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EVALUATION OF THE IMMUNOMODULATORY PROPERTIES OF *EUPHORBIA TRIGONA* -AN *IN VITRO* STUDY

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ABSTRACT

Background: The use of *Euphorbia trigona* is well documented in the traditional Indian medicinal system of Ayurveda. Four organic extracts of its latex and aerial parts, selected on the basis of primary bioactivity, *viz* anti swarming property, were evaluated for their effect on cells involved in both innate and adaptive immunity. Results: A remarkable increase in the extent of neutrophil extra cellular traps (NETs) formation by neutrophil polymorhonuclear granulocytes as visualized by fluorescence microscopy was observed in the presence of the extracts. The stimulation index for freshly isolated human lymphocytes as well as leukemic T cell line Jurkat J6 treated with *E. trigona* extracts ranged from 1.214 ± 0.23 to 2.667 ± 0.15, showing a significant ($p<0.05$) stimulatory effect of the extracts on lymphocytes. TNF α synthesis was not induced by the extracts. Conclusion: The results obtained in the present work indicate that *E. trigona* cotains bioactive components that stimulate both innate and adaptive immunity. The toxicity evaluation studies using normal mouse fibroblast cell line L929 showed that the *E. trigona* extracts are non-toxic at immunostimulant doses, validating its use in Ayurveda.

INTRODUCTION

The genus *Euphorbia* comprising about 8910 species[1] is one of the most divergent genera in the plant kingdom. It is distributed in both temperate and tropical regions and several species are used in folk medicine the world over [2,3]. Many *Euphorbia* species such as *E.segetalis, E. thymifolia, E. kansui, E. tirucalli* have been reported to possess antimicrobial, antiviral and anticancer properties [4,5,6] and a variety of bioactive phytochemicals such as tannins, flavonoids, terpenoids and lactones have been isolated from these plants[7,8,9,10]. A few species have been investigated for their immunomodulatory activity and both immunosuppressive and immunostimulatory activities have been reported[11,12]. However, no work has been done on the anti-infective and immunomodulatory properties of *E. trigona* which was selected for the present study on the basis of an ethno pharmacological rationale.

E. trigona is prescribed in the traditional Indian medicinal system of Ayurveda for the treatment of respiratory infections, urinary tract infections and gonorrhea. A previous study in our lab on the latex of this plant has revealed that it has remarkable anti-swarming properties against *Proteus mirabilis* and *Pseudomonas aeruginosa*[13]. Swarming is a rapid and coordinated translocation of a bacterial population over solid surfaces, and known to be associated with hyper expression of microbial virulence factors resulting in an elevated resistance to antibiotics. Apart from infections, *E. trigona* is also used to treat tumors and warts, intestinal parasites, rheumatoid arthritis, hepatitis, inflammation etc. and is known to be extremely effective. Its efficacy in such a diverse range of ailments suggests that it may possess beneficial immunomodulatory properties which may lead to stimulation of the immunological defense mechanism.

Immunostimulants are envisaged to enhance the host resistance against not only infections but also against cancer, allergy and autoimmune disorders. These compoundss are inherently non specific in nature but they can act through both the innate and adaptive arms of the immune system. The neutrophil polymorphonuclear granulocytes (PMNs) are the major component of the innate immune response. They form the first line of defense against an invading pathogen or its virulence factors- ingesting and degrading foreign bodies by the process of phagocytosis[14]. Apart from this well known function, it has been recently reported that activated neutrophils are capable of forming extra cellular structures mainly composed of their chromatin material. These structures, referred to as Neutrophil Extra cellular Traps or NETs[15], are involved in enhancing the efficiency of neutrophils to remove bacteria/ viruses or their virulence factors, ensuring that those bacteria which are not phagocytosed are killed extracellularly. Therefore any substance that could induce NET formation can be of immense therapeutic potential.

The T-lymphocytes are an integral part of the adaptive immune system and their activation/ proliferation along with cytokine production on stimulation with antigens plays an important role in mopping up pathogens and their virulence factors [16].

In order to rationally apply immunostimulants of plant origin, verification of clinical efficacy and research on mechanisms underlying immunomodulation are necessary. In the present study, the effect of *E. trigona* latex as well as whole plant extracts on *in vitro* lymphocyte proliferation, TNF α secretion, and NETs formation was studied to explore the possible immunomodulatory effects of this plant. This may serve to establish a scientific basis for its time- tested efficacy in the treatment of several ailments. A preliminary phytochmical analysis of the extracts was also carried out.

MATERIALS AND METHODS

Plant material and extract preparation:

Whole plant extracts: The aerial parts of *E. trigona* were washed, air dried in the shade and ground to a fine powder. 5 grams of the powder was subjected to successive extraction in a Soxhelet apparatus using solvents (150ml each) with increasing polarity, *viz.* petroleum ether, chloroform, ethyl acetate and ethanol. All the extracts were dried in a vacuum concentrator, the residues resuspended in sterile 10% DMSO (BIOGENE Reagents Inc., CA, USA) and used for the assays.

Latex extracts: 0.5 ml of freshly withdrawn latex was extracted twice with 5 ml aliquots of each solvent. The two extracts were pooled, dried in a vacuum concentrator, resuspended in sterile 10% DMSO and used for the assays.

Controls were set up with each assay using 10% DMSO.

Phytochemical Analysis and primary bioactivity determination: Qualitative analytical methods were used to identify the phytoconstituents present in the whole plant extracts. Tests were carried out for sterols, alkaloids, tannins, flavonoids and saponins[17,18].

