Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(5):160-166

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Screening of antimicrobial activity of ethanolic extracts from raw materials containing alkaloids

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ABSTRACT

This paper presents data on screening of antimicrobial properties of extracts from the raw materials (18 plants) containing alkaloids. The microbiological method of studying antimicrobial properties of extracts, agar well diffusion method, has been applied; special mathematic method of comparison of antimicrobial properties of extracts vector theory has been applied. The most active extracts have been selected; they have antimicrobial activity of medium strength: from Phellodendron amurense bark; Macleaya cordata herb; Nuphar lutea root; Corydalis marshal tubers; Belladonna herb; Solanum dulcamara herb and fruits. A potential to use in practice of the extracts obtained from Echinops sphaerocephalus seeds and Cynoglossum herb as antimicrobial medicinal products has been shown. Low levels of antimicrobial activity have been demonstrated by the extract obtained from Peganum harmala herb. Data show significant antimicrobial properties of numerous kinds of raw materials that contain alkaloids and high possibility of their use in complex phytochemical medicinal products, but with certain restrictions and thorough side effect examination.

Keywords: Antimicrobial activity, ethanolic extracts, alkaloids.

INTRODUCTION

At the moment, issues of infectious dermatological, stomatological and otorhinolaryngologic diseases treatment have not lost their actuality. Recently, synthetic medicaments have been overwhelmingly used for treatment of most infectious diseases caused by pathogenic types of microorganisms: antiseptics; antibiotics, sulfanilamides, derivatives of other synthetical groups; antifungal antibiotic, etc.; anti-viral medications, not taking into consideration natural antimicrobial agents: extracts from plants, animals, and insects [1]. However, upward trend of formation and development of highly purified neogalenic medicinal products is currently observed [2].

One of the promising plant groups, which according to the literature data should have certain antimicrobial, anti-fungal, antivirus, antiparasitic, and insecticidal properties, is the group of plants containing alkaloids [3-6]. The main representatives of alkaloids in the plants studied are represented below: tropane, steroid, quinoline, aporphine, isoquinoline, indole, quinolizidine, alkaloids and pyrrolizidine.

The objective of this work is to conduct screening of antimicrobial activity of hydro-ethanolic extracts from some plant species containing alkaloids and to select most promising plants in order to conduct their further study and include them into complex phytocompositions.
In order to achieve the objective stated above, it was necessary to complete several tasks:

- to obtain alcohol-water extracts from the plants, to study some of their technological parameters and to pass them over in order to study their antimicrobial activity;

- to conduct studies of antimicrobial activity of the extracts obtained;
- to compare the complex index of antimicrobial activity of extracts and to select the most active ones.

EXPERIMENTAL SECTION

Collection of Plant material. Plant raw material for studies was acquired during 2013-2014 in OJSC Chemist's shop "Medicinal plants", Kharkov, and in the company "Medicinal herbs, extracts, oils", Sole proprietor Lyubimaya, Kharkov, Ukraine.

Preparation of plant extract. Ethanol 70±1 % vol. was used for extraction. Plant raw material/extragent ratio: 1:7 (mass: vol.), extraction temperature: 27±2 ^{o}C, method of extraction: maceration during 24 hours of infusion. Raw material crushing was carried out with grinder type DEX DCG 8 WH, manufactured by "Dexkee Elec-Technology Co., LTD"; sifting of the necessary fraction (0.1-0.5 mm) was carried out with laboratory sieves SLM-200, mesh size: 0.1 and 0.5.

Extract density was determined according to the method, described in [7]. Relative measurement error composed no more than 0.6 % in five parallel determinations.

Extracts extraneous substance content was determined according to the method, described in paper [8]. Relative measurement error composed no more than 0.6 % in five parallel determinations.

Antimicrobial activity method. Antimicrobial activity of ethanolic extracts was determined using agar well diffusion method, with determination of diameters of microorganism growth inhibition zones [9]. This method has several advantages over the other possible methods straight away: technological accessibility; it measures the antimicrobial activity of the amount of extraneous substance without alcohol; it allows predicting of activity dependence (the diameter of growth inhibition zone) on the extraneous substances concentration in the extract.

