Effects of rhamnocitrin 4-β-D-galactopyranoside, isolated from *Astragalus hamosus* on toxicity models *in vitro*

Magdalena Kondeva-Burdina, Ilina Krasteva[1], Mitka Mitcheva

Laboratory for Drug Metabolism and Drug Toxicity, Department of Pharmacology, Pharmacotherapy and Toxicology, [1]Department of Pharmacognosy, Faculty of Pharmacy, Medical University of Sofia, Sofia, Bulgaria

ABSTRACT

Background: *Astragalus hamosus* L. (Fabaceae) is used in herbal medicine as emollient, demulcent, phrodisiac, diuretic, laxative, and good for inflammation, ulcers, and leukoderma. It is useful in treating irritation of the mucous membranes, nervous affections, and catarrh. **Objective:** Rhamnocitrin 4-β-D-galactopyranoside (RGP), isolated from *A. hamosus*, was investigated for its possible protective effect on different models of toxicity *in vitro* on sub-cellular and cellular level. **Materials and Methods:** The effects of RGP were evaluated on isolated rat brain synaptosomes, prepared by Percoll reagent and on rat hepatocytes, isolated by two-stepped collagenase perfusion. **Results:** In synaptosomes, RGP had statistically significant protective effect, similar to those of silymarin, on 6-hydroxy (OH)-dopamine-induced oxidative stress. These results correlate with literature data about protective effects of kempferol and rhamnocitrin on oxidative damage in rat pheochromocytoma PC12 cells. In rat hepatocytes, we investigate the effect of RGP on two models of liver toxicity: Bendamustine and cyclophosphamide. In these models, the compound had statistically significant cytoprotective and antioxidant activity, similar to those of silymarin. **Conclusion:** According to these results, we can suggest that such cytoprotective effect of RGP might be due to an influence on bendamustine and cyclophosphamide metabolism in rat hepatocytes. In isolated rat hepatocytes, in combination with bendamustine and cyclophosphamide and in 6-OH-dopamine-induced oxidative stress in isolated rat synaptosomes, RGP, isolated from *A. hamosus*, was effective protector and antioxidant. The effects were closed to those of flavonoid silymarin-the classical hepatoprotector and antioxidant.

Key words: Antioxidant activity, *Astragalus hamosus*, cytoprotection, flavonol glycoside, hepatocytes, synaptosomes

INTRODUCTION

Bendamustine is a bi-functional alkylating agent with cytotoxic activity against human ovarian and breast cancers *in vitro*. Bendamustine as monotherapy or as part of combination chemotherapy protocols for first-line or subsequent treatment produced objective response rates of 61-97% in patients with Hodgkin's disease or nonHodgkin's lymphoma (NHL). In patients with multiple myeloma, a bendamustine/prednisone regimen produced a higher rate of complete response and more durable responses than a mephalan/prednisone regimen. Substitution of bendamustine for cyclophosphamide in a standard first-line cyclophosphamide, vincristine, and prednisolone regimen yielded similar response rates in patients with advanced low grade NHL. Substituting bendamustine for cyclophosphamide in the cyclophosphamide, methotrexate, and fluorouracil protocol prolonged remission from 6.2 to 15.2 months in patients with metastatic breast cancer.[1,2]

Roué *et al*. found that bendamustine cytotoxicity was mediated by the generation of reactive oxygen species (ROS), leading to oxidative stress.[3]

Cyclophosphamide is an anticancer pro-drug that is dependent on cytochrome P450 metabolism for its therapeutic effectiveness. As a result of its metabolism the toxic metabolites acrolein and chloroacetaldehyde are formed. They lead to oxidative stress and DNA-damage.[4,5]

Address for correspondence:
Prof. Ilina Krasteva, Department of Pharmacognosy, Faculty of Pharmacy, 2 Dunav St., 1000, Sofia, Bulgaria.
E-mail: ikrasteva@pharmfac.net

Dopamine metabolism and oxidation produce both ROS and reactive quinines, which lead to oxidative stress. These species are implicated in dopamine neurotoxicity and neurodegeneration.[6]

Astragalus hamosus L. (Fabaceae) is used in herbal medicine as emollient, demulcent, phrodisiac, diuretic, laxative, and good for inflammation, ulcers, and leukoderma. It is useful in treating irritation of the mucous membranes, nervous affections, and catarrh.[7]