The primary bioactivity of the extracts was determined by the anti- swarming assay[19] using *Proteus mirabilis* and *Pseudomonas aeruginosa* obtained from Institute of Microbial Technology, Chandigarh, India. Briefly, swarm agar [13] plates containing the extracts (50µg/ml) were centrally inoculated with 5µl of an overnight broth culture of the test organisms and incubated at 37^0C for 24 hours. Plates containing an equal volume of 10% DMSO served as control. The reduction in the diameter of the swarm zone in the presence of the extracts as compared to the solvent control plates was noted and expressed as % reduction.

The anti- bacterial activity of the extracts was determined by well diffusion method using Mueller-Hinton agar.

Immunomodulatory effects: The lymphocyte proliferation assay was performed using leukemic T cell line Jurkat J6 (obtained from National Centre for Cell Sciences, Pune, India) as well as human peripheral blood lymphocytes (PBL) and the NETs formation assay was performed using freshly cultured human PMNs to assess modulation of the adaptive and innate immune system respectively *in vitro*

PMN and PBL Isolation:

Neutrophil polymorphonuclear granulocytes (PMN) and PBLs were isolated according to the standard mononuclear leukocyte separation method by Boyum[20] which was modified for neutrophil separation by Ferrante and Thong[21]. Heparinized whole blood was layered on top of a density gradient material (Ficoll/Hypaque) and subjected to a centrifugation at 1500rpm for 30min at 4ºC. Since erythrocytes (RBC) and PMN are denser than the gradient material, they penetrate the gradient material and sediment to the bottom of the centrifuge tube. The buffy coat was carefully aspirated and used for PBL culture in RPMI 1640 medium containing 10% fetal bovine serum (FBS).The RBC and granulocyte pellet was resuspended in Hanks balanced salts solution (HBSS) and overlaid on 5% Dextran in phosphate buffered saline (PBS). RBCs were allowed to sediment to the bottom of the centrifuge tube by keeping the centrifuge tube at 45º angle for 45 min at 4ºC. The PMN-rich supernatant was carefully aspirated; the cells were washed with HBSS (Invitrogen, Carlsbad, CA, USA) and resuspended in RPMI 1640(Invitrogen) for NETs assay.

The cell viability determined by Trypan blue (MP Biochemicals, Vannes, France) dye exclusion test was over 95%.

Lymphocyte proliferation assay and cytokine production: This assay was performed using leukemic T cell line Jurkat J6 as a model cell line and freshly cultured human peripheral blood lymphocytes (PBLs). The MTT assay for mitochondrial activity was used to estimate lymphocyte proliferation [22] in the presence/absence of *E. trigona* whole plant as well as latex extracts. Briefly, PBLs or Jurkat cells were suspended (1-5 x 10^5cells/ml) in complete RPMI 1640 medium containing 10% fetal bovine serum. The extracts were added in a concentration gradient ranging from 5-80 µg/ml. PHA and 10% DMSO were used as control. The cells were cultured in triplicate at 37^0C for 24-48 hours in a humidified atmosphere containing 5% CO2. Thereafter, the spent medium was replaced by fresh RPMI medium containing 5% FBS and MTT (1mg/ml), and incubated at 37^0C for 4hours. The MTT-formazan crystals were solubilised in DMSO and lymphocyte proliferation was assessed by spectrophotometric measurements of O.D at

570 nm in an ELISA reader. The absorbance values were used to calculate the stimulation index by the formula

$$SI = \text{O.D of sample - O.D of control / O.D of control}$$

The spent media or culture supernatants were reserved to qualitatively estimate TNF α production [23] using the cell line L929(obtained from National Centre for Cell Sciences, Pune, India) to which it is toxic.

NETs formation: The induction of NETs formation by *E. trigona* whole plant as well as latex extracts was studied as per the protocol described by Brinkman *et al*[24]. Briefly, the NETs induction ability of *E. trigona* extracts was evaluated by exposing the PMNs to extracts at concentrations of 5-80 µg/ml in RPMI 1640 with and without 2% heat inactivated donor serum. The cell suspensions were incubated at 37^0C in a humidified environment with 5% CO2 in air for 30-60 minutes, fixed with methanol, stained with propidium iodide(10-20 µg/ml) in PBS for 10 minutes, washed with HBSS and observed under Motic fluorescent microscope. 25 µM phorbol myristatic acid (PMA) which is known to induce NETs formation was used in the positive control set-up. An uninduced fraction of PMNs in RPMI medium served as the negative control. To confirm the presence of DNA in NETs, separate fractions of PMNs induced with the extracts and with PMA (MP Biochemicals) were briefly exposed to DNase I before fixing with methanol.

Cytotoxicity Evaluation: The toxicity of all the *E. trigona* extracts was evaluated by the MTT assay using the normal mouse fibroblast cell line L929. The cell monolayer was exposed to a concentration gradient of the petroleum ether and ethanol extracts (2-80 µg/ml) supplemented in DMEM medium containing 10% FBS and incubated in triplicate at 37^0C for 24-48 hours in a humidified atmosphere containing 5% CO2. Thereafter, the spent medium was replaced by fresh DMEM medium containing 5% FBS and MTT (1mg/ml), and incubated at 37^0C for 4hours. The formazan crystals were solubilised in DMSO and absorbance measured at 570 nm in an ELISA reader.

