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Article

Hepatoprotective and antioxidant effects of alcesefoliside from *Astragalus monspessulanus*

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The hepatoprotective potential of alcesefoliside (AF) from *Astragalus monspessulanus* was investigated. Iron sulphate/ascorbic acid (Fe^{2+}/AA) lipid peroxidation was induced in rat liver microsomes and pre-incubated with AF and silybin (100, 10 and 1 μmol). Pronounced effects were observed in 100 μmol. *In vivo* experiments were carried out on rats, challenged orally with carbon tetrachloride (CCl_4) alone and after pre-treatment and followed by curative treatment with AF (10 mg/kg). The activity of the serum and antioxidant enzymes, together with reduced glutathione (GSH) levels and malonedialdehyde (MDA) quantity were measured. Microsomal incubation with Fe^{2+}/AA increased MDA production. The pre-incubation with AF reduced the formation of MDA, comparable to silybin. These findings were supported by the *in vivo* study where CCl_4-induced liver damage was discerned by significant increase in serum enzymes and in MDA production as well as by GSH depletion and reduced antioxidant enzymes activity. The AF pre-treatment and consecutive curative treatment normalizes the activity of the serum and antioxidant enzymes alike, as well as the levels of GSH and MDA. Histological examination of AF-treated livers showed a decrease in the abnormal accumulation of lipids in hepatocytes as well as reduced alterative changes in their structure in a model of CCl_4-induced toxicity.

Keywords: Hepatoprotective activity. Antioxidant activity.

INTRODUCTION

Oxidative stress is described as a perturbation of redox signalling which results from an excessive reactive oxygen species generation. Reactive oxygen species (ROS) play an important role in the pathogenesis of various disorders such as atherosclerosis, liver disorders, lung and kidney damage, aging and diabetes mellitus (Singh *et al*., 2008; Simeonova *et al*., 2019). Free radicals are generated in cells by normal metabolism as well as by environmental factors such as ultraviolet radiation, pollutants, X-rays and xenobiotics. There are many studies reporting the key role of the oxidative stress in the hepatic injury, induced by CCl_4. It has been found that chlorinated free radicals, formed *via* cytochrome P450 bio activation of CCl_4, such as trichloromethyl ($\cdot CCl_3$) and trichloromethyl peroxyl ($\cdot OOCCl_3$) radicals, impair the hepatocytes, inducing morphological changes in the endoplasmic reticulum, Golgi apparatus, plasma membrane, and mitochondria of the targeted cells, leading to apoptosis (Li *et al*., 2019). Membrane disintegration of hepatocytes results in subsequent release of aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH) and γ-glutamyltransferase (GGT) as well as decreased endogenous antioxidant defence system (Singh *et al*., 2008; Simeonova *et al*., 2014).

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A number of plants, including those of the genus *Astragalus*, have been shown to possess a number of pharmacological properties varied from immunostimulant effects, anti-bacterial and antiviral properties to hepatoprotective, anti-inflammatory activity, cardiovascular tonic effects and others The antioxidant properties of the *Astragalus* species are mainly attributed to the flavonoids as one of the main constituents (Pistelli, 2002). Flavonoids have been isolated from many species of the genus *Astragalus* (Krasteva *et al*., 2015).

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 (Shkondrov *et al*., 2015; Simeonova *et al*., 2015; Vitcheva *et al*., 2013; Simeonova *et al*., 2010). Alcesefoliside (quercetin-3-*O*-α-L-rhamnopyranosyl-(1→2)-[α-L-rhamnopyranosyl-(1→6)]-β-D-galactopyranoside) is a rare flavonol tetraglycoside with cytoprotective activity in a model of *tert*-butylhydroperoxide induced oxidative stress in isolated rat hepatocytes (Krasteva *et al*., 2015). The flavonol showed neuroprotective activity *in vitro* (on non-enzyme induced lipid peroxidation) and *in vivo* (on carbon tetrachloride-induced encephalopathy) (Simeonova *et al*., 2019). The compound was isolated in the present study in significantly large quantity from *A. monspessulanus* L. which allowed the current research.