According to recommendations of the WHO the following test strains of microorganisms were used for evaluation of antimicrobial activity of medicinal products: *Staphylococcus aureus ATCC 25923*, *Escherichia coli ATCC 25922*, *Pseudomonas aeruginosa ATCC 27853*, *Proteus vulgaris ATCC 4636*, *Bacillus subtilis ATCC 6633*, and *Candida albicans ATCC 885/653* [10].

Preparation of the microbial suspension of microorganisms was carried out using the device Densi-La-Meter (PLIVA-Lachema production, the Czech Republic; wave length 540 nm). The suspension was prepared in accordance with the instruction manual that is applied to the device and according to the informational letter about health care system innovation of Ukraine. Synchronization of the cultures was conducted using low temperature (4º). Microbial burden compiled 10^7 colony forming unit per 1 mL (CFU/mL) of the medium and was determined in accordance with the McFarland standard. 18-24-hour microorganism culture was taken into work. Müller-Hinton agar was used for the studies.

Determination antimicrobial activity of medicinal products was conducted with agar well diffusion method. "Starving", not sown media were used in the lower layer (agar-agar, water, salts). The lower layer represents a supporting structure that is 10.0 mL volume, on which 6 thin-walled stainless steel cylinders with diameters of 8 mm and height of 10 mm were set strictly horizontally. The upper layer that consists of nutritious agar medium, melted and cooled to 40º and to which a correspondent standard of daily test microbe cultivation was included, was poured round the cylinders. Preliminary, the upper layer was well intermixed until the indiscrete mass was formed. After its freezing, the cylinders were taken out with the help of sterile forceps, and the studied substance, taking into account its volume (0.30 mL), was placed into the wells formed. Medium volume for the upper layer was 15.0 mL. The dishes were parched at room temperature for 30-40 minutes and placed into the thermostat for 18-24 hours.

The following criteria were applied when evaluating of antibacterial properties: absence of microorganism growth inhibition zones around the well, and also inhibition zones up to 10 mm indicates to the fact that the microorganism is not sensitive to the medicinal product or concentration inserted into the well; growth inhibition zones with diameter of 10-15 mm indicates to the low sensitivity of the cultivation to the antibacterial substance concentration studied; growth inhibition zones with diameter of 15-25 mm are regarded as an indicator of microorganism sensitivity to the medicinal product concentration studied; growth inhibition zones, the diameter of which exceeds 25 mm, indicate to high sensitivity of microorganisms to the medicinal products studied.

Calculation methods. Statistical processing of the results has been conducted in accordance with the Article of State Pharmacopoeia of Ukraine "Statistical analysis of chemical experiment results" [8] with the help of add-on

"Data analysis" of MS Excel 2013 package. Diameters of microorganism growth inhibition zones were measured using measuring bar with measurement error of ±0.1 mm. Assuming that the variation of diameters of microorganism growth inhibition zones occurs in accordance with the normal distribution law, calculation of the average arithmetic diameter and its measurement error was performed with correction for small samples using Student criterion with confidence level of 0.95 and number of degrees of freedom of 5.

In order to conduct calculation of the complex index antimicrobial activity of medicinal product, a vector theory was used [12]. Calculation of the complex index antimicrobial activity of medicinal product and its error was performed using the following formulas:

$$A = \sqrt{\left(a_1 \cdot \frac{D_1}{25}\right)^2 + \ldots + \left(a_6 \cdot \frac{D_6}{25}\right)^2} \quad (1)$$

$$\Delta A = \sqrt{\left(\frac{{a_1}^2 \cdot D_1 \cdot \Delta D_1}{25^2}\right)^2 + \ldots + \left(\frac{{a_6}^2 \cdot D_6 \cdot \Delta D_6}{25^2}\right)^2} \quad (2)$$

where A is a complex index antimicrobial activity of medication, dimensionless value, (index efficiency ranges: 1.0-1.5 the medicinal product has weak antimicrobial activity; 1.5-2.5 the medicinal product has medium antimicrobial activity; more than 2.5 the medicinal product has strong antimicrobial activity);

a_1, a_2, a_3, a_4, a_5, a_6 are weighing coefficients of microorganism strain significance in the disease, in order to simplify, we took them as a one unit, however, application data from the research of prevalence degree of microorganisms in affected people can also be used;