Statistical analysis: All experiments were performed in triplicate. Results were expressed as mean$\pm$ standard error. Statistical significance was analyzed using Students *t* test. A *p* value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Phytochemical Analysis and primary bioactivity determination:

Phytochemical analysis was carried to determine the category of the potential bioactive compounds. The extracts showed the presence of sterols, alkaloids, tannins, flavonoids and saponins (Table 1). Alkaloids were found to be present in all the extracts while tannins were present in all except the chloroform extract. Sterols were detected only in the petroleum ether and ethanol extracts. Flavonoids were present in the petroleum ether extract only whereas saponins were detected only in the ethanol extract. Although no clear phytochemical structure-bioactivity relationship has been established so far, a relatively high percentage of effective plant derived immunostimulants have been reported to belong to the alkaloids, phenolics, quinones, saponins and terpenoids[25], all of which were found to be present in some or other *E. trigona* extracts.

The anti-swarming property of *E. trigona* whole plant as well as latex extracts was selected to determine primary bioactivity as swarming motility is known to be extremely important in the establishment and spread of infections[26] especially those caused by opportunistic pathogens such as *P. mirabilis* and *Ps. aeruginosa.* All the *E. trigona* whole plant extracts demonstrated remarkable inhibition of swarming motility of both *P mirabilis* and *Ps. aeruginosa* (fig 1). The chloroform and ethanol extracts were found to be equally effective against both the organisms, showing 38-55% reduction in the diameter of the swarm zone. The anti-swarming properties of the latex extracts have been demonstrated in our earlier work[13] and are similar to the whole plant extracts. However, conventional antibacterial activity in the form of growth inhibition zones was not observed at extract concentrations where significant anti-swarming activity was present. Hence it seems likely that though the *E. trigona* extracts are not bactericidal or bacteriostatic they have other bioactivities such as anti- swarming and presumably immunomodulatory properties which appear to play a major role in the control and eradication of pathogens.

Immunomodulatory effects: The immunomodulatory effects of all the extracts on the adaptive and innate immune system were estimated by studying lymphocyte proliferation and NETs formation respectively, in the presence of *E. trigona* whole plant as well as latex extracts.

The immune system is involved in processing of antigens and removal of microorganisms and their virulence factors. Several Ayurvedic formulations are known to be potent immunostimulants and a few studies have been made previously on the effects of some *Euphorbia* species on cells of the immune system. Xu *et al*[27], have studied the immunomodulatory properties of *E. ebracteolata. E. aellenii* and *E pubescens*[28,29] were

also reported to possess positive immunomodulatory properties attributed to their diterpenes. On the other hand, immunosuppressive[30] effects of an ethyl acetate fraction from *E. royleana* have also been observed. However, no previous reports are available on the immunomodulatory properties of *E. trigona.* In the present study we demonstrate that the *E. trigona* whole plants as well as latex extracts have significant beneficial influence on cells of both the innate and adaptive arms of the immune system *in vitro.*

Effect of *E. trigona* on Lymphocyte proliferation: The *in vitro* immunomodulatory properties of the *E. trigona* whole plant and latex extracts at a concentration range of 10-60 µg/ml were tested using T- cell line Jurkat J6 as well as freshly cultured human PBLs. The activation and proliferation of T-lymphocytes is extremely important in the control of microbial infections, especially in conditions where suppression of normal immune response is observed. Comparison of the lymphocyte proliferation in extract treated and untreated cells revealed substantial increase in cell numbers of both Jurkat and PBLs in the presence of petroleum ether and ethanol extracts only. The chloroform and ethyl acetate extracts were not found to stimulate lymphocyte proliferation. The highest SI of 2.667$\pm$ 0.151 was observed in case of PBLs treated with petroleum ether latex extract (20µg/ml). A marked increase in SI was observed up to an extract concentration of 20µg/ml in case of cells treated with petroleum ether and ethanol latex extracts (Fig. 2, Fig. 3), showing a significant ($p<0.05$)dose dependent immunostimulatory effect of *E. trigona* on the adaptive immune system cells *in vitro.* This is in close agreement with the observation of Amirghofran *et al* [31] who have also reported stimulation of lymphocytes by the methanolic extract of *E.cheiradenia.* Negative SI was observed at higher latex extract concentrations of 40-60µg/ml indicating their potency at low doses and cytotoxicity at higher concentrations. *Euphorbia* latex is known to be toxic causing skin irritation and is administered carefully by practitioners of Ayurvedic medicine. The SI of both Jurkat and PBLs treated with whole plant extracts (Fig. 4, Fig. 5) were generally found to be higher than cells treated with latex extracts. The maximum SI s were observed for Jurkat as well as PBLs treated with petroleum ether and ethanol extracts at an extract concentration of 10µg/ml, with a significant($p<0.05$) dose dependent decrease in SI as the cells were exposed to an increasing concentration gradient of extracts from 10-60 µg/ml. However, negative SIs were not observed in case of cells treated with whole plant extracts at higher extract concentration. Thus, the whole plant extracts appear to be far less toxic than latex extracts although they too possess potent lymphoproliferative properties. Studies on other *Euphorbia* species such as *E. nerifolilia*[32], *E.marginata*[33] and *E. characias* [34] have demonstrated mitogenc activity on murine spleen lymphocytes as well as human T-lymphocytes.

