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Full Length Research Paper

In vitro immunoinhibitory effects of Setarud on human B lymphocyte

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Setarud is a new herbal medicinal product which is composed of alcoholic extracts of three local plants supplemented with selenium. Preliminary results obtained from *in vitro* and *in vivo* investigations have demonstrated immune modulating activity for Setarud. In this study, the *in vitro* effects of Setarud were investigated on different biological functions of normal human B lymphocytes. Human B lymphocytes were isolated from peripheral blood and stimulated with Toll-like receptor (TLR) 7/8 (R848) and TLR9 (CpG) agonists in presence or absence of different dilutions of Setarud. The effects of Setarud on proliferation, immunoglobulin (Ig) production, IL-10 secretion and expression of the costimulatory molecule CD86 were subsequently studied by 3H-thymidine uptake, ELISA and flow cytometry, respectively. The extract induced a dose-dependent inhibitory effect on all biological functions of TLR stimulated B lymphocytes, particularly at 1/5000 and 1/10000 dilutions. A significant inhibition in proliferation of B lymphocytes stimulated by R848 ($p<0.05$) and CpG ($p<0.01$) was observed. IL-10 and Ig production were also inhibited in CpG ($p<0.05$ and $p<0.01$, respectively) and R848 ($p<0.01$ and $p<0.05$, respectively) stimulated B lymphocytes at 1/5000 dilution of Setarud. The level of CD86 expression in B lymphocytes treated with 1/5000 dilution of Setarud was significantly lower than untreated B lymphocytes ($p<0.05$). We observed a significant correlation between all biological functions which were found to be downregulated by Setarud in TLR stimulated B lymphocytes. The dose-dependent inhibitory effect of Setarud on TLR stimulated B lymphocytes implies its potential therapeutic implication in B lymphocyte mediated autoimmune diseases and B-cell malignancies.

Key words: Setarud, herbal extract, immunomodulation, B lymphocyte, immunoglobulin, IL-10, CD86.

INTRODUCTION

Natural medicinal products have been traditionally used for treatment of various complications in eastern countries, particularly China, India and Iran since the immemorial time (Ram et al., 2009). Nowadays, a large collection of herbal products and extracts is widely being employed in these countries for therapeutic interventions in both traditional and modern clinical centers (Davicino et al., 2007). Traditional medicine has made a significant contribution to the Western pharmacopeia, but the scientific basis for biological activity of most herbal medicinal products is obscure (Wang et al., 2008). Extracts of several plants have shown impressive spectrum of biological activities as well as immunomodulatory effects (Martino et al., 2010).

Herbal extracts have long been considered as suitable candidates for immune interventions, due to their safety, low cost, availability and their potential functional activity

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as immunomodulators. The majority of the anti-cancer candidates which are routinely subjected to preliminary anti-cancer screening are derived from botanical sources (Cragg and Newman, 2005).

In some countries, traditional medicine is used to meet the primary health care need and to treat patients with a variety of infectious diseases, such as AIDS.

Several natural products have been found to inhibit unique enzymes and proteins crucial to the life cycle of viruses, however, the mechanism of anti-viral activities of many natural products is unknown (Wang et al., 2006).

A mixture of local herbal extracts (*Tanacetum vulgare, Rosa canina,* and *Urtica dioica*) enriched with selenium (Se), collectively known as Setarud, has recently demonstrated positive effects on the immune system (Kheyrandish et al., 2009).

T. vulgare L. (Asteraceae) has traditionally been used to treat migraine, neuralgia and rheumatism. The ethyl acetate extract and the isolated fraction, parthenolide, from this plant protected Vero cells from herpes simplex virus (HSV-1) infection *in vitro* (Onozato et al., 2009). The extract of *R. canina* has also been reported to have antiviral properties (Mouhajir et al., 2001). Oral immunomodulator Dzherelo (Immunoxel) which has been reported to be effective for the management of HIV infection contains concentrated aqueous-alcohol extract from different medicinal plants, including *R. canina* (Nikolaeva et al., 2008). The third herbal component of Setarud, *U. dioica L.*, is a common plant with antioxidative and other immunomodulatory effects which produces allergenic substances causing oedema and inflammation in humans (Yener et al., 2009; Akbay et al., 2003). Selenium is an essential trace element required for the proper functioning of the immune system, and appears to be involved in counteracting the HIV virulence and AIDS progression (Arthur et al., 2003; Kupka et al., 2004; Rayman, 2000).

