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Effect of alkaloidal fraction of *Elaeocarpus ganitrus* Roxb. seeds on murine *in vitro* immune parameters

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ABSTRACT

The alkaloidal fraction of *Elaeocarpus ganitrus* seeds (EGAF) was prepared and evaluated for *in vitro* effects on murine cells for release of immune mediators and cell proliferation. The EGAF was tested at concentrations ranging between 6.5-832µg/ml. The release of mediators assayed from peritoneal exudates cells (PEC) include nitric oxide (NO), superoxide (NBT reduction), lysosomal and myeloperoxidase. The fraction was also evaluated for cell proliferation in sulforhodamine B (SRB) assay on murine PEC, splenocytes and bone marrow cells. The data was statistically compared by using one way ANOVA followed by Dunnet's multiple comparisons test. The fraction showed significant stimulation (P<0.05) of NBT reduction, NO release and myeloperoxidase activity of PEC cells. The effect on lysosomal enzyme activity was not significant. Significant proliferation (P<0.05) was observed in SRB assay with PEC and bone marrow cells. The effect of EGAF was not significant on proliferation of splenocytes. Positive control, phytohaemagglutinin showed stimulation of release of all the tested mediators and proliferation of immune cells. In conclusion, the EGAF showed *in vitro* stimulation of immune mediators from PEC and proliferation of immune cells.

Keywords: Nitro blue tetrazolium; macrophages; myeloperoxidase; sulforhodamine B

INTRODUCTION

Plants of the genus *Elaeocarpus* have been reported to be of use as traditional medicines, particularly in India. *Elaeocarpus ganitrus* (syn: *Elaeocarpus sphaericus*; Elaeocarpaceae) is a tree found in the Himalayan region of India. The fruits of this plant are commonly known as Rudraksha and have been used in Ayurvedic traditional medicine for the treatment of mental diseases, epilepsy, asthma, hypertension, arthritis and liver diseases [1, 2, 3].

E. ganitrus contains alkaloids, flavonoids, glycosides and steroids. Previous chemical studies of *Elaeocarpus sphaericus* have yielded mainly indolizidine alkaloids. Seven alkaloids including (-)-isoelaeocarpiline, (+)-isoelaeocarpiline, (+)-epiisoelaeocarpiline, (-)-epielaeocarpiline, (+)-epialloelaeocarpiline, (-)-alloelaeocarpiline and pseudoepi-isoelaeocarpiline have been isolated from *E. sphaericus* [4, 5]. Rudrakine, (-)-elaeocarpine, (-)- isoelaeocarpine alkaloids have also been isolated from *E. ganitrus* [1,6]. Since alkaloids are major secondary metabolites present in *E. ganitrus* it was considered worthwhile to evaluate the *in vitro* effects of its alkaloidal fraction for immune response on murine cells.

MATERIALS AND METHODS

Plant material and preparation of the alkaloidal fraction

Authenticated seeds of *Elaeocarpus ganitrus* Roxb were obtained as a gift sample from Rudraksha Research and Testing Laboratory (RRTL), Mumbai, India. The seeds were collected in July 2008. The voucher specimen (No. 2008/07/03) was deposited at the Herbarium of department. The seeds were coarse powdered in cutter and grinding mill. Powdered seeds of *Elaeocarpus ganitrus* (4 kg) were defatted with petroleum ether (60-80^{0}C) and then extracted with boiling methanol by soxhlation. The yield of methanol extract (EGM) was 10.85% w/w and stored in a desiccator. Sodium hydroxide (5%w/v) was added to EGM and was kept on rotary shaker for 2h to convert alkaloids to free bases. In separating funnel chloroform (equal volume) was added to this and shaken intermittently. Chloroform phase containing alkaloids, resins, fats and waxes was separated from aqueous phase containing acids, glycosides, sugars and pigments. To the chloroform phase, dilute HCl (1M) was added (partitioned) and shaken well to convert alkaloids to alkaloidal salts. Then aqueous (acidic) phase (containing alkaloidal salts) and chloroform phase (containing resins, fats and oils) were separated. To the aqueous acidic phase 5%NaOH was added to basify and shaken well to neutralize alkaloids. Alkaloids were partitioned into chloroform in separating funnel by shaking it well. Chloroform layer was evaporated to give mixture of alkaloids of *E. ganitrus* (EGAF). The alkaloids were tested by Dragendroff reagent test [7].

