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Research Article

EVALUATION OF ANTINOCICEPTIVE ACTIVITY OF ETHANOLIC EXTRACT OF *AZADIRACHTA INDICA* LEAVES

K. Kanagasanthosh[1*], V. Kavirajan[2], S. Shanmugapriyan[2]

1. Chennai Medical College Hospital and Research Centre, Trichy, Tamil Nadu.
2. Meenakshi Medical College Hospital and Research Institute, Kanchipuram, Tamil Nadu.

***Corresponding author's Email:** kanagasanthosh@gmail.com

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ABSTRACT

Objective: The present study was carried out to investigate the antinociceptive activities of the ethanol extract of Azadirachta indica leaves (Neem) on experimental animal models at two different dose levels of 200mg/kg and 400mg/kg.

Materials and methods: Swiss albino mice (25-30g) of either sex were selected for this study. Each group consisted of six animals. Antinociceptive activity of ethanol extract was evaluated by well-established models like formalin test; acetic acid induced writhing and tail immersion method in mice.

Results: Oral administration of the ethanol extract of Azadirachta indica leaves (200,400mg/kg) significantly ($P<0.05$) attenuated the early and late phase of formalin induced paw licking response in mice. Further it exhibited significant inhibition of ($P<0.05$) acetic acid induced writhing test for both the dosage and increased the withdrawal latency time in tail immersion test at the dose of (200, 400mg/kg).

Conclusion: These observations suggest that, the ethanol extract of Azadirachta indica leaves has potent antinociceptive activities, which explains the basis of its use in traditional medicine to manage acute and chronic pain. Further investigations are required to identify the active principle responsible for this beneficial effect.

Keywords: *Azadirachta indica* leaves, formalin test, acetic acid induced writhing, tail immersion.

INTRODUCTION

Traditional remedies which include herbal medicines, acupuncture and spiritual therapies, have been used for millennia by various people to treat acute and chronic illnesses [1]. Wide spread use of herbal remedies and healthcare preparations have been described in the ancient texts like Vedas and Bible. In many developing countries, they remain the most accessible and most commonly used form of medical care. While pharmaceutical modern medicines are commonly used in developed countries to treat a vast range of infectious diseases and chronic conditions, patients in developing countries continue to rely on traditional medicines for several reasons [1, 2].

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 [3]. Every part of the tree has been used as traditional medicine for household remedy against various human ailments, from antiquity. Neem has been extensively used in ayurveda, unani and homoeopathic medicine and has become a cynosure of modern medicine [3, 4].

Information obtained from some traditional medicine practitioners and some of their patients reveals that the Neem leaf is used for the treatment of pain and inflammation. Therefore the present study was undertaken to

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investigate the scientific basis of antinociceptive activity of Azadirachta indica leaves, if any, of this notion.

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.

Animals:

Adult Swiss albino mice of either sex weighing between 25 - 30 g (Animal house, Meenakshi Medical College Hospital and Research institute, 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 September 2012 to February 2013 at Department of Pharmacology, Meenakshi Medical College Hospital and Research Institute, Kanchipuram.

Drugs and Extract administration:

Morphine (Pharma chemical laboratories, India), Tween 80, acetic acid and formalin were used in the study. The ethanolic extract of Azadirachta indica leaves was prepared as a uniform suspension using 1% tween 80 for oral administration in experimental animals.

Method for the evaluation of antinociceptive effect:

Acetic acid induced abdominal constrictions:

According to the method by Koster et al., 1959, we evaluated our study [5]. Acetic Acid (0.6%) 0.1 ml/10 g was injected intraperitoneally in all mice. Response was observed as abdominal contraction and relaxation with hind limb extension for 15 min. The mice were divided into four groups each containing six animals. Group I (vehicle) received 1ml/100gm of 1%v/v tween 80 orally, sixty minutes before acetic acid challenge. Group II (Standard) received morphine (1 mg/kg, s.c) 30 min before acetic acid injection. Group III, IV, received ethanolic extract of Azadirachta indica leaves in the dose of 200 mg/kg and 400 mg/kg orally respectively 60 min before acetic acid injection. Mice were observed for 15 min to count the number of writhings in each group. The abdominal constriction induced by acetic acid after different treatment was compared with that of the vehicle.

