Original research paper

MICROBIOLOGICAL SAFETY EVALUATION AND BIOLOGICAL PROPERTIES OF THE *ALLIUM URSINUM* SAUCE (ORIGINAL PRODUCT FROM KUČAJ MOUNTAIN)

Katarina G. Mladenović*[1], Mirjana Ž. Grujović[1], Ljiljana R. Čomić[1], Aleksandar Glišović[2]

[1]University of Kragujevac, Faculty of Science, Department of Biology and Ecology, Radoja Domanovića 12, 34000 Kragujevac, Republic of Serbia

[2] „Our paradise garden“, Kneza Mihajla 278, 34000 Kragujevac, Republic of Serbia

*Corresponding author:
Phone: +38134336223
E-mail address: katarina.mladenovic@pmf.kg.ac.rs

ABSTRACT: In this paper, it was described the physical-chemical characteristics of the *Allium urisnum* sauce, an original product made from the wild garlic, collected from the Kučaj mountain (Serbia). The microbiological safety of the product was examined. Also, for the first time, the influence of *A. urisnum* sauce on the growth of pathogenic bacteria and probiotics was examined, *in vitro*. The influence of *A. urisnum* sauce on the biofilm formation of selected bacteria was also studied. The results indicated that the sauce does not contain inappropriate microorganisms. Antimicrobial activity of sauce against planktonic forms of 18 bacteria was determined by using the disc diffusion and the microdilution method by determining the inhibition zones as well as minimum inhibitory concentration (MIC) and the minimum microbiocidal concentration (MMC). The antibiofilm activity was determined by using the crystal violet method. The sauce showed antimicrobial activity on *Staphylococcus aureus* and *Pseudomonas aeruginosa*. It was also showed the weak antibiofilm activity against *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* ATCC 70063. These preliminary results indicate the possibility of introducing the original product for commercial purposes. Also, these results contribute to the knowledge about the safety properties of this product and its impact on human health.

Key words: *Allium ursinum*, anti*biofilm, microbiological safety, physical-chemical analysis*

INTRODUCTION

Allium ursinum (fam. Amaryllidaceae) is a plant known as wild garlic, which is used in traditional medicine, of wide-spread distribution in Europe and Asia (Malinauskaitė and Šaluchaitė 2018). In Serbia, it appeared as autochthonous specie (Igić et al., 2010). Due to the presence of sulfur-containing compounds, *A. ursinum* has a smell like garlic, which are the most characteristic constituents in the *Allium* genera (Sobolewska et al., 2015).

Based on the literature, *A. ursinum* has been examined in several aspects in recent years. Sobolewska et al. (2015) have studied the phytochemistry and pharmacological properties of *A. ursinum.* They concluded that *A. ursinum* is a very important plant because it possesses the chemicals compounds which possess the possibility of application for therapeutic purposes. Mihaylova et al. (2012) evaluated the antioxidant and antimicrobial activity of the typical Bulgarian spice “levurda” (*A. ursinum* L.). This plant had been used for centuries in folk medicine and food flavoring. Also, they determined the chemical composition and antimicrobial activity of wild garlic *A. ursinum* of Bulgarian origin (Ivanova et al. 2009). Lupoae et al. (2013) investigated the antimicrobial activity of extracts made from wild garlic (*A. ursinum*) from Romanian spontaneous flora. Influence of different wild-garlic (*A. ursinum*)

extracts on the gastrointestinal system: spasmolytic, antimicrobial and antioxidant properties, were determined (Pavlović et al., 2017). Based on the results of the effect on the planktonic growth, it was determined the effect of *Allium sativum* on biofilm formation of bacteria (Mohsenipour and Hassanshahian, 2015). Biofilm formation is an important property of bacteria and its use has been associated with the protection of organisms against environmental stresses (Nessner Kavamura and Soares de Melo. 2014). *Allium* extract has been considered a natural preservative or food additive, and it can be used as an additional method for control of the development of pathogens (Gull et al., 2012). In the study conducted by Znamirowska (2017), it was confirmed the use of wild garlic (*A. ursinum*) plant in the conservation of kefirs from sheep's milk.

