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IN VITRO IMMUNE RESPONSE OF SAPONIN RICH FRACTION OF *BACOPA MONNIERI*, LINN.

Hule A.K. and Juvekar A.R.*
Department of Pharmaceutical Sciences and Technology, University Institute of Chemical Technology (UICT), Nathalal Parikh Marg, Matunga (East), Mumbai-400019, M.S. India.

Corres author: arj@udct.org
Phone No: +91 22 2414 5616, Ext. 2215
Mobile No: +91 9870009227,Fax No.: +91 22 2414 5614

ABSTRACT: The saponin rich fraction (SRF) of *Bacopa monnieri* were prepared and evaluated for in vitro immune response using murine immune cells. Thioglycollate medium elicited peritoneal exudates cells (PEC) were used to study *in vitro* effects of the fraction (SRF) on release of immune mediators. SRF was evaluated at different concentrations (832 – 6.5µg/ml) for release of nitric oxide (NO), superoxide (NBT reduction), lysosomal and myeloperoxidase assays on PEC. SRF was also screened for cytotoxicity in sulforhodamine B (SRB) assay on murine isolated peritoneal macrophages, splenocytes and bone marrow cells. The data was statistically compared by using one way ANOVA followed by Dunnet's multiple comparisons test. The fraction showed significant (P<0.05) stimulation of release of NO at 416µg/ml (stimulation index, SI=1.67) and 208µg/ml (SI=2.14). NBT reduction were significantly stimulated at 52µg/ml (SI=1.34) by the fraction as compared to control (SI=1). Lysosomal enzyme activity was stimulated by the fraction at 104µg/ml (SI=1.89). Myeloperoxidase activity were significantly increased at 832µg/ml (SI=1.43) and 416µg/ml (SI=1.36) of the fraction with respect to control wells (SI=1). Phytohemagglutinin-M (positive control) showed stimulation of release of all the tested mediators. The SRF showed stimulation of macrophages (208µg/ml), splenocytes (832 and 416µg/ml) and bone marrow cells (208µg/ml) in SRB assay. In conclusion, saponin rich fraction of *Bacopa* exhibited *in vitro* stimulatory effects on release of immune mediators from murine peritoneal cells and proliferative effects on immune cells *in vitro*.
KEYWORDS: *Bacopa monnieri*; saponin; nitric oxide; myeloperoxidase; splenocytes; SRB

INTRODUCTION

Bacopa monnieri (Linn) Wettstein, is also referred to as *Bacopa monniera*, *Gratiola monniera, Herpestis monniera*, *Moniera cuncifolia*, water hyssop, and Brahmi (family: *Scrophulariaceae*). In the ancient Indian system of medicine, viz., Ayurveda, B. monnieri has been classified under medhyarasayana, i.e., medicinal plants rejuvenating intellect and memory. The ancient classical Ayurvedic treatises, viz., Charak samhita, Susruta samhita, and Astanga hrdaya, have prescribed B. monnieri for the promotion of memory, intelligence, and general performance[1].

Traditionally, *Bacopa* was used as a brain tonic to enhance memory development, learning, concentration, and to provide relief to patients with anxiety or epileptic disorders. The plant has also been used in India and Pakistan as a cardiac tonic, digestive aid, and to improve respiratory function in cases of bronchoconstriction[2]. Antioxidant properties of *Bacopa* may offer protection from free radical damage in cardiovascular disease and certain types of cancer[3].

The major chemical constituents isolated and characterized from *Bacopa* are dammarane type of triterpenoid saponins with jujubogenin or pseudojujubogenin moieties as aglycone units. The composition of bacoside A has been established as a mixture of four triglycosidic saponins, viz. bacoside A_3, bacopaside II, bacopaside X and bacosaponin C[4,5]. Other major bacopasaponins are bacoside B, bacopaside I, bacopaside N2 and the minor components were bacopasaponin F, bacopasaponin E, bacopaside N1, bacopaside III, bacopaside IV and bacopaside V[6]. *Bacopa* also contains alkaloids viz. brahmine, nicotine and herpestine[2]. Other constituents of *Bacopa* are *p*-hydroxy benzmethanol, *p*-hydroxy benzoic acid, ursolic acid, lupeol, 28-hydroxylupeol, Stigmasterol-3-O-β-D-glucopyranoside, β-Daucosterin, ampelozigenin, 3,4-

dimethoxycinnamic acid, feruloyl glucoside, rosavin, quercetin, apigenin, luteolin, zizyotin, loliolide[7].

