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Behavioral, Morphological and Histopathological Effects of Sublethal Doses of Quercetin on the Species *Polycelis felina* (Dalyell)*

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The effect of quercetin was studied on the multi-eyed flatworm *Polycelis felina* (Daly.) in laboratory conditions. This is a very suitable test-organism available year-round in nature, easily maintained in the laboratory. The results showed that depending on the dose and recovery period quercetin caused locomotory (behavioral) changes in treated animals including aggregation in groups, resting and unsynchronized movements and twisting of particular body parts. Morphological changes in the form of depigmentation of certain body parts were the result of damage or deterioration of reticular and parenchymal cells. Numerous basophilic bodies representing parts of damaged and decomposed cells were determined in the gastroderm between the third and seventh day after treatment. All histological preparations revealed significant deterioration of cellular material, and therefore a damaging effect of quercetin on the multi-eyed flatworm *Polycelis felina* (Daly.). We found an increased number of neoblasts and reticular cells on the third day after treatment, so we concluded that quercetin in the applied doses had a stimulating effect on cell division of neoblasts and reticular cells of treated flatworms.

Key words: Quercetin; planarian; depigmentation; deformations; neoblasts, reticular cells.

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Quercetin is one of the most commonly used phenolic flavonoid compounds. Although absent in algae, flavonoids are one of the largest and most widespread groups of secondary plant metabolites (ERLUND 2002). They exert a wide range of biological effects. These effects are the results of the modulating activity of enzymes and nuclear receptors, effects on gene expression and on chelation of metal ions. Quercetin (3,5,7, 3', 4'-pentahydroxyflavone) is one of the most abundant flavonoids in plant food. Various anti-bacterial, anti-inflammatory and anticarcinogenic mechanisms of quercetin in the cell are associated with flavonoid compounds. Numerous mechanisms of quercetin on cell cycle, differentiation and apoptosis are associated with modulating the activity of enzymes, disruption of DNA structure and gene expression. Quercetin can cause programmed cell death, and this effect is connected with the accumulation of tumor suppressor protein p53, which may be associated with causing the break up of DNA strands by hydrogen peroxide (YAMASHITA *et al.* 1990), by topoisomerase (HODEK *et al.* 2002), inhibition of certain types of protein kinases (FORMICA & REGELSON 1995), and inhibition of expression of HSP (heat shock protein) at elevated temperatures (HOSOKAWA *et al.* 1992).

Quercetin is a very strong antioxidant substance and has a very positive effect on the human body. It is present in numerous plant species (onion, cabbage, apples, berries). Quercetin shows antioxidant, antiallergic, antiviral, and anticancer effects. It interferes with the endocrine system and has a germinative effect. It is effective in various dis-

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eases – leukemia, breast, ovary and liver cancer, and in certain doses the positive effect of quercetin is in blocking the growth of malignant cells and DNA synthesis (ERLUND 2002).

However, quercetin may have a mutagenic effect, which depends on experimental conditions, i.e. the presence of oxygen ions (FORMICA & REGELSON 1995).

Flavonoids affect the reproduction of plants and animals. Rutin, quercetin, red wine and some herbal teas that contain these flavonoids showed a weak genotoxic effect on *Drosophila* (GRAF *et al.* 1994), but show no effect on segregation of chromosomes during meiosis in the same species (SCHRAMM *et al*. 1998). It accelerates metamorphosis and causes histopathological changes in fatty tissue in larvae (ŠARIĆ *et al*. 2007). As a kinase inhibitor, quercetin has an effect on meiosis of oocitae in the bivalve *Spisula solidissima.* It inhibits the phosphorylation of proteins that influence the firing of nuclear membrane during meiosis.

Whether the effect of flavonoids is positive, negative or neutral depends on the temperature and the presence or absence of chlorogenic acid (STAMP & YANG 1996).

Quercetin at low concentrations can stimulate the division of cancer cells dependent on estrogenic receptors. Quercetin shows antiproliferative and cytostatic activity at higher concentrations *in vitro* on various types of cancer cells (FORMICA & REGELSON 1995; HODEK *et al.* 2002), which gives hope in the treatment of malignant disease.

The multi-eyed flatworm is a very suitable test-organism in similar studies because it is available year-round in nature, in the laboratory is easily maintained and has active mitotic and undifferentiated cells – neoblasts that are easily observed under a light microscope.

