ACTA BIOLOGICA SLOVENICA
LJUBLJANA 2010 Vol. 53, Št. 2: 53–60

In vitro propagation of *Lilium martagon* L. var. *cattaniae* Vis. and evaluation of genotoxic potential of its leaves and bulbs extracts

In vitro razmnoževanje in ocena genotoksičnosti izvlečkov listov in čebul taksona *Lilium martagon* L. var. *cattaniae* Vis.

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Abstract: *Lilium martagon* L. var. *cattaniae* Vis. (*Liliaceae*) is endemic plant of Dinaridi mountain. In this work we established protocol for fast in vitro propagation and multiplication of *Lilium martagon* var. *cattaniae*. The aim was to enable fast production of plant material as potential source of pharmaceutically valuable secondary metabolites. Seeds of *L. martagon* var. *cattaniae* were germinated on a Murashige and Skoog basal medium with a supplement of 0.15 mg/l gibberellic acid (GA_3), and multiplication was performed on MS medium supplemented with 0.1 mg/l gibberellic acid (GA_3), 0.2 mg/l indole-3-butyric acid (IBA) and 0.5 mg/l 6-benzylaminopurine (BAP). We used ultrasound assisted extraction to prepare extracts of leaves and bulbs of *Lilium martagon* var. *cattaniae*, which were evaluated for their genotoxic potential using Allium test and cytokinesis-block micronucleus test in human lymphocytes culture. There was statistically significant difference between all used concentrations of lilium extracts and control on proliferation of cells of root tip of onion (*Allium cepa*). In cytokinesis-block micronucleus test no statistically significant difference between frequencies of analyzed parameters in samples treated with tested concentrations of extracts and control was obtained.

Keywords: *in vitro* culture, ultrasound assisted extraction, micronuclei, genotoxic, *Lilium martagon* L. var. *cattaniae* Vis.

Izvleček: *Lilium martagon* L. var. *cattaniae* Vis. (*Liliaceae*) je endemični takson Dinaridov. Članek podaja načrt za hitro in vitro propagacijo in multiplikacijo taksona *Lilium martagon* L. var. *cattaniae*. Cilj je bil omogočiti hitro propagacijo rastlinskega materiala, ki je potencialni izvor metabolitov, pomembnih za farmacijo. Semena taksona *Lilium martagon* var. *cattaniae* smo kalili na Murashige in Skoog (MS) hranljivi podlagi, kateri smo dodali 0,15 mg/l giberelinske kisline (GA_3). Multiplikacija je prav tako potekala na MS podlagi, ki smo ji dodali 0,1 mg/l giberelinske kisline (GA_3), 0,2 mg/l indol-3-maslene kisline (IBA) in 0,5 mg/l 6-benzilaminopurina (BAP). Za pripravo izvlečkov listov in korenin taksona *Lilium martagon* var. *cattaniae* smo uporabili ultrazvočno ekstrakcijo. Za ugotavljanje genotoksičnega potenciala teh izvlečkov je bil uporabljen Allium test in citokinetski blok mikronukleus test na humanih limfocitih. Različne koncentracije izvlečkov lilij so imele značilen vpliv na proliferacijo celic korenin čebule (*Allium cepa*) glede na kontrolo. Pri citokinetskem blok mikronukleus testu ni bilo značilnih razlik med frekvencami analiziranih parametrov pri vzorcih obravnavanih z različnimi koncentracijami izvlečkov in kontrolo.

Ključne besede: *in vitro* kultura, ultrazvočna ekstrakcija, mikronukleusi, genotoksičnost, *Lilium martagon* L. var. *cattaniae* Vis.

Introduction

Lilium martagon L. var. *cattaniae* Vis. *Liliaceae* is an endemic plant species of Bosnia and Herzegovina (Šilić 2007). According to Flora Europaea, (Matthews 1980) Turk's-cap lilies in Europe are represented by: *L. martagon* L., *L. chalcedonicum* L., *L. pomponium* L., *L. pyrenaicum* Gouan. and *L. carniolicum* Bernh. ex Koch. In addition there are also endemic European taxa with unclear taxonomic status: *L. albanicum* Griseb., *L. bosniacum* (G. Beck) Beck ex Fritsch and *L. jankae* A. Kerner from the *L. carniolicum* complex, and *L. cattaniae* (Vis.) Vis. from the *L. martagon* complex (Matthews 1980).

