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Obtaining and Research of Callus Mass of *Gentiana lutea L* Roots.

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ABSTRACT

The results of introduction to the culture *in vitro Gentiana lutea L.* are provided. The influence of phytohormones on growth processes of plant cells is researched, the optimal conditions for cultivation of *Gentiana lutea L.* are defined and chosen. Biomass extract is obtained and is researched on the presence in it the biologically active substances and antimicrobial activity.

Keywords: *Gentiana lutea L.*, callusogenesis, phytohormones, callus, explant, extract, antimicrobial activity.

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INTRODUCTION

Currently, important research is that, which provide us with the new range of medicines based on medicinal plants. Search for the advanced plants among native flora is today an urgent task of modern pharmacy. Interesting objects of study include representatives of family Gentianaceae, namely *Gentiana lutea L. Gentiana lutea L.* – this is unique, very rare, one of the most popular medicinal herbs of Ukrainian Carpathians, which is extensively used by official and folk medicine. Perennial herb of 50-120 cm height may be met in Ukraine only in Carpathians, namely - Chornohora, Svydovets, Marmaroski Alpy, Polonyna Borzhava, Horhany [7,13].

Complex allopathic and homeopathic medications of foreign producers are registered and widely used in the pharmaceutical market of Ukraine, which contain extracts of *Gentiana lutea L.* roots (Herbion stomach drops, Herbal balm and Dr. Theiss Schweden bitter, Aflubin, Limphomiosot). They are used at diseases of the gastrointestinal tract, for overall body strengthening. Though, there is not enough information about the microbocide properties of *Gentiana lutea L.*.

Ukrainian producers of medicines do not make drugs from *Gentiana lutea L.*, as this type is rare mountain European type, which is on the verge of extinction and is listed in the Red Book of Ukraine. Raw materials are very limited and are not subject to commercial operation[2].

As an alternative promising source of biomass obtaining is the use of biotechnological methods for growing of *Gentiana lutea L. in vitro conditions* on artificial nutrient environment. The resulting biomass contains biologically active substances peculiar to this plant.

Various ways of introduction to the culture in vitro of *Gentiana lutea L.* are described in the literature, as well as the development of culture media content, culture obtaining of isolated roots, plant micropropagation [6,10].

The aim of the article is to develop a method for biomass obtaining from the root tissue of *Gentiana lutea L.,* biomass research on the content of biologically active substances (BAS); comparative analysis of callus biomass and plant material of *Gentiana lutea L.*; extracts obtaining of callus biomass, plant material and studying their antimicrobial properties on different strains of microorganisms.

MATERIALS AND METHODS

The research material was medicinal herb – root of *Gentiana lutea L.*, gathered on the hill Vysokyi khrebet of Ihrovets, Horhany, Bohorodchanskyi district, Ivano-Frankivsk region in June-July 2012-2014, 1750 meters above sea level.

Root tissue of *Gentiana lutea L.* was washed with soap and water, sterilized with 30% solution of hydrogen peroxide for 20 and 40 minutes and planted in Petri caps on the agar nutrient medium of Murasyhe-Scoog with half-salt content. For the callusogenes is induction the kinetics and different concentrations of BAP, 2,4-D and NAA were used. Callusogenes is frequency was determined by the ratio of the explants number to their total quantity [1].

The cultivation duration was 40 days. All experiments were performed in three repetitions. Research results were worked out statistically. Cells callus mass was determined gravimetrically. It was originally taken, then dried on leaves at 58 ± 2^0C.. The dried biomass was analyzed and used to produce extracts.

Index of growth (IP) according to dry mass was determined by the formula W= (M-m)/m, where: W – relative growth; M – mass in 20 days; M – initial weight [1, 8].

The comparative analysis of the *Gentiana lutea L.* root callus mass was done according to the following indicators: humidity, total ash, ash insoluble in HCl, extractives content (20%-th , 40%-th, 70%-th, 96%-th ethanol), the content of flavanoids, alkaloids and tannins according to methods SPU [5,14].

Roots extracts of *Gentiana lutea L.* and callus mass was prepared by method of exhaustive extraction in Soxhlet apparatus. 20%-th, 40%-th, 70%-th and 96%-th ethanol was used as extractant. The optimal ratio between the raw material and extractant 1:20 [12]

The defining of qualitative and quantitative content of biologically active substances in the derived extracts was conducted.

Qualitative analysis of the flavonoids content was carried out through the following reactions: cyanide reaction, the reaction with a 10% solution of iron (III) chloride, reaction with 10% solution of basic acetate of lead; tannins: the reaction with the solution of iron ammonium alum, reaction with bromine water.

