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Study of Antimicrobial Activity of the Callus Tissue Salvia Pratensis L. (Lamiaceae) In Vitro

Elena Maslova
Belgorod State National Research University
Russia, Belgorod
maslova@bsu.edu.ru

Tatyana Glodik
Belgorod State National Research University
Russia, Belgorod
gtania98@mail.ru

Valeria Semykina
Belgorod State National Research University
Russia, Belgorod
valeria.semikina@yandex.ru

Natalya Gulya
Biotech company "NoviStem"
Russia, Belgorod
gulia.natalya@yandex.ru

Tatyana Perelygina
Belgorod State National Research University
Russia, Belgorod
perelygina@bsu.edu.ru

***Abstract*—In this paper, we studied the antimicrobial activity of callus tissue Salvia pratensis L. obtained in vitro against *Escherichia coli* and *Staphylococcus aureus*. The most effective sterilizing agents for disinfection of plant explants with their introduction into in vitro culture were selected. The composition of the nutrient medium for the cultivation of callus tissues of *S. pratensis* has been optimized. A comparative analysis for the antibacterial activity of plant extracts obtained from callus tissues and intact plants was carried out, and as a result it was found that extracts from callus tissue demonstrate the most pronounced antibacterial properties in concentrations of 100% and at a dilution of 1:10, in contrast to extracts from leaves and flowers of an intact plant.**

Keywords—callus tissue, antibacterial activity, S. pratensis, plant extracts, in vitro, gram-negative, gram-positive microorganisms

I. INTRODUCTION

Antibiotic resistance is one of the main problems facing humanity. In this regard, there is an increasing need for the search for new antimicrobial agents. Plant materials are one of these sources and they do not cause resistance [1]. Nowadays the modern preparation and production of biologically active substances from plant materials with antimicrobial, anti-inflammatory, antioxidant effects are more and more actively associated with the use of various biotechnological techniques.

In recent years, special attention of researchers has been given to the study of a significant number of representatives of Lamiaceae or Labiatae family. A number of authors studied plant species with pronounced antimicrobial properties, such as *Teucrium polium* L., *Mentha piperita* L., *Rosmarinus officinalis* L., *Ballota nigra* L., *Salvia aethiopis* L., *Salvia stepposa* Shost., *Ocimum* [2-9]. The problem of the limitations of natural raw materials, the distribution range of the species, the dependence of gathering raw materials on geographical, climatic and seasonal factors leads to the search for alternative plant sources. The development of plant cell and tissue engineering methods allows inducing the formation of callus tissue of plants in vitro.

Obtaining cell cultures in vitro enables the synthesis of environmentally friendly plant biomass, which possesses or produces valuable biologically active substances with antibacterial, antioxidant, antiviral and anti-cancer properties. Previously, the authors investigated the antimicrobial properties of callus cultures of *Ajuga genevensis* L., and the high antibacterial activity of the extracts in relation to *E.coli* was proved [10]. A high antimicrobial activity of the callus culture of *Mentha arvensis* L. against bacteria of the genus *Proteus* was also revealed [11]. Using the medicinal plant *Salvia officinalis* L. as an example, the regenerative ability of callus tissue of this species was studied [12] and the conditions for obtaining callus and somatic embryogenesis of plant explants were determined [13]. The data indicate the increasing importance of the use of plant cell cultures to obtain a number of biologically active compounds or substances with valuable properties.

Meadow sage (*Salvia pratensis* L.) is a perennial plant with biologically active substances in its essential oil [14]. Sage contains borneol, cineole, α and β thujone, as well as aliphatic components, oxygen-containing sesquiterpenes, sesquiterpene hydrocarbons, oxygen-containing monoterpenes, monoterpene hydrocarbons. In total, in the composition of *S. pratensis* 28 compounds, mainly monoterpenoids with the sabinene component (21%) were identified [15].

The importance of *S. pratensis* in human use is great, alongside with closely related species of medicinal sage (*S. officinalis*) and nutmeg (*S. sclarea*). It has such properties as anti-inflammatory, antimicrobial, expectorant [16].

The aim of the research is to obtain callus tissue of *Salvia pratensis* L. in vitro, determine its antibacterial activity, and make a comparison.

II. EXPERIMENTAL

As materials and objects of research we used - callus tissue and intact plants (flowers and leaves) of *S. pratensis*, growing in Belgorod region, as well as test objects gram-negative bacteria of the species *Escherichia coli* (VKPM-

M17 strain) and gram-positive – *Staphylococcus aureus* (MDC 5233 strain).

