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Research Article

Effects of Cadmium and *Acanthopanax senticosus* Extract on the Activities of Immune Cells in Mice Spleen

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ABSTRACT

Cadmium is an environmental hazard which suppress humoral and cellular immune response of immune cells. *Acanthopanax senticosus* (Rupr. et Maxim.) Harms (AS) is a widely used adaptogen containing triterpenoid saponins, flavonoids and polysaccharides. AS improves immune response enhancing activities of immune cells. The aim of this study was to investigate the ability of liquid extract of AS to moderate immunotoxic effects of cadmium. The effects of AS extract on accumulation of Cd as well as on levels of splenic immune cells after chronic treatment with $CdCl_2$ were investigated. BALB/c mice were periodically injected intraperitoneal (i.p.) for 6 weeks with $CdCl_2$ and AS extract solutions of two different concentrations in deionized water: 0.05 LD_{50} AS; 0.10 LD_{50} AS; 0.05 LD_{50} Cd^{2+}; 0.05 LD_{50} Cd^{2+} and 0.05 LD_{50} AS; 0.05 LD_{50} Cd^{2+} and 0.10 LD_{50} AS. Control group mice were periodically injected i.p. with the saline solution. The levels of macrophages, T lymphocytes and B lymphocytes were determined by immunohistochemistry using, respectively, CD68, CD3 and CD20 antibodies. The results showed that periodical injection of $CdCl_2$ and $CdCl_2$ together with AS caused increase of Cd^{2+} concentration in the spleen as compared to control and AS groups. Injections of cadmium increased the number of macrophages. AS extract and/or Cd^{2+} did not change the number of T lymphocytes. AS extract significant increase the number of B lymphocytes in comparison to control group, furthermore the number of B lymphocytes was higher in Cd+AS 0.1 LD_{50} group than in mice injected with $CdCl_2$ only.

Keywords: Cadmium; *Acanthopanax senticosus*; Mice; Spleen

INTRODUCTION

Cadmium is a heavy metal of considerable toxicity with destructive impact on most organ systems. There is evidence for potential roles of cadmium in chronic liver diseases and hepatocellular carcinoma risk [1]. Cadmium accumulation in the spleen, liver and kidney is associated with degeneration and inflammatory changes [2]. Cadmium induces tissues injury trough creating oxidative stress [3,4], epigenetics changes in DNA expression [5,6]. Post-natal Cd exposures induce cell cycle arrest and apoptosis in splenocytes [7].

Cadmium is able to induce apoptosis of lymphoid cells, and suggest that this phenomenon may contribute to its immunotoxic effect in vivo [8]. Cadmium affects immune response and seems to have a clear carcinogenic effect in human

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[9]. Lymphocyte proliferation and natural killer cell activity are suppressed by Cd [10]. Cadmium suppress both humoral and cellular immunity with simultaneous increase in the level of blood corticosterone and aldosterone, involvement of adrenal hormones in cadmium induced immunosuppression suggesting that cadmium activates the corticosteroid associated immunoregulatory circuit [11]. Cadmium potentiates oxidative stress followed by mitochondrial-caspase dependent apoptotic pathway. It causes thymic atrophy and splenomegaly, in addition to immuno-suppression and modulation of humoral and/or cellular immune response [12,13].

The results on the distribution of blood lymphocyte subsets suggest that cadmium inhibits the humoral and cellular immune response with the lower doses of the metal used, and opposite effects were detected with the higher doses [14]. Chronic exposure to Cd produced dose- and time-dependent splenomegaly and thymic atrophy [15]. Overall, cadmium has been shown to enhance humoral immune responses at low levels of exposure, whereas higher ones may result in either no effect or decreased antibody production. Similarly, phagocytosis, natural killer cell activity and host resistance toward experimental infections were markedly impaired in most instances. [16]. Studies of the immunomodulatory effects of Cd in experiment animals are inconsistent probably due to varying doses, route of administration, length of Cd exposure, and differences in the sensitivity of immune system of different animal species [14-17]. Cd shows little if any immune-stimulating potential when injected alone [17] Costimulatory effects contrasts with immunosupressive properties for this metal [2,16].

