Analgesic and Anti-Inflammatory Activity of Hydro-Alcoholic Extract of *Azadirachta Indica* Leaf

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Summary

The methanol extract of its leaf was investigated for its anti-inflammatory and analgesic activities in animal models. The hydro-alcoholic extract, ethyl acetae and n-butanol fractions at 100 mg/kg body weight reduced significantly the formation of oedema induced by carrageenan. In the acetic acid-induced writhing model, the extract and the fractions had a good analgesic effect ($P<0.01$) characterized by a reduction in the number of writhes when compared to the control. In tail flick method, the extract and all the fractions at 100 mg/kg showed significant activity ($P<0.01$) after 30 minutes. These results were also comparable to those of indomethacin, the reference drug used in this study. Acute toxicity test showed that the plant may be safe for pharmacological uses. This study has provided some justification for the folkloric use of the plant in several communities for conditions such as pain and inflammations.

Key words: analgesic, anti-inflammatory, carrageenan, histamine, indomethacin.

Introduction

Inflammation is the complex biological response of vascular tissues to harmful stimuli including pathogens, irritants, or damaged cells. It is a protective attempt by the organism to remove the injurious stimuli as well as initiate the healing process for the tissue (1). The process of inflammation is necessary for healing of wounds, however if not controlled, lead to onset of diseases like vasomotor rhinnorhoea, rheumatoid arthritis and atherosclerosis (2). Inflammation is characterised by classical signs edema, erythrema, pain, heat, and subsequently loss of function. Inflammatory models are of two types, acute and chronic inflammatory model. Acute models are designed to test drugs that modulate erythema, changes in vascular permeability, leukocyte migration and chemotaxis, phagocytosis poly-morpho nuclear leucocytes and other phagocytic cells, measurement of local pain, antipyretic activity and local analgesic action (3). Chronic models are designed to find drugs that may modulate the disease process and these include sponge and pellet implants and granuloma pouches which deposit granulation tissue, adjuvant induced arthritis and monoarticular arthritis which have an immune etiology (4). *Azadirachta indica* (Maliaceae) is a tree which has been used for a long time in agriculture and medicine. *Azadirachta indica* is an indigenous plant widely distributed in India. The medicinal properties of the plant Azadirchata indica were studied by several workers. The antipyretic effect, (5) antimalarial effect, (6) antitumour effect, antiulcer effect, antidiabetic effect, (7) antifertility effect, effect on the central nervous system and cadiovascular effect (8) were some of the studies of the earlier workers.

Despite the progress made in medical research during the past decades, the treatment of many serious diseases is still problematic. Chronic inflammatory diseases remain one of the world's major health problems (9). Inflammation is the response of living tissues to injury. It involves a complex array of enzyme activation, mediator release, extravasations of fluid, cell migration, tissue breakdown and repair (10). Inflammation has become the focus of global scientific research because of its implication in virtually all human and animal diseases. The conventional drugs used to ameliorate this phenomenon are either too expensive or toxic and not commonly available to the rural folks that constitute the major populace of the world (11). This study therefore seeks to examine *Azadirachta indica* for anti-inflammatory activity and analgesic effects since pain is one of the cardinal signs of inflammation.

Materials and Methods

Plant material

The leaves of *Azadirachta indica* were collected in December 2010 from Bhubaneswar, Odisha.. The plant material was taxonomically identified by Dr. P. C. Panda, Scientist, Regional Plant Resource Centre, Bhubaneswar, India. The voucher specimen and the herbarium were preserved in the Department of Pharmacognosy, Siksha O Anusandhan University, Bhubaneswar, India for future reference.

Extraction

The leaves were dried under shade and crushed into coarse powder. Coarse powder was extracted by cold maceration method at room temperature (24-26ºC) using methanol:water (1:1). The extract thus obtained was concentrated in rotary flash vacuum evaporator and further dried in vacuum oven. The hydromethanolic extract was subjected to fractionation with ethyl acetate and n-butanol. The fractions were dried in vacuum desiccator until use. Preliminary phytochemical studies of hydroalcoholic extract of *Azadirachta indica* leaf revealed the presence of alkaloids, triterpenoids, tannins and flavonoids.

