ORIGINAL ARTICLE

The immunomodulatory activity of ethanolic extracts from *Galium verum* L. herb.

Imunomodulační aktivita etanolových extraktů z *Galium verum* L. herb.

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Summary

The present article discusses the results of the research into the phytochemical profile and immunomodulatory activity of *Galii veri* herba (*Galium verum* L., *Rubiaceae*) ethanolic extracts. The extracts under study were obtained from the plant material by means of maceration technique with 20% aqueous ethanol solution (*fluid extract I)*, 60% aqueous ethanol solution (*fluid extract II)* and 96% ethanol (*fluid extract III)* on heating. In the substances obtained, the content of hydroxycinnamic derivatives, flavonoids and polyphenols were determined spectrophotometrically; polysaccharides were quantified gravimetrically; the immunomodulatory activity of the substances under study was determined in the reaction of lymphocyte blast transformation (RLBT). It was established that *fluid extract I* contained 2.4% polysaccharides, 3.1% hydroxycinnamic derivatives, 0.24% flavonoids, and 2.9% polyphenols; *the fluid extract II* contained 4.13% hydroxycinnamic derivatives, 0.16% flavonoids, and 3.84% polyphenols; *fluid extract III* contained 2.7% hydroxycinnamic derivatives, 0.18% flavonoids and 2.7% polyphenols. All the extracts under study possessed a marked stimulant effect on the transformation activity of the immunocompetent blood cells. The highest immunomodulatory activity was established for 96% ethanol extract: the percentage of lymphocytes proliferating in RLBT under the influence of this extract increased by 6.77– 8.04 times in comparison with their spontaneous transformation and by 1.14–1.36 times in comparison with phytohemagglutinin. The results obtained give grounds for further research in the mechanisms of the immunomodulatory activity of the extracts of *G. verum* herb.

Key words: ethanolic extracts • *Galium verum* L. • immunomodulatory activity • lymphocyte blast transformation

Souhrn

V tomto článku jsou diskutovány výsledky výzkumu fytochemického profilu a imunomodulační aktivity etanolických extraktů *Galli veri* herba (*Galium verum* L., *Rubiaceae*). Studované extrakty se získaly z rostlinného materiálu macerací za tepla 20% vodným roztokem ethanolu (tekutý extrakt I), 60% vodným roztokem ethanolu (tekutý extrakt II) a 96% ethanolem (tekutý extrakt III). Obsah hydroxycinnamických derivátů, flavonoidů a polyfenolů byl stanoven spektrofotometricky; polysacharidy byly kvantifikovány gravimetricky; imunomodulační aktivita studovaných látek byla stanovena reakcí blastické transformace lymfocytů (RLBT). Bylo zjištěno, že tekutý extrakt I obsahoval 2,4 % polysacharidů, 3,1 % hydroxycinnamických derivátů, 0,24 % flavonoidů a 2,9% polyfenolů; tekutý extrakt II obsahoval 4,13 % hydroxycinnamových derivátů, 0,16 % flavonoidů a 3,84 % polyfenolů; tekutý extrakt III obsahoval 2,7 % hydroxycinnamických derivátů, 0,18 % flavonoidů a 2,7 % polyfenolů. Všechny studované extrakty vykazovaly výrazný stimulační účinek na transformační aktivitu imunokompetentních krevních buněk. Nejvyšší imunomodulační aktivita byla stanovena pro 96% ethanolový extrakt: procento lymfocytů proliferujících v RLBT vlivem tohoto extraktu se zvýšilo o 6,77–8,04krát ve srovnání s jejich spontánní transformací a 1,14–1,36krát ve srovnání s fytohemaglutininem. Získané výsledky dávají

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důvody pro další výzkum mechanizmů imunomodulační aktivity extraktů z natě *G. verum*.

