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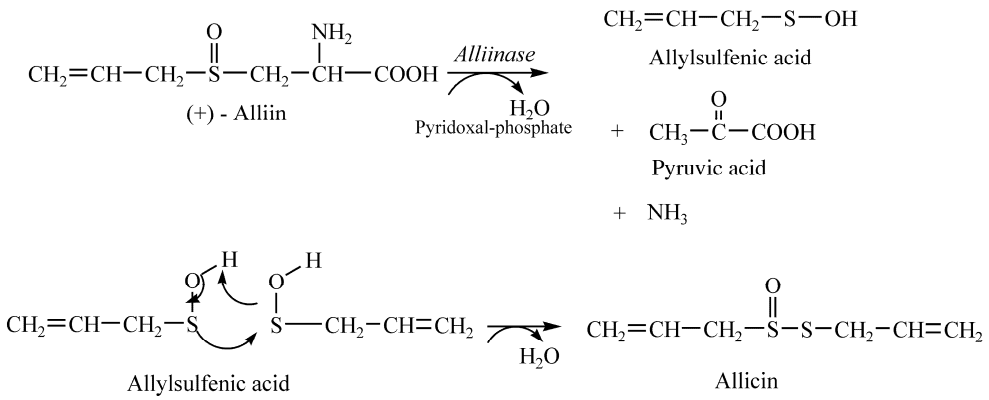
# THERMAL DEGRADATION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF THE SYNTHESIZED ALLICIN AND ALLICIN INCORPORATED IN GEL

*The main carriers of the pharmacological activity of garlic (Allium sativum L.) are organic sulfur compounds, the most important among them being allicin, a sulfenic acid thioester, or allylthiosulfonate. In this paper, the identification of synthesized and purified allicin was determined by using various spectroscopic methods (UV/Vis, FTIR and NMR). An HPLC method was developed for the detection and determination of the allicin content. The thermal degradation of allicin by using FTIR method was monitored. The method for the production of allicin gel based on Carbopol 940 (poly(acrylic acid)) was elaborated. The antimicrobial activity of pure allicin and allicin incurporated into gel by using a disk diffusion method was determined. In order to determine the antioxidant activity of allicin DPPH testing was done and it was proved that with low concentrations (1 mg·cm$^{-3}$) a high DPPH radicals scavenging capacity (90%) was achieved.*

The main carriers of the pharmacological activity of garlic (*Allium sativum* L.) are organic sulfur compounds, the most important among them being allicin (allyl thiosulfinate). It is formed postmortem, as a secondary metabolite, under the influence of alliinase on genuine alliin in the bulb (Scheme 1) [1–6].

Regarding the chemical composition, allicin is a thioester of sulfenic acid, or allylthiosulfinate. It is an oily liquid, light yellow in color, with a characteristic garlic smell [6]. Pharmacologically, allicin is the most important and the most active substance and it is found in the fresh aqueous extract of garlic [3,14,15].




*Scheme 1. Transformation of (+)-alliin in the garlic bulb under the influence of alliinase.*

It can also be obtained in a synthesis process, whereby allylsulfide is used as a precursor. The basis of the synthesis reaction is oxidation, and various oxidation agents, such as hydrogen peroxide [4,7–10], *m*-chloro--perbenzoic acid [11], and magnesium monoperoxy hydrate can be used as the oxidation means [12]. In this work [13], detailed investigations of the mechanisms and the kinetics of allicin synthesis from allyldisulfide and hydrogen peroxide as oxidation agents in an acid medium were carried out. This synthesis, taking place in accordance with a radical mechanism, can be represented by a cumulative chemical reaction (Scheme 2).