The aim of the present research was to examine the antioxidant and hepatoprotective properties of alcesefoliside (AF) using both *in vitro* model of non-enzyme induced lipid peroxidation in isolated liver microsomes and *in vivo* model of CCl_4-induced liver damage in rats. The effects of AF were compared with silybin and silymarin, used as positive controls for the *in vitro* and *in vivo* experiments, respectively.

MATERIAL AND METHODS

Plant material, extraction and isolation of alcesefoliside

The aerial parts of *A. monspessulanus* were collected in May 2016 from Rodopi Mountain, close to the town of Dzhebel, Bulgaria, at coordinates 41°32′56.00″ N, 25°21′47.43″ E. The plant was identified by Dr. D. Pavlova from the Department of Botany, Faculty of Biology, Sofia University, where a 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 main fractions (I-IX). Fraction III was chromatographed over Sephadex LH-20 with MeOH as eluent and six subfractions (A1-A6) were collected. Subfraction A4 was purified by repeated LPLC over ODS C_{18} with MeOH-H_2O (40:60, v/v) as an eluent and further subjected to isocratic semi-preparative HPLC using a mobile phase MeCN-H_2O (14:86, v/v) to give 435 mg AF (98% purity, HPLC) (Figure 1). The compound was identified by comparing the experimental and reported ^1H and ^{13}C NMR data (Krasteva *et al*., 2015).

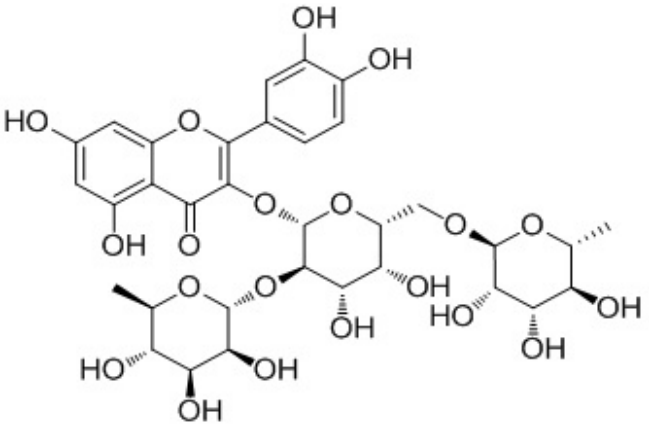

FIGURE 1 - Structure of alcesefoliside.

Chemicals

All the reagents used were of analytical grade. Carbon tetrachloride, as well as other chemicals, ascorbic acid, silybin, silymarin, beta-nicotinamide adenine dinucleotide 2`-phosphate reduced tetrasodium salt (NADPH), reduced glutathione (GSH), oxidized glutathione (GSSG), glutathione reductase (GR), 2,4-dinitrochlorobenzene (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).

Animals

Male Wistar rats with body weight 200–250 g were used. The rats were housed in Plexiglas cages (3 per cage) in a 12/12 light/dark cycle, under standard laboratory conditions (ambient temperature 20 °C ± 2 °C and humidity 72% ± 4%) with free access to water and standard pelleted rat food 53-3, produced according ISO 9001:2008. 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 of farm № 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.

Experiments on isolated rat liver microsomes

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 AF – both are pure compounds and share a flavonoid structure. Liver microsomes were isolated from untreated rats (Guengerich, 1987). Briefly, the livers of the animals, sacrificed by cervical decapitation were removed after perfusion with ice-cold 1.15% KCl and then homogenized with ice-cold 0.1 M Tris-potassium buffer, pH 7.5 (1:4), using a glass homogenizer with Teflon pestle. The prepared liver homogenate was centrifuged at 10 000 x *g* for 20 min to obtain the post mitochondrial fraction which was then centrifuged at 105 000 x *g* for 60 min to obtain microsomal fraction. 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 (Lowry *et al*., 1951) and adjusted to 1 mg protein/mL. The isolated microsomes were pre-incubated with AF and silybin, as a positive control at three consequently decreased equimolar concentrations: 100 μmol, 10 μmol, and 1 μmol. The pre-incubation was performed at 37 °C for 15 min. The reaction was started with 20 mM solution of iron sulphate and 0.5 mM ascorbic acid (Mansuy *et al*., 1986). The reaction was stopped with a mixture of 25% TCA and 0.67% TBA at 20 min after lipid peroxidation (LPO) initiation. The quantity of MDA was assessed (Mansuy *et al*., 1986).