This species is named after Maria Cattani Selebam who showed differences between *Lilium cattaniae* Vis. (Vis.) and *Lilium martagon* L. to R. Visiani so he published in year 1872 that it is new species (Šilić 2007). This species is used as medicinal plant in Mediterranean area. It represents an important resource both for phytochemical and pharmacological research (Redžić 2010). Bulbs of its closest species *Lilium martagon* L. posses cardiotonic properties and are used in the treatment of dysmenorrhoea (Khare 2007), liver diseases in both humans and animals in Northern Albania (Pieroni et al. 2005). Bulbs of *Lilium martagon* L. are used externaly for ulcers (Khare 2007). There are studies confirming presence of anticarcinogenic components, such as jatropham which is antileucemic agent, in *Lilium martagon* L. The presence of kaempferol, quercetin and isorhamnetin was identified in *L. martagon* (Eisenreichová et al. 2004). HPLC analysis should be done to characterise crude leaf and bulb extracts of *Lilium martagon* L. var. *cattaniae* Vis. for major secondary metabolites.

Culture *in vitro* is revolutionary methodology useful in development of synthesis and accumulation of natural products and possible method for modification of products (Remington 2005). This is especially important when endemic and rare plants are used as a source of medically active substances. Bulbous plants, like lilies, have proved to be ideal for tissue culture, as their regeneration potential is usually high. Tissue culture has been applied to the propagation of lilies since the late 1950's. Nowadays, lilies are one of the most important bulbous crops produced in tissue culture also in an industrial scale. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products (Pelkonen 2005).

When preparing herbal extracts, method of extraction plays very important role. In history many different methods of extraction have been developed. Non-conventional extraction techniques have gained more attention recently, and one of these techniques is ultrasonically assisted extraction. In the area of inter-phase mass transfer, solid-liquid extractron appears to be most greatly enhanced by the application of ultrasonic waves. The mechanism believed to be primarily responsible for the larger increases is the cell disruption brought about by cavitation. Cavitation can result when high-intensity acoustic waves are passed through liquids producing small bubbles in the liquid. On collapse, the contents of the bubbles are compressed to very high temperatures and are capable of producing shock waves (Chendke et al. 1975).

Ultrasonic extraction is simple, low cost in terms of solvent used and less time consumed. This method promotes better penetration of solvent into plant particles and uses low extraction temperature which affects the stability of active components (Rouhani et al. 2009). Ultrasonic processing is still in its infancy and requires a great deal of future research (Dolatowski et al. 2007).

It is very important that herbal extracts used in traditional medicine, are not toxic, or that its toxicity is under defined limits. Remarkable aspect of toxicity is genotoxicity. Numerous tests can be used in studying genotoxicity. In this study we used Allium test and cytokinesis-block micronucleus test in human lymphocytes culture, to evaluate genotoxicity potential of *L. martagon var. cattaniae* leaves and bulbs extracts.

Materials and methods

Plant material

Seeds of *Lilium martagon* L. var. *cattaniae* Vis. used in this study as starting material were provided by dr.sci. Edina Muratovic. Seeds were collected on Borova Glava, 1100 m, Bosnia and

Herzegovina. Voucher specimens are deposit at Department of Biology, Faculty of Science, University in Sarajevo.

In vitro culture of *Lilium martagon* L. var. *cattaniae* Vis.

For *L. martagon var. cattaniae* seed germination, commercial MS (Murashige and Skoog, 1962) medium (*Duchefa*, *Netherlands*) was used. Medium pH was adjusted to 6.5. Seeds were sterilized and germinated according to protocol given by Parić et al. 2008. Five to six cm long lilies explants were cut into smaller which contained 1-3 bulbs. Explants were inoculated on MS medium supplemented with 0.1mg/l GA_3, 0.2 mg/l IBA (indole butiric acid) and 0.5 mg/l BAP (benzyl amino purine). Explants were cultivated for three weeks, subcultivated for three times and then collected for extraction.

