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Determination of Antioxidant Activity and Toxicity of *Sambucus nigra* Fruit Extract Using Alternative Methods

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Summary

The aim of the study is to measure the *in vitro* antioxidant activity of elderberry (*Sambucus nigra*) fruit extract and to study its toxicity in a plant model system with regard to its possible application in food and agricultural industry. The antioxidant capacity of *Sambucus nigra* fruit powder was quantified by a photochemiluminescence method. The study of phytotoxicity of aqueous solutions of powder extract was performed using *Allium cepa* as a test organism. Photochemiluminescence determinations showed a very high antioxidant capacity of the product but also revealed its cytotoxic effect, along with mitodepressive activity and even inhibiton of mitosis at the preprophase stage when the fruit extract was used at higher concentrations. Aqueous solutions of the fruit powder have a reasonably expressed mutagenic activity *in vivo* on the radicles of *Allium cepa*, especially when they are used at a concentration of 1 g/dL for a prolonged time (48 h). At lower concentrations (0.1 g/dL), however, the mutagenic effect was not observed any more. The conclusion of our study is that *Sambucus nigra* fruit extract powder has a very high *in vitro* antioxidant activity and no mutagenic effects at low concentrations, which makes it recommendable for applications in the food industry.

Key words: *Sambucus nigra*, photochemiluminescence, antioxidant capacity, mitotic index, phytotoxicity

Introduction

Anthocyanins have received increasing attention during the last fifteen years due to their activities as safe and potent antioxidants (*1*), because of which they are considered important nutraceuticals (*2*). Moreover, their attractive bright purple colour and water solubility, along with their positive therapeutic effects, make berry anthocyanins potential substitutes for synthetic foodstuff colourants (*3*).

A number of studies have already shown that anthocyanins have positive therapeutic properties and their various biological activities have been extensively reviewed (*1,4*). They are claimed to provide beneficial effects in reducing the incidence of cardiovascular diseases (*5,6*), cancer, hyperlipidaemia and other chronic diseases (*7,8*), as well as ocular deficiencies (*9*). Recent studies have

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indicated that anthocyanins may even be neuroprotective agents and could also be beneficial in ameliorating ethanol neurotoxicity (*10*).

Despite the comparatively large amount of studies about their effects, different aspects regarding their way of action at molecular level still remain unclear, taking into account their rather low bioavailability and short time of action within the organism (*11*). Also, there are insufficient data about a possible toxicity of anthocyanin extracts (*12*). Up to now, only a few papers have reported the effects of anthocyanins or anthocyanin-rich extracts on the overall gene expression (*13–15*). Before promoting them as safe dietary supplements some more investigations about possible toxic effects should be performed, taking the safety of the supplements into account as the key concern (*16*).

The objective of the present study is to evaluate possible cytotoxic and genotoxic effects of anthocyanin-rich elderberry (*Sambucus nigra*) concentrate using an alternative method (*Allium* test) to assess its antioxidant activity evaluated by chemiluminometry.

Materials and Methods

Fruit extract and its preparation

The analyzed material consisted of *Sambucus nigra* fruit extract powder prepared by BerryPharma AG (Leichlingen, Germany). The powder was obtained by spray drying with corn maltodextrin (dextrose equivalent of 8–10 %, according to the manufacturer's declaration) of an elderberry extract obtained by membrane separation. The final product had an anthocyanin content of 10 %, expressed as cyanidin-3-glucoside according to the manufacturer's label.

Determination of antioxidant capacity using chemiluminometry

The antioxidant capacity of the product was measured using a photochemiluminometer PHOTOCHEM® (Analytik Jena AG, Jena, Germany) according to the recommended protocols (*17,18*). For the experiment, a stock solution of elderberry extract in methanol (0.05 g/dL) was prepared. Subsequently, four batches of 5, 10, 20 and 30 μL of stock solution were made.

The antioxidant capacity of the samples was quantified by comparison with the standard, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) calibration curve expressed as equivalent (TE) units of standard substance.

For additional fine tuning of the antioxidant capacity of elderberry extracts, two standard compounds, known as powerful antioxidants, quercetin and rutin, both obtained from Fluka (Sigma-Aldrich, St. Louis, MO, USA), were used as primary reference standards. Measurements for both these substances were performed in the same way as it was done for the elderberry samples.

