EVALUATION OF *IN VIVO* IMMUNOMODULATORY ACTIVITY OF AQUEOUS AND ETHANOLIC EXTRACT OF *EULOPHIA NUDA* L.

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ABSTRACT

Objective: The aim of this study was to evaluate the *in vivo* immunomodulatory activity of an aqueous and ethanolic extract of dried tubers of *Eulophia nuda* (ENA and ENE).

Methods: Effect of both the extracts was evaluated on delayed-type hypersensitivity (DTH) response, serum antibody response, and cyclophosphamide-induced myelosuppression in Swiss albino mice.

Results: The extracts showed stimulation of DTH reaction in mice in response to T-cell dependent antigen by both ENE () and ENA (); significant increase in serum antibody titer at of ENA (768.01) and ENE (768.33) extract at 200 mg/kg, compared to control group (213.33); Combined treatment of ENA+Cytochromes P450 [CYP]-25 mg/kg and ENE+CYP-25mg/kg (50, 100, and 200 mg/kg) doses of ENA and ENE each with 25 mg/kg resulted in restoration of bone marrow activity as compared with CYP treatment alone.

Conclusion: Both specific and non-specific immunostimulating properties of the ENE and ENA tubers in *in vivo* experimental methods suggest its therapeutic usefulness in immunocompromised conditions.

Keywords: *Eulophia nuda L.*, Peritoneal macrophages, Nitric oxide, Carbon clearance, Ovalbumin antibody titer.

INTRODUCTION

The immune system is known to be involved in the etiology as well as pathophysiological mechanisms of many diseases [1]. Ayurveda gives emphasis on promotion of health - A concept of strengthening host defenses against different diseases [2]. Rasayana plants are particularly recommended for the treatment of immune disorder [3], Ayurveda (with particular reference to plants) may play an important role in modern health care, particularly where satisfactory treatment is not available. There is need to evaluate the potential of Ayurvedic remedies to the advantage of counteract side effect of modern therapy and compare the cost-effectiveness of certain therapies vis-a-vis modern therapeutic schedules [4]. Development of agents capable of moving "patients" immune system from a state of immune deficiency to one or more normal function would likely to have a significant impact on disease and the patient it affects. Such agent would not be a cure but would control the manifestation and course of disease [5-7].

Eulophia nuda L., Orchidaceae family, is a perennial, terrestrial herb with underground tubers found in central and Southeast Asian regions. In India, this plant is found in the tropical Himalayas, from Nepal to Assam, and in Deccan from Konkan southwards. The tubers are reported to be used against tumors, scrofulous glands of the neck, bronchitis, blood disease, and as a vermifuge [8-12]. In Thailand, this orchid is used in traditional medicines for the treatment of skin rash. Raw tubers are eaten for curing rheumatoid arthritis [13]. Recently, it is reported to be demulcent and anthelmintic in action [14]. Underground part (tubers) also called as Salep is used as an aphrodisiac drug [15]. They are also used to treat acidity, piles, and stomach complaints [16,17].

METHODS

Plant material and preparation of extract
Fresh tubers of *E. nuda L.* were collected from Bhimashankar region of the Western Ghats in Pune district, India, in May 2012 and authenticated from Blatter Herbarium, St. Xavier's College, Mumbai - 400 001. The voucher specimen number is 20831; specimen sample was deposited in the herbarium of the institute for future reference. The tubers were thoroughly washed cut into small pieces and dried at controlled temperature of 45°C and powdered. The powder was then extracted with boiling ethanol under soxhlation to give an ethanolic extract of *E. nuda* (ENE) and similarly with boiling water under soxhlation to give an aqueous extract of *E. nuda* (ENA). The extracts were evaporated to dryness. The yield of ethanolic and water extract was obtained 7.5% and 9% for ENE and ENA extract, respectively.

Preliminary phytochemical screening
The ENE and ENA were subjected to preliminary phytochemical screening [18] for the detection of various plant constituents.

Experimental animals
Swiss albino mice were obtained from National Toxicology Centre, Pune. The animals were acclimatized for 10 days before being used for the experiments. They were housed in a room with controlled temperature (23±20°C) and a 12-h light/12-h dark cycle. The animals were fed with standard pellet diet and water *ad libitum*.

The experimental protocols were approved by the Institutional Animal Ethics Committee of the National Toxicology Centre, Pune, and conducted according to the guidelines of the Committee for the Purpose of Control and Supervision on Experiments on Animals, India (Research project No. 159).

