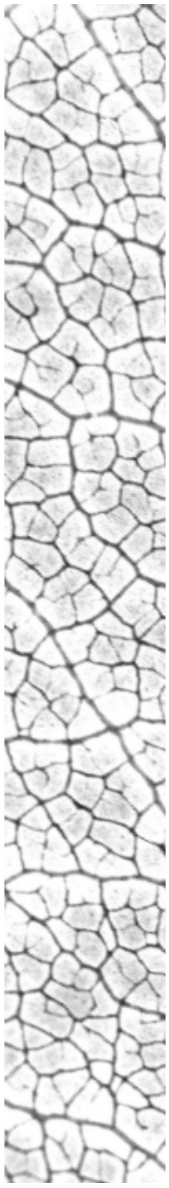

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Study of antimicrobial activity and technology optimization of *Calendulae flos* galenicals

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Abstract
The article presents materials on optimization of manufacture technology for *Calendulae flos* galenicals with medium level of antibacterial activity. For antibacterial study of extracts, we used agar well diffusion method. In our research, we utilized six test-strain microorganisms: *E. coli* ATCC 25922, *S. aureus* ATCC 25923, *P. vulgaris* ATCC 4636, *P. aeruginosa* ATCC 27853, *C. albicans* ATCC 885/653, and *B. subtilis* ATCC 6633. We have found that the maximum level of extracts' antimicrobial activity is achieved in the range of ethanol content in the extractant from 70 to 97 % v/v. Basing on these results, we have suggested a highly effective filtration technology of extraction for manufacture of liquid extract and tincture with medium level of antimicrobial activity from *Calendulae flos*. Based on HPLC analysis of extracts we detected the following main groups of compounds: quercetin and caffeic acid of derivatives. We have found that the antimicrobial activity of galenicals have good correlation with dry residue concentration but not with the rutin and chlorogenic acid concentration.

Keywords: *Calendulae flos*, antimicrobial activity, galenicals, technology

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INTRODUCTION

Development and practical application of antimicrobial drugs for local wound treatment for infectious injury of skin and mucous membrane is a rather urgent issue (McCulloch and Kloth 2010).

A good alternative to mono- or combination therapy for local treatment of infectious injury of skin and mucous membrane is to use galenicals in the form of tinctures and, in particular, extracts that may be used for development of different drug dosage forms (ointments, solutions, aerosols, sprays, tablets, etc.).

Calendula officinalis is a herblike plant belonging to *Asteraceae* family, which has been used for a long time in medical practice in different countries around the world. Drugs from *Calendulae flos* exhibit multiple pharmacological effects: anti-inflammatory, wound healing, antispasmodic, antibacterial, antiviral, anticancer, sedative, choleretic, etc. (Basch et al. 2006, Efstratiou et al. 2012, Khalid and Teixeira da Silva 2012, Sampiev and Hochava 2010, Ukiya et al. 2006, Yoshikawa et al. 2001).

In our previous article, we studied antimicrobial activity of *Calendulae tinctura* from different manufacturers of Ukraine and the Russian Federation, and we have concluded that this drug dosage form has a low level of antimicrobial activity and supposed the necessity to modify the technology and, possibly, pharmacopoeia norm for tincture manufacture to increase its antimicrobial activity (Boyko et al. 2016).

We have also called attention to such a significant and understudied issue as a choice of optimal ethanol concentration for obtaining a tincture and extract with maximum antimicrobial activity from *Calendulae flos*.

The aim of this article is to substantiate and suggest an optimal production technology of galenics with antimicrobial activity from *Calendulae flos*.

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Table 1. Main parameters of the validation method of analysis and suitability of HPLC system for determination of chlorogenic acid and rutin

Parameter	Pharmacopoeia limitation (Russian State Pharmacopoeia 2018)	Chlorogenic acid*	Rutin*
Retention time, min	-	6.3±0.3	15.9±0.7
Asymmetry coefficient	0.8-1.5	0.9	0.84
Separation coefficient	≥1.5	2.2	1.6
RSD of peak's area, %	≤2.0	0.8	1.5
LOD, g/ml	-	$2.2 \cdot 10^{-5}$	$2.3 \cdot 10^{-5}$
LOQ, g/ml	-	$6.5 \cdot 10^{-5}$	$6.8 \cdot 10^{-5}$
Determination coefficient, r^2	≥0.98	0.9999	0.9993
Calibration linear equation, $C(g/ml)=f(S(mAU \cdot s))$	-	$C=(2.92\pm0.04) \cdot 10^{-7} \cdot S$	$C=(4.90\pm0.18) \cdot 10^{-7} \cdot S$

* Note. The mean value and its error (X±ΔX) were calculated at repeat count n=3 and significance level P=0.95

MATERIALS AND METHODS

Plant Material

For our study, we used standard plant raw material of *Calendulae flos* from Chemists shop "Medicinal plants", Kharkov, Ukraine, 70 g, No. 100116, expiration date 07/2017.