Antioxidant and biochemical parameters in rats

For 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.

Group 1 – control animals, treated with olive oil (1.25 mL/kg, p.o.).
Group 2 – animals treated with AF alone (10 mg/kg, p.o./ 21 days) (Simeonova *et al*., 2019).
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 in olive oil, 1.25 mL/kg, p.o.) on the 7th day (Ahn *et al*., 2007)
Group 5 – animals treated with AF (10 mg/kg, p.o/7 days). On the 7th day, 90 minutes after the last treatment the animals were challenged with CCl_4 (10% solution in olive oil, 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 minutes after the last treatment the animals were challenged with CCl_4 (10% solution in olive oil, 1.25 mL/kg, p.o.) and after that treated with silymarin in the same dose for additional 14 days.

On the 22nd day of the experiment blood for biochemical analysis was collected from the tail vain

of all animals then the animals were sacrificed by decapitation and livers were taken for biochemical assays and histopathology. For all following experiments the excised livers were washed out with cold saline solution (0.9% NaCl), blotted dry, weighed, and homogenized with appropriate buffers.

Animals were made comfortable in a restrainer while maintaining the temperature around at 35 to 40 °C. Lidocaine cream (2%) was applied on the surface of the tails 30 min before the experiment. Blood was collected using a capillary tube containing ethylene glycol tetra-acetic acid. Serum was separated by centrifugation in a bench centrifuge (Eppendorf MiniPlus) at 10 000 x *g* for 10 min, at 4 °C. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT) activities were measured, using commercially available standard diagnostic kits using an automatic chemistry analyser (BS-120, Mindray, Shenzhen, China).

Oxidative damage was determined by measuring MDA equivalents (Polizio, Pena, 2005). GSH was assessed by measuring of non-protein sulfhydryls after precipitation of proteins with trichloroacetic acid (TCA) (Bump, Taylor, Brown, 1983). The antioxidant enzymes activity was measured in the supernatant of 10% homogenates, prepared in 0.05 M phosphate buffer (pH 7.4). The protein content of liver homogenate was measured (Lowry *et al*., 1951). Catalase activity was determined by measuring the decrease in absorbance at 240 nm and expressed as μM/mg (Aebi, 1974). Superoxide dismutase (SOD) activity was measured (Misra, Fridovich, 1972). Glutathione peroxidase activity (GPx) was assessed by NADPH oxidation, using a coupled reaction system consisting of glutathione, GR, and cumene hydroperoxide (Tappel, 1978). Glutathione reductase activity (GR) was measured spectrophotometrically at 340 nm (Pinto, Mata, Lopez-Barea, 1984) by following NADPH oxidation. GST was measured using CDNB as a substrate (Habig, Pabst, Jakoby, 1974).

Histopathological examination

For light microscopic evaluation, liver tissues were fixed in 10% buffered formalin, embedded in paraffin and then thin sections (5 μm) were subsequently stained with haematoxylin/eosin (H&E) for general histological features determination (Bancroft, Gamble, 2008). Sections were studied under light microscope Euromex BioBlue. Histological evaluation was performed on liver sections of each animal (n = 6) and the liver damage expressed as a per cent.

