ANTIMICROBIAL ACTIVITY OF SILVER NANOPARTICLES SYNTHESIZED USING PLANT EXTRACTS

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Abstract. The aim of the study was to determine the antimicrobial activity of silver nanoparticles synthesized using the oak bark and juniper berry extracts against foodborne pathogenic bacteria. The peaks of localized surface plasmonic resonance in UV-VIS spectrum confirmed biosynthesis of silver nanoparticles synthesized using plant extracts. The antimicrobial activity of silver nanoparticles was determined by the agar diffusion method against the reference strains *Escherichia coli* ATCC 25922, *Bacillus cereus* ATCC 11778, *Listeria monocytogenes* ATCC 19111, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* subsp. *enterica* serovar *typhimurium* ATCC 13076 cultures and 10 cultures of *Bacillus cereus* isolated from food products. The control samples (10 % of oak bark and juniper berry extracts) did not inhibit the growth of bacteria mentioned above. The silver nanoparticles synthesized using plant extracts exhibited a broad antimicrobial spectrum against tested reference strains and cultures isolated from food products. Gram-positive bacteria were more sensitive to silver nanoparticles than gram-negative bacteria (maximum diameters of inhibition zones were 14.8±0.1 mm and 12.0±0.2 mm, respectively). Silver nanoparticles synthesized using juniper berry extracts showed a higher antimicrobial activity against the foodborne cultures *B. cereus* (diameter of inhibition zones ranged from 14.0±0.1 mm to 14.8±0.1 mm) than against the reference culture *B. cereus* ATCC 11778. Silver nanoparticles synthesized using oak bark extracts demonstrated approximately 1.5 times stronger inhibition to reference culture of *B. cereus* ATCC 11778 than against the cultures of *B. cereus* isolated from food products.

Keywords: silver nanoparticles, plant extracts, antimicrobial activity, pathogenic bacteria.

Introduction. Nanotechnology is a new interdisciplinary field of science and technology, which develops, investigates and uses nanoparticles, nanocomposites or nanostructures in new products or technological processes (Miller, 2007). Unique optical, mechanical, catalytic properties of nanoparticles allow to use them in medicine, pharmacy, and food production or packaging. Chemical and physical technologies are used to synthesize nanoparticles, however, they are rather expensive, and their by-products and waste are toxic and harmful to the environment (Yu, 2007; Mallic et al., 2005; Liu & Lin, 2004; Smetana et al., 2005). Since physical, chemical and catalytic properties of nanoparticles are determined by their size, shape and the surrounding environment, the issues of nanoparticle stability and stabilization remain very relevant. As an alternative to chemical and physical nanoparticle technologies, scientists suggest adopting "green nanoparticle synthesis" by using various biological systems, such as yeasts, microfungi, bacteria, fruit and plant extracts (Kowshik et al., 2003; Senapati et al., 2005; Shahverdi et al., 2007). Plant extracts are the most commonly studied biological system for biosynthesis of metal nanoparticles (Narayanan & Sakthivel, 2008; Chandran et al., 2006; Dubey et al., 2010a). Nanoparticles have been synthesized using various plant extracts: curry tree (*Murraya koenigii*) (Philip et al., 2011a), mangosteen (*Mangosteen*) and Indian mango (*Mangifera indica*) leaves (Veerasamy et al., 2011; Philip, 2011b), common tansy (*Tansy*) fruits (Dubey et al., 2010b), *Jatropha curcas* (Bar et al., 2009), Ceylon cinnamon (*Cinnamomum zeylanicum*) leaves (Smitha et al., 2009), Chinese tea plant (*Camellia sinensis*) (Nestor et al., 2008), true aloe (*Aloe vera*) (Chandran et al., 2006), mushrooms (Philip, 2009c), honey (Philip, 2009d) and others. Silver nanoparticles, synthesized using biological materials, have a large surface area, are small in size and have high optical dispersion. Such synthesized particles may be used as spectrally selective coatings for solar panels, as optical sensors (Schultz et al., 2000), catalysts for chemical reactions, biomarkers and antibacterial agents. A large specific surface area of silver nanoparticles ensures maximum contact with the environment (Krutyakov et al., 2008).