Acanthopanax senticosus (Rupr. et Maxim.) Harms (Araliaceae), also called Siberian Ginseng, Eleutherococcus senticosus is a widely used traditional Chinese herb that could invigorate qi, strengthen the spleen, and nourish kidney in the theory of Traditional Chinese Medicine [18]. Several kinds of chemical compounds have been reported, including triterpenoid saponins, lignans, coumarins, and flavones, among which, phenolic compounds such as syringin and eleutheroside E, were considered to be the most active components [18]. *Acanthopanax senticosus* (AS) appears to alter the levels of different neurotransmitters and hormones involved in the stress response, chiefly at the hypothalamic-pituitary-adrenal axis [19]. Eleutherosides have also been reported to bind to receptor sites progestins, oestrogens, mineralcorticoids, and glucocorticoids in vitro and therefore may theoretically exert many pharmacological actions important for the body's stress response [20].

AS possess antioxidant, anti-stress, antiulcer, anti-irradiation, anticancer, anti-inflammatory and hepatoprotective activities [18,21,22]. *Acanthopanax senticosus* Harms aqueous extracts (ASE) injection attenuated the morphological injury of liver and increased the activity of antioxidant enzymes and the ratio of GSH/GSSG in serum and liver. ASE protect against oxidative stress which may be generated via the induction of Nrf2 and related antioxidant enzymes [23]. AS increases splenocyte proliferation in weaned piglets, thereby sustaining an increase with the production of immunoglobulins [24]. AS has antitumor activity to inhibit tumor metastasis prophylactically as well as therapeutically and its antitumor effect is associated with activation of macrophages and NK cells. AS have anti-metastatic activity through the activation of macrophages and NK cells [25,26]. It was found that intravenous (i.v.) administration of AS dramatically inhibit metastasis of colon carcinoma cells in a dose-dependent manner. *In-vitro* analysis showed AS to enhance the proliferation of splenocytes. AS also stimulates peritoneal macrophage, which was followed by the production of various cytokines such as IL-1beta, TNF-alpha, IL-12 and IFN-gamma [27].

The aim of this study was to investigate the ability of liquid extract of AS to moderate immunotoxic effects of cadmium. The effects of AS extract intraperitoneal (i.p.) injections on accumulation of Cd as well as on levels of macrophages, T lymphocytes, and B lymphocytes in mouse spleen after chronic treatment with $CdCl_2$ i.p. injections were investigated.

MATERIALS AND METHODS

Preparation of the extract from *Acanthopanax senticosus* (AS) roots

The extract from roots of AS was prepared from raw material [28] imported from Poland. Briefly, 1 kg dry AS roots was ground into 3-mm particles that were then evenly moistened with 1 L 40% aqueous ethanol, and then were placed in a closed vessel for 24 h. The moistened roots were then placed in a percolator to which 1 L of 40% aqueous ethanol was then poured to yield a 1:1 extract. The materials were heated for 24 h, after which the inert fibrous part of the material was removed and pressed; the pressed liquid was then mixed with materials that had been solubilized. The final material was then filtered. Yield analyses indicated that 1 ml of extract contained 0.151 g dried material (i.e., 151 mg AS/ml= final concentration).

Animals

Animals experiments were performed using male BALB/c mice (6 weeks old, weighing 20-25 g) obtained from the Vivarium of the Institute Immunology of Vilnius University (Vilnius, Lithuania). All mice were housed in facilities maintained at 22 ± 1°C with a 55 ± 10% relative humidity and a 12-h light/dark cycle. All mice had ad libitum access to standard rodent food during the studies. Mice were weighed weekly and euthanized 6 weeks after treatments according to the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. All protocols here were approved by the Lithuanian Commission for Ethics in Laboratory Animal Use, the State Food and Veterinary Service (license number 0028).

Experiment protocol

Sample Collection Experiments were carried out on 6-week-old outbred male white laboratory mice BALB/c weighing 20 to 25 g. The intraperitoneal injections (i.p.) of $CdCl_2$ and AS extract solutions were performed as follows. Experimental mice (10 mice in each group) were periodically injected i.p. for 6 weeks (three times a week) with metal salts and AS extract solutions of

