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Impact of the *Haberlea rhodopensis* extract on the innate immune system and response in rabbits following KLH-hemocyanin immunization and cyclophosphamide treatment

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Abstract: The aim of the present investigation was to study the impact of *Haberlea rhodopensis* extract on the innate immune response in rabbits. The complement and lysozyme activity, percent phagocytosis, and phagocytic index were studied. The analysis of the results showed that the treatment with *Haberlea rhodopensis* extract, cyclophosphamide, and KLH renders a remittent effect upon the classical pathway of activation of the complement system and the lysozyme concentration in the rabbit blood serum. These same factors have no direct effect on the phagocytic activity in the investigated animals. The obtained data show limited effect of *Haberlea rhodopensis* extract on rabbits' natural immunity.

Key words: *Haberlea rhodopensis*, cyclophosphamide, complement, lysozyme, phagocytic activity

1. Introduction

The plants that possess medicinal properties could prove to be useful in the development of novel drugs derived from natural products. The interest in plant-based medical preparations has been growing due to their therapeutic potential and safety, and as an alternative to the conventional medicines. It has been shown for a large number of plants that they possess a therapeutic effect that can be explained with their antioxidant, antiinflammatory, and antitumor potentials (1–3).

In a number of plant products immunomodulatory activity has been found (4). Some of these products contain proteins, lectins, flavonoids, polysaccharides, and certain other substances, which have a stimulant effect upon the immune system (5). Plants like *Panax ginseng*, *Viscum album*, *Tinospora cordifolia*, *Boerhaavia diffusa*, *Withania somnifera*, *Ocimum sanctum*, and *Curculigo orhcioides* (6–10) are well-documented and proven immunomodulatory agents.

Haberlea rhodopensis Friv. (Gesneriaceae) is a Balkan relict endemic plant, whose natural habitats are the Rhodopi mountains, Sredna Gora, and the central Balkans. It is well known that this plant has the ability to survive in unfavorable environmental conditions. *Haberlea rhodopensis* still remains relatively unstudied and there exist no data about its phytochemical content. In some other species of Gesneriaceae, however, flavones, flavonoids, ghanins, zeaxanthin, beta carotene, ascorbate, and glutathione have been described (11). Some other authors have considered the role of polysaccharides and lipids in the mechanism of anabiosis (12). The presence of antibacterial activity in the *Haberlea rhodopensis* total extract has been reported (13). Multiple isoforms of several antioxidant enzymes in the leaves of the resurrection plant *Haberlea rhodopensis* were identified, and the presence of 6 isoforms of superoxide dismutase (1 form of the nonspecific guaiacol peroxidase) and 2 of ascorbate peroxidase was shown (14). A radioprotective potential of the *Haberlea rhodopensis* extract was reported in an in vitro experiment with irradiated rabbit lymphocytes (15).

Due to the fact that there exist no data about the immunomodulatory action of the *Haberlea rhodopensis* extract, we set it as a task to study its impact on the complement activity in rabbits treated with cyclophosphamide and immunized with KLH-hemocyanin.

2. Materials and methods

2.1. Experimental animals

For our experiments we used male rabbits of the New Zealand white breed, which were supplied by the educational farm of Trakia University. The rabbits were of

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the same age (4 months old) and approximately the same weight (3–3.5 kg). The animals were bred in individual cages and were fed on a standard mixture.

2.2. Plant extract from *Haberlea rhodopensis*

The total extract was prepared by maceration of the plant leaves for 48 h in 70% aqueous-ethanol solution and a subsequent alcohol distillation in a vacuum evaporator until a drug/liquid phase ratio of 5:1 was reached (1 mL of the extract contained 0.12 g of extracted substances).

2.3. Experimental design

The experimental animals were subdivided into 4 groups (n = 5) as follows:

Group 1 – treated with *Haberlea rhodopensis* (HR; 0.24 g/kg) and cyclophosphamide (CP; 50 mg/kg), and immunized with KHL (hemocyanin) (50 μg to each animal).

Group 2 – treated with CP (50 mg/kg) and immunized with KHL (hemocyanin) (50 μg to each animal).