It is considered that the pharmacological activities of *Bacopa monnieri* are attributed to saponin compounds present in the alcoholic extract of the plant[4]. Therefore, aim of the present investigation was to prepare and evaluate saponin rich fraction of *Bacopa monnieri* for i*n vitro* immune response on isolated murine cells for release of immune mediators and cell viability.

EXPERIMENTAL

PLANT MATERIAL

Aerial parts of *Bacopa monnieri* were collected from Botanical Garden of Mumbai University Kalina campus, in August 2008 and authenticated by botanist of the Institute. The voucher specimen (No. 2008/08/02) was deposited in the herbarium of the Institute.

EXTRACTION FOR SAPONIN RICH FRACTION

This was carried out as per the method described earlier[4] with some modifications. The air dried and powdered plant material (400g) was extracted with methanol by soxhlation for 6h. The methanol extract was concentrated under vacuum. The dark colored residue (78g) was refluxed with n-butanol for 2h and n-butanol soluble constituents were separated by filtration. The n-butanol layer was sequentially washed with distilled water, alkali (2% KOH) and distilled water again. The n-butanol layer was evaporated and dried under vacuum to obtain a dark green color powder (14g). Charcoal treatment was given to the powder and the filtrate was further dried under vacuum to give saponin rich fraction (10.5g). The fraction was tested for froth test, Libermann-Burchard test and Molisch test.

ANIMALS

Swiss albino mice were procured from Haffkine Bio-pharmaceuticals Ltd., Mumbai. The animals were acclimatized for 10 days before being used for the experiments. They were housed in a room with controlled temperature ($23+2^0$C) and a 12h light/ 12h dark cycle. The animals were fed with standard pellet diet ('Amrut' brand, M/s. Nav Maharashtra Chakan oil mills Ltd., Pune, India) and water *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee of Institute and conducted according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

CHEMICALS

Fluid thioglycollate medium, fetal bovine serum (FBS), Streptomycin, penicillin, Roswell Park Memorial Institute (RPMI) 1640 medium, Triton-X-100 and HEPES buffer were procured from Himedia Pvt. Ltd. India. Phytohemagglutinin-M (PHA), nitroblue tetrazolium (NBT) AND sulforhodamine B (SRB) were procured from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

ISOLATION OF PERITONEAL EXUDATE CELLS AND CULTURE CONDITIONS

Peritoneal exudates cells (PEC) were isolated from mice which were injected intraperitoneally (i.p.) with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100mg/ml streptomycin (complete RPMI). Total cell numbers were counted using hemocytometer, and percentages of neutrophils and macrophages were determined by differential counting of Wright-Giemsa-stained cytospins (100–200 cells per sample) using a light microscope. Then the cells were adjusted to required cell count per ml and plated on a 96-well flat-bottom culture plate (Tarsons Products Pvt. Ltd., India) and then incubated for 2h at 37^0C in a 5% CO_2 humidified incubator. After removing the nonadherent cells, the mono-layered cells were treated with 20µl of *Bacopa* extract (832 – 6.5 µg/ml) dissolved in complete RPMI medium containing 0.1% DMSO (dimethyl sufoxide) and maintained for 24h at 37^0C in a 5% CO_2 humidified incubator[8,9]. Following assays were performed on these incubated cells. All the experiments were performed triplicate.

NITRITE ASSAY

Nitrite accumulation was used as an indicator of nitric oxide (NO) production in the medium as previously described[10]. Cell-free supernatant (50µl) from 24h incubated PEC ($5x10^5$ cells/ml) was mixed with 50µl of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. The optical density (OD) was measured at 540 nm with a microplate reader (ELX800MS, BioTek Instruments Inc., USA). PHA (10µg/ml) was used as a positive control. Nitrite concentrations were determined from standard curve of sodium nitrite in culture conditions. Stimulation index (SI) was calculated as the nitrite concentrations ratio of the treated and control cells.

