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# THE PROTECTIVE POTENTIAL OF THE HABERELEA RHODOPENSIS EXTRACT AGAINST THE GENOTOXIC EFFECTS AND LIPID PEROXIDATION INDUCED BY CYCLOPHOSPHAMIDE IN RABBITS

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**ABSTRACT**

The aim of the present study was to investigate the protective potential of the Haberlea Rodopensis (HR) extract against the genotoxic effect and lipid peroxidation induced by cyclophosphamide (CP) in rabbits. From the performed comet assay it is clearly seen that CP induces damage in the DNA molecule. Such damage is detected and registered as a 48% increase of DNA in the comet tails. The HR extract also induces DNA breaks in the cells, but with a far lesser effect, i.e. 27%. When the experimental animals are simultaneously treated with CP and HR extract, the effects of CP are fully eliminated. A firm verification for this is the fact that the comet size goes beyond the size of the comets in the group, treated solely with the HR extract, although it never reaches the comet size in the control group.

The results from determining the MDA levels show a considerable elevation of this parameter in the group treated with CP – at the 24$^{th}$ hour the level of MDA is 6.23±0.42 ($p<0.001$) and on the 8$^{th}$ day - 5.72±0.61 ($p<0.01$) when compared with the control group (4.38±0.8). When the rabbits are treated in a combined manner (HR+CP), a tendency for a decrease in the registered levels of MDA is observed in the time interval 4$^{th}$ hour – 8$^{th}$ day. Significant differences in the levels of MDA in the differently treated groups are registered at the 24$^{th}$ hour following treatments. In this interval the level of MDA in the group treated with CP - 6.23±0.42 authentically exceeds the level in the group treated with HR+CP - 5.2±0.6 ($p<0.01$).

The performed study shows that the HR extract does possess antigenotoxic properties and also decreases the level of lipid peroxidation in rabbits, treated with the alkylating agent cyclophosphamide.

**Key words:** cyclophosphamide, extract Haberlea Rhodopensis, comet assay, MDA**.**

## INTRODUCTION

Cyclophosphamide (CP) is an anti-tumour chemotherapy agent which damages the DNA matrix. Apart from being a bi-functional alkylating agent, CP has a cytotoxic effect upon all rapidly proliferating cell populations through a decrease in the diphosphopiridin nucleotide in the cancer cells; CP also renders an immunosuppressive effect since it reduces the number of the immunoglobulin-synthesizing B-lymphocytes (1, 2).

The cytotoxic action of CP is based on the interaction of its alkylating metabolites with the DNA molecule. It is generally thought that the cytotoxic effect of CP is due to the formation of crosslinks in DNA via the covalent bondage of the powerfully reactive alkyl groups of the alkylating nitrogen

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mustard. It is well known that one of the CP metabolites – the acrolein- induces the oxidative stress, which thereafter damages DNA, the lipids and proteins (3).

The cytotoxic effect of CP is independent of the cell cycle and is phase unspecific (4). In a number of experiments in *in vivo* and *in vitro* models it was found that CP has a very well expressed mutagenic activity inducing chromosome aberrations (CA), micronuclei (MN), and sister chromatid exchange (SCE) (5, 6).

Haberlea rhodopensis Friv. from the Gesneriaceae genera, Didymocarpeae subfamily, is a Balkan relict endemite, mainly spread in the Rhodopi mountains (along the banks of the Chaia river in the region of Bachkovo, along the banks of Arda river in the regions of Kardzhali and Studen Kladenetz dams, The Wonderous Bridges in the Trigrad defile, village of Gela, Smolyan district, and certain areas in Greece), and also in some parts of Sredna Gora and the Central Blakan.

HR is a relatively unstudied plant species. The main efforts of researchers have always been directed at elucidating the mechanism of anabiosis, since some of them suggest that in the case the term "pokilohydria" is more appropriate to be used. Poikilohydria means an anabiotic property in certain plants to survive in a fully viable and renewable cycle through a total vegetation desiccation (desiccation tolerance), and after, at the supply with water to be able to fully revive their viability.

The phytochemical characteristics of the Gesneriaceae family are barely known. There are data that the different genera in this family contain flavonoids, anthocyanins, caffeoyl phenylethanoid flavonoids, tannins, zeaxanthins, and also ascorbate (7, 8). During systemic chemical investigations and tests of a number of representatives of the Gesneriaceae family it was found, via X-ray analysis and magnetic resonance imaging (MRI), that the basic compound in Ramonda myconii is the phenylethanoid glucoside myconosid, which from a chemical point of view is indeed 2-(3,4-dihydroxyphenyl) 3-caffeoyl-β-allopyranoside (9). It has been additionally reported that the same compound is mainly to be found in the Ramonda serbica and HR species, and in the latter the caffeic acid residue is dehydrated. It has also been ascertained that the HR extract possesses an antibacterial, anticlastogenic and a high antioxidant activity (10, 11, 12).

