IMMUNOGENIC MODULATIONS INDUCED BY PROSPECTIVE ANTIMALARIAL HERBAL EXTRACTS IN MURINE MODEL

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ABSTRACT

Keeping in view the ever increasing problem of drug resistance and affordability of the antimalarial drugs by the poor mass, herbal medicines can become an important and alternative sustainable strategy for malaria treatment. Aqueous extracts of three Himalayan herbs *Equisetum arvense, Artemisia vulgaris* and *Centella asiatica*, with reported antimalarial property were screened for clinical efficacy against a local strain of *Plasmodium vivax* antigen in murine model. *E. arvense* extract was consistent in boosting phagocytic activity, nitric oxide generation, acid phosphatase and alkaline phosphatase activities in the peritoneal macrophages. The effectiveness of the rest herbals was discrete. A need for further detailed investigation to evaluate the clinical efficacy of these herbals seems essential.

KEYWORDS

Antimalarial herb; phagocytic index; nitric oxide; acid phosphatase; alkaline phosphatase.

INTRODUCTION

In the tropical countries of the world, malaria continues to be one of the leading threats to the human life (Snow *et al.,* 2005). This challenging scenario necessitates development of an alternative approach to cope with this dynamic disease. Although Indian biodiversity supports a huge medicinal floral resource, the clinical efficacies of most such herbals are not yet established through scientific screening. In this present study, three common Darjeeling Himalayan herbs having ethnobotanical reports of antimalarial property were tested. The herbals were screened in preventive as well as curative approach to evaluate their exact utilitarian benefits.

Phagocytosis is a primitive immune effector mechanism and phagocytic clearance of *Plasmodium* laden HRBC is an important line of innate defense mechanism against malaria (Stevenson and Riley, 2004). And efficient phagocytic clearence of *P. falciparum* infected HRBC by murine macrophage is in report (Kodjo *et al.,* 2005). During the formation of L-citruline from L-arginine by nitric oxide synthase, nitric oxide (NO) is produced as a reactive nitrogen intermediate (Ischiropoulos et al., 1992). NO is considered as a dependable innate immune molecule as it has the ability to kill pathogens itself or by combining with superoxide (O_2^-) to form peroxynitrite, a strong bactericidal agent (Bogdan, 2001).

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Acid phosphatase (AP) and alkaline phosphatase (ALP) are important hydrolytic lysosomal enzymes that plays critical role in maintaining cellular homeostasis and cellular immunity. It is reported that on the brush bordered cell membrane, AP inhibits the membrane attached NADPH oxidase activity and thereby suppresses oxidative burst (generation of H_2O_2 and O_2^-) by the immune cells (Glew et al., 1988). AP is considered as a major hydrolytic enzyme that acts in phagocytic vesicles to degrade endocytosed particles. Besides, AP plays an important role in the detoxification process of toxic compounds entering the body (Zheng et al., 2007). On the other hand, ALP has often been implicated in phosphorylative transfer of extracellular molecules against concentration gradients at cell membranes (Monin and Rangneker, 1974). ALP being an enzyme supposedly involved in transfer of extracellular substances, it is probable that this enzyme was either utilized as extracellular hydrolytic enzyme or are involved in secretion of other hydrolytic enzymes at the cell membrane.

The aqueous extracts of the herbals were examined for their possible role in increasing phagocytic activity of the murine peritoneal macrophages. They were also screened for probing their ability to stimulate NO generation, AP and ALP activities in the murine peritoneal macrophages so as to evaluate the possible immunogenic role of the herbals against *P. vivax* antigenic insult.

MATERIALS AND METHODS

Preparation of plant extracts

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The three annual herbs *Equiserum arvense* (Family: Equisetaceae), *Artemisia vulgaris* (Family: Asteraceae) and *Centella asiatica* (Family: Apiaceae) were tested. Fresh green leaves of all the three plants were collected, dried under shade and pulverized into powder. The pulverized powder forms of the plant tissue (5 gm each) were stirred separately in distilled water (100ml) overnight at room temperature and the final aqueous extracts were prepared by Soxhlet extraction (Bhat and Surolia, 2001), evaporated to dryness and stored in dry, airtight, sterilized glass vials.

