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EXPERIMENTAL ARTICLES

Characterization and Antimicrobial Activity of the Bioactive Metabolites in Streptomycete Isolates[1]

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Abstract—Twenty different streptomycete isolates were obtained from soils of southeast Serbia. Five isolates identified as *Streptomyces hygroscopicus* (SH100, SH101, SH102, SH103, and SH104) showed strong activity against *Botrytis cinerea*, a parasite found in domestic vines. These isolates were extensively studied for their in vitro antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeasts and fungi, and also antiviral activity against *Herpes simplex*. The results indicated that the obtained isolates were highly active against *Botrytis cinerea, Candida albicans*, and *Herpes simplex*, with an inhibition zone at ≥31 mm. The structure of the bioactive components was determined using elemental analysis, as well as UV/VIS, FTIR, and TLC.

Key words: Antimicrobial activity, Antibiotics, Streptomycetes.

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INTRODUCTION

Streptomycetes, Gram (+) filamentous bacteria, are widely distributed in a variety of natural and man-made environments and constitute a significant component of the microbial population in most soil [1]. The results of extensive screening have been the discovery of about 4000 antibiotic substances from bacteria and fungi, many of which have found applications in human medicine, veterinary medicine, and agriculture. Most of them are produced by streptomycetes [2]. Most streptomycetes and also other actinobacteria produce a diverse array of antibiotics, including aminoglycosides, macrolides, β-lactams, peptides, polyenes, polyether, tetracyclines, etc. [3]. In searching for new antibiotics, over 1000 different bacteria (including actinobacteria), fungi, and algae have been investigated. To prevent exponential emergence of microorganisms becoming resistant to the clinically available antibiotics already marketed, the periodic replacement of existing antibiotics is necessary. In the present study, the isolation and characterization, as well as the antimicrobial activity, of local streptomycete isolates were studied.

MATERIALS AND METHODS

Isolation of microorganisms. For screening, soil samples were collected from various locations in southeast Serbia from 2000 to 2002. Soils of depth ranging from 0.25 to 1.5 m were collected. Several diverse habitats in different areas were selected for isolation of streptomycete strains. These habitats included agricultural soil, forest soils, and river soils.

For the screening procedure, soil samples of 1 g were suspended in 100 ml distilled water and then incubated in an orbital shaker incubator at 28°C at 150 rpm for 30 min. The mixtures were allowed to settle and then serial dilutions up to 10–6 were prepared. In accordance with the crowded plate technique [4], from each dilution 0.1 ml was taken and spread evenly over the surface of medium plates (in triplicate) with a sterile L-shaped glass rod and then incubated at 30°C for seven days. Plated dilutions that gave 20–200 colonies were chosen for further isolation. Repeated streaking on medium plates purified the bacterial colonies that showed a Streptomyce-like appearance. For isolation of streptomycete strains from soil samples, the following agarized media were used: Czapek's agar, Starch-dextrose agar, Bennett's agar, Nutrient agar, and Glucose-asparagine agar (Difco) [5]. Pure cultures were obtained from selected colonies for repeated subculturing on starch-dextrose agar.

Characterization of the isolates. Some diagnostic characters of highly active isolates were determined following the directions given in the probabilistic identification matrix of Williams [6] and Bergey's Manual of Systematic Bacteriology [7]. A Willcox probability matrix

[1] The text was submitted by the authors in English.

Table 1. Antimicrobial activity of the five highly active streptomycete isolates

Test organisms	Zone of inhibition, mm				
	Isolates				
	SH100	SH101	SH102	SH103	SH104
Botrytis cinerea	35	27	34	35	35
Staphylococcus aureus ATTC6538	8	14	6	32	32
Staphylococcus epidermis MU29	25	18	27	31	31
Bacillus anthracis	32	21	18	22	22
Bacillus subtilis	7	14	8	25	25
Enterobacter aerogenes RSKK750	22	5	4	7	7
Escherichia coli	8	4	8	18	18
Pseudomonas aeruginosa RSKK102	32	7	18	7	7
Proteus mirabilis	16	8	15	7	7
Candida albicans	31	14	32	22	22
Saccharomyces cerevisiae RSKK102	34	9	31	17	17
Herpes simplex	35	18	38	31	31

was used to assign and identify isolates where scores of 0.8 and above indicated a positive identification [8].

