

THE EFFECT OF THE EXTRACT OF RHIZOME AND ROOT OF HELLEBORE (*HELLEBORUS ODORUS* W. ET K.) ON PARAMETERS OF WHITE BLOOD COUNT AND DEGREE OF PHAGOCYTOSIS IN WISTAR RATS

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(Received 15th December 2009)

*The objective of this research was to study the effects that the extract of rhizome and root of *Helleborus odorus* W. et K. (*Ranunculaceae*) can have on modifications in the parameter values of white blood cells count and degree of phagocytosis by peritoneal macrophages and neutrophil granulocytes in Wistar rats. The trial was conducted on 28 rats divided into 4 groups with 7 animals in each group. To the control group of rats sterile physiological solution in the quantity of 0.25 mL/100 g BW was applied intramuscularly. For the purpose of monitoring the effect of the extract of rhizome and root of hellebore (HE) during a time period, the HE was applied intramuscularly to rats in a dose of 10 mg/100 g BW, while the blood samples for analysis were taken after 24h, 48h and 72h.*

The consequence of intramuscular application of HE was an increased count of total leukocytes in all trial groups, the most expressed leukocytosis being registered 24h after application of HE. Statistically significant higher value in the count and percent of neutrophil granulocytes in the blood was recorded 24h after treatment in relation to the control and two other trial groups ($p < 0.001$), among which a statistical significance was not established. The extract of hellebore rhizome and root has led to lymphopenia, resulting in the increase of the neutrophil/lymphocyte index in the trial groups 24h and 48h after treatment. The application of HE had no significant effect on the count of monocytes in treated animals. The applied extract has caused a significant increase in the degree of phagocytosis by residing peritoneal macrophages and neutrophil granulocytes in blood.

Key words: degree of phagocytosis, hellebore, hematological parameters, rats

INTRODUCTION

In the health care of animals a single or combined medical herb substances are applied for the purpose of prophylaxis, treatment of the onset stages of disease or as a supplement in medical therapy. By application of herbal preparations it is possible to stimulate the functions of the immune system and potentiate its defensive capacities. In recent years, immunostimulatory activity has been reported in a number of medicinal and other plants (Mungantiwar *et al.*, 1997; Makare *et al.*, 2001; Goel *et al.*, 2002; Schepetkin and Quinn, 2006).

A herbal drug *Helleborus* sp. is represented by a dried rhizome of dark brown to black colour, thickly overgrown by thin, dark grey roots (*Hellebori rhizoma et radix*), and is being extracted in spring or in autumn. *Hellebori rhizoma et radix* composed of various active components including bufadienolides (Wissner and Kating, 1974; Muhr *et al.*, 1995), steroidal saponins (Ribár *et al.*, 1986; Vladimirov *et al.*, 1991), lipids (Colombo *et al.*, 1991), ecdysones (Colombo and Tomè, 1993; Rosselli *et al.*, 2009) and alkaloids (Slavík *et al.*, 1987), has long been used in traditional folk veterinary medicine. Because of its irritating influence on skin and mucous membrane, the rhizome of hellebore is, in ethnoveterinary medicine, used in "herbal treatment" for a great number of farm animals (horses, pigs, sheep). Unspecific irritable therapy by means of transcutaneous implant of hellebore rhizome is conducted for the purpose of prophylaxis in uninfected animals (Tucakov, 1996), immature animals and in cases of diminished appetite (Bogdan *et al.*, 1989; 1990-a), as well as in acute stages of chronic diseases (Tosevski *et al.*, 2004).

It was confirmed by a number of research studies that the extract of rhizome and root of hellebore displays stimulatory effects on the immune system although some immunosuppressive effects were described, as well. Bolte *et al.* (1992) studied a proinflammatory influence of the extract of *Helleborus* sp. in rats, rabbits, horses and dogs and determined the inflammatory effect to be proportional to the given concentration, dose and way of application. The same authors mention some trial results that suggest the immunomodulatory influence of rhizome and root of *Helleborus* L. when applied simultaneously with antisalmonellosis vaccine in calves.

