Pharmacogn. Commn. 2016; 6(1) 42-47
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcogcommn.org

Original Article

Antimicrobial Activity of the Genus *Galium* L.

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ABSTRACT

Introduction: The common use of *Galium* L. plants (family Rubiaceae Juss.) in Ukrainian folk medicine for treating infectious diseases became the basis for the study of antimicrobial activity of lipophilic complexes obtained from the *Galium* species. **Materials and Methods:** The samples of herbs of *Galium verum*, *Galium salicifolium, Galium dasypodum, Galium aparine, Galium carpaticum and Galium pseudomollugo* harvested at flowering stage became the objects of the present research. Lipophilic complexes have been obtained by the exhaustive extraction of herbal drugs with chloroform in the Soxhlet apparatus. The complexes have been used as 2% alcohol solutions in 96% ethyl alcohol. The study was conducted *in vitro* by the agar diffusion method. Standard strains of micro-organisms were used to assess the antimicrobial activity. **Results:** *Staphylococcus aureus* was most sensitive to the studied lipophilic complexes. *Escherichia coli* and *Proteus vulgaris* showed low sensitivity. *Pseudomonas aeruginosa* and *Bacillus subtilis* have shown moderate sensitivity. The highest activity against *Candida albicans* has been observed in complexes of *Galium aparine, Galium dasypodum, Galium pseudomollugo*. Conversly, *Galium verum* manifested no activity against *Candida albicans*. The minimum bactericidal and inhibitory concentrations were determined. The correlation between the content of individual groups of biologically active substances and the level of activity of the complexes was established. All complexes were non-toxic. **Conclusion:** The results obtained provided us the basis for further in-depth studies of antimicrobial and antifungal activity of the lipophilic complexes of the genus *Galium* L.

Key words: Antimicrobial activity, Antifungal activity, Bedstraw genus, Lipophilic complexes, Toxicity.

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DOI : 10.5530/pc.2016.1.8

INTRODUCTION

The genus *Galium* L. is the largest genus of tribe *Rubieae* subfamily *Rubioideae* of Madder family (*Rubiaceae* Juss.), and in the world flora is represented by more than 600 species distributed in extratropical of Eurasia, North and South America, North America, Australia; Arctic highlands, subtropical and tropical climatic zones.[1-4] About 50 species of Bedstraws grow in Ukraine.

In the official medicine of Ukraine the herb of Lady's Bedstraw or Yellow Bedstraw–*Galium verum* L. is used. A tincture from Lady's Bedstraw herb is a constituent of the Ukrainian domestic combined herbal remedy "Tazalok ™", which is used in the treatment of menstrual disorders, premenstrual syndrome, algodysmenorrhea, dysmenorrhea and climacteric disorders.[5] Countervailing preclinical and clinical trails have shown the efficacy of this remedy in the treatment of hormonal dysfunctions and associated pathologies.[6]

Cleavers or «goosegrass» (*Galium aparine* L.) is mentioned in The British Herbal Pharmacopoeia.[7] This species is traditionally used to treat a variety of skin ailments, light wounds and burns.

The tincture from *Galium aparine* herb is a component of a number of antihomotoxic remedies manufactured by German company Biologische Heilmittel Heel GmbH–"Galium-Heel" (the composition of this remedy is represented by tinctures from *Galium album* Mill. and *Galium aparine* L. herbs); "Ginseng compositum"; "Cutis compositum"; "Thyreoidea compositum (Rx)"; "Tonsilla compositum" and "Ubichinon compositum".

The extracts from *Galium album* Mill. and *Galium verum* L. leaves are applied topically to treat wounds.[8] Numerous experimental studies of the scientists from many countries confirm the antimicrobial properties of *Galium* species.[9-12]

In Ukranian folk medicine, *Galium* species are widely used in the treatment of skin infections, infections of respiratory, genitourinary systems and sepsis.[8]

Despite this, the antimicrobial activity of Ukranian *Galium* species is poorly reported. The wide use of *Galium* species in the folk medicine for the treatment of infectious diseases was the basis for the study of antimicrobial activity of complexes obtained from the studied plants.