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Animals

Swiss albino mice were procured from Haffkine Bio-pharmaceuticals Ltd., Mumbai. The animals were acclimatized for 10 days before being used for the experiments. They were housed in a room with controlled temperature (23±2^{0}C) and a 12h light/ 12h dark cycle. The animals were fed with standard pellet diet ('Amrut' brand, M/s. Nav Maharashtra Chakan oil mills Ltd., Pune, India) and water *ad libitum.* The experimental protocols were approved by the Institutional Animal Ethics Committee of Institute and conducted according to the guidelines of Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India.

Chemicals

Phytohemagglutinin-M (PHA), nitroblue tetrazolium (NBT) and

sulforhodamine B (SRB) were procured from Sigma Aldrich (St. Louis, MO, USA). Fluid thioglycollate medium, fetal bovine serum (FBS), Streptomycin, penicillin, Roswell Park Memorial Institute (RPMI) 1640 medium, Triton-X-100 and Tris buffer were procured from Himedia Pvt. Ltd. India. All other chemicals used were of analytical grade.

Isolation of peritoneal exudate cells and culture conditions

Peritoneal exudates cells (PEC) were isolated from mice which were injected intraperitoneally (i.p.) with 2 ml of 4% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100mg/ml streptomycin (complete RPMI). Total cell numbers were counted using hemocytometer, and percentages of neutrophils and macrophages were determined by differential counting of Wright-Giemsa-stained cytospins (100–200 cells per sample) using a light microscope. Then the cells were adjusted to required cell count per ml and plated on a 96-well flat-bottom culture plate (Tarsons Products Pvt. Ltd., India) and then incubated for 2h at 37^0C in a 5% CO_2 humidified incubator. After removing the non-adherent cells, the mono-layered cells were treated with 20µl of EGAF fraction (832 – 6.5 µg/ml) dissolved in complete RPMI medium containing 10% DMSO (dimethyl sufoxide) and maintained for 24h at 37^0C in a 5% CO_2 humidified incubator [8, 9]. Following assays were performed on these incubated cells. PHA (10µg/ml) was used as a positive control. All the experiments were performed in triplicate.

Nitrite assay

Nitrite accumulation was used as an indicator of nitric oxide (NO) production in the medium as per the procedure described earlier [10]. Cell-free supernatant (50µl) from 24h incubated PEC ($5x10^5$ cells/ml) was mixed with 50µl of Griess reagent (1% sulfanilamide, 0.1% napthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. The optical density (OD) was measured at 540 nm with a microplate reader (ELX800MS, BioTek Instruments Inc., USA). Nitrite concentrations were determined from standard curve of sodium nitrite in culture conditions. Stimulation index (SI) for nitrite release was calculated as the nitrite concentrations ratio of the treated and control cells.

Nitro blue Tetrazolium (NBT) dye reduction

The NBT dye reduction assay was carried out as per the procedure described earlier [11]. Briefly, 50µl of 0.3% NBT solution in phosphate buffered saline (pH 7.4) (PBS) were added to the 24h incubated PEC (1×10^6 cells/ml) with EGAF. The mixture was further incubated at 37^0C in a 5% CO_2 humidified incubator for 1h. The adherent cells were rinsed vigorously with complete RPMI medium, and washed four times with 200µl Methanol. After air-dried, formazan-deposits were solubilized in 120µl of 2M KOH and 140µl of DMSO. After homogenization of the contents of the wells, the OD was read at 630 nm by using a microplate reader. The NBT reduction stimulation index (SI) was calculated as the OD ratio of the treated and control cells.

Cellular lysosomal enzyme activity

The cellular lysosomal enzyme activity of PEC cells was evaluated by measuring acid phosphatase activity as per method described earlier [10]. Briefly, 24h after incubation at 37°C in humidified 5% CO_2, the medium was removed by aspiration and 20µl of 0.1% Triton X-100 were added to each well. A fter 15 minutes incubation, 100 µl of 10mM *p*-nitrophenyl phosphate (*p*NPP) and 50 µl of 0.1 M citrate buffer (pH 5.0) were added. Further the plates were incubated for 1h and 0.2 M borate buffer (150 µl, pH 9.8) was added. The OD was measured at 405 nm by using a microplate reader. The phagocytic stimulation index (SI) was calculated as the OD ratio of the treated and control PEC cells.