The percent inhibition of abdominal constrictions produced by different groups was calculated using the formula:

$$[C – T / C] X 100$$

C = Number of abdominal constriction in vehicle treated group.

T = Number of abdominal constriction in treatment group.

Formalin Test:

According to the method by Tjolsen et al., 1992 we evaluated our study [6]. The animals were divided into four groups each containing six mice. Group I (vehicle) received 1ml/100gm of 1%v/v tween 80 orally, sixty minutes before formalin challenge. Group II (Standard) received morphine (10 mg/kg, s.c) 30 min before formalin injection. Group III and IV, received ethanolic extract of Azadirachta indica leaves in the dose of 200 and 400 mg/kg orally respectively 60 min before formalin challenge.

Fifty µl of 0.5% formalin was injected subcutaneously into the plantar surface of the left hind paw of the mouse. It reacts with a licking or biting response of injected paw. The time spent in paw licking or biting the injected paw was recorded every 5 min for a period of 30 min. The summation of responses of first ten minutes was taken as acute phase and ten to thirty minutes was counted as chronic phase.

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The percentage inhibition of paw licking time compared with vehicle treatment was calculated using the formula:

[C-T / C] X100

C = Biting/Paw licking response time (seconds) in vehicle treated group

T = Biting/Paw licking response time (seconds) in treatment group.

Tail Immersion Test:

According to the method described by Sewell and Spencer, 1976, we evaluated our study [7]. Prior to analgesic experiments, the animals were screened for the sensitivity test by immersing the tail of the mice gently in hot water maintained at 55° C ± 0.5° C . The animals withdrawing the tail from hot water within five sec were selected for the study. The time taken to flick the tail was taken as the reaction time. A cut off period of 10sec was maintained to prevent thermal injury to the tail. The reaction time was measured just before the administration of test substances at 0 min then at an interval of 30 min up to a period of 90 min. Swiss albino mice of either sex were used for the study. Then animals were divided into 4 groups each containing 6 mice. Group I (vehicle) received 1ml/100gm of 1%v/v tween 80 orally. Group II (Standard) received morphine (10 mg/kg, s.c) 30 min before experiment. Group III, IV, received ethanolic extract of Azadirachta indica leaves in the dose of 200 and 400 mg/kg orally respectively 60 min before experiment. The reaction time was recorded at 0 min, 30 min, 60 min and 90 min.

Maximum protective effect (% MPE) for increase in latency period of each dose was calculated by the following formula,

(%MPE) = { [(Test latency – control latency) / (Cut off time – control latency)] × 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:

Acetic acid induced abdominal constrictions: The mean number of abdominal constrictions in vehicle treated control animals was 37.0 ± 0.85. A significant reduction in the number of abdominal constrictions was recorded in morphine treated mice ($p < 0.05$) and the percentage inhibition of nociception was 75.67%. A dose dependent reduction in the number of abdominal constrictions was noticed after the administration of ethanolic extract of Azadirachta indica leaves. The reduction was significant with 200mg/kg and 400mg/kg ($p < 0.05$) of the extract. In the above doses, the percentage inhibition of nociception was 48.64% and 56.75 respectively, which is shown in (Table: 1).

Formalin – induced nociception:

In vehicle treated control animals the paw licking response time was 64.0 ± 1.15 sec in early phase (0-10 min) and 126.0 ± 1.13 sec in the late phase (10-30 min). In morphine treated animals the paw licking response time was significantly reduced both in the early and late phase ($p<0.05$). Treatment with ethanolic extract of Azadirachta indica leaves significantly reduced the paw licking response time in acute and chronic phase in a dose dependent fashion ($p<0.05$). The magnitude of reduction in paw licking response time appeared to be more in chronic phase than the acute phase. (Table: 2).

