Revolutionizing the effect of *Azadirachta indica* extracts on edema induced changes in C-reactive protein and interleukin-6 in albino rats: *in silico* and *in vivo* approach

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**Abstract.** OBJECTIVE: The aim of the present study is to determine the *in vivo* and *in silico* anti-inflammatory effect of *Azadirachta indica* (*A. indica*) in carragenan-induced rats and its blood biomarkers. *A. Indica* (Neem) is a widely used medicinal plant across the world, especially in Pakistan. Neem leaves have been traditionally used for the synthesis of drugs and treatment of a wide variety of diseases.

MATERIALS AND METHODS: In this study, sixty albino rats (160-200 g) were divided into 4 groups: control (group I), standard (group II), ethanolic and aqueous (group III and IV) at doses of 50, 100, 200 and 400 mg/kg.

RESULTS: Ethanolic and aqueous extracts showed maximum inhibition in paw size at the 5th hour (400 mg/kg). Similarly, biomarkers measured, including Interleukin-6 and C-reactive protein, exhibited significant anti-inflammatory activity at the highest dose of 400 mg/kg in both experimental groups but were more distinct in the group treated with ethanolic extracts. Correlation between C-reactive protein (CRP) and interleukin-6 (IL-6) showed positive correlation in group III, while negative in group IV. Similarly, positive and negative correlations were observed between CRP biomarkers and paw size in group III and IV, and the same results were also shown in the case of IL-6 and paw size. In molecular docking, the binding energy value of protein CRP and IL-1β with the identified ligands quercetin and nimbinosterol showed (-8.2 kcal/mol and -7.7 kcal/mol) the best binding affinity as compared to standard drug diclofenac with -7.0 kcal/mol binding energy respectively.

CONCLUSIONS: In conclusion, *in silico* and *in vivo* analysis revealed that the extracts of *A. indica* leaves can be used as an effective drug to manage inflammation.

Key Words: 
*A. indica*, Albino rats, Inflammatory biomarkers, Molecular docking

Introduction

*A. indica* (Neem) belongs to the Meliaceae family and is a natural source of a variety of medicinal compounds. Fruits, seeds, oil, leaves, roots, bark, and nearly all other portion of this tree possesses antiviral, anti-inflammatory, anti-ulcer,
antifungal, anti-plasmodial, antiseptic, antipyretic, and anti-diabetic properties. Neem leaves contain limonoids, such as nimbin, nimbanene, another ascorbic acid, n-hexacosanol, and asparagine acid that possess effective anti-inflammatory activity. Medicinal plants have been used to cure various diseases for a long time. In Unani, Siddha, and Ayurveda, Neem (A. indica) is a well-known plant utilized for various ailments, including bacterial and viral infections. According to the World Health Organization (WHO), 60% population uses medicinal plants regularly to treat various diseases, and about 40% population is using such plants in pharmaceutical activities. It is a natural source of many medicinal compounds that possess effective immunomodulatory, anti-inflammatory, and antihyperglycemic effects through biochemical pathways. Phenolic compounds, azadirachtin, nimbidin, alkaloids, triterpenoids, carotenoids, saponins, flavonoids, and glycosides are phytochemicals present in A. indica that possesses anti-inflammatory properties.

Inflammation is the body’s first immune response and a complicated biological mechanism to cell injury and vascularized tissue; however, uncontrolled, and chronic inflammation can be harmful to tissues. Inflammation is a pathological response in which plasma fluids and blood cells assemble locally in living organisms. However, it is a defense mechanism in which complex actions and cytokines involved in the inflammatory reaction can originate, sustain, or accelerate medical problems. In recent years, medicinal plants have increased internationally in various regions of the world, and the literature showed that the immense therapeutic potential has been utilized traditionally. Medicinal plants with therapeutic properties are thought to be a useful source of unknown chemical compounds. However, inflammation-related diseases are becoming more well known, and the complexity of the currently available anti-edematous drugs poses a major cause. Therefore, the development of new, potent anti-edematous drugs with fewer serious side effects is reliable. Carrageenan-induced edema is used to examine the role of natural products in preventing the met-abolic alterations linked with acute inflammation. Interleukin-6 (IL-6) and C-reactive protein (CRP) are basically inflammatory biomarkers, each with pro-inflammatory effects. IL-6 exhibits more potent anti-inflammatory than pro-inflammatory effects, while CRP production in the liver is stimulated by IL-6. CRP is deposited at the sites of tissue damage and inflammation and plays an important role in inflammation. Bioinformatics provides essential evidence to recognize the method of ligands binding to targets and helps to choose the best ligands for additional experimental proof. Bioinformatics utilizes computational tools to solve biological problems. The goal of the present study is to evaluate the anti-inflammatory properties of ethanolic and aqueous extracts from A. indica leaves in a rat model, assess associated blood biomarkers, and study the phytochemical constituents of A. indica using in silico approach.