D_1, … D_6 and ΔD_1, … ΔD_6 are diameters and their errors of growth inhibition zone of the examined microorganism strains: *S. aureus ATCC 25923, E. coli ATCC 25922, P. aeruginosa ATCC 27853, P. vulgaris ATCC 4636, B. subtilis ATCC 6633, and C. albicans ATCC 885/653,* mm;

25 is a virtual indicator of the microorganism growth inhibition zone, in mm, that has an optimal value (between medium and strong sensitivity), however, an indicator of conventional medicinal product for the certain strain may be used under the same determination conditions, as medicinal products studied;

ΔA is an error of the complex index antimicrobial activity of medicinal product.

For comparison of A (research) and B (standard) objects/drugs (vectors) with each other, cosine of the angle between them, which equals correlation coefficient r and denotes linear connection power between the parameters in mathematical statistics, may also be used, apart from their absolute value (size). Square of correlation coefficient (r^2) denotes objects (vectors) similarity level. Correlation coefficient (cosine of the angle) between vectors may be calculated using the following formula (3):

$$\cos\gamma = r = \frac{\sum\left[a_i^A \cdot D_i^A \cdot a_i^B \cdot D_i^B\right]}{\sqrt{\sum\left[a_i^A \cdot D_i^A\right]^2} \cdot \sqrt{\sum\left[a_i^B \cdot D_i^B\right]^2}} \quad (3)$$

RESULTS AND DISCUSSION

Data on the results of the studies that were carried out in order to determine antimicrobial activity of extracts from plant raw material containing alkaloids using agar well diffusion method is represented in Table 1.

Table 1: Antimicrobial activity of the extracts studied, determined by agar well diffusion method

Name of the raw material	Diameters of growth inhibition zones in mm; repeat count, n=6, P=0.95					
	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. *Aristolochia clematitis* herb	19.2±0.6	19.0±0.4	12.6±0.5	13.4±0.7	20.1±0.7	18.3±0.6
2. *Atropa belladonna* herb	15.8±0.4	17.2±0.6	14.4±0.5	14.3±0.4	17.5±0.8	14.7±0.5
3. *Berberis vulgaris* root	20.4±0.5	19.5±0.7	14.0±0.6	14.1±0.7	17.7±0.4	18.3±0.4
4. Capsicum tincture	19.5±0.7	19.2±0.8	15.7±0.4	16.4±0.6	18.6±0.3	16.3±0.8
5. *Chelidonium majus* herb	20.7±0.8	growth	growth	growth	21.4±0.4	17.0±0.5
6. *Corydalis marshal* tubers	24.9±0.6	23.7±0.4	13.4±0.8	13.6±0.7	25.3±0.5	growth

As it may be seen from the results of Table 1, performing a quick analysis and selecting the most active antimicrobial extracts is somewhat complicated, for example, it is difficult to determine indexes of *Belladonna* herb and *Nuphar* root extracts. *Belladonna* extract influences all the microorganism test-strains, and *Nuphar* root extract does not influence two test-strains, but according to the quantitative indexes, it excels *Belladonna* extract. The vector theory, which had been applied to the analysis of antimicrobial characteristics of some stomatological and antiseptic medicinal products by the authors for the first time, helped to solve this problem [11, 12].

Data on the screening of antimicrobial activity of the extracts from the raw material containing alkaloids using vector theory are represented in Table 2.

Table 2: Antimicrobial activity of the extracts

No	Name of the raw material	Complex index of antimicrobial activity, *A*	$\cos\gamma = r$*	*Extraneous substances concentration in the extract, g/g*	*Extract density, g/mL*
1.	*Aristolochia clematitis* herb	1.70±0.02	0.99	0.0306	0.893
2.	*Atropa belladonna* herb	1.54±0.02	0.99	0.0201	0.893
3.	*Berberis vulgaris* root	1.72±0.02	0.99	0.0182	0.888
4.	Capsicum tincture	1.73±0.02	0.99	-	-
5.	*Chelidonium majus* herb	1.37±0.02	0.70	0.0339	0.899
6.	*Corydalis marshal* tubers	1.87±0.02	0.88	0.0294	0.892
7.	*Cynoglossum officinale* herb	1.50±0.02	0.99	0.0228	0.894
8.	*Echinops sphaerocephalus* seeds	1.47±0.02	0.90	0.0232	0.890
9.	*Glaucium flavum* herb	1.70±0.03	0.90	0.0195	0.886

* results of correlation coefficient are shown with consideration for the formula (3) in which virtual indexes, accepted throughout all the strains of 25 mm, are supplied instead of real indexes of growth inhibition zones for the comparative medicinal product.