Silver is an effective antimicrobial agent against bacteria, viruses and microfungi, although its antimicrobial mechanism has not been fully understood yet (Sharma et al., 2009). For more than a century colloidal solutions of silver nanoparticles have been produced using silver nitrate solutions. In Lithuania, there is lack of data in scientific literature on antimicrobial activity of silver nanoparticles synthesized by plant extracts against bacteria.

The antimicrobial activity of silver nanoparticles synthesized using oak bark and juniper berry extracts has been investigated in this research. Active substances of oak bark and juniper berries are known to have good antimicrobial properties (Aleknienė, 2013), so silver nanoparticles could be used to enhance these properties.

The aim of the study was to synthesize silver nanoparticles using aqueous extracts of oak bark and juniper berries, and silver nitrate, as well as to examine the antimicrobial activity of silver nanoparticles against certain pathogenic bacteria found in food products.

Materials and methods. Plant extracts were prepared from 10 g of oak bark and 5 g of juniper berries (*Dr. P. Karvelis'* therapy-phytotherapy enterprise, Šutoniai village, Lithuania) using 45 ml and 100 ml of distilled water respectively for extraction. Oak bark was heated for 2 h at 70 °C, juniper berries – for 90 min at 85 °C. Obtained extracts were filtered through a black-band filter (pore size 8-12 µm) (*Filtrak,* Germany). The synthesis of silver nanoparticles was carried out by adding 50 µl of 0.5 M $AgNO_3$ solution and various amounts of 10 % oak bark and juniper berry extracts to 10 ml of distilled water.

Optical properties of plant extracts with silver nanoparticles were evaluated using "Ocean Optics USB 4000 UV-VIS spectrometer" (*Ocean Optics*, Inc., USA), designed to measure the dependence of absorbance of solutions. Wavelength range of the spectometer was 200–900 nm and resolution – 1.5 nm.

Active acidity of primary extracts (control solutions) and extracts with synthesized silver nanoparticles was measured by "HI 98103Checker® pH Tester" pH meter (*Hanna instruments*, Inc., USA), with measurement error of ±0.2.

Antimicrobial activity of silver nanoparticles synthesized using plant extracts was detected by agar diffusion method, using cultures of reference strains (*Listeria monocytogenes* ATCC 19111, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923, *Salmonella enterica* subsp. *enterica* serovar *typhimurium* ATCC 13076, *Esherichia coli* ATCC 25922) and ten cultures of *B. cereus* isolated from food products. The cultures were stored in "Viabank" system (*Medical Wire & Equipment,* UK) at minus 72 °C. During the experiment, the cultures were grown at the optimal temperatures (30–37 °C) on agar slants, using PCA medium (Plate Count Agar, *Liofilchem,* Italy). The grown-up bacterial cultures were washed off from the agar with sterile saline solution, and the density of cell suspension of each culture was adjusted according to McFarland standard No 0.5. 1 ml of the prepared suspension was added to the medium, melted before and cooled to 45 °C, and was mixed thoroughly. The prepared mixture of bacteria cell suspension and the medium was poured into 90 mm Petri dishes, 10 ml each. After the medium had solidified and agar surface had dried, wells of 8 mm diameter were made in the plates and filled with 50 µl of the examined solution. Antimicrobial effect against the bacteria cultures was evaluated after 24 h of growth (at the optimal temperature) according to the diameter (in mm) of inhibition zones around the wells. If inhibition zones failed to form around the wells, the test solution had no antimicrobial effect against the tested bacteria culture.

B. cereus isolated from food products onto MYP agar (MYP Agar Base, *Oxoid,* UK) were identified using the pyrosequencing method. DNA of the bacteria were extracted automatically in the *QIAcube* machine according to the manufacturer's instructions, using *QiAmp DNA mini* (*Qiagen*, GmbH, Germany) reagent kit. DNA amplification was carried out using *BlackLight Sepsis* (*2BBlackbio,* Spain) kit (which includes three types of tubes containing necessary reagents and primers suitable for bacteria DNA amplification (V1, V2, V3)) according to the manufacturer's instructions. Sequencing of the amplified DNA was carried out by automatic *PyroMark Q24* (*Qiagen,* GmbH, Germany) machine, using *Gold24* reagent kit of nucleotide, enzyme and substrate mixtures (*Qiagen,* GmbH, Germany) according to the manufacturer's instructions.