Semmar *et al.* (2002) found that in other *Astragalus* species were found several flavonol glycosides.[8]

Flavonoids-secondary metabolites found ubiquitously in plants-are the most common group of polyphenolic compounds consumed by humans as dietary constituents. Flavonoids have been reported to have anti-allergic, anti-inflammatory, antimicrobial, antioxidant, and anticancer activities.[9,10]

Previous phytochemical study of the aerial part of *A. hamosus* afforded the isolation of new flavonol glycoside 7-O-methyl-kaempferol-d-galactopyranoside (rhamnocitrin 4'-*β*-d-galactopyranoside [RGP]) and known flavonols hyperoside, isoquercitrin, and astragalin.[11] Rutin, astragalin, and isoquercitrin have been also obtained in callus and suspension cultures of the plant.[12]

Saleem *et al.* (2013) found a hepatoprotective activity of flavonoid RGP, obtained from leaves of *A. hamosus* L. against N-diethylnitrosamine-induced hepatic cancer.[13] Hong *et al.* found that flavonoids kempferol (isolated from tea, broccoli, grapefruit, cabbage, beans, tomato, strawberries, grapes, apples) and rhamnocitrin (kempferol 7-O-methyl ether) revealed protective effects on oxidative damage in rat pheochromocytoma PC12 cells induced by a limited supply of serum and hydrogen peroxide (H_2O_2). They suggest that kaempferol and rhamnocitrin can augment cellular antioxidant defense capacity, at least in part, through regulation of heme oxygenase-1 gene expression and mitogen-activated protein kinase signal transduction.[14]

Based on the information available, the objective of the following study was to investigate the possible protective and antioxidant effects of flavonoid RGP, isolated from *A. hamosus* on different toxicity models *in vitro*.

MATERIALS AND METHODS

Chemicals and reagents

In our experiments, pentobarbital sodium (Sanofi, France), N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid) (HEPES) (Sigma Aldrich, Germany), NaCl (Merck, Germany), KCl (Merck), D-glucose (Merck), $NaHCO_3$ (Merck), KH_2PO_4 (Scharlau Chemie SA, Spain), $CaCl_2 \cdot 2H_2O$ (Merck), $MgSO_4 \cdot 7H_2O$ (Fluka AG, Germany), collagenase from *Clostridium histolyticum* type IV (Sigma Aldrich), albumin, bovine serum fraction V, minimum 98% (Sigma Aldrich), ethylene glycol tetraacetic acid (Sigma Aldrich), 2-thiobarbituric acid (TBA) (4,6-dihydroxypyrimidine-2-thiol) (Sigma Aldrich), trichloroacetic acid (TCA) (Valerus, Bulgaria), 6-hydroxydopamine (Merck), 2,2'-dinitro-5,5'-dithiodibenzoic acid (DTNB) (Merck), lactate dehydrogenase (LDH) kit (Randox, UK), D(+) sucrose (Fluka, Germany), NaH_2PO_4 (Merck), $MgCl_2 \cdot 6H_2O$, Percoll (Sigma Aldrich), (3-[4,5-dimethylthiazol -2-yl]-2, 5diphenyl-tetrazolium bromide) (Sigma Aldrich), dimethyl sulfoxide (DMSO) (Valerus, Bulgaria) were used.

Plant material

The plant material of *A. hamosus* was collected in June 2006 in Northeastern parts of Bulgaria. The plant was identified by Dr. D. Pavlova from the Department of Botany, Faculty of Biology, Sofia University, where voucher specimen had been deposited (SO 102680).

Extraction and isolation

Air-dried powdered aerial parts of the plant (1 kg) were defatted with *n*-hexane and extracted with MeOH/H_2O (9:1) and (1:1). The extracts were filtrated, concentrated under reduced pressure, and successively partitioned with $CHCl_3$, EtOAc, and *n*-BuOH. A flavonol glycoside was isolated by Sephadex LH-20 column chromatography and crystallization with MeOH from the ethyl acetate extract. Based on the chemical and spectral data, the structure of the compound was established as 7-O-methyl-kaempferol-d-galactopyranoside or RGP [Figure 1]. Details of isolation and identification of the flavonoid have been published previously.[11]