Acute oral toxicity studies
Acute oral toxicity studies were carried out for ENA and ENE as per the organization for economic cooperation and development guideline number 423 [19,20].

Chemicals
Ovalbumin, Freund's complete adjuvant (FCA), and bovine serum albumin (BSA) were procured from Bangalore Genei, India. Streptomycin,

penicillin, Roswell Park Memorial Institute (RPMI) 1640 medium, and HEPES buffer were procured from HiMedia Pvt., Ltd., India. Fetal bovine serum (FBS) and phytohemagglutinin-M (PHA) were procured from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

In vivo phagocytic activity by carbon clearance assay

Phagocytic activity of both extracts was determined as per the method described by Yan *et al.* 2007 [21]. Mice were divided into nine groups, of six each. The vehicle control group received (0.5% sodium carboxymethyl cellulose [NaCMC]). Positive control group received immunosin 50 mg/kg. Negative control group received cyclophosphamide 10 mg/kg. Mice in the treatment groups were administered orally ENA and ENE extracts each (50, 100, and 200 mg/kg) suspended in vehicle daily for 20 days. Colloidal carbon solution, Rotring ink® (Hamburg, Germany) was diluted with normal saline (1:8), and injected (0.01 ml/g body weight) was through tail vein to each mouse 24 h after the last dose. Blood samples were drawn from the retro-orbital plexus under ether anesthesia at 2 and 15 min after injection. Blood (25 μl) was mixed with 0.1 % sodium carbonate (2 ml) for the lysis of erythrocytes OD was recorded at 660 nm. The phagocytic index (K) was calculated using the following equation:

$$K = \frac{\ln OD1 - \ln OD2}{T2 - T1}$$

Where, OD1 and OD2 are the optical densities at times T1 and T2, respectively [22].

Immunization and treatment

Mice were divided into nine groups of six each. The vehicle control group received 0.5% NaCMC and positive control group received immunosin 50 mg/kg. Negative control group received cyclophosphamide 10 mg/kg; while mice in the treatment groups were administered with the ENA and ENE extract each (50, 100, and 200 mg/kg, p.o.) in vehicle daily for 20 days. On the 14th day, the animals were immunized subcutaneously with ovalbumin (3 mg) dissolved in normal saline emulsified with an equal volume of FCA.

Delayed type hypersensitivity (DTH) response

To assess the DTH response, mice were challenged subcutaneously with 25 μg ovalbumin in 25 μl normal saline in the left hind footpad 7 days after the immunization. The right hind footpad was injected with 25 μl vehicle and served as control. The increase in footpad thickness was measured 24 h after the challenge with the help of a digimatic caliper (Mitutoyo Corporation, Japan) [23].

Detection of serum antibody response

Blood was collected from mice through retro-orbital plexus after 7 days of immunization and serum was separated under centrifugation. Serum antibody titers for quantification of serum IgG to ovalbumin were estimated by ELISA as described earlier [23]. Flat bottom polystyrene plates were coated with 12.5 μg of ovalbumin dissolved in 100μl of sodium carbonate buffer (pH 9.6) at 4°C for 12 h. The coated plates were washed 3 times with phosphate buffer saline (0.15 M, pH 7.2) containing 0.05% TWEEN-20 (PBS-Tw). The wells were incubated with 100μl of 1% BSA in sodium carbonate buffer at 37°C for 1 h. Serial dilutions of mouse serum samples in PBS-Tw were prepared, and 100μl was incubated with coated wells for 1 h at 37°C. After washing, diluted (1:2000) anti-mouse IgG conjugated with peroxidase (100 μl) was added, and the plates were incubated at 37°C for 1 h. The enzyme activity was determined by addition of TMB/H_2O_2. The enzyme reaction was stopped by addition of 50 μl, 8 N sulfuric acid, and the absorbance was measured at 450 nm. Endpoint antibody titers were expressed as the reciprocal of the highest dilution of the test serum samples showing 3 times more OD as compared with control samples.