Klíčová slova: ethanolické extrakty • *Galium verum* L. • imunomodulační aktivita • blastická transformace lymfocytů

Introduction

The study of the development, progression and correction of conditions accompanied by malfunction of the immune system is an important challenge medical science faces today. Extensive research in theoretical and clinical immunology has identified a multitude of diseases caused by the dysfunction of various components of the immune system and requiring an immune-corrective therapy.

For the correction of immunodeficiency conditions, modern medicine has developed a wide range of medicinal products of both synthetic and natural origin. Noteworthily, developed of medicinal products of medicinal origin constitutes an especially promising direction[1)].

The immunomodulators of plant origin, as opposed to the synthetic ones, display a number of advantages, i.e., a mild immunomodulating effect, a low toxicity, an activation of immune system functions due to biologically active compounds of a holistic effect, etc.[2–4)].

In this connection, in recent years most research has been focused on the potential immunostimulatory properties of herbal phenolic compounds[5–7)]. It was established that chlorogenic, gallic, ellagic, caffeic, protocatechuic acid and salicylic acid stimulate the production of the immunoglobulins class G. Well known is the effect of caffeic acid on the humoral component of the immune system[8)].

For *in vitro* assessment of the substances' influence on the proliferative activity of the cells of the immune system, a reaction of lymphocytes blast transformation (RLBT) is often used. It consists in the ability of small peripheral blood lymphocytes to transform into large, undifferentiated blast cells within 72–96 hours after a primary contact with mitogens, such as phytohemagglutinin (PHA), etc., or after the second contact with microbial tissue antigens. Thus, RLBT under the influence of mitogens is viewed as an indicator of the functional activity of the T- and B-components of the immune system[9)].

Previously, we studied the immunomodulatory activities of *Asperula* L. species and of a fluid water extract from *Galium verum* L., *Rubiaceae* family[10, 11)].

The aim of the present article is to discuss the effect of the fluid ethanolic extracts from *G. verum* on the functional activity of lymphocytes in the reaction of lymphocyte blast transformation.

Experimental part

Plant materials

G. verum herb was harvested at full flowering stage in the Kharkiv region (Ukraine) in the summer of 2016. Herbarium samples (No. 20062016-25062016) are deposited at the Department of Pharmacognosy (National University of Pharmacy, Ukraine).

Equipment

Spectrophotometer Evolution™ 60S UV-Visible (Thermo Fisher Scientific, USA), electronic analytical scales AN 100 "Axis" (AXIS, PL), electrical temperature chamber TC80M-3 (Medlabortekhnika, UA), centrifuge OPN-3 (Phizpribor, RU), microscope ZEISS Primo Star (ZEISS, DE), pipette Thermo Scientific, Lait series 1–200 µl (Thermo Fisher Scientific, USA), pipette Thermo Scientific, Lait series 1–50 µl (Thermo Fisher Scientific, USA), pipette Thermo Scientific, Lait series 1–1000 µl (Thermo Fisher Scientific, USA), pipette Thermo Scientific, Lait series 1–20 µl (Thermo Fisher Scientific, USA), CO_2 incubator (Binder, DE), bioanalyzer Agilent 2100 (Agilent, DE).

Chemicals

96% Ethanol and purified water used during extraction complied with requirements of the State Pharmacopoeia of Ukraine[12, 13)]; chemicals used for the phytochemical screening and quantification of main groups of biologically active compounds (BACs): ethanol (ACS reagent, Fisher Scientific, USA), hydrochloric acid, p.a. (Sobstar, Zaporizhia, UA), acetic acid, puriss. (PJSC AZOT, UA), lead (II) acetate, p.a. (Unikhim Ltd., RU), aluminium chloride, p.a., granulated zinc, p.a. (PC Uralskiy zavod khimicheskih reaktivov, RU), ferric (III) chloride, puriss. (Sigma-Aldrich, USA), gallic acid, chlorogenic acid and rutin were of analytical grade (Merck, DE).

