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Immunomodulatory Activities of *Dendrophthoe falcata* (L.f) Ettingsh in Experimental Animals: *In vitro* and *In vivo* Investigations

S. P. Pattanayak*and P. M. Mazumder

Division of Pharmacology, Birla Institute of Technology (BIT), Ranchi 835215, India

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Abstract

In the present study, an attempt was made to screen immunomodulatory activity of the hydroalcoholic extract (HEDF) of *Dendrophthoe falcata* (L.f.) Ettingsh (Loranthaceae), an Indian Ayurvedic plant, on different arms of the immune system. HEDF was evaluated for immunological function by studying delayed type hypersensitivity (DTH) to sheep RBCs, nitric oxide (NO) release from murine peritoneal macrophages, phagocytic activity of polymorphonuclear (PMN) cells *in vitro* and reticuloendothelial system *in vivo*, plaque forming cell response of splenic lymphocytes to sheep erythrocytes, haemagglutination antibody titer and neutrophil adhesion test. Significant increase in NO production by mouse peritoneal macrophages was detected in culture supernatants indicated increased phagocytic activity of macrophages. After post oral administration of HEDF in three doses of 250, 475 and 950 mg/kg body weight, a significant increase in phagocytic activity of PMN cells/reticuloendothelial system, stimulation of neutrophil function and splenic antibody secreting cells, were also noticed. Stimulation of humoral immune response was further observed with elevation in haemagglutination antibody titer. Heightened DTH reaction suggested convincing evidence for activation of cellular immune system. Present study thus confirms the immunomodulatory activity of the hydroalcoholic extract of *D. falcata* and the immunomodulatory responses were found to be dose dependent manner.

Keywords: Dendrophthoe falcata; Antibody titer; Neutrophil adhesion; Phagocytic activity.

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1. Introduction

The modulation of immune response with the aid of various medicinal plants in order to alleviate certain diseases is an active area of interest. Immunomodulation using medicinal plants can provide an alternative to conventional chemotherapy for a variety of diseases, especially when the host defence mechanism has to be activated under the conditions of impaired immune response. The use of plant products as immunomodulators is still in a developing stage. There are several herbs used in the indigenous systems of medicine that may modulate the body's immune system. A verity of plant-derived materials such as

* *Corresponding author:* sppattanayak@bitmesra.ac.in

lectins, peptides, phenolics, flavonoids, polysaccharides, tannins and saponins have been reported to modulate the immune system [1 – 3].

Dendrophthoe falcata (L.f) Ettingsh (Loranthaceae), commonly known as 'Banda' (Hindi) is an evergreen shrub with bark smooth grey, leaves opposite unequal, thick 1.6 - 25.4 cm long, flowers single, orange-red or scarlet softly pubescent, berries soft ovoid-oblong, 1.3 cm diameter and indigenous to India, Sri Lanka, Thailand, Indo-china, Australia. The aerial parts are used in wounds, menstrual troubles, asthma, psychic disorders, and pulmonary tuberculosis by the tribal of India. Leaf paste is used in skin diseases. Its paste is applied on boils, setting dislocated bones and extracting pus. The plant has been scientifically proved to have anti-lithic, diuretic, wound healing, antimicrobial, *in vitro* and *in vivo* antioxidant activities [4 – 6]. Administration of the extract, BALB/c mice was found to increase the total number of WBC and polymorphonuclear leukocytes [4]. Recently our research group reported the effects of *D. falcata* in lowering the incidence of breast tumor growth in the experimental female Wistar rats [7, 8]. The plant is also proved to have antifertility efficacy in Wistar female rats [9]. The plant *D. falcata* was also reported to possess *in vitro* cytotoxic activity of on human breast adenocarcinoma cells (MCF 7) and *in vivo* antitumor activity against ehrlich ascites carcinoma in swiss albino mice [10, 11]. The merit of the traditional use of *D. falcata* has also been reported by the isolation and identification of several possible active chemical constituents such as β-sitostirol, stigmasterol, kaempferol, quercetin–3-O-rhamnoside, rutin, quercetin, myricetin and their glycosides, (+)-catechin, leucocyanidin, gallic acid, chebulinic acid and some pentacyclic triterpenes, kaempferol-3-O-α-L-rhamnopyranoside and quercetin-3-O-α-L-rhamnopyranoside, etc.[4]. In view of the various claims about the efficacy of the plant, the present experimental study was designed to evaluate the effect of hydroalcoholic extract of *D. falcata* on different arms of the immune system.