Chemicals and Solvents

Ethanol with concentrations of 26, 43, 56, 72, 82, 97±1 % v/v was used as an extractant.

Ukrainian pharmacopoeia standards of chlorogenic acid and rutin were used, the content ≥98.0 %.

Antimicrobial Assay

For antimicrobial activity study, we used agar well diffusion method. For the study, we used six test-strain microorganisms: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Proteus vulgaris* ATCC 4636, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 885/653, and *Bacillus subtilis* ATCC 6633.

The microbial burden was 10^7 CFU/ml for upper-layer of the medium. The volume of test extracts was 0.275±0.025 ml. The diameter of wells was 10 mm, and the total width of layers of agar was 4.0±0.5 mm. Mueller-Hinton agar was used for bacteria. Sabouraud agar was used for *Candida albicans*. Determination of antimicrobial activity of extracts was carried out on two layers of the solid medium in Petri dishes.

HPLC Analysis

The chromatographic studies were carried out with a chromatograph by "*Agilent Technologies 1200 Infinity*", the USA. Chromatographic process was carried out under the following conditions: mobile phase (A): 1% water solution of formic acid, second mobile phase (B): ethanol in linear gradient elution regime; chromatographic column: *Supelco Ascentis express* C_{18} *2.7μm × 100 mm × 4.6 mm*; mobile phase velocity: 0.5 ml/min; temperature of the chromatographic column: +35 °C; sample volume: 1 μl. Detailed description of chromatography conditions is presented in article (Zhilyakova et al. 2017).

The main parameters of the validation method of analysis and suitability of HPLC system for determination of licuroside and glycyram are presented in **Table 1**.

Method of Dry Residue and Density Determination

Dry residue and density in the extract were determined by a gravimetric method in accordance with general pharmacopoeia monograph *Tinctures* GPhA.1.4.1.0019.15 of the Russian State Pharmacopoeia (XIV edition 2018).

Extraction Methods

Simple maceration: transfer 1.0 or 2.0 g of milled plant raw material (accurately weighed) into a flask and add 10.0 ml of the extractant and weigh for higher accuracy; then seal the flask and macerate at 24±1°C for 24 h.

After maceration, the extract was decanted and centrifuged at 13,000 rpm. The quantitative analysis of chlorogenic acid and rutin were carried out by reverse phase high performance liquid chromatography (RP HPLC).

Filtration method: transfer 10.0 g of milled plant raw material (accurately weighed) into a percolator (bulk density was equal 23.0±0.5 ml), set it into the thermostat with temperature 25.0±0.2 °C. The volume flow rate of the extractant was 0.32±0.03 ml/min, it was set up by infusion pump UN 2/50. The extracts were collected into 10.0-ml volumetric flasks (plant raw materil / extract ratio was 1:1 w/v), weighted and analysied for dry residue, density, chlorogenic acid and rutin.

RESULTS AND DISCUSSION

Antimicrobial Studies

In the first series of experiments, optimal ethanol concentration with maximum extract's antimicrobial activity was determined. The results of antimicrobial study with the use of different concentrations of ethanol for *Calendulae flos* are shown in **Table 2**. Weight / volume ratio of plant raw material to extractant in this case was 1:5 w/v.

