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PHARMACOLOGY AND TOXICOLOGY

Evaluation of the Gastroprotective Effect of the Flavonoid Complex from *Lychnis chalcedonica* L. on the Models of Experimental Ulcerogenesis

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Using rat and mouse models of neurogenic, ethanol-induced, and indometacin-induced damage to the gastric mucosa we demonstrated that course preventive treatment with flavonoid complex from aerial parts of *Lychnis chalcedonica* L. increased the resistance of gastric mucosa to ulcerogenic factors of different etiology. The gastroprotective effect of the phytocomplex in a dose range of 16-1600 μg/kg was comparable with that of the reference drug plantaglucide and was superior to that of the reference drugs eleutherococcus extract and methyluracil in the therapeutic doses. The antiulcerogenic activity of *Lychnis chalcedonica* flavonoid complex considerably exceeded activity of *Lychnis chalcedonica* L. extract demonstrated in our previous experiments.

Key Words: *gastroprotective effect; flavonoids; Lychnis chalcedonica L.*

Gastroduodenal diseases are among the pathologies that can be successfully treated with herbal preparations in the form of monotherapy or in combination with other therapeutic measures [3,5,7].

Our previous experiments on the models of neurogenic and aspirin-induced ulcerogenesis demonstrated a pronounced antiulcer effect of unfractionated *Lychnis chalcedonica* extract containing a complex of major and rare ecdysteroids, triterpene glycosides, alkaloids, polyphenols, and flavonoids [4]. These findings allow considering the above phytocomplexes as the basis for the creation of substances for gastroenterological practice. It was shown that the complex of flavonoids isolated from the aerial part of *Lychnis chalcedonica* produces anti-inflammatory and analgesic effects comparable to those of diclofenac sodium [13].

Our aim was to study the antiulcer effect of a complex of flavonoids from the aerial part of *Lychnis chalcedonica* on conventional models of neurogenic, indomethacin-induced, and ethanol-induced ulcerogenesis in mice and rats.

MATERIALS AND METHODS

The experiments were carried out on male outbred CD1 mice (n=118, age 7-8 weeks, weight 25-26 g), outbred male Wistar rats (n=98, age 7-8 weeks, weight 200-220 g, first category, conventional animals). The animals were obtained from the Department of Experimental Biomodelling of the E. D. Goldberg Research

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Institute of Pharmacology and Regenerative Medicine (health certificate is available). Animal housing and design of the experiments were approved by the Bioethical Committee of the E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine (Protocol JACUC No. 96092015. September 9, 2015) and complied with Directive 2010/63/EU of the European Parliament and the Council of the EU (On the Protection of Animals used for Scientific Purposes, September 22, 2010) and the Order No. 199n of the Ministry of Health of the Russian Federation (On Approval of the Rules of Good Laboratory Practice, April 1, 2016).

The complex of flavonoids (flavonoids) was obtained in the Laboratory of Phytochemistry of the Tomsk State University by repeated extraction of the air-dry raw material of the aerial part of *Lychnis chalcedonica* L. with 70% ethyl alcohol, followed by filtration, concentration under vacuum, dilution with water (1:2), selective extraction of flavonoids with n-butyl alcohol, recrystallization from 95% ethanol. The composition of flavonoids was analyzed by HPLC on a Shimadzu LC-20AD liquid chromatograph (photodiode array detector, chromatographic column Perfect Sil Target ODS-3; 4.6×250 mm, packing particle size 5 μm). Phenolic compounds were identified using standards (Sigma-Aldrich, ≥95% purity). The identification of the compounds showed that the complex includes apigenin di-C-glycosides and mono-C-glycosides vitexin, neovitexin, and isovitexin [8].

The doses were chosen based on the results of studies of the anti-inflammatory and analgesic effects of *Lychnis chalcedonica* flavonoids [8,13]. The solution of flavonoids was injected *per os* in a dose range of 12-1600 μg/kg in a volume of 0.3 ml/20 g body weight to mice or 16-1600 μg/kg in a volume of solvent 0.6 ml/200 g body weight to rats. The reference drugs were 500 mg/kg methyluracil (Biokhimiya) for mice, 263 mg/kg plantaglucide (Vifitekh) for rats, and 5 ml/kg eleutherococcus liquid extract (Vifitekh) for mice. These doses of the reference drugs corresponded to the mean therapeutic dose for humans [7,10]. The animals were deprived of food 24 h before treatment, but were allowed free access to water. The test substances were injected once daily through a tube *per os* for 4-5 days, the last dose was administered 1 h before ulcer induction. The negative control group received an equivalent volume of the solvent, purified water (FS.2.2.0020.15) by the same schedule. The animals were removed from the experiment by CO_2 overdose.