Effect of *E. trigona* on NETs formation: In order to assess the effect of the *E. trigona* extracts on the innate immune system, PMNs were exposed to the extracts *in vitro*. A relatively recently recognized pathogen killing mechanism (NETs formation) was found to be activated in the presence of petroleum ether and ethanol extracts only (Fig. 8a, Fig. 9a).These extracts triggered NETs formation at 10 µg/ml and 20 µg/ml in the presence as well as absence of serum. The PMNs are conventionally known to be involved in the removal of microorganisms by phagocytosis where the invading pathogen is ingested and killed by exposing it to lytic enzymes and reactive oxygen species[35]. This mechanism seems to have its limitations as the PMNs can handle only a few pathogens at a time. The formation of NETs which are composed of their chromatin material enables the PMNs to eliminate higher loads of pathogens. Moreover, the half life of circulating neutrophils is known to be short. This additional mechanism may have evolved to ensure bacterial killing even after the neutrophils' death. Although DNA forms the major structural framework, several proteins such as histones and proteases [36] are associated with the NETs which ensure an efficient killing of the entrapped microbes. NETs are known to be induced by bacterial lipopolysaccharide, IL-8 and PMA [37]. In our previous work [38], we have found that Nisin, a bacterial lantibiotic also induces NETs formation. The petroleum ether and ethanol *E. trigona* extracts (whole plant as well as latex) were found to stimulate NETs formation in isolated human PMNs at extract concentration of 10µg/ml. After stimulation, some PMNs were briefly treated with DNase I before fixing with methanol. None of the stimulated PMNs subjected to DNase I treatment (Fig.8b & Fig.9b) showed NETs, confirming the presence of DNA in the NETs.

NET s formation was not visualized in case of the chloroform and ethyl acetate extract treated PMNs. Interestingly, lymphocyte proliferation too was not observed in case of chloroform and ethyl acetate extract treated PBLs and Jurkat cells. Obviously, the immunostimulant activity cannot be attributed to the alkaloids or tannins which were detected in all the extracts. The phytochemical groups detected only in the petroleum ether and ethanol extracts *viz,* sterols, flavonoids and saponins were responsible for the immunomodulatory properties observed in the present study.

Cytotoxicity assay: Since immunostimulants are often administered over a longer period of time, it must be ensured that they are safe and do not generate adverse side effects. Only the ethanol and petroleum ether extracts (latex as well as whole plant), which demonstrated significant immunomodulatory activity were subjected to an evaluation of thejr cytotoxicity and the IC $_{50}$ values were calculated with the help of the graph (Fig. 10) The IC $_{50}$ values were found to be 18µg/ml, 42µg/ml, 71µg/ml and 79µg/ml for the petroleum ether whole plant extract, petroleum ether latex extract, ethanol latex extract and ethanol whole plant extract respectively. Thus, these extracts are non-toxic to normal mouse fibroblast cell line L929 as well as PBLs and Jurkat at extract concentrations

where considerable immunostimulant activity was observed Moreover, none of the *E. trigona* extracts induced the formation of TNF α which is generally implicated in adverse reactions [39]. Hence these can presumably be considered safe for usage, although further *in vivo* studies and clinical trials are essential.

Thus the overall results of our work indicate the immunostimulatory effect of *E. trigona* on cells of the innate as well as adaptive immune system, in addition to its anti- swarming properties. This dual effect may explain the observed efficacy of this plant in the treatment of several ailments ranging from bacterial and viral infections to parasite infestation, tumors and immune diseases. Medicinal plants are increasingly being used in combination with chemotherapy when mixed infections, nosocomial infections or MDR infections are to be treated. Plant based immunostimulants are also an attractive adjunct in the management of chronic diseases, especially in conditions where there is suppression of normal immune response such as for patients on steroids or those suffering from AIDS. The present investigation into the mechanism of action of *E. trigona* extracts will not only scientifically validate and justify its traditional use in Ayurveda but also help in new drug development/modification of currently used drugs. In conclusion, *E. trigona* has definite potential for further investigation as anti-infective and immunomodulatory agents.

TABLE 1. PHYTOCHEMICAL ANALYSIS OF *E. TRIGONA* EXTRACTS

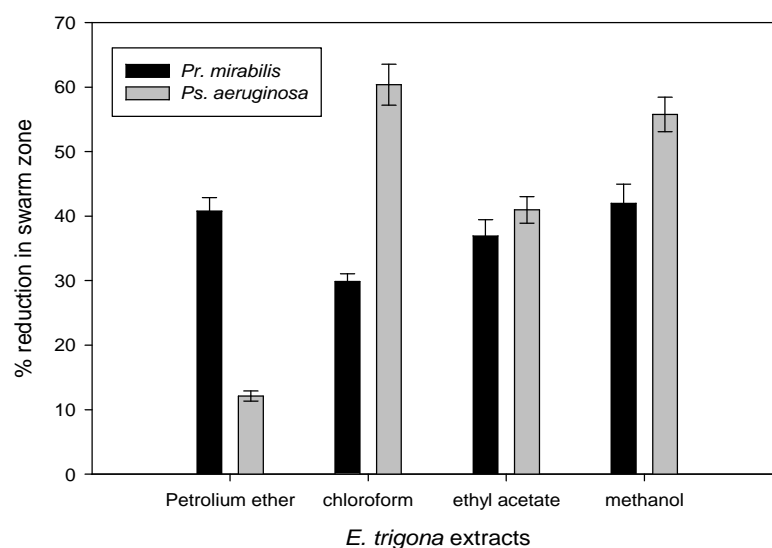| Plant Constituents	Test	Petroleum ether extract	Chloroform extract	Ethyl acetate extract	Ethanol extract
Sterols	Salkowaski test	+	-	-	+
Alkaloids	Mayer's test	+	+	+	+
	Wagner's test	+	+	+	+
Tannins	Ferric chloride test	+	-	+	+
Flavonoids	Shinoda test	+	-	-	-
Saponins	Foam test	-	-	-	+

Fig. 1 Effect of *E. trigona* whole plant extracts on swarm zone of *Pr. mirabilis* and *Ps. aeruginosa*