Since many investigations so far were conducted to the examinations of*. Allium ursinum* extracts and their biological properties, the aims of this study were to examine the physical-chemical properties and the safety aspect of the *A ursinum* sauce, as an original product from Kučaj mountain (Serbia). Also, the aim was to examine its impact on the planktonic growth and ability of biofilm formation of selected bacteria in order to investigate its impact on human health. The results from this study give the first evidence about the possibility of using this product for commercial purposes.

MATERIALS AND METHODS

Chemical

Resazurin was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany). Crystal violet was obtained from Fluka AG (Buchs SG, Switzerland). Liquid media, a Mueller– Hinton broth and pure agar were purchased from Torlak (Belgrade, Serbia). Ethanol was purchased from Hemmos (Belgrade, Serbia), while methanol purchased from Zorka Šabac (Šabac, Serbia). Tween 20 was purchased from SERVA electrophoresis GmbH D-69115 (Heidelberg, Germany).

Plant material

The *Allium ursinum* leaf was collected from the Kučaj Mountain. Kučaj is a mountain in eastern Serbia and belongs to a group of Carpathian-Balkan mountains. It has the direction of the north-east-southwest at a length of 40 km and the elevation is 1284 m. A sampling of the plant was done before the sun zenith and after the sun rose (around 11 o'clock in the morning), due to the highest concentration of medicinal ingredients on the periphery of the leaf at that time interval.

The technological process of making the *A. ursinum* sauce

The fresh leaf was crushed, and the preservation was done with organic sunflower oil and Himalayan salt. Any other processing of the shimmer before flowering (drying, freezing and heat treatment) are not able to save the alliin. After a few hours after treatment, evaporation of the alliin leads to the degradation of the allicin, so the healing properties are rapidly lost (about 2% per hour). For 200 g *A. ursinum* sauce (oscillations are present up to 5%), was required: 140 g of the leaf, 35 ml of sunflower oil, 5 g of salt. The four months old sample of the sauce was stored in darkness at refrigerator temperature in the laboratory of Department of Biology and Ecology, Faculty of Science, University of Kragujevac (Serbia). The sample of sauce was.

Determination of the physical-chemical properties of the *A. ursinum* sauce

Physical-chemical tests (chemistry and energy efficiency of products – the content of water, dry matter, fat, protein, carbohydrates, the content of saturated fatty acids (C6:0, C8:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0, C21:0, C22:0, C23:0, C24:0), sodium chloride) were performed according to accredited methods for testing the food of the Institute for Public Health Kragujevac, Serbia (Rulebook on the declaration,

labelling, and advertising of food (The Official Rules, No. 19/2017, 16/2018), rules on the quality of soups, sauces, food supplements, and related products (The Official Rules, No. 41/93).

Microbiological safety of *A. ursinum* sauce

Microbiological safety (the presence of *Escherichia coli,* aerophilic mesophilic bacteria, molds and yeasts, *Bacillus cereus*) of *A. ursinum* sauce was performed according to the accredited methods of the Institute for Public Health Kragujevac, Serbia (Law on Food Safety (The Official Rules, No. 41/2009) and corresponding by-laws and food safety report H 5917, SRPS EN ISO16649-2:2008, SRPS EN ISO7932:2009, SRPS EN ISO215272:2011, SRPS EN ISO4833-1:2017).

Preparation of the *A. ursinum* sauce samples and bacterial strains for biological examinations

The initial concentration of 20 mg/ml was used to test the antimicrobial activity of *A. ursinum* sauce. The sauce was dissolved in concentrated DMSO (10% of total volume) and then diluted with a liquid medium (up to 100% of the total volume). Concentrated DMSO is bactericidal, therefore a solvent control was set up, confirming that 10% DMSO does not have a negative effect on the growth of microorganisms. To stabilize the *A ursinum* sauce*,* Tween 20 was used (10% of the total volume). The overnight cultures of the following bacterial species were used: isolates (*Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium, Salmonella enterica, Proteus mirabilis, Pseudomonas aeruginosa*, *Enterococcus faecalis, Staphylococcus aureus*)*,* standard strains (*Escherichia coli* ATCC 25923, *Klebsiella pneumoniae* ATCC 70063, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 9027, *Enterococcus faecalis* ATCC 29212, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538) and probiotic strains (*Bacillus subtilis* IP 5832, *Lactobacillus plantarum*, *Bifidobacterium animalis* subsp. *lactis*). All clinical isolates were a generous gift from the Institute of Public Health, Kragujevac, Serbia. The other microorganisms (ATCC strains and probiotics) were provided by the Microbiology Laboratory, Faculty of Science, University of Kragujevac, Serbia. The bacterial strains were kept in glycerol stock at -80°C until use.

