

Anti-diabetes and neuroprotection potential and primary safety studies of *Isatis tinctoria* L. hydroalcoholic leaf extract

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Abstract

Objectives

Natural plant raw materials, previously underestimated in therapeutics, are becoming the subject of research for new applications in medicine. In our research, the hydroalcoholic extract of *Isatis tinctoria* leaf, rich in flavonoid compounds such as vicianin-2 and quercetin, was examined as a potential antidiabetic and neuroprotective agent.

Methods

The effect of the extract and its main flavonoid compounds on protein glycation, alpha-glucosidase activity, and acetylcholinesterase activity was tested. *In vitro*, in the mouse hippocampal neuronal cell line and *in vivo*, using a mouse model, the safety of the extract was screened for.

Key findings:

Our experiments demonstrated significant inhibition of protein glycation, alpha-glucosidase activity, and acetylcholinesterase activity by the extract, in a concentration dependent manner. The extract had a strong reducing effect, did not exhibit cytotoxicity up to a concentration of 25 mg/mL. Intraperitoneal administration of the extract to mice did not have negative effects on body mass, locomotor activity, coordination, and liver cell integrity.

Conclusions

Our research sheds a new light on this raw material and deepens knowledge of its activity. This may result in the recognition of its therapeutic effects and even in its introduction in the modern treatment of diseases characterized by pathological changes associated with hyperglycemia, oxidation, and inflammation.

1. Introduction

Diabetes mellitus (DM) is an endocrine disease characterized by dysfunctional metabolism of lipids and carbohydrates by insulin, a pancreatic hormone, leading to an increase in blood glucose levels [1]. DM is a global health crisis that deeply affects the personal, social, and physical well-being of the population. Current therapeutic strategies for glycaemic control include insulin and its analogues, sulphonylureas, biguanides, thiazolidinediones, and α -glucosidase inhibitors [2]. Glycation inhibitors are a potential drug class for the management of disorders caused by hyperglycemia, however, to date, none of the tested compounds (aminoguanidine was the closest to success) have undergone clinical trials [3].

Emerging evidence highlights the strong link between DM and Alzheimer's disease (AD) and is lately often referred to as "type 3 diabetes", because of the crucial role of insulin in cognitive function and neurodegenerative processes involving memory elaboration and neuronal integrity [4]. The connection between the two pathologies is further complicated by inflammation, oxidative stress, and glycation, which are exacerbated in DM, in addition to significantly contributing to the development of AD [5].

Plant-derived drugs with antioxidant, anti-inflammatory and antibacterial properties have gained attention due to their numerous advantages, such as low toxicity, mild side effects, as well as multiple pharmacological targets [6]. Therefore, the search for natural products or phytochemicals is growing, with the possibility that they may become acceptable alternatives or complementary agents to existing pharmacological treatment [7].

In view of this, *Isatis tinctoria* L. (IT) is a plant of great interest as it exhibits various beneficial properties closely related to its phytochemical profile. It is well known for its use as a Chinese herb in the treatment of inflammatory diseases, viral and bacterial infections in Traditional Chinese Medicine [8]. *Isatis tinctoria* was recognized as a pharmacopoeial plant and introduced into official European phytomedicine in 2011 [9]. In the pharmacopoeial monograph, *Isatis indigotica* was listed as another name for *Isatis tinctoria*. This record was maintained until the current 10th edition of the European Pharmacopeia [10]. When describing *Isatidis radix* (BǎnLánGēn) and *Isatidis folium* (DàQīngYè), many of the researchers following the Chinese pharmacopoeia claim that these raw materials originate from *I. indigotica* [11, 12]. Nevertheless, some new works consider them to be two different species [13]. Despite considering them as separate species, the European Pharmacopeia deems them to be equivalent for the purposes of obtaining the raw material. The European monograph concerns *Isatidis radix*. However, *Isatidis folium* is not included, which necessitates research into the biological activity of the leaf extract in animal models and into the safety of its use.

Recently, the increasing demand for the use of IT for its medicinal properties has driven a renewed interest in the promising biological activities ascribed to its bioactive compounds, which include alkaloids, phenolic compounds, and glucosinolates [14]. As a result, in the last decade, extracts obtained from IT leaves and roots have been the subject of numerous phytochemical studies and investigations into their therapeutic potential in animal and human diseases [15]. Studies have mainly focused on analysing the lipophilic extract of IT and evaluating its strong anticancer and anti-inflammatory properties in various animal models [16]. Isolated compounds such as the alkaloids tryptanthrin, indolinone, and indirubin have been widely examined for their beneficial health effects [17–20].

However, to date, few investigations have been carried out on the biological activity of the polar constituents of this species [15, 21–24]. The phenolic compounds of the hydroalcoholic extract (MeOH 70) of IT leaves were first characterized by Taviano et al., and next Miceli et al., revealed a strong correlation between antioxidant activity and flavonoids and phenolic acids [22]. Among the flavonoids identified in the said extract, vicenin-2 was the most abundant flavone, followed by isovitexin and apigenin glucosides, whereas quercetin was the most abundant flavonol. The polar extract showed good

radical scavenging and ferrous ion chelating activity, as well as moderate reduction power, ascribed to the high concentration of flavonoids [21]. Interestingly, vicenin-2, also known as 6,8-di-C-glucoside of apigenin, has been reported to possess a wide variety of pharmacological activity including anti-cancer, anti-inflammatory, antioxidant, and hepatoprotective properties [25]. An *in vivo* study, conducted in a carrageen-induced rat paw oedema model, demonstrated that vicenin-2 blocks the production of inflammatory mediators such as tumor necrosis factor- α (TNF- α) and nitric oxide (NO) through the inhibition of nuclear factor-kB (NF-kB) [26]. In addition, interesting findings come from a study focusing on the antidiabetic potential of vicenin-2 as a result of the strong inhibition of α -glucosidase, aldose reductase, and protein tyrosine phosphatase 1B. These three enzymes have become important therapeutic targets in the treatment of diabetes mellitus [25]. Furthermore, a notably increasing number of studies highlight the therapeutic potential of apigenin, a natural flavone, in the management of neurodegenerative disorders. A recent experiment conducted in a rat model of AD, revealed the ability of apigenin to inhibit glycogen synthase kinase-3 beta, a molecule that plays a key role in the generation of amyloid beta plaques and neurofibrillary tangles, leading to neuroprotective effects [27].

Little information is available on the historical use of IT in European countries since its introduction to Europe from China and Japan in ancient times is only described by Hamburger [28]. The raw material used in the experiments in this work comes from natural sites in Italy and has been identified as *Isatis tinctoria*. In view of the absence of information on the action and activity of the European raw material, such studies are most needed and recommended.

In our previous article, we showed that a single administration of IT hydroalcoholic leaf extract reverses anxiety-like behavior caused by the acute restraint stress paradigm through a mechanism partially dependent on the modulation of neuroinflammation, oxidative stress, and NO reduction [24]. In this manuscript, taking into account that IT hydroalcoholic leaf extract has high concentrations of vicenin-2 (6,8-di-C-glucoside of apigenin) or quercetin [21], which reduce protein glycation [25, 29] and significantly inhibit the activity of enzymes involved in the breakdown of complex carbohydrates in the digestive tract, such as amylase [30] or α -glucosidase [25, 31], we determined the impact of the tested extract on protein glycation and on α -glucosidase activity. We compared the effect of IT extract to the effect of its main active compounds, i.e., vicenin-2 and quercetin (Fig. 1 shows the structures of these compounds). Moreover, we examined the effect of the IT extract on acetylcholinesterase activity. We also assessed the *in vitro* and *in vivo* safety of IT hydroalcoholic leaf extract. Confirmation of safety is extremely important in the early-stage development of a therapeutic agent. Products that have significant side effects are excluded from further research, saving time, money, and lives of animals. Such screening using safety assays is necessary to select the appropriate doses for future testing in specific indications and to clearly confirm the potential for *in vivo* use.