Fig. 1 Reduction in swarm zone diameter of *Ps. aeruginosa* and *Pr. mirabilis* in the presence of *E. trigona* whole plant extracts. Extract concentration: 50μg/ml
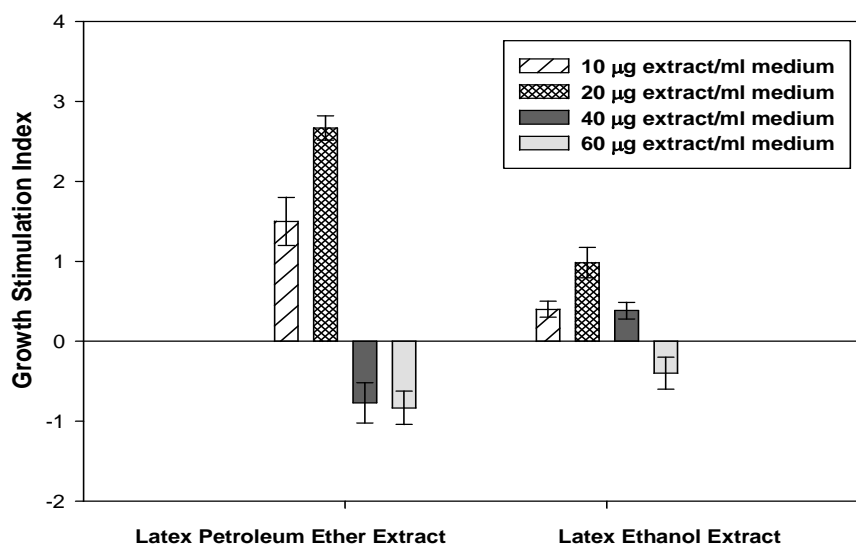
Fig. 2 *In vitro* stimulation of proliferation of Jurkat cells by *E. trigona* latex extracts
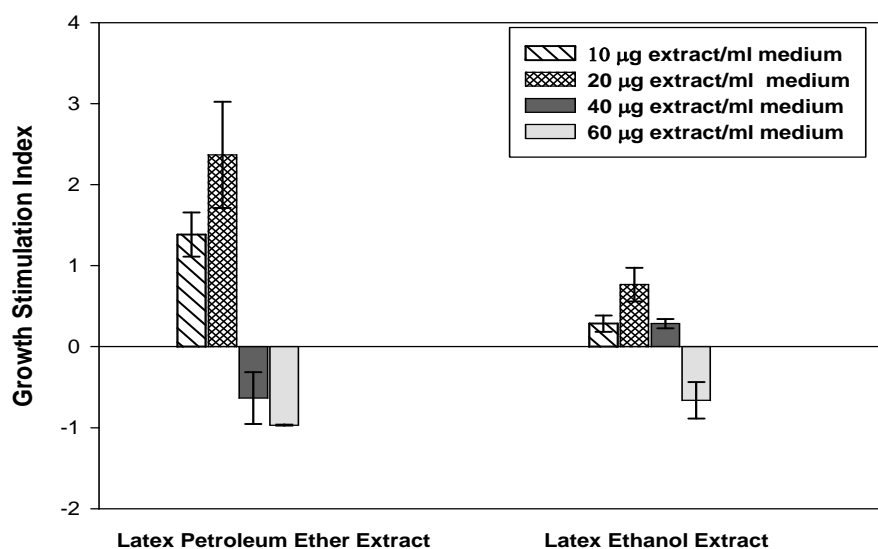
Fig. 2 Lymphoproliferation induced by *E. trigona* latex extracts: Jurkat J6 cell suspensions were incubated in the presence/ absence of a concentration gradient of petroleum ether and ethanol extracts of the latex. After 24 hours, lymphocyte proliferation was estimated by the MTT assay and the SI calculated using O.D values of treated and control set-ups. Data represent means $\pm$ SEM of triplicate determinations.
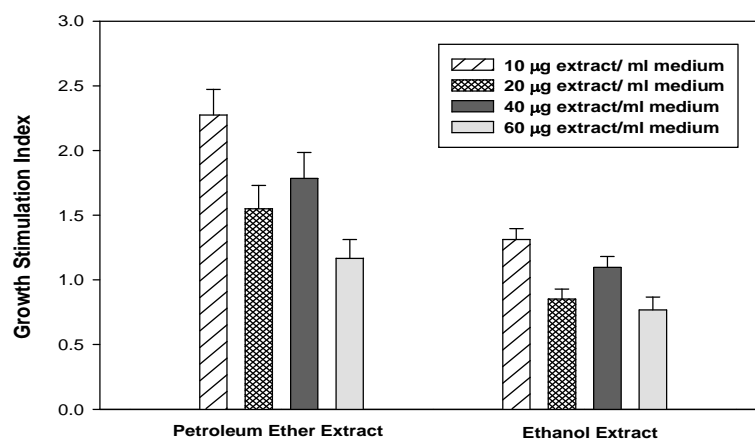
Fig. 3 *In vitro* stimulation of proliferation of human lymphocytes by *E. trigona* latex extracts

