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GREEN SYNTHESIS OS SILVER NANOPARTICLES USING METHANOLIC EXTRACTS OF GENTIANA SEPTEMFIDA, ERYSIMUM CONTRACTUM AND CHELIDONIUM MAJUS AND EVALUATION OF ANTIBACTERIAL, ANTIFUNGAL AND CYTOTOXIC ACTIVITIES OF OBTAINED AGNPS

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ვერცხლის ნანონაწილაკების მწვანე სინთეზი Gentiana Sepemfida-ს, Erysimum Contractum-ისა და Chelidonium majus-ის მეტანოლური ექსტრაქტების გამოყენებით და მიღებული AGNP- ების ანტიბაქტერიული, სოკოსსაწინააღმდეგო და ციტოტოქსიური მოქმედებების შეფასება

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რეზიუმე

მეტალის ნანონაწილაკების ბიოსინთეზი მარტივი, ეფექტური, ეკოლოგიურად სუფთა და იაფი გზაა. ეს არატოქსიკური მეთოდია, ბიოსინთეზირებული ნანონაწილაკები ძირითადად ბიოთავსებადია და მათი გამოყენება შესაძლებელია ბიოსამედიცინო საშუალებებში. ცნობილია, რომ ვერცხლის ნანონაწილაკებს (AgNPs) გააჩნიათ ძლიერი ანტიბაქტერიული მოქმედება პათოგენური ბაქტერიების წინააღმდეგ და ავლენენ ანტიფუნგალურ, ანტივირუსულ აქტივობას, ამიტომ ისინი შეიძლება სასარგებლო იყოს არა მხოლოდ ბაქტერიების წინააღმდეგ საბრძოლველად.

მოცემულ კვლევაში ვერცხლის ნანონაწილაკების ბიოსინთეზი წარიმართა Gentiana septemfida-ს, Erysimum contractum-ისა და Chelidonium majus-ის მეთანოლიანი ექსტრაქტების გამოყენებით. მიღებული ნანონაწილაკების ანტიბაქტერიული და ფუნგიციდური აქტივობა შეფასდა გრამუარყოფითი Escherichia coli, გრამდადებითი Staphylococcus aureus და Candida albicans წინააღმდეგ. ასევე, მათი ციტოტოქსიკურობა შეფასდა ადამიანის ფილტვის კარცინომის (A-549), მსხვილი ნაწლავის ადენოკარცინომის (DLD-1) და ჯანმრთელი ადამიანის კანის ფიბრობლასტების (WS1) უჯრედულ ხაზებზე.

შედეგებმა აჩვენა ანტიბაქტერიული, სოკოს საწინააღმდეგო და ციტოტოქსიკური ეფექტები, სხვადასხვა ხარისხით თითოეული ტიპისთვის. ვერცხლის ნანონაწილაკების ბიოსინთეზი მარტივი, ეკონომიური, ეკოლოგიურად სუფთა მეთოდია და მიღებულ ნანონაწილაკებს აქვთ ანტიბაქტერიულ, სოკოს საწინააღმდეგო და ციტოტოქსიკურ აგენტებად გამოყენების პოტენციალი.

Introduction.

According to modern data, the most promising approach to the synthesis of silver nanoparticles is so-called Green Synthesis. Research around this topic focuses on the development of an efficient and environmentally friendly method for the synthesis of metal nanoparticles using green chemistry. The green approach of the synthesis of silver nanoparticles has several advantages over chemical and physical methods [11]. First of all, it is very simple, effective, eco-friendly and inexpensive way because we use bio-resources that can be used as a reducing, as well as stabilizing and caping agent [18], doesn't need to provide additional means for this purpose. Does not require high temperature and pressure. It is a non-toxic method due to small or zero consumption of hazardous materials on the surface of nanomaterials. The process is characterized by low energy costs [19]. In addition, biosynthesized nanoparticles are primarily biocompatible and can be used in biomedical applications [4,14]. In scientific literature biosynthesis of silver nanoparticles is described using plants, [2] bacteria, [8,9] and algae [5].