It is active against a great number of bacteria, viruses, fungi, and many other parasites [16–18]. In low concentrations, it inhibits the growth of *Staphylococcus*, *Streptococcus*, *Bacillus*, *Brucella*, *Vibrio* and *Candida* species [7,19]. Its antimycotic activity is stronger than that of nistatin and other antimycotics [16, 20–24]. It shows a virucidal activity against *Herpes simplex* type 1 and 2, *Parainfluenza* virus type 3, *Vaccinia* virus, *Vesicular stomatitis* virus, and *Human rhinovirus* type 2 [25]. Besides the antimicrobial activity, allicin has an important role in clinical use in prevention of cancer and cardiovascular diseases and shows an outstanding antioxidant activity [18,26,27].

Numerous investigation results definitely show that, without doubt, garlic and its phyto-preparations have a clinical activity when properly prepared. As for synthetic active principles of garlic as a human medicinal for-

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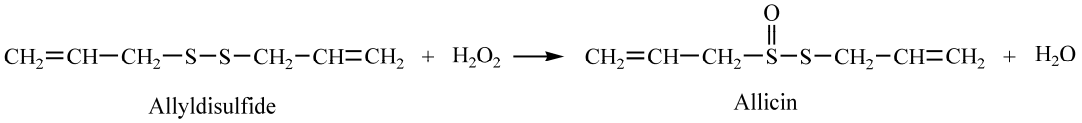


*Scheme 2. Synthesis of allicin from allyl disulfide with hydrogen peroxide.*

mulation, a new issue important for the therapeutic application of any medicine arises: the action and safety during administration. Namely, there is an old saying: "When asserting that a substance has no side effects, there is no certainty that it has the principal effect either". The investigations of acute toxicity of allicin have shown that, with rats [28], the $LD_{50}$ value amounts to 60 mg·kg$^{-1}$ *i.v.* (when administered intravenously), and 120 mg·kg$^{-1}$ *s.c.* (for a subcutaneous administration). The application of high doses of allicin can cause a number of disorders in the organism. The enhanced activity of liver lipase and $\alpha$-glucan-phosphorilase, or a decreased activity of glucoso-6-phosphatase, *i.e.* hepato-toxicity may occur. High dosages of allicin may bring about a complete inhibition of growth, disorders of thyroid gland function, hyperplasia (goiter), allergic reactions on the skin and mucous and suffocation. With patients with profuse hemorrhages, bleeding is further increased [28]. Data presented in reference works also confirm the interaction of allicin with other medicines. The use of allicin is especially dangerous with patients who are under constant medication for life supporting and vital functions (diabetics, patients with blood pressure disorders and increased levels of cholesterol in blood, patients suffering from malignant diseases and with organic disorders of the digestive system). Therefore, the answer to the question is: allicin can be used in human medicine as a medicine with given contraindications such as allergy to *allium* species, vital functions disorders, stomach and intestinal diseases, uncontrolled bleeding, and intolerance to odor [28].

In this paper, allicin was synthesized, its thermal degradation was analyzed and the antioxidant and antimicrobial activity was determined. In order to investigate the possibilities of making a preparation for external use, allicin was incorporated into a gel which was also determined and antimicrobial activity to the microbes investigated.

## EXPERIMENTAL

### Chemicals

Allyldisulfide (80%, $\rho$ = 1.008 g cm$^{-3}$, $M$ = 146.8 g mol$^{-1}$) was purchased from Aldrich Chemicals Co., hydrogen peroxide (30%, $\rho$ = 1.11 kg dm$^{-3}$, $M$ = 34.01 g mol$^{-1}$) from Riedel-de Haën, Germany; 2.2-diphenyl-1--picrylhydrazyl radical (DPPH) from Sigma Chemicals Co., (St. Louis, MO, USA). Other reagents used in this work were of analytical and HPLC grade.

### Allicin synthesis

Allicin was synthesized from allyldisulfide by the procedure described elsewhere (yield: 73%) [13].

### Preparation of gel with allicin

Carbopol® 940 (0.05 g) was dispersed in water (2 cm$^3$) until a complete homogenization, and then another 5 cm$^3$ of water were added. Allicin was dissolved in isopropanol (290 mg/3.2 cm$^3$) and the solution was added to the prepared dispersion with continuous mixing. The obtained mixture was neutralized by 5% aqueous solution of sodium hydroxide (0.25 g/5 cm$^3$) and left to swell at room temperature, with periodical mixing, until the gel was formed.