Fig. 3 Lymphoproliferation induced by *E. trigona* latex extracts: PBL cell suspensions were incubated in the presence/ absence of a concentration gradient of petroleum ether and ethanol extracts of the latex. After 24 hours, lymphocyte proliferation was estimated by the MTT assay and the SI calculated using O.D values of treated and control set-ups. Data represent means $\pm$ SEM of triplicate determinations
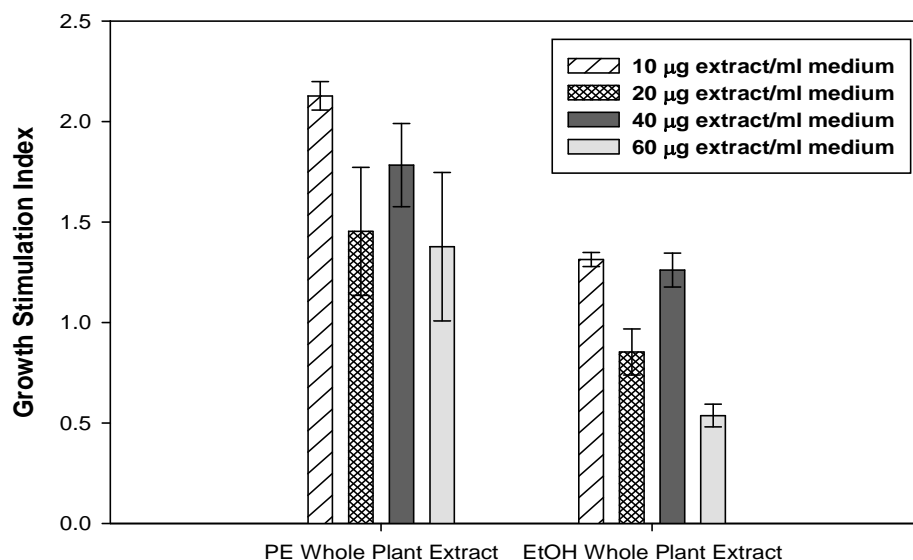
Fig. 4 *In vitro* stimulation of proliferation of Jurkat cells by *E. trigona* whole plant extracts

Fig. 4 Lymphoproliferation induced by *E. trigona* whole plant extracts: Jurkat J6 cell suspensions were incubated in the presence/ absence of a concentration gradient of petroleum ether and ethanol extracts of the whole plant. After 24 hours, lymphocyte proliferation was estimated by the MTT assay and the SI calculated using O.D values of treated and control set-ups. Data represent means $\pm$ SEM of triplicate determinations.

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Fig. 5 *In vitro* **stimulation of human lymphocytes by** *E. trigona* **whole plant extracts**

Fig. 5 Lymphoproliferation induced by *E. trigona* whole plant extracts: PBL cell suspensions were incubated in the presence/ absence of a concentration gradient of petroleum ether and ethanol extracts of the whole plant. After 24 hours, lymphocyte proliferation was estimated by the MTT assay and the SI calculated using O.D values of treated and control set-ups. Data represent means $\pm$ SEM of triplicate determinations.
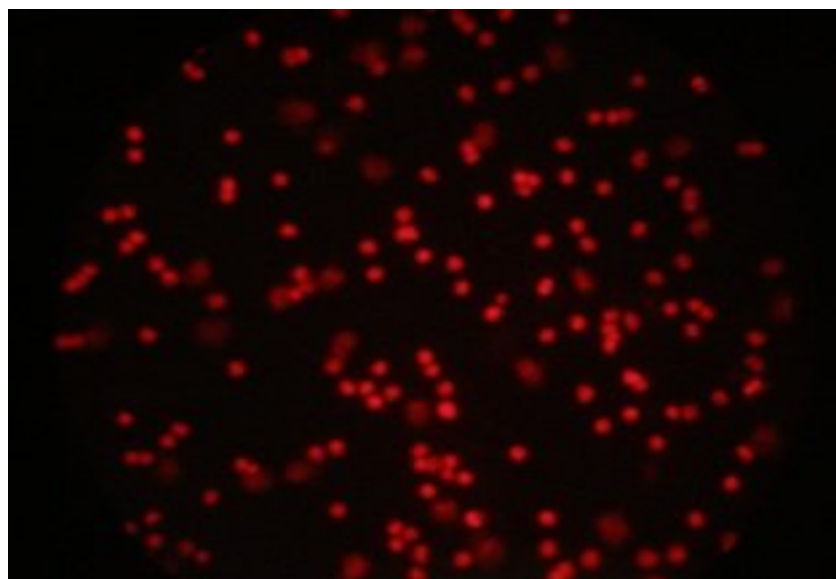
Fig. 6   PMNs treated with 10% DMSO (Solvent control)
Fig. 6 Visualization of NETs induction by fluorescence microscopy: PMNs exposed to 10% DMSO do not form NETs.
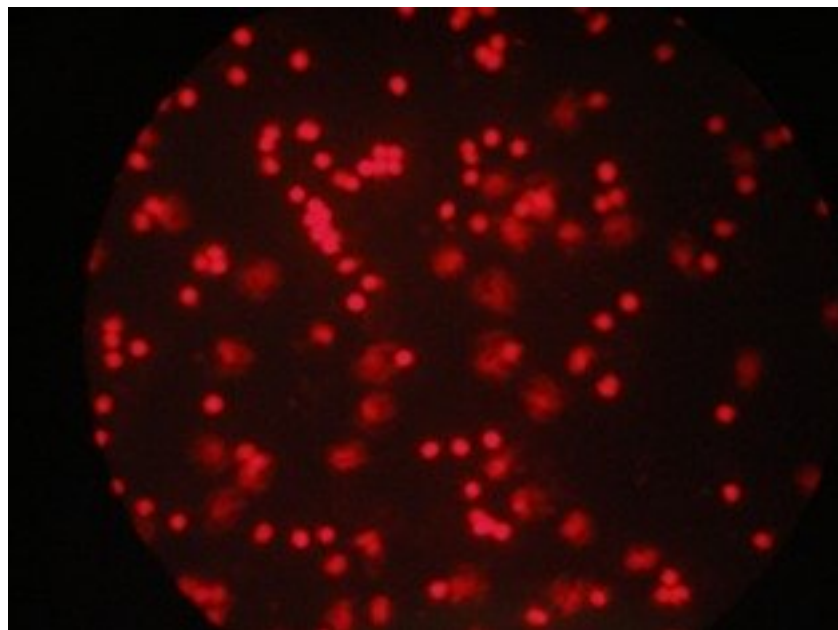
Fig.7   PMNs treated with PMA (Positive control)