For the quantitative determination of flavonoids, alkaloids and tannins in the studied extracts of *Gentiana lutea L.,* the spectrophotometric method was used in terms of routines for flavonoids and gallic acid for tannins [5, 12, 14].

Antimicrobial activity was determined on the standard strains of microorganisms *Candida albicans* (ATCC 668653), *Staphylococcus aureus* (ATCC 25923 (F-49)), *Pseudomonas aeruginosa* (ATCC 27853 (F-51), *Staphilococcus epidermidis* (191), *Proteus vulgaris* (152), *Escherichia coli* (ATCC 25922), *Bacillus licheniformis* (ВКПМ-7038) and clinical strains of microorganisms *Staphylococcus aureus, Escherichia coli, Candida albicans*. Thus, the method of diffusion in Agar was used with the use of standard culture media (MPB, MPA, Saburo). Evaluation of antimicrobial activity of extracts was carried out, taking into account the bactericidial action of ethanol [4,11].

Statistical analysis was performed using the computer program STATISTICA-8 and statistical functions package of the Microsoft Excel program. The mean arithmetic value M was determined, the arithmetic mean error m, number of observations n, t-criteria of Student [12].

RESULTS AND DISCUSSION

It is known that the main factors, which influence the callusogenesis induction include the composition of culture medium and cultivation conditions [3,11].

Research of environments MS i MS/2 showed, that the process of callus creation was little influenced by concentrations of micro and macro salts, both environments induce callus creation. The ratio of phytohormones is very important in nutrient media. During the use of combination BAP+NAA there was not much impact on callus growth. The combination use BAP+2,4-D stimulated the formation of callus of pale yellow colour, loose consistency that quickly grew. During the experiment it was set that the most optimal environment for the cultivation of *Gentiana lutea L.* was the environment MS/2 with the addition of 0,1 мg/l BAP i 1,0 mg/l 2,4-D (table.1). On the 58% of planted explants the formation of callus took place. Cultivation was carried out at the temperature of 23, illumination 2000, photoperiod 16 h/8.

Table1: Intensity of callusogenesis of *GentianaluteaL.* depending on the content of culture medium,%.

MS 2,0 mg/l BAP 0,4 mg/lNAA	MS 0,1 mg/lBAP 1,0 mg/l 2,4-D	MS/2 2,0 mg/lBAP 0,4 mg/lNAA	MS/2 0,1 mg/lBAP 1,0 mg/l 2,4-D
10,4	68,3	15,1	89,4
8,5	75,4	20,5	85,1
9,2	51,2	14,8	92,4

Dry biomass, grown for 40 days is a porous mass, relatively strong, with bumpy surface and strong edges. Pieces with size of 0,7-1,6 cm, of undefined shape, yellow color, specific smell, bitter taste. Weight of callus mass is 156 g per 1 liter of culture medium corresponding to weight of 6-8 years old wild plant.

Result of callus mass analysis, grown on a solid nutrient medium (cultivation term - 40 days), and yellow gentian root (5-6 years) are provided in the table 2.

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Table 2: Comparative analysis of callus mass of *GentianaluteaL* root and root of *GentianaluteaL*..

Indicator	Callus mass of root of *GentianaluteaL.*	Root of *GentianaluteaL.*
Humidity	5,88	7,83
Total ash	10,42	5,18
Ash, insoluble inHCl	0,34	-
Extract plants (%):		
Ethanol		
20%-th	9,1	9,4
40%-th	9,06	9,46
70%-th	9,04	9,03
96%-th	5,23	5,27
Content ofBAS:		
Flavanoids	2,06	0,41
Tannins	1,68	0,42

As the table shows, callus mass grown on agar environment, according to the main phytochemical indicators, is not inferior to plant material.

Extracts, obtained from callus mass of *Gentiana lutea L.* root and root of *Gentiana lutea L*., were studied on the content of extractives, flavonoids and tannins. As a result of the research it is found that the amount of extractives in the extracts of callus mass of *Gentiana lutea L*. is slightly smaller than in the extract of *Gentiana lutea L*. root. The results are shown in the table 3.

Table 3: The content of extractives in the studied.

Researched extract	Extract content, %	
	Extract of callus mass *GentianaluteaL.*	Extract of root *GentianaluteaL.*
20%	4,96	5,23
40%	5,43	5,72
70%	5,12	5,41
96%	2,58	2,97

Quantitative content of flavonoids for the callus mass extract of *Gentiana lutea L.* root and root extract of *Gentiana lutea L.* was respectively – 1,96% and 0,37%: tannins – 1,41% i 0,28% respectively.