Neurogenic ulcerogenesis was modeled in mice by partial 24-h immobilization with forceps by the skin on the neck, which led to the development of all types of ulcerative lesions of the gastric mucosa (GM) in animals [3,10]. Indomethacin-induced lesions in GM were induced in mice by administration of indomethacin *per os* in a dose of 20 mg/kg twice (with an interval of 4 h), the number and size of destructive changes were determined in 16 h [3]. In rats, indomethacin-induced ulcerogenesis was modeled by single oral administration of indomethacin in a dose of 60 mg/kg in 1 ml saline; the gastroprotective effect of flavonoids was assessed 6 h after administration of the ulcerogen [3]. Ethanol damage to GM in rats was modeled by single peroral administration of 95% ethanol (1 ml/200 g body weight); the effect was assessed 1 h after the administration of ulcerogen [3].

At necropsy, the stomachs were removed, opened along the lesser curvature, washed with cold saline and, the number and area of destruction were determined using a special magnifier under bright illumination; the lesions were differentiated into punctate (diameter <0.05 mm), strip-like, and large (diameter >2 mm). The mean number of ulcerations per animal and the percentage of animals with ulcers in the group were calculated. Pauls index (PI) was determined as an integral indicator of the number of destruction by the formula: (mean number of ulcers)×(% animals with ulcers):100%. Antiulcer activity (AUA) of the test drugs was determined as the ratio of PI in the control group to PI in the experimental group. The test agent was considered active at AUA ≥2. The degree of damage (DD) to the gastric mucosa was evaluated by calculating the total length/area of ulcerative lesions (mm/mm^2) in each rat. The severity of damage (SD,%) was calculated using the formula: $SD=(DD_{experiment}/DD_{control})\times 100\%$.

The results were statistically processed by the methods of variation statistics using the Statistica 6.0 software (StatSoft, Inc.). The mean (X) and standard error of the mean (SEM) were calculated. The differences between the groups were tested using the nonparametric Mann—Whitney U test and angular Fisher transform. The differences were considered significant at $p\leq 0.05$ [6].

RESULTS

Models of neurogenic (partial immobilization), chemical (ethanol), and drug-induced (indomethacin) damage to GM in mice and rats share some pathogenetic mechanisms, but they are not identical and therefore they complement each other in the search and development of drugs (methods of correction) for gastroenterological practice [3].

Partial immobilization led to the development of all types of ulcerative GM lesions in 100% mice of the negative control group (Table 1). Eleutherococcus extract (AUA=3.6) significantly decreased both the number of animals with ulcers and mean number

TABLE 1. Antiulcerogenic Effect of *Lychnis chalcedonica* Flavonoids in Models of Neurogenic and Indomethacin Damage to GM of in Outbred Male CD1 Mice ($X \pm m$)

Group	Mice with ulcers, %	Number of ulcers per mouse			Mean number of ulcers per mouse	PI	AUA
		small	strip-like	large			
Model of neurogenic ulceration (4-day course)							
Negative control (*n*=9)	100	3.1±0.9	0.6±0.2	3.1±0.7	6.8±1.4	6.80	0
Eleutherococcus, 5 ml/kg (*n*=10)	70^{++}	2.6±0.9	0	0.1±0.1**	2.7±0.9**	1.89	3.60
Flavonoids 12 μg/kg (*n*=10)	70^{++}	3.6±1.2	0.3±0.2	0.5±0.3**	4.4±1.6	3.08	2.20
16 μg/kg (*n*=10)	30^{++}	0.5±0.3*	0	0**	0.5±0.3**	0.15	45.30
20 μg/kg r (*n*=10)	80^{+}	2.6±0.7	0.1±0.1	0**	2.7±0.7**	2.16.	3.10
24 μg/kg (*n*=10)	50^{++}	0.5±0.3	0.1±0.1	0.1±0.1**	0.7±0.3**	0.35	19.40
Model of indomethacin-induced ulcer (4-day course)							
Negative control (*n*=10)	100	14.6±1.5	3.3±0.9	0.8±0.3	18.7±1.8	18.70	0
Methyluracil, 500 mg/kg (*n*=10)	90	6.0±1.5**	0.1±0.1**	0.2±0.2	6.3±1.5**	5.67	3.30
Flavonoids 32 μg/kg (*n*=9)	89	2.2±0.6**	0**	0.1±0.1	2.3±0.6**	2.07	9.00
160 μg/kg (*n*=10)	80^{++}	2.0±0.7**	0.2±0.2**	0.1±0.1	2.3±0.6**	1.84	10.20
1600 μg/kg (*n*=8)	100	3.4±0.6**	0.1±0.1*	0.1±0.1	3.6±0.5**	3.64	5.10

