



Study of effects *Aloe vera* extract supplemented feed on hematological and immunological indices of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of this study was to evaluate the effects of *Aloe vera* extract (AE) on the immunity responses and hematological parameters in rainbow trout (*Oncorhynchus mykiss*) fry to develop alternative drug to chemotherapeutics and antibiotics in aquaculture. 1200 rainbow trout (*Oncorhynchus mykiss*) fry weighing 2 ± 0.2 gram were randomly allocated into two treatment groups including: 1) placebo-treated group (control), 2) *Aloe vera* extract-treated group, each of three replicates. The fishes were hand-fed once a day with diet medicated AE or placebo at a rate of 1% of feed weight in the first feeding for 8 weeks. At the end of every two weeks 24 hrs after feeding, some of hematological and immunological parameters were analyzed. The results showed that serum total protein, albumin and globulin, respiratory burst activity, phagocytic activity and serum lysozyme activity vary among the two treatment groups which were found to be higher in AE-treated group ($p < 0.05$). However there were no significant differences in hematological parameters between two groups. It was concluded that supplementation of AE at a rate of 1% registered higher immunological responses in compared to placebo group. Therefore, supplementation of AE in fish diets would enhance the nonspecific immunity responses and would be use particularly at time of outbreaks.

Keywords: Herbal immunostimulant, Medicinal plants, *Aloe vera*, Fish

INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is the most preferred coldwater species in aquaculture industry of Iran. Achieving sustainable development in rainbow trout culture, to maintain the health status of this fish is of major importance. Fish pathogenic organisms are a serious threat to economic viability of any aquaculture practice. Currently, the use of antibiotics for the prophylaxis and treatment of diseases leading to the development of antibiotic resistant bacterial strains, accumulation of residue in cultured fish and environmental problems. Therefore, a new approach to immunotherapy is actively used to prevent or treat fish diseases. In this regard, extensive research has been carried out to test various immunostimulants including medicinal plants which they have found to be effective in fish. It has been found that use of medicinal herbs in fish diets enhance the immune system against infections with various bacteria, especially (Ahamad et al., 2011; Subeena Begum and Navaraj, 2012; Maqsood et la., 2011; Castro et al., 2008).

Aloe vera (synonym: *Aloe barbadensis* Miller) belonging to family liliaceae is widely distribution in the tropical and subtropical regions of the world. Most of *Aloe* species are indigenous to Africa, but now have wide distribution in the tropical and subtropical regions of the world (Mahdavi et al., 2013). The genus *Aloe* contains over 400 different species and *Aloe barbadensis* Miller is considered to be the most biologically active. Cosmetic and some medicinal products are made from the mucilaginous tissue in the center of the *Aloe vera* leaf and called *Aloe vera* gel. The peripheral bundle sheath cells of *Aloe vera* produce intensely bitter, yellow latex, commonly termed aloe juice, of sap, or aloes. *Aloe vera* sap and gel are often confused. Unlike aloes, *Aloe vera* gel contains no anthraquinones, which are responsible for the strong laxative effects of aloes. However, total leaf extracts may contain anthraquinones. Although most commercially- available products are based on the gel, the British Pharmacopoeia does not contain an entry for *Aloe vera* gel but it does describe aloes (Marshall, 1990). Acemannan and the other constituents of *Aloe vera* have been found to improve macrophage activity as much as tenfold, enhance macrophage effectiveness in modulating the entire immune system, in stimulating, producing, and releasing antibodies (Baseline of Health Foundation, 2002). The objective of the present study was to evaluate the effects of *Aloe vera* extract (AE) on various parameters of non-specific immunity responses including respiratory burst activity, phagocytic activity of blood leukocytes, serum lysozyme activity, total plasma protein level and some of haematological indices in rainbow trout (*Oncorhynchus mykiss*) to develop alternative drug for the prevention or the treatment of diseases in aquaculture.