As a result of conducted research antimicrobial action is defined of root callus mass extract of *Gentiana lutea* (20°) as well as the root extract of *Gentiana lutea* (20°) concerning relation to standard strains *B. licheniformis, P. aeruginosa, C.albicans*, *S. aureus*, *S. epidermidis*, *E. coli* and clinical strains *C.albicans*; root extract *Gentiana lutea* (40°) concerning relation to strains *P. aeruginosa, P.vulgaris;* root callus mass extract of *Gentiana lutea* (40°) to strain *P. Aeruginosa;* extract *Gentiana lutea* (70°) concerning clinical strains *E.coli,* root callus mass extract of *Gentiana lutea* (70°) concerning clinical strains *E.coli, S. aureus;* extract *Gentiana lutea* (96°) and callus mass extract *Gentiana lutea* (96°) concerning standard strains *B. licheniformis, S. epidermidis, P. aeruginosa* and clinical strains *S. aureus, E. coli, C. albicans.* The research results are provided in the table 4.

CONCLUSIONS

The optimal conditions for the best accumulation of callus mass from the root tissue of *Gentiana lutea L.* the culture medium MS/2 was used with addition of 0,1 mg/l BAP and 1,0 mg/l 2,4-D. Output of callus mass was 156 g. per 1 liter of culture medium, corresponding to the mass of root of 6-8 years old wild plant.

The obtained callus mass is studied on the content of BAS and the comparative analysis is carried out concerning the content of BAS in the plant root of *Gentiana lutea L.*.

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Table 4: Antimicrobial activity of extract *GentianaluteaL* root and extractcallus mass *GentianaluteaL*.P < 0,05

Microorganisms strains	Zones of growth holdback (mm)											
	96°			70°			40°			20°		
	Root	Callus mass	Control	Root	Callus mass	Control	Root	Callus mass	Control	Root	Callus mass	Control
Standard strains												
Candida albicans	9 ± 0,25	9± 0,3	5± 0,2	6 ± 0,3	7,5 ± 0,2	4± 0,2	6 ± 0,2	5,5 ± 0,2	4± 0,2	9 ± 0,2	12 ± 0,2	0
Staphylococcus aureus	8 ± 0,2	9 ± 0,3	6± 0,2	5,5 ± 0,4	7 ± 0,3	5± 0,2	6,5 ± 0,3	7 ± 0,3	5± 0,2	9 ± 0,3	10 ± 0,4	0
Staphylococcus epidermidis	10 ± 0,3	14 ± 0,4	6± 0,2	6 ± 0,2	5 ± 0,2	4± 0,2	5 ± 0,25	5 ± 0,2	4± 0,2	9 ± 0,2	11 ± 0,2	0
Escherichia coli	8 ± 0,2	8 ± 0,25	4± 0,2	7 ± 0,25	6 ± 0,25	5± 0,2	7 ± 0,2	6 ± 0,25	5± 0,2	8 ± 0,4	13 ± 0,3	0
Bacillus licheniformis	12 ± 0,35	15 ± 0,4	7± 0,2	5 ± 0,4	6,5 ± 0,2	4± 0,2	5 ± 0,4	6,5 ± 0,2	4± 0,2	10 ± 0,2	15 ± 0,2	0
Proteus vulgaris	6 ± 0,2	7 ± 0,2	5± 0,2	7,5 ± 0,2	8 ± 0,25	5± 0,2	10 ± 0,2	9 ± 0,25	5± 0,2	5 ± 0,25	6 ± 0,35	0
Pseudomonas aeruginosa	10 ± 0,3	14 ± 0,3	5± 0,2	7,7 ± 0,3	7 ± 0,3	4± 0,2	10 ± 0,3	15 ± 0,4	4± 0,2	10 ± 0,2	14 ± 0,2	0
Clinical strains												
Candida albicans	10± 0,3	15 ± 0,4	5± 0,2	5 ± 0,2	5,5 ± 0,2	4± 0,2	5 ± 0,2	5,5 ± 0,2	4± 0,2	12 ± 0,3	15 ± 0,2	0
Staphylococcus aureus	9± 0,25	14 ± 0,3	4± 0,2	8 ± 0,25	13± 0,2	4± 0,2	7 ± 0,25	8 ± 0,3	5± 0,2	5 ± 0,2	6 ± 0,25	0
Escherichia coli	9± 0,25	14 ± 0,35	5± 0,2	10 ± 0,3	14± 0,4	5± 0,2	5,5 ± 0,2	6 ± 0,2	4± 0,2	6 ± 0,4	7 ± 0,3	0

The antimicrobial features of the studied extracts from callus mass and roots of *Gentiana lutea L.* are defined concerning the standard and clinical strains of the several microorganisms. The obtained results of experimental research provide the possibility to continue scientific research on this theme and are perspective for the pharmaceutical science.

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