The aim of the present study was to investigate the protective potential of the Haberlea Rodopensis extract against the genotoxic effect and lipid peroxidation induced by cyclophosphamide (CP) in rabbits.

## MATERIAL AND METHODS

The experiments were performed on 20 rabbits that were distributed in four groups as follows: Group 1 – controls; Group 2 – treated with HR; Group 3 – treated with CP; Group 4 – treated with HR+CP. The treatment with HR (concentration 0.12 g/kg b.w.) was carried out via intramuscular (i.m.) administration in three consecutive days (group 2, group 4). The treatment with CP (50 mg/kg b.w.) was performed via intramuscular (i.m.) administration one hour after the last application of HR (group 3, group 4). The blood samples were obtained at the $4^{th}$, $24^{th}$ and $72^{nd}$ hour and on the $8^{th}$ day after the CP administration.

### Comet assay (SCGE – comet assay)

The antigenotoxic activity of the extract was assessed via gel electrophoretic assay for DNA damage in the lymphocytes of rabbit peripheral blood. In order to do this, we applied the alkaline version of the comet assay (13) and a modified experimental protocol (14).

**Blood sample harvesting and cell processing:** The blood samples for the comet assay were obtained from the ear marginal vein of the rabbits in sterile test-tubes containing 30 U/mL heparin at the $4^{th}$ hour after the administration of CP and were placed on ice. 1 mL of each sample was transferred in a 1.5 mL test-tube (Eppendorf) and was rotated at 1000 rpm on a centrifuge Hermle Z – 233 M for 3 min. at 4°C. The cell sediment was suspended in phosphate buffer-saline (PBS) of Sigma - USA.75 μl of the cell suspension was mixed with 100 μl 1.4 % low-melting agarose (LMA) from Boehringer Manheim (Germany) for mounting upon the microscopic slides.

**Preparation of the microscopic slides:** The above-described suspension of cells and agarose was spread as micro-gel upon a microscopic slide. Following the agarose polymerization, the slides with the micro-gel were dipped in a lysing solution (1 M NaCI, 50

mM EDTA pH 8, 30mM NaON,) for 60 min and then were dipped in a denaturation solution (30mM NaON, 10 mM EDTA pH 8; pH 12.6) for 30 min.

**Electrophoresis:** The prepared microscopic slides were placed in a horizontal container for the gel electrophoresis. The container was filled with 1100 ml solution for electrophoresis (10mM EDTA and 30 mM NaOH, pH 8; pH 12.6) up to a level approximately 0.25 cm above the slides. The electrophoresis was performed at $10^0$C for 20 min. at 0.45 V/cm with a compact electricity source (Power Pack P 25 Biometra Analytic GmbH). After the electrophoresis the slides were taken out of the container. Tris buffer (0.5 M Tris, pH 7.5) was added in drops, gradually and carefully, so that the alkalinity be neutralized and the slides were left for neutralization for 5 min. following the process of neutralization, the slides with the gel were consecutively fixed in 70% and 95% ethanol, each step 5 min, and then were dried at room temperature.

**Staining:** DNA of the comets was dyed with the fluorescent stain SYBR Green. The slides were coverslipped, placed in a humid hermetically closed chamber and analyzed in 3 to 4 hours.

In order to visualize the DNA damage, the slides were observed under a microscope and photographed at a magnification x 1000 by using a x1000 objective (oil immersion) on a fluorescent microscope (Leitz Ortoplan, VARIO ORTHOMAT 2, Germany) through a 450-490 nm filter.

Up to 50 images from each slide, or about 1000 objects as a whole, were assessed and analyzed from the samples, obtained 4 hrs. after the treatment.

**Measurement of the malondialdehyde (MDA) levels.**

The general quantity of the products from the peroxide oxidation was determined in plasma by using the thiobarbiturate assay, which is based on the spectrophotometric measurement ($\lambda$=532 nm) of the products yielding a positive reaction with the thiobarbituric acid (TBA) with a basic component MDA (15). In brief: 1 ml deproteinized plasma is mixed with 0.25 ml freshly prepared 1% solution of thiobarbituric acid (TBA). The mixture is heated for 90 min. at 95$^o$C. After cooling down it is measured spectrophtometrically at 532 nm ($\varepsilon$ =1.55 x $10^5$ $M^{-1}cm^{-1}$). The amount of MDA in the tested sample is determined in μM.

