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Original Article

# Influence of *Azadirachta indica* leaf extracts on tumor necrosis factor-α and interleukin-6 in albino rats and its computational analysis

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**Abstract**

The current study was designed to investigate the effect of *A. indica* (Neem) leaf extracts (ethanolic and aqueous) in yeast-induced pyrexia and acetic acid-induced writhing in rat models to evaluate the antipyretic and analgesic biomarkers and its phytochemical screening with computational analysis. For the antipyretic activity model 60 albino rats (160-200g) of either sex were divided into 4 groups and all groups were injected with yeast to induce pyrexia. Out of 4 groups, first group (control) consisted of 6 rats, treated with normal saline, the second group (standard) comprised 6 rats, treated with paracetamol. Third and fourth experimental groups consisted of 48 rats, treated with *A. indica* leaf ethanolic and aqueous extracts at doses of (50, 100, 200 and 400mg/kg b.w). For analgesic activity group division was the same and all groups were injected with acetic acid to induce pain TNF-α and IL-6 levels were measured using ELISA kits after blood samples were taken and serums were separated. An acute toxicity study was performed. In molecular docking, nimbandiol and nimbolide were used as ligand molecules to target protein Tnf-α and IL-6. In both activities at the dose of 400mg/kg, group III showed significant inhibition ($p<0.05$). Biomarkers showed significant results at the dose of 400mg/kg. Phytochemical screening was performed to reveal the existence of various active constituents. In molecular docking, nimbandiol and nimbolide showed -5 and -5.3 binding energies respectively, as compared to the standard drug paracetamol with -4.2 binding energy to TNF-Alpha protein. Therefore, *A. indica* extracts can be used as a valuable drug for the treatment of pain and fever.

**Keywords:** *Azadirachta indica*, Induced fever and pain, Screening, Biomarkers, Molecular docking

## 1. Introduction

Neem is an exclusive source of a wide range of chemicals with different chemical structures. Despite the fact that neem leaf extracts have long been utilized for medical purposes, modern pharmaceuticals require considerable study into pharmacotherapeutics mechanism of action, toxicity and bioactivity, as well as clinical trials and rigorous standardization [1]. The majority of plant components, including leaves, roots, fruits, seeds and bark contain antipyretic, antiseptic, anti-inflammatory, antiviral, anti-ulcer and antifungal chemicals [2]. *A. indica* leaves include carbohydrates, phenols, flavonoids, tannins, alkaloids, glycosides, triterpenoids, saponins and proteins according to phytochemical analysis [3]. Pain is a subjective unpleasant sensation that occurs when tissues are damaged or are on the verge of being damaged. Chemicals produced in the area because of cell damage may cause pain directly or indirectly by stimulating nerve terminals that mediate pain [4]. Furthermore, it is widely assumed that immunological or central nervous system cells generate cytokines that directly sensitize peripheral nociceptors. TNF, Bradykinins and interleukins all influence the transducing ability of free nerve terminals (ILs), prostaglandins (PGs) cause hyperalgia and pain [5]. Cuts, burns, sprains, earaches and headaches as well as fevers, can all benefit from neem's pain-relieving, anti-inflammatory and fever-reducing components [6]. Pyrexia is caused by a variety of nerve pathways and substances, including IL-6 and TNF- α. TNF-α and IL-6 levels were shown to be elevated following yeast-induced fever, according to data gathered from animal model studies. This meant that the amounts of these parameters might be used to assess a drug's antipyretic activity [7].

Molecular docking is a prominent approach in medicinal chemistry research since it predicts the binding orientations of ligands into a receptor-binding site. It is now es-

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sential for the development of various rational drug design strategies, such as structural-based virtual screening for discovering innovative candidates and a thorough understanding of the most important chemical components that guide ligand-protein interactions in relevant biological targets [8].

## 2. Materials and methods

### 2.1. Plant collection and preparation of *A. indica* leaves extract

*A. indica* leaves that were mature and fresh were taken from the University of Punjab, Lahore and confirmed by the staff members of the botany department. After the identification, cotton was used to clean the leaves, which were then desiccated at room temperature. Extraction of plant material was done by cold maceration method [9].

