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Alcesefoliside protects against oxidative brain injury in rats

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ABSTRACT

This study investigated the possible antioxidant and neuroprotective effects of alcesefoliside, isolated from *Astragalus monspessulanus* L., Fabaceae, against carbon tetrachloride (CCl^4)-induced brain injury in Wistar rats. Iron sulphate/ascorbic acid lipid peroxidation was induced in rat brain microsomes and pre-incubated with alcesefoliside and silybin. Male rats were treated *in vivo* with alcesefoliside and with silymarin alone; animals challenged with CCl_4; and pre-treated with alcesefoliside or silymarin in respective doses for 7 days, challenged with CCl_4, followed by curative treatment (additional 14 days). The activity of acetylcholine esterase and the antioxidant enzymes: superoxide-dismutase, catalase, glutathione-peroxidase, glutathione reductase and glutathione-*S*-transferase as well as the biomarkers of oxidative stress malondialdehyde and reduced glutathione were measured. The alcesefoliside pre-treatment and consecutive curative treatment normalizes the activity of the antioxidant enzymes as well as levels of malondialdehyde and reduced glutathione. The observed effects on tissue level correlate with the histopathological observations of the brain. They were comparable to the effects of silymarin, used as a positive control. The results showed that alcesefoliside has a neuroprotective effect against CCl_4-induced brain toxicity in rats.

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Introduction

To investigate the pathological role of brain oxidative stress in neurodegenerative diseases, experimental models are widely used. Carbon tetrachloride (CCl_4) undergoes metabolic bio activation to CCl_3• (thrichloromethyl) radical which further leads to free radical generation in the tissues such as liver, brain, kidney, heart, lung, testis, blood, *etc.* (Dashti et al., 1989). Due to this mechanism, CCl_4 is used in experimental toxicology as a model substance that mimics oxidative stress in many pathophysiological situations. Cells normally have a number of mechanisms to resist against damage induced by free radicals, *i.e.* endogenous antioxidant scavengers such as glutathione (GSH), and antioxidant enzymes. Exogenous natural dietary antioxidants include vitamin A, C, and E, carotenoids, flavonoids, and polyphenols. Flavonoids are glycosylated in their natural dietary forms (Rice-Evans, 2001). In recent years, there has been an increasing interest in investigating the positive pharmacological properties of flavonoids, isolated from plants, because they can prevent injury caused by ROS. In our laboratory an extensive research, both *in vitro* and *in vivo* on the hepatoprotective and antioxidant properties of different *Astragalus* species has been carried out (Simeonova et al., 2010, 2015; Vitcheva et al., 2013). Carbon tetrachloride (CCl_4)-induced hepatotoxicity is a well-known model for understanding the pathogenesis of some chronic liver diseases, and consequently of hepatic encephalopathy (Jiménez et al., 1992). Liver metabolism of CCl_4 causes formation of reactive oxygen and nitrogen species, making it suitable to evaluate the role of pro-oxidant and antioxidant mechanisms, as well as the influence of different types of natural biologically active substances on the latter mechanisms (Tirkey et al., 2005).

Astragalus monspessulanus L., Fabaceae (Montpellier Milk Vetch) is a clump-forming perennial herb from Fabaceae, found in Bulgarian flora. Our previous studies led to isolation of two novel flavoalkaloids and several flavonoids, including the rare alcesefoliside (**1**) from the overground parts of the plant (Krasteva et al., 2015). In a model of CCl_4-induced liver damage the *n*-butanol extract of the plant displayed hepatoprotective properties (Simeonova et al., 2015). Further investigation of the antioxidant effects of **1** in *tert*-butylhydroperoxyde (*t*-BuOOH)-induced oxidative stress in isolated rat hepatocytes showed its

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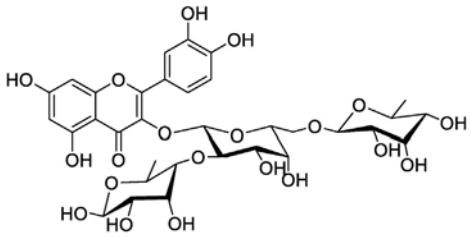
cytoprotective activity (Krasteva et al., 2015). Recently, thirteen known compounds, including seven flavonoids were identified in an extract from aerial parts of *A. monspessulanus* and their antioxidant potential was evaluated *in vitro* (Bourezzane et al., 2018).