Cyclophosphamide-induced myelosuppression

This experiment was studied according to the procedure described earlier with some modifications [24]. Mice were divided into nine

groups of six each. The vehicle control group received (0.5% NaCMC); positive control group received immunosin 50 mg/kg. Negative control group received cyclophosphamide 25 mg/kg, while mice in the treatment groups were administered with the ENA and ENE extract each (50, 100, and 200 mg/kg, p.o.) in vehicle daily for 19 days. On 17th, 18th, and 19th day of study, all the animals except in the vehicle control group were injected with cyclophosphamide (25 mg/kg, i.p.,) 1 h after administration of the extract or vehicle. Blood samples were collected on day 20, and total blood cell count was determined using hematology analyzer (Arcus, Diatron, Wien Austria).

Statistical analysis

Results are expressed as mean±SEM. Data were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons test with p<0.05 as the criteria for significance.

RESULTS

Extraction

The yield of ethanolic and water extract was obtained 9.5% and 11% for ENE and ENA, respectively. The extract showed the presence of phytosterols, saponins, proteins, alkaloids, glycosides, and flavonoids.

Pharmacological activity

In vivo phagocytic activity by carbon clearance assay

In vivo phagocytic activity of ENA and ENE extracts was determined by the carbon clearance assay in mice. The results are presented in Table 1. The phagocytic index (K) for ENA extract was significantly higher (p<0.05) at 50 mg/kg (15.86%), 100 mg/kg (32.06%), and 200 mg/kg (35.47%), and for ENE extract was significantly higher (p<0.05) at 50 mg/kg (14.8%), 100 mg/kg (31.9%), and 200 mg/kg (41.12%) dose levels as compared to control group. Positive control immunosin (50 mg/kg) showed 71.2% higher K compared to the control group (Fig. 4).

DTH response in ovalbumin immunized mice

To examine the effect of ENA and ENE extracts on the cellular immune system, its activity was investigated as DTH reaction to ovalbumin immunized mice. The DTH response, i.e. difference in footpad thickness of mice is shown in Table 1. Both aqueous and ethanolic extracts produced a significant increase in the DTH response to ovalbumin at all the tested dose levels. Increase in DTH reaction in mice in response to T cell-dependent antigen revealed the stimulatory effect of aqueous and ethanolic extract on T cells (Fig. 2). Immunosin 50 mg/kg and ENA and ENE 100 and 200 mg/kg (p<0.05) compared to control group.

Detection of serum antibody response to ovalbumin

Humoral response to ovalbumin was studied by ELISA antibody titer assay. Mice treated with different doses of the ENA and ENE extract showed an increase in the antibody titer in a dose-dependent manner. There was significant increase in serum antibody titer at 200 mg/kg (768.01) (p<0.05) of ENA extract compared to control group (213.33) and significant increase in serum antibody titer at 200 mg/kg (768.33) (p<0.05) of ENE extract compared to the control group (213.33) (Table 1). Positive control immunosin (50 mg/kg) showed (1195.00) (p<0.05) compared to control group (Fig. 3).

Cyclophosphamide-induced myelosuppression

There was a significant reduction (p<0.05) in total white blood cell (WBC) count of cyclophosphamide (25 mg/kg) treated mice (8.792 × 10³ cells/cmm) as compared to vehicle control group (13.38 × 10³ cells/cmm). Cyclophosphamide dose of 25 mg/kg caused a significant reduction in the WBC, red blood cell (RBC), hemoglobin, and platelet count. Combined treatment of ENA+cytochromes P450 [CYP]–25 mg/kg and ENE+CYP–25 mg/kg (50, 100, and 200 mg/kg) doses of ENA and ENE each with 25 mg/kg resulted in the restoration of bone marrow activity as compared with CYP treatment alone.

DISCUSSION

Immunomodulation through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense

Table 1: Effects of ENE and ENA on phagocytic index, DTH, and antibody titer

S. No.	Treatment group (dose mg/kg)	Phagocytic index (K)	DTH mm ($\times 10^{-2}$)	Antibody titer
1	Vehicle control	0.0561±0.0014	23.67±0.49	213.3±26.9
2	CYP 10	0.0264±0.0010	18.67±0.88	149.3±21.3
3	Immunosin-50	0.0960±0.0010	38.63±0.89	1195.0±170.6
4	ENA 50	0.0650±0.0025	26.33±0.76	298.7±42.6
5	ENA 100	0.0741±0.0013	29.33±0.49	341.3±53.9
6	ENA 200	0.0760±0.0021	31.67±0.49	768.6±114.5
7	ENE 50	0.0644±0.0011	26.17±0.48	341.3±53.9
8	ENE 100	0.0740±0.0014	27.83±0.45	426.7±53.9
9	ENE 200	0.0831±0.0013	29.17±0.48	768.0±114.5