** the arithmetic mean of the parameter (X) and standard deviation of the parameter (S) for the sample were calculated from the condition $X \geq 3S$.

According to the data from Table 2, we may easily define the complex index of antimicrobial activity (*A*), for example for medicinal products with average power of antimicrobial activity its indexes are situated in a range of *A*=1.5÷2.5. From Table 2 it is seen that such medicinal products include extracts from the following materials: *Phellodendron amurense* bark *A*=2.29; *Macleaya cordata* herb *A*=1.94; *Nuphar lutea* root *A*=1.88; *Corydalis marshal* tubers *A*=1.87; *Vinca minor* leaf *A*=1.75; *Berberis vulgaris* root *A*=1.72; *Aristolochia clematitis* herb *A*=1.70; *Glaucium flavum* herb *A*=1.70; *Lycopodium A*=1.64; *Atropa belladonna* herb *A*=1.54; *Solanum dulcamara* herb and fruits *A*=1.51.

The mean result for the complex index of antimicrobial activity for the most of extracts from plants containing alkaloids is *A*=1.60 (70% vol. ethanol at a ratio of raw material: extracting agent – 1:7 wt.:vol.) and may range from 0.74 to 2.47. The mean result of the correlation coefficient is *r*=0.89, and can range from 0.58 to 0.99. The mean result of the concentration of extractives in extracts is C = 0.0273 g/g of extract, and may range from 0.0055 to 0.0492 g/g of extract. The mean result of the extract density is ρ = 0.893 g/mL, and can range from 0.882 to 0.905 g/mL.

In order to illustrate antimicrobial characteristics of the extracts studied, their indexes may be compared with those of some official phytopreparations and synthetical medicinal products. The indexes of antimicrobial characteristics (complex index of antimicrobial activity and correlation coefficient), calculated according to the data of papers [12, 13], are indicated in Table 3.

Table 3: Antimicrobial activity of industrial medicinal products

No	Name of the product	Complex index of antimicrobial activity, *A*	*cosγ = r*	*Extraneous substances concentration in the extract, g/g*	*Extract density, g/mL*
1	**2**	**3**	**4**	**5**	**6**
1	*Sophora japonica tincture*	2.05	0.97	0.1554	0.999
2	*Mentha piperita tincture*	2.12	0.99	0.0011	0.843
3	*Eucalyptus tincture*	1.5	0.82	0.0505	0.895
4	*Calendula tincture*	1.29	0.99	0.0262	0.896
5	*Propolis tincture*	1.2	0.81	0.0444	0.875
6	*Paeonia tincture*	1.03	0.71	0.0196	0.953
7	*Echinacea tincture*	0.84	0.58	0.0182	0.938
8	*Sanguiritrin*	1.91	0.9	-	-

[1] *Sophora japonicum* tincture, (1:2) on 48 % ethanol, CJSC "Pharmaceutical factory "Viola", Zaporozhe, Ukraine, batch No 040213, expiry date 02/2015;
[2] *Mentha piperita* tincture, (1:20) on 90 % ethanol and 5 % peppermint oil, CJSC "Pharmaceutical factory "Viola", Zaporozhe, Ukraine, batch No 080213, expiry date 08/2015;
[3] *Eucalyptus* tincture, (1:5) on 70 % ethanol, public enterprise "Lugansk regional "Pharmacy" pharmaceutical factory, Lugansk, Ukraine, batch No 10313, expiry date 04/2018;
[4] *Calendula* tincture, (1:10) on 70 % ethanol, public enterprise "Lugansk regional "Pharmacy" pharmaceutical factory, Lugansk, Ukraine, batch No 51212, expiry date 01/2017;
[5] *Propolis* tincture, (1:10) on 80 % ethanol, public enterprise Kiev Regional Council "Pharmaceutical factory", Kiev, Ukraine, batch No 151012, expiry date 10/2014;
[6] *Paeonia* tincture, (1:10) 40% ethanol, "State public enterprise "Pharmaceutical factory" LLC, Zhytomyr, Ukraine, batch No 110313, expiry date 03/2016;
[7] *Echinacea purpurea* rootstock tincture with roots of fresh ones, (1:5) on 48 % ethanol, "Pharmaceutical factory" LLC, Zhytomyr, Ukraine, batch No 70613, expiry date 06/2016.
[8] "*Sanguiritrin*" – alcoholic solution 0.2 % *Macleaya cordata* alkaloids, CJSC "Pharmcenter VILAR", Russia, batch No 070612, expiry date 06 / 2015;
[9] "*Chlorhexidine bigluconate*" – chlorhexidine bigluconate water solution 0.5 mg/mL, public enterprise "Lugansk regional "Pharmacy" pharmaceutical factory, Lugansk, Ukraine, batch No 220213, expiry date 03 / 2016;

