



Original Contribution

**MODULATORY EFFECTS OF TOTAL EXTRACT OF
HABERLEA RHODOPENSIS AGAINST THE CYCLOPHOSPHAMIDE
INDUCED GENOTOXICITY IN RABBIT LYMPHOCYTES IN VIVO**

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ABSTRACT

The Balkan region endemite *Haberlea rhodopensis* is a world record-holder in desiccation tolerance. Regarding the variable biological activities of *Haberlea rhodopensis*, it is important to study its antimutagenic activity in somatic cells *in vivo*. New Zealand white rabbits were intramuscular (i.m.) administered with total extract of HR at a dose of 100 mg/kg b. wt. for two consecutive days then treated with cyclophosphamide (i.m.) at a dose of 50mg/kg b. wt. Twenty-four hours thereafter blood was taken from all animals and samples were prepared from peripheral lymphocytes for chromosomal aberrations (CA) and sister chromatid exchanges (SCEs).

Extract of *Haberlea Rhodopensis* inhibited the frequency of SCEs induced by Cyclophosphamide (CP) in peripheral lymphocytes. This inhibition reached to 43.84% (HR-100 mg /kg b.wt.). The number of chromosomal aberrations induced by CP in rabbits peripheral lymphocytes decreased significantly ($p < 0.001$). The percentage of inhibition of chromosomal aberrations was 42,85 %.

The activities of SOD and the contents of MDA were also tested by the colorimetric methods. The results showed that CP at a dose of 50 mg/kg, i.m. significantly inhibited the activities of SOD and increased MDA contents in rabbits blood. On the other hand, HR antagonized the reduction of the activities of SOD and GPx, and the increase in MDA contents.

In conclusion, the results of this *in vivo* study show that HR has antimutagenic potential against carcinogen CP.

Keywords: *Haberlea Rhodopensis*, cyclophosphamide, Chromosomal aberrations, SCE, oxidative stress, SOD, MDA.

INTRODUCTION

There are many reports that the hazardous environmental chemical capable induces genotoxic and cancerogenic effects on the mammary. The main mechanism is producing of species free radicals that induces damages to critical macromolecules such as DNA and different other molecules and therefore cause mutagenesis. Many natural compounds in plants have been reported to have potential antimutagenic and anticancerogenic effects (1-3).

The Balkan region endemite *Haberlea rhodopensis* is a world record-holder in desiccation tolerance. Unfortunately, till now there are no strong historical and ethnobotanical data confirming the ancients' knowledge and use of *Haberlea* as medicinal attribute. However, one of the local plant names in the Rhodopi mountains is "shap" (food and mouth disease) which is considered as confirmation that the local people were using the plant against animal diseases (4). Other studies discuss the role of lipids and polysaccharides in the mechanism of anabiosis, as well as the presence of powerful antioxidant systems whose role is to prevent the oxidative peroxidation of the cellular lipids and proteins

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(5, 6). The thermo- and photo stability of the photosynthesizing system of *H. rhodopensis* have also been investigated under different conditions, and also the role of this system in maintaining the desiccation tolerance in the plant (7, 8). High antioxidant activity was found in total extract of HR by Radev et al (9). Recently, we reported that total extract of HR had a protective effect on genotoxicity induced by gamma irradiation in vitro (10).

Cyclophosphamide (CP) is commonly used chemotherapeutic and immunosuppressive agent for the treatment of a wide range of neoplastic as well as some autoimmune diseases. With increased success rate of cancer treatment, due in part to the aggressive use of high combination drug therapies, there has been growing concern about the long term side effects (carcinogenic) of these alkylating agents and other neoplastic drugs. There are several reports indicating the carcinogenic effects of CP in humans and animals (11, 12).

In this study by using chromosome aberrations (CA) and sister chromatid exchanges (SCE) assays method we have analyzed the modulatory effect of extract of HR against CP induced genotoxicity in rabbit lymphocytes in vivo. The CA test in cultured mammalian cells is one of the sensitive methods to predict environmental mutagens and/or carcinogens. SCE technique is considered as an important method for the cytogenetic mutagenicity testing. Even though SCE is generally more sensitive indicator of genotoxic effects than structural aberrations (13), which however is not adequate to replace classical methods of analysis of structural chromosomal aberrations. Both CA and SCE assays methods are widely used as the parameters for testing the modulatory effects of natural compounds on drug and chemical induced genotoxicity.

The aim of the present study is to investigate the influence of *Haberlea rhodopensis* extract against the cyclophosphamide induced genotoxicity in rabbit lymphocytes in vivo.

