VOL.3 NO.2September 2014 - ISSN 2233 – 1859

Operations Research Society in Bosnia and Herzegovina

Southeast Europe Journal of Soft Computing

Available online: www.scjournal.com.ba

IUS Soft Computing Research Group

GENOTOXICITY ANALYSIS OF FROMILID AND METHOTREXATE USING *ALLIUM* TEST

Merisa Ćenanović[1]

[1]International University of Sarajevo, Faculty of Engineering and Natural Sciences, Hrasnicka Cesta 15, Ilidža 71210 Sarajevo, Bosnia and Herzegovina

Article Info

Article history:
Received Sep 2014
Received in revised form Nov 2014

Keywords:
Target Tracking and Identification, Pose Estimation, Automatic Target Recognition, Statistical Hypothesis Testing

Abstract

Ground Moving Target Indicator (GMTI) and High Resolution Radar (HRR) can track position and velocity of ground moving target. Pose, angle between position and velocity, can be derived from kinematics estimates of position and velocity and it is often used to reduce the search space of a target identification (ID) and Automatic Target Recognition (ATR) algorithms. Due to low resolution in some radar systems, the GMTI estimated pose may exhibit large errors contributing to a faulty identification of potential targets. Our goal is to define new methodology to improve pose estimate. Besides applications in target tracking, there are numerous commercial applications in machine learning, augmented reality and body tracking.

1. INTRODUCTION

Due to increased environmental contamination, humans and other living beings are increasingly exposed to a large amount of chemicals that can have a negative effect on genetic material. Among them, many can be classified as genotoxic factors or genotoxins. Genotoxins are chemical, physical and biological agents that disturb the structure, organization and functioning of genetic material or changing cellular processes vital for integrity and/or function of a genome. Such effect of genotoxins is known as genotoxicity. Genotoxic factors can be classified as biological (e.g. bacterial decomposition products, certain viruses etc.), physical (e.g. ionizing and non-ionizing radiation, extreme temperature variations etc.), chemical factors (e.g. alkylating agens, nitrogenous bases analogs, drugs etc.). In this study, accent was on chemical factors from group of drugs (fromilid and methotrexate); (Sofradzija et al., 1989).

Genotoxicity studies are designed to determine potential mutagens that can cause mutations in genetic material. A large number of assay systems, especially *in vitro* systems, have been devised to detect the genotoxic effects of different substances. Genotoxicity test results are usually taken as indicators for mutagenic effects. However, the differential responses to chemical substances between animal and plant

assays can be considered through differences in their metabolism. To an extent, results of plant bioassays can reveal potential health hazards in humans (Adeyemo et al., 2013). The chromosome assays on plants are rapid, inexpensive and do not require elaborate laboratory facilities and a wide range of genetic endpoints are available. The chromosomes of plants and animals are morphologically similar, and appear to respond to treatment with mutagens in a similar way to those of mammals and other eukaryotes (Nefic et al., 2013).

Among plant test systems, *Allium cepa L.* is one of the most commonly used species for the study of chromosomal aberrations. The growing root tips of the onion, *Allium cepa L.* provide a readily available source of material for studying the damaging effects of chemicals on chromosomes. Meristem tissue of onion root tips contain a great number of cells in mitosis and diploid number of

chromosomes is 16 (2n=16). Chromosomes are relatively large, approximately 10μm long, so chromosomal aberrations can be relatively easily detected. The decrease in the mitotic index (MI) of *Allium cepa* meristem cells can be considered as a reliable method to determine the presence of genotoxic agents in the environment (Nefic et al. 2013).

The aim of this study was to evaluate the genotoxic potential of fromilid and methotrexate using the *A. cepa* test (Fiskesjö, 1985, Fiskesjö, 1993).

2. METHODOLOGY

Fromilid is a semi-synthetic macrolide antibiotic derived from erythromycin. It is used to treat bacterial infections in different parts of the body. It is also used in combinations with other medicines to treat duodenal ulcers caused by *Helicobacter pylori*. Fromilid inhibits bacterial protein synthesis by binding to the bacterial 50S ribosomal subunit. Binding inhibits peptidyl transferase activity and interferes with amino acid translocation during the translation and protein assembly process. Fromilid may be bacteriostatic or bactericidal depending on the organism or drug concentration. The chemical name of fromilid is 6-O-methylerythromycin. The molecular formula is $C_{38}H_{69}NO_{13}$ which corresponds to a molecular weight of 747.95 (Vasil'ev and Zvenigorodskaia, 2002).