Myeloperoxidase activity assay

This assay was carried out as per the procedure described earlier[12]. Briefly, 24h incubated PEC cells ($5x10^5$cells/ml) were washed three times with fresh complete RPMI medium. Then the mixture (100 µl) of *o*-phenylenediamine (0.4 g/ml) and 0.002% H_2O_2 in phosphate-citrate buffer (pH 5.0) was added to each well. The reaction was stopped after 10 min using 0.1 N H_2SO_4 and OD were measured at 490 nm. The myeloperoxidase stimulation index (SI) was calculated as the OD ratio of the treated and control PEC.

Sulforhodamine B (SRB) assay on isolated murine peritoneal exudates cells

Peritoneal exudates cells (PEC) were isolated as explained above and were cultured (1×10^5cells/well) in complete RPMI and incubated for 2 h at 37ºC in 5% CO_2 atmosphere. The SRB assay was performed as per the procedure described earlier [13]. In brief, the EGAF was dissolved in complete RPMI containing 10% DMSO were added at various concentrations (832-6.5µg/ml) in triplicate. After 24 h incubation, cells were fixed by adding ice-cold 20% trichloroacetic acid (TCA) and incubating for 1 h at 4ºC. The plates were washed with distilled water, air-dried and stained with SRB solution (0.4% w/v in 1% acetic acid) for 30 min at room temperature. Unbound SRB was removed by washing thoroughly with 1% acetic acid and the plates were air-dried. The bound SRB stain was solubilized with 100µl of 10mM Tris buffer (pH-10.0), and the OD was read at 540 nm. The PEC proliferation index (PI) of EGAF was calculated as the OD ratio of the treated and control wells.