All work and experiments with plant explants, callus tissues and microorganisms were carried out in laminar boxes "Lamsystems" class II and class III protection, A2-A3 type (manufactured by Russia, ZAO Laminar Systems) in compliance with aseptic rules [17]. Callus tissues were obtained by introducing into the culture in vitro plant explants (seeds) of *S. pratensis* gathered in Belgorod Region during the active fruiting of plants in late June - early July 2017-2018. Plant explants underwent phased sterilization. As sterilizers lysoformin 3000 (3%, 5%), biocide (3%, 5%), sodium hypochlorite (5%, 2.5%), chloramine B (5%), silver nitrate (0.5 %, 0.1%) were used. Then, seedlings obtained from seeds were wounded in order to induce callusogenesis and cultivated on modified nutrient media of various compositions with the addition of various concentrations of phytohormones IAA, BAP and sucrose (20, 30 g/l) [18]. Callus tissues were cultured in a thermostat at a temperature of 23.5° C and passaged every 3-4 weeks for fresh nutrient in order to propagate and maintain cultures.

Plant extracts of callus tissue and intact *S. pratensis* plants were obtained using the method of preparation of alcoholic extracts [10]. After receiving a 100% plant extract, the procedure for obtaining serial dilutions was carried out. To obtain a 1:10 dilution, 1 ml of a 100% plant extract was taken and placed using an automatic dispenser in the first tube with 9.3 ml of autoclaved distilled water. To obtain a 1: 100 dilution, 1 ml of the solution was taken from a 1:10 dilution and added to a test tube with 9.3 ml of distilled water. Subsequent dilutions of 1: 1000 and 1: 10000 were prepared in a similar manner [19]. Cell cultures of passage 4 were used to prepare plant extracts from callus tissue.

The study of antimicrobial activity, based on the disk diffusion method, was carried out. Daily diurnal cultures of *E. coli* (VKPM-M17 strain) and *S.aureus* (MDC 5233 strain) were preliminarily obtained on oblique agar-agar using the nutritional medium GRM; microorganism suspensions were prepared according to standard methods [20]. Filter disks with a diameter of 14 mm were impregnated with the studied solutions (extracts). For control, a 40% solution of ethyl alcohol (included in the extracts) and antibiotic solutions were used. The control antibiotic gentamicin was used for *E. coli*, and ceftriaxone was used for *S. aureus*. The diameters of zones of growth inhibition of microorganisms were evaluated by standard indicators [20]. Data processing was performed with the help of the statistical analysis software package Microsoft Excel. The following statistical characteristics were used: arithmetic mean (x), mean error (Sx). To assess the significance of differences between the control and experimental groups, the Fisher test was used [21].

III. RESULTS AND DISCUSSION

Obtaining callus tissues of *S. pratensis* was carried out by introducing into the culture in vitro plant explants, seeds by nature. To determine the most effective sterilizing agent, *S. pratensis* plant explants were sterilized with five disinfecting solutions: lysoformin 3000 with a concentration of 3% and 5%, biocide with a concentration of 3% and 5%, sodium hypochlorite with a concentration of 5% and 2.5%, chloramine B - 5%, silver nitrate concentration of 0.5% and 0.1%. The exposure time of each disinfectant was 10 and 15 minutes.

As a result, it was found that the most effective sterilizing solution for introducing *S. pratensis* plant explants into the culture in vitro is a 3% biocide when exposed to it for 10 minutes (Table I, Fig. 1).

With this sterilization mode, the maximum number of viable plant explants (43.3%) in relation to sterile (80%) was obtained. However, an increase in the exposure time of this sterilizer to 15 minutes at a given concentration led to a decrease in seed viability (20% viable from 96.67% sterile). The use of this sterilizer in higher concentrations of 5% for 15 minutes had a detrimental effect on the viability of plant explants, which did not germinate at all, but were not covered by infection, because seed sterility was 100%. Reducing the exposure time of 5% biocide to 10 minutes led to a slight decrease in sterile explants (96.67%), but their viability increased significantly (out of 96.67% sterile viable was 20%). Therefore, the use of this sterilization regimen is very effective for obtaining seedlings in an in vitro culture. It is also possible to use 5% chloramine B as a sterilizing agent with an exposure time of 15 minutes (20% viable) and 5% sodium hypochlorite for 10 minutes. However, the latter sterilization regime provides a small amount of 13.33% of viable plant explants.

