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Research Article

EVALUATION OF *IN VITRO* IMMUNOMODULATORY ACTIVITY OF AQUEOUS AND ETHANOLIC EXTRACT OF *EULOPHIA NUDA* LINDL

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ABSTRACT

Objective: The aim of this study was to evaluate the *in vitro* immunomodulatory activity of aqueous and ethanolic extract of dried tubers of *Eulophia nuda*.

Methods: Effect of both the extracts was evaluated at various concentrations (832–6.5 µg/ml) for secretion of mediators such as nitric oxide (NO), superoxide, lysosomal enzyme, and myeloperoxidase activity of isolated murine peritoneal macrophages.

Results: The extracts showed stimulation of NO, statistically significant at 832 µg/ml (SI 1.739) for ENA and at 832 µg/ml (stimulation index [SI] 1.662) for ENE; significant stimulation on lysosomal enzyme release for ENA at 832 µg/ml (SI 1.404) and ENE at 832 µg/ml (SI 1.513); myeloperoxidase activity was statistically significant for ENA at 832 µg/ml (SI 1.728) and ENE at 832 µg/ml (SI 1.770).

Conclusion: *In vitro* phagocytic index showed significant results and thus proving the need for confirmation through *in vivo* studies.

Keywords: *Eulophia nuda* L., Peritoneal macrophages, Nitric oxide, Lysosomal enzyme, Myeloperoxidase.

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INTRODUCTION

The immune system is identified as a major system to be involved in the pathophysiological as well as etiological mechanisms of many diseases and disorders [1]. Ayurveda, which literally means "knowledge of life," offers importance on promotion of health, which is a concept of reinforcement of host defense system against different diseases and disorders [2]. In Rasayana, a branch of the Ayurveda, plants are predominantly suggested for the treatment, prevention, and care of various immune diseases and disorders [3]. Ayurveda (with specific reference to plants) may play a significant role in modern health care, chiefly when acceptable treatment is not available in allopathic medicinal system, either due to toxic side effects or due to cost of therapy. There is essential requirement to evaluate the therapeutic potential of Ayurvedic remedies to take as an advantage to counteract the side effects of modern therapy and relate the cost efficiency 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].

Eulophia nuda L. is a rare and endangered orchid and perennial terrestrial herb from Orchidaceae family. It is found in central and Southeast Asian regions. This plant is found in Nepal to Assam, which are parts of tropical Himalayas. In India, it is found in Deccan region, Konkan Southwards, Maharashtra, Rajasthan, Himachal Pradesh, Tamil Nadu, Kerala, Uttarakhand, Madhya Pradesh, Orissa, Jharkhand, and West Bengal. The plant contains tubers, which are reported to have activity against tumors, blood disease, scrofulous glands of the neck, bronchitis, and as a vermifuge [6-10]. It is commonly employed in Thailand's traditional medicinal system for the treatment of skin rash. Raw tubers are eaten for curing rheumatoid arthritis [11]. It is also reported to have demulcent and anthelmintic action [12]. Underground parts (tubers), which are also known as Salep, are used as an aphrodisiac drug [13]. They are also employed in the treatment of acidity, piles, and stomach complaints [14]. A compound extracted from the *E. nuda* "9,10-dihydro-2,5-dimethoxyphenanthrene-1,7-diol" demonstrated significant the growth suppressive activity against human cancer cells [15]. So far, no reports are found for immunomodulatory activity of the plant and the objective of our present study is to evaluate the *in vitro* immunomodulatory activity of *E. nuda* tuber.

METHODS

Plant material and preparation of extract

Fresh tubers of *E. nuda* L. were collected from Western Ghats in Bhimashankar region of in Pune, India, in May 2012. The authentication of tubers is done 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 cleaned and washed thoroughly. Then, they are cut into small pieces and dried at controlled temperature 45°C and powdered. The powder was then extracted with boiling ethanol under soxhlation to give *E. nuda ethanolic extract* (ENE) and similarly with boiling water under soxhlation to give *E. nuda aqueous extract* (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 ethanolic and aqueous extracts of tubers of *E. nuda* were subjected to preliminary phytochemical screening [16] for the detection of various plant constituents.