MATERIALS AND METHODS

Plant collection and extraction

The objects of study were herbs of 5 *Galium* species: *Galium verum* L., *Galium salicifolium* Klokov, *Galium dasypodum* Klokov, *Galium aparine* L, *Galium carpaticum* Klokov and *Galium pseudomollugo* Klokov, harvested in Kharkiv and Ivano-Frankivsk regions in the full-flowering phase in the summer of 2013-2014.

Air dried herbal drugs were cut into small pieces and ground to a fine powder. Then 20 gramms of each herb have been soaked in 200 ml of chloroform. Lipophilic fractions were obtained by exhaustive extraction of herbal drugs with chloroform in the Soxhlet apparatus. The mixtures have been filtered. The filtrates were subsequently evaporated using vacuum rotary evaporator and air dried at 40°C.

Amounts of 20 grams of powdered *Galium verum* herb were extracted with different solvents and three extracts have been obtained by adding 200 ml of water, 70% ethyl alcohol and chloroform respectively.

Qualitative phytochemical studies

The quantification of chlorophylls and carotenoids was performed by the spectrophotometric method on a spectrophotometer HP 8453 UV-VIS,

Hewlett Packard company, USA.[13]

The composition and content of lipophilic compounds were determined by chromatography-mass spectrometry as described below.

To study carboxylic acids composition 50 mg of dry substances with an internal standard (tridecane, 50 mg in hexane) and 1.0 ml of methylating agent (14% BCl_3 in methanol, Supelco 3-3033) have been placed in 2 mL vial. The mixture has been kept in a sealed vial during 8 hours at 65°C. During this time the fatty oil was completely extracted from the herbal drugs, hydrolyzed to fatty acids and their methylation occured. Simultaneously free carboxylic acids were methylated. The reaction mixture has been filtered, diluted with 1 ml of distilled water. To extract methyl esters of carboxylic acids 0.2 ml of dichloromethane were added, the mixture was stirred for an hour and then the obtained extract of methyl esters was chromatographed.[14]

The research of volatile compounds. 0.5 g of lipophilic fractions have been placed in a 20 mL vial, internal standard (tridecane, 50 mg in hexane), 10 ml of water were added and mixture was distiled off for 2 hours using a reflux condenser. After cooling the system a condensate on the inner surface of the reflux condenser was washed with 3 ml of ultra-pure pentane in a dry 10 mL vial. The solution was concentrated by blowing (100 mL/min) with especially pure nitrogen to a residual volume of 10 ml of the extract, which was completely taken away by chromatographic syringe. Further concentration of the samples was carried out directly in the syringe to the volume of 2 μl.

The injection of tests in chromatographic column has been conducted in the *splitless* mode. Agilent Technology 6890N chromatograph with mass spectrometric detector 5973N have been used. The analysis conditions: chromatographic capillary column DB-5 (to determine the components before methylation) and INNOWAX (to determine the components after methylation) with the length of column–30 m, an internal diameter – 0.25 mm, carrier gas–helium, a speed of carrier gas – 1.2 ml/min. The temperature of thermostat–50°C programming 4°C /min to 320°C.[15,16]

Obtained spectra had been analyzed as based on general laws of fragmentation of organic compounds molecules under the action of the electron impact, as by comparing results with the data from mass spectra libraries NIST02 and WILEY 2007 with the total amount of more than 470000 spectra and using identification programs AMDIS and NIST. The content of compounds have been calculated in relation to the internal standart.

Antibacterial screening

Test microorganisms

According to WHO recommendations for preliminary assessment of antibacterial and antifungal activity we used test-strains of *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 4636, *Bacillus subtilis* ATCC 6633 and *Candida albicans* 885-663.

Evaluation of antimicrobial activity

Substances were used as 20 g/l solutions in 96% ethyl alcohol. *In vitro* study was performed by agar well diffusion method.[17-19] Microbial loading has been to 10^7 microbial cells in 1 ml of medium and was determined visually and by optical turbidity standard by McFarland.[17,19]

The bacterial cultures were grown on nutrient agar at t=37°C to determine the antimicrobial action. The period of microorganisms culturing was 24 hours. We have used Saburo medium to determine the antifungal activity of substances. The microorganisms sensitivity was measured as a radius of inhibition zone in mm.

