

**NARCISSUS CV. HAWERA LEAVES' EXTRACT CLEAVES  
DNA IN *CHLAMYDOMONAS REINHARDTII* BY  
INDUCING DSBS**

Petya Parvanova<sup>1✉</sup>, Teodora Todorova<sup>1</sup>, Borianna Sidjimova<sup>2</sup>,  
Milena Nikolova<sup>2</sup>, Strahil Berkov<sup>2</sup>, Stephka Chankova<sup>1</sup>

Received on June 23, 2023

Presented by D. Ivanov, Corresponding Member of BAS, on September 26, 2023

**Abstract**

The aim of this study is to go inside the mode of action of *Narcissus* cv. Hawera leaves' extract on *Chlamydomonas reinhardtii* by analyzing its chemical composition, genotoxic, mutagenic, and DNA damaging effects.

GC/MS analysis of the methanol extract shows that alkaloids comprise about 23.93% of the total ion current (TIC). Considering the alkaloid pattern, lycorine is the major alkaloid (8.53% of TIC), followed by 6-*epi*-mesembrenol (4.59% of TIC), galanthamine (2.63% of TIC), and mesembrenone (2.01% of TIC). Other compounds in the extract are mono- and di-saccharides, organic acids, and fatty acids.

Based on our experiments, both well-expressed genotoxic and DNA damaging potential of *Narcissus* cv. Hawera total leaves' extract was revealed for the first time. The most pronounced effect is obtained after the treatment with concentrations equal to or higher than 500 µg/ml. Cell survival decrease in a dose-dependent manner correlates with increased levels of induced DNA double-strand breaks (DSBs) but does not correspond with statistically confirmed mutagenic action. A good correlation is obtained between the levels of DSBs induced and cell survival rate.

Here, for the first time, experimental evidence that *Narcissus* cv. Hawera leaves' extract can cleave DNA of *Chlamydomonas reinhardtii* inducing DSBs was reported by us. Our finding could be considered a good reason for further

---

<https://doi.org/10.7546/CRABS.2024.03.04>

investigation of its biocidal capacity on other test systems for the purposes of “green” practice.

**Key words:** *Chlamydomonas reinhardtii*, *Narcissus* cv. Hawera total leaves extract, genotoxic, mutagenic, DNA damaging effects

**Introduction.** For decades biocidal products are widely applied in different areas of modern society – agriculture, pharmacology, cosmetics, as disinfectants and certain preservatives, etc., providing various benefits to man – high standard of health, improved quality of industrial processes and goods [1, 2]. At the same time it is well documented that biocidal products harmfully affect both the environment and human health [3].

Following the recommendations for “best practices” and to avoid negative effects of synthetic biocidal products an elaboration of eco-friendly plant-based biocides is considered more ecologically friendly [4]. Currently, various plant products – extracts and oils – are used in medicine and pharmacy, due to their advantages – antimicrobial potential, slight or no toxicity to animals and humans, and fast biodegradation in natural environments [1]. Bearing in mind the fact that data on their biocidal capacity are scarce, we turned our attention to well-known plant extracts widely used in folk medicine.

*Narcissus* cv. Hawera provokes interest due to its unique alkaloid pattern consisting of a specific group of compounds, the so-called *Sceletium*-type alkaloids, along with typical Amaryllidaceae alkaloids [5]. This artificial hybrid between two *Narcissus* species is registered as cv. Hawera and widely cultivated in the Netherlands and England [5]. Little is currently known about the mode of action of *Narcissus* cv. Hawera leaves’ extracts. An alkaloid fraction from this plant possesses anxiolytic and antidepressant-like activities as well as potent acetylcholinesterase (AChE) inhibitory and antineoplastic activities [5]. Further studies on the genotoxic, mutagenic, DNA damaging, etc. effects on plants, animals, and humans are of great importance for the evaluation of its potential for application as biocidal product.

In the present study, we hypothesized that the alkaloid-rich *Narcissus* cv. Hawera total leaves’ extract will possess well-expressed DNA damaging effect on a test-system *Chlamydomonas reinhardtii*.

Here, by combining microbiological, genetic and molecular methods we aimed to go inside the mode of action of *Narcissus* cv. Hawera leaves’ extract on *Chlamydomonas reinhardtii* (robust plant cell model) by analyzing its chemical composition, genotoxic, mutagenic, and DNA damaging effects.