Test-organisms. *Staphylococcus aureus, Staphylococcus epidermis, Bacillus anthracis, Bacillus subtilis, Proteus mirabilis, Pseudomonas aeruginosa, Escherichia coli, Enterobacter aerogenes, Botrytis cinerea, Saccharomyces cerevisiae, Candida albicans*, and *Herpes simplex* (Table 1) were used to determine the antimicrobial and antiviral activity of the isolated strains. All these test-organisms were obtained from the Institute of Health Protection (Leskovac, Serbia).

In vitro screening of isolates for antagonisms. The antimicrobial activity of streptomycete isolates was tested using the diffusion method [9]. For the growth of test-bacteria, solid medium was used, containing dextrose, 1 g/l; meat extract 1.5 g/l; yeast extract, 3 g/l; kazein, 4 g/l; peptone, 6 g/l; agar, 15 g/l; and distilled water, pH 7.6. Yeasts and fungi were grown on medium of the following composition: tripton, 15 g/l; soya–peptone, 5 g/l; NaCl, 5 g/l; agar, 15 g/l; and distilled water, pH 5.6. Filter paper disks (Cellulose disk Antibiotica—Test Blattchen Shleicher and Schuell, Germany, 12.5 mm in diameter) were impregnated with samples of liquid cultures of highly active strains, dried, and placed onto plates (in the center of a petri dish), previously seeded with test organisms. After incubation at 30°C for 48 hours, the incubation zones were measured (mm).

The antimicrobial activity of the active compounds was tested at a concentration of 70 μg disc^{-1} against test organisms (Table 1) by the same method.

Antiviral activity against *Herpes simplex* was generated in the microbiological laboratory of the "Zdravlje–Actavis" pharmaceutical company, Leskovac.

Fermentation and extraction. The highly active isolates were grown in a 500-cm^3 shake flask containing 100 cm^3 of the culture medium (0.8 g NaCl, 1 g NH_4Cl, 0.1 g KCl, 0.1 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g $CaCl_2 \cdot 2H_sO$, 2 g glucose, 3 g yeast extract in 1 l of distilled water, pH 7.3). The fermentation medium was inoculated with 5% (v/v) of a preculture after 48 h growth and incubated at 28°C for 168 hours under standard conditions of aeration and agitation (200 rpm). The biosynthetic activity of isolates was followed by testing the antibiotic activity every 12 h during the fermentation. For this procedure, each sample was mixed with an equal volume of methanol and the mixture was shaken for two hours and filtered. The filtrate was assayed by the paper disk method using *Botrytis cinerea* as a test organism. As maximum antibiotic production was observed on the seventh day of incubation, fermentation was terminated on this day and the broth was centrifuged at 4000 rpm for 15 min to separate the mycelial biomass. For the extrac-

Table 2. Some common cultural characteristics of the highly active streptomycete strains

Medium	Growth	Aerial mycelium	Substrate mycelium (Reverse)	Soluble pigment
Czapek's agar	Good	Abundant, early brown white, later brownish gray	Pale yellowish brown	None
Starch-dextrose agar	Good	Abundant, early brown white, later dark brownish gray	Brownish gray	None
Bennett's agar	Good	Abundant, early brown white to brownish gray, later becomes moist and exhibits brownish black patches, gradually spreading over the whole surface	Pale yellowish brown	None
Nutrient agar	Good	No aerial mycelium	Pale yellowish brown	None
Glucose-asparagine agar	Good	Abundant, early brown white, later brownish gray	Pale yellowish brown	None

tion of the antibiotic from the culture, supernatants were used: *n*-butanol, *n*-hexane, and ethyl acetate.