Statistical methods

Statistical analysis was performed using statistical programme 'MEDCALC'. Results are expressed as mean ± SEM for six rats in each group. The significance of the data was assessed using the non-parametric Mann–Whitney *U* test. Values of $p \leq 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

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

The effect of AF on MDA quantity assessed in Fe^{2+}/AA LPO model is shown in Figure 2. Neither AF nor silybin changed the MDA levels in pure microsomes after incubation (Figure 2 A). Microsomal incubation with Fe^{2+}/AA mixture led to a statistically significant increase in MDA production by 218% ($p < 0.05$). The pre-incubation of the microsomes with AF (100, 10, 1 μmol) prior to initiation of 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 59% ($p < 0.05$) after pre-incubation with AF. Pre-incubation with equimolar concentrations with silybin, however, resulted in better antioxidant activity. The MDA production measured in microsomes incubated with at 100 μmol silybin was reduced by 67% ($p < 0.05$) (Figure 2B).

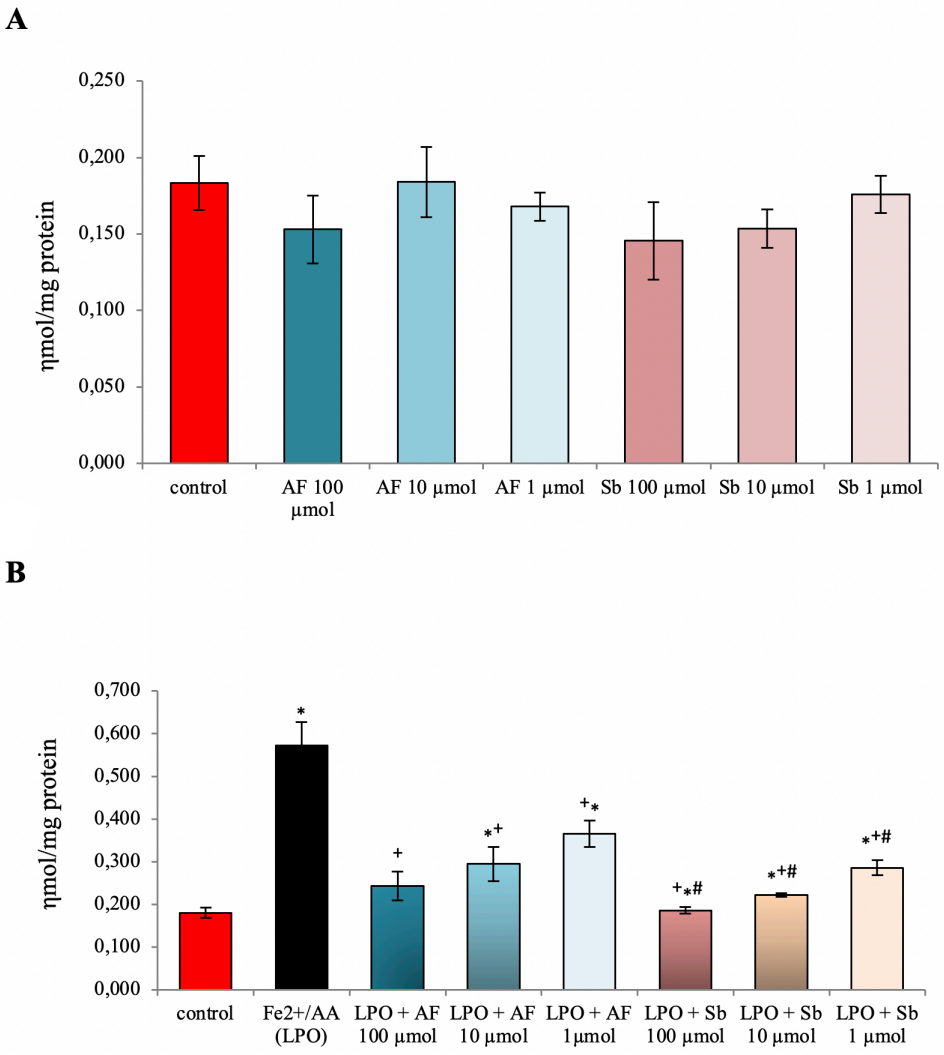

FIGURE 2 - Effect of alcesefoliside (AF) and silybin (Sb) on MDA quantity assessed in pure microsomes (2A) and in microsomes with Fe^{2+}/AA - induced LPO (2B). Mean ± SEM (n = 6). *$p < 0.05$ *vs* control; $^{+}p < 0.05$ *vs* induced LPO; $^{\#}p < 0.05$ *vs* respective equimolar concentration AF.