2. Materials and Methods

2.1. Plant material

The leaves were picked from IT growing in the wild around Acireale (Catania, Sicily, Italy). The dried plant material was extracted according to the procedure reported by Taviano et al. [22]. Finally, the hydroalcoholic extract (MeOH 70%) was evaporated to dryness in vacuum. The yield, referred to as 100g of dried plant material, was 24.32%. Prof. Salvatore Ragusa confirmed the taxonomic identity of the plant material. The specimens are deposited in the Herbarium of the Department of Scienze della Salute, University "Magna Graecia" of Catanzaro (Italy), under accession number no. 327/11. Phytochemical analysis of the extract was previously performed by HPLC-PDA-ESI-MS

2.2. In vitro protein glycation

2.2.1. Preparation of samples of glycated BSA

The formation of glycated BSA was determined using the modified method of Vinson and Howard, 1996 [72], with minor modifications. In brief, BSA (10 mg/mL) in 100 mM sodium phosphate buffer (pH 7.4) was incubated with fructose solution (0.5mM), 0.02% sodium azide, (used to prevent bacterial growth), and the tested solutions. Quercetin was used as the reference compound. Only BSA in the same buffer without fructose was a blank. The reaction mixtures were incubated for twenty one days (at 37°C) and were then assayed to determine the quantity of advanced glycation end products.

2.2.2. Formation of advanced glycation end products

The fluorescence intensity of the reaction products was determined using spectrofluorometric detector POLAR star Omega (BMG Labtech, Germany), wavelengths of excitation and emission: 340 nm, 440 nm. The results are presented as fluorescence intensity and are expressed as advanced glycation end products (AGEs) units: 1U = fluorescence of 0.5M fructose solution.

2.3. Alpha-glucosidase inhibition assay

The effect of the tested extract on alpha-glucosidase activity was measured using a previously described method [25], with minor modifications. In brief, 100 µL of tested solution in 100 mM phosphate buffer (pH 6.8), 20 µL 0.2 U/mL alpha-glucosidase in 100 mM phosphate buffer (pH 6.8), and 100 µL of 100 mM phosphate buffer (pH 6.8) were added to a plate. The plate was incubated at 37°C for 15 min. The enzymatic reaction was initiated by the addition of 50 µL of 2.5 mM p-nitrophenyl alpha-D-glucopyranoside (p-NPG, Merck, Germany). The plate was incubated at 37°C for 20 min, and the reaction was stopped by adding 50 µL of 0.1 mol/L sodium carbonate solution.. The absorbance ($\lambda = 405$ nm) was measured using a plate reader (ThermoFisher Scientific, USA). Vicenin-2, quercetin and acarbose were used as reference compounds. The inhibition percentage was calculated using the formula:

$$I [\%] = \frac{Ac - As}{Ac} \times 100\%$$

where Ac is the absorbance of control, and As is absorbance of the sample.

2.4. Acetylcholineesterase inhibition assay

The effect of the tested extract on acetylcholineesterase (AChE) activity was measured as described by [29], with minor modifications. The reaction mixture contained: 60 µL of 100 mM sodium phosphate buffer (pH 8.2), 50 µL of the tested sample solution, 50 µL of 5,5'-dithiobis(2-nitrobenzoic acid) (6mM, Merck, Germany) and 20 µL of 0.5 U/mL AChE solution (Merck, Germany). It was incubated at room temperature (10 min). The reaction was initiated by the addition of 20 µL of acetylthiocholine iodide (25 mM, Merck, Germany). The absorbance was measured at $\lambda = 412$ nm after 5 min, using a plate reader (ThermoFisher Scientific, USA). The inhibition percentage was calculated using the formula:

$$I [\%] = 100 - \left(\frac{As * 100}{Ac} \right)$$

where Ac is the absorbance of the control, and As is absorbance of the sample.

Tactine, vicien-2, and quercetin (Merck, Germany) were used as reference compounds.

2.5. Reduction assay (ferric reducing antioxidant power, FRAP) – in vitro

The total antioxidant activity of the IT extract was determined by spectrophotometric determination of the reduced iron concentration. The assay was performed as described by Benzie and Strain [61] with some modifications [73, 74]. The assay was carried out as follows 10 µL of the extract or solvent and 300 µL of the reagent were added to the following composition: ten parts of 0.3 M sodium acetate buffer (pH 3.7), one part of 2,4,6-tris(2-pyridyl)-s-triazine solution (0.01 M, Merck, Germany) and one part of $\text{FeCl}_3 \times 6 \text{H}_2\text{O}$ solution (0.02 M). The absorbance was measured after 10 min of incubation (room temperature, 593 nm). Deionized water with FRAP solution was used as a blank. Ascorbic acid was used as the reference compound. The results for the tested extract were presented as the amount of reduced iron (III) ions. $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ salt was used for the construction of a standard curve (Fig. 2).

2.6. Cytotoxicity assay – in vitro

The extract was tested at six concentrations ranging from 25 to 1 mg/mL using the mouse hippocampal neuronal cell line (HT-22).

HT-22 were cultured in Dulbecco's modified Eagle's Medium-high glucose (DMEM, Glutamx, ThermoFisher Scientific, USA) supplemented with 10% heat inactivated fetal bovine serum (ThermoFisher Scientific, USA), 100 µg/mL streptomycin (Merck, Germany), and 100 IU/mL penicillin (Sigma Aldrich). The cells were placed in a 96-well culture plate (2×10^4 cells per well, Falcon) and were grown for 24 hours in the sterile condition (37°C, 5% CO_2). Subsequently, different concentrations of the

extract were added and incubated for 24 hours. Cell viability was determined using both the Presto Blue and the ToxiLight assays.

2.6.1. Presto Blue assay

The Presto Blue reagent (ThermoFisher Scientific, USA) was used to evaluation of cell viability [75]. After 24 hours of incubation with the extract, 10 μ l of PrestoBlue reagent was added and incubated for 15 minutes at 37°C. The fluorescence intensity (wavelengths of excitation and emission: 340 nm, 440 nm) was measured in a plate reader (POLARstar Omega, BMG Labtech). The results were provided as the percentage of live cells with respect to the vehicle sample.

2.6.2. ToxiLight assay

The ToxiLight reagent (Lonza, Switzerland), which measures membrane damage, was used to evaluation of cell viability [76]. It quantitatively measures the release of adenylate kinase from the membrane of damaged cells. After 24 hours of treatment, the supernatant was transferred to a new plate and the adenylate kinase detection reagent was added. Luminescence was measured after 5 minutes of incubation in a plate reader (POLARstar Omega, BMG Labtech). The results were provided as the percentage of damaged/lysed cells.

2.7. Animals

All animal care and experimental procedures were carried out in accordance with the European Union and Polish legislation acts concerning animal experimentation and were approved by the Local Ethics Committee of Jagiellonian University of Cracow, Poland (Permissions No: 473/2020, date: 16.12.2020; No: 545B/2021, date: 21.07.2021).

