

# Antinociceptive and Antioxidant Activities of Methanolic Extract of Leaves of *Azadirachta Indica* (Neem).

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## Abstract

Ethnopharmacological relevance

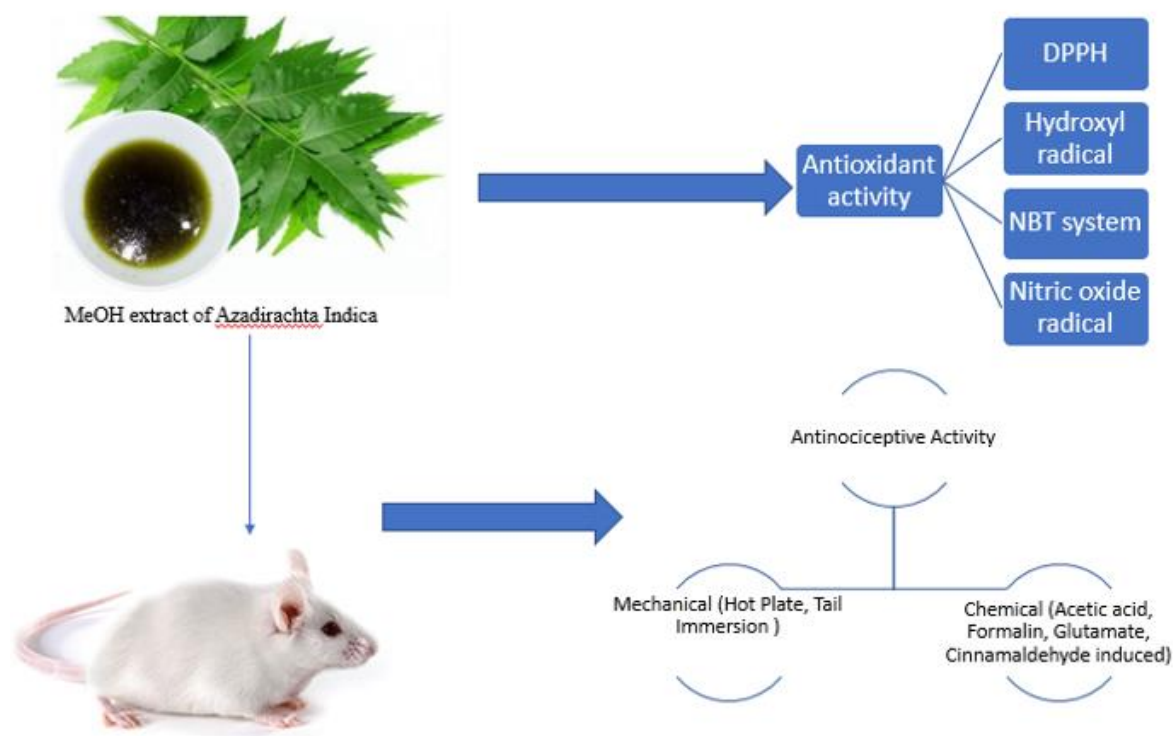
*Azadirachta indica* (Neem) is a communal plant of *Meliaceae* family called Neem Or *Kadunimb* in Maharashtra, India. Neem stated anti-inflammatory through regulation of proinflammatory enzyme activities with *COX* and *LOX* enzyme. Previous studies show that *Azadirachta Indica*(neem) and its chief constituents play essential role in anticancer management via the modulation of different molecular pathways including NF- $\kappa$ B, p53, PI3K/Akt, Bcl-2, pTEN and VEGF. Many parts of the plant are traditionally used in the treatment of various pharmacological action, the analgesic activity of Neem Seed Oil has already reported but Neem Leaves.

Methods: The antinociceptive activity of *Azadirachta Indica* Leaves (AZIL) was examined using heat-induced-mechanical (hot-plate and tail-immersion test) and chemical-induced (acetic acid, formalin, glutamic acid, cinnamaldehyde) nociception models in mice at 50,100, and 200 mg/kg doses. ATP-sensitive K<sup>+</sup> channel pathway, cyclic guanosine monophosphate (cGMP) pathway and involvement of opioid system was also tested using glibenclamide, methylene blue and naloxone/morphine respectively. The methanolic extract of leaves of *A. Indica* was assessed by using different *in vitro* antioxidant models of screening like scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, nitric oxide radical, superoxide anion radical, and hydroxyl radical.

Results: AZIL showed antinociceptive activity and antioxidant activity. In both hot plate and tail immersion tests AZIL significantly increases the latency to the thermal stimuli. In acetic acid-induced writhing test the extract repressed the number of abdominal writhing. Similarly, AZIL produced substantial dose-dependent inhibition of paw licking in both neurogenic and inflammatory pain induced by intraplantar injection of formalin. As well, AZIL also expressively withdrawn cinnamaldehyde-induced pain and the glutamate-induced pain in mice. It was also proved that pretreatment with naloxone significantly reversed the antinociception produced by AZIL in mechanical tests signifying the involvement of opioid system in its effect. Furthermore, administration of methylene blue, enhanced AZIL induced antinociception while glibenclamide, an ATP-sensitive K<sup>+</sup> channel antagonist, could not converse antinociceptive activity induced by AZIL.

