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Full Length Research Paper

In vitro immunomodulation effects of the aqueous and protein extracts of *Berberis hispanica* Boiss and Reut. (Family Berberidaceae)

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Immunomodulatory activities of aqueous and protein extracts of *Berberis hispanica* were evaluated *in vitro*. Both extracts at 1 mg/ml showed no cytotoxicity effect on thymocytes, splenocytes and macrophages cells. These extracts strongly inhibited *in vitro* the mitogen proliferative response of thymocyte and spleen cells. Humoral immunity measured by plaque-forming cell assay and evolution of complement activity showed an increase of plaque-forming cell formed and complement activity after addition of protein extract, where aqueous extract induced a significant diminution only in plaque-forming cell. In addition, the same extracts elicited a significant decrease in phagocytic capacity without affecting macrophage cells viability. In the allogenic mixed lymphocyte reaction test, plant extracts mainly increased lymphocyte cytotoxic activity towards chicken red bloods cells. The present data support that *Berberis hispanica* extracts showed an immunosuppressive action on proliferation and biological effects of T-cell and B-cell. This suggests a possible use of aqueous extract in auto-immune diseases therapy. However, the protein extract, highly stimulates allogenic mixed lymphocyte which suggest an interesting use in cancer therapy.

Key words: *Berberis hispanica*, immunomodulation, plaque-forming cell, allogenic mixed lymphocyte phagocytosis.

INTRODUCTION

The immune system is involved in the etiology, as well as pathophysiologic mechanisms of many diseases. Modulation of the immune responses to alleviate various diseases has been of interest for many years (Sharma and Charaka, 1983). Medicinal plants are a rich source of substances which are claimed to induce immune-modulation of immunity cells (Sainis et al., 1997). The traditional medicinal system in Morocco, describes certain plants which strengthen the immune system (Bellakhdar, 1997, 2008; Merzouki et al., 1997). *Berberis hispanica* (family Berberedaceae) is a deciduous thorny shrub. An infusion of the stem bark of *B. hispanica* has been used in traditional medicine to treat the gastrointestinal affections, inflammation, liver and biliary disorders (Bellakhdar, 1997). Herbalists indicate also the use of the plant in cancer medication. In the same Berberis species, the active constituents of *B. hispanica* are alkaloids and the major compound is Berberine (Bellakhdar, 1997; Chevallier, 2001; Ali and Khan, 1978).

Berberine and several Berberis species show a wide range of biochemical and pharmacological activities such as in amebiasis, cholera and diarrhea (Sack and Froehlich, 1982; Said, 1996), possess analgesic (EL Hamsas EL Youbi et al., 2011) and antipyretic effects (Yesilada and Küpeli, 2002) and were reported to exhibit antiarrhythmic and anti-tumor effects (Yamamoto et al., 1993; Fukuda et al., 1999). Immunomodulatory activities are also suggested recently for some compounds of Berberis species. These activities include anti-inflammatory action (Iizuka et al., 2000; Ivanovska and Philipov, 1996), inhibition of cytokine secretion, alteration of T-cell differentiation (Ivanov et al., 2006). In this work we aimed to investigate the effects of aqueous and protein extracts of the stem bark of *B. hispanica* "of Moroccan origin" on *in vitro* immunological responses modulation by studying the proliferation of lymphocytes,

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macrophages and their biological functions.

MATERIALS AND METHODS

Chemical materials and preparation

All chemicals products used in this study were purchased from Sigma (St Louis, MO, USA).

Experimental animals

The bred Zemmouri males' rabbits weighing 2.5 kg were housed under a 12 h light/dark cycle in a temperature-controlled room (22 to 24°C) and used for *in vitro* investigations. Rabbits had free access to standard chow and water. The animal experiments were used according to national ethical laws.

Plants material and extraction

The plant material constituted of the stem bark of *Berberis hispanica*, bought from Immouzer-Mermoucha in Morocco. The specimen of plant was identified and a voucher specimen was deposited at the herbarium of the National Institution of Medicinal and Aromatic Plants under the number exciccata n° INP76. After identification, two kinds of *B. hispanica* extracts were prepared. Aqueous extract was obtained with maceration method. So, 10 g of powder was dissolved in 100 ml of phosphate buffer saline (PBS, 150 mM, pH 7.4) and stirring during 2 h. The suspension was centrifuged (15 min at 2700 g 4°C) and the supernatant sterilized by filtration through 0.22 µm nitrocellulose filters and protein extract was obtained by 40% (w/v) ammonium sulfate precipitation (England and Seifter, 1990).