NITROBLUE TETRAZOLIUM (NBT) DYE REDUCTION

The NBT dye reduction assay was carried out as described previously[11]. Briefly, 50µl of 0.3% NBT solution in phosphate buffered saline (pH= 7.4) (PBS) were added to the 24h incubated PEC (1×10^6 cells/ml) with saponin fraction. The mixture was further incubated at 37^0C in a 5% CO_2 humidified incubator for 1h. The adherent cells were rinsed vigorously with complete RPMI medium, and washed four times with 200µl Methanol. After air-dried, formazan-deposits were solubilized in 120µl of 2M KOH and 140µl of DMSO. After homogenization of the contents of the wells, the OD was read at 630 nm by using a microplate reader. The stimulation index (SI) was calculated as the OD ratio of the treated and control cells.

CELLULAR LYSOSOMAL ENZYME ACTIVITY

The cellular lysosomal enzyme activity of PEC cells was evaluated by measuring acid phosphatase activity as described earlier[10]. Briefly, 24h after incubation at 37°C in humidified 5% CO_2, the medium was removed by aspiration and 20µl of 0.1% Triton X-100 were added to each well. After 15 minutes incubation, 100 µl of 10mm *p*-nitrophenyl phosphate (*p*NPP) and 50 µl of 0.1 M citrate buffer (pH 5.0) were added. Further the plates were incubated for 1h and 0.2 M borate buffer (150 µl, pH 9.8) was added. The OD was measured at 405 nm by using a microplate reader. The Phagocytic stimulation index (SI) was calculated as the OD ratio of the treated and control PEC cells.

MYELOPEROXIDASE ACTIVITY ASSAY

This assay was carried out as per the as per the procedure described earlier[12, 13]. Briefly, 24h incubated PEC cells ($5x10^5$cells/ml) were washed three times with fresh complete RPMI medium. Then the mixture (100 µl) of *o*-phenylenediamine (0.4 g/ml) and 0.002% H_2O_2 in phosphate-citrate buffer (pH 5.0) was added to each well. The reaction was stopped after 10 min using 0.1 N H_2SO_4 and optical density (OD) was measured at 490 nm. The myeloperoxidase activity was measured as stimulation index (SI) which was calculated as the OD ratio of the treated and control macrophages.

SULFORHODAMINE B (SRB) ASSAY ON ISOLATED MURINE PERITONEAL EXUDATES CELLS

Peritoneal exudates cells (PEC) were isolated as above and were cultured (1×10^5cells/well) in complete RPMI and incubated for 2 hr at 37ºC in 5% CO_2 atmosphere. The SRB assay was performed as per the procedure described earlier by Vichai et al (2006)[14]. In Brief, the SRF of Bacopa was dissolved in complete RPMI containing 0.1% DMSO were added at various concentrations (832-6.5µg/ml) in triplicate. After 24 h incubation, cells were fixed by adding ice-cold 20% trichloroacetic acid (TCA) and incubating for 1 hr at 4ºC. The plates were washed with distilled water, air-dried and stained with SRB solution (0.4%w/v in 1% acetic acid) for 30 min at room temperature. Unbound SRB was removed by washing thoroughly with 1% acetic acid and the plates were air-dried. The bound SRB stain was solubilized with 100 µl of 10mM Tris buffer (pH-10.0), and the optical density was read at 540 nm. The effect of SRF on cell count was calculated as proliferative index (PI) as the OD ratio of the treated and control wells.

SRB ASSAY ON SPLENOCYTES

The mice were euthanized by cervical dislocation. The spleens were removed aseptically, stripped of fat and placed in a complete RPMI medium. Single-cell suspensions were obtained by gentle homogenization of mouse spleen in a tissue homogenizer. The spleen cells were separated from the debris; erythrocytes were lysed by hypotonic solution and then washed twice (5 min at 800g at 4^0C). Isolated cell suspensions were washed three times in the culture medium. Splenocyte viability was assessed by trypan blue dye exclusion[15]. The viability of the cells used throughout was > 95%. SRB assay for these splenocytes (1×10^5cells/well) was carried out as described above.