TABLE I. THE EFFECT OF STERILIZING SOLUTIONS ON PLANT EXPLANTS *S. PRATENSIS*

Sterilizing solution and its concentration	Sterilization duration (minutes)	Sterile explants number (%)	Viable explants number (%)
Lysoformin 3000 (5%)	10	61,67±2,3	0,0±0,0
Lysoformin 3000 (5%)	15	96,67±3,6	0,0±0,0
Biocide (3%)	10	80,0±3,09	43,3±1,67
Biocide (3%)	15	96,67±3,61	20,0±0,77
Biocide (5%)	10	96,67±3,61	20,0±0,77
Biocide (5%)	15	100,0±0,0	0,0±0,0
Sodium hypochlorite (5%)	10	96,67±3,61	13,33±0,51
Sodium hypochlorite (5%)	15	100,0±0,0	0,0±0,0
Sodium hypochlorite (2,5%)	10	96,67±3,61	0,0±0,0
Sodium hypochlorite (2,5%)	15	81,67±3,09	0,0±0,0
Chloramine B (5%)	10	96,67±3,61	0,0±0,0
Chloramine B (5%)	15	100,0±0,0	20,00±0,77
Silver nitrate (0,1%)	10	96,67±3,61	0,0±0,0
Silver nitrate (0,1%)	15	96,67±3,61	0,0±0,0

The use of all other sterilization regimes with lysoformin 3000, sodium hypochlorite and silver nitrate in various concentrations and exposure times is not advisable, since when using them, the percentage of viable seeds is close to zero.

Using the Fisher Test, it was found out that at a significance level of P> 0.05 all values of viable and sterile explants *S. pratensis*, presented in table I, are statistically significant.

Viable isolated cultures obtained by sterilization were grown on various modified culture media (MR_1 and MR_2) to induce callusogenesis and to obtain callus tissues. As a result, it was found that MR_2 medium is a more optimal medium for cultivating callus tissue of *S. pratensis* (Fig. 2), since callus tissue growth was initiated on it at subsequent passaging stages, which was not observed on MR_1 medium.

To conduct a comparative analysis of the antimicrobial properties, plant extracts were obtained from callus tissues cultured in vitro and as well as from flowers and leaves of intact plants collected in Belgorod Region. The results of the antimicrobial effect of the extract from the flowers, leaves of an intact plant and callus tissue of *S. pratensis* on microorganisms of the species *E. coli* and *S. aureus* are presented in Table II and diagrams (Fig. 3, 4).

As it can be seen from table II and fig. 3 a 100% plant extract from the flowers of the intact *S. pratensis* plant and its dilution 1:10 have weak antimicrobial activity against *E. coli*. The plant extract from the flowers of the intact plant *S. pratensis* does not show antibacterial activity against *S. aureus*, since the control exceeded almost all the values of the studied extracts and their dilutions, except for 100%, which is 0.14 units higher than the control value (Table II , fig. 4).

100% plant extract from the leaves of the plant *S. pratensis* and its dilution 1:10 have weak antibacterial activity against E. coli. In relation to *S. aureus*, only 100% extract from the leaves of the plant *S. pratensis* exhibits weak antimicrobial activity.

The results of the antimicrobial effect of the extract from callus tissue of *S. pratensis* on microorganisms of the *E. coli* species show that the 100% extract, as well as its dilution 1:10 of the callus culture of *S. pratensis* demonstrate antimicrobial activity significantly exceeding the control values. The remaining dilutions were lower than the control value; therefore, they did not show antimicrobial activity against *E. coli*. In relation to *S. aureus*, 100% callus tissue extract demonstrates strong antimicrobial activity, while its dilutions of 1:10 and 1: 100 have weak antimicrobial activity. The indicators of all other dilutions were below the control value; therefore, they did not possess antibacterial properties.

Based on the data above, 100% extract from callus tissue *S. pratensis* and some of its dilutions have high antimicrobial activity against *E. coli* and *S. aureus* than the extracts from flowers and leaves of an intact plant.