Experimental animals

Male Wistar rats (body weight, 200-250 g) were used. Rats were housed in plexiglass cages (three per cages) in a 12/12 light/dark cycle, temperature 20 ± 2°C. Food and water were provided *ad libitum*. Animals were purchased from the National Breeding Centre, Sofia, Bulgaria. All experiments
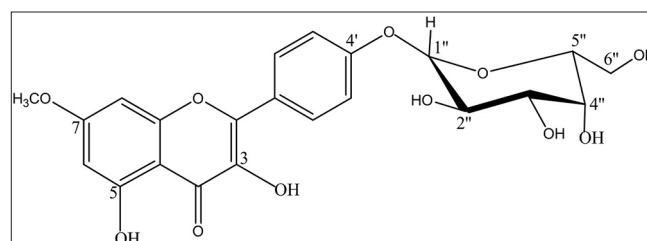
Figure 1: Structure of rhamnocitrin 4'-β-D-galactopyranoside

were performed after at least 1 week of adaptation to this environment.

The experimental procedures were approved by the Institutional Animal Care and Use Committee at the Medical University-Sofia, Bulgaria. The principles stated in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS 123) were followed strictly throughout the experiment.

Isolated hepatocytes are a well-controlled, biological model system with high drug-metabolizing capacities. This *in vitro* system is included in the battery of recommended tests from the European Centre for the Validation of Alternative Methods (ECVAM).

The main goal of ECVAM is to promote the acceptance of alternative methods, which are important for reducing, refining and replacing the use of laboratory animals.[15]

Isolation and incubation of hepatocytes
Rats were anesthetized with sodium pentobarbital (0.2 ml/100 g). *In situ* liver perfusion and cell isolation were performed as described by Fau *et al.* with modifications.[16,17]

After portal catheterization, the liver was perfused with HEPES buffer (pH = 7.85) +0.6 mM EDTA (pH = 7.85), followed by HEPES buffer (pH = 7.85), without any addition and finally HEPES buffer, containing collagenase type IV (50 mg/200 ml) and 7 mM $CaCl_2$ (pH = 7.85). The liver was excised, minced into small pieces and hepatocytes were dispersed in Krebs-Ringer-bicarbonate (KRB) buffer (pH = 7.35) +1% bovine serum albumin.

Cells were counted under the microscope and the viability was assessed by Trypan blue exclusion (0.05%).[16] Initial viability averaged 89%.

Cells were diluted with KRB, to make a suspension of about 3×10^6 hepatocytes/ml. Incubations were carried out in flasks, containing 3 ml of the cell suspension (i.e. 9×10^6 hepatocytes) and were performed in a 5% CO_2 + 95% O_2 atmosphere.[16] Hepatocytes were incubated with 60 μM bendamustine and cyclophosphamide.[18]

Isolation and incubation of synaptosomes
Synaptosomes were prepared by brains from adult male Wistar rats, as previously described by Taupin *et al.*[19] The brains were homogenized in 10 volume of cold buffer 1, containing: 5 mM HEPES and 0.32 M sucrose (pH = 7.4).

The brain homogenate was centrifuged twice at $1000 \times g$ for 5 min at 4°C. The supernatant was collected and

centrifuged 3 times at $10,000 \times g$ for 20 min at 4°C. The pellet was re-suspended in ice-cold buffer 1.

The synaptosomes were isolated by using Percoll reagent to prepare the gradient. Synaptosomes were re-suspended and incubated in buffer 2, containing: 290 mM NaCl, 0.95 mM $MgCl_2 \cdot 6H_2O$, 10 mM KCl, 2.4 mM $CaCl_2 \cdot H_2O$, 2.1 mM NaH_2PO_4, 44 mM HEPES, and 13 mM D-glucose. Incubations were performed in a 5% CO_2 + 95% O_2 atmosphere.

The content of synaptosomal protein was determined according to the method of Lowry *et al.* using serum albumin as a standard.[20]

Synaptosomes visibility' measured by (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide)-test, described by Mungarro-Menchaca *et al.*[21]
After incubation with the compounds, synaptosomes were treated with (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (0.5 mg/ml) for 1 h in 37°C. After incubation they were centrifuged at $15,000 \times g$ for 1 min. The formed formasan crystals were dissolved in DMSO. The extinction was measured spectrophotometrically at λ = 580 nm.

Lactate dehydrogenase release
Lactate dehydrogenase release in isolated rat hepatocytes was measured as described by Fau *et al.*[22]

The cells were centrifuged at $500 \times g$ for 1 min and the supernatant was taken for measuring the LDH activity. The activity was measured by using LDH kit (Randox). About 20 μl from the cell supernatant was added in 180 μl from the mixture of the kit (buffer A + buffer B). The activity is measured spectrophotometrically at 340 nm.

