Cytokine Mediated Immunomodulatory Properties of Selected Sri Lankan Medicinal Plants

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Abstract
Present study has tested the immunomodulatory properties of Ethanolic extracts of roots of Clerodendrum infortunatum Linn., Croton laccifer Linn. And Solanum surattense Burm. f, rhizomes of Alpinia calcarata Rosc. and tubers of Cyperus rotundus Linn. in a rat model. Tested plant extracts induced significantly (P<0.05) higher inflammatory response and shorter recovery periods in paw edema assay and significantly(P<0.05) increased the production of cytokinesIL-1 beta, IL-4, IL-6, IL-10, IL-12, IFN-γ and TNF-α in a dose dependent manner. Tested plant extracts also resulted a significant (P<0.05) development of immunological memory and higher granulocyte adhesion percentages and significantly (P<0.05) up regulated granulocyte and monocyte counts. In contrast with control group the plant extract treated groups showed higher survival ratios when treated with immune suppressor Cyclophosphamide indicating a potential tolerance against immunosuppression. Taken together these findings indicated that studied plant extracts are positive immunomodulators in all aspects of the study.

Key words; Immunomodulation, Inflammatory response, Cytokines, Medicinal plan

1. Introduction

The application of plants as therapeutic agents has been practicing by a significant percentage of the world population in developing countries and more than 150000 plant species have been studied for their bioactive substances against various diseases (Loc and Kiet, 2011). Immunomodulators are substances that alter the immune response by augmenting or reducing the ability of the immune system to produce antibodies or sensitized cells that recognize and react with the antigen that induced their production (Marie, 2013; Owen et al., 2013; Mans et al., 2000).

Evaluation of the cellular arm of immune system is obviously important for the diagnosis and monitoring of cell-mediated immune deficiencies (Fletcher et al., 1997; Stites et al., 1997), diagnosis of infection (Bothamley, 2002) and monitoring the effects of immunomodulators (Whiteside and Henk, 2002). Leukocyte extravasation, cytokine mediated cellular communication and phagocytosis collectively employ in eliciting an inflammatory reaction.

In immunomodulatory research the strength of inflammatory response is expressed in terms of paw edema under the conditions of antigen induced inflammation (Stites et al., 1997; Yadav et al., 2011). Leukocyte extravasation; the migration of leukocytes in blood stream to the site of infection is crucial for the immune response which is a complex process mediated by intercellular adhesion and messenger molecules. Enhancement of adhesion molecules results effective recruitment of leukocytes to the affected site and leukocyte adhesion test is used as a tool to signify the adhesion capabilities of leukocyte at primary level in research (Wilkinson et al., 1978).

Cell-to-cell communication is maintained via cytokine networks and they play a key role in modulation of both innate and adaptive immune responses (Thao et al., 2008). Modulation of cytokine production may be vital for effective immune response and it is a key concept in cytokine therapy. Cytokines such as IL-1 and TNF-α act as key mediators in inflammation and IL-4, IL-6, IL-10, IL-12 and IFN-γ exert their actions mainly on T and B lymphocytes (Male et al., 2007; Thao et al., 2008).

Immunological memory is the capacity of the immune system to elicit the immune response for a previously encountered antigen through the activation of antigen specific B and T lymphocytes or long lasting antibodies (Thao et al., 2008). Thus modulation of immune memory would enable the immune system to function efficiently in the cases of repeated exposure to same pathogen. Immuno restorative potential of botanicals is experimentally evaluated under the conditions of suppressed immunity using agents such as Cyclophosphamide and resistance against immunosuppression indicates the potential applicability of compounds as immune boosters (Yadav et al., 2011).

As claimed by Sri Lankan practitioners *Clerodendrum infortunatum* Linn.*, Croton laccifer*Linn.*, Solanum surattense* Burm. f.*, Cyperus rotundus* Linn. And *Alpinia calcarata* Rosc. are powerful immunomodulators. Potential medicinal value of these plants was described elsewhere ((Jayaweera, 1981; Jayaweera, 1982; Rahman and Zaman, 1989; Yusuf et al., 1994; Sharma et al., 2001; Pullaiah, 2002; Kiritikar et al., 2003; Prajapati et al., 2003; Sharma, 2003; Uddin et al., 2006; Arambewela et al., 20102012; Arawwawala et al., 2012; Sannigrani et al., 2009; Praveen et al., 2012). It was hypothesized that these plants might induce immune system via modulating cytokine expression. Thus the present study was mainly focused to study the immunomodulatory cytokine expression and associated immune responses of rats in response to the treatments of these plant extracts.

2. Materials and methods

2.1. Plant materials

Root barks of *Clerodendrum infortunatum*, *Croton laccifer*, and *Solanumsurattense*, tubers of *Cyperusrotundus* and rhizomes of *Alpiniacalcarata* collected from natural habitats were identified and

authenticated by Prof. PiyalMarasinghe, Chief Botanist, National Research Medicinal Plant Garden, Haldummulla, Sri Lanka. Voucher specimens were preserved at Department of Zoology, University of Ruhuna, Sri Lanka for future reference.

2.2.   Preparation of plant extracts

Plant materials were cleaned with distilled water and dried at 25^0C under shade. The   dry materials were crushed into a fine powder and macerated with 80% ethanol for 72 hours with frequent shaking. The solvent phase was isolated by centrifugation and evaporated   to dryness under reduced pressure. The crude residues were sealed in air tight containers and stored at -20^0C for further use.