The evaluation of microorganisms sensitivity was performed on the following criteria:

- no inhibition zone and areas of inhibition zone to 10 mm pointed out that the microorganism is not sensitive to the studied substance or substance's chosen concentration;
- inhibition zones with diameter 10-15 mm have shown low sensitivity of microorganism to the tested substance concentration;
- inhibition zones with diameter 15-25 mm have been regarded as an indicator of sufficient sensitivity of the microorganism to the tested substance;
- inhibition zones with diameter exceeding 25 mm have shown high sensitivity of microorganisms to the studied substances.

Determination of minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC)

To quantify the antimicrobial and antifungal activity of lipophilic fractions and determine the minimum inhibitory concentration, the serial dilutions method has been used.[19-21] The method is based on titration of studied antibacterial agent in liquid culture medium by serial dilution of a certain volume of liquid in the first tube to the following ones using control – the medium which does not contain the drug. Suspension of daily agar bacterial cultures of 5×10^5 microbial cells in 1 ml has been put in all test tubes.

The account of results has been carried out 16-18 hours later by assessing the growth inhibition of microorganisms in test tubes containing the appropriate drug dilutions. The last tube with the growth inhibition (clear broth) has met the minimum inhibitory concentration of the substance in relation to the test strain. We have taken on a dense nutrient medium to assess the bactericidal properties of the substance from 2-3 last test tubes with the lack of growth. In 24-48 hours of incubation in a thermostat the lowest concentration of antibiotic drug in tube, bacterial inoculation from which gave no growth was considered as the minimum bactericidal concentration. We have used the meat broth as a media for all bacterial strains and Sabouraud medium for *Candida albicans*.

As the reference drug we have used "Chlorophyllipt"–domestic Ukrainian antibiotic drug of plant origin, 1 ml of which contains 0.02 g of the thick extract from Eucalyptus leaves and is used as an antiseptic, antibacterial and anti-inflammatory agent for the treatment of diseases caused by antibiotic resistant staphylococcus.[22]

Toxicity screening

To determine the acute toxicity we have used 42 nonlinear white laboratory mice of both sexes weighing 20–22 g, aged 2.0–2.6 month. The studies have been conducted on 7 groups of laboratory animals: group 1 (n=6) – has been a control one – the animals which have been intragastrically administered with distilled water; 2–7 groups (n=6) – the animals, which by means of intragastric probe received aqueous suspension of the studied substances at doses that corresponded to different classes of substances toxicity: 50 mg/kg, 500 mg/kg and 5000 mg/kg in the volume of 0.8 ml each.[23-25] The observations have been conducted for 14 days. The evaluation of the acute toxicity has been performed by the signs of clinical toxicity of test animals, including their general conditions, the functional state of the skin and count the number of animals that have died.[25]

Statistical analysis

Results have been processed statistically by Glantz S.[26]

RESULTS AND DISCUSSION

Qualitative phytochemical studies

The highest yield of lipophilic complex from the herbal drugs has been observed for *Galium carpaticum* (7.69%). During the phytochemical research of lipophilic complexes we have found that the highest content of

chlorophylls (7.69%), carotenoids (0.58%) and aromatic acids (0.06%) was in the complex from *Galium carpaticum*; the highest content of dibasic acids (0.29%) – in the complex from *Galium aparine*; the highest content of unsaturated fatty acids (2.88%) and the content of fatty acids (7.50%) were established for the complex from *Galium salicifolium*; the maximum content of volatile compounds (14.70%) and content of terpenoids (12.20%) were found in the complex from *Galium verum*; the highest content of steroid compounds (0.33%) has been found in the the complex from *Galium dasypodum* (Table 1).

Antimicrobial activity

The preliminary study of antimicrobial activity has begun with the study of water, alcohol (70%) extracts and chloroform complex of Lady's Bedstraw herb (Table 2).