Glutathione depletion
At the end of the incubation, isolated rat hepatocytes were recovered by centrifugation at 4°C, and used to measure intracellular reduced glutathione (GSH), which was assessed by measuring nonprotein sulfhydryls after precipitation of proteins with TCA, followed by measurement of thiols in the supernatant with DTNB. The absorbance was measured at 412 nm.[16]

Malondialdehyde assay
Hepatocyte suspension (1 ml) was taken and added to 0.67 ml of 20% (w/v) TCA. After centrifugation, 1 ml of the supernatant was added to 0.33 ml of 0.67% (w/v) TBA and heated at 100°C for 30 min. The absorbance was measured at 535 nm, and the amount of TBA-reactants was calculated using a molar extinction coefficient of malondialdehyde (MDA) 1.56×10^5/M/cm.[16]

Glutathione level in synaptosomes, described by Robyt *et al.*[23]

Glutathione was determined with the Ellman reagent (DTNB), which forms color complexes with-SH group at pH = 8 with maximum absorbance at 412 nm.

The synaptosomes were centrifuged $500 \times g$ for 1 min and the sediment was used for measuring GSH level. The sediment was precipitated with 5% TCA, after that was centrifuged for 10 min at $4000 \times g$ and the level of GSH in the supernatant was measured with DTNB spectrophotometrically at 412 nm.

The biochemical parameters were determined by spectrophotometric methods using a Spectro UV-VIS Split spectrophotometer.

Statistical analysis

Statistical analysis was performed using statistical program "MEDCALC". Results are expressed as mean ± standard error of mean for six experiments. The significance of the data was assessed using the nonparametric Mann–Whitney test. Values of $P \leq 0.05$; $P \leq 0.01$ and $P \leq 0.001$ were considered as statistically significant. Three parallel samples were used.

RESULTS

In isolated rat hepatocytes, RGP, administered alone, revealed toxic effects, as statistically significant decreased cell viability and GSH level, increased LDH leakage and MDA level, compared with control [Tables 1 and 2]. The compound was less toxic on the examined parameters compared to silymarin. The effects were concentration dependent.

Hepatocytes incubation with bendamustine (60 µM) and cyclophosphamide (60 µM) resulted in statistically significant reduction of cell viability by 33% and 43%; increased LDH leakage with 59% and 100%, respectively.

In combination with bemdamustine and cyclophosphamide, RGP revealed better cytoprotective effect on cell viability and had weaker protective effect on decreasing LDH leakage, compared with silymarin [Table 3].

Hepatocytes incubation with bendamustine (60 µM) and cyclophosphamide (60 µM) resulted in statistically significant depletion of cell GSH by 76% and 72% and increased MDA level by 194% and 184%, respectively.

In combination with bendamustine, RGP, and silymarin revealed more prominent protective effect on GSH level, than in combination with cyclophosphamide and had statistically significant closer antioxidant activity, while with

cyclophosphamide, this activity was weaker than those of silymarin [Table 4].

In isolated rat synaptosomes, RGP, administered alone, revealed toxic effects, as statistically significant decreased synaptosomes' viability and GSH level, compared with control [Table 5]. The compound was more toxic on the examined parameters compared with silymarin.

The incubation of rat synaptosomes with 6-hydroxy (OH)-dopamine (150 µM) resulted in statistically significant decreased of viability and depletion of GSH by 29% and 74%, respectively.

In combination with 6-OH-dopamine, RGP and silymarin statistically significantly reduced the damage caused by neurotoxic agent and preserved synaptosomes' viability and GSH level [Table 6]. RGP had weak protective effect on the examined parameters, compared to those of silymarin.