### 2.2. Experimental rats

Albino rats of either female and male sex (140- 200g) were used for antipyretic, analgesic and acute toxicity activity. For experimental study, albino rats were brought from the animal house of the Institute of Molecular Biology and Biotechnology (UOL). Rats were placed in polypropylene cages at the University of Lahore's animal house. Rats were fasted for some time before being used in experiments. After that, they were given distilled water and balanced feed [10].

### 2.3. Drugs used in the experiment

*A. indica* leaf extracts, Paracetamol, Diclofenac, Normal saline, Yeast, Acetic acid and distilled water.

### 2.4. Analgesic Activity Model

The writhing process occurs in rats, by the injection of acetic acid. It was injected to find out the potential of extract of *A. indica* in the pain process. But before 1 hour of the experiment, normal saline (10ml/kg) was administered intraperitoneally to group I animals. The rats in group II were given a dose of diclofenac 10ml/kg. The rats in groups III and IV were given different quantities of ethanolic and aqueous extracts of *A. indica* at doses of 50, 100, 200, and 400 mg/kg, respectively. The stopwatch was used, for counting the writhes. The rats were placed into different cages during activity. Complete activity was performed within 30 minutes [5].

$$\text{Analgesic activity} = \frac{Nc - Nt}{Nc} \times 100$$

Where,
Nt = treated group writhe
Nc = control group writhe

### 2.5. Antipyretic Activity Model

All the groups of test animals were injected with yeast below the nape of neck in order to induce fever. After injecting yeast, the fever developed after 21 hours and the highest temperature was 101.2 Fahrenheit. In group I, rats were injected with normal saline (10ml/kg) below the nape of the neck. The rats in group II were given a 10ml/kg dosage of paracetamol. The rats in groups III and IV were given different quantities of ethanolic and aqueous extracts of *A. indica* at doses of 50, 100, 200, and 400 mg/kg, respectively. After the time interval of 1, 2, 3 and 4 hours the body temperature (rectum) of rats was measured with digital thermometer [11].

For calculating the anti-pyretic activity, the formula is given below

$$\text{Inhibition} = \frac{B - Cb}{B - A} \times 100$$

Where,
B = temperature after fever induction
A = original body temperature
Cb = temperature after 1, 2, 3 and 4 hours

### 2.6. Acute toxicity study

A total of 20 rats were divided into four groups, each with five animals. Overnight fasting rats were given a single dosage of neem leaf extract (500, 1000, and 2000 mg/kg), while the control group was given distilled water (5ml/kg). Individual animals were examined for 48 hours for any behavioural and neurological changes such as diarrhoea, tremors, convulsions, lacrimation, salivation, sleep and feeding behaviour that could indicate acute toxicity. The observation period was prolonged to 14 days to look for any signs of mortality.

### 2.7. Qualitative and Quantitative phytochemical analysis

Analysis was carried out using standard protocols [12].

### 2.7.1 *In-silico* antipyretic and analgesic activity

To begin the *in-silico* investigation, various bioinformatics software was employed to support *A. indica* antipyretic and analgesic activity. Chemskech, chimaera, pymol, pyrx, depth residue, and discovery studio were used among the computational tools. TNF-alpha and IL-6 protein were chosen as target proteins for ligands in molecular docking studies. Protein 3D structure was evaluated using the Protein Data Bank (PDB). The 3D structure of the ligand molecules was assessed using the Pubchem website. Active sites were discovered in depth residue. PyRx was utilized for virtual screening to find a ligand molecule for a certain protein. For the complex's 3D construction, the Chimera tool was employed.

### 2.8.Statistical Analysis

Data were first subjected to homogeneity of variance to check normality then proceeded to one-way ANOVA using PROC GLM in SAS software (version 9.1). Duncan's multiple range test was applied for the comparison of significant treatment means considering $p < 0.05$.