2. Materials and Methods

2.1. *Plant material and extract preparation*

Fresh aerial parts of *D. falcata* growing on the host plant *Azadirachta indica* were collected in the month of March from the thick forest areas of Similipal biosphere reserve, Mayurbhanj district of Orissa, India. *D. falcata*(L.f)Ettingsh (Loranthaceae) was authenticated from Botanical Survey of India, Hawrah, West Bengal, India (vide access no. CNH/I-I/32/2010/Tech.II/237-2). One set of the herbarium has been preserved in our laboratory for future reference. The aerial parts were air-dried, pulverized to a coarse powder in a mechanical grinder, passed through a 40 mesh sieve and extracted in a soxhlet extractor with ethanol-water (8:2). The extract was decanted, filtered with Whatman No. 1 filter paper and concentrated at reduced pressure below 40 ^{0}C through rota vapour to obtain dry extract (20.6% w/w). Hydroalcoholic extract from the aerial parts of *D. falcata* (HEDF) was kept at 4^{0}C for further use. The dried HEDF was dissolved in phosphate buffer saline (pH 7.4) and filtered through 0.22µm membrane filter (Millipore, USA).

2.2. *Phytochemical screening*

An attempt was also made to observe the presence and absence of different phytochemical constituents in the hydroalcoholic extract. The tests for flavonoids, sterols and tannins were carried out by using the methods previously described by Tona *et al*. [12]. Tests were also carried out for the identification of terpenes, alkaloids, glycosides, fixed oil, proteins, carbohydrates and polysaccharides in HEDF, following the standard methods [13, 14].

2.3. *Experimental animals*

Swiss albino female mice (20 – 30g) were selected for the experiment. The experimental animals were used after acclimatization to the laboratory environment for a 7-day period. They were kept in the departmental animal house at 26±2 ^{0}C at relative humidity 44–55% and light dark cycles of 10 and 14 hr, respectively. Animals were provided with rodent diet and water *ad-libitum*. The animal experiment was performed according to the institute's ethical committee approval and guidelines Reg no. 621/02/ac/CPCSEA of Birla Institute of Technology, Mesra, India under the proposal approval no. BIT/PH/IAEC/05/2008.

2.4. *Dosing of animals*

From the reported acute toxicity data, the LD_{50} (oral) for the hydroalcoholic extract of *D. falcata* was found to be 4.55g/kg [9]. Three groups of animals each consisting of six mice received HEDF intragastrically in doses of 250 mg/kg low dose (~ 0.05 × LD_{50} oral), 475 mg/kg medium dose (~ 0.1 × LD_{50} oral) and 950 mg/kg high dose (~ 0.2 × LD_{50} oral) [9] for different pre-treatment periods. The control group was treated with equal volume of phosphate buffer saline (pH 7.4); where as native group was devoid of any treatment. At the end of the pre-treatment phase, the animals were subjected to immunological screening using the following experimental models: delayed type hypersensitivity (DTH) to sheep RBCs, nitric oxide (NO) release from murine peritoneal macrophages, phagocytic activity of PMN cells *in vitro* and reticuloendothelial system *in vivo*, plaque forming cell response of splenic lymphocytes to sheep erythrocytes, haemagglutination antibody titer and neutrophil adhesion test in mice. No mortality or any toxic effects were observed in the above mentioned doses of HEDF during the treatment period.