Due to the nature of its ingredients, Setarud exerted positive effects on the immune system, serum lipid profile, and showed hepatoprotective activity in animals (Farzamfar, 2008). Preliminary studies have indicated safety and efficacy of Setarud in laboratory animals and potential immunomodulatory effects in HIV-infected patients (Kheyrandish et al., 2009). Its acute and chronic toxicities have been assessed in animals and in a Phase I clinical trial in HIV-infected patients in order to determine maximum tolerated dose (MTD) (Kheyrandish et al., 2009). Potential mutagenicity and genotoxic properties of Setarud have also been evaluated. It does not display any mutagenic properties (Farzamfar, 2008). Protective effects of Setarud on development of diet-induced hypercholesterolemia and induced hepatotoxicity have been determined (Azonov et al., 2008; Khorshid et al., 2008). Herbal ingredients of Setarud may exert anti-inflammatory, anti-viral and immune stimulating properties (Kheyrandish et al., 2009). This study was performed to determine the *in vitro* immunomodulatory effects of Setarud on human B lymphocytes. Our findings have demonstrated a dose-dependent inhibitory effect of Setarud on different aspects of B lymphocyte function.

MATERIALS AND METHODS

Isolation of peripheral blood mononuclear cells and B cells

A consent letter was taken from ten healthy volunteers participating in this study. 50 to 70 ml of peripheral blood was collected into heparin-containing tubes and mixed at a ratio of 1:1 with RPMI-1640 culture medium (Gibco-BRL, NY, USA).

Peripheral blood mononuclear cells (PBMC) were isolated using Histopaque (Sigma, ST Louis, USA) density-gradient centrifugation. Isolation of B cells was performed by negative selection using a B cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) with magnetic cell sorting (MACS) system according to the manufacture's instruction (Gharagozlou et al., 2005). Cell viability was uniformly greater than 90% as determined by trypan blue dye exclusion. B cell purity was assessed by flow cytometry using FITC-labelled anti-CD19 monoclonal antibody (DAKO, Glostrup, Denmark).

Effect of Setarud on B cell Proliferation

Enriched B cells (10^5 cells/well) were cultured in 96-well flat-bottom plates (Greiner, Frickenhausen, Germany) in a total volume of 200 µl of complete culture medium [RPMI-1648 supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL), penicillin (100 IU) and streptomycin (100 µg/ml) (Gibco-BRL] per well. Cultured cells were stimulated with 1.25 µg/ml R848, a highly water-soluble derivation of the imidazoquinoline compound (CL097, *Invivo*Gene, San Diego, USA), and 2.5 µg/ml CpG-2006, a single stranded phosphorothioate stabilized oligodeoxynucleotide (TCGTCGTTTTGTCGTTTTGTCGTT) (Manufactured by Eurofins MWG Operon, Ebersberg, Germany) in the presence or absence of Setarud (IMOD™ , Pars Roos Co., Tehran, Iran) at different dilutions for 72 h.

Preparation of Setarud and its ingredients have been described in detail elsewhere (Kheyrandish et al., 2009). Briefly, leaves and small stems of *U. dioica* and *T. vulgare* and fruits of *R. canina* were dried in a dark place for 3 to 4 days at 42°C and broken into small pieces. Then, they were separately packed into glass vessels and extracted with 96% ethanol at 42°C for 30 days. After that, *R. canina* and *U. dioica*/*T. vulgare* extracts were enriched with Se. After 24 h incubation at 42°C, the mixture was sequentially passed through a millipore 5.0, 0.45 and 0.22 µm filters, and dispensed into sterile vials for research use. The concentration of selenium was 5 mg/125 mg of extract. Cells were labeled with 1 µCi of [^{3}H] thymidine (Amersham, Buckinghamshire, United Kingdom) for the last 18 h of culture and then harvested. [^{3}H] Thymidine incorporation was measured by β scintillation counter as previously described (Kazemi et al., 2008). All cell treatments were performed in triplicate.

Flow cytometric analysis of B cell activation

After 40 h incubation in the presence or absence of Setarud at different dilutions, cells were washed with 1 ml of PBS containing 1% FCS and 0.5 mM EDTA and subsequently incubated for 45 min at 4°C with a PE-conjugated anti CD86 antibody (BD, New Jersey, USA). Cells were then fixed with 1% paraformaldehyde in cold PBS and finally examined by Partec PAS II flow cytometer (Partec, Munster, Germany). B cells were gated by the forward and side-