Methotrexate is a cytostatic and a folate analog metabolic inhibitor. It is used to treat choriocarcinoma, leukemia in the spinal fluid, osteosarcoma, breast cancer, lung cancer, non-Hodgkin lymphoma, and head and neck cancers. Methotrexate prevents cells to use folate in order to synthesize DNA and RNA molecules. It inhibits the dihydro-pholic acid reductase enzyme which reduces dihydrofolate into tetra-hydropholate in order to be used by cells in nucleotide synthesis. Methothrexate interferes with DNA synthesis, DNA repair systems and replication. The molecular formula of methotrexate is $C_{20}H_{22}N_{18}O_5$ which corresponds to a molecular weight of 454.45 (Olsen, 1991).

The root tip (meristem) cells of onion were used to test the potentially genotoxic effects of fromilid and methotrexate. The *Allium* test was carried out according to Fiskesjö protocol (Fiskesjö, 1985, Fiskesjö, 1993, Fiskesjö, 1997), with some modifications. To perform this test, onion bulbs that were cultivated without application of herbicides or fungicides were used. Healthy onion bulbs of the approximately same size were used in the test. Onion bulbs then have been grown in water, at room temperature. Adequate root growth should be obtained in 3-4 days. When the newly emerged roots were 2 cm in length, they were ready for treatment with the test drug. The bulbs were then transferred to Petri dishes with freshly prepared fromilid and methotrexate solutions with appropriate concentrations for each treatment. A group of six bulbs of onion (*Allium cepa L.*) for each treatment were used. Roots of onion have been treated with a series of concentrations of 100, 500 and 1000 μg/ml (for fromilid treatment) and 1, 10, 50 and 100 μg/ml (for methotrexate treatment) in three different time periods: 4, 8 and 12 hours for each concentration (Anirban et al. 2013). Freshly prepared solutions of fromilid and methotrexate should be used. Treatments took place in the dark. A negative control has been treated with distilled water. After 4, 8 and 12 hours, the roots of each bulb were extracted and fixed in freshly prepared and cool fixative mixture, containing glacial acetic acid and absolute ethanol (3:1, v/v) for 24 hours.

For preparation of root tips chromosome slides, acetoorcein squash technique has been used. The root tips of bulbs were hydrolysed in 1N hydrochloric acid (HCl) at 60^0C for 4-5 min. The purpose of hydrolization with HCl is to dissolve the cell wall of analyzed cells. From HCl the roots were transferred to distilled water and left for a few minutes. The roots were then transferred on a clean microscope slide. Three to four root tips were used for each slide. On the slide, tips were crushed in a drop of 2% acetoorcein (Sigma-Aldrich, USA) with the taper and squashed under a cover microscopic slip. The pressure was applied above several layers of filter paper, with avoidance of sideway movements of the microscope cover slip. Eight slides were prepared for each concentration of selected drugs and the control. For cytogenetic analysis of mitotic cells a microscope (Janaval) was used, at 1000x magnification.

The mitotic index (MI) represents the total number of dividing cells in relation to the number of analysed cells in a cell cycle. A minimum of 1000 cells were analyzed for MI and expressed as percentage of total number of examined cells undergoing mitosis. The frequency of CAs was expressed as a number of aberrant cells per 100 cells examined. A hundered cells were analyzed for each bulb in experimental group and the number of aberrant cells in each experimental group is compared with the values from the control group.

As mentioned previously, we analyzed the effect of methotrexate and fromilid on mitotic activity of onion cells, expressed through mitotic index (MI). The frequency of irregular mitotic phases is calculated by analysis of one hundred cells compared to control, and using Chi-square test at $p<0.001$, $p<0.01$, $p<0.05$ and $p<0.02$ significance levels, and 95% confidence level. Statistical analysis (Chi-square test) was also done using MedCalc ver. 12.5.0.0 statistical software package.

3. RESULTS

The study showed concentration-dependent effects on *MI* of *Allium cepa* root tip meristem cells treated with different concentrations of fromilid (100, 500 and 1000 μg/ml) in time periods of 4, 8 and 12 hours, comparing to group of control cells.