Preparation of extracts

As a solvent, ethanol at various concentrations (20%, 60% and 96%) was used; the extraction was carried out at a general ratio of the plant material : solvent of 1 : 10 on heating with reflux. The extraction was repeated three times under the same conditions (30 min each). The extracts obtained were combined, concentrated on a vacuum rotary evaporator to the ratio of plant material – finished product of 1 : 1.

Preliminary phytochemical screening of G. verum herb fluid ethanolic extracts

The preliminary phytochemical screening was performed using generally accepted methods and techniques of phytochemical analysis[13, 14)]. Glycosides and aglycons of flavonoids were determined in extracts from *G. verum* herb in the reactions of identification: cyanidine reaction by Bryant (yellow-red colouring of the aqueous phase and yellow-hot colouring of the octal phase), the reaction with 3% solution of iron (III) chloride (dark green colour of flavonols, flavones); the reaction with an alkaline solution (bright yellow colour); the reaction with 5% solution of aluminium chloride (yellow-green colouring); the boric-acid reaction (yellow colouring on detection of 3- and 5-hydroxyflavones and 5-hydroxyflavanones); the reaction with ammonia (flavones, flavonols, flavanones and flavanonols dissolve with formation of yellow co-

lour, which, when heated, changes to orange or brown colour).
To determine tannins, the reactions of the sediment were carried out with 1% gelatine solution, 1% solution of quinine hydrochloride, 10% solution of basic acetate of lead. The group of tannins was detected by the reaction with a solution of iron ammonium alum.

Quantification of main groups of BACs
In 20% aqueous ethanol extract, polysaccharides were quantified gravimetrically after complete drying at room temperature taking into account the loss on drying[15, 16]. In all the fluid ethanolic extracts from *G. verum* herb, the sum of the hydroxycinnamic derivatives was determined by direct spectrophotometry (as chlorogenic acid, $\lambda = 325$ nm) according to Yezerska et al.[17], Spagnol et al.[18]; flavonoids were quantified by the method of differential spectrophotometry with aluminium chloride (as rutin, $\lambda = 410$ nm)[19]; polyphenols were quantified by direct spectrophotometry (as gallic acid, $\lambda = 270$ nm) according to Kovalyova et al.[20]. All assays were performed in triplicate.

Study of immunomodulatory activity
To assess the immunomodulatory activity of the extracts obtained, *in vitro* RLBT with an adequate resolution was used[21, 22].
As a sample for substance testing, the mononuclear cells (lymphocytes) removed from venous heparinized blood (donated blood, Kharkiv Regional Blood Banking Centre, UA) by ficoll-verographine gradient density centrifugation (density 1.077 g/mL) (Research and Production Enterprise "PanEco", RU) by the standard technique[23], were used (Protocol of Committee on Biomedical Ethics of SO "Mechnikov Institute of Microbiology and Immunology" No. 2 of May 16, 2017).
The cells obtained were cultured in medium 199 with addition of 10% bovine foetal serum (Thermo Fisher Scientific, BR), 2 mM L-glutamine (Altera Holding, RU), 100 μg/mL gentamicin (LEK (CZ). A suspension of 1 million cells per 1 mL of the culture medium with an addition of substances was incubated for 15–18 hours in a thermostat at 37 °C, in a 5% CO_2 atmosphere with saturated water vapour.
The intensity of the proliferative reaction was evaluated by the indices of DNA (deoxyribonucleic acid) synthesis activation recorded by the treatment of samples with anti-BrdU (5-bromo-2'-deoxyuridine) Antibody (3H579) monoclonal antibodies (Santa Cruz Biotechnology, USA) at a concentration of 100 mg/mL. After the final sample preparation, numerical data on the total number of cells and the percentage of blast forms in the samples were established for the flow cytometric analysis with fluorescence detection.
Before the RLBT, the extracts were prepared in ratios of 1/200, 1/20, 1/10 (solvent – distilled water). 100 μL of substances were added to 100 μL of primary cultures of immunocompetent cells. The mitogenic stimulation of lymphocytes by PHA (Research and Production Enterprise "PanEco", RU) at the concentration of 2.5 μg/mL was performed as the control. RLBT without the addition of the substances under study (spontaneous blast transformation) was also evaluated.