Materials and Methods

1. Experimental animals: 20 male New Zealand white rabbits (bred in the animal facilities of the Trakia University) were used in the study. They were in good health, 5-month-old and weighed between 3.5 and 4.0 kg at the beginning of the experiment. Animals were housed in individual cages and maintained under standard conditions of temperature,

humidity and light. All animals received food and water *ad libitum*.

2. Preparation of H. Rhodopensis extract: Leaves of *H. rhodopensis* were collected from plants growing in their natural habitat (the vicinity of Bachkovo, Bulgaria) after license of Ministry of environment and water of Bulgaria. Leaves were cutted into small pieces and dried in room temperature for 1 month. After grinding the dry matter were macerated for 6 hours in 70% ethyl alcohol and were percolated for 48 h. Primary extract was concentrated by evaporation of ethanol in a vacuum environment to reach a ratio of 5% ethanol and 95% water. The obtained extract was filtered through filter paper to remove emulsified substances, chlorophyll and other particles. The extract was standardized in accordance with the method for determining the relative density (Bulgarian Pharmacopoeia Roll 2, p.19). The amount of extracted substances ranged between 0.098 and 0.113 g/cm³ (average 0.105 g/cm³).

3. Chemicals:

Endoxan (cyclophosphamide monohydrate) was purchased from BAXTER Oncology GmbH, Germany. All other chemicals used were of analytical grade.

4. Treatment:

Animals were divided into 4 groups of 5 animals each. Group I were used as negative control.

Group II as positive control, were treated with Extract of HR/ kg b.wt. for 2 consecutive days. Group III were treated with cyclophosphamide (CP) at 50 mg/kg b.wt. intramuscular (i.m.), . Group IV were treated with Extract of HR 100 mg/ kg b.wt. for 2 consecutive days and cyclophosphamide (CP) at 50 mg/kg b.wt. intramuscular (i.m.); on the second day, 2 hours after HR. Blood was taken 24h after the last treatments.

5. Sister chromatid exchanges (SCEs):

The blood samples were obtained from the ear marginal vein with heparin in a concentration of 30 U/mL. Immediately after the blood was taken, it was transported to the laboratory for cytogenetic analysis, processed by the method of Hungerford 1965. Differential staining of sister chromatids was done according to Pery and Wolf, (14). 30 well spread metaphases were analyzed per rabbit to determine the frequency of SCEs/cell.

2. 5. Chromosome aberrations

Preparation of lymphocyte cultures: Micromethod of EVANS HJ, (15) with

modification for rabbits was used. 0.5 mL of whole heparinised blood was incubated in 7 mL RPMI 1640 medium, 3 mL heat-inactivated normal calf serum, 0.2 mL reconstituted PHA, 100E/mL penicillin, and 50 µg/mL gentamicin. The cultivation flasks were thermostated in the dark at 39^o C. Each group included two cultivation flasks from each donor.

All cultures were incubated for 48 hours. Colcemid at a final concentration of 0.2µg/ ml was added at 46 h to block the cells at metaphase stage. At the end of the 48th hour from the beginning of lymphocyte incubation, chromosomal preparations for detection of chromosomal aberrations were prepared.

100 well spread metaphases were scored for each rabbit for chromosome aberrations.

Evaluation of the activity of HR to reduce SCEs and chromosomal aberrations induced by CP was carried out according to **Madrigal-Bujaidar et al. (16)** formula as follows: Inhibitory index = [1- (HR and CP – control)/ (CP - control)] X100

SOD and MDA assays

The SOD activity determined as described by SUN Y. with some modifications (17). The xanthine/xanthinoxidase system was used for superoxide anion production. This anion reduces nitrobluetetrasole (NBT) to formasan, which was monitored at 560 nm. SOD in the samples removes the superoxide anion and suppresses the reduction. The reduction rate was used to measure of SOD activity.

One unit of SOD activity was determined as that enzyme quantity which causes 50% suppression of NBT reduction to formasan.

MDA contents, as the end product of fatty acid peroxidation.

The method of thiobarbituric acid, which measures MDA-reactive products, was used Plaser and Cushman (18). In brief, 1 ml plasma, 1 ml physiological solution, and 1 ml 25% trichloroacetic acid were mixed and centrifuged at 2,000 rpm for 20 min. One mililitre proteinfree supernatant was mixed with 0.25 ml 1% thiobarbituric acid and heated at 95^oC for 1 h. After cooling, the intensity of pink colour of the end fraction product was determined at 532 nm.

2. 6. Statistical analysis:

The significance of the results from the negative control data and between HR with CP comparing to CP alone was calculated using t-test for SCEs and Chi-square test (2X2 contingency table) for chromosomal aberrations.