Determination of cytotoxic and genotoxic effects using the Allium test

Allium test was used to assess the cytotoxic and genotoxic effects (*19–21*). *Allium cepa* is a plant particularly sensitive to the harmful effects of different substances. The inhibition of *Allium* root growth and the changes occuring in chromosomes following the treatment with a certain substance can clearly demonstrate toxic effects of the substance in question. Also, as the chromosomes of *Allium cepa* are relatively large and few, it is easy to observe them under light microscope. Apart from that, the root meristems contain a large number of dividing cells. Therefore, our test uses meristematic plant cells to investigate the mechanisms that permit extrapolation to animal cells, thus providing information regarding possible genotoxic effects of these chemicals in mammals and especially in humans (*22,23*).

The *Sambucus nigra* fruit powder was dissolved in distilled water at different concentrations (0.1, 0.5 and 1 g/dL). The *Allium cepa* bulbs were submerged in these solutions for 6, 24 and 48 h, and tap water was used as control. Fragments of young roots were fixed for 16 h at 4 °C with a mixture of ethanol/acetic acid in a volumetric ratio of 3:1, followed by a mild acid hydrolysis in 1 M HCl solution. The roots were coloured by the Feulgen method using Schiff solution treatment for 90 min followed by washing with tap water. The slides were prepared by squashing and the samples were analyzed under a light microscope (Epifluorescent Microscope Fluo 2 with a Bel Photonics DV-1300 digital camera, BEL Engineering s.r.l., Monza, Italy). For each slide ten microscopic fields with good density of cells for mitotic index calculation and another ten different microscopic fields for abnormal interphases and chromosomal aberrations were studied.

The formula used for the mitotic index (*MI*) calculation was:

$$MI = N(\text{cells at mitosis})/N(\text{analyzed cells}) \cdot 100 \quad /1/$$

Results and Discussions

Antioxidant capacity of S. nigra extract

A very high antioxidant capacity was observed in the four batches of stock solution used for determinations according to the protocols (Table 1). The values of stock solutions of 20 and 30 μL were too high and therefore could not be properly measured. The lowest sample volume in which the antioxidant activity was measured was 10 μL (5 μg of elderberry extract powder), which was equivalent to 0.747 nmol of Trolox.

The antioxidant capacity of the two standard substances (quercetin and rutin) showed high values as well: 5 μg of quercetin had an antioxidant activity similar to 2.654 nmol of TE, and 5 μg of rutin showed an antioxidant activity of 1.824 nmol of TE (Table 1). These data are supported by the information presented by Heim *et al.* (*24*), according to which quercetin and rutin have a high Trolox-equivalent antioxidant capacity (TEAC) value, reflecting stronger antioxidant activities.

There are many studies supporting the high antioxidant power of anthocyanins from different sources, measured by several methods which are in agreement with our presented data (*24–27*). Up until now, a generally accepted or standardized method has not existed to accurately evaluate the antioxidant capacity of biological

Table 1. Antioxidant capacities of four volumes of elderberry extract stock solution in comparison with standard solutions

No.	Sample	*V*(sample) / μL	TEAC/*V*(sample) / nmol/μL	Observations
1	γ(elderberry extract stock solution)=0.05 g/dL	5	0.048	sample is too diluted
2	γ(elderberry extract stock solution)=0.05 g/dL	10	0.747	optimal volume and concentration selected
3	γ(elderberry extract stock solution)=0.05 g/dL	20	3.299	sample is too concentrated
4	γ(elderberry extract stock solution)=0.05 g/dL	30	9.241	sample is too concentrated
5	γ(quercetin)=0.01 g/dL	5	1.327	optimal volume and concentration selected
6	γ(rutin)=0.01 g/dL	5	0.912	optimal volume and concentration selected

samples of different origin. Recently, in order to choose the most effective one, the performances of several methods have been compared (*27*,*28*). In general, the authors maintain that the chemical content of the samples does not always correlate with their antioxidant power (*28*). For more accurate estimations in the future, a more appropriate method for antioxidant power measurement, correlating with the biological activity rather than with the chemical content, could be useful.

In comparison with the information provided by other authors (*27*,*28*), our extract seems to have a very high antioxidant capacity at very low concentrations.

Cytotoxic and genotoxic effects of S. nigra extract

In our study we observed normal chromosomes and also normal cell division behaviour in the untreated control roots, with a mitotic index of 15.8. The test roots, however, were shorter and less numerous, so that even macroscopically certain differences to the control became evident.