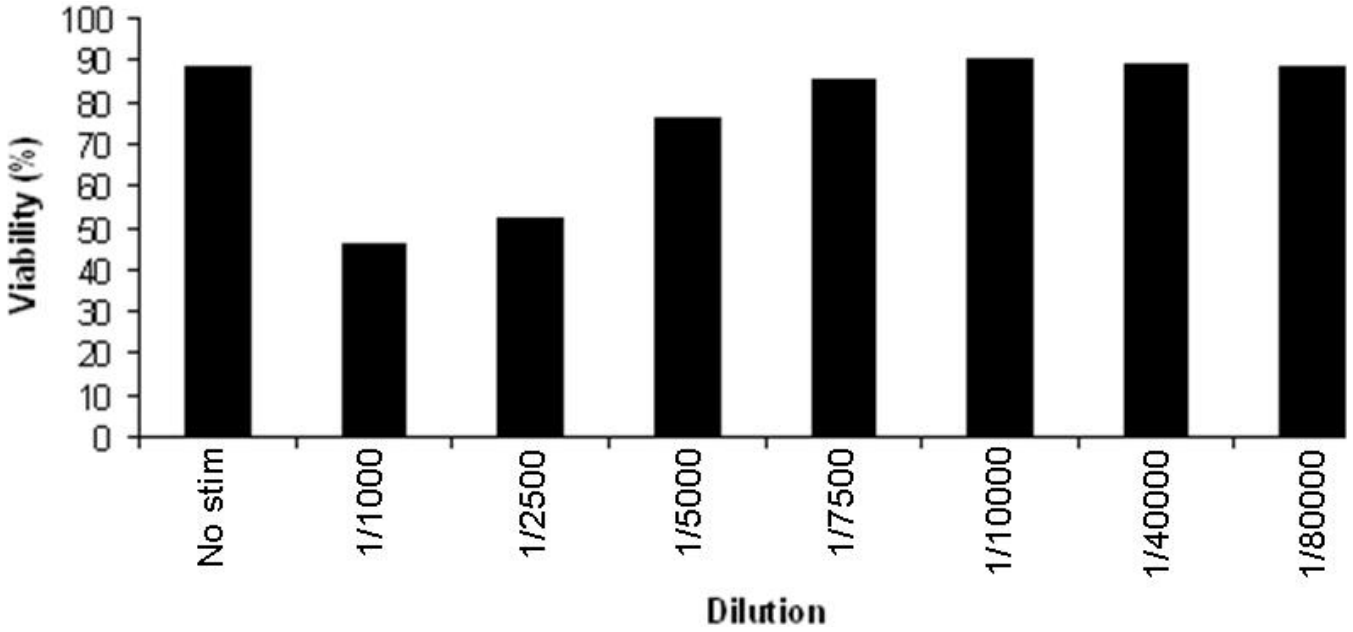

Figure 1. Dose-dependent cytotoxicity of Setarud in unstimulated B cells. No stim (No stimulation) represents untreated B cells.

scatter gating method for the analysis of the lymphocyte population. Typically 10,000 cells were analyzed. Fluorochrome conjugated isotype control antibody of irrelevant specificity served as negative controls (BD, New Jersey, USA).

Quantification of Ig in culture supernatant

A sandwich enzyme-linked immunosorbent assay (ELISA) was used for the measurement of human Ig secreted in the culture supernatant. Affinity purified rabbit anti human Ig (prepared in our lab) was coated at 10 µg/ml on flat-bottom 96-well microtiter polystyrene plates (Maxisorp, Nunc, Roskilde, Denmark), overnight at 4ºC. The wells were then washed thrice with PBS containing 0.05% Tween 20 (PBS/T) (Sigma, St Louis, USA), and then non-specific sites were blocked by adding 100 µl of PBS/T containing 3% skim milk (Fluka, Zwijndrecht, the Netherlands), at 37ºC for 1.5 h. After blocking, wells were washed with PBS/T and the culture supernatant from cultured B cells was added to the plate.

Following 1.5 h incubation at 37ºC and washing with PBS/T, appropriate dilution of affinity-purified horseradish peroxidase-conjugated rabbit anti-human Ig (prepared in our lab) was added to the plate. Following 1.5 h incubation at 37°C, wells were washed with PBS/T and incubated with TMB (3,3′,5,5′-Tetramethylbenzidine) substrate solution. The reaction was finally stopped with 2 M HCl, and the optical density (OD) measured at 450 nm. Concentration of secreted human Ig was measured by extrapolation from a standard curve constructed using known concentrations of human Ig.

Quantification of IL-10:

The level of secreted IL-10 was measured in supernatants collected from stimulated and unstimulated cells using a commercial sandwich ELISA kit, as recommended by the manufacturer (eBioscience, San Diego, CA, USA).

Statistics

Statistical analyses were performed using the SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used for comparisons between multiple treatments and Student's t test for comparisons between two treatments. Comparisons with probability values of less than 0.05 were considered to be significant.

RESULTS

Effect of Setarud on viability of unstimulated B cells

Setarud induced a dose-dependent cytotoxicity in negatively enriched unstimulated B cells. Remarkable cytotoxic effect was observed at 1/1000 and 1/2500 dilutions, while other dilutions showed no significant cytotoxicity (Figure 1).

Anti-proliferative effects of Setarud on TLR-activated B cells

To study the immunomodulatory effect of Setarud on different functional aspects of human B cells, the extract was added to TLR-stimulated B cells at dilutions not inducing significant cytotoxicity. Thus, 1/5000 and higher dilutions were examined.