Animals

Adult albino rats (150–200 g) and albino mice (20–25 g) of both sexes were obtained from the experimental animal facility of Siksha 'O' Anusandhan University, Bhubaneswar, Odisha. After randomization into various groups and before initiation of experiment, the rats and mice were acclimatized for a period of 7 days under standard environmental conditions of temperature (25 ± 2º C), relative humidity (35-60%) and dark/light cycle (12/12h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water ad libitum. All experimental procedures were reviewed and approved by University Animal Ethics Committee, Siksha O Anusandhan University.

Acute toxicity test

The acute toxicity of *Azadirachta indica* hydro-alcoholic extract was determined in rats according to the method of Hilaly *et al.*(12) with slight modifications. Rats fasted for 16h were randomly divided into groups of six rats per group. Graded doses of the extract (200, 400, 800, 1600 and 3200mg/kg p.o.) were separately administered to the rats in each of the groups by means of bulbed steel needle. All the animals were then allowed free access to food and water and observed over a period of 48h for signs of acute toxicity. The number of deaths within this period was recorded.

Determination of analgesic activity

Acetic acid induced writhing in mice

A group of mice were injected intraperitoneal (ip) with 0.lml/l0mg of 0.3 % (v/v) acetic acid. The mice exhibiting the writhing movements (stretching of hind limbs and bending of trunk) were selected for the study. The albino mice were divided into five groups (n = 6). Group I received normal saline (2 ml/kg), group II received indomethacin (10 mg/kg b.w. p.o.) group III, IV and V received hydro-alcoholic extract, ethyl acetate and n-butanol fractions at the doses of 100 mg/kg b.w., p.o. respectively. 30 min after indomethacin and test drugs administration, group II, III, III and IV received acetic acid (1% v/v, 10 ml/kg b.w., i.p.) and writhing reflex was noted for the period of 30 min (13).

Table 1: Analgesic activity of *Azadirachta indica* by acetic acid induced writhing method

Treatment and dose (mg/kg)	Number of writhing			% inhibition		
	0-10 min	10-20 min	20-30 min	0-10 min	10-20 min	20-30 min
Vehicle	23.53±0.874	25.88±0.569	13.76±0.668	-	-	-
Indomethacin (10)	9.78±1.07	13.11±0.852**	5.16±1.24**	58.43	49.34	62.5
Hydro-alcoholic extract (100)	16.67±0.783	18.82±1.58*	8.58±0.821**	29.15	27.27	37.64
Ethyl acetate fraction (100)	11.67±0.574	16.61±0.789**	7.82±1.04**	50.40	35.81	43.16
n-butanol fraction (100)	10.35±1.87	15.04±0.846**	6.89±0.656**	56.01	41.88	49.92

The values are represented as mean ± standard error of mean (SEM). Statistical significance was analyzed by One way ANOVA with Dunnett;s T-test. *P* values of < 0.05 were considered as statistically significant.

Hot plate method

The hot plate test was carried out as described by previous workers (14). Five groups of mice (n = 6) were treated orally with hydro-alcoholic extract (100 mg/kg), ethyl acetate (100 mg/kg), n-butanol (100 mg/kg), indomethacin (10 mg/kg) and normal saline (2 ml/kg). Mice were placed on a hot plate (Bibby Sterilin, UK) maintained at 55 ± 1°C and the reaction latency (in seconds) for licking of hind paw or jumping noted. The mice which reacted within 15 sec and which did not show large variation when tested on four separated occasions were selected for studies. Recordings were taken before treatment with the different drugs and 30, 60 and 90 minutes post treatment. Results were expressed as the difference between the baseline reaction latency and the reaction latency at recorded times.

Table 2: Analgesic activity of *Azadirachta indica* by hot plate method

Treatment and dose (mg/kg)	Mean latency (s) before and after drug administration(s)				% inhibition		
	0 min	30 min	60 min	90 min	30 min	60 min	90 min
Vehicle (2ml/kg)	3.46±1.07	2.57±1.68	3.82±1.32	2.17±1.78	-	-	-
Indomethacin (10)	1.94±0.683	6.97±1.38**	10.28±0.688**	14.16±0.674**	63.12	62.80	84.67
Hydro-alcoholic extract (200)	2.07±1.45	3.17±1.89*	4.58±0.861**	7.84±1.08**	18.92	46.69	63.77
Ethyl acetate fraction (400)	2.63±0.843	4.97±0.532*	6.78±1.14**	10.33±0.726**	48.28	43.68	78.99
n-butanol fraction (600)	2.93±0.673	6.07±1.02**	9.14±1.43**	12.9±0.663**	57.66	58.20	83.17

The values are represented as mean ± standard error of mean (SEM). Statistical significance was analyzed by One way ANOVA with Dunnett;s T-test. *P* values of < 0.05 were considered as statistically significant.