The animals were obtained from the Animal House of the Jagiellonian University Pharmaceutical Faculty. Six-week-old male Albino Swiss mice, CD-1, weighing 20–22 g, were used. They were kept in environmentally controlled rooms, in standard cages lit by artificial light for 12 hours per day. The animals had free access to food and water, except for during the acute experiment. The randomly established experimental groups consisted of 8 mice.

2.8. Extract dosage and administration schedule

The extract was administered intraperitoneally (i.p.) to mice, at the following doses: 50 mg/kg, 100 mg/kg, or 500 mg/kg b.w. These are the same doses of *Isatis tinctoria* leaf extract described in our previous manuscript [24]. The vehicle (1% Tween 80; Sigma-Aldrich, Poland) was administered i.p. at a volume of 10 mL/kg. Thirty minutes after the first administration, the chimney and Rotarod tests were performed. Seven days later, 30 minutes after the second administration, locomotor activity was measured. Exactly one hour after administration of the extract, plasma was collected for biochemical testing.

2.9. Chimney test

The mice were trained before the test and only those animals that could get out of the chimney in less than 1 minute were used in the experimental stage. The selected mice were placed in a horizontal tube (length 25cm, diameter 2.5cm), which was next set up vertical, in such a way that the mice could only exit by climbing backwards until they reached the other end. Motor impairment was established to be the inability of the mice to exit the tube within 60 seconds. The number of animals unable to climb backwards within 60 seconds was recorded and was expressed as the % of animals that could not climb out [77]. The mice were injected with the extract 30 minutes prior to the chimney test.

2.10. Rotarod test

In brief, the mice were trained on the Rotarod apparatus (May Commat RR0711, Turkey; rod diameter: 2cm) prior to the experiment. During each training session, the mice were placed on the rotating rod (10 rpm, constant speed) twice for 3 minutes. The experiment was carried out 24 hours after the last training session. On the day of the test, the mice were injected with the extract, and 30 minutes after administration, tested on the chimney test, followed by the Rotarod test (10 rpm, constant speed). Motor impairment was regarded as the inability to remain on the rotating rod for 60 seconds and was expressed as the percentage of animals that fell off the rotating rod [78].

2.11. Change of body weight after administration of the tested extract

Mice were weighed prior to test extract administration and 24 hours after administration, on a weighing scale suitable for small animals (K-PZ Waagen, Germany).

2.12. Locomotor activity test after single administration of test extract

Locomotor activity was individually recorded for each animal using specifically designed activity cages made of clear Perspex (40 cm × 40 cm × 31 cm, Activity Cage 7441, Ugo Basile, Italy). The cages were fitted with I.R. horizontal beam emitters connected to a counter, which records the light-beam interruptions. Prepared suspensions of the extract were administered as i.p. injections 30 minutes before testing. The control animals received i.p. injections of vehicles. Each mouse was placed in the cage for a 30-minute habituation period (immediately after administration). Afterwards, the number of photo beam interruptions was measured for 5 minutes, which is the same observation period used in other tests.

2.13. Plasma collection

At the end of the experiment, 60 minutes after IT or vehicle administration and 20 minutes after ip administration of heparin (2500 units/mouse; Polfa Warszawa S.A., Poland), blood was collected after decapitation. It was centrifuged at 600 x g for 15 min (4°C) in order to obtain plasma. It was frozen in liquid nitrogen and placed in a freezer (-80°C) until biochemical assays were performed.

2.14. Biochemical analysis

To determine alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γ GTP), alanine aminotransferase (AlaT), and asparagine aminotransferase (AspaT) activity in plasma, a standard enzyme spectrophotometric tests (Biomaxima S.A., Poland) were used. Absorbance was measured at a wavelength of 405 nm (ALP, γ GTP) or 340 nm (AlaT, AspaT).

2.15. The Ferric Reducing Ability of Plasma (FRAP) - a measure of “Antioxidant Power”

The assay was performed as described by Benzie and Strain [61] with some modifications [79]. The FRAP working solution was prepared before the start of the analysis, using 0.3 mol of acetate buffer (pH 3.6), 0.01 mol of 2,4,6-tripyridyl-s-triazine (Merck, Germany) in 0.04 mol of HCl (POCH, Poland) and 0.02 M $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ in water (iron III) (Chempur, Poland), which were mixed in a volumetric ratio of 10:1:1 and protected from light. Subsequently, 20 μL of the plasma sample tested, brain homogenate, or $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ solution was mixed with 180 μL of the FRAP working solution. The mixtures obtained were incubated at 37°C for 30 minutes and absorbance was measured at 593 nm. $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ (Chempur, Poland) was used to create a standard curve (100–1000 $\mu\text{mol/L}$). Deionized water containing FRAP solution was used as the blank.

2.16. Statistical analysis

Statistical calculations were performed using GraphPad Prism 9 software (GraphPad Software, USA). Results are expressed as mean + standard deviation (SD) or mean + $\Delta/2$, where Δ is a width of the 95% confidence interval (CI), $n = 6-8$. Statistical significance was calculated using one-way ANOVA, Dunnet or Tuckey post hoc test. Differences were considered statistically significant at: $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

3. Results

3.1. Influence on bovine serum albumin (BSA) glycation

IT extract caused a reduction in the formation of advanced glycation products (AGEs). Incubation of the extract at concentrations 25 mg/mL, 20 mg/mL and 15 mg/mL with BSA and 0.5M fructose solution for three weeks significantly reduced the formation AGEs compared to the control sample, i.e. BSA and 0.5M fructose. The results are shown in Fig. 3.

The active compound in the IT extract, vicienin-2, also significantly inhibited the glycation of BSA at concentrations 10^{-5} and 10^{-6} M. Quercetin, the reference compound used, reduced the formation of AGEs to a comparable extent as vicienin-2.

3.2. Influence on alpha-glucosidase activity

IT extract inhibited α -glucosidase activity in a concentration-dependent manner. The results are shown in Fig. 4. The highest concentration of IT extract tested, 1 mg/mL, inhibited the activity of the enzyme by approximately 50%, and a concentration ten times lower inhibited it by less than 10%. This effect was stronger than that of the selected compounds - vicenin-2 and quercetin.

The active compounds from the IT extract, vicenin-2 and quercetin, also inhibited α -glucosidase activity at concentrations 10^{-5} , 10^{-6} and 10^{-7} M. However, the effect was only about 15% at the highest concentration used (10^{-5}) and was half as weak compared to the inhibition at the lowest concentration of acarbose used (10^{-7}). Additionally, inhibition by the IT extract was about 5.5 times weaker in comparison to the inhibition at the same concentration by acarbose (10^{-5}). Acarbose, the reference compound used, inhibited α -glucosidase activity in a concentration-dependent manner.

3.3. Influence on acetylcholinesterase (AChE) activity

IT extract inhibited acetylcholinesterase activity at all the tested concentrations. The results are shown in Fig. 5. At the highest concentration, 1 mg/mL, the IT extract inhibited the activity of the enzyme by approximately 25%, at half the maximum concentration it inhibited the activity of AChE by about 20%, and at lower concentrations, 0.25 mg/mL and 0.1 mg/mL, inhibition was below 5%. The inhibitory activity determined for the two highest concentrations of IT extract was similar to that of the selected compounds - vicenin-2 and quercetin, at concentrations 10^{-4} to 10^{-6} , but lower than inhibitory activity of tacrine.