Isolation of active substance. The solvent was added to the supernatant in 1 : 2 proportion. The extracts was concentrated *in vacuo* and adsorbed on a silica gel (Kieselgel 100, 0.063–0.2 mm, Merck) column (60 × 1 cm). After washing with chloroform and chloroform-methanol (3 : 1), active components were eluted with chloroform-methanol (1 : 1) and (1 : 3). The eluate, which contained the active components, was combined, concentrated, and further purified on a Sephadex LH-20. The active substances were eluted with methanol and yielded 16 g of a yellow powder. Afterwards, the TLC plates were air-dried. Bands were scraped from the plates with a spatula under UV light, extracted with methanol, and filtered through Whatman no. 5 paper. Each band was bioassayed using *B. cinerea* and the active bands were purified again on TLC using the same solvent system and visualized using UV light or the iodine–sulfuric-acid color reaction [10]. The R_f factor for each band was measured. Each isolated band was also dissolved in methanol, and its UV absorption spectra was measured with a *Perkin-ElmerLambda* 15 UV/VIS spectrophotometer to determine the λ_{max} of the band. Microanalysis for carbon, hydrogen, and nitrogen was carried with a Carlo Erba 1106 microanalyser. The FTIR spectra were recorded using a Michaelson Bomen MB-100 series spectrophotometer, using a KBr pellet (1/100 mg) and the liquid technique.

RESULTS AND DISCUSSION

A total of 20 different isolates of actinomycetes were recovered from 33 soil samples. Antibacterial activity was exhibited by 44.5% of the total isolates, while the five isolates exhibit very strong activity. Analysis of the morphological and some cultural characteristics of the five isolates especially active against *Staphylococcus epidermis, Candida albicans, Botrytis cinerea*, and *Herpes simplex* (Table 1) allowed us to determinate their probable taxonomic classification in genus *Streptomyces*, according to the directions given in Bergey's Manual of Systematic Bacteriology (7). In order to determine their taxonomic status, more detailed characterization studies were carried out. Some of the cultural and physiological characteristics of the strains are given in Tables 2 and 3. Through a critical study of all the selected strains on the various differential media (the best among them is starch–dextrose agar [5]), it was discovered that all of the cultures had certain fundamental characteristics in common [6]. The most fundamental of these are the following: (i) Sporiferous appendages arise as short side branches of the main hyphae and generally terminate in tight spirals of two to many turns (Figs. 1c, 1e); frequently, there is also an evident tendency towards clustering of sporiferous structures (Figs. 1a, 1b, 1d, 1e). (ii) On media which promote abundant sporulation, all strains studied were found to produce spores which were a brownish-gray color en masse (iii).

All strains studied developed, to some degree, on one or more media which promote sporulation, characteristic

Table 3. Some common physiological characteristics of the highly active streptomycete strains

Property	Result
Temperature for growth	21–37°C
pH range for growth	5.5–8.5
Production of melanoid pigment	Negative
Tyrosinase reaction	Negative
Nitrate reduction	Negative
Starch hydrolysis	Positive
Gelatin liquefaction	Positive, strong
Milk peptonization	Positive
Cellulose reaction	Negative

moist, glistening, dark hygroscopic patches in the aerial mycelium. Comparative analysis of some morphological, cultural, and physiological properties of highly active streptomycete strains led to the designation of the species as probably *Streptomyces hygroscopicus*, with Willcox probability scores of 0, 8, and above.

The following work deals with the characterization of the bioactive metabolites from streptomycete isolates, which was selected on the basis of its strong antimicrobial activity against the test organisms. Different solvents were used and tested for the extraction of the antibiotic from the culture supernatant. The following solvents were used: *n*-butanol, *n*-hexane, and ethyl acetate to determine the ideal solvent for extraction of the antibiotic from the cultures supernatant. As it can be seen, the most appropriate solvent was *n*-butanol, since the yield of antibiotic with it is the highest one (Table 4). The UV spectral data for the *n*-buthanol extracts of selected active fermented broths of active strains are shown in Table 5. Maximum absorbance peaks range between 215–270 nm and the characteristics of absorption peaks indicate a mostly polyene nature (Fig. 2). Isolation of active substance from *n*-butanol extracts of culture supernatant of the strain no. SH100 was carried out by consecutive procedures with the use of adsorbance on a silica gel column, washing with chloroform and chloroform-methanol (3 : 1), and further purification on Sephadex. A methanol solution of purified samples was analyzed by silica TLC plates with a benzene : methanol (85 : 15) mixture. The active components were marked as component A with an Rf value of 0.70 and component B with an Rf value of 0.78.