two different concentrations in deionized water: (I) 0.05 LD_{50} AS (corresponding to AS ethanol extract (1:1) 3.65 mL/kg and dry extract - 0.55 g/kg body mass) (II) 0.10 LD_{50} AS (corresponding to AS ethanol extract 7.3 mL/kg and dry extract - 1.07 g/kg body mass). (III) 0.05 LD_{50} Cd^{2+} (corresponding to Cd^{2+} 0.16 mg/kg body mass). (IV) 0.05 LD_{50} Cd^{2+} and 0.05 LD_{50} AS. (V) 0.05 LD_{50} Cd^{2+} and 0.10 LD_{50} AS. For an ethanol extract of AS the intraperitoneal LD_{50} in mice was 14.5 mL/kg [29]. The Effect of Eleutherococcus Senticosus (Rupr. et Maxim.) Maxim. Extract on Cadmium-Induced Toxicity in Mice. J Food Drug Anal 19 (4):523-527). For the solution of $CdCl_2$ the intraperitoneal LD_{50} in mice was 3.2 mg Cd/kg body mass. To determine the median lethal dose LD_{50} was calculated using the following formula: $lgLD_{50}$=lgDN – δ (Σ Li) y. where DN is the highest dose of the study substance administered to mice, δ is the logarithm of the ratio between the doses of the substance administered, and Li is the ratio between the number of dead mice and the number of mice used to determine the dose effect [30]. Acute toxicity of ibogaine and noribogaine. Medicina 44: 984-988). Control group mice were periodically injected i.p. with the same volume of saline solution. The mice were weighed weekly and decapitated after 6 weeks according to the rules defined by European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (License N. 0028).

Detection of Cd in spleen

The concentration of Cd^{2+} in tissue specimens from spleen was determined on electrothermal graphite furnace atomic absorption spectrophotometer (Perkin-Elmer Zeeman/3030, Perkin-Elmer Life and Analytical Sciences, Inc., Wellesley, MA, USA). The venous blood was obtained using the single-use syringes with anticoagulant. Tissue specimens were dissolved with 0.125 M NaOH at 90°C, and the digests were diluted till the appropriate volume with twice distilled water. The modified method, as described by Schlemmer [31], was employed for the analysis of heavy metals in biological samples (1989).

Immunohistochemical detection of splenic macrophages, T and B lymphocytes

Each second subset spleen was fixed in 10% neutral-buffered formalin for 48 h, then placed in paraffin blocks and sectioned at 3 μ monasilanized surface. Sections were then deparaffinized and rehydrated using a Varistain Gemini slide stainer (Thermo Scientific, Runcorn, UK). The sections were then washed with distilled water and heated in Tris/Ethylenediamine tetraacetic acid (EDTA) buffer, pH 9, for 8 min at 110°C in a Histoprocessor RHS-1 microwave (Milestone, Sorisole, Italy). A Shandon cover plate system (Thermo Scientific) was then employed for immunohistochemical labelling.

Specifically, after blocking the activity of endogenous peroxidases with a specific blocking solution, the slides were incubated with primary antibody (Dako Cytomation, Glos-trup, Denmark) for 1 h. The specific rabbit antimouse antibodies (all from Abcam, Cambridge, UK) used were a 1:300 dilution of anti-CD68 (clone KP1); a 1:300 dilution of anti-CD3; or a 1:200 dilution of anti-CD20 (clone EP459Y). After primary incubation and rinsing away of unbound antibody, the samples underwent sequential 30-min incubations with Advance HRP Link and Advance HRP Enzyme reagents (Dako). Binding of the primary antibodies was detected using a Liquid DAB + Substrate-Chromogen System (Dako). Lastly, each section was counterstained with Mayer ' s hematoxylin (JT Baker, Phillipsburg, NJ) and then mounted using xylene-based medium (Consul-Mount TM; Thermo Scientific). Each slide was examined by light microscopy, and histological images were taken using a DP-70 Olympus digital camera (Olympus Corp., Tokyo, Japan). Analysis of the cells in each slide was then performed using Image-Pro AMS 6.0.0 software.

STATISTICAL ANALYSIS

Results were expressed as mean ± standard error of mean. A Student's t-test with a Bonferroni correction was applied for the comparisons of Cd levels. Nonparametric Kruskal-Wallis and Mann-Whitney tests were used for evaluation of differences in the areas (%) of macrophages and T and B lymphocytes in spleens of mice in each group. The level of statistical significance was set at $p<0.05$.

RESULTS AND DISCUSSION

Cd^{2+} concentration in mouse spleen

Mice were injected using various solutions of $CdCl_2$ and AS extract for 6 weeks. Mice of the control group were injected with saline solution. One day after the final treatment, mice were euthanized, and spleen extracted.