Table 2. Antimicrobial activity of extracts from *Calendulae flos* obtained with different concentrations of ethanol

No.	Ethanol concentration	Dry residue concentration, % wt.	Growth inhibition zone diameters of test microorganisms, in mm*					
			S. aureus ATCC 25923	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*P. vulgaris* ATCC 4636	*B. subtilis* ATCC 6633	*C. albicans* ATCC 885-653
1	26 % v/v	6.2±0.3	13.7±1.9	12.6±2.2	12.0±2.3	12.3±1.6	13.4±1.8	Growth
2	43 % v/v	5.8±0.3	15.8±2.0	14.0±2.1	13.7±1.8	14.2±1.6	16.9±1.9	13.6±2.1
3	72 % v/v	5.7±0.3	17.0±1.7	16.4±2.0	14.0±2.3	17.1±1.8	18.2±1.8	15.3±1.6
4	97 % v/v	3.3±0.2	17.2±2.2	16.3±1.8	14.1±1.6	16.6±2.3	17.7±1.7	15.9±2.0
5	Control ethanol from 26 to 97 % v/v		Growth	Growth	Growth	Growth	Growth	Growth

* Statistical calculations are carried out with repeat counts n=3 and significance level P=0.95

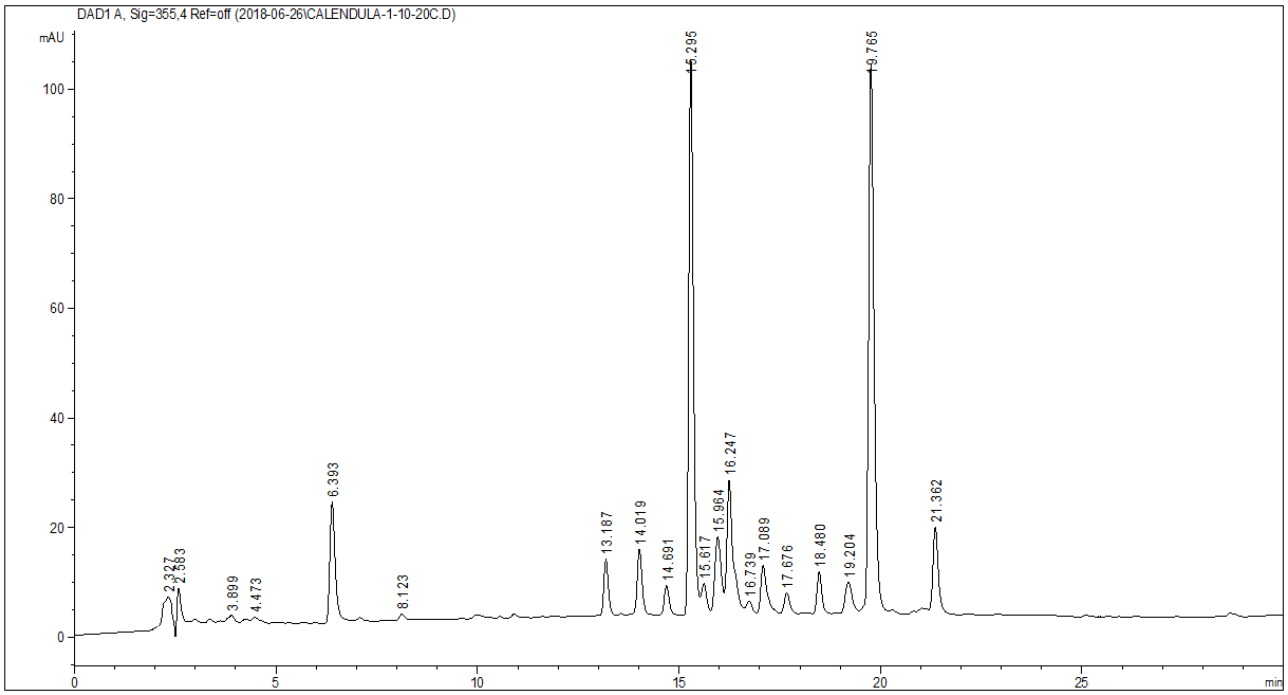

Fig. 1. Chromatogram of the extract with 72 % v/v ethanol (diode-array detection at λ = 355 nm)

As it can be seen from **Table 2**, in general, the greater ethanol concentration in hydro-ethanol mixture, the greater antimicrobial activity of extracts, and at the same time, concentration of dry residue in the extract tends to decrease with increasing ethanol concentration in the extractant. It is interesting to note that optimal ethanol concentration for the tincture is 72 % v/v and the one for the extract is 97 % v/v.

Phytochemical Studies

In the second series of experiments HPLC analysis of extracts were carried out. The result of HPLC analysis of the extract based on ethanol 72 % v/v is shown in **Fig. 1**.

As it can be seen from chromatogram in **Fig. 2**, more than ten substances were detected in the extract. Typical spectra of dominant substances are shown in **Fig. 2**.