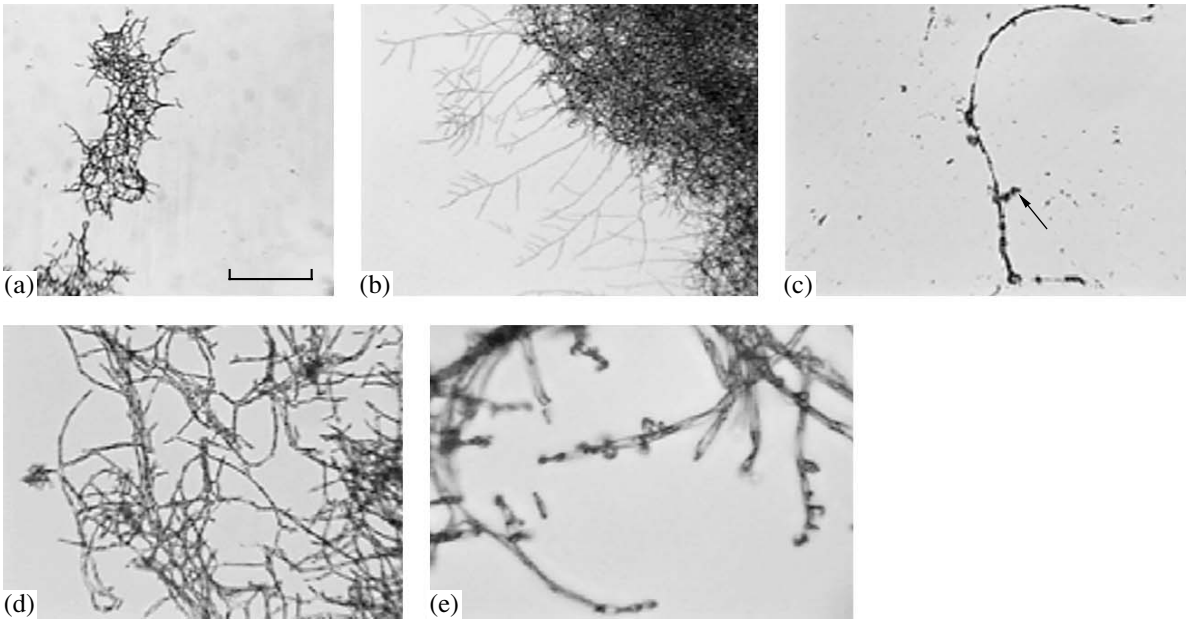

Fig. 1. Sporulating aerial mycelium of highly active streptomycete strains (on starch-dextrose agar medium): (a) SH101 (enlargement 40×), (b) SH100 (enlargement 100×), (c) SH102 (enlargement 100×), tight spiral(arrow), (d) SH103 (enlargement 400×) (e) tight spirals of spore chains of strain SH104 (enlargement 1000×).

Elementary analysis of the active component A is a follows (in %):

For $C_{18}H_{34}NO_8$ (M.W. 392)

anal. calcd. (%):	C 55.10;	H 8.67;	O 32.65;	N 3.57.
Found (%):	C 55.13;	H 8.74;	O 32.48;	N 3.61.

Elementary analysis of active component B is a follows:

For $C_{18}H_{35}NO_8$ (M.W. 393)

anal. calcd. (%):	C 54.96;	H 8.90;	O 32.56;	N 3.56.
Found (%):	C 54.91;	H 8.97;	O 32.49;	N 3.60.

The UV spectral data of components A and B exhibited strong absorption at λ_{max} = 217 and 221 nm in methanol, suggesting a carbon–carbon double bond (Fig. 3). The purified component A and component B exhibit great and equal activity against the tested microorganisms, especially against *Botrytis cinerea* (MIC value is 1 μg cm^{-3}) (Table 6).

The FTIR spectrum of the component A exhibits absorption at ~3335 cm^{-1} and 1029 cm^{-1}, which indicates hydroxyl groups, and at 1653 cm^{-1}, indicating a double bond (Figure 4). The FTIR spectrum of the component B exhibits absorption at ~3396 cm^{-1} and 1029 cm^{-1}, which indicates hydroxyl groups, and at 1652 cm^{-1}, indicating a double bond (Fig. 5).

The spectral analysis indicates a polyene characteristic of the isolated antibiotics. The molecular weight of the active components was determined from the elementary analysis and mass spectrum. The mass spectrum of active component A showed the molecular ion peak at *m/e* 392, and the molecular ion peak at *m/e* 393 for active component B, which is in good agreement with the molecular formula $C_{18}H_{34}NO_8$ and $C_{18}H_{35}NO_8$.