SRB ASSAY ON BONE MARROW CELLS

Mice were killed and femur bones were removed aseptically. Cell suspension was prepared by means of flushing. The mixture was centrifuged and the cell pellets were washed twice and resuspended in complete RPMI medium. The cell numbers were determined by a hemocytometer and cell viability was tested by trypan-blue dye exclusion technique[16]. SRB assay for these bone marrow cells (1×10^5cells/well) was carried out as described above.

STATISTICAL ANALYSIS

Results expressed as Mean ± SEM for triplicate assays. Data were analyzed by one way ANOVA followed by Dunnet's multiple comparisons test using GraphPad InStat software. Values of $P<0.05$ were the criteria for statistical significance.

RESULTS

EXTRACTION AND PHYTOCHEMICAL TESTS

The yield of the saponin rich fraction of the dry and powdered aerial parts of *Bacopa monnieri* was 2.6%w/w. This fraction gave positive froth test for saponins, Libermann-Burchard test for triterpenes and Molisch test for sugars.

NITRITE ASSAY

The ability of macrophages to induce the release of nitric oxide (NO) was measured through nitrite, which is a stable breakdown product of NO. Activation of murine peritoneal cells caused accumulation of nitrite in the culture medium (Fig. 1). The PEC exposed to saponin fraction of *Bacopa* produced increasing amount of nitrite without concentration dependent manner, indicating the fraction as an effective NO inducer. In all three experiments performed, *Bacopa* fraction induced nitrite production in significantly higher ($P<0.05$) amounts at 416µg/ml (SI=1.67), 208µg/ml (SI= 2.14) and 104µg/ml (SI= 1.59) concentrations than control. PHA (positive control) also showed significant increase ($P<0.05$) in nitrite release (SI= 2.36). High NO production is an indication of macrophage activation.

NITROBLUE TETRAZOLIUM (NBT) DYE REDUCTION

The *in vitro* phagocytic effect of the fraction (832 – 6.5 µg/ml) on the reduction of NBT dye by isolated PEC cells is presented in Fig. 1. Significant ($P<0.05$) suppression was observed at 832µg/ml (SI=0.67) and stimulation at 52µg/ml (SI=1.34) with respect to control (SI=1). Positive control, PHA showed significant stimulation of NBT reduction (SI= 1.73).

CELLULAR LYSOSOMAL ENZYME ACTIVITY

The effect of saponin rich fraction of *Bacopa* on cellular lysosomal enzyme activity of murine PEC cells is

presented in Fig. 1. Lysosomal enzyme activity was significantly stimulated (P<0.05) by the fraction at 104µg/ml (SI=1.89) and by PHA (SI=2.19) as compared to control wells (SI=1).

MYELOPEROXIDASE ACTIVITY

The effect of sasponin rich fraction of *Bacopa* on myeloperoxidase activity of murine PEC cells is presented in Fig. 1. Significant increase (P<0.05) in the myeloperoxidase activity was observed for the fraction at 832µg/ml (SI = 1.43) and 416µg/ml (SI = 1.36) as compared to control wells (SI=1). Positive control, PHA showed significant stimulation (P<0.05) with stimulation index (SI) of 1.59.

SRB ASSAY ON ISOLATED MURINE PERITONEAL EXUDATES CELLS

The effect of saponin rich fraction (SRF) of *Bacopa* on murine peritoneal exudates cells is presented in Table 1. The effect of the fraction on exudates cells in SRB assay was not dose-dependent. The proliferation index for SRF was significant at 208µg/ml (PI=1.56) and for PHA (10µg/ml) was 1.72.

SRB ASSAY ON SPLENOCYTES

The effect of saponin rich fraction (SRF) of *Bacopa* on murine splenocytes is presented in Table 1. SRF showed significant proliferation of splenocytes in SRB assay at 416 (PI=1.44) and 832µg/ml (PI=1.39). Positive control, PHA (10µg/ml) showed significant proliferation (PI=1.49).

SRB ASSAY ON BONE MARROW CELLS

The effect of saponin rich fraction (SRF) of *Bacopa* on murine bone marrow cells is presented in Table 1. The effect of SRF was not dose-proportionate on bone marrow cells in SRB assay. The significant proliferation was observed with SRF at 208µg/ml (PI=1.34) and with PHA at 10µg/ml (PI=1.52).