### Ultraviolet/visible spectroscopy (UV/Vis)

UV/Vis spectra were recorded in methanol on a Varian Cary-100 Conc. UV/Vis spectrophotometer in 1 cm thick quartz cuvettes. $\lambda_{max}$ in Vis area for 2,2-diphenyl-1-picrylhydrazyl radical is 517 nm.

### Infrared Fourier transformation (FTIR)

FTIR spectra of the synthesized allicin were made on a Bomem Hartmann & Braun MB-series spectrophotometer between KBr plates with a 0.1 mm thick layer in wavelength bands from 4000 to 400 cm$^{-1}$. The stability and transformations of the synthesized allicin at various temperatures, 70 and 80 °C, were also monitored by FTIR method with corresponding cells thermostatted at given temperatures.

### $^1$H-NMR and $^{13}$C-NMR spectrometry

$^1$H-NMR and $^{13}$C-NMR spectra of allicin were recorded on Bruker AC 250 E apparatus with operational frequencies of 250 and 62.5 MHz, respectively, in a 5 mm dia. glass cuvette at room temperature by the impulse method with multiple impulse repetitions to record $^{13}$C-NMR spectra.

### High pressure liquid chromatography (HPLC)

The content of synthesized allicin was determined by HPLC method on an Agilent 1100 apparatus under the following conditions: Zorbax Eclipse XDB-C18, 4.6 mm×250 mm, 5μm; column temperature: 20 °C; eluent: acetonitrile/water = 80:20; mobile phase flow: 1 cm$^3$/min; injected volume: 20 μl; detector: DAD Agilent 1200, detection at 205 nm.

### DPPH test

DPPH test was used to determine the antioxidant activity of the synthesized allicin. The test is based on

the use of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), which has the ability of reacting with the molecules showing the antioxidant activity, whereby a stable hydrogenized molecule DPPH-H is formed. Since this involves a color reaction, the test is based on measuring the adsorbance of samples on UV/Vis spectrophotometer at wavelength of 517 nm in corresponding solvent (MeOH).

A number of solutions of different concentrations ranging from 0.1 to 12.5 mg·cm$^{-3}$ were prepared from the basic allicin solution (250 mg/20 cm$^3$ MeOH), and their adsorbance was determined at 517 nm. The methanol solution of DPPH radical with $3\times10^{-4}$ mol dm$^{-3}$ concentration (1 cm$^3$) was added to the allicin solutions (2.5 cm$^3$) of various concentrations, and their absorbance was measured at the same wavelength. The absorbance of the pure methanol solution of DPPH radical diluted to the given ratio (1 cm$^3$ of DPPH radical of given concentration with the addition of 2.5 cm$^3$ of methanol) was determined under the same conditions. Methanol was used as the blank sample. Before measuring the adsorbance, all the samples were subjected to 20 min incubation at room temperature in the dark. The free radicals scavenging capacity was determined according to the relation given below:

$$\text{DPPH radical scavenging capacity (\%)} = 100 - ((A_U - A_B)(100/A_K)) \quad (1)$$

where $A_U$ is the sample absorbance (allicin methanol solution treated by DPPH radical solution) at 517 nm; $A_B$ is the blank sample adsorbance (allicin methanol solution not treated by DPPH radical solution) at 517 nm; $A_K$ is the control sample adsorbance (1 cm$^3$ of DPPH radical $3\times10^{-4}$ mol·dm$^{-3}$ concentration + 2.5 cm$^3$ of methanol) at 517 nm [29–33].

**Antimicrobial activity**

A disk diffusion method was used for the microbiological investigations of allicin and allicin incurporated in gel. The allicin concentration per disk was 30 μg of allicin/50 μl gel or isopropanol. The samples were left to incubate for 24 h at 37 °C for bacteria, and for 36 h at 25 °C for fungi.