Materials and Methods

Sample Collection and Preparation of A. indica Extract
Fresh leaves of A. indica were collected from different botanical areas of Lahore and identified by the staff members of the botany department of The University of Punjab, Lahore. For plant material extraction, the cold maceration method was used. Experimental Rats
Albino rats of either sex weighing (160-200 g) were purchased from the University of Lahore and kept in polypropylene cages in the animal house of The University of Lahore. This study was approved by the Ethics Committee of “The University of Lahore”, Lahore. Before experimental work, rats were kept in fasting condition and then were given distilled water and balanced feed.

Drugs Used in the Experiment
A. indica ethanolic and aqueous extracts were prepared at concentrations of 50, 100, 200, and 400 mg/kg and were given to group III (ethanolic extract) and group IV (aqueous extract). Diclofenac (100 mg/kg) and normal saline (10 mg/kg) were given to group I (control) and group II (standard), respectively. 100 mg/kg carrageenan was used to induce inflammation.

Procedure
Carrageenan-induced paw edema
1% carrageenan of 100 mg/kg was injected into all rat groups in the left hind paw in the sub-planter region to induce edema. Paw sizes were taken before and after the injection of carrageenan.

Grouping and Treatment Schedule
Group I was the control group, group II was the standard group, group III was treated with...
ethanolic extract, and group IV was treated with Aqueous extract.

In group I (control), rats were injected with normal saline (10 ml/kg) in the sub-planter region of the hind paw. Paw size was measured. At first, paw size increased due to edema, but after 4-5 hours, it started to decrease and got normalized after 18 hours.

In group II (standard), rats were injected with diclofenac (100 mg/kg) in the sub-planter region of the hind paw. At first, paw size was increased due to edema, but after injecting diclofenac, it began to decrease and got normalized after 3 hours.

In groups III (ethanolic extract) and IV (aqueous extract), *A. indica* extracts at doses of 50, 100, 200, and 400 mg/kg were administrated. The formula to calculate the anti-inflammatory activity is given below:

\[
\% \text{ inhibition} = \frac{\text{Control mean} - \text{treated mean}}{\text{Control mean}} \times 100
\]

Collection of blood samples and assessment of inflammatory biomarkers

At the end of the experiment, blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes via cardiac puncture without anticoagulant and left for 10 minutes. Tubes were centrifuged at 4,000 rpm/min for 10 minutes, and obtained serum was kept at -20°C for further treatment in Eppendorf. C-reactive protein and IL-6 were examined using commercially available ELISA kits (DiaMetra, Spello, PG, Italy).

In silico anti-inflammatory activity of *A. indica*

Different computational tools for in silico studies, including ChemSkech, chimera, pymol, pyrx, depth resi-due, and discovery studio, were used to maintain the anti-inflammatory effect of *A. indica*.

Selection and Preparation of Ligands

Three ligands such as quercetin, nimbosterol, and diclofenac were selected for the docking studies. The 3D structure of the ligand molecule was assessed using the PubChem, a database of chemical molecules and their activities against the biological assays website and ChemSketch.

Protein preparation and prediction of active site

CRP and IL-1β, two proteins involved in the inflammatory cascade, were chosen for docking studies. 3D structures were copied using Swissport database and opened in Chimera, and one IL-1β chain was chosen out of four. Before gathering the proteins, all extra water molecules, metals, and ions were removed. The protein structures were saved in a PDB file. In the depth residue, the active sites of IL-1β and CRP proteins were found.