Tail flick method

Before the study, Swiss albino mice were screened for sensitivity test by placing the tip of the tail on the radiant heat source. Any animals that held to withdraw its tail in 5 second were rejected from the study. The selected animals were divided into four groups of six rats each. Each animal of the groups received one of the following extract (200mg/kg & 400mg/kg), Pentazocine (30mg/kg) and 2% w/v of Gum acacia (2ml/kg) in normal saline intraperitoneally. Analgesia was assessed with a tail flick apparatus (Analgesiometer). The basal reaction time was measured initially and another set of four measures were taken as 15, 30, 45,60, 90 and 120 minutes interval and the reaction of the animals considered as the post – drug reaction time. A cut-off period of 10sec. was observed to prevent tissue damage of the tail of the animals (15). The results are tabulated in table 3.

Table 3: Analgesic activity of *Azadirachta indica* by tail flick method

Treatment and dose (mg/kg)	Basal Reaction Time (seconds)	Reaction Time (seconds)					
		15 min	30 min	45 min	60 min	90 min	120 min
Vehicle (2 ml/kg)	3.9±0.23	3.9±0.28	4±0.23	4±0.21	4.1±0.28	4.1±0.24	4.2±0.29
Indomethacin (10)	3.24±0.54	6.97±0.67	9.04±0.36**	10.54±0.37**	11.84±0.69**	12.86±0.52**	10.42±0.58
Hydro-alcoholic extract (200)	3.7±0.26	5.4±0.37	6.5±0.58*	7.3±0.56*	8.7±0.42*	9±0.28*	7.8±0.42
Ethyl acetate fraction (200)	3.9±0.28	5.6±0.37	6.8±0.47*	8.3±0.52**	9.4±0.34*	9.6±0.27*	8.1±0.29
n-butanol fraction (200)	4.1±0.28	6.9±0.43	8.5±0.45*	9.4±0.22**	9.6±0.28**	9.9±0.52*	8.3±0.24

The values are represented as mean ± standard error of mean (SEM). Statistical significance was analyzed by One way ANOVA with Dunnett;s T-test. *P* values of < 0.05 were considered as statistically significant.

Determination of anti-inflammatory activity

Carrageenan induced rat paw oedema

The rats were divided into five groups containing six rats in each group. Group I served as control which received 0.9% normal saline in 3% Tween 80 [2ml/kg]), group II received), indomethacin (10 mg/kg body weight), group III, IV and V received hydro-alcoholic extract, ethyl acetae and n-butanol fractions respectively. Acute inflammation was produced by the sub-plantar administration of 0.1 ml of 1% carrageenan in normal saline that contained Tween 80 in the right paw of rats. The paw volume was measured at 0, 1, 2 and 3 h after carrageenan injection using a micrometer screw gauge. Increases in the linear diameter of the right hind paws were taken as an indication of paw oedema. Oedema was assessed in terms of the difference in the zero time linear diameter of the injected hind paw and its linear diameter at time *t* (i.e. 60, 120, 180 min) following carrageenan administration (10).

Table 4: Anti-inflammatory activity of *Azadirachta indica* by carrageenan induced rat paw oedema method

Treatment and dose (mg/kg)	Mean increase in paw volume (ml)			% inhibition of paw oedema		
	1 h	2 h	3 h	1 h	2 h	3 h
Vehicle (2 ml/kg)	0.46±0.108	0.73±0.187	0.94±0.865	-	-	-
Indomethacin (10)	0.16±0.821	0.22±0.452**	0.28±0.369**	65.21	69.86	70.21
Hydro-alcoholic extract (200)	0.31±0.865	0.48±0.572*	0.58±0.371**	32.60	34.24	38.29
Ethyl acetate fraction (400)	0.21±0.662	0.29±0.384**	0.35±0.289**	54.34	60.27	62.76
n-butanol fraction (600)	0.18±0.652	0.24±0.447**	0.29±0.583**	60.86	67.12	69.14

The values are represented as mean ± standard error of mean (SEM). Statistical significance was analyzed by One way ANOVA with Dunnett;s T-test. *P* values of < 0.05 were considered as statistically significant.

