



The study of the antimicrobial activity of CO₂ extracts of *Plantago major* and *Acorus calamus*

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ABSTRACT

Given article deals with the results of the study of antimicrobial activity and microbiological purity of CO₂ extracts of *Plantago major* and *Acorus Calamus*. It has been found that both extracts exhibit antimicrobial activity. Thus *Plantago major* extract have a broad spectrum of activity, and all cultures of microorganisms are sensitive to this extract. *Acorus calamus* extract has activity against gram-positive bacteria cultures as well as antifungal activity against *Candida albicans*. Both samples meet the requirements of the Pharmacopeia of Ukraine in terms of "microbiological purity of non-sterile drugs." These extracts can be used to develop formulations with antimicrobial properties.

Key words: CO₂ extract, *Plantago major*, *Acorus Calamus*, antimicrobial activity, microbiological purity.



INTRODUCTION

Currently is proposed and actively developed the technology of vegetable raw materials extraction by compressed and liquefied gases. Biologically active substances in CO₂ - extracts are in a natural environment consisting of gums, oils, waxes. Therefore, they are more active, and their therapeutic efficiency is much higher.

Obtained using the proposed technology extracts are completely natural, as evidenced by their chemical analysis. In addition, attracting is also environmental friendliness of the process, because carbon dioxide is not toxic, and it is almost completely removed from extract in the final stages of the technological cycle, and it does not require any additional measures. Another interesting fact is that the process parameters provide a unique microbiological purity of the obtained products, satisfying the most stringent current requirements on the use of plant extracts in food, perfume, cosmetic and pharmaceutical industries. In the course of our research were obtained CO₂- extracts of *Acorus calamus* and *Plantago major*. Antimicrobial activity, as well as microbial purity of the resulting products has been studied.

MATERIALS AND METHODS

The studies of antimicrobial activity of the extracts have been performed at the Department of Biotechnology of the National University of Pharmacy. For the analysis were obtained samples of extracts:

No 1: CO₂ - extract of *Plantago major*;

No 2: CO₂- extract of *Acorus calamus*.

The antimicrobial activity of the extracts samples was studied *in vitro* by the method of diffusion in agar well which is based on the ability of active substances to diffuse into the agar previously seeded by microorganisms cultures [1]. All studies were conducted in strict aseptic conditions, using a laminar box (cabinet of biological safety AS2-4E1 "Esco", Indonesia).

As a test cultures using microorganisms from the American Typical Culture Collection (ATCC - American Typical Culture Collection): Gram-positive bacteria *Staphylococcus aureus* ATCC 25293, *Bacillus subtilis* spore culture ATCC 6633, a Gram-negative *Escherichia coli* culture ATCC 25922. antifungal activity was determined with respect to the yeast-like fungi *Candida albicans* ATCC 885-653 [2].

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Index of antimicrobial activity is the size of the delay growth of test microorganisms zones that is formed in agar medium on Petri dishes. The diameter of the zones of growth inhibition considering the wells diameter was measured with accuracy of 1 mm, while focused on the complete absence of visible growth.

For research was used DSA suspension of bacterial microorganisms in saline, and a two-day culture of yeasts. Microbial load was 1×10^7 of microbial colony forming units in 1 ml culture medium (CFU / ml) [1].

In Petri dishes mounted on a horizontal plane were placed 10 ml of uninfected "hungry" AGV agar (for the upper layer when using bacterial cultures used the meat-peptone agar (MPA), working with yeast-like fungi - agar Saburo). After solidification of this agar layer on its surface at an equal distance from each other and from the edge of the cup were placed a sterile steel cylinders (height $10,0 \pm 0,1$ mm, outer diameter $8,0 \pm 0,1$ mm) and was poured melted and chilled to $45-48^\circ\text{C}$ upper agar layer with cultures of microorganisms in an amount of 15 ml (13.5 ml melted agar and 1.5 ml of a microbial suspension with a microorganism load $1 \cdot 10^7$ CFU / ml). After cooling and solidification of the upper layer of the culture medium cylinders was removed with sterile forceps and in the formed wells were poured studied extracts samples (0.25-0.3 ml) [1].