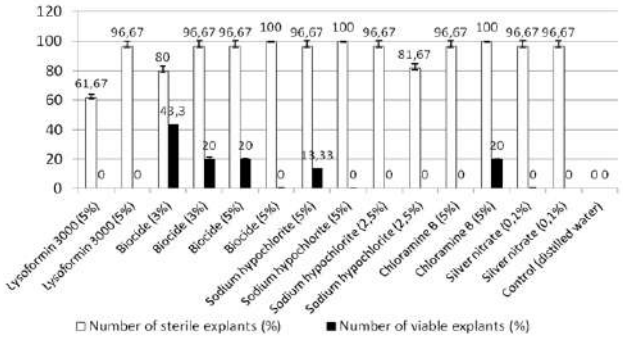
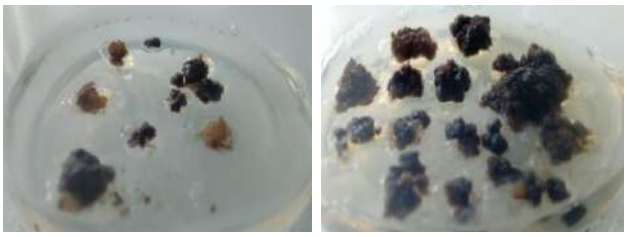


Fig. 1. The effect of sterilizing solutions on the ratio of sterile and viable explants of the species *S. pratensis.*



a) b)

Fig. 2. Callus tissue of *S. pratensis* on media: a) MR_1 at 1 month of cultivation (1 passage); b) MR_2 at 4 months of cultivation (4 passage).

TABLE II. THE EFFECT OF PLANT EXTRACTS FROM FLOWWERS, LEAVES OF AN INTACT PLANT AND CALLUS TISSUE OF *SALVIA PRATENSIS* ON MICROORGANISMS *E.COLI* AND *S.AUREUS*

Extract concentration	Diameters of the zones of growth inhibition of microorganisms, mm					
	Intact plant				Callus tissue	
	Flowers		Leaves			
Microorganisms	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
100%	1,13±0,37	1,5±0,5	0,962±0,32	1,56±0,52	9,4±3,1	13,7±4,5
1:10	0,93±0,31	1,2±0,4	0,93±0,31	1,2±0,4	7,23±2,41	9,3±3,1
1:100	0,0±0,0	1,0±0,33	0,0±0,0	1,0±0,33	1,03±0,34	6,7±2,3
1:1000	0,0±0,0	0,916±0,3	0,0±0,0	0,0±0,0	0,82±0,27	2,4±0,8
1:10000	0,0±0,0	0,53±0,17	0,0±0,0	0,0±0,0	0,32±0,1	1,25±0,41
control	0,36±0,1	1,36±0,4	0,3±0,1	1,375±0,45	3,9±1,3	3,86±1,28
antibiotic	9,5±3,06	10,2±3,4	8,5±2,8	9,2±3,06	8,6±2,8	8,4±2,8

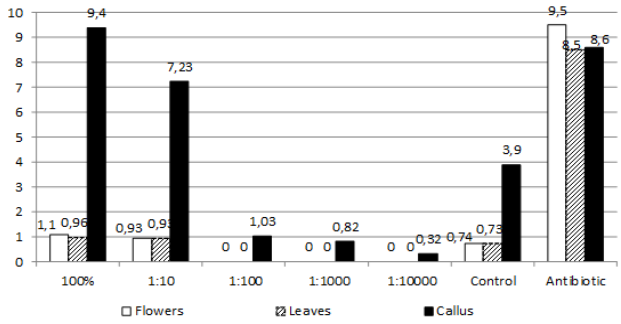


Fig. 3. Comparative analysis of the antimicrobial activity of extracts of *S. pratensis* from flowers, leaves and callus tissue in relation to *E. coli.*

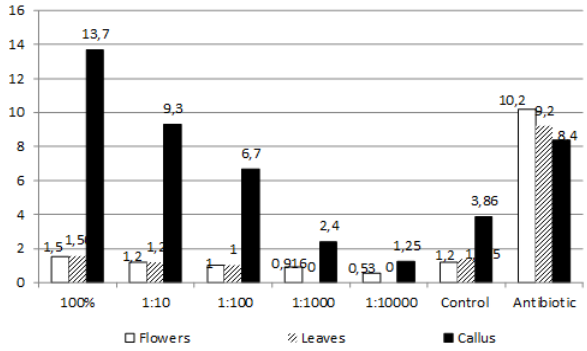


Fig. 4. Comparative analysis of the antimicrobial activity of extracts of *S. pratensis* from flowers, leaves and callus tissue in relation to *S. aureus*.

IV. Conclusion

Thus, the data obtained indicate that the cultivation of callus culture is advisable to obtain antibacterial substances, which exist in a much smaller amount in the leaves and flowers of an intact plant. This research serves as the basis for further study of callus tissue of *S. pratensis* in different passages of cultivation and testing it on a wider range of microorganisms.

