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Research Article

EVALUATION OF ACUTE TOXICITY, ANTI-INFLAMMATORY ACTIVITY AND PHYTOCHEMICAL SCREENING OF ETHANOLIC EXTRACT OF *AZADIRACHTA INDICA* LEAVES

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ABSTRACT

Objective: The present study was carried out to investigate the acute toxicity and anti-inflammatory activities of the ethanol extract of Azadirachta indica leaves (Neem) on experimental animal models.

Materials and methods: Adult wistar rats (150 - 200g) of either sex were selected for this study. Each group consisted of six animals. Anti-inflammatory activity of ethanol extract was evaluated by well-established model like Carrageenan induced rat paw edema at two different dose levels of 200mg/kg and 400mg/kg. Swiss albino mice of weight 25 – 30g were used for acute toxicity.

Results: A maximum of 89% inhibition of paw edema was noted in diclofenac treated animals at the end of six hours. Oral administration of the ethanol extract of Azadirachta indica leaves (200, 400mg/kg) significantly ($P<0.05$) inhibit the carrageenan induced paw edema when compare with control. The maximum percentage inhibition of paw edema at 6th hour by the above doses of Azadirachta indica were 46% and 60% respectively. In acute toxicity study no mortality were observed in mice with the maximum dose of 2000mg/kg.

Conclusion: The present study indicates that oral administration of both the doses of Azadirachta indica leaves shows dose dependant improvement in the anti-inflammatory activity. The extract lowers the carrageenan induced rats paw oedema. Further pharmacological and biochemical investigation are essential to elucidate the mechanism of action.

Keywords: Azadirachta indica leaves, acute toxicity study, anti-inflammatory study, phytochemical analysis.

INTRODUCTION

One among the major world health problem is inflammatory disorders [1, 2]. Inflammation is considered as a pathophysiological response and defence mechanism that helps body to protect itself against infection, burn, toxic chemicals, allergens or other noxious stimuli [3]. An uncontrolled and persistent inflammation may results in many chronic illnesses and the mediators involved in the inflammatory reaction can induce, maintain or aggravate many diseases, which initiate and perpetuate the pain reaction [4]. Inflammation has become the focus of global scientific research because of its implication in virtually all human and animal diseases. Drugs that are currently used for the management of inflammatory conditions are non-steroidal anti-inflammatory drugs (NSAID) and corticosteroids. NSAID remain the mainstay of therapy to treat a wide variety of inflammatory diseases. The most common adverse effects of NSAID are in gastrointestinal tract leading to epigastric distress, ulceration, perforation and gastrointestinal bleeding, on the other hand long term use of corticosteroids results in immunosuppressant, which causes various microbial infections. Therefore to alleviate the sign and symptoms of inflammation, novel anti-inflammatory drugs lacking these side effects are being

researched as alternatives to NSAID and corticosteroids. Attention is being focused on the investigation of the efficacy of plant-based drugs used in the traditional medicine as well as synthetic compounds for this purpose.

Neem (Azadirachta indica A. Juss) is a member of the Mahogany family. It has similar properties to its close relative, Melia azederach. The word Azadirachta is derived from the Persian azaddhirakt (meaning 'noble tree'). Neem is an evergreen tree, cultivated in various parts of the Indian subcontinent [5]. Neem, the versatile medicinal plant is the unique source of various types of compounds having diverse chemical structure. Although extracts from neem leaves have medicinal applications from time immemorial, modern drugs can be developed after extensive investigation of its bioactivity, mechanism of action, pharmacotherapeutics, toxicity and after proper standardization and clinical trials. In fact, time has come to make good use of centuries-old knowledge on neem through modern approaches of drug development.

MATERIALS AND METHODS:

Plant Collection and Identification:

The leaves of Azadirachta indicia collected from Meenakshi Medical College Hospital and Research institute (MMCH&RI) campus, Kanchipuram, were shade dried for a week. The plant was taxonomically identified and authenticated in C.L Bhaid Metha college of Pharmacy, Chennai.

Preparation of Ethanolic Extract:

The dried leaves were ground into powder form and stored in an airtight container. About 1kg powder was then macerated in 5 litres of 90% ethanol for 7 days at room temperature with occasional stirring. The ethanol extract of the plant was collected in a separate container and concentrated under reduced pressure below 50 °C through rotatory vacuum evaporator. The concentrated extract was a blackish green colour residue (40g) which was stored in a refrigerator. The extract was subjected to various phytochemical and pharmacological evaluations.

