WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 5, 1402-1413. Research Article ISSN 2277– 7105

ASSESSMENT OF CYTOTOXIC EFFECT OF METHANOLIC EXTRACTS OF *LEUCAS ASPERA* AND *TAGETES ERECTA* USING CULTURED HUMAN MONONUCLEAR CELLS

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Article Received on
19 March 2017,
Revised on 09 April 2017,
Accepted on 29 April 2017
DOI: 10.20959/wjpr20175-8460

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ABSTRACT

Natural products have been the most successful source of potential drug leads. Natural products have been used since ancient times and in folklore for the treatment of many diseases and illnesses. Peripheral Blood Mononuclear Cells (PBMC) was engaged for assessing the toxicity of various concentrations (100 – 500 µg/ml) of methanolic leaf extract of *L. aspera* and methanolic flower extract of *T. erecta*. Trypan blue exclusion assay was performed for different time peroid 24, 48 and 72 h. Results of the present study suggest that the lymphocytes when treated with (100 µg/ml and 200 µg/ml) *L. aspera*, the percentage of viability was 71.84 ± 2.91% and 66.66 ± 5.36% and 69.5 ± 0% and 66 ± 9.61% after incubating at 24 and 48 h respectively. In the case of *T. erecta*, percentage of cell viability upon treatment with 100 µg/ml and 200 µg/ml of methanolic extract at 24 and 48 h was found to be 90 ± 0% and 81.88 ± 0% (24 h) and 81.8 ± 0% and 81.2 ± 1.05% (48 h) respectively. Hemolysis assay was performed on red blood cells and cells treated with *L. aspera* extract (100 µg/ml and 200 µg/ml) did not show any increase in the absorbance at 540 nm. Whereas, cells treated with *T. erecta* at a 500 µg/ml exhibited only marginal increase in the absorbance value (0.246) when compared with

100 µg/ml (0.116). The above results suggest that the methanolic extract of did not exhibit toxic effects even at the concentration of 200 µg/ml**.**

KEYWORDS: *Leucas aspera, Tagetes erecta,* Peripheral Blood Mononuclear Cells (PBMC), Tryphan blue, Cell viability, Menthanolic extract.

INTRODUCTION

The need for new and useful compounds to provide assistance and relief in all aspects of the human condition is ever-growing. Drug resistance in bacteria, the appearance of life-threatening viruses, chronic diseases in persons with organ transplants, incidence of fungal infections in the world's population all emphasizes the inadequacy to cope with these medical problem.[1] Natural products have been the most successful source of potential drug leads. Natural products have been used since ancient times and in folklore for the treatment of many diseases and illnesses. The earliest records of natural products were found from Mesopotamia (2600 B.C.) which documented oils from *Cupressus sempervirens* and *Commiphora* species which are still used today to treat coughs, colds and inflammation. Pharmacological records suggest that more than 700 plant-based drugs are now being used to treat various ailments.[2] Herbalists and traditional healers have used botanical herbs as medicines for the treatment and prevention of diseases. For example, *Artemisia douglasiana*, Besser plant, popularly known as "matico" is a medicine for gastric ulcer and for treatment of external skin diseases.[3] Various extracts derived from the seeds of *Pongamia pinnata* (Karanj) decreased pentobarbitone sleeping time, and similar properties were exhibited by its roots. The acetone soluble fraction of petroleum ether extract of *Lawsonia inermis* (Mehendi) leaves decreased lithium induced head twitches. Oral administration of Rasayana group of drugs (from Ayurveda) were found to significantly increase total WBC count, bone marrow cellularity, natural killer cell and antibody dependent cellular cytotoxicity in gamma radiation exposed mice.[4]