[10] "*Hexoral*" – 0.1 % hexetidine water solution, Famar Orleans, France, batch No D06179KR, expiry date 09 / 2014.

As it may be seen from the data of Table 3, only four industrial tinctures from *Sophora japonicum* A=2.05, *Mentha piperita* A=2.12, *Eucalyptus* A=1.50 (but the tincture from *Eucalyptus* does not influence two microorganism of six test strains, r=0.82, r^2=0.67) and *Capsicum* A=1.73 as well as *Sanguiritrin* A=1.91 and *Chlorhexidine bigluconate* A=2.07 correspond to the group of medium strength of antimicrobial activity. Whereby it is important to note, that the tincture from *Sophora japonicum* that was prepared in the ratio of 1:2, is an almost fluid extract with the quantity of extraneous substances of 15 % weight, and to the tincture from *Mentha piperita,* that is prepared in the ratio of 1:20, peppermint oil is added (5 % weight), which leads to such significant antimicrobial characteristics of the medicinal product.

Comparing complex indexes of antimicrobial activity of the extracts studied with those of industrial medicinal products, we may come to the conclusion about potentially high perspective of herbal raw material usage with some alkaloids as antimicrobial agents (13 of 18 plants studied show antimicrobial characteristics of medium strength). However, because of high biological activity of alkaloids over different body systems (nervous, GIT, cardiovascular, etc.), their application for mouth cavity is irrational and in some cases may lead to serious side effects. When such combination is useful, for example, for external application, they may be added to the formulation without significant reduction of antimicrobial characteristics for complex phytopreparations.

CONCLUSION

In order to study antimicrobial characteristics of extracts from herbal raw material containing alkaloids, a range of plants of interest was determined on the basis of literature data to the number of 18 items.

The most active of all the extracts having antimicrobial characteristics of medium strength from *Phellodendron amurense* bark; *Macleaya cordata* herb; *Nuphar lutea* root; *Corydalis marshal* tubers; *Berberis vulgaris* root; *Vinca minor* leaf; *Ariseolochia clemaeieis* herb; *Glaucium flavum* herb; *Atropa belladonna* herb; *Solanum dulcamara* herb and fruits were selected. Extracts from *Echinops sphaerocephalus* seeds and *Cynoglossum officinale* herb are promising. The extract from *Peganum harmala* herb has the worst results among the plants studied.

The mean result of the complex index of antimicrobial activity for the most of extracts from plants containing alkaloids is A=1.61 (70% vol. ethanol at a ratio of raw material: extracting agent – 1:7 wt.:vol.) and may range from 0.74 to 2.47. The mean result of the correlation coefficient is r=0.89, and can range from 0.58 to 0.99. The mean result of the concentration of extractives in extracts is C=0.0273 g/g of extract, and may range from 0.0055 to 0.0492 g/g of extract. The mean result of the extract density is ρ=0.893 g/mL, and can range from 0.882 to 0.905 g/mL.

Research data show significant antimicrobial characteristics of many species of herbal raw material containing alkaloids (13 of 18 extracts from the raw material demonstrated antimicrobial characteristics of medium strength) and potentially high perspective of their usage in complex phytopreparations, but with certain restrictions and thorough side effect examination.

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