Allium cepa root tip meristem cells treated with fromilid solutions (100μg/ml conc./4 and 8 hours; 500μg/ml /4 hours time periods) showed decrease in MI compared to control group of cells. Decrease in MI was statistically significant in 100μg/ml (4 hours time period) treatment ($p<0.01$). In the rest of the treatments with fromilid (100μg/ml – 4 hours; 500μg/ml – 8 and 12 hours; 1000μg/ml – all selected time periods) *A.cepa* root tip cells showed increase in MI compared to control group of cells.

Increase in MI was statistically significant in treatments with 500μg/ml concentration (8 and 12 hours; $p<0.01$) and 1000μg/ml (4,8, 12 hours; $p<0.02$) time periods (Table 1 and 2).

Analysis of micropreparations and *irregular mitotic phases* showed that a number of irregular prophases and metaphases were gradually increased. In case of gradual increase of irregular prophases, the increment was not statistically significant comparing to control cell group. The increment of irregular metaphases comparing to control group was significant ($p<0.001$), in 1000μg/ml concentration in all time periods (4, 8, 12 hours). The number of irregular anaphases was also increased comparing to control cell group (all concentrations, 12 hours time period at $p<0.001$). Exception was only the treatment with concentration of 100μg/ml (4 hours time period), where the number of irregular anaphases is decreased compared to control cell group, but with no statistical significance. The number of irregular telophases is also increased comparing to control cell group in 100 μg/ml concentration (12 hours time period) and 500 μg/ml concentration (4, 8 hours time period), but with no statistical significance.

Cytogenetic analysis of micropreparations showed various types of *chromosomal (CAs) and numerical aberrations (NAs)* in all selected concentrations (100, 500, 1000μg/ml) and time periods (4, 8, 12 hours). Detected CAs comprise vacuolization of chromosomes, atypical spiralisation, disturbance of chromosomal kynetics (such as lagging chromosomes, falling out of spindle apparatus), agglutination and stickiness of chromosomes, C-mitosis. Regarding NAs, polyploid cells were observed. The occurrence of observed aberrations in onion root tip meristem was concentration-dependent, with higher occurrence in treatments with 500 and 1000 μg/ml fromilid solution, in all selected time periods. (Figure 1).

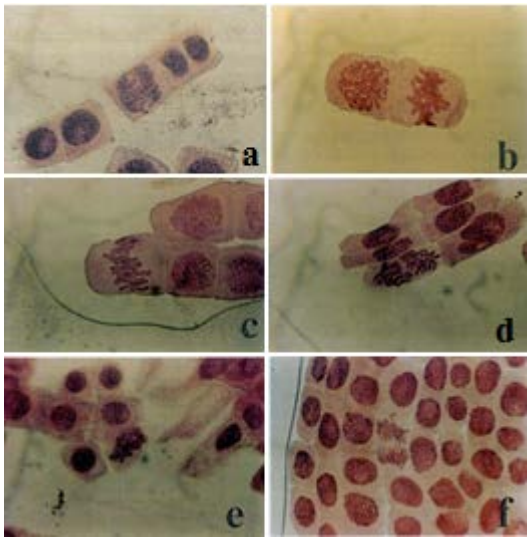

Figure 1. Photomicrographs of aberrations induced by Fromilid in root tip cells of *Allium cepa*; a) stickiness of chromosomes; b) unequal spiralisation; c) vacuolization; d) polyploid cell; e) C-mitosis, f) abnormal kynetics.

Table 1.	**Mitotic index (MI) of *Allium cepa* root tip cells exposed to different concentrations of fromilid**		
Concentration of Fromilid solution (μg/ml)	***4 hours***	***8 hours***	***12 hours***
Control (0)	13.4		
100	9.9**	13.2	14.0
500	13.3	17.6*	20.2*
1000	14.4	13.8	16.2***
Legend: Statistically significant at p<0.001, p<0.01**, p<0.02*** when compared to untreated control*			

Table 2.	**Changes in mitotic activity of *A. cepa* root tip cells treated with fromilid compared to control group**		
Treatment length (in hours)	***100 μg/ml***	***500 μg/ml***	***1000 μg/ml***
4	-26%*	-0.7%	5.8%
8	-1.4%	31.3%**	1.4%
12	4.4%	50.7%**	19.1%***
Legend: Statistically significant at p<0.001, p<0.01**, p<0.02*** (-) Decreased mitotic activity compared to untreated control*			

The study of onion root meristem treated with methotrexate (concentrations 1, 10, 50 and 100 μg/ml) and selected time periods (4, 8 ans 12 hours) showed decrease in MI, comparing to control group of untreated cells. Mitotic activity of cells was greatly reduced, what is especially observed in longer time periods of treatment with methotrexate, and most of the cells were in interphase of the cell cycle. Decrease of MI was statistically significant at all concentrations and time periods ($p<0.001$) except at 1μg/ml concentration (4 hours time period) as showed in Table 3 and 4.