MATERIALS AND METHODS

Preparation of Aloe Vera extract

The plant of *Aloe vera* was procured from Medicinal Plants Production Cooperation of Havin and plant species was identified and confirmed by a botanist. The leaves were collected and washed in sterile distilled water and evacuated from gel. The leaves were separately shade-dried for 10 day till weight constancy was achieved. The sample was powdered in an electric blender. The extract was prepared with the standard method of percolation. To do this, chopped dried plant leaves in 80% ethanol were percolated for 72 hours. Then, the slurry was filtered with Whatman No. 1 filter paper and centrifuged for 5 min at 5000 rpm. The filtrate obtained from ethanol using a rotary device, the excess solvent was separated from the extract. These crude extract was stored at 4°C until use

Supplementation of the normal diet with dried Aloe vera extract

The formulated fish feed was prepared using the normal fish diet (50% crude protein, 18% crude lipid, 1.9% fiber, 1.3% total phosphorus, 8.3% ashes, and 14.8% nitrogen free extract) with dried *Aloe vera* extract or placebo at a ratio 1% of weight food and mixing part by part in a drum mixer. Sufficient water along with the oil ingredients were then added to make a paste of each diet. After it was pelleted and allowed to cool dry. The pellets were air dried and stored in air tight containers until fed.

Fish and experimental conditions

1200 rainbow trout weighing 2 ± 0.2 gram were used. All experiments were carried out in 1,000 liter round concrete ponds with a continuous water flow of 2.5 liter per second. The fish were kept at an ambient, including uncontrolled water temperature of $15\pm 1^\circ\text{C}$, dissolved oxygen of 7.2 ± 0.2 mg l⁻¹ and pH 8 ± 0.3 . After 2 weeks adaptation, fish were randomly allotted in two groups including an experimental group and a control group, in triplicate was maintained in 6 concrete ponds each containing 200 fish. Each group was hand-fed once a day with diet medicated 1% of *Aloe vera* extract, or placebo (70% lactose, 10 % starch and 20 % talc) prepared in the laboratory at a rate 2% of body weight for 8 weeks and three times with normal diet.

Bleeding and serum collection

During bleeding, fish were rapidly netted, tranquillized with 50 mg/l of tricaine methane sulfonate (MS₂₂₂, Sigma chemical Co. St. Louis, MO, USA). Fish were bled from caudal vein by cutting the tail with a scalpel. A total number of 15 blood samples were collected from 15 fish in each group (5 samples from each replicate) at the end of every 2 weeks, 24 h after final feeding period. The blood pooling of 5 fish from each replicate divided into 2 halves. Half collected in serological tubes containing a pinch of lithium heparin powder, shaken gently and kept at 4°C to test hematological parameters. Other half collected in tubes without of anticoagulant and allowed to clot at 4°C for 2hrs to test serological parameters. The clot was spun down at 2000 rpm for 10 min to separate the serum. The serum collected by micropipette and was stored in sterile Eppendorf tubes at -20°C until used for assay.

Hematological assay

Blood sample was analyzed with routine methods adopted in fish hematology (Blaxhall and Daisley, 1973). The total red blood cell counts (RBC $\times 10^6/\mu\text{l}$) were determined in a 1:200 dilution of the blood sample in Hayem's solution and total white blood cell counts (WBC $\times 10^3/\mu\text{l}$) in a 1:20 dilution of the blood sample with a Neubauer hemocytometer. The hematocrit (Hct) and leucocrit percentages were determined in duplicate by using micro hematocrit-heparinized capillary tubes of 75 μl volume and a micro hematocrit centrifuge at 15000 rpm for 5 min (Goldenfarb et al., 1971). The percentages of erythrocyte (hematocrit) and leucocyte (leucocrit) volumes were calculated by overlaying the tubes on a sliding scale hematocrit reader.

The hemoglobin (Hb g/dl) concentrations were determined by the cyanomethaemoglobin method (Valery et al., 1991) using a haemoglobin reagent set (Ziest Chem Diagnostics). The all the values of red blood cell indices, the mean values of cell haemoglobin (MCH pg), cell haemoglobin concentration (MCHC %), and cell haemoglobin volume (MCV fl) were calculated according to Wintrobe formulae (Wintrobe, 1933). The differential leukocytes count was carried out using blood smears stained with Wright-Giemsa. The percentage composition of leukocytes was determined based on their identification characters listed by Ivanava (Ivanava, 1983).

Biochemical assay

Serum total protein content was estimated photo metrically by citrate buffer and bromocresol green (BCG) dye binding method (Shaziya and Goyal, 2012) using the kit (total protein and albumin kit, Pars Azmun Company, Iran). Albumin was determined BCG binding method. The absorbance of standard and test were measured against blank in a spectrophotometer at 546 nm. Globulin level was calculated by subtracting albumin values from total serum protein. Albumin/globulin (A/G) ratio was calculated by dividing albumin values by globulin values.