## RESULTS AND DISCUSSION

### Comet assay

In the treated animals, unlike the controls, clearly defined comets were obtained and visualized. These comets were with comparatively equal size and density in the rabbits treated in the same way but differed in length and density in the differently treated animals. Comets were also registered in the control animals **(Fig. 1).**

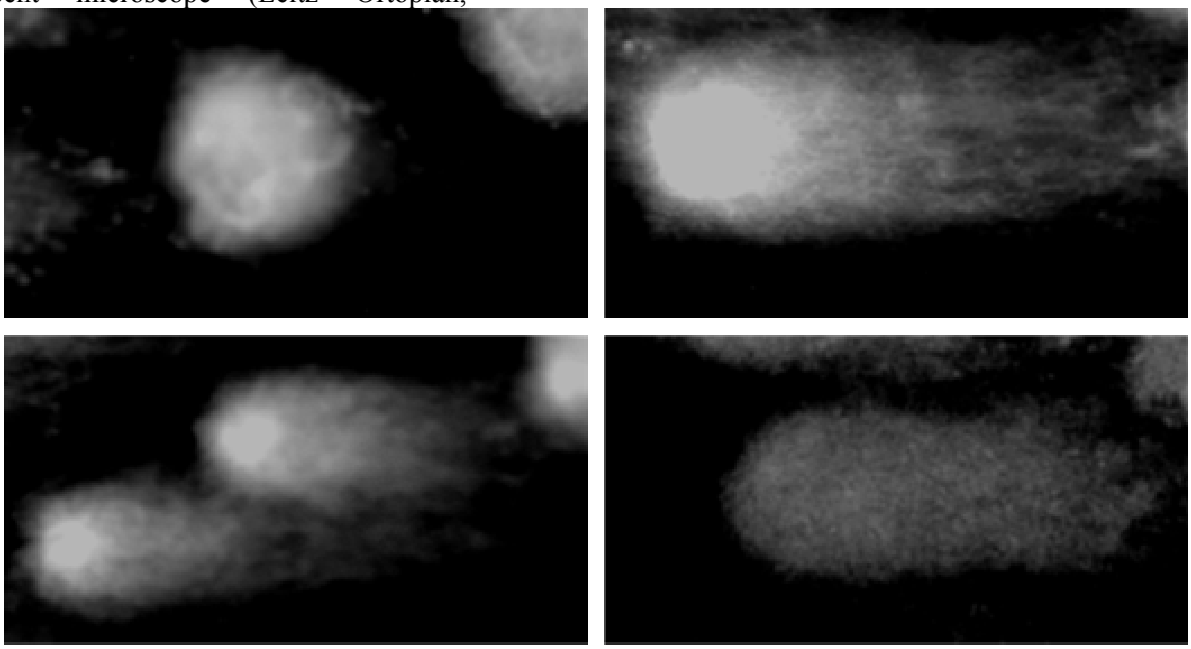


**Figure 1.** Comets from lymphocytes after treatment of rabbits:
a - control, untreated; b - treated with CP;
c – treated with HR; d – treated with CP+ HR.

The results from the measurements and the statistical processing at the 4th hour after the treatments are presented in **Table 1.**

**Figure 2** presents the data from **Table 1.** It illustrates the average length of the comets, derived in the individual treatments.

***Table 1.*** *Comet tail length, derived from the blood samples of the control and treated rabbits after performing the comet assay*

| Tail Length (px) | | | | |
|---|---|---|---|---|
| **Rabbits No.** | **Controls** | **HR** | **CP** | **HR+CP** |
| 1 | 30,95 | 35, 34 | 45, 58 | 31, 21 |
| 2 | 22,75 | 35, 34 | 43, 66 | 36, 18 |
| 3 | 21, 92 | 36, 00 | 42, 45 | 33, 66 |
| 4 | 27, 63 | 39, 79 | 43, 09 | 28, 82 |
| 5 | 31 | 39, 39 | 40, 66 | 43, 6 |
| mean | **26, 85** | **37, 172** | **43, 0875** | **34, 694** |
| SD | 4, 59 | 2, 23 | 2, 07 | 5, 68 |