DTH: Delayed-type hypersensitivity. ENE: Ethanolic extracts of *E. nuda L.*, ENA: Aqueous extracts of *E. nuda L.*, *E. nuda*: *Eulophia nuda*, CYP: Cytochromes P450

Table 2: Effects of ENA and ENA on total WBC, RBC count, hemoglobin concentration, and platelet count

S. No.	Treatment group (dose mg/kg)	Total WBC count in thousand (cmm)	RBC count in (%) million (cmm)	Hemoglobin concentration in g	Platelet count in thousands (cmm)
1	Vehicle Control	13.38±0.315	7.19±0.153	12.06±0.192	498.29±3.42
2	CYP (25)	8.792±0.206	5.88±0.105	7.81±0.302	432.43±3.71
3	(Immunosin (50)+CYP (25)	12.030±0.163	8.09±0.181	12.12±0.193	540.24±1.08
4	ENA (50)+CYP (25)	9.260±0.140	5.98±0.190	10.65±0.142	451.26±4.91
5	ENA (100)+CYP (25)	10.540±0.280	6.34±0.226	11.15±0.169	482.15±4.27
6	ENA (200)+CYP (25)	11.470±0.203	7.13±0.168	11.35±0.207	495.23±1.59
7	ENE (50)+CYP (25)	9.341±0.160	6.00±0.076	10.91±0.296	430.41±2.27
8	ENE (100)+CYP (25)	10.643±0.240	6.79±0.187	11.04±0.129	456.46±0.67
9	ENE (200)+CYP (25)	10.900±0.090	6.73±0.155	11.35±0.188	475.84±1.19

ENE: Ethanolic extracts of *E. nuda L.*, ENA: Aqueous extracts of *E. nuda L.*, E. nuda: *Eulophia nuda*, CYP: Cytochromes P450, WBC: White blood cell, RBC: Red blood cell
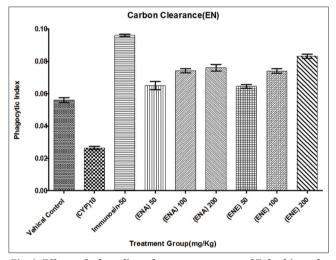

Fig. 1: Effects of ethanolic and aqueous extracts of Eulophia nuda L. on carbon clearance

Fig. 2: Effects of ethanolic and aqueous extracts of Eulophia nuda L. on delayed-type hypersensitivity

mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy [25]. There is a growing interest in identifying herbal immune modulators ever since their possible use in modern medicine has been suggested [26]. The main objective of the study was to investigate the immune modulatory effects of ENA and ENE.

Macrophages have been known to play an important role in the host protection against a wide range of tumors and microorganisms. Macrophages also present antigen to lymphocytes during the development of specific immunity and serve as supportive accessory cells to lymphocytes. When activated, macrophages increase the phagocytic activity and release various materials such as cytokines and reactive intermediates and then carry out non-specific immune responses. There has been great interest in reactive nitrogen intermediates, nitric oxide (NO), because of its antibacterial and antitumor effect [27]. NO mediates diverse functions, including vasodilatation, neurotransmission, and inflammation [28]. A very
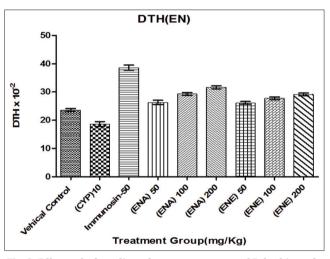
high NO production indicates increased phagocytosis and bactericidal activity, which is supported by the data, presented in Fig. 1. Macrophages play an important role in defense mechanism against host infection and in killing tumor cells. Higher reduction of nitroblue tetrazolium (NBT) dye by ENA and ENE extract represents a higher activity of oxidase enzyme, reflecting stimulation of phagocytes in proportion to intracellular killing. For lysosomal enzyme activity, the transformation of para-nitrophenylphosphate to colored compound by the acid phosphatase of the stimulated macrophages correlates to the extent of degranulation in phagocytosis [6,29]. Phagocytosis of particles by macrophages is usually accompanied by a burst of oxidative metabolism allowing the generation of reactive oxygen species which can be detected through an assay based on the reduction of NBT [30]. The effect of various concentrations of ENA and ENE extracts on the reduction of NBT dye and lysosomal enzyme activity response of macrophages was studied for phagocytic assay. ENA and ENE extracts appeared to produce phagocytic stimulation with dose-response relationship in lysosomal enzyme activity evaluation.
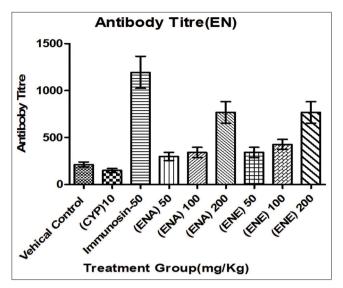
Fig. 3: Effects of ethanolic and aqueous extracts of Eulophia nuda L. on serum antibody response to ovalbumin