The search for anti-cancer agents from plant sources started in the 1950s and over 50% of the drugs in clinical trials are available for anticancer activity. The discovery and development of the vinca alkaloids, vinblastine and vincristine and the isolation of the cytotoxic podophyllotoxins have known to show antitumor and antiancancer activites. This led to the discovery of many novel chemotypes including the taxanes and camptothecins.[5] Green tea was found to contain simple phenols and phenolic acids, quinones, flavones, flavonoids, flavonols and tannins has excellent antimicrobial activity against almost all

microorganisms.[6] Various plant medicines and health products have been accepted by people from all over the world as it helps in improving the quality of life, disease prevention and treatment of chronic diseases and geriatric diseases.[7]

Tagetes (Compositae) is a genus of herbs, commonly known as marigold, native of Mexico and other warmer parts of America and naturalized elsewhere in the tropics and subtropics.[8] All parts of the plant, from root to seed possess a multitude of phytochemical secondary metabolites and also responsible for repellent activity.[9] *Tagetes erecta* originated in North and South America and are widely cultivated in other Asian countries like Bhutan, China, Nepal and India. It is known as 'marigold' in English, 'thulukka samanthi' in Tamil and 'genda' in Hindi. *Tagetes erecta* is a medicinal plant which has a high therapeutic value in the field of medicine for treating hiccups, dermatitis, athlete's food, colitis and wound burns. The plant has been used to treat stomach ache, diarrhoea, liver problems, vomiting, indigestion, toothache, chest pain, rheumatic pains, cold, bronchitis, ulcer, diseases of the eye and uterus and to expel worms from the body. The leaves are used for treating kidney problems, muscular pain and as an application on boils. The infusion of this plant is used against rheumatism, cold and bronchitis.[10]

Leucas aspera commonly known as 'Thumbai' is distributed throughout India from the Himalayas down to Ceylon. The plant is used traditionally as an antipyretic and insecticide. Medicinally, it has been proved to possess various pharmacological activities like antifungal, antioxidant, antimicrobial, antinociceptive and cytotoxic activity. Reasearch carried out in these plants reveal the presence of various phytochemical constituents mainly triterpenoids, oleanolic acid, ursolic acid and β-sitosterol, nicotine, sterols, glucoside, diterpenes, phenolic compounds.[11] However, before exploring the bioactive potentials in them, it is crucial to understand the toxicity profiles of these natural products. Therefore in the present study the cytotoxic potential of methanolic extracts of *Leucas aspera* and *Tagetes erecta* using PBMC and RBC.

MATERIALS AND METHODS

Peripheral blood collection

Peripheral Blood was collected from healthy adult volunteers from which, the Peripheral Blood Mononuclear Cells (PBMC) were isolated. About 5 mL of the collected blood was mixed with an anticoagulant Ethylene diamine tetra acetic acid (EDTA).

Isolation of PBMC

PBMC were isolated according to[12] by density gradient centrifugation using the Lymphocyte separation medium. Briefly, the anticoagulant containing blood was centrifuged at 2200 rpm for 15 min. The white layer formed intermittently was taken carefully and washed by using RPMI-1640 (Roswell Park Memorial Institute) medium at 2200 rpm for15 min. The cell viability was assessed by trypan blue exclusion assay and the PBMC were adjusted to 1×10^6 cells/mL.

Culturing of PBMC

The PBMC isolated were cultured in RPMI 1640 supplemented with 10% Fetal bovine serum (FBS) and 1X antibiotic solution (100 U/ml penicillin or 100 mg/ml streptomycin) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Preparation of *L. aspera* and *T. erecta* extract

Extracts were prepared according to [13]. Fresh leaves of *L. aspera* and flowers of *T. erecta* were washed with alcohol, water and dried under shade. The air dried leaves and flowers were powdered and subjected to cold extraction with methanol (MeOH) at 37°C in orbital shaker for 72 h. The extracts were dried under reduced pressure under vacuum dessicator until dryness was completely removed and the percentage of yield was calculated. The dried extract was dissolved in distilled water and used for further analysis. The yield of the extract was calculated as below:

$$\text{Yield of the extract} = \frac{\text{Weight of the beaker with extract} - \text{Weight of the empty beaker}}{\text{Weight of the sample in grams}} \times 100$$

Assessment of Cytotoxicity using Trypan Blue Exclusion assay and MTT assay using PBMC.