2.3.   Experimental animals

Female albino Wistar rats of 8 months old and in weight range of 200-230 g were obtained from medical research institute, Colombo, Sri Lanka. The rats were accommodated in the animal care facility of Department of Zoology, University of Ruhuna under standard housing conditions. Animals were granted free access to standard pellet diet and water.

2.4.   Selection of Doses

The crude extracts were suspended in normal saline and tested at 1000 mg/kg with no observed adverse effects. Thus the doses were selected as 50, 100, 200 mg/kg/day PO for further studies.

2.5.   Treatment protocol

Animals were randomly divided into groups with twelve animals in each group and subjected for treatments as shown in the table 2.

2.6.   Preparation of antigen

Fresh blood was collected in to an EDTA coated tube from a chicken sacrificed in a local slaughter house. Blood was centrifuged at 500g for 5 minutes. Plasma was removed and the resultant cell mass was washed five times with sterile saline (0.9% NaCl W/V) and the cell concentration was adjusted to 7.5x 10^{10} cells per milliliter in order to use as the antigen.

2.7.   Experiments

2.7.1. Treatments

For all the experiments rats were treated for ten days continuously according to the treatment protocol unless otherwise mentioned. On 10th day blood samples were collected from the tail vein into EDTA coated tubes after two hours from the last dose unless otherwise mentioned (Animka et al., 2010; Papiya et al., 2012).

2.7.2. Inflammatory response assay

Inflammatory response assay was carried out as described elsewhere (Patel et al., 2012; Joelson et al., 1994; Vogel et al., 2002). On the 15th day of treatment all the rats were challenged with 0.02 mL of antigen suspension at sub plantar region of right hind paw. Thickness of the foot pads were measured using Vernier caliper and recorded as a function of time.

2.7.3. Hematological parameters

Blood samples were analyzed using automated 3- differential veterinary hematology analyzer (GN-2200 Vet) as instructed by the manufacturer.

2.7.4. Granulocyte adhesion assay

Blood samples were collected and analyzed for differential leukocyte count using GN -2200 Vet hematology analyzer. Blood samples were incubated with nylon fibers at 80 mgmL^{-1} at 37° C. for 15 minutes. Incubated blood samples were analyzed again for differential leukocyte count (Wilkinson et al., 1978). Percentage adhesion was calculated as follows,

$$\text{Granulocyte adhesion} = \left[\frac{(\text{Gran1} - \text{Gran2})}{\text{Gran1}}\right] \times 100\%$$

Where
Gran 1 = granulocyte count before incubation with nylon fibers
Gran2 = granulocyte count after incubation with nylon fibers

2.7.5. In vivo cytokine assay

The rats were treated in accordance to treatment plan for a period of 15 consecutive days. On the 15th day rats in the vehicle control group were randomly divided into two groups and labeled as negative control and positive control. Inflammation was induced in all the treatment groups with positive control group at right hind paw by injecting 0.02 mL of antigen suspension at sub-plantar region. The negative control group received no antigen. After three hours from first antigen challenge that blood some samples of each group were taken from the tail vein into EDTA coated tubes. Blood samples were centrifuged at 1000g for 15 minutes at 6^{0}C in order to isolate the plasma. Plasma samples were analyzed for IL-1 beta, IL-4, IL-6, IL-10, IL-12, TNF-α and IFN-γ using enzyme linked immunosorbent assay (ELISA) kits produced by Cusabio biotech Co. Ltd, China according to the protocol developed by the manufacturer.

2.7.6. Immunological memory assay

The rats received no treatment after the first antigen challenge and seven days later rats were challenged with the same antigen suspension in same volume at the right hind foot pad. Thickness of foot pads were measured as a function of time.

2.7.7. Cyclophosphamide induced immunosuppression.

Rats from all groups received the treatment in accordance with the treatment protocol for 15 consecutive days. On the 15th day, two hours after the treatment Cyclophosphamide was given at a dose of 50 mgKg^{-1} to all individuals orally. Blood samples were collected prior to and 3 days after the administration of cyclophosphamide and analyzed for total and differential leukocyte count (Animka et al., 2010).

2.8. Statistical analysis

Results were expressed as mean ± standard deviation of mean. Data were analyzed by One-Way ANOVA followed by Turkey multiple comparison test using SPSS version 19. $P < 0.05$ was considered as the measure of statistical significance.

3. Results and Discussion

3.1. Inflammatory response assay

As indicated by inflammatory response assay treated rats reported a higher inflammatory response coupled with shorter recovery periods in comparison with control group (Supplementary figure S1).

3.2. Hematological parameters

3.2.1. *Differential Leukocyte count*

Effect of treatments on differential white blood cell count is shown in figure 1. The effect was dose dependent and higher doses of each drug has significantly increased (P<0.05) granulocyte and monocyte count and hence total WBC counts in comparison with the control group. Further the effect of treatments on lymphocyte count was not significant (P>0.05).

Neutrophils are a type of phagocytes normally found in the blood stream during the beginning (acute) phase of inflammation, particularly as a result of bacterial infection, exposure to a foreign body and some cancers (lacro et al., 2004; Waugh and Wilson, 2008). Neutrophils are one of the first responders of inflammatory cells to migrate towards the site of inflammation. Eosinophils are active participants in innate and adaptive immune responses to parasite helminth infections (Thao et al., 2008) while basophils are important in allergic reactions of adaptive immune responses. All the five plant extracts exhibit significant mediation (P<0.05) on increasing monocyte count at higher doses. Monocyte in the blood stream migrate into tissues and differentiate into resident macrophages or dendritic cells that play a key role in as active phagocytic cells against foreign particles and pathogens and scavengers of cellular debris (Swirski, 2009). Plant extracts did not exert a significant (P>0.05) effect on lymphocyte count. In this context as the plant extracts used in this study are capable of up regulating the key factors of cellular immune response such as granulocytes and monocytes in rat model they can be applied therapeutically in order to trigger leukocyte count to mount a potent immune response against infections.