Myeloperoxidase, a heme protein secreted by neutrophils and macrophages, which uses the oxidizing potential of H_2O_2 to convert chloride ion into hypochlorous acid (HOCl). A potent bactericidal agent, HOCl is a critical component of host defenses against invading bacteria, fungi, and viruses [7,31]. The increase in the stimulation index of myeloperoxidase by the exposure of ENA and ENE extracts indicates the enhanced defense capability of these cells to pathogenic organisms. The release of immune mediators from murine peritoneal macrophages was significantly stimulated by the exposure of the extract. Murine isolated peritoneal macrophages incubated with the ENA and ENE extracts at different concentrations ranging between 832 and 6.5 µg/ml for 24 h, showed significant activation of macrophages by modulating the secretion of various mediators including NO, lysosomal enzyme, and myeloperoxidase activity. This suggests that ENA and ENE extracts can effectively strengthen innate immunity against foreign particles [32].

The process of phagocytosis involves certain body cells, known as phagocytes, which ingest and removes microorganisms, malignant cells, inorganic particles, and tissue debris [33]. Phagocytosis and killing of invading microorganisms by macrophages constitute body's primary line of defense. Macrophages are an integral part of the immune system, acting as phagocytic, microbicidal, and tumoricidal effector cells [34].
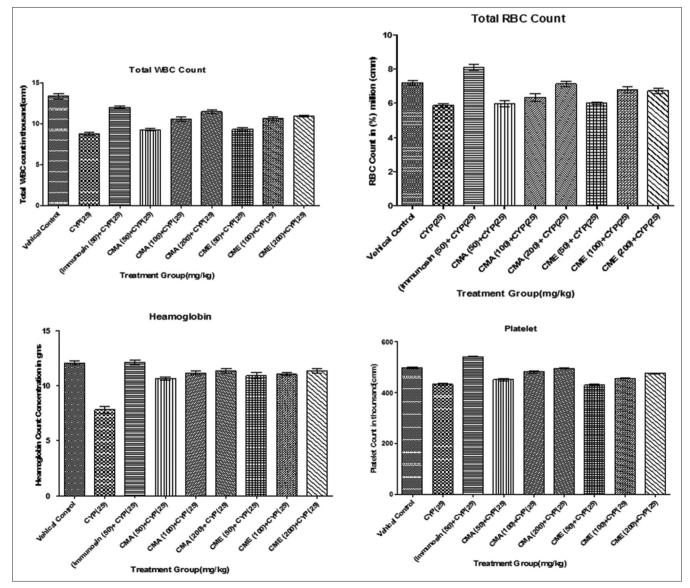

Fig. 4: Effects of ethanolic and aqueous extracts of Eulophia nuda L. on total white blood cell, RBC count, hemoglobin concentration, and platelet count

Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response [27]. In view of the pivotal role played by the macrophages, ENA and ENE extracts were also evaluated for it's *in vivo* effect on macrophage phagocytic activity. The increase in carbon clearance, i.e. phagocytic index by NNA and ENE extracts reflects the enhancement of phagocytic function of mononuclear macrophage and thus non-specific immunity. This indicates that ENA and ENE extracts were able to activate murine peritoneal macrophages, and hence phagocytic assays *in vitro* and *in vivo*.