The following bacteria were used as the test microbes: *Staphilococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, and fungi: *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404.

*B-1 Bacto antibiotic medium 1 dehydrated* (Difco laboratories, Detroit, MI, USA) was used as the substrate for the bacteria, and Tripton soja-agar (Torlak Institute of Immunology and Virology, Belgrade, Serbia) for fungi.

## RESULTS AND DISCUSSION

For the structural characterization of synthesized and purified allicin, various instrumental techniques were used, such as UV, FTIR, $^1$H-NMR and $^{13}$C-NMR.

UV (methanol, 43 μg cm$^{-3}$): 198 nm originating from $\pi\rightarrow\pi^*$ transition of the isolated C=C bond and $n\rightarrow\sigma^*$ transition from S=O group, and the adsorption peak with significantly weaker intensity, appearing at 254 nm and originating from $n(p_y)\rightarrow\pi^*$ transition of C=C group present in the allicin molecule.

FTIR (0.1 mm thin layer method on KBr): 1634 cm$^{-1}$ (a medium intensity band originating from stretching of terminal C=C bond valence vibrations), 1087 cm$^{-1}$ (strong, stretching of S=O group), 3083 cm$^{-1}$ (C–H stretching asymmetric of $=CH_2$), 2978 cm$^{-1}$ (C–H stretching symmetric of $=CH_2$), 1423 and 1326 cm$^{-1}$ (δ C–H deformation of $=CH_2$) and 988 and 925 cm$^{-1}$ (γ C–H deformation of $=CH_2$).

$^1$H-NMR (250 MHz, $CDCl_3$, $\delta$ / ppm): 3.25–3.50 (2H, CC**H**$_2$SO), 3.50–3.60 (2H, SC**H**$_2$C), 5.16–5.30 (2H, C**H**$_2$C), 5.80–5.96 (1H, $CH_2$C**H**$CH_2$).

$^{13}$C-NMR (62.5 MHz, $CDCl_3$, $\delta$ / ppm): 33.2 (3b, S**C**$H_2$CH), 53.6 (3a, CH**C**$H_2$SO), 118.5 (1b, CH**C**$H_2$), 124.0 (1a, **C**$H_2$CH), 125.3 (2b, $CH_2$**C**H$CH_2$), 132.6 (2a, $CH_2$**C**H$CH_2$). The type of proton and carbon (1a, 2a, 3a, 1b, 2b and 3b) is shown in Fig. 1.

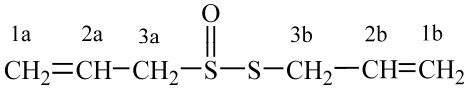


*Figure 1. The structure of allicin with the indicated types of hydrogen and carbon atoms for NMR.*

For the detection and determination of the allicin content, an HPLC method has been developed. By choosing the corresponding conditions of column and the mobile phase, the retention time ($R_t$) of 2.992 min was determined for allicin. The allicin content, *i.e.*, the allicin concentration is proportional to the peak area in the chromatogram. There is a part of the peak area that is a linear function of allicin concentration to 500 μg·cm$^{-3}$, *i.e.*, to the peak area of 8000 mAU*s, for which the coefficient of linear correlation $R$ = 0.998. The straight line equation for the linear function is:

$$A = 149.203 + 17.207c \quad (2)$$

where $A$ is the peak area, mAU*s, $c$ is the concentration of allicin in μg cm$^{-3}$. The allicin detection limit in the solution by this method is about 0.06 μg cm$^{-3}$.

Allicin is an unstable molecule and it can degrade under the influence of various factors. In this paper, allicin degradation under the influence of temperature was monitored by FTIR spectrometry. Namely, to monitor the allicin degradation a band in the IR spectrum origi-

nating from S=O valence vibrations at 1087 $cm^{-1}$ (indicative of allicin) was chosen.