DISCUSSION

Saponin compounds of the *Bacopa monnieri* are considered as active constituents of the plant[4]. Therefore it was considered worthwhile to assess the effect of the saponin rich fraction of *Bacopa* on isolated murine peritoneal exudates cells on secretion of mediators involved in elicitation of immune response. The saponin rich fraction of *Bacopa* extract showed positive froth test for saponins, Libermann-Burchard test for triterpenes and Molisch test for sugars confirms that the fraction contains triterpenoid saponin glycosides.

The isolated murine peritoneal exudate cell preparations composed of ~30% macrophages, ~60% neutrophils, and 7–10% lymphocytes[9]. These cells are important immune effector cells required in maintaining disease free state or elevating disturbed immune state. Macrophages actively participate as cellular effectors of non-specific host defense. Antigen stimulation transforms the precursor monocytes into macrophages, which subsequently eliminate pathogens via phagocytosis[17, 18].

Nitric oxide (NO) has been shown to be the principal effector molecule produced in macrophages by inducible nitric oxide synthase (iNOS) for cytotoxic activity and can be used as a quantitative index of macrophage activation[19, 20]. Agents that modulate the activity of NO may be of considerable therapeutic value[21]. NO mediates diverse functions, including vasodilatation, neurotransmission and inflammation[22]. The fact that saponin rich fraction of *Bacopa* extract induce the release of NO from macrophages indicates that it is acting as general macrophage function activator.

The reduction of NBT to insoluble blue formazan was used as a probe for superoxide generation, although it is not entirely specific for O_2^- radical[23]. The NBT reduction assay estimates the ability of neutrophils, and macrophages to produce oxygen radicals (O_2^-, OH^-, O_3, H_2O_2). The ability of macrophages to kill the pathogenic microbes is probably one of the most important mechanisms of protection against disease[24]. The fraction showed suppression at higher concentration (832µg/ml) and stimulation at lower concentration (52µg/ml). This dual effect of *Bacopa* fraction on NBT dye reduction suggests concentration dependent activity. For lysosomal enzyme activity, the transformation of *p*-NPP to coloured compound by the acid phosphatase of the stimulated macrophages correlates to the extent of degranulation in phagocytosis[25]. The effects of various concentrations of saponin rich fraction of *Bacopa* extract on NBT dye reduction and lysosomal enzyme activity response of peritoneal exudate cells were studied for phagocytic assay. Saponin fraction appeared to produce phagocytic modulation without dose response relationship in these assays.

Neutrophils and macrophages secrete a heme protein, myeloperoxidase, which uses the oxidizing potential of H_2O_2 to convert chloride ion into hypochlorous acid (HOCl). A potent bactericidal agent, HOCl is a critical component of host defenses against invading bacteria, fungi, and viruses[26]. Stimulation of myeloperoxidase activity by saponin fraction of *Bacopa* suggests its role in defense system. Modulation of the secretion of these mediators of immune system by the saponin rich fraction of *Bacopa* suggests that it can effectively strengthen innate immunity against foreign agents[27].

The sulforhodamine B (SRB) assay is one of the most widely used method for *in vitro* cell viability/ cytotoxicity screening. The assay relies on the ability of the SRB to bind to protein components of the cells that has been fixed to tissue culture plates by trichloroacetic acid (TCA). SRB is a bright pink aminoxanthene dye with two sulphonic groups that bind to basic amino acid residue under mild acidic conditions, dissociate under basic conditions. Washing with water then looses the disulfide bond of protein and make it free to bind with SRB dye. At last, addition of TRIS-buffer makes medium alkaline, leads to release of SRB dye from disulfide bond and give pinkish red color which is then measured by ELISA plate reader. As the binding of SRB is stoichiometric, the amount of the dye extracted from stained cells is directly proportional to the cell mass[14]. Murine isolated peritoneal macrophages, splenocytes and

bone marrow cells when exposed to saponin rich fraction (SRF) of *Bacopa monnieri* and assayed for SRB assay showed significant proliferation of these cells. The saponin rich fraction was non-toxic to these cells at tested concentrations (832-6.5µg/ml). This indicates that the fraction may stimulate the immune system by stimulating production of these cells.