PBMC were incubated with different concentrations (100-500 μg/mL) of methanolic extracts of *L. aspera* and *T. erecta* at 37°C with 5% CO_2 in water jacketed incubator (NAPCO, INCUB 5420-1) for 24 h. The cell viability was checked at various time intervals (24 h, 48 h, and 72 h) and the loss of viability was compared with 1mM DimethylSulfoxide (DMSO). Trypan blue exclusion assay was performed according to.[14] The stained and unstained cells were counted and the percentage of viability was calculated using the following formula:

$$\text{Viable cells} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100$$

Hemolysis Assay.

The hemolysis assay was performed according to.[12] Blood was collected from healthy volunteers and RBC was freshly isolated from the blood. The cells were washed thrice with freshly prepared 150 mM NaCl. After centrifugation at 2500 rpm for 15min, the supernatant was removed and the cells were resuspended in 100 mM sodium phosphate buffer. 200 μL of RBC solution was mixed with different concentrations of *L. aspera* and *T. erecta* extracts (100- 500 μg/mL) and was made to equal 1mL with phosphate buffer. Triton X- 100 and sodium phosphate buffer (with milli Q water) were used as positive and negative controls, respectively. Tubes were then incubated at 37°C in a water bath for 30 min. After incubation, the cells were centrifuged at 2500 rpm for 15 min. The amount of hemoglobin released was taken as a measure of cell lysis. The supernatant was collected and the absorbance was measured at 540 nm in a UV-Vis spectrophotometer.

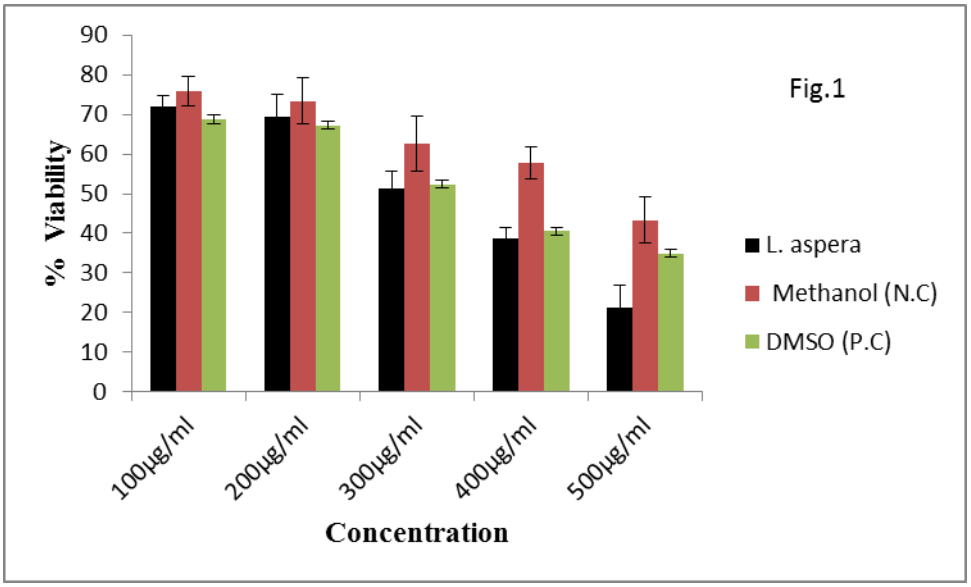
STATISTICAL ANALYSIS

Experimental results concerning this study were represented as mean ± S.D of three parallel measurements.