Polycelis felina (Daly.) belongs to the kingdom Animalia, phylum Platyhelminthes, class Turbellaria, order Tricladida, family Planariidae. *Polycelis felina* (Daly.) is a wild, free-living flatworm, and is usually found in springs, clean streams and lakes of Europe. It inhabits waters rich in oxygen, at a temperature of 6^{o}C to 15^{o}C, a well tolerated and fast water flow. The planarian's body is covered with a single-layer epidermis and with real epidermal cells with numerous cilia on the ventral and lateral sides, and with glandular cells, which produce rhabdites. Rhabdites are rod formations, followed by ejection from the body, that produce mucus whose main function is to protect the animal from predators. Epidermal cells are located at the basal membrane, followed by a muscular layer. Spaces between muscles and internal organs form the parenchyma or mesenchyma. Large reticular cells and smaller, spindle-shaped or round neoblast cells constitute the parenchyma. Neoblasts are the only cell type in planarians that can divide mitotically (VILLAR & SCHAEFFER 1993). They behave like embryonic cells (HANSEN *et al.* 1993), are totipotent and can differentiate into any of the 12-14 specialized planarian cell types (BAGUNA & ROMERO 1981). This is a unique phenomenon in the animal world and a very useful adaptive mechanism, caused by the constant presence of a number neoblasts that are in the G2 phase of the cell cycle and after the stimulus immediately enter mitosis (BAGUNA 1974; BAGUNA 1975). There is evidence that neoblasts are involved in planarian regeneration. The multi-eyed flatworm *Polycelis felina* (Daly.) has another type of specialized parenchyma cell called reticular cells that are phagocytic and are capable of migration. They engulf old and damaged cells, and in their cytoplasm contain numerous glycogen granules (MORITA & BEST 1984).

The aim of this study was to determine the effect of sublethal doses of quercetin in the planarian species *Polycelis felina* (Daly.), on the behavior, morphology and cytohistological changes under laboratory conditions.

Material and Methods

Polycelis felina (Daly.) was collected from Gračani pond in Zagreb, Croatia. Animals were of comparable size and developmental stage. Their lengths varied between 12-15 mm. Four groups of planarians were used in the experiment. Each group consisted of 10 individuals. They were kept in a Petri dish of 60 ml volume, dimensions 5.5 cm in diameter and 3.5 cm in height. Animals were treated with concentrations of 0.2, 0.3, and 0.6 g/l of quercetin (Sigma, Aldrich, Switzerland) for 24 hours. These concentrations were determined experimentally as sublethal doses of a potentially lethal substance. In lower concentrations we did not notice any changes. Higher concentrations than those applied in this experiment were lethal to planarians. The first group of planarians served as a control and was kept in clean aquarium water. Quercetin was dissolved in a dimethyl sulfoxide (DMSO) concentration of 0.1 %. After 24 hour treatment all planarians were washed out in clean aquarium water and transferred into clean aquarium water afterwards. Over the next 15 days of the experiment, planarians were kept in a refrigerator at a temperature of 7°C. Aquarium water was changed daily.

During the experiment we daily monitored the following parameters: mortality, locomotory (behavioral) and morphological changes using a magnifying glass.

For histological examination with a magnifying glass, we selected two visibly damaged planarians from each concentration and two from the control group. We analyzed animals on the first, second, third and seventh day during the recovery period. Planarians were fixed in Bouin's fixative (15 ml of a saturated solution of picric acid, 5 ml of 36% formaldehyde and 1 ml of glacial acetic acid) for 24 hours. After fixation, planarians were dehydrated in an ethanol series of ascending grades (70%, 80%, 96%, 100%), cleared in chloroform, and afterwards embedded in paraplast. Each tissue block was cut with a microtome in 7 micron thick sections and mounted onto glass slides using glycerin gelatin.

After deparaffination with xylene, slides were rehydrated in an ethanol series of descending grades and then stained with Meyer's solution and toluidine blue.

Stained slides were dehydrated in an ethanol series of ascending grades and embedded in Canada balsam. Microscopic slides were analyzed with a Nikon Eclipse E 600 light microscope. Micrographs were recorded with a digital camera (Nikon DXM 1200).