3.2.2. *Effect on red blood cells and related parameters*

Test extracts did not show any significant effect on red blood cells and associated parameters including RBC count, Haemoglobin (HGB), Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), Red Cell Distribution Width standard deviation (RDW-SD) and Red Cell Distribution Width coefficient of variation (RDW-CV). The drugs exert significant effect on none of these parameters (P>0.05) (Supplementary figureS2).

3.2.3. *Effect on platelet related parameters*

There was no significant effect of drugs on platelet associated parameters such as Platelet count, Mean Platelet Volume (MPV), Platelet Distribution Width (PDW), Plateletcrit (PCT) and Platelet Large Cell Ratio (P-LCR) (Supplementary figureS3).

3.3. Granulocyte Adhesion

Migration of phagocytic cells such as neutrophils and monocytes into sites of tissue damage or infection leaving the blood stream (extravasation) is a multi-step process mediated by adhesion interactions between endothelial cells and leukocytes (Kenneth et al., 2012; Thao et al., 2008). Expression of key endothelial adhesion molecules (P-selectins, ICAM-1 and VCAM-1) occurs under the influence of inflammatory cytokines such as TNF-α, IFN-γ and IL-1 (Laudes et al., 2004; Thao et al., 2008; Whalen et al., 2000).

In the present study plant extracts significantly (p<0.05) up regulated granulocyte adhesion to nylon fibers in comparison with the control group (Figure 2). This may be due to up regulation of expression of certain adhesion molecules on granulocytes accompanied by the treatment.

3.4. In vivo cytokine assay

Immune response is a complicated process cooperated by multiple components of immune system against immune invaders. Cytokines are a diverse group of products mainly of immune cells that play a vital role as

signaling molecules in cellular communication. The effect of treatments on the expression of key inflammatory cytokines namely Interleukin-1 beta (IL-1 beta), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-12 (IL-12), Interferon- gamma (IFN-γ) and Tumor Necrosis Factor-alpha (TNF-α) were measured after the chick red blood cell induced inflammation (Figure 3). The treatment significantly ($p<0.05$) up regulated the expression of cytokines in comparison with the negative control (vehicle control received no drug treatment or antigen) and positive control (Vehicle control received no drug treatment but the antigen). In this consideration the observed enhancement in inflammatory response, granulocyte adhesion and immunological memory mediated by treatment may occur under the influence of increased expression of above cytokines.

Components of both innate and adaptive immune systems may respond to certain antigens via the process of inflammation. It is a rapid and non-specific process that initiates prior of specific immune response. In the inflammatory response assay the capability of plant extracts to modulate inflammatory cytokines was assessed under the conditions of induced inflammation. Inflammatory response is characterized by increased blood supply carrying more leukocytes and plasma molecules, increased permeability of capillaries allowing exudation of plasma proteins and increased migration of leukocytes to the affected tissue (Male et al., 2007). In a case of exposure to a foreign antigen the innate immune response is triggered by monocyte/macrophage recognition of specific microbial pathogens by a family of pathogen-associated molecular pattern (PAMP) receptors (Modlin et al., 1999; Underhill, 2003; Underhill and Ozinsky, 2002; Vasselon and Detmers, 2002). Activated macrophages produce TNF- α, IL-1, IL-6 and IL-12 (Bone et al., 1997; Evans, 1996). In order to initialize the inflammatory response. IL-1 and TNF-α induce vasodilation and increased vascular permeability increasing blood flow to the affected site resulting edema redness and heat (Thao et al., 2008). The results of inflammatory response assay showed higher rates of initial paw edema in treated groups in contrast with the control. As suggested by the results of in vivo cytokine assay increased production of TNF-α and IL-1 beta may support this observation. IL-6 results fever via the production of acute phase proteins and IL-12 mediates NK cell stimulation (Thao et al., 2008). The higher recovery rates of paw edema in treated rats in comparison with control group may suggest a more powerful immune attack in treated rats against the injected antigen. Results showed elevated granulocyte and monocyte counts and probable up regulation of expression of leukocyte-endothelial adhesion molecules under the influence of elevated TNF-α, IL-1 beta and IFN-γ levels in plasma (Laudes et al., 2004; Thao et al., 2008; Whalen et al., 2000) facilitating effective leukocyte homing in the site of inflammation in treated rats. Thus it can be concluded that the cumulative effect of all these events may be responsible for higher rates of initiation of paw edema and shorter recovery periods in treated subjects in comparison with the untreated group.

3.5. Immunological memory assay

Immunological memory is an important feature in adaptive immune response in contrast with innate immunity. In the exposure to a particular antigen in first place it may create a defensive reservoir of antigen specific antibodies and memory lymphocytes (T cells and B cells) through which the memory is maintained for prolonged periods (Thao et al., 2008). Thus the attack against the antigen becomes rapid and rigorous in subsequent exposures. In the case of induction of inflammation with the same antigen at same dose after seven days from initial challenge in the absence of drug treatment, treated animals reported significantly higher ($p<0.05$) rate of inflammation and shorter recovery periods in comparison with the values of first challenge and the control (Supplementary figureS4). This implies that plant extract treated rats exerted more rapid and forceful immune attack on the antigen on its second exposure. In comparison with the first exposure control group also showed a slight progress but it was not effective as in the treated rats. These data indicate that the treatments can induce a certain kind of memory build up in rats. Even though the

mechanism is unclear this may occur due to existence of specific antibodies or development of memory cells against the antigen.