Parasite collection and extraction

Blood samples infected with healthy asexual stage of *P. vivax* were collected from patients infected with a local (South 24-Pargana, West Bengal) strain. The donors had no earlier history of malaria infection and had not taken any antimalarial drug. The blood samples were carried to the laboratory maintained at 4^0C. The degree of parasitamia was accounted by microscopic inspection of Giemsa-stained thin blood smears. The parasite laden cells were resuspended in chilled phosphate buffer saline (PBS), pH 7.2, and washed thrice by centrifugation. The parasites were extracted from the infected cells employing tonic stress by suspending them in chilled distilled water for 30 min. The extracted parasites were collected from the buffy coat by repeated washing and centrifugation of the ruptured cell aliquots. The final parasite pellets were resuspended in 1 ml PBS aliquots. The work was designed in accordance with the guidelines of the institutional (University of Calcutta) norms of animal handling and care.

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Animal model and dose

Swiss albino mice (average body weight 35 gm; identical age group of 3 months belonging to female sex) were chosen for the experiments. The median lethal dose (LD_{50}) values of all the three herbals were determined after Karber (Turner, 1965). (Table I) and a dose of 125mg/ kg body weight was considered for administration; as for the parasites, a uniform dose of 3 ml/kg body weight was administered. The doses were formulated to evaluate the preventive as well as curative efficacy of the herbals. For preventive strategy, separate batches of mice were injected intraperitoneally with the three herbals thrice with an interval of 3 days between two doses; thereafter the same animals were injected with parasite extracts intravenously with identical time intervals of dose administration. For the curative approach, sets of Swiss albino mice were first injected intravenously with the parasite extracts thrice within same time intervals followed by thrice intraperitoneal herbal injection. During treatment the mice were fed with standard pellets and water *ad libitum* and were kept in 12h day-night cycle.

Collection of peritoneal macrophage

All the cytological preparations were done under sterilized conditions. Peritoneal macrophages were collected from the sacrificed mice and washed in chilled PBS, pH 7.2 supplemented with 0.01M EDTA and 1% glucose by centrifugation. The macrophages were resuspended to 10^6 cells/ml in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg /ml streptomycin.

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Culture of yeast

Baker's yeast (*Saccharomyces cerivisiae*, West Mill Foods, Maidenhead, Berks, UK) cells were killed by boiling for 1 hour and the resulting cell suspension was washed thrice in pH 7.4 TBS/Ca^{2+} (20mM Trizma base, 77 mM NaCl, 10 mM $CaCl_2$) by centrifugation at 650 x g for 10 min. The washed cells were then resuspended at a concentration of 10^7cells/ ml in Grace's Insect Medium (GIM; Himedia).

Preparation of cell lysate

Macrophages suspended in DMEM supplemented with 10% fetal calf serum at a density of 10^6 cells/ml were centrifuged and the pellet was collected. The cell pellet was lysed with 0.1% TritonX-100 solution under ice for 30 min; the aliquots were centrifuged (2000 x g) and the supernatants were collected.

Phagocytic index analysis

The phagocytic efficiency of the peritoneal macrophages was examined by challenging the murine peritoneal macrophages with yeast suspension *in vitro* over slide. To the adherent monolayer of macrophages, 0.1 l of yeast (1 x 10^7cells/ ml) were added and incubated at 37^0C in a humid chamber for 2 h. After incubation, the monolayer was washed with PBS (pH 7.2), stained with Giemsa's stain and observed under microscope (Olympus, BH2). Not less than 200 fields were examined for each slide and following data were accounted: number of phagocytic macrophage, total number macrophage and number of yeast particles engulfed by each macrophage. The data were calculated and represented

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in terms of phagocytic index (PI) (Elssner *et al.*, 2004), where, PI= (particles/cell x phagocytic cells /100). The experiment was repeated thrice.