## 3. Results

### 3.1. Antipyretic activity and effect of *A. indica* on TNF-α and IL-6

Fever was treated with *A. indica* aqueous and ethanolic extracts at various doses (50, 100, 200 and 400 mg/kg). When compared to the control group I, the extracts had considerably lower temperatures 1 to 4 hours after yeast injection. The higher dose of *A. indica* (400mg/kg) produced the most significant ($p<0.05$) percent inhibitory activity, which was comparable to that of the standard medication paracetamol. While ethanolic group III results at the dose of 400mg/kg showed 100% inhibition in fever resulting in more distinct curative as compared to aqueous group IV. Interleukin-6 and TNF-alpha concentrations in the plasma were increased as a result of yeast injection. In both ethanolic and aqueous extracts, TNF-alpha and IL-6 marker values differ considerably between treatment groups. There was significant upregulation in control

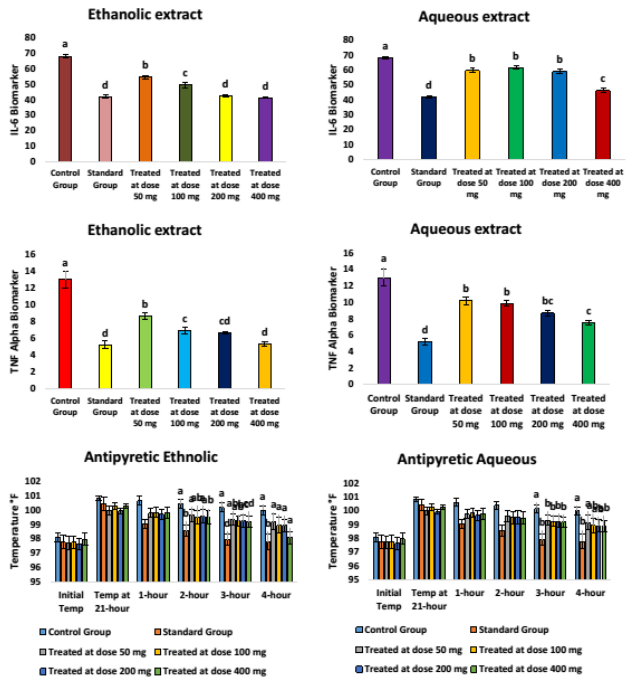


**Fig. 1.** TNF Alpha and IL-6 showing significant upregulation and downregulation and temperature of rats (F°) at 1-4 hours post yeast injection in groups I, II, III and IV.

group I and downregulation in groups III and IV at the concentration of 400mg/kg (Fig. 1).

### 3.2. Analgesic activity

In analgesic activity, ethanolic leaf extract of *A. indica* at a dose of 400mg/kg showed significant results (P<0.05) in comparison with the aqueous extract at doses of 50mg/kg, 100mg/kg, 200mg/kg and 400mg/kg. The dose of ethanolic leaf extract reduced writhing more significantly (P<0.05) in albino rats as compared to standard drug diclofenac (Table 1).

### 3.3. Acute toxicity

The acute toxicity results revealed that the ethanolic extracts had an excellent safety profile, as no mortality or toxicity-related symptoms were found in the rats at the highest dosage (2000mg/kg). The animals did not show any changes in their gross behaviour or associated stereotypical symptoms. After two weeks of treatment, the activities of ALT, AST and ALP in group IV were higher (P< 0.05) than the group II and III. The concentration of bilurubin, total protein and albumin in group II was higher (P< 0.05) than the group III and IV (Table. 2).

### 3.4. Phytochemical screening

The results of qualitative and quantitative phytochemical screening of the aqueous and ethanolic leaf extracts of *A. indica* showed the presence of tannins, saponins, phenols, alkaloids, flavonoids and glycosides. But protein was absent both in aqueous and ethanolic extract. Quantitative phytochemical screening showed that ethanolic extract showed high quantity of phytochemicals as compared to aqueous extract (Fig. 2).

### *3.5. In-silico* analysis

The molecular binding score of the phytochemicals of *A. Indica* and the standard drug paracetamol against target TNF-Alpha protein and IL-6 with scores -4.2 and -5 kcal/mol and -5.3. While the nimbolide exhibited the highest score among the three (Fig. 3).