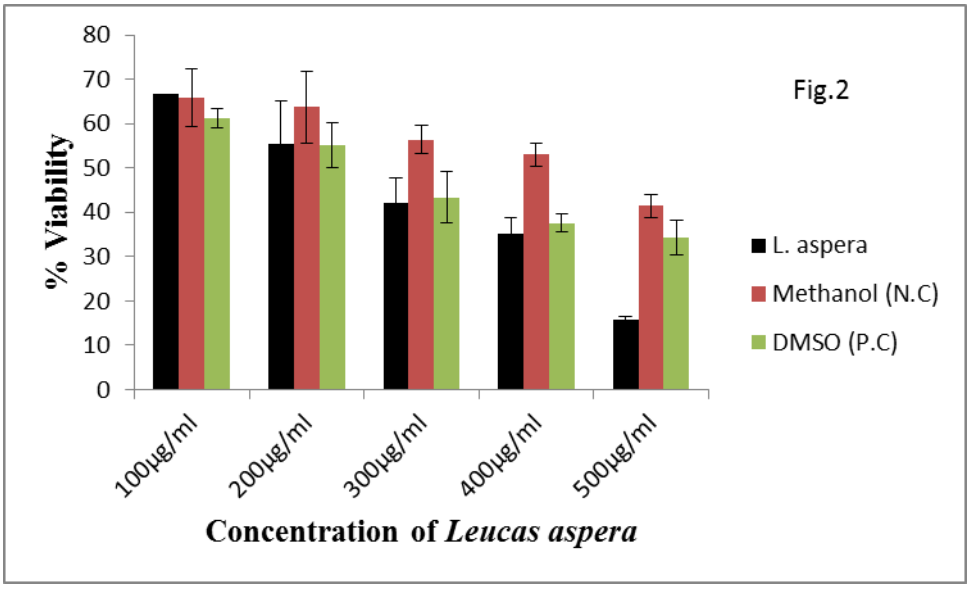
RESULTS

Assessment of cytotoxic effects of *L. aspera* on cultured human mononuclear cells

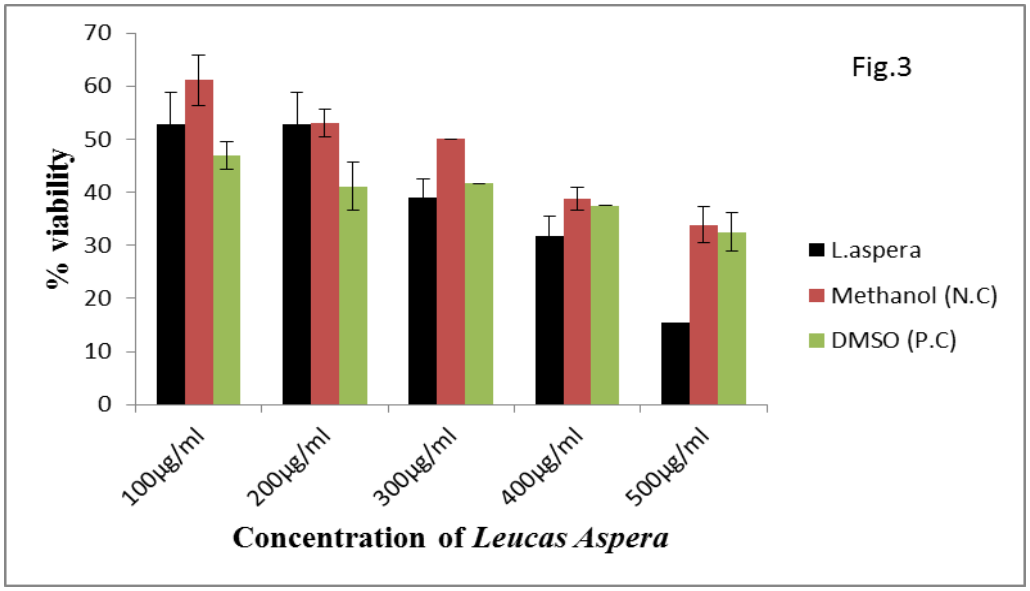
To examine the effect of *L. aspera* on the cultured lymphocytes, the cell viability assay was performed after 24, 48 and 72 h. At the concentration of 100 µg/ml, the viability was retained after incubating for 24 and 48 h (Fig. 1-3) and the viability was about 71.83± 2.91 and 66.66 ± 5.36% respectively. This result suggests that the extract did not exhibit toxic effects at the concentration of 100 µg/ml even after incubating at 48 h. However, at the concentration of 500 µg/ml, the viability was reduced noticeably and the percentage of viability was drastically reduced for 24, 48 and 72 h which suggest that methanolic leaf extract of *L. aspera* exhibited toxic effects in PBMC (Fig. 1-3), when treated with higher concentrations (500 µg/ml). Dimethyl sulphoxide (DMSO) was used as positive control whereas Methanol was used as a negative control (Fig 1-3).