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Determination of Antibacterial Activity of *Artemísia Absínthium* L. (Asteraceae) Extracts

Elena Maslova
Belgorod State National Research University
Belgorod, Russia
maslova@bsu.edu.ru

Valeria Semykina
Belgorod State National Research University
Belgorod, Russia
valeria.semikina@yandex.ru

Tatyana Glodik
Belgorod State National Research University
Belgorod, Russia
gtania98@mail.ru

Tatyana Perelygina
Belgorod State National Research University
Belgorod, Russia
perelygina@bsu.edu.ru

Margarita Petrosyan
Yerevan State University
Yerevan, Armenia
margaritpetrosyan@gmail.ru

***Abstract*—In this study, plant extracts of *Artemisia absinthium* L. were produced. Herbal extracts from leaves and flowers were evaluated for antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. As a result, it was found that plant extracts from *Artemisia asbinthium* flowers have the greatest antibacterial properties. Their use as a substance of plant origin is the most appropriate.**

Keywords—antibacterial activity, plant extract, microorganisms, Artemísia absínthium, Escherichia coli, Staphylococcus aureus

I. INTRODUCTION

The search and rational use of valuable biological resources in the pharmaceutical industry still remains an urgent task nowadays. It is known that medicinal plant material with sufficient pharmacological effectiveness is less toxic and has less allergenic capacity.

A. absínthium L. is a deserving phytoncidic medicinal plant for medicine and pharmacy. *Artemísia absínthium L.* is a perennial wild plant with a characteristic specific aroma and a very bitter taste. It contains a variety of biologically active compounds such as: bitter glycosides (absintin and anabsintin), flavonoids, an essential oil consisting of terpenoids, phytoncides, alkaloids, capillin, vitamins, organic acids, ascorbic acid, saponins, carotene, mineral salts and tannins. The presence of at least 15 amino acids, 9 of which are irreplaceable, has been discovered in the grass and wormwood meal by the method of HPLC; 9 of which are irreplaceable, a wide range of trace elements has been determined, the presence of 62 compounds such as fatty acids, sterols, alcohols, heavy terpenoids has been discovered [1-3].

Previously, the authors had found the bactericidal, fungistatic, and antipersistent effect of various wormwood species *A. abrotanum, A. dracunculus, A. austriaca A. obtusiloba, A. pontica, A. glauca* [4].

Due to the emergence of antibiotic-resistant strains of microorganisms, there is a need to search for new sources of raw materials with microbial properties. In this regard, plant organisms are of great interest, which can serve as a basis or substance for the development of agents with antibacterial activity, to which resistance does not emerge.

The aim of the research was to study the antibacterial activity of plant extracts obtained from flowers and leaves of *A. absinthium.*

II. EXPERIMENTAL

As objects of study intact plants (flowers and leaves) of *Artemísia absínthium L.,* test objects of gram-negative bacteria of the *Escherichia coli* species, and gram-positive bacteria of the species *Staphylococcus aureus* were used.

Intact plants were gathered during mass flowering in the summer of 2019 (Belgorod region, Korochansky district, the village of Gremyachye, the right bank of the Koren river). Plant extracts were produced by maceration of leaves and flowers of plants separately, with the use of the method of alcoholic extracts preparation [5-7].

After getting a 100% plant extract of the studied species, the procedure for obtaining serial dilutions was carried out. To obtain a 1:10 dilution, 1 ml of a 100% plant extract was taken and placed using an automatic dispenser in the first tube with 9.3 ml of autoclaved distilled water. To obtain a 1: 100 dilution, 1 ml of the solution was already taken from a 1:10 dilution and added to a test tube with 9.3 ml of distilled water. Subsequent dilutions of 1: 1000 and 1: 10000 were prepared in a similar way [8].

The study of antibacterial activity, based on the method of diffusion into agar with filter discs, was carried out. Diurnal cultures of *E. coli* (strain VKPM-M17) and *S. aureus* (strain MDC 5233) were obtained on oblique agar-agar in the nutritional medium GRM; microorganism suspensions were prepared according to standard methods [7-8]. To determine the antimicrobial properties of plant extracts, the nutritional medium GRM was poured into sterile Petri plates; after the solidification 100 mcl of a suspension of microorganisms were inoculated using a continuous lawn method with a Drigalski spatula. Then, in each Petri plate pre-sterile filter disks were placed on which

the studied solutions were applied and the diameters of the zones of inhibition of microorganisms' growth were taken into account. As a control, a 40% solution of ethyl alcohol (a component of the extracts) and antibiotic solutions were used. The antibiotic cefepime was used for *E. coli*, and levofloxacin for *S. aureus*. [9]. The data obtained were statistically processed with the use of the statistical functions of the Microsoft Office Excel 2007 application with AtteStat 8 add-ons. The accuracy of the studies was verified by the Fisher Test [10].