3.6. Cyclophosphamide induced immunosuppression.

As suggested by Cyclophosphamide treatment plant extracts were capable of enhancing survival rate under the conditions of induced immunosuppression (Figure 4). According to the results of immunosuppression assay conducted to evaluate the resistance to immunosuppression both treated and control groups showed down regulation of WBC counts in same extent with no significant difference. Treated groups showed enhanced survival rate in contrast with the control group even though the extent of reduction of WBC is same as the control. This suggests that the plant extracts show a certain life enhancing potential under the conditions of weakened immunity and further investigations may be required to express the mechanistic basis of survival.

4. Conclusion

Withhin the scope of findings of the present study the tested plants C. infortunatum Linn, C. laccifer Linn, S. surattenseBurm.f, C. rotunduslinn and A. calcarataRosc. contain potentially bioactive compounds that significantly up regulate several key landmarks of immune function including cell mediated immunity, leukocyte adhesion and cytokine modulation.

5. Acknowledgements

6. References

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7. Tables and Figures

Table 2. Treatment protocol.

Treatment			
Experimental drug	**Dose/ (mg.Kg^{-1}.day^{-1})**	**Group name**	**Number of Rats**
Clerodendruminfortunatum (Roots)	50	C/inf/EE/050	12
	100	C/inf/EE/100	12
	200	C/inf/EE/200	12
Croton laccifer (Roots)	50	C/lac/EE/050	12
	100	C/lac/EE/100	12
	200	C/lac/EE/200	12
Solanumsurattense (Roots)	50	S/sur/EE/050	12
	100	S/sur/EE/100	12
	200	S/sur/EE/200	12
Cyperusrotundus (Tubers)	50	C/rot/EE/050	12
	100	C/rot/EE/100	12
	200	C/rot/EE/200	12
Alpiniacalcarata (Rhizomes)	50	A/cal/EE/050	12
	100	A/cal/EE/100	12
	200	A/cal/EE/200	12
Control			
Vehicle	**Volume per dose/ mL**	**Group name**	**No. of Rats**
Normal saline	1.0	Vehicle Control	12
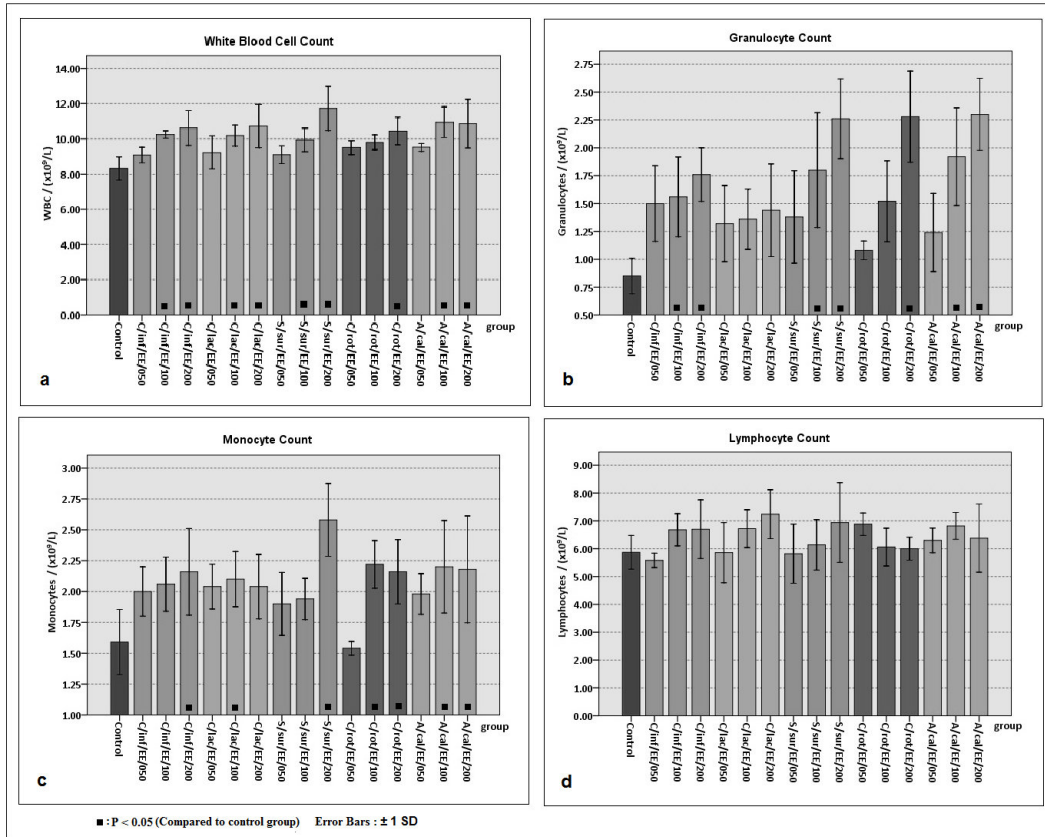
Figure 1. Effect of tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day PO) on differential leukocyte count in comparison with the control rats. a: Total WBC count; b: granulocyte count; c: Monocyte count; d: Lymphocyte count.
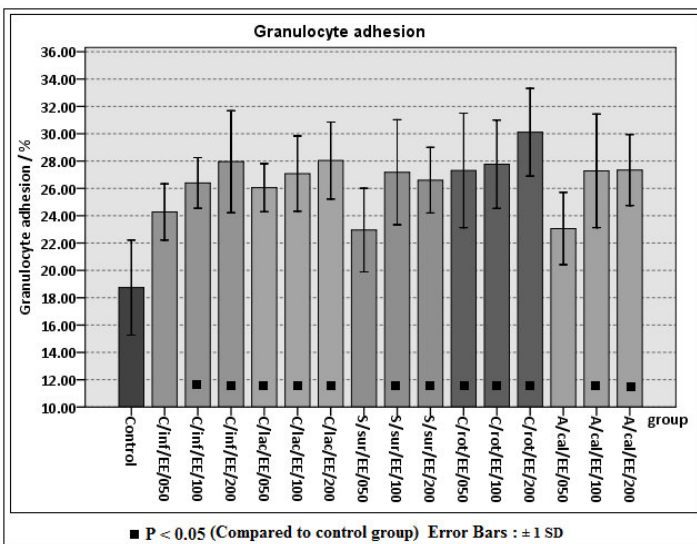