Changes in antioxidant profile and biochemical parameters on Wistar rats

During treatment, there were no observed changes 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.

The effect of AF on CCl_4-induced alteration in serum enzyme activity is shown in Table I. A significant ($p < 0.05$) increase in the activity of serum ALT, AST, ALP and GGT was observed in rats challenged with CCl_4 as follows: AST was increased by 65%, ALT – by 53%, ALP – by 49% and GGT – by 39%. The treatment with AF resulted in significant ($p < 0.005$) decreases in enzyme activities, compared to the CCl_4 only group. The effect of AF was comparable to those of silymarin.

The effect of AF and silymarin on hepatic lipid peroxidation and antioxidant enzymes against CCl_4-induced toxicity in rats is presented in Table II. Compared to the control group, CCl_4 administration induced significant pro-oxidant effects, discerned by a marked increase, by 41% ($p < 0.05$) in MDA production and decrease in GSH levels by 50% ($p < 0.05$). In addition, a decrease ($p < 0.05$) in antioxidant enzyme activity as follow: CAT by 48%, SOD by 36%, GPx by 48% and GST by 46% was also observed. Compared to CCl_4-only group, the treatment

with AF (21 days in total) resulted in significant decrease in MDA quantity by 23% ($p < 0.05$) and an increase ($p < 0.05$) in GSH levels and CAT activity by 77%, in SOD activity by 53%, in GPx by 51%, in GR by 38% and in GST – by 66%. The antioxidant and hepatoprotective effect of AF was comparable to those of silymarin.

TABLE I - Effect of AF and silymarin (SL) pre-treatment on CCl_4-induced alterations in serum enzyme activities

Enzyme, U/L	Control	CCl_4	SL	CCl_4 + SL	AF	CCl_4 + AF
AST	183.9 ± 32	302 ± 23*	170±25	195.6±12+	183±12	200.6±20+
ALT	56.7 ± 5.7	86.7 ±10.8*	65.3±0.62	57.15±3.7+	54.5±21	56.4±1.4+
ALP	338.7 ± 39	506±10.14*	363±79	395.6±64+	299±84	380±23.5+
GGT	2.1 ± 0.43	2.93±0.3*	2.03±0.3	1.60±0.32+	1.4±0.9	1.66±0.35+

Mean ± SEM (n = 6). *Significant difference from control values (Mann–Whitney U-test, p < 0.05); +Significant difference from CCl4-treated group (Mann–Whitney U-test, p < 0.05).

TABLE II - Effect of AF and silymarin (SL) treatment on hepatic liver peroxidation and antioxidant profile in rats challenged with CCl4

Group	Control	AF	Silymarin	CCl_4	AF+CCl_4	SL+CCl_4
MDAa	3.92±0.08	3.94±0.05	3.88±0.16	5.54±0.24*	4.27±0.24+	4.03±0.17*+
GSHa	6.08±0.30	6.19±0.23	6.85±0.19	3.02±0.21*	5.34±0.3+	5.93±0.29+
CATb	79.8±5.9	84.9±4.7	86.2±2.1	41.3±1.9*	73.0±3.0+	71.3±7.6+
SODc	0.238±0.03	0.225±0.02	0.220±0.02	0.152±0.01*	0,233±0.03+	0.232±0.02+
GPxc	0.402±0.05	0.383±0.03	0.428±0.04	0.243±0.03*	0.367±0.05+	0.360±0.07*+
GRc	0.224±0.02	0.203±0.01	0.229±0.02	0.116±0.01*	0.160±0.01*+	0.178±0.01*+
GSTb	1.427±0.1	1.468±0.26	1.418±0.12	0.767±0.11*	1.274±0.04+	1.343±0.12+