Sephadex-chromatography column

Protein extract was separated on sephadex (G-100) chromatography column (40 × 2 cm, W, Facher bround). Elution was performed by PBS at rate of 120 ml per hour. Fractions of 2 ml were collected and their protein concentration was determinate by measuring absorbance at 280 nm.

Cells materials and culture

Cells suspension used in this study were obtained from rabbit sacrificed. Briefly, spleens and thymus were removed aseptically from animals and then suspensions prepared by pressing the organs through a fine wire mesh. These cell suspensions were washed by centrifugation (2 to 16 P, Sigma) repeated in RPMI and the red blood cells were lysed by 154 mM ammonium chloride. The number of viable cells was determined microscopically by trypan blue exclusion test. The culture used RPMI medium (without glucose) supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 10% (v/v) FCS (fetal calf serum) and antibiotics (ampicillin 100 U/ml and streptomycin 100 mg/ml).

Phagocytosis cells

The phagocyte test was realized as notified before by EL Hamsas EL Youbi et al. (2010b). Briefly, macrophages were obtained from spleen cells preparation (see before). 100 µl of spleen cells suspension at 10^6 cells/ml was added in 96 well plates that are incubated at 37°C during 3 h for adherence of macrophages. Thereafter, supernatant was removed and every well-plate was washed twice with sterile PBS. The phagocyte test was conducted using neutral red as indicator of phagocyte action of macrophages. In every well-plates, 100 µl of RPMI (with 10% (v/v) FCS) containing 0.075% of neutral red and 10 µl of plant extracts (or PBS in the blank) were added and then plates incubated for 2 h. Finally, after removing supernatant and washing them three times, their reaction were stopped with a solution containing acetic acid (1M) /ethanol (1:1 v/v). Phagocytosis was evaluated by measuring absorbance at 540 nm. Proliferation of adherent macrophages was measured as described using colorimetric MTT test.

Cell proliferation assay

Cells proliferation (splenocytes, thymocytes and macrophages) were measured by the MTT assay (Mosman, 1983) as described by Daoudi et al. (2008) and EL Hamsas EL Youbi et al. (2010a). Briefly, cells suspensions were plated at 5000 cells/well in 96 well plates and incubated, in BINDER incubator, at 37°C in humidified chamber under an atmosphere of 95% air and 5% CO_2 for 72 h. Plant extracts was added to cells before their incubation at the concentration of 1 mg/ml for aqueous and protein extracts. Thereafter, 10 µl of MTT solution (5 mg/ml in PBS) was added. After 3 h of incubation, the supernatant was removed and replaced by 100 ml of dimethyl sulfoxide (DMSO). Finally, the optical density was measured through wavelength at 570 nm using the spectrophotometer (C-CIL 1010, Sigma).

Antibody production (plaque-forming cell assay)

The plaque forming cell (PFC) assay was performed using the method of Cunningham (1965) with some modification (Daoudi et al., 2008). Rabbits were previously immunized with mouse red blood (MRB). The spleen was removed, cleaned free of extraneous tissues and a single cell suspension of 10^6 cells/ml was prepared from it in RPMI medium. For PFC assay, the MRB was prepared at a cell density of 5×10^8 cells/ml in NaCl 0.9%. One milliliter of MRB in medium was added to 1 ml of spleen cells suspension with or without plant extracts and then introduced in Cuningham chambers. The chambers were sealed with paraffin and incubated at 37°C for 2 h. The PFC were counted under a light microscope and expressed as PFC per 10^6 spleen cells (Raisuddin et al., 1991).

Allogenic mixed lymphocyte reaction (MLR)

Thymus from rabbit was aseptically removed and placed in complete RPMI medium. Thymocytes were then obtained as described for splenocytes. Chicken red blood cells (CRBC) were obtained from chicken and were washed by NaCl 0.9 % before being used. For MLR assay, the CRBC were prepared at a cell density of 5.10^5 cells/ml in PBS. 50 µl of CRBC in medium was added to 1 ml of thymocytes (1×10^6 cells/ml) flowed by addition of 100 µl/well in 96-well-plates culture plates as a final volume. Cell mixture was then incubated in presence or absence of plant extracts, for 24 h at 37°C in humidified atmosphere. Cytotoxicity of thymocytes against CRBC was evaluated by measuring the liberation of CRBC hemoglobin in the medium by determining the absorbance at 640 nm.