The dependence of the peak area variation normalized with the peak area maximum at 1087 $cm^{-1}$, *i.e.*, $c_A/c_{A0}$ on the time of the allicin exposure to temperature of 70 and 80 °C represents an exponentially decreasing dependence, Fig. 2.

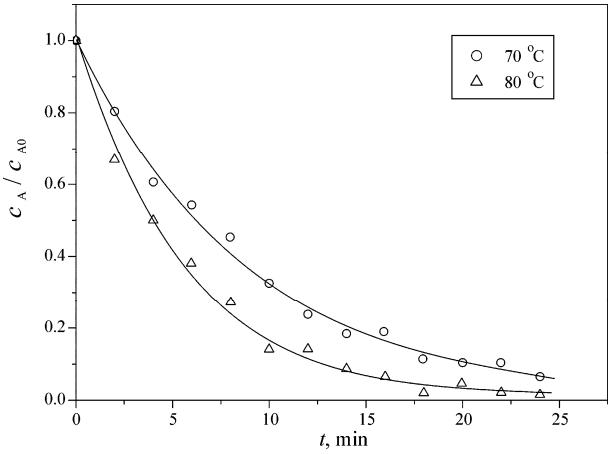


*Figure 2. Dependence of normalized peak area at 1087 $cm^{-1}$, i.e., $c_A/c_{A0}$ on time at 70 and 80 °C.*

To determine the kinetic parameters for thermal degradation of allicin the $n^{th}$-order equation for the reaction rate is used:

$$-\frac{dc_A}{dt} = kc_A^n \tag{3}$$

The introduction of normalized concentration in this equation, *i.e.*, the use of $c_A/c_{A0}$ instead of $c_A$, yields:

$$-\frac{d\left(\frac{c_A}{c_{A0}}\right)}{dt} = k\left(\frac{c_A}{c_{A0}}\right)^n \tag{4}$$

By taking the logarithm of Eq. (4) and by using differential method of the analysis of data on reactant concentration variation in time, whereby the normalization of data was made by dividing the maximum value of the area at the beginning of the reaction, we obtain:

$$\ln\left(-\frac{dc_A}{c_{A0}dt}\right) = \ln k + n\ln\left(\frac{c_A}{c_{A0}}\right) \tag{5}$$

which is equivalent to Eq. (6):

$$\ln\left(-\frac{dc_A}{dt}\right) = \ln\left(\frac{k}{c_{A0}^{n-1}}\right) + n\ln c_A \tag{6}$$

Eq. (5) is a linear equation which enables the determination of kinetic parameters of the allicin transformation reaction, *i.e.*, the rate constant of the degradation reaction ($k$), and the order of the reaction ($n$). In Fig. 3 the linear dependence of the function $\ln(-dc_A/dt)$ on $\ln(c_A)$ is given, where the reaction order is determined from the slope, and the value $(k/c_{A0}^{n-1})$ is determined from the intersection at ordinate. According to the results shown, one can see that the reaction of the allicin thermal degradation is a reaction of the first order. Since the reaction order for allicin transformation is 1, then $c_{A0}^{n-1} = c_{A0}^0 = 1$ and $k/c_{A0}^{n-1} = k$.

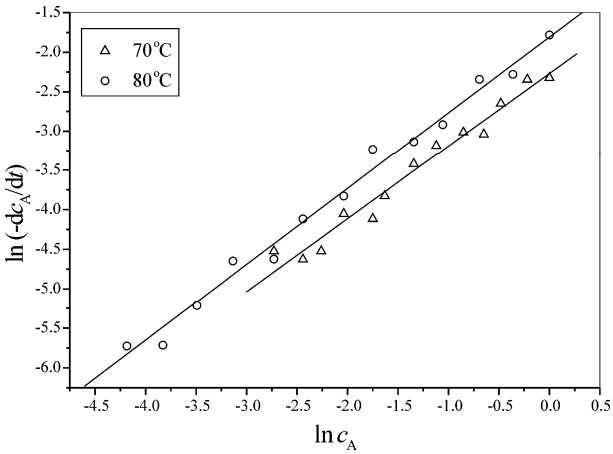


*Figure 3. Determination of the kinetic parameters of allicin degradation at 70 and 80 °C.*

By using values of the rate constant for two different temperatures according to Arrhenius expression:

$$k = Ae^{-\frac{Ea}{RT}} \tag{7}$$

we calculated the activation energy and the pre-exponential factor (the results are given in Table 1).