Results

Morphological and locomotory (behavioral) changes

On the first day after treatment, mortality was not detected in any of the groups of the treated planarians. Moreover, mortality was not noticed throughout the experiment. Morphological or locomotory changes were not recorded in the control group; the animals were not depigmented on the outer body parts. They were moderately active in swimming, were not disoriented, and no contractions of the body were noticed. Planarians showed locomotory (behavioral) changes in all three applied concentrations of quercetin. About 50% of planarians treated with 0.2 and 0.3 g/l of quercetin and 80% of planarians treated with 0.6 g/l of quercetin did not move as individuals in control groups did, but formed groups and stood motionless on the bottom of the dish (Table 1). Animals twisted the whole body. About 10% of treated planarians in all concentrations had small wounds, recesses and depigmented areas on the body surface (Table 2). Auricles were damaged in planarians treated with 0.3 g/l of quercetin, they were not pointed out, and in some planarians damage was present on the posterior parts of the body. Twenty percent of the planarians treated with the lowest concentration of quercetin had their pharynx thrown out. On the second day after treatment 50% of planarians with 0.6 g/l of quercetin remained motionless on the bottom of the dish. Depigmented areas were still visible in 30% of the treated planarians. On the third day after treatment 20% of the animals showed uncoordinated movements and twisting of the whole body and 30% of the animals had depigmented areas. Small wounds were not visible by means of a stereomicroscope. On the fourth and fifth day after treatment, planarians moved onto the walls of the dish as in the control group. Lighter areas were visible on outer body parts. After 15 days about 10% of the treated animals had deformations on auricles or posterior parts of the body.

Table 1

Contraction of planarians in the experiment (%)

g/l quercetin / hours of recovery	24	48	72	360
0.2	50	30	20	20
0.3	50	30	20	10
0.6	80	50	10	20

Table 2

Depigmentation of planarians in the experiment (%)

g/l quercetin / hours of recovery	24	48	72	360
0.2	10	30	40	20
0.3	10	20	30	20
0.6	30	30	30	30

Histopathological changes

All the treated planarians showed similar histopathological changes. As in morphological and locomotory (behavioral) observations, these changes were most intensive during the first five days after the treatment. Animals treated with higher concentrations showed more intensive changes in comparison to the control (Fig. 1A).

On the first day after treatment we noted damage to the outer mucous layer and epidermis, as well as