The obtained data have shown that the aqueous extract of Lady's Bedstraw herb showed no antibacterial activity, alcohol (70%) extracts obtained at the ratio of herbal drugs: extractant 1:5 and 1:10 were also unpromising for further investigation of their antimicrobial activity.

In contrast, lipophilic (chloroform) extract has shown a significant level of antimicrobial activity. It has given the basis for further search of antimicrobial substances among chloroform complexes obtained from different species.

The results of research of antimicrobial and antifungal activity of lipophilic complexes (Table 3) have shown that *Staphylococcus aureus* was highly sensitive to lipophilic complexes, but lipophilic complex from *Galium aparine* has shown the highest activity; *Escherichia coli* by contrast, has shown less sensitivity to all studied complexes; *Pseudomonas aeruginosa* has been rather sensitive to all studied complexes and highly sensitive to the complex from *Galium aparine*; *Bacillus subtilis* has been highly sensitive to the complex from *Galium verum* and *Galium carpaticum*, very sensitive to the complex from *Galium salicifolium*, *Galium aparine* and *Galium pseudomollugo* and insensitive to the complex from *Galium dasypodum*; *Proteus vulgaris* has been highly sensitive to the complex from *Galium dasypodum*, sensitive enough to the complexes from *Galium verum* and *Galium carpaticum* and has low sensitivity to the rest of complexes.

Candida albicans has appeared to be highly sensitive to the complex from *Galium aparine, Galium dasypodum, Galium pseudomollugo* and generally insensitive to the complex from *Galium verum*.

The results of MBC and MIC determination of lipophilic complexes are given in Table 4. Comparing MBC and MIC parameters of "Chlorophyllipt" and lipophilic complexes we have established that with respect to *Staphylococcus aureus* MBC of *Galium dasypodum*, *Galium aparine*, *Ga-*

Table 1: Phytochemical profile of lipophilic complexes from *Galium* L. species

The lipophilic complex	Yield, %	Content, %									
		Chlorophylls	Carotenoids	Carboxylic acids					Terpenoids	Steroids	The content of volatile compounds
				dicarboxylic	aromatic	saturated fatty	unsaturated fatty	total fatty acids content			
Galium verum	3.09	1.62±0.08	0.53±0.01	0.26	0.04	4.75	1.87	6.62	12.20	-	14.07
Galium salicifolium	4.52	1.65±0.08	0.08±0.002	0.05	0.02	4.62	2.88	7.50	0.02	0.043	0.19
Galium dasypodum	6.89	5.70±0.21	0.33±0.01	0.06	0.03	2.01	2.07	4.09	0.29	0.33	1.67
Galium aparine	3.02	4.10±0.20	0.46±0.01	0.29	0.04	0.95	1.68	2.64	0.68	0.04	4.79
Galium carpaticum	7.69	6.10±0.23	0.58±0.01	0.21	0.06	0.64	0.45	1.09	0.11	-	1.02
Galium pseudomollugo	4.03	2.33±0.11	0.33±0.01	0.10	0.05	1.44	1.30	2.74	0.21	0.18	1.34

Table 2: Antimicrobial activity of *Galium verum* L. herb extracts

No	Substance (solvent)	Zone of growth inhibition, mm, (M±m), p≤0,05					
		Staphylococcus aureus 15923	*Escherichia coli* 25922	*Pseudomonas aeruginosa* 2789	*Bacillus subtilis* 6633	*Proteus vulgaris* 4636	*Candida albicans* 885-563
1	Alcohol extract (70% ethanol) (1:5)	-	-	-	-	-	10.0±0.1
2	Alcohol extract (70% ethanol) (1:10)	-	-	-	10.0±0.1	10.0±0.2	10.0±0.2
3	The aqueous extract (1:10)	-	-	-	-	-	-
4	Chloroform extract (20 g/l alcohol solution)	30.3±0.4	12.0±0.1	21.2±0.2	20.2±0.3	16.1±0.3	-
5	Chloroform extract (50 g/l alcohol solution)	32.4±0.3	13.2±0.2	20.2±0.3	30.3±0.4	15.1±0.2	-

Note. "-" – no zone of growth inhibition.