The active compounds from the IT extract, vicenin-2 and quercetin, inhibited AChE activity at concentrations 10^{-4} , 10^{-5} and 10^{-6} M. However, this effect was only about 30 – 20% at the highest concentration used (10^{-4}) and was weak compared to activity of tacrine at the same concentration.

Tacrine, the reference compound used, inhibited AChE activity in a concentration-dependent manner.

3.4. Toxicity Study and Antioxidant Assays

3.4.1. Cytotoxicity

The extract was not cytotoxic at all the concentrations tested. Cell viability was determined using the Presto Blue assay, the number of cells in the control (vehicle, water) being 100% live cells (Fig. 6a) and in the ToxiLight assay, 100% damaged cells being present in the lysis sample (Fig. 6b).

3.4.2. Reduction activity

In the FRAP assay, IT extract caused a concentration-dependent reduction of iron (III) ions. 1 gram of extract reduced 60 mmol Fe^{3+} to 140 mmol (Fig. 7a).

Ascorbic acid was used as the reference compound. An increase in absorbance, proportional to the concentration of reduced iron (0.1–1 mM) was determined, indicating an increase in total antioxidant activity. The maximum activity of ascorbic acid occurred a concentration of 1 mM (Fig. 7b).

3.5 Influence on the body mass of mice

The tested extracts did not have a significant effect on the body mass of mice during a 24-hour period, after one intraperitoneal administration. The results are shown in Fig. 8.

3.6 Influence on the locomotor activity of mice

The locomotor activity of mice decreased significantly after administration of 50 mg/kg b.w. ($F(3,28) = 3.948, p = 0.0182, n = 8$ of IT leaves extract). The results are shown in Fig. 9.

3.7 Influence on motor coordination

IT extract did not affect motor coordination in the Rotarod and chimney tests. Results are shown in Table 1. Only one mouse from the group treated with IT extract at a dose of 100 mg/kg b.w. did not exit the chimney within 1 minute. However, we observed that in this group, the exit times were significantly longer compared to the control group ($F(3,20) = 2.697, p = 0.0733, n = 6$). We assigned a time of 60 seconds to the mouse that did not come out of the chimney. Results are shown in Fig. 10.

Table 1
The effect of IT on motor coordination in the Rotarod and Chimney test in mice.

Treatment	Rotarod test [% of animals that could not stay on a rotating rod for at least 1 minute]	Chimney test [% of animals that did not climb out after 1 minute]
1% Tween 80	0	0
IT 500 mg/kg	0	0
IT 100 mg/kg	0	16.67
IT 50 mg/kg	0	0

IT – *Isatis tinctoria* extract. n = 6 animals per group.

3.8 Influence on hepatic enzyme activity in plasma

After administration of IT extract only a significant increase in alkaline phosphatase (AP) activity was determined in comparison to the plasma activity determined for the control group ($F(3,28) = 3.414, p = 0.0310, n = 8$). The activity of the other enzymes determined in plasma of the treated group were

comparable to the activity of these enzymes in the plasma of the control group. Results are shown in Fig. 11.

2.9 Influence on the total antioxidant activity of plasma

The extract of IT leaves administered to mice at a dose of 500 mg/kg b.w. had a significant antioxidant effect one hour after intraperitoneal administration ($F(3, 28) = 7.116$, $p = 0.0011$, $n = 8$). Results are shown in Fig. 12.

4. Discussion

Our research demonstrates the anti-diabetic and neuroprotective potential of *Isatis tinctoria* L. hydroalcoholic leaf extract for the first time. It shows that the effect of the extract on the reduction of protein glycation is significant. Additionally, IT extract inhibits alpha-glucosidase activity in a concentration dependent manner and AchE activity at some concentrations. We also show the results of preliminary *in vivo* safety studies and show that intraperitoneal administration of hydroalcoholic extract of IT leaf at doses from 50 mg/kg b.w. to 500 mg/kg, is most likely safe for the organism. Moreover, the tested extract has no cytotoxic properties, even at concentrations characterized by a strong reducing effect.

Neurodegenerative, cardiovascular, and metabolic diseases have a significant negative effect on the quality of life and life span of the aging population. The increased accumulation of damaged proteins is responsible for the formation of bulky aggregates and plaques typical of the aforementioned diseases. Type 2 DM is one of the most important risk factors responsible for age-related cognitive impairment [32]. The pathophysiology of AD is attributed to numerous factors such as cholinergic dysfunction, amyloid/tau toxicity and oxidative stress/mitochondrial dysfunction [33]. Interestingly, clinical evidence considers hyperglycemia to be one of the risk factors for the development and progression of AD [34]. Consequently, plant products that inhibit AchE activity, alpha-glucosidase activity, and protein glycation, in addition to exhibiting antioxidant properties, have anti-diabetic and neuroprotective potential.

Numerous medicinal herbs and dietary plants, containing polyphenols, were found to prevent protein glycation in *in vitro* studies [35–38]. The anti-glycating activity of many flavonoids, including quercetin, quercitrin, genistein, kaempferol, naringin, rutin, and propyl gallate, has been documented [39–44]. Phenolic acids could effectively protect BSA from glycation *in vitro* [45–48].

The IT extract we chose for our research contains many different compounds in various concentrations, including e.g.: vicianin-2 (7.51 mg/g), flavone-di-glucoside (6.42 mg/g), isovitexin (5.46 mg/g), stellarin-2 (4.69 mg/g), apigenin-di-glucoside (4.51 mg/g), apigenin-glucoside (4.68 mg/g), isoscoparin (3.89 mg/g), quercetin (1.86 mg/g), neochlorogenic acid (1.84 mg/g), sinapic acid (1.49 mg/g) and kaempferol (1.48 mg/g) [21]. Some of these compounds have proven anti-glycation and antioxidant activity; and an inhibitory effect on some enzyme activity, e.g. alpha-glucosidase or AchE. Despite the inhibition of

protein glycation by the extract being less intense than the inhibition exerted by the main compounds (vicenin-2 and quercetin) of this extract at the tested concentrations, it is still detectable.

Earlier studies clearly demonstrate that the accumulation of advanced glycation end products (AGEs) in tissue is the leading cause of age-related degeneration, atherosclerosis, and diabetic complications such as neuropathy, retinopathy, cardiomyopathy, and nephropathy [49, 50]. Recent studies suggest that AGEs interact with specific receptors (RAGE) in the plasma membrane, modifying gene expression, changing intracellular signal transmission, and promoting the release of free radicals and pro-inflammatory molecules [51, 52]. Therefore, inhibition of AGE formation is a potential therapeutic target for preventing the progression of the aforementioned complications of diabetes and protecting against the development of age-related cognitive impairment and neurodegenerative disorders.

Alpha-glucosidase is an enzyme located in the digestive tract which is responsible for breaking down carbohydrates to facilitate the absorption of monosaccharides. Inhibition of alpha-glucosidase activity can reduce diabetic complications [53, 54], making its inhibition by the main compounds in IT, vicenin-2, quercetin, of great value [25]. In our study, IT leaf extract was more active than these compounds at almost all the tested concentrations. The inhibition of alpha-glucosidase shows the potential of IT extract in the management of hyperglycemic states, diabetes, obesity, and neurodegenerative diseases.

AchE plays a key role in the hydrolysis of acetylcholine in the regulation of cholinergic neurotransmission [55]. Inhibitors of this enzyme can delay the progress of mental illness, thus providing a rational therapeutic alternative in the treatment of Alzheimer's disease. Our study shows that IT extract has a weak inhibitory effect on AchE. Vicenin-2 and quercetin also possess weak activity in this regard. The effect on AchE activity can be considered as an additional benefit of this extract in the management of neurodegenerative disorders.