Disk diffusion method

For screening of the antimicrobial activity of the *A. ursinum* sauce, the disc diffusion method was used. Mueller-Hinton agar was spilled into petri dishes and a solid wellss of 8 mm diameter were made under sterile conditions. Then, the plates were inoculated with bacteria (suspension density of 1.5 x 10^8 CFU/ml), using sterile swab cotton sticks. After the inoculation of the bacteria, 100 µl of *A. ursinum* sauce samples was added into wells. The prepared samples were left in incubation for 24 hours at 37°C. The results were interpreted by measuring the diameter of activity (the appearance of the inhibitory zone, in mm).

Microdilution method

The bacterial sensitivity on the sauce samples of *A. ursinum* was based on the determination of the minimum inhibitory concentration (MIC) and minimum microbiocidal concentration (MMC). Minimal inhibitory concentrations are determined by using the microdilution method (Sarker et al., 2007). The microdilution method was performed in microtiter plates with 96 wells under sterile conditions. From the overnight cultures, grown on nutrient agar, of the tested strains of bacteria, the suspensions were prepared in a sterile saline solution. For the standardization of bacterial suspensions, the Mc Farland standard number 0.5 was used, which indicates the bacteria density of 1.5 x 10^8CFU/ml. A series of double dilutions of the tested sauce in the range of 0.156 mg/ml to 20 mg/ml was prepared in 96 wells microtiter plates. The total volume in the well was 100 µl. In each well, were added 10 µl of suspension of the tested bacteria. The microtiter plates were incubated at 37°C for 24 hours. Resazurin,

an indicator of cell growth, was added in order to read the results of the inhibitory effect of the sauce on the growth of bacteria. The color change of the blue-purple indicator in pink indicates the emergence of growth. The lowest concentration at which there was no change in the color of the indicator was determined as the minimum inhibitory concentration. Minimum microbiocidal concentrations were determined by screening samples from the wells of the microtiter plate in which no growth was observed after 24 hours on a solid nutrient medium. After incubation, the lowest concentration at which growth was not observed (colonies) was considered to be the minimum microbiocidal concentration. Each experiment contains growth control, sterility control and positive control (antibiotic). Each test is done in duplicate and the results are shown as the mean value. Ampicillin and tetracycline (Sigma Chemicals Co., USA), dissolved in a nutrient liquid medium, were used as reference compounds.

The influence of the *A. ursinum* sauce on the biofilm formation

The experiment was performed according to the method described by O'Toole and Kolter (1998). The bacterial strains *K. pneumoniae*, *K. pneumoniae* ATCC 70063, *P. aeruginosa, S. aureus, S. aureus* ATCC 6538 were used. A microtiter plate of 98 wells (Sarstedt, Germany) was used, and in each of them, 100 µl Mueller-Hinton broth was added. A 100 µl of the tested sauce sample was added to the first row of wells, with an initial concentration of 20 mg/ml, from which there were doubled dilutions to 0. 156 mg/ml. Then 10 µl of bacterial suspension (0.5 McFarland) was added to each well. The inoculated microtiter plates were incubated at 37°C for 48 hours. The rest of the experiment was performed according to the method described in Muruzović et al., (2016). Control of sterility of the broth and control of growth of tested bacteria were established. Two antibiotics were used as positive controls: Vancomycin and Tetracycline. All the tests were done in triplicate and the mean value was presented.