To detect *B. cereus* enterotoxins, commercial BCET-RPLA kit (*Oxoid,* UK) was used. The cultures were prepared for testing according to the methodology specified in the manufacturer's instructions.

Statistical analysis of test findings was carried out using SPSS 16 package. Tukey's HSD test was used in order to determine the significant difference ($p<0.05$) amongst inhibition zone sizes against different microorganisms. The tests were repeated 3 times.

Results. Formation and stability of silver nanoparticles synthesized using different amounts of oak bark and juniper extracts in aqueous colloidal solution was confirmed using UV-VIS spectral analysis. The peaks of localized surface plasmonic resonance (LSPR) in UV-VIS spectrum (Figs. 1 and 2) confirmed biosynthesis of silver nanoparticles.

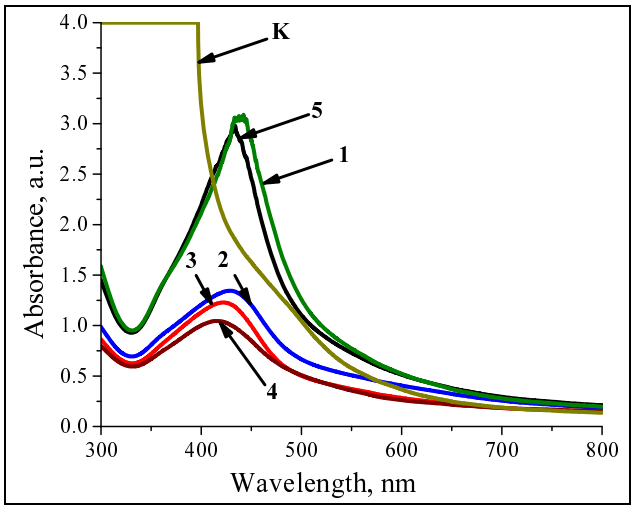

Fig. 1. **UV-VIS spectra of silver nanoparticles synthesized using oak bark extract, when the volume ratio of $AgNO_3$ and oak bark extract was:** 1 – 1:2, 2 – 1:1, 3 – 2:1, 4 – 10:3, 5 – 1:3, K – control (10 % of oak bark extract)

Absorption peaks of silver nanoparticles synthesized using oak bark extract solutions were obtained at wavelength of 420 nm to 442 nm. Maximum absorption peaks were characteristic of silver nanoparticles in samples 1 and 5. Absorption peaks of other samples were of lower intensity. Absorption peak of the control sample (10 % of oak bark extracts) was noticed at 395 nm wavelength. Based on the data of the UV-VIS spectrum, it was found that peak maximum of the LSPR of silver nanoparticles was dependent on the amount of oak bark extract. An increase in the amount of the extract by 3 times moved LSPR by 9 nm and the intensity of the LSPR

peak increased by 7.4 %. Reducing the amount of oak bark extract led the intensity of the LSPR to decrease: 2 and 4 times less amount of oak bark extract reduced LSPR intensity by 71.1 %, and 73.5 %, respectively.

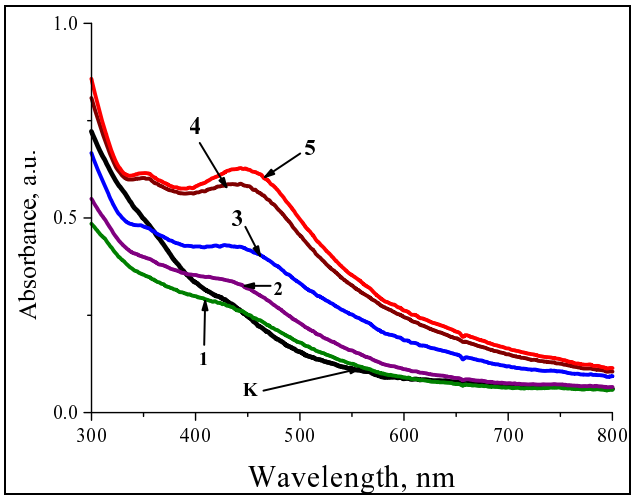

Fig. 2. **UV-VIS spectra of silver nanoparticles synthesized using juniper berry extract, when the volume ratio of $AgNO_3$ and juniper berry extract was:** 1 – 1:1, 2 – 1:2, 3 – 1:4, 4 – 1:6, 5 – 1:8, K – control (10 % of juniper berry extract)