As it can be seen from **Figs. 1** and **2**, the following groups of substances were detected in the extract by PR HPLC method: caffeic acid derivatives (retention times: 6.4, 16.0, 19.2 min), quercetin derivatives (retention times: 13.2, 14.0, 15.3, 16.2, 21.4 min), and kaempferol derivative (retention time: 19.8 min). Furthermore, chlorogenic acid and rutin were dominant compared to other compounds.

The values of peak's area and concentration for the main biologically active substances in extracts from *Calendulae flos* by RP HPLC analysis with different ethanol concentration are presented in **Table 3**.

As it can be seen from **Table 3**, the highest concentrations of chlorogenic acid and rutin were detected in ethanol with concentration from 43 to 81 % v/v. It is interesting to note that concentration of these substances in ethanol 97 % v/v in three times lower than that in ethanol 72 % v/v.

Comparing data of phytochemical analysis and antimicrobial activity, we have made a conclusion that antimicrobial effect of extracts does not correlate with concentration of chlorogenic acid and rutin because antimicrobial activity of extracts based on ethanol 72 and 97 % v/v is equal, but the concentration of these substances is three-fold different.

However, it is should be noted that antimicrobial activity of ethanol-water extracts have good correlation with dry residue concentration. This fact requires additional studies for identification of the active

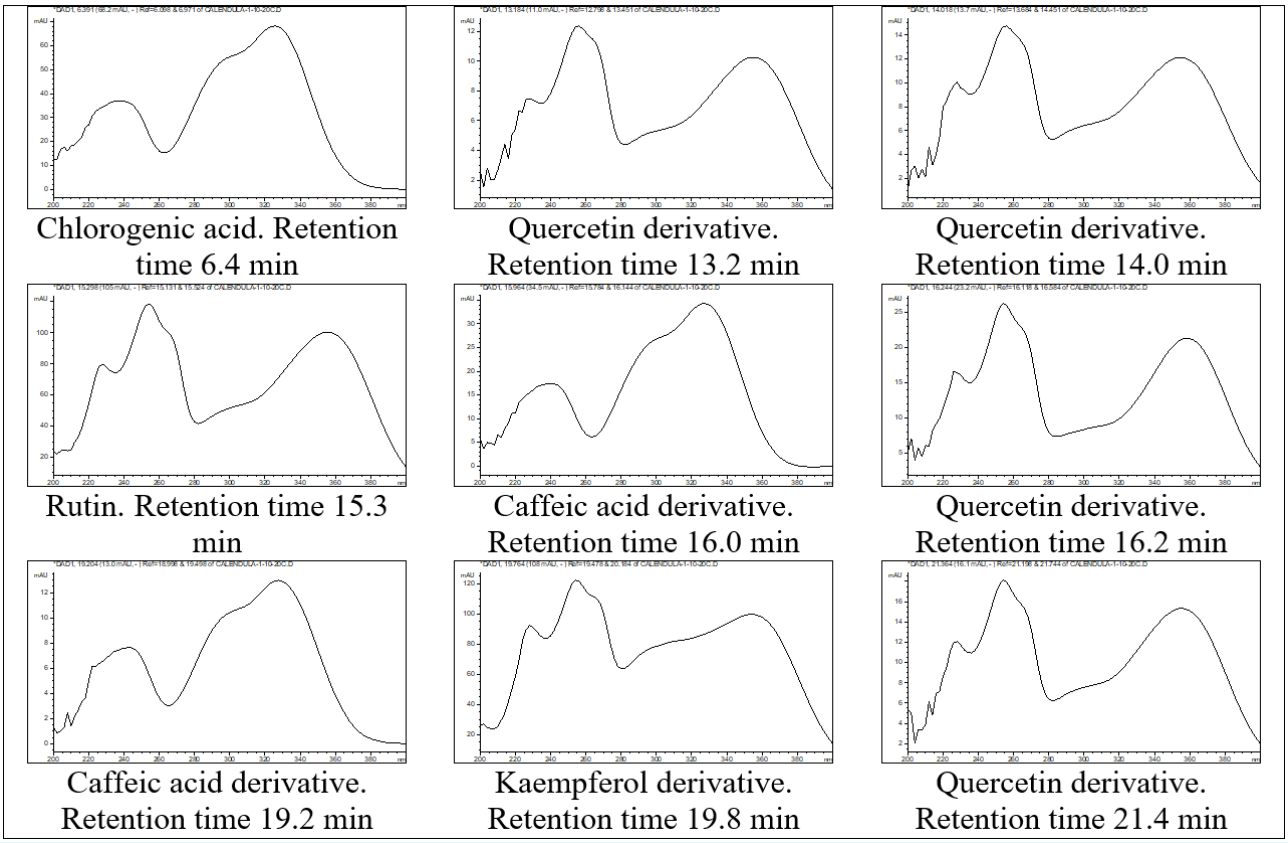

Fig. 2. Typical UV-spectra and retention times of dominant substances detected in the extract by PR HPLC method