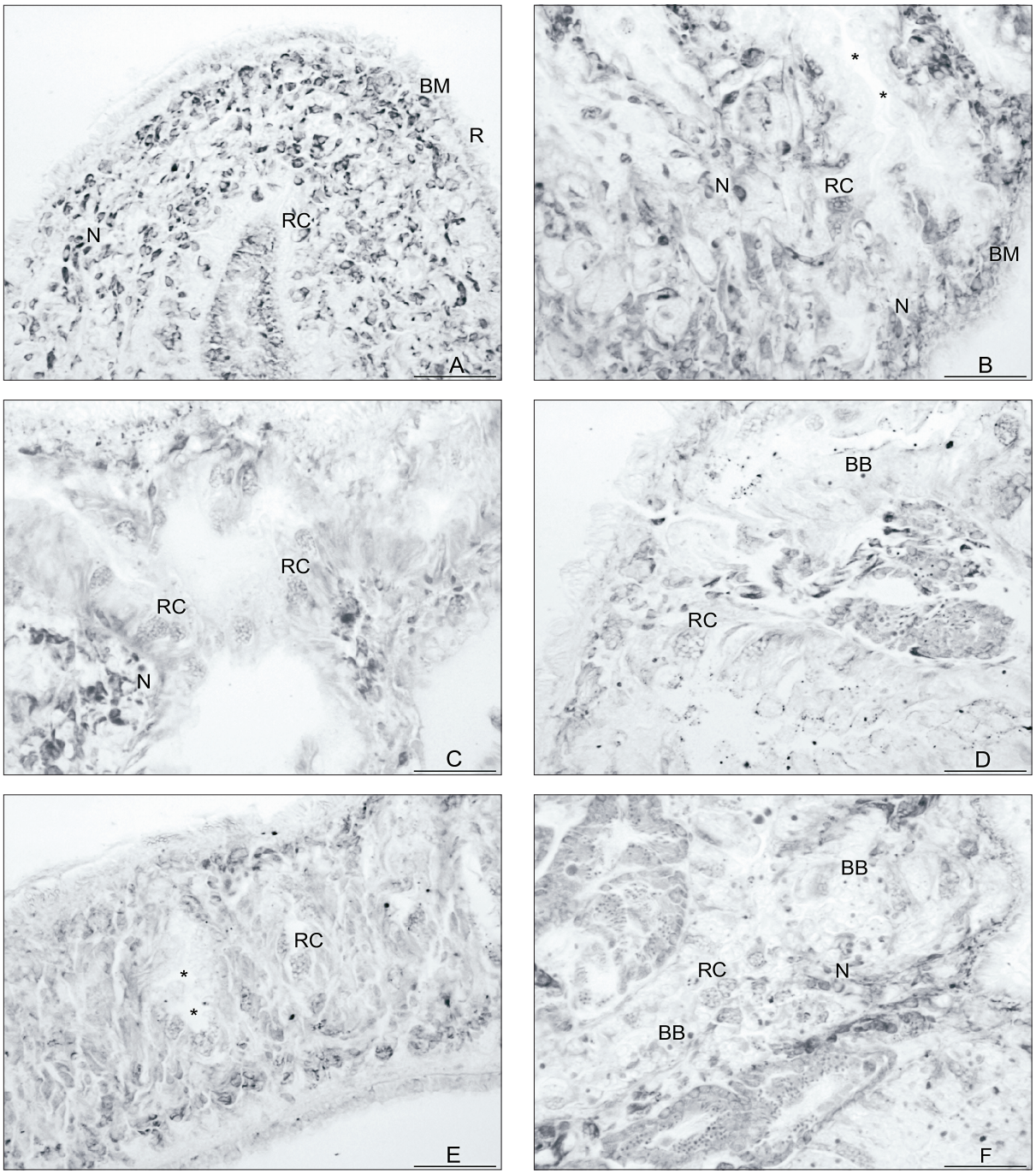

Fig. 1. Longitudinal section of the planarian tissue. Toluidine blue. Bars – 20 microns. A) Control. B) treated with 0.2 g/l of quercetin and fixed on the second day after the treatment. Damage to the basal membrane (BM), visible empty areas within parenchyma (*), number of neoblasts (N) and reticular cells (RC) increased. C) treated with 0.6 g/l of quercetin and fixed on the third day after treatment. Increased number of reticular cells (RC) with large white vacuoles. Numerous neoblasts (N) present. D) treated with 0.2 g/l of quercetin and fixed on the seventh day after treatment. Most reticular cells (RC) contain large, bright vacuoles. Basophilic bodies (BB) present in parenchyma. E) treated with 0.3 g/l of quercetin and fixed on the seventh day after treatment. Recovering epidermal layer. Increased number of reticular cells (RC). Numerous basophilic bodies (BB) present in parenchyma. Visible empty areas (*) within parenchyma. F) treated with 0.6 g/l of quercetin and fixed on the seventh day after treatment. Numerous basophilic bodies (BB) present in parenchyma. Neoblasts (N). Decreased number of reticular cells (RC) with large, white vacuoles.
BB – basophilic body, BM – basal membrane, N – neoblast, R – rhabdite, RC – reticular cell, * – empty areas (without cells in parenchyma)

splitting of ectodermal cells from the basal membrane throughout most of the body of the treated planarians in all three applied concentrations. Rhabdite damage was obvious in some areas of the treated planarians. Particular areas of parenchyma were filled with mucous and disintegrated cells. On the second and third day after treatment, the outer mucous layer was completely destroyed and rhabdites were degraded. The basal membrane was also damaged so there was no boundary between the surface and inner layer. Large areas without cells were visible within the parenchyma. In the vicinity of these areas neoblasts were present in higher numbers. Some neoblasts were in the process of differentiation. Numerous reticular cells with large and abundant vacuoles were visible; reticular cells in that number were not present in the control (Fig. 1B, C). Neoblasts showed differentiation towards the reticular cells in the parenchyma. Compared to the control, cells of weaker contrast were present in the testes of planarians treated with 0.2 g/l of quercetin. Changes were not noticed in egg cells. On the seventh day after treatment, regeneration of the epidermal layer occurred and rhabdites in these places were scarce in comparison to the control. The number of neoblasts was lower compared to the control. The number of reticular cells in lower concentrations increased, while their number in the highest concentration decreased. Most of the reticular cells were filled with light vacuoles and numerous basophilic bodies were present in parenchyma as a result of damaged cells. Empty areas without cells were visible within the parenchyma (Fig. 1D, E, F).