Protein-ligand docking

PyRx was used for virtual screening to find ligand molecules for a specific protein or the potential of an anti-inflammatory drug. In this technique, the target proteins CRP and IL-1β, as well as ligand molecules, were chosen from PDB files and attached to the receptor protein’s binding site. Similarly, the discovery studio tool was used in this method to create the structure of protein-ligand molecules and the 3D structure of complex for high-quality observation.

Statistical Analysis

Data were subjected to homogeneity of variance to check normality and preceded to one-way ANOVA using PROC GLM in SAS software (Cary, North Carolina, USA) (version 9.1). Duncan’s multiple range test, Pearson correlation method, and Dunnett’s t-test were used considering \( p < 0.05 \).

Results

The anti-inflammatory effect of *A. indica* ethanolic and aqueous extracts against paw edema was determined with the following groups: group I (control) consisted of 6 rats, treated with normal saline, group II (standard) consisted of 6 rats, treated with diclofenac, groups III and IV, both experimental groups, comprised of 48 rats, treated with *A. indica* ethanolic and aqueous extracts correspondingly, at doses of 50, 100, 200 and 400 mg/kg.

Effect of A. Indica on Paw Sizes in Rats

All groups showed a significant increase in paw edema volume injected with 1% carrageenan. Group I showed a remarkable increase in paw volume (45.17 ± 1.66, 47.83 ± 2.29, 55.67 ± 2.58, 64.17 ± 1.66, and 62.33 ± 1.67 after 1 to 5 hours of injection. While groups III and IV, treated with *A. indica* extracts at different concentrations (50, 100, 200, and 400 mg/kg), reduced paw edema at 1 to 5 hours as compared to group I, which showed an improvement in the edema volume as compared to group I. The improvement was more pronounced at the 5th hour in a dose-dependent manner with 40.11, 45.80, 48.24, and 50.13% inhibition closer to
Table I. Mean paw size and % age inhibition of paw size by groups I (control), II (standard), III (Ethanolic extract of A. indica) in carrageenan-induced paw edema of rats.

<table>
<thead>
<tr>
<th>Treatment Groups</th>
<th>1-hour</th>
<th>2-hour</th>
<th>3-hour</th>
<th>4-hour</th>
<th>5-hour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>45.83 ± 1.74</td>
<td>49.17 ± 1.96</td>
<td>57.17 ± 2.7</td>
<td>64.50 ± 2.11</td>
<td>61.50 ± 1.98</td>
</tr>
<tr>
<td>Group II</td>
<td>33.67 ± 1.61</td>
<td>36.00 ± 1.15</td>
<td>37.00 ± 1.46</td>
<td>30.67 ± 1.67</td>
<td>26.50 ± 0.76</td>
</tr>
<tr>
<td>Dose 50 mg/kg</td>
<td>39.17 ± 1.19</td>
<td>40.33 ± 0.71</td>
<td>41.50 ± 1.34</td>
<td>37.83 ± 0.40</td>
<td>36.83 ± 0.40</td>
</tr>
<tr>
<td>Dose 100 mg/kg</td>
<td>37.50 ± 0.76</td>
<td>37.33 ± 0.71</td>
<td>39.33 ± 0.80</td>
<td>34.00 ± 0.45</td>
<td>33.33 ± 0.49</td>
</tr>
<tr>
<td>Dose 200 mg/kg</td>
<td>34.50 ± 0.76</td>
<td>36.33 ± 1.12</td>
<td>40.50 ± 1.18</td>
<td>37.33 ± 0.08</td>
<td>31.83 ± 1.11</td>
</tr>
<tr>
<td>Dose 400 mg/kg</td>
<td>34.50 ± 0.76</td>
<td>36.17 ± 1.30</td>
<td>39.83 ± 1.08</td>
<td>33.17 ± 1.05</td>
<td>30.67 ± 0.67</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Mean paw size and % age of paw size inhibition by groups I, II, and IV (Aqueous extract of A. indica) in carrageenan-induced paw edema of rats. The letters on different means differ significantly at $p < 0.05$ ($a > b > c > d$).