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In continuation of our ongoing investigations, we aimed to extend the knowledge on antioxidant and neuroprotective properties of alcesefoliside (**1**) using both *in vitro* model of non-enzyme induced lipid peroxidation in isolated brain microsomes from Wistar rats and *in vivo* model of CCl_4-induced brain damage in rats.

Materials and methods

Plant material, extraction and isolation

The aerial parts of *Astragalus monspessulanus* L., Fabaceae, were collected in May 2016 from Rodopi Mountain and identified by Dr. D. Pavlova from the Department of Botany, Faculty of Biology, Sofia University, where voucher specimen was deposited (SO-107533). The air-dried and powdered plant material was exhaustively extracted with 80% MeOH under reflux. The extract was filtrated, concentrated under reduced pressure, and successively partitioned with $CHCl_3$, EtOAc, and *n*-BuOH. The *n*-BuOH extract (53.6 g) was separated on a Diaion HP-20 column eluting with H_2O-MeOH (100:0 → 0:100, v/v) to give nine fractions (I-IX). Fraction III was chromatographed over Sephadex LH-20 with MeOH as eluent to give six subfractions (A1-A6). Subfraction A4 was purified by repeated low pressure liquid chromatography (LPLC) over octadecyl silica gel (ODS C_{18}) with MeOH-H_2O (40:60, v/v) and further subjected to isocratic semi-preparative HPLC using mobile phase MeCN-H_2O (14:86, v/v) to give alcesefoliside (**1**) in considerable quantity (435 mg). The compound was identified by comparing the experimental with previously reported ^1H and ^{13}C NMR data (Krasteva et al., 2015).

Animals

Male Wistar rats (body weight 150–180 g) were housed in plexiglass cages (three per cage) in a 12/12 light/dark cycle, ambient temperature 20 ± 2 °C and humidity 72 ± 4%, with free access to water and standard pelleted rat food 53-3. The animals were purchased from the National Breeding Centre, Sofia, Bulgaria. Seven days' acclimatization was allowed before the commencement of the study and a veterinary physician monitored the health of the animals regularly. Vivarium (certificate of registration № 0072/01.08.2007) was inspected by the Bulgarian Drug Agency in order to check the husbandry conditions (№ A-11-1081/03.11.2011). All performed procedures were approved by the Bulgarian Food Safety Agency (BFSA) and the principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) (Council of Europe, 1991) were strictly followed throughout the experiment.

Chemicals

All the reagents used were of analytical grade. Carbon tetrachloride, ascorbic acid, beta-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), 1-chloro-2,4-dinitrobenzene (CDNB), and cumene hydroperoxide were purchased from Sigma Chemical Co. (Taufkirchen, Germany). 2,2-Dinitro-5,5-dithiodibenzoic acid (DTNB) was obtained from Merck (Darmstadt, Germany).

In vitro *methods*

Silybin, the major component (70%) of the flavolignan mixture, named silymarin, was used as a reference substance for the *in vitro* study. Its effects would be better comparable to those of alcesefoliside (**1**) – both are pure compounds and share a flavonoid structure. In previous studies, silybin and silymarin proved to penetrate the blood brain barrier (Kondeva-Burdina et al., 2018).

Preparation of brain microsomes

Brain microsomes were isolated from untreated rats (Voirol et al., 2000). Immediately after decapitation, brains were excised, rinsed in cold (4 °C) buffer A (0.32 M sucrose, 50 mM KH_2PO_4, 1 mM EDTA, 0.1 mM dithiothreitol, pH 7.4). Whole brains of several rats were pooled and used for the preparation of subcellular fractions and biochemical assessment. All procedures were done on ice and all the centrifugations were carried out at 4 °C. The samples were homogenized in 10 vol. of cold 1 mM Tris HC1 buffer (pH 7.4) containing 154 mM NaCl. The microsomal fraction was prepared from the homogenate in 10 vol. of cold 0.32 M sucrose containing 1 mM phosphate buffer (pH 7.4). The prepared brain homogenate was centrifuged at 17,000 × *g* for 25 min and then pelleted by centrifugation at 105,000 × *g* for 1 h. The microsomal pellets were re-suspended in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% glycerol. The content of microsomal protein was determined using bovine serum albumin as a standard and adjusted to 0.2 mg protein/ml (Lowry et al., 1951).