Fig. 2. Effect of tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day PO) on Granulocyte adhesion to nylon fibers in comparison with the control rats.
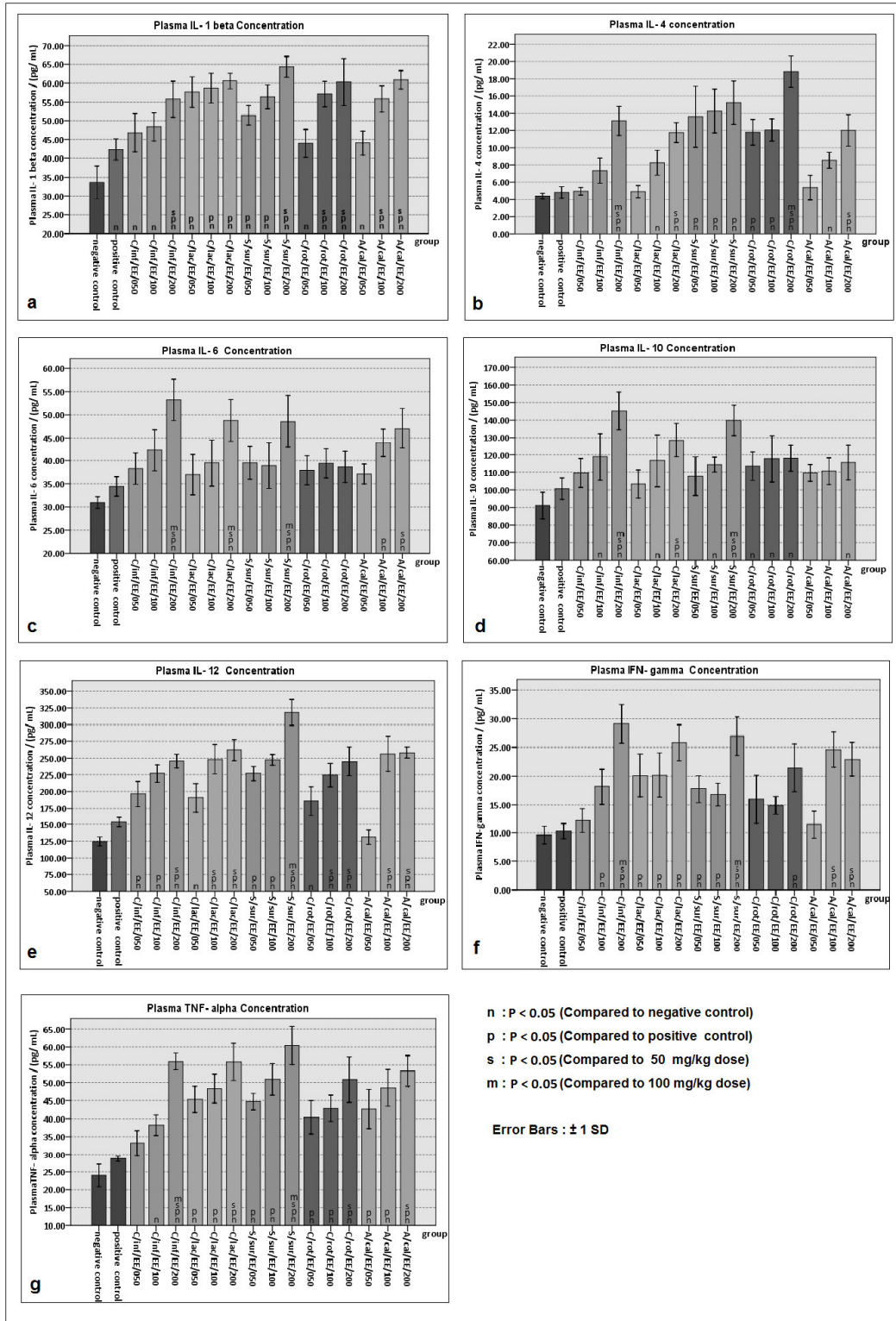
Figure 3. Effect of tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day PO) on expression of inflammatory cytokines in response to antigen induced inflammation in comparison with the control rats. a: IL-1β; b: IL-4; c: IL-6; d: IL-10; e: IL-12; f: IFN-γ; g: TNF- α.