Compared to controls, a decrease in the mitotic index was evident in all variants. The highest percentage of dividing cells was in the prophase, followed at some distance by telophase. The smallest number of cells in metaphase and anaphase was recorded in variants 6, 7, 8 and 9. In controls as well as in the treated variants, the predominance of prophase and telophase over the metaphase and anaphase was evident (Table 2). The highest percentage of dividing cells was registered in variant no. 1 (14.2 %), shown in Table 2. The largest number of cells (34) in prophase was registered in variant 1, the largest number of cells (29) in telophase in variant 6. The highest number of metaphasic cells (7) was evident in variants 1 and 3, followed by variants 2, 4 and 5, with six cells in metaphase. Most of the anaphasic cells (8) were in variant 1, followed by variants 2 and 3 with seven cells.

The mitotic index decreased especially in the case of 1 g/dL of *Sambucus nigra* fruit extract powder solution after 48 h of treatment, supporting the idea that cell division, compared to controls, is slowed down (Fig. 1). These microscopic observations are in agreement with the macroscopic results (root number and size) mentioned above.

Chromosomal aberrations are relatively small, randomly distributed, probably depending on the concentration of *Sambucus nigra* fruit extract powder and treatment period. The phytotoxicity test revealed a minor cytotoxic effect, with a mitodepressive activity and inhibition of mitosis at the preprophase stage. There were cells with large nuclei and uncoiled forms, cells with pyknotic chromosomes with uncoiled and sticky forms, cells with anaphase or telophase bridges as a result of errors in chromosome separation, or cells with heterochromatic or vacuolated nuclei. Moreover, during prolonged tratment, plasmolysis of the cells and chromatin condensation were observed.

After treatment with the solution of 0.1 g/dL of *Sambucus nigra* fruit extract for 6 h, the cells with big nuclei

Table 2. Number of analysed cells for citogenetic studies regarding the effects of *Sambucus nigra* fruit extract powder on cell division

Variant	Total studied cells	Total interphase cells		Total division cells		Total prophase cells		Total metaphase cells		Total anaphase cells		Total telophase cells	
	N (total cells)	*N* (cells)	(*N*(cells)/*N*(total cells))/%	*N* (cells)	(*N*(cells)/*N*(total cells))/%	*N* (cells)	(*N*(cells)/*N*(total cells))/%	*N* (cells)	(*N*(cells)/*N*(total cells))/%	*N* (cells)	(*N*(cells)/*N*(total cells))/%	*N* (cells)	(*N*(cells)/*N*(total cells))/%
Control	500	421	84.2	79	15.8	28	5.6	15	3.0	16	3.2	20	4.0
V1	500	429	85.8	71	14.2	34	6.8	7	1.4	8	1.6	22	4.4
V2	500	432	86.4	68	13.6	29	5.8	6	1.2	7	1.4	26	5.2
V3	500	431	86.2	69	13.8	32	6.4	7	1.4	7	1.4	23	4.6
V4	500	432	86.4	68	13.6	31	6.2	5	1.0	5	1.0	27	5.4
V5	500	436	87.2	64	12.8	27	5.4	5	1.0	4	0.8	28	5.6
V6	500	439	87.8	61	12.2	24	4.8	3	0.6	5	1.0	29	5.8
V7	500	441	88.2	59	11.8	31	6.2	3	0.6	3	0.6	22	4.4
V8	500	448	89.6	52	10.4	27	5.4	2	0.4	4	0.8	19	3.8
V9	500	453	90.6	47	9.4	25	5.0	3	0.6	2	0.4	17	3.4

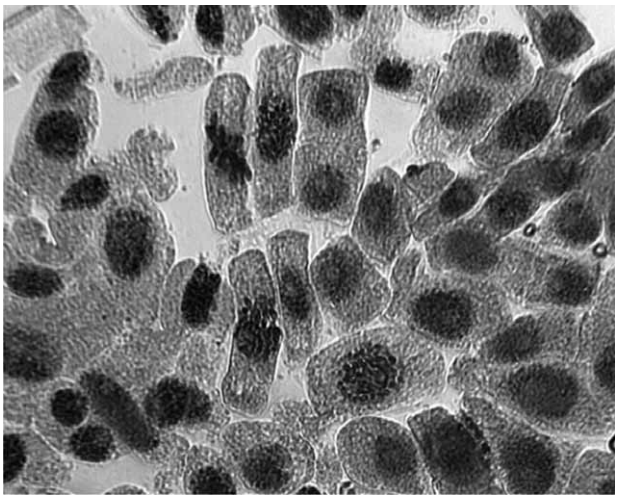

Fig. 1. Root meristematic cells of *Allium cepa* control variant. Cells are in prophase, metaphase and telophase