Iron-ascorbate (Fe^{2+}/AA) induced lipid peroxidation

The isolated microsomes were pre-incubated with **1** and silybin at three consequently decreased equimolar concentrations: 100, 10, and 1 μmol. The pre-incubation was performed at 37 °C for 15 min. Lipid peroxidation (LPO) was induced by incubating microsomes (0.2 mg protein/ml) with 84 μM $FeSO_4$ and 400 μM ascorbic acid in 1 mM Tris HC1, 154 mM NaCl, 0.1 mM EDTA, pH 7.4. Aqueous solutions of $FeSO_4$ and ascorbic acid were prepared immediately before their use. Samples were incubated in a water bath, at 37 °C, for 40 min (Mansuy et al., 1986). The reaction was stopped with mixture of 25% TCA and 0.67% TBA at 20 min after LPO initiation and MDA quantity was assessed (Deby and Goutier, 1990).

In vivo *methods*

Design of the in vivo *experiment*

A total of 36 animals were randomly allocated into six experimental groups, each consisting of six animals ($n = 6$). The route of administration of all compounds was oral gavage. Silymarin was used as a reference since there is a well-established and ethically approved model at our laboratory (Simeonova et al., 2010; 2015).

Group 1 – control animals, treated with saline (0.5 ml/g bw) for 21 days.

Group 2 – animals treated with AF alone (10 mg/kg/p.o/21 days).

There is no data present for doses of alcesefoliside (**1**) in animal models. As seen from the *in vitro* study, 10 μmol/ml was the mean effective concentration. This was extrapolated *via* the molecular mass of **1**, resulting in approximate dose of 10 mg/kg. Additionally,

the dose of **1** was confirmed on literature data for rutin, which is chemically similar to AF (Lima Gonçalves et al., 2013).

Group 3 – animals treated with silymarin (100 mg/kg/*p.o.*/21 days) (Habbu et al., 2008)

Group 4 – animals challenged with CCl_4 (10% solution, 1.25 ml/kg *p.o.*) on the 7th day of the experiment (Ahn et al., 2007).

Group 5 – animals treated with AF (10 mg/kg/*p.o.*/7 days). On the 7th day, 90 min after the last treatment the animals were challenged with CCl_4 (10% solution, 1.25 ml/kg *p.o.*) and after that treated with AF in the same dose for additional 14 days.

Group 6 – animals treated with silymarin (100 mg/kg/*p.o.*/7 days). On the 7th day, 90 min after the last treatment the animals were challenged with CCl_4 (10% solution, 1.25 ml/kg *p.o.*) and after that treated with silymarin in the same dose for additional 14 days.

On the day 22 of the experiment the animals were sacrificed by decapitation and brains were taken for biochemical assays and histopathology. For all following experiments the excised brains were washed out with cold (4 °C) saline solution (0.9% NaCl), blotted dry, weighed, and homogenized with appropriate buffers.

Assessment of biochemical parameters in whole brain

Our experiment was carried out in whole brain. Therefore no particular brain structures were identified and isolated. Briefly, the procedure we followed was as follows: after decapitation of six ($n = 6$) rats from each group, the brains were taken out, measured, and divided into five parts, one for measurement of AChE activity, one for assessment of MDA quantity, one for GSH levels assessment, one for measurement of antioxidant enzymes, and one for histopathological examination. The brain samples were consequently homogenized with the respective buffers.

Preparation of brain tissue extracts and assessment of acetylcholinesterase enzyme (AChE) activity

The brains were minced and homogenized in 10 vol of 0.1 M phosphate buffer (pH 7.4) and aliquots of homogenates of the individual rat brain of the various treatment groups were taken and used to measure AChE activity by Elman's method (Ellman, 1959), where thiocholine, produced by acetylcholinesterase enzyme (AChE), reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) to form a colour product (with A_{max} 412 nm), proportional to the AChE activity. AChE activity was calculated and expressed as μmol/min/mg protein, using the molar extinction coefficient of 13.600 M^{-1} cm^{-1}. The protein content of brain homogenate was assessed (Lowry et al., 1951) with bovine serum albumin as a standard.

Preparation of brain homogenate for assessment of malondialdehyde (MDA)

The brains were homogenized with 0.1 M phosphate buffer and EDTA, pH = 7.4 (1:10). Lipid peroxidation was determined by measuring the rate of production of thiobarbituric acid reactive substances (TBARS) (expressed as malondialdehyde (MDA) equivalents) as described by with slight modifications (Polizio and Pena, 2005). Briefly one vol. of the brain homogenate was mixed with 1 ml 25% trichloroacetic acid (TCA) and 1 ml 0.67% thiobarbituric acid (TBA). Samples were then mixed thoroughly, heated for 20 min on a boiling water bath, cooled, and centrifuged at 4000 × *g* for 20 min. The absorbance of supernatant was measured at 535 nm against a blank that contained all the reagents except the tissue homogenate. MDA concentration was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed in nmol/g wet tissue.