Bogdan *et al.* (1993) applied the extract of rhizome and root of *H. purpurascens* W. et K. to undeveloped fattening lambs with a complete metabolic disorder, and to sheep before their exposure to antigen. In lambs a significant increase in body mass and decrease in mortality rate was realized whilst in sheep an expressed leukocytosis with neutrophilia was ascertained which is deemed important for the increase of antimicrobial protection. The results of the research of Tosevski *et al.* (1999, 2004) pointed out the fact that parenteral application of the extract of a whole plant of *H. odorus* W. et K. can influence the metabolism of hepatocytes, muscular cells, cells of bone marrow and mastocytes (*BTC-bufaenolides target cells*) and can provoke acceleration of metabolic processes in piglets and sheep. Bolte *et al.* (2001) proved that purified extract of *Helleborus* sp. can be effective in strengthening postvaccinal immunological response in calves and bullcalves by activating both specific, as well as unspecific defensive

mechanisms. The same authors confirmed that purified extract of *Helleborus* sp. can provoke a modification of immuno response in cases of primary or secondary immunodeficiency, then can stimulate cellular multiplication, the activation of macrophages and releasing of IL-2, TNF and interferon.

An expressed leukocytosis and a positive effect on reproductive traits, fertility and number of live born piglets were determined after the extract of rhizome and root of *H. odorus* W. *et* K. had been applied to gilts in the time of their sexual maturity (Ristoska *et al.*, 2002). Milanović *et al.* (2004) studied the effect of the extract of the rhizome and root of *H. odorus* W. *et* K. on the immuno system of Wistar strain rats and confirmed a significant leukocytosis and granulocytosis.

MATERIAL AND METHOD

The Wistar rats, male and female, at the age of 2 months, average body weight of about 200 grams, were divided into groups of 7 animals each. In order to determine the duration of the effect of the extract of hellebore rhizome and root (HE), the trial animals were given intramuscularly, in the hind limb, a liquid extract of rhizome and root of hellebore in the concentration of 10 mg/100 g BW, while a blood sample was taken by cardiac puncture after 24h (Group II), 48h (Group III) and 72h (Group IV). To the rats in the control group (Group I) a physiological solution in the quantity of 0.25 mL/100 g BW, which corresponded to the volume of the applied extract of hellebore rhizome and root, was applied intramuscularly.

A grounded rhizome with roots of *H. odorus* W. *et* K. was extracted in the apparatus for a continual extraction according to Soxhlet dissolutions of arising polarity: petroleum ether, chloroform and methanol. The extracted plant material was then dried in a stream of cool air and afterwards extracted twice by water. By pairing the united water extracts a dry extract of yellow-ocher colour was obtained.

The parameter values of white blood count: count of total leukocytes (Le), count of neutrophil granulocytes (GR), percent of neutrophil granulocytes (GR%), count of lymphocytes (Lym) and count of monocytes (Mo) were determined in full heparinised blood by means of a standard laboratory procedure on automatic haematologic analyser Arcus Diatron®, Gmbh Wien, Austria. A neutrophil-lymphocytes index was calculated by means of the formula: $I = (\text{count of neutrophile granulocytes} : \text{count of lymphocytes}) \times 100$.

Determination of the degree of phagocytosis of residing (non-stimulated) peritoneal macrophages was carried out by means of HRP and phenol-red micro-titre method according to Pick and Mizel (1981). A degree of phagocytosis of neutrophil granulocytes in full blood sample was determined by the test of phagocytosis of latex particles, a method according to Matusiewicz and Urbankowska (1991).

RESULTS AND DISCUSSION

During the trial, the count of total leukocytes in the blood of rats belonging to the control group was in the range of physiological limits and in accordance with

reference values suggested by Moore (2000). A significant increase of leukocytes count after the application of HE was also similar to some literature citations. An average count of total leukocytes in the blood of control group of rats was $10.14 \pm 2.37 \times 10^9/L$. In the blood of rats treated intramuscularly by hellebore extract (10 mg/100 g BW) an average value of total count of leukocytes was: $13.37 \pm 1.34 \times 10^9/L$ after 24h, $10.37 \pm 1.61 \times 10^9/L$ after 48h and $10.76 \pm 1.34 \times 10^9/L$ after 72h. A trial group to which HE was applied had a greater count of leukocytes after 24h in relation to control group (FR) by 31.85%, what is statistically significant at the level of $p < 0.01$. An increased count of leukocytes was also recorded 48h and 72h after treatment of rats by HE, but those differences were not statistically significant in relation to the control group, whilst in relation to the value determined after 24h the count of leukocytes was significantly lower ($p < 0.01$) (Figure 1).