Microscopic analysis of *irregular mitotic phases* of onion cells treated with solutions of methotrexate with selected concentrations, showed increase in number of irregular prophases and metaphases in respect to control. The increase of irregular metaphases comparing to control group was statistically significant (at $p<0.05$), in 1 μg/ml and 50μg/ml concentration in 4 and 8 hours time periods. The number of irregular anaphases was decresead, except in treatments with 10 μg/ml and 100 μg/ml (8 and 4 hours time period respectively), with no statistical significance. The increased number of irregular telophases was observed only in 4 hours treatment and 100 μg/ml concentration, with no statistical significance comparing to control.

The CAs in *Allium cepa* root tip meristem cells after exposure to methotrexate in selected time periods (4, 8 and 12 hours) were: anaphase bridges, breaks, chromosome lagging and stickiness, distinctive vacuolization, abnormal spiralisation, polyploidy, C-mitosis. Cytological aberrations observed after treatment with methotrexate were: micronucleus formation and abnormal kinetics of chromosomes. Also, morphological changes were

observed such as degradation of nucleus, cytoplasm destruction, even the cells themselves (Figure 2).

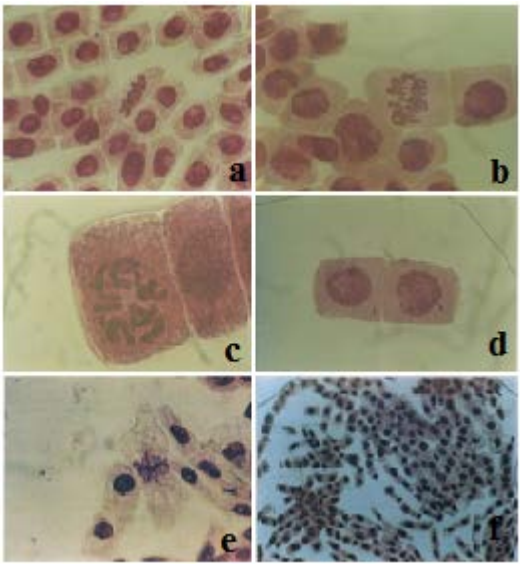

Figure 2. Photomicrographs of aberrations induced by Methotrexate in root tip cells of *Allium cepa*; a) agglutination of chromosomes; b) abnormal kinetics; c) C-mitosis; d) Micronucleus formation; e) vacuolization (with cytoplasm destruction); f) Inhibited mitotic activity.

Table 3.	**Mitotic index (MI) of *Allium cepa* root tip cells exposed to different concentrations of methotrexate**		
Concentration of Methotrexate solution (µg/ml)	***4 hours***	***8 hours***	***12 hours***
Control (0)	18.1		
1	15.9	7.7*	5.4*
10	11.3*	8.1*	2.4*
50	8.9*	3.9*	2.0*
100	9.1*	1.3*	0.5*
Legend: Statistically significant at p<0.001, p<0.01**, p<0.02*** when compared to untreated control*			

Table 4.	**Changes in mitotic activity of *A. cepa* root tip cells treated with methotrexate solutions compared to control group**			
Treatment length (in hours)	***1 µg/ml***	***10 µg/ml***	***50 µg/ml***	***100 µg/ml***
4	-12.9%	-37.5%*	-51.3%*	-50.2%*
8	-57.4%*	-55.2%*	-78.6%*	-92.8%*

Table 4.	**Changes in mitotic activity of *A. cepa* root tip cells treated with methotrexate solutions compared to control group**			
Treatment length (in hours)	***1 µg/ml***	***10 µg/ml***	***50 µg/ml***	***100 µg/ml***
12	-70.1%*	-87.6%*	-89.0%*	-97.2%*
Legend: Statistically significant at p<0.001, p<0.01**, p<0.02*** compared to untreated control* *(-) Decreased mitotic activity compared to untreated control*				

Comparative genotoxicity analysis of Fromilid and Methotrexate

For comparative genotoxicity analysis of fromilid and methotrexate, the concentration of 100 µg/ml was selected, and analyzed in 4, 8 and 12 hours time periods.