of abnormal shape and cytokinesis with longitudinal separation of daughter cells evolved (Fig. 2). This may be due to some disequilibrium as a consequence of the accumulation of genetic material in too large quantities. The majority of the cells were in interphase, prophase and cytokinesis as a consequence of the treatment with a solution of 0.1 g/dL of *Sambucus nigra* fruit extract powder solution for 24 h. Later, after 48 h of treatment, abnormal shape of nuclei developed more frequently.

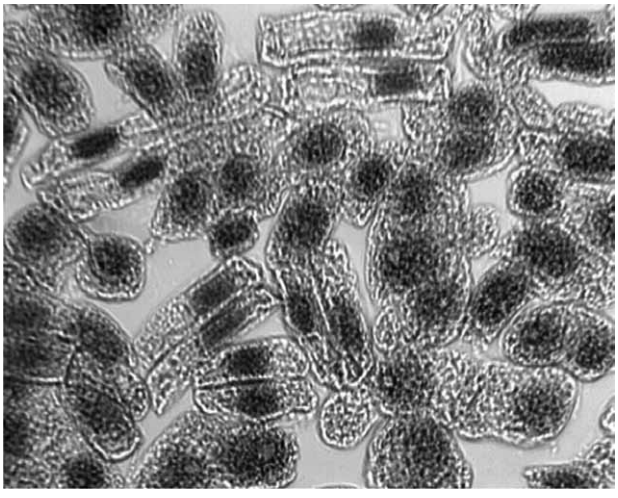

Fig. 2. Root meristematic cells of *Allium cepa* treated with 0.1 g/dL of fruit extract powder solution for 6 h. Cells are in prophase, telophase and cytokinesis with longitudinal separation of daughter cells

Following the treatment with *Sambucus nigra* fruit extract powder solution at a concentration of 0.5 g/dL for 6 h, cells in prophase with pyknotic chromosomes, cells with uncoiled and sticky chromosome forms, cells with micronuclei, and cells with vacuolated nuclei were observed (Fig. 3). After treatment with a solution of 0.5 g/dL of *Sambucus nigra* fruit extract powder for 24 h, the majority of cells were in interphase, prophase, telophase and cytokinesis with plasmolysed cells in prophase and

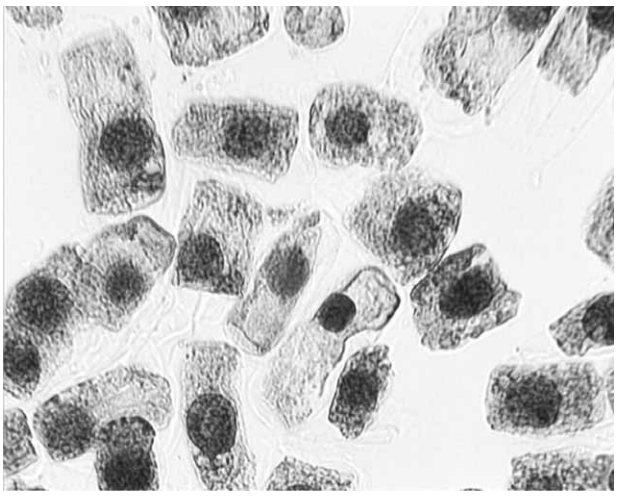

Fig. 3. Root meristematic cells of *Allium cepa* treated with 0.5 g/dL of fruit extract powder solution for 6 h. Cells are in prophase with pyknotic chromosomes, with uncoiled and sticky form and micronuclei

cytokinesis with longitudinal separation of daugther cells. Consequently, after 48 h of treatment, plasmolysis became more frequent, with plasmolysed cells observable in the interphase, prophase and telophase.