Estimation of NO activity

The activity of NO was measured as the amount of the nitrite released from the macrophages of the mouse using Griess reagent after the modified protocol of Green *et al.*, (1982). Macrophage and liver tissue lysates were incubated with equal volume of Griess reagent (1% Sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% orthophosphoric acid) at 37^0C for 30 min in a humid chamber. The absorbance was recorded in a colorimeter (Erma Inc., AE-11M, Tokyo) at 530nm against a standard blank. The activity of NO was determined using a standard curve of sodium nitrite. The experiment was repeated thrice.

Estimation of AP activity

AP (EC 3.1.3.2) activity of the cell lysates was estimated after Michell *et al.* (1970) and the corresponding protein content was estimated after Lowry et al. (1951). The enzyme acted on 5mM p-nitrophenol phosphate in 50 mM sodium acetate buffer (pH 5.0) for 30 min at 37^0C in a humid chamber. The reaction was halted with 0.1(N) sodium hydroxide (NaOH) for 30 min and the absorbance was measured at 420nm in a colorimeter (Erma Inc., AE-11M, Tokyo). The entire experiment was repeated thrice. The acid phosphatase activity was measured using a standard curve of p-nitrophenol.

Estimation of ALP activity

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ALP (EC 3.1.3.1) activity of the experimental lysates was estimated after Michell *et al.* (1970) and the corresponding protein content was estimated after Lowry et al. (1951). The enzyme reacted with 5mM PNPP in 50mM Glycine buffer (pH 10.0) and 10 mM $MgCl_2$ for 30 min at 37^0C in a humid chamber. The reaction was stopped with 0.02 (N) NaOH for 30 min and the absorbance was measured at 420nm in a colorimeter (Erma Inc., AE-11M, Tokyo). The enzyme activity was measured using a standard curve of p-nitrophenol. The entire experiment was repeated thrice. The enzyme activity was measured using a standard curve of p-nitrophenol.

Estimation of protein

The estimation of the protein content of all the samples were done after the method of Lowry *et al.* (1951) using a standard curve of bovin serum albumin.

Statistical analysis of data

Statistical data analysis was carried out using Student's t-test. Differences were considered significant at $P < 0.05$, $P < 0.01$, $P < 0.001$. Data was presented as the mean ± standard error (S.E).

RESULTS AND DISCUSSION

In the present study, all the three herbals exhibited the ability to enhance the phagocytic activity of the macrophage in both preventive and curative strategies. Particularly in case of preventive strategy, *E. arvense* and *A. vulgaris* extracts exhibited high potency in enhancing phagocytic efficiency of the murine macrophage (Fig 2.). Thus

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probability of phagocytic clearance of parasite laden HRBC by the herbal induced macrophage *in vivo*, seems quite promising.

Fig 1 to be inserted here

Fig 2 to be inserted here

In the past two decades, NO mediated pathogen killing and superoxide anion remediation has earned recognition as an effective immune strategy in the animal world (Bogdan, 2001). Malaria is associated with increased number of macrophages in the bone marrow liver and spleen (Wernsdorfer and McGregor, 1988). While NO itself might not be inhibitory for the development of the parasites, its downstream products possibly generated by the macrophages do have antiplasmodial activity (Helmby and Troye-Blomberg, 2000). Here, the NO-activity of murine peritoneal was vitalized under induction of the parasite extract with respect to the control. The NO-activity attained optimum intensity in the animal sensitized with *E. arvense* extract both in preventive as well as curative approaches. The other herbals seem not to have any notable effect in inducing NO-activity (Fig 3). Herbal extracts of *E. arvense* thus exhibits a high potential to stimulate NO mediated immunity in the host thereby aiding to combat oxidative stress and scavenge pathogenic invasion. Although not very consistent, the other herbal extracts showed moderate or discrete power to stimulate NO activity in the murine immune system.