Fig. 7 Visualization of NETs induction by fluorescence microscopy: NETs formation induced in PMNs treated with 25 µM PMA, a known NETs inducer
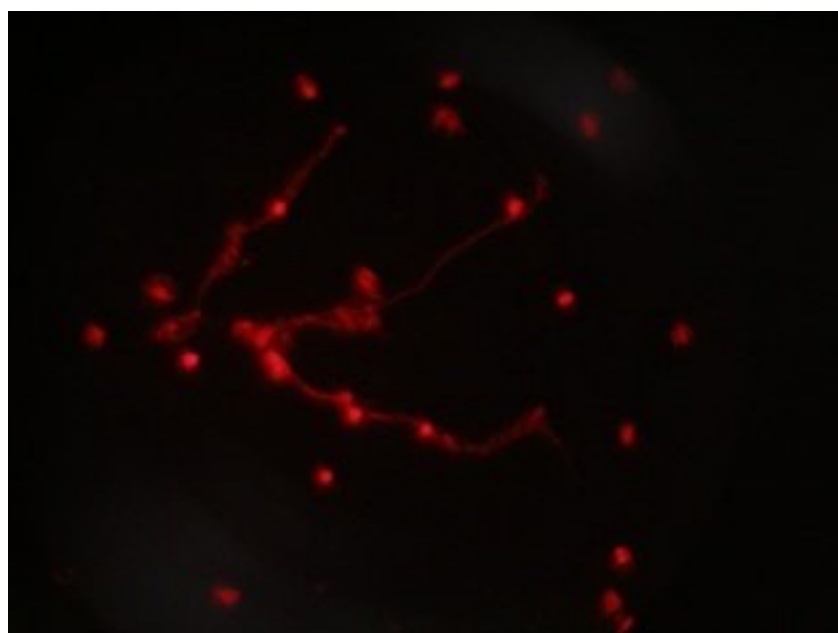

Fig. 8(a)   PMNs treated with Ethanol extract

Fig.8(a) Visualization of NETs induction by fluorescence microscopy: A remarkable increase in the extent of NETs formation in the presence of *E. trigona* ethanol extracts
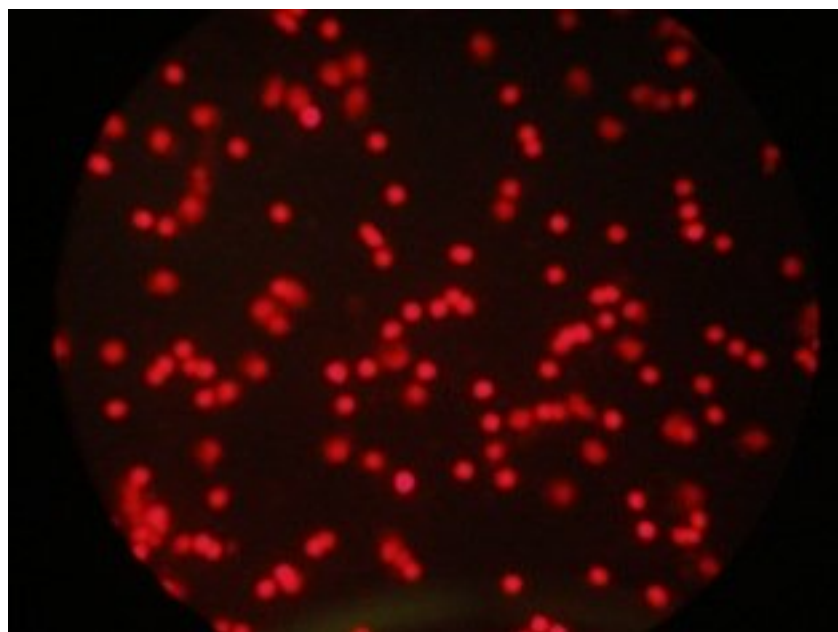
Fig. 8 (b)   PMNs treated with Ethanol extract after DNase I treatment

Fig.8(b) Visualization of NETs induction by fluorescence microscopy: Absence of NETs in PMNs exposed to *E. trigona* ethanol extracts and treated with DNase I before fixing
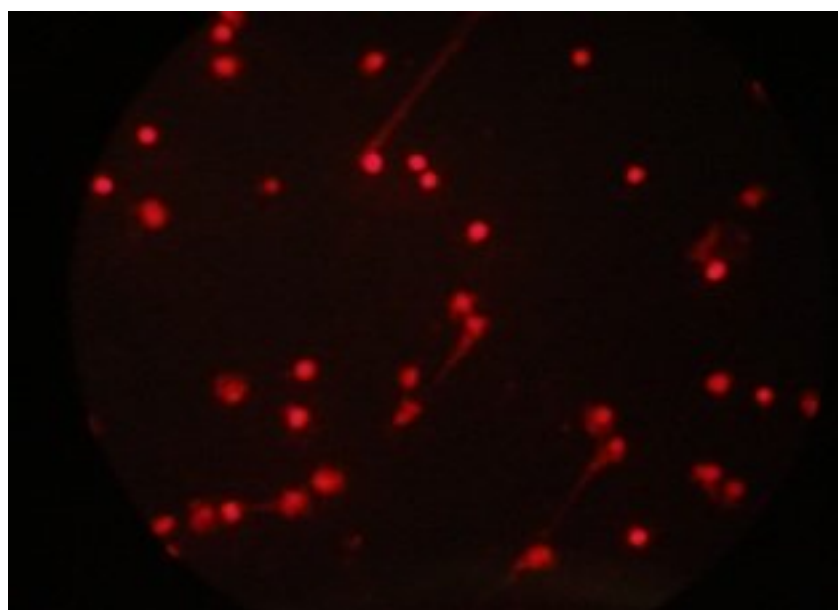