**Table 1.** % Inhibition of pain by ethanolic and aqueous extracts of *A. Indica* and diclofenac on acetic acid-induced pain in rats.

| Treatment | Ethanolic | Aqueous |
|---|---|---|
| Control | $18.83^{a} \pm 0.31$ (0%) | $18.83^{a} \pm 0.31$ (0%) |
| Standard | $10.83^{d} \pm 0.83$ (-42%) | $10.83^{c} \pm 0.83$ (-42%) |
| Treated at dose 50mg/kg | $16.50^{b} \pm 0.65$ (-12%) | $18.25^{a} \pm 0.48$ (-3%) |
| Treated at dose 100mg/kg | $13.25^{c} \pm 0.85$ (-30%) | $16.00^{b} \pm 0.41$ (-15%) |
| Treated at dose 200mg/kg | $9.75^{d} \pm 0.48$ (-48%) | $10.75^{c} \pm 0.48$ (-42%) |
| Treated at dose 400mg/kg | $6.25^{e} \pm 0.48$ (-66%) | $8.50^{d} \pm 0.29$ (-54%) |
| p-value | < 0.0001 | < 0.0001 |

Superscripts on different means within row differ significantly at $p \leq 0.05$.

**Table 2.** Effect of acute toxicity by ethanolic extracts of *A. indica* and normal saline in rats.

| Parameter | Control | Dose A | Dose B | Dose C | p-value |
|---|---|---|---|---|---|
| AST (µL) | $10.00^{d}\pm1.30$ | $43.80^{c}\pm7.65$ | $82.60^{b}\pm5.16$ | $184.20^{a}\pm10.47$ | < 0.0001 |
| ALT(µL) | $9.20^{c}\pm1.07$ | $35.00^{b}\pm8.35$ | $43.40^{ab}\pm3.36$ | $49.60^{a}\pm1.57$ | < 0.0001 |
| ALP(µL) | $57.40^{d}\pm7.92$ | $109.40^{c}\pm7.07$ | $147.00^{b}\pm8.50$ | $204.40^{a}\pm5.16$ | < 0.0001 |
| Bilirubin (mg/dl) | $1.92^{b}\pm0.08$ | $2.56^{a}\pm0.30$ | $0.48^{c}\pm0.14$ | $0.38^{c}\pm0.11$ | < 0.0001 |
| Totalprotein (g/dl) | $7.94^{a}\pm0.09$ | $7.86^{a}\pm0.66$ | $6.08^{b}\pm0.25$ | $6.16^{b}\pm0.27$ | 0.0028 |
| Albumin (g/dl) | $3.86^{b}\pm0.21$ | $5.66^{a}\pm0.89$ | $1.78^{c}\pm0.12$ | $1.46^{c}\pm0.10$ | < 0.0001 |

Superscripts on different means within row differ significantly at $p \leq 0.05$
Dose A = 500mg/kg; Dose B = 1000mg/kg; Dose C = 2000mg/kg

| Phytochemical Component | Ethanol Extract | Aqueous Extract |
|---|---|---|
| Alkaloids | + | + |
| Sponins | + | + |
| Tannins | + | + |
| Flavonoids | + | + |
| Glycosides | + | + |
| Protien | – | – |
| Phenols | + | + |