Statistical analysis
All statistical analyses were carried out in accordance with the requirements of the State Pharmacopoeia of Ukraine using Microsoft Office Excel 2007[16, 24]. Differences between groups were statistically analysed using one-way analysis of variance (ANOVA). The results were expressed as mean ± standard *deviation* (SD). P values less than 0.05 were considered statistically significant.

Results and discussion

Phytochemical screening of G. verum herb fluid ethanolic extracts
The phytochemical screening of *G. verum* herb fluid ethanolic extracts revealed the presence of flavonoids (flavonols and flavones) and condensed tannins and the results obtained correspond with previous studies[25–27].

Quantification of main groups of BACs
The content of main groups of BACs in G. verum herb fluid ethanolic extracts is given in Table 1.

Table 1. The content of main groups of BACs in G. verum herb fluid ethanolic extracts

Extract	Group of BACs (%)			
	Polysaccharides	Hydroxycinnamic derivatives	Flavonoids	Polyphenols
Extract **I** (the extragent – 20% aqueous ethanol solution)	2.4 ± 0.04	3.1 ± 0.05	0.24 ± 0.01	2.9 ± 0.05
Extract **II** (the extragent – 60% aqueous ethanol solution)	–	4.13 ± 0.06	0.16 ± 0.01	3.48 ± 0.06
Extract **III** (the extragent – 96% ethanol)	–	2.7 ± 0.05	0.14 ± 0.01	2.7 ± 0,05

– this group of BACs is not contained in extracts

The highest content of hydroxycinnamic derivatives is found in fluid extract **II** and it is comparable with the content of hydroxycinnamic derivatives in the fluid water extract **II**), the lowest content of this group of BACs being found in the 96% ethanol extract. Among the fluid ethanolic extracts, the content of flavonoids is the highest in the extract obtained with 20% aqueous ethanol solution, and it is significantly lower than that in the fluid water extract **II**). The content of polyphenols is the highest in the extract obtained with 60% aqueous ethanol solution and it is slightly higher than that in the water extract **II**). The data obtained display some differences from those reported by other researchers[28, 29], which may result from various factors, such as growth conditions of the plants under study or the methods of extraction and analysis.

In vitro reaction of lymphocyte blast transformation

The research presented is the first known study of the immunomodulatory activity of *G. verum* herb fluid ethanolic extracts.

It was established that all substances under study considerably stimulate the transformational activity of peripheral blood mononuclear cells. Under the influence of the extracts under study, 41.7–66.7% of mononuclear cells were involved in the proliferation process, which indicates the stimulating effect of the substances on T- and B-lymphocytes (Table 2). The activity under the influence of substances increases from 33.4% (extract **II** at a dilution of 1/200) to 58.4% (extract **III** at a dilution of 1/20) compared with spontaneous lymphocyte blast transformation.

The highest activity was established for extract **III** at a dilution of 1/20, its activity being by 58.4% higher than that of the lymphocyte spontaneous transformation and by 17.5% higher than that of the reference substance PHA. Somewhat lower indices were established for the extract at a dilution of 1/200, its activity being by 54.8% higher than that of the lymphocyte spontaneous transformation and by 13.9% higher than that of PHA; the lowest activity was exhibited at a dilution of 1/10, its activity being by 47.9% higher than that of the lymphocyte spontaneous transformation and by 7.0% higher than that of PHA.

Extract **I** showed a significant activity at a dilution of 1/20 (its activity was by 52.5% higher than that of the lymphocyte spontaneous transformation and by 11.6% higher than that of PHA), at a dilution of 1/10 (its activity was by 49.3% higher than that of the lymphocyte spontaneous transformation and by 8.4% higher than that of PHA), at a dilution of 1/200 (its activity was by 46.9% higher than that of the lymphocyte spontaneous transformation and by 6.0% higher than that of PHA).