Optical measurements of solutions of silver nanoparticles synthesized using juniper berry extract showed that absorption peaks were formed at wavelength of 442 nm to 456 nm. Peaks of the tested samples in UV-VIS spectrum were wide and according to Murray and Barnes (2007) silver nanoparticles were polydispersed. When the amount of the juniper berry extract was increasing, the intensity of LSPR peaks increased from 0.017 a.u. to 0.144 a.u. Since absorption is proportional to the concentration of the nanoparticles, it is concluded that the number of silver nanoparticles in a colloidal solution increases in proportion to the amount of juniper berry extract added. However, small silver nanoparticles aggregate into silver nanocomposites, and dipole LSPR are characteristic of them. Silver nanoparticles from samples 4 and 5 had two LSPR peaks in the UV-VIS spectrum (Fig. 2). They showed that small silver nanoparticles in these samples aggregated into silver nanorods.

Colloidal solutions of silver nanoparticles formed after reduction of silver ions, whereas, during the reduction the pH of the solutions decreases. Following the biosynthesis of silver nanoparticles using various amounts of plants extracts, active acidity (pH) of all examined samples was determined. The pH of the primary 10 % of oak bark extract was 5.11, whereas, the 0.5 M $AgNO_3$ solution – 6.5. The pH of silver nanoparticles solutions synthesized using different amounts of oak bark extract ranged from 4.17 to 3.65 (Fig. 3).

The decrease of active acidity with the increase in extract amount confirmed that silver ions in the plant extracts were reduced and silver nanoparticles were formed. After repeated pH measurements of all solutions several days later, pH decrease did not exceed 0.2 measurement units. It confirmed that the silver nanoparticle solutions were stable.

Following the synthesis of silver nanoparticles in oak bark and juniper berry extracts, antimicrobial properties of these nanoparticles against gram-positive and gram-negative reference cultures, and food-borne enterotoxic and non-enterotoxic *B. cereus* were investigated. All presumtive *B. cereus* isolated from food products onto MYP agar were identified according to 16S DNA gene sequences as *B. cereus* species. 5 cultures, grown at 37 °C in a brain-heart infusion broth, produced diarrheal enterotoxins, the remaining cultures did not produce.

Control (10 % extracts of oak bark and juniper berries) used for the synthesis of silver nanoparticles was antimicrobial inactive against the tested bacterial cultures. Aqueous $AgNO_3$ solution, obtained by adding 50 µl 0.5 M $AgNO_3$ to 10 ml distilled water, also had no inhibitory effect against the tested bacterial cultures.

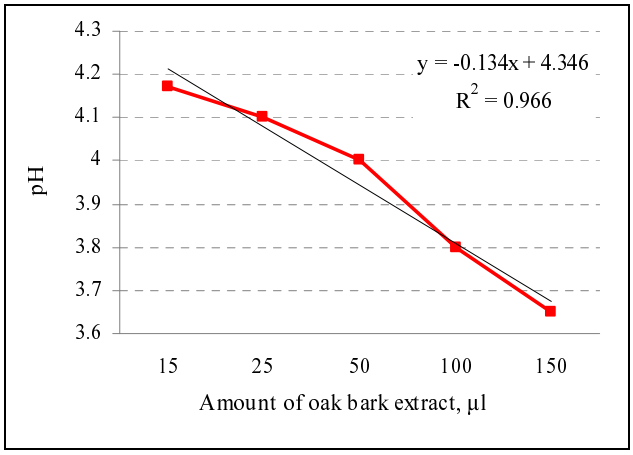

Fig. 3. **The changes of the active acidity of solutions of silver nanoparticles synthesized using different amounts of oak bark extract**