**Material and methods. Material and extraction.** *Narcissus* cv. Hawera plants were grown in soil at the greenhouse of the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences (IBER – BAS). Leaves from flowering plants were cut, dried, and then extracted with CH<sub>3</sub>OH for 24 h in a proportion of 1:10. After the filtration, the solvent was evaporated under vac-

uum to obtain a total dry extract [6,7]. A methanol solution with a concentration of 10 mg dry extract per millilitre was prepared for gas chromatographic/mass spectral analysis (GC-MS).

**Gas chromatographic/mass spectral (GC/MS) analysis.** The GC/MS spectra were recorded on a Thermo Scientific Focus GC coupled with a Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m  $\times$  0.25 mm  $\times$  0.25  $\mu$ m) was used. The temperature programme was: 100–180 °C at 15 °C/min, 180–300 °C at 5 °C/min, and 10 min hold at 300 °C. The injector temperature was 250 °C. The flow rate of carrier gas (Helium) was 0.8 ml/min. The split ratio was 1:10. 1  $\mu$ l of the solution was injected.

The GC/MS spectra of alkaloids in the total extract were deconvoluted by AMDIS 2.64 software (NIST, National Institute of Standardization and Technology, Gaithersburg, MD) before their comparison with those of standard compounds or literature data as described by [5].

**Test-system cultivation.** *C. reinhardtii* strain 137 C (+) (WT) due to its benefits [8] was used to evaluate the potential genotoxic, mutagenic, and DNA-damaging capacities of *N. cv. Hawera* wild-type. At standard cultivation conditions the strain was grown in GC 400 Growth chamber to the end of the exponential and the beginning of the stationary phase [8].

**Genotoxicity. Spot test** is applied in order to reveal the potential genotoxic effect based on a spot intensity depending on the exposure time and concentrations range. The cell suspensions with a density of  $1 \times 10^6$  cells/ml were treated for 2 or 24 h with concentrations in the range of 5–1000  $\mu$ g/ml of *N. cv. Hawera* total leaves' extract. The spot intensity in treated samples was compared with those in the control samples – dimethyl sulphoxide DMSO (0.1%), and Sager-Granick liquid medium (SG) and the herbicide Paraquat (PQ) (Methyl Viologen, Sigma-Aldrich) – a well-known inducer of oxidative stress as a positive control. Paraquat (PQ) treatment conditions – the concentration of 5  $\mu$ M and 2 h exposure time have been defined previously in our pilot experiments.

**Survival fraction (SF).** The survival fraction was analyzed by “clonal assay”, based on colony-forming ability as described in [9]. Cell suspensions with a density of  $1 \times 10^6$  cells/ml were treated with the following concentrations – 250, 500, 750, and 1000  $\mu$ g/ml for 2 h. Three levels of lethality were calculated – LD<sub>20</sub>, LD<sub>37</sub>, and LD<sub>50</sub> by the Reed–Muench method [10].

**Mutagenic potential.** The potential mutagenic activity of the extract was revealed microscopically (stereomicroscope Carl Zeiss at 10-fold magnification) by the test of “visible mutations”. Five basic types of mutant colonies – small-size, pigment, morphological, sector, and mosaic are informative for induced and realized genetic events. Calculating formulas and Mutagenic index (MI) were described in [8].

**Double-strand breaks (DSB) induction.** The constant field gel electrophoresis (CFGE) as described in detail by us [11] was applied using digital

camera and Syngene software (Gene Tool Analyser G: Box Syngene). DSBs were calculated as  $FDR = \text{DNA released} / (\text{DNA in well} + \text{DNA released})$  [12].

**Statistical analysis.** Data were expressed as mean  $\pm$  standard error (SEM) and statistically assessed by One-way ANOVA with Tukey multiple comparison tests and Two-way ANOVA with Bonferroni's post-test (GraphPad Prism 6.04 software, San Diego, USA). Pearson Correlation analysis was performed in order to establish the relationship between the survival fraction and DNA double-strand break levels (GraphPad Prism 6.04 software, San Diego, USA). Data were presented as a coefficient of determination ( $R^2$ ) and linear correlation coefficient ( $r$ ) and considered statistically significant when  $P \leq 0.05$ .