RESULTS AND DISCUSSION

Physical-chemical characteristics of the *A. ursinum* sauce

The results of physical-chemical composition of *A. ursinum* sauce, as an original product, were presented for the first time in this paper. The *A. ursinum* sauce corresponds to the Rulebook on the declaration, labeling and advertising of food (The Official Rules, No. 19/2017, 16/2018). The *A. ursinum* sauce had a dense, non-homogeneous consistency, green with herbs (crushed *A. ursinum*). The smell and taste were inherent in the product, remainded at garlic. The content of water, fat, protein and other parameters and their measured values were shown in Table 1.

Table 1.
Physical-chemical analysis of the *A. ursinum* sauce

Parameters	Measuring unit	Found value	Test method
Water content	%	43.30	EL.021[1]
Dry matter content	%	56.70	EL.021[1]
Fat content	g/100g	29.70	У.05.46[2]
Protein content	g/100g	2.18	У.05.46[2]
Content of carbohydrates	g/100g	22.84	У.05.46[2]
The energetic value of the sample	KJ/kcal/100g	1524.2/362.9	У.05.46[2]
Total content of sugar	g/100g	1.87	EL.021[1]
Content of saturated fatty acids	g/100g	2.69	SRPS EN ISO 12966-1:2015 GC / MCD
Content of sodium chloride	g/100g	1.98	У.05.88[3]

[1]Analysis of food products (Trajković et al., 1983); [2]Determination energetic value of food; [3]Determination of the sodium chloride content

Microbiological safety of the *A. ursinum* sauce

According to the parameters from Law on Food Safety (Official Rules, No. 41/2009) and the corresponding food safety report H 5917, the founded number of investigated bacteria is lower than alowed. The results of the microbiological examination of the product are shown in Table 2. Based on the results, it could be concluded that *A. ursinum* sauce is safe for human consumption.

Table 2.
Microbiological analysis of the *A. ursinum* sauce

Microbiological parameters	Measuring unit	Allowed value	Founded value	Test method
Escherichia coli	cfu/g	$<10^2$	<10	SRPS EN ISO16649-2:2008
Aerophilic mesophilic bacteria	cfu/g	$<5.0 \times 10^6$	<10	SRPS EN ISO4833-1:2017
Molds and yeasts	cfu/g	$<10^4$	<10	SRPS EN ISO215272:2011
Bacillus cereus[1]	cfu/g	$<10^4$	<10	SRPS EN ISO7932:2009

[1]*Incubation temperature of MYP Agar (Mannitol Egg Yolk Polymyxin Agar) is 30°C*

Antimicrobial activity of *A. ursinium* sauce – disk diffusion and microdilution method

The antimicrobial activity of the *A. ursinum* sauce, as the original product, for the first time was investigated in this study, using the disk diffusion and microdilution methods. Pure sunflower oil showed no antimicrobial activity. According to the disk diffusion method, tested bacteria showed no sensitivity to the *A. ursinum* sauce, except *P. aeruginosa* (14 mm), *S. aureus* (16 mm) and *S. aureus* ATCC 6538 (14 mm) (Table 3). The turbid zone of inhibition was noticed for the mentioned bacteria. According to the microdilution method, tested bacterial strains were not sensitive to the tested concentrations of *A. ursinum* sauce. The results of the sensitivity of tested bacteria to antibiotics are shown in Table 4.

Table 3.
Antimicrobial activity of the *A. ursinum* sauce – disc diffusion method

Tested species	Zone of growth inhibition given in mm (millimetre)
E. coli	/
E. coli ATCC 25923	/
K. pneumoniae	/
K. pneumoniae ATCC 70063	/
S. typhimurium	/
S. enterica	/
P. mirabilis	/
P. mirabilis ATCC 12453	/
P. aeruginosa	14 mm (T*)
P. aeruginosa ATCC 9027	/
E. faecalis	/
E. faecalis ATCC 29212	/
B. subtilis IP 5832	/
B. subtilis ATCC 6633	/
L. plantarum	/
B. animalis subsp. *lactis*	/
S. aureus	10 mm (T*)
S. aureus ATCC 6538	14 mm (T*)