Further investigations are needed in order to determine the structure of active components and their antibiotic spectra, and to study the active substances from other highly active streptomycete strains.

The conducted investigation of actinomycete isolates from soils of various locations in southeast Serbia showed that almost half of the isolates had antimicrobial activity. The significance of antibiotic activity to the soil microbial community and the potential effects of antibiotic-producing bacteria on plant health, especially in agricultural soils, is apparent. The istribution of the antibiotic inhibition phenotype of streptomycetes with great antibacterial and antifungal activity was noted. Isolated highly active streptomycete strains were determined as probably *S. hygroscopicus* (related to the group of synonyms of species *S. violaceusniger*) [7]. It is known that some streptomycetes, described as *S. hygroscopicus*, formed anti-

Table 4. Different solvents used for extraction of antibiotic substance

Solvents used for extraction	Yield of the antibiotic substance, mg dm^{-3}
n-butanol	1.75
ethyl acetate	0.84
n-hexane	0.52

Table 5. UV spectral data of *n*-butanol extracts of fermentation broths of five highly active streptomycete strains

Strain	λ_{max}, nm	Shoulder, nm
SH100	221, 262	274
SH101	226, 246	281
SH102	216	252, 317
SH103	221–250	272, 313
SH104	226–250	260–300

Table 6. MIC values of active components A and B

Test organisms	MIC values, μg cm^{-3}	
	Component A	Component B
Botrytis cinerea	1	1
Staphylococcus aureus ATTC6538	8	8
Bacillus subtilis	10	10
Escherichia coli	8	8
Candida albicans	2	2
Herpes simplex	2	2