Complement test

The effect of *B. hispanica* extracts on complement production is based on inhibition or activation of hemolysis of mouse red blood

cells (MRBC) by the complement pathway in the presence of anti-MRBC antibodies prepared in immunized rabbits. Firstly, MRBC were washed twice with NaCl 0.9% and a 1% cell suspension was prepared. MRBC (suspension at 0.5%) was diluted in NaCl 0.9% with addition of complement (guinea pig complement serum, 10%, ALFABiotech, USA) and serum containing anti-MRBC antibodies. MRBC were then incubated on a 96 well-plates in the presence or absence of plant extracts at 37°C during 90 min. After incubation, 100 µl of the supernatants were transferred to a new plates and the absorbance at 540 nm measured using a micro-plate reader.

Statistical analysis

Each experimental condition was realized at least in triplicate (n = 3). Data were expressed as the mean ± standard deviation (S.D.). Statistical analyses were carried out using the student's t test. Differences were considered statistically significant at $p<0.05$.

RESULTS

Effect of aqueous and protein extracts on splenocytes and thymocytes proliferation

Concerning proliferation assay, in the absence of mitogen agent, the total aqueous extract (AE) at 1 mg/ml stimulates the proliferation of splenocyte without modifying thymocyte proliferation where the protein extract (PE, 1 mg/ml) enhanced the proliferation of both cells (Figures 1a and 1b). For the stimulation of cell proliferation, we have used two pathways, the mitogen Concanavalin-A (Con-A, 4 µg/ml) on thymocytes and the protein kinase-C activator PMA (6 µg/ml) for splenocytes. In the presence of these mitogens, there is an inhibition of proliferation for both cells: splenocytes + PMA = (AE: 21%; PE: 52%), N = 5; thymocytes + Con-A = (AE: 58%; PE: 49%), N = 5 (Figures 1a and 1b). In these conditions, we observe that the AE and PE with mitogens agents (PMA or Con-A) induce an important suppression of both cells proliferation.

Chromatography of protein extract

Three peaks were obtained when whole soluble protein extract is passed through a Sephadex G100 chromatography column. The molecular weight of the three peaks is 75 kDa for F1 and 20 kDa for F2. The F3 is formed by fractions with PM less than 5 kDa. The elution pattern of protein peaks and recovery are reproducible in different sets of experiments. A complete elution profile is shown in Figure 2.

Effect of protein fractions on splenocyte and thymocyte proliferation

Results obtained (Figures 3a and 3b) indicate that the protein fractions, exhibited a different profile on cell proliferation. On thymocyte cells, in absence of Con-A, the protein fractions; F1 and F3 at 0.1 mg/ml show no effect but F2 at 0.1 mg/ml stimulate thymocyte proliferation. On splenocytes, all fractions stimulate cell proliferation with a high effect to F2 (Figure 3b). On thymocytes and in the presence of Con-A (Figure 3a), the F1 fraction induce the stimulation effect, Con-A + F1 = 24%. While, the F2 and F3 fractions blocked the mitogenic effect of Con-A compared to their respective control: Con-A + F2 = 2%; Con-A + F3 = 4% of response. In contrast, on splenocytes, these fractions do not block phorbol-12-myristate-13-acetate (PMA) action. We still observe a stimulation of the proliferation by this mitogen (Figure 3b). The highly stimulating effects were observed under F1 and F3 compared to that observed with F2: PMA + F1 = 557%, PMA + F2 = 650%, PMA + F3 = 590% of response.

Effect on phagocytosis

The results of the effect of *B. hispanica* on macrophage functions are presented in Figures 4A, AE and PE. Extracts show a decrease in phagocytic activity without cytotoxicity on macrophages proliferations (Figure 4b). In contrast, the protein fractions F1, F2 and F3 elicit a significant decrease in the phagocytic activity which was related to an inhibition of macrophage proliferation (Figures 4a and 4b).