DISCUSSION

In experimental toxicology the *in vitro* systems play an important

Table 1: Effects of rhamnocitrin 4-β-D-galactopyranoside (Rh) and silymarin (10 µM, 100 µM), administered alone, on trypan blue exclusion and LDH leakage in isolated rat hepatocytes

Group	Trypan blue exclusion (%)	Effect (%) vs control	LDH leakage (µmol/min/ 10^6 cells)	Effect (%) vs control
Control	81±5.9	100	0.232±0.04	100
10 µM Rh	69±6.8*	↓15	0.286±0.01***	↑23
100 µM Rh	48±2.3***	↓41	0.369±0.02***	↑59
10 µM S	66±5.4*	↓19	0.359±0.04***	↑55
100 µM S	47±6.2***	↓42	0.414±0.03***	↑78

*$P<0.05$; ***$P<0.001$ vs control

Table 2: Effects of rhamnocitrin 4-β-D-galactopyranoside (Rh) and silymarin (10 µM, 100 µM), administered alone, on GSH depletion and lipid peroxidation in isolated rat hepatocytes

Group	GSH level (nmol/ 10^6 cells)	Effect (%) vs control	MDA (nmol/ 10^6 cells)	Effect (%) vs control
Control	25±2.7	100	0.173±0.01	100
10 µM Rh	18±2.8**	↓28	0.164±0.01*	↓5
100 µM Rh	13±0.8***	↓48	0.184±0.02*	↑6
10 µM S	17±1.6**	↓32	0.166±0.003*	↓4
100 µM S	12±0.5***	↓52	0.195±0.003*	↑13

*$P<0.05$; **$P<0.01$; ***$P<0.001$ vs control

Table 3: Effects of rhamnocitrin 4-β-D-galactopyranoside (Rh) and silymarin (100 μM) in models of cytotoxicity on trypan blue exclusion and LDH leakage in isolated rat hepatocytes

Group	Trypan blue exclusion (%)	Effect (%) vs toxic agent	LDH leakage (μmol/min/10^6 cells)	Effect (%) vs toxic agent
Control	81±5.9		0.232±0.04	
60 μM Bendamustine	54±1.3***	100	0.369±0.01***	100
100 μM Rh+60 μM B	73±2.5+++	↑35	0.320±0.02+	↓13
100 μM S+60 μM B	59±2.4+	↑9	0.269±0.03+++	↓27
60 μM Cyclophosphamide	46±0.5***	100	0.464±0.01***	100
100 μM Rh+60 μM C	60±1.6+++	↑30	0.393±0.03+++	↓15
100 μM S+60 μM C	49±1.3+	↑7	0.295±0.05+++	↓36

***$P<0.001$ vs control. +$P<0.05$; +++$P<0.001$ vs toxic agent

Table 4: Effects of rhamnocitrin 4-β-D-galactopyranoside (Rh) and silymarin (100 μM) in models of cytotoxicity on GSH depletion and lipid peroxidation in isolated rat hepatocytes

Group	GSH level (nmol/10^6 cells)	Effect (%) vs toxic agent	MDA (nmol/10^6 cells)	Effect (%) vs toxic agent
Control	25±2.7		0.173±0.1	
60 μM Bendamustine	6±0.9***	100	0.508±0.03***	100
100 μM Rh+60 μM B	10±1.7+	↑67	0.184±0.01+++	↓64
100 μM S+60 μM B	11±2.7++	↑83	0.173±0.02+++	↓66
60 μM Cyclophosphamide	7±1.3***	100	0.492±0,2***	100
100 μM Rh+60 μM C	11±1.5++	↑57	0.262±0.03+++	↓47
100 μM S+60 μM C	12±0.5++	↑71	0.175±0.01+++	↓64

***$P<0.001$ vs control. +$P<0.05$; ++$P<0.01$; +++$P<0.001$ vs toxic agent

Table 5: Effects of rhamnocitrin 4-β-D-galactopyranoside (Rh) and silymarin (100 μM), administered alone on synaptosomes' vibility and GSH depletion, compared to Silymarin

Group	Synaptosomes' viability (%)	Effect (%) vs control	GSH level (nmol/mg protein)	Effect (%) vs control
Control	0.113±0.003	100	0.121±0.003	100
100 μM Rh	0.089±0.003*	↓21	0.059±0.002*	↓51
100 μM S	0.092±0.005*	↓19	0.072±0.003*	↓40

*$P<0.05$ vs control

role for the investigation of xenobiotic biotransformation and reveal the possible mechanisms of toxic stress and its protection. There are different *in vitro* systems for investigating metabolism on sub-cellular and cellular level. These systems help for the reduction, replacement and refinement of the experimental laboratory animals.

Rhamnocitrin 4'-*β*-d-galactopyranoside, isolated from *A. hamosus*, administered alone in isolated rat hepatocytes and synaptosomes, showed toxic effects, comparable to those of silymarin.