Conclusion: Based on the results of the present study it can be said that AZIL keeps significant antinociceptive activity which acts in both central and peripheral mechanisms.

## 75 Graphical Abstract



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79 **Key words:** Antinociception, antioxidant, Azadirachta Indica, DPPH assay, Opioids system.

80 **Abbreviations:**

81 AZIL : Azadirachta indica leaves

82 COX: cyclooxygenase

83 LOX: lipoxygenase

84 pTEN: phosphatase and tensin homolog

85 VEGF: vascular endothelial growth factor

86 DPPH: 2,2-diphenyl-1-picrylhydrazyl.

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## 1. Introduction

*Azadirachta Indica* (Neem) is an evergreen, fast growing ancient tree, generally known as Miracle tree. Neem has an excellent of medicinal properties and active ingredients like *azadirachtin*, *nimbidin*, *flavonoids*, *alkaloids*, *saponins*, *tannins* which play vital role in various pharmacological actions [6]. *Azadirachta indica* has become significant in the global context today because it offers answers to the key concerns facing mankind. Since ancient time man is in search of remedies for pain. Pain is the commonest symptom that takes a patient to the hospital. Traditional medicine provides a substitute through which this quest can be fulfilled. Neem is a fast-growing perennial tree, is a native of Indian subcontinent, Africa, America. It is acknowledged for over 4000 years now and is called ‘arishtha’ in Sanskrit meaning ‘perfect, reliever of sickness, hence it has been acclaimed as ‘Sarbarogaribarini’. This miracle tree’ Neem also exhibits antibacterial, antiviral, antioxidant, anti-mutagenic, immunomodulatory, anti-inflammatory, anti-hyperglycemic, antiulcer, antimalarial, antifungal, and anticarcinogenic properties [6,8] Chemical constitutes which is demanded to possess analgesic and anti-inflammatory properties. NSAID’s and opioids are used nowadays, is limited by its own side effects. The analgesic activity using the Neem Seed Oil (NSO) has already been done but not the *Azadirachta indica* Leaf Extract (AZIL) [8]. Hence the present study is done to evaluate the analgesic effect of AZIL on albino mice as well determine antioxidant properties of AZIL via different models.

## 2. Chemicals

All chemicals and solvents were of analytical grade and obtained from Loba Chemicals Ltd, Mumbai. 1,1-diphenyl,2-picryl hydrazyl (DPPH) was obtained from Sigma, USA. The other chemicals used were sodium nitroprusside, sulphanilamide, *O*-phosphoric acid, nachyl ethylene diamine dihydrochloride, glacial acetic acid, nitroblue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), riboflavin, and Fe-EDTA. morphine sulphate, diclofenac sodium, naloxone (Lobachem, Mumbai), trichloroacetic acid (TCA), methanol, formalin, cinnamaldehyde, methylene blue, L-glutamic acid (Merck, Germany), glibenclemide, and DMSO (Lobachem, Mumbai). UV Visible spectrophotometer (Shimadzu 1700) was used for recording the spectra.

### 2.1. Experimental animals

Swiss albino mice (20–25 g) of either sex were obtained from National Institute of Bioscience (1091/GO/bt/S/07/CPCSEA), Dist- Pune (Maharashtra) and were adapted for 10 days under standard housing conditions (23±2°C; 50-55% RH with 12:12 h light/dark cycle). approved by Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The animals had free access to mice food (Lipton Gold Mohr, India) and water. The animals were habituated to laboratory conditions for one week prior to the experimental protocol to minimize any nonspecific stress. The experimental protocol was approved by the Institutional Animal Ethics Committee by Gourishankar Institute of Pharmaceutical education and research Limb Satara, India (Approval no. GIPER/2017-18/09).

## 2.2.Plant material and extract preparation

The leaves of *A.Indica* were collected from Satara region, Maharashtra, India in November 2017. The collected samples were then identified by Botany Department, Yashwantrao Chavan College of Science, Satara, India. Powdered dried leaves (50.75g) were soaked with 250 ml of methanol with occasional stirring at  $23\pm 2^{\circ}\text{C}$  for 72 Hrs [13]. The extract was then filtered with the help of sterilized cotton filter and Buchner funnel. The solvent was removed by rotary evaporator and 20g extract was obtained (Yield 39.40%). This crude extract was used for the further studies.

### 2.2.1. Phytochemical screening

AZIL was qualitatively tested for the detection of carbohydrates, saponins, flavonoids, tannins, alkaloids, glycosides, reducing sugars by standard procedures (See Supplement).