CONCLUSION

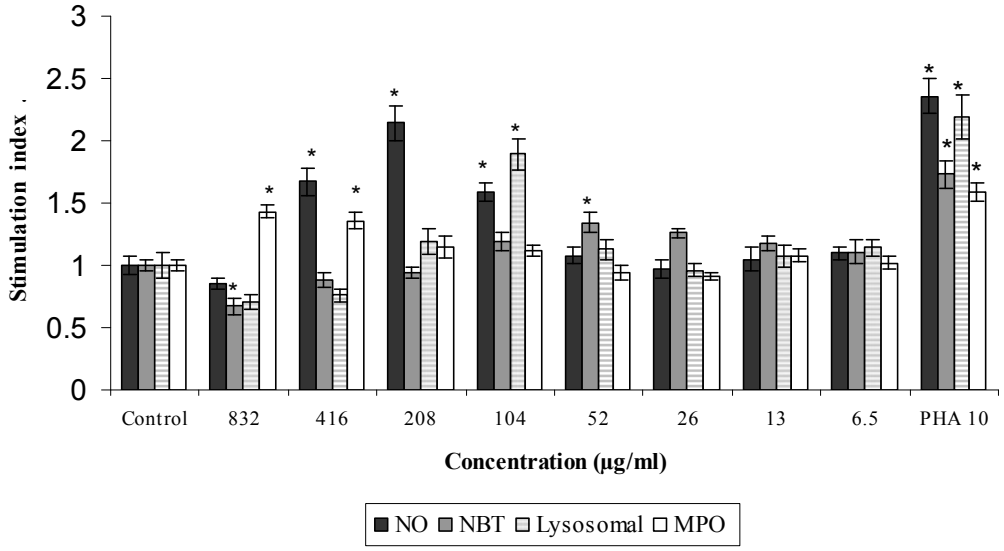
In conclusion, our results suggest that the fraction containing triterpene saponin glycosides are able to activate murine peritoneal exudates cells (consisting mainly macrophages and neutrophils), resulting in the increased production of various immune mediators and was able to stimulate viability of immune cells. These results suggest that the activity of the fraction may be mediated through upregulation of secretory molecules in macrophages and neutrophils and this fraction plays role in activation of these cells. Further studies with animal models are necessary to clarify how this activation/modulation occurs and to what extent it occurs in intact live system.

Table 1. Effect of saponin rich fraction of *Bacopa m.* on cell viability of murine peritoneal macrophages, splenocytes and bone marrow cells in SRB assay

Groups (Concentration -µg/ml)	Macrophage PI	Splenocytes - PI	Bone Marrow Cells - PI
Control	1.00±0.09	1.00±0.09	1.00±0.05
SRF-832	0.79±0.07	1.39±0.13*	1.05±0.07
SRF-416	0.94±0.10	1.44±0.11**	1.21±0.11
SRF-208	1.56±0.06**	1.07±0.08	1.34±0.10*
SRF-104	1.31±0.05	1.10±0.06	0.96±0.05
SRF-52	1.11±0.06	1.16±0.04	0.93±0.06
SRF-26	0.88±0.07	0.86±0.05	1.04±0.04
SRF-13	0.90±0.05	0.93±0.06	0.89±0.07
SRF-6.5	0.79±0.06	0.90±0.04	0.84±0.05
PHA-10	1.72±0.13**	1.49±0.12**	1.52±0.10*

The data represent Mean ± SEM of triplicate experiments.
SRF - saponin rich fraction of *Bacopa monniera*, PI – proliferation index, PHA - Phytohemagglutinin-M
* $P<0.05$ and ** $P<0.01$ vs. control group.

Figure 1: In vitro effect of saponin rich fraction of *Bacopa M.* on release of nitric oxide and NBT reduction, lysosomal and myeloperoxidase enzyme activity of isolated peritoneal exudates cells



The data plotted represent Mean ± SEM of triplicate experiments.
NO – Nitric oxide; NBT - Nitroblue tetrazolium; MPO – myeloperoxidase assay
* $P<0.05$ and ** $P<0.01$ vs. control group.

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