Petri dishes with crops placed in an incubator - bacterial cultures at $32,5 \pm 2,5^\circ\text{C}$ for 18-24 h, culture yeasts at $22,5 \pm 2,5^\circ\text{C}$ for 48 h. The diameters of the zones of inhibition of microbial growth characterize the antimicrobial activity of the samples.

When studying microbiological purity of samples was used method of extracts the State Pharmacopoeia of Ukraine (1.4, p. 5.1.4 - microbiological purity of non-sterile medicines, p. 171), which allows objectively evaluate the quality characteristics of the samples on the basis of experimentally obtained statistically processed results [2, 3]. Estimation of microbiological contamination degree of the drug include the identification the total number of aerobic mesophilic bacteria (TAMC) and total yeasts and molds (TYMC) 1 g extracts, establishing the absence of bacteria *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*. In 1 g of non-aqueous medicinal products for oral and rectal administration the total number of aerobic microorganisms (TAMC) may be not more than 103 CFU (colony forming units); the Total Combined Yeast and Mould Count (TYMC) not

more than 102 CFU) [3]. To check the suitability of determination methods of total viable aerobic microorganisms as test-strains were used the following bacteria from the American Type Culture Collection (ATCC): *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, *Aspergillus brasiliensis* ATCC 16404 [3].

According with the requirements of the Ukrainian Pharmacopoeia was used following dense and liquid nutrient medium: Casein soy agar (to determine the number of live bacteria), Sabouraud dextrose agar (to determine the number of fungi), soybean casein broth (preincubation for in determining the presence of certain microorganisms), manitn-saline agar (for identification of bacteria *Staphylococcus aureus*), cetrimide agar (to identify *Pseudomonas aeruginosa*), Mac Conkey agar (for bacteria detection of *Escherichia coli*).

RESULTS AND DISCUSSIONS

The diameter of the microorganism growth characterizes the antimicrobial activity of the experimental samples as follows:

- absence of growth inhibition zones of the microorganisms around the wells, as well as growth inhibition zone diameter less than 10 mm was evaluated as the insensitivity of microorganisms to the extract samples introduced into the wells;
- the zone of growth inhibition diameter of 11-15 mm were evaluated as weak sensitivity of the culture to the active ingredients of the samples extracts;
- the zone of growth inhibition diameter 15-25 mm - strain sensitive to the sample;
- the zone of growth inhibition, with a diameter more than 25 mm, testified to the high sensitivity of microorganisms to the sample extracts.

The result of studies of the extracts samples antimicrobial activity for the various cultures of microorganisms shown in table 1.

The experimentally obtained data showed that the sample №1 (CO₂ *Plantago major* extract) exhibits antimicrobial activity against all the strains of the used microorganisms (Gram-positive and Gram-negative bacteria), and also has activity against yeasts *Candida*. Thus, the extract №1 has a broad spectrum of action and all cultures of microorganisms are sensitive to this extract (diameter of the zones of microbial growth delay from 16 to 22 mm). Sample №2 (CO₂ *Acorus calamus* extract) exhibits activity against gram-positive bacterial cultures: *Staphylococcus aureus* -

18-19 mm, *Bacillus subtilis* - 17-18. Regarding *Escherichia coli* gram-negative culture activity was not observed. Culture yeasts *Candida albicans* is sensitive to the action of sweet flag extract, which is manifested in the antifungal activity.

Thus, the sample extracts №1 (CO₂ extract of *Plantago major*) and №2 (CO₂ extract *Acorus calamus*) are promising for further developing of formulations with antimicrobial properties.

When determining the microbiological purity of the analyzed extracts in order to prevent errors in the evaluation of results, preliminary studies it was found that all the samples of the extracts have antimicrobial activity. For neutralization of the antimicrobial action have been prepared diluted extracts 1:10 by adding a buffer solution with sodium chloride and peptone pH 7.0. After dilution of the extracts (1:10) antimicrobial activity in all of the samples was not observed.