**Results. GC/MS analysis.** The GC/MS analysis revealed that the alkaloids comprise about 23.93% of the total ion current (TIC, Fig. 1A, B) of the total methanol extract. Mono- and di-saccharides, organic acids, and fatty acids were also found. Considering the alkaloid pattern, lycorine was the major alkaloid (8.53% of TIC), followed by 6-*epi*-mesembrenol (4.59% of TIC), galanthamine (2.63% of TIC), and mesembrenone (2.01% of TIC).

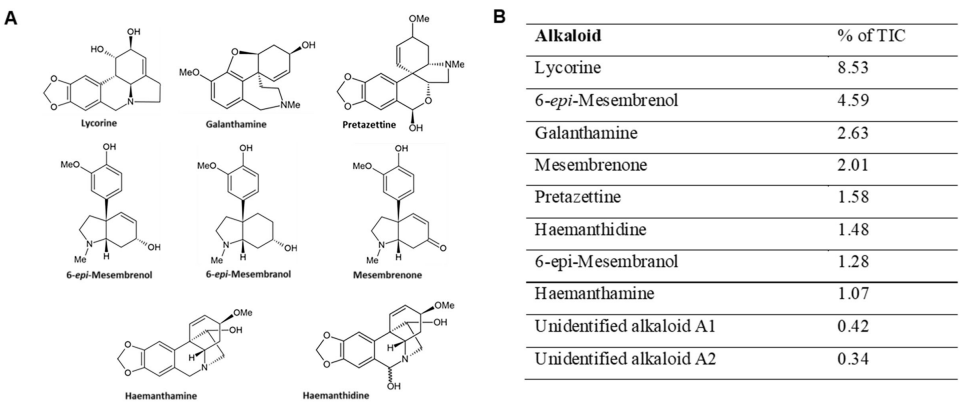


Fig. 1. Structures of the detected alkaloids in *Narcissus* cv. Hawera leaves' methanol extract (A). Alkaloids in *Narcissus* cv. Hawera leaves' methanol extract detected by GC-MS (B)

**Genotoxicity screening. Spot test** is the first step for the revealing concentrations and exposure time-dependent decrease in spots intensity (Fig. 2A, B). This effect was the most pronounced in samples treated with 1000  $\mu\text{g/ml}$  for 2 and 24 h.

**Survival fraction (SF).** Further, two hours exposure time was given to evaluate the SF based on 137 (C+) strain colony-forming ability. A dose-dependent decrease in the survival fraction was revealed after the treatment with concentrations equal to or higher than that of 500  $\mu\text{g/ml}$  (Fig. 3A). No statistically significant genotoxic effect of the lowest concentration of the extract – 250  $\mu\text{g/ml}$  was found. The treatment with 500  $\mu\text{g/ml}$  *Narcissus* cv. Hawera extract resulted in a 33.7% decrease in cell survival ( $P < 0.001$ ). The results were comparable

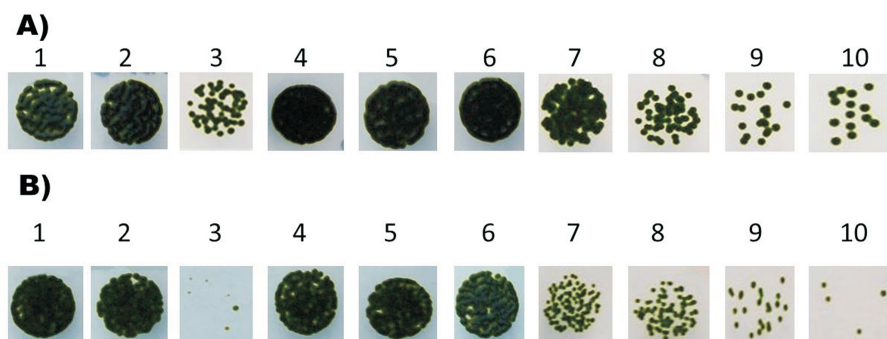


Fig. 2. Spot-test with *Narcissus* cv. Hawera extract at 2 h (A) and 24 h (B) exposure time; Samples: 1 – CSG – Control Sager–Granick liquid medium; 2 – CDMSO – Control dimethyl sulphoxide (DMSO); 3 – 5  $\mu$ M PQ – positive control; tested concentrations – 4–5  $\mu$ g/ml; 5 – 50  $\mu$ g/ml; 6 – 100  $\mu$ g/ml; 7 – 250  $\mu$ g/ml; 8 – 500  $\mu$ g/ml; 9 – 750  $\mu$ g/ml, and 10 – 1000  $\mu$ g/ml