Immunological assay**Separation of leukocytes from the blood**

Leucocytes for assay were separated from each blood sample by density-gradient centrifugation. One milliliter of histopaque 1.119 (Sigma) containing 100 μl of bacto hemagglutination buffer, pH 7.3 (Difco, USA) was dispensed into siliconised tubes. 1 ml of a mixture of 1.077 density histopaque and hemagglutination buffer and 1 ml of blood was carefully layered on the top. The sample preparations were centrifuged at 2500 rpm for 15 min at 4°C. After centrifugation, plasma was collected and stored at -80°C for future analysis; separated leukocytes were gently removed and dispensed into siliconised tubes, containing phenol red free Hanks Balanced Salt Solution (HBSS, Sigma). Cells were then washed twice in HBSS and adjusted to 2×10^6 viable cells/ml.

Respiratory burst activity

Respiratory burst activity of isolated leukocytes was quantified by reduction of ferricytochrome C (Secombes, 1990). Briefly, 100 μl of leukocyte suspension and an equal volume of cytochrome C (2 mg/l

in phenol red free HBSS) containing phorbol 12-myristate 13-acetate (PMA, Sigma) at 1µg/ml were placed in triplicate in micro titer plates. In order to test specificity, another 100µl of leukocyte suspensions and solutions of cytochrome c containing PMA and superoxide dismutase (SOD, Sigma) at 300 U/ml were prepared in triplicate in micro titer plates. Samples were then mixed and incubated at room temperature for 15 min. Extinctions were measured at 550 nm against a cytochrome C blank in a multiscan spectrophotometer. Readings were converted to nmoles O₂ by subtracting the O.D. of the PMA/SOD treated supernatant from that treated with PMA given alone for each sample, and converting O.D. to n moles O₂ by multiplying by 15.87. Final results were expressed as nano moles O₂ produced per 10⁵ blood leukocytes.

Phagocytosis assay

Phagocytosis activity of blood leukocytes was determined spectrophotometrically according to Seeley *et al.*, (1990). This assay involves the measurement of congo red-stained yeast cells which have been phagocytised by cells. To perform the assay, 250µl of the leukocyte solution was mixed with 500µl of the congo red-stained and autoclaved yeast cell suspension (providing a yeast cell: leukocyte ratio of 40:1). The mixtures were incubated at room temperature for 60 min. Following incubation, 1 ml ice-cold HBSS was added and 1 ml of histopaque (1.077) was injected into the bottom of each sample tube. The samples were centrifuged at 2500 rpm for 5 min to separate leukocytes from free yeast cells. Leukocytes were harvested and washed two times in HBSS. The cells then were resuspended in 1 ml trypsin-EDTA solution (5.0g/l trypsin and 2.0g/l EDTA, Sigma) and incubated at 37°C overnight. The absorbance of the samples was measured at 510 nm using trypsin-EDTA as a blank.

Serum lysozyme assay

In this study, an assay based on the lysis of *Micrococcus lysodeikticus* was used to determining the lysozyme activity. Serum lysozyme activity was measured spectrophotometrically according to the method Parry *et al.*, (1965). Briefly, 0.02% (w/v) lyophilized *Micrococcus lysodeikticus* in 0.05 mM solution phosphate buffer (pH 6.2) was used as substrate. 10µl of fish serum was added to 250µl of bacterial suspension and reduction in absorbance at 490 nm was determined after 0.5 and 4.5 min of incubations at 25°C using a microplate reader. One unit of lysozyme activity was defined as the amount of enzyme causing a decrease in absorbance of 0.001 per min.

Statistical analysis

All results for each parameter measured were expressed as means±standard errors, and were compared at each time point using Student's *t*-test for independent data. Significant differences between experimental groups were expressed at a significance level of P <0.05. All analyses were carried out on 15 fish per group.

RESULTS

Hematological analysis

Dietary *Aloe vera* extract incorporated test diets had no significant (p <0.05) effect on red blood cell count (RBC), white blood cell count (WBC), differential leukocytes count (monocytes, lymphocytes and neutrophils), hematocrit (Hct), hemoglobin (Hb), the all the values of red blood cell indices, the mean values of cell hemoglobin (MCH pg), cell hemoglobin concentration (MCHC %), and cell hemoglobin volume (MCV fl) at the end of none of the identical two weeks after feeding in compared to placebo group (Table 1).