Zone appearance (T - turbid zone of inhibition; / - no zone of inhibition)*

Table 4.
Antimicrobial activity of the *A. ursinum* sauce – microdilution method

Species	***A. ursinum* sauce**		**Ampicillin**		**Tetracycline**	
	MIC	**MMC**	**MIC**	**MMC**	**MIC**	**MMC**
K. pneumoniae	>20	>20	>128	>128	4	32
E. coli	>20	>20	2.1	1.2	2	6
E. coli ATCC 25922	>20	>20	0.37	0.5	4	6
P. aeruginosa	20	>20	>128	>128	>128	>128
P. aeruginosa ATCC 27853	>20	>20	>128	>128	4	32
P. mirabilis	>20	>20	>128	>128	>128	>128
P. mirabilis ATCC 12543	>20	>20	n.d	n.d	n.d	n.d
S. typhimurium	>20	>20	2	2	2	2
S. enterica	>20	>20	1	1	2	4
E. faecalis	>20	>20	4	6	1	6
E. faecalis ATCC 39212	>20	>20	0.25	0.75	1.5	3
S. aureus	>20	>20	< 0.06	< 0.06	< 0.06	< 0.06
S. aureus ATCC 6538	20	>20	0.25	0.75	1.5	3
B. subtilis ATCC 6633	>20	>20	3	4	0.25	0.37
L. plantarum	>20	>20	n.d	n.d	n.d	n.d
B. animalis subsp. *lactis*	>20	>20	< 0.06	0.12	4	8
B. subtilis IP 5832	>20	>20	8	16	< 0.06	< 0.06

Minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) values given in mg/ml for sause and µg/ml for antibiotics; n.d-not determined

A. ursinum was used in medicine both for internal and external treatments. Various studies have been done in order to investigate and confirm the antimicrobial effect of *A. ursinum* extract on bacteria *in vitro* (Ivanova et al., 2009; Synowiec et al., 2010; Mihaylova et al., 2012). The results of our research showed that *A. ursinum* sauce does not have antimicrobial activity on the members of the Enterobacteriaceae family. It was showed antimicrobial activity for the members of genera *Staphylococcus. S. aureus* is the most important cause of infectious diseases in humans. This microorganism is a part of the commensal microbiota, causing opportunistic infections under appropriate conditions (Tohidpour et al., 2010). Potential danger of the *S. aureus* originates due to its ability to develop the antibiotic resistance (Tanaka et al., 2004). In addition to *in vitro* studies of *A. ursinum,* it was performed a study of antimicrobial activity of two garlic species (*A. sativum* and *A. tuberosum*) against staphylococci infection, as well as *in vivo* study in rats. Venâncio et al. (2017) concluded that both garlic species possess molecules related to antimicrobial properties. Both species were able to reduce staphylococcal infection. No additive or complementary effect was observed by adding *A. sativum* extract with amoxicillin. Antimicrobial activity of *A. ursinum* probably originates from sulfur-containing compounds (Sobolewska et al., 2015). Alline is a natural ingredient and an integral part of fresh garlic. It is a derivate of amino acid cysteine, which converts into allicin under the action of the enzymes alliinase. Allicin is a precursor of sulfur compounds, responsible for the smell and some of the pharmacological properties of garlic. Once exposed to atmospheric air, allicin is converted into diallydissulphinate, which had an antimicrobial activity (Wang et al., 2011; Khodavandi et al., 2011). *A. ursinum* sauce in our study showed limited and selective antimicrobial activity, probably due to the fact that secondary metabolites could not be dissolved in sunflower oil, which was used as a medium of sauce.

Influence of the *A. ursinum* sauce on the bacterial biofilm formation

The antibiofilm activity of the *A. ursinum* sauce was investigated in this study for the first time, by using the crystal violet method. The results are shown in Table 5. The *A. ursinum* sauce showed a limited influence on the ability of biofilm formation of tested bacteria. *A. ursinum* sauce showed no influence on biofilm formation of *S. aureus* ATCC 6538. Bic_{50} for

S. aureus and K. *pneumoniae* were 20000 µg/ml. For *K. pneumoniae* ATCC 70063, it was 14972 µg/ml and for *P. aeruginosa*, 11385 µg/ml.