[1]. Cytotoxic evaluation of methanolic leaf extract of *L. aspera* by trypan blue exclusion assay after incubation for 24 h



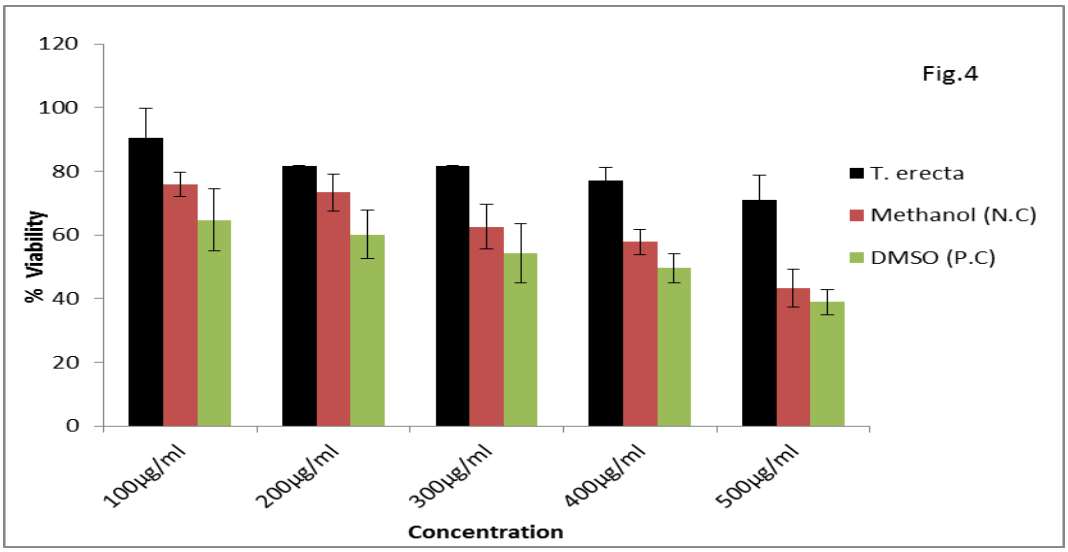
[2]. Cytotoxic evaluation of methanolic leaf extract of *L. aspera* by trypan blue exclusion assay after incubation for 48 h