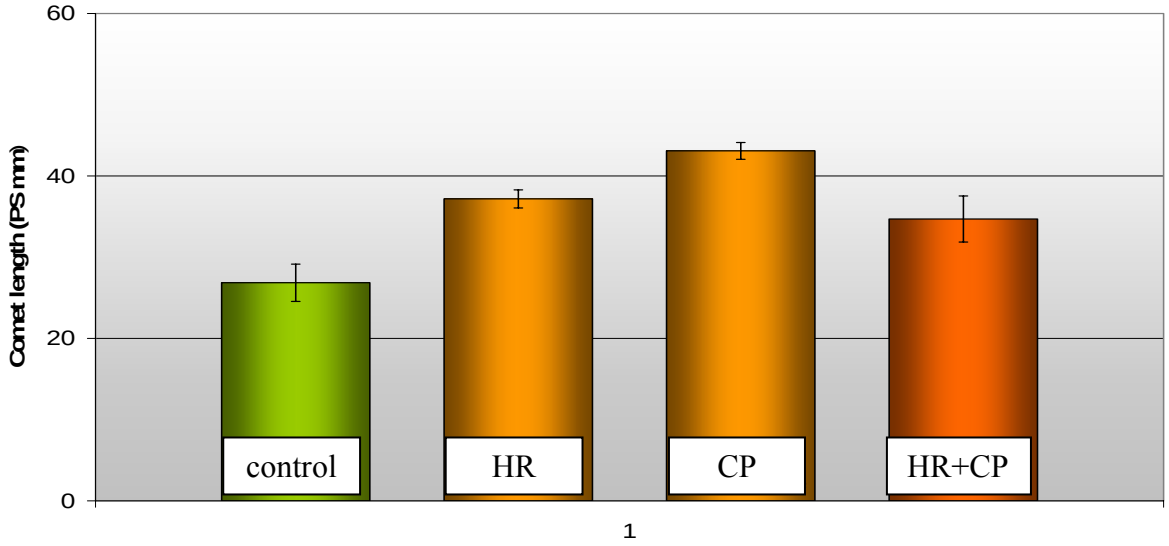



**Figure 2**. Tail length of the comets in the control and treated groups, subject to administration of CP and HR extract.

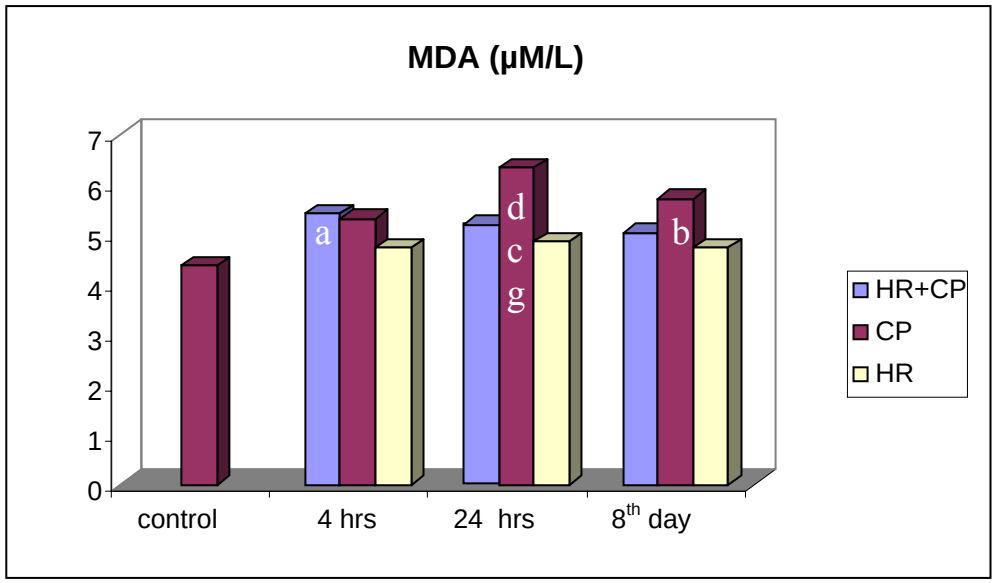
From the performed comet assay it is clearly seen that CP induces damage in the DNA molecule. Such damage is detected and registered as a 48% increase of DNA in the comet tails. This effect should be foreseen since it is well known that CP is a cytostatic agent which evokes the formation of DNA adducts (covalent bonds within the DNA double helix), and also protein-DNA covalent bonds.

The HR extract also induces DNA breaks in the cells, but with a far lesser effect, i.e. 27%.