Preliminary Qualitative Phytochemical Analysis:

The extract was subjected to preliminary phytochemical screening for the presence or absence of phytoconstituents of the following tests such as, alkaloids, carbohydrates, steroids, proteins, tannins, phenols, flavanoids and glycosides [6].

Animals:

Adult wistar rats of either sex weighing between 150 - 200 g obtained from animal house, MMCH&RI, Kanchipuram, were housed in a standard cage at 25° C in a 12/12 h light and dark cycle, and were supplied with food and water ad libitum. Experiments were carried out between 0900 and 1500 hours to maintain uniformity. In all the experimental studies each group consisted of six animals. The care and maintenance of the animals were carried out as per the approved guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The research protocols were approved by the Institutional Animal Ethical Committee (IAEC) Approval letter No: T.C/COL/1130/2012/ HOS F – 378, dated 23-01-2012. The study was undertaken for 6 months from March 2012 to August 2012 at department of pharmacology, MMCH&RI, Kanchipuram.

Drugs and Extract administration:

Carrageenan from (SD Pharmaceuticals, India), was used in the study. The ethanolic extract of Azadirachta indica leaves were prepared as a uniform suspension using 1% tween 80 for oral administration in experimental animals.

Acute Oral Toxicity Study:

Acute oral toxicity study was performed according to Organization for Economic Cooperation and Development (OECD 423) guidelines. The acute oral toxic class method is a stepwise procedure with 3 animals per step. The method is based on biometric evaluation with fixed doses and the results allow the substance to be ranked and classified according to the Globally Harmonized System (GHS) for the classification of extracts which causes acute toxicity [7].

Swiss albino male/female mice weighing 25-30g were used for the study. Tween 80 1% v/v was used as vehicle to suspend the ethanolic extract of Azadirachta indica leaf. Mice were divided in control and test groups of three each. The test groups received a single oral dose of alcoholic extract of Azadirachta indica leaf at a dose of 20 mg/kg, 200mg/kg or 2000mg/kg. Control group received 1%v/v of tween 80. Food was withheld for a further 3 to 4 hours after oral administration of drugs. The incidence of mortality was checked in the first 24 hours and the study was extended up to 14 days.

Carrageenan Induced Hind Paw Edema in Rats:

According to the method by Winter et al., 1962, we evaluated our study [8]. Adult wistar rats of either sex (150 – 180 g) were divided in four groups of 6 animals each. Group I (vehicle) received 1%v/v tween 80 (1ml/100gm) orally. Group II (standard) received diclofenac (10mg/kg/i.p) as a reference drug half an hour prior to carrageenan injection. Group III and IV received orally ethanolic extract of Azadirachta indica leaves 200mg/kg and 400mg/kg respectively sixty minutes prior to carrageenan injection.

To induce paw edema 0.1ml of 1% suspension of carrageenan in normal saline was injected to the sub planter region of left hind paw. The diameter of paw was measured by using digital vernier calliper before administration of carrageenan at hourly intervals up to six hours after the administration of carrageenan. The difference between the initial and subsequent readings was calculated as mean increase in paw diameter which is a measure of the edema. This was compared with vehicle treatment.

The difference of values between treated animals and control group is calculated for each time interval and evaluated statistically. Edema (ΔT) was calculated as follows [9]

$$\Delta T = Tt - T0$$

Tt is the left hind paw thickness in mm at time t

T0 is the left hind paw thickness before sub plantar injection.

% reduction of edema was calculated as follows,

Mean increase in paw diameter in vehicle group(C) --- mean increase in paw diameter in drug treated group (T)/ mean increase in paw diameter vehicle group x 100

([C – T / C] x 100).

Statistical analysis:

The data was analyzed employing Instat 3 graph pad prism. Results of various experiments are expressed as mean ± standard error of mean of six animals in each group. The data was subjected to one-way ANOVA and significance was calculated by employing Dunnett's t-test. A p value <0.05 was considered statistically significant.

RESULTS:

Phytochemical screening:

The preliminary phytochemical screening of the extract shows the presence of glycosides, phenols, volatile oils, flavanoids, tannins, terpenoids, proteins, carbohydrates, in the leaves extract of Azadirachta indica. (Table - 1)

Acute toxicity study:

No significant changes were observed in the behavioural or autonomic responses in mice after treatment with different dosses of Azadirachta indica leaf extract. There was no mortality in these animals during the observational period of 14 days.