Biochemical analysis

Aloe vera extract had significant (p<0.05) effect in increase of total protein (TP), albumin (AL), and globulin (GL), at the end of the identical every two weeks after feeding in compared to placebo group (Table 2). The maximum level of total protein, albumin and globulin were recorded on week 8 of

exposure duration. However, albumin/globulin ratio was not exhibited significant differences in compared to placebo group at the end of the identical every two weeks after feeding in compared to placebo group ($p>0.05$; Table 2).

Immunological analysis

Respiratory burst activity

The respiratory burst activity significantly ($p<0.05$) enhanced in fish fed with 1% of *Aloe vera* extract supplementation feed at the end of the identical every two weeks after feeding in compared to placebo group (Fig. 1). The maximum activity was observed on week 2 of exposure duration.

Phagocytic activity

Phagocytic activity of blood leucocytes significantly ($p<0.05$) enhanced in fish treated with 1% of *Aloe vera* extract supplementation feed at the end of the identical every two weeks after feeding in compared to placebo group (Fig. 2). However, there was significant difference only on week 2 of exposure duration in compared two groups.

Lysozyme activity

Lysozyme activity significantly ($p<0.05$) enhanced in fish fed with 1% of *Aloe vera* extract supplementation feed at the end of the identical every two weeks after feeding in compared to placebo group on weeks 2, 4 and 6 of exposure duration (Fig. 3). The maximum activity was recorded on 6 week of exposure duration. Although, lysozyme activity on week 8 was recorded higher in AE group in compared to placebo group, but there was no significant difference.

DISCUSSION

Dietary medicinal plant extracts as immunostimulants elevate non-specific defenses against pathogens during period of stress. Hematological assay may provide an index of the physiology status of fish. This study indicates the effects of dried *Aloe vera* extract on the hematological parameters and immunological responses in rainbow trout (*Oncorhynchus mykiss*). In the present study, the hematological parameters such as RBC, hemoglobin, hematocrit, and the values of red blood cell indices including MCH, MCHC and MCV were no significant differences at the end of identical two weeks period after feeding when compared to placebo group. These results are in consistent with the results obtained of Alisha *et al.*, who reported common carp treated with dietary *Aloe vera* supplementation were no significant differences in RBC and Hct (Alishahi *et al.*, 2010), or RBC and Hb (Farahi *et al.*, 2012) in rainbow trout. Also, in our study, there were no significant differences in WBC and differential leukocytes counts between experimental and control groups. In the present study, in dietary supplemented *Aloe vera* extract group enhanced total plasma protein, albumin and globulin values in comparison with placebo group. Similar results were reported in rainbow trout fed with garlic (Nya, 2009), ginger (Nya and Austin, 2009_a), lipopolysaccharide (Nya and Austin, 2009_b), *Laurus nobilis* (Bilen and Bulut, 2010), and *Coggyria coggyria* (Bilen *et al.*, 2011). Serum proteins are various humoral elements of the non-specific immune system, measurable total protein, albumin and globulin levels suggest that high concentrations are likely to be a result of the enhancement of the non-specific immune response of fish. So, this study revealed that *Aloe vera* extracts incorporated diets helped to increase the humoral elements in the serum. Globulin is the main resource of immunoglobulin production, thus its enhancement in serum provide immunostimulatory potential. Albumin globulin ratio does not indicate significantly differences in treated group in compare to control group. Similar result was reported in *Cyprinus carpio* fingerlings of treated levamisole (Maqsood *et al.*, 2009). Also, the results obtained of this study indicated an enhancement in respiratory burst activity in treated group in comparison with placebo group, which are in agreement the results of some of studies with dietary immunostimulants used in various fish species (Yin