Effect of plant extracts on humoral immunity

The effect of *B. hispanica* on humoral immunity is evaluated by PFC assay and complement assay. The results were shown in Figures 5 and 6. Berberis aqueous extract at 1 mg/ml result in a significant decrease in PFC formed (AE = 76% of control) where the PE at 1 mg/ml enhance the number of PFC (PE = 219% of control). The results of complement assay were shown in Figure 6; both extracts are capable of stimulation of complement activity. PE induces an increase 136.9% more than that of AE with 124.9% compared to control.

Effect of plant extract on allogenic mixed lymphocyte reaction

The immune-potency of aqueous and protein extracts of *B. hispanica* on lymphocyte T cytoxicity were assessed using MLR test. So the results prove that the AE at 1 mg/ml has no significant effect where PE at 1 mg/ml shows an increase in MLR activity with a higher effect of PE (62%) compared to AE (4%).

DISCUSSION

The results indicate that the aqueous and protein extracts

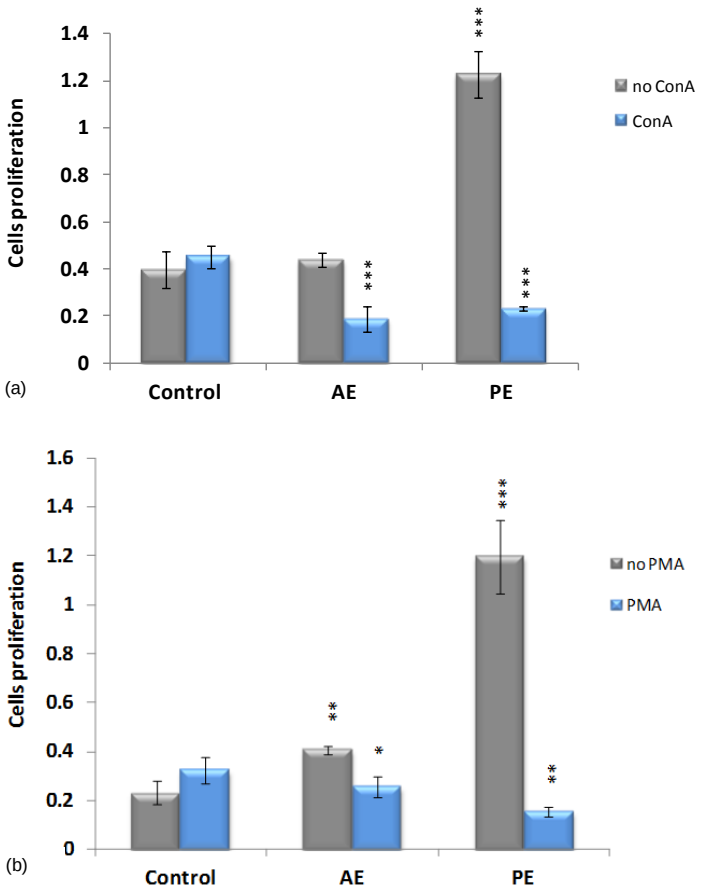

Figure 1. Effects of *B. hispanica* aqueous and protein extracts on thymocytes proliferation (a), on splenocytes proliferation (b). AE: aqueous extract at 1 mg/mL, PE: protein extract at 1 mg/ml. Control means cells without extracts plant. Values represent the mean ± S.D of five animals. *p<0.05, **p<0.01, ***p< 0.001 when compared with the control.

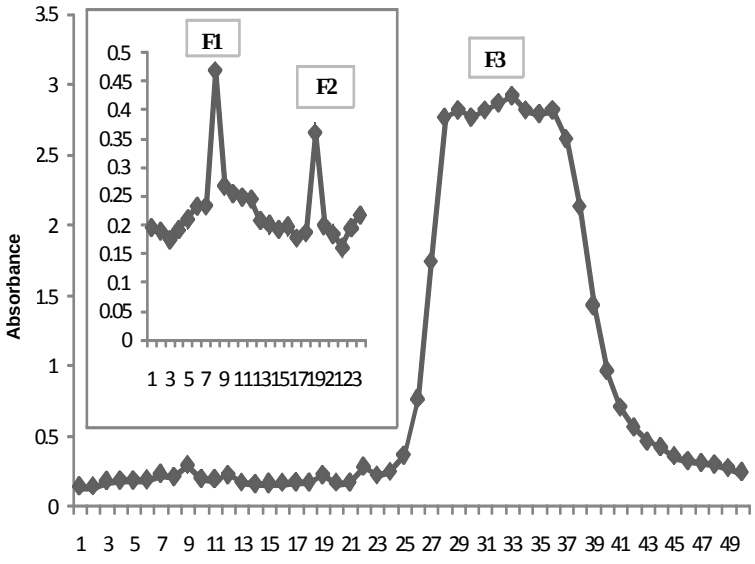

Figure 2. Chromatography profile of *B. hispanica* protein separation on G100 sephadex column.