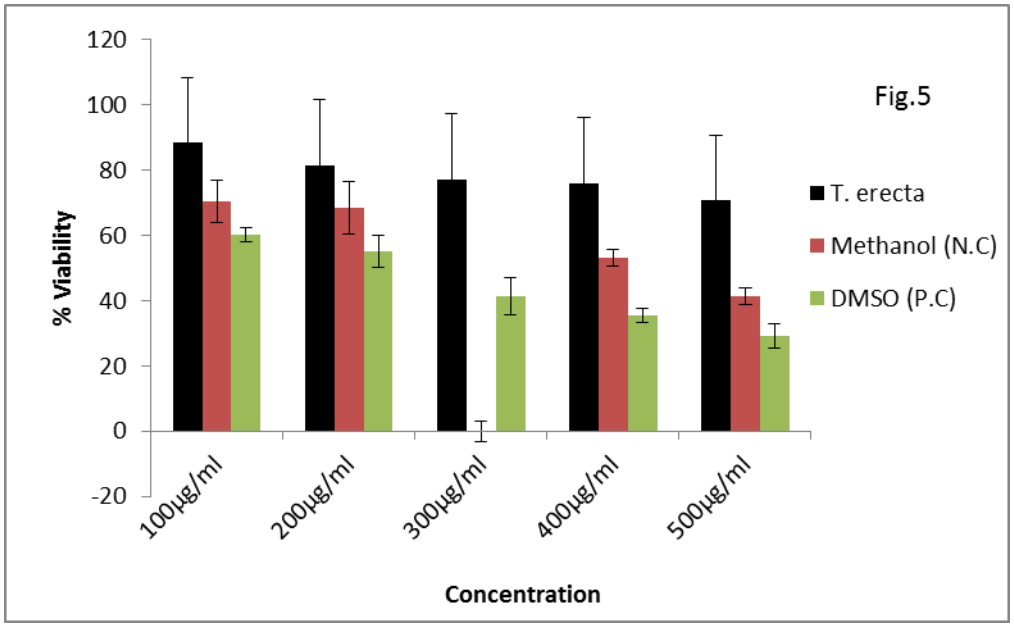
[3]. Cytotoxic evaluation of methanolic leaf extract of *L. aspera* by trypan blue exclusion assay after incubation for 72 h

Evaluation of cytotoxic effect of *T. erecta* on cultured mononuclear cells

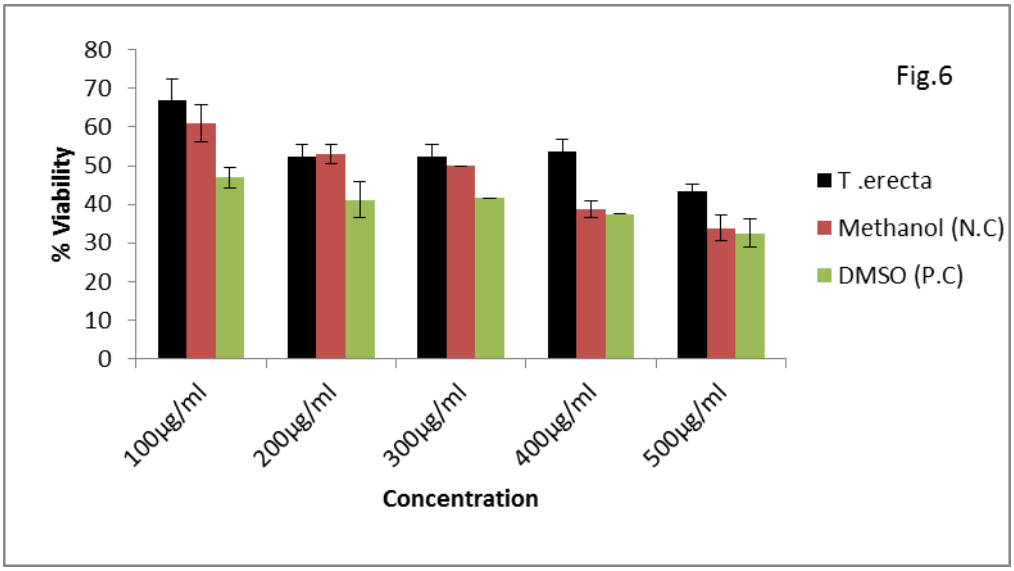
The cytotoxic effect of *T. erecta* on the cultured mononuclear cells was analyzed by tryphan blue exclusion assay. The cell viability assay was performed after 24, 48 and 72 h. The viability of cells was retained (88.37 ± 4.37%) even after 48 h of incubation of 100 µg/ml of methanolic flower extract of *T. erecta* (Fig. 4 & 5). Similar to *L. aspera*, methanolic extract of *T. erecta* exhibited toxic effects after incubation at 72 h (Fig.6).