Results

The hydro-alcoholic extract, ethyl acetae and n-butanol fractions of *Azadirachta indica* induced significant decrease in the number of writhes when compared to the control (Table 1). The hydro-alcoholic extract, ethyl acetae and n-butanol fractions at 100 mg/kg b.wt. exhibited % age inhibition of 37.66, 43.16 and 49.92 respectively whereas indomethacin showed 62.5% inhibition. The results were found to be statistically significant ($p<0.01$).

Results of hotplate test are presented in Table 2 for the hydro-alcoholic extract and fractions of *Azadirachta indica* leaf. The % age nociception inhibition of stimulus by hydro-alcoholic extract, ethyl acetae and n-butanol fractions at the dose level of 100 mg/kg was 63.77, 78.99 and 83.17 respectively at 90 minutes. The results were found to be statistically significant ($p<0.01$).

In tail flick method, the hydro-alcoholic extract, ethyl acetae and n-butanol fractions of *Azadirachta indica* leaf at a dose of 100mg/kg showed significant activity of 6.5, 6.8 and 8.5 respectively ($P < 0.05$) after 30 minutes whereas indomethacin at a dose of 10mg/kg showed significant analgesic activity of 9.04 after 30 minutes ($P < 0.01$) (Table 3).

The results obtained as mean increase in paw volume and %age inhibition are shown in Table 4. The results shown % age inhibition of paw edema by the hydro-alcoholic extract, ethyl acetae and n-butanol fractions of *Azadirachta indica* in dose (100 mg/kg) were 38.29, 62.76 and 69.14 % respectively at 3 h after carrageenan administration. The % age inhibition of paw edema by indomethacin (10 mg/kg) was found as 70.21 at 3 h. The results were found to be statistically significant ($p<0.01$).

Discussion

The preliminary phytochemical analysis showed that the hydro-alcoholic extract of *Azadirachta indica* leaf revealed the presence of alkaloids, triterpenoids, tannins and flavonoids. The flavonoids are known to possess Anti-inflammatory activity by inhibiting the cyclooxygenase responsible for synthesis of inflammatory prostaglandins (16). Acetic acid induced writhing in mice attributed visceral pain finds much attention of screening analgesic drugs (17). The crude extracts of both the plants showed significant analgesic action compared to the reference drug indomethacin. Pain sensation in acetic acid induced writhing method is elicited by triggering localized inflammatory response resulting release of free arachidonic acid from tissue phospholipid(18) via cyclooxygenase (COX), and prostaglandin biosynthesis (19). In other words, the acetic acid induced writhing has been associated with increased level of PGE2 and PGF2α in peritoneal fluids as well as lipoxygenase products (20). The acetic acid induced writhing method was found effective to evaluate peripherally active analgesics. The agent reducing the number of writhing will render analgesic effect preferably by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition (21).

Carrageenan-induced oedema involves the synthesis or release of mediators at the injured site. These mediators include prostaglandins, especially the E series, histamine, bradykinins, leucotrienes and serotonin, all of which also cause pain and fever (22). Development of oedema induced by carrageenan is commonly correlated with early exudative stage of inflammation (23). Since carrageenan-induced inflammation model is a significant predictive test for anti-inflammatory agents acting by the mediators of acute inflammation (24). the results of this are an indication that *Azadirachta indica* can be effective in acute inflammatory disorders.

Conclusion

Since the plant extract reduced significantly the formation of oedema induced by carrageenan as well as reduced the number of writhes in acetic acid induced writhing models the *Azadirachta indica* leaf extract exhibited anti-inflammatory and analgesic activities. Again, no mortality was recorded in the acute toxicity study; it showed that the plant is safe for use. The study has thus provided some justification for the folkloric use of the plant in several communities for conditions such as stomachache, pain and inflammations.

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