2.5. *Delayed type hypersensitivity reaction*

To determine the effect of the drugs on cell-mediated immunity, the delayed hypersensitivity to sheep RBC was assessed in mice by following the method reported by Ray *et al*. [15] and Liew [16]. Animals were sensitised with 10% sheep RBC (1 × 10 ^{8} cells) at day 0 and day 7 subcutaneously (s.c.). These animals were divided into four

groups: Group I (control) was administered with PBS; Group II – IV were treated with 250, 475, 950 mg/kg HEDF (p.o.) on days - 4, - 2, 0, 2, 4, 6, 8. On day 9, all groups were challenged with 1×10^8 sheep RBCs, intradermally into the left footpad of each mouse, while PBS was injected into right hind paw. The increase in footpad thickness was measured at 24, 48, 72 and 96h after sheep RBC challenge and expressed as mean percent increase in paw volume (plethysmometrically). The percentage increase in the paw volume was taken as a measure of the delayed hypersensitivity to sheep RBC.

2.6. *Isolation of peritoneal macrophage and culture conditions*

Peritoneal macrophages were obtained from mice that had been injected intraperitoneally 3 days previously with 2 ml of 3% thioglycollate medium (Himedia, India). Three days later, the peritoneal exudates were collected in RPMI-1640, the exudates were centrifuged at 1000 rpm at 25 ºC for 20 min and erythrocytes were lysed by hypotonic lysis. The mixture was centrifuged and the cell pellets were washed twice and resuspended in RPMI-1640 medium. The cell numbers were determined by a hematocytometer and cell viability was tested by trypan-blue dye exclusion technique. The collected cells were then adjusting to required cell counts per ml, and seeded into a 96-well plate with RPMI-1640 containing 10% fetal bovine serum (FBS), 20 µM 2-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. The cells were cultured at 37℃ for 2 hr under a humidified atmosphere of 95% CO_2. The growth medium was replaced to a sample dissolved in the medium, and then it was maintained for 24 hr under the same condition.

2.6.1. *Nitric oxide assay*

NO production was determined by assaying culture supernatants for nitrite using Griess reagent by the method of Keller *et al*. [17]. PEC (adherent cells) at 5×10^6 cells/ml was incubated with different concentration of drug and Phytohemagglutinin for 24 hr at 37°C in 5% CO_2 atmosphere. Cell-free supernatant (75 µl) was mixed with 75 µl of griess reagent (sulphanilamide 1%, phosphoric acid 5%, napthylethylenediamine dihydrochloride 0.1%) and incubated at room temperature for 10 min, cells incubated with Phytohemagglutinin (100 µg/ml) were used as a positive control. After incubation, the absorbance of the wells was determined by using ELISA reader (VERSAmax, Molecular Devices) equipped with 540nm filter. Nitrite concentration was determined using dilutions of sodium nitrite in culture medium as standard.

2.7. *Phagocytic activity*

2.7.1. *Carbon clearance test*

HEDF was administered intragastrically for 5 consecutive days to different treated groups. Control was given equal volume of PBS (pH 7.4) and native group was devoid of

any treatment. After 48 hr of last dose, animals were injected via the tail vein with colloidal carbon (Indian ink, Camel), which was diluted with PBS (pH 7.4) to eight times before use (10 µl/g BW). Blood samples were drawn from the retro-orbital plexus at 0, 3, 6, 9, 12 and 15 min. The blood (25 µl) was dissolved in 0.1% sodium carbonate (2ml) and the absorbance was measured at 660 nm [18]. The phagocytic index, *K*, was calculated by equation:

$$K = (ln\ OD_1 - ln\ OD_2)/(T_2 - T_1)$$

where OD_1 and OD_2 depict the optical densities at times T_1 and T_2, respectively.