The ability of pathogenic bacteria to form biofilms, which are complexly organized communities of bacteria attached to various surfaces and surrounded by a matrix made up of extracellular polysaccharides, proteins, nucleic acids, and other macromolecules synthesized by bacteria, is a serious

medical problem [12,17]. Biofilms can contain a variety of bacteria, fungus, and protozoa in addition to the microorganisms of one species. Biofilms are now widely acknowledged as the main form of bacterial survival in natural environment. Resistance to medications is several times (10–1000 times) stronger in bacteria living in biofilms than in bacteria developing as plankton, i.e., unattached microorganisms. (Jamal et al. 2018).

Biofilms can be found on a wide range of medical equipment and implants, having a huge impact on medicine. Novel alternative chemicals or techniques are urgently needed due to the lack of efficient anti-biofilm antibiotics. Silver nanoparticles (AgNPs) are known to have antibacterial effects against pathogenic bacteria and also against bacteria exhibiting resistance against antibiotics, sometimes having synergistic action in complex with antibiotics [10]. Besides, it has to be mentioned that AgNPs have shown antifungal activities and can be helpful not only for fighting against bacteria. [1,21] Modern data suggest that they can be used to develop effective remedies against biofilms containing various types of micro-organisms [13]. To obtain silver nanoparticles biosynthesis using plant extracts is the most economical and promising method. Plants contain various compounds that serve as the reducing agent of metal cations including silver [20]. Such AgNPs can be used in medicine for antimicrobial and antitumor therapy [22]. Also, they have found to have antiviral effect against SARS-CoV-2 virus [3,6].

In given study silver nanoparticles were biosynthesized using methanolic extracts of crested gentian (Gentiana septemfida), Erysimum contractum and the greater celandine (Chelidonium majus). Obtained nanoparticles were evaluated for antibacterial and fungicidal activities against Gram-negative Escherichia coli (ATCC 25922), Gram-positive Staphylococcus aureus (ATCC 25923) and Candida albicans (ATCC 10231). Also, their cytotoxic effects were tested on human lung carcinoma A-549 (ATCC #CCL-185), colon adenocarcinoma DLD-1 (ATCC #CCL-221) and healthy human skin fibroblasts WS1 (ATCC CRL-1502) cell lines

Materials and Methods

Plant extracts used for biosynthesis of silver nanoparticles

Gentiana septemfida, Erysimum contractum and *Chelidonium majus* were collected in Georgia and authenticated by T. V. Oproshanskaya from Botany departments at National University of Pharmacy, Kharkiv. This paper presents the results of experiments involving the methanolic extracts prepared from aerial parts of above listed plants. These extracts contributed to the efficient formation of AgNPs during the reduction of silver cations from silver nitrate. Extracts were obtained by maceration of dry plant shoots in pure methanol, with ration 1:9 for 24 hours. Afterwards the plant leftover was removed and extracts were filtered.

Phytochemistry of plant extracts

For determination of phytochemical content of prepared plant extracts the gas chromatography, LC/MS/MS and HPLC Analysis were conducted. (At the Toxicology and Chemical Expertise laboratory of Levan Samkharauli Court Expertise National Bureau, Georgia) in the following conditions. Tandem chromate-mass spectrometry – device: Agilent Technologies 7000 GC/MS/MS Triple Quad; column - Elite 5-MS; 30MX250 μm X 0, 25 μm; furnace temperature: 600C-3100C (program regime); injector temperature - 2500C; transfer line temperature - 3100C; airborne - helium 1ml/m; ionization source El-70 ev; scanning regime TIC. For the purpose of identification of the target substance in the object under study, mass spectrums of the peaks existing on chromatographs were compared with the mass spectrums of the substances existing in the database (NIST 2016).

Sample Preparation for LC/MS/MS and HPLC Analysis: The methanolic extracts were filtered through a 0.45 μm nylon filter. A 10 μl volume extract was injected into the HPLC column for analysis by LC-DAD-MS/MS. Method was performed by using an Agilent technologies 1290 Infinity LC system consisting a DAD and coupled to Agilent technologies 6460 Triple quadrupole LC/MS. The column was a 200 mm X 4 mm 3 μm particle size Zorbax Eclipse C18, maintained at 350C and protected with a UHPLC GUARD Zorbax Eclipse column of the same material.