The effect of the ENA and ENE extracts on cell-mediated immunity (CMI) was evaluated through DTH reaction to ovalbumin, a T cell-dependent antigen. CMI responses are critical to defend against infectious organisms, infection of foreign grafts, tumor immunity, and DTH reaction (Miller, 1991). Therefore, increase in DTH reaction in mice in response to T cell-dependent antigen revealed the stimulatory effect of ENE and ENA (Table 2). The T and B cell cognate interaction provides an optimal signal for B cell differentiation and antibody production toward T dependent antigen (ovalbumin) [33]. The augmentation of the humoral immune response to ovalbumin by ENA and ENE extracts, as evidenced by an increase in the antibody titer in mice indicated the enhanced responsiveness of T and B lymphocyte subsets, involved in the antibody synthesis [35].

A high degree of cell proliferation renders the bone marrow a sensitive target, particularly to cytotoxic drugs. In fact, bone marrow is the organ most affected during any immunosuppressive therapy, especially with cyclophosphamide. Loss of stem cells and the inability of the bone marrow to regenerate new blood cells results in thrombocytopenia and leukopenia [36,37]. Since *E. nuda* augmented the circulating antibody titer, it was thought worthwhile to evaluate its effect on peripheral blood count and cyclophosphamide-induced immunosuppression. The administration of *E. nuda* significantly ameliorated the total WBC count, RBC count, hemoglobin, and platelet count and also restored the myelosuppressive effects induced by cyclophosphamide (Table 2). The present investigation suggests that *E. nuda* may stimulate both cellular and humoral immune responses. Further studies to elucidate the exact immunostimulatory mechanism of *E. nuda* need to be explored.

CONCLUSION

The studies have demonstrated specific and non-specific immunostimulating properties of the ENE and ENA tubers in *in vivo* experimental methods. This suggests its therapeutic usefulness in immunocompromised conditions.

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AUTHORS' CONTRIBUTION

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Dr. (Mrs.) Vanita G. Kanase collected the data, prepared the protocol, conduct the research and assesses the manuscript. Mr. Diptesh T. Patil prepared Manuscript, provided the statistical assessment of data and suggested the necessary changes, and helps in designing manuscript.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interests regarding the publication of this paper.