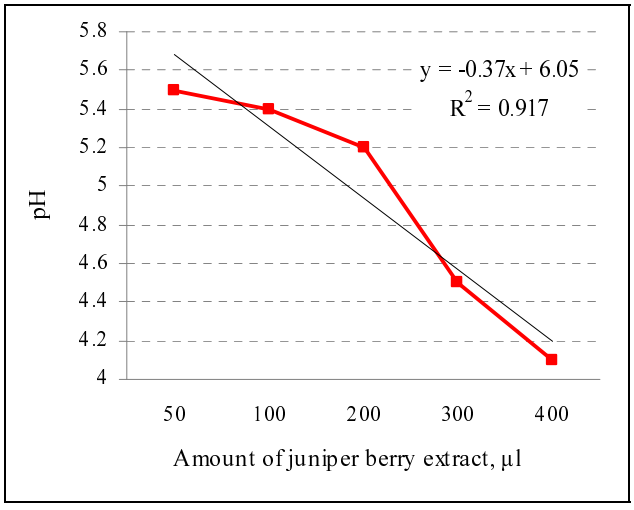

Fig. 4. **The changes of the active acidity of solutions of silver nanoparticles synthesized using different amounts of juniper berry extract**

The decrease of active acidity of solutions of silver nanoparticles synthesized using various amounts of juniper berry extract is provided in Fig. 4. The pH of the primary 10 % of juniper berry extract was 6.22.

LSPR peak maximums in UV-VIS spectra of silver nanoparticles (Fig. 2) were obtained when $AgNO_3$ and oak bark extract volume ratios were 1:2 and 1:3 (samples 1 and 5), therefore, only these solutions were used to detect antimicrobial activity against the bacterial cultures (Table 1).

Table 1. **Antimicrobial activity of silver nanoparticles synthesized using different amounts of oak bark extract against tested bacterial cultures**

Target bacteria	Diameter of inhibition zones, mm		
	Sample variant		
	K*	1	5
E. coli ATCC 25922	0.00±0.00	10.00±0.10	11.00±0.10
S. aureus ATCC 25923	0.00±0.00	11.00±0.05	12.00±0.10
S. typhimurium ATCC 13076	0.00±0.00	9.00±0.10	11.00±0.05
L. monocytogenes ATCC 19111	0.00±0.00	12.00±0.10	12.00±0.10
B. cereus ATCC 11778	0.00±0.00	14.00±0.10	14.00±0.10
Enterotoxic *B. cereus*	0.00±0.00	10.90±0.10	11.20±0.05
Non-enterotoxic *B. cereus*	0.00±0.00	10.40±0.10	10.70±0.15

K* – control solution (10 % of oak bark extract)

Inhibitory effect of silver nanoparticles against reference cultures of *L. monocytogenes* and *B. cereus* was not dependent on the amount of oak bark extract. The findings showed that the silver nanoparticle solution, in which $AgNO_3$ and oak bark extract volume ratio was 1:3 (sample 5), was more antimicrobially active ($p<0.05$) against reference strains of *E. coli*, *S. aureus* and *S. typhimurium*. Meanwhile, when silver nanoparticle concentrations in the oak bark extract varied, inhibition of the growth of foodborne enterotoxic and non-enterotoxic *B. cereus* cultures had no statistically significant differences.

The 10 % control juniper berry extract had no effect against the tested bacterial cultures, meanwhile, solutions of silver nanoparticles synthesized using this plant extract inhibited the growth of all reference strains, as well as foodborne *B. cereus* cultures (Table 2).

Table 2. **Antimicrobial activity of silver nanoparticles synthesized using different amounts of juniper berry extract against tested bacterial cultures**