Note. *n* is the number of animals. PI is Pauls index, AUA is antiulcer activity. $^{+}p<0.05$, $^{++}p<0.01$ (φ Fisher's test), $^{*}p<0.05$, $^{**}p<0.01$ (Mann—Whitney *U* test) in comparison with negative control group.

of destructions. Flavonoids in a dose of 12 μg/kg (AUA=2.2) significantly ($p<0.01$) decreased in the mean number of animals with ulcers (to 70%) and the number of mice with large destructions (by 6.2 times) relative to the corresponding values in the negative control group (Table 1). The use of flavonoids in a dose of 16 μg/kg decreased the incidence of ulcers to 30% ($p<0.01$), suppressed the genesis of strip-like destructions and large ulcers, resulting in a gastroprotective effect of 45.3 points (Table 1). Administration of flavonoids in a dose of 20 μg/kg reduced the number of mice with ulcers by 20%, the mean number of destructions by 2.5 times ($p<0.01$), and suppressed the formation of large ulcers. Increasing the dose to 24 μg/kg led to an increase in the gastroprotective effect from 3.1 (20 μg/kg) to 19.4 points, due to a significant ($p<0.01$) decrease in large and small defects, number of mice with ulcerative destruction (by 50%) and the mean number destruction (by 9.7 times) relative to the corresponding values of the negative control (Table 1).

Under conditions of indomethacin-induced damage, the reference drug methyluracil exhibited pronounced gastroprotective effect (AUA=3.3); in the negative control group, ulcers were detected in 100% mice (Table 1). The use of flavonoids in a dose of 32 μg/kg reduced the number of mice with small destructions by 6.6 times ($p<0.01$), suppressed the formation of strip-like ulcers, and reduced the mean number of ulcers per animal by 8.1 times ($p<0.01$) in comparison with the negative control group (Table 1). Increasing the dose from 32 to 160 μg/kg led to an increase in the gastroprotective effect of the phytocomplex (AUA=10.2) due to a decrease in the number of mice with ulcers (to 80%; $p<0.01$) and the mean number of destructions (by 8.1 times; $p<0.01$). Increasing the dose to 1600 μg/kg led to a 5.2-fold decrease in the mean number of all types of ulcerative destruction ($p<0.01$). Positive changes led to significant gastroprotective activity (AUA=5.2), but it was 2-times lower than that observed after administration of 160 μg/kg phytocomplex (AUA=10.2) (Table 1). More potent antiulcerogenic efficacy of the phytocomplex in all doses in comparison with methyluracil is worthy of note.

Our previous study of the effect of single oral administration of indomethacin in a dose of 60 μg/kg on the morphological structure of GM and the small intestine in rats revealed a pronounced inflammatory process and significant destructive restructuring in GM and the "lower" gastrointestinal regions [1]. In this experiment, administration of indomethacin in a similar regimen induced significant damage to GM in 100% rats. The gastroprotective activity of the reference drug plantaglucide consisted in a significant decrease in the number and area of large ulcers and the mean number of ulcers per rat, which resulted in a decrease in the degree (by 1.8 times; $p<0.05$) and severity (by 43%) of damage in comparison with the corresponding values in the negative control group (Tables 2, 3). The antiul-

cerogenic effect of flavonoids (16, 160, and 1600 μg/kg) manifested in a significant decrease in the number and area of large ulcers. The degree and severity of damage to GM in rats treated with the phytocomplex in a dose of 16 μg/kg decreased by 1.7 times ($p<0.01$) and 42%, respectively, in comparison with the values of the negative control group; in animals receiving 160 μg/kg phytocomplex, these parameters decreased by 1.8 times ($p<0.01$) and 43%; for the dose of 1600 μg/kg, the corresponding shifts were by 2.2 times ($p<0.01$) and 54% (Tables 2, 3).