Extraction

Bulbs and leaves were separated and plant material was dried in a flow of hot air, chopped and extracted with water. First, plant material was soaked in distilled water (1:20) for 2 hours. After that mixture was put in ultrasound bath (Iskra UZ4R) for 25 minutes. The mixture was put in a dark place for 22 hours with frequent shaking, filtrated and vacuum dried on temperature 30 ºC and pressure 50 mbar. Dried extracts were held on room temperature above silica gel until they were used.

Testing of genotoxic potential

Allium test

Fresh, healthy, equal-sized bulbs of a commercial variety of *Allium cepa* L. were selected. Just before use, the outer scales of the bulbs were carefully removed and the brownish bottom plates were scraped away without destroying the root primordia. Four concentrations (0.1mg/ml, 0.5mg/ml, 1mg/ml and 5mg/ml) of dried extracts of leaves and bulbs were used in the experiment. For every concentration and for negative control two bulbs were taken. They were put in water on room temperature and in shadow for 48 hours. After that, bulbs were put in extracts for 24 hours (control bulbs were left in water). Then root tips were fixated in ice-cold acetic acid: ethanol 1:3 on 4 ºC for few hours. Root tips were hydrolyzed with 1 N HCl at 60 ºC for 7 min and after that washed in distilled water. For every concentration two slides were prepared. Three root tips were taken for every slide, the meristematic cell region was removed by cutting 2 mm from the root cap, this section was set on a clean slide, macerated with metal stick, immersed in drop of 1% lacto-propion orcein and squashed under a cover glass, excess color was removed with filter paper and after that, borders of cover glass were paraffinated. Slides were observed under 40× magnification. On every slide 1000 cells were analyzed for frequency of cells in interphase, prophase, methaphase, anaphase and telophase. Mitotic index (MI) was calculated as percentage of mitotic cells in all analyzed cells.

Cytokinesis-block micronucleus test

According to results of Allium test two concentrations of extracts (0,1 mg/ml and 1 mg/ml) were chosen for micronucleus test in human lymphocytes. Micronucleus test was performed on blood samples from four persons. Donors of blood were not on therapy with medicines and did not suffer any chronic disease in past 6 months, they were not smokers and age was between 24 and 27 years. Two donors were female and two donors were male. The study was conducted in accordance to ethical principals.

Cytokinesis-block micronucleus assay was performed according to protocol and scoring criteria given by Fenech and coworkers (2003) and Fenech (2000). Blood was cultivated for 72 hours on RPMI medium. After 24 hours of cultivation extracts were added. After 45 hours of cultivation citohalazin B was added (4.5 µg/ml).

Estimation of lymphocytes proliferation was done by calculating nuclear division index (NDI) according to Eastmond and Tucker (1989). The calculation was made according a formula: NDI= [M1 + 2(M2) + 3(M3) + 4(M4)]/N, where M1-M4 represent cells with one to four nucleus, N is total number of viable cells analyzed.

Statistical analysis

Z-test and ANOVA followed by pair-wise comparisons with Newmans-Keuls Multiple

comparison test were calculated for statistical analysis, using Winks 4.5 Professional software (TexaSoft, Cedar Hill, TX, USA). Level of significance was $p \leq 0.05$.

Results and discussion

In vitro propagation and multiplication of Lilium martagon L. var. cattaniae Vis.

In vitro cultivation is very important tehnique for production of significant natural products in pharmacy. One of the aims is to produce big amount of plant material in the shortest time. If the starting material is seed, germination is a very important factor, and it can be improved using appropriate protocol for *in vitro* germination. Seeds of *Lilium* species generally have deep dormancy. Removing of seed coats and cutting seeds allowed germination, showing that dormancy of *L. bosniacum* and *L. martagon* var. *cattaniae* was induced by the presence of the testa (Parić et al. 2008). On different species of *Lillium* sp. it is determined that removal of seed testa increases germination. It is assumed that, besides it is physical barrier, testa contains physiological system that maintains dormancy of seed (Pelkonen 2005). In this work sterilized seeds were germinated on MS medium with GA_3. After 21 day of incubation all seeds germinated.