Biosynthesis of Silver Nanoparticles

100 ml of each previously prepared extracts were placed into a 250 ml volume flask and placed on magnetic stirrer. Equal amount of 7mM silver nitrate solution was added slowly under continuous stirring

and the flasks were left covered with lid on stirrers for 1h. For further synthesis of silver nanoparticles (AgNPs) the mixtures were then left in dark place, at 21^{O}C for 24 hours. The mixture was afterwards centrifuged at 13,000 rpm for 10 min to remove methanolic extracts together with supernatant. The precipitate containing nanoparticles was diluted in distilled water and the process was repeated two times to obtain purified AgNPs. To analyze the characteristics and biological activities of synthesized nanoparticles further assays were performed.

UV-vis Spectroscopy

The biosynthesis of silver nanoparticles was detected using i9 UV-VIS spectrophotometer (Hanon Instruments). This method is based on detection of plasmon resonance which is characteristic for AgNPs in the range of specific wavelength, which mostly varies between 380-450nm [16]. Absorbance of prepared samples was scanned in the wavelength range of 200-500 nm.

Bacterial strains, growing media and conditions

The in vitro antimicrobial activity of silver nanoparticles was tested against gram-negative Escherichia coli (ATCC 25922), gram-positive Staphylococcus aureus (ATCC 25923). Bacterial strains were provided by the Chicoutimi Hospital, Saguenay, Canada.

Bacteria were stored at -80^{0}C until use. For culturing, all bacteria were placed in a nutrient broth base (Difco) for 16–18 h at 37^{0}C; The cellular density of the inoculum was measured via optical density, measured at 600 nm for E. coli (Pal et al. 2007), 660 nm for S. aureus (Kaatz et al. 2000), using a Multiskan™ GO Spectrophotometer (Thermo Fisher Scientific). Based on the results the inoculum was re-diluted in the nutrient broth to obtain the required bacterial concentration.

Cell cultures

The human lung carcinoma A-549 (ATCC #CCL-185), colon adenocarcinoma DLD-1 (ATCC #CCL-221) and healthy human WS1 (ATCC CRL-1502) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, USA). The A-549, DLD-1, cell lines were grown in Minimum Essential Medium with Earle's salts, while WS1 cell line was grown in Dulbecco's modified Eagle's medium (Mediatech Cellgro®, Herndon, USA). Both Media were supplemented with 10% fetal calf serum (Hyclone, Logan, USA), solution of vitamins (1X), sodium pyruvate (1X), non-essential amino acids (1X), penicillin (100 IU) and streptomycin (100 g/ml) (Mediatech Cellgro®). Cells were cultured in a humidified atmosphere at 37 ºC in 5% CO2.

Evaluation of Antibacterial and Antifungal Activities

The antibacterial and antifungal activity assays of biosynthesized silver nanoparticles were performed using described method /17/. Activities were evaluated using a modified microdilution method. Exponentially growing bacteria were plated in 96-well flat-bottom microplates (Greiner, Bio-one) at a density of 5× 103 Gram-negative Escherichia coli (ATCC 25922) or 3.5 × 104 Gram-positive Staphylococcus aureus (ATCC 25923) per well in 100 µL of nutrient broth (Difco) or 2 × 103 Candida albicans (ATCC 10231) per well in 100 µL of Sabouraud dextrose (Difco). Increasing concentrations of compounds solubilized in Biotech DMSO, then diluted in nutrient broth or Sabouraud dextrose were then added (100 µL per well). The final concentration of DMSO in the culture medium was maintained at 0.1% (volume/volume) to avoid solvent toxicity. The plates were incubated for 24 h at 37 °C. Absorbance was read using a Varioskan Ascent plate reader (Thermo Electron) at 600 nm for bacteria and 540 nm for yeasts. Gentamycin and Amphotericin B were used as control. The MIC50 is determined as the lowest concentration of silver nanoparticles resulting in 50% inhibition of bacterial and fungal growth and MIC90 for the lowest concentration of AgNPs resulting in 90% inhibition.

Cytotoxicity assay

Exponentially growing cells were plated at a density of 5 x 103 cells per well in 96-well microplates (Costar, Corning inc.) in 100 µl of culture medium and were allowed to adhere for 16 hours before treatment. Then, 100 µl of increasing concentrations of silver nanoparticles mixed with DMSO (Sigma-Aldrich) were added. The final concentration of solvent in the culture medium was maintained at 0.5% (volume/volume) to avoid solvent toxicity. The cells were incubated for 48 h. Cytotoxicity was assessed using the resazurin reduction test as described by O'Brien [15] and Hoechst method. Fluorescence was measured on an automated 96-well Fluoroskan Ascent Fl plate reader (Labsystems) using an excitation

wavelength of 530 nm and an emission wavelength of 590 nm. Cytotoxicity was expressed as the concentration of extract or compound inhibiting cell growth by 50% (IC50). Etoposide was used as control in cytotoxicity assay.