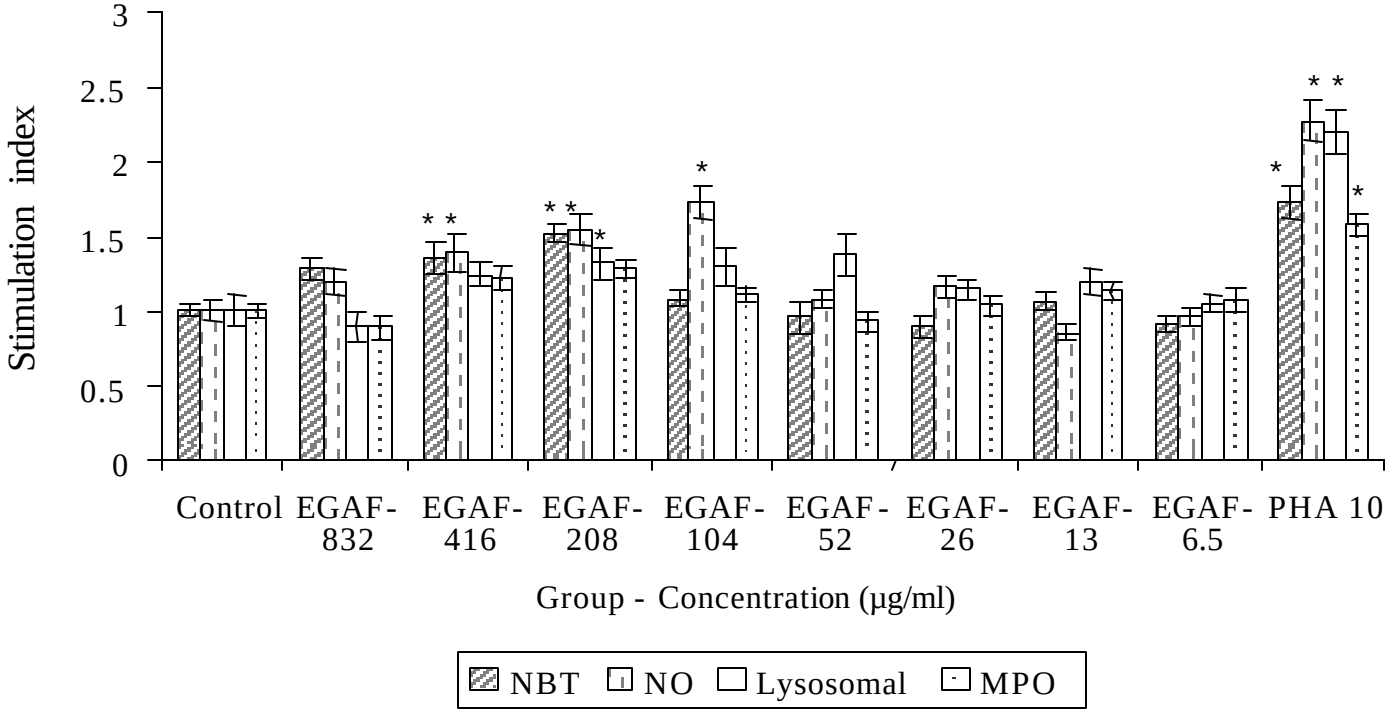
SRB assay on splenocytes

The mice were euthanized by cervical dislocation. The spleens were removed aseptically, stripped of fat and placed in a complete RPMI medium. Single-cell suspensions were obtained by gentle homogenization of mouse spleen in a tissue homogenizer. The spleen cells were separated from the debris; erythrocytes were lysed by hypotonic solution. Isolated cell suspensions were washed three times in the culture medium. Splenocyte viability was assessed by trypan blue dye exclusion [14]. SRB assay for these splenocytes (1×10^5cells/well) was carried out as described above. The splenocytes proliferation index (PI) of EGAF was calculated as the OD ratio of the treated and control wells.

SRB assay on bone marrow cells

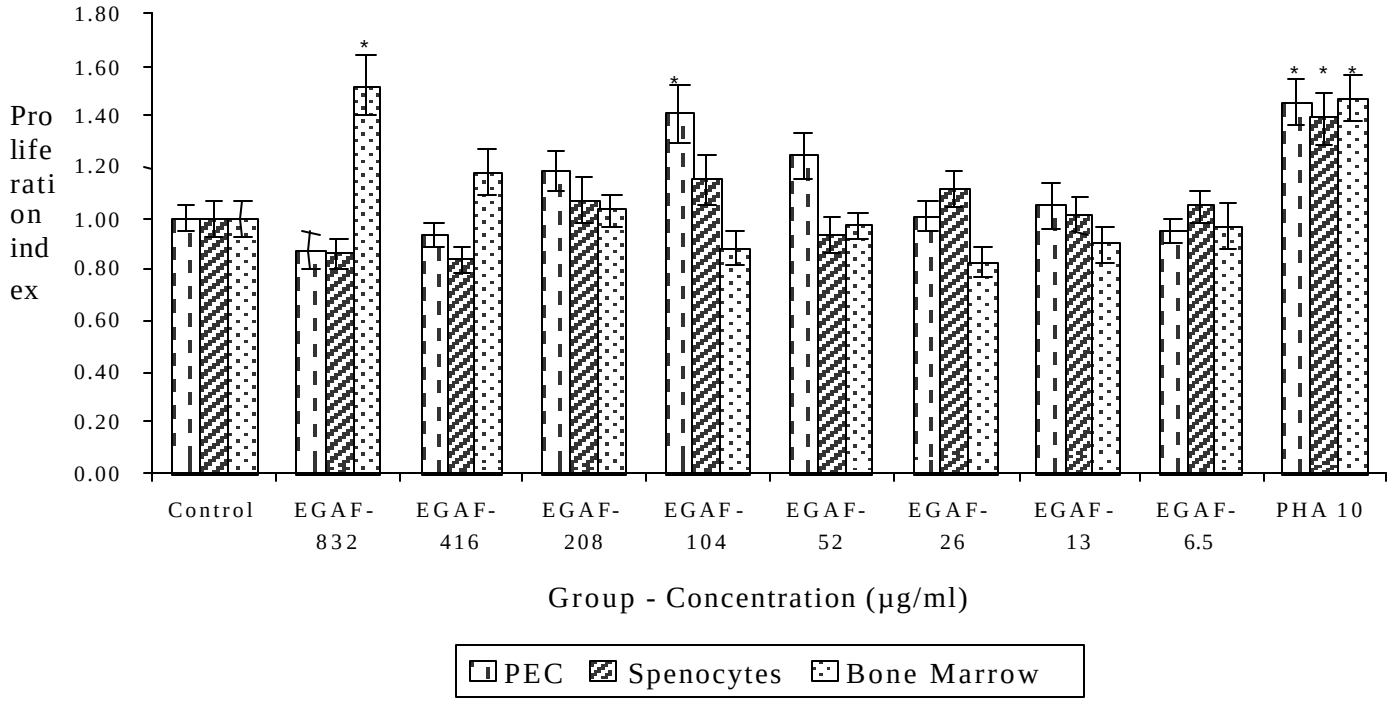
Mice were killed and femur bones were removed aseptically. Cell suspension was prepared by means of flushing. The mixture was centrifuged and the cell pellets were washed twice and resuspended in complete RPMI medium. The cell numbers were determined by a hemocytometer and cell viability was tested by trypan-blue dye exclusion technique [15]. SRB assay for these bone marrow cells (1×10^5cells/well) was carried out as described above. The bone marrow cells proliferation index (PI) of EGAF was calculated as the OD ratio of the treated and control wells.