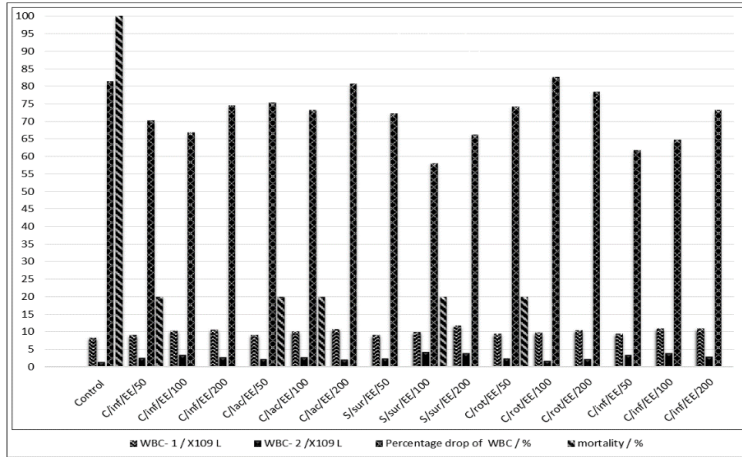
Fig. 4. Effect of tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day PO) on white blood cells and mortality in response to cyclophosphamide induced immunosuppression in comparison with the control rats. WBC-1: WBC count prior to administration of cyclophosphamide; WBC-2: WBC count after 72 hours frm administration of cyclophosphamide
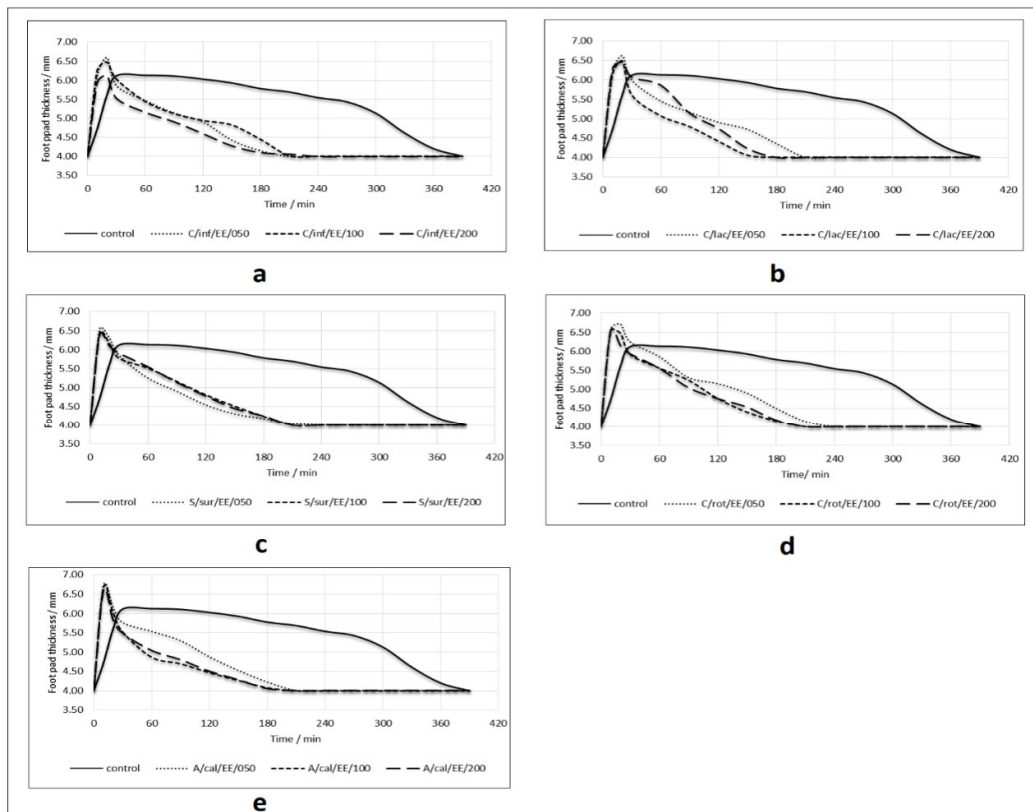
Supplementary figures