Figure 1. A. Paw sizes of rats (mm) at 1-5 hours post carrageenan injection in group I, group II, and Group III extracts of A. indica. B. % Inhibition of edema by A. indica ethanolic extracts and diclofenac against carrageenan-induced rats paw edema at the end of 1-5 hours; analyzed by one-way ANOVA followed by DMR test. The letters on different means differ significantly at $p < 0.05$ ($a > b > c > d$).
Anti-inflammatory properties of ethanolic and aqueous extracts from *A. indica* leaves

*Table II.* Mean paw size and % age of paw size inhibition by groups I, II, and IV (Aqueous extract of *A. indica*) in carrageenan-induced paw edema of rats.

<table>
<thead>
<tr>
<th>Reaction Time with Mean ± S.D (% inhibition) per hour</th>
<th>Treatment Groups</th>
<th>1-hour</th>
<th>2-hour</th>
<th>3-hour</th>
<th>4-hour</th>
<th>5-hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>45.83± 1.74</td>
<td>49.17± 1.96</td>
<td>57.17± 2.71</td>
<td>64.50± 2.11</td>
<td>61.50± 1.98</td>
</tr>
<tr>
<td></td>
<td>Group II</td>
<td>33.67± 1.61</td>
<td>36.00± 1.15</td>
<td>37.00± 1.46</td>
<td>30.67± 1.67</td>
<td>26.50± 0.76</td>
</tr>
<tr>
<td></td>
<td>Dose 50 mg/kg</td>
<td>45.83± 1.30</td>
<td>47.83± 1.35</td>
<td>47.17± 1.76</td>
<td>45.83± 1.01</td>
<td>43.50± 0.76</td>
</tr>
<tr>
<td></td>
<td>Dose 100 mg/kg</td>
<td>46.00± 0.73</td>
<td>48.17± 0.10</td>
<td>48.17± 1.22</td>
<td>43.83± 0.49</td>
<td>41.67± 0.67</td>
</tr>
<tr>
<td></td>
<td>Dose 200 mg/kg</td>
<td>42.83± 1.30</td>
<td>44.83± 1.14</td>
<td>45.67± 1.4</td>
<td>41.00± 1.34</td>
<td>38.33± 0.88</td>
</tr>
<tr>
<td></td>
<td>Dose 400 mg/kg</td>
<td>39.00± 0.97</td>
<td>42.17± 0.95</td>
<td>45.17± 0.95</td>
<td>34.83± 1.08</td>
<td>35.33± 0.71</td>
</tr>
</tbody>
</table>

The significantly different means within the column differ at *p* < 0.05 (*a > b > c > d*).

![Figure 2A](image1.png) ![Figure 2B](image2.png)

*A. indica* ethanolic extract (group III) showed the most pronounced effect as compared to *A. indica* aqueous extracts (group IV), as shown in Table II (Figure 2A-B).

**Effect of *A. Indica* on Immunological and Biochemical Parameter**

*A. indica* extracts significantly (*p* < 0.05) reduced the elevated levels of CRP and IL-6.
in blood. CRP biomarkers values significantly differ in group III (4.70 ± 0.01) levels of CRP and IL-6 in blood. CRP biomarker values significantly differ in groups III and IV (7.85 ± 0.01) as compared to group I (control) with elevated levels of CRP (9.59 ± 0.20). IL-6 values also differ significantly (p < 0.05) at different doses in extract-treated groups III (12.77 ± 0.02) and IV (15.58 ± 0.01) as compared to group I control (17.13 ± 0.10), as shown in Table III (Figure 3A-D). The most significant (p < 0.05) effect of both extracts was observed at dose 400 mg/kg closer to group II (standard) treated with diclofenac drug (2.43 ± 0.09 and 10.40 ± 0.07) in a dose-dependent manner, where p-value < 0.0001.

**Comparison Study of Biomarker CRP**

By comparing CRP biomarker in groups III and IV doses vs. groups I and II, a significant p < 0.001 mean difference between treated groups at different doses were observed. Group III treated at dose 400 mg/kg vs. control and standard group has mean differences -4.8900 and 2.26667, respectively. group IV, treated at dose 400 mg/kg vs. control and standard group, has a mean difference -1.7350 and 5.42167, respectively, as shown in Table IV.