A limitation of our study is the difficulty in drawing a direct comparison between the potency of the extract and the potency of the main compounds. For that reason, we consider the results to be preliminary, since the main compounds isolated from IT and the reference compounds (acarbose, tacrine) were used in molar concentrations so that their activity could be compared, which cannot be done for the extract.

Few *in vivo* studies showing the effects of various IT leaf extracts on the organism are presented in the literature [20, 24, 56, 57], and these mainly concern their anti-inflammatory effect. Moreover, most of the manuscripts focus on the *in vitro* activities of the various extracts of this plant with interesting therapeutic properties [15, 21, 58].

Oxidative stress is characteristic of many diseases including, type 2 diabetes mellitus, atherosclerosis, hypertension, chronic obstructive pulmonary disease, major depression, Alzheimer's disease, inflammation, and cancer, making the development of effective antioxidant therapies of great importance [59]. Free radicals are oxidizing agents, causing tissue damage. Uncontrolled oxidative activity is the basis of various pathological conditions and can be countered by antioxidant treatment.

Nonetheless, oxidants may exert beneficial effects by regulating cell signaling cascades [60]. In an *in vitro* study, we demonstrated the absence of cytotoxic activity by the tested extract on HT-22 cells, even at concentrations with significant reducing power. Subsequently, in plasma collected from animals, we determined the plasma reduction force by determining the reduction force of Fe³⁺ ions, which is interpreted as the total antioxidant force of plasma [61]. IT extract administered to mice at a dose of 500 mg/kg b.w. had a significant antioxidant effect one hour after a single intraperitoneal administration. This is an important property of the extract *in vivo*.

From the simple measurements of basal body weight after administration of the plant extract/compound, basic conclusions on the effects on the organism can be drawn. Since animals that feel somehow unwell after the administration of an agent have reduced locomotor activity [62, 63], and consume less food, they may lose significant body mass. Weight loss can also be the result of increased activity and calorie expenditure. It should be noted that such changes in the mice can be noticed in a short timeframe because they constantly consume food throughout the day [64]. Furthermore, it is worth noting that body weight is a dynamic parameter and that daily fluctuations, are within the range of approximately 2–3% (in control animals).

In our research, we measured body weight prior to and 24 hours after the administration of IT extract, weighed the amount of food consumed, and measured the influence on locomotor activity. The extract tested did not have a significant effect on mouse body weight. Interestingly, after administration of the extract at doses of 500 and 50 mg/kg b.w., a slight decrease in body weight was established. In all groups, the mice ate a similar amount of food within 24 h, that is, 20g/24h/6 mice $\pm$ 1 g. However, locomotor activity, after administration of the leaf extract at a dose of 50 mg/kg b.w., significantly decreased in comparison to the spontaneous activity determined in the control group. This observed decrease in activity could explain the slightly lower weight gain observed in this group after administration of the extract. A limitation of these studies is the short time (5 minutes) allocated to determination of the effect of IT extract on spontaneous activity, therefore further studies should be carried out to monitor spontaneous activity for e.g., 24 hours after administration of the tested extracts. An interesting result is that IT extract only has a sedative effect at the lowest dose used. These differences in the effect on spontaneous activity may be attributed to the differences in the action of the compounds in the extract at different concentrations. At lower doses, certain compounds may have significant activity (sedation), whereas, at higher doses, other compounds may become active and suppress or counteract the effects of the compounds acting at lower doses.

In our previous research, we demonstrated that IT hydroalcoholic leaf extract can reduce stress-induced behavioral disturbances by regulation of neuro-nitrosative, neuro-oxidative, and neuroimmune pathways [24]. However, given the reports, some centrally acting compounds or drugs, e.g.: antidepressants, stress relievers, and anxiolytics, may negatively affect motor coordination [65, 66]. In view of this, we conducted preliminary safety studies to reduce the use of animals later on, since products that disturb motor coordination are excluded from further studies. The results obtained from the motor coordination tests clearly show that at the doses used, the IT extract does not significantly disturb the motor coordination

of mice. However, it should be taken into account that at a dose of 100 mg/kg b.w., the extract significantly prolonged the time of exit of mice from the chimney compared to the time of exit from the chimney of the control mice - which suggests that at this dose, the activity of the compounds in the extract is the least safe. This coincides with the observations described in the earlier manuscript, where a dose of 100 mg/kg b.w. was considered the least favorable [24].

As part of the safety studies conducted, we also performed assays on plasma collected from animals to determine the activity of enzymes such as gamma-glutamyl transpeptidase (gGT), alkaline phosphatase (AP), alanine aminotransferase (AlaT), and aspartate aminotransferase (AspaT). These were done to check the acute effect of the tested extracts on liver cells (liver integrity or hepatocellular damage).

Liver toxicity is one of the most common difficulties encountered in drug development. However only 43% of the toxicities seen in rodents are observed humans, as was established by a retrospective study on the toxicity of pharmaceuticals in development [67]. Enzymes synthesized in cells perform specific functions intracellularly, and few are secreted into the extracellular space. Each tissue has its own specific enzyme profile. The presence of intracellular enzymes in body fluids may indicate tissue damage (damage to the cell membrane – reversible, or cell breakdown - irreversible). The current best-practice recommendation for nonclinical safety assessment is that a minimum of four serum parameters is used to assess hepatocellular (a minimum of two markers) and hepatobiliary (a minimum of two markers) injury [68, 69]. Any two markers of glutamate dehydrogenase activity, sorbitol dehydrogenase activity, alanine transaminase activity, and aspartate transaminase activity, can be used to evaluate hepatocellular injury, whilst any two of gamma-glutamyl transferase activity, alkaline phosphatase activity, total bile acids and 5'-nucleotidase, total bilirubin can be used to assess hepatobiliary injury [70].

In our study, we determined the activity of four enzymes in plasma 60 minutes after administration of the test extract, similarly as other authors earlier [71]. After administration of the IT extract, only a significant increase in AP activity was determined in comparison to the activity determined for the control group. This difference was only observed at a dose of 100 mg/kg b.w. ip. Since plasma levels of AP increase as the patency of the bile duct is reduced, AP is widely used in nonclinical and human clinical settings as a marker of cholestatic liver injury [70]. In the absence of an increase in the other measured enzymes, especially gGT, hepatic disorders are excluded. It is known that fluctuations of AP need to be interpreted cautiously in nonclinical settings since there is an intestinal isoform of AP whose activity transiently increases postprandially [68].

5. Conclusions

The presented preliminary studies show that IT hydroalcoholic leaf extract exhibits antiglycation, anti-alpha-glucosidase, anti-AchE, antioxidant, anti-inflammatory properties and therefore also has anti-diabetic and neuroprotective potential. Additionally, *in vitro*, and *in vivo* safety studies in mice confirm that the tested extract does not cause coordination disorders and does not significantly affect body mass and liver enzyme activity. Depending on the dose used, it can have a sedative effect (which may be

beneficial in some conditions). It has no cytotoxic effect on hippocampal cells, even at concentrations with significant reducing power. Therefore, hydroalcoholic extract of this plant may be considered for further research in the management of disorders linked to oxidative stress.