Carrageenan induced hind paw edema:

Administration of carrageenan in the plantar surface of the rat hind paw increased the paw diameter gradually over the period of six hours. Treatment with Azadirachta indica extract resulted in dose-dependent reduction in carrageenan evoked hind paw edema and differed significantly ($P<0.01$) among the different groups of rats. The two doses of plant extract showed statistically significant ($P<0.01$) inhibitory effect on "mean increase in paw diameter" at all the time intervals (1st h, 2nd h, 3rd h, 4th h, 5th h, and 6th h) as shown in (Table 2). The effect was dose dependent and time dependent. Treatment with diclofenac significantly attenuated this increase at all periods of observation.

At the end of 5th hour and 6th hour of carrageenan administration, Azadirachta indica exhibited maximum % inhibition of paw diameter ($P<0.05$) by 34%, 46% and 50%, 60%, at the two doses of plant extract 200 mg/kg and 400 mg/kg respectively, however % inhibition of paw diameter was less than that of standard drug diclofenac 74%, 89% ($P<0.05$) at the dose of 10 mg/kg (Table 2, Fig. 1).

DISCUSSION:

Acute toxicity of Azadirachta indica was investigated with the objective to detect any possible adverse effect. In acute toxicity testing no mortality was observed in mice even in a dose of 2g/kg of ethanolic extract of Azadirachta indica which indicates the safe nature of the extract as it correlates with the traditional use for centuries.

The anti-inflammatory effect of Azadirachta indica was evaluated by studying inflammatory response produced in rodents by injecting carrageenan in the hind paw. Carrageenan is a phlogistic agent and upon administration produces edema. Carrageenan-induced paw edema is known for its classic biphasic effect [10].

Table - 1: Phyto chemical constituents of *Azadirachta indica* leaves

Chemical constituents	**Present (+) / Absent (-)**
Glycosides	Present
Phenols	Present
Flavanoids	Presents
Alkaloids	Absent
Tannins	Present
Terpenoids	Present
Sterols	Absent
Proteins	Present
Fat	Present
Carbohydrates	Present
Volatile oils	Present

Table - 2: Effect of Ethanolic Extract of *Azadirachta indica* Leaves on Carrageenan Induced Paw Edema in Rats:

	Mean increase in paw diameter (mm)					
Treatment	**1st hour**	**2nd hour**	**3rd hour**	**4th hour**	**5th hour**	**6th hour**
Vehicle (1% Tween 80 v/v, p.o)	0.82±0.03	1.11±0.02	1.21±0.03	1.35±0.02	1.43±0.02	1.48±0.02
Diclofenac sodium 10mg/kg, s.c	0.76±0.02*	0.86±0.03*	0.79±0.01*	0.53±0.02*	0.38±0.02*	0.16±0.03*
Azadirachta indica ethanolic extract 200mg/kg, p.o	0.75±0.03*	1.08±0.02*	1.15±0.01*	1.08±0.04*	0.95±0.03*	0.80±0.02*
Azadirachta indica ethanolic extract 400mg/kg, p.o	0.80±0.02*	0.95±0.02*	1.00±0.03*	0.88±0.02*	0.72±0.01*	0.60±0.02*

Each value represents the mean ± SEM of 6 observations

*$p < 0.05$ compared with vehicle, one way ANOVA and Dunnett's t – test.