B cells stimulated with CpG and R848 were sensitive to the inhibitory effects of Setarud, but at variable levels. At 1/5000 and 1/10000 dilutions of the extract, a significant inhibition in proliferation of B cell cultures stimulated by

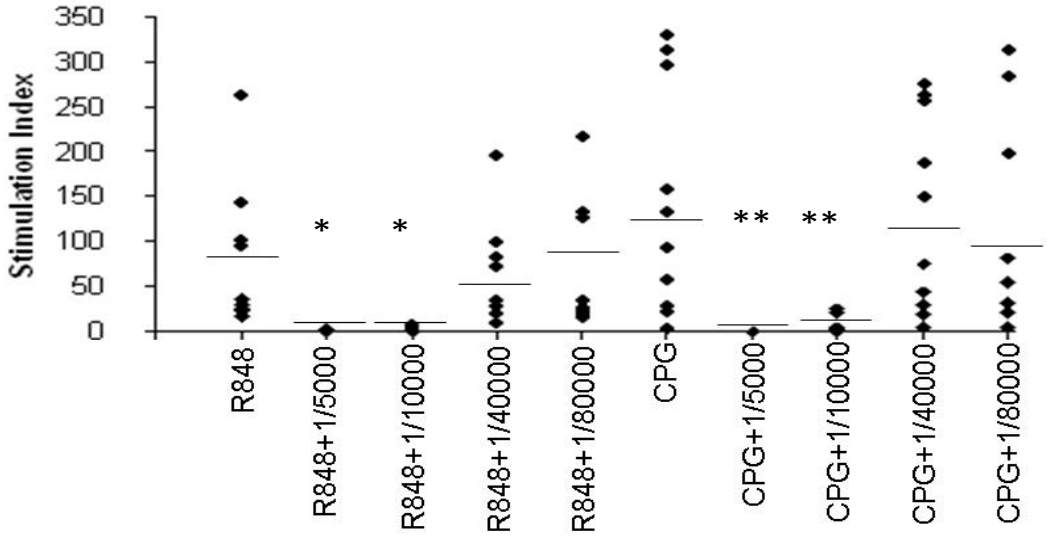

Figure 2. Antiproliferative effect of Setarud on B cells activated with TLR agonists. Horizontal lines represent mean of data; Significant differences are shown as *(P= 0.05-0.01) and ** (P<0.01)

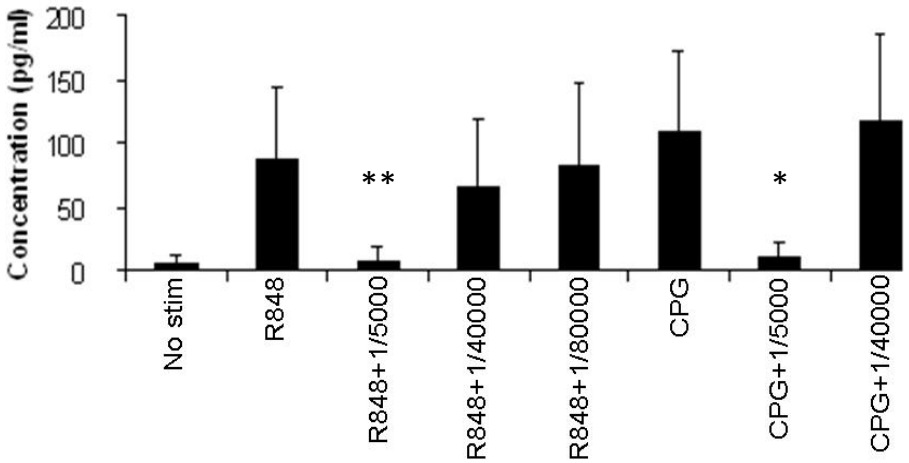

Figure 3. The effect of Setarud on IL-10 secretion by CpG and R848 stimulated B cells. Vertical bars represent standard deviation (SD); No stim: No stimulation.

R848 (p=0.01 for 1/5000 and p=0.021 for 1/10000) and CpG (p=0.006 for 1/5000 and p=0.007 for 1/10000) was observed (Figure 2). The stimulation indices of dilutions greater than 1/40000 were not significantly different from Setarud untreated B cells.

Effect of Setarud on IL-10 production by TLR-stimulated B cells

Incubation of enriched B cells for 72 h with Setarud in presence of CpG or R848 resulted in a remarkable inhibition of IL-10 production. This inhibitory effect was dose dependent. Significant differences were observed at 1/5000 dilution of Setarud (p<0.05 and p<0.01 for CpG and R848, respectively). There were no significant differences of IL-10 level secreted in presence of 1/40000 and 1/80000 dilutions of Setarud and CpG. 1/10000 and 1/80000 dilutions of Setarud were not employed in this set up to minimize the load of work (Figure 3).