2.7.2. *In vitro phagocytic activity of polymorphonuclear (PMN) cells*

At the end of the drug treatment phase, 2 drops of blood were collected on a clean, dry glass slide and placed in a moist chamber to permit adherence of PMN cells, after which the clot was gently removed without disturbing the adherent polymorphonuclear (PMN) cells. This layer of PMNs was covered with a suspension of *C. albicans* (MTCC 227, Microbial Type Culture Collection & Gene Bank, Institute of Microbial Technology, Chandigarh, India) (10^6 candida/ml) and incubated for 1hr [19]. The slide was then stained with Giemsa stain and the effect of HEDF on phagocytic activity was expressed as the percentage of cells showing phagocytosis and the average number of Candida per PMN.

2.8. *Haemagglutination antibody titer*

HEDF was administered in different doses to different groups of animals on -4, -2, 0, 2, 4 days. Control group received equal volume of PBS (pH 7.4). Animals in all groups were injected i.p. 0.2 ml of 5×10^9 sheep RBC on day 0. Blood samples were collected from retro-orbital plexus on day 7. Antibody titer was determined following the procedure reported by Nelson and Mildenhall [20]. To two-fold dilution of serum samples made in 25 µl volumes of normal saline containing 0.1% BSA (BSA saline) in V bottom haemagglutination plates (Tarsons, India) were added 25 µl of 0.1% suspension of sheep RBC in BSA saline. After thorough mixing sheep RBCs were allowed to settle at room temperature for 90 min until control wells showed small button of cells (negative pattern). The value of the highest serum dilution causing visible haemagglutination was considered as the antibody titer.

2.9. *Plaque forming cell (PFC) assay*

The spleen cells of sheep RBC immunized HEDF treated mice were separated in RPMI-1640 medium, washed twice and suspended in same medium (10^6 cells/ml). Glass petridishes were layered with 1.2% agarose in 0.15 M sodium chloride to form bottom layer. A mixture of 2 ml agarose (0.6%) in RPMI-1640 medium, 0.1 ml suspension of

20% sheep RBC and 1×10^6 spleen cells in a volume of 0.1 ml was poured over the bottom layer of agarose followed by an incubation period of 90 min at 37 ^oC [21]. 2 ml of 1:9 diluted fresh rabbit serum was added to petridish and plate was reincubated for 40 min to allow the formation of plaques. The number of plaque was counted immediately and values are expressed as counts per 10^6 spleen cells.

2.10. *Neutrophil adhesion test*

On 14th day of drug treatment, blood samples were collected (before challenge) by puncturing the retro-orbital plexus into heparinized vials and were analysed for total leukocyte counts and differential leukocyte counts by fixing blood smears and staining with field stain I and II-Leishman's stain. After initial counts, blood samples were incubated with 80 mg/ml of nylon fibres for 15 min at 37 ^oC. The incubated blood samples were again analysed for total leukocyte count and differential leukocyte count. The product of total leukocyte count and % neutrophil gives neutrophil index (NI) of blood sample. Percent neutrophil adhesion was calculated as:

$$\text{Neutrophil adhesion (\%)} = 100 \times (NI_u - NI_t) / NI_u$$

where NI_u is neutrophil index of untreated blood sample, and NI_t is neutrophil index of treated blood sample [22].

2.11. *Statistical analysis*

Pharmacological data were analysed by one-way analysis of variance (ANOVA) followed by Dunnett's *t* test with equal sample size. The difference was considered significant when p value < 0.05. All the values were expressed as mean ± standard error mean (S.E.M.).

3. Results and Discussion

3.1. *Phytochemical screening*

The results of the preliminary phytochemical screening of the crude hydroalcoholic extract revealed the presence of steroids, terpenes, glycosides, tannins, proteins, flavonoids, carbohydrates, polysaccharides and fixed oil.