Fig 3 to be inserted here

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Phosphatases are enzymes, which catalyze the liberation of orthophosphate from complex organic phosphorus compounds and an organic moiety and are thus believed to have an essential function in the nutrient dynamics. The herbal extracts of *C. asiatica* and *E. arvense* boosted the AP activity maximally in the macrophages of the mice in the preventive strategy (Fig 4). In the curative approach, all the herbal extracts were found to be effective in stimulating the enzyme activity. While *C. asiatica* and *A. vulgaris* seem to stimulate this enzyme activity maximally in the preventive approach, *E. arvense* extract did the same in the curative view.

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E. arvense and *C. asiatica* enhanced the ALP activity optimally in the preventive approach *A. vulgaris* extract had identical effects in the curative strategy (Fig 5). In the whole study, the aqueous extracts of *E. arvense* had a positive induction in stimulating the examined immune parameters and thus demands more detailed investigation to validate its prospect as an antimalarial prescribe.

Acknowledgement:

We acknowledge the kind help provided by Mr. Pradyumna Patra, Mr. Anjan Chowdhury and Mr. Kalyan Naskar at Malaria B/S Collection Laboratory, Canning S/D Hospital, South 24-Parganas, West Bengal, India.

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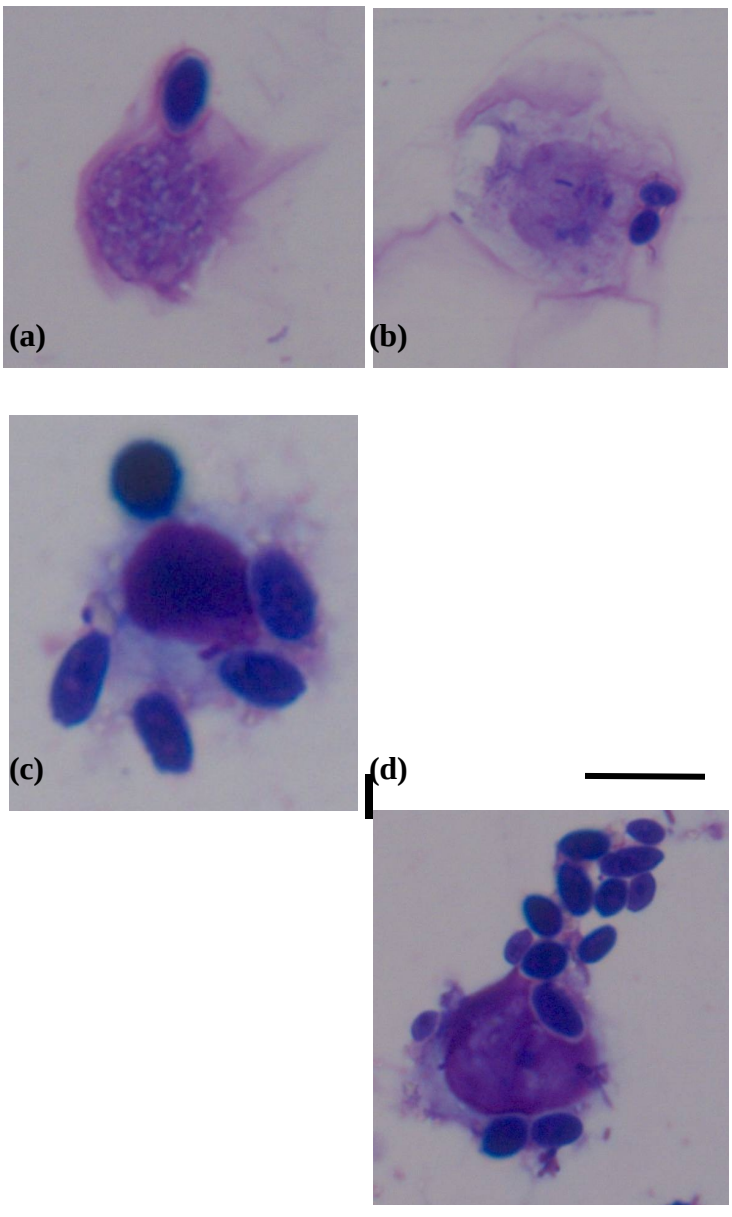