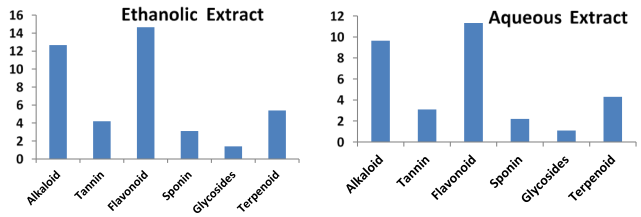


**Fig. 2.** Qualitative and Quantitative analysis of neem leaves extracts.

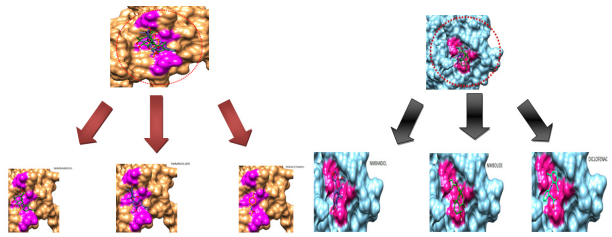

**Fig. 3.** Docking of TNF-α and IL-6 with 3 ligand molecules.

## 4. Discussion

Medicinal plants have been gaining more interest because of their beneficial properties for the living organisms. [13-25]. The goal of this research was to investigate whether *A. indica* ethanolic and aqueous leaf extracts could help with pain and fever. Phytochemical analysis of aqueous and ethanolic extracts of *A. indica* revealed the presence of different chemical components. The analgesic and antipyretic properties of leaf extract were investigated in albino rats using acetic acid and brewer's yeast to induce pain and fever. In this investigation, subcutaneous injection of yeast solution significantly increased rectal temperature after 21 hours, however, treatment with neem leaf extract resulted in a considerable decrease in rectal temperature. Our study correlated to the other findings explaining the leaf extract significantly reduced yeast-induced fever in rats [26]. The present investigation showed that the ethanolic leaf extracts of *A. indica* showed a dose-dependent significant 100% inhibition in fever at the dose of 400mg/kg. Also, the standard (paracetamol) was found to reduce pyrexia better than the plant extract at 1000, 250 and 125 mg/kg. However, at 500 mg/kg the extract was observed to have an improved % reduction in pyrexia (up to 150%) than the standard (paracetamol) [27]. We found that the ethanol leaf extract was found to have a better antipyretic effect than the aqueous leaf extract. Our study correlated to the previous study showing that the capacity of *A. indica* leaf extracts to block prostaglandin synthetase activity or decrease the rise of interleukin-1 production following interferon creation may account for the considerable reduction in baker's yeast-enhanced body temperature in rats [5].

This investigation showed that the presence of high amount of alkaloids, and flavonoids relatively high amount in ethanolic leaf extract may be liable for the greater antipyretic effects compared to aqueous leaf extract. This finding agrees with the report in which the ethanolic extract was found to contain alkaloids, tannins and terpenes in high amounts and thus greater antipyretic effects on rats than aqueous extract [28].

In the acetic acid-triggered writhing model, the natural release of arachidonic acid and prostaglandins is the cause of pain. Acetic acid stimulates the release of some endogenous chemicals that stimulate pain receptor nerve endings [29]. In other words, elevated levels of PGE2 and PGF2 in peritoneal fluids, as well as lipoxygenase products, have been linked to acetic acid-induced writhing [30]. In our study, the pain was significantly ($p<0.05$) reduced in the experimental groups treated with *A. indica* aqueous and ethanolic extracts. The presence of alkaloids, flavonoids, nimbolide and nimbandiol in *A. indica* leaf extract was discovered to be responsible for its analgesic and antipyretic properties in this study.

In the acute toxicity study, the rats were given ethanolic neem extracts in low to high dosages to determine the level of acute toxicity. The current investigation did not observe sedation, convulsions, diarrhea and itching in the rats. There was no fatality discovered during the 48-hour examination. The antipyretic activity of *A. Indica* leaf extract was evaluated using blood biomarkers (IL-6 and TNF-α) in control and experimental groups at various concentrations. After yeast injection, serum levels of IL-6 and TNF-alpha increased significantly in the control group. This is consistent with a previous study that found that IL-6 and TNF-a mRNA levels rose in rats with yeast-induced fever [31].

Computational approaches to drug discovery and development are effective and time-efficient. The protein-ligand docking elucidates the inhibition mechanism as well as the selectivity and efficacy of the ligand as an inhibitor [32]. Our study evidenced that the phytoconstituents such as nimbandiol and nimbolide present in *A. indica* have utmost inhibitory potential against TNF-alpha and IL-6 and could be practiced to manage the fever disorders. Our results are related to previous findings in which nimbolide demnostrated the inhibiting potential against fever [33-34].

## 5.Conclusion

From the above consequences, our study concluded that the ethanolic and aqueous extracts of *A. indica* can be used as a potential drug for pain and fever. We determined that ethanolic extract of *A.indica* showed significant results in both activities. In our present study, we concluded that *A. indica* is an efficient and protected drug in the treatment of pain and fever is attributed to the potentially bioactive phytochemicals like, flavonoids and alkaloids in neem leaf.

## Conflict of Interest

The authors declare no conflict of Interest.

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