Table 5.
The influence of *A. ursinum* sauce on the bacterial biofilm formation

Species	***A. ursinum* sauce**	**Vancomycin**	**Tetracycline**
	BIC_{50}		
S. aureus	20000	n.d.	300
S. aureus ATCC 6538	>20000	62.6	n.d.
K. pneumoniae	20000	n.d.	n.d.
K. pneumoniae ATCC 70063	14972	n.d.	n.d.
P. aeruginosa	11385	733.8	2715.7

Biofilm inhibitory concentration (BIC_{50}) values given as µg/ml; n.d – not determinated

Bacteria that had the ability of biofilm formation are more resistant to antibiotics or other antimicrobials than bacteria in the planktonic form (Schlag et al., 2007). The influence of *A. sativum* on the biofilm formation of *S. aureus* was investigated. Mohsenipour and Hassanshahian (2015) confirmed the ability of garlic extracts to inhibit the biofilm formation of these bacteria. According to their results, the antimicrobial potential of this plant was confirmed, and the extracts of this plant are suitable choices against pathogenic microorganisms. In our study, *A. ursinum* sauce showed BIC_{50} values for *K. pneumoniae* ATCC 70063 and *P. aeruginosa* at very hight concentrations.

CONCLUSION

Wild garlic or *A. ursinum*, is widely consumed by humans in many forms, as a fresh or like a sauce or like a spice. Many studies so far indicated that extracts of *A. ursinum* could be used as additional means for the treatment of infections caused by pathogenic microorganisms or as a means of preventing their occurrence. But there is no study of some products from wild garlic. The sauce tested in this study presents the original product, made from wild garlic from the Kučaj mountain. Based on the results presented in this paper, the *A. ursinum* sauce is safe for human consummation. Also, the results indicated that the sauce had no significant antimicrobial activity. Therefore, the sauce can be consumed as a spice or food supplement.

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ПРОЦЕНА МИКРОБИОЛОШКЕ ИСПРАВНОСТИ И БИОЛОШКА АКТИВНОСТ *ALLIUM URSINUM* СОСА (ОРИГИНАЛНОГ ПРОИЗВОДА СА ПЛАНИНЕ КУЧАЈ)

Катарина Г. Младеновић[*1], Мирјана Ж. Грујовић[1], Љиљана Р. Чомић[1], Александар Глишовић[2]

[1]Универзитет у Крагујевцу, Природно-математички факултет, Институт за биологију и екологију, Радоја Домановића 12, 34000 Крагујевац, Република Србија
2 „Наша рајска башта“, Кнеза Михајла 278, 34000 Крагујевац, Република Србија

Сажетак: У овом раду описане су физичко-хемијске карактеристике *Allium ursinum* соса, оригиналног производа који је направљен од дивљег белог лука (сремуша), сакупљеног са планине Кучај (Србија). Испитана је микробиолошка исправност производа. Такође, по први пут је испитиван утицај сремуш соса на раст патогених бактерија и пробиотика, *in vitro*. Такође је проучаван утицај сремуш соса на формирање биофилма одабраних бактерија. Резултати су показали да сос не садржи штетне микроорганизме. Антимикробна активност соса на 18 бактерија испитана је коришћењем диск дифузионе и микроразблажења методе; мерењем зона инхибиције као и одређивањем минималне инхибиторне концентрације (МИК) и минималне бактерицидне концентрације (МБК). Антибиофилм активност је испитана методом са кристал виолетом. Сремуш сос је показао антимикробно деловање на *Staphylococcus aureus* и *Pseudomonas aeruginosa*. Такође је показано слабо антибиофилм деловање на *Pseudomonas aeruginosa* и *Klebsiella pneumoniae* ATCC 70063. Ови прелиминарни резултати указују на могућност коришћења оригиналног производа у комерцијалне сврхе. Такође, ови резултати доприносе сазнању о безбедносним својствима овог производа и његовом утицају на здравље људи.

Кључне речи: *Allium ursinum*, антибиофилм, микробиолошка исправност, физичко-хемијске карактеристике