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Fig 1. Phagocytic murine macrophahes engulfing yeast particles in (a) control (b) *P. vivax* sensitized (c) *E. arvensi* sensitized (d) *C. asitica* sensitized mice. (Magnification: 1000 x; Scale: 10µm)

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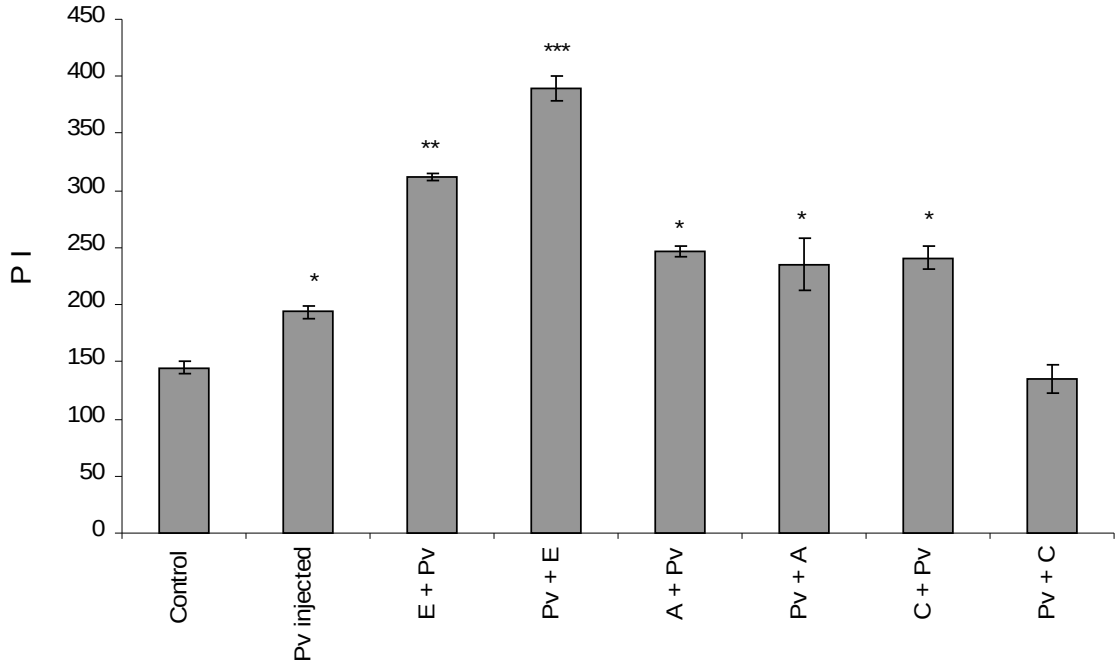

Fig 2. Phagocytic index (PI) of peritoneal macrophage from mice treated with *P. vivax* (Pv) antigen and herbals *E. arvense* (E), *A. vulgaris* (A) *and C. asiatica* (C) in preventive and curative approaches, challenged with yeast particles. Data presented as mean ± S.E. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