Chemicals

From Bangalore Genei Private Limited, the following compounds were obtained - Freund's complete adjuvant, ovalbumin, nitroblue tetrazolium (NBT), tetramethyl benzedrine/hydrogen peroxide (TMB/H_2O_2), and bovine serum albumin. From Hi-Media Pvt., Ltd., India, following chemicals were procured - penicillin, streptomycin, HEPES buffer, and Roswell Park Memorial Institute (RPMI) 1640 medium. From Sigma-Aldrich (St. Louis, MO, USA) following chemicals were procured - phytohemagglutinin-M (PHA) and fetal bovine serum (FBS). All other chemicals used were of analytical grade.

Isolation of peritoneal macrophage and culture conditions

The mice were subjected to injection of 2 ml of 4% (w/v) fluid thioglycollate medium through intraperitoneal route. After 3 days, mice undergo peritoneal lavage with 10 ml of RPMI 1640 medium. By this procedure, peritoneal macrophages were extracted and isolated. These extracted cells were washed with RPMI 1640. Further, they are subjected to culture medium containing RPMI 1640 supplemented with 100 µg/ml streptomycin (complete RPMI), 100 U/ml penicillin, 2 mM L-glutamine, and 10% FBS. Hemocytometer was employed to evaluate the macrophage count. Trypan blue dye exclusion technique was employed to determine the cell viability. The cells were adjusted to prerequisite cell count per ml. A 96-well flat-bottom culture plate (Tarsons Products Pvt., Ltd., India) was used to plate on these macrophage cells. Incubation of the plate was carried out at 37°C in a 5% CO_2 humidified incubator for 2 h. The non-adherent cells were removed. The monolayered macrophages were treated with both the extracts separately (832–6.5 µg/ml) dissolved in complete RPMI medium containing 20% DMSO. It was maintained for 24 h at 37°C in a 5% CO_2 humidified incubator [17].

Following *in vitro* assays were performed on these incubated cells. For these *in vitro* assays, PHA (10 µg/ml) was used as a positive control. All the results of these experiments were triplicated.

Nitrite assay

Nitric oxide (NO) production was indicated by the increased accumulation of nitrite concentration in the medium [18]. After incubating the macrophages (5×105 cells/ml) for 24 h, the cell-free supernatant (50 µl) was collected and mixed with 50 µl of Griess reagent (0.1% naphthyl ethylenediamine dihydrochloride, 2% phosphoric acid, and 1% sulfanilamide). The resulting solution was incubated for 10 min at room temperature. A microplate reader (ELX800MS, BioTek Instruments Inc., USA) was used to measure the optical density at 540 nm with. Standard curve of sodium nitrite in culture conditions was used to determine nitrite concentrations. The nitrite concentrations ratio of the treated and control macrophages was used in calculation of stimulation index (SI) for nitrite release.

NBT dye reduction assay

The 24 h incubated cells (1×106 cells/ml) with MCM extract were mixed with 50 µl of 0.3% NBT solution in PBS (phosphate-buffered saline, pH 7.4), and the mixture was further incubated in CO_2 incubator. After incubation for 1 h, the adherent macrophages were washed and cleaned vigorously with complete RPMI medium, and again washed 4 times with 200 µl methanol. Formazan deposits were solubilized in 120 µl of 2 M KOH and 140 µl of DMSO, after air drying. Homogenization was carried out. These homogenized contents of the wells were subjected for the estimation of optical density. The optical density was read at 630 nm using a microplate reader. The optical density ratio of the control and treated macrophages was employed in calculation of SI [19].

Cellular lysosomal enzyme activity

Measurement of acid phosphatase activity was utilized to evaluate the cellular lysosomal enzyme activity of macrophages. Macrophages were incubated at 37°C in 5% CO_2 for 24 h with MCM. After that, the supernatant was removed by aspiration and 20 µl of 0.1% Triton X-100 (Hi-Media, India) were added to each well. After 15 min' incubation, 100 µl of 10 mM *p*-nitrophenyl phosphate and 50 µl of 0.1 M citrate buffer (pH 5.0) were added. Then, the plates were incubated for 1 h. To these incubated plates, 0.2 M borate buffer (150 µl, pH 9.8) was added. A microplate reader was used to measure the optical density at 405 nm. The optical density ratio of the control and treated macrophages were used to calculate the phagocytic SI [19].