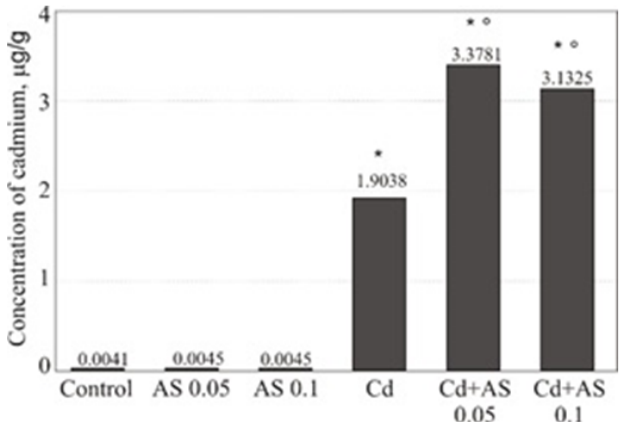

Figure 1: Cd levels in spleen of mice. Mice were injected i.p. using various solutions of AS extract and CdCl2 for 6 weeks: 0.05 LD_{50} AS; 0.10 LD_{50} AS; 0.05 LD_{50} Cd2+; 0.05 LD_{50} Cd^{2+} and 0.05 LD_{50} AS; 0.05 LD_{50} Cd^{2+} and 0.10 LD_{50} AS. Control group mice were periodically injected i.p. with the same volume of saline solution. One day after the final treatment, mice were euthanized, and spleen extracted. *p< 0.05 comparing the Cd group to the control, AS 0.05 and AS 0.1. °p<0.05 comparing Cd+AS 0.05 and Cd+AS 0.1 group to the Cd group.

Cd^{2+} concentration in spleen of mice periodically injected i.p. for 6 weeks with solution of $CdCl_2$ and two different concentrations of AS extract was evaluated. There were no significant differences in Cd^{2+} concentration in the spleen of control and AS group. Periodical injection of $CdCl_2$ for 6 weeks

caused significant increase of Cd^{2+} concentration in the spleen as compared to control and AS groups. It is of interest that periodical injection of $CdCl_2$ together with AS caused significant increase of Cd^{2+} concentration in the spleen as compared to Cd^{2+} group. Spleen of mice injected with Cd^{2+}+AS 0.05 LD_{50} and Cd^{2+}+AS 0.1 LD_{50} contained 1.8-fold and 1.6-fold, respectively, higher Cd^{2+} concentration than mice injected with $CdCl_2$ only (Figure 1).

Immunohistochemical evaluation of macrophages, T lymphocytes and B lymphocytes in spleen

Immunohistochemical evaluation of macrophages, T lymphocytes and B lymphocytes in spleen of mice periodically injected i.p. for 6 weeks with solution of $CdCl_2$ and two different concentrations of AS extract was evaluated. Obtained data showed, that the number of macrophages in cadmium group was higher comparing to the control, AS 0.05, AS 0.1, Cd +AS 0.05 and Cd+AS 0.1group. AS extract of concentration 0.05 LD_{50} and 0.1 LD_{50} did not change the number of macrophages in comparison with control group, but the number of macrophages was smaller in mice injected using solution Cd +AS 0.05 LD_{50} and Cd +AS 0.1 LD_{50} than in mice injected with $CdCl_2$ only (Figure 2).

AS extract of concentration 0.05 LD_{50} and 0.1 LD_{50} and $CdCl_2$ only did not change the number of T-lymphocytes in comparison with control group, but the number of T-lymphocytes was smaller in mice injected with Cd +AS 0.05 LD_{50} and Cd +AS 0.1 LD_{50} than in mice injected with $CdCl_2$ only. (Figure 3).

