RESEARCH PAPER

Evaluation of Anti-Inflammatory Activity of Azadirachta Indica in Wistar Rats

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ABSTRACT

Introduction: Various compounds obtained from Azadirachta indica have diverse spectrum of medicinally important actions, including antibacterial, antifungal, antiviral, antiulcer, hypoglycemic, antifertility, antimalarial, hepatoprotective, antioxidant, anticarcinogenic, insecticidal, anti-inflammatory, analgesic and antipyretic activities.

Objective: To screen the anti-inflammatory activity of Azadirachta indica in wistar rats.

Materials & Methods: Wistar rats weighing between 120-150 gm of either sex was fasted overnight and divided into 3 groups of 6 each. Group 1 is a control group in which administered 1ml N saline orally. Group 2 is a standard group in which administered the Naproxen 100 mg/kg orally. Group 3 is a test-group in which administered alcoholic extract of leaves of Azadirachta indica 200mg/kg orally. Acute inflammation was produced by injecting formalin intraperitoneally and sub-acute inflammation by implanting cotton wool pellets subcutaneously in healthy adult wistar rats. Mean peritoneal exudates volume and increment in dry weight granuloma of cotton pellet for various groups were calculated and expressed in ml and mg respectively. The percentage inhibition of volume of exudates and dry weight granuloma of cotton pellet was calculated and compared.

Results: Naproxen treated & Azadirachta indica treated group showed statistically significant inhibition of peritoneal exudate volume ($p < 0.001$) when compared to control. Similarly, the alcoholic extract of Azadirachta indica treated group also exhibited statistically significant decrease in granuloma dry weight ($p < 0.001$) when compared to control group. It was found that anti-inflammatory effect of alcoholic extract of leaves of Azadirachta indica was comparable to naproxen ($p > 0.05$) in subacute model of inflammation.

Conclusions: A. Indica (Ethanol extract of leaf) showed anti-inflammatory property by significantly reducing volume of exudates in formalin induced peritonitis (acute inflammation) and dry weight of granuloma of cotton pellet (subacute inflammation) when compared to control groups.

Keywords: Azadirachta indica, Anti-inflammatory, Formalin induced peritonitis, Naproxen, Cotton pellet.

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Introduction

Inflammation apart from being an important defense mechanism is disabling the accompaniment of many medical illnesses. Inflammation is a part of a complex biological response of vascular tissues to harmful stimuli such as pathogens, damaged cells, or irritants.[1] Drug therapy of inflammation has always been debatable. As of now, the drugs most commonly used in the treatment are glucocorticoids and Non-Steroidal Anti-inflammatory Drugs (NSAIDs). Most of these drugs are known to pose more or less deleterious side effects. NSAIDs are among the largest groups of anti-inflammatory drugs used all over the world and are also found to cause the 30% of all adverse drug reactions (ADRs).[2] NSAIDs are largely responsible for ADRs leading to hospital admissions or causing ADRs within the hospital episode. Hence there is always scope for continuous research to identify more effective and safer agents in the therapy of inflammation.

Azadirachta indica (Neem) is perhaps the most useful traditional medicinal plant in India. Various compounds obtained from Azadirachta indica have diverse spectrum of medicinally important actions, including antibacterial, antifungal, antiviral, antiulcer, hypoglycemic, antifertility, antimalarial, hepatoprotective, antioxidant, anticarcinogenic, insecticidal, anti-inflammatory, analgesic and antipyretic activities.[3] Hence this study was designed to evaluate the anti-inflammatory effect of alcoholic extract of leaves of Azadirachta indica.

Material and methods

Permission of the Institution Animal Ethics Committee was obtained prior to the commencement of the study. The study was conducted according to CPCSEA guidelines. Animals were housed in polypropylene cages with stainless steel top grills having facilities for providing food in the form of pellets and filtered water and paddy husk was used as the bedding. Regulated conditions were maintained with temperatures 18^0C-29^0C, humidity 30-70% and a 12-hours light-dark cycle. Animals were housed under standard laboratory conditions with free access to filtered water and commercial animal feed in the form of pellets.

A) Animals: Wistar rats of either sex weighing between 120-150 grams were procured from the central animal house, J. N. Medical College, Belgaum and were acclimatized to 12:12 hour light - dark cycle for 10 days prior to the day of experimentation.