Myeloperoxidase activity assay

Isolated macrophages were also subjected to the evaluation of the myeloperoxidase activity [20]. Macrophages (5×105 cells/ml) were washed 3 times with fresh complete RPMI medium after incubation period of 3 hrs. Then, each well was filled with the mixture (100 µl) of *o*-phenylenediamine (0.4 g/ml) and 0.002% H_2O_2 in phosphate-citrate buffer (pH 5.0). After 10 min, using 0.1 N H_2SO_4, the reaction was stopped and measurement of optical density was carried out at 490 nm. The myeloperoxidase SI was calculated as the optical density ratio of the control and treated cells.

Statistical analysis

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

RESULTS

Extraction

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

Nitrite assay on isolated peritoneal macrophages

The nitrite level (NO) produced in cell culture supernatants was measured at 24 h of treatment, showing that ENA extracts induced nitrite production in statistically significant higher at 832 µg/ml (SI 1.739), 416 µg/ml (SI 1.551), 208 µg/ml (SI 1.452), and 104 µg/ml (SI 1.331) (Table 1), and ENE extract induced nitrite production in statistically significant higher, 832 µg/ml (SI 1.884), 416 µg/ml (SI 1.831), 208 µg/ml (SI 1.809), 104 µg/ml (SI 1.671), 52 µg/ml (SI 1.545), and 26 µg/ml (SI 1.483) concentrations than controls in all three experiments performed. PHA (positive control) also showed significant increase in nitrite release (SI 2.013). The result of this study is shown in Tables 1 and 2 as well as Figs. 1 and 2.

NBT dye reduction activity

The *in vitro* phagocytic effects of different concentrations of ENA extract on the reduction of NBT dye activity of macrophages are presented in Table 1. The effect of ENA extract showed significant stimulation on NBT reduction at 832 µg/ml (SI 1.540), 416 µg/ml (SI 1.379), 208 µg/ml (SI 1.367), 104 µg/ml (SI 1.315), 52 µg/ml (SI 1.316), 26 µg/ml (SI 1.215), and 13 µg/ml (SI 1.161), and ENE extract showed significant stimulation

Table 1: *In vitro* effect of ENA on release of nitric oxide, NBT reduction, lysosomal, and myeloperoxidase activity of isolated macrophages

Sr. No.	Concentration (µg/ml)	SI			
		NO	NBT	Lysosomal	MPO
1	Control	1.000±0.01	1.000±0.01	1.000±0.03	1.000±0.01
2	ENA (832)	1.739±0.06	1.540±0.03	1.404±0.07	1.728±0.01
3	ENA (416)	1.551±0.03	1.379±0.01	1.399±0.07	1.695±0.02
4	ENA (208)	1.452±0.05	1.367±0.01	1.388±0.05	1.725±0.01
5	ENA (104)	1.331±0.03	1.315±0.03	1.676±0.11	1.730±0.02
6	ENA (52)	1.261±0.05	1.316±0.04	1.391±0.03	1.601±0.04
7	ENA (26)	1.205±0.04	1.215±0.02	1.420±0.05	1.541±0.07
8	ENA (13)	1.094±0.03	1.161±0.03	1.467±0.04	1.427±0.04
9	ENA (6.5)	1.009±0.04	1.023±0.01	1.483±0.06	1.375±0.03
10	PHA (10)	2.012±0.05	1.910±0.02	1.971±0.07	1.788±0.02

SI: Stimulation index, NO: Nitric oxide, NBT: Nitroblue tetrazolium, MPO: Myeloperoxidase, PHA: Phytohemagglutinin, ENA: *Eulophia nuda* aqueous

Table 2: *In vitro* effect of ENE on release of nitric oxide, NBT reduction, lysosomal, and myeloperoxidase activity of isolated macrophages