Effect of Setarud on immunoglobulin (Ig) secretion by B cells

Supernatants of CpG and R848 stimulated B cells in the

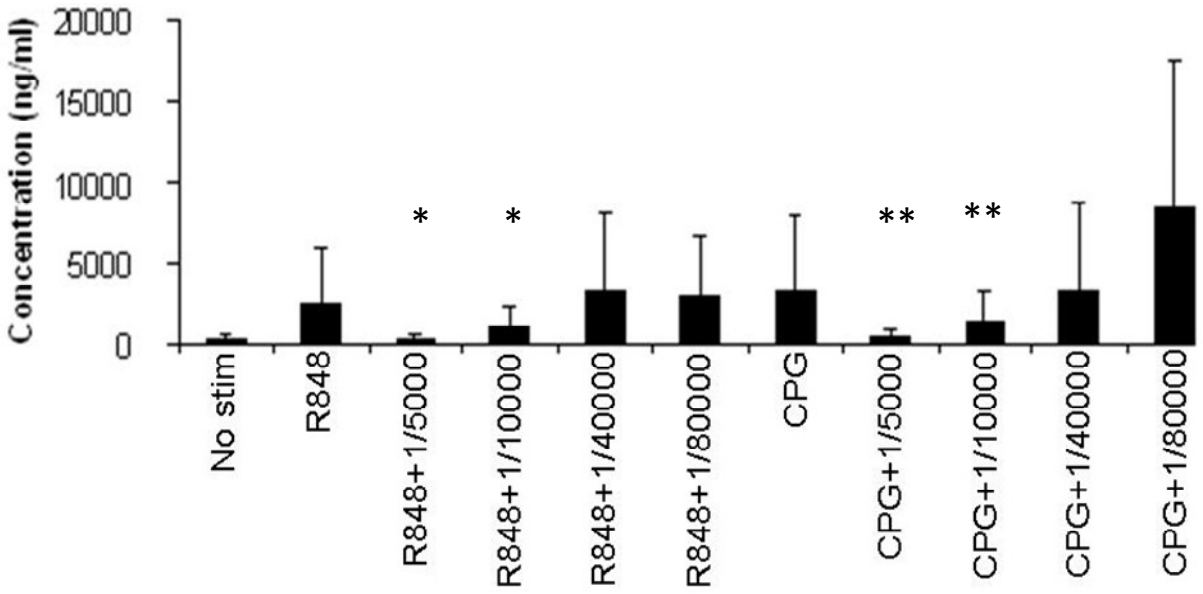

Figure 4. The effect of Setarud on Ig secretion by CpG and R848 stimulated B cells. Asterics indicates statistical significant differences compared to Setarud untreated TLR-activated cultures; Vertical bars represent SD.

presence or absence of Setarud was assayed for human Ig.

As shown in Figure 4, Setarud dose-dependently inhibited CpG and R848 induced Ig secretion at dilutions of 1/5000 ($p<0.01$ for CpG and $p<0.05$ for R848) and 1/10000 ($p<0.01$ for CpG and $p<0.05$ for R848). Higher dilutions of Setarud did not affect Ig secretion, though the 1/80000 dilution increased Ig secretion in CpG stimulated B cells, but the difference was not statistically significant (Figure 4).

Setarud down regulates expression of CD86 on B cells

CpG and R848 stimulation results in B cell activation and enhanced surface expression of the costimulatory molecule CD86, Representative flow cytometry results obtained for one sample are presented in Figure 5. We observed a decrease in mean fluorescence intensity (MFI) and expression level of CD86 on TLR activated B cells in presence of 1/5000 and 1/10000 dilutions of Setarud, though the effect of only 1/5000 dilution was significant ($p<0.05$) (Figure 6).

Correlation between different B cell biological functions affected by Setarud

A significant correlation was observed between all biological functions inhibited by Setarud ($p<0.01$-$P<0.0001$, $r = 0.349$-0.833). The highest correlation was obtained between IL-10 secretion and B cell proliferation ($r = 0.833$, $p<0.001$) as well as IL-10 secretion and Ig production ($r = 0.739$, $p<0.001$). The least correlation was obtained for CD86 expression and IL-10 secretion ($r = 0.349$, $p = 0.004$). Representative correlation data is presented in Figure 7.

DISCUSSION

Several studies have demonstrated the effects of herbal extracts on the immune system (Martino et al., 2010). Suppression of immune response by medicinal plant products as a possible therapeutic measure has recently become a subject of extensive investigation (Zhang and Sun, 2009). The function and activation status of the immune cells could be up-regulated or down-regulated by different compounds, including naturally occurring metabolites in herbal products. An understanding of the specific effect of herbal products on different subsets of immune cells is essential and prerequisite to explore their therapeutic potential in the management of inflammatory disorders, tumors, infections, and immune disorders (Nworu et al., 2009). In this study we examined the immunomodulatory effect of Setarud on different aspects of B cell function.