[4]. Cytotoxic evaluation of methanolic flower extract of *T. erecta* by trypan blue exclusion assay after incubation for 24 h



[5]. Cytotoxic evaluation of methanolic flower extract of *T. erecta* by trypan blue exclusion assay after incubation for 48 h

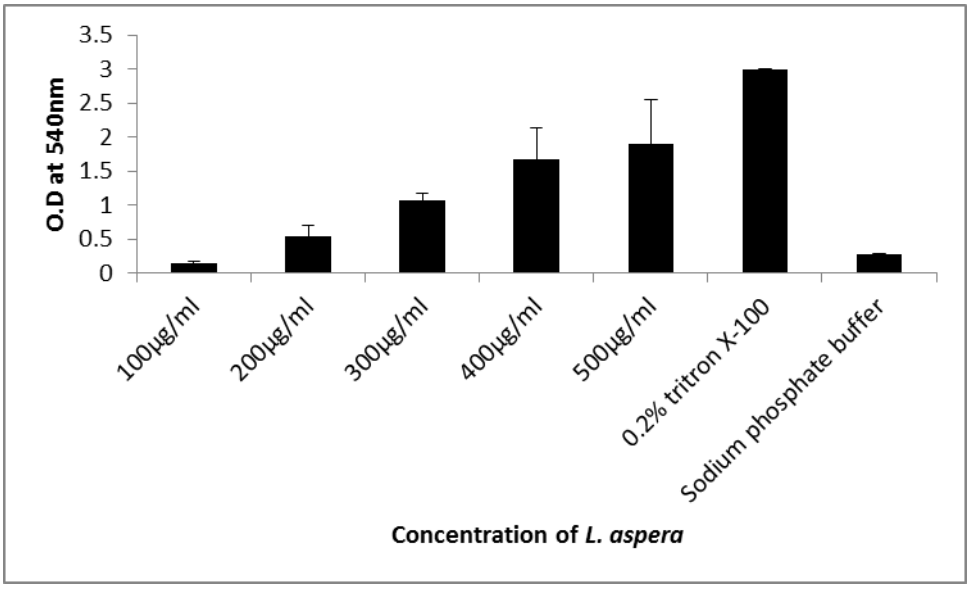


[6]. Cytotoxic evaluation of methanolic flower extract of *T. erecta* by trypan blue exclusion assay after incubation for 72 h.

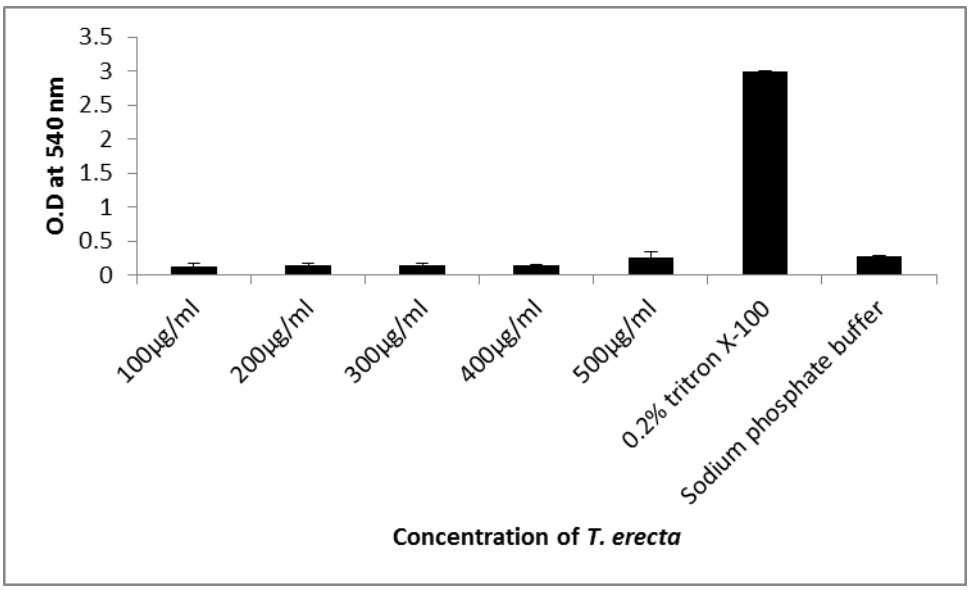
Evaluation of Membrane Disintegrating Effects of methanolic extract of *L. aspera and T. erecta*