Figure 1. Effect of alkaloidal fraction of E. ganitrus (EGAF) on release of mediators from isolated peritoneal exudates cells



*The data plotted represent Mean ± SEM (n=3). NBT assay- Nitroblue tetrazolium; NO assay – Nitric oxide; MPO – myeloperoxidase assay. * P<0.05 vs. control group.*

Figure 2. Effect of alkaloidal fraction of E. ganitrus (EGAF) on cell viability (PI) in SRB assay on isolated murine cells



*The data plotted represent Mean ± SEM (n=3). PEC - peritoneal exudates cells, PHA – Phytohemagglutinin. * P<0.05 and ** P<0.01 vs. control group.*

Statistical analysis

Results expressed as Mean ± SEM for triplicate assays. Data were analyzed by one way ANOVA followed by Dunnet's multiple comparisons test using GraphPad InStat software against control samples. Values of $P<0.05$ were the criteria for statistical significance.

RESULTS

Alkaloidal fraction of *Elaeocarpus ganitrus* (EGAF)

The fraction of *Elaeocarpus ganitrus* showed positive test for Dragendroff reagent indicating presence of alkaloids. The yield of the alkaloidal fraction was 0.45%w/w.

Nitrite assay

The ability of macrophages to induce the release of nitric oxide (NO) was measured through nitrite, which is a stable breakdown product of NO. The effect of EGAF on release of nitrite in the culture medium from murine peritoneal cells is presented in Fig. 1. The PEC exposed to EGAF showed significant stimulation ($P<0.05$) at 416µg/ml (SI 1.39), 208µg/ml (SI 1.54) and 104µg/ml (SI 1.73). Positive control, PHA showed significant stimulation ($P<0.05$) of nitric oxide stimulation index (SI) of 2.27.

Nitro blue Tetrazolium (NBT) dye reduction

The *in vitro* phagocytic effect of EGAF on the reduction of NBT dye by isolated PEC cells is presented in Fig. 1. The fraction showed significant ($P<0.05$) stimulation of NBT reduction at 416µg/ml (SI 1.35) and 208µg/ml (SI 1.52). PHA (Positive control) showed significant stimulation of NBT reduction (SI 1.73).

Cellular lysosomal enzyme activity

The effect of EGAF on cellular lysosomal enzyme activity of murine PEC cells is presented in Fig. 1. EGAF has not shown any significant effect on the cellular lysosomal enzyme activity. PHA (positive control) showed significant stimulation of lysosomal enzyme activity (SI 2.19) at 10µg/ml.

Myeloperoxidase activity

The effect of EGAF on myeloperoxidase activity of murine PEC cells is presented in Fig. 1. EGAF showed significant stimulation of myeloperoxidase activity of PEC at 208µg/ml (SI 1.28) as compared to control wells. Positive control, PHA showed significant stimulation ($P<0.05$) with stimulation index (SI) of 1.59.

SRB assay on isolated murine peritoneal exudates cells (PEC)

The effect of EGAF on cell viability of murine peritoneal exudates cells (PEC) was evaluated in SRB assay is presented in Figure 2. Significant proliferation ($P<0.05$) of PEC was observed at 104µg/ml (PI 1.41) of EGAF. At other tested concentrations, the fraction had no significant effect on viability of PEC. The positive control (PHA-10µg/ml) showed significant ($P<0.05$) proliferation of PEC (PI 1.45).

SRB assay on splenocytes

The effect of EGAF on viability of murine splenocytes in SRB assay is presented in Figure 2. EGAF has not shown any suppressive or proliferative effect on murine splenocytes at all the tested concentrations ranging between 6.5µg/ml to 832µg/ml. Positive control, PHA (10µg/ml) showed significant ($P<0.05$) proliferation (PI 1.39) of splenocytes as compared to control wells.

SRB assay on bone marrow cells

The effect of EGAF on viability of murine bone marrow cells was tested in SRB assay and is presented in Figure 2. The fraction produced significant proliferation ($P<0.05$) of these cells at 832µg/ml (PI 0.58) as compared to untreated control well. Positive control, PHA (10µg/ml) showed significant ($P<0.01$) stimulation of splenocytes (PI 1.47) as compared to control wells.