Results for SOD and MDA are presented as mean and standard deviation (SD). Statistical analysis was conducted by ANOVA with multiple comparison test. $p < 0.05$ was considered statistically significant.

RESULTS

3. 1. Sister chromatid exchanges:

Sister chromatid exchanges analysis is a rapid objective method of observing reciprocal exchanges between sister chromatids. In the present study, the frequencies of SCEs/cell induced by total extract of HR Exceed 13 percent frequencies of SCEs/cell in control group. Nevertheless when rabbits were treated with HR two days and CP on the second day (2 hours after treatment with HR), frequencies of SCEs/cell decreased significantly compared to CP treated group $p < 0.001$. The mean percentage of SCEs/cell was reduced from **18.55±1.09** with 50 mg/kg b.wt. CP to **10.41±0.58 with** 100 mg/kg. b.wt. HR for two days and 50 mg/kg b.wt CP on the second day.

Table 1. Frequency of sister chromatid exchanges (SCEs) in rabbit peripheral lymphocytes, after treatment with CP and Extract of HR .

Treatment mg/kg b.w.	No of metaphases scored	total No of SHE ^a	SHE/ cells ^b Mean ±SD	Inhibitory index%
I. control	150	906	6.03±0.44	
II. HR (200 mg)	150	1026	6.82±0.23**	
III. CP (50 mg)	150	2783	18.55±1.09***	
IV. HR(200 mg) +CP(50 mg)	150	1563	10.41±0.58*** ••	65

a. The total number of chromosomes is 6600

b. The total number of scored cells is 150 (5 animals/ group)

** $p < 0.01$, *** $p < 0.001$: Significance compared to control; •• $p < 0.001$: Significance compared to CP group (t- test).

The inhibitory index for used dose of HR was **65%**. The number of SCEs/chromosome were recorded (**Table 1**)

3. 2. Chromosomal aberrations:

Table 2 show the number and percentage of the chromosomal aberrations in control and treated animals.

Table 2. Number and mean percentage of chromosomal aberrations in rabbits peripheral lymphocytes after treatment with CP and extract of HR.

Treatment mg/kg b.wt.	No of meta-phases	Aber. Cells	Single Frag	Double Frag	Dicentric +rings	Exchanges	Total aberrations No. (%)	Inhib. index%
I. control	500	6	4	1	-	1	6 1.20	
II. HR (100 mg)	500	9	7	2	-	1	10 ^{n.s} 2.0	
III. CP (50 mg)	500	63	61	6	3	6	76*** 15.2	
IV. HR(100mg) +CP(50mg)	500	38	37	5	1	3	46** 9.2	42,85

*** $p < 0.001$: Significance compared to control

n.s.: not significant; ** $p < 0.01$,: Significance compared to CP group (X^2 - test).

The number of total aberrations in treated with extract of HR for two consecutive days was higher, than the control group, but not significantly. The percentage of aberrant cells in rabbits treated with cyclophosphamide was statistically significant higher in comparing to the control group $p < 0.001$. Administration of extract of HR and CP resulted in reduced number of the chromosomal aberrations $p < 0.01$ in comparing to CP treated group.

The value of inhibitory index for chromosome aberrations was 42, 85%.

3. 3. Effect of HR on CP-induced changes in SOD activity and MDA contents in rabbits blood.

As shown in **Fig. 1** and **Fig. 2** there was no significant difference in the activity of SOD and MDA contents in rabbits that received only HR compared to the control rabbits ($P > 0.05$). The activity of SOD was substantially decreased ($P < 0.001$) and MDA levels increased ($P < 0.001$) in CP group as compared to the control group. Pre-treatment of HR could inhibit the decrease of the activity of SOD and the increase in MDA levels induced by CP.

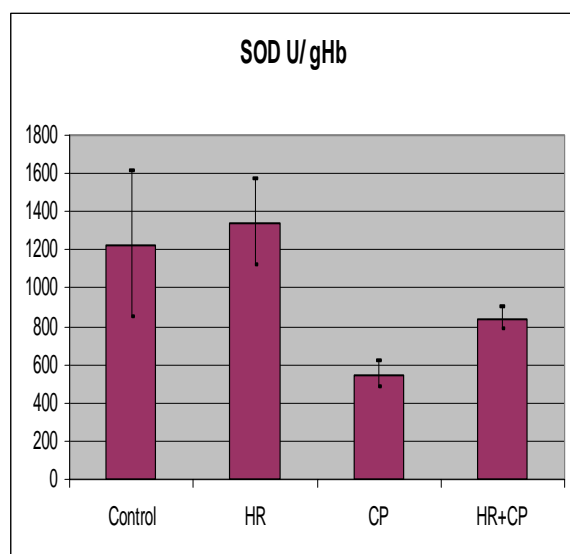


Fig. 1. Changes in SOD activity.