to those after the treatment with the positive control 5  $\mu$ M PQ (SF = 0.745). Both treatments revealed a similar reduction in cell survival. On the other hand, concentrations in the range between 750 and 1000  $\mu$ g/ml resulted in around 70% SF, forming a “plateau” region that could be interpreted as the presence of concentrations forming one group of action, or similar DSBs repair rejoining [13].

One of the most promising strategies for comparing the genotoxic/phytotoxic capacity of different substances/extracts is the calculation of the levels of lethality. Here, LD<sub>20</sub> = 352  $\mu$ g/ml, LD<sub>37</sub> = 482  $\mu$ g/ml, and LD<sub>50</sub> = 598  $\mu$ g/ml were calculated after the treatment with *N. cv. Hawera* total leaves extract.

As the next steps, we have to analyze the mutagenic and DNA-damaging potential measured as DSBs.

**Mutagenicity.** In the present work, 0.074% spontaneous mutation frequency was calculated (data not shown). The induced mutation frequency was found to vary between 0.086 and 0.127% depending on the test concentrations.

The mutagenic index was calculated to be lower than 2.5 suggesting no mutagenic effect.

**Induction of DNA double-strand breaks.** As a next step, the DNA damaging potential of *Narcissus* cv. Hawera extract measured as induction of DNA DSBs was evaluated (Fig. 3A, B).

Statistically significant increased DSBs levels were observed after the treatment with concentrations equal to or higher than 500  $\mu$ g/ml. The most pronounced this effect was after the treatment with the highest tested concentration – 1000  $\mu$ g/ml (about two-fold higher levels than the spontaneous ones).

From our point of knowledge this is the first experimental evidence that *Narcissus* cv. Hawera leaves’ extract can cleave *Ch. reinhardtii* DNA by inducing DSBs in a concentration depending manner.

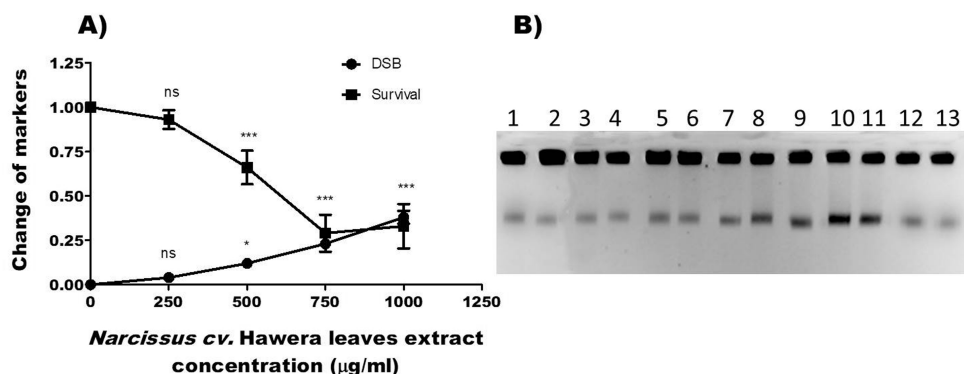


Fig. 3. Survival curve and levels of primary induced double-strand breaks in DNA of *Chlamydomonas reinhardtii* after the treatment with different concentrations – 250, 500, 750, and 1000 µg/ml of *Narcissus cv. Hawera* total leaves extract at 2 h exposure. Where the standard errors are not presented, they are equal to or smaller than the symbols of the graphic. Values represent the mean fraction of DNA released (FDR). Data are presented as mean  $\pm$  SEM. All the results are from at least three independent experiments (ns  $P > 0.05$ ; \*  $P < 0.05$ ; \*\*\*  $P < 0.001$ ) (A). Inverted gel electrophoresis image of the levels of primary induced DNA double-strand breaks in *Chlamydomonas reinhardtii* (where 1, 2, 3 – control; 4, 5 – 250 µg/ml; 6, 7 – 500 µg/ml; 8, 9 – 750 µg/ml; 10, 11 – 1000 µg/ml and 12, 13 – 5 µM PQ) (B)

No statistically significant difference between DSB levels measured after the treatment with 500 µg/ml *Narcissus cv. Hawera* extract and those calculated for the positive control 5 µM PQ (FDR = 0.09) was observed. Statistically significant higher DSB levels after the treatment with 750 µg/ml and 1000 µg/ml *Narcissus cv. Hawera* extract were detected compared to those found in 5 µM PQ ( $P < 0.05$ ).