Preparation of brain homogenate for reduced glutathione (GSH) assessment

GSH was assessed by measuring nonprotein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA) (Bump et al., 1983). Briefly, brains were homogenized in 5% TCA (1:10) and centrifuged for 20 min at 4000 × *g*. The reaction mixture contained 0.05 ml supernatant, 3 ml 0.05 M phosphate buffer (pH = 8), and 0.02 ml DTNB reagent. The absorbance was determined at 412 nm and the results were expressed as nmol/g wet tissue.

Preparation of brain homogenates for antioxidant enzyme activity measurement

Known amounts of brain were rinsed in ice-cold physiological saline and minced with scissors. Ten per cent homogenates were prepared in 0.05 M phosphate buffer (pH = 7.4) and centrifuged at 7000 × *g* and the supernatant was used for antioxidant enzymes assay. Analyses were performed in triplicate and the average values were used. Protein content was measured by the Lowry's method (Lowry et al., 1951). Catalase activity was assessed (Aebi, 1974). Briefly, 10 μl of homogenate was added to 1990 μl of H_2O_2 solution (containing 6.8 μl of 30% H_2O_2 + 1983.2 μl 0.05 M phosphate buffer, pH = 7.4). CAT activity was determined by monitoring the H_2O_2 decomposition which was measured spectrophotometrically by the decrease in absorbance at 240 nm. Enzyme activity was calculated using a molar extinction coefficient of 0.043/mM^{-1}/cm^{-1} and expressed as μM/min/mg protein. Superoxide dismutase activity (SOD) was measured (Misra and Fridovich, 1972) following spectrophotometric autoxidation of epinephrine at pH 10.4, 30 °C, using the molar extinction coefficient of 4.02/mM^{-1}/cm^{-1}. The incubation mixture contained 50 mM glycine buffer, pH 10.4. The reaction was started by the addition of epinephrine. SOD activity was expressed as nmol of epinephrine prevented from autoxidation after addition of the sample. Glutathione peroxidase (GPx) activity was measured by NADPH oxidation, using a coupled reaction system consisting of glutathione, glutathione reductase, and cumene hydroperoxide (Tappel, 1978). Briefly, 100 μl of enzyme sample was incubated for 5 min with 1.5 ml 0.05 M phosphate buffer (pH 7.4), 100 μl 1 mM EDTA, 50 μl 1 mM GSH, 100 μl 0.2 mM NADPH, and 1 unit of glutathione reductase. The reaction was initiated by adding 50 μl cumene hydroperoxide (1 mg/ml) and the rate of disappearance of NADPH with time was determined by monitoring its absorbance at 340 nm. Results were expressed as nmol/min/mg protein using the molar extinction coefficient of 6.22 mM^{-1} cm^{-1}. Glutathione reductase (GR) activity was measured by following NADPH oxidation spectrophotometrically at 340 nm and using an extinction coefficient of 6.22 mM^{-1} cm^{-1} (Pinto et al., 1984). The incubation mixture contained 0.05 M phosphate buffer, pH = 7.4, 2.5 mM GSSG, and 125 μM NADPH at 30 °C. Results were expressed as nmol/min/mg protein.

Glutathione-*S*-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate (Habig et al., 1974). The incubation mixture containing 1.6 ml 0.05 M phosphate buffer, 100 μl 1 mM GSH, 100 μl 1 mM EDTA, and 100 μl homogenate was incubated for 15 min at 37 °C. After the incubation, 100 μl 1 mM CDNB was added and the increase in absorbance with time was recorded at 340 nm. Enzyme activity was measured using an extinction coefficient of 9.6×10^3/M^{-1}/cm^{-1} and was expressed as nmol of CDNB-GSH conjugate formed/min/mg protein.