Results and discussion

Using GC/MS detection following compounds were identified:

***Gentiana septemfida* methanolic extract:** Terpinene 4-acetate 5.42, trans-p-Mentha-2,8-dienol 5.94, Benzoic acid 6.15, Camphor 6.2, Geranyl isovalerate 6.47, D-Verbenone 6.77, Decylic Alcohol 7.0, alfa.-Copaene 7.85, β-copaene 7.91, α-acorenol 8.08, Caryophyllene 8.17, Palustrol 8.42, β-Lactose 9.84

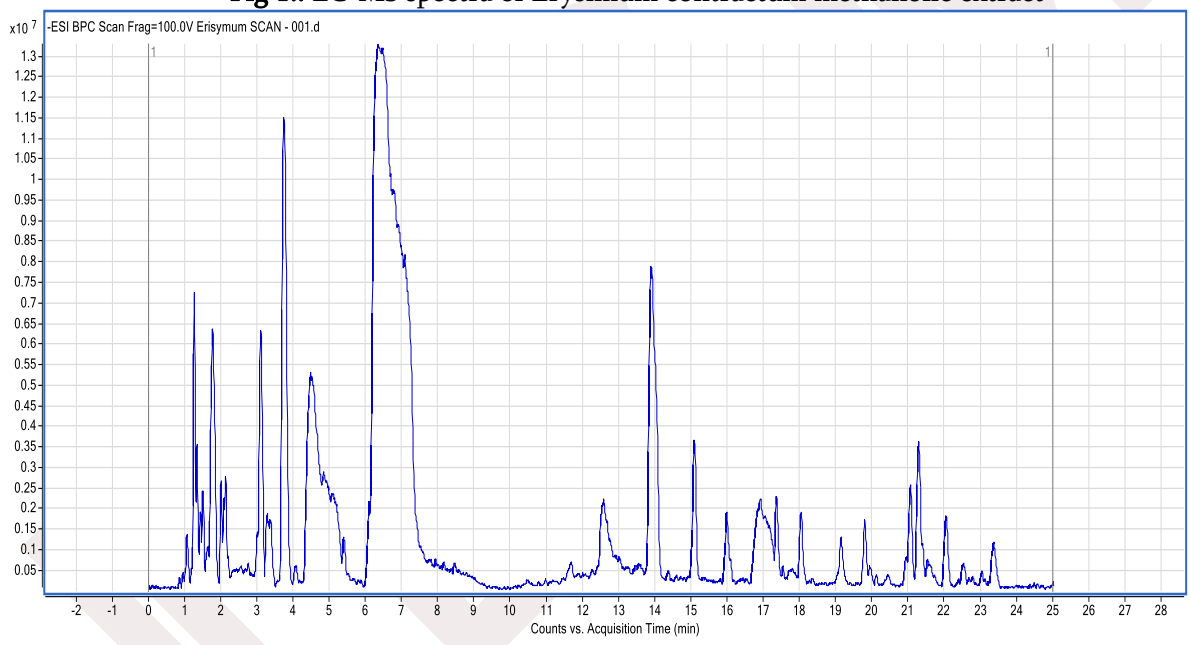
***Erysimum contractum* methanolic extract:** Vanillin lactoside 7.93, alfa-Copaene 7.85, Caryophyllene, Oleic Acid 11.49,

***Chelidonium majus* methanolic extract:** Caryophyllene, α-Himachalene, α-Yalangene, lavandulyl acetate, Decyl alcohol, D-(+)-Carvone, Linalyl acetate (Bergamiol), D-Verbenone, . β-Linalool, β-copaene, Camphor, Dihydroactinidiolide, (±)-Stylopine,

Polyphenolic compounds of *Erysimum contractum* identified with LC-MS/MS:

(609-301)- Rutine, (447-301)- Querc-3-O-ramn, (353-173)-4-CQA(4-O-caffeoylquinic acid), (305-225)-Gallocatechin, (301-151)- quercetin, (269-151)-Apigenine, (255-213)-Pinocembrin, (253-209)- Chrysin, (179-161)- Caffeic acid, (167-123)-Vanillic acid, (153-109)-Protocatechuic acis.