et al., 2009; Bilen and Bulut, 2010). Respiratory burst activity is considered as an important indicator of non-specific defense in fish, which is a measure of the increase of oxidation level in phagocytes stimulated by foreign agents (Liaghat et al., 2011). Respiratory burst and phagocytosis response by phagocytes in blood present a major antibacterial defense mechanism in fish (Harikrishnan et al., 2010). The main cells involved in phagocytosis in fish are neutrophils and macrophages. These cells remove bacteria mainly by the production of reactive oxygen species (ROS) during a respiratory burst. In addition, neutrophils possess myeloperoxidase in their cytoplasmic granules, which in the presence of halide and hydrogen peroxidase kills bacteria by halogenations of the bacterial cell wall. Moreover, these cells possess lysozymes and other hydrolytic enzymes in their lysosomes (Uribe et al., 2011). Similarly, macrophages can produce nitric oxide in mammals and can be as potent antibacterial agents, peroxy nitrates and hydroxyl groups (Secombes, 1996). The phagocytic activity of the blood leucocytes in *Aloe vera* extract-treated group was significantly higher than the placebo group. This result supports enhanced phagocytic activity of leucocytes in rainbow trout by other medicinal herbs (Düğenci et al., 2003; Haghighi and Sharif Rohani, 2013). Also, in this study, an increasing trend in lysozyme activity has been shown which is in agreement with several reports indicating the role of herbal immunostimulants in enhancing lysozyme activity (Rao et al., 2006; Choi et al., 2008). Lysozyme is a humoral component of the non-specific defense mechanism which has the ability to prevent the growth of bacteria by splitting β -1, 4 glycosidic bonds in the peptidoglycan of bacterial cell walls. In conclusion, supplementation of AE in aquaculture diets would be use to enhance non-specific immune system in fish. Therefore, further studies are necessary for effective use of *Aloe vera* extract with optimal dose, suitable duration, and method of administration.

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Table 1: The haematological parameters, WBC, RBC, Hct, Hb, MCH, MCV, MCHC, neutrophil, monocyte, and lymphocyte of rainbow trout fed with 1% placebo (PL) or *A. vera* extract, (AE) in feed for 8 weeks.

Week	Groups	WBC(10^3 /mL)	RBC(10^6 /mL)	Hct(%)	Hb(g/dL)	MCH(pg)	MCV(fL)	MCHC(%)	Neut(%)	Mon(%)	Lymp(%)
2	PL	2.20±0.7	0.87±.06	26.00±3.3	8.90±0.6	102.3±2.1	298.8±12	3.42±0.3	12.33±0.6	1.66±0.7	86.0±1.1
	AE	2.65±0.3	0.94±.06	27.33±1.5	9.20±0.7	97.87±1.1	290.7±12	3.36±0.6	13.00±0.3	2.00±0.2	85.0±0.1
4	PL	2.43±0.3	0.91±.06	28.00±5.2	9.46±1.6	103.9±5.6	307.6±9	3.37±0.8	13.00±0.3	1.66±0.3	85.3±0.3
	AE	3.01±0.7	1.05±.06	30.00±5.2	10.76±1.3	102.4±7.3	285.7±14	3.20±0.9	12.00±0.5	2.33±0.3	86.0±0.9
6	PL	2.95±0.2	1.23±.06	25.00±3.7	8.46±1.5	68.78±4	203.2±5	3.38±0.4	11.00±0.6	2.33±0.5	86.6±1.9
	AE	3.56±0.1	1.20±.04	26.00±3.3	8.63±1.6	71.91±1.6	216.6±6	3.27±0.3	11.33±0.3	3.00±0.2	85.7±1.6
8	PL	3.40±0.3	1.35±.03	29.00±3.3	9.63±1.9	71.33±4.2	214.8±9	3.32±0.6	11.66±0.6	2.00±0.1	86.4±1.2
	AE	3.60±0.2	1.46±.05	30.50±2.5	9.93±1.6	68.01±3.6	208.9±14	3.25±0.5	12.66±0.2	2.33±0.3	85.0±0.5

Data are expressed as mean±SE (n=15). No significant differences were observed in the *A. vera* treated groups relative to the placebo group at the end of the identical every two weeks after feeding ($P>0.05$). Neut: neutrophil; Mon: Monocyte; Lymp: Lymphocyte.

Table 2: Changes in the serum total protein, albumin, globulin and albumin/globulin ratio of rainbow trout after feeding with 1% placebo, or *A. vera* extract for 8 weeks.