B cells are major contributors of the humoral immune response. Upon stimulation with a specific antigen or a polyclonal activator such as a mitogen or TLR agonist, they proliferate and are subsequently activated and differentiated into Ig secreting lymphocytes. Activated B cells express higher levels of costimulatory molecules, such as CD86 and CD83 and in addition to Ig, they secrete a variety of cytokines such as IL-10 (Hayashi et

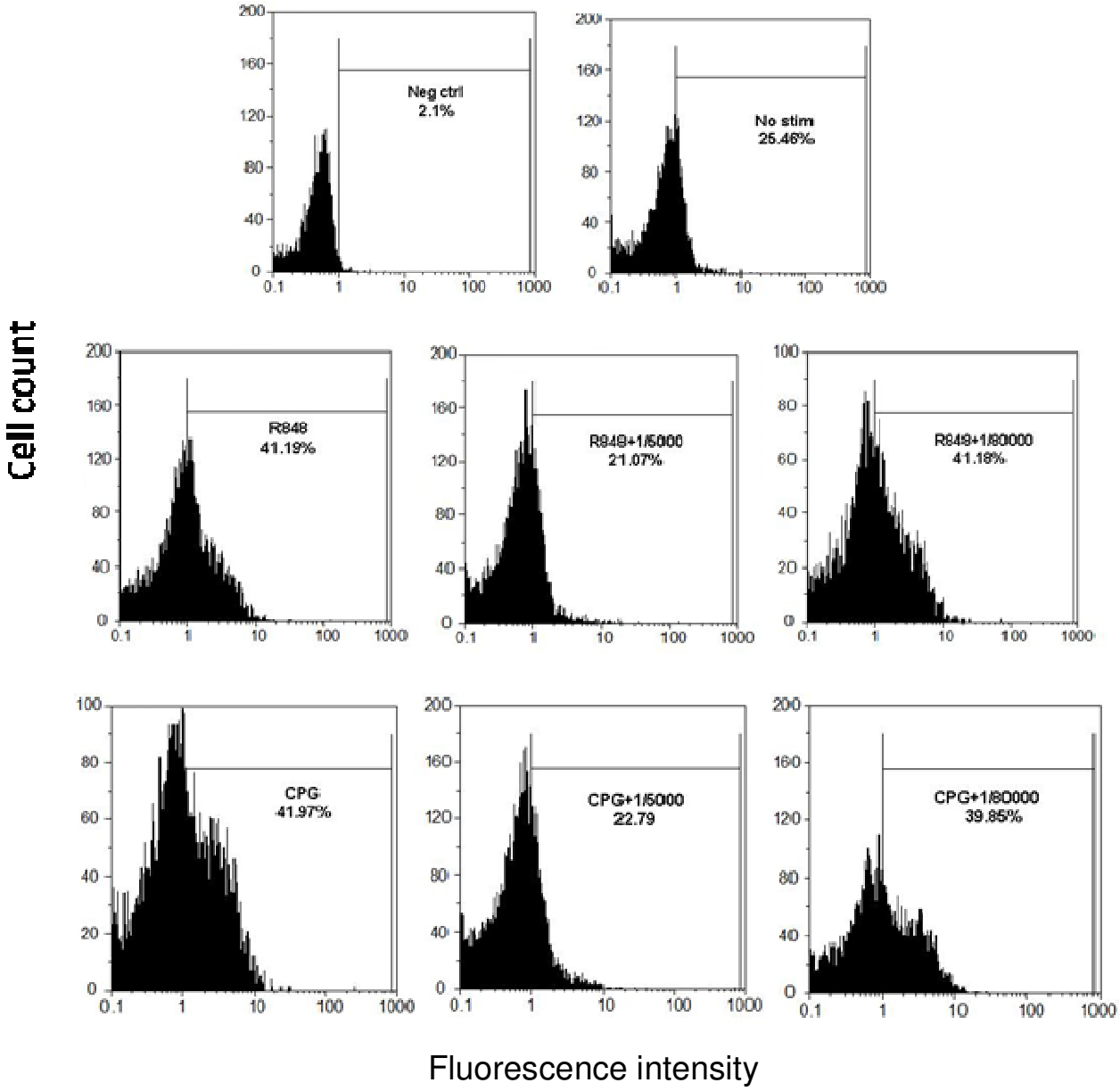

Figure 5. Representative flow cytometric results of CD86 expression in TLR stimulated B cells in presence or absence of Setarud. Neg ctrl: negative control.

al., 2010; Carpenter et al., 2009; Jeannin et al., 1997).

Setarud is an alcoholic extract of three local plants, *T. vulgare, R. canina,* and *U. dioica* enriched with selenium. The mechanism of action of Setarud and its immune cell target is not known, though T cells are thought to be potentially involved (Mohraz et al., 2009). Due to its promising immunonopotentiating effects, Setarud has recently been patented (Abdollahi, 2010). Interpretation of the existing literature suggests that Setarud might improve the immune system of HIV patients by potentiating the function and count of CD4 T cells (Mohraz et al., 2009). Among those who had CD4 count less than 400 cells per ml, treatment with Setarud for 3 months significantly increased the number of their CD4 cells in comparison with those who did not receive any treatment (Mohraz et al., 2009).

No significant adverse effect due to Setarud was observed after one month of follow-up (Kheyrandish et al., 2009).