**Comparison Study of Biomarker IL-6**

By comparing IL-6 biomarkers at different doses, group III, treated at dose 400 mg/kg vs. control and standard, has a mean difference

### Table III. Effect of *A. indica* and diclofenac CRP (ng/ml) in ethanolic (group III) and aqueous extracts (group IV).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRP (ng/ml)</th>
<th>IL-6 protein (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group III</td>
<td>Group IV</td>
</tr>
<tr>
<td>Control Group</td>
<td>9.59±0.20</td>
<td>9.59±0.20</td>
</tr>
<tr>
<td>Standard Group</td>
<td>2.43±0.09</td>
<td>2.43±0.09</td>
</tr>
<tr>
<td>Dose 50 mg/kg</td>
<td>5.26±0.01</td>
<td>8.25±0.01</td>
</tr>
<tr>
<td>Dose 100 mg/kg</td>
<td>5.15±0.01</td>
<td>8.29±0.10</td>
</tr>
<tr>
<td>Dose 200 mg/kg</td>
<td>4.88±0.02</td>
<td>7.99±0.04</td>
</tr>
<tr>
<td>Dose 400 mg/kg</td>
<td>4.70±0.01</td>
<td>7.85±0.01</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

The means with different superscripts (a > b > c > d > e) within the column differ significantly.

### Table IV. Comparisons of CRP biomarker means through Dunnett’s *t*-test in groups III and IV.

<table>
<thead>
<tr>
<th>Treatment comparison</th>
<th>Difference between means</th>
<th>Simultaneous 95% confidence limits</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose 50 vs. group I</td>
<td>-4.3300</td>
<td>-4.6666</td>
<td>***</td>
</tr>
<tr>
<td>Dose 100 vs. group I</td>
<td>-4.4367</td>
<td>-4.7732</td>
<td>***</td>
</tr>
<tr>
<td>Dose 200 vs. group I</td>
<td>-4.7100</td>
<td>-5.0466</td>
<td>***</td>
</tr>
<tr>
<td>Dose 400 vs. group I</td>
<td>-4.8900</td>
<td>-5.2266</td>
<td>***</td>
</tr>
<tr>
<td>Dose 50 vs. group II</td>
<td>2.82667</td>
<td>2.66968</td>
<td>***</td>
</tr>
<tr>
<td>Dose 100 vs. group II</td>
<td>2.72000</td>
<td>2.5631</td>
<td>***</td>
</tr>
<tr>
<td>Dose 200 group II</td>
<td>2.44667</td>
<td>2.28698</td>
<td>***</td>
</tr>
<tr>
<td>Dose 400 vs. group II</td>
<td>2.26667</td>
<td>2.10698</td>
<td>***</td>
</tr>
</tbody>
</table>

**Group IV (Aqueous extracts)**

<table>
<thead>
<tr>
<th>Treatment comparison</th>
<th>Difference between means</th>
<th>Simultaneous 95% confidence limits</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose 50 vs. group I</td>
<td>-1.2950</td>
<td>-1.6695</td>
<td>***</td>
</tr>
<tr>
<td>Dose 100 vs. group I</td>
<td>-1.3417</td>
<td>-1.7162</td>
<td>***</td>
</tr>
<tr>
<td>Dose 200 vs. group I</td>
<td>-1.5983</td>
<td>-1.9728</td>
<td>***</td>
</tr>
<tr>
<td>Dose 400 vs. group I</td>
<td>-1.7350</td>
<td>-2.1095</td>
<td>***</td>
</tr>
<tr>
<td>Dose 50 vs. group II</td>
<td>5.86167</td>
<td>5.62359</td>
<td>***</td>
</tr>
<tr>
<td>Dose 100 vs. group II</td>
<td>5.81500</td>
<td>5.57693</td>
<td>***</td>
</tr>
<tr>
<td>Dose 200 group II</td>
<td>5.55833</td>
<td>5.32026</td>
<td>***</td>
</tr>
<tr>
<td>Dose 400 vs. group II</td>
<td>5.42167</td>
<td>5.18359</td>
<td>***</td>
</tr>
</tbody>
</table>

Comparison of the experimental group IV with the control and standard group, our results showed significant difference where *** = p < 0.001.
Anti-inflammatory properties of ethanolic and aqueous extracts from *A. indica* leaves

-4.35667 and 2.37500, respectively. Group IV, treated at dose 400 mg/kg vs. control and standard groups, has a mean difference -1.55000 and 5.18167, respectively, as shown in Table V.