3.2. *DTH reaction*

The delayed type hypersensitivity that was measured in this study has a few major components – sensitization, release of cytokines and inflammation. DTH reaction is characterised by large influxes of non-specific inflammatory cells, in which the macrophage is a major participant. It is a type IV hypersensitivity reaction that develops

when antigen activates sensitized T-cells. Activation of T cells by antigen presented through appropriate antigen presenting cells results in the secretion of various cytokines including interleukin-2, interferon-γ, macrophage migration inhibition factor and tumor necrosis factor-β [23]. The overall effects of these cytokines are to recruits macrophages into the area and activate them, promoting increased phagocytic activity vis-a-vis increased concentration of lytic enzymes for more effective killing. Several lines of evidence suggest that DTH reaction is important in host defence against parasites and bacteria that can live and proliferate intracellularly. The T-cell mediated DTH response to sheep RBC (percentage increase in paw volume) showed a dose-dependent increase due to the treatment with HEDF. With doses of 250, 475 and 950 mg/kg, the DTH response were 27.475 ± 0.234 %, 37.381 ± 0.456 % and 39.912 ± 0.759 %, respectively in comparison with corresponding values of 20.279 ± 0.336 % for PBS-treated control group after 24hr (Fig. 1). The differences in DTH response were statistically significant ($p < 0.05$) as compared to control group up to 96hr of study. Thus, treatment of HEDF enhanced DTH reaction, suggesting heightened infiltration of macrophages to the inflammatory site. This supports a possible role of HEDF in assisting cell-mediated immune response.

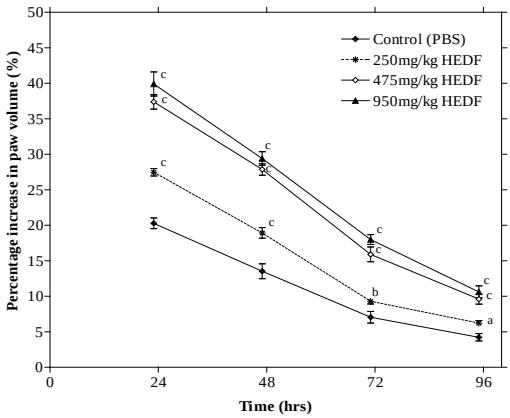

Fig. 1. Effect of *D. falcata* on cellular immunity (delayed type hypersensitivity to sheep RBCs). HEDF: Hydroalcoholic extract of *D. falcata*; PBS: phosphate buffer saline (pH 7.4); Values are expressed as mean ± S.E.M.; [a]: $p < 0.05$; [b]: $p < 0.01$; [c]: $p < 0.001$ (compared to control).

3.3. *NO assay*

Increase in the nitrite production has a significant effect on the macrophages function. NO is synthesised by NO synthase (NOS) [24] and mediates diverse functions, including vasodilation, neurotransmission and inflammation [25]. NO has been shown to be the principal effecter molecule produced by macrophages for cytotoxic activity and can be used as quantitative index of macrophage activation [26]. It increases the phagocytic activity of the macrophages. The extract showed significant ($p < 0.05$) increase in the NO

production from peritoneal macrophage at 208, 416 and 832 µg/ml with 29.56%, 39.62% and 55.76%, respectively (Fig. 2).

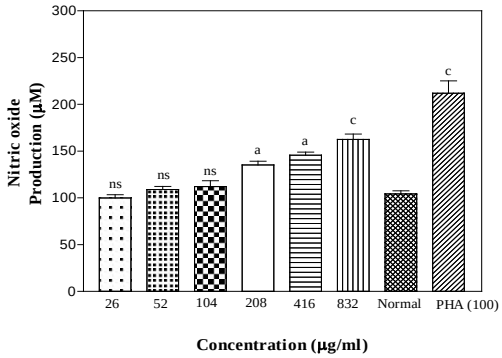

Fig. 2. Effect of HEDF on the nitrite production of peritoneal macrophages. Values as % of control, mean ± SEM. [a]: $p<0.005$; [b]: $p<0.01$; [c]: $p<0.001$: significantly different from vehicle control group.