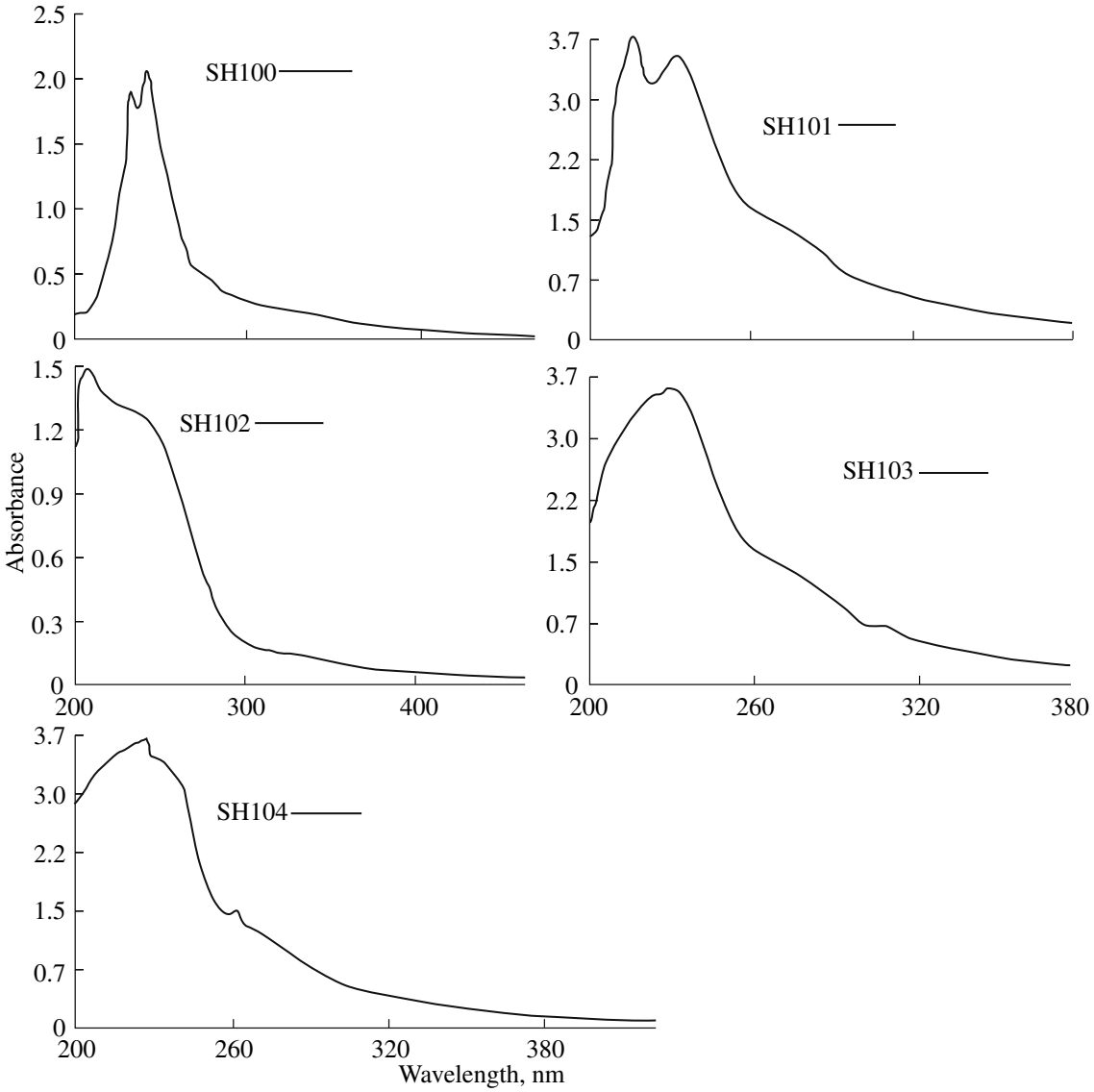

Fig. 2. UV spectra of n-buthanol extracts of fermentation broth of five highly active streptomycete strains SH100, SH101, SH102, SH103, SH104.

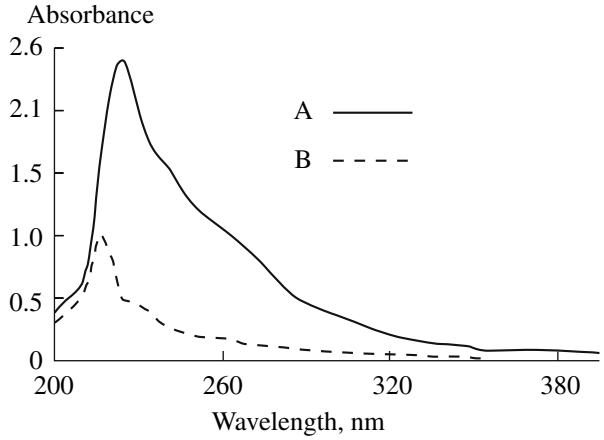

Fig. 3. The UV spectrum of the active components A and B in methanol.

biotics with great antibacterial and antifungal activity of polyenic nature [11, 12]. It should be noted that the capacity of streptomycetes to form bioactive substances of polyenic nature is quite widespread. Thus, the spectral data of active extracts for isolated streptomycetes from Mugla province [13], and for some aquatic actinomycetes obtained by Swaadoun et al. [14], indicated their polienic nature.

Antibiotic substances of highly active investigated streptomycete strains obtained from soils of southeast Serbia also had a polienic nature and showed great activity against *Botrytis cinerea* (MIC value is 1 μg cm^{-3}), a parasite found in domestic vines. This fact has undoubted biotechnical significance. On the domestic market, biofungicides, which are not toxic, and teratogenes do not exist. A preparation developed from the inves-