**Correlation Study**

Analyzing correlation studies is crucial to understanding biological processes. Correlation analysis is frequently used to examine, quantify, and analyze the correlation between observed data. Correlation was significant at $p \leq 0.05$. According to Pearson’s correlation, in group III there was a negative correlation between biomarker CRP and IL-6 ($p < 0.05$) in the control and standard groups, and at all doses 50, 100, 200, and 400 mg/kg, there was a positive correlation correspondingly. In group IV, there was a strong positive correlation at higher doses.
of 200 and 400 mg/kg. A positive and negative correlation was observed between IL-6 marker and paw size, where the correlation was significant at \((p < 0.05)\). A positive and negative association was evident with increasing dose rates at 100, 200, and 400 mg/kg i.e., 0.02, -0.48, and 0.13 in group III and 0.82, -0.33, and 0.25 in group IV, correspondingly.

Based on the results, CRP marker and paw sizes illustrated a positive correlation of 0.25 and 0.68, in the control and standard group. At the 4th hour, there was a positive correlation at doses of 50 and 100 mg/kg as (0.30) and (0.002). However, the correlation was weakly negative at dose of 200 and strongly negative at 400 mg/kg i.e., -0.14 and -0.47, correspondingly. At the end of 4th hour, there was a negative correlation between the biomarker IL-6 and paw sizes \((p < 0.05)\) in the experimental group IV at doses 50, 100, and 200 as (-0.14), (-0.88) and (-0.71), respectively as shown in Table VI.

In Silico Analysis

The potential of \textit{A. indica} to target the cytokines IL-1β and CRP was examined \textit{in silico}. The binding energy of quercetin and nimbosterol with CRP and IL-6 showed much better docking energy results as compared to the standard drug diclofenac.

Study of protein structure evaluation and ligands in the binding pocket

IL-1β has one chain, CRP has five chains. All ligands and proteins were added to the grid box.

### Table V. Comparisons of IL-6 biomarker means through Dunnett’s t-test.

<table>
<thead>
<tr>
<th>Treatment comparison</th>
<th>Difference between means</th>
<th>Simultaneous 95% confidence limits</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose 50 vs. group I</td>
<td>-3.96667</td>
<td>-4.15947</td>
<td>-3.77836</td>
</tr>
<tr>
<td>Dose 100 vs. group I</td>
<td>-3.97000</td>
<td>-4.16281</td>
<td>-3.77719</td>
</tr>
<tr>
<td>Dose 200 vs. group I</td>
<td>-4.21333</td>
<td>-4.40614</td>
<td>-4.02053</td>
</tr>
<tr>
<td>Dose 400 vs. group I</td>
<td>-4.35667</td>
<td>-4.54947</td>
<td>-4.16386</td>
</tr>
<tr>
<td>Dose 50 vs. group II</td>
<td>2.76500</td>
<td>2.63064</td>
<td>2.89936</td>
</tr>
<tr>
<td>Dose 100 vs. group II</td>
<td>2.76167</td>
<td>2.62731</td>
<td>2.89603</td>
</tr>
<tr>
<td>Dose 200 group II</td>
<td>2.51833</td>
<td>2.38397</td>
<td>2.65269</td>
</tr>
<tr>
<td>Dose 400 vs. group II</td>
<td>2.37500</td>
<td>2.24064</td>
<td>2.50936</td>
</tr>
</tbody>
</table>

In comparison of the experimental group IV with the control and standard group, our results showed significant difference where *** \(= p < 0.001\).