3.4. *Phargocytic activity*

Phagocytosis represents an important innate defence mechanism against ingested particulates including whole pathogenic microorganisms. The specialized cells that are capable of phagocytosis include blood monocytes, neutrophils, and tissue macrophages. Once the particulate material is ingested to phagosomes, the phagosomes fuse with lysosomes and the ingested material is then digested. The carbon clearance test was conducted to establish phagocytic activity of reticuloendothelial system after drug treatment [27]. Phagocytic index (PI) was significantly ($p < 0.01$) increased after HEDF administration. The PI for different doses of HEDF were ranging from 0.2 ± 0.02 for low dose 250 mg/kg BW to 0.48 ± 0.02 for high dose 950 mg/kg BW. Thus, enhancement uptake of particulate matter with the treatment of HEDF is evident from carbon clearance

Table 1. Effect of HEDF treatment on phagocytic index (carbon clearance test) and on the phagocytic activity of blood PMN cells.

Group (treatment)	Phagocytic index (carbon clearance)	Phagocytic activity of PMN cells	
		PMN showing phagocytosis (%)	Average no. of Candida/PMN
Native (no treatment)	0.05 ± 0.003	71.7 ± 3.21	2.7 ± 0.20
Control (PBS)	0.07 ± 0.004	72.2 ± 2.82	2.6 ± 0.24
0.25 g/kg BW (HEDF)	0.20 ± 0.02[b]	81.26 ± 0.87[a]	3.8 ± 0.21[a]
0.475 g/kg BW (HEDF)	0.35 ± 0.03[b]	89.84 ± 1.39[b]	4.0 ± 0.31[b]
0.95 g/kg BW (HEDF)	0.48 ± 0.02[b]	94.2 ± 1.47[b]	4.4 ± 0.25[b]

HEDF: Hydroalcoholic extract of *D. falcata*; PBS: phosphate buffer saline (pH 7.4); PMN: polymer-phonuclear cells; values are as mean ± S.E.M.; [a]: $p < 0.05$; [b]: $p < 0.01$ (compared to native group).

test. During the *in vitro* phagocytic activity test the higher doses of HEDF increased the phagocytic activity of PMN cells as evidenced by an increase in the average number of *Candida* per PMN cell ($p < 0.01$) and improvement in the percentage of PMN cells showing phagocytosis ($p < 0.01$), while the low dose showed moderately less activity ($p < 0.05$) (Table 1).

3.5. *Haemoglutination antibody titer and PFC assay*

The humoral immunity was enhanced by HEDF treatment to mice, which was observed during the haemoglutination antibody titer. The antibody titer was determined to establish the humoral response against sheep RBC. At neutral pH, red blood cells possess negative ions cloud that makes the cells repel from one another, this repulsive force is referred to as zeta potential. Because of its size and pentameric nature, IgM can overcome the electric barrier and get cross-link red blood cells, leading to subsequent agglutination [28]. A dose related increase in haemogglutination antibody titer was observed with three doses of HEDF (Table 2) compared to control group. These observations reflect that HEDF treatment elevated the humoral immune response in mice. In PFC assay, HEDF administration for 5 days beginning from the day of sensitization with Sheep RBC produced 23.19 – 51.9% increase in antibody secreting cells in mouse spleen (Table 2). Effect was significant ($p < 0.01$) compared to control group at all tested doses.

Table 2. Effect of HEDF on haemaglutination antibody titer, humoral immunity (plaque forming cell assay) and total leukocyte count.

Group (treatment)	Haemagglutination antibody titer	Plaque forming cells/10^6 spleen cells
Native (no treatment)	49.65±7.2	503.36±13.29
Control (PBS)	51.2 ±7.83	526.0±7.21
250 mg/kg BW (HEDF)	140.8±31.35	648.0±13.91[b]
475 mg/kg BW (HEDF)	179.2±22.29[a]	764.0±10.2[c]
950 mg/kg BW (HEDF)	204.8±31.4[b]	799.0±19.6[c]

HEDF: Hydroalcoholic extract of *D. falcata*; PBS: phosphate buffer saline (pH 7.4); values are as mean ± S.E.M.; [a]: $p < 0.05$; [b]: $p < 0.01$; [c]: $p < 0.001$ (compared to control).