Histopathological examination

The brain samples from all experimental animals were 1 cm thick, fixed with 10% neutral buffered formalin and then washed

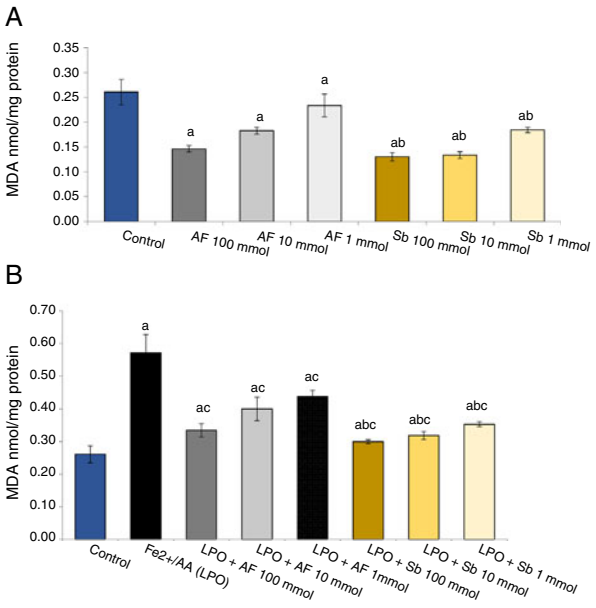

Fig. 1. Effect of alcesefoliside (AF) and silybin on MDA quantity assessed in pure microsomes (**A**) and in microsomes with Fe^{2+}/AA - induced LPO (**B**). Data are expressed as mean ± SD of four different experiments; [a] Significant difference from control values (Mann–Whitney *U*-test, $p < 0.05$); [b] Significant difference from respective equimolar concentration AF (Mann–Whitney *U*-test, $p < 0.05$); [c] Significant difference from induced LPO (Mann–Whitney *U*-test, $p < 0.05$); [a] $p < 0.05$ vs control; [b] $p < 0.05$ vs respective equimolar concentration AF; [c] $p < 0.05$ vs induced LPO.

under running tap water for 24 h. The tissues were dehydrated by series of passages through increasing concentrations of ethanol then cleared with xylene and immersed in melted paraffin for 12 h. The paraffin blocks were sliced on a rotating microtome (5 μm), dewaxed, rehydrated in decreasing concentrations of ethanol and stained with Haematoxylin-Eosin. Microscopic examination and micro photographing were performed with a microscope Euromex BioBlue (Bancroft and Gamble, 2002). Normal brain tissue consisting of typical grey and white matter, of healthy neurons and normal circulation was the criteria for the absence of CCl_4-induced damage. For evaluation of the pathological effects, both degenerative changes in the nerve cells and haemodynamic lesions in the brain tissue were chosen as criteria for damage.

Statistical analysis

For the *in vitro* experiments the statistical analysis included ANOVA and the Student's *t*-test. The results were presented as mean ± SD of six animals per group. For each of the examined parameters, three parallel samples were used. Statistical program 'MEDCALC' was used for analysis of the *in vivo* data. The results are expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the nonparametric Mann–Whitney *U* test. For both statistical methods, values of $p \leq 0.05$ were considered statistically significant.

Results

Ascorbic-iron (Fe^{2+}/AA) induced lipid peroxidation

Alcesefoliside (**1**) as well as silybin after incubation decreased the MDA levels in pure microsomes in concentration dependent manner (Fig. 1). The effect was more pronounced at higher concentrations. At concentration 100 μmol and 10 μmol alcesefoliside decreased statistically significant the level of MDA with 44% ($p < 0.05$) and 34% ($p < 0.05$) respectively. This reduction of MDA formation is commeasurable with the effect of silybin. Microsomal incubation with Fe^{2+}/AA mixture led to a statistically significant increase in MDA production by 119% ($p < 0.05$). The pre-incubation of the microsomes with **1** (100, 10 and 1 μmol) prior to initiation of lipid peroxidation (LPO) reduced the formation of MDA in a concentration-dependent manner. The most prominent effect was observed at 100 μmol. Compared to the Fe^{2+}/AA group, the MDA production measured at 100 μmol, was reduced by 42% ($p < 0.05$) after pre-incubation with AF. Pre-incubation with equimolar concentrations with silybin, resulted in almost the same antioxidant activity. The MDA production measured in microsomes incubated with at 100 μmol silybin was reduced by 48% ($p < 0.05$) (Fig. 1B).

In vivo *study*

During treatment, there were no changes observed in behaviour or in food and water consumption among the animals in either the control or treated groups. All animals survived until the end of the treatment period.