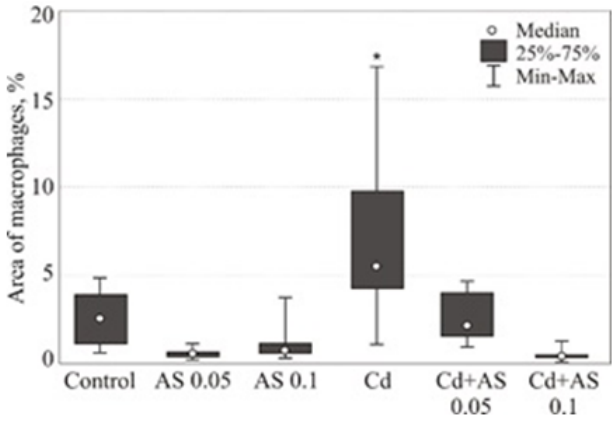

Figure 2: The total area of macrophages in spleen (as %) of mice. Mice were injected i.p. using various solutions of AS extract and $CdCl_2$ for 6 weeks: 0.05 LD_{50} AS; 0.10 LD_{50} AS; 0.05 LD_{50} Cd2+; 0.05 LD_{50} Cd2+ and 0.05 LD_{50} AS; 0.05 LD_{50} Cd2+ and 0.10 LD_{50} AS. Control group mice were periodically injected i.p. with the same volume of saline solution. One day after the final treatment, mice were euthanized, and spleen extracted. *p< 0.05 comparing the Cd group to the control, AS 0.05, AS 0.1, Cd+AS 0.05 and Cd+AS 0.1group.

The data of AS influence on B lymphocytes activity show, that AS extract of concentration 0.05 LD_{50} and 0.1 LD_{50} and $CdCl_2$ statistically significant increase the number of B-lymphocytes in comparison to control group, but the number of B-lymphocytes was smaller in mice injected with Cd +AS 0.05 LD_{50} and statistically significant higher Cd +AS 0.1 LD_{50} than in mice injected with $CdCl_2$ only (Figure 4).

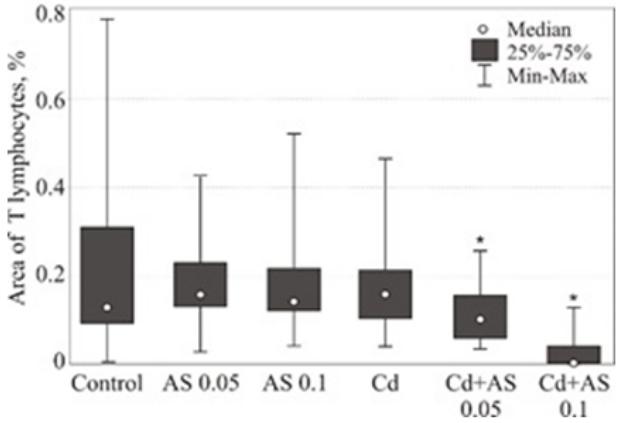

Figure 3: Total area of T-lymphocytes in spleen (as %) of mice. Mice were injected i.p. using various solutions of AS extract and CdCl2 for 6 weeks: 0.05 LD_{50} AS; 0.10 LD_{50} AS; 0.05 LD_{50} Cd^{2+}; 0.05 LD_{50} Cd^{2+} and 0.05 LD_{50} AS; 0.05 LD_{50} Cd^{2+} and 0.10 LD_{50} AS. Control group mice were periodically injected i.p. with the same volume of saline solution. One day after the final treatment, mice were euthanized, and spleen extracted. * p<0.05 compared Cd+AS 0.05 and Cd+AS 0.1 group to the Cd group.

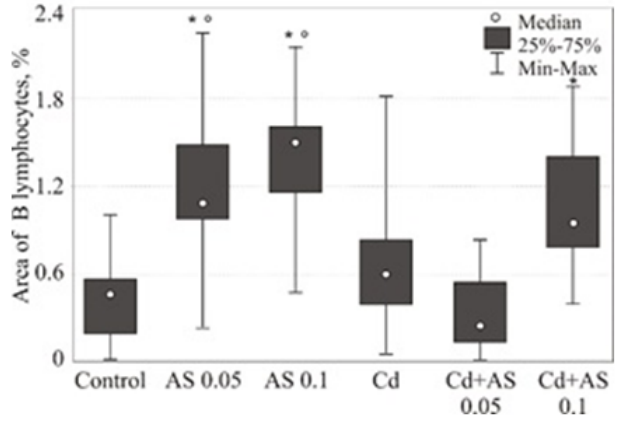

Figure 4: Total area of B-lymphocytes in spleen (as %) of mice. Mice were injected i.p. using various solutions of AS extract and $CdCl_2$ for 6 weeks: 0.05 LD_{50} AS; 0.10 LD_{50} AS; 0.05 LD_{50} Cd^{2+}; 0.05 LD_{50} Cd^{2+} and 0.05 LD_{50} AS; 0.05 LD_{50} Cd^{2+} and 0.10 LD_{50} AS. Control group mice were periodically injected i.p. with the same volume of saline solution. One day after the final treatment, mice were euthanized, and spleen extracted. * p<0.05 compared AS 0.05, AS 0.1 and Cd+AS 0.1 group to the control, Cd and Cd+AS 0,1groups. ◦ p<0.05 compared AS 0.05 and AS 0.1 group to the Cd+AS 0.05 and Cd +AS 0.1 group.