Table 3: Antimicrobial activity of *Galium* L. species lipophilic complexes using agar diffusion method

The studied object	Zone of growth inhibition, mm, (M±m), p≤0,05					
	Staphylococcus aureus 25923	*Escherichia coli* 25922	*Pseudomonas aeruginosa* 27853	*Bacillus subtilis* 6633	*Proteus vulgaris* 4636	*Candida albicans* 885-663
Galium verum	32.4±0.3	13.2±0.2	20.2±0.3	30.3±0.4	15.1±0.2	-
Galium salicifolium	30.3±0.4	12.1±0.2	20.2±0.3	20.0±0.3	13.1±0.2	10.0±0.1
Galium dasypodum	32.3±0.3	12.0±0.1	15.0±0.1	11.0±0.1	32.3±0.3	30.3±0.2
Galium aparine	35.4±0.1	10.0±0.1	26.0±0.4	23.1±0.3	14.2±0.1	33.1±0.2
Galium carpaticum	28.4±0.1	14.3±0.3	22.2±0.3	30.3±0.2	16.1±0.3	13.2±0.2
Galium pseudomollugo	30.2±0.4	13.8±0.2	18.2±0.3	20.0±0.3	14.1±0.2	32.5±0.4
Chloroform	-	-	-	-	-	-
96% ethyl alcohol	-	-	-	-	-	-

Note. "-" – no zone of growth inhibition.

Table 4: The degree of antimicrobial and antifungal activity of *Galium* L. species lipophilic complexes

The studied object	MBC, µg/ml					
	Staphylococcus aureus 25923	*Escherichia coli* 25922	*Pseudomonas aeruginosa* 27853	*Bacillus subtilis* 6633	*Proteus vulgaris* 4636	*Candida albicans* 885-663
Galium verum	125.0	500.0	250.0	250.0	500.0	-
Galium salicifolium	125.0	500.0	250.0	250.0	500.0	500.0
Galium dasypodum	62.5	500.0	500.0	>1000.0	125.0	62.5
Galium aparine	62.5	500.0	125.0	250.0	500.0	62.5
Galium carpaticum	125.0	500.0	250.0	62.5	500.0	>1000.0
Galium pseudomollugo	62.5	500.0	250.0	125.0	500.0	62.5
"Chlorophyllipt"	125.0	500.0	250.0	250.0	250.0	>2000.0
	MIC, µg/ml					
Galium verum	62.5	250.0	125.0	125.0	250.0	>1000.0
Galium salicifolium	62.5	250.0	125.0	125.0	250.0	250.0
Galium dasypodum	31.25	250.0	250.0	500.0	62.5	31.25
Galium aparine	31.25	250.0	62,5.0	125.0	250.0	31.25
Galium carpaticum	62.5	250.0	125.0	31.25	250.0	>1000.0
Galium pseudomollugo	31.25	250.0	125.0	62.5	250.0	31.25
"Chlorophyllipt"	31.25	250.0	125.0	125.0	125.0	>1000.0

lium pseudomollugo complexes were half, other complexes were equal to "Chlorophyllipt"; MBC of the these complexes were the same as in "Chlorophyllipt" and in the rest of the complexes – were twice as much.

The parameters have been found at the level of "Chlorophyllipt" in all studied complexes in relation to *Escherichia coli.* In relation to *Pseudomonas aeruginosa*, MBC and MIC of *Galium aparine* complex were half the "Chlorophyllipt", for *Galium dasypodum* complex they were twice the "Chlorophyllipt", and the rest of complexes equal to the level of "Chlorophyllipt".

In relation to *Bacillus subtilis,* the lipophilic complex from *Galium carpaticum* had the lowest MBC and MIC, compared to "Chlorophyllipt" they were approximately 4 fold lower and for the complex from *Galium pseudomollugo* they were half the "Chlorophyllipt". MBC and MIC of *Galium dasypodum* were 4 times higher than for "Chlorophyllipt"; the rest of the complexes were at the level of "Chlorophyllipt".