Having in mind the similar trend in cell survival curve, correspondence between low cell survival and increased DSB levels exists could be suggested (Fig. 3A). In order to evaluate the contribution of DSBs to cell survival, a linear correlation analysis was conducted. A very strong negative linear correlation was obtained between both the FDR and SF ( $r = -0.9970$ ;  $R^2 = 0.9941$ ,  $P < 0.01$ ). Increased levels of DSB correlate with a decrease in the SF. This result indicates that both events are most probably related.

**Discussion.** Here, an attempt was made to gain insight into the mode of action of total leaves' extract of *Narcissus cv. Hawera* by evaluating its genotoxic, mutagenic and/or DNA damaging potential.

Our finding that concentrations equal to or higher than those inducing the LD<sub>50</sub> possess a well-marked genotoxic and DNA-damaging capacity expands the current knowledge of the spectrum of damage induced by natural products from narcissus plants – toxic at high doses [14], cytotoxic [15], clastogenic [16], cell cycle arrest and gene expression blocking. These effects are commonly related to the high alkaloid content. Our GC/MS analysis revealed both – 23.93% total

alkaloid content and lycorine – 8.53% content (the major constituent) of *Narcissus* cv. Hawera leaves extract. Previously, it is indicated [17] that lycorine is a powerful inhibitor of ascorbic acid (L-Asc) biosynthesis, cell growth, cell division, and organogenesis in higher plants, algae, yeasts. It is already known that some alkaloids may induce the production of hydrogen peroxide and singlet molecular oxygen thus leading to toxic action resulting in induction of DNA damage; photo-system damage; cell division inhibition and oxidative damage; lipid peroxidation in different cells such as cyanobacteria, *Escherichia coli*, mouse spleen cells [18]. Approximately 10 years ago Hu et al. [19] described the inhibition capacity of narciclasine on root growth of lettuce seedlings by DNA damage-induced cell cycle arrest.

In our experiments, various types of spontaneous and induced “visible mutations” are scored but calculating the mutagenicity index, no mutagenic effect at the tested concentrations of the plant extract is obtained. Our results demonstrated no correlation between the genotoxic and mutagenic capacity of *Narcissus* cv. Hawera in *Chlamydomonas reinhardtii* 137 C.

**Conclusion.** Here, for the first time, we report experimental evidence that *Narcissus* cv. Hawera leaves’ extract can cleave DNA of *Chlamydomonas reinhardtii* inducing DSBs. Our finding could be considered a good reason for further investigation of its biocidal capacity on other test systems for the purposes of “green” practice.

## REFERENCES

- [1] CZAJA K., K. GÓRALCZYK, P. STRUCICKI et al. (2015) Biopesticides – towards increased consumer safety in the European Union, *Pest. Manag. Sci.*, **71**(1), 3–6.
- [2] THIVIYA P., N. GUNAWARDENA, A. GAMAGE et al. (2022) Apiaceae family as a valuable source of biocidal components and their potential uses in agriculture, *Horticulturae*, **8**(7), 614.
- [3] RICHMOND E. (2018) Glyphosate: A review of its global use, environmental impact, and potential health effects on humans and other species, *J. Environ. Stud. Sci.*, **8**, 416–434.
- [4] RAJPUT P., A. THAKUR, P. DEVI (2020) Emerging agrochemicals contaminants: current status, challenges, and technological solutions, Chapter 5. In: (Ed. M. N. V. Prasad) *Agrochemicals Detection, Treatment and Remediation*, Oxford, UK, Butterworth-Heinemann, 117–142.
- [5] BERKOV S., D. PECHLIVANOVA, R. DENEV et al. (2021) GC-MS analysis of Amaryllidaceae and Scelletium-type alkaloids in bioactive fractions from *Narcissus* cv. Hawera, *Rapid. Commun. Mass. Spectrom.*, **35**(14), e9116.
- [6] BERKOV S., E. OSORIO, F. VILADOMAT et al. (2020) Chemodiversity, chemotaxonomy and chemoecology of Amaryllidaceae alkaloids, *Alkaloids: Chem. Biol.*, **83**, 113–185.
- [7] BERKOV S., L. GEORGIEVA, B. SIDJIMOVA et al. (2021) *In vitro* propagation and biosynthesis of Scelletium-type alkaloids in *Narcissus pallidulus* and *Narcissus* cv. Hawera, *South African Journal of Botany*, **136**, 190–194.