Tail immersion test:

The mean reaction time in the vehicle treated mice during the observation periods of 30, 60 and 90 min were shown in (Table: 3). Morphine treated group showed significant increase in latency period after 30 min. The ethanolic extract of Azadirachta indica leaf in doses of 200mg/kg and 400mg/kg also showed significant increase in latency period after 30min when compared with the vehicle treated mice. A marked increase in maximal protective effect (MPE) was noted after treatment with morphine and to a lesser degree with Azadirachta indica extracts. For morphine treated group the %MPE at 30, 60 and 90min were 73%, 79% and 94% respectively. In a dose of 200mg/kg of Azadirachta indica the %MPE at 30, 60 and 90min were 20%, 26% and 28% respectively. For 400mg/kg of Azadirachta indica at 30, 60 and 90min the %MPE were 24%, 28% and 36% respectively. (Fig: 1).

DISCUSSION:

In order to estimate the antinociceptive property of any new substance using behavioural nociceptive tests it is essential to employ different tests which differ in stimulus quality,

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Table 1: Effect of Ethanolic Extract of *Azadirachta indica* Leaf Extract on Acetic Acid Induced Abdominal Constrictions in Mice

Treatment	Dose	No of writhing Mean ± SEM	% Inhibition
Vehicle, p.o.	------	37.0 ± 0.85	-----
Morphine, s.c.	10mg/kg	9.0 ± 0.55*	75.67%
***Azadirachta indica* ethanolic extract, p.o.**	200mg/kg	19.0 ± 0.57*	48.64%
	400mg/kg	16.0 ± 1.03*	56.75%

Each value represents the mean ± SEM of 6 observations,
*p < 0.05 compared with vehicle, one way ANOVA and Dunnett's t – test.

Table 2: Effect of Ethanolic Extract of *Azadirachta indica* Leaves Extract on Formalin Induced Nociception in Mice

Treatment	Dose	Early phase	% Inhibition	Late phase	% Inhibition
		Paw licking / biting time in seconds			
Vehicle	0.1ml/10g, p.o	64.0 ± 1.15	----	126.0 ± 1.13	----
Morphine	10mg/kg, s.c	9.0 ± 0.36*	85.93	7.0 ± 0.81*	94.44
***Azadirachta indica* leaves ethanolic extract**	200mg/kg, p.o	33.0 ± 1.35*	48.93	30.0 ± 0.68*	76.19
	400mg/kg, p.o	30.0 ± 0.68*	53.12	22.0 ± 0.93*	82.53

Each value represents the mean ± SEM of 6 observations,
*p < 0.05 compared with vehicle, one way ANOVA and Dunnett's t – test.

Table 3: Effect of Ethanolic Extract of *Azadirachta indica* Leaves Extract on Hot Water Tail Immersion in Mice

Treatment	Dose	Tail withdrawal latency time (seconds)			
		Time of observation			
		0 min	30 min	60 min	90 min
Vehicle (Tween80,1%v/v)	0.1ml/10g, p.o	1.5 ± 0.22	2.3 ± 0.21	2.0 ± 0.25	2.5 ± 0.22
Morphine	10mg/kg, s.c	1.8 ± 0.56	7.8 ± 0.91*	8.3 ± 0.84*	9.5 ± 0.22*
***Azadirachta indica* leaves ethanolic extract**	200mg/kg, p.o	1.6 ± 0.33	3.3 ± 0.42*	3.8 ± 0.47*	4.0 ± 0.36*
	400mg/kg, p.o	1.8 ± 0.36	3.8 ± 0.62*	4.1 ± 0.30*	4.8 ± 0.40*

Each value represents the mean ± SEM of 6 observations,
*p < 0.05 compared with vehicle, one way ANOVA and Dunnett's t – test.