*Table 1. Values of kinetic parameters for allicin thermal degradation reaction*

| Temperature, K | Kinetic parameters | |
|---|---|---|
| | $k$ / $min^{-1}$ | $n$ |
| 343 | 0.10 | 0.92 |
| 353 | 0.16 | 0.96 |
| $Ea$ / kJ $mol^{-1}$ | 46 | |
| $A$ / $min^{-1}$ | $1.2\times10^6$ | |

Figure 4 gives the results of the allicin antioxidant activity. The graph shows that the degree of free DPPH radical scavenging increases with the increase of the allicin concentration. A high level of radical neutralization is achieved with the allicin concentration of 1 mg $cm^{-3}$, and it amounts to about 90%. This indicates that allicin is a molecule type with a marked antioxidant activity, and as such, it can find its place in the production of pharmaceutical preparations. A decrease in the initial DPPH concentration by 50% was defined as $EC_{50}$. Butylated hydroxylotene (BHT) is a compound which has

excellent antioxidant characteristics and was used here as a reference substance. Its $EC_{50}$ value is 0.021 mg cm$^{-1}$ while for synthesized allicin $EC_{50}$ value was determined to be 0.37mg cm$^{-3}$.

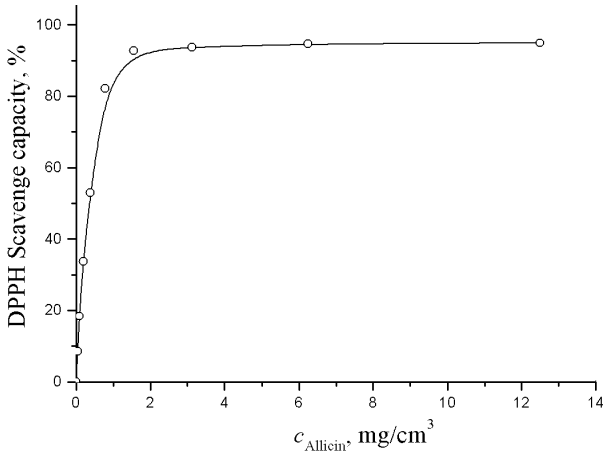


*Figure 4. Antioxidant activities of allicin methanol solutions.*

The results of antimicrobial activities of the synthesized allicin and allicin incorporated in gel for the microorganisms tested are given in Table 2.

Based on the results shown it can be seen that allicin reacts against all the tested microbes and that there are almost no differences between the activities of the pure allicin and the allicin incorporated in gel. This, further, shows that Carbopol based gel does not induce the reduction of the allicin activity, and presents a favorable formulation for the production of preparations for external application. Also, it shows that the gel itself has no influence on the growth of microorganisms, *i.e.*, it has no inhibition zones. Among the bacteria tested, *Staphylococcus aureus* ATCC 6538 was the most susceptible, followed by *Escherichia coli* ATCC 8739, while the least susceptible was *Pseudomonas aeruginosa* ATCC 9027. *Candida albicans* ATCC 10231 was the most susceptible one among the fungi, and *Aspergillus niger* ATCC 16404 the least susceptible.