Cd exposure generates oxidative stress and decreases the inflammatory responses in a murine macrophage cell line [32]. Exposure to very low concentrations of Cd reduces cell viability and results in cell death by apoptosis and necrosis [33]. ROS are generated following acute Cd overload and play important roles in tissue damage [34]. ROS is a critical mediator of Cd-induced apoptosis and that cadmium may compromise splenic immune function by accelerating apoptosis [35]. Cd exposure induce cell cycle disturbance and apoptosis in splenocytes [7] and liver [36]. Cadmium suppresses lymphocyte proliferation [10], causes thymic atrophy and splenomegaly [12].

Extracts of *Acanthopanax senticosus* improve immune responses [24], inducing cytokine actions of interleukin-1 beta and interleukin 6 [37]. *Acanthopanax senticosus* polysaccharides (ASPS) directly increase the proliferation and differentiation of B cells, and the cytokine production of macrophage, but not the proliferation and cytokine production of T cells [26].

Our results showed that periodical injection of $CdCl_2$ for 6 weeks caused the increase of concentration of Cd in the spleen as compared to control. However, injection of Cd and AS extract caused higher increase of Cd concentration in the spleen as compared to Cd group. Cd-bound to metallothionein (MT) is responsible for Cd accumulation in tissues and the long biological half-life of Cd in the body [36]. Repeated injections of Cd produce hematotoxic and immunotoxic effects, and intracellular MT protects against Cd-induced effects [15]. AS evaluate level of glutathione (GSH) [20]. Cadmium could make bonds to the thiol groups (SH) on the GSH molecules. This Cd-SH interaction also may explain how metallothionein, like GSH, plays a protective role against Cd-induced toxicity [38].

According to our results injections of AS extract or AS extract and $CdCl_2$ did not change the number of the macrophages in comparison to control group, whereas the number of macrophages significantly increased in mice injected with $CdCl_2$ only. Cadmium accumulation in the spleen, liver and kidney is associated with degeneration and inflammatory changes. Cadmium causes significant suppression of humoral and cell mediated immune response in mice which could be due to its cytotoxic action on liver, kidney and immune cells [2]. On the other hands cadmium, has been shown to enhance humoral immune responses at low levels of exposure. By contrast, cell-mediated immunity was more consistently shown to be depressed [16]. It is possible that i.p. injections generated inflammation processes in spleen. As a result of these processes there was increase of the number of macrophages. However, AS extract could diminish the inflammatory reactions in the mice spleen because AS possesses anti-inflammatory activities [18,21,22], inhibits reactive oxygen species production by mouse peritoneal macrophages in vitro and in vivo and may be partly responsible for the antiinflammatory function [39].

Injections of AS extract or $CdCl_2$ did not change the number of T lymphocytes in comparison to control group. On the other hands AS extract and $CdCl_2$ statistically significant increased the number of B lymphocytes in comparison to control group. Our results correlate with studies of Han et al. who confirmed that extracts of *Acanthopanax senticosus* improved immune responses enhancing activities of B cells and phagocytosis of macrophages rather than T cells (2003). Furthermore, it is shown that acute cadmium administration to rats exerts immunosuppressive effect on T lymphocytes in spleen. [40]. Dietary ASPS have potent immunomodulatory activity by improving lymphocyte proliferation [41,42]. ASPS activate B cells and macrophages by interacting with toll-like receptors and leading to the subsequent activation of mitogen-activated protein kinases and NF-kappa B [26].

In conclusion, studies showed that long-term injections of extract of AS (0.1 LD_{50} and 0.05 LD_{50}) combined with $CdCl_2$ (0.05 LD_{50}) leads to the significant increase of cadmium concentration in spleen of experimental mice. Data demonstrated that AS decreases the activity of macrophages and T lymphocytes induced by cadmium. On the other hands AS increase the activity of B lymphocytes.

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The authors declare that they have no conflict of interest.

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