Table 3. The values of peak's area and concentration for main biologically active substances in extracts from *Calendulae flos*

Compound (λ, nm)	Retention time, min	Peak's area of compound, mAU·s					
		Ethanol, % v/v					
		26	43	59	72	81	97
1	2	3	4	5	6	7	
1. Chlorogenic acid (325 nm)	6.4±0.3	375±19	641±32	685±34	689±34	640±32	158±8
2. Chlorogenic acid concentration, mg/ml		1.10±0.06	1.87±0.09	2.0±0.1	2.0±0.1	1.87±0.09	0.46±0.02
3. Ruin (355 nm)	15.3±1.0	617±31	867±43	877±44	871±44	797±40	226±11
4. Rutin concentration, mg/ml		3.02±0.15	4.3±0.2	4.3±0.2	4.3±0.2	3.9±0.2	1.11±0.06

* Note. The mean value and its confidence interval (Mean±SEM) are calculated with repeat counts n=3 and significance level P=0.95. Plant raw material / extractant ratio 1:10 w/v

substance in the extract that exhibits antimicrobial activity of extracts from *Calendulae flos.*

Technological Studies

In the third step of our experiments, we studied the yield of dry residue, chlorogenic acid and rutin from the plant raw material under conditions of the filtration method of extraction. The results are presents at **Fig. 3**.

As it can be seen from the graph in **Fig. 3**, the yield of rutin from the plant raw material into the first drain (plant raw material / extract ratio 1:1 w/v, time of draining 78 min), was 75±4 %. The yield of dry residue and chlorogenic acid was lower and equaled to 59±3 %.

The yield of rutin, chlorogenic acid and dry residue from the plant raw material into the fifth drain (plant raw material / extract ratio 1:5 w/v, time of draining 203 min), was 100±5 %.

Fig. 4 presents the dependencies of relative concentration of chlorogenic acid, rutin and dry residue in the drains on their number.

As it can be seen from **Fig. 4**, the largest concentration of BAS was detected in the first drain (1:1 w/v), moreover, the concentration of dry residue reached the value up to 19.4 % w/v which is typical for liquid extracts. It is interesting to note that to obtain the extract with equivalent parameters it is possible to use re-percolation or evaporation methods.

These data demonstrate the advantages of using the filtration method to obtain the galenicals with antimicrobial activity, such as a liquid extract (1:1 w/v) and tincture (1:5 w/v) from *Calendulae flos* using ethanol 70-97 % v/v.

The extract obtained (1:1 w/v) is a useful semi-product for development of semisolid dosage forms, for example, ointments and gels for local application in the