## CONCLUSIONS

A structural characterization of synthesized and purified allicin was carried out by using UV/Vis, FTIR and NMR. For the detection and determination of the allicin content, a highly reproductive and accurate analytical HPLC method was elaborated according to which the retention time ($R_t$) of allicin was 2.992 min. FTIR method was used to determine the kinetic parameters of thermal destruction of allicin for two different temperatures, 343 and 353 K ($k_1$ = 0.10 min$^{-1}$, $k_2$ = 0.16 min$^{-1}$, $n_1$ = 0.92, $n_2$ = 0.96, respectively, $E_a$ = 46 kJ mol$^{-1}$, $A$ = = 1.2×10$^6$ min$^{-1}$). A procedure for the preparation of Carbopol 940 based gel with allicin was developed.

The microbiological analysis of pure allicin and allicin incorporated in gel showed the same activity against the tested microbes. The most susceptible microorganism among the bacteria was *Staphylococcus aureus* ATCC 6538, and *Candida albicans* ATCC 10231 among the fungi.

A DPPH test was used to determine the neutralization degree of a stable DPPH radical, and for the allicin concentration of 1 mg cm$^{-3}$ it amounted to about 90%, which indicates that it is a good antioxidant agent. The $EC_{50}$ value of allicin methanol solution was 0.37 mg cm$^{-3}$.

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*Table 2. Growth inhibition zone values for the microorganisms tested for pure allicin and allicin in gel*

| Microorganisms | Growth inhibition zone values for the microorganisms, mm | |
|---|---|---|
| | Pure allicin | Allicin in gel |
| Bacteria | | |
| *Staphylococcus aureus* ATCC 6538 | 39 | 40 |
| *Escherichia coli* ATCC 8739 | 37 | 38 |
| *Pseudomonas aeruginosa* ATCC 9027 | 20 | 19 |
| Fungi | | |
| *Candida albicans* ATCC 10231 | 41 | 41 |
| *Aspergillus niger* ATCC 16404 | 35 | 34 |

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## IZVOD

ANTIOKSIDATIVNA I ANTIMIKROBNA AKTIVNOST SINTETISANOG ALICINA I ALICINA INKORPORIRANOG U GEL

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(Naučni rad)

Glavni nosioci farmakološkog dejstva belog luka (*Allium sativum* L.) jesu organo-sumporna jedinjenja od kojih je najvažniji alicin, tioestar sulfenske kiseline, ili alil-tiosulginat. U ovom radu izvršena je sinteza alicina (aliltiosulfinata) oksidacijom alildisulfida kiselim vodonik-peroksidom. Sintetisani alicin je prečišćen višestepenom ekstrakcijom sa dietiletrom. Identifikacija prečišćenog alicina izvršena je primenom različitih spektroskopskih metoda (UV/Vis, FTIR, NMR). Za detekciju i kvalitativno i kvantitativno određivanje alicina razvijena je HPLC metoda. Termička razgradnja alicina praćena je primenom FTIR metode. Odabrana je traka koja potiče od valencionih vibracija S=O grupe koja je indikativna za alicin. Određene su vrednosti kinetičkih parametara za reakciju degradacije alicina koja predstavlja reakciju prvog reda. Razrađen je postupak za izradu gela sa alicinom na bazi Carbopola 940. Mikrobiološka analiza čistog alicina i alicina u gelu je pokazala istu antimikrobnu aktivnost na ispitivane mikrobe što ukazuje da je izabrana formulacija gela pogodna i da ne utiče na umanjenje aktivnosti alicina. Od bakterija najosetljivija je *Staphylococcus aureus* ATCC 6538, a od gljiva *Candida albicans* ATCC 10231. Antioksidativna aktivnost alicina određena je primenom DPPH testa. Za koncentraciju alicina od 1 mg $cm^{-3}$ ona iznosi oko 90%, što ukazuje da je alicin dobar antioksidativni agens. Vrednost $EC_{50}$ za rastvor alicina u metanolu iznosi 0,37 mg $cm^{-3}$.

Ključne reči: Alicin • Termička razgradnja • Antioksidativna aktivnost • Antimikrobna aktivnost • HPLC • UV/Vis • FTIR • NMR

Key words: Allicin • Thermal degradation • Antioxidant activity • Antimicrobial activity • HPLC • UV/Vis • FTIR • NMR