For analysis were taken 2.0 g of the extract test sample was added to the buffer solution of sodium chloride and peptone pH 7.0 to a final volume 20 ml (1:10 dilution). In a Petri dish 9 cm in diameter was added 15 ml of casein-soya agar or Sabouraud-dextrose agar at a temperature from 45 to 50 ° C, culture media was allowed to cool.

One ml of the test dilutions (1:10) were added into tubes containing 4 ml of melted and cooled to a temperature not more than 45 ° C agar medium. The tube contents were rapidly mixed and transferred into a Petri dish with the prepared first layer of the nutrient medium. By a quick shake of the Petri dishes evenly distributed top layer of the medium. For each dilution were prepared three Petri dishes for each culture medium.

Dishes with casein-soya agar were incubated at 30-35 ° C 5 days dishes with Sabour and dextrose agar were incubated at 20-25 ° C for 7 days. For each nutrient medium was calculated arithmetic mean value the number of colonies, and was determined the number of CFU per gram of medicament.

Incubation of prepared extracts samples (1:10 dilution) for manitno-salt agar (temperature 30-

35°S - 72 hours), cetrimid agar (temperature 30-35 ° C - 72 hours) and MacConkey agar (30-35 ° C - 72 hours) showed the absence of colonies, which corresponds to the result "no bacteria *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* in 1 g of the test samples of extracts."

The results of microbiological purity of the extracts samples studies are shown in the table 2.

Thus, it is experimentally proved that the samples of extracts №1 and №2 did not reveal the presence of bacteria *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*.

Found that the total number of fungi (TYMC) is less than 10 CFUs / g in all extracts samples (№1 CO₂ extract of *Plantago major* №2 CO₂ extract of *Acorus calamus*).

The number of bacteria (TAMC) in 1 g of the test samples of extracts is 10 CFUs / g for sample №2 (CO₂ extract of *Acorus calamus*).

For sample №1 (CO₂ extract of *Plantago major*) number of bacteria (TAMC) in 1 g of the test samples of extracts is less than 10 CFUs / g.

The results show that extracts samples №1 and №2 meet the requirements of the Pharmacopeia of Ukraine in terms of "microbiological purity of non-sterile drugs."

Conclusions.

Based on these studies we can conclude that the resulting CO₂ extracts of *Plantago major* and *Acorus Calamus* exhibit antimicrobial activity. Thus *Plantago major* extract have a broad spectrum of activity, and all cultures of microorganisms are sensitive to this extract (growth inhibition zone ranges from 16 to 22 mm). *Acorus calamus* extract has activity against gram-positive bacteria cultures: *Staphylococcus aureus* - 18-19 mm, *Bacillus subtilis* - 17-18, as well as antifungal activity against *Candida albicans*. Both samples meet the requirements of the Pharmacopeia of Ukraine in terms of "microbiological purity of non-sterile drugs." These extracts can be used to develop formulations with antimicrobial properties.

Table 1: Antimicrobial activity of the extracts samples

Samples	Cultures of microorganisms			
	S.aureus	B. subtilis	E. coli	C. albicans
	Zone of microorganisms growth delay diameter, mm			
№1. CO ₂ - extract of <i>Plantago major</i>	18-19	18-19	16-17	21-22
№2. CO ₂ - extract of <i>Acorus calamus</i>	18-19	17-18	-	19-20

“ – “ – no zone of growth inhibition of microorganisms.

Table 2: The results of the microbial purity control of extracts

Sample	Amount of sample	Dilution	The total number of microorganisms in 1 g of the extract		Microorganisms		
			bacteria (TAMC) CFU / g	Fungi (TYMC) CFU / g	E. coli	Staph. aureus	Ps. aeruginosa
№1 CO ₂ extract of plantago major	2,0 g	1:10	<10	<10	no growth	no growth	no growth
№2 CO ₂ extract of Acorus calamus	2,0 g	1:10	10	<10	no growth	no growth	no growth

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