Target bacteria	Diameter of inhibition zones, mm					
	Sample variant					
	K*	1	2	3	4	5
E. coli ATCC 25922	0.00±0.00	11.00±0.02	12.00±0.05	10.50±0.08	11.00±0.10	11.00±0.05
S. aureus ATCC 25923	0.00±0.00	13.00±0.50	12.00±0.82	13.00±0.60	13.00±0.40	12.50±0.15
S. typhimurium ATCC 13076	0.00±0.00	11.00± 0.20	11.00±0.51	11.00±0.55	11.50±0.20	12.00±0.20
L. monocytogenes ATCC 19111	0.00±0.00	14.00± 0.70	14.00±0.40	14.00±0.30	15.00±0.20	14.50±0.20
B. cereus ATCC 11778	0.00±0.00	11.00±0.10	9.50±0.15	9.00±0.10	9.00±0.05	9.00±0.05
Enterotoxic *B. cereus*	0.00±0.00	14.00±0.10	14.20±0.10	14.30±0.10	14.40±0.10	14.60±0.10
Non-enterotoxic *B. cereus*	0.00±0.00	14.00±0.20	14.20±0.10	14.40±0.12	14.60±0.15	14.80±0.10

K* – control solution (10 % of juniper berry extract)

It was found that silver nanoparticles synthesized using various amounts of juniper berry extracts were the most effective in inhibiting *L. monocytogenes* and *S. aureus* reference strains ($p<0.05$). *B. cereus* cultures isolated from food products were statistically more sensitive – silver nanoparticle solutions had an approximately 1.5 stronger inhibitory effect against these cultures, as compared to the reference *B. cereus* strain

culture. No statistically significant differences of inhibitory effectiveness ($p>0.05$) were found between enterotoxic and non-enterotoxic *B. cereus* isolated from food products, when comparing the effects of the same silver nanoparticles synthesized using juniper berry extracts solutions against these cultures. No pattern was found to show that the effect of silver nanoparticles synthesized using various amounts of juniper berry extract on the cultures depended on the active acidity of the solutions. Optimum inhibitory effects of silver nanoparticles varied against different cultures.

Discussion. Reduction of silver nanoparticles in oak bark extracts is most likely to occur due to tannins present in the extract. Although tannin acid is a weak reducer and forms germs of nanoparticles, the reduction of silver ions may be possible because of products of tannic acid hydrolysis – glucose, gallic acid (Puišo et al., 2012a). Previous studies have showed that tannic acid does not act as a stabilizer of silver nanoparticles (Mačionienė et al., 2012), therefore, silver nanoparticles are most likely stabilized by the components present in the oak bark.

Juniper berries contain more than 30 % of carbohydrates, 10 % of resin, essential oils, various acids, flavonoids and other substances. Silver nanoparticles formed in a juniper berry extracts were stabilized by chemical compounds of essential oils and fatty acids. Previous studies have found that when using 10 % juniper berry extract dissolved in ethanol for synthesis, silver nanoparticles do not form in the solution (Puišo et al., 2012b). It confirms that silver ions in the juniper berry extracts are reduced by water soluble chemical compounds present in juniper berries.

The effect of silver nanoparticles in plant extracts against gram-positive (*S. aureus*, *L. monocytogenes*, *B. cereus*) and gram-negative (*E. coli*, *S. typhimurium*) reference strains and foodborne *B. cereus* cultures had a wide spectrum of antimicrobial activity. Literature provides conflicting statements on the effect of nanoparticles on gram-positive and gram-negative bacteria. Shrivastava et al. (2007) have discovered that gram-negative bacteria are more resistant to the effect of silver nanoparticles. On the other hand, Kong and Jang (2008) and Peticae et al. (2008) have proved that silver nanoparticles have equal inhibitory effect against both gram-positive and gram-negative strains. Gram-positive bacteria should be more sensitive to the effect of antimicrobial agents due to the fact that their cell walls have a different structure. The cell walls of gram-positive bacteria have a thick layer of peptidoglycan, consisting of linear polysaccharides chains cross-linked by short peptides thus forming rigid structure. The gram-negative bacteria cell wall is much more complex. It consists of a rigid peptidoglycan layer that is much thinner than that found in the gram-positive cells, overlaid by an outer membrane containing a diversity of structures. Therefore, it is harder for nanoparticles to penetrate through the layer of membrane of gram-negative bacteria (Shrivastava et al., 2007). During the study gram-positive bacteria were sensitive to the effect of silver nanoparticles synthesized using various plant extracts.