Group 3 – treated with HR (0.24 g/kg) and immunized with KHL (hemocyanin) (50 μg to each animal).

Group 4 – immunized with KHL (hemocyanin) (50 μg to each animal).

The animals in Groups 1 and 3 were given an intramuscular injection of the *Haberlea rhodopensis* extract (0.12 g/kg). On the second day, the rabbits of Groups 1 and 3 were reinjected with *Haberlea rhodopensis* extract (0.12 g/kg), and those in Group 1 were treated with cyclophosphamide 1 h after the administration of the *Haberlea rhodopensis* extract. The animals in Group 2 were treated only with cyclophosphamide and this step was performed simultaneously with the treatment in Group 1. The animals of all groups were immunized with KHL (hemocyanin) (50 μg per animal).

The blood samples were obtained from the marginal auricular vein before treatment (autocontrol), 24 h after treatment with the *Haberlea rhodopensis* extract and cyclophosphamide, and also on days 7, 14, and 21 after the immunization.

2.4. Methods

2.4.1. Lysozyme determination

Serum lysozyme concentrations were determined by the method of Lie et al. (16). Briefly, 20 mL of 2% agarose (ICN, UK, Lot 2050) dissolved in phosphate buffer (70 mM Na_2HPO_4 and NaH_2PO_4, pH 6.2) was mixed with a 20-mL suspension of a 24-h culture of *Micrococcus lysodeikticus* at 67 °C. This mixture was poured out in petri dishes (14 cm in diameter). After solidifying at room temperature, 32 wells were made (5 mm in diameter). Fifty microliters of undiluted sera were poured out in each well. Eight standard dilutions (from 0.025 to 3.125 mg/L) of lysozyme (Veterinary Research Institute, Veliko Tarnovo, Bulgaria) were used in the same quantity as well. The samples were incubated for 20 h at 37 °C and lytic diameters were measured. The final lysozyme concentrations were calculated using a special computer program developed at Trakia University and were expressed as mg/L.

2.4.2. CPCA determination

The classical pathway of complement activation (CPCA) was tested by the method of Stelzner and Stain (17). Each serum sample was first diluted by mixing 30 μL of serum with 170 μL of veronal-veronal Na buffer (in final concentrations: 146 mM NaCl, 1.8 mM 5,5-diethylbarbituric acid sodium salt, 3.2 mM 5,5-diethylbarbituric acid, 15 mM $CaCl_2$, and 0.8 mM $MgCl_2$). In U-bottomed plates (Flow Laboratories, UK), 5 other dilutions from each diluted serum were again prepared in veronal-veronal Na buffer: 3/20, 3/80, 3/160, 3/320, and 3/640. Next, 100 μL of buffer and 100 μL of 1% sheep erythrocyte suspension sensitized with hemolytic antibodies were added to each well. After incubation for 1 h at 37 °C, samples were centrifuged at 150 × *g* for 3 min at room temperature (23 °C). Thereafter, 150 μL of each supernatant was removed and placed in flat-bottomed plates for measurement of optic density at 540 nm with a Sumal-PE2 ELISA reader (Karl Zeiss, Germany). The final CPCA activity was calculated using a special computer program developed at Trakia University and was expressed as CH50 units (CH50 units correspond to 50% of complement-induced hemolysis of applied erythrocytes).

2.5. Statistical analysis

Data were analyzed using the fixed-effect MANOVA model (Program STATISTICA, StatSoft, Inc., USA).

3. Results

The results for the CPAC system are presented in Table 1. The result analysis showed statistically significant differences in the complement activity in the group that underwent combined treatment (HR + CP). At 24 h after treatment the value of this parameter of the innate immunity reached 278.65 ± 5.15, which is 17% higher than the complement activity before the treatment. The complement activity was 14% higher in this group on day 14 after treatment. Best expressed was the influence of the combined treatment (HR + CP) on day 21, when the complement activity was the highest at 284.78 ± 18.56.

A well-documented increase in the complement activity on day 14 after treatment was also observed in the group treated only with cyclophosphamide: 285.34 ± 20.27 ($P < 0.05$).