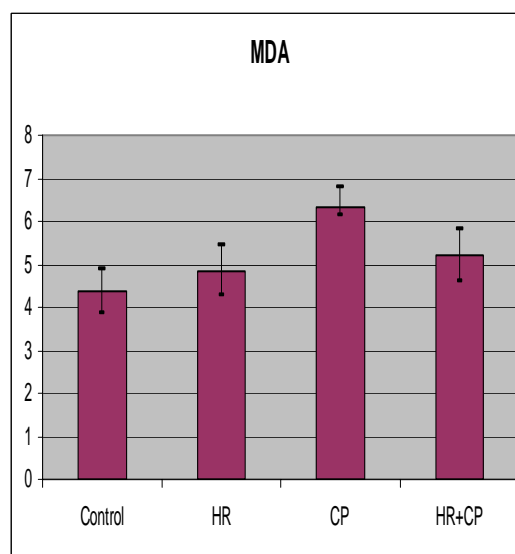


Fig. 2. Changes in MDA levels.

DISCUSSION

Chemotherapy with CP can cause secondary tumors in humans by activating hepatic mixed function oxidases. Phosphoramidate mustard, the major antineoplastic metabolite of CP, is an alkylating agent that induces a variety of changes in DNA (19, 20) through its ability to form labile covalent DNA adducts and cross-linkages (21).

Compounds with antioxidant properties can have antioxidant and oxidant effects that are dose-dependent (22, 23). As shown in our study the increase of the cytogenetic parameter (SCEs) is statistically significant, but the level of chromosomal aberrations in rabbit lymphocytes was not significantly higher when animals were treated with 100 mg/kg b.wt. extract of HR (only) for two consecutive days compared with the control group.

From the other hand extract of HR effectively protected cells against the genotoxicity (SHEs) and clastogenicity (CA) of CP. With the treatment schedule used here, it was not possible to determine whether the protection offered by the extracts was related to a reduction in the efficacy of CP (by affecting the pharmacokinetics of this compound), or to a direct effect on the cells themselves. However, previous studies have shown that natural antioxidants can prevent the oxidation of biomolecules, including DNA, without decreasing the effectiveness of chemotherapeutic compounds. According to Liu *et al.* (24) and Berger (25), an antioxidant dietary supplement can reduce the level of oxidative damage to DNA and protect normal cells against the adverse side-effects of some chemotherapeutic agents. Therapy with antioxidants concomitant with chemotherapy reduces the frequency and severity of adverse effects associated with many drugs in cancer patients, thereby allowing the treatment to be continued (the toxicity of anti-cancer drugs is a frequent limitation to their extended use) (26). As shown here, HR protected against oxidative DNA damage caused by CP, which agrees with the ability of antioxidants to inhibit chemical mutagenesis *in vivo* and with studies showing that plant extracts contain antioxidants that can protect DNA against damage by ROS (27, 28, 29, 30).

Free radicals exert the toxic effects by acting on biologic macromolecules such as DNA,

membrane proteins and lipids. Oxidative stress occurs when the generation of free radicals increases or the capacity to scavenge free radicals and repair of oxidatively modified macromolecules decreases, or both (31). The activities of SOD, CAT and GPx play significant roles in preventing oxidative stress. SOD is the only known enzyme that uses superoxide radicals as a substrate to produce hydrogen peroxide (H₂O₂) and oxygen (32). Moreover, MDA, a final indicator of lipid peroxidation, is widely used as to reflect oxidative stress and cell membrane damage (33). CP has a pro-oxidant character and its treatment is associated with induction of oxidative stress by the generation of free radicals (34). Some studies have shown that the activities of anti-oxidant enzymes decrease and lipid peroxidation increase after CP treatment (35). The present studies showed that CP decreased the activities of SOD and increased lipid peroxidation levels, but the treatment with CP+ HR reduces negative effect of the treatment with CP only.

In conclusion: This study investigates cytogenetic activity of total extract of HR on chromosomes by two main cytogenetic biomarkers chromosomal aberrations and sister chromatid exchanges. Using of CP as DNA alkylating agent makes severe mutations in chromosomes gives the ability to fairly judge on the cytogenetic activity of HR in RABBITS *in vivo*. This work explores the antioxidant and antigenotoxic activity of HR, and its antimutagenic effects in reducing and preventing the DNA damages, which can be induced by carcinogens in somatic cells. The underlying molecular mechanisms now require attention.

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