Fig 1.: LC-MS spectra of Erysimum contractum methanolic extract



Alkaloids identified in *Chelidonium majus*:

Allocryptpine, Berberine, Palmatine, Sangvinarine, Chelidonine, Protopine, Coptisine, Tetrahydrocoptisine, Tetrahydroberberine, Cheleritrine

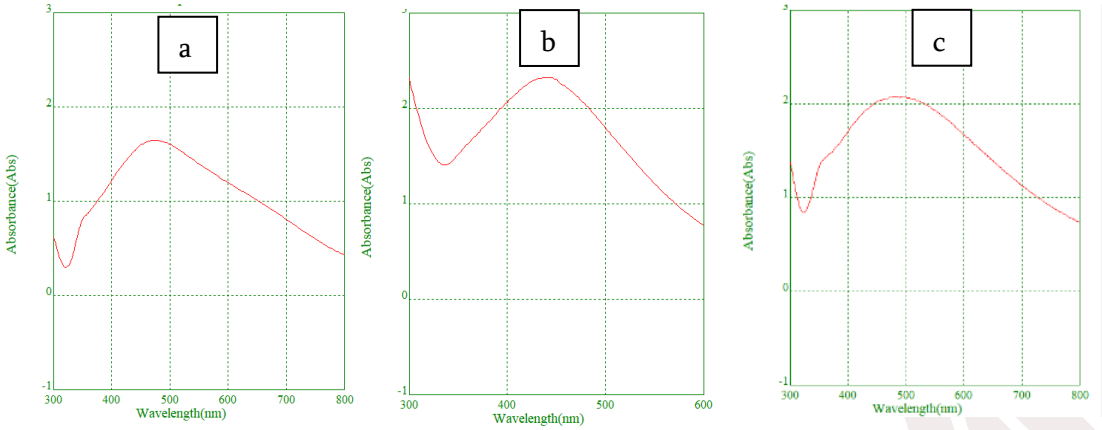
Biosynthesis of AgNPs

The first sign of biosynthesis of silver nanoparticles was the rapid change of color while the $AgNO_3$ solution was added to the extracts. Obtained brownish color became darker after passing 24 hours. It indicates that silver ions present in $AgNO_3$ solution were reduced.

UV-vis Spectroscopy

Recorded UV-Vis spectra proved formation of silver nanoparticles in all of tested samples. For silver nanoparticles biosynthesized by G.septemfida methanolic extract (G-AgNPs) absorption peak was shown at 470 nm. In case of silver nanoparticles biosynthesized by E.contractum methanolic extract (E-AgNPs) absorption peak was recorded at 445nm and for silver nanoparticles biosynthesized by C. majus methanolic extract (C-AgNPs) absorption peak was recorded at 490 nm. These absorption peaks are characteristic for plasmon resonance which occurs in silver nanoparticles and thus they serve as evidence for successful biosynthesis.

Fig 2.: Characteristic plasmon resonance detected by UV-vis Spectroscopy
(a: G-AgNPs; b: E-AgNPs; c: C-AgNPs)



Antibacterial and Antifungal Activity

The results of antibacterial and antifungal activity assay showed that biosynthesized silver nanoparticles have good antibacterial action on E. coli and S. aureus, which is extremely high in case of silver nanoparticles biosynthesized by the extract of Erysimum contractum, which also exposed good inhibition again C. albicans at low concentration. The results are shown in the table 1.

Table 1. Results of antibacterial and antifungal activity assay of silver nanoparticles: G-AgNPs for biosynthesized by extract of Gentiana septemfida; E-AgNPs for Erysimum contractum and C-AgNPs for Chelidonium majus.

Sample	IC50 (µg/ml) E. coli	IC50 (µg/ml) S. aureus	IC50 (µg/ml) C.albicans	IC90 (µg/ml) E. coli	IC90 (µg/ml) S. aureus	IC90 (µg/ml) C.albicans
G-AgNPs	1,96 ± 0,07	5,5 ± 0,2	192 ± 1	3,4 ± 0,1	5,8 ± 0,2	198 ± 0,4
E-AgNPs	<1,563	2,02 ± 0,08	85,1 ± 0,5	<1,563	2,6 ± 0,1	90,5 ± 0,5
C-AgNPs	7,4 ± 0,8	22,7 ± 0,6	>200	15 ± 2	23,7 ± 0,4	>200
Gentamycin	0,016 ± 0,001	0,047 ± 0,008		0,045 ± 0,004	0,07 ± 0,01	
AmphotericinB			0,41 ± 0,06			0,55 ± 0,08