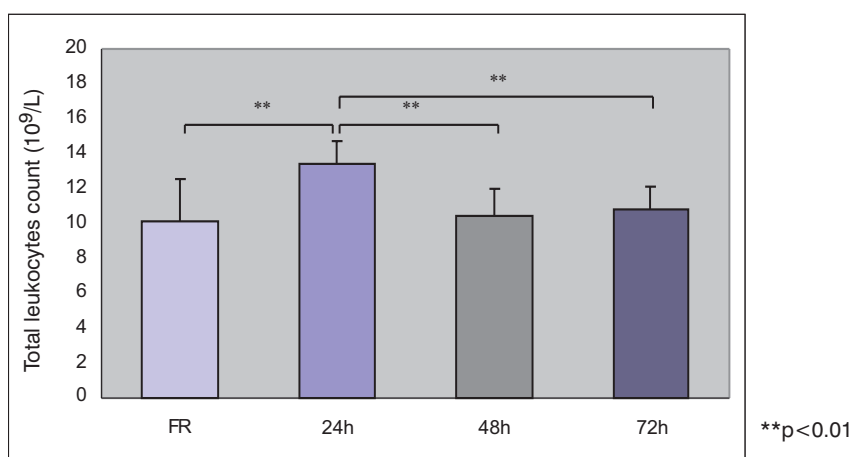


Figure 1. Total leukocytes count ($10^9/L$)

A pronounced leukocytosis was also noticed by Bogdan *et al.* (1989, 1990-a) 24h after transcutaneous implantation of rhizome of *Helleborus* sp. in cattle necklace, horses chest skin and sheep and pigs auricle. An insignificant increase in the number of total leukocytes in the blood of rats 48h and 72h after application of HE is in accordance with the results obtained by these authors by studying the count of leukocytes 48h, 96h and 144h after the implantation of hellebore rhizome to all species of animals. Comparing the effects obtained in sheep by implantation of rhizome *H. purpurascens* L. and injecting 3 variants of *H. purpurascens* L, Bogdan *et al.* (1990-b) confirmed that the most pronounced leukocytosis was observed 24h after the implantation of rhizome, the order of efficiency being as follows: 4% extract of saponosides, 4% decoction and 1‰ decoction. An inflammatory reaction manifested by an occurrence of oedema and spotted bleedings at the place of injection, was perceived in rats 24h after intramuscular

application of the extract of *Helleborus* sp. (Bolte *et al.*, 1992). Tosevski *et al.* (1999, 2004) recorded, after the application of extract of *Helleborus odorus* W. *et* K. to piglets in the age of 35 days and 52 days, a significant increase in the leukocytes count in the blood after 7, 14 and 21 days. A determined increase in the count of total leukocytes 24h after treatment of rats by HE in our trial is similar to the increase of this value stated by Milanović *et al.* (2004). In the trial on Wistar rats by the application of water extract of hellebore diluted by saline solution in proportion of 1:2 this author also registered a significant leukocytosis. High values in the count of total leukocytes after HE application can be compared with the results of Ristoska *et al.* (2002) who, after the application of the extract of hellebore to gilts, also recorded a significant increase in the value of the count of total leukocytes in relation to reference values.

Treatment with aqueous extract of *Boerhaavia diffusa* Linn. (Nyctaginaceae) also has been shown to induce leukocytosis with predominant neutrophilia (Mungantiwar *et al.*, 1997).

Pritchett and Corning (2004) suggest the limit of $1.3-3.6 \times 10^9/L$ as reference values in the count of neutrophil granulocytes in the blood of rats. In our trial, count of neutrophil granulocytes in the blood of rats of control group was $2.49 \pm 0.54 \times 10^9/L$, what is in accordance with the values suggested by these authors. In all trial groups in which the effect of HE during the time periods (24h, 48h i 72h) was monitored on the modifications in the count of neutrophil granulocytes in the blood of rats a greater value in relation to the control group was registered. Mean values as regards the count of neutrophil granulocytes in the blood of rats given the hellebore extract were as follows: $6.43 \pm 0.80 \times 10^9/L$ after 24h, $2.86 \pm 1.51 \times 10^9/L$ after 48h, $2.55 \pm 0.36 \times 10^9/L$ after 72h. A statistically highly significant difference was confirmed only between a control group and 24h after application of HE ($p < 0.001$). In this group the count of neutrophil granulocytes was higher by 158.23%. A statistically significantly higher value in the count of neutrophil granulocytes in blood was recorded 24h post-treatment in relation to other two trial groups ($p < 0.001$), among which a statistical significance was not recorded (Figure 2).