- [8] DIMITROVA M., E. DIMOVA, Z. MITROVSKA et al. (2007) Testing of polluted soil samples for genotoxic potential using *Chlamydomonas reinhardtii*, *Algological Studies*, 111–121.
- [9] CHANKOVA S., J. A. MATOS, F. SIMOES, P. E. BRYANT (2005) The adaptive response of a new radio-resistant strain of *Chlamydomonas reinhardtii* correlates with increased DNA double-strand break rejoining, *Int. J. Radiat. Biol.*, **81**(7), 509–514.
- [10] LIDANSKI T. (1988) Statistical methods in biology and agriculture, Sofia, Zemizdat, 374 pp (in Bulgarian).
- [11] CHANKOVA S. G., P. E. BRYANT (2002) Acceleration of DNA-double strand rejoining during the adaptive response of *Chlamydomonas reinhardtii*, *Radiats. Biol. Radioecol.*, **42**(6), 606–603.
- [12] CHANKOVA S. G., N. P. YURINA, E. G. DIMOVA et al. (2009) Pretreatment with heat does not affect double-strand breaks DNA rejoining in *Chlamydomonas reinhardtii*, *J. Therm. Biol.*, **34**(7), 332–336.
- [13] CHANKOVA S. G., E. DIMOVA, M. DIMITROVA, P. E. BRYANT (2007) Induction of DNA double-strand breaks by zeocin in *Chlamydomonas reinhardtii* and the role of increased DNA double-strand breaks rejoining in the formation of an adaptive response, *Radiat. Environ. Biophys.*, **46**, 409–416.
- [14] MOROVVATI H., N. ARMAND (2020) Investigate the effect of alcoholic extract of bulbs of narcissus (*Narcissus tazetta* L.) on indexes of renal function, *KAUMS Journal (FEYZ)*, **24**(1), 56–62.
- [15] UTHIRAPATHY S., A. TAHSIN (2021) Evaluation of Genotoxic effects of a Hydro-alcoholic extract of flowers of Nargis (*Narcissus tazetta* L.), *Eurasian J. Sci. Eng.*, **7**(2), 39–48.
- [16] MOHAMMED B. M., M. AHMED (2018) Evaluating the genotoxicity enhancement of the antileukemic drug 6-mercaptopurin when combined with Iraqi *Nerium oleander* and *Narcissus tazetta* extracts *in vivo*, *J. Biotech. Research Center*, **12**(1), 92–102.
- [17] BASTIDA J., R. LAVILLA, F. VILADOMAT (2006) Chemical and biological aspects of Narcissus alkaloids, *Alkaloids: Chem. Biol.*, **63**, 87–179.
- [18] SHAO J., R. LI, J. E. LEPO, J. D. GU (2013) Potential for control of harmful cyanobacterial blooms using biologically derived substances: Problems and prospects, *J. Environ. Manag.*, **125**, 149–155.
- [19] HU Y., J. LI, L. YANG et al. (2014) Inhibition of root growth by narciclasine is caused by DNA damage-induced cell cycle arrest in lettuce seedlings, *Protoplasma*, **251**, 1113–1124.

<sup>1</sup>*Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences,  
2 Gagarin St, 1113 Sofia, Bulgaria  
e-mails: petq\_parvanova@abv.bg, tedi\_todorova@yahoo.com,  
stephanie.chankova@yahoo.com*

<sup>2</sup>*Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences,  
Akad. G. Bonchev St, Bl. 23, 1113 Sofia, Bulgaria  
e-mails: sidjimova@yahoo.com, mtihomirova@gmail.com/mtihomirova@mail.bg,  
berkov\_str@yahoo.com*