DISCUSSION

The alkaloidal fraction of *Elaeocarpus ganitrus* seeds (EGAF) was prepared from the methanol extract of *E. ganitrus* and evaluated for *in vitro* immune response on isolated immune cells. Thioglycollate medium elicited isolated murine peritoneal exudates cell (PEC) preparations composed of ~30% macrophages, ~ 60% neutrophils, and 7–10 % lymphocytes [9]. These cells are important immune effector cells required in maintaining disease free-state or elevating disturbed immune state. Macrophages actively participate as cellular effectors of non-specific host defense. Antigen stimulation transforms the precursor monocytes into macrophages, which subsequently eliminate pathogens via phagocytosis [16]. Macrophages are the main proinflammatory cells that respond to invading pathogens by releasing many pro-inflammatory molecules, including short-living free radical nitric oxide (NO). NO is synthesized from L-arginine by NO synthase (NOS) in numerous types of cells. In mammals, 3 distinct isoforms of NOS have been cloned: endothelial, neuronal, and inducible NOS (iNOS). Among these isoforms, iNOS plays an important role in the regulation of cytotoxic responses [17]. Agents that modulate the activity of NO may be of considerable therapeutic value [14]. NO mediates diverse functions, including vasodilatation, neurotransmission and inflammation [18]. The alkaloidal fraction has shown stimulation of nitric oxide release from PEC indicating protective property against pathogens.

The formation of insoluble blue formazan by reduction of NBT was used as a probe for superoxide generation, although it is not entirely specific for O_2^- radical [19]. The NBT reduction assay estimates the ability of neutrophils, and macrophages to produce various oxygen radicals viz. O_2^-, OH^-, O_3, H_2O_2. The ability of macrophages to kill the pathogenic microbes is probably one of the most important mechanisms of protection against disease [20]. In lysosomal enzyme activity, the transformation of *p*-NPP to coloured compound by the acid phosphatase of the stimulated macrophages correlates to the extent of degranulation in phagocytosis [21]. The alkaloidal fraction of *E. ganitrus* has shown significant stimulation of NBT reduction and non-significant increase of lysosomal enzyme activity of PEC indicating enhanced phagocytic capability of these cells.

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 [22]. The increase in the stimulation index of myeloperoxidase by the exposure of alkaloidal fraction indicates enhanced defense capability of these cells to pathogenic organisms. The release of immune mediators from peritoneal exudates cells i.e. mainly from macrophages and neutrophils were significantly stimulated by the exposure of alkaloidal fraction of *E. ganitrus* to these cells.

SRB assay is one of the most widely used methods for assessing *in vitro* cell viability. This assay relies on the ability of the SRB to bind to protein components of the cells that has been fixed to tissue culture plates by trichloroacetic acid (TCA). SRB is a bright

pink aminoxanthene dye with two sulphonic groups that bind to basic amino acid residue under mild acidic conditions, dissociate under basic conditions. Washing with water then looses the disulfide bond of protein and make it free to bind with SRB dye. At last, addition of Tris-buffer makes medium alkaline, leads to release of SRB dye from disulfide bond and give pinkish red color which is then measured by ELISA plate reader. As the binding of SRB is stoichiometric, the amount of the dye extracted from stained cells is directly proportional to the cellmass[13]. Murine isolated peritoneal exudate cells, splenocytes and bone marrow cells when exposed to the alkaloidal fraction of *E. ganitrus* (EGAF) and assayed for SRB assay.

The viability of PEC containing mainly macrophages and bone marrow cells was stimulated by EGAF indicating its immunostimulant nature at these concentrations. EGAF has shown no effect on viability of splenocytes at all the tested concentrations. Phytohemagglutinin (PHA), the positive control used in the experiments showed stimulation of immune parameters in all these assays indicates stimulation of immune system *in vitro*. Considering these effects, the alkaloidal fraction *E. ganitrus* may be proved as an immunostimulator for application. Further detailed studies are necessary to carry out with different cells and cell lines to explore its immunomodulatory potential.

CONCLUSION

In conclusion, our results of in vitro assays suggests, immunostimulant effects of the alkaloidal fraction *E. ganitrus.* Further studies with animal models are necessary to clarify how this modulation occurs and to what extent it occurs *in vivo*.

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