Fig. 9(a)   PMNs treated with petroleum ether extract

Fig. 9(a) Visualization of NETs induction by fluorescence microscopy: A remarkable increase in the extent of NETs formation in the presence of *E. trigona* petroleum ether extracts
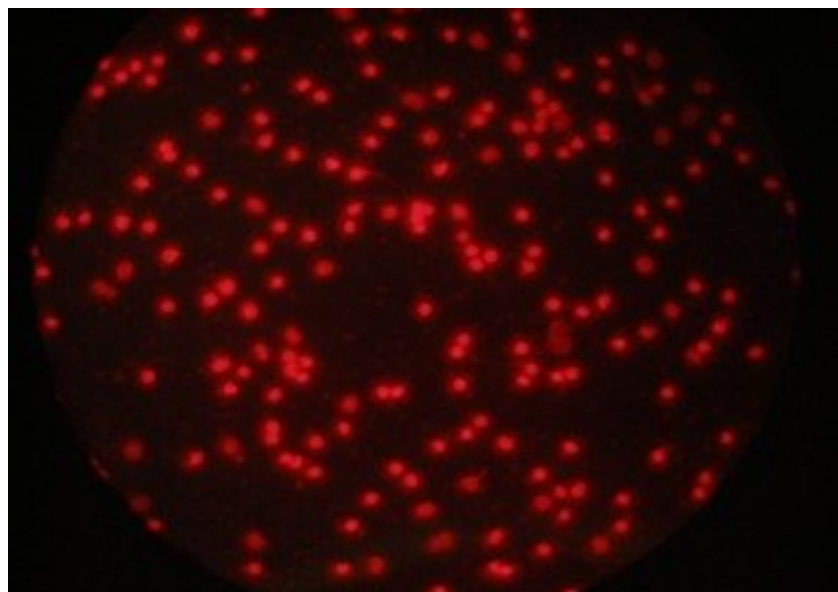
Fig. 9(b) PMNs treated with petroleum ether extract after DNase I treatment
Fig. 9(b) Visualization of NETs induction by fluorescence microscopy: Absence of NETs in PMNs exposed to *E. trigona* petroleum ether extracts and treated with DNase I before fixing

Fig. 10 Effect of *E. trigona* extracts on L929

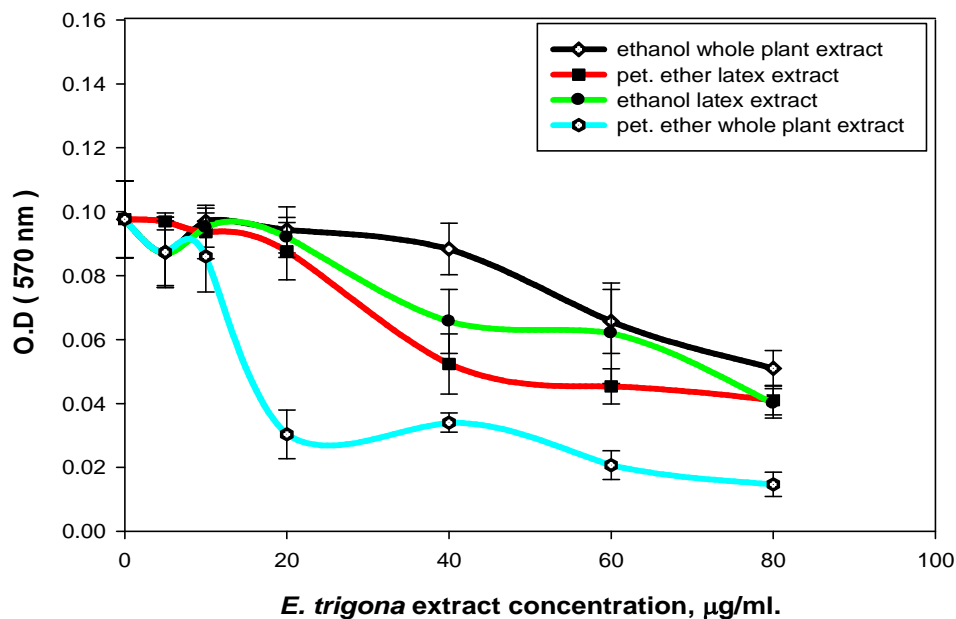

E. trigona extract concentration, μg/ml.

Fig. 10 Cytotoxicity assay: MTT-based cytotoxicity evaluation of the petroleum ether and ethanol (latex as well as whole plant) extracts of *E. trigona* using L929, showing reduction in cell viability with an increase in extract concentration as reflected in the decrease in O.D values. Data represent means $\pm$ SEM of triplicate determinations.

ACKNOWLEDGEMENTS

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