## 3. *In vitro* antioxidant activity

### 3.1.DPPH assay

To 1 ml extract of different concentrations, 1 ml solution of DPPH (0.1 mM) was added. An equal amount of MeOH and DPPH solution served as control. After 20 mins of incubation in the dark, absorbance was measured at 517 nm. 1g Ascorbic acid was used as standard. The experiment was done in triplicate and the percentage scavenging was calculated. [21]

### 3.2.Scavenging of nitric oxide radical

Nitric oxide was produced from sodium nitroprusside and measured by Griess reaction. Sodium nitroprusside (5mM) in standard phosphate buffer saline solution (0.75mM, pH 7.4) was incubated with different concentrations of (0.05-1mg/ml) of the methanolic extract dissolved in phosphate buffer saline after that the tubes were incubated at  $25^{\circ}\text{C}$ . after 5 Hrs 0.5 ml of solution was removed and diluted with 0.5 ml of Griess reagent (2g of 1% sulphanilamide, 2 g of 0.1% naphthyl ethylenediamine dihydro chloride and 5 ml of 2% O-phosphoric acid). The absorbance was read at 546 nm. Ascorbic acid was used as standard. The experiment was done in triplicate.[7,9,19]

### 3.3.Hydroxyl radical scavenging activity

AZIL with different concentrations (0.05-1mg/ml) were taken in different test tubes and evaporated on water bath. 1 ml DMSO, 0.5 ml of EDTA, and 1 ml Fe-EDTA were added and the reaction was started by adding 0.5 ml ascorbic acid to each of the test tubes. Test tubes were capped firmly and heated in water bath at  $75-95^{\circ}\text{C}$  for 20 m. after that the reaction was completed by addition of 1 ml of ice-cold TCA(17.5%, w/v) in every test tube and kept at RT for 5 to 10 min. The formaldehyde was measured by adding 2.5 ml Nash's reagent. This reaction mixture was kept aside for 15 min for color development. [5,17] Intensity of yellow color was measured spectrophotometrically at 412 nm. Ascorbic acid was used as standard.

### 3.4.Scavenging of superoxide radical by riboflavin-NBT system

The assay was constructed on the capacity of the sample to inhibit blue formazan formation by scavenging the superoxide radicals generated in the riboflavin-NBT system. The reaction mixture contains 50 mM phosphate buffer pH 7.6, 20 g riboflavin, 12 mM NBT. Reaction was started by enlightening the test samples of the extract (0.05-1 mg/ml). The absorbance was measured at 590 nm [3].

## 4. Drugs and treatments

The control group orally administered by deionized water (0.1 mL/mice) 30 min before the experiments. The positive control group intraperitoneally received standard drug morphine in hot plate, and tail immersion test at the dose of 2 mg/kg acetyl salicylic acid (100 mg/kg) in acetic acid-induced writhing and diclofenac sodium (10 mg/kg) in formalin induced licking, glutamate-induced paw licking, cinnamaldehyde-induced licking test 15 min before the experiments. AZIL was administered orally at the doses of 50, 100, and 200 mg/kg 30 min before the experiments. To assess the involvement of opioid-mediated antinociceptive activity, naloxone was administered (at the dose of 1 mg/kg) 10 min before morphine sulfate (2 mg/kg) or AZIL (50, 100, and 200 mg/kg) running in the hot plate and tail immersion test. Methylene blue (15 mg/kg) and glibenclamide (5 mg/kg) were intraperitoneally injected 15 min before control administration to evaluate the involvement of cyclic guanosine monophosphate (cGMP) and ATP-sensitive K<sup>+</sup> channel pathway respectively. All the doses of drugs and AZIL were prepared using ddH<sub>2</sub>O.

## 5. Antinociceptive analysis

### 5.1.Hot plate test

The mice that showed forepaw licking, withdrawal response within 15 s on hot plate (55 ± 0.5°C). Mice were fasted overnight with water given ad libitum. The animals were treated with morphine or AZIL and were placed on Eddy's hot plate kept at a temperature of 55 ± 0.5°C. A cut off period of 20 s was maintained to avoid paw tissue damage [14,21]. The response in the form of forepaw licking, withdrawal of the paw(s) or jumping was recorded on 30, 60, 90, and 120 min following treatment.

### 5.2.Tail immersion test

To evaluate the central analgesic property the tail immersion test was performed. This method is based on the observation that morphine like drugs prolongs the tail withdrawal time from hot water in mice [14,21]. 1-2 cm of tail of the mice pretreated with morphine/AZIL were immersed in warm water kept constant at 50 ± 0.5°C. The latency between tail submersion and refraction of tail was recorded. A latency period of 20 s was maintained to avoid tail tissue damage in mice. The latency period of the tail-withdrawal response was taken, as the index of antinociception and was determined at 30, 60, 90, and 120 min after the administration of morphine or AZIL.

### 5.3.Acetic acid-induced writhing test



Acetic acid-induced writhing test was performed to evaluate the peripheral antinociceptive activity of AZIL in chemical-induced pain. The mice were treated with drug or AZIL and then the writhing was induced by injecting 0.5% acetic acid after 10 and 20 min, respectively, at the dose 10 ml/kg body weight. Five min after the injection of acetic acid, the mice were observed, and the number of writhing was counted for 30 min [15]. The contractions of the abdomen, elongation of the body, twisting of the trunk and/or pelvis ending with the extension of the limbs were considered as complete writhing.

#### 5.4. Glutamate-induced nociception

20  $\mu$ L of glutamate (10  $\mu$ M/paw) was injected into the ventral surface of the right hind paw of mice 30 min after AZIL treatment and 15 min after injection of diclofenac sodium. The mice were observed for 10 min following glutamate injection and number of paw licking was counted as an indication of nociception [4,17].