3.6. *Nautrophil adhesion test*

Migration of neutrophils from the blood stream requires a firm adhesion, which is mediated through the interactions of the β2 integrins present on the neutrophils [29]. The β2 integrin stored in the cell granules to be up regulated for a firm adherence [30]. In the

current study, HEDF when orally administered significantly ($p< 0.001$) increased the adhesion of neutrophil to nylon fibers, which correlates with the process of migration of cells in blood vessels. The neutrophil adhesion was significantly increased with all the treated groups when compared with untreated control indicating possible imunostimulant effect (Fig. 4). This may be due to the up-regulation of $\beta 2$ integrins.

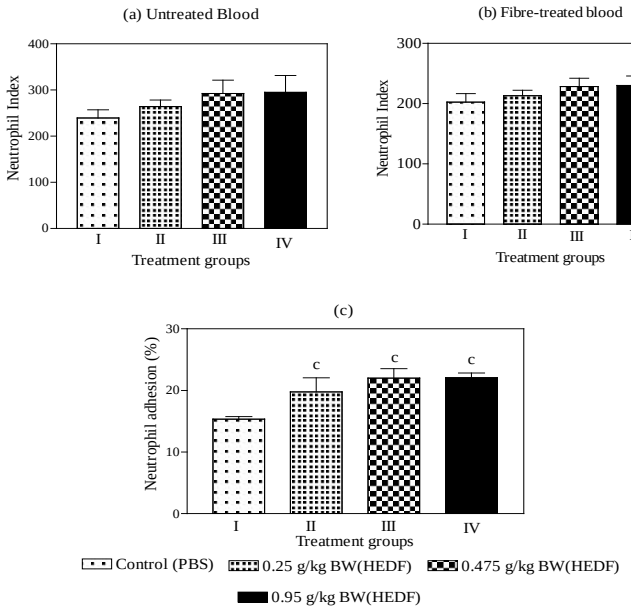

Fig. 4. Effect of HEDF on neutrophil adhesion in mice. (a), (b): Neutrophil index for untreated and fibre-treated blood respectively and (c): Percentage of neutrophil adhesion. HEDF: Hydroalcoholic extract of *D. falcata*; PBS: phosphate buffer saline (pH 7.4); Values are expressed as mean ± S.E.M.; ^{c}: $p < 0.001$ (neutrophil adhesion of treated groups II, III and IV compared to control group I).

4. Conclusion

It is convincing to assume that the hydroalcoholic-extract of *D. falcata* contains triterpenoids [4], which are reported to be responsible for the formation of nanoconstructs largely referred to as immunostimulatory complexes (ISCOMS). These ISCOMS may presumably be held responsible for stimulation and potentiating of overall immune response. It is likely that some of the other proteins/peptides (constituents of *D. falcata* [31, 4] could had entrapped into these nanoconstructs. The latter could have functionally presented them to the macrophages as well as to the cytosol components of dendritic cells (antigen presenting cells). It is reported that antigen-iscom complex triggers immune response through secretion of IL-2 and IFN-γ, which are ultimately responsible for Th-1 and Th-2 cellular responses [32]. As well, HEDF revealed the presence of

polysaccharides, which are ideal candidates for therapeutics with immunomodulatory and antitumor effects [33]. The result of this study was also in agreement with the reports that, plant phenolics and saponins (components of *D. falcata* [4]) are effective immunomodulators [3].

Based on the above results and analysis, it can be concluded that HEDF has the potential to stimulate cell-mediated immunity as well as humoral immunity and it may be a potential therapeutic candidate in several immunosupressed clinical conditions. And the immunomodulatory responses were found to be a dose dependent manner. Further detailed studies on its mode of immune action and active constituents isolation are in progress.

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