 min. The OGTT evaluates the ability to respond appropriately to a glucose change. For the OGTT, mice are typically fasted overnight, which provokes a catabolic state of metabolism, and reduction of liver glycogen stores. The longer the fast lasts, it decreases the metabolic rate and enhances glucose usage in mice, which is in contrast to humans. As the feeding patterns in mice also do not mimic human behaviour, it may be thus more physiological to perform an OGTT after a short fast (31). Our results suggest that increased levels of glucose tolerance may be due to increased secretion of insulin, and therefore indicates that the Prunus spinosa L. flower extract possesses a hypoglycaemic effect.

In long term hyperglycemia regulation, Prunus spinosa L. flower extract supplementation in diabetic mice decreased fasting blood glucose level and postprandial glucose tolerance which showed improvement exhibiting similar pattern as normal mice with peak increase at 30 min. Results in vivo indicate that Prunus spinosa L. flower extract displayed a good concentration-dependent inhibitory effect on serum alpha amylase activity. Inhibition of alpha amylase activities has been demonstrated with polyphenols from plants in many researches (4, 5, 14, 32). The elevated insulin secreting in hyperglycemic group treated with Prunus spinosa L. flower extract agrees with in vivo results, shown in Fig. 5, and strengthening the evidence that the Prunus spinosa L. flower extract acts as a stimulator of insulin secretion.

Some studies suggested that polyphenols from Prunus spinosa L. flower extract reduce hyperglycemia through various mechanisms. Polyphenols from plants can inhibit alpha amylase and alpha glucosidase activity, stimulate insulin secretion from the β cells, balance glucose release from the liver and promote glucose uptake in the insulin sensitive tissue (29). Dong Kwon Yang et Hyung-Sub Kang (33) reported that combined treatment of quercetin and resveratrol in streptozotocin induced diabetic rats maintained the activities of hepatic glucose metabolic enzymes and structure
of pancreatic β-cells, significantly decreased glucose levels. Consumption of tea (Camellia sinesis L.) and coffee (Coffea Arabica L.) has been related to a lower risk of hyperglycemia by improvement of glucose tolerance, insulin sensitivity and insulin secretion, reduction of glucose intestinal uptake and regulation of glycemic homeostasis (4). Furman et al. (28) reported the antihyperglycemic effect of caffeic acid in diabetic mice by reduction of blood glucose and increased plasma insulin. Chukwuma et al. (5) reported that combined acute treatment in vivo of chlorogenic and caffeic acid have glucose tolerance improvement effect as well as plant extracts which contained equal amounts of both phenolic acids, suggesting a synergistic influence of phenolic acids. Previous studies on the other plants used the similar therapeutic approaches for lowering blood glucose and enhancing pancreatic β-cell function, peripheral tissue insulin sensitivity and inhibition of digestive enzymes involved in carbohydrate metabolism.

Our results confirm that the Prunus spinosa L. flower extract has similar antihyperglycemic effects as described by Ben Salem et al. (6) which suggest that ethanol extract of Cynara scolymus significantly decreased (p<0,001) the α-amylase levels in serum of diabetic rats, reduced blood glucose rate in the treated group compared to diabetic rats after 28 day of treatment. Similar results were obtained by Tang et. al (34) which have shown that polyphenols from Punica granatum L. flower improved insulin sensitivity on diabetic rats, approved by OGTT and ITT insulin tolerance tests.

Varshney et al. (35) have shown in vivo that tested flavonoids quercetin, rutin myricetin and kaempferol (all applied in doses 25 mg/kg bm/day) significantly enhanced insulin sensitivity in streptozotocin induced diabetic mice by a decline in glucose level with increasing time. Kaempferol gave the greatest effects on improving blood glucose level, glucose tolerance and insulin sensitivity. Alkhalidy (36) reported that after 4 weeks of oral administration of kaempferol reduced fasting blood glucose levels reduced and that improved insulin sensitivity in diet induced obese C57BL/6 mice.