The lowest activity was established for extract II: at a dilution of 1/20 its activity was by 42.9% higher than that of the lymphocyte spontaneous transformation and by 2.0% lower than that of PHA; at a dilution of 1/10 its activity was by 38.5% higher than that of the lymphocyte spontaneous transformation and by 2.4% lower than that of PHA; at a dilution of 1/200 its activity was by 33.4% higher than that of the lymphocyte spontaneous transformation and by 7.5% lower than that of PHA.

On average, the studied substances (at the concentration of 100 μL) showed the most potent stimulation on the functional activity of immunocompetent cells at a dilution of 1/20, which corresponds with the data obtained in the study of the immunomodulatory activity of the water extract from *G. verum* herb and its components[11].

Only extract **III** (the extragent – 96% ethanol) at dilutions of 1/20 and 1/200 possesses almost the same stimulant effect on the functional activity of lymphocytes as

Table 2. The effect of fluid ethanolic extracts of G. verum on the indices of lymphocyte blast transformation (X ± m), n = 5

Extract	**Dilution of the extract**	**RLBT, %**
Extract **I** (the extragent – 20% aqueous ethanol solution)	1/200	55.2 ± 2.3
	1/20	60.8 ± 2.5*
	1/10	57.6 ± 2.6
Extract **II** (the extragent – 60% aqueous ethanol solution)	1/200	41.7 ± 2.3
	1/20	51.2 ± 2.4
	1/10	46.8 ± 2.6
Extract **III** (the extragent – 96% ethanol)	1/200	63.1 ± 2.2*
	1/20	66.7 ± 2.4*
	1/10	56.2 ± 2.3
PHA	–	49.2 ± 2.3
Spontaneous RLBT	–	8.3 ± 0.6

PHA – phytohemagglutinin, RLBT – the reaction of lymphocyte blast transformation

* P < 0.05 in comparison with the indices of control

the water extract; however, at a dilution 1/10 its effect is somewhat lower[11]. The levels of the immunomodulatory activity established for extracts **I** and **II** are lower than that of the water extract. The activity of extract **I** (the extragent – 20% aqueous ethanol solution) is comparable with the activity of the polysaccharide complex[11].

While the immunomodulatory effects of a number of plants of the *Rubiaceae* family have been reported in several studies[10, 30, 31], there is only one study on the immunomodulatory effects of the species of genus *Galium – Galium mite*[32]. Although the inhibitive effect of the plant on lymphocyte proliferation via induction of apoptosis has been described, there are no reports regarding the constituents of *G. mite.*

A number of previous studies have established the presence of flavonoids and hydroxycinnamic acids in the herb *G. verum*[33, 34]. As flavonoids are known to exhibit immunostimulatory and/or immunosuppressive effects[35, 36], this could account for the results of the immunological analysis of the plant extracts.

Up to date, there have been no publications testifying for the immunomodulatory activity of extracts from *G. verum*. Our data, however, correlate with those of the immunostimulatory effects of extracts containing polyphenolic compounds, i.e. hydroxycinnamic acids, flavonoids, etc., from other plants, such as, *Trigonella foenum graecum* and *Leonurus cardiaca*[37–40].

The data obtained indicate that ethanolic extracts from *G. verum* herb intensively stimulate the blast transformation of lymphocytes at the initial low level, which gives grounds for further research into the influence of these extracts on the blast transformation of lymphocytes at the initial high level.

Conclusion

First-obtained fluid ethanolic extracts from *G. verum* herb were studied for their chemical composition and immunomodulatory activity. All ethanolic extracts from *G. verum* herb significantly stimulate the transformational activity of immunocompetent blood cells, with 96% ethanolic extract being most active. The percentage of lymphocytes proliferating in RLBT under the influence of 96% ethanolic extract increased by 8.04–6.77 times compared with the spontaneous transformation and by 1.36–1.14 times compared with PHA. The data obtained give grounds for further research into the mechanisms of immunomodulatory activity of extracts from *G. verum* herb.

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