#### 5.5. Formalin-induced nociception

Mice were injected with 10  $\mu$ L of a 2.5% formalin solution (0.92% formaldehyde) made up in saline into the subplantar region of the right hind paw 60 min after AZIL treatment and 10 min after injection of diclofenac sodium. Licking of the injected paw was recorded as nociceptive response from 0-5 min (neurogenic phase) and 10-20 min (inflammatory phase) after formalin injection [14,12].

#### 5.6. Cinnamaldehyde-induced nociception

10  $\mu$ L of cinnamaldehyde (10 nM/paw prepared in saline) were injected intraplantarly in the ventral surface of the right hind paw. Animals were observed individually for 5 min following cinnamaldehyde injection. The amount of time spent licking the injected paw was recorded with a chronometer and was considered as indicative of nociception. The animals were treated with AZIL orally 1 hr before cinnamaldehyde injection. Control animals received vehicle (10 ml/kg orally) [4].

### 6. Analysis of the possible mechanism of action of AZIL

#### 6.1. Involvement of opioid system

The possible participation of the opioid system in the antinociceptive effect of AZIL was examined by injecting naloxone hydrochloride (2 mg/kg i.p.), a non-selective opioid receptor antagonist, 15 min prior to the administration of either morphine or AZIL. Then the hot plate and tail immersion latencies were measured at 30, 60, 90 and 120 min with the same cut off time of 20 s for the safety of animals [11].

#### 6.2. Involvement of cyclic guanosine monophosphate (cGMP) pathway

To verify the possible involvement of cGMP pathway in the antinociceptive action caused by AZIL the mice were pretreated with methylene blue (20 mg/kg), a non-specific inhibitor of

Nitric oxide/guanylyl cyclase, intraperitoneally 15 min before administration of either diclofenac sodium or AZIL. Then the nociceptive responses against 0.6% acetic acid injection were observed for 30 min, starting from 5 min post injection. The numbers of abdominal writhing were counted as indication of pain behavior [11].

### 6.3. Involvement of ATP-sensitive K<sup>+</sup> channel pathway

Possible contribution of K<sup>+</sup> channel in the antinociceptive effect of AZIL was evaluated by previously described method [16,17]. The mice were pre-treated with glibenclamide (10mg/kg), an ATP-sensitive K<sup>+</sup> channel inhibitor, intraperitoneally 15 min before administration of either diclofenac Sodium or AZIL. Following the injection of acetic acid, the animals were immediately placed in a Perspex chamber and the number of writhing was recorded for 30min, starting from 5 min post injection.

## 7. Statistical analysis

The results are presented as MEAN±SEM. The statistical analysis of the results was performed using one-way analysis of variance (ANOVA) followed by Dunnett's test or Bonferroni's test as appropriate using SPSS 25.0 software. Differences between groups were considered significant at a level of  $p < 0.001$  and  $p < 0.05$ . The results of the tail immersion and hot plate tests were given with percentage of the maximal possible effect (%MPE), which was calculated using the following formula.

$$\%MPE = \frac{[(Postdrug\ latency) - (Predrug\ latency)]}{[(Cut\ off\ time) - (predrug\ latency)]} \times 100$$

## 8. Results

### 8.1. Phytochemical screening

The preliminary screening revealed the presence of alkaloid, carbohydrate, glycoside, steroid, flavonoid, saponin and tannin in AZIL (Table no.1).

#### 8.1.2. Acute toxicity

From the acute toxicity test it has been found that the LD<sub>50</sub> of AZIL is 758.58 mg/kg in mice.

#### 8.1.3. Antioxidant activity

Six concentrations ranging from 0.05 to 1 mg/ml of the methanolic extract of *A. indica* were tested for their antioxidant potential using different *in vitro* models. Study indicate that free radicals were scavenged by test compounds at different concentrations (0.05, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml). The maximum inhibitory concentration (IC<sub>50</sub>) in nitric oxide



radical, DPPH, superoxide, hydroxyl radical scavenging activity,  $IC_{50}$  were found to be 6.38, 6.65, 9.21, and 4.35 mg/ml, respectively. The antioxidant model and percentage scavenging of each concentration of extract and standard are shown in Table 2.

#### 8.1.4. Hot plate test

The antinociceptive effect of AZIL and morphine in hot plate test are given in Table 3. AZIL at 100 and 200 mg/kg doses significantly increased the reaction time to the thermal stimulus ( $p < 0.05$ ). The antinociceptive effect was dose-dependent as we observed stronger effect at 200 mg/kg dose than 50 mg/kg dose. Morphine showed highest latency at all the observation periods. The extract also showed significant increase in latency to the thermal stimuli at 50, 100, and 200 mg/kg doses ( $p < 0.05$ ). Naloxone exerted significant ( $p < 0.05$ ) antagonistic effect on the antinociceptive activity of AZIL and morphine.