In relation to *Proteus vulgaris,* the MBC and MIC values of *Galium dasypodum* complex were half and the rest complexes were had twice the MBC and MIC of "Chlorophyllipt". In relation to *Candida albicans,* the complexes

from *Galium dasypodum*, *Galium aparine*, *Galium pseudomollugo* had the lowest values.

In relation to *Pseudomonas aeruginosa,* a positive correlation between antimicrobial activity of lipophilic complexes and the content of dicarboxylic acids (K=0.77), as well as a negative correlation between the content of steroid compounds (K=-0.78) were observed. For *Bacillus subtilis,* a negative correlation (K=-0.90) between antimicrobial activity and steroid compounds content was observed. In relation to *Proteus vulgaris,* a positive correlation (K=0.82) between the level of antimicrobial activity of lipophilic complexes and steroid compounds content was found.

Quantification of toxicity

Experimental data have suggested that mice treated with the studied complexes had no differences from the control group in terms of the central and autonomic nervous system, cardiovascular system, urinary system, gastrointestinal tract, optic apparatus, mucous membranes, skin and hair. Lethal cases have not been observed among the mice of the experimental and control groups. The lack of lethality among the mice can suggest that the LD_{50} values of the studied complexes exceed the maximum dose that has been used in the experiment – LD_{50}> 5000 mg/kg. This value of LD_{50} allowed us designate the studied complexes to the V class of toxicity – practically non-toxic substances according to the classification by Stefanov O.V.[25]

CONCLUSION

On average, the substances under study display more efficiency in reference to Gram-positive microorganisms and slightly less efficiency in reference to Gram-negative strains. Low toxicity and a broad spectrum of antimicrobial activity indicate that lipophilic substances derived from *Galium* species can be used as a template to develop new drugs with manifested antimicrobial and antifungal activities.

ACKNOWLEDGEMENT

The authors thank Nataliya V. Kashpur for consultative assistance while conducting research of antibacterial activity and toxicity studies. Authors thank Boris A. Vinogradov and Stanislav O. Uliyancev for helping establish composition and content of lipophilic compounds.

CONFLICTS OF INTEREST

The authors have no conflicts of interest.

ABBREVIATION USED

WHO: World Health Organization; **MBC:** Minimum bactericidal concentration, **MIC:** Minimum inhibitory concentration; **LD_{50}:** Median lethal dose.

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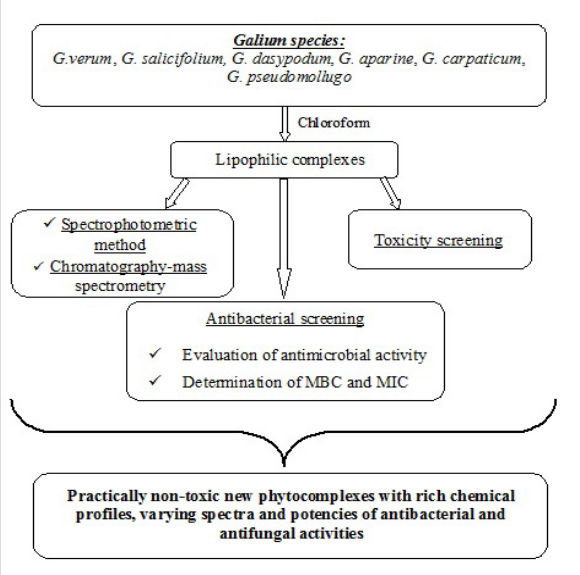
SUMMARY

- Complexes of lipophilic compounds from all studied Galium species are active against Gram-possitive and Gram-negative bacteria.
- Lipophilic complexes form *G. aparine*, *G. dasypodum* and *G. pseudomollugo* exhibit potent anifungal activity against Candida albicans.
- Obtained complexes are practically non-toxic and they are considered prospect for further in-depht research using clinical strains of microorganisms and fungi.

PICTORIAL ABSTRACT



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