An average percent of neutrophil granulocytes in the blood of the control group of rats was 25.01 ± 4.80 , what is within the limits of reference values which according to Moor (2000) for this category of rats are: $16.6 \pm 5.7\%$ (males) and $15.3 \pm 5.7\%$ (females). A percent of neutrophil granulocytes in the blood of the trial rats in which the effect of HE was monitored during the time periods was from 48.09% (after 24h) being statistically significant in relation to the control group of animals ($p < 0.001$), to 26.53% (after 48h) and 23.77% (after 72h) which represent a statistically not significant difference in relation to the control group. The values regarding the percent of neutrophil granulocytes in the blood of the group 24h after application of HE was significantly higher in relation to other trial groups ($p < 0.001$), among which a statistical significance was not confirmed (Figure 3).

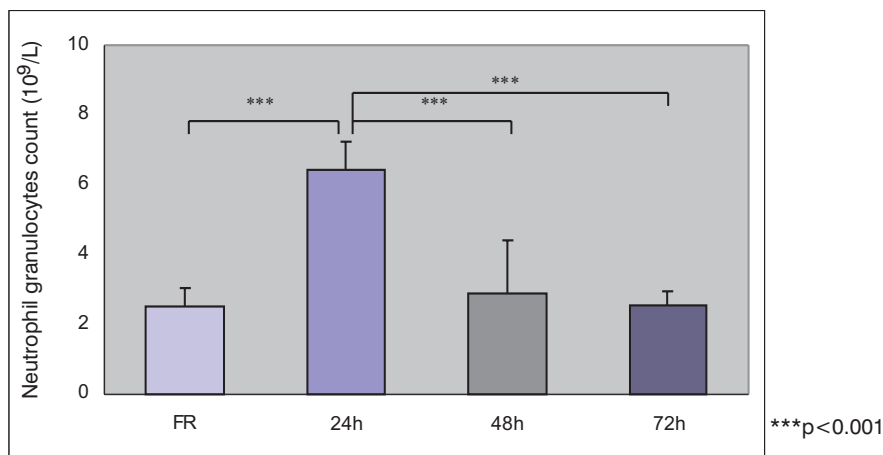


Figure 2. Neutrophil granulocytes count ($10^9/L$)

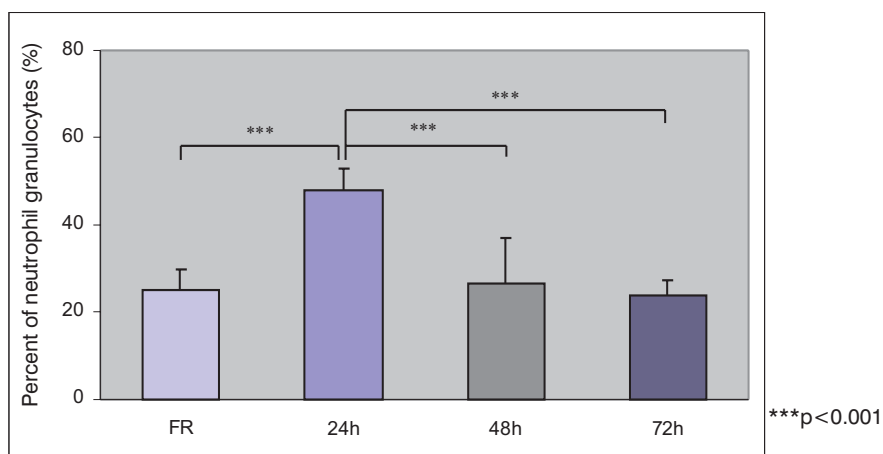


Figure 3. Percent of neutrophil granulocytes (%)

Our results correspond to the results of a greater number of authors who also recorded an increase in count and percent of neutrophil granulocytes in the blood of animals treated by HE. Bogdan *et al.* (1989, 1990-a, 1990-b, 1993) determined a significant increase in the count and percent of neutrophil granulocytes in a number of animal species (cattle, horses, pigs and sheep) 24h post-implantation of rhizome or application of the extract of hellebore. However, different to our research they registered a significant increase of neutrophil granulocytes after 48h, 96h and 144h as well. This could be explained by differences in the applied doses and mode of extraction. The authors also

perceived the modification in blood count relationship in some types of leukocytes in the leukocyte formula, in favour of neutrophil granulocytes whose mobilisation was stimulated the most. Granulocytosis, suggested by Milanović *et al.* (2004), after the application of HE water solution diluted by saline solution in proportions 1:2, 1:4 and 1:8 and applied to rats also corresponds to our results.