On the model of ethanol-induced ulcerogenesis, the use of 96% ethanol induced destructive processes in GM of 100% rats with a predominant formation of large and striped ulcers (Tables 2, 3). Course preventive administration of plantaglucide led to a decrease in the mean number of destructions (by 1.5 times; $p<0.01$) due to a decrease in the number of large ulcers (by 3.5 times; $p<0.05$). When flavonoids were used in a dose of 16 μg/kg, the Pauls index (2.1 points) indicated a gastroprotective effect, while the degree and severity of damage to GM tended to increase relative to the negative control group. Administration of flavonoids in doses of 160 and 1600 μg/kg prevented the formation of large destructions: their number and area decreased by 3.5 and 5.3 times ($p<0.05$), respectively, which resulted in a decrease in the degree and severity of damage to GM in rats (Tables 2, 3). At the same time, the integral indicator of the antiulcer effect of flavonoids was 1.6 (160 μg/kg) and 2.0 (160 μg/kg), *i.e.* was comparable with that of plantaglucide.

In recent published reports, apigenin was tested in experimental (diabetes, oncology, Alzheimer's disease, amnesia, and depression) and clinical (Alzheimer's disease, insomnia, knee osteoarthritis, neuroses, and depression) studies with course administration in a dose range of 2.5-50 mg/kg [12,14,15]. In our study, *Lychnis chalcedonica* flavonoids administered *per os* exhibited pronounced antiulcer activity in a dose range of 16-1600 μg/kg. It should be noted that in two models of ulcerogenesis in mice, a wave-like dose—effect relationship was revealed: positive results were obtained with a low (16 μg/kg) and high (1600 μg/kg) doses of flavonoids with a decrease in activity at intermediate doses. The revealed type of dependence may testify in favor of the polypotent mechanism of action of *Lychnis chalcedonica* flavonoids [9,14,15]. In addition, more pronounced gastroprotective effect of the flavonoid complex was observed in experiments on mice, which can be due to interspecies differences in the sensitivity of mice and rats to the tested substances and experimental influences [2].

Modeling of ulcerogenesis triggers a cascade of pathological factors, including changes in the neurohumoral status of animals, stimulation of the carbonic anhydrase mechanism of hydrochloric acid secretion, epithelial apoptosis, the formation of free radicals, the release of interleukins, lysosomal enzymes, and

TABLE 2. Antiulcerogenic Effect of *Lychnis chalcedonica* Flavonoid Complex in Models of Indomethacin- and Ethanol-Induced Damage to GM in Outbred Male Wistar Rats ($X±m$)

Group	Rats with ulcers, %	Number of ulcers per rat			Mean number of ulcers per rat	PI	AUA
		small	strip-like	large			
Indomethacin-induced ulcer model (5-day course)							
Negative control (n=10)	100	10.4±2.8	9.8±1.3	17.9±2.3	38.1±4.4	38.1	—
Plantaglucide, 263 mg/kg (n=10)	100	13.0±2.1	8.6±1.3	6.1±1.1**	27.7±1.8*	27.7	1.4
Flavonoids 16 μg/kg (n=10)	100	17.9±2.4*	6.4±1.5	9.0±1.6**	33.3±2.9	33.3	1.1
160 μg/kg (n=10)	100	16.8±2.9	8.3±1.5	6.4±1.1**	31.5±4.2	31.5	1.2
1600 μg/kg (n=10)	100	19.4±3.1*	6.8±1.5	5.3±1.0**	31.5±3.0	31.5	1.2
Ethanol-induced ulcer model (5-day course)							
Negative control (n=10)	100	10.8±3.4	10.7±1.6	11.6±4.0	33.1±4.5	33.1	—
Plantaglucide, 263 mg/kg (n=10)	100	7.2±2.2	11.3±1.1	3.3±1.8*	21.8±3.6*	21.8	1.5
Flavonoids 16 μg/kg (n=8)	100	0±0*	10.0±1.3	5.5±1.4	15.5±1.6*	15.5	2.1
160 μg/kg (n=10)	100	4.2±2.3	12.9±1.3	3.3±1.1*	20.4±2.7*	20.4	1.6
1600 μg/kg (n=10)	100	5.1±1.7	9.5±1.5	2.2±0.9*	16.8±1.9*	16.8	2.0

Note. n is the number of animals. PI is Pauls index, AUA is antiulcer activity. *$p<0.05$, **$p<0.01$ in comparison with negative control (Mann—Whitney U test).