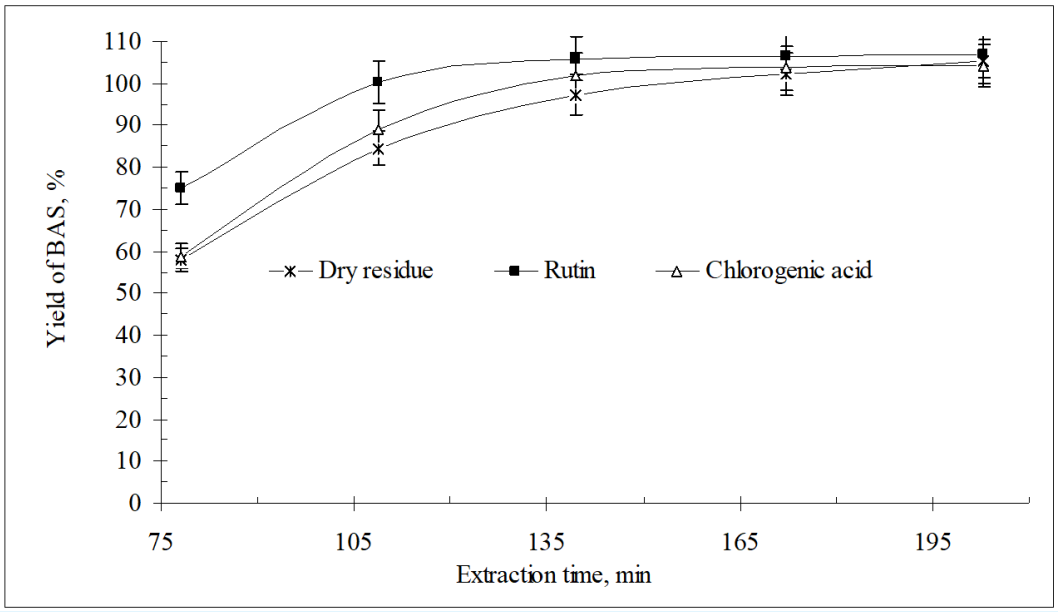

Fig. 3. Dependency of the yield of biologically active substances (BAS) on time. Filtration method of extraction. Repeat counts *n*=3 and significance level *P*=0.95

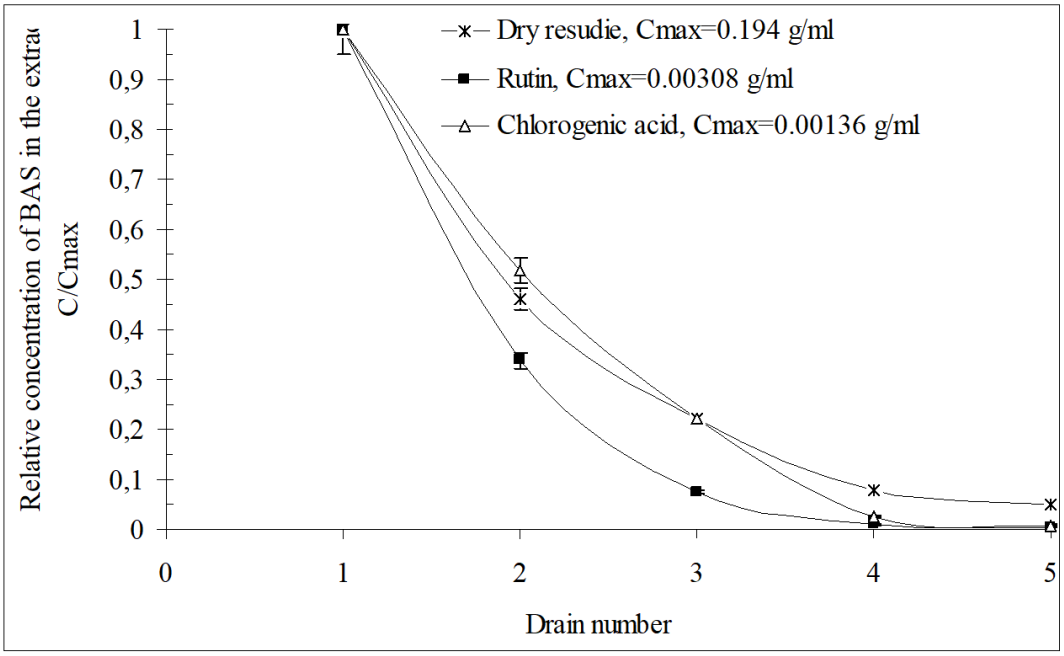

Fig. 4. Dependency of relative concentration of BAS in the drains on their number

dentistry and/or dermatology, which will be our task in the future studies.

CONCLUSIONS

We have studied antimicrobial activity of hydro-ethanol extracts obtained with different concentrations of ethanol; we have found that the maximum level of extracts' antimicrobial activity is achieved in the range of ethanol content in the extractant from 70 to 97 % v/v. We have noted that optimal ethanol concentration for the tincture is 70±5 % v/v and the one for the liquid extract is 97 % v/v. Basing on these results, we have suggested a highly effective filtration technology of extraction for manufacture of a liquid extract and tincture with medium level of antimicrobial activity from *Calendulae flos*. Based on HPLC analysis of extracts we have detected the following main groups of compounds: quercetin and caffeic acid of derivatives. It was found that the antimicrobial activity of galenicals have good correlation with dry residue concentration but not with the rutin and chlorogenic acid concentration.

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