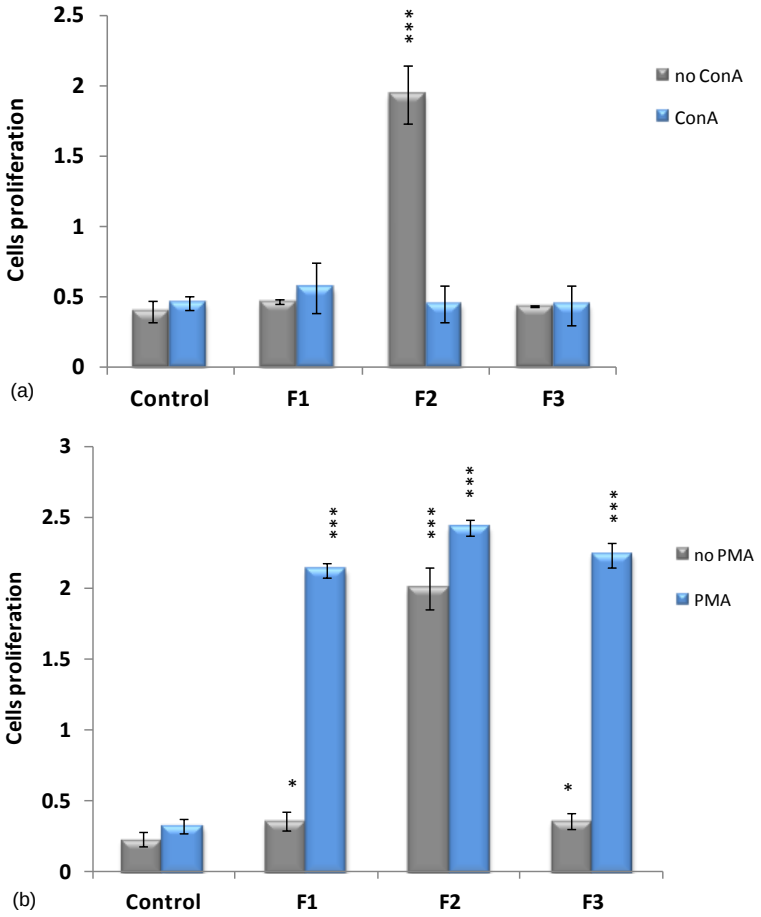

Figure 3. Effects of B. hispanica protein fractions on thymocyte proliferation (a), on splenocyte proliferation. (b) Protein Fractions at 0.1 mg/ml. Control means cells without plant extracts. Values represent the mean ± S.D of five animals. *p<0.05, ***p< 0.001 when compared with the control.

of *B. hispanica* at 1 mg/ml have no cytotoxicity effect on the three cells studied, splenocytes, thymocytes and macrophages. In addition, the protein extract induce a high stimulation of thymocyte and splenocyte proliferation. In those conditions, these extracts inhibit the mitogen-proliferation of splenocytes and thymocytes and reduced the phagocytosis of macrophages. This indicates that *B. hispanica* exert a general immunosuppression by blocking the humoral and cellular immunity reactions without affecting cell viability. It was shown before that Berberis species contain major compounds such as berberine and berbamine (Döpke, 1963; Di et al., 2003). These products induce an immunosuppression of T-lymphocyte activities. Ren et al. (2008) observes that berbamine inhibit T-lymphocyte proliferation and IFN-γ secretion in treated mice with autoimmune encephalomyelitis. Differentiation of T-cell was also altered by berberine in NOD mice (Cui et al., 2009). This reduction of lymphocyte activities is obtained without affecting cell viability. High quantity of alkaloid was reported in our aqueous extract (data not shown), which explain the immunomodulatory of AE as cited in literature (Li et al., 1989; Ivanovska et al., 1999).