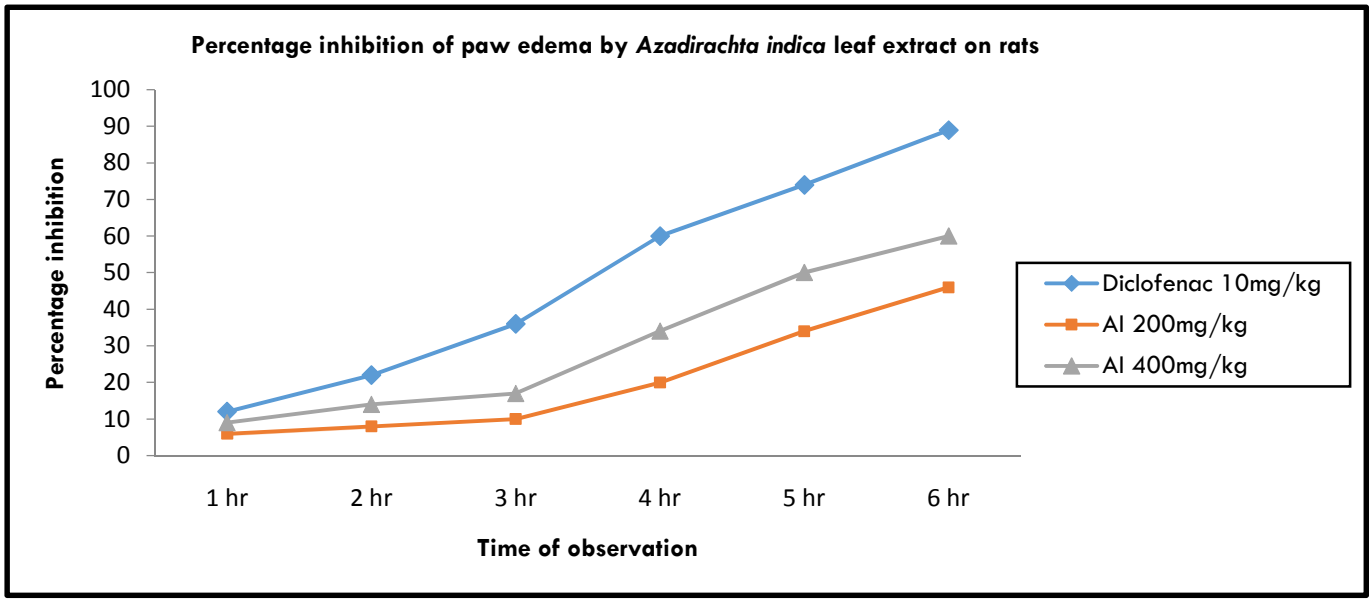

Figure – 1: Percentage inhibition of paw edema by *Azadirachta indica* leaf extract on rats

Edema represents the early phase of inflammation and a number of mediators have been identified to be released in a sequential manner. There is an initial release of histamine and 5-hydroxytryptamne producing an increased vascular permeability followed by release of kinins further contributing to the increased vascular permeability and finally, the prostaglandins and slow reacting substance are released to maintain the increased vascular permeability by histamine, 5-hydroxytryptamine and kinins [11]. The carrageenan-induced paw edema model in rats is known to be sensitive to cyclooxygenase inhibitors and has been used to evaluate the effect of non-steroidal anti-inflammatory agents, which primarily inhibit the cyclooxygenase involved in prostaglandin synthesis [12].

Carrageenan-induced hind paw edema is the standard experimental model of acute inflammation. Administration of carrageenan in the plantar surface of the rat hind paw increased the paw diameter gradually over the period of six hours. Treatment with diclofenac significantly attenuated this increase at all periods of observation (Table 1). A maximum of 89% inhibition of paw edema was noted in diclofenac treated animals at the end of six hours. Treatment with Azadirachta indica extract also attenuated the increase in paw diameter after carrageenan administration. The effect was dose dependent and time dependent. The maximum percentage inhibition of paw edema at 6th hour for 200mg/kg and 400mg/kg doses of Azadirachta indica were 46% and 60% respectively.

Previous studies have reported the potent antinociceptive and anti-inflammatory activity exerted by higher doses of Azadirachta indica leaves at 400mg, 500mg, 750mg and 1g respectively by Khosla et al., 2000; Zaman et al., 2009; Mossadek et al., 2008, [13,14,15]. The present results are in agreement with the above previous reports and identified significant anti-inflammatory activity even at lower dosage of ethanolic extract.

The preliminary phytochemical screening of the extract showed the presence of glycosides, phenols, volatile oils, flavanoids, tannins, terpenoids, proteins and carbohydrates, in the leaves extract of Azadirachta indica. Therefore, it is assumed that these compounds may be responsible for the observed anti-inflammatory activity. The mechanisms of anti-inflammatory activity may be related to the antiphlogestic action of the tannins. Flavonoids and other phenolic compounds of plant origin have been reported as antioxidants and as scavengers of free radicals. Antioxidants can also exert anti-inflammatory effects [16].

On the basis of these findings, it may be inferred that ethanolic extract of Azadirachta indica has potent anti-inflammatory activity. Further investigations are anticipated to identify the active components and lead to their further clinical use.

CONCLUSION:

The search for safer drugs to treat inflammation is necessitated by serious adverse effects to the drugs used in current therapeutic practice. The present study was an attempt to investigate the medicinal claims of Azadirachta indica, an herb widely used in Indian traditional system of medicine against pain and inflammation. Our study highlighted the importance of detailed investigations on traditional medicine to identify novel classes of drugs which will be safe and effective in many disease states.

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