The data presented here confirms that the extract is not toxic to B cells at dilutions higher than 1/5000. Considering the fact that concentration of the effective materials in the alcoholic extracts was 312 mg/ml, the level of the effective material in 1/5000 working dilution

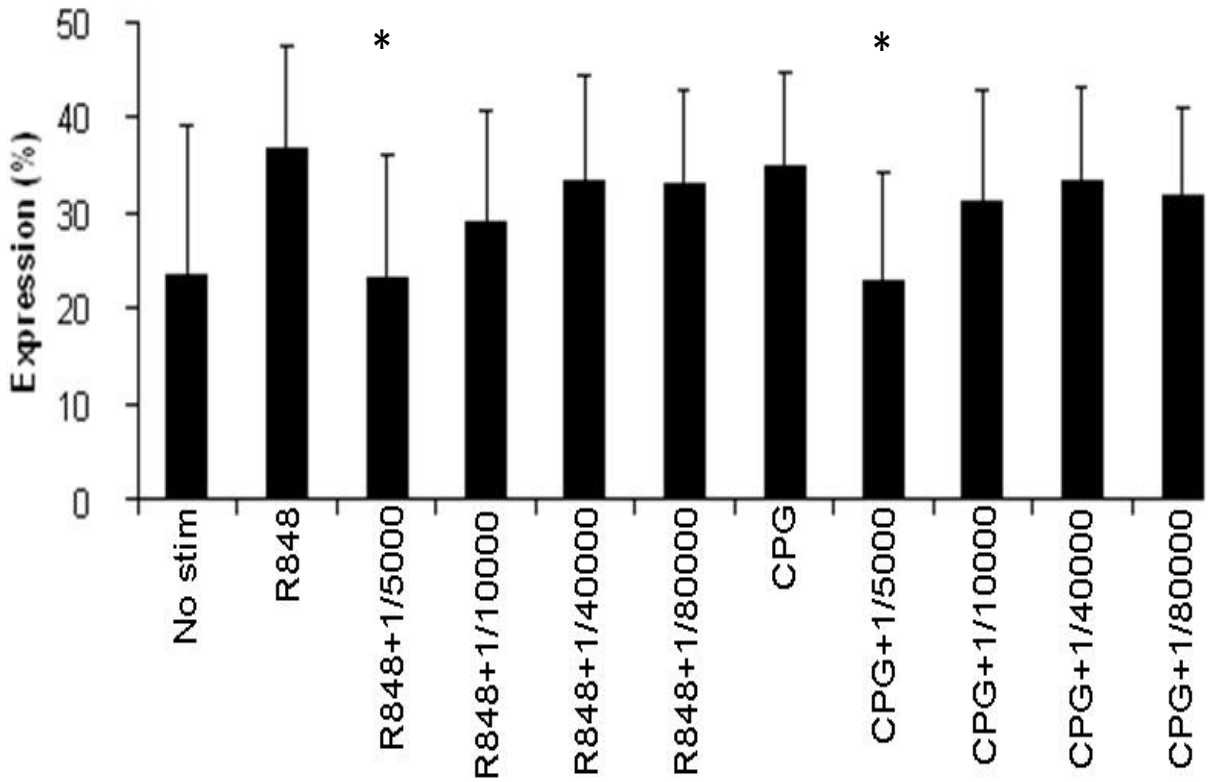

Figure 6. Expression of CD86 in B cells stimulated with TLR agonist in presence or absence of different dilutions of Setarud. Asteric indicates statistical significant differences compared to Setarud TLR-activated cultures; Vertical bars represent SD.

would be equal to 0.062 mg/ml.

Since cytotoxic effect of Setarud is increased at dilutions lower than 1/5000 or concentrations higher than 0.062 mg/ml in human B cells, this level of toxicity is to be taken into consideration when this product is prescribed for patients. Our results obtained in enriched B cells suggest that the extract may induce its inhibitory effect in the absence of accessory cells. This observation implies that the activity of Setarud is delivered directly to B cells and is not mediated via the production of negative signals from accessory cells (macrophages or dendritic cells). Setarud also inhibited B-cell proliferation induced by potent TLR agonists, including R848 (TLR7/8) and CpG (TLR9). Human B cells constitutively express both TLR9 and TLR7 molecules and could be vigorously stimulated by the corresponding TLR agonists (Bernasconi et al., 2003; Bekeredjian-Ding et al., 2005; Smits et al., 2008; Jiang et al., 2007; Dasari et al., 2005). The extract induced a dose-dependent inhibitory effect on proliferation, expression of costimulatory molecules, Ig and IL-10 secretion in TLR stimulated B lymphocytes, particularly at 1/5000 and 1/10000 dilutions.

Viability experiments in unstimulated B cells revealed that low dilutions of Setarud (up to 1/2000) induce substantial cytotoxicity (Figure 1). No substantial cytotoxicity was observed at dilutions higher than 1/5000. Thus, we decided to determine the biological effect of Setarud on B cells at dilutions higher than 1/5000. We observed a significant positive correlation between all biological functions which were found to be downregulated by Setarud in TLR stimulated B cells. However, the correlation between CD86 expression and the other parameters tested in this study was less significant. This could be due to the constitutive expression of CD86 on human B cells (Lenschow et al., 1996).