Regarding effects on mitotic activity, it was observed that methotrexate significantly decreased MI comparing to fromilid, for selected concentration treatment and in all selected time periods. Analysis of irregular mitotic phases in onion root tip meristem, showed increase in total number of irregular phases in treatment with fromilide comparing to control group of cells, in all selected time periods. Also, it showed decrease in total number of irregular phases in treatment with methotrexate, except in 4 hours treatment, where the total number was increased comparing to control group. Regarding the effects of methotrexate, overall decrease can be explained by strong cytotoxic effects of a drug and consequently, strong inhibition of mitotic activity since most of the cells were in interphase of the cell cycle.

Also, regarding comparison of obtained results, it should be taken into consideration that fromilid and methotrexate belong to a different group of drugs, and affect different molecular processes in the cell.

4. DISCUSSION

Allium cepa root tip test is used to detect potential genotoxicity of chemical substances (Kumar et al. 2007, Abu and Mba, 2011, Adeyemo et al. 2013) . *A. cepa* test is widely used and repeatedly suggested as a standard test. Positive results of *Allium* test should be seen as a warning signal and indication that the tested chemical may constitute a potential health risk (Fiskesjo, 1985). Since the chromosomes of plants and animals are morphologically similar, and appear to respond to treatment with mutagens in a similar way to those of mammals and other eukaryotes, *A. cepa* test can also be used to predict genotoxicity in human DNA (Tadesco and Laughinghouse, 2012).

In this study, *Allium* test enables the assessment of different genetic endpoints which occur as a result from exposure to selected drugs (fromilid and methotrexate).

Allium cepa root tip cells treated with fromilid solution with 100 µg/ml (4 and 8 hours time period), as well as with 500 µg/ml solution (4 hours) showed decreased mitotic activity comparing to control. The results obtained with above mentioned concentrations

indicate cytotoxic effect of fromilid on analyzed cells. Interesting was that treatment with 500 μg/ml (8 and 12 hours time period) and 1000 μg/ml (4,8 and 12 hours time period) seem to have stimulative mitogenic effect on onion root meristem cells with respect to control value. This increase in mitotic activity indicates that fromilid in these concentrations and time periods did not show cytotoxic effects. The study also showed that a number of irregular phases was overall increased compared to control group, what could probably be connected with increased mitotic index in treatments with higher concentrations.

Various CAs were detected (such as vacuolization of chromosomes, atypical spiralisation, disturbance of chromosomal kinetics, agglutination and stickiness, C-mitosis). NAs were also detected in form of polyploid cells. Available literature and other data sources proved to be rather scarce regarding analysis of genotoxic effects of fromilid, so other data from studies regarding genotoxicity of other antibiotics, of which some belong to the same class of drugs as fromilid (group of macrolid antibiotics), were used. Based on available data, it can be said that fromilid has potential genotoxic effects on *A. cepa* root tip cells, in form of structural and numerical CAs (Scaglione et al., 1993).

Microscopic analysis of *Allium cepa* meristem cells treated with methotrexate showed mitodepressive effect on cell division of onion, which was statistically significant at all selected concentrations and time periods in comparison to control group values, except in treatment with 1μg/ml and 4 hours time period. Most of the cells were in interphase of the cell cycle. The observed results can probably be explained with high toxicity of this cytostatic (Podbielkowska et al.,1980). The number of irregular phases were increased compared to control group, but was concentration and time dependent. Various CAs were detected (such as chromosome lagging and stickiness, anaphase bridges and breaks, distinctive vacuolization, abnormal spiralisation, C-mitosis, formation of MNi, abnormal chromosomal kynetics). Also NAs were detected in form of polyploid cells. Morphological mutations observed were: degradation of cell nucleus, cytoplasm destruction and cell lysis. Among available reports on the genotoxicity assessments of methotrexate in literature, that seemed to be rather scarce, there is a limited information on the potential cytotoxic and genotoxic effects of methotrexate in eukaryotic systems (Finnete, et al. 2000, Choudhury, et al. 2000; Rice et al. 2004). Based on results from this study, it can be said that methotrexate has potential genotoxic and cytotoxic effects on genetic material.