Our results are similar to those stated by Bolte *et al.* (1992) who studied immunostimulatory effects of HE in calves. The experiment which Bolte *et al.* (2001) some time later repeated on calves and bullcalves confirmed a significant increase in the count and percent of neutrophil, eosinophil and basophil granulocytes on 7, 14 and 21 days after the application of HE. Tosevski *et al.* (1999, 2004) recorded a significant increase in the count and percent of neutrophil granulocytes in the blood of piglets at the age of 35 days on 7 and 14 days after the application of HE, and in piglets at the age of 52 days on 14 and 21 day after the HE treatment. These results differ from ours only in the fact that the effect we have perceived was short-lived. Ristoska *et al.* (2002) reported that 24h after the application of HE to gilts the count and percent of neutrophil granulocytes remains within the limits of reference values, which is in contrast to the results of our research.

In the course of the trial the mean values in the count of lymphocytes in the blood of examined rats ranged from $6.05 \times 10^9/L$ (24h after application of HE) to $7.38 \times 10^9/L$ (72h after the application of HE) and were within the physiological limits ($5.6-8.3 \times 10^9/L$) suggested by Pritchett and Corning (2004). The value in the lymphocytes count in the blood of rats of the control group was lower only in relation to the lymphocytes count recorded in the group 72h after application of HE, whilst in other trial groups the lymphocytes count was higher, although determined differences were not significant. At the expiration of 72h after the application of HE a significantly higher count of lymphocytes in relation to the group of 24h after application of HE ($p < 0.05$) was recorded. The lowest value in the count of lymphocytes 24h after application of HE is proportional to the increase in the count of neutrophil granulocytes which is most pronounced in this group (Figure 4).

The results obtained 24h and 48h after the treatment with HE correspond to the results of Ristoska *et al.* (2002) who also determined that the count of lymphocytes remains within the limits of reference values 24h after the application of HE to gilts. A significant increase in the count of lymphocytes 72h after the application of HE to rats is partly in accordance with the results of Tosevski *et al.* (1999, 2004) who established that the application of HE to piglets at the age of 35 days caused an increase in the count of lymphocytes by 87% after 7 days and by 83% after 14 days in relation to the recorded value before HE application. It is interesting to note the observation of Tosevski *et al.* (2004) that in piglets at the age of 52 days the same dose and the same mode of application of HE as in previous studies brought about no change in the value regarding the count of lymphocytes and it has remained the same although somewhat reduced at day 14 and day 21 of the experiment.

Dirsch *et al.* (1993) confirmed that hellebrin, β -ecdysone and 5α -hydroxyecdysone from *H. purpurascens* W. *et* K. can produce supreme

proliferation of lymphocytes whilst a steroid saponine can have a stimulating effect therein. *In vitro* applied water extract of *H. niger* L. in different concentrations can influence immunocompetent cells in such a way that they induce the exchange of sister chromatides (SCE) in the culture of peripheral blood mononuclear cells (PBMC) of healthy persons and provoke the proliferation of isolated lymphocytes (Büsing and Schweizer, 1998).

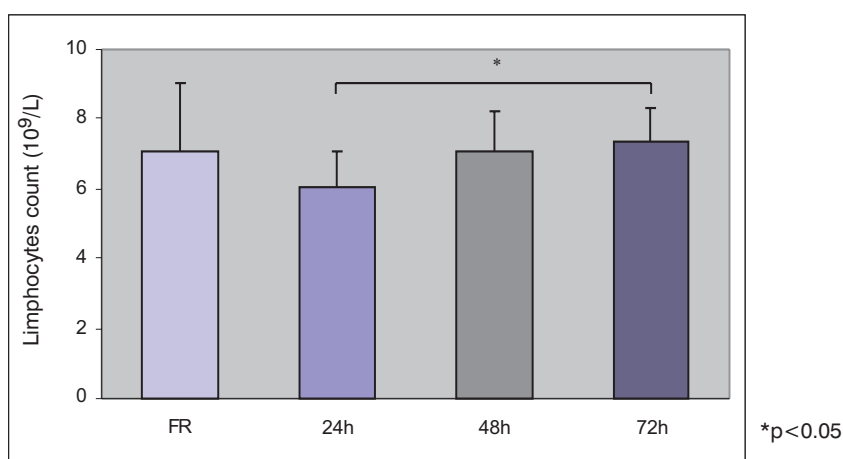


Figure 4. Lymphocytes count ($10^9/L$)

In all trial groups treated with hellebore extract except for the group at 72h after application of HE, the increase in neutrophil/lymphocyte index, in relation to the control group in which Ne/Lym index was 37.17 ± 11.66 , was observed. The highest confirmed Ne/Lym index was registered 24h after application of HE (109.22 ± 24.24), and it was higher by 193.84% in relation to the control group ($p < 0.001$). A difference in the average value of Ne/Lym index 24h after treatment with HE in relation to other trial groups was statistically significant ($p < 0.001$) (Figure 5).