### Table VI. Correlation between Paw sizes, CRP and IL-6 in group III (ethanolic group) and group IV (aqueous extract).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CRP and IL-6</th>
<th>IL-6 and paw sizes</th>
<th>CRP and Paw sizes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group III</td>
<td>Group IV</td>
<td>Group III</td>
</tr>
<tr>
<td>Control</td>
<td>-0.19</td>
<td>-0.19</td>
<td>0.70</td>
</tr>
<tr>
<td>Standard</td>
<td>-0.54</td>
<td>-0.54</td>
<td>0.02</td>
</tr>
<tr>
<td>Dose 50 mg/kg</td>
<td>0.51</td>
<td>0.75</td>
<td>0.37</td>
</tr>
<tr>
<td>Dose 100 mg/kg</td>
<td>0.25</td>
<td>-0.89</td>
<td>0.02</td>
</tr>
<tr>
<td>Dose 200 mg/kg</td>
<td>0.95</td>
<td>0.81</td>
<td>-0.48</td>
</tr>
<tr>
<td>Dose 400 mg/kg</td>
<td>0.91</td>
<td>0.84</td>
<td>0.13</td>
</tr>
</tbody>
</table>

IV (aqueous extract).
Anti-inflammatory properties of ethanolic and aqueous extracts from *A. indica* leaves

In Figure 4A, CRP is represented by golden color and the binding pocket with purple color and ligand molecules. Quercetin is presented with grey, nimbosterol with sea green, and diclofenac with green color. In Figure 4B, IL-1β protein is represented by green color; the binding pocket is orange; ligand molecules quercetin is silver; nimbosterol is sea green, while diclofenac is green color.

**Discussion**

The present study was determined to assess the *in vivo* and *in vitro* anti-inflammatory effects of ethanolic and aqueous extract of *A. indica* plant. The medicinal systems of Ayurveda are accepted as traditional system with significant research on clinical, therapeutics and pharmacology. Carrageenan-induced paw edema is responsive to cyclooxygenase inhibitors and used to test the efficacy of non-steroidal anti-inflammatory drugs that predominantly inhibit the cyclooxygenase implicated in prostaglandin biosynthesis, protease, and lysosomes within 1 to 6 hours. This period is useful to assess the anti-inflammatory effects of medicinal plants. In a previous study, MEAI inhibited edema during the first and second phase of inflammation by inhibiting prostaglandin release and suppressing histamine, serotonin, and bradykinin.
According to our findings, all doses of groups III (ethanolic extract) and IV (aqueous extract) have been in-volved in the suppression of edema. Maximum reduction was observed at 400 mg/kg, which exhibited 50.13% and 42.55% inhibition of edema and was almost effective as the standard drug diclofenac with (56.91%) inhibition. A. indica can suppress eicosanoid production by inhibiting both cyclooxygenase (COX) and lipooxygenase (LOX), while thymoquinone (TQ) has been exposed to reduce lipid peroxidation and A. indica also inhibits macrophage nitric oxide generation. As a result, it can be confirmed that a high concentration of A. indica components lowers inflammation, while a smaller concentration has negative effects. Results of other studies35-38 were concurrent with our findings. A. indica extracts contain many phytochemicals, including flavonoids, triterpenes, and tannins which are responsible for showing significant findings in anti-inflammatory activity in a dose-dependent manner. Group III showed the strongest anti-inflammatory potential. Several previous studies35-37 concluded that phytochemicals present in Neem decrease edema by suppressing the activity of STAT-1 and NF-κβ.

CRP is an acute-phase protein generated in the liver and secreted into the bloodstream in reaction to IL-6 stimulation during an inflammatory phase. The concentration of CRP in plasma increases considerably when IL-6 levels rise. We evaluated the medicinal effects of A. indica in the regulation of normal systematic de-pending on the outcomes of inflammatory biomarkers. According to earlier research, CRP plays various es-sential roles during an inflammatory episode. It binds to injured, necrotic, and microbial cells, increases neutrophil and macrophage phagocytosis, and activates the complement system, which aids in the mainte-nance of inflamm-a-tion40. In our results, group I (control) remarkably raised the levels of CRP and IL-6 biomarkers and groups III (ethanolic extract) and IV (aqueous extract) showed significant inhibition of edema at the dose of 200 mg/kg and 400 mg/kg. In groups III and IV, a higher dose of the extract (400 mg/kg) ex-hibited a higher inhibitory effect on IL-6 than the lower dose (50 mg/kg). For inflammation, few cells are engaged to promote the production of pro-inflammato-ry chemical mediators [IL, IL-6, and Tumor Necrosis Factor alpha (TNF-α)]. Nimbo-lide is a plant-derived phytochemical that inhibits the expression of inflam-matory cytokines such as Interleukin (IL, IL-6, IL-8, and IL-12) and nu-
clear factor (TNF-α). In our results, there was a positive correlation between the biomarker interleuken-6 and C-reactive protein in group IV (aqueous extract). Similarly, other reports45-47 are in concurrence with our data set showing that classic and trans pathways together take part in an inflammatory condition. All those pathways are diverse. Through classical signaling in the liver, IL-6 upregulates the production of CRP, with lipolysis. IL-6 correlates with the severity of other inflammatory processes interceding inflam-matory reactions.