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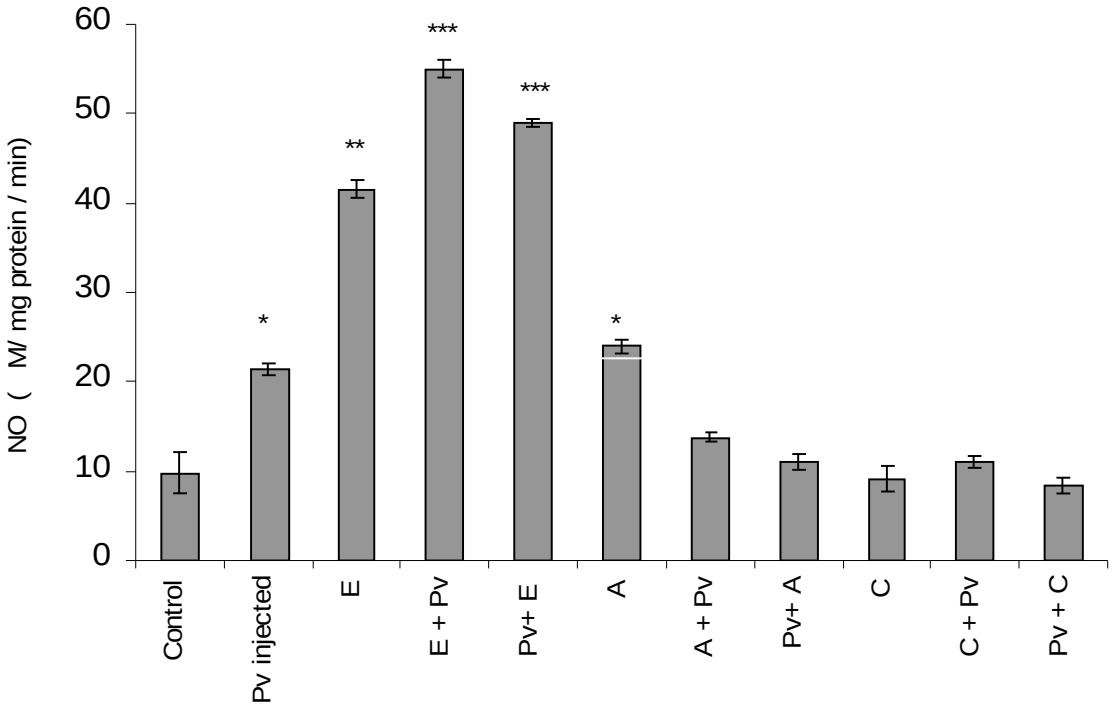

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286Fig 3. Nitric oxide (NO) activity of peritoneal macrophage from mice treated with *P. vivax*

287(Pv) antigen and herbals *E. arvense* (E), *A. vulgaris* (A) *and C. asiatica* (C) in preventive

288and curative approaches. Data presented as mean ± S.E. *$P < 0.05$, **$P < 0.01$, ***$P <$

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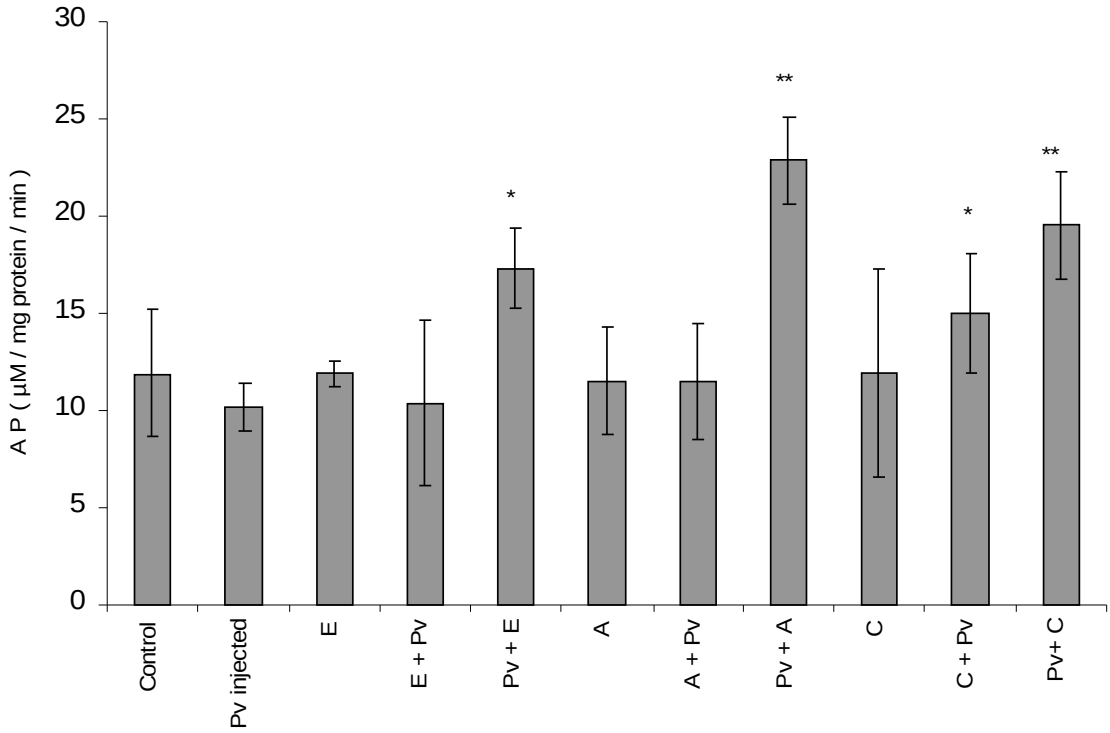