After treatment with a solution of 1 g/dL of *Sambucus nigra* fruit extract powder solution for 6 h, cells with pyknotic chromosomes, with uncoiled and sticky form, plasmolysed cells in prophase and metaphase, cells with heterochromatinised nuclei in prophase, cells with pyknotic chromosomes in prometaphase and cells with anaphase or telophase bridges (Fig. 4) were observed. Treatment with a solution of 1 g/dL of *Sambucus nigra* fruit extract

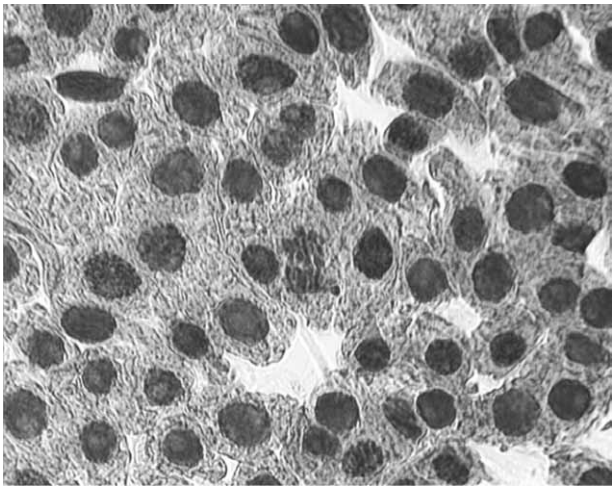

Fig. 4. Root meristematic cells of *Allium cepa* treated with 1 g/dL of fruit extract powder solution for 6 h. Cells are in prophase with heterochromatic nuclei, prometaphase with pyknotic chromosomes and anaphase bridges

powder solution for 24 h led to interphase, prophase, telophase and heterochromatinised nuclei, pyknotic chromosomes, anaphase bridges and abnormal nuclei shapes in the majority of cells (Fig. 5). In this last variant, nuclei

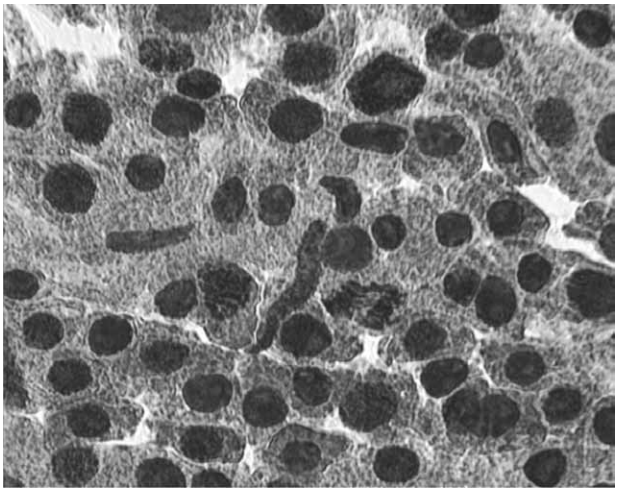

Fig. 5. Root meristematic cells of *Allium cepa* treated with 1 g/dL of fruit extract powder solution for 24 h. Cells are in prophase with anaphase bridges and abnormal shape of nuclei

of irregular shape and size were observed and chromosomes appeared either big with a relaxed chromatin, or small but presenting a compact chromatin but with irregular shape. Finally, after 48 h, cells in prophase with heterochromatinised nuclei, prometaphase and metaphase with pyknotic chromosomes and anaphase bridges evolved (Figs. 6 and 7).

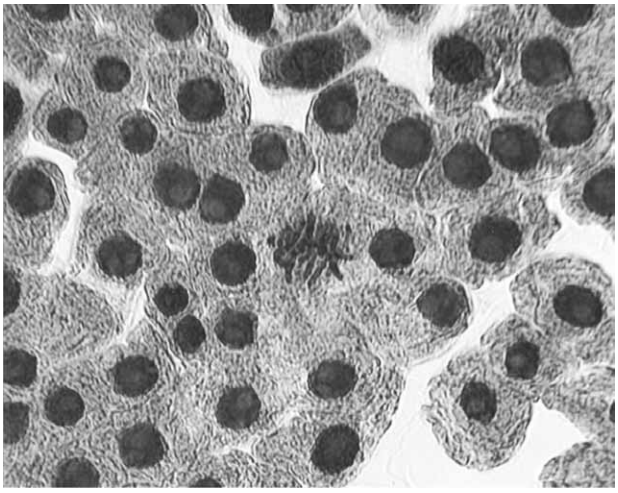

Fig. 6. Root meristematic cells of *Allium cepa* treated with 1 g/dL of fruit extract powder solution for 48 h. Cells are in prophase with heterochromatic nuclei, and metaphase with pyknotic chromosomes and normal telophase

Moreover, treatment with a solution of 1 g/dL of *Sambucus nigra* fruit extract powder solution for 48 h led to a frequent decrease in mitotic division, probably as an additional result of high concentration and long period of treatment.