CONCLUSIONS

The obtained results show that consumption of Prunus spinosa L. flower extract, both short term (in min, OGTT) or repeatedly in long term (during 10 days) has the ability to slightly but significantly raise blood sugar concentration in metabolically healthy (normoglycemic) mice. The conclusion of this study is that the daily 10 days intake of Prunus spinosa L. flower extract has a potential protective effect on hyperglycaemia in C57BL/6 mice by lowering blood glucose, improving glucose tolerance, enhancing insulin secretion and inhibiting serum α-amylase activity. Based on the current investigations Prunus spinosa L. flower extract may be a useful support in hyperglycaemia management and also its complications.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHORS’ CONTRIBUTION

I. Crnić participated in designing and performing experiments, processing and interpreting data, and preparation of manuscripts. T. Frančić, P. Dragičević and V. Balta participated in performing experiments and processing data. V. Dragović-Uzelac participated in interpreting data. D. Đikić and I. Landeka Jurčević took part in designing and performing experiments, interpreting data, preparation of manuscript, writing and revising the manuscript, and the final approval of the version to be published.

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Fig.1. The experimental design. The experiment consisted of two segments: Segment I. glucose, insulin and amylase measurement on the 1\textsuperscript{st}, 5\textsuperscript{th} and 10\textsuperscript{th} day of treatment with \textit{Prunus spinosa} L. flower extract and the Segment II. the glucose challenge of treatment groups (oral glucose tolerance test-OGTT test) and determination of glucose levels within 120 min after glucose and extract intake on the 1\textsuperscript{st}, 5\textsuperscript{th} and 10\textsuperscript{th} experimental days. The baseline group in OGTT test portrays the glucose dynamics within 20 min in experimental groups without the glucose intake and day 0 is the glucose challenge in experimental groups prior to treatment with \textit{Prunus spinosa} L. flower extract. Day 1\textsuperscript{st} is glucose challenge 24 h after \textit{Prunus spinosa} L. flower extract treatment (single dose) and day 5\textsuperscript{th} and 10\textsuperscript{th} are the glucose challenge (OGTT) after 5 and 10 repeated \textit{Prunus spinosa} L. flower extract doses.
Fig. 2. Sugar content analysis in *Prunus spinosa* L. flower extract
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C - Control  
PSE - Prunus spinosa L. flower extract  
AL - Alloxan  
AL+PSE - Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract
Fig. 3. Serum blood glucose levels in experimental groups of C57BL/6 mice measured on the a) the 1st experimental day, after single dose b) on the 5th experimental day, after five daily doses and c) on the 10th experimental day, after ten consecutive daily repeated doses of Prunus spinosa L. flower extract (PSE) intake. The values are means ± SD. (n=6 for each group). C-Control, PSE-Prunus spinosa L. flower extract, AL-Alloxan, AL+PSE- Alloxan challenged hyperglycemic group treated with Prunus spinosa L. flower extract. *, **, *** The statistically significant differences (p ≤0.05) between the different groups is shown with lines above the columns.
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Fig. 4. Oral glucose tolerance test (OGTT) in experimental groups of C57BL/6 mice challenged with single oral glucose intake and daily repeated doses of *Prunus spinosa* L. flower extract (PSE) treatment. The blood glucose concentration within 2 h (120 min). a-e) represents the pharmacokinetic (PK) curves of blood glucose in treatment groups and f-j) shows the area under the curves (AUC) of associated PK curves within the same row. The values are means ± SD. (n=6 for each group). C-Control, PSE-*Prunus spinosa* L. extract, AL-Alloxan, AL+PSE-combined treatment with Alloxan and PSE. *, **, ***The statistically significant differences (p ≤0.05) between the different groups are shown with lines above the columns.
Fig. 5. Levels of alpha amylase in serum in C57BL/6 mice a-e a) day 1st b) day 5th c) day 10th. Levels of insulin in serum in C57BL/6 mice d-f d) day 1st e) day 5th f) day 10th. Daily repeated doses of Prunus spinosa L. flower extract (PSE) intake. The values are means ± SD. (n=6 for each group). C-Control, PSE-Prunus spinosa L. flower extract, AL-Alloxan, AL+PSE-combined treatment with Alloxan and...
PSE. *, **, ***The statistically significant differences ($p \leq 0.05$) between the different groups is shown with lines above the columns.