B) Drugs and Chemicals:

1. Naproxen: Naproxen is a nonsteroidal anti-inflammatory drug (NSAID) of the propionic acid class. It is a nonselective COX inhibitor.[4]

IUPAC name (+)-(S)-2-(6-methoxynaphthalen-2-yl) propanoic acid.
Formula C14H14O3
Molar mass 230.259 g/mol

A well-known NSAID with analgesic, anti-inflammatory, and antipyretic qualities, naproxen is used to treat a variety of illnesses, including osteoarthritis, kidney stones, gout, rheumatoid arthritis, psoriatic arthritis, migraine, ankylosing spondylitis, menstrual cramps, tendinitis, and bursitis. Primary dysmenorrhea is also treated with it.[5] In this study naproxen was used as a standard anti-inflammatory drug and results obtained with the test compound (alcoholic extract of Azadirachta indica leaves) were compared with those of naproxen. Naproxen was procured from RPG Life Sciences Ltd., Ankleshwar.

2. Alcoholic extract of Azadirachta indica: The ethanolic extract of neem leaf was prepared according to the procedure described by Chattopadhyay.[6] Air-dried powder (1 kg) of A. indica leaves was mixed with 3L of 70% ethyl alcohol and kept at room temperature for 36 h. The slurry was stirred intermittently for 2 hours and left overnight. The mixture was then filtered, and the filtrate was concentrated under reduced pressure (bath temperature 50˚C) and finally dried in a vacuum desiccator. The residue collected (yield 48g/kg of neem leaf powder) was a thick paste, green in color. The extract was suspended in normal saline to obtain a final concentration of 20 mg/ml and used for the experiment.

3. Formalin: This is 37% by weight of formaldehyde gas in water. It is colorless and pungent in odor and is highly irritant to mucous membrane. In this study, 1% formalin is used for intraperitoneal injections to produce acute peritonitis model of acute inflammation.

4. Ether (Diethyl ether): It is a highly volatile liquid, produces irritating vapors which are inflammable and explosive. Ether is a potent anesthetic, produces good analgesia and marked muscle relaxation by reducing ACh output from motor nerve endings. In this study, ether is used to produce anesthesia while performing suturing in subacute models of inflammation, ie. Cotton pellet granuloma method.

5. Normal Saline: Normal saline is used as a suspending agent and drug vehicle both for alcoholic extract of A. indica leaves and naproxen; and administered in control group without any drug.

C) Equipment/Appliances:

- Mouth gag: Used to facilitate the introduction of feeding tube into the stomach of rats.
- Feeding tube: Used for oral administration of standard drug and the test compound.
- Tuberculin syringe: Used for injecting small volume of formalin (1 ml.) into peritoneal cavity of rats.
- 4 Skin suturing and dissection sets: Used for subcutaneous implantation of cotton wool pellets.
- Cotton wool pellets: Sterile cotton wool pellets each weigh 20 mg used for subcutaneous implantation in albino rats for inducing granuloma formation.
- Hot air oven: Used to dry the cotton pellet with granuloma tissue after extraction from rats.
- Electronic weighing scale: used to weigh cotton pellets before and after implantation in granuloma method.

Methods: Acute inflammation was produced by injecting formalin intraperitoneally and sub-acute inflammation by implanting cotton wool pellets subcutaneously in healthy adult wistar rats as described below.

FORMALIN INDUCED PERITONITIS IN RATS[7]

This method comprises the measurement and comparison of the volume of inflammatory exudates formed after intraperitoneal injection of formalin in different groups of rats. wistar rats weighing between 120-150 gm of either sex was fasted overnight and divided into 3 groups of 6 each.

Group 1- Control group; administered 1ml N saline orally.

Group 2- Standard group; administered Naproxen 100 mg/kg orally.[8]

Group 3- Test group; administered alcoholic extract of leaves of Azadirachta indica 200mg/kg orally.[9]

After one hour of oral administration, acute inflammation peritonitis was induced in all the rats of control, Standard, and test group by administering 1 ml of 1% formalin intraperitoneally. Four hours later the animals were sacrificed, abdominal cavities were opened and the peritoneal exudates volume was measured by placing the rat on a glass funnel as shown in figure 1. Mean peritoneal exudates volume for various groups were calculated and expressed in ml. The percentage inhibition of volume of exudates was calculated using the following formula,

$$\text{Percentage inhibition of peritoneal exudates formation} = 1 - \left(\frac{\text{Mean volume of peritoneal exudate in treated group}}{\text{Mean volume of peritoneal exudate in control group}} \right) \times 100$$