5. CONCLUSIONS

The results obtained in this study indicate that fromilid induced genotoxic effects in form of chromosomal (vacuolization of chromosomes, atypical spiralisation, disturbance of chromosomal kinetics – such as lagging chromosomes, falling out of spindle apparatus), agglutination and stickiness of chromosomes, C-mitosis) and numerical (polyploidy cells) aberrations. It was also shown that fromilid in higher concentrations has mitogenic effects, and causes increase in mitotic index values.

The results also showed that methotrexate possess increased inhibitory and mitodepressive effects on cell division. It also induced strong genotoxic effects in form of chromosomal (anaphase bridges, breaks, chromosome lagging and stickiness, distinctive vacuolization, abnormal spiralisation, polyploidy, C-mitosis), cytological (micronucleus formation, abnormal kinetics), morphological (degradation of nucleus, cytoplasm destruction, even cell lysis) aberrations.

The aberrations found in *Allium cepa* meristem cells are indicators of genotoxic potential of fromilid and methotrexate, suggesting a need for safe dose administration of these drugs in human medicine. Also, there is a need for further *in vitro* and *in vivo* studies of mutagenic effects of selected drugs. In future researches, it would be interesting to have *Allium cepa* test compared to other genotoxicity tests.

REFERENCES

Abu NE, Mba KC (2011) Mutagenicity testing of pharmaceutical effluents on Allium cepa root tip meristems. J Toxicol Environ Health Sci 3:44-51.

Adeyemo OA, Farinmade AE (2013) Genotoxic and cytotoxic effects of food flavor enhancer, monosodium glutamate (MSG) using Allium cepa assay. Afr J Biotechnol 12:1459-1466.

Anirban P, Sudipa N, Kaushik S (2013) Cytological effects of Blitox on Root Mitosis of Allium cepa L. IJSRP 3:1-7.

Choudhury RC, Ghosh SK, Palo AK (2000) Cytogenetic toxicity of methotrexate in mouse bone marrow. Environ Toxicol Phar 8:191-196.

Finette BA, Homans AC, Albertini RJ (2000) Emergence of genetic instability in children treated for leukemia. Science 288:514-517.

Fiskesjó G (1985) The *Allium* test as a standard in environmental monitoring. Hereditas 102:99-112.

Fiskesjö G (1993) *Allium* test I: A 2-3 day plant test for toxicity assessment by measuring the mean root growth of onions (*Allium cepa* L.). Environ Toxicol Water Qual 8:461-470.

Fiskesjö G (1997) Allium test for screening chemicals; Evaluation of cytological parameters. In: Wang W, Gorsuch JW, Hughes JS (ed) Plants for environmental studies. Lewis, New York, pp 308-333.

Kumar G, Pannerselvam N (2007) Cytogenetic studies of food preservative in *Allium* cepa root meristem cells. Med Biol 14:60-63.

Nefic H (2013) Chromosomal and nuclear alterations in root tip cells of Allium cepa L. induced by Alprazolam. Med Arh 67:388-392

Olsen EA (1991) The pharmacology of methotrexate. J Am Acad Dermatol 25:306-318.

Podbielkowska M, Waleza M, Zobel A (1980) Influence of methotrexate on respiration and ultrastructure of meristematic cells from *Allium cepa* L. roots. Acta Soc Bot Pol 50:563-566.

Rice SC, Vacek P, Homans AH, Messier T, Rivers J, Kendall H, Finette BA (2004) Genotoxicity of therapeutic intervention in children with acute lymphocytic leukemia. Cancer Res 64:4464-4471.

Scaglione F, Demartini G, Dugnani S, Fraschini F (1993). A new model examining intracellular and extracellular activity of amoxicillin, azithromycin, and clarithromycin in infected cells.Chemotherapy 39:416-423.

Sofradžija A, Hadžiselimović R, Maslić E (1989) Genotoksičnost pesticida. Svjetlost, Sarajevo.

Tadesco SB, Laughinghouse HD (2012) Bioindicator of genotoxicity: The Allium cepa test. In: Srivastava J (ed) Environmental contamination, In Tech. pp 137-157.

Vasil'ev IuV, Zvenigorodskaia LA (2002) Fromilide (clarithromycin) in eradication of Helicobacter pylori in patients with duodenal ulcer. Antibiot Khimioter 47:16-8.

Corresponding Author: Merisa Ćenanović
mcenanovic@ius.edu.ba; mcenanovic2@gmail.com