Lipid peroxidation, cell glutathione and acetylcholinesterase activity

Compared to the control group, CCl_4 administration induced significant pro-oxidant effects, discerned by a marked increase, by 72% ($p < 0.05$) in MDA production, and decrease in reduced glutathione (GSH) levels by 45% ($p < 0.05$). In addition, a significant ($p < 0.05$) decrease in AChE activity by 66% was seen (Table 1). Treatment with AF, administered alone decreased the MDA formation in the brain with 29% ($p < 0.05$) and increased the GSH level with 45% ($p < 0.05$), compared to control group. Compared to the CCl_4 treated group, AF administration (21 days) significantly decreased the MDA content by 24% and increased the GSH level by 60%. However, the effect of SM was more pronounced, because it decreased the formation of MDA by 37% ($p < 0.05$) and increased the quantity of the cell protector GSH by 93% ($p < 0.05$), compared to CCl_4 intoxicated group. The administration of both compounds AF and SM, did not affect the AChE activity in CCl_4 treated group.

Antioxidant enzyme activity

Compared to the control group, CCl_4 administration induced a significant ($p < 0.05$) decrease in antioxidant enzyme activity as follow: CAT by 50%, SOD by 48%, GPx by 47%, GR by 43%, and GST by 24% (Table 2). Compared to CCl_4-only group, the treatment with AF (21 days in total) resulted in significant increase in CAT activity by 54%, in SOD activity by 39%, in GPx by 64%, in GR by 70% and in GST – by 22%. The antioxidant and neuroprotective effect of alcesefoliside was comparable to those of silymarin.

Histopathology

In the brain tissue of the animals from the control group normal histological architecture was observed. The grey and white matters were composed primarily of myelinated axons, oligodendroglia, astrocytes, and microglia. In the grey matter the ganglion cells were also located. Clear spaces in white matter surrounding large axons (artefacts formed when the lipid components of myelin lamellae were dissolved by solvents in the process of embedding tissue in paraffin for sectioning) were visible (Fig. 2A). CCl_4 treatment resulted in brain haemorrhages, degenerative changes in ganglion and Purkinje cells of the cerebellum. Cells with blurred, granular, eosinophilic cytoplasm with unclear and displaced nuclei were visible. Also presence of haemodynamic disorders in the brain substance like haemorrhagic lesions with accumulation of blood (erythrocytes) in the brain parenchyma were observed (Fig. 2B).

Table 1
Effect of alcesefoliside (**1**) and silymarin treatment on brain lipid peroxidation (MDA), cell glutathione (GSH) and acetylcholinesterase (AChE) activity in rats challenged with CCl_4.

Groups	MDA[a]	% v/s control	% vs CCl_4	GSH[a]	% v/s control	% vs CCl_4	AChE[b]	% v/s control	% vs CCl_4
Control	3.23 ± 0.38	100		1.32 ± 0.19	100		0.93 ± 0.09	100	
1	2.62 ± 0.45	−29		1.92 ± 0.22[c]	+45		0.96 ± 0.17	+3	
SL	1.61 ± 0.20[c]	−50		2.08 ± 0.14[c]	+58		0.90 ± 0.15	−3	
CCl4	5.56 ± 0.35[c]	+72	100	0.73 ± 0.15[c]	−45	100	0.32 ± 0.07[c]	−66	100
1 + CCl_4	4.23 ± 0.16[c,d]	+31	−24	1.17 ± 0.14[d]	−11	+60	0.40 ± 0.09[c]	−57	+25
SL + CCl_4	3.53 ± 0.07[d]	+9	−37	1.41 ± 0.13[d]	+7	+93	0.43 ± 0.05[c]	−54	+34

[a] nmol/g tissue.
[b] μmol/min/mg protein.
[c] Significant difference from control values (Mann–Whitney *U*-test, $p < 0.05$).
[d] Significant difference from CCl_4-treated group (Mann–Whitney *U*-test, $p < 0.05$).