#### 8.1.5. Tail immersion test

The antinociceptive activity of AZIL and morphine in tail immersion test has been given in Table 4. AZIL at all three doses (50, 100, and 200 mg/kg) significantly increased the latency period to hot-water induced thermal stimuli ( $p < 0.001$ ) in a dose-dependent manner. Morphine showed highest latency, however, the extract also showed significant latency at 50, 100, and 200 mg/kg doses ( $p < 0.001$ ) at different observation time. Naloxone exerted significant ( $p < 0.05$ ) antagonistic effect on the antinociceptive activity of AZIL at all three doses and of morphine throughout the observation periods. (Table 4)

#### 8.1.6. Acetic acid-induced writhing test

The effect of oral administration of AZIL using the abdominal constriction test in mice is shown in table 5. It was found that AZIL was able to inhibit the nociceptive effects induced by acetic acid compared to the control group (Deionized water) at the doses of 50, 100, and 200 mg/kg, respectively ( $p < 0.001$ ). The percentage inhibition of constrictions was calculated as 69.94% (Acetyl salicylic acid, 100 mg/kg), 18.55% (AZIL, 50 mg/kg), 41.95% (AZIL, 100 mg/kg), and 50% (AZIL, 200 mg/kg).

#### 8.1.7. Formalin test

AZIL produced a dose-related inhibition of formalin induced nociception and caused inhibition of both neurogenic (0–10 min) and inflammatory (10–30 min) phases of formalin-induced licking test at the doses of 50, 100, and 200 mg/kg when compared with control group (Deionized water) (Table 7). Antinociceptive effect was more pronounced in the second phase of this model of pain. Diclofenac sodium (10 mg/kg, i.p.) reduced formalin induced nociception in both phases ( $p < 0.001$ ).

#### 8.1.8. Glutamate-induced nociception

The antinociceptive activity induced by oral administration of AZIL was dose-dependent. It showed that AZIL at the doses of 50, 100, and 200 mg/kg produced significant prohibition of the glutamate-induced nociception test (Table 6). Diclofenac sodium (10 mg/kg) was used as a

standard drug, which showed 82.14 % inhibition of licking as compared to the control group. All treatments displayed significant antinociceptive activity compared together with the control group (Deionized water).

### 8.1.9. Cinnamaldehyde-induced nociception

The result of cinnamaldehyde-induced nociception showed that administration of AZIL at 50, 100, and 200 mg/kg dose produced dose-dependent inhibition of the cinnamaldehyde-induced neurogenic nociception with the percentage of inhibition of 30.37%, 65.18% and 72.68%, respectively (Table 8). Only 50 mg/kg AZIL treatment showed no significant difference compared with the control group, at the same time as the rest presented noteworthy antinociceptive activity ( $p < 0.001$ ).

### 8.1.10. Involvement of cyclic guanosine monophosphate (cGMP) Pathway

The study showed at the effects of 50, 100, and 200 mg/kg AZIL and methylene blue (20 mg/kg) treatments. Methylene blue administration expressively inhibited acetic acid-induced abdominal writhing (Fig. 1). Given together, methylene blue significantly increased AZIL (200 mg/kg) induced antinociception compared to the control group.

### 8.1.11. Involvement of ATP-sensitive K<sup>+</sup> channels pathway

This study appeared at the effects of 50, 100, and 200 mg/kg AZIL and glibenclamide (10 mg/kg) treatments. It was found out that glibenclamide administration alone did not alter abdominal writhing count when assessed through the injection of 0.6% acetic acid (Fig. 2). When given together, the antinociceptive activity of AZIL was noticeably decreased by glibenclamide at the doses of 50, 100 and 200 mg/kg, respectively.

**Table No. 1 Phytochemical analysis of AZIL**

Sr.No	Phytochemicals	Water	Methanol
1	Alkaloids	++	+++
2	Flavonoids	++	+++
3	Saponins	+	+
4	Tannins	+	++
5	Reducing sugars	++	+++
6	Glycosides	++	+++
7	Phenol	+	++
8	Terpanoids	-	+
+: Low; ++: Medium; +++: High, -: Absent			

390 **Table No. 2 Antioxidant activity of Azadiracta Indica**

Sr.No	Antioxident models	% RSA of AA 200mg/ml (Control)	Azadiracta indica extract (mg/ml)						
			0.05	0.1	0.2	0.4	0.8	1.0	IC <sub>50</sub>
1	DPPH*	76.35	29.35	34.15	47.65	49.32	53.647	61.25	07.65
2	Nitric Oxide	78.65	41.36	44.36	51.26	69.35	70.32	74.36	06.38
3	Hydroxyl radicle	61.37	37.65	47.62	52.36	56.65	61.26	73.95	04.35
4	Superoxide radicle	83.65	43.65	51.65	61.65	67.96	74.64	84.36	09.21

391 AA- Ascorbic acid, \*-1,1-diphenyl, 2-picryl hydrazyl

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393 **Table No. 3 Effect of AZIL extract in hot plate test**