In the available literature data we found no records regarding the values of Ne/Lym index after the application of HE or implantation of *Helleborus* L rhizome.

A number of monocytes in the blood of the control group rats was $0.57 \times 10^9/L$, whilst in the blood of trial animals this value ranged from $0.48 \times 10^9/L$ to $0.93 \times 10^9/L$. A determined difference in mean values in the count of monocytes was not statistically significant, neither between the control group and groups 24h, 48h and 72h after application of HE, nor among the self same groups treated by HE in the course of time period (Figure 6).

The absence of statistical significance in the examined groups of rats during the trial is in accordance with the results obtained on piglets at the age of 35 days (Tosevski *et al.*, 1999, 2004). Ristoska *et al.* (2002) established a very pronounced monocytosis 24h and 48h after the application of HE to gilts. Tosevski *et al.* (2004)

also noted an increase in the count of monocytes in piglets at the age of 52 days being 1.4 times higher 14 days after the application of HE and by 1.2 times higher at day 21. of trial.

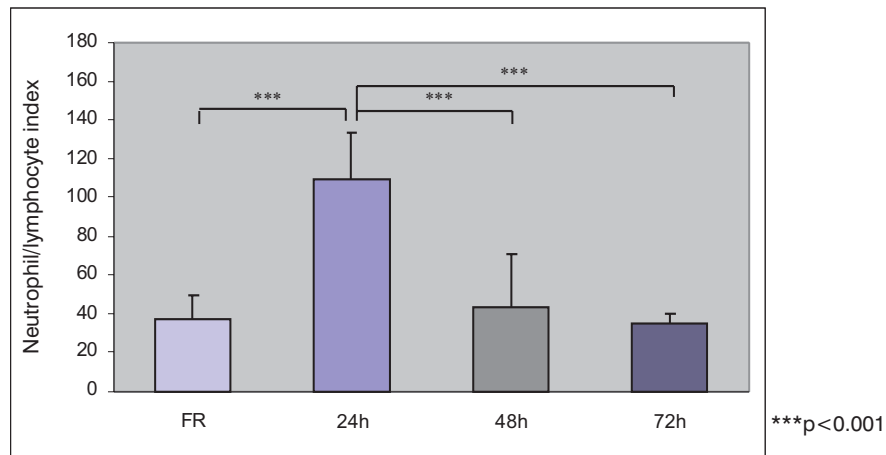


Figure 5. Neutrophil/lymphocyte index

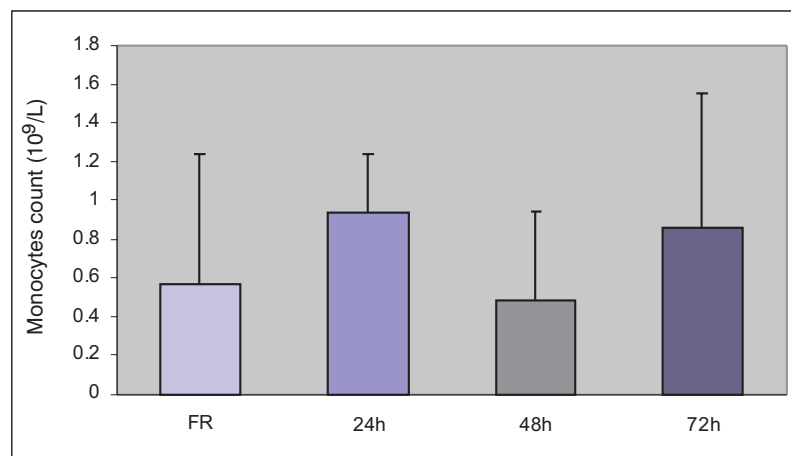


Figure 6. Monocytes count (10⁹/L)