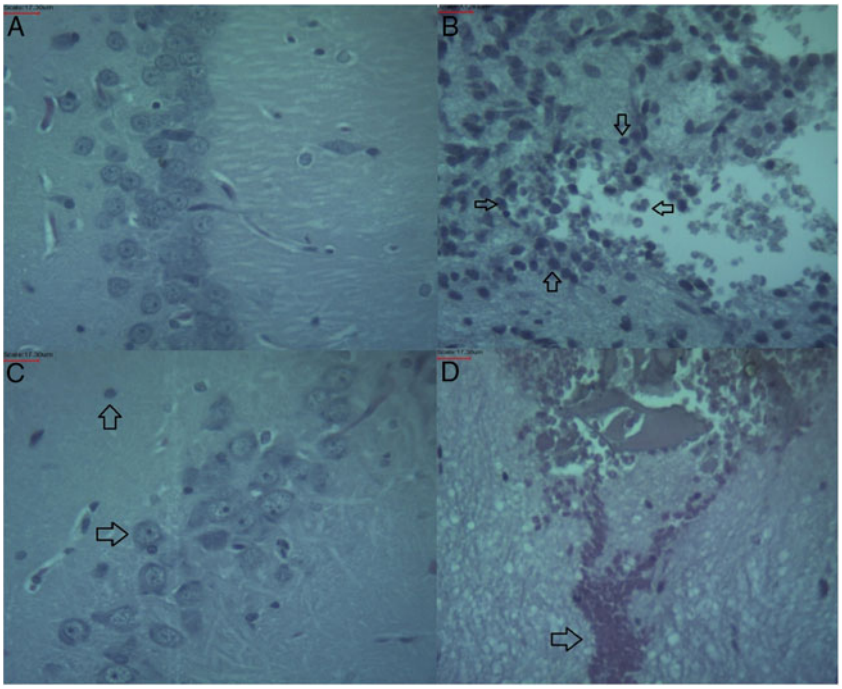

Fig. 2. Histopathology (H&E stain, scale bars 17.30 mm); **A**: Control; **B**: Hemisphere of a rat treated with CCl_4. Accumulation of erythrocytes, glial and mononuclear cells; **C**: Hemisphere of a rat treated with CCl_4 and protected with AF. Intact ganglion and glial cells; **D**: Hemisphere of a rat treated with CCl_4 and protected with Silymarin. Accumulation of erythrocytes in the brain parenchyma.

Pre-treatment of the animals with AF resulted in neuroprotective effect against CCl_4-induced brain damage. In the brains of the animals pre-treated with alcesefoliside and then challenged with CCl_4 no hemodynamic lesions and degenerative-necrotic changes in the ganglion and glial cells were observed (Fig. 2C). However, single haemorrhages were found in the brain parenchyma of the animals, protected with Silymarin, indicating weaker protection in this group, compared to rats pre-treated with AF (Fig. 2D).

Discussion

Since endogenous antioxidant defences are not always completely effective, and since exposure to damaging environmental factors is increasing, it seems reasonable to propose that exogenous antioxidants could be effective in diminishing the cumulative effects of oxidative damage. However, the therapeutic use of most of these compounds is limited since they do not cross the blood brain barrier (BBB). That is why, any novel biologically active substance with antioxidant potential, especially from the group of flavonoids, should be investigated for neuroprotection with potential to cross the BBB after systemic administration. Flavonoids were reported to penetrate BBB (Kondeva-Burdina et al., 2014).

In the *in vitro* experiment, carried out in isolated brain microsomes, alcesefoliside (**1**) exerted concentration-dependent antioxidant activity in pure microsomes as well as in microsomes with Fe^{2+}/AA-induced lipid peroxidation. The effect was discerned by MDA quantity reduction and was almost similar to those of silybin (Fig. 1B). The decreased lipid peroxidation in non-treated brain microsomes might be explained by the fact that brain has a higher level of MDA when compared to other organs. The observed effect of **1** was similar to those of the positive control silybin, which antioxidant activity is well documented. One of the main mechanisms through which silybin exerts an antioxidant activity, is its ability to inhibit the formation of superoxide anion and nitric oxide (NO) radicals. There are also data that silybin serves as ROS scavenger (Borsari et al., 2001). On the basis of our results showing similar antioxidant potential of alcesefoliside (**1**) and silybin and based on the chemical similarity, we suggest also similar *in vitro* antioxidant mechanisms.

Based on the antioxidant activities of alcesefoliside (**1**) proved in the *in vitro* part of our study we further investigated the biological activity of **1** against CCl_4-induced brain damage in male Wistar rats. Animals challenged with CCl_4 had increased MDA production, depleted GSH level and reduced AChE and antioxidant

Table 2
Effect of alcesefoliside (**1**) and silymarin treatment on antioxidant profile in rats challenged with CCl_4.