Declarations

Author Contributions: Conceptualization, and methodology, M.K.; formal analysis, M.K., N.N., M.B., M. G-L., R.H., P.Z., A.M., E.T.; investigation, M.K., N.N., M.B.; resources, I.K., N.M., S.R.; data curation, M.K.; writing - original draft preparation, M.K., N.N., M.B., M.G-L., I.K., K.M.; writing - review and editing, M.K., P.Z., N.N., A.A, I.K., N.M., E.R.L., J.S.; project administration, M.K.; visualization, M.K; funding acquisition, M.K., I.K.; supervision, M.K., I.K.; All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Local Ethics Committee for Experiments on Animals of the Jagiellonian University in Krakow (Permissions No: 473/2020, date: 16.12.2020; No: 545B/2021, date: 21.07.2021).

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Conflicts of Interest: All authors declare that they have no conflicts of interest.

Sample Availability: The *Isatis tinctoria* L. hydroalcoholic leaf extract was earlier characterized to full details of the chemical composition. The data have been presented in previous manuscripts (see Miceli et al., 2017[21], Taviano et al., 2018 [22]). Samples of the extract are available from the authors.

Data availability statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures

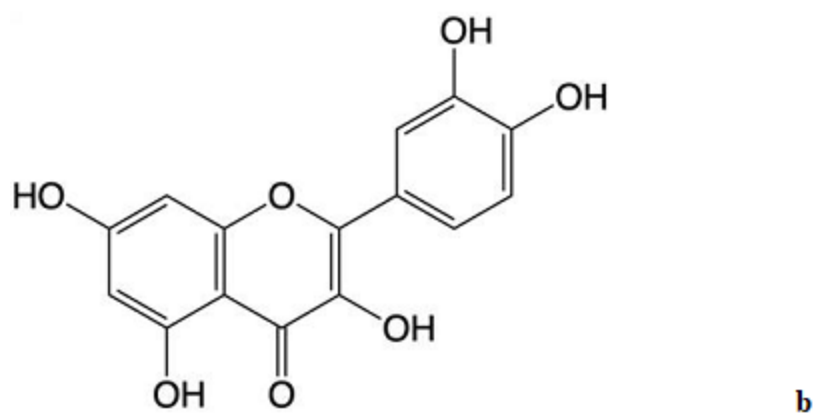
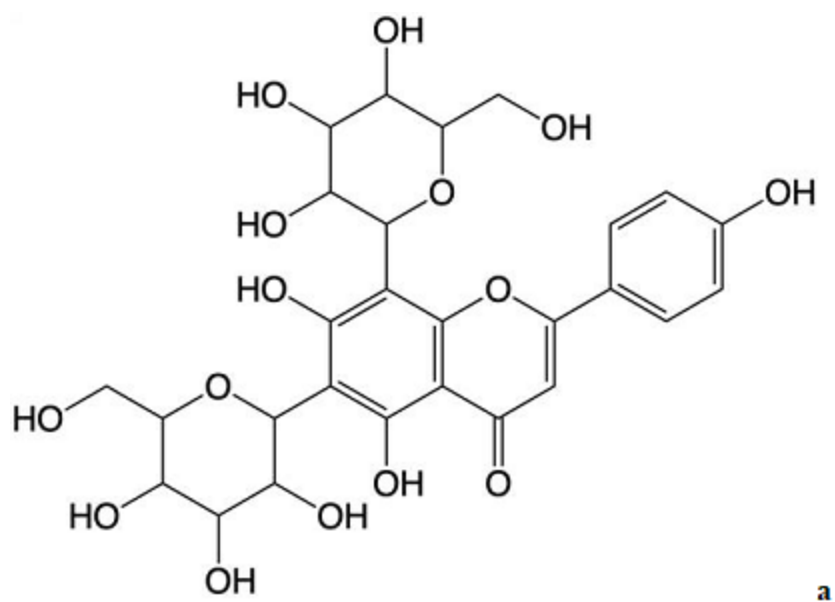


Figure 1

(a) Vicenin-2 structure, (b) quercetin structure.

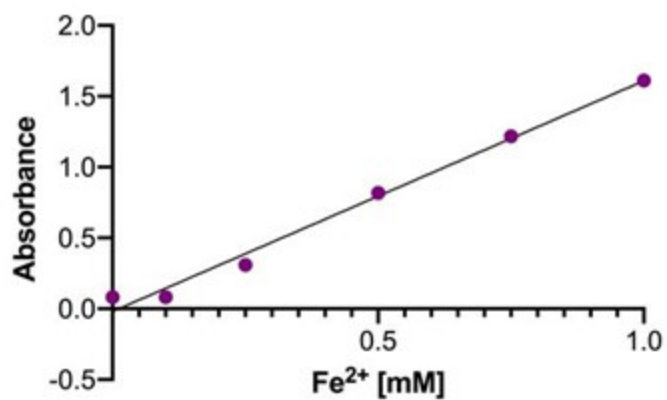


Figure 2

Standard absorbance curve of Fe²⁺.

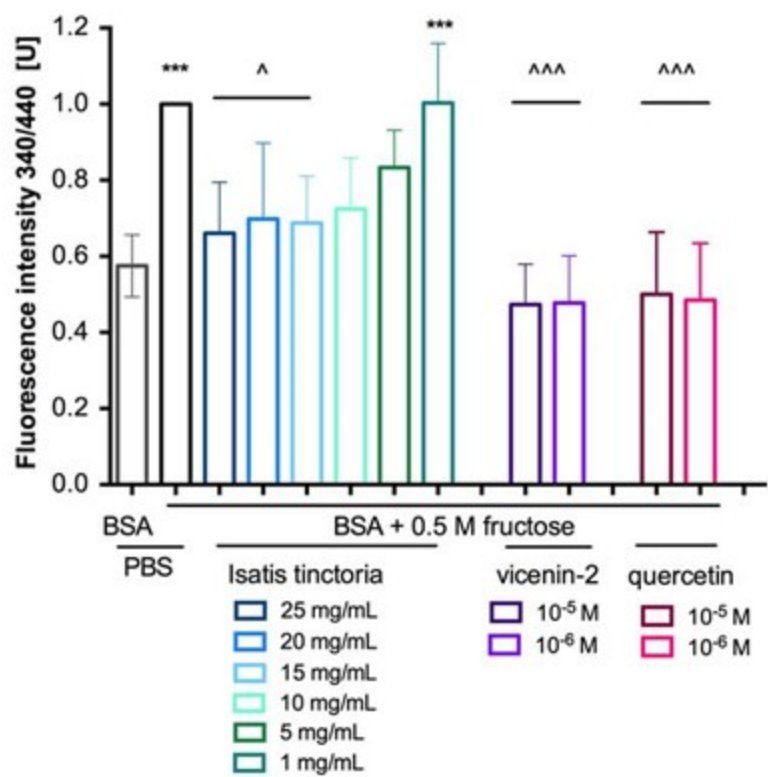


Figure 3

Influence of different concentrations of IT leaves extract on BSA glycation after 3 weeks of incubation. Mean $\pm$ SD from three separate duplicated experiments, n=3. To calculate the significance of differences between the groups one-way ANOVA followed by Tukey's post hoc test was used. * Significant difference vs BSA+PBS group, ^ significant difference vs BSA+Fructose group. Significance level: ^ p <0.05, ***, ^^^ p<0.001.