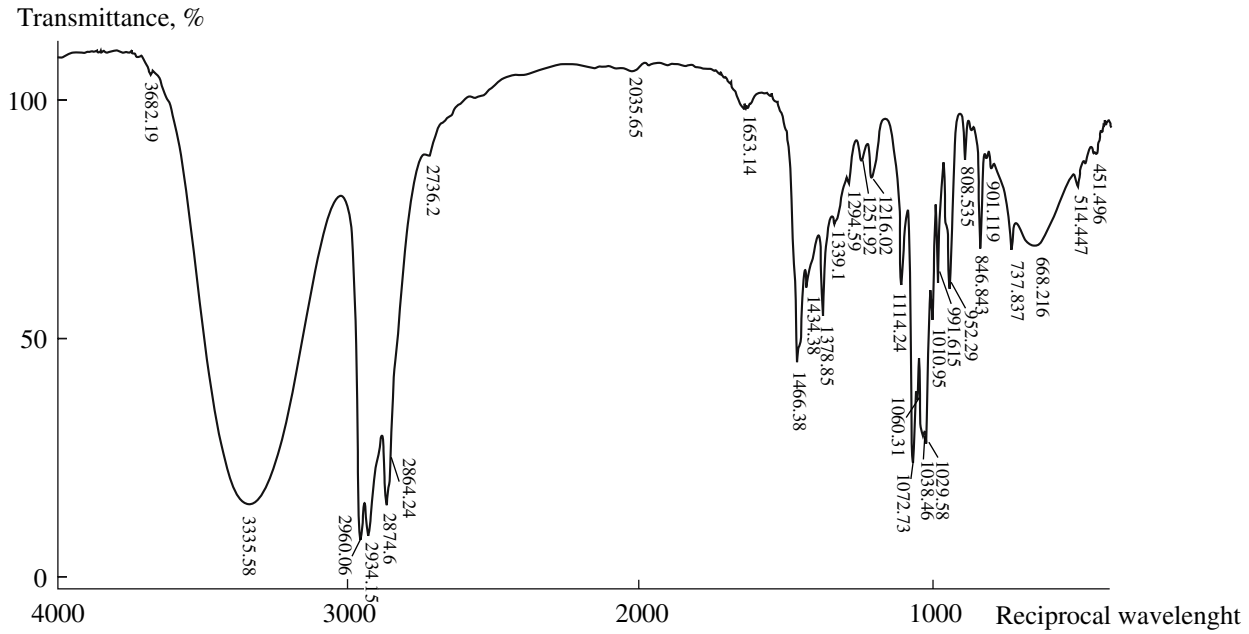

Fig. 4. The FTIR spectrum of the component A.

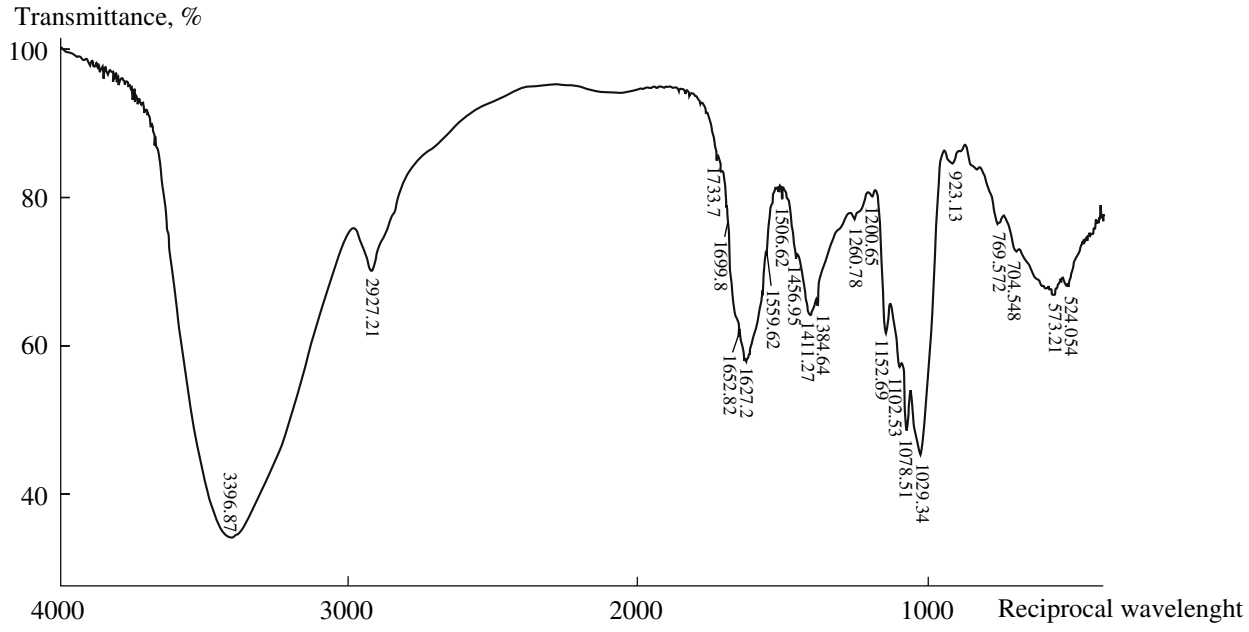

Fig. 5. The FTIR spectrum of the component B.

tigated isolates would have great advantages over existing commercial preparations.

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