According to our results, computational studies determined the required geometry of two molecules with identified structures. Our findings were associated with the efficiency of bioactive substances and their in-teractions help to stabilize protein-ligand complexes. In molecular docking studies, the protein CRP with the identified ligands quercetin and nimbo-sterol showed the best binding affinity -8.2 kcal/mol and -7.72 kcal/mol, and IL-1β rep-resented the binding affinity -4.5 kcal/mol and -3 kcal/mol. The standard drug, di-clofenac with interacting protein, exhibited the binding affinity -7.02 kcal/mol and -4.4 kcal/mol. The results of this docking study confirmed the binding potential of the reported constituents of quercetin and nimbos-terol with CRP and IL-1β, which plays a chief role in anti-inflamm-a-tory activity42-48.

**Conclusions**

The use of nonsteroidal (NSAID) or steroidal (SAID) anti-inflammatory drugs could be bene-ficial in treating a variety of controversial dis-or-ders, but their side effects are the leading cause of acute and chronic prob-lems. The impact of A. indica is a combined and synergistic reaction of several classes of phytoconstituents, rather than a single ingredient with a variety of polarities that work by blocking the COX-1 and COX-2 en-zymatic activity, as well as IL-1, CRP, IL-6, and TNF-α production is inhibited. As a result, thera-pies based on a safe natural source mediator with fewer side effects are required, and extracts of A. indica leaves can be used as an effective drug to treat disorders such as inflammation.

**Informed Consent**

Not applicable.
Anti-inflammatory properties of ethanolic and aqueous extracts from A. indica leaves

Availability of Data and Materials
All the data generated in this research study has been included in the manuscript.

Authors’ Contributions
Conceptualization: Ammara, Sobia, and Sohail.; methodology, Abid and Tariq.; software, Ahellah.; validation, Rewaa.; formal analysis, Nehad and Manal investigation, Nouf and Amnah.; resources, Ammara, Sobia, Nureen and Sohail.; data curation, Abid and Tariq writing—original draft preparation, Majid and Abdulhakeem.; writing-review and editing, Nahaa, Rewaa, Nehad, Nouf, Manal, Amnah, Majid, Abdulhakeem. Anas S Dablool, Saad Alghamdi; visualization, Ammara, Sobia, Nureen and Sohail; supervision, Tariq.; project administration, Nureen; funding acquisition, Nahaa.

Ethics Approval
Ethical approval for this research study was approved by the Ethical Committee Institute of Microbiology and Biotechnology (IMBB) University of Lahore under letter No. CRiMM/22/Research/145, dated 02/12/2022.

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Conflicts of Interest
The authors declare no conflict of interest.

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References
10) Saleem A, Afzal M, Naveed M, Makhdoom SI, Mazhar M, Aziz T, Khan AA, Kamal Z, Shahzad M, Alharbi M, Alshammar A. HPLC, FTIR and GC-MS Analyses of Thymus vulgaris Phytochemicals Executing In Vitro and In Vivo Biological Activities and Effects on COX-1, COX-2 and
Gastric Cancer Genes Computationally. Molecules 2022; 27: 8512.


43) Alzohairy MA. Therapeutics Role of Azadirachta indica (Neem) and Their Active Constituents in Diseases Prevention and Treatment. Evid Based Complement Alternat Med 2016; 2016: 7382506.