Discussion

In the conducted experiment quercetin caused locomotory, morphological and histological changes. Mortality was not detected in any of the groups of planarians. Quercetin was dissolved in dimethyl sulfoxide (DMSO), the concentration of 0.1% in the test solution. It was found that DMSO in this concentration did not induce toxic or behavioral changes in planaria (PAGÁN *et al.* 2006). It has been established that numerous xenobiotics and essential compounds in sublethal doses cause locomotory (behavioral), morphological and histological changes, from the cellular to the tissue level, depending on the doses and duration of treatment (FRANJEVIĆ *et al.* 2000; KALAFATIĆ *et al.* 2004a; KALAFATIĆ *et al.* 2004b; KOVAČEVIĆ *et al.* 2009).

It is known that quercetin in particular doses affects the reproduction of plants and animals. Vinegar flies fed with quercetin have more numerous offspring and a shortened larval stage, although quercetin does not affect chromosome segregation in this species. Changes in the structure of fat tissue in *Drosophila* were also established in treatment with quercetin (ŠARIĆ *et al*. 2007).

The most intense locomotory (behavioral), morphological and cytohistological changes were noticed during the first three days after treatment.

On the third day after treatment an increased number of neoblasts and reticular cells was detected suggesting that quercetin in the applied doses exerted a stimulating effect on neoblast division and differentiation in reticular cells. Namely, xenobiotics in certain concentrations exhibit a stimulative effect on the division of particular cells (KOVAČEVIĆ *et al.* 2009).

All the applied concentrations of quercetin caused locomotory (behavioral) changes but the most pronounced effect was observed with the 0.6 g/l dose. Planarians were contracted and remained motionless on the bottom of the dish during the first days after treatment. The outer mucous layer, the first barrier for toxicants, was damaged in a high number of treated planarians. By contractions of the body, planarians try to prevent the toxicant from entering the body. Grouping, lowered locomotory activity and contraction of the damaged body parts also represent behaviors that decrease toxicant entrance into the body (HORVAT *et al.* 2005; KOVAČEVIĆ *et al.* 2009).

Histopathological changes occurred in parallel to the pronounced morphological and locomotory changes. Damaged areas and the deterioration of the majority of the cellular material were noticed on the second and third day after treatment. These areas were filled with mucous and dead cells. We assume that exactly these areas observed under the stereomicroscope were visible as depigmented areas on the planarian body. During this period undifferentiated totipotent cells (neoblasts) showed the highest intensity of differentiation into reticular cells. The number of reticular cells with numerous large vacuoles increased on the third day after treatment in all analyzed planarians. We suggest that reticular cells phagocytized the molecules of quercetin and damaged body parts that led to increased vacuolization. For example, accumulation of aluminum in reticular cells may take place (KOVAČEVIĆ *et al.* 2009). An increased number of basophilic bodies, retained in a certain number also on the seventh day after treatment, represented the cellular structures of damaged cells. Permanent deformations were noticed in 10 % of treated planarians after regeneration of damaged body parts (KOVAČEVIĆ *et al.* 2009) that could be a consequence of mutations by intensive mitotic and differentiative processes, established in treatments with other xenobiotics (KALAFATIĆ & TOBORŠAK 1998; KOPJAR *et al.* 1997).

The occurrence of permanent deformations after the regenerative processes supports the fact that quercetin in sublethal doses in a smaller number of individuals also disturbs normal processes in division and differentiation of cells. However, we cannot exclude other factors such as starvation.

Since we have shown the damaging effect of quercetin, our next step is to determine the maximum concentration that does not cause toxic changes in the activity of enzymes, lipids and DNA molecules in the cell. For this purpose, we will trace the activity of glucose-6-phosphate dehydrogenase, then free radical damage to DNA by comet assay, and protein damage. We plan to measure carbonyl activity and lipid peroxidation to show damage in lipid membranes.

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References

BAGUNA J. 1974. Dramatic mitotic response in planarians after feeding, and a hypothesis for the control mechanism. J. Exp. Zool. **190**: 117-122.

BAGUNA J. 1975. Mitosis in the intact and regenerating planarian *Dugesia meditarranea* n. sp. II. Mitotic studies during regeneration, and a possible mechanism of blastema formation. J. Exp. Zool. **195**: 65-80.

BAGUNA J., ROMERO R. 1981. Quantitative analysis of cell types during growth, degrowth and regeneration in the planarians *Dugesia mediterranea* and *Dugesia tigrina.* Hydrobiologia **84**: 181-194.

ERLUND I. 2002. Chemical analysis and pharmacokinetics of the flavonoids quercetin, hesperetin and naringenin in humans. [Academic dissertation]. National Public Health Institute, Department of Applied Chemistry and Microbiology, University of Helsinki. Http://www.ktl.fi/publications/2002/a27.pdf.