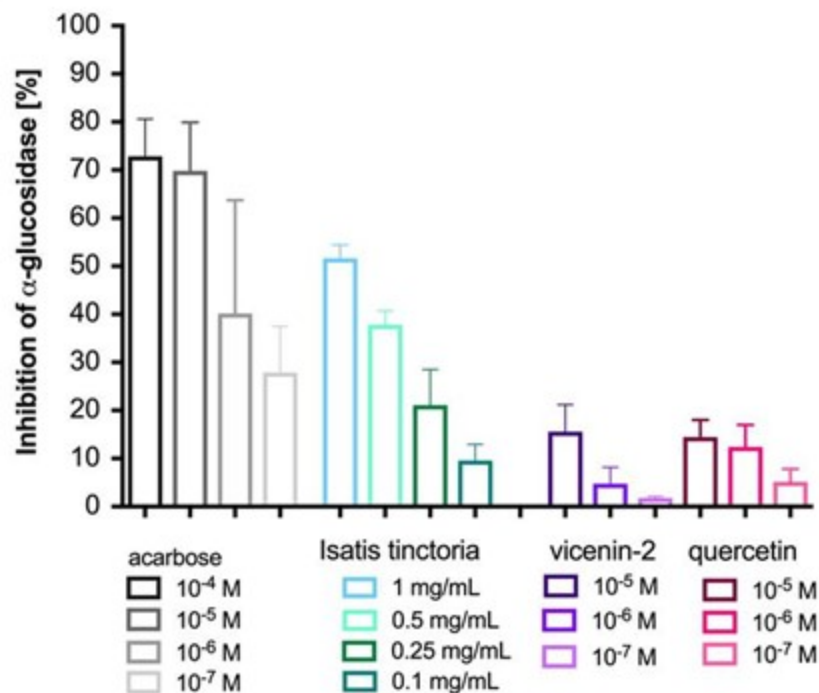


Figure 4

Influence of different concentrations of IT leaves extract on alpha-glucosidase activity. Mean $\pm$ SD from three separate duplicated experiments, n=3.

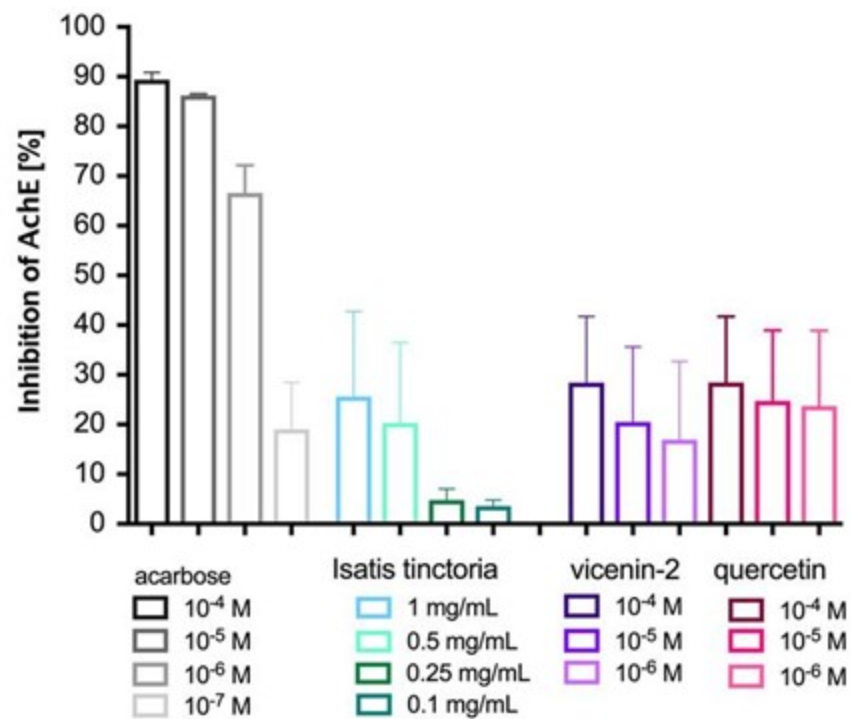


Figure 5

Influence of different concentrations of IT leaves extract on acetylcholinesterase activity. Mean $\pm$ SD from three separate duplicated experiments, n=3.

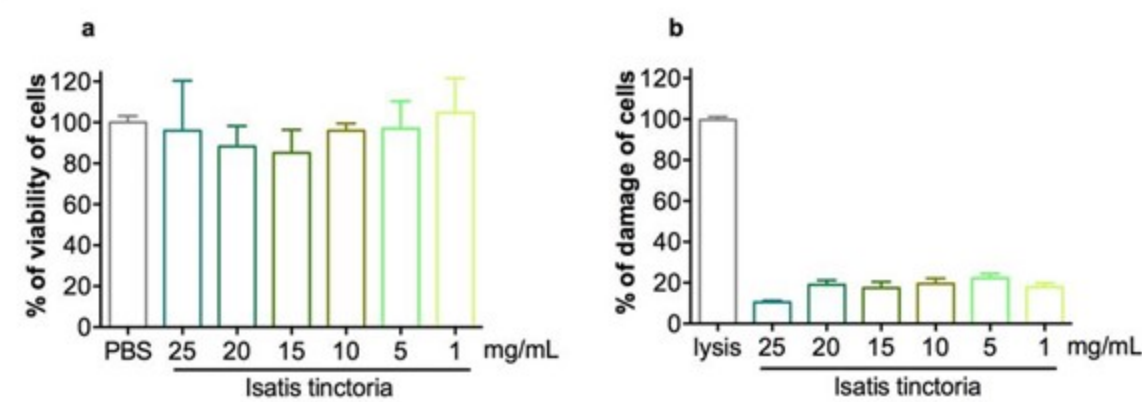


Figure 6

Cytotoxic effect of different concentrations of IT leaves extract on HT-22 cells after 24 hours of incubation: the percentage of live cells vs vehicle sample - Presto Blue assay (a), the percentage of damaged cells vs lysis sample - ToxiLight assay (b). Mean $\pm$ SD from two separate duplicated experiments, n=2.

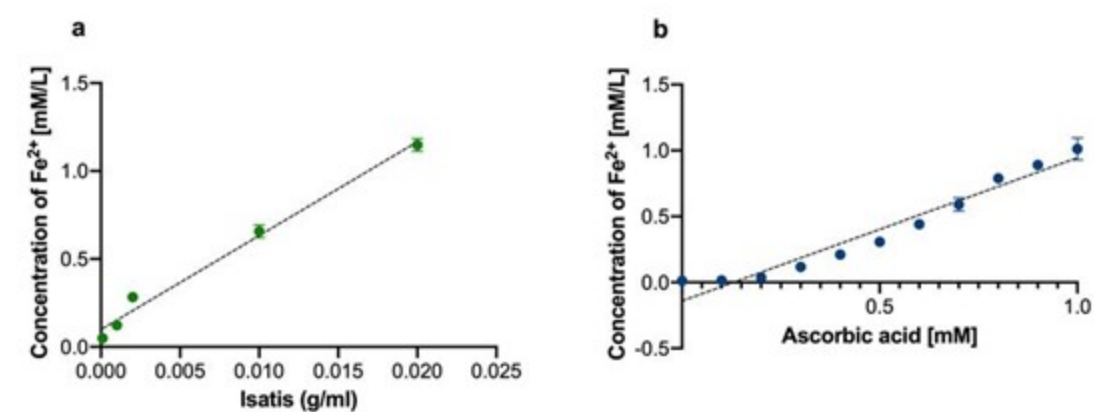


Figure 7

Antioxidant effect of IT leaves extract (a) or ascorbic acid (b) in FRAP assay. The results are presented as the mean $\pm$ SD from two separate duplicated experiments.