REFERENCES

1. Sharma P. Charak Samhita. 1st ed. Varanasi: Chaukhambha Orientalia; 1983. p. 54.
2. Thatte U, Dahanukar S. Ayurveda and contemporary scientific thought. Trends Pharmacol Sci 1986;7:247-51.
3. Dhanukar SA, Thatte UM, Rege NM. In: Wagner H, editor. Immunomodulatory Agents from Plants. Basel, Switzerland: Birkhauser Verlag; 1999. p. 289-323.
4. Dahanukar S, Thatte U. Current status of ayurveda in phytomedicine. Phytomedicine 1997;4:359-68.
5. Gottlieb A, Gottlieb M, Scholes V. Reconstitution of immune function in AIDS/ARC. Concept Immunopathol 1987;4:261-74.
6. Kanase V, Jain B, Yadav P. Evaluation of *in-vitro* immunomodulatory activity of aqueous and ethanolic extract of *Capparis mooni*. Int J Pharm Bio Sci 2013;4:344-52.
7. Kanase V, Jain B, Yadav P. Evaluation of *in-vitro* immunomodulatory activity of hydroalcoholic extract of *Ceropegia Bulbosa* Roxb. Int J Res Sci Innov 2016;3:36-40.
8. Kirtikar K, Basu B. Indian Medicinal Plants. Vol. IV. Varanasi: Lalit Mohan Basu; 1918. p. 2405.
9. The Wealth of India: A Dictionary of Indian Raw Materials and Industrial Products. Vol. III). New Delhi: Publications and information Directorate; 1966. p. 221-2.
10. Chopra R, Nayar S, Chopra I. Glossary of Indian Medicinal Plants. New Delhi: Council of Scientific and Industrial Research; 1956. p. 221.
11. Cooke T. The Flora of the Presidency of Bombay. Calcutta: Botanical Survey of India; 1967. p.197-8.
12. Nadkarni K. Indian Materia Medica. Mumbai: Popular Prakashan Private Ltd; 1976. p. 519.
13. Mali P, Bhadane V. Some rare plants of ethnomedicinal properties from Jalgaon district of Maharashtra. Int J Green Pharm 2008;2:76-8.
14. Singh A, Duggal S. Medicinal orchids: An overview. Ethnobotanical leaflets 2009;13:351-63.
15. Jagdale S, Shimpi S, Chachad D. Pharmacological studies of 'salep'. J Herbal Med Toxicol 2009;3:153-6.
16. Mahekar P, Yadav S. Medicinal Plants of South Western Maharashtra. In: Pullaiah T, editor. Biodiversity in India. New Delhi, India: Regency Publications; 2006. p. 561.
17. Shriram V, Kumar V, Kishor P, Suryawanshi S, Upadhyay A, Bhat M. Cytotoxic activity of 9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol from *Eulophia nuda* against human cancer cells. J Ethnopharmacol 2010;128:251-3.
18. Trease G, Evans W. Textbook of Pharmacognosy. 12th ed. London: Balliere, Tindall 1989. p. 343-83.
19. OECD Guidelines for the Testing of Chemicals: Guideline 423: Acute Oral Toxicity.; 2002. p. 1-14.
20. Litchfield J, Wilcoxon F. A simplified method of evaluating dose-effect experiments. J Pharm Exp Ther 1949;96:99-113.
21. Yan Y, Wanshun L, Baoqin H, Changhong W, Chenwei F, Bing L, *et al*. The antioxidative and immunostimating properties of d-glucosamine. Int Immunopharmacol 2007;7:29-35.
22. Lee S, Suk K. Heme oxygenase-1 mediates cytoprotective effects of immunostimulation in microglia. Biochem Pharmacol 2007;74:723-9.
23. Popov S, Golovchenko V, Ovodova R, Smirnov V, Khramova D, Popova G, *et al*. Characterisation of the oral adjuvant effect of lemnan, a pectic polysaccharide of *Lemna minor* L. Vaccine 2006;24:5413-9.
24. Ghule B, Murugananthan G, Nakhat P, Yeole P. Immunostimulant effects of *Capparis zeylanica* Linn. leaves. J Ethnopharmacol 2006;108:311-5.
25. Wagner H. Immunostimulatory drugs of fungi and higher plants. In: Hikino NR, Farnsworth H, editors. Economic and Medicinal Plant Research. Vol. I. London: Academic Press; 1984. p. 113-53.
26. Tiwari U, Rastogi B, Singh P, Saraf D, Vyas S. Immunomodulatory effects of aqueous extract of *Tridax procumbens* in experimental animals. J Ethnopharmacol 2004;92:113-9.
27. Lowenstein CJ, Snyder SH. Nitric oxide, a novel biologic messenger. Cell 1992;70:705-7.
28. Saraphanchotiwitthaya A, Ingkaninan K, Sripalakit P. Effect of *Bacopa monniera* Linn. extract on murine immune response *in vitro*. Phytother Res 2008;22:1330-35.
29. Benencia F, Courrèges MC, Coulombié FC. *Trichilia glabra*: Effect on the phagocytic activity and respiratory burst response of peritoneal macrophages. Immunopharmacology 1999;41:45-53.
30. Brown, K., Brunt, E. and Heinecke, J. (2001). Immunohistochemical Detection of Myeloperoxidase and Its Oxidation Products in Kupffer Cells of Human Liver. The American Journal of Pathology, 159(6), pp.2081-2088.
31. Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, *et al*. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 1992;256:225-8.
32. Miller LE. In: Ludke HR, Peacock JE, Tomar RH, editors. Manual of Laboratory Immunology. London: Lea and Febiger; 1991. p. 1-18.

33. Stanilova S, Zhelev Z, Dobreva Z. Preliminary studies on the immunomodulatory effect of the C3 binding glycoprotein isolated from *Cuscuta europea*. Int J Immunopharmacol 2000;22:15-24.
34. Son E, Moon E, Rhee D, Pyo S. Stimulation of various functions in murine peritoneal macrophages by high mannuronic acid-containing alginate (HMA) exposure *in vivo*. Int Immunopharmacol 2000;1:147-54.
35. Shrestha P, Handral M. Evaluation of immunomodulatory activity of extract from rind of *Nephelium lappaceum* fruit. Int J Pharm Pharm Sci 2016;9:38.
36. Lee S, Chary M, Salehi I, Bansal R. Immune-mediated *Adalimumab-induced* thrombocytopenia for the treatment of ulcerative colitis. Int J Pharm Pharm Sci 2018;7:456-8.
37. Agarwal R, Diwanay S, Patki P, Patwardhan B. Studies on immunomodulatory activity of *Withania somnifera* (*Ashwagandha*) extracts in experimental immune inflammation. J Ethnopharmacol 1999;67:27-35.