These data support the idea that a solution of 1 g/dL of *Sambucus nigra* fruit extract powder solution caused a minor decrease of normal root growth of higher plants as a consequence of negative effects of cell division of the meristematic cells in *Allium cepa*.

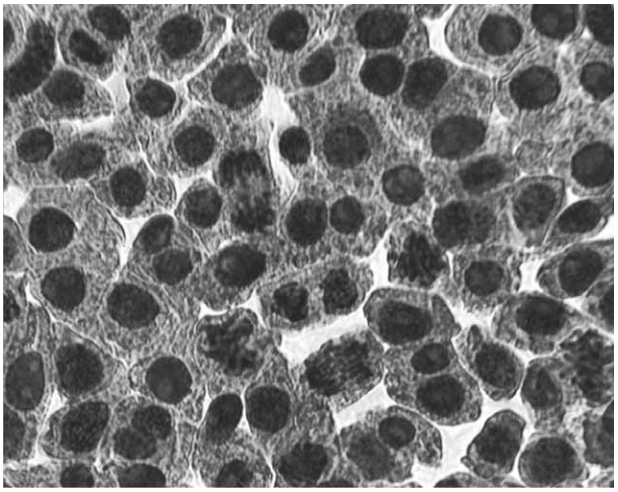

Fig. 7. Root meristematic cells of *Allium cepa* treated with 1 g/dL of fruit extract powder solution for 48 h. Cells are in metaphase with pyknotic chromosomes and anaphase bridges

The studied concentrated solutions of *Sambucus nigra* fruit extract powder may have, according to our results, the following negative effects: slowing down of the cell division rate (Figs. 2–7), a dehydration effect at cell level frequently inducing cell plasmolysis, expressed more drastically at higher concentrations or in longer treatments, heterochromatinisation during prophase (Figs. 4 and 6), changes of nucleus shapes into elongated (Fig. 5) or abnormal ones, cells in different cell cycle stages with pyknotic chromosomes and uncoiled and sticky form (Figs. 3, 4, 6 and 7), cells with anaphase or telophase bridges as a result of errors in chromosome separation (Figs. 4, 5 and 7), cells in cytokinesis with longitudinal separation of daugther cells (Fig. 2), cells in prophase with pyknotic chromosomes, nuclei with vacuolisations and micronuclei (Figs. 3 and 4). Thus, our data agree with other findings, postulating a prooxidant effect responsible for the cytotoxic and mutagenic effects observed in some flavonoids (*24*).

Conclusions

The results of the *Allium* test used in this study show that the treatments of aqueous solutions of *Sambucus nigra* fruit extract powder led to a decrease in cell division. A decrease in the mitotic index was observed using all combinations of concentration and exposure times compared to controls. The treated roots were fewer and smaller in length than those in the control.

The treatment with 0.1 g/dL of *Sambucus nigra* fruit extract powder solution for 6 h caused an occurence of big nuclei with abnormal shape and cytokinesis with longitudinal separation of daughter cells as a consequence of the accumulation of genetic material in too large quantities. The treatment with 1 g/dL of *Sambucus nigra* fruit extract powder solution for 48 h led to a frequent decrease in mitotic division and also to cells in prophase with heterochromatinised nuclei, prometaphase and metaphase with pyknotic chromosomes. Also, anaphase bridges evolved, probably as an additional result of high concentration and long period of treatment.

It is clear that the intensity of the mutagenic effect depends on the concentration of the fruit extract and on the duration of the treatment. Cytogenetic tests using *Allium cepa* reveal a decrease in the mitotic index after the treatment with the solutions of *Sambucus nigra* fruit extract powder. Mitosis analyses show a comparatively low number of aberrations during interphases and chromosomal aberrations identified in different mitotic stages, the division process being hardly affected. Evidently, these microscopic observations are supported macroscopically, since the root size and number of roots clearly diminished.

Therefore, *Sambucus nigra* fruit extract powder solutions had mutagenic activity, but only at higher concentrations. Taking into account the high antioxidant capacity observed at very low concentrations of the extract, the conclusion should be that its benefical activity as antioxidant stress protector would be easily fulfilled without any dangers or side effects when used in small amounts.

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