ISSN: 2395-0536 Impact Factor- 2.95*

INTERNATIONAL JOURNAL OF PHARMACEUTICAL RESEARCH AND NOVEL SCIENCES

IJPRNS

METHOD *IN VITRO* ANTI-INFLAMMATORY ACTIVITY AND ANTI-NOCICEPTIVE ACTIVITY EVALUATION OF *TRIBULUS TERRESTRIS*

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ABSTRACT

Aim is to study and compare the anti-nociceptive activity of extract of *T. terrestris* in LPS induced inflammation in RAW264.7 macrophages. In *in vitro*, alkaloid enriched fraction of *T. terrestris*, showed significant increase in cell viability in LPS induced inflammatory model

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INTRODUCTION

Neuropathic pain is defined as neural damage in nervous system which leads to pain and loss of function. International association for the study of pain (IASP) has given a new definition pain initiated or caused by primary lesion, dysfunction or transitory perturbation of the peripheral or central nervous system . Neuropathic pain includes peripheral and central neuropathic pain where peripheral neuropathic pain results from lesions to the peripheral nervous system (PNS) caused by mechanical trauma, metabolic diseases, neurotoxic chemicals, infection, or tumor invasion and involves multiple pathophysiological changes both within the PNS and in the CNS . Central neuropathic pain most commonly results from spinal cord injury, stroke, or multiple sclerosis . Pathophysiology of neuropathic pain involves two processes appear to be major general contributors to developing neuropathic pain: the balance between compensatory and decompensatory reactions of the nervous system to neural damage, and a genetic background that either enhances or protects an individual from the establishment of neuropathic pain. Many of the changes that occur in response to neural injury are potentially adaptive: removal of cell and myelin debris, changes in receptors that counterbalance the loss of input, other alterations that dampen ion fluxes and metabolic stress after the acute injury, recruitment of anti-apoptotic survival strategies to prevent neuronal cell death, induction of axonal growth and sprouting, synaptic remodeling and remyelination and where the mechanism of neuropathic pain includes ectopic impulse generation, ectopic transduction, central sensitization, Low threshold Aβ-Fiber mediated pain, Disinhibition, structural changes, neurodegeration and chronic pain, neuro-immune interaction. Here pain produced may arise both from ectopic activity in nociceptors and from low-threshold large myelinated afferents due to central sensitization and altered connectivity in the

spinal cord. Also neuropathic pain involves a profound switch in sensitivity such that low-intensity input can generate pain, a disruption of the normal pattern of pain specificity. Here it involves some fibres includes such as Aδ and C fibers which associated to generation of neuropathic pain (1-4).

MATERIALS AND METHODS

Animals

Adult male wistar rats(200-250g) were obtained from the animal house. The rats were grouped and housed in polypropylene cages (38 X 23 X 10cm) with not more than 6 animals per cage. They were maintained at an ambient temperature of 25+1°C. Animals had free access to food and water. The animals were acclimatized to laboratory condition for one week prior to experiments. The experimental protocols were approved by Institutional Animal Ethics Committee and conducted according to the guidelines of the Committee for the Purpose of Supervision and Control of Experiments on Animals (CPCSEA), Government of India.

T. terrestris (Nerunjil) Hydroalcoholic Extract

T. terrestris alcoholic extract used in this study was prepared as per Ayurvedic principle. The sub fraction of the prepared extract was carried to extract the plant and enrich the alkaloidal fraction.

In vitro studies

Mammalian cell culture

RAW 264.7 (Murine macrophage cell line) was obtained from NCCS Pune. It was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% serum (FBS), amphotericin (3 µg/ml), gentamycin (400 µg/ml), streptomycin (250 µg/ml) and penicillin (250 units/ml) at 37°C in a carbon dioxide incubator at 5% CO_2.

Preparation of media and other reagents required for cell culture

Minimum Essential Medium (MEM) was prepared as follows

The powdered media was dissolved in 900 ml of sterile glass - distilled or Millipore water in an autoclaved glass conical flask under sterile conditions. The antibiotics were added in the concentration as mentioned above and stirred well. 3.7 g of Sodium bicarbonate was added into the flask and stirred until it gets dissolved completely. 10% FBS was added and mixed well. The liquid was slowly poured into the upper portion of a Media sterilisation unit (Corning) and filtered through a 0.2µ filter under negative pressure. The medium was immediately stored at 4°C.

Drugs and LPS Preparation

Extracts were subjected to solubility test with different organic solvents, finally get dissolved in dimethyl sulphoxide (DMSO), 25 mg/100 µl stock was prepared. The desired doses such as of 0.01 to 0.5µg/ml were prepared from the stock using culture medium. Lipopolysaccharide (LPS) in the dose of 1mg/ml was prepared with distilled water.