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291 Fig 4. Acid phosphatase (AP) activity in macrophage of mice treated with *P. vivax* (Pv)

292 antigen and herbals *E. arvense* (E), *A. vulgaris* (A) *and C. asiatica* (C) in preventive and

293 curative approaches. Data presented as mean ± S.E. *$P < 0.05$, **$P < 0.01$.

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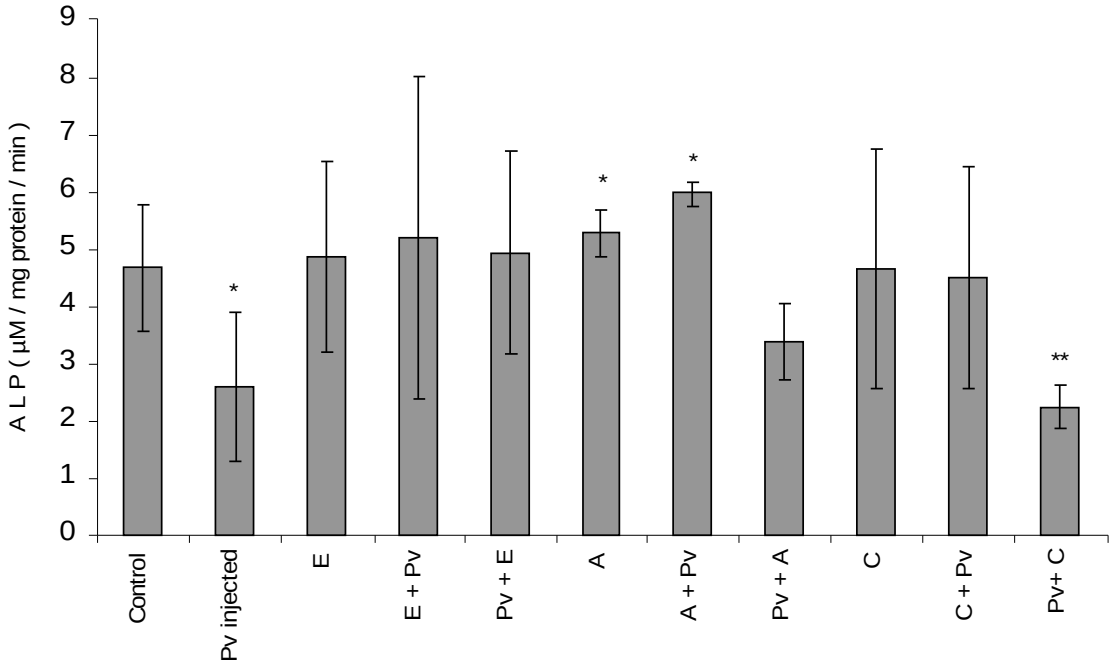

Fig 5. Alkaline phosphatase (ALP) activity in macrophage of mice treated with *P. vivax* (Pv) antigen and herbals *E. arvense* (E), *A. vulgaris* (A) *and C. asiatica* (C) in preventive and curative approaches. Data presented as mean ± S.E. **P* < 0.05, ***P* < 0.01.

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313 Table1. Median effect concentrations (LD_{50}) values mice against herbal extracts

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Extracts of herbs	***LD_{50} (mg /Kg body weight/ 15 days)***
Equiserum arvense	4325.75 ± 5.078
Artemisia vulgaris	3317.61 ± 6.426
Centella asiatica	4217.79 ± 7.403

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