FORMICA J. V., REGELSON W. 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem. Toxicol. **33**: 1061-1080.

FRANJEVIĆ D., KRAJNA A., KALAFATIĆ M., LJUBEŠIĆ N. 2000. Toxic effects of copper upon the planarian *Polycelis felina* (Daly.). Periodicum Biologorum. **102**: 283-287.

GRAF A., ALONSO MORAGA A., CASTRO R., DIAZ CARRILLO E. 1994. Genotoxicity testing of different types of beverages in the drosophila wing somatic mutation and recombination test. Food Chem. Toxicol. **32**: 423-430.

HANSEN L. G., TEHSEEN W. M., FOLEY G. L., SCHAEFFER D. J. 1993. Modification by polychlorinated biphenyls (PCBs) of cadmium induced lesions in the planarian model, *Dugesia dorotocephala*. Biomed. Environ. Sci. **6**: 367-384.

HODEK P., TREFIL P., STRIBOROVA M. 2002. Flavonoids-potent and versatile biologically active compounds interacting with cytochromes P450. Mini review. Chem. Biol. Interact. **139**: 1-21.

HORVAT T., KALAFATIĆ M., KOPJAR N., KOVAČEVIĆ G. 2005. Toxicity testing of herbicide norflurazon on aqatic bioindicator species-the planarian *Polycelis felina* (Daly.). Aquatic Toxicology. **73**: 342-352.

HOSOKAWA N., HIRAYOSHI K., KUDO H., TAKECHI H., AOIKE A., KAWAI K., NAGATA K. 1992. Inhibition of the activation of heat shock factor in vivo and in vitro by flavonoids. Mol. Cell Biol. **12**: 3490-3498.

KALAFATIĆ M., TOBORŠAK S. 1998. Effects of chromium upon neoblast division in the regenerates of *Polycelis felina*. Biologia **53**: 321-325.

KALAFATIĆ M., KOPJAR N., BESENDORFER V. 2004a. The impairments of neoblast division in regenerating planaria *Polycelis felina* (Daly.) caused by *in vitro* treatment with cadmium sulfate. Toxicology in Vitro **18**: 99-107.

KALAFATIĆ M., KOPJAR N., BESENDORFER V. 2004b. The influence of mercuric chloride on neoblast division in regenerating planarian *Polycelis felina* (Daly.). Water Air Soil Pollut. **156**: 195-210.

KOPJAR N., KALAFATIĆ M., BESENDORFER V. 1997. Mitotic and chromosomal disturbances in the planarian *Polycelis felina* caused by manganese. Biologia **52**: 469-474.

KOVAČEVIĆ G., GREGOROVIĆ G., KALAFATIĆ M., JAKLINOVIĆ J. 2009. The effect of Aluminium on the Planarian *Polycelis felina* (Daly.). Water Air Soil Pollut. **196**: 333-344.

MORITA M., BEST J. B. 1984. Electron microscopic studies of planarian regeneration. IV. Cell division of neoblasts in *Dugesia dorotocephala*. J. Exp. Zool. **229**: 425-436.

PAGÁN O. R., ROWLANDS A. L., URBAN K. R. 2006. Toxicity and behavioral effects of dimethylsulfoxide in planaria. Neuroscience Letters **407**: 274-278.

SCHRAMM D. D., COLLINS H. E., HAWLEY R. S., GERMAN J. B. 1998. Unaltered meiotic chromosome segregation in *Drosophila melanogaster* raised on a 5% quercetin diet. Food Chem. Toxicol. **36**: 585-589.

STAMP N. E., YANG Y. L. 1996. Response of insect herbivores to multiple allelochemicals under different thermal regimes. Ecology **77**: 1088-1102.

ŠARIĆ A., KALAFATIĆ M., RUSAK G., KOVAČEVIĆ G., FRANJEVIĆ D., GUTZEIT H. O. 2007. Postembryonic development of *Drosophila melanogaster* under the influence of quercetin. Entomol. News **118**: 235-240.

VILLAR D., SCHAEFFER D. J. 1993. Morphogenic action of neurotransmitters on regenerating planarians – a review. Biomed. Envirom. Sciences **6**: 327-347.

YAMASHITA Y., KAWADA S., NAKANO H. 1990. Introduction of mammalian topoisomerase II dependent DNA cleavage by nonintercalative flavonoids, genistein and orobol. Biochem. Pharmacol. **39**: 737-744.