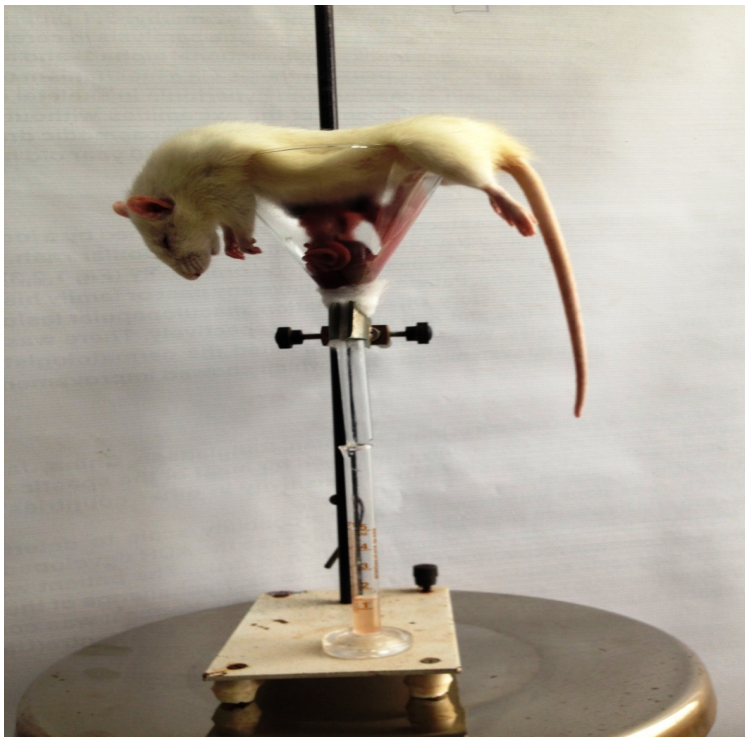


Figure 1: COLLECTION OF EXUDATES (Experimental Peritonitis Model in Wistar Rats)

The percentage inhibition of peritoneal exudate formation in Normal saline, Naproxen & Alcoholic extract of leaves of Azadirachta indica treated rats was determined and compared.

COTTON PELLET INDUCED GRANULOMA[10]

The method of Meir R et al, 1950 with some modification was adopted here to study the chronic inflammatory reaction induced by subcutaneous implantation of cotton pellets into the rats. This method involves comparison of inhibition of granuloma formation that occurs as an inflammatory response to a foreign body. Albino rats weighing between 120-150 gm of either sex was fasted overnight and divided into 3 groups of 6 each.

Group 1- Control group; administered 1ml N saline orally.

Group 2- Standard group; administered Naproxen 100 mg/kg orally.

Group 3- Test group; administered alcoholic extract of leaves of Azadirachta indica 200mg/kg orally.

After one hour the rats were subjected to light ether anesthesia and small incision of length 1 cm were made in the axillary region of both forefeet and groin region of both hindfeet. Sterile cotton pellets each weighing 20 mg were implanted in these areas (4 pellets in each rat) and the wounds were sutured with black silk thread as shown in figure 2 (A&B). The animals were maintained in clean cages and were allowed food and water ad libitum throughout the period of experimentation. From the 2nd day control, standard and test group rats were orally administered 1ml N saline, Naproxen 100 mg/kg and alcoholic extract of leaves of Azadirachta indica 200mg/Kg respectively, once a day till 5th day. On the 6th day the animals were sacrificed and the implanted cotton wool pellets with granuloma were removed from all the rats of control, standard & test groups.

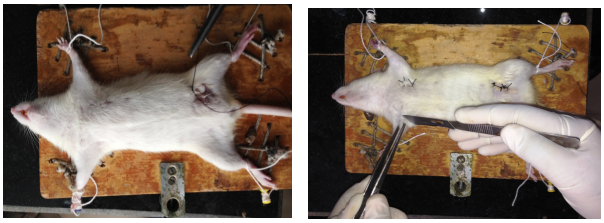


Figure 2 (A&B): IMPLANTATION OF COTTON WOOL PELLETS (Cotton-Wool Pellet Granuloma Model in Wistar Rats)

The increment in the dry weight of pellets (by calculating the difference between the dry weights of cotton wool pellets recorded before implantation and after removal) was taken as a measure of granuloma formation. The granuloma formation in Naproxen and Azadirachta indica treated groups was compared with control group. The inhibition of granuloma formation assesses anti-inflammatory activity. The percentage inhibition of granuloma formation in Naproxen & Alcoholic extract of leaves of Azadirachta indica treated rats was determined using following formula,

$$\text{Percentage inhibition of granuloma dry weight} = 1 - \left(\frac{\text{Mean Dry weight of granuloma in treated group}}{\text{Mean Dry weight of granuloma in control group}} \right) \times 100$$

The percentage inhibition of granuloma formation in Naproxen & Alcoholic extract of leaves of Azadirachta indica treated groups was determined and compared. The results observed are indicated in table X under the results section.