Figure S1. Variation of foot pad thickness of treated (tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day po)) and control rats with time in response to antigen (chick Red blood cells) induced paw edema.   a: *C. infortunatum* Linn; b: *C. laccifer Linn*; c: *S. surattense*Burm.f.; d: *C. rotundus*; e: *A. calcarata*Rosc.
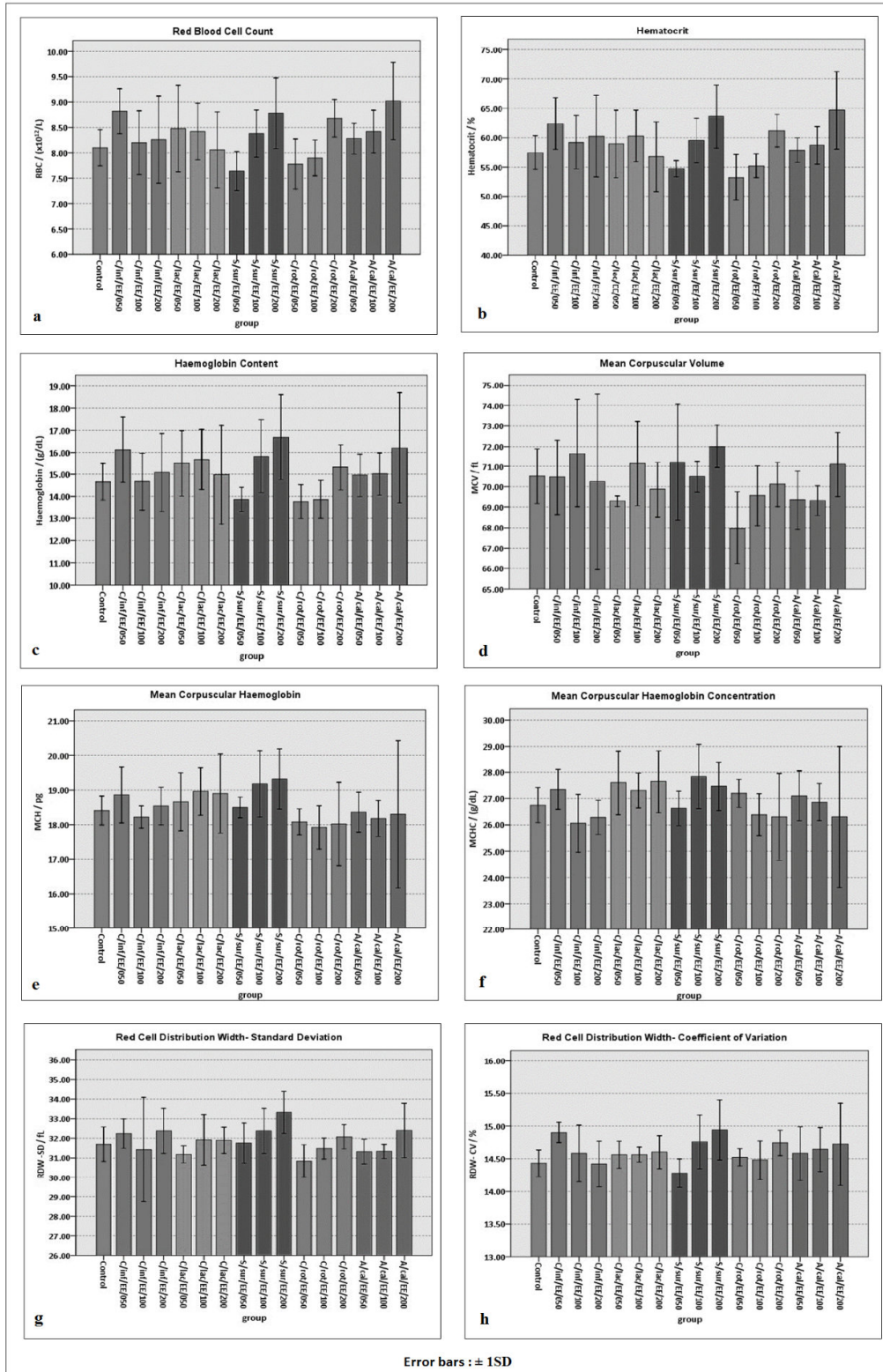
Fig. S2. Effect of tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day PO) on Red blood cell indices in comparison with the control rats. a: RBC count; b: Hematocrit; c: Hemoglobin content; d: MCV; e: MCH; f: MCHC; g: RDW-SD; h: RDW-CV
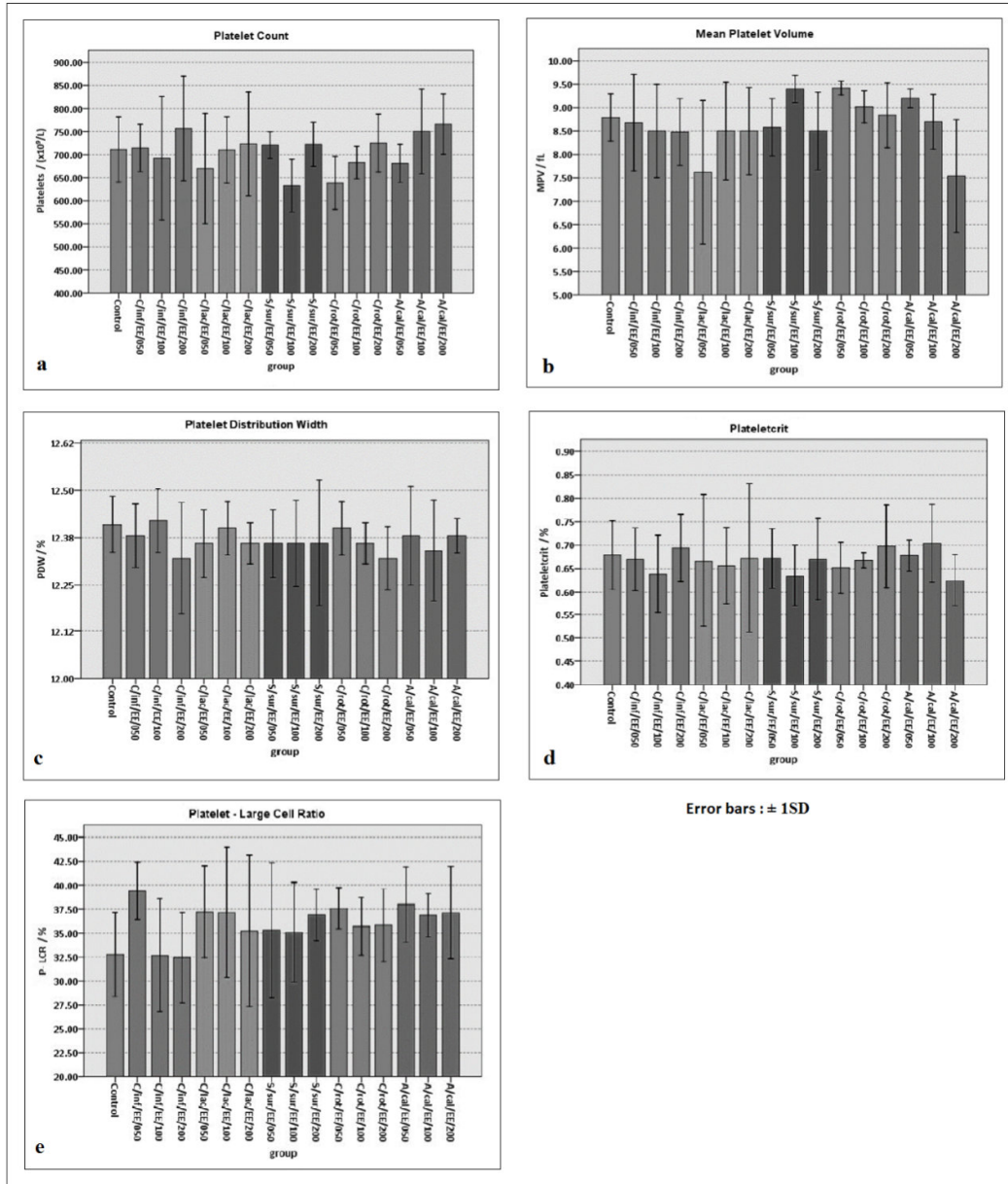