A research on the degree of phagocytosis of residing (non-stimulated) peritoneal macrophages, by determining the level of production of H₂O₂ was conducted 24h after the intramuscular application of sterile physiological solution (FR) to a control group of rats and application of HE (10 mg/100 g BW) to a trial

group. The average H_2O_2 concentration values (nM/mg protein) after the stimulation of macrophages by different concentrations of PMA in the control group of rats ranged from 12.80 (after stimulation with 0 nM PMA) to 38.74 that is 32.36 (after stimulation with 25 nM PMA ie. 50 nM PMA). In the trial group of rats 24h after the application of HE, after stimulation of macrophages by different concentrations of PMA, the values ranged from 6.29 (after stimulation with 0 nM PMA) to 88.53 (after stimulation with 50 nM PMA). These results suggest a dose-dependent trend in the production of H_2O_2 depending on the concentration of PMA, reaching the "plateau" values at the concentration of 25 nM in the control group and 50 nM in the trial group of rats.

A significantly higher production of H_2O_2 (nM/mg proteins) created by macrophages after stimulation by 0 nM PMA was recorded in the control group in relation to the groups to which HE was applied, the level of significance being $p < 0.01$. The difference in mean values in the concentrations of H_2O_2 (nM/mg proteins) created by macrophages was not statistically significant between the control and trial groups after stimulation of macrophages with 6 nM PMA. Statistically significant differences ($p < 0.05$) were confirmed after stimulating the production of H_2O_2 (nM/mg proteins) by macrophage with 12.5 nM PMA, 25 nM PMA and 50 nM PMA, whereas in all cases a significantly greater concentration was established in the group of rats to which HE was applied in relation to the control group (Figure 7).

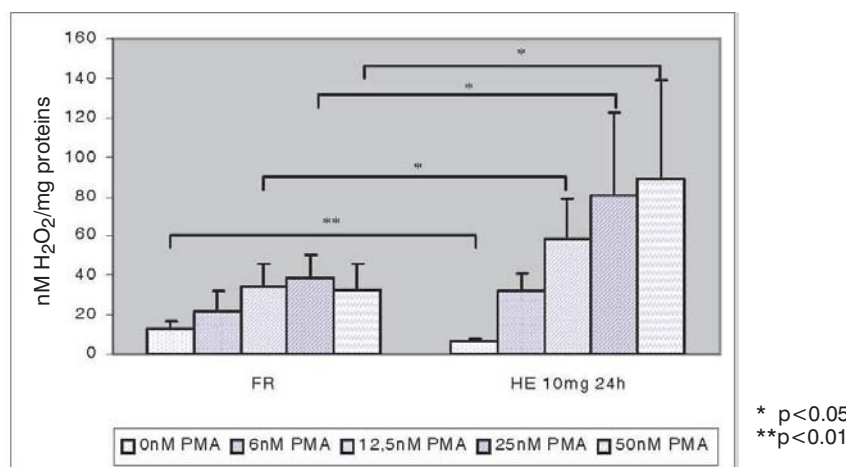


Figure 7. Concentration of H_2O_2 (nM/mg proteins) created by residing (non-stimulated) peritoneal macrophages

On the basis of these records it can be concluded that the treatment of rats with the extract of rhizome and root of hellebore has led to a significant increase in the production of H_2O_2 by residing peritoneal macrophages. Analysing a highly purified extract of *H. purpurascens* W. *et* K. (HP 12) Kerek (1997) confirmed that it

has a stimulatory influence on rats' macrophages. Also, the application of the aqueous extract of *Boerhaavia diffusa* Linn. (Nyctaginaceae) to Swiss albino mice has resulted in the stimulation of the phagocytic and bactericidal capacity of macrophages and neutrophil granulocytes (Mungantiwar *et al.*, 1997). Different immunoactive plant polysaccharides can activate neutrophils and macrophages and enhance secretion of pro-inflammatory mediators such as cytokines, eicosanoids and enzymes. Pectic polysaccharides from *Selene vulgaris* (Caryophyllaceae) and galactomannan from *Trigonella foenum-graecum* (Fabaceae) were shown to increase the uptaking capacity of rat peritoneal resident macrophages (Schepetkin and Quinn, 2006). Oral administration of Echinacea extracts resulted in increased phagocytic activity of rats' alveolar macrophages and increased phagocytic index (Goel *et al.*, 2002).