Data presentation and statistical analysis

Results were demonstrated as mean ± SEM. Statistical evaluation was done by using GraphPad Instat software version 3.06. Statistical tests were carried out by unpaired t test and analysis of variance, followed by a post hoc Tukey's test. In interpreting the results, $p \leq 0.05$ was as statistically significant.

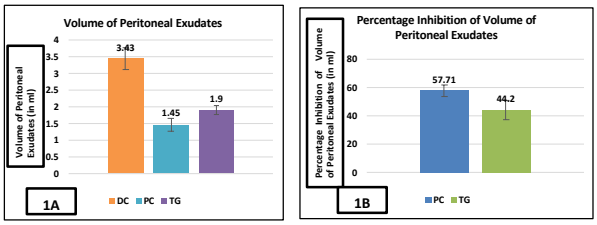
Results

In the present study, alcoholic extract of Azadirachta indica leaves was investigated for its possible anti-inflammatory effect, in acute and sub-acute models of inflammation in albino rats of either sex.

Acute Inflammation (formalin induced experimental peritonitis):

The mean peritoneal exudate volume in milliliters (ml) for control group after 4-hour interval was 3.43 ± 0.33. The corresponding mean peritoneal exudate volume in naproxen treated group was 1.45 ± 0.18, with the calculated percentage inhibitions of 57.71 ± 4.11 %. Naproxen treated group showed statistically significant inhibition of peritoneal exudate volume ($p < 0.001$) when compared to control as shown in graph 1A.

The mean peritoneal exudate volume in milliliters (ml) for A. indica treated test group after 4-hour interval was 1.9 ± 0.14, with the calculated percentage inhibitions of 44.20 ± 6.97 %. Azadirachta indica treated group showed statistically significant inhibition of peritoneal exudate volume ($p < 0.001$) when compared to control as shown in graph 1B.



Graph 1(A&B): Assessment of acute inflammation by formalin induced experimental peritonitis in Rats

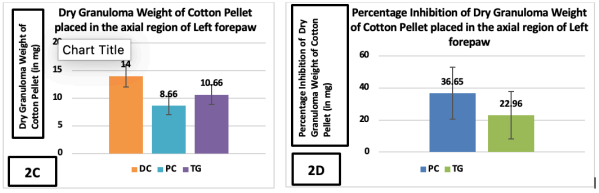
The above results clearly show the anti-inflammatory effect of alcoholic extract of leaves of Azadirachta indica in acute model of inflammation when compared to control. Further anti-inflammatory effect of A. indica group was compared with that of naproxen group. There was a statistically significant difference in mean peritoneal exudate volume of A. indica group when compared to mean peritoneal exudates volume of naproxen ($P < 0.05$) group. It shows that the anti-

inflammatory effect of A. indica was inferior to naproxen in acute model of inflammation.

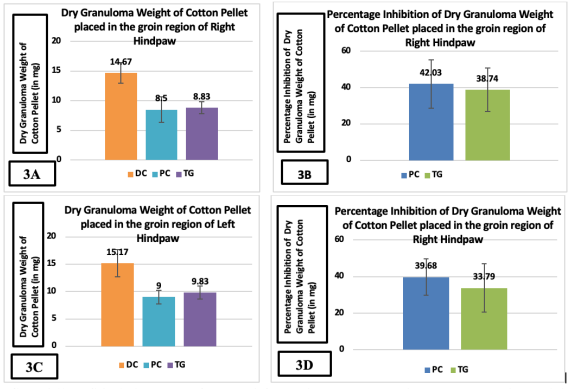
Subacute inflammation (cotton pellet induced granuloma model):

The mean dry weight of five-day old granuloma placed in all the four paws, expressed in milligrams (mg), in control group was 17.83 ± 1.16, 14 ± 2, 14.67 ± 1.75, & 15.17 ± 2.48 (Right forepaw, left forepaw, right hind-paw & left-hand paw respectively). In Naproxen treated group, cotton pellet placed all the paws showed significantly decreased dry weight granuloma when compared to control group with a p value <0.001 as shown in table 2 and graph 2A, 2B, 2C & 2D. Similarly, the alcoholic extract of Azadirachta indica treated group also exhibited statistically significant decrease in granuloma dry weight ($p < 0.001$) when compared to control group as shown in table 2 and graph 3A, 3B, 3C & 3D. Similar findings were was observed for percentage inhibition of naproxen & Azadirachta indica treated group when compared to control group.