MTT assay (5, 6)

RAW 264.7 cell lines were maintained in Minimum Essential Medium (MEM) supplemented with 10% serum(FBS), amphotericin (3 µg/ml), gentamycin (400 µg/ml), streptomycin (250 µg/ml) and penicillin (250 units/ml) in a carbon dioxide incubator at 5% CO_2. 5000-10000 cells/well were added in 96 well plates from well grown culture medium, the viability was tested using trypan blue dye with help of haemocytometer and 95% of viability was confirmed. After 24 hrs, the LPS (1- 400µg/ml) were incubated with Cultured RAW cell lines separately for 24 hrs. After incubation, the LPS containing medium was removed and the medium was changed again for all groups and 10 µl of MTT (5 mg/ml stock solution) was added and the plates were incubated for an additional 4 hrs. The medium was discarded and the formazan blue, which was formed in the cells, was dissolved with 150 µl of DMSO. The optical density was measured at 595 nm. The percentage toxicity was calculated by using following formula

% Toxicity = (LPS treated cells/LPS untreated cells) x 100

RESULTS AND DISCUSSION

IC_{50} determination of LPS and morphological observations by Phase contrast microscopy

LPS (1 - 400 µg/ml) was incubated in RAW 264.7 cells for 24 hours. After 24 hrs, IC_{50} of the LPS was calculated through graphical determinations (Fig-1) and it was found to be 70 µg/ml. RAW 264.7 cells are small, highly light refractive, round shaped, macrophage cells, growing densely and forming focal accumulation. Treatment of LPS in RAW 264.7 cells resulted in morphological changes like reduced cell density, reduced differentiation of cells when compared to the control cells. Treatment of alkaloid enriched fraction of *T. terrestris* at the doses of 0.01 to 0.5 µg/ml with LPS, protects the cell from the LPS toxicity in a dose dependent manner and restores the morphology of cells.

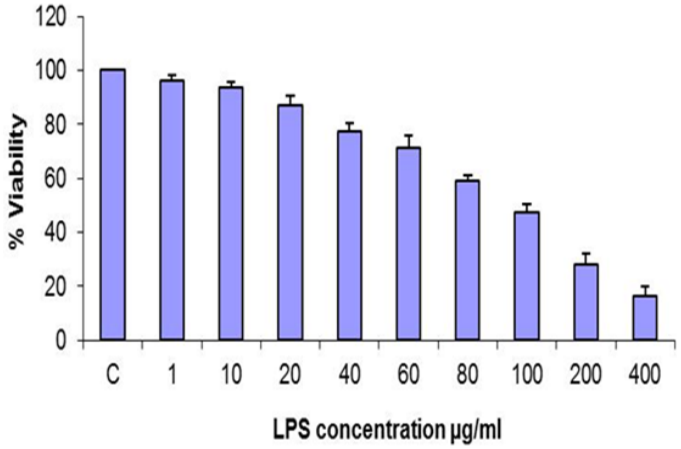

Fig-1 IC_{50} value for LPS dose determination

Effect of T. terrestris extracts treatment on cell viability in without LPS treated RAW 264.7 cells

To observe the dose dependent activity of AEF in various doses (5, 1, 0.5, 0.1, 0.05, 0.025, 0.01µg/ml) in DMSO was incubated in RAW 264.7 cell lines for 24 hours without LPS. After 24 hours values are seen and plotted in graph. Here it shows 0.05µg/ml shows significant effect ($p<0.05$) compare to other doses (fig-2)

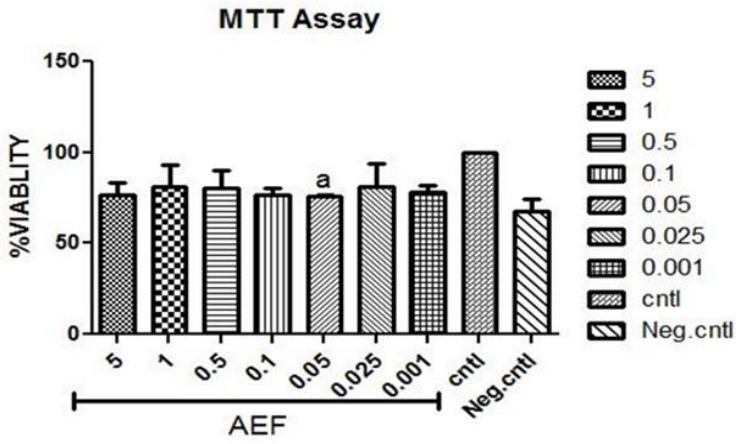

Fig-2 Effect of AEF dose dependent in *T. terrestris* on cell viability in without LPS treated RAW 264.7 cell lines

Paw licking/biting time

Effect of treatment on acute phase (0-10min)

Pregabalin (10 mg/kg) treated rats showed significant reduction ($p<0.01$) in paw licking/biting time as compared to negative control rats. Treatment of alkaloid enriched fraction of *T. terrestris* showed significant reduction in paw

licking/biting at 50 mg/kg ($p<0.05$) and 100 mg/kg ($p<0.001$) in comparison to negative control rats. Mother extract of *T. terrestris* (50 and 100 mg/kg) treated rats did not show any significant changes in paw licking/biting behaviour in comparison to negative control rats.

T. terrestris mother extract and alkaloid enriched fraction had a significant anti-nociceptive and anti-inflammatory effect in acute conditions, when tonic pain was induced by using formalin in rats. In the formalin test, the cumulative time spent in biting/licking the paw after intraplantar injection with 1% formalin is used as an outcome parameter and nociception index and produces a biphasic behavioural reaction. The nociception induced by formalin is associated with injured tissue and it is believed that it more closely resembles clinical pain in comparison to other tests. The acute phase (0-10 min) is short-lived and initiates immediately after injection, being characterized by C-fiber activation due to peripheral stimuli.

CONCLUSION

In *in vitro*, alkaloid enriched fraction of *T. terrestris*, showed significant increase in cell viability in LPS induced inflammatory model. In *in vivo* formalin *T. terrestris* test showed dose dependent decrease in the paw licking/biting and elevation in both the phases. The study has been concluded that the *T. terrestris* mother extract and alkaloid enriched fraction exhibits dose dependent decrease in nociception in both *in vitro* and *in vivo* models.

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