Week	Groups	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Albumin/globulin ratio (g/dL)
2	Placebo	3.26±0.03	1.31±0.01	1.95 ±0.02	0.67±0.01
	Aloe vera extract	3.58±0.03*	1.44±0.03*	2.14±0.01*	0.67±0.02
4	Placebo	3.59±0.05	1.49±0.04	2.10 ±0.02	0.70±0.04
	Aloe vera extract	3.73±0.05*	1.59±0.02*	2.14±0.03*	0.74±0.02
6	Placebo	3.87±0.09	1.53±0.02	2.34 ±0.03	0.65±0.03
	Aloe vera extract	4.28±0.07*	1.63±0.04*	2.65 ±0.04*	0.61±0.02
8	Placebo	3.88±0.05	1.60±0.05	2.28 ±0.04	0.70±0.03
	Aloe vera extract	4.82±0.02*	1.86±0.06*	2.96 ±0.02*	0.62±0.03

Data are expressed as mean±SE (n=15). *: $P<0.05$ compared with the placebo at the end of identical every two weeks.

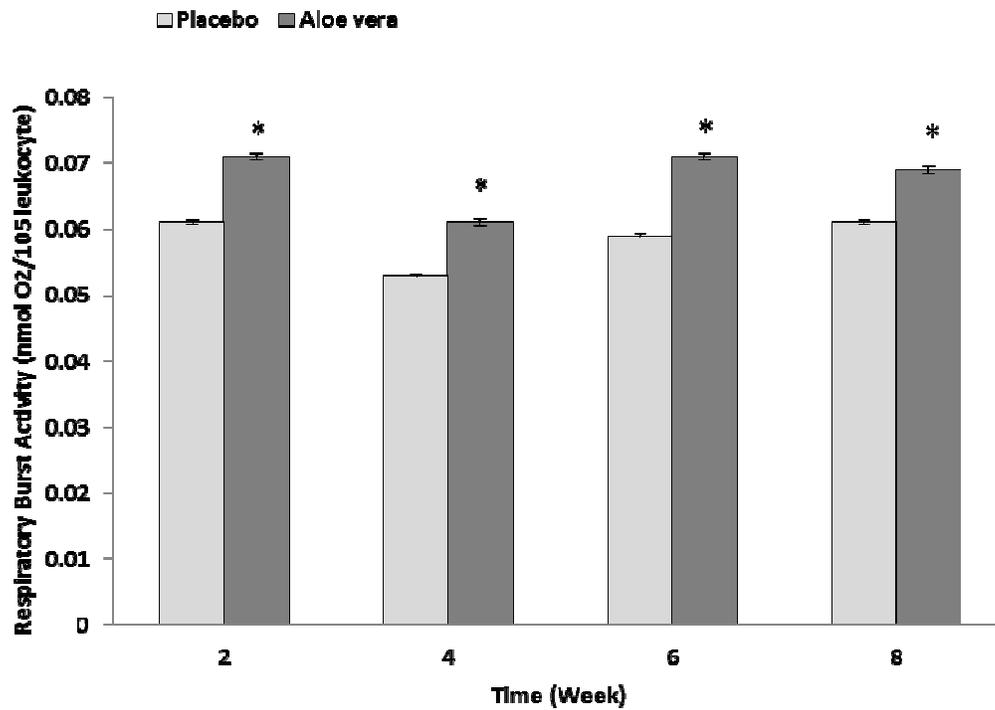


Figure 1 Respiratory burst activity of different experimental groups observed on different weeks.

Data are expressed as mean±SE (n=15). Asterisks indicate significantly different from placebo in the same week. * $P < 0.05$.