Treatment	Dose (mg/kg)	Latency period (Seconds) (%MPE)				
		Pre-treatment	30 Min	60 Min	90 Min	120 Min
Control	0.1ml/mice	9.1 ±0.35	10.25±1.1 *	12.65±0.6	14.68±0.7	14.99±2.6 **
Morphine	2	10.2 ±0.10	9.91±0.67(-15.15)	12.78±0.12 (5.18)	15.54±0.51 (6.69)	15.98±0.09(7.48)
Nalaxone(NLX)	1	11.68 ±0.8	11.31±1.1	10.87±1.3	9.84±0.98	9.03±0.21
Control+NLX	1	11.35 ± 0.3	9.97±1.54	9.57±0.12	11.65±0.68	12.08±1.5
Morphine+NLX	2+1	11.98 ± 0.2	12.36±0.54(2.10)	12.78±0.51 (1.66)	13.14±1.3(1.48)	13.57±0.65(1.47)
AZIL	50	9.54 ± 0.22	9.99±0.25 (2.25)	10.24±0.24 (1.40)	12.35±0.78 (3.51)	12.96±0.67(3.12)
	100	10.35 ± 0.6	10.94±0.58(3.00)	12.25±0.59 (2.38)	13.65±1.4(4.14)	14.85±0.60(4.10)
	200	10.96 ± 0.7	11.58±0.24(5.3)	12.22±0.14 (2.56)	13.52±0.11 *(3.23)	15.17±0.65(3.86)
AZIL+NLX	50+1	11.61 ± 0.1	11.72±0.12(0.59)	11.97±0.25 (0.74)	12.03±0.84 (0.53)	12.68±0.27(0.98)
	100+1	12.13 ±0.54	12.43±0.30(1.67)	12.97±0.68 (1.75)	13.37±0.44 (1.59)	13.96±0.29(1.69)
	200+1	13.03 ± 0.5	13.12±0.21(0.53)	13.26±0.32 (0.48)	13.54±0.56 (0.66)	13.87±0.11(0.79)

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Each value is presented as the mean  $\pm$  SEM (n = 5). AZIL =Azadiracta Indica Leaves extract;  
NLX = Naloxone.

\*p < 0.05 compared with the control group (Dunnett's test).

**Table No. 4 Effect of AZIL extract in tail immersion test**

Treatment	Dose (mg/kg)	Latency period(Seconds) (%MPE)				
		Pre-treatment	30 Min	60 Min	90 Min	120 Min
Control	0.1ml/mice	3.39 $\pm$ 0.22	3.68 $\pm$ 0.2	3.72 $\pm$ 1.3	3.81 $\pm$ 0.65	3.89 $\pm$ 0.80
Morphine	2	4.11 $\pm$ 0.1	5.84 $\pm$ 0.9 (6.68)	7.84 $\pm$ 0.2 (6.67)	8.63 $\pm$ 0.54*(4.71)	6.95 $\pm$ 0.5* (2.45)
Nalaxone(NLX)	1	5.51 $\pm$ 0.5	4.98 $\pm$ 0.1	4.54 $\pm$ 0.64	4.11 $\pm$ 0.25	3.95 $\pm$ 0.67
Control+NLX	1	4.21 $\pm$ 0.5	4.65 $\pm$ 0.51	4.98 $\pm$ 0.68	5.67 $\pm$ 0.21*	5.87 $\pm$ 0.63
Morphine+NLX	2+1	3.97 $\pm$ 0.54	4.14 $\pm$ 0.41(0.58)	4.36 $\pm$ 0.84(0.66)	4.57 $\pm$ 0.51(0.60)	4.21 $\pm$ 0.09* (0.20)
AZIL	50	3.33 $\pm$ 0.23	3.87 $\pm$ 0.81(2.02)	4.25 $\pm$ 0.10 (1.62)	5.11 $\pm$ 0.2* (5.89)	5.00 $\pm$ 0.61(1.43)
	100	3.61 $\pm$ 0.5	4.02 $\pm$ 0.24(1.55)	5.21 $\pm$ 0.58(2.83)	6.21 $\pm$ 0.87*(3.00)	5.31 $\pm$ 0.82(1.46)
	200	3.84 $\pm$ 0.43*	4.51 $\pm$ 0.29(2.56)	5.98 $\pm$ 0.16(3.81)	6.44 $\pm$ 0.17 (4.01)	5.64 $\pm$ 0.26(1.54)
AZIL+NLX	50+1	3.29 $\pm$ 0.55	3.61 $\pm$ 0.18(1.19)	3.89 $\pm$ 0.27(1.05)	3.94 $\pm$ 0.67 (0.7)	4.06 $\pm$ 0.95 (0.65)
	100+1	3.86 $\pm$ 0.14	3.72 $\pm$ 0.34(-0.53)	3.44 $\pm$ 0.68(-0.72)	3.94 $\pm$ 0.24*(0.09)	3.11 $\pm$ 0.19 (-0.64)
	200+1	2.62 $\pm$ 0.87	2.55 $\pm$ 0.21(-0.25)	2.43 $\pm$ 0.41 (-0.33)	2.96 $\pm$ 0.54*(0.41)	2.21 $\pm$ 0.12 (-0.34)

Each value is presented as the mean  $\pm$  SEM (n = 5). AZIL =Azadiracta Indica Leaves extract;  
NLX = Naloxone.