In the group treated with the *Haberlea rhodopensis* extract, the complement activity in the period up to day 21 considerably outmatched its activity before treatment, but the difference was not statistically significant.

When comparing the results among the groups, we found the lowest complement activity in the group immunized with KLH, even though in this particular case the difference was not statistically significant.

Table 1. CPAC activity after treatment with cyclophosphamide and HR extract in rabbits immunized with KHL.

	HR + CP	CP	HR	KLH
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Before treatment	237.15 ± 8.32	237.15 ± 8.32	237.15 ± 8.32	237.15 ± 8.32
24 h	278.65 ± 5.15**	264.62 ± 17.61	254.67 ± 9.45	261.99 ± 21.76
Day 7	248.39 ± 6.98	228.40 ± 28.03	257.53 ± 10.15	218.03 ± 16.17
Day 14	271.79 ± 2.72*	285.34 ± 20.27*	268.83 ± 15.62	246.92 ± 27.33
Day 21	284.78 ± 18.56**	247.69 ± 17.22	260.73 ± 17.17	249.24 ± 32.01

*: P < 0.05, **: P < 0.01 vs. before treatment.

Table 2 illustrates the dynamics in the lysozyme values depending on the type of treatment in the experimental groups.

At 24 h following treatment, the lysozyme value was the highest in the group treated with a combination of substances (HR + CP). The measured value of 1.90 ± 0.38 mg/L was significantly higher than (P < 0.001) the lysozyme activity before treatment (0.67 ± 0.76 mg/L). Following the dynamics of this value of the innate immunity of the group (HR + CP) in Table 2, it can be seen clearly that on day 7 the lysozyme activity was the highest at 2.05 ± 0.31 mg/L (P < 0.001 vs. before treatment), while on day 14 it decreased significantly to 0.78 ± 0.13 mg/L (P < 0.05 vs. day 7), and again on day 21 it was higher when compared both with the value before treatment (P < 0.01) and that on day 14.

Cyclophosphamide treatment significantly increased the lysozyme activity on day 7 (2.25 ± 1.05; P < 0.05) and on day 21 (1.29 ± 0.36; P < 0.05).

The results in rabbits treated with the *Haberlea rhodopensis* extract show the stimulant effect of the extract, which is well expressed in the significant elevation of the lysozyme activity at 24 h (1.36 ± 0.42; P < 0.05) and on day 7 (1.39 ± 0.44; P < 0.05) and day 21 (1.34 ± 0.39; P < 0.05).

3.1. Phagocytosis and phagocytic index

The results of the treatment effects of cyclophosphamide and *Haberlea rhodopensis* extract in rabbits immunized with KLH on the percent phagocytosis and phagocytic index are presented in Tables 3 and 4.

The combined effect of the *Haberlea rhodopensis* extract and cyclophosphamide upon the percent of phagocyting leukocytes (percent phagocytosis) is expressed through the increased values of this parameter during almost the whole experimental period. Unlike the tendency in this group, in the rest of the experimental groups the percent phagocytosis was comparable to that before treatment. The group differences were not statistically significant.

Almost no variation was registered in the values of the phagocytic index (Table 4).

4. Discussion

When considering the dynamics of the CPAC activity in the group of rabbits treated with the *Haberlea rhodopensis* extract and cyclophosphamide, certain documentable changes in the activity of this parameter can be observed. It is interesting to point out the remittance in the CPAC activity when compared with that before the treatment. We found an increase in the activity at 24 h and a decrease

Table 2. Lysozyme activity after treatment with cyclophosphamide and HR extract in rabbits, immunized with KLH.

	HR + CP	CP	HR	KLH
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Before treatment	0.667 ± 0.76	0.667 ± 0.76	0.667 ± 0.76	0.667 ± 0.76
24 h	1.90 ± 0.38***	0.83 ± 0.09	1.36 ± 0.42*	0.81 ± 0.366
Day 7	2.05 ± 0.31***	2.25 ± 1.05*	1.39 ± 0.44*	1.51 ± 0.54*
Day 14	0.78 ± 0.13	0.67 ± 0.19	0.83 ± 0.24	0.41 ± 0.08
Day 21	1.82 ± 0.49**	1.29 ± 0.36*	1.34 ± 0.39*	0.86 ± 0.21

*: P<0.05, **: P<0.01, ***: P<0.001 vs. before treatment.