Human erythrocytes were used for the evaluation of membrane disintegrating activity of *L. aspera* and *T. erecta* extracts. RBC treated with various concentrations of *L. aspera* leaf extract (100-500 μg/ml) exhibited the least absorbance of 0.149 ±0.016 and 0.537± 0.16 for 100 and 200 μg/ml respectively. (Fig.7). A complete loss of membrane integrity was

observed in the cells treated with 0.2% Triton X-100, as the absorbance at 540 nm was significantly high ($P < 0.05$) and increased to 2.998 ± 0.0005. In case of *T. erecta*, the absorbance observed for (100 and 200 μg/ml) was 0.116 ± 0.05 and 0.132 ± 0.035 respectively. Even for 500 μg/ml the absorbance value was recorded to be 0.246 ± 0.1 (Fig .8). The values are relatively low even for the highest concentration. Therefore, the results suggest that the both *L. aspera* and *T. erecta* did not interrupt or damage the RBC membrane even at the concentration of 200 μg/ml.



[7]. Membrane disintegrating effects of mentholic leaf extract of *L. aspera* by hemolysis assay



[8]. Membrane disintegrating effects of methanolic flower extract of *T. erecta* by hemolysis assay

DISCUSSION

Nature has been a resource of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources.[15] In recent times, focus on plant research has increased all over the world and a large body of evidence has been collected to show immense potential of medicinal plants used in various traditional systems.[16] Therefore, it is crucial to assess the safety evaluation on these extracts before undertaking further studies on other therapeutic efficacies.[17] Hence in the present study, the cytotoxic effects of plant extract of *L. aspera* and *T. erecta* was analyzed on cultured mononuclear cells. The toxic effects of methanolic extracts of these traditional plants were analyzed for various time period 24 h, 48 h and 72 h. In the case of *L. aspera*, the methanolic leaf extract did not exhibit toxic effects, at the concentration of 100 µg/ml and 200 µg/ml even after incubating for 72 h. However, at the concentration of 500 µg/ml, the extract exhibited toxic effects and reduced the number of viable cells, after incubating for 72 h.

In the case of *T. erecta*, where the methanolic extract did not exhibit toxic effects at the concentration of 100 µg/ml even after incubating for 72 h. However, upon treatment with 500 µg/ml for 24 and 48 h[17], the mononuclear cells showed lesser loss of viability, even at the highest concentration of 500 µg/ml. However, the methanolic flower extracts reduced the viability of cells after incubating for 72 h at the concentration of 500 µg/ml. Hence the extract exhibited time- dependent cytotoxic effect after 72 h on PBMC.

Erythrocytes are simple blood cell type without any sub-cellular organelles, hence they can be exploited for testing *in vitro* toxicity of selected compounds by measuring the release of their hemoglobin content, which is generally represented as an index of cell membrane damage. A significant increase in the absorbance was observed, when the cells were treated with 0.2% Triton X-100, which indicates a complete hemolysis pattern, whereas the cells treated with methanolic leaf extracts of *L. aspera* (100 and 200 µg/ml) did not show any increase in the absorbance at 540 nm . Similar results were observed for R. Manivannan et al.[18] The methanolic flower extracts of *T. erecta* did not affect the membrane integrity of RBC which suggests that the extracts *T .erecta* did not damage the membrane of the erythrocytes even at the concentration of 200 µg/ml.[17]

CONCLUSION

In the present study, the cytotoxic effects of methanolic extracts of *L. aspera* and *T. erecta* were evaluated. The outcome of the present study suggests that both the extracts did not

possess cytotoxicity towards PBMC. Since both the extracts did not alter the membrane of Red blood cells, the methanolic extract of both the extracts were not found to be toxic to human mononuclear cells.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the management of Srimad Andavan Arts & Science College for providing the financial support and infrastructure facility.

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