Optimal combination and concentrations of growth regulators are necessary to achieve wanted processes on cellular level and wanted growth of the whole plant. There are many works about which combinations of GA_3, BAP and IBA are good for growth and development of plants *in vitro*. For example Petrović and Jačimović-Plavšic (1992) proved that the best development and propagation of axillary buds of *Aronia melanocarpa Elliot* was achieved on MS medium supplemented with 0.1 mg/l GA3, 0.1 mg/l IBA and 0.5 mg/l BAP. In this work thirty days after germination plants were transferred to MS medium containing of GA_3, IBA and BAP. Three multiplications lasting 20 days were performed and 86 g of fresh material was obtained.

Selected method of extraction significantly

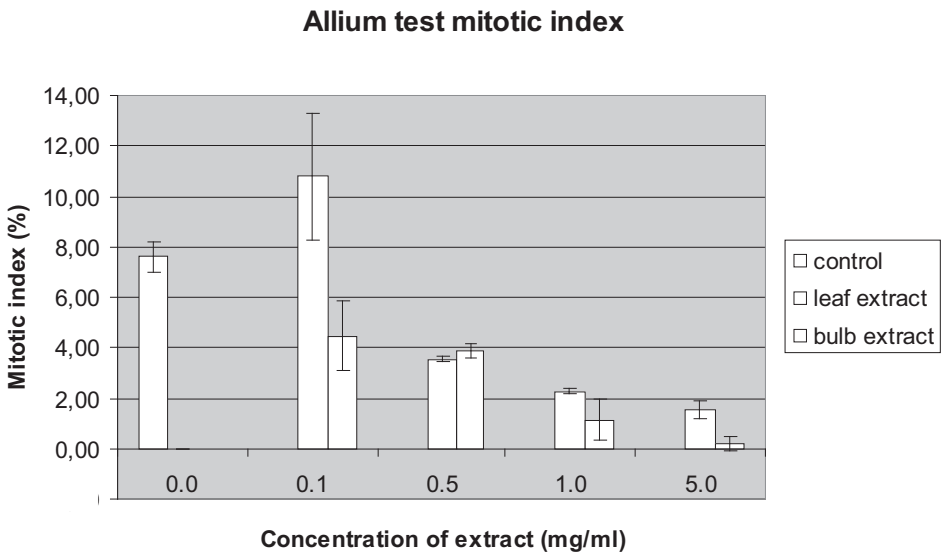

Figure 1: Mitotic index of cells of root tip of onion after treatment with extracts of *L. martagon* var. *cattaniae* and control. The data represent the means ± SD.

Slika 1: Mitotski indeks celic korenin čebule po obravnavanju z izvlečki taksona *L. martagon* var. *cattaniae* v primerjavi s kontrolo. Rezultati so predstavljeni kot povprečje ± SD.

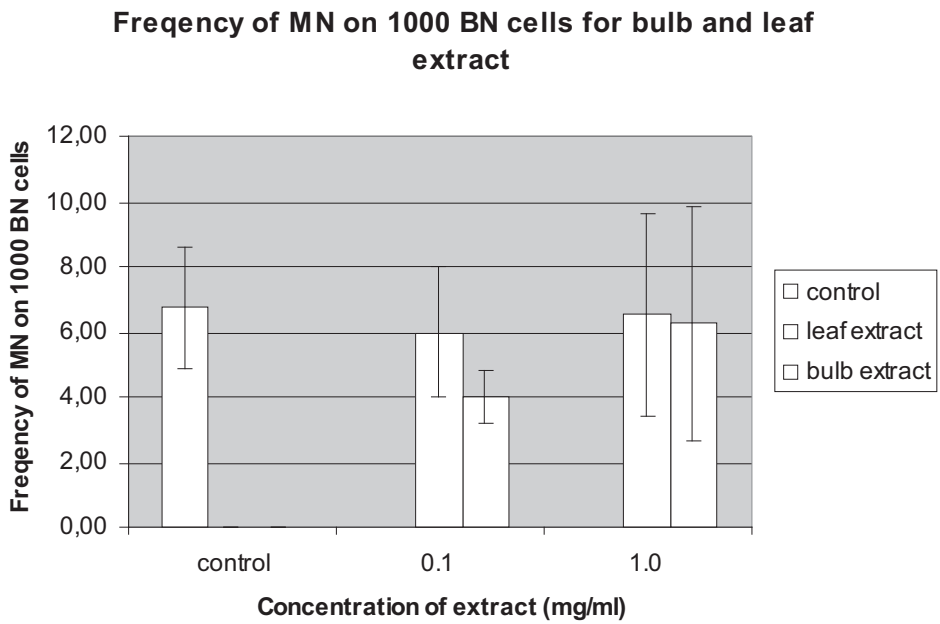

Figure 2: Frequency of MN on 1000 BN cells after treatment with extracts of *L. martagon* var. *cattaniae* and control. The data represent the means ± SD.