Nevertheless, the results show that the combined addition of protein extract and mitogens induce a response smaller than that recorded by protein extract or mitogens alone, which can explain the high antagonism between the compounds of protein extract and mitogen action. This antagonism has been observed with Con-A stimulation or PMA. And since PMA acts on protein kinase-C (PKC), we can postulate that the protein extract of *B. hispanica* acts at intracellular level at a PKC after-step. From the protein extract, we have identified three protein fractions; F1 (75 kDa), F2 (20 kDa), and F3 (5 kDa) as the major compounds of PE. Interestingly, the effect of these fractions does not show the same effects of PE on thymocytes, splenocytes and macrophages proliferations. These results are likely to be explained by some agonist and/or antagonist between fractions protein may be supposed, other compounds fractions proteins

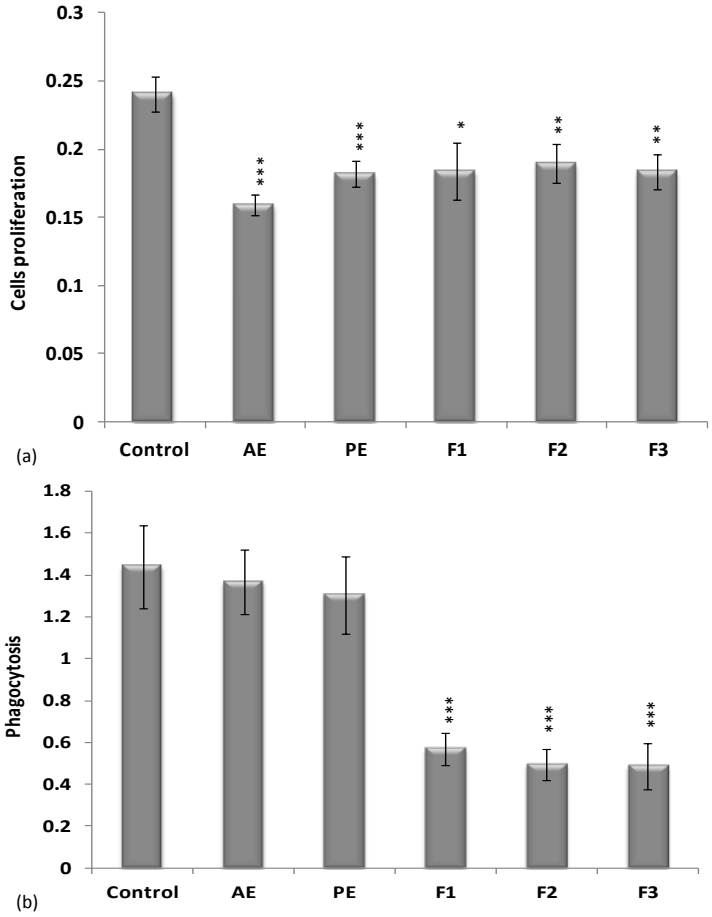

Figure 4. (a) Effects of *B. hispanica* on macrophage phagocytosis, assessed by Neutral red assay, and on macrophage proliferation. (b) assessed by MTT assay. AE: aqueous extract at 1 mg/ml, PE: protein extract at 1 mg/ml and F: protein fractions used at 0.1 mg/ml. Control means cells without plant extracts. Values represent the mean ± S.D of five animals. *p<0.05, **p<0.01, ***p< 0.001 when compared with the control.

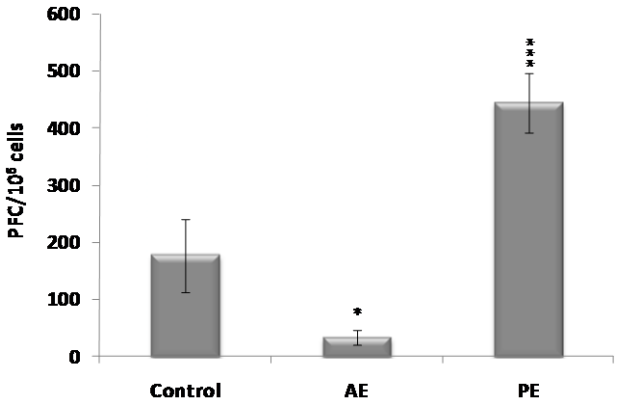

Figure 5. Effects of B. hispanica extracts on the humoral immunity as assessed by the plaque-forming cell assay. AE: aqueous extract at 1 mg/ml, PE: protein extract at 1 mg/ml. Control: PFC observed without plant extracts. Values represent the mean ± S.D of five animals. *p< 0.05, ***p< 0.001 when compared with the control animals.

