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

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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 non-Hodgkin'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]
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′,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 (H₂O₂).[1] 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.[12]

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), NaHCO₃ (Merck), KH₂PO₄ (Scharlau Chemie SA, Spain), CaCl₂·2H₂O (Merck), MgSO₄·7H₂O (Fluka AG, Germany), collagen 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), NaH₂PO₄ (Merck), MgCl₂·6H₂O, Percoll (Sigma Aldrich), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-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/H₂O (9:1) and (1:1). The extracts were filtrated, concentrated under reduced pressure, and successively partitioned with CHCl₃, 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 were provided.
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 CaCl₂ (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 × 10⁶ hepatocytes/ml. Incubations were carried out in flasks, containing 3 ml of the cell suspension (i.e. 9 × 10⁶ hepatocytes) and were performed in a 5% CO₂ + 95% O₂ 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 × g for 5 min at 4°C. The supernatant was collected and centrifuged 3 times at 10,000 × 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 MgCl₂, 6H₂O, 10 mM KCl, 2.4 mM CaCl₂, H₂O₂ 2.1 mM NaH₂PO₄, 44 mM HEPES, and 13 mM D-glucose. Incubations were performed in a 5% CO₂ + 95% O₂ 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 × 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 × 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 sulphydryls 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 × 10⁵/M/cm.[10]
Glutathione level in synaptosomes, described by Robyt et al.\(^1\)\(^2\)\(^3\)

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 × 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 × 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 role...
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***                 | 135                      | 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***                 | 130                      | 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<0.01 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*                     | 167                      | 0.184±0.01***        | ↓64                      |
| 100 µM S+60 µM B       | 11±2.7**                    | 183                      | 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**                    | 157                      | 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’ viability 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 (H₂O₂).[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...
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. Later Shimada et al. proved that some flavonoids (galangin, kaempferol, chrysin, apigenin, and genistein) revealed inhibitory activity on human CYP1A2.

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. There are literature data that in human liver microsomes, some flavonoids exerted inhibitory effects on CYP3A activity.

Lahouel et al. found that flavonoids – diosmine and quercetin 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 capture of the reactive metabolites of the studied drugs.

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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