III. RESULTS AND DISCUSSION

The test results of plant extracts obtained by us from *A. absinthium* plants gathered in Belgorod Region revealed the manifestation of antibacterial activity against two strains of microorganisms *E. coli* and *S. aureus* (Table I).

Screening of plant extracts obtained from *A. absinthium* flowers shown in Fig. 1 made it possible to determine sensitivity to microorganisms *E. coli and S. aureus*. Moreover, *S. aureus* turned out to be the most sensitive, while *E. coli* revealed less sensitivity to plant extracts obtained from flowers of intact plants as compared to the control indices. Ethanol extracts of *A. absinthium* flowers inhibit the growth of *E. coli* with a zone of 13.4 (100% extract) -15.6 (1: 100 dilution) mm, thereby exhibiting weak antibacterial sensitivity, the difference with the control was 9.1 mm. Also, plant extracts from flowers are highly sensitive *to S. aureus*, inhibiting their growth with delay zones of up to 17.3 mm.

The results of testing ethanol plant extracts from the leaves of *A. absinthium* revealed a high sensitivity to E. coli and S. aureus compared to the control (Fig. 2).

TABLE I. INHIBITION OF GROWTH OF TEST ORGANISMS BY PLANT EXTRACTS FROM FLOWERS AND LEAVES OF *A. ABSINTHIUM*

Extract concentration	The diameters of the zones of growth inhibition of microorganisms, mm			
	Flowers		Leaves	
	E.coli	*S.aureus*	*E.coli*	*S.aureus*
100%	13,4	16	17,5	17,6
1:10	9,4	16,3	23,5	15,4
1:100	15,6	9	12,8	8,5
1:1000	8,7	17,3	15	13,5
Control	6,5	0	4	6
Antibiotic	8,5	17,6	8,2	17,6

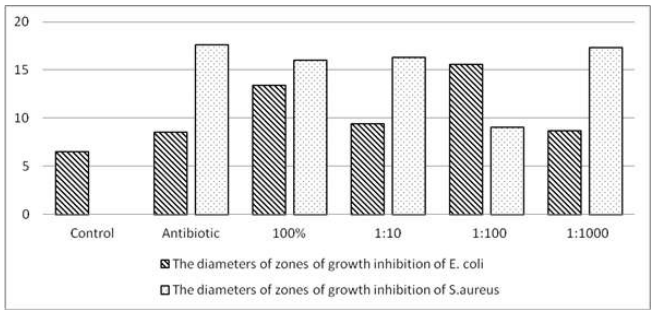

Fig. 1. The effect of plant extract from the flowers of *A. absinthium* on the microorganisms *E. coli* and *S. aureus.*

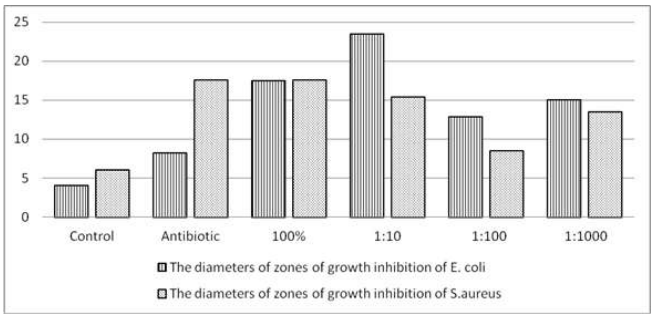

Fig. 2. The effect of plant extract from the leaves of *A. absinthium* on the microorganisms *E. coli* and *S. aureus.*

Zones of growth inhibition of *E. coli* up to 23.5 mm were recorded, with a control of 4 mm. Zones of growth inhibition of *S. aureus* with plant extracts were 17.6 mm, the difference with control was 11.6 mm. Thus, plant extract from leaves in different concentrations reveals antibacterial activity with high sensitivity to this microorganism.

IV. CONCLUSION

Thus, the results obtained revealed the manifestation of the antibacterial activity of alcoholic plant extracts of various dilutions of *A. absínthium* agaist gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria. These data indicate a need for further pharmacological studies, expanding the species composition of microorganism strains, with the purpose of obtaining plant-based antimicrobial agents.

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