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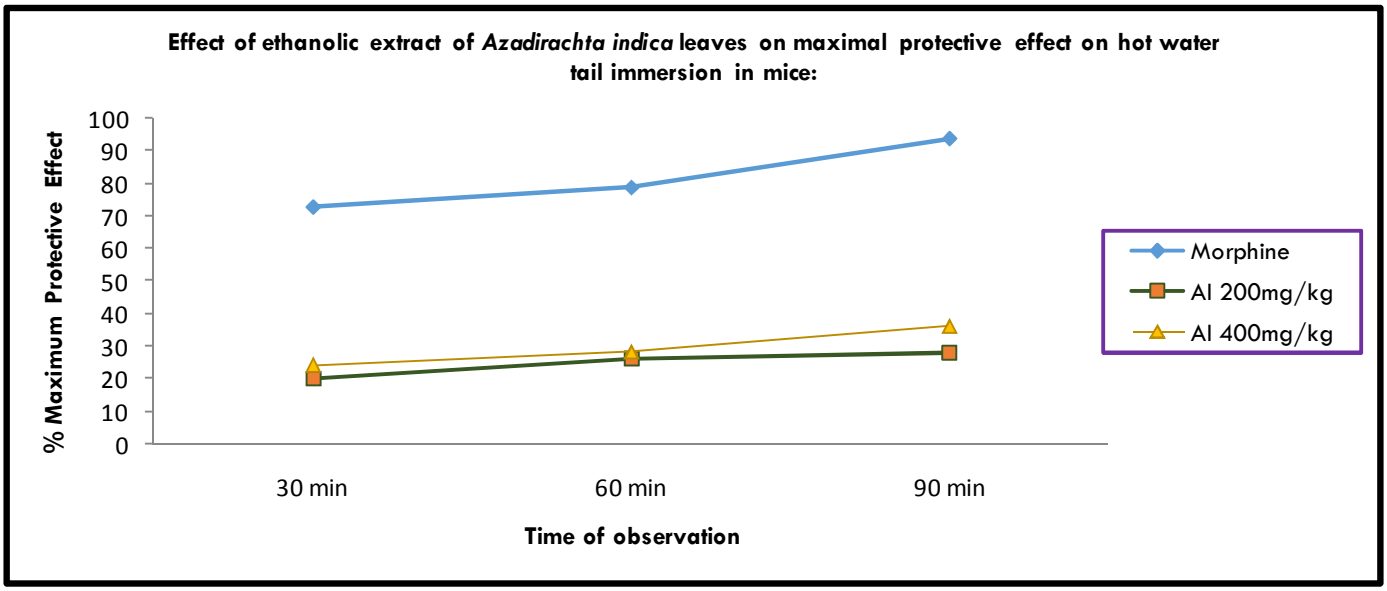

Figure 1: Effect of ethanolic extract of *Azadirachta indica* leaves on maximal protective effect on hot water tail immersion in mice

Maximum protective effect (% MPE) calculated by the following formula,
(%MPE) = [(Test latency – control latency) / (Cut off time – control latency)] × 100

intensity and duration [8]. Selection of mice for the present study enabled us to investigate the antinociceptive effect of ethanolic extract of Azadirachta indica leaves in three different types of nociception; viz: visceral nociception (acetic acid induced abdominal constriction assay), thermal nociception (hot water tail immersion assay), neurogenic and inflammatory nociception (formalin nociception).

Acetic acid induced abdominal constriction assay is regarded as a very sensitive method employing minimal noxious stimulus and even weaker analgesics can be detected from this method [5], it is simple, reliable and also affords rapid evaluation of peripheral type of antinociceptive action [9]. Acetic acid causes pain by liberating endogenous substances such as serotonin, histamine, prostaglandins (PGs), bradykinin and substance P, which stimulate nerve endings [10]. Local peritoneal receptors are postulated to be involved in the abdominal constrictions response. The significant reduction in both the doses of ethanolic extract of Azadirachta indica leaves suggests that it is a potent antinociceptive compound and the analgesic effect may be mediated by the inhibition of synthesis and release of PGs and other endogenous substances.