Cytotoxicity assay

The cytotoxicity assay of biosynthesized silver nanoparticles against human lung carcinoma A-549 (ATCC #CCL-185), colon adenocarcinoma DLD-1 (ATCC #CCL-221) and healthy human WS1 (ATCC CRL-1502) cell lines showed that among AgNPs obtained from three different plant methanolic extracts the ones biosynthesized by Erysimum contractum has strong cytotoxic action, highest of all and in the case of A-549 cells the inhibition occurs at lot lower concentration than with Etoposide. Detailed results are shown in the table 2.

Table 2. Results of cytotoxicity assay of silver nanoparticles: G-AgNPs for biosynthesized by extract of Gentiana septemfida; E-AgNPs for Erysimum contractum and C-AgNPs for Chelidonium majus.

Sample	Resazurine A-549	Resazurine DLD-1	Resazurine WS-1	Hoechst A-549	Hoechst DLD-1	Hoechst WS-1
G-AgNPs	79 ± 15 µg/ml	30 ± 2 µg/ml	27,3 ± 0,3 µg/ml	52 ± 14 µg/ml	28 ± 2 µg/ml	30 ± 4 µg/ml
E-AgNPs	16 ± 2 µg/ml	18 ± 3 µg/ml	16 ± 2 µg/ml	9,5 ± 0,5 µg/ml	17 ± 2 µg/ml	16 ± 2 µg/ml
C-AgNPs	>200 µg/ml	90 ± 2 µg/ml	95,7 ± 0,2 µg/ml	85 ± 18 µg/ml	73 ± 2 µg/ml	74 ± 3 µg/ml
Etoposide	27 ± 14 µM	14 ± 2 µM	3,7 ± 0,8 µM	2,0 ± 0,3 µM	1,7 ± 0,3 µM	0,5 ± 0,1 µM

Cytotoxic activity study of Erysimum contractum methanolic extract can be found in scientific literature [7]. The extract itself has certain cytotoxic effect on the same cell lines used in given research, which is probably due to alkaloids found in it. It might be suggested that residuals of methanolic extract on silver nanoparticles causes the effects given below but comparison of results shows that AgNPs have much stronger cytotoxic action against DLD-1 and WS-1 cell lines. On one hand is proves that biosynthesized silver nanoparticles have strong cytotoxic action but on the other hand the fact that healthy human fibroblasts (WS-1) are affected can be marked as disadvantage of AgNPs.

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GREEN SYNTHESIS OS SILVER NANOPARTICLES USING METHANOLIC EXTRACTS OF GENTIANA SEPTEMFIDA, ERYSIMUM CONTRACTUM AND CHELIDONIUM MAJUS AND EVALUATION OF ANTIBACTERIAL, ANTIFUNGAL AND CYTOTOXIC ACTIVITIES OF OBTAINED AGNPS

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SUMMARY

Biosynthesis of metallic nanoparticles is simple, effective, eco-friendly and inexpensive way. It is a non-toxic method. Biosynthesized nanoparticles are primarily biocompatible and can be used in biomedical applications. Silver nanoparticles (AgNPs) are known to have strong antibacterial effects against pathogenic bacteria and have shown antifungal, antiviral activities so they can be helpful not only for fighting against bacteria. In given study silver nanoparticles were biosynthesized using methanolic extracts of *Gentiana septemfida, Erysimum contractum* and *Chelidonium majus*. Obtained nanoparticles were evaluated for antibacterial and fungicidal activities against Gram-negative Escherichia coli, Gram-positive Staphylococcus aureus and Candida albicans. Also, their cytotoxic effects were evaluated on human lung carcinoma A-549, colon adenocarcinoma DLD-1, and healthy human skin fibroblasts WS1 cell lines. The results show antibacterial, antifungal and cytotoxic effects at different level for each type. Biosynthesis of silver nanoparticles is simple, cost effective, eco-friendly method and obtained nanoparticles have potential to be used as antibacterial, antifungal and cytotoxic agents.

Keywords: Silver nanoparticles, Eco-friendly, E. contractrum, Ch. Majus, G. septemfida