Further alcoholic extract of leaves of Azadirachta indica was compared with subacute anti-inflammatory effect of naproxen. It was found that anti-inflammatory effect of alcoholic extract of leaves of Azadirachta indica was comparable to naproxen ($p > 0.05$) in sabacute model of inflammation.



Graph 2 (A, B, C&D): Assessment of subacute inflammation by cotton pellet induced granuloma in Rats (Fore paw – Right & Left)



Graph 3 (A, B, C & D): Assessment of subacute inflammation by cotton pellet induced granuloma in Rats (Hind paw – Right & Left)

Discussion and Conclusion

Nature itself has been a source of medicinal agents for thousands of years from which an impressive number of modern drugs have been isolated, many based on their use in traditional medicine. It has been observed that the original source of many important pharmaceuticals in current use have been plants used by indigenous people. It has been reported that about 64% of the total global population remains dependent on traditional medicine and medicinal plants for provision of their health-care needs. To promote the use of medicinal plants as potential sources of antimicrobial compounds, it is pertinent to thoroughly investigate their composition and activity and thus validate their use.[11] Azadirachta indica (Neem) is perhaps the most useful traditional medicinal plant in India. Various compounds obtained from Azadirachta indica have diverse spectrum of medicinally important actions, including antibacterial, antifungal, antiviral, antiulcer, hypoglycemic, antifertility, antimalarial, hepatoprotective, antioxidant, anticarcinogenic, insecticidal, anti-inflammatory, analgesic and antipyretic activities.[3] Hence the present study was conducted to evaluate the effect of alcoholic extract of A. indica leaves in acute and sub-acute models of inflammation, in wistar rats.

Results of the present study clearly indicate that A. indica leaves show significant anti-inflammatory activity in acute and sub-acute models of inflammation when compared to control group. Observations of the present study are in agreement with the earlier reports that have shown anti-inflammatory activity in different extracts from various parts of Azadirachta indica.[11,12,13]

The exact mechanism of anti-inflammatory action is still unclear. The study by Chattopadhyay showed that the test compound extract produces dose dependent inhibition of the inflammatory edema produced by 5HT and PGE, but its extract failed to suppress histamine and bradykinin induced edema.[6] These observations support the conclusion that A. indica has anti-inflammatory action probably by its anti 5HT and anti-PGE activity. Between the two edemogens A. indica exhibited greater action against PGE. Therefore A. indica resembles indomethacin like drugs. Neem also plays role as anti-inflammatory via regulation of proinflammatory enzyme activities including cyclooxygenase (COX), and lipoxygenase (LOX) enzyme.[14]

Acute model of inflammation:

In acute model of inflammation, ie. Formalin induced peritonitis; the results obtained indicate that the anti-inflammatory activity of naproxen is of higher order while that of the test compound (ethanolic extract of

leaves of a. indica) is promising. The "p" values of the results obtained are suggestive of statistical significance. Observations of the present study agree with the earlier reports showing statistically significant anti-inflammatory activity of Azadirachta indica leaves extract when compared to control group in paw edema model of acute inflammation by Kumar et al and Kanagasanthosh et al.[13]

Subacute model of inflammation:

The results obtained in sub-acute model of inflammation by granuloma method are suggestive of suppression of inflammatory activity of alcoholic extract of A. indica leaves, maybe by inhibiting any of the proinflammatory cytokines or inflammatory pathways. Compared with control group the anti-inflammatory activity of both standard and test groups is statistically significant. The "p" values of the results obtained are suggestive of statistical significance. Results of the present study are in accordance with the earlier studies showing significant anti-inflammatory activity of alcoholic leave extract of A. indica in granuloma method by Chattopadhyay et al and Neem oil by K D et al.[6,12] Further studies are required to support these findings in humans as the animal data cannot be directly extrapolated on humans.

Conclusions: A. Indica (Ethanol extract of leaf) showed anti-inflammatory property by significantly reducing volume of exudates in formalin induced peritonitis (acute inflammation) and dry weight of granuloma of cotton pellet (subacute inflammation) when compared to control groups.

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None

Conflict of Interest

None

Funding Source

None

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