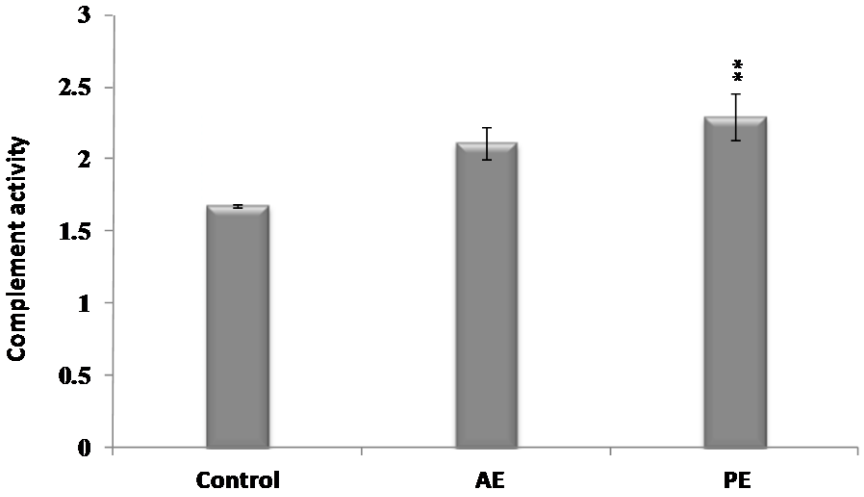

Figure 6. Effects of B. hispanica extracts on complement activity. AE: aqueous extract at 1 mg/ml, PE: protein extract at 1 mg/ml. Control: complement activity without plant extracts. Values represent the mean ± S.D of five animals. **p<0.01 when compared with the control.

probably are responsible of these effects reported by PE. On cellular immunity, we observe that the AE and PE extracts do not modify the macrophage viability but they reduce the phagocytosis. On other part, these extracts enhance the lymphocyte cytotoxicity. This finding suggests that *B. hispanica* extracts are a potent stimulator of lymphocyte cytotoxicity against allogenic cells. A part of explanation is the stimulatory effect of PE on the thymocyte proliferation observed before; on the contrary, a Berbamine from *Berberis* species exert a suppressive effect on MLR and significantly prolong allograft survival compared with untreated transplanted mice demonstrated *in vivo* (Luo et al., 1998). This indicates that stimulation of MLR observed in our conditions is not related to berbamine.

The PFC assay is considered to be one of the most highly predictive single assays to assess potential modulation of the humoral immune response, which quantifies the number of B cells producing SRBC-specific IgM (Wilson et al., 1999). The inhibition observed by aqueous extract on PFC without modifying significantly complement activity suggests a possible inhibitory effect of AE compounds on antibodies production. Comparable data were revealed by *in vitro* and *in vivo* experiments in mice (Ivanovska and Philipov, 1996). In same conditions, PE enhanced complement activity and high PFC; this indicates firstly, that proteins of *B. hispanica* stimulated probably the production of antibodies by B-lymphocytes. Secondly, other AE constituents of *B. hispanica* antagonist effects of the PE on PFC and complement activities. In contrast, a study conducted by Ivanovska and Philipov (1996) *in vitro* show that Berberine and their fraction alkaloid Bv1 inhibit complement activation in normal human serum, while fraction alkaloid Bv2, enhance the haemolysis after 30 min pre-incubation time. However, we can conclude that the Berberine is partly responsible for the activity of the AE and the PE has significant pharmacological effects in comparison with other constituents of the plant.

Conclusion

The aqueous extract shows a reduction of all immunity cell proliferation and their activities. This suggests that aqueous compounds are highly immunosuppressive. In contrast, PE induces an inhibition of mitogenic-induced-proliferation of lymphocytes and splenocytes without effect on macrophage proliferation. Under this extract we observe an increase of antibody production and of MLR. These funding indicate a possible use of PE extract to enhance humoral immunity and lymphocyte toxicity in different diseases which can confirm the traditional use of *B. hispanica*.

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