When the experimental animals are simultaneously treated with CP and HR extract, the effects of CP are fully eliminated. A firm verification for this is the fact that the comet size goes beyond the size of the comets in the group, treated solely with the HR extract, although it never reaches the comet size in the control group.

**Determination of the MDA plasma concentration**

The results from determining the levels of the lipid peroxidation parameter – malondialdehyde (MDA) – are presented in **figure 3.**




**a**-p<0.05; **b**- p<0.001; **c**- p<0.001 vs. control group
**d**- p<0.05 vs. 4 hrs; **g**- p<0.01 vs HR+CP

**Figure 3.** Dynamics of MDA in the interval before treatment up to the 8th day after treatment.

The results from determining the MDA levels show a considerable elevation of this parameter in the group treated with CP – at the 24th hour the level of MDA is 6.23±0.42 (p<0.001) and on the 8th day - 5.72±0.61 (p<0.01) when compared with the control group (4.38±0.8). From the obtained results it can be inferred that CP, in the concentration administered, increases the levels of lipid peroxidation. When the rabbits are treated in a combined manner (HR+CP), a tendency for a decrease in the registered levels of MDA is observed in the time interval 4th hour – 8th day, although such differences have not been statistically proved. In this group, an authentic elevation in comparison with the control group is registered only at the 4th hour 5.42±0.41 (p<0.05), while at the 24th hour and on the 8th day the MDA levels are almost next to normal. The sole administration of HR extract does not alter the levels of MDA during the investigated period of time.

Significant differences in the levels of MDA in the differently treated groups are registered at the 24th hour following treatments. In this interval the level of MDA in the group treated with CP - 6.23±0.42 authentically exceeds the level in the group treated with HR+CP - 5.2±0.6 (p<0.01), and in the group, treated solely with HR - 4.86±0.5 (p<0.01).

The general analysis of the results in the different groups and in dynamics shows that in the case of combined treatment lipid peroxidation, induced by the effects of CP, is significantly reduced.

In the present study, through performing a comet assay, we were able to obtain proof for the genotoxic effect of cyclophosphamide, as well as for the reduction of this effect by the total extract of Haberlea Rhodopensis. From the comet analysis it becomes clear that cyclophosphamide evokes damage in the DNA molecule. Such damage is detected as a 48% increase of DNA in the tails of the analyzed comets. When the rabbits were subject to a combined treatment with cyclophosphamide and Haberlea Rhodopensis extract, the genotoxic effect of cyclophosphamide was almost entirely eliminated.

Studies on a large number of plant extracts prove the antigenotoxic properties of their extracts against well-known clastogens and mutagens (16, 17).

Cyclophosphamide possesses pro-oxidative properties and via the generation of free radicals it evokes oxidative stress (18). Certain studies show that after treating with cyclophosphamide the activity of the antioxidant enzymes decreases, while the levels of lipid peroxidation increase (19). According to Halliwell, et al, (20) the increased lipid peroxidation is also another reason for the inactivation of the cellular antioxidant systems.

The observed in the present study alterations in the plasma levels of MDA, which serve as a

biological marker for oxidative stress, are in full agreement with the literature data cited above. Therefore, the formation of free radicals is one of the possible mechanisms through which CP and its metabolites enhance their toxic effect in various tissues (18). The effect from the application of the extract from Haberlea Rhodopensis, prior to treatment with CP, is expressed in a decrease in the MDA blood levels of the experimental animals. Due to the fact that oxidative stress is one of the mechanisms for the toxic effect of CP, our results show that the protective properties of Haberlea Rhodopensis against the cyclophosphamide-induced alterations in the antioxidant systems most probably also contribute for its protective effect against CP genotoxicity.

According to Liu et al., (21) and Berger, (22), the addition of antioxidants to the therapeutic scheme decreases the oxidative damage of DNA and protects the healthy cells from the adverse effects, caused by certain chemotherapeutic agents. The application of antioxidants in the course of chemotherapy decreases the frequency and gravity of its adverse effects (23). The experimental results fro our study show that the Haberlea Rhodopensis extract decreases the oxidative DNA damage, caused by CP, a fact that fully confirms the property of the antioxidants to suppress chemical mutagenesis *in vivo* and are in agreement with other studies, showing that plant extracts contain antioxidants which protect DNA from damage caused by ROS (24, 25, 26, 27).

## CONCLUSION

The performed study shows that the HR extract does possess antigenotoxic properties and also decreases the level of lipid peroxidation in rabbits, treated with the alkylating agent cyclophosphamide.

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