\*p < 0.05 compared with the control group (Dunnett's test).

**Table No. 5 Effect of AZIL on acetic acid-induced writhing in Mice**

Sr no	Treatment	Dose(mg/kg)	No of Writhings	Inhibition(%)
1	Control	0.1 ml/mice	37.3 ± 3.2	-
2	Acetyl salicylic acid	100	11.21 ± 2.1	69.94
3	AZIL	50	30.58 ± 1.3*	18.55
		100	21.65 ± 1.1*	41.95
		200	18.65 ± 1.8**	50.00

\* $P < 0.05$ , \*\* $P < 0.01$  vs. control, [Values are mean ± SE from six animals in each group.]

\* $p < 0.001$  compared with the control group (Dunnett's test).

**Table No. 6 Effect of AZIL in glutamate-induced nociception**

Sr no	Treatment	Dose(mg/kg)	Licking Time (Sec)	Inhibition(%)
1	Control	0.1 ml/mice	121.6 ± 4.2	-
2	Diclofenac Sodium	10	39.65 ± 3.1	67.39
3	AZIL	50	107.00 ± 1.3**	12.00
		100	64.36 ± 1.1*	47.07
		200	51.23 ± 1.8*	57.87

\* $P < 0.05$ , \*\* $P < 0.01$  vs. control, [Values are mean ± SE from six animals in each group.]

\* $p < 0.001$  compared with the control group (Dunnett's test).

**Table No. 7 Effect of AZIL in formalin-induced nociception**

Sr no	Treatment	Dose(mg/kg)	Early Phase (0-10 Min)		Late phase (10-30 min)	
			Licking Time (Sec)	Inhibition (%)	Licking Time (Sec)	Inhibition (%)
1	Control	0.1 ml/mice	101.23 ± 4.2	-	31.65 ± 0.36	
2	Diclofenac Sodium	10	21.65 ± 3.1	78.61	5.65 ± 0.76	82.14
3	AZIL	50	73.68 ± 1.3**	27.21	26.69 ± 1.2*	15.67
		100	59.27 ± 1.1*	41.50	13.84 ± 0.98	56.27
		200	33.33 ± 1.8*	67.07	8.6 ± 0.35**	72.82

\* $P < 0.05$ , \*\* $P < 0.01$  vs. control, [Values are mean ± SE from six animals in each group]

\* $p < 0.001$  compared with the control group (Dunnett's test).

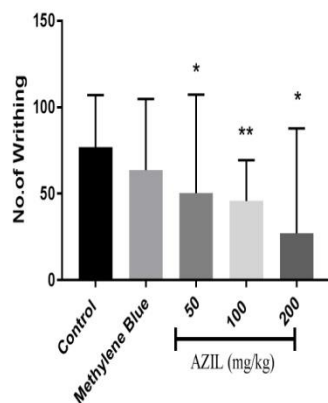
**Table No 8 Effect of AZIL on cinnamaldehyde-induced nociception**

Sr no	Treatment	Dose(mg/kg)	Licking Time (Sec)	Inhibition(%)
1	Control	0.1 ml/mice	62.05 ± 0.65	-
2	Diclofenac Sodium	10	9.6± 0.10*	83.92
3	AZIL	50	43.2± 1.3**	30.37
		100	21.6± 0.35	65.18
		200	16.95± 1.8*	72.68

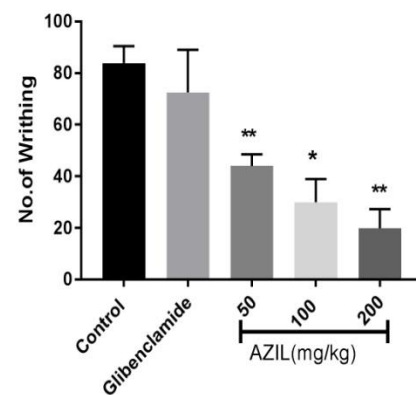
\* $P < 0.05$ , \*\* $P < 0.01$  vs. control, [Values are mean ± SE from six animals in each group.]

\* $p < 0.001$  compared with the control group (Dunnett's test).

**Involvement of Cyclic Guanosine Monophosphate (cGMP) pathway**



**Involvement of ATP-sensitive K<sup>+</sup> channel pathway**



**Figure No 1 and 2.** Effects of AZIL on involvement of cyclic guanosine monophosphate (cGMP) pathway and ATP-sensitive K<sup>+</sup> channel pathway. Values are presented as mean ± SEM (n = 5). \*\* $p < 0.001$  compared with the control group (ANOVA followed by post hoc Dunnett's test)