The treatment of isolated rat brain synaptosomes with 6-OH-dopamine is a convenient *in vitro* sub-cellular system for the investigation of processes, which play role in the

neurodegenerative disease, including Parkinson's and Alzheimer's disease. The mechanism of 6-OH-dopamine neurotoxicity includes the formation of ROS and reactive metabolites, as a result of its metabolism in mitochondria of the nerve cells.[6]

The mechanism of the destruction of the nerve terminals is thought to involve oxidation of 6-OH-dopamine to a *p*-quinone, the production of a free radical or of superoxide anion. The reactive intermediate reacts covalently with the nerve terminal and permanently inactivates it.[22] In rat brain synaptosomes, prepared by using Percoll reagent, the flavonoid RGP had statistically significant protective effect, similar to those of silymarin on 6-OH-dopamine-induced oxidative stress.

These results correlate with literature data about protective effects of kaempferol and rhamnocitrin on oxidative damage in rat pheochromocytoma PC12 cells induced by a limited supply of serum and hydrogen peroxide (H_2O_2).[14]

Isolated liver cells are used as a suitable model for evaluation of the cytoprotective effect of some perspective biologically active compounds, both newly synthesized and plant isolated.

Pre-incubation of the hepatocytes with RGP significantly protected against bendamustine and cyclophosphamide

Table 6: Effects of rhamnocitrin 4-β-D-galactopyranoside (Rh) and silymarin (100 µM) in 6-OH-dopamine (6-OH-D)-induced oxidative stress on synaptosomes` vibility and GSH depletion

Group	Synaptosomes viability (%)	Effect (%) vs 6-OH-D	GSH level (nmol/mg protein)	Effect (%) vs 6-OH-D
Control	0.113±0.003		0.121±0.03	
150 µM 6-OH-D	0.08±0.001*	100	0.031±0.001**	100
100 µM Rh+150 µM 6-OH-D	0.100±0.003+	↑25	0.083±0.001++	↑168
100 µM S+150 µM 6-OH-D	0.106±0.003+	↑33	0.097±0.001+	↑213

*$P<0.05$; **$P<0.01$ vs control. +$P<0.05$; ++$P<0.05$ vs 6-OH-D

toxicity. This compound, during bendamustine- and cyclophosphamide-induced hepatotoxicity, preserved the cell viability and significantly decreased LDH leakage in the medium, compared to the toxic agents. On cellular GSH, RGP had protective effect in combination with bendamustine and cyclophosphamide. Bendamustine and cyclophosphamide caused an elevation of the lipid peroxidation (LPO) marker MDA. In combination with the toxic agent, RGP significantly decreased the level of MDA.

Some authors found that in human liver microsomes, CYP1A2 played role in the bendamustine oxidation, producing two toxic metabolites.[24,25] Later Shimada *et al.* proved that some flavonoids (galangin, kaempferol, chrysin, apigenin, and genistein) revealed inhibitory activity on human CYP1A2.[26]

Cyclophosphamide is metabolized by hepatic cytochrome P450 via two major pathways. The first involves 4-hydroxylation to the active metabolite and is carried out predominantly by CYP2B6. The alternative pathway involves a CYP3A4-mediated *N*-dechloroethylation of cyclophosphamide to form the inactive metabolite and the toxic by-product chloroacetaldehyde.[5] There are literature data that in human liver microsomes, some flavonoids exerted inhibitory effects on CYP3A activity.[27]

Lahouel *et al.* found that flavonoids – diosmine and quercetine protected against vinblastine, cyclophosphamide and paracetamol toxicity by inhibition of LPO and increasing liver glutathione concentration. They suggested that increased glutathione concentration was a result of activation of the turnover of the glutathione and enzymes, stimulating particularly glutathione-*S*-transferases, permitting the captation of the reactive metabolites of the studied drugs.[28]

Based on the information available and according to our results, we can suggest that such cytoprotective effect of RGP might be due to an influence on bendamustine and cyclophosphamide metabolism and on LPO process and liver glutathione concentration in rat hepatocytes.

CONCLUSION

In isolated rat hepatocytes, in combination with bendamustine and cyclophosphamide and in 6-OH-dopamine-induced oxidative stress in isolated rat synaptosomes, RGP, isolated from *A. hamosus*, was effective protector and antioxidant. The effects were close to those of flavonoid silymarin – the classical hepatoprotector and antioxidant.

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