TABLE 3. Influence of *Lychnis chalcedonica* Flavonoids on the Severity of Indomethacin- and Ethanol-Induced Damage to GM in Outbred Male Wistar Rats (*X*±*m*)

Group	Area of ulcers per rat, mm²			Degree of injury, mm²	Severity of injury, %
	small	strip-like	large		
Indomethacin-induced ulcer model (5-day course)					
Negative control (*n*=10)	2.0±0.6	32.0±5.0	56.2±7.1	90.3±6.4	100
Plantaglucide, 263 mg/kg (*n*=10)	2.6±0.4	29.4±5.0	19.2±3.5**	51.1±6.4*	57
Flavonoids 16 μg/kg (*n*=10)	3.5±0.5*	20.8±5.2	28.3±5.0**	52.6±6.6**	58
160 μg/kg (*n*=10)	3.3±0.6	27.7±7.7	20.1±3.5**	51.1±8.1**	57
1600 μg/kg (*n*=10)	3.8±0.6*	21.3±5.4	16.7±3.1**	41.8±6.7**	46
Ethanol ulcer model (5-day course)					
Negative control (*n*=10)	2.1±0.7	78.4±28.9	36.5±12.6	116.9±27.0	100
Plantaglucide, 263 mg/kg (*n*=10)	1.4±0.4	77.6±9.6	10.4±5.8*	89.4±8.5	76
Flavonoids 16 μg/kg (*n*=8)	0±0**	180.5±31.1*	17.3±4.4	197.8±32.9	169
160 μg/kg (*n*=10)	0.8±0.5	96.1±13.5	10.4±3.5*	107.3±13.5	92
1600 μg/kg (*n*=10)	1.0±0.3	87.6±17.9	6.9±2.8*	95.5±18.7	82

Note. *n* is the number of animals. *$p<0.05$, **$p<0.01$ in comparison with negative control (Mann—Whitney *U* test).

tumor necrosis factor [3,5,14]. These perturbations can induce changes in all types of metabolism, local disturbances of secretion and motility of the stomach and duodenum, deterioration of regional blood flow and, ultimately, the development of destructive processes in the gastroduodenal zone [3,5]. A possible mechanism of the antiulcerogenic activity of the phytocomplex can be improvement of the protective barrier function of the supraepithelial mucous layer of the stomach due to regulation of the redox processes, stabilization of cell membranes, and modulation of enzyme and receptor activity [4,9,12,14,15]. The antioxidant effect of apigenin is associated with a decrease in the expression of adhesion molecules, increased production of antioxidant enzymes such as glutathione peroxidase, catalase, and SOD [9,14,15]. In *in vitro* systems, flavonoids enhance the expression of genes encoding phase II enzyme, blocking the NADPH oxidase complex and increasing the expression of Nrf-2 nuclear translocation. Once in the bloodstream (C-forms of apigenin are characterized by good bioavailability), flavonoids are involved in diverse processes of cell signaling, gene expression, and various metabolic transformations [9,15]. It is known that apigenin can interact with cytokine receptors coupled with G-protein (GPCR), tyrosine kinase (RTK), and transmembrane proteins [9,15]. The anti-inflammatory effect of apigenin is associated with inhibition of nitric oxide production via suppression of NO synthase expression and COX-2 activity [14]. The anti-inflammatory and analgesic activity of the *Lychnis chalcedonica* flavonoid complex demonstrated in our previous experiments related to its inhibitory effect on mediators (prostaglandins and kinins) of the late phase of the inflammatory process is an evidence of *in vivo* pharmacological action of C-forms of apigenin flavones [13]. Currently, it is believed that the anti-inflammatory effect of apigenin is due to suppression of the expression of the CD40 differentiation cluster and secretion of proinflammatory cytokines (TNFα, IFNγ, IL-6, *etc*.) by immunocompetent cells [14]. In this case, the development of these effects is based on the blockade of phosphorylation of Akt, protein kinases c-Raf, ERK1/2, p38, and JNK, as well as inactivation of the nuclear transcription factor NF-κB and proteins of the STAT family. This leads to violation of the expression of genes responsible for the production of proinflammatory cytokines and adhesion molecules [11]. In general, ample data are obtained (mainly in *in vitro* experiments) on the molecular mechanisms of action of apigenin and its C-glycosylated forms. Nevertheless, there is still no detailed understanding of the pharmacodynamics of these flavonoids *in vivo* [9,14,15].

Thus, a complex of flavonoids extracted from the aerial part of *Lychnis chalcedonica* and administered *per os* in a dose range of 16-1600 μg/kg exhibits a pronounced antiulcer effect in models of neurogenic, chemical, and drug-induced damage to GM in mice and rats. Activity of the phytocomplex exceeds the effect of the reference preparations *Eleutherococcus* extract and methyluracil in therapeutic doses, but is similar to the effectiveness of plantaglucide. The antiulcer effect of the flavonoid complex significantly exceeds the previously identified antiulcerogenic

activity of the whole extract of *Lychnis chalcedonica*, which allows us to conclude that further study of this phytocomplex is promising as a candidate for a drug for a gastroenterological clinic.

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