Groups	CAT[a]	% vs control	% vs CCl_4	SOD[b]	% vs control	% vs CCl_4	GPx[b]	% v/s control	% vs CCl_4	GR[b]	% v/s control	% vs CCl_4	GST[c]	% v/s control	% vs CCl_4
Control	22.7 ± 2.5	100		0.330 ± 0.02	100		0.68 ± 0.06	100		3.11 ± 0.2	100		0.89 ± 0.06	100	
1	19.4 ± 2.0	−15		0.339 ± 0.01	+3		0.79 ± 0.09	+16		3.34 ± 0.19	+7		1.08 ± 0.15	+21	
Silymarin	22.8 ± 2.9	0		0.332 ± 0.02	+1		0.89 ± 0.04[c]	+31		3.49 ± 0.23	+12		1.20 ± 0.09[c]	+35	
CCl_4	11.3 ± 1.6[c]	−50	100	0.172 ± 0.02[c]	−48	100	0.36 ± 0.05[c]	−47	100	1.78 ± 0.18[c]	−43	100	0.68 ± 0.07[c]	−24	100
1 + CCl_4	17.4 ± 0.6[c,d]	−23	+54	0.239 ± 0.03[c,d]	−28	+39	0.59 ± 0.08[d]	−13	+64	3.02 ± 0.15[d]	−3	+70	0.83 ± 0.05[d]	−7	+22
SL + CCl_4	19.5 ± 1.3[d]	−14	+73	0.231 ± 0.02[c,d]	−30	+34	0.69 ± 0.09[c,d]	+1	+92	3.34 ± 0.13[d]	+7	+88	0.90 ± 0.04[d]	+1	+32

[a] μmol/min/mg protein.
[b] nmol/min/mg protein.
[c] Significant difference from control values (Mann–Whitney *U*-test, $p < 0.05$).
[d] Significant difference from CCl_4-treated group (Mann–Whitney *U*-test, $p < 0.05$).

enzyme activity: CAT, SOD, GPx, GR, and GST (Tables 1 and 2). These results are in good correlation with previous findings (Makni et al., 2012). The observed tissue biochemical changes are confirmed by the histological changes: haemorrhages, degenerative changes in ganglion cells and Purkinje cells of the cerebellum, induced by CCl_4 (see Fig. 2B). Alcesefoliside, administered alone for 21 days, did not produce remarkable changes in investigated biochemical brain parameters, especially on antioxidant enzyme activities. However, it produced protective effect on the brain after CCl_4-induced damage most probably due to the increased permeability of the BBB – a result of CCl_4 injury. It was showed that rats with CCl_4-induced cirrhosis of the liver revealed structural changes in the haematoencephalic barrier presenting as peri- and endocapillary changes and changes in vascular peduncles of astrocytes (Mishchenko et al., 1993). Compared to CCl_4 group, the 21-day treatment with AF (7-day treatment before challenge with CCl_4, followed by 14-day curative treatment) ameliorated the neurotoxic effect induced by the toxicant. This was evidenced by a significant decrease of MDA production and increase in GSH levels as well as by preserving antioxidant enzyme activities (CAT, SOD, GPx, GR, and GST) (Tables 1 and 2). These effects of alcesefoliside might be attributed to its flavonoid structure. Flavonoids can prevent injury caused by ROS in various ways. The main way is the direct scavenging of free radicals (*i.e.* superoxides). The effects of AF *in vivo* were compared with silymarin, a preferable positive control substance with proven antioxidant and neuroprotective properties (Borah et al., 2013). Silymarin has been implicated in protecting neurons against oxidative stress (Nencini et al., 2007). Alcesefoliside (**1**) brain protection was supported also by the prevention of CCl_4 caused histopathological changes (see Fig. 2C). Its effect was similar to those of the effect of silymarin on this level. On the other hand, single lesions and erythrocyte infiltrations were still visible in the brain tissue of rats, protected with Silymarin. This was not found in animals, protected with **1**, which give us a reason to believe in stronger neuroprotective activity of **1**. Radical scavenging activities could be assumed for **1**.

Under the conditions of our study, AF showed antioxidant activity in *in vitro* model of Fe^{2+}/AA-induced lipid peroxidation in isolated brain microsomes and *in vivo* neuroprotective activity against CCl_4-induced brain injury in rats.

Authors' contributions

IK collected the plant material and designed the study. AS carried out the extraction and purification of AF. RS and VV performed the *in vivo* experiment and wrote the first draft. MKB performed *in vitro* experiment. GP and VM performed histological examination of the tissues. All authors contributed to the critical revision of the manuscript.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflicts of interest

The authors declare that they have no conflict of interest.

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