Inclusion of other costimulatory molecules such as CD83 which are mainly induced following stimulation could enhance the correlation of this marker with other parameters included in this study.

Analysis of the signaling pathways involved in TLR9 and TLR8 activation has demonstrated induction of several downstream signaling molecules which collectively results in activation of mitogen-activated protein (MAP) kinases and NF-Kb (Takeda and Akira, 2004). NF-Kb is an inducible transcription factor which plays an evolutionarily conserved and critical role in triggering and coordinating both innate and adaptive immune responses (Tripathi and Aggarwal, 2006).

Setarud seems to counteract these signaling pathways. Direct inspection of these pathways is necessary to explore the mechanism of action of Setarud and its

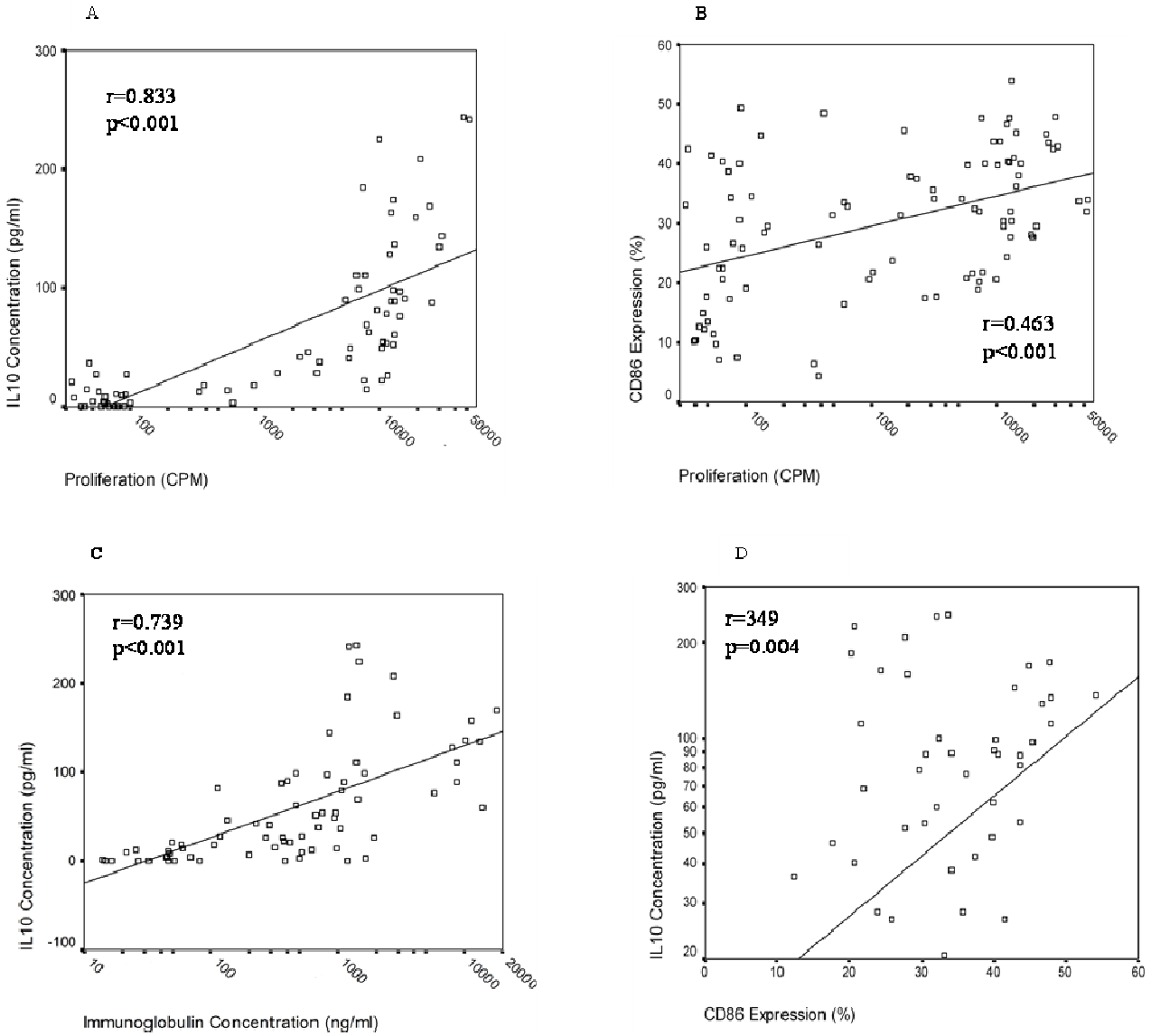

Figure 7. Representative correlation data obtained for IL-10 secretion, Ig production, cell proliferation and CD86 expression by B cells treated with Setarud.CPM: Count per minute.

inhibitory effect on TLR signaling.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper

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