This study elaborates that oral administration of AZIL produce a potent and dose-dependent antinociceptive effect in mechanical induced and chemical nociception models even AZIL acts centrally and peripherally [2]. The important increase in latency time in hot plate by AZIL ( $p < 0.05$ ) at different doses submits the central antinociceptive activity of AZIL. The effect experiential in the tail immersion test, which is particularly for centrally acting analgesics, carried out by hot plate test [17]. To find out the possible mechanism of action of AZIL we analyzed the effect of naloxone, a non-selective opioid receptor antagonist Vs the antinociceptive effect of AZIL. The reverse effect was determinative against morphine in both the hot plate and tail immersion test. These studies confirm that the antinociceptive effect of AZIL may occur through opioid receptors at the spinal and supraspinal level. Some studies reveled mu and delta opioid receptors are involved in spinal mechanism, and mu 1 and 2-opioid receptors may



mediate mainly supraspinal analgesia [10]. However, it can be predicted that the central antinociceptive effect of AZIL may be protuberant on mu-opioid receptors. Peripheral antinociceptive activity evaluated by acetic acid induced writhing test. Due to releasing pain mediators writhing test is globally accepted as a model visceral pain. Oral administration of AZIL produced significant decrease in acetic acid-induced writhing. IP administration of acetic acid increases the level of bradykinin cyclooxygenase, Interleukin 1,8 beta, lipoxygenase, prostaglandins, histamines, TNF-alpha in the peripheral tissue fluid [14]. The inhibition of writhing response maintains the peripheral antinociceptive effect of AZIL [15,18]. In formalin test AZIL presented important antinociceptive activity in both neurogenic (early phase) and inflammatory (late phase). Formalin-induced pain is constantly inhibited by analgesic and anti-inflammatory drugs like morphine, diclofenac sodium. This study shows AZIL significantly decreases the cinnamaldehyde-induced pain, which probably involved with TRPA1 receptor located in C-fibers reducing the pain [16]. Presence of alkaloids, glycosides, steroids, carbohydrates, saponins, tannins and flavonoids revealed by Preliminary phytochemical screening. Flavonoids suppress the intracellular calcium level elevation, as well as the release of pro-inflammatory mediators (TNF alpha, IL 1 beta). In future our laboratory interested to find out role of flavonoids in antinociceptive mechanism. In addition, we investigate the involvement of cGMP pathway in the antinociceptive effect of AZIL. Nociceptive activity is depending on activation or deactivation of cGMP [16]. Intracellular cGMP concentrations are regulated by the action of guanylyl cyclase and by the rate of degradation by cGMP-specific phosphodiesterase. Nitric oxide elevates level of cGMP by activation of soluble GCs, which impacts physiological functions including pain and analgesia. cGMP acts on the ion-channels directly or through the activation of protein kinases and phosphodiesterase [1]. Future study includes determination of involved pathways in nociception which might helpful to provide therapeutic strategy in disease condition and provide appropriate use of medicinal plant.

## 9. Conclusions

It can be concluded that AZIL possesses important antinociceptive activity in both chemical and heat induced pain models (mechanical) in mice. The antinociceptive effect of AZIL is most likely mediated via inhibition of peripheral mediators and central inhibitory mechanisms. These results support the traditional use of this plant indifferent painful conditions. Further investigations are required to perceive the mechanisms of action of AZIL and to identify the active constituents that may be used as a lead compound for new drug development.

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## 12. Ethics approval

All the experimental mice were treated following the Ethical Principles and Guidelines from The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) The Institutional Animal Ethical Committee (1988/PO/Re/S/17/CPCSEA) of Gourishankar Institute of Pharmaceutical education and Research approved all experimental rules.

## 13. Conflict of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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## 15. Supplement I

### A. Phytochemical screening methods

Preliminary phytochemical screening of the AZI Leaves extracts was carried out using standard procedures described by Trease and Evans.

#### Test for saponins

One mL of the tepal extract was diluted with distilled water to 20 mL and shaken in a graduated cylinder for 15 minutes. The formation of one centimeter layer of foam indicates the presence of saponins.

#### Test for phenols

A small amount of the ethanolic extract was taken with 1 mL of water in a test tube and 1 to 2 drops of Iron III chloride ( $\text{FeCl}_3$ ) was added. A blue, green, red or purple color is a positive test.

#### Test for glycosides

A small amount of alcoholic extract was taken in 1 mL of water in a test tube and a few drops of aqueous NaOH were added. A yellow coloration indicates the presence glycosides.

#### Test for flavonoids

One to five drops of concentrated hydrochloric acid (HCl) were added to little amount of ethanolic extract of the plant material. Immediate development of a red colour indicates the presence of flavonoids.

#### Test for alkaloids

Two mL of extract was taken in a test tube and then 0.2 mL dilute HCl was included, followed by 1 mL of Meyer's reagent. A yellowish coloration indicates alkaloid's presence.

#### Test for tannins

Five mL of the tepal extract was placed in a test tube and then 2 mL of 5 % of  $\text{FeCl}_3$  solution was added. A greenish-black precipitate indicates the presence of tannins.

#### Test for terpenoids

In a test tube containing 2 mL of chloroform, 0.5 mL of extract was added. This is then followed by the addition of 3 mL conc.  $\text{H}_2\text{SO}_4$  which forms a layer. Reddish brown coloration of the interface indicates terpenoids.

641 **Test for reducing sugars**

642  
643 To 0.5 ml of extract solution, 1 ml of dihydrogen monoxide and 5-8 drops of Fehling's solution  
644 was integrated at boiling and observed for brick red precipitate.  
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