Table 3. Percent phagocytosis depending on the type of treatment in the experimental animal groups.

	HR + CP	CP	HR	KLH
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Before treatment	77.84 ± 1.24	77.84 ± 1.24	77.84 ± 1.24	77.84 ± 1.24
24 h	79.02 ± 1.49	75.0 ± 1.91	76.8 ± 1.95	77.6 ± 2.03
Day 7	79.2 ± 1.49	78.4 ± 2.03	76.0 ± 1.78	77.6 ± 2.71
Day 14	77.0 ± 1.91	77.6 ± 2.71	76.8 ± 1.49	82.0 ± 2.0
Day 21	81.0 ± 1.91	77.6 ± 2.03	76.0 ± 2.82	76.8 ± 1.49

Table 4. Phagocytic index in rabbits treated with the *Haberlea rhodopensis* extract and CP.

	HR + CP	CP	HR	KLH
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
Before treatment	1.34 ± 0.04	1.34 ± 0.04	1.34 ± 0.04	1.34 ± 0.04
24 h	1.48 ± 0.05	1.39 ± 0.02	1.392 ± 0.08	1.42 ± 0.07
Day 7	1.44 ± 0.05	1.49 ± 0.1	1.41 ± 0.03	1.46 ± 0.06
Day 14	1.46 ± 0.06	1.49 ± 0.05	1.40 ± 0.05	1.62 ± 0.06
Day 21	1.58 ± 0.01	1.48 ± 0.04	1.43 ± 0.09	1.43 ± 0.07

back to the baseline values on day 7. Our opinion is that the phenomenon could be explained with the simultaneous action of both antigens on the rabbit immune system. The administered vaccine evokes a primary immune response and as a result antibodies against it (most probably from the IgG and IgM classes) are formed. The newly synthesized antigen–antibody complex is the triggering factor for the activation of CPAC. On day 7 a decrease back to the baseline values of CPAC was registered due to its exhaustion. The process continued, however, after a most probable mobilization of the immune system. This should be the case since the components of CPAC are synthesized in various cells and tissues. On days 14 and 21 we found significantly higher CPAC activity, which suggests that the rabbit organism had been fighting against the administered antigens. It is quite possible that the *Haberlea rhodopensis* extract and cyclophosphamide caused a slight immunosuppressive effect on the CPAC in the period until day 7 (18–21). Good proof of this is the CPAC value on day 7 in the group treated only with cyclophosphamide, where the values were also the lowest for the whole experimental period. According to the obtained data, the impact of the *Haberlea rhodopensis* extract alone in the respective group was insignificant. Therefore, the documentable intragroup differences were a result of the simultaneous action of the extract and cyclophosphamide. In the group treated with KLH, the CPAC values also showed a certain remittance, although the value differences are insignificant and thus we cannot make the conclusion that they were due to the impact of the substance.

It can be seen from the obtained results that the lysozyme concentration in all 4 experimental groups kept changing in the same manner: it increased on day 7, then decreased on day 14 and returned to the baseline value on day 21. These one-directional changes show that the applied antigens have the same influence upon lysozyme and its serum concentration.

The lack of documentable differences (intragroup and intergroup) shows that the applied "factors" (*Haberlea rhodopensis* extract, cyclophosphamide, and KLH) do not have definite impact on the innate cellular immunity. This in part can be explained by the fact that the cells constituting the innate cellular immunity and possessing phagocytic properties are not prone to influences that are not particularly intense. In our opinion this is an evolutionary adaptation that helps the organisms survive better in the present conditions of the environment.

In conclusion we should point out that the application of *Haberlea rhodopensis* extract could not be used as a strong immunomodulation for boosting rabbits' humoral and cellular natural immunity.

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