Mean ± SEM (n = 6). *Significant difference from control values (Mann–Whitney U-test, p < 0.05);
+Significant difference from CCl4-treated group (Mann–Whitney U-test, p < 0.05); anmol/g tissue; bnmol/min/mg protein; cμmol /min/mg protein

Pathological study

The livers of animals from the control group were with brownish-red colour, smooth capsular surface and well-formed lobar structure. The livers of the animals treated with CCl_4 were enlarged with rounded edges, bulges on incision, greasy and yellowish in colour. The consistency was soft and friable. Animals from AF and Silymarin treated groups had livers, macroscopically similar to control. Livers of animals, protected with AF and treated with CCl_4 were with normal gross morphological appearance without visible lesions. The same finding was observed for those animals, protected with Silymarin and treated with CCl_4.

Analysis of the liver weight was conducted. There was an increase in liver's weight of the CCl_4-treated animals (10.40 ± 0.56 g), compared to the non-treated group (9.00 ± 0.21 g). The livers of animals, treated with

AF were slightly lighter (9.33 ± 0.66 g) in comparison to SL treated (10.00 ± 0.30 g). A similar finding was observed in the liver of rats, treated in combination of AF and CCl_4 (9.67 ± 0.35 g), compared to the combined treatment of SL and CCl_4 (10.00 ± 0.29 g). Results are expressed as a mean of six livers' weight. It was found that the weight were in correlation with the histopathological findings.

Necrotic changes and abnormal accumulations of lipids in hepatocytes' cytoplasm were not present in the control group (Figure 3A). In the liver tissue of animals, treated with CCl_4, both degenerative and necrotic changes in hepatocytes were abundant. Changes expressed as hepatocellular necrosis, microvesicular accumulations of fats, localized in the center of acini, predominated in the liver of CCl4-treated rats. In the individual cells or group of cells, lytic changes were observed in the nucleus of the hepatocytes also as perivascular and mononuclear aggregations (Figure 3B). Due to the deparafinisation with xylene, lipid accumulation was visualised as optically empty spaces (Bancroft, Gamble, 2008). AF and SL treatment resulted in normal architecture of the liver parenchyma and hepatocytes, in which microscopically visible changes were absent (Figure 3C and 3D). In liver tissue from animals, treated with CCl_4 and SL, abnormal accumulation of small lipid droplets were visible in some cells (Figure 3F), though those changes were not present in animals treated with CCl_4 and AF (Figure 3E). Histology quantitation was performed and the observed results were correspondent to the pathological findings (Figure 4).