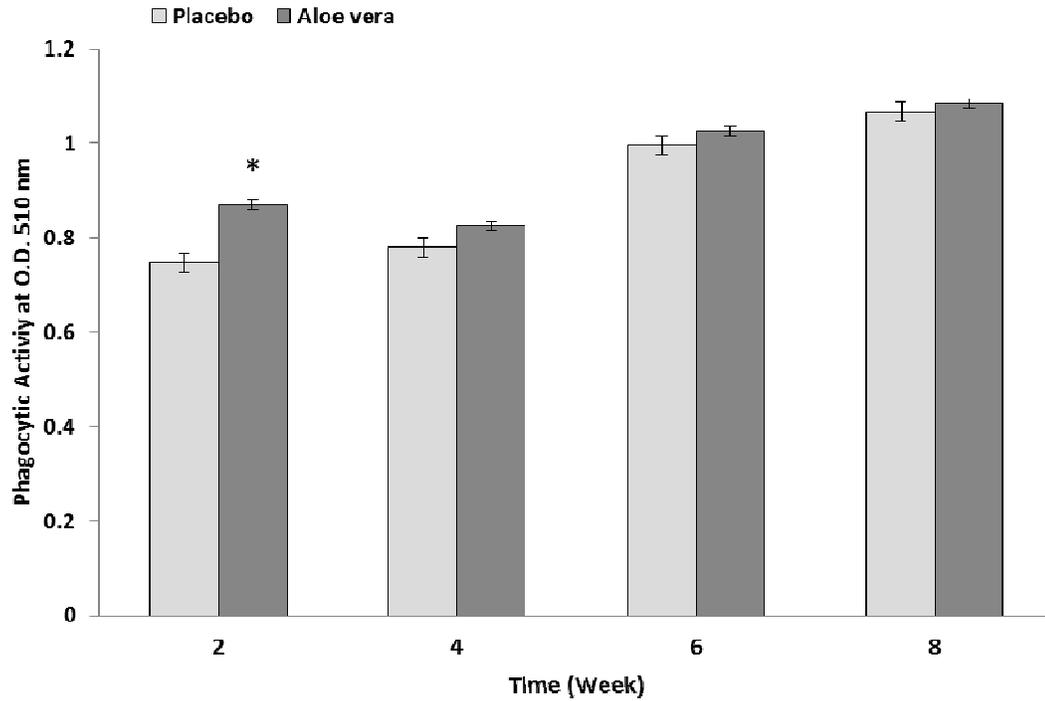


Figure 2 Phagocytic activity of different experimental groups observed on different weeks.

Data are expressed as mean±SE (n=15). Asterisk indicates significantly different from placebo in the same week. * $P < 0.05$.

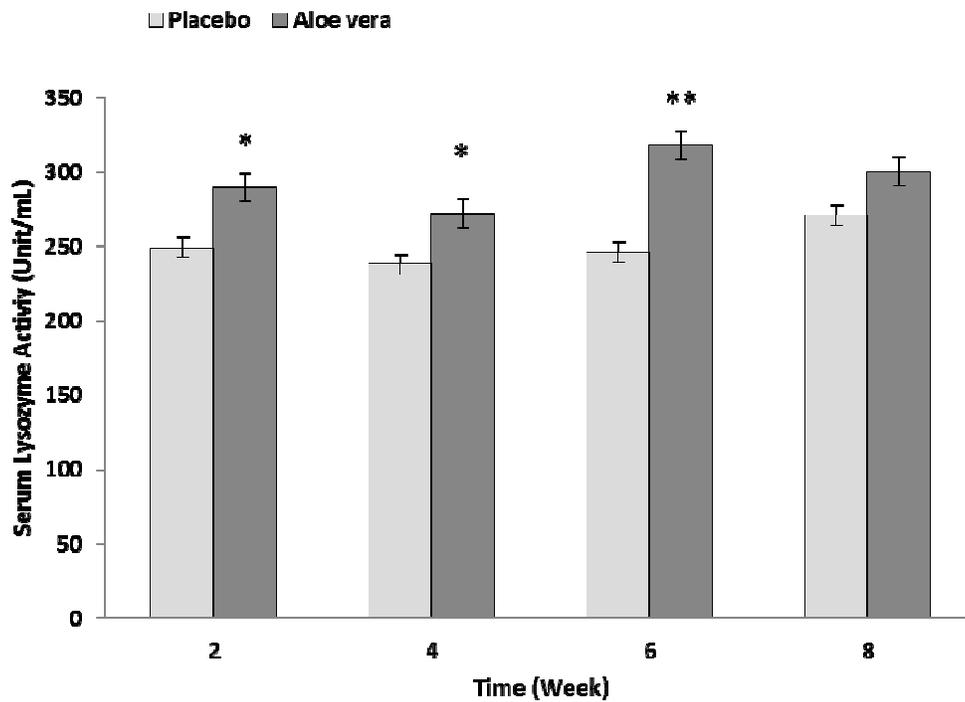


Figure 3 Serum lysozyme activity of different experimental groups observed on different weeks.

Data are expressed as mean \pm SE (n=15). Asterisks indicate significantly different from placebo in the same week. * $P < 0.05$; ** $P < 0.001$.