Fig. S3. Effect of tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day PO) on Red blood cell indices in comparison with the control rats. a: platelet count; b: MPV; c: PDW; d: Plateletcrit; e: P-LCR
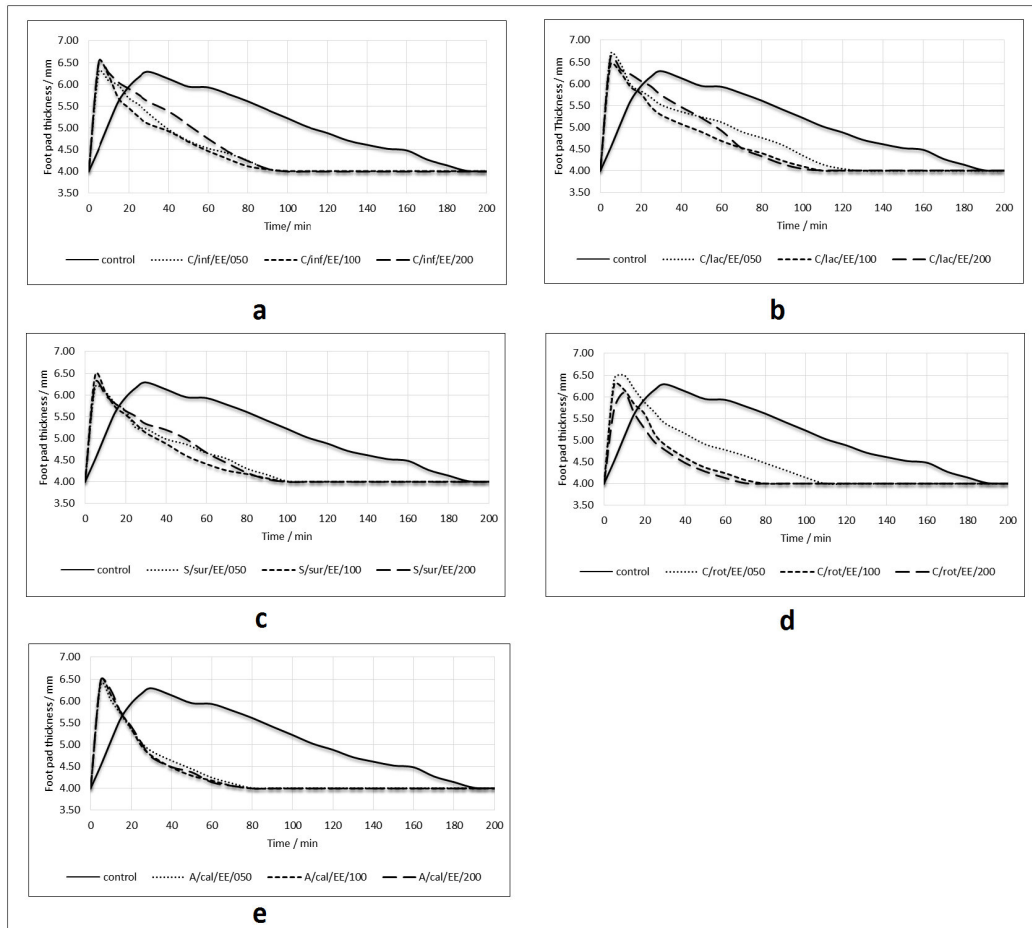
Fig. S4. Variation of foot pad thickness of treated (tested ethanolic plant extracts at doses (50, 100, 200 mg/kg/day po)) and control rats with time in secondary response to antigen (chick Red blood cells) induced paw edema after 7 days from first challenge in the absence of treatment.

a: C. infortunatum Linn; b: C. laccifer Linn; c: S. surattenseBurm.f.; d: C. rotundus; e: A. calcarataRosc.