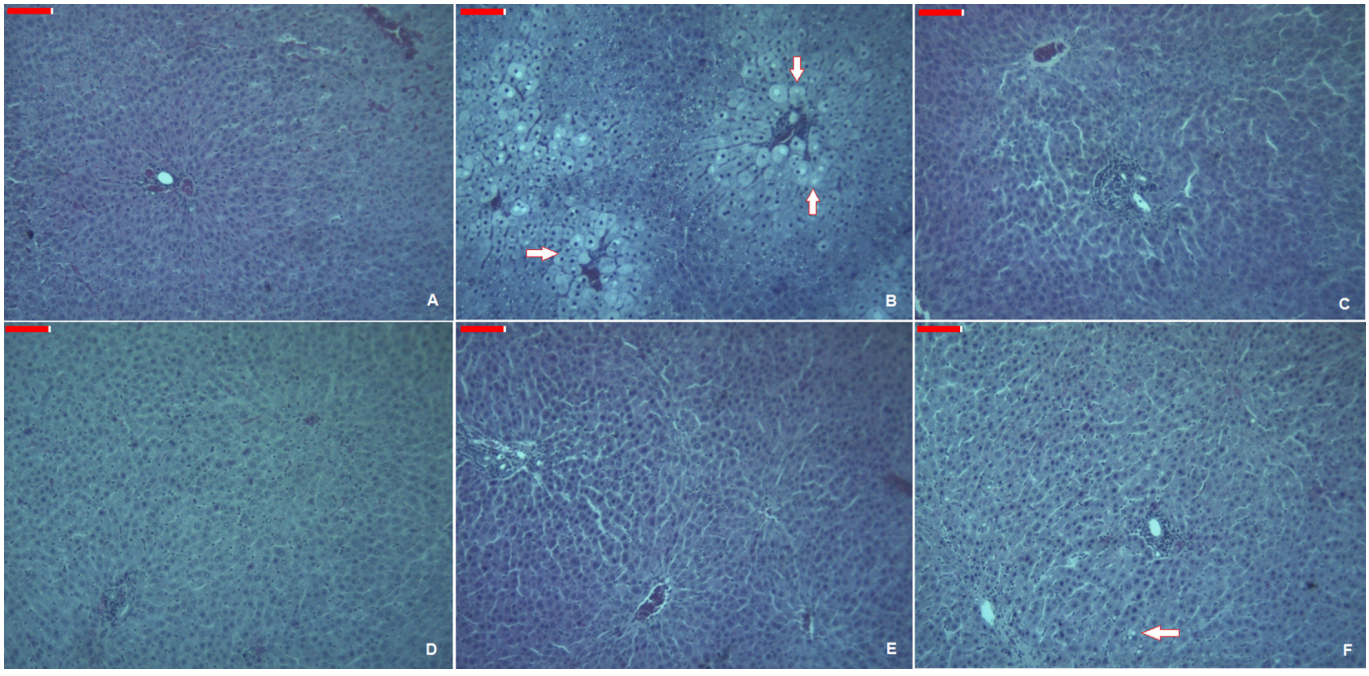

FIGURE 3 - Microscopic observations of livers stained with H&E (scale bars 66.67 µm, on 40x): **A.** Liver tissue from control rats treated with olive oil alone revealed normal cellular architecture; **B.** Liver tissue of CCl_4 – treated group revealed a presence of centriacinar and micro vesicular accumulation of fats; **C.** Liver tissue of AF treated group – normal cellular architecture; **D.** Liver tissue of silymarin treated group – normal cellular architecture; **E.** Liver tissue from rats challenged with CCl_4 after 7-day pre-treatment and consecutive 14 days curative treatment with AF revealed lower deposition of fats in the hepatocytes. Hepatic necrosis was not observed; **F.** Liver tissue from rats challenged with CCl_4 after 7-day pre-treatment and consecutive 14 days' curative treatment with silymarin revealed micro vesicular deposition of fats only in individual hepatocytes.

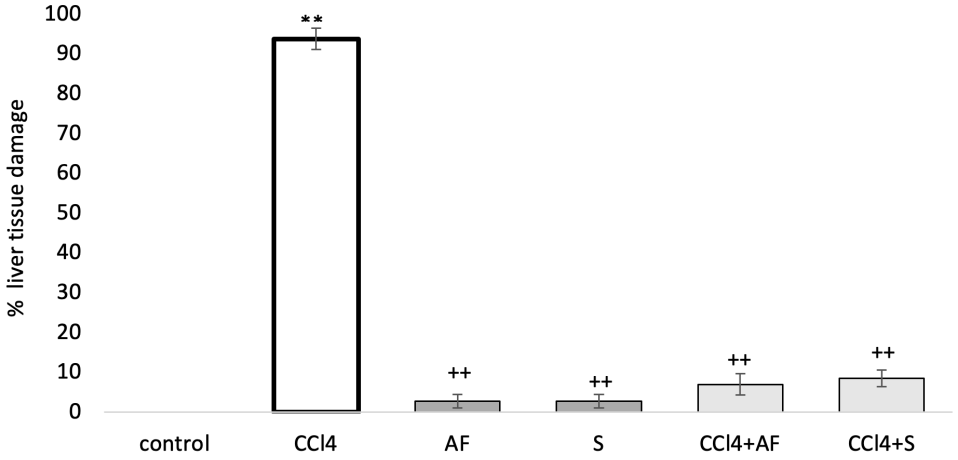

FIGURE 4 - Graphical representation of the histological analysis. $^{**}p < 0.01$ *vs* control (non-treated animals); $^{++}p < 0.01$ *vs* CCl_4.