According to literary data, active agents present in oak bark and juniper berries have good antimicrobial properties, however, the study showed that 10 % control plant extracts had no effect on the examined reference strains and *B. cereus* cultures isolated from food, while, silver nanoparticle solutions had an inhibitory effect. *B. cereus* cultures isolated from food products were statistically more resistant to silver nanoparticles synthesized using oak bark extract than the reference *B. cereus* strain culture. McMahon et al. (2007) have found that the sensitivity of pathogens to antimicrobial agents decreases because of the effect of stressors (low pH, high NaCl concentration) in food. It might have led to lower sensitivity of *B. cereus* cultures isolated from food products to silver nanoparticle solutions.

Antimicrobial effect of various extracts with silver nanoparticles has been widely investigated by scientists. Studies most often use *E. coli*, *S. aureus*, *P. aeruginosa* of clinical origin (Bozanica et al., 2011; Jain et al., 2009; Koraa et al., 2010; Veerasamy et al., 2011). A study has been conducted to investigate the antimicrobial activity of acalypha (*Acalypha indica*) leaf extract with silver nanoparticles against *E. coli*. It has been found that the minimum inhibitory concentration (MIC) against these gram-negative bacteria was 10 µg/ml, whereas, MIC of $AgNO_3$, which was added to the primary extract for nanoparticle synthesis, against *E. coli* was 20 µg/ml. Lower MIC of silver nanoparticles as compared to $AgNO_3$ may exist because of smaller size of the nanoparticles (Schultz, 2000). Prema and Roju (2009) have detected antibacterial effect of silver nanoparticles (reduced by sodium citrate) against *Shigella* and *Bacillus* sp. The scientists concluded that the inhibitory effect of silver nanoparticles depends on the concentration and size of the nanoparticles, as well as on the initial number of bacteria examined: the reduction in the number of viable cells and the increase in the number of nanoparticles in solutions are correlated (Suriya et al., 2011).

The high bactericidal activity is certainly due to the silver cations released from Ag nanoparticles that act as reservoirs for the Ag+ bactericidal agent. Big changes in the membrane structure of bacteria as a result of the interaction with silver cations lead to the increased membrane permeability of the bacteria (Dibrov et al., 2002; Sondi et al., 2004).

The data on the antimicrobial effect of silver nanoparticles on certain cultures have shown that these nanoparticles could be used in practice to inhibit the growth of microorganisms. For example, using silver nanoparticles in film production could lead to the creation of antimicrobial packaging for food products.

Conclusions

1. The formation of silver nanoparticles was confirmed using UV-VIS spectral analysis. Peak maximums of localized surface plasmonic resonance of silver nanoparticles were obtained at wavelength of 420 nm to 442 nm, when the volume ratios of 0.5 M $AgNO_3$ and 10 % of oak bark extract were 1:2 and 1:3. Peaks of dipole localized surface plasmonic resonance of silver nanoparticles were obtained at 442 nm to 456 nm

wavelengths, when the volume ratios of 0.5 M $AgNO_3$ and 10 % of juniper berry extract were 1:6 and 1:8.

2. Silver nanoparticles synthesized using oak bark and juniper berry extracts had an antimicrobial effect against all *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 19111, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* serovar *typhimurium* ATCC 13076 reference strains and *B. cereus* cultures isolates from food products. Gram-positive bacteria were more sensitive to the effect of silver nanoparticles synthesized in plant extracts than gram-negative ones (maximum diameters of inhibition zones were 14.8±0.1 mm and 12.0±0.2 mm, respectively).

3. Silver nanoparticles synthesized using oak bark extract, with the volume ratio of 0.5 M $AgNO_3$ and 10 % of oak bark extract of 1:3, were more antimicrobially active against reference strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Salmonella enterica* subsp. *enterica* serovar *typhimurium* ATCC 13076 than when the ratio of the silver nitrate and the extract was 1:2.

4. Silver nanoparticles synthesized using juniper berry extract were more antimicrobially active against foodborne *B. cereus* cultures (the diameter of the inhibition zone varied from 14.0±0.1 mm to 14.8±0.1 mm) than against the culture of reference strain *B. cereus* ATCC 11778; silver nanoparticles synthesized using oak bark extract had an approximately 1.5 stronger inhibitory effect against the culture of reference strain *B. cereus* ATCC 11778 than against *B. cereus* cultures isolated from food products.

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