A research on the degree of phagocytosis of neutrophil granulocytes by determining the phagocyte index was carried out 24h after intramuscular application of sterile physiological solution to a control group of rats and application of HE (10 mg/100 g BW) to a trial group. A significantly higher percent ($p < 0.05$) of neutrophil granulocytes containing at least 3 granules of latex particles in rats to which HE was applied (30.04 ± 6.29) in relation to the rats in the control group (23.76 ± 2.89) was recorded (Figure 8).

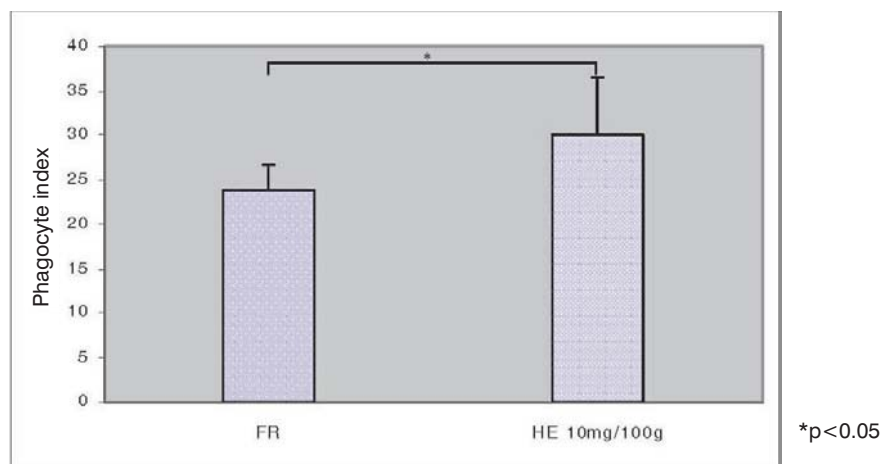


Figure 8. Phagocyte index of neutrophil granulocytes

Our results obtained on rats correspond to the results of Bogdan *et al.* (1989, 1990-a). These authors say that the index of phagocytosis in sheep was 0-21 before the implantation of rhizome of *Helleborus* L. and 0-34 after the implantation. It means that under the influence of the implants of hellebore the value of an overall capacity of phagocytosis of neutrophil granulocytes has increased more than 6 times in relation to the value before the implantation. The determined difference was statistically significant after 48h ($p < 0.01$).

It can be assumed that the immunostimulatory influence of HE is realized by at least three mechanisms: by increasing the count of leukocytes, by increasing the count of neutrophil granulocytes and by stimulating the phagocytosis by neutrophil granulocytes and macrophages.

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UTICAJ EKSTRAKTA RIZOMA I KORENA KUKUREKA (*HELLEBORUS ODORUS* W. ET K.) NA PARAMETRE BELE KRVNE SLIKE I STEPEN FAGOCITOZE KOD WISTAR PACOVA

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SADRŽAJ

Cilj ovog rada je bio da se ispita uticaj ekstrakta rizoma i korena *H. odorus* W. et K. na promenu vrednosti parametara bele krvne slike i stepen fagocitoze od

strane peritonealnih makrofaga i neutrofilnih granulocita kod pacova soja Wistar. Ogled je izveden na 28 pacova podeljenih u 4 grupe po 7 jedinki. Kontrolnoj grupi pacova je intramuskularno aplikovan sterilan fiziološki rastvor u količini od 0,25 ml/100 g TM. U cilju praćenja efekta ekstrakta rizoma i korena kukureka (EK) u toku vremena, pacovima je intramuskularno aplikovan EK u dozi od 10mg/100g TM, a krv za analizu je uzimana posle 24h, 48h i 72h.

Intramuskularna aplikacija EK imala je za posledicu povećanje broja ukupnih leukocita u svim oglednim grupama, pri čemu je najizraženija leukocitoza registrovana 24h nakon aplikovanja EK. Statistički značajno veća vrednost broja i procenta neutrofilnih granulocita u krvi zabeležena je 24h posle tretmana u odnosu na kontrolnu i ostale dve ogledne grupe ($p < 0,001$), između kojih nije utvrđena statistička značajnost. Ekstrakt rizoma i korena kukureka doveo je do nastanka limfopenije, što je imalo za posledicu povećanje neutrofilno/limfocitnog indeksa u oglednim grupama 24h i 48h nakon tretmana. Aplikacija EK nije značajno uticala na broj monocita kod tretiranih životinja. Upotrebljeni ekstrakt doveo je do značajnog povećanja stepena fagocitoze od strane rezidentnih peritonealnih makrofaga i neutrofilnih granulocita krvi.