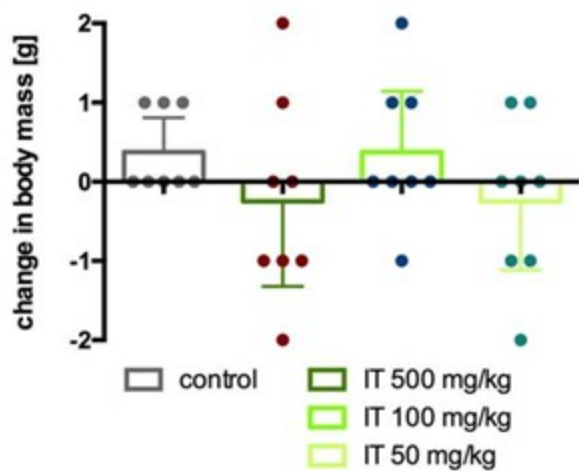


Figure 8

Changes in mice body mass after administration of IT leaves extract. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's post hoc test was used to calculate the significance of differences between the groups; $n = 8$.

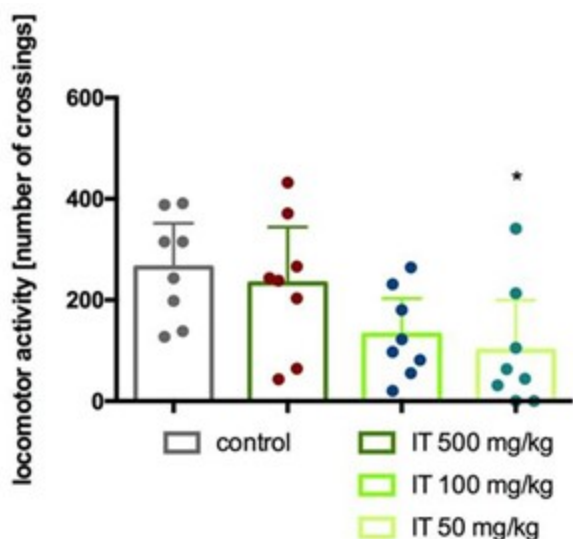


Figure 9

Locomotor activity of mice after administration of IT leaves extract. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's post hoc test was used to calculate the significance of differences between the groups; $n = 8$. * Significant difference vs control group, significance level: $*p < 0.05$

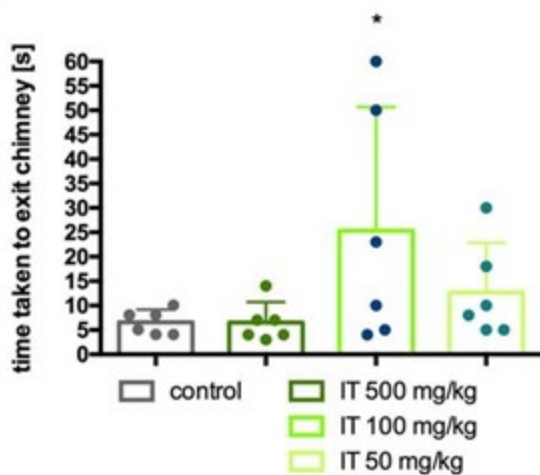


Figure 10

Escape time from chimney after administration of IT leaves extract. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's post hoc test was used to calculate the significance of differences between the groups; $n = 6$. * Significant difference vs control group, significance level: $*p < 0.05$

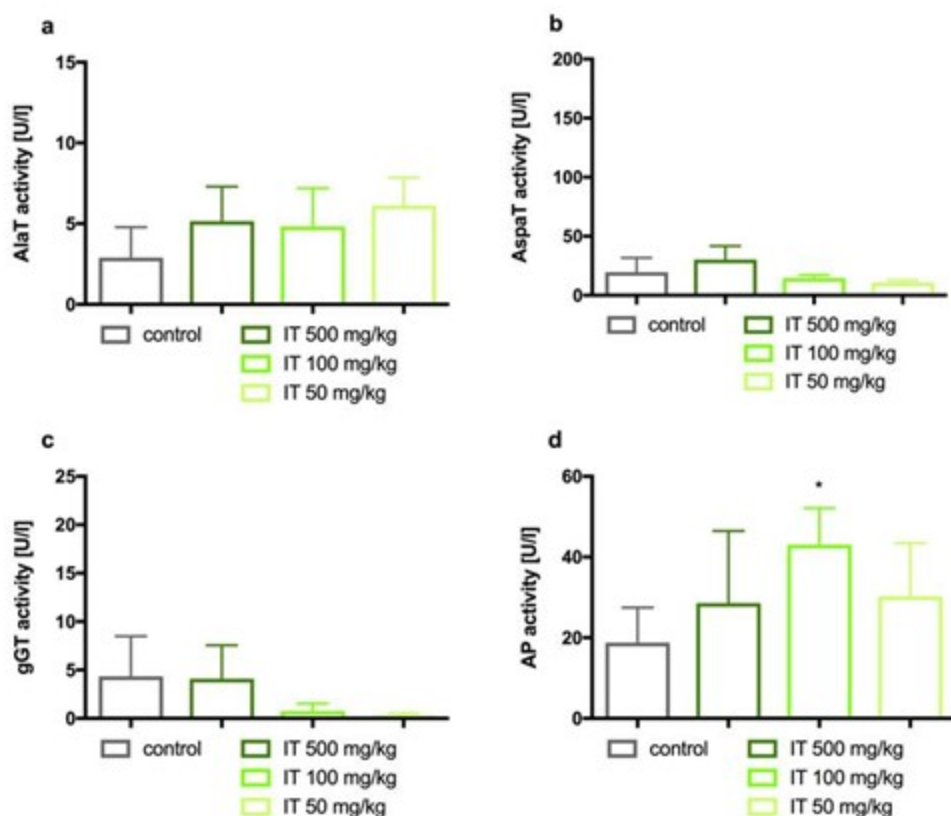


Figure 11

Plasma enzyme activity: alanine aminotransferase (AlaT, **a**), aspartate aminotransferase (AspaT, **b**), gamma-glutamyl transpeptidase (gGT, **c**) and alkaline phosphatase (AP, **d**) after administration of leaves

extract. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's post hoc test was used to calculate the significance of differences between the groups; n = 7-8. * Significant difference vs control group, significance level: *p <0.05

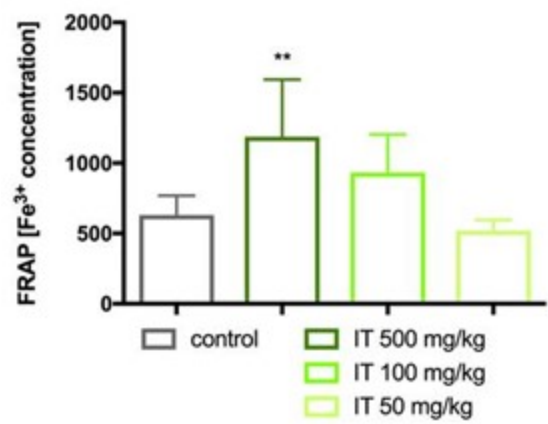


Figure 12

Total plasma antioxidant power 1 hour after administration of IT leaves extract to mice. The results are presented as the mean of the measurements $\pm \Delta/2$, where Δ is the width of the 95% confidence interval (CI). One-way ANOVA followed by Dunnett's post hoc test was used to calculate the significance of differences between the groups; n = 6-8. * Significant difference vs control group, significance level: ** p<0.001