Natural plant-derived products containing mostly flavonoids are being investigated as a source of antioxidants due to their ability to neutralize different types of oxidizing species including superoxide anion, hydroxyl radical, or peroxy-radicals. Experimental studies showed that the antioxidant properties of flavonoids are attributed to their scavenging activity, acting as a hydrogen atom donor to the free radical during the oxidation-reduction reaction (Younes, Siegers, 1981).

In the *in vitro* experiment, carried out in isolated rat microsomes, AF exerted concentration-dependent antioxidant activity against Fe^{2+}/AA-induced lipid peroxidation. The effect was discerned by MDA quantity reduction and was similar to those of silybin (Figure 2B). The antioxidant effect of silybin 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 (Loguercio, Festi, 2011). There are also data that silybin serves as an iron chelator (Borsari *et al*., 2001). On the basis of our results showing similar antioxidant potential of AF and silybin we suggest also similar *in vitro* antioxidant mechanisms.

Based on the data from the *in vitro* part of our study we further investigated the biological activity of AF against CCl_4-induced liver damage in male Wistar rats. Hepatotoxicity caused by CCl_4 is due to enzymatic bio activation and formation of $CCl_3^{\bullet}$, which subsequently induces lipid peroxidation and disturbs the structure and function of the cellular membranes. Due to the well-established mechanism of CCl_4-induced toxicity, the latter is frequently used in the experimental toxicology as a model of hepatic damage (Simeonova *et al*., 2015; Shkondrov *et al*., 2015). In our study, rats challenged with CCl_4 had an increased activity of plasma transaminases (ALT and AST), ALP and GGT (Table I) as well as increased MDA producti on, depleted GSH level and reduced activity of the antioxidant enzymes: CAT, SOD, GPx, GR, and GST (Table II). These results are in good correlation with the results from our previous studies (Simeonova *et al*., 2015; Vitcheva *et al*., 2013) as well as with the findings of other researchers (Timbrell, 2000; Lin *et al*., 2008; Weber, Boll, Stampfl, 2003). The observed plasma and tissue biochemical changes are confirmed by the histological changes: lymphocytes infiltration, steatosis and centrilobular necrosis, induced by CCl_4 (Figure 3B).

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 hepatotoxic effect induced by the toxicant. This was evidenced by a significant lowering of MDA quantity and increase in GSH levels as well as by preserving antioxidant enzyme activities (CAT, SOD, GPx, GR, GST) (Table II). The increased by CCl_4 activity of the plasma enzymes (ALT, AST, ALP and GGT) was also normalized by AF (Table I).

AF liver protection was supported also by the prevention of CCl_4 caused histopathological changes. AF lowered the fat deposition in the hepatocytes and prevented the development of necrosis (Figure 3E). The effects of AF i*n vivo* were compared with silymarin, a preferable positive control substance with proven antioxidant, immunomodulatory, anti-fibrotic, anti-proliferative, and antiviral properties. AF exerted similar to silymarin effects on serum, tissue and histopathological levels.

For the first time the *in vivo* antioxidant and hepatoprotective effects of AF were studied due to the large amount of the compound isolated. There are a lot of studies regarding those effects for flavonoids, but none for AF in particular. Moreover, the fact that the species is introduced in *in vitro* culture gives the basis for large scale production of this compound by biotechnological means (Zdraveva *et al.*, 2017). Under the conditions of our study, AF showed antioxidant activity in *in vitro* model of Fe^{2+}/AA-induced lipid peroxidation in isolated liver microsomes and *in vivo* hepatoprotective activity against CCl_4-induced liver injury in rats. We conclude that the observed beneficial effects of AF are no doubt related to its phenolic nature. Similar to other phenolic compounds we assume that it is capable to stabilize free radicals and reactive oxygen species by electron donation and in this way to maintain the integrity of the hepatocyte membrane and to impede the entrance of toxic substances or xenobiotics. We also suggest that being similar to silymarin, AF may also affect intracellular glutathione, which prevents lipid peroxidation of the membranes.

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