Slika 2: Frekvenca MN na 1000 BN celic po obravnavanju z izvlečki taksona *L. martagon* var. *cattaniae* v primerjavi z kontrolo. Rezultati so prikazani kot povprečje ± SD.

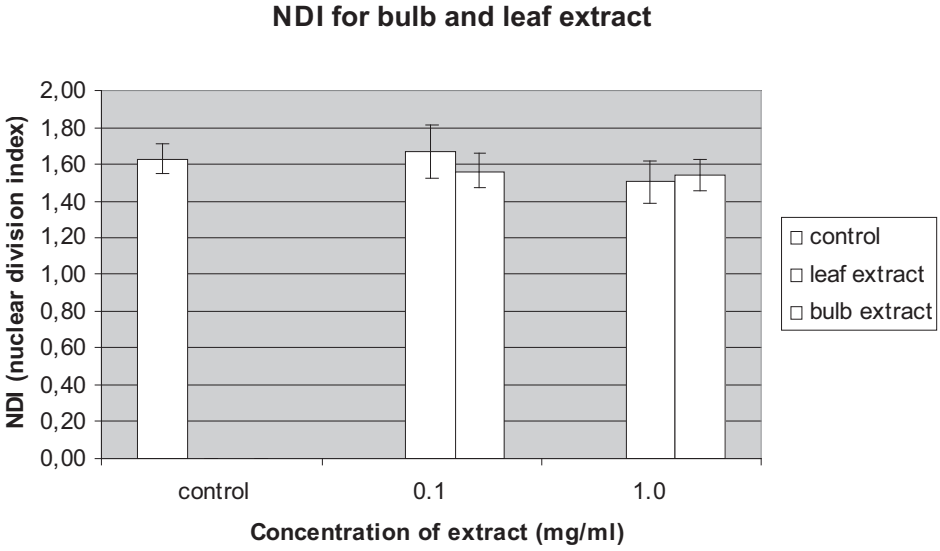

Figure 3: Nuclear division index after treatment with extracts of *L. martagon* var. *cattaniae* and control. The data represent the means ± SD.

Slika 3: Indeks delitve nukleusa po obravnavanju z izvlečki taksona *L. martagon* var. *cattaniae* v primerjavi z kontrolo. Rezultati so predstavljeni kot povprečje ± SD.

affects composition of obtained extracts. Ultrasound assisted extraction attracts more attention recently because of many benefits, primarily better penetration of solvent into plant particles and low extraction temperature which affects the stability of active components (Rouhani et al. 2009).

Weng and coworkers (2004) were doing extraction of loganin in wine on room temperature and they achieved concentration of 48 mg/l after 30 days, while using ultrasound assisted extraction the concentration of 50 mg/l was achieved after 2 days.

In this work total 9.85g of dried material was obtained (4.88g from leaves and 4.97g from bulbs). After extraction, 1.54 g of leaf powder and 1.12 g of bulbs powder was obtained.

Allium test

Four concentrations of extracts (0.1 mg/ml, 0.5 mg/ml, 1 mg/ml and 5 mg/ml) were examined. Mitotic index (MI), presenting percentage of cells in mitosis in total number of analyzed cells, was determined (Figure 1)

It was established that there were statistically significant differences between mitotic index in control and all tested samples.

In vitro micronucleus test on human lymphocytes

Binucleated cells (BN) with one and two micronuclei (MN) were observed. Frequency of MN on 1000 BN cells are presented in Figure 2.

Statistical evaluation of data using Z-test has shown that there is no statistically significant difference in frequency of micronuclei (MN) on 1000 binucleated (BN) cells among control and examined concentrations of leaf and bulb extracts.

Results of calculating NDI for controls and treated cultures are presented in Figure 3.