Sr. No.	Concentration (µg/ml)	SI			
		NO	NBT	Lysosomal	MPO
1	Control	1.000±0.02	1.000±0.01	1.000±0.01	1.000±0.01
2	ENE (832)	1.884±0.04	1.662±0.03	1.513±0.004	1.770±0.08
3	ENE (416)	1.831±0.04	1.608±0.02	1.397±0.02	1.758±0.08
4	ENE (208)	1.809±0.03	1.447±0.02	1.394±0.01	1.738±0.07
5	ENE (104)	1.671±0.05	1.293±0.02	1.577±0.01	1.510±0.05
6	ENE (52)	1.545±0.03	1.183±0.02	1.500±0.07	1.572±0.02
7	ENE (26)	1.483±0.02	1.154±0.02	1.394±0.02	1.534±0.05
8	ENE (13)	1.332±0.02	1.087±0.01	1.462±0.06	1.542±0.04
9	ENE (6.5)	1.138±0.03	1.013±0.02	1.417±0.04	1.403±0.07
10	PHA (10)	2.013±0.02	1.910±0.01	1.971±0.07	1.965±0.01

SI: Stimulation index, NO: Nitric oxide, NBT: Nitroblue tetrazolium, MPO: Myeloperoxidase, PHA: Phytohemagglutinin, ENE: *Eulophia nuda* ethanolic

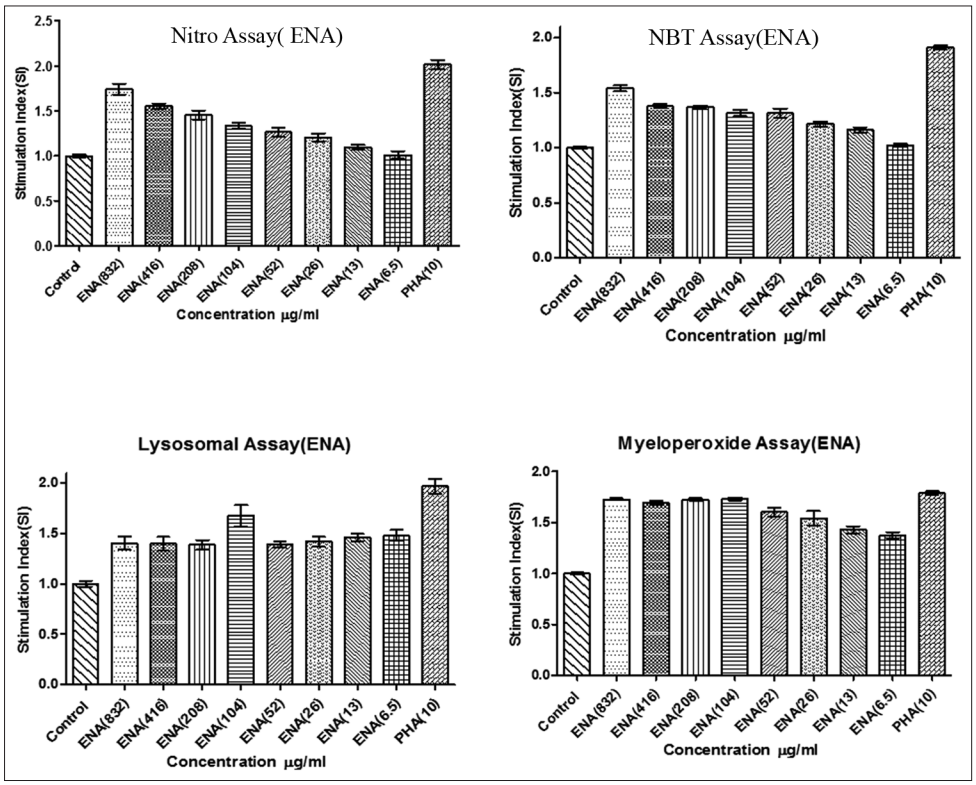

Fig. 1: *In vitro* effect of *Eulophia* nuda aqueous on release of nitric oxide, nitroblue tetrazolium reduction, *lysosomal*, and *myeloperoxidase* activity of isolated macrophages

at 832 µg/ml (SI 1.662), 416 µg/ml (SI 1.608), 208 µg/ml (SI 1.447), 104 µg/ml (SI 1.293), 52 µg/ml (SI 1.183), and 26 µg/ml (SI 1.154). Positive control, PHA showed significant stimulation of NBT reduction (SI 1.910). The result of this study is shown in Tables 1 and 2 as well as Figs. 1 and 2.