Formalin induced nociception measures the ability of the substance to attenuate moderate continuous pain generated by injured tissue [6]. The acute and chronic phases of formalin nociception are considered to represent neurogenic and inflammatory pain behaviour respectively. This procedure has also been employed for studying the antinociceptive response in diabetic animals to study chronic pain [11, 12, 13]. The early phase of formalin response is attributed to direct stimulation of nociceptors [14]. The late phase is due to an inflammatory reaction caused by tissue injury leading to the release of histamine, serotonin, prostaglandin and excitatory amino acids [15]. Centrally acting analgesic drugs like narcotic analgesics inhibit both the phases while peripherally acting drugs such as steroids and NSAID suppress mainly the late phase.

Treatment with Azadirachta indica extract significantly reduced the early phase of formalin response (Table - 2). The reduction in paw licking time is almost similar with both the doses of Azadirachta indica. Drugs that inhibit the early phase of formalin nociception are considered to be useful in neuropathic pain. The present observation on Azadirachta indica also suggests such a possibility. The degree of inhibition in the late phase of formalin nociception was much higher when compared to early phase in both the doses of Azadirachta indica leaves. This observation may suggest a more preferential and predominant effect of ethanolic extract of Azadirachta indica leaves on inflammatory pain.

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Hot water tail immersion test employs a high degree of thermal nociception and compounds exhibiting good nociceptive effect in this method may be considered as potent analgesics [7]. Thermal painful stimuli are known to be selective to centrally acting analgesic drugs. In the present study, a dose dependent increase in reaction time after treatment with the both the doses of ethanolic extract of Azadirachta indica leaves indicates the efficacy of these compounds in a model of thermal nociception.

Similar study by Khosla et al from Haryana in the year 2000, on aqueous extract showed significant antinociceptive properties at higher dose of 500 mg/kg, other earlier studies of the plant neem (Azadirachta indica) leaf extract by Zaman et al from Bangladesh in the year 2009, identified the potent antinociceptive properties of ethanolic extract at higher doses [16, 17]. Our study shows significant antinociceptive properties at the lowest dose of 200mg/kg and 400mg/kg when compare to earlier other two studies.

The acetic acid induced nociception and the late phase of formalin nociception are considered to represent the inflammatory pain response [6, 8]. Significant attenuation of both the above responses by both the doses of ethanolic extract of Azadirachta indica leaves suggests that these compounds may be more effective in inflammatory pain. Significant inhibition of acetic acid induced nociception, both the phases of formalin nociception, and thermal nociception by ethanolic extract of Azadirachta indica leaves indicate that it may be effective in pain of different origin.

Our earlier study of the plant extract Azadirachta indica leaves, the phytochemical screening of the compound showed the presence of glycosides, phenols, volatile oils, flavanoids, tannins, terpenoids, proteins and, carbohydrates [18]. Therefore, it is assumed that these compounds may be responsible for the observed analgesic activity. Flavonoids were reported to have a role in analgesic activity primarily by targeting prostaglandins. There are also reports on the role of tannins in anti-nociceptive activity. Besides, alkaloids are well known for their ability to inhibit pain perception. Tannins are important compounds known to be potent cyclooxygenase-1 inhibitors and with anti-phlogistic activity [19].

CONCLUSION:

The present study assumes significance in that it has identified the ethanolic extract of Azadirachta indica leaves in its two different dosage with potent antinociceptive effect, which might be potentially useful as such one of the alternative analgesic herbal drugs. Further investigation of this active ingredients may strengthen the pharmacological profile in general. Hence, its use in home remedies as pain killer is justified and substantiating its traditional usage in India.

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