All extracts induced reduction of NDI comparing to control, except leaf extract with concentration 0.1 mg/ml which in two blood samples induced growth of NDI. After statistical evaluation of data using Independent Group Analysis ANOVA test it was determined that there was no statistical significance in NDI values between control and examined concentrations of leaf and bulb extracts. Using Newman-Keuls multiple comparison it was determined that there was no significant difference among all examined samples.

Conclusions

- Sterile germination of *Lilium martagon* var. *cattaniae* seed on MS medium with addition of 0.15 mg/l GA3 with removal of testa and border parts of endosperm was successful so in this work 100% germination was achieved.
- In this work multiplication was done on MS medium with 0.1mg/l GA3, 0.2mg/l IBA and 0.5mg/l BAP and satisfying results were achieved.
- Results of ultrasound assisted extraction were very good because from 4.88g of dried leaf 1.536g of dried extract was achieved, and from 4.97g of dried bulbs 1.117g of dried extract was achieved.
- There was statistically significant difference between all used concentrations of lilium extracts and negative control (water) on proliferation of cells of root tip of onion (*Allium cepa*). Leaf extract of 0.1 mg/ml significantly increased proliferation was significantly decreased. All concentrations of leaf extract significantly decreased proliferation, and effect was bigger with higher concentrations.
- Results of proliferation in micronucleus test on human lymphocytes are similar to those in allium test, but in this case there is no statistically significant difference between used extracts and control. To explain mechanisms with which extracts change proliferation it would be necessary to do chemical analysis of extracts and do some more investigations.
- Used extracts didn't show genotoxic properties under experimental conditions.

Povzetek

Lilium martagon L. var. *cattaniae* Vis. (*Liliaceae*) je endemični takson Dinaridov

(Šilić 2007). Čebula se največ uporablja v ljudski medicini na območju Mediterana. Pomembna je za fitokemijske in farmakološke raziskave. Čebula vrste *Lilium martagon* L. ima kardiotonične lastnosti in se uporablja pri obravnavanjih dismenoreje (Khare 2007) in za zdravljenje jetrenih bolezni pri ljudeh in živalih v Severni Albaniji (Pieroni et al. 2005). Čebula vrste *Lilium martagon* se uporablja za zunanje rane (Khare 2007), nekatere raziskave pa so pokazale prisotnost antikancerogenih komponent (Eisenreichová et al. 2004). *In vitro* kultura se uporablja za masovno produkcijo in tako za ohranjanje endemičnih sort lilij. Kaljenje semen taksona *L. martagon* var. *cattaniae* je potekalo na MS hranljivi podlagi, kateri 0,15 mg/l giberelinske kisline (GA_3). Kalitev je bila 100%. Za multiplikacijo smo uporabili MS podlago ter dodali 0,1 mg/l giberelinske kisline (GA_3), 0,2 mg/l indol-3-maslene kisline (IBA) ter 0,5 mg/l 6-benzilaminopurina (BAP). Po treh tednih kultivacije smo rastline posušili in material uporabili za ekstracijo. Uporabljena je bila ultrazvočna ekstrakcija. Rezultati ultrazvočne ekstrakcije so bili odlični, saj smo iz 4,85 g suhih listov dobili 1,54 g suhega izvlečka, iz 4,9 g suhe čebule pa 1,12 g suhega izvlečka. Za ugotavljanje genotoksičnega potenciala izvlečkov lista in čebul taksona *L. martagon* var. *cattaniae* smo uporabili Allium test in citokinetski-blok mikronukleus test na humanih limfocitih *in vitro*. V primerjavi s kontrolo so vse koncentracije izvlečkov lilij statistično značilno vplivale na proliferacijo celic korenin čebule (*Allium cepa*). Izvleček listov v koncentraciji 0,1 mg/ml je značilno povečal proliferacijo, medtem ko so imele vse druge koncentracije negativne vplive. Učinek se je povečeval z višjanjem koncentracije. Izračun NDI (nucelar division index) mikronukleus testa ni pokazal značilnih razlik.

Acknowledgements

This work was supported by Institute for Genetic Engineering and Biotechnology, Sarajevo. We are thankful to dr. sci. Edina Muratovic for providing seeds of *Lilium martagon* L. var. *cattaniae* Vis. used in this study as starting material.

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