Cellular lysosomal activity assay

The effect of ENA extract showed significant stimulation on lysosomal enzyme release at 832 µg/ml (SI 1.404), 416 µg/ml (SI 1.399), 208 µg/ml (SI 1.388), 104 µg/ml (SI 1.676), 52 µg/ml (SI 1.391), 26 µg/ml (SI 1.420), 13 µg/ml (SI 1.467), and 6.5 µg/ml (SI 1.483), and in ENE extract showed significant stimulation at 832 µg/ml (SI 1.513), 416 µg/ml (SI 1.397), 208 µg/ml (SI 1.394), 104 µg/ml (SI 1.577), 52 µg/ml (SI 1.500), 26 µg/ml (SI 1.394), 13 µg/ml (SI 1.462), and 6.5 µg/ml (SI 1.417); positive control, PHA showed significant stimulation ($p<0.05$) of NBT reduction (SI 1.971). The result of this study is shown in Tables 1 and 2 as well as Figs. 1 and 2.

Myeloperoxidase activity assay

The effect of ENA and ENE extract on myeloperoxidase activity of macrophages is presented in Table 1 and Fig. 1, respectively. The ENA extract showed significant ($p<0.05$) stimulation of myeloperoxidase activity of macrophages at 832 µg/ml (SI 1.728), 416 µg/ml (SI 1.695), 208 µg/ml (SI 1.725), 104 µg/ml (SI 1.730), 52 µg/ml (SI 1.601), 26 µg/ml (SI 1.541), 13 µg/ml (SI 1.427), and 6.5 µg/ml (SI 1.375); positive control, PHA showed significant stimulation ($p<0.05$) of NBT reduction (SI 1.788) as compared to control wells and in case of ENE extract at 832 µg/ml (SI 1.770), 416 µg/ml (SI 1.758), 208 µg/ml (SI 1.738), 104 µg/ml (SI 1.510), 52 µg/ml (SI 1.572), 26 µg/ml (SI 1.534), 13 µg/ml (SI 1.542), and 6.5 µg/ml (SI 1.403) as compared to control wells. Positive control, PHA showed significant stimulation with SI value of 1.788. The result of this study is shown in Tables 1 and 2 as well as Figs. 1 and 2.

DISCUSSION

Disease-free state may be maintained by stimulation or suppression of the immune system, and the procedure of achieving it is known as immunomodulation. Supportive therapy to conventional chemotherapy can be achieved by agents that activate host defense mechanisms in the presence of an impaired or compromised immune responsiveness [21].

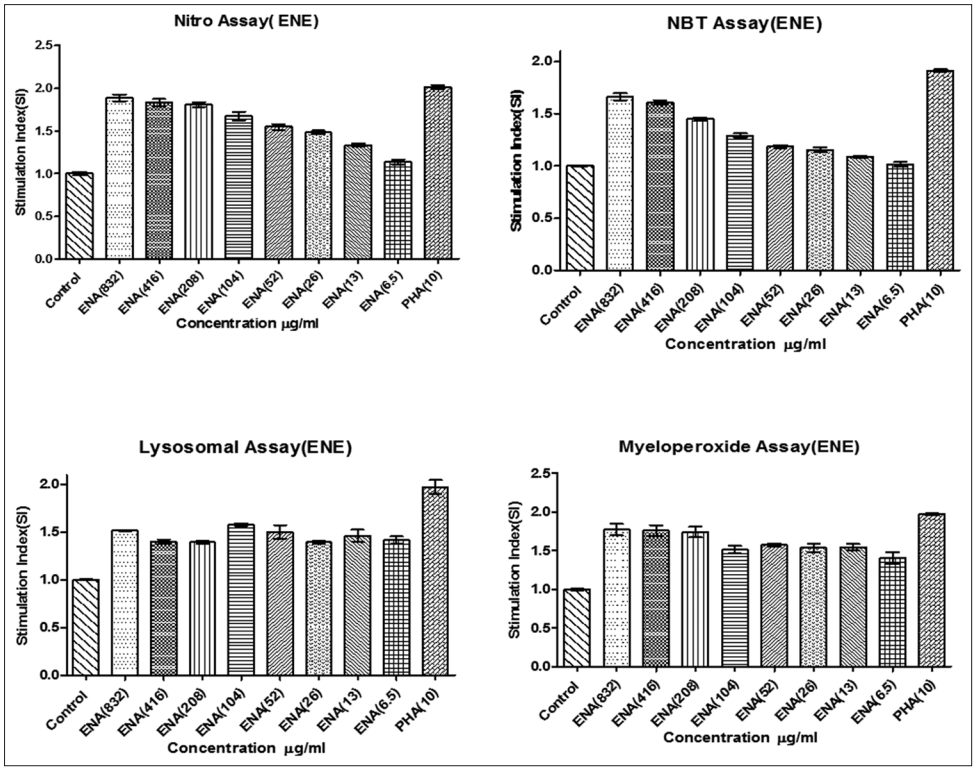

Fig. 2: *In vitro* effect of *Eulophia nuda ethanolic* on release of nitric oxide, *nitroblue tetrazolium* reduction, *lysosomal*, and *myeloperoxidase* activity of isolated macrophages

There is a rising interest in identifying and isolating the immunomodulators from herbal origin ever since there is a possible use of them in the modern medicine system [22]. The main objective of this study was to investigate and demonstrate the immunomodulatory effects of ethanolic and aqueous extract of *E. nuda*.

Macrophages have been known to play an essential role in the protection of the host cells against a wide range of microorganisms and tumors. 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. Reactive nitrogen intermediates, NO, are in interest of researchers because of their antibacterial and antitumor effect [23]. Diverse functions including vasodilatation, inflammation, and neurotransmission are maintained by NO [24]. Increased phagocytosis and bactericidal activity are indicated by a very high NO production, which is supported by the data, presented in Fig. 1. Macrophages kill tumor cells, and by this, they play an important role in defense mechanism against host infection. Higher reduction of 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 *p*-NPP to colored compound by the acid phosphatase of the stimulated macrophages correlates to the extent of degranulation in phagocytosis [25]. 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 [26]. The effect of various concentrations of ENA and ENE extract on the reduction of NBT dye and lysosomal enzyme activity response of macrophages was studied for phagocytic assay. ENA and ENE extract appeared to produce phagocytic stimulation with dose–response relationship in lysosomal enzyme activity evaluation.

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 [27]. The increase in the SI of myeloperoxidase by the exposure of ENA and ENE extract indicates 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 extract at different concentrations ranging between 832 and 6.5 µg/ml for 24 h showed a significant activation of macrophages by modulating the secretion of various mediators including NO, lysosomal enzyme, and myeloperoxidase activity. This suggests that ENA and ENE extract can effectively strengthen innate immunity against foreign particles [28-31].

The process of phagocytosis involves certain body cells, known as phagocytes, which ingests and removes microorganisms, malignant cells, inorganic particles, and tissue debris [29-32]. 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 [30]. Through interaction with lymphocytes, macrophages play an important role in the initiation and regulation of immune response. In view of the pivotal role played by the macrophages, ENA and ENE extract were evaluated for its *in vivo* effect on macrophage phagocytic activity.

CONCLUSION

The studies have demonstrated immunostimulating properties of both the ethanol and aqueous extract of *E. nuda* L. tubers in various *in vitro* experimental methods. Further studies to elucidate the exact immunostimulatory mechanism of *E. nuda* L. need to be explored. *In vivo* studies also need to be done to correlate with the *in vitro* results.

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AUTHOR'S 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. Kanse collected the data, prepared the protocol, conducted the research